

ALTERATIONS IN VITAMIN D METABOLISM
WITH CHRONIC INFLAMMATION: POTENTIAL
IMPLICATIONS IN INFLAMMATION-INDUCED
BONE LOSS

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CHAPTER I

INTRODUCTION

Background

Vitamin D is well established for maintaining calcium homeostasis and preserving skeletal health (1,2). The major source of vitamin D is derived through cutaneous synthesis following exposure to ultraviolet light from the sun. Diet has become an alternative approach for humans to meet their vitamin D requirements (3,4). Although vitamin D synthesis is initiated from the skin, the synthesis of the active form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), is completed in the kidney. This active form of vitamin D exerts its effects on target tissues such as bone, intestine, and immune cells primarily through the nuclear hormone vitamin D receptor (VDR) (4-6). Vitamin D binding proteins (DBPs) are vitamin D metabolite carriers in the serum. The regulation of vitamin D metabolism can be affected at the gene transcription level. Functionally, vitamin D has been shown to have protective effect on bone, cardiovascular, and immune system (1,7-10).

Serum 25-(OH)D₃ is an indicator of systemic vitamin D status. It is used as measure of vitamin D status, however, the levels used to define deficiency, insufficiency, or optimal vitamin D status remain controversial (11,12). According to Whiting and Calvo, serum 25-(OH)D₃ less than 30 nmol/L is defined as vitamin D deficiency,

insufficiency ranges from 40-80 nmol/L, and 90-120 nmol/L serum 25-(OH)D₃ is defined as the optimal (12). In contrast, Zittermann and colleagues (13) defined vitamin D deficiency as 0-25 nmol/L, insufficiency as 25-50 nmol/L, hypovitaminosis as 50-70 nmol/L, adequacy as 70-250 nmol/L, toxicity as >250 nmol/L.

Deficiency of vitamin D is not only found in populations living in low ambient UV environments, for instance in the northern part of the United States (6,14,15), Canada (16), France (17), Ireland (18), and New Zealand (19), but it is also found in populations living in the sunny countries such as Australia, Africa, India and Pakistan (20). Populations from these countries may be deficient in vitamin D due to insufficient sun exposure, clothing habits, hyper-pigmentation, air pollution which prevent ultraviolet (UVB) exposure to skin as well as insufficient intake of vitamin D (21). Further, Awumey and colleagues (22) showed that fibroblasts of Indian-Americans had higher activity level of 24-hydroxylase (24-OHase) compared to controls, suggesting the increased to vitamin D catabolism may contribute to vitamin D deficiency. At risk populations for vitamin D deficiency include the elderly (1,23,24), those with darkly pigmented skin (25), hospitalized patients (24), and women receiving anti-osteoporotic therapy (15). Vitamin D deficiency is often related to rickets in children (16,26), however, osteoporosis (27), cardiovascular disease, certain cancers, type 1 and type 2 diabetes mellitus are diseases associated with vitamin D deficiency in adults and the elderly (28,29).

The benefits of vitamin D to bone have been well established. Vitamin D prevents (2) and reduces (30) the risk of hip fracture when calcium supplementation is added. Zhu and colleagues (31) showed that a combination of vitamin D and calcium

supplementation is effective in reducing bone turnover rate in elderly women living in a sunny climate. Further, vitamin D combined with calcium supplementation has been shown to reduce the risk of falls in older women (32). A randomized controlled trial in adults showed that daily vitamin D supplementation ≥ 800 IU reduced risk of hip fracture and nonvertebral fracture compared with calcium and placebo (33).

Renal production of $1,25(\text{OH})_2\text{D}_3$ is regulated by parathyroid hormone (PTH), serum calcium and phosphorous (34). The relationship between 25-(OH)D_3 and PTH is considered to be inverse (35,36). Low serum 25-(OH)D_3 in response to elevated PTH could result in accelerated bone turnover which may lead to bone loss, osteoporosis, and fracture (36-39). The decrease in vitamin D may be attributed to decreased expression of renal $1\text{-}\alpha$ hydroxylase ($1\alpha\text{-OHase}$) (40). As renal function deteriorates, the body fails to maintain proper calcium and phosphorous balance. The disturbances in calcium and phosphorous metabolism cause parathyroid glands to secrete excess PTH, which leads to secondary hyperparathyroidism (SHPT) (23). SHPT is responsible for decreased bone mineral density (BMD) and increased bone turnover, fractures, bone pain, marrow fibrosis and eventual erythropoietin resistance in kidney patients (21). SHPT associated with vitamin D deficiency also increases the risk of fracture, particularly femoral neck fracture (29).

In addition to protecting the bone and cardiovascular system, vitamin D has various effects on cellular proliferation and on immune function (10). Many of the biological effects of vitamin D are mediated through the vitamin D receptor (VDR) (41) which are present in most of the cells of the immune system, including the activated T- and B-lymphocytes, macrophages, and dendritic cells (4,42). Both macrophages and

dendritic cells can act as antigen presenting cells (APCs), however, dendritic cells are the primary APCs responsible for T-lymphocytes activation (43) compared to macrophages (44). Furthermore, as macrophages and dendritic cells differentiate, they both demonstrate similar patterns of expression for cytochrome P450 family 27 (CYP27), the vitamin D activating enzyme, and VDR (43,44). Studies on vitamin D and autoimmune diseases showed that $1,25(\text{OH})_2\text{D}_3$ is an effective suppressor of inflammatory bowel disease (IBD), rheumatoid arthritis (RA), multiple sclerosis (MS), type 1 diabetes mellitus (45), and experimental autoimmune encephalomyelitis (46).

Previously, a chronic low-grade inflammation model was developed in our laboratory to examine the effects of subclinical inflammation on bone loss (47). In the first study, chronic inflammation was induced in 3-month-old male Sprague-Dawley rats exposed to 90-days of lipopolysaccharide (LPS) and resulted in decreased bone mineral density (BMD) in femur and vertebra. Bone loss was primarily observed in the trabecular regions compared to the mid-diaphysis area which is primarily cortical bone. In addition to bone loss, over time LPS exposure in Sprague-Dawley rats fibrous tissue developed around the arterioles, consistent with vascular disease. Elevation of neutrophil and monocyte counts in peripheral blood was demonstrated over time and the up-regulation of tumor necrosis factor (TNF)- α , interleukin (IL)- 1β , and cyclooxygenase (COX)-2 at 90 days suggested an on-going inflammatory response. A subsequent study, was performed using adult male C57BL/6J mice and similar results were shown relative to the bone and vascular pathology as observed in the rat model (48). TNF- α was up-regulated in the same regions of the bone and vascular wall. Although chronic elevation of pro-inflammatory mediators, bone loss and vascular pathology have been shown in this

animal model, it is not known if the pathology results from the direct effects of these inflammatory mediators alone or if their effects on other factors such as vitamin D status may play a role.

Humans with inflammatory conditions such as inflammatory bowel disease (IBD) (49-52), type 1 and type 2 diabetes mellitus (53,54), RA were found to have compromised vitamin D status (55,56). TNF- α was one of the major contributors to the manifestation of these diseases which is found in the serum, synovial tissue and fluid (57-60). Previous *in vivo* studies have indicated that the production of inflammatory cytokines (i.e., TNF- α) induced via slow-release LPS pellets was up-regulated in the cardiovascular wall and decreased trabecular bone (47,48). Thus, it is interesting to examine whether the up-regulation of TNF- α , as well as other inflammatory cytokines, alter local and/or systemic vitamin D metabolism.

Therefore, the purpose of this study is to examine the extent to which chronic inflammation alters vitamin D metabolism. Due to the role of the kidney and liver in vitamin D synthesis and catabolism, we will examine the alterations in key genes encoding for vitamin D activating and deactivating enzyme, 1- α -hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1) in animals chronically exposed to LPS. Additionally, the spleen will be used as a site to examine local vitamin D metabolism due to its high monocytes content (61,62). It should be noted that monocytes/macrophages CYP27B1 have been shown to produce 1,25(OH) $_2$ D $_3$ in the late phase of activation, thus it seems that vitamin D has a local rather than systemic effect on monocytes/macrophages (41). The following hypotheses will be tested accomplish the overall purpose of this study.

Hypotheses (Experiment 1)

1. Over time (i.e., 30 and 90 days) chronic inflammation induced with increasing dose of LPS in adult C57BL/6J mice will alter genes encoding for vitamin D activating and deactivating enzyme, 1- α -hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1), in hepatic tissue.
2. LPS will dose dependently reduce circulating vitamin D metabolite (25-(OH)D₃) in adult male C57BL/6J mice over time.
3. LPS-induced pro-inflammatory cytokine (e.g. TNF- α) will down-regulate genes encoding vitamin D activating and deactivating enzymes in the spleen, a monocyte rich tissue, in adult male C57BL/6J mice.

Hypotheses (Experiment 2)

1. Short-term (14-days) inflammation induced with increasing dose of LPS (0, 0.1 or 0.2 mg LPS /kg body weight/day) in adult C57BL/6J mice will alter genes encoding for vitamin D activating and deactivating enzyme, 1- α -hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1), in hepatic and renal tissues.
2. LPS will dose dependently reduce circulating vitamin D metabolite (25-(OH)D₃) in adult male C57BL/6J mice.
3. LPS-induced pro-inflammatory cytokine (e.g. TNF- α) will down-regulate genes encoding vitamin D activating and deactivating enzymes in the spleen, a monocyte rich tissue, in adult male C57BL/6J mice.

Objectives (Experiment 1)

1. To assess the relative abundance of mRNA of for genes encoding for vitamin D activating and deactivating enzymes in C57BL/6J mice, quantitative real-time PCR (qRT-PCR) will be used. RNA will be extracted from the liver and results will be expressed relative to controls.
2. To access circulating vitamin D metabolites, 25-(OH)D₃ and 1,25(OH)₂D₃, in mice exposed to low-grade chronic inflammation in plasma using an enzyme-linked immunoassays.
3. To determine relative expression of genes encoding for vitamin D activating and deactivating enzymes in the spleen, a monocyte rich tissue, using quantitative real-time PCR (qRT-PCR).

Objectives (Experiment 2)

1. To assess genes encoding for vitamin D activating and deactivating enzymes in C57BL/6 mice using quantitative real-time PCR (qRT-PCR). RNA will be extracted from the liver and kidney and results will be expressed relative to controls.
2. To access circulating vitamin D metabolites, 25-(OH)D₃ and 1,25(OH)₂D₃, in mice exposed to low-grade chronic inflammation in plasma, an enzyme-linked immunoassay will be used.
3. To determine relative expression of genes encoding for vitamin D activating and deactivating enzymes in the spleen, a monocyte rich tissue, quantitative real-time PCR (qRT-PCR) techniques will be used to examine the relative abundance.

Limitations

As with most scientific investigations, the experiments performed as a part of this overall project were not without limitations. For instance, experiment 1 was a previously conducted experiment that was not designed to address our questions on inflammation and vitamin D as described here. It was designed to examine the dose-dependent effects of subclinical inflammation on bone loss over time. Because of this, kidney samples were not procured in this experiment. We would have preferred to have kidney tissue from experiment 1, though we utilized tissue samples (i.e., liver and spleen) from the experiment to examine the relationship of inflammation and vitamin D.

In experiment 1, we utilized slow release-pellets to deliver LPS subcutaneously. The amount of LPS delivered in experiment 1 was approximately 3 μg over 24 hours. In contrast, LPS that we used in experiment 2 was delivered via intraperitoneal injection. The amount of LPS delivered in experiment 2 was approximately 4 μg in a single dose. Therefore, the difference in the amount of LPS and the duration of the LPS release into the animals might cause variation in gene expression levels. Furthermore, a single dose of 4 μg of LPS injection caused immediate and definite changes in the liver that might not be achievable with a slow-release pellet that was designed to deliver 3 μg of LPS over 24 hours. Hence, in these studies, two different approaches to delivering LPS were utilized to examine the effects of chronic low-grade inflammation on vitamin D metabolism. Due to the different techniques of LPS delivery, we cannot compare the effects of LPS on key vitamin D hydroxylation enzymes and VDR expression between the two experiments. However, our data would provide insight into what effects occur at what methods of LPS delivery.

Another limitation in our study was that we only utilized one time point (14 days) in experiment 2. Thus, we could not address what would happen to the gene expression associated with key vitamin D hydroxylation enzymes and vitamin D levels after the two-week time point.

CHAPTER II

REVIEW OF LITERATURE

Introduction to Osteoporosis

Osteoporosis is defined as a skeletal disease characterized by a low bone mass and bone microarchitectural deterioration with a consequent increase in bone fragility and susceptibility to fracture (63). The World Health Organization (WHO) has established criteria for osteoporosis in postmenopausal women based on measurements of bone mineral density (BMD) expressed in terms of the number of standard deviations (SD) from the normal value or **T** score (64). The diagnostic categories are 1) normal $T \geq -1.0$, 2) osteopenia $-2.5 < T < -1.0$, 3) osteoporosis $T \leq -2.5$, 4) established or severe osteoporosis $T \leq -2.5$ in the presence of one or more fragility fractures. These criteria have since been accepted and extensively used.

Although osteoporotic fracture may occur at various skeletal sites, the most common sites include the hip, spine, and wrist (65). In the United States, 44 million women and men are at risk for osteoporosis with 55% of these individuals being over the age of 50. It is estimated that the prevalence of this disease in women over the age of 50 will increase to 41 million in 2020, whereas the incidence in men is likely to reach over 17 million (65). Currently, greater than 1.5 million osteoporosis-related fractures occur each year. Among the individuals that experience these fractures, it is estimated that

500,000 will be hospitalized, 800,000 will visit an emergency room, 2.6 million will see a physician, and 180,000 will be placed in a nursing home (66). The increased incidence of osteoporosis-related fractures has become a burden to the healthcare system. In 2005, it was estimated that the incidence of fracture was more than 2 million at a total cost of \$19 billion. In 2006, the direct healthcare costs associated with osteoporosis ranged from 12 to 18 billion dollars (66). By 2025, it is predicted that fractures will increase to more than 3 million per year and the annual projected fracture costs will be \$25.3 billion (67). The lifetime risk of osteoporotic fracture in the Western world is 40-50% chance in women and 13-22% in men (68).

Factors that contribute to the risk of osteoporosis include, but are not limited to, low-peak bone mass, low dietary intake of calcium and vitamin D, hormonal factors (e.g. abnormality of PTH secretion), tobacco smoking, physical inactivity, race, small body frame size, and a personal or family history of fracture (65,69). Recently, increasing evidence has demonstrated that chronic inflammatory conditions such as periodontitis and RA are also associated with osteoporosis (70,71). Studies have shown that vitamin D status is a very important dietary factor associated with bone health in young and old females and males as well as in various ethnic groups. For instance, adolescent girls with high vitamin D status (≥ 74.1 nmol/L) were shown to have higher BMD, lower bone turnover markers, and lower parathyroid hormone (PTH) levels compared to those with lower vitamin D status (72). A double-blind, randomized, controlled trial, demonstrated that calcium supplementations combined with vitamin D positively affected BMD and BMC in peri- and post-menopausal women (73). In a cohort study of elderly men, serum 25-hydroxyvitamin D₃ (25-(OH)D₃) of elderly men was found to be a determinant of

BMC, cortical thickness, and biomechanical parameters of the femoral neck (74). Furthermore, young men, 25-(OH)D₃ were also found to be a significant determinant of BMC and BMD (74). Serum 25-(OH)D₃ was also found to be positively associated with bone health in older, community-dwelling men (75), and in white men (76). In non-Hispanic white adults over the age of 65, serum 25-(OH)D₃ was inversely related to hip fracture risk (77). Beneficial effects of vitamin D on bone health have also been demonstrated in women in the United Kingdom (U.K.), South Asia, and United Arab Emirates (UAE). South Asian women of Pakistani Muslim origin living in the U.K. with serum 25-(OH)D₃ insufficiency/deficiency were found to have reduced bone mass at the hip and wrist (78), while vitamin D deficiencies in women living in UAE were found to be associated with increased bone turnover (79). Hence, it is evident that vitamin D is an important nutrient for maintaining skeletal health in a variety of populations.

Vitamin D Status

Currently, 25-(OH)D₃ and 1,25-dihydroxyvitamin D (1,25(OH)₂D₃), two vitamin D metabolites, are used as indicators of vitamin D status. Serum 25-(OH)D₃ is the vitamin D metabolite most frequently used to assess vitamin D status because it corresponds to cutaneous production and food intake of vitamin D (80). Moreover, serum 25-(OH)D₃ has a longer half-life (10.4 days) (81) compared to 1,25(OH)₂D₃ with a half-life of 4-6 hours (82). Although circulating 1,25(OH)₂D₃ has not been considered a part of the routine vitamin D status clinical assessment, it has been suggested that in the future evaluation of 1,25(OH)₂D₃ may have merit (83).

Individuals at greatest risk of vitamin D insufficiency and deficiency include the elderly, hospitalized patients, and young adults with minimal exposure to sunlight or limited vitamin D intake (24). Vitamin D status among the elderly is recognized as one of the most significant public health concerns because the synthesis of vitamin D in the skin decreases with age and the renal conversion of 25-(OH) D₃ to 1,25(OH)₂D₃ is less efficient resulting in compromised vitamin D status (23,84).

Vitamin D status varies among the elderly populations from the United States and Europe (Scandinavia, Central, and Western Europe) (37). Even among both community-dwelling and institutionalized elderly populations in the U.S. and Europe, serum 25-(OH)D₃ levels are different. Comparing serum 25-(OH)D₃ in community-dwelling with institutionalized elderly population enable researchers to determine whether different duration of sun exposure would affect vitamin D synthesis in these populations. Community-dwelling elderly populations from the U.S. have an average serum 25-(OH)D₃ of 71-86 nmol/L, whereas 25-(OH)D₃ in a similar group of Europeans is 21-55 nmol/L. In the U.S., the institutionalized elderly have serum 25-(OH)D₃ levels of 53-45 nmol/L compared to 9-37 nmol/L for those from Europe. Lips and colleagues (37) reported that patients with hip fracture in the U.S. had serum 25-(OH)D₃ levels of approximately 32 nmol/L, whereas those in Europe were 19-46 nmol/L. Generally, the minimum level of serum 25-(OH)D₃ for fracture prevention ranges between 50-80 nmol/L.

There continues to be some controversy related to definition of insufficient, deficient, optimal, and toxic vitamin D status (**Table 1**). For fracture prevention, the recommended serum 25-(OH)D₃ concentrations ranged from 50-80 nmol/L, but some

researchers recommended 70-80 nmol/L (11). Alternatively, BMD can be used to estimate optimal vitamin D levels. 25-(OH)D₃ levels approximately 94 nmol/L was shown to be the preferred level for maintaining total hip BMD in young and older populations in the U.S. (49). Although the optimum level varies, experts recognize that the intake of at least 20-25 µg (800-1000 international unit, IU)/day of vitamin D is necessary for older men and women to reach serum 25-(OH)D₃ of 75 nmol/L for fracture prevention (11). For optimal health benefits, both children and adults should maintain > 75 nmol/L of 25-(OH)D₃ (85).

Table 1. General Classification of Vitamin D Status

Classification	Serum 25-(OH)D₃ (nmol/L)
Insufficiency	40-80 (12)
	25-50 (13)
	51-74 (87)
Deficiency	<30 (12)
	0-25 (13)
Optimum level	90-120 (12)
	70-250 (13)
	>75 (11, 32)
Toxicity	>250 (13)
	>375 (87)

Circulating 25-(OH)D₃ >375 nmol/L is defined as vitamin D intoxication (85). Vitamin D intoxication is characterized by elevated plasma vitamin D concentration that may result in hypercalcemia (85,86). In addition, high amounts (i.e., ≥ 600 nM) of “free” 1,25(OH)₂D₃ displaced from vitamin D-binding protein (DBP), such as in the case of patients with vitamin D toxicity may also cause hypercalcemia (87,88). Hypercalcemia may present with symptoms mimicking acute myocardial infarction such as fatigue, anorexia, and chest pain (89). Both the physicians and the manufacturers should be certain that vitamin D supplementation prescribed and manufactured are at the appropriate levels to prevent vitamin D intoxication (90).

Sources of Vitamin D

The recommended daily intake assumed to be adequate, (i.e., adequate intake or AI), in 50-70 years old is 10 µg (400 IU)/day; the AI for adults over the age of 70 is 15 µg (600 IU)/day. The tolerable upper intake level (UL) for vitamin D among adults in the U.S. is 50 µg (2000 IU)/day. It should be noted that sunlight exposure can provide an adult up to 250 µg (10,000 IU)/day of vitamin D, which exceeds the UL. However, excessive sun exposure does not result in vitamin D intoxication because previtamin D₃ and vitamin D₃ can be photolyzed or photodegraded to noncalcemic products (e.g. 5,6-transvitamin D₃, suprasterol I, and suprasterol II) which are biologically inactive (91,92). It is not known whether these products have any biological significance, however, 5,6-transvitamin D₃ analog had been shown to stimulate intestinal calcium transport and modestly stimulate mobilization of calcium from the bone of anephric rats (93). Sun exposure is the major source of vitamin D, however, vitamin D (i.e., D₂ and D₃,

ergocalciferol and cholecalciferol respectively) can also be obtained from foods (5).

Vitamin D₂ is synthesized from ergosterol, a provitamin, to vitamin D₂, and is derived from the irradiation of plant sterols and converted to ergocalciferol when exposed to ultraviolet (UVB) radiation (5). Although there are limited foods that contain vitamin D (i.e., D₂ and D₃), some of the best dietary sources of vitamin D₃ (cholecalciferol) include fish (e.g. salmon, mackerel, and sardines), fish liver oils, egg yolk, and liver (86). Some wild mushrooms contain D₂ and meat contains small amounts of D₃. Most of the naturally occurring vitamin D-rich foods are not consumed on a regular basis, therefore, Americans depend on vitamin D-fortified foods such as breakfast cereals, milk, margarine, and orange juice to meet their daily vitamin D requirements (61,94).

Generally, vitamin D status in the U.S. is better compared to Europe (95), Canada, Australia, and New Zealand because of the availability of fortified foods (96). Although vitamin D-fortified foods provide the greater part of dietary intake in the U.S., vitamin D deficiency has been attributed to low fortification level in foods (5), insufficient intake of these foods, reduced consumption of milk and dairy products (97). Even though dietary sources of vitamin D have been recognized, their (vitamin D₂ and D₃) content in foods have not been developed. Hence, the Nutrient Data Laboratory (NDL) at the Beltsville Human Nutrition Research Center, Agricultural Research Service, United States Department of Agriculture has recently begun to collaborate with vitamin D experts to analyze vitamin D content of food samples and to develop acceptable values for vitamin D₂ and D₃. The outcome of this work will be used in the National Nutrient Database as Standard Reference for acceptable values (98).

Other Factors Influencing Vitamin D Status

Diet is recognized as an important factor for vitamin D status, but environmental and personal factors may also play a role. For example, latitude, season, time of day, the amount of ozone and cloud, aerosol, and albedo (i.e., reflectivity of a surface) are among the environmental factors that affect vitamin D status. Skin type, age, clothing, and the use of sunscreen are some of the personal factors that affect the synthesis of vitamin D and hence affect vitamin D status. In addition to that, premature birth, malabsorption, and obesity also contribute to the alteration in vitamin D status (99).

Vitamin D Metabolism

Sunlight is the primary source of vitamin D for humans (3,4). As skin is exposed to ultraviolet radiation, 7-dehydrocholesterol (7-DHC) found in the epidermal layer of the skin is converted to previtamin D₃. A thermal reaction further converts previtamin D₃ to vitamin D₃ at the epidermal layer. Vitamin D₃ then enters the circulation and is transported by a high-affinity binding protein, vitamin D binding protein (DBP), to fat for storage, or to the liver where it is further metabolized. In the hepatocytes (100), vitamin D-25-hydroxylase (25-OHase) metabolizes vitamin D₃ to 25-(OH)D₃ by adding a hydroxyl group on carbon 25 (101). CYP27A1 is the gene encoding 25-OHase, which is expressed primarily in the liver but also in the kidney and other tissues (100). CYP27A1 is a multifunctional cytochrome P450 enzyme found in the mitochondria for catabolism and removal of cholesterol from cells (102) as well as the synthesis of vitamin D₃ (103-106). Due to its functions on cholesterol-27-hydroxylation and 25-hydroxylation of vitamin D₃, CYP27A1 also known as sterol 27-hydroxylase, P450c27 and mitochondrial

vitamin D₃ 25-hydroxylase (103-107). As indicated previously, 25-(OH)D₃ is used as an indicator of vitamin D status as it leaves the liver, re-enters the circulation, and is transported to the kidney for further hydroxylation (6).

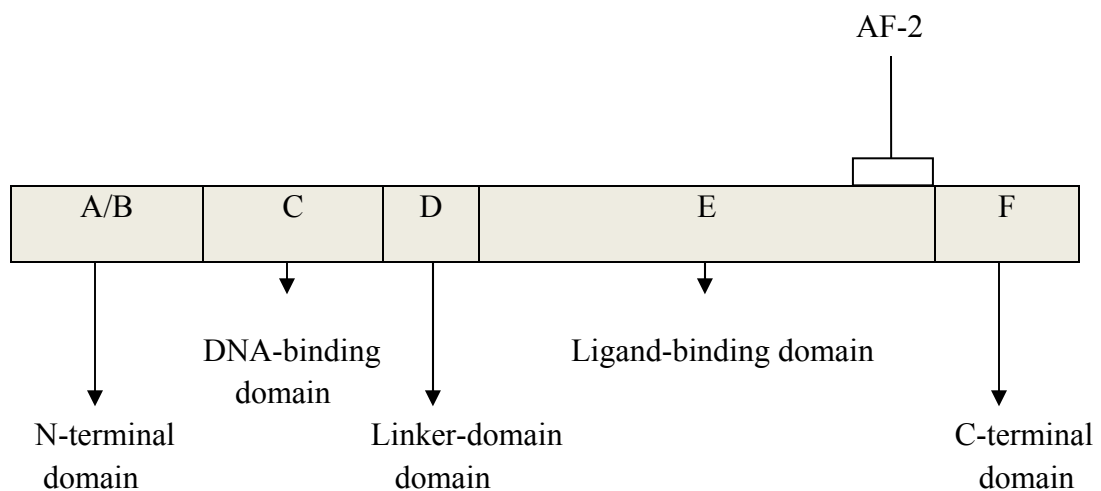
In the kidney, 25-(OH)D₃ is converted to 1,25(OH)₂D₃ in cells within the kidney by 25-hydroxyvitamin D₃-1 α -hydroxylase (1 α -OHase) present mainly in the proximal convoluted tubule (3,6,29,108). Cytochrome P450, family 27, subfamily b, polypeptide 1 (CYP27B1) is the gene responsible for the 25-hydroxylation of 25-(OH)D₃ to 1,25(OH)₂D₃ (109). It is not only expressed in the kidney, but is also expressed in bone (110), macrophages (111), intestine (colon), pancreas (islets), and placenta (112). 1,25(OH)₂D₃ re-enters the circulation and is transported via vitamin D binding proteins (DBP) to the target tissues, such as bone, intestine, heart, pancreas, and immune cells (e.g. T- and B-lymphocytes) that expresses VDR (4,6,46). Regulation of vitamin D homeostasis also requires 25-hydroxyvitamin D-24-hydroxylase (24,25(OH)₂D₃). CYP24A1, the cytochrome P450, family 24, subfamily A, polypeptide 1, is a gene encoding the enzyme 24,25(OH)₂D₃ in the kidney that is involved in the catabolism of 1,25(OH)₂D₃ and 25-(OH)D₃ when 1,25(OH)₂D₃ is sufficiently available (113). CYP27B1 and CYP24A1 are vital genes encoding vitamin D enzymes that tightly regulate the production of vitamin D hormone in the body (114). By studying these genes, we are able to get a better understanding on vitamin D regulation in the cellular level.

Vitamin D Receptor (VDR) and Functions

VDR is a member of the steroid nuclear hormone receptor superfamily involved in the regulation of gene expression (115). While VDR was traditionally recognized for its expression on tissues such as bone, small intestine, heart, and pancreas, it is now known to be expressed on a variety of cells including T- and B-lymphocytes and monocytes (4,29,46). 1,25-(OH)₂D₃ binds to the cytoplasmic VDR in target tissues causes the rejection of corepressors (e.g. nuclear receptor corepressor) (116). It then translocates to the nucleus and heterodimerizes with retinoid-X receptor (RXR), a member of the superfamily of nuclear hormone receptors which is activated by the vitamin A metabolite 9-*cis*-retinoic acid (9-*cis* RA) (116-118). The binding increases the affinity of the VDR/RXR heterodimeric complex for the vitamin D response element (VDRE), which is a specific sequence of nucleotides in the promoter region of vitamin D target genes (119). The binding of VDR/RXR heterodimeric complex to VDRE in the promoter region induces transcriptional activation or suppression (120). VDRE can be found thousands of basepairs distal from the transcription initiation site; coactivators have been associated with the bending of DNA following their binding to VDR/RXR. However, it is unclear whether coactivators are essential to connect VDR/RXR heterodimeric complex to its VDRE (121). To date, the two coactivator complexes that have been identified are the steroid receptor activator complex (SRC), consisting of p160 family of SRC1, 2 and 3 coactivators, and the vitamin D receptor interacting protein complex (DRIP) (122,123). These coactivators bind to specific domain of the VDR for transcription activation.

In order to understand transcriptional activation, one must be familiar with the structure of VDR (**Figure 1**). VDR is composed of: 1) an A/B region that contains the ligand-independent AF-1 transactivation domain; 2) a C region containing the DNA-binding domain; 3) a D region that includes the linker domain; and 4) an E region containing the ligand-binding domain, the dimerization surface and the ligand-dependent AF-2 transactivation domain (124). The SRC coactivator interacts with AF-2 transactivation domain and recruits histone acetyltransferases (HATs) and methyltransferases (MeTs) to the VDR forming a multisubunit complex interacting with VDRE, destabilizing the interaction of DNA and the histone core, facilitating transcription (123,125). Similarly, DRIP coactivator interacts with AF-2 transactivation domain and recruits RNA polymerase II to the transcription initiation site (125).

Figure 1. The Structural Organization of VDR



Vitamin D's Classical Role in Calcium Homeostasis

The traditional role of vitamin D is to optimize the absorption of calcium and phosphorous from the intestinal lumen and increase the reabsorption of calcium from the kidneys. Vitamin D promotes the formation and mineralization of bone and is essential for the maintenance of mineralized skeleton (4). Calcium is transported by a vitamin D-dependent transcellular pathway, which is highly expressed in the proximal intestine (126). The process begins with luminal calcium entering duodenal enterocytes through a calcium channel, transient receptor potential cation channel, subfamily V, member 6 (TRPV6) located on the apical membrane of the enterocyte surface (127). Calcium is transported by calbindin 9k, a cytosolic calcium transport protein, from the apical membrane to the basolateral membrane, where vitamin D-dependent plasma membrane Ca(2+)-ATPase (PMCA1) pump is located (126,127). The regulation of intestinal and renal calbindin 9K is highly dependent on VDR and was suggested to be an important mediator for calcium reabsorption in the kidney (128). The transcription of calbindin 9k and TRPV6 are enhanced by the binding of vitamin D to VDR (129).

The small intestine, kidney, bone, and parathyroid glands are the tissues most involved in the maintenance of systemic calcium homeostasis. Vitamin D functions together with the peptide hormone, PTH, which regulates serum calcium and inorganic phosphate levels through its effects on the kidney and bone (130,131). The relationship of 25-(OH)D₃ to PTH is characterized as inverse relationship in that when 25-(OH)D₃ is adequate, PTH is suppressed (35,36). In contrast, low serum 25-(OH)D₃ in response to elevated PTH promotes calcium resorption from the skeleton, enhances bone loss, and increases the risk of fracture (37).

Studies have shown that vitamin D plays a crucial role in skeletal health. Traditionally, vitamin D deficiency has been recognized as a cause of severe bone deformities or rickets in children (132). Rickets in children or osteomalacia in adults is a metabolic bone disease caused by a defect in phosphate and/or vitamin D metabolism (133). In osteomalacia, the amount of osteoid or the organic matrix of bone, is in excess to adequate bone mineralization. These children manifest defective mineralization of bone and the cartilaginous matrix of the growth plate. Impaired bone mineralization in osteomalacia may lead to osteoporosis, which then predisposes an individual to fractures (133). In postmenopausal women, vitamin D deficiency has been reported in association with acute hip fracture (39). Irish postmenopausal women were shown to have low vitamin D status (< 50 nmol/L), but not vitamin D deficiency during late wintertime (134). Low serum concentration of 25-(OH)D₃ (≤ 12 ng/mL) was associated with increased risk of osteoporotic fracture in persons aged 65-75 years in the Netherlands (135) and may be associated with lower forearm BMD in young Japanese women (136). Various clinical studies have been conducted to further examine the relation of vitamin D and skeletal health. Clinical studies in the elderly have shown that bone loss is prevented and bone density is improved when supplemented with 400-800 IU/day of vitamin D, given alone or in combination with calcium. Furthermore, osteoporotic fractures in the elderly were prevented with daily supplementation of 400-800 IU of vitamin D and 700-800 mg of calcium (137). Vitamin D supplementation with added calcium improves bone mineral density and reduces markers of bone turnover in both young and old populations (31,138).

Studies involving animal models have been used to examine the role of vitamin D in bone metabolism. For instance, the role of vitamin D receptor (VDR) was examined in VDR gene knockout (KO) mice (139,140). Before weaning, the VDR KO mice did not exhibit defects in development and growth, however, after weaning, VDR KO mice did show defects in development and growth, resulting resistance to $1,25(\text{OH})_2\text{D}_3$ action (139,140). The growth plate of the femur from 1α -OHase-ablated (CYP27B1) mice were expanded with disorganized cartilage layers, demonstrating that 1α -OHase is an essential enzyme for bone metabolism (141). Dardenne and colleagues (141) further investigated the effect of $1,25(\text{OH})_2\text{D}_3$ administration on the 1α -OHase-ablated mice and showed that $1,25(\text{OH})_2\text{D}_3$ was effective in restoring biomechanical properties of bone tissue and reducing pseudo-vitamin D deficiency rickets (PDDR). Vitamin D was shown to prevent binge alcohol-induced bone loss in Sprague-Dawley rats and the $1,25(\text{OH})_2\text{D}_3$ /VDR system is required for optimal osteoclastic bone resorption and osteoblastic bone formation (142-144). Based on animal studies, both VDR and CYP27B1 involved in vitamin D metabolism are critical for bone metabolism and the maintenance of skeletal health.

Inflammation

Inflammation is a defense reaction occurring in the body in response to infection (145) and tissue damage (146) and may be characterized as either acute or chronic (147). The major characteristics of the inflammatory response includes vasodilation to increase blood flow to the site of infection; increased vascular permeability to allow soluble mediators to diffuse across the endothelial barrier and to enter the site of infection; and

accumulation of immune cells (e.g. monocytes/macrophages, T- and B-lymphocytes, and granulocytes) at the infected area to reduce tissue damage (148). Once monocytes enter the site of infection, they may differentiate into macrophages with phagocytic activity. Chemical mediators such as lymphokines (i.e., cytokines secreted by activated lymphocytes) are required for the activation of macrophages which serve to either promote or suppress inflammation depending on their phenotype (149).

T-lymphocytes, type I T helper (Th1) and type II T helper (Th2) cells are subsets of T-lymphocytes that produce interferon (IFN)- γ , interleukin (IL)-2, tumor necrosis factor (TNF)- β and IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 respectively (150). Cytokines produced by both Th1 and Th2 cells demonstrate phagocyte-dependent protective capabilities, with Th1 cytokines responsible for macrophages activation and cell-mediated immunity, while Th2 cytokines responsible for antibody production, eosinophil activation, and macrophage functions inhibition (151). Pro-inflammatory cytokines produced by Th1 cells and anti-inflammatory cytokines produced by Th2 cells are the two major groups of cytokines involved in inflammation (152).

Bone Metabolism in Normal Conditions

In order to understand the response of bone to inflammation, one must first have a basic understanding of bone metabolism under normal conditions. In the adult skeleton, bone is constantly remodeled by a process known as resorption by osteoclasts followed by bone formation by osteoblasts (153). Under normal condition, osteoclast breakdown or resorb bone, forming dish-shaped cavities known as lacunae. Osteoblasts will then deposit new bone (i.e., osteoid) at the site of the lacunae until it is fully replaced with new

bone (153). In addition, the process of bone resorption and formation is tightly regulated by receptor activator of nuclear factor kappa B/ receptor activator of nuclear factor kappa B ligand/osteoprotegerin (i.e., RANK/RANKL/OPG) system (153). The ligand for receptor activator for NF- κ B (RANKL) is expressed by osteoblasts and activated T-lymphocytes and binds to its receptor RANK which is expressed on osteoclast precursors and dendritic cells (154). The binding of RANKL to RANK allows osteoclast to differentiate and to become mature bone resorpting cells (154). Osteoprotegerin (OPG), serves as a soluble decoy receptor of RANKL, and can inhibit RANK-RANKL interaction (155,156). When RANK-RANKL interaction occurs and mature osteoclasts are formed, they attach to the bone surface forming a ruffled border enclosure so that hydrogen ions and proteolytic enzymes can be released within a confined environment. These two events in turn lead to the activation of tartrate-resistant acid phosphatase (TRAP) and cathepsin K (CATK), thus initiating bone resorption (157).

Bone Metabolism in Inflammatory Conditions

Inflammation can cause an imbalance in normal bone remodeling usually in favor of bone resorption exceeding new bone formation (158). Such alterations in bone metabolism result in bone loss (i.e., osteopenia) or osteoporosis and further complicate many chronic inflammatory conditions such as rheumatoid arthritis, periodontitis, certain cancers and HIV. Clinical studies have demonstrated that patients with rheumatoid arthritis (RA), an inflammatory joint disease, exhibit a significant decrease in BMD including lumbar spine and femur BMD (159,160). These changes in BMD coincided with elevated pro-inflammatory cytokines, in particular IL-1 β , IL-6, and TNF- α , in the

inflamed synovium and synovial fluid of these patients (161,162). More recently, the pro-inflammatory cytokine IL-17, which is produced predominantly by T-lymphocytes, was also found to be associated with RA (163). Further investigation into the effects of inflammation on bone in RA patients, have shown that urinary and serum markers of bone resorption (i.e., pyridinoline, deoxypyridinoline, N-telopeptides and C- telopeptides of type I collagen) were significantly increased in these patients (164,165). The serum bone formation marker, procollagen type I C-terminal telopeptide (ICTP) was elevated in RA patients, but not procollagen type I C-terminal propeptide (PICP) or procollagen Type I N-terminal propeptide (PINP) (166). These findings confirmed that in RA, bone resorption is accelerated and bone formation is decreased or remain unchanged.

Another chronic inflammatory disease, periodontitis has also been associated with bone or tooth loss (167). Femur BMD was found to be positively correlated with interproximal (i.e., between the adjoining surface) alveolar bone loss (168). Patients with inflamed gingival tissue tend to have increased circulating levels of pro-inflammatory cytokines including IL-1 β , IL-6, and TNF- α (169-171). These cytokines in addition to cyclooxygenase (COX)-2 tend to be highly expressed in patients with inflamed gingival tissue (170,172,173). Therefore, gingival inflammation is likely to serve as a source of pro-inflammatory mediators that produce local as well as systemic effects on skeletal tissue.

In terms of bone loss in other chronic inflammatory conditions such as HIV and breast cancer, decreased in BMD is common (174,175). Patients with HIV have been shown to have decreased BMD of the hip, lumbar spine, proximal femur, and total body (176,177). Biochemical markers of bone metabolism suggest that these alterations in

bone density result from increases in bone resorption and decreases in bone formation (178,179). Additionally, decreased BMD is often reported with cancers of the breast and lung that is associated with osteolytic metastatic bone disease (180-182). Other examples exist in the literature of patient populations who suffer from osteoporosis associated with chronic inflammatory diseases or conditions including, atherosclerosis (183), pancreatitis (184), inflammatory bowel disease (IBD) (185), and lupus (186).

Animal Models of Chronic Inflammation and Bone Loss

Animal models have provided a great deal of insight into the relationship between chronic inflammation and bone. For instance, a rat model of periodontitis-induced bone loss showed a significant increase in alveolar bone resorption (187). Pro-inflammatory cytokines involved in bone loss such as TNF- α , IL-1 β , IL-6 (188,189), and IL-17 (190) have been reported in animal arthritis models. RANKL was significantly elevated in the joints (local) and serum (systemic) of arthritic rats, however, local and systemic bone loss were prevented when RANKL was inhibited by OPG (191). RANKL knockout mice showed a significant decrease in bone resorption compared to the control, confirming that RANKL is an essential factor for osteoclast differentiation in bone erosion associated with arthritis (192). *In vivo*, elevated numbers of osteoclasts were reported in subchondral bone, which is situated beneath cartilage and is the common erosion site near synovial inflammatory tissue. Osteoblasts were found primarily along the cortical surface, a more distant site, confirming that bone resorption activity is higher than bone formation activity at inflammatory sites. Furthermore, the authors also demonstrated that

blockade of TNF- α decreased osteoclast numbers and enhanced osteoblast numbers, favoring bone formation (193).

LPS is the major constituent of the cell wall of Gram-negative bacteria such as *E. coli* 0157:H7 and *Salmonella* (194), whose function is to maintain the membrane structure. LPS induces an immune response, and to be highly immunogenic. The immunogenic response of LPS begins when LPS in the circulation binds to the liver-derived lipopolysaccharide binding protein (LBP), an acute phase protein (195). This LPS-LBP complex interacts with the co-receptor cluster of differentiation (CD14) present on the surface of monocytes/macrophages, subsequently facilitating its interaction with the plasma membrane protein toll like receptor 4 (TLR4) (195). The activated TLR4 recruits the adaptor protein myeloid differentiation factor (MyD88), which in turn recruits IL-1 receptor associated kinases 1 (IRAK1) and IRAK4 (196,197). Upon recruitment or binding of IRAK4 to the receptor complex, the IRAK1 is phosphorylated, which in turn facilitates the interaction of TNF receptor-associated factor 6 (TRAF6) (198,199). When the IRAK1-IRAK4-TRAF6 complex dissociates from TLR4, TLR4 activates downstream kinases such as transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1), TAK1-binding protein 1 and 2 (TAB1 and TAB2) (200). This eventually activates mitogen-activated protein kinase (MAPK) and nuclear factor- κ B (NF- κ B) pathways and thereby regulates gene expression (201). Due to the efficacy of LPS in creating downstream inflammatory signaling pathways, it has been used to initiate immune response in animals, human, and cell culture studies (47,202,203).

A model of low-grade chronic inflammation was developed in our laboratory to study the influence of subclinical inflammation on bone loss (47). In this study, the

continuous inflammatory response in the animals was confirmed by elevation in neutrophils and monocytes throughout the 90-day study period and up-regulation of pro-inflammatory such as TNF- α , IL-1 β , and COX-2 in the bone at the end of the study. This model of inflammation in Sprague-Dawley male rats was shown to produce a decrease in BMD and trabecular bone microarchitecture at 3 months of age (47). The bone loss occurred in conjunction with increases in TNF- α , IL-1 β and COX-2 protein expression in the bone. Moreover, in C57BL/6J mice this model was shown to produce significant bone loss in 30 days, which appeared to be mediated by up-regulating TNF- α (48). Hence, it appears that TNF- α plays a significant role in bone response to chronic inflammation in this model. Therefore, we propose to use the chronic LPS model of low-grade chronic inflammation to study the effects of inflammation on systemic and local vitamin D metabolites in mice. Additionally, we will study the potential role of TNF- α in mediating these effects.

The Discovery of Vitamin D's Relation to Inflammation

In addition to the role of vitamin D in calcium homeostasis, vitamin D also plays an important role in the immune system. Two of the early events that led to this discovery of this relationship were the observation that several immune cells (e.g. T-lymphocytes, monocytes and macrophages) expressed VDR (4,42,47) and that vitamin D was shown to modulate macrophage and lymphocyte functions associated with tuberculosis (TB) (204). The stage of maturation of monocytes and macrophages determine the levels of production of 1,25(OH) $_2$ D $_3$ and the expression of VDR. An *in vitro* study utilizing peripheral mononuclear cells (PMC) showed that the capacity to synthesize 1,25(OH) $_2$ D $_3$,

but not 24,25(OH)₂D₃ and to express VDR, is enhanced when blood monocytes differentiated into macrophages. This indicates the importance of the maturation stage of monocytes and macrophages in 1,25(OH)₂D₃ production (43). In 2000, Veldman and colleagues reported that VDR was highly expressed in T-lymphocytes, but not B-lymphocytes, and suggested that vitamin D directly regulates T-lymphocyte function (205). For some time it has been known that the prevalence of new cases of TB increased during the colder seasons when limited sun exposure, resulting in limited cutaneous synthesis of vitamin D, and low serum levels of vitamin D were reported. Originally, exposure to UV light successfully cured tubercular infection in tuberculosis patients before the availability of drugs. However, it was unclear whether vitamin D was actually involved with immune function or whether these effects were mediated by other mechanisms.

In the 1980s, patients with tuberculosis were shown to have low serum concentration 25-(OH)D₃ compared to the healthy controls (206). Studies were conducted to determine the potential relationship between vitamin D and tuberculosis. Using *in vitro* studies, 1,25(OH)₂D₃ was shown to effectively modulate the cytokine response to tuberculosis antigens (207). Incubation of human monocytes in 1,25(OH)₂D₃ at a concentration of 10⁻⁹ mol/L resulted in anti-tuberculosis activity (204). Exposure of mature macrophages to 1,25(OH)₂D₃ at 4 µg/ml (1040 nmol/L) was able to activate macrophages to express tuberculo-immunity, where the activated macrophages are able to kill intracellular tubercle bacilli (208). Another study showed that 1,25(OH)₂D₃ metabolism leads to activation of macrophages and suppresses the intracellular growth of TB, indicating that vitamin D can modulate macrophage function (209). A more recent

study showed that vitamin D's anti-microbial activity against tuberculosis is dependent on cathelicidin induction (210). Cathelicidins are antimicrobial peptides of the innate immune system that have an anti-inflammatory role by down-regulating inflammation via inhibition of TLR4 signaling (211). As mentioned previously, vitamin D was used to treat TB in sanatoriums before the antibiotics came in to use, however, the relationship of vitamin D and the treatments of TB remain unclear. Later, vitamin D was shown to modulate macrophage and lymphocyte associated with TB. Since macrophage and lymphocyte are involved in inflammatory response, thus vitamin D is considered to play a role in inflammation.

Vitamin D and Inflammatory Conditions

The relationship of vitamin D and inflammation has been shown clinically. For example, patients with inflammatory bowel disease (IBD) were found to have low BMD, in the lumbar spine, total hip, and total body in accordance to low serum 25-(OH)D₃ concentrations (49-52). Suppressed vitamin D level has also been reported in diabetes, which is considered to have an inflammatory component (53,54). Epidemiological studies indicate that patients with type 1 diabetes mellitus have lower serum concentrations of 25-(OH)D₃ and 1,25(OH)₂D₃ than controls (212,213). Vitamin D insufficiency is considered a risk factor for diabetes, particularly type 2 (13). Furthermore, peripheral blood monocytes from type 2 diabetics were shown to have a pro-inflammatory profile (i.e., elevated TNF- α , IL-6, IL-1, IL-8, COX-2, intercellular adhesion molecule (ICAM)-1 and B7-1 or CD80 molecule) and the expression of several of these cytokines (i.e., TNF- α , IL-6, IL-1, IL-8) were down-regulated with the addition of 1,25(OH)₂D₃ (57).

Suzuki and colleagues (214) showed that type 2 diabetics have low serum 25-(OH)D₃. These studies showed that diabetic patients not only demonstrate inflammatory conditions but also display low vitamin D.

Patients with RA were found to have low serum concentration of 25-(OH)D₃ (55). A clinical study conducted on male and female patients with RA between the ages of 18-80 years showed that high disease activity in these patients is negatively correlated with serum levels of 1,25(OH)₂D₃ (56). Research showed that, aging decreases the activity of the kidney to produce 1,25(OH)₂D₃ from 25-(OH)D₃ (23,84), however, the authors found only weak negative correlation between age and serum 1,25(OH)₂D₃ in this patient population, indicating disease activity may be affecting 1,25(OH)₂D₃ (56). A study comparing Northern (Estonia) and Southern (Italy) European RA patients showed that serum concentrations of 25-(OH)D₃ in female RA patients from Northern (Estonia) Europe were negatively correlated with RA disease activity score (215). A subsequent study demonstrated that oral treatment with 2 µg/day of 1,25(OH)₂D₃ lowered the risk of RA (216).

The relationship between inflammation and vitamin D has been explored by using animal models. In an experimental murine model of colitis, VDR knockout (KO) mice were shown to have severe IBD symptoms (217). Cantorna and colleagues (218) utilized IL-10 KO mice, which exhibit IBD symptoms, to study the effects of 1,25(OH)₂D₃ on IBD. The mice were made vitamin D deficient, vitamin D sufficient or supplemented with 1,25(OH)₂D₃ and the severity of IBD development was examined. The authors showed that vitamin D deficiency aggravates symptoms of IBD as indicated by severe diarrhea and weight loss. No severe diarrhea or weight loss were shown in vitamin D

sufficiency, however, vitamin D supplementation ameliorated symptoms of IBD in the IL-10 KO mice (218). The beneficial effects of vitamin D was further analyzed in a multiple sclerosis (MS) mouse model, where 1,25(OH)₂D₃ was found to reverse experimental autoimmune encephalomyelitis (EAE) by inhibiting chemokines (219), iNOS, and monocyte recruitment into the CNS (220). Furthermore, treatment with 1,25(OH)₂D₃ was shown to prevent acute liver rejection following transplantation in both Sprague-Dawley and Wistar rats by inhibiting IFN- γ expression and increasing IL-10 expression. These data indicate that 1,25(OH)₂D₃ induces secretion of cytokines from Th2, which has to anti-inflammatory role, protect liver function and alleviate liver rejection (221).

In vitro studies have also provided insight into the relationship between vitamin D and immune function. Immune stimuli such as LPS and IFN- γ have been shown to regulate macrophages 1- α hydroxylase (CYP27B1) expression, the enzyme that converts 25-(OH)D₃ to 1,25(OH)₂D₃ (222). Furthermore, LPS can attenuate 1,25(OH)₂D₃/VDR functions and may affect the ability of 1,25(OH)₂D₃ to induce myloid differentiation into monocytes/macrophages (223). Liu and colleagues (224) demonstrated that activation of toll-like receptors (TLR) TLR2/1, in human monocytes and macrophages up-regulated the expression of VDR and CYP27B1 genes inducing the antimicrobial peptide cathelicidin to kill intracellular *mycobacterium tuberculosis*. The activated VDR results in up-regulation of cathelicidin antimicrobial peptide (CAMP), which has direct antimicrobial effects on tuberculosis. Further analysis of human monocytes cultured in serum containing media, in the presence and absence of 25-(OH)D₃ demonstrated that 25-(OH)D₃ is required for cathelicidin expression. This study demonstrated there is a

strong link between vitamin D, TLR, and antimicrobial responses of cathelicidin in immune response (224).

Vitamin D, Bone, and Inflammation

Most of the immune cells, in particular antigen-presenting cells (macrophages and dendritic cells) and activated T-lymphocytes, express VDR (225,226) in addition to express 1α -OHase (222,227) and 24OHase (228). The 1α -OHase found in these immune cells is identical to those found in the kidney and is essential for the hydroxylation of 25-(OH)D₃ to 1,25(OH)₂D₃ (222,227). Renal 1α -OHase is regulated by PTH and 1,25(OH)₂D₃ itself, whereas the immune cells 1α -OHase is controlled by immune signals, with IFN- γ and Toll-like receptor (TLR) agonists being powerful stimulators (222). The classical actions of vitamin D to maintain calcium and phosphate homeostasis and bone mineralization (4), is in contrast to the nonclassical actions of vitamin D affecting the immune system and cellular differentiation (229). Vitamin D deficiency or insufficiency is associated with increased risk of chronic inflammatory conditions such as TB and leprosy (230-232), which may be caused by the decrease in monocyte activation and differentiation into macrophages induced by activated vitamin D (43). Various factors including vitamin D stimulate the transcription and production of RANKL, indicating that vitamin D has significant effects on the RANK/RANKL/OPG system in regulating bone metabolism. In terms of the relationship between vitamin D and RANK/RANKL/OPG system in human study, vitamin D supplementation was shown to suppress osteoclastogenesis by decreasing the production of RANKL and by stimulating osteoblast function via alkaline phosphatase (ALP) and bone specific ALP in

postmenopausal women (233). The study also suggested that vitamin D is significantly involved in RANK/RANKL/OPG system contribute to the treatment of osteopenia/osteoporosis.

Inflammation is the primary factor in various chronic diseases including osteoporosis, diabetes mellitus, HIV, RA, IBD, and ankylosing spondylitis, to mention a few. Furthermore, there is a link between chronic inflammatory process and serum concentration of 25-(OH)D₃ in that hypovitaminosis D is observed in many of these conditions. A cohort drawn from the Baltimore Hip Studies demonstrated that women above 65 years of age with hip fracture were found to have vitamin D deficiency (i.e., 25-(OH)D₃ < 15 ng/mL or 37.5 nmol/L) and elevated IL-6, suggesting the elevation of pro-inflammatory cytokines may be associated with vitamin D deficiency (234). In a meta-analysis, serum concentrations of 25-(OH)D₃ were found be associated with anti-inflammatory effects and reduced susceptibility to gingival inflammation (235).

Studies have shown that prolonged or chronically ill patients had increased bone resorption and osteoblast dysfunction (236,237) and had very low serum concentrations of 25-(OH)D₃ and 1,25(OH)₂D₃ (236,238,239). Often, lack of vitamin D contributes to bone loss in prolonged or chronically ill patients. Studies showed the negative correlation between serum 1,25(OH)₂D₃ and TNF- α may impair 1 α -OHase and aggravate its activity (238,239). It is known that kidney has the most abundant CYP27B1, which is the gene encoding 1 α -OHase, thus the dysfunction of 1 α -OHase may alter CYP27B1. It would be of interest to examine whether chronic inflammation alters CYP27B1 in the tissue, especially the kidney, of animals undergoing chronic low-grade inflammation.

While clinical studies have demonstrated an association between inflammation and vitamin D, animal studies have been used to further examine this relationship. An *in vivo* study utilizing male Sprague-Dawley rats showed that high intestinal VDR is positively correlated with high 1,25(OH)₂D₃ activity (240). In 1 α -OHase (CYP27B1) deficient mice, hypocalcemia, secondary hyperparathyroidism, retarded growth, and skeletal abnormalities (rickets) were observed in addition to a reduction in CD4⁺ (helper) and CD8⁺ (cytotoxic) T-lymphocytes. All of these symptoms are associated with defective immune function, further supporting that 1 α -OHase is essential in skeletal homeostasis and regulation of immune function (241). While VDR knockout (KO) mice have provided insight into the role of 1,25(OH)₂D₃ in monocytes/macrophage differentiation, monocytes/macrophage differentiation can also occur in the absence of VDR, suggesting monocytes/macrophage can differentiate via other mechanisms (242). Another animal study utilizing Sprague-Dawley rats showed that bone expressed CYP27B1 and CYP24A1, where CYP24A1 expression is regulated by the synthesis of 1,25(OH)₂D₃ in bone and 1,25(OH)₂D₃ synthesis is associated with bone mineralization (243). A dextran sodium sulfate (DSS)-induced mouse model of colitis showed a 5-fold decrease in renal CYP27B1 and lower serum 1,25(OH)₂D₃ (244). These studies indicate that chronic inflammatory conditions are associated with low vitamin D and that low vitamin D is related to defective immune function.

The role of LPS, a bacterial toxin, on vitamin D system has been examined *in vitro* utilizing THP-1 cells, a human blood monocytic cell line (223). LPS decreased the expression of the VDR protein and down-regulated VDR function. Furthermore, LPS impaired 1,25(OH)₂D₃/VDR by decreasing the ability of 1,25(OH)₂D₃ to induced

myeloid differentiation into monocytes/macrophages and thus decreasing the ability of bacteria killing by innate immune system(223). LPS is a potent stimulator of myeloid/macrophage cell production of TNF- α , which involved in chronic inflammatory disease such as RA and Crohn's disease (60). Currently, there are lack of studies examining the relationship between LPS and vitamin D metabolites *in vivo*. The role of liver and kidney in the systemic synthesis of 1,25(OH) $_2$ D $_3$ is well known, but more recently local synthesis has been demonstrated *in vitro*. *In vitro* human blood mononuclear cells were used to show that monocyte-derived macrophages and monocyte-derived dendritic cells are able to hydroxylate vitamin D $_3$ into 1,25(OH) $_2$ D $_3$. These data suggests that local 1,25(OH) $_2$ D $_3$ synthesis occurs in myeloid cells of the skin and gut (245). Based on this evidence that 1,25(OH) $_2$ D $_3$ is synthesized locally in various tissues and LPS impairs 1,25(OH) $_2$ D $_3$ /VDR functions (222, 244), it is reasonable to anticipate that LPS-induced chronic inflammation would affect local and/or systemic production of vitamin D.

To date, we are not aware of any studies that have examined the relationship between the effects of LPS-induced chronic inflammation on local and systemic vitamin D metabolism *in vivo*. The availability of 1,25(OH) $_2$ D $_3$ in tissues is dependent on genes encoding vitamin D activating and deactivating enzymes, CYP27B1 and CYP24A1 (246). Therefore, by examining genes such as CYP27B1 and CYP24A1 in tissues especially in liver, spleen, and kidney *in vivo* we will gain insight into the manner by which chronic inflammation affects key hydroxylation enzymes involved in vitamin D metabolism and whether chronic inflammation alters vitamin D metabolism.

CHAPTER III

METHODOLOGY

Animals and Diet

Male C57BL/6J mice used in these experiments (Jackson Laboratory, Bar Harbor, ME) were housed in an environmentally controlled animal care facility (22-25°C temperature, 12 light:12 dark conditions) and allowed to acclimate for 5-7 days prior to the start of the experiments. Mice were fed semi-purified AIN-93M diet and had access to reverse osmosis (RO) water *ad libitum* throughout both studies (47,247). All procedures were approved by the Oklahoma Christian University of Arts and Sciences or Oklahoma State University Institutional Animal Care and Use Committees.

Study Design for Experiment 1

Experiment 1 was designed to evaluate the dose and time response of LPS-induced low-grade chronic inflammation in the animals using slow-release pellets. Twelve-week-old male C57BL/6J mice were randomly assigned to three groups (n= 12 mice/group) for a study duration of 30 or 90 days: LPS1=0.01 mg LPS /kg body weight/day, LPS2=0.1 mg LPS /kg body weight/day), or Control (0 mg LPS/ kg body weight/day). Slow-release pellets (Innovative Research of America, Sarasota, FL)

LPS (*E. coli* Serotype 0127:B8; chromatographically pure, Sigma, St. Louis, MO) were implanted subcutaneously in the interscapular region of C57/BL6J mice. The minor surgery required that mice be anesthetized using an intramuscular injection of a ketamine/xylazine cocktail (10/1 mg/ kg body weight). The site of the incision (i.e., ~2 cm distal to the interscapular region) was cleaned with 70% ethanol and shaved, and sterile forceps were used to make a subcutaneous tunnel for pellet placement. The incision was closed with a single suture and mice were allowed to recover under a warming light prior to return to their cage.

At the end of the 30 and 90 day study period, animals were fasted for 12 hours prior to necropsy and then anesthetized (10/1 mg/kg body weight ketamine/xylazine cocktail) and sacrificed by exsanguinations via the carotid artery. Blood was collected in an EDTA-coated Pasteur pipet and bone specimens were dissected and cleansed of all soft tissues. Blood samples were centrifuged at 4000 rpm (3220 x g) at 4°C (Eppendorf Centrifuge, Refrigerated Model 5810 R, Hamburg, Germany) for 20 minutes and aliquots of plasma were stored at -80°C for later analyses. Soft-tissues (i.e., liver and spleen) were immediately frozen in liquid nitrogen.

Study Design for Experiment 2

Experiment 2 was designed to investigate the short-term effects of LPS on the animals using a daily intraperitoneal (i.p.) injection. Animals were allowed to acclimate for 5-7 days and given semi-purified AIN-93M diet prior to the start of the study. Thirty, 16-week old male C57BL/6J mice were randomly assigned to one of the three

groups (n=10 mice/group): control=0 mg LPS /kg body weight/day), LPS1=0.1 mg LPS /kg body weight/day or LPS2=0.2 mg LPS /kg body weight/day). In the current study, LPS (*E. coli* Strottype 0127:B8; chromatographically pure, Sigma, St. Louis, MO Sigma) was administered i.p. at approximately the same time each day for 14 consecutive days. Body weights were recorded every 3 days throughout the 14-day study period.

At the end of the 14-day study period, animals were fasted for 12 hours prior to necropsy. Animals were then anesthetized with an intramuscular (i.m.) injection of ketamine/xylazine cocktail (10/1 mg/ kg body weight), then body composition, whole body BMD, BMC, and bone mineral area (BMA) were determined by dual-energy x-ray absorptiometry (DXA) using a GE Lunar PIXImus (GE Medical Systems, Madison, WI). The instrument was calibrated according to the manufacturer's protocol prior to the whole body scans and quality control, was demonstrated by a coefficient of variation for BMD of <1% and a percent fat +/- 1.0 SD. Animals were then sacrificed by exsanguination via the carotid artery. Whole blood was collected in EDTA-coated Pasteur pipet and blood smears were prepared. Whole blood samples were centrifuged at 4000 rpm (3220 x g) at 4°C for 20 minutes and aliquots of plasma were stored at -80°C for later analyses. Soft-tissues including the liver, spleen, and kidney were carefully isolated, weighed, and immediately placed in liquid nitrogen.

Blood Leukocyte Counts

Whole blood was collected at the termination of the study for total and differential white blood cell (WBC) counts. 25 µL whole blood was added to microcentrifuge tube containing 475 µL of diluting buffer (1:20 dilution), which was prepared with 98 mL

deionized water, 1 mL glacial acetic acid, 1 mL 1% gentian violet and the whole blood/diluting buffer mixtures were stored at room temperature. Cell counts were performed using Neubauer hemacytometer (Bright-Line™, Hausser Scientific, Buffalo, NY) under a light-microscope.

Assessment of Bone Microarchitecture using Microcomputed Tomography (μ CT)

Tibia specimens from Experiment 1 were scanned to determine the alterations in trabecular metaphyseal and cortical bone microarchitecture using x-ray microcomputed tomography (μ CT40, SCANCO Medical, Switzerland). For trabecular bone analyses, the proximal end of tibia was scanned using an isotropic voxel size of 6 μ m in a 2048 x 2048 matrix to acquire 175 slices (6 μ m/slice) distal to the growth plate. Trabecular region was analyzed by placing contours beginning 25 slices from the growth plate to incorporate only the secondary spongiosa and 100 slices (600 μ m) of the trabecular region were evaluated within the volume of interest (VOI). The trabecular parameters that were assessed included trabecular bone volume expressed per unit of total volume (BV/TV), trabecular number (TbN), trabecular thickness (TbTh), trabecular separation (TbSp), structure model index (SMI), bone connectivity density (Conn Den), and linear x-ray attenuation coefficient (Lin Atten).

For cortical analyses, the mid-shaft of the tibia was scanned and 20 slices (120 μ m) were evaluated within the VOI. Cortical bone parameters were assessed at the tibia mid-shaft, including cortical thickness, area, and porosity and medullary area.

Assessment of Plasma 25-hydroxyvitamin D₃ using ELISA

Plasma 25-hydroxyvitamin D (25-(OH)D₃) was assessed using an enzyme immunoassay (ELISA) (Immunodiagnostic Systems, Fountain Hills, AZ). Reagents were prepared according to the manufacturer's protocol. The colorimetric assay was read on a Synergy HT multi-detection microplate reader (BIO-TEK, Winooski, VT) and absorbance of each well at 450 nm was determined using BIO-TEK KC4 (software version 3.4). This assay had a sensitivity of 5 nmol/L (i.e. 2 ng/mL) with intra and inter-assay coefficients of variation at < 8% and < 10% respectively.

RNA Isolation

RNA was isolated from the liver and spleen in Experiment 1 and from the liver, spleen, and kidney in Experiment 2. RNA from the liver and kidney were isolated with the following protocol. Five to six specimens from each treatment group were randomly selected for quantitative real-time polymerase chain reaction (qRT-PCR) analyses. Total RNA was isolated from tissues using STAT-60 reagent according to the manufacturer's instructions (Tel-Test, Inc., Friendswood, Texas). In short, the tissue was homogenized in the RNA STAT-60 reagent using a polytron homogenizer (VWR PowerMax™ Advanced Homogenizing Systems, 200, Thorofare, NJ). The homogenate was allowed to incubate for 5 minutes at room temperature to permit the complete dissociation of ribonucleoprotein complexes. Next, 0.2 ml of chloroform per ml of the RNA STAT-60 was added to the homogenate and shaken vigorously for 15 seconds, and incubate at room temperature for 2-3 minutes. The homogenate was centrifuged at 12,000 x g for 15 minutes at 4°C. Following centrifugation, the aqueous (upper) phase was transferred to a

fresh microtube and 0.5 mL isopropanol /mL of STAT-60 was added for RNA precipitation. The sample remained on ice for 10 minutes and then centrifuged at 12,000 x g for 10 min at 4°C. Supernatant was removed and the precipitated RNA pellet was washed with 75% ethanol and centrifuged (12,000 x g) for 15 min at 4°C. At the end of the procedure, the RNA pellet was briefly air-dried for 2-3 min and then resuspended in diethylpyrocarbonate (DEPC)-treated water. The purity of RNA was determined by optical density at 260 and 280nm spectrophotometry NanoDrop spectrophotometer (ND-1000, version 3.1.2, Wilmington, DE).

In contrast, RNA from the spleen were isolated using a RNA Mini Kit (PureLink™, Invitrogen Corp., Carlsbad, CA). Reagents were prepared and RNA was isolated according to the manufacturer's protocol. DNase (PureLink™, Invitrogen Corp., Carlsbad, CA) was used for on-column digestion of DNA to obtain DNase free total RNA. Similar to the RNA from the liver and kidney, the purity of spleen RNA was determined by optical density measured at 260 and 280 on NanoDrop spectrophotometer (ND-1000, version 3.1.2, Wilmington, DE).

Analysis of Gene Expression using Quantitative Real-time PCR

For qRT-PCR analysis, 2 µg RNA was treated with DNase I (Roche, Penzberg, Germany) and reverse-transcribed using SuperScript II according to the manufacturer's protocol. qRT-PCR was performed on 7900HT Fast Real-time PCR System (Sequence Detection System Version 2.3, Applied Biosystems, Foster City, CA) using SYBR Green Chemistry. PCR reactions (10 µl) were performed in duplicate on a 384-well plate with 10 µl per well. Results were evaluated by the comparative cycle number at threshold (C_T)

method (User Bulletin No. 2; Perkin-Elmer Life Sciences) using cyclophilin as the invariant control gene, Cyclophilin b (247). Primer sequences for genes of interest are provided in **Table 2**.

Statistical Analysis

Differences in treatment groups were determined using multiple paired t-tests with SAS statistical software (version 9.1.2; SAS Institute Inc., Cary, NC). Data are presented as means \pm standard error of mean (SEM) and $p < 0.05$ were considered statistically significant for all analyses.

Table 2. *Mus musculus* Primer Sequences.

Symbol	Name	Accession Number	Primer Sequence (5' → 3')
LPS Receptor Complex			
TLR4	Toll-like receptor 4	NM_021297	F 5'ACTGTTCTTCTCCTGCCTGACA3' R 5'TGATCCATGCATTGGTAGGTAATA3'
CD14	CD14 antigen	NM_009841	F 5'GCCGCCACCGCTTCT3' R 5'ACACGTTGCGGAGGTTCA3'
Pro-inflammatory Cytokines			
MIP-1α	Macrophage inflammatory protein-1alpha	NM_011337	F 5'TTCATCGTTGACTATTTTGAAACCA3' R 5'GCCGGTTTCTCTTAGTCAGGAAa3'
STAT3	Signal transducer and activator of transcription 3	NM_213659	F 5'CCATTGACCTGCCGATGTC3' R 5'CTCAGCACCTTACCAGTTATTTC3'
TNF-α	Tumor necrosis factor-alpha	NM_013693	F 5'CTGAGGTCAATCTGCCCAAGTAC3' R 5'CTTACAGAGCAATGACTCCAAAG3'
IL1-β	Interleukin 1 beta	NM_008361	F 5'CAACCAACAAGTGATATTCTCCTAG3' R 5'GATCCACACTCTCCAGCTGCA3
IL-6	Interleukin 6	NM_031168	F 5'GAGGATAACCACTCCCAACAGACC3' R 5'AAGGCATCATCGTTGTTCATACA3'
Anti-inflammatory Cytokines			
IL-10	Interleukin 10	NM_010548	F 5'GGTTGCCAAGCCTTATCGGA3' R 5'ACCTGCTCCACTGCCTTGCT3'
IL-4	Interleukin 4	NM_021283	F 5'ACAGGAGAAGGGACGCCAT3' R 5'GAAGCCCTACAGACGAGCTCA3'
TGF-β1	Transforming growth factor, beta 1	NM_011577	F 5'GACCCTGCCCTATATTTGGA3' R 5'CCGGGTTGTGTTGTTGTAGA3'
Vitamin D Metabolism and Internal Control			
CYP27A1	Cytochrome P450, family 27, subfamily a, polypeptide 1	NM_024264	F 5'GCCTCACCTATGGGATCTTCA3' R 5'TCAAAGCCTGACGCAGATG3'
CYP27B1	Cytochrome P450, family 27, subfamily b, polypeptide 1	NM_010009	F 5'AGCAGCTCCTGCGACAAGAA3' R 5'CGTTAGCAATCCGCGAAGCA3'
CYP24A1	Cytochrome P450, family 24, subfamily a, polypeptide 1	NM_009996	F 5'TGCCCTATTTAAAGGCCTGTCT3' R 5'CGAGTTGTGAATGGCACACTT3'
VDR	Vitamin D receptor	NM_009504	F 5'GGCTTCCACTTCAACGCTATG3' R 5'ATGCTCCGCCTGAAGAAAC3'
Cyclo	Cyclophilin b	NM_011149	F 5'TGGAGAGCACCAAGACAGACA3' R 5'TGCCGGAGTCGACAATGAT3'

CHAPTER IV

FINDINGS

EXPERIMENT 1: LPS-induced Inflammation via Slow-Release Pellets Over 30 and 90 Days

Body Weight

Animals were weighed every other week and no alterations in body weight between treatment groups were observed over the 30- and 90-day study periods (*Data not shown*).

Bone Microarchitecture Properties

At 30 days, animals in LPS2 (0.1 mg LPS /kg body weight/day) group exhibited a significant decrease in trabecular BV/TV, TbTh, and Conn Denn, and a significant increase in SMI. No alterations were observed in TbN and TbSp at this time point. After 30-days of LPS exposure, cortical porosity of tibial mid-diaphysis was also increased in animals from the LPS2 group. The increased in medullary area was the only alteration observed in the cortical bone following 90-day LPS exposure (**Table 3**).

Plasma 25-hydroxyvitamin D₃

Plasma 25-(OH)D₃ was assessed to determine the extent to which LPS-induced inflammation at 30 and 90 days altered circulating vitamin D. At 30 days, neither LPS1 nor LPS2 altered plasma 25-(OH)D₃ (**Figure 2**). Unfortunately, plasma 25-(OH)D₃ data at 90 days was not available due to insufficient sample volume.

Gene Expression Analysis by qRT-PCR

At the end of 30 days of LPS exposure using slow-release pellets, hepatic TLR4, CD14 and pro- and anti-inflammatory cytokines in both LPS-treated groups were not altered (**Figure 3 A**). Similarly, hepatic genes encoding vitamin D metabolism were not altered by 30-day LPS exposure (**Figure 3 B**). At the end of 90 days of LPS exposure using slow-release pellets, IL-10 (pro-inflammatory) and IL-1 β (anti-inflammatory) cytokines, were significantly down-regulated in LPS1 group (**Figure 4A**). Hepatic CYP27A1 was significantly down-regulated by LPS2 (**Figure 4 B**).

Following 30-day LPS exposure, alterations in inflammatory mediators, genes involved in the activation and deactivation of vitamin D and the VDR were assessed in the spleen. Splenic TLR4, CD14 and pro- and anti-inflammatory cytokines were not altered due to LPS following 30-day (**Figure 5 A**). Similarly, 30-day LPS exposure did not alter splenic genes encoding vitamin D metabolism (**Figure 5 B**). At the end of 90 days LPS exposure, LPS2 tended to increase ($p = 0.06$) splenic TNF- α whereas splenic IL-6 was elevated in the LPS1 compared to control groups and an intermediate effect was observed on IL-6 in response to LPS 2 (**Figure 6 A**). Splenic CYP27B1 significantly

down-regulated in LPS2 group whereas splenic VDR tended to decrease ($p = 0.07$) in LPS2 group at the end of 90 days LPS exposure (**Figure 6 B**).

EXPERIMENT 2: LPS-induced Inflammation via Intraperitoneal Injections Over 14 Days

Body and Tissue Weights

The body weights of animals were not significantly altered over the course of the 14-day study period (**Table 4**). No alterations were observed in the spleen and kidneys weights of animals in the two LPS-treated groups compared to controls (**Table 4**).

Bone Density and Composition

DXA scans were performed prior to necropsy to determine if the low grade inflammation induced by LPS injections affected bone density and composition. Whole body bone parameters including, BMD, BMC, and BMA were not altered following 14-day of LPS treatment which may have been anticipated due to the short study duration. In the case of body composition, lean and fat mass, and percent body fat remained unchanged at the end of the 14-day study period (**Table 5**).

Blood Leukocyte Counts

Blood leukocytes were determined light-microscopy to assess if LPS injections altered the systemic inflammatory state. Mean total white blood cells (WBC) (n = 8- 10 per group) were: Control $4.30 \times 10^6 \pm 0.94$, LPS1 $4.74 \times 10^6 \pm 0.58$, and LPS2 4.00×10^6

± 0.36 . There was no significant difference in the WBC among the three different LPS treatment groups. WBC differentials showed LPS1 (0.1 mg LPS /kg body weight/day) significantly increased monocytes after 14 days of LPS administration (**Figure 7 A**). No alterations were observed in percentage of neutrophils, neutrophils/monocytes ratios (**Figure 7 B and C**), eosinophils and basophils for the two LPS-treated groups.

Plasma 25-hydroxyvitamin D₃

The dose-dependent effects of LPS exposure over time were assessed in the circulating 25-(OH)D₃. Plasma 25-(OH)D₃ of animals receiving LPS1 and LPS2 were significantly elevated compared to controls. No significant difference was found in plasma 25-(OH)D₃ of LPS1 and LPS2 (**Figure 8**).

Gene Expression Analysis by qRT-PCR

CD14 and TLR4 gene expression was assessed to determine if an LPS response occurred following 14-day LPS administration. Hepatic CD14 and TLR4 gene expression was significantly up-regulated in LPS1 and LPS2 compared to controls, confirming the LPS response (**Figure 9 A**). The pro-inflammatory macrophage inflammatory protein-1 α (MIP-1 α) and downstream effector STAT3 were not altered with either dose of LPS (**Figure 9 A**).

To assess the inflammatory response after 14 days of LPS injection, hepatic gene expression of TNF- α , IL-1 β , IL-6 (pro-inflammatory) and IL-10, IL-4, and TGF- β (anti-inflammatory cytokines) were evaluated. Hepatic IL-10 and TGF- β expression was significantly up-regulated in LPS2 compared to controls (**Figure 9 B**). No significant

alteration was seen in hepatic TNF- α , IL-1 β , IL-6, and IL-4 expression (**Figure 9 B**). Genes involved in vitamin D metabolism (CYP27A1 and VDR) in hepatic tissue were assessed following 14-day LPS treatment and no significant alterations were observed (**Figure 9 C**).

The inflammatory response to LPS in the spleen was assessed by the expression of TLR4, CD14, TNF- α , IL-1 β , IL-6, and IL-10. Splenic IL-1 β tended to decrease ($p = 0.05$) in LPS1 group and no significant alterations were observed in LPS receptor complex, and the other pro- and anti-inflammatory cytokines analyzed (**Figure 10 A**). Splenic VDR expression tended to be decreased ($p=0.08$) with LPS2 (**Figure 10 B**).

In addition to the determining the influence of 14 days of LPS on liver and spleen gene expression, kidney, another major site of vitamin D metabolism and activity was examined. The pro-inflammatory cytokine IL1- β expression in the kidney was up-regulated in LPS1 but not in LPS2 (**Figure 11 A**). LPS1 and LPS2 significantly down-regulated kidney CYP27B1 expression compared to controls, however, kidney CYP27B1 expression was not different between the two LPS-treated groups (**Figure 11 B**). Furthermore, kidney CYP24A1 expression significantly elevated in LPS1 compared to controls but not in LPS2 suggesting that LPS1 may increase the catabolism of 1,25(OH) D_3 (**Figure 11 B**).

Table 3. Trabecular and Cortical Bone Microarchitectural Properties of the Proximal Tibia Metaphysis and the Tibia Mid-diaphysis following 30 and 90 Days of LPS Treatment.

Parameters	30 days			90 days		
	Control	LPS 1	LPS 2	Control	LPS 1	LPS 2
<u>Trabecular Bone</u>						
BV/TV (%)	19.9 ± 1.7 ^a	14.6 ± 2.5 ^a	11.6 ± 0.9 ^b	16.9 ± 1.5	18.1 ± 1.7	13.0 ± 2.3
TbN (1/mm ³)	5.33 ± 0.09 ^a	4.67 ± 0.30 ^b	4.92 ± 0.13 ^a	4.42 ± 0.19 ^a	5.05 ± 0.12 ^b	4.53 ± 0.10 ^a
TbTh (mm)	0.058 ± 0.003 ^a	0.049 ± 0.003 ^b	0.045 ± 0.002 ^b	0.059 ± 0.003 ^a	0.055 ± 0.003 ^a	0.049 ± 0.003 ^b
TbSp (mm)	0.18 ± 0.04 ^a	0.22 ± 0.02 ^b	0.20 ± 0.01 ^a	0.23 ± 0.01 ^a	0.19 ± 0.01 ^b	0.22 ± 0.01 ^a
<u>Cortical Bone</u>						
Cort Th (mm)	0.25 ± 0.11	0.24 ± 0.01	0.23 ± 0.00	0.24 ± 0.01	0.24 ± 0.01	0.24 ± 0.01
Cort Area (mm ²)	0.86 ± 0.04	0.81 ± 0.05	0.72 ± 0.01	0.81 ± 0.04	0.83 ± 0.03	0.83 ± 0.05
Medullary Area (mm ²)	0.034 ± 0.003	0.037 ± 0.002	0.037 ± 0.001	0.029 ± 0.003 ^a	0.037 ± 0.001 ^b	0.035 ± 0.001 ^b
Cort Porosity (%)	3.8 ± 0.2 ^a	4.5 ± 0.3 ^b	4.8 ± 0.2 ^b	3.5 ± 0.4	4.3 ± 0.2	4.1 ± 0.2

Control (0 mg LPS /kg body weight/day), LPS 1 (0.1 mg LPS /kg body weight/day), and LPS 2 (0.2 mg LPS /kg body weight/day) on mean body weight of animals. Values for a given parameter that do not share the same superscript at 30 or 90 days are statistically different ($p < 0.05$). Values are mean ± SE, (n = 12/ group).

Table 4. Body and Tissue Weights following 14 Days of LPS Treatment.

	Control (n = 10)	LPS 1 (n=8)	LPS 2 (n = 10)
Body Weight (g)			
Baseline	27.94 ± 0.52	27.59 ± 0.56	28.55 ± 0.56
Final	26.73 ± 0.58	25.94 ± 0.69	27.63 ± 0.61
Tissue Weight (g)			
Spleen	0.08 ± 0.01	0.08 ± 0.01	0.09 ± 0.00
Kidney	0.29 ± 0.01	0.30 ± 0.01	0.31 ± 0.01

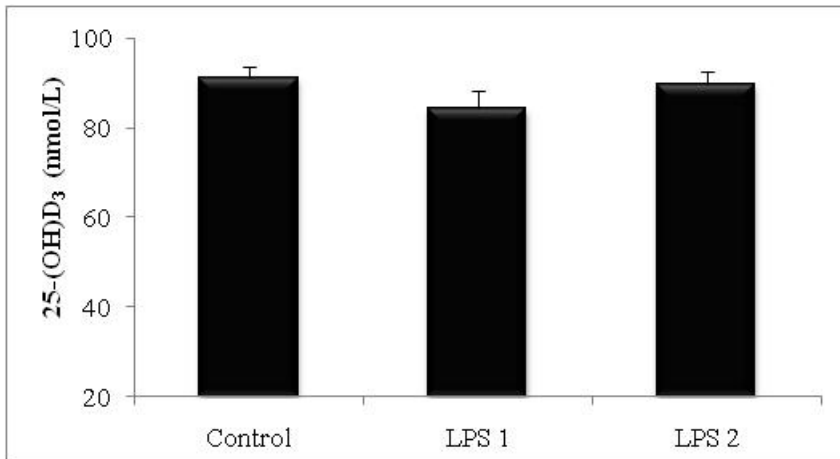
Control (0 mg LPS /kg body weight/day), LPS 1 (0.1 mg LPS /kg body weight/day), and LPS 2 (0.2 mg LPS /kg body weight/day) on mean body weight of animals. Values are mean ± SE.

Table 5. Bone Densitometry and Body Composition Parameters of Mice following 14 days of LPS Treatment.

Parameters	Control	LPS 1	LPS 2
Bone Densitometry			
BMA (cm²)	12.381 ± 0.35	11.924 ± 0.39	12.158 ± 0.16
BMC (g)	0.706 ± 0.03	0.662 ± 0.02	0.681 ± 0.01
BMD (g/cm³)	0.056 ± 0.001	0.055 ± 0.000	0.055 ± 0.000
Body Composition			
Lean (g)	20.32 ± 0.704	19.66 ± 0.676	21.88 ± 0.561
Fat (g)	5.84 ± 0.2	5.61 ± 0.1	5.60 ± 0.2
Fat (%)	22.36 ± 0.7	22.34 ± 0.7	20.34 ± 0.5

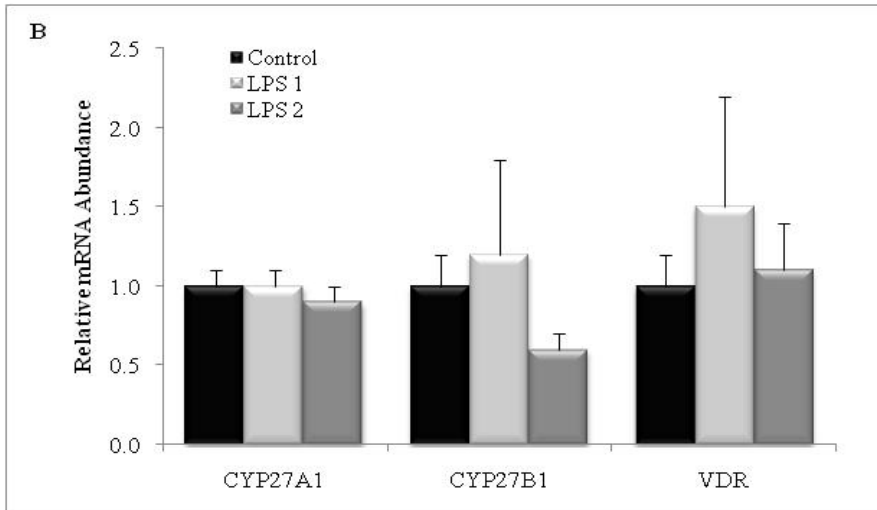
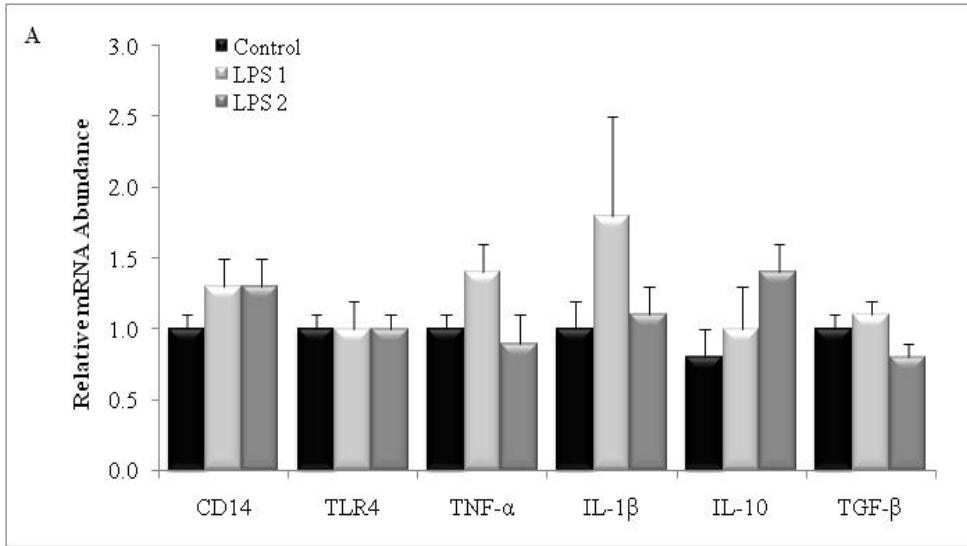
Control (0 mg LPS /kg body weight/day), LPS1 (0.1 mg LPS /kg body weight/day), and LPS2 (0.2 mg LPS /kg body weight/day). Values are mean ± SE.

Figure 2.



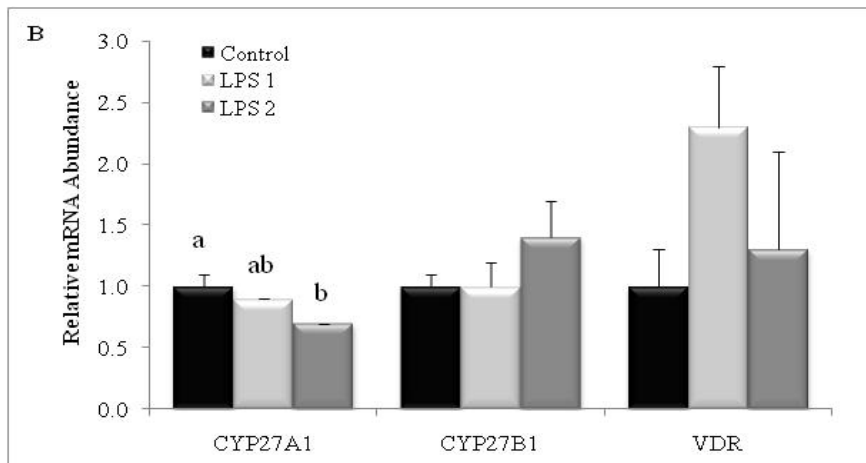
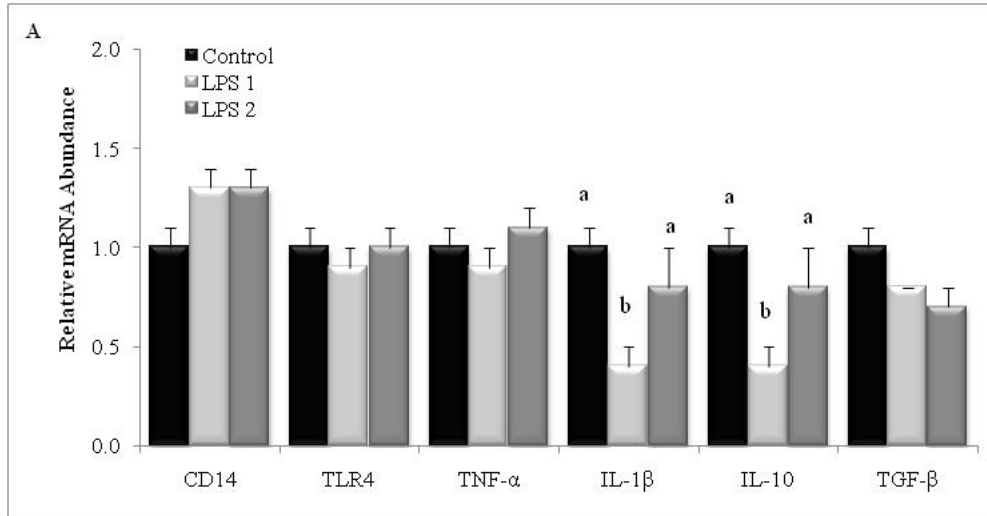
Plasma 25-(OH)D₃ following 30 Days of LPS Treatment. Control (0 mg LPS/ kg body weight/day), LPS 1 dose (0.01 mg LPS /kg body weight/day), and LPS 2 (0.1 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group (n=6-7/ group).

Figure 3.



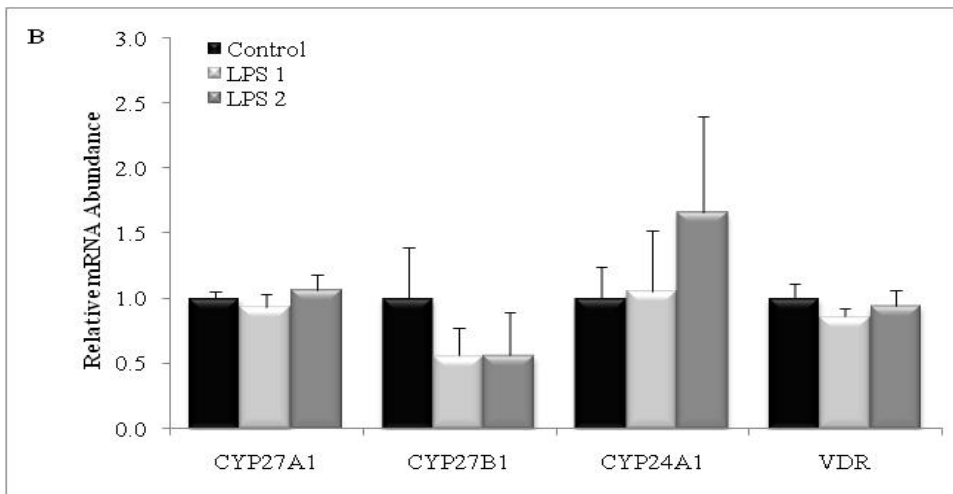
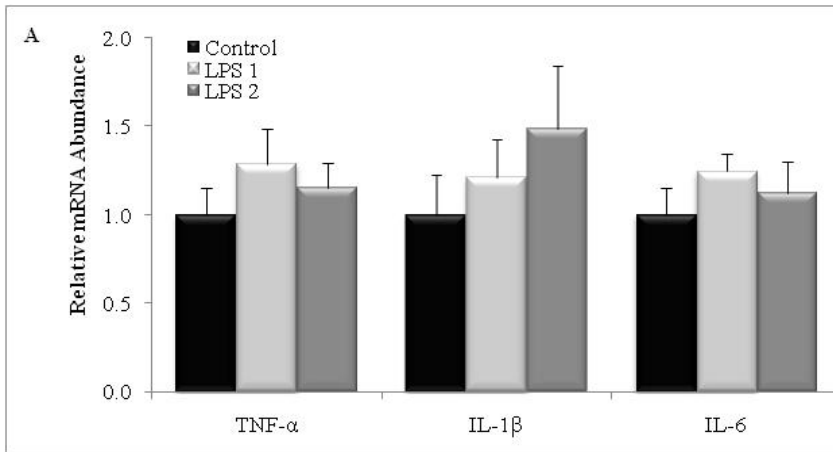
Hepatic Gene Expression Associated with (A) CD14, TLR4 and Inflammatory Mediators, and (B) CYP27A1, CYP27B1, and VDR following 30 Days of LPS Treatment. Control (0 mg LPS/ kg body weight/day), LPS 1 dose (0.01 mg LPS /kg body weight/day), and LPS 2 (0.1 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group (n= 4-5/group).

Figure 4.



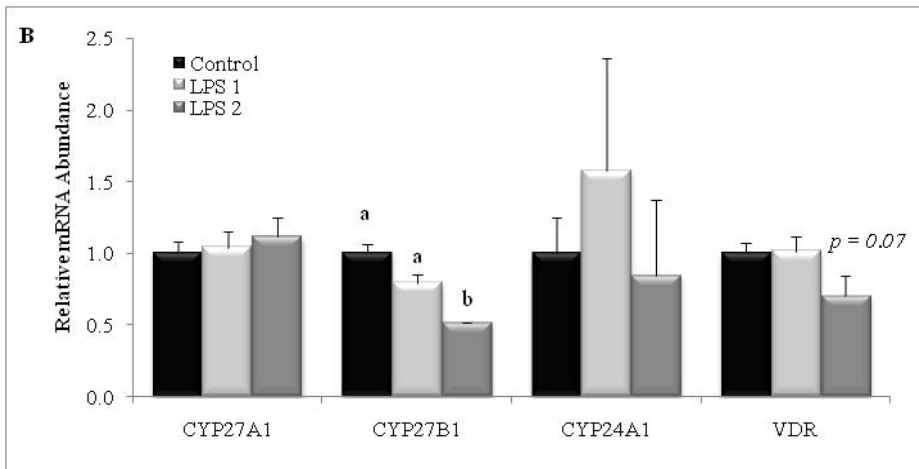
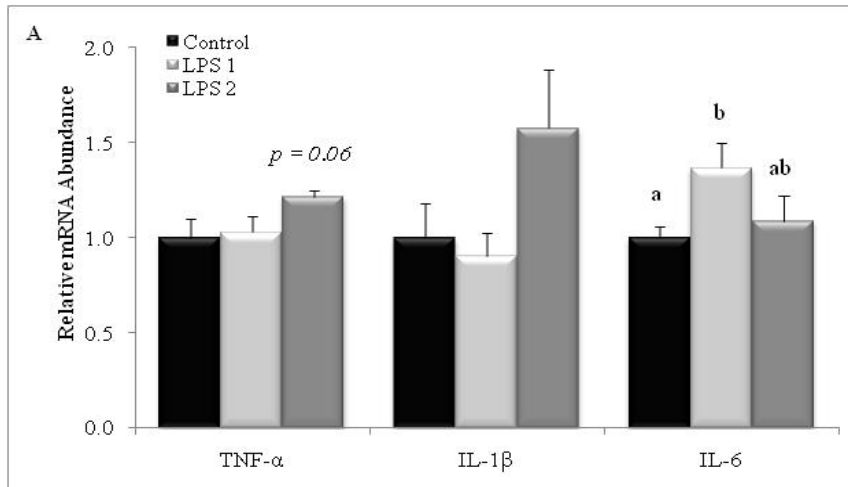
Hepatic Gene Expression Associated with (A) CD14, TLR4 and Inflammatory Mediators, and (B) CYP27A1, CYP27B1, and VDR following 90 Days of LPS Treatment. Control (0 mg LPS/ kg body weight/day), LPS 1 dose (0.01 mg LPS /kg body weight/day), and LPS 2 (0.1 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group. Bars for a given gene without superscript letter are not statistically different ($p < 0.05$) (n= 4- 6/group).

Figure 5.



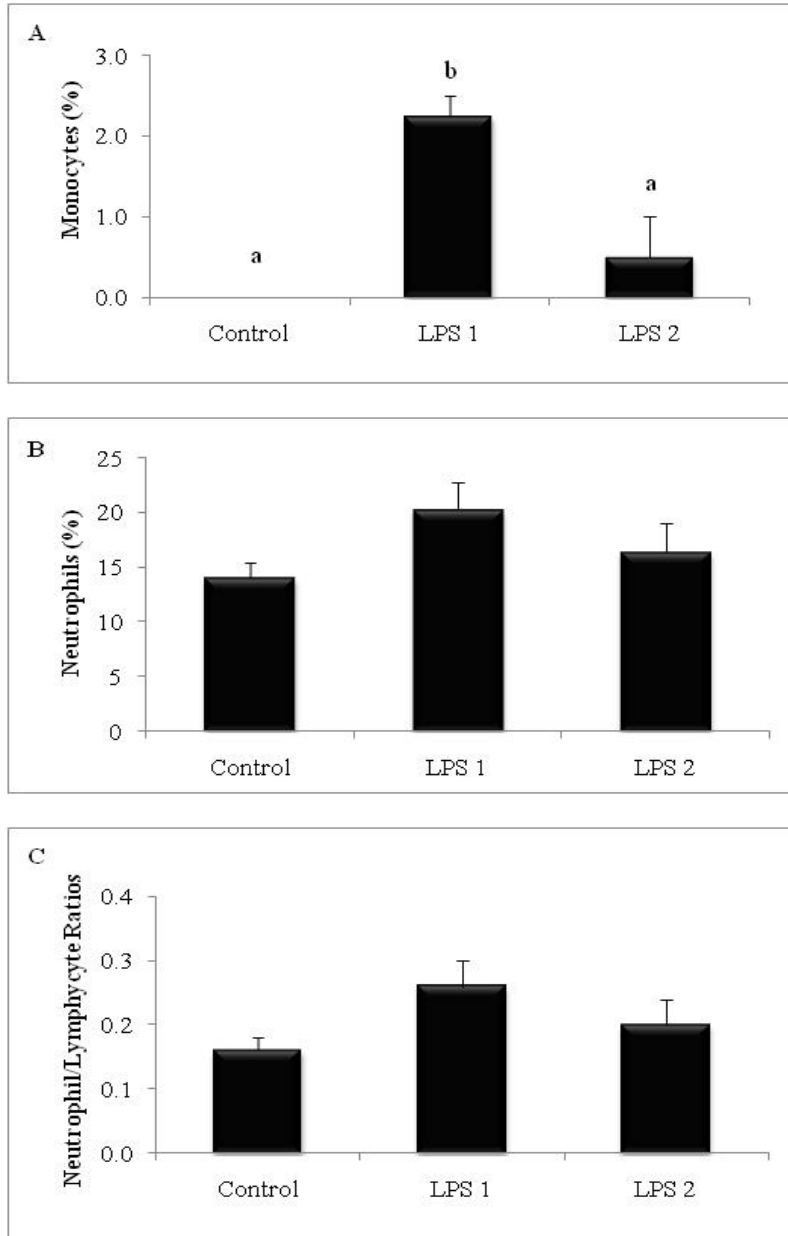
Splenic Gene Expression Associated with (A) Inflammatory Mediators, and (B) CYP27A1, CYP27B1, CYP24A1, and VDR following 30 Days of LPS Treatment. Control (0 mg LPS/ kg body weight/day), LPS 1 dose (0.01 mg LPS /kg body weight/day), and LPS 2 (0.1 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group. Bars for a given gene without superscript letter are not statistically different ($p < 0.05$) (n = 4- 5/ group).

Figure 6.



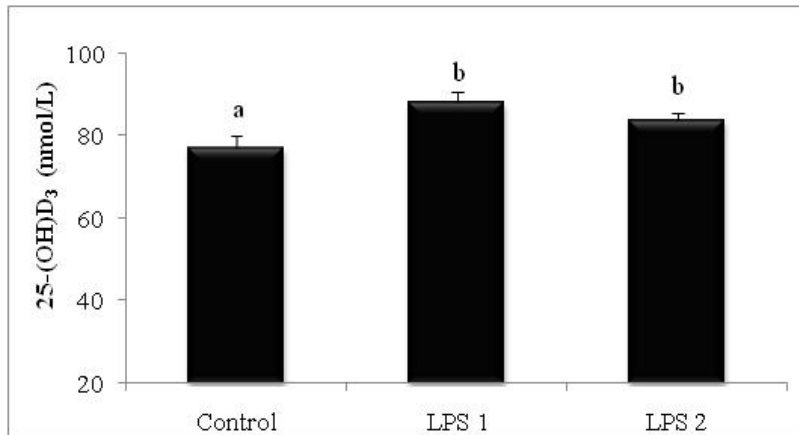
Splenic Gene Expression Associated with (A) Inflammatory Mediators, and (B) CYP27A1, CYP27B1, CYP24A1, and VDR following 90 Days of LPS Treatment. Control (0 mg LPS/ kg body weight/day), LPS 1 dose (0.01 mg LPS /kg body weight/day), and LPS 2 (0.1 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group. Bars for a given gene without superscript letter are not statistically different ($p < 0.05$) (n = 4- 5/group).

Figure 7.



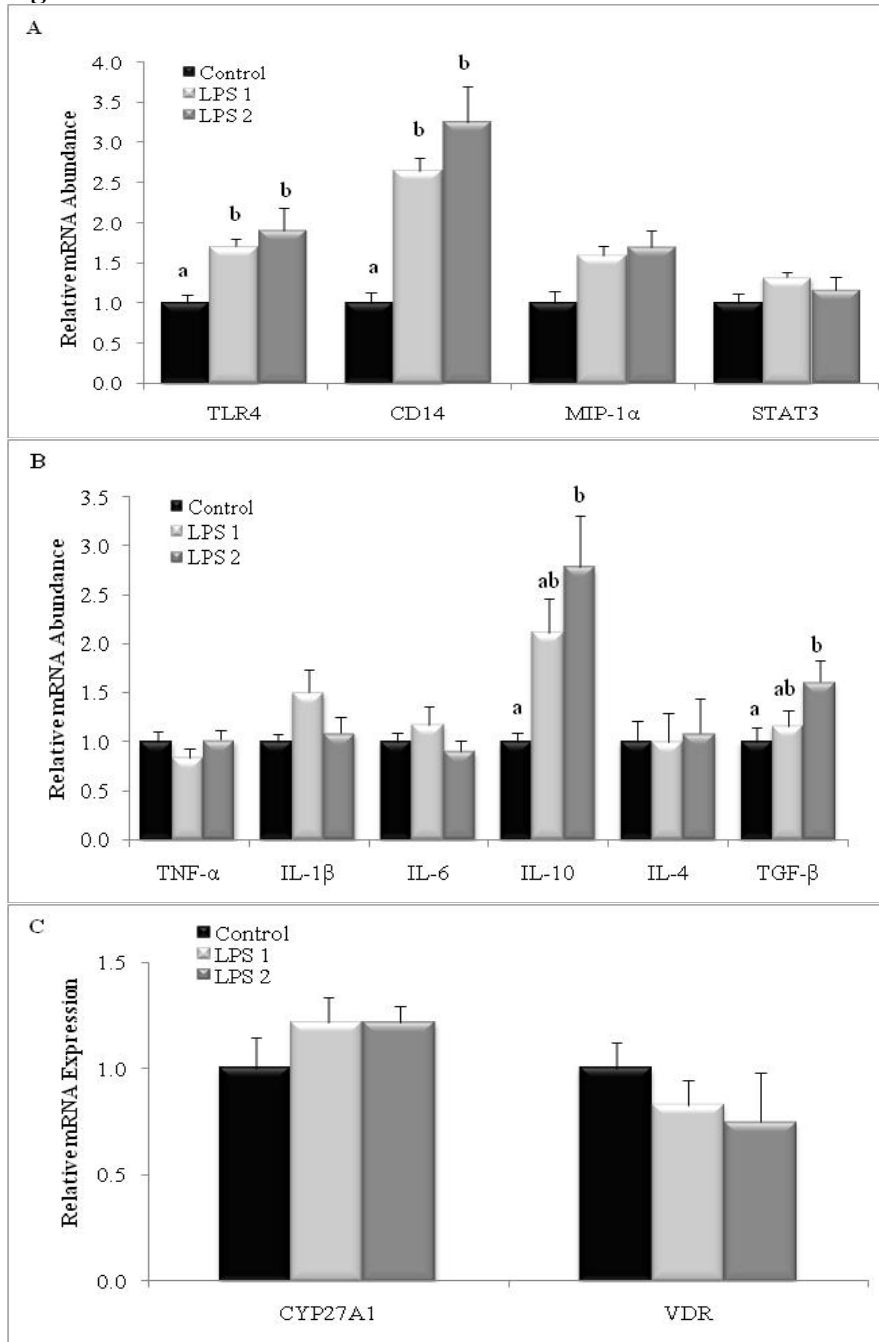
Alterations in Percentage of (A) Monocytes, (B) Neutrophils, (C) Neutrophils/Monocytes Ratios After 14 Days of LPS Treatment. Control (0 mg LPS /kg body weight/day), LPS 1 (0.1 mg LPS /kg body weight/day), and LPS 2 (0.2 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group. Bars without superscript letter are not statistically different ($p < 0.05$) (n=4 mice/group).

Figure 8.



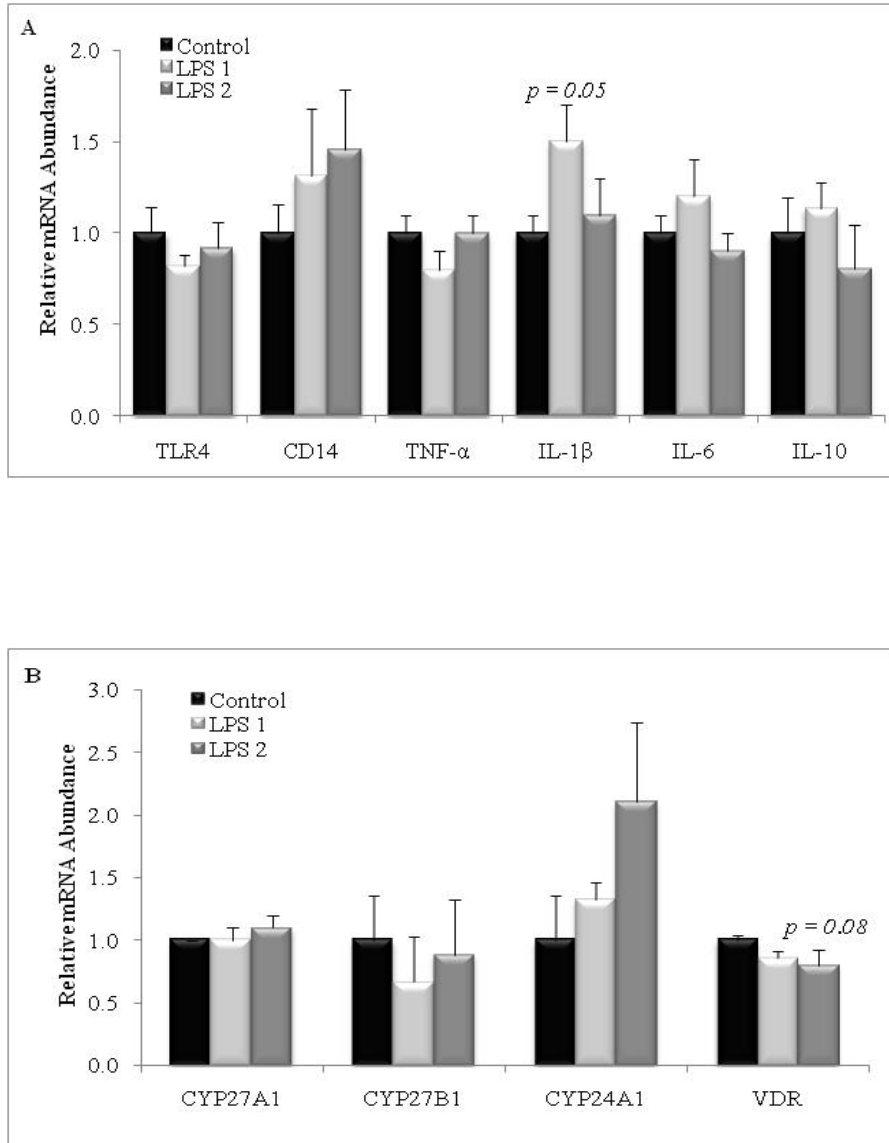
Plasma 25-(OH)D₃ following 14 Days of LPS Treatment. Control (0 mg LPS /kg body weight/day), LPS 1 (0.1 mg LPS /kg body weight/day), and LPS 2 (0.2 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group. Bars that do not share the same superscript letter are statistically different ($p < 0.05$) (n=8-10 mice/group).

Figure 9.



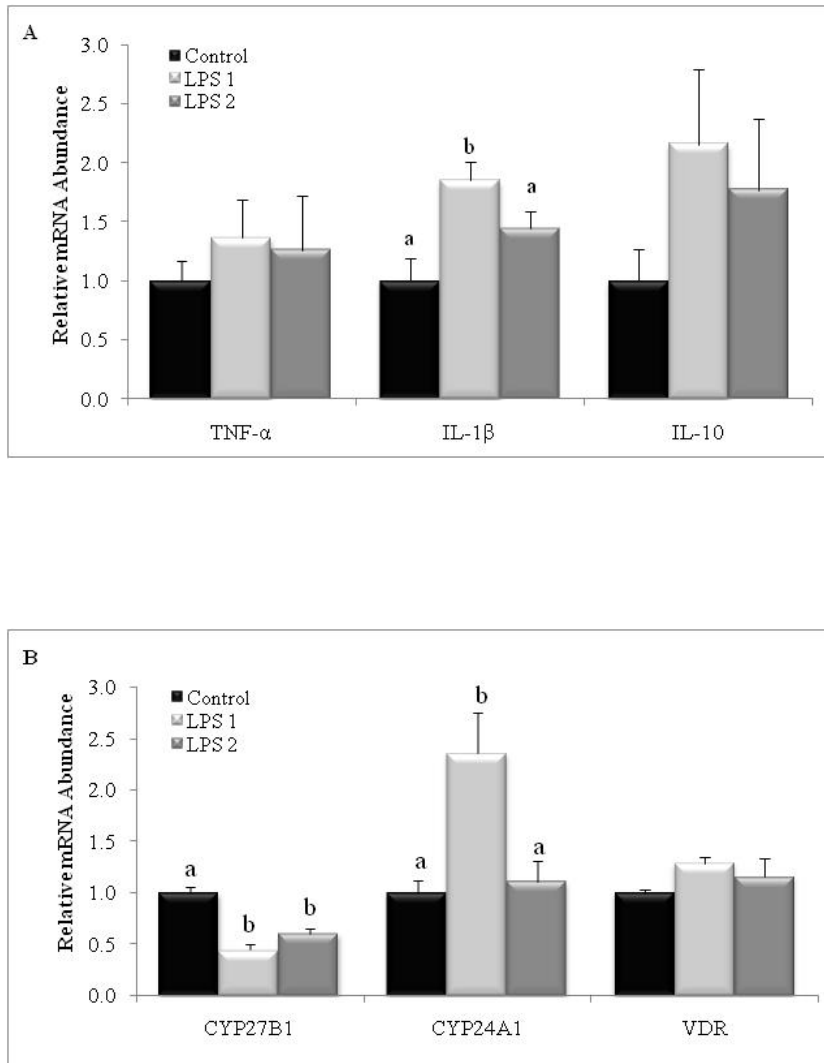
Hepatic Gene Expression Associated with (A) TLR4, CD14, MIP-1 α , and STAT3, (B) Inflammatory Mediators, and (C) CYP27A1 and VDR following 14 Days of LPS Treatment. Control (0 mg LPS /kg body weight/day), LPS 1 (0.1 mg LPS /kg body weight/day), and LPS 2 (0.2 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group. Bars of a given gene that do not share the same superscript letter are statistically different ($p < 0.05$) ($n = 5-6$ /group).

Figure 10.



Splenic Gene Expression Associated with (A) TLR4, CD14, and Inflammatory Mediators, and (B) CYP27A1, CYP27B1, CYP24A1, and VDR following 14 Days of LPS Treatment. Control (0 mg LPS /kg body weight/day), LPS 1 (0.1 mg LPS /kg body weight/day), and LPS 2 (0.2 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group. Bars of a given gene without superscript letter are not statistically different ($p < 0.05$) ($n = 6$ per group).

Figure 11.



Renal Gene Expression Associated with (A) Inflammatory Mediators and (B) CYP27B1, CYP24A1, and VDR following 14 Days of LPS Treatment. Control (0 mg LPS /kg body weight/day), LPS 1 (0.1 mg LPS /kg body weight/day), and LPS 2 (0.2 mg LPS /kg body weight/day). Bars represent the mean \pm SE for each treatment group. Bars of a given gene that do not share the same superscript letter are statistically different ($p < 0.05$) ($n = 5-6$ /group).

CHAPTER V

DISCUSSION

The overall objective of this study was to examine the effects of low dose LPS administration *in vivo* on inflammatory pathways and gene expression of VDR and key hydroxylation enzymes involved in vitamin D metabolism. Our laboratory previously reported that LPS delivered via slow-release pellets was shown to induce a low-grade chronic inflammation that resulted in bone loss in skeletally mature rat and mouse (47,48). Ninety days of LPS exposure in male Sprague-Dawley rats was associated with a decrease in femoral and vertebral BMD, with greater bone loss in the trabecular compared to the cortical regions (47). Additionally, fibrous tissue surrounding the coronary arterioles was observed, consistent with microvascular disease. Elevation of peripheral neutrophils and monocytes determined by differential white blood cell counts indicated that a continuous inflammatory response had occurred. Similar bone and vascular pathology were observed in C57BL/6J mice in a shorter-time period (i.e. 28 days).

In the current study, we were interested in determining if alterations in vitamin D metabolism were involved in the relationship between LPS, bone, and vascular pathology. To begin to explore this relationship, we first examined the inflammatory

response looking at TLR4, CD14 and pro- and anti-inflammatory cytokines in the liver and spleen. Studies have shown that LPS stimulates pro-inflammatory responses of Kupffer cells or resident hepatic macrophages, enhances TLR4 signaling and contributes to *in vivo* hepatic inflammation and injury (107,248-250). These studies suggested that LPS acts via TLR4 to initiate inflammatory response. Thus we wanted to verify the inflammatory response by examining TLR4 expression. We have shown here that in the hepatic tissue, 14 days of low dose LPS injection resulted in up-regulation of IL-10 and TGF- β , which coincided with increased CD14 and TLR4 gene expression. The LPS-induced up-regulation of hepatic CD14 in our study is similar to the response observed in human monocytes, where CD14 was shown to be up-regulated by LPS (251). *In vivo* LPS delivered via osmotic mini-pumps was shown to up-regulate hepatic IL-10 and TGF- β , which was consistent with our observation in the 14 days of low dose LPS injection study (252). LPS was shown dose-dependently increased TNF- α in isolated Kupffer cells from male Sprague-Dawley rats (107), however, hepatic TNF- α mRNA expression was not increased in our study. The lack of changes in hepatic TNF- α , IL-1 β and IL-6 mRNA levels may in part, be due to the suppression of IL-10, which was significantly up-regulated in hepatic tissue along with TGF- β in the low dose LPS group. The up-regulation of IL-10 has been shown to protect mice from septic shock (253). Pre-treatment of IL-10 *in vivo* was shown to reduce the LPS-induced release of TNF- α , verifying the efficacy of IL-10 in suppressing TNF- α production (254). Similarly TGF- β was shown to inhibit *in vitro* the macrophage response to LPS by down-regulating CD14 (255).

Furthermore, pro- and anti-inflammatory cytokines exhibit different peak and nadir times, thus choosing a time point that coincided with the nadir time would yield low gene expression levels. *In vivo* study of mice administered a single dose of 5 mg/kg LPS has shown that, circulating TNF- α and IL-10 as well as their hepatic mRNA expression peaked at 1.5 hour following the injection. After a nadir at 6 hour, a second peak was shown in 8 and 12 hours (256). At 24 hours, circulating TNF- α was decreased following administration of a single dose of 9 mg/kg LPS (257). In terms of hepatic IL-6 expression, it was increased within 2 hours after a single dose of LPS injection (5 mg/kg) and declined to normal level 8 hours post-injection (258). Keeping in mind that our samples were procured and analyzed 20 hours post injection, we might not observed an increase in hepatic TNF- α and IL-6 mRNA expression. Furthermore, we did not measure the circulating TNF- α and its expression in the supernatant, however, we measured its expression in the tissue.

In addition to the alterations in gene expression in the liver, we also evaluated the response of the spleen, a tissue that tends to have high numbers of monocytes (62,202). Monocytes/macrophages were shown to express CYP27B1 (62) and are the main producers of TNF- α in normal mice (257). Monocytes differentiate into macrophages, which then perform scavenging activities and at the same time enhance the release of inflammatory mediators. This response was demonstrated *in vitro*, in LPS-stimulated splenic macrophages isolated from mice in which, LPS activated macrophages and enhanced TNF- α release (263). We found that in response to 14 days of LPS administration via intraperitoneal injection, the only splenic inflammatory mediator that tended to be increased was IL-1 β in the group receiving the low dose of LPS. Thirty and

ninety days of LPS exposure did not alter splenic TNF- α , IL-1 β , however, at 90 days, splenic IL-6 was increased by LPS exposure. The low level of expression is likely related to the variability of time response of inflammatory mediators after LPS administration.

To investigate how LPS influenced vitamin D metabolism, we examined genes associated with hydroxylation with CYP27A1, CYP27B1, and CYP24A1 of vitamin D activation and deactivation and the expression of VDR in the liver, spleen, and kidney. The hepatic CYP27A1 is the major gene that encodes for 25-OHase, which is required for the hydroxylation of vitamin D₃ to 25-(OH)D₃ which is the most abundant circulating form of vitamin D. Fourteen days and 30 days of LPS-induced chronic inflammation did not alter hepatic CYP27A1, however, at 90 days its expression level was decreased in higher dose of LPS. This may imply that hepatic CYP27A1 would be decreased after an extended period of exposure time. The short-term effect (i.e. within hours) of LPS on hepatic sterol 27-hydroxylase (CYP27A1) was demonstrated in Syrian hamsters and C57BL/6J mice, where LPS was shown to down-regulate the activity and hepatic mRNA expression of CYP27A1 in Syrian hamsters at 24 hours and in C57BL/6J mice at 16 hours (264). Pramanik and colleagues (223) demonstrated that LPS down-regulated VDR and impaired vitamin D function in human THP-1 blood monocytes at 24 hours. However, our results from the spleen showed that 14 and 90 days LPS administration tended to decrease splenic VDR, but no changes were observed at 30 days. At 90 days CYP27B1 expression was decreased in the spleen. These data suggest that low doses of LPS do not alter local vitamin D metabolism in the spleen at 30 days, but after 90 days some changes had occurred.

The kidney was also examined due to its major role in the synthesis of 1,25(OH)₂D₃ (CYP27B1) and the catabolism of 1,25(OH)₂D₃ and 25-(OH)D₃ (CYP24A1). We found that the up-regulation of renal IL-1β coincides with the unaltered renal VDR expression. At 14 days, gene expression for CYP27B1 in the kidney was down-regulated, indicating low doses of LPS may suppress gene expression in a relatively short period of time. Additionally, renal CYP24A1, the intrinsic mechanism by which 1,25(OH)₂D₃ and 25-(OH)D₃ are catabolized, was up-regulated. Bland and colleagues (265) showed a significant increase of 24-OHase activity in human cortical collecting duct cells following incubation with LPS, coincides with the expression of TLR4 and CD14. Although we did not examine renal TLR4 and CD14, we verified the inflammatory response by examining hepatic TLR4, CD14, pro- and anti-inflammatory cytokines expression. We observed a significant increase in CYP24A1 expression following 14 days of LPS administration. At the 14-day time point, although we observed that renal CYP24A1 was increased in low dose LPS, its increase did not decrease plasma 25-(OH)D₃. It seems that CYP24A1, at least at this time point, did not affect plasma 25-(OH)D₃. However, the decrease in CYP27B1 gene expression at 14 days may be due to the direct effect of LPS. It is unclear what the response would be to LPS exposure longer than 30 days. Unfortunately, we were not able to determine if the alterations in gene expression occurred in response to 30 and 90 days of LPS administered via LPS pellets due to the fact that kidney samples were not procured at the time of necropsy. The relationship between chronic LPS exposure (i.e., ≥30 days) to vitamin D metabolism in the kidney remains to be elucidated.

In addition to examining the changes in tissue level, a classical approach was used to determine the effects of LPS on circulating vitamin D. Fourteen days of LPS administration increased plasma 25-(OH)D₃, but no alteration were observed with the pellets at 30 days. Plasma 25-(OH)D₃ seemed to not to be affected by the up-regulation of CYP24A1 at 14 days. This may perhaps be due to the majority of CYP24A1 being used to catabolize plasma 1,25(OH)₂D₃ instead of plasma 25-(OH)D₃. However, this is speculation on our part and unfortunately data for plasma 1,25(OH)₂D₃ is not available at this time. The increase in plasma 25-(OH)D₃ may also attributed to the unaltered hepatic CYP27A1, which is involved in converting vitamin D₃ to 25-(OH)D₃. CYP27A1 was strongly express in the macrophages derived from human blood monocytes (245), thus local expression of CYP27A1 in the macrophages within the liver may contribute to the increased plasma 25-(OH)D₃. Mice with inactivated CYP27A1 had normal plasma 1,25(OH)₂D₃, indicating CYP27A1 was not critical for plasma 1,25(OH)₂D₃ (266). When LPS was infused (0.0, 0.5, 1.0, and 1.5 µg/kg body weight) into dairy cows over a period of 100 minutes, both plasma 25-(OH)D₃ and 1,25(OH)₂D₃ were not affected by intravenous LPS infusion, however, when comparing all other doses of LPS combined to controls plasma 1,25(OH)₂D₃ tended to decrease (267). This study showed that LPS at minimal doses did not decrease plasma 25-(OH)D₃ *in vivo*. Similarly, we may anticipate that minimal doses of LPS may not decrease plasma 25-(OH)D₃ in mice. However the mechanism of how LPS influence plasma 25-(OH)D₃ and 1,25(OH)₂D₃ remains to be elucidated.

Based on the results of these studies, we conclude that low grade chronic inflammation induced by LPS influences vitamin D metabolism over time. Further

studies are needed to better characterize these effects over time utilizing either the slow release pellet system or injection and to further investigate the mechanisms involved.

CHAPTER VI

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Summary

The purpose of our study was to examine the effects of low dose LPS administration *in vivo* on inflammatory pathways and gene expression of VDR and key hydroxylation enzymes involved in vitamin D metabolism. In experiment 1, twelve-week-old male C57BL/6J mice (n=12/group) were assigned to three doses of LPS (0, 0.01, 0.1 mg LPS /kg body weight/day) delivered via slow-release pellets for 30 or 90 days. In experiment 2, sixteen-week-old male C57BL/6J mice (n=30) were administered LPS (0, 0.1, 0.2 mg LPS /kg body weight/day i.p.) for 14 days. We showed that two weeks of LPS exposure did not produce significant changes in pro-inflammatory cytokines but increased anti-inflammatory cytokines IL-10 and TGF- β and TLR4 and CD14 in the liver. Inflammatory mediators in the liver did not change at 30 days but IL-1 β and IL-10 were decreased with higher LPS dose at 90 days. Two weeks low dose LPS tended to increase ($p = 0.05$) splenic IL-1 β and significantly increased renal IL-1 β . No changes were observed in inflammatory mediators after 30 days of LPS exposure, however, low dose LPS significantly increased IL-6 and higher LPS dose tended to increase ($p = 0.06$) TNF- α in 90 days. In terms of the effects of LPS on the key vitamin D hydroxylation enzymes, no changes were observed in the liver and the spleen after two

weeks of LPS administration, however, splenic VDR tended to decrease ($p = 0.08$) in higher LPS dose. After two weeks of LPS administration, both doses of LPS suppressed renal CYP27B1 while lower LPS dose enhanced renal CYP24A1. Thirty days of LPS exposure did not alter any key vitamin D hydroxylation enzymes and VDR expressions in the liver and spleen, however higher LPS dose decreased hepatic CYP27A1 and splenic CYP27B1 and tended to decrease ($p = 0.07$) splenic VDR after 90 days of LPS exposure. At two weeks, plasma 25-(OH)D₃ increased in low dose of LPS but did not change after 30 days LPS exposure. These data suggest that LPS-induced low grade chronic inflammation alter gene expression of VDR and key hydroxylation enzymes involved in vitamin D metabolism over time.

Conclusions

Based on the results of these studies, we conclude the following for each of the hypotheses tested:

1. Over time (i.e., 30 and 90 days) inflammation induced with increasing dose of LPS in adult C57BL/6J mice will alter genes encoding for vitamin D activating and deactivating enzyme, 1- α -hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1), in hepatic tissue.

This hypothesis was rejected due to the lack of an effect of either dose of LPS on hepatic CYP27B1 and CYP24A1 at 30 and 90 days. It is important to note that CYP27B1 and CYP24A1 expression was not abundant in the liver. CYP27B1 is required for the 25-hydroxylation of 25-(OH)D₃ to 1,25(OH)₂D₃ while CYP24A1 is required to catalyze 1,25(OH)₂D₃ to 1,24,25(OH)₃D₃ or 25-(OH)D₃.

to 24,25(OH)₂D₃ (1) and both are highly expressed in the kidney (2). Therefore, having access to kidney samples and being able to examine the expression of genes encoding for these enzymes in the kidney instead of the liver in future experiments would enable us to determine the effects of chronic inflammation induced by LPS on the vitamin D activating and deactivating enzymes.

2. LPS will dose dependently reduce circulating vitamin D metabolites in adult male C57BL/6J mice over time.

We rejected this hypothesis because the circulating vitamin D metabolite (i.e., 25-(OH)D₃) was not affected by increasing dose of LPS following 30 days exposure. The inability to evaluate plasma 25-(OH)D₃ at 90 days prevented us from determining if the decrease in hepatic CYP27A1 expression, which is required to synthesize circulating 25-(OH)D₃ (3-6), would have resulted in decreased circulating 25-(OH)D₃ at this time point.

3. LPS-induced pro-inflammatory cytokine (e.g. TNF- α and IL-6) will down-regulate genes encoding vitamin D activating and deactivating enzymes in the spleen, a monocyte rich tissue, in adult male C57BL/6J mice.

This hypothesis is accepted because splenic CYP27B1 and VDR were down-regulated to some degree by the higher LPS dose at 90 days, suggesting that LPS at this dose has modest effects on local splenic CYP27B1 expression. These effects were seen in 90 days of LPS2 exposure and corresponded to the increased ($p = 0.07$) of splenic TNF- α and an intermediate effect of IL-6.

With regard to Experiment 2, the following hypotheses were tested:

1. Short-term (i.e., 14-days) induced inflammation with increasing dose of LPS (0, 0.1, 0.2 mg LPS /kg body weight/day) in adult C57BL/6J mice will alter genes encoding for vitamin D activating and deactivating enzyme, 1- α -hydroxylase (CYP27B1) and 24-hydroxylase (CYP24A1), in hepatic and renal tissues.

As a result of Experiment 2, we rejected this hypothesis. Fourteen-days LPS treatment did not alter hepatic CYP27B1 and CYP24A1, which may be suppressed by the up-regulation of IL-10 and TGF- β . The low abundance of these genes in the hepatic tissue may not be detectable by qRT-PCR. However, renal CYP27B1 was significantly decreased in LPS1 following two-week LPS treatment which may contribute to the up-regulation of renal IL-1 β and CYP24A1. As to whether which of these genes (renal IL-1 β and CYP24A1) are the major mediators of these effects will require further investigation.

2. LPS will dose dependently reduce circulating vitamin D metabolites in adult male C57BL/6J mice over time.

We rejected this hypothesis because circulating 25-(OH)D₃ was significantly increased in LPS1 group and no changes were seen in LPS2 (0.2 mg LPS /kg body weight/day) group. Circulating 25-(OH)D₃ is synthesized by CYP27A1, thus the conservation of hepatic CYP27A1 may contribute to the increased circulating 25-(OH)D₃ in LPS1 group.

3. LPS-induced pro-inflammatory cytokine (e.g. TNF- α) will down-regulate genes encoding vitamin D activating and deactivating enzymes in the spleen, a monocyte rich tissue, in adult male C57BL/6J mice.

This hypothesis was rejected because TNF- α did not seem to down-regulate vitamin D activating and deactivating enzymes in the spleen at 14 days. However, VDR expression tended to be decreased ($p=0.08$) with the higher LPS dose (LPS2). Experiment 2 suggests that LPS1 administered via intraperitoneal injection for 2 weeks suppressed renal CYP27B1 gene expression and has modest effects on VDR expression, indicating chronic inflammation affects vitamin D metabolism at the gene level.

Future Directions

To better understand if the effects of LPS on vitamin D metabolism play a role in the bone loss associated with chronic inflammation, future studies should be designed to examine alterations in gene expression in renal tissue and plasma 1,25(OH) $_2$ D $_3$ over time (e.g. 4 and 6 weeks). Previously, we observed bone loss in the animals after 30 and 90 days of LPS exposure, but could not determine if there were alterations in gene expression associated with vitamin D metabolism in the kidney or changes in circulating vitamin D metabolites due to the fact that renal tissue and plasma samples were not available. Utilizing a model of LPS exposure over time may allow us to correlate the changes in gene expression that occur in the kidney and systemic vitamin D status to bone loss.

Additionally, a KO mouse model should be used to examine if the alterations in vitamin D metabolism are involved in the relationship between bone loss, and key pro-inflammatory cytokines such as TNF- α . For example, a TNF- α KO mouse implanted with slow-release pellets or given intraperitoneal LPS would provide information if TNF- α is the major pro-inflammatory cytokine that causes bone loss in trabecular and cortical bone and decreases gene expression of vitamin D hydroxylation enzymes in bone and soft-tissues. Additionally, this would allow us to examine whether TNF- α is causing a decrease in plasma 25-(OH)D₃ by down-regulating hepatic CYP27A1 and plasma 1,25(OH)₂D₃ by down-regulating renal CYP27B1, when comparing plasma 25-(OH)D₃ and 1,25(OH)₂D₃ between TNF- α KO and wild type mice. A CYP27B1 KO mouse model may not be an appropriate model to address the question of our study because it is known that CYP27B1 KO mice experience skeletal abnormalities and decrease immune function. Exposing the animals to LPS may exacerbate bone loss and may eventually shorten the lifespan of the animals.

In experiment 2, we utilized only one time point (i.e., 2 weeks) to examine the effects of low dose LPS administration *in vivo* on inflammatory pathways and vitamin D activating and deactivating enzymes and VDR gene expression. Furthermore, we do not know if 2 weeks of LPS treatment causes bone loss. To further confirm if LPS treatment alters gene expression of vitamin D hydroxylation enzymes and bone metabolism, we should utilize additional time points such as 4 and 6 weeks in the same model (i.e., administering LPS i.p.).

The effects of LPS on vitamin D and bone metabolism should be examined utilizing vitamin D insufficient animals. Exposing the vitamin D insufficient animals and

normal animals with different doses of LPS via slow-release pellets or injection at various time points (e.g. 2, 4, 6 weeks), would allow us to examine whether over time chronic inflammation decrease gene expression of vitamin D hydroxylation enzymes and increase bone loss. We may also examine whether vitamin D insufficiency exacerbate inflammatory conditions, which in turn, further decrease vitamin D status. Furthermore, the effects of LPS-induced inflammation on local synthesis of vitamin D may be compared between vitamin D insufficient and normal animals by examining CYP27B1 in bone and in monocytes from spleen.

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APPENDICES

Oklahoma State University Institutional Animal Care and Use Committee (IACUC)

Protocol Expires 7/31/2011

Date : Tuesday, October 28, 2008

Animal Care and Use Protocol (ACUP)

HE-08-3

Proposal Alterations in Vitamin D Metabolism with Chronic inflammation and the Resulting Effects on Bone

Principal Investigator:

Brenda J. Smith
Nutritional Sciences
301 HES
Campus

Reviewed and

Processed as: Special Review

Modification

Approval Status Recommended by Reviewer(s) : Approved

The requested modifications in personnel and change in study design have been approved. The protocol is amended to test the effects of two doses of LPS without the TNF-Alpha inhibitor. The first dose is 0.1 mg LPS/kg body weight/d and the additional dose is 0.2 mg LPS/kg body weight/d. Two researchers have been added to the personnel list: Lawrance Chandra and Weiyu Jiang.

Signatures

Dr. Charlotte Ownby, IACUC Chair

Tuesday, October 28, 2008
Date

cc: Department Head, Human Environmental Sciences
LAR

Approvals are valid for three calendar years, after which time a request for renewal must be submitted. Any modifications to the research project, course, or testing procedures must be submitted for review and approval by the IACUC, prior to initiating any changes. Modifications do not affect the original approval period. Modification approvals are valid for the duration of the protocol approval (see protocol expiration date). Approved projects are subject to monitoring by the IACUC. OSU is a USDA registered research facility and maintains an Animal Welfare Assurance document with the Public Health Service Office of Laboratory Animal Welfare, Assurance number AA3722-01.

VITA

Yin Foong Lim

Candidate for the Degree of

Master of Science

Thesis: ALTERATIONS IN VITAMIN D METABOLISM WITH CHRONIC
INFLAMMATION: POTENTIAL IMPLICATIONS IN INFLAMMATION-
INDUCED BONE LOSS

Major Field: Nutritional Sciences

Biographical:

Personal Data: Born in Negeri Sembilan, Malaysia, the daughter of Siak Siaw
Lim and Lin Tai Chow.

Education: Received Bachelor of Science degree in Nutritional Sciences, with
option in Dietetics from Oklahoma State University, Stillwater,
Oklahoma in May, 2007. Completed the requirements for the Master of
Science degree with a major in Nutritional Sciences at Oklahoma State
University, Stillwater, Oklahoma in July, 2009.

Experience: Employed by Oklahoma State University, Department of
Nutritional Sciences as a graduate research assistant, 2007 to present.

Professional Memberships: American Dietetic Association
American Society of Nutrition

Name: Yin Foong Lim

Date of Degree: July, 2009

Institution: Oklahoma State University

Location: Stillwater, Oklahoma

Title of Study: ALTERATIONS IN VITAMIN D METABOLISM WITH CHRONIC
INFLAMMATION: POTENTIAL IMPLICATIONS IN
INFLAMMATION-INDUCED BONE LOSS

Pages in Study: 101

Candidate for the Degree of Master of Science

Major Field: Nutritional Sciences

Scope and Method of Study: Animal Study

Findings and Conclusions:

Inflammatory conditions are often associated with compromised serum 25-(OH)D₃ status and previous *in vitro* studies have shown that inflammatory cytokines may be involved. The objective of this study was to examine the effects of low dose LPS *in vivo* on inflammatory mediators and gene expression of VDR and hydroxylation enzymes involved in vitamin D metabolism. In experiment 1, 12-week-old male C57BL/6J mice (n=12/group) were randomly assigned to LPS (0, 0.01, and 0.1 mg/kg bw/d) delivered by slow release pellets over 30 and 90 days. In experiment 2, 16-week-old male C57BL/6J mice (n=10/group) were administered (i.p.) LPS (0, 0.1, 0.2 mg/kg bw/d) for 14 days. Body weight was not altered during either study. Low dose LPS increased hepatic TLR4, CD14, and IL-10 after 14 days. No alterations were observed in hepatic genes of interest after 30 days, but IL-10 and IL-1 β were decreased after 90 days. Splenic IL-1 β tended to increase ($p = 0.05$) after 14 days and IL-6 was increased in low dose LPS after 90 days. Hepatic CYP27A1 gene expression was decreased after 90 days, but not after 14 and 30 days. High dose LPS tended to decrease ($p=0.08$) splenic VDR after 14 days, and decreased CYP27B1 after 90 days. Renal CYP27B1 was decreased and CYP24A1 was increased after 14 days. A significant increase in plasma 25-(OH)D₃ was observed after 14 days, but not 30 days. Our data suggest that chronic low dose LPS has modest effects on VDR, decreases renal CYP27B1, and increases renal CYP24A1. Further studies should further explore how LPS alters renal gene expression of cytochrome P450 enzymes involved in vitamin D metabolism over time.

ADVISER'S APPROVAL: Dr. Brenda J. Smith
