

# Redirection of Human Autoreactive T-Cells Upon Interaction With Dendritic Cells Modulated by TX527, an Analog of 1,25 Dihydroxyvitamin D<sub>3</sub>

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**The active form of vitamin D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), is a potent immunomodulator known to affect T-cells through targeting antigen-presenting cells such as dendritic cells (DCs). We studied the effects of a novel nonhypercalcemic 1,25(OH)<sub>2</sub>D<sub>3</sub> analog, TX527, on DC differentiation, maturation, and function with respect to stimulation of a committed human GAD65-specific autoreactive T-cell clone. Continuous addition of TX527 impaired interleukin (IL)-4 and granulocyte/macrophage colony-stimulating factor (GM-CSF)-driven DC differentiation as well as lipopolysaccharide (LPS) and interferon-γ (IFN-γ)-induced maturation into Th1-promoting DC (DC1), as characterized by marked changes in DC morphology and abrogation of IL-12p70 release upon CD40 ligation. Addition of TX527 during maturation did not affect DC morphology but significantly changed DC cytokine profiles. The potential of treated DCs to alter the response pattern of committed autoreactive T-cells was found to depend on the timing of TX527 exposure. Continuously TX527-treated DCs significantly inhibited T-cell proliferation and blocked IFN-γ, IL-10, but not IL-13 production, whereas DCs treated during maturation failed to inhibit T-cell proliferation but affected IL-10 and IFN-γ production. Collectively, we provide evidence that nonhypercalcemic TX527 is a potent in vitro DC modulator, yielding DCs with the potential to change cytokine responses of committed autoreactive T-cells. *Diabetes* 51: 2119–2125, 2002**

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1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; APC, antigen-presenting cells; DC, dendritic cell; DC1, Th1-promoting DC; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte/macrophage colony-stimulating factor; IFN-γ, interferon-γ; IL, interleukin; IMDM, Iscove's modified Dulbecco's medium; LPS, lipopolysaccharide; MFI, mean fluorescence intensity.

**B**ecause preventing and curing type 1 diabetes are priorities in health care, much effort is put into gaining insight into the pathogenesis of the disease. In the NOD mouse model, it is clear that primary prevention of type 1 diabetes, e.g., autoimmunity itself, is easily achievable (1). Secondary prevention and especially prevention of recurrence of autoimmune diabetes after islet transplantation are much harder to realize. In the latter situation, an already activated immune system needs to be silenced or preferentially redirected (2). One of the protocols that are able to prevent diabetes in NOD mice, even when started after insulinitis is present, includes the use of 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), the biologically active form of vitamin D<sub>3</sub> (3,4). Although intervention in humans is not yet possible, epidemiologic data support the hypothesis that vitamin D status is an important risk factor for the development of type 1 diabetes, because dietary vitamin D supplementation during infancy was shown to be associated with a reduced risk of type 1 diabetes later in life (5).

1,25(OH)<sub>2</sub>D<sub>3</sub> is a secosteroid hormone that has several distinct functions in both bone and calcium/phosphate metabolism as well as in the regulation of immune responses (6). A variety of cells within the immune system are known to express vitamin D receptors, including antigen-presenting cells (APCs) and activated T-cells (7). 1,25(OH)<sub>2</sub>D<sub>3</sub> directly affects proliferation and polarization of naive murine T-cells by preventing the development of Th1 cells (8). However, inhibition of Th1 development by 1,25(OH)<sub>2</sub>D<sub>3</sub> is thought to be mediated primarily via its action on APCs, leading to reduced IL-12 production after 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment (9). In addition, 1,25(OH)<sub>2</sub>D<sub>3</sub> was recently shown to affect the maturation, activation, and survival of human DCs, leading to impaired priming of alloreactive T-cell responses in vitro (10–12). These results demonstrate that APCs, in particular DCs, are primary targets for the immunomodulatory activity of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

Because hypercalcemia seriously hampers clinical application of 1,25(OH)<sub>2</sub>D<sub>3</sub>, however, much effort has been put into the development of 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs with reduced hypercalcemic capacity (13). Protective effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs have been studied extensively in the NOD mouse, demonstrating prevention of both insulinitis and diabetes (14). Moreover, progression to clinically

overt diabetes and recurrence of diabetes after syngeneic islet transplantation has been prevented by analogs of  $1,25(\text{OH})_2\text{D}_3$  (15). Both studies point out that  $1,25(\text{OH})_2\text{D}_3$  analogs interfere with progression of autoimmune diabetes, i.e., changing an ongoing autoimmune reaction, which suggests an effect on committed autoreactive T-cells. Immunomodulatory agents, such as  $1,25(\text{OH})_2\text{D}_3$ , that are able to shift dominant pathogenic (Th1 associated) T-helper responses (16) and possibly induce regulatory T-cells (17) are extremely interesting.

The aim of the present study was to analyze whether incubation of DCs with TX527, a nonhypercalcemic analog of  $1,25(\text{OH})_2\text{D}_3$ , could alter DCs in such a manner that a change in the response pattern of an already committed GAD65-autoreactive T-cell clone is induced. The T-cell clone used in this study was derived from a prediabetic type 1 diabetes patient and recognizes naturally processed GAD65 as well as a defined peptide epitope in the context of HLA-DR3 (18). First we studied phenotypic and functional changes in DCs induced by TX527 or  $1,25(\text{OH})_2\text{D}_3$  treatment during various stages of their development from monocytes. In addition, we determined proliferative and cytokine responses of T-cells cocultured with nontreated TX527 or  $1,25(\text{OH})_2\text{D}_3$ -treated immature and mature DCs.

## RESEARCH DESIGN AND METHODS

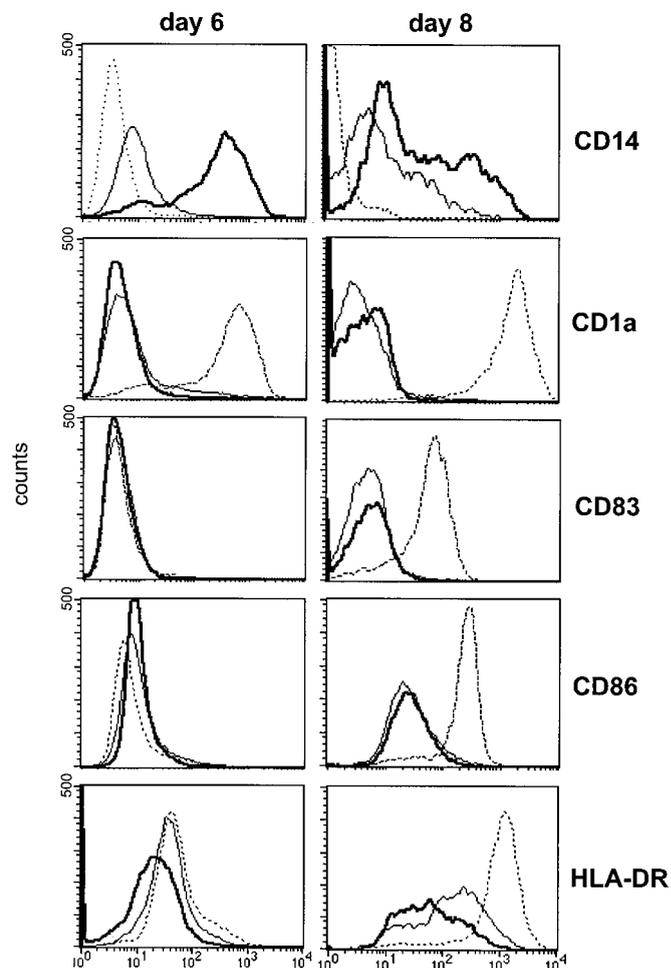
**Generation of human DCs.** Human HLA-DR3 homozygous peripheral blood mononuclear cells were isolated by Ficoll gradient from HLA-typed buffy coats, obtained from healthy blood donors. Monocytes were obtained via positive selection on a MACS column after staining peripheral blood mononuclear cells with CD14-MicroBeads according to the supplier's protocol (Milteny Biotec via CLB, Amsterdam, the Netherlands). Isolated monocytes ( $0.5 \times 10^6/\text{ml}$ , routinely >90% pure) were subsequently cultured for 6 days in RPMI 1640 medium (Gibco Life Technologies, Breda, The Netherlands) supplemented with 10% heat-inactivated FCS, 2 mmol/l glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, 1,000 units/ml recombinant human IL-4 (Strathmann Biotec AG, Hannover, Germany), and 800 units/ml recombinant human granulocyte/macrophage colony-stimulating factor (GM-CSF) (Leucomax, Novartis Pharma, Arnhem, the Netherlands) in 24-well tissue culture plates (Costar, Cambridge, MA). Medium and cytokines were refreshed every 3 days. At day 6, when DCs had obtained an immature phenotype, DC1 maturation was induced by addition of 100 ng/ml *Escherichia coli*-derived lipopolysaccharide (LPS) (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands), 1,000 units/ml recombinant human IFN- $\gamma$  (Peprotech, Rocky Hill, NJ), and 800 units/ml GM-CSF. DCs were harvested after 48 h for further analysis.

In some experiments, macrophages were generated in parallel to DCs by culturing monocytes in RPMI 1640 medium supplemented with 10% FCS, 2 mmol/l glutamine, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 50 ng/ml recombinant human M-CSF (Peprotech) in 24-well tissue culture plates. Medium and cytokines were refreshed at days 3 and 6.

**$1,25(\text{OH})_2\text{D}_3$  or TX527 treatment of DCs.**  $1,25(\text{OH})_2\text{D}_3$  was obtained from J.P. Vandeveld (Duphar, Weesp, the Netherlands). The analog TX527 (19-nor-14,20-bisepi-23-yne- $1,25(\text{OH})_2\text{D}_3$  (19,20) was synthesized by M. Vandewalle and P. De Clercq (University of Ghent, Belgium) and further obtained from Théraxem S.A. (Monaco). Both  $1,25(\text{OH})_2\text{D}_3$  and TX527 were used at a final concentration of  $10^{-8}$  mol/l and refreshed at days 3 and 6, respectively. They were added to the culture medium either during the complete culture period from monocyte to immature DCs to mature DCs or only during the maturation phase.

**Analysis of DC surface markers, cytokine production, and phagocytosis capacity.** For evaluating DCs' surface expression, DCs were incubated for 30 min at 4°C with the following FITC- or PE-conjugated murine antibodies (all from Becton Dickinson, San Jose, CA): CD1a, CD14, CD83, CD86, CD80, CD54, HLA-DR, CD40, and control mIgG, all diluted in PBS containing 0.1% BSA. After extensive washing, DCs were subsequently analyzed on a FACS Calibur (Becton Dickinson).

Because mature DC1 cells are unresponsive to bacterial or viral compounds (21),  $4 \times 10^5$  DCs were incubated with  $4 \times 10^5$  CD154 expressing L cells (provided by Dr. C. van Kooten, Department of Nephrology, LUMC, the Netherlands, [22]) in the presence of 1,000 units/ml recombinant human IFN- $\gamma$

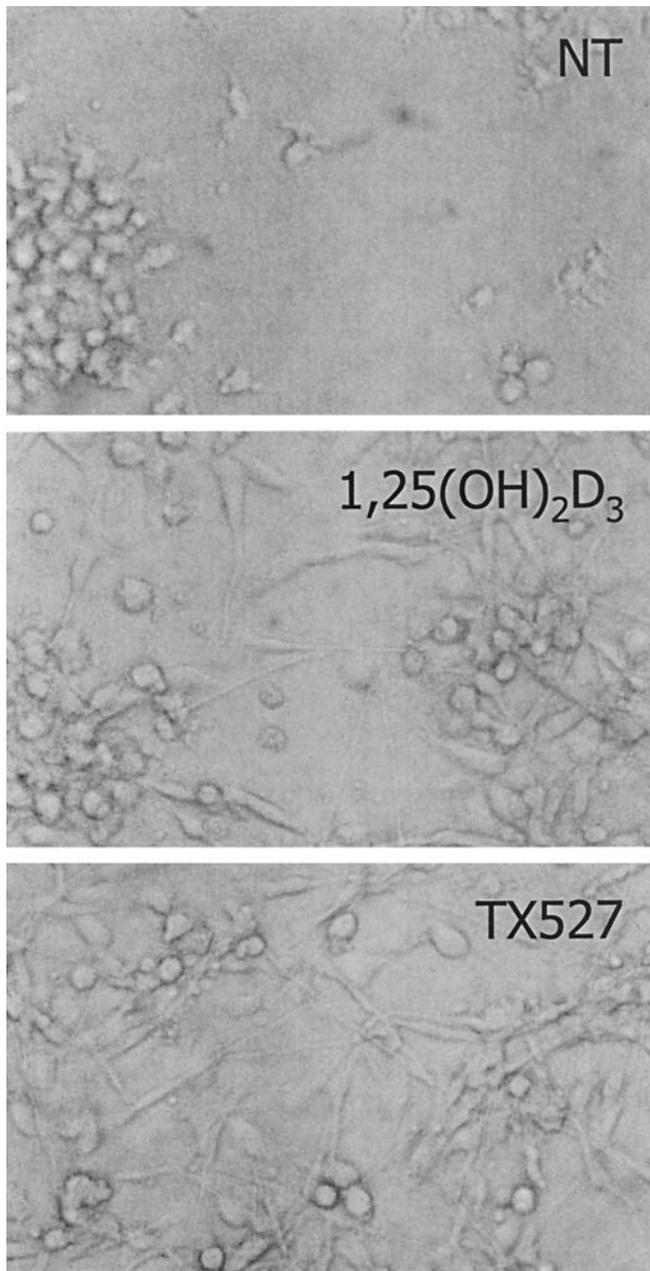


**FIG. 1.** Characterization of cell surface markers on human immature (day 6) and mature DCs (day 8) generated from isolated monocytes in the presence of GM-CSF and IL-4. DC cultures were either nontreated (dotted line) or treated with  $10^{-8}$  mol/l  $1,25(\text{OH})_2\text{D}_3$  (solid thin line) or TX527 (solid thick line). DC maturation was induced by addition of LPS and IFN- $\gamma$ .

to trigger cytokine production by untreated  $1,25(\text{OH})_2\text{D}_3$  or TX527 modulated DCs. After 24 h, supernatants were collected and cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA) by using commercial kits specific for IL-12p70 and IL-10 (R&D Systems, Minneapolis, MN).

Fluorescein-labeled zymosan particles (*Saccharomyces cerevisiae*; Molecular Probes Europe, Leiden, the Netherlands) were used to evaluate the phagocytosis capacity of variously treated DCs and macrophages. Briefly, the various cell suspensions were incubated for 2 h with fluoresceinated particles (1–100 particles/cell), after which the cells were washed and dissolved in 100  $\mu\text{l}$  of Trypan Blue. After extensive washing, the cells were resuspended in 2% paraformaldehyde and analyzed by FACS. Phagocytosis capacity is expressed as percentage of fluorescein-positive cells.

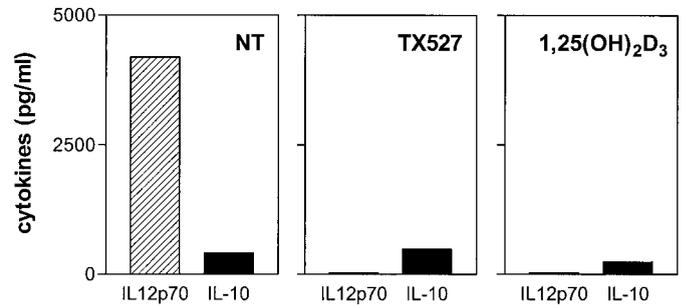
**T-cell assays.** To test the T-cell stimulatory capacities of nontreated- $1,25(\text{OH})_2\text{D}_3$  or TX527-treated DCs, we used a GAD65-specific T-cell clones derived from a prediabetic individual, which recognizes naturally processed GAD65 antigen (Diamyd, Stockholm, Sweden) and the minimal GAD65 epitope p (339–352) in the context of HLA-DR3, as described previously (18). For proliferation or cytokine induction,  $1 \times 10^4$  T-cells were incubated with an equal number of variously treated DCs, diluted in Iscove's modified Dulbecco's medium (IMDM), supplemented with 2 mmol/l glutamine and 10% pooled human AB serum. T-cells were cultured for, respectively, 3 days (proliferation) or 24 h (ELISpot) in 96-well flat-bottom plates (Costar, Cambridge, MA). After 3 days, cultures were pulsed with [ $^3\text{H}$ ]thymidine (0.5  $\mu\text{Ci}/\text{well}$ ) for another 18 h, after which  $^3\text{H}$  incorporation was measured by liquid scintillation counting. Results are expressed as means  $\pm$  SD of triplicate wells. For detection of the T-cell cytokines IL-2, IFN- $\gamma$ , IL-10, and IL-13, T-cells were



**FIG. 2.** Morphology of variously treated mature DCs. Cultures were either nontreated (NT) or continuously treated with  $10^{-8}$  mol/l  $1,25(\text{OH})_2\text{D}_3$  or TX527.

harvested by gently rinsing the wells and washing the collected T-cells in a large volume of IMDM (23). T-cells were subsequently plated on anti-cytokine antibody-precoated ELISA plates and cultured for 2 h (IL-2), 5 h (IFN $\gamma$ , IL-13), or overnight (IL-10) in IMDM supplemented with 2% pooled human AB serum at 37°C 5% CO $_2$ . After lysis of the cells with ice-cold deionized water, the plates were washed with PBS/0.05% Tween-20 and incubated with biotinylated detector antibody for 1 h at 37°C, followed by a second incubation with gold-labeled anti-biotin antibody (GABA) for 1 h at 37°C. All antibodies were diluted in PBS/1% BSA. After extensive washing with PBS/0.05% Tween-20, the plates were developed according to the manufacturer's protocol (U-CyTech, Utrecht, the Netherlands). Spots were counted on an Olympus microscope and analyzed with Olympus Micro Image 4.0 software (Paes Nederland, Zoeterwoude, the Netherlands). Results are expressed as means  $\pm$  SD of triplicate wells.

**Statistical analysis.** The Mann-Whitney *U* test was used to compare results obtained from untreated DCs with either TX527- or  $1,25(\text{OH})_2\text{D}_3$ -treated DCs in various experiments. Significance was defined at the 0.05 level.



**FIG. 3.**  $1,25(\text{OH})_2\text{D}_3$  or TX527 treatment affects DC cytokine production induced by CD40 triggering. Variously treated DCs were cultured for 24 h with CD40L-transfected L cells after which supernatant was harvested for the determination of IL-12p70 (▨) or IL-10 (■) by ELISA.

## RESULTS

**Effects of TX527 or  $1,25(\text{OH})_2\text{D}_3$  on morphological features and functional profile of human dendritic cells.** To compare the effects of TX527 and its parent compound  $1,25(\text{OH})_2\text{D}_3$  on in vitro DC generation, we added TX527 or  $1,25(\text{OH})_2\text{D}_3$  to monocytes that were cultured with GM-CSF and IL-4 for 6 days to generate immature DCs. On the basis of earlier findings (10,24), the concentration chosen in our study was  $10^{-8}$  mol/l for both drugs, although TX527 was found to be equally potent at 10- to 100-fold lower concentrations (data not shown).

Addition of  $1,25(\text{OH})_2\text{D}_3$  or TX527 impaired downregulation of the monocyte marker CD14 and interfered with upregulation of the skin DC marker CD1a when compared with untreated immature DCs (Fig. 1, left). Levels of MHC class II (HLA-DR) as well as costimulatory molecules CD86, CD80, CD54, and CD40 were unchanged compared with nontreated immature DCs (data not shown for CD80, CD54, and CD40). The DC maturation marker CD83 was obviously not expressed on nontreated TX527 or  $1,25(\text{OH})_2\text{D}_3$ -treated immature DCs.

After exposure to LPS and IFN- $\gamma$  to induce DC maturation into DC1 (25), TX527- or  $1,25(\text{OH})_2\text{D}_3$ -treated DCs continued to express CD14 but failed to upregulate CD1a or CD83 (Fig. 1, right). In addition, TX527 or  $1,25(\text{OH})_2\text{D}_3$  treatment resulted in a 10-fold lower expression of HLA-DR and CD86, whereas expression of CD80, CD40, or CD54 was not different from control mature DCs (data not shown).

This TX527- or  $1,25(\text{OH})_2\text{D}_3$ -induced altered DC phenotype was accompanied by typical morphological features (Fig. 2). Upon culture for 6 days with GM-CSF and IL-4, DCs became nonadherent and expressed protruding veils (data not shown). After the addition of LPS and IFN- $\gamma$ , maturing DCs formed large clusters of nonadherent cells with even more pronounced cytoplasmic projections, whereas continuous addition of TX527 and, to a lesser extent,  $1,25(\text{OH})_2\text{D}_3$  yielded small clusters of mostly adherent growing spindle-shaped cells.

In addition to the induction of phenotypic differences, prolonged TX527 or  $1,25(\text{OH})_2\text{D}_3$  treatment was found to affect DC cytokine production induced by CD40 triggering via CD40L-expressing cells (Fig. 3). Untreated DCs express high levels of IL-12p70, whereas TX527 or  $1,25(\text{OH})_2\text{D}_3$  treatment almost completely abrogated IL-12p70 production. The production of IL-10 was not different from untreated DC.

TABLE 1  
Capacity of macrophages and variously treated dendritic cells to take up fluorescein-labeled zymosan particles by phagocytosis

	Fluorescein-positive cells (%)
Macrophage	40
Mature DC	15
1,25(OH) <sub>2</sub> D <sub>3</sub> -DC	15
TX527-DC	17
Immature DC	37

Because TX527 treatment in particular yielded adherent growing cells, which lack the expression of typical DC markers, e.g., CD1a and CD83, and more closely resemble macrophages, we compared the phagocytosis capacity of variously treated DCs and macrophages generated in parallel (Table 1). The phagocytosis capacity of TX527- or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DCs did not differ from untreated mature DCs. All three types of DCs showed a lower capacity to take up fluorescent particles when compared with macrophages or immature DCs.

Taken together, TX527 and its parent compound 1,25(OH)<sub>2</sub>D<sub>3</sub> affect the differentiation of DCs from monocytes and their subsequent maturation, yielding morphologically altered cells that lack DC-specific features and express lower levels of cell surface molecules involved in antigen presentation. Moreover, TX527- or 1,25(OH)<sub>2</sub>D<sub>3</sub>-modulated immature DCs were unable to convert into classical mature DC1 cells upon withdrawal of TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> and addition of LPS + IFN- $\gamma$ , as determined by FACS analysis and analysis of IL-12p70 levels induced

by CD40 ligation (data not shown), demonstrating that the effects of both drugs on DC differentiation are persistent. **Stimulation of autoreactive T-cells by TX527- or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DCs.** Because lower levels of MHC class II and CD86 expression as well as reduced cytokine production by TX527- or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DCs could affect the immune-stimulatory potential of these DCs, we tested proliferative and cytokine responses of a GAD65-specific T-cell clone upon coculture with variously treated DCs. Typically, this T-cell clone displays a Th0 cytokine profile when stimulated with peptides that contain the 339–352 sequence, i.e., IFN- $\gamma$ , IL-10, and IL-13 (18). To exclude any (selective) effects of TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> on T-cells (8,24), we washed DCs extensively before addition to T-cell cultures.

Because immature DCs are most efficient in processing complete protein into peptides (26), we cocultured variously treated immature DCs with T-cells in the presence of GAD65 protein or the minimal peptide epitope 339–352 (Fig. 4). TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> pretreatment of DCs had no significant effect on GAD65-induced proliferative responses of the T-cells, whereas cytokine responses were completely blocked ( $P < 0.001$ ). When the minimal peptide epitope was directly loaded onto DCs, proliferation of the T-cell clone was again not influenced by TX527 pretreatment of DCs. However, peptide-induced cytokine responses were differently affected by TX527 pretreatment, varying from complete abrogation of IL-10 ( $P < 0.001$ ), significantly reduced IFN- $\gamma$  ( $P = 0.04$ ), and unchanged IL-13 production, whereas 1,25(OH)<sub>2</sub>D<sub>3</sub> pretreat-

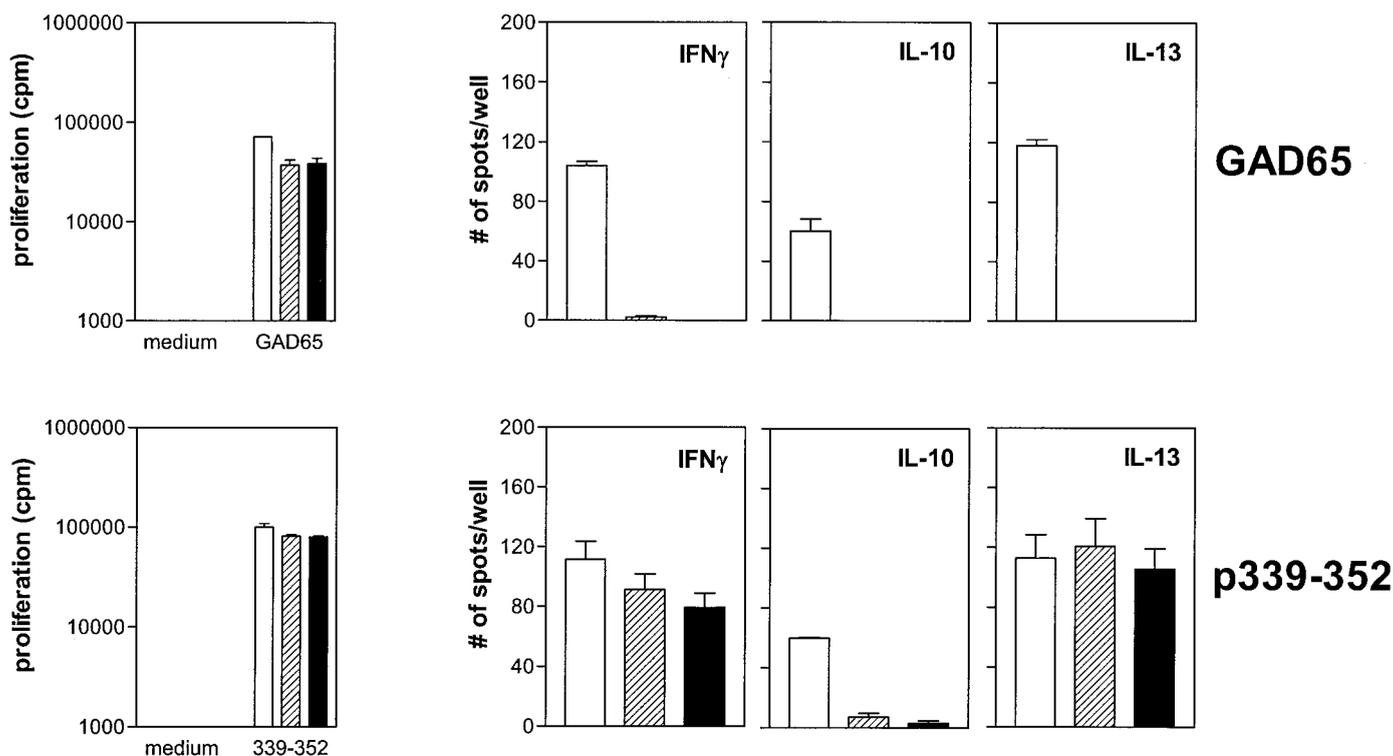


FIG. 4. Modulation of autoreactive mol/I-cell responses by 1,25(OH)<sub>2</sub>D<sub>3</sub>- or TX527-treated immature DCs. GAD65-specific T-cells were cocultured with either nontreated (□) or 10<sup>-8</sup> mol/l 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated (▨) or TX527-treated (■) immature DCs in the presence of 10 μg/ml GAD65 protein (top) or 0.2 μg/ml GAD (339–352) peptide (bottom) for the induction of T-cell proliferation and cytokine production. A representative experiment of two is shown.

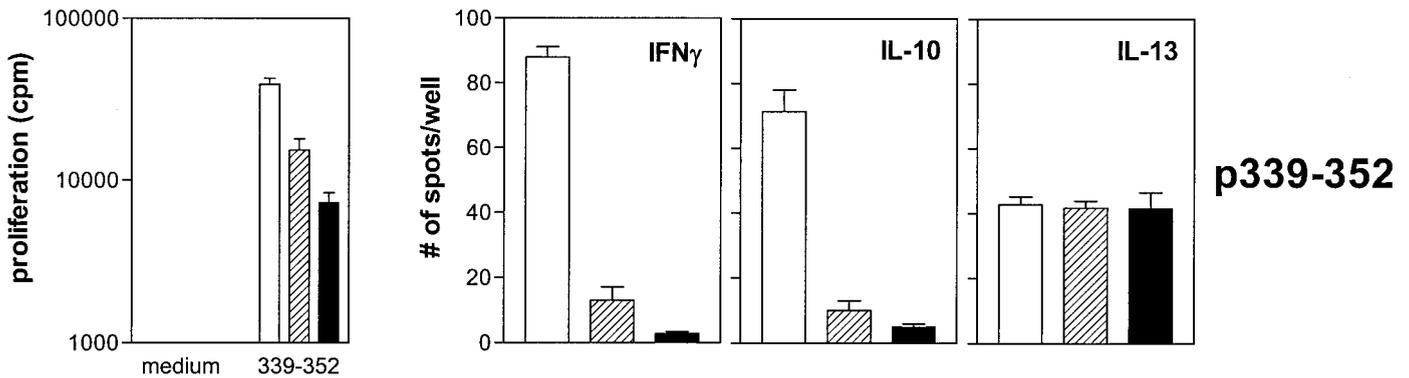


FIG. 5. Effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> or TX527 treatment on T-cell stimulatory capacity of mature DCs. Mature DCs were generated from isolated monocytes in the absence (□) or presence of 10<sup>-8</sup> mol/l 1,25(OH)<sub>2</sub>D<sub>3</sub> (▨) or TX527 (■), after which GAD65-specific T-cells were added. T-cells were cultured in the presence of 0.2 μg/ml GAD (339–352) peptide for the induction of T-cell proliferation and cytokine production. A representative experiment of five is shown.

ment of DCs was found to block IL-10 levels ( $P = 0.002$ ) without significantly affecting IFN- $\gamma$  or IL-13.

Mature DCs, however, do not have the capacity to process proteins into peptides efficiently. Therefore, the T-cell clone was incubated only with its minimal peptide epitope in the presence of variously treated DCs (Fig. 5). APCs that were generated from monocytes in the continuous presence of TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> induced significantly lower proliferation ( $P = 0.002$  for TX527 vs.  $P = 0.009$  for 1,25(OH)<sub>2</sub>D<sub>3</sub>) and remarkably affected the cytokine profile of the T-cell clone. Both IFN- $\gamma$  and IL-10 were almost completely inhibited ( $P = 0.002$ ), whereas IL-13 was not affected. Of both drugs, TX527 pretreatment induced the most pronounced inhibitory effects on the response pattern of the committed T-cell.

**DC phenotype and function upon addition of TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> only during the maturation phase.** To evaluate the effects of TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> on the process of LPS- and IFN- $\gamma$ -induced DC maturation (phase II), we added TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> during the last 2 days of culture of immature DCs generated in the presence of GM-CSF and IL-4 only. Introduction of TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> resulted in upregulation of CD14 on a low percentage of mature DCs (mean fluorescence intensity [MFI] 225.73 in TX527- and MFI 129.76 in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DCs vs. MFI 49.38 in control untreated DCs). Addition of TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> did not affect expression of CD1a, CD83, CD80, CD86, CD40, CD54, or HLA-DR, because levels of these markers expressed on TX527- or 1,25(OH)<sub>2</sub>D<sub>3</sub>-modulated DCs were identical to levels expressed by nontreated mature DCs (data not shown). Nevertheless, DC cytokine profiles released upon CD40 triggering were significantly altered (Fig. 6). In contrast to untreated mature DCs, which express high levels of IL-12p70 and low levels of IL-10, TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment during maturation resulted in DCs that were unable to secrete IL-12p70 but secreted four- to eightfold increased levels of IL-10 upon CD40 ligation. Thus, TX527, as well as the parent compound, redirects mature DCs, resulting in functionally different DCs compared with nontreated mature DCs.

**Profile of autoreactive T-cells incubated with DCs exposed to TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> during the maturation phase only.** Despite that TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment did not affect peptide-induced proliferative re-

sponses of the T-cell clone, cytokine responses were affected to various extents (Fig. 7). Again, TX527-modulated DCs were more effective than 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DCs in redirecting cytokine responses, as demonstrated by a complete reduction in IL-10 ( $P < 0.001$ ) and significant reduction in IFN- $\gamma$  ( $P = 0.002$ ) release. 1,25(OH)<sub>2</sub>D<sub>3</sub>-pretreated DCs significantly affected IL-10 and IL-13 ( $P = 0.002$ ) but not IFN- $\gamma$  production.

## DISCUSSION

In this study, we demonstrated that TX527 is a potent 1,25(OH)<sub>2</sub>D<sub>3</sub> analog that is capable of targeting DCs at various stages throughout their in vitro generation from monocytes. TX527 was found to be biologically active in a broader range (10<sup>-7</sup> to 10<sup>-10</sup> mol/l) when compared with its parent compound 1,25(OH)<sub>2</sub>D<sub>3</sub> (10<sup>-7</sup> to 10<sup>-8</sup> mol/l), yielding DCs with the highest potential to alter the cytokine profile of a committed autoreactive T-cell clone. This modulating effect was mediated via the antigen-presenting DCs, because TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> was not added during T-cell cultures.

Most classical immunomodulators and immune suppressants modulate T-cells through direct interaction with T-cell proliferation or cytokine production. Very few strategies also affect antigen-presenting DCs, the central cell of the immune system able to polarize the immune system

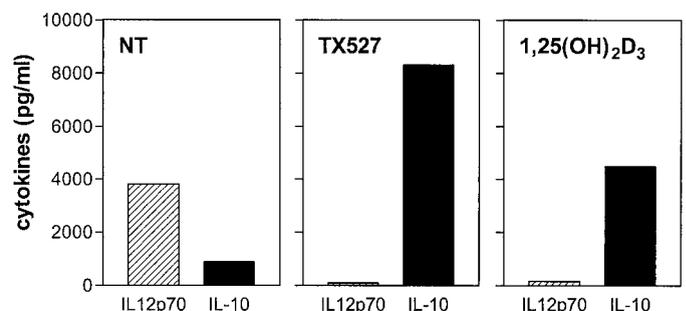


FIG. 6. Abrogation of IL-12p70 and enhancement of IL-10 secretion upon CD40 ligation of DCs treated with 1,25(OH)<sub>2</sub>D<sub>3</sub> or TX527 during maturation only. Immature DCs were generated from isolated monocytes, after which maturation was induced in the absence or presence of 10<sup>-8</sup> mol/l 1,25(OH)<sub>2</sub>D<sub>3</sub> or TX527. Variously treated mature DCs were subsequently cocultured with CD40L-expressing cells, after which supernatant was collected for determination of IL-12p70 (▨) or IL-10 (■) by ELISA.

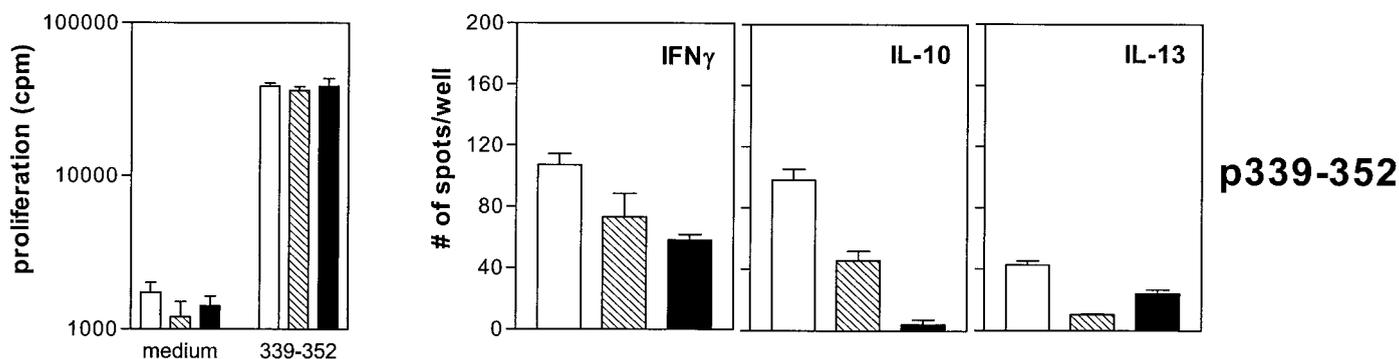


FIG. 7. 1,25(OH)<sub>2</sub>D<sub>3</sub> or TX527 treatment during maturation yields DCs that marginally affect GAD65-committed T-cells. Immature DCs were matured in the absence (□) or presence of 10<sup>-8</sup> mol/l 1,25(OH)<sub>2</sub>D<sub>3</sub> (▨) or TX527 (■). After 48 h, DCs were cocultured with GAD65-specific T-cells in the absence or presence of 0.2 μg/ml GAD (339–352) peptide for the induction of proliferation and cytokine production. A representative experiment of three is shown.

into different directions, e.g., Th1 versus Th2 (27). The active form of vitamin D<sub>3</sub>, 1,25(OH)<sub>2</sub>D<sub>3</sub>, is one of the few products available (10,12), together with glucocorticoids (28–31), that is able to change DC differentiation and function *in vitro*.

In the present study, we demonstrated that a novel nonhypercalcemic 1,25(OH)<sub>2</sub>D<sub>3</sub> analog, TX527, is able to modify significantly the phenotype of DCs as characterized by major changes in cell surface marker expression, morphology, and cytokine profile. In contrast to another study (12), we demonstrated that TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment does not simply abrogate differentiation and maturation but leads to the emergence of a different cell that lacks typical DC features. Moreover, these modulated DCs were not able to equalize the high phagocytosis capacity that is observed for untreated macrophages or immature DCs. This is a strong argument against the postulated 1,25(OH)<sub>2</sub>D<sub>3</sub>-mediated arrest in the immature stage of DC development (11). It also confirms that 1,25(OH)<sub>2</sub>D<sub>3</sub> or TX527 does not simply mediate differentiation toward macrophages (12). In the past, however, a clear, positive effect of 1,25(OH)<sub>2</sub>D<sub>3</sub> on the differentiation of macrophages was shown under different culture conditions (32). Monocytes may be considered as relative immature precursors with multiple differentiation potentials, depending on the microenvironment during differentiation (33). *In vitro*, cytokines and inflammatory substances play an important role in the final determination of whether monocytes will acquire DC, macrophage, osteoclastic, or other characteristics and functions (34,35). Additional analysis of phenotype and functionality will be necessary to characterize fully the cell type generated by continuous presence of TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub>.

*In vivo*, 1,25(OH)<sub>2</sub>D<sub>3</sub> analogs induce an immune shift that is characterized by downregulation of Th1 cytokines and upregulation of Th2 cytokines in response to pancreatic autoantigens such as GAD65 (16). Our *in vitro* data on committed human autoreactive T-cells indicate that TX527 treatment of DCs indeed inhibited IFN- $\gamma$  release but did not increase Th2-linked IL-13 production. It is conceivable that this partial discordance with the *in vivo* data results from a direct action of 1,25(OH)<sub>2</sub>D<sub>3</sub> on T-cells during *in vivo* treatment, because 1,25(OH)<sub>2</sub>D<sub>3</sub> was shown to promote directly Th2-associated cytokine production in the absence of APCs (8).

Another important finding in our study is the redirection by TX527-modulated DCs of committed T-cells toward a different cytokine profile without completely blocking their proliferative capacity. The persistence of the proliferative capacity suggests that the autocrine stimulation of IL-2 was unaffected. Indeed, IL-2 production was not changed from that of autoreactive T-cells stimulated with untreated DCs (data not shown). This finding indicates that for complete blockade of committed autoreactive T-cells, a combined immunosuppressive therapy that targets both IL-2-dependent (e.g., cyclosporine, tacrolimus, or dacliximab [36]) and -independent pathways (TX527 or 1,25(OH)<sub>2</sub>D<sub>3</sub>) of T-cell activation would be preferred. This is in accordance with data on prevention of recurrent autoimmune disease after syngeneic islet transplantation in diabetic NOD mice treated with a combination of a vitamin D<sub>3</sub> analog and cyclosporine, whereas the individual drugs were less effective (15).

In addition, treatment with 1,25(OH)<sub>2</sub>D<sub>3</sub> in combination with the IL-2-independent drug mycophenolate mofetil was recently shown to induce transplantation tolerance associated with an increased frequency of regulatory T-cells that bear the IL-2 receptor (CD152<sup>+</sup>CD4<sup>+</sup>CD25<sup>+</sup> [17]). Mycophenolate mofetil is a selective lymphocyte inhibitor that blocks the progression of T-cells into the S phase without affecting the IL-2 pathway (24). The IL-2 pathway is known to be critical in the development of CD25-expressing regulatory T-cells (37) that mediate transplantation tolerance (17) and control autoimmunity in NOD mice (38).

1,25(OH)<sub>2</sub>D<sub>3</sub>- or analog-induced changes in cytokine profile of committed T-cells may *in vivo* result in impaired autoimmune effector cells. In addition, naive T-cells were shown to become hyporesponsive through priming by 1,25(OH)<sub>2</sub>D<sub>3</sub>-modulated DCs (10,12). When extrapolating these *in vitro* data to the *in vivo* situation, where the generation of suppressor cells by 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs has been confirmed in different settings (4,17), these studies collectively define 1,25(OH)<sub>2</sub>D<sub>3</sub>, especially its nonhypercalcemic analogs, as ideal immunosuppressants in autoimmunity and as candidate drugs to be combined with typical anti-T-cell immunosuppressants to intervene in hyperactivated immune systems, e.g., in islet transplantation. We conclude that a new structurally modified analog of vitamin D<sub>3</sub>, TX527, modulates morphologi-

cal features and cytokine production of human DCs, yielding altered DCs that redirect an already committed T-cell clone toward a cell with a reduced pathogenic potential.

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