SUPPLEMENTARY APPENDIX

Reduced frequencies and functional impairment of dendritic cell subsets and non-classical monocytes in myelodysplastic syndromes

Nathalie van Leeuwen-Kerkhoff,[1] Theresia M. Westers,[1] Pino J. Poddighe,[2] Giovanni A.M. Povoleri,[3] Jessica A. Timms,[4] Shahram Kordasti,[4,5#] Tanja D. de Gruijl[6#] and Arjan A. van de Loosdrecht[1#]

[1]Department of Hematology, Amsterdam University Medical Centers, Vrije Universiteit Amsterdam, Cancer Center Amsterdam, the Netherlands; [2]Department of Clinical Genetics, Amsterdam University Medical Centers, Vrije Universiteit Amsterdam, the Netherlands; [3]Department Inflammation Biology, King's College London, Center for Inflammation Biology and Cancer Immunology, London, UK; [4]Systems Cancer Immunology Lab, Comprehensive Cancer Center, King's College London, London, UK; [5]Dipartimento Scienze Cliniche e Molecolari, UNIVPM, Ancona, Italy and [6]Department of Medical Oncology, Amsterdam University Medical Centers, Vrije Universiteit Amsterdam, Cancer Center Amsterdam, the Netherlands

[#]SK, TDG and AAL contributed equally as co-senior authors.

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Correspondence: *ARJAN A. VAN DE LOOSDRECHT* - a.vandeloosdrecht@amsterdamumc.nl

Supplementary methods

Patient and control samples

IPSS and IPSS-R risk groups of 150 and 136 patients, respectively, could be calculated (Table 1). According to the IPSS-R, 29 patients were considered very low risk, 48 low risk, 32 intermediate, 15 high and 12 very high risk. The 2016 World Health Organization (WHO) classification was available for 163 patients. Eleven patients could be classified as MDS with single lineage dysplasia (MDS-SLD), 65 as MDS with multilineage dysplasia (MDS-MLD), 8 as MDS with ringed sideroblasts with single lineage dysplasia (MDS-RS-SLD), 31 as MDS with ringed sideroblasts with multilineage dysplasia (MDS-RS-MLD), 24 as MDS with excess blasts-1 (MDS-EB-1) and 24 patients had MDS with excess blasts-2 (MDS-EB-2).

Sample processing and isolation

Mononuclear cells were isolated by density centrifugation using Ficoll-Paque medium (GE Healthcare, Uppsala, Sweden). For functional experiments, cDC from fresh samples were sorted with a BD FACSAria™ (BD Biosciences, San Jose, USA) as previously described.[10,11] Slan+ monocytes were magnetically isolated using a M-DC8 isolation kit (Miltenyi Biotec). For microarray experiments, all cells were flowcytometrically sorted from frozen samples. Sorted cells showed >92% purity and were either used directly after sort or, in case of microarray experiments, stored in Trizol (Life Technologies, Carlsbad, USA) until further use (see for clinical data table S1). Flow cytometric analysis was performed on fresh total white blood cells after erythrocyte lysis.

Flow cytometry

Frequencies of cell subsets were evaluated by flow cytometry. First, erythrocytes of BM- or PB-derived samples were lysed (BD Pharm Lyse, BD Biosciences). Then cells were incubated with monoclonal antibodies (mAb): M-DC8-FITC, CD303-FITC (Miltenyi Biotec, Utrecht, The Netherlands), CD16-PE (Beckman Coulter, Brea, USA), CD1c-Pe-Cy7 (eBioscience, San Diego, USA), CD141-APC (Miltenyi Biotec), CD11c-PerCP-Cy5.5, CD14-APC-H7, CD19-APC-H7, HLA-DR-V450 and CD45-KO (all, BD Biosciences) and analyzed on a flow cytometer (FACSCanto™, BD Biosciences). Data was analyzed using the FlowJo software program (Tree star, Ashland, OR, USA).

Fluorescence in situ hybridization (FISH)

Frozen vials were rapidly thawed and stained with a mAb cocktail as mentioned above including CD34. cDC2, slan+ monocytes, CD34+ myeloid progenitor cells and B cells were flow cytometrically sorted (BD FACSAria^{TM}). Cells were collected in small Eppendorf tubes and further processed for interphase FISH analysis. They were fixed with 3:1 methanol/acetic acid and transferred to a microscopic slide. Interphase FISH was performed on each sorted cell subset using probes LSI EGR1(5q31)/D5S23,D5S721(5p15.2) Dual Color Probe Set, LSI D7S486(7q31)/CEP7, and LSI CEP8 (D8Z2) (all probes from Abbott Molecular, Des Plaines, IL). For each probe 100 cells were investigated. In samples with less than 100 cells on the slide, all cells were evaluated.

Functional assays and multidimensional mass cytometry

The maturation capacity of BM-derived cDC2 and slan+ monocytes was assessed by comparing the expression levels of co-stimulatory (CD80 and CD86) and HLA-DR molecules at baseline and after overnight TLR-stimulation with a combination of two TLR ligands, i.e. LPS (100 ng/ml, Sigma-Aldrich, St. Louis, USA) and R848 (3 µg/ml, Enzo Life Sciences, Farmingdale, USA). Culture supernatants were collected for the detection of cytokines using the human enhanced sensitivity cytometric bead array (CBA) flex sets (BD Biosciences). An allogeneic mixed leukocyte reaction (MLR) was performed to test the T cell proliferation induction capacity of different cell subsets. HD-derived peripheral blood lymphocytes from one donor were labelled with 1 µM carboxyfluorescein succinimidyl ester (CFSE, Life Technologies). Thereafter they were co-cultured with isolated cDC1, cDC2 or slan+ monocytes in a 1:10 ratio. Before co-culture DC were stimulated overnight with a combination of TLR ligands: R848 + Poly I:C (25 µg/ml, Sigma-Aldrich) for cDC1 and LPS + R848 for cDC2 and slan+ monocytes. The percentage of CFSE-dilution was analyzed by flow cytometry and used as a measure for CD4+ and CD8+ T cell proliferation.

A multi-parameter deep-phenotyping strategy, known as cytometry by time-of-flight (CyTOF), was used for T cells cultured in the presence of MDS-derived or healthy PB-derived slan+ monocytes. These monocytes were obtained from fresh samples of two MDS patients and two HD using magnetic bead isolation. They were incubated and stimulated overnight. Next, total HD-derived naive CD4+ T cells were magnetically isolated (Miltenyi Biotec, Utrecht, The Netherlands). Thereafter, they were co-

cultured with slan+ monocytes on a plate pre-coated with CD3 in a ratio of 1:10 for 5 days. T cells from day zero or cultured in the presence of slan+ monocytes were stained with an antibody panel containing cell surface markers, transcription factors and cytokines.

Data was acquired on a Helios mass cytometer (Fluidigm) and were analyzed using Cytobank.[30] Dimensionality reduction of data was carried out using t-Distributed Stochastic Neighbor Embedding (t-SNE)[31] on gated CD3+ live T-cells for each experiment. The FlowSOM algorithm was applied to the dimensionality reduced data, using Self-Organizing Maps (SOMs) to assess marker expression on all cells to identify clusters within the population (FlowSOM settings: 100 clusters and 15 metaclusters).[32]

Microarray transcriptional analysis

After running the samples, microarray signals were normalized using the RMA algorithm in the Affymetrix® Expression Console software (Affymetrix®). Quality checks and background noise correction were performed. The Transcriptome Analysis Console (TAC) Software version 4.0 (Affymetrix) was used for transcript filtering, identification of differentially expressed genes (DEGs; using fold change [FC] levels of <-2.5 or >2.5, a false discovery rate (FDR) <0.05 and a gene level p-value <0.05) and hierarchical clustering. Pathway analyses were performed with the STRING v10.5 database (http://string-db.org/). Gene Set Enrichment Analysis (GSEA) was carried out using the free available GSEA software of the Broad Institute (http://software.broadinstitute.org) and the GO geneset database from the Molecular Signatures Database-MsigDB.

Table S1. MDS samples used in functional experiments and transcriptional profiling

MDS ID	IPSS-R	WHO2016	Functional experiments	Transcriptional profiling
MDS5	Low	SLD	maturation, cytokines, MLR	no
MDS6	-	ICUS	maturation, cytokines	no
MDS7	High	EB-II	maturation, cytokines	no
MDS8	-	RS-CMML	cytokines	no
MDS9	Intermediate	MLD	maturation, cytokines, MLR, CyTOF	no
MDS10	Low	MLD	maturation, cytokines, MLR	no
MDS11	-	ICUS	cytokines, MLR	no
MDS12	Low	Del(5q)	cytokines	no
MDS13	Low	SLD	maturation, cytokines, MLR	no
MDS14	Low	Del(5q)	maturation, cytokines	no
MDS1	Low	RS-MLD	CyTOF	yes
MDS2	Intermediate	RS-MLD	-	yes
MDS3	Very low	RS-MLD	-	yes
MDS4	Low	RS-MLD	-	yes

Abbreviations: CMML, chronic myelomonocytic leukaemia; EB, excess blasts; ICUS, idiopathic cytopenia of undetermined significance; IPSS-R, Revised International Prognostic Scoring System; MLD, multilineage dysplasia; MLR, mixed lymfocyte reaction; RS, ring sideroblasts; SLD, single lineage dysplasia; WHO, World Health Organisation.