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Dietary Supplementation With High Doses of Regular Vitamin D_3 Safely Reduces Diabetes Incidence in NOD Mice When Given Early and Long Term

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High doses of the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], prevent diabetes in the NOD mouse but also elicit unwanted calcemic side effects. Because immune cells themselves can convert vitamin D_3 into 1,25$(OH)_2D_3$ locally, we hypothesized that dietary vitamin D_3 can also prevent disease. Thus, we evaluated whether dietary administration of high doses of regular vitamin D_3 (800 IU/day) during different periods of life (pregnancy and lactation, early life [3–14 weeks of age], or lifelong [3–35 weeks of age]) safely prevents diabetes in NOD mice. We found that only lifelong treatment raised serum 25-hydroxyvitamin D_3 from 173 nmol/L in controls to 290 nmol/L, without inducing signs of calcemic or bone toxicity, and significantly reduced diabetes development in both male and female NOD mice. This diabetes protection by vitamin D_3 correlated with preserved pancreatic insulin content and improved insulitis scores. Moreover, vitamin D_3 treatment decreased interferon-γ–positive $CD8^+$ T cells and increased $CD4^+(CD25^+)FoxP3^+$ T cells in pancreatic draining lymph nodes. In conclusion, this study shows for the first time that high doses of regular dietary vitamin D_3 can safely prevent diabetes in NOD mice when administered lifelong, although caution is warranted with regards to administering equivalently high doses in humans.

Type 1 diabetes is recognized as an autoimmune-mediated disorder with a variable prodromal phase characterized by the progressive loss of the insulin-producing β-cells in the pancreatic islets in genetically at-risk individuals (1,2). Several facts support a critical role for environmental factors that trigger the development of type 1 diabetes. Leading environmental candidates include exposure to enteroviruses, early introduction of wheat, and insufficient vitamin D levels. The prevalence of type 1 diabetes increases with latitude of residence and decreased sunlight exposure, whereas exposure to ultraviolet light, known to induce vitamin D production in the skin, is associated with the lower incidence of type 1 diabetes in countries closer to the equator (3,4). These observations strengthen the hypothesis that an inadequate vitamin D status due to insufficient sun exposure, dietary uptake, and/or abnormalities in its metabolism may increase the risk of type 1 diabetes (5). Vitamin D deficiency indeed increases the onset and severity of autoimmune type 1 diabetes in at-risk children (6) and also in the NOD mouse (7,8). Moreover, most epidemiological data, based on dietary questionnaires, suggested that vitamin D supplementation during pregnancy, infancy, or early adulthood might be associated with a reduced risk of type 1 diabetes later in life (6,9,10). It has been suggested that vitamin D in early life is important for gut maturation, thereby reducing permeability for agents/proteins that can act as potent antigenic stimuli (11,12).

Despite these exciting results, several areas of controversy remain in this domain, and randomized, double-blinded,

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placebo-controlled trials with extended observation periods in humans are limited. Moreover, most of the human studies have several shortcomings, including recall bias, absence of repeated 25(OH)D measurements, and lack of a quantifiable evaluation of intake of vitamin D from food or supplements or data on sunlight exposure. So far, convincing evidence is missing on whether dietary vitamin D as D_2 (ergocalciferol, from plants) or D_3 (cholecalciferol, from animals) can reduce or prevent type 1 diabetes. Moreover, what formulation, dose, route, or duration of treatment is ideal to intervene in the diabetes process remains unclear.

In a small pilot study from our group, NOD mice treated with cholecalciferol at a daily dose of 1,000 IU (25 μg), given intraperitoneally (i.p.) from 3 until 70 days of age, were unaffected in their diabetes presentation (13), whereas the active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$, 5 μg/kg given i.p. on alternative days] from weaning until 200 days of age clearly reduced the development of autoimmune diabetes, although this inhibition occurred only at doses that also caused mild hypercalcemia and bone demineralization (7,14). In another study, oral administration of 50 ng $1,25(OH)_2D_3$ per day reduced diabetes incidence compared with vitamin D–deficient NOD mice (8).

In the current study, we evaluated the effects of high-dose oral vitamin D_3 supplementation administered at various intervals in life—during pregnancy and neonatal life as well as during infancy (early life) and adulthood (lifelong)—in safety, immune modulation, and the development of diabetes in already vitamin D–sufficient NOD mice.

RESEARCH DESIGN AND METHODS

Animals

NOD mice, originally obtained from Professor Wu (Department of Endocrinology, Peking Union Medical College Hospital, Beijing, China), were housed and bred in animal facility of the Katholieke Universiteit (KU) Leuven since 1989. Housing of NOD mice occurred under semibarrier conditions, and animals were fed sterile food and water ad libitum. NOD mice were screened for the onset of diabetes by evaluating glucose levels in urine (Clinistix; Bayer Diagnostics, Tarrytown, NY) and venous blood (Accu-Chek Aviva; Roche Diagnostics Belgium, Vilvoorde, Belgium). Mice were diagnosed as diabetic when having positive glycosuria and two consecutive blood glucose measurements above 200 mg/dL. At the time of the experiments, the breeding stock had a diabetes incidence of 84% in female and 38% in male mice. Animals were maintained in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Experimental Design

NOD mice were randomly assigned to be fed a vitamin D_3–sufficient control diet or a vitamin D_3–supplemented diet during three periods of life. The control diet consisted of the Ssniff R/M-H diet (BioServices BV, Uden, the Netherlands) plus 1% calcium, 0.7% phosphorus, and 1,000 IU vitamin D_3/kg diet (4 IU/day based on a consumption of 4 g chow/20 g body wt [BW] daily). The vitamin D_3–supplemented diet consisted of the Ssniff R/M-H diet plus 1% calcium, 0.7% phosphorus and 200,000 IU vitamin D_3/kg diet (800 IU/day). The different feeding periods were early life (age 3–14 weeks), lifelong (age 3–35 weeks), and during pregnancy and neonatal life (until the end of lactation). All mice were monitored until 35 weeks of age. The research protocol of this study was approved and performed in accordance with the KU Leuven Ethics Committees (Leuven, Belgium).

Calcium and Bone Parameters

At 35 weeks of age, blood was collected by heart puncture and a femur was removed. Serum and the femur were stored at −20°C until biochemical determinations were performed. Calcium content of the femur, measured on HCl-dissolved bone ashes, and of the serum was analyzed by SYNCHRON Clinical Systems (Beckman Coulter, Analis SA, Suarlée, Belgium) and corrected for dry weight (bone). Phosphate levels in the serum were analyzed by the same system. Levels of osteocalcin and $25(OH)D_3$ were measured using an in-house (15) and $25(OH)D_3$ RIA kit (DiaSorin NV, Anderlecht, Belgium), respectively.

Microcomputed Tomography

Microcomputed tomography (μCT) analysis of the femur was performed ex vivo using the high resolution SkyScan 1172 system (settings: 50 kV, 200 μA, 0.5-mm Al filter, 5-μm pixel size; Bruker-microCT, Kontich, Belgium) as previously described (16). In brief, serial tomographs, reconstructed from raw data using the cone-beam reconstruction software (NReconx v.1.4.4.0; Bruker-microCT), were used to calculate trabecular and cortical parameters, respectively, from the metaphyseal and middiaphyseal area. A bone standard was used for the calibration of bone-density measurements. Measurements were performed in the Laboratory of Clinical and Experimental Endocrinology, KU Leuven, according to the guidelines of the American Society for Bone and Mineral Research.

Glucose Tolerance Test

Glycemia was measured in tail vein blood using a glucometer before and after (5, 15, 30, 60, and 120 min) an intraperitoneal injection of 2 g/kg BW D-glucose monohydrate dissolved in sterile PBS.

Histology, Immunohistochemistry, and Insulin Determination

Severity of insulitis was assessed by histological screening of pancreatic sections of at least four animals per group imbedded in paraffin taken from experimental NOD mice at 35 weeks of age. Tissue sections (6 μm) from formalin-fixed paraffin-embedded pancreata of each animal were cut and collected 100 μm apart, then stained with hematoxylin and eosin. Islets were observed under light microscopy

at original magnification ×20 or ×40, enumerated, and graded by an independent investigator in blinded fashion. At least 25 islets per pancreatic sample were scored for islet infiltration as follows: 0, no infiltration; 1, peri-insulitis; 2, lymphocyte infiltration in less than 50% of the islet; 3, lymphocyte infiltration in more than 50% of the islet; or 4, completely destroyed islets.

Immunofluorescence detection of FoxP3-expressing cells was performed on 6-μm-thick paraffin tissue sections. Briefly, after antigen retrieval (10 mmol/L citrate buffer [pH = 6], 10 min), sections were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-FoxP3 primary antibody (5 μg/mL, clone FJK-16a; eBioscience) in Tris-buffered saline/proteinase I with 5% BSA (overnight, 4°C), followed by AlexaFluor (AF) 488–conjugated rabbit anti-FITC antibody (12.5 μg/mL; Molecular Probes, Invitrogen). For signal amplification, AF 488–conjugated anti-rabbit IgG was used (8 μg/mL; Molecular Probes). Next, insulin was revealed using guinea pig anti-swine antibody (0.3 mg/mL, 60 min; DakoCytomation) and AF 555–conjugated goat anti-guinea pig IgG (4 μg/mL, 60 min; Molecular Probes). The other half of each pancreas was used for insulin content determination as described (17).

Flow Cytometric Analysis

Single-cell suspensions of spleen and pancreatic lymph nodes (PLN) were prepared from mice at 35 weeks of age. The following antibodies were used for staining: CD4 (GK1.5), CD8 (53-6.7), CD25 (PC61.5), CD11b (M1/70), CD11c (N418), I-A^k (10-3.6, cross-reactive with I-A^{g7} of NOD mice), the programmed death 1 ligand (MIH5), interferon-γ (IFN-γ), FoxP3, and matching isotype controls (all eBioscience). Cells were analyzed in a Gallios flow cytometer with Kaluza software (Beckman Coulter).

Statistical Analysis

Graphs were plotted and statistics calculated with GraphPad Prism software (GraphPad Software, La Jolla, CA). Differences in the incidence of diabetes were assessed using the Mantel-Cox log-rank test. Results were expressed as mean ± SEM if normally distributed or as median and interquartile range if not normally distributed. The Student *t* test or Mann-Whitney test were used for comparison between groups, respectively. *P* values of <0.05 were considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

RESULTS

Safety of Vitamin D_3 Supplementation

On the basis of dose-titration studies (data not shown), we selected 800 IU/day (40,000 IU/kg BW/day) as a dose raising the vitamin D levels into the "highly vitamin D–sufficient" zone [25(OH)D_3 concentration >230 nmol/L], without inducing hypercalcemia or bone decalcification. Only in mice that were treated lifelong (from 3 until 35 weeks of age), serum 25(OH)D_3 concentration increased to a mean concentration of 290 nmol/L (236–351 nmol/L) in male and female NOD mice at 35 weeks of age compared with 173 nmol/L (125–224 nmol/L) in the group fed the control diet (Table 1). This protocol did not perturb normal weight evolutions and did not cause significant alterations in serum phosphate or calcium levels (Table 1). However, the 25(OH)D_3 concentrations in mice supplemented with vitamin D_3 during pregnancy and lactation or during early life (from 3 until 14 weeks of age) were comparable to mice fed the control diet until 35 weeks of age (Supplementary Fig. 1).

Histological analysis of kidney and heart of the vitamin D_3 group revealed no calcifications in aorta and renal arteries (data not shown). To verify whether vitamin D_3 supplementation affected bone metabolism, serum osteocalcin and bone parameters were evaluated. Serum osteocalcin concentrations were not altered by lifelong vitamin D_3 supplementation (Table 1), indicating no alterations in bone turnover. Moreover, bone dry weight and the ratio of calcium-to-bone dry weight was similar to controls in the vitamin D_3 group at the end of the observation period (Table 1). The bone microstructure analysis by μCT showed that dietary vitamin D_3 did not affect bone-mineral density and did not impair cortical or trabecular bone architecture

Table 1—Vitamin D and calcium metabolism after vitamin D_3 supplementation in NOD mice

	35 weeks of age			
	Females		Males	
Parameters	Controls	800 IU	Controls	800 IU
Weight (g)	26.5 ± 0.5	26.0 ± 0.7	32.2 ± 0.4	33.6 ± 3.2
Serum 25(OH)D_3 (nmol/L)	184.5 ± 6.1	301.3 ± 20.9***	160.3 ± 5.1	280.0 ± 10.9***
Serum calcium (mmol/L)	2.4 ± 0.1	2.6 ± 0.1	2.6 ± 0.2	2.6 ± 0.1
Serum phosphate (mmol/L)	3.2 ± 0.3	3.7 ± 0.3	3.3 ± 0.2	3.0 ± 0.2
Serum osteocalcin (ng/mL)	52.2 ± 5.8	49.5 ± 5.8	35.4 ± 4.1	39.3 ± 3.2
Bone dry weight (mg)	51.1 ± 0.9	52.7 ± 0.7	51.5 ± 5.4	56.3 ± 0.9
Calcium-to-bone dry weight	0.30 ± 0.21	0.36 ± 0.21	0.33 ± 0.01	0.31 ± 0.02

Summary data (mean ± SEM) from female and male (*n* = 4–5 per experiment). NOD mice were fed normal chow (4 IU vitamin D, control) or chow supplemented with 800 IU/day vitamin D_3. Statistical significance was calculated by the Student *t* test. $^{***}P < 0.001$ vs. controls.

(Fig. 1*A* and *B*). The vitamin D_3–supplemented diet did not affect any of the analyzed bone parameters (Fig. 1*B*). Overall, long-term vitamin D_3 supplementation was tolerated well, and we found no indications of vitamin D toxicity or adverse metabolic side effects.

Vitamin D_3 Supplementation Reduces Diabetes Incidence in NOD Mice When Given Early and Lifelong

We next assessed the effect of vitamin D_3 on diabetes development in the NOD mouse. Vitamin D_3 was given prophylactically (800 IU/day) during pregnancy and lactation (until 3 weeks of age), during early life (from 3 until 14 weeks of age), or lifelong from early life onwards (from 3 until 35 weeks of age). Only lifelong vitamin D_3 supplementation could significantly inhibit diabetes development in female and male NOD mice, resulting in a 52% reduction in diabetes incidence in females and a 58% reduction in males compared with mice fed the control diet (Fig. 2*A*). Only the long-term supplementation was effective in controlling diabetes progression, because shortening the period of vitamin D_3 treatment from 3 until 14 weeks of age caused a loss of diabetes protection by vitamin D_3: in females, the diabetes incidence in vitamin D_3–supplemented mice was comparable to the control group (Fig. 2*B*), whereas in males, vitamin D_3 supplementation demonstrated a trend to decreased diabetes development, yet this was not significant (Fig. 2*B*). When given during pregnancy and lactation, vitamin D_3 supplementation had no effect on diabetes development in female or male offspring (Fig. 2*C*).

Lifelong Vitamin D_3 Supplementation Preserves β-Cell Function and Reduces Severe Insulitis

Prevention of diabetes by vitamin D_3 suggests preservation of sufficient β-cell mass and/or function. To test this, we first did an intraperitoneal glucose tolerance test and found that the disease-free vitamin D_3 group controlled a glucose bolus with similar efficiency as normoglycemic NOD mice fed the control diet (Fig. 3*A*). Second, the insulin content of pancreata harvested from disease-free vitamin D_3–supplemented mice was comparable to amounts present in normoglycemic controls. Importantly, pancreata of disease-free vitamin D_3–supplemented animals contained significantly more insulin than pancreata of diabetic NOD mice fed the control diet at 35 weeks of age (Fig. 3*B*). Third, scoring of the insulitis revealed that immune infiltration in islets worsened in the control group, whereas less severe insulitis, including a higher number of insulitis-free islets, were observed in most of the vitamin D_3–supplemented mice (Fig. 3*C* and *D*). Taken together, treatment efficiency was reflected not only in parameters measuring β-cell function and mass but also in the grade of insulitis.

Vitamin D_3 Supplementation Decreases Effector $CD8^+IFN\text{-}\gamma$ and $CD4^+IFN\text{-}\gamma$ T Cells and Increases $CD4^+(CD25^+)FoxP3^+$ Regulatory T Cells

We found that lifelong vitamin D_3 supplementation (from 3 until 35 weeks of age) did not cause immune depletion because the total number of lymphocytes in the spleen and PLN was similar to values found in sex-matched NOD

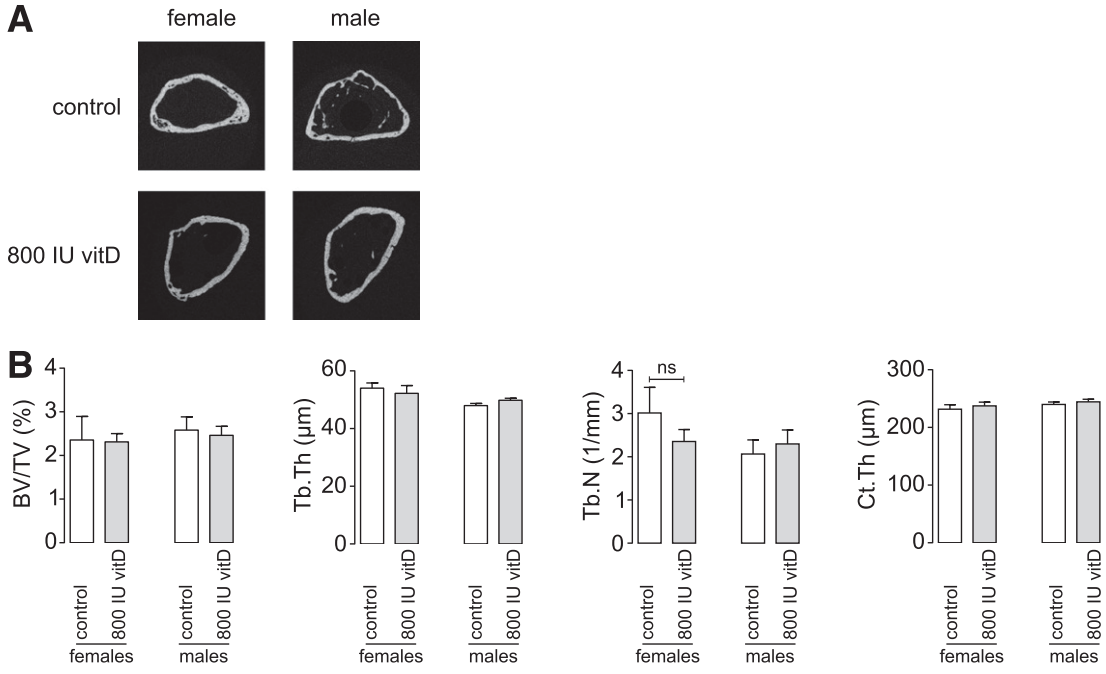

Figure 1—Dietary vitamin D_3 supplementation does not affect bone parameters. *A*: μCT-based visualization of trabecular bone in femur from control (*upper panels*) and vitamin D_3–supplemented NOD mice (*lower panels*). *B*: From left to right: quantification of the trabecular bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and cortical thickness (Ct.Th) in control mice and long-term 800 IU/day vitamin D_3–supplemented mice at 35 weeks of age. Statistical significance was calculated using Student *t* test. ns, not significant.

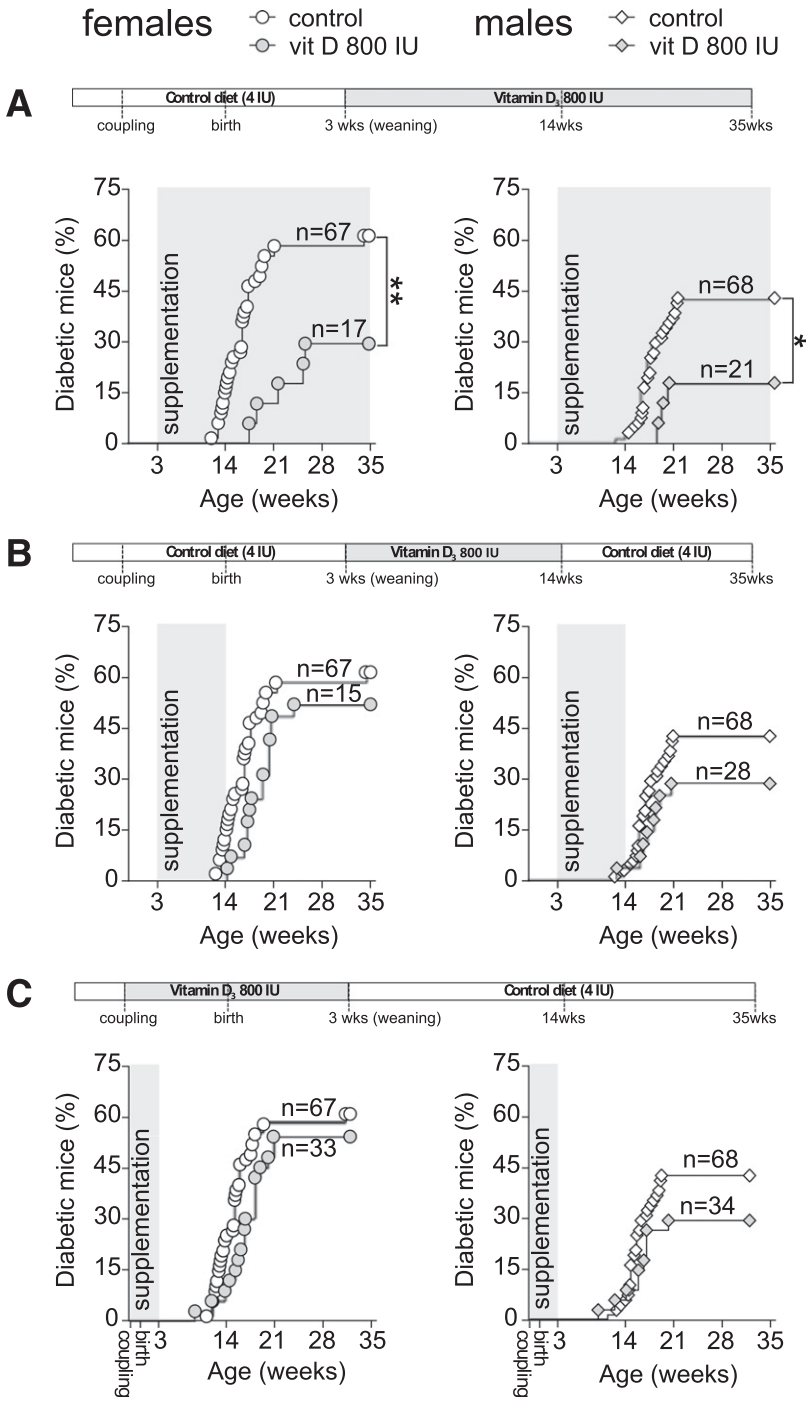

Figure 2—Lifelong vitamin D_3 supplementation reduces diabetes incidence in NOD mice. Female and male NOD mice were fed control chow until 35 weeks of age or fed chow supplemented with 800 IU/day vitamin D_3 per from 3 until 35 weeks of age (lifelong) (*A*), from 3 until 14 weeks of age (early life) (*B*), or during coupling until 3 weeks of age (pregnancy and lactation) (*C*). Dietary regimens are depicted graphically; in gray is the period when mice received the vitamin D_3–supplemented diet. Mice with two consecutive measurements of blood glucose levels >200 mg/dL were considered diabetic. Statistical significance was calculated vs. NOD mice fed control chow by Mantel-Cox log-rank test. $^*P < 0.05$; $^{**}P < 0.01$.

mice fed the control diet (Fig. 4). Also, there were no significant differences in the frequencies of dendritic cell and macrophage subsets in spleen and PLN between the vitamin D_3 and the control group (data not shown). Likewise, expression of the antigen-presenting molecule MHC class II I-A^{g7} and the programmed death 1 ligand on antigen-presenting cells was unaltered in mice receiving dietary vitamin D_3 supplementation (data not shown).

We did not observe differences in the absolute number and proportions of $CD4^+$ and $CD8^+$ T cells in the spleen

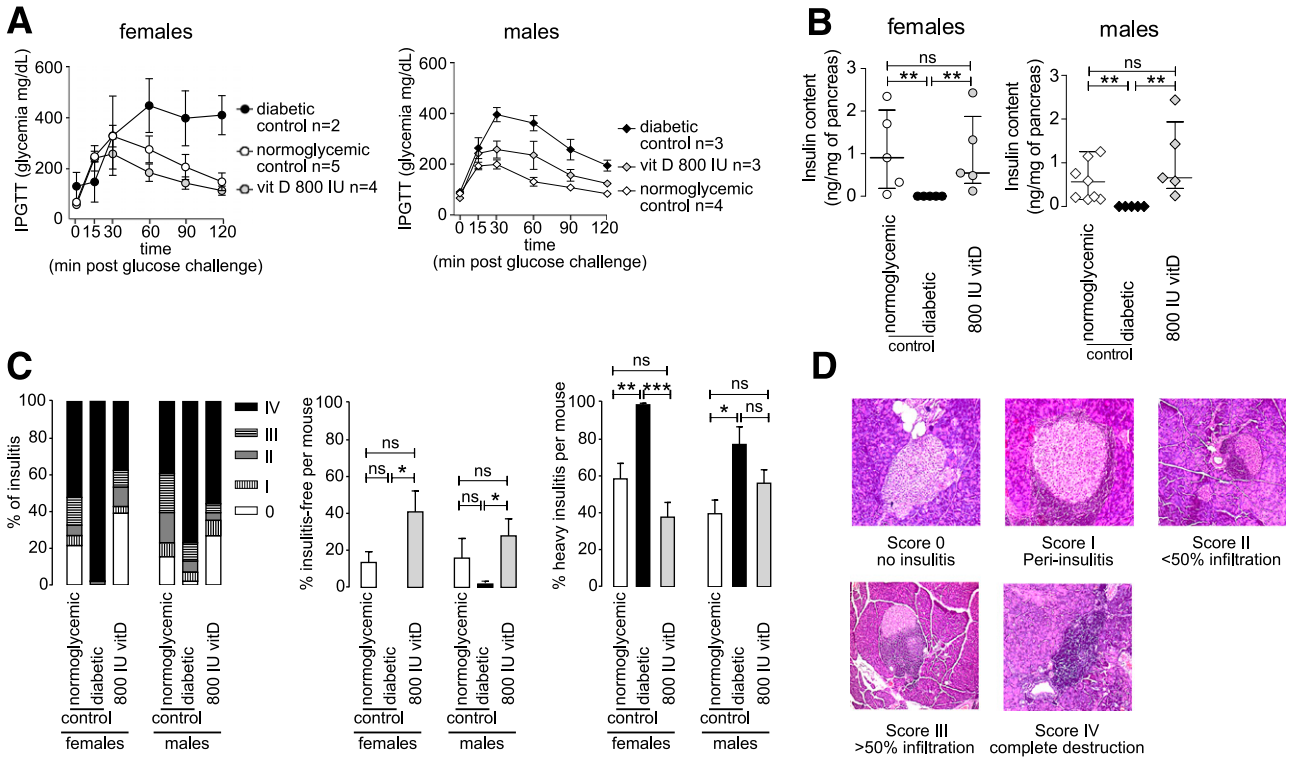

Figure 3—Lifelong vitamin D_3 supplementation preserves insulin content and decreases severe insulitis. *A*: Intraperitoneal glucose tolerance tests (IPGTT) were performed at the end of the observation period (at 35 weeks of age) in experimental groups, as indicated. *B*: Evaluation of insulin content in pancreatic extracts from experimental groups by ELISA, as indicated. Data are expressed as ng/mg of pancreas. Graphs show the median and interquartile range. Statistical significance between two groups was calculated using the Mann-Whitney test. ns, not significant; $^{**}P < 0.01$. *C*: Pancreatic sections of indicated groups were stained with hematoxylin and eosin, and insulitis was scored as indicated in RESEARCH DESIGN AND METHODS. *From left to right* graphs show the percentage of insulitis, percentage of insulitis-free islets, and percentage of islets with heavy insulitis in vitamin D_3–supplemented mice vs. control normoglycemic and diabetic mice. Statistical significance between two groups was calculated using the Mann-Whitney test; ns, not significant; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. *D*: The following score was used for the evaluation of insulitis: 0 (white, intact islets), I (vertical lines, peri-insulitis), II (gray, <50% infiltration), III (horizontal lines, >50% infiltration), IV (black, complete destruction).

and PLN after vitamin D_3 supplementation (Fig. 4 and Supplementary Fig. 2). However, lifelong vitamin D_3 supplementation induced a shift in the balance between effector T cells (Teff) and regulatory T cells (Tregs). As such, vitamin D_3 supplementation significantly decreased the frequency as well as the total number of $CD8^+$ T cells producing IFN-γ in the spleen and PLN of female and male NOD mice (Fig. 5*A* and Supplementary Fig. 3). Moreover, the proportions and the absolute numbers of $CD4^+$ T cells expressing IFN-γ were lower in the spleen of vitamin D_3–supplemented female mice and in the PLN of vitamin D_3–supplemented male mice compared with sex-matched NOD mice fed the control diet (Fig. 5*B* and Supplementary Fig. 3).

Expression of FoxP3, a transcription factor associated with Treg differentiation and function (18), is presently the most reliable marker to identify Tregs in mice. Female and male NOD mice receiving lifelong vitamin D_3 supplementation showed a significantly higher frequency of $CD4^+FoxP3^+$ Tregs in the PLN but not in the spleen compared with sex-matched mice fed the control diet (Fig. 6*A*). Analysis of CD25 expression on $CD4^+FoxP3^+$ T cells revealed that this increase concerned activated Tregs, which were increased not only in the spleen but also in the PLN of vitamin D_3–treated mice (Fig. 6*B*) (19,20). Of note, vitamin D_3 supplementation during pregnancy and lactation or during early life (from 3 until 14 weeks of age) did not increase the percentages of $CD4^+(CD25^+)$ $FoxP3^+$ T cells in the spleen or PLN (Supplementary Fig. 4).

Enumeration of $FoxP3^+$ cells in the pancreas revealed higher numbers of $FoxP3^+$ T cells in the islet infiltrates of vitamin D_3–supplemented mice, especially in the females, compared with diabetic controls (Fig. 7), suggesting these Tregs suppress autoreactive responses not only in the PLN but also at the site of inflammation.

DISCUSSION

Vitamin D is a prohormone that is well known for its role in calcium homeostasis and bone mineralization and metabolism, but accumulating data suggest additional roles in many important body functions, including the regulation of innate and adaptive immune responses. In humans, the daily vitamin D intake recommended by the

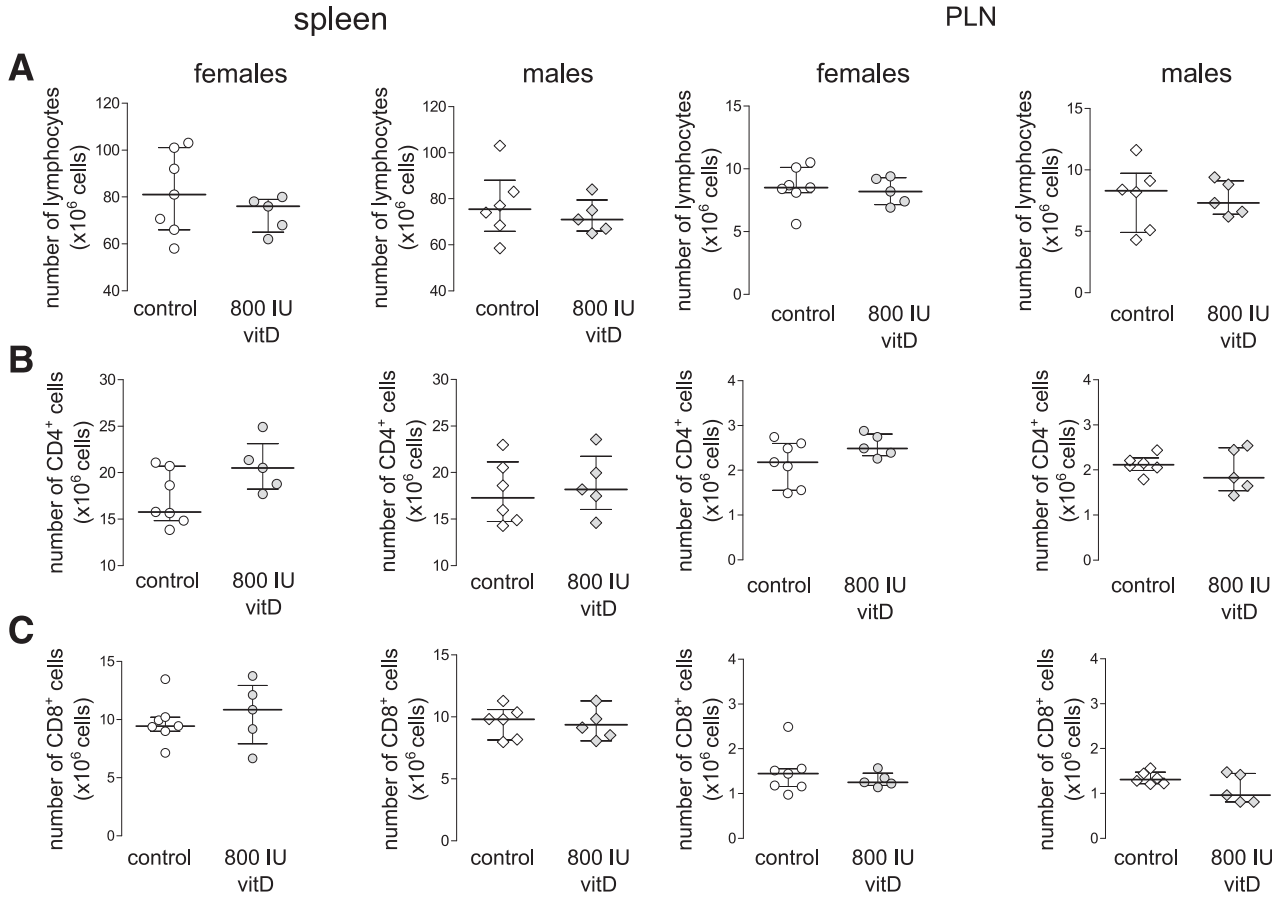

Figure 4— Unaltered lymphocyte number and subsets in spleen and PLN of mice after lifelong vitamin D_3 supplementation. Spleen and PLN were harvested from NOD mice (n = 5–7) at 35 weeks of age. The absolute number of lymphocytes (*A*), $CD4^+$ (*B*), and $CD8^+$ T-cell subsets (*C*) were measured by flow cytometry in the spleen (*left panels*) and PLN (*right panels*) of control and vitamin D_3–supplemented mice. Graphs show median and interquartile range. Statistical significance was calculated by the Mann-Whitney test.

Institute of Medicine is 400 IU/day for children younger than 1 year, up to 600 IU/day for all individuals between 1 and 70 years, assuming already some sunlight exposure, and 800 IU/day for individuals older than 70 years (21). However, many clinicians suggest that for a good health state and to allow extraskeletal benefits, the daily supply of vitamin D from all sources should be greater than 800 IU.

Until now, data are very limited on whether dietary vitamin D supplements can in fact modify the course of autoimmune diseases such as type 1 diabetes in humans. These studies often lack precise information on the dosage and regimen of vitamin D supplementation, which can lead to contradictory results. Meta-analysis of records from four case-control studies and one cohort study implies that the risk of type 1 diabetes development was significantly reduced in children treated with regular vitamin D between 7 and 12 months of age compared with those who were supplemented before 7 months of age or not supplemented (5,22). A recent meta-analysis of two cohort studies and six case-control studies on vitamin D intake during early life corroborated these findings (23). The importance of maternal vitamin D intake during pregnancy is also not clear: a case-control study in Norway indicated that the risk of type 1 diabetes was higher in the offspring of women with the lowest 25(OH)D concentrations (10), whereas other studies did not find any correlation between maternal serum 25(OH)D values and type 1 diabetes risk in the offspring (24,25). Only properly designed, randomized, and long-term trials will bring the definitive answer.

Here, we provide the first evidence that only early and lifelong vitamin D_3 supplementation with 800 IU/day, which significantly increased serum 25(OH)D_3 levels above sufficiency and corrected the immunological defects in diabetes-prone mice that already had sufficient, even high, vitamin D_3 levels, safely reduced diabetes development. Vitamin D_3 supplementation prevented severe insulitis and preserved β-cell mass best. In contrast, a short and early intervention (age 3–14 weeks) with regular vitamin D_3 was less protective, in line with our previous study showing that short-term intraperitoneal administration of 1,000 IU of vitamin D_3 to NOD mice early in life, corresponding to neonatal life and childhood in man, was insufficient to protect against diabetes (13).

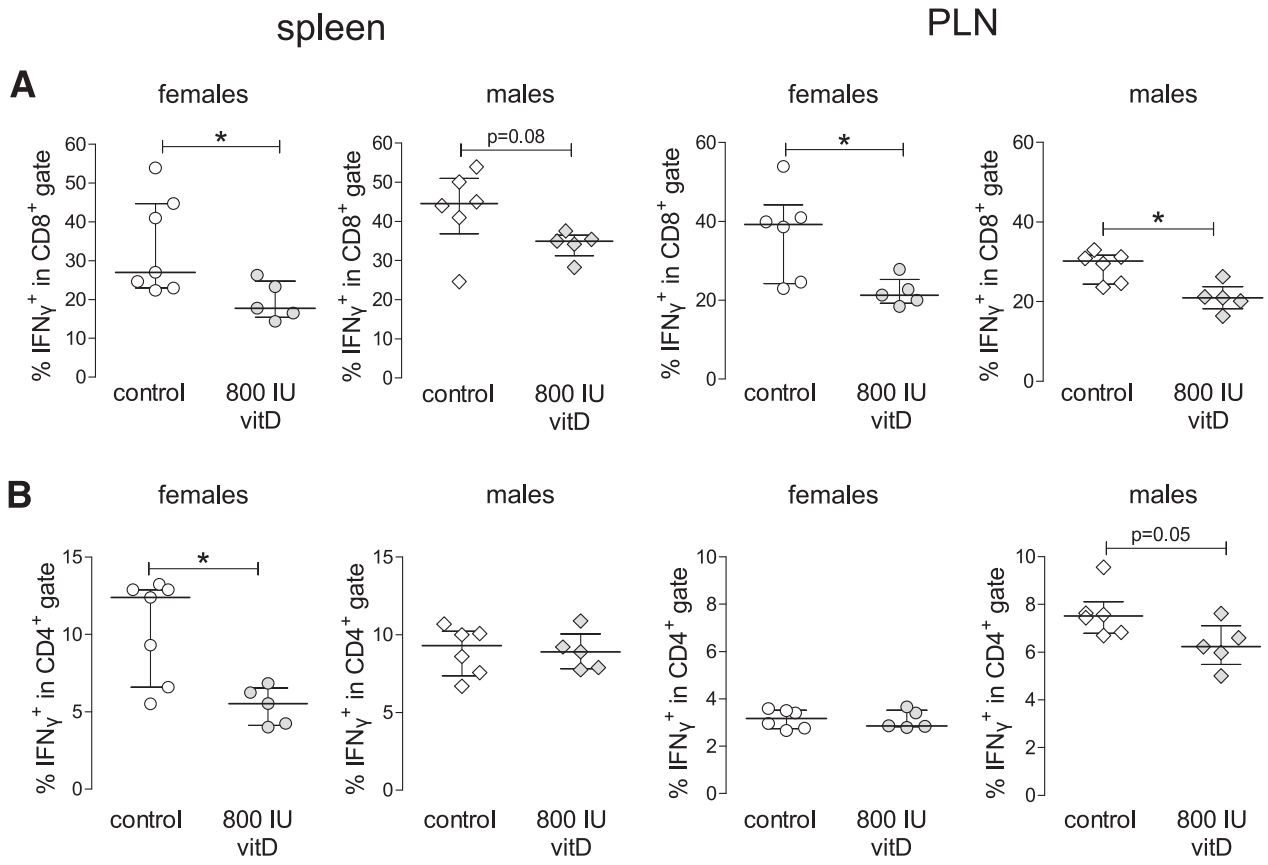

Figure 5—Decrease of $CD8^+IFN\text{-}\gamma^+$ T cells in spleen and PLN after lifelong vitamin D_3 supplementation. Spleen and PLN were harvested from NOD mice (n = 5–7) at 35 weeks of age. The frequencies of $CD8^+IFN\text{-}\gamma^+$ (*A*) and $CD4^+IFN\gamma^+$ (*B*) T-cell subsets were measured by flow cytometry and are displayed as the percentage of IFN-γ^+ in $CD8^+$ (*A*) or $CD4^+$ (*B*) gate in the spleen (*left panels*) and PLN (*right panels*) of control and vitamin D_3–supplemented mice. Graphs show median and interquartile range. Statistical significance was calculated by the Mann-Whitney test. *$P < 0.05$.

Of note, we did not evaluate the effects of vitamin D supplementation at a later stage in life (e.g., age 14–35 weeks) because we demonstrated previously that a late intervention with the bioactive vitamin D_3 metabolite (from 14 weeks of age, when insulitis is present) failed to prevent disease (26).

Oral administration of vitamin D may favor regulation of tight junction proteins necessary to maintain mucosal integrity (27). Moreover, Ooi et al. (28) demonstrated that oral bioactive vitamin D_3 can regulate the composition of the gut bacterial microflora and that deficiency of vitamin D or its receptor can cause impaired epithelial integrity, dysbiosis, increased inflammation, and more severe experimental colitis. Active vitamin D also regulates the development and function of regulatory invariant natural killer T cells and intraepithelial CD8αα lymphocytes in the gut (28,29). These data imply that vitamin D supplementation might be a way to modulate the gut microflora and its microbiome and, consequently, regulates gastrointestinal innate and adaptive immune responses.

Recently, Roy et al. (30) suggested that not just the antagonistic interaction between Treg and Teff cells but also their continuous regulation by vitamin D ultimately determines the outcome of an (auto)immune disease. This implies that optimal vitamin D concentrations are vital in keeping the balance between Treg and Teff cells in individuals (or animals) with an underlying immune dysfunction. In fact, female and male NOD mice fed a vitamin D_3–sufficient control diet have normal concentrations of serum $25(OH)D_3$ (mean, 173 nmol/L) and still present a high incidence of diabetes. Thus, our data suggest that $25(OH)D_3$ concentrations of more than 230 nmol/L may be required to modulate immune dysregulation in NOD mice. Although further studies are needed, a recent report from the Institute of Medicine indicated that serum 25 $(OH)D_3$ concentrations in humans above 125 nmol/L may result in potential adverse reactions and there should be caution against overtreatment (21).

Several studies in human subjects evaluated the influence of administering vitamin D to pregnant and/or breastfeeding women on the risk of developing type 1 diabetes in their children (31,32). Despite some early studies suggesting a protective effect of vitamin D, a meta-analysis by Zipitis and Akobeng (22) concluded that supplementing pregnant women with vitamin D

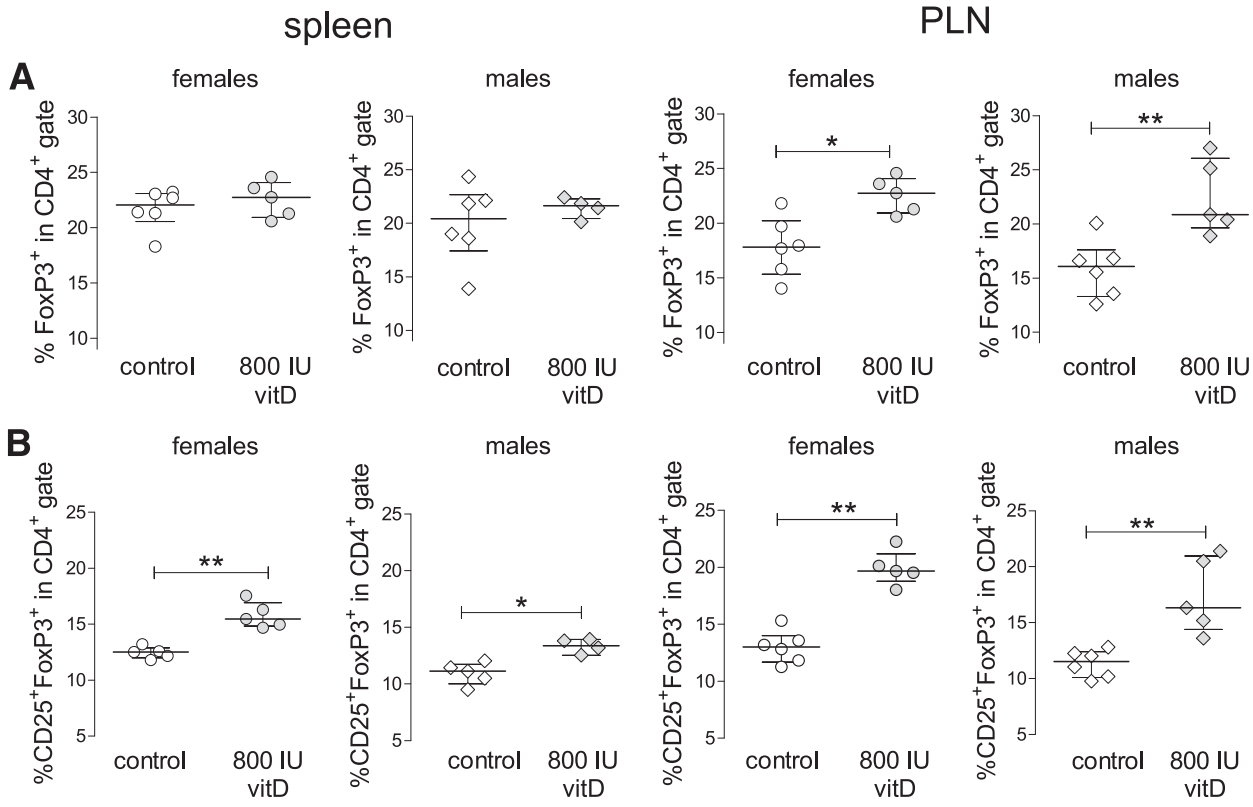

Figure 6—Lifelong vitamin D_3 supplementation increases Treg frequencies. At 35 weeks of age, spleen (*right panels*) and PLN (*left panels*) were harvested from NOD mice (n = 5 to 6), and the frequency of Tregs was determined as the percentage of $FoxP3^+$ in the $CD4^+$ fraction (*A*) and activated Tregs as the percentage of $CD25^+FoxP3^+$ in the $CD4^+$ gate (*B*) using flow cytometry. Graphs show median and interquartile range. Statistical significance was calculated using the Mann-Whitney test. $^*P < 0.05$; $^{**}P < 0.01$.

had no implications on type 1 diabetes development in their infants. Our current observations in NOD mice are consistent with this. Moreover, our data showing that vitamin D_3 supplementation during lactation failed to prevent disease in NOD mice are in line with an earlier study showing that 16 IU/day vitamin D from conception until 10 weeks of age does not suffice to interfere with disease progression in NOD mice (33). It is suggested that during this early period, the adaptive immune system has not yet fully matured and thus escapes the immunomodulatory action of vitamin D. As such, supplementation with vitamin D_3 during pregnancy and lactation in already vitamin D_3–sufficient mothers will not have immunoregulatory actions on the hyperactivity of effector immune cells against self-antigens. We also found that our vitamin D_3 protocol given from early life on and lifelong did not cause immune depletion because the total number of lymphocytes and T-cell subsets (e.g., CD4, CD8) in the spleen and PLN remained unchanged. However, vitamin D_3 supplementation dampened Th1 responses, which are often associated with autoimmune responses (34), and skewed the Teff-to-Treg balance in favor of Tregs in diabetes-prone mice. Interestingly, the increase of Tregs concerned activated Tregs according to CD25 expression (20,35). Our observations are supported by several in vitro and in vivo findings. The active form of vitamin D exerts a marked inhibitory effect on the adaptive immune cells by inhibiting Th1 polarization (36) and triggering the emergence of Tregs with the functional capacity to suppress activation and proliferation of Teff cells (37,38). Here, we report that upon vitamin D_3 supplementation, these Treg (subsets) preferentially accumulate in draining PLN and pancreatic islets. Our group had already documented that a vitamin D analog had the capacity to imprint human T cells with a specific homing signature favoring migration to sites of inflammation (37).

Finally, caution is warranted when interpreting these data, because not only lifelong administration of vitamin D_3 was necessary for protection but also extremely high doses were needed, namely, more than 200 times the amount of vitamin D normally present in the daily amount of food of a mouse. Converting these doses to humans is difficult, and we advocate that rather the serum concentrations of $25(OH)D_3$ reached after supplementation versus baseline in the mice should be considered. The values achieved in this study (mean, 290 nmol/L) are well above the present advocated targets of 50–100 nmol/L for humans (39), but it is important to keep in mind that control NOD mice are already very vitamin D–sufficient [serum $25(OH)D_3$ concentrations >150 nmol/L]. If we apply the estimate of Heaney et al. (40) that a 40 IU/day increment in intake raises $25(OH)D_3$ by 0.7 nmol/L in

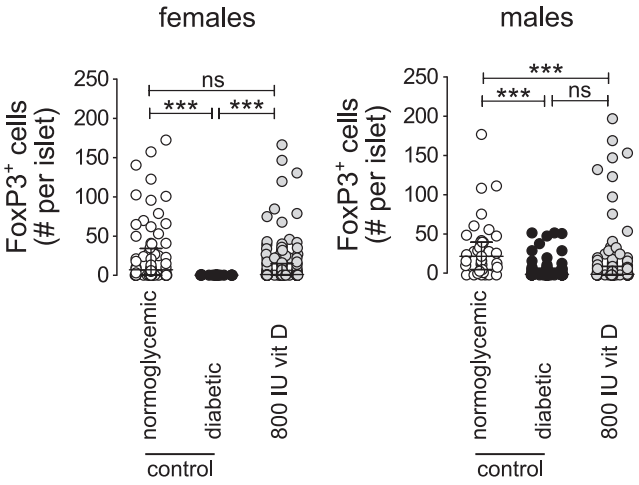

Figure 7—Increased numbers of FoxP3$^+$ cells in the pancreatic infiltrates of lifelong vitamin D_3–supplemented mice. Number of FoxP3$^+$-expressing cells in pancreatic infiltrates as determined by manual counting of FoxP3$^+$ cells on immunostained paraffin sections (n = 6–14). Scatter plots show the median with interquartile range. Statistical significance was calculated using the Mann-Whitney test. ns, not significant; ***$P < 0.001$.

healthy men with a mean baseline value of 70 nmol/L, then a vitamin D_3 dose of 12,500 IU/day or more will be needed to raise serum 25(OH)D_3 concentrations to 290 nmol/L. These doses are thus completely out of the range of the suggested supplements for bone health (39) and may lead to calcemic side effects when administered long-term. At present, it is thus not advised to systematically administer these high doses in humans, because randomized intervention trials will be needed to demonstrate or refute the potential of these mega doses in diabetes prevention in humans.

In conclusion, lifelong dietary supplementation with high doses of regular vitamin D_3 brings 25(OH)D_3 concentrations above 230 nmol/L, prevents diabetes in NOD mice in a safe way, and is accompanied by induction of Tregs. This preclinical study confirms the potential of exploiting the vitamin D system in the prevention of type 1 diabetes in humans, but the fact that only lifelong supplementation with high doses altered disease presentation warns for overly optimistic statements on duration of treatment.

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