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Involvement of PU.1 in the transcriptional regulation of TNF-α

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ABSTRACT

PU.1 is a myeloid- and lymphoid-specific transcription factor that serves many important roles in the development and specific gene regulation of hematopoietic lineages. Mast cells (MC) and dendritic cells (DC) express PU.1 at low and high levels, respectively. Previously, we found that enforced expression of PU.1 in MC resulted in acquisition of DC-like characteristics, including repression of several IgE-mediated responses due to reduced expression of IgE-signaling related molecules. In contrast, PU.1 overexpression in MC up-regulated TNF-α production in response to IgE- and LPS-stimulation suggesting that PU.1 positively regulates TNF-α expression. However, the role of PU.1 in the expression of TNF-α is largely unknown. In the present study, the effects of PU.1 on the TNF-α promoter in mouse bone marrow-derived (BM) MC and DC were studied. Real-time PCR, ELISA, and chromatin immunoprecipitation assays indicated that the kinetics and magnitude of TNF-α expression levels following LPS- or IgE-stimulation are related to the amount of PU.1 binding to the promoter. In brief, higher and delayed up-regulation of TNF-α promoter function was observed in DC, whereas there were lower and rapid responses in MC. When PU.1-overexpressing retrovirus vector was introduced into MC, the amount of PU.1 recruited to the TNF-α promoter markedly increased. The knockdown of PU.1 in BMDC by siRNA resulted in a reduction of TNF-α protein produced from LPS-stimulated BMDC. These observations indicate that PU.1 transactivates the TNF-α promoter and that the amount of PU.1 binding on the promoter is associated with promoter activity.

Introduction

The transcription factor, PU.1 belongs to the Ets-family, which possesses the Ets-domain as a DNA-binding region. PU.1 is involved in the development and specific gene regulation of the myeloid- and lymphoid-lineages, including macrophages, dendritic cells (DC), neutrophils, mast cells (MC), and lymphoid cells [1–7].

Although PU.1 is involved in specific gene expression in MC [7,8], the level of PU.1 expression itself is lower in MC than that in monocytes, including DC [9]. We previously found that enforced expression of PU.1 in MC or its progenitors induces various monocyte (DC-like)-characteristics, including specific gene expression and morphological changes [10–12]. This suggests that the level of PU.1 expression determines cell fate between MC and DC. MC that overexpress PU.1 exhibit enhanced expression of LPS-mediated cytokine production [10–13]. Although several IgE-mediated responses are suppressed by PU.1 overexpression, TNF-α production increases in response to IgE-stimulation as well as LPS-stimulation. These studies indicate that PU.1 may up-regulate the signaling pathway under TLR4 and/or that PU.1 may transactivate the TNF-α promoter in a stimulation-independent manner. Although PU.1 is reported to recognize the essential element in the TLR4 promoter [14] and to control alternative TLR4 promoter usage [15], overexpression of PU.1 does not affect the surface TLR4 expression level on MC [12]. In addition, the TLR4-signaling pathway was not identified through clustering analysis of microarray data, whereas the FcεRI-signaling pathway was significantly down-regulated in MC that overexpress PU.1 [13]. Based on these observations, we hypothesized that PU.1 directly transactivates the TNF-α promoter. Although several studies of the structure of the TNF-α promoter have been reported [16–19], the role of PU.1 in TNF-α promoter function is largely unknown.

In the present study, the involvement of PU.1 in TNF-α promoter function was analyzed. TNF-α expression levels were measured using ELISA and RT-PCR, and PU.1 recruitment to the promoter was monitored using the chromatin immunoprecipitation

Abbreviations: BM, bone marrow-derived; ChIP, chromatin immunoprecipitation; DC, dendritic cell; MC, mast cell.

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(ChIP) assay. This was conducted under various conditions of the cells, in which PU.1 was overexpressed by infection with retrovirus carrying PU.1 cDNA, or in which PU.1 expression was knocked down by introduction of PU.1 siRNA.

Materials and methods

Cells. To generate BMMC, BM cells prepared from BALB/c mice (Japan SLC, Hamamatsu, Japan) were grown in RPMI 1640 medium (Sigma–Aldrich) supplemented with 10% heat-inactivated FBS, 100 μM 2-ME, 10 μM MEM nonessential amino acids solution (Sigma–Aldrich), 100 U/ml penicillin, 100 μg/ml streptomycin, and pokeweed mitogen-stimulated spleen-condition medium (PWM-SCM) [20] for 3–5 weeks, as previously described [12]. BMDC were prepared from BALB/c BM cells with culture in RPMI 1640 supplemented with 10% FBS, 100 μM 2-ME, 10 μM MEM nonessential amino acids solution, antibiotics, and 10 ng/ml mouse rGM-CSF (PeproTech) based on a previously reported method [21].

LPS and IgE stimulation. LPS (from *Escherichia coli*; Sigma–Aldrich) was used to stimulate BMMC or BMDC via TLR4. Stimulation of BMMC via FcεRI was performed with mouse anti-DNP-IgE (BD Pharmingen) and anti-mouse IgE (R35–72; BD Pharmingen) as described previously [13,22].

Quantification of mRNA by real-time PCR. Total RNA prepared from BMMC or BMDC using an RNeasy Micro Kit (QIAGEN) was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The amount of mRNA of TNF-α, PU.1, and GAPDH was quantified using a 7500 Real-Time PCR System (Applied Biosystems) with TaqMan Gene Expression Assays (Applied Biosystems; #Mm00443258_m1 for TNF-α, #Mm00488140 for PU.1, and rodent GAPDH #4308313) and TaqMan Universal Master Mix (Applied Biosystems). Each expression level of TNF-α or PU.1 was defined as the ratio to GAPDH by calculating the cycle threshold (Ct) values in amplification plots using 7500 SDS software as described previously [23].

Determination of TNF-α *production level by ELISA.* The amount of TNF-α protein in culture medium was measured using a Quantikine ELISA Kit (R&D Systems) [13].

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was performed as previously described [24,25]. Anti-PU.1 goat IgG (#D-19, #sc-5949, Santa Cruz Biotechnology) and goat IgG (#02-6202, Invitrogen) were used. Chromosomal DNA was quantified with a 7500 Rea-Time PCR System using the following synthesized primers and TaqMan probes (Applied Biosystems): for TNF-α promoter (−132/−71), forward primer TNF-α-132F (5′-CCGCTTCCTCCACATG AGA-3′), reverse primer TNF-α-71R (5′-TCATTCAACCCTCGGAAAA CTT-3′), and TaqMan probe TNF-α-112P (5′-FAM-CATGGTTTTCTCC ACCAAG-MGB-3′).

PU.1 overexpression with retrovirus vector. Retrovirus vector carrying PU.1 cDNA and its mock vector were generated by transfection of packaging cells Plat-E with plasmids pMXs-puro-PU.1 [13] and pMXs-puro, respectively. BMMC was then transfected with each retrovirus vector and maintained in the presence of puromycine to select transfectants as described previously [10,12,13].

siRNA-mediated inhibition of PU.1 expression. PU.1 siRNA (Stealth Select RNAi, #MSS247676) and control siRNA (Stealth RNAi Negative Universal Control, #45-2001) were purchased from Invitrogen. A 5 μl aliquot of 20 μM siRNA was introduced into 1×10^5 BMDC with a Mouse Macrophage Nucleofector Kit (Amaxa GbmH, Koeln, Germany) using Nucleofector II (Amaxa) set at Y-001 as described in our previous reports [24,26].

Results

Transcription kinetics of TNF-α in BMDC and BMMC

In order to analyze the kinetics of the transcription levels, the amount of TNF-α mRNA in BMDC and BMMC was measured at various time points following LPS or IgE stimulation. The level of TNF-α mRNA in LPS-stimulated BMDC reached a maximum at 1 h post-stimulation of approximately 70-fold that of the control cell level without stimulation (Fig. 1A, left). In IgE-stimulated BMMC, the level of TNF-α mRNA rapidly increased to a maximum value at 30 min of approximately 30-fold that of control cell level and then markedly decreased to 3 h post-stimulation (Fig. 1A, right). In LPS-stimulated BMMC, the level of TNF-α mRNA increased by 2.5-fold that of the control cell level; a much smaller increase than those seen in the LPS-stimulated BMDC and IgE-stimulated BMMC (Fig. 1A, middle). These results suggest that TNF-α transcription rapidly peaked and then decreased to the basal level in IgE-stimulated BMMC in contrast to sustained up-regulation in LPS-stimulated BMDC.

At the same time, the concentration of TNF-α in the culture medium released from cells was also measured (Fig. 1B). The TNF-α concentration released from DC was markedly higher than that from either IgE-stimulated MC or LPS-stimulated MC, while LPS-stimulated BMDC showed the highest response. Conversely, the concentration of TNF-α released from LPS-stimulated BMMC was the lowest, indicating that the kinetics and magnitude of TNF-α protein production from stimulated cells are somewhat affected by transcriptional regulation.

Specific binding of PU.1 to the TNF-α promoter in BMDC and BMMC

A ChIP assay was performed to examine whether PU.1 binds to the TNF-α promoter regions on chromosomal DNA in BMDC and BMMC. As shown in Fig. 2A, the amount of chromosomal DNA around the TNF-α promoter immunoprecipitated with anti-PU.1 Ab was significantly higher than that with control Ab in non-stimulated BMDC or BMMC. The relative input unit of specific binding of PU.1 in BMDC (Fig. 2A, left) was higher than those in LPS- and IgE-stimulated BMMC (Fig. 2A, middle and right, respectively). The PU.1-binding level on the promoter was transiently up-regulated following LPS- or IgE-stimulation reaching a maximum value at 30 min post-stimulation in LPS-stimulated BMDC, and at 15–30 min in LPS- or IgE-stimulated BMMC, and rapidly decreased to control cell levels at 60 min post-stimulation in BMMC. In contrast, the increased binding remained present at 60 min post-stimulation in BMDC. Such significant binding of PU.1 was not observed when a non-related control region was analyzed (data not shown). These results indicate that PU.1 constitutively binds the TNF-α promoter in BMDC and BMMC and that the amount of PU.1 binding to these promoters parallels the expression magnitude and kinetics. When the PU.1-binding motifs GGAA and AGAA [8] were searched for in the promoter region, nine motifs were found in 200 bp of the TNF-α promoter, which include the essential elements required for LPS- or cytomegalovirus-induced activation in a monocytic cell line [16–19] (Fig. 2B). Therefore, PU.1 may bind the promoter region via these multiple motifs.

Occupancy of the TNF-α promoter by PU.1 in PU.1-overexpressing MC

When PU.1 was overexpressed in BMMC, LPS-induced TNF-α production dramatically increased and reached the same level of production as that from BMDC [13]. Considering the present results, the occupancy of the TNF-α promoter with PU.1 may have been increased by enforced expression of PU.1. To confirm this

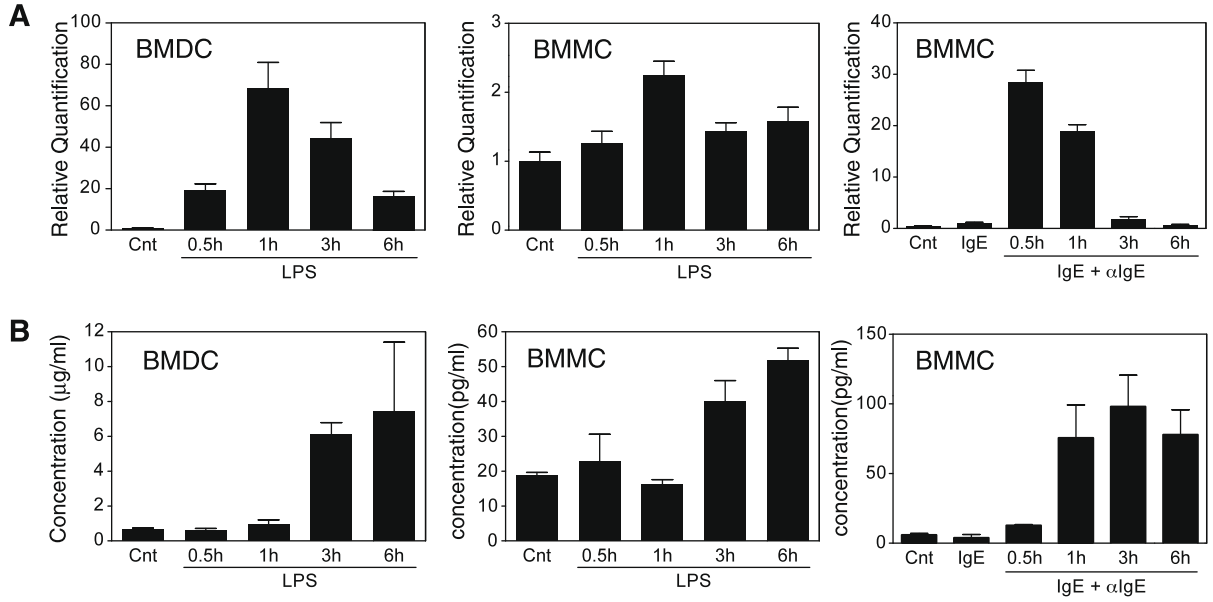

Fig. 1. TNF-α expression levels in stimulated BMDC and BMMC. (A) Fold increase of TNF-α mRNA in LPS- or IgE-stimulated BMDC and BMMC. Messenger RNA levels of TNF-α normalized against GAPDH are presented as the ratio to that without LPS (left and middle) or to that of IgE alone without anti-IgE Ab (right). Data represent average ± SD of triplicate samples. A representative result from three independent experiments is shown. Cnt: control cells without any additives for stimulation in all figures. (B) The concentration of TNF-α protein released from stimulated BMDC and BMMC. Data represent average ± SD of triplicate samples. Results are representative of three independent experiments.

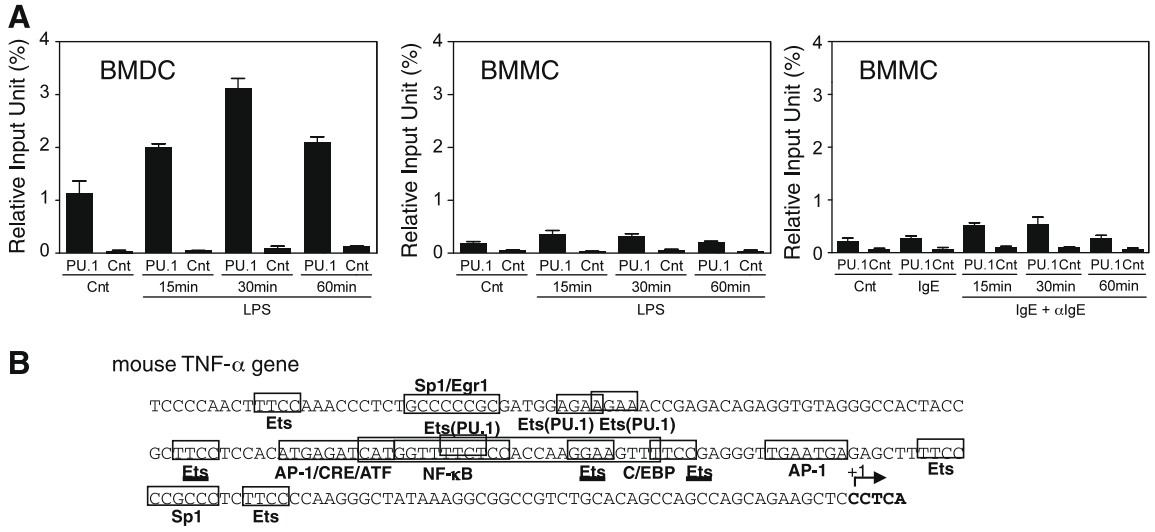

Fig. 2. PU.1 recruitments to the TNF-α promoter. (A) The amount of PU.1 binding to the TNF-α promoter in stimulated BMDC and BMMC. Data represent mean ± SD of PCR performed three times with duplicate samples for each ChIP. Similar results were obtained in two additional ChIP experiments. PU.1, anti-PU.1 Ab; Cnt, control Ab. (B) Nucleotide sequence of the mouse TNF-α promoter. *Cis*-enhancing elements identified in the human TNF-α promoter and conserved in the mouse TNF-α promoter are shown [16–18]. Ets boxes marked with underlined "Ets" were identified to be *cis*-enhancing elements in those studies. Additional consensus Ets-family protein binding sequences (GGAA) and PU.1-bindable sequences (AGAA) are boxed and marked with Ets and Ets (PU.1), respectively [8].

hypothesis, a ChIP assay was performed using retrovirus-transfected MC. The prepared BMMC with overexpression of PU.1 exhibited a high level of TNF-α production in response to LPS stimulation, equivalent to that of BMDC, while retaining weak expression of TLR4 (data not shown), as seen in our previous studies [12,13]. The relative input unit of PU.1 in the mock transfectants was moderately increased to approximately 0.5% by LPS stimulation (Fig. 3), which is consistent with the data of normal BMMC (Fig. 2A, middle). The occupancy of the TNF-α promoter by PU.1 in BMMC was significantly increased by transfection of PU.1-expressing retrovirus, and a further increase was observed following LPS-stimulation (Fig. 3). These results suggest that higher occupancy of the TNF-α promoter with PU.1 is one of the mechanisms that caused the higher production of TNF-α in the PU.1 transfectants.

Effect of PU.1 knockdown by siRNA on TNF-α and IL-6 production from stimulated BMDC

The results above demonstrate binding of PU.1 to the TNF-α promoter and an association between the amount of PU.1 on the promoter and the TNF-α expression level in stimulated BMDC and BMMC. Based on these observations, we performed PU.1 knockdown by siRNA to evaluate the effect of PU.1 in the expression of TNF-α. Introduction of PU.1 siRNA maintained the reduc-

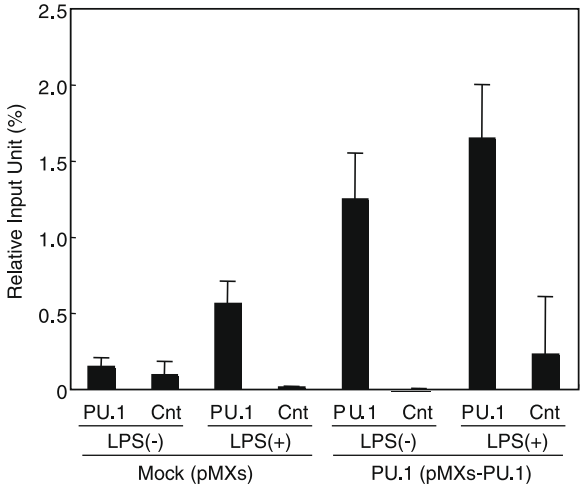

Fig. 3. Occupancy of PU.1 on the TNF-α promoter in PU.1-overexpressing cells. ChIP assays were performed using retrovirus transfectants carrying mock or PU.1 cDNA. Data represent mean ± SD of three PCRs with duplicate samples. Similar results were obtained in two additional ChIP experiments. PU.1, anti-PU.1 Ab; Cnt, control Ab.

tion of PU.1 mRNA levels at 5% of the control siRNA-introduced BMDC levels 24–48 h after electroporation (Fig. 4A). At 24 h post-siRNA treatment, the concentration of TNF-α protein in the culture medium of BMDC was determined at various time points following LPS-stimulation. As shown in Fig. 4B, the amount of TNF-α protein produced from PU.1 siRNA-introduced BMDC was markedly lower at 3 and 6 h. Such suppression was not observed in the concentration of IL-6 released from LPS-stimulated BMDC (Fig. 4C). This observation suggests that PU.1 is involved in the production of TNF-α in BMDC.

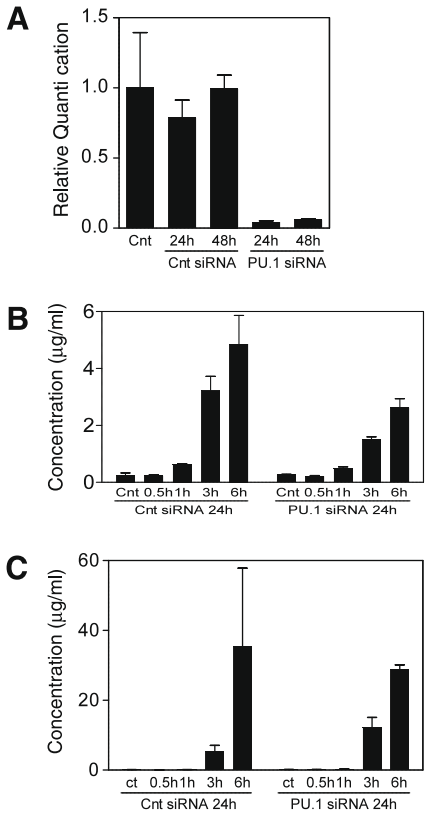

Fig. 4. Effect of PU.1 knockdown on the production of TNF-α from BMDC. The amount of PU.1 mRNA in siRNA-treated BMDC (A). Data represent mean ± SD of triplicate samples. Results are representative of three independent experiments. The concentration of TNF-α (B) and IL-6 (C) in culture medium of siRNA-treated BMDC. Data represent mean ± SD of two independent experiments with triplicate samples. Similar results of suppressed protein production were observed in another experiment.

Discussion

Several Ets-related motifs are present in the mouse TNF-α promoter, as shown in Fig. 2B. Three of them (labeled with underlined "Ets" in Fig. 2B) have been previously identified as *cis*-enhancing elements in LPS-stimulated activation of the TNF-α promoter in a human macrophage cell line [16,17]. The veracity of *in vitro* binding of PU.1 to these elements is controversial. In brief, the underlined Ets motif located at the most upstream region was reported to be bound to PU.1 in an EMSA experiment, but without provision of data [17]; however, PU.1 did not bind to this site in a DNaseI footprinting experiment even when binding of other Ets-family proteins, Ets-1 and Elk-1, was possible [16]. This Ets motif was also required for cytomegalovirus-mediated activation of the TNF-α promoter in monocytes and PU.1 was suggested to be a candidate transcription factor recognizing this element, although the binding of PU.1 against this sequence was not confirmed [19]. In the present study, we demonstrated the binding of PU.1 on the TNF-α promoter region of chromosome DNA in primary living DC by the ChIP assay and the involvement of PU.1 in TNF-α production by siRNA.

Introduction of PU.1 siRNA suppressed TNF-α production from LPS-stimulated BMDC. We cannot completely dismiss the possibility that PU.1 siRNA also down-regulated the expression and/or function of TLR4-signaling pathway-related molecules, although this pathway was not previously identified to be affected by PU.1 overexpression in MC [13]. However, we believe that PU.1 functions as a transactivator of the TNF-α promoter, based on the effect of PU.1 siRNA on TNF-α production and the lack of effect of PU.1 siRNA on the IL-6 promoter. In addition, IgE-mediated TNF-α production was also up-regulated, even under the conditions of IgE-signaling suppression, which cause a reduction in several IgE-mediated responses [13]. The TNF-α promoter in the PU.1-overexpressing cells was further occupied by PU.1 in the ChIP assay with retrovirus transfectants, also suggesting that TNF-α is the direct target of PU.1.

Previously, we found that PU.1-overexpressing MCs acquired high capacity for LPS-induced production of TNF-α and IL-6 to a similar degree [13], whereas in the present study PU.1 siRNA suppressed TNF-α expression but did not affect IL-6 expression in LPS-stimulated DCs. This discrepancy suggests that PU.1 acts both directly and indirectly in LPS-induced stimulation. PU.1 is directly involved in TNF-α transcription via direct interaction with the promoter in a TNF-α promoter-specific manner. The indirect effect of PU.1 may manifest as follows: an as yet unidentified molecule, which positively regulates LPS-induced stimulation signaling for expression of TNF-α and IL-6 in a similar manner, may be up-regulated by PU.1 in MCs but may not be down-regulated by PU.1 siRNA in DCs. Further studies to clarify the exact mechanism of the indirect effect of PU.1 are required to address this discrepancy in the results.

In the present study, the amount of PU.1 binding to the TNF-α promoter was increased following LPS-stimulation. LPS is known to activate PU.1 function by modulating the phosphorylation state of Ser in macrophages [27], which may one of the mechanisms of the rapid recruitment of PU.1 toward the promoter. In addition, LPS up-regulates the PU.1 protein levels in monocytes [27] and MCs [12]. Therefore, PU.1 involvement in LPS-induced transactivation of the TNF-α promoter may be mediated through at least two pathways.

DC introduced with PU.1 siRNA still possessed TNF-α producing capacity, although production was reduced. This remaining capacity may mean that the contribution of PU.1 to TNF-α promoter function is not as high as that of other transcription factors. Previously, several transcription factors, including the AP-1 family, Sp1, NF-kB, Egr-1, NF-ATp, Ets-1, and Elk-1, have been identified as binding to the TNF-α promoter [16,18]. To evaluate the degree of contribution of each transcription factor, the TNF-α production level should be compared among cells in the presence of each siRNA. Monocytes, macrophages, DC, T- and B-cells, MC, and fibroblasts are known to produce TNF-α. The expression of PU.1 is restricted in some of the myeloid- and lymphoid-lineages, and is detected in monocytes/macrophages/DC-lineage, the T/B-lineages, and MC, which is consistent with the TNF-α production profile. This observation may support the possible involvement of PU.1 in TNF-α promoter function. Considering the absence of PU.1 in fibroblasts, the effects of PU.1 on TNF-α promoter function may be restricted in TNF-α producing-hematopoietic cells, and this promoter may be regulated by a different combination of transcription factors in fibroblasts.

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