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Role of natural killer cell subsets and natural cytotoxicity receptors for the outcome of immunotherapy in acute myeloid leukemia

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In a phase IV trial, 84 patients (age 18–79) with acute myeloid leukemia (AML) in first complete remission (CR) received cycles of immunotherapy with histamine dihydrochloride (HDC) and low-dose human recombinant interleukin 2 (IL-2) for 18 months to prevent leukemic relapse. During cycles, the treatment resulted in expansion of $CD56^{bright}$ ($CD3^{-}/16^{-}/56^{bright}$) and $CD16^{+}$ ($CD3^{-}/16^{+}/56^{+}$) natural killer (NK) cells in the blood along with increased NK cell expression of the natural cytotoxicity receptors (NCRs) NKp30 and NKp46. Multivariate analyses correcting for age and risk group demonstrated that high $CD56^{bright}$ NK cell counts and high expression of NKp30 or NKp46 on $CD16^{+}$ NK cells independently predicted leukemia-free survival (LFS) and overall survival (OS). Our results suggest that the dynamics of NK cell subsets and their NCR expression may determine the efficiency of relapse-preventive immunotherapy in AML.

Introduction

AML is characterized by the rapid accumulation of abnormal myeloid cells in the bone marrow and other organs. In the United States, AML is the most common form of acute leukemia in adults with 20,000 estimated new cases in 2015.[1] The current therapy comprises induction chemotherapy aiming at reducing the burden of the leukemia to microscopically undetectable levels (CR) and consolidation chemotherapy aiming at eradicating residual malignant cells. Younger AML patients with high-risk disease may also be subjected to allogeneic stem cell transplantation, which is usually performed in the post-consolidation phase. Despite advances in AML therapy in recent decades, the 5-year survival rate is in the range of 25–40%, which is significantly explained by a high incidence of leukemic relapse.[2-5] Novel therapies, including strategies to prevent relapse in the post-chemotherapy phase, are highly warranted.

Myeloid leukemic cells are frequently susceptible to the cytotoxicity of NK cells. The results of clinical studies imply that a deficient NK cell-mediated cytotoxicity and reduced NK cell counts in the blood are common in AML and that NK cell functions may determine the risk of relapse and survival.[6-14] The claim that NK cell function is relevant to the course of AML is further bolstered by reports of a low incidence of relapse in allo-transplanted patients with donor/recipient mismatches of killer immunoglobulin-like receptors (KIR) and HLA, whose leukemic cells are incapable of inhibiting alloreactive NK cells via KIR/HLA interactions[15,16] (reviewed in ref. 17).

Human NK cells comprise two main cellular phenotypes that differ in function and in their expression of the cell surface markers CD16 and CD56.[18] In healthy subjects, 90–95% of blood NK cells are $CD16^{+}/56^{+}$ (here referred to as $CD16^{+}$ NK cells) and 5–10% are $CD16^{-}/56^{bright}$ cells ($CD56^{bright}$ NK cells). $CD16^{+}$ NK cells are cytotoxic to several histiotypes of malignant cells, including myeloid leukemic cells, whereas $CD56^{bright}$ NK cells are only weakly cytotoxic and are widely accepted to be precursors to the cytotoxic $CD16^{+}$ NK cells.[19-22] NK cell cytotoxicity is regulated by activating and inhibitory NK cell receptors and their cognate ligands on leukemic cells.[23-26] The main inhibitory receptors encompass the family of KIRs and the NKG2A – CD94 heterodimer, whereas the main activating receptors comprise the NCRs (NKp46, NKp30, and NKp44) and NKG2D.[23-26]

The insufficiency of NK cells in AML and the purported prognostic role of NK cells have inspired the design of NK cell-based immunotherapies, including the use of NK cell-activating

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cytokines and the adoptive transfer of NK cells, for relapse prevention.[13,27] In a phase III trial, immunotherapy with histamine dihydrochloride and low-dose interleukin-2 (HDC/IL-2) was shown to prevent relapse in patients with AML who had achieved CR and completed the phase of consolidation chemotherapy.[28] The IL-2 component of this regimen aims at promoting antitumor functions of NK cells, whereas the HDC component aims at reducing the production of immunosuppressive reactive oxygen species (ROS) from malignant and non-malignant myeloid cells, and thus at rescuing NK cells from ROS-induced inactivation and apoptosis.[29-32] *In vitro* and *in vivo* studies imply that NK cells are essential for the anti-neoplastic efficacy of HDC, used as a single agent or in conjunction with IL-2[31,33,34]; however, a systematic analysis of the immunomodulatory properties of treatment with HDC/IL-2 within the framework of a clinical trial has not been carried out.

Here we report the results of a phase IV trial (Re:Mission trial; NCT01347996) in patients with AML who received immunotherapy with HDC/IL-2 for relapse control. Our results suggest that subsets of NK cells and their NCR expression are induced during immunotherapy and that these aspects of NK cell biology herald favorable outcome in terms of relapse risk and survival.

Results

Immunotherapy with HDC/IL-2 triggers accumulation of NK cell subsets in the blood

AML patients in first CR received 10 consecutive 3-week cycles of HDC/IL-2 in the post-consolidation phase. Peripheral blood was collected before and after treatment cycles 1 and 3, and analyzed for NK cell content and phenotype. The treatment schema is outlined in **Fig. 1**. Treatment with HDC/IL-2 induced a 3-fold increase in the absolute number of blood NK cells during cycle 1. An increment of NK cell counts was observed in 46/47 evaluable patients. The expanded NK cells comprised $CD56^{bright}$ and $CD16^{+}$ NK cells (**Fig. 2**, with gating strategies shown in **Fig. S1**). NK cell numbers in the blood declined between the end of treatment cycle 1 and the start of cycle 3. $CD56^{bright}$ cell counts typically returned to pre-treatment levels, whereas $CD16^{+}$ NK cell counts remained modestly elevated at the onset of cycle 3 as compared with levels at the onset of cycle 1 ($p < 0.01$, paired t-test; $n = 46$). A renewed accumulation of $CD56^{bright}$ and $CD16^{+}$ NK cells in the blood was observed during treatment cycle 3 (**Fig. 2**).

Induction of NCR expression on NK cells

During cycle 1 the median fluorescence intensity (MFI) of NKp30 and NKp46 expressed by $CD16^{+}$ NK cells increased by 30% and 50%, respectively (**Figs. 3B, D**). The MFI of NCRs on $CD16^{+}$ NK cells increased during cycle 1 in 50/56 evaluable patients for NKp30 and in 43/56 patients for NKp46. For $CD56^{bright}$ cells, the results were discordant with a pronounced induction of NKp30 and no induction of NKp46 during cycle 1 (**Figs. 3A, C**). As was observed for NK cell counts, the expression intensity of these NCRs declined during the resting period between cycles. However, for $CD56^{bright}$ cells the NKp30 expression remained significantly elevated at the onset of cycle 3 compared with levels at the onset of cycle 1 and was significantly higher at the end of cycle 3 than at the end of cycle 1 ($P < 0.05$, paired t-test; $n = 42$). The NKp30 expression on $CD56^{bright}$ cells, as well as the NCR expression on $CD16^{+}$ NK cells, increased during cycle 3 to an extent similar to that recorded during cycle 1 (**Fig. 3**).

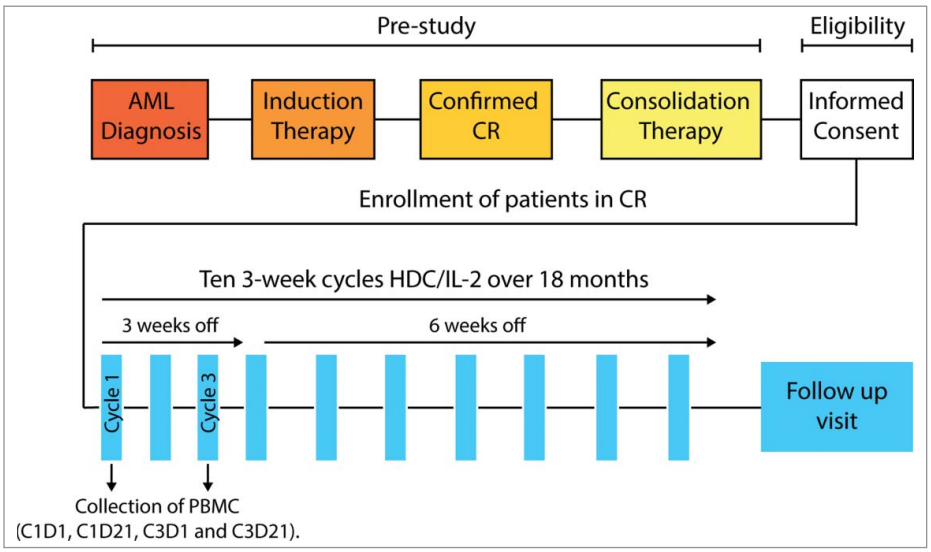

Figure 1. Overview of the Re:Mission phase IV trial. AML patients in first complete remission (CR) were evaluated for eligibility after induction and consolidation chemotherapy. Eligible patients received 3-week cycles of HDC/IL-2 over 18 months. Peripheral blood mononuclear cells (PBMC) were collected before and after cycles 1 and 3. The protocol specified additional follow-up for 6 months after completing the last treatment cycle.

Impact of NK cell counts and NCR expression on LFS and OS

In analyses of the impact of NK cell markers on clinical outcome (reflected by leukemia-free survival, LFS, and overall survival, OS), patients were dichotomized at the median with respect to NK cell counts in the blood or NCR expression (MFI) on NK cells and analyzed for LFS and OS using the logrank test. Hazard ratios were determined by univariate Cox regression analyses and multivariate Cox analyses adjusted for age and risk groups. There was a strong correlation between relapse and death – one patient (59 years old) died without a preceding relapse (from myocardial infarction at 19 months after the onset of treatment) – emphasizing that relapse is a major risk factor for death for AML patients in CR.[35,36]

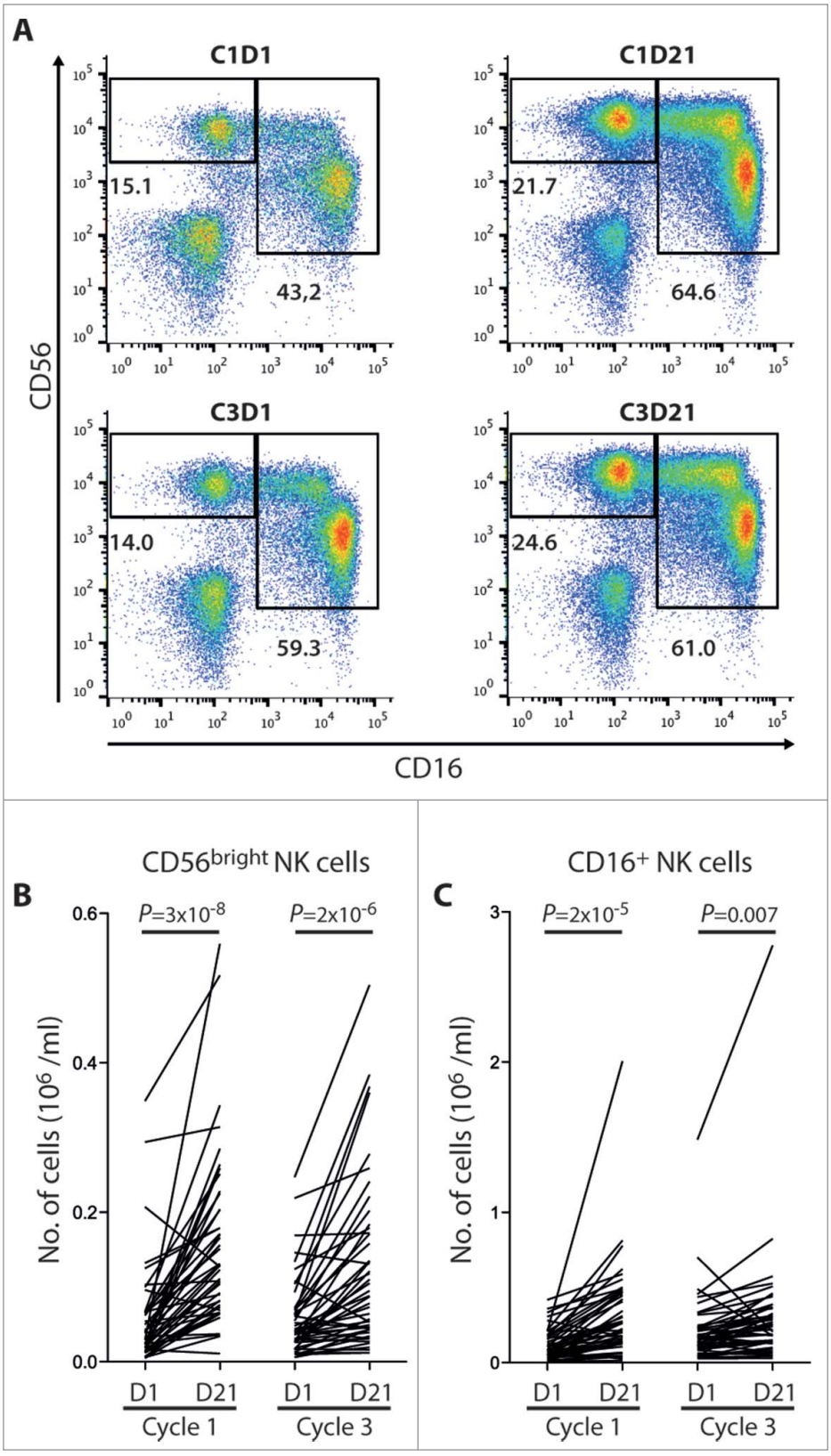

Figure 2. Induction of NK cell subsets in the blood during immunotherapy with HDC/IL-2. (**A**) shows representative dot plots of the expression intensity of CD16 and CD56 on $CD3^-$ cells in samples obtained at the onset of cycle 1 (C1D1), at the end of cycle 1 (C1D21), at the onset of cycle 3 (C3D1) and at the end of cycle 3 (C3D21). Figures indicate the percentage of $CD56^{bright}$ and $CD16^+$ NK cells of CD3- lymphocytes. (**B and C**) show blood counts of $CD56^{bright}$ and $CD16^+$ NK cells before and after cycles 1 and 3. Treatment with HDC/IL-2 increased blood counts of $CD56^{bright}$ cells and $CD16^+$ NK cells during cycle 1 ($n = 47$) and cycle 3 ($n = 46$; Student's paired *t*-test).

At the onset of the first cycle of therapy, high counts of $CD56^{bright}$ NK cells predicted LFS, which translated into an improved OS (**Figs. 4A, B** with multivariate analysis shown in **Table 1**). In contrast, $CD16^+$ NK cell blood counts at onset of cycle 1 did not influence outcome (**Fig. 4C**). Also, LFS and OS were not significantly impacted by the magnitude of the increment of $CD56^{bright}$ or $CD16^+$ NK cell counts during the first cycle of therapy (data not shown) or by the absolute counts of these NK cell populations on day 21 of cycle 1 (**Figs. 4D–F**).

A high (above median) expression of NKp46 on $CD16^+$ NK cells at the onset of cycle 1 was associated with a favorable outcome (**Figs. 5A–C** and **Table 1**). The magnitude of NCR induction during cycle 1 did not predict LFS or OS. However, patients with high NKp46 MFI on $CD16^+$ NK cells on day 21 of cycle 1 showed a favorable outcome (**Fig. 5E, F** and **Table 1**). A similar trend was observed for patients with high NKp30 expression before and after cycle 1 (**Fig. 5D**). There were also trends toward a favorable outcome in patients with high NKp30 and NKp46 expression on $CD56^{bright}$ NK cells (**Fig. S2**).

Treatment-induced accumulation of $CD16^+$ NK cells predicts outcome

At the onset of therapy, there was a skewed ratio of $CD56^{bright}$ cells to $CD16^+$ cells in favor of $CD56^{bright}$ cells (median ratio = 0.58 on cycle 1, day 1 vs. <0.1 in healthy subjects), which is in agreement with previous studies.[37,38] The increase of $CD16^+$ NK cell counts between the onset of treatment in cycle 1 and the start of cycle 3 resulted in a shift of the distribution of NK cell subtypes in blood toward a

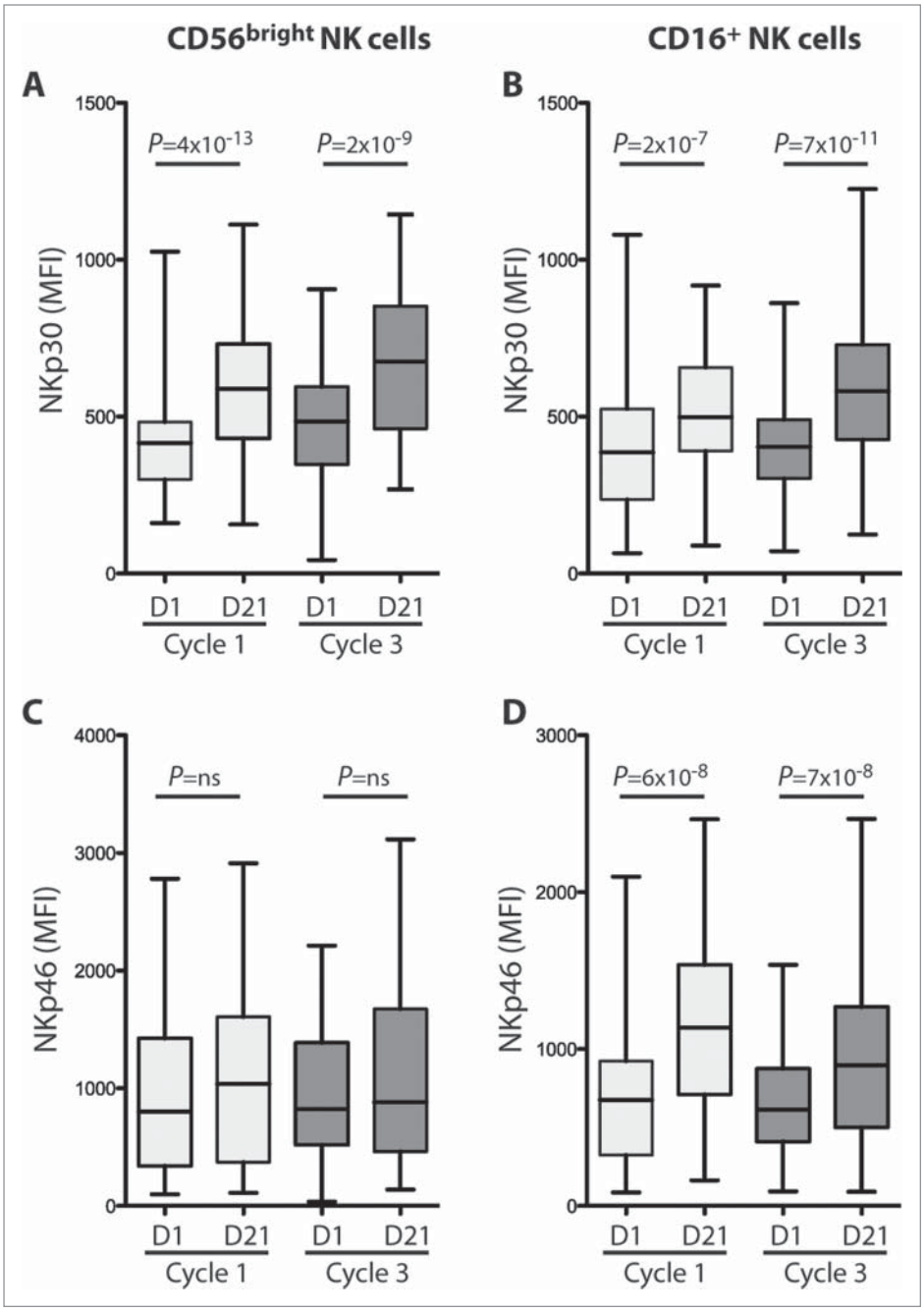

Figure 3. Induction of NCRs during immunotherapy with HDC/IL-2. The box plots show the median fluorescence intensity (MFI) of NKp30 and NKp46 expression on $CD56^{bright}$ (**A** and **C**) and $CD16^{+}$ NK cells (**B** and **D**) at indicated time points (D1 = day 1). NKp30 was induced in both NK cell subsets during cycle 1 ($n = 56$) and during cycle 3 ($n = 48$). NKp46 was significantly up-regulated on $CD16^{+}$ NK cells during both cycles, while no significant induction of NKp46 was observed on $CD56^{bright}$ cells (Student's paired *t*-test).

normalized ratio of $CD56^{bright}$ to $CD16^{+}$ NK cells between cycle 1, day 1 and cycle 3, day 1 (0.58 vs. 0.28, median ratios, $p = 0.001$, signed rank test, $n = 46$). When assessing the induction of $CD16^{+}$ NK cells in relapsing and non-relapsing patients followed for at least 18 months, a significant induction was observed only in patients who remained in remission (**Fig. 6A**). In line with this finding, there was a trend toward a favorable LFS and OS in patients with a high maintained level of $CD16^{+}$ NK cells at the onset of cycle 3 (**Figs. 6B, C** and **Table 1**).

***Post-hoc* analyses of lymphocyte increment vs. LFS in a phase III trial**

In an attempt to confirm that $CD16^{+}$ NK cells accumulated in the blood between the onset of cycles 1 and 3, we performed *post-hoc* analyses of the results of a phase III trial (M0201 trial) that compared outcomes in AML patients in CR who were randomly assigned to receive HDC/IL-2 ($n = 160$) or standard-of-care ($n = 160$).[28] Lymphocyte counts in the blood, but not lymphocyte phenotypes, were captured before and during treatment cycles. In the phase III trial, the absolute lymphocyte counts increased between onset of treatment (C1D1) and the start of cycle 3 in HDC/IL-2-treated patients ($p = 0.004$), but not in untreated control patients (**Fig. 6D**).[39] The increment of lymphocyte counts between treatment cycles in this phase III trial likely reflected an accumulation of $CD16^{+}$ NK cells, since $CD16^{+}$ NK cells accounted for the majority (median 53%) of the lymphocyte increment between the onset of cycles 1 and 3 in the current phase IV Re:Mission trial using the identical treatment regimen. In agreement with the results in the Re:Mission trial, the lymphocyte induction between the onset of treatment and the start of cycle 3 in HDC/IL-2 treated patients in the phase III trial was predictive of LFS and OS (**Figs. 6E, F**). No such correlations were observed in the corresponding control patients ($p > 0.5$, data not shown).

Discussion

The purported role of NK cells for the outcome of AML has inspired attempts at pharmacologically enhancing NK cell functions to improve clearance of leukemic cells and thus preventing relapse. Such immunotherapy seems intuitively attractive for patients in CR who have completed consolidation chemotherapy and thus carry a minimal burden of leukemia. However, most previous attempts to boost the functions

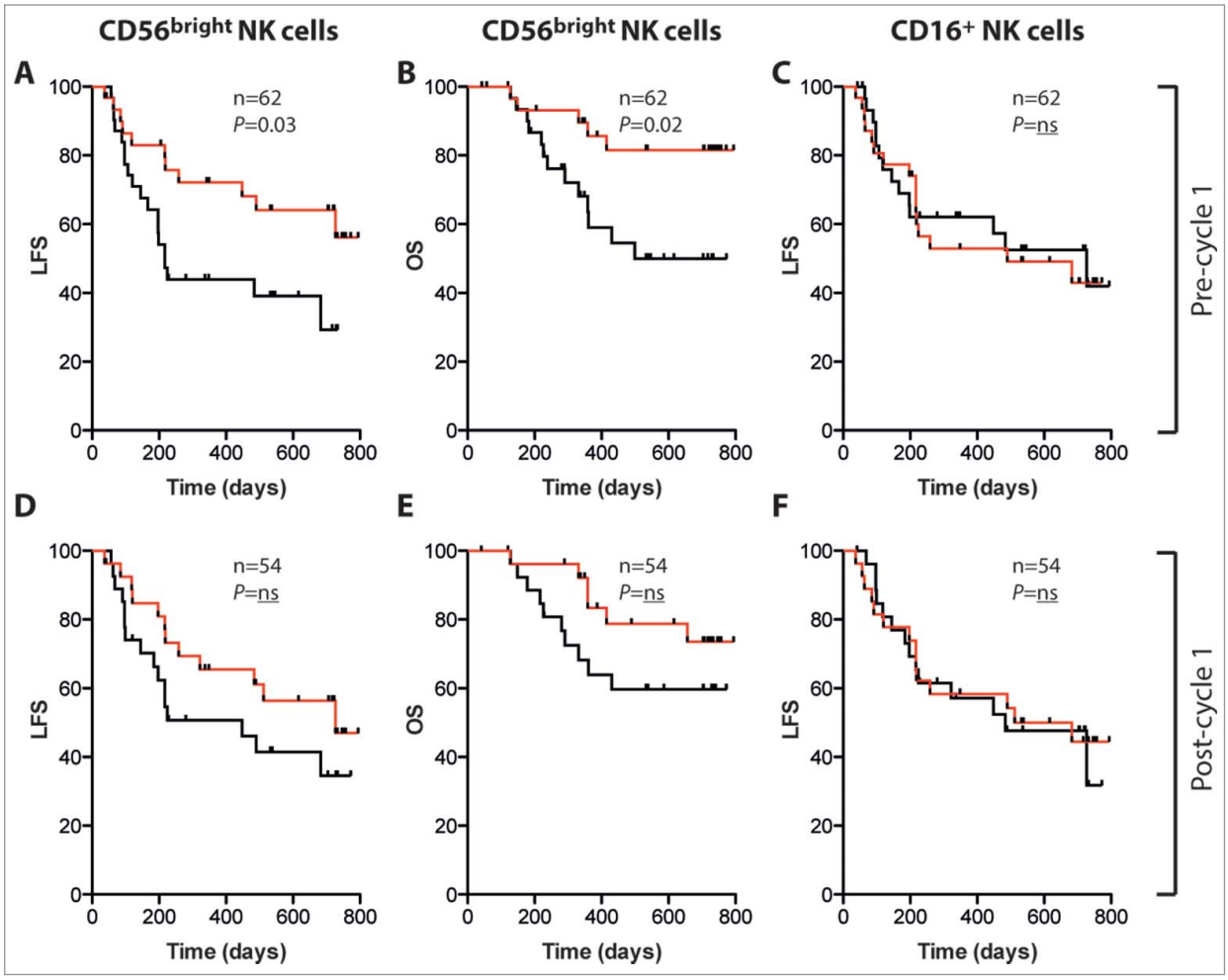

Figure 4. Impact of NK cell subsets on LFS and OS in AML patients receiving HDC/IL-2. Patients were dichotomized based on above (red) or below (black) median blood cell counts of CD56^{bright} (**A, B** and **D, E**) or CD16^{+} (**C** and **F**) NK cells before or after one cycle of treatment and analyzed with regard to LFS (**A**, **C**, **D** and **F**) and OS (**B** and **E**). LFS and OS were analyzed using the logrank test.

of endogenous NK cells for relapse prevention in AML have been unsuccessful. For example, monotherapy with IL-2, a prototypic NK cell-activating cytokine, in the post-consolidation phase of AML did not significantly prevent relapse or prolong the duration of CR in any of six randomized trials.[40-45] The inefficiency of IL-2 monotherapy to prevent relapse is supported by the results of meta-analyses comprising a total of >1,400 IL 2-treated patients.[36,46]

HDC is assumed to improve the anti-leukemic efficiency of NK cell activators by targeting immunosuppressive ROS.[29-32]

Table 1. Multivariate analysis of LFS and OS. LFS or OS in patients with above or below median values of each variable as determined by univariate and multivariate Cox regression analyses. In the multivariate analyses, hazard ratios were corrected for age and risk group classification.

	Univariate analysis			Multivariate analysis		
Variable	**Hazard ratio**	**Confidence interval**	**p-value**	**Hazard ratio**	**Confidence interval**	**p-value**
No. of CD56^{bright} NK, C1D1, LFS	0.44	0.20-0.94	0.033	0.40	0.15-1.02	0.053
No. of CD56^{bright} NK, C1D1, OS	0.31	0.10-0.87	0.025	0.22	0.06-0.74	0.014
NKp30 expression CD16^{+} NK, C1D1, LFS	0.58	0.27-1.21	0.15	0.32	0.13-0.78	0.011
NKp46 expression CD16^{+} NK C1D1, LFS	0.46	0.22-0.97	0.040	0.41	0.18-0.89	0.024
NKp46 expression CD16^{+} NK C1D1, OS	0.26	0.09-0.75	0.012	0.26	0.08-0.75	0.013
NKp46 expression CD16^{+} NK, C1D21, LFS	0.33	0.15-0.69	0.003	0.34	0.15-0.75	0.007
NKp46 expression CD16^{+} NK, C1D21, OS	0.22	0.07-0.62	0.004	0.29	0.09-0.88	0.028
Induction of CD16^{+} NK, C1D1-C3D1, LFS	0.34	0.12-0.92	0.032	0.38	0.12-1.20	0.098

LFS or OS in patients with above or below median values of each variable as determined by univariate and multivariate Cox regression analyses. In the multivariate analyses, hazard ratios were corrected for age and risk group classification.

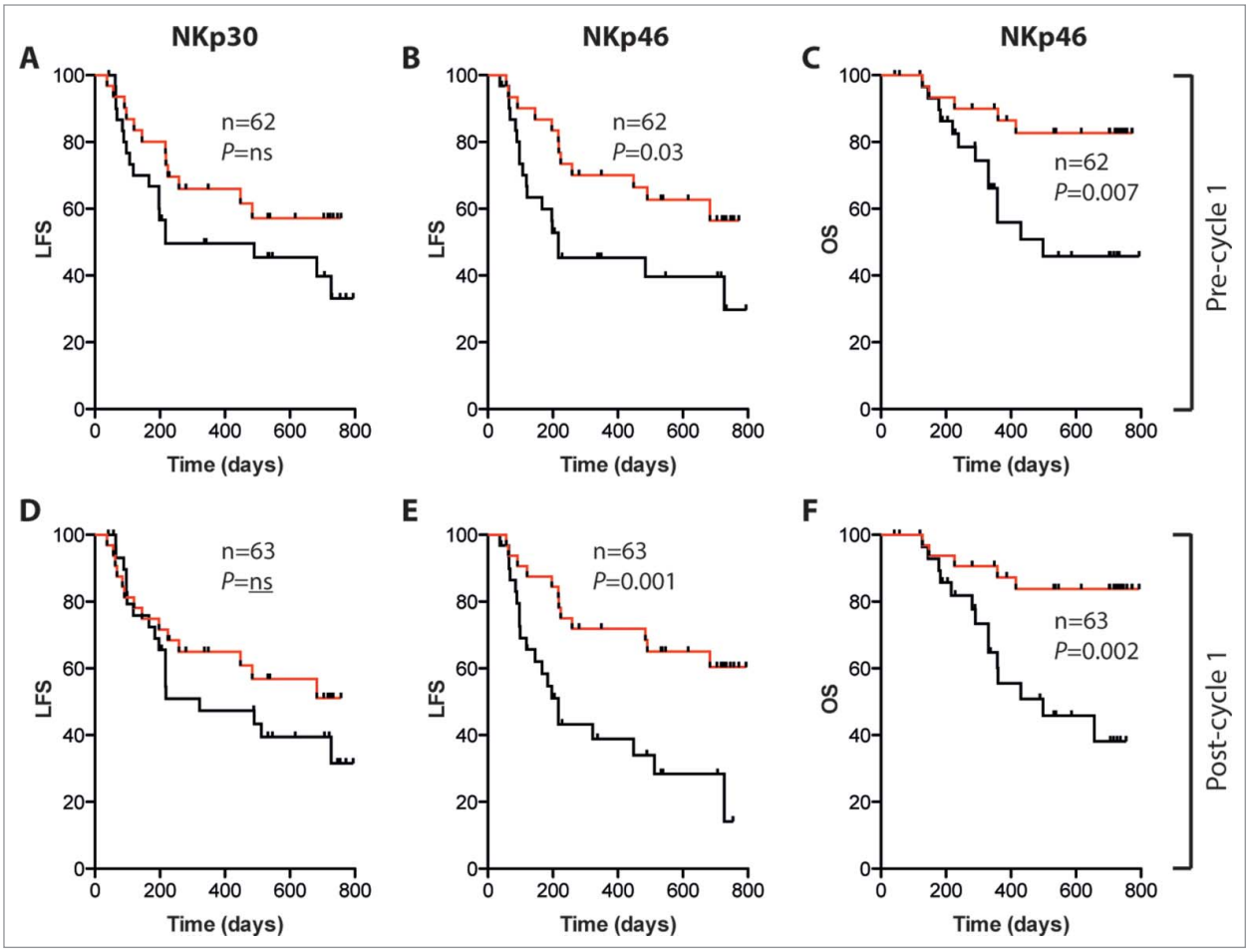

Figure 5. Impact of NCR expression on LFS and OS in AML patients receiving HDC/IL-2. Patients were dichotomized based on above (red) or below (black) median expression (MFI) of NKp30 (**A** and **D**) or NKp46 (**B**, **C**, **E** and **F**) on $CD16^+$ NK cells before or after first treatment cycle. LFS and OS were analyzed using the logrank test.

These toxic oxygen derivatives, which are formed by the NADPH oxidase (NOX2) of several subsets of myeloid cells, are pivotal effector molecules in anti-microbial defense but have also been ascribed a role as negative regulators of cellular immunity by inducing dysfunction and apoptosis in adjacent NK cells and T cells.[30,31,47] HDC blocks the activity of NOX2 by targeting H_2-type histamine receptors. The resulting reduction of extracellular release of ROS from myeloid cells rescues NK cells from inhibition and apoptosis and thus promotes activation of anti-leukemic properties of IL-2 and other NK cell-activating compounds.[29-31] The synergy between IL-2 and HDC in activating NK cells *in vitro* [31,48] and in reducing the growth of NK cell-sensitive tumors in murine models *in vivo* [33] formed the basis for the evaluation of the clinical efficacy of HDC/IL-2 in several forms of cancer.[49,50] A phase III trial with 320 non-transplanted AML patients in CR in the post-consolidation phase showed a significantly improved LFS and a reduced incidence of relapse among patients randomly assigned to receive HDC/IL-2 vs. standard-of-care, in particular among patients in their first CR.[28] This regimen has not yet been evaluated in patients who have not achieved CR.

A main finding in the present study was that the administration of HDC/IL-2 to patients with AML pronouncedly augmented NK cell counts in the blood. The induction of NK cell counts was noted during each evaluated 21-d cycle and comprised NK cells of the $CD16^+$ and $CD56^{bright}$ phenotypes. Attempts to define the impact of NK cell phenotypes in the blood on LFS and OS revealed that above-median counts of $CD56^{bright}$ NK cells at onset, but not at later time points, predicted a favorable outcome as did treatment-induced accumulation of $CD16^+$ NK cells between the start of cycle 1 and start of cycle 3. The validity of the latter finding was supported by *post-hoc* analyses of the results of a previous phase III trial, where induction of lymphocytes between cycles 1 and 3 was significantly associated with LFS and OS in patients randomized to receive HDC/IL-2 but not in untreated control patients.[39] Since $CD56^{bright}$ NK cells are assumed to be the immediate precursors of $CD16^+$ NK cells,[19-22] we hypothesize from these findings that the anti-leukemic efficacy of HDC/IL-2 immunotherapy results, at least in part, from initial activation and expansion of $CD56^{bright}$ NK cells that subsequently differentiate into cytotoxic $CD16^+$ cells. The finding of a

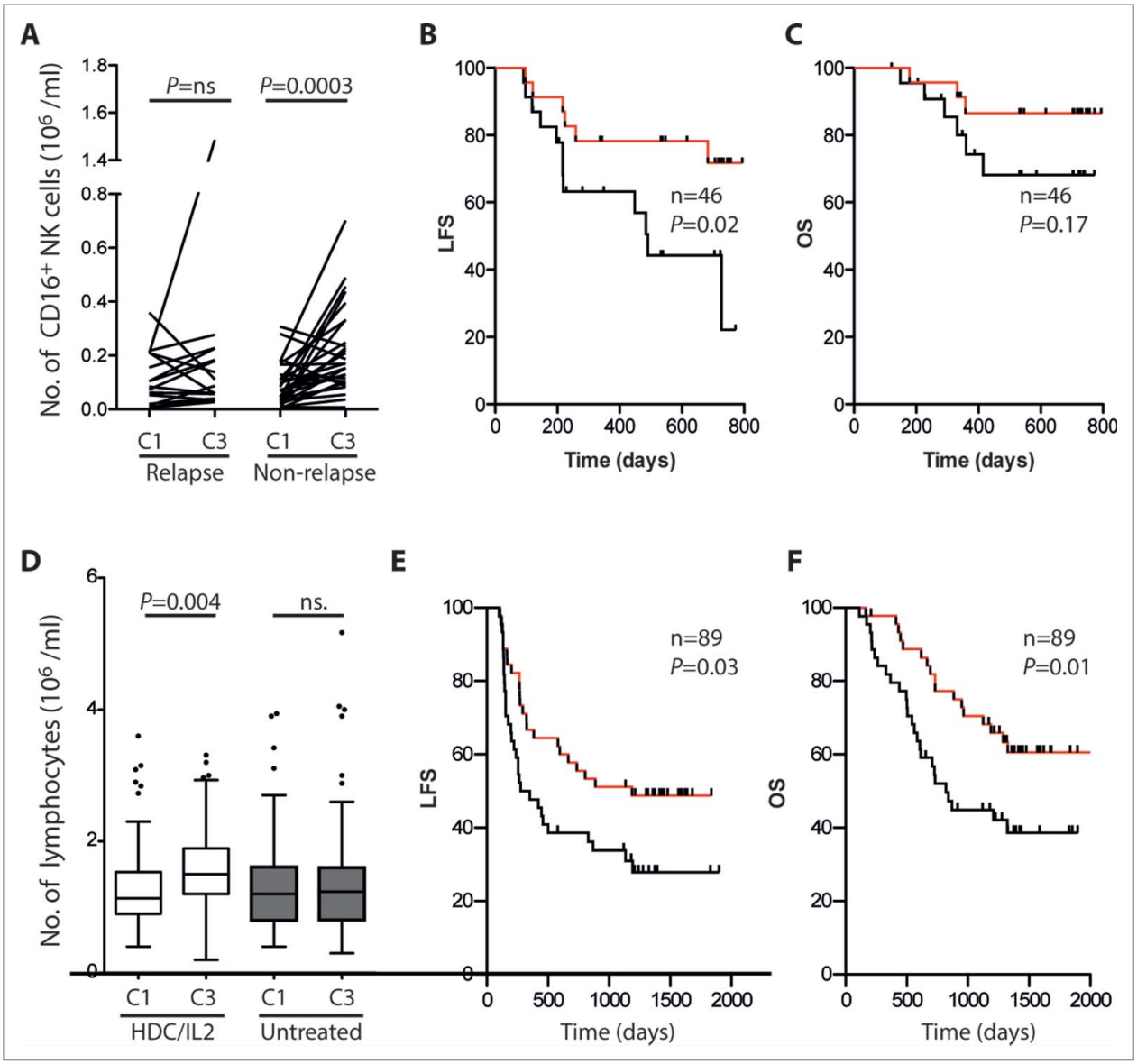

Figure 6. Impact of sustained expansion of CD16$^+$ NK cells on LFS and OS. (**A**) shows the absolute blood counts of CD16$^+$ NK cells on day 1 of cycles 1 and 3 (C1D1 and C3D1) in relapsing ($n = 16$) and non-relapsing ($n = 16$) patients followed for more than 18 months. In (**B**) and (**C**), patients were dichotomized based on above (red) or below (black) the median induction of CD16$^+$ NK cells between C1D1 and C3D1. LFS (**B**) and OS (**C**) were analyzed using the logrank test. Panels (**D**)–(**F**) show *post-hoc* results from a phase III AML trial (0201), where patients in CR were randomized to receive HDC/IL-2 or no treatment (control). (**D**) shows lymphocyte counts at the onset of treatment and at the onset of cycle 3 (C1D1 and C3D1) in HDC/IL-2-treated patients and controls, analyzed using the Student's paired *t*-test. In (**E**) and (**F**), HDC/IL-2-treated patients were dichotomized based on above (red) or below (black) median induction of lymphocytes between C1D1 and C3D1. LFS (**E**) and OS (**F**) were analyzed using the logrank test.

significantly increased ratio of CD16$^+$ to CD56^{bright} NK cell counts between the first days of cycles 1 and 3 supports that CD56^{bright} cells may have differentiated into cytotoxic CD16$^+$ cells during immunotherapy.

The expression of NKp30 on subsets of NK cells during treatment cycles largely paralleled the induction of NK cell counts in blood. Thus, the MFI of NKp30 was markedly increased during cycles 1 and 3 on CD16$^+$ NK cells as well as on CD56^{bright} cells, and the NKp30 expression remained modestly elevated on CD56^{bright} NK cells at the onset of cycle 3 when compared with the level of expression at the start of therapy. NKp46 expression was strongly induced on CD16$^+$ cells but not on CD56^{bright} cells and no between-cycles induction of NKp46 was observed in any of the NK cell subtypes. Despite these differences in the dynamics of NCR expression during immunotherapy, a high expression of NKp30 or NK46 on CD16$^+$ NK cells at onset and/or after the first cycle of therapy was positively and significantly associated with LFS and OS. The validity of these findings is bolstered

by previous studies showing that newly diagnosed AML patients frequently show deficient expression of NCRs with negative impact on OS.[7]

In our study, we did not observe significant correlations between outcome and in-cycle induction of NCR expression when analyzing the entire study population, and it thus remains uncertain whether the favorable outcome in patients with high NCR expression was related to the immunotherapy or a reflection of variable baseline NCR expression among patients. However, *post-hoc* analyses of patients with low NCR expression at onset of therapy supported that treatment-induced NCR expression was associated with a favorable outcome. In this subgroup, induction of NKp46 expression on $CD56^{bright}$ cells during the first treatment cycle was significantly associated with a favorable outcome (**Fig. S3**).

We propose that the dynamics of NK cell subsets and their NCR expression may contribute to the anti-leukemic efficiency of immunotherapy with HDC/IL-2 in AML. While the exploratory nature of these findings should be emphasized, it is conceivable that AML patients with an intact NCR expression may benefit from relapse-preventive immunotherapy with HDC/IL-2, or other strategies aimed at boosting NK cell function and, also, that NCR expression may be a valid biomarker in future trials evaluating immunotherapy in AML.

Patients and Methods

Patients

Eighty-four patients aged 18 years or older (age 18–79, median 61) with *de novo* or secondary AML in first CR were eligible for enrollment. In this multicenter study, induction and consolidation courses were given as per each participating center. For all patients, the induction courses included cytarabine, either as continuous infusion 7 d or in intermediate doses (2000–3000 mg bid) for 3–5 d. Anthracyclines used in the induction courses were daunorubicin ($n = 77$), whereas in 7 cases idarubicin and fludarabine were used. Ten patients received cytarabine with other additional chemotherapy including etoposide ($n = 7$), amsacrine ($n = 1$) and ozogamizin ($n = 1$). Twenty patients achieved CR after two induction courses.

The number of post-remission courses were 1 ($n = 15$), 2 ($n = 20$), 3 ($n = 37$), 4 ($n = 5$), or 5 ($n = 1$).Typically, consolidation courses included cytarabine and an anthracycline; daunorubicin ($n = 45$), idarubicin ($n = 10$) or mitoxantrone ($n = 2$). Four patients received cytarabine and other drugs (etoposide,ozogamizin, methotrexate). In 15 patients the consolidation courses comprised high or intermediate doses of cytarabine as the single drug. Six patients did not receive any post-remission therapy, of these 5 had received 2 induction courses to achieve CR. Precise data on consolidation were missing in 2 patients.

The baseline characteristics of participating patients and their impact on LFS are shown in **Table S1**. Inclusion criteria included adequate renal, cardiac, and pulmonary functions along with a performance status (according to Eastern Cooperative Oncology Group [ECOG] criteria) of 0 to 1. Any previous induction or consolidation therapy, including autologous bone marrow transplantation, was allowed with the exception of allogeneic transplantation (performed or planned). Other exclusion criteria included FAB-M3 AML, class III or class IV cardiac disease, other active malignancies and severe hypersensitivity reactions. Elapsed time from dates of CR and the completion of consolidation chemotherapy were not to exceed 6 and 3 months respectively. The mean time from CR to study entry was 121 d.

Study design and objective

This single-armed multicenter phase IV study (Re:Mission, NCT01347996) enrolled 84 patients at 20 European centers between September 2009 and August 2012. The primary endpoint included assessment of the quantitative and qualitative pharmacodynamic effects of HDC/IL-2 on the immune responses of T and NK cells, including NCR expression. An interim analysis report based on data available on May 21st 2013 was submitted by the study sponsor to the European Medicines Agency (EMA). The analyses of NK cell markers vs. outcome are based on data for LFS available at this date. The trial was approved by the Ethics Committees of each participating institution, and was conducted in accordance with the Helsinki Declaration. All patients gave written informed consent before enrollment.

Treatment and dosing

Patients received 10 consecutive 3-week cycles of HDC/IL-2 with 3 weeks off treatment in cycles 1 to 3, and 6 weeks off treatment in cycles 4 to 10. The treatment continued for a total of 18 months or until the patients relapsed, died, discontinued therapy because of adverse events, withdrew consent, or became lost to follow-up. In each cycle, patients received HDC (0.5 mg; EpiCept Corporation) subcutaneously bid and human recombinant IL-2 (aldesleukin; 16,400 U/kg; Chiron Corporation) subcutaneously bid (**Fig. 1**). The scheduled total follow-up time was 2 years, and all patients included for the present analyses were followed until the closing date for this analysis.

Definitions and response criteria

Relapse was defined as at least 5% blast cells in the bone marrow or extramedullary leukemia. LFS was defined as the time in days from the first day of treatment with HDC/IL-2 to relapse or death from any cause. OS was defined as corresponding time to death regardless of cause. Risk groups were classified according to recommendations by the European LeukemiaNet.[51]

Sampling of peripheral blood and flow cytometry

Peripheral blood was collected before and after treatment cycles 1 and 3, i.e. on day 1 and day 21 of cycle 1 (C1D1 and C1D21), and on day 1 and day 21 of cycle 3 (C3D1 and C3D21). The patient blood was collected into BD Vacutainer CPT tubes with sodium citrate (BectonDickinson, Stockholm, Sweden), and within two hours PBMCs were isolated by density centrifugation. The prepared PBMCs were cryopreserved at local sites in CryoMaxx S cryopreservation media (PAA), and shipped on dry ice to the central laboratory for immunological assessment

(at the Sahlgrenska Cancer Center, University of Gothenburg, Sweden). The central laboratory operates under Good Clinical Practice (GCP) conditions and passed a GCP-inspection by the EMA in August 2013 (2013/014). In addition it forms part of the Good Laboratory Practice (GLP) accredited laboratories at the Department of Clinical Virology, which is reviewed by the Swedish Board for Accreditation and Conformity Assessment. Samples were available from 79 out of 84 participating patients.

Samples were analyzed flow cytometrically within 30 months of collection, with a median time until analysis of 15 months. The frozen PBMC samples were thawed quickly and washed in warm medium. Samples were first stained with LIVE/DEAD Fixable Yellow Stain (Invitrogen) in PBS. After washing, cells were stained with a cocktail of antibodies in PBS containing 0.5% BSA and 0.1% EDTA. The following anti-human monoclonal antibodies were purchased from BD Biosciences (Stockholm, Sweden): anti-CD3-FITC (clone: HIT3a), CD4-APCH7 (RPA-T4), CD8-PerCpCy5.5 (SK1), CD16-V450 (3G8) CD56-APC (B159). The antibodies to NCRs NKp30-PE (AF29-4D12), NKp46-APC (9E2) were purchased from Miltenyi Biotec. Samples were collected via a 4-laser BD LSRFortessa SORP (405, 488, 532, and 640 nm; BD Biosciences). Data were analyzed with FACSDiva Version 6 software (BD Biosciences) or FlowJo Version 8.4 software (TreeStar). All available samples were analyzed. If an analysis failed according to pre-defined criteria (experimental failure, few cells, below 25% cellular viability), a second sample was thawed for re-analysis. In 18 cases for C1D1 samples and in 12 cases for C1D21 samples, also the second attempt failed to generate data, and these samples were excluded from analysis. Differential counts of whole blood were performed at local sites and were utilized to calculate absolute counts of blood NK cells.

Analyses of lymphocyte counts vs. outcome in a phase III trial

In an open-label, randomized phase III study (NCT00003991), 320 AML patients were enrolled after induction/consolidation therapies and randomly assigned to an HDC/IL-2 arm (with treatment schedule and dosing identical to patients in the Re:Mission Trial) or a control arm (no treatment).[28] Lymphocyte counts were captured before treatment as well as before and after treatment cycles. For the present study, the prognostic impact of an increment in lymphocyte counts during therapy was determined by dichotomizing patients by high or low (by the median) lymphocyte counts followed by analysis of LFS and OS in these groups by the logrank test.

Statistics

As specified in the statistical plan, paired *t*-test was used for single comparisons of NK cell phenotypes. In exploratory analyses the impact of NK cell-related markers on outcome were determined by dichotomizing patients by high or low (by the median) NK cell counts or NCR expression intensity (median fluorescence intensity, MFI) followed by analysis of LFS and OS in these groups by the logrank test. Parameters that significantly predicted LFS and/or OS using the logrank test were further analyzed by univariate and multivariate Cox regression analysis. In the multivariate analyses, hazard ratios were corrected for age and risk group classification (**Table 1**). For practical reasons, the first day of treatment was set to day 0 in all survival analyses, also in those that evaluated immunological status on day 1 in cycle 3. Since the time between first day of treatment and C3D1 is constant for all patients (84 d), this has no consequence for the conducted analyses. All indicated p values are 2-sided.

Disclosure of Potential Conflicts of Interest

Authors KH and MB are past or present consultants to the study sponsor (Meda Pharma). Author KH holds patents protecting the use of histamine dihydrochloride in cancer immunotherapy. Authors AM, RF, and FBT have received honoraria and/or travel grants from the study sponsor. The other authors declare no conflict of interest.

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