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Vitamin D3 modulates the function of chicken macrophages

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ABSTRACT

Vitamin D_3 is known to modulate both innate and adaptive immune responses in mammals, but there is little information on its effects on avian immune system cells. Here, we studied the effects of vitamin D_3 on chicken macrophages. Chicken macrophages expressed vitamin D receptor (VDR) and lipopolysaccharide (LPS) stimulation increased their VDR expression. Macrophages were treated with $1,25(OH)_2D_3$ in the presence or absence of Toll-like receptor ligands, such as LPS and Pam_3CSK_4. Subsequently, macrophage activation was assessed by measuring nitric oxide (NO) and expression of CXCL8 and interleukin (IL)-1β. In addition, changes in major histocompatibility complex (MHC)-II and CD86 were examined. Treatment of cells with $1,25(OH)_2D_3$ increased the ability of macrophages to respond to stimuli and produce NO, but vitamin D3 alone did not activate macrophages and resulted in the down-regulation of CD86, MHC-II, CXCL8 and IL-1β. These findings suggest that vitamin D3 has an immunomodulatory role in chicken macrophages.

1. Introduction

The role of vitamin D_3 in calcium and phosphorus metabolism is crucial for its well-documented involvement in bone and egg shell formation in layer hens (de Matos, 2008; DeLuca, 1988; Fleming, 2008). After vitamin D_3 enters the body through feeding or photosynthesis in epidermal cells, it undergoes two hydroxylation steps. The first step occurs in the liver, where it is changed into 25-hydroxy vitamin D3 ($25(OH)D_3$). The next step takes place in the kidney where it is changed into 1,25-dyhydroxyvitamin D_3 ($1,25(OH)_2D_3$), with the latter being considered the active metabolite (Norman and Hurwitz, 1993). It has been shown that $1,25(OH)_2D_3$ interacts with the vitamin D receptor (VDR), which is a member of the nucleus hormone receptor superfamily (Haussler et al., 1998). The expression of the VDR by almost all known cell types reveals the broad effects of this vitamin on different systems of the body, including the immune system (Provedini et al., 1983; Veldman et al., 2000).

In relation to the effect of vitamin D_3 on the immune system, various studies have demonstrated a predominately immunoregulatory role (Van Etten and Mathieu, 2005). For example, in humans $1,25(OH)_2D_3$ has been shown to be efficacious in the prevention and control of various autoimmune conditions (Cantorna, 2000; Cantorna et al., 1996, 1998; Lemire et al., 1992; Zella et al., 2003). Studies into the mechanisms of these effects have demonstrated that vitamin D_3 impacts both the innate and acquired immune systems.

The effect of vitamin D_3 on the innate immune system is demonstrated by the fact that vitamin D_3 diminishes the function of human dendritic cells by decreasing maturation, antigen presentation and production of cytokines, such as interleukin (IL)-12 and IL-23 (D'Ambrosio et al., 1998; Lang and Samaras, 2012). Additionally, an increase in human macrophage differentiation and production of cathelicidin and β-defensin has been observed after Vitamin D_3 treatment (Wang et al., 2004). Another study demonstrated that antimicrobial peptides are only produced by macrophages cultured in human serum with sufficient levels of vitamin D_3 (Fabri et al., 2011). In addition, macrophages treated with $1,25(OH)_2D_3$ had altered expression of various cytokines and chemokines, such as IL-12, CXCL8, IL-6, and tumor necrosis factor (TNF)-α (D'Ambrosio et al., 1998; Hakim and Bar-Shavit, 2003; Ryynänen and Carlberg, 2013). Macrophages are able to adjust the level of vitamin D_3 through the hydroxylation of $25(OH)D_3$ to $1,25(OH)_2D_3$. The enzyme responsible for the hydroxylation, 1,α hydroxylase, is expressed in macrophages in response to immune stimuli and is not influenced by negative feedback (Van Etten and Mathieu, 2005). On the other hand, production of 24-hydroxylase ($1,25(OH)_2D_3$ degrading enzyme) by macrophages enables the macrophages to regulate the level of this metabolite (Chen and DeLuca, 1995). The ability to control the production and degradation of vitamin D_3 suggests its importance for macrophage function and homeostasis.

In the case of the acquired immune system, expression of granulocyte-macrophage colony-stimulating factor (GM-CSF), which

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results in inflammatory responses, is down regulated by exposing T lymphocytes to 1,25(OH)2D3 (Tobler et al., 1987). Moreover, the effects of vitamin D_3 on dendritic cells, may determine the outcome of polarization of T cells into T helper (Th)1 or Th2 cells (Penna and Adorini, 2000). A reduction in the production of Th1 cytokines, such as interferon (IFN)-γ, and increases in Th2 cytokines, such as IL-4, IL-5, IL-10 and IL-13 have been described in humans (Helming et al., 2005; Lang and Samaras, 2012; Tiosano et al., 2013). Furthermore, vitamin D_3 regulates T cell development and migratory patterns of these cells (Di Rosa et al., 2011). In addition to the effects on T cells, evidence suggests that vitamin D_3 is important in the proliferation of activated B cells. Notably, it was demonstrated that the active metabolite of vitamin D_3 inhibits the ongoing proliferation of activated B cells and induces their apoptosis, whereas initial cell division is unaffected (Chen et al., 2007; Di Rosa et al., 2011). Therefore, the effect of vitamin D_3 on the function of the immune system has been established, however more research in this area is needed.

In chickens, limited information on the effects of vitamin D_3 on the immune system is available. Aslam and coworkers (1998) demonstrated that vitamin D_3 deficiency in broiler chickens resulted in a decrease in cell-mediated immune responses with no effect on antibody production. More recently, chicken CD4+ cells were shown to have the potential to alter the amount of active vitamin D_3 in their local environments by altering the expression of key enzymes involved in vitamin D_3 metabolism (Shanmugasundaram and Selvaraj, 2012). More recently, up-regulation of nitrite production and modulation of cytokine production in chicken macrophages and monocytes, have been reported after treatment of cells with 25(OH) D3 and LPS (Morris and Salveraj, 2014). The main objective of the present study was to elucidate the direct effects of the active metabolite of vitamin D_3, on the behavior of chicken macrophages, an important cellular component of the innate immune system.

2. Materials and methods

2.1. Cells

The MQ-NCSU cell line is derived from chicken macrophages (Qureshi et al., 1990) and was generously provided by Dr. Rodriguez-Lecompte (University of Prince Edward Island). The cells were maintained in 1:1 combination of McCoy's 5A modified medium and L-15 Leibovitz medium supplemented with 8% fetal bovine serum, 10% chicken serum, 1% tryptose phosphate broth, 1% sodium pyruvate, 2 mM L-glutamine, 200 U/ml penicillin, and 80 μg/ml streptomycin. During experiments, cells were cultured in DMEM-culture medium containing 10% fetal bovine serum, 200 U/ml penicillin, 80 μg/ml streptomycin, and 50 μg/ml gentamicin. Throughout cells were maintained at 41 °C in a humidified 5% CO_2 environment.

2.2. 1, 25-dihydroxyvitamin D_3

1,25-dyhydroxyvitamin D_3 ($1,25(OH)_2D_3$; Sigma) was used to treat MQ-NCSU cells. $1,25(OH)_2D_3$ was re-suspended in 95% EtOH at a concentration of 10×10^4 nM. Cells were treated with various concentrations of $1,25(OH)_2D_3$ ranging from 100 to 1×10^{-3} nM.

2.3. Treatment of MQ-NCSU cells

2.3.1. Nitric oxide production

A series of pilot studies were performed to see if $1,25(OH)_2D_3$ could potentiate TLR-mediated NO production by chicken macrophages. For this purpose different doses (100 – 10^{-3} nM) of $1,25(OH)_2D_3$ were tested with different doses of lipopolysaccharide (LPS) from *Escherichia coli* 0111: B4 (1 ng/ml to 1 μg/ml; Sigma) and Pam3-CSK (1 ng/ml to 1 μg/ml; Invivogen). Pretreatment with $1,25(OH)_2D_3$ was compared to co-administration of $1,25(OH)_2D_3$ and the TLR-L (data not shown). Pretreatment of cells with $1,25(OH)_2D_3$ for 24 hours at a concentration of 10 and 100 nM followed by administration of 5 ng/ml or 10 ng/ml of LPS and Pam3-CSK was selected.

To assess the NO production capacity of MQ-NCSU cells, with or without stimulation of $1,25(OH)_2D_3$, and/or TLR-L, 5×10^5 cells/well were seeded in 24 well plates and incubated for 2 hours. Then 10 nM of $1,25(OH)_2D_3$, EtOH (control) and culture medium (control) were added and the cells were incubated for an additional 24 hours. Subsequently, the cells were washed twice with DMEM-culture and LPS and Pam3-CSK were added at a concentration of 5 ng/ml and incubated for 6 hours. The plates were washed and 1 ml of DMEM-culture was added to each well and incubated for another 18 hours, before collecting the samples. For each group, four technical repeats were considered.

2.3.2. VDR gene expression

MQ-NCSU cells were seeded at a density of 1×10^6 cells/well (900 μl/well) in 24 well plates. After 2 hours of incubation, 100 μl of 1 μg/ml of LPS or DMEM (control) was added to the relevant wells. Cells were harvested at 0, 1, 2 and 4 hours by discarding the medium and adding 1 ml of TRIzol® reagent (Life Technologies Inc.) and then stored at –80 °C until RNA extraction.

2.3.3. CXCL8 and IL-1β gene expression

MQ-NCSU cells at 1×10^6 cells/well (900 μl/well) were seeded in 24 well plates and incubated for 2 hours. Then, 100 μl of DMEM, EtOH, or 1,25 $(OH)_2D_3$ (10 nM) was added to the appropriate wells and the cells were incubated for 24 hours. Then the cells were washed and subsequently, 1 ml of DMEM culture containing 5 ng/ml of LPS was added to the each well and the cells were harvested for RNA extraction at 0, 2, 8 and 24 h after activation, as described above.

2.4. Nitric oxide (NO) quantification

NO produced by MQ-NCSU cells was quantified using the Promega Griess assay kit according to manufacturer's instructions (Promega Corporation, USA).

2.5. RNA extraction, cDNA synthesis and real-time PCR

Total RNA was isolated from MQ-NCSU cell cultures using TRIzol® reagent (Life Technologies) and DNase treated with the DNA-free kit (Life Technologies). The concentration of RNA was quantified using the NanoDrop ND2000 (Thermo, Rockford, IL, USA). cDNA synthesis from mRNA template was carried out according to the Super Script First-Strand protocol (Invitrogen, Carlsbad, CA, USA), with oligo dT primers. cDNA samples were diluted 1:10 prior to real-time PCR.

To quantify relative gene expression, real-time PCR was performed with the LightCycler 480 (Roche, Laval, QC, Canada) as described previously (St Paul et al., 2013). The primers used are detailed in Table 1. PCR efficiency (E) is determined by the equation $E = 10^{-1/\text{slope}}$ using standard curves generated from plasmids containing a cloned copy of the amplicon. The gene expression relative to β-actin of each sample was calculated using Pfaffl's formula (Pfaffl, 2001): relative gene expression $= (E_T)^{\Delta Cp\ \text{target[calibrator-sample]}} / (E_R)^{\Delta Cp\ \text{reference[sample-calibrator]}}$, where E_T is the efficiency of the target gene, and E_R is the efficiency of the reference gene, β-actin.

2.6. Flow cytometry

MQ-NCSU cells (1×10^6 cells/ml) were cultured in the presence or absence of $1,25(OH)_2D_3$ (100 nM) for 24 h and cultured in the presence of LPS from *E. coli* 0111: B4 (10 ng/ml) or medium only