B-cell activating factor (BAFF) plays a role in the mechanism of action of a tolerogenic peptide that ameliorates lupus

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Received 6 August 2008; accepted with revision 22 December 2008
Available online 1 February 2009

Abstract Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by dysregulated immune responses mediated by T and B cells. A tolerogenic peptide, designated hCDR1, ameliorated the serological and clinical manifestations of SLE in mouse models of lupus. We investigated the role of B-cell activating factor (BAFF) in the beneficial effects of hCDR1. BAFF production was reduced in hCDR1-treated mice in association with diminished production of dsDNA-specific autoantibodies and proteinuria levels. In addition, IFN-γ and IL-10, which induce BAFF secretion, were down-regulated in hCDR1-treated mice. The reduced levels of BAFF correlated with a lower rate of maturation and differentiation of B cells, and with a decrease in integrin expression and anti-apoptotic gene expression by B cells. Moreover, BAFF signaling through the NF-κB pathways was inhibited in hCDR1-treated mice. Thus, down-regulation of BAFF plays a role in the mechanism of action by which hCDR1 ameliorates lupus manifestations.

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KEYWORDS
B-cell activation factor; B-cell populations; Marginal zone; Systemic lupus erythematosus; Tolerogenic peptide

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by dysregulated immune responses mediated by T and B cells, leading to increased production of pathogenic autoantibodies, against several self antigens. The latter are associated with clinical manifestations in various organs [1–3]. Several strains of mice that spontaneously develop an SLE-like disease were reported, of which the [New Zealand Black (NZB) × New Zealand White (NZW)]F₁ female mice are the most widely used [4]. In addition, our laboratory established a model of experimentally induced SLE in different susceptible mouse strains that are not SLE prone [5–7]. A peptide, designated hCDR1, based on the complementarity determining region (CDR)1 [8] of a human monoclonal anti-DNA antibody, was designed and synthesized in our laboratory and was shown to ameliorate the serological and clinical manifestations of SLE, in both spontaneous and induced models of lupus [9]. Furthermore, hCDR1 was shown to reduce the secretion and the expression of the pathogenic cytokines interferon (IFN)–γ, interleukin (IL)-10, IL-1β, and tumor necrosis factor (TNF)α, while up-regulating the immunosuppressive cytokine, transforming growth factor (TGF)β [9]. Looking at the T-cell
compartment, studies aimed at elucidating the mechanisms of action of hCDR1 indicated that treatment with hCDR1 resulted in the induction of functional CD4^+CD25^+ regulatory T cells, which were shown to play a key role in mediating the clinical amelioration of the SLE-affected mice [10]. In addition to the immunomodulating effect of T-cell-derived cytokines [10,11], treatment with hCDR1 reduced the rates of apoptosis of T cells of the diseased mice by affecting the p21/Ras [12], Fas-FasL [13], and Bcl-xL [14] signaling pathways.

B cells play an important role in the pathogenesis of SLE. They produce autoantibodies that mediate tissue injury, they function as antigen-presenting cells (APCs) that present epitopes of self antigens to autoreactive T cells, and they produce soluble mediators involved in the organization of lymphoid tissues and in the initiation and perpetuation of inflammatory processes [15,16]. B-cell activating factor (BAFF, also known as BlyS or TALL-1) is a member of the TNF superfamily that regulates B-cell survival and autoreactivity [17]. BAFF is found either on the cell surface or is released in a soluble form after cleavage. BAFF binds to three receptors: transmembrane activator and calcium modulator ligand interactor (TACI), B-cell maturation antigen (BCMA), and BAFF receptor or BR3 [17,18]. A proliferation-inducing ligand (APRIL) is a homologous molecule to BAFF, that binds only to TACI and BCMA and shares many functions with BAFF, although it cannot facilitate the survival of B cells, a function that depends on the interaction of BAFF with BAFF receptor [19]. Mice overexpressing BAFF [BAFF-Transgenic (Tg) mice] develop an autoimmune disease with hallmarks resembling human SLE, including autoantibodies against dsDNA, circulating immune complexes, and severe membranoproliferative glomerulonephritis [20]. Serum levels of BAFF and APRIL were shown to rise in autoimmune diseases, including SLE and rheumatoid arthritis [21,22], and blockade of BAFF and a proliferation-inducing ligand using soluble fusion proteins of BAFF receptors prevented autoimmunity in animal models of the disease [23,24].

Transitional type 1 (T1) B cells are the earliest bone marrow-derived B-cell precursors emigrating to the spleen. These cells develop into transitional type 2 (T2), mature follicular, and finally to marginal zone (MZ) B cells [25]. In BAFF knockout mice, B cell development is blocked mainly at the T1 stage [26], whereas expanded T1 and T2 transitional B-cell populations were observed in BAFF-Tg mice [27]. Moreover, BAFF overexpression markedly increased the number of splenic CD21^hiCD23^- B-cells, which represent the dominant B cell population of the MZ compartment. The latter cells are the major source of autoreactive B cells, producing pathogenic IgG autoantibodies [27]. BAFF was shown to retain these B cells in the MZ by up-regulating the expression of the integrins αL(β2) and α4(β1) that recognize the vascular cell adhesion molecule (VCAM-1), and the intercellular adhesion molecule (ICAM)-1 on stromal cells [28]. Furthermore, transcription factors of the nuclear factor kappa B (NF-kB) family play an important role in B-cell maintenance [29]. Five NF-kB proteins have been described in mammalian cells: RelA, c-Rel, RelB, p50, and p52. Prior to activation, homo- and heterodimers of these proteins are retained in the cytoplasm by their association with inhibitory proteins of the inhibitory kappa B (IkB) family [30]. Activation of NF-kB occurs by a variety of different agents, mainly through induced degradation of the IkB proteins, or of IkB-homologous regions in p105 and p100, and the consequent translocation of NF-kB dimmers to the nucleus [31]. Two major pathways leading to NF-kB activation were described, the canonical and alternative pathways. B-cell receptor triggering leads to canonical NF-kB activity, whereas BAFF induces both pathways [30].

In the present study, we determined the effect of hCDR1 on BAFF and BAFF-induced functions, and assessed the role of these effects in the amelioration of SLE in (NZB×NZW)F1 mice. We demonstrated that the expression and secretion of BAFF were reduced in the hCDR1-treated mice, and that down-regulation of BAFF was associated with diminished production of dsDNA-specific autoantibodies and proteinuria levels. In addition, IFN-γ and IL-10, that induce BAFF secretion, were down-regulated in hCDR1-treated mice. The lower levels of BAFF were associated with the down-regulation of T1, T2, and MZ B cells, and with a decrease in integrin [lymphocyte function-associated antigen (LFA)-1, α4, β1] expression. Furthermore, the expression of anti-apoptotic genes by B cells was inhibited in hCDR1-treated mice, and reversed following the addition of BAFF. Moreover signaling through the NF-kB pathways was shown to be involved in the inhibition of BAFF by hCDR1. Thus, the down-regulation of BAFF plays a role in the mechanism of action by which hCDR1 ameliorates lupus manifestations.

Materials and methods

Mice

Female (NZB×NZW)F1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and Balb/c mice were obtained from Harlan (Jerusalem, Israel). The study was approved by the Animal Care and Use Committee of the Weizmann Institute of Science.

Synthetic peptides

A peptide, GYYWSWirQPGkGEEWIG, designated hCDR1 [32], that is based on the sequence of CDR1 of a human anti-DNA monoclonal antibody bearing the 16/6Id [8], was synthesized (solid-phase synthesis by F-moc chemistry) by Polypeptide Laboratories (Torrance, CA) and was used in this study. A peptide containing the same amino acids as hCDR1, with a scrambled order (scrambled peptide), SKGIPpyGGWP-WEGWRYEI, was used as a control.

Treatment of mice with hCDR1

Mice at the age of 6–7 months were divided into three groups (n = 8 to 12) and injected subcutaneously (s.c.) once a week for 10–13 weeks with the vehicle, Captisol® (sulphobutyl–l-cyclodextrin, CyDex Inc., Lenexa, KS), with hCDR1 (50 μg/mouse) or with the scrambled peptide (50 μg/mouse).

ELISA

For the detection of anti-dsDNA antibodies, maxisorb microtiter plates (Nunc, Roskilde, Denmark) were coated with poly-L-lysine (5 μg/ml) (Sigma), followed by coating
with λ phage dsDNA (5 μg/ml) (Boehringer Mannheim, Mannheim, Germany). After incubation with various sera dilutions, horseradish peroxidase (HRP) goat anti-mouse-IgG (γ-chain-specific, Jackson ImmunoResearch, West Grove, PA) was added to the plates and was followed by the addition of the substrate, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma, Rehovot, Israel).

Cytokine secretion was detected in the supernatants of splenocytes (5×10⁶/ml) that were incubated for 48 h in enriched medium. IFN-γ and IL-10 were determined using OptEIA sets (PharMingen, San Diego, CA) according to the manufacturer’s instructions.

Serum BAFF levels were determined using an ELISA detection kit for soluble mouse BAFF (ApoTech, San-Diego CA) according to the manufacturer’s instructions.

Antibodies and reagents

The following reagents were used in the study. FITC anti-mouse CD21/CD35 (clone eBio8D9), biotin anti-mouse CD11a (IntegrinαxL, LFA-1a) (clone M17/4), allophyocyanin anti-mouse/human CD45R (B220) (clone RA3-68B2), FITC anti-mouse IgD (clone 11-26c), PE anti-mouse CD23 (FcγRII, clone B384), biotin anti-mouse CD24 (clone M1/69) (eBioscience, San Diego, CA). Rat anti-IgM PE (clone 1B4B1) (SBA, Birmingham, AL), Streptavidin-Cy-Chrome (BD Biosciences, San Diego, CA). Biotin anti-mouse CD49d (clone 9C10), allophyocyanin anti-mouse/rat CD29 (clone HM β 1-1) (Biolegend, San Diego, CA, USA). BAFF soluble mouse recombinant (ApoTech, San Diego CA). PE anti-mouse Bcl-xL (clone H-5) and its isotype control (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-mouse BAFF antibody (clone 121808) (R and D systems, Minneapolis, MN), antibodies to p65 and p52, β-actin (clone C-2) and Lamin B1 (clone-20) (Santa Cruz).

Flow cytometry analysis

Splenocytes (1×10⁶ cells) were incubated with the relevant antibodies according to the manufacturers’ instructions, and analyzed by FACS (Becton Dickinson, Franklin Lakes, NY). For intracellular staining, the cells were incubated with a fixation solution, then washed, and resuspended in permeabilization solution (Serotec).

Annexin V and propidium iodide (PI) staining

Cells were analyzed using the Phosphatidyl Serine Detection Kit (IQ Products), according to the protocol supplied by the manufacturer. Cells were analyzed by FACS.

Separation of B cells

B cells were isolated from spleens of the experimental mice using CD19 MicroBeads, MidiMACS separator, and MS column (Milteny Biotec, Bergisch Gladbach, Germany). Briefly, cells were magnetically labeled with anti-CD19 MicroBeads, and the cell suspension was then loaded onto MS columns that were placed in the magnetic field of a MACS separator. The labeled CD19⁺ cells were retained in the column, and were eluted after removing the column from the magnetic field.

**Cell lysates and Western blot analysis**

Nuclear or whole cell lysates of spleen and separated B cells (50×10⁶/ml) were prepared as described [33]. Lysates were applied to SDS-PAGE by using 10% polyacrylamide and transferred to a nitrocellulose membrane. The membranes were reacted with the relevant antibodies followed by incubation with the second antibody coupled to HRP. Detection was conducted by the ECL method. Protein expression was determined by photodensitometry using the NIH Image program. Densitometric units were calculated and presented as the percentage of the respective totals.

**Detection of proteinuria**

Proteinuria was measured by a standard semiquantitative test, using an Albustix kit (Bayer Diagnostic, Newbury, UK). Results were graded according to the manufacturer’s instructions as negative, + (0.3 g/l), ++ (1 g/l), +++ (3 g/l), and ++++ (≥ 20 g/l).

**Real-time RT-PCR**

Total RNA was isolated from splenocytes and then RNA was reverse-transcribed to prepare cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega). The resulting cDNA was subjected to real-time RT-PCR according to the manufacturer’s instructions (Roche, Mannheim, Germany). Briefly, a 20 μl reaction volume contained 3 mM MgCl₂, LightCycler HotStart DNA SYBR Green I mix (Roche), specific primer sequences (forward and reverse, respectively) were as follows: β-actin, 5′-GGACCGTGAGACATCCG-3′, and 5′-CAGTAAAGTCGCCGCT-3′; BAFF, 5′-AGCGAGTGTCTCCGTACC-3′, and 5′-TTTTGTATAGTCGGCGTG-3′; Pim-2, 5′-GGACCGTGCTTAGG-3′, and 5′-CCGCATAGGTGCAG-3′; Bcl-xL, 5′-GGACCCGCTATCCAGAG-3′, and 5′-GCAATTGCCGCTAGAG-3′. β-actin levels were used to normalize the expression levels of the other genes.

**Statistical analysis**

The Mann-Whitney and unpaired Student’s t-tests were used for evaluating the significant differences between groups. Values of P≤0.05 were considered significant.

**Results**

**Treatment with hCDR1 results in the down-regulation of serum BAFF secretion levels**

Since the expression of BAFF was reported to be elevated in patients with SLE and in lupus mouse models [21–23], it was of interest to determine the effect of treatment with hCDR1 on BAFF secretion. To this end, (NZB×NZWF)F₁ mice, at the age of 6.5 months, were treated with weekly injections (50 μg/mouse) of hCDR1, the control scrambled peptide or the vehicle, and their sera were tested for BAFF levels at the end of the experiment (after 13 injections). Young (2 months
old) (NZB×NZW)F₁, and old Balb/c (8 months old) mice served as controls. In addition, we determined the lupus-associated manifestations (e.g., anti-dsDNA antibody titers in the sera, and proteinuria) of the treated mice. As can be seen in Fig. 1A (a representative experiment) and 1B (mean of four experiments), the secreted levels of BAFF were significantly higher in SLE-afflicted (NZB×NZW)F₁ mice, as compared with disease-free young (NZB×NZW)F₁ mice, or with age-matched Balb/c control mice. Treatment with hCDR1 significantly reduced the serum levels of BAFF (325 ng/ml and 299 ng/ml in the vehicle- and scrambled peptide-treated mice, respectively versus 187 ng/ml in the hCDR1 treated mice, Fig. 1A). Figs. 1C and D present the mean secretion of anti-dsDNA autoantibodies, and mean levels of proteinuria, respectively, in four experiments. As seen, the down-regulated BAFF secretion, following treatment with hCDR1, was associated with a significant reduction of autoantibodies and proteinuria levels.

**Treatment with hCDR1 down-regulates the expression of BAFF and the secretion of IFN-γ and IL-10 in spleens of (NZB×NZW)F₁ mice**

BAFF affects mainly the peripheral B-cell population [25,26]. Therefore, we tested BAFF expression in the spleens of SLE-afflicted mice, and determined the effect of treatment with hCDR1 on its expression. To this end, spleen cells of SLE-afflicted (NZB×NZW)F₁ mice that were treated with weekly injections (50 μg/mouse) of hCDR1, the scrambled peptide, or the vehicle were taken at the end of the experiment (after 13 injections). The mRNA prepared from the spleen cells was analyzed for the gene expression of BAFF by real-time RT-PCR (Fig. 2A). Fig. 2A demonstrates the percentage of BAFF gene expression relative to the vehicle-treated group (considered as 100%). Three independent experiments were done. (B) Western blots of BAFF expression (representative of three experiments performed). (C) Mean secretion of IFN-γ in supernatants of splenocytes of three experiments measured by ELISA. (D) Mean secretion of IL-10 in spleen cell supernatants of three experiments measured by ELISA. P values of hCDR1 group as compared with the vehicle-treated group.

![Figure 1](image1.png)

**Figure 1** Treatment with hCDR1 down-regulates BAFF secretion. (NZB×NZW)F₁ mice were treated with weekly injections of hCDR1, the control scrambled peptide or the vehicle. Young (2 months old) (NZB×NZW)F₁, and old (8 months old) Balb/c mice served as controls. (A) BAFF levels measured in sera (dilution 1:50) of individual mice of a representative experiment out of four performed. (B) Mean levels of BAFF relative to levels in sera of vehicle-treated mice (considered as 100%), of four experiments. (C) Mean secretion of anti-dsDNA autoantibodies of four experiments performed. (D) Mean production of proteinuria of four experiments performed. P values for hCDR1-treated mice compared with the vehicle-treated group.

![Figure 2](image2.png)

**Figure 2** The effect of treatment with hCDR1, on the expression of BAFF and on IFN-γ and IL-10 secretion. At the end of treatment experiments, spleen cells of the mice were either tested for BAFF gene and protein expression, or incubated for 48 h in an enriched medium to analyze their supernatants for IFN-γ and IL-10. (A) Real-time RT-PCR for BAFF gene expression presented as mean percent relative to the vehicle-treated group (considered as 100%). Three independent experiments were done. (B) Western blots of BAFF expression (representative of three experiments performed). (C) Mean secretion of IFN-γ in supernatants of splenocytes of three experiments measured by ELISA. (D) Mean secretion of IL-10 in spleen cell supernatants of three experiments measured by ELISA. P values of hCDR1 group as compared with the vehicle-treated group.
controls. As shown in Figs. 2C and D, hCDR1 significantly inhibited the secretion of IFN-γ (vehicle—971 pg/ml, scrambled peptide—800 pg/ml, hCDR1—467 pg/ml) and IL-10 (vehicle—1875 pg/ml, scrambled peptide—2123 pg/ml, hCDR1—1108 pg/ml). Thus, the down-regulated production of BAFF following treatment with hCDR1 might be, at least partially, due to the diminished secretion of IFN-γ and IL-10.

**Treatment with hCDR1 down-regulates the maturation and differentiation of B cells of (NZB×NZW)F₁ mice**

Since BAFF plays a major role in the maturation of B cells [25,26], we looked for changes in the population of mature B cells in the spleens of untreated and hCDR1-treated SLE-afflicted mice. To this end, spleens of (NZB×NZW)F₁ mice were taken at the end of a treatment experiment (after 13 injections), and were stained for IgM, IgD, B220, CD21, CD23, and CD24. Fig. 3 presents the results of the staining of a representative experiment out of four performed. As can be seen, treatment with hCDR1 resulted in a significant reduction in the number of spleen cells (Fig. 3A1) and in the B220 (Fig. 3A2) cell counts. Fig. 3A3 demonstrates the percentage of mature (IgM⁺ IgD⁺) B-cells, out of the total B-cell population, and Fig. 3A5 presents representative dot plots of the latter. The absolute numbers of mature B cells are presented in Fig. 3A4. It can be seen that the percentage of IgM⁺ IgD⁺ cells was significantly reduced in hCDR1-treated mice, as is also documented in the numbers of IgM⁺ IgD⁺ cells. It is also evident from the dot plots shown in Fig. 3A5 that the diminished number of mature B cells in the hCDR1-treated mice was similar to that observed in old, free-of-disease Balb/c mice. We further tested the effect of treatment with hCDR1, on the B-cell intermediate differentiation stages, defined as T1 (B220⁺CD21⁻CD23⁻CD24⁺) and T2 (B220⁺CD21⁻CD23⁺CD24⁺). Fig. 3B1–B5 present the percentages, as well as the absolute numbers, of B cells at the T1 and T2 stages in the different treated groups. The percentages of T1 and T2 cells out of a total of B220 cells diminished significantly (6.9% T1 and 4.2% T2 in the vehicle-treated groups versus 3.8% T1 and 2.7% T2 in hCDR1-treated mice), following treatment with hCDR1 (Figs. 3B1 and B3). Similar results are shown for the total numbers of T1 and T2 cells, whereas treatment with the scrambled peptide did not affect the latter (Figs. 3B2 and B4). Fig. 3B5 shows representative dot plots of the latter.

**Reduction of MZ B cells in hCDR1-treated (NZB×NZW) F₁ mice**

Since there is an enlarged compartment of MZ B cells in autoimmune diseases [18,25], and since BAFF can up-regulate the accumulation of self-reactive B cells (CD21⁺CD23⁻) in the MZ area, it was of interest to determine whether the lower levels of BAFF in the hCDR1-treated mice affect the size of the CD21⁺CD23⁻ B-cell population. To this end, spleen cells of (NZB×NZW)F₁ mice treated as above were stained with anti-CD23 and CD21 antibodies. Fig. 4 presents representative dot plots of CD21⁺CD23⁻ B-cell
staining (Fig. 4A), and two graphs that illustrate the percentage and the absolute numbers of these MZ B cells, out of the total B-cell population (Figs. 4B and C), for five experiments performed. The dot plots clearly demonstrate the suppressive effect of hCDR1 on the size of the CD21⁺CD23⁻ B-cell population (vehicle—9.64%, hCDR1—4.25%, scrambled—8.1%, old Balb/c—2%). The mean percentage and the total number of MZ B cells shown in Fig. 4B and C confirm the significant effect of hCDR1. Treatment with the scrambled peptide had a negligible effect on this population (Figs. 4B and C). Thus, hCDR1 down-regulates the MZ B-cell population, which is elevated in lupus.

Reduced expression of integrins in MZ B cells of hCDR1-treated mice

Retention of B cells, within the MZ is reported to depend on the expression of α4, β1, and LFA-1 integrins, which can be up-regulated by BAFF [27,28]. Since treatment with hCDR1 inhibited BAFF, it was of interest to test the effect of hCDR1 on the expression of the above integrins. Thus, at the end of treatment experiments of (NZB × NZW)F1 mice, spleens were taken for staining of LFA-1, α4, β1, CD21, and CD23 markers. Figure 5 demonstrates the expression of LFA-1 (A), α4 (B), and β1 (C) on CD21⁺CD23⁻ gated cells. As can be seen in the figure, (representative of three experiments performed), the expression of all the tested integrins was reduced following treatment with hCDR1. Thus, inhibition of integrins could contribute to the lower MZ B-cell population in the hCDR1-treated mice.

Reduced survival of MZ B cells of hCDR1-treated mice

BAFF-induced survival molecules (e.g., Pim-2 and Bcl-xL) were reported to be involved in the accumulation of B cells in the MZ compartment [28]. We tested the expression of the anti-apoptotic molecules Pim-2 and Bcl-xL, and the effect of treatment with hCDR1 on their expression. At the end of treatment experiments, B cells isolated from spleens were analyzed for the expression of Pim-2 and Bcl-xL genes, by real time RT-PCR. Fig. 6 presents the mean levels of Pim-2 (A) and Bcl-xL (B) gene expression relative to the expression in the vehicle-treated group (defined as 100%). As can be seen, the mean (results of three experiments) expression of Pim-2, and especially of Bcl-xL genes, was significantly reduced in spleen-derived B cells of hCDR1-treated mice, as compared with B cells of the vehicle or the scrambled peptide-treated mice. The down-regulative effect of hCDR1 on Bcl-xL expression in B cells was confirmed at the protein level.
using flow cytometry (Fig. 6C). To test the role of BAFF in the mode of action of hCDR1, we incubated B cells of the hCDR1-treated mice with and without BAFF (1 μg/ml) for 24 h, and determined the Pim-2 and Bcl-xL gene expression thereafter. Mean (of two experiments) gene expression of Pim-2 (A) and Bcl-xL (B) in isolated B cells determined by real-time RT-PCR, expressed as the percentage relative to vehicle (considered as 100%). (Aa and Bb) present the mean gene expression of Pim-2 and Bcl-xL in hCDR1-treated mice (hCDR1 treated B cells without BAFF were considered as 100%), following addition of BAFF. Mean (of three experiments) of Bcl-xL protein expression (C) and the rate of apoptosis (D) in B cells as determined by flow cytometry.

**Inhibition of NF-kB signaling in B cells of hCDR1-treated mice**

Because BAFF was reported to induce the expansion of MZ B cells, and the up-regulation of integrins by the activation of the alternative NF-kB signaling pathway [27], we analyzed the effect of hCDR1 on BAFF-induced as well as the constitutive alternative NF-kB pathway. At the end of treatment experiments, B cells were isolated from spleens and nuclear extracts were prepared and probed for p65 levels, to analyze the classical NF-kB pathway. As can be seen in Fig. 7A, the expression of constitutively generated p65 was down-regulated in nuclear extracts of B cells of hCDR1-treated mice, as compared with the levels measured in lysates from vehicle or scrambled peptide-treated mice. Fig. 7B shows that p52 was markedly down-regulated in lysates of B cells of hCDR1-treated mice (88, 73, and 31, densitometric units for vehicle, scrambled peptide and hCDR1-treated mice, respectively). Furthermore, B cells isolated from spleens of SLE-affected (NZB×NZW)F1 (8 months old) mice were incubated for 24 h in either medium or in the presence of hCDR1 (25 μg/ml) or the scrambled peptide (25 μg/ml), with and without BAFF (1 μg/ml) and the expression of p52 was determined in lysates of these cells. Fig. 7C presents the Western blots and the measured densitometric units (representative of two experiments). As can be seen, the expression of p52 was down-regulated in B cells that were incubated with hCDR1, as compared with the levels determined in cells cultured in the presence of medium or the control scrambled peptide. However, the addition of BAFF to B cells co-cultured with hCDR1 increased the...
expression of p52 by a rate (38%) similar to that observed for B cells co-cultured with medium (36%). Thus, inhibition of NF-κB signaling by hCDR1 can be partially reversed by the addition of BAFF. It is noteworthy that the addition of BAFF (1 μg/ml, 24 h) to the cell cultures resulted in an increased survival rate of B cells since, as shown in Fig 7D, lower rates of apoptosis were determined in those cells cultured in the presence of BAFF, as compared with cultures to which BAFF was not added.

Discussion

The main findings of this study are that hCDR1 decreased the expression of BAFF in association with the diminished production of dsDNA-specific autoantibodies, proteinuria, and of the pathogenic cytokines IFN-γ and IL-10. Inhibition of BAFF also resulted in decreased numbers of T1 and T2 B cells and of MZ B cells. The latter expressed lower levels of integrins (LFA-1, α4, β1) and anti-apoptotic (Pim-2, Bcl-xL) genes than B cells of the vehicle or the scrambled peptide-treated mice. Furthermore, addition of BAFF reversed the inhibition of the anti-apoptotic genes. Moreover, BAFF appeared to be involved in the inhibition of the NF-κB classical and alternative pathways by hCDR1. It is thus suggested that the suppression of BAFF, which is a crucial regulator of B-cell development, maturation, and survival, plays a role in the ameliorative effects of hCDR1 in SLE-affected (NZB × NZW)F1 mice.

BAFF overexpression was reported to be associated with autoimmune and lymphoproliferative disorders such as SLE [21,22], rheumatoid arthritis [22], Sjogren's syndrome [35], and multiple myeloma [36]. In SLE patients, as in lupus-prone mouse models, serum levels and gene expression of BAFF were reported to be significantly higher than in healthy controls [21–23]. In normal mice, the exogenous addition of BAFF enhanced antibody responses to antigen challenge, whereas treatments that blocked BAFF inhibited those responses [26,37,38]. In agreement, hCDR1, which ameliorated the serological and the clinical manifestations of SLE in the spontaneous and induced mouse models [9], significantly reduced the serum levels and the gene expression of BAFF (Figs. 1 and 2). The effect of hCDR1 was specific since the scrambled control peptide did not significantly affect the expression and secretion of BAFF. The reduction in BAFF levels was associated with diminished dsDNA specific autoantibody titers and proteinuria levels. In agreement, blocking of BAFF in animal models of autoimmune diseases (arthritis, spontaneous B lymphocyte autoimmunity, and SLE) reduced autoantibody production and disease manifestations [24,39,40].

Both IFN-γ and IL-10 that are pathogenic cytokines in SLE, were reported to stimulate BAFF expression by macrophages, monocytes, and dendritic cells [25,34]. Indeed, we detected a significant and specific reduction of IFN-γ and IL-10 secretion in hCDR1-treated mice, in association with the reduced BAFF expression. Thus, it is possible that the down-regulated levels of IFN-γ and IL-10 played a role in diminishing the levels of BAFF.

Reports on the effects of BAFF in Tg mice indicate that BAFF induces T1, T2, and MZ B-cells in the periphery, and in BAFF-deficient mice the maturation of B cells is inhibited [41–43]. Moreover, BAFF was reported to regulate B-cell survival [27]. In our study we observed a significant and specific reduction in the mature (lgM+lgD+), the transitional (T1 and T2), and the MZ B-cell populations in spleens of hCDR1-treated mice (Figs. 3 and 4). It is likely that the reduced BAFF levels following treatment with hCDR1 led to the decrease in T2 and MZ B-cell populations. The reduction in T1 B cells, that were shown not to be affected by BAFF [26], is probably due to additional effects of hCDR1 that are distinct from BAFF blockade. For example, the administration of CTLA4-Ig to SLE-affected mice was reported to result in down-regulating of T1 B cells in their spleens [24]. Indeed, treatment with hCDR1 was shown to up-regulate the expression of CTLA-4 in the (NZB × NZW)F1-diseased mice [10,44].

An enlarged MZ B-cell compartment was reported to be associated with autoimmune diseases in murine models [27,45]. The accumulation of self-reactive B-cells (CD21+CD23+) in the MZ compartment was shown to be regulated by BAFF-induced increased expression of LFA-1 and α4β1 integrins [46,47]. In the present study, we demonstrated that treatment with hCDR1 reduced the numbers of MZ B cells, and reduced the expression of LFA-1 and α4β1 integrins (Figs. 4 and 5).

Reduced survival signals may also lead to diminished numbers of MZ B cells in the MZ compartment. BAFF was reported to up-regulate the expression of Bcl-xL through the activation of NF-κB alternative and classical pathways, and to induce the expression of Pim-2 mainly through the NF-κB alternative pathway [27]. In our study, we demonstrated that the mRNA levels of Bcl-xL and Pim-2 in B cells of hCDR1-treated mice were significantly and specifically reduced, whereas the rate of B-cell apoptosis was elevated (Fig. 6). The addition of BAFF reversed these effects (Figs. 6 and 7). Therefore, we suggest that the inhibition of BAFF by hCDR1 led to the reduction of both integrins and B-cell survival, resulting in reduced numbers of B cells in the MZ.

The mechanisms by which BAFF regulates B-cell maturation and survival are not completely clear; nevertheless, the NF-κB classical and alternative pathways mediate most of its functions [27,29]. The effects of BAFF on transitional B cells were found to depend mainly on the NF-κB alternative pathway, whereas the classical pathway was shown to be essential for Ig class switching [27]. Moreover, the disruption of both the alternative and the classical NF-κB pathways resulted in a loss of most of the CD21+CD23– B-cells [27]. In the present study, we showed that treatment with hCDR1 resulted in the down-regulation of both the alternative and the classical pathways. However, the addition of BAFF to B cells co-cultured with hCDR1 resulted in a moderate (38%) elevation of p52 levels, suggesting that the inhibition of p52 is mediated at least partially by the suppression of BAFF production. Note that the NF-κB classical signaling pathway was shown to be down-regulated in T cells, as well, following treatment with hCDR1 [11].

In summary, we suggest that the mechanisms of action by which hCDR1 suppresses autoreactive B cells involve BAFF in a series of steps. Thus, hCDR1, which binds MHC class II and inhibits TCR signaling [11], down-regulates the expression and secretion of IFN-γ and IL-10 (known to activate BAFF expression), leading to reduced levels of serum and peripheral BAFF expression. Reduction of BAFF leads to the
down-regulation of the T1, T2, and MZ B-cell populations, which play a role in disease progression. The reduction of BAFF production results in diminishing levels of integrins and survival signals following treatment with hCDR1, which leads to reduced numbers of self-reactive B cells in the MZ compartment. Finally, inhibition of both NF-kB signaling pathways by hCDR1 might affect all the above events. Thus, in addition to the prominent inhibitory effects on T cells [10–14,44], hCDR1 immunomodulates B-cell populations through its effects on BAFF.

References


