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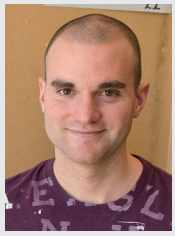

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"…data suggest that at least a subset of the genetic predisposition to Crohn's disease results from defects in the detection and/or processing of intracellular pathogens by the innate immune system."

Vitamin D has attracted increasing interest in recent years in both the scientific literature and the popular press. While discovered as the cure for nutritional rickets and studied mostly for its role in calcium homeostasis, work over the last 20–25 years has revealed that vitamin D has a broad range of activities, including a role as a cancer chemopreventive agent [1] and as an important regulator of immune system function [2]. Vitamin D is obtained from limited dietary sources [3] as well as from the photochemical conversion of 7-dehydroxycholesterol in the skin in the presence of sufficient solar ultraviolet B (UVB) radiation. With increasing latitude, surface solar UVB irradiation is insufficient to induce cutaneous vitamin D_3 synthesis for periods around the winter solstice of 6 months or even more at the latitudes of northern Europe or Scandinavia, a period that is known as 'vitamin D winter'. UVB-induced vitamin D_3 synthesis is also strongly influenced by skin colour [3]. Lack of cutaneous vitamin D synthesis owing to of lack of sun or sun avoidance, coupled with vitamin D-poor diets, have contributed to widespread vitamin D insufficiency or deficiency [4].

The term vitamin D refers collectively to vitamin D_2 (ergocalciferol), which is fungal in origin, and vitamin D_3 (cholecalciferol), which is obtained from animal sources or irradiation of skin. Vitamin D compounds are constitutively hydroxylated largely in the liver to form 25-hydroxyvitamin D (25D), the major circulating form and marker of vitamin D status. The hormonal form of vitamin D, 1,25-dihydroxyvitamin D (1,25D), is generated by 1α-hydroxylation of 25D by the enzyme CYP27B1. Signaling by 1,25D occurs through binding to the vitamin D receptor (VDR), a member of the nuclear receptor family of ligand-regulated transcription factors.

Vitamin D signaling & innate immunity

Recent research has begun to unravel important roles of vitamin D in the regulation of innate immunity [2]. Unlike the adaptive immune system, the innate immune system is responsible for nonspecific defense against pathogens. Stimulation of this system triggers the release of cytokines and chemokines, which are important for the culmination of the innate immune response, as well as recruiting and activating cells of the adaptive immune system to the site of infection. In response to bacterial pathogens, the innate immune response includes the production and release of antimicrobial peptides (AMPs) [5]. In data first published by our group, treatment of several cell lines or primary cell cultures with 1,25D induced the expression of two AMPs, human β-defensin 2 (DEFB2/DEFB4/HBD2) and cathelicidin AMP (CAMP) [6]. Moreover, conditioned media from 1,25D-treated cells acquired the capacity to kill bacteria, including the pathogen *Pseudomonas aeruginosa*. While the effect of 1,25D alone on DEFB2/HBD2 was only modest, recent work detailed later has shown that 1,25D in combination with other signaling pathways leads to robust stimulation of DEF2/HBD2 expression. By contrast, induction of CAMP was

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particularly strong and subsequent follow-up studies have shown that CAMP expression is widely regulated by 1,25D both *in vitro* and *in vivo* [7–10].

> "Recently our group found that transcription of the *NOD2* gene was stimulated directly by the 1,25D-bound vitamin D receptor."

The innate immune system uses pattern recognition receptors to detect the presence of conserved molecular motifs characteristic of certain families of pathogens. Toll-like receptors (TLRs) are transmembrane proteins that induce an innate immune response after detecting components of extracellular microbes, such as bacterial lipopolysaccharide by TLR4, or lipoprotein in the case of TLR2. Importantly, TLR2/1 stimulation by *Mycobacterium tuberculosis* lipoprotein induces the expression of both the VDR and CYP27B1; stimulated macrophages thus acquire the capacity to respond to circulating levels of 25D, underlying the central role of vitamin D signaling in human innate immune responses [7]. The 1,25D produced stimulates expression of CAMP, which colocalizes with mycobacteria in infected macrophages [7].

Crohn's disease, NOD2 & autophagy

Crohn's disease (CD) is a chronic inflammatory bowel disease. While widely thought of as an autoimmune disease, autoantibodies have yet to be detected, and its symptoms are more likely the result of a dysregulation of intestinal innate immunity [11]. Its etiology is poorly defined, but recently a rich list of genetic susceptibility markers has been identified, and it is likely that a combination of these and environmental cues or infections trigger the full manifestation of CD. Variants in the *NOD2/CARD15/IBD1* locus are associated with the strongest risk of development of CD [12]. *NOD2* encodes a protein that is a member of a family of intracellular pattern recognition receptors [13]. NOD2 recognizes modified forms of muramyl didpeptide (MDP), a lysosomal breakdown product of bacterial peptidoglycan. Recent work by one of our team has shown that NOD2 is particularly sensitive to the *N*-glycolyl form of MDP produced by mycobacteria [14]. Remarkably, major *NOD2* variants associated with CD contain frameshift or point mutations that encode proteins incapable of recognizing MDP, and are thus inactive signaling molecules. *ATG16L1* and *IRGM*, both of which encode proteins required for autophagy, are also CD susceptibility loci [15–17]. Autophagy is a process that employs autophagosomes to target other damaged organelles, proteins and a number of intracellular pathogens for degradation in lysosomes.

Recent studies have linked NOD2 function to autophagy [18,19]. Activated NOD2 recruits ATG16L1 to the cell membrane at the site of bacterial entry [18]. ATG16L1 is an essential component of protein complexes that control autophagy [20]. Stimulation of NOD2 induced autophagy and clearance of pathogen. However, the introduction of mutations common to CD for either *NOD2* or *ATG16L1* abrogated this effect, strongly suggesting a functional role for these mutations in the pathogenesis of CD [18]. Taken together, these data suggest that at least a subset of the genetic predisposition to CD results from defects in the detection and/or processing of intracellular pathogens by the innate immune system.

Vitamin D, NOD2, CD & autophagy

Recently our group found that transcription of the *NOD2* gene was stimulated directly by the 1,25D-bound VDR. Regulation of NOD2 expression by 1,25D is noteworthy for several reasons. Signaling through NOD2 induces the function of the NF-κB transcription factor, which in turn induces expression of *DEFB2/HBD2* [21]. We found that pretreatment with 1,25D to induce NOD2, followed by incubation with MDP, led to stimulation of NF-κB function and synergistic induction of *DEFB2/HBD2* expression [21]. Significantly, this synergism, along with induction of NF-κB function, was absent in macrophages from patients homozygous for inactivating *NOD2* mutations. Taken together with previous work, these data demonstrated that 1,25D is both a direct and indirect inducer of the NOD2–DEFB2/HBD2 innate immune pathway.

These findings have several implications for CD. Notably, the region encoding DEFB2/HBD2 has been identified as a CD susceptibility locus. The gene exists in multiple copies in a cluster on chromosome 8, and the specific number of copies shows high variation in healthy patients, with the median number of copies being four. Analysis of copy number in CD patients showed that cases of colonic CD, but not ileal CD, were associated with a decrease in copy number, with less than three copies significantly increasing the risk of developing colonic CD [22].

> "The observation that *NOD2* is a 1,25D target gene also links vitamin D signaling to autophagy. Stimulation of NOD2 expression by 1,25D implies that it would boost autophagy at least in part by enhancing NOD2 function."

Several defensins are important for maintaining an antimicrobial barrier to intestinal mucosa, which is important in the control of bacterial flora and defense against the adherence of intracellular pathogens. Ten defensins have been characterized in humans: six α-defensins and four β-defensins. *NOD2* mutations have been largely linked to ileal CD and have been associated with a reduced level of expression of α-defensins HD-5 and HD-6 in isolates of ileal Paneth cells [23]. The increase in NOD2 expression in response to 1,25D as seen in human intestinal epithelial cells (immortalized cells isolated from intestinal epithelial crypts) could have implications in the restoration of the AMP mucosal barrier in CD patients that are heterozygous for the *NOD2* mutation, by elevating their levels of sensitivity to MDP [21], although this has yet to be investigated.

The observation that *NOD2* is a 1,25D target gene also links vitamin D signaling to autophagy. Stimulation of NOD2 expression by 1,25D implies that it would boost autophagy at least in part by enhancing NOD2 function. In addition, recent work has shown that 1,25D-stimulated CAMP production enhanced autophagy in mycobacteria-infected macrophages [24]. Previous studies revealed that CAMP expressed in 1,25D-treated cells colocalized

with mycobacteria in phagolysosomal structures [4]. Ablation of CAMP expression decreased the number of autophagosomes in 1,25D-treated cells [24]. While this study is intriguing, it is not clear whether CAMP functioned to enhance autophagy directly or indirectly by reducing bacterial viability due to its AMP activity.

The effect of 1,25D alone on CAMP expression is strong [6–9]. Thus, the effects of 1,25D-induced CAMP on autophagy may be at least partially independent of NOD2 function. This raises the possibility that enhanced CAMP expression may be sufficient to induce clearance of intracellular pathogens despite mutations in the NOD2 pathway common in CD. Notably, in this regard, adherent-invasive *Escherichia coli* has been detected in ileal lesions in CD. Normal autophagy is sufficient in the clearance of this pathogen, but in the absence of functional ATG16L1 and IRGM, adherent-invasive *Escherichia coli* is able to proliferate [25]. In addition, there has been a positive correlation between the detection of *Mycobacterium avium* substrain *paratuberculosis* in biopsies from CD patients as compared with control samples taken from patients with ulcerative colitis [26]. While neither has yet to be directly implicated in the etiology of CD, their persistent presence might explain some of the inflammatory complications that manifest in CD.

Vitamin D & CD in the clinic

Bowel resections are a common practice in the treatment of CD and, coupled with malabsorption, pose a limitation on dietary vitamin D uptake, which occurs largely in the duodenum and jejunum. With vitamin D deficiency widespread in the normal population (defined as serum levels of 25D below 50 nM), achieving sufficiency (serum concentration higher than 75 nM [4]) poses a significant hurdle for vitamin D therapy in CD. The importance of this is emphasized by the identification of vitamin D deficiency as a high-risk factor for the development of inflammatory bowel disease [27]. Curiously, a patient case report of a 57-year-old woman with a long history of CD, having undergone three bowel resections, was suffering from bone and muscle pain. She was administered 600 IU of vitamin D daily for 36 months and showed no change in condition, and had serum levels of 25D that fell into the range of insufficiency. Following this, she was exposed to UVB in a tanning bed for 10 min three times a week. After 4 weeks, her serum 25D and calcium levels were elevated into the normal range, and after 6 months there was relief of bone and muscle pain [28]. While this sort of anecdotal evidence falls short of scientific rigor, and says nothing as to the mechanism of action of vitamin D in the alleviation of symptoms (whether it was responsible for the induction of an immune response and downstream clearance of an infection that was central to the manifestation of the disease, or if the pain was simply due to the decalcification of tissue that was rescued after serum levels were restored), it reveals a potential avenue for vitamin D therapy for CD under conditions where typical dietary supplementation has failed. Alternatively, it may be possible to develop synthetic VDR agonists that are more efficiently absorbed than natural vitamin D compounds [29].

Conclusion & future directions

The convergence of vitamin D-mediated modulation of innate immunity and CD raises a number of questions that merit further investigation. What are the organisms whose intracellular fate depends most heavily on this NOD2-autophagy pathway, and is a specific pathogen responsible for disease or rather is a dysregulated immune response generated against a complex microbial population? What are the consequences of these infections for the host, and how exactly would they result in a state of chronic inflammation that is predominantly localized to the GI tract? Can these findings in any way explain the therapeutic benefit of TNF-α inhibitors and other immunomodulators used to treat this disease and, more generally, why would immune-suppressive drugs be efficacious if the primary defect is an immune deficiency? Finally, most specific to the vitamin D aspect, do these findings suggest that those who are in most need of vitamin D-mediated immunity are those in whom the pathway is defective, or rather, can one envision a means of supplementing and circumventing this pathway to the benefit of the patient?

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