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Genetic determination of the role of PU.1 in macrophage gene expression

Myungsoo Joo [a,*], Minjae Kwon [a], Anser C. Azim [b], Ruxana T. Sadikot [b], Timothy S. Blackwell [a], John W. Christman [b]

[a] Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University Medical Center, B1222 MCN, Nashville, TN 37232-2650, USA
[b] Section of Pulmonary, Critical Care and Sleep Medicine, University of Illinois and the Jesse Brown Veterans Affairs Medical Center, Chicago, IL 60612, USA

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ABSTRACT

PU.1, an Ets family transcription factor, mediates macrophage effector function in inflammation by regulating gene expression. But, the extent and nature of PU.1 function in gene expression has not been genetically determined because ablation of PU.1 gene abolishes macrophage development. Here, we epigenetically suppressed PU.1 by stably expressing PU.1 specific siRNA in macrophages, and determined the effect of PU.1 deficiency on expressions of key inflammatory genes: Toll-like receptor 4 (TLR4), cyclooxygenease-2 (COX-2), and macrophage inflammatory protein-1α (MIP-1α). PU.1-silenced cell lines expressed lower TLR4 mRNA and COX-2 protein, but higher MIP-1α protein, than controls. Over-expression of PU.1 suppressed lipopolysaccharide-induced MIP-1α production. PU.1 occupied proximal and distal cognate sites in the endogenous MIP-1α promoter, but dissociated only from the distal sites in response to lipopolysaccharide, suggesting a novel negative regulatory mechanism by PU.1. Together, our results defined PU.1 function in differentially regulating expressions of TLR4, COX-2, and MIP-1α.

PU.1, an Ets transcription factor exclusively expressed in myeloid cells, determines the development of macrophages, as shown in PU.1 gene knockout mice that have no macrophage [1]. In macrophages, PU.1 has been shown to induce expressions of various inflammatory genes including Toll like receptor 4 (TLR4) [2], cyclooxygenas-2 (COX-2) [3], and macrophage inflammatory protein-1α (MIP-1α) [4]. But, this function has not been genetically determined due to the close genetic link between PU.1 and macrophage development. Nevertheless, these results suggest that PU.1 is an essential transcription factor in macrophage-mediated innate immunity.

Macrophages are essential cells in innate immunity. They sense pathogens and then initiate inflammatory responses [5], which is conferred, in part, by abundant expression of TLR4, a receptor for bacterial endotoxin (lipopolysaccharide: LPS) [6]. Thus, mice harboring defective TLR4 are more susceptible to bacterial infection [7]. LPS binding to TLR4 activates a series of transcription factors such as NF-κB [6], C/EBP-β [8], and PU.1 [3], resulting in induction of inflammatory genes including COX-2 [6] and MIP-1α [4]. Although PU.1, activated by LPS, may induce TLR4, it is unclear whether LPS treatment leads to expression of functional TLR4.

COX-2 plays an important role in inflammation by catalyzing the production of prostanoids that have diverse effects on inflammation, such as recruitment of inflammatory cells, increase of vascular permeability, and induction of vasodilation [9–11]. Thus, transcriptional regulation of COX-2 gene expression has been studied extensively, showing that CREB, NF-κB, and C/EBP-β are major transcription factors for COX-2 expression in various cell types [12–15]. In addition, PU.1 was suggested as a macrophage specific factor for COX-2 expression in macrophages [3], but another *in vitro* study failed to relate PU.1 and COX-2 expression [16]. Therefore, whether or not PU.1 regulates COX-2 expression in macrophages remains controversial.

MIP-1α is a C–C chemokine that binds to G protein coupled receptors CCR3 and CCR5 and recruits diverse inflammatory effector cells including macrophages [17]. A previous report located a PU.1 binding site in the promoter of MIP-1α, along with C/EBP-β. In that study, although over-expression of C/EBP-β supports MIP-1α expression, over-expression of PU.1, failed to do so, and elevated PU.1 expression was not correlated with MIP-1α production in spleen focus-forming virus-induced murine erthroleukemias (MEL) cells [4]. Thus, despite the biochemical, *in vitro* binding capability of PU.1 to the MIP-1α promoter, it remains unknown whether or not PU.1 is functionally involved in MIP-1α expression.

Here, by epigenetically suppressing PU.1 in macrophages, we examined the role of PU.1 in TLR4 expression and in TLR4-mediated COX-2 and MIP-1α expressions. Due to low transfection efficiency in macrophages, it was necessary to generate macrophage cell lines that stably express siRNA specific for PU.1. Our re-

* Corresponding author. Fax: +1 615 340 2347.
E-mail address: Myungsoo.Joo@vanderbilt.edu (M. Joo).

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sults defined the role of PU.1 in regulating expression of those genes, in which PU.1 functioned as a positive and a negative regulator.

Materials and methods

Cell culture. A murine macrophage cell line, RAW 264.7 (ATCC, Rockville, MD), was maintained in DMEM (Cellgro) containing 10% fetal bovine serum (FBS) (Hyclone). LPS treatment was described previously [3].

RNAi plasmid construct, plasmids, and transfection. A pair of oligonucleotides designed by a software, targeting at from +115 to +135 nt of the murine PU.1 gene, was inserted into pSUPER.retro.puro plasmid (OligoEngine, Seattle, WA): the forward, 5′-GATCCCCGC CATAGCGATCACTACTGTTCAAGAGACAGTAGTGATCGCTATGGCTTT TTGGAAA-3′ and the reverse, 5′-AGCTTTTCCAAAAAGCCATAGCG ATCACTACTGTCTCTTGAACAGTAGTGATCGCTATGGCGGG-3′. Plasmids were purified by Endo-free Maxiprep kit (Qiagen). RAW264.7 transfected with GenePORTER 2 (Gene Therapy Systems, San Diego, CA) was selected and maintained under 2 μg/ml of Puromycin (SIGMA). The plasmids encoding PU.1 and C/EBP-β were gifts from Dr. Atchison (University of Pennsylvania) and Dr. Sealy (Vanderbilt University), respectively.

Western Blotting. Total cell lysate were prepared as described previously [3]. Proteins separated by SDS–PAGE were analyzed by appropriate antibodies with enhanced chemoluminescence (ECL plus, Amersham). Antibodies for α-PU.1 (rabbit polyclonal), α-p65 (rabbit polyclonal), α-IκBα (rabbit polyclonal), α-actin antibody, and α-Ets-1/2 (goat polyclonal) were obtained from Santa Cruz Biotechnology, and α-murine COX-1 and -2 were from Cayman Chemical.

RT-PCR. Total RNA was prepared by RNeasy kit per the protocol of the manufacturer (Qiagen). 2 μg of RNA was used for cDNA synthesis. Actin cDNA from each sample was used to normalize the samples for differences in PCR efficiency. TLR4 mRNA quantity was determined by using end-point dilution PCR, including three serial 1–5 dilutions (1:1, 1:5, 1:25, and 1:125) of RT products for PCR amplification. To avoid genomic DNA contamination, equal amounts of RNA from each sample were PCR amplified without RT reaction. cDNA was amplified with *Taq* polymerase (Perkin-Elmer) and appropriate primers at 94 °C for 40 s, 60 °C for 30 s, and 72 °C for 40 s for 35 cycles with an initial 4 min denaturation at 95 °C and final 10-min extension at 72 °C. The primers for TLR4 were 5′-GGAAGTTTCTCTGGACTAACAAGTTTAGA-3′ and 5′-AAATTG TGAGCCACATTGAGTTTC-3′. The primers for β-actin were 5′-AGAG GGAAATCGTGCGTGAC-3′; and 5′-CAATAGTGATGACCTGGCCGT-3′.

Flow cytometry analysis. One million cells pre-incubated with normal IgG were stained with either phycoerythrin-conjugated anti-TLR4/MD2 or isotypic IgG (Santa Cruz Biotechnology) at 4 °C for 45 min in a medium (DMEM medium, 10% newborn calf serum), and analyzed by a FACScan flow cytometer (Becton–Dickinson, Mountain View, CA, USA) and CellQuest software.

Luciferase assay. Cells were transfected with an NF-κB firefly luciferase reporter construct, along with a tk-*Renilla* luciferase construct. Dual luciferase assay was performed per the protocol of the manufacturer (Promega).

Chromatin immunoprecipitation (ChIP) assay. Reagents and assay procedure were described previously [18]. DNA was amplified as follows: 94 °C for 240 s; 30–32 cycles at 94 °C for 40 s, 54 °C for 40 s, and 72 °C for 60 s; and final elongation at 72 °C 10 min. The primers used for the distal sites were 5′-ACACTGGATAA CTGCTTACTTT-3′ and 5′-AGTACACTCATAACATTGGT GA-3′, and those for the proximal site 5′-GTGGCCTAGTCACTTTGCG-3′ and 5′-CAGCTCTCAACTCGTGACC-3′. Each experiment was performed at least three times independently.

Results

PU.1 up-regulates TLR4 mRNA expression

To silence PU.1 expression by siRNA in macrophages, to which transfection is poor, we stably transfected RAW264.7 cells with an empty host vector plasmid or a plasmid encoding siRNA specific for the murine PU.1 gene, and the transfected cells were selected under 2 μg/ml of Puromycin. As shown in Fig. 1A, PU.1 expression was suppressed in two independent PU.1 siRNA cell lines, PU 5.7 and PU 5.9, while a stable transfectant with the empty vector plasmid, PU 4.11, expressed PU.1 similar to the parental cell line, RAW264.7. To examine whether PU.1 siRNA cross-silences other Ets family transcription factors, we measured expressions of Ets-1 and -2 in PU 5.7, and found no differences in their expressions (Fig. 1B). These results show that our PU.1 siRNA cell lines specifically suppressed PU.1 expression without affecting related proteins.

Next, to examine whether PU.1 regulates expression of TLR4 mRNA, we extracted total RNA from the PU.1-silenced cell lines and analyzed it by semi-quantitative RT-PCR. As shown in Fig. 1C, PU 5.7 expressed a low level of TLR4 mRNA expression compared to RAW264.7 (left panel). Similarly, PU 5.9 expressed less TLR4 mRNA than PU 4.11 (right panel). We also performed similar experiment to measure MD2 mRNA, and found no significant changes in the PU.1-silenced cell lines compared to controls (data not shown). Together, these data indicate that PU.1 positively regulates TLR4 mRNA expression.

Decrease of TLR4 mRNA does not affect TLR4 signaling

Since TLR4 needs to complex with a cofactor, MD2, for its cell surface presentation and responsiveness to LPS [19,20], we examined whether low TLR4 mRNA expression affects the cell surface

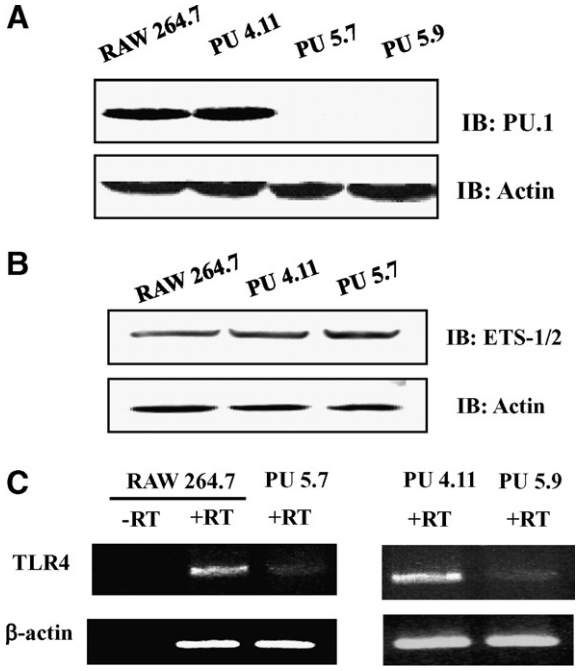

Fig. 1. PU.1 up-regulates TLR4 mRNA expression. (A) Silencing PU.1 expression in siRNA cell lines, PU 5.7 and PU 5.9, was determined by Western blot with α-PU.1 antibody (top panel) and α-actin antibody for internal controls (bottom panel). (B) Potential cross-silencing was examined in PU 5.7 by Western blot with α-ETS ½ antibodies. (C) TLR4 mRNA expression was examined by semi-quantitative RT-PCR of PU 5.7 (left panel) and PU 5.9 (right panel). Included was PCR for TLR4 without RT reaction (−RT) to exclude genomic DNA contamination. β-Actin as internal controls was similarly analyzed.

expression of TLR4/MD2 and responsiveness to LPS. First, for determination of the cell surface expression of TLR4, the cells were treated with LPS for different periods, stained with phycoerythrin-conjugated TLR4/MD2 antibody, and subsequently analyzed by FACS. As shown in Fig. 2A and B, the surface expression of TLR4/MD2 complex, at a steady state and during LPS treatment, was not significantly different between the parental and PU.1 siRNA cell line, PU 5.7.

Next, in order to determine responsiveness to LPS, the cells were transfected with an NF-κB-luciferase reporter construct and

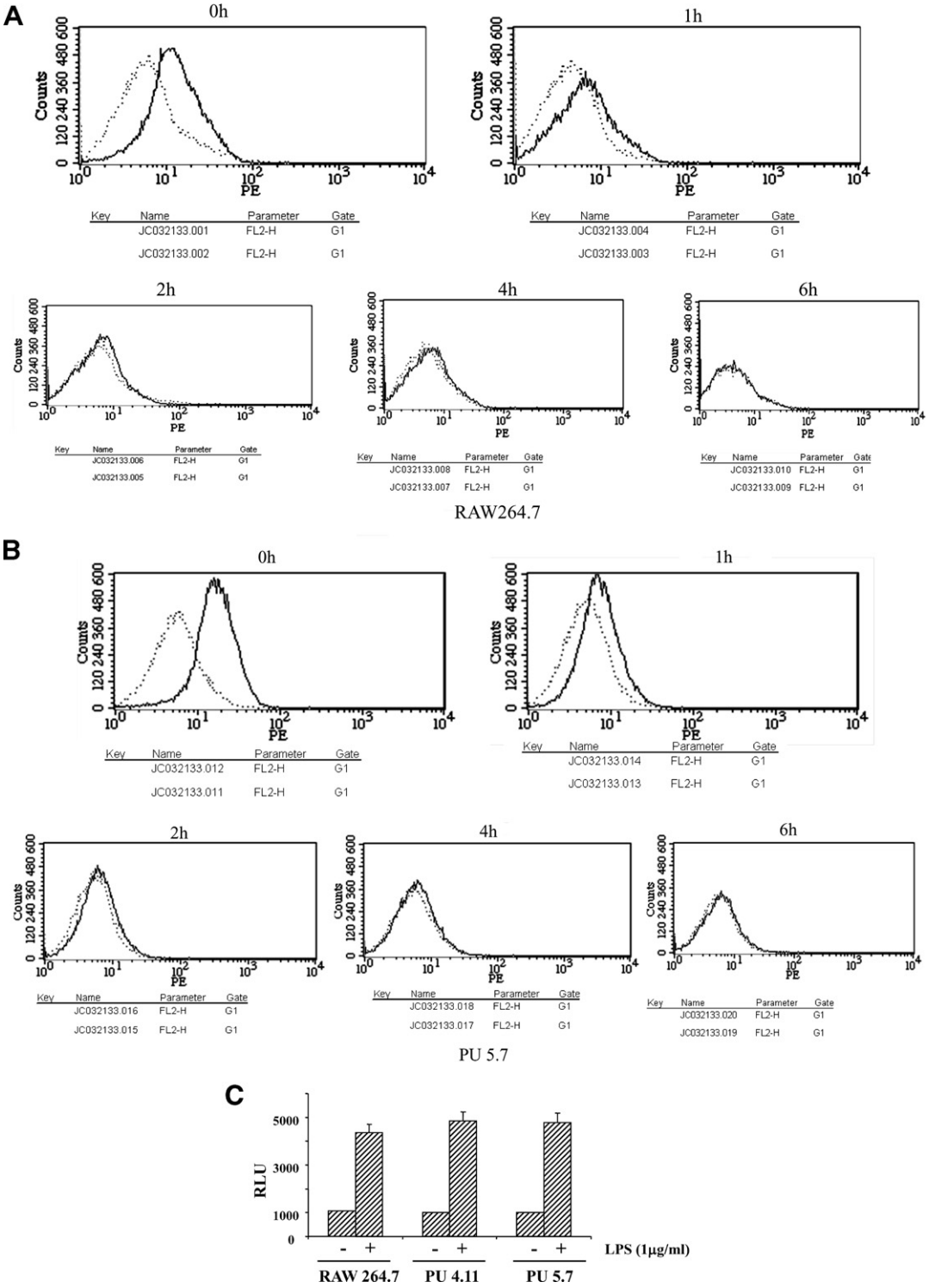

Fig. 2. Expression of cell surface TLR4 and NF-κB activation in the PU.1-silenced cell line. Surface expression of TLR4/MD2 was determined in RAW 264.7 cells (A) and PU 5.7 cells (B) by FACS analyses. The cells differentially treated with LPS (0.1 μg/ml) were fixed and stained with α-TLR4/MD2 antibody. The dotted line represents staining with PE-conjugated isotypic IgG and solid line does for with PE-conjugated TLR4/MD2 antibody. (C) NF-κB activity of PU.1-silenced cell line was measured by a reporter assay. The cells transfected with an NF-κB-luciferase and a tk-*Renilla* reporter constructs were treated with LPS (1 μg/ml) for 4 h. The results were an average of triplicate settings, and experiment was performed three times independently.

subsequently treated with LPS for NF-κB activation, an indicative of activated TLR4 signaling. As shown in Fig. 2C, NF-κB activation was not different from controls, indicating intact responsiveness to LPS. Similar results were also obtained in another PU.1-silenced cell line, PU 5.9 (data not shown). Together, these results suggest that although PU.1 deficiency results in a reduction of a steady level of TLR4 mRNA, this low TLR4 mRNA expression is sufficient for maintaining the cell surface presentation and function of TLR4.

PU.1 regulates early COX-2 expression

Previously, we showed that over-expression of PU.1 enhances COX-2 expression elicited by LPS treatment [3], which was, however, not supported by a recent *in vitro* study conducted in another laboratory [16]. These results prompted us to test whether or not PU.1 regulates COX-2 expression in macrophages by using the PU.1 siRNA cell lines. We treated the cells with LPS for up to 8 h, and analyzed COX-2 expression by Western blotting. As shown in Fig. 3, the PU.1 siRNA cell line, PU 5.7, expressed lower level of COX-2 expression than controls at 2 and 4 h after LPS treatments (compare lanes 2 and 3 to lanes 5 and 6). But, at 8 h after LPS treatment, COX-2 expression in PU 5.7 was not different from PU 4.11. On the other hand, COX-1 expression was not different in these two cell lines regardless of LPS treatment (data not shown). We obtained similar results using two additional PU.1-silenced cell lines, PU 5.6 and PU 5.9, (data not shown). Together, these results demonstrate that PU.1 regulates early expression of COX-2 in macrophages.

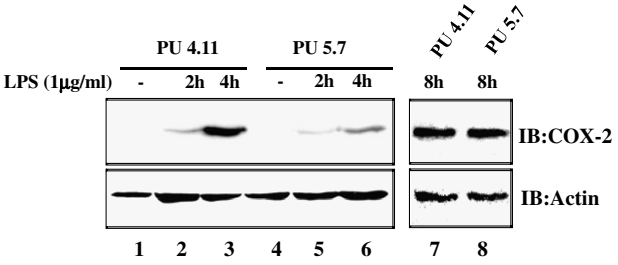

Fig. 3. PU.1 up-regulates COX-2 expression. COX-2 expression in PU.1-silenced cell lines was determined by Western blot analysis. The cells were treated with LPS (1 μg/ml) for up to 8 h, and total cell lysate was analyzed by immunoblotting for COX-2 (top panels) and actin (bottom panels).

PU.1 down-regulates MIP-1α expression

Since it remains unknown whether or not PU.1 functionally regulates MIP-1α expression, we determined the role of PU.1 in MIP-1α expression. First, since a previous report showed that C/EBP-β is involved in MIP-1α expression [4], we tested whether over-expression of C/EBP-β induces MIP-1α in macrophages. As shown in Fig. 4A, transfection of RAW264.7 with a C/EBP-β expressing plasmid induced MIP-1α (lanes 3 and 4), suggesting that, consistent with the previous report [4], C/EBP-β supports MIP-1α expression. Next, to determine the role of PU.1 in LPS-induced MIP-1α expression, we treated the PU.1 silenced cells, PU 5.7, with LPS for different periods, and measured MIP-1α expression by Western blotting. As shown in Fig. 4B, unlike control (lane 1), PU 5.7 cells expressed MIP-1α without LPS treatment (lane 4), which was further enhanced by LPS treatment (compare lanes 2 and 3 to lanes 5 and 6), suggesting that PU.1 functions as a suppressor of MIP-1α expression. To test this possibility, we transfected RAW 264.7 cells with increasing amounts of a PU.1 expressing plasmid prior to LPS treatment for 4 h, and analyzed MIP-1α expression by Western blotting. As shown in Fig. 4C, over-expression of PU.1 suppressed MIP-1α expression. Together, these results suggest that PU.1 is a negative regulator of MIP-1α expression.

In order to elucidate a novel suppressive mechanism by PU.1, we examined PU.1 binding to the endogenous MIP-1α promoter in response to LPS treatment. First, we analyzed the murine MIP-1α promoter sequence by the TFSEARCH program (version 1.3, Tokyo University, Japan), which located, within an 1.5 kb-long promoter, three c-Ets sites: two sites, from −949 to −959 nt and from −1078 to −1090 nt, designated as distal binding sites, and one site, from −342 to −352 nt, as a proximal binding site. Next, to examine whether PU.1 utilizes these sites, we performed chromatin immunoprecipitation (ChIP) assay. RAW cells were treated with LPS for different periods, and fixed to cross-link DNA and

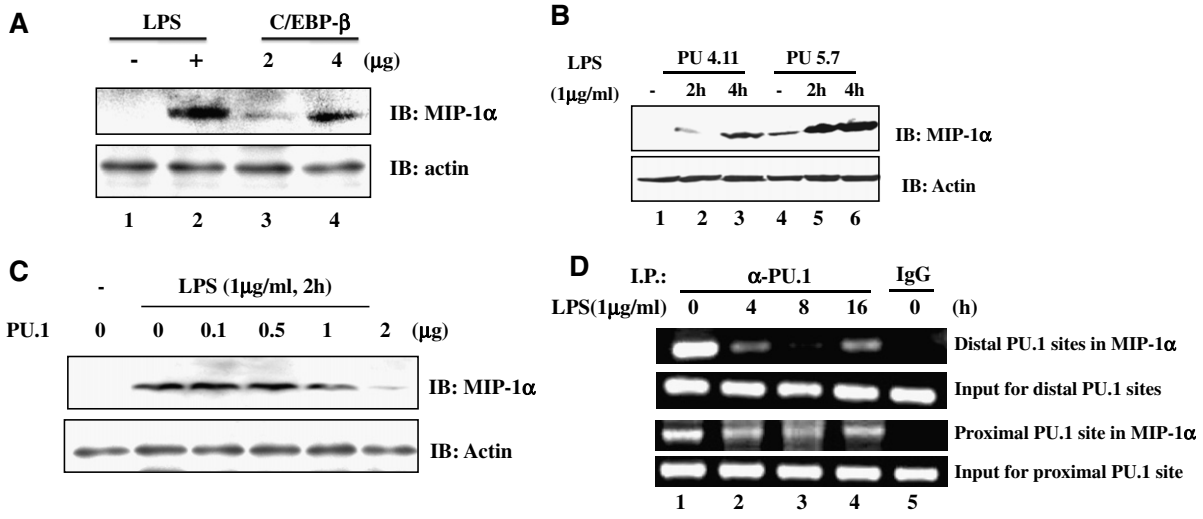

Fig. 4. PU.1 negatively regulates MIP-1α expression. (A) RAW 264.7 cells were transfected with pcDNA3.1 (lanes 1 and 2) or a plasmid encoding C/EBP-β (lanes 3 and 4) for 48 h. Transfection was normalized with pcDNA3.1 to 4 μg. Total cell lysate was analyzed by Western blot for MIP-1α (top panel) and actin (bottom panel). (B) MIP-1α expression in PU 5.7 following LPS treatment was determined by Western blot analysis. (C) RAW 264.7 cells were transfected with increasing amounts of a PU.1-expressing plasmid, and the transfected cells were treated with LPS for 2 h. MIP-1α expression was determined by Western blot analysis. (D) PU.1 binding to the endogenous MIP-1α promoter was analyzed by ChIP assay. PU.1 bound to DNA was immunoprecipitated by α-PU.1 antibody (lanes 1 to 4), and co-precipitated DNA was analyzed by PCR for distal PU.1 sites (top two panels) and proximal site (bottom two panels). Included was an isotypic IgG to exclude nonspecific immunoprecipitation (lane 5).

bound transcription factors. For precipitation of PU.1 bound DNA, the nuclear fraction was collected, sonicated, and added with α-PU.1 antibody. DNA precipitated with PU.1 was eluted and amplified by the specific sets of primers flanking either the distal binding sites or the proximal binding site. As shown in Fig. 4D, PU.1 bound to these binding sites in an unstimulated condition (lane 1). While constitutively binding to the proximal site regardless of LPS treatment (third panel from the top), PU.1 lost binding to the distal sites at early time points (lanes 2 and 3) but returned to the sites at a late time point after LPS treatment (lane 4). Taken together, these results suggest that differential binding of PU.1 to these sites, in response to LPS, is associated with down-regulation of MIP-1α expression.

Discussion

Bacterial infection is associated with severe sepsis, the 10th leading cause of death in USA [21], which is characterized by an uncontrolled, adverse host immune reaction that is independent of resolving bacterial infection [22]. Given the key role of macrophages in innate immunity, in which PU.1 is a central transcription factor [23], elucidating PU.1 function in regulation of inflammatory gene expression could provide a clue to control excessive inflammation that results in organ dysfunction. But, due to its close link with macrophage development [1], the precise function of PU.1 has not been genetically determined. In this study, we attempted to circumvent this issue by epigenetically suppressing PU.1 expression and to genetically determine PU.1 function in regulating inflammatory gene expression such as TLR4, COX-2, and MIP-1α.

LPS bound TLR4 activates PU.1, and PU.1 has been shown to increase TLR4 transcription [2]. Thus, it is conceivable that LPS treatment increases TLR4 expression and thereof responsiveness to LPS, which, however, remains controversial [24,25]. Consistent with the published results [2,24], our data clearly show that PU.1 positively regulated TLR4 gene expression. But, as published by other laboratories [26,27], our results also show that LPS treatment decreased the level of the cell surface TLR4, and we found no evidence that the level of the cell surface TLR4 was recovered during LPS treatment (data not shown). It seems that a low level of TLR4 mRNA did not affect the level of cell surface TLR4 and its signaling. Thus, our results suggest that LPS treatment does not enhance, rather suppresses, TLR4 function.

COX-2, induced by inflammatory stimuli, regulates inflammation, and aberrant expression of COX-2 is associated with cancer [28]. Numerous studies have shown that COX-2 expression results from a complex interplay among key transcription factors such as NF-κB, C/EBP-β, and PU.1. Since the involvement of PU.1 is controversial [16], we addressed this by using the PU.1-silenced cell lines. Our data show that deficiency of PU.1 resulted in decreased COX-2 protein expression at early, but not at later time point, indicating that the impact of PU.1 on COX-2 expression was time dependent. It is notable that the PU.1-silenced cell lines exhibited intact TLR4 signaling, excluding the possibility that reduced COX-2 expression is due to impaired TLR4 signaling. In conjunction with our previous report showing that PU.1 binds to two different sites in the endogenous COX-2 promoter in response to LPS, we conclude that PU.1 is involved in COX-2 expression.

MIP-1α, rapidly induced by LPS and TNF-α, promotes inflammation by recruiting immune effector cells [17]. Although previous study implicated PU.1 in MIP-1α gene expression [4], neither high expression of PU.1 in MEL cells [4] nor GM-CSF treatment to increase PU.1 expression [29] resulted in MIP-1α expression, leaving the role of PU.1 in MIP-1α expression unknown. Our results suggest that PU.1 suppresses MIP-1α gene expression. Consistent with this, over-expression of PU.1 suppressed MIP-1α expression elicited by LPS treatment. Thus, our results provide explanation why expression of PU.1 failed to induce MIP-1α.

Dual, opposing effects of a transcription factor are not unprecedented. Yin-Yang 1 (YY1) has been documented as a dual regulator of transcription [30,31]. This function is in part determined by acetylation or deacetylation of YY1, which are mediated by histone acetylases (HATs) and histone deacetylases (HDACs), respectively [30,31]. Likewise, PU.1 interacts with HATs and HDACs [32,33]. As yet, it is unknown whether the acetylation status of PU.1 is associated with its dual activity.

In summary, we attempted to define the role of PU.1 in macrophage effector function. To that end, we established PU.1 loss-of-function cell lines by utilizing siRNA. The PU.1-silenced cell lines maintained functional TLR4 signaling, which allowed us to study PU.1 function in inflammatory gene expression following LPS treatment. Our data demonstrated that PU.1 positively regulates TLR4 and COX-2 expression but negatively regulates MIP-1α. Our study also supplies evidence that siRNA approaches in macrophages could provide important tools to decipher functions of PU.1 and other regulatory factors in macrophages.

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