the case of the untreated patients might due to a multiplicity of CML subclones.

CML patients develop imatinib resistance through either Bcr-Abl dependent or independent mechanisms. The most characterized and frequent mechanism is the acquisition of point mutations within the kinase domain of the Bcr-Abl gene, and some of the mutations such as T315I are potent predictors for outcome. However, even in those patients who have some mutations other than a few restricted mutations such as T315I and F317L, we cannot accurately predict the efficacy of TKIs. Furthermore, nearly half of the patients resistant to imatinib have no mutations in Bcr-Abl, which indicates that other mechanisms are also important for the acquisition of drug-resistance. Thus, we need other information for selecting TKIs. In this study, 4 patients carried point mutations in this region. Samples from 3 of them had RI values compatible with the predictive outcomes from the mutations. Notably, the RI values of the other sample contradicted the response of the mutation, but accorded with the actual response of the patient. From these points of view, the system described here can be utilized as another powerful predictor than IC50s for Bcr-Abl mutations.

The immunoblot system described here has the capacity to detect TKI-resistant subclones, including CML cells with Bcr-Abl mutations. In addition, our strategy seems to evaluate Bcr-Abl activity more directly than the cellular IC50 and require smaller population of TKI-resistant subclones than Bcr-Abl sequence analysis. Thus, when used together with the cellular IC50 values and Bcr-Abl sequence, this immunoblot system should help improve the treatment of patients with CML.

Conflict of interest

The authors state that they have no conflict of interest.

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Myeloid neoplasm-related gene abnormalities differentially affect dendritic cell differentiation from murine hematopoietic stem/progenitor cells

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ABSTRACT

Dendritic cells (DCs) play important roles in tumor immunology. Leukemic cells in patients with myeloid neoplasms can differentiate into DCs *in vivo* (referred to as *in vivo* leukemic DCs), which are postulated to affect anti-leukemia immune responses. We established a reproducible culture system of *in vitro* FLT3 ligand-mediated DC (FL-DC) differentiation from murine lineage$^-$ Sca-1$^+$ c-Kit^{high} cells (LSKs), which made it possible to analyse the effects of target genes on steady-state DC differentiation from hematopoietic stem/progenitor cells. Using this system, we analysed the effects of various myeloid neoplasm-related gene abnormalities, termed class I and class II mutations, on FL-DC differentiation from LSKs. All class II mutations uniformly impaired FL-DC differentiation maintaining a plasmacytoid DC (pDC)/conventional DC (cDC) ratio comparable to the control cells. In contrast, class I mutations differentially affected FL-DC differentiation from LSKs. FLT3-ITD and a constitutively active form of Ras (CA-N-Ras) yielded more FL-DCs than the control, whereas the other class I mutations tested yielded less FL-DCs. Both FLT3-ITD and FLT3-tyrosine kinase domain (TKD) mutation showed a comparable pDC/cDC ratio as the control. CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα showed a severe decrease in the pDC/cDC ratio. CA-STAT5 and CA-MEK1 severely inhibited pDC differentiation. FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ aberrantly induced programmed death ligand-1 (PD-L1)-expressing DCs. In conclusion, we have established a simple, efficient, and reproducible *in vitro* FL-DC differentiation system from LSKs. This system could uncover novel findings on how myeloid neoplasm-related gene abnormalities differentially affect FL-DC differentiation from murine hematopoietic stem/progenitor cells in a gene-specific manner.

1. Introduction

DCs are professional antigen-presenting cells, and are the only cell type that can prime naive T cells. Hence, DCs play a pivotal role in innate and adaptive immunities as well as tolerance [1,2]. There are at least two major subsets of DCs, cDCs and pDCs [3]. cDCs possess numerous dendrites and exhibit high expression of major histocompatibility complex class II (MHC II), thereby enabling the stimulation of naïve T cells in the presence of appropriate costimulation. By contrast, pDCs have no or few dendrites and exhibit a plasmacytoid round morphology and low expression of MHC II and costimulatory molecules. Therefore, pDCs are poor stimulators of naïve T cells.

In tumor immunology, deregulation of differentiation, maturation, and function of DCs is thought to contribute to the inhibition of anti-tumor immunity, thereby facilitating disease progression [4–6]. DCs in cancer tissue and cancer-draining lymph nodes often display an immature phenotype both in tumor-bearing animals and in patients with cancer [5–7]. These immature DCs are reported to often induce tolerance by presenting antigens to T cells [2,5,8]. Moreover, the immunosuppressive milieu created by tumors frequently causes a decrease in the numbers of cDCs with no or little effect on the numbers of pDCs, which are known to play important roles in the maintenance of tolerance [6,7]. It has also been shown in mouse models that tumors themselves produced tumor-specific tolerance by pDCs or cDCs through expression of indoleamine 2,3-dioxygenase (IDO) or PD-L1 (also called B7-H1), respectively [9,10]. Thus, DC abnormalities in malignant tumors are characterised by the deregulation of the maturation states, subsets, or functions of DCs.

In contrast to non-hematopoietic malignancies, leukemic cells from patients with acute myeloid leukemia (AML) can differentiate into DCs *ex vivo* in the presence of granulocyte macrophage colony-stimulating factor (GM-CSF) with or without interleukin (IL)-4 [11]. These cells retain leukemic gene abnormalities of the original leukemic cells, hence enabling presentation of known and potentially unknown leukemia-associated antigens (LAAs) [12]. Therefore, in the case of AML, leukemia-derived DCs *ex vivo* (*ex*

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vivo leukemic DCs), which are sometimes referred to as AML-DCs or AML-derived DCs, have been used in DC immunotherapy. In addition to *ex vivo* leukemic DCs, populations of spontaneously differentiated DCs *in vivo* from leukemic cells (*in vivo* leukemic DCs) exist in patients with AML [13,14], chronic myeloid leukemia (CML) [15], and myelodysplastic syndrome (MDS) [16,17]. Because DCs derived from normal cells *in vivo* (*in vivo* normal-origin DCs) are present in these patients, one must discriminate the three different types of DCs that may be present in cases of hematopoietic neoplasm: *in vivo* leukemic DCs, *in vivo* normal-origin DCs, and *ex vivo* leukemic DCs. Although *in vivo* leukemic DCs are thought to have LAAs [13,14] and postulated to affect anti-leukemia immune responses, there have been few reports detailing concise examinations about their subsets, maturation state, or function [13–17].

By contrast, much work has been done on *ex vivo* leukemic DCs [11,18], which are cultured in the presence of GM-CSF. However, the concentration of GM-CSF under steady-state conditions is low or undetectable and *in vitro* GM-CSF-mediated DCs (GM-DCs) are regarded as monocyte-derived DCs, which are induced only under *in vivo* inflammatory states [3,19]. By contrast, the FLT3 ligand (FL) is a crucial cytokine for steady-state DC development *in vivo*, and *in vitro* FL-mediated DCs (FL-DCs) are close equivalents to *in vivo* steady-state splenic DCs [3,19,20]. In patients with leukemia, steady-state conditions refer to the early phase of the disease or the phase of minimal residual disease after therapy. Therefore, it is important to examine the properties of *in vivo* leukemic DCs under steady-state conditions, which are postulated to affect host immune responses in a LAA-specific manner. In this study, we aimed to establish a culture method to induce FL-DCs from LSKs, which are the target for leukemic transformation. We selected FL as a cytokine to induce DCs *in vitro*, which is in contrast to previous studies that have used GM-CSF. This system enabled us to evaluate the direct effects of myeloid neoplasm-related gene abnormalities on FL-DC subsets, their maturation state, and function. Our results reveal novel functions of myeloid neoplasm-related gene abnormalities as direct immune modifiers.

2. Materials and methods

2.1. Mice

C57BL/6 mice (6–9 weeks of age) were used for DC cultures. BALB/c mice (10–13 weeks of age) were used for the mixed leukocyte reaction. All animals were maintained in a pathogen-free barrier facility and handled according to institutional guidelines.

2.2. Antibodies and flow cytometry

Single-cell suspensions were treated with an Fc receptor-blocking antibody (2.4G2, BD Biosciences, San Jose, CA). When Fc receptor blocking was inadequate, purified isotype control antibodies were added to 2.4G2. Cells were subsequently stained with monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) or phycoerythrin-Cy7 (PE-Cy7). Biotinylated antibodies were detected using FITC conjugated streptavidin. The following antibodies were purchased from BD Biosciences. Gr-1 (RB6-8C5), Mac-1 (M1/70), Ter119 (TER-119), CD3ε (145-2C11), B220 (RA3-6B2), CD11c (HL3), I-A^b (AF6-120.1), CD40 (3/23), CD80 (16-10A1), CD86 (GL1), NK1.1 (PK136), CD172a (Sirp-α, P84), biotin CD24 (M1/69), c-Kit (2B8), Sca-1 (Ly6A/E, D7). The antibodies specific for PDCA-1 (eBio129c) and PD-L1 (MIH5) were purchased from eBioscience (San Diego, CA). The antibody specific for CCR9 (FAB2160P) was purchased from R&D systems (Minneapolis, MN). FACS analysis and cell sorting were performed using the FACS Canto II (BD Biosciences) and the FACS Aria (BD Biosciences) machines respectively.

2.3. Isolation of LSKs

Bone marrow cells extracted from 6- to 9-week-old C57BL/6 mice were mixed with CD117 MicroBeads (Miltenyi Biotech, Germany), and then $CD117^+$ cells were isolated with the autoMACS Separator (Miltenyi Biotech). The cells were stained with PE-conjugated antibodies specific for the lineage (Gr-1, Mac-1, Ter119, CD3ε, and B220) markers, APC-conjugated c-Kit-, and PE-Cy7-conjugated Sca-1-monoclonal antibodies. After washing, the cells were resuspended in 7-amino-actinomycin (7-AAD) (Calbiochem, San Diego, CA)-containing buffer. The 7-AAD^- lineage^- Sca-1^+ c-Kit^{high} cells were subsequently sorted using the FACS Aria (BD Biosciences). The purity of LSKs was consistently greater than 97%.

2.4. In vitro DC culture

Purified LSKs were cultured in a 48-well flat-bottom culture plate at a density of $4–10 \times 10^4$ cells/well in IMDM (GIBCO BRL, Grand Island, NY) containing 10% (vol/vol) fetal bovine serum (FBS), penicillin (100 units/ml, Nacalai Tesque, Kyoto, Japan), streptomycin (100 μg/ml, Nacalai Tesque, Kyoto, Japan), murine SCF (100 ng/ml, R&D), and murine TPO (100 ng/ml, R&D) at 37 °C in a humidified air containing 5% CO_2 (pre-culture phase, Fig. 1A). After 48 h, cells were washed, resuspended in IMDM (GIBCO) containing 10% FBS, murine FLT3-ligand (FL) (100 ng/ml, R&D), sodium pyruvate (1 mM, GIBCO), and 2-ME (100 μM, WAKO, Osaka, Japan), seeded in a 96-well round-bottom culture plate at a density of 3×10^3 cells/well, and cultured at 37 °C in a humidified air containing 10% CO_2 for the indicated period (DC-induction phase, Fig. 1A). In an *in vitro* GM-CSF/IL-4-mediated DC (GM/IL-4-DC) culture from LSKs, FL was substituted for GM-CSF (20 ng/ml, R&D) and IL-4 (10 ng/ml, R&D) during the DC-induction phase.

2.5. Plasmid constructs

Using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), we constructed a murine FLT3-internal tandem duplication (ITD) that contained a tandem insertion of Arg-Glu-Tyr-Glu-Asp-Lys between amino acids 602/603, similar with the previously reported FLT3-ITD [21]. The primer sequences for FLT3-ITD were as follows: 5′-agg-gaa-tat-gaa-gac-ctt-3′ (forward), 5′-aag-gtc-ttc-ata-ttc-cct-3′ (reverse). Murine FLT3 wild type (FLT3-WT) [22], murine FLT3-ITD, murine $FLT3^{Asp838Val}$ (FLT3-TKD) [22], N-$Ras^{Gly12Asp}$ (CA-N-Ras) [23], murine c-$Kit^{Asp814Val}$ (c-Kit-TKD) [24], FIP1L1/PDGFRα [25], TEL/PDGFRβ [25], AML1/ETO [26], PML/RARα [27], CBFβ/MYH11 [28], AML1dC [29] were each subcloned into a murine stem cell virus-internal ribosome entry site-EGFP (pMie) vector. pMYs-IRES-EGFP, a retrovirus expression vector, was kindly provided by Dr. T. Kitamura (University of Tokyo, Tokyo, Japan). STAT3c [30], 1*6-STAT5A [31], constitutive active form of MEK1 (CA-MEK1) (Invitrogen, Carlsbad, CA, USA), and membrane-targeted p110 [32] were each subcloned into pMYs-IRES-EGFP.

2.6. Preparation of retroviral particles

Conditioned medium containing high titer retroviral particles was prepared as reported previously with some modifications [33]. Briefly, each retroviral vector was cotransfected with vesicular stomatitis virus glycoprotein-, and gag/pol-expression plasmids into 293T cells by Lipofectamine 2000 (Invitrogen). After 48 h, the

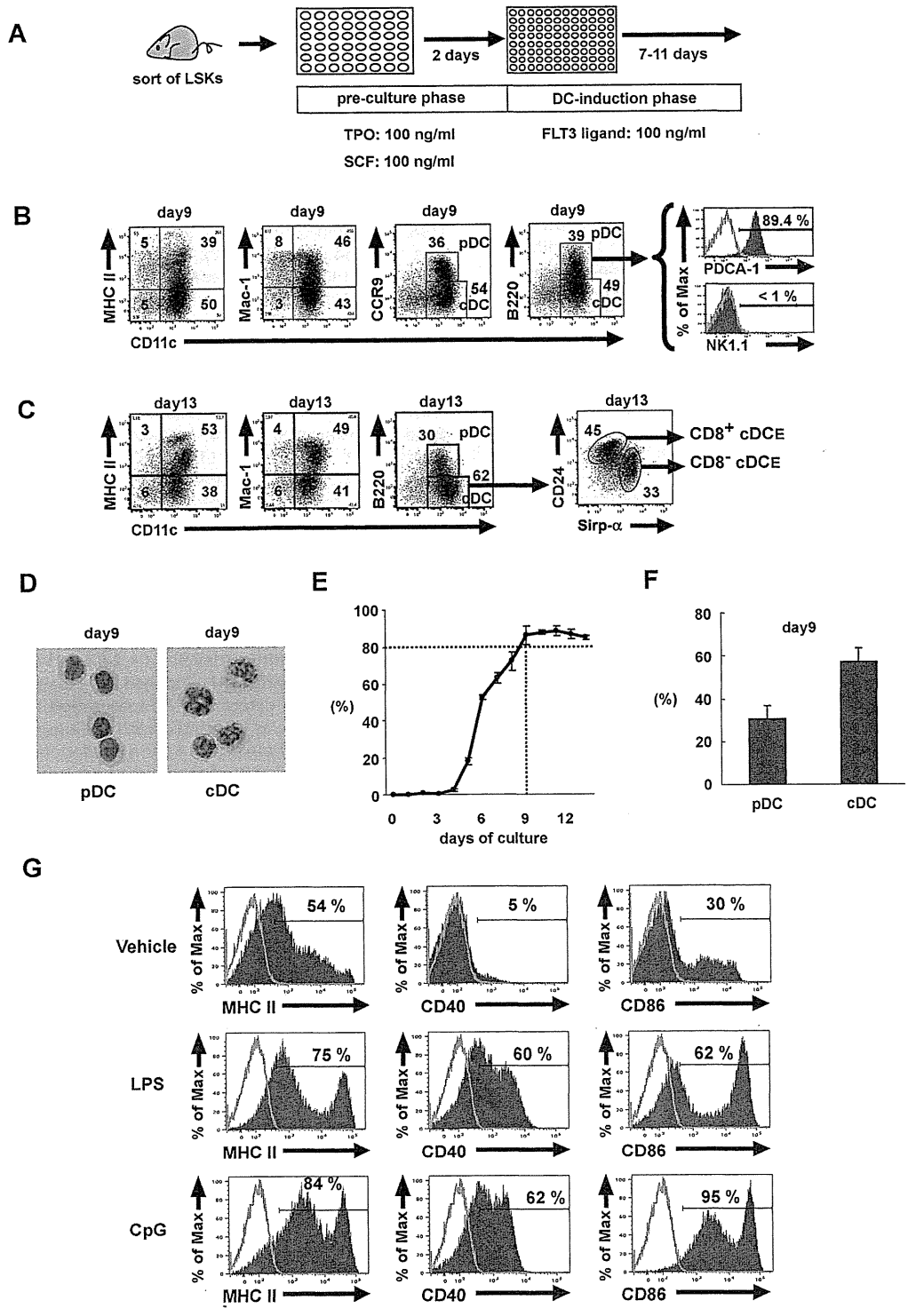

Fig. 1. FL-DCs from LSKs are phenotypically and morphologically similar to FL-DCs from whole bone marrow cells. (A) Schema of an *in vitro* FL-mediated DC differentiation system from LSKs. After 9 (B) or 13 (C) days of culture, cells were collected and the expression of the indicated surface markers was analysed by flow cytometry. (D) After 9 days in culture, FACS-sorted pDCs ($CD11c^+B220^+CCR9^+$) or cDCs ($CD11c^+B220^-CCR9^-$) cells were spun onto slides, and stained with May-Giemsa. Images are shown at ×400 original magnification. (E) Cells were harvested at the indicated day of culture and the surface expression of CD11c was analysed by flow cytometry. (F) After 9 days in culture, cells were collected and the expression of CD11c and B220 was analysed by flow cytometry. The bars show the proportion of pDCs ($CD11c^+B220^+$ cells) and cDCs ($CD11c^+B220^-$ cells) in cultured cells respectively. Numbers in the dot plots represent percentage of cells. (G) After 9 days in culture, cells were stimulated with CpG (1 μM), LPS (1 μg/ml), or left unstimulated. After 24 h, cultured cells were stained with the indicated surface markers and analysed by flow cytometry on the gated $CD11c^+$ cell population. Filled and open histograms show specific staining and back ground staining respectively.

culture supernatant was collected, concentrated 100-fold in volume, filtered (0.45 μm), and aliquoted for storage at −80 °C until use.

2.7. Retroviral transduction into murine hematopoietic stem/progenitor cells

Purified LSKs were cultured in IMDM medium (GIBCO) containing 10% FBS, penicillin (100 unit/ml), streptomycin (100 μg/ml), murine SCF (100 ng/ml, R&D), and murine TPO (100 ng/ml, R&D) in a flat-bottom 48-well culture plate at 37 °C in a humidified air containing 5% CO_2. After 24 h, cells were seeded into a flat-bottom 48-well culture plate coated with Retronectin (TAKARA BIO, Shiga, Japan) at a density of 1.5–2.0 × 10^5 cells/well, and then infected with each retrovirus in the same medium containing protamine sulphate (10 μg/ml, Sigma, St Louis, MO). The cells were cultured at 37 °C in a humidified air containing 5% CO_2 for 24 h after which the cells were washed and subjected to the DC-induction phase.

2.8. Allogeneic mixed leukocyte reaction (MLR)

Varying numbers of FACS-sorted, irradiated (30 Gy) *in vitro* FL-mediated $CD11c^+$ or $EGFP^+CD11c^+$ cells were plated in a 96-well round-bottom culture plate with 5 × 10^4 BALB/c splenic $CD4^+$ T cells that were immunomagnetically selected using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec). The purified $CD4^+$ T cells were suspended in a final volume of 150 μl RPMI 1640 (Nacalai Tesque) supplemented with 10% FBS, 2-ME (50 μM), penicillin (100 units/ml, Nacalai Tesque), streptomycin (100 μg/ml, Nacalai Tesque) prior to the mixing reaction. Cells were cultured for 4 days and pulsed with 1 μCi $[^3H]$-thymidine (Amersham Biosciences, Buckinghamshire, UK) per well during the last 16 h of culture. $[^3H]$-thymidine incorporation was measured on a β-plate counter.

2.9. ELISA

To evaluate IFN-α production, more than 97% of purified FL-DCs that were immunomagnetically selected with CD11c microbeads (Miltenyi Biotec), were cultured for 24 h at a density of 5 × 10^4 cells/200 μl in a 96-well round-bottom culture plate in IMDM medium supplemented with 10% FBS, penicillin (100 units/ml, Nacalai Tesque), streptomycin (100 μg/ml, Nacalai Tesque), 2-ME (50 μM), and sodium pyruvate (1 mM, GIBCO). Cells were stimulated with 1 μM of CpG-A-ODN (5′-ggTGCATCGATGCAggggggG-3′; small letters indicate bases with phosphorothioate-modified backbones), lipopolysaccharide (LPS) (Escherichia coli O55:B5, 1 μg/ml, Sigma, St Louis, MO), or vehicle. Cultured supernatants were assayed using an IFN-α ELISA Kit (R&D systems).

2.10. Cell count

All cultured cells in each well were harvested at 3 or 9 days of culture, and the absolute cell numbers and the proportion of $EGFP^+$ cells were analysed by flow cytometry. Fold increase in $EGFP^+$ cells was obtained by dividing the mean absolute cell numbers of $EGFP^+$ cells in 8 wells at 9 days of culture by the number at 3 days of culture. Data are shown as mean ± standard deviation (SD) from at least three independent assays.

2.11. Statistics

Data are shown as mean ± standard deviation (SD), and the Student *t*-test was used to compare two groups of samples.

3. Results

3.1. Establishment of an in vitro FL-mediated DC differentiation system from LSKs

Our initial aim was to establish a reproducible *in vitro* FL-mediated DC (FL-DC) differentiation system from LSKs for the purpose of assessing the influence of target genes on steady-state DC differentiation. *In vitro* FL-mediated $CD11c^+$ cells from LSKs were subdivided into $CD11c^+B220^+CCR9^+$ cells (pDC phenotype) and $CD11c^+B220^-CCR9^-$ cells (cDC phenotype) (Fig. 1B) [34–37]. $CD11c^+B220^+$ cells also express PDCA-1 but not NK1.1 [38], which is compatible with the pDC phenotype. Consistent with previous studies on FL-DCs from whole bone marrow cells [3,20], FL-DCs from LSKs did not express CD4 or CD8α (data not shown). Additionally $CD11c^+B220^-$ cells could be subdivided into $CD24^{high}CD11b^{low}Sirp\alpha^{low}$ ($CD8^+$ cDC_E) and $CD24^{low}CD11b^{high}Sirp\alpha^{high}$ ($CD8^-$ cDC_E) cells (Fig. 1C), equivalent with $CD8\alpha^+$ and $CD8^-$ cDCs found *in vivo* respectively [20]. FL-DCs from LSKs expressed none or low levels of other lineage markers such as Gr-1, Ter119, CD3ε, CD19, and NK1.1 (data not shown). FL-DCs from LSKs were morphologically compatible with the FL-DCs from whole bone marrow cells (Fig. 1D). After 9 days of culture, greater than 80% of cultured cells were consistently CD11c positive (Fig. 1E). In addition, the proportion of pDCs ($CD11c^+B220^+$ cells) and cDCs ($CD11c^+B220^-$ cells) were reproducibly consistent (pDCs; 30.4 ± 6.4%, cDCs; 57.1 ± 6.4%, pDCs/cDCs ratio; 0.55 ± 0.19) (Fig. 1F). Similar to a previous study using a DC culture from whole bone marrow cells [39], a maximal number of DCs was obtained after 9–10 days of culture (day 9: 7.1 ± 2.8 × 10^4 cells/well), which is similar to the kinetics of FL-induced DC expansion *in vivo* [40]. We next sought to determine the maturation ability of FL-DCs from LSKs. FL-DCs was associated with the upregulation of MHC II and costimulatory molecules upon stimulation with LPS or CpG (Fig. 1G). Similar to *in vitro* DC cultures from whole bone marrow cells, $CD11c^+$ cells included many pre-DCs, defined as $CD11c^+MHC\ II^-$ cells (Fig. 1B and G). Pre-DCs are considered late-stage precursors, and differentiate with a minimal number of divisions into exclusively DC subsets [41,42]. Consistent with this, almost all pre-DCs immediately differentiated into $CD11c^+MHC\ II^+$ cells after 24 h stimulation with CpG or LPS (Fig. 1G). Therefore, we classified all $CD11c^+$ cells, including pre DCs, as FL-DCs. The pDC/cDC ratio did not significantly differ between 9 and 13 days in culture, whereas the proportion of pre-DCs at 13 days of culture was decreased compared to the proportion of pre-DCs at 9 days in culture (Fig. 1B and C). These results were reproducible in at least four independent experiments.

3.2. FL-DCs from LSKs are functional

We next determined whether FL-DCs from LSKs were functional. FL-DCs from LSKs efficiently stimulated allogeneic $CD4^+$ T cells (Fig. 2A). FL-DCs from LSKs yielded a large amount of type I interferon upon CpG-stimulation (Fig. 2B). These results indicated that FL-DCs from LSKs were functionally competent DCs.

3.3. FL-DC differentiation from LSKs is deregulated in a myeloid neoplasm-related gene abnormality-specific manner

Mohty et al. reported the quantitative imbalance of *in vivo* DC subsets in patients with AML and divided the patients into three groups according to the proportion of pDCs and cDCs [13], however, the cause for this heterogeneity was unknown. AMLs have heterogeneous myeloid neoplasm-related gene abnormalities, that are termed class I and class II mutations, which contribute to deregulated signal transduction pathways and myeloid differentiation impairment respectively [43]. On the other hand, DC

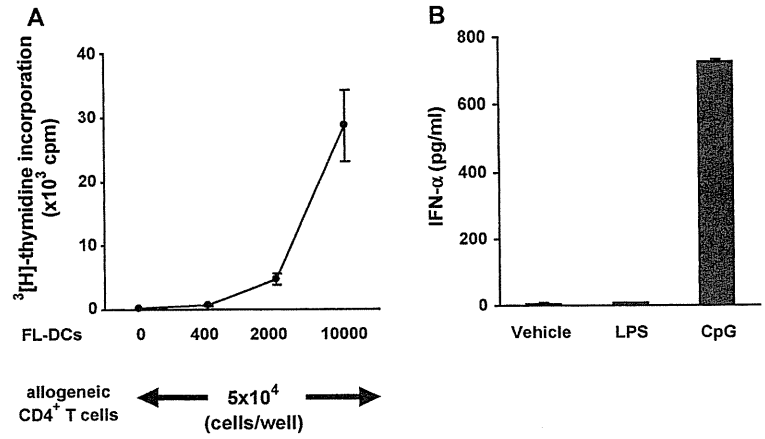

Fig. 2. FL-DCs from LSKs are functional. (A) Allogeneic CD4$^+$ T cells (5 × 10^4 cells) from BALB/c mice were co-cultured with graded numbers of irradiated (30Gy) FACS-sorted FL-DCs (CD11c$^+$ cells) from C57BL/6 mice for 4 days. Proliferation was measured by [^{3}H]-thymidine incorporation. Mean ± SD is shown. Experiments were repeated at least three times with similar results. (B) After 9 days in culture, FL-DCs were stimulated with CpG (1 μM), LPS (1 μg/ml), or left unstimulated in a total volume of 100 μl. After 24 h, the supernatants were harvested and assayed using ELISA.

differentiation is crucially regulated by STAT3/5 and in part associated with myeloid differentiation [3,44]. From these observations, we hypothesised that myeloid neoplasm-related gene abnormalities themselves might cause the quantitative imbalance found in *in vivo* leukemic DC subsets. Therefore, we determined whether myeloid neoplasm-related gene abnormalities affected FL-DC differentiation from LSKs. We selected FLT3-ITD, FLT3-TKD, CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα as representatives of class I mutations, and AML1/ETO, PML/RARα, CBFβ/MYH11, and AML1dC as representatives of class II mutations. We investigated how myeloid neoplasm-related gene abnormalities affected the absolute cell numbers of EGFP$^+$ cells (Fig. 3A). Compared with the mock population, all class I mutations except for FIP1L1/PDGFRα increased the number of EGFP$^+$ cells to various extents ($p < 0.01$). AML1/ETO and CBFβ/MYH11 yielded less EGFP$^+$ cells than mock population ($p < 0.05$). PML/RARα and AML1dC yielded comparable EGFP$^+$ cells to the mock population. We then focused on the proportion of whole FL-DCs (EGFP$^+$CD11c$^+$ cells in EGFP$^+$ cells). Compared to the mock population, all myeloid neoplasm-related gene abnormalities showed a significant decrease in the proportion of whole FL-DCs from LSKs ($p < 0.00001$) (Fig. 3B and C). Class II mutations uniformly displayed a mild decrease in the proportion of whole FL-DCs from LSKs (the proportion of EGFP$^+$CD11c$^+$ cells in EGFP$^+$ cells: 35.6–55.8%). By contrast, class I mutations exhibited variability (from mild to severe) in the decrease of the proportion of whole FL-DCs from LSKs (the proportion of EGFP$^+$CD11c$^+$ cells in EGFP$^+$ cells: 9.4–55.2%). From these data, we calculated the fold increase in whole FL-DC yields by multiplying the fold increase in EGFP$^+$ cells by the proportion of FL-DCs (the mean fold increase in EGFP$^+$ cells × the mean proportion of whole FL-DCs). FLT3-ITD and CA-N-Ras showed increased FL-DC yields than the mock population (Fig. 3D). The remaining class I mutations and all class II mutations exhibited decreased FL-DC yields. Lastly, we focused on the DC subsets, pDCs and cDCs. Compared with mock, CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα mutations displayed a severe decrease in the proportion of pDCs, with almost all EGFP$^+$CD11c$^+$ cells being cDCs, indicating a severe decrease in the pDC/cDC ratio (Fig. 3B and E). By contrast, FLT3-WT, FLT3-ITD, FLT3-TKD, and all class II mutations displayed comparable pDC/cDC ratios as the mock population. Taken together, class II mutations consistently yielded fewer FL-DCs from LSKs, and exhibited comparable pDC/cDC ratios with the control population. In contrast, class I mutations exhibited a variety of patterns regarding the whole FL-DC yields and their pDC/cDC ratios. FLT3-ITD and FLT3-TKD exhibited a comparable pDC/cDC ratio with the control, regardless of the difference in whole FL-DC yields. CA-N-Ras displayed a marked increase in the FL-DC yield and a severe decrease in the pDC/cDC ratio. c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα exhibited less whole FL-DC yields and a severe decrease in the pDC/cDC ratio.

3.4. CA-N-Ras-, c-Kit-TKD-, TEL/PDGFRβ-, and FIP1L1/PDGFRα-expressing FL-DCs from LSKs showed distinct differentiation patterns from FL-DCs derived from LSKs

Two distinct types of DCs exist, steady-state and inflammatory DCs, the equivalents of which can be induced *in vitro* by FL or GM-CSF/IL-4 and are termed FL-DCs and GM/IL-4-DCs, respectively [3,19]. GM/IL-4-DCs are considered to be monocyte-derived DCs and composed of only cDCs. By contrast, FL-DCs include both pDCs and cDCs. Moreover, precursors of steady state DCs and FL-DCs are distinct from monocytes [19,45], suggesting that FL-DC and GM/IL-4-DC differentiation are distinct pathways. We focused on the differentiation pattern of FL-DCs and GM/IL-4-DCs from LSKs (Fig. 4A). In the presence of FL, almost all cultured cells began to express CD11c and Mac-1$^{-/dim}$ during 4–7 days in culture, and then they began to differentiate into CD11c$^+$Mac-1$^-$ and CD11c$^+$Mac-1$^+$ cells. In the presence of GM-CSF and IL-4, almost all cultured cells initially expressed Mac-1 and subsequently expressed CD11c after 7 days in culture. Consistent with previous reports, GM/IL4-DCs lacked pDCs (B220$^+$CD11c$^+$ cells) throughout the culture period (Fig. 4A). We next investigated the effects of CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα on the differentiation pattern of FL-DCs from LSKs (Fig. 4B). FL-DC differentiation from LSKs expressing CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, or FIP1L1/PDGFRα was characterised by the initial expression of Mac-1 during 4–7 days in culture, followed by the delayed expression of CD11c on Mac-1$^+$ cells. Among these four mutations, CA-N-Ras induced a significant proportion of CD11c$^+$Mac-1$^+$ cells at 7 days in culture. FL-DCs from LSKs expressing CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, or FIP1L1/PDGFRα generated few pDCs throughout the culture period.

3.5. Active forms of STAT5 and MEK1 severely impaired pDC differentiation from LSKs

We next examined why class I, but not class II, mutations caused variable patterns of FL-DC differentiation from LSKs. Class I, but not class II, mutations constitutively activated various sig-

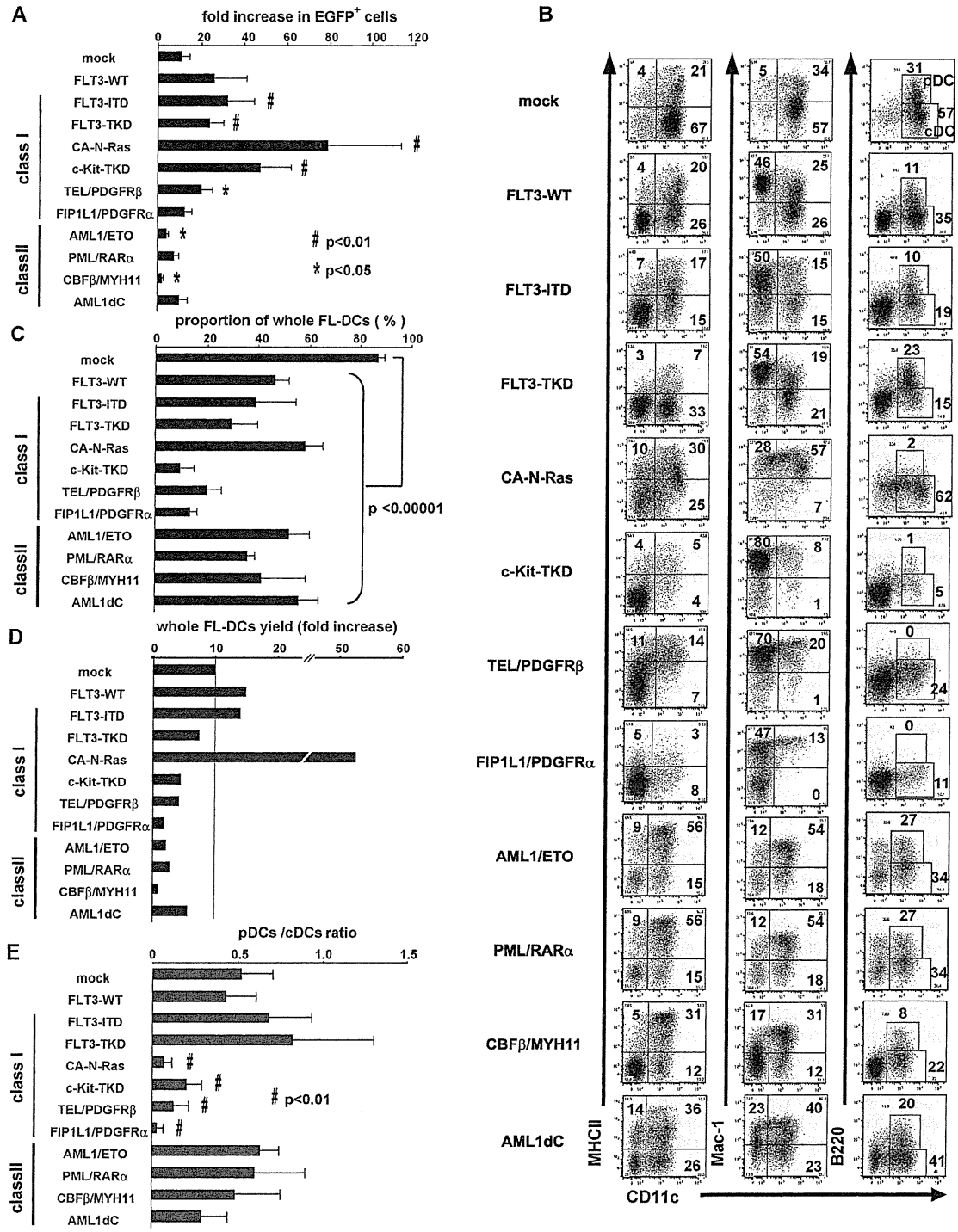

Fig. 3. FL-DC differentiation from LSKs is deregulated in a myeloid neoplasm-related gene abnormality-specific manner. During the pre-culture phase, cells were transduced with the indicated myeloid neoplasm-related gene abnormality. After 9 days in culture, cells were collected, and the absolute cell numbers and expressions of the indicated surface markers were analysed by gating on the EGFP$^+$ cells by flow cytometry (A–C, and E). Numbers in the dot plots represent percentage of cells. The bars represent fold increase in EGFP$^+$ cells (fold increase in absolute cell numbers of EGFP$^+$ cells from 3 to 9 days in culture) (A), the proportion of FL-DCs (EGFP$^+$CD11c$^+$ cells in EGFP$^+$ cells) (C), FL-DC yields (the mean fold increase in EGFP$^+$ cells × the mean proportion of whole FL-DCs) (D), and pDCs (EGFP$^+$CD11c$^+$B220$^+$ cells)/cDCs (EGFP$^+$CD11c$^+$B220$^-$ cells) ratio (E), respectively. Data are representative of at least three experiments with similar results. Mean ± SD is shown. * and # indicate $p < 0.05$ and $p < 0.01$ respectively.

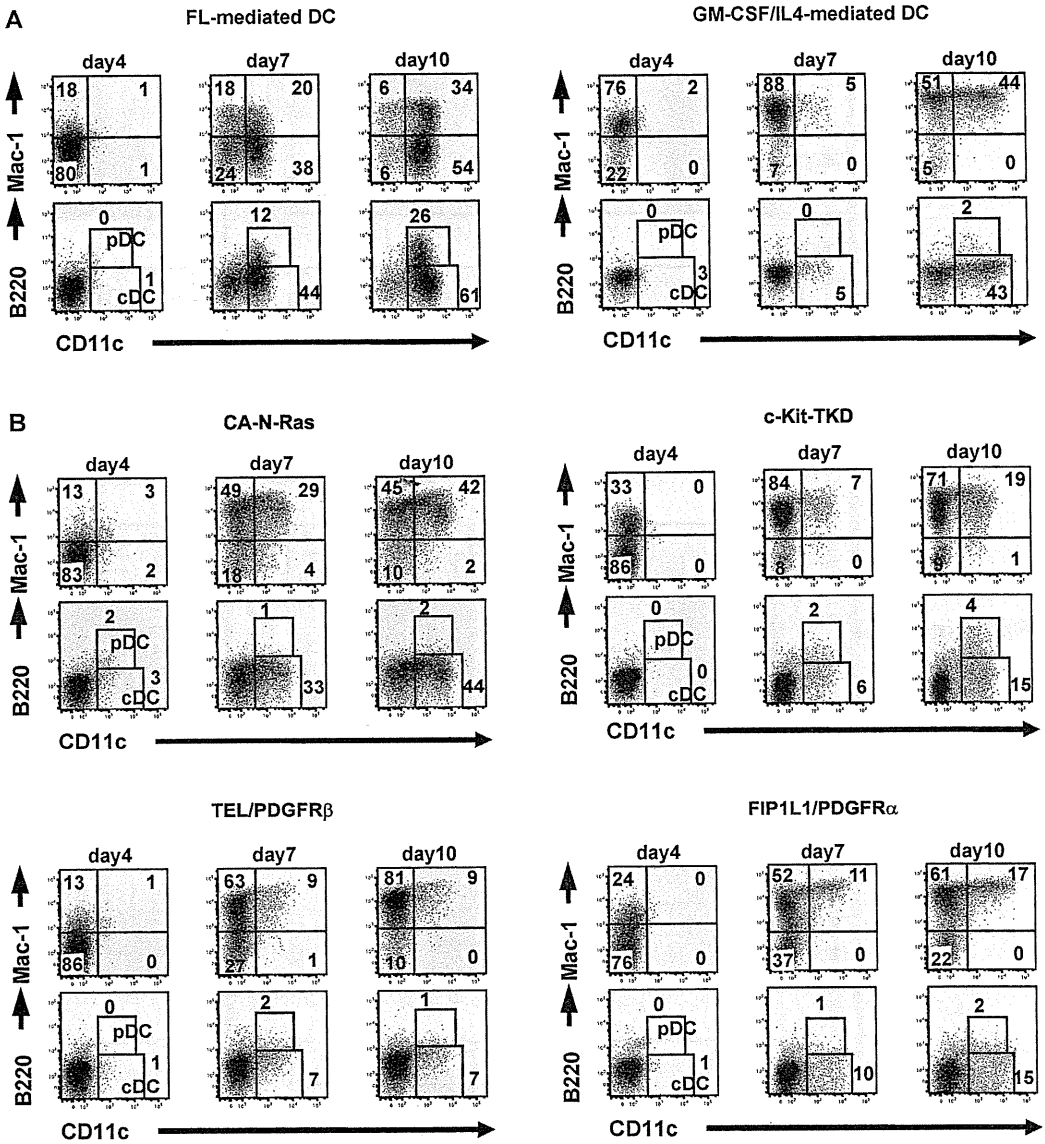

Fig. 4. CA-N-Ras-, c-Kit-TKD-, TEL/PDGFRβ-, and FIP1L1/PDGFRα-expressing FL-DCs from LSKs showed distinct differentiation patterns. (A) After the indicated days in culture in the presence of FL or GM-CSF with IL-4, cells were collected and the expression of the indicated surface markers was analysed by flow cytometry. (B) During the pre-culture phase, cells were transduced with the indicated myeloid neoplasm-related gene abnormality. After the indicated days of culture in the presence of FL, cells were collected and the expression of the indicated surface markers was analysed on the gated EGFP$^+$ cell population by flow cytometry. Numbers in the dot plots represent percentage of cells.

nal transduction pathways [43,46]. Therefore, we hypothesised that constitutively activated signals might generate the heterogeneous patterns of FL-DC differentiation from LSKs. We evaluated the influence of activated forms of signaling molecules on FL-DC differentiation from LSKs. We selected STAT3c, membrane-targeted p110, 1*6-STAT5A, and CA-MEK1 as a constitutively active form of STAT3, PI 3-kinase, STAT5, and MAP-kinase pathways, respectively. Compared with control cells, CA-STAT3 and CA-PI 3-kinase displayed a slight decrease in the proportion of whole FL-DCs from LSKs and was associated with a modest increase in the pDC/cDC ratio (Fig. 5). By contrast, CA-STAT5 and CA-MEK1 displayed a severe and mild decrease in the proportion of whole FL-DCs from LSKs, respectively. Both mutations resulted in a, severe decrease in the pDC/cDC ratio (Fig. 5).

3.6. Expression patterns of MHC II and costimulatory molecules on FL-DCs from LSKs are heterogeneous among class I mutations

We determined whether this heterogeneity in DC differentiation seen in class I mutations influenced the DC maturation state. DC maturation is associated with up-regulation of MHC II and costimulatory molecules [47]. We therefore screened the surface expression of MHC II and costimulatory molecules such as CD40, CD80, and CD86 on FL-DCs from LSKs bearing myeloid neoplasm-related gene abnormalities. Overall, expressions of MHC II, CD80, and CD86 on whole FL-DCs induced by class I mutations were higher than those in control cells with several exceptions such as MHC II expression on DCs induced by FLT3-TKD and FIP1L1/PDGFRα (Fig. 6A and B). None of the class I mutations induced expression

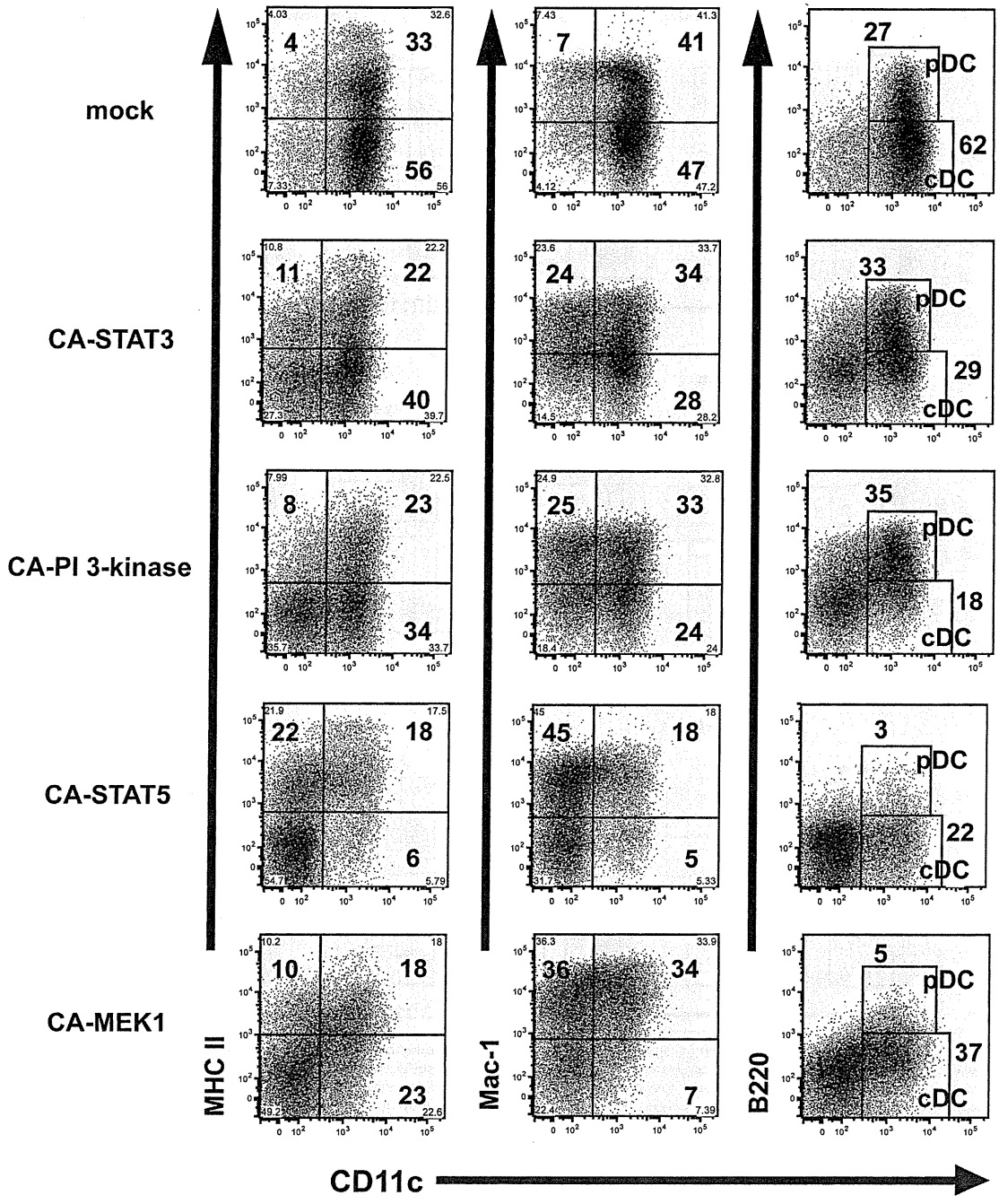

Fig. 5. Both active forms of STAT5 and MEK1 severely impaired pDC differentiation from LSKs. During the pre-culture phase, cells were transduced with the indicated activated form of signaling molecule. After 9 days in culture, cells were collected and the expression of the indicated surface markers was analysed by flow cytometry on the gated EGFP$^+$ cell population. Data are representative of at least two experiments with similar results. Numbers in the dot plots represent percentage of cells.

of CD40 on whole FL-DCs from LSKs (data not shown). In addition, we determined whether these differences in expression of MHC II and costimulatory molecules on FL-DCs from LSKs between class I mutations affected the ability to stimulate allogeneic CD4$^+$ T cells. Among class I mutations, we selected FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ as representatives for an allogeneic MLR assay, because they showed contrasting patterns of whole FL-DC yields (Fig. 3D), pDC/cDC ratios (Fig. 3E), differentiation patterns (Fig. 4), and expression patterns of MHC II and costimulatory molecules (Fig. 6A and B). Both CA-N-Ras- and TEL/PDGFRβ-expressing FL-DCs from LSKs efficiently stimulated allogeneic CD4$^+$ T cells, possibly resulting from high expressions of MHC II and costimulatory molecules (Fig. 6C). By contrast, despite FLT3-ITD-expressing FL-DCs from LSKs exhibiting a relatively higher expression of MHC II and costimulatory molecules than those of mock cells, FLT3-ITD-expressing FL-DCs stimulated allogeneic CD4$^+$ T cells at comparable

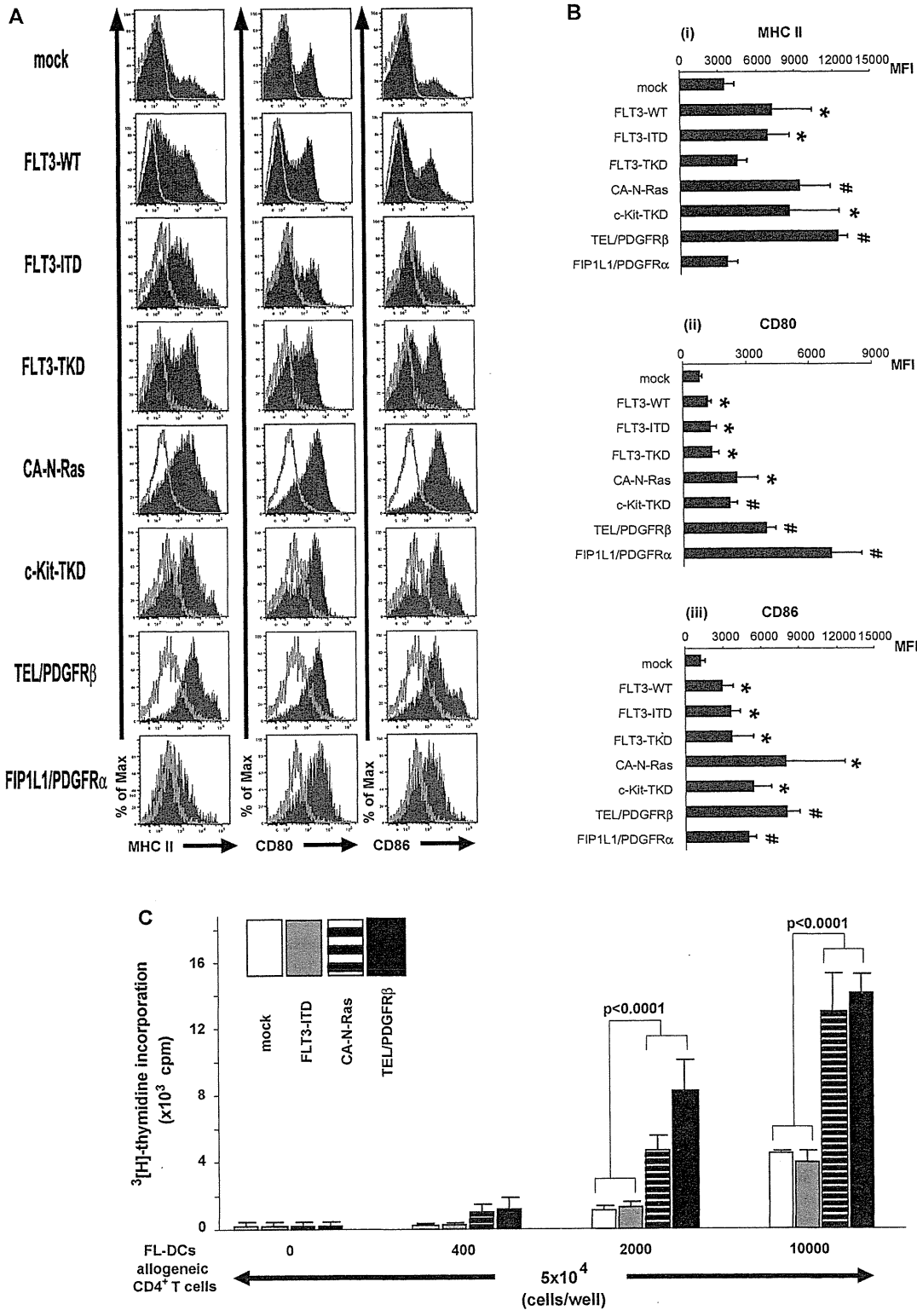

Fig. 6. Expression patterns of MHC II and costimulatory molecules on FL-DCs from LSKs are heterogeneous among class I mutations. During the pre-culture phase, cells were transduced with the indicated class I mutation. After 9 days in culture, cells were collected and the expression of MHC II and costimulatory molecules was analysed by flow cytometry on the gated EGFP⁺CD11c⁺ cell population. (A) Filled and open histograms show specific staining and back ground staining respectively. (B) The bars represent mean fluorescence intensity (MFI) of surface expression of MHC II (i), CD80 (ii), and CD86 (iii) respectively. (C) Allogeneic CD4⁺ T cells from BALB/c mice were co-cultured with graded numbers of irradiated (30 Gy) FACS-sorted EGFP⁺ FL-DCs from LSKs from C57BL/6 mice for 4 days. Proliferation was measured by [³H]-thymidine incorporation. Data are representative of at least three experiments with similar results. Mean ± SD is shown. * and # indicate $p < 0.05$ and $p < 0.01$ respectively.

level as control cells (Fig. 6C). Taken together, the class I mutations tested displayed heterogeneous expression patterns of MHC II and costimulatory molecules on FL-DCs from LSKs.

3.7. FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ aberrantly induced PD-L1-expressing DCs

The interaction between programmed death-1 (PD-1) and PD-L1 results in diminished anti-tumor T-cell responses in both solid tumors [10,48–51] and hematological malignancies [52–54]. Therefore, we determined whether class I mutations induced expression of PD-L1 on FL-DCs from LSKs. We selected FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ as representatives of class I mutations. FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ induced PD-L1-expressing DCs, whereas the mock population did not induce PD-L1-expressing DCs (Fig. 7). In FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ, the proportion of FL-DCs expressing PD-L1 was increased in an EGFP-intensity dependent manner, suggesting that the induction of PD-L1-expressing DCs correlated with the expression of each myeloid neoplasm-related gene abnormality.

4. Discussion

Little is known about *in vivo* DCs in patients with myeloid leukemia [13–17,55–57] compared with *ex vivo* leukemic DCs [11,18]. This may be a result from several obstacles in the study of *in vivo* DCs [58]. DCs are a relatively rare population *in vivo* compared with other hematopoietic cells [59]. In addition, the expression of aberrant markers such as CD7, CD19, or CD56 is often detected [60–62], and may be retained on *in vivo* leukemic DCs in AML cases. Therefore, it is difficult to phenotypically identify *in vivo* leukemic DCs, which are identified by the negative selection using surface markers such as CD3, CD14, CD16, CD19, and CD56 [13,16]. Furthermore, in hematological malignancies, two distinct types of DCs *in vivo* exist that differ in their origin, "*in vivo* leukemic DCs" and "*in vivo* normal-origin DCs". To date it is not possible to isolate viable *in vivo* leukemic DCs from *in vivo* normal-origin DCs until genetic analysis for leukemia-specific markers such as fluorescence *in situ* hybridisation, sequence, or polymerase chain reaction is performed. Thus, study into *in vivo* DCs in leukemia cases is challenging and may explain the relatively small number of studies that have investigated the significance of *in vivo* leukemic DCs.

In order to address this problem, we established a reproducible FL-mediated *in vitro* DC differentiation system from LSKs, which imitates the differentiation process of *in vivo* leukemic DCs. Our system is characterised by two points distinct from previous studies. First, the system recapitulates steady-state DC differentiation. In tumor immunology, once tolerance to tumor-associated antigens (TAAs) has been established, immunisation with TAA, even with the use of mature DCs merely enhances TAA-specific immunosuppression [63–66], suggesting that early TAA-specific immune responses critically affect subsequent tumor control. We believed it was important to analyse the properties of *in vivo* leukemic DCs during the early phase of the disease or at the phase of minimal residual disease after therapy, which is regarded as the steady-state condition in DC development. Therefore, in our DC differentiation system, we selected FL, a crucial cytokine in steady-state DC differentiation [3]. Second, we developed a DC differentiation system from murine LSKs, but not from whole bone marrow cells. Leukemic cells develop from hematopoietic stem cells or progenitor cells that have acquired various genetic abnormalities. These myeloid neoplasm-related gene abnormalities are characterised primarily as growth/survival promoting abnormalities or differentiation blocking abnormalities, and are described as class I or class II mutations, respectively [43]. These mutations classes have also been identified as prognostic factors, as demonstrated for FLT3-ITD [67,68]. Obviously, *in vivo* leukemic DCs should have the same origin and genetic abnormalities as leukemic blasts. Therefore, we transduced abnormal myeloid neoplasm-related abnormal genes into hematopoietic stem cells or progenitor cells (which are target cells for leukemic transformation) and subsequently induced these cells into DCs in the presence of FL. For this purpose, we used murine LSKs as the initial cell population for DC differentiation. At present, there is a well-established culture method to generate FL-DCs from whole bone marrow cells [19,20,39], which are equivalent with steady-state splenic DCs [3,19,20]. Because the cell density is important for this culture method [39], we selected a 96-well round-bottom rather than flat-bottom culture plate during the DC-induction phase to maintain a constant cell density. Consequently, we were able to establish a simple, efficient, and reproducible FL-DC differentiation system from LSKs without additional cytokines or feeder cells (Fig. 1A–F), thereby enabling a comparative study between various genetic manipulations.

In this study, we have found that class I mutations differentially affected FL-DC differentiation from LSKs regarding the fold increase in whole FL-DCs, and the pDC/cDC ratio (Fig. 3A–E). The effects of these mutations on FL-DC differentiation differed from those on myeloid differentiation, a process that is inhibited primarily by class II mutations [43]. A time course study of FL-DC differentiation showed that CA-N-Ras, c-Kit-TKD, TEL/PDGFRβ, and FIP1L1/PDGFRα induced a transition from Mac-1$^+$CD11c$^-$ to Mac-1$^+$CD11c$^+$ to varying degrees, which differed from the control FL-mediated DC differentiation pathway (Fig. 4A and B). These findings suggest that class I mutations deregulate FL-mediated DC differentiation or may convert it into GM-CSF/IL-4-mediated DC differentiation to various degrees despite the cells being cultured in the presence of FL. Previous studies showed that DC differentiation is regulated by FL and GM-CSF primarily through the activation of STAT3 via FLT3 and STAT5 via the GM-CSF receptor, respectively [3,69]. Thus, steady-state DC differentiation is composed of both pDCs and cDCs and is maintained by FL, of which activation of STAT3 is indispensable. By contrast, DC differentiation in the inflammatory state which produces only cDC, is mediated primarily by GM-CSF, in which the activation of STAT5 by GM-CSF plays an important role in promoting GM-CSF-mediated DC differentiation and inhibiting FL-mediated DC differentiation. In DC differentiation in the inflammatory state, activation of STAT3 is dispensable. In addition, it is postulated that FL- or GM-CSF-mediated DC differentiation is determined by the balance between the activations of STAT3 and STAT5 [69,70], which leads to the difference in pDC/cDC ratio. Consistent with these observations, CA-STAT5, but not CA-STAT3, impaired FL-DC, particularly pDC, differentiation in our system (Fig. 4). In addition, CA-MEK1 inhibited FL-DC, particularly pDC, differentiation. This inhibition is similar to that seen by CA-N-Ras, suggesting that the Ras/MAP kinase pathway plays a role in the impairment of pDC differentiation (Fig. 4). Because class I mutations constitutively and simultaneously activate multiple signal pathways [43], we speculate that these mutations differentially regulate FL-DC differentiation, possibly via their specific targets and extent of activation of each signal transduction pathway. Further studies will be required to investigate how each class I mutation and its deregulated signal transduction pathway is involved in FL-DC differentiation.

Mohty et al. [13] reported that patients with AML showed various patterns of quantitative imbalances in the proportions of circulating myeloid DCs (mDCs) (the human counterpart for murine cDCs) and pDCs. They classified these various patterns into three groups. Group I showed similar proportions with healthy volunteers. Group II included three subgroups: mDC expansion, pDC expansion, and mDC and pDC expansion. Group III showed no detectable DC subsets. The heterogeneous DC proportions in

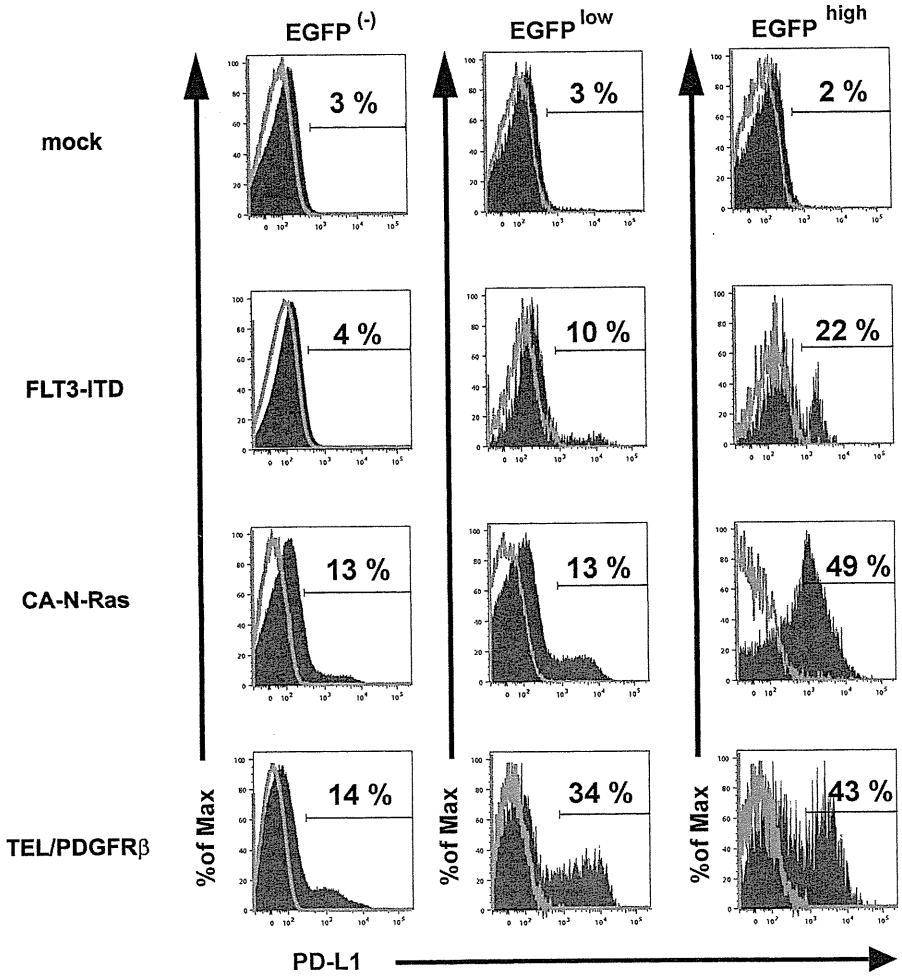

Fig. 7. FLT3-ITD, CA-N-Ras, and TEL/PDGFRβ aberrantly induce PD-L1-expressing DCs. During the pre-culture phase, cells were transduced with the indicated gene. After 9 days in culture, cells were collected and the expression of PD-L1 was analysed by flow cytometry on the gated $EGFP^{-}CD11c^{+}$, $EGFP^{low}CD11c^{+}$ and $EGFP^{high}CD11c^{+}$ cell population. Filled and open histograms show specific staining and back ground staining respectively. $EGFP^{low}$ and $EGFP^{high}$ cells were defined as those showing lower and higher fluorescence intensity than the MFI of all $EGFP^{+}$ cells, respectively. Data are representative of at least three experiments with similar results.

AML patients may partly reflect the various effects that myeloid neoplasm-related gene abnormalities have on DC differentiation as we have shown. However, particularly during the manifestation of the disease, *in vivo* DC differentiation may be affected by various mechanisms/factors such as abnormal secreted cytokine levels [7,56,71,72] and DC distributions [73]. Therefore, we should reassess the changes in *in vivo* DCs in relation to myeloid neoplasm-related gene abnormalities in patients with myeloid neoplasms.

DCs play a pivotal role in determining the balance between T cell immunity and tolerance to tumor cells [5,6,74]. Generally, mature and immature DCs contribute to T cell immunity and tolerance, respectively. pDCs usually display an immature phenotype and often facilitate tumor progression through various mechanisms [6,7,9] such as induction of T cell anergy or deletion, induction of T cells with regulatory property, IDO-mediated tryptophan catabolism, or induction of PD-L on IDO-negative DCs by IDO-positive pDCs. In this study, we have shown that FLT3-ITD specifically retained pDC differentiation from LSKs and showed relatively immature phenotype among class I mutations tested. In addition, FLT3-ITD aberrantly induced PD-L1-expressing DCs. Therefore, FLT3-ITD may work as an inducer of *in vivo* leukemic DCs with tolerogenic function among class I mutations, which may be one reason for it being a poor prognostic factor [67,68]. Therefore, whether leukemic DCs bearing FLT3-ITD have tolerogenic property needs to be further investigated. In contrast, both CA-N-Ras- and TEL/PDGFRβ-expressing FL-DCs exhibited a mature phenotype among the class I mutations tested and efficiently stimulated allogeneic T cells. Therefore, these mutations may have immunogenic properties. However, in certain immunological contexts, mature DCs can also contribute to tolerance by inducing T cells with regulatory properties [47,64]. In addition, both CA-N-Ras and TEL/PDGFRβ aberrantly induced PD-L1-expressing DCs. Therefore, how *in vivo* leukemic DCs bearing CA-N-Ras or TEL/PDGFRβ affect host immune responses needs to be further elucidated. In summary, the differentiation, maturation, and function of FL-DCs from LSKs are deregulated by myeloid neoplasm-related gene abnormalities, particularly class I mutations. Therefore, in patients with myeloid neoplasms, how myeloid neoplasm-related gene abnormalities (particularly class I mutations and associated deregulated signal transduction pathways), affect host immune system, tolerance and immunity, through *in vivo* leukemic DCs needs to be further examined. If *in vivo* leukemic DCs have tolerogenic prop-

erties, they may be candidate targets for therapy. Conversely, if *in vivo* leukemic DCs have immunogenic property, their inhibition may lead to disease progression.

In conclusion, here we have found the possible novel function inherent in myeloid neoplasm-related gene abnormalities, that is, the differentiation, maturation, and function of *in vivo* leukemic DCs may be differentially affected by myeloid neoplasm-related gene abnormalities themselves.

Acknowledgements

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