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Tolerogenic Dendritic Cells Induced by Vitamin D Receptor Ligands Enhance Regulatory T Cells Inhibiting Allograft Rejection and Autoimmune Diseases

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Abstract Dendritic cells (DCs) not only induce but also modulate T cell activation. 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] induces DCs with a tolerogenic phenotype, characterized by decreased expression of CD40, CD80, and CD86 costimulatory molecules, low IL-12 and enhanced IL-10 secretion. We have found that a short treatment with 1,25$(OH)_2D_3$ induces tolerance to fully mismatched mouse islet allografts that is stable to challenge with donor-type spleen cells and allows acceptance of donor-type vascularized heart grafts. This effect is enhanced by co-administration of mycophenolate mofetil (MMF), a selective inhibitor of T and B cell proliferation that has also effects similar to 1,25$(OH)_2D_3$ on DCs. Graft acceptance is associated with an increased percentage of $CD4^+CD25^+$ regulatory cells in the spleen and in the draining lymph node that can protect 100% of syngeneic recipients from islet allograft rejection. $CD4^+CD25^+$ cells, able to inhibit the T cell response to a pancreatic autoantigen and to significantly delay disease transfer by pathogenic $CD4^+CD25^-$ cells, are also induced by treatment of adult nonobese diabetic (NOD) mice with 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D_3 (BXL-698). This treatment arrests progression of insulitis and Th1 cell infiltration, and inhibits diabetes development at non-hypercalcemic doses. The enhancement of $CD4^+CD25^+$ regulatory T cells, able to mediate transplantation tolerance and to arrest type 1 diabetes development by a short oral treatment with VDR ligands, suggests possible clinical applications of this approach. J. Cell. Biochem. 88: 227–233, 2003.

Key words: vitamin D analogues; tolerance; regulatory T cells; immunoregulation

The active form of vitamin D, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), is a secosteroid hormone that binds to the vitamin D receptor (VDR), a member of the superfamily of nuclear receptors for steroid hormones, thyroid hormone, and retinoic acid [Haussler et al., 1998; Norman et al., 2001], agents that have important effects on the immune system. The presence of VDR in most cell types of the immune system [Provvedini et al., 1983] prompted the investigation of the potential for VDR ligands as immunomodulatory agents able to control the immune response, and results accumulated over the past 20 years have clearly shown their capacity to exert powerful immunoregulatory activities [Mathieu and Adorini, 2002].

In addition to direct effects on T cell activation, VDR ligands modulate with different mechanisms the phenotype and function of antigen-presenting cells (APCs), and in particular of dendritic cells (DCs). In vitro and in vivo experiments have shown that VDR ligands induce DCs to acquire tolerogenic properties that favor the induction of regulatory rather than effector T cells. These intriguing actions of VDR ligands have been demonstrated in several experimental models and could be exploited, in principle, to treat a variety of human inflammatory conditions.

MAJOR TARGET CELLS OF VDR LIGANDS IN THE IMMUNE SYSTEM: ANTIGEN-PRESENTING CELLS AND T CELLS

APCs and, in particular, DCs are key targets of VDR ligands, both in vitro and in vivo. Earlier indications for the capacity of 1,25$(OH)_2D_3$ to

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target APCs were corroborated by its ability to inhibit the production of IL-12 [Lemire et al., 1995; D'Ambrosio et al., 1998], an APC-derived cytokine critical for Th1 cell development. More recent work has demonstrated that VDR ligands inhibit the differentiation and maturation of DCs [Berer et al., 2000; Griffin et al., 2000; Penna and Adorini, 2000; Piemonti et al., 2000; Canning et al., 2001], a critical APC in the induction of T cell-mediated immune responses. These studies, performed either on monocyte-derived DCs from human peripheral blood or on bone-marrow derived mouse DCs, have consistently shown that in vitro treatment of DCs with VDR ligands leads to downregulated expression of the costimulatory molecules CD40, CD80, CD86 and to decreased IL-12 and enhanced IL-10 production, resulting in decreased T cell activation. The abrogation of IL-12 production and the strongly enhanced production of IL-10 highlight the important functional effects of $1,25(OH)_2D_3$ and its analogs on DCs and are, at least in part, responsible for the induction of DCs with tolerogenic properties. The prevention of DC differentiation and maturation as well as the modulation of their activation and survival leading to DCs with tolerogenic phenotype and function, and to T cell hyporesponsiveness, certainly play an important role in the immunoregulatory activity of $1,25(OH)_2D_3$. These effects are not limited to in vitro activity: $1,25(OH)_2D_3$ and its analogs can also induce DCs with tolerogenic properties in vivo, as demonstrated in models of allograft rejection by oral administration directly to the recipient [Gregori et al., 2001] or by adoptive transfer of in vitro-treated DCs [Griffin et al., 2001]. Tolerogenic DCs induced by a short treatment with $1,25(OH)_2D_3$ are probably responsible for the capacity of this hormone to induce $CD4^+CD25^+$ regulatory T cells that are able to mediate transplantation tolerance [Gregori et al., 2001].

T lymphocytes have also been shown to be direct targets for VDR ligands, in particular Th1-type cytokines, such as IL-2 and IFN-γ. $1,25(OH)_2D_3$ inhibits IL-2 secretion by impairing the transcription factor NF-AT complex formation because the ligand-bound VDR complex binds to the distal NF-AT binding site of the human IL-2 promoter [Alroy et al., 1995; Takeuchi et al., 1998]. IFN-γ is directly inhibited by $1,25(OH)_2D_3$ through interaction of the ligand-bound VDR complex with a vitamin D responsive element in the promoter region of the cytokine [Cippitelli and Santoni, 1998]. $1,25(OH)_2D_3$ has been recently shown to enhance the development of Th2 cells via a direct effect on naïve $CD4^+$ cells [Boonstra et al., 2001], and this could also account for the beneficial effect of VDR ligands in the treatment of autoimmune diseases and possibly also allograft rejection.

A novel aspect of the multiple effects of VDR ligands on T cells is provided by the induction of cells with suppressive and regulatory properties. Regulatory T cells are induced by the modulation of APCs that promotes DCs with tolerogenic phenotype and function, both in vitro [Griffin et al., 2000; Penna and Adorini, 2000; Piemonti et al., 2000] and in vivo [Gregori et al., 2001]. A short treatment with $1,25(OH)_2D_3$ and mycophenolate mofetil (MMF), a selective inhibitor of T and B cell proliferation [Allison and Eugui, 1996] that also modulates APCs [Mehling et al., 2000], induces tolerance to islet allografts associated with an increased frequency of $CD4^+CD25^+$ regulatory T cells able to adoptively transfer transplantation tolerance [Gregori et al., 2001]. In addition, a combination of $1,25(OH)_2D_3$ and dexamethasone has been shown to induce human and mouse naïve $CD4^+$ T cells to differentiate in vitro into regulatory T cells [Barrat et al., 2002]. In contrast to the previously described Tr1 cells [Groux et al., 1997], these cells produced only IL-10 but no IL-5 and IFN-γ, and retained strong proliferative capacity. $1,25(OH)_2D_3$ and dexamethasone induced the development of IL-10-producing T cells also in the absence of APCs, with IL-10 acting as a positive autocrine factor and, upon transfer, the IL-10-producing T cells could prevent central nervous system inflammation [Barrat et al., 2002].

In conclusion, although APCs, and in particular DCs, appear to be primary targets for the immunomodulatory activities of VDR ligands, they can also act directly on T cells. The capacity of VDR ligands to target APCs and T cells depends on VDR expression by both cell types and more importantly, on the presence of common targets in their signal transduction pathways.

REGULATORY T CELLS INDUCED BY MMF AND 1,25 DIHYDROXYVITAMIN D_3 TREATMENT MEDIATE TRANSFERABLE TRANSPLANTATION TOLERANCE

Based on the in vitro results suggesting that VDR ligands induce DCs with tolerogenic

properties, we have analyzed the ability of $1,25(OH)_2D_3$, administered alone or in combination with MMF to induce transplantation tolerance. We have shown that $1,25(OH)_2D_3$ induces tolerance to islet allografts associated with downregulated costimulatory molecules on DCs and macrophages surrounding the graft [Gregori et al., 2001]. The tolerogenic phenotype expressed by DCs recruited to the transplant area of tolerant mice was paralleled by their profoundly reduced IL-12 production upon interaction with $CD4^+$ cells and by their inability to induce full activation of $CD4^+$ cells. These results indicate that low-molecular weight compounds able to induce DCs with tolerogenic properties can be used to induce peripheral tolerance to allografts.

We first analyzed the ability of $1,25(OH)_2D_3$ and MMF, administered alone or in combination, to inhibit islet allograft rejection. Pancreatic islets isolated from C57BL/6 (B6) mice were transplanted under the kidney capsule of BALB/c mice rendered diabetic by a single injection of streptozotocin. Recipient mice were treated from day −1 to 30 with MMF (100 mg/kg p.o. daily) and/or $1,25(OH)_2D_3$ (5μg/kg p.o. 3 ×/week). The mean rejection time in vehicle-treated recipients was 23 ± 3 days. MMF and $1,25(OH)_2D_3$ administered alone prolonged islet graft survival, but only in about 50% of the recipients. Conversely, over 80% of the mice treated with both drugs showed long-term (>70 days) islet graft acceptance (Table I). We next challenged BALB/c recipients showing long-term (>70 days) allograft acceptance with i.p. injection of 10^6 donor-type B6 spleen cells (Table I). Recipient mice treated with peri-transplant administration of anti-CD4 mAb accommodated the islet graft but were not tolerant because all mice rejected the graft after challenge with a mean survival time of 14 ± 2.4 days.

Forty percent of allografts accepted under the cover of MMF alone were resistant to rejection upon challenge, confirming the tolerogenic properties of MMF in this model, and $1,25(OH)_2D_3$ had even superior activity. Combined treatment with MMF and $1,25(OH)_2D_3$ resulted in

TABLE I. Tolerance Induction by Combined Treatment With Mycophenolate Mofetil (MMF) and $1,25(OH)_2D_3$

		n	% Graft survival	*P* value
C57BL/6 into BALB/c islet allografts (day 0)[a]				
Treatment				
None		25	0[b]	
Anti-CD4 (day −1, 0, 1, 2)		5	100[b]	< 0.001
$1,25(OH)_2D_3$ (day −1 to 30)		21	48[b]	0.0048
MMF (day −1 to 30)		23	52[b]	< 0.0001
MMF + $1,25(OH)_2D_3$ (day −1 to 30)		20	85[b]	< 0.0001
C57BL/6 spleen cell challenge (day 70)[c]				
Treatment				
Anti-CD4 (day −1,0,1,2)		5	0[d]	
$1,25(OH)_2D_3$ (day −1 to 30)		7	53[d]	ns
MMF (day −1 to 30)		9	33[d]	ns
MMF + $1,25(OH)_2D_3$ (day −1 to 30)		18	72[d]	0.007
Vascularized heart graft (day 100)[e]				
Strain combination				
Donor	Recipient			
C57BL/6	Naïve BALB/c	6	0[f]	
C_3H	Tolerant BALB/c	4	0[f]	ns
C57BL/6	Tolerant BALB/c	5	80[f]	0.0043

[a]Long-term islet allograft survival induced by MMF and $1,25(OH)_2D_3$ treatment. BALB/c mice were rendered diabetic by a single injection of streptozotocin (250 mg/kg i.v.) and transplanted with 350 B6 islets. Recipient mice were treated with MMF (100 mg/kg p.o. daily) and/or ($1,25(OH)_2D_3$ 5 mg/kg p.o. 3 ×/week) from day −1 to 30. The function of islet allografts was monitored 2 ×days/week by blood glucose measurement.
[b]At day 70.
[c]Percent islet graft survival after B6 spleen cell challenge. Recipient mice were treated with MMF (100 mg/kg p.o. daily) and/or $1,25(OH)_2D_3$ (5 mg/kg p.o. 3 ×/week) from day −1 to 30. Alternatively, recipient mice were treated at day −1,0, 1, and 2 with anti-Cd4 mAb i.p. (10 mg/kg/day). Mice with functioning islet grafts 70 days after transplantation were injected i.p. with 106 B6 spleen cells. The function of islet allografts was monitored 2 ×/week by blood glucose measurement.
[d]At day 100.
[e]Vascularized heart graft survival. Mice still normoglycemic 4 weeks after spleen cell challenge were transplanted with B6 (donor type) or C_3H (third party) vascularized heart grafts. As controls, naïve BALB/c mice were transplanted with B6 (allograft) hearts. Heart function was monitored daily by palpation and islet graft function was monitored 2 ×/week by blood glucose measurement. *P* values were calculated by Fisher's exact test.
[f]At day 140.

resistance to rejection upon challenge in 73% of allografts (Table I). Mice that continued to show islet graft function for 4 weeks after challenge were transplanted, 100 days after the initial islet graft, with a vascularized heart from B6 (donor-type) or C_3H (third party) mice. Results in Table I show that naïve BALB/c mice rejected B6 heart grafts in 10 days, whereas only one tolerant mouse out of five rejected the heart graft, 25 days after transplant. In contrast, tolerant BALB/c mice rejected a third-party heart in 10–12 days.

$CD4^+$ spleen cells from tolerant mice prevented donor-type islet allograft rejection in all recipients, indicating an active and effective tolerogenic mechanism. Tolerant mice displayed an increased percentage of $CD4^+CD25^+$ regulatory cells both in draining lymph nodes (13.3% vs. 6.7%) and in the spleen (32.8% vs. 16.4%), compared to acutely rejecting mice (Table II). Moreover, tolerant mice presented a lower percentage of $CD45RB^{high}$ cells and an increased percentage of $CD45RB^{low}$ cells compared to acutely rejecting mice (Table II). An increased expression of CD152 was found in $CD4^+CD25^+$ regulatory T cells isolated from tolerant mice compared to acutely rejecting mice, both in draining lymph nodes (37% vs. 17%) and spleens (35.5% vs. 20%). To evaluate the ability of $CD4^+CD25^+$ regulatory T cells to prevent allograft rejection, we transferred $CD4^+CD25^-$ (4×10^6/mouse) or $CD4^+CD25^+$ (5×10^5/mouse) T cells isolated from tolerant mice into naïve diabetic BALB/c mice, 2 days before donor-type (B6) islet transplant. $CD4^+CD25^+$ cells prevented islet allograft rejection while $CD4^+CD25^-$ cells did not, indicating an active mechanism of tolerance induction mediated by $CD4^+CD25^+$ regulatory T cells [Gregori et al., 2001].

In conclusion, our results demonstrate that a short treatment with MMF/$1,25(OH)_2D_3$ can induce transferable tolerance to islet grafts. Tolerance is associated with the induction of macrophages and DCs with a tolerogenic phenotype and with an increase of $CD4^+CD25^+$ regulatory T cells. The enhancement of $CD4^+CD25^+$ regulatory T cells by VDR ligands may provide a new paradigm, applicable to the prevention of allograft rejection in humans.

VDR LIGAND ENHANCES REGULATORY T CELLS AND ARRESTS AUTOIMMUNE DIABETES IN NONOBESE DIABETIC (NOD) MICE

The NOD mouse that spontaneously develops type 1 diabetes with a pathogenesis similar to the human disease represents a useful model for the study of autoimmune diabetes [Atkinson and Leiter, 1999]. Several effector mechanisms leading to specific islet β-cell destruction have been identified, including cytotoxic $CD8^+$ lymphocytes and macrophages [Benoist and Mathis, 1997], both of which are regulated by IL-12-dependent T helper 1 (Th1) cells [Trembleau et al., 1995]. The activation of Th1 cells specific for β-cell autoantigens could reflect defective elimination of autoreactive T cell clones [Ridgway et al., 1996], inefficient mechanisms of peripheral tolerance [Delovitch and Singh, 1997], enhanced IL-12 production [Adorini, 2001], or impaired suppressive mechanisms [Salomon et al., 2000].

Agents like $1,25(OH)_2D_3$ and its analogs are able to inhibit in vivo IL-12 production and

TABLE II. Increased Percentage of $CD4^+CD25^+$ T Regulatory Cells in Tolerant Mice

	Kidney LN $CD4^+$ cells		Spleen $CD4^+$ cells	
	Acutely rejecting	Tolerant upon $CD4^+$ transfer	Acutely rejecting	Tolerant upon $CD4^+$ transfer
CD25	6.7	13.3[a]	16.4	32.8[a]
CD69	11.9	6.5	17.4	20.4
$CD45RB^{low}$	15.1	20.1	40.7	57.8
$CD45RB^{high}$	35.2	30.1	57.6	42.2

[a]It represents ≥ 2-fold increase in marker expression.
$Cd4^+$T cells from untreated acutely rejecting (30 days after transplantation) and tolerant mice upon $CD4^+$ cell transfer (190 days after transplantation) were stained with mAbs specific for the indicated surface molecules and analyzed by flow cytometry.
Acquisition was performed on $CD4^+$cells.

Th1 development [Mattner et al., 2000], and to enhance $CD4^+CD25^+$ regulatory T cells [Gregori et al., 2001] may, therefore, be beneficial in the treatment of type 1 diabetes. $1,25(OH)_2D_3$ itself reduces the incidence of insulitis [Mathieu et al., 1992] and prevents type 1 diabetes development [Mathieu et al., 1994], but only when administered to NOD mice starting from 3 weeks of age, before the onset of insulitis. $1,25(OH)_2D_3$ was found ineffective in preventing progression of diabetes in NOD mice when given from 8 weeks of age, when NOD mice present a well-established insulitis [Mathieu et al., 1997]. However, a combined treatment of 8-week-old NOD mice with the $1,25(OH)_2D_3$ analog MC 1288 and cyclosporine A reduced the incidence of disease, although neither treatment alone was effective [Casteels et al., 1998a].

In contrast, we have recently identified the $1,25(OH)_2D_3$ analog 1,25-dihydroxy-16,23Z-diene-26,27-hexafluoro-19-nor vitamin D_3 (BXL-698) that is able, as a short-course monotherapy, to treat at non-hypercalcemic doses the ongoing type 1 diabetes in the adult NOD mouse (Fig. 1A) by effectively blocking the progression of insulitis (Fig. 1B). This property is likely due, at least in part, to the increased metabolic stability of this analog against the inactivating C-24 and C-26 hydroxylations, and the C-3 epimerization [Uskokovic et al., 2001], resulting in a 100-fold more potent immunosuppressive activity compared to $1,25(OH)_2D_3$. A short treatment with non-hypercalcemic doses of BXL-698 inhibits IL-12 production and pancreatic infiltration of Th1 cells while increasing the frequency of $CD4^+CD25^+$ regulatory T cells in pancreatic lymph nodes, arresting the immunological progression and preventing the clinical onset of type 1 diabetes in the NOD mouse [Gregori et al., 2002].

Protection from type 1 diabetes was found associated with a selective decrease of Th1 cells in the pancreatic lymph nodes and in the pancreas, without a marked deviation to the Th2 phenotype (Fig. 2). The frequency of $CD4^+CD25^+$ cells in the pancreatic lymph nodes of BXL-698-treated NOD mice was two-fold higher compared to untreated 8 weeks old and to age-matched vehicle-treated controls. These cells were anergic, as demonstrated by their impaired capacity to proliferate and secrete IFN-γ in response to TCR ligation, inhibited the T cell response to the pancreatic

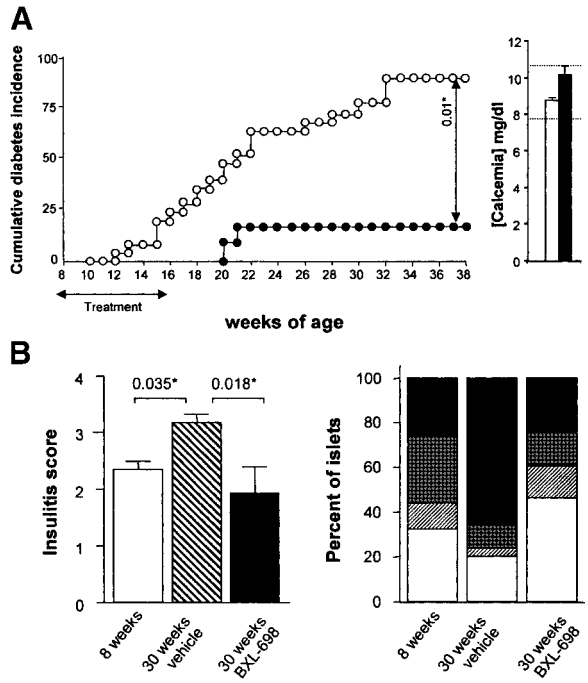

Fig. 1. BXL-698 administration to 8-week-old nonobese diabetic (NOD) mice inhibits type 1 diabetes development. **A**: NOD mice were treated 5 ×/week with vehicle (open circles, n = 16) or with 0.03 μg/kg BXL-698 p.o. (filled circles, n = 12) from 8 to 16 weeks of age. Diabetes development was monitored twice weekly by measurement of blood glucose levels. The *P* value was calculated by Mann–Whitney U test. Serum calcium levels (means ± SE) were measured in vehicle-treated (open bars) and BXL-698-treated (filled bars) NOD mice after 40 administrations. Stippled lines indicate the range of normal serum calcium levels. **B**: Decreased insulitis in BXL-698-treated NOD mice. Histological scoring of insulitis was performed on pancreas sections stained with hematoxylin/eosin from untreated 8 weeks old, and NOD mice at 30 weeks of age treated with BXL-698 or vehicle from 8 to 16 weeks of age. Each bar represents the mean score of about 40–50 islets/mouse (**left panel**). The results refer to the mean values ± SE of eight mice/group, except for BXL-698-treated mice where three mice were scored. The *P* values were calculated by Mann–Whitney U test. The severity of the infiltrate is shown in the **right panel**. Islets from the same mice were scored for absence of insulitis (open bars), peri-insulitis (striped bars), moderate insulitis with < 50% infiltration of the islets (stippled bars), and severe insulitis with > 50% of infiltration of the islets (filled bars).

autoantigen IA-2, and delayed disease transfer by pathogenic $CD4^+CD25^-$ cells [Gregori et al., 2002).

Immature DCs have been shown to induce $CD4^+$ cells with regulatory properties [Jonuleit et al., 2000] and arrest of DCs at the immature stage induced by BXL-698 treatment could account for the enhanced frequency of $CD4^+CD25^+$ cells. $CD4^+CD25^+$ regulatory T cells appear to play an important role in controlling the progression of type 1 diabetes in

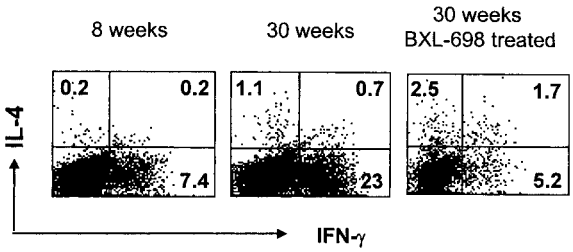

Fig. 2. Cytokine production by pancreas-infiltrating $CD4^+$ cells. Pancreas-infiltrating $CD4^+$ cells were obtained from untreated 8-week-old or 30-week-old nonobese diabetic (NOD) mice treated 5 ×/week p.o. from 8 to 16 weeks of age with vehicle or with 0.03 μg/kg BXL-698. Positively selected $CD4^+$ cells (2×10^5 cells/well) were stimulated with PMA and ionomycin and analyzed by flow cytometry for IFN-γ (abscissa) and IL-4 (ordinate) production. Acquisition was performed on $CD4^+$ cells. Percentage of positive cells, set according to the isotype-matched controls (not shown), are indicated in the top corner of each quadrant.

NOD mice because a low level of $CD4^+CD25^+$ T cells correlates with exacerbation and acceleration of the disease [Salomon et al., 2000]. It is likely that this cell population is more relevant than Th2 cells in disease control, although both could contribute to protection. Indeed, $1,25(OH)_2D_3$ can induce regulatory cells with disease-suppressive activity in the NOD mouse [Mathieu et al., 1994] and a disease-preventing $1,25(OH)_2D_3$ analog could deviate pancreas-infiltrating cells to the Th2 phenotype [Casteels et al., 1998a]. In addition, the pro-apoptotic activity of $1,25(OH)_2D_3$ and its analogs can restore the defective sensitivity to apoptosis of NOD lymphocytes [Casteels et al., 1998b], leading to a more efficient elimination of potentially dangerous autoimmune effector cells. The increased apoptosis induced by $1,25(OH)_2D_3$ and its analogs in DCs [Penna and Adorini, 2000] and T cells [Casteels et al., 1998b] has been observed after different apoptosis-inducing signals, and could help to explain why short-term treatments with these agents afford long-term protection and promote tolerance induction.

The observation that ongoing type 1 diabetes in the adult NOD mouse can be arrested by a relatively short course of treatment with a $1,25(OH)_2D_3$ analog [Gregori et al., 2002] suggests that a similar treatment may also inhibit disease progression in prediabetic or newly diagnosed type 1 diabetes patients. Polymorphisms of the vitamin D receptor gene have been associated with type 1 diabetes in different populations [Chang et al., 2000; Pani et al., 2000], and epidemiological studies have shown a higher incidence of the disease in northern than in southern latitudes [Green et al., 1992], suggesting a possible involvement of a $1,25(OH)_2D_3$ deficiency in the pathogenesis of type 1 diabetes. This is further supported by a large population-based case-control study showing that the intake of vitamin D contributes to a significantly decreased risk of type 1 diabetes development [The EURODIAB Substudy 2 Study Group, 1999], providing an additional rationale for a clinical testing of VDR ligands in the treatment of autoimmune diabetes.

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