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Circulating myeloid and lymphoid precursor dendritic cells are clonally involved in myelodysplastic syndromes

L Ma[1], M Delforge[1], V Van Duppen[1], G Verhoef[1], B Emanuel[2], M Boogaerts[1], A Hagemeijer[2] and P Vandenberghe[1,2]

[1]*Laboratory for Experimental Hematology, University of Leuven, Leuven, Belgium; and* [2]*Center for Human Genetics, University of Leuven, Leuven, Belgium*

Circulating myeloid and lymphoid precursor dendritic cell (pDC) counts were determined in peripheral blood from 22 patients with myelodysplastic syndromes (MDS) by a single-platform flow cytometric protocol. The absolute count of myeloid and lymphoid pDC, as well as their relative number (as proportion of mononuclear cells or total leukocytes) was significantly lower in MDS (*n*=22) than in healthy controls (*n*=41). In 11 patients with chromosomal aberrations, purified pDC were examined by interphase fluorescence *in situ* hybridization. This revealed clonal involvement of myeloid as well as lymphoid pDC in all of them. These data therefore strongly suggest that myeloid and lymphoid pDC share a common precursor. Whether reduced peripheral blood counts of pDC contribute to the immunological abnormalities observed in MDS remains to be investigated.
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Introduction

Fluorescence *in situ* hybridization analysis and X-chromosome inactivation studies have established myelodysplastic syndromes (MDS) as clonal diseases of myelopoiesis, with clonal involvement encompassing B lymphocytes in only a minority of cases.[1–6] Yet, MDS, especially with trisomy 8 and/or HLA-DR15, can respond to T-cell targeting immunosuppression.[7–12] This paradoxical observation sparked renewed interest in T-cell mediated mechanisms comodelling the presentation of MDS, and, in particular, in T-cell interactions with antigen-presenting cells (APC).

Dendritic cells (DC) are professional APC that regulate immune responses, clonal deletion or anergy in T-cells. Distinct types, DC1 and DC2, can be distinguished in the human and mouse, based on their development, immunophenotype or function.[13,14] Many authors have cultured CD14(+) monocytes with GM-CSF and IL-4 with or without TNF-α to obtain *monocyte-derived* DC1, but CD34(+) stem cells, or CD11c(+) myeloid pDC in the blood constitute other less extensively studied sources of DC1.[15,16] On the other hand, blood CD11c(–) pDC, variably referred to as lymphoid (derived) pDC, pre-DC2 or plasmacytoid monocytes develop to DC2 after culture with IL–3 and CD40L.[17]

In the past, Rigolin *et al*[12] have shown that *monocyte-derived* DC were clonally involved in eight cases of MDS (four refractory anemia, four refractory anemia with excess blasts) with karyotype abnormalities. Furthermore, their yield after GM-CSF/IL-4 culture was decreased, and functional impairments were found. In contrast, the yield and immunophenotypic maturation of monocyte-derived DC in CMML seems less dramatically altered.[18] More recently, it was reported that myeloid or lymphoid pDC numbers can be unaltered, decreased or increased dramatically in patients with AML.[19] In the present study, we measured absolute counts of circulating myeloid and lymphoid pDC in MDS, with a highly accurate single-platform flow cytometric procedure. In addition, we investigated clonality on purified flow-sorted pDC populations in patients with cytogenetic markers.

Correspondence: Dr P Vandenberghe, Center for Human Genetics, University of Leuven, Herestraat 49, 3000 Leuven, Belgium; E-mail: peter.vandenberghe@uz.kuleuven.ac.be
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Materials and methods

Monoclonal antibodies

CD14-FITC, CD16-FITC, CD33-PE, Cy-Chrome (CY)-conjugated anti-HLA-DR and mouse IgG2a isotype-CY control were purchased from Pharmingen (San Diego, CA, USA). FITC-lineage-cocktail (CD3, CD14, CD16, CD19, CD20, CD56), CD3-FITC, CD4-PE, CD19-PE, CD123 (Anti-IL-3Rα)-PE, CD14-PE, HLA-DR-PerCP, CD8-PerCP, CD14-PerCP, Simultest (CD3-FITC, CD16/CD56-PE) and the mouse isotype controls IgG1-FITC, IgG2a-FITC, IgG1-PE, IgG2a-PE, IgG2a-PerCP were purchased from Becton Dickinson Immunocytometry Systems (BDIS, Erembodegem, Belgium).

Study subjects

A total of 22 patients with MDS, previously untreated except with supportive erythrocyte transfusions or Vitamin B_6, were studied (Table 1). The diagnosis of MDS was based on peripheral blood counts, cytology of peripheral blood and bone marrow (BM) according to the FAB classification[20] and conventional cytogenetic analysis. Normal values for myeloid and lymphoid pDC counts were determined in 41 healthy volunteers aged 20–65 years.

Measurement of peripheral blood count of pDC

Circulating pDCs were identified with a single-platform three-color flow cytometric analysis, as recently described (21). Briefly, 100 μl whole blood was immunostained in TruCount tubes (BDIS), followed by lysis in 2.0 ml of FACS lysing solution (BDIS). With a forward scatter threshold allowing detection of TruCount beads, a live gate excluding débris is drawn. For each experiment, 60 000 events were acquired on a FACScan and analyzed using CellQuest 3.1 software (BDIS). Among mononuclear cells, myeloid pDC are identified as CD14(–)/CD16(-) and CD33(+)/HLA-DR(+) cells. Lymphoid pDC are identified as lineage (dim/–), CD123(+) /HLA-DR(+) mononuclear cells, as previously reported.[21,22] The number of pDC was calculated

Table 1 Clinical, hematological and cytogenetic characteristics of 22 MDS patients

Case	Sex/age (years)	FAB[a]	Bone marrow karyotype	Leukocyte count ($\times 10^{-9}$/l)	Hemoglobin (g/l)	Platelet count ($\times 10^{-9}$/l)	Therapy
1	F/70	RA	46,XX,del(5)(q14q33)[8]/46,XX[2]	1.7	89	117	—
2	M/75	RA	46,XY,del(5)(q14q34)[4]/ 46,XY,del(5)(q23q34)[3]/46,XY[3]	3.9	107	90	Erythrocyte transfusions
3	F/62	RA	46,XX,del(5)(q12q34)[8]/46,XX[2]	3.5	65	127	Erythrocyte transfusions
4	M/76	RA	46,XY,del(20)(q11)[10]	2.8	105	137	Erythrocyte transfusions
5	M/70	RA	46,XY,del(20)(q11)[10]	1.6	91	157	—
6	M/79	RA	46,XY,del(20)(q12)[3]/46,XY[3]	5.5	96	259	—
7	M/69	RA	47,XY,+8[6]/46,XY[4]	1.7	138	148	—
8	F/58	RA	47,XX,+8[7]/46,XX[3]	5.8	94	99	Vit B_6
9	M/65	RA	46,XY[10]	3.4	112	122	—
10	M/67	RA	46,XY[10]	2.5	109	171	Vit B_6
11	M/77	RA	46,XY,del(7)(q21q31)[4]/46,XY[6]	4.9	105	234	Vit B_6
12	F/74	RA	46,XX[11]	2.4	91	127	Vit B_6
13	M/54	RARS	46,XY[10]	2.6	68	348	Vit B_6, erythrocyte transfusions
14	F/80	RARS	46,XX[10]	3.0	111	169	Vit B_6, corticosteroids, erythrocyte transfusions
15	M/79	RARS	46,XY[11]	5.9	94	201	Vit B_6
16	M/61	RARS	46,XY[10]	3.1	118	129	rHu-EPO, filgrastim
17	F/61	RARS	46,XX,del(11)(q21)[4]/46,XX[6]	8.0	123	98	Erythrocyte transfusions
18	F/76	RARS	46,XX,del(13)(q13q21)[2]/ 46,XX[8]	4.8	97	69	Erythrocyte transfusions
19	F/75	RARS	46,XX,del(13)(q13q21)[3]/ 46,XX[9]	7.2	113	94	—
20	M/66	RARS	46,XY[10]	4.2	108	238	Vit B_6
21	F/79	RAEB	46,XX[10]	5.6	107	151	—
22	M/78	RAEB	46,XY[10]	3.0	141	35	Cyclosporin A

[a]Diagnosis according to FAB classification.[20]

as follows: pDC/μl = ((events in the pDC gate) $\times$ (number of the beads per tube))/((events in the bead gate) $\times$ (sample volume (μl))).[21]

Cytogenetic analysis was performed on unstimulated short-term bone marrow cultures. Analysis of 10 R-banded metaphases was carried out and reported according to the International System for Human Cytogenetic Nomenclature.[23]

Fluorescence-activated cell sorting

A total of 11 patients with structural or numerical chromosomal aberrations were selected for interphase FISH. Peripheral blood mononuclear cells (PBMC), obtained by density centrifugation over Lymphoprep (Nycomed Pharma, Oslo, Norway), were immunostained. They were sorted by FACSVantage (BDIS) to obtain purified myeloid pDC (CD33 (+)/HLA-DR(+), CD14/CD16(-/dim)), lymphoid pDC (CD123(+)/HLA-DR(+), lineage (−/dim)), monocytes (CD14(+)), natural killer (NK) cells (CD16(+)/CD56(+), CD3/CD14(–)), helper T lymphocytes (CD3(+)/CD4(+)), cytotoxic T lymphocytes (CD3(+)/CD8(+)) and B lymphocytes (CD19(+),CD3(−)). Unfixed cells (3000–5000) were sorted directly onto slides and fixed in methanol/acid (3:1). Purity always exceeded 98% as verified by separate immunophenotyping. For May–Grünwald–Giemsa staining, cytospin preparations of sorted myeloid and lymphoid pDC were used.

FISH

For interphase FISH, the following probes were used: LSI EGR1 (5q31)/D5S721, D5S23 (a Spectrum Orange-labelled probe for 5q31, and a Spectrum Green-labelled probe for 5p15.2);. CEP 8 DNA Probe Kit (chromosome 8 centromere); LSI MLL Dual Color Break Apart Rearrangement Probe (11q23); LSI 13 (RB1) Spectrum Orange probe (13q14); LSI D20S108 Spectrum Orange probe (20q12) (all from Vysis Inc., Downers Grove, IL, USA). Procedures were performed as described by the manufacturer with slight modifications. Fluorescence was visualized with Leitz DMRB fluorescence microscope with a cooled black and white CCD camera (Photometrics) run by Smartcapture software (Vysis). In total, 200 nuclei were scored per specimen by independent observation. In all experiments, background was less than 5.5%.

Statistical analysis

Statistical analysis was performed with the parametric unpaired *t*-test. A value of $P<0.05$ was considered statistically significant. Data were expressed as mean$\pm$s.d.

Results and discussion

We measured absolute numbers of myeloid and lymphoid pDC in peripheral blood of 22 MDS patients and 41 healthy volunteers (Figure 1). In MDS patients, the mean number of circulating myeloid pDC was $7.1\pm3.9\times10^6$/l (range 0.2–14.2×10^6/l), compared with $17.0\pm5.7\times10^6$/l (range 8.4–31.1×10^6/l) in normal healthy controls ($P<0.0001$). The mean number of circulating lymphoid pDC in MDS patients was $4.2\pm3.3\times10^6$/l (range 0.2–12.1×10^6/l), compared with $8.7\pm4.1\times10^6$/l (range 1.8–19.8×10^6/l) in healthy controls ($P<0.0001$) (Figure 2a). The number of myeloid and lymphoid pDC expressed as a fraction of mononuclear cells (Figure 2b) or of total leukocytes (not shown) was also substantially and

significantly decreased in comparison with normal controls. Subset analysis of age groups aged 21–35 years, 36–50 years, 51–65 years, and more than 65 years revealed significant differences of myeloid and lymphoid pDC counts between MDS and control in age-matched cohorts of 51–65 years. In contrast, no significant differences between age cohorts were found in the MDS group, nor in healthy controls, as previously reported for healthy volunteers.[21,24] pDC counts in male *vs* female subjects, or in transfused *vs* untransfused patients, were not significantly different (not shown). Therefore, the data convincingly indicate that MDS patients have significantly reduced numbers of myeloid as well as lymphoid pDC.

The quantitative decrease of circulating myeloid and lymphoid pDC raised the question whether both these cell populations are clonally involved and part of the malignant clone. This question was addressed in a subset of 11 patients with karyotypic abnormalities (Table 2). After flow sorting of the cell subset of interest onto slides, interphase FISH was applied with probes for chromosome 5, 8, 11, 13 or 20. In all cases

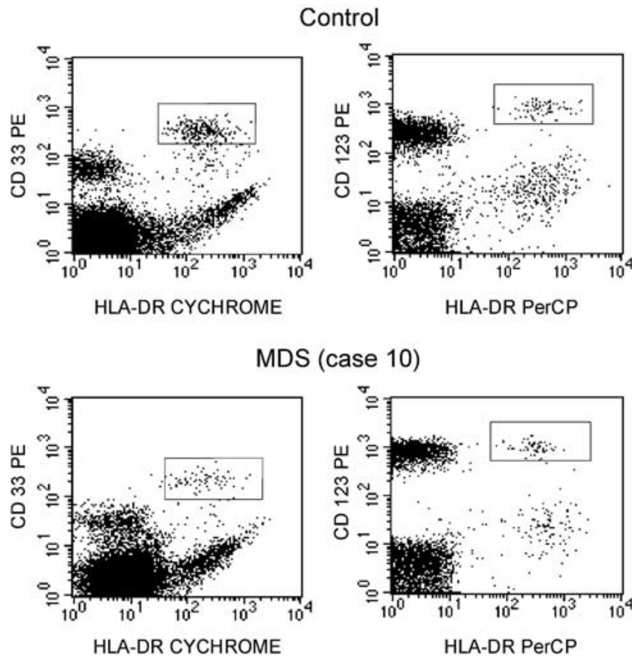

Figure 1 Identification of circulating pDC subsets by flow cytometry. PBMCs isolated from healthy subjects and MDS patients were analyzed by three color immunostaining in a lyse-no-wash protocol. A live gate excluding débris was set and for each plot 60 000 events were collected. With gating on CD14/16 (dim/negative) mononuclear cells, myeloid pDC can be identified as CD33(+) HLA-DR(+) cells (left column). Lymphoid pDC are identified as lineage dim/negative, CD123(+)/HLA-DR(+) mononuclear cells (right column). Upper panels represent a normal control, lower panels show a representative case of MDS (case 10).

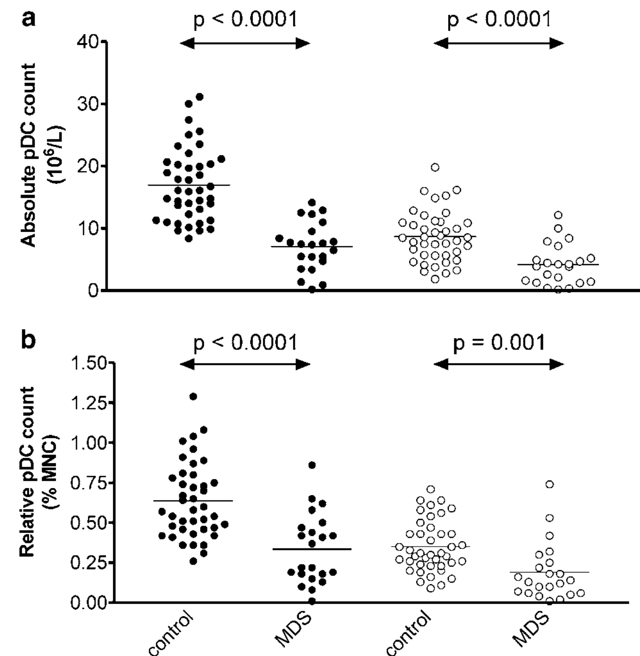

Figure 2 Counts of circulating precursor (pDC) subsets in the peripheral blood. The count of circulating myeloid pDC (solid circles) and lymphoid pDC (open circles) was determined by flow cytometry in 41 healthy controls and 22 MDS cases. Plots represent the distribution of absolute pDC counts (a) and of relative pDC counts (% of mononuclear cells) (b). The horizontal lines represent mean values.

Table 2 Interphase FISH study of precursor dendritic cells (pDC), monocytes, T lymphocytes and B lymphocytes in MDS

		% of cells with abnormal FISH pattern[a]				
Case	*Probe*	*Myeloid pDC*	*Lymphoid pDC*	*Monocytes*	*Helper T cells*	*NK cells*
1	LSI EGR1 (5q31)/D5S721, D5S23	92.0	95.5	79.0	7.0	74.5
2	LSI EGR1 (5q31)/D5S721, D5S23	98.5	96.5	52.0	2.0	19.0
3	LSI EGR1 (5q31)/D5S721, D5S23	95.5	94.0	88.0	1.0	51.5
4	LSI D20S108 (20q12)	93.5	83.5	—	—	97.0
5	LSI D20S108 (20q12)	80.5	93.0	—	—	—
6	LSI D20S108 (20q12)	20.0	18.5	16.0	3.0	11.0
7	CEP8	90.0	82.5	95.0	0.5	16.5
8	CEP8	26.5	23.0	26.5	0.5	5.0
17	LSI MLL	53.0	42.5	32.5	3.0	26.0
18	LSI 13 (RB1) 13q14	52.0	29.5	7.5	3.0	13.0
19	LSI 13 (RB1) 13q14	18.5	14.5	25.0	2.0	9.0

[a]Interphase FISH was carried out on flow-sorted populations as indicated. The percentage of cells with the numerical or structural anomaly was scored and is given in the table. FISH results on sorted B cells and cytotoxic T cells are not shown.
— = not done.

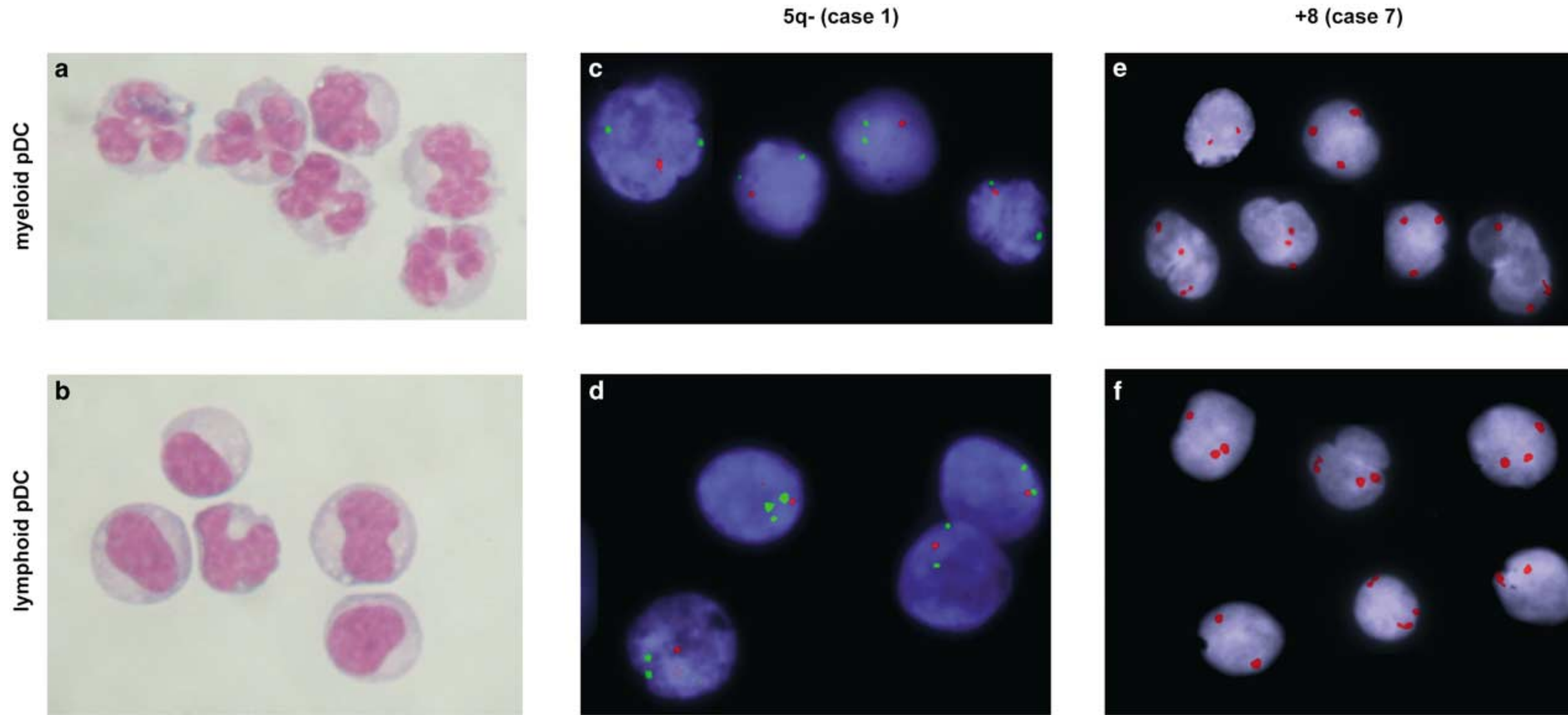

Figure 3 FISH study of the clonality of myeloid and lymphoid pDC in MDS. May–Grünwald–Giemsa staining of flow-sorted myeloid and lymphoid pDC (a, b). FISH data reveal loss of one red signal (5q31) while two green signals (5p15) remain (c, d). Three red signals (CEP8) are visualized (e, f).

examined, distinctively abnormal FISH patterns were observed in a substantial proportion of myeloid as well as lymphoid pDC (Figure 3). The percentage of abnormal cells was variable from patient to patient, but the involvement of myeloid and lymphoid pDC within single patients seemed to correlate with one another. Cases 6, 8 and 19 still had a major proportion of karyotypically normal cells in unstimulated bone marrow cultures and had proportionately fewer myeloid and lymphoid pDC with abnormal FISH patterns. In nine patients, FISH studies on flow-sorted peripheral blood monocytes or natural killer (NK) cells revealed abnormal FISH indicative of clonal involvement, consistent with previously published reports concerning clonality of monocytes and more recently NK cells in MDS.[25] On the other hand, again consistent with the literature, normal FISH patterns were found in helper T cells (Table 2), cytotoxic T cells and B cells (not shown).[1–6,25]

To the best of our knowledge, these are the first data reporting absolute pDC numbers in MDS-type refractory anemia of refractory anemia with ringed sideroblasts. With a highly reproducible single-platform procedure, we show that absolute and relative counts of circulating myeloid as well as lymphoid pDC in MDS patients are reduced about 50% compared with healthy volunteers. Relatively few literature data are available on myeloid and lymphoid pDC numbers in health and disease. In addition, all available data were obtained by more error-prone methods utilizing at least two platforms (i.e. flow cytometry and hematological cell counters[18]), or even involving culture steps.[12] Contrary to AML where different patterns are found,[19] the absolute numbers of myeloid and lymphoid pDC are uniformly decreased in the MDS group analysed here. Despite the relatively mild cytopenias, the alterations in both pDC compartments were quantitatively important, and comparable with the reduction seen in polymorphonuclear fractions (polymorphonuclear cells in control *vs* MDS: 3.67×10^9/l (2.04–6.3×10^9/L) *vs* 1.78×10^9/l (0.12–4.34×10^9/l), $P < 0.0001$).

The normal developmental origin of myeloid and lymphoid pDC is a debated issue, with some studies indicating that myeloid and lymphoid pDC originate from common myeloid progenitors,[26,27] and others showing that either cell population can be generated *in vivo* along both myeloid and lymphoid pathways.[28] The clonality of circulating myeloid and lymphoid pDC in MDS has not yet been formally studied. Our data convincingly show clonal involvement of circulating myeloid and lymphoid pDC in all patients with cytogenetic abnormalities in this series. A recent study in newly diagnosed AML also found clonal involvement in lymphoid and myeloid pDC.[19] As such, they support the notion that both pDC subsets derive from a clonally involved cellular precursor, which they have in common with peripheral blood granulocytes, monocytes and NK cells[5] (see also the review by Knuutila[29]). It remains uncertain whether the latter cellular precursor is distinct from committed lymphoid progenitors: as suggested for 5Q-MDS, clonally involved pluripotent hematopoietic stem cells can also be deficient in generating mature lymphocytes. Even with a normal capacity to generate mature lymphocytes, the absence of clonality in peripheral T- and B lymphocytes could be explained by longevity of nonclonally involved lymphoid progenitors and lymphocytes, and the limited sensitivity of FISH and X-chromosome inactivation assays.[30] Finally, substantial numbers of pDC1 and pDC2 were found to have normal FISH patterns: this could indicate either the persistance of nonclonally involved cells in the pool of shared DC precursors, contributing to pDC development, or that a fraction of myeloid or lymphoid pDC is derived from other nonclonally involved types of precursors. Further studies will be required to investigate these issues and whether the clonal involvement of the DC compartment contributes to the abnormalities in the T-lymphocyte compartment in MDS.

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