A peptide based on the complementarity-determining region 1 of an autoantibody ameliorates lupus by up-regulating $CD4^+CD25^+$ cells and TGF-β

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Communicated by Michael Sela, Weizmann Institute of Science, Rehovot, Israel, April 25, 2006 (received for review March 2, 2006)

Systemic lupus erythematosus is an autoimmune disease characterized by autoantibodies and systemic clinical manifestations. A peptide, designated hCDR1, based on the complementarity-determining region (CDR) 1 of an autoantibody, ameliorated the serological and clinical manifestations of lupus in both spontaneous and induced murine models of lupus. The objectives of the present study were to determine the mechanism(s) underlying the beneficial effects induced by hCDR1. Adoptive transfer of hCDR1-treated cells to systemic lupus erythematosus-afflicted $(NZB\times NZW)F_1$ female mice down-regulated all disease manifestations. hCDR1 treatment up-regulated (by 30–40%) $CD4^+CD25^+$ cells in association with $CD45RB^{low}$, cytotoxic T lymphocyte antigen 4, and Foxp3 expression. Depletion of the $CD25^+$ cells diminished significantly the therapeutic effects of hCDR1, whereas administration of the enriched $CD4^+CD25^+$ cell population was beneficial to the diseased mice. Amelioration of disease manifestations was associated with down-regulation of the pathogenic cytokines (e.g., IFN-γ and IL-10) and up-regulation of the immunosuppressive cytokine TGF-β, which substantially contributed to the suppressed autoreactivity. TGF-β was secreted by $CD4^+$ cells that were affected by hCDR1-induced immunoregulatory cells. The hCDR1-induced $CD4^+CD25^+$ cells suppressed autoreactive $CD4^+$ cells, resulting in reduced rates of activation-induced apoptosis. Thus, hCDR1 ameliorates lupus through the induction of $CD4^+CD25^+$ cells that suppress activation of the autoreactive cells and trigger the up-regulation of TGF-β.

cytokines | Foxp3 | immunomodulating peptide | regulatory T cells | systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of Ab against nuclear antigens and damage to multiple organs including kidneys, CNS, joints, and skin (1). Several strains of mice that spontaneously develop an SLE-like disease were reported, of which the $(NZB\times NZW)F_1$ female mice are the most widely used (2, 3). In addition, our laboratory has established a model of experimentally induced SLE in different susceptible strains of mice (4–6).

A peptide, designated hCDR1, based on the complementarity-determining region (CDR) 1 (7) of a human anti-DNA mAb, was shown to ameliorate the serological and clinical manifestations in both the spontaneous and induced models of SLE and to reduce the secretion and expression of the pathogenic cytokines IFN-γ, IL-10, IL-1β, and TNF-α (the latter in the induced model) while up-regulating the immunosuppressive cytokine TGF-β (8).

It has become increasingly evident that peripheral tolerance is mediated by suppressor T cells with a regulatory function (9, 10). The best-characterized are the $CD4^+CD25^+$ cells, which constitute 5–10% of the $CD4^+$ cells (11). $CD4^+CD25^+$ cells are naturally occurring, whereas adaptive regulatory $CD4^+CD25^-$ cells with suppressive capacity may be induced in the periphery in response to tolerogenic stimuli (11, 12). Nevertheless, several *in vitro* and *in vivo* studies indicated that $CD25^+$ cells might also be generated in the periphery (13–20). Recently it was reported that the number of $CD4^+CD25^+$ cells is diminished in patients with SLE as well as in $(NZB\times NZW)F_1$ female mice with established lupus (21–23), thus suggesting a role for these cells in regulating the disease.

In the present study we attempted the elucidation of the mechanism(s) underlying the ameliorating effects of treatment with hCDR1 on SLE manifestations. We demonstrated that the inhibitory effects of hCDR1 can be adoptively transferred to mice with established lupus by cells originating from young, healthy $(NZB\times NZW)F_1$ female mice that were treated with hCDR1. $CD4^+CD25^+$ cells were up-regulated in the hCDR1-treated cell population and were found to play a crucial role in ameliorating the serological and clinical parameters of SLE. This improvement was achieved by suppressing the activation of the $CD4^+$ cells and by triggering the up-regulated secretion of TGF-β, which was shown to play a key role in down-regulating SLE manifestations.

Results

Adoptive Transfer of Spleen Cells from Mice Treated with hCDR1 to $(NZB\times NZW)F_1$ Mice with Established Disease Is Beneficial. To determine whether the beneficial effects of hCDR1 can be transferred by cells of treated mice, we first performed adoptive transfer experiments. Thus, 2-mo-old, disease-free, $(NZB\times NZW)F_1$ mice were injected with hCDR1 s.c. (50 μg per mouse) for 3 alternating days. Two control groups of young mice were treated with the vehicle or with a scrambled (control) peptide. Splenocytes (20×10^6 per mouse) from the different groups were injected i.p. to respective groups of 8-mo-old $(NZB\times NZW)F_1$ mice with established disease. Disease severity of the recipient mice was similar in all groups as assessed by their anti-dsDNA autoantibody titers and proteinuria levels. Fig. 1 summarizes the clinical effects of the transferred cells on lupus-like manifestations at the end of a 2-wk follow-up and represents one experiment of five performed. As demonstrated, the production of dsDNA-specific autoantibodies as well as elevated proteinuria levels and the formation of glomerular immune complex deposits (ICD) were significantly reduced in SLE-afflicted mice that were injected with the hCDR1-treated spleen cells compared with recipients of cells treated with the scrambled peptide or with the vehicle.

Treatment with hCDR1 Results in an Up-Regulation of $CD4^+CD25^+$ Cells. Because $CD4^+CD25^+$ cells are the most characterized immunoregulatory T cells and because these cells were shown to be protective against autoimmune responses, we studied their possible role in the mode of action of hCDR1. For this purpose, three groups of 2-mo-old $(NZB\times NZW)F_1$ mice were treated with hCDR1, the vehicle, or a scrambled peptide. In all experiments the magnitude of $CD4^+CD25^+$ cells ranged between 3% and 9%. Treatment with

Conflict of interest statement: M.S. serves on the Board of Directors of Teva Pharmaceutical Industries, which supported this study.

Abbreviations: CDR, complementarity-determining region; CTLA-4, cytotoxic T lymphocyte antigen 4; FasL, Fas ligand; ICD, immune complex deposits; SLE, systemic lupus erythematosus; PE, phycoerythrin.

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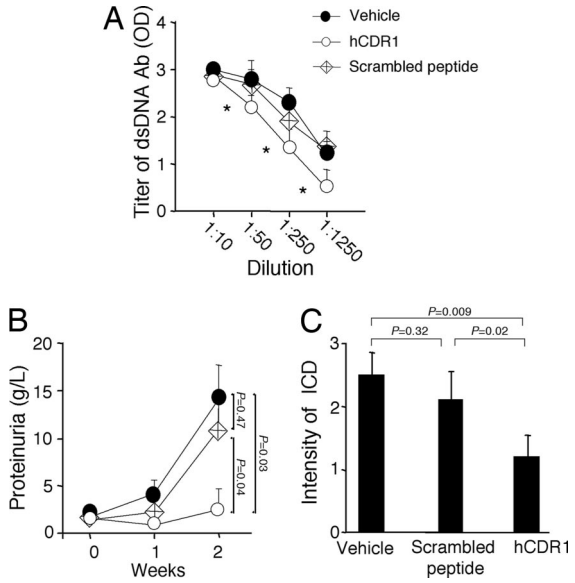

Fig. 1. Amelioration of murine lupus by adoptive transfer of cells from hCDR1-treated mice. Two-month-old (NZB×NZW)F_1 female mice were treated with three s.c. injections of hCDR1, the vehicle, or a scrambled control peptide. Splenocytes of the different groups were injected i.p. (20×10^6 per mouse) to respective groups of 8-mo-old (NZB×NZW)F_1 mice with established lupus. (*A*) Titers (mean OD ± SD) of dsDNA-specific Ab (*, $P < 0.05$) in individual sera of recipient mice ($n = 6$–10 mice per group) 2 wk after cell transfer. (*B*) Kinetics of proteinuria levels (mean grams per liter ± SD) in the recipient mice. (*C*) Mean intensity ± SD of ICD of kidney sections of all recipient mice per treatment group. Results represent one of five independent experiments.

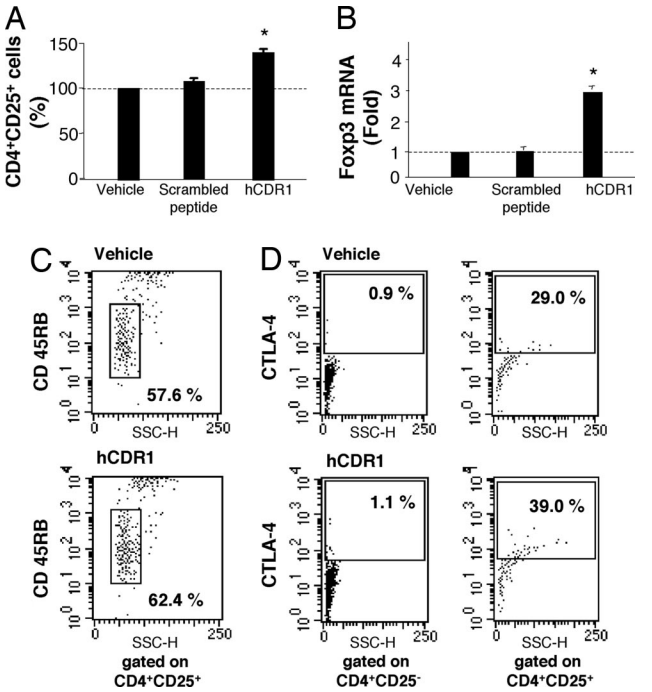

Fig. 2. Induction and characterization of $CD4^+CD25^+$ cells after treatment with hCDR1. Two-month-old (NZB×NZW)F_1 female mice ($n = 3$ mice per group) were treated with three s.c. injections of hCDR1, the vehicle, or a scrambled peptide. Spleen cells were then pooled and examined for cell-surface and intracellular markers by flow cytometry. (*A*) Percentage of $CD4^+CD25^+$ cells compared with that of vehicle-treated mice that was considered as 100%. Shown are results of five experiments (mean percentage ± SD). (*B*) Foxp3 mRNA expression. Shown are mean ± SD values of five independent experiments. Results were normalized to β-actin expression and are presented relative to the vehicle-treated mice (represented by the dashed line). (*C*) Expression of $CD45RB^{low}$ in $CD4^+CD25^+$ cells. (*D*) Expression of CTLA-4 on $CD4^+CD25^-$-gated and $CD4^+CD25^+$-gated cells. *, $P < 0.05$.

hCDR1, unlike treatment with the scrambled peptide, up-regulated these cells by 1–2%. The latter was associated with a 1.5- to 2-fold increase in mean fluorescence intensity. Fig. 2*A*, which represents the mean values of five experiments, indicates a 30–40% up-regulation of $CD4^+CD25^+$ cells compared with the vehicle-treated population. The latter was also accompanied by a 3-fold increase in the mRNA expression of Foxp3 (Fig. 2*B*), which indicated the development of $CD4^+CD25^+$ regulatory T cells (24, 25). The expression of regulatory characteristic markers [$CD45RB^{low}$ and cytotoxic T lymphocyte antigen 4 (CTLA-4)] on $CD4^+$ cells was higher as a result of hCDR1 treatment. Triple staining of the $CD4^+CD25^+$ cell population with the latter two markers indicated that their up-regulation was associated with an increase of $CD4^+CD25^+$ cells (Fig. 2 *C* and *D*). As shown in Fig. 2*D*, CTLA-4 was expressed almost exclusively on $CD4^+CD25^+$ cells, and its levels were higher in hCDR1-induced $CD4^+CD25^+$ cells. Thus, it appears that treatment with hCDR1 up-regulates the immunoregulatory $CD4^+CD25^+$ cells.

hCDR1-Induced $CD4^+CD25^+$ Cells Play an Important Role in Ameliorating Lupus-Like Manifestations. Because treatment with hCDR1 resulted in an up-regulation of $CD4^+CD25^+$ cells, it was of major importance to determine whether the hCDR1-induced $CD4^+CD25^+$ cells play a role in ameliorating lupus manifestations. To this end, we performed experiments for depletion and enrichment of $CD25^+$ cells that were further transferred into SLE-afflicted recipient mice. Splenocytes of hCDR1-treated mice, as well as the latter, depleted of or enriched with $CD25^+$ cells, were transferred into old SLE-afflicted (NZB×NZW)F_1 mice. Splenocytes of five control groups of donors were used as well (Fig. 3): untreated mice, scrambled peptide-treated mice, vehicle-treated mice, vehicle-treated mice depleted of $CD25^+$ cells, and vehicle-treated mice enriched with $CD25^+$ cells. Fig. 3*A*, which represents the results of six independent experiments, demonstrates that the up-regulated $CD4^+CD25^+$ cells of the hCDR1-treated mice (lane f) or enrichment of $CD4^+CD25^+$ cells of the latter origin (lane h) were accompanied by 3- and 8-fold increased Foxp3 mRNA expression, respectively, compared with the vehicle-treated cells (Fig. 3*A*, lane c). Foxp3 mRNA was also up-regulated in enriched $CD4^+CD25^+$ cells of the vehicle-treated mice (Fig. 3*A*, lane e), although to a lesser extent.

The above eight cell populations were injected (20×10^6 cells per mouse) into respective groups of 8-mo-old (NZB×NZW)F_1 mice with established manifestations of lupus. A representative experiment shows a significant decrease in the dsDNA Ab as well as down-regulated secretion of IFN-γ and IL-10 in the groups of old mice that were injected with hCDR1-treated splenocytes, or the latter enriched with $CD4^+CD25^+$ cells (Fig. 3 *B* and *C*, lanes f and h). Depletion of $CD4^+CD25^+$ cells abrogated the inhibitory effects of hCDR1 (Fig. 3 *B* and *C*, lane g). Administration of $CD4^+CD25^+$-enriched cells of vehicle-treated mice (Fig. 3*A*, lane e) did not diminish the levels of dsDNA-specific Ab (Fig. 3*B*, lane e) or the secretion of the pathogenic cytokines (Fig. 3*C*, lane e). In addition, depletion of the $CD4^+CD25^+$ cells from the vehicle-treated cells (Fig. 3*A*, lane d) did not substantially modulate autoantibody titers or the levels of secreted cytokines (Fig. 3 *B* and *C*, lane d versus lanes a–c). The kidney function of the diseased mice was similarly affected. SLE-afflicted mice that were administered with cells of the three control groups (Fig. 3 *D* and *E*, groups a–c) had high levels of proteinuria and glomerular ICD. In contrast, adoptive transfer of hCDR1-treated cells, as well as of hCDR1-treated cells enriched with $CD4^+CD25^+$ cells, significantly lowered the proteinuria levels

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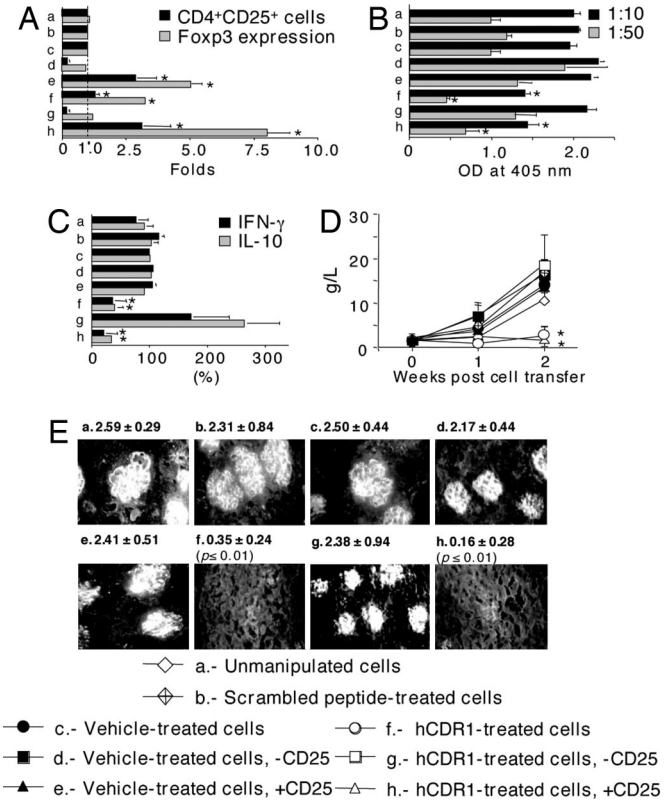

Fig. 3. Ameliorating effects of hCDR1-induced $CD4^+CD25^+$ cells. Cells of hCDR1- or vehicle-treated mice were either unmanipulated or depleted or enriched for CD25 cells and then transferred to SLE-afflicted (NZB×NZW)F_1 mice. (*A*) Donor $CD4^+CD25^+$ cells and Foxp3 mRNA expression. Shown are mean ± SD values of six independent experiments. Results are presented relative to the vehicle-treated mice (represented by the dashed line). (*B*) Titers (mean OD ± SD) of dsDNA-specific Ab in sera (diluted 1:10 and 1:50) obtained from recipient mice of each of the eight groups described ($n = 5$–8 mice per group) 2 wk after the cell transfer. Results represent one of six independent experiments performed. (*C*) Constitutive secretion of IFN-γ and IL-10. Results are relative to levels measured in the supernatants of splenocytes from mice that were injected with the vehicle-treated cells (100% = 210 ± 23 pg/ml for IFN-γ and 415 ± 32 pg/ml for IL-10). Shown are mean values ± SD of four independent experiments. (*D*) Kinetics of proteinuria levels (mean grams per liter ± SD) in the recipient mice. Results represent one of six independent experiments. (*E*) Immunohistology of kidney sections of representative mice of each group are demonstrated. (Magnification: ×400.) Also shown are the mean intensity values ± SD of kidney sections of all mice per treatment group. *, $P \leq 0.05$.

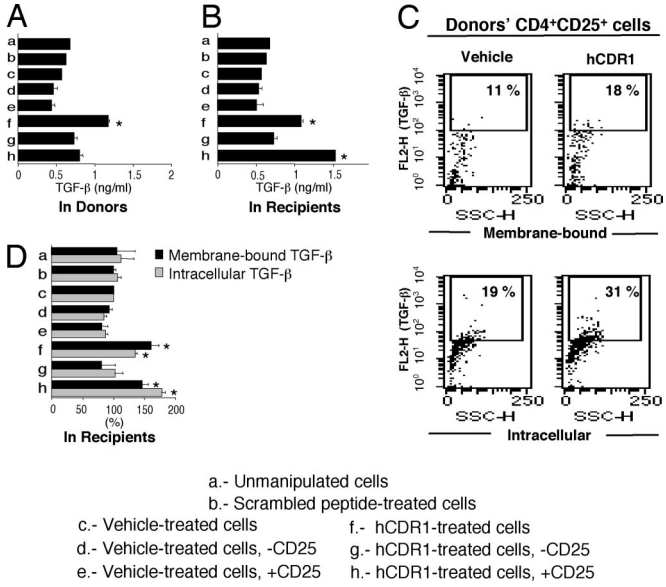

Fig. 4. The status of TGF-β in $CD4^+CD25^+$ and in affected $CD4^+$ cells. Levels of secreted TGF-β were determined in the supernatants of splenocytes of the donor treatment groups (*A*, $n = 5$ mice per group) and the different groups (*B*, $n = 5$–8 mice per group) of recipient mice. (*C*) Staining of donor splenocytes of hCDR1- and vehicle-treated mice for the presence of membrane-bound and intracellular TGF-β in $CD4^+CD25^+$-gated cells. Dot plots are representative of one of two experiments performed. (*D*) Splenocytes of the different groups of recipient mice were stained for membrane-bound and intracellular TGF-β in $CD4^+$ cells. Percentages of stained cells were compared with those found on cells of recipients of vehicle-treated cells (considered as 100% and determined to be 9.5 ± 0.1% for membrane-bound TGF-β and 40.0 ± 4.0% for intracellular TGF-β). *, $P \leq 0.05$.

and the intensity of glomerular ICD (Fig. 3 *D* and *E*, groups f and h). The efficacy of the enriched hCDR1-induced $CD4^+CD25^+$ cells was demonstrated in dose-dependent experiments. Thus, as low as 10^6 and 10^5 enriched $CD4^+CD25^+$ cells down-regulated proteinuria to levels observed after transfer of 20 and 10 million splenocytes of hCDR1-treated cells, respectively. Furthermore, the latter was confirmed by the significant reduction of ICD determined in the kidneys of recipients of 10^6 and 10^5 enriched $CD4^+CD25^+$ cells. No significant difference could be observed in the sustained high levels of proteinuria and ICD after the transfer of either enriched or depleted $CD4^+CD25^+$ cells originating from vehicle-treated cells (Fig. 3 *D* and *E*, groups d and e).

hCDR1-Induced $CD4^+CD25^+$ Cells Promote the Secretion of TGF-β by Recipient-Derived $CD4^+$ Cells. It was of interest to find out whether the up-regulated TGF-β in hCDR1-treated mice could be related to the hCDR1-induced $CD4^+CD25^+$ cells. Fig. 4*A* shows the levels of TGF-β in the supernatants of splenocytes of the donor (disease-free) mice. It can be seen that splenocytes of donor mice, which were treated with hCDR1, secreted elevated levels of TGF-β, as compared with those of control groups. Depletion of $CD4^+CD25^+$ cells led to diminished secretion of the latter; however, enrichment with $CD4^+CD25^+$ cells did not result in a significant increase of this cytokine. Nevertheless, when the treated cells were injected into old SLE-afflicted mice, splenocytes of the recipients of the enriched (hCDR1-treated) $CD4^+CD25^+$ cell population secreted the highest levels of TGF-β (Fig. 4*B*). Hence, it appears that $CD4^+CD25^+$ cells originating from hCDR1-treated mice affect another subset or subsets of cells to secrete TGF-β rather than secreting elevated levels of this cytokine by themselves. Nevertheless, the hCDR1-induced $CD4^+CD25^+$ cells had a significantly ($P \leq 0.05$) higher expression of both membrane-bound and intracellular TGF-β as compared with the expression by $CD4^+CD25^+$ cells of vehicle-treated mice (Fig. 4*C*).

To find the cell source of the elevated TGF-β levels, we determined the expression of membrane-bound and intracellular TGF-β in potential producers. In comparison to the control groups, significantly higher levels of expression of both membrane-bound and intracellular TGF-β could be observed mainly in $CD4^+$ cells from recipients of hCDR1-treated cells and from recipients of hCDR1-treated cells that were enriched with $CD4^+CD25^+$ cells (Fig. 4*D*). The expression of TGF-β in macrophages and in apoptotic cells was not affected in the eight groups of the recipient mice described. Because $CD4^+$ cells from the recipients of hCDR1-treated cells or of the enriched hCDR1-induced $CD4^+CD25^+$ cells expressed high levels of TGF-β, it is likely that these $CD4^+$ cells also secreted TGF-β.

Suppression by hCDR1-Induced $CD4^+CD25^+$ Cells Is Mediated by Means of TGF-β. To further assess the suppressive efficacy of hCDR1-induced $CD4^+CD25^+$ immunoregulatory cells, we used enriched $CD4^+CD25^+$cells from either hCDR1- or vehicle-treated

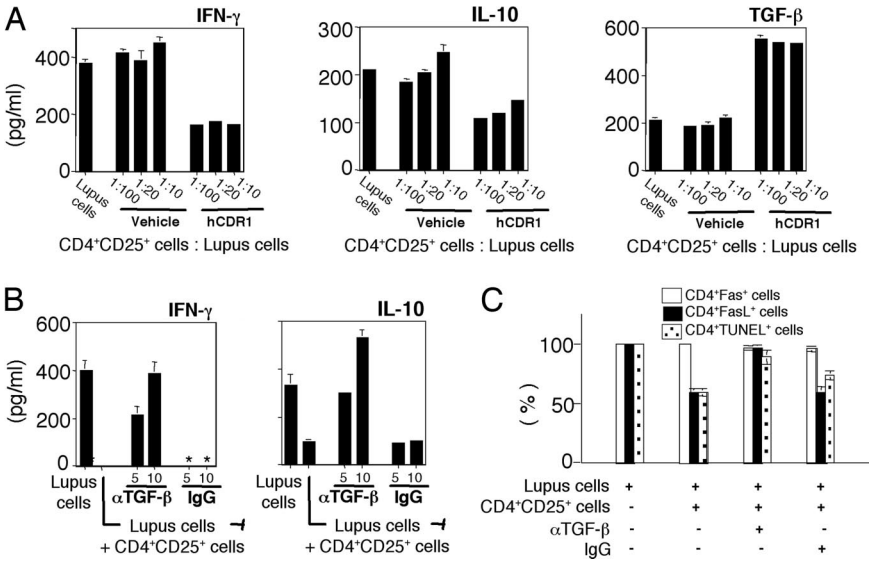

Fig. 5. Suppression by hCDR1-induced $CD4^+CD25^+$ cells is mediated by means of TGF-β. Triplicate of spleen cells (10^5 cells, designated lupus cells) of 8-mo-old $(NZB\times NZW)F_1$ female mice ($n = 5$) were cocultured with various numbers of hCDR1-induced or vehicle-induced $CD4^+CD25^+$ cells. (*A*) Lupus cells (10^5 cells) were cultured alone or together with various numbers of hCDR1-induced or vehicle-induced $CD4^+CD25^+$ cells for 36 h. Supernatants were collected and assessed for secreted cytokines. Lupus cells (10^5 cells) were cocultured with hCDR1-induced $CD4^+CD25^+$ cells (1,000 cells) for 36 h in the presence of anti-TGF-β neutralizing mAb (5 or 10 μg/ml) or its IgG isotype control. (*B*) Effect on cytokine secretion. (*C*) Effect on activation-induced apoptosis.

mice. Each of the two groups of enriched $CD4^+CD25^+$ cells was coincubated (in three different ratios) with splenocytes (designated "lupus cells") of 8-mo-old $(NZB\times NZW)F_1$ mice with established lupus. Fig. 5*A* shows that coincubation with hCDR1-induced $CD4^+CD25^+$ cells resulted in a significant decrease of the pathogenic cytokines IFN-γ and IL-10, whereas the levels of the immunosuppressive cytokine TGF-β were elevated (Fig. 5*A*). This effect was achieved with all three concentrations of the hCDR1-induced $CD4^+CD25^+$ cells, the lowest being 1:100. Coincubation with vehicle-induced $CD4^+CD25^+$ cells had no effect on the cytokine profile.

To determine the role of TGF-β in the inhibitory effect of the hCDR1-induced $CD4^+CD25^+$ cells, lupus cells (10^5 cells) were coincubated with 10^3 hCDR1-induced $CD4^+CD25^+$ cells for 36 h with or without anti-TGF-β neutralizing mAb (5 or 10 μg/ml) and its IgG isotype control. Fig. 5*B* demonstrates that both concentrations of anti-TGF-β mAb abrogated the ability of the hCDR1-induced $CD4^+CD25^+$ cells to down-regulate IFN-γ and IL-10, whereas the isotype control used did not interfere with the activity of hCDR1-induced $CD4^+CD25^+$ cells. Furthermore, it can be seen in Fig. 5*C* that suppression of $CD4^+$ lupus cells by the hCDR1-induced $CD4^+CD25^+$ cells, as indicated by a significant ($P < 0.0001$) reduction of Fas ligand (FasL) and apoptosis (determined by TUNEL), was also mediated by TGF-β.

hCDR1-Induced $CD4^+CD25^+$ Cells Suppress the Activation of Lupus $CD4^+$ Cells *in Vivo*. We further measured the expression of Fas and FasL (26) and the rate of apoptosis and in $CD4^+$ cells of the different groups of recipient mice to confirm the effect of $CD4^+CD25^+$ cells on activation-induced apoptosis of the $CD4^+$ cells of SLE-afflicted mice. Administration of hCDR1-treated cells or the latter enriched with $CD4^+CD25^+$ cells significantly down-regulated the rate of TUNEL[+] (Fig. 6*A*) and FasL expression (Fig. 6*B*) on $CD4^+$ cells in comparison with the control groups. In contrast, when lupus-afflicted mice were injected with hCDR1-treated cells that were depleted of $CD4^+CD25^+$ cells, the rate of apoptotic $CD4^+$ cells and FasL-expressing cells was remarkably high (Fig. 6). None of these changes occurred in response to depletion or enrichment with the $CD4^+CD25^+$ cells of the vehicle-treated mice. These results were reproduced in three independent experiments.

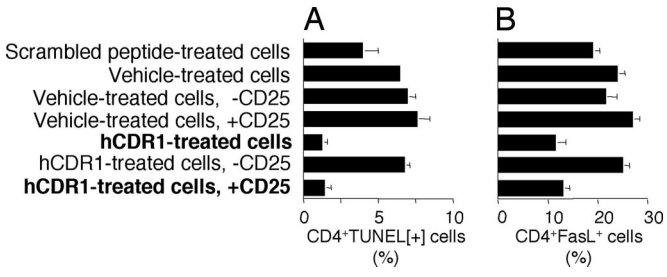

Fig. 6. hCDR1-induced $CD4^+CD25^+$ cells reduce activation-induced cell death of $CD4^+$ cells. (*A*) Two weeks after the transfer of the various cell populations, the mice ($n = 5$–8 mice per group) were killed, and $CD4^+$ spleen-derived cells were stained for apoptosis by using the TUNEL technique. (*B*) Cells from each group were double-stained for CD4 and FasL. Shown are representative results of one experiment of three performed.

Discussion

The main findings of this study are that amelioration of the clinical and serological manifestations of SLE after treatment with hCDR1 is, at least partially, the consequence of the induction of immunoregulatory $CD4^+CD25^+$ cells. These cells were found to suppress $CD4^+$ cells not through their deletion by apoptosis but rather by down-regulating their state of activation and by up-regulating the secretion of the immunosuppressive cytokine TGF-β by $CD4^+$ cells of the recipient mice. This cascade of events is triggered directly by the hCDR1-induced $CD4^+CD25^+$ cells. Thus, this study is an *in vivo* demonstration of the induction of $CD4^+CD25^+$ immunoregulatory cells by a CDR-based peptide that ameliorates lupus manifestations in association with cytokine immunomodulation.

We showed here that cells of hCDR1-treated mice could actively transfer the inhibitory capacity of hCDR1 into mice with established lupus. The latter suggested the presence of a subpopulation of regulatory cells with suppressive activity in the splenocytes of hCDR1-injected mice. Several types of regulatory cells of the immune system are recognized. The $CD4^+CD25^+$ regulatory T cells are the best characterized and are known to be protective against the development of autoimmunity. We therefore studied the mechanistic role of $CD4^+CD25^+$ regulatory T cells regarding the ameliorative effects of hCDR1 on SLE. Indeed, treatment with hCDR1 resulted in the up-regulation of $CD4^+CD25^+CD45RB^{low}$ cells (Figs. 2 and 3*A*) with regulatory characteristics such as CTLA-4 and TGF-β and of Foxp3 mRNA, which is selectively expressed in these cells (24, 25). In light of the fact that treatment with the scrambled control peptide, as well as with the vehicle, had no effect on the magnitude of the $CD4^+CD25^+$ cell population, and because a 3-fold-higher expression of Foxp3 mRNA was determined exclusively in the hCDR1-treated cells, our data suggest that

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treatment with hCDR1 results in the peripheral generation of $CD4^+CD25^+$ immunoregulatory cells.

The relevance and importance of hCDR1-induced $CD4^+CD25^+$ cells were demonstrated in *in vitro* and *in vivo* settings. Thus, the clinical amelioration combined with the reduction of activated $CD4^+$ lupus cells and of the pathogenic cytokines IFN-γ and IL-10 occurred only in the presence of hCDR1-induced $CD4^+CD25^+$ cells. This effect was also demonstrated after a 10-wk direct treatment with hCDR1 of SLE-afflicted $(NZB\times NZW)F_1$ mice (A.S. and E.M., unpublished data). In agreement, previous reports have shown the induction of $CD4^+CD25^+$ cells with regulatory functions in other systems, including models of lupus (13–20). The hCDR1-induced $CD4^+CD25^+$ cells reported in the present study are highly effective because as little as 10^5 enriched cells were still protective after transfer to SLE-afflicted recipient mice.

Antigenic specificity of $CD4^+CD25^+$ regulatory cells was reported under both autoimmune and infectious conditions (19, 20, 27–29). The specificity of hCDR1-induced $CD4^+CD25^+$ cells is presumed for several reasons. First, injection of $CD4^+CD25^+$ cells of either naïve (healthy) donors or of mice treated with a control peptide into mice with established lupus had no beneficial effects. Furthermore, treatment of SLE-afflicted mice with an enriched $CD4^+CD25^+$ cell population from vehicle-treated donors neither improved the clinical condition of the mice nor modulated their pattern of cytokine secretion or state of cellular activation. These results rule out the possibility of a quantitative replenishment in the number of regulatory cells as an explanation for lupus amelioration. In contrast, a small number of hCDR1-induced $CD4^+CD25^+$ cells effectively suppressed the clinical manifestations and the secretion of pathogenic cytokines. Furthermore, whereas adoptive transfer of cells from vehicle-treated donors that were depleted of $CD25^+$ cells did not affect the severity of the disease, the transfer of hCDR1-treated cells that were depleted of $CD4^+CD25^+$ cells resulted in a more severe kidney disease in the recipient mice, associated with an up-regulated secretion of IFN-γ and IL-10 (Fig. 3 *C* and *D*). Moreover, in another model of experimental SLE, inhibition of the specific *in vitro* proliferation of cells from mice immunized with an anti-DNA mAb that bears an idiotype designated 16/6Id (4–6) could be achieved only by the transfer of splenocytes from mice that were treated with hCDR1, but not with a dual altered peptide ligand (18), which was reported to down-regulate myasthenogenic manifestations (H.Z. and E.M., unpublished data). Collectively, these data indicate that the hCDR1-induced $CD4^+CD25^+$ cells have unique qualitative characteristics that enable them to specifically suppress lupus-associated manifestations.

Treatment with hCDR1 has always been associated with an up-regulation of the secretion and expression of the immunosuppressive cytokine TGF-β (8). The latter correlated with the amelioration of lupus manifestations. Here we have shown that the secretion of TGF-β depends on the presence of hCDR1-induced $CD4^+CD25^+$ cells (Fig. 4 *A* and *B*). Although $CD4^+CD25^+$ cells were reported in some studies to function independent of TGF-β (28, 29), others showed that immune suppression *in vivo* depended on the presence of TGF-β (30–33). Furthermore, Thompson and Powrie (34) reported that *in vivo* suppression by $CD4^+CD25^+$ cells from TGF-$\beta^{-/-}$ donor mice could still be achieved when TGF-β, clearly derived from other cell types, was present. Indeed, only in the presence of other cell types, shown here to be $CD4^+$ cells of the recipient mice (Fig. 4*D*), were the levels of TGF-β elevated, as demonstrated after the transfer of either hCDR1-treated cells or the latter enriched with $CD4^+CD25^+$ cells. Neutralization of TGF-β abrogated the effects of hCDR1-induced $CD4^+CD25^+$ cells on the secretion of cytokines and activation-induced apoptosis (Fig. 5 *C* and *D*), thus supporting a central role for TGF-β in mediating the suppression.

It is possible that the hCDR1-induced $CD4^+CD25^+$ cells, by means of membrane-bound or soluble forms of TGF-β and/or by means of engagement of CTLA-4, may raise the threshold for TCR activation, reported to be lower in lupus cells (35). We therefore suggest that hCDR1-induced $CD4^+CD25^+$ cells cause the "silencing" of $CD4^+$ cells as indicated by reduced expression of FasL, consequently with a reduced rate of activation-induced apoptosis (26), rather than causing the depletion of the latter by means of apoptosis. Taken together, our results indicate a key role for $CD4^+CD25^+$ cells in the mechanism of action of hCDR1, although other cell types and mechanisms (36, 37) may be involved as well. Based on the present report, we suggest that inhibition by the hCDR1-induced $CD4^+CD25^+$ cells is mediated through TGF-β, which is secreted by other T cells that are affected by the immunoregulatory cells. The up-regulated secretion of TGF-β and the down-regulation of activated $CD4^+$ cells are associated with a decrease in the pathogenic cytokines IFN-γ and IL-10. Eventually, the suppression of $CD4^+$ lupus cells by the hCDR1-induced $CD4^+CD25^+$ cells enables the clinical improvement of the SLE-afflicted mice.

Materials and Methods

Mice. Female $(NZB\times NZW)F_1$ mice were purchased from The Jackson Laboratory. All experiments were approved by the Animal Care and Use Committee of the Weizmann Institute of Science.

Synthetic Peptides. A peptide, GYYWSWIRQPPGKGEEWIG, designated hCDR1, based on the CDR1 of the human anti-DNA mAb that bears a major idiotype, 16/6Id (7, 38), was synthesized (solid-phase synthesis by F-moc chemistry) by Polypeptide Laboratories (Torrance, CA) and used in this study. A peptide containing the same amino acids as hCDR1, with a scrambled order (scrambled peptide), SKGIPQYGGWPWEGWRYEI, was used as a control. hCDR1 (Edratide) is currently under clinical development for the treatment of human SLE by Teva Pharmaceutical Industries (Netanya, Israel).

Treatment of Mice with hCDR1. Two-month-old $(NZB\times NZW)F_1$ female mice were treated with s.c. injections of hCDR1 (50 μg per mouse) a total of three times on alternating days. Control groups of young mice were treated with the scrambled peptide or with the vehicle alone [Captisol, sulfobutylether β-cyclodextrin, a solvent designed by CyDex (Lenexa, KS) to enhance the solubility and stability of drugs].

Depletion and Enrichment of $CD4^+CD25^+$ Cells. Depletion and enrichment of $CD25^+$ cells were performed by using the StemSep system (StemCell Technologies). Briefly, splenocytes (100×10^6) of mice treated with hCDR1 (50 μg per mouse) were incubated with anti-CD25-biotinylated mAb (clone 7D4; Southern Biotechnology Associates). The cells were further incubated with an anti-biotin tetrameric complex (StemCell Technologies) followed by incubation with magnetic beads (StemCell Technologies). The cells that were eluted from a column (StemCell Technologies), which was placed within a magnet stand, were collected. Depletion rate of $CD25^+$ cells was >90%. Next, the column was removed from the magnet stand and washed, and the eluted cells (≈80% $CD4^+CD25^+$ cells) were collected.

Measurement of dsDNA-Specific Ab. Briefly, Maxisorb microtiter plates (Nunc) were coated with polyL-lysine (5 μg/ml) (Sigma) followed by coating with λ phage dsDNA (5 μg/ml) (Boehringer Mannheim). After the plates were blocked, the sera were added. Goat anti-mouse IgG (γ-chain-specific) conjugated to horseradish peroxidase (Jackson ImmunoResearch) was added. Plates were incubated with the substrate 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) and read at 405 nm with an ELISA reader.

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

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

Ab and Reagents. The following antibodies were used in the study: anti-CD4-phycoerythrin (PE) (clone GK1.5), anti-CD4-allophycocyanin (clone L3T4), anti-CD25-FITC (clone 7D4), anti-CTLA-4-PE (clone 1B8), anti-CD8-FITC (clone 53-6.7), and their matched isotype controls (obtained from Southern Biotechnology Associates). Anti-CD45RB-PE (clone 16A), anti-Fas-PE (clone Jo2), anti-FasL-PE (clone MFL3), and their matched isotype controls were purchased from Pharmingen. Anti-TGF-β1-PE Ab (clone TB21) was obtained from IQ Products (Groningen, The Netherlands). Biotinylated chicken anti-TGF-β1 and anti-TGF-β1, -β2, and -β3 neutralizing mAb (clone 1D11) and its isotype control were purchased from R & D Systems. Streptavidin labeled with PE or FITC and anti-F 4/80 Ab (clone CI:A3-1) with its matched isotype control were obtained from eBioscience (San Diego). Fixation and permeabilization solutions for intracellular staining were obtained from Serotec.

Cytokine Detection by ELISA. Splenocytes (5×10^6 cells per well) were incubated in enriched medium for 48 h and 72 h. IFN-γ and IL-10 were determined in the supernatants by ELISA using OptEIA sets (Pharmingen) according to the manufacturer's instructions. For the detection of TGF-β, the plates were coated with a recombinant human TGF-β sRII/Fc chimera (R & D Systems). Supernatants were added after activation of latent TGF-β1 to immunoreactive TGF-β1, a biotinylated anti-human TGF-β1 Ab was added thereafter, and the assay was developed according to the manufacturer's instructions (R & D Systems).

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

TUNEL Assay. Apoptosis, as demonstrated by fragmented DNA, was determined by using the *In Situ* Death Detection Kit (Roche, Indianapolis) based on TUNEL technology, according to the protocol supplied by the manufacturer. Cells were analyzed by FACS.

***In Vitro* Assays.** Enriched ($\approx$80%) $CD4^+CD25^+$ cells obtained from mice treated with either hCDR1 or the vehicle were coincubated (in different ratios) for 18–48 h with splenocytes (10^5 cells) taken from 8-mo-old (NZB$\times$NZW)F_1 female mice with established lupus.

Real-Time RT-PCR. The mRNA levels of Foxp3 were analyzed by real-time RT-PCR by using LightCycler (Roche, Mannheim, Germany). Total RNA was isolated from splenocytes, and then RNA was reverse-transcribed to prepare cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega). The resulting cDNA was subjected to real-time PCR according to the manufacturer's instructions. Briefly, a 20-μl reaction volume contained 3 mM $MgCl_2$, LightCycler HotStart DNA SYBR Green I mix (Roche), specific primer pairs, and 5 μl 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. Primer sequences (forward and reverse, respectively) were used as follows: Foxp3, 5′-taccacaatatgcgaccc-3′ and 5′-ctcaaattcatctacggtcc-3′; β-actin, 5′-gacgttgacatccgtaaag-3′. The relative expression of Foxp3 normalized to β-actin levels was determined.

Statistical Analysis. Mann–Whitney and unpaired Student's *t* tests were used for evaluating the significant differences between treated and untreated groups. Values of $P \leq 0.05$ were considered significant.

This work was supported by Teva Pharmaceutical Industries.

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