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The Role of Apoptosis in the Ameliorating Effects of a CDR1-Based Peptide on Lupus Manifestations in a Mouse Model

Amir Sharabi, Dror Luger, Hava Ben-David, Molly Dayan, Heidey Zinger, and Edna Mozes

Experimental systemic lupus erythematosus (SLE) can be induced in mice following immunization with an anti-DNA mAb expressing a major Id, 16/6Id. Treatment with a peptide, designated human CDR1 (hCDR1; Edratide), that is based on the sequence of CDR1 of the 16/6Id ameliorated disease manifestations. In the present study, we investigated the roles of apoptosis and related molecules in BALB/c mice with induced experimental SLE following treatment with hCDR1. A higher state of activation and increased rate of apoptosis were found in lymphocytes of SLE-afflicted mice as compared with healthy controls. The latter effects were associated with up-regulated caspase-8 and caspase-3, and down-regulated Bcl-xL. The ameliorative effects of hCDR1 were associated with down-regulation of caspase-8 and caspase-3, up-regulation of Bcl-xL, and a reduced rate of apoptosis. Treatment of diseased mice with an apoptosis-reducing compound that inhibited caspasas down-regulated the secretion of the pathogenic cytokine IFN-γ and lowered the intensity of glomerular immune complex deposits and the levels of proteinuria. Furthermore, coincubation of Bcl-xL inhibitors with hCDR1-treated cells abrogated the ability of hCDR1 to reduce the activation state of lymphocytes and to down-regulate the secretion of IL-10 and IFN-γ. Moreover, the Bcl-xL-expressing CD4+CD25+ cells from hCDR1-treated mice induced the expression of Bcl-xL in CFSE-labeled CD4+CD25− cells of the SLE-afflicted mice. Thus, the reduction of apoptosis and the up-regulation of Bcl-xL, which plays an apparent role in tolerance induction, contribute to at least part of the beneficial effects of hCDR1 on lupus manifestations. The Journal of Immunology, 2007, 179: 4979–4987.
Materials and Methods

Mice

BALB/c female mice were purchased from Harlan, and BWF1 female mice were purchased from The Jackson Laboratory. All experiments were approved by the Animal Care and Use Committee of the Weizmann Institute of Science.

Synthetic peptide

A peptide, GYYWSWIRQPPGKGEEWIG, designated hCDR1, based on CDR1 of the human anti-DNA mAb that bears a major Id, 16/6Id (15), was synthesized (solid-phase synthesis by F-moc chemistry) by Polypeptide Laboratories and used in this study. hCDR1 (Edratide) is currently under clinical development for the treatment of human SLE by Teva Pharmaceutical Industries.

Monoclonal Abs

The human anti-DNA 16/6Id (IgG1/κ) was secreted by hybridoma cells (15) that were grown in culture and purified by a protein G-Sepharose column (Pharmacia Fine Chemicals).

Induction of experimental SLE

Two-month-old naive BALB/c mice were immunized with the human mAb 16/6Id in CFA and boosted 3 wk later with the 16/6Id in PBS (5).

Treatment of mice with hCDR1

SLE-affected, 16/6Id-immunized BALB/c mice (3 mo after boost, when clinical manifestations are observed) and SLE-affected BWF1 female mice (at the age of 7–8 mo) were treated with 10 weekly s.c. injections of either hCDR1 (50 μg/mouse) or the vehicle alone. The vehicles that were used in BALB/c mice and in BWF1 mice, respectively, were PBS and Captisol (sulfobutylether β cyclodextrin that has been designed by CyDex to enhance the solubility and stability of drugs).

Treatment with carbobenzoxy-valyl-alanyl-aspartyl-(β-o-methyl) fluoromethylektoine (ZVD-fmk)

ZVD-fmk (Enzyme Systems Products) was dissolved in DMSO and diluted in PBS. The treatment group received daily s.c. injections of ZVD-fmk at 5 μg/g weight in 0.2 ml for 19 days (corresponding to the last 3 of 10 weekly injections with hCDR1). Control animals were injected with the corresponding volume of the PBS-DMSO diluent.

Depletion and enrichment of CD4+CD25+ cells

Depletion and enrichment of CD25+ cells were performed using the StemSep system (StemCell Technologies). Briefly, the cells were incubated with anti-CD25-biotinylated mAb (clone 7D4; Southern Biotechnology Associates). The cells were further incubated with anti-biotin tetrameric complex (StemCell Technologies), followed by incubation with magnetic beads (StemCell Technologies). The eluted cells were collected, and depletion rate of CD25+ cells was above 90%. The positively selected cells (~80% CD4+CD25+ cells) were collected thereafter.

CFSE labeling of CD25+ effector cells

CellTrace CFSE Cell Proliferation Kit ( Molecular Probes) was used for CFSE labeling of CD25+ cells, according to the manufacturer’s protocol.

Measurement of dsDNA-specific Ab

Anti-dsDNA Abs were detected using λ phage dsDNA, as previously described (13).

Proteinuria

Proteinuria was measured by a standard semiquantitative test, using an Albustix kit (Bayer Diagnostic).

Immunohistology

For the detection of immune complex deposits (ICD), frozen cryostat kidney sections (6 μm) were incubated with FITC-conjugated goat anti-mouse IgG (γ-chain specific) (Jackson ImmunoResearch Laboratories). Staining was visualized using a fluorescence microscope. The intensity of ICD was graded as follows: 0, no ICD; 1, low intensity; 2, moderate intensity; and 3, high intensity of immune complexes. ICD analysis was performed by 2 persons blinded to whether mice belong to control or experimental groups.

Cytokine detection by ELISA

Splenocytes (5 × 10^6 cells/well) were incubated in enriched medium for 48 h. IFN-γ and IL-10 were determined in the supernatants by ELISA using OptEIA sets (BD Pharmingen), according to the manufacturer’s instructions.

Ab and reagents

The following Abs were used in the study; anti-CD4 PE (clone GK1.5), anti-CD4 allopheycocyanin (clone L3T4), anti-CD25 FITC (clone 7D4), anti-CD19 FITC (clone 6D5), and their matched isotype controls were obtained from Southern Biotechnology Associates. Anti-CD45RB PE (clone 16A), anti-CD69 PE (clone H1.2F3), anti-active caspase-3 FITC, and their matched isotype controls were purchased from BD Pharmingen. Anti-Bcl-xL PE (clone H-5) and its isotype control were purchased from Santa Cruz Biotechnology. Anti-Foxp3 FITC (clone FK1-16s) and its isotype control were purchased from eBioscience. CaspGlow Fluorescein active caspase-8 staining kit was purchased from BioVision Research Products. Fixation and permeabilization solutions for intracellular staining were obtained from Serotec.

Flow cytometry

Briefly, cells (1 × 10^6 cells) were incubated with the relevant Ab and analyzed by FACS. For intracellular staining, the cells were incubated with a fixation solution, washed, and resuspended in permeabilization solution (Serotec).

Annexin V/propidium iodide (PI) staining

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

TUNEL assay

Aptoptosis, as demonstrated by fragmented DNA, was determined by using the In Situ Death Detection Kit (Roche) based on TUNEL technology, according to the protocol supplied by the manufacturer, as previously described (13).

Preparation of cell lysates

LN cells (50 × 10^6/ml) were incubated for 10 min on ice in the presence of cold lysis buffer containing the following: 50 mM HEPES, 150 mM NaCl, 1.5 mM MgCl2, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM Na-orthovanadate, 30 mM Na-pyrophosphate, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin (pH 7.2).

Western blot analysis

Lysates were boiled in the presence of sample buffer. Equal amounts of proteins were separated on SDS-PAGE by using 10% polyacrylamide and transferred to nitrocellulose membrane. After blocking, the membrane was reacted with Bcl-xL mAb (clone H-5; Santa Cruz Biotechnology), Bcl-2 mAb (clone C-2; Santa Cruz Biotechnology), and tubulin mAb (clone B-5-1-2; Sigma-Aldrich). The membrane was further incubated with the second Ab coupled to HRP. Detection was conducted by ECL method. Protein expression was determined by densitometry using the NIH Image program.

In vitro assays

In the Bcl-xL inhibition experiments, lymphocytes (5 × 10^6/well) were incubated in enriched medium for 36 h. Cells derived from hCDR1-treated, 16/6Id-immunized mice were incubated in the presence of two doses (25 and 100 mM) of Bcl-2 inhibitor (Calbiochem), which inhibits Bcl-xL, or two doses (5 and 25 mM) of HA14-1 (Calbiochem), which inhibits Bcl-2 and Bcl-xL (16). In the regulatory-effector cell experiment, enriched (~80%) CD4+CD25+ cells obtained from mice treated with either hCDR1 or the vehicle were coincubated (in different ratios) for 36 h with CFSE-labeled CD25+ effector cells (5 × 10^6 cells/well) taken from mice afflicted with lupus.

Real-time RT-PCR

The mRNA levels were analyzed by real-time RT-PCR using LightCycler (Roche). Total RNA was isolated from lymphocytes, and then RNA was reverse transcribed to prepare cDNA using Moloney murine leukemia virus reverse transcriptase (Promega). The resulting cDNA was subjected to real-time RT-PCR, according to the manufacturer’s instructions. Primer sequences (forward and reversed, respectively) were used as follows:
capase-8 (5′-acatacccaactcaga-3′, 5′-gtggatatcacaacg-3′), capase-3 (5′-tctcgtctgta-3′, 5′-gctactggcctc-3′), Bcl-xL (5′-ggacgcgtcataca-3′, 5′-ctgtgcctggag-3′), Bcl-2 (5′-cattgtctgataggc-3′, 5′-atacgeacgttaa-3′), Bad (5′-gacacgtacacac-3′, 5′-gtaataagcgcg-3′), Bak (5′-tataacacgcgtacgc-3′, 5′-ggttacaacagttg-3′), Bax (5′-tctcgcgtgatggca-3′, 5′-gggctcctgtagg-3′), β-actin (5′-gaagttgacatccagaa-3′). β-actin levels were used for normalizing the expression levels of the other genes.

Statistical analysis

Mann-Whitney and unpaired Student’s t tests were used for evaluating the significant differences between the treated and untreated groups. Values of p = 0.05 were considered significant.

Results

Treatment with hCDR1 results in reduced apoptosis, down-regulated caspase-8 and caspase-3, and up-regulated Bcl-xL

It was of interest to determine the status of apoptosis in mice with induced experimental SLE, and to determine the effect of a specific treatment with hCDR1 on the latter. To this end, naive BALB/c mice were immunized with the human anti-DNA mAb, 16/6Id, followed by a boost injection 3 wk later. Three months after the boost, when lupus-associated manifestations (e.g., anti-dsDNA Ab in the sera, and proteinuria) were detected, the mice were divided into two groups and treated with 10 weekly s.c. injections of either PBS or hCDR1 (50 μg/mouse/wk), respectively. Naive BALB/c mice that were immunized and boosted with PBS were used as a control group and were treated with 10 weekly s.c. injections of PBS. Assessment of apoptosis rates of lymphocytes derived from inguinal LN of the immunized mice was based on annexin V/PI staining and on the TUNEL technique, and mean results of apoptotic rates determined before and after 5 and 10 treatment injections of three independent experiments are presented in Fig. 1. An increase in the apoptotic rate was determined in the cells of 16/6Id-immunized mice that were treated with the vehicle PBS in comparison with the cells of control, PBS-immunized mice (Fig. 1, A and B). However, treatment with hCDR1 resulted in an apoptotic rate similar to that observed for cells of control mice. The rates of apoptosis in hCDR1-treated, PBS-immunized mice were not different from those of PBS-treated, PBS-immunized mice.

We then measured the mRNA expression of apoptosis-related molecules in the lymphocytes of mice of the above groups. The mean results of three independent experiments are shown in Fig. 1C for three of the molecules, namely, the proapoptotic caspase-8 and caspase-3, and the antiapoptotic Bcl-xL. As shown, mRNA expression of caspase-8 and caspase-3 was significantly higher (p = 0.007) and the mRNA expression of Bcl-xL was lower (p = 0.02) than in PBS-immunized mice. In contrast, administration of hCDR1 to 16/6Id-immunized mice resulted in a significant down-regulation of caspase-8 and caspase-3 and in a substantial up-regulation of Bcl-xL expression after 10 injections (Fig. 1C). Note that, in contrast to the modulating effects of hCDR1 in the 16/6Id-immunized mice, hCDR1 treatment had no significant effect on the expression of the three apoptosis-related molecules in the PBS-immunized mice.

Intracellular staining for active caspase-8 and active caspase-3 was performed on the cells stained for expression of CD4 (T cells) and CD19 (B cells); the results are shown in Fig. 1D. We thus found that the active forms of caspase-8 and caspase-3 in CD4+ cells were enhanced in the 16/6Id-immunized, PBS-treated mice as compared with their expression in PBS-immunized, PBS-treated mice. In CD19+ cells, the active caspase-8 was increased in the 16/6Id-immunized, PBS-treated mice, although the active form of caspase-3 was barely detected in all treatment groups (Fig. 1D). Expression of Bcl-xL was much more prominent in CD4+ than in CD19+ cells (30 vs 3% cells, respectively). Treatment with hCDR1 significantly elevated the expression of Bcl-xL in CD4+ cells. The expression of Bcl-xL in CD19+ cells was reduced to levels comparable with those in healthy controls. Fig. 2 shows that mRNA expression (Fig. 2A) and protein levels (Fig. 2B) of Bcl-2 were hardly changed by the treatment with hCDR1. In addition, mRNA expression of several proapoptotic molecules (i.e., Bak, Bad, and Bax) was moderately down-regulated. Collectively, our data indicate that caspase-8, caspase-3, and Bcl-xL are mainly affected by the treatment with hCDR1.

Inhibitory effect of ZVAD-fmk and hCDR1 on the manifestations of experimental SLE

To test the relevance of caspase inhibition in inducing the ameliorative effects of hCDR1, we treated 16/6Id-immunized mice with a caspase inhibitor, ZVAD-fmk. Thus, 16/6Id-immunized mice (n = 5–7 mice/group) with established lupus were treated with 10 weekly s.c. injections of hCDR1 (50 μg/mouse), or with...
daily s.c. injections of ZVAD-fmk (5 µg/g body weight) during the last 19 days of the experiment. All mice in the experimental cohort were evaluated individually for all parameters. The dot plots presented in Fig. 3 demonstrate the apoptotic rate of a representative mouse, as determined by annexin V/PI staining (Fig. 3A1) and by the TUNEL technique (Fig. 3B1). The mean apoptotic rates of the individual mice (n = 5–7) per group for both methods are also presented in the figure. A significant decrease in the rate of lymphocyte apoptosis from ~40% (annexin V staining) and 10% (TUNEL technique) in 16/6Id-immunized, PBS-treated mice to 1% for both methods was found in cells of all mice that were immunized with 16/6Id and treated with ZVAD-fmk. Furthermore, hCDR1 treatment reduced the frequency of apoptotic cells to levels that were comparable to those found in healthy controls (PBS-immunized, PBS-treated mice). Thus, we confirmed the in vivo activity of ZVAD-fmk, and the ability of hCDR1 to reduce the rate of apoptosis to that measured in healthy controls.

The clinical parameters of each mouse were evaluated, and the values, determined at the end of the experiment, are presented in Fig. 4. As shown in Fig. 4A, the levels of anti-dsDNA Ab in the sera of 16/6Id-immunized (PBS-treated) mice were high as compared with PBS-immunized, PBS-treated mice. Administering hCDR1 to 16/6Id-immunized mice resulted in reduced autoantibodies against dsDNA (p = 0.004), unlike treatment with ZVAD-fmk, which had no significant effect on the autoantibody titers. In contrast, treatment with ZVAD-fmk had beneficial effects on the kidney disease (e.g., proteinuria and ICD) of the 16/6Id-immunized mice, as observed for hCDR1 (Fig. 4, B and C).

Effects of hCDR1 and ZVAD-fmk on the activation state of lymphocytes of mice with experimental SLE

Expression of the early activation marker, CD69, in T and B lymphocytes derived from the different treatment groups was determined for each mouse in the experiment. Representative dot plots of individual mice and column graphs representing the mean values of CD69 expression in the lymphocytes of each treatment group are shown in Fig. 5. It can be seen that expression of CD69 in T and B cells was enhanced in the SLE-afflicted mice (PBS treated), as compared with healthy controls (PBS immunized, PBS treated). However, whereas treatment of mice with induced experimental SLE (16/6Id-immunized mice) with hCDR1 resulted in a significant down-regulation of CD69 in T and B cells, treatment with ZVAD-fmk led to a marked up-regulated expression of CD69 in both types of lymphocytes.

We also determined the effects of treatment with ZVAD-fmk and hCDR1 in mice that were injected only with PBS. As shown in Fig. 5, the expression of CD69 in T and B lymphocytes from hCDR1-treated mice was comparable to that of the healthy controls, in contrast to the effect of ZVAD-fmk, which resulted in a significant up-regulation of CD69.

**FIGURE 2.** The effect of treatment with hCDR1 of SLE-afflicted mice on members of the Bcl-2 family. BALB/c mice with experimental SLE were divided into two groups (n = 5/group) and treated with 10 weekly s.c. injections of either PBS or hCDR1 (50 µg/mouse), respectively. A, Bcl-2 mRNA expression. Results were normalized to β-actin expression and are presented relative to the 16/6Id-immunized, PBS-treated mice (considered as 100%). B, Western blot analysis of Bcl-2 expression. C, The change in Bax, Bak, and Bad mRNA expression in response to hCDR1, relative to PBS-treated mice. Results were normalized to β-actin expression. Mean results (±SD) of two independent experiments are shown.

**FIGURE 3.** Effects of ZVAD-fmk and hCDR1 on apoptosis of lymphocytes from SLE-afflicted mice. Mice with experimental SLE (n = 5–7 mice/group) were treated with 10 weekly s.c. injections of PBS, or hCDR1 (50 µg/mouse), or with daily s.c. injections of ZVAD-fmk (5 µg/g of body weight) during the last 19 days of the experiment. PBS-immunized, PBS-treated mice were used as controls. All of the mice in the experimental cohort were evaluated individually at the end of the treatment course. Lymphocytes derived from LN of individual mice per treatment group were stained for annexin V and PI (A1) and according to the TUNEL technique (B1) and analyzed by FACS. Representative dot plots are shown. Mean values (±SD) of annexin V+/PI- cells (A2) and TUNEL+ cells (B2) are shown for all mice per treatment group. The results presented are after the reduction of the background staining obtained with the matched isotype controls. *, p < 0.05.

**FIGURE 4.** Clinical effects of ZVAD-fmk and hCDR1 on mice on mice with experimental SLE. The mice from each treatment group (n = 5–7 mice/group) were evaluated individually at the end of the treatment course. A, Levels (mean OD ± SE) of dsDNA-specific Ab in individual sera (dilution 1/250) of mice were measured by ELISA. B, Proteinuria levels (mean g/L ± SE) in individual mice were measured by a standard semiquantitative test by using an Albustix kit. C, Mean intensity (±SE) of ICD of kidney sections of all mice per treatment group. *p < 0.005, †p < 0.05 as compared with SLE-afflicted mice that were treated with PBS.
Effects of hCDR1 and ZVAD-fmk on the cytokine profile of mice with experimental SLE

It was of interest to compare the effect of treatment with hCDR1 and ZVAD-fmk on the cytokine profile of the treated mice. As shown in Fig. 6, levels of IFN-γ/H9253 and IL-10 were up-regulated significantly in supernatants of the SLE-afflicted mice (PBS treated), as compared with healthy controls (PBS-immunized, PBS-treated mice). Administering ZVAD-fmk to the diseased mice resulted in the abrogation of IFN-γ from the supernatants and in a mild up-regulation of IL-10 secretion. This effect of ZVAD-fmk was also confirmed in PBS-immunized mice. Treatment of the SLE-afflicted mice with hCDR1 significantly down-regulated the secretion of both IFN-γ/H9253 and IL-10.

Reduced activation state of lymphocytes from SLE-afflicted mice by treatment with hCDR1 is Bcl-xL dependent

The association between the activation state of lymphocytes and the up-regulated expression of Bcl-xL following hCDR1 treatment was studied by using Bcl-2 inhibitor (the inhibitor of Bcl-xL) and HA14-1 (the inhibitor of Bcl-2 and Bcl-xL). To this end, $5 \times 10^6$ lymphocytes originating from the hCDR1-treated mice and the PBS-treated mice were incubated in enriched medium for 36 h in the presence of either Bcl-2 inhibitor or HA14-1. The protein levels of Bcl-xL, determined by Western blotting, are shown in Fig. 7A. It can be seen that the...
The role of Bcl-xL in the immunomodulation of the cytokine profile in SLE-affected mice following treatment with hCDR1. Mice (n = 5–7 mice/group) with experimental SLE were treated with 10 weekly s.c. injections of PBS or hCDR1 (50 µg/mouse). PBS-immunized, PBS-treated mice were used as controls. At the end of the treatment course, splenocytes were harvested and pooled from each group, and incubated (5 × 10^7/well) in enriched medium in the presence of the 16/6Id for 36 h. In addition, splenocytes from hCDR1-treated mice were also incubated in the presence of two doses of two inhibitors of Bcl-xL (25 and 100 mM for Bcl-2 inhibitor; 5 and 25 mM for HA14-1). Cytokine levels were measured in the supernatants by ELISA; the values represent the mean (pg/ml ± SD) of cytokine production by duplicate cultures. The results were reproducible in three experiments. *, p < 0.005 as compared with levels in supernatants of splenocytes from SLE-affected mice.

IL-10. Thus, we concluded that Bcl-xL plays a significant role in the ability of hCDR1 to down-regulate IFN-γ and IL-10.

Treatment with hCDR1 up-regulates Bcl-xL expression in CD4^+CD25^+ regulatory cells

Because Bcl-xL plays a role in the suppressive effects of hCDR1 on the lymphocytes’ state of activation and on the secretion of the pathogenic cytokines, we further attempted to determine Bcl-xL expression in CD4^+CD25^+ regulatory cells. As shown in Fig. 9A, the expression of Bcl-xL in mice treated with hCDR1 was increased by ~70% in the hCDR1-induced CD4^+CD25^+ Foxp3-expressing cells as compared with CD4^+CD25^+ cells of PBS-treated mice. These results were reproducible in three independent experiments (Fig. 9B). Next, lymphocytes from either PBS- or hCDR1-treated, 16/6Id-immunized mice were separated into two cell populations that were either depleted of or enriched with CD4^+CD25^+ cells, and the protein levels of Bcl-xL were quantified by Western blotting. The results in Fig. 9C reveal that the protein levels of Bcl-xL were higher in the Foxp3-expressing CD4^+CD25^+ cells of hCDR1-treated mice relative to PBS-treated mice. Also, the levels of Bcl-xL were up-regulated in CD4^+CD25^+ cells in response to treatment with hCDR1 (Fig. 9C). Our data thus indicate that treatment with hCDR1 up-regulates the expression of Bcl-xL in both the CD25^+ and CD25^- subsets of CD4^+ cell populations.
hCDR1-induced CD4+CD25+ cells lead to the expression of Bcl-xL in CD4+CD25+ cells of SLE-afflicted mice

It was of interest to determine whether CD4+CD25+ regulatory cells affect the expression of Bcl-xL on effector CD4+CD25− cells of SLE-afflicted mice. To this end, mice in which experimental SLE was induced by the 16/6ld (n = 5/group) were treated with 10 weekly s.c. injections of either PBS or hCDR1. Pooled lymphocytes from each group were either depleted of or enriched with CD4+CD25+ cells (see Materials and Methods). Following CD25 cell depletion, the cells (designated as effector cells) from the vehicle-treated mice were labeled with CFSE, and thereafter incubated for 36 h with enriched CD4+CD25+ cells (at 3 ratios) from either the vehicle- or the hCDR1-treated mice. The expression of Bcl-xL in the CFSE-labeled CD4+CD25+ cells was determined by FACS. Left end, Representative histograms in which gray contour indicates the expression of Bcl-xL in effector cells alone, and the effects after addition of CD4+CD25+ cells; Treg indicates CD4+CD25+ regulatory T cells. Right end, Mean results (±SD) of three experiments showing the Bcl-xL expression in CFSE-labeled CD4+CD25− cells before and after incubation with CD4+CD25+ cells. (E, Effector CD4+CD25− cells; Treg, indicates CD4+CD25+ regulatory cells of SLE-afflicted mice following treatment with a tolerogenic (hCDR1) peptide.)*, p < 0.05 as compared with incubation of E alone; †, p < 0.01 comparing the effects of PBS- and hCDR1-Treg on E cells.

Discussion

The main findings of this study are that the underlying mechanism by which hCDR1 ameliorates lupus manifestations involves, at least in part, the effects on apoptosis-related molecules affecting the apoptotic process. The decreased rate of apoptosis in response to hCDR1 led to reduced secretion of the pathogenic cytokines IFN-γ and IL-10. Treatment with hCDR1 up-regulated the expression of Bcl-xL in CD4+CD25+ regulatory cells, which resulted in the elevation of this antiapoptotic molecule in CD4+ effector cells. To the best of our knowledge, this is the first report that demonstrates induced expression of Bcl-xL in effector T cells by regulatory T cells of SLE-afflicted mice following treatment with a tolerogenic (hCDR1) peptide.

The relevance of increased apoptosis to SLE was implicated in several studies (17, 18). Accordingly, apoptotic cells constitute a potential source of autoantigen to which autoimmune responses may be initiated, thereby leading to the development of autoimmunity. In this study, we found that the frequency of apoptosis was significantly higher in the diseased mice as compared with healthy controls (Fig. 1, A and B, and Fig. 3), and was associated with an increased activation state of T and B lymphocytes as well (Fig. 5). Thus, mice with experimentally induced SLE exhibited increased rates of activation-induced apoptosis. Two families of proteins, namely, caspases and Bcl-2-associated proteins, mediate activation-induced cell death. The Fas signaling pathway, in which Fas-FasL interactions are followed by caspase-8 activation, is known to play a central role in activation-induced cell death of mature T cells (19). Indeed, lymphocytes from the diseased mice had up-regulated expression of caspase-8 and caspase-3 as compared with healthy controls (Fig. 1C). The association reported in this study between caspase-8 expression and lymphocyte activation is supported by studies indicating a role for caspase-8 in the immune activation of naive lymphocytes, and in T cell proliferation (20, 21). In this study, we found that treatment with hCDR1 reduced the rate of apoptosis and the expression of caspase-8 and caspase-3, in association with clinical and serological amelioration of the SLE manifestations.

ZVAD-fmk is a peptide-based inhibitor of the IL-1β-converting enzyme family that is active against several, but not all caspases (22). Caspase-8, in particular, is the initiating caspase in the Fas signaling pathway that is effectively inhibited by irreversible binding of ZVAD-fmk to the active site cysteine Cys287 of caspase-8 (16, 23). Treatment of the diseased mice with ZVAD-fmk indicated that caspase inhibition, which resulted in a substantially reduced level of apoptosis, could reduce the glomerular deposition of immune complexes, although to a lesser extent than treatment with hCDR1. In agreement, such an effect of ZVAD-fmk on glomerular ICD was also reported in IFN-γ-transgenic mice with a lupus-like syndrome (24). Collectively, caspase-mediated apoptosis may be involved in the pathogenesis of SLE, and hence, it is likely that the

FIGURE 10. hCDR1-induced CD4+CD25+ cells lead to expression of Bcl-xL in CD4+CD25+ cells of SLE-afflicted mice. (A) In the presence of experimental SLE (A) and 8-mo SLE-afflicted BWF1 mice (n = 5–7/group) (B) were treated with 10 weekly s.c. injections of either the vehicle (PBS or Captisol) or hCDR1. Then, LN (from 16/6ld-immunized mice)- or spleen (from BWF1 mice)-derived cells were pooled from each group. Following CD25 cell depletion, the cells (designated as effector cells) from the vehicle-treated mice were labeled with CFSE, and thereafter incubated for 36 h with enriched CD4+CD25+ cells (at 3 ratios) from either the vehicle- or the hCDR1-treated mice. The expression of Bcl-xL in the CFSE-labeled CD4+CD25+ cells was determined by FACS. Left end, Representative histograms in which gray contour indicates the expression of Bcl-xL in effector cells alone, and the effects after addition of CD4+CD25+ cells at a ratio of 1:50 (dashed line), 1:10 (black line), or 1:1 (gray line). Right end, Mean results (±SD) of three experiments showing the Bcl-xL expression in CFSE-labeled CD4+CD25− cells before and after incubation with CD4+CD25+ cells. (E, Effector CD4+CD25− cells; Treg, indicates CD4+CD25+ regulatory T cells.) *, p < 0.05 as compared with incubation of E alone; †, p < 0.01 comparing the effects of PBS- and hCDR1-Treg on E cells.
observed down-regulation of caspase-8 and caspase-3 as well as the rate of apoptosis could play an important role in the mechanism of action of hCDR1. In contrast, whereas treatment with hCDR1 resulted in a reduced activation state of the lymphocytes and in decreased secretion of the pathogenic cytokines (IFN-γ and IL-10), treatment with ZVAD-fmk rather increased the activation state of the lymphocytes and reduced only the secretion of IFN-γ while sustaining the up-regulated secretion of IL-10, a cytokine with potent effects on B cell proliferation and differentiation (25). The latter effects could explain the inability of ZVAD-fmk to reduce the titers of Ab against dsDNA in the diseased mice (Fig. 4), thus indicating that this drug affects only partially lupus manifestations. Treatment with hCDR1 was shown to reduce significantly the dsDNA-specific Ab levels; however, this effect was less prominent than that on the kidney disease. Indeed, the high levels of anti-dsDNA Abs do not always correlate with renal damage (26–29). Furthermore, in agreement with our findings, Seery et al. (24) showed that, whereas treatment with ZVAD-fmk did not affect significantly the levels of anti-dsDNA Ab in comparison with non-treated mice, it still attenuated the kidney disease (24). It is likely that the reduced rate of apoptosis following treatment with ZVAD-fmk, which results in a lower load self Ag, down-regulates formation of immune complexes and thus improves the renal function.

The expression of FasL, like the expression of caspase-8, is increased in lymphocytes from both mice and humans afflicted with SLE (14, 30). FasL was shown to participate in lupus renal injury by inducing apoptosis in endothelial cells in the peritubular capillaries and in the tubular epithelium (31, 32). Neutralization of FasL in SLE-prone BWF1 mice was shown to prevent the development of lupus nephritis (33). Furthermore, we have previously shown that the inhibited expression of FasL following treatment with hCDR1 or after FasL neutralization resulted in reduced levels of IFN-γ and IL-10 and elevated levels of TGF-β in the supernatants of splenocytes from SLE-affected BWF1 mice (14). These results further indicate a significant role for the Fas signaling pathway in SLE.

Bcl-2 and Bcl-xL molecules belong to the Bcl-2 family of proteins and act as negative regulators of Fas-mediated apoptosis in lymphocytes by blocking caspase activation (34). The expression of Bcl-xL in LN cells of the SLE-affected mice was down-regulated, whereas treatment with hCDR1 up-regulated it significantly (Fig. 1C). The effect of treatment with hCDR1 on Bcl-2 was negligible (Fig. 2). Expression of Bcl-xL was much more prominent in CD19+ cells than in CD4+ cells (Fig. 1D). However, treatment with hCDR1 down-regulated the elevated levels of Bcl-xL on B cells of diseased mice. In agreement, bcl-2 transgenic mice, in which the expression of Bcl-2 was enforced in B-lymphoid cells, were shown to develop SLE-like manifestations (35).

The suppressive functions of Bcl-xL on lupus-associated responses were demonstrated on a few levels. Thus, the reduced activation state of T and B lymphocytes in response to hCDR1 treatment was abolished following inhibition of Bcl-xL (Fig. 7). In agreement, it was shown that cross-linking of CTLA-4, a molecule with a contrasting signal to that of the costimulation molecule CD28 during T cell activation, could result in B-cell 2 induction (36). Moreover, Bcl-xL and Bcl-2 molecules, by themselves, could affect upstream NF-κB and result in the inhibition of NF-κB activation, thereby leading to the prevention of inflammation (37). Indeed, treatment with hCDR1 was shown to down-regulate NF-κB activity in association with inhibition of T cell function (38). Furthermore, the decreased secretion of the pathogenic cytokines following treatment with hCDR1 was reversed when Bcl-xL was blocked (Fig. 8). Finally, the Bcl-xL molecule was highly expressed in hCDR1-induced CD4+ CD25+ regulatory cells (Fig. 9). The latter regulatory T cells were reported previously to down-regulate SLE manifestations mainly via the interactions with CD4+ cells (11, 12). In agreement, suppressive regulatory T cells were reported to be resistant to apoptosis due to up-regulated expression of either Bcl-2 or Bcl-xL (39–41). Furthermore, we showed in the present study that the CD4+ CD25+ regulatory cells from hCDR1-treated mice elicited the up-regulated expression of Bcl-xL in CD4+ CD25+ cells (Fig. 10). Thus, Bcl-xL is suggested to be a key molecule in tolerance induction that mediates at least a few of the beneficial effects of hCDR1 upon lupus manifestations. Altogether, because apoptosis appears to be involved in the pathogenesis of SLE, the reduced rate of apoptosis in SLE-affected mice, as seen following treatment with hCDR1, is of importance for the amelioration of disease manifestations.

Disclosures
The authors have no financial conflict of interest.

References


