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Downregulation of PU.1 Leads to Decreased Expression of Dectin-1 in Alveolar Macrophages during *Pneumocystis* Pneumonia▽

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Dectin-1 is an important macrophage phagocytic receptor recognizing fungal β-glucans. In this study, the mRNA levels of the Dectin-1 gene were found to be decreased by 61% in alveolar macrophages (AMs) from *Pneumocystis*-infected mice. The expression of Dectin-1 protein on the surface of these cells was also significantly decreased. By fluorescence *in situ* hybridization, mRNA expression levels of the transcription factor PU.1 were also found to be significantly reduced in AMs from *Pneumocystis*-infected mice. Electrophoretic mobility shift assay showed that PU.1 protein bound Dectin-1 gene promoter. With a luciferase reporter gene driven by the Dectin-1 gene promoter, the expression of the PU.1 gene in NIH 3T3 cells was found to enhance the luciferase activity in a dose-dependent manner. PU.1 expression knockdown by small interfering RNA (siRNA) caused a 63% decrease in Dectin-1 mRNA level and 40% decrease in protein level in AMs. Results of this study indicate that downregulation of PU.1 during *Pneumocystis* pneumonia leads to decreased expression of Dectin-1 in AMs.

Pneumocystis pneumonia (PCP) is a common opportunistic disease among immunocompromised individuals, such as in patients infected with HIV (27). Although the incidence of PCP has declined following the introduction of highly active antiretroviral therapy, it remains an important cause of morbidity and mortality in HIV-infected patients (20). Understanding the host defense mechanisms against *Pneumocystis* infections is crucial for the development of effective treatments and prophylaxis for PCP. Many studies have shown the importance of CD4 and CD8 T lymphocytes in adaptive immunity (11, 28), but the roles of cells such as alveolar macrophages (AMs) in innate immunity against the infection are not fully understood.

AMs are key effector cells in the first line of defense against pulmonary pathogens (8). Rats depleted of AMs are susceptible to *Pneumocystis* infection (19). The ability of AMs to recognize and respond to an invading pathogen is essential for controlling the infection. For this purpose, AMs express receptors which recognize pathogen-associated molecular patterns (PAMPs). The major carbohydrates of the fungal cell wall β-glucan and mannan are PAMPs of *Pneumocystis* organisms.

Dectin-1 is a type II transmembrane protein which binds β-1,3 and β-1,6 glucans. It has been shown to be involved in AM recognition, nonopsonic phagocytosis, and killing of *Pneumocystis* organisms both *in vitro* and *in vivo*. RAW 264.7 macrophages overexpressing Dectin-1 bind *Pneumocystis* organisms at higher levels than control cells (31). A fusion protein consisting of the extracellular domain of Dectin-1 linked to the Fc portion of murine IgG1 increases macrophage-dependent killing of *Pneumocystis* (25). Two recent reports show that Dectin-1-deficient mice have compromised clearance of *Pneumocystis* (26) and *Candida albicans* (32), as well as attenuated macrophage inflammatory responses to these organisms. These studies suggest a critical role of Dectin-1 in the control of *Pneumocystis* infection. In this study, we found that the expression levels of Dectin-1 in AMs during PCP were decreased due to reduced expression of the transcription factor PU.1.

MATERIALS AND METHODS

Mouse model of PCP. C57BL/6 mice were obtained from Harlan (Indianapolis, IN). All mice used in this study were female, 6 to 8 weeks of age, and 18 to 20 g in weight. Animal studies were approved by the Indiana University Animal Care and Use Committee and carried out under the supervision of veterinarians. Immunosuppression of mice was achieved by intraperitoneal injection of 0.3 mg anti-CD4 monoclonal antibody (MAb; clone GK1.5; Harlan, Indianapolis, IN) once a week until the mice were sacrificed. Three days after initial injection, mice were transtracheally instilled with 2×10^6 *Pneumocystis* organisms in 50 μl sterile phosphate-buffered saline (PBS). *Pneumocystis* organisms were obtained from heavily infected lungs of dexamethasone-immunosuppressed mice and isolated as previously described (38). Immunosuppressed, uninfected mice were used as controls. Tetracycline was added to the drinking water (9.2 g/liter) to prevent bacterial infections in the mice.

AM isolation. AMs were isolated by bronchoalveolar lavage (BAL). Mice were anesthetized by intramuscular injection of 0.02 ml ketamine cocktail (ketamine hydrochloride, 80 mg/ml; acepromazine, 1.76 mg/ml; atropine, 0.38 μg/ml) and then sacrificed by cardiac exsanguination. Lungs were lavaged with 1 ml sterile saline at a time through an intratracheal catheter until 10 ml of BAL fluid was recovered from each mouse as described previously (38). The BAL fluid was centrifuged at $300 \times g$ for 10 min to pellet AMs. The pelleted cells were suspended and cultured in a 12-well culture plate at 37°C with 5% CO_2, at a concentration of 3×10^5 cells per well in 1 ml RPMI 1640 medium (Sigma

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Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum, 1 mM pyruvate, 1% nonessential amino acids, 14 mM glucose, 17.9 mM $NaHCO_3$, 10 mM HEPES, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. After 1 h of incubation, nonadherent cells were washed off with PBS.

RNA isolation and real-time RT-PCR. Total RNA was isolated from AMs using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA concentration and purity were determined by spectrophotometry. cDNA was synthesized from the total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and random primers; 0.2 μg of total RNA was used for each reaction with a total reaction volume of 20 μl. The reaction mixtures were incubated at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. Two microliters of each cDNA product was used for quantitative PCR analysis. Real-time reverse transcription-PCR (RT-PCR) for Dectin-1 and PU.1 was performed using the Assays-on-Demand gene expression kits containing two unlabeled PCR primers and a 6-carboxyfluorescein (FAM)-labeled TaqMan probe (Applied Biosystems, Foster City, CA) on a Smartcycler (Cepheid, Sunnyvale, CA). Since the expression of the ribosomal protein S8 (RPS8) is not affected by *Pneumocystis* infection, RPS8 mRNAs were assayed in an identical manner as an internal control as described previously (38). Data from each sample were normalized to RPS8 gene expression and were shown as fold increase or percentage relative to control cells.

Flow cytometry of Dectin-1 expression on AMs. AMs were lavaged from uninfected or *Pneumocystis*-infected mice. After blocking in 5% bovine serum albumin for 1 h, the cells were stained with phycoerythrin (PE)-labeled macrophage-specific antibody Mac3 (R&D), followed by fluorescein isothiocyanate (FITC)-labeled anti-Dectin-1 monoclonal antibody (R&D) for 1 h. Separate sets of cells were stained with PE- or FITC-labeled IgG isotype control antibody. After being washed twice with 4 ml PBS, the stained cells were analyzed with a BD FACScan flow cytometer (BD Biosciences). Gate R1 was used to count Mac3-positive cells (macrophages), which included less than 0.01% of cells stained with PE-labeled isotype control antibody. Dectin-1 surface expression levels were analyzed in the R1-gated cells. Gate R2 was used to count Dectin-1-positive cells, which included less than 0.01% of cells stained with FITC-labeled isotype antibody.

***In situ* hybridization of PU.1 in AMs.** The fluorescent probes were made by *in vitro* transcription using the FISH Tag RNA Multicolor kit (Invitrogen). A 256-bp fragment of the PU.1 cDNA was first amplified by PCR using the following primers: sense primer, *AAG*-^{347}*CTT*CCCTGAGAACCACTTCA^{366}; antisense primer, *GGA*-^{602}*TCC*ATCAGACACCTCCAG^{585}, where italicized nucleotides are recognition sequences of HindIII and BamHI, respectively. The PCR product was cloned into pCRII-TOPO vector. Recombinant plasmids containing the 256-bp inserts of the PU.1 fragment in both orientations were selected and verified by sequencing. To transcribe the PU.1 fragment, the plasmid for making the sense RNA probe was linearized by HindIII digestion, and that for making the antisense RNA probe was linearized by BamHI digestion. The linearized plasmid DNA was purified and used for *in vitro* transcription with T7 RNA polymerase and Alexa Fluor 488-labeled UTP provided in the kit.

For *in situ* hybridization, AMs (5×10^5) from mice with or without PCP were cytospun onto a microscopic slide, fixed with 4% paraformaldehyde in PBS for 20 min, and permeabilized with 0.1% Triton X-100 also in PBS for 10 min at room temperature. After proteinase K digestion, cells were prehybridized overnight at 55°C in hybridization buffer (50% formamide, 5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 100 μg/ml fragmented salmon testis DNA, 50 μg/ml heparin, 0.1% Tween 20 in phosphate-buffered Tris), followed by hybridization with fluorescence-labeled RNA probe (1 μg/ml) in hybridization buffer at 55°C for 16 h. The slides were washed three times with PBS and mounted with SlowFade Gold antifade reagent (Invitrogen). The fluorescence images were taken with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY).

Western blotting. AMs (1×10^6 cells per group) from uninfected or *Pneumocystis*-infected mice were lysed with a protein extraction buffer (RIPA buffer: 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate) containing 1% protease inhibitor cocktail (Sigma). Protein concentration was determined by using the RC DC protein assay kit (Bio-Rad). Equal amounts of protein from each sample were electrophoresed in a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. PU.1 protein levels were determined by Western blotting using anti-PU.1 antibody (Cell Signaling Technology, Danvers, MA) and were normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Densitometry analysis of results on X-ray films was performed by using ImageJ software (http://rsb.info.nih.gov/ij/).

EMSA. Two double-stranded, biotin-labeled oligonucleotides corresponding to nucleotide positions −454 to −437 and −109 to −92 of the Dectin-1 gene, each containing a different PU.1 binding site located in the Dectin-1 promoter, were used for electrophoretic mobility shift assay (EMSA). Biotin-labeled consensus double-stranded PU.1-binding oligonucleotide probe (5′-TGAAAGAG GAACTTGGT-3′) was used as the positive control. The recombinant PU.1 protein was a gift from Mark Kaplan (Indiana University School of Medicine). PU.1 binding was determined by using the LightShift chemiluminescent EMSA kit (Pierce, Rockford, IL). Briefly, 5 ng of PU.1 protein was incubated with 20 fmol of biotin-labeled oligonucleotides in a buffer containing 2 mM HEPES, 15 mM NaCl, and 5% glycerol at 4°C overnight. Unrelated protein EBNA was used as negative control. The reaction mixture was then electrophoresed in a 6% polyacrylamide gel in 0.5× Tris-boric acid-EDTA buffer. DNA in the gel was transferred to a nylon membrane, followed by UV cross-linking. The membrane was incubated with streptavidin-horseradish peroxidase (HRP) conjugate, and biotin-labeled DNA was detected by incubating the membrane with chemiluminescent HRP substrate and exposing the membrane to an X-ray film. Cold competition was carried out by adding 10 pmol of unlabeled PU.1-binding oligonucleotides (5′-TGAAAGAGGAACTTGGT-3′) to the reaction mixture.

Plasmid constructs and luciferase assay. The 1.5-kb DNA fragment containing the Dectin-1 gene promoter was amplified by PCR from mouse genomic DNA using the primer set 5′-ATAAGGGCTTTGTTCCTAAGGACT-3′ and 5′-TATAAAATAACTGCCAGGATGAGG-3′. Plasmid pGL-Dectin1 was constructed by cloning of the amplified fragment into the firefly luciferase reporter vector pGL4.14 (Promega). The PU.1-expressing plasmid pFLAG-PU.1 was constructed in the same manner using pFLAG vector (Promega). The plasmid constructs were verified by sequencing. NIH 3T3 cells were cotransfected with 1.0 μg pGL-Dectin1 and various amounts of pFLAG-PU.1. To normalize for transfection efficiency, the cells were cotransfected with *Renilla* luciferase reporter plasmid pRL-TK (Promega). Plasmids were introduced into NIH 3T3 cells by transfection using Transfectin (Bio-Rad). Forty-eight hours after transfection, cells were lysed and assayed for luciferase activity using the Dual-Glo luciferase assay system (Promega).

PU.1 gene silencing by siRNA in AMs. AMs (0.5×10^6) were placed in each well of a 24-well plate in 1 ml Dulbecco modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum, 1 mM pyruvate, 1% nonessential amino acids, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were cultured for 12 h at 37°C with 5% CO_2. The culture medium was refreshed 15 min before transfection with 100 μl of complete DMEM. Silencer predesigned and validated small interfering RNA (siRNA) for PU.1 (Ambion) was used to knock down PU.1 gene expression. Silencer validated nontargeting siRNA (Ambion) was used as negative-control siRNA. For each well, 0.375 μg of PU.1 siRNA or control siRNA was mixed with 6 μl of HiperFect transfection reagent (Qiagen) in 100 μl of OPTI medium without serum and incubated at room temperature for 10 min. The mixture was then added to the well containing the cells. After an incubation for 6 h at 37°C with 5% CO_2, 400 μl of complete DMEM was added to each well. Cells were harvested 72 h after transfection.

PU.1 and Dectin-1 gene silencing by siRNA and phagocytosis assay in RAW 264.7 cells. RAW264.7 cells (mouse macrophage cell line; 0.5×10^6) were placed in each well of a 4-well chamber slide in 0.5 ml complete DMEM. PU.1 or the Dectin-1 gene was knocked down as described above, using Silencer predesigned and validated siRNA for PU.1 or Dectin-1 (Ambion). Forty-eight hours after transfection, the culture medium was refreshed with 0.5 ml of complete DMEM containing 0.5×10^7 FITC-labeled zymosan particles. Cells were incubated for 1 h at 37°C with 5% CO_2. After being washed vigorously three times with sterile PBS, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Chambers were removed, and the slide was coverslipped using SlowFade Gold antifade reagent (Invitrogen) and examined under fluorescence microscopy. Cell association index representing the number of FITC-labeled zymosan particles bound to or phagocytosed per 100 macrophages was determined on at least 500 cells in two separate slides.

Statistics. Data were presented as means ± standard deviations (SDs) of the indicated number of experiments. Differences between groups were determined using the two-tail Student *t* test and were considered statistically significant if P was <0.05.

RESULTS

Decreased Dectin-1 expression in AMs from mice with PCP. AMs are defective in phagocytosis during PCP (16). To investigate whether this defect is due to an abnormality in a certain macrophage receptor, the expression of Dectin-1 in AMs was examined since it is a major phagocytic receptor for β-glucan. AMs (0.5×10^6) from mice that had been infected with *Pneumocystis* for 2, 4, and 6 weeks were used. Equal numbers of

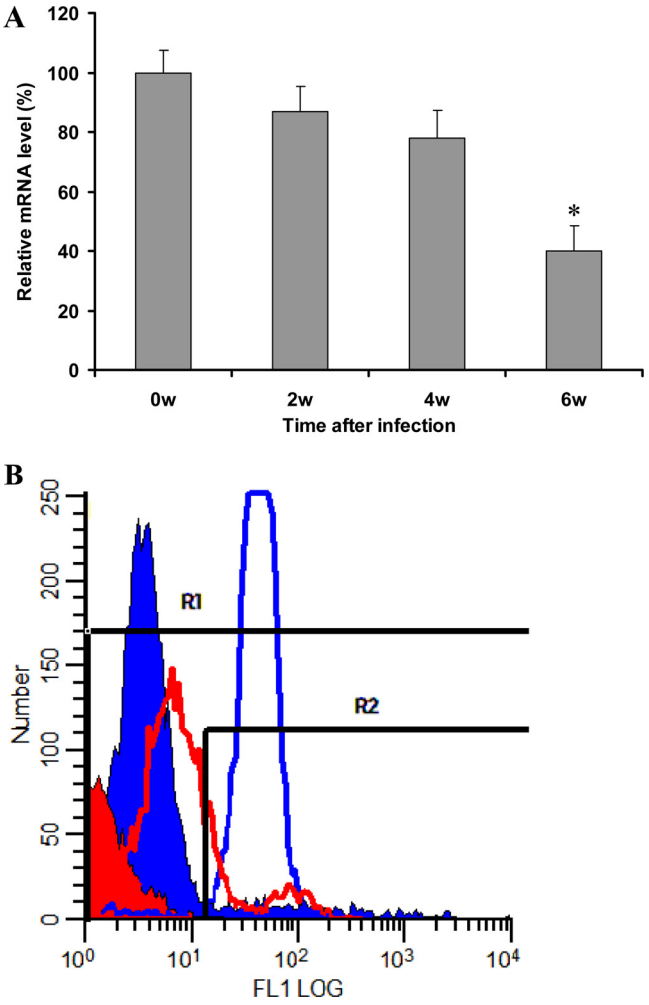

FIG. 1. Downregulation of Dectin-1 in AMs during PCP. (A) Real-time RT-PCR analysis of Dectin-1 mRNA in AMs. Total RNA isolated from the AMs was subjected to real-time RT-PCR analysis for Dectin-1. RPS8 was coamplified as an internal control in each assay. The mRNA expression levels of Dectin-1 in AMs from *Pneumocystis*-infected mice (2, 4, and 6 weeks after infection) are shown as percentages relative to that in AMs from uninfected mice. Bars represent means ± SDs for 5 or 6 mice of each group. The asterisk indicates $P < 0.05$ compared to control group. (B) Flow cytometry analysis of Dectin-1 protein on the surface of AMs. BAL fluid cells were stained with PE-labeled macrophage-specific antibody Mac3 and FITC-labeled anti-Dectin-1 monoclonal antibody. FL1 stands for the logarithm of FITC intensity. The blue and red lines represent anti-Dectin-1 antibody-stained AMs from uninfected and *Pneumocystis*-infected mice (6 weeks), respectively. The filled peaks represent the isotype control antibody-stained AMs. Gate R1 was used to include all AMs, and gate R2 was used to define Dectin-1-positive cells. Percentage of Dectin-1-positive cells was calculated by dividing the number of Dectin-1-positive cells in the R2 gate by the number of AMs in the R1 gate in each condition.

AMs from immunosuppressed, noninfected mice were used as control. Real-time RT-PCR results (Fig. 1A) showed that Dectin-1 mRNA level gradually decreased as infection progressed over time, and the decrease did not become statistically significant until 6 weeks after infection. *Pneumocystis* infection caused a 61% ± 7.1% decrease in Dectin-1 mRNA level in AMs ($P < 0.05$) at 6 weeks after infection. Dectin-1 protein

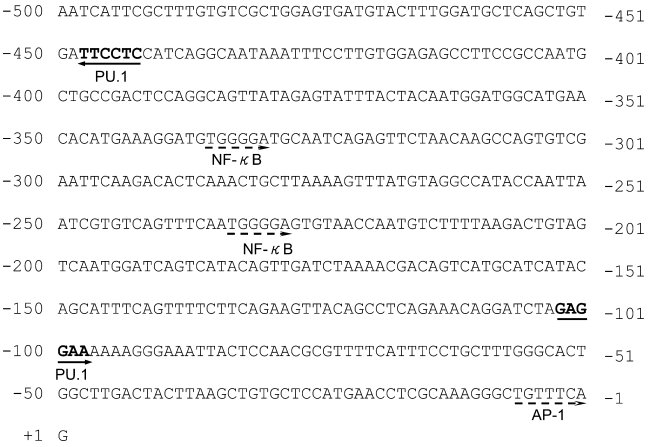

FIG. 2. Analysis of Dectin-1 promoter. The region within 500 bp upstream from the translation initiation codon of the Dectin-1 gene was analyzed for possible transcription factor binding sites using the program Pattern Search for Transcription Factor Binding Sites (PATCH) public 1.0.

expression on the surface of AMs was examined at 6 weeks after infection by flow cytometry to determine the percentage of Dectin-1-positive cells (R2) in the AMs (R1). Results showed that only 18% of AMs from PCP mice express Dectin-1 on their surface, whereas 95% of AMs from control noninfected mice were positive for Dectin-1 (Fig. 1B), suggesting that Dectin-1 expression in AMs is downregulated during PCP.

Decreased PU.1 expression in AMs from mice with PCP. To understand how the expression of the Dectin-1 gene is regulated, the region within 500 bp upstream from the translation initiation codon of the Dectin-1 gene was analyzed for possible transcription factor binding sites using the program Pattern Search for Transcription Factor Binding Sites (PATCH). This analysis revealed two potential binding sites for the ETS family transcription factor PU.1 (GAGGAA) located at nucleotide positions −98 and −443, relative to the translation initiation codon of the Dectin-1 gene (Fig. 2). Potential binding sites for other transcription factors such as AP-1 (at −6) and NF-κB (at −233 and −336) were also found. To investigate whether PU.1 regulates the Dectin-1 gene, PU.1 mRNA expression level was first determined by real-time RT-PCR at 2, 4, and 6 weeks after infection. The results show that the PU.1 mRNA level of AMs was also decreased gradually after *Pneumocystis* infection and that the decrease became statistically significant after 4 weeks of infection (Fig. 3A). The expression of the PU.1 gene in AMs during PCP (6 weeks after infection) was also determined. *In situ* hybridization results showed that abundant PU.1 mRNAs were present in both cytoplasm and nuclei of normal AMs. In contrast, only scant PU.1 mRNAs were seen in the cytoplasm of AMs from PCP mice, indicating that PU.1 mRNA levels were significantly decreased in AMs from PCP mice (Fig. 3B). The protein expression of Dectin-1 in AMs was also decreased during PCP, and densitometry analysis of Western blots showed an approximately 70% decrease in the protein level of PU.1 (Fig. 3C).

Binding of PU.1 to Dectin-1 promoter. To determine whether PU.1 binds to the two potential PU.1-binding sites located at positions −98 and −443, EMSA was performed using the two

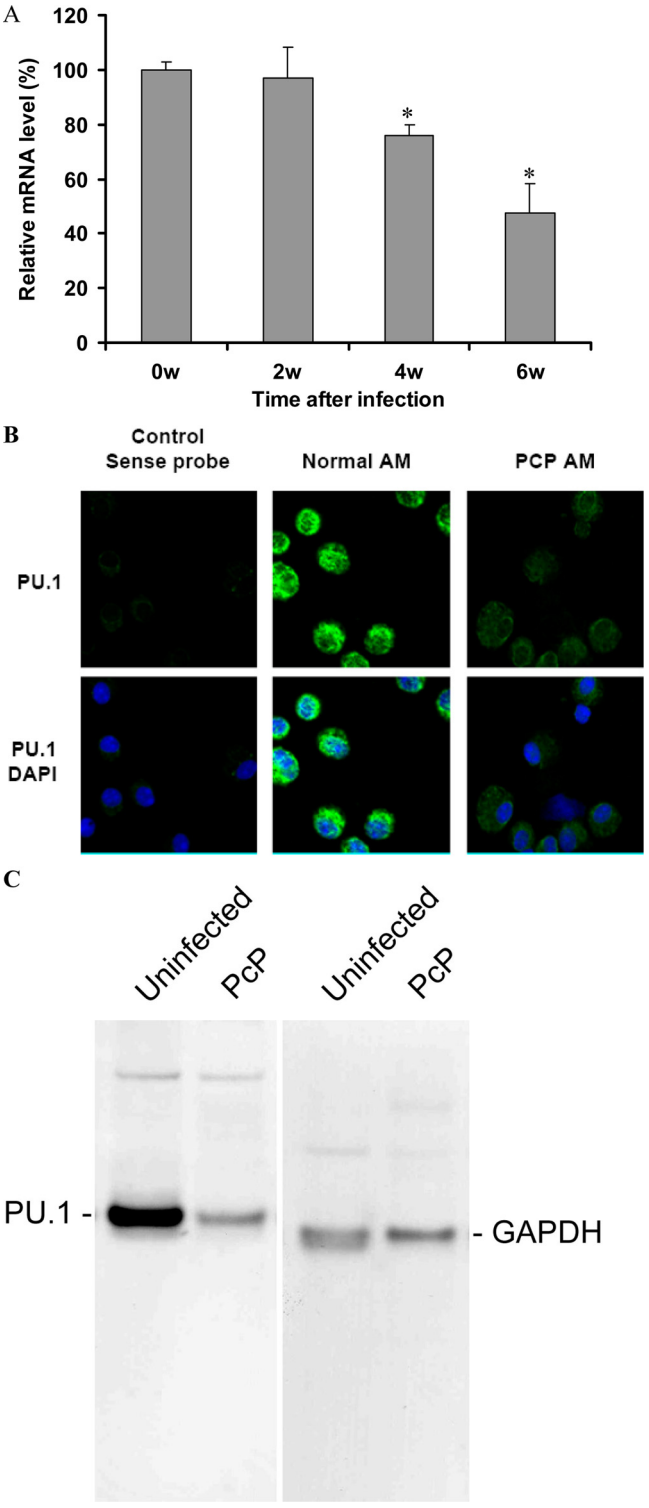

FIG. 3. Downregulation of PU.1 in AMs during PCP. (A) Real-time RT-PCR analysis of PU.1 mRNA in AMs. Total RNA isolated from the AMs was subjected to real-time RT-PCR analysis for PU.1. RPS8 was coamplified as an internal control in each assay. The mRNA expression levels of PU.1 in AMs from *Pneumocystis*-infected mice (2, 4, and 6 weeks after infection) are shown as percentages relative to those in AMs from uninfected mice. Bars represent means ± SDs for 5 or 6 mice of each group. The asterisks indicate $P < 0.05$ compared to control group. (B) AMs from mice without or with PCP for 6 weeks were permeabilized and reacted with FITC-labeled antisense PU.1 probe. FITC-labeled sense probe was used as the negative control. PU.1 mRNA levels were examined under a fluorescence microscope at a magnification of ×400. DAPI, 4′,6-diamidino-2-phenylindole. (C) Equal amounts of total protein from mice with or without PCP were electrophoresed on a 10% SDS-polyacrylamide gel. Protein concentration of PU.1 was determined by Western blotting using anti-PU.1 antibody. GAPDH was used as an internal loading standard. Data presented are representative of 5 or 6 mice per group.

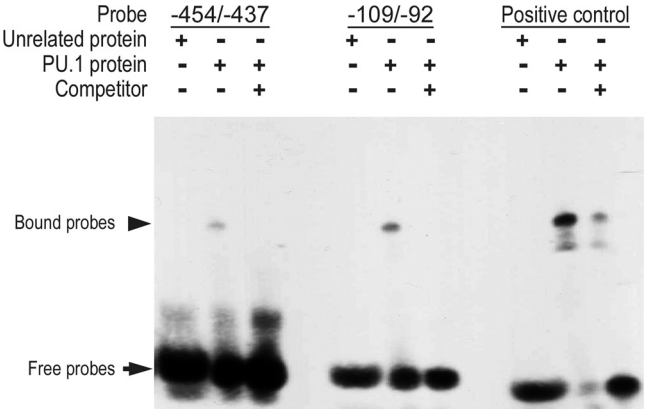

FIG. 4. Binding of PU.1 to Dectin-1 promoter. EMSA was performed using the two oligonucleotide probes containing the putative PU.1 binding sites in Dectin-1 promoter and a recombinant PU.1 protein. The oligonucleotide containing a known PU.1-binding site was used as the positive control. For each reaction, a negative control with addition of an unrelated protein, EBNA, instead of PU.1 protein was included. Cold competition for each probe was done by adding unlabeled oligonucleotides with the known PU.1-binding site to the reaction mixture.

oligonucleotide probes containing the putative PU.1 binding sites and a recombinant PU.1 protein (2). The oligonucleotide containing a known PU.1-binding site was used as the positive control. For each reaction, a negative control with addition of an unrelated protein, EBNA, instead of PU.1 protein was included. Cold competition for each probe was done by adding unlabeled oligonucleotides with the known PU.1 binding site to the reaction mixture. Addition of PU.1 protein to the reaction resulted in a band shift with both oligonucleotides (−98 and −443) of the Dectin-1 promoter, as well as the positive-control probe (Fig. 4). This result suggests that the PU.1 protein binds to Dectin-1 promoter. The binding was specific since the shifted bands were almost completely competed out by unlabeled probes containing the known PU.1-binding sequence. In addition, the unrelated protein EBNA did not bind to the probes.

Transactivation of Dectin-1 promoter by PU.1. To investigate whether the PU.1-binding sites in the Dectin-1 promoter are functional and whether PU.1 regulates the expression of Dectin-1, the effects of PU.1 on the activity of the Dectin-1 gene promoter were examined. The plasmid pGL-Dectin1 containing a luciferase reporter gene driven by the Dectin-1 gene promoter (Fig. 5A), together with the PU.1-expressing plasmid pFLAG-PU.1, was introduced into NIH 3T3 cells. NIH 3T3 cells instead of macrophages were chosen for this experiment because they do not normally express PU.1; therefore, the influence of endogenous PU.1 can be avoided. Cells were lysed

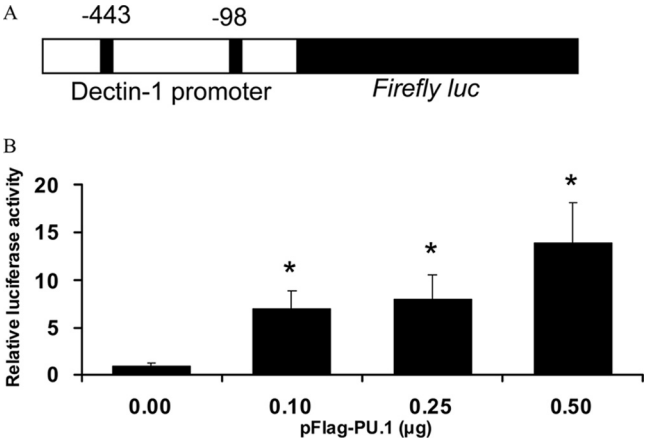

FIG. 5. Enhancement of the promoter activity of the Dectin-1 gene by PU.1. The plasmid pGL-Dectin1 containing the luciferase reporter gene driven by the Dectin-1 promoter containing two putative PU.1-binding sites (black bar) (A), together with the PU.1-expressing plasmid pFLAG-PU.1 at different doses (0.1, 0.25, and 0.5 μg), was introduced into NIH 3T3 cells. Dectin-1 promoter activity was determined by measuring luciferase activity in the cell lysates (B). Bars represent SDs of three independent assays. The asterisks indicate $P < 0.05$ compared to the control group.

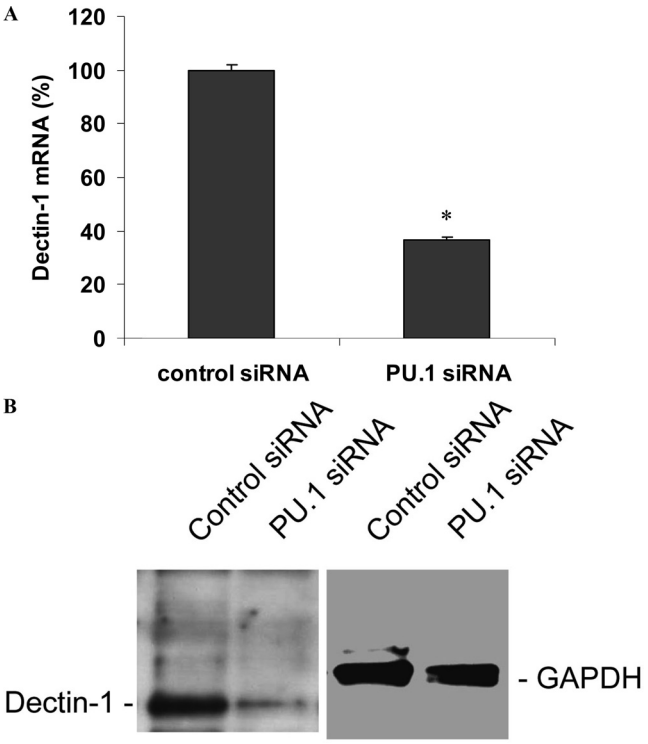

FIG. 6. Effect of PU.1 siRNA on the expression of Dectin-1. Normal AMs (5×10^5) were transfected with PU.1 siRNA or control siRNA. Dectin-1 gene expression was examined at the mRNA level by real-time PCR (A) and at the protein level by Western blotting (B). Similar results were obtained in three independent experiments. The asterisk indicates $P < 0.05$ compared to control group.

48 h after the transfection, and the Dectin-1 promoter activity was determined by measuring luciferase activity in the cell lysates. NIH 3T3 cells that were cotransfected with pGL-Dectin1 and pFLAG were used as the negative control. The luciferase activity of negative-control cells was set as 1. As shown in Fig. 5B, cotransfection of NIH 3T3 cells with 0.1, 0.25, and 0.5 μg of pFLAG-PU.1 increased the luciferase activity by 7.0-, 7.9-, and 13.8-fold, respectively ($P < 0.05$), indicating that PU.1 was successfully expressed from pFLAG-PU.1 and that PU.1 activates the expression of Dectin-1 in a dose-dependent manner.

Decreased Dectin-1 expression in AMs by PU.1 knockdown. To confirm that PU.1 regulates the expression of Dectin-1, 0.5×10^6 normal AMs were transfected with PU.1 siRNA, and Dectin-1 expression was determined 72 h after the transfection. AMs transfected with scrambled siRNA were used as controls. Real-time PCR results (Fig. 6A) showed that the Dectin-1 mRNA level in AMs with PU.1 knockdown was only 37% ± 2.1% of that of control AMs ($P < 0.05$), and Western blotting results revealed that the Dectin-1 protein level in AMs transfected with PU.1 siRNA was only 60% of that of control AMs (Fig. 6B). These data indicated that PU.1 knockdown in AMs caused a significant decrease in Dectin-1 expression and suggest that PU.1 is essential for Dectin-1 gene expression in AMs.

Decreased phagocytosis in mouse macrophage line RAW 264.7 cells after PU.1 or Dectin-1 gene knockdown. To investigate the roles of Dectin-1 and PU.1 in the phagocytosis of macrophages, mouse macrophage line RAW 264.7 cells were transfected with PU.1 or Dectin-1 siRNA, and phagocytic activities were determined 48 h after transfection by incubating the cells with FITC-labeled zymosan particles. Zymosan is a cell wall preparation of *Saccharomyces cerevisiae* and has been widely used to study phagocytosis (30). Zymosan is composed of β-glucans, mannans, mannoproteins, and chitin. Each of these compounds has been identified as a *Pneumocystis* cell wall component (5, 9, 18). As shown in Fig. 7, the control group had a cell association index of 19.95 ± 7.76 particles per 100 cells; the cell association index was reduced by 75% (4.92 ± 4.66) ($P < 0.05$) in the group transfected with PU.1 siRNA and by 74% (5.26 ± 2.45) ($P < 0.05$) in the group transfected with Dectin-1 siRNA.

DISCUSSION

β-Glucan is a major component of the cell wall of many fungal pathogens. It contributes to cellular structure and rigidity of the organism. Dectin-1 is a pattern recognition receptor of macrophages. It recognizes β-glucans on the surface of fungal pathogens and initiates phagocytosis of the organisms by macrophages (31). Phagocytosis of *Pneumocystis* organisms by AMs is defective during PCP (16). The mechanism by which AMs become defective in phagocytosis during PCP is not completely clear. A possible cause of this defect is aberrant expression of macrophage receptors. Since Dectin-1 is a major macrophage receptor and has been shown to play a critical role in phagocytosis of *Pneumocystis* organisms (26), we examined its expression at both mRNA and protein levels. Results of this study showed that the transcription of the Dectin-1 gene was decreased by 61% in AMs from mice infected with *Pneumocystis* compared to those from uninfected mice (Fig. 1A). We also found that only 18% of the AMs from mice with PCP had Dectin-1 on their surface (Fig. 1B). Since many factors can

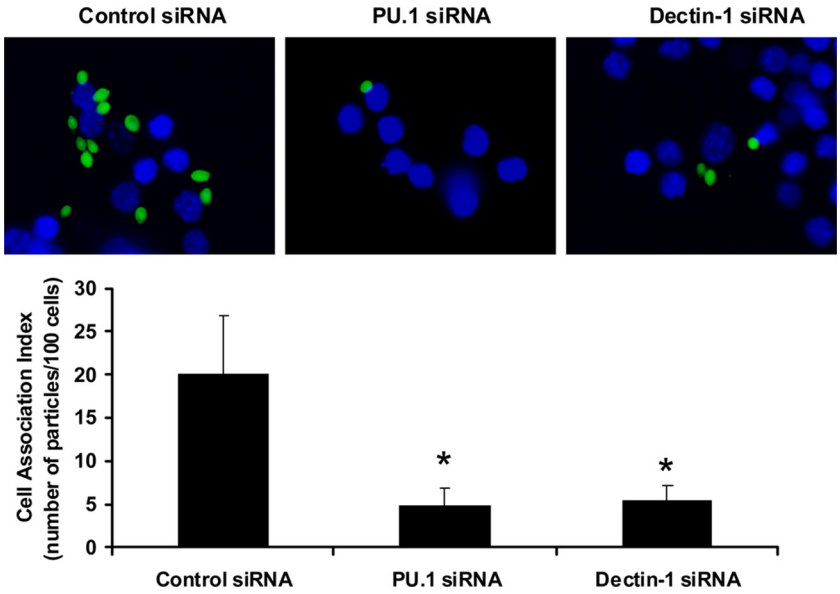

FIG. 7. Effects of PU.1 or Dectin-1 knockdown on phagocytic activity of macrophages. RAW 264.7 cells (5×10^5) were transfected with PU.1 siRNA, Dectin-1 siRNA, or control siRNA. The phagocytosis assay was performed by incubating transfected cells with 5×10^6 FITC-labeled zymosan particles for 1 h. The cells were examined under a fluorescence microscope at a magnification of ×400 and evaluated by cell association index (CAI) representing the number of FITC-labeled zymosan particles bound to or phagocytosed per 100 macrophages. Bars represent means ± SDs for each condition in 500 cells on two separate slides. The asterisks indicate $P < 0.05$ compared to control group.

influence Dectin-1 synthesis, including posttranslational modification (13) and alternative splicing (4, 37), it is not surprising that the change in Dectin-1 surface expression levels determined by flow cytometry does not completely agree with that of Dectin-1 mRNA levels determined by real-time RT-PCR in AMs. It has been shown that the expression level of Dectin-1 in primary macrophages affects the ability of these cells to recognize and respond to fungi (36). We also found that Dectin-1 gene knockdown in the mouse macrophage cell line RAW 264.7 leads to decreased phagocytosis of zymosan particles (Fig. 7). Given the abundance of β-glucan in *Pneumocystis* cell wall and the significant role of Dectin-1 in the recognition of *Pneumocystis* organisms, it is conceivable that the decreased Dectin-1 expression results in a defect in phagocytosis by AMs during PCP.

As Dectin-1 is a newly defined β-glucan receptor in macrophages, the regulation of Dectin-1 expression has not been well studied. It has been shown that Dectin-1 expression is highly upregulated by granulocyte-macrophage colony-stimulating factor (GM-CSF) and by the cytokines interleukin-4 (IL-4) and IL-13, which induce alternative macrophage activation (36). MyD88$^{-/-}$ macrophages have decreased surface expression of Dectin-1 compared to wild-type macrophages. However, addition of factors such as vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein 1 (MCP-1) resulted in equivalent expression of Dectin-1 in macrophages from both MyD88$^{-/-}$ and wild-type mice (10). These results suggest that multiple pathways are involved in the regulation of Dectin-1 gene expression.

To investigate why Dectin-1 expression is decreased in AMs during PCP, we first examined how Dectin-1 expression is regulated. We searched for transcription factor binding sites in the promoter region of the Dectin-1 gene and found two PU.1 binding sites (Fig. 2). Potential binding sites for transcription factors AP-1 and NF-κB were also identified. Since previous studies showed that the expression levels of both AP-1 and NF-κB are upregulated during *Pneumocystis* or other fungal infections (33–35), they are not likely the cause of Dectin-1 downregulation during PCP. PU.1 is present in myeloid cells and is essential for production of macrophages and other hematological lineage cells (3, 22) as well as terminal differentiation of macrophages (12). PU.1 has been shown to regulate the expression of many macrophage-specific surface molecules such as macrophage colony-stimulating factor receptor (23), Fc receptor (7), scavenger receptor (21), mannose receptor (6), and integrin subunits CD11b/CD18 (24). Our results showed, for the first time, that PU.1 also regulates Dectin-1 expression in AMs. Using electrophoretic mobility shift assays, we demonstrated that both PU.1 binding sites in Dectin-1 promoter can be bound by PU.1 (Fig. 4). A luciferase reporter gene driven by the Dectin-1 promoter was shown to respond to PU.1 in a dose-dependent manner (Fig. 5). Surprisingly, we found that PU.1 expression in AMs during PCP was downregulated by 70% (Fig. 3). Furthermore, knocking down PU.1 expression in AMs from normal mice caused a significant decrease in Dectin-1 mRNA and protein levels in these cells (Fig. 6). Although these results do not indicate that PU.1 directly affects Dectin-1 expression, as many genes in the cell also have PU.1-binding sites, the results do suggest that the PU.1-binding sites in the Dectin-1 promoter are functional and that PU.1 can affect Dectin-1 expression. Although decreased Dectin-1 expression in AMs during PCP could possibly be the reason for defective phagocytosis against *Pneumocystis* organisms, the defect in phagocytosis appears to be general, not specific to *Pneumocystis* organisms, as our previous study showed that AMs from PCP animals are defective in phagocytosis of both

Pneumocystis organisms and latex beads (17). A mechanism that involves the function or expression of many different receptors is needed to explain the global defect of phagocytosis in AMs. It has been shown that lack of PU.1 expression correlates with decreased maturation, differentiation, and surfactant metabolism in AMs from patients with pulmonary alveolar proteinosis (1). Another study also showed that the absence of PU.1 correlated with functional defects in AMs, including decreased surfactant catabolism, cell adhesion, phagocytosis, bacterial killing, pathogen recognition receptor expression, and inflammatory cytokine signaling (29). Our data in this study showed that PU.1 knockdown in macrophages leads to decreased phagocytic activity (Fig. 7). Our finding of PU.1 downregulation during PCP suggests that AM terminal differentiation may be disrupted, leading to the reduced number of AMs during PCP that we have observed previously (14).

The expression levels of PU.1 and Dectin-1 genes in AMs were gradually decreased as PCP progressed, but the changes did not become statistically significant until the late stage of the disease (6 weeks). This is consistent with the chronic nature of this disease, as our previous study showed that *Pneumocystis*-infected mice started to lose body weight and show symptoms at approximately 6 weeks after initiation of infection (35).

Further study of the cause of PU.1 downregulation during PCP is needed. Based on results of previous studies, GM-CSF appears to play a major role in the regulation of PU.1 expression. It has been found that PU.1 expression was markedly reduced in AMs from $GM^{-/-}$ mice but was restored by selective expression of GM-CSF in the lungs (29). Exogenous GM-CSF treatment also upregulated PU.1 expression in AMs from patients with pulmonary alveolar proteinosis (1). Our previous study showed that *Pneumocystis*-infected animals had 75% lower levels of GM-CSF in the BAL fluids than did uninfected animals (15). It is conceivable that the decreased GM-CSF levels cause the downregulation of PU.1, leading to decreased expression of Dectin-1 in AMs during PCP.

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