1α,25-Dihydroxyvitamin D_3 Induces an Autoantigen-Specific T-helper 1/T-helper 2 Immune Shift in NOD Mice Immunized With GAD65 (p524–543)

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Prevention of type 1 diabetes in NOD mice by 1,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is accompanied by a T-helper (Th) 1/Th2 cytokine shift in the pancreas. The aim of this study was to investigate whether this immune shift also occurs outside of the pancreas and whether it is limited to autoantigen-specific immune responses. NOD mice treated with 1α,25$(OH)_2D_3$ (5 μg/kg every 2 days) or control vehicle were immunized with GAD65 (p524–543) or ovalbumin (OVA) in the rear footpads. First, we examined T-cell proliferation and cytokine production (via enzyme-linked immunosorbent assay) of draining lymph node cells in vitro with or without peptide rechallenge. Although no differences in proliferation were measured between control and 1α,25$(OH)_2D_3$-treated mice after in vitro GAD65 rechallenge, a marked shift in cytokine secretion profile was seen in 1α,25$(OH)_2D_3$-treated mice: interleukin-4 was increased (37 ± 5 vs. 21 ± 12 pg/ml in controls, $P < 0.005$), whereas γ-interferon levels were decreased (6 ± 3 vs. 9 ± 3 ng/ml in controls, $P < 0.05$). This shift was absent in OVA-primed mice. Second, we measured cytokine profiles by reverse transcriptase–polymerase chain reaction in popliteal lymph nodes at different time points after priming with GAD65 or OVA in vivo. A marked Th1/Th2 shift occurred in 1α,25$(OH)_2D_3$-treated mice after in vivo priming with GAD65. Again, this shift was absent after OVA immunization. Finally, we measured cytokine profiles after rechallenge with a panel of autoantigens (GAD65, heat shock protein 65, insulin B-chain) and control antigens (OVA, keyhole limpet hemocyanine, myelin proteolipid protein, tetanus toxin) and confirmed the Th1/Th2 shift in autoantigen-injected mice but not in control antigen-injected mice. In conclusion, the immune deviation induced by 1α,25$(OH)_2D_3$ in NOD mice can also be induced in the peripheral immune system but is limited to pancreatic autoantigens. *Diabetes* 49:1301–1307, 2000

Type 1 diabetes is an autoimmune disease characterized by the immune system's destruction of the insulin-producing β-cells of the islets of Langerhans in the pancreas. In the NOD mouse, a widely used animal model for the study of type 1 diabetes, lymphocytic infiltration into the pancreatic islets (insulitis) is the primary lesion and eventually progresses to overt diabetes by the age of 3 to 4 months. Although several diabetogenic islet autoantigens have been described, among which are GAD, insulin B-chain (Ins-B), and heat shock protein (hsp), the exact trigger initiating the disease is not yet known. GAD, however, is particularly relevant, because autoantibodies and T-cell–mediated responses to GAD are strongly correlated with the disease process. Indeed, already in 4-week-old NOD mice (concurrent with the onset of insulitis), antibodies and spontaneous proliferative T-cell responses to native GAD65 and GAD65 peptides (p509–528 and p524–543) have been reported (1,2). Moreover, diabetes in NOD mice can be prevented by tolerization of the mice to GAD by intrathymic (2), intravenous (1), or intraperitoneal injection (3,4) or intranasal administration (5,6). Recently, the importance of GAD65-reactive T-cells in triggering diabetes was directly shown by their ability to adoptively transfer diabetes to NOD/SCID mice (7). Moreover, β-cell–specific suppression of GAD expression in antisense GAD transgenic NOD mice blocked the generation of diabetogenic T-cells and prevented diabetes (8). Finally, also in human disease, GAD65 appears to be a major β-cell autoantigen because both antibodies and T-cells reactive against GAD65 can be identified in most type 1 diabetic patients or relatives at risk (9–11).

Characteristic cytokine secretion profiles allow the classification of activated T-cells into 2 subsets of T-helper (Th) cells: Th1 and Th2. Th1 cells are mainly involved in cell-mediated immune responses characterized by inflammation, cytotoxicity, and delayed type hypersensitivity. They are induced by the macrophage-derived cytokine interleukin (IL)-12 (12,13) and produce IL-2, γ-interferon (IFN-γ), tumor necrosis factor (TNF)-α , and TNF-β. Th2 cells, on the other hand, act mainly

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CFA, complete Freund's adjuvant; ELISA, enzyme-linked immunosorbent assay; hsp, heat shock protein; IFN-γ, γ-interferon; IL, interleukin; Ins-B, insulin B-chain; KLH, keyhole limpet hemocyanine; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; OVA, ovalbumin; PBS, phosphate-buffered saline; PLN, popliteal lymph node; PLP, myelin proteolipid protein; RT-PCR, reverse transcriptase–polymerase chain reaction; TGF, transforming growth factor; Th, T-helper; TNF, tumor necrosis factor; TT, tetanus toxin; VDR, vitamin D receptor.

as mediators of humoral immunity by activating B-cells and antibody production and downregulating Th1 cells. Th2 cells produce high levels of IL-4, IL-5, IL-10, IL-13, and transforming growth factor (TGF)-β. Increasing evidence is accumulating that type 1 diabetes and several other autoimmune diseases may be mediated by Th1 lymphocytes and cytokines and that protection or prevention of the disease can be achieved by shifting the immune response in the Th2 direction (14–17).

In view of potential therapeutic interventions, great efforts have been made in the search for immunomodulators that can inhibit Th1 and promote Th2 responses, thus causing an immune shift. In this regard, several studies recently showed a direct regulatory effect of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], the activated form of vitamin D, on the transcription of various cytokine genes, including IL-12 (18), IL-2 (19,20), IFN-γ (21), granulocyte-macrophage colony-stimulating factor (22), IL-8 (23), IL-4 (24), and TGF-β (25), through interaction with the vitamin D response elements nuclear factor of activated T-cells, NFκB, or AP-1 sites in the respective promoter regions. Furthermore, 1α,25$(OH)_2D_3$ and its analogs have been shown to manifest immunomodulatory activities in experimental models of autoimmunity such as type 1 diabetes (26–30) and experimental autoimmune encephalomyelitis (24,31–33). Our group has previously demonstrated that both insulitis and diabetes can be prevented or arrested in NOD mice by administration of 1α,25$(OH)_2D_3$ (26) or its nonhypercalcemic analogs (29). Moreover, recurrence of disease after syngeneic islet transplantation can be prevented by administration of these nonhypercalcemic analogs in combination with low doses of cyclosporin A (30). These studies also demonstrate that 1α,25$(OH)_2D_3$ induces an immune shift locally in the pancreas as well as in transplanted islet grafts that is characterized by downregulation of the Th1 cytokines and upregulation of Th2 cytokines.

The aim of this study was to investigate whether the local immune shift induced by administration of 1α,25$(OH)_2D_3$ is associated with a Th1/Th2 shift in the peripheral immune system and whether this immune shift is inducible for all antigens or is restricted to autoantigen-specific T-cell responses. Therefore, T-cell proliferation and Th1/Th2 cytokine profiles were examined after immunization of the NOD mice with GAD65 (p524–543), a diabetes-relevant peptide, or with ovalbumin (OVA), which is considered to be a diabetes-irrelevant peptide. A clear Th1/Th2 shift was observed in GAD-immunized mice and in mice immunized with other pancreatic autoantigens (e.g., hsp and Ins-B), whereas no shift was observed in the case of control antigens (OVA, keyhole limpet hemocyanine [KLH], tetanus toxin [TT], and myelin proteolipid protein [PLP]).

RESEARCH DESIGN AND METHODS

Animals. NOD mice were originally obtained from Prof. C.Y. Wu (Beijing), were further bred in our animal house, and were kept under semibarrier conditions (34). Diabetes incidence by the age of 200 days in our stock colony at the time of the study was 72% in female mice and 23–26% in male mice. Balb/c mice were purchased from Harlan (Horst, the Netherlands). Mice were fed regular diet and water ad libitum. Nondiabetic mice aged 7–9 weeks of both sexes were used in the experiments.

Proteins and peptides. Mouse GAD65 (p524–543) (SRLSKVAPVIKARMMEY GTT), hsp65 peptide (PALDSLTPANED), and TT (p830–843) (QYIKANSKIGITE) contained a $CONH_2$-group and were more than 75% pure (Eurogentec, Luik, Belgium). OVA grade V (98% purity), KLH, bovine Ins-B, and PLP peptide (amino acids 135–151) were purchased from Sigma (Bornem, Belgium).

Treatment regimen. NOD mice were treated with 1α,25$(OH)_2D_3$ from 3 weeks of age until death. The drug was suspended in arachis oil and administered at a dose of 5 μg/kg i.p. every 2 days. Control NOD mice received the treatment vehicle.

Proliferation assays. Mice were immunized in the rear footpads with 100 μg GAD65 (p524–543) or 100 μg OVA emulsified in complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI) at a 1:1 concentration. Control mice were injected with phosphate-buffered saline (PBS) emulsified in CFA. After 11 days, popliteal lymph nodes (PLNs) were isolated and dispersed into a single cell suspension. Cells were cultured in flat-bottom 96-well plates at a concentration of 4×10^6 cells/ml (200 μl/well) in RPMI-1640 medium supplemented with 10% fetal calf serum and 5×10^{-5} mol/l 2-mercaptoethanol. GAD65 (p524–543) was added at a final concentration of 4.5 μmol/l (10 μg/ml). Similarly, cells were cultured at a concentration of 1×10^6 cells/ml (200 μl/well) with the addition of 22 μmol/l (1 mg/ml) OVA. Experiments were performed in triplicate. For the last 18 h of a 72-h culture, cells were pulsed with 1 μCi [^{3}H]thymidine. The cells were then harvested, and the incorporated radioactivity was assayed by liquid scintillation counting. Data are means ± SD in counts per minute.

Enzyme-linked immunosorbent cytokine assays. Lymphocytes were cultured as described above (see PROLIFERATION ASSAYS) either alone or in the presence of 4.5 μmol/l GAD65 (p524–543) or 22 μmol/l OVA. Cell supernatants were harvested after 48 h of culture, were separated from contaminating cells by centrifugation at 930*g* for 5 min, and were stored at –20°C until used. Cytokine production was determined using the Duoset enzyme-linked immunosorbent assay (ELISA) system for mouse IL-4 and IFN-γ (Genzyme, Leuven, Belgium) according to the manufacturer's instructions.

Cytokine analysis by real-time reverse transcriptase–polymerase chain reaction. At 7–9 weeks of age, both control and 1α,25$(OH)_2D_3$-treated NOD mice (4–10 mice for each experimental setup) were injected in the rear footpads with 100 μg GAD65 (p524–543) or 100 μg OVA emulsified in CFA. Draining PLNs were removed for total RNA extraction 4 and 10 days after this primary injection.

In a separate experiment, control and 1α,25$(OH)_2D_3$-treated NOD mice were given a primary injection and a booster injection (20 days after the first injection) in the rear footpads using 100 μg of GAD65 (p524–543), hsp, Ins-B, PLP, OVA, KLH, or TT emulsified in CFA. All mice received a primary injection at 7–9 weeks of age, except for the Ins-B–immunized mice, which were primed at 15 weeks of age. PLNs were removed for RNA extraction 0, 1, 2, or 7 days after the booster injection.

Total RNA was extracted using TRIzol (Life Technologies, Gaithersburg, MD). A constant amount of 3 μg target RNA was reverse transcribed using 100 U Superscript II RT (Life Technologies) at 42°C for 80 min in the presence of 5 μmol/l oligo$(dT)_{16}$.

Real-time quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) was performed for IL-2, IL-4, IL-5, IL-10, IL-12 (p40), IL-13, IFN-γ, TGF-β, and β-actin as described before (35). Briefly, PCR reactions were performed in the ABI Prism 7700 Sequence Detector (Perkin Elmer/Applied Biosystems, Foster City, CA). The system uses the 5′ nuclease activity of the Taq polymerase to cleave a nonextendable dual-labeled fluorogenic probe. Fluorescent emission is measured continuously during the PCR reaction. Therefore, PCR amplification and detection are performed in a single step.

PCR amplifications were performed in a total volume of 25 μl containing 0.5 μl cDNA sample; 1× buffer A (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 10 mmol/l EDTA, 60 nmol/l passive reference 1); 200 μmol/l dATP, dCTP, and dGTP; 400 μmol/l dUTP; 3–9 mmol/l $MgCl_2$; 100–200 nmol/l of each primer; 0.625 U AmpliTaqGold; and 0.25 U AmpErase Uracil N-Glycosylase (Perkin Elmer/Applied Biosystems). Each reaction also contained 100 nmol/l of the corresponding detection probe (35). Each PCR amplification was performed in triplicate wells using the following conditions: 2 min at 50°C and 10 min at 94°C, followed by a total of 40 or 45 2-temperature cycles (15 s at 94°C and 1 min at 60°C).

For the generation of standard curves, plasmid clones containing a partial cDNA sequence of the target DNA were constructed by cloning the corresponding PCR fragments into pGEM Teasy plasmid vector (Promega, Madison, WI). The exact identity of the cloned fragments was confirmed by sequence analysis (Pharmacia, Uppsala, Sweden). Serial dilutions from the resulting plasmid clones were used as standard curves, each containing a known amount of template copy number (35).

Statistics. The equal-variance *t* test was used for statistical analysis. Significance was defined at the 0.05 level. Data are means ± SD for proliferation and cytokine ELISA assays or as means ± SE for cytokine RT-PCR results.

RESULTS

In vitro proliferative responses of PLN cells after immunization with GAD65 (p524–543) or OVA. In a first experiment, GAD65 (p524–543) or OVA was injected into the rear footpads, and the proliferation capacity of PLN cells was measured in vitro without peptide restimulation. After immuniza-

tion with GAD65 (p524–543), a significant increase in proliferation was observed when culturing the PLN cells in vitro in all NOD mice compared with PBS-injected NOD mice. This spontaneous proliferation was not influenced by $1\alpha,25(OH)_2D_3$ because comparable levels were measured in the $1\alpha,25(OH)_2D_3$-treated and control NOD groups ($P < 0.005$ vs. PBS-injected mice for both). No such spontaneous proliferative capacity was seen in Balb/c mice (Fig. 1*A*). These results confirm previously reported data on the spontaneous proliferative responses of GAD65-specific T-cells in 4-week-old NOD mice (1,2). No spontaneous proliferative responses were observed in OVA-immunized mice compared with PBS-injected control mice in either NOD or in Balb/c mice (Fig. 2*A*).

We further examined the proliferation of PLN cells in the same groups after in vitro rechallenge with GAD65 (p524–543) or OVA, respectively. Strong proliferative responses were observed on in vitro rechallenge with GAD65 (p524–543) or OVA (Figs. 1*B* and 2*B*) in all mouse strains examined. After in vitro rechallenge with GAD65 (p524–543), significantly higher proliferative responses were observed in all NOD mice than in Balb/c mice ($P < 0.05$). No differences in proliferative capacity were noted between $1\alpha,25(OH)_2D_3$-treated and control NOD mice (NS). On rechallenge with OVA, however, a significantly higher proliferation was evident in the $1\alpha,25(OH)_2D_3$-treated NOD group versus control NOD mice ($P < 0.01$) and Balb/c mice ($P < 0.005$). This observation confirms previous data indicating that $1\alpha,25(OH)_2D_3$ does not induce a generalized immunosuppression at that dose.

Cytokine production of in vitro proliferated PLN cells after in vivo GAD65 (p524–543) or OVA immunization. In view of the importance of inducing Th1 versus Th2 responses in the development of type 1 diabetes and the strong proliferative responses induced in the PLN cells by GAD65 (p524–543) and OVA, we further investigated the pattern of antigen-specific cytokine production in the stimulated cells. This was performed to test whether $1\alpha,25(OH)_2D_3$, which did not influence the proliferative capacity of the PLN cells, had an influence on the cytokine response to GAD65 or OVA. PLN cells from $1\alpha,25(OH)_2D_3$-treated and control NOD mice as well as from Balb/c mice in vivo immunized with GAD65 (p524–543) or OVA were cultured for 48 h in vitro with or without peptide rechallenge.

In the culture supernatant of spontaneously proliferating cells from GAD65 (p524–543)–immunized mice, no IFN-γ and relatively low IL-4 levels were measured in $1\alpha,25(OH)_2D_3$-treated and control NOD mice as well as in Balb/c mice (Fig. 3).

After in vitro restimulation with GAD65 (p524–543), however, high levels of IFN-γ were measured in the culture supernatant of cells from control NOD mice compared with Balb/c mice ($P < 0.05$), whereas treatment with $1\alpha,25(OH)_2D_3$ significantly decreased IFN-γ production ($P < 0.05$ vs. control NOD group) and reached levels comparable with those in Balb/c mice (NS vs. Balb/c) (Fig. 3*A*). IL-4 production, on the contrary, was significantly increased in PLN cells from $1\alpha,25(OH)_2D_3$-treated mice ($P < 0.005$ vs. control NOD mice, $P < 0.05$ vs. Balb/c mice) (Fig. 3*B*). Treatment of NOD mice with $1\alpha,25(OH)_2D_3$ thus clearly induces a shift from Th1 toward Th2 cytokine production, which corrects for the known deficiencies in IL-4 production in NOD mice (36,37).

After in vivo immunization with OVA, IFN-γ levels were only detectable in low concentrations after restimulation with the protein. A clear skewing of the immune system toward a Th1 cytokine profile with higher IFN-γ levels and lower IL-4 levels could be seen in all NOD mice compared with the Balb/c mice. The fact that the Th1/Th2 shift induced by $1\alpha,25(OH)_2D_3$ that was clear for GAD65 could not be observed for the diabetes-neutral OVA is noteworthy (Fig. 4).

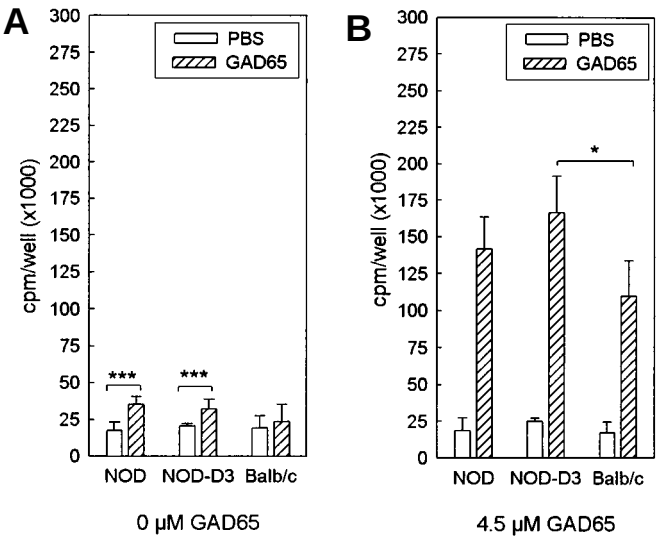

FIG. 1. GAD65-specific proliferative immune responses of PLN cells. Control (NOD) mice, $1\alpha,25(OH)_2D_3$-treated (NOD-D3) NOD mice, and Balb/c mice were immunized in the rear footpads with 100 μg GAD65 (p524–543) or PBS emulsified in CFA. After 11 days, PLN cells were purified and cultured at a concentration of 4×10^6 cells/ml for 72 h in vitro without rechallenge (*A*) or in the presence of 4.5 μmol/l GAD65 (p524–543) (*B*). During the last 18 h of a 72-h culture, the cells were pulsed with 1 μCi [^{3}H]thymidine. Data are means ± SD in counts per minute from lymphocyte cultures of 4 (GAD65) or 2 (PBS) individual mice, each performed in triplicate wells. *$P < 0.05$; ***$P < 0.005$.

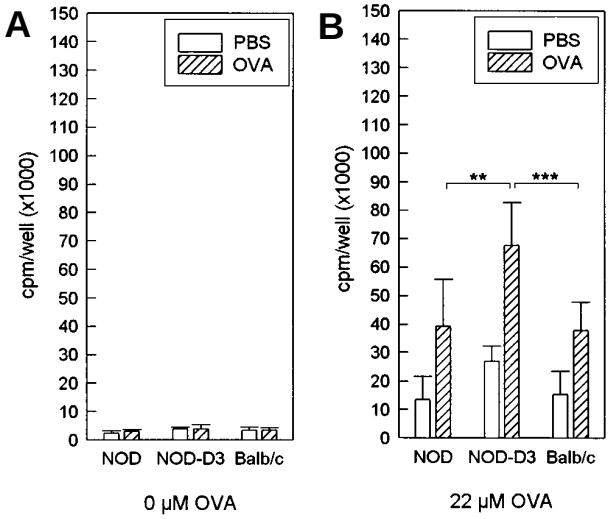

FIG. 2. OVA-specific proliferative immune responses of PLN cells. Control (NOD) mice, $1\alpha,25(OH)_2D_3$-treated (NOD-D3) NOD mice, and Balb/c mice were immunized in the rear footpads with 100 μg OVA or PBS emulsified in CFA. After 11 days, PLN cells were purified and cultured at a concentration of 1×10^6 cells/ml for 72 h in vitro without rechallenge (*A*) or in the presence of 22 μmol/l OVA (*B*). Data are means ± SD in counts per minute from lymphocyte cultures for 3–7 different mice, each performed in triplicate wells. **$P < 0.01$; ***$P < 0.005$.

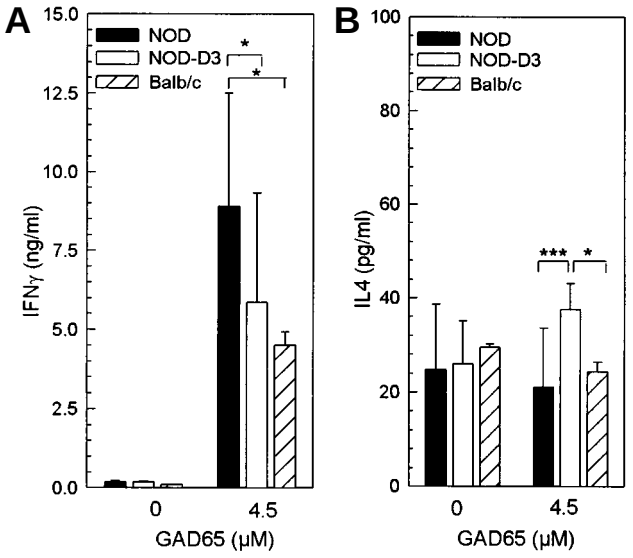

FIG. 3. IFN-γ and IL-4 production by PLN cells after immunization with GAD65 (p524–543). Control (NOD) mice, 1α,25(OH)$_2$D$_3$-treated (NOD-D3) NOD mice, and Balb/c mice were immunized in the rear footpads with 100 μg GAD65 (p524–543) emulsified in CFA. After 11 days, PLN cells were purified and cultured at a concentration of 4 × 10^6 cells/ml for 48 h without restimulation or in the presence of 4.5 μmol/l GAD65 (p524–543). IFN-γ (*A*) and IL-4 (*B*) production in culture supernatant was quantified by ELISA. Data are means ± SD for 7–10 different mice. **P* < 0.05; ****P* < 0.005.

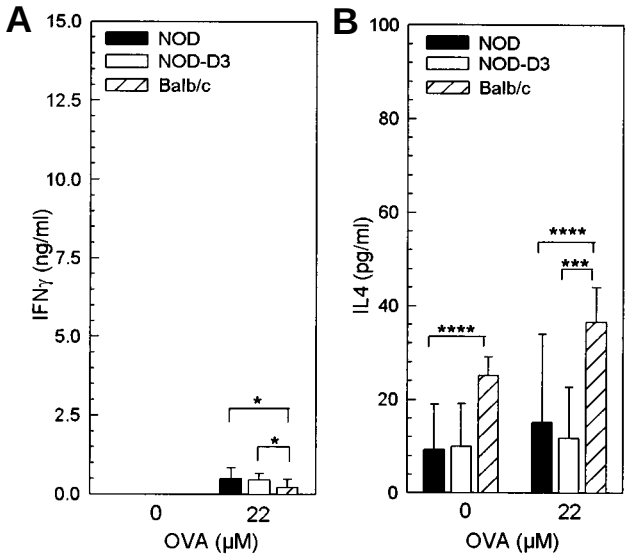

FIG. 4. IFN-γ and IL-4 production by PLN cells after immunization with OVA. Control (NOD) mice, 1α,25(OH)$_2$D$_3$-treated (NOD-D3) NOD mice, and Balb/c mice were immunized in the rear footpads with 100 μg OVA emulsified in CFA. After 11 days, PLN cells were purified and cultured at a concentration of 1 × 10^6 cells/ml for 48 h without restimulation or in the presence of 22 μmol/l OVA. IL-4 (*A*) and IFN-γ (*B*) production in culture supernatant was quantified by ELISA. Data are means ± SD for 5–10 different mice. **P* < 0.05; ****P* < 0.005; *****P* < 0.001.

In vivo cytokine mRNA expression of PLN cells of NOD mice immunized with GAD65 (p524–543) or OVA. To examine whether the pattern of cytokine production observed in vitro was reflected in the peripheral immune system in vivo, we quantified mRNA levels of different cytokines in PLN cells after in vivo immunization with GAD65 (p524–543) or OVA. We measured the major Th1-inducing cytokine (IL-12), cytokines specific for a Th1 response (IFN-γ and IL-2), and cytokines specific for a Th2 response (IL-4, IL-5, IL-10, IL-13, and TGF-β). Cytokine mRNA levels were measured in PLN cells at different time points after in vivo priming (days 0, 4, and 10) with GAD65 (p524–543) or OVA in 1α,25(OH)$_2$D$_3$-treated and control NOD mice.

After in vivo priming of control NOD mice with GAD65 (p524–543), a major increase in IL-12 was observed, with mRNA levels at 4 days postimmunization that were ~10-fold the baseline level (day 0) (Fig. 5*C*). These high levels were maintained and even slightly increased until 10 days postimmunization. Interestingly, significantly lower mRNA levels were measured in the 1α,25(OH)$_2$D$_3$-treated group, both at 4 days ($P < 0.05$) and at 10 days ($P < 0.05$) postimmunization compared with control NOD mice. This agrees with the reported inhibitory effect of 1α,25(OH)$_2$D$_3$ on the transcription of IL-12 by direct recognition of regulatory sequences in its promoter region (18,31). The 2 Th1 cytokines tested, IFN-γ and IL-2, were slightly upregulated at day 4 postimmunization in control NOD mice. Interestingly, for both of these cytokines, this upregulation was completely prevented by administration of 1α,25(OH)$_2$D$_3$, which resulted in significantly lower levels of IFN-γ ($P < 0.05$) and IL-2 ($P < 0.05$) in the 1α,25(OH)$_2$D$_3$-treated group compared with controls (Fig. 5*B*).

For the Th2 cytokine IL-4, on the contrary, treatment with 1α,25(OH)$_2$D$_3$ resulted in a significant transcriptional upregulation, also 4 days after GAD65 (p524–543) immunization, compared with control NOD mice ($P < 0.001$) (Fig. 5*A*). IL-5 and IL-13, 2 other Th2 cytokines tested, were detected at very low levels at all time points, and no differences in regulation were observed for treatment with 1α,25(OH)$_2$D$_3$ (data not shown). Also for IL-10, although very highly expressed, similar mRNA levels were measured in 1α,25(OH)$_2$D$_3$-treated and control NOD mice (data not shown).

Similar studies performed after in vivo priming with the diabetes-irrelevant protein OVA showed no significant differences between control and 1α,25(OH)$_2$D$_3$-treated mice for all cytokines tested (data not shown).

In vivo cytokine mRNA expression of PLN cells of NOD mice immunized with a panel of autoantigens and control antigens. To investigate whether this immune deviation induced by 1α,25(OH)$_2$D$_3$ can be observed for all autoantigens, a panel of diabetes-relevant autoantigens (GAD65, Ins-B, hsp65), nonislet self-antigens (PLP), and foreign antigens (OVA, KLH, TT) was tested. The mRNA levels of Th1 and Th2 cytokines were measured by RT-PCR in PLN cells with these antigens at different time points after rechallenge (days 0, 1, 2, and 7).

On in vivo rechallenge with all autoantigens and control antigens performed 20 days after the primary injection, IL-12 mRNA levels were very low, and no differences were noted between 1α,25(OH)$_2$D$_3$-treated and control NOD mice (data not shown). Strikingly, IFN-γ transcription was clearly decreased in 1α,25(OH)$_2$D$_3$-treated mice throughout the period analyzed after rechallenge with all 3 autoantigens (Fig. 6*A–C*). In contrast, on rechallenge with GAD65, all Th2 cytokines except IL-10 were upregulated by 1α,25(OH)$_2$D$_3$, an effect that was very pronounced for IL-4 (Fig. 6*D*). After rechallenge with Ins-B or hsp, on the other hand, no differ-

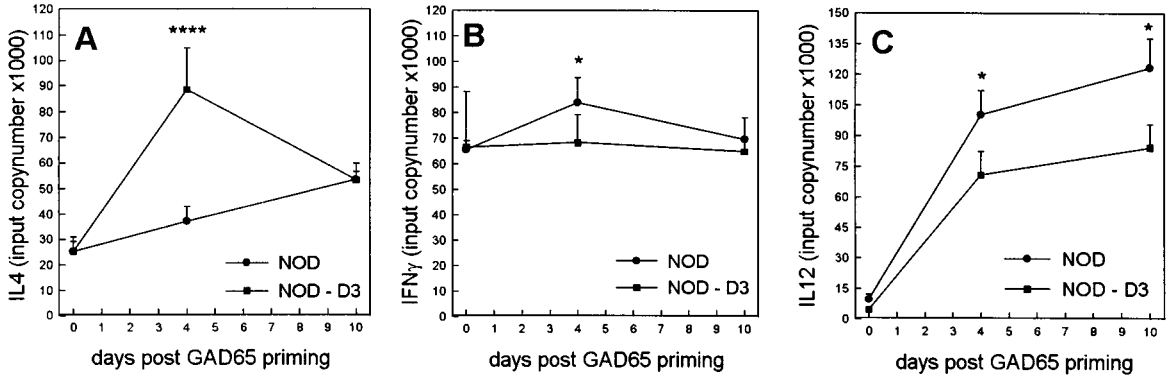

FIG. 5. In vivo cytokine mRNA expression in PLN after GAD65 (p524–543) immunization. Control (NOD) and 1α,25(OH)$_2$D$_3$-treated (NOD-D3) NOD mice were immunized in the rear footpads with 100 µg GAD65 (p524–543) emulsified in CFA. At 0, 4, and 10 days after the primary injection, PLN was isolated for total RNA extraction. The cDNA synthesis and quantitative PCR were performed as described in RESEARCH DESIGN AND METHODS. The level of cytokine mRNA is expressed as relative input copy number normalized to β-actin. Data are means ± SE of 4 (at day 0), 10 (at day 4), or 7 (at day 10) different mice for IL-4 (*A*), IFN-γ (*B*), and IL-12 (*C*) levels after GAD65 (p524–543) priming. *P < 0.05; ****P < 0.001.

ences in Th2 cytokine levels were measured between 1α,25(OH)$_2$D$_3$-treated and control NOD mice (Fig. 6*E* and *F*).

When calculating ratios of IFN-γ/IL-4, a clear Th1 to Th2 shift was observed in 1α,25(OH)$_2$D$_3$-treated mice after rechallenge with all autoantigens tested. This shift, however, is the result of both a decrease in Th1 cytokines in combination and a clear increase in Th2 cytokines on GAD65 rechallenge, whereas on rechallenge with Ins-B and hsp, this shift results mainly from an inhibition of Th1 cytokines by 1α,25(OH)$_2$D$_3$.

The cytokine profiles observed after rechallenge with the nonislet self-antigen PLP and the control antigens OVA, KLH, and TT were identical between 1α,25(OH)$_2$D$_3$-treated and control NOD mice (data not shown).

DISCUSSION

Studies have established that 1α,25(OH)$_2$D$_3$ and its analogs can prevent type 1 diabetes in the NOD mouse (26,29,38) as well as prevent recurrence of the autoimmune disease after syngeneic islet transplantation (30). From the data in these studies, the induction of suppressor cells by administration of 1α,25(OH)$_2$D$_3$, although playing a role, is clearly not the only mechanism of protection against diabetes. Additionally, 1α,25(OH)$_2$D$_3$ treatment leads to better elimination of effector cells by inducing an immune shift with lower Th1 cytokine levels locally in the inflamed islets of the native pancreas as well as in transplanted islets.

To acquire more information on the immune effects of 1α,25(OH)$_2$D$_3$ in general, we investigated whether the previously documented local immune shift induced by administration of 1α,25(OH)$_2$D$_3$ is associated with a Th1/Th2 immune shift in peripheral lymphocytes and whether it is restricted to autoantigen responses. This study conclusively demonstrates that 1α,25(OH)$_2$D$_3$ does not induce general immunosuppression. Indeed, PLN cells from 1α,25(OH)$_2$D$_3$-treated mice proliferated equally as well as control PLN cells in response to the islet autoantigen GAD65 and even better in response to the β-cell–irrelevant protein, OVA. This confirms previous findings on the normal proliferative capacity of splenocytes from 1α,25(OH)$_2$D$_3$-treated mice after in vitro concanavalin A stimulation (29). When analyzing the cytokine profiles produced by cells proliferating in response to GAD65, a Th1 to Th2 shift was noted in NOD mice treated with 1α,25(OH)$_2$D$_3$. Interestingly, this immune shift appears specific for GAD65-autoreactive T-cells because it is not seen on rechallenge with OVA. These results were confirmed and even more extensively demonstrated in vivo. In this study, 1α,25(OH)$_2$D$_3$ clearly caused a downregulation of IL-12 and the 2 Th1 cytokines tested (IFN-γ and IL-2) early after GAD65 priming. This decreased IL-12 expression is not surprising because 1α,25(OH)$_2$D$_3$ can directly inhibit IL-12 transcription (18). Because of the central role of IL-12 as the major Th1-inducing cytokine, downregulation of its expression is, as expected, accompanied by a similar decrease in IFN-γ and IL-2. Moreover, a direct regulation of IL-2 and IFN-γ by 1α,25(OH)$_2$D$_3$ has been described (20,21). On the other hand, a clear increase in Th2 cytokines can be observed. This may be in part because of IL-12 downregulation, but recently a direct upregulation of IL-4 and TGF-β has been described by Cantorna et al. (24) in another model of autoimmunity, experimental autoimmune encephalomyelitis.

A similar immune deviation was observed when mice were rechallenged with a panel of pancreatic autoantigens (GAD65, Ins-B, hsp), whereas no immune shift was observed on rechallenge with several control or nonislet self-antigens. However, for insulin and hsp, this immune deviation was exclusively because of an inhibition of the Th1 cytokines, especially IFN-γ. This again suggests that 1α,25(OH)$_2$D$_3$ primarily acts by inhibiting the Th1 pathway and eventually results in a Th1 to Th2 shift.

In all experiments, immunizations were performed with peptides or proteins emulsified in CFA. Although a possible influence of CFA itself on the Th1/Th2 balance cannot be ruled out when using this system (39–41), the immune shift induced by 1α,25(OH)$_2$D$_3$ is only evident in autoantigen/CFA-immunized mice and not in control antigen/CFA-immunized mice, which rules out a major role for CFA on the Th1/Th2 shift in the present experiments.

An intriguing observation is the fact that this shift is seen only for the autoantigens relevant in the NOD setting and not for the foreign antigens or a self-antigen not relevant to diabetes induction in the NOD mouse (e.g., PLP). No definitive explanation is available yet for this phenomenon, but one may lie in the fact that the autoantigens and control antigens differ in the way they are presented in the immune system of

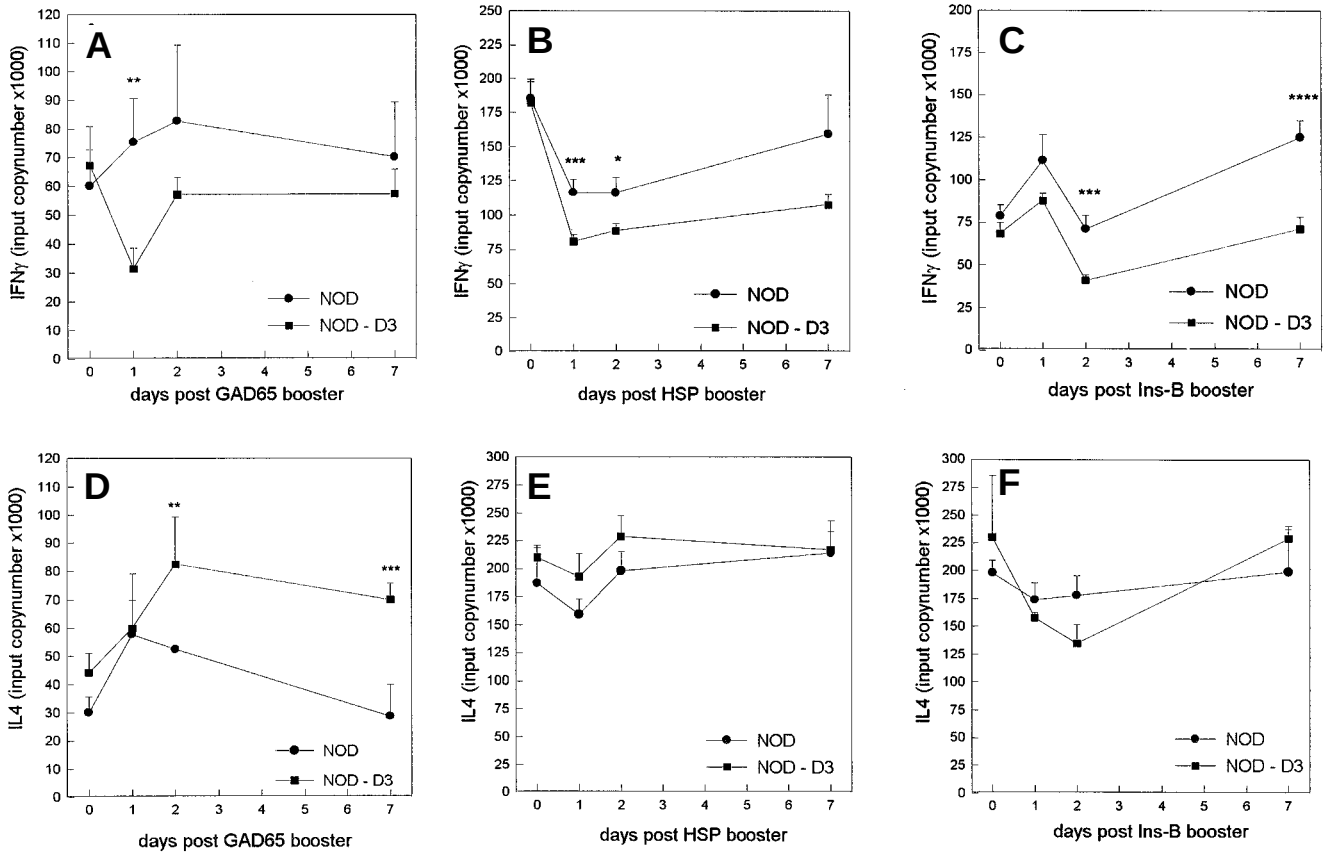

FIG. 6. In vivo IFN-γ and IL-4 mRNA expression in PLN after rechallenge with a panel of autoantigens. Control (NOD) and 1α,25$(OH)_2D_3$-treated (NOD-D3) NOD mice were given a primary and recall injection (20 days after the primary injection) in the rear footpads with 100 µg GAD65, hsp, Ins-B, OVA, KLH, TT, or PLP emulsified in CFA. At 0, 1, 2, and 7 days after in vivo peptide rechallenge, PLN was isolated for total RNA extraction. The cDNA synthesis and quantitative PCR were performed as described in RESEARCH DESIGN AND METHODS. The level of IFN-γ or IL-4 mRNA is expressed as relative input copy number normalized to β-actin. Each point is the mean ± SE of 7–10 different mice. *A*, *B*, and *C*: IFN-γ levels after GAD65, hsp, and Ins-B rechallenge; *D*, *E*, and *F*: IL-4 levels after GAD65, hsp, and Ins-B rechallenge. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$.

NOD mice. Because the autoantigens must be considered endogenous peptides, in contrast with the exogenous foreign proteins or nonislet self-peptide PLP, differences in sensitivity to 1α,25$(OH)_2D_3$ may exist in the different presentation pathways. A first important difference is that, for the NOD mice used in the study, the experimental "priming" with an autoantigen will already be a rechallenge of initial autoantigen encounters during the first weeks of life, whereas, for the foreign antigens and for PLP, the experimental contact with the peptide is a true priming. This is an important point because lymphocytes only acquire vitamin D receptor (VDR), and thus become sensitive to 1α,25$(OH)_2D_3$, after activation (42,43). Preliminary data on the presence of VDR confirm this view because much higher levels of VDR are observed in GAD65-primed NOD mice than in OVA-primed NOD mice, which indicates a higher state of activation in GAD65-responsive lymphocytes (data not shown). Our finding that 1α,25$(OH)_2D_3$ treatment in insulin-immunized mice only induced a Th1/Th2 shift in 15-week-old NOD mice and not in 8-week-old NOD mice (data not shown) supports this hypothesis. Indeed, T-cells responsive to insulin develop spontaneously in NOD mice only from 8 weeks of age on, versus GAD65 and hsp-reactive T-cells, which both arise at an earlier age (1). Other differences between endogenous and exogenous peptide presentation lie in the location of initial presentation (centrally in the thymus for endogenous peptides vs. peripherally for exogenous peptides), the type of cells performing the primary presentation or rechallenge (dendritic cells, macrophages, B-cells), and the amount of antigen presented. Each of these pathways may be differently sensitive to 1α,25$(OH)_2D_3$. The influence of the antigen-presenting cell responsible for the initial presentation is of particular importance because the antigen-presenting cell plays a central role in determining the direction of the immune response, and these cells are important targets for 1α,25$(OH)_2D_3$ (31,44,45).

In conclusion, these results show that 1α,25$(OH)_2D_3$ treatment does not induce a general immunosuppressive state because T-cells maintain their normal proliferative capacity. However, the results are highly indicative of an immune shift from a Th1 to a Th2 profile, which is limited to a subset of T-cells with reactivity for autoantigens. Treatment with 1α,25$(OH)_2D_3$ may therefore induce an autoantigen-specific "protective" Th2 cell population not only at the site of the β-cell attack as previously reported but also in the peripheral immune system. Thus, 1α,25$(OH)_2D_3$ treatment may be of potential therapeutic value in the primary or secondary prevention of type 1 diabetes by providing a minimally invasive means of specifically altering the pathogenic autoimmune responses.

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