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

**Running head:** NK cell deficiency in MDS

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Abstract

Natural Killer cells are well known to mediate anti-leukemic responses in myeloid leukemia but their role in myelodysplastic syndromes is not well understood. Here, in a cohort of newly diagnosed patients (n=75), widespread structural and functional natural killer cell defects were identified. Firstly, one subgroup of patients (13%) had a selective deficiency of peripheral natural killer cells (count < 10/mm^3 blood) with normal frequencies of T and natural killer-like T cells. Natural killer cell-deficient patients were predominantly found in high-risk subgroups and were significantly associated with poor prognosis. In the second subgroup, representing the majority of patients (76%), natural killer cells were present but exhibited poor cytotoxicity. The defect was strongly associated with reduced levels of perforin and granzyme B. Notably, natural killer cell function and arming of cytotoxic granules could be fully reconstituted by *in vitro* stimulation. Further phenotypic analysis of these patients revealed an immature natural killer cell compartment that was biased towards CD56^{bright} cells. The residual CD56^{dim} cells exhibited a significant increase of the unlicensed NKG2A*KIR* subset and a striking reduction in complexity of the KIR repertoire. Taken together, these results suggests that the widespread defects in natural killer cell function of myelodysplastic patients are mostly due to either unsuccessful or inefficient generation of mature, functionally competent natural killer cells, which might contribute to disease progression due to impaired immune surveillance.
Introduction

Myelodysplastic syndromes (MDS) constitute a heterogeneous group of bone marrow disorders, which are characterized by dysfunctional hematopoietic progenitor cells and a propensity of evolution to acute myeloid leukemia (AML). According to the WHO classification system, different MDS subgroups are distinguished based on the degree of dysplasia, the frequency of ring sideroblasts, and the number of medullary and/or peripheral blasts. While the majority of patients is initially diagnosed with low-grade disease, approximately two thirds of patients eventually succumb to multi-lineage cytopenia or transformation to leukemia. The risk of tumor progression can be estimated by the international prognostic scoring system (IPSS), classifying patients into four groups (low, intermediate 1 and 2, or high risk) based on cytogenetic, morphological, and clinical criteria. The etiology and pathophysiology of MDS, which is the most common hematopoietic malignancy of the elderly (age > 70y), remain incompletely defined.

The role of immunological determinants in MDS are poorly understood. It is known that a subgroup of patients responds to immunosuppressive treatment. However, immunosuppression could compromise proper immune surveillance for aberrant hematopoietic progenitor cells and favor expansion of the malignant clone. In this regard, the role of natural killer (NK) cells is of rising interest. NK cells can exert graft-versus-leukemia responses as previously shown in the setting of haploidentical stem cell transplantation for acute myeloid leukemia. NK cell function is determined by a balance of stimulatory and inhibitory receptors surveying the organism for signs of viral infections, cellular stress, and malignant transformation. To this end, NK cells express a variety of stimulatory receptors such as NKp30, NKp46, and NKG2D, recognizing, among others, stress-induced and tumor-associated ligands. Stimulatory signals are balanced by arrays of inhibitory receptors such as killer cell Ig-like receptors (KIR) and the NKG2A receptor, which sense expression of various MHC class I molecules. This system enables NK cells to identify and eliminate tumor variants escaping MHC-restricted, adaptive immune control by downregulation of MHC class I, a mechanism referred to as “missing self” recognition.

With regard to MDS, increased NK-cell mediated cytotoxicity was found in one study, while several other studies reported impaired NK cell function in peripheral blood and
bone marrow. Kiladjian et al. found decreased cytotoxicity, proliferation and increased apoptosis of peripheral NK cells without changes in expression of inhibitory or stimulatory NK cell receptors. Impaired cytotoxicity was also seen by Epling-Burnette et al., who attributed this to a lower frequency of NKG2D-expressing NK cells in peripheral blood. In a recent study, Carlsten et al. associated decreased cytotoxicity with decreased expression of DNAM-1 and NKG2D in NK cells from bone marrow but not peripheral blood. Overall, the underlying mechanisms for defective peripheral NK cell function remain elusive.

In the present study, a thorough phenotypic and functional analysis of NK cells was performed in a cohort of newly diagnosed MDS patients. In the majority of patients, NK cell defects were found and could be attributed either to an overall lack of NK cells, which was strongly associated with high-risk MDS subtypes and poor prognosis or more frequently to the presence of NK cells with an immature phenotype, which were characterized by non-armed granules and an immature NK cell receptor repertoire.
Methods

Patients and controls
Peripheral blood was obtained from 75 newly diagnosed MDS patients (age: 41-90, mean: 71) and 30 age-matched healthy control donors (age: 51-90, mean: 72). Informed consent was obtained from all patients and donors according to the Declaration of Helsinki. The study was ethically approved by the local institutional review board. Patient characteristics and classification of MDS according to WHO criteria are given in Table 1. Peripheral blood mononuclear cells (PBMC) were isolated from patients and healthy donors using density gradient centrifugation with Biocoll Separating Solution (Biochrom, Berlin, Germany) and subsequently frozen and stored in liquid nitrogen for later analysis.

Antibodies
The following fluorescence-labeled monoclonal antibodies (mAb) were used: CD56-PE, PC5 or APC (N901), CD3-ECD or PC5 (UCHT1), CD158a/h-APC (EB6), CD158b1/b2/j-APC-Alexa Fluor 750 (GL183), CD159a-PE (NKG2A, Z199), NKG2D-PE (ON72), CD62L-PC5 (DREG56) all from Beckman Coulter, CA, USA. CD158e1-FITC (DX9), CD57-FITC (HCD57), Granzyme B-FITC (GB11), Perforin-PE (dG9), CD107-APC or FITC (H4A3) and IFN-gamma FITC (B27) were purchased from Biolegend, CA, USA and CD56-PE-vio770 (AF12-7H3) from Miltenyi Biotec, Bergisch Gladbach, Germany. Flow cytometric analyses were performed on FACSCanto I (BD Biosciences, NJ, USA) using FACS Diva 5.0.1 software.

CD107a and interferon-gamma assay
Thawed PBMC were cultured overnight in RPMI 1640 containing 10% FBS, 5% human serum type AB (Biochrom), and 1000 U/mL Interleukin-2 (Novartis, Basel, Switzerland) in a concentration of 1x10⁶ cells/mL. PBMC and K562 target cells were mixed at an effector/target (E/T) ratio of 10:1 (5x10⁵:5x10⁴) in a volume of 200 µl (in a 96-well plate). For analysis of cytotoxic granule mobilization, CD107a mAb was added prior to incubation. To determine spontaneous degranulation, a control sample without target cells was included. After 1 h incubation, 2 µl of 2mM Monensin (Biolegend) was added and incubated for a further 5 h. Finally, the cells were washed in PBS (Lonza) and stained with mAbs (CD56, CD3). For measurement of intracellular interferon-gamma, effector and K562 target cells were co-incubated for 6
h. After the first hour, Brefeldin A (Sigma-Aldrich, Missouri, USA) was added at a concentration of 10 µg/ml. Cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) and stained intracellularly with anti-IFN-gamma mAb.

**Intracellular staining of granzyme B and perforin**

PBMC of MDS patients and healthy age-matched donors were stained with fluorescence-labeled CD56 and CD3 surface marker. After washing with PBS, cells were intracellularly stained, using intracellular staining kit (BD Biosciences) and granzyme B and perforin mAb. Expression of granzyme B and perforin was then determined in CD56<sup>dim</sup> NK cells by flow cytometry.

**Cytotoxicity assay**

K562 target cells were stained with CFDA-SE (Vybrant® CFDA-SE Tracer Kit, Invitrogen, CA, USA). PBMC (overnight cultured with IL-2 1000 U/ml) and stained target cells were mixed at a ratio of 10:1 in a volume of 200 µl. The following controls were prepared: unstained K562 and two wells with stained K562, one as a control for CFDA-SE staining and one to determine spontaneous lysis of K562 cells. After 6 h incubation, cells were harvested and stained with propidium iodide (PI, BD Biosciences). Frequency of CFSE<sup>+</sup>PI<sup>+</sup> K562 cells was determined by flow cytometry. Specific lysis of K562 was calculated as: lysis of K562 with effector cells – spontaneous lysis of K562.

**Statistical analysis**

All tests (Fisher’s exact test, t-test) were performed at the two-sided .05 significance level. All statistical analyses were performed using GraphPad Prism software.

Additional methods regarding cell lines, apoptosis assay, KIR genotyping, and NK cell stimulation are detailed in *supplementary methods*. 
Results

A subgroup of MDS patients exhibits profound NK cell deficiency
In order to characterize the role of NK cells in MDS, peripheral blood NK and T cells were analyzed from 75 patients, which were newly diagnosed with MDS and consequently had not received any prior disease-specific treatment (Table 1). NK cell frequencies (CD56^+CD3^-) were moderately reduced in MDS patients compared to healthy age-matched donors as shown by flow cytometric analysis (Figure 1A). Notably, a subgroup of cases (n=15) exhibited unusually low NK cell frequencies <1% (normal range 2-20%). Remarkably, we could not detect any donors with NK cell frequencies <1% in the age-matched control group and also not in a second, larger group of 116 healthy adult donors of undefined age (supplemental Figure 1). Frequency reduction was associated with an even more significant reduction in NK cell numbers. The most severe cases were classified and subsequently referred to as “NK cell deficient” (n=10; NK cell count < 10/mm^3 blood). In NK cell-deficient patients discrete NK cell populations were typically not detectable by flow cytometry based on expression of CD3, CD56 (Figure 1D) or CD16 (data not shown). Since NK cells are believed to be generated from T/NK common progenitors, we also analyzed T cells (CD3^+) and NK-like T cells (CD56^+CD3^-). Notably, overall frequencies and cell counts in T and NK-like T cells were comparable to healthy age-matched controls (Figure 1B-C). Direct comparison of NK and T cell counts in MDS samples demonstrates that the observed reduction was NK cell specific and that the NK cell-deficient group exhibited normal T cell (Figure 1E) and NK-like T cell counts (data not shown). In order to rule out that the observed reduction of NK cells in MDS patients was due to a selective loss of NK cells during thaw/freeze or storage procedures, we repeated the analysis with PBMC freshly drawn from three MDS patients with NK cell deficiency. The NK cell-deficient phenotype seen in the frozen samples could be confirmed in all three fresh samples (data not shown). Longitudinal analyses of three cases (first analysis in 2009, second analysis in 2012) revealed a stable NK cell-deficient phenotype over time (data not shown).

NK cell deficiency is associated with high-risk MDS subgroups and poor prognosis
We next asked if NK cell deficiency was associated with specific disease subgroups and/or prognosis as defined by the most recent WHO and IPSS classification
systems, respectively. Indeed, NK cell-deficient patients were overrepresented in the WHO RAEB I/II subgroup (31%), which has the highest risk for progression to AML, compared to the RCMD (11.6%) and RA/RARS subgroups (0%) with a lower risk profile (Figure 2A). Moreover, NK cell-deficient patients were strongly associated with IPSS high-risk groups (5% in the Low/Int1 vs. 44% in Int2/High groups, p<0.001), (Figure 2B). In general, NK cell counts (Figure 2A-B) and frequencies (Figure 2C-D) were significantly lower in the WHO and IPSS high-risk subgroups RAEB I/II and Int2/high, respectively. Again, T cells exhibited no association with disease subgroups (data not shown).

Functionally-deficient NK cells of MDS patients exhibit defects in the killing machinery

Whereas it was not possible to reliably assess NK cell function in NK cell-deficient patients, CD107 mobilization could be readily determined in MDS patients without NK cell deficiency. No difference in CD107 mobilization in response to K562 target cells was observed in MDS patients compared to healthy age-matched donors (Figure 3A). In contrast, killing of target cells was significantly decreased in MDS patients (Figure 3B). Plotting of NK cell frequency against specific lysis of K562 revealed that decreased target cell lysis in MDS patients was only weakly correlated to low frequency of NK cells among PBMC (supplemental Figure 2). Of note, no correlation of CD107 mobilization with target cell killing was found (Figure 3C-D). Besides cytotoxicity, intracellular IFN-gamma production was significantly reduced in response to stimulation with K562 (supplemental Figure 3).

Since NK cells of MDS patients showed no change in degranulation activity but impaired cytotoxicity, we next asked if granules were properly armed with cytotoxic molecules. Intracellular staining demonstrated that granzyme B and even more perforin loading of granules were substantially reduced in CD56dim NK cells of MDS patients (Figure 3E). In most cases, both factors were downregulated simultaneously (Figure 3F). Importantly, the small subgroup of MDS patients with functional NK cells (cytotoxicity >20%) had high granzyme B/perforin expression levels. A similar correlation between granzyme B/perforin expression levels and cytotoxicity was found when enriched NK cells were used (Supplemental Figure 4). However, there was a subgroup of MDS patients (≈15%) that had impaired killing in spite of normal
frequencies of granzyme B/perforin containing NK cells suggesting additional unidentified mechanisms of functional repression.

**In vitro stimulation of NK cells resolves functional deficiency**

In order to determine whether functional deficiency of NK cells can be resolved *in vitro*, NK cells of five MDS patients with functional deficiency and three healthy age-matched donors were subjected to an IL-2 based stimulation protocol. Importantly, NK cells could be expanded to a similar extent (10-100 fold in 10 days) in MDS patients and controls (Figure 4A). This observation was in contrast to a previous study showing lack of NK cell proliferation in MDS patients. However, here a different stimulation protocol was applied including irradiated feeder cells (supplementary methods). Notably, the perforin and granzyme B deficiency of MDS NK cells was fully reversible (Figure 4B). Consequently, *in vitro* stimulation of functionally deficient NK cells restored cytotoxicity against K562 to control levels (Figure 4C).

**Phenotypic analyses reveals immature differentiation state of NK cells in MDS**

To further elucidate possible mechanisms for the widespread functional NK cell defects in MDS, we next analyzed NK cell maturation state and receptor repertoires. MDS patients with functional defects showed a significant increase in frequency of CD56\textsuperscript{bright} NK cells, representing an immature, less cytotoxic NK cell subset\textsuperscript{14} as well as a decrease in CD56\textsuperscript{dim} NK cells (Figure 5A-B). In contrast, patients with normal NK cell function had CD56\textsuperscript{bright} frequencies that were comparable to controls. Analysis of cell counts revealed that in fact the CD56\textsuperscript{bright} subset was unchanged in terms of absolute cell numbers (Figure 5C) whereas the more mature CD56\textsuperscript{dim} subset was strongly decreased in functionally deficient patients compared to cases with normal NK cell function and healthy controls (Figure 5D). Notably, the decrease in CD56\textsuperscript{dim} NK cells was significantly associated (p=0.0004) with reduced target cell killing (Figure 5E).

We next investigated if the selective decrease of mature NK cells in functionally deficient MDS patients could be due to increased apoptosis of CD56\textsuperscript{dim} NK cells. However, no differences in spontaneous cell death of CD56\textsuperscript{dim} or CD56\textsuperscript{bright} subsets were found in comparison to age-matched controls (Supplemental Figure 5A-B).
Additionally, no correlation between apoptosis of NK cells and the NK cell count of MDS patients was observed (Supplemental Figure 5C).

CD56<sup>dim</sup> NK cells can be further separated on the basis of KIR/NKG2A expression into four consecutive maturation stages. MDS patients exhibited an unusually high frequency of NKG2A<sup>-</sup>KIR<sup>-</sup> CD56<sup>dim</sup> NK cells (Figure 6A). This subset represents an immature differentiation stage that is functionally not educated and is generally hypo-responsive to various stimuli. Furthermore, the two KIR-expressing “late” stages (NKG2A<sup>+</sup>KIR<sup>+</sup> and NKG2A<sup>-</sup>KIR<sup>+</sup>) were less represented in MDS patients. KIR-expressing NK cells, which are essentially a combination of the two “late” stages, showed significantly decreased frequency as well as absolute numbers in patients (data not shown).

To further refine the analysis of NK cell maturation states, clonal KIR expression patterns of CD56<sup>dim</sup> NK cell were analyzed. During NK cell differentiation, KIR expression is regulated in a sequential mode with KIR2DL2/3 representing the first inhibitory KIR and subsequent expression of KIR3DL1 and KIR2DL1. The analysis revealed a strong decrease of NK cells expressing the “late” KIR2DL1 or KIR3DL1 as their only KIR, whereas the “early” KIR2DL2/3 frequencies were increased in MDS patients (Figure 6B). Direct comparison of NK cells selectively expressing one of the three inhibitory KIR (“single KIR” expressing NK cells) for the major HLA class I-encoded ligands (Bw4, C1, C2) reveals a strong bias towards KIR2DL2/3 expression (t-test: p <0.0001) and a significant underrepresentation of KIR2DL1 and KIR3DL1 in the KIR repertoires of patients (Figure 6C). Similar effects were seen when cases were stratified according to KIR genotype, i.e. in patients homozygous for group A haplotypes and those having one or two B haplotypes (B/x; supplemental Figure 6). Furthermore, NK cells expressing multiple KIR, a hallmark of experienced NK cells, were significantly decreased in the repertoires of patients (Figure 6D). Further analysis of other markers associated with distinct stages of NK cell differentiation revealed that CD62L, which is associated with naïve NK cells, was significantly increased in MDS patients (Supplemental Figure 7). Furthermore, expression of CD16, a marker of mature NK cells, was significantly decreased in MDS patients (Supplemental Figure 7). Low expression of CD16 could specifically be attributed to the CD56<sup>dim</sup> subset of NK cells (Supplemental Figure 8). Of note, the stimulatory receptors NKG2D and NKp30 were not differentially expressed in either
NK subset. Altogether, these data reveal a profound deficiency of fully matured NK cells in the majority of MDS patients.
Discussion

Ineffective hematopoiesis is a hallmark of MDS and affects single or multiple myeloid lineages depending on the MDS subtype. It is less clear in how far lymphopoiesis is also affected by the impaired differentiation capacity of aberrant hematopoietic progenitor cells or a compromised stem cell microenvironment. So far, several previous reports found deficient NK cell responses in MDS against various autologous or allogeneic tumor targets. The present study suggests that in the majority of cases NK cell defects can be attributed to inefficient or defective NK cell differentiation, either leading to a predominantly immature NK cell compartment or an overall lack of NK cells.

Three different MDS patient groups could be defined on the basis of NK cell number and function: a small group with normal NK cell number and function, a major group of patients with normal or moderately decreased NK cell number and impaired function, and an NK cell-deficient group. The latter subgroup, in which NK cells were rare or undetectable (PBMC: 1-10 NK cells/mm$^3$) was preferentially found in the high-risk WHO subgroups RAEB I/II and showed a significantly elevated IPSS risk score. These observations are in line with a recent study that found strongly reduced NK cell frequencies in a high-risk (presumably pretreated) cohort of MDS patients undergoing unrelated HSCT. Since NK cells are an important component of immunological tumor surveillance, it is tempting to speculate that a lack of NK cells in MDS patients increases the risk for leukemic progression to AML. In this regard, NK cells were previously implicated in relapse control following allogeneic HSCT for myeloid leukemia. Moreover, recent studies have associated certain KIR gene linkage groups with decreased relapse and improved outcome in HSCT for AML. Future analysis of larger MDS cohorts and a longer follow-up will be necessary to show if NK cell deficiency is an independent prognostic parameter for progression to leukemia and/or overall clinical outcome.

The majority of MDS patients had normal or moderately decreased NK cell numbers while exhibiting impaired cytotoxicity confirming several previous reports. In order to better understand the underlying mechanisms, we analyzed NK cell effector functions on the clonal level. Unexpectedly, cell surface mobilization of CD107 was not affected in MDS patients in spite of strongly decreased target cell cytotoxicity.
Moreover, intracellular analysis of perforin and granzyme B revealed significantly decreased expression of either one or both molecules. Normal levels of NK cell cytotoxicity were only seen in patients expressing high levels of granzyme B and perforin. These observations strongly suggest that the lack of armed granules is causally involved in the decreased cytotoxicity of NK cells in MDS. Notably, a small subset (15% of cases) exhibited decreased cytotoxicity in spite of normal levels of granzyme B and perforin (Figure 3F). In these cases, the cause for defective function has yet to be elucidated.

The above observations illustrate certain limitations of the CD107 assay that in recent years emerged as an established surrogate marker for cytotoxic activity of T and NK cells.\textsuperscript{23,24} In functionally competent NK cells, mobilization of CD107 to the cell surface correlates with release of granule content and subsequent target cell cytotoxicity. However, if cytotoxic granules are not properly armed with perforin or granzyme B, NK cells might still mobilize CD107 to the cell surface but would not be able to kill. Indeed, in MDS patients NK cells with insufficiently armed granules still mobilized CD107 to the cell surface (supplemental Figure 9). These observations might not only be relevant for MDS but also for other hematological or pathological settings in which NK cell function might be similarly compromised by deficient arming of cytotoxic granules, which then would not be detected by standard CD107 mobilization assay.

Several lines of evidence suggest that the observed functional defects are related to the immaturity of the NK cell compartment in MDS patients. Firstly, the distribution of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} subsets was biased towards the immature non-cytotoxic CD56\textsuperscript{bright} subset. Whereas CD56\textsuperscript{bright} frequencies increased, the CD56\textsuperscript{dim} subset was strongly diminished. The CD56\textsuperscript{dim} count significantly correlated with lack of cytotoxicity. Secondly, among CD56\textsuperscript{dim} NK cells, MDS patients show a significant increase in KIR\textsuperscript{−}NKG2A\textsuperscript{−} cells. It was previously shown that this CD56\textsuperscript{dim} subset is immature and can be differentiated in vitro into the other three subsets in a sequential fashion starting with NKG2A\textsuperscript{−}KIR\textsuperscript{−} followed by the NKG2A\textsuperscript{−}KIR\textsuperscript{+} subset and finally NKG2A\textsuperscript{−}KIR\textsuperscript{+} NK cells.\textsuperscript{15} Importantly, KIR\textsuperscript{−}NKG2A\textsuperscript{−} cells are not licensed due to their lack of HLA class I-specific inhibitory receptors and thus exhibit poor cytotoxicity.\textsuperscript{16,25} In contrast, the more mature KIR expressing stages KIR\textsuperscript{+}NKG2A\textsuperscript{−} and KIR\textsuperscript{+}NKG2A\textsuperscript{−} are less abundant and consequently overall KIR expression was
significantly reduced in MDS patients. Thirdly, analysis of clonal KIR expression demonstrates a bias towards expression of “early” KIR in MDS patients: KIR repertoires were dominated by expression of KIR2DL3, which is the first inhibitory KIR that is expressed during sequential acquisition of KIR.\textsuperscript{17,18,26} KIR2DL1 and KIR3DL1, which are expressed at later stages of KIR repertoire development were strongly diminished in patients. Moreover, the frequency of NK cells expressing multiple inhibitory KIR, which represent a more advanced stage of NK cell development, was diminished, too.\textsuperscript{27}

Notably, the above-described phenotypic characteristics resemble those of neonatal NK cells. Similar to NK cells in MDS, neonatal NK cells exhibit an elevated frequency of CD56\textsuperscript{bright} NK cells, increased expression of NKG2A in the CD56\textsuperscript{dim} subset, and a KIR repertoire biased towards KIR2DL3.\textsuperscript{28} A further parallel is that effector functions of neonatal NK cells are initially reduced compared to adult NK cells but are quickly catching up upon stimulation with IL-2 similar to what is observed for NK cells in MDS (Figure 4). It is also revealing that the phenotypic changes described here such as increased frequency of the CD56\textsuperscript{bright} subset is actually opposite to the changes observed in the NK cell compartment during healthy ageing.\textsuperscript{29} Thus, although MDS is a disease of the elderly, NK cell repertoires are clearly phenotypically and functionally immature.

It can only be speculated about the mechanisms leading to the lack of mature, functionally competent NK cells in MDS. Notably, similar analysis of NK cells in AML and CML patients revealed no such changes of the CD56\textsuperscript{bright} and CD56\textsuperscript{dim} subsets, suggesting that the underlying mechanisms are specific for MDS.\textsuperscript{30,31} We could not detect a significant increase in spontaneous apoptosis of CD56\textsuperscript{dim} and CD56\textsuperscript{bright} NK cells in MDS patients, making it unlikely that the observed lack of mature NK cells is due to selective cell death of the CD56\textsuperscript{dim} NK cell subset (supplemental Figure 5). One possibility that was not thoroughly addressed in this study is that the malignant clone contributes to altered NK cell differentiation of NK cell progenitors. Indeed, clonal markers were detected in variable fractions of NK cells in some studies,\textsuperscript{11,32} but not others.\textsuperscript{12} Our preliminary analysis confirms participation of the MDS clone in the NK cell compartment of del5q patients (data not shown). So far it is unknown if and how clonal alterations can influence the efficiency of NK cell differentiation and/or NK cell function. Given the fact that functional NK cell defects and
developmental immaturity were found across all MDS subtypes and including many different genetic aberrations, it appears unlikely that clonal involvement is the sole cause.

An alternative, but not mutually exclusive, possibility is that NK cell differentiation is disturbed due to a lack of stromal support in the stem cell microenvironment of MDS patients. In this regard, it was previously shown that efficient generation of mature, KIR-expressing NK cells requires the presence of suitable supportive stromal cells.\textsuperscript{17} Moreover, it was recently shown that mesenchymal stem cells (MSC) of MDS patients have a substantially diminished capacity to support myeloid differentiation across all disease subtypes and moreover that hematopoietic stem and progenitor cells (HSPC) from MDS patients can regain their differentiation potential when cultured on MSC from healthy controls.\textsuperscript{33}

In conclusion, we could associate defective NK cell responses with NK cell maturation defects in the majority of MDS patients. Functional NK cell defects were reversible in vitro by IL-2-induced stimulation. In this context, clinical treatment of MDS patients with Lenalidomide or other derivatives of Thalidomide was previously shown to improve NK cell function and proliferation via increased T cell-based IL-2 production.\textsuperscript{34} It would be interesting to find out if immunomodulatory drug treatment is also effective in NK cell-deficient patients. The majority of these patients have a high-risk profile with increased risk for progression to leukemia and might particularly profit from improved NK cell-based immune surveillance. Finally, it should also be considered if MDS patients could benefit from adoptive NK cell-based immunotherapy. This might be a valuable option for patients with poor prognosis in order to improve immunosurveillance for leukemic cells as well as for patients with secondary immunodeficiency, e.g. experiencing recurrent viral infections.
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Authorship contributions
M.H. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript; A.M. analyzed data and wrote the manuscript; J.F., A.K., X.Z., and K.S. conducted experiments and analyzed data; U.G., R.H., and N.G. designed the study, provided samples, and critically read the manuscript; MU conceived, directed, and supervised the study, designed and analyzed experiments, and wrote the manuscript.

Conflict of Interest Disclosures
The authors declare no competing financial interests.
References


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<th>Table 1: Characteristics of MDS patients</th>
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* RA, refractory anemia; RARS, refractory anemia with ring sideroblasts; RCMD, refractory cytopenias with multilineage dysplasia; RAEB, refractory anemia with excess blasts
Figure Legends:

**Figure 1: Selective NK cell deficiency in a subset of MDS patients.** Frequencies (left panels) and cell counts (right panels) of NK cells (A), T cells (B) and NK-like T cells (C) were analyzed in MDS patients (n=75) and healthy age-matched donors (n=30). NK cells were defined as CD56+CD3- cells within the lymphocyte gate. NK cell numbers/mm³ were calculated as: (absolute number of lymphocytes) x (frequency of NK cells). The dashed horizontal line in right panel A demarcates the NK cell-deficient cases. Each dot represents one individual, and horizontal bars represent mean values. (D) Representative flow cytometric dot plots showing expression of CD56 and CD3 cells in PBMC from an NK cell-deficient patient (left), and a patient with normal NK cell frequency (right). (E) Cell counts of NK cells versus T cells in MDS patients (n=75). Statistical significance was determined by two-tailed t-test (*p<0.05, ***p<0.001).

**Figure 2: NK cell deficiency correlates with poor prognosis.** Absolute NK cell counts (A) and NK cell frequency (C) in patients within WHO subclasses RA/RARS (n=8), RCMD (n=43), and RAEB I/II (n=13). Statistical significance for subclasses was calculated by one-way ANOVA (*p<0.05). NK cell counts (B) and frequency (D) according to the IPSS score, separated into a low/Int1 (n=59) and an Int2/high (n=16) risk group. Statistical significance was determined by two-tailed t-test (*p<0.05). Each dot represents one individual, and horizontal bars represent mean values. The dashed horizontal lines in A and B demarcate the NK cell-deficient cases.

**Figure 3: Impaired NK cell cytotoxicity in MDS patients associates with low levels of granzyme B and perforin.** (A) K562-induced degranulation of NK cells following IL-2 stimulation were measured in 30 patients and 20 healthy age-matched donors with flow cytometry (CD3-CD56+CD107+). (B) Specific lysis of K562 target cells at a ratio of 10:1 E/T using IL-2 stimulated PBMC from patients (n=43) and healthy donors (n=20). Patients with lysis <20% were considered as functionally deficient. (C) Degranulation of NK cells versus specific lysis of K562 target cells in MDS patients (n=30). (D) CD107 expression levels and K562 lysis are shown for a representative MDS patient and a healthy age-matched donor. Spontaneous degranulation of NK cells (upper panel) and spontaneous lysis of K562 (lower panel) are depicted by filled histograms. (E) Box plots showing intracellular staining of
granzyme B and perforin in CD56<sup>dim</sup> NK cells of 37 MDS patients (light grey) and 20 healthy donors (dark grey). (F) Correlation between granzyme B and perforin expression in CD56<sup>dim</sup> NK cells of 37 MDS patients. 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%). Error bars represent standard deviation. Statistical significance was determined by two-tailed t-test (**p<0.01, ***p<0.001).

**Figure 4: Reversibility of deficient NK cell function.** (A) IL-2 induced expansion of NK cells from MDS (n=5, dashed lines) and healthy age-matched individuals (n=3, solid lines) for 10 days. (B) Cumulative frequency of granzyme B/perforin on days 0 and 10 of NK cell expansion (C) Cytotoxicity of NK cells measured on day 10 in comparison with initial cytotoxicity.

**Figure 5: Selective reduction in CD56<sup>dim</sup> NK cell numbers leading to increased CD56<sup>bright</sup> frequency in MDS patients.** Frequency of CD56<sup>bright</sup> (A) and CD56<sup>dim</sup> NK cells (B), as well as absolute number of CD56<sup>bright</sup> (C) and CD56<sup>dim</sup> NK cells (D) were determined in MDS patients with functionally deficient NK cells (n=35, defined as specific lysis of K562 <20%), functional NK cells (n=8, defined as specific lysis of K562 ≥20%) and healthy age-matched donors (n=30). Statistical significance was determined by two-tailed t-test (*p<0.05, ***p<0.001). (E) Correlative analysis of CD56<sup>dim</sup> NK cell count and specific lysis of K562 in 43 MDS patients (linear regression analysis, p=0.0004). Error bars represent standard deviation.

**Figure 6: Immaturity of CD56<sup>dim</sup> NK cells in MDS patients.** (A) Frequency of four NK cell subpopulations according to expression of KIR and/or NKG2A. (B) Frequency of clonal combinations of KIR2DL1, KIR2DL2/3, KIR3DL1, and NKG2A receptors for patients and healthy donors, ordered according to number of expressed receptors. (C) Pie charts showing frequency of CD56<sup>dim</sup> NK cells expressing a given single KIR (KIR2DL1, KIR2DL2/3, or KIR3DL1) in healthy donors (left) and patients (right). NK cells without KIR or other KIR constellations were not considered here. (D) Frequency of NK cells expressing a given number of KIR. Analyses were performed with MDS patients (n=30) and healthy age-matched donors (n=20). Error bars represent standard deviation. Statistical significance was determined by two-tailed t-test (*p<0.05, **p<0.01, ***p<0.001).
Figure 1

A) [% NK cells (log)]

B) [% T cells (log)]

C) [% NK-like T cells (log)]

D) [NK cells/mm³ (log)]

E) [T cells/mm³ (log)]
Figure 2

A) NK cells/mm$^3$ (log)

- RA/RARS
- RCMD
- RAEB I/II

B) NK cells/mm$^3$ (log)

- low/Int1
- Int2/high

NK cell deficient (p<0.001)

C) % NK cells (log)

- RA/RARS
- RCMD
- RAEB I/II

D) % NK cells (log)

- low/Int1
- Int2/high
Figure 4

A) NK cell count

B) % granzyme B + % perforin

C) % specific lysis of K562

healthy  MDS

day 0  day 10  day 0  day 10  day 0  day 10
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$^{\text{dim}}$ (A) and CD56$^{\text{bright}}$ 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$^{\text{dim}}$ and CD56$^{\text{bright}}$ NK cells. Frequency of CD16 on CD56$^{\text{dim}}$ and CD56$^{\text{bright}}$ 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

Figure S2

Figure S3

Figure S4

Figure S5
Figure S6

A) 

B) 

% of CD56dim NK cells

0 20 40 60 80

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

A) healthy

B) MDS

% of CD56dim NK cells

0 20 40 60

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

A) healthy

B) MDS