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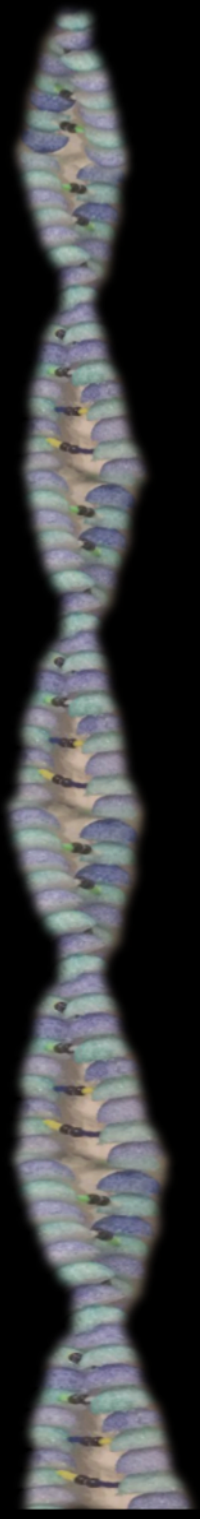

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Excerpts from Expert

A Novel Mechanistic Insight into the Role of Vitamin D Deficiency in Cardiovascular Diseases

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Cardiovascular diseases (CVDs) are the major cause of mortality and morbidity in the USA as well as globally. According to the World Health Organization (WHO), CVDs take the lives of 17.7 million people every year, 31% of all global deaths (1). The cardiovascular diseases consist of hypertension, ischemic heart diseases, strokes, peripheral vascular disease, heart failure, rheumatic heart disease, congenital heart disease, and cardiomyopathies. Extensive basic and clinical research in this field have dramatically advanced our understanding of these diseases and helped develop several novel and potent therapies. Several pioneer and state-of-the-art genetic studies such as Framingham Heart Studies have provided crucial cues for the involvement of not only the genetic component but also lifestyle in the pathogenesis of CVDs. Even with these positive developments, CVDs remain highly prevalent globally and are still the number one killer of mankind (2). This suggests that our understanding of these diseases is incomplete. Recent epidemiological and retrospective studies highlighted that Vitamin D (Vit-D) deficiency, which is widely prevalent in the western world, is strikingly associated with CVDs, suggesting a potential role of Vit-D in the development of CVDs (2,3,4,5,6).

Vitamin D consists of two forms, Ergocalciferol (Vitamin D2), and cholecalciferol (Vitamin D3). Vitamin D2 is obtained through our diet via certain vegetables or through oral over the counter (OTC) supplements. Vitamin D3 is synthesized in the skin mainly from exposure to ultraviolet B rays from the sun (3). There are some food sources such as oily fish and variably fortified foods (milk, juices, yogurts, cereals, and soy), as well as some OTC supplements from where Vitamin D3 can be obtained (7). Vitamin D3 is metabolized in liver into 25-hydroxyvitamin D (cholecalciferol), the main circulating form of Vitamin D. In the kidney 25-hydroxyvitamin D undergoes hydroxylation further to form 1, 25-dihydroxyvitamin D, the active form of Vit-D. The average concentration of Vit-D in the human body is estimated by measuring the concentration of the 25-hydroxyvitamin D in the circulation. The non-toxic yet sufficient concentration of Vit-D in the human body is 80 nmol/L. Vitamin D toxicity is reported to occur at 500 nmol/L or higher (8). The average daily intake to achieve sufficient levels of Vit-D is 1000-2000 IU/day of supplemental cholecalciferol.

The active form of Vit-D (1,25-dihydroxycholecalciferol) binds to intracellular receptors, Vit-D receptors (VDR) which forms a complex with its co-activator retinoid receptor (RXR). The VDR-RXR complex then migrates into the nucleus and functions as a transcription factor to modify the transcription of several target genes (9). The

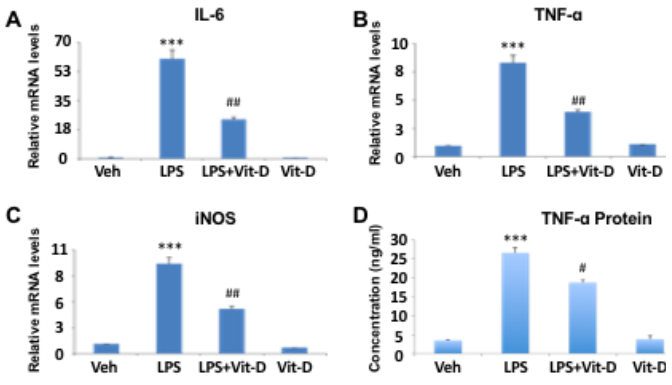

Fig. 1. LPS treatment increased mRNA levels of pro-inflammatory genes *IL-6* (A; 60 ± 6-fold), *TNF-α* (B; 8 ± 0.7-fold), and *iNOS* (C; 10 ± 0.7-fold) in RAW cells. Pretreatment with 10 nM Vit-D but not 0.1 or 1 nM significantly inhibited LPS-induced increases in expression levels of *IL-6* (24±2-fold), *TNF-α* (4 ± 0.3-fold), and *iNOS* (4.7 ± 0.3-fold versus LPS alone). Vit-D also suppressed LPS-induced TNF-α protein production by macrophages (D; 18 ± 0.8 versus 27 ± 1.4 ng/ml). *** p<0.001 vs. vehicle, # and ## p<0.05 and p<0.01 vs. LPS alone.

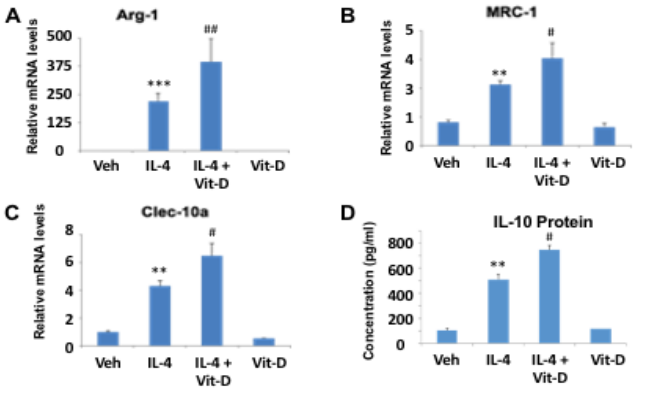

Fig. 2. IL-4 treatment induced expression levels of anti-inflammatory genes *Arg-1* (A; 93 ± 18-fold), *MRC-1* (B; 2 ± 0.1-fold), and *Clec10a* (C; 2.2 ± 0.2-fold). Pretreatment with Vit-D dose-dependently potentiated IL-4-induced increases in *Arg-1, MRC-1*, and *Clec10a* mRNA levels. Vit-D at 10 nM markedly increased expression levels of *Arg-1* (287 ± 51-fold), *MRC-1* (6 ± 0.8-fold), and *Clec10a* (6 ± 0.8-fold versus IL-4 alone) induced by IL-4. Vit-D also increased IL-4-induced IL-10 protein production (D; 510 ± 41 versus 748 ± 36 pg/ml). ** and *** p<0.01 and p<0.001 vs. vehicle, # p<0.05 vs. IL-4 alone.

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active form of Vit-D acts as a hormone in the body, regulating more than 200 genes. Historically, Vit-D is known to play an essential role in bone metabolism and regulation of calcium absorption, maintaining bone homeostasis. While Vit-D deficiency is one of the top three deficiencies in the U.S. (10) it can be caused by many factors such as weight, skin, pigmentation, sex, age, and environment.

Cross-sectional studies have indicated that Vit-D deficiency is correlated with an increased risk of CVDs (11). Previously, it's been reported that the incidence and mortality rates from coronary heart disease (CHD) demonstrated a strong seasonal pattern with higher rates in the winter (12). Individuals with CVDs tend to have lower Vit-D levels than others. This shows more during the winter season, when Vit-D levels (and sunlight) are hypothetically at their lowest (13). Moreover, blood pressure appears to increase as one moves away from the equator (13). Conversely, Vit-D deficiency (<20 ng/ml) was associated with higher prevalence of CHD and peripheral vascular disease. Several cardiovascular risk factors such as hypertension, diabetes, high basal metabolic index (BMI) and low-density lipoprotein (LDL) increase with Vit-D deficiency (11). Cases of self-reported angina and heart failure also increase in Vit-D deficiency. In patients undergoing coronary angioplasty, Vit-deficiency imparted a 50% increase in fatal stroke (14). In another study, it was observed that when Vit-D levels are optimal in aging patients, they are 20% more likely to not develop hypertension (15). Collectively, these studies and observations strongly argue for a crucial role of Vit-D in the maintenance of cardiovascular functions and normal physiology.

In contrast, several studies have highlighted beneficial effects of Vit-D supplementation in cardiovascular diseases. For example, Vit-D (400 IU) plus calcium supplementation given during an 8-week period in elderly German women significantly reduced systolic blood pressure by 9% (16). UVB exposure to skin significantly reduced systolic and diastolic blood pressure after 6 weeks of therapy compared to those subjects receiving UVA (17). Additionally, oral administration of calcitriol ameliorates atherosclerosis in mice by inducing regulatory T cells (18). Collectively, these clinical and preclinical investigations underscore an important physiological role of Vit-D in maintaining normal cardiovascular function. It is now evident that Vit-D supplementation may have important therapeutic effects against CVDs besides its beneficial effect in normal bone metabolism. This evidence warrants for detailed mechanistic and systematic studies to understand the underlying molecular mechanism of Vit-D and to explore its full therapeutic potential. Our team at the Chicago State University is trying to understand how Vit-D contributes to CVDs and its underlying mechanism.

Recent studies have provided ample evidence suggesting that cardiovascular diseases are chronic inflammatory conditions wherein inflammation critically contributes to the pathology of the disease (19,20,21). We propose that Vit-D attenuates inflammation by inhibiting macrophages-mediated inflammation-the primary cause of systemic inflammation. Macrophages can exist in many states, the M1-state, which is pro-inflammatory state; M2-state, an anti-inflammatory state; and several M2 subsets, including M2a, M2b, M2c, and M2d. To address a role of Vit-D on macrophages-mediated inflammation, we cultured and treated RAW 264.7 cells—a murine macrophage cell line—with LPS or IL-4 to induce M1 and M2 polarization in the absence or presence of Vit-D. We determined the mRNA and protein levels of M1 and M2 marker genes (22) using quantitative RT-PCR and ELISA kits, respectively.

Our investigation revealed that LPS treatment increased mRNA levels of pro-inflammatory cytokines IL-6 (60±6-fold), TNF-α (8±0.7-fold), and iNOS (10±0.7-fold) in RAW cells. Pretreatment with 10 nM Vit-D but not 0.1 or 1 nM significantly inhibited LPS-induced increases in expression levels of IL-6 (24±2-fold), TNF-α (4±0.3-fold), and iNOS (4.7±0.3-fold) as compared to LPS treatment alone ($P<0.05$). Vit-D also suppressed LPS-induced TNF-α protein production by macrophages (18±0.8 versus 27±1.4 ng/ml, $p<0.05$). The data demonstrate that Vit-D inhibits M1 macrophage polarization and thereby reduces M1-mediated inflammation.

To investigate whether Vit-D impacts M2 polarization, we investigated the effect of Vit-D on IL-4-induced M2 polarization. Our data revealed that IL-4 treatment induced expression levels of anti-inflammatory genes Arg-1 (93±18-fold), MRC-1 (2±0.1-fold), and Clec10a (2.2±0.2-fold). Pretreatment with Vit-D dose-dependently potentiated IL-4-induced increases in Arg-1, MRC-1, and Clec10a mRNA levels. Vit-D at 10 nM markedly increased IL-4-induced expression levels of Arg-1 (287±51-fold), MRC-1 (6±0.8-fold), and Clec10a (6±0.8-fold) as compared to IL-4 alone($p<0.05$). Further, Vit-D increased IL-4-induced IL-10 protein production (510±41 versus 748±36 pg/ml, $p<0.05$) as compared to the saline treatment. The data suggest that Vit-D promotes M2 polarization and thereby increases anti-inflammatory cytokine production.

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This data demonstrate that Vit-D can attenuate inflammation by inhibiting proinflammatory state of macrophages and promoting anti-inflammatory state of macrophages. It is to be noted that Vit-D independently inhibited LPS-induced M1 polarization and augmented M2 IL-4-induced formation, suggesting a dual action of Vit-D in macrophages. It is prudent to mention here that macrophages exhibit a spectrum of phenotype in the body, including M1, M2a, M2b, and M2c states (23). In addition, several other cell types such as Th17, Th1 cells, and vascular smooth muscle cells contribute to inflammation CVDs, including atherosclerosis and obesity. Therefore, further studies are warranted to address a role of Vit-D in other macrophage subsets and different immune cells. Vit-D has been shown to protect against atherosclerosis, diabetes, and insulin resistance in animal models (18,24,25). These effects were associated with increased cholesterol efflux from macrophages, activation of regulatory T-cells, and promotion of M2 phenotype in macrophages. It is conceivable that Vit-D confers protective effects against CVDs by reducing inflammation by different immune cell types. Collectively, our data suggest that Vit-D supplementation inhibits the M1 state of macrophages and promotes their M2 phenotype. Thus Vit-D attenuates macrophages-mediated inflammation by decreasing M1/M2 ratio in macrophages. In conclusion, we propose that Vit-D deficiency contributes to chronic cardiovascular diseases by promoting systemic inflammation via favoring proinflammatory phenotype of macrophages, the principal cells that regulate inflammation.

Methods

M1 and M2 polarization in RAW 264.7 macrophages

To investigate a role of Vit-D on macrophages phenotype and macrophage-mediated inflammation, we cultured RAW 264.7 cells (ATCC, Manassas, VA), a murine macrophage cell line in Dulbecco's Modified Eagle Media (DMEM) and pre-treated them with different doses of Vit-D (0.1,1, and 10 nM) for 8 hours. The cells were then differentiated into pro-(M1) or anti-inflammatory (M2) states by treatment with lipopolysaccharide (LPS, 10 ng/ml, Sigma, St. Louis, MO) and IL-4 (10 nM, Sigma, St. Louis, MO), respectively, for 24 hours. Total cellular RNA was extracted by using Trizol (Invitrogen, Carlsbad, CA) and cDNA was synthesized using Superscript III first strand synthesis kit (Invitrogen).

RNA extraction and quantitative real-time PCR analysis

Total cellular RNA was extracted using Trizol (Invitrogen, Carlsbad CA). cDNA was synthesized using Superscript III first strand synthesis kit (Invitrogen). The mRNA expression levels of M1 and M2 marker genes were measured by quantitative real-time PCR analysis (qRT-PCR) in a Mastercycler RealPlex 2 (Eppendorf, Enfield, CT) using Kapa Sybr Fast or probe fast qPCR mix (Kapa Biosystems) using conventional Sybr primers. Changes in relative gene expression were normalized to 18S mRNA levels and were determined using the relative Ct method.

Cytokines measurements

The protein levels of TNF-a and IL-10 were measured in the culture supernatant by ELISA kits (Biolegend, San Diego, CA) by following the manufacturer's instructions. Supernatant was collected 24 hours after LPS/IL-4 treatment and frozen at -80 C until used for measurements.

Statistical analysis

All data are expressed as mean ± standard errors of mean (SEM). Differences between two groups or two treatments were compared with a Student's two-tailed t test. Differences between more than two groups or two treatments were compared with a One-way ANOVA followed by Dunnett's t-test. P values <0.05 were considered statistically significant.

References:

1. Cardiovascular Diseases (CVD), May 2017, World Health Organization. https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds). Accessed on Nov 16, 2020.
2. Zittermann A. Vitamin D in preventive medicine: are we ignoring the evidence? 2003, *Br J Nutr*; 89:552-72.
3. Wang C. Role of vitamin d in cardiometabolic diseases. 2013, *J Diabetes Res*; 2013:243934.
4. Gysemans CA, et al., Mathieu C: 1,25-Dihydroxyvitamin D3 modulates expression of chemokines and cytokines in pancreatic islets: implications for prevention of diabetes in nonobese diabetic mice. 2005, *Endocrinol*; 146:1956–64.
5. De Vita F, et al., Maggio M: Relationship between vitamin D and inflammatory markers in older individuals. 2014, *Age* (Dordr); 36:9694.
6. Laird E, , et al., Ward M: Vitamin D deficiency is associated with inflammation in older Irish adults. 2014, *J Clin Endocrinol Metab;* 99:1807-15.
7. Kennel KA, et al., Hurley DL. Vitamin D deficiency in adults: when to test and how to treat. 2010, *Mayo Clin Proc*; 85:752-8.

8. Holick MF. Vitamin D status: measurement, interpretation, and clinical application. 2009, *Ann Epidemiol*;19:73-8.
9. Bikle D. Vitamin D: Production, Metabolism, and Mechanisms of Action. [Updated 2017 Aug 11]. *In: Feingold KR, Anawalt B, Boyce A, et al., editors*. Endotext [Internet]. South Dartmouth (MA): MDText.com, Inc.; 2000.
10. CDC's Second Nutrition Report: A comprehensive biochemical assessment of the nutrition status of the U.S. population;https://www.cdc.gov/nutritionreport/pdf/4page_%202nd%20nutrition%20report_508_032912.pdf.Accessed on Nov 16, 2020.
11. Judd SE, Tangpricha V: Vitamin D deficiency and risk for cardiovascular disease. 2009, *Am J Med Sci*; 338:40-4.
12. Pell JP, Cobbe SM: Seasonal variations in coronary heart disease. 1999, *QJM*; 92:689-96.
13. Lindqvist PG: Cardiology Patient Page. On the possible link between vitamin D deficiency and cardiovascular disease: should we D-lighten our lives? 2014, *Circulation*;129:e413-4.
14. Kendrick J, et al., Chonchol M: 25-Hydroxyvitamin D deficiency is independently associated with cardiovascular disease in the Third National Health and Nutrition Examination Survey. 2009, *Atherosclerosis*; 205:255-60.
15. Meehan M, Penckofer S: The Role of Vitamin D in the Aging Adult. 2014, *J Aging Gerontol;* 2:60-71.
16. Pfeifer M, et al., Minne HW: Effects of a short- term vitamin D(3) and calcium supplementation on blood pressure and parathyroid hormone levels in elderly women. 2001, *J Clin Endocrinol Metab*; 86:1633-7.
17. Krause R, et al., Hopfenmüller W: Ultraviolet B and blood pressure. 1998, *Lancet*; 352:709-10.
18. Takeda M, et al., Sasaki N: Oral administration of an active form of vitamin D3 (calcitriol) decreases atherosclerosis in mice by inducing regulatory T cells and immature dendritic cells with tolerogenic functions. 2010, *Arterioscler Thromb Vasc Biol*; 30:2495-503.
19. Lopez-Candales A, et al., Hernandez-Suarez DF: Linking Chronic Inflammation with Cardiovascular Disease: From Normal Aging to the Metabolic Syndrome. 2017, *J Nat Sci*; 3:e341.
20. Ridker PM, et al., Thuren T: CANTOS Trial Group. Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease. 2017, *N Engl J Med*; 377:1119-31.
21. Hansson GK: Inflammation, atherosclerosis, and coronary artery disease. 2005, *N Engl J Med*; 352:1685-95.
22. Shahid M, et al., Wu MX: IEX-1 deficiency induces browning of white adipose tissue and resists diet-induced obesity. 2016, *Scientific Reports*; 6:24135.
23. Mantovani A, et al., Locati M: The chemokine system in diverse forms of macrophage activation and polarization. 2004, *Trends in immunology*; 25:677-86.
24. Oh J, et al., Bernal-Mizrachi C: Deletion of macrophage Vitamin D receptor promotes insulin resistance and monocyte cholesterol transport to accelerate atherosclerosis in mice. 2015, *Cell Reports*; 10:1872-86.
25. Yin K, et al., Agrawal DK: Vitamin D Protects Against Atherosclerosis via Regulation of Cholesterol Efflux and Macrophage Polarization in Hypercholesterolemic Swine. 2015, *Arteriosclerosis, thrombosis, and vascular biology*; 35:2432-42.

Author Contributions

MS wrote the manuscript, conceived the project, and designed, performed and analyzed the experiments. FK, AA, TO, LJ, and EH performed and analyzed the experiments.

Competing Financial Interest. The authors have no competing financial interest with regard to the presented study.

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