

Role of PU.1 in MHC class II expression through transcriptional regulation of class II transactivator pI in dendritic cells

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Background: PU.1 is a hematopoietic cell-specific transcription factor belonging to the Ets family. We hypothesized that PU.1 is involved in MHC class II expression in dendritic cells (DCs).

Objective: The role of PU.1 in MHC class II expression in DCs was analyzed.

Methods: Transcriptional regulation of the DC-specific pI promoter of the class II transactivator (CIITA) gene and subsequent MHC class II expression was investigated by using PU.1 small interfering RNA (siRNA) and reporter, chromatin immunoprecipitation, and electrophoretic mobility shift assays.

Results: PU.1 siRNA introduction suppressed MHC class II expression, allogeneic and syngeneic T-cell activation activities of bone marrow-derived DCs (BMDCs) with reduction of CIITA mRNA driven by the DC-specific promoter pI, and MHC class II mRNA. The chromatin immunoprecipitation assay showed constitutive binding of PU.1 to the pI region in BMDCs, whereas acetylation of histone H3 on pI was suppressed by LPS stimulation in parallel with shutdown of CIITA transcription. PU.1 transactivated the pI promoter through *cis*-elements at -47/-44 and -30/-27 in a reporter assay and to which PU.1 directly bound in an electrophoretic mobility shift assay. Acetylation of histones H3 and H4 on pI was reduced in PU.1 siRNA-introduced BMDCs. Knockdown of interferon regulatory factor 4 or 8, which is a heterodimer partner of PU.1, by siRNA did not affect pI-driven CIITA transcription or MHC class II expression.

Conclusion: PU.1 basally transactivates the CIITA pI promoter in DCs by functioning as a monomeric transcription factor and by affecting histone modification, resulting in the subsequent expression and function of MHC class II. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: PU.1, class II transactivator, dendritic cells, MHC class II

MHC class II molecules are cell-surface glycoproteins that play important roles in the adaptive immune system by presenting peptide from exogenously derived antigen to CD4⁺ T cells through interaction with the T-cell receptor. MHC class II expression is largely restricted to professional antigen-presenting cells and thymic epithelial cells. Other nonhematopoietic cells, including fibroblasts, astrocytes, endothelial cells, epithelial cells, and keratinocytes, are induced to express MHC class II by means of IFN- γ stimulation. The cell type-specific and cytokine-induced expression of MHC class II is regulated by a cofactor termed class II transactivator (CIITA).¹ Human *CIITA* and murine *C2ta* genes possess 4 (pI, pII, pIII, and pIV) and 3 (pI, pIII, and pIV) cell type-specific promoters, respectively.^{1,2} Initially, studies on the differential use of each promoter simply indicated that pI, pIII, and pIV are mainly used in dendritic cells (DCs), B cells, and IFN- γ -stimulated cells, respectively.²⁻⁴ Subsequent studies have revealed that the use of CIITA promoters is not so simple. For example, pIII also functions in activated human T cells⁵ and murine plasmacytoid dendritic cells (pDCs)⁶ in addition to B cells. The molecular mechanisms by which pIII and pIV are regulated have been vigorously studied, and the following transcription factors have been identified to regulate these promoters: signal transducer and activator of transcription 1 (STAT1), E47, PU.1, interferon regulatory factor (IRF) 4, AML2/3, cyclic AMP response element binding (CREB), and BLIMP1 for pIII and STAT1, USF1, IRF1, and IRF2 for pIV (summarized in Reith et al¹). In contrast, the mechanism of transcriptional regulation of pI is largely unknown, despite the importance of DCs as antigen-presenting cells. Therefore in the present study we analyzed pI function in DCs with a focus on the role of the transcription factor PU.1.

The hematopoietic lineage-specific transcription factor PU.1 that belongs to the Ets family is involved in lymphoid and myeloid cell development and specific gene regulation. PU.1 knockout mice show incomplete hematopoietic cell development, including the abolition of macrophage and B-cell production and a delay in neutrophil and T-cell production.⁷⁻⁹ Analyses of PU.1 knockout mice also indicate a role for PU.1 in the development of DCs.^{10,11} In our previous studies overexpression of PU.1 in mast cells or their progenitors caused morphologic and functional changes toward DCs.^{12,13} We also found that PU.1 small interfering RNA (siRNA) suppressed MHC class II expression on DCs in

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Abbreviations used

BMDC:	Bone marrow–derived dendritic cell
cDC:	Conventional dendritic cell
ChIP:	Chromatin immunoprecipitation
CIITA:	Class II transactivator
CREB:	Cyclic AMP response element binding
DC:	Dendritic cell
EMSA:	Electrophoretic mobility shift assay
FITC:	Fluorescein isothiocyanate
GAPDH:	Glyceraldehyde-3-phosphate dehydrogenase
IRF:	Interferon regulatory factor
pDC:	Plasmacytoid dendritic cell
siRNA:	Small interfering RNA
STAT1:	Signal transducer and activator of transcription 1

our recent study regarding PU.1 as a transcriptional regulator of CD80 and CD86.¹⁴ On the basis of these observations, we hypothesized that PU.1 is a key transcription factor for gene expression in DCs and is a candidate regulator of MHC class II expression in DCs. The present study demonstrates the critical role of PU.1 in the transactivation of CIITA pI and the subsequent expression and function of MHC class II in DCs.

METHODS**Mice**

BALB/c and C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan), and DO11.10 mice were purchased from the Jackson Laboratories (Bar Harbor, Me). All animal experiments were performed according to the approved guidelines of the Institutional Review Board of Juntendo University School of Medicine, Tokyo, Japan.

Cells

Whole bone marrow cells from BALB/c mice were grown in RPMI 1640 medium (Sigma-Aldrich, St Louis, Mo) supplemented with 10% heat-inactivated FBS, 100 μ mol/L 2-mercaptoethanol, 10 μ mol/L MEM nonessential amino acids (Sigma-Aldrich), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10 ng/mL murine rGM-CSF (PeproTech, London, United Kingdom) for 10 days to generate bone marrow–derived dendritic cells (BMDCs; see Fig E1 in this article's Online Repository at www.jacionline.org) based on a previously reported method.¹⁵ CD11c⁺ BMDCs were isolated by using the MACS separation system with anti-mouse CD11c MicroBeads and an autoMACS (all from Miltenyi Biotech, Tübingen, Germany). LPS (1 μ g/mL from *Escherichia coli*, Sigma-Aldrich) was added into the culture medium to stimulate BMDCs.

Quantification of mRNA by means of real-time PCR

Total RNA prepared from BMDCs with an RNeasy kit (QIAGEN, Hilden, Germany) was reverse transcribed with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, Calif). The mRNA level of PU.1, MHC class II, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was quantified by using a Step-One Real-Time PCR system (Applied Biosystems) with TaqMan Gene Expression Assays (Applied Biosystems; no. Mm01270606_m1 for PU.1, no. Mm00772352_m1 for MHC class II, and rodent GAPDH no. 4308313) and TaqMan Universal Master Mix (Applied Biosystems). A TaqMan gene expression product to detect CIITA mRNA under the control of the pI promoter was custom made by Applied Biosystems using the forward primer CIITA-pIF (5'-AGGCTCC CACGGTAGAGA-3'), the reverse primer CIITA-pIR (5'-CCATGGTGGCA CACAGACTA-3'), and the TaqMan probe CIITA-pIM (5'-FAM-ACAGG GACCATGGAGACCA-MGB-3'). The level of expression of each mRNA

was evaluated relative to that of GAPDH as the housekeeping gene by calculation of cycle threshold values, as described previously.^{16,17}

Knockdown of PU.1, IRF4, and IRF8 expression by siRNA

PU.1 siRNA (Stealth Select RNAi, Sfp11-MSS247676, Sfp11-MSS247678, and Sfp11-MSS277025), IRF4 siRNA (Irf4-MSS205499, Irf4-MSS205500, and Irf4-MSS205501), IRF8 siRNA (Irf8-MSS236846, Irf8-MSS236847, and Irf8-MSS236848), and control siRNA (Stealth Negative Universal Control, no. 45-2001) were purchased from Invitrogen (Carlsbad, Calif). A 5- μ L aliquot of 20 μ mol/L siRNA was introduced into 1×10^6 BMDCs with a Mouse Macrophage Nucleofector kit (Lonza, Basel, Switzerland) with a Nucleofector II (Lonza) set at Y-001.

Flow cytometry

Fluorescein isothiocyanate (FITC)– or phycoerythrin-conjugated anti-mouse I-A^d antibody (AMS-32.1, no. 553547; BD PharMingen, San Diego, Calif) was used to stain cell-surface MHC class II after blocking Fc receptors with 2.4G2 (BD PharMingen). Cell-surface fluorescence was analyzed by means of FACSCalibur flow cytometry (BD PharMingen), as described previously.^{13,16}

T-cell stimulation activity

BMDCs were subjected to syngeneic and allogeneic T-cell proliferation assays 24 hours after transfection of PU.1 or control siRNA. Briefly, after mitomycin C treatment, siRNA-introduced BMDCs were cocultured with CD4⁺ T cells from DO11.10 spleens in the presence of ovalbumin 323-339 peptide or with CD4⁺ T cells from C57BL/6 spleens, as described previously.^{12,13,18} Thymidine uptake was measured after 42 hours of coculture by pulsing with tritiated thymidine for the last 18 hours of coculture.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed as previously described.^{16,19} Anti-PU.1 goat IgG (D-19, no. sc-5949; Santa Cruz Biotechnology, Santa Cruz, Calif), anti-acetyl–histone H3 rabbit IgG (no. 06-599; Upstate Biotechnology, Lake Placid, NY), anti-acetyl–histone H4 rabbit IgG (no. 06-866, Upstate Biotechnology), goat IgG (no. 02-6202, Invitrogen), and rabbit IgG (no. 02-6102, Invitrogen) were used. Quantitative PCR of chromosomal DNA was performed by using the same method as used for mRNA quantification and the following primers and TaqMan probes: for the pI promoter (–96/–6), forward primer pI-96 (5'-CCTAACCCA TTCCGTTTCATCA-3'), reverse primer pI-6R (5'-ACCTCTGTGTGCA GTGTAGCCTTCT3'), and TaqMan probe pI-64P (FAM-5'-CCTGCACCG GAATG-3'-MGB) were used. The ratio of a specific DNA fragment in each immunoprecipitate to that fragment in the DNA before immunoprecipitation (input DNA) was calculated from each cycle threshold value and is represented as a percentage.

Western blotting

The cells were lysed with RIPA buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP-40, 0.25% sodium deoxycholate, and a protease inhibitor cocktail; Thermo Scientific, Rockford, Ill) and were solubilized in Laemmli SDS sample buffer. Cell lysates were resolved on a 12.5% SDS polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, Mass). The same anti-PU.1 antibody used for the ChIP assay, anti-actin murine mAb (C-2, Santa Cruz Biotechnology), and anti-CIITA rabbit polyclonal antibody (no. ab49132; Abcam, Cambridge, Mass) were used as the primary antibodies. Peroxidase-conjugated anti-rabbit IgG, anti-mouse IgG, and anti-goat IgG antibodies (GE Healthcare, Buckinghamshire, United Kingdom) were used as the secondary antibodies. The membranes were soaked with Luminata Forte Western HRP Substrate (Millipore), and signals were detected with the LAS-4000 (FujiFilm, Tokyo, Japan).

Luciferase assay

A series of reporter plasmids carrying various lengths of the 5' flanking region of the *C2ta* form I gene were constructed by using PCR and restriction endonucleases as follows. Briefly, $-564/+54$ and $-331/+54$ regions of the *C12ta-pI* gene were amplified from BALB/c genomic DNA by using PCR with murine CIITA-pI-F1 and murine CIITA-pI-F2 as forward primers, respectively, and murine CIITA-pI-R as the reverse primer (see Table E1 in this article's Online Repository at www.jacionline.org). These DNA fragments were inserted into *Bgl*II/*Hind*III sites of pGL4-Basic (Promega, Madison, Wis). Other constructs corresponding to specific deletions in the promoter region ($-443/+54$, $-285/+54$, $-239/+54$, $-212/+54$, $-179/+54$, $-127/+54$, $-74/+54$, and $-6/+54$) were generated from the abovementioned plasmids through introduction of *Bgl*II recognition sequences at appropriate sites by using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif) with the appropriate oligonucleotides and their complementary oligonucleotides (see Table E1). Each 5' site was then removed by means of *Bgl*II digestion and self-ligation. Mutant reporter plasmids lacking each PU.1-binding sequence were generated by using a QuikChange site-directed mutagenesis kit and the appropriate synthesized oligonucleotides (see Table E1). A plasmid, pCR-PU.1, which was generated in our previous study,²⁰ and its empty vector, pCR3.1 (Invitrogen), were used for coexpression analysis.

Cells of the simian kidney cell line CV-1 (1×10^6 ; RIKEN Cell Bank, Tsukuba, Japan) were transfected with 500 ng of reporter plasmid, 100 ng of expression plasmid, and 0.5 ng of pRL-null (Promega) with FuGENE6 (Roche Diagnostics, Indianapolis, Ind), according to the manufacturer's instructions. In the case of RAW264.7 cells, 2 μ g of reporter plasmid and 0.5 ng of pRL-null were introduced with FuGENE6. Luciferase activity in cells harvested 20 to 24 hours after transfection was measured, as described previously.¹⁶

Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was performed, as described previously.^{19,21} Probes were prepared by annealing 2 synthesized 5'-FITC-labelled oligonucleotides (Invitrogen) with complementary sequences. The sequence of the sense strand of the probe corresponding to nucleotides $-62/-20$ upstream of the transcription start site in the pI promoter was 5'-GCACCGGAATGAGGAAACATTCCTAAGAAGGA-GAAGGCTACA-3'. The PU.1 protein was prepared with a TnT T7 Quick coupled transcription/translation system (Promega) by using pCR-PU.1 as the template. The anti-PU.1 antibody and control IgG, which were used for ChIP assay and Western blotting, were used.

RESULTS

Effect of PU.1 siRNA on pI-mediated CIITA expression

The cofactor CIITA functions as a master regulator of MHC class II expression. In conventional dendritic cells (cDCs) CIITA transcription is mainly driven by the pI promoter among 3 CIITA promoters: pI, pIII, and pIV. Therefore to evaluate the effect of PU.1 siRNA on the transcription of MHC class II and pI-driven CIITA (hereafter referred to as pI-CIITA), their mRNA levels were analyzed by using real-time PCR. We first confirmed that the PU.1 mRNA level was decreased to approximately 10% of the control level by PU.1 siRNA and that this reduction in the PU.1 mRNA level continued for at least 96 hours after siRNA transfection in the presence and absence of LPS stimulation (Fig 1, A). The pI-CIITA mRNA level in PU.1 siRNA-introduced BMDCs was less than 10% of that in control BMDCs from 48 to 96 hours after transfection (Fig 1, B). PU.1 siRNA reduced the MHC class II mRNA level by more than 50% at 48 hours after transfection and by approximately 80% at 96 hours compared with control values (Fig 1, C). Furthermore, LPS stimulation downregulated the mRNA levels of PU.1, pI-CIITA, and MHC class II. These

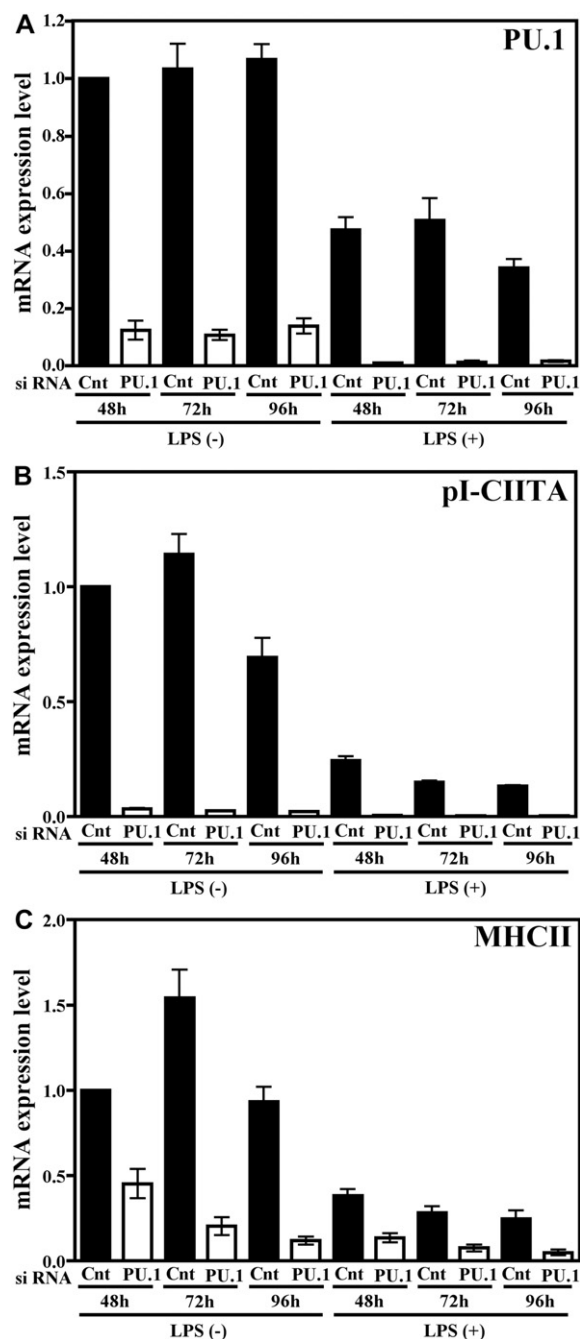


FIG 1. Effects of PU.1 knockdown by siRNA on the transcription levels of PU.1, pI-CIITA, and MHC class II in BMDCs. BMDCs were divided into 2 aliquots at 48, 72, or 96 hours after introduction of PU.1 siRNA (PU.1) or its control siRNA (Cnt) and were cultured for an additional 24 hours in the presence (LPS [+]) or absence (LPS [-]) of LPS. The mRNA expression level of PU.1 (A), pI-CIITA (B), and MHC class II (C) in these BMDCs is displayed as the ratio of mRNA levels versus those seen in control siRNA-introduced nonstimulated BMDCs at 48 hours. Data represent means $\pm$ SEMs of 2 independent experiments with triplicate samples.

results indicate that PU.1 siRNA reduced both pI-CIITA and MHC class II mRNA levels and that the reduction in MHC class II levels was delayed compared with levels of CIITA. Furthermore, LPS stimulation reduced the mRNA level of PU.1, pI-CIITA, and MHC class II in parallel.

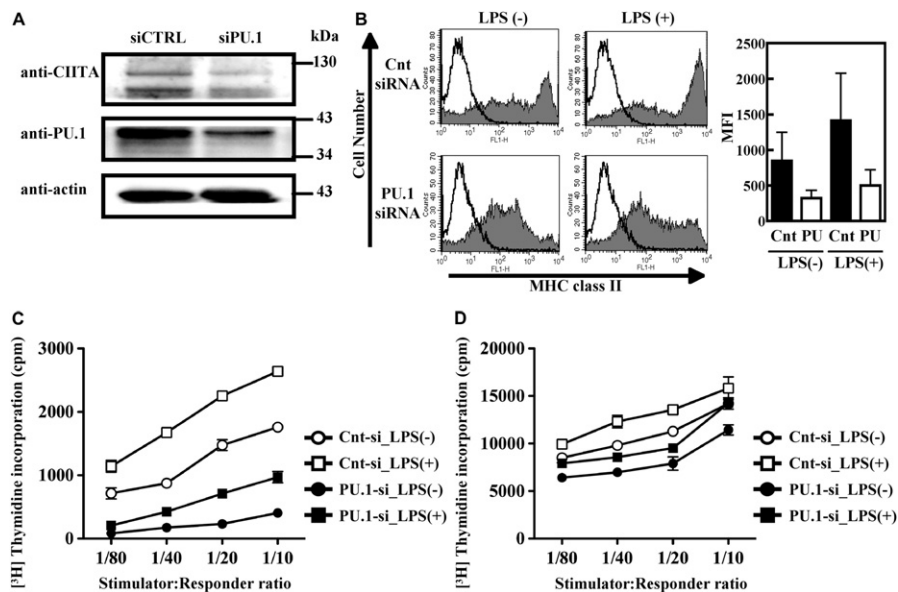


FIG 2. Effect of PU.1 siRNA on the cell-surface expression and function of MHC class II in BMDCs. **A**, Protein levels of CIITA and PU.1 in whole-cell lysates of BMDCs transfected with control siRNA (*siCTRL*) or PU.1 siRNA (*siPU.1*) for 48 hours were assayed by means of Western blot analysis. Actin was probed as a loading control. **B**, Cell-surface expression of MHC class II. BMDCs transfected with control siRNA (*top*) or PU.1 siRNA (*bottom*) were stained with FITC-conjugated anti-I-A^d murine IgG2b antibody (*shaded histograms*) or FITC-conjugated murine IgG2b (*unshaded histograms*) after stimulation with LPS (*right*) or without stimulation (*left*). A representative result of 3 independent experiments is shown. The graph shows means $\pm$ SEMs of mean fluorescence intensity (MFI) obtained from 3 experiments with duplicate samples. **C** and **D**, Allogeneic (**C**) and syngeneic (**D**) T cell-stimulating activity of PU.1 knockdown BMDCs. T-cell activity (*responder*) by BMDCs (*stimulator*) was determined by means of assay of T-cell proliferation with tritiated thymidine.

PU.1 knockdown by siRNA suppresses MHC class II expression and function

Western blotting and flow cytometric analyses were performed to evaluate the effect of PU.1 siRNA on the protein expression of PU.1, CIITA, and MHC class II. Western blotting confirmed that PU.1 siRNA reduced the PU.1 protein level and subsequent CIITA protein expression (Fig 2, A). Flow cytometric analysis showed that cell-surface expression of MHC class II was downregulated by PU.1 siRNA introduction (Fig 2, B, left). A suppressive effect of PU.1 siRNA on MHC class II expression was still observed in LPS-stimulated BMDCs, the MHC class II expression of which was increased compared with that of nonstimulated DCs (Fig 2, B, right).

The ability of PU.1 siRNA- or control siRNA-introduced BMDCs to stimulate T-cell proliferation was assayed to examine MHC class II-mediated function in PU.1 knockdown BMDCs. BMDC-T cell-stimulating activity was significantly reduced by PU.1 knockdown in an allogeneic interaction between BALB/c DCs and B6 T cells (Fig 2, C) and in a syngeneic system by using DO11.10 and ovalbumin (Fig 2, D).

These results indicate that PU.1 is involved in the expression and function of MHC class II in DCs.

PU.1 is recruited to the CIITA pI promoter in BMDCs

The role of PU.1 in transactivation of the pIII promoter in B cells is well characterized.^{22,23} In contrast, to date, the involvement of PU.1 in pI function in DCs is largely unknown. Therefore a ChIP assay was performed to examine whether PU.1 is recruited

to the pI region on the chromosome in DCs. The amount of DNA immunoprecipitated with the anti-PU.1 antibody was significantly higher than that immunoprecipitated with control antibody under both nonstimulated and stimulated conditions, suggesting that PU.1 constitutively binds to the CIITA-pI promoter region (Fig 3, A, and see Fig E2 in this article's Online Repository at www.jacionline.org). The ChIP assay for PU.1 on the pI promoter showed no specific binding of PU.1 to the pI promoter in bone marrow-derived mast cells (see Fig E3, A, in this article's Online Repository at www.jacionline.org), suggesting that the binding of PU.1 to the pI is cell type specific. Although LPS stimulation increases cell-surface MHC class II expression on DCs, transcription of CIITA and subsequent MHC class II transcription are suppressed by LPS stimulation, as shown in Fig 3, B through D, and Fig E3, B. These results are consistent with the data in Fig 1, although the effect is more striking, possibly because of the absence of electroporation for siRNA transfection in the experiments in Fig 3. Among 3 CIITA isoforms, pI-CIITA was the most strongly suppressed (Fig 3, C, and see Fig E3, B), which is consistent with the previous observation of LPS-stimulated BMDCs.²⁴ PU.1 also constitutively binds to pIII and pIV as well (see Fig E3, C). Western blotting showed that consistent with the decreased CIITA mRNA level, the CIITA protein level was also reduced by LPS stimulation in DCs. In contrast, the density of the PU.1 protein band was moderately increased and was accompanied by the appearance of an additional upper band in Western blotting of the whole-cell lysate of stimulated DCs (Fig 3, E). When the histone acetylation state of the pI promoter region was analyzed by using the ChIP assay, histones H3 and H4

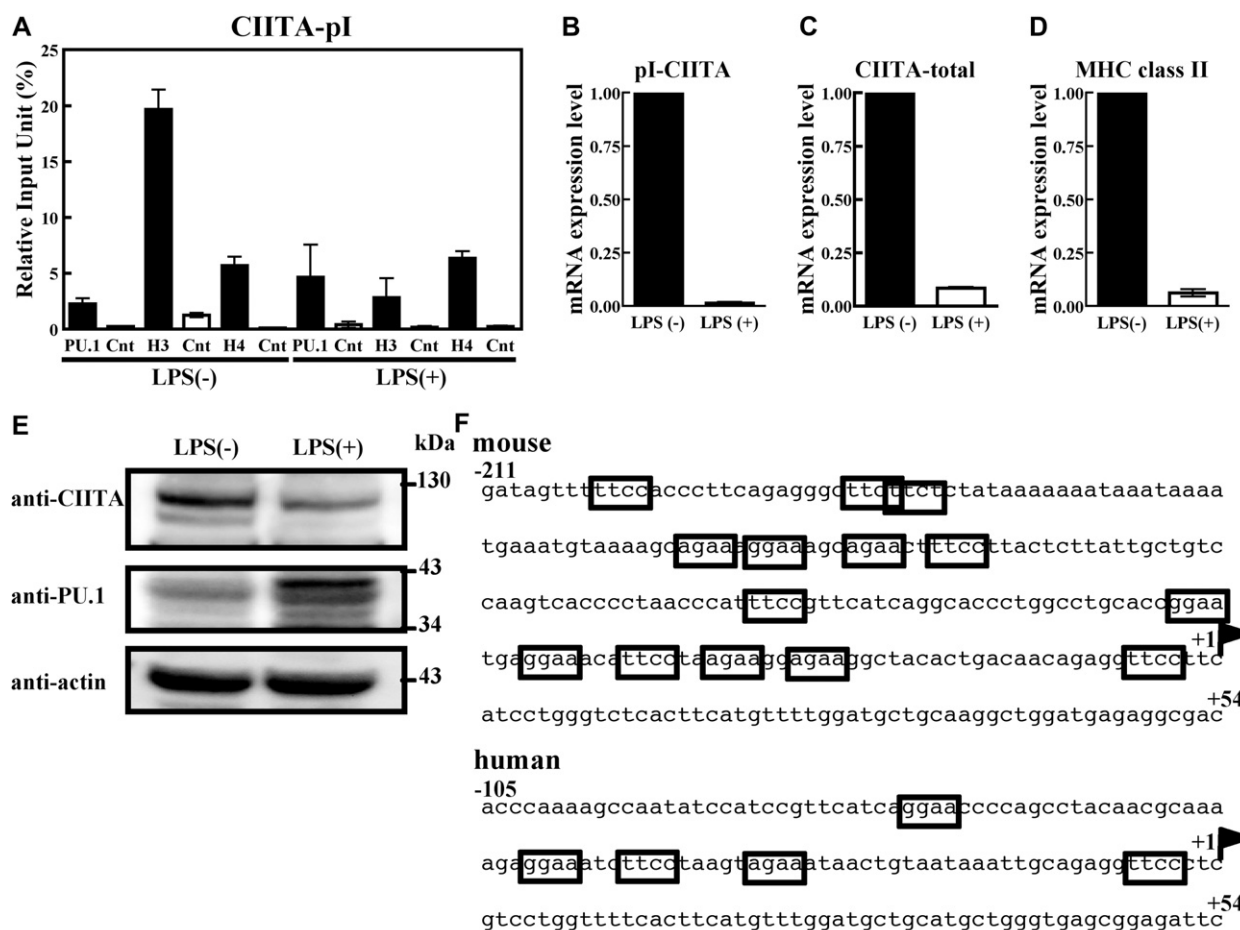


FIG 3. PU.1-binding and histone acetylation status of the CIITA-pI promoter in BMDCs. **A**, Quantitative analysis of PU.1 binding to the pI promoter and the acetylation status of histones H3 and H4 in the pI promoter by using ChIP assays. *LPS*(+), Preincubated with 1 μ g/mL LPS for 1 hour; *LPS*(-), without LPS. *Solid bar*, Specific antibody; *open bar*, control (*Cnt*). **B-D**, Quantitative real-time PCR analysis of the mRNA expression of CIITA driven by pI (Fig 3, B) total CIITA (Fig 3, C), and MHC class II (Fig 3, D). The expression level of each mRNA in BMDCs 24 hours after LPS stimulation (*LPS*(+)) is shown as a ratio versus the level in nonstimulated BMDCs (*LPS*(-)). In Fig 3, A-D, the results are expressed as means $\pm$ SEMs of 2 independent experiments with duplicate samples. **E**, Western blot analysis of protein levels of CIITA and PU.1 in LPS-stimulated (*LPS*(+)) or nonstimulated (*LPS*(-)) BMDCs. **F**, Nucleotide sequences of the murine and human CIITA-pI promoters. The consensus Ets family protein-binding sequence GGAA and the AGAA sequence to which PU.1 can bind²⁰ are boxed. The EICE sequence GGAANNGAAA to which the PU.1/IRF4 or PU.1/IRF8 heterodimers bind is underlined.²⁵ +1, Transcription start site.

were acetylated. LPS stimulation reduced the acetylation of H3 but not that of H4 (Fig 3, A). These results demonstrate that PU.1 constitutively binds to the pI promoter and that LPS-induced downregulation of CIITA transcription and translation and subsequent MHC class II mRNA transcription is reflected by H3 acetylation of the pI promoter region.

PU.1 transactivates the pI promoter through binding to the -74/-7 region

The ChIP assay targeting -96/-6 indicated that PU.1 is recruited to the CIITA-pI promoter region, in which several PU.1-binding sequences are detected (Fig 3, F).^{20,25} To evaluate the effects of PU.1 on CIITA-pI promoter activity, we performed a reporter assay using the CV-1 cells, which were previously shown to be useful for evaluation of the transactivating activity of hematopoietic cell-specific transcription factors in coexpression

analyses.^{14,16} Luciferase activity driven by the pI promoter region -564/+54 was significantly upregulated by the coexpression of exogenous PU.1. Other shorter promoter regions, from -443/+54 to -74/+54, which had deletions of various lengths at the 5' site, were still transactivated by PU.1 (Fig 4, A). When nucleotides upstream of -7 were deleted from the reporter plasmid, luciferase activity driven by the remaining -6/+54 region was not upregulated by exogenous PU.1. Fold increases in the transcription activity of -212/+54 and -239/+54 induced by exogenous PU.1 were high compared with those of -74/+54 and other constructs longer than -238/+54, suggesting the possible presence of additional PU.1-responsive elements in -212/-75 and of silencing elements in -564/-240. Regardless, the result shows a critical requirement of -74/-7 for the response to exogenous PU.1, suggesting that the pI promoter possesses a PU.1-responsive *cis*-element or *cis*-elements that reside within the region -74/-7.

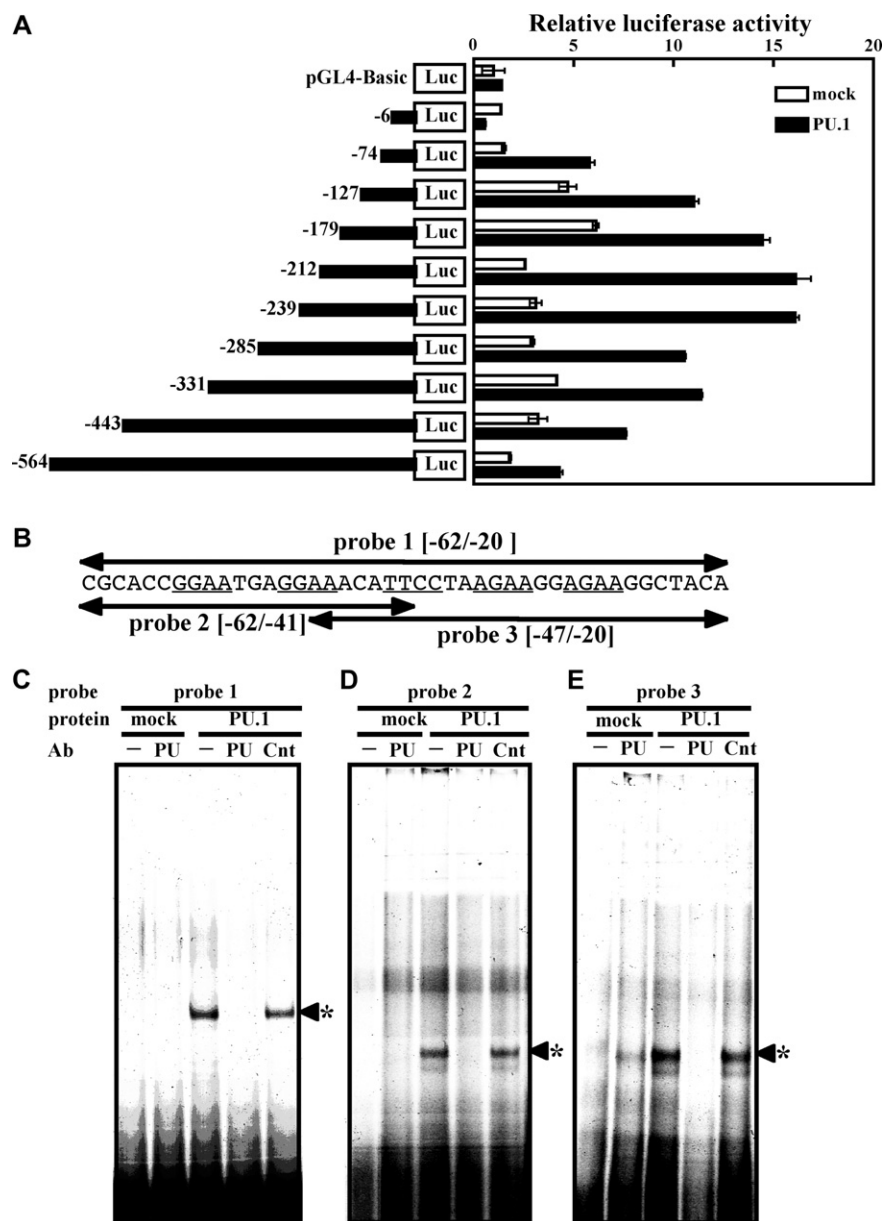


FIG 4. PU.1 transactivates the CLTA-pI promoter through direct binding to a region just upstream of the transcription start site. **A**, PU.1-coexpressing reporter assay. Relative luciferase activity is displayed as the ratio of luciferase activity versus that seen in cells cotransfected with pGL4-Basic and mock vector. Data represent means $\pm$ SEMs of 3 independent experiments performed with quadrant samples. **B**, Nucleotide sequences of the probes used for EMSAs (**C**, **D**, and **E**). EMSA data are typical results obtained in one of 3 independent experiments. *In vitro* transcription/translation was performed with the pCR3 empty vector (*mock*) or pCR3-PU.1 (*PU.1*) as a template. *-*, Without antibody (*Ab*); *PU*, with anti-PU.1 goat IgG antibody; *Cnt*, with control goat IgG. Specific bands corresponding to complexes of PU.1 and the probe are marked with an asterisk.

PU.1 directly binds the pI promoter through PU.1-binding sequences in the region -62/-20

The results of the reporter assay indicated the presence of a PU.1-responsive *cis*-element or *cis*-elements in the region -74/-7 of the pI promoter. An EMSA was performed with the -62/-20 pI region as a probe to determine whether PU.1 directly binds to the pI promoter because 3 typical Ets family-binding sequences (GGAA) and 2 AGAA sequences that are recognized by PU.1²⁰ are located between -56 and -27 in this -74/-7 region

(Fig 4, B). When the *in vitro*-translated PU.1 protein was mixed with the FITC-labeled double-stranded -62/-20 region (probe 1), a specific band appeared on an electrophoretic gel, which disappeared in the presence of the anti-PU.1 but not the control antibody (Fig 4, C, asterisk), suggesting that the PU.1 protein directly binds to the -62/-20 region. In EMSA with a nuclear extract from BMDCs (see Fig E4 in this article's Online Repository at www.jacionline.org), a band was observed that disappeared on addition of the anti-PU.1 antibody, but no other bands of lower

mobility were detected. This observation might suggest that PU.1 binds to this probe as a monomer but not as a dimer with IRF4 or IRF8. A specific PU.1 band was still observed in EMSA using 2 other probes, $-62/-41$ (probe 2) or $-47/-20$ (probe 3), as shown in Fig 4, D and E, respectively. These results indicate that PU.1 recognizes and binds to the $-62/-20$ region in the pI promoter through at least 2 different sites.

Identification of *cis*-enhancing elements in the pI promoter for PU.1 transactivation

EMSA were performed with competitive oligonucleotides to further identify PU.1-binding sequences in the $-62/-20$ region. When wild-type competitor (WT2) with the same sequence as probe 2 was added, the specific band shift corresponding to the complex of PU.1 and the probe DNA disappeared (Fig 5, B, left). This specific band remained in the presence of the mutant competitor Mut B, whereas addition of WT2 and Mut A reduced the band intensity, suggesting that PU.1 binds to GGAA at the 3' site ($-47/-44$). Similarly, in the case of probe 3, the specific band disappeared by addition of wild-type, Mut C, or Mut D, whereas this band remained in the presence of Mut E, indicating that PU.1 binds probe 3 through AGAA at the 3' site ($-30/-27$). Reporter plasmids carrying the $-74/+54$ CIITA-pI promoter with the same mutation as Mut A, B, or E (Fig 5, A) were generated to evaluate the effect of these identified PU.1-binding sites on promoter activity. The promoter with the Mut A mutation exhibited similar luciferase activity as the wild-type $-74/+54$ promoter, which was transactivated by coexpression of PU.1 (Fig 5, C, left). In contrast, luciferase activity driven by mutant promoters lacking either the PU.1-binding sequence GGAA ($-47/-44$, Mut B) or AGAA ($-30/-27$, Mut E) was low and was not transactivated by PU.1 similarly to the promoter-less control plasmid. The critical role of $-47/-44$ and $-30/-27$ in pI promoter activity was confirmed with a reporter assay using RAW264.7 monocyte cells that contain endogenous PU.1 (Fig 5, C, right). On the basis of these results, it is concluded that PU.1 transactivates the CIITA-pI promoter through direct binding to GGAA at $-47/-44$ and AGAA at $-30/-27$.

Effect of PU.1 on epigenetic regulation of the pI

It was recently reported that PU.1 is required for chromatin modification of the IL-9 promoter in a T_H subtype-specific manner.²⁶ Briefly, the acetylation state of histone H3 on the IL-9 promoter in T_H9 cells, which is higher than those of other T_H subtypes, is reduced to a half by PU.1-conditional knockout. Therefore the effects of PU.1 on the histone acetylation state of the pI promoter in DCs were analyzed by using ChIP assays. A significantly higher amount of chromosomal DNA around the pI region was immunoprecipitated by anti-acetylated H3 or anti-acetylated H4 antibodies compared with control IgG, even when control siRNA was introduced into BMDCs (Fig 5, D). PU.1 siRNA treatment decreased the degree of acetylation of histone in the pI promoter to approximately 50% in H4 acetylation with a possible reduction in H3 acetylation. One of the mechanisms explaining the relatively small effect of PU.1 siRNA on H3 acetylation degree might be the effect of electroporation, which could reduce the H3 acetylation around pI because H3 acetylation of control siRNA transfectants (Fig 5, D) is markedly lower than that of normal DCs (Fig 3, A), as seen in the difference

in mRNA levels observed between Fig 1 and Fig 3, B through D. Regardless, this result indicates that PU.1 is involved in the regulation of pI by affecting the degree of its histone acetylation state in addition to its role as a transactivator through *cis*-enhancing elements.

Effect of IRF4 and IRF8 siRNAs on MHC class II expression

PU.1 binds and transactivates target genes in 2 ways, as a monomer and as a heterodimer with IRF4 or IRF8. IRF4 and IRF8 siRNAs were introduced into BMDCs to evaluate the involvement of IRF4 and IRF8 in PU.1-mediated expression of pI-CIITA and MHC class II. Although both IRF4 and IRF8 siRNAs markedly reduced their target mRNA levels, transcripts of MHC class II and pI-CIITA were downregulated in PU.1 siRNA-transfected DCs but not in IRF4 or IRF8 siRNA-transfected DCs (Fig 6, A). Interestingly, PU.1 siRNA also reduced IRF4 mRNA levels. We used 2 additional PU.1 siRNAs to exclude the possibility of off-target effects of PU.1 siRNA and found that all 3 PU.1 siRNAs knocked down PU.1 mRNA. The levels of IRF4 mRNA and MHC class II mRNA in transfectants were decreased in parallel with the degree of PU.1 mRNA reduction, whereas the amount of IRF8 mRNA was not reduced by any PU.1 siRNA (Fig 6, B), suggesting that PU.1 transactivates the IRF4 promoter. Although additional siRNAs for IRF4 and IRF8 were similarly used to confirm the effect of IRF4/IRF8 siRNA (Fig 6, B), IRF4 siRNAs 2 and 3 and IRF8 siRNA 2 did not suppress the transcription of pI and MHC class II, as did PU.1 siRNAs 1, 2, and 3 (IRF4 siRNA 2 might not be functional). The marked reduction in mRNA levels for MHC class II, pI, and IRF4 in the IRF8 siRNA 3 transfectant might be due to a nonspecific effect because IRF8 siRNAs 1 and 2 did not reduce these mRNA levels, even though their knockdown of IRF8 was similar to that of siRNA 3. Additionally, BMDCs transfected with IRF4 or IRF8 siRNAs exhibited a similar expression level of cell-surface MHC class II as seen on control siRNA transfectants, whereas MHC class II expression was markedly downregulated on PU.1 siRNA-transfected DCs (Fig 6, C and D). These results suggest that IRF4 and IRF8 are not involved in PU.1-mediated regulation of pI-CIITA and MHC class II expression.

DISCUSSION

MHC class II expression is an important hallmark of professional antigen-presenting cell DCs. Cell type-specific expression of MHC class II is restricted by the transcription cofactor CIITA, the expression of which is regulated by 3 promoters in its gene. Although the first promoter, pI, is predominantly used for CIITA expression in DCs, the molecular mechanism of pI function is largely unknown, which is in contrast to that of the other 2 promoters, pIII and pIV, the structure and regulation of which in some lineages, including lymphoid cells and nonhematopoietic cells, have been vigorously studied.

The studies with PU.1-deficient mice indicated that PU.1 is necessary for all DCs¹⁰ or myeloid-derived DCs.¹¹ However, because PU.1 regulates the gene expression of several developmentally important receptors, including GM-CSF²⁷ and Flt3,²⁸ the research with PU.1-deficient mice had hardly clarified the involvement of PU.1 in the regulation of each DC-specific gene. In our recent analysis of the role of PU.1 in CD80 and CD86

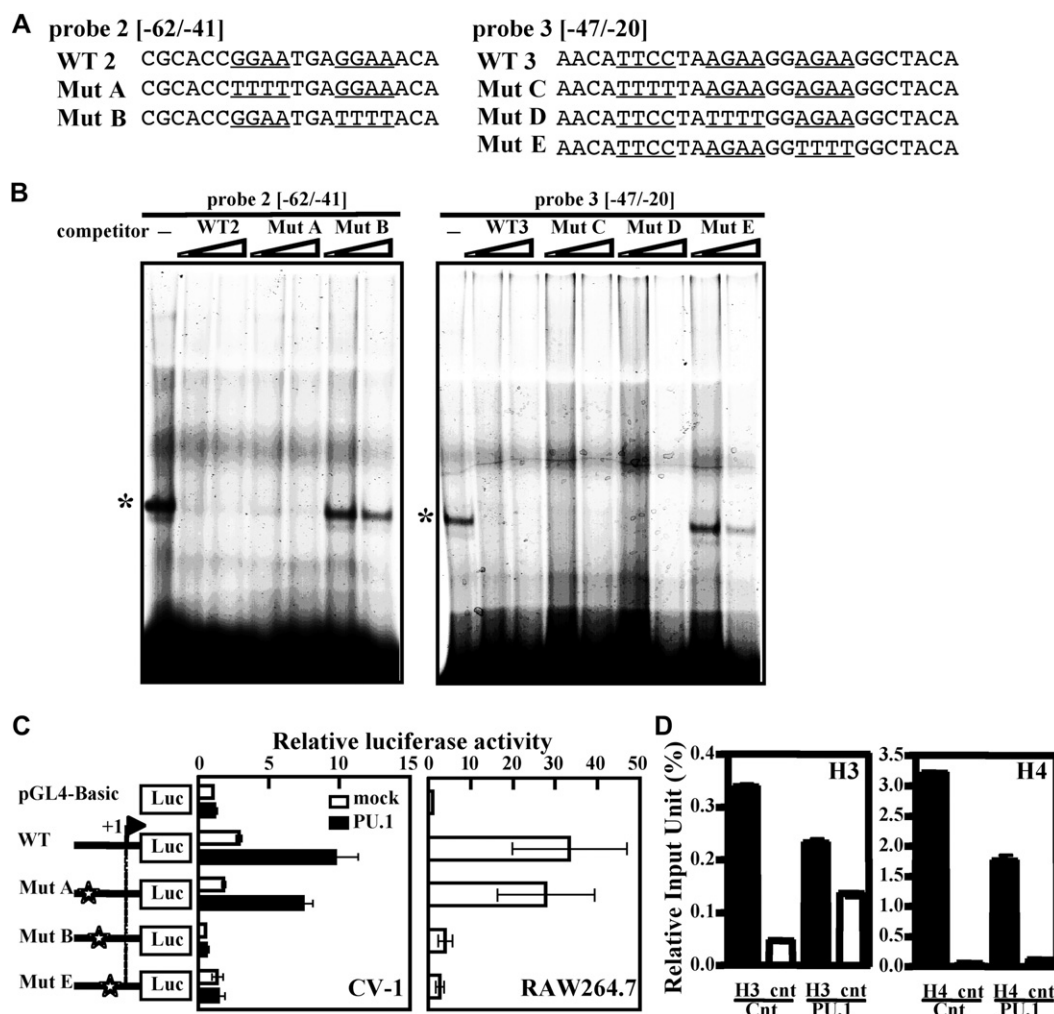


FIG 5. Identification of *cis*-enhancing elements in the CIITA-pI promoter that mediate transactivation by PU.1. **A**, Nucleotide sequences of competitive oligonucleotides used for EMSAs. WT2 and WT3 are self-competitors, and Mut A, B, C, D, and E are mutant competitors lacking one of the GGAA or AGAA sequences. **B**, EMSAs with competitors to identify PU.1-binding sites. An excess amount of competitor together with a 30- or 60-fold molar concentration of probe DNA was added to each protein-DNA mixture. Specific bands corresponding to complexes of PU.1 and the probe are marked with an asterisk. A typical result of 3 independent experiments is shown. **C**, Reporter assay with mutant promoters to identify *cis*-enhancing elements. WT, pGL4 reporter plasmid carrying the -74/+54 region of the CIITA-pI promoter; Mut A, B, and E, reporter plasmids carrying the same promoter region but mutated as in Fig 5, A. *Left*, Coexpression analysis with CV-1 cells; *right*, reporter assay without exogenous PU.1 with RAW264.7 as the host cell. Data represent means $\pm$ SEMs of 3 independent experiments with quadrant samples. **D**, Role of PU.1 in the histone acetylation status of the pI promoter. The histone H3 (*left*) and H4 (*right*) acetylation status of the pI promoter was analyzed by using a ChIP assay. Cnt, Control siRNA-introduced DCs; PU.1, PU.1 siRNA-introduced DCs. H3, Anti-acetyl H3 rabbit IgG; H4, anti-acetyl H4 rabbit IgG; Cnt, control rabbit IgG. The results are expressed as means $\pm$ SEMs of 2 independent experiments with triplicate samples.

promoter activity in DCs, we found that PU.1 siRNA suppressed MHC class II expression on DCs.¹⁴ This observation prompted us to examine the role of PU.1 in CIITA-pI promoter activity in DCs. In the present study ChIP assays, reporter assays, and EMSA analyses revealed that PU.1 constitutively binds CIITA-pI and transactivates CIITA-pI through direct binding to GGAA at -47/-44 and AGAA at -30/-27. The histone acetylation state of the pI promoter reflected the transcription levels of CIITA and MHC class II, which are high in immature DCs and are shut down in mature DCs. PU.1 is involved in regulation of the basal expression of CIITA and MHC class II in both immature and mature DCs. As expected, PU.1 knockdown by siRNA suppressed

pI-CIITA transcription and subsequent MHC class II expression and function. An effect of IRF4 and IRF8 siRNAs on CIITA-pI and MHC class II expression was not observed, indicating that PU.1 regulates CIITA-pI as a monomeric transcription factor but not as a heterodimer. Furthermore, PU.1 siRNA introduction into DCs showed that PU.1 not only functions as a transcription factor but also modulates the histone acetylation state of the CIITA-pI promoter region. On the basis of these observations, we conclude that PU.1 is a key molecule for regulation of the expression of MHC class II through pI-CIITA transcription.

It is well known that PU.1, along with other transcription factors, including STAT1, E47, IRF4, AML2/3, and CREB,

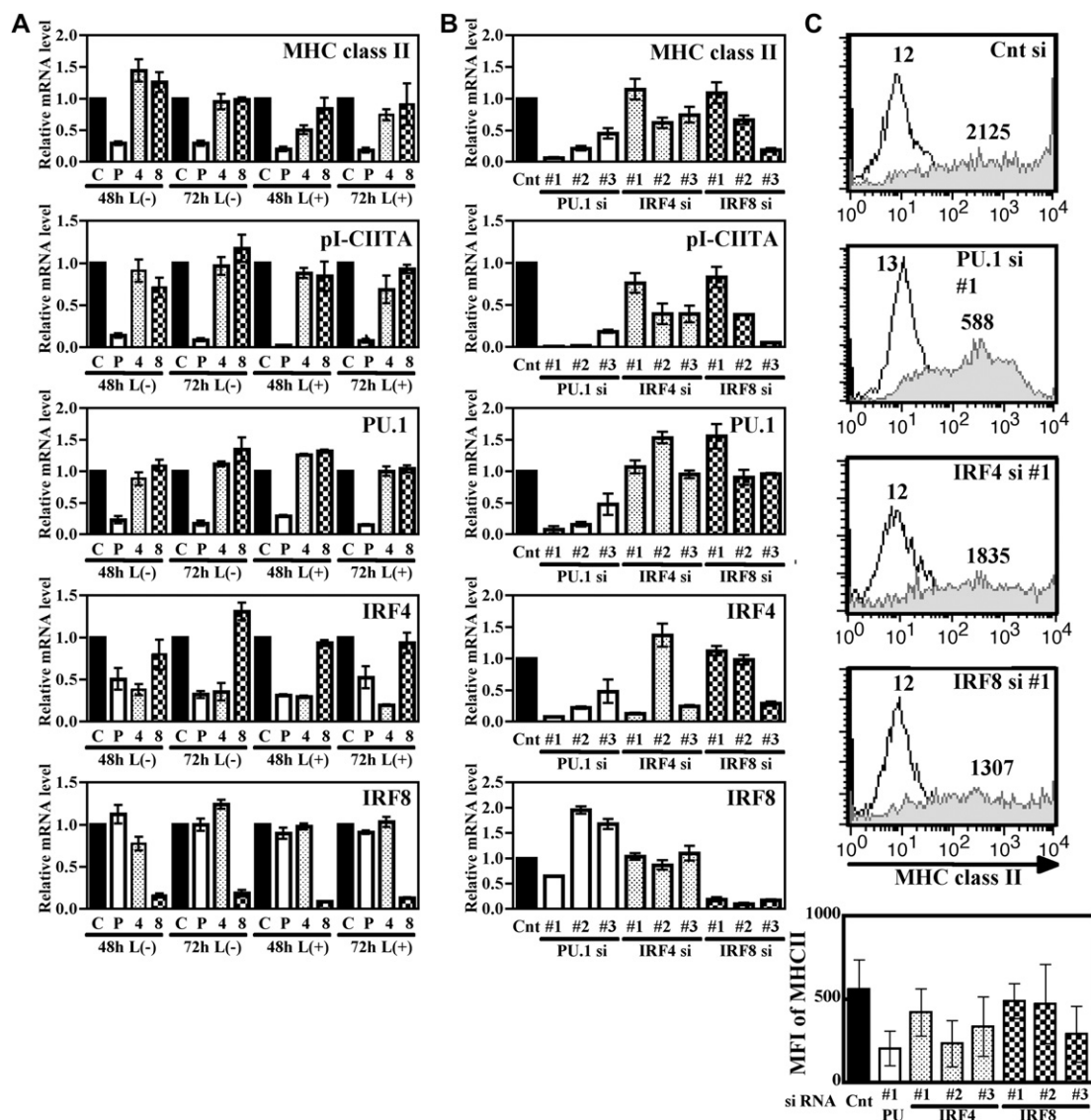


FIG 6. Effects of siRNA against IRF4 and IRF8 on MHC class II expression. **A**, mRNA expression levels of MHC class II (top panel), pI-CIITA (second panel), PU.1 (third panel), IRF4 (fourth panel), and IRF8 (bottom panel) in cells transfected with control siRNA (C), PU.1 siRNA (P), IRF4 siRNA (4), and IRF8 siRNA (8) and treated with (L+) or without (L-) LPS. Cells were harvested after 48 or 72 hours of culture after siRNA introduction. For LPS stimulation of DCs, 1 μ g/mL LPS was added at 24 hours before harvest. Relative mRNA levels are displayed as a ratio to those seen in each control siRNA-introduced DC. The results are expressed as means $\pm$ SEMs of 3 independent experiments with duplicate samples. **B**, The effect of 2 siRNAs of different sequences (2 and 3) in addition to sequence 1 corresponding to siRNA in Fig 6, A, were used with the same condition as that of 48 hours of L(-) in Fig 6, A. **C**, Flow cytometric analysis of the expression level of MHC class II on siRNA-introduced DCs. Shaded histograms, phycoerythrin-conjugated anti-I-A^d murine IgG2b antibody; unshaded histograms, phycoerythrin-conjugated murine IgG2b. The numbers on each histogram indicate the mean fluorescence intensity. A representative result of 3 independent experiments is shown. The data in the graph are means $\pm$ SEMs of mean fluorescence intensity values obtained from 2 experiments with duplicate samples.

positively regulates the CIITA-pIII promoter.^{1,5,22,29} PU.1 is also involved in CIITA-pIII activation through binding to a distal element that modifies chromatin conformation, which is necessary for CIITA expression.²³ In our previous studies the expression of some monocyte/DC-specific genes, including MHC class II, was induced by exogenously expressed PU.1 in mast cells.^{13,30} PU.1 bound to all of the CIITA promoters in these cells, and the histones in these promoters were highly acetylated after PU.1 overexpression, although only the pIV-driven CIITA transcript

was detected.³⁰ In the present study PU.1 regulated pI-CIITA by means of transactivation of the promoter and through enhancement of the promoter histone acetylation state. Therefore PU.1 participates in the expression of CIITA through multiple effects on the CIITA gene promoter.

The present study demonstrated that the effects of LPS stimulation on the expression and localization of PU.1 are complicated. LPS stimulation of DCs reduces the PU.1 mRNA level, but in contrast, the PU.1 protein level is increased, whereas

PU.1 binding to the pI promoter is constitutively observed. Thus the transcription of PU.1 does not reflect the total amount of PU.1 protein, and the recruitment of PU.1 to the chromosome does not parallel the total amount of PU.1 protein. Because LPS stimulation transiently upregulated the PU.1 protein level in mast cells in our previous study, an increase in PU.1 protein level in response to LPS stimulation might be common in some lineages.¹³ Although the mechanism that controls the dynamics of PU.1 has not yet been clarified, posttranslational modification could be one regulatory mechanism that might help to explain these complicated observations. Phosphorylation is a candidate modification of PU.1 because it has been recently reported that PU.1 phosphorylation promotes DC differentiation.³¹ Thus phosphorylation might explain the additional upper band of PU.1 observed in our Western blot analyses of LPS-stimulated DCs (Fig 3, E) and that observed in a previous study of LPS-stimulated mast cells.¹³ Alternatively, ubiquitination might be another candidate modification because MHC class II protein survival is prolonged by blocking of MHC class II ubiquitination in activated DCs, even when MHC class II transcription is suppressed.³² As for the histone modification function of PU.1, although the role of PU.1 on the acetylation degree of H3 and H4 in pI was demonstrated by using siRNA experiments, the degree of H3 acetylation was reduced by LPS stimulation, even in continuous but variable binding of PU.1 to pI. Therefore the presence of PU.1 might be necessary but not sufficient for full acetylation of H3. Further detailed analysis will be required to fully understand PU.1-mediated gene expression in DCs.

Although knockdown of PU.1 reduced the pI-CIITA mRNA and protein expression, PU.1 remained bound to the pI promoter in LPS-stimulated DCs, the pI-CIITA mRNA level of which was quite low compared with that of unstimulated DCs. In a previous study Landmann et al²⁴ demonstrated that transcriptional repression of CIITA in mature DCs is mediated by histone deacetylation of the entire *MHC2TA* regulatory region, which was partly confirmed in the present ChIP assay as a decrease in H3 acetylation state (Fig 3, A). Therefore PU.1 might play a physiologic role in the maintenance of CIITA and subsequent MHC class II expression in a nonstimulated/immature state but not reflect an increase in MHC class II expression after cellular activation in DCs.

During preparation of this article, a study regarding the involvement of positive regulatory domain 1 and IRF8/PU.1 in human CIITA-pI promoter activity in monocyte-derived DCs was published.³³ In that study it was reported that the PU.1 monomer bound to the human CIITA-pI promoter region through 2 candidate Ets motifs just upstream of the transcription start site and that the IRF8/PU.1 dimer bound to the 5'-untranslated region through an EICE sequence. Although the Ets motifs are highly conserved between human and murine genes (as were the Ets motifs detected in the present study, which were mutated [Mut B and Mut C] in Fig 5), the typical PU.1/IRF4- or PU.1/IRF8-binding sequence GGAANNAGAAA that is present in the 5'-untranslated region of the human pI gene is not found in the murine gene (Fig 3, F). In the present study no effect of IRF8 siRNA on the mRNA levels of pI-CIITA and MHC class II or on the cell-surface protein level of MHC class II was detected, which is in contrast to the critical role of PU.1 in MHC class II expression. Therefore the involvement of IRF8 might differ between human subjects and mice, between DC subtypes, or both. There have been many studies regarding the effect of IRF4 or IRF8 on DC development. However, a significant effect of IRF4 or IRF8 siRNAs

on MHC class II expression was not detected under the experimental conditions of the present study (Fig 6, C), even though we paid careful attention to offset effects, and nonspecific effects of the siRNA used. The knockdown of target mRNA at the terminus of BMDC development by siRNA might cause a different effect than the knockout of the target gene throughout BMDC development. Therefore a further detailed analysis of the role of IRF4/IRF8 will be required. IRF4 and IRF8 might not function as PU.1 partners in PU.1 heterodimer, but there might also be cross-talk between PU.1 and IRF4, IRF8, or both. Such cross-talk is suggested by the data showing that PU.1 siRNA reduced IRF4 mRNA levels, indicating that IRF4 expression is under the control of PU.1. We will perform further study to determine the involvement of PU.1 and IRF4 and IRF8 in various subtypes of human DCs in the near future. Regardless of the relationship between PU.1 and IRF4/IRF8, it is commonly observed in both human and murine DCs that PU.1 transactivates the pI promoter through the 2 motifs GGAA and AGAA, which are highly conserved between human and murine genes.

CIITA promoter use is different between pDCs and cDCs: briefly, pIII is used in pDCs, whereas pI is used in cDCs.⁶ Although transcription factors regulating pIII in pDCs have not been identified, considering that PU.1 regulates pIII in B cells²² and activated T cells⁵ and that PU.1 is expressed in pDCs,³⁴ pIII might be transactivated by PU.1 in pDCs as well. Therefore PU.1 might be a common target of factors that control MHC class II and CIITA expression in pDCs and cDCs. It will be interesting to understand the mechanism by which PU.1 distinguishes CIITA promoters depending on cell type. Further detailed analyses of the regulation of the CIITA-pI promoter by PU.1 and identification of other molecules regulating the CIITA-pI promoter will be required to reveal the mechanism of CIITA expression in DCs.

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Clinical implications: PU.1 plays a critical role in MHC class II expression in DCs through the regulation of CIITA expression. Briefly, PU.1 directly binds to and transactivates the CIITA-pI promoter and is involved in chromosomal modification.

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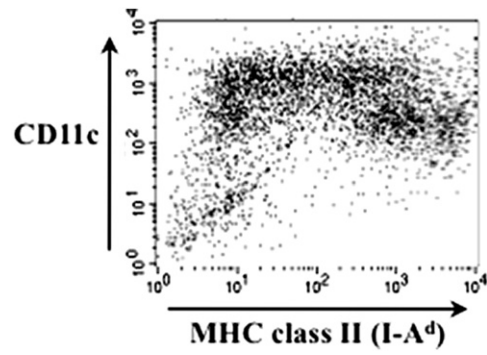


FIG E1. Flow cytometric analysis of BMDCs. Cells generated from whole bone marrow cells after 10 days of culture were confirmed as BMDCs by means of staining with FITC-conjugated anti-I-A^d and phycoerythrin-conjugated anti-mouse CD11c.

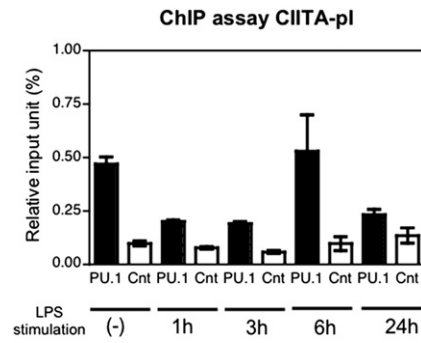


FIG E2. Monitoring of PU.1 binding to the pl promoter over time after LPS stimulation by using the ChIP assay. *Solid bars*, Anti-PU.1 antibody; *open bars*, control (*Cnt*) antibody. The degree of PU.1 binding to the pl promoter at 1, 3, 6, and 24 hours after LPS stimulation was measured.

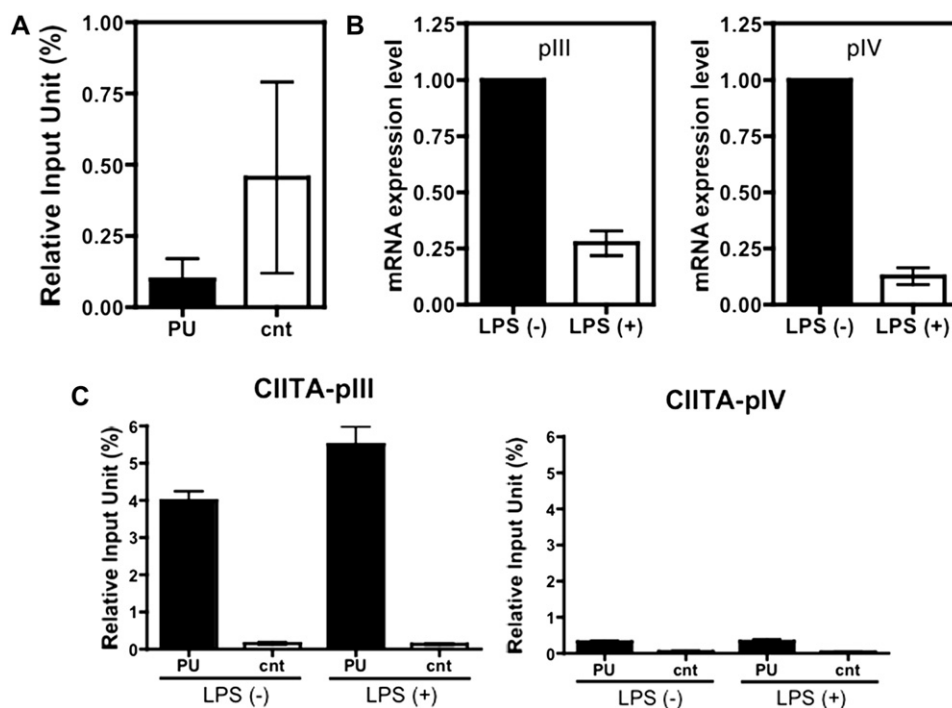


FIG E3. **A**, ChIP assay of PU.1 binding to the pI promoter in mast cells. The immunoprecipitate of murine bone marrow–derived mast cells obtained by using an anti-PU.1 antibody (*PU*) or control antibody (*cnt*) was amplified by using primers targeting the pI promoter region. **B**, The mRNA level of CIITA driven from the pIII and pIV promoters in BMDCs 24 hours after LPS stimulation (*LPS* [+], *open bars*) is shown as a ratio versus the level seen in nonstimulated BMDCs (*LPS* [–], *solid bars*). **C**, ChIP assay of PU.1 binding to the pIII and pIV promoters in BMDCs. The immunoprecipitate of BMDCs obtained by using anti-PU.1 antibody (*PU*) or control antibody (*cnt*) was amplified by using primers^{E1} targeting the promoter regions of pIII (*left*) and pIV (*right*). *LPS* (–), Without LPS stimulation; *LPS* (+), 1 hour after LPS stimulation.

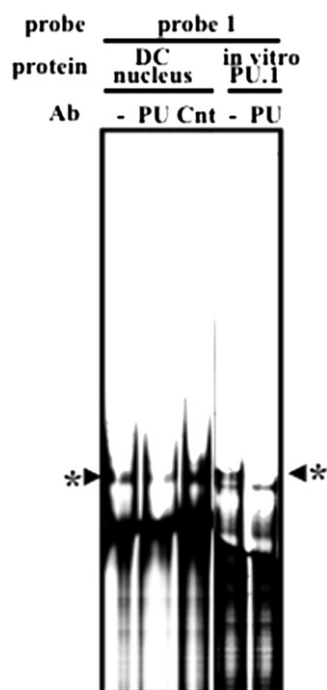


FIG E4. An EMSA with nuclear extracts of BMDCs. An EMSA was performed with nuclear extracts of BMDCs (DC nucleus) or an *in vitro* transcription/translation reaction mixture using PU.1 cDNA as template (*in vitro* PU.1). Specific bands corresponding to complexes of PU.1 and the probe are marked with an *asterisk* and an *arrowhead*. The mobility of the band obtained with *in vitro* PU.1 and the probe is slightly lower than that obtained with PU.1 in the DC nucleus and the probe because *in vitro* PU.1 is fused with 2× Flag-tag at the N-terminus, which increases its molecular weight compared with endogenous PU.1. Nuclear proteins were extracted from BMDCs, as described previously.^{E2,E3}

TABLE E1. Nucleotide sequences of synthesized oligonucleotides for reporter plasmid construction

mCIITA-pI-F1	5'- CCAGATCT TGTGAGCCACCATGCTGGC TCCAGC-3'
mCIITA-pI-F2	5'- CCAGATCT CGGTGCTGGGGATTGAACC CAAGGC-3'
mCIITA-pI-R	5'- GGAAGCTT GTCGCCTCTCATCCAGCCT TGCAGC-3'
mCIITA-pI-443-F	5'-GCAGTCTTGTGT AGATCT GGGAAGG CTTGA-3'
mCIITA-pI-443-R	5'-TCAAGCCTTCCC AGATCT ACACAAG ACTGC-3'
mCIITA-pI-285-F	5'-AGATGCAAAACC AGATCT TCCCCAA GTGAG-3'
mCIITA-pI-285-R	5'-CTCACTTGGGGA AGATCT GGTTTTG CATCT-3'
mCIITA-pI-239-F	5'-TTTTTTTTTAAC AGATCT ACTAATCT GTAA-3'
mCIITA-pI-239-R	5'-TTACAGATTAGT AGATCT GTAAAAA AAAA-3'
mCIITA-pI-212-F	5'-AATAATCTGCCC AGATCT GATAGTTT TTCC-3'
mCIITA-pI-212-R	5'-GGAAAACTCTC AGATCT GGGCAGA TTATT-3'
mCIITA-pI-179-F	5'-TCAGAGGGCTTC AGATCT ATAAAAAA ATAA-3'
mCIITA-pI-179-R	5'-TTATTTTTTTAT AGATCT GAAGCCCTC TGA-3'
mCIITA-pI-127-F	5'-AGAAAGGAAAGC AGATCT TTCCCTTAC TCTT-3'
mCIITA-pI-127-R	5'-AAGAGTAAGGAA AGATCT GCCTTCC TTTCT-3'
mCIITA-pI-74-F	5'-ACCCATTTCCGT AGATCT GGCACCCT GGCC-3'
mCIITA-pI-74-R	5'-GGCCAGGGTGCC AGATCT ACGGAAA TGGGT-3'
mCIITA-pI-6-F	5'-TACACTGACAAC AGATCT TCCTTCATC CTG-3'
mCIITA-pI-6-R	5'-CAGGATGAAGGA AGATCT GTTGTCAG TGTA-3'
pI-74-MutA-F	5'-ACCCTGGCCTGCACC TTTT TGAGGAAA CATTCT-3'
pI-74-MutA-R	5'-AGGAATGTTTCTCTAAAAAGGTGCAGG CCAGGGT-3'
pI-74-MutB-F	5'-CCTGCACCGGAATG ATTT TACATTCTTA AGAAGG-3'
pI-74-MutB-R	5'-CCTTCTTAGGAATGTAAATCATTCCGG TGCAGG-3'
pI-74-MutE-F	5'-ACATTCTTAAGAAGG TTTT TGGCTACAC TGACAAC-3'
pI-74-MutE-R	5'-GTTGTCAGTGTAGCCAAAACCTTCTTAG GAATGT-3'

*Bgl*II or *Hind*III recognition sequences are shown in boldface, and mutated sequences are shown in italics.

mCIITA, Murine CIITA.