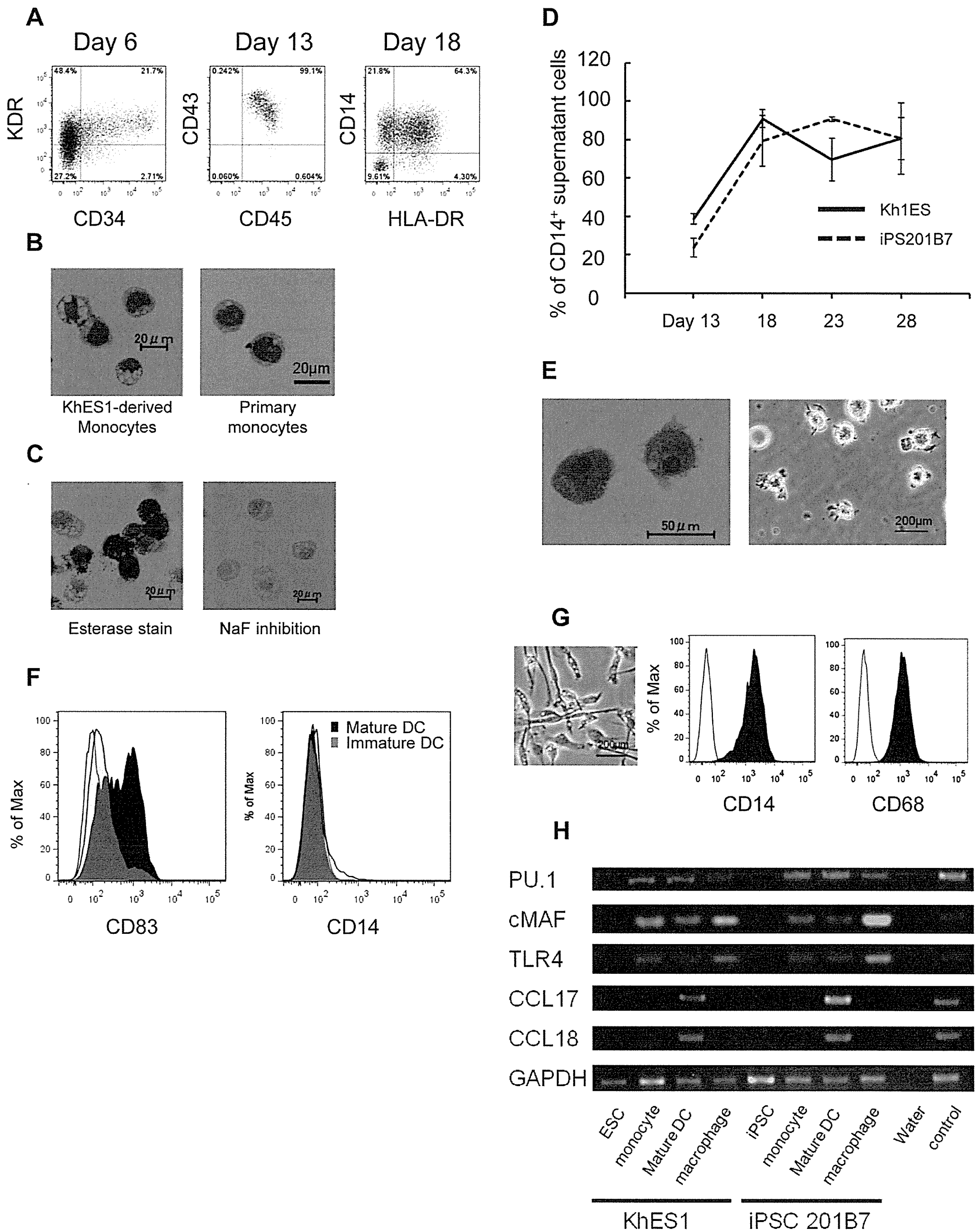

Figure 2. Phenotype analysis and gene expression pattern of monocytic lineage cells derived from pluripotent stem cells. (A) Flow cytometric analysis of monocytic lineage cells derived sequentially from pluripotent stem cells. An analysis of adherent cells on day 6 and supernatant cells on day 13 and 18 is shown. (B) May-Giemsa staining of $CD14^{+}$ monocyte-like cells derived from KhES1 on day 16 (left) and primary human monocytes (right). (C) Esterase staining for $CD14^{+}$ monocyte-like cells derived from KhES1 on day 16. (D) The percentage of $CD14^{+}$ cells within the total floating cells derived from KhES1/iPS-201B7 was evaluated from day 13 to day 28. (E) May-Giemsa staining (left) and phase contrast image (right)

of mature DCs derived from pluripotent stem cells. (F) Flow cytometric analysis of immature/mature DCs derived from pluripotent stem cells. (G) Phase contrast image and flow cytometric analysis of macrophages derived from pluripotent stem cells.(H) RT-PCR analysis of monocytic lineage cells derived from KhES1/iPS-201B7 clones for expression of monocytic lineage marker genes (*PU.1, c-MAF, TLR4, CCL17* and *CCL18*). Peripheral blood monocytes and peripheral blood monocyte-derived mature DCs were used as positive controls.(A–C, E–G) The data from KhES1-derived cells are shown as representative.
doi:10.1371/journal.pone.0059243.g002

Step 3: generation of hematopoietic cells with hematopoietic cytokines. The cytokines in StemPro-34 medium were switched to the step-3 cytokine cocktail composed of SCF (50 ng/mL), IL-3 (50 ng/mL), TPO (Thrombopoietin) (5 ng/mL), M-CSF (50 ng/mL), and Flt-3 ligand (50 ng/mL), on day 6. Thereafter, the medium was changed on day 10.

Step 4: monocytic lineage-directed differentiation with Flt-3 ligand, GM-CSF and M-CSF. The cytokines in StemPro-34 medium were switched to the step-4 cytokine cocktail composed of Flt-3 ligand (50 ng/mL), GM-CSF (25 ng/mL), and M-CSF (50 ng/mL) on day 13–15. The medium was changed every 3–4 days. The $CD14^+$ monocytic lineage-directed cell fraction in supernatant was positively sorted by autoMACS pro (Miltenyi Biotec) with CD14 MicroBeads (Miltenyi Biotec) on days 15–28.

Step 5: differentiation into DCs (step 5-1) and MPs (step 5-2) from $CD14^+$ monocytic lineage-cells. $CD14^+$ cells sorted by autoMACS pro (1.5×10^6 cells per well in a 6-well plate with Ultra-Low Attachment Surface (CORNING)) were cultured in StemPro-34 medium supplemented with GM-CSF (25 ng/mL) and IL-4 (40 ng/mL), with a medium change 4 days later, for differentiation into DCs (step 5-1). LPS (100 ng/mL, InvivoGen) and TNFα (0.2 ng/mL) were added for the last 2 days of the 7 day DC differentiation culture to promote maturation of DCs. $CD14^+$ cells (1.5×10^6 cells per well in a 6-well tissue culture plate) were cultured in RPMI-1640 medium (Sigma) supplemented with 10%

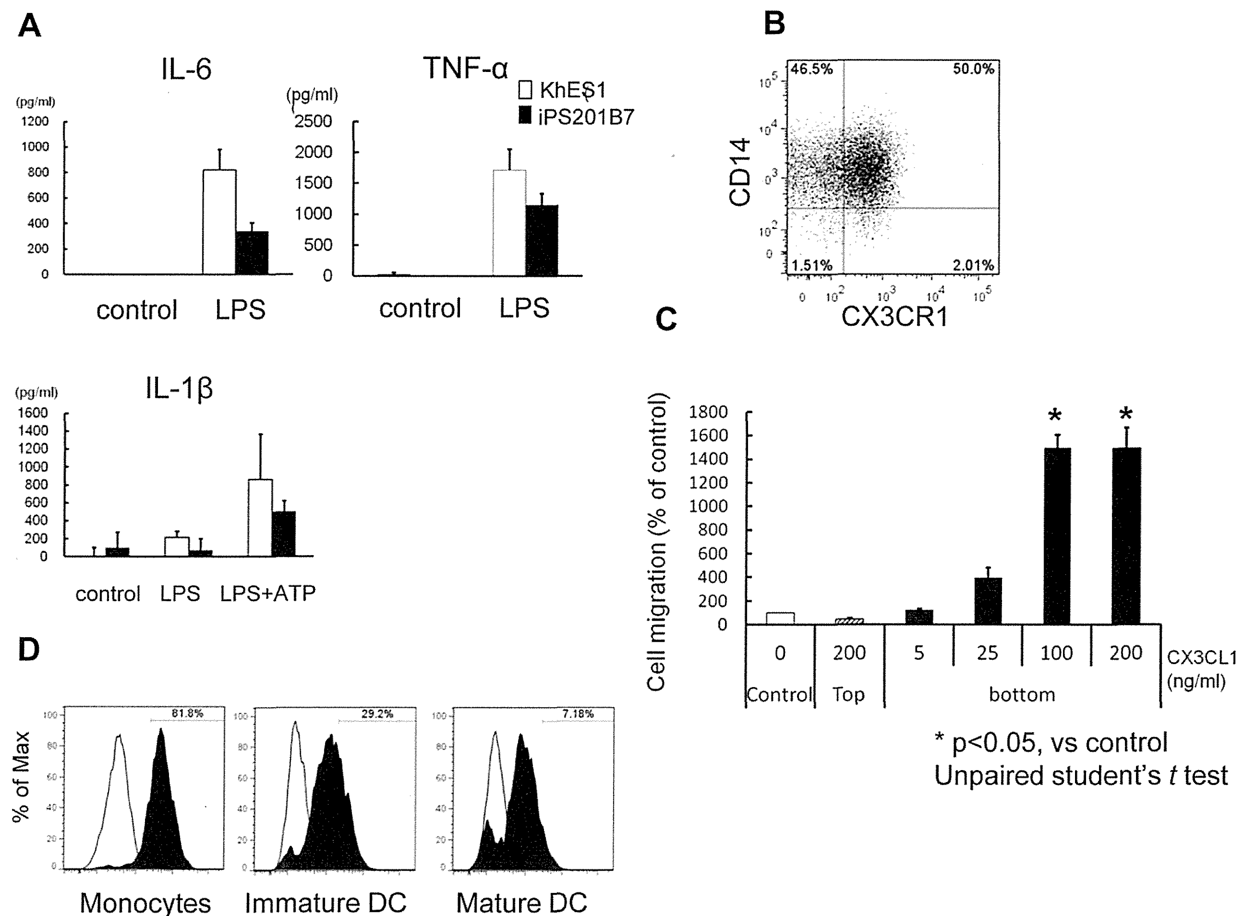

Figure 3. Functional assays for monocytes derived from pluripotent stem cells. (A) The levels of IL-6 and TNFα in supernatants of PS-Mo culture medium 4 hours after LPS stimulation. The levels of IL-1β were measured 4 hours after LPS stimulation with/without an additional 30-minute ATP stimulation. (B) Flow cytometric analysis of CX3CR1 on PS-Mo. (C) Chemotaxis assay of PS-Mo for CX3CL1 (fractalkine) using a trans-well migration assay. After the addition of CX3CL1 into either the bottom or top of the trans-well chamber, PS-Mo were applied and incubated for 5 hours at 37°C. (D) Antigen uptake was evaluated in monocytes, immature DCs and mature DCs derived from pluripotent stem cells by examining the fluorescence intensity of Alexa fluor 488-conjugated ovalbumin 45 minute after incubation at 37°C (black). Control samples (white) were kept on ice. (B–D) The data of KhES1-derived cells are shown as representative. PS-Mo: monocyte derived from pluripotent stem cells.
doi:10.1371/journal.pone.0059243.g003

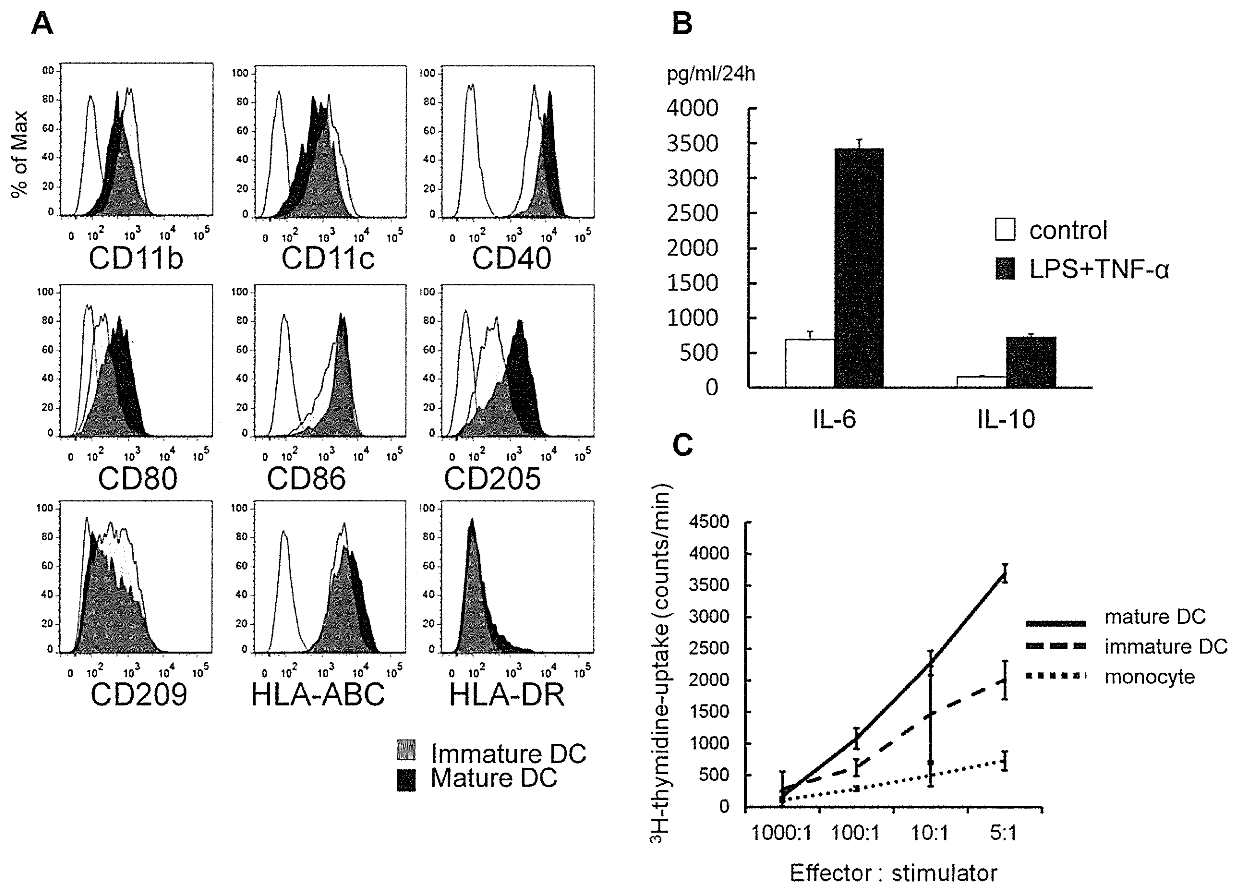

Figure 4. Functional assays for dendritic cells derived from pluripotent stem cells. (A) Flow cytometric analysis of immature/mature DCs derived from pluripotent stem cells. (B) The levels of IL-10 and TNFα in supernatants of culture medium with PS-DCs 24 hours after LPS stimulation. (C) The proliferation of allogeneic naïve T cells (1×10^5 cells per well) co-cultured with 40 Gy-irradiated stimulator cells for 3 days was evaluated. The proliferation of naïve T cells in the last 16 hours was measured by ^{3}H-thymidine uptake. (A–C) The data of KhES1-derived cells are shown as representative.
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fetal bovine serum (FBS) and M-CSF (100 ng/mL) for 7 days with a medium change at day 4, for differentiation into macrophages (step 5-2). IFNγ (20 ng/ml) or IL-4 (20 ng/ml) was added for another day to promote differentiation into M1 or M2 macrophages, respectively.

Flow Cytometric Analysis

Flow cytometric analysis data were collected using the MACS Quant™ Analyzer (Miltenyi Biotec) and then analyzed utilizing the FlowJo software package (Treestar). The following antibodies were purchased from BD Biosciences: CD11b-FITC, CD11c-APC, CD34-PE, CD40-PE, CD43-FITC, CD80-PE, CD83-PE, CD86-FITC, CD205-Alexa fluor 647, CD206-FITC, CD209-PE, HLA-ABC-FITC and HLA-DR-FITC. CD14-APC and CD45-APC antibodies were purchased from Beckman Coulter. CD163-APC antibody was purchased from R&D systems. KDR (CD309)-Alexa fluor 647 and CX3CR1-PE antibodies were purchased from Biolegend.

May-Giemsa Staining and Esterase Staining

Cells were seeded onto glass slides by CYTOSPIN 4 (Thermo Scientific) and stained with May-Grunwald and Giemsa staining solution (MERCK) and Esterase staining solution (Muto pure chemicals) following the manufacturer's instructions.

RNA Extraction and RT-PCR Analysis

RNA samples were prepared using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Typically, 500 ng of total RNA were subjected to reverse transcription (RT) with a Sensiscript-RT kit (Qiagen). RT-PCR was performed for the evaluation of the expression of monocytic lineage marker genes such as *PU.1, MAF, TLR4, CCL17* and *CCL18* using the primers in **Table S1**. [21–22] Peripheral blood monocyte-derived mature DCs/macrophages were generated from peripheral $CD14^+$ monocytes using the step 5-1/5-2 cytokine cocktails in 10% FBS-supplemented RPMI-1640 for use as positive controls.

Cytokine Assay

Concentrations of cytokines (IL-1β, IL-6, IL-10, IL-12p70 and TNFα) in supernatants were analyzed with FlowCytomix kits (Bender MedSystems) following the manufacturer's instructions. The IL-1β, IL-6 and TNFα levels in the culture supernatants of pluripotent cell-derived monocytes (PS-Mo) were analyzed in three settings, (1) culture in RPMI-1640 medium supplemented with 10% FBS and LPS (100 ng/ml) for 4.5 hours, (2) as in (1) but with

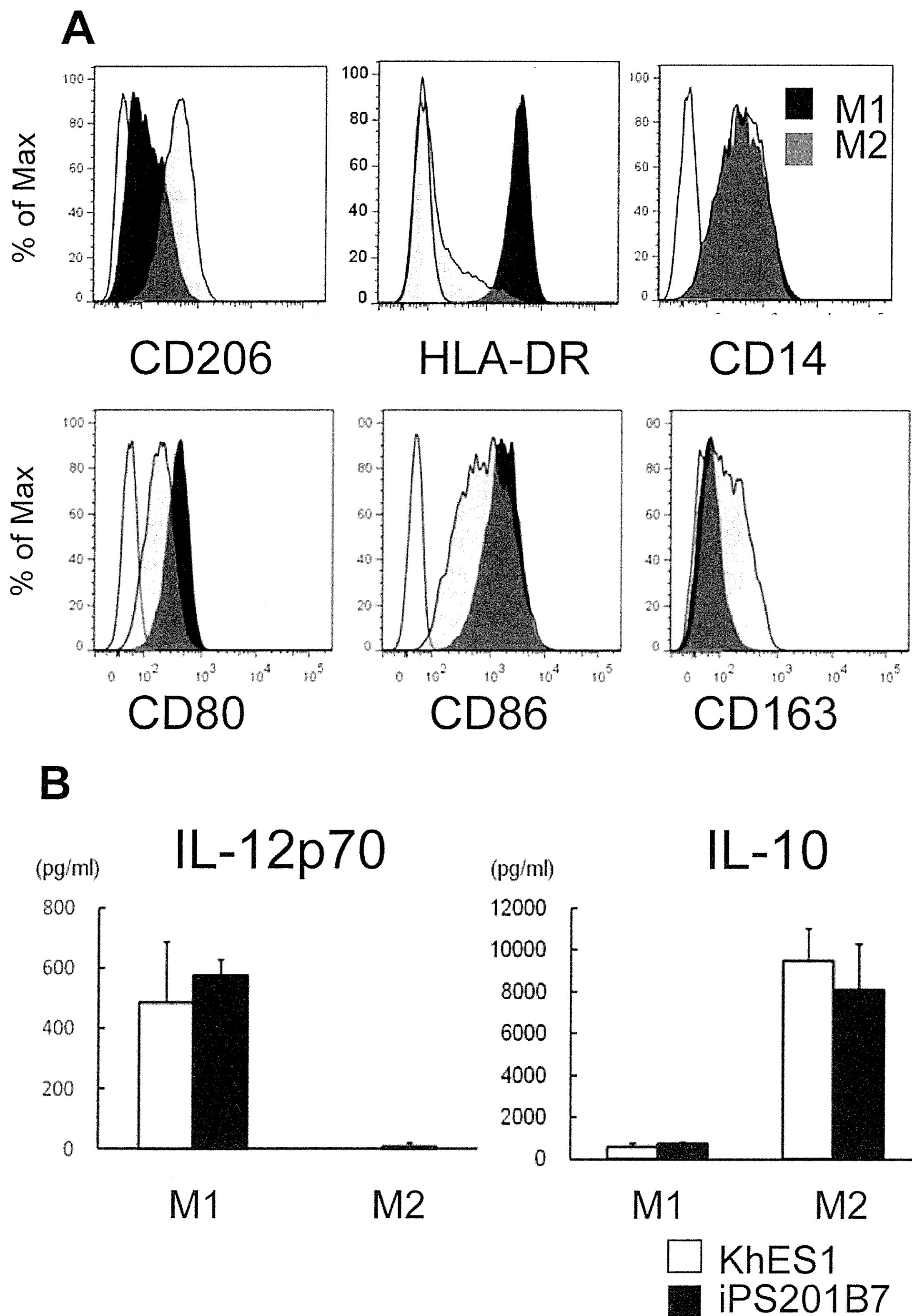

Figure 5. Functional assays for M1/M2 macrophages derived from pluripotent stem cells. (A) Flow cytometric analysis of M1/M2 macrophages derived from pluripotent stem cells. (B) The levels of IL-12p70 and IL-10 in supernatants of culture medium with M1/M2 macrophages derived from pluripotent stem cells 24 hours after LPS stimulation. The data of KhES1-derived cells are shown as representative.
doi:10.1371/journal.pone.0059243.g005

the addition of ATP (1 mM) for the last 30 min, (3) without LPS or ATP for 4.5 hours, to evaluate the production pattern of IL-1β in response to LPS plus ATP. [23].

The levels of IL-6, IL-10, IL-12p70 and TNFα in the supernatants of M1 or M2 macrophage culture were measured 24 hours after LPS (100 ng/ml) stimulation.

Chemotaxis Assay

PS-Mo chemotaxis was evaluated using a trans-well migration assay with 8-µm pore size inserts (BD Biosciences). After CX3CL1 (fractalkine; R&D systems) was added to either the bottom or top of the chamber, serum-starved PS-Mo were loaded onto the inserts which were placed into 24-well plates containing RPMI-1640 and then incubated at 37°C for 5 hours. [24] Cell migration was measured by flow cytometry as previously reported: equivalent amounts of counting beads were added to each sample and the ratios of PS-Mo to the counting beads were calculated. [25].

Antigen Uptake Assay

The antigen uptake capacity of monocytic lineage cells was evaluated as previously described. [26] Briefly, the cells were collected and stored on ice for 10 min. PS-Mo, pluripotent cell-derived immature DCs (PS-imDCs) and pluripotent cell-derived mature DCs (PS-mDCs) (5×10^4 cells) were incubated with Ovalbumin Alexa fluor 488 Conjugate (Molecular Probes) at 0.1 mg/ml at 37°C or on ice for 45 min. Ice-cold FACS buffer was added in order to stop the reaction, samples washed twice, and the fluorescence intensity analyzed by flow cytometry.

Mixed Leukocyte Reactions

Allogeneic naïve T cells (1×10^5 cells per well) were purified from umbilical cord blood mononuclear cells using naïve $CD4^+$ T cell isolation kits (Miltenyi Biotec) and then co-cultured with 40 Gy-irradiated stimulator cells (PS-Mo, PS-imDC, and PS-mDC) in 96-well round bottomed culture plates for 3–5 days. ^{3}H-methylthymidine (25 uCi/ml, Moravek Biochemicals and Radiochemicals) was added to the culture medium of 10% FBS-supplemented RPMI-1640 for the last 16 hours. The cells were harvested onto a filter mat (Perkin Elmer) and the ^{3}H methylthymidine uptake determined using a scintillation counter (MicroBeta TriLux, Perkin Elmer).

Ethical Considerations

This study was approved by the Ethics Committee of Kyoto University and written informed consent was obtained from each healthy volunteer.

Statistics

Data are presented as the mean ± S.D. and the statistical significance of the differences between cultures were evaluated by Student's *t*-test.

Results

Differentiation of ESCs/iPSCs into Dendritic Cells and Macrophages via Monocyte-like Cells

A KDR^+CD34^+ hemangioblast-like population was detected in adherent cell clusters on day 6 (steps 1,2), and around 95% of supernatant cells were $CD43^+CD45^+$ hematopoietic cells on days 13–15 (step 3; **Figure 2A**). [17] Floating cells were recovered every 3–4 days in step 4 (**Figure S1**); the majority of these cells were $CD14^+$ monocyte-like cells (**Figure 2A**). These pluripotent cell-derived monocytes (PS-Mo) were similar to peripheral blood monocytes in morphology (**Figure 2B**). PS-Mo are positive for Esterase staining which was inhibited by NaF (**Figure 2C**). The percentages of PS-Mo in floating cells were constantly about 50–90% between day 18–28 (**Figure 2D and Figure S2A**). The yield of PS-Mo per 100 mm culture dish starting with about 30 colonies was $1.3\times10^6\pm0.3\times10^6$ at each step-4 medium exchange.

To derive DCs, PS-Mo were purified by magnetic sorting, and differentiated into $CD14^-CD83^-$ immature DCs (PS-imDCs) with the step 5-1 cytokine cocktail in 5 days (**Figure 2E**). PS-imDCs were stimulated with LPS and TNFα for an additional 2 days, which further differentiated them into $CD14^-CD83^+$ mature DCs (PS-mDCs) (**Figure 2F**). The differentiation efficiency of mature DCs from PS-Mo was comparable to that from primary monocytes (7.7%±0.9% vs. 16.5%±1.0%, p = 0.20, unpaired Student's *t*-test). PS-Mo also had the potential to differentiate into macrophages (PS-MPs) with the step 5-2 cytokine cocktail. PS-MPs are morphologically comparable to primary monocyte-derived macrophages and they express typical surface markers such as CD14 and CD68 (**Figure 2G and Figure S3A,B**).

We confirmed that PS-Mo, pluripotent cell-derived DCs (PS-DCs), and PS-MPs expressed monocytic lineage-specific genes (**Figure 2H and Figure S2B**). [22,27] Collectively, by using this protocol, sufficient numbers of monocytic cell lineage cells can be obtained from a small number of human ESCs/iPSCs.

Functional Assays for Monocytes Derived from ESCs/iPSCs

Next, we evaluated the functional activity of pluripotent cell-derived monocytic lineage cells. PS-Mo robustly produced the pro-inflammatory cytokines IL-6 and TNFα after LPS stimulation (**Figure 3A, Figure S3C**). Secretion pattern of IL-1β from PS-Mo with two stepwise signals LPS and ATP were similar to primary monocytes (**Figure 3A, Figure S3D**). [23,28].

PS-Mo expressed CX3CR1, implying chemotactic responses to CX3CL1 (fractalkine) (**Figure 3B**). PS-Mo migration in trans-well assays increased with increasing doses of CX3CL1 in the lower compartment of the chamber (**Figure 3C**). This phenomenon was not due to chemokinesis, but chemotaxis, because CX3CL1 in the top compartment could not induce PS-Mo migration into the lower compartment of the chamber. [24] We next compared the antigen uptake ability of PS-Mo, PS-imDCs, and PS-mDCs by incubating them with Ovalbumin Alexa fluor 488 Conjugate. [26] PS-Mo had the highest ability to take up antigen and as DCs matured they lost their ability to endocytose antigens (**Figure 3D**).

Functional Assays for DCs Derived from ESCs/iPSCs

For evaluating functions of PS-DCs, we first confirmed that patterns of expression of cell surface markers on PS-imDCs/mDCs were comparable to those on primary dendritic cells (**Figure 4A, Figure S4A**). When stimulated with LPS and TNFα, PS-DCs also produced almost comparable amounts of pro-inflammatory and anti-inflammatory cytokines (**Figure 4B, Figure S4B**).

To test the ability of PS-DCs to activate naïve T cells, we next co-cultured allogeneic naïve T cells with PS-DCs and PS-Mo. As shown in Figure 4C, PS-mDCs had the most potent capacity to stimulate allogeneic T cell proliferation and this dose-response relationship was comparable to that observed with PB-DCs (Figure S4C).

Functional Assays for Macrophages Derived from ESCs/iPSCs

Using this technique, we obtained morphologically typical macrophage-like cells that adhered firmly to the culture dish. To test whether these PS-MPs possessed functional plasticity like primary macrophages, we tried to polarize them into M1 or M2 state by treating them with IFNγ or IL-4, respectively. PS-MPs exhibited typical surface markers that were characteristic of primary M1 or M2 macrophages (**Figure 5A, Figure S5A**). The M1 cytokine pattern is typically IL-12^{high} and IL-10^{low}, whereas the M2 pattern is IL-12^{low} and IL-10^{high}. [5] Pluripotent cell-derived M1 and M2 macrophages (PS-M1/M2) also exhibited cytokine profiles that were comparable to those generated from primary monocytes (**Figure 5B, Figure S5B**).

Discussion

We have established a novel differentiation system for monocytic cells from human ES and iPS cells. Since macrophages and dendritic cells are usually obtained *in vitro* from monocytes, the most important point of the evaluation is to establish whether monocytes differentiated from ESCs/iPSCs are functionally comparable to primary monocytes. In several functional assays, PS-Mo indeed proved to be comparable to primary monocytes, and importantly, PS-DCs and PC-MPs from PS-Mo were also functionally comparable to their primary counterparts.

Although complete M1/M2 macrophage polarization still requires aserum-containing medium, the present results prove that the current method can precisely manipulate macrophages that have the potential to differentiate into M1/M2 macrophages. The cytokine profiles of PS-M1/M2 were also comparable to those of primary M1/M2 macrophages. The expression patterns of surface markers in PS-DCs after LPS stimulation and of PS-MPs after M1/M2 polarization were almost identical to those of DCs/MP derived from primary monocytes. However, the level of IL-10 in PS-DCs after stimulation was higher than that in primary DCs and the expression levels of HLA-DR in PS-DCs/MP were low in comparison with those in DCs/MP derived from primary monocytes. Therefore, further improvement of culture conditions such as the use of a modified medium and cytokine cocktail will be needed.

Several embryonic body methods and feeder cell co-culture methods for PS-DCs/MP differentiation have already been reported. [7,27,29–30] These methods show relatively poor-reproducibility because of the use of xenogeneic feeder cells and/or serum. In an earlier report which describes a protocol that can derive macrophages and dendritic cells from human iPSCs in feeder- and serum-free manner, [7] the authors did not fully characterize the monocytes and noted that PS-DCs/MP were generated only from two of the five iPSC clones tested. The current culture system simply propagated progenitor cells in 2-dimensional cultures without passage or sorting, and floating PS-Mo and PS-DCs/MP could be obtained repetitively from all five ESC/iPSC clones tested (**Figure S2 and S6**). These monocytic cells derived from disease- or patient- specific iPSC would be useful tools for the examination of disease pathologies and for drug discovery in immunological disorders such as autoimmune diseases, immunodeficiencies and autoinflammatory syndromes. However, even in our protocol, there are subtle clonal variations of timing of differentiation such as the day of step 3 to 4 switching which is determined by the emergence of CD43^{+}CD45^{+} cells (day 13–15, data not shown). Fine adjustment of the protocol for each ESC/iPSC clone seemed to further improve the yield of monocytes.

iPSC technology is overcoming immunological and ethical concerns in regenerative medicine using human pluripotent cells. Furthermore, a number of disease-associated iPSCs generated from patients with immunological disorders have been reported. [15,31–34] Because patient- or disease-specific iPS cells will be an important resource for unraveling human immunological disorders, a robust and simple hematopoietic differentiation system that can reliably mimic in vivo hematopoiesis is necessary for this purpose. Our simple and robust protocol to produce monocytic cells is therefore expected to be useful for regenerative medicine and studies of immunological disorders.

Supporting Information

Figure S1 Image of floating hematopoietic cells derived from iPS cells Phase contrast image of floating hematopoietic cells derived from iPS-201B7 at day 21 (step 4).
(PDF)

Figure S2 Phenotype analysis and gene expression pattern of monocytic lineage cells derived from 3 additional pluripotent stem cell lines. (A) The percentage of CD14+ cells within the total floating cells derived from 3 iPSC clones (253G4, CIRA188Ai-W2, and CB-A11) was evaluated from day 13 to day 28. (B) RT-PCR analysis of monocytic lineage cells derived from 253G4, CIRA188Ai-W2, and CB-A11 clones for expression of monocytic lineage marker genes (c-MAF, TLR4, and CCL17). Peripheral blood monocytes and peripheral blood monocyte-derived mature DCs were used as positive controls.
(PDF)

Figure S3 Characteristics of primary monocytes and macrophages. (A) Phase contrast image and (B) flow cytometric analysis of macrophages derived from primary monocytes. (C) The levels of IL-6 and TNF-α in supernatants of primary monocyte culture medium 4 hours after LPS stimulation. (D) The levels of IL-1β were measured 4 hours after LPS stimulation with/without an additional 30-minute ATP stimulation.
(PDF)

Figure S4 Characteristics and functional assays of dendritic cells derived from primary monocytes. (A) Flow cytometric analysis of immature/mature DCs derived from primary monocytes. (B) The levels of IL-10 and TNF-α in supernatants of culture medium with primary-DCs 24 hours after LPS stimulation. (C) The proliferation of allogeneic naïve T cells (1×105 cells per well) co-cultured with 40 Gy-irradiated stimulator cells for 3 days was evaluated. The proliferation of naïve T cells in the last 16 hours was measured by 3H-thymidine uptake.
(PDF)

Figure S5 Characteristics and functional assays of M1/M2 macrophages derived from primary monocytes. (A) Flow cytometric analysis of M1/M2 macrophages derived from primary monocytes. (B) The levels of IL-12p70 and IL-10 in supernatants of culture medium with M1/M2 macrophages derived from primary monocytes 24 hours after LPS stimulation.
(PDF)

Figure S6 Replication assays for 3 additional pluripotent stem cell lines. (A) Phase contrast image (left) and May-Giemsa staining (right) of mature DCs derived from iPSC clones. (B) Phase contrast image of macrophages derived from iPSC clones. (C) Flow cytometric analysis of immature/mature DCs and macrophages derived from iPSC clones.
(PDF)

Table S1 Primers for RT-PCR.
(PDF)

Acknowledgments

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Author Contributions

iPSC establishment: MDY IA. Conceived and designed the experiments: MDY AN HG TH TN MKS. Performed the experiments: MDY ST SN YM TT JI FHO. Analyzed the data: MDY AN TY KO TN MKS. Wrote the paper: MDY AN TY MKS.

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PROGRESS IN HEMATOLOGY

Efficacy and resistance of molecularly targeted therapy for myeloid malignancies

Mechanisms of action and resistance to all-*trans* retinoic acid (ATRA) and arsenic trioxide (As_2O_3) in acute promyelocytic leukemia

Akihiro Tomita · Hitoshi Kiyoi · Tomoki Naoe

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Abstract Since the introduction of all-*trans* retinoic acid (ATRA) and arsenic trioxide (As_2O_3) for the treatment of acute promyelocytic leukemia (APL), the overall survival rate has improved dramatically. However, relapse/refractory patients showing resistance to ATRA and/or As_2O_3 are recognized as a clinically significant problem. Genetic mutations resulting in amino acid substitution in the retinoic acid receptor alpha (RARα) ligand binding domain (LBD) and the PML-B2 domain of PML-RARα, respectively, have been reported as molecular mechanisms underlying resistance to ATRA and As_2O_3. In the LBD mutation, ATRA binding with LBD is generally impaired, and ligand-dependent co-repressor dissociation and degradation of PML-RARα by the proteasome pathway, leading to cell differentiation, are inhibited. The PML-B2 mutation interferes with the direct binding of As_2O_3 with PML-B2, and PML-RARα SUMOylation with As_2O_3 followed by multimerization and degradation is impaired. To overcome ATRA resistance, utilization of As_2O_3 provides a preferable outcome, and recently, a synthetic retinoid Am80, which has a higher binding affinity with PML-RARα than ATRA, has been tested in the clinical setting. However, no strategy attempted to date has been successful in overcoming As_2O_3 resistance. Detailed genomic analyses using patient samples harvested repeatedly may help in predicting the prognosis, selecting the effective targeting drugs, and designing new sophisticated strategies for the treatment of APL.

Keywords APL · PML-RARα · ATRA · Arsenic trioxide (As_2O_3) · Drug resistance

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Introduction

Almost two decades ago, the prognosis of acute promyelocytic leukemia (APL) was critically poor due to fatal coagulation disorders at diagnosis [1, 2]. Even with conventional chemotherapy using anthracyclines, more than 70 % of APL patients showed poor prognosis [3, 4]. After introduction of all-*trans* retinoic acid (ATRA) in the clinical setting in combination with conventional chemotherapy, the prognosis of APL has improved dramatically, with the result that more than 85 % of patients now achieve complete remission (CR) and nearly 70 % of patients can be cured [5–8]. Since 1994, the marked effectiveness of As_2O_3 in APL patients, even in relapsed patients after combination therapy with ATRA, has been confirmed [9–12]. When As_2O_3 is utilized as a single agent, ~70 % of patients can be cured, whereas nearly 90 % of patients can be cured if As_2O_3 is utilized in combination with ATRA [13, 14]. Although outcomes of APL treatment with ATRA and/or As_2O_3 in combination with conventional chemodrugs have improved, relapsed/refractory patients are still observed in the clinical setting and drug resistance to ATRA and As_2O_3 has been recognized as a critical problem.

More than 98 % of APL patients carry the t(15;17) translocation, which results in fusions of the retinoic acid

receptor alpha (RARα) gene with the promyelocytic leukemia (PML) gene, *PML-RARα* (Fig. 1) [15–17]. A very limited number of patients, showing APL phenotype without t(15;17), exhibit a variety of X-RARα fusions (Fig. 1) [18–25]. Interestingly, some patients expressing X-RARα show clinical resistance to ATRA and/or As_2O_3. Previous reports have indicated that both ATRA [26, 27] and As_2O_3 [28–30] have rigorously defined molecular targets, an improved understanding of their molecular mechanisms of action and resistance may be important to further improving clinical outcomes in APL treatment.

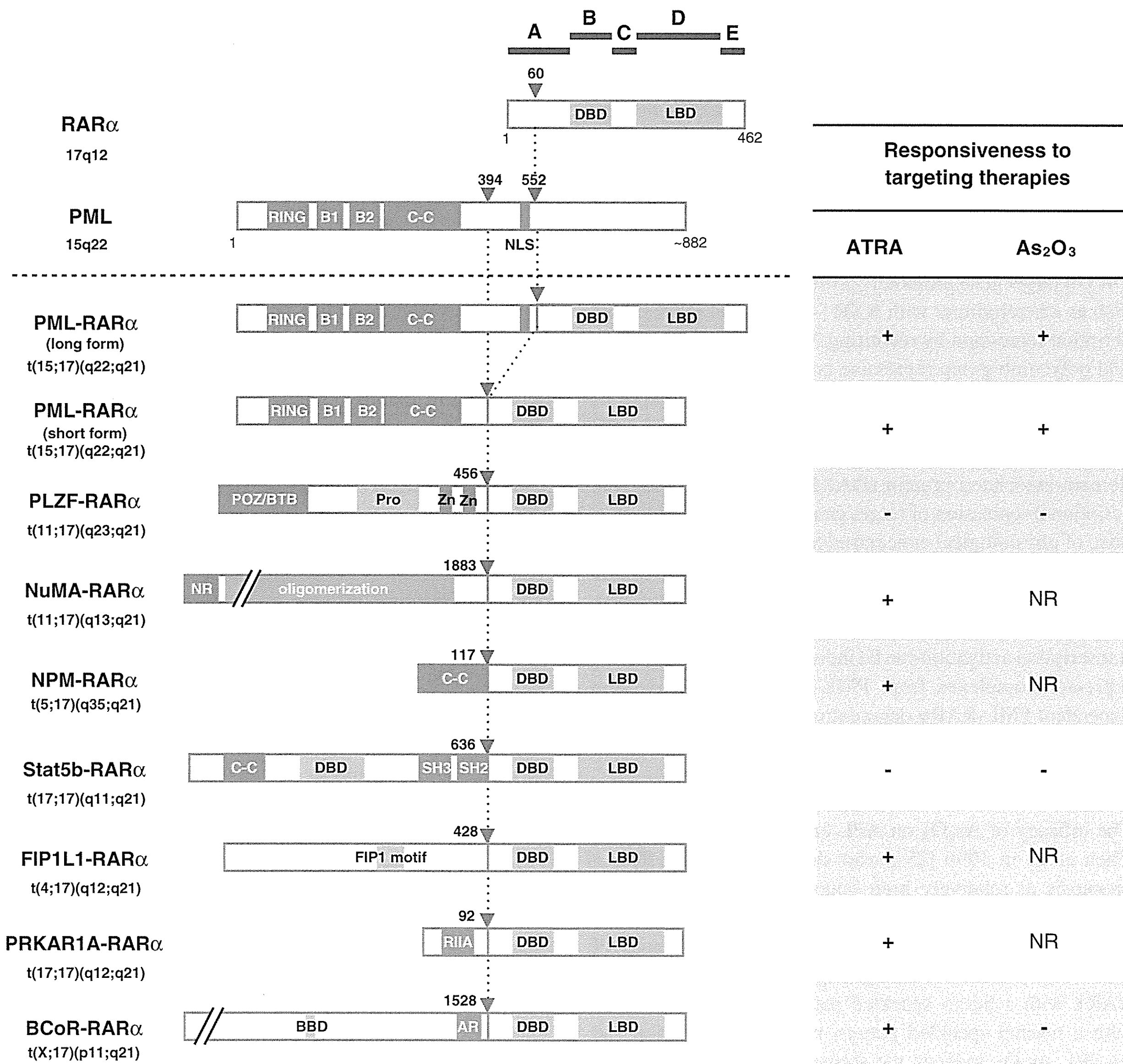

Fig. 1 Schematic representation of PML-RARα and X-RARα fusion protein confirmed in APL. Chromosomal translocations resulting in the fusion protein are also indicated under the name of fusion protein. Long and short forms of PML-RARα with or without nuclear localizing signal (NLS) are reported [86]. ATRA and As_2O_3 responsiveness in the clinical setting and/or in vitro analyses is indicated in the *right panel*. *Gray triangles* indicate break points of chimeric protein. *Numbers* indicate the amino acid positions. *A* to *E* functional domains in RARα, *DBD* DNA binding domain, *LBD* ligand binding domain, *RING* really interesting new gene finger domain, *B1 and B2* B-box motifs, *C–C* coiled-coil domain, *POZ/BTB* pox virus and zinc finger/BR–C, ttk and bab domain, *Pro* proline rich domain, *Zn* zinc finger domain, *NR* nuclear reassembly, *RIIA* dimerization domain, *BBD* BCL6-binding domain, *AR* ankyrin repeats, + sensitive, − resistant, *NR* not reported

Mechanisms of action of molecular targeting drugs to APL cells

ATRA

Wild-type RARα is a nuclear hormone receptor that binds to consensus sequence DR5 (five bases spaced between two AGGTCA motifs) in target gene promoters, normally as heterodimer with retinoid X receptor (RXR) [31–33]. Without ligands, ATRA and 9-*cis* retinoic acid, RAR-RXR heterodimer induces transcription repression throughout chromatin remodeling by recruiting transcription co-repressors, such as N-CoR/SMRT large protein complexes, that contain histone deacetylases (HDACs) [27, 34–37] and histone methyltransferases [38–40]. In the presence of ligand ($\sim 10^{-7}$ M), the co-repressor complexes dissociate from RAR-RXR, and transcriptional de-repression and activation are induced [34–37, 41]. PML-RARα binds to DR5 of target gene promoters primarily as a homodimer, but also as a heterodimer with RXR [42, 43], and induces transcription repression by recruiting N-CoR/SMRT complexes and polycomb group repressive complex 1 and 2 (PRC1/2) [39, 40], which contain histone methyl transferases, in the absence of ligands [27] (Fig. 1). PML-RARα can be SUMOylated at K160 of the PML protein to recruit death domain-associated protein (DAXX), resulting in the transcriptional repression of target genes [44]. Even in the presence of physiological concentration of ligand (10^{-7} M), the co-repressor complex still binds with PML-RARα and the transcriptional repression cannot be dissolved. In the presence of pharmacological concentration of ATRA (10^{-6} M), transcription activation can be induced by dissociation of co-repressor complexes from PML-RARα and proteasome-dependent PML-RARα degradation [45–47].

As_2O_3

The efficacy of As_2O_3 on APL cells was first reported by Chen et al. in 1996 [28], who showed the dual effect of apoptosis at relatively high concentrations (0.5–2 μM/L) and partial differentiation at low concentrations (0.1–0.5 μM/L) in both ATRA-responsive and ATRA-resistant APL cells. As_2O_3 induces the targeting of nucleoplasmic PML-RARα with a micro speckled pattern into nuclear bodies with a normal speckled pattern prior to degradation [30, 48–50]. As_2O_3 induces the formation of reactive oxygen species (ROS) [30], which induce multimerization of PML-RARα through intermolecular disulphide crosslinks at PML B1-domain (Fig. 2) and PML-RARα SUMOylation by ubiquitin-conjugating enzyme 9 (UBC9) [30]. A recent report indicated that As_2O_3 directly binds with PML at the C–C motif in the PML B2-domain, and that PML SUMOylation can be induced by enhancement of UBC9 binding at the PML RING domain [29, 30, 50]. SUMOylated PML recruits RING finger protein 4 (RNF4), which is known as a SUMO-dependent ubiquitin ligase [51], and polyubiquitylated PML-RARα can be degraded by ubiquitin–proteasome pathway [29, 49, 51].

Molecular mechanisms of drug resistance in APL cells

From the molecular mechanisms of ATRA and As_2O_3 effectiveness as indicated above, several mechanisms of drug resistance have been speculated [52]. In this section, we outline the molecular mechanisms of resistance that are thought to be significant from the clinical perspective.

RARα fusion proteins in APL

In very limited cases with APL phenotype, *RARα* translocations with *X*-genes other than *PML* (*PLZF* [18], *NuMA* [19], *NPM* [20], *STAT5b* [21, 53], *FIP1L1* [22], *PRKAR1A* [23, 24], and *BCOR* [25]) resulting in the production of X-RARα fusion protein have been reported (Fig. 1). PML-RARα forms mainly homodimers, and it has been reported that homodimerization of PML-RARα is critical for the pathogenesis of APL [42, 43]. Sternsdorf et al. [54] indicated that forced homodimerization of RARα induces ALP-like leukemia in a mouse model, indicating that the dimerization domain of the fusion protein may be critical to the induction of leukemogenesis by X-RARα. In fact, homodimerization through specific domains (coiled-coil; PML-, NPM-, and STAT5b-, POZ/BTB; PLZF-, RIIA; PRKAR1A-, and so on) has been confirmed in all X-RARα proteins. Interestingly, in PML-, PRKAR1A- [24], and BCOR-RARα [25], heterodimerization with RXR is also important for transformation and/or RARE binding.

Since those chimeric proteins all hold RARα DNA binding domain (DBD) and ligand binding domain (LBD), ATRA responsiveness is speculated in all cases. However, ATRA resistance has been confirmed clinically in cases showing *PLZF-RARα* [18, 34, 41] and *STAT5b-RARα* [21, 53, 55] fusions. One explanation for ATRA resistance is that the N-CoR/SMRT-corepressor complex interacts with PLZF, even in the presence of pharmacological concentration of ATRA, such that transcriptional de-repression cannot occur at RARα target gene promoters [34, 41]. The molecular mechanisms of ATRA resistance in STAT5b-RARα-expressing cells has not been fully explicated. Wild-type Stat5b is localized in cytoplasm, but STAT5b-RARα aberrantly localizes in nucleus [21]. STAT5b is a component of the janus kinase (JAK)-STAT signaling pathway, and phosphorylation of STAT5b by JAK causes homodimerization and translocation into the nucleus, where it acts as a transcription factor [56]. Aberrant transcription

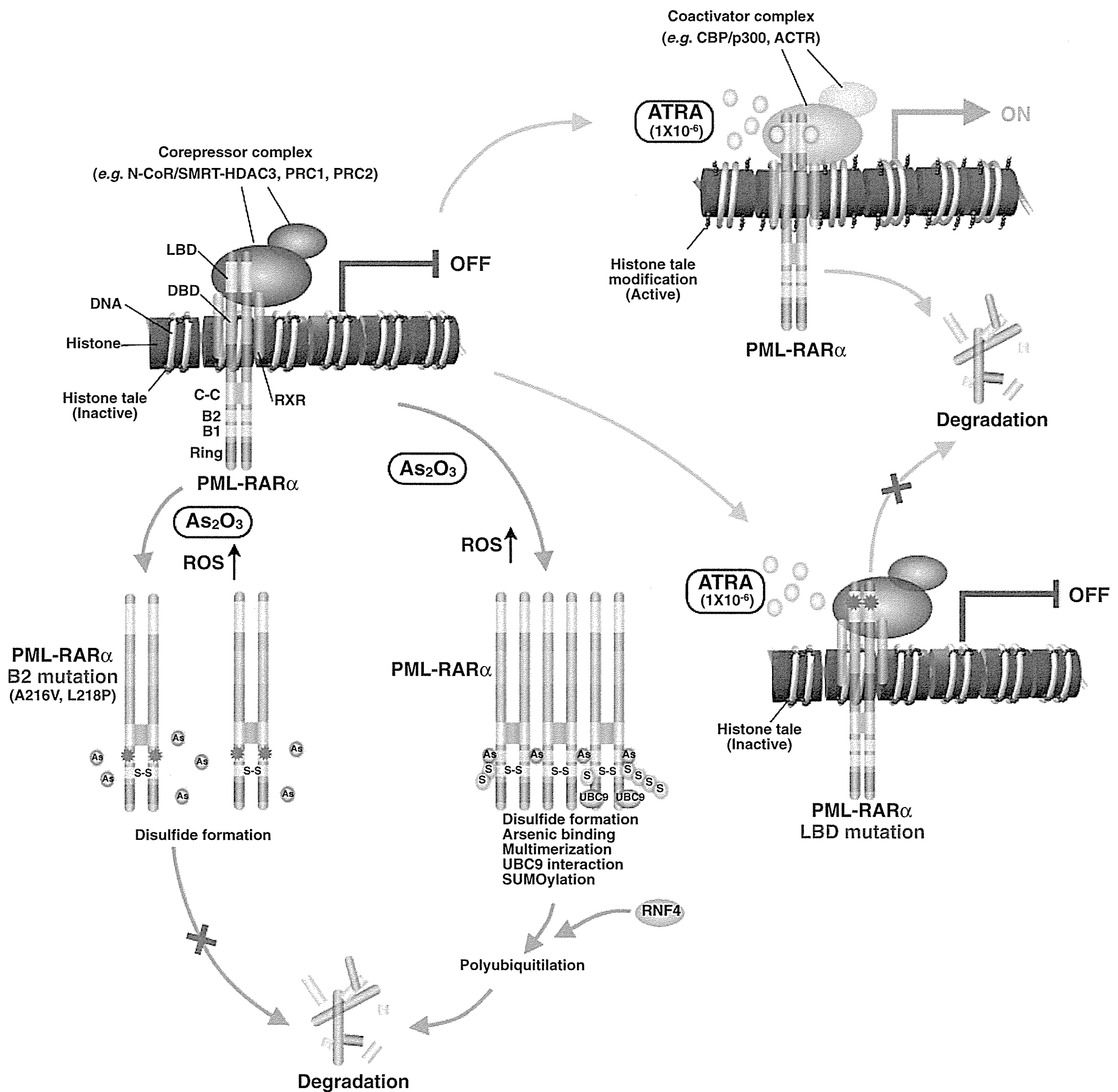

Fig. 2 Molecular mechanisms of action and resistance to ATRA and As_2O_3 in APL cells. PML-RARα are found mainly as homodimers through the C–C domain of PML, and partially as heterodimers with RXR. PML-RARα binds with target gene promoter in the absence of ligand, and recruits co-repressor complexes, such as N-CoR/SMRT complexes containing histone deacetylases (e.g. HDAC3) [34–37, 41] and PRC1/2 complex containing histone methyltransferases (e.g. EZH2) [39] to repress the gene expression. Histone tail deacetylation and/or methylation are related to transcription repression. In the presence of pharmacological concentration (1×10^6 μM) of ligand (ATRA), co-repressor complexes are dissociated from RARα, while co-activator complexes containing histone acetyltransferases (e.g. p300/CBP) are recruited, and transcription activation occurs. In the cases of PML-RARα with LBD mutations, ligand binding with LBD is interfered and co-repressor dissociation does not occur in the presence of pharmacological concentrations of ATRA. In the presence of As_2O_3, the formation of reactive oxygen species (ROS) is induced, and PML intermolecular disulfide crosslinks through B1 domain, that induce multimerization, and SUMOylation of PML by ubiquitin-conjugating enzyme 9 (UBC9) occur. As_2O_3 directly bind with PML-B2 domain and enhancing UBC9 binding and SUMOylation of PML. SUMOylated PML recruits RING finger protein 4 (RNF4), and is polyubiquitylated by RNF4, and proteasome-dependent degradation occurs. If PML-RARα has PML-B2 mutation, direct binding of As_2O_3 with PML is impaired, and polyubiquitylation and degradation are perturbed

regulation of STAT5b target genes in addition to RARα target genes by STAT5b-RARα may be related to ATRA resistance.

On the other hand, As_2O_3 resistance in clinical setting was observed in patients expressing PLZF- [57, 58], STAT5b- [55], and BCoR-RARα [25]. The As_2O_3-binding C–C motif is confirmed in PML-B2 domain, and As_2O_3 binding is critical for the multimerization followed by PML-RARα degradation [29, 30, 42]. Lack of As_2O_3 binding sites in X-RARα protein may be one explanation of loss of As_2O_3 responsiveness. However, no direct effect of As_2O_3 on RARα has been reported.

Mechanisms of resistance to ATRA

A number of mechanisms have been proposed to explain ATRA resistance in APL patients expressing PML-RARα, such as amino acid substitution in RARα LBD domain by genetic mutations, increased catabolism of ATRA, presence of cytoplasmic retinoic acid binding protein (CRABP), and abnormal ATRA delivery to the cell nucleus. Only genetic mutations on the RARα LBD domain in PML-RARα have been confirmed as an ATRA-resistant mechanism, from both clinical observations and in vitro molecular analyses [59–66]. Genetic mutations (missense, nonsense, and deletions) on RARα LBD domain have been confirmed in ATRA-resistant patients and APL cell lines, which grow despite pharmacological concentrations of ATRA, as summarized in Fig. 3. These mutations accumulate in the three subregions (zones I, II, and III in Fig. 3) of the LBD domain [66]. Gallagher et al. [66] reported that *PML-RARα* LBD mutation was confirmed 18 of 45 (40 %) relapse patients treated with ATRA/chemotherapy. In vitro analyses using ATRA-resistant NB4 cells (NB4-R1, -R2 [67], -R4 [60], and -RA [61]) and mutated-*PML-RARα* expressing Cos-1 cells [65] indicated that ATRA binding affinity with mutated PML-RARα was generally lower than that with PML-RARα without mutations, due to conformational changes in LBD. Furthermore, ligand-dependent N-CoR/SMRT co-repressor release and co-activator recruitment (e.g. ACTR histone acetyltransferase), which are critical for the transcriptional activation of genes with RARE sites and morphological cell differentiation, was impaired under the therapeutic dose of ATRA [60, 65, 67].

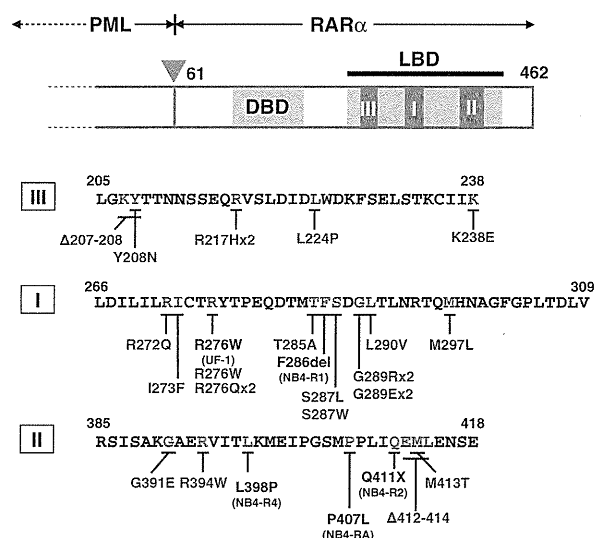

Fig. 3 Genetic mutations resulting in amino acid substitution in PML-RARα LBD confirmed in clinically ATRA-resistant patients and ATRA-resistant cell lines. Mutations are confirmed in 3 cluster regions (zones I to III) in RARα-LBD [66]. *Red letters* indicate amino acids substituted in specific patients and/or cells. Amino acid substitutions and deletions in ATRA-resistant patients are indicated in *blue letters*. Substitution in ATRA-resistant *cell lines* indicated in *black*. Names of *cell lines* are indicated in *brackets*. The position of the mutation is described with reference to normal amino acid sequence of RARα1 [31]

To overcome ATRA resistance, a number of therapeutics has been tested in vitro and in vivo. Several clinical reports indicated that As_2O_3 rescue most of relapsed/refractory patients treated with ATRA/chemotherapy [9–12, 68]. Am80, a synthetic retinoid that shows higher binding affinity with PML-RARα than ATRA, is utilized in the clinical setting [69–71]. Am80 is approximately 10 times more potent than ATRA as an in vitro inducer of differentiation in NB-4 and HL60 cells, and is chemically more stable than ATRA [72, 73]. Histone deacetylase (HDAC) inhibitors [74], such as sodium butyrate (NaF), valproic acid (VPA), and trichostatin A (TSA), have been utilized with ATRA and are expected to transcriptionally activate PML-RARα target genes to inhibit co-repressors complexes that contain HDACs [75–77]. Another approach to overcoming the resistance uses other molecular targeting therapeutics, such as gemtuzumab ozogamicin (GO), an anti-CD33 monoclonal antibody linked with calicheamicins [78, 79].

Molecular mechanisms of resistance to As_2O_3

Even for relapsed/refractory patients following treatment with ATRA/chemotherapy, As_2O_3 therapy is highly effective, with a complete remission rate of more than 80 % [80–82]. Although the CR rate is high even in relapsed patients, resistance to As_2O_3 treatment has been recognized as a clinically critical problem. Information on As_2O_3 resistance remains limited compared with that on ATRA resistance.

Recently, we reported two cases showing clinical As_2O_3 resistance after treatment with ATRA/chemotherapy, which exhibited missense mutations leading to substitution of amino acids in the PML-B2 domain in PML-RARα [50, 68, 83]. One patient with the M3 variant, expressing PML-

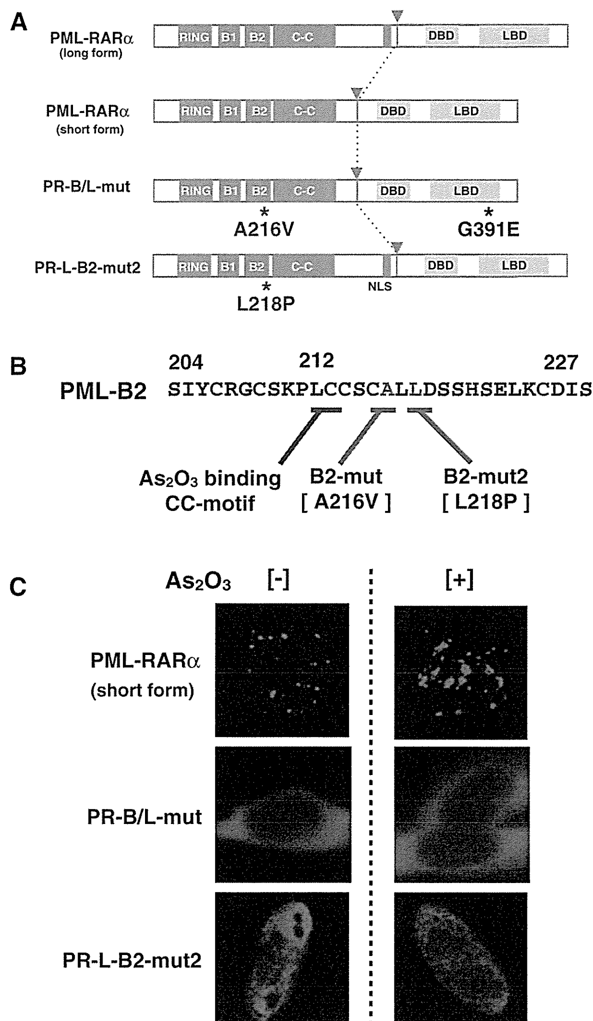

Fig. 4 Genetic mutations resulting in amino acid substitution in PML-B2 domain confirmed in clinically As_2O_3 resistant APL patients. **a** Schematic representation of PML-RARα chimeric protein with B2-domain mutation. One patient held PML-B2 mutation (A216V) and RARα-LBD mutation (G391E) on short form PML-RARα (PR-B/L-mut), and another patient held PML-B2 mutation (L218P) on long form PML-RARα [68]. **b** As_2O_3 direct binding dicysteine motif (C212/C213) [29, 30] and mutated positions in As_2O_3-resistant patients (C216 and L218) occur quite close to each other. **c** Flag-tagged PML-RARα short form, PR-B/L-mut, and PR-B2-mut2 were over expressed in HeLa cells with or without As_2O_3. Over expressed PML were detected by immunofluorescence staining using anti-Flag antibody. When using PML-RARα short form without As_2O_3, PML body was confirmed in the microspeckled pattern in cytoplasm. After incubation with As_2O_3, PML bodies showed macro granular patterns. When using PR-B/L-mut or PR-B2-mut2, the PML body showed diffuse pattern in cytoplasm or nucleus. No difference was seen with/without As_2O_3

RARα short form without nuclear localizing signal (NLS) [84], showed ATRA and As_2O_3 resistance at his terminal stage. Significant clonal expansion of *PML-RARα* mutant leading to A216V (PML-B2 domain mutation) and G391E (RARα-LBD mutation) was confirmed in leukemia cells harvested at the terminal stage (Fig. 4a, b). In vitro analysis using wild-type and mutant PML-RARα (PR-B/L-mut)-expressing HeLa and HL60 cells indicated that PML-RARα (short form) localized in cytoplasm as micro speckled pattern without As_2O_3, and as a macro granular pattern after adding As_2O_3 (Fig. 4c; PML-RARα). In contrast, PR-B/L-mut localized in cytoplasm with diffuse pattern without As_2O_3, and no change was confirmed in the presence of As_2O_3(Fig. 4c; PR-B/L-mut). Another case carried an L218P mutation, also in the PML-B2 domain (PR-B2-mut2), in PML-RARα long form with NLS. PML-RARα long form localized in nucleus, while PR-B2-mut2 was diffusely localized in the nucleus. No change was confirmed with or without As_2O_3 (Fig. 4c; PR-B2-mut2). Further in vitro analysis using PML-RARα overexpressed HeLa cells indicated that SUMOylation of PR-B/L-mut and PR-B2-mut2 after As_2O_3 treatment was strictly impaired. Recent reports have indicated that direct As_2O_3 binding to PML-B2 domain is critical for the serial reaction including SUMOylation, multimerization, and degradation [29, 30]. Jeanne et al. conclude that dicysteine C212/C213 in PML-B2 domain may be the direct As_2O_3 binding motif. From these results, genetic mutations identified in As_2O_3-resistant patients resulting in A216V and L218P may contribute to As_2O_3 resistance through impairment of direct As_2O_3 binding to PML-RARα due to conformational changes in As_2O_3 binding sites. Further accumulation of patients for genetic analyses is required for confirming the clinical significance of PML-B2 domain mutations in As_2O_3 resistance.

Conclusion

Although the overall survival of APL has been significantly prolonged since the introduction of ATRA and As_2O_3, relapse/refractory disease due to ATRA and/or As_2O_3 resistance remains a serious clinical problem. Additional genetic mutations in PML-RARα and another gene, such as FLT3-ITD or TP53 [66, 85], may contribute to disease progression and drug resistance in APL. Detailed genomic analyses using clinical samples harvested repeatedly from patients may help for predicting prognosis, selecting effective targeting drugs, understanding molecular backgrounds, and designing sophisticated new therapeutic strategies.

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