Impaired cytotoxicity associated with defective natural killer cell differentiation in myelodysplastic syndromes

Maryam Hejazi,1 Angela R. Manser,1 Julia Fröbel,2 Andrea Kündgen,2 Xiaoyi Zhao,1 Kathrin Schönberg,1 Ulrich Germing,2 Rainer Haas,2 Norbert Gattermann,2 and Markus Uhrberg1

1Institute for Transplantation Diagnostics and Cell Therapeutics, Medical Faculty, Heinrich-Heine University Düsseldorf; and 2Department of Hematology, Oncology and Clinical Immunology, Medical Faculty, Heinrich-Heine University Düsseldorf, Germany

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Correspondence: Markus.Uhrberg@med.uni-duesseldorf.de
Supplementary methods

Cell lines
The HLA class I-deficient target cell line K562 was grown in DMEM (Gibco, CA, USA) supplemented with 10% FBS and 1% Penicillin/Streptomycin/L-Glutamine (Gibco).

Annexin V apoptosis assay
Apoptosis of NK cells in MDS patients and healthy age-matched donors was determined using the Annexin V detection Kit (Biolegend). Briefly, PBMCs were stained with fluorescence-labeled CD56 and CD3 surface marker. After washing with PBS, Annexin V-FITC and 7-AAD was added. Apoptotic NK cells were identified as Annexin V+ 7-AAD− NK cells by flow cytometry.

KIR genotyping
Genomic DNA was isolated from blood of MDS patients and healthy age-matched donors via QiaAmp DNA Blood Mini Kit (Qiagen). KIR genotyping was performed by polymerase chain reaction with sequence specific primer (PCR-SSP) as reported previously.¹

NK cell stimulation
1x10⁵ CD3-depleted PBMC (CD3 Microbeads, Miltenyi Biotech) were incubated in a 24-well tissue culture plate with 1x10⁵ irradiated K562 and 1x10⁶ irradiated feeder PBMC (pooled from 3 different donors) with 1000 U/ml IL-2 in RPMI 1640, 10% FBS and 5% human serum type AB. Medium was exchanged every two days with fresh medium.

Supplementary figure legends:

Figure S1: Decreased frequency of NK cells in MDS patients. Frequency of NK cells (CD56^+CD3^-) in healthy adult donors (n=116; age 18-50) compared to MDS patients (n=75). Statistical significance was determined by two-tailed *t*-test (***p<0.001).

Figure S2: Correlation between frequency of NK cells and cytotoxicity in MDS patients. Specific lysis of K562 is plotted versus frequency of NK cells among PBMC of MDS patients (linear regression analysis, p=0.136).

Figure S3: Reduced IFN-γ production in MDS patients. Intracellular IFN-γ production by IL-2-stimulated NK cells after co-culture with K562 in patients (n=10) and healthy age-matched donors (n=7). Statistical significance was determined by two-tailed *t*-test (*p<0.05).

Figure S4: Association between cytotoxicity and levels of granzyme B and perforin in MDS patients. NK cells of 5 MDS patients and one healthy age-matched donor were enriched to 80-90% purity (EasySep™ Human NK Cell Enrichment Kit, Stemcell Technologies). Cytotoxicity as well as intracellular staining of granzyme B and perforin were performed as described in the Methods section. Filled dots represent patients with low NK cell function (specific lysis of K562 <20%) and open dots patients with normal NK cell function (specific lysis of K562 ≥20%). The open triangle represents a healthy age-matched controls with normal NK cell function. (linear regression analysis, p=0.0019).

Figure S5: Annexin V-determined apoptosis of CD56_{dim} and CD56_{bright} NK cell. PBMC of MDS patients (n=20) and healthy age-matched donors (n=13) were thawed. Annexin V apoptosis assay was performed and the frequency of
apoptotic cells (Annexin V^+7-AAD^-) was determined in the CD56^{dim} (A) and CD56^{bright} NK cell (B). (C) Correlation between frequency of apoptotic NK cells and absolute number of NK cells in MDS patients (linear regression analysis, p=0.585).

**Figure S6: KIR repertoires in MDS patients and healthy age-matched donors according to presence of group A and B haplotypes.** Frequency of 16 KIR (KIR2DL1, KIR2DL2/3 and KIR3DL1) and NKG2A receptor combinations, ordered according to number of expressed receptors in MDS patients (A/A: n=10, B/x: n=20) and healthy age-matched donors (A/A: n=6, B/x: n=14) with A/A haplotype (A) and B/x haplotype (B). Statistical significance was determined by two-tailed \( t \)-test (*p<0.05, **p<0.01).

**Figure S7: Increased expression of CD62L on NK cells in MDS.** Surface expression of CD57, CD62L, NKG2D, NKp30 and CD16 on NK cells of MDS patients (n=16) and healthy age-matched donors (n=10). Statistical significance was determined by two-tailed \( t \)-test (*p<0.05).

**Figure S8: Expression of CD16 on CD56^{dim} and CD56^{bright} NK cells.** Frequency of CD16 on CD56^{dim} and CD56^{bright} NK cells of 15 MDS patients and 5 healthy age-matched controls. Statistical significance was determined by two-tailed \( t \)-test (*p<0.05).

**Figure S9: Reduced granzyme B and perforin content in CD107^+ NK cells of MDS patients.** Box plots showing frequencies of granzyme B (A) and perforin (B) expressing NK cells when restricting analysis to the CD107^+ subset. Intracellular granzyme B and perforin were analyzed in CD56^+CD107^+ cells following stimulation with K562. Statistical significance was determined by two-tailed \( t \)-test (*p<0.05, **p<0.01, ***p<0.001).
Supplementary figures:

**Figure S1**

![Graph showing % IFN-gamma+ NK cells in healthy vs MDS](image1)

**Figure S2**

![Graph showing % specific lysis of K562 vs % NK cells](image2)

**Figure S3**

![Bar graph showing % IFN-gamma+ NK cells in healthy vs MDS](image3)

**Figure S4**

![Graph showing % perforin+ CD56dim NK cells vs % granzyme B+ CD56dim NK cells](image4)

**Figure S5**

**(A) & (B)**

![Scatter plots showing % apoptotic CD56dim NK cells in healthy vs MDS](image5)

**(C)**

![Scatter plot showing % apoptotic NK cells vs NK cells/mm³](image6)
Figure S6

A) % of CD56<sup>dim</sup> NK cells

- KIR-NKG2A
- KIR2DL1
- KIR2DL2/3
- KIR3DL1
- NKG2A
- KIR2DL1_KIR2DL2/3
- KIR2DL1_KIR3DL1
- KIR2DL1_NKG2A
- KIR2DL2/3_KIR3DL1
- KIR2DL2/3_NKG2A
- KIR3DL1_NKG2A
- KIR2DL1_KIR2DL2/3_KIR3DL1
- KIR2DL1_KIR2DL2/3_NKG2A
- KIR2DL1_KIR3DL1_NKG2A
- KIR2DL2/3_KIR3DL1_NKG2A
- KIR2DL1_KIR2DL2/3_KIR3DL1_NKG2A

B) % of CD56<sup>dim</sup> NK cells

- KIR-NKG2A
- KIR2DL1
- KIR2DL2/3
- KIR3DL1
- NKG2A
- KIR2DL1_KIR2DL2/3
- KIR2DL1_KIR3DL1
- KIR2DL1_NKG2A
- KIR2DL2/3_KIR3DL1
- KIR2DL2/3_NKG2A
- KIR3DL1_NKG2A
- KIR2DL1_KIR2DL2/3_KIR3DL1
- KIR2DL1_KIR2DL2/3_NKG2A
- KIR2DL1_KIR3DL1_NKG2A
- KIR2DL2/3_KIR3DL1_NKG2A

Legend:
- Healthy
- MDS

* Significant difference
** Highly significant difference
**** Extremely significant difference