Myelodysplasia-associated immunophenotypic alterations of bone marrow cells in myeloma: are they present at diagnosis or are they induced by lenalidomide?

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Online Supplementary Design and Methods

Immunophenotypic studies and flow cytometric score

Whole BM samples ($2x10^6$ cells in 100 μL/test) were stained for cell surface markers using a stain-lyse-and-then-wash direct immunofluorescence technique, while intracellular stainings (nuclear (n) and cytoplasmic (Cy)) were performed after cell fixation and permeabilization, using the Fix and Perm reagent kit (Invitrogen, Carlsbad, CA, USA). The following combinations of monoclonal antibodies (MAb) in 4 color stainings (fluorescein isothiocyanate, FITC; phycoerythrin, PE; peridinin chlorophyll protein, PerCPCy5.5; allophycocyanin, APC) were systematically used: HLA-DR, CD117, CD45, CD34; HLA-DR, CD123, CD45, CD34; CD11b, CD13, CD45, CD34; CD61, CD33, CD45, CD34; nTdT, CyMPO, CD45, CD34; CD15, CD16, CD45, CD34; CD19, CyCD79a, CD45, CD34; CD65, 7.1, CD45, CD34; CD36, CD64, CD45, CD34-CD14; IREM-2, CD14, CD45, CD34; CD2, CD56, CD45, CD34; CD71, CD235a, CD45, CD34; and CyCD3, CD7, CD45, CD34. Immediately after staining, sample aliquots were measured in a FACSCalibur flow cytometer (Becton Dickinson Biosciences [BDB], San Jose, CA, USA) using the CellQUEST software program (BDB) for $3x10^4$ events corresponding to whole BM cellularity per sample aliquot. Data were analyzed with the INFINICYT™ program (Cytognos SL, Salamanca, Spain). Total $CD34^+$ BM precursors were identified and counted according to their light scatter characteristics (forward light scatter, FSC; sideward light scatter, SSC). Positivity for CD34 and dim CD45 expression followed the ISHAGE guidelines, as described in detail elsewhere.[1] Additionally, $CD117^+/CD34^-$ precursors were identified in an SSC *versus* CD117 bivariate dot plot histogram after excluding $CD34^+$ HPC, as illustrated in Figure 1C in the main text. The following subsets of $CD117^+/CD34^-$ precursors were identified: a) neutrophil-committed precursors ($CD45^{lo}/SSC^{lo/int}$); and b) erythroid-committed precursors ($CD45^-/SSC^{very\text{-}lo}$); c) maturing $CD34^-$ mast cells were identified as those $CD45^+$ events being $CD117^{hi}$ with heterogeneous HLA-DR expression ($HLA\text{-}DR^{-\,to\,+}$). In addition to $CD34^+$ and/or $CD117^+$ cells, the following subsets of $CD34^-/CD117^-$ hematopoietic BM cells were identified: a) neutrophil ($CD45^{lo}/SSC^{int/hi}$); b) erythroid ($CD45^-/SSC^{very\text{-}lo}$); c) monocytic ($CD36^{-/hi}/CD64^{hi}/CD45^{int/hi}/CD14^{lo/hi}$); d) B-cell ($CD45^{lo}/SSC^{very\text{-}lo}/CD19^+/CyCD79a^+$); e) plasmacytoid dendritic cell (pDC) ($HLA\text{-}DR^{hi}/CD123^{hi}/CD45^{int}$); and f) basophil-maturing cells ($HLA\text{-}DR^-/CD123^{hi}/CD45^{int}$). The specific immunophenotypic characteristics of mature lymphocytes were taken as a standard to define the relative position of the different compartments of the BM precursors in a dot plot of CD45 against SSC, and to establish cut-off levels defining the lower level of expression of myeloid-associated antigens.

Among $CD34^-$ neutrophil lineage cells, four different stages of increasing maturation were defined on the basis of the reactivity of the CD11b and CD13 antigens: stage I, $CD13^{hi}/CD11b^-$ myeloblasts; stage II, $CD13^{lo/int}/CD11b^-$ promyelocytes; stage III, $CD13^{lo/int}/CD11b^+$ myelocytes and metamyelocytes; and stage IV, $CD13^{hi}/CD11b^+$ bands and mature neutrophils. In turn, mature monocytes were identified as those cells displaying a $CD36^{hi}$, $CD64^{hi}$, $CD45^{int/hi}$, $CD14^{hi}$ immunophenotype while their CD34- precursors were defined as being $CD36^{-/lo}/CD64^{hi}/CD45^{int}/CD14^{lo/int}$. The presence of aberrant phenotypes and/or other maturation-associated alterations were defined for each BM cell population on the basis of numerical and phenotypic deviations greater than the mean ± 2 standard deviations (SD) from the normal BM profiles. Accordingly, for each individual immunophenotypic variable analyzed (n=83) in each BM sample, a score of 0, 0.5, 1 or 2 was given when the values were, respectively, within ± 2 SD, ± 2-3 SD, ± 3-4 SD, ± >4 SD of the mean values found for that parameter among the normal BM samples analyzed.

Immunophenotypic variables studied

Up to 83 different immunophenotypic variables were investigated in each case: a) immature cell compartments: percentage of $CD34^+$, $CD34^-/CD117^+$, $CD34^-/CD117^+$ neutrophil-committed precursors and of $CD34^-/CD117^+$ erythroid-committed precursors, percentage of $CD34^-$ B cells and expression (MFI) of CD19 and Cy-CD79a in the latter population; b) maturing neutrophils: percentage of $CD34^-$ neutrophil lineage cells, and MFI of FSC, SSC, CD45, CD15, CD16, CyMPO, CD11b, CD13,

CD33, CD64, CD65, percentage of CD16^{hi}, CD64^{hi}, CD14^{+} and CD56^{+} aberrant cells together with the percentage of neutrophil cells in the maturation stages I, II, III and IV (see above) and MFI of CD45, SSC, CD11b and CD13 within each of these four neutrophil maturation stages; c) monocytic maturation: percentage of monocytic cells, monocytic precursors (CD14$^{-/int}$), and of mature (CD14^{hi}) monocytes, and MFI of FSC, SSC, CD14, CD36, CD45 and CD64 within each of these subsets, together with overall reactivity of monocytic cells for CD11b, CD13, CD15, CD33, CD65, CyMPO, HLA-DR, percentage of IREM-2^{+} cells and of CD2 and CD56 aberrant monocytic cells; d) erythroid maturation: percentage of nucleated red blood cells (NRBC), percentage of aberrant CD36$^{-/lo}$ cells and MFI of CD71, CD235a and CD36 on the NRBC compartment; e) basophil and pDC: percentage of basophils and pDC, and expression levels (MFI) of CD123 in both groups of cells.

References

1. Sutherland DR, Anderson L, Keeney M, Nayar R, Chin-Yee I. The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering. J Hematother. 1996;5(3):213-26.

Online Supplementary Table S1. Flow cytometry immunophenotypic scores in normal and reactive bone marrow (BM) *versus* patients with myelodysplastic syndrome (MDS), symptomatic multiple myeloma (MM), and smoldering MM (SMM) at diagnosis and after nine cycles of Len/Dex.

Immunophenotypic score	Normal BM (N = 20)	Reactive BM (N = 20)	MDS (N = 56)	Symptomatic MM at diagnosis (N = 15)	SMM at diagnosis (N = 18)	SMM after Len/Dex (N = 22)
< 2	100%	100%	0	9 (60%)	16 (89%)	17 (77%)
≥ 2 to 9	-	-	16 (29%)	5 (33%)	4 (22%)	7 (32%)
≥ 10	-	-	40 (71%)	1 (7%)	0 (0%)	0 (0%)
Overall score	0±0	0.6±0.6 (0-1.5)	16±8 (2.5-40)	1.5±2.8 (0-10)	0.3±0.7 (0-2)	0.6±1.0 (0-3)

Results expressed as number of cases and percentage (in brackets). A score of 1 or 2 was given when the value obtained for each of the parameters in the upper panel was within ± 3-4 SD and ± >4 SD of the means for that parameter among all normal BM samples analyzed, respectively. Immunophenotypic parameters analyzed in each individual included: a) **immature cell compartments** (percentage of total CD34^{+}, total CD34^{-}/CD117^{+}, CD34^{-}/CD117^{+} neutrophil-committed and erythroid-committed precursors, percentage of CD34^{-} B-cells and expression of CD19 and Cy-CD79a), b) **neutrophil maturation** (percentage of total neutrophil lineage cells and expression of FSC, SSC, CD45, CD15, CD16, CyMPO, CD11b, CD13, CD33, CD64, CD65, percentage of CD16^{hi}, CD64^{hi} and of CD14^{+} and CD56^{+} aberrant cells, percentage of neutrophil maturation stages I-IV and expression of CD45, SSC, CD11b and CD13), c) **monocytic maturation** (percentage of total monocytic lineage cells, monoblasts, mature monocytes and expression of FSC, SSC, CD14, CD36, CD45 and CD64, total monocytic CD11b, CD13, CD15, CD33, CD65, CyMPO, HLA-DR, percentage of IREM-2^{+} cells and aberrant expression of CD2 and CD56), d) **erythroid maturation** (percentage of red blood cells, expression of CD71, CD235a, CD36 and percentage of aberrant CD36$^{-/lo}$ cells) and, e) **basophil and pDC maturation** (percentage of cells and expression of CD123).

No significant differences were found between symptomatic and smoldering MM patients at diagnosis, or between smoldering MM patients before and after treatment.

Online Supplementary Table S2. Distribution of hematopoietic bone marrow (BM) cell populations in patients with symptomatic multiple myeloma (MM) and smoldering multiple myeloma (SMM) at diagnosis, and SMM cases after induction therapy with nine courses of lenalidomide plus dexamethasone (Len/Dex).

Cell populations	Symptomatic MM at diagnosis (N = 15)	SMM at diagnosis (N = 18)	*P**	SMM after Len/Dex (N = 22)	*P***
$CD34^+$ HPC	0.7 (0.08-3.0)	0.6 (0.2-1.8)	0.8	0.6 (0.09-3.0)	0.5
#MPO^+	21 (9-38)	16 (5-29)	0.1	14 (3-36)	0.6
#TdT^+	6 (0-33)	16 (0-45)	0.06	11 (0-67)	0.8
$CD34^-$ / $CD117^+$ HPC	1.3 (0-6.7)	0.8 (0.2-2.7)	0.5	0.8 (0- 2.9)	0.9
#Neutrophil-committed	51 (0-80)	56 (16-88)	0.6	60 (20-95)	0.7
#Erythroid-committed	47 (0-80)	41 (12-84)	0.9	40 (5-80)	0.9
Neutrophils	48 (22-64)	55 (33-69)	0.5	49 (29-82)	0.3
#Stage I	2 (0.2-8)	1 (0.3-5)	0.1	2 (0-6)	0.07
#Stage II	14 (3-31)	8 (0.2-19)	0.02	14 (0-22)	0.04
#Stage III	31 (1-47)	24 (2-52)	0.4	33 (0-46)	0.2
#Stage IV	50 (28-92)	65 (32-97)	0.2	49 (31-99)	0.09
Monocytes	4.4 (0.9-13)	2.9 (1.3-20)	0.2	4.6 (1.0-15)	0.07
Erythroblasts	12 (3.0-35)	6.3 (1.0-28)	0.03	11 (0.6-26)	0.02
Dendritic cells	0.1 (0-1.0)	0.2 (0.1-0.6)	0.2	0.2 (0-0.6)	0.6
Basophils	0.2 (0.08-0.9)	0.3 (0.06-1.0)	0.8	0.4 (0.05-1.5)	0.01
B cells	0.8 (0.2-6.2)	1.7 (0.3-3.9)	0.07	0.5 (0.2-13)	0.005
#Pro-B precursors	6.5 (0-21)	5.5 (0-12)	0.8	9.0 (0-40)	0.2
#Pre-B precursors	14 (0-42)	22 (0-57)	0.6	16 (0-90)	0.6
#Mature B cells	81 (35-100)	76 (37-100)	0.3	76 (63-100)	0.7

** MM vs. SMM at diagnosis; ** SMM at diagnosis vs. SMM after induction. Results expressed as median (range) percentage of the specific cell population within the whole BM cellularity, excluding myelomatous plasma cells; N #: median (range) percentage of the specific cell subpopulation from the overall corresponding cell population; HPC: hematopoietic precursor cells.*

Online Supplementary Table S3. Detailed immunophenotypic characterization of the 8 smoldering multiple myeloma (SMM) patients with paired bone marrow (BM) samples obtained at diagnosis and after nine cycles of lenalidomide plus dexamethasone (Len/Dex).

Patient	Diagnosis	After nine cycles of Len/Dex
#1	No alterations	No alterations
#2	No alterations	No alterations
#3	No alterations	Isolated multilineage ($CD14^+$ expression in neutrophils and $CD2^+$ expression in monocytes)
#4	No alterations	No alterations
#5	Isolated unilineage (MPO^{dim} expression in neutrophils)	Isolated unilineage (MPOlo expression in neutrophils)
#6	No alterations	No alterations
#7	No alterations	Isolated unilineage ($CD2^+$ expression in monocytes)
#8	No alterations	Isolated multilineage ($CD14^+$ expression in neutrophils and $CD36^{neg}$ asynchronous expression in nucleated red blood cells)