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

Amir Sharabi*, Heidy Zinger*, Maya Zborowsky*, Zev M. Sthoege†, and Edna Mozes*

*Department of Immunology, Weizmann Institute of Science, Rehovot 76100, Israel; and †Department of Medicine B, Kaplan Hospital, Rehovot 76100, Israel

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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-affected (NZB × NZW)F1 female mice down-regulated all disease manifestations. hCDR1 treatment up-regulated (30–40%) CD4+CD25+ cells in association with CD45RB+T, 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 autoreactive CD4+ T cells of treated mice. 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 × NZW)F1 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 × NZW)F1 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 × NZW)F1 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 × NZW)F1 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 × NZW)F1 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 × 10⁶ per mouse) from the different groups were injected i.p. to respective groups of 8-mo-old (NZB × NZW)F1 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 × NZW)F1 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...
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peptide. Splenocytes of the different groups were injected i.p. (20 × 10^6 per mouse) to respective groups of 8-mo-old (NZB × NZW)F1 mice with established lupus. (A) Titors (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.

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. 2A, 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. 2B), which indicated the development of CD4^+CD25^+ regulatory T cells (24, 25). The expression of regulatory characteristic markers [CD45RBlow 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 the increase of CD4^+CD25^+ cells (Fig. 2C and D). As shown in Fig. 2D, 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-affected recipient mice. Splenocytes of hCDR1-treated mice, as well as the latter, depleted of or enriched with CD25^+ cells, were transferred into old SLE-affected (NZB × NZW)F1 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. 3A, 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. 3A, lane c). Foxp3 mRNA was also up-regulated in enriched CD4^+CD25^+ cells of the vehicle-treated mice (Fig. 3A, 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)F1 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. 3B and C, lanes f and h). Depletion of CD4^+CD25^+ cells abrogated the inhibitory effects of hCDR1 (Fig. 3B and C, lane g). Administration of CD4^+CD25^+ -enriched cells of vehicle-treated mice (Fig. 3A, lane e) did not diminish the levels of dsDNA-specific Ab (Fig. 3B, lane e) or the secretion of the pathogenic cytokines (Fig. 3C, lane e). In addition, depletion of the CD4^+CD25^+ cells from the vehicle-treated cells (Fig. 3A, lane d) did not substantially modulate autoantibody titers or the levels of secreted cytokines (Fig. 3B and C, lane d versus lanes a–c). The kidney function of the diseased mice was similarly affected. SLE-affected mice that were administered with cells of the three control groups (Fig. 3D 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.
Results represent one of six independent experiments performed. Results are presented relative to the vehicle-treated group (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 < 0.05. Levels of this cytokine by themselves. Nevertheless, the hCDR1-enriched 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-enriched CD4+CD25+ cells had a significantly (P < 0.05) higher expression of both membrane-bound and intracellular TGF-β as compared with the expression by CD4+CD25+ cells from recipient mice (Fig. 4C).

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. 4D). 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
mice. Each of the two groups of enriched CD4+CD25+ cells was cocultured (in three different ratios) with splenocytes (designated “lupus cells”) of 8-mo-old (NZB×NZW)F1 mice with established lupus. Fig. 5A shows that coculture with hCDR1-induced CD4+CD25+ cells resulted in a significant decrease of the patho-
egenic cytokines IFN-γ and IL-10, whereas the levels of the immunosuppressive cytokine TGF-β were elevated (Fig. 5A). This effect was achieved with all three concentrations of the hCDR1-induced CD4+CD25+ cells, the lowest being 1:100. Coculture 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 (10^6 cells) were cocultured with 10^5 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. 5B 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. 5C 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 downregulated the rate of TUNEL+ cells (Fig. 6A) and FasL expression (Fig. 6B) 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. 6B). 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.

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+CD25+ 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+CD45RBlow cells (Figs. 2 and 3A) 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...
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-affected (NZB×NZW)F1 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 106 enriched cells were still protective after transfer to SLE-affected 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 naive (healthy) donors or of mice treated with a control peptide into mice with established lupus had no beneficial effects. Furthermore, treatment of SLE-affected 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 16/6ld (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 (18), which was reported to down-regulate myasthenic 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-β−/− 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), 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-affected mice.

Materials and Methods

Mice. Female (NZB×NZW)F1 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, 16/6ld (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×NZW)F1 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 CD4+CD25+ cells were performed by using the StemSep system (StemCell Technologies). Briefly, splenocytes (100 × 106) 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 poly-l-lysine (5 μg/ml) (Sigma) followed by coating with A 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-ethylthiazoline-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).
**Immunohistochemistry.** 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-phycocerythrin (PE) (clone GKL1.5), anti-CD4-allophycocyanin (clone L3T4), anti-CD25-FLTC (clone 7D4), anti-CTLA-4-PE (clone 1B8), anti-CD8-FLTC (clone 53-6.7), and their matched isotype controls (obtained from Southern Biotechnology Associates). Anti-CD45RB-PE (clone Jo2), anti-Fas-PE (clone MF3), and their matched isotype controls were purchased from Pharmingen. Anti-TGF-β1-PE Ab (clone TB21) was obtained from Bio-Rad (Hercules, CA) and anti-TGF-β2, and anti-β3 neutralizing mAb (clone 1D11) and its isotype control were purchased from R & D Systems. Streptavidin labeled with PE (catalogue no. SA2101) was purchased from R&D Systems. For the detection of TGF-β, OptEIA sets (Pharmingen) according to the manufacturer's instructions. Briefly, 20-μl reaction volume contained 3 mM MgCl₂, 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'-ctacaactactatggccac-3' and 5'-ctcaaatatctagggcct-3'; β-actin, 5'-ggtgtgacgaactgaa-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 ≤ 0.05 were considered significant.

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