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Developmental vitamin D_3 deficiency induces alterations in immune organ morphology and function in adult offspring☆

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ABSTRACT

Vitamin D_3 deficiency and insufficiency are common in women of child-bearing age. This may be cause for concern because vitamin D_3 is a well known regulator of immune function and epidemiological evidence has suggested that immune disorders, including autoimmune diseases, could have developmental origins. However, it is not known whether a developmental deficiency in vitamin D_3 could lead to persistent changes in the immune system in adult offspring. Given the prominence of receptors for vitamin D_3 within immune cells we hypothesised that the developmental absence of vitamin D_3 may alter thymic development and thus produce associated functional changes in T cells. We have developed a model of developmental vitamin D_3 (DVD) deficiency in Sprague–Dawley rats, in which the vitamin D_3 deficiency is transient and restricted to gestation. First we demonstrate that DVD deficiency induced an increase in central but not peripheral immune organ size. Second when stimulated, lymphocytes from DVD-deficient rats exhibit a pro-inflammatory phenotype. This is the first study to show that a transient vitamin D_3 deficiency restricted to gestation can persistently alter aspects of immune phenotype and function in the adult offspring. Given an increased incidence of vitamin D_3 deficiency in women of child-bearing age these findings may be highly relevant for autoimmune disorders with a developmental basis.

1. Introduction

In recent decades there has been intense interest in the immunomodulatory effects of the active form of vitamin D_3, 1,25 dihydroxyvitamin D_3 (henceforth referred to as vitamin D_3) [1,2]. The receptor for vitamin D_3 (VDR) is present in cells of the immune system [3] including macrophages, dendritic cells and resting and activated T cells [4]. Certain immune cells also have the ability to locally synthesise vitamin D_3 [5] suggesting that vitamin D_3 can signal directly within these cells.

Epidemiological studies have linked many different clinical disorders with exposure to low vitamin D_3 [6]. It has been postulated that low vitamin D_3 during gestation and early life may alter the development of the foetal immune system thereby leaving the individual vulnerable to the development of immune-related disorders such as diabetes and multiple sclerosis in later life [7,8]. To the best of our knowledge only one study has examined immune function when vitamin D_3 deficiency was limited to gestation only [9]. We have developed a model of gestational developmental vitamin D_3 (DVD) deficiency in Sprague–Dawley rats [10]. In this model dietary vitamin is re-introduced at birth and is present in the diet through postnatal life. Importantly, this model shows no signs of hypocalcaemia or any aspect of a rachitic phenotype with calcium, phosphorus, parathyroid hormone and vitamin D_3 levels being normal in the adult DVD-deficient rats [11].

Hypovitaminosis D_3 is prevalent in modern populations largely due to a change in diet and outdoor activity. This appears to be of particular concern in young women. A study of non-institutionalised women of child-bearing age in the USA found that 19% of 20–39 year olds were deficient in vitamin D_3 [12]. Pregnant women are at an increased risk of vitamin D_3 deficiency due to the increased calcium requirements for foetal growth and a reduction in outdoor activity (and thus UVB exposure), particularly in the third trimester [13,14]. The aim of this study was to broadly characterize the immune system of the adult DVD-deficient rat. Given the prominence of receptors for vitamin D_3 within immune cells, in particular the thymus [15], we hypothesise that the developmental absence of this vitamin will induce changes in how immune

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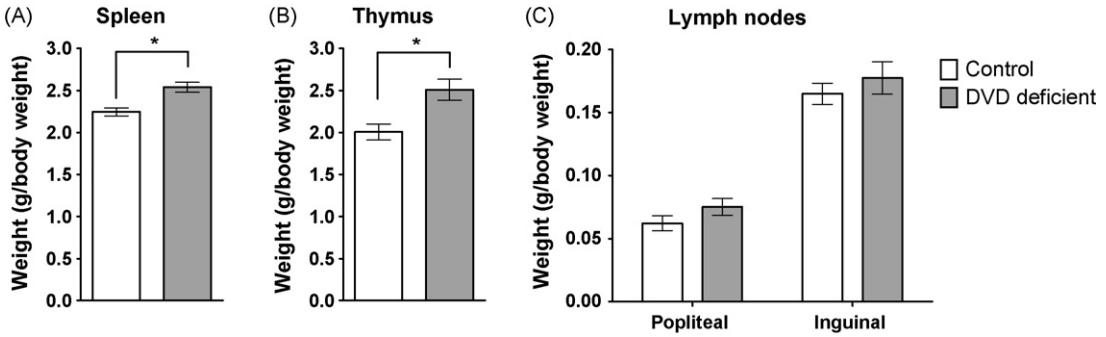

Fig. 1. Weight of spleen, thymus and popliteal and inguinal lymph nodes from male and female, control and DVD-deficient rats. Weights are normalised against individual rats' body weights, to control for the difference in body weight between male and females. Data presented as mean ± SEM. $n = 19–21$ per group (spleen), $n = 10–11$ per group (thymus, popliteal, inguinal). $^*p < 0.05$.

organs like the thymus develop and produce associated changes in resultant T cell function.

2. Materials and methods

2.1. Animals

Our model of maternal vitamin D_3 deficiency has been previously described [11]. Briefly female Sprague–Dawley rats are raised on a vitamin D_3 deficient diet (0.45% calcium and 0.3% phosphorus, Dyets Inc., PA, USA) for 6 weeks prior to mating and during gestation. Within 12 h of birth vitamin D_3 deficient dams were switched to a normal vitamin D_3 containing rat chow (Dyets Inc., PA, USA). Control dams were fed a vitamin D_3—normal diet (Dyets Inc., PA, USA) throughout pregnancy and post-birth. Offspring from both dietary groups were weaned at P21, and male and female, control and DVD-deficient rats were examined at 10–12 weeks of age. All procedures were performed with approval of the University of Queensland Animal Ethics Committee under the guidelines of the National Health and Medical Research Council of Australia.

2.2. Tissue and blood collection

Rats were killed by lethal injection (sodium pentobarbitone, 325 mg/mL, Virbac, Australia). Whole blood was collected via cardiac puncture. Spleen and thymus were dissected, weighed and placed on ice in basal media (Dulbecco's modified Eagle medium (DMEM) with 500 mg/L D-glucose and 110 mg/mL sodium pyruvate) supplemented with 2 g/L sodium bicarbonate, 10 mM HEPES, 60 mg/L penicillin, 100 mg/L streptomycin, 216 mg/L L-glutamine, 50 μM mercaptoethanol and 10% heat inactivated foetal calf serum (Gibco, USA), disassociated to single cell suspensions using a 70 μm nylon cell strainer (Becton Dickson Falcon, USA) and resuspended in fresh media. Right and left popliteal and inguinal lymph nodes were dissected, weighed and discarded. Peripheral blood mononuclear cells (PBMC) and splenocytes were purified using the Ficoll-Paque density gradient separation method. EDTA anti-coagulated blood samples were used to obtain a complete blood count using a Coulter counter (Coulter Corporation, FL, USA). A white blood cell differential was determined from microscopic analysis of whole blood smears, obtained using Automated Wright Stain (Sigma–Aldrich, USA) with an Ames Hema-Tek Automated Slide Stainer (Bayer Corporation, USA).

2.3. Immunophenotyping

PBMCs and splenocytes were immunophenotyped for B and T cells, T cell subsets (helper, cytotoxic, activated), macrophages and natural killer cells. The percentage of mature and immature T cells was examined in thymus. Standard fluorescent antibody labelling and flow cytometry techniques were used [16].

2.4. PBMC cytokine stimulation and cell culture

PBMCs were resuspended at a density of 1×10^6 cells/mL and incubated in basal media with or without 10 ng/mL PMA (Sigma, USA) and 250 ng/mL ionomycin (Sigma, USA) for 48 h at 37 °C in 5% CO_2. After 48 h, cells and supernatant were collected separately. Cell viability and activity was determined in one cohort of unstimulated and stimulated cells with the tetrazolium (MTS) assay (Cell Titre 96 "AQ_{ueous} One Solution" Promega, USA). The cytokine content of the supernatant was assessed using commercial ELISA kits against rat IL-2, IL-10, TNF-α and IFN-γ (all Biosource, Invitrogen, USA). To control for week-to-week variations in cell density during *in vitro* experiments, cytokine production for each rat was normalised against the MTS value for cell viability.

2.5. Statistics

DVD deficiency does not induce an alteration in adult body weight [11], however, to control for individual differences in body weight, immune organ weights are presented and analysed normalised against body weight. Results were analysed using SPSS (SPSS Inc., Chicago, USA). A two-way ANOVA was used to determine the main effects of "Sex" and "Maternal Diet" on experimental parameters. There was no main effect of Sex thus, for clarity, data are presented pooled for sex in all graphical representations. Threshold for statistical significance was set as $P < 0.05$.

3. Results

3.1. Immune organ weight

There was a significant effect of Maternal Diet on immune organ weight. Both spleen ($F_{1,66} = 14.2$, $P < 0.01$, Fig. 1A) and thymus weights ($F_{1,39} = 25.3$, $P < 0.01$, Fig. 1B) were increased in DVD-deficient rats. This enlargement was not seen in the peripheral lymph tissue, with popliteal and inguinal lymph node weights unchanged (Fig. 1C).

3.2. Immunophenotyping

There was no effect of DVD deficiency on the percentage of B and T immune cell subsets found in the blood, spleen or thymus ($n = 13–19$ per group (blood and spleen), $n = 8–12$ per group (thymus)). There was also no effect of Maternal Diet on the complete blood count or white blood cell differential (data not shown).

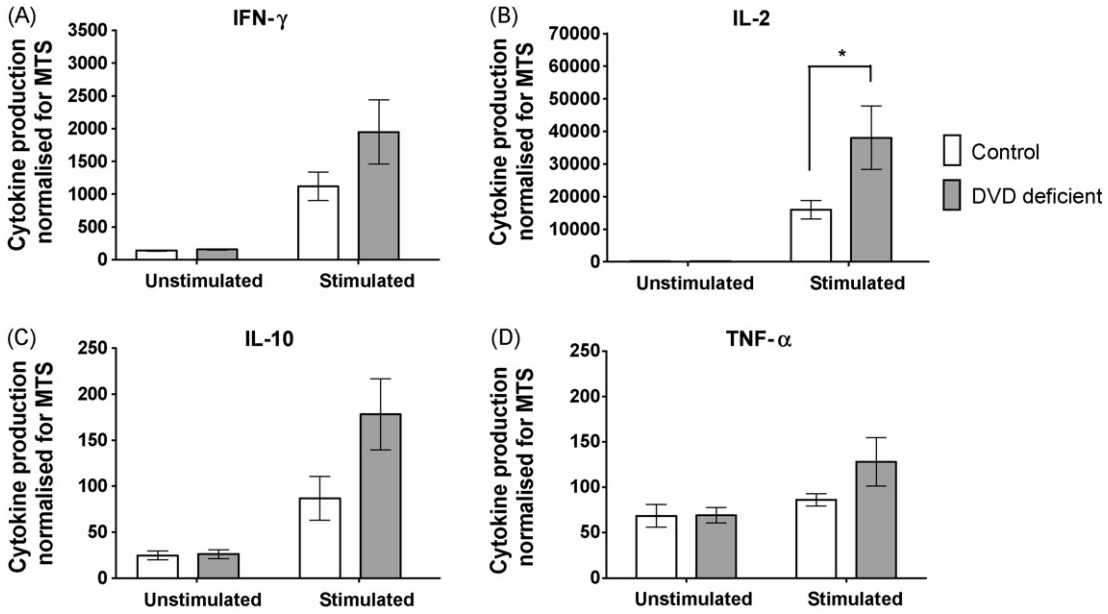

Fig. 2. Cytokine production in stimulated peripheral blood mononuclear cells (PBMCs). PBMCs (1×10^6 cells/mL) were stimulated with 10 mg PMA and 250 ng ionomycin and incubated for 48 h at 37 °C and 5% CO_2. Supernatants were collected and analysed using commercial enzyme-linked immunosorbent assay kits for interferon-gamma (IFN-γ), interleukin-2 (IL-2), IL-10 and tumour necrosis factor alpha (TNF-α). Cytokine production was normalised for cell viability, activity and week-to-week variations in plating density using a commercial tetrazolium-MTS assay. Data presented as mean ± SEM. $n = 14$ per group, $^*P < 0.05$.

3.3. *In vitro cytokine production*

As expected, lymphocytes stimulated with PMA/ionomycin produced robust cytokine responses compared to unstimulated cells (Fig. 2). DVD deficiency resulted in an increase in cytokine production in cultured lymphocytes, specifically in IL-2 ($F_{1,26} = 4.7$, $P < 0.05$, Fig. 2B). Numerically, DVD deficiency also appeared to increase production for all cytokines assayed, with the difference in IL-10 approaching significance ($F_{1,26} = 4.0$, $P = 0.055$, Fig. 2C). This increase was not due to an alteration in the number of cells plated or their survival in culture, as assessed by MTS assay (data not shown). Similarly there was no difference in percentage of activated (CD25+) T cells after stimulation (data not shown). Therefore the increase in cytokine production represents an increased output per cell rather than a simple change in cell integrity or number.

4. Discussion

The main finding of this study was that transient, gestational vitamin D_3 deficiency induced subtle, persistent changes in the immune system of the adult offspring. When examined at adulthood, DVD-deficient rats had mildly enlarged thymus and spleens. Enlargement of the spleen is associated with a range of disease states. For example, clinically defined splenomegaly has been associated with viral infections and chronic autoimmune diseases [17] as well as disease states in which the spleen must cope with an increased workload, abnormal blood flow, congestion or infiltration. However, the complete blood count performed in this study gave no indication that DVD-deficient rats suffered from any of these disorders. Only one previous study has examined the effect of vitamin D_3 deficiency on spleen weight [18]. Although spleen enlargement was qualitatively described in that study, these rats were also severely deficient in calcium and phosphorus. It is unlikely the enlarged spleens described in the current study are due to calcium and phosphorus deficiency as these minerals are normal in both the neonate [10] and adult DVD-deficient rat [11].

Thymic hyperplasia has also been observed in certain autoimmune and cortisol disorders. The use of thymic T cells in bioassays for 1,25 dihydroxyvitamin D_3 is strongly suggestive of a role for vitamin D_3 signalling in this organ [15]. Consistent with these data is the finding that exposure to high levels of vitamin D_3 leads to a 50% *decrease* in thymus weight, a selective change that was not evident in any other organ (although this effect may have been secondary to an alteration in extracellular calcium) [19]. At a molecular level, over-expression of insulin-like growth factor II (IGF-II) in transgenic mice is known to induce a selective increase in thymus weight [20]. Vitamin D_3 is known to inhibit this growth factor in a variety of cells [21]. Therefore, it is feasible that a failure to inhibit IGF-II induced thymus growth during development may represent a plausible mechanism for the thymic hyperplasia seen in the current study. However, vitamin D_3 also inhibits the actions of a range of other growth factors such as tumour and nerve growth factors, important for organogenesis throughout the body. Future studies need to explore if IGF-II or other immune organ-specific growth factors could mediate the increase in immune organ size identified in the adult DVD-deficient rats.

The current study has also assessed the responsiveness of a heterogenous population of PBMCs to a well known, cytokine-inducing stimulus [22]. Given there was no alteration in cell viability/metabolic activity or T cell activation status, it would appear that DVD deficiency has induced an increase in the amount of cytokine produced per cell rather than an increase in T cell survival, metabolism or activation.

The elevation in pro-inflammatory cytokine response in lymphocytes from rats developmentally deprived of vitamin D_3 is consistent with the vitamin's known role in the suppression of pro-inflammatory cytokines. Exogenous vitamin D_3 application inhibits IL-2 production and proliferation in T cells [23]. Importantly, exogenous IL-2 is able to reverse the anti-proliferative effect of vitamin D_3, suggesting that vitamin D_3 could inhibit proliferation via an IL-2 dependent pathway [24]. There is no known similar repressive mechanism for IL-10 production. However, it is likely that the increase in IL-10 described in cells from the DVD-deficient rat may be secondary to the general increase in Th1-associated pro-inflammatory cytokines. The fact that IL-10 production continued to rise for up to 96 h after PMA/ionomycin exposure, long after pro-inflammatory cytokine production had peaked (48 h), is consistent with this idea (data not shown). Finally, other investigators have

shown that when vitamin D_3 is added to mouse splenocyte cultures the production of IFN-γ, IL-2 and IL-10 is reduced [25]. These data mirror the *inverse* findings described in the current study in which vitamin D_3 was *removed* during development.

Much of the current work on vitamin D_3 status and immune function is centred on the ability of vitamin D_3 to attenuate symptoms within a variety of animal models of autoimmune disorders including: (a) experimental autoimmune encephalitis as a model of multiple sclerosis [26]; (b) IL-10 knock-out mice as a model of inflammatory bowel disease [27] and (c) the non-obese diabetic mouse as a model of Type 1 Diabetes [28]. Vitamin D_3 is being explored as a potential treatment for all of these disorders [27,29]. The addition of vitamin D_3 is known to directly and indirectly produce fewer and less active Th1 cells, which leads to a suppression of autoimmunity [30]. Our findings of increased production of the pro-inflammatory cytokine IL-2 in DVD-deficient rats represents the first direct experimental support for the idea that DVD deficiency may predispose an animal to enhanced autoimmunity later in life.

If these findings in the DVD-deficient rat model translate to humans, this study could have important clinical implications for the etiopathogenesis of autoimmune disorders. First, low prenatal vitamin D_3 has been proposed as a candidate risk factor for multiple sclerosis [31]. Second, a study based on a large Finnish birth cohort found that vitamin D_3 supplementation during the first year of life was associated with a significantly decreased risk of Type 1 Diabetes [8] and a significantly increased risk of atopy and allergic rhinitis at age 31 [32]. This finding is supported by a study using a genetically predisposed mouse model of Type 1 Diabetes, in which vitamin D_3 deficiency for the first 14 weeks of life led to an increase in the number of mice presenting with disease symptoms at 35 weeks of age [33]. Though preliminary, these studies demonstrate that the environmental and genetic risk factors for vitamin D_3 status and receptor expression have the potential to significantly affect adult immune function.

5. Conclusions

Examined collectively, the findings of the current study are broadly consistent with what is known about vitamin D_3's effects on the immune system. An increase in central immune organ size is suggestive of enhanced capacity for immune cell production and/or turnover. An enhanced capacity to produce pro-inflammatory cytokines suggests an immune system which is primed for a Th1 cell-mediated response to infection. Studies exploring the impact of DVD deficiency on *in vivo* cell-mediated immunity are now warranted. It would also be of interest to examine if the model of DVD deficiency affects experimental outcomes in animal models of multiple sclerosis and inflammatory bowel disease.

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