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The Suppression of Murine Lupus by a Tolerogenic Peptide Involves Foxp3-Expressing CD8 Cells That Are Required for the Optimal Induction and Function of Foxp3-Expressing CD4 Cells

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A peptide, designated human CDR1 (hCDR1), that is based on the CDR1 of an anti-DNA Ab ameliorates systemic lupus erythematosus (SLE) in murine models via the induction of CD4+CD25+ regulatory T cells (Tregs). In the present study, the involvement of CD8 Tregs in the mode of action of hCDR1 was investigated in SLE-afflicted (NZB × NZW)F1 mice and in SJL mice following immunization with the lupus-inducing anti-DNA mAb that bears a common Id, 16/6Id. Treatment with hCDR1 up-regulated Foxp3-expressing CD8+CD28+ Tregs in association with clinical amelioration of lupus manifestations. Furthermore, the in vivo depletion of the latter cells diminished the clinical improvement and the inhibitory effects of hCDR1 on the secretion of IFN-γ and resulted in the up-regulation of IL-10. However, the stimulatory effect of hCDR1 on the secretion of TGF-β was not affected by the CD8 Tregs. In the absence of CD8 Tregs, CD4+CD25+ Tregs were unable to expand in the hCDR1-treated mice, and the expression of Foxp3 was reduced, thereby interfering further with the suppressive function of CD4+CD25+ Tregs as determined in the in vitro assays. However, CD8 cells from hCDR1-treated mice that were adoptively transferred into SLE-affected mice led to up-regulation of CD4+CD25+ cells with intensified Foxp3 expression in the recipient mice. Thus, a functional link between two subsets of Tregs is demonstrated in which CD8+CD28+ Tregs are required for both the optimal expansion and function of lupus ameliorating hCDR1-induced CD4+CD25+ Tregs.

CD8+CD28- cells were shown to be essential for the induction and the optimal suppressive function of CD4+CD25+ cells following treatment with hCDR1. Thus, interactions between the two hCDR1-induced subsets of Tregs result in the effective suppression of SLE-associated responses.

Materials and Methods

Mice
Female (New Zealand Black × New Zealand White)F1 (BWFI) mice and female inbred SJL mice were purchased from The Jackson Laboratory. The study was approved by the Animal Care and Use Committee of the Weizmann Institute of Science, Rehovot, Israel.

Synthetic peptides
A peptide, GYYWSWIRQPAGKGEWIG (hCDR1), based on the CDR1 of the human anti-DNA mAb (5) bearing the 16/6Id was synthesized (solid phase synthesis by Fmoc chemistry) by Polypeptide Laboratories and used in this study. A peptide containing the same amino acids as hCDR1 in a scrambled order (scrambled peptide), SKGIFQYGWPWEGWRYEI, was used as a control. The control peptide binds MHC class II with an avidity similar to that of hCDR1.

Monoclonal antibodies
The human anti-DNA 16/6Id (IgG1/k) was secreted by hybridoma cells (5) that were grown in culture and was purified by a protein G-Sepharose column (Pharmacia Fine Chemicals). The following Abs were used for immunofluorescent staining of cells: anti-CD4-PE (clone GK1.5), anti-CD4-allophycocyanin (clone L3T4), anti-CD25-FITC (clone JD4), anti-CD8-FITC (clone 53-6.7), anti-CD19-FITC (clone 6D5), anti-CTLA-4-PE (clone 1B8); these Abs and their matched isotype controls were obtained from Southern Biotechnology Associates. Anti-CD25-PE (clone 37.51), anti-CD69-PE (clone H1.2F3), anti-Fas-L-PE (clone MFL3), and their matched isotype controls were purchased from BD Pharmingen. Anti-Foxp3-FITC (clone FJK-16s Set) was purchased from eBioscience. Anti-Blc-xL-PE (clone H-5) and its isotype control were purchased from Santa Cruz Biotechnology.

Immunization with 16/6Id and treatment with hCDR1
SJL mice were immunized with 1 μg of the human mAb 16/6Id in CFA and were either not injected or injected s.c. with hCDR1 (50 μg/mouse) or the control scrambled peptide concomitant with the 16/6Id immunization.

Proliferation assays
Ten days after immunization with 16/6Id, mice were euthanized and inguinal lymph node (LN)-derived cells were used for proliferation assays as previously described (9).

Treatment of SLE-affected BWFI mice with hCDR1
Eight-month-old BWFI female mice were treated during a 10-wk period with weekly s.c. injections of hCDR1 (50 μg/mouse), the scrambled peptide, or the vehicle alone (Capsitol (sulfobutyl ether-β-cyclodextrin), a solvent designed by CyDex to enhance the solubility and stability of drugs).

In vivo depletion of CD8+ cells
SJL mice were injected i.p. with 250 μg of anti-CD8 mAb (20) on days −4, 0, and 4 relative to the day of immunization with 16/6Id (counted as day 0). SLE-affected BWFI mice were injected i.p. with 250 μg of anti-CD8 mAb every 4 days during the first and the last 3 wk of the experiment and once a week during the 4-wk period in between. Depletion rate (>91%) was verified by FACS. Total counts of CD4+ T cells and CD19+ B cells were not significantly changed following the depletion.

Isolation of CD8 cells
Cells were labeled with the BD Imag Mouse CD8 T Lymphocyte Enrichment Set-DM and separated (~90% purity) on the BD Imagnet according to the manufacturer’s protocol (BD Biosciences).

Depletion and enrichment of CD4+CD25+ cells
The procedure for depletion and enrichment of CD25+ cells is described elsewhere (11).

Measurement of dsDNA-specific Ab
Anti-dsDNA Abs were detected using λ phase dsDNA, as previously described (9).

Cytokine production and detection by ELISA
Supernatants of splenocytes (5 × 10^6/ml) were removed after 48 h (for detecting IFN-γ and IL-10) and 72 h (for detecting TGF-β) incubation and analyzed, as previously described (9).

FACS analysis
Cells were incubated with the relevant Ab and analyzed by FACS (BD Biosciences). For intracellular staining the cells were incubated with a fixation solution, washed, and resuspended in permeabilization solution (Serotec).

Detection of proteinuria
Proteinuria was measured by a standard semiquantitative test using an Al-bustix kit (Bayer Diagnostic).

Immunohistology
Renal immune complex deposits (ICD) were determined as described (11). The intensity of the ICD was graded as follows: 0, no ICD; 1, low intensity; 2, moderate intensity; and 3, high intensity of ICD. ICD analysis was performed by two persons blinded to whether mice belonged to the control or the experimental group.

Statistical analysis
The Student’s t test and the Mann-Whitney U test were used. Values of p ≤ 0.05 were considered significant.

Results

The effect of treatment with hCDR1 on the development of CD8 Tregs
The frequency of two subsets of Tregs was determined in LN-derived lymphocytes of 2-mo-old SJL mice 10 days following immunization with 16/6Id in CFA concomitantly with s.c. injections of either hCDR1 (50 μg/mouse) or the vehicle. As shown in Fig. 1A, treatment with hCDR1 resulted in the up-regulation of CD4+CD25+ cells in which the expression of Foxp3 was also elevated compared with that in the vehicle-injected mice (Fig. 1B). Fig. 1C demonstrates that the absolute numbers of CD4+CD25+Foxp3+ cells were raised from 3.5 million in the vehicle-treated group to ~6 million cells in the hCDR1-treated group. In addition, injection of hCDR1 resulted in an increase of >50% in the total CD8+ cell population compared with the vehicle-injected mice. Yet, the CD8+CD28− cell compartment (Fig. 1D) that expressed Foxp3 (Fig. 1E) was the most affected. The mean absolute number of CD8+CD28−Foxp3+ cells was 10-fold higher following treatment with hCDR1 compared with the vehicle-treated group (Fig. 1F). Note that the treatment of unimmunized mice with hCDR1 resulted in Treg levels that were similar to those observed in 16/6Id-immunized, hCDR1-treated mice, namely 5.6 ± 0.3% ([5.5 ± 0.6] × 10^6 cells) and 11.8 ± 1.9% ([2.8 ± 0.6] × 10^6 cells) for CD4+ and CD8+ Foxp3-expressing Tregs, respectively. The CD8-expressing subset of cells that was the most affected by hCDR1 belonged to the T cell compartment, because 20% of the LN cells of hCDR1-treated mice were CD8+CD25+ cells (Fig. 1, G and H) whereas the frequency of CD8+ dendritic cells (DC) (CD8+CD11c−) was <0.5%. Moreover, the expression of Foxp3, which is required for regulatory activity, was almost exclusively determined in CD8-expressing T cells and not in DC.

In addition to the short-term experiments, we followed the kinetics of Tregs in BWFI mice that spontaneously develop manifestations of SLE. In these experiments, mice with an advanced disease (proteinuria levels ≥3 g/L) were divided into three groups

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Depletion of CD8 cells abrogates hCDR1-suppressive effects on IFN-γ and IL-10 secretion

We measured the levels of cytokines in the supernatants of splenocytes derived from 16/6Id-immunized mice that were concomitantly injected with anti-CD8 neutralizing mAb. A representative cytokine profile of one (of three) short-term experiment can be seen in Fig. 3A. The levels of the pathogenic cytokines IFN-γ and

FIGURE 2. Kinetics of CD8 and CD4 Tregs in BWF1 mice. Eight-month-old SLE-afflicted BWF1 mice (n = 15–17 mice/group) were treated once a week for 10 wk with hCDR1, a control scrambled peptide (50 μg/mouse), or the vehicle. After 3, 5, and 10 injections the splenocytes from mice of each group (n = 5/time point) were stained and analyzed for the presence of Foxp3-expressing CD8^+ CD28^- and CD4^+ CD25^+ cells. Mean percentages and absolute numbers (±SD) of two independent experiments are shown. *, p < 0.05 compared with the vehicle-treated group.

FIGURE 3. Depletion of CD8^+ cells abrogates hCDR1-suppressive effects on IFN-γ and IL-10. A, Splenocytes (5 × 10^6/ml) from individual 16/6Id-immunized mice in each group were incubated in enriched medium in the presence of 16/6Id (25 μg/ml) for 48 h (for detecting IFN-γ and IL-10) and 72 h (for detecting TGF-β). The levels of secreted cytokines were measured in the supernatants by ELISA. Mean levels (±SD) in individual mice in each treatment group are shown. The results represent one of three experiments performed. B, Eight-month-old SLE-afflicted BWF1 mice were divided into six groups (n = 5–7 mice/group) and treated with 10 weekly injections of vehicle alone, hCDR1 alone, or the latter injected with either the anti-CD8 mAb or control IgG. After 10 wk, the splenocytes (5 × 10^6/ml) of individual mice were incubated in enriched medium for 48 and 72 h. Resultsrepresent the mean cytokine concentration of supernatants of individual mice per treatment group. *, p < 0.05 compared with the vehicle-treated group.

(n = 15–17 mice/group) for 10 weekly s.c. injections of hCDR1 (50 μg/mouse), scrambled (control) peptide (50 μg/mouse), or the vehicle. Fig. 2 shows the percentages and the mean absolute numbers of CD8^+ CD28^- Foxp3^- cells and CD4^+ CD25^+ Foxp3^- cells of two independent experiments during the treatment period. It can be seen in the figure that the number of CD8^+ CD28^- Foxp3^- cells was significantly up-regulated in the hCDR1-treated mice whereas no significant changes could be determined in the scrambled peptide-treated mice in comparison to the vehicle-treated mice. The number of CD4^+ CD25^+ Foxp3^- cells also increased significantly in the SLE-afflicted mice that were treated with hCDR1.

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IL-10 were significantly reduced in 16/6Id-immunized, hCDR1-treated mice as compared with vehicle-treated mice. Administration of anti-CD8 mAb to the experimental mice abrogated the ability of hCDR1 to down-regulate IFN-γ, whereas injection of an IgG control to hCDR1-treated mice did not interfere with the down-regulation of this cytokine. Notably, the levels of IL-10 in either the vehicle-treated mice or the hCDR1-treated mice that received anti-CD8 mAb were substantially elevated, whereas treatment with IgG did not interfere with the suppressive activity of hCDR1 (Fig. 3A). The secretion of TGF-β was up-regulated following treatment with hCDR1 and was not affected by the depletion of CD8 cells (Fig. 3A). The results with the 16/6Id-immunized mice were confirmed in a long-term treatment experiment performed on SLE-affected BWF1 mice (n = 5–7/group) that were analyzed individually (Fig. 3B).

**Depletion of CD8+ cells interferes with the clinical ameliorative effects of hCDR1**

The impact of CD8 depletion on the hCDR1 therapeutic effects was also assessed in the SLE-affected BWF1 mice. As shown in Fig. 4A, the titers of Ab against dsDNA were reduced following treatment with hCDR1 (with or without the injection of control IgG); however, administration of anti-CD8 mAb to the diseased mice abrogated the inhibitory effect of hCDR1 on the production of these Ab. The levels of proteinuria in the vehicle-treated mice rose progressively (from 8 to 16 g/L) during the follow-up period. Depletion of CD8+ cells in these mice resulted in similar high rates of proteinuria. Administration of the control IgG moderately decreased proteinuria levels; however, compared with vehicle-only treated group, the differences were not significant (Fig. 4B). Treatment with either hCDR1 or hCDR1 and IgG resulted in significantly lower levels of proteinuria. However, injection of anti-CD8 mAb into hCDR1-treated mice partially interfered with the beneficial effect of hCDR1 on the levels of proteinuria (Fig. 4B). The mean ICD intensity in the glomeruli of mice is presented in Fig. 4C. It can be seen that the injection of anti-CD8 mAb to hCDR1-treated mice abrogated the effect of hCDR1 in comparison to the injected control IgG, which did not interfere with the suppressive effect of hCDR1. It is noteworthy that the mortality rate of vehicle- and hCDR1-treated mice was 40 and 10%, respectively, in all experiments performed in this study. Depletion of CD8 cells did not affect significantly the survival rate of the mice.
Depletion of CD8+ cells in hCDR1-treated mice interferes with the up-regulation and function of CD4+CD25+Foxp3+ cells

We investigated the effect of the depletion of CD8 cells on the induction of CD4+CD25+Foxp3+ cells in hCDR1-treated, SLE-affected BWF1 mice. The expression of Foxp3 in CD4+CD25+ cells is depicted in Fig. 5A1, which shows representative histograms of individual mice from the different groups; the mean results of all mice per treatment group are summarized in Fig. 5A2. Both the percentage and the absolute number of double-positive CD4 and CD25 cells were higher in the hCDR1-treated group relative to the-vehicle injected mice (e.g., 8.3 ± 0.6 vs 6.7 ± 0.9%; p < 0.05), and these results were not significantly different following treatment with anti-CD8 mAb (Fig. 5A2). However, it can be seen in the figure that the percentages of CD4+CD25+ cells that express Foxp3 were reduced following depletion of CD8 cells in both the vehicle- and the hCDR1-treated groups. The intensity of Foxp3, which was higher in the hCDR1-induced CD4 Tregs, was also lower following depletion of the CD8 cells in both the vehicle- and the hCDR1-treated groups. Similar effects of CD8 depletion on the induction of CD4+CD25+Foxp3+ cells were observed in the 16/6ld-immunized mice that were treated with hCDR1 (data not shown).

To assess the function of hCDR1-induced CD4+CD25+ cells in the CD8-depleted mice, splenocytes (5 × 10^6/well) derived from individual vehicle-treated, SLE-affected BWF1 mice injected with either the anti-CD8 mAb (n = 5) or IgG (n = 5) were incubated alone or together with hCDR1-induced CD4+CD25+ cells (0.5 × 10^6/well) isolated from mice (n = 5) injected with the anti-CD8 mAb (i.e., hCDR1/Treg/antiCD8 cells) or from mice (n = 5) injected with IgG (i.e., hCDR1/Treg/IgG cells). Thereafter, the levels of secreted cytokines in the supernatants of the culture mixtures were determined. As shown in Fig. 5B, the levels of the pathogenic cytokines IFN-γ and IL-10 in the supernatants of splenocytes from the vehicle-treated, anti-CD8 mAb-injected mice were significantly higher (p < 0.05) than those from mice injected with IgG. However, the incubation of vehicle-treated cells that were either depleted or not depleted of CD8 cells with hCDR1/Treg/IgG cells significantly down-regulated the secretion of the pathogenic cytokines. Isolated hCDR1/Treg/anti-CD8 cells were significantly less effective in diminishing the secretion of IFN-γ (p = 0.02 for hCDR1/Treg/IgG cells as compared with hCDR1/Treg/anti-CD8 cells) and did not affect at all the secretion of IL-10 (Fig. 5B). The levels of TGF-β in supernatants of splenocytes from the vehicle-treated mice were not affected by CD8 depletion, and incubation with hCDR1-induced CD4+CD25+ cells of either anti-CD8 or IgG-injected mice resulted in the up-regulation of this cytokine.

CD8 cells from hCDR1-treated mice possess regulatory characteristics

We investigated the direct suppressive effects of CD8 cells. To this end, 2-mo-old SJL mice (n = 3) were treated with 3 s.c. injections of hCDR1 (50 μg/mouse) or the vehicle on alternating days during 1 wk. Thereafter, CD8 cells were isolated from the LN (~90% purity, as shown in Fig. 6A). Whereas 70% of the CD8 cells from
hCDR1-treated mice were determined to negatively express the costimulatory molecule CD28 and to mainly express Foxp3, only 40% of the vehicle-derived CD8 cells were CD28 negative with a minute expression of Foxp3. Furthermore, the CD8 cells (at a ratio of 1:5) from hCDR1- and not from the vehicle-treated mice inhibited the proliferation of 16/6Id-stimulated LN-derived cells from 16/6Id-immunized mice (Fig. 6B). In contrast, CD8 cells derived from hCDR1-treated mice were incapable of suppressing polyclonal proliferation of spleen-derived lymphocytes of naive mice following their stimulation with anti-CD3 and anti-CD28 mAb (Fig. 6B2), suggesting the specificity of the activity of the hCDR1-derived CD8 Tregs. In addition, the enhanced secretion of IFN-γ and IL-10 by splenocytes from 16/6Id-immunized mice was significantly reduced (Fig. 6C) after incubation with isolated CD8 cells (at ratios of 1:50 and 1:5) from hCDR1- and not from vehicle-treated mice. It should be noted that CD8 Tregs from hCDR1-treated, SLE-affected BWF1 mice exhibited similar in vitro suppressive effects, because the baseline rates of 25.9 ± 1.5% activated CD4+ cells (i.e., CD4+CD69+ cells) in the spleens of diseased mice could be reduced significantly to levels of 18.0 ± 2.7% in the presence of the hCDR1-derived CD8 Tregs in the coculture.

**CD8 cells from hCDR1-treated mice play a role in the induction of Foxp3-expressing CD4+CD25+ cells**

We analyzed the status of CD4+CD25+Foxp3+ cells in the diseased mice following the administration of CD8 cells from hCDR1-treated mice. For this purpose, the total cell population and isolated CD8 cells were taken from spleens of 2-mo-old hCDR1-treated mice and isolated CD4 cells (7 × 10^6/mouse) from splenocytes of the hCDR1-treated or vehicle-treated groups were adoptively transferred to SLE-affected BWF1 mice (n = 4–8/group). Three weeks later splenocytes of the recipient mice were assessed individually for the presence of CD4+CD25+ cells (representative dot plots) and their expression of Foxp3 (representative histograms, in which gray histograms indicate staining with the isotype control). A. Recipients of total cell population. B. Recipients of isolated CD8 cells. C. Percentages of CD4+CD25+ cells that express Foxp3 (left) and the mean fluorescence intensity (MFI) of Foxp3 expression in CD4+CD25+ cells (right). Shown are the mean results (±SD) of individual recipient mice per group from a representative experiment of two performed. *, p < 0.05 compared with the recipients of vehicle-treated cells.

In each experiment there were 4–8 recipient SLE-affected BWF1 mice in each group. The titers of anti-dsDNA Ab were reduced in recipients of the total cell population from hCDR1-treated mice, but this effect was abolished when the cell population was depleted of CD8 and CD4+CD25+ cells. Further, the titers of dsDNA-specific Ab were significantly reduced following the administration of hCDR1-induced CD4+CD25+ cells, whereas the titers remained high when hCDR1-induced CD8 cells were injected (Fig. 8). In regard to the renal disease, SLE-affected mice that received the total cell population from hCDR1-treated mice had a significant improvement in the levels of proteinuria and a substantial lower degree of glomerular ICD, but these effects were abrogated when both CD8 and CD4+CD25+ cells were depleted. It is also seen in Fig. 8 that the administration of hCDR1-induced CD4+CD25+ cells resulted in diminished levels of proteinuria and ICD, whereas a suppressive effect of hCDR1-induced CD8 cells was demonstrated in these experiments only on the level of ICD (Fig. 8). The effect of the CD8 cells on ICD, although significant, was less pronounced than that of hCDR1-induced CD4+CD25+ cells.

**Comparison between hCDR1-induced CD8 and CD4+CD25+ cells in ameliorating SLE**

Because CD8 cells of hCDR1-treated mice were shown to be capable of inducing Foxp3-expressing CD4+CD25+ cells, it was of importance to assess the inhibitory competence of the former cells and to compare it to that of CD4+CD25+ cells. To this end, BWF1 mice with established manifestations of lupus (baseline proteinuria levels of 4 g/L) were injected i.p. with one of the following four types of cell populations originating from the spleens of either hCDR1- or vehicle-treated, free-of-disease, BWF1 mice: total cell population (20 × 10^6/mouse), total cell population depleted of CD8 and CD4+CD25+ cells (20 × 10^6/mouse), isolated CD8 cells (7 × 10^6/mouse), and isolated CD4+CD25+ cells (7 × 10^6/mouse). Three weeks following the adoptive transfer of the cells, the mice were clinically evaluated. The results of two independent adoptive transfer experiments are combined and shown in Fig. 8.

**FIGURE 7.** hCDR1-derived CD8 cells play a role in the induction of Foxp3-expressing CD4+CD25+ cells. Two-month old BWF1 mice (n = 5/group) were injected with three s.c. injections of hCDR1 (50 μg/mouse) or the vehicle on alternating days. Total cell population (20 × 10^6/mouse) and isolated CD8 cells (7 × 10^6/mouse) from splenocytes of the hCDR1-treated or vehicle-treated groups were adoptively transferred to SLE-affected BWF1 mice (n = 4–8/group). Shown are the mean results (±SD) of individual recipient mice per group from a representative experiment of two performed. *, p < 0.05 compared with the recipients of vehicle-treated cells.
hCDR1-induced CD8 cells were less effective in cytokine inhibition in comparison to hCDR1-induced CD4+CD25+ cells.

**hCDR1-derived CD8 cells do not induce tolerogenic-associated molecules that play a role in SLE amelioration**

The ability of the two types of regulatory cells to induce two major tolerogenic-associated molecules, namely CTLA-4 and Bcl-xL, which are of importance in the mechanism of action of hCDR1 (10, 12), was assessed. Fig. 10 demonstrates that the adoptive transfer of a total cell population from hCDR1-treated (free-of-antibodies) BWF1 healthy donors as compared with cells from vehicle-treated donors (Fig. 10, A1–C1). Mean percentages (±SD) of cells expressing the relevant molecules. A2–C2, Representative histograms of individual mice. *, p < 0.05 compared with the recipients of total cell population from the vehicle-treated group.

**Discussion**

The main findings of this study are that Foxp3-expressing CD8+CD28− cells play an important role in the amelioration of SLE manifestations following treatment with the tolerogenic peptide hCDR1. The latter cells are essential for the induction and the optimal function of CD4+CD25+ regulatory cells, suggesting that interactions involving the two cell populations lead to the observed suppression of SLE-associated responses in mice that are treated with hCDR1.

T lymphocytes with inhibitory capabilities are important in regulating immune responses, and when the suppressive cells are deficient or interference of the suppressor function of the cells occurs, autoimmunity might emerge (21). Of the T lymphocytes, abnormalities in regulatory CD4 and CD8 cells are reported in SLE patients and in lupus-prone mice (11, 16, 18, 19, 22–25). The role of CD8 cells in the pathogenesis of SLE was demonstrated in previous studies. Hence, depletion of CD8 cells accelerated the development of SLE in genetically prone (NZB × BXB)F1 mice
CD8 Tregs ARE REQUIRED FOR POTENT hCDR1-INDUCED CD4 Tregs

(26) and in mice with 16/6Id-induced disease (27). Likewise, CD8 Tregs with a C57BL/6 background that is resistant to SLE induction (28) were shown to be susceptible to the experimental disease (29). In addition, when the 16/6Id-immunized mice were injected with inhibitory CD8 cells specific for 16/6Id, the disease manifestations were prevented (30).

The present study investigated the effects of treatment with hCDR1 on the status of CD8 cells in two settings of lupus-associated responses, namely in short-term experiments 10 days following the immunization of nonautoimmune-prone mice with the SLE-inducing mAb 16/6Id and in long-term experiments in lupus-prone BWF1 mice with a full-blown disease. Treatment of both 16/6Id-immunized mice and BWF1 mice that have established SLE with hCDR1 resulted in a significant up-regulation in two subsets of Tregs (Figs. 1 and 2), namely in Foxp3-expressing CD8+CD28- cells (i.e., CD8 Tregs) and in Foxp3-expressing CD4+CD25+ cells (CD4 Tregs). CD8+ DC were reported to also have a tolerance-inducing role (31); however, the results of the present study indicated that the vast majority of the hCDR1-induced Foxp3-expressing CD8 cells belonged to the T cell compartment, whereas the numbers of CD8+ DC were negligible (Fig. 1, G and H).

The significant role of CD8 Tregs in the mechanism of action of hCDR1 was demonstrated in CD8 cell depletion experiments. Thus, hCDR1 treatment of SLE-afflicted mice that were depleted of CD8 cells failed to reduce the production of autoantibodies against dsDNA and to ameliorate the renal disease. Further, in vivo depletion of CD8 cells abrogated the down-regulatory effects of hCDR1 on the secretion of IFN-γ and IL-10 in the SLE-prone mice, and the replenishment of CD8 cells from hCDR1-treated donors in diseased mice resulted in a diminished secretion of IFN-γ and IL-10 in the recipient mice. Both cytokines play a role in the pathogenesis of SLE (32, 33) and, therefore, the involvement of CD8 Tregs in mediating the suppressive effects of hCDR1 on the cytokines further emphasizes the significant role of these cells. In contrast, the adoptive transfer of CD8 cells from hCDR1-treated healthy donors into diseased mice revealed that the cells’ direct effect was not complete because the titers of anti-dsDNA Ab were not reduced in the recipient mice, while the glomerular ICD were decreased but without improving the levels of proteinuria. In agreement with this, a partial clinical effect of CD8 Tregs on manifestations of SLE was demonstrated in a nucleosomal peptide-based protocol for tolerance induction (19). In contrast to the effect of CD8 Tregs, the adoptive transfer of CD4 Tregs from hCDR1-treated donors was more prominent in ameliorating the disease manifestations of SLE-afflicted mice (Figs. 8–10 and Ref. 11). Hence, it appears that hCDR1-induced CD8 Tregs act not as dominant suppressor cells but rather play a role in the development of CD4 Tregs that mediate the inhibitory effects of hCDR1 on SLE manifestations. An effect of CD8 Tregs on CD4 Tregs was also suggested to be beneficial in another model of autoimmunity, namely, experimental autoimmune encephalomyelitis (34). It is noteworthy that treatment with hCDR1 or transfer of hCDR1-induced CD8 Tregs might have also an effect on non-T cell populations such as DC; however we focused in the present study on the interactions between the populations of Tregs.

Evidence for interactions between CD8 and CD4 Tregs are as follows: 1) CD4 Tregs from hCDR1-treated healthy donors that were depleted of CD8 cells were significantly less effective in diminishing the secretion of IFN-γ and IL-10 by splenocytes of SLE-afflicted BWF1 mice; 2) the depletion of CD8 cells in hCDR1-treated mice interfered with both the expansion of CD4+CD25+ cells and the intensity of Foxp3 expression (Fig. 5); 3) the CD8 cells in hCDR1-treated mice were directly involved in the induction of CD4 Tregs because the adoptive transfer of CD8 cells from hCDR1-treated healthy donors into diseased mice resulted in the up-regulation of CD4+CD25+ cells and in the increased intensity of the expression of Foxp3 (Fig. 7). Our data thus indicate, for the first time, a functional link between these two cell populations in circumstances of a tolerogenic therapeutic regimen for SLE.

The specificity of hCDR1-induced CD8 Tregs is suggested for several reasons. First, CD8 Tregs of naive mice that were treated with the vehicle were unable to either suppress 16/6Id-triggered proliferation or down-regulate the secretion of the pathogenic cytokines (Fig. 6, B1 and C). Furthermore, hCDR1-induced CD8 Tregs did not inhibit polyclonal lymphocyte proliferation (Fig. 6B2). In addition, the adoptive transfer of hCDR1 and not of vehicle-derived CD8 cells down-regulated the expression of FasL in CD4 cells (Fig. 10C), the secretion of the pathogenic cytokines (Fig. 9), and the ICD in the kidneys (Fig. 8). These results are supported by our previous studies showing that hCDR1 did not inhibit immune responses to nonrelevant Ag (35).

It is likely that hCDR1-induced CD8 Tregs that were taken from CD8 cell-depleted mice were unable to decrease the high titers of autoantibodies in the diseased mice due to their inability to suppress the secretion of IL-10, a cytokine with potent activity on B cell proliferation and differentiation (36). Likewise, the ability of CD8 Tregs that were induced in hCDR1-treated mice to attenuate the glomerular ICD in the SLE-afflicted recipient mice could be the result of suppressed secretion of IFN-γ. In support of this, it has been demonstrated that the down-regulation of IFN-γ by itself was sufficient to decrease ICD (32). An additional explanation for the incomplete clinical effects of CD8 Tregs is the fact that the hCDR1-derived CD8 Tregs, in contrast to hCDR1-derived CD4 Tregs, were unable to up-regulate the expressions of CTLA-4 and Bcl-xL in CD4 cells of the SLE-afflicted mice (Fig. 10). In this regard, we have previously shown the important contribution of the major tolerogenic molecules CTLA-4 and Bcl-xL in mediating the suppressive effects of CD4 Tregs in the hCDR1-treated mice (10, 12).

TGF-β is considered to be a key factor that is essential for suppression of autoreactivity. Under tolerization protocols, this immunosuppressive cytokine was shown to be secreted by different types of cells, including CD4 cells (11), CD8 cells (18), and DC (37). In this study we demonstrated that the secretion of TGF-β may be elicited even in the absence of CD8 cells (Fig. 3). Also, hCDR1-induced CD4 Tregs from CD8 cell-depleted healthy donors were capable of up-regulating the secretion of TGF-β when cocultured with splenocytes from CD8 cell-depleted diseased mice (Fig. 5). These results are in line with our previous studies, showing that the CD4 cells of SLE-afflicted mice were the main source for TGF-β following the adoptive transfer of hCDR1-induced CD4 Tregs (11). The results of the present study suggest that the role of TGF-β is limited. Thus, even when the secretion of TGF-β was not affected in hCDR1-treated, SLE-afflicted mice, only a partial clinical response (i.e., preventing further deterioration of proteinuria levels) could be observed in the absence of CD8 cells. In accordance with this, Singh et al., using another Ig VH peptide for the treatment of SLE, reported recently that TGF-β could not be the sole element for inhibiting autoreactivity (18).

Our results clearly indicate that CD8 Tregs, which constitute the majority of CD8 cells in the hCDR1-treated mice, were required for the optimal expansion and function of hCDR1-induced CD4 Tregs. Accordingly, the induction of CD8 Tregs should precede the generation of CD4 Tregs. The latter was also suggested by Suciu-Foca and colleagues, indicating that such a sequence of events could promote infectious tolerance (38). Alternatively, the
induction of CD4⁺/CD8⁺ T cells in the hCDR1-treated mice could be followed by the generation of CD8 Tregs because of the ability of the CD4⁺/CD25⁺ T cells to trigger the secretion of TGF-β (Fig. 5 and Ref. 11), which is of importance for the development of the CD8 Tregs (39, 40). It may be concluded that for an optimal effect of hCDR1 on lupus manifestations, the induction and interactions between the two cell populations of Tregs are required.

Disclosures
The authors have no financial conflict of interest.

References