Amelioration of murine lupus by a peptide, based on the complementarity determining region-1 of an autoantibody as compared to dexamethasone: Different effects on cytokines and apoptosis

Amir Sharabi, Asher Haviv, Heidy Zinger, Molly Dayan, Edna Mozes*

Department of Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

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Abstract A peptide (hCDR1) based on the sequence of the complementarity-determining region-1 of an anti-DNA autoantibody ameliorates clinical manifestations of lupus. We analyzed the beneficial effects of hCDR1 when given alone or in combination with dexamethasone, while comparing the mechanisms of action of the latter. Treatment with either hCDR1 or dexamethasone, or a combination of the latter significantly reduced titers of dsDNA-specific autoantibodies, levels of proteinuria, and intensity of glomerular immune complex deposits. Both drugs down-regulated the secretion and expression of IFN-γ and IL-10, but only treatment with hCDR1 up-regulated TGF-β. While both drugs reduced the expression of Fas ligand (FasL) and caspase 8, treatment with hCDR1 resulted in reduced whereas dexamethasone administration resulted in increased rate of apoptosis. Furthermore, down-regulation of FasL appeared to play a role in cytokine modulation. We conclude that specific treatment with hCDR1 ameliorates murine lupus via distinct mechanisms of action than those of dexamethasone.

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KEYWORDS
Apoptosis; Cytokines; Dexamethasone; Fas pathway; Immunomodulation; (NZBxNZW)F1 mice; Peptides; Systemic lupus erythematosus

Abbreviations: CDR, complementarity-determining region; hCDR1, human CDR1; FasL, Fas ligand; Id, idiotype; NZB, New Zealand Black; NZW, New Zealand White; SLE, systemic lupus erythematosus; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling.

* Corresponding author. Fax: +972 8 9344141.
E-mail address: edna.mozes@weizmann.ac.il (E. Mozes).

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**Introduction**

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the dysregulation of immune responses mediated by T and B cell lymphocytes. This results in the vast production of autoantibodies against several self antigens and further development of immune complexes, which deposit within the kidneys and other organs as well [1]. Our laboratory established a model of murine SLE induced in naive mice by active immunization with a pathogenic anti-DNA monoclonal antibody that bears the 16/6 idiotype (Id) of either human or mouse origin [2,3]. The immunized mice develop a clinical SLE-like disease manifested by high levels of autoantibodies, including anti-dsDNA antibodies [2]. Leukopenia, proteinuria, and immune-complex glomerular kidney disease are also observed in the immunized mice [2,3]. Further, mice with experimental SLE, shared common features with (New Zealand Black (NZB) × New Zealand White (NZW)) F1 mice that are known to develop the disease spontaneously. High homology was found between the variable regions coding for the heavy and light chains of anti-DNA monoclonal antibody of the mice with induced SLE and (NZBxNZW)F1 mice [4].

Peptides based on the sequences of the complementarity-determining region (CDR)-1 and 3 of either a murine (5G12) or human anti-DNA, 16/6Id* monoclonal antibodies [5,6] were designed and synthesized. The peptides, shown to interact and affect T cells, were capable of down-regulating autoimmune responses associated with SLE [5,7,8]. Furthermore, the peptides were capable of either preventing or treating an established disease that was either induced or developed spontaneously [7,9–11]. Moreover, hCDR1 and hCDR3 were shown to inhibit efficiently and specifically the 16/6Id-induced in vitro proliferation and interleukin (IL)-2 production of peripheral blood lymphocytes of SLE patients [12]. hCDR1 was shown to immunomodulate the cytokine profile found in SLE-affected mice by down-regulating the secretion and expression of the pathogenic cytokines IL-1, IL-10, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α and by up-regulating the secretion of the immunosuppressive cytokine transforming-growth factor (TGF)-β [7]. Furthermore, hCDR1 was demonstrated to affect specifically T cell adhesion, chemotaxis, and proliferation [8,13].

Currently, glucoctosteroids are frequently used for the treatment of lupus. Their down-regulating effects are generally explained by their ability to suppress immune responses that are mediated by T and B cell lymphocytes as well as effector functions of monocytes and neutrophils [14]. The main disadvantage of steroids, however, is their broad immunosuppressive effect as well as the development of serious adverse side effects following their long-term use. In contrast, treatment with CDR-based peptides is highly specific and is aimed at down-regulating the SLE-associated autoreactive responses only.

In the present study, we investigated the effects and mechanisms of action of the well-studied specific immunomodulator hCDR1 by comparing it to those of the commonly used long acting steroid, dexamethasone. While treatment with hCDR1 is as effective as dexamethasone in ameliorating clinical manifestations of SLE, both means of treatment differ in their mechanisms of action as demonstrated by the resultant cytokine profiles, rates of apoptosis, and both apoptotic and non-apoptotic functions of Fas ligand (FasL).

**Materials and methods**

**Mice**

Female (NZBxNZW)F1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), and BALB/c female mice were purchased from Harlan (Jerusalem, Israel). The study has been approved by the Animal Care and Use committee of the Weizmann Institute of Science.

**Synthetic peptide**

A peptide with the following sequence GYYWSWIRQPGK-GEEWIG (hCDR1) based on the CDR1 of the human anti-DNA monoclonal antibody [6] bearing the 16/6Id was synthesized (solid phase synthesis by F-moc chemistry) by Polypeptide laboratories (LA, USA) and used in this study. A peptide with scrambled order of the amino acids of the hCDR1, SKGPQYGWPWEGWRYEIQ (‘scrambled peptide’) was synthesized and used as a control. hCDR1 (TV-4710, Edratide) is currently under clinical development for human SLE by Teva Pharmaceutical Industries Ltd.

**Monoclonal antibody**

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

**Treatment of mice with hCDR1 and dexamethasone**

A preliminary dosing study using 0.5, 1, and 2 μg/mouse of dexamethasone indicated that, although the 3 doses used down-regulated SLE-associated responses, the 2 μg dose demonstrated the most efficient and reproducible inhibitory effect and therefore was used in this study.

(NZBxNZW)F1 female mice at the age of 6 months were divided into 6 groups (n = 7–12 mice/group) and treated with 10 weekly subcutaneous injections as follows: vehicle Captisol® (Sulfoxbutylether beta cyclodextrin that has been designed by CyDex to enhance the solubility and stability of drugs), hCDR1 (50 μg/mouse), scrambled peptide (50 μg/mouse), dexamethasone [(9α-Fluoro-16α-methylprednisolone); Sigma; 2 μg/mouse], hCDR1 and dexamethasone, a 5-week treatment with a combination of hCDR1 and dexamethasone followed by a 5-week treatment with hCDR1 alone. In the short-term experiment, BALB/c mice were immunized intradermal with the human anti-DNA monoclonal antibody, 16/6Id (1 μg/mouse, in CFA) concomitant with the injection of either hCDR1 (50 μg/mouse, in PBS), dexamethasone (2 μg/mouse), or both.

**Proliferation of lymph node-derived cells of 16/6Id-immunized BALB/c mice**

All assays were performed in triplicate in flat-bottomed microtiter plates (Falcon, Becton Dickinson, Oxnard, CA,
USA). Popliteal lymph node cells (5 \times 10^5/well) from each treatment group were cultured in enriched RPMI-1640, supplemented with 1% normal mouse serum in the presence of various concentrations (0.1–10 \mu g/well) of the 16/6Id. The cultures were incubated in 7.5% CO_2 at 37°C for 96 h before [\textsuperscript{3}H] thymidine (0.5 \muCi of 5 Ci/mmol) (Nuclear Research Center, Negev, Israel) was added, and 16 h later plates were harvested and radioactivity was counted.

Cytokine production and detection by ELISA

Spleen cells (5 \times 10^6/ml) of (NZBxNZW)F1 mice or lymph node cells (3 \times 10^6/ml) of BALB/c mice that were immunized with the 16/6Id were incubated with either enriched medium or hCDR1 (25 \mu g/ml), or the 16/6Id for the immunized mice (25 \mu g/ml). Supernatants were removed after 48 h and 72 h and analyzed for cytokine content. IFN-\gamma and IL-10 were determined by ELISA using OptEIA sets 48 h and 72 h and analyzed for cytokine content. IFN-\gamma and IL-10 were determined by ELISA according to the manufacturer’s instructions. For the detection of TGF-\beta, plates were coated with recombinant human TGF-\beta1 sRII/Fc chimera (R&D Systems, Minneapolis, USA). Supernatants were added after activation of latent TGF-\beta1 to immunoreactive TGF-\beta1 according to the manufacturer (R&D Systems, Minneapolis, USA). Thereafter, a biotinylated anti-human TGF-\beta1 antibody was added and the assay was developed according to the manufacturer’s instructions (R&D Systems, Minneapolis, USA).

ELISA for the detection of anti-dsDNA antibodies

Maxisorb microtiter plates (Nunk, Denmark) were coated with poly-L-lysine (5 \mu g/ml) (Sigma, St Louis, MO), followed by coating with lambda phage dsDNA (5 \mu g/ml) (Boehringer, Mannheim). After incubation with different dilutions of sera, horseradish peroxidase-labeled goat anti-mouse IgG (\gamma chain-specific; Jackson Immuno Research, West Grove, PA) was added to the plates, followed by the addition of the substrate, ABTS (Sigma). Results were read at 405 nm using an ELISA reader.

Real-time PCR

Levels of mRNA of cytokines were analyzed by quantitative real-time RT-PCR using LightCycler (Roche, Germany). Total RNA was isolated from spleen cells that were pooled (n = 9–12 mice) from each treatment group of the (NZBxNZW)F1 mice. RNA was reverse-transcribed to prepare cDNA using M-MLV reverse transcriptase (Promega, Madison, WI). The resultant cDNA was subjected to real-time PCR according to the manufacturer’s instructions. Briefly, 20 \mu l reaction volume contained 3 mM MgCl_2, LightCycler HotStart DNA SYBR Green I mix (Roche), specific primer pairs, and 5 \mu l of cDNA. PCR conditions were as follows: 10 min at 95°C followed by 35–50 cycles of 15 s at 95°C, 15 s at 60°C, and 15 s at 72°C. Cytokine primer sequences (forward and reverse, respectively) were used as follows: \beta-actin (5'-gtgcagttgactcgg-3', 5'-cagtaactccgcct-3'), caspase 8 (5'-actaacccaccactcgg-3', 5'-tgtggtatcttacagcaga-3'), IFN-\gamma (5'-gaacgtcatacactgc-3', 5'-ctgacgcttggtgg-3'), IL-10 (5'-aacctcgttgacctct-3', 5'-caccatacagaaagc-3'), and TGF-\beta1 (5'-gaccccccattgctg-3', 5'-gccctgatatcgcct-3'). \beta-actin levels were used for normalization while calculating the expression levels of all other genes.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay

Apoptosis, as evidenced by fragmented DNA, was determined using the In Situ Death Detection Kit (Roche, Indianapolis, IN) based on TUNEL technology, according to the protocol supplied by the manufacturer. Cells were analyzed by FACS, with forward and side scatter gates adjusted to include all cells and to exclude debris. Each sample was accompanied by a negative control, consisted of UTP labeled with fluorescein, and a positive control, consisted of DNase I, grade I (Roche) that was added prior to the TUNEL staining.

Staining with antibodies to Fas and FasL

Spleen cells (1 \times 10^6 cells/tube) were stained with monoclonal antibody to PE-conjugated Fas (CD95, clone Jo2) or PE-conjugated FasL (CD178, clone MFL3, Pharmingen) for 30 min at 4°C. Each sample was also stained with the appropriate IgG isotype control (Pharmingen). Cells were thereafter analyzed by FACS.

In vitro assays

Spleen cells (0.5 \times 10^6 cells/tube) taken from 8-month-old (NZBxNZW)F1 female mice with established lupus were co-incubated either with anti-mouse FasL monoclonal antibody (C57BL/6 gld anti-mFasL-transfected L5178Y T lymphoma, clone Kay-10, Pharmingen), or with splenocytes (in a 1:25 ratio) obtained from 2-month-old (NZBxNZW)F1 female mice subcutaneously treated with hCDR1 (50 \mu g/mouse, 3 injections in 1 week), or with both anti-mouse FasL monoclonal antibody and the hCDR1-treated cells, during 36 h and thereafter assessed for content of secreted cytokines.

Detection of proteinuria

Proteinuria was measured by a standard semi-quantitative test, using an Albustix kit (Bayer Diagnostic, Newbury, UK). Results were graded according to the manufacturer as: negative, + = 0.3 g/l, ++ = 1 g/l, +++ = 3 g/l, or ++++ = \geq 20 g/l.

Immunohistology

Mice were sacrificed 2–3 weeks after the end of treatment, and kidneys were removed and frozen immediately in liquid nitrogen. Frozen cryostat sections (6 \mu m) were air-dried and fixed in acetone. For the detection of Ig deposits, sections were incubated with FITC-conjugated goat anti-mouse IgG (\gamma chain-specific) (Jackson Immuno Research). Staining was visualized using a fluorescence microscope.
Statistical analysis

To evaluate the significance of the differences between groups, the Student’s t test and the non-parametric Mann–Whitney test were used. Values of \( P \leq 0.05 \) were considered significant.

Results

hCDR1 and dexamethasone have similar beneficial effects on lupus manifestations

Female (NZBxNZW)F1 mice at the age of 6 months (when lupus-like manifestations are already present) were treated weekly during a 10 week period with subcutaneous injections of the hCDR1 (50 \( \mu \)g/mouse), dexamethasone (2 \( \mu \)g/mouse), or both. An additional group received both hCDR1 and dexamethasone for 5 weeks whereas for the rest of the treatment period, hCDR1 only was administered to the mice. A control group of mice was treated with the vehicle alone.

Fig. 1 demonstrates results of a representative experiment. It can be seen in the figure that treatment with hCDR1 resulted in a reduction in the titers of anti-dsDNA autoantibodies as did the treatment with dexamethasone alone, when compared to mice treated with the vehicle or the control (scrambled) peptide (Fig. 1A). Co-treatment with hCDR1 and dexamethasone (either a 5 or a 10 week course) also decreased the levels of anti-dsDNA autoantibodies.

The effect of the different treatment protocols on the kidney disease of the mice was assessed by measuring the levels of proteinuria and by analyzing the kidneys for the presence of immune complex deposits. Fig. 1B shows that while proteinuria in vehicle or in control peptide-treated mice increased with time, its levels remained low in all groups of hCDR1- and/or dexamethasone-treated mice, and the differences were statistically significant after the fifth weekly treatment. In addition, Fig. 2 demonstrates that the improvement in proteinuria was associated with a significant reduction in the intensity of the glomerular immune complex deposits in the hCDR1 and/or dexamethasone-treated groups.

The effects of treatment with hCDR1 and/or dexamethasone on the pattern of secreted and expressed cytokines

It was of interest to compare the effect of treatment with hCDR1, dexamethasone, or both on the cytokine profile of the treated mice. The results presented in Fig. 3 summarize 4 experiments performed and demonstrate that the high secreted levels of IFN-\( \gamma \) and IL-10 (Fig. 3A) in mice with established SLE decreased dramatically after treatment with hCDR1, dexamethasone, or both means given in combination. The reduction of the latter cytokines by hCDR1 was specific since treatment with a control (scrambled) peptide resulted in an increase rather than a decrease of IFN-\( \gamma \) and IL-10 levels. Fig. 3A depicts also the levels of TGF-\( \beta \) secreted by splenocytes of mice of the different groups. It can be seen that whereas splenocytes of hCDR1-treated mice secreted significantly \( (P \leq 0.003) \) higher levels of TGF-\( \beta \) than splenocytes of the vehicle-treated mice, treatment with dexamethasone affected minimally the secretion of the latter cytokine. The combined treatment of hCDR1 and a 5-week administration of dexamethasone resulted in a significant higher secretion of TGF-\( \beta \) than in the control groups (Fig. 3A). Treatment with a control peptide did not modulate the secretion of TGF-\( \beta \). To further confirm the above results of cytokines secretion, each group of cells was assessed for mRNA expression of the relevant cytokines. Results of cytokine gene expression demonstrated in Fig. 3B are in agreement with the cytokine secretion data.

hCDR1 and dexamethasone down-regulate the proliferative responses of BALB/c mice and immunomodulate cytokine secretion

The effect of dexamethasone was compared to that of hCDR1 in an additional model, namely in BALB/c mice
that were immunized with the monoclonal anti-DNA 16/6Id shown to be capable of inducing experimental SLE [2]. The immunized mice were treated concomitantly with subcutaneous injections of hCDR1 (50 μg/mouse, administered in PBS), dexamethasone (2 μg/mouse), or both drugs. Lymph node-derived cells were taken from the experimental mice 10 days later and their ability to proliferate in vitro in the presence of the 16/6Id or to secrete cytokines following in vitro triggering with the 16/6Id was tested. Fig. 4A shows that treatment with hCDR1 or with dexamethasone concomitant with the immunization with 16/6Id resulted in a significant inhibition of cell proliferation. Co-treatment with both hCDR1 and dexamethasone led to the most prominent inhibition. Both hCDR1 and dexamethasone reduced the levels of IFN-γ (Fig. 4B). However, hCDR1, but not dexamethasone up-regulated TGF-β levels (Fig. 4C). As shown in Fig. 4C, dexamethasone alone did not affect the secretion of TGF-β as compared to mice immunized with the 16/6Id. It is also shown in the figure that levels of TGF-β in supernatants of lymph node cells of mice treated with dexamethasone and hCDR1 were similar to those of mice injected with hCDR1 alone. Thus, the results of the short-term experiments in BALB/c mice confirmed the data of long-term experiments of treatment of the SLE-prone (NZBxNZW)F1 mice indicating that only hCDR1 up-regulated TGF-β.

hCDR1 reduces whereas dexamethasone increases rates of apoptosis

A disruption of apoptosis and subsequent clearance of cellular debris are hypothesized to be in the essence of the pathogenesis of some autoimmune diseases, including SLE. In order to determine the effect of treatment on apoptosis in SLE-affected mice, we documented the frequency of cells undergoing apoptosis and the involvement of the Fas pathway. Fig. 5A shows that the expression of Fas was comparable for both young and old (diseased) mice. However, a significant enhanced expression (of about 40%) in FasL was determined in old SLE-affected mice in comparison to young healthy mice. The latter was reproducible in 4 individual experiments. Concerning the effect of treatments, neither hCDR1 nor dexamethasone caused a significant change in the surface expression of Fas (Fig. 5A). Nevertheless, FasL expression was reduced in all treatment groups (Fig. 5A; c–f versus a) but not in the group treated with the control peptide (Fig. 5A; b versus a). As can be seen in Fig. 5B, the rate of apoptosis as determined by TUNEL was 2–3-fold higher in SLE-affected mice than in young healthy controls. A reduced number of TUNEL+ cells was determined in spleens of hCDR1-treated mice as compared to vehicle-treated mice (Fig. 5B; a versus c). Treatment with the control peptide did not affect the rate of apoptosis (Fig. 5B; a versus b). However, the number of TUNEL+ cells was...
increased after treatment with dexamethasone (Fig. 5B; a versus d). Co-treatment of both drugs resulted in an intermediate number (but lower than in the old mice) of TUNEL\[+\] cells (Fig. 5B; e). Discontinuation of dexamethasone for half the time of treatment while continuing with hCDR1 treatment led to a number of TUNEL\[+\] cells that was lower than that observed for vehicle or control peptide treatments (Fig. 5B; f versus a and b). These results were reproducible in four individual experiments. To confirm the effect of FasL on the rate of apoptosis, we measured mRNA expression of caspase 8. As demonstrated in Fig. 5C, all treatment groups had a lower expression of caspase 8 mRNA as compared to the vehicle or scrambled peptide-treated groups. Indeed, the expression of caspase 8 was associated with that of FasL. Thus, treatment with hCDR1 led to a decrease, whereas treatment with dexamethasone resulted in an increase in the rate of apoptosis.

The hCDR1-mediated reduction of FasL plays a role in the cytokine secretion

Since FasL is suggested to be involved not only in the apoptotic process, and because treatment with hCDR1 resulted in a 40% decrease in FasL expression, it was of interest to find out whether the latter affects cytokine secretion. To this end, splenocytes (5 x 10^5 cells) originating from 8-month-old SLE-afflicted mice (‘lupus’ cells) were co-incubated for 36 h with splenocytes (2 x 10^4 cells) from 2-month-old (NZBxNZW)F1 mice that were pre-treated with hCDR1 (50 mg/mouse, 3 subcutaneous injections in 1 week). As can be seen in Fig. 6 (left end), co-incubation with hCDR1-treated cells resulted in down-regulation of IFN-\gamma and IL-10, and in up-regulation of TGF-\beta, in comparison to incubation of ‘lupus’ cells alone. Fig. 6, right end, shows that incubation of ‘lupus’ cells in the presence of different concentrations of anti-FasL neutralizing monoclonal antibody (0.3, 1.5, 3, or 15 mg) resulted in a cytokine modulation similar to that observed following incubation with hCDR1-treated cells. No such effects were observed when the isotype control was present in the medium (Fig. 6, right end). Further, no additive effect, to that of hCDR1-treated cells, could be determined when the anti-FasL monoclonal antibody was present in the incubation medium.
antibody was present in the co-culture of ‘lupus’ cells and hCDR1-treated cells (Fig. 6, right end). It, thus, appears that FasL that is reduced by hCDR1 treatment is also involved in the down-regulation of IFN-γ and IL-10, and the up-regulation of TGF-β.

Discussion

The main findings of this study are that the specific treatment with hCDR1 is at least as effective as that with dexamethasone. The mechanisms underlying the therapeutic effects of the two drugs are partially distinct. Under the combined treatment of hCDR1 and dexamethasone, the latter drug does not interfere with hCDR1 activity. Both drugs down-regulate the secretion of IFN-γ and IL-10. Yet, treatment with hCDR1 results in an up-regulated TGF-β secretion whereas treatment with dexamethasone has a minor effect on the latter cytokine. Both drugs reduce the expression of FasL; however, hCDR1 decreases whereas dexamethasone increases the rate of apoptosis. Further, in addition to the apoptosis-related effects of FasL, neutralization of FasL, is demonstrated to reverse the pathogenic profile of cytokines as achieved with hCDR1 treatment.

Anti-DNA antibodies are involved in the pathogenesis of the immune glomerulonephritis in SLE in mice and in human patients [15,16]. Here, we demonstrated that treatment with hCDR1 resulted in lower titers of anti-DNA antibodies in the sera as did treatment with dexamethasone, or with a combination of both means (Fig. 1A). Further, all treatment groups responded with a significant reduction of proteinuria levels and of immune complex deposits in the kidneys in comparison to the untreated mice (Figs. 1B and 2).

Cytokines have been suggested to play a central role in the immune dysregulation observed in experimental models of SLE, in lupus-prone mice, and in SLE patients [17–19]. Both IFN-γ and IL-10 were reported to be involved in the pathogenesis of lupus in SLE models as well as in patients [18,20–24]. In this study, we showed that treatment with either hCDR1 or dexamethasone, and a combined treatment with both drugs reduced significantly the levels of IFN-γ and IL-10 (Fig. 3). Indeed, steroids were reported to down-regulate the latter cytokines, although they mainly affect the secretion of the Th1 type cytokines [25,26].

The importance of TGF-β in SLE was shown in several studies. In SLE patients, the high levels of IgG were attributed, in part, to the low levels of TGF-β [27]. Likewise, TGF-β gene knockout mice were shown to rapidly develop a lethal syndrome of lymphocyte hyperactivity and autoantibodies together with lupus-like disease [28,29]. Moreover, in MRL/lpr/lpr mice, this cytokine was capable of decreasing the production of autoantibodies [30], and in NZBxNZWF1 mice, the improvement in clinical manifestations was correlated with increased mRNA expression of TGF-β [31]. In the present study, we demonstrate that the production of TGF-β is influenced differently by the two types of drugs. Thus, while hCDR1 treatment resulted in the up-regulation of TGF-β, dexamethasone treatment resulted in unchanged or reduced levels of the latter cytokine (Fig. 3). This particular pattern was demonstrated in supernatants as well as in mRNA expression. It was also confirmed in short-term experiments in BALB/c mice immunized with the SLE inducing autoantibody, 16/6id (Fig. 4), and in mice with experimental SLE induced by the 16/6id that were treated with hCDR1 [7]. Further, treatment of naive BALB/c mice and young, free-of-disease (NZBxNZWF1 mice with hCDR1 markedly up-regulated the secretion of TGF-β by splenocytes of the treated mice (unpublished data). Furthermore, up-regulated levels of TGF-β following treatment
with hCDR1 were shown to be involved in the inhibition of T cell migration, adhesion, and proliferation \[8,13\], and in mediating the suppressive effects of hCDR1-induced CD4+CD25+ regulatory cells (unpublished data). Thus, whereas TGF-\(\beta\) is not involved in the mechanism of action of dexamethasone, this immunosuppressive cytokine plays a central role in mediating the ameliorative effects of hCDR1.

Dysregulated apoptosis has been demonstrated for autoimmune diseases, including SLE. Thus, administration of apoptotic lymphocytes to (NZBxNZW)F1 mice resulted in accelerated onset of SLE-like manifestations in these mice \[32\]. Further, lymphocytes of SLE patients exhibited increased rate of apoptosis that was also correlated with disease activity \[33,34\]. We showed here an increased rate of apoptosis that was associated with increased expression of FasL in SLE-afflicted (NZBxNZW)F1 mice (Fig. 5). In agreement, several reports indicated high levels of functional FasL in activated T cells originated from SLE patients \[35\]. In hCDR1-treated mice, amelioration in clinical status was associated with a reduction in rate of apoptosis, as reflected by TUNEL staining. Analyzing the Fas/FasL system indicated that this could be explained, at least partially, by the reduced expression of FasL, as the expression of caspase 8 was reduced too while no changes were observed in the levels of Fas (Fig. 5). Dexamethasone, which also caused a reduced FasL expression, still resulted in higher rates of apoptosis. This finding strikingly presents the dual role of FasL as a trigger for apoptosis in T lymphocytes \[36\], but also as a pro-inflammatory signal \[37–40\]. Thus, administration of steroids, which are pro-apoptotic and anti-inflammatory agents, leads to reduced expression of the pro-inflammatory FasL but with increased rate of apoptosis, which further supports in this case the involvement of apoptotic signaling pathways other than the Fas/FasL system. In agreement, studies in knockout mice demonstrated differences between corticosteroid-induced apoptosis and Fas-induced apoptosis \[41\]. Thus, although the two apoptosis pathways are caspase-dependent, the Fas-induced apoptosis requires the involvement of caspase 8 whereas the corticosteroid-induced apoptosis may depend alternatively on caspase 3.

FasL, in addition to its involvement in the apoptotic process, plays a role in the modulation of cytokine profile. Indeed, neutralization of FasL affected splenocytes of SLE-afflicted (NZBxNZW)F1 mice to reduce the secretion of IFN-\(\gamma\) and IL-10 and to enhance the secretion of TGF-\(\beta\) (Fig. 6). The same effect could be achieved by co-incubation of hCDR1-treated cells and ‘lupus’ cells, thus suggesting that the cytokine modulation by hCDR1 is likely to be affected by the reduced expression of FasL. Likewise, FasL was reported to affect the kidney disease of (NZBxNZW)F1 mice because

**Figure 6** Down-regulated expression of FasL by hCDR1 is associated with modulation of cytokines profile. Triplicate of spleen cells (5 \(\times\) 10\(^3\)) from 8-month-old (NZBxNZW)F1 mice (n = 3), designated as ‘lupus’ cells, were co-incubated for 36 h with splenocytes (2 \(\times\) 10\(^4\) cells) from 2-month-old mice (n = 3) that were pre-treated subcutaneously with hCDR1 (50 \(\mu\)g/mouse, 3 injections in 1 week), or with the latter in the presence or absence of either anti-FasL neutralizing monoclonal antibody or its isotype control. Levels of secreted cytokines in the supernatants were measured by ELISA for each concentration of antibody (presented in logarithmic scale). Results are of one representative experiment out of two performed.

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**Figure 7** Schematic illustration of the effects of hCDR1 and dexamethasone. (A) Effects of hCDR1. (B) Effects of dexamethasone. Dashed line arrows represent alternative pathways.
its neutralization prevented the development of lupus nephritis [42].

The rate of apoptosis may be influenced not only by FasL expression but also by modulation of the cytokine profile. For instance, in SLE patients with active disease, the high levels of IL-10 were reported to augment T cell death in vivo [43]. In addition, IFN-γ, which is up-regulated in lupus, was shown to mediate its responses through the induction of subset of genes, namely, IFN-stimulated-genes that are involved, among other biological responses, in the apoptotic process [44]. Hence, the reduction of the latter cytokines by hCDR1 may lead to the observed lower apoptosis rates. In contrast, in the case of dexamethasone, apoptosis was unaffected by the down-regulated IL-10 and IFN-γ, and the observed higher rates may be attributed to the classic genomic activation [41]. In addition, TGF-β1 was shown to inhibit the expression of FasL and to reduce Fas-induced apoptosis [45,46]. Also, T cells of TGF-β1-deficient mice were reported to undergo increasing rates of apoptosis [47]. Therefore, the up-regulation of TGF-β1 by hCDR1 may mediate the reduction of apoptosis as well. In contrast, TGF-β1 does not play a role in the mechanisms of action of dexamethasone since this drug minimally affected its secretion and expression. Hence, it appears that the down-regulation of IFN-γ and IL-10 and the up-regulation of TGF-β1, in addition to the reduced expression of FasL, may contribute to the reduced rate of apoptosis following treatment with hCDR1.

In summary, in the present study, we demonstrate that hCDR1 and dexamethasone down-regulate lupus manifestations by different mechanisms of action. As illustrated in Fig. 7, treatment with either hCDR1 or dexamethasone results in reduced secretion of IFN-γ and IL-10, but only treatment with hCDR1 leads to an increased secretion of TGF-β1. The effect on cytokine secretion may be, at least in part, due to the reduced expression of FasL. The combined effect of cytokine modulation and FasL reduction leads to the decrease in the apoptosis rate in the lupus-afflicted mice, in response to treatment with hCDR1. In contrast, treatment with dexamethasone, which also down-regulated IFN-γ and IL-10 but did not affect TGF-β1, leads to an increase in the apoptosis rate suggesting the existence of another pathway that is different from that of hCDR1 (Fig. 7B, dashed line). The mechanism of action as well as its specificity distinguishes treatment with hCDR1 from that with dexamethasone. Thus, hCDR1 is an effective specific candidate for the treatment of SLE.

References

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