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

Mandy Boontanrart [a], Samuel D. Hall [a], Justin A. Spanier [b], Colleen E. Hayes [c], Julie K. Olson [d,*]

[a] Department of Neurological Surgery, School of Medicine and Public Health, University of Wisconsin-Madison, United States
[b] Department of Medicine, Center for Immunology, University of Minnesota, United States
[c] Department of Biochemistry, University of Wisconsin-Madison, United States
[d] Department of Diagnostic and Biological Sciences, University of Minnesota, United States

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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.

* Corresponding author at: Department of Diagnostic and Biological Sciences, University of Minnesota, 515 Delaware Street SE, 17-164B Moos Health Sciences Tower, Minneapolis, MN 55455, United States.
E-mail address: jkolson@umn.edu (J.K. Olson).

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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

for RT-PCR kit in a final volume of 100 μl (Clontech). Real- time PCR was conducted in triplicate with Faststart DNA Master SYBR Green kit (Roche Molecular Biochemicals). Briefly, 0.5 μM primers, 2 mM $MgCl_2$, 1× Faststart SYBR Green reagent, and 2 ml of cDNA were combined. The primers for the cytokines, chemokines, and effector molecules were previously described (Olson et al., 2001; Olson and Miller, 2004). The primers for VDR, Cyp27b1, and Cyp24a1 were previously described (Spanier et al., 2012). Real time PCR was conducted on a Rotorgene 6000 (Qiagen) using hot start with cycle combinations, 40 cycles: 95 °C for 15 s; 60 °C for 2 s; 72 °C for 15 s, followed by a melt from 75 °C to 95 °C. Quantitation of the mRNA was based on standard curves derived from cDNA standards for each primer pair. Positive and negative cDNA controls were used for each cytokine using cells known to express or not express the specific cytokine. Samples from different groups were normalized based on expression of β-actin. All samples were run in triplicate for each primer pair. Statistical analysis comparison between groups was determined using Student's *t* test ($p < 0.05$).

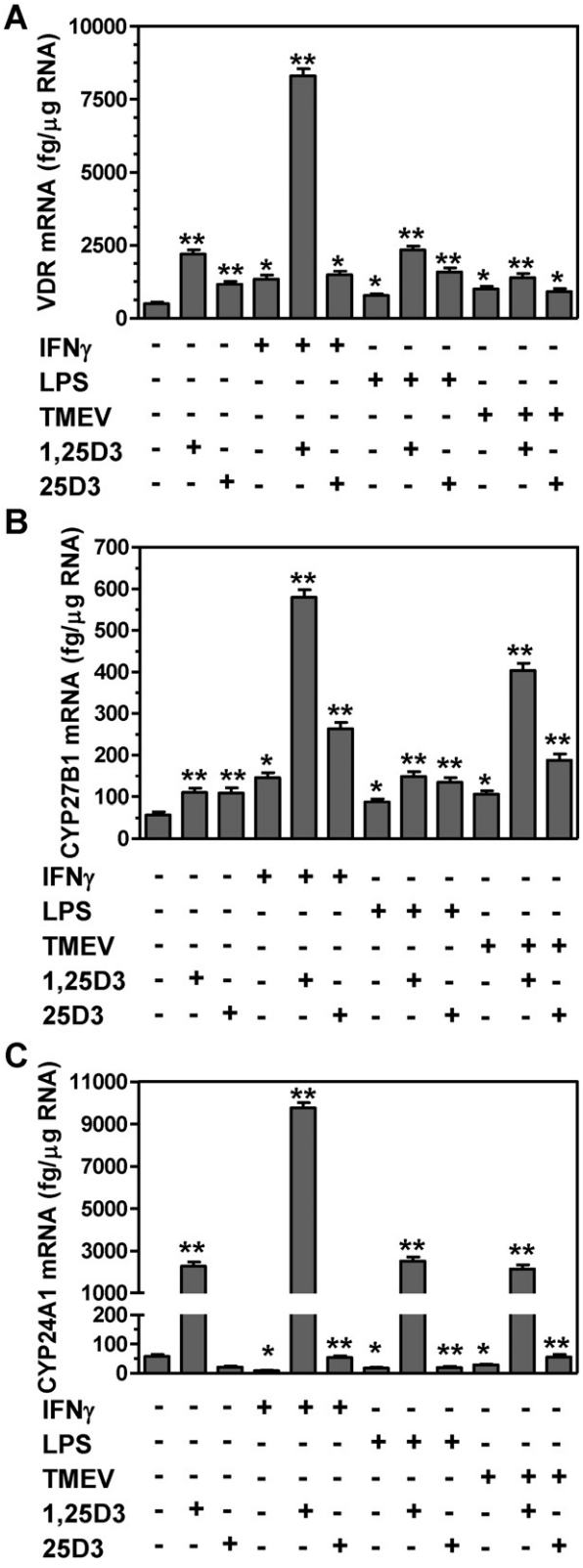

Fig. 1. Microglia expression of VDR, *Cyp27b1*, and *Cyp24a1* is altered during microglia activation. Primary microglial cultures were derived from neonatal SJL mice. The cultures were stimulated with IFNγ (100 U/ml) or LPS (5 μg/ml) or infected with TMEV at (MOI of 4) and $1,25(OH)_2D_3$ (10 nM) was added to the cultures. After 24 h, RNA was isolated from the cells, DNAse treated, converted to cDNA, and used in real time PCR with primers for VDR (A), *Cyp27b1* (B), or *Cyp24a1* (C). The concentration was based on standards for each set of primers and groups were normalized based on expression of β-actin. The significant difference (*) between unstimulated microglia and stimulated microglia, with IFNγ, LPS, or TMEV was determined by Student's *t* test ($p < 0.05$). The significant difference (**) between each stimulated sample control treated compared with $1,25(OH)_2D_3$ treated for that stimulation was determined by Student's *t* test. This is a representative graph from three individual repeated experiments.

3. Results

3.1. Microglia express VDR, Cyp27b1, and Cyp24a1

First, we wanted to determine whether microglia express VDR which is essential for $1,25(OH)_2D_3$ responsiveness. Primary microglia were isolated from neonatal SJL mice, as previously described (Olson et al., 2001). The microglia were either unstimulated, stimulated with IFNγ or LPS, or infected with TMEV for 24 h in the absence or presence of $1,25(OH)_2D_3$ (10 nM) and then examined for the expression of VDR (Fig. 1). Microglia expressed the VDR constitutively, and in the presence of $1,25(OH)_2D_3$, VDR expression was increased (Fig. 1A and Table 1). Microglia stimulated with IFNγ increased the expression of VDR, and the addition of $1,25(OH)_2D_3$ further increased the expression of VDR by 6 fold compared to IFNγ-stimulated microglia alone. Microglia stimulated with LPS increased the expression of VDR which suggests that signaling through TLR4 increases the expression of VDR. The addition of $1,25(OH)_2D_3$ to the LPS-stimulated microglia further increased the expression of VDR by 3 fold compared to LPS-stimulation alone. Microglia infected with TMEV increased the expression of VDR which suggests that signaling through the innate immune receptors or cytokines may upregulate VDR expression during virus infection. The addition of $1,25(OH)_2D_3$ to the TMEV-infected microglia further increased the expression of VDR by 2 fold compared to TMEV-infected alone.

Next, microglia were analyzed for expression of *Cyp27b1* which encodes the 1α-hydroxylase that converts $25(OH)D_3$ to $1,25(OH)_2D_3$ (Fig. 1B). Microglia constitutively expressed *Cyp27b1*, and *Cyp27b1* expression was increased in the presence of $1,25(OH)_2D_3$ (Fig. 1B and Table 1). IFNγ-stimulated microglia increased the expression of *Cyp27b1* and further increased the expression in the presence of $1,25(OH)_2D_3$ by 4 fold compared to IFNγ-stimulated microglia alone. LPS-stimulated microglia slightly increased the expression of *Cyp27b1* compared to unstimulated microglia but the addition of $1,25(OH)_2D_3$ further increased the expression of *Cyp27b1* by 2 fold compared to LPS-stimulated microglia alone. TMEV-infected microglia also increased the expression of *Cyp27b1* compared to uninfected microglia and expression of *Cyp27b1* was further increased with the addition of $1,25(OH)_2D_3$ by 4 fold compared to TMEV-infected microglia alone.

Finally, microglia were analyzed for the expression of *Cyp24a1* which encodes the 24-hydroxylase that breaks down $1,25(OH)_2D_3$ to prevent toxic levels from accumulating in the cells (Fig. 1C and Table 1). Microglia constitutively expressed low levels of *Cyp24a1* but it was highly increased in the presence of $1,25(OH)_2D_3$. IFNγ stimulation reduced the expression of *Cyp24a1* in microglia by 7 fold compared

Table 1
Microglia expression of VDR, Cyp27b1, Cyp24a1.

	Unstim	Unstim 1,25D	Unstim 25D3	IFNγ	IFNγ 1,25D	IFNγ 25D3	LPS	LPS 1,25D	LPS 25D3	TMEV	TMEV 1,25D	TMEV 25D3
VDR	1.00	4.43	2.34	2.70	16.77	3.01	2.01	4.72	3.21	2.03	2.80	2.05
CYP27B1	1.00	2.01	2.01	2.59	10.34	4.69	2.00	2.64	2.39	2.08	7.20	3.34
CYP24A1	1.00	39.07	0.58	0.15	168.31	0.93	0.21	43.31	0.43	0.47	36.86	0.98

Fold difference based on expression in unstimulated microglia.

to unstimulated microglia. However, the addition of $1,25(OH)_2D_3$ highly increased the expression of *Cyp24a1* in IFNγ-stimulated microglia with a 4-fold increase compared to unstimulated microglia with $1,25(OH)_2D_3$. LPS stimulation of microglia also reduced the expression of *Cyp24a1* in microglia by 3 fold compared to unstimulated microglia while the addition of $1,25(OH)_2D_3$ increased the expression of *Cyp24a1* in the LPS-stimulated microglia similar to unstimulated microglia. TMEV infection reduced the expression of *Cyp24a1* in microglia by 2 fold compared to uninfected microglia, however the addition of $1,25(OH)_2D_3$ to the infected cells increased the expression of *Cyp24a1* similar to uninfected microglia with $1,25(OH)_2D_3$.

These results show that microglia express the VDR, *Cyp27b1*, and *Cyp24a1* which creates an intracrine system within microglia for converting circulating vitamin D3, $25(OH)D_3$, to the active form, $1,25(OH)_2D_3$. Interestingly, the expression of VDR and *Cyp27b1* can be increased when microglia are activated with IFNγ or LPS or infected with TMEV and further increased in the presence of $1,25(OH)_2D_3$. These results also show that activation of microglia by either IFNγ or LPS or infection with TMEV decreased the expression of *Cyp24a1*, however the addition of $1,25(OH)_2D_3$ increased the expression of *Cyp24a1* especially in IFNγ-stimulated microglia. These results suggest that activation of microglia increases the responsiveness of microglia to vitamin D3 by increasing VDR and *Cyp27b1* expression and by decreasing *Cyp24a1* expression which degrades $1,25(OH)_2D_3$. However, high amounts of $1,25(OH)_2D_3$ leads microglia to increase *Cyp24a1* to prevent cytotoxic levels from accumulating in the cells.

3.2. Vitamin D3 alters the immune response by activated microglia

We have previously shown that microglia stimulated with IFNγ increased the expression of pro-inflammatory cytokines, such as IL-6, IL-12, and TNFα, effector molecules, such as iNOS, and chemokines, such as MIP-1α (Olson et al., 2001; Olson and Miller, 2004). We have also shown that microglia express all the TLRs and respond to stimulation through TLRs, including LPS, by increasing the expression of pro-inflammatory cytokines, effector molecules, and chemokines (Olson and Miller, 2004). Further, we have previously shown that microglia infected with TMEV express high levels of type I interferons, IFNα and IFNβ, and pro-inflammatory cytokines as well as chemokines and effector molecules (Olson et al., 2001; Olson and Miller, 2004). As shown above, microglia expressed VDR and *Cyp27b1* enabling microglia to respond to vitamin D3, and this expression was increased by stimulation with IFNγ or LPS or infection with TMEV making microglia more responsive to vitamin D3. Thus, we wanted to determine whether vitamin D3 can alter the immune response by microglia following stimulation with IFNγ or LPS or infection with TMEV. Microglia were stimulated with IFNγ or LPS or infected with TMEV in the presence of $1,25(OH)_2D_3$ or $25(OH)D_3$

IFNγ-stimulated microglia increased the expression of pro-inflammatory cytokines, IL-6, IL-12 and TNFα, which was reduced by the presence of either $1,25(OH)_2D_3$ or $25(OH)D_3$ (Fig. 2). Most importantly, IFNγ-stimulated microglia have a slight increase in the expression of IL-10 however this was greatly increased in the presence of either

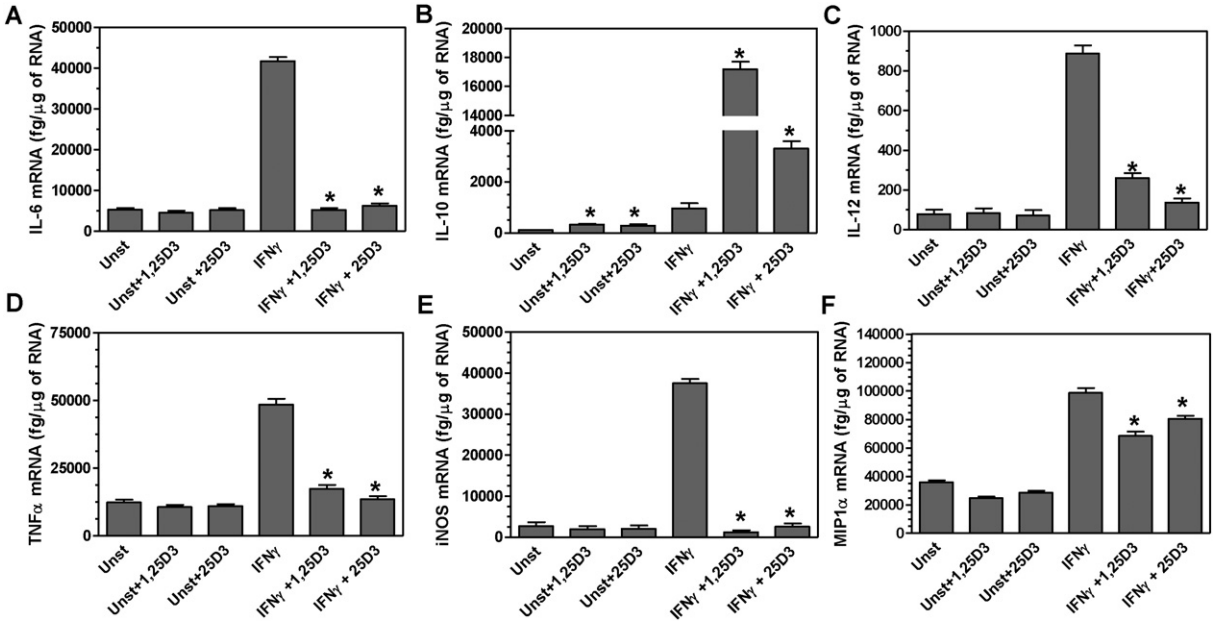

Fig. 2. Vitamin D3 alters immune activation of microglia following IFNγ stimulation. Primary microglial cultures were derived from neonatal SJL mice. The cultures were unstimulated or stimulated with IFNγ (100 U/ml) in the presence of $1,25(OH)_2D_3$ (10 nM), or $25(OH)D_3$ (10 nM). After 24 h, RNA was isolated from the cells, converted to cDNA and used in real time PCR with primers for IL-6 (A), IL-10 (B), IL-12 (C), TNFα (D), iNOS (E), and MIP-1α (F). The concentration was based on standards for each set of primers and groups were normalized based on expression of β-actin. The significant difference (*) between the IFNγ-stimulated microglia and the $1,25(OH)_2D_3$ (10 nM), or $25(OH)D_3$ (10 nM) treated cells was determined by Student's *t* test ($p < 0.05$). This is a representative graph from four individual repeated experiments.

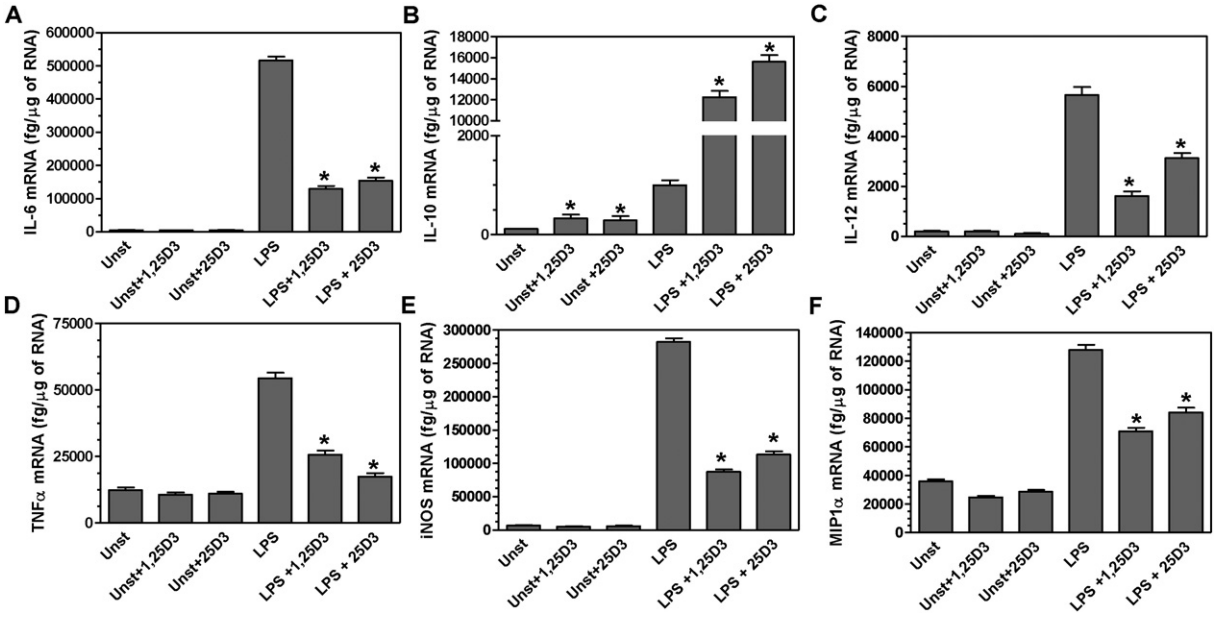

Fig. 3. Vitamin D3 alters innate immune activation of microglia following LPS stimulation. Primary microglial cultures were derived from neonatal SJL mice. The cultures were unstimulated or stimulated with LPS (5 μg/ml) in the presence of $1,25(OH)_2D_3$ (10 nM), or $25(OH)D_3$ (10 nM). After 24 h, RNA was isolated from the cells, converted to cDNA and used in real time PCR with primers for IL-6 (A), IL-10 (B), IL-12 (C), TNFα (D), iNOS (E), and MIP-1α (F). The concentration was based on standards for each set of primers and groups were normalized based on expression of β-actin. The significant difference (*) between the LPS stimulated microglia and the $1,25(OH)_2D_3$ (10 nM), or $25(OH)D_3$ (10 nM) treated cells was determined by Student's *t* test ($p < 0.05$). This is a representative graph from four individual repeated experiments.

$1,25(OH)_2D_3$ or $25(OH)D_3$. IFNγ-stimulated microglia increased the expression of effector molecule, iNOS, and this expression was reduced by either $1,25(OH)_2D_3$ or $25(OH)D_3$. Finally, IFNγ-stimulated microglia increased the expression of chemokine, MIP-1α, and this expression was slightly reduced by vitamin D3, either $1,25(OH)_2D_3$ or $25(OH)D_3$.

LPS- stimulated microglia increased the expression of pro-inflammatory cytokines, IL-6, IL-12, and TNFα, which was reduced in the presence of either $1,25(OH)_2D_3$ or $25(OH)D_3$ (Fig. 3). Most interestingly, LPS-stimulated microglia express low levels of IL-10 but the expression of IL-10 was greatly increased by $1,25(OH)_2D_3$ or $25(OH)D_3$. LPS-stimulated microglia also increased the expression of effector molecule, iNOS, which was reduced in the presence of vitamin D3, either $1,25(OH)_2D_3$ or $25(OH)D_3$. Finally, LPS-stimulated microglia increased the expression of chemokines, MIP-1α, which was reduced in the presence of $1,25(OH)_2D_3$ or $25(OH)D_3$.

TMEV infection of microglia induces the expression of large amounts of type I interferons, IFNα and IFNβ, and interestingly, the addition of vitamin D3, $1,25(OH)_2D_3$ or $25(OH)D_3$, increased the expression of both IFNα and IFNβ (Fig. 4). TMEV-infected microglia increased the expression of pro-inflammatory cytokines, IL-6, IL-12 and TNFα, and this expression was reduced in the presence of either $1,25(OH)_2D_3$ or $25(OH)D_3$. Most importantly, TMEV-infected microglia expressed IL-10 and this level was greatly increased by vitamin D3, either $1,25(OH)_2D_3$ and $25(OH)D_3$. TMEV-infected microglia also increased the expression of effector

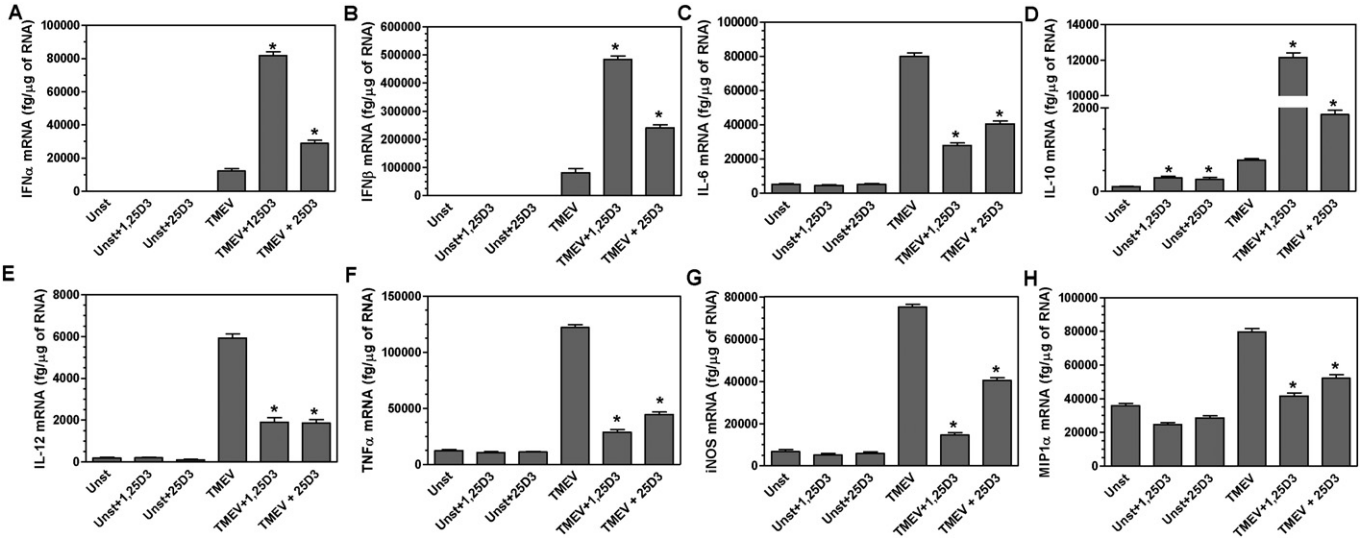

Fig. 4. Vitamin D3 alters the immune response during TMEV infection of microglia. Primary microglial cultures were derived from neonatal SJL mice. The cultures were infected with TMEV (MOI 4) in the presence of $1,25(OH)_2D_3$ (10 nM), or $25(OH)D_3$ (10 nM). After 24 h, RNA was isolated from the cells, converted to cDNA and used in real time PCR with primers for IFNα (A), IFNβ (B), IL-6 (C), IL-10 (D), IL-12 (E), TNFα (F), iNOS (G), and MIP-1α (H). The concentration was based on standards for each set of primers and groups were normalized based on expression of β-actin. The significant difference (*) between the TMEV infected microglia and the $1,25(OH)_2D_3$ (10 nM), or $25(OH)D_3$ (10 nM) treated cells was determined by Student's *t* test ($p < 0.05$). This is a representative graph from four individual repeated experiments.

molecules, iNOS, which was reduced in the presence of either $1,25(OH)_2D_3$ or $25(OH)D_3$. Finally, TMEV infected microglia increased the expression of chemokines, MIP-1α, which was reduced by vitamin D3.

These results suggest that vitamin D3 present during activation of microglia by IFNγ, LPS, or TMEV infection decreases the expression of pro-inflammatory cytokines, effector molecules, and chemokines while increasing the expression of IL-10, an anti-inflammatory cytokine. Further, vitamin D3 increased the expression of type I interferons by microglia during TMEV infection. Overall, these results show that vitamin D3, either in the circulating form or the active form, can alter the immune response by microglia which demonstrates that microglia can convert the circulating form into the active form for binding to the VDR which then alters the expression of cytokines, chemokines, and effector molecules.

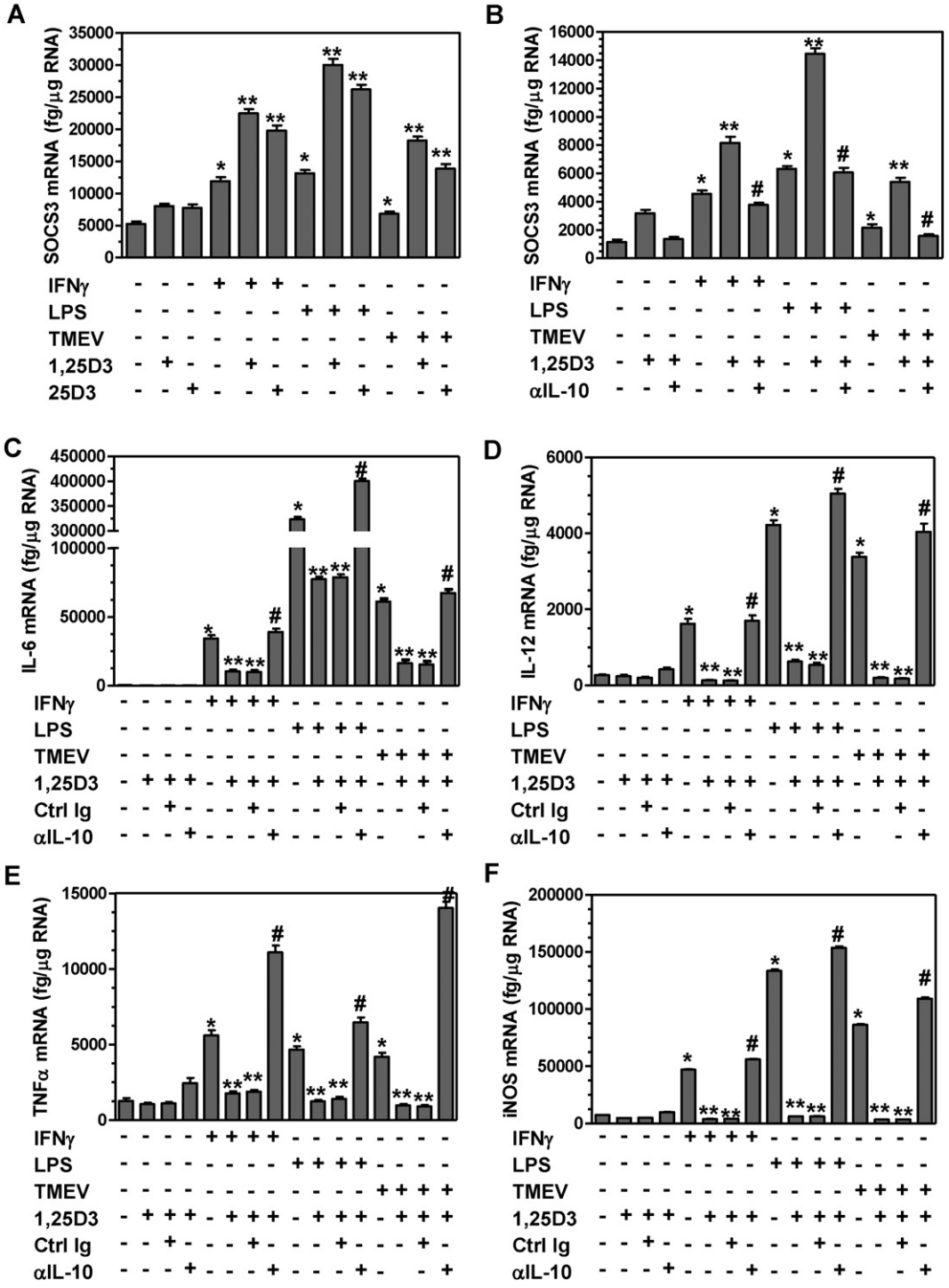

Fig. 5. Vitamin D3 induced expression of IL-10 increased SOCS3 and reduced pro-inflammatory cytokine expression in activated microglia. Primary microglial cultures were derived from neonatal SJL mice. The cultures were stimulated with IFNγ or LPS, or infected with TMEV in the presence of $1,25(OH)_2D_3$ (10 nM) or $25(OH)D_3$ (10 nM) and with antibody to IL-10 (50 ng/ml) or control antibody (50 ng/ml). After 24 h, RNA was isolated from the cell, converted to cDNA and used in real time PCR with primers for SOCS3 (A and B), IL-6 (C), IL-12 (D), TNFα (E), and iNOS (F). The significant difference (*) between the unstimulated and stimulated microglia, the significant difference (**) between stimulated microglia without or with vitamin D3, and the significant difference (#) between stimulated microglia with vitamin D3 and with control antibody or with antibody to IL-10, were determined by Student's *t* test ($p < 0.05$). This is a representative graph from three individual repeated experiments.

3.3. Vitamin D3 increases the expression of SOCS3 in an IL-10 dependent manner which reduces the expression of pro-inflammatory cytokines

Microglia stimulated with IFNγ or LPS or infected with TMEV had increased expression of IL-10 in the presence of vitamin D3. Likewise, the activated microglia had reduced expression of pro-inflammatory cytokines, especially IL-6 and TNFα, in the presence of vitamin D3. Previous studies have shown that IL-10 can reduce the activation of macrophage by activating suppressor of cytokine signaling-3 (SOCS3) (Qin et al., 2006). SOCS3 is a negative regulator of Janus kinases (JAKs) which are involved in the signaling pathway for pro-inflammatory cytokines, such as IL-6 (Baker et al., 2009). SOCS3 expression has previously been shown to be induced in microglia following IFNγ or LPS stimulation (Baker et al., 2009). Thus, we wanted to determine whether vitamin D3-induced expression of IL-10 was increasing the expression of SOCS3 in microglia (Fig. 5). Microglia stimulated with IFNγ had a slight increase in SOCS3 expression, however, SOCS3 expression was increased in the presence of vitamin D3, both $25(OH)D_3$ and $1,25(OH)_2D_3$. Microglia stimulated with LPS also slightly increased SOCS3 expression but the expression was further increased by vitamin D3.Microglia infected with TMEV slightly increased the expression of SOCS3, however, vitamin D3 greatly increased the expression of SOCS3. Next, we wanted to determine whether IL-10 secreted from the microglia was binding to the IL-10 receptor on the microglia to increase the expression of SOCS3 (Fig. 5). Neutralizing antibody to IL-10 was added to the stimulated microglia in the presence of vitamin D3. Microglia stimulated with IFNγ or LPS or infected with TMEV in the presence of vitamin D3 had reduced expression of SOCS3 when antibody to IL-10 was added to the cells compared to control antibody. Thus, these results show that vitamin D3 increased the expression of SOCS3 in activated microglia and that the increased SOCS3 expression was dependent on IL-10 binding to its receptor

Next, we wanted to determine whether IL-10 was required for the reduction in the expression of pro-inflammatory cytokines in activated microglia treated with vitamin D3 (Fig. 5). Vitamin D3 reduced the expression of pro-inflammatory cytokines, IL-6, IL-12, and TNFα, and effector molecule, iNOS, in microglia stimulated with IFNγ or LPS, however, when IL-10 was neutralized, the expression of the pro-inflammatory cytokines and effector molecules returned to levels at or above microglia stimulated in the absence of vitamin D3. Similarly, vitamin D3 reduced the expression of pro-inflammatory cytokines by TMEV-infected microglia, and when IL-10 was neutralized, the expression of pro-inflammatory cytokines and effector molecules were increased to levels similar to TMEV-infected microglia in the absence of vitamin D3. Therefore, these results show that vitamin D3 induced secretion of IL-10 from the microglia which then bound to the IL-10 receptor and subsequently reduced the expression of pro-inflammatory cytokines and effector molecules.

Next, we wanted to determine whether IL-10 induction of SOCS3 was involved in the reduction of pro-inflammatory cytokine expression in activated microglia by silencing SOCS3 (Fig. 6). Vitamin D3 reduced the expression of cytokines, IL-6, IL-12, and TNFα, and effector molecule, iNOS, in microglia stimulated with IFNγ or LPS, however, when SOCS3 was

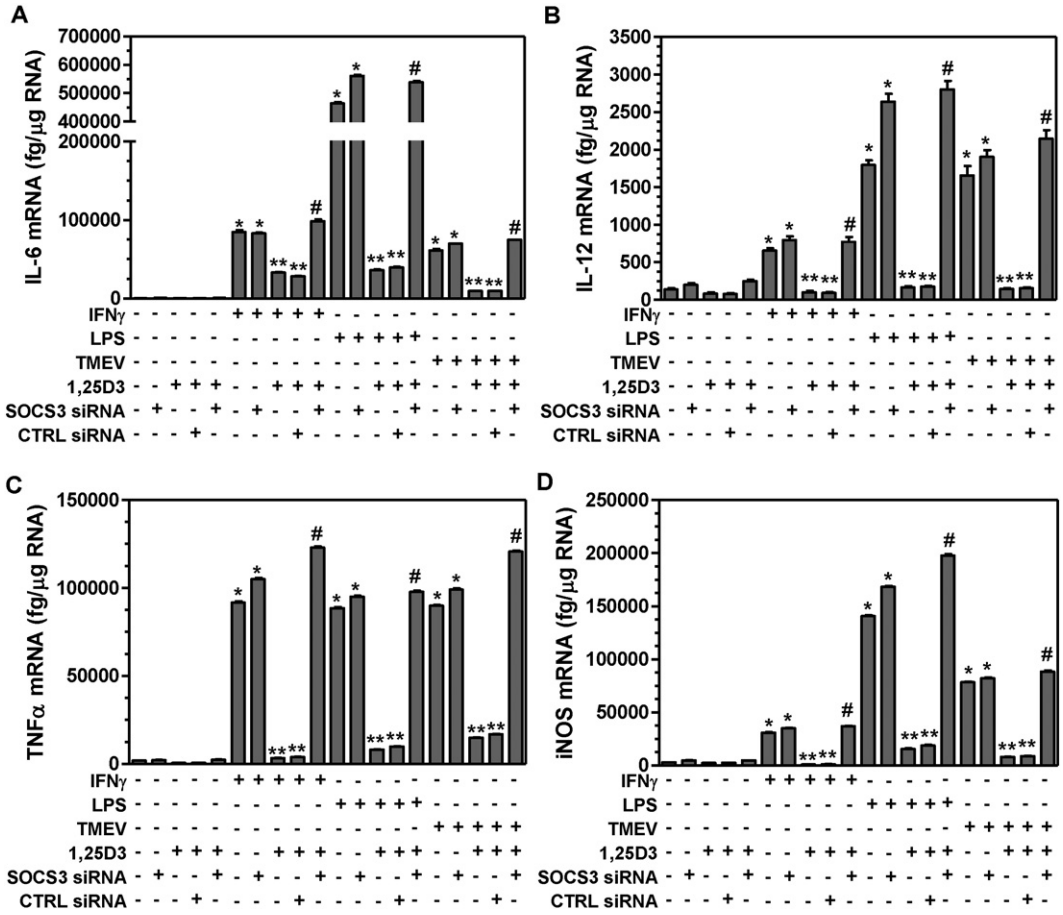

Fig. 6. Vitamin D3 reduced expression of pro-inflammatory cytokines in activated microglia was dependent on SOCS3. Primary microglial cultures were derived from neonatal SJL mice. The primary cultures were transfected with siRNA for SOCS3 (5 μM) or control siRNA (5 μM), and the cultures were stimulated with IFNγ or LPS, or infected with TMEV in the presence of $1,25(OH)_2D_3$ (10 nM). After 24 h, RNA was isolated from the cell, converted to cDNA and used in real time PCR with primers for IL-6 (A), IL-12 (B), TNFα (C), and iNOS (D). The significant difference (*) between the unstimulated and stimulated microglia, the significant difference (**) between stimulated microglia without or with vitamin D3, and the significant difference (#) between stimulated microglia with vitamin D3 and with control siRNA or with siRNA for SOCS3, were determined by Student's *t* test ($p < 0.05$). This is a representative graph from three individual repeated experiments.

silenced, the expression of pro-inflammatory cytokines returned to levels at or above microglia stimulated in the absence of vitamin D3. This was due to IFNγ and LPS inducing some SOCS3 expression in the absence of vitamin D3. Likewise, vitamin D3 reduced the expression of IL-6, IL-12, TNFα, and iNOS in TMEV-infected microglia, however, when SOCS3 was silenced, the expression of pro-inflammatory cytokines returned to levels similar to TMEV infected microglia in the absence of vitamin D3. These results show that SOCS3 reduced the expression of pro-inflammatory cytokines and effector molecules following vitamin D3 treatment of activated microglia. Thus, vitamin D3 increased the expression of IL-10 which led to the expression of SOCS3 that subsequently reduced the expression of pro-inflammatory cytokines and effector molecules.

4. Discussion

Vitamin D3 has been suggested to have immune modulating properties; however, vitamin D3 must be processed into an active form before having an effect on the immune response. Vitamin D3 circulates in the blood as $25(OH)D_3$ and must be converted to the hormonally active $1,25(OH)_2D_3$ by the enzyme 1α-hydroxylase encoded by the *Cyp27b1* gene. Only the active form, $1,25(OH)_2D_3$, binds to the VDR which then forms a complex with retinoid X receptor. This complex translocates to the nucleus where it 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, high levels of $1,25(OH)_2D_3$ induces *Cyp24a1* expression which encodes 24-hydroxylase that degrades $1,25(OH)_2D_3$ to prevent vitamin D toxicity within the cell. Previous studies have suggested that the vitamin D serum binding protein limits access of the active form, $1,25(OH)_2D_3$, to the CNS (Gascon-Barre and Huet, 1983). In addition, previous studies have suggested that $25(OH)D_3$ can be converted into the active form $1,25(OH)_2D_3$ in the CNS but the cellular source for this conversion has not been determined (Spach and Hayes, 2005). Our studies show that microglia express *Cyp27b1*, and that microglia can convert $25(OH)D_3$ into the active form $1,25(OH)_2D_3$, as demonstrated by their responsiveness when $25(OH)D_3$ was added to the cells. Thus, these results show that microglia have an intracrine system whereby the cells convert $25(OH)D_3$ to $1,25(OH)_2D_3$ which can then bind to VDR in the cell. Further, these results demonstrate that microglia may be the cellular source for converting circulating vitamin D3, $25(OH)D_3$, into active form, $1,25(OH)_2D_3$, in the CNS during normal conditions.

Microglia can be activated to express cytokines, chemokines, and effector molecules in the presence of IFNγ which is secreted by infiltrating immune cells during an inflammatory immune response (Olson et al., 2001; Olson and Miller, 2004). Activated microglia can play an important role in the adaptive immune response in the CNS by expressing pro-inflammatory cytokines and by processing and presenting antigens to $CD4^+$ T cells (Olson et al., 2001; Olson and Miller, 2004). Microglia stimulated with IFNγ increased the expression of VDR and *Cyp27b1* suggesting that inflammatory mediator IFNγ can increase the responsiveness of microglia to vitamin D3. Further, IFNγ reduced the expression of *Cyp24a1* in microglia which degrades the active form of vitamin D3, $1,25(OH)_2D_3$, leading to increased levels of $1,25(OH)_2D_3$ and promoting the response to vitamin D3. IFNγ-stimulated microglia in the presence of $1,25(OH)_2D_3$ greatly increased the expression of VDR, *Cyp27b1*, and *Cyp24a1* which suggests a synergistic relationship between IFNγ and $1,25(OH)_2D_3$. These results correlate with our recent study showing that mice lacking IFNγ have a reduced expression of VDR, including in the microglia (Spanier et al., 2012). Most interestingly, IFNγ-stimulated microglia in the presence of $1,25(OH)_2D_3$ decreased the expression of pro-inflammatory cytokines and increased the expression of IL-10. Similar results were observed when $25(OH)D_3$ was added to the IFNγ-stimulated microglia demonstrating that microglia convert the circulating form of vitamin D3 into the active form which binds to VDR altering the expression of cytokines, chemokines, and effector molecules in the IFNγ-stimulated microglia. These results suggest that IFNγ modulates the expression of VDR and *Cyp27B1* in microglia to enable microglia to be more responsive to vitamin D3. As a consequence, vitamin D3 reduced the expression of pro-inflammatory cytokines and increased the expression of IL-10 in IFNγ-stimulated microglia suggesting that vitamin D3 may act as a control mechanism for microglia activation during an inflammatory immune response.

Microglia express innate immune receptors, TLRs, and act as sensors for the CNS by detecting infection and injury (Olson and Miller, 2004). We have previously shown that microglia are activated by LPS to secrete pro-inflammatory cytokines (Olson and Miller, 2004). Our current studies show that LPS increased the expression of VDR in microglia as well as increased *Cyp27b1*. Interestingly, LPS stimulation of microglia reduced the expression *Cyp24a1* which degrades $1,25(OH)_2D_3$, thus leading to higher levels of $1,25(OH)_2D_3$ for the cells. The addition of $1,25(OH)_2D_3$ to LPS-stimulated microglia reduced the expression of pro-inflammatory cytokines and increased the expression of IL-10. Further, our results show that $25(OH)D_3$ can be converted to $1,25(OH)_2D_3$ by LPS-activated microglia via *Cyp27b1* expression to decrease the expression of pro-inflammatory cytokines. These results suggest that vitamin D3 can alter microglia activation through innate immune receptors by reducing the expression of pro-inflammatory cytokines and increasing the expression of anti-inflammatory cytokines. Previous studies have suggested that vitamin D3 can reduce the expression of TLRs which may contribute to the reduced expression of pro-inflammatory cytokines (Choi et al., 2011). However, our results show that IL-10 was increased in LPS-stimulated microglia in the presence of $1,25(OH)_2D_3$ suggesting that vitamin D3 may be mediating its affect directly through transcriptional regulation of cytokines.

TMEV infection of microglia activates microglia through multiple innate immune receptors to secrete cytokines, chemokines, and effector molecules (Olson et al., 2001; Olson and Miller, 2004). The most highly expressed innate immune cytokines following virus infection are type I interferons, IFNα and IFNβ. Type I interferons are directly antiviral by inhibiting virus replication but also have several immune modulatory functions. TMEV-infected microglia increased the expression of VDR and *Cyp27b1* but decreased the expression of *Cyp24a1*. Therefore, TMEV-infected microglia can convert $25(OH)D_3$ to $1,25(OH)_2D_3$ and inhibit the degradation of $1,25(OH)_2D_3$ which would promote sustained responsiveness to vitamin D3. TMEV infection in the presence of $1,25(OH)_2D_3$ decreased expression of pro-inflammatory cytokines and increased the expression of IL-10 similar to the IFNγ and LPS-stimulated microglia, however, TMEV-infected microglia also increased the expression of type I interferons. Similarly, $25(OH)D_3$ was converted to $1,25(OH)_2D_3$ by the TMEV-infected microglia which lead to decreased expression of pro-inflammatory cytokines and increased expression of type I interferons and IL-10. These results suggest that vitamin D3 can alter microglia activation following virus infection by reducing the expression of pro-inflammatory cytokines and increasing the expression of IL-10. Most significantly, vitamin D3 altered the microglia immune response to virus infection by increasing the expression of type I interferons, IFNα and IFNβ, which are critical for the antiviral immune response. Similarly, a recent study demonstrated that vitamin D3 could alter the antiviral response in hepatocytes to hepatitis C virus infection (Gal-Tanamy et al., 2011). Thus, the increased expression of type I interferons in the presence of vitamin D3 may suggest an important role for vitamin D3 in viral immunity.

Interestingly, vitamin D3 increased the expression of IL-10 in microglia stimulated with IFNγ or LPS or infected with TMEV. Thus, we were interested in determining whether IL-10 may be mediating the anti-inflammatory effects of vitamin D3. A previous study showed that IL-10 could induce the expression of SOCS3 in microglia (Qin et al., 2006). SOCS3 was initially determined to inhibit signaling by the IL-6 family of cytokines by targeting JAKs for degradation (Baker et al., 2009). SOCS3 interacts with the common receptor subunit of the IL-6 cytokine family, gp130, through its SH2 domain inhibiting the tyrosine kinase activity of JAKs directly through its kinase inhibitory region

(KIR) and targeting JAKs for degradation via proteosomal degradation (Boyle et al., 2009; Lang et al., 2003; Lehmann et al., 2003). Activation of JAKs leads to phosphorylation of STAT transcription factors which promote the expression of immune molecules, including cytokines. The IL-6 receptor cytokine family activates JAKs that then activate STAT3, thus SOCS3 is an inhibitor of STAT3 signaling. However, SOCS3 has also been determined to inhibit signaling in the NFκB pathway and enhancing signaling through the MAPK pathway (Baetz et al., 2004; Cacalano et al., 2001). SOCS3 has a short half-life of about 1–2 h and thus must be constantly turned over through transcription (Siewert et al., 1999). Most interestingly, our results show that microglia increased the expression of SOCS3 in the presence of vitamin D3. Our results further show that SOCS3 expression was dependent on IL-10 binding to its receptor and that IL-10 was also required for the reduction of pro-inflammatory cytokine expression. Thus, vitamin D3 increases the expression of IL-10 which increases the expression of SOCS3. Further, to determine whether SOCS3 was reducing the expression of pro-inflammatory cytokines in activated microglia, SOCS3 was silenced using siRNA. Silencing SOCS3 in the presence of vitamin D3 returned the levels of pro-inflammatory cytokines to those observed in the activated microglia without vitamin D3. These results suggest that vitamin D3 reduced the inflammatory response by activated microglia through increased expression of IL-10 which then induced SOCS3 expression.

Overall, our results show that vitamin D3 can alter the immune activation of microglia which may have implications in the immune response in the CNS during infection and disease. Acute activation of microglia during infection, injury, or disease is essential to protect and restore the CNS environment. However, chronic inflammation or activation that skews too far to the inflammatory response may be detrimental to the CNS and has been associated with neurological diseases. Therefore, vitamin D3 may be a control mechanism by which the inflammatory immune response by microglia is appropriately downregulated following the acute insult returning microglia to homeostatic conditions. Thus, modulating the levels of vitamin D3 may have an effect on microglia activation and on development and progression of CNS diseases.

Multiple sclerosis is a demyelinating disease that is associated with a pro-inflammatory immune response in the central nervous system (CNS). Activated microglia have been identified in the inflammatory lesions which form in areas of demyelination in the brain and spinal cord of MS patients (Henderson et al., 2009; van and Amor, 2009). A recent retrospective study showed that people who had lower serum levels of vitamin D3 were more likely to develop MS than those with normal levels of vitamin D3 (Munger et al., 2006). Several studies in MS patients have suggested an association between serum $25(OH)D_3$ and MS disease activity where low levels of $25(OH)D_3$ are associated with relapses and higher levels of $25(OH)D_3$ are associated with disease remission (Simpson et al., 2010; Smolders et al., 2008) (Correale et al., 2009; Soilu-Hanninen et al., 2005). Most interestingly, a recent report determined that a cohort of MS patients in Canada have a mutation in the *CYP27B1* gene resulting in low levels of $1,25(OH)_2D_3$ in the serum (Ramagopalan et al., 2011). Further, a genome wide association study found an association of MS with genetic regions containing vitamin D metabolism genes, CYP27B1 and CYP24A1 (Sawcer et al., 2011). More importantly, small clinical trials have shown supplementation with vitamin D3 reduced relapse rates and reduced the number of lesions in MS patients (Kimball et al., 2007; Mahon et al., 2003). More recently, a pilot clinical study has been conducted where MS patients currently receiving IFNβ were supplemented with high dose vitamin D3. The early results show that high dose vitamin D3 has an immunomodulatory shift toward an anti-inflammatory cytokine profile (Smolders et al., 2010). Our results show that microglia convert $25(OH)D_3$ into the active form $1,25(OH)_2D_3$ in the CNS, and that activated microglia in the presence of vitamin D3 reduced the expression of pro-inflammatory cytokines and increased the expression of IL-10 and IFNβ. Therefore, vitamin D3 may be downregulating microglia activation thus reducing the inflammatory immune response in the CNS during MS. Finally, microglia may also represent the cellular source for converting the circulating form of vitamin D3 into the active form in the CNS for more effective treatment during MS.

Microglia become more sensitized with age and exhibit a chronic low level inflammatory activation in aged individuals. Microglia in the aged CNS have a more rapid response to express pro-inflammatory mediators compared to microglia from younger CNS (Streit and Xue, 2010). Microglia acquire an activated phenotype with age by upregulating MHC class II and costimulatory molecules (Frank et al., 2010; Von et al., 2010). Interestingly, as the pro-inflammatory cytokines increase in the aged brain, the anti-inflammatory cytokines, including IL-10, are decreased in the aged brain (Dilger and Johnson, 2008). Thus, aging influences microglia activation by skewing the response toward the pro-inflammatory response associated with several neurological diseases in the aging population.

Alzheimer's disease is a dementia associated with the aging population and is identified by the presence of beta-amyloid plaques and neurofibrillary tangles in the brain. Alzheimer's disease is marked by a prominent inflammatory immune response which is driven by microglia activation toward a pro-inflammatory immune response (Lue et al., 2010). Studies have shown that an increased amount of microglia activation is associated with neuronal damage and more severe dementia in patients with similar amounts of plaques in the brain (Lue et al., 1996; Lue et al., 1999). Interestingly, vitamin D3 deficiency has been associated with Alzheimer's disease, and higher $25(OH)D_3$ serum levels correlated with higher cognitive abilities in patients with probable disease (Evatt et al., 2008; Oudshoorn et al., 2008). Parkinson's disease is another neurodegenerative disease associated with elderly and involves the loss of dopaminergic neurons in the substantia nigra leading to severe movement disorder and disability. Injured neurons release μ-calpain, α-synuclein, and matrix metalloproteinase 3, and neuromelanin which activate microglia toward a pro-inflammatory immune response (Lull and Block, 2010). Neuroinflammation is an important component of the pathogenesis of Parkinson's disease and may be contributing to progressive neuronal damage (Tufekci et al., 2012). Interestingly, vitamin D3 insufficiency has been associated with development of Parkinson's disease (Evatt et al., 2008, 2011). Our results show that vitamin D3 reduces the pro-inflammatory immune response by microglia. Thus, one mechanism by which vitamin D3 may be protective in Alzheimer's disease and Parkinson's disease is by reducing the inflammatory immune response by microglia and returning microglia to homeostatic levels following acute activation, thus preventing chronic inflammation associated with neurological diseases.

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