

## Vitamin D deficiency in early life accelerates Type 1 diabetes in non-obese diabetic mice

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### Abstract

**Aims/hypothesis.** 1,25-dihydroxyvitamin D<sub>3</sub>, the active form of vitamin D, prevents Type 1 diabetes in non-obese diabetic (NOD) mice. Epidemiological data show a threefold increase in human Type 1 diabetes when vitamin D deficiency was present in the first months of life. To evaluate whether a similar dietary deficiency affects diabetes incidence in NOD mice, we generated NOD mice with vitamin D deficiency in early life.

**Methods.** Breeding pairs of NOD mice, as well as their offspring (test mice), were kept in surroundings devoid of ultraviolet light and were fed a vitamin D-depleted diet for 100 days. Mice were followed for 250 days.

**Results.** At 250 days, 35% (12/35) male and 66% (22/33) female vitamin D-deficient mice were diabetic compared to 15% (6/40,  $p=0.05$ ) and 45% (13/29,

$p<0.01$ ) of the control mice. At 100 days no difference in insulinitis was seen, but more vitamin D-deficient mice were glucose intolerant. Higher IL1 expression was detected in islets of vitamin D-deficient mice and their peritoneal macrophages had an aberrant cytokine profile (low IL1 and IL6, high IL15). Thymus and lymph nodes of vitamin D-deficient mice contained less CD4<sup>+</sup>CD62L<sup>+</sup> cells.

**Conclusion/interpretation.** Vitamin D status increases the expression of Type 1 diabetes in NOD mice. Our data in NOD mice, as well as human epidemiological data, point to the importance of preventing vitamin D deficiency in early childhood. Controlling this dietary factor could be an easy and safe way to reduce the incidence of Type 1 diabetes in subjects who are genetically at risk. [Diabetologia (2004) 47:451–462]

**Keywords** Vitamin D · Type 1 diabetes · NOD · Islets · Macrophage · Prevention · Rickets

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**Abbreviations:** 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> · 25(OH)D<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub> · BW, body weight · HBSS, Hanks' balanced salt solution · IP, interferon-inducible protein · IPGTT, intra-peritoneal glucose tolerance test · iNOS, inducible nitric oxide synthase · LPS, lipopolysaccharide · MCP, monocyte chemo-attractant protein · MIP, macrophage inflammatory protein · MLR, mixed lymphocyte reaction · NOD, non-obese diabetic · RPMI, Roswell Park Memorial Institute · UV, ultraviolet

Environmental factors have since long been implicated in the pathogenesis of autoimmune diseases such as Type 1 diabetes [1, 2]. Candidates can be sought in diet, lifestyle, viral infections, but the clear north-south gradient in prevalence of this and other autoimmune diseases in Europe has often suggested a role for sunlight as a modulator of autoimmunity [3, 4]. Also the observation that children with completely different genetic backgrounds often convert to the diabetes incidence of the country they live in, suggests that an environmental factor is involved [5, 6].

The discovery of the presence of receptors for the active form of vitamin D, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) in several cells of the immune system, such as macrophages, dendritic cells, B lymphocytes and activated T lymphocytes has strengthened the idea that vitamin D might function as a physiological im-

mune modulator [7]. In different animal models of autoimmune diseases administration of high doses of  $1,25(\text{OH})_2\text{D}_3$  is able to prevent disease [8, 9, 10, 11, 12] and in recent years both T lymphocytes and antigen presenting cells, especially dendritic cells, have been identified as main targets for  $1,25(\text{OH})_2\text{D}_3$  in the immune system [13].

Several epidemiological studies have shown a correlation between supplements of regular vitamin D in early life and a protection against Type 1 diabetes [14, 15, 16]. In most studies the vitamin D status of the population at start was not clear, whereby the observed protection was either due to the restoration of a vitamin D-deficient status or to a true supplementary effect of extra vitamin D being given to already vitamin D-sufficient children. In one study a subgroup of children was described with overt rickets in the first year of life [17]. In these children a threefold increase in diabetes prevalence in later life was observed. Since the effect of vitamin D deficiency was much greater than the effect of vitamin D supplementation in the other children, we hypothesised that mainly the vitamin D-deficient status in a genetically predisposed background might trigger autoimmunity, whereas high doses of active vitamin D hormone ( $1,25(\text{OH})_2\text{D}_3$ ) or an analogue would be needed to prevent the disease in vitamin D-sufficient subjects.

Our aim was to investigate the effects of vitamin D deficiency in utero and in early life on the prevalence of Type 1 diabetes in later life in an animal model of Type 1 diabetes, the NOD mouse. We discovered a clear increase in diabetes incidence in mice that were vitamin D deficient in the early weeks of life. Important changes in macrophage behaviour, namely a disturbed cytokine profile, might contribute to early beta-cell damage and a more aggressive presentation of the disease in genetically at risk subjects.

## Material and methods

### Animals

NOD mice have been bred in our animal facility since 1989, when they were obtained from Professor Wu (Beijing, China). At the time of the study, the diabetes incidence in our colony at the age of 250 days was 55% in female and 21% in male mice. With the purpose of inducing vitamin D deficiency in NOD mice, young (3-week-old) NOD male and female mice were kept in ultraviolet (UV) light-free surroundings and received a vitamin D-depleted diet containing 2% calcium and 1.25% phosphorus (TD 87095, Harlan Teklad Test Diets, Madison, Wis., USA). With this regime, the concentration of 25-hydroxy-vitamin  $\text{D}_3$  ( $25(\text{OH})\text{D}_3$ ) dropped below detection limit in male and female mice ( $<2.5$  ng/ml) by 8 weeks of age. After this was documented, breeding pairs were formed. Their offspring, the mice used in this study, were also kept in UV-free surroundings. After weaning, they received the same diet as their parents. Control mice were kept under similar conditions except their chow was supplemented with 2200 IU/kg vitamin D (control

chow, Harlan Teklad Test Diets, Madison, Wis., USA), reaching the daily requirements for vitamin D. From 100 days of age, all mice were fed control chow.

Mice were screened two times weekly for diabetes onset by measuring glucose in urine (Clinistix, Bayer Diagnostics, Tarrytown, N.Y., USA) and in blood obtained from a tail vein (Glucocard, Menarini, Florence, Italy) in glucosuric mice. Mice were considered diabetic when having glucosuria and hyperglycaemia ( $>11.10$  mmol/l) on two consecutive days.

C57Bl/6 and BALB/c mice were purchased from Harlan CPB (Zeist, the Netherlands). All experiments were approved by the ethics commission from the KU Leuven.

### Immune function of vitamin D-deficient and control NOD mice

*Fluorescence-activated cell sorter (FACS) analysis of spleens, thymuses and lymph nodes.* Spleens, thymuses and mesenteric lymph nodes were removed aseptically from diethylether anaesthetised control and vitamin D-deficient mice that were 100 days of age, and dispensed through a metal mesh in PBS to obtain single-cell suspensions. The following antibodies were used for standard staining: aCD3, aCD4, aCD8, aCD25, aCD62L, aB220, aCD11b, aCD11c, aCD54 mAbs (Caltag Laboratories, Burlingame, Calif., USA; PharMingen, Hamburg, Germany; Serotec, Oxford, UK). Cells were incubated for 30 min at  $4^\circ\text{C}$  with  $10\ \mu\text{l}$  of PE-labelled mAb and  $5\ \mu\text{l}$  FITC-labelled mAb. After washing twice, the cells were fixed with 1% paraformaldehyde. Analysis was done on a FACSsort cytometer (Becton Dickinson, Dendermonde, Belgium). False positive cells were excluded by isotype-matched irrelevant mAb staining through similar procedures.

*In vitro cell cultures.* After aseptic removal, spleens and thymuses were placed in sterile Petri dishes containing Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco, Paisley, Scotland, UK) and were disrupted by mechanical teasing. For mixed lymphocyte reaction (MLR) culture, splenocytes ( $5 \times 10^6/\text{ml}$ ,  $5 \times 10^5/\text{well}$ ) from the responder population were co-cultured, after T-cell enrichment by a passage through nylon wool, with irradiated (30 Gy,  $^{60}\text{Co}$  source) splenocytes from the stimulator population (C57Bl/6 mice) for 5 days at a R/S ratio of 1:1. Cells were cultured in triplicate in flat-bottomed 96-well microplates in complete culture medium (RPMI 1640 medium supplemented with 10% FCS and  $5 \times 10^{-5}$  mol/l 2-mercapto-ethanol) at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$  and air. Four days later,  $1\ \mu\text{Ci}$  [ $^3\text{H}$ ] thymidine (Radiochemical Centre Amersham, Buckinghamshire, UK) was added to each well. After incubation for another 18 h, cells were harvested on glass filter paper using a semi-automated cell harvester (Skatron Instruments SA, Lier, Norway) and the incorporation of labelled nucleotide was calculated by liquid scintillation counting in a  $\beta$ -counter (Packard, Meriden, CT).

In parallel, thymocytes and splenocytes were cultured in flat-bottomed 96-well microplates at a concentration of  $1 \times 10^6$  cells/ml in complete culture medium for anti-CD3 T-cell stimulation. Anti-CD3 mAb (Serotec) was added in a final concentration of  $0.75\ \mu\text{g}/\text{ml}$ . Experiments were done in triplicate. For the last 18 h of a 72-h culture, cells were pulsed with  $1\ \mu\text{Ci}$  [ $^3\text{H}$ ] thymidine. The cells were then harvested and the incorporated radioactivity was assayed by liquid scintillation counting.

Results of T-cell proliferation assays are expressed as stimulation index (SI = mean counts per min (cpm) of stimulated cells/mean cpm of non-stimulated cells/mean cpm of non-stimulated cells)  $\pm$  SEM.

**In vitro macrophage function.** For analysis of macrophage function, whole blood and peritoneal macrophages were used in parallel tests. Peritoneal macrophages were harvested as described previously [7]. Briefly, mice were anaesthetised with diethylether and injected intraperitoneally with 10 ml of RPMI 1640 medium. After 1 min, medium was collected from the peritoneum with an 18g needle into a sterile tube. Macrophage purity reached above 90% when assessed by flow cytometry, using the mAb CD11b (Serotec). Cells were counted and suspended to a concentration of  $1 \times 10^6$  cells/ml in complete culture medium. For macrophage chemotactic evaluation, the ability of binding casein was measured as described previously [7]. Chemotactic capacity was expressed as percent of fluorescein-positive cells. Respiratory oxidative burst capacity was evaluated using a commercial kit (BURTEST, Orpegen Pharma, Heidelberg, Germany). The test was carried out by following the instructions from the manufacturer. Results were expressed as percentage of fluorescein-positive cells. Parallel tests were done to assess macrophage function in whole blood. Around 1 ml of blood was collected from ether-anaesthetised mice by heart puncture. Chemotaxis was evaluated in a similar manner as was done for peritoneal macrophages. In addition, formyl-Nle-Leu-Phe-Nle-Tyr-Lys fluorescein derivative (fNlpntl) was also used to evaluate chemotaxis capacity of neutrophils, as described before [7]. Phagocytosis capacity was measured by flow cytometry using PHAGOTEST (Orpegen Pharma). The test was done following the instructions given by the manufacturer.

For cytokine analysis, peritoneal macrophages were suspended in RPMI 1640 and 10% FCS to reach a final concentration of  $2.5 \times 10^5$  cells per ml and were stimulated either with LPS (10  $\mu$ l/ml, Sigma, St. Louis, Mo., USA) or with LPS plus IFN $\gamma$  (100 U/ml, Peprotech, Rocky Hill, N.J., USA). Cells were incubated for either 6 h or 24 h. For mRNA analysis, cells were harvested and used immediately for RNA extraction. Until use, mRNA was stored at  $-80^\circ\text{C}$ .

#### Calcium and bone parameters in vitamin D-deficient and control NOD mice

At 100 and at 250 days of age, blood was collected by heart puncture under diethylether anaesthesia. Serum samples for calcium, osteocalcin, 25(OH) $D_3$  and 1,25(OH) $_2D_3$  were stored at  $-20^\circ\text{C}$  prior to their measurement. At the same time, a tibia was collected and stored at  $-20^\circ\text{C}$  for measurements of bone calcium content. Calcium (in serum and tibia) was measured using a photo-colorimetric method (Sigma Chemical, St. Louis, Mo., USA). Osteocalcin was measured by an in-house RIA as described [18]. Serum 25(OH) $D_3$  and 1,25(OH) $_2D_3$  were measured by ELISA (IDS Boldon, Tyne & Wear, UK) and RIA (ICN Biomedicals, Costa Mesa, Calif., USA) respectively.

#### Beta-cell function in vitamin D-deficient and control NOD mice

**Preparation and culture of pancreatic islets.** Islets were isolated from the pancreas of male and female vitamin D-deficient and control NOD mice as described [19]. Briefly, islets were isolated from the pancreas by collagenase digestion in HBSS followed by hand picking under a stereomicroscope.

For insulin secretion and content, freshly isolated islets (five islets in each well) were incubated in 300  $\mu$ l Krebs-Ringer bicarbonate buffer with 16 mmol/l HEPES for 2 h in the presence of various D (+) glucose concentrations (0–30 mmol/l). After

incubation, insulin was measured in both islet supernatant and homogenate by ELISA (Mercodia, Uppsala, Sweden).

For mRNA analysis, islets from at least five animals per group were prepared from 2- to 12-week-old male and female control and vitamin D-deficient NOD mice as well as from 4-week-old male and female BALB/c mice. Two independent experiments were done. Islets were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  until use.

**Intra-peritoneal glucose tolerance test (IPGTT).** Glycaemia was measured in tail vein blood using a glucometer (Gluco-card, Menarini, Florence, Italy) before and after (5, 15, 30, 60 and 120 min) an intra-peritoneal injection of 3 g D (+) glucose monohydrate/kg BW, dissolved in sterile PBS.

#### Histology

Pancreases from all experimental groups at 100 and 250 days were embedded in optimal cutting temperature (OCT) medium, snap-frozen in 2-methyl-butane 99% + chilled in liquid nitrogen and stored at  $-80^\circ\text{C}$  until processing [20]. 5- $\mu$ m cryostat sections were stained with Mayer's haematoxylin and eosin. By light microscopy, insulinitis was scored by an experienced blinded observer as follows: 0, islets free of insulinitis; 1, islets with peri-insulinitis; 2, islets with lymphocyte infiltration in less than 50% of the area; 3, islets with lymphocyte infiltration in more than 50% of the area; 4, islets with lymphocyte infiltration in more than 90% of the area and severe beta-cell destruction.

#### Real time reverse transcriptase polymerase chain reaction (RT-PCR) analysis of various cell types

Total RNA was extracted using the available commercial High Pure RNA isolation kit (Roche Diagnostics Indianapolis, Ind., USA) for peritoneal macrophages and islets and the SV total RNA isolation System (Promega, Madison, Wis., USA) for pancreases. cDNA synthesis was done as described [21, 22]. Each RT-PCR reaction contained 0.5  $\mu$ l cDNA sample, 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 10 mmol/l EDTA, 60 nmol/l Passive Reference 1200  $\mu$ mol/l dNTPs, 3–9 mmol/l MgCl $_2$ , 100 to 900 nmol/l of each primer, 100 nmol/l TaqMan probe, and 0.625 U AmpliTaq Gold. PCR amplifications were done in an ABI Prism 7700 SDS (Applied Biosystems, Foster City, Calif., USA) for 10 min at  $94^\circ\text{C}$  followed by a total of 40 to 45 two-temperature cycles. The primer sequences used here; IL1 $\beta$ , IL4, IL6, IL12 $_{p40}$ , IL15, iNOS, TNF $\alpha$ , TGF $\beta$ , IFN $\gamma$ , MIP3 $\alpha$ , MCP1, IP10, fractalkine and  $\beta$ -actin are described elsewhere [21]. Quantification was made using a standard curve (cDNA plasmids) as described [22]. Results were expressed as the ratio of copy number target to copy number  $\beta$ -actin.

#### Statistical analysis

Statistical analyses were made using the following tests when appropriate: Kaplan Meier Survival test, log-rank test, chi-squared test, two-way analysis of variance (ANOVA) test (with post Student's *t* tests), the area under the curve (AUC) with ANOVA test, the Mann-Whitney U test and the Student's *t* test. A *p* value .05 (or in case of multiplicity of testing, 0.01) was considered to be significant. The software NCSS 2000 (Kaysville, Utah) was used.

**Table 1.** Metabolic parameters at 100 and 250 days of age in mice

		D-deficient NOD male	Control NOD male	D-deficient NOD female	Control NOD female
Serum calcium (mg/dl)	100d	9.1±0.5 (n=14)	9.5±0.3 (n=7)	10.0±0.4 (n=14)	9.4±0.5 (n=5)
	250d	9.5±0.4 (n=13)	8.9±0.5 (n=10)	10.5±0.4 (n=11)	10.2±0.2 (n=11)
25(OH)D <sub>3</sub> (ng/ml)	100d	9.6±1.6 (n=11) <sup>a</sup>	73.9±6.2 (n=8)	7.3±1.3 (n=11) <sup>a</sup>	56.8±7.8 (n=5)
	250d	54.2±2.3 (n=5)	56.8±8.4 (n=5)	52.8±2.8 (n=5)	43.9±2.5 (n=5)
Bone calcium (mg)	100d	7.89±0.46 (n=5)	8.39±1.14 (n=5)	7.73±0.37 (n=5)	9.29±0.70 (n=5)
	250d	8.47±0.34 (n=9)	8.66±0.18 (n=13)	7.83±0.36 (n=8)	8.43±0.32 (n=8)
Osteocalcin (ng/ml)	100d	61.6±4.1 (n=10)	67.2±4.1 (n=6)	72.8±8.3 (n=10)	82.1±7.8 (n=5)
	250d	29.6±5.0 (n=8)	25.6±2.6 (n=8)	58.1±11.1 (n=5)	52.1±7.3 (n=9)

Data expressed as means ± SEM

<sup>a</sup>*p*<0.00001 compared to control mice of the same sex

## Results

### Evaluation of vitamin D deficiency in NOD mice

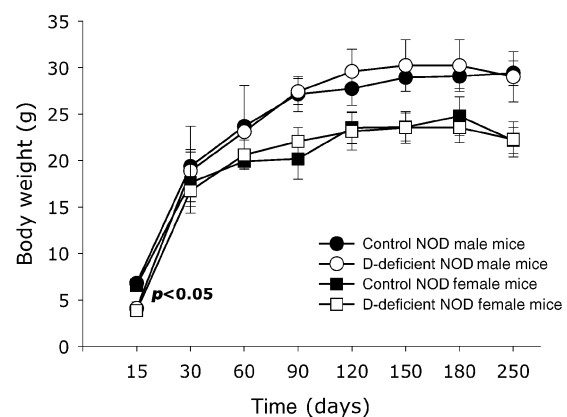
A significant difference in body weight was observed between NOD mice exposed in utero to either normal or low vitamin D when measured at 2 weeks of age (6.56±0.63 vs 3.85±0.56 g in female mice, *p*<0.0001 and 6.81±0.57 vs 4.08±0.49 g in male mice, *p*<0.0001), suggesting that vitamin D deficiency induced a growth retardation in utero or during lactation. After weaning however, no difference could be seen between control and vitamin D-deficient mice within male and female mice (Fig. 1).

At 100 days of age, no differences were noted in serum calcium, osteocalcin or bone calcium between control and vitamin D-deficient NOD mice (Table 1). Serum 25(OH)D<sub>3</sub> values were dramatically decreased in vitamin D-deficient animals of both sexes (Table 1). Also the concentration of the activated form of vitamin D, 1,25(OH)<sub>2</sub>D<sub>3</sub>, was very low in vitamin D-deficient mice (19.7±2.4 vs. 78.4±16.9 pg/ml in control mice, *p*<0.05).

At 250 days of age, when vitamin D was already restored in the diet since 100 days of age, all metabolic parameters returned to normal, confirming the transient nature of the vitamin D deficiency (Table 1).

### Effect of vitamin D deficiency in early life on insulinitis and diabetes incidence in NOD mice

No difference was observed in insulinitis prevalence, with insulinitis already being present in 9/10 and 9/9 vitamin D-deficient male and female NOD mice versus 9/9 and 9/10 control male and female mice (NS) at 100 days. Also the severity of insulinitis was comparable between the groups (score 1.8±0.3 and 2.7±0.3 in vitamin D-deficient male and female mice respectively, vs score 2.7±0.4 and 2.9±0.2 in control mice, NS). At 250 days of age, this severity increased to

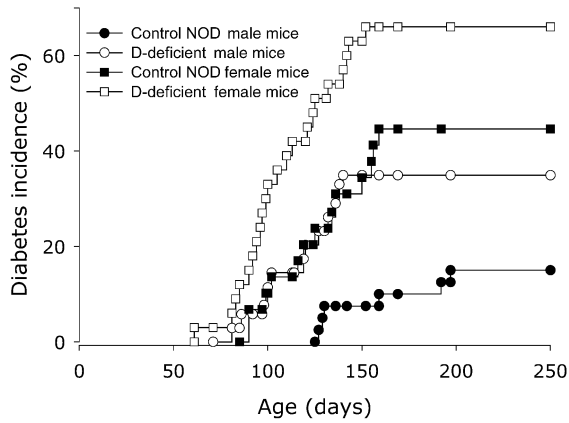


**Fig. 1.** Body weight of vitamin D-deficient and control NOD mice. Body weight from vitamin D-deficient and control NOD mice were evaluated over a period of 250 days. Note that vitamin D-deficient mice are smaller at 2 weeks of age while no difference can be seen after 1 month. Data expressed as means ± SEM. At least ten mice per group were weighed at each time point

score 3.9±0.4 and 3.6±0.9 in vitamin D-deficient male and female mice respectively, vs score 3.7±0.7 and 4.0±0.0 in male and female control mice, with insulinitis present in 8/8 and 9/9 male and female vitamin D-deficient mice respectively vs 9/9 and 8/8 in male and female control mice (NS).

Diabetes presentation was more aggressive in mice with vitamin D deficiency in early life, with an earlier onset and a higher final incidence (Fig. 2). While control male mice developed diabetes from 127 days of age (mean of 152 days), reaching a maximum incidence of 15% (6 out of 40 mice) by 250 days of age, NOD male mice with vitamin D deficiency in early life started developing diabetes at 71 days (mean 113 days, *p*<0.0005), reaching an incidence of 35% (12 out of 35 mice) at 250 days of age (*p*=0.05). A similar phenomenon occurred in female mice, where the controls developed diabetes from 85 days of age (mean 127 days) reaching 45% (13 out of 29 mice) by 250 days of age and vitamin D-deficient mice had diabetes from 61 days of





**Fig. 2.** Diabetes incidence in vitamin D-deficient and control NOD mice. Diabetes incidence is clearly higher in NOD mice that were submitted to vitamin D deficiency in utero and early life. A higher incidence and an earlier disease onset were present in the vitamin D-deficient mice. Vitamin D-deficient NOD male mice reached an incidence of 35% (12 out of 35 mice) against 15% (6 out of 40) of control NOD male mice ( $p=0.05$ ). Vitamin D-deficient NOD female mice presented the highest incidence reaching 66% (22 out of 33) while control NOD female mice appear with 45% (13 out of 29,  $p<0.01$ ). Statistically significant differences were obtained by Kaplan Meier-life table analysis (log-rank test) and chi-square test

age (mean 108 days,  $p<0.0005$ ) reaching 66% (22 out of 33 mice) at the end of the study ( $p<0.01$ ).

**Immune function of vitamin-D deficient and control NOD mice**

*Phenotyping of immune cells.* Thymic composition was only slightly altered in vitamin D-deficient mice at 100 days of age, with clearly lower amounts of single positive CD4 (in females) and CD8 cells (in males and female mice) and higher amounts of double positive CD4/CD8 cells (in females) (Table 2). Of interest, CD4<sup>+</sup>CD62L<sup>+</sup> cells, claimed to be regulator cells, were scarcer in thymuses of vitamin D-deficient mice, however only reaching the level of significance in female mice ( $p<0.05$  vs control female mice).

Cellularity was markedly decreased in the spleens of both female and male vitamin D-deficient mice ( $p<0.05$ ), but in the peripheral murine system only subtle difference could be detected between vitamin D-deficient and control NOD mice (Table 2). In mesenteric lymph nodes again lower counts of CD4<sup>+</sup>CD62L<sup>+</sup> cells were found ( $p<0.05$  in female mice), while a clearly higher amount of CD8<sup>+</sup> cells were present ( $p<0.05$  in males and female mice). Ex vivo analysis of the surface marker profile of CD11<sup>+</sup> dendritic cells in thymus, spleen and lymph nodes did not show any differences.

*Analysis of T and B lymphocyte function.* No severe impairment in T-cell proliferation was observed. Splenocytes from vitamin D-deficient mice proliferated to

**Table 2.** Cellular composition of lymphoid organs of vitamin D-deficient and control NOD mice at 100 days of age

	Thymus				Spleen				Mesenteric Lymph Node			
	D-deficient NOD		Control NOD		D-deficient NOD		Control NOD		D-deficient NOD		Control NOD	
	male (n=8)	female (n=6)	male (n=8)	female (n=7)	male (n=10)	female (n=10)	male (n=10)	female (n=10)	male (n=5)	female (n=5)	male (n=5)	female (n=5)
Leukocytes ( $\times 10^6$ cells)	51±13	72±29	46±13	81±9	78±18 <sup>a</sup>	118±18 <sup>a</sup>	190±22	208±33	20±9	37±16	45±13	49±34
CD4 (%)	10.0±1.2	9.3±2.2 <sup>a</sup>	10.6±2.2	14.0±3.0	27.9±2.6	29.7±3.5	28.7±5.2	28.5±4.6	51.8±0.5	52.4±2.9	54.2±2.5	56.0±2.2
CD8 (%)	3.2±2.0 <sup>b</sup>	3.2±1.0 <sup>b</sup>	5.2±0.9	5.1±1.5	12.8±1.7	13.2±1.9	12.2±2.9	13.0±3.3	26.6±0.5 <sup>a</sup>	29.4±3.1 <sup>a</sup>	20.8±1.9	21.7±1.8
CD4CD8 (%)	78.1±2.7	78.2±3.3 <sup>a</sup>	75.7±2.6	71.2±4.7	3.8±2.2	3.0±1.3	2.4±0.7	3.0±2.0	3.4±0.4 <sup>a</sup>	3.3±0.3 <sup>a</sup>	1.7±1.0	1.5±0.7
CD4-CD8 <sup>-</sup> (%)	5.7±2.3	6.4±1.7	5.7±1.1	6.3±1.7	NA	NA	NA	NA	NA	NA	NA	NA
CD4CD25 (%)	3.3±1.6	3.6±0.3	2.3±0.5	4.1±1.9	6.6±3.5	6.7±2.1	8.7±4.8	5.6±1.5	14.0±4.4	11.5±2.7	10.6±3.2	12.3±5.4
CD4CD62L (%)	9.7±2.4	12.4±1.5 <sup>a</sup>	14.1±1.5	17.4±1.0	18.1±3.4	21.5±7.0	21.4±5.8	20.2±4.7	15.7±4.3	5.3±2.4 <sup>c</sup>	20.6±12.8	32.9±7.3
CD11c <sup>+</sup> (%)	2.8±1.1 <sup>d</sup>	1.2±0.7	1.1±0.2	1.1±0.2	2.6±1.7	1.7±0.8	2.7±1.1	2.6±0.9	0.9±0.1	1.0±0.4	1.1±0.4	1.1±0.2
B220(%)	3.8±1.2	3.5±0.8	2.9±1.1	2.7±1.4	37.3±9.9	35.4±7.2	35.4±6.4	33.2±6.0	21.6±6.1	26.7±9.2	20.2±6.5	17.9±3.6
MAC1(%)	0.9±0.5	1.0±0.5	0.7±0.4	0.7±0.5	17.7±5.3	19.0±3.7	18.9±9.2	17.9±4.0	3.3±1.4	3.3±1.2	3.6±1.1	3.6±2.2

Data expressed as means ± SD; NA, not applicable

<sup>a</sup>  $p<0.005$ , <sup>b</sup>  $p<0.02$  when compared to control mice of the same sex; <sup>c</sup>  $p<0.05$ , <sup>d</sup>  $p<0.002$  compared to control mice of both sexes

**Table 3.** In vitro macrophage function in mice at 100 days of age

	D-deficient NOD male (n=10)			Control NOD male (n=10)			D-deficient NOD female (n=10)			Control NOD female (n=10)		
	Peritoneal		Whole blood	Peritoneal		Whole blood	Peritoneal		Whole blood	Peritoneal		Whole blood
	Macro-phage	Neutro-phil	Macro-phage	Macro-phage	Neutro-phil	Macro-phage	Macro-phage	Neutro-phil	Macro-phage	Macro-phage	Neutro-phil	Macro-phage
Chemotaxis casein (%)	82.0±4.5	70.5±16.3	87.7±9.4	80.2±0.4	68.9±19.2	79.7±8.1	71.3±8.0	88.2±10.4	88.3±10.8	77.1±2.7	87.4±11.2	87.2±13.2
Chemotaxis filpntl (%)	NA	44.7±27.1	NA	NA	32.1±24.2	NA	NA	37.5±18.4	NA	NA	36.5±15.3	NA
Phagocytosis (%)	ND	34.1±7.7	29.6±10.9	ND	38.0±14.3	31.6±11.8	ND	37.3±12.6	38.9±13.0	ND	34.4±9.3	29.5±10.8
Respiratory Burst (%)	45.5±5.5 <sup>a</sup>	NA	NA	60.9 ± 1.8	NA	NA	40.3±10.0 <sup>b</sup>	NA	NA	53.2±7.1	NA	NA

Data expressed as means ± SEM

NA, not applicable; ND, not done

<sup>a</sup>  $p < 0.001$  compared to male control mice, <sup>b</sup>  $p < 0.05$  compared to female control mice

the same extent to different stimuli such as allogeneic lymphocytes and anti-CD3. Of note, however, is the somewhat lower SI in MLR in vitamin D-deficient mice. Vitamin D-deficient mice had a SI of  $34.0 \pm 15.2$  in male ( $n=4$ ) and  $57.3 \pm 23.2$  in female mice ( $n=4$ ), whereas in control NOD mice, SI was  $72.3 \pm 15.4$  and  $118.5 \pm 17.4$  in male ( $n=4$ ) and female mice ( $n=4$ ) respectively, but this value did not reach statistical significance.

No difference in antibody profile could be detected in 100-day-old vitamin D-deficient and control mice (IgG<sub>1</sub> and IgG<sub>2a</sub>).

*In vitro analysis of macrophage function.* No differences in chemotactic or phagocytic capacity were found between the groups (Table 3). The ability to form free radicals was, however, decreased in peritoneal macrophages from vitamin D-deficient mice (Table 3). Exposing peritoneal macrophages to LPS or a mixture of LPS and IFN $\gamma$  further highlighted defects present in the macrophages of the vitamin D-deficient mice. Indeed, a severe deficiency in IL1 (Fig. 3a) and IL6 (Fig. 3b) response to these stimuli was observed, whereas the IL15 (Fig. 3c) response was greatly enhanced. No differences were observed for iNOS (Fig. 3d), TNF $\alpha$ , TGF $\beta$  or IL12<sub>p40</sub>.

#### Beta-cell function in vitamin D-deficient and control NOD mice

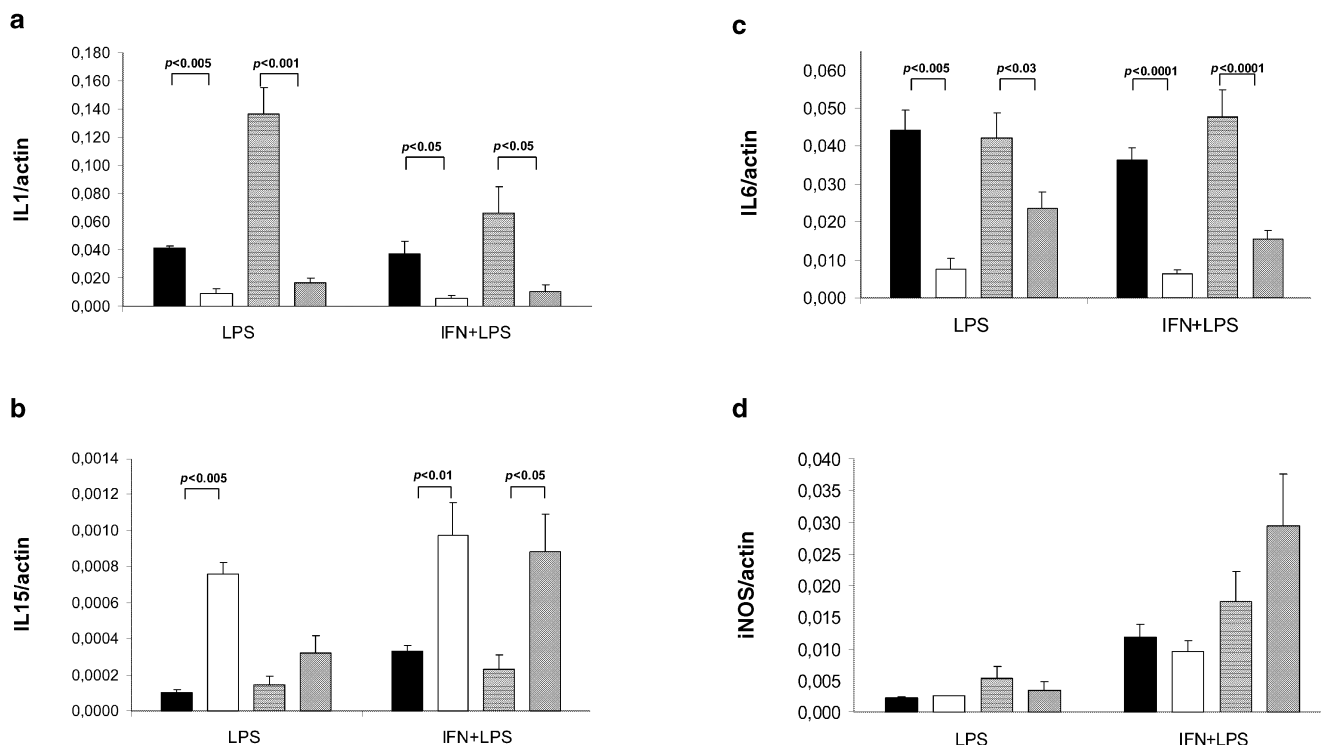
In order to detect impaired glucose tolerance at early age, IPGTTs were done at 100 days of age in non-diabetic mice (Fig. 4). Basal glycaemia (0 min) was comparable between vitamin D-deficient and control mice ( $5.37 \pm 0.59$  vs  $6.63 \pm 0.56$  mmol/l in males and  $5.53 \pm 0.69$  vs  $5.41 \pm 0.88$  mmol/l in females, NS). After the intra-peritoneal glucose challenge a different response pattern was observed between the two groups in both sexes ( $p < 0.05$  and  $p < 0.05$  for female and male mice, respectively), reaching severely impaired values mainly in male mice (Fig. 4).

When isolated islets from 100-day-old mice were exposed to an in vitro glucose challenge (20 mmol/l), no defect in either insulin content or secretion could be detected in the vitamin D-deficient mice compared with both male and female control mice.

#### Intra-islet cytokine and chemokine expression

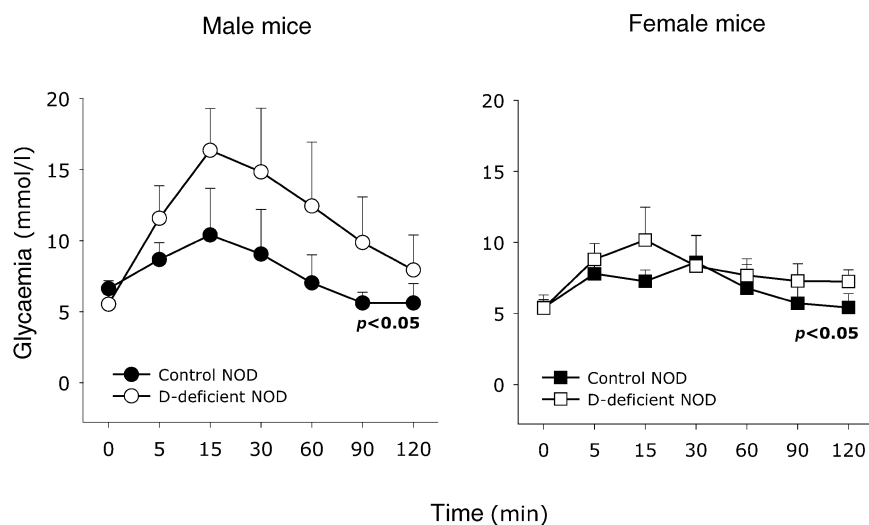
Whole pancreases were analysed at 100 days of age to assess cytokine expression profile. No difference in expression of IFN $\gamma$  and IL4 were observed between vitamin D-deficient and control NOD mice.

Chemokine expression was analysed in pancreatic islets from 2- to 12-week-old vitamin D-deficient and control NOD mice matched for sex. As previously



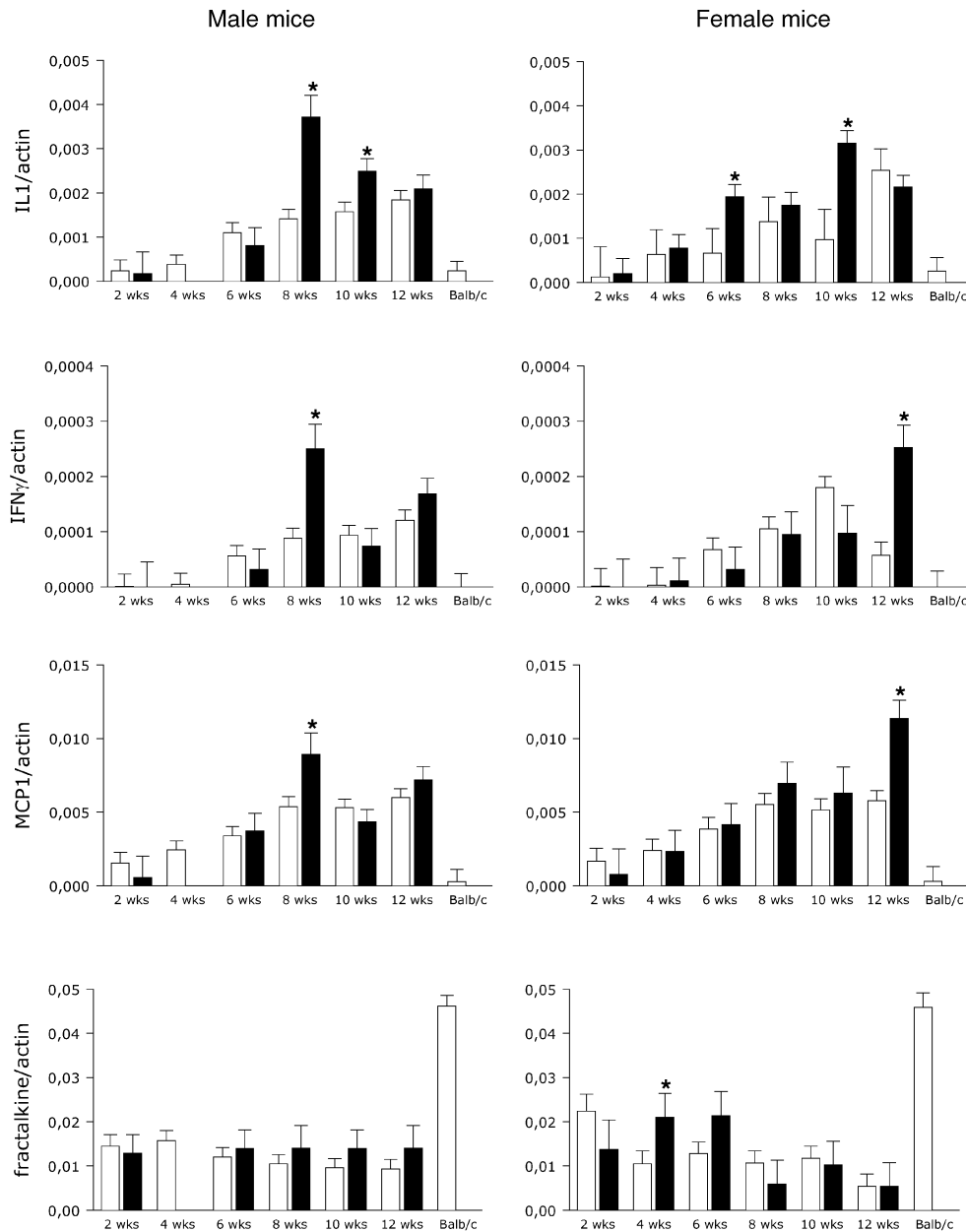
**Fig. 3.** Cytokine mRNA expression in peritoneal macrophages. Real-time RT-PCR was used to determine cytokine mRNA expression in peritoneal macrophages harvested from vitamin D-deficient and control NOD mice. Cells were incubated for 6 h and challenged either with LPS or with LPS plus IFN $\gamma$ . Data is expressed as mean of target copies divided by  $\beta$ -actin copies  $\pm$

SEM. At least five mice were used to compose each group. *White bar* Control NOD male, *black bar* Vitamin D-deficient NOD male, *hatched bar* Control NOD female, *grey bar* Vitamin D-deficient NOD female. (a) IL1 expression; (b) IL6 expression, (c) IL15 expression, (d) iNOS expression



**Fig. 4.** IPGTTs in vitamin D-deficient and control NOD mice. Mice were challenged intra-peritoneally with 3 g of glucose and glycaemia was accessed at 5, 15, 30, 60 and 120 min. Basal glycaemia was comparable between the groups while glucose intolerance was observed in vitamin D-deficient mice in the minutes subsequent. Data expressed as means  $\pm$  SEM

observed, control male and female NOD mice showed a gradual increase in expression of pro-inflammatory cytokines (IL1 $\beta$  and IFN $\gamma$ ) and chemokines (MCP1, IP10 and MIP3 $\alpha$ ), coinciding with the increase in insulinitis score (Fig. 5). For all cytokines and chemokines tested, mRNA expression levels were increased in vitamin D-deficient as well as in control NOD mice as compared to the BALB/c controls (p<0.05), except for fractalkine, where an already known NOD defect was confirmed. When comparing vitamin D-deficient and



**Fig. 5.** Intra-islet cytokine and chemokine expression. Real-time RT-PCR was used to assess mRNA expression of cytokines and chemokines in isolated islets of vitamin D-deficient and control NOD mice ( $n \geq 5$ ). Data is expressed as mean of target copies divided by  $\beta$ -actin copies  $\pm$  SEM from two independent experiments. \* $p < 0.05$ , when vitamin D-deficient mice is compared to control NOD mice. *Black bar* Control NOD; *white bar* Vitamin D-deficient NOD

control NOD mice, IL1 levels were up-regulated from 6 and 8 weeks of age in the rachitic groups. In general, no major differences were noted for IFN $\gamma$  and chemokine (MCP1 and fractalkine) mRNA expression in islets between vitamin D-deficient and control mice (Fig. 5). Also no difference was seen in IP10 and MIP3 $\alpha$  expression levels between vitamin D-deficient and control NOD mice.

## Discussion

The presentation of Type 1 diabetes in humans as well as in NOD mice is dependent on an interplay between genetic and environmental factors. Several studies point towards vitamin D as being one of the modulating environmental factors [17]. Our study shows that in NOD mice that are genetically at risk of developing Type 1 diabetes, vitamin D deficiency in early life leads to a more aggressive presentation of the disease, with an earlier onset, and a higher final incidence of the disease. We created a model of subtle vitamin D deficiency in early life, with only a measurable vitamin D deficiency in blood, but no effect on calcium concentration in serum or bone calcium content. We deliberately chose this model of temporary slight vitamin D deficiency, since this is most proba-



bly the reflection of the vitamin D status in many infants and small children, even in areas with high exposure to sunlight, since infants often are shielded from UV B exposure. Although vitamin D supplements are advised in many countries, the application is far from strictly controlled and many times, these supplements are omitted or administered irregularly. Nutritional rickets that has never been completely eradicated in some countries and may be reappearing in industrialised countries [23] could be one of the factors contributing to the higher incidence of Type 1 diabetes in the past two decades [24]. A recent Finnish epidemiological study [17] has shown a clear association between overt rickets in the first year of life in this genetically high-risk population and the development of diabetes in later life. We induced vitamin D deficiency in early life in a NOD colony with an intermediate risk for diabetes, anticipating an increase in diabetes incidence. The vitamin D deficiency in our mice was already present in utero, since the parents of the mice studied were already put on a vitamin D-free diet from 3 weeks of age. This vitamin D-deficient status was then maintained until 100 days of age as shown by the low concentration of  $25(\text{OH})\text{D}_3$ . This vitamin D deficiency in early life in the genetically predisposed NOD mouse was accompanied by a more aggressive presentation of Type 1 diabetes. Diabetes onset was earlier and final incidence was increased in females as well as in the more diabetes-resistant male mice. Switching mice to a normal diet at 100 days of age avoided the development of overt rickets in mice with disturbances in bone and calcium homeostasis. In adult life however the higher aggressiveness of the disease persisted and a higher total incidence was registered in both sexes. At 250 days of age, when the study was terminated, no calcium or bone abnormalities could be found in the previously vitamin D-deficient mice.

Analysis of the immune system of the vitamin D-deficient mice at 100 days of age, time when animals were switched to normal food, showed major abnormalities in macrophage behaviour. The differences were, however, not situated in impaired phagocytosis or chemotaxis, previously observed in severe rickets [7, 25, 26, 27]. The near normal phagocytic and chemotactic capacity confirms again the relevance of our model, where we did not create severely rachitic mice, but only induced a vitamin deficiency frequently observed in children. This deficiency resulted, however, in an impressively disturbed inflammatory behaviour of the NOD macrophages, with lower respiratory burst capacity and a disturbed cytokine profile with increased IL15 mRNA expression and extremely low IL1 and IL6 expression. This low expression of IL1 and IL6 is in agreement with previous reports in rachitic animals [28]. Abnormalities in macrophages have been implicated in the pathogenesis of Type 1 diabetes, with these cells being one of the first to

be present in insulinitis [29, 30]. An abnormal inflammatory profile of these cells could lead to aspecific beta-cell damage, thus triggering a beta-cell destructive cascade. Previously, NOD mice were shown to have severe defects in their macrophage differentiation and function [31]. In this context, NOD macrophages are known to have a defect in IL1 expression upon stimulation with LPS [32]. Vitamin D-deficient mice generated in this study presented with an even bigger defect in IL1 and also IL6 secretion, probably due to a defect in macrophage differentiation in NOD mice lacking vitamin D in the diet. High IL15 mRNA expression on the other hand possibly contribute to the survival of autoreactive T lymphocytes [33], and could thus be implicated in the worsening of the disease. These abnormal cytokine profiles of macrophages from vitamin D-deficient NOD mice might play a crucial role in the higher incidence of Type 1 diabetes in vitamin D-deficient mice. A characteristic of badly differentiated NOD macrophages is the fact that they produce a low amount of IL1 when exposed to stimuli such as LPS *in vitro*, but accumulate locally in the islets where they produce inappropriately high amounts of cytokines in the presence of apoptotic cells that should have been cleared silently [34]. Vitamin D deficiency clearly aggravated this macrophage maturation problem.

In accordance with the abnormal macrophage cytokine profile, we found high pro-inflammatory cytokine expression locally in the islets of vitamin D-deficient NOD mice compared to control NOD mice. Recently we and others have observed that in islets of NOD mice, but also in pure rat or human beta cells, inflammatory cytokines can trigger the expression of chemokines, thus probably contributing to the accumulation of more immune cells in the islets and precipitating beta-cell destruction [35, 36, 37]. In our study, no significant differences could be observed in local islet chemokine expression between vitamin D-deficient and control mice. However, the observation of a higher intra-islet IL1 expression taken together with the histological observation of a similar degree of insulinitis in control and vitamin D-deficient mice, suggests a higher activation status of the infiltrating immune cells and thus a more aggressive presentation of the disease.

Vitamin D deficiency also affected lymphocyte profiles, with clearly lower counts of splenocytes in vitamin D-deficient mice and a disturbed thymocyte profile. In the thymus, lower percentages of single positive CD8 T lymphocytes and higher numbers of double positive CD4/CD8 cells were present, possibly indicating a maturational problem. Of interest are the lower counts of CD4<sup>+</sup>CD62L<sup>+</sup> lymphocytes in thymus as well as in peripheral lymph nodes. Vitamin D-deficiency seems to further decrease the number of these cells in the NOD mice. CD4<sup>+</sup>CD62L<sup>+</sup> are considered regulator cells, since they are able to prevent diabetes

transfer in NOD-scid recipients [38] and their lower numbers in rachitic mice might contribute again to the more aggressive presentation of the disease.

Another interesting feature presented by the vitamin D-deficient NOD mice is their pattern of growth. Vitamin D-deficient NOD mice are lighter and smaller at 15 days of age but they reach the same weight as control mice around weaning at 30 days of age. This lower weight during the first days of life, probably due to the vitamin D deficiency of the dams, followed by a more rapid weight gain in the weeks thereafter might be another factor contributing to the diabetes presentation since also in humans, rapid growth and weight gain are implicated in Type 1 diabetes acceleration through increased metabolic demand [39, 40, 41].

Vitamin D deficiency not only affects the immune system, but it can also lead to beta-cell dysfunction. As shown previously, severe rickets leads to decreased insulin synthesis, insulin secretion and even overt insulin deficiency in vivo [42, 43, 44]. In the present study islets isolated from vitamin D-deficient mice at 100 days of age had an insulin synthetic and secretory capacity comparable to islets from control NOD mice. In vivo, however, clear glucose intolerance was observed already at 100 days of age in female and even more pronounced in male vitamin D-deficient NOD mice. There are several reports on the link between vitamin D deficiency and the pathogenesis of insulin resistance, impaired glucose intolerance and even Type 2 diabetes in humans [45, 46]. Therefore the impaired glucose intolerance found in the vitamin D-deficient NOD mice at 100 days of age might be explained in part by an increased insulin resistance in these animals, since insulin secretion and synthesis were comparable to those found in vitamin D-sufficient NOD mice.

Previously we have shown that in order to intervene with vitamin D in vitamin D-sufficient mice in a preventive way, the use of regular vitamin D is insufficient [47] and long-term treatment with the activated form of the molecule,  $1,25(\text{OH})_2\text{D}_3$ , or a synthetic analogue is needed [48]. The use of  $1,25(\text{OH})_2\text{D}_3$ , and even of the synthetic analogues that have been designed to reduce the hypercalcaemic properties of the original molecule and to enhance its immune effects, still presents the danger of metabolic side effects, being hypercalcaemia and bone decalcification. These dangers are real, as chronic treatment with these products is necessary. At present several groups are considering dietary interventions using regular vitamin D supplements in early life in populations at risk for Type 1 diabetes. The effectiveness of these trials might be enhanced by selecting the trial subjects more carefully by measuring the vitamin D status of the subjects at baseline. Based on our data from NOD mice, we expect that giving vitamin D supplements to subjects with normal vitamin D levels could lack protective power, while preventing vitamin D deficiency

in an at risk population might have dramatic effects on diabetes incidence. A weakness of our study is that we are unable to distinguish between the effects of vitamin D deficiency in utero or during the first weeks of life.

In conclusion, transient vitamin D deficiency in utero and in early life leads to a more aggressive presentation of Type 1 diabetes in NOD mice. An aberrant macrophage cytokine profile, a defect in  $\text{CD4}^+\text{CD62L}^+$  cells in combination with a higher islet inflammatory cytokine expression might trigger the disease more rapidly in mice genetically at risk. Vitamin D deficiency is thus an environmental factor that influences the expression of autoimmune Type 1 diabetes in NOD mice. If our NOD mouse data can be extrapolated to the human situation, preventing vitamin D deficiency in early life might be an easy and safe way to reduce the incidence of Type 1 diabetes.

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