A transcription factor PU.1 is critical for Ccl22 gene expression in dendritic cells and macrophages

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The chemokine CCL22 is predominantly produced by dendritic cells (DCs) and macrophages. CCL22 acts on CCR4-expressing cells including Th2 and Treg. Although a correlation between the CCL22-CCR4 axis and allergic diseases has been established, the mechanism of monocyte lineage-specific Ccl22 gene expression is largely unknown. In the current study, we investigated transcriptional regulation of the Ccl22 gene in DCs and macrophages. Using reporter assays, we identified the critical cis-enhancing elements at 21/−18 and −10/−4 in the Ccl22 promoter. Electrophoretic mobility shift assays proved that transcription factor PU.1 directly binds to the cis-elements. Knockdown of PU.1 markedly decreased Ccl22 expression in bone marrow-derived DCs (BMDCs) and BM macrophages (BMDMs). Chromatin immunoprecipitation assays revealed that PU.1 bound to the Ccl22 promoter in not only BMDCs and BMDMs, but also splenic DCs and peritoneal macrophages. LPS stimulation increased the amount of PU.1 recruited to the promoter, accompanied by upregulation of the Ccl22 mRNA level, which was diminished by Spi1 knockdown. We identified similar cis-elements on the human CCL22 promoter, which were bound with PU.1 in human monocytes. Taken together, these findings indicate that PU.1 transactivates the Ccl22 gene in DCs and macrophages by directly binding to the two elements in the promoter.

Chemokines are chemo-attractant proteins which regulate the migration of different subsets of leukocytes during immune responses. CCL22 (also called macrophage-derived chemokine, MDC) is one of the chemokines mainly produced by myeloid cells such as macrophages and DCs in the steady state. CCL22 and a closely related chemokine, CCL17, tandemly locate on human chromosome 16 and mouse chromosome 8. These chemokines share the same receptor, CCR4, predominantly expressed by Th2 cells. Therefore, these chemokines are considered to be key mediators in the development of Th2-dominant diseases such as atopic dermatitis (AD) and asthma. Indeed, serum levels of CCL22 and CCL17 are significantly elevated in AD patients. It has been reported that CCL22 and CCR4 are also expressed in several types of tumor cells and Foxp3+ Treg, respectively. This CCL22-CCR4 axis is thought to attract Treg into the tumor microenvironment to evade the immune attack. A CCR4 antagonist is useful for alleviating immune suppression and preventing tumor growth.

PU.1 is a transcription factor specifically expressed in hematopoietic lineages. It is well known that expression level of PU.1 determines the cell fate during development of B cells, macrophages, and granulocytes from hematopoietic stem cells. A study using PU.1 reporter mice demonstrated that PU.1 is highly expressed in myeloid DCs, in contrast to a low level expression of PU.1 in plasmacytoid DCs. PU.1 binds to Ets motifs as a monomer and binds to EICE motifs as a heterodimer PU.1/IRF4 or PU.1/IRF8. C/EBPα and β, and c-Jun are also known to form a complex with PU.1. We have demonstrated that PU.1 regulates the expression of DC-characteristic genes including Cd80, Cd86, Cita, Tnf-α, and Tnfsf4. Moreover, previous studies have shown that PU.1 is involved in macrophage-specific gene expression.

Despite its critical involvement in several allergic diseases, the expression mechanism of the Ccl22 gene is not fully understood. In the current study, we found that PU.1 transactivates the Ccl22 gene both in DCs and macrophages via the two Ets motifs in the promoter.

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showed that the luciferase activities were apparently detected as long as the segment from −21 to +18 (termed Site 1), and TTCTTCT containing two overlapping Ets-like motifs at −27/−1 region of the promoter plays a pivotal role in the gene expression of Ccl22 in both DCs and Macrophages. Then, we searched possible transcription factor-binding sites in this region using a database, JASPAR (http://jaspar.genereg.net/), and found that three Ets motifs: a typical Ets motif GGAA at −21/−18 (termed Site 1), and TTCTTCT containing two overlapping Ets-like motifs at −10/−4 (termed Site 2), are located within this region (Fig. 1c). To assess the necessity of these putative transcription factor-binding sites for promoter activity, we constructed mutant reporter vectors lacking Site1 and/or Site2. Although the introduction of a mutation at either Site 1 or Site 2 did not affect the luciferase activity, double mutation of both sites significantly reduced the activity (Fig. 1d). These results indicate that two sites containing Ets motifs, −21/−18 and −10/−4, are critical for transcrptional activity of the Ccl22 promoter.

Results

Ccl22 promoter activity in DC- and Macrophage-cell lines. To determine the region critical for promoter activity, we generated a series of reporter vectors, and carried out luciferase assays by transfection of monocyte cell lines with the reporter plasmids. A luciferase assay using JAWSII, a mouse DC cell line, as the host showed that the luciferase activities were apparently detected as long as the segment from −27 to +108 were contained (Fig. 1a). A similar result was obtained by a reporter assay using a mouse macrophage cell line, RAW264.7 (Fig. 1b). These results indicate that −27/−1 region of the promoter plays a pivotal role in the gene expression of Ccl22 in both DCs and Macrophages. We then searched possible transcription factor-binding sites in this region using a database, JASPAR (http://jaspar.genereg.net/), and found that three Ets motifs: a typical Ets motif GGAA at −21/−18 (termed Site 1), and TTCTTCT containing two overlapping Ets-like motifs at −10/−4 (termed Site 2), are located within this region (Fig. 1c). To assess the necessity of these putative transcription factor-binding sites for promoter activity, we constructed mutant reporter vectors lacking Site1 and/or Site2. Although the introduction of a mutation at either Site 1 or Site 2 did not affect the luciferase activity, double mutation of both sites significantly reduced the activity (Fig. 1d). These results indicate that two sites containing Ets motifs, −21/−18 and −10/−4, are critical for transcriptional activity of the Ccl22 promoter.

Figure 1. Analysis of cis-element in the Ccl22 promoter. JAWSII cells (a) and RAW264.7 cells (b) were transfected with reporter plasmids for renilla luciferase as an internal control and with reporter plasmids for firefly luciferase containing various lengths of the mouse Ccl22 promoter. (c) Sequences of the −27/−1 region of the mouse Ccl22 promoter. Two Ets motifs, designated Site1 and Site 2, are indicated in bold. (d) JAWSII cells were transfected with reporter plasmids of WT or mutant promoters lacking the Ets motif(s) at the indicated sites. All results are shown as means ± S.D.s (n = 3). Similar results were obtained in three independent experiments. *p < 0.05.
the transactivation effect of PU.1 on promoter activity (Fig. 2e). This result indicates that PU.1 transactivates the mouse Ccl22 gene through binding to Site 1 and Site 2.

**PU.1/Spi1 knockdown by siRNA transfection.** In order to determine the role of PU.1 in the expression of the Ccl22 gene, we introduced Spi1 siRNA into JAWSII and RAW264.7 cells. The introduction of Spi1 siRNA markedly decreased mRNA levels of Spi1 and Ccl22 in JAWSII (Fig. 3a) and RAW264.7 (Fig. 3b). Further, we confirmed the effect of Spi1 knockdown on Ccl22 expression in primary mouse DCs and macrophages generated from bone-marrow cells by cultivation in the presence of GM-CSF and M-CSF, respectively. Under the PU.1-knocked-down condition (both of mRNA and protein levels), the Ccl22 mRNA level was significantly decreased in BMDCs (Fig. 3c) and BMDMs (Fig. 3d). Furthermore, ELISA showed that the amount of CCL22 protein produced from BMDCs (Fig. 3e) and BMDMs (Fig. 3f) was markedly reduced by PU.1 knockdown. Although CCL22 production is enhanced during polarization to alternative activation macrophages (AAM), mRNA levels of other AAM makers, Fizz-1 and Ym-1 were not decreased but rather increased by Spi1 knockdown (Supplemental Fig. 2), suggesting that reduction of Ccl22 expression in Spi1 knockdown cells is not simply due to a reduction of these cells from a less of an AAM phenotype. To evaluate the effect of Spi1 knockdown-mediated suppression of CCL22 production on the capacity to cause migration of Th2 cells, we performed a migration assay. We observed that the number of Th2 cells migrated to Spi1-knocked-down DCs was moderately lower than that to control DCs (Supplemental Fig. 3). We introduced other Spi1 siRNAs into BMDCs to exclude the possibility of an off-target effect ofSpi1 siRNA and found that these two siRNAs also significantly suppressed the Ccl22 mRNA level in parallel with the Spi1 knockdown level (Supplemental Fig. 4). Taken together, these results suggest that PU.1 is involved in the gene expression of mouse Ccl22 in DCs and macrophages.

**Binding of PU.1 to the mouse Ccl22 promoter in DCs and macrophages.** To investigate whether PU.1 binds to the Ccl22 gene in CCL22-expressing cells, we performed ChIP assays using BMDCs (Fig. 4a) and
BMDMs (Fig. 4b). A significant amount of PU.1 binding to $-17/+52$ was detected in DCs and macrophages, whereas PU.1 did not bind to further upstream regions, $-1780/-1714$ and $-1080/-1008$ (Fig. 4a,b). These results demonstrate the specific binding of PU.1 to the proximal region of the transcription start site of the mouse Ccl22 gene.

Furthermore, we carried out ChIP assays using DCs and macrophages freshly isolated from mice. As expected, significant and specific binding of PU.1 to $-17/+52$ of the Ccl22 promoter was detected in splenic DCs (Fig. 4c).
and peritoneal macrophages (Fig. 4d). These results indicate that PU.1 trnasactivates the Ccl22 gene by binding to the proximal region of the promoter in DCs and macrophages in vivo.

**Involvement of IRFs in the gene expression of Ccl22.** In addition to the role as a monomeric transactivation factor, PU.1 also regulates the target genes forming a heterodimer with IRF4 or IRF8. In order to investigate the involvement of IRFs in the transcriptional regulation of Ccl22, we introduced siRNA against either Irf4 or Irf8 into BMDCs. Quantitative PCR showed that Irf4 knockdown significantly decreased the mRNA level of Ccl22 under the condition, in which levels of Irf4 mRNA and IRF4 protein were reduced (Fig. 5a). In contrast, Irf8 siRNA did not affect the Ccl22 mRNA level even though levels of Irf8 mRNA and IRF8 protein were decreased (Fig. 5b). To examine whether IRF4 co-localizes to the minimum promoter region of the Ccl22 gene with PU.1, we performed a ChIP assay using an anti-IRF4 Ab and quantified the amount of the immunoprecipitated chromatin (Fig. 5c). The amounts of immunoprecipitated chromatin were determined by quantitative PCR targeting the −17/+52 region of the Ccl22 promoter. Data are expressed as the percentage of the input for each ChIP assay. (d) mRNA level (left) and protein level (right) of PU.1 in control (siNega) cells. (e) PU.1 binding degree to −17/+52 in control (siNega) cells determined by ChIP assay. Results are shown as means + S.D.s (n = 3). Similar results were obtained in three independent experiments. *p < 0.05.

**Effect of TLR-mediated stimulation on the recruitment of PU.1 to the gene.** DCs and macrophages dramatically alter their gene expression in response to stimulation signaling such as TLR ligands. As shown in Fig. 6a, the mRNA level of Ccl22 was markedly increased in response to LPS stimulation, in contrast to a slight increase in the Ccl17 mRNA level. Thus, we performed a ChIP assay to investigate whether the LPS-induced upregulation of Ccl22 transcription reflects the PU.1 binding ability to the Ccl22 promoter in DCs. As shown in Fig. 6b, the PU.1 binding level at the region −17/+52 in mature BMDCs was approximately 1.8-fold of that of immature BMDCs. We also confirmed that the LPS-induced increase of the Ccl22 mRNA level was significantly attenuated by siRNA-mediated Spi1 knockdown (Fig. 6c). These results demonstrate that PU.1 recruitment to the Ccl22 promoter is augmented by LPS-dependent maturation, thereby increasing the transcription of Ccl22.

**Involvement of PU.1 in expression of the human CCL22 gene.** To investigate whether the role of PU.1 in CCL22 expression in mouse monocytes is translatable to human biology, we evaluated the effect of SPI1 siRNA on CCL22 expression levels in human monocyte cells, THP-1. As shown in Fig. 7a, SPI1 siRNA effectively
suppressed SPI1 mRNA level and subsequently reduced CCL22 mRNA level. When nucleotide sequence of the human CCL22 gene was compared with that of mouse, we found that the elements similar to Site1 and Site2 exist on the human CCL22 promoter (Fig. 7b). By ChIP assay, we confirmed that PU.1 bound to this region in human monocyte cells (Fig. 7c). These results suggest that PU.1 is a common transactivator for the CCL22 (Ccl22) gene in human and mouse.

Discussion

The Ets family transcription factor PU.1 is essential for myeloid cell development. Several studies have demonstrated that PU.1 plays critical roles in DC and macrophage functions. In the current study, we provided evidence that PU.1 is involved in CCL22 expression in DCs and macrophages.

Using a reporter assay, we demonstrated that transcriptional regulatory elements of the Ccl22 gene located within −27/+/108 and two putative PU.1 binding elements were critical for transcriptional activity. Ccl22 expression was markedly decreased by Spi1 knockdown in all investigated cell types. ChIP assays showed that PU.1 bound to the proximal region of the Ccl22 promoter. From these results, we demonstrate that PU.1 is one of the most important transcription factors in the gene expression of Ccl22. Although transcriptional activity was significantly suppressed by mutation of the two PU.1 binding motifs in the luciferase assay, the activity was still higher...
than that of those without the promoter. This result indicates that another transcription factor(s) is involved in the gene expression of Ccl22 by binding within the −277/+108 region. Indeed, CCL22 is also expressed in non-hematopoietic cells, such as keratinocytes, which do not express PU.1. Previous studies have demonstrated that NF-kB and STAT1 are involved in the IFN-γ/TNF-α-dependent upregulation of CCL22 in human keratinocytes21,22. Considering that our reporter assay was carried out in a steady state, these molecules were not likely to enhance the transcriptional activity via binding to the −277/+108 region. It will be intriguing to further investigating which transcription factors regulate Ccl22 gene expression.

Even though ChIP assays were carried out using JAWSII and BMDCs without inducing maturation, we detected significant amount of PU.1 binding to the Ccl22 promoter. These results suggest that PU.1 plays a role as an activator of basal transcription of the Ccl22 gene in DCs, which produce a large amount of CCL22 even in the absence of inflammatory signaling. We also exhibited that PU.1 binding levels at the identified region were increased in response to LPS stimulation and subsequent upregulation of Ccl22 was significantly attenuated by Spi1 knockdown. These results suggest that PU.1 also plays an important role in the transactivation of Ccl22 upon DC maturation.

Here we used BMDMs and peritoneal macrophages as macrophage models. Macrophages are classified into two groups, M1 macrophages and M2 macrophages, depending on their expression of surface markers, cytokines, and chemokines. Ccl22 is one of the M2 macrophage-markers, and BMDMs generated with M-CSF are thought to exhibit an M2-like phenotype23. Indeed, we demonstrated that PU.1 is involved in the transcriptional regulation of Ccl22 by binding to the promoter in BMDMs. Recent studies have demonstrated that characteristic transcription factors like Spi-C and GATA6 are essential for differentiation of splenic and peritoneal macrophages, respectively24,25, suggesting that tissue-resident macrophages are functionally distinct. Although we showed that PU.1 marginally bound to the Ccl22 promoter in peritoneal macrophages, it is possible that PU.1 binding level is altered depending on where macrophages reside. Further analyses are required to elucidate this.

Previously, Chang et al. demonstrated that PU.1 promotes the development of Th926 and subpopulation of Th27 that express CCL22. PU.1 has also been described in promoting alternative activation of macrophages that produce CCL2228. In the current study, it was revealed that the CCL22 (Ccl22) gene, which has been reported to be under the control of PU.1, is directly transactivated by PU.1 via the cis-elements. Although Th cells were not examined in our study, the CCL22 gene may be a direct target of PU.1 in Th cells.

Previous studies have demonstrated that serum levels of CCL22 and CCL17 are significantly elevated and correlated with disease severity in patients with Atopic dermatitis29-31. Moreover, administration of CCR4 blocking antibody to the airway inflammation model abolished asthmatic symptoms32. According to these studies, targeting the interactions between CCL17/CCL22 and CCR4 may be a useful approach for controlling allergic diseases. We previously reported that injection of Spi1 siRNA significantly suppressed contact hypersensitivity of mice33. Considering that Th2-related genes such as Tnsf4 and Ccl22 are also transactivated by PU.1 in DCs, Spi1 knockdown may be a favorable strategy for allergic diseases.

Materials and Methods

Mice and cells. Bone marrow-derived DCs (BMDCs) and macrophages (BMDMs) were generated from BALB/c mice (Japan SLC, Hamamatsu, Japan) as previously described16,33,34. Mouse DC line JAWSII, mouse macrophage line RAW264.7, and human monocyte cell line THP-1 were maintained as previously described13,16,18. All animal experiments were performed in accordance with the approved guidelines of the Institutional Review Board of Tokyo University of Science, Tokyo, Japan. The Animal Care and Use Committees of Tokyo University of Science specifically approved this study.

Isolation of splenic DCs and peritoneal macrophages. CD11c microbeads and an auto MACS Pro separator (Miltenyi Biotech, Tubingen, Germany) were used to isolate DCs from mouse spleen. Peritoneal macrophages were obtained as adhesive cells by incubating mouse peritoneal cells with 10% FCS-DMEM for 1 hour.

Introduction of siRNA into cells. Spi1 siRNA (Stealth Select RNAi, Sfpi1-MSS247676), Ifr4 siRNA (MSS205499), Ifr8 siRNA (MSS236848), SPI1 siRNA (SFP11-HSS186060) and control siRNA purchased from Invitrogen (Carlsbad, CA) were introduced into BMDCs or BMDMs as previously described16. Mouse DC line JAWSII, mouse macrophage line RAW264.7, and human monocyte cell line THP-1 were introduced into BMDCs or BMDMs as previously described16. Mouse DC line JAWSII, mouse macrophage line RAW264.7, and human monocyte cell line THP-1 were introduced into BMDCs or BMDMs as previously described16. Mouse DC line JAWSII, mouse macrophage line RAW264.7, and human monocyte cell line THP-1 were introduced into BMDCs or BMDMs as previously described16. Mouse DC line JAWSII, mouse macrophage line RAW264.7, and human monocyte cell line THP-1 were introduced into BMDCs or BMDMs as previously described16.

Quantitative RT-PCR. Quantitative RT-PCR was performed as previously described16. The TaqMan primers are listed as follows: Spi1, Mm00488142_m1; Ifr4, Mm00516431_m1; Ifr8, Mm00492567_m1; Gapdh, Mm00805843_m2; SPI1, Mm00488142_m1; GAPDH, Mm00805843_m2; GAPDH, Mm00805843_m2. The nucleotide sequences of primers used for detection of the mouse Cld2 and human CCL22 mRNAs are as follows: mCcl22-forward, 5′-AGCCTGGGTAGGCTTGTGTGAT-3′, mCcl22-reverse, 5′-CCTGGGATCGGCACAGATA-3′, hCCL22-forward, 5′-CCCTACGGCGCCAACAT-3′, hCCL22-reverse, 5′-AAGCCTGGCGTTTGGAT-3′.

Western blotting. Western blotting analysis was performed as previously described20. Following antibodies were used: anti-PU.1 antibody (D-19), anti-IRF4 antibody (M-17), and anti-IRF8 antibody (C-19), (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin antibody (AC-15, Sigma-Aldrich).

ELISA. The concentration of mouse CCL22 protein was determined using an ELISA kit (MCC220, R&D systems, Minneapolis, MN).

Luciferase assay. A series of reporter plasmids carrying the wild type or mutant mouse Cld2 promoters were generated as previously described18 using primer sets listed in Supplemental Table 1.
Transfections of JAWSII, RAW264.7, and 293 T cells were performed as previously described. After 48 hours, luciferase activity was determined using ARVO Light (Perkin Elmer, Waltham, MA) and a Dual-luciferase assay kit (Promega), as previously described.

**EMSA.** Nuclear proteins were prepared from JAWSII cells. PU.1 protein was synthesized using a TNT T7 quick-coupled transcription/translation system (Promega) and pCR-PU.1 as a template. After incubation of the proteins and fluorescein-labeled oligonucleotides, electrophoretic mobility shift assay was performed as previously described.

**ChIP assay.** ChIP assays were performed as previously described. Antibodies against PU.1 and IRF4 were the same as those used in Western blotting analysis. Goat IgG (#02-6202, Invitrogen) was used as control antibody. The amount of immunoprecipitated or input DNA was quantified by a real-time PCR system using the primer sets (see Supplementary Table 2).

**Statistical analysis.** Statistical analysis was performed as previously described.

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**Author Contributions**

T.Y. designed research, performed experiments, and prepared the manuscript; S.N., K.N., Y.U. and K.K. performed experiments and analyzed data; C.N. designed research and prepared the manuscript.

**Additional Information**

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