ROLE OF VDR IN HOST IMMUNE RESPONSE TO *PORPHYROMONAS GINGIVALIS* INFECTION

by

GULAM YEZDANI

PING ZHANG, CHAIR
AMJAD JAVED
JANET KATZ
SUZANNE MICHALEK

A THESIS
Submitted to the graduate faculty of The University of Alabama at Birmingham, in partial fulfillment of the requirements for the degree of Master of Science

BIRMINGHAM, ALABAMA

2011
ROLE OF VDR IN HOST IMMUNE RESPONSE TO *PORPHYROMONAS GINGIVALIS* INFECTION

GULAM YEZDANI

ORAL BIOLOGY

ABSTRACT

*Porphyromonas gingivalis* is one of the etiologic factors of periodontal disease, a chronic inflammatory disorder characterized by the destruction of periodontal connective tissue and the subsequent loss of alveolar bone. Epidemiological and genetic studies have indicated an association between periodontal disease and the vitamin D system. However, little is known regarding how vitamin D signaling regulates the inflammatory immune process of this disease. The purpose of the study was to understand the role of the vitamin D receptor (VDR) in regulating the host response to *P. gingivalis* infection by using a dual chamber mouse model. These chambers served both as infection and sample collection sites. Chamber exudates of wild type (WT) and VDR knock out (VDR⁻/⁻) mice were sampled for neutrophil and monocyte levels, bacterial clearance and levels of TNF-α, IL-6, IL-12p40, and IL-10 cytokines. In addition, serum cytokines, *P. gingivalis* specific CD4⁺ T cell cytokine responses and serum antibody responses were assessed. Our results showed similar levels of neutrophil and monocyte infiltration in WT and VDR⁻/⁻ mice following *P. gingivalis* infection. However, VDR⁻/⁻ mice exhibited a significantly higher level of bacteria than WT mice. Furthermore, significant higher
levels of IL-6, IL-10, IL-12p40 and TNF-α were observed in the chamber exudates of VDR<sup>−/−</sup> mice. Although a similar level of serum IL-6 was seen in WT and VDR<sup>−/−</sup> mice 4 hr after <i>P. gingivalis</i> infection, 24 hr post infection, the levels were increased in VDR<sup>−/−</sup> mice. Assessment of <i>P. gingivalis</i>-specific CD4<sup>+</sup> T cell cytokine responses suggested that in the presence of the VDR a Th1 response was favored following <i>P. gingivalis</i> infection. Furthermore, VDR<sup>+/−</sup> mice exhibited lower levels of serum IgG anti-<i>P. gingivalis</i> antibodies than that observed in WT mice. Taken together, these results suggest that VDR deficient mice have an increased susceptibility to <i>P. gingivalis</i> infection, implicating the VDR in the control of bacterial growth and in modulating inflammatory responses that could be detrimental to the host during infection by <i>P. gingivalis</i>. These findings provide valuable insight on the role of the VDR in <i>P. gingivalis</i> infection.
DEDICATION

I dedicate this thesis to my parents, brothers and sisters who supported every aspect of my education and pushed me further. To my wife whose love, support and prayers kept me strong throughout.
ACKNOWLEDGMENTS

I would like to acknowledge several people who helped to make this thesis possible. Dr. Ping Zhang, who is my mentor and chair of my committee, has helped me throughout the research progress, thank you. I am thankful for my committee members Dr. Jannet Katz, Dr. Suzanne Michalek, Dr. Amjad Javed for their kind assistance on the project. My thanks go to Qingan Xu and Amit Ashtekar for their help and guidance throughout my graduate work. My lab mates Mike, Dalia also deserve vast amount of praise for their help and support. Special thanks and I am grateful to Dr. Jannet Katz and Dr. Ping Zhang who gave me motivation, listened and helped me with the difficulties I faced at the beginning of my masters programme. Also many thanks to Dr. Suzanne Michalek, for everything she provided to me in my research. Last person I would acknowledge is Greg for his excellent technical assistance. These people I will always be indebted my life. Thank you all.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>viii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>Immune system, vitamin D and its effects on disease</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin D receptor</td>
<td>3</td>
</tr>
<tr>
<td>Vitamin D receptor knockout mice</td>
<td>4</td>
</tr>
<tr>
<td>Periodontal apparatus</td>
<td>6</td>
</tr>
<tr>
<td>Periodontal disease</td>
<td>7</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>8</td>
</tr>
<tr>
<td>Rationale to use mouse chamber model</td>
<td>9</td>
</tr>
<tr>
<td>MATERIAL AND METHODS</td>
<td>9</td>
</tr>
<tr>
<td>RESULTS</td>
<td>15</td>
</tr>
<tr>
<td>DISCUSSION</td>
<td>18</td>
</tr>
<tr>
<td>GENERAL LIST OF REFERENCES</td>
<td>21</td>
</tr>
<tr>
<td>APPENDIX: NOTICE OF APPROVAL FROM INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE</td>
<td>33</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Periodontal structure</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Infection and sample collection diagram</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>Cytokine production in bone marrow-derived macrophages</td>
<td>26</td>
</tr>
<tr>
<td>4</td>
<td>Neutrophil and monocyte infiltration</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>Bacterial culture</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>Cytokine production in chamber exudates</td>
<td>29</td>
</tr>
<tr>
<td>7</td>
<td>Cytokine production in serum</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>CD4⁺ T cell cytokine production</td>
<td>31</td>
</tr>
<tr>
<td>9</td>
<td>Serum antibody to <em>P. gingivalis</em></td>
<td>32</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
<td></td>
</tr>
<tr>
<td>VDR&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>vitamin D receptor knock out</td>
<td></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor alpha</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
<td></td>
</tr>
<tr>
<td>Th</td>
<td>t helper</td>
<td></td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
<td></td>
</tr>
<tr>
<td>BMMs</td>
<td>bone marrow derived macrophages</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
<td></td>
</tr>
</tbody>
</table>
INTRODUCTION

Immune function, vitamin D and its effects on disease.

The main function of the immune system is to protect the host from infection by pathogenic organisms. It also serves to rid the body of altered self cells, the uncontrolled division of which can lead to cancer. Therefore, the immune system is comprised of several organs and cell types, which communicate through cell surface and soluble molecules such as cytokines and chemokines. Host responses involve the innate and adaptive immune system (9, 11). The innate system is constitutively present and deals non-specifically with the invading pathogen, whereas the adaptive system involves the activation and differentiation of antigen specific CD4$^+$ or CD8$^+$ T cells and antibody secreting B cells and is characterized by a delayed response tailored to the pathogen that induces memory (32). CD4$^+$ T cell are important in responses to bacteria, fungi and parasites, and ensures that subsequent reinfection by the same pathogen is dealt quickly before symptoms develop. Loss of immunity results in recurrent infections and severe immunodeficiencies that can also lead to death. Equally, inappropriate is the excessive activation of the immune response. Thus the immune system can be viewed as a double edge sword and maintaining equilibrium is essential for a state of health (7, 11, 48).

Vitamins have been known to have an effect on the host, its deficiencies have been attributed to diseases, affecting overall host immune mechanism. Of lately vitamin D as such have been studied in the context of immunity (1, 3, 25, 32). Such information could
be useful in the design of treatments for active disease. Although vitamin D has been traditionally associated with calcium and phosphate homeostasis, in recent years, it has been shown to be involved in host immunity, influencing both the innate and adaptive arms of the response (3, 25, 27).

Important co-relations between vitamin D and the immune system were observed in the 1960’s when patients having a chronic granulomatous disorder such as sarcoidiosis, tuberculosis or histoplasmosis, had high levels of circulating vitamin D in their blood (32). It is an accepted fact that prior to the development of drugs, the treatment of choice for tuberculosis was sunlight. This was proven by Lie et al. (49, 39) who showed that vitamin D had antimicrobial properties that could inhibit the growth of *Mycobacterium tuberculosis* in macrophage cultures. Furthermore, low levels of the principal vitamin D metabolite (25-hydroxy vitamin D) in the circulation, is associated with a high incidence of autoimmune disorders e.g., multiple sclerosis, inflammatory bowel disease and diabetes (7). Many investigators have reported that a functioning VDR system helps control inflammation and modulates the immune system in certain types of cancers and infectious diseases (27). Furthermore, genetic studies have shown an association between VDR gene polymorphisms and periodontal infections (12, 14, 26). It has been shown that a defective VDR codon causes an increase susceptibility to generalized aggressive periodontitis moreover ongoing periodontal studies are using the VDR gene risk marker as a susceptibility test to periodontal disease (2). Human studies of vitamin D have shown that its deficiency is directly responsible for a higher incidence of periodontal diseases and further, VDR ligands have been used for the treatment of periodontal diseases (12, 2, 14).
**Vitamin D receptor**

The vitamin D receptor (VDR) was discovered in 1969 as a binding protein and was later cloned and sequenced in 1987 (6) and further investigations led to the discovery of its binding (38) to the active vitamin D hormone, which confirmed that the receptor and 1,25-(OH)$_2$D$_3$ functions like many other steroid hormones, acts by binding to and activating a nuclear transcription factor (5). VDR expression has been identified in every human tissue and nearly all nucleated cells express VDR (32).

**VDR structure**

The VDR is a molecule of approximately 50-60 kDa depending on the species. The VDR protein consists of 3 major domains, an N-terminal DNA-binding domain, a ligand binding domain and a C-terminal transaction domain which can mediate co-factor interaction (24). The most conserved domain is the DNA binding domain, which is composed of 2 zinc fingers. The first zinc finger confers specificity for DNA binding to the vitamin D responsive elements (VDREs) while the second zinc finger is the region for the heterodimerization to the retinoid X receptor (RXR) (5, 35, 13). The ligand binding domain is responsible for the binding to 1,25(OH)$_2$D$_3$. Interaction with 1,25(OH)$_2$D$_3$ causes the VDR to heterodimerize with the RXR, inducing or inhibiting transcription of the target gene. Although the VDR is capable of dimerizing with other nuclear receptors, it has been observed to prefer interaction with RXR (25, 30). Thus the VDR is a member of the nuclear receptor family of transcription factors. These receptors function as ligand activated, transcriptional regulatory proteins. VDR is localized in both the cytosol and nucleus, and accumulates in the nucleus in response to 1,25(OH)$_2$D$_3$ binding. It has been
seen that mutations of this receptor results in hereditary vitamin D resistant rickets. In humans, multiple polymorphisms of the VDR gene have been identified and thoroughly studied that link the VDR to disorders like cancer and autoimmune diseases (10, 27, 25). Data obtained from VDR\(^{-/-}\) has been instrumental in elucidating the physiological actions of VDR. VDR\(^{-/-}\) animal models have the full phenotype of severe vitamin D deficiency.

**Vitamin D receptor knockout (VDR\(^{-/-}\)) mice**

VDR\(^{-/-}\) mice are viable and fertile with a normal survival of approximately 14 months. The RNA isolated from these mice shows a truncated deletion of the second zinc finger coding region followed by a premature termination codon, resulting in the absence of receptor protein (30). At 21 days of age these mice, show hypocalcemia, hypophosphatemia, hyperparathyroidism, increased serum parathyroid hormone, abnormal blood mineral levels, and growth retardation (5). After at 4 weeks of age, VDR\(^{-/-}\) mice exhibit perioral and periorbital alopecia that progresses over the entire body by 4 months of age. Introducing a rescue diet enriched for calcium, phosphorus, and lactose to young mice prevents hyperparathyroidism, rickets, and osteomalacia, but not alopecia.

**Vitamin D metabolism**

Vitamin D is obtained through diet, supplements, and exposure to sunlight. Vitamin D\(_2\) (ergocalciferol) is found in plants and fungi, and vitamin D\(_3\) (cholecalciferol) in meats (5, 32). Vitamin D\(_3\) is also produced endogenously when the skin is exposed to ultraviolet light. Vitamin D\(_3\) is hydroxylated at the 25-position by the hepatic vitamin D\(_3\) hyrdroxylase enzyme (CYP27A1) to yield 25-hydroxyvitaminD\(_3\) (25-hydroxycholecalciferol), the major form of vitamin D in the circulation (4). This 25-hydroxyvitaminD\(_3\) is further hydroxylated to the final form of 1,25-hydroxyvitamin D with
the help of enzyme 1α-hydroxylase (CYP27B1) occurring exclusively in the kidneys to form the active vitamin D metabolite 1,25-dihydroxyvitaminD$_3$ also known as calcitriol (32). This 1,25-dihydroxyvitaminD$_3$ binds to the vitamin D receptor VDR, a nuclear receptor highly expressed in the target organs of calcium homeostasis, such as the intestine, bone, kidney parathyroid glands and cells of the immune system (24, 32). The ligand bound VDR activates transcription by heterodimerization with RXRs, which is essential for high-affinity DNA binding to cognate vitamin D response elements VDREs located in the regulatory regions of 1,25D target genes. The VDR/RXR heterodimer recruits numerous coregulatory proteins which control histone modifications, chromatin remodeling and RNA polymerase II binding and transcriptional initiation (3, 4, 5, 7, 32). There is strong evidence that shows vitamin D signaling in the innate and adaptive immune systems. The VDR is present in most cells of the immune system, including neutrophils, macrophages, dendritic cells and T and B lymphocytes (1, 28, 36). Studies have shown that inflammatory bowel disease (IBD), arthritis and multiple sclerosis in VDR$^{-/-}$ mice have a negative influence on the outcome of the diseases (7, 17). However, other infection studies showed that vitamin D has no effect on the susceptibility of mice to infections with herpes simplex virus or Candida albicans (5) and an unsubstantiated link between vitamin D deficiency and cases of tuberculosis (18, 39, 40) has been noted. Experimentally, vitamin D deficiency and host resistance to infectious diseases have not been studied extensively. Surprisingly, little is known about the effect of the vitamin D status on the ability of the host to fight infections. Moreover, little is known about the role of vitamin D and 1,25(OH)$_2$D$_3$ in regulating immune responses to infectious diseases.
Periodontal apparatus

The periodontal ligament is the fibrous connective tissue structure, with neural and vascular components, that joins the cementum covering the root to the alveolar bone. The periodontal ligament serves primarily a supportive function by attaching the tooth to the surrounding alveolar bone proper. (Fig. 1) This function is mediated primarily by the principal fiber groups (horizontal, oblique, apical and interradicular) of the periodontal ligament that form a strong fibrous union between the root cementum and the bone (37). The periodontal ligament also serves as a shock-absorber by mechanisms that provide resistance from light, moderate and heavy forces. Light forces are cushioned by intravascular fluid that is forced out of the blood vessels. Moderate forces are absorbed by extravascular tissue fluid that is forced out of the periodontal ligament space into the adjacent marrow spaces (41). The heavier forces are taken up by the principal fibers. The periodontal ligament also serves as a reservoir by providing cells that are able to form all the tissues that make up the attachment apparatus i.e. bone, cementum and the periodontal ligament. In addition, the periodontal ligament has a sensory function by nerve endings that are primarily receptors for pain and pressure, and it also provides a nutritive function that maintains the vitality of its various cells via dental arteries that enter the ligament through the alveolar bone.
Periodontal disease

Periodontitis is a highly prevalent, chronic immune inflammatory disease of the periodontium that results in progressive loss of the periodontal ligament, and adjacent supporting alveolar bone. It is initiated by specific bacteria within the dental plaque biofilm which induces a host immune response thought to be implicated in disease pathogenesis. Porphyromonas gingivalis, a gram negative anaerobe is thought to be involved in the etiology of periodontal disease (42, 47). Its virulence factors including lipopolysacchride, fimbriae, hemagglutinis, hemolysins and proteolytic enzymes allow for the colonization, initiation and progression of the disease process. Although P. gingivalis can be detected in periodontally healthy people, it is only at very low numbers (17, 33).

The host immune inflammatory response involves the participation of various molecules, such as cytokines, prostaglandins and host enzymes released from immune and non-
immune cells. These factors further increase the extent of inflammation and contribute to tissue destruction. It is well known that proinflammatory cytokines produced by host cells play an important role in periodontal tissue destruction (16). Cytokines produced in response to periodontal pathogens like *P. gingivalis* are believed to act not only in host defense, but also in periodontal tissue breakdown (41). Activated lymphocytes, macrophages and neutrophils infiltrate periodontal tissue and secrete inflammatory mediators such as interleukin-1 (IL-1) and prostaglandin E2. In addition, T helper1 (Th1) and Th2 cells, up-regulate the production of the pro-inflammatory IL-6 and TNF-α, cytokines that can induce alveolar bone resorption (44).

Keeping in view all the epidemiological and experimental studies of VDR influencing infectious disease stated above, I wanted to investigate if VDR has any role in periodontal infection, so hence forth I state my hypothesis for the present study

**Hypothesis**

In the present study, we hypothesized that VDR plays an immunomodulator role to *P. gingivalis* infection. We investigated the role of VDR in response to the periodontal pathogen *P. gingivalis* using a dual subcutaneous chamber model of infection in WT and VDR$^{-/-}$ mice. To investigate this hypothesis we had 2 aims:

1. **To assess the role of the VDR in the cellular host immune response to *P. gingivalis* infection in vivo**

We evaluated the neutrophil and monocyte infiltration, bacterial clearance and CD4$^+$ T cell cytokine production and antibody production.
2. To assess how the VDR signaling in macrophage respond to *P. gingivalis* in vitro

To do this we stimulated macrophages in vitro for the production of pro-inflammatory IL-6, TNF-α and IL-12p40 and anti-inflammatory cytokine production IL-10 in WT and VDR<sup>-/-</sup> mice.

**Rationale to use mouse chamber model**

The subcutaneous chamber model allows the sampling of the chamber contents throughout the course of infection for microbiological, immunological and cytological examination (20, 21, 28, 36,). Thus allowing for examination of specific host factors produced locally in response to bacterial challenge during *P. gingivalis* infection. Although, the lesions are not located in the oral cavity, this model is acceptable for examining bacterially induced infections that are localized. This is well established model used in periodontal-related studies (36).

**MATERIALS AND METHODS**

**Bacteria**

*P. gingivalis* ATCC 33277 was cultured and maintained on enriched trypticase soy agar (ETSA) plates containing trypticase soy agar, 1% yeast extract, 5% defibribrinated sheep blood, 5 µg/ml of hemin, and 1 µg/ml of menadione at 37°C in an anaerobic atmosphere of 10% H<sub>2</sub>, 5%CO<sub>2</sub>, and 85% N<sub>2</sub>. For the preparation of *P. gingivalis* for cell stimulation, the bacteria were harvested, centrifuged, and washed in PBS. The number of bacteria (CFU/ml) was determined by measuring the optical density (OD) at 580 nm and extrapolating using a standard curve.
**Mice**

C57BL/6 wild type (WT) and VDR<sup>−/−</sup> mice were bred and maintained within an environmentally controlled pathogen free animal facility at the University of Alabama at Birmingham with the genotyped checked by polymerase chain reaction. Female mice, age 8-12 weeks old were used in these studies. All studies were performed according to the National Institute of Health guidelines, and protocols were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

**Generation and stimulation of bone marrow-derived macrophage**

Femurs and tibiae from C57BL/6 WT and VDR<sup>−/−</sup> mice (8 to 10 weeks old) were removed and dissected free from adhering soft tissue. After soaking the bones in 70% ethanol for 2 min and rinsing then in PBS, both ends were cut and the marrow was flushed out with PBS using a 25-gauge needle. Single-cell suspensions were prepared by mechanically dispersing the bone marrow through a 100-μm cell strainer. Erythrocytes were lysed using M-lysis buffer (R&D systems, Minneapolis, MN). Bone marrow cells were cultured in α-10 medium (α-MEM, 10% FCS, 1 x PenStrep) in a humidified 7.5% CO<sub>2</sub> incubator at 37°C overnight. The next day, non-adherent cells were harvested and cultured with α-10 medium supplemented with 10% culture supernatant from CMG14-12 cells as the source of macrophages colony stimulating factor (M-CSF). After 5 days, floating cells were removed and attached cells were used as BMMs. BMMs (2x10<sup>5</sup> cells/well) were cultured in 96-well plates in a α-10 medium supplemented with 10% M-CSF. Cultures were stimulated with live *P. gingivalis* at an multiplicity of infection
(MOI) of 50 or were left untreated. Culture supernatants were harvested after 24h to assess IL-6, IL-10, IL-12p40 and TNF-α levels by ELISA.

**Dual chamber model**

Two coils / mouse were surgically implanted in the dorsolumbar region approximately 10mm apart from each other, to create subcutaneous chambers in C57BL/6 WT and VDR⁻/⁻ female mice (8-10 weeks old). These coils were made of stainless steel surgical wire that was 10 mm long and 5 mm in diameter. After a two week healing period, mice were infected with *P. gingivalis* and cellular responses assessed by sampling the exudates in the chamber (14).

**Experimental design**

The implanted chambers in WT and VDR⁻/⁻ mice were infected with *P. gingivalis* suspended in PBS (0.1 ml; 5 x10⁹ CFU/ml) on day 0 and a second infection was given on day 14th. (Fig. 2)

Exudates were collected from one chamber at 24 h after the first infection, from the other chamber 48 h and from both chambers on day 7. An aliquot of chamber fluid was immediately analyzed for viable *P. gingivalis* and leukocytes. The remaining fluid was centrifuged and the supernatant stored at -20°C for the future analysis of cytokines. In addition, cytokines were assessed in serum by collecting blood samples from the facial vein prior to infection and at different time points following infection. Serum was obtained after centrifugation and stored at -20°C until cytokine or specific antibody responses were analyzed.
An aliquot of chamber fluid samples was serially diluted in PBS and streaked on ETSA plates and incubated at 37°C under anaerobic conditions. Black pigmented colonies were counted after 7-10 days of incubation, and the number of colony forming units (CFUs) in was determined.

**Leukocyte counts**

A differential leukocyte count was performed in the chamber fluid was performed by microscopic examination of cytospin preparations. Each sample was diluted (1:2000) in PBS and 100 µl of this dilution was loaded in the cytospin funnel and centrifuged at 2000 rpm for 3 minutes (Shandon III cytocentrifuge). Cytospin preparations were stained with Diff-Quik stain set (Dada Behring, Inc., Newark, DE), and neutrophils and monocytes/macrophages present in the chamber exudates were identified by their nuclear morphology.
Isolation and stimulation of CD4⁺ T cells

Fourteen days after the second infection, mice were sacrificed and spleens were aseptically removed for in vitro assessment of the *P. gingivalis* specific CD4⁺ T cell cytokine response. Specifically, single-cell suspension from spleens were prepared by mechanically dispersing the tissues through 40 µm cell strainers (BD Labware, Franklin Lakes, NJ) into Hank’s balanced salt solution (HBSS) supplemented with 10% fetal calf serum. Erythrocytes were removed from the single cell suspensions by using M-lyse buffer (R&D Systems, Minneapolis, MN). Cells were then washed, suspended in RPMI 1640 supplemented with 10% fetal calf serum, 2mM L-glutamine, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 1.5 mg/ml of sodium bicarbonate, 50 µg/ml of gentamicin sulfate, 25mM HEPES, 50 U/ml of penicillin, and 50 µg/ml of streptomycin (complete medium), and counted in a hemacytometer with trypan blue to estimate viability. CD4⁺ T cells were purified by negative selection using a mouse CD4⁺ T cell subset column according to the manufacture’s protocol (R&D System, Minneapolis, Minn). For the preparation of feeder cells, splenocytes from naïve WT and VDR⁻/⁻ mice were irradiated with 3000 rad for 42 minutes. Equal numbers of CD4⁺ T cells and irradiated feeders were co-cultured in 96-well plates in RMPI 1640 media supplemented with 10% heat inactivated FCS at 37°C in a humidified 7.5% CO₂ incubator. Cultures were immediately stimulated with *P. gingivalis* at an MOI of 10 and culture supernatant harvested after 5 days incubation for cytokine analysis.

Quantification of antibody response

The levels of total IgG and subclass IgG subclass specific antibodies in the serum samples were determined by an ELISA, as previously described (47). Briefly, polystyrene
Maxisorp 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated with formalin-killed *P. gingivalis* (100 µl of 5 x 10⁸ CFU/ml) in borate-buffer saline (BBS; 100 Mm NaCl, 50 mM boric acide, 1.2 mM Na₂B₄O₇, AND 0.02% azide, pH8.2) at 4°C overnight. Total IgG levels in serum samples was determined by coating plates with goat anti-mouse IgG antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL). Plates were washed and blocked for 4 h at room temperature with 0.01 M phosphate buffer (pH 7.2) containing 0.5 M NaCl and 0.15% Tween 20. Serial two fold dilution of serum samples were added in duplicate and plates were incubated at 4°C overnight. Afterwards plates were washed with 0.01 M phosphate buffer containing 0.5 M NaCl and 0.015% Tween 20 (pH 7.2) and peroxidase-conjugated goat anti-mouse IgG or IgG subclass-specific antibodies (Southern Biotechnology Associates, Inc) were added followed by addition of o-phenylenediamine substrate with H₂O₂. Optical density was measured at a wavelength of 490 nm. The concentration of antibodies were calculated by interpolation on calibration curves generated at the same time using a mouse immunoglobulin reference serum (ICN Biomedical, Aurora, OH) and constructed by a computer program based on a four-parameter logistic algorithm (Softmax/ Molecular Devices Corp, Menlo Park, CA)

**Cytokine analysis**

The levels of TNF-α, IL-6 (eBiosciences, San Diego, CA) IL-10 and IL-12p40 (R&D Systems) in chamber fluids and BMMs culture supernatants, as well as the levels of IL-4, IL-5(R&D Systems) and IFN-γ (eBiosciences) in CD4⁺ T cell culture supernatants were assessed by an ELISA, according to the manufacturer’s instructions.
Statistical analysis

The significance of difference between groups was evaluated by ANOVA and the Tukey multiple comparison test using the Instat program (GraphPad, San Diego, Calif.) differences between groups were considered significant at the level of $P < 0.05$.

RESULTS

Cytokine production by BMMs following *P. gingivalis* stimulation

To address the role of the VDR in the regulation of *P. gingivalis*-induced inflammatory response, we first assessed the induction of cytokines by *P. gingivalis* stimulated BMMs from WT and VDR/− mice. The levels of IL-6, IL-12p40 and TNF-α in VDR/− BMM cultures following *P. gingivalis* stimulation were significantly higher ($P < 0.05$) than the levels in WT cultures (Fig. 3). However, *P. gingivalis* induced a significantly lower ($P < 0.05$) level of the anti-inflammatory cytokine IL-10 in VDR/− cell cultures compared to that of WT cell cultures. These results suggest that VDR plays a regulatory role in the *P. gingivalis*–induced cytokine response of macrophages by downregulating the production of pro-inflammatory cytokines and potentiating the production of anti-inflammatory cytokine IL-10.

Infiltration of neutrophils and monocytes into the chambers following *P. gingivalis* challenge

Neutrophils and monocyte are initial responders in periodontal infections and 1, 25(OH)2D3 can affect these cells (32). To understand the role of VDR in regulating the cellular response to *P. gingivalis* infection, WT and VDR/− mice were infected with 5 x 10⁸ CFU of *P. gingivalis* in each subcutaneous chamber. Chamber exudates were collected at 24 h,
72 h and day 7 for neutrophil and monocyte counts. The total leukocyte count in the chamber fluids of WT and VDR\(^{-/-}\) control mice (PBS only injected in the chambers) was very low (data not shown). However following \textit{P. gingivalis} infection, a significantly higher number of leukocytes was observed in both WT and VDR\(^{-/-}\) mice; yet there was no significant difference in the neutrophil or macrophage count between the two groups (Fig. 4). These results indicate that VDR does not play a role in regulating the recruitment of leukocytes following \textit{P. gingivalis} infection.

**Analysis of chamber fluid for bacterial killing**

To determine the anti-bacterial effect of the VDR on \textit{P. gingivalis} chamber fluids were collected from WT and VDR\(^{-/-}\) mice at 24 h, 48 h and day 7 after \textit{P. gingivalis} infection. A decrease in the number of recovered \textit{P. gingivalis} was observed in the chamber fluid from both the WT and VDR\(^{-/-}\) mice at 24 h to day 7 (Fig. 5). However, the number of \textit{P. gingivalis} in VDR\(^{-/-}\) mice was significantly higher (\(P < 0.05, P < 0.05\)) than that of WT mice at all times tested. No bacterium was recovered from chamber fluids of control non-infected (data not shown). These results suggest that mice deficient in VDR has an impaired ability to clear \textit{P. gingivalis} from the chambers compared to WT mice.

**Cytokines levels in the chamber fluid following \textit{P. gingivalis} infection**

Since the VDR has been shown to play a role in the inflammatory host response, we next investigated the cytokine profile in the chamber exudates after \textit{P. gingivalis} challenge. Chamber fluids from non-infected mice had no detectable amounts of tested cytokines (data not shown). However, \textit{P. gingivalis} infection induced the production of cytokines in WT and VDR\(^{-/-}\) mice (Fig. 6). In the WT group, IL-6, IL-10 and TNF-\(\alpha\) cytokine levels peaked at 24 h post infection, but by 48 h the cytokine levels had decreased significantly.
However, levels of IL-12p40 remained the same at both time points in WT mice. Levels of TNF-α and IL-6 cytokines were similar 24 h and 48 h in VDR−/− mice, whereas IL-10 and IL-12p40 gradually increased from 24 h to 48 h. However, VDR−/− mice showed cytokines levels that were significantly higher ($P < 0.001$, $P < 0.001$) than those of WT mice. These results demonstrate that VDR down-regulate the inflammatory cytokine response to *P. gingivalis* infection.

### Serum cytokine profile

The serum cytokine response following *P. gingivalis* infection of the subcutaneous chambers was also assessed. Our results showed similar levels of serum IL-6 in WT and VDR−/− mice at 4 h after *P. gingivalis* infection (Fig. 7), however the level were significant higher ($P < 0.001$) in the VDR−/− than WT mice at 48 h post-infection. Minimal levels of serum TNF-α, IL-12p40, IL-10 were detected at 24 h and 48 h in WT and VDR−/− mice (data not shown).

### CD4⁺ T cell cytokine profile

T-cells, especially CD4⁺ T cells, are implicated in periodontal inflammation and tissue destruction (40). Furthermore it has been shown that CD4⁺ T cells can be direct targets of vitamin D (7). Thus we investigated the production of cytokines by splenic CD4⁺ T cell from infected mice following restimulation of cells with *P. gingivalis* in vitro. We found a markedly elevated levels IFN-γ in the culture supernatant of both WT and VDR−/− CD4⁺ T cell cultures (Fig. 8). However, the levels of IFN-γ in VDR−/− supernatants was significantly lower ($P < 0.05$) than that in WT cultures. Levels of IL-4 and IL-5 were undetectable in WT and VDR−/− cultures. These results suggest that VDR may favor a Th1
response following a *P. gingivalis* infection. However, further studies need to be taken to confirm this result.

**Serum anti-*P. gingivalis* antibody levels**

To understand the role of VDR in regulating the antibody response to *P. gingivalis*, serum samples from infected WT and VDR\(^{-/}\) mice were assessed for specific IgG, IgG1 and IgG2b antibody levels. Very low levels of total IgG1 and IgG2b anti-*P. gingivalis* specific antibodies were detected in WT and VDR\(^{-/}\) mice at 7 and 14 days after the first infection (Fig. 9). However, two weeks following the second infection (day 28), significantly higher levels of total IgG and of IgG1 and IgG2b specific antibodies were observed. Nevertheless WT mice showed significantly higher levels of total IgG (\(P < 0.001\)), IgG1 (\(P < 0.01\)) and IgG2b (\(P < 0.05\)) antibodies than VDR\(^{-/}\) mice. These results suggest that VDR plays a role in the up-regulation of serum IgG, IgG1 and IgG2b specific antibodies following *P. gingivalis* infection.

**DISCUSSION**

The active form of vitamin D, 1,25(OH)\(_2\)D\(_3\), not only plays an essential role in bone metabolism, but also facilitates phagocytosis by neutrophils (9, 25, 31, 45) and monocytes (3, 4, 18) as well as monocyte differentiation (25,31), thereby exerting a great influence on the host immune function. However it is not known if the VDR plays a role in the host response to periodontal pathogens such as *P. gingivalis*. In the present study using a mouse subcutaneous chamber infection model, we have shown the involvement of the VDR on various aspects of the host immune response to *P. gingivalis*. 
Polymorphisms in the human VDR gene have been associated with increased resistance or susceptibility to a number of infectious diseases. Case-control studies suggested links between certain alleles of the VDR gene and susceptibility to periodontal disease (2, 11, 12, 14, 26), tuberculosis (39), leprosy, dengue fever and hepatitis B virus-induced chronic hepatitis. Furthermore, strong evidence shows that TLR1/2 signaling in human macrophages, leads to an up-regulated expression of the VDR that in turn induced the expression of the antimicrobial peptide cathelicidin, which was shown to mediate intracellular killing of \textit{Mycobacterium tuberculosis} (31, 39, 40). In this experiment we investigated the ability of the host to clear bacteria and disease progression; we observed that VDR\textsuperscript{−/−} mice showed significantly enhanced containment of these bacteria in the chambers as compared to their wild-type. This was evident in accordance to our culture data that shows VDR deficiency or inefficiency would contribute to increased bacteria burden. In our further experiment highlights our observation of up regulated levels of pro-inflammatory cytokine production of IL-6, TNF-\(\alpha\), IL12p40 present at the local infection site, as well as cytokine increase of IL-6 in serum of VDR\textsuperscript{−/−} mice following \textit{P. gingivalis} challenge. This relevance of our findings in vivo was further analyzed in vitro where macrophages were stimulated with \textit{P. gingivalis} which showed a similar response. Our findings are also supported and correlative with another study of salmonella infection where in VDR\textsuperscript{−/−} mice exhibited a similar pro-inflammatory bias after \textit{Salmonella} infection and had increased bacterial burden and mortality (46). Studies show that Th1 and Th2 cell responses have a tremendous influence on the outcome of \textit{P. gingivalis} infection, wherein Th1 response dramatically results in increased bone resorption (15, 19, 44). We wanted to observe the cytokine profile
induced by *P. gingivalis* in CD4+ T cell cultures to help us understand the effect of the VDR on T cell cytokine production (33, 34). We compared the production of the Th2 associated cytokines (IL-4, IL-5) and Th1 associated cytokine production (IFN-γ) by CD4+T cells from infected VDR−/− and wild-type mice after *in vivo* stimulation with *P. gingivalis* antigen. It has been shown in recent data that VDR can inhibit the secretion of cytokines that are involved in the activation and differentiation of a subset of T helper cells (2). Autoimmune in vivo models of mice has shown that VDR inhibits the induction of Th1 and directly inhibits IFN-γ production and prevents IgG2 antibody responses (23). Our results indicate that WT mice had an IFN-γ cytokine up regulation, demonstrating that VDR−/− mice neither displayed neither enhanced Th1 nor elevated Th2 responses in this infection model. This result does support studies with leishmania major infection in which higher IFN-γ was seen in VDR−/− (16). Furthermore we wanted to evaluate if the effects of VDR have on antibody responses and reflects which Th response. With regards to the serum IgG and subclass, higher IgG1 and IgG2b responses against *P. gingivalis* were observed in WT mice, suggesting VDR supports Th1 response (29, 33, 34). This view is inconsistent with our results from many other infection studies that showed a preferential IgG2b or Th2 response after infection in presence of VDR signaling.

In summary, we concluded that VDR contributes in regulating host immune system against *P. gingivalis* infection. It plays a important and complex anti inflammatory role at the infected site suppressing inflammation and helps clear bacteria. It supports our hypothesis that it modulates inflammation, but many questions remain still unanswered for the adaptive responses and more further research would be needed to evaluate VDR’s role in *P. gingivalis* induced antibody and T cell responses.
REFERENCES


FIG. 3. **Cytokines production from BMMs.** Cytokines produced in culture supernatant of bone marrow-derived macrophages (BMMs), after 24 h stimulation with *Porphyromonas gingivalis* (MOI=50). * indicates significant differences at $P < 0.05$. between *P. gingivalis* infected and non infected groups.
FIG. 4. Differential count of neutrophil and monocytes in the chamber fluids. These were carried out on smears stained using Diff-Quik stain, and cells were identified by cell morphology. The chamber fluid samples were obtained at 24hr, 48hr and 7th day. Results represent the mean count at 10 different fields under 10 x magnifications.
FIG. 5. Bacterial count in chamber exudates. Chambers fluid samples obtained at 24h, 48h and 7 days after infection with *P. gingivalis* were cultured on ETSA plates following incubation, the number of CFUs were enumerated. Results represent the arithmetic mean ± SEM of samples from four mice / strain. *, indicate significant differences between the WT and VDR-/- mice at *P* < 0.05.
FIG. 6. Chamber fluid cytokine profile. Cytokines produced at 24 h and 48 h after *P. gingivalis* chamber challenge were assessed by ELISA. The data represent cytokine levels for pooled serum samples. Results represent the arithmetic mean ± SEM of four mice / strain. *** indicate significant differences between the groups of mice at \( P < 0.001 \), respectively.
**FIG. 7. Serum cytokine profile.** Cytokines produced at 4 h and 24 h after *P. gingivalis* infection via the implanted chambers. The data represent cytokine levels of pooled mice serum. Results represent the arithmetic mean ± SEM of four mice / strain. ** *** indicate significant differences between the groups of mice at $P < 0.001$, respectively.
FIG. 8. CD4\(^+\) T cells cytokine profile. Cytokine production of IL-4, IL-5 and IFN-\(\gamma\) from culture supernatant of CD4\(^+\) T cell from P. gingivalis infected WT and VDR\(^{-/-}\) mice, which were stimulated in vitro with P. gingivalis at an (MOI=10) for 24 h. Anti-CD3 and anti-CD28 antibodies were used as positive control for CD4\(^+\) T cell. Results represent the arithmetic mean ± SEM of four mice / strain. *, indicate significant differences between the groups of mice at \(P < 0.05\).
FIG. 9. Serum antibody response to *Porphyromonas gingivalis* of WT and VDR⁻/⁻ mice. Antibody levels at 7, 14, and 28 days after *P. gingivalis* infection via the implanted chamber. Results represent the arithmetic mean ± SEM of four mice / strain. *, ** and *** indicate significant differences between the groups of mice at  $P < 0.05$, $P < 0.01$ and $P < 0.001$. 
APPENDIX

NOTICE OF APPROVAL FROM INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

Notice of Approval for Protocol Modification

DATE: July 1, 2010

TO: Jannet Katz, D.D.S., Ph.D.
BBRB-713 2170
FAX: 934-1426

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Mechanisms of Immune Modulation and Periodontal Disease
Sponsor: NIH
Animal Project Number: 090608175

On July 1, 2010, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the modification as described: Personnel - Gulam Yezdani; Procedures - steel coil insertion. The sponsor for this project may require notification of modification(s) approved by the IACUC but not included in the original grant proposal/experimental plan; please inform the sponsor if necessary. The following species and numbers of animals reflect this modification.

<table>
<thead>
<tr>
<th>Species</th>
<th>Use Category</th>
<th>Number in Category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>B</td>
<td>Zero - Procedural modification only</td>
</tr>
<tr>
<td>Mice</td>
<td>A</td>
<td>300</td>
</tr>
</tbody>
</table>

Animal use is scheduled for review one year from June 2009. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files.

Refer to Animal Protocol Number (APN) 090608175 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
CH19, Suite 403
933 19th Street
205.934.7692
FAX: 205.934.1188

Mailing Address:
CH19 Suite 403
1530 3RD AVE.S
BIRMINGHAM AL 35294-0019