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

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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 caspases 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.

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ystemic lupus erythematosus (SLE) is an autoimmune disease characterized by dysregulation of immune responses mediated by T and B cell lymphocytes. Typical manifestations of the disease include the production of autoantibodies against nuclear Ag and the malfunction of kidneys due to the deposition of immune complexes. Several mouse models are available for studying SLE. In the (New Zealand Black × New Zealand White)F₁ (BWF₁) mouse model, clinical lupus-like manifestations develop spontaneously in female mice beginning at the age of 3–4 mo (4). In another model, experimental SLE can be induced in naive, non-SLE-prone mice by immunization with anti-DNA mAb that expresses the major Id, designated 16/6Id, of either human or mouse origin (5, 6). The 16/6Id-immunized mice produce high levels of autoantibodies (e.g., anti-dsDNA Ab, anti-nuclear Ab, etc.) and develop other clinical manifestations, including kidney damage, resulting in proteinuria (5, 6).

The pathogenesis of SLE is still unknown, but it can be partly explained by the waste-disposal theory (7). Accordingly, autoimmune immune responses develop against self Ag, which accumulate owing to increased rates of apoptosis concomitantly with a limited capacity for clearance of the apoptotic bodies that contain those self Ag. The essence of this theory implies that inappropriate regulation of immune responses affects not only the clearance of apoptotic debris and immune complexes, but also the activation state of lymphocytes and the competency of regulatory T cells (2, 3, 8).

Pepides based on sequences of CDR1 and CDR3 of the murine and human 16/6Id mAb were synthesized and shown to interact with autoreactive T cells, and to down-regulate autoimmune immune responses associated with SLE (9, 10). The beneficial effects of the two peptides were associated with a down-regulated secretion of IFN-γ, IL-10, and TNF-α, and with an up-regulation of TGF-β (9, 10). Furthermore, the ameliorative effects of a CDR1-based peptide of the human 16/6Id mAb, designated human CDR1 (hCDR1; Edratide), on SLE-afflicted BWF₁ mice are mediated by inducing tolerance through the generation of specific CD4⁺CD25⁺ regulatory T cells (11, 12). The observed effects of treatment with hCDR1 include a reduced rate of apoptosis, involving at least two signaling pathways, as follows: first, the JNK activity along the p21Ras/MAPK, which is reduced following treatment with hCDR1 (13); second, the Fas pathway in which down-regulation of Fas ligand (FasL) in CD4⁺ T cells is also shown to mediate the effects on the cytokine profile of the treated mice (11, 14).

In the present study, we investigated whether the effects of hCDR1 on apoptosis contributed to ameliorating of disease manifestations in mice with induced experimental SLE. We show in this study that treatment of mice with hCDR1 led to a diminished rate of apoptosis due to the down-regulation of caspase-8 and caspase-3, and the up-regulation of Bcl-xL in association with clinical improvement of the diseased mice.

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3 Abbreviations used in this paper: SLE, systemic lupus erythematosus; BWF₁, (New Zealand Black × New Zealand White)F₁ (BWF₁) mouse model, clinical lupus-like manifestations develop spontaneously in female mice beginning at the age of 3–4 mo (4). In another model, experimental SLE can be induced in naive, non-SLE-prone mice by immunization with anti-DNA mAb that expresses the major Id, designated 16/6Id, of either human or mouse origin (5, 6). The 16/6Id-immunized mice produce high levels of autoantibodies (e.g., anti-dsDNA Ab, anti-nuclear Ab, etc.) and develop other clinical manifestations, including kidney damage, resulting in proteinuria (5, 6).

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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, GYYWSWRQPPGKGEWIG, 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 (Edrataide) 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 carboxenzoxy-valyl-alanyl-aspartyl-(β-o-methyl) fluoromethylketone (ZVAD-fmk)**

ZVAD-fmk (Enzyme Systems Products) was dissolved in DMSO and diluted in PBS. The treatment group received daily s.c. injections of ZVAD-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 (λ-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 two 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 allophycocyanin (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 FJK-16s) and its isotype control were purchased from eBioscience. CaspGlow Fluoroscein 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**

Apatosis, as demonstrated by fragmented DNA, was detected by 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 EGTA, 1% 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 infected 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:
caspase-8 (5'-acataccccaattgac-3', 5'-gtggattattgatac-3'), caspase-3 (5'-cctcgcgtgctgc-3', 5'-gcaattgtcatcgtt-3'), Bcl-xL (5'-gggccttctct-3', 5'-gtgctcagctttg-3'), Bad (5'-gctgatgccggtg-3', 5'-gtatacggcagc-3'), Bak (5'-tattacccgggctc-3', 5'-tggtaccagcttc-3'), Bax (5'-tcatggcctggag-3', 5'-ggtagccttggc-3'), Bcl-2 (5'-cactcaacaagag-3', 5'-aatgctttgtttc-3'), -actin (5'-ccgagtagtcgcctc-3', 5'-ggccagtagtcgcctc-3').

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), respectively. Naïve 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 that 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., Bax, Bak, and Bad) 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 ameliorating 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...
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 protein. 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.

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.
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 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 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

FIGURE 5. Effects of ZVAD-fmk and hCDR1 on the activation state of lymphocytes from 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. Top, Representative dot plots of stained lymphocytes from individual mice per treatment group. Bottom, Mean values (±SD) of all mice per treatment group. The results presented are after the reduction of the background staining obtained with the matched isotype controls. A, Lymphocytes derived from LN of individual mice per treatment group were double stained with CD4 (FITC conjugated) and CD69 (PE conjugated) and analyzed by FACS. B, Lymphocytes derived from LN of individual mice per treatment group were double stained with CD19 (FITC conjugated) and CD69 (PE conjugated) and analyzed by FACS. *, $p < 0.05$.

FIGURE 6. Effects of ZVAD-fmk and hCDR1 on the cytokine profile of SLE-afflicted mice. The mice from each treatment group ($n = 5–7$ mice/group) were evaluated individually at the end of the treatment course. Splenocytes ($5 \times 10^6$/well) from each mouse per treatment group were incubated in enriched medium for 48 h in the presence of 16/6Id, and the content of cytokines in the supernatants was measured by ELISA. Mean values (pg/ml ± SE) are presented. *, $p < 0.05$; †, $p < 0.001$ as compared with SLE-afflicted mice that were treated with PBS.

FIGURE 7. The effects of Bcl-xL inhibitors on the state of activation of lymphocytes from SLE-afflicted mice after 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 control. At the end of the treatment course, LN-derived lymphocytes were pooled ($5 \times 10^6$/well) from each group and incubated in enriched medium in the presence of 16/6Id for 36 h, with or without two types of inhibitors of Bcl-xL (e.g., Bcl-2 inhibitor and HA14-1). A, Western blot analysis of Bcl-xL expression. B, Expression of early activation marker, CD69 (PE-conjugated), in CD4+ (FITC-conjugated), and CD19+ (FITC-conjugated) lymphocytes was analyzed by FACS. Gray contour indicates the effects following treatment with either PBS (left end) or hCDR1 (right end). Fine and coarse black lines indicate the effects in the presence of low and high concentrations of the inhibitors, respectively (25 and 100 mM for Bcl-2 inhibitor; 5 and 25 mM for HA14-1). C, Mean values (±SD) of three independent experiments regarding the expression of CD69 in CD4+ and CD19+ cells. *, $p < 0.05$. 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...
levels of Bcl-xL were indeed elevated in the 16/6Id-immunized mice adopted because the levels of Bcl-xL in cells from both treatment groups were significantly down-regulated. Representative histograms of one experiment of three performed showing the T and B lymphocyte expression of CD69 are illustrated in Fig. 7B. In PBS-treated mice, the high expression of CD69 in the lymphocytes was further increased when Bcl-2 inhibitor (100 mM) or HA14-1 (25 mM) was added to the medium (Fig. 7B, left end). Likewise, the reduced expression of CD69 in T and B cells of the hCDR1-treated mice was reversed in the presence of the low and more prominently in the high concentrations of either inhibitor (Fig. 7B, right end). The mean results of three experiments for the lymphocytes’ CD69 expression in response to the inhibitors of Bcl-xL are shown in Fig. 7C. Based on these experiments, it appears that Bcl-xL plays a role in determining the state of activation of the cell.

Bcl-xL plays a key role in the ability of hCDR1 to down-regulate IFN-γ and IL-10

The role of Bcl-xL in the immunomodulation of the cytokine profile by hCDR1 was studied as well. Splenocytes (5 × 10⁶/well) of mice from the various treatment groups were incubated in enriched medium for 36 h. Two concentrations of each of the two inhibitors, Bcl-2 inhibitor and HA14-1, were added to the culture medium of the cells derived from hCDR1-treated mice. Fig. 8 presents the cytokine levels measured in a representative experiment of three performed. Levels of IFN-γ and IL-10 were quantified by Western blotting. The results in Fig. 9A show that both inhibitors of Bcl-xL were competitive because the levels of Bcl-xL in cells from both treatment groups were significantly down-regulated. Representative histograms of one experiment of three performed showing the T and B lymphocyte expression of IFN-γ are illustrated in Fig. 7A. In PBS-treated mice, the high expression of IFN-γ in the lymphocytes was further increased when Bcl-2 inhibitor (100 mM) or HA14-1 (25 mM) was added to the medium (Fig. 7A, left end). Likewise, the reduced expression of IFN-γ in T and B cells of the hCDR1-treated mice was reversed in the presence of the low and more prominently in the high concentrations of either inhibitor (Fig. 7A, right end). The mean results of three experiments for the lymphocytes’ IFN-γ expression in response to the inhibitors of Bcl-xL are shown in Fig. 7B. Based on these experiments, it appears that Bcl-xL plays a role in determining the state of activation of the cell.

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 was increased in 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 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. 9D). 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.

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.
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 16/6id (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 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.

**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 imitated, 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 Cys237 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
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 (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-afflicted 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-afflicted 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 less 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 Bcl-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-afflicted 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


