Proceedings of the Nutrition Society (2012), **71**, 62–66 doi:10.1017/S0029665111003193
 First published online 14 October 2011

A meeting of the Nutrition Society hosted by the Irish Section jointly with the American Society for Nutrition was held at University College Cork, Republic of Ireland on 15–17 June 2011

70th Anniversary Conference on 'Vitamins in early development and healthy aging: impact on infectious and chronic disease'

Symposium 3: Vitamin D and immune function: from pregnancy to adolescence

Vitamin D, invariant natural killer T-cells and experimental autoimmune disease

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Vitamin D is an important regulator of the immune system in general and multiple sclerosis in particular. Experimentally (i), invariant natural killer T (iNKT) cells have been shown to be important suppressors of autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE; an animal model of multiple sclerosis). Conversely, in experimental allergic asthma iNKT cells are required for disease induction and are therefore pathogenic. The active form of vitamin D (calcitriol) suppresses EAE. The development of EAE symptoms is accelerated in vitamin D deficiency. Interestingly experimental asthma is less severe in vitamin D deficiency although there is no effect of calcitriol on disease severity. The data suggest that an important target of vitamin D in EAE and asthma are the iNKT cells. Vitamin D and/or vitamin D receptor deficiency results in the impaired development of iNKT cells. Vitamin D is critical very early during development of the immune system. Low levels of vitamin D *in utero* resulted in significantly reduced numbers of iNKT cells that failed to recover when calcitriol was used to supplement neonatal or adult mice. The data suggest that one of the consequences of early vitamin D deficiency is a reduction in the numbers of iNKT cells that develop. The iNKT cells are required for the beneficial effects of calcitriol in EAE. The important role of vitamin D on iNKT cells could impact the development of human immune-mediated diseases including multiple sclerosis and asthma.

Vitamin D: Immune function: Multiple sclerosis: Asthma: NKT cell

Vitamin D is a fat-soluble vitamin that can be made in the skin following light exposure of the skin. 7-Dehydrocholesterol is converted to pre-vitamin D_3[1]. Vitamin D_3 that is either made in the skin or ingested from the diet is then hydroxylated to form the circulating form of vitamin D, calcidiol[1]. Calcidiol is also largely inactive although it can bind to the vitamin D receptor (VDR) but with a low affinity[1]. Calcidiol is converted in the kidney by the Cyp27B1 1 alpha-hydroxylase to the high-affinity VDR ligand, calcitriol[1]. Although the classic function of vitamin D is in the maintenance of calcium homoeostasis, the discovery of the VDR in cells of the immune system sparked research aimed at understanding why immune cells express the VDR.

Vitamin D and immune function

Early experiments added calcitriol to peripheral blood mononuclear cells and observed that T-cells in the cultures

Abbreviations: DP, double positive; EAE, experimental autoimmune encephalomyelitis; KO, knockout; MS, multiple sclerosis; iNKT, invariant natural killer T; Th, T helper; Treg, regulatory T; VDR, vitamin D receptor.

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had decreased proliferation and secreted less IL-2 and interferon-γ[2,3]. All T-cell subsets that have been examined express the VDR at a low level and following activation expression of the VDR is up-regulated[4]. Several direct and indirect targets of vitamin D have been identified. Cytokine secretion by Th (T helper) 1 and Th17 cell subsets is inhibited by calcitriol[5,6]. Calcitriol- or VDR-deficient T-cells are predisposed to produce IL-17 and interferon-γ[5,7]. Conversely, FoxP3 + regulatory T (Treg) cells are induced to develop *in vitro* and *in vivo* with calcitriol treatment[8,9]. The effects of calcitriol on Th2 cell development and function is less clear with investigators showing inhibition of IL-4 production and induction of IL-4 production using different models and systems[10–12].

VDR knockout (KO) mice have provided a valuable tool for studying the immune system. VDR KO mice have normal numbers of conventional T-cells[13]. There are more memory T-cells that are predisposed to develop into Th1 and Th17 cells in VDR KO *v.* wild-type mice[5]. VDR KO Th2 cells are able to develop normally *in vitro*[11,14]. Treg cells do not require VDR expression for either development or function[15]. Invariant natural killer T (iNKT) cells require expression of the VDR since they fail to develop in VDR KO mice[16]. In addition, the iNKT cells from VDR KO mice are functionally defective and secrete significantly less IL-4 and interferon-γ[16]. VDR KO mice have high Th1 and Th17 responses, no change in Th2 or Treg cells and very low iNKT cells.

Vitamin D and multiple sclerosis

MS (multiple sclerosis) is an autoimmune disease where T-cells target the central nervous system. The development of experimental autoimmune encephalomyelitis (EAE; an animal model of MS) results because of a Th17- and Th1-mediated immune attack on the central nervous system[17]. Other T-cell responses inhibit the development of Th17 and Th1 cells and are therefore important negative regulators of EAE. Negative regulators of EAE include iNKT cells and Treg cells[18]. Patients with MS have fewer iNKT cells and Treg cells and remission from symptoms is associated with the increased number and function of these cell types[19].

Epidemiological data suggest that there may be a link between vitamin D status and MS in human subjects[20]. Low level of circulating vitamin D was linked to increased disability scores in MS patients[21]. Both sun exposure and vitamin D supplements during childhood and adolescence were shown to correlate with MS incidence north of the Arctic Circle, and these factors were also linked to time of MS onset[22,23]. Participants in the nurse's health study who were in the highest quintile of vitamin D intakes had 40% less MS[24]. There is evidence for a role of vitamin D in the aetiology and severity of MS in human subjects.

Experimentally vitamin D deficiency accelerates the development of EAE[25]. In addition, calcitriol inhibits EAE and suppression is associated with a reduction in Th1, and Th17 cell responses[5,6]. Calcitriol treatment of mice resulted in the increased numbers of Treg cells isolated[26]. Recent data also show that calcitriol and vitamin D are positive regulators of iNKT cells[13,16]. Together the data suggest that improved vitamin D status would have a beneficial effect on multiple cell types important in the pathology of MS.

Vitamin D and asthma

Like MS, asthma is also an immune-mediated disease. Unlike MS, in asthma the pathogenic T-cells are of the Th2 cell and iNKT variety. IL-4, IL-5 and IL-13 are the disease-causing cytokines in asthma pathology[27]. iNKT cells have been shown to be involved in several different experimental models of asthma[28]. Allergic-induced airway hyperresponsiveness required IL-4 and IL-13 producing iNKT cells[29]. iNKT cell-deficient mice fail to develop experimental allergic asthma[29]. Conversely, Treg cells are important suppressors of asthma development and therapies that induce Treg cells are effective ways to suppress experimental asthma[30].

The role of vitamin D in asthma has been studied by several different groups. There are conflicting data about the role of vitamin D in Th2 and experimental asthma regulation. Calcitriol has been shown to both increase and inhibit IL-4 production from Th2 cells[10–12]. Various symptoms of experimental allergic asthma were increased, decreased or not changed with calcitriol treatment[31–33]. Our data suggest that calcitriol treatment had no effect on experimental asthma development[33]. VDR KO mice failed to develop experimental allergic asthma but the failure to develop asthma was not because of defective Th2 cells[14]. VDR KO Th2 cells were found to develop normally and to induce asthma when transferred to wild-type mice[14]. VDR KO mice have normal numbers of functional Treg cells[15]. VDR expression was shown to be critical in the lung epithelium[14]. In addition, iNKT cells require the VDR for both development and function. The failure of VDR KO mice to develop experimental asthma is a result of a complex set of factors that include defective iNKT cells and normal functional Treg cells[14,15]. In addition, there is an immune extrinsic requirement for the VDR in the lung epithelium[14]. The effect of calcitriol on Th2 cells and experimental asthma is harder to dissect but the data suggest that perhaps Th2 responses are less affected by changes in vitamin D than Th1 responses.

Vitamin D regulation of invariant natural killer T-cell function

iNKT cells have two distinct points at which vitamin D and the VDR are required. iNKT cells diverge from conventional T-cells at the CD4/CD8 double-positive (DP) stage (Fig. 1). The iNKT cell precursors rearrange their T-cell receptor and can be stained with CD1d tetramers (bound to ligands including α-galactoceramide). After expressing the invariant T-cell receptor the iNKT cell precursors mature by down-regulating CD24 to become $DP^{dim}/CD24^-$ and then as the iNKT cell precursor diverges from conventional T-cells it undergoes rapid proliferation (Fig. 1). Following proliferation the S0 iNKT cells undergo three additional modifications (S1: $CD44^-$; S2: $CD44^+$;

https://doi.org/10.1017/S0029665111003193 Published online by Cambridge University Press

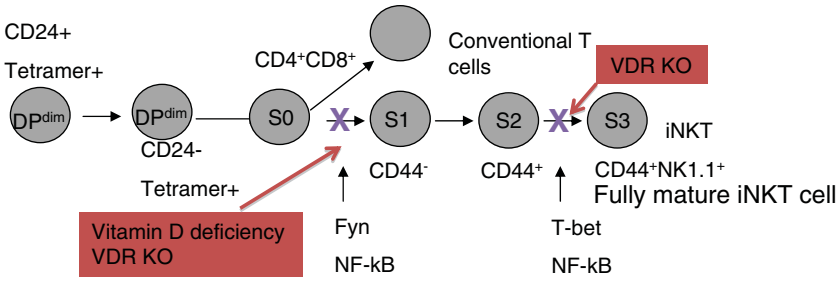

Fig. 1. (Colour online) Vitamin D and vitamin D receptor (VDR) targets in invariant natural killer T (iNKT) cell development[13,16]. iNKT cells develop in the thymus following several different phenotypic changes. The earliest iNKT cell precursor, DP^{dim} expresses the invariant T-cell receptor (tetramer$^+$) and CD24$^+$. The early iNKT cells down-regulate CD24 and diverge from the other CD4/CD8 DP cells that go on to become conventional T-cells. Expression of two transcription factors (Fyn and NF-κB) is important in the movement of iNKT cells from stage (S) 0 to S1. Vitamin D and VDR deficiency affect the number of iNKT cells that rapidly expand and enter the S1 stage in maturation. There is no effect of vitamin D deficiency on the further maturation of iNKT cells. VDR knockout (KO) iNKT cells have an additional block in maturation at the S2 stage and fail to fully develop into mature iNKT cells. T-bet and NF-κB expression is associated with the transition of iNKT cells from S2 to S3. VDR KO iNKT cells express significantly less T-bet than there fully mature S3 wild-type counterparts[16].

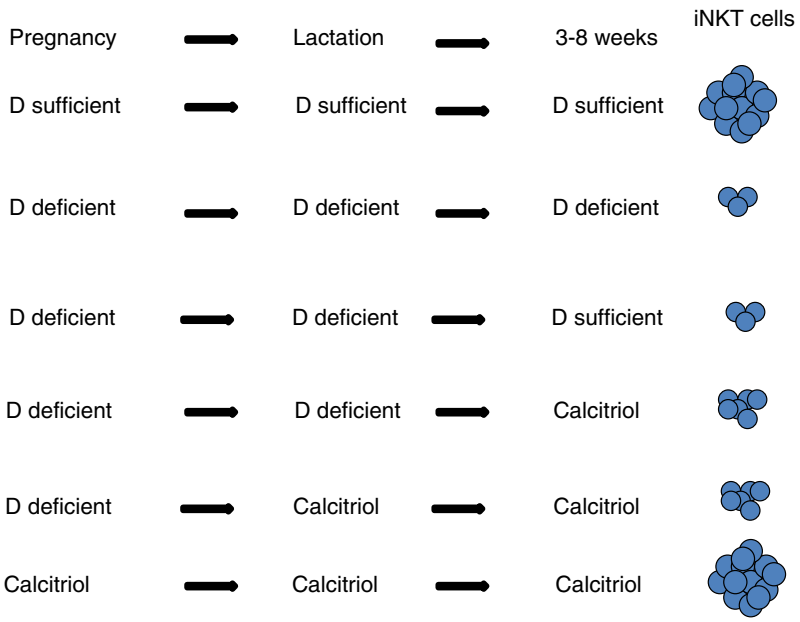

Fig. 2. (Colour online) Gestational effects of vitamin D deficiency on invariant natural killer T (iNKT) cells[13]. Vitamin D-sufficient, vitamin D-deficient or calcitriol-supplemented diets were fed to mice during three different windows of time: pregnancy, lactation (0–3 weeks), following weaning (3–8 weeks of age). The vitamin D-sufficient or calcitriol-treated throughout mice had the highest numbers of iNKT cells. Vitamin D-deficient throughout or switching to D sufficient diets from 3 to 8 weeks had the fewest iNKT cells. Supplementing D-deficient mice with calcitriol from 3 to 8 weeks or during lactation until 8 weeks increased iNKT cell numbers somewhat but not to the level found in the vitamin D-sufficient mice.

S3: CD44$^+$NK1.1$^+$) that result in mature iNKT cells that exit the thymus (Fig. 1).

VDR KO mice have fewer iNKT cells. The iNKT cells that remain in VDR KO mice are blocked at the stage just before they fully develop and exit the thymus (Fig. 1)[34]. Most of the iNKT cells in the VDR KO mouse are blocked at S2 and the immature iNKT cells produce less cytokines than their wild-type counterparts[16]. The iNKT cells in

https://doi.org/10.1017/S0029665111003193 Published online by Cambridge University Press

vitamin D-deficient mice are fewer than those from vitamin D-sufficient mice[13]. Unlike the result from the VDR KO mice, vitamin D-deficient iNKT cells are functionally normal and the frequency of iNKT cells in S2 and S3 stages of maturation are similar to the frequencies in vitamin D-sufficient mice[13]. The expansion defect in vitamin D-deficient and VDR KO mice was a result of the increased apoptosis of early DP^{dim} $CD24^{+}$ iNKT cells (Fig. 1)[13]. In the absence of vitamin D and the VDR fewer iNKT cells are produced (Fig. 1)[16]. In addition, the VDR is required for the full maturation of the iNKT cells (Fig. 1)[16]. There is one pathway in iNKT cell development that is regulated by both vitamin D and the VDR; which is the expansion and proliferation of early iNKT cell precursors. In addition, expression of the VDR also affects the last stage in iNKT cell maturation.

Vitamin D status is affected by season. Furthermore, Tsang *et al*. showed that children born in the summer started out with high levels of calcidiol that went down to low levels 6 months later in winter[35,36]. Conversely, children born in winter started out with low levels of calcidiol that increased 6 months later in summer[35,36]. We used mice to model these changes in calcidiol levels and looked at the effect of changing levels of vitamin D on iNKT cell numbers. The offspring from vitamin D-deficient breeders was maintained vitamin D-deficient throughout life and at 8 weeks the mice had very few iNKT cells compared with vitamin D-sufficient mice (Fig. 2)[13]. A series of experiments were carried out to supplement vitamin D or calcitriol between the age of 3 and 8 weeks. Vitamin D had no effect on iNKT cell numbers when given from age 3 to 8 weeks (Fig. 2)[13]. Conversely, calcitriol increased the numbers of iNKT cells but not to the level found in vitamin D-sufficient mice (Fig. 2)[13]. Earlier treatment with calcitriol given at birth and through 8 weeks of age also failed to recover iNKT cell numbers to those in vitamin D-sufficient mice (Fig. 2)[13]. Treating breeders and offspring with calcitriol throughout gestation resulted in the same numbers of iNKT cells as vitamin D-sufficient mice (Fig. 2)[13]. Vitamin D is required early *in utero* for normal iNKT cell numbers to develop in mice. There is a gestational effect of vitamin D on early iNKT cell precursors that cannot be recovered later with vitamin D or calcitriol treatment. Early changes in vitamin D status can affect immune function.

Conclusions

Experimental models of Th1- and Th17-mediated autoimmune diseases like MS are affected by changes in vitamin D status. iNKT cells in mice absolutely require vitamin D for both function and development. There are two different targets for vitamin D and the VDR in the development of iNKT cells. These iNKT cells are early producers of cytokine that have been shown to inhibit several models of experimental autoimmunity and to be important in the development of inflammation in the lung. The requirement of murine iNKT cells for vitamin D early during gestation might help to explain why vitamin D status is linked to MS in human subjects. The effects of vitamin D in the immune system depend on the tissue being targeted as well as the protective and pathologic mechanisms involved in the disease.

Acknowledgements

We thank the members of the Center for Molecular Immunology and Infectious Diseases for lively discussion. This work was supported by the National Institute of Neurological Disorders and Stroke NS067563 and the National Center for Complementary and Alternative Medicine and the Office of Dietary Supplements AT005378. M. T. C., J. Z. and L. Y. were equal contributors to the manuscript, collected the literature, wrote the literature and made the figures. The authors declare no financial or commercial conflict of interest.

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