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 Uhrberg[1]

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

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. 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 deficiency of these cells was significantly associated with poor prognosis. In the second subgroup, comprising 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 repertoire of killer cell immunoglobulin-like receptors. Taken together, these results suggest that the widespread defects in natural killer cell function occurring in patients with myelodysplastic syndromes are mostly due to either unsuccessful or inefficient generation of mature, functionally competent natural killer cells, which might contribute to disease progression through 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 for evolution into acute myeloid leukemia.[1] According to the World Health Organization (WHO) classification system, different MDS subgroups are distinguished based on the degree of dysplasia, the frequency of ring sideroblasts, and the number of bone marrow and/or peripheral blasts.[2] Although most patients are initially diagnosed with low-grade disease, approximately two-thirds of patients eventually succumb to multi-lineage cytopenia or transformation to leukemia.[3] The risk of tumor progression can be estimated by the International Prognostic Scoring System (IPSS), classifying patients into four risk groups (low, intermediate 1 and 2, or high) based on cytogenetic, morphological, and clinical criteria.[4] The etiology and pathophysiology of MDS, which is the most common hematopoietic malignancy of the elderly (subjects aged >70 years), 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.[5] In this regard, the role of natural killer (NK) cells is of increasing interest. NK cells can produce graft-*versus*-leukemia responses as previously shown in the setting of haploidentical stem cell transplantation for acute myeloid leukemia.[6] 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.[7] Stimulatory signals are balanced by arrays of inhibitory receptors such as killer cell immunoglobulin-like receptors (KIR) and the NKG2A receptor, which sense expression of various major histocompatibility complex (MHC) class I molecules. This system enables NK cells to identify and eliminate tumor variants escaping MHC-restricted, adaptive immune control by down-regulation of MHC class I, a mechanism referred to as "missing self" recognition.[8,9]

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.[10-13] 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.[11] 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.[12] 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.[13] Overall, the underlying mechanisms for defective peripheral NK cell function remain elusive.

 doi:10.3324/haematol.2014.118679
The online version of this article has a Supplementary Appendix.
Manuscript received on October 13, 2014. Manuscript accepted on February 9, 2015.
Correspondence: Markus.Uhrberg@med.uni-duesseldorf.de

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 patients with newly diagnosed MDS (age, 41-90 years; mean 71 years) and 30 age-matched healthy control donors (age, 51-90 years; mean 72 years). 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. The patients' 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 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 interferon-γ 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 a FACSCanto I (BD Biosciences, NJ, USA) using FACS Diva 5.0.1 software.

Table 1. Characteristics of the MDS patients.

Healthy controls	Total
Age: 51-90 (mean 72), years	30
MDS patients	**75**
Sex	
Male	45
Female	30
Age, years	
40-60	7
60-70	22
70-80	30
> 80	16
WHO subtypes	
Refractory anemia	3
Refractory anemia with ring sideroblasts	5
Refractory cytopenias with multilineage dysplasia	43
Refractory anemia with excess blasts	13
Chronic monomyelocytic leukemia	6
5q- syndrome	4
Unclassified MDS	1
IPSS	
Low	35
Intermediate 1	24
Intermediate 2	11
High	5

CD107a and interferon-gamma assay

Thawed PBMC were cultured overnight in RPMI 1640 containing 10% fetal bovine serum, 5% human serum type AB (Biochrom), and 1000 U/mL interleukin-2 (Novartis, Basel, Switzerland) in a concentration of $1x10^6$ cells/mL. PBMC and K562 target cells were mixed at an effector/target (E/T) ratio of 10:1 ($5x10^5$:$5x10^4$) in a volume of 200 μL in a 96-well plate. For analysis of cytotoxic granule mobilization, CD107a monoclonal antibody was added prior to incubation. To determine spontaneous degranulation, a control sample without target cells was included. After incubation for 1 h, 2 μL of 2 mM Monensin (Biolegend) were added and incubated for a further 5 h. Finally, the cells were washed in phosphate-buffered saline (Lonza) and stained with monoclonal antibodies (CD56, CD3). For measurement of intracellular interferon-γ, 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-interferon-γ monoclonal antibody.

Intracellular staining of granzyme B and perforin

PBMC from MDS patients and healthy age-matched donors were stained with fluorescence-labeled CD56 and CD3 surface marker. After washing with phosphate-buffered saline, cells were intracellularly stained, using an intracellular staining kit (BD Biosciences) and granzyme B and perforin monoclonal antibodies. Expression of granzyme B and perforin was then determined in $CD56^{dim}$ NK cells by flow cytometry.

Cytotoxicity assay

K562 target cells were stained with CFDA-SE (Vybrant® CFDA-SE Tracer Kit, Invitrogen, CA, USA). PBMC (cultured overnight with interleukin-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 incubation for 6 h, cells were harvested and stained with propidium iodide (BD Biosciences). The frequency of K562 cells double positive for CFSE and propidium iodide was determined by flow cytometry. Specific lysis of K562 cells was calculated as: lysis of K562 cells with effector cells – spontaneous lysis of K562 cells.

Statistical analysis

All tests (Fisher exact test, t-test) were performed at the two-sided 0.05 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 the *Online Supplementary Methods*.

Results

A subgroup of patients with myelodysplastic syndromes exhibits profound natural killer cell deficiency

In order to characterize the role of NK cells in MDS, we

analyzed peripheral blood NK and T cells from 75 patients who were newly diagnosed with MDS and consequently had not received any prior disease-specific treatment (Table 1). NK cell ($CD56^+CD3^-$) frequencies were moderately lower in MDS patients than in 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 or in a second, larger group of 116 healthy adult donors of undefined age (*Online Supplementary Figure S1*). Reduced NK cell frequency 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 of T and NK-like T cells were comparable to those of healthy, age-matched controls (Figure 1B,C). Direct comparison of NK and T-cell counts in MDS samples demonstrated that the observed reduction was NK cell-specific and that the NK cell-deficient group had 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 the thawing/freezing 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 was 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*).

Natural killer cell deficiency is associated with high-risk subgroups of myelodysplastic syndromes and poor prognosis

We next investigated whether 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 refractory anemia with excess blasts I/II subgroup (31%), which has the highest risk for progression to acute myeloid leukemia,

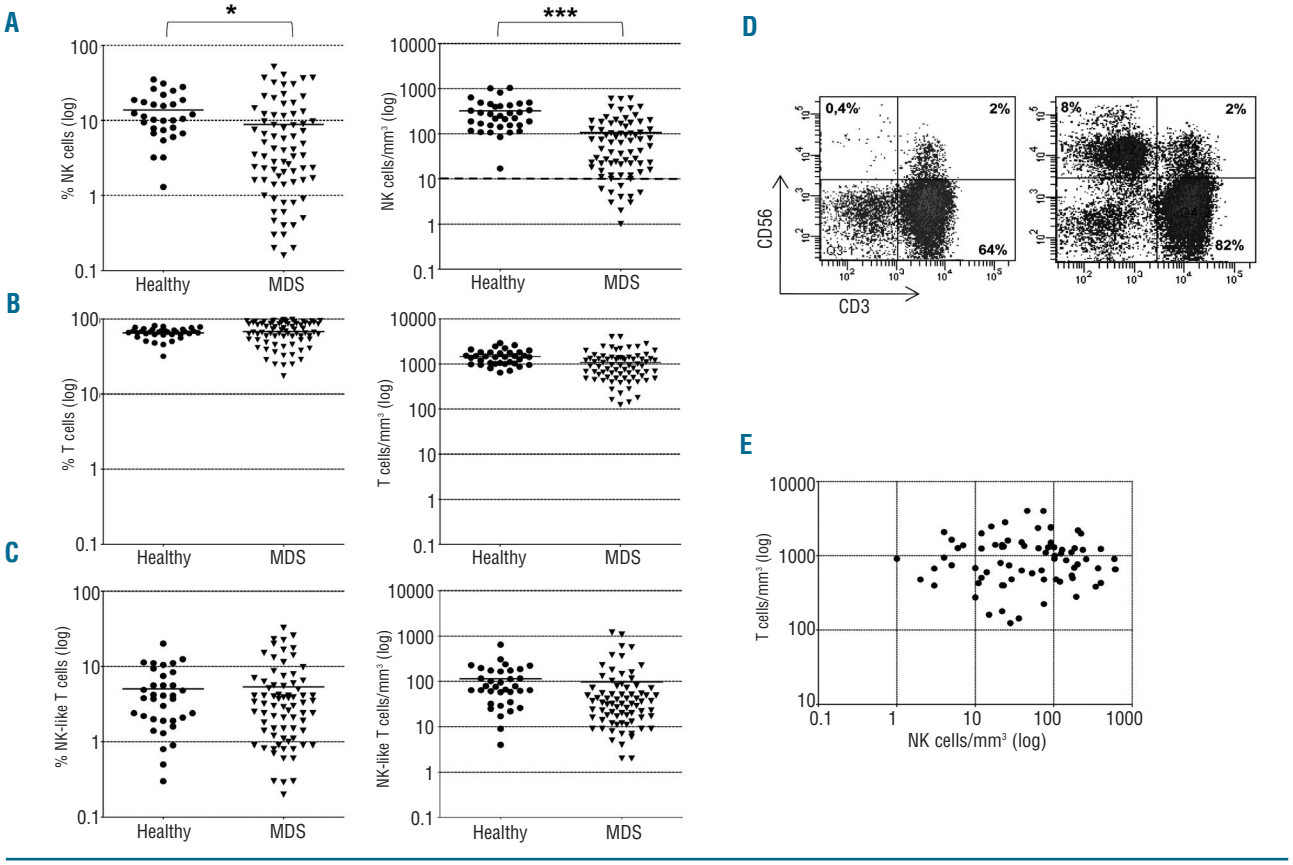

Figure 1. Selective NK cell deficiency in a subset of MDS patients. Frequencies (left panels) and cell counts (right panels) of (A) NK cells, (B) T cells and (C) NK-like T cells were analyzed in MDS patients (n=75) and healthy age-matched donors (n=30). NK cells were defined as $CD56^+CD3^-$ cells within the lymphocyte gate. NK cell numbers/mm^3 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 a 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 a two-tailed t-test (*P<0.05, ***P<0.001).

compared to the subgroups refractory cytopenia with multilineage dysplasia (11.6%) and refractory anemia/refractory anemia with ring sideroblasts (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/intermediate 1 risk groups *versus* 44% in the intermediate 2/high risk 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, refractory anemia with excess blasts I/II and intermediate 2/high, respectively. Again, T cells exhibited no association with disease subgroups (*data not shown*).

Functionally deficient natural killer cells from patients with myelodysplastic syndromes exhibit defects in their 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 was observed in CD107 mobilization in response to K562 target cells between MDS patients and healthy, age-matched donors (Figure 3A). In contrast, killing of target cells was significantly decreased in MDS patients (Figure 3B). Plotting NK cell frequency against specific lysis of K562 cells revealed that decreased target cell lysis in MDS patients was only weakly correlated with a low frequency of NK cells among PBMC (*Online Supplementary Figure S2*). Of note, no correlation was found between CD107 mobilization and target cell killing (Figure 3C,D). Besides cytotoxicity, intracellular interferon-γ production was significantly reduced in response to stimulation with K562 cells (*Online Supplementary Figure S3*).

Since NK cells from MDS patients showed no change in degranulation activity but did have impaired cytotoxicity, we next investigated whether granules were properly armed with cytotoxic molecules. Intracellular staining demonstrated that granzyme B and even more perforin loading of granules was substantially reduced in $CD56^{dim}$ NK cells from MDS patients (Figure 3E). In most cases, both factors were down-regulated simultaneously (Figure 3F). Importantly, the small subgroup of MDS patients with functional NK cells (cytotoxicity >20%) had high levels of granzyme B/perforin expression. A similar correlation between granzyme B/perforin expression levels and cyto-

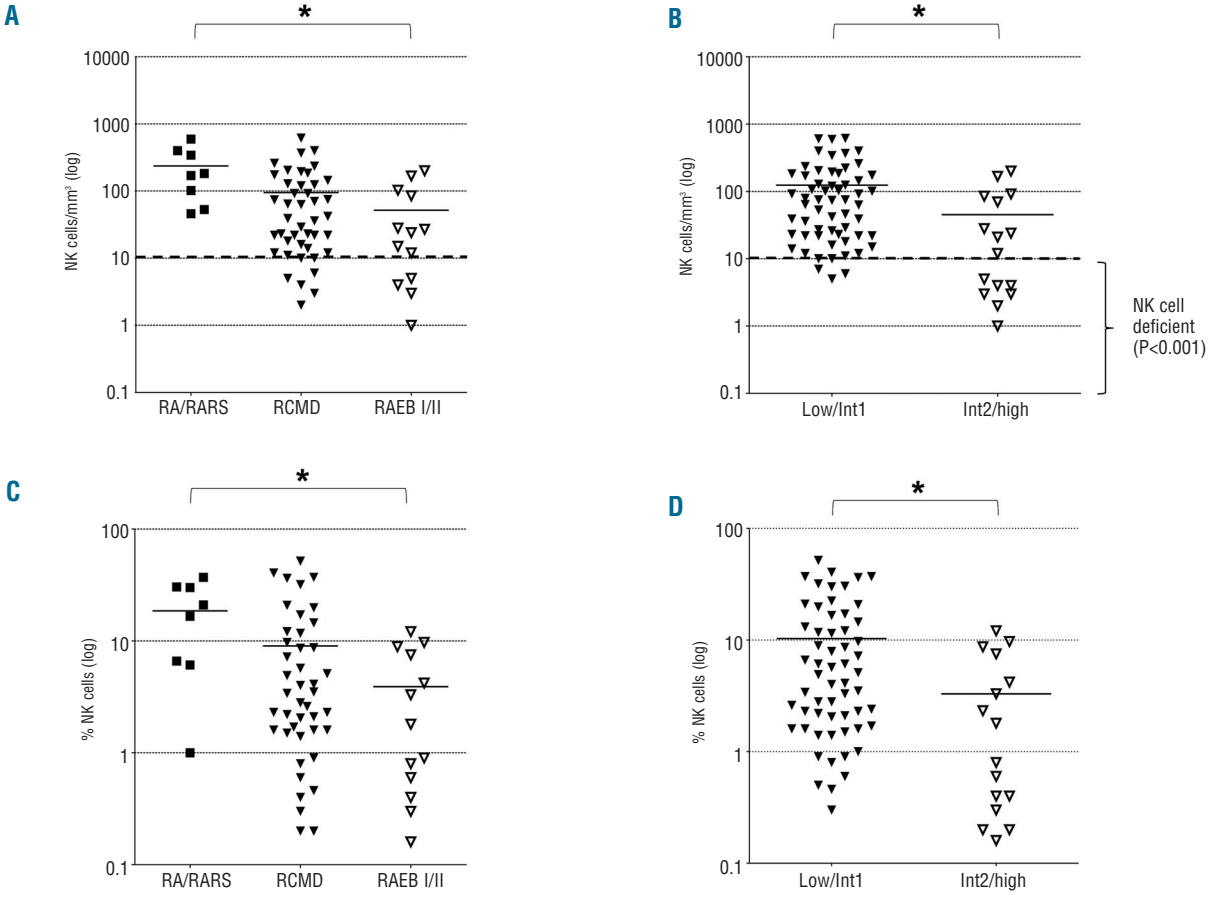

Figure 2. NK cell deficiency correlates with poor prognosis. (A) Absolute NK cell counts and (C) NK cell frequency in patients within WHO subclasses refractory anemia/refractory anemia with ring sideroblasts (RA/RARS) (n=8), refractory cytopenias with multilineage dysplasia (RCMD) (n=43), and refractory anemia with excess blasts (RAEB) I/II (n=13). Statistical significance for subclasses was calculated by one-way ANOVA (*$P<0.05$). (B) NK cell counts and (D) frequency according to the IPSS score, separated into low/intermediate 1 (Int1) (n=59) and intermediate2 (Int2)/high (n=16) risk groups. Statistical significance was determined by a 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.

toxicity was found when enriched NK cells were used (*Online Supplementary Figure S4*). However, there was a subgroup of MDS patients (≈15%) who 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 natural killer cells resolves functional deficiency*

In order to determine whether functional deficiency of NK cells can be resolved *in vitro*, NK cells from five MDS patients with functional deficiency and from three healthy age-matched donors were subjected to an interleukin-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 that in a previous study showing lack of NK cell proliferation in MDS patients.[11] However, a different stimulation protocol, including irradiated feeder cells, was applied here (*Online 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 cells to control levels (Figure 4C).

Phenotypic analyses revealed immature differentiation state of natural killer cells in myelodysplastic syndromes

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 $CD56^{bright}$ NK cells, representing an immature, less cytotoxic NK cell subset,[14] as well as a decrease in $CD56^{dim}$ NK cells (Figure 5A, B). In contrast, patients with normal NK cell function had $CD56^{bright}$ frequencies that were comparable to those of controls. Analysis of cell counts revealed that the $CD56^{bright}$ subset was unchanged in terms of absolute cell numbers (Figure 5C) whereas the more mature $CD56^{dim}$ 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 $CD56^{dim}$ NK cells was significantly associated (P=0.0004) with reduced target cell killing (Figure 5E).

We next investigated whether the selective decrease of mature NK cells in functionally deficient MDS patients could be due to increased apoptosis of $CD56^{dim}$ NK cells. However, we found no differences in spontaneous cell

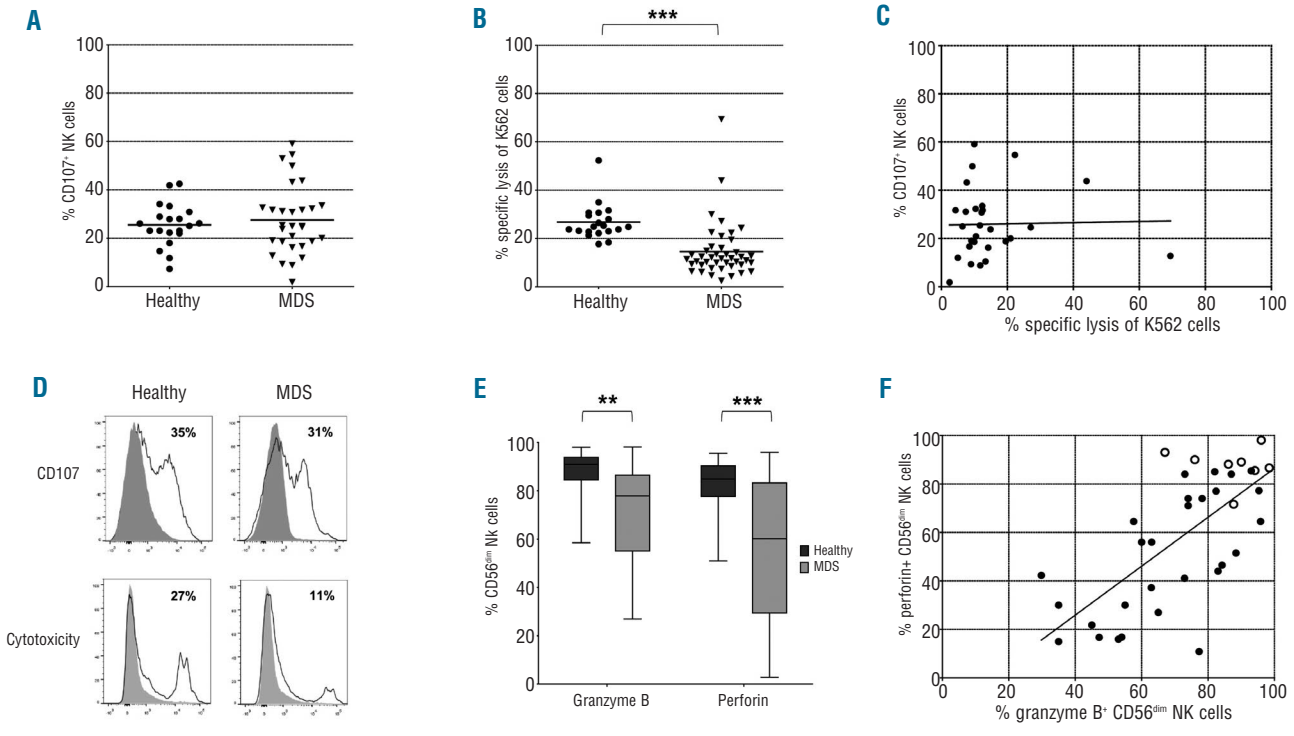

Figure 3. Impaired NK cell cytotoxicity in MDS patients is associated with low levels of granzyme B and perforin. (A) K562-induced degranulation of NK cells following interleukin-2 stimulation was measured in 30 patients and 20 healthy age-matched donors with flow cytometry ($CD3^-CD56^+CD107^+$). (B) Specific lysis of K562 target cells at an E/T ratio of 10:1 using interleukin-2 stimulated PBMC from patients (n=43) and healthy donors (n=20). Patients with <20% lysis were considered to have a functional deficiency. (C) Degranulation of NK cells *versus* specific lysis of K562 target cells in MDS patients (n=30). (D) CD107 expression levels and K562 cell 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 cells (lower panel) are depicted by filled histograms. (E) Box plots showing intracellular staining of granzyme B and perforin in $CD56^{dim}$ NK cells from 37 MDS patients (light gray) and 20 healthy donors (dark gray). (F) Correlation between granzyme B and perforin expression in $CD56^{dim}$ NK cells from 37 MDS patients. Filled dots represent patients with low NK cell function (specific lysis of K562 cells <20%) and open dots patients with normal NK cell function (specific lysis of K562 cells ≥20%). Error bars represent standard deviation. Statistical significance was determined by a two-tailed t-test (**P<0.01, ***P<0.001).

death of CD56^{dim} or CD56^{bright} subsets between patients and age-matched controls (*Online Supplementary Figure S5A,B*). Additionally, no correlation between apoptosis of NK cells and the NK cell count of MDS patients was observed (*Online Supplementary Figure S5C*).

CD56^{dim} NK cells can be separated on the basis of KIR/NKG2A expression into four consecutive maturation stages.[15] MDS patients had an unusually high frequency of NKG2A^{-}KIR^{-} CD56^{dim} NK cells (Figure 6A). This subset represents an immature differentiation stage that is functionally not educated and is generally hyporesponsive to various stimuli.[16] Furthermore, the two KIR-expressing "late" stages (NKG2A^{+}KIR^{+} and NKG2A^{-}KIR^{+}) were less represented in MDS patients. Both the frequency and

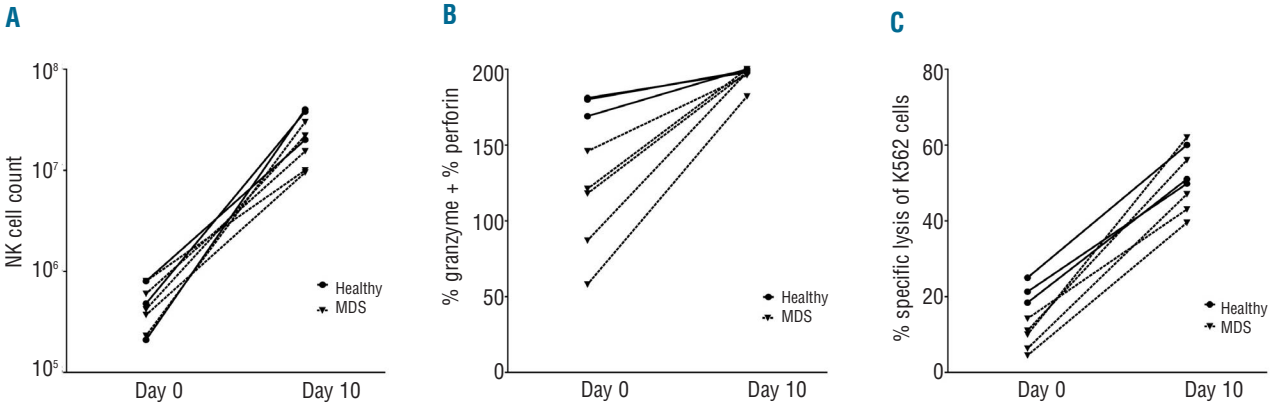

Figure 4. Reversibility of deficient NK cell function. (A) Interleukin-2-induced expansion of NK cells from MDS patients (n=5, dashed lines) and healthy age-matched individuals (n=3, solid lines) for 10 days. (B) Cumulative frequency of granzyme B/perforin on days 0 and 10 of NK cell expansion (C) Cytotoxicity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

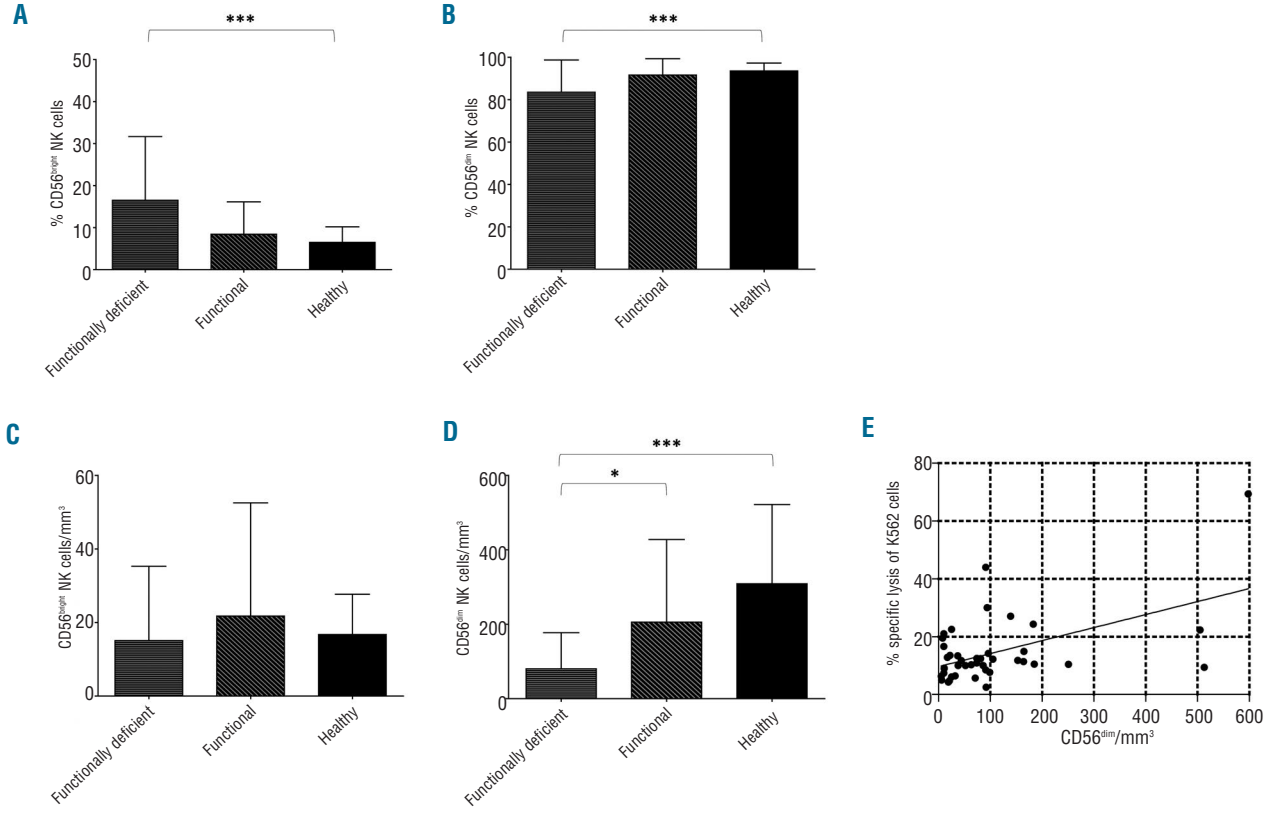

Figure 5. Selective reduction in CD56^{dim} NK cell numbers leading to increased CD56^{bright} frequency in MDS patients. Frequency of (A) CD56^{bright} and (B) CD56^{dim} NK cells, as well as (C) absolute number of CD56^{bright} and (D) CD56^{dim} NK cells were determined in MDS patients with functionally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

absolute numbers of KIR-expressing NK cells, which are essentially a combination of the two "late" stages, were significantly decreased in patients (*data not shown*).

In a further analysis of NK cell maturation states, clonal KIR expression patterns of CD56^{dim} NK cell were analyzed. During NK cell differentiation, KIR expression is regulated in a sequential mode with KIR2DL2/3 representing the first inhibitory KIR; KIR3DL1 and KIR2DL1 are expressed subsequently.[17,18] The analysis revealed a strong decrease of NK cells expressing the "late" KIR2DL1 or KIR3DL1 as their only KIR, whereas the frequencies of the "early" KIR2DL2/3 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, Ct2) revealed 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 where stratified according to KIR genotype, i.e. into patients homozygous for group A haplotypes and those having one or two B haplotypes (B/x; *Online Supplementary Figure S6*). 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 (*Online Supplementary Figure S7*). Furthermore, expression of CD16, a marker of mature NK cells, was significantly decreased in MDS patients (*Online Supplementary Figure S7*). Low expression of CD16

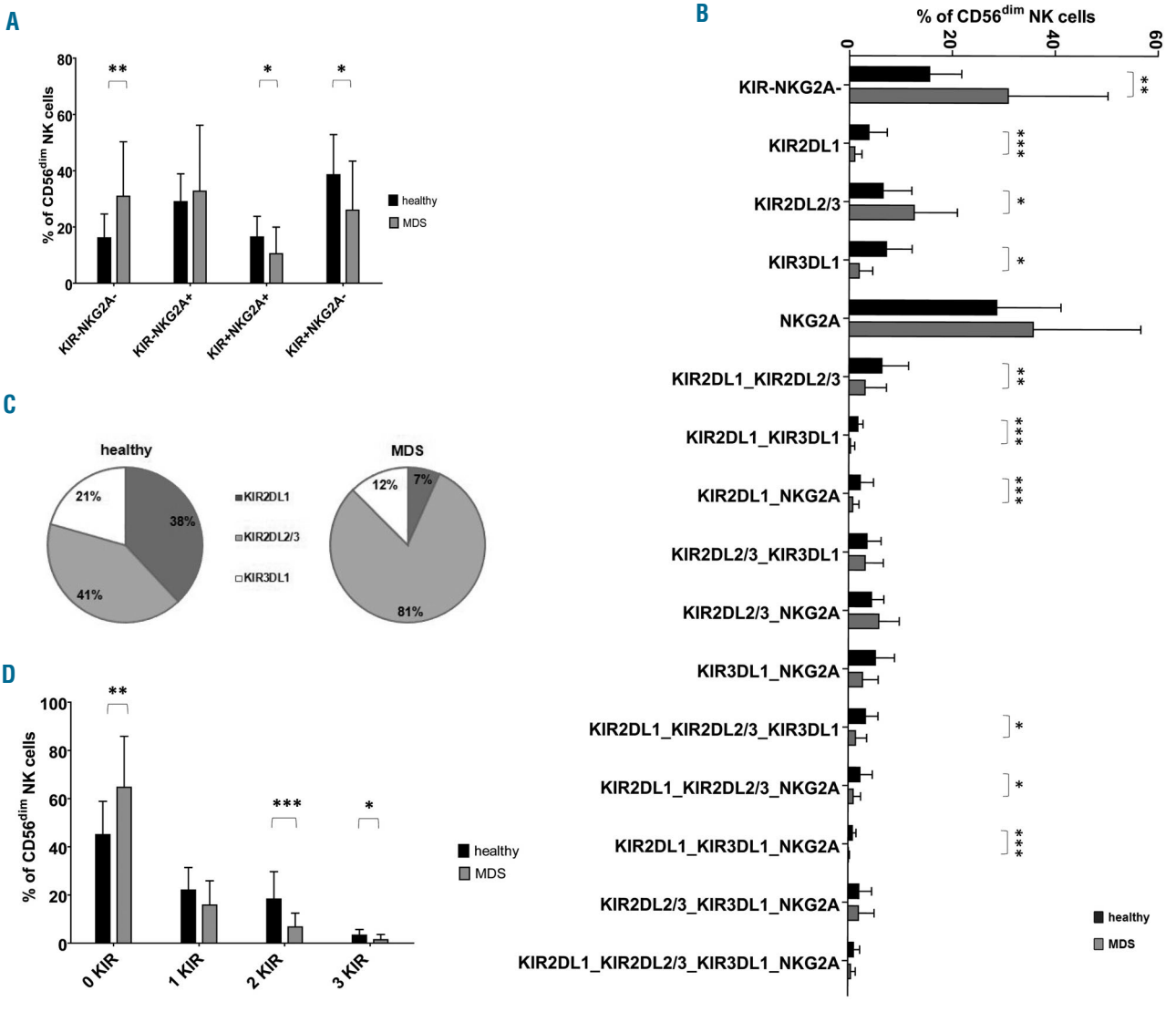

Figure 6. Immaturity of CD56^{dim} NK cells in MDS patients. (**A**) Frequency of four NK cell subpopulations according to expression of KIR and/or NKG2A. (**B**) Frequency of clonal combinations of KIR2DL1, KIR2DL2/3, KIR3DL1, and NKG2A receptors for patients and healthy donors, ordered according to number of expressed receptors. (**C**) Pie charts showing frequency of CD56^{dim} 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 a two-tailed t-test (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

could specifically be attributed to the CD56^{dim} subset of NK cells (*Online Supplementary Figure S8*). Of note, the stimulatory receptors NKG2D and NKp30 were not differentially expressed in either NK subset. Collectively, 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 to what extent lymphopoiesis is also affected by the impaired differentiation capacity of aberrant hematopoietic progenitor cells or a compromised stem cell microenvironment. Several previous studies in MDS found deficient NK cell responses against various autologous or allogeneic tumor targets.[11,12,19,20] The present study suggests that NK cell defects can, in the majority of cases, be attributed to inefficient or defective NK cell differentiation, leading either to a predominantly immature NK cell compartment or to an overall lack of NK cells.

Three different groups of MDS patients could be defined on the basis of NK cell number and function: a small group with normal NK cell numbers and function, a large group of patients with normal or moderately decreased NK cell number and impaired function, and an NK cell-deficient group. This last group of patients, in whom NK cells were rare or undetectable (PBMC: 1-10 NK cells/mm^3), was preferentially found in the high-risk WHO subgroups of refractory anemia with excess blasts I/II and had a significantly elevated IPSS risk score. These observations are in line with those of a recent study that found strongly reduced NK cell frequencies in a high-risk (presumably pretreated) cohort of MDS patients undergoing hematopoietic stem cell transplantation from unrelated donors.[20] 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 of leukemic progression to acute myeloid leukemia. In this regard, NK cells were previously implicated in relapse control following allogeneic hematopoietic stem cell transplantation for myeloid leukemia.[21] Moreover, recent studies have associated certain KIR gene linkage groups with decreased relapse and improved outcome in patients undergoing hematopoietic stem cell transplantation for acute myeloid leukemia.[22] Future analysis of larger MDS cohorts and a longer follow-up will be necessary to show whether 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 understand the underlying mechanisms better, 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) had decreased cytotoxicity despite normal levels of granzyme B and perforin (Figure 3F). In these cases, the cause of the defective function has yet to be elucidated.

The above observations illustrate certain limitations of the CD107 assay, which has emerged in recent years as an established surrogate marker for cytotoxic activity of T and NK cells.[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, NK cells with insufficiently armed granules still mobilized CD107 to the cell surface in MDS patients (*Online Supplementary Figure S9*). These observations might not only be relevant for MDS but also for other hematologic or pathological settings in which NK cell function might be similarly compromised by deficient arming of cytotoxic granules, which would not then be detected by a 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^{bright} and CD56^{dim} subsets was biased towards the immature non-cytotoxic CD56^{bright} subset. Whereas CD56^{bright} frequencies increased, the CD56^{dim} subset was strongly diminished. The CD56^{dim} count significantly correlated with lack of cytotoxicity. Secondly, among CD56^{dim} NK cells, MDS patients had a significant increase in KIR$^-$ NKG2A$^-$ cells. It was previously shown that this CD56^{dim} subset is immature and can be differentiated *in vitro* into the other three subsets in a sequential fashion starting with NKG2A$^+$KIR$^-$ followed by the NKG2A$^+$KIR$^+$ subset and finally NKG2A$^-$KIR$^+$ NK cells.[15] Importantly, KIR$^-$ NKG2A$^-$ cells are not licensed due to their lack of HLA class I-specific inhibitory receptors and thus exhibit poor cytotoxicity.[16,25] In contrast, the more mature KIR-expressing stages KIR$^+$NKG2A$^+$ and KIR$^+$NKG2A$^-$ are less abundant and consequently overall KIR expression was significantly reduced in MDS patients. Thirdly, analysis of clonal KIR expression demonstrated 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.[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 also diminished.[27]

Notably, the above-described phenotypic characteristics resemble those of neonatal NK cells. Like NK cells in MDS, neonatal NK cells have an elevated frequency of CD56^{bright} NK cells, increased expression of NKG2A in the CD56^{dim} subset, and a KIR repertoire biased towards KIR2DL3.[28] A further parallel is that effector functions of neonatal NK cells are initially reduced compared to those of adult NK cells but quickly catch up upon stimulation with interleukin-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^{bright} subset, are actually the opposite of the changes observed in the NK cell compartment during healthy

aging.[29] Thus, although MDS is a disease of the elderly, NK cell repertoires are clearly phenotypically and functionally immature.

The mechanisms leading to the lack of mature, functionally competent NK cells in MDS can only be speculated. Notably, similar analyses of NK cells in patients with acute or chronic myeloid leukemia revealed no such changes of the $CD56^{bright}$ and $CD56^{dim}$ subsets, suggesting that the underlying mechanisms are specific to MDS.[30,31] We did not detect a significant increase in spontaneous apoptosis of $CD56^{dim}$ and $CD56^{bright}$ NK cells in MDS patients, making it unlikely that the observed lack of mature NK cells is due to selective cell death of the $CD56^{dim}$ NK cell subset (*Online Supplementary Figure S5*). 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,[11,32] but not in others.[12] Our preliminary analysis confirmed 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.[17] Moreover, it was recently shown that mesenchymal stem cells from MDS patients have a substantially diminished capacity to support myeloid differentiation across all disease subtypes and that hematopoietic stem and progenitor cells from MDS patients can regain their differentiation potential when cultured on mesenchymal stem cells from healthy controls.[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 interleukin-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 interleukin-2 production.[34] It would be interesting to determine whether immunomodulatory drug treatment is also effective in NK cell-deficient patients. The majority of these patients have a high-risk profile with increased risk of progression to leukemia and might particularly profit from improved NK cell-based immune surveillance. Finally, it should also be considered whether MDS patients could benefit from adoptive NK cell-based immunotherapy. This might be a valuable option for patients with a poor prognosis in order to improve immunosurveillance for leukemic cells as well as for patients with secondary immunodeficiency, e.g. experiencing recurrent viral infections.

Acknowledgments

We would like to thank all individuals who voluntarily donated blood for this study. We also thank Dr. Jürgen Rack for providing samples from healthy age-matched donors. This work was supported by funds from the Deutsche José Carreras Leukämie-Stiftung e.V. (to NG and MU), by the Deutsche Krebshilfe (to UG, NG, RH, and MU) and the Deutsche Forschungsgemeinschaft (research grant UH 91/7-1 to MU).

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

References

1. Germing U, Kobbe G, Haas R, et al. Myelodysplastic syndromes: diagnosis, prognosis, and treatment. Dtsch Arztebl Int. 2013;110(46):783-790.
2. Campo E, Swerdlow SH, Harris NL, et al. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. Blood. 2011;117(19):5019-5032.
3. Neukirchen J, Schoonen WM, Strupp C, et al. Incidence and prevalence of myelodysplastic syndromes: data from the Dusseldorf MDS-registry. Leuk Res. 2011;35(12):1591-1596.
4. Greenberg P, Cox C, LeBeau MM, et al. International scoring system for evaluating prognosis in myelodysplastic syndromes. Blood. 1997;89(6):2079-2088.
5. Barrett AJ, Sloand E. Autoimmune mechanisms in the pathophysiology of myelodysplastic syndromes and their clinical relevance. Haematologica. 2009;94(4):449-451.
6. Velardi A. Natural killer cell alloreactivity 10 years later. Curr Opin Hematol. 2012;19(6):421-426.
7. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. Nat Immunol. 2008;9:495-502.
8. Kärre K, Ljunggren HG, Piontek G, et al. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. Nature. 1986;319(6055):675-678.
9. Moretta A, Locatelli F, Moretta L. Human NK cells: from HLA class I-specific killer Ig-like receptors to the therapy of acute leukemias. Immunol Rev. 2008;224:58-69.
10. Chamuleau ME, Westers TM, van Dreunen L, et al. Immune mediated autologous cytotoxicity against hematopoietic precursor cells in patients with myelodysplastic syndrome. Haematologica. 2009;94(4):496-506.
11. Kiladjian JJ, Bourgeois E, Lobe I, et al. Cytolytic function and survival of natural killer cells are severely altered in myelodysplastic syndromes. Leukemia. 2006;20(3):463-470.
12. Epling-Burnette PK, Bai F, Painter JS, et al. Reduced natural killer (NK) function associated with high-risk myelodysplastic syndrome (MDS) and reduced expression of activating NK receptors. Blood. 2007;109(11):4816-4824.
13. Carlsten M, Baumann BC, Simonsson M, et al. Reduced DNAM-1 expression on bone marrow NK cells associated with impaired killing of CD34+ blasts in myelodysplastic syndrome. Leukemia. 2010;24(9):1607-1616.
14. Nagler A, Lanier LL, Cwirla S, et al. Comparative studies of human FcRIII-positive and negative natural killer cells. J Immunol. 1989;143(10):3183-3191.
15. Juelke K, Killig M, Thiel A, et al. Education of hyporesponsive NK cells by cytokines. Eur J Immunol. 2009;39(9):2548-2555.
16. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity. 2006;25(2):331-342.
17. Miller JS, McCullar V. Human natural killer cells with polyclonal lectin and immunoglobulinlike receptors develop from single hematopoietic stem cells with preferential expression of NKG2A and KIR2DL2/L3/S2. Blood. 2001;98(3):705-713.
18. Fischer JC, Ottinger H, Ferencik S, et al. Relevance of C1 and C2 epitopes for hemopoietic stem cell transplantation: role for sequential acquisition of HLA-C-specific inhibitory killer Ig-like receptor. J Immunol. 2007;178(6):3918-3923.
19. Porzsolt F, Heimpel H. Natural killer cell activity in preleukaemia. Lancet. 1982;1(8269):449.
20. Gleason MK, Ross JA, Warlick ED, et al. CD16xCD33 bispecific killer cell engager (BiKE) activates NK cells against primary MDS and MDSC CD33+ targets. Blood. 2014; 123(19):3016-3026.

21. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science. 2002;295(5562):2097-2100.
22. Cooley S, Weisdorf DJ, Guethlein LA, et al. Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia. Blood. 2010;116(14):2411-2419.
23. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. J Immunol Methods. 2003;281(1-2):65-78.
24. Uhrberg M. The CD107 mobilization assay: viable isolation and immunotherapeutic potential of tumor-cytolytic NK cells. Leukemia. 2005;19(5):707-709.
25. Cooley S, Xiao F, Pitt M, et al. A subpopulation of human peripheral blood NK cells that lacks inhibitory receptors for self-MHC is developmentally immature. Blood. 2007;110(2):578-586.
26. Schonberg K, Sribar M, Enczmann J, et al. Analyses of HLA-C-specific KIR repertoires in donors with group A and B haplotypes suggest a ligand-instructed model of NK cell receptor acquisition. Blood. 2011;117(1):98-107.
27. Bjorkstrom NK, Riese P, Heuts F, et al. Expression patterns of NKG2A, KIR, and CD57 define a process of CD56dim NK-cell differentiation uncoupled from NK-cell education. Blood. 2010;116(19):3853-3864.
28. Schonberg K, Fischer JC, Kogler G, et al. Neonatal NK-cell repertoires are functionally, but not structurally, biased toward recognition of self HLA class I. Blood. 2011;117(19):5152-5156.
29. Le Garff-Tavernier M, Beziat V, Decocq J, et al. Human NK cells display major phenotypic and functional changes over the life span. Aging Cell. 2010;9(4):527-535.
30. Stringaris K, Sekine T, Khoder A, et al. Leukemia-induced phenotypic and functional defects in natural killer cells predict failure to achieve remission in acute myeloid leukemia. Haematologica. 2014;99(5):836-847.
31. Chen CI, Koschmieder S, Kerstiens L, et al. NK cells are dysfunctional in human chronic myelogenous leukemia before and on imatinib treatment and in BCR-ABL-positive mice. Leukemia. 2012;26(3):465-474.
32. Miura I, Kobayashi Y, Takahashi N, et al. Involvement of natural killer cells in patients with myelodysplastic syndrome carrying monosomy 7 revealed by the application of fluorescence in situ hybridization to cells collected by means of fluorescence-activated cell sorting. Br J Haematol. 2000;110(4):876-879.
33. Geyh S, Oz S, Cadeddu RP, et al. Insufficient stromal support in MDS results from molecular and functional deficits of mesenchymal stromal cells. Leukemia. 2013;27(9):1841-1851.
34. Hayashi T, Hideshima T, Akiyama M, et al. Molecular mechanisms whereby immunomodulatory drugs activate natural killer cells: clinical application. Br J Haematol. 2005;128(2):192-203.