Functional analysis of PU.1 domains in monocyte-specific gene regulation

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Abstract The Ets family transcription factor PU.1 is required for the development of various lymphoid and myeloid cell lineages, and regulates the expression of several genes in a cell type-specific manner. Recently we found that overproduction of PU.1 in mouse bone marrow-derived mast cell progenitors induced the expression of monocyte-specific genes. This prompted us to analyze the functions of each domain of PU.1 in monocyte-specific gene expression, using transfection of mast cell progenitors with a series of retrovirus vectors for overexpression of various truncation mutants. Both the acidic region and the Ets domain of PU.1 were required for expression of monocyte-specific genes, and for enhanced interleukin-6 production in response to lipopolysaccharide. The Gln-rich region was suggested to be involved in expression of both MHC class II and F4/80. On the other hand, when PU.1 protein lacking the PEST domain was produced in the progenitor cells, expression of monocyte-specific genes was substantially enhanced, suggesting that the PEST domain plays a negative role in monocyte-specific gene expression.
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Key words: PU.1; MHC class II; CD11b; CD11c; F4/80; PEST

1. Introduction

PU.1 is an Ets family transcription factor involved in myeloid and lymphoid cell lineage development. The requirement for PU.1 in generation of these lineages was demonstrated by a PU.1-knockout mouse, in which macrophage and B cell production was abolished, and production of neutrophils and T cells was delayed [1–3]. Recent analysis of the PU.1-knockout mouse revealed that PU.1 was required for the development of myeloid dendritic cells (DCs) [4,5]. PU.1 is expressed in multipotent, interleukin (IL)-3-dependent hematopoietic progenitor cells, B cells, macrophages, mast cells, and neutrophils [6]. In mast cells PU.1 is also shown to regulate expression of the high affinity receptor for IgE (FcεRI) [7], which plays an important role in IgE-mediated allergic reactions. In addition, PU.1 is involved in mast cell-specific gene regulation in combination with transcription factors GATA-1 and -2 in a mast cell-specific manner [7–9]. Thus, PU.1 may play a primary role in the allergic response in mast cells.

It has also been reported that PU.1 determines the lineage commitment of macrophage/B cell [10] and macrophage/neutrophil cell fates [11] in a dose-dependent manner, and overproduction of PU.1 in an erythroid cell line induced a lineage switch to myelomonocytic cells [12]. In our recent analysis, overproduction of PU.1 in mast cell progenitors induced monocyte-specific gene expression and caused morphological changes [13], suggesting that the expression level of PU.1 determines cell fates between mast cells and monocyte as in the case of macrophage/B cells and macrophage/neutrophils.

PU.1 contains four functional domains (regions), the acidic region, the Gln-rich region, the PEST domain, and the Ets domain. PU.1 binds to target DNA via the Ets domain at the C-terminus, and the acidic and Gln-rich regions are believed to be necessary for transactivation [6]. However, the function of each domain is different depending on the target genes and/or the differentiation stage of cells. In this study, we analyzed the function of each domain of PU.1 in monocyte-specific gene expression by using retrovirus vectors expressing various truncation mutants of PU.1.

2. Materials and methods

2.1. Cells

Bone marrow cells prepared from BALB/c mice (Japan SLC, Hamamatsu, Japan) were grown in RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 100 μM minimum essential medium, non-essential amino acids (Invitrogen, San Diego, CA, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM 2-mercaptoethanol, and 10% pokeweed mitogen-stimulated spleen-conditioned medium [14]. A retrovirus packaging cell line, Plat-E [15], was maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml puromycin (Sigma-Aldrich), and 10 μg/ml blasticidin (Funakoshi, Tokyo, Japan).

2.2. Constructs

A plasmid, pMX-puro-PU.1 [13], was used to generate retrovirus vector to overproduce wild-type PU.1. To construct the plasmids for expression of PU.1 deletion mutants, pMX-puro-PU.1-ΔAcid, pMX-puro-PU.1-ΔGln, pMX-puro-PU.1-ΔPEST, and pMX-puro-PU.1-ΔEts, DNA fragments corresponding to the desired domains were deleted after introduction of restriction endonuclease recognition sequences at the corresponding positions of pCR-2F-PU.1 by site-directed mutagenesis.

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Abbreviations: DC, dendritic cell; IRF, interferon regulatory factor; LPS, lipopolysaccharide; TLR, Toll-like receptor

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2.3. Transfection

Infection of bone marrow-derived cells was performed with previously reported methods [13,16,17]. In brief, each plasmid of the pMX-puro series was transiently introduced into Plat-E with Fugene6 (Roche Diagnostics, Indianapolis, IN, USA) to obtain culture media containing infectious viruses. Bone marrow cells cultivated for 2 weeks under the conditions described above were incubated with the infectious supernatants for 2 days in the presence of 10 μg/ml of polybrene (Sigma-Aldrich). Infected cells were selected by cultivating in the presence of 1.2 μg/ml or puromycin for 10–20 days.

2.4. Flow cytometric analysis

Fc receptors on the cell surface were blocked with 2.4G2 (BD PharMingen, San Diego, CA, USA) before staining. The anti-mouse antibodies used were FITC-conjugated anti-I-A^d, anti-CD11b, anti-CD11c, and anti-F4/80, and PE-conjugated anti-c-kit, all of which were purchased from BD PharMingen. Staining of cells was performed as previously described [18,19], and cell surface expression of each molecule was analyzed by FACSCalibur (BD Biosciences, Mountain View, CA, USA).

2.5. Cytokine measurements

Concentrations of IL-6 in the culture supernatant were determined by ELISA kits after incubation for 6 h with or without lipopolysaccharide (LPS) (from *Escherichia coli*; Sigma-Aldrich) according to the manufacturer's instructions (Genzyme Techne, Minneapolis, MN, USA).

2.6. Cytochemical analysis and electron microscopy

Cells were cytocentrifuged onto glass slides and stained with May-Grünwald-Giemsa staining solution (Muto Pure Chemicals, Tokyo, Japan). Transfected cells were fixed with 2.5% glutaraldehyde in 0.1 M pH 7.4 Sørensen phosphate buffer for 1 h at 4°C, dehydrated in graded acetone solutions, and embedded in Epok-812 (OKEN, Ohken shoji, Tokyo, Japan).

3. Results

3.1. Effect of truncation of PU.1 on expression of monocyte-specific gene

To elucidate the roles of domains/regions of PU.1 in monocyte-specific gene expression, we transfected murine bone marrow-derived progenitor cells with retrovirus vectors carrying wild-type and a series of PU.1 mutants (Fig. 1A). In this system, wild-type and PU.1 mutants were overproduced in the cells (Fig. 1B).

As we recently reported, overproduction of wild-type PU.1 induced the expression of monocyte-specific genes, such as MHC class II, CD11b, CD11c, and F4/80, and repressed the expression of c-kit [13] (Fig. 2). Cells infected with a virus expressing PU.1-ΔEts or PU.1-ΔAcid showed profiles similar to those of the cells infected with the mock virus (Fig. 2). This observation indicates that the Ets domain and the acidic region are required for activating monocyte-specific gene expression and for repressing mast cell-specific gene expression as observed in the PU.1-overproduction experiment. Because the Ets domain and the acidic region of Ets family transcription factors are thought to play roles in DNA binding and transactivation, respectively, we concluded that PU.1 binds target sequences by its Ets domain and exerts its transcription activation ability derived from the acidic region.

Overproduction of the mutant that lacked the PEST domain (PU.1-ΔPEST) gave expression profiles similar to those observed with wild-type PU.1 for MHC class II, F4/80, and c-kit. However, the PU.1-ΔPEST construct exhibited higher expression of CD11b and CD11c. In contrast, deletion of the Gln-rich region dramatically decreased expression of MHC class II and F4/80 but did not affect expression levels

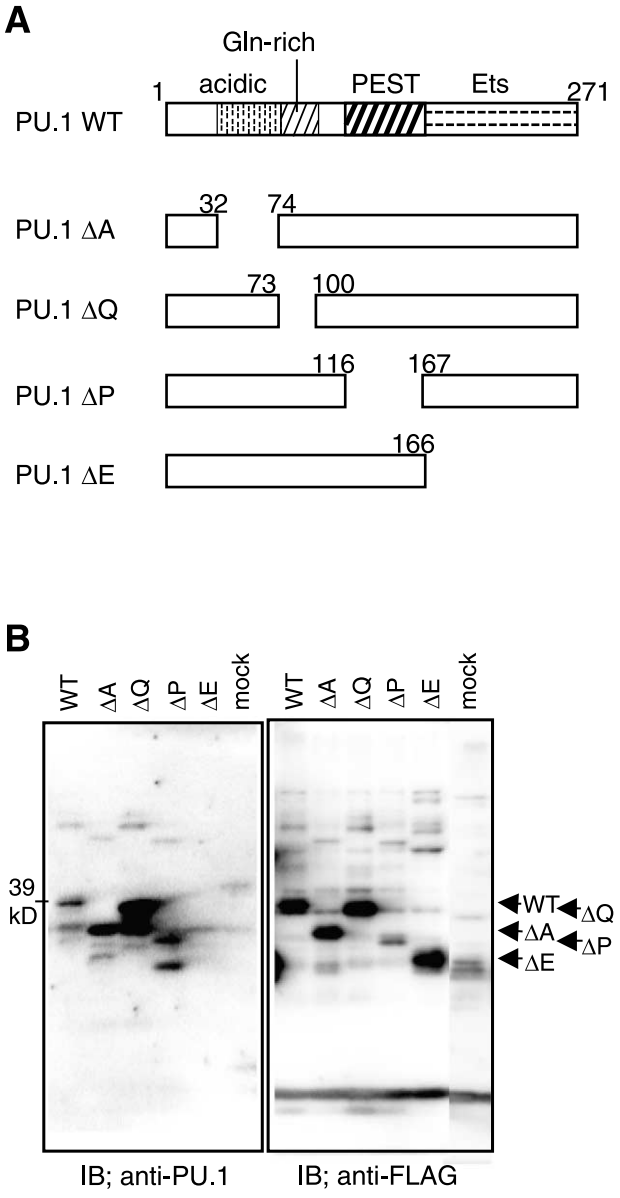

Fig. 1. PU.1 and its derivatives. A: Structure of wild-type or various mutants of PU.1. WT, wild-type; ΔA, PU.1-ΔAcid (lacking the acidic region); ΔQ, PU.1-ΔGln (lacking the Gln-rich region); ΔP, PU.1-ΔPEST (lacking the PEST domain); ΔE, PU.1-ΔEts (lacking the Ets domain). B: Western blotting analysis for PU.1. Lysates (5×10^5 cells per lane) were analyzed by using anti-PU.1 or anti-FLAG antibody.

of CD11b and CD11c (Fig. 2). These results suggest that each domain (region) of PU.1 has an individual role in expression of these markers.

3.2. IL-6 production in response to LPS

Toll-like receptors (TLRs) are highly conserved proteins and recognize specific compounds, like LPS, derived from pathogenic microorganisms. TLR4 signaling activated by LPS induces monocytes to produce proinflammatory cytokines including IL-6 [20]. Therefore, IL-6 production by LPS stimulation in the PU.1-overproducing cells could be an index showing that the progenitor cells acquired monocyte-like features. To examine whether the transfected cells responded to LPS, we analyzed the production of IL-6 in

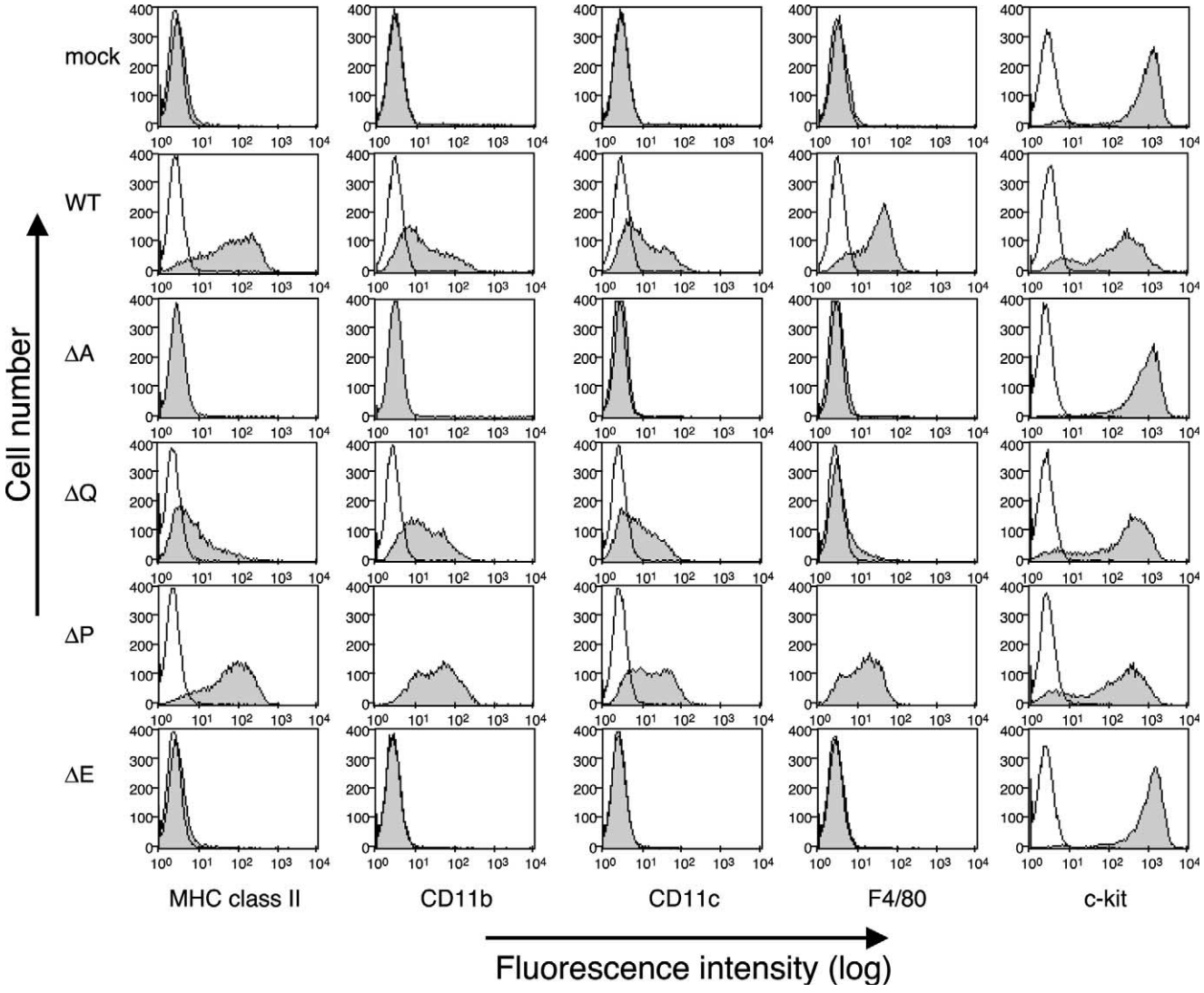

Fig. 2. Phenotypes of bone marrow-derived cells transfected with retrovirus carrying wild-type or various mutant PU.1 cDNAs. Typical results are shown. Similar results were obtained in five other independent experiments.

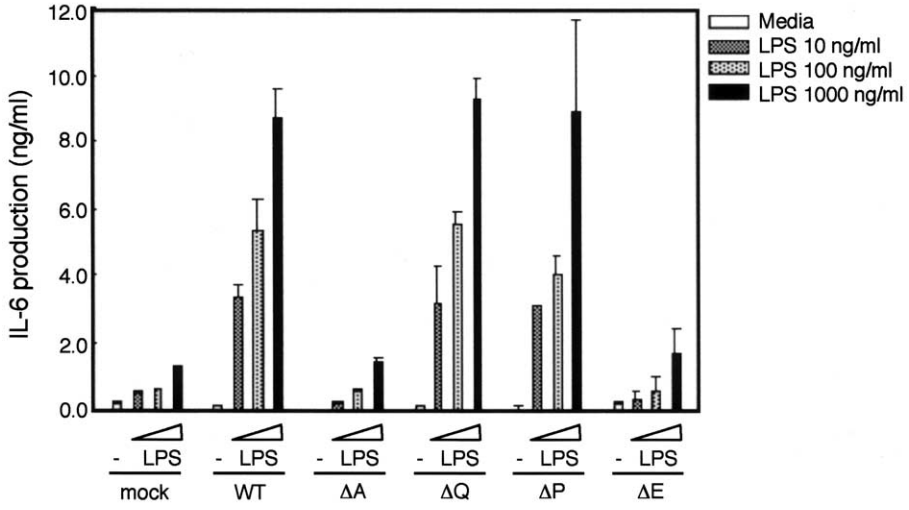

Fig. 3. Responses of transfected cells to stimulation with LPS. IL-6 production by cells stimulated with LPS. Data represent mean ± S.D. of more than three independent experiments, each performed in duplicate.

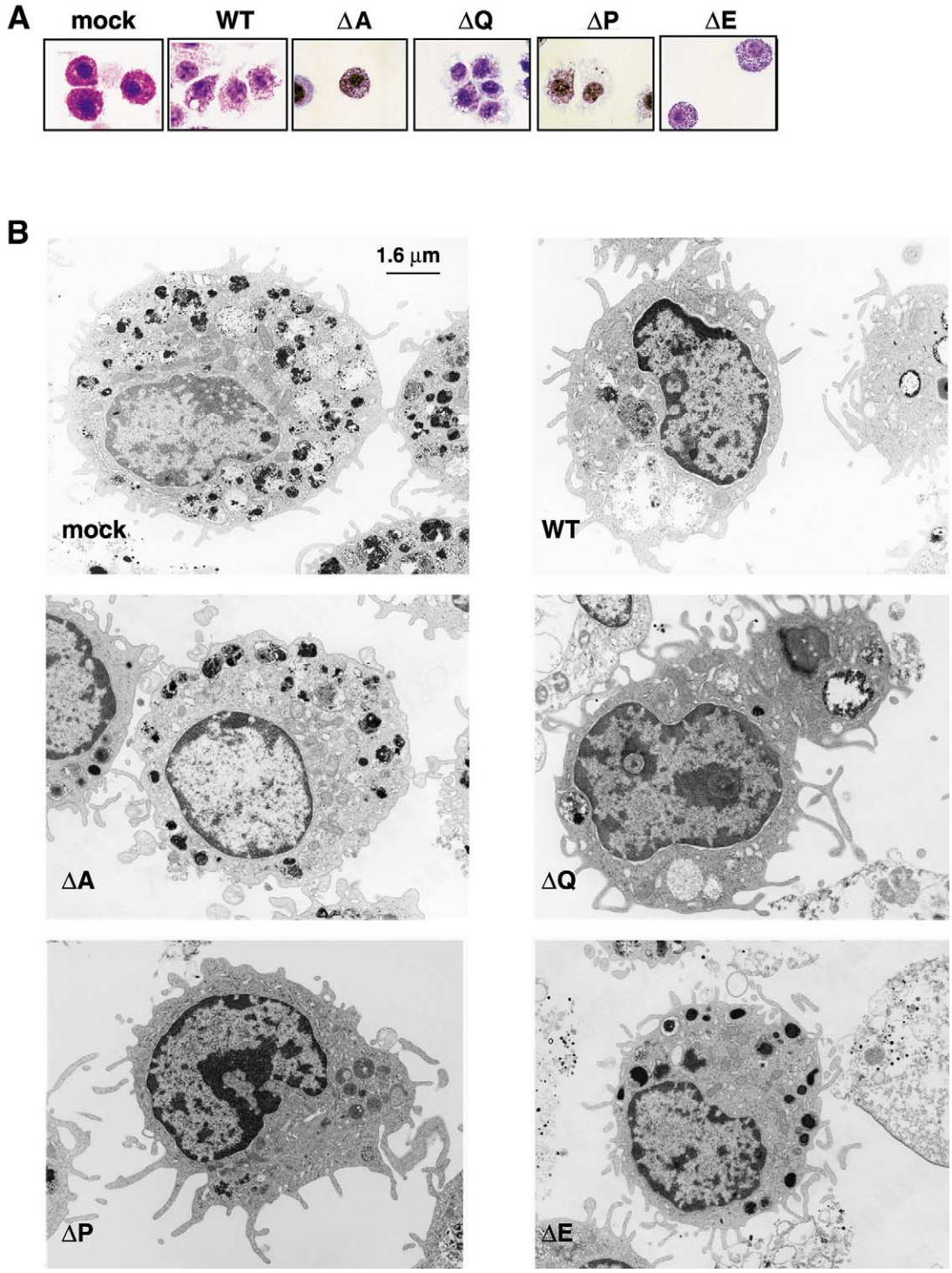

Fig. 4. Morphology of bone marrow-derived cells transfected with retrovirus carrying wild-type or various mutant PU.1 cDNAs. A: May-Grünwald-Giemsa staining of cytospun cells. ×400. B: Electron micrographs of cells. Magnification is 6000× and bar = 1.6 μm.

the transfected cells after LPS stimulation (Fig. 3). The IL-6 production was markedly increased in cells producing wild-type PU.1, PU.1-ΔGln, and PU.1-ΔPEST. In contrast, the IL-6 production level was significantly lower in the cells infected with the virus directing the overexpression of PU.1-ΔAcid or PU.1-ΔEts, similar to the case of mock-transfected cells.

3.3. Morphological analysis

The morphology of each cell was analyzed by May-Grünwald-Giemsa staining of cytospun samples and by electron microscopy (Fig. 4A,B). When transfected with the virus expressing the PU.1-ΔAcid and PU.1-ΔEts genes, the cells were found to contain numerous granules (Fig. 4A), which is typical of mast cells. On the other hand, cells that overexpressed wild-type PU.1, PU.1-ΔGln, and PU.1-ΔPEST contained few granules but showed monocyte-like morphology characterized by vacuoles in the cytoplasm, eccentric nuclei, and polarized lamellipodia (Fig. 4A), as observed for mouse macrophages

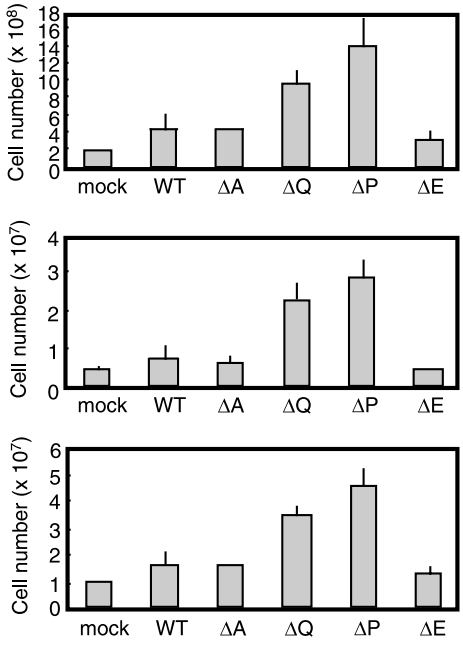

Fig. 5. Effects of wild-type and mutants PU.1 on proliferation of infected cells. Cells were counted after cultivation for 20 days in the presence of puromycin.

and DCs [21,22]. Under electron microscopy, larger veils and lamellipodia extending from cell bodies were observed in cells overexpressing wild-type PU.1, PU.1-ΔGln, or PU.1-ΔPEST but not in the cells overexpressing PU.1-ΔAcid and PU.1-ΔEts (Fig. 4B). From these results we concluded that the Ets domain and the acidic region of PU.1 are required for function of this molecule in morphological change.

3.4. Overproduction of PU.1 increases cell number

When the progenitor cells were infected with the retrovirus carrying cDNA of wild-type PU.1, an increase (1.5–2 times) in cell number was observed (Fig. 5). A marked increase in cell number was also observed in the cells infected with a retrovirus carrying PU.1-ΔGln, and PU.1-ΔPEST, whereas an apparent increase in cell number was not observed upon overproduction of PU.1-ΔEts. These results suggest that PU.1 accelerates survival and proliferation of hematopoietic progenitors through the functions of the acidic region and the Ets domain, and that the Gln-rich region and the PEST domain repress the activating function of PU.1.

4. Discussion

Recently, we found that overproduction of PU.1 induced monocyte-specific gene expression in mast cell progenitors and caused morphological changes [13]. In the present study, we observed the enhancement of IL-6 production in response to LPS stimulation by the overproduction of PU.1. To elucidate a role of each domain of PU.1 in these effects, we transfected mast cell progenitors with a series of retrovirus vectors directing the expression of various truncation mutants. The acidic region and the Ets domain were required for induction of both the monocyte-specific gene expression and the morphological changes. The involvement of the Ets domain in macrophage development, mediating effects such as the enhanced expression of Mac-1 (CD11b/CD18) and F4/80 and morphological change, has also been demonstrated in a similar PU.1 truncation analysis [23]. However, in that study, it was reported that the acidic region was not required for the development of macrophages, although the requirement of the acidic region for the transactivating function of PU.1 was also observed in several other studies [10,24–26]. Contradictory results are also found in the functional analysis of the Gln-rich region. The Gln-rich region was shown to be required for the function of PU.1 in most cases [10,12,23,25], but there is also a report that denies the involvement of the region in the function [26]. In our study using a retrovirus overexpression system, the Gln-rich region was critically required for the expression of F4/80 and involved in the expression of MHC class II, but not required for the expression of CD11b and CD11c, suggesting the involvement of the region in a certain kind of monocyte-specific gene expression. We currently cannot explain these controversial results. We may assume that each domain of PU.1 has different functions, depending on target gene, lineage and developmental stage of the cells producing PU.1.

The PEST domain of PU.1 is reported to serve as the interacting domain with transcription factors such as interferon regulatory factor (IRF) family transcription factors, IRF-4, and ICSBP/IRF-8 [27]. We showed that deletion of the PEST domain did not decrease the expression of monocyte-specific genes, such as MHC class II and F4/80, nor did it affect the morphological changes in our PU.1-overproducing system, suggesting that the domain is not involved in the changes in the gene expression profile and the cell morphology. However, the deletion of the PEST domain resulted in enhanced expression of CD11b and CD11c. This result suggests that the PEST domain may have a function to repress the expression of the genes through association with transcription factors such as IRF-4 and ICSBP, both of which are expressed in lymphoid and myeloid cells [28–30]. The production level of PU.1-ΔPEST was shown to be lower than that of wild-type and other mutants by Western blotting analysis with anti-FLAG antibody (Fig. 1B and not shown). This suggests that the changes induced by PU.1-ΔPEST may have been underestimated. It should be noted that the PU.1 mutant lacking the PEST domain also has a higher potential for inducing the proliferation of DC-like monocytes. This finding may suggest that masking the function of the PEST domain by some way might be adapted for preparation of DCs, which is expected to be used in immunotherapeutic approaches for treating allergies, autoimmune diseases, infectious diseases, and cancer.

Deletion of the Gln-rich region or the PEST domain caused a marked increase in transfected cell numbers. The effect is intriguing, because wild-type PU.1 does not show such a significant effect on cell numbers, which is in contrast with the observation that wild-type and both PU.1 mutants show the same phenotype in morphology and IL-6 assay. This may suggest unidentified functions of the Gln-rich region or the PEST domain of PU.1 in cell proliferation or apoptosis. At present, we cannot explain the effect of the deletion on the increase in cell number. Further detailed analysis such as estimation of cell death (apoptosis) and proliferation rate in various cell population will be required to elucidate the mechanism.

The mechanism of enhanced expression of IL-6 in response to LPS in PU.1-overproducing cells is unclear. A previous

report suggested that PU.1 recognizes the promoter of TLR4 [31]. Therefore one might imagine that the stimulatory signal of LPS was amplified in the cells through overproduction of TLR4. However, this was not the case in this study, because the level of cell surface expression of TLR4 was not affected by overproduction of PU.1 (preliminary observation). Previously it was reported LPS stimulation caused functional and conformational changes of PU.1 through phosphorylation of specific Ser residues [32]. This suggests the possibility that PU.1 is downstream of a TLR4-mediated signal cascade. Indeed, further analysis will be required to clarify the role of PU.1 in the production of IL-6.

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