Published OnlineFirst July 6, 2018; DOI: 10.1158/2326-6066.CIR-17-0711

Research Article

Cancer Immunology Research

Cytomegalovirus Serostatus Affects Autoreactive NK Cells and Outcomes of IL2-Based Immunotherapy in Acute Myeloid Leukemia

Elin Bernson[1], Alexander Hallner[1], Frida E. Sander[1], Malin Nicklasson[2], Malin S. Nilsson[1], Karin Christenson[1], Ebru Aydin[1], Jan-Åke Liljeqvist[2], Mats Brune[3], Robin Foà[4], Johan Aurelius[1,3], Anna Martner[1], Kristoffer Hellstrand[1], and Fredrik B. Thorén[1]

Abstract

Human cytomegalovirus (CMV) infection is reported to promote NK cell differentiation and education. The CMV-induced generation of highly differentiated adaptive-like NK cells has been proposed to affect favorably on the maintenance of remission in patients with acute myeloid leukemia (AML) after allogeneic stem cell transplantation (allo-SCT). The impact of CMV infection and adaptive-like NK cells on relapse and survival of patients with AML not receiving allo-SCT remains unknown. We assayed CMV IgG serostatus to determine past CMV infection in 81 nontransplanted AML patients who were receiving relapse-prevention immunotherapy comprising histamine dihydrochloride and low-dose interleukin-2 (HDC/IL2; NCT01347996). CMV seropositivity correlated negatively with leukemia-free and overall survival of patients receiving HDC/IL2, but did not correlate with outcomes in a contemporary control cohort. Analysis of outcome after stratification of patients based on concordant or discordant killer immunoglobulin-like receptor (KIR) and HLA genotypes implied that the negative impact of CMV seropositivity was restricted to patients lacking a ligand to inhibitory KIRs (iKIR). Previous CMV infection was also associated with fewer NK cells expressing only nonself iKIRs (NS-iKIR). We propose that CMV-driven NK cell education depletes the population of NS-iKIR NK cells, which in turn reduces the clinical benefit of relapse-preventive immunotherapy in AML. *Cancer Immunol Res; 6(9); 1110–9. ©2018 AACR.*

Introduction

Human cytomegalovirus (CMV) belongs to the family of herpesviruses and establishes persistent infection in humans (1). Reactivation of CMV is a common complication in immunosuppressed patients (2) and contributes to morbidity and mortality associated with allogeneic stem cell transplantation (allo-SCT) in patients with acute myeloid leukemia (AML; ref. 3). Preemptive antiviral therapy has made CMV reactivation manageable in most AML cases (3). Moreover, several studies suggest that CMV reactivation after allo-SCT in AML is associated with reduced relapse risk (4–6). The mechanism underlying the purported protective effects of CMV infection on outcome of allo-SCT is unknown, but may involve effects of CMV infection on the distribution and function of the immune cells that eliminate AML cells (7–9). Individuals harboring IgG antibodies against CMV (CMV-seropositive individuals, CMV^+) as a sign of past CMV infection show elevated numbers of differentiated adaptive $CD57^+NKG2C^+$ NK cells in blood (10–13), and CMV-induced reactivation after allo-SCT is associated with rapid reconstitution of such differentiated NK cells (3, 14–17).

NK cells are implicated in the defense against hematopoietic malignancies, including AML, also in nontransplanted patients (18, 19). Downregulation of activating NK cell receptors, including the natural cytotoxicity receptors (NCR) NKp46 and NKp30, is associated with poor prognosis in AML (19–21). NK cell cytotoxicity is also regulated by inhibitory receptors such as killer immunoglobulin-like receptors (KIR) and NKG2A-CD94 (NKG2A) that recognize cognate HLA molecules, thus preventing NK cells from attacking healthy cells. As NK cells mature, their expression profile of inhibitory receptors changes from an $NKG2A^+KIR^-$ phenotype to gradual inclusion of KIRs along with loss of NKG2A (22). NK cells acquire KIRs in a partly stochastic fashion, and the NK cell repertoire thus comprises an array of cells equipped with varying numbers and combinations of inhibitory receptors (23).

Approximately two thirds of humans lack one or more KIR ligands for their cognate inhibitory receptors, resulting in a substantial fraction of NK cells expressing only inhibitory KIRs for nonself HLA (NS-iKIR NK cells; refs. 24–27). Under resting conditions, these potentially autoreactive, "unlicensed" NK cells have limited cytotoxic capacity (28, 29). However, immune perturbations, especially in the context of cancer, may activate these NK cells to become effector cells, as they do not express inhibitory receptors that can be targeted by malignant cells (26, 30–33).

[1]TIMM Laboratory, Sahlgrenska Cancer Center, University of Gothenburg, Gothenburg, Sweden. [2]Department of Infectious Diseases, University of Gothenburg, Gothenburg, Sweden. [3]Department of Hematology, University of Gothenburg, Gothenburg, Sweden. [4]Hematology, Department of Cellular Biotechnologies and Hematology, Sapienza University, Sapienza, Italy.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

Corresponding Author: Fredrik B. Thorén, University of Gothenburg, Box 425, 40530 Gothenburg, Sweden. Phone: 46-70-928-9056; E-mail: fredrik.thoren@gu.se

doi: 10.1158/2326-6066.CIR-17-0711

Published OnlineFirst July 6, 2018; DOI: 10.1158/2326-6066.CIR-17-0711

Little is known regarding the role of CMV in patients who do not undergo allo-SCT. In this study, we asked whether CMV IgG serostatus, as a reflection of a past CMV infection, correlated with relapse risk and survival in two cohorts of nontransplanted AML patients, one of which received postconsolidation immunotherapy comprising IL2, an activator of T cells and NK cells, and histamine dihydrochloride (HDC), which targets the production of immunosuppressive reactive oxygen species from myeloid cells (34, 35). Based on the effect of CMV on NK cell differentiation and education (10), we hypothesized that a skewed NK cell repertoire might affect the outcome for patients receiving immunotherapy dependent on NK cell function (36). Our results show that CMV seropositivity was associated with high relapse risk and poor survival in AML patients receiving HDC/IL2. After IL2 stimulation *in vitro*, NS-iKIR NK cells from CMV$^-$ donors constituted a substantial fraction of the responding cells against primary HLA-matched AML blasts. We propose that CMV-driven NK cell differentiation depletes autoreactive NS-iKIR NK cells in blood, which in turn reduces the clinical benefit of relapse-preventive immunotherapy.

Patients, Materials, and Methods

Patients

This study included two nontransplanted cohorts of AML patients. A first cohort included patients enrolled in the single-arm multicenter phase IV study (Re:Mission, NCT01347996, www.clinicaltrials.gov; $n = 84$ patients; age 18–79, all in first complete remission, CR). Of 84 patients enrolled in the Re:Mission trial, 3 patients withdrew consent. The remaining patients received 10 scheduled 3-week cycles of HDC/IL2 for 18 months. Follow-up time was at least 2 years, when all patients were censored for relapse and death. Primary endpoints included assessment of quantitative and qualitative pharmacodynamic effects of HDC/IL2 by monitoring T and NK cell phenotypes before and after treatment cycles. The herein reported aspects of NK cell biology versus clinical outcome (leukemia-free survival, LFS; and overall survival, OS) were performed *post hoc*. A detailed account for induction and consolidation therapy, exclusion criteria, treatment, and dosing is found in previous study reports (20, 21). Patients were risk-classified according to recommendations issued by the European LeukemiaNet (37). The trial was approved by the Ethical Committees of each participating institution, and all patients gave written informed consent before enrollment. A second cohort of 76 contemporary, nontransplanted AML patients (age 22–81), diagnosed and treated at Sahlgrenska University Hospital during 2007–2013, was included in the study to compare the impact of CMV serostatus on outcome of patients receiving or not receiving immunotherapy. In this cohort, 22 patients did not achieve CR after induction chemotherapy and serum serology result was lacking for 1 patient. Hence, 53 patients in CR1 were included in comparisons with the Re:Mission trial cohort. For patient characteristics, see Supplementary Table S1.

Flow cytometry analyses

Cryopreserved peripheral blood mononuclear cells collected from the Re:Mission trial patients before and after the first and third treatment cycles, i.e., cycle 1, day 1 (C1D1) and cycle 1, day 21 (C1D21), cycle 3, day 1 (C3D1), and cycle 3, day 21 (C3D21) were analyzed by flow cytometry at the Sahlgrenska Cancer Center, University of Gothenburg, Sweden. The following anti-human monoclonal antibodies were used for NK cell phenotyping: anti-CD3-APC-H7 (clone: SK7), CD14-APC-H7 (MφP9), CD16-BV786 (3G8), CD56-BV711 (NCAM1), CD57-BV605 (NK-1), CD107a-BV510 (H4A3), CD19-APC-H7 (SJ25C1; all from BD Biosciences), NKG2C-Alexa Flour 488 (134591; R&D Systems), NKG2A-PE (Z199), KIR2DL1/S1-Pe-Cy7 (EB6B), KIR2DL2/L3/S2-Pe-Cy5.5 (GL 183; all from Beckman Coulter), KIR3DL1-APC (DX9; BioLegend) and LIVE/DEAD fixable yellow stain (Life Technologies). Stained samples were acquired using a 4-laser BD LSRFortessa SORP flow cytometer (BD Biosciences). Data were analyzed using the FlowJo software, version 10.1r5 (TreeStar) or the FACSDiva software, version 8.0.1 (BD Biosciences). Samples with less than 25% viability were excluded.

KIR/HLA genotyping and CMV serology

KIR and KIR ligands were analyzed using the One Lambda KIR SSO Genotyping Test and the Olerup SSP KIR HLA Ligand kit, respectively, as described elsewhere (33). Patients were dichotomized based on concordance/discordance between their KIR and HLA genotypes. Patients with "all ligands present" harbor all class I ligands for their set of inhibitory *KIR* genes. Patients lacking an HLA ligand for one self-expressed inhibitory KIR were considered "missing ligand." In this algorithm, the major inhibitory KIR/HLA receptor ligand pairs were considered, i.e., KIR2DL1-HLA-C2, KIR2DL2/3-HLA-C1, and KIR3DL1-HLA Bw4.

Serum samples were obtained from 81 patients in the Re:Mission trial. CMV serology was determined by the accredited anti-CMV IgG assay (Architect, Abbot) at the Virology Laboratory at Sahlgrenska University Hospital (38). One patient showed CMV DNA in serum at onset of immunotherapy. In the control cohort, CMV serology results were available from 75 of 76 patients.

Degranulation responses in NK cells

Fifty-eight paired serum samples and leukopacks from healthy donors were obtained from the Blood Center at Sahlgrenska University Hospital. Donors lacking either HLA-C1 or HLA-C2 were identified using the Olerup SSP KIR HLA Ligand kit. Experiments were performed with all CMV$^-$ donors lacking either HLA-C1 or C2 and a corresponding number of CMV$^+$ donors. PBMCs were isolated using density gradient centrifugation and NK cells were isolated from donors lacking a KIR2DL ligand using MACS NK cell isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. NK cells were incubated overnight in the presence or absence of IL2 (500 or 1,000 U/mL) and coincubated in the presence of a CD107a antibody with Daudi lymphoblastoid target cells precoated with NKp46- and CD2-triggering antibodies, K562 cells, or with sorted HLA-matched primary AML blasts. Daudi cells, preincubated with antibodies to NKp46 (9E2, Miltenyi Biotec) and CD2 (RPA-2.10, Thermo Fisher), were subjected to NK cells in the presence of a BUV395-conjugated antibody to CD107a (H4A3, BD Biosciences) for 2 hours. In experiments using AML samples, blasts were sorted with CD45-BV786 (HI30, BD Biosciences) on a 3-laser BD FACSAria III (BD Biosciences), stained with CellTrace violet (Invitrogen), and then subjected to HLA-matched NK cells for 4 hours in the presence of CD107a antibody. NK cells were stained with a flow cytometry panel covering antibodies to NKG2A, KIR2DL1/S1, KIR2DL2/L3/S2, and KIR3DL1 (see above). Degranulation responses were determined in NKG2A$^{+/-}$ single KIR$^+$ NK cells expressing either KIR2DL1 or KIR2DL2/L3. Donors

Published OnlineFirst July 6, 2018; DOI: 10.1158/2326-6066.CIR-17-0711

lacking 3DL1 extracellular expression were excluded from the analyses as intracellularly retained 3DL1 may affect licensing.

Daudi cells were kindly provided by Ka-Wei Tang, University of Gothenburg, and were authenticated using Multiplex Cell Authentication by Multiplexion. Cells were used up to passage 30.

Statistical analyses

Analyses of the impact of CMV serostatus or NK cell phenotypes on LFS and OS were performed using the log-rank test. For the Re:Mission cohort, LFS is defined as the time in days from start of immunotherapy with HDC/IL2 to relapse or death from any cause, while OS is defined as the corresponding time to death, available at the trial closing date, i.e., when all patients had been followed for 24 months (18 months of treatment and 6 months of additional follow-up). For the control cohort, not receiving immunotherapy, OS is defined as time in days from diagnosis to death, while LFS is defined as time in days from achieved CR to relapse or death of any cause. For both cohorts, all remaining patients were censored after 730 days. Relapse was defined as at least 5% blast cells in the bone marrow or presence of extramedullary leukemia. To determine the impact of NK cell subsets or markers on outcome, samples were dichotomized by the median frequency or expression level at single time points if not otherwise stated. In analyses of the impact of frequency of NS-iKIR on clinical outcome, cutoff values were defined as the median of NS-iKIR NK cells among all patients at the specified time point (1.53% and 2.44%) with a frequency range of 0%–36.7% and 0%–26.2% for C1D1 and C3D1, respectively. Parameters that significantly predicted LFS or OS using the log-rank test were analyzed by univariable and multivariable Cox regression analysis. Covariates including age, risk group classification, number of induction courses required to achieve CR (1 or >1) and number of consolidation courses (0–2 or >2) were tested in univariable analyses. Covariates with *P* values below 0.1 in univariable analyses (age and number of induction courses) were included in the multivariable analysis (Table 1). Mann–Whitney test was used for single comparisons of NK cell phenotypes. For comparison of degranulation in NK cell subsets, paired (comparisons within the same group) or unpaired (comparisons between groups) Student *t* test was used.

Results

CMV$^+$ AML patients have altered NK cell profiles

CMV$^+$ individuals reportedly present with a more differentiated NK cell repertoire comprising elevated frequencies of highly differentiated NK cells (10, 11). To address the impact of CMV IgG serostatus on the NK cell repertoire in the postconsolidation phase and during subsequent cytokine-based immunotherapy, we analyzed the expression of the major inhibitory KIRs (iKIR), NKG2A, CD57, and NKG2C in samples from patients with AML undergoing relapse-preventive immunotherapy (gating strategy shown in Supplementary Fig. S1). As reported elsewhere, a 3-week treatment cycle with HDC/IL2 triggered expansion of CD56^{bright} NK cells in blood (21). Between treatment cycles, the peripheral blood NK cell population contracted and the relative frequencies of CD56^{bright} and CD56^{dim} subsets returned to baseline levels, although absolute counts of CD56^{dim} NK cells remained elevated (21). NK cells from CMV$^+$ patients expressed NKG2C to a higher extent than CMV$^-$ patients at onset of treatment (C1D1), with the frequency remaining elevated in CMV$^+$ patients during the course of immunotherapy. By contrast, despite higher CD57 expression in CMV$^+$ individuals at onset of treatment, there was no significant difference in CD57$^+$ NK cell frequency between CMV$^+$ and CMV$^-$ individuals at later time points during HDC/IL2 treatment (Fig. 1A and B). However, the highly differentiated KIR$^+$CD57$^+$NKG2C$^+$ NK cell subset was significantly more prominent in CMV$^+$ patients (Fig. 1C and D; Supplementary Fig. S2). Thus, the NK cell repertoire in CMV$^+$ AML patients was skewed toward differentiated cells before and during immunotherapy as compared with CMV$^-$ patients.

CMV infection has been reported to promote NK cell acquisition of self-specific iKIRs, thus increasing the fraction of cells that are educated through interactions with self-HLA (10). By combining flow cytometry data with each patient's HLA genotype, we determined the frequency of NKG2A$^+$ or NKG2A$^-$ NK cells expressing only iKIRs for nonself HLA (NS-iKIR NK cells). NKG2A$^-$ NS-iKIR$^+$ NK cells comprise a subset referred to as unlicensed, or uneducated, NK cells (29), whereas NKG2A$^+$ NS-iKIR$^+$ NK cells are licensed through NKG2A–HLA-E interactions but devoid of KIR-mediated inhibition. The NS-iKIR NK cell subset was reduced in frequency in CMV$^+$ AML patients both before start of immunotherapy and at onset of the third treatment cycle, with a similar trend in frequency levels of the NKG2A$^-$ NS-iKIR subset (Fig. 1E; Supplementary Fig. S3).

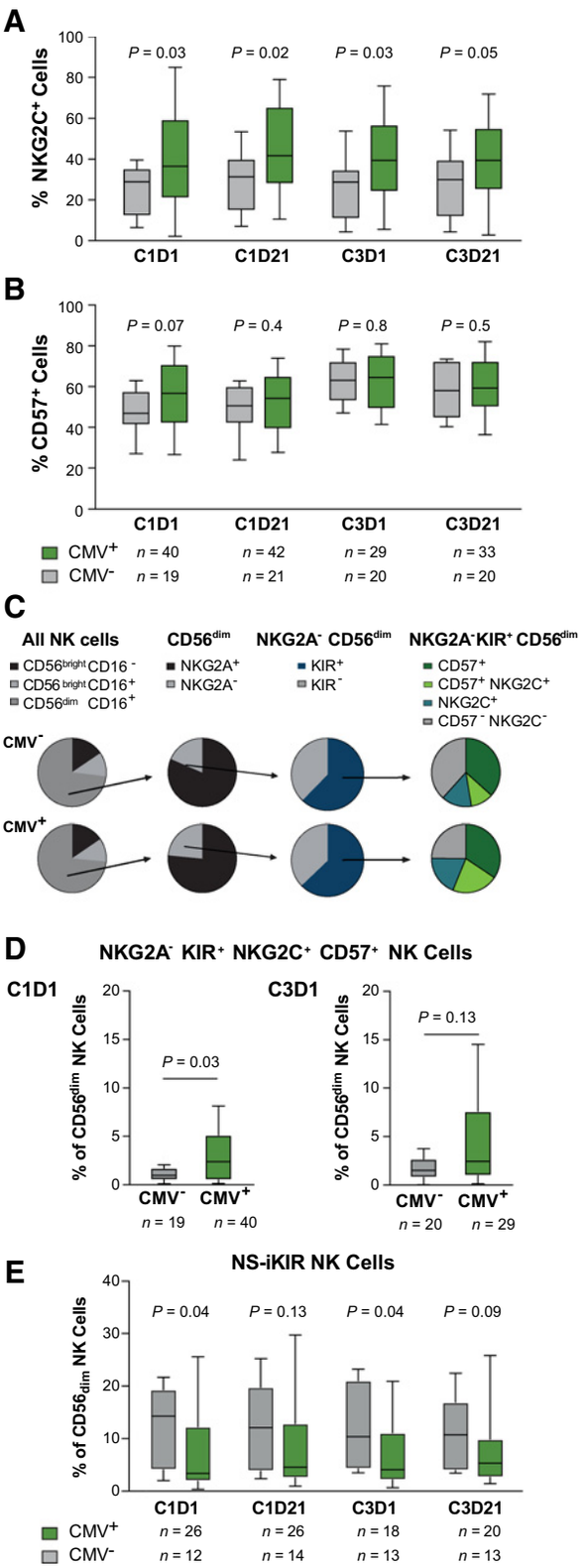
CMV$^+$ serostatus affects negatively outcome in patients receiving immunotherapy

Studies suggest that highly differentiated CMV-induced NK cells are important antileukemic effector cells in AML patients undergoing allo-SCT (4, 16, 39). However, other studies propose that NS-iKIR NK cells might exert antineoplastic effector functions after immunomodulatory therapy (26, 30, 32, 33). The high proportion of highly mature, adaptive NK cells along with the low proportion of NS-iKIR NK cells in CMV$^+$ patients led us to examine the impact of CMV serostatus on clinical outcome. Within the Re:Mission trial, 58 patients (70%) were seropositive for CMV IgG and 25 were seronegative, which reflects the average distribution of CMV seropositivity among healthy age-matched individuals (40). The prevalence of CMV seropositivity was higher among relapsing against nonrelapsing patients (80% versus 54%; Supplementary Table S1) and Kaplan–Meier analysis of LFS and OS implied that CMV seropositivity was associated with reduced LFS and OS in patients receiving HDC/IL2 immunotherapy (Fig. 2A and B, multivariable analyses in Table 1). We also examined the impact of CMV serostatus in a cohort of

Table 1. Univariable and multivariable analyses of the impact of CMV serostatus on LFS and OS

	Univariable analysis			Multivariable analysis		
Variable	**Hazard ratio**	**Confidence interval**	***P***	**Hazard ratio**	**Confidence interval**	***P***
CMV serostatus LFS	0.41	0.19–0.89	0.02	0.46	0.21–1.01	0.05
CMV serostatus OS	0.26	0.08–0.87	0.03	0.28	0.08–0.96	0.04

Published OnlineFirst July 6, 2018; DOI: 10.1158/2326-6066.CIR-17-0711



contemporary nontransplanted AML patients who did not receive postconsolidation immunotherapy ($n = 53$). We observed an association between CMV serostatus and LFS or OS in patients receiving HDC/IL2 but not in the patients who had not received immunotherapy (Fig. 2C and D).

Negative impact of CMV infection restricted to patients with missing ligand genotype

To understand why CMV seropositivity was associated with poor outcome for AML patients receiving postconsolidation immunotherapy, we first investigated whether presence of $KIR^+CD57^+NKG2C^+$ differentiated NK cells affected clinical outcome in the Re:Mission trial cohort. Relapse risk did not differ between patients dichotomized by above or below median frequency of highly differentiated NK cells (Supplementary Fig. S4). We and others have previously reported that a genotypic discordance between the individual's *KIR* and *HLA* genotypes, i.e., a "missing ligand genotype," where an iKIR lacks its corresponding HLA ligand, may be associated with superior outcome in different malignancies (26, 30, 32, 33). As a common denominator in these studies was that patients received an immune-perturbing treatment, we speculated that NS-iKIR NK cells, which are only present in individuals with a missing ligand genotype, might become activated to exert cytotoxicity against malignant cells upon immunotherapy. We investigated whether the clinical effect of a missing ligand genotype differed between CMV^+ and CMV^- patients during HDC/IL2 immunotherapy. As shown in Fig. 3A and B, a missing ligand genotype was only associated with a significantly favorable outcome in CMV^- patients. Furthermore, CMV serostatus only had a negative impact on LFS in patients with a missing ligand. Thus, CMV^- patients with a missing ligand genotype showed superior clinical outcome compared with all other patients (Fig. 3C and D).

These results suggest that NS-iKIR NK cells may constitute an effector population during HDC/IL2 immunotherapy (33) and that CMV-driven depletion of NS-iKIR NK cells adversely affects outcome. To address this possibility, we compared degranulation responses *in vitro* among single KIR^+ NK cells in CMV^- or CMV^+ donors with a missing ligand genotype. For these studies, NK cell degranulation was determined in response to HLA-deficient K562 cells or in a redirected assay where HLA-deficient Daudi cells were preincubated with triggering antibodies to CD2 and NKp46. In accordance with previous studies (29, 32, 33, 41), educated S-KIR–expressing NK cells displayed greater degranulation responses to HLA-deficient target cells as compared with less educated NS-iKIR populations of NK cells without prior stimulation (Fig. 4A). The relatively hyporesponsive NS-iKIR NK cells degranulated significantly more in response to IL2 in both assays. These assays however ignore the HLA interactions with inhibitory

Figure 1.

NK cell differentiation status in CMV-seronegative or -seropositive AML patients receiving HDC/IL2 therapy. **A** and **B,** Frequency of $NKG2C^+$ (**A**) or $CD57^+$ (**B**) cells among $CD56^{dim}$ $NKG2A^-$ KIR^+ NK cells during treatment. **C,** Pie charts show the fraction of NK cells from CMV^- and CMV^+ patients that fulfill the differentiation criteria $CD16^+56^{dim} > NKG2A^- > KIR^+ > CD57^{+/-} > NKG2C^{+/-}$, from samples collected before immunotherapy, $n = 59$. **D,** Box plots depict percentage (%) of $KIR^+CD57^+NKG2C^+$ NK cells at C1D1 and C3D1 in CMV^- and CMV^+ patients. **E,** Box plots depict percentage (%) of NS-iKIR NK cells at onset (C1D1) and during HDC/IL2 therapy in CMV^- and CMV^+ patients. Mann–Whitney test; box plots show 10th to 90th percentiles.

Published OnlineFirst July 6, 2018; DOI: 10.1158/2326-6066.CIR-17-0711

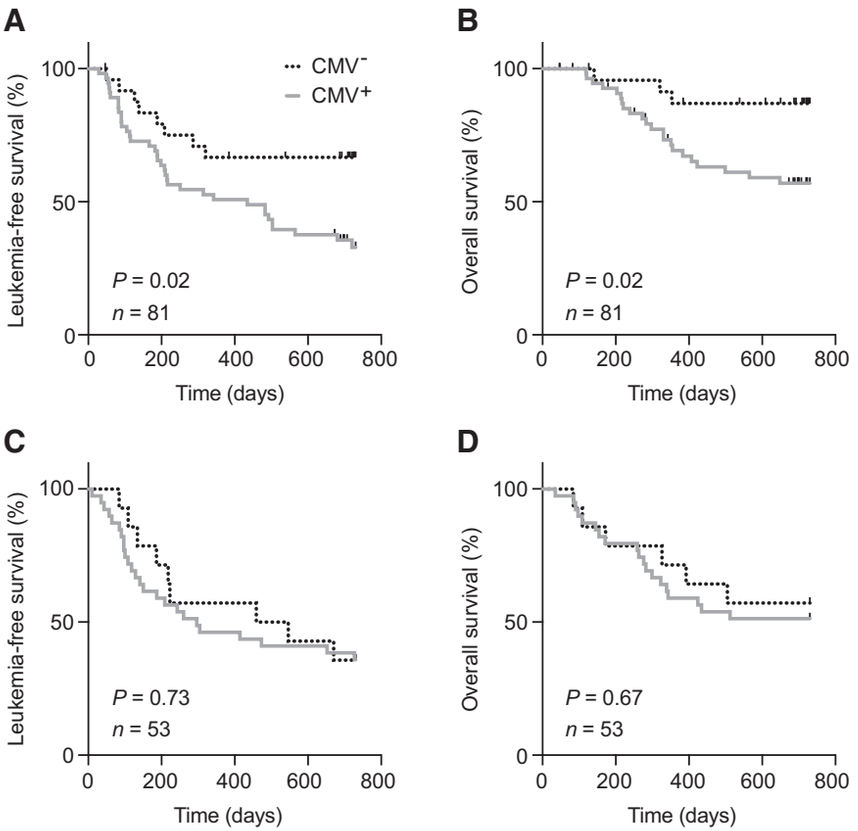


Figure 2.
Impact of CMV serostatus on clinical outcome. **A** and **B,** Leukemia-free (**A**) and overall (**B**) survival for AML patients in the Re:Mission trial that are CMV^- or CMV^+. **C** and **D,** Leukemia-free (**C**) and overall (**D**) survival for a historical control cohort of nontransplanted patients with AML.

receptors. Thus, we next performed a degranulation assay with NK cells toward HLA-matched primary AML blasts. After IL2 stimulation, NS-iKIR NK cells degranulated significantly more than S-iKIR NK cells in response to these primary leukemic cells (Fig. 4A). In further experiments, we divided the NK cell donors into two groups based on CMV serostatus. The percentage of NS-iKIR NK cells that degranulated in the redirected Daudi cell assay was similar in CMV^- and CMV^+ donors, indicating that there was no inherent defect in the NS-iKIR NK cell subset in CMV^+ donors (Fig. 4B). However, as the size of the NS-iKIR subset was larger in CMV^- donors, the degranulating NS-iKIR NK cells comprised a higher fraction of responding NK cells in CMV^- donors as compared with CMV^+ donors (Fig. 4C and D; 4% versus 8% in the Daudi cell assay; 5% versus 10% in the assay with primary AML blasts).

Next, we determined the impact of the number of NS-iKIR NK cells on the course of disease among CMV^+ versus CMV^- patients who received postconsolidation immunotherapy. For these analyses, LFS was compared in the Re:Mission trial patients with above versus below median frequency of NS-iKIR NK cells. As shown in Fig. 5A and B, a high frequency of NS-iKIR NK cells was beneficial in CMV^- patients in terms of LFS at onset of immunotherapy and before the third treatment cycle. Similar results were obtained when only unlicensed $NKG2A^-$ NS-iKIR NK cells were taken into consideration (Supplementary Fig. S4). No such trends were observed in CMV^+ patients (Fig. 5C and D). Instead, high proportions of $NKG2C^+$ NK cells were found to be protective in CMV^+ but not in CMV^- patients (Supplementary Fig. S5).

Discussion

The objectives of this study were to clarify whether CMV serostatus affected the outcome for nontransplanted AML patients in CR and, also, to elucidate the potential mechanisms involved with reference to KIR/HLA interactions and the maturation status of NK cells. In accordance with previous reports, a past CMV infection, as reflected by presence of CMV IgG, was associated with a more differentiated NK cell repertoire in blood as exemplified by elevated levels of highly differentiated $CD57^+NKG2C^+$ NK cells (10, 11). These differentiated, adaptive NK cells have been ascribed antineoplastic effector functions, and several immunotherapeutic regimens exploiting this subset of NK cells have been proposed (42–44). However, we did not observe any correlation between high percentage of $CD57^+NKG2C^+$ NK cells and favorable clinical outcome in nontransplanted AML patients who received HDC/IL2 for relapse prevention, although high levels of $NKG2C^+$ NK cells were associated with beneficial LFS in CMV-seropositive patients. By contrast, we observed that CMV seropositivity was associated with poor clinical outcome during IL2-based immunotherapy.

The negative impact of CMV seropositivity was not observed in a contemporary control cohort of nontransplanted AML patients who received similar induction and consolidation chemotherapy but did not receive HDC/IL2 in the postconsolidation phase, suggesting that the negative impact of a past CMV infection was linked to the efficacy of immunotherapy. We have reported a clinical benefit for AML patients with a missing KIR ligand genotype during HDC/IL2 immunotherapy (33). In these patients, a fraction of NK cells did not express any

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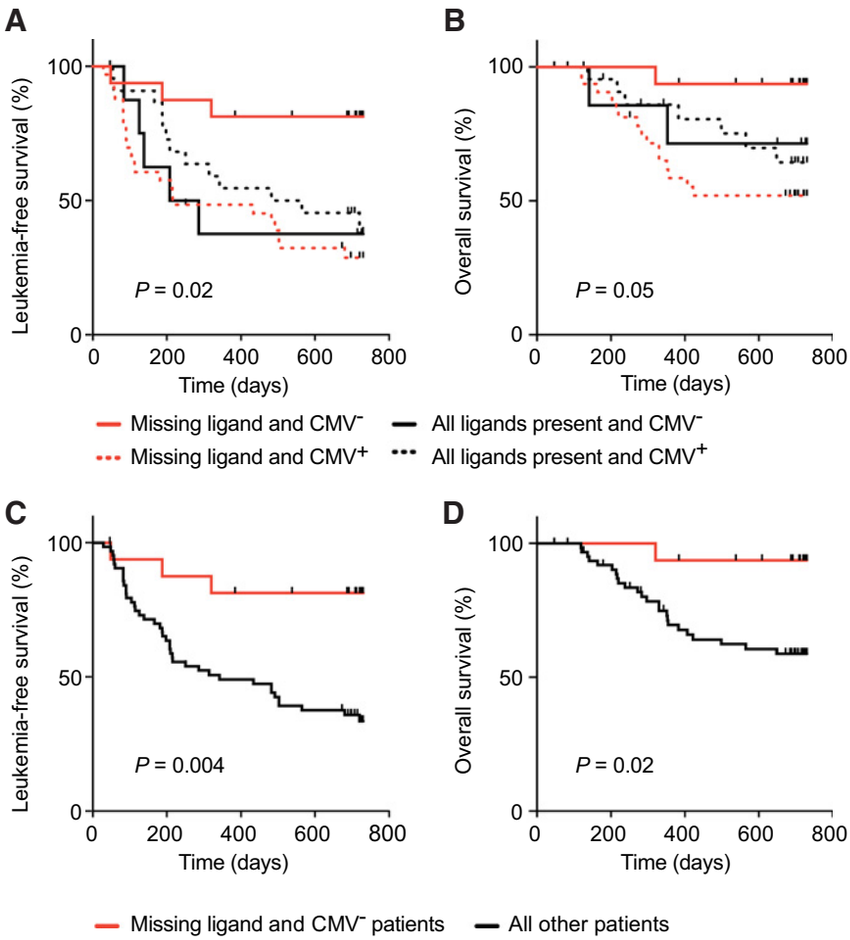

Figure 3.

Impact of a missing ligand genotype and CMV serostatus on outcome of AML patients receiving HDC/IL2. **A** and **B,** Leukemia-free (**A**) and overall (**B**) survival for patients with a "missing ligand" or "all iKIR ligands present" genotype, and that are either CMV^+ ($n = 33$ and 23, respectively) or CMV^- ($n = 17$ and 8, respectively). **C** and **D,** Leukemia-free (**C**) and overall (**D**) survival of patients with a missing iKIR ligand that are CMV^- compared with all other patients ($n = 17$ and 64, respectively).

iKIRs for self-HLA, but instead one (or two) iKIRs for nonself HLA molecules (NS-iKIR NK cells). A subset of these NS-iKIR NK cells also lacked expression of NKG2A, thus constituting a potentially autoreactive NK cell subset. The unlicensed NK cell subset is hyporesponsive at steady state, but proinflammatory conditions may activate these cells to become uninhibited effector cells (28, 45). The relevance of this NK cell subset is highlighted by studies reporting a benefit from a missing KIR ligand genotype after immunomodulatory therapies in malignancies (26, 30, 32).

As CMV drives differentiation of NK cells, the infection will induce not only acquisition of NKG2C and CD57 but also expression of additional KIRs; in fact, CMV specifically promotes expansion of NK cell subsets expressing self-KIRs (10). Induction of differentiation in NS-iKIR NK cell subsets entails acquisition of a self-reactive KIR; such induction will deplete the unlicensed subset and make NK cells receive more inhibitory signals when interacting with HLA-expressing malignant cells. In *in vitro* experiments using HLA class I–deficient target cells, the presence of a licensing S-KIR is associated with elevated cytotoxicity (41, 46). However, this experimental design may neglect the negative impact of an inhibitory receptor in a context in which the putative target cells have intact expression of HLA class I. AML blasts are reported to express HLA class I at a level comparable to that of healthy hematopoietic cells (33, 47–49). Thus, in a clinical setting where the malignant cells express HLA, immune activation may trigger NS-iKIR NK cells to break tolerance to self and exert antileukemic effector functions against malignant cells. In this setting, enhanced CMV-driven differentiation of NK cells may deplete a pool of important antileukemic effector cells.

To shed further light on these mechanisms, we identified CMV^+ and CMV^- blood donors with a missing ligand genotype and evaluated degranulation responses in NK cell subsets *in vitro*. Upon IL2 stimulation, the otherwise hyporesponsive NS-iKIR NK cells degranulated significantly more than S-KIR NK cells when exposed to HLA-matched AML blasts. Due to the higher percentage of NS-iKIR NK cells in CMV-seronegative donors, the fraction of NS-iKIR NK cells among responding cells was twice as high in CMV^- donors as compared with CMV^+ donors. In the Re:Mission trial, we also observed that the frequency of both $NS\text{-}iKIR^+$ NK cells and unlicensed $NKG2A^-NS\text{-}iKIR^+$ NK cells was decreased in CMV^+ AML patients. Accordingly, only CMV^- patients benefited from carrying a missing ligand genotype, whereas CMV^+ individuals, in whom CMV drives depletion of NS-iKIR NK subsets, had no such benefit. Thus, CMV^- patients with a missing ligand genotype, i.e., patients with high levels of potentially autoreactive NS-iKIR NK cells, had better prognoses than all other patients. In support of these genotype-based results, analyses of the NK cell repertoire showed that above median percentage of NS-iKIR cells was associated with LFS among CMV-seronegative patients. No

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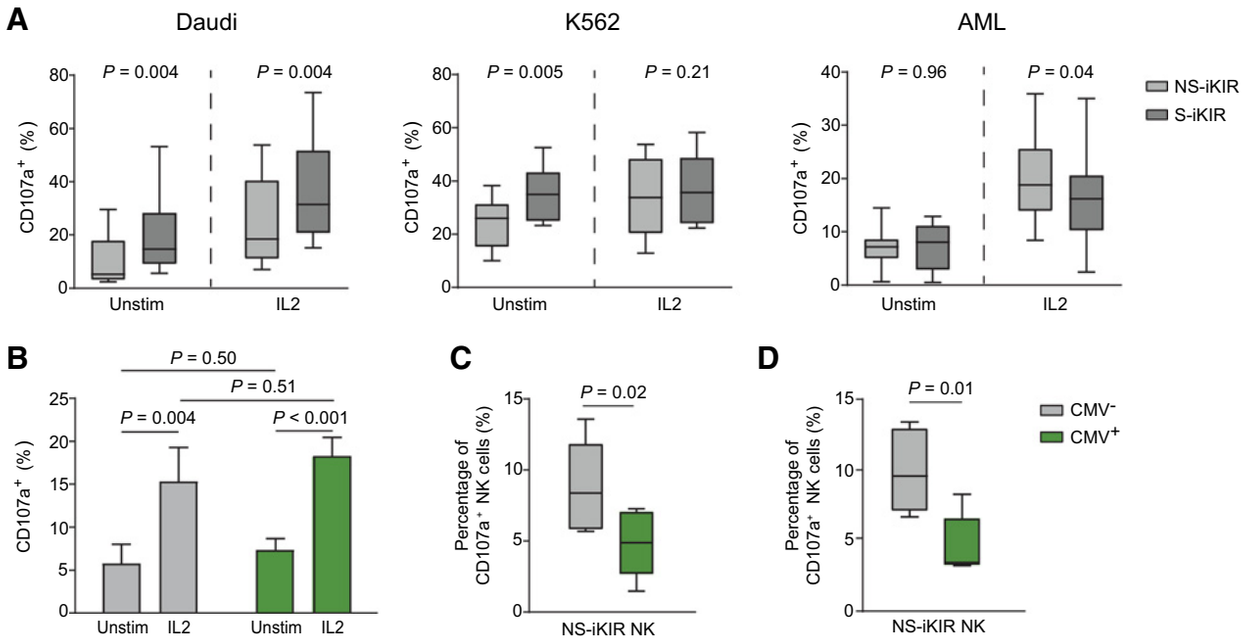

Figure 4.

Degranulation responses in single KIR$^+$ NK cells. **A,** Frequency of CD107a$^+$ NS-iKIR or S-iKIR NK cells in degranulation assays toward Ab-coated Daudi cells ($n = 10$), K562 cells ($n = 10$), or primary AML blasts ($n = 10$ and $n = 14$ for unstimulated and IL2-stimulated, respectively). **B,** Frequency of CD107a$^+$ NS-iKIR NK cells toward Ab-coated Daudi cells in CMV$^-$ ($n = 6$) and CMV$^+$ donors ($n = 7$). **C** and **D,** Percentage of CD107a$^+$ NS-iKIR NK cell response of total CD107a$^+$ NK cell response toward Ab-coated Daudi cells (**C**) or primary AML blasts (**D**) in CMV$^-$ ($n = 6$; **C**, $n = 5$; **D**) and CMV$^+$ donors ($n = 7$; **C**, $n = 5$; **D**).

protective effect of presence of NS-iKIR NK cells was observed among CMV-seropositive patients. There are several possible explanations for this finding. One possibility is that there are not only quantitative but also qualitative differences between NS-iKIR NK cells depending on the CMV serostatus. Another possibility is that the relative antileukemic contribution by different effector cell populations may be context-dependent, and that other populations come into play in CMV-seropositive individuals. For example, we found high proportions of NKG2C$^+$ NK cells to be protective in CMV-seropositive but not in seronegative patients. High levels of NKG2C$^+$ NK cells were found both among NS-iKIR^{hi} and NS-iKIR^{low} patients, which limits our ability to clarify the effects of NS-iKIR cells.

Our study suggests that cytokine stimulation may activate autoreactive NS-iKIR NK cells to exert effector functions against leukemic cells. Adoptive transfer of allogeneic memory-like NK cells, generated by cytokine stimulation with IL12, IL15, and IL18 *ex vivo*, has shown promise in AML (50). The efficacy of this treatment has been attributed to a memory-like NK cell subset, but this cytokine regimen was reported to increase cytotoxicity of unlicensed NK cells (51). Thus, it remains to be determined if the reported clinical benefit is due to the highly differentiated cells or maybe also due to the more immature NS-iKIR NK cell subsets.

In the cohort of nontransplanted patients who did not receive postconsolidation immunotherapy, CMV serostatus did not affect clinical outcome. The control cohort included a larger fraction of adverse risk patients, and the distribution of these patients was skewed toward CMV$^+$ patients. However, no impact of CMV serostatus was observed in the control cohort when excluding high-risk patients. The results are compatible with the view that CMV-driven differentiation only becomes disadvantageous under proinflammatory conditions. In the absence of cytokine-based immunotherapy, NS-iKIR NK cells may remain largely inactive. A CMV-induced reduction of this subset of cells would then not adversely affect outcome, and other NK cell subsets, such as highly differentiated NKG2C$^+$ NK cells, may be more important effector cells.

In allo-transplanted AML patients, studies have reported CMV reactivation as associated with reduced relapse risk (4–6). The proposed mechanism of the protective effect seen at CMV reactivation is related to the expansion of adaptive NK cells (16) that exert a graft-versus-leukemia effect (17). In a KIR–HLA mismatch setting, adaptive donor NK cells may also display alloreactivity against leukemic blasts that lack an HLA ligand expressed in the donor, thereby allowing for NK cell–mediated killing of the leukemic blasts. In contrast to the above-mentioned reports suggesting that CMV infection may diminish the relapse risk of allo-transplanted AML patients, we observed that CMV seropositivity was associated with inferior survival in nontransplanted AML patients who received HDC/IL2 for relapse prevention. Notably, in reports from allogeneic transplantation settings, CMV reactivation, rather than CMV seropositivity, was associated with favorable outcome after allo-SCT (4). An explanation of these results could be that upon CMV reactivation, virus may infect leukemic blasts, inciting an ensuing antiviral response, including expansion of memory-like NK cells and T cells that may also contribute to relapse prevention in the transplantation setting (52).

In conclusion, our results indicate (i) that a past CMV infection predicts increased risk of relapse in AML patients receiving HDC/

Published OnlineFirst July 6, 2018; DOI: 10.1158/2326-6066.CIR-17-0711

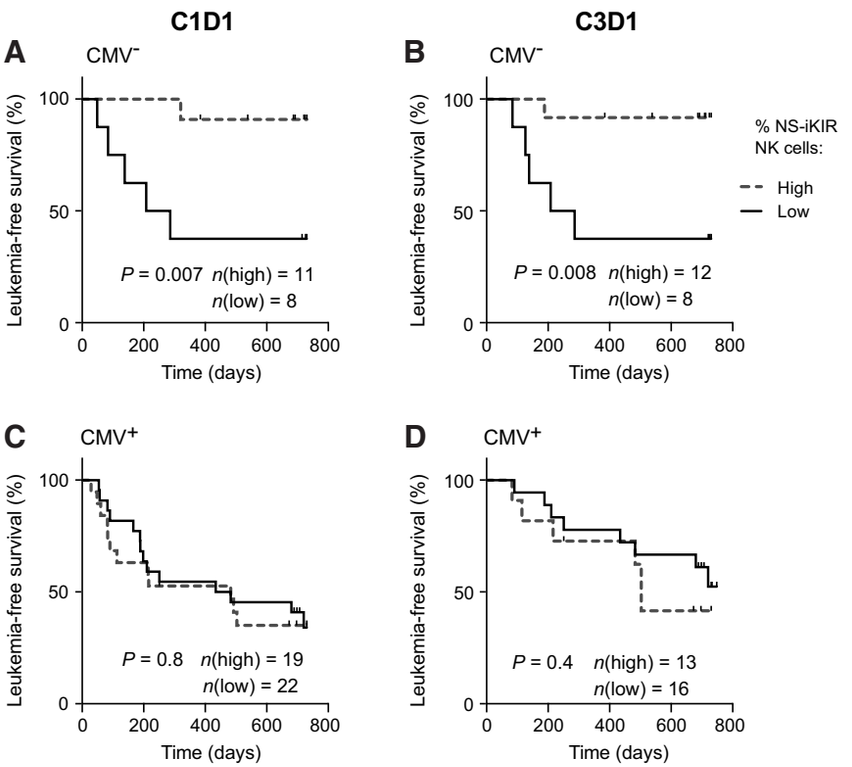

Figure 5.

Leukemia-free survival for AML patients receiving HDC/IL2 therapy with above or below median frequency of NS-iKIR NK cells before HDC/IL2 treatment start (C1D1; **A, C**) or at onset of third treatment cycle (C3D1; **B**, **D**), in patients who are CMV^- (**A**, **B**) or CMV^+ (**C**, **D**).

IL2 immunotherapy for relapse prevention and (ii) that CMV-induced depletion of NS-iKIR NK cells contributes to this effect. Our study lends support to the emerging concept that autoreactive NS-iKIR NK cells may constitute effector cells in cancer, and thus, further studies of strategies to harness NK cell autoreactivity are warranted.

Disclosure of Potential Conflicts of Interest

F.E. Sander, J. Aurelius, A. Martner, K. Hellstrand and F.B. Thoren are authors of issued or pending patents protecting the use of histamine dihydrochloride (HDC) in cancer immunotherapy. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: J. Aurelius, K. Hellstrand, F.B. Thorén
Development of methodology: E. Bernson, A. Hallner, F.E. Sander
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E. Bernson, A. Hallner, F.E. Sander, M. Nicklasson, K. Christenson, E. Aydin, J.-Å. Liljeqvist, M. Brune
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Bernson, A. Hallner, M.S. Nilsson, K. Christenson, E. Aydin, J.-Å. Liljeqvist, A. Martner, K. Hellstrand
Writing, review, and/or revision of the manuscript: E. Bernson, A. Hallner, F.E. Sander, M. Nicklasson, M.S. Nilsson, M. Brune, R. Foà, A. Martner, K. Hellstrand, F.B. Thorén
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Christenson
Study supervision: J. Aurelius, A. Martner, F.B. Thorén

Acknowledgments

This work was supported by Meda Pharma (Clinical Study Sponsor), the Swedish Research Council, Swedish Society for Medical Research (SSMF), Swedish Cancer Society, the Swedish state via the ALF agreement, Erna and Victor Hasselblad Foundation, IngaBritt and Arne Lundberg Foundation, Assar Gabrielsson Foundation, Lars Hierta Memorial Foundation, Lion Cancer Foundation West, Wilhelm and Martina Lundgren Research Foundation, BioCARE–a National Strategic Research Program, and the Sahlgrenska Academy at University of Gothenburg.

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Received December 11, 2017; revised April 13, 2018; accepted June 26, 2018; published first July 6, 2018.

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Published OnlineFirst July 6, 2018; DOI: 10.1158/2326-6066.CIR-17-0711

Cancer Immunology Research

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Elin Bernson, Alexander Hallner, Frida E. Sander, et al.

Cancer Immunol Res 2018;6:1110-1119. Published OnlineFirst July 6, 2018.