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# Opposite effects of PU.1 on mast cell stimulation

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ABSTRACT

An Ets-family transcription factor PU.1 is involved in the development and specific gene regulation of hematopoietic cells. PU.1 also determines the commitment between several lineages via its expression level. Although enforced expression of PU.1 in mast cells (MC) induced expression of monocyte-specific markers and morphological change from MC to monocytes, especially dendritic cells (DC), in the previous report, intracellular events caused by PU.1 are largely unknown. In the present study, effect of PU.1 on IgE- and LPS-mediated stimulation degrees was analyzed. The amounts of IL-6, IL-13, and TNF-α produced from LPS-stimulated MC were markedly increased by overexpression of PU.1. In contrast, IL-6 and IL-13 production levels in response to IgE were reduced by PU.1, whereas that of TNF-α was up-regulated. β-Hexosaminidase release as a means of degranulation was decreased in PU.1 transfectants. When eicosanoid generation in response to IgE-stimulation was analyzed, overexpression of PU.1 reduced leukotriene $C_4$ ($LTC_4$) release, but enhanced $PGD_2$ production. Microarray analysis suggested that expression of FcεRI signal pathway related molecules were suppressed in PU.1 overexpressing MC as well as DC. These observations indicate that up-regulation of PU.1 suppresses expression of FcεRI signal transduction-related intracellular molecules, but increases the potential of transcription activity of monocyte characters.



Transcription factor PU.1 plays important roles in the development of myeloid- and lymphoid-lineages and specific gene regulation. PU.1 is expressed in lymphoid cells, macrophages, dendritic cells (DC), neutrophils, and mast cells (MC) in a cell type-specific manner [1], and required for the development of these lineages [2–7]. In addition, the expression level of PU.1 determines cell fate between different lineages, including B cells/macrophages [8] and neutrophils/macrophages [9]. In our recent studies, PU.1 exhibited the potential for determination of commitment between MC/monocytes [10–12]. In brief, we found that overexpression of PU.1 in MC and its progenitors induced the expression of monocyte-specific genes and caused the morphological change from MC to DC/macrophages. It is also demonstrated that overexpression of PU.1 in $CD34^+$ human myeloid progenitors triggered development of Langerhans cells (LC) [13].

Although the effect of PU.1 on expression of cell-specific markers and morphological change is drastic, detailed information of the effect for expression of other genes that involve the function of MC is remains unclear. In the present study, we compared the responses against IgE- and LPS-mediated activations between normal MC and PU.1-introduced MC. The production levels of IL-6 and IL-13 in response to stimulation through FcεRI were reduced by overexpression of PU.1; in contrast, that of TNF-α was up-regulated. The amounts of cytokines produced from LPS-stimulated cells were markedly increased by PU.1. Degranulation degree and leukotriene $C_4$ ($LTC_4$) production as early event of FcεRI-cross linking were suppressed, whereas $PGD_2$ production was enhanced. The FcεRI pathway was identified to be significantly down-regulated pathway in PU.1-overexpressing MC as well as DC by microarray analysis, whereas cell surface expression levels of FcεRI and TLR4 were comparable between MC and PU.1-overexpressing MC in FACS analysis. From these observations, we concluded that PU.1 down-regulated expression of intracellular molecules related to the FcεRI signal cascade, but up-regulated potential of cytokine and PG transcription activities.

## Materials and methods

*Cells and retrovirus transfection.* Complementary DNA encoding PU.1 was introduced into bone marrow-derived MC (BMMC),

*Abbreviations:* BM, bone marrow-derived; DC, dendritic cells; LC, langerhans cells; LT, leukotriene; LPS, lipopolysaccharoide; MC, mast cells; PG, prostagrandine.

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which was developed from bone marrow cells of BALB/c (Japan SLC, Hamamatsu, Japan) after 3 weeks culture in RPMI 1640 (Sigma–Aldrich) supplemented with 10% heat-inactivated FBS (Sigma–Aldrich), 100 μM 2-ME, 10 μM MEM nonessential amino acids solution (Invitrogen), antibiotics, and 10% pokeweed mitogen-stimulated spleen-condition medium [14], using retrovirus vector system as described in our previous report [10]. In brief, a plasmid pMXs-puro-PU.1 was constructed by insertion of BamHI/NotI fragment from pMX-puro-PU.1 [11] to BamHI/NotI-digested pMXs-puro (kindly provided by Dr. T. Kitamura) [15]. Each of pMXs-puro (mock vector) and pMXs-puro-PU.1 (for the expression of PU.1) was transiently introduced into a retrovirus packaging cell, Plat-E [16], with Fugene6 (Roche Diagnostics). BMMC was incubated with harvested culture medium of Plat-E containing infectious viruses for 2 days and cultured for an additional 10–20 days in the presence of puromycin to selectively obtain transfectants [10]. BMDC was prepared from BALB/c bone marrow cells with culture in RPMI 1640 supplemented with 10% FBS, 100 μM 2-ME, 10 μM MEM nonessential amino acids solution, antibiotics, 10 ng/ml mouse rGM-CSF (PeproTech), and 10 ng/ml mouse rIL-4 (PeproTech) based on a previously reported method [17,18].

*Flow cytometric analysis.* PE-conjugated anti-I-$A^d$, and FITC-conjugated anti-CD11c Abs were purchased from (BD Pharmingen), and PE/Cy7-conjugated anti-c-kit Ab (BioLegend), FITC-conjugated anti-FcεRIα Ab (eBioscience), and PE-conjugated anti-TLR4 Ab (abcam, Tokyo, Japan) were also used. Cells were stained with Abs in the presence of 2.4G2 for blocking of Fc receptors and analyzed by a FACSCalibur flow cytometer (BD Bioscience).

*GeneChip expression analysis.* Total RNAs were extracted from three samples; PU.1-overexpresing BMMC, control BMMC, and BMDC were used as templates for the reactions of the One-Cycle Target Labeling method using Affymetrix kits. In brief, double-stranded cDNA was reverse transcribed from 1 μg total RNA with a Poly-A RNA Control Kit and a One-Cycle cDNA Synthesis Kit, and then the biotinylated cRNA was *in vitro* transcribed from cDNA with an IVT Labeling Kit. After cRNA fragmentation, a reaction cocktail prepared with a Hybridization Control Kit was hybridized with an Affymetrix GeneChip microarray, Mouse Genome 430 2.0 Array, according to the manufacturer's instruction. Washing and staining, and scanning of each chip was performed with using the Fluidics Station 400/250, and the GeneChip Scanner 3000, respectively. Transcription levels of whole genes were determined after normalization with each GAPDH value using the Affymetrix GeneChip® Operating Software. Hierarchical clustering analysis was performed for genes, which were selected considering significance ($p < 0.05$) determined by one-way ANOVA statistic analysis with three independent data.

*LPS and IgE stimulation.* Stimulation via FcεRI was performed according to our previously reported methods [10,19] with a modification of anti-mouse IgE usage instead of Ag. In brief, $0.5–3 \times 10^5$ cells sensitized with mouse anti-DNP-IgE (BD Pharmingen) for 1 h were stimulated with 0.5 μg/ml anti-mouse IgE (R35-72; BD Pharmingen). For activation via TLR4, LPS (from *Escherichia coli*; Sigma–Aldrich) was used. Each culture medium was harvested after 30 min incubation for determination of concentration of $PGD_2$ and $LTC_4$, 3 h for TNF-α, and 6 h for IL-6 and IL-13. The concentration of $PGD_2$ and $LTC_4$ was determined by ELISA kit (Cayman Chemical Co., Ann Arbor, MI). ELISA kits of Genzyme Techne (Minneapolis, MN) were also used for measuring TNF-α, IL-6, and IL-13 concentration. Bete-hexosaminidase activity in Tyrode's buffer at 30 min after stimulation with IgE/anti-IgE was also determined as described previously [10,19] to investigate degranulation activity.

## Results

### *Preparation of BMMC-transfectants infected by retrovirus carrying PU.1 cDNA*

To generate BMMC possessing enforced expression of PU.1, BMMC at 3 weeks culture from BM was transfected with retrovirus carrying PU.1 cDNA or empty vector. After an additional 2 weeks culture in the presence of puromycine, cell surface expression of lineage-specific molecules was analyzed by FACS (Fig. 1). PU.1 induced marked expression of MHC class II and CD11c on MC (Fig. 1A) and suppressed expression of c-kit (Fig. 1B, top), consistent with the previous study [10]. In contrast, significant difference was not observed in cell surface expression levels of FcεRI and TLR4 between mock and PU.1-overexpressing cells (Fig. 1B). Transfectants were used for the following experiments after confirmation of the cell surface expression.

### *PU.1 down-regulates FcεRI-mediated production of IL-6 and IL-13, but up-regulates that of TNF-α as well as LPS-stimulation signal*

To evaluate the effect of PU.1 on MC activation, the amounts of cytokines produced from IgE- or LPS-stimulated MC were determined (Fig. 2). Production levels of IL-6 and IL-13 from IgE-stimulated MC were significantly suppressed by expression of exogenous PU.1. In contrast, surprisingly, the amount of TNF-α released from PU.1-overexpressing MC in response to IgE-stimulation was markedly higher compared with that of control MC. In addition, overexpression of PU.1 up-regulated all cytokine levels generated from MC in response to LPS-stimulation. These results suggest that PU.1 amplified stimulation activity via TLR4 but exhibited different effects on expression of cytokines through FcεRI signal cascades in a target-dependent manner.

### *Effects of PU.1 on eicosanoids generation and degranulation in response to IgE-stimulation*

Stimulation of MC by cross-linking of FcεRI with IgE and antigens (or anti-IgE Ab) elicits not only transcription of cytokine genes, but also secretory granule exocytosis and immediate eicosanoid generation. To examine the effect of PU.1 on these early events, β-hexosaminidase activity and amounts $PGD_2$ and $LTC_4$ in culture supernatant of IgE-stimulated MC 30 min following cross-linking were determined (Fig. 3). β-Hexosaminidase activity that reflects degranulation degree was suppressed by overexpression of PU.1 (Fig. 3A) as in the previous study [10]. The concentration of $PGD_2$ in supernatant was markedly increased by enforced expression of PU.1 (Fig. 3B), whereas the amount of $LTC_4$ was decreased in supernatant of PU.1 transfectants (Fig. 3C). These results suggest that PU.1 exhibited an opposite effect on early events in IgE-stimulated MC; in brief, PU.1 suppressed degranulation and $LTC_4$ production, but enhanced $PGD_2$ generation.

### *Identification of a set of genes that were suppressed by PU.1*

The effect of PU.1 on FcεRI-mediated stimulation was suppressor for degranulation, and IL-6, IL-13, and $LTC_4$ production but was a positive amplifier for TNF-α and $PGD_2$ production. To elucidate the complex effect of PU.1, we evaluated whole genes by comparing expression levels among control MC, PU.1-overexpressing MC, and BMDC using DNA microarray technology. From approximately 45,000 probes on mouse whole genome GeneChip, 2411 probes showing significantly different expression levels were obtained by one-way ANOVA analysis on probes passed through detection call filtering. Hierarchical clustering analysis indicated that 542

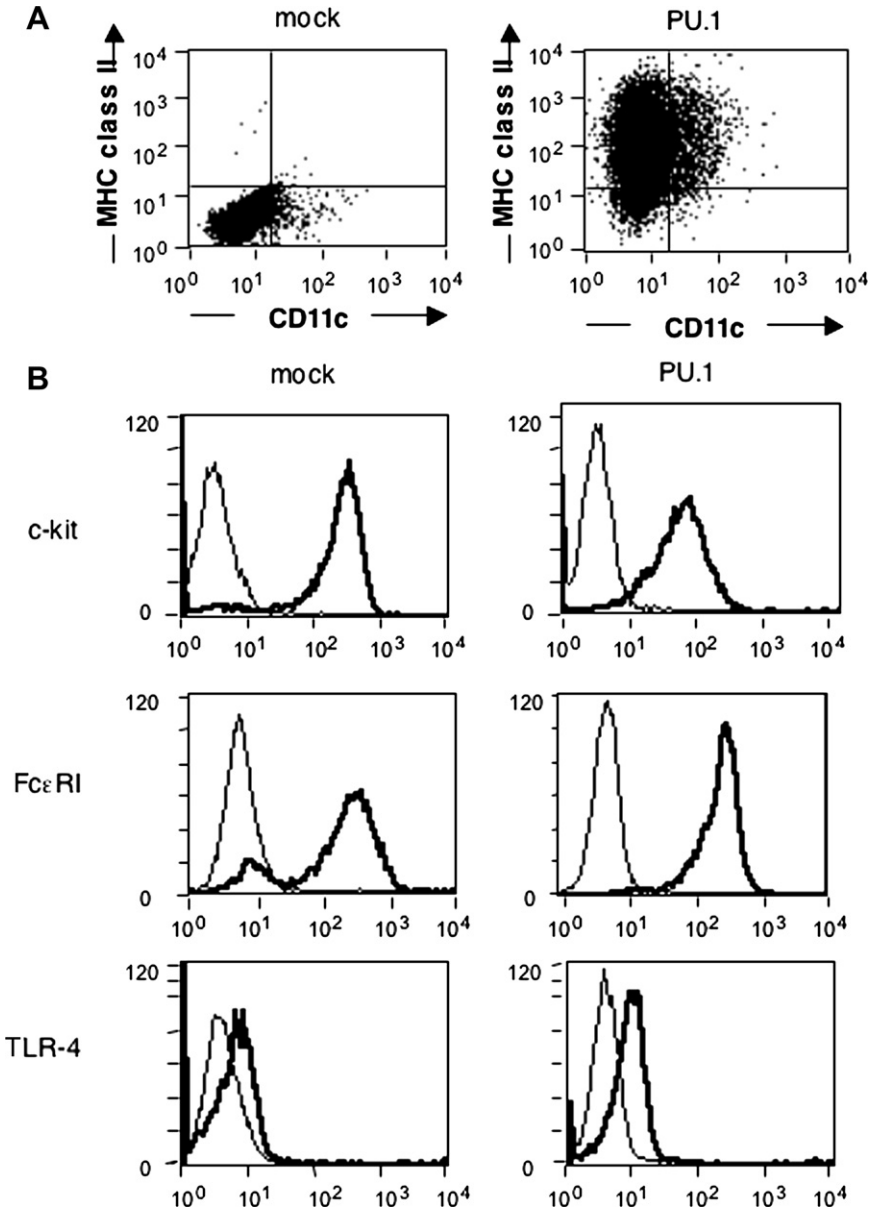



**Fig. 1.** Cell surface expression levels of lineage-specific markers and receptors for IgE and LPS. (A) PU.1-mediated induction of DC-specific cell surface markers expression. Transfectants with pMXs-puro or pMXs-puro-PU.1 were selected through 14 days culture in the presence of puromycin, and were double stained with PE-labeled anti-I-A$^d$ Ab and FITC-labeled anti-CD11c Ab. (B) Expression levels of c-kit, FcεRI, and TLR4. Thick-line histogram represent cells with each Ab. Thin-line histogram indicates control with 2.4G2 alone. Mock (left panel), control BMMC transfected with empty vector pMXs-puro; PU.1 (right panel), BMMC transfected with pMXs-puro-PU.1. A representative result of five independent experiments is shown in A and B.

members were localized in a cluster characterized at a high level in MC, low level in DC, and down-regulated by PU.1 overexpression (Fig. 4). When KEGG pathway analysis was performed, FcεRI signaling pathway was found ($p < 0.05$; http://www.genome.jp/kegg/pathway/has/hsa04664.html). Gene symbols significantly found in the FcεRI signaling pathway and their signal intensities were shown to the right of the expression profiles in Fig. 4. This result suggests that expression of intracellular molecules relating to FcεRI-signal transduction was down-regulated by PU.1.

## Discussion

PU.1 plays important roles in hematopoietic cell development and determines cell fate. We found that enforced expression of PU.1 in MC and MC-progenitors caused several changes toward monocytes, including induction of specific gene expression and morphological change in the previous studies [10–12]. In the present study, DNA microarray analysis was performed to evaluate the effect of PU.1 on expression levels of whole genes. FcεRI pathway was identified to be down-regulated by PU.1 according to pathway analysis using one-way ANOVA-treated microarray data. Degranulation, $LTC_4$ release, and production of IL-6 and IL-13 in response to IgE-stimulation were suppressed by enforced expression of PU.1 in MC, suggesting that FcεRI signal transduction was weakened due to the reduced expression of intracellular molecules relating to this signal pathway. These observations indicate that up-regulation of PU.1 suppresses intracellular MC-characters.

Interestingly, in contrast to IL-6 and IL-13, the amount of TNF-α released from IgE-stimulated MCs was up-regulated by PU.1. One possible explanation for this opposite appearance is the difference of gene expression mechanism that TNF-α, which accumulated within granules, is also rapidly released by degranulation in addition to production of TNF-α induced by stimulation-dependent transactivation, whereas the promoters of IL-6 and IL-13 are

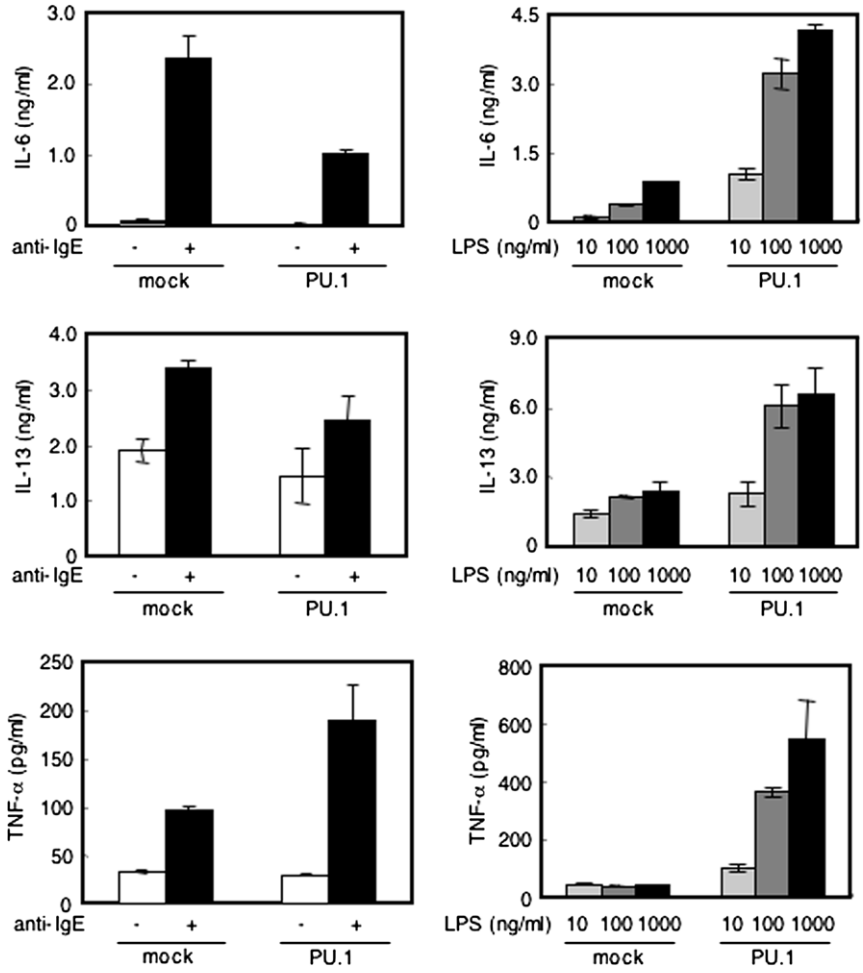



**Fig. 2.** Effect of PU.1 on production levels of cytokines from MC in response to stimulations by IgE and LPS stimulation. IL-6 and IL-13 concentrations in culture medium after 6 h of stimulation and TNF-α after 3 h were measured by ELISA. Each representative result performed with quadrant was shown as means ± SD. Similar results were observed in the other three independent experiments.

strictly dependent on IgE-mediated induction. This hypothesis coincides with the TNF-α mRNA level in microarray data, a 6-fold increase by overexpression of PU.1 in MC even in a resting state (data not shown). Previously, it was reported that PU.1 bound to *cis*-enhancing element in TNF-α promoter, which was essential for transactivation by LPS-stimulation in monocytes [20]. Therefore, enforced expression of PU.1 may directly amplify TNF-α promoter activity by binding to the critical element in the promoter. Regardless, further detailed analysis to evaluate the role of PU.1 on TNF-α promoter function will be required to clarify the opposite effects.

BMMC generate mainly $PGD_2$ and $LTC_4$ in response to IgE-stimulation immediately via different *de novo* synthesis pathways: $PGD_2$ via the cyclooxygenase pathway and $LTC_4$ via the 5-lipoxygenase pathway. Increase of $PGD_2$ generation in PU.1-overexpressing MC suggests that PU.1 is involved in the cyclooxygenase pathway. The immediate synthesis of $PGD_2$ is mediated by PG endoperoxidase H synthase (PGDH)-1 and hematopoietic $PGD_2$ synthase (hPGDS). Transcription level of hPGDS was increased 8-fold by enforced expression of PU.1 in microarray data (not shown). This up-regulation of hPGDS may explain the reason for increased production of $PGD_2$ in PU.1-overexpressing MC. A transcription factor MITF was essential for transactivation of mouse hPGDS promoter in MC in a previous report [21]. Considering that PU.1 regulates gene expression not only alone, but also with MITF in synergistic manner through interaction [22,23], PU.1 may up-regulate hPGDS promoter activity via affecting MITF function.

In the previous report, PU.1 was identified to bind a *cis*-enhancing element, which is highly conserved between human and mouse, and essential for TLR4 gene promoter activity [24]. However, expression level of TLR4 was not increased by exogenous PU.1 in this study, suggesting that other transcription factors are still required for up-regulation of TLR4 at the level of DC. One of candidates is IRF-8/ICSBP, which binds a target sequence with forming heterodimer with PU.1 and is also identified to involve in TLR4 gene promoter function [24]. Transcription level of IRF-8/ICSBP was slightly increased by PU.1-overexpression (2.5-fold), but was quietly lower than that of DC (35-fold compared with normal MC) (data not shown). Co-expression of PU.1 and IRF-8/ICSBP may be required to clarify the necessity of these two transcription factors for full TLR4 promoter activity. Even though TLR4 expression level was comparable, LPS-induced cytokine production was up-regulated by enforced expression of PU.1. These results suggest that function and/or expression of intracellular molecules relating LPS-stimulation signal were enhanced by PU.1, resulting in obtaining monocyte-like characters. However, TLR4-signaling pathway was not found by pathway analysis, suggesting that one or a few key molecules in TLR4-signaling cascade was a target of PU.1 in contrast to the case of FcεRI-signaling pathway. So far, we cannot identify the putative molecules by searching the microarray data. We will continue further detailed analysis to clarify the target of PU.1, which regulates magnitude of LPS-stimulation signaling.

Effect of enforced expression of PU.1 is mainly suppressive for mast cell characters and an amplifier for monocyte characters. In

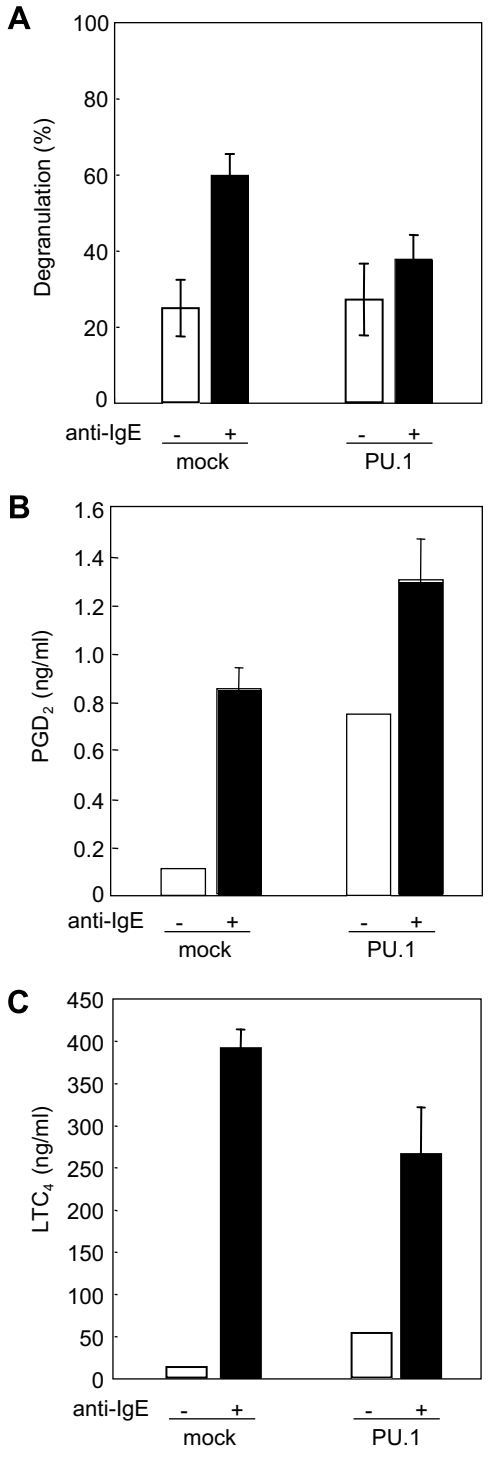
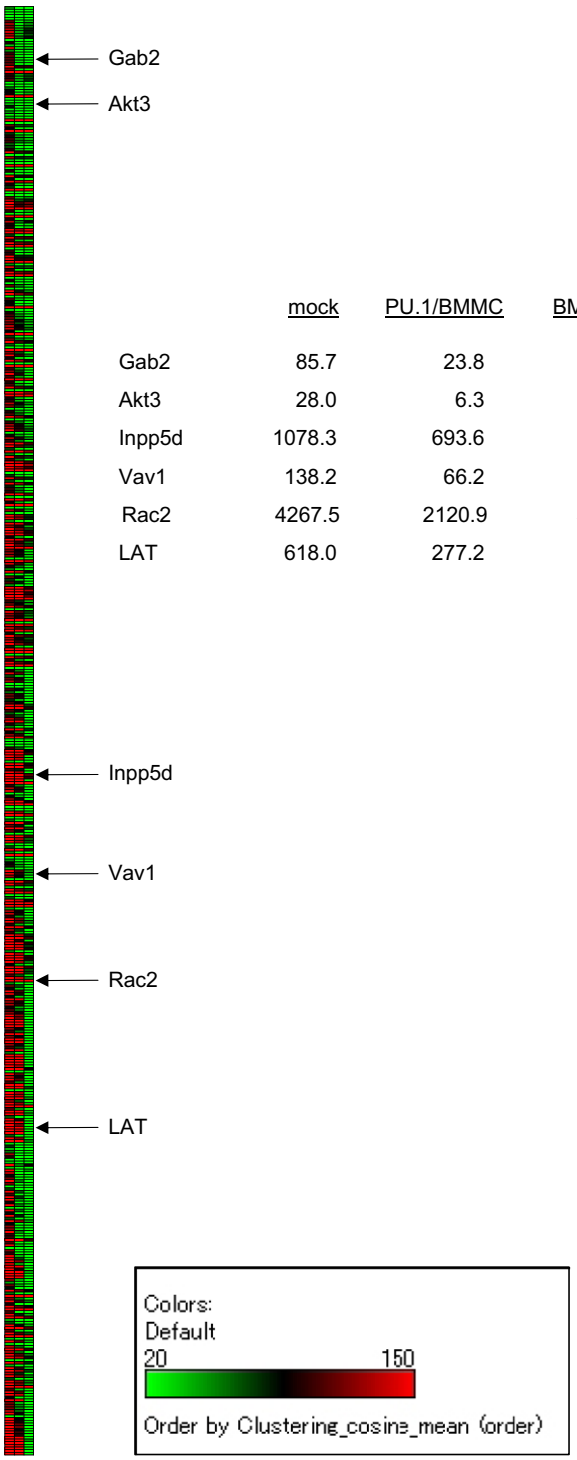



**Fig. 3.** Generation of eicosanoids and degranulation degree. (A) β-Hexosaminidase release. β-Hexosaminidase activity in supernatant was measured after 30 min incubation from stimulation by IgE/anti-IgE (+) or IgE alone (−). $PGE_2$ (B) $LTC_4$ (C) generation levels. Each concentration in culture medium after 30 min of stimulation by IgE/anti-IgE (+) or IgE alone (−) was measured by ELISA. A result performed with triplicate was shown as means ± SD and similar result was observed in another independent experiment in A, B, and C.




| | mock | PU.1/BMMC | BMDC |
|---|---|---|---|
| Gab2 | 85.7 | 23.8 | 19.7 |
| Akt3 | 28.0 | 6.3 | 13.5 |
| Inpp5d | 1078.3 | 693.6 | 81.2 |
| Vav1 | 138.2 | 66.2 | 27.3 |
| Rac2 | 4267.5 | 2120.9 | 590.2 |
| LAT | 618.0 | 277.2 | 8.5 |

**Fig. 4.** Gene expression profiles in BMMC, PU.1-overexpressing cells, and BMDC. Representation of mRNA expression levels in control BMMC (left), PU.1-overexpressing BMMC (middle), and BMDC (right). One row of colored bars represents one gene and the color bars capture the magnitude of the response for each gene according to the scale shown. A cluster of genes that were highly expressed in BMMC, a low level in BMDC and reduced by overexpression of PU.1 is shown.

some points such as production of TNF-α and $PGD_2$ in response to IgE-stimulation, the effect of PU.1 appears to be complex. However, considering that TNF-α and $PGD_2$ are abundantly produced from activated monocytes, increased transcription magnitude of these genes may be reasonable. We will further study regulation of PU.1 function in activated monocytes to clarify this observation.

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