

# A 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> Analog Enhances Regulatory T-Cells and Arrests Autoimmune Diabetes in NOD Mice

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**Type 1 diabetes is a chronic progressive autoimmune disease characterized by mononuclear cell infiltration, dominated by interleukin-12 (IL-12)-dependent Th1 cells, of the pancreatic islets, with subsequent destruction of insulin-producing  $\beta$ -cells. Here, we demonstrate that treatment of adult nonobese diabetic (NOD) mice with an analog of 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>, an immunomodulatory agent preventing dendritic cell maturation, decreases lipopolysaccharide-induced IL-12 and  $\gamma$ -interferon production, arrests Th1 cell infiltration and progression of insulinitis, and inhibits diabetes development at nonhypercalcemic doses. Arrest of disease progression is accompanied by an enhanced frequency in the pancreatic lymph nodes of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells that are able to inhibit the T-cell response to the pancreatic autoantigen insulinoma-associated protein 2 and to significantly delay disease transfer by pathogenic CD4<sup>+</sup>CD25<sup>-</sup> cells. Thus, a short treatment of adult NOD mice with an analog of 1,25-dihydroxyvitamin D<sub>3</sub> inhibits IL-12 production, blocks pancreatic infiltration of Th1 cells, enhances CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells, and arrests the progression of type 1 diabetes, suggesting its possible application in the treatment of human autoimmune diabetes. *Diabetes* 51:1367–1374, 2002**

**T**he nonobese diabetic (NOD) mouse, which spontaneously develops type 1 diabetes with a pathogenesis similar to the human disease, represents a useful model for the study of autoimmune diabetes (1). Several effector mechanisms leading to specific  $\beta$ -cell destruction have been identified, including cytotoxic CD8<sup>+</sup> lymphocytes and macrophages (2), both of which are regulated by interleukin-12 (IL-12)-dependent T-helper 1 (Th1) cells (3). The activation of Th1 cells specific for  $\beta$ -cell autoantigens could reflect defective elimination of autoreactive T-cell clones (4), inefficient

mechanisms of peripheral tolerance (5), enhanced IL-12 production (6), or impaired suppressive mechanisms (7).

Studies using different autoimmune disease models have evidenced a subset of CD4<sup>+</sup> T-cells, characterized by constitutive expression of CD25, with immunosuppressive activity. CD4<sup>+</sup>CD25<sup>+</sup> T-cells fail to proliferate and to secrete cytokines in response to polyclonal or antigen-specific stimulation and inhibit the activation of responsive T-cells, probably via cell-cell contact rather than secretion of soluble factors (8,9). Transfer of CD4<sup>+</sup>CD25<sup>+</sup> T-cells inhibits the induction of different autoimmune diseases, such as the autoimmune syndrome induced by day 3 thymectomy in genetically susceptible mice (10), inflammatory bowel disease (11), and type 1 diabetes in thymectomized rats (12) and NOD mice (7). Thus, CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells may control type 1 diabetes in prediabetic NOD mice.

An agent able to both inhibit IL-12 production and Th1 development and enhance CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells may therefore be beneficial in the treatment of type 1 diabetes. 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> [1,25(OH)<sub>2</sub>D<sub>3</sub>], the activated form of vitamin D<sub>3</sub>, is a secosteroid hormone that not only plays a central role in bone and calcium metabolism, but also modulates the immune response via specific receptors expressed in antigen-presenting cells (APCs) and activated T-cells (13). 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs have been shown to inhibit autoimmune diseases in animal models, such as experimental allergic encephalomyelitis (14–16), murine lupus (17), collagen-induced arthritis (18,19), and type 1 diabetes (20). They are clinically used in the treatment of psoriasis, a Th1-mediated autoimmune disease of the skin, where they show efficacy comparable to topical steroids (21). Polymorphisms of the vitamin D receptor gene have been associated with type 1 diabetes in different populations (22,23). In addition, epidemiological studies have shown a higher incidence of the disease in northern than in southern latitudes (24), suggesting a possible involvement of a 1,25(OH)<sub>2</sub>D<sub>3</sub> deficiency in the pathogenesis of type 1 diabetes. This is supported by a large population-based case-control study showing that the intake of vitamin D<sub>3</sub> contributes to a significantly decreased risk of type 1 diabetes development (25).

1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits IL-12 production (26), likely via inhibition of nuclear factor- $\kappa$ B (27), and IL-12 has been shown to exert an important role in the development of type 1 diabetes in the NOD mouse (3,28) and in humans (29). Thus, 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs could represent interest-

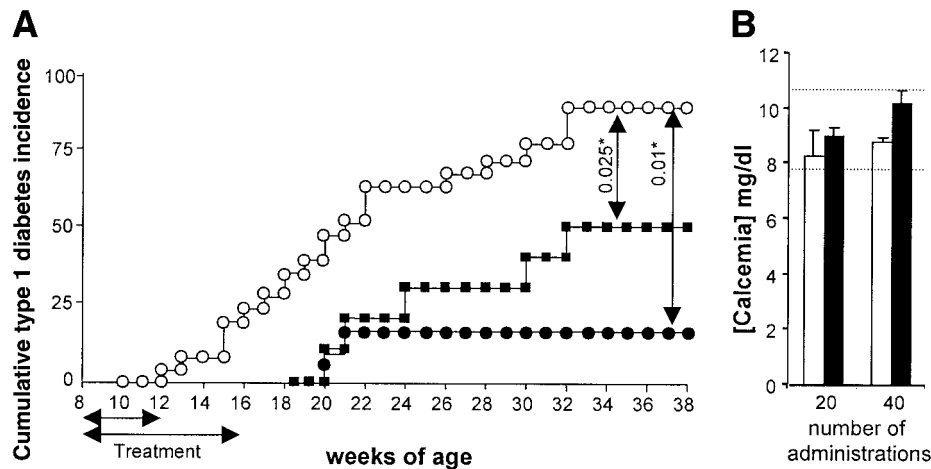
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APC, antigen-presenting cell; DC, dendritic cell; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; IA-2, insulinoma-associated protein 2; IFN- $\gamma$ ,  $\gamma$ -interferon; IL, interleukin; LPS, lipopolysaccharide; mAb, monoclonal antibody; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>; PE, phycoerythrin; PMA, phorbol myristic acid; Ro 26-2198, 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D<sub>3</sub>; TCR, T-cell receptor.



**FIG. 1. A:** Ro 26-2198 administration to 8-week-old NOD mice inhibits type 1 diabetes development. NOD mice were treated five times per week with vehicle (○,  $n = 16$ ) or with 0.03  $\mu\text{g}/\text{kg}$  Ro 26-2198 per os (●,  $n = 12$ ) from 8 to 16 weeks of age, or from 8 to 12 weeks of age (■,  $n = 10$ ). Diabetes development was monitored twice weekly by measurement of blood glucose levels. The  $P$  values were calculated by Mann-Whitney  $U$  test. **B:** Serum calcium levels (means  $\pm$  SE) were measured in vehicle-treated (□) and Ro 26-2198-treated (■) NOD mice after 20 or 40 administrations. Stippled lines indicate the range of normal serum calcium levels.

ing candidates to modulate APCs (30), leading to inhibition of IL-12-dependent Th1 cells and type 1 diabetes development.

Results in this paper demonstrate that a short course of treatment with the 1,25(OH)<sub>2</sub>D<sub>3</sub> analog 1 $\alpha$ ,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D<sub>3</sub> (Ro 26-2,198) inhibits IL-12 production and pancreatic infiltration of Th1 cells while increasing the frequency of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells in pancreatic lymph nodes, arresting the immunological progression and preventing the clinical onset of type 1 diabetes in the NOD mouse.

## RESEARCH DESIGN AND METHODS

**Mice.** NOD/Lt and NOD.SCID female mice were purchased from Charles River Laboratories (Calco, Italy). All mice were kept under specific pathogen-free conditions. Glucose levels in the tail venous blood were quantified using an EUROFlash glucometer (Lifescan, Issy les Moulineaux, France). A diagnosis of diabetes was made after two sequential glucose measurements higher than 200 mg/dl. Serum calcium was determined using a colorimetric assay (Sigma). **Treatments.** Ro 26-2198, was dissolved in ethanol and then diluted in miglyol 812. NOD/Lt mice were dosed orally five times per week with vehicle (miglyol 812) alone or containing Ro 26-2198.

**Immunohistology.** Pancreata were snap-frozen in Tissue Tek (Miles Laboratories, Elkhart, IN), and 5- $\mu\text{m}$ -thick sections were stained with hematoxylin and eosin. The insulinitis score was quantified as follows: 0, no insulinitis; 1, peri-insulinitis; 2, insulinitis in <50% of the islet; 3, insulinitis in >50% of the islet. A mean score was calculated from 40–50 islets per individual pancreas. In addition, pancreas cryostat sections were stained with biotinylated monoclonal antibodies (mAbs) directed against CD4, CD8, B220, CD11b, or CD11c (all from PharMingen), followed by streptavidin-peroxidase conjugate. For insulin detection, pancreas cryostat sections were stained with anti-porcine insulin (Sigma) followed by PAP (Sigma). 3-Amino-9-ethylcarbazole (Dako, Carpinteria, CA) was used as chromogen and hematoxylin as a counterstain.

**Flow cytometric analysis.** Stainings were performed in the presence of 100  $\mu\text{g}/\text{ml}$  mouse IgG using the following mAbs, all from PharMingen: CyChrome-streptavidin, fluorescein isothiocyanate (FITC)-anti-CD4 (L3T4), phycoerythrin (PE)-anti-CD8 (53-6.7), PE-anti-B220 (RA3-6B2), FITC-anti-CD11b (MI/70), PE-anti-CD11c (HL-3), PE-anti-CD45RB (16A), PE-anti-CD38 (rat IgG2a), and biotinylated anti-CD25 (7D4). Cells were analyzed with a FACScan flow cytometer equipped with CellQuest software.

**Intracellular staining for cytokine production.** Cells were stained for  $\gamma$ -interferon (IFN- $\gamma$ ), IL-4, IL-10, and IL-2 as previously described (31). Briefly, cells were preincubated for 10 min with PBS/FCS/saponin and then incubated with FITC-labeled rat anti-mouse IFN- $\gamma$  (XMGL2) and PE-labeled rat anti-IL-2 (JE56-5H4), rat anti-mouse IL-4 (11B11), or PE-labeled rat anti-mouse IL-10 (JES5-16E3). Isotype controls were FITC- and PE-labeled rat IgG1

(R3-34). After 30 min, cells were washed twice with PBS/FCS/saponin and then with PBS containing 5% FCS without saponin to allow membrane closure. The cell surface was then stained with CyChrome-labeled anti-CD4 (L3T4) for 15 min at room temperature. Analysis was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with CellQuest software, and 10,000 events were acquired.

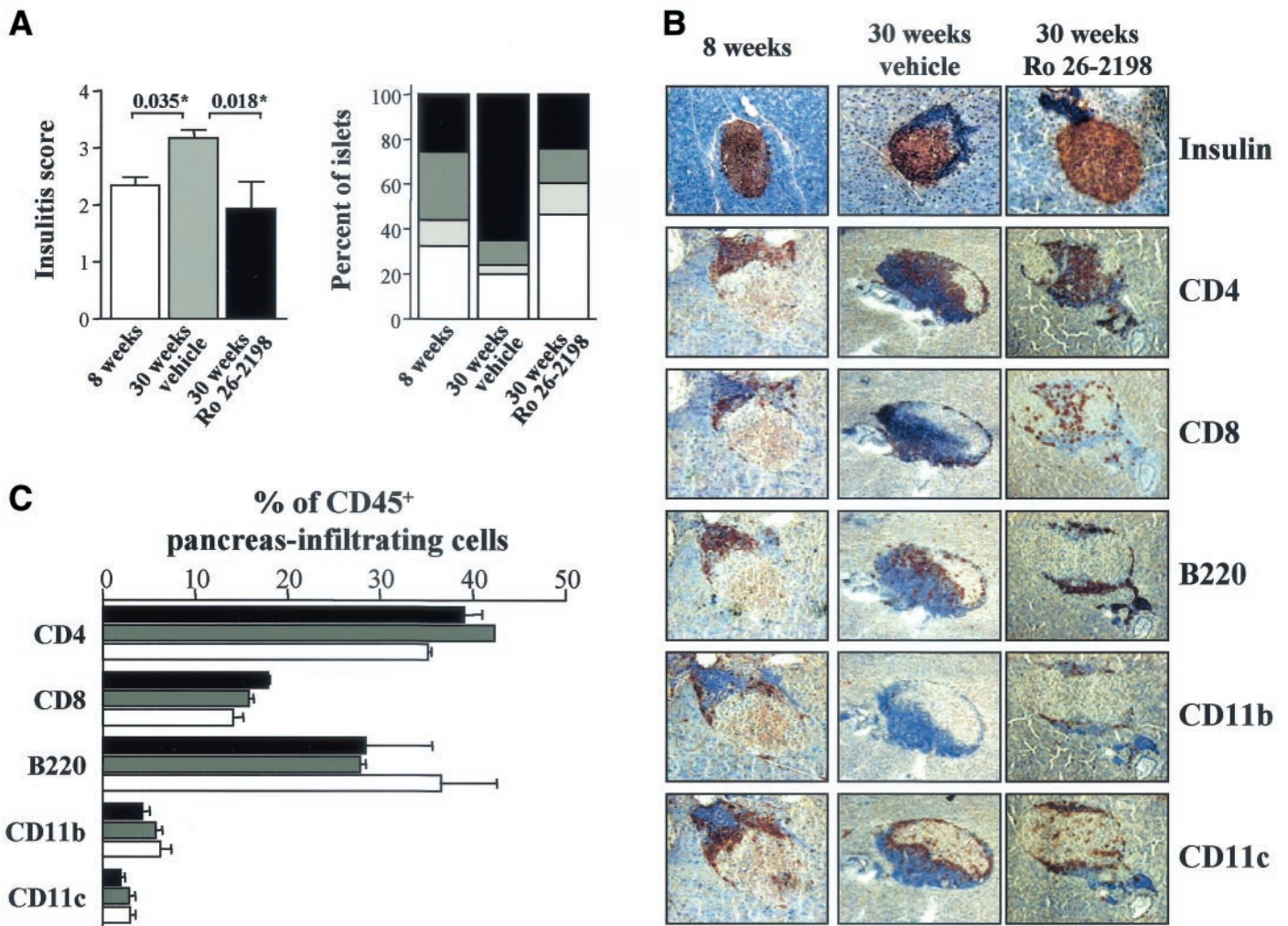
**Cell cultures.** Total spleen cells (10<sup>6</sup> cells/well) from vehicle- or Ro 26-2198-treated mice were cultured for 48 h in flat-bottom 96-well plates (Costar) with the indicated concentrations of insulinoma-associated protein 2 (IA-2). The intracellular domain (amino acids 601–979) of mouse IA-2 was expressed in *Escherichia coli* as a glutathione-S-transferase fusion protein and purified by affinity chromatography on glutathione-Sepharose, followed by thrombin cleavage to recover >98% pure recombinant mouse IA-2, as previously described (32). Single-cell suspensions from pancreatic lymph nodes or spleen were incubated with anti-CD4 mAb-coated microbeads and applied onto Mini-MACS columns (Miltenyi) to obtain CD4<sup>+</sup> cells. Splenic or pancreatic lymph node CD4<sup>+</sup>CD25<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup> cells were sorted with a MultiSort kit (Miltenyi). Purified CD4<sup>+</sup> cells (6  $\times$  10<sup>5</sup>/well) were cultured for 48 h in round-bottom 96-well plates (Costar) precoated with 3  $\mu\text{g}/\text{ml}$  purified anti-T-cell receptor (TCR) mAb (HB 218; American Type Culture Collection, Manassas, VA) with or without 100 units/ml IL-2 and/or 10  $\mu\text{g}/\text{ml}$  anti-CD28 mAb. Cultures were performed in synthetic HL-1 medium (Ventrex Laboratories, Portland, ME) supplemented with 2 mmol/l L-glutamine and 50  $\mu\text{g}/\text{ml}$  gentamicin (Sigma). To measure cell proliferation, cultures were pulsed 8 h before harvesting with 1  $\mu\text{Ci}$  [<sup>3</sup>H]thymidine (40 Ci/nmol; The Radiochemical Center, Amersham, U.K.). Incorporation of [<sup>3</sup>H]-thymidine was measured by liquid scintillation spectrometry.

**IL-12 and IFN- $\gamma$  production in vivo.** Lymphokine production was induced in 8-week-old female NOD mice by intraperitoneal injection of 400  $\mu\text{g}/\text{mouse}$  *Salmonella abortus equi* lipopolysaccharide (LPS; Sigma). Ro 26-2198 was administered 72, 48, 24, and 1 h before LPS. Blood samples were collected 3 h post-LPS injection, and serum cytokines were measured by enzyme-linked immunosorbent assay (ELISA).

**Quantification of cytokines.** Cytokines were quantified by two-site ELISA. IFN- $\gamma$  was determined using paired mAbs from PharMingen, as previously described (32). ELISA for IL-12p75 was performed as previously described (33) with mAbs kindly provided by Dr. Maurice K. Gately (Hoffman-La Roche, Nutley, NJ). Detection limits were 15 pg/ml for both cytokines.

**Cell transfer.** Nucleated spleen cells were incubated with biotinylated anti-CD25 mAb (7D4) followed by streptavidin-conjugated beads and positively selected using Mini-MACS columns (Miltenyi Biotec). Pancreatic lymph node cells were incubated with anti-CD4 mAb-coated Microbeads (Miltenyi Biotec) and positively selected using Mini-MACS columns (Miltenyi Biotec). Purified cells were mixed as indicated and adoptively transferred by intravenous injection into NOD.SCID mice.

**Statistical analysis.** All statistical analyses were performed using the nonparametric Mann-Whitney  $U$  test.



**FIG. 2.** Decreased insulinitis in Ro 26-2198-treated NOD mice. **A:** Histological scoring of insulinitis was performed on pancreas sections stained with hematoxylin/eosin from untreated 8-week-old mice and from NOD mice at 30 weeks of age that had been treated with Ro 26-2198 or vehicle from 8 to 16 weeks of age. Each bar represents the mean score of ~40–50 islets per mouse (left panel). The results refer to the mean values  $\pm$  SE of eight mice per group, except for Ro 26-2198-treated mice, where three mice were scored. The *P* values were calculated by Mann-Whitney *U* test. The severity of the infiltrate is shown in the right panel. Islets from the same mice were scored for absence of insulinitis ( $\square$ ), peri-insulinitis (light gray), moderate insulinitis with <50% infiltration of the islets (dark gray), and severe insulinitis, with >50% of infiltration of the islets ( $\blacksquare$ ). **B:** Consecutive sections of the same islets were stained with anti-CD4, anti-CD8, anti-B220, anti-CD11b (Mac-1), and anti-CD11c (N418) mAbs. In addition, islets were stained for insulin. **C:** Cytofluorimetric analysis of pancreas-infiltrating cells. Pancreas-infiltrating cells isolated from untreated 8-week-old NOD mice and from vehicle- and Ro 26-2198-treated NOD mice at 30 weeks of age were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry. Acquisition was performed on CD45<sup>+</sup> cells. Bars represent the mean  $\pm$  SE from three separate experiments.

## RESULTS

**Ro 26-2198 administration inhibits the development of insulinitis and type 1 diabetes.** To analyze the ability of Ro 26-2198 to inhibit type 1 diabetes development, NOD female mice were treated daily with Ro 26-2198 (0.03  $\mu$ g/kg per os) or vehicle from 8 to 12 or 16 weeks of age, and glycemia levels were monitored until 38 weeks of age. The incidence of disease was significantly lower in mice treated with Ro 26-2198 compared with controls, and the longer treatment was most effective (Fig. 1). Of the vehicle-treated controls, ~90% were diabetic by 38 weeks of age, compared with only 50% of mice treated with Ro 26-2198 from 8 to 12 weeks of age. A longer treatment, from 8 to 16 weeks of age, afforded higher protection, and only 16% of NOD mice developed type 1 diabetes. Ro 26-2198 did not induce hypercalcemia, even after 40 administrations. Because 0.03  $\mu$ g/kg per os was the maximum dose of Ro 26-2198 that did not induce

hypercalcemia, higher doses were not tested. Ro 26-2198 administered at 0.01  $\mu$ g/kg did not affect diabetes development (data not shown). Different schedules of Ro 26-2198 administration (0.03  $\mu$ g/kg per os) were also tested. Treatment (five times per week) from 8 to 10 or from 8 to 11 weeks of age did not significantly reduce diabetes development, whereas Ro 26-2198 given three times per week from 8 to 16 weeks of age prevented diabetes in 50% of NOD mice, showing a significant disease reduction compared with controls (*P* = 0.006). Therefore, an effective treatment of diabetes requires administration of Ro 26-2198 at the highest nonhypercalcemic dose for at least 4 weeks.

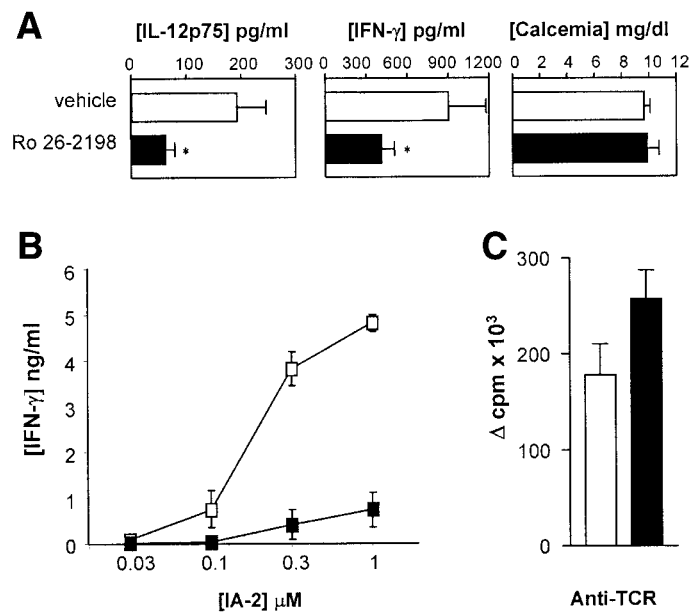
The score and severity of insulinitis was similar between 8-week-old untreated controls and Ro 26-2198-treated NOD mice at 30 weeks of age, whereas this had significantly progressed in vehicle-treated mice (Fig. 2A). These data indicate that Ro 26-2198 treatment is able to halt the

recruitment of infiltrating cells into the pancreatic islets. Functional islet  $\beta$ -cells, demonstrated by the positive staining for insulin, were more frequent in both 8-week-old and Ro 26-2198-treated mice at 30 weeks of age, compared with vehicle-treated NOD mice at 30 weeks of age (Fig. 2B). Immunohistochemical analysis showed a similar pattern of islet infiltration in 8-week-old and Ro 26-2198-treated NOD mice at 30 weeks of age, comprising  $CD4^+$  and  $CD8^+$  T-cells,  $B220^+$  B cells,  $CD11b^+$  macrophages, and  $CD11c^+$  dendritic cells (DCs). Conversely, a more florid infiltrate was observed in vehicle-treated 30-week-old NOD mice (Fig. 2B). The pancreas-infiltrating cells were analyzed by flow cytometry, and no difference in the percentage of  $CD4^+$ ,  $CD8^+$ ,  $B220^+$   $CD11b^+$ , and  $CD11c^+$  cells was found among the three groups (Fig. 2C), indicating that Ro 26-2198 treatment blocked the progression of insulinitis without modifying the cellular composition of the infiltrate.

**Ro 26-2198 administration inhibits LPS-induced IL-12 and IFN- $\gamma$  production and the Th1 response to the pancreatic autoantigen IA-2.** The IFN- $\gamma$  production induced by LPS administration is largely IL-12 dependent, and we have previously shown that the LPS-induced serum levels of both cytokines can be inhibited by a short treatment with  $1,25(OH)_2D_3$  or its analogs (16). We thus tested the capacity of Ro 26-2198 to inhibit LPS-induced IL-12 and IFN- $\gamma$  production in vivo. Mice were pretreated with vehicle or with 0.03  $\mu$ g/kg Ro 26-2198 administered per os daily for 4 days before LPS injection. Ro 26-2198 did not induce hypercalcemia, as demonstrated by the serum calcium levels similar to controls, but inhibited significantly the LPS-induced IL-12p75 and IFN- $\gamma$  production (Fig. 3A), with a potency  $\sim$ 100-fold higher than  $1,25(OH)_2D_3$  (16 and data not shown).

We have also analyzed the ability of Ro 26-2198 to inhibit the spontaneous T-cell response to the intracytoplasmic region (amino acids 601–979) of the tyrosine phosphatase-like IA-2 protein, an autoantigen associated with type 1 diabetes in both humans and the NOD mouse (32). Spleen cells from unprimed NOD mice treated with Ro 26-2198 from 8 to 12 weeks of age secreted much lower levels of IFN- $\gamma$  in response to IA-2, compared with spleen cells from vehicle-treated controls (Fig. 3B). In contrast, splenic T-cells from Ro 26-2198- or vehicle-treated mice proliferated similarly to TCR ligation, indicating the lack of a generalized immunosuppressive effect.

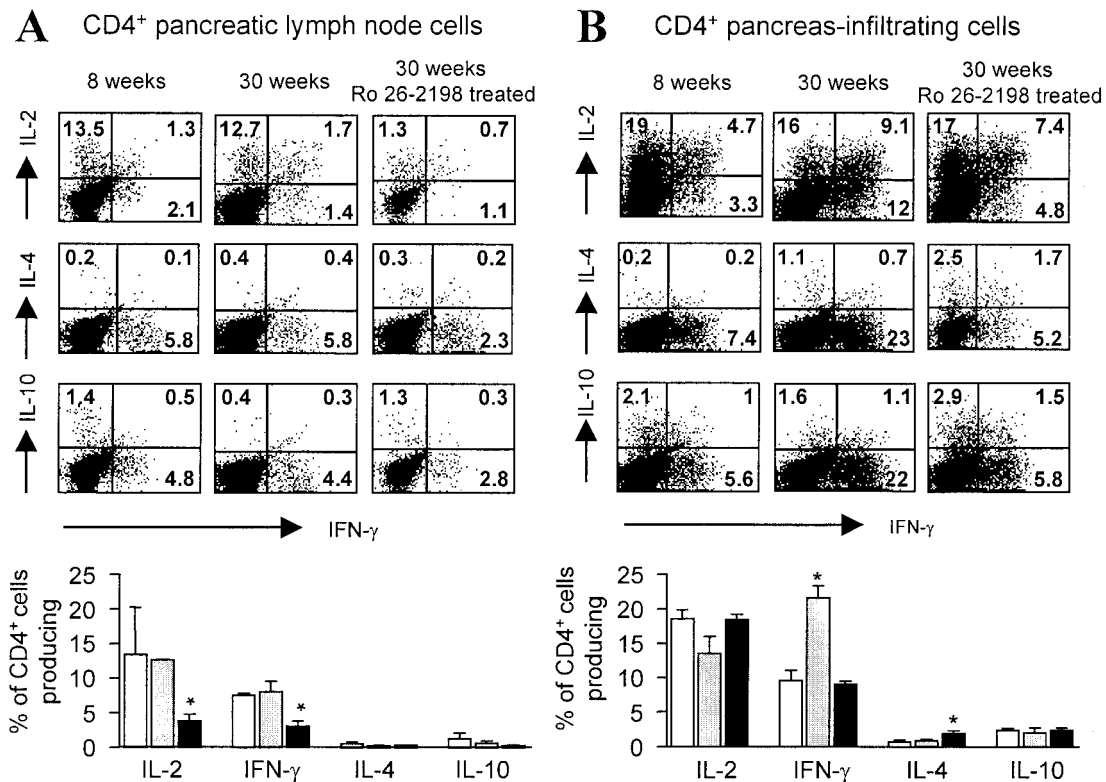
**Reduced Th1 cells in pancreatic lymph nodes and pancreas-infiltrating cells from Ro 26-2198-treated NOD mice.** To identify mechanisms accounting for the reduced type 1 diabetes development in Ro 26-2198-treated NOD mice, we analyzed the cytokines produced by  $CD4^+$  cells of 8- and 30-week-old NOD mice treated from 8 to 16 weeks of age with Ro 26-2198 or vehicle. Splenic  $CD4^+$  T-cells from Ro 26-2198- or vehicle-treated NOD mice displayed a similar cytokine production pattern (data not shown). In contrast, analysis of  $CD4^+$  T-cells from pancreatic lymph nodes revealed an average ( $n = 4$ ) threefold reduction of IL-2 and of IFN- $\gamma$ -producing cells in Ro 26-2198-treated compared with either 30-week-old vehicle-treated or 8-week-old NOD mice, indicating a decrease in Th1 cells (Fig. 4).  $CD4^+$  pancreas-infiltrating cells showed a similar percentage of IL-2-producing cells



**FIG. 3.** Ro 26-2198 treatment inhibits IL-12-dependent IFN- $\gamma$  production in vivo and the ex vivo induced IFN- $\gamma$  secretion in response to IA-2. **A:** Ro 26-2198 (0.03  $\mu$ g/kg) was administered orally in miglyol to NOD mice 72, 48, 24, and 1 h before LPS injection. IFN- $\gamma$ , IL-12p75, and serum calcium levels were quantified 3 h post-LPS injection. Bars represent the mean  $\pm$  SE of 10 mice per group. The  $P$  values were calculated by Mann-Whitney  $U$  test. \* $P < 0.05$  vs. vehicle-treated NOD mice. **B:** Splenocytes ( $10^6$  cells/well) from individual 12-week-old NOD mice treated with vehicle or Ro 26-2198 from 8 to 12 weeks of age were stimulated with the indicated concentrations of IA-2. After 48 h of culture, IFN- $\gamma$  secretion was measured by two-site ELISA. Values represent the mean  $\pm$  SE of IFN- $\gamma$  levels secreted in response to rIA-2 from five individual mice. **C:** Positively selected  $CD4^+$  spleen cells were stimulated with plate-coated anti-TCR mAb. Cells were pulsed with [ $^3$ H]-thymidine during the last 8 h of a 48-h culture. Data are presented as the means  $\pm$  SE of [ $^3$ H]-thymidine incorporation ( $\Delta$  cpm) from triplicate cultures; background values were  $790 \pm 100$  cpm.

in all three groups, but the IFN- $\gamma$ -producing cells were significantly increased in vehicle-treated mice, whereas they were similarly lower in 8-week-old and Ro 26-2198-treated NOD mice. These results indicate that Ro 26-2198 can arrest the recruitment of IFN- $\gamma$ -producing Th1 cells into the pancreas, and this effect is still clearly seen 14 weeks after treatment withdrawal. No increase in IL-10-producing  $CD4^+$  cells was observed, and a modest, though significant, increase in IL-4-producing cells ( $0.7 \pm 0.2\%$  in 8-week-old NOD mice and  $0.9 \pm 0.2\%$  in vehicle-treated vs.  $2.5 \pm 0.3\%$  in Ro 26-2198-treated NOD mice,  $P = 0.02$  by Mann-Whitney  $U$  test, means of four experiments) was detected.

**Ro 26-2198 treatment enhances the frequency of  $CD4^+CD25^+$  cells.**  $CD4^+CD25^+$  T-cells possess regulatory activity and have been implicated in the control of autoimmune responses in several models (8,9), including type 1 diabetes in the NOD mouse (7). Analysis of pancreatic lymph node cells revealed in Ro 26-2198-treated compared with 8-week-old or vehicle-treated 20-week-old NOD mice, respectively, a significantly increased percentage of  $CD4^+CD25^+$  and  $CD4^+CD38^+$  cells. No difference was seen in CD152 expression between  $CD4^+CD25^+$  and  $CD4^+CD25^-$  cells from either vehicle- or Ro 26-2198-treated NOD mice (data not shown). Pancreatic lymph node cells from Ro 26-2198-treated NOD mice contained a slightly lower percentage of  $CD45RB^{\text{high}}$  and a significantly

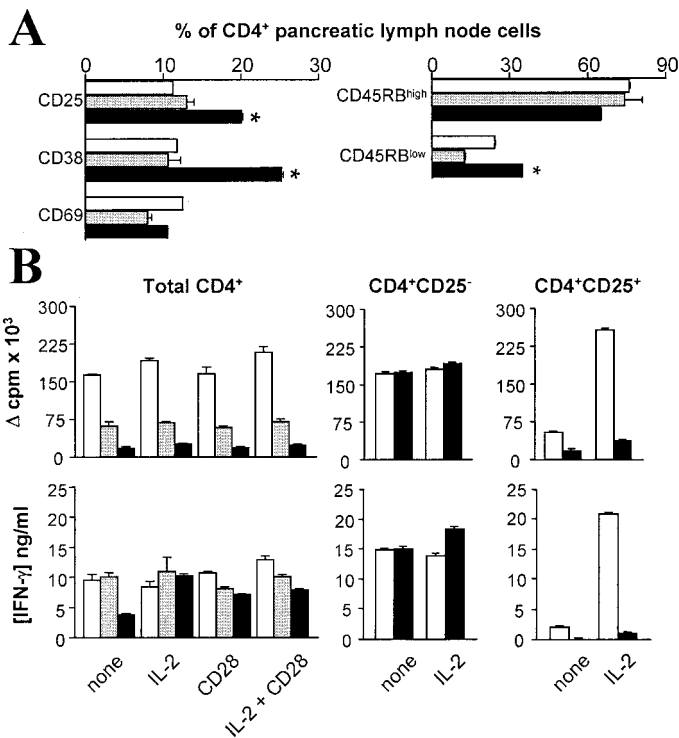


**FIG. 4.** Cytokine production by pancreatic lymph node cells and pancreas-infiltrating CD4<sup>+</sup> cells. Pancreatic lymph node (A) and pancreas-infiltrating (B) CD4<sup>+</sup> cells were obtained from untreated 8- or 30-week-old NOD mice treated 5 times per week per os from 8 to 16 weeks of age with vehicle or 0.03  $\mu$ g/kg Ro 26-2198. Positively selected CD4<sup>+</sup> cells ( $2 \times 10^5$  cells/well) were stimulated with PMA and ionomycin and analyzed by flow cytometry for IFN- $\gamma$  (abscissa) and IL-2, IL-4, or IL-10 (ordinate) production. Acquisition was performed on CD4<sup>+</sup> cells. Percentage of positive cells, set according to the isotype-matched controls (not shown), are shown in the top corner of each quadrant. Dot plots are from one representative experiment out of four performed. Bar graphs represent the means  $\pm$  SE of four independent experiments using pooled cells from three to four mice. The *P* values were calculated by Mann-Whitney *U* test. \**P* < 0.05 vs. 8-week-old NOD mice.

higher percentage of CD45RB<sup>low</sup>CD4<sup>+</sup> cells than 8-week-old and 20-week-old vehicle-treated NOD mice (Fig. 5A). Interestingly, double-positive CD25<sup>+</sup>CD38<sup>+</sup>CD4<sup>+</sup> cells (17 vs. 8 or 7%, respectively) were selectively increased in the pancreatic lymph node of Ro 26-2198–treated compared with 8-week-old and 20-week-old vehicle-treated NOD mice (data not shown). These results indicate that Ro 26-2198 administration leads to a selective increase of CD4<sup>+</sup> cells with a regulatory phenotype in the pancreatic lymph node of NOD mice that persists for at least 1 month after treatment withdrawal.

**Suppressive activity of CD4<sup>+</sup>CD25<sup>+</sup> cells.** The profound reduction of IL-2–producing cells in the pancreatic lymph nodes of Ro 26-2198–treated NOD mice was paralleled by the strongly decreased proliferative response to insolubilized anti-TCR mAb. Pancreatic lymph node CD4<sup>+</sup> cells from 20-week-old Ro 26-2198–treated NOD mice proliferated 4 times less than age-matched controls and 10 times less than cells from 8-week-old mice (Fig. 5B). The proliferation in response to TCR ligation was not restored by the addition of IL-2, alone or together with anti-CD28 mAb, indicating a profoundly anergic state of these cells. However, addition of IL-2 and/or anti-CD28 mAb restored the lower IFN- $\gamma$  secretion in pancreatic lymph node cells from Ro 26-2198–treated mice. In contrast to CD4<sup>+</sup> cells from pancreatic lymph nodes, splenic CD4<sup>+</sup> T-cells isolated from Ro 26-2198–treated mice or age-matched and 8-week-old controls, stimulated with insolubilized anti-TCR mAb, showed a similar proliferative response (Fig.

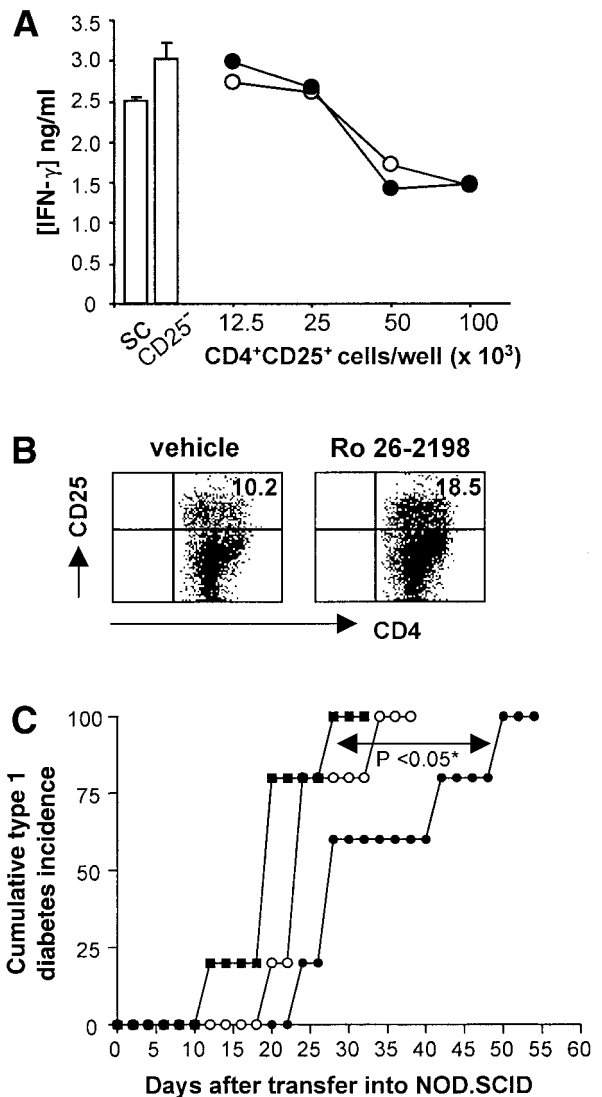
3B) and IFN- $\gamma$  production (data not shown). This indicates a selective effect of Ro 26-2198 treatment on IL-2 and IFN- $\gamma$  secretion by pancreatic lymph node CD4<sup>+</sup> cells stimulated via TCR ligation, consistent with the results obtained by stimulation with phorbol myristic acid (PMA) and ionomycin (Fig. 3). CD4<sup>+</sup>CD25<sup>–</sup> pancreatic lymph node cells from Ro 26-2198– or vehicle-treated NOD mice showed a similar proliferative response and IFN- $\gamma$  production to TCR ligation, indicating that removal of the CD4<sup>+</sup>CD25<sup>+</sup> population was sufficient to restore T-cell responsiveness. CD4<sup>+</sup>CD25<sup>+</sup> pancreatic lymph node cells from Ro 26-2198–treated NOD mice proliferated less to insolubilized anti-TCR mAb than age-matched vehicle-treated mice. Moreover, the proliferation of CD4<sup>+</sup>CD25<sup>+</sup> pancreatic lymph node cells from Ro 26-2198–treated mice was not restored by the addition of IL-2 compared with vehicle treated cells, indicating that Ro 26-2198 treatment induced a long-lasting, profound anergic state selectively in CD4<sup>+</sup>CD25<sup>+</sup> pancreatic lymph node cells. These cells could inhibit the IFN- $\gamma$  production in response to IA-2 and appeared to be, in this respect, functionally similar to CD4<sup>+</sup>CD25<sup>+</sup> cells from vehicle-treated mice (Fig. 6A). A higher frequency of CD4<sup>+</sup>CD25<sup>+</sup> cells, as previously observed (Fig. 5A), was found in the pancreatic lymph nodes of Ro 26-2198–treated NOD mice compared with controls (Fig. 6B). The higher frequency of CD4<sup>+</sup>CD25<sup>+</sup> cells was paralleled by a higher efficiency of total CD4<sup>+</sup> cells in delaying the capacity of CD4<sup>+</sup>CD25<sup>–</sup> cells to transfer type 1 diabetes into NOD.SCID recipients (Fig. 6C).



**FIG. 5.** Ro 26-2198 treatment enhances the frequency of CD4<sup>+</sup>CD25<sup>+</sup> cells. **A:** Positively selected CD4<sup>+</sup> T-cells were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry. Acquisition was performed on CD4<sup>+</sup> cells. Bars represent the percent of lymph node CD4<sup>+</sup> T-cells expressing the indicated surface molecules from untreated 8-week-old (□) or 20-week-old mice treated five times per week from 8 to 16 weeks of age with vehicle (▨) or with 0.03 μg/kg Ro 26-2198 (■). Data are presented as the means ± SE of three separate experiments. The *P* values were calculated by Mann-Whitney *U* test. \**P* < 0.05 vs. 8-week-old NOD mice. **B:** Proliferation and IFN-γ secretion after TCR ligation. Pancreatic lymph node CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>-</sup>, and CD4<sup>+</sup>CD25<sup>+</sup> cells from the same mice were positively selected and stimulated ( $6 \times 10^5$  cells/well) with plate-bound anti-TCR mAb (10 μg/ml) in the presence of 100 units/ml IL-2 and/or 5 μg/ml anti-CD28 mAb. Cells were pulsed with [<sup>3</sup>H]-thymidine during the last 8 h of a 48-h culture. Data are presented as the means ± SE of thymidine incorporation (Δ cpm) from triplicate cultures; background values were  $790 \pm 100$  cpm. After 48 h of culture, IFN-γ secretion was quantified by ELISA. Results, presented as the means ± SE of triplicate cultures, are from one representative experiment out of four performed.

## DISCUSSION

In this study, we have shown that a short-term administration of the 1,25(OH)<sub>2</sub>D<sub>3</sub> analog Ro 26-2198 to adult NOD mice inhibits IL-12 production, blocks Th1 infiltration in the pancreatic islets and the progression of insulinitis, and prevents type 1 diabetes development at nonhypercalcemic doses. Protection from type 1 diabetes was found to be associated with a selective decrease of Th1 cells in the pancreatic lymph nodes and in the pancreas, without a marked deviation to the Th2 phenotype. The frequency of CD4<sup>+</sup>CD25<sup>+</sup> cells in the pancreatic lymph nodes of Ro 26-2198-treated NOD mice was twofold higher compared with untreated 8-week-old and age-matched vehicle-treated controls. These cells were anergic, as demonstrated by their impaired capacity to proliferate and secrete IFN-γ in response to TCR ligation, inhibited the T-cell response to the pancreatic autoantigen IA-2, and delayed disease transfer by pathogenic CD4<sup>+</sup>CD25<sup>-</sup> cells. Thus, Ro 26-2198 administration enhances the frequency of CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells in pancreatic lymph nodes



**FIG. 6.** CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells inhibit the response to IA-2 and control the diabetogenic potential of CD4<sup>+</sup>CD25<sup>-</sup> cells. **A:** Positively selected pancreatic lymph node CD4<sup>+</sup>CD25<sup>+</sup> cells from 16-week-old NOD mice treated five times per week from 8 to 16 weeks of age with vehicle (○) or with 0.03 μg/kg Ro 26-2198 (●) were cultured with CD25-depleted spleen cells ( $10^6$  cells/well) from 8-week-old NOD mice in the presence of 0.3 μmol/l rIA-2. After 48 h of culture, IFN-γ secretion was measured by two-site ELISA. **B:** Positively selected CD4<sup>+</sup> pancreatic lymph node cells isolated from 20-week-old NOD mice treated five times per week from 8 to 16 weeks of age with vehicle or with 0.03 μg/kg Ro 26-2198 were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry. Acquisition was performed on CD4<sup>+</sup> cells. **C:** Eight-week-old NOD.SCID mice (five mice/group) were injected with  $4 \times 10^6$  CD25-depleted splenocytes from newly diabetic NOD mice alone (■) or together with  $2 \times 10^6$  CD4<sup>+</sup> cells isolated from 20-week-old NOD mice treated five times per week from 8 to 16 weeks of age with vehicle (○) or with 0.03 μg/kg Ro 26-2198 (●). Diabetes development was monitored twice weekly by measurement of blood glucose levels. The *P* value was calculated by Mann-Whitney *U* test.

and arrests the progression of type 1 diabetes in NOD mice.

Two checkpoints have been defined in the pathogenesis of type 1 diabetes in the NOD mouse. The first controls the onset of insulinitis, starting at ~3 weeks of age, the second exerts its activity at ~8–12 weeks of age, controlling the switch to overt disease (34). The first checkpoint regulates the composition of APC populations (35) and the expres-

sion of integrins and adhesion molecules that increase the islet-homing potential of T-cells. The second checkpoint controls the transition from insulinitis to diabetes via several nonexclusive mechanisms, including APCs; cytokines; Th1/Th2 balance; modulation of surface receptors such as CD152, CD25, and NK-like receptors; recruitment of pathogenic cells; and number and function of regulatory cells (34). IL-12 could play a role at both checkpoints. IL-12 administration to 2- or 8-week-old NOD mice induced premature onset of the disease mediated by pancreas-infiltrating CD4<sup>+</sup> cells with Th1 phenotype (3,32). However, targeting IL-12 with a specific antagonist induced a deviation of pancreas-infiltrating CD4<sup>+</sup> cells toward the Th2 phenotype and prevented type 1 diabetes when administered at 3 weeks of age, but it had little effect when administered to 8-week-old NOD mice (31). This suggests that IL-12 is critical at checkpoint 1 and dispensable at checkpoint 2. Therefore, to interfere with type 1 diabetes development in adult NOD mice, we looked for an agent able not only to inhibit IL-12, but also to exert other immunoregulatory activities.

1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs are immunoregulatory agents able to prevent Th1-mediated autoimmune diseases (36). 1,25(OH)<sub>2</sub>D<sub>3</sub> reduces the incidence of insulinitis (37) and prevents type 1 diabetes development (20), but only when administered to NOD mice starting from 3 weeks of age, before the onset of insulinitis. 1,25(OH)<sub>2</sub>D<sub>3</sub> was found ineffective in preventing progression of diabetes in NOD mice when given from 8 weeks of age, when NOD mice present a well-established insulinitis (38). However, a combined treatment of 8-week-old NOD mice with the 1,25(OH)<sub>2</sub>D<sub>3</sub> analog MC 1288 and cyclosporine A reduced the incidence of disease, although neither treatment alone was effective (39). In contrast, the 1,25(OH)<sub>2</sub>D<sub>3</sub> analog Ro 26-2198 is able, as a monotherapy, to treat the ongoing type 1 diabetes in the adult NOD mouse, effectively blocking the disease course. This property is likely due, at least in part, to its increased metabolic stability against the inactivating C-24 and C-26 hydroxylations and the C-3 epimerization (40), resulting in a 100-fold more potent immunosuppressive activity compared with 1,25(OH)<sub>2</sub>D<sub>3</sub>.

1,25(OH)<sub>2</sub>D<sub>3</sub>, in addition to inhibiting IL-12 production, decreases antigen-induced T-cell proliferation (41) and cytokine production (42). APCs and, in particular, DCs are primary targets for the immunosuppressive activity of 1,25(OH)<sub>2</sub>D<sub>3</sub> (30,43–46). 1,25(OH)<sub>2</sub>D<sub>3</sub> inhibits differentiation, maturation, activation, and survival of DCs, leading to impaired activation of alloreactive CD4<sup>+</sup> T-cells, which show downregulated CD154 and upregulated CD152 expression (30). These results may help to explain the profoundly anergic state of pancreatic lymph node CD4<sup>+</sup> cells in Ro 26-2198-treated NOD mice. 1,25(OH)<sub>2</sub>D<sub>3</sub> not only inhibits IL-12 but also greatly enhances IL-10 secretion by DCs (30). These two effects, coupled with the downregulation of costimulatory molecule expression by DCs (30,43–45), could account for the increase in regulatory T-cells observed in the pancreatic lymph nodes of treated mice. DCs can be not only immunogenic but also tolerogenic (47), and immature DCs have been shown to induce CD4<sup>+</sup> cells with regulatory properties (48). Administration of 1,25(OH)<sub>2</sub>D<sub>3</sub> induces DCs with a tolerogenic phenotype and promotes tolerance to allografts associated

with an increased percentage of CD4<sup>+</sup>CD25<sup>+</sup> regulatory cells that could adoptively transfer transplantation tolerance (49). This is consistent with the present results, suggesting that the arrest of DCs at the immature stage induced by Ro 26-2198 treatment limits T-cell costimulation, leading to enhanced frequency of CD4<sup>+</sup>CD25<sup>+</sup> cells.

The presence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells with suppressive activity has been demonstrated in several animal models of autoimmune diseases (8,9). CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells appear to play an important role in controlling the progression of type 1 diabetes in NOD mice, because a low level of CD4<sup>+</sup>CD25<sup>+</sup> T-cells correlates with exacerbation and acceleration of the disease (7). It is likely that this cell population is more relevant than Th2 cells in the control of type 1 diabetes, although both could contribute to protection from disease. 1,25(OH)<sub>2</sub>D<sub>3</sub> can induce regulatory cells with disease-suppressive activity in the NOD mouse (20), and a disease-preventing 1,25(OH)<sub>2</sub>D<sub>3</sub> analog could deviate pancreas-infiltrating cells to the Th2 phenotype (39). Our results show that pancreatic lymph node CD4<sup>+</sup> cells from Ro 26-2198-treated NOD mice contain an enhanced frequency of CD25<sup>+</sup> cells that are able to significantly delay the capacity of CD4<sup>+</sup>CD25<sup>-</sup> pathogenic T-cells to transfer disease into NOD.SCID recipients. Conversely, Th2 cells were not observed in pancreatic lymph nodes from treated mice, although a small percentage of Th2 cells was found in the pancreas.

In conclusion, we have demonstrated that ongoing type 1 diabetes in the adult NOD mouse can be arrested by a relatively short course of treatment with a 1,25(OH)<sub>2</sub>D<sub>3</sub> analog, which is able to induce a selective inhibition of Th1 and an increased frequency of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells in pancreatic lymph nodes. These results, coupled with the recently documented presence of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T-cells in humans (50), suggest that a similar treatment may also inhibit disease progression in prediabetic or newly diagnosed type 1 diabetic patients.

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