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Session 2: Micronutrients and the immune system Mechanisms underlying the effect of vitamin D on the immune system

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Vitamin D and the vitamin D receptor (VDR) have been shown to be important regulators of the immune system. In particular, vitamin D and VDR deficiency exacerbates experimental autoimmune diseases such as inflammatory bowel disease (IBD). IBD develops due to an immune-mediated attack by pathogenic T-cells that overproduce IL-17 and IFN- γ and a few regulatory cells. VDR knockout mice have twice as many T-cells making IL-17 and IFN- γ than wild-type mice. In addition, vitamin D and the VDR are required for normal numbers of regulatory T-cells (iNKT and CD8 $\alpha\alpha$) that have been shown to suppress experimental IBD. In the absence of vitamin D and the VDR, autoimmunity occurs in the gastrointestinal tract due to increased numbers of IL-17 and IFN- γ secreting T-cells and a concomitant reduction in regulatory T-cells.

Vitamin D: Regulatory T cells: Inflammatory bowel disease: Multiple sclerosis

The incidence of immune-mediated diseases such as multiple sclerosis and inflammatory bowel disease (IBD) has increased in developed countries over the last 50 years. To explain the increased incidence of immune-mediated diseases as well as the geographical restriction of these diseases to the developed world, the hygiene hypothesis has been put forward. The hygiene hypothesis states that reduced exposure to microbial components results in immune dysregulation and T-cell responses that drive immune-mediated disease. We propose that other factors in the environment in addition to the hygiene hypothesis are important in the development of the immune response leading to multiple sclerosis and IBD. We propose that decreased outdoor activity, increased pollution and diets that lack adequate vitamin D have combined to create large fluctuations in vitamin D status in developed countries and especially in populations that experience winter^(1,2). The vitamin D hypothesis proposes that vitamin D regulates the development and function of the immune system and that early childhood and prenatal changes in vitamin D status affect the resultant immune response and the development of autoimmune diseases^(3,4). Here we will describe the

current understanding of the mechanisms by which vitamin D regulates experimental autoimmunity.

Vitamin D

A major source of vitamin D results from its manufacture via a photolysis reaction in the skin, and vitamin D available from sunlight exposure is significantly less in northern climates and especially low during the winter^(5,6). In addition, dietary intake of vitamin D is problematic since there are few foods that are naturally rich in vitamin D. There is mounting evidence for a link between vitamin D availability either from sunshine or from diet and the prevalence of autoimmune diseases⁽⁷⁾. The use of supplemental vitamin D (500–600 IU) is associated with a 40% reduction in the risk of developing multiple sclerosis⁽⁸⁾. In addition, vitamin D deficiency is common in patients with autoimmune diseases⁽³⁾. The amount of vitamin D in the environment (food, sun or supplements) might influence the development and function of specific arms of the immune system. With our present lifestyles that feature

decreased activity outside and diets naturally low in vitamin D, it seems that the amount of vitamin D people are exposed to from both diet and sunshine has become more variable^(1,2). We propose that supplementing people with high but safe⁽⁹⁻¹¹⁾ doses of vitamin D (800 IU/d) would decrease the incidence of autoimmunity by increasing the availability of a substrate to make active vitamin D (1,25(OH)₂D₃).

Vitamin D and immune regulation

The function of vitamin D is regulation of Ca homeostasis and thus bone formation and resorption. The discovery of the vitamin D receptor (VDR) in cells of the immune system and the presence of the 1 α -25(OH) vitamin D₃ hydroxylase in dendritic cells and macrophages suggest that locally produced 1,25(OH)₂D₃ has regulatory autocrine and paracrine properties at the site of inflammation⁽¹²⁾. Synthesis of active vitamin D requires the 1 α hydroxylase, which catalyses the conversion of 25(OH)D₃ to 1,25(OH)₂D₃. The actions of 1,25(OH)₂D₃ are mediated by its binding to the VDR, which acts as a transcription factor to modulate the expression of genes in a tissue-specific manner. The VDR is a member of the steroid/hormone superfamily of nuclear transcription factors and is constitutively expressed in a variety of immune cells⁽¹³⁾. Resting T-cells express low levels of VDR, which are upregulated following activation⁽¹⁴⁾.

The active form of vitamin D (1,25(OH)₂D₃) has been recognized as an immunosuppressive agent that ameliorates the pathogenesis of T helper 1-mediated autoimmune diseases including IBD and experimental autoimmune encephalomyelitis (EAE; a murine model of multiple sclerosis)^(7,15). Furthermore, vitamin D deficiency and VDR deficiency have been shown to exacerbate experimental IBD in three different mouse models⁽¹⁶⁻¹⁸⁾. The increase in T regulatory cells caused by 1,25(OH)₂D₃ both *in vitro* and *in vivo* has been suggested as a mechanism underlying the ability of 1,25(OH)₂D₃ to suppress autoimmunity^(19,20). In addition, genome-wide screening techniques suggest that VDR polymorphisms are associated with increased susceptibility to both Crohn's disease⁽²¹⁾ and ulcerative colitis⁽²²⁾ in human subjects.

Vitamin D and experimental inflammatory bowel disease

Mice lacking the VDR do not develop overt symptoms or present histological evidence of IBD even when they are housed in conventional facilities. However, increased expression of IL-1 β and TNF- α in the colon of young (5 week old) and old (9 month old) VDR knockout (KO) mice when compared to age-matched wild-type (WT) mice suggests that VDR deficiency results in chronic and low-grade inflammation in the gastrointestinal tract⁽¹⁷⁾. T-cells from VDR KO mice have been shown to exhibit a more pathogenic phenotype than WT T-cells. Specifically, VDR KO T-cells express an inflammatory phenotype, proliferate twice as much in a mixed lymphocyte reaction and transfer a more severe form of IBD than their WT counterparts⁽¹⁷⁾.

In part the increased pathogenicity of the VDR KO T-cells was shown to be a result of increased IFN- γ ⁽¹⁷⁾ and IL-17 (unpublished IL-17 results). VDR KO mice have heightened immune responses and inflammation in the colon, which suggest that the absence of the VDR predisposes to the development of IBD.

FoxP3+ T regulatory cells

CD4+ T-cells from VDR KO mice failed to suppress IBD, whereas WT CD4+ T-cells were effective in suppressing the same model of IBD⁽²³⁾. T regulatory cells that express the transcription factor FoxP3+ and produce IL-10 are important for the maintenance of self-tolerance and the suppression of IBD. It has been shown that a combination of 1,25(OH)₂D₃ and dexamethasone induces IL-10-producing T regulatory cells *in vitro*⁽¹⁹⁾. Furthermore, *in vivo* 1,25(OH)₂D₃ treatment of experimental autoimmune diabetes induces a population of T regulatory cells that correlates with protection of the mice from diabetes⁽²⁰⁾. The percentages of FoxP3+ T regulatory cells in the VDR KO and WT mice were determined. The numbers of T regulatory cells in the spleen, thymus or intraepithelial lymphocytes (IEL) of VDR KO and WT mice were not different. T regulatory cells were tested *in vitro* for functional suppression of naïve T-cell proliferation to CD3 antibodies. T regulatory cells from VDR KO mice were as effective as WT T regulatory cells in suppressing proliferation of both WT and VDR KO T-cells⁽²³⁾. T regulatory cells were sorted from VDR KO and WT mice and tested *in vivo* for suppression of IBD induced by WT naïve T-cell transfers to T- and B-cell-deficient Rag KO mice. Either WT or VDR KO T regulatory cells suppressed IBD development when they were transferred at the same time as the naïve T-cells⁽²³⁾. The T regulatory cells from VDR KO mice function to suppress proliferation *in vitro* and IBD *in vivo*. Therefore, it seems that expression of the VDR is not required for the development or function of T regulatory cells.

Invariant NKT-cells

NKT-cells are thought to be amongst the earliest producers of cytokines in an immune response and have been shown to influence a wide variety of different diseases⁽²⁴⁾. NKT-cells are narrowly defined as T-cells that express NK lineage receptors and an $\alpha\beta$ T-cell receptor (TCR). The majority of NKT-cells express an invariant TCR (iNKT) and are responsive to a marine sponge sphingolipid, α -galactoceramide (α GalCer). NKT-cells play an important regulatory role in several models of autoimmunity, infection, cancer and atherosclerosis^(25,26). Because NKT cell activation induces an early production of IL-4, NKT cell activation has been shown to delay the onset and reduce the symptoms of EAE and experimentally induced colitis⁽²⁷⁻²⁹⁾. In addition, transgenic mice that overexpress NKT-cells are protected from the development of EAE⁽³⁰⁾.

To investigate whether vitamin D affects *in vivo* NKT cell function, VDR KO, WT and 1,25(OH)₂D₃-fed WT mice were injected with α GalCer. Feeding WT mice

1,25(OH)₂D₃ for 1 week prior to αGalCer injection increased IFN-γ and IL-4 production in the serum. VDR KO mice injected with αGalCer produced significantly less IFN-γ and IL-4 in the serum than both the WT and 1,25(OH)₂D₃-fed WT mice⁽³¹⁾. The numbers of NKT-cells in the thymus, spleen and liver of WT and VDR KO mice were determined using CD1d-αGalCer tetramer staining. The percentages of iNKT-cells were significantly lower in VDR KO mice thymus, liver and spleen compared to WT mice⁽³¹⁾.

iNKT-cells develop predominately in the thymus, with the final step in maturation (conversion to NK1.1 expression) occurring in both the thymus and peripheral lymphoid tissues. The majority of VDR KO iNKT-cells failed to express NK1.1 and therefore were not fully matured⁽³¹⁾. To determine whether the residual VDR KO iNKT-cells are functionally different from WT iNKT-cells, cytokine production from iNKT-cells was assessed at the single-cell level by intracellular staining. Less than 3% of the iNKT-cells from the spleen of VDR KO mice made IL-4 and 25% made IFN-γ⁽³¹⁾. Conversely, 15% of spleen-derived WT iNKT-cells produced IL-4 and 46% produced IFN-γ⁽³¹⁾. VDR KO mice have fewer, less mature iNKT-cells and the residual iNKT-cells from VDR KO mice are defective for production of both IL-4 and IFN-γ.

CD8α/T-cell receptor αβ intraepithelial lymphocytes

The gut contains a large number of T-cells and, unlike cells in the periphery, many of those T-cells express CD8α either alone or in combination with CD4 or CD8αβ⁽³²⁾. Expression of CD8α on T-cells decreases the sensitivity of those cells to antigen^(32,33). The presence of CD8α T-cells is thought to help maintain tolerance to the bacterial and food antigens that abound in the gastrointestinal tract^(32,33). IEL were isolated from the small intestine of VDR KO and WT mice and stained for the presence of CD8α. We found that the total number of IEL isolated, the percentage of CD4 and the percentage of CD8αβ T-cells were not different between the VDR KO and WT IEL⁽²³⁾. However, the percentage of VDR KO CD8α and TCRβ/CD8α IEL was about half that in the WT IEL⁽²³⁾. More importantly, the TCRβ/CD4/CD8α-positive population is missing in the VDR KO IEL⁽²³⁾. The percentage of TCRγδ/CD8α or NK1.1/CD8α was not different between WT and VDR KO IEL.

Intracellular staining for IL-10 in WT IEL showed that 13.6% of the cells produced IL-10, while only 0.8% of the VDR KO IEL produced IL-10⁽²³⁾. The majority of the IL-10 produced was from the CD8α IEL in both the WT and VDR KO mice⁽²³⁾. Therefore, the amount of IL-10 secretion in the IEL corresponds to the numbers of CD8α cells that are present. The IEL and CD8α expressing IEL from VDR KO mice fail to produce IL-10 that is known to suppress IBD *in vivo*.

The ability of VDR KO T-cells to home to the intestinal epithelium was tested *in vivo*. Rag KO mice were injected with a 1:1 mixture of CD45.1 WT and CD45.2 VDR KO cells from either the mesenteric lymph node or IEL. Staining for TCRβ and CD45.1 was used to identify T-cells

from VDR KO mice (TCRβ+ and CD45.1-) or WT (TCRβ+ and CD45.1+) in the Rag KO recipients. Reconstitution of the spleens of Rag KO mice was 53% VDR KO cells and 46% WT T-cells, and the values were not significantly different⁽²³⁾. Conversely, reconstitution of the IEL of the Rag KO mice resulted in only 14% VDR KO compared to 85% WT T-cells⁽²³⁾. We conclude that VDR KO T-cells repopulate the spleen but fail to home to the intestine.

CD8α T-cells are regulatory cells that suppress IBD symptoms in the gut^(32,33). Expression of the VDR is important for CD8α expression, local production of IL-10 and homing of the T-cells to the gut⁽²³⁾.

Conclusions

Increased autoimmunity in VDR KO mice is a result of the increased pathogenicity of naive T-cells and deficiency in two regulatory T-cell populations^(23,31). The CD8α T-cells are specific for the gut and maintain gastrointestinal homeostasis by inhibiting immune responses to gut antigens. The iNKT-cells are early cytokine producers that link the innate and adaptive immune responses. iNKT-cells have been shown to play a regulatory role in experimental autoimmunity including EAE and IBD. Conversely, the FoxP3+ T regulatory cell does not require expression of the VDR for developing normally. Protective T-cells require expression of the VDR and vitamin D for developing and functioning normally. The effects of vitamin D on the immune system will then be most severe for diseases like EAE and IBD that are regulated by iNKT-cells and CD8α T-cells.

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