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*J Immunol* published online 22 February 2013
http://www.jimmunol.org/content/early/2013/02/22/jimmunol.1203273

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/02/22/jimmunol.1203273.DC1

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1,25-Dihydroxyvitamin D Promotes Negative Feedback Regulation of TLR Signaling via Targeting MicroRNA-155–SOCS1 in Macrophages

Yunzi Chen,*,†,1 Weicheng Liu,*,† Tao Sun,† Yong Huang,*, Youli Wang,*, Dilip K. Deb,*, Dosuk Yoon,*, Juan Kong,*,† Ravi Thadhani, ‡ and Yan Chun Li*,†,§

The negative feedback mechanism is essential to maintain effective immunity and tissue homeostasis. 1,25-dihydroxyvitamin D (1,25(OH)2D3) modulates innate immune response, but the mechanism remains poorly understood. In this article, we report that vitamin D receptor signaling attenuates TLR-mediated inflammation by enhancing the negative feedback inhibition. Vitamin D receptor inactivation leads to hyperinflammatory response in mice and macrophage cultures when challenged with LPS, because of microRNA-155 (miR-155) overproduction that excessively suppresses suppressor of cytokine signaling 1, a key regulator that enhances the negative feedback loop. Deletion of miR-155 attenuates vitamin D suppression of LPS-induced inflammation, confirming that 1,25(OH)2D3 stimulates suppressor of cytokine signaling 1 by downregulating miR-155. 1,25(OH)2D3 downregulates bic transcription by inhibiting NF-kB activation, which is mediated by a bC cis-DNA element located within the first intron of the bic gene. Together, these data identify a novel regulatory mechanism for vitamin D to control innate immunity. The Journal of Immunology, 2013, 190: 000–000.

Macrophages play a key role in innate immune response. Upon stimulation, activated macrophages release a panel of proinflammatory mediators including cytokines and chemokines to initiate inflammatory response. Inflammatory reaction protects the host from pathogenic microorganisms; however, oversustained inflammation is detrimental and can cause tissue damage and even death of the host. Thus, negative feedback mechanisms are in place to control the duration and intensity of inflammatory reaction (1). The suppressor of cytokine signaling (SOCS) family of proteins are key components of the negative feedback loop that regulates the intensity, duration, and quality of cytokine signaling (2). As feedback inhibitors of inflammation, SOCS proteins are upregulated by inflammatory cytokines and, in turn, block cytokine signaling by targeting the JAK/STAT pathway (3). SOCS1 is a well-established key negative regulator of LPS-induced inflammation (4, 5) and inhibits the proinflammatory pathways of cytokines such as TNF-α, IL-6, and INF-γ (6). SOCS1 in T cells and macrophages has been shown to prevent lethal inflammation in mice (7, 8). SOCS1 can also inhibit LPS-induced inflammatory response by directly blocking TLR-4 signaling (4, 5) via targeting IL-1R–associated kinase (IRAK)1 and IRAK4 (9).

MicroRNAs (miRNAs) are a class of naturally occurring, small, noncoding RNAs of ~22 nucleotides that control gene expression by translational repression or mRNA degradation (10). In recent years, miRNA-155 (miR-155) has emerged as a critical regulator of innate immunity and TLR signaling (11). miR-155 is encoded by a noncoding gene known as bic (12) and is highly inducible in macrophages in response to TLR ligands such as LPS and poly(I:C) (13, 14). As miR-155 targets SOCS1 in activated macrophages (15), leading to blockade of the negative feedback loop, the induction of miR-155 during macrophage activation serves to maximize and prolong the inflammatory process. Suppression of miR-155, therefore, will de-repress the negative feedback mechanism resulting in attenuation of inflammatory reaction.

1,25-dihydroxyvitamin D [1,25(OH)2D3], the active metabolite of vitamin D, is a pleiotropic hormone. The activity of 1,25(OH)2D3 is mediated by the vitamin D receptor (VDR), a member of the nuclear receptor superfamily (16). 1,25(OH)2D3 has potent immunomodulatory activities in both innate and adaptive immunity (17, 18). The best-known activity of vitamin D in the regulation of innate immunity is to stimulate antimicrobial peptide production in macrophages (19). TLR4 activation by bacterial infection increases local production of 1,25(OH)2D3 and VDR in macrophages, and this local 1,25(OH)2D3-VDR signaling induces the expression of antimicrobial peptide cathelicidin to kill bacteria (19, 20). This mechanism, however, cannot explain the anti-
inflammatory action of vitamin D, in which 1,25(OH)2D3 down-regulates proinflammatory cytokines and chemokines in macrophages and other cells (21, 22). Little is known about the molecular basis of this anti-inflammatory mechanism. In this study, we provide evidence that the VDR signaling in macrophages limits inflammatory response by targeting the miR-155-SOCS1 pathway, resulting in heightened negative feedback inhibition of TLR-4 signaling.

Materials and Methods

Animals and treatment

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Chicago. VDR−/− mice were reported previously (23), and miR-155−/− mice (24) were purchased from The Jackson Laboratory (stock no. 007745). All mice, including wild-type (WT) controls, were in C57BL/6 background. Mice were used experimentally at 2–4 mo of age. To induce sepsis, we injected mice with one dose of LPS (O111:B4, Sigma L2630; 20 mg/kg i.p.). To study the anti-inflammatory effect of vitamin D, we pretreated mice daily with vehicle (60:30:10 propylene glycol:water:ethanol) or noncalcemic vitamin D analog paricalcitol (19-nor-1,25-dihydroxyvitamin D2, 200 ng/kg; provided by Abbott Laboratories) for 1 wk (i.p. injection) before LPS (20 mg/kg) challenge. Blood was collected from tail vein at indicated times after LPS treatment for serum cytokine measurement. Spleen and peritoneal macrophages were harvested at the end of the experiment for protein and RNA analyses.

Cell culture and treatment

HEK293T, L929, and RAW264.7 cells were grown in DMEM supplemented with 10% FBS. Peritoneal macrophages were harvested and plated on six-well plates in DMEM containing 10% FBS. Unattached cells were removed after overnight culture. Bone marrow–derived macrophages (BMDMs) were cultured as described previously (25). In brief, mouse bone marrow cells were plated in DMEM supplemented with 10% FBS after RBCs were lysed with 10 mM NH4Cl (pH 8.0). After overnight culture, the unattached cells were replated and differentiated into BMDMs in 30% L929 conditioned media. Human PBMCs were isolated by Ficoll-Hypaque (Pharmacia) gradient centrifugation of buffy coats from healthy donors and suspended in complete RPMI 1640 medium containing 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B. The cells were then allowed to adhere in culture flasks for 1 h at 37 °C in humidified 5% CO2. The nonadherent cells were removed, and the remaining adherent cells were harvested and cultured at a density of 1×104 cells/ml in complete RPMI 1640 medium. Macrophage cultures were usually treated with 100–200 ng/ml LPS for 0–72 h with or without overnight pretreatment with 20 nM 1,25(OH)2D3 as specified in each experiment, followed by isolation of total RNAs, lysates, and/or media supernatants for various assays.

miRNA arrays

Total RNAs were extracted from RAW264.7 cells treated with LPS (100 ng/ml) in the presence or absence of 1,25(OH)2D3 (20 nM) overnight. miRNA profiling was performed using the mirCURY LNA miRNA Arrays (Exiqon, Vedvaek, Denmark) according to manufacturer’s standard protocols. The arrays were scanned with GenePix4000B scanner using the manufacturer’s recommended settings. The raw data were extracted using GenePix Pro and imported into GeneSpring GX10 for analyses. The raw and normalized miRNA array data were deposited in the Gene Expression Omnibus database (accession no. GSE43300; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE43300).

Cytokine quantitation

TNF-α and IL-6 concentrations in the serum or culture media were determined by ELISA using commercial ELISA kits obtained from BioLegend (San Diego, CA).

RT-PCR

Total cellular RNAs were extracted using TRIzol reagents (Invitrogen, Carlsbad, CA). First-strand cDNAs were synthesized using a ThermoScriptRT kit (Invitrogen) or a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) for miRNA analyses. Conventional PCRs were carried out in a BioRad DNA Engine (BioRad). Real-time PCRs (qPCRs) were performed in a Roche LightCycler 480II Real-Time PCR System, using SYBR green PCR reagent kits (Clontech) for miRNA transcript quantitation or TaqMan OpenArray Real Time PCR Master Mix (Applied Biosystems) for miR-155 quantitation. Relative amount of transcripts was calculated using the 2−ΔΔCt formula as described previously (26). All PCR primers are reported in Table I.

Northern blot

Northern blot analysis of miR-155 was conducted according to a previously published method (27). In brief, total RNAs were separated on 6% polyacrylamide gels containing formaldehyde and transferred onto a nylon membrane. Hybridization was performed at 42 °C using LNA mmu-miR-155 probe (Exiqon) labeled with [32P]-dATP. The 32P-labeled U6 probe (Exiqon) was used as an internal control.

Western blot

Proteins were separated by SDS-PAGE and electroblotted onto Immobilon-P membranes. Western blotting analyses were carried out as previously described (28). The Abs used in this study included SOCS1 (38-5200, rabbit polyclonal; Zymed Laboratories), VDR (sc-1009, rabbit polyclonal; Santa Cruz), IκB kinase α/β (IKKα/β; sc-7607, rabbit polyclonal; Santa Cruz), IκB kinase α, β, and ε (IKKα/β; sc-7607, rabbit polyclonal; Santa Cruz), p65 (sc-109, rabbit polyclonal; Santa Cruz), and β-actin (A2066, rabbit polyclonal; Sigma).

Cell transfection

Cell transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instruction. Cells were transfected with pCDH-miR-155 plasmid kindly provided by Dr. Mo (Southern Illinois University School of Medicine) or mmu-miR-155 oligo mimic (Thermo Scientific Dharmacon) to overexpress miR-155. RAW264.7 cells or peritoneal macrophages were transfected with 50 nM mmu-miR-155 hairpin inhibitor (Thermo Scientific Dharmacon) to knock down miR-155 or were transfected with 50 nM p65-specific small interfering RNA (Thermo Scientific Dharmacon) to silence p65.

Kinase assay

IKK kinase was performed as described previously (29). Cell lysates were prepared from RAW264.7 cells treated with LPS in the presence or ab-

FIGURE 1. VDR inactivation leads to hyperresponsiveness to LPS. (A) Survival curves of WT and VDR-null (VDRKO) mice after i.p. injection of LPS at 20 mg/kg; n = 7–8, p < 0.001 by log rank test. (B and C) Serum TNF-α (B) and IL-6 (C) concentration in WT and VDRKO mice at 0 and 24 h after LPS challenge. **p < 0.001 versus 0 h; ###p < 0.001 versus WT; n = 7–8. (D–F) Time course of TNF-α (D), IL-6 (E), or IL-1β (F) transcript induction after LPS (100 ng/ml) treatment in BMDMs derived from WT and VDRKO mice, n = 3; *p < 0.05, **p < 0.01, ***p < 0.001 versus WT.
sequence of 1,25(OH)₂D₃ were immunoprecipitated with anti-IKKγ Abs (Santa Cruz). The precipitant was incubated with recombinant GST-IκBα (1-54) (Clontech) in the presence of γ-[³²P]-ATP, and ³²P-labeled GST-IκBα (1-54) was detected by autoradiography.

Luciferase reporter assays

The DNA fragment from –587 to +263 in mouse bic gene was PCR amplified with specific primers (Table I) and cloned into pGL3-Basic luciferase reporter plasmid (Promega), generating pGL-bic-Luc. Reporter plasmid carrying mutations at the +87 intronic B site (pGL-bicmut-Luc, mutated from 5'-GGAgAcTTCC-3' to 5'-GGAgAcTTCC-3') was generated by site-directed mutagenesis. RAW264.7 cells were transfected with these plasmids or pNF-κB–Luc (control) using Lipofectamine 2000 (Invitrogen). After overnight culture, the transfected cells were treated with LPS in the presence or absence of 1,25(OH)₂D₃ (20 nM). Luciferase activity was measured after 24 h using Promega luciferase assay system.

EMSA probes

bic in situ binding was confirmed by competition with an excess amount of the unlabeled probe, a mutant probe 5'-ATCGTGAGTTGGAAATTTCCCAACAGGAGATGG (underlined is the B site). Specificity of the protein–DNA interaction was confirmed by competition with an excess amount of the unlabeled probe, a mutant probe 5'-ATCGTGAGTTGGAAATTTCCCAACAGGAGATGG (underlined is the B site). Specificity of the protein–DNA interaction was confirmed by competition with an excess amount of the unlabeled probe, a mutant probe 5'-ATCGTGAGTTGGAAATTTCCCAACAGGAGATGG (underlined is the B site). Specificity of the protein–DNA interaction was confirmed by competition with an excess amount of the unlabeled probe, a mutant probe 5'-ATCGTGAGTTGGAAATTTCCCAACAGGAGATGG (underlined is the B site).

ChIP primers

bic promoter-5-Sal1
CGGTCGACCTGGGAATCAAACCTAGGC
bic promoter-3-Nco1
CGGCCATGGCAGCATACAGCCTTCAGC

EMSA probes

bic intronic κB-1
TTCTTGAGTTGGAAATTTCCCAACAGGAGATGG
bic intronic κB-2
ATCGTGAGTTGGAAATTTCCCAACAGGAGATGG
bic κB mutant-1
TTCTTGAGTTGGAAATTTCCCAACAGGAGATGG
bic κB mutant-2
ATCGTGAGTTGGAAATTTCCCAACAGGAGATGG

ChIP primers

bic-κB-1
AGTCCTTCTCTCATCAACTATGAGCC
bic-κB-2
CTGCCCCTTCTCTATAGCTCCT

Statistical analysis

Data values were presented as means ± SD. Statistical analyses were performed using unpaired two-tailed Student t test or one-way ANOVA as appropriate, with p < 0.05 being considered significant.

FIGURE 2. VDR activation inhibits LPS-induced inflammatory reaction. (A) BMDMs were pretreated with vehicle (V) or paricalcitol (P; 200 ng/kg daily i.p.) for 1 wk before being challenged with saline control or LPS (20 mg/kg). Serum TNF-α (A) or IL-6 (B) concentration was measured at 24 h after LPS injection. ***p < 0.01; ###p < 0.001 versus V; n = 7. (C) BMDMs were pretreated with ethanol (EtOH) or 1,25(OH)₂D₃ (1,25-VD; 20 nM) for 24 h before being exposed to LPS (100 ng/ml). The time course of TNF-α (C) and IL-6 (D) release into the media was determined by ELISA. ***p < 0.001 versus 0 h; ###p < 0.001 versus corresponding EtOH; n = 3.

Table I. Primers and oligonucleotide probe sequences used in the study

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1,25(OH)2D3 and its analog suppress inflammatory response of macrophages when the VDR signaling is inactivated. The innate immune response is dysregulated and oversustained in the WT counterpart (Fig. 1D–F). These observations indicate that responsiveness of VDR survived after 120 h (Fig. 1A). Consistent with the hyperresponsiveness of VDR−/− mice, within 24 h of LPS treatment, the induction of serum TNF-α and IL-6 was much more robust in VDR−/− mice compared with WT mice (Fig. 1B, 1C). Because macrophages play a critical role in innate immunity (31), we directly examined the impact of VDR inactivation on the response of macrophages to LPS. Time-course studies showed that exposure of BMDMs to LPS led to much more robust and prolonged production of proinflammatory cytokines TNF-α, IL-6, and IL-1β quantified by qPCR (Table I) in VDR−/− BMDMs compared with the WT counterpart (Fig. 1D–F). These observations indicate that the innate immune response is dysregulated and oversustained in macrophages when the VDR signaling is inactivated.

1,25(OH)2D3 and its analog suppress inflammatory response

To validate the importance of VDR in innate immunity, we compared responsiveness of BMDMs to LPS led to much more robust and prolonged production of proinflammatory cytokines TNF-α, IL-6, and IL-1β quantified by qPCR (Table I) in VDR−/− BMDMs compared with WT BMDMs (Fig. 2A, 2B). Among the miRNAs that were induced the most by LPS and suppressed the most by 1,25(OH)2D3, miR-155 was on the top of the list (Table II), with 1,25(OH)2D3 suppressing miR-155 in RAW264.7 cells. Similar regulations were seen for bic (Fig. 2C, 2D). 1,25(OH)2D3 also inhibited LPS-induced TNF-α, IL-1β, and IL-6 expression in RAW264.7 cells, a murine macrophage cell line (Supplemental Fig. 1A).

**Results**

**VDR inactivation leads to hyperresponsiveness to LPS**

To understand the role of VDR in innate immunity, we compared the response of WT and VDR−/− mice to LPS challenge. After one dose of LPS (20 mg/kg i.p.), >40% of VDR−/− mice died within 48 h and all died by 96 h; in contrast, 60% of WT mice still survived after 120 h (Fig. 1A). Consistent with the hyperresponsiveness of VDR−/− mice, within 24 h of LPS treatment, the induction of serum TNF-α and IL-6 was much more robust in VDR−/− mice compared with WT mice (Fig. 1B, 1C). Because macrophages play a critical role in innate immunity (31), we directly examined the impact of VDR inactivation on the response of macrophages to LPS. Time-course studies showed that exposure of BMDMs to LPS led to much more robust and prolonged production of proinflammatory cytokines TNF-α, IL-6, and IL-1β quantified by qPCR (Table I) in VDR−/− BMDMs compared with the WT counterpart (Fig. 1D–F). These observations indicate that the innate immune response is dysregulated and oversustained in macrophages when the VDR signaling is inactivated.

**1,25(OH)2D3 and its analog suppress inflammatory response**

To validate the importance of VDR in inflammatory regulation, we examined the effects of VDR activation in mice and macrophages. WT mice were challenged with LPS after pretreatment with vehicle or a noncalcemic vitamin D analog paricalcitol for 1 wk (1,25(OH)2D3 was not used as it can induce hypercalcemia). Paricalcitol pretreatment substantially suppressed LPS-induced serum TNF-α and IL-6 concentrations (Fig. 2A, 2B). Similarly, in LPS-treated WT BMDMs, the time-dependent induction of TNF-α and IL-6 release was markedly attenuated in the presence of 1,25(OH)2D3 (Fig. 2C, 2D). 1,25(OH)2D3 also inhibited LPS-induced TNF-α, IL-1β, and IL-6 expression in RAW264.7 cells, a murine macrophage cell line (Supplemental Fig. 1A).

**Vitamin D–VDR signaling suppresses miR-155 in macrophages**

As a growing number of miRNAs have emerged as regulators of immune response (32), we postulated that vitamin D downregulates inflammation via targeting miRNAs. To test this hypothesis, we analyzed miRNA profiling in RAW264.7 cells treated with LPS in the presence or absence of 1,25(OH)2D3. Among the miRNAs that were induced the most by LPS and suppressed the most by 1,25(OH)2D3, miR-155 was on the top of the list (Table II), with 1,25(OH)2D3 suppressing ~50% of the LPS induction. Northern blot (Fig. 3A, 3B) and qPCR analyses (Fig. 3C) validated that 1,25(OH)2D3 substantially suppressed LPS-induced miR-155 in RAW264.7 cells. Similar regulations were seen for bic transcript (Fig. 3D). Time-course studies confirmed that the time-dependent induction of bic transcript and miR-155 by LPS was suppressed by 1,25(OH)2D3 in RAW264.7 cells (Fig. 3E–3F), as well as in BMDMs from WT mice (Supplemental Fig. 1B, 1C).

We further examined the effect of VDR inactivation on miR-155. At baseline, bic transcript and miR-155 levels were very low in WT peritoneal macrophages, but they were elevated in VDR−/− macrophages (Fig. 4A, 4B). After LPS stimulation, the time-dependent induction of bic and miR-155 was much more robust and prolonged in VDR−/− BMDMs compared with WT BMDMs (Fig. 4C, 4D). Together, these observations demonstrate that vitamin D–VDR signaling suppresses miR-155 in macrophages.

**FIGURE 3.** 1,25(OH)2D3 downregulates miR-155. (A and B) Northern blot (A) and PhosphoImaging quantitation (B) of premiR-155 and miR-155 in RAW264.7 cells stimulated with LPS for 24 h in the presence of EtOH (E) or 1,25(OH)2D3 (1,25VD). (C) Real-time PCR quantitation of miR-155 in RAW264.7 cells stimulated with LPS for 24 h in the presence of EtOH or 1,25(OH)2D3. (D) RT-PCR assessment of bic transcript in RAW264.7 cells stimulated with LPS in the presence EtOH vehicle or 1,25-VD. (E and F) Time course of bic transcript (E) and miR-155 (F) induction in RAW264.7 cells stimulated with LPS in the presence of EtOH or 1,25(OH)2D3. The bic transcript and miR-155 were quantified by qPCR. ***p < 0.001 versus the rest; n = 3.
BMDMs, quantitated by qPCR. LPS induction of bic transcript (mice. (A) Northern blot analysis of baseline miR-155 in peritoneal macrophages harvested from WT and VDRKO mice. (C and D) Time course of LPS induction of bic transcript (C) and miR-155 (D) in WT and VDRKO BMDMs, quantitated by qPCR. **p < 0.001 versus WT. n = 3.

Vitamin D inhibits inflammatory cytokines and miR-155 in human PBMCs

Because differences were reported in vitamin D’s immunoregulatory activities between mice and humans, we assessed the relevance of vitamin D regulation of miR-155 in human PBMCs. As shown in Fig. 5, 1,25(OH)2D3 markedly blocked not only the induction of TNF-α and IL-6 by LPS (Fig. 5A, 5B), but also that of bic transcript and miR-155 (Fig. 5C, 5D) in human PBMCs.

Vitamin D–VDR signaling upregulates SOCS1 via suppressing miR-155

Because SOCS1 is a proved target of miR-155, we expected that vitamin D inhibition of miR-155 would release the suppression on SOCS1, whereas VDR inactivation would depress SOCS1. Indeed, we observed that the time-dependent induction of SOCS1 by LPS was further enhanced by 1,25(OH)2D3 treatment in RAW264.7 cells (Fig. 6A, 6B). In contrast, LPS was not able to induce SOCS1 protein in VDR−/− BMDMs (Fig. 6C, 6D), likely because of overexpression of miR-155. To assess the in vivo phenotype of macrophages after LPS challenge, we examined peritoneal macrophages immediately harvested from LPS-treated mice. SOCS1 was highly induced in the macrophages from WT mice as expected, but SOCS1 induction was hardly detectable in the macrophages obtained from VDR−/− mice (Fig. 6E, 6F). These results indicate that vitamin D–VDR signaling targets the miR-155-SOCS1 pathway in macrophages. Given the critical role of SOCS1 in the negative feedback regulation, these data suggest that vitamin D–VDR signaling limits inflammatory response by promoting the negative feedback action.

Upregulation of miR-155 is required for hyperinflammatory reaction in VDR-null macrophages

To explore the role of miR-155 in vitamin D regulation of inflammation, we asked whether miR-155 upregulation was responsible for the hyperresponsiveness of VDR−/− macrophages to LPS stimulation. As expected, in VDR−/− peritoneal macrophages, LPS induced much more robust and more prolonged secretion (Fig. 7B, 7C) and mRNA expression (Fig. 7D, 7E) of TNF-α and IL-6 compared with WT cells. When LPS-induced miR-155 upregulation was attenuated using an miR-155–specific miRIDIAN hairpin inhibitor (Fig. 5A), the time-dependent induction of TNF-α and IL-6 was also markedly attenuated at the protein (Fig. 7B, 7C) and transcript (Fig. 7D, 7E) levels. The induction of bic, however, was not affected by miR-155 knockdown as expected (Fig. 7F). These results confirm that miR-155 overproduction is a major cause for the hyperinflammation seen in VDR-null macrophages.

We also asked whether ectopic overexpression of miR-155 could abrogate vitamin D inhibition of inflammation. Indeed, lentiviral overexpression of miR-155 in RAW264.7 cells led to upregulation of TNF-α, but this TNF-α upregulation could no longer be suppressed by 1,25(OH)2D3 (Supplemental Fig. 2A–D). Together, these data suggest a critical role of miR-155 in vitamin D regulation of macrophage inflammatory response.

miR-155 is required for vitamin D inhibition of inflammation

Next, we used the miR-155–null mouse model (24) to further assess the role of miR-155 in vitamin D regulation of innate immunity. WT and miR-155−/− mice were pretreated with vehicle or paricalcitol daily for 1 wk before LPS challenge. As expected, paricalcitol substantially blocked the induction of serum TNF-α and IL-6 in LPS-treated WT mice at 16 h. In miR-155−/− mice, however, the induction of serum TNF-α and IL-6 was significantly lower compared with WT mice; interestingly, paricalcitol failed to inhibit these proinflammatory cytokines (Fig. 8A, 8B). In vitro time-course experiments showed that 1,25(OH)2D3 markedly suppressed LPS-induced TNF-α and IL-6 transcripts in WT BMDMs. In miR-155−/− BMDMs, the induction of these cytokines was less robust, but 1,25(OH)2D3 had little effect on the expression of these cytokines (Fig. 8C, 8D). As expected, LPS induction of SOCS1 was much more robust in miR-155−/− macrophages compared with WT macrophages (Fig. 8E). Whereas 1,25(OH)2D3 markedly elevated SOCS1 in WT BMDMs as expected, it failed to induce SOCS1 in miR-155−/− macrophages (Fig. 8F, 8G). Consistently, paricalcitol could induce splenic SOCS1 in WT mice but not in miR-155−/− mice (Supplemental Fig. 3A, 3B). Together, these data provide compelling evidence that vitamin D–VDR signaling maintains SOCS1 levels primarily via suppressing miR-155. As a result, the negative feedback inhibition loop is maintained. Therefore, vitamin D limits inflammatory response by targeting the miR-155-SOCS1 pathway.
FIGURE 6. VDR signaling enhances SOCS1 induction. (A and B) Time course of SOCS1 protein induction (A) and its quantitation (B) in RAW264.7 cells stimulated with LPS in the presence of ethanol (EtOH) or 1,25(OH)2D3 (1.25VD). (C and D) Time course of SOCS1 protein induction (C) and quantitation (D) in WT and VDR-null (VDRKO) BMDMs stimulated with LPS for 0–24 h. (E and F) SOCS1 protein levels (E) and quantitation (F) in peritoneal macrophages immediately harvested from WT and VDRKO mice treated with saline (−) or LPS (+). **p < 0.01, ***p < 0.001.

1,25(OH)2D3 downregulates bic transcription via blocking NF-κB activation

It has been speculated that NF-κB is involved in the upregulation of miR-155 (33), but no functional κB cis-DNA elements have been identified in the mouse bic gene promoter. We found that LPS induction of bic transcript could be blocked by NF-κB inhibitor Bay 11-7082 (Supplemental Fig. 4A), and that small interfering RNA silencing of p65 abrogated LPS-induced bic expression in RAW264.7 cells (Supplemental Fig. 4B, 4C), confirming the importance of NF-κB activation in the induction of bic/miR-155. Through careful in silico analysis, we identified a putative κB cis-element within the first intron of the bic gene (Fig. 9A) that shared >95% homology to the canonical κB sequence. ChIP assays showed that LPS induced p65 binding to this site, and the binding was attenuated by 1,25(OH)2D3 (Fig. 9B, 9C). EMSA showed that, like the canonical κB probe, this intronic κB probe interacted with p65/p50 (Fig. 9D, lanes 1, 3), and the binding was competed off by an excess amount of unlabeled canonical κB probe (lanes 8, 9) or WT bic intronic κB probe (lanes 4, 5), but not by a mutant intronic κB probe (lanes 6, 7).

We then cloned a DNA fragment covering −587 to +263 from the bic gene that contains the intronic κB site into luciferase reporter vector pGL3, and also generated a mutant construct that carried mutations at the intronic κB site (Fig. 9E). Luciferase reporter assays showed that LPS markedly stimulated luciferase activity in RAW264.7 cells transfected with the WT luciferase reporter, and the stimulation was attenuated by 1,25(OH)2D3; however, mutations at the κB site eliminated LPS induction, as well as 1,25(OH)2D3 inhibition (Fig. 9F). These results confirmed the functionality of this intronic κB cis-element and revealed that this κB element mediated the downregulation of bic gene transcription by 1,25(OH)2D3. In fact, IKK assays showed that 1,25(OH)2D3 attenuated LPS-induced IκBα phosphorylation in macrophages (Fig. 9G), which explains why 1,25(OH)2D3 stabilized IκBα protein reported previously (34, 35), and immunostaining confirmed that 1,25(OH)2D3 blocked LPS-induced p65 nuclear translocation in RAW264.7 cells (Fig. 9H). Therefore, 1,25(OH)2D3 indeed is able to block NF-κB activation. Together, these data demonstrate that 1,25(OH)2D3 downregulates bic transcription by blocking NF-κB activation (Fig. 10).

Discussion

Inflammatory response is regulated by negative feedback mechanisms to avoid excessive reaction. SOCS1 is a critical negative regulator because it blocks the JAK/STAT signaling pathway of proinflammatory cytokines (2, 3, 6) and suppresses IRAK4 in the TLR4 signaling pathway (9, 36, 37). During endotoxin-induced inflammatory reaction, the rapid increase in miR-155 suppresses SOCS1 translation, which allows inflammatory reaction to proceed and sustain without resistance (Fig. 10). In this study, we demonstrated that vitamin D–VDR signaling downregulates bic transcription by blocking NF-κB activation, leading to reduction in miR-155. As a result, SOCS1 translation is increased, which enhances the negative feedback regulation of inflammatory response (Fig. 10). We conclude that this is the molecular basis of a novel anti-inflammatory mechanism whereby vitamin D–VDR signaling limits TLR4-mediated inflammation.
Vitamin D is a well-known immune modulator and affects both innate and adaptive immunities (18). 1,25(OH)2D3 inhibits T cell activation and proliferation (38), and suppresses IL-2 and IFN-γ production in Th1 cells (39, 40). In contrast, 1,25(OH)2D3 promotes Th2 cell differentiation and increases IL-4 production (41, 42). 1,25(OH)2D3 inhibits Ag-presenting dendritic cell differentiation and suppresses the production of IL-12, a cytokine crucial for the development of Th1 cells (43). 1,25(OH)2D3 also inhibits Th17 cell response because of its capacity to inhibit IL-6 and IL-23 production (44, 45), and induces differentiation and expansion of FOXP3+ regulatory T cells (46). Recent studies reported that VDR is required for NK T cell development (47), TCR signaling, and T cell activation (48). With regard to innate immunity, Liu et al. (19) have demonstrated an antimicrobial capability of the local macrophage vitamin D–VDR system. TLR4 activation triggered by bacteria induces VDR expression and local 1,25(OH)2D3 biosynthesis in macrophages, which, in turn, stimulate antimicrobial peptide cathelicidin expression to suppress bacterial growth. In this report, we revealed a novel regulatory mechanism for the VDR signaling to control TLR4-mediated inflammation. This anti-inflammatory mechanism is mediated by miR-155 downregulation, which de-represses SOCS1, leading to heightened negative feedback regulatory action. The importance of miR-155 in vitamin D regulation of innate immunity was confirmed by the experiments using the miR-155–null model and the miR-155 hairpin inhibitor in VDR-null macrophages. Consequently, in the absence of VDR, LPS triggers a hyper and sustained inflammatory response in macrophages, primarily because of deregulation of the negative feedback loop. This is caused by miR-155 upregulation leading to excessive suppression of SOCS1. Together, these data demonstrate that the vitamin D–VDR signaling attenuates TLR4-mediated inflammatory response. SD represents 1,25(OH)2D3 or its analogs.
mediated inflammatory reaction by upgrading the negative feed-back regulation. This conclusion predicts potential detrimental effects of vitamin D deficiency, under which condition inflammatory reaction may be unchecked and sustained. This may, in part, explain the association of vitamin D deficiency and inflammatory disorders in humans (49–52).

Our data show that vitamin D–VDR signaling inhibits miR-155 by a transcriptional mechanism. We identified a functional kB cis-DNA element within the first intron of the mouse bic gene and proved that this intronic kB site interacts with NF-kB and mediates the upregulation of bic gene transcription upon TLR4 activation. 1,25(OH)2D3 suppresses LPS-induced bic gene transcription by blocking the activation of NF-kB, namely, by inhibiting IKK activity leading to IκBα stabilization and arrest of p65/p50 nuclear translocation. However, exactly how vitamin D regulates NF-kB remains unclear, and the detailed molecular mechanism remains to be fully defined in future studies.

As an important regulator in the immune system, miR-155 has multiple targets involved in immune functions. In addition to SOCS1, miR-155 also represses SHP1 and C/EBPβ (53, 54), two key negative regulators of IL-6 signaling pathway. It has been reported that miR-155 can also attenuate TLR4 signaling by targeting IKKe (55). Thus, it is postulated that vitamin D may modulate immune response by also regulating SHP1, C/EBPβ, and/or IKKe via miR-155. This speculation warrants further investigations. Moreover, miR-155 has been detected in a variety of immune cells including B cells (24), T cells (56), macrophages (13), dendritic cells (57) and hematopoietic progenitor cells (58). Given the broad functionalities of miR-155, it is possible that vitamin D may regulate immune activities of other immune cells through miR-155. In this regard, this study has opened new avenues to explore various immunomodulatory mechanisms of vitamin D.

Acknowledgments

We thank Dr. Yinyuan Mo (Southern Illinois University School of Medicine, Springfield, IL) for providing pCDH-hmR155 plasmid.

Disclosures

The authors have no financial conflicts of interest.

References