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Vitamin D3 alters microglia immune activation by an IL-10 dependent SOCS3 mechanism

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ABSTRACT

Microglia become activated immune cells during infection or disease in the central nervous system (CNS). However, the mechanisms that downregulate activated microglia to prevent immune-mediated damage are not completely understood. Vitamin D3 has been suggested to have immunomodulatory affects, and high levels of vitamin D3 have been correlated with a decreased risk for developing some neurological diseases. Recent studies have demonstrated the synthesis of active vitamin D3, 1,25-dihydroxyvitamin D3, within the CNS, but its cellular source and neuroprotective actions remain unknown. Therefore, we wanted to determine whether microglia can respond to vitamin D3 and whether vitamin D3 alters immune activation of microglia. We have previously shown that microglia become activated by IFNγ or LPS or by infection with virus to express pro-inflammatory cytokines, chemokines, and effector molecules. In this study, activated microglia increased the expression of the vitamin D receptor and *Cyp27b1*, which encodes the enzyme for converting vitamin D3 into its active form, thereby enhancing their responsiveness to vitamin D3. Most importantly, the activated microglia exposed to vitamin D3 had reduced expression of pro-inflammatory cytokines, IL-6, IL-12, and TNFα, and increased expression of IL-10. The reduction in pro-inflammatory cytokines was dependent on IL-10 induction of suppressor of cytokine signaling-3 (SOCS3). Therefore, vitamin D3 increases the expression of IL-10 creating a feedback loop via SOCS3 that downregulates the pro-inflammatory immune response by activated microglia which would likewise prevent immune mediated damage in the CNS.

1. Introduction

Microglia are central nervous system (CNS) resident immune cells that originate from primitive macrophage progenitors in the yolk sac and populate the CNS during early embryogenesis (Saijo and Glass, 2011). Microglia play a very important role in immune surveillance of the CNS due to the blood brain barrier limiting immune cell infiltration under healthy conditions. We have previously shown that microglia act as sensors in the CNS expressing many innate immune receptors, such as Toll-like receptors (TLRs), that detect foreign pathogens as well as self-tissue damage (Olson and Miller, 2004). The activation of microglia through the innate immune receptors leads to expression of cytokines, chemokines, and effector molecules. The activation of the innate immune response directly contributes to the development of the adaptive immune response (Olson et al., 2001; Olson and Miller, 2004). The innate immune chemokines attract immune cells from the periphery, and the innate immune cytokines activate the infiltrating immune cells, including the $CD4^+$ T cells. In addition, innate immune activation of microglia leads to the expression of MHC class II and the ability of microglia to present antigens to $CD4^+$ T cells (Olson et al., 2001; Olson and Miller, 2004).

Activated microglia have been identified in the CNS during several neurological diseases, including multiple sclerosis (MS), Alzheimer's disease, and Parkinson's disease (Henderson et al., 2009; van and Amor, 2009). (Lue et al., 2010; Tansey and Goldberg, 2010). An acute inflammatory response in the CNS is important for responding to infection or injury, however, chronic inflammation has been associated with neurological diseases. The activation of microglia in the CNS has been well documented, however the mechanisms involved in resolving the inflammatory activation of microglia and returning microglia to homeostatic conditions has not been as well documented.

Vitamin D3 is synthesized in the skin from 7-dehydrocholesterol upon exposure to sunlight, ultraviolet irradiation, or ingested through supplemented diet. Vitamin D is transported in the blood by the vitamin D binding protein to the liver where it is hydroxylated by 25-

Abbreviations: CNS, central nervous system; LPS, lipopolysaccharide; MS, multiple sclerosis; TMEV, Theiler's murine encephalomyelitis virus; VDR, vitamin D receptor; $25(OH)D_3$, 25-hydroxyvitamin D3; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3.

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hydroxylase enzyme into 25-hydroxyvitamin D3 (25(OH)D_3). The 25(OH)D_3 is the circulating form that can be converted to the hormonally active 1,25-dihydroxyvitamin D3 (1,25,$(OH)_2D_3$) by the enzyme 1α-hydroxylase encoded by the *Cyp27b1* gene. The cells in the kidney, lung, and placenta, as well as macrophage and dendritic cells have been determined to express *Cyp27b1* for converting 25(OH)D_3 to 1,25(OH)D_3 (Holick, 2007). The active form, 1,25$(OH)_2D_3$, binds to the vitamin D receptor (VDR) forming a complex with retinoid X receptor, and this complex then translocates to the nucleus. In the nucleus the complex can bind to the promoter region of targeted genes and can interact with other transcription factors leading to repression or activation of transcription (Pike and Meyer, 2010). Further, 1,25$(OH)_2D_3$ induces *Cyp24a1* expression which encodes 24-hydroxylase that degrades 1,25$(OH)_2D_3$ to prevent vitamin D toxicity. A previous study showed that cultured microglia from rats can convert 25(OH)D_3 into 1,25$(OH)_2D_3$, however, the expression of the enzymes required for conversion and degradation of vitamin D3 were not examined (Neveu et al., 1994).

The active form of vitamin D3, 1,25$(OH)_2D_3$, has been shown to have immunomodulatory effects (Alroy et al., 1995; Cippitelli and Santoni, 1998; Towers and Freedman, 1998; Hayes et al., 2015), and vitamin D3 insufficiency has been correlated with development of neurological diseases (Annweiler et al., 2009; Fernandes de Abreu et al., 2009). Multiple sclerosis is a demyelinating disease that is associated with an inflammatory immune response in the CNS where inflammatory lesions can be observed in patients that contain immune cells surrounding areas of demyelination (Barnett et al., 2009). Most importantly, microglia have been identified in pre-lesions and activate inflammatory lesions in MS patients (Henderson et al., 2009; van and Amor, 2009). The causative agent for MS has not been determined. However, environmental factors, such as low vitamin D levels, have been associated with disease development, especially during childhood (Ebers, 2008; Simpson et al., 2011). A recent retrospective study of people in the military showed that people with lower serum levels of vitamin D3 developed MS more frequently than people who had high serum levels of vitamin D3 (Munger et al., 2006). Another environmental factor that may contribute to development of MS is a virus infection acquired during the first 15 years of life (Kurtzke, 1993). The specific virus associated with development of MS has not been determined, however, many studies have focused on members of the herpesvirus family, especially Epstein Barr virus (EBV) (Pender, 2011; Trojano and Avolio, 2009).

Theiler's murine encephalomyelitis virus (TMEV)-induced demyelinating disease shares immunological and pathological similarities to MS and is used as an animal model of disease. TMEV infection of susceptible mice (SJL strain) leads to the development of a persistent infection in the microglia/macrophage population in the CNS associated with development of demyelinating disease. The clinical signs of demyelinating disease begin around 35 days post infection and continue to progress, eventually leading to paralysis and death (Lipton, 1975). TMEV-infected microglia become activated immune cells participating in both the innate and adaptive immune response during the virus infection and subsequent demyelinating disease (Olson et al., 2001; Olson and Miller, 2004).

Activated microglia have been associated with neurological diseases, and low levels of vitamin D3 have been associated with increased incidence of neurological diseases. Thus, we proposed that microglia convert circulating vitamin D3 into the active form of vitamin D3 which may then attenuate the inflammatory immune response by microglia and reduce the development and progression of neurological diseases. First, microglia were shown to express VDR, *Cyp27b1*, and *Cyp24a1*, and microglia activated by IFNγ, LPS, or TMEV infection increased the expression of VDR and *Cyp27b1* enabling them to be more responsive to vitamin D3. Most interestingly, microglia exposed to vitamin D3, either 25(OH)D_3 or 1,25$(OH)_2D_3$, at the time of immune activation with IFNγ, LPS, or TMEV had reduced expression of pro-inflammatory cytokines and increased expression of IL-10. IL-10 has been shown to have anti- inflammatory properties and is associated with a switch from Th1 type response to a Th2 type T cell response (Chabot et al., 1999; O'Keefe et al., 1999). Recently, IL-10 has been shown to reduce pro-inflammatory cytokine expression through the induction of suppressor of cytokine signaling-3 (SOCS3) (Qin et al., 2006). Vitamin D3 increased the expression of SOCS3 in microglia in an IL-10 receptor dependent manner to reduce the expression of pro-inflammatory cytokines. These results show that microglia metabolize circulating 25(OH)D_3 into active 1,25$(OH)_2D_3$, thereby microglia have a complete intracrine pathway of 1,25$(OH)_2D_3$ synthesis that is further induced upon activation. More importantly, this vitamin D3 intracrine signaling pathway altered the immune activation of microglia by promoting the expression of IL-10 which increased the expression of SOCS3 and suppressed the expression of pro-inflammatory cytokines. These results suggest that vitamin D3 may aid in resolving the inflammatory immune response by microglia which may have a beneficial impact on activated microglia during neurological diseases.

2. Materials and methods

2.1. Mice

Pregnant SJL/J mice (15–17 days) were purchased from Harlan Laboratories (Madison, WI). The mice were housed at the University of Minnesota Research Animal Resource Center according to the university and ACUC approved protocols. Neonatal mice, age 1–3 days, were used for the microglia isolation.

2.2. Isolation and culture of microglia cells

Isolation of primary glial cultures from neonatal mice was performed, as previously described (Olson et al., 2001). Briefly, brains were removed from 1 to 3 day old mice, and the meninges were removed. The left and right hemispheres of the brain were gently dissociated in a nylon mesh bag. The cells were resuspended in DMEM-F12 media (Lonza) supplemented with 10% FCS (Invitrogen Life Technologies) and 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen Life Technologies). The cells were seeded in poly(D-lysine) (Sigma-Aldrich) coated tissue culture flasks and incubated at 37 °C. After 10–14 days of incubation, microglia were removed from the astroglial layer by shaking the flasks in an orbital shaker for 24 h. The primary microglia were removed from the flask and resuspended in DMEM (Invitrogen Life Technologies) supplemented with 20% FCS and 3 ng/ml rGM-CSF (R&D Systems). The microglia were seeded in 24 well plates coated with poly(D-lysine). Microglia were activated by adding rIFNγ (100 U/ml) or LPS (5 μg/ml) (Sigma-Aldrich) to the media for 24 h. Microglia were infected with the BeAN strain of TMEV at a multiplicity of infection of 5 in serum-limited DMEM for 24 h as previously described (Olson et al., 2001; Olson and Miller, 2004). The primary cultures were either unstimulated, stimulated with IFNγ or LPS, or infected with TMEV in the absence or presence of 1,25$(OH)_2D_3$ (10 nM) or 25(OH)D_3 (10 nM) (Sigma-Aldrich). The concentration for vitamin D3 in cultures was based on a dose response analysis as well as physiological levels. The primary cultures were cultured with neutralizing antibody to IL-10 (50 ng/ml) based on a dose response analysis or isotype control antibody (eBioscience). The primary cultures were transfected with siRNA for SOC3, SMARTpool SOCS3 siRNA (5 μM), or siCONTROL (Dharmacon) using Dharmafect 4 following the protocol provided by Dharmacon.

2.3. RNA isolation and real-time PCR analysis

Microglia were scraped from the culture wells and RNA was isolated from the cell using SV Total RNA Isolation kit which contains a DNAse reaction (Promega). First strand cDNA was generated from 1 μg of total RNA from the microglia using oligo$(dT)_{12-18}$ primers and Advantage