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A Peptide That Ameliorates Lupus Up-Regulates the Diminished Expression of Early Growth Response Factors 2 and 3

Uri Sela,*† Molly Dayan,* Rami Hershkoviz, † Ofer Lider,‡* and Edna Mozes‡*

Expansion of autoreactive T cells and their resistance to anergy was demonstrated in systemic lupus erythematosus (SLE). A pair of transcription factors, early growth response 2 (Egr-2) and 3 (Egr-3), are negative regulators of T cell activation that were shown to be important in anergy. A peptide (designated hCDR1 for human CDR1) based on the CDR-1 of an anti-DNA Ab ameliorated SLE in both induced and spontaneous lupus models. Our objectives were to determine the expression levels of Egr-2 and Egr-3 in autoreactive T cells following immunization with the lupus-inducing anti-DNA Ab that bears a common Id designated 16/6Id and also in a full-blown SLE and to determine the effect of hCDR1 on these transcription factors. We demonstrated diminished expression levels of Egr-2 and Egr-3 mRNA both early after immunization with the 16/6Id and in SLE-afflicted (NZB × NZW)F1 (New Zealand Black and New Zealand White) mice. Furthermore, by down-regulating Akt phosphorylation and up-regulating TGFβ secretion, treatment with hCDR1 significantly up-regulated Egr-2 and Egr-3 expression. This was associated with an increased expression of the E3 ligase Cbl-b. Inhibition of Akt in T cells of immunized mice decreased, whereas silencing of the Egr-2 and Egr-3 in T cells of hCDR1-treated mice increased IFN-γ secretion. Thus, hCDR1 down-regulates Akt phosphorylation, which leads to up-regulated expression of T cell Egr-2 and Egr-3, resulting in the inhibition of IFN-γ secretion that is required for the maintenance of SLE. The Journal of Immunology, 2008, 180: 1584–1591.

Early growth response (Egr)2 and 3 belong to a family of four transcription factors with highly conserved zinc finger DNA-binding domains that were initially discovered as genes up-regulated in response to growth factors (1). Recently Egr-2 and Egr-3 were found to be up-regulated in T cell anergy, and the overexpression of these transcription factors was associated with the inhibition of T cell activation (2, 3). Anergy refers to a state of unresponsiveness induced in dangerous lymphocytes during Ag presentation as a means of preserving self-tolerance. This state is generally reversible upon exposure to exogenous IL-2 (4). Anergy can be induced in T cells by delivery of a strong TCR signal in the absence of costimulation or by stimulation with a low affinity ligand in the presence of costimulation (5). Akt provides a costimulatory signal for and can be activated by either CD28 (6) or other costimulatory molecules such as leukocyte function Ag 1 (LFA-1) and 4-1BB (7, 8). In addition, Akt can be activated following cross-linking of the TCR (9).

Systemic lupus erythematosus (SLE) is a disease characterized at early stages by the expansion of autoreactive T cells that trigger polyclonal B cell activation with subsequent hypergammaglobulinemia and organ injury due to immune complex deposits and cell infiltration (10–12). Recently it was established that autoreactive T cells in lupus are resistant to anergy (13). An increased PI3K signaling pathway with the up-regulation of T cell phosphorylated Akt (p-Akt) was found in both human and mouse models for SLE (14, 15). The increased PI3K activity was found to induce infiltrating lymphoproliferative disorder and autoimmune renal disease with an increased number of T lymphocytes (16), while inhibition of this pathway blocked the glomerulonephritis and prolonged mice survival (15).

Experimental SLE can be induced in naive, non-SLE prone mice by their immunization with monomeric anti-DNA Abs that express the major Id designated 16/6Id (17, 18). The 16/6Id-induced disease in mice is manifested by high levels of autoantibodies (anti-dsDNA and anti-nuclear protein antibody) and by SLE-associated clinical symptoms (17, 18). Furthermore, a peptide based on the sequences of the CDR1 of the human 16/6Id was shown to down-regulate in vitro and in vivo autoreactive T cell responses and to ameliorate the clinical manifestations of spontaneous (NZB × NZW)F1 (NZB/W; New Zealand Black and New Zealand White mice) and induced models of SLE in mice (19–23). The latter was associated with down-regulation of the cytokines that play a key role in the pathogenesis of lupus (e.g., IFN-γ, IL-10, and IL-1) and with an up-regulation of the immunosuppressive cytokine TGFβ (20, 21, 23). Moreover, treatment with hCDR1 inhibited T cell interactions with the extracellular matrix, including adhesion and chemotaxis, by down-regulating ERK phosphorylation (24). The inhibition of phosphorylation of this kinase was also found to be involved in 16/6Id-stimulated proliferation via down-regulating the expression and function of a pair of adhesion and costimulatory
molecules, LFA-1 and CD44. IFN-γ was also found to play an important role in the 16/6Id-stimulated proliferation (25) and to be associated with down-regulation of TCR signaling, T-bet expression and NFκB activation (26).

Because T cells in lupus resist anergy (13), the induction of anergy in these cells is of major importance. Therefore, the objective of the present study was to determine whether human CDR1 (hCDR1), which has been shown to be beneficial in lupus, affects anergy. Further, we wanted to find out whether the effects of hCDR1 are mediated by the two transcription factors Egr-2 and Egr-3 that were found to play an important role in anergy. We show here that treatment with hCDR1-induced anergy in autoreactive T cells as it down-regulated their IL-2 mRNA expression and proliferation, with reversibility of the latter upon IL-2 addition. The effect of hCDR1 was associated with the inhibition of Akt phosphorylation and the up-regulated Egr-2 and Egr-3 mRNA expression that down-regulated IFN-γ secretion.

Materials and Methods

Mice

Mice of the BALB/c inbred strain were obtained from Harlan and female mice were used at the age of 8–10 wk. NZB/W female mice were purchased from The Jackson Laboratory. This study has been approved by the Animal Care and Use Committee of the Weizmann Institute of Science.

Synthetic peptides

A peptide based on the sequence of the CDR (CDR1; Ref. 27) of the human mAb that bears the major Id, designated 16/6Id (GYW SWIRQGPPKGEWEIG), was synthesized by Polypeptide Laboratories using solid phase synthesis by Fmoc (N-(9-fluorenylmethoxycarbonyl) chemistry. hCDR1 (eductate) is under clinical development for the treatment of SLE by Teva Pharmaceutical Industries. As a control, we used a peptide containing the amino acids of hCDR1 (SKGIPQYGGWPWEGWRYEIG), which was designated hCDR1. This peptide binds HMC class II in a similar avidity to that of hCDR1 (our unpublished data).

Abs and reagents

The human anti-DNA mAb that bears the 16/6Id (IgG1/κ) was previously described (28). The Ab is secreted by hybridoma cells that are grown in culture and purified by using a protein G-Sepharose column (Amersham Pharmacia). Abs to Ser473-phosphorylated Akt (p-Akt) and anti-Akt were obtained from Cell Signaling Technology. Recombinant mouse TNFα, IFN-γ and IL-2 were obtained from Sigma-Aldrich, Egr-2, Egr-3, Cbl-b, and tubulin were obtained from Santa Cruz Biotechnology. Anti-Egr-2, anti-Egr-3, anti-Cbl-b, and anti-tubulin (as a control for the total protein loaded), Immunoexpression protein bands were visualized using labeled secondary Abs and enhanced ECL system. The results were also demonstrated as densitometric histograms that were calculated as a percentage of total protein using the NIH Image program.

Western blot analysis

For preparation of whole cell lysate, T cells that were either unstimulated or in vitro stimulated with 16/6Id were lysed as described previously (24). Samples containing equal amounts of protein were separated by electrophoresis on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked and probed with anti-general Akt Ab and its phosphorylated form, anti-Egr-2, anti-Egr-3, anti-Cbl-b, and anti-tubulin (as a control for the total protein loaded). Immunoreactive protein bands were visualized using labeled secondary Abs and the enhanced ECL system. The results were also demonstrated as densitometric histograms that were calculated as a percentage of total protein using the NIH Image program.

Real-time RT-PCR

The levels of mRNA of IL-2, IFN-γ, Egr-2, and Egr-3 were determined by quantitative real-time RT-PCR using the LightCycler system (Roche). Reverse transcription into complementary DNA was performed by using Moloney murine leukemia virus reverse transcriptase (Promega). Real-time RT-PCR was performed according to the manufacturer’s instructions. Briefly, 20 μl of reaction volume contained 3 mM MgCl2, LightCycler HotStart DNA SYBR Green I mix (Roche), specific primer pairs, and 5 μl of cDNA. PCR cycling conditions were 10 min at 95°C followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The following primer sequences were used (forward and reverse respectively): IL-2 (5′-ctccagctctgtagag-3′ and 5′-gtagctcacagggct-3′), IFN-γ (5′-gaaggcaactc-3′ and 5′-ctgaagctgtgagg-3′), Egr-2 (5′-ctgttgctgctg-3′ and 5′-gagggtgcctagag-3′), Egr-3 (5′-gctgcagccgctgc-3′ and 5′-ctcctgctgcagccg-3′), and β-actin (5′-ctgttgctgctg-3′ and 5′-gagggtgcctagag-3′). The levels of β-actin were used for normalization while calculating the expression levels of other genes. The results are expressed as the relative expression levels for each gene.

Statistical analysis

The nonparametric Mann-Whitney U test was used for statistical analyses of the data. A value of p ≤ 0.05 was considered statistically significant.
vitro addition of rIL-2 abrogated the hCDR1-induced down-regulation of Egr-2 and Egr-3 mRNA relative to 16/6Id (as measured by real-time RT-PCR). Results are expressed as mean cpm ± SD. Depicted are the results of one experiment representative of three that yielded similar results.

Results

Addition of exogenous rIL-2 abrogates the hCDR1-induced down-regulation of the 16/6Id-triggered proliferation

The inhibitory effect of hCDR1 is shown by its ability to specifically inhibit 16/6Id-triggered proliferation. To find out whether the hCDR1-induced inhibition of proliferation is associated with the down-regulation of IL-2, we determined the mRNA expression of the latter cytokine in mice that were immunized with 16/6Id and treated with hCDR1. Thus, two groups of mice were immunized with 16/6Id and one of the groups was concomitantly treated with hCDR1. Ten days later we measured IL-2 mRNA levels in T cells derived from the two groups of mice by using real-time RT-PCR. As shown in Fig. 1A, treatment with hCDR1 significantly down-regulates IL-2 mRNA expression. Table I demonstrates that the observed down-regulation is specific, because a control peptide (scrambled) did not inhibit IL-2 mRNA expression in T cells from 16/6Id-immunized mice. Furthermore, when the hCDR1 was injected as a treatment to mice immunized with IgG (used as a specificity control) it did not down-regulate the IL-2 mRNA expression.

Next, to find out whether the hCDR1-induced down-regulation of the 16/6Id-triggered proliferation can be reversible, rIL-2 was added to LN cells derived from mice that were immunized with 16/6Id and treated with hCDR1. Fig. 1B demonstrates that the in vitro addition of rIL-2 abrogated the hCDR1-induced down-regulation of the 16/6Id-triggered proliferation. These results may suggest that T cell anergy might be part of the mechanism by which hCDR1 exerts its inhibitory effect.

hCDR1 down-regulates Akt phosphorylation and IFN-γ production by T cells

p-Akt is an important kinase that plays a key role in T cell survival, activation, and proliferation (29, 30). Therefore, we wanted to find out whether Akt plays a role in the mechanism of action of hCDR1. Thus, we studied the effect of in vivo treatment with hCDR1 on the 16/6Id-stimulated Akt phosphorylation in T cells. To this end, T cells derived from mice that were immunized with 16/6Id without and with concomitant injection of hCDR1 were incubated with irradiated splenocytes derived from normal mice (as a source for APCs) and in the presence or absence of 16/6Id stimulation (25 μg/ml). Cells were then collected and the extent of Akt phosphorylation was determined in their lysates. As shown in Fig. 2A, in vivo treatment with hCDR1 down-regulated both in vitro 16/6Id-stimulated and unstimulated Akt phosphorylation as compared with T cells derived from 16/6Id-immunized mice that were not treated with hCDR1.

We have previously shown (26) that treatment with hCDR1 down-regulated IFN-γ secretion from T cells. To find out whether the down-regulation in Akt phosphorylation may contribute to the decreased IFN-γ secretion, we studied the effect of inhibition of the PI3K pathway on IFN-γ secretion. To this end, T cells derived from 16/6Id-immunized mice were incubated with irradiated normal splenocytes in the presence of 16/6Id stimulation with or without wortmannin (PI3K inhibitor). Fig. 2B demonstrates that in vitro incubation with wortmannin significantly down-regulated T cell IFN-γ secretion. As shown in Fig. 2C, in vitro addition of wortmannin also significantly decreased the mRNA expression of IFN-γ as measured by real-time RT-PCR.

Egr-2 and Egr-3 expression is down-regulated in T cells of 16/6Id-immunized mice and up-regulated by hCDR1

The transcription factors Egr-2 and Egr-3 are negative regulators of T cell activation that are up-regulated in anergy and inhibit T cell functions (2). Therefore, we studied the expression of these transcription factors in T cells of 16/6Id-immunized mice. As shown in Fig. 3A, I and B, I, Egr-2 and Egr-3 mRNA expressions were significantly down-regulated in resting T cells derived from 16/6Id-immunized mice compared with resting normal mice. In vitro stimulation with 16/6Id significantly up-regulated the mRNA expression of these factors in T cells derived from 16/6Id-immunized mice and to a much lesser extent (nonsignificant) in T cells derived from normal mice. Nevertheless, the expression of Egr-2 and Egr-3 mRNA was significantly decreased in T cells of 16/6Id-immunized mice compared

Table I. hCDR1 inhibits specifically 16/6Id-stimulated IL-2 mRNA expression

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Treatment</th>
<th>IL-2 Relative Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>16/6Id</td>
<td>hCDR1</td>
<td>0.55</td>
</tr>
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<td>16/6Id</td>
<td>hCDR1</td>
<td>1.76</td>
</tr>
<tr>
<td>hIgG</td>
<td>hCDR1</td>
<td>1.71</td>
</tr>
<tr>
<td>16/6Id Scrambled peptide</td>
<td>hCDR1</td>
<td>1.2</td>
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</tbody>
</table>
with T cells of unimmunized mice. Moreover, in vivo treatment with hCDR1 significantly up-regulated T cell mRNA expression of Egr-2 and Egr-3 (Fig. 3, C.1 and D.1). Similar results were observed in Western blot analysis as shown in Fig. 3, A.2, B.2, C.2, and D.2. The observed hCDR1-induced up-regulation in Egr-2 and Egr-3 mRNA expressions was found to be specific because, as demonstrated in Table II, a control peptide (scrambled) did not up-regulate Egr-2 and Egr-3 mRNA expressions in T cells from 16/6Id-immunized mice. In addition, when the hCDR1 was injected as treatment to mice immunized with the control Ag human IgG it did not up-regulate the Egr-2 and Egr-3 mRNA expressions.

**Inhibition of PI3K up-regulates Egr-2 and Egr-3 mRNA expressions in T cells**

Because treatment with hCDR1 down-regulated Akt phosphorylation, we studied the effect of inhibition of the PI3K signaling pathway on the mRNA expressions of Egr-2 and Egr-3 in T cells. To this end, T cells derived from 16/6Id-immunized mice were incubated with irradiated normal splenocytes in the presence of 16/6Id stimulation with or without wortmannin. As demonstrated in Fig. 4, incubation with wortmannin significantly up-regulated the mRNA expressions of Egr-2 and Egr-3 in T cells derived from 16/6Id-immunized mice. Fig. 4 also demonstrates that the in vitro
addition of rIL-2 (shown to be down-regulated by hCDR1) abrogated the effect of wortmannin on the mRNA expression of Egr-2 and Egr-3.

**In vitro inhibition of the transcription factors Egr-2 and Egr-3 abrogates hCDR1-induced down-regulation of IFN-γ secretion**

To find out whether hCDR1-induced up-regulated Egr-2 and Egr-3 mRNA expressions may contribute to the inhibition of IFN-γ secretion, we transfected T cells derived from hCDR1-treated mice with siRNA specific to these transcription factors. The latter cells were electroporated with Egr-2- or Egr-3-specific siRNA and a control siRNA. Control groups of T cells derived from 16/6Id-immunized mice with or without in vivo treatment with hCDR1 were electroporated without siRNA. After incubation in recovery medium, the cells were washed and reincubated with irradiated syngeneic splenocytes from normal mice in the presence of 16/6Id and IFN-γ secretion in the supernatants was determined by ELISA (mean ng/ml ± SD). B–E, Real-time RTPCR was performed immediately after incubation in recovery medium for Egr-2 (B) and Egr-3 (D) mRNA expression. Results express Egr-2 and Egr-3 mRNA relative to β-actin. Whole cell lysates, prepared after overnight incubation with splenocytes and 16/6Id, were applied to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti Egr-2 (C) or Egr-3 (E). Abs against total tubulin served as a control for the protein loaded. The percentage of expression of the total protein loaded is presented. Depicted are the results of one experiment representative of three performed; *, p < 0.05.

**FIGURE 5.** In vitro silencing of the transcription factors Egr-2 and Egr-3 abrogates hCDR1-induced down-regulation of IFN-γ secretion. A, T cells, purified from 16/6Id-immunized BALB/c mice concomitant with hCDR1 treatment, were electroporated with Egr-2- or Egr-3-specific siRNA and a control siRNA as described in Materials and Methods. Control groups of T cells derived from 16/6Id-immunized mice with or without in vivo treatment with hCDR1 were electroporated without siRNA. The cells were incubated in recovery medium and reincubated (48 h) with irradiated syngeneic splenocytes from normal mice in the presence of 16/6Id and IFN-γ secretion in the supernatants was determined by ELISA (mean ng/ml ± SD). B–E, Real-time RT-PCR was performed immediately after incubation in recovery medium for Egr-2 (B) and Egr-3 (D) mRNA expression. Results express Egr-2 and Egr-3 mRNA relative to β-actin. Whole cell lysates, prepared after overnight incubation with splenocytes and 16/6Id, were applied to SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with anti Egr-2 (C) or Egr-3 (E). Abs against total tubulin served as a control for the protein loaded. The percentage of expression of the total protein loaded is presented. Depicted are the results of one experiment representative of three performed; *, p < 0.05.

**FIGURE 4.** Inhibition of PI3K up-regulates Egr-2 and Egr-3 mRNA expression in T cells. T cells purified from LN cells derived from 16/6Id-immunized mice were incubated with or without wortmannin, washed, and then incubated with irradiated (3,000 rad) syngeneic splenocytes from normal mice in the presence of 16/6Id (25 μg/ml) with or without rIL-2 (10 ng/ml). The mRNA expressions of Egr-2 (A) and Egr-3 (B) were determined by real-time RT-PCR. Results present the Egr-2 and Egr-3 mRNA expressions relative to β-actin. Depicted are the results of one experiment representative of four performed. *, p < 0.05.

**Table II.** hCDR1 up-regulates specifically 16/6Id-stimulated Egr-2 and Egr-3 mRNA expression

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Treatment</th>
<th>Egr-2 Relative Expression</th>
<th>Egr-3 Relative Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>16/6Id</td>
<td>hCDR1</td>
<td>7.02</td>
<td>3.83</td>
</tr>
<tr>
<td>16/6Id</td>
<td>Scrambled peptide</td>
<td>1.14</td>
<td>1.5</td>
</tr>
<tr>
<td>hIgG</td>
<td>hCDR1</td>
<td>1.42</td>
<td>1.0</td>
</tr>
<tr>
<td>hIgG</td>
<td>hCDR1</td>
<td>1.84</td>
<td>0.91</td>
</tr>
</tbody>
</table>

*a* Mice were immunized with either the 16/6Id or human IgG and treated concomitantly with hCDR1 or the control peptide (scrambled). T cells were stimulated in vitro (see Materials and Methods) in the presence of irradiated normal APCs and the immunizing agent (25 μg/ml) and were evaluated for Egr-2 or Egr-3 mRNA expression (relative to β-actin) using real-time RT-PCR. Shown are the results of one experiment representative of three that yielded similar results.
Egr-3-specific siRNA was significantly up-regulated (2.2- and 1.6-fold, respectively) compared with T cells of hCDR1-treated mice that were only electroporated or electroporated and transfected with a control siRNA. The levels of IFN-γ secreted following siRNA transfection specific to either Egr-2 or Egr-3 was almost similar to that secreted by electroporated T cells derived from 16/6Id-immunized mice. Fig. 5, B and D, demonstrate the effective down-regulation of Egr-2 and Egr-3 mRNA expressions following transfection of T cells derived from hCDR1-treated mice with Egr-2- and Egr-3-specific siRNA. Fig. 5, C and E, show up-regulation of Egr-2 and Egr-3 protein levels following in vivo treatment with hCDR1 and their down-regulation by the specific siRNA. It should be noted that there was no down-regulation but rather an up-regulation of Egr-2 mRNA expression in Egr-3-specific siRNA-transfected T cells. The same was true for Egr-3 mRNA expression in Egr-2 specific siRNA-transfected T cells (data not shown).

Egr-2 and Egr-3 mRNA expression is down-regulated in T cells of NZB/W SLE-affected mice and up-regulated by treatment with hCDR1. Treatment with hCDR1 was shown to up-regulate TGFβ secretion (24, 26). To study whether TGFβ may contribute to the hCDR1-induced up-regulation in Egr-2 and Egr-3 mRNA expression, T cells derived from 16/6Id-immunized mice were incubated with TGFβ (250 pg/ml, 24 h) and with irradiated normal splenocytes in the presence or absence of 16/6Id (25 μg/ml). Whole cell lysate was applied to SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and immunoblotted with anti-Cbl-b. Abs against tubulin served as a control for the protein loaded. The percentage of expression of the total protein loaded is presented in columns. Depicted are the results of one experiment representative of three performed; *, p < 0.05.
Table III. hCDR1 up-regulates Egr-2 and Egr-3 mRNA expression in NZB/W mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Egr-2 Relative Expression (Mean ± SEM)</th>
<th>Egr-3 Relative Expression (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.14 ± 0.92</td>
<td>1.02 ± 0.53</td>
</tr>
<tr>
<td>hCDR1</td>
<td>7.2 ± 2.98</td>
<td>7.08 ± 3.56</td>
</tr>
</tbody>
</table>

* Two groups (four mice/group) of SLE-afflicted NZB/W mice (8 mo old) were treated with either the vehicle (Captisol) or hCDR1. T cells, purified from individual mice, were evaluated for Egr-2 or Egr-3 mRNA expression using real-time RT-PCR. Results are mean mRNA expression of individual mice relative to β-actin.

1 p < 0.005 compared to vehicle.

2 p < 0.001 compared to vehicle.

normal old aged matched (8 mo) BALB/c mice were compared for the expression of Egr-2 and Egr-3. Fig. 7, A and B, demonstrate a significant down-regulation in Egr-2 and Egr-3 mRNA expression in T cells of old SLE-afflicted mice compared to young NZB/W mice or to normal old BALB/c mice. Treatment of the SLE-afflicted NZB/W mice with hCDR1 significantly up-regulated the expression of both Egr-2 and Egr-3 transcription factors, whereas treatment with a control peptide did not affect their expression (Fig. 7, C and D). Table III, which shows the mean values of mRNA of individual mice, confirms the significant up-regulation of Egr-2 and Egr-3 mRNA expressions following treatment with hCDR1.

**Discussion**

The main findings of this study are that hCDR1 (edratide) inhibited T cell proliferation and IFN-γ secretion by inducing anergy. The latter was associated with inhibition of Akt phosphorylation and up-regulation of the mRNA expression of two negative regulators of TCR activation, namely Egr-2 and Egr-3, which we found to be basically down-regulated in SLE. Similarly, these transcription factors were up-regulated by TGFβ that is elevated following treatment with hCDR1. To the best of our knowledge, this is the first demonstration that the negative regulators Egr-2 and Egr-3 are down-regulated in lupus and can be immunomodulated. Furthermore, these results also suggest a role for Akt in cell activation and proliferation through a negative effect on Egr-2 and Egr-3 expressions, leading to the prevention of anergy.

The beneficial effects of hCDR1 on the clinical manifestations of SLE were shown to be associated with down-regulation of pathogenic cytokines, including IFN-γ, and with an increase in the immunosuppressive cytokine TGFβ (23). We have recently shown (24, 25) that already 10 days following immunization with the 16/6Id, long before the appearance of serological and clinical manifestations of experimental lupus, treatment with hCDR1 diminished specifically T cell responses including chemotaxis, adhesion, and proliferation in association with the inhibition of ERK phosphorylation (24, 25). The present study added to our understanding of the mechanism underlying the beneficial effects of hCDR1 in relation to anergy and the T cell negative regulators Egr-2 and Egr-3.

We have shown previously that already in the very early stages of disease induction, hCDR1 inhibited specifically 16/6Id-stimulated T cell proliferation in association with the inhibition of ERK phosphorylation (25). In the present study we showed that treatment with hCDR1 down-regulated T cell IL-2 mRNA expression already 10 days after immunization with the 16/6Id, while the addition of IL-2 to T cells derived from hCDR1-treated mice abrogated the hCDR1-induced inhibition of T cell proliferation. These results may suggest that hCDR1 also exerts its beneficial effect by inducing T cell anergy. Inhibition of autoreactive T cell proliferation and anergy induction in lupus is of major importance, because the proliferation and the expansion of autoreactive T cells were found to be correlated with disease severity (10), and it was reported recently that autoreactive T cell in lupus are resistant to anergy (13).

T cell Akt phosphorylation was found to be up-regulated in both human and mouse models for SLE (14, 15), while inhibition of PI3K signaling pathway blocked the glomerulonephritis and prolonged mice survival (15). In addition, Akt was shown to provide a costimulatory signal for the up-regulation of IL-2 and IFN-γ secretions (6) and to play a key role in T cell survival, activation, and proliferation (29, 30). In agreement with this, we demonstrated in this study that treatment with hCDR1 down-regulated Akt phosphorylation in T cells and that the inhibition of this signaling pathway was associated with the down-regulation of IFN-γ secretion and expression (Fig. 2A). Inhibition of the latter cytokine is of major importance, because IFN-γ was shown to contribute to the 16/6Id-stimulated proliferation. Indeed, addition of this cytokine to T cells derived from hCDR1-treated mice abrogated the hCDR1-induced inhibition of proliferation (25).

Akt can be phosphorylated following stimulation of either the TCR, CD28 (9), or other costimulatory molecules including LFA-1 (7, 8). Indeed, we have shown recently (26) that treatment with hCDR1 down-regulated LFA-1 expression and function and inhibited TCR signaling, probably by acting as a partial agonist. Egr-2 and Egr-3 are transcription factors that were found recently to be up-regulated in T cell anergy, and their overexpression was associated with the inhibition of T cell activation (2, 3). However, very little is known about their expression in autoimmune diseases and particularly in lupus. In this study we demonstrate for the first time that the expression of these transcription factors is down-regulated in T cells derived from mice immunized with 16/6Id in the very early stages of SLE induction, already 10 days after immunization, as compared with normal BALB/c mice (Fig. 3, A and B). In addition, these two transcription factors are probably important also in a fully established disease of the spontaneous model, as their expression is down-regulated in old (8 mo) SLE-afflicted NZB/W mice in comparison with young NZB/W mice or normal age-matched BALB/c mice (Fig. 6, A and B). Moreover, we demonstrated that the expressions of Egr-2 and Egr-3 can be modulated, because treatment with hCDR1 specifically up-regulated both factors in early stages after immunization with 16/6Id as well as in an established lupus in NZB/W mice (Figs. 3, C and D, and 6, C and D, and Table II). The latter up-regulation in Egr-2 and Egr-3 expression in SLE-afflicted NZB/W mice was accompanied by improvement in the serological, clinical, and immunohistological manifestations of the disease (data not shown). The mechanism by which hCDR1 induces increased expression of these transcription factors is probably through the inhibition of Akt phosphorylation, as in vitro inhibition of PI3K signaling pathway by wortmannin significantly up-regulated Egr-2 and Egr-3 mRNA expression in T cells derived from 16/6Id-immunized mice (Fig. 4). Furthermore, Akt was reported to contribute to cell survival by various mechanisms (30, 31). The up-regulation of Egr-2 and Egr-3 mRNA expression in T cells following the inhibition of PI3K signaling pathway by wortmannin may support a role for Akt in anergy prevention as a possible additional mechanism. The up-regulation of Egr-2 and Egr-3 mRNA expressions following treatment with hCDR1 is of major functional importance because it probably contributes to the hCDR1-induced inhibition of T cell IFN-γ secretion, a cytokine that is elevated in lupus and plays a key role in the pathogenesis of the disease (27, 32–34). Indeed, transfection of T cells derived from mice that were treated with hCDR1 concomitant with 16/6Id immunization with siRNA specific for either Egr-2 or Egr-3 abrogated the inhibition by hCDR1 of IFN-γ secretion by T cells (Fig. 5).
TGFβ was shown to be up-regulated following treatment with hCDR1 and to participate in its mechanism of action, including down-regulation of T cell adhesion, migration, and 16/6Id-stimulated proliferation and IFN-γ secretion (23–26). In the present study (Fig. 6A and B), the addition of TGFβ up-regulated the mRNA expressions of Egr-2 and Egr-3. In addition, Akt was reported (35, 36) to inhibit TGFβ signaling by sequestering Smad3 and therefore leading to inhibition of its function, whereas TGFβ prevents the formation of Akt-Smad3 complexes. Indeed, treatment with hCDR1 down-regulated Akt phosphorylation and thus, probably by regulating the sensitivity to TGFβ, provided an additional pathway for the up-regulation of Egr-2 and Egr-3 mRNA expression. Furthermore, overexpression of Egr-2 and Egr-3 was associated with the increased expression of Cbl-b. Indeed, treatment with hCDR1 up-regulated the protein expression of this E3 ubiquitin ligase.

In conclusion, the apparent down-regulation of murine autoreactive T cell functions, including IFN-γ secretion, is at least partially a consequence of the ability of hCDR1 to induce anergy. This is associated with specific up-regulation of T cell-negative regulators, namely Egr-2 and Egr-3, which are down-regulated in lupus-associated autoreactive T cells. The hCDR1-induced down-regulation of Akt phosphorylation is probably through the inhibition of TCR signaling (26) and costimulation (e.g., LFA-1, 25). Inhibition of Akt phosphorylation leads to up-regulation of Egr-2 and Egr-3 expressions, and the latter transcription factors subsequently inhibit IFN-γ secretion by T cells. The hCDR1-induced up-regulation of Egr-2 and Egr-3 mRNA expressions was found both early in disease induction (10 days after immunization with 16/6Id) and in SLE afflicted NZB/W mice. Therefore, the above-described mechanisms probably play a role in the amelioration of SLE manifestations following treatment with hCDR1.

Disclosures

The authors have no financial conflict of interest.

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