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Natural killer-cell counts are associated with molecular relapse-free survival after imatinib discontinuation in chronic myeloid leukemia: the IMMUNOSTIM study

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ABSTRACT

Despite persistence of leukemic stem cells, patients with chronic myeloid leukemia who achieve and maintain deep molecular responses may successfully stop the tyrosine kinase inhibitor imatinib. However, questions remain unanswered regarding the biological basis of molecular relapse after imatinib cessation. In IMMUNOSTIM, we monitored 51 patients from the French Stop IMatinib trial for peripheral blood T cells and natural killer cells. Molecular relapse-free survival at 24 months was 45.1% (95% CI: 31.44%-58.75%). At the time of imatinib discontinuation, non-relapsing patients had significantly higher numbers of natural killer cells of the cytotoxic CD56^{dim} subset than had relapsing patients, while CD56^{bright} natural killer cells, T cells and their subsets did not differ significantly. Furthermore, the CD56^{dim} natural killer-cell count was an independent prognostic factor of molecular-relapse free survival in a multivariate analysis. However, expression of natural killer-cell activating receptors, *BCR-ABL1*⁺ leukemia cell line K562-specific degranulation and cytokine-induced interferon-gamma secretion were decreased in non-relapsing and relapsing patients as compared with healthy individuals. After imatinib cessation, the natural killer-cell count increased significantly and stayed higher in non-relapsing patients than in relapsing patients, while receptor expression and functional properties remained unchanged. Altogether, our results suggest that natural killer cells may play a role in controlling leukemia-initiating cells at the origin of relapse after imatinib cessation, provided that these cells are numerous enough to compensate for their functional defects. Further research will decipher mechanisms underlying functional differences between natural killer cells from patients and healthy individuals and evaluate the potential interest of immunostimulatory approaches in tyrosine kinase inhibitor discontinuation strategies. (*ClinicalTrials.gov Identifier NCT00478985*)

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Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasia caused by the fusion of the *BCR* and *ABL1* genes, as the result of the acquired reciprocal t(9;22)(q34;q11) translocation. In the early 2000s, imatinib, the first ATP-competitive inhibitor of the BCR-ABL1 oncoprotein, revolutionized the management of CML, providing most patients a dramatic progression-free survival benefit.¹ Since then, newer generations of tyrosine kinase inhibitors (TKI) have been developed in order to overcome some of the drawbacks of imatinib, but imatinib remains one of the key initial therapies for newly diagnosed patients.²

When imatinib treatment is addressed appropriately, life expectancy of adult patients diagnosed with chronic-phase CML (CP-CML) is close to that of the general population.^{3,4} However, the current recommendation is to administer treatment lifelong because of the inability of imatinib and other TKI to eliminate quiescent leukemic stem cells.^{5,8} This recommendation represents a substantial challenge with respect to long-term safety, quality of life and economic burden. Therefore in the past few years, clinical trials have investigated the feasibility of discontinuing imatinib treatment in patients with sustained deep molecular responses. In the pioneering STIM trial, patients on imatinib therapy for a minimum of 3 years in whom *BCR-ABL1* transcripts were undetectable for at least 2 years had a probability of maintaining deep molecular responses without any treatment of about 40%, challenging the statement that TKI may never be stopped.⁹ These findings were rapidly corroborated by the independent TWISTER trial.¹⁰ However, a definitive cure remains uncertain in patients who do not relapse. Indeed, serial assessments with reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) showed that peripheral blood *BCR-ABL1* transcripts could be detected in patients who successfully stopped imatinib, albeit in low amounts.⁹ The use of genomic DNA-based PCR as a monitoring tool revealed that patients continued to harbor the *BCR-ABL1* gene after discontinuation of imatinib, even when the corresponding transcripts were undetectable.¹¹ In patients who had been off TKI therapy for several years, *BCR-ABL1* transcripts could be amplified in CD34⁺ cell-derived colony-forming cells and long-term culture-initiating cells despite undetectable residual disease in the peripheral blood.⁸ Altogether, these results indicate that a reservoir of primitive leukemic cells persists in most if not all TKI-treated patients regardless of outcome after treatment discontinuation.

There is great clinical interest in trying to identify patients who are more likely to succeed in discontinuing imatinib in order to minimize potential risks of a leukemic rebound and to avoid undesirable drug-withdrawal symptoms.¹² So far, the search for clinical variables predictive of outcome has been challenging but factors such as the Sokal score, duration of therapy, depth of molecular response and duration of deep molecular response have provided some insights into the probability of successful imatinib discontinuation in several studies.^{9,13,14} However, biological factors directing the fate of residual leukemic cells once TKI pressure is released are unclear. Given the susceptibility of CML to adaptive and innate immune cellular attack, an efficient autologous anti-CML response might help to control the leukemic load beyond cessation

of TKI treatment.^{15,16} We designed and conducted an ancillary biological study within the STIM trial, named IMMUNOSTIM, with the goal of analyzing peripheral blood T cells and natural killer (NK) cells and investigated whether immune parameters were associated with molecular relapse-free survival.

Methods

Patients

IMMUNOSTIM is a sub-study of the STIM trial approved by French health authorities (NCT00478985).⁹ Written informed consent was given in agreement with the Declaration of Helsinki. Imatinib was stopped after ≥ 3 years of therapy and ≥ 2 years of undetectable *BCR-ABL1* transcripts. Stringent monitoring by RT-qPCR was performed after imatinib discontinuation to detect a molecular relapse.⁹ The assay sensitivity was ≥ 4.5 log. Consecutively detectable peripheral blood *BCR-ABL1* transcripts showing a ≥ 1 log increase or loss of a major molecular response [*BCR-ABL1/ABL1* internationally standardized (IS) ratio $\leq 0.1\%$] defined molecular relapse and triggered imatinib resumption. In IMMUNOSTIM, heparinized blood was collected at baseline, bi-monthly for 6 months then every 6 months until 24 months unless imatinib was resumed. Healthy donors were recruited through the Paris Saint-Louis Blood Donation Center and gave informed consent. Experiments were performed in a centralized fashion, allowing ≤ 48 h from blood collection to processing.

Immunophenotyping

Patients' whole blood cell counts were determined using a Sysmex XS 1000i analyzer. T cells and NK cells were quantified by dual-platform flow cytometry using monoclonal antibodies recognizing CD3, CD4, CD127, CD25, CD8, CD45RA, CCR7, CD27, CD56, CD16 and NKG2D (*Online Supplementary Methods*). Peripheral blood mononuclear cells (PBMC) were purified with Ficoll density-gradient centrifugation and cryopreserved in liquid nitrogen. NK-cell receptor expression was studied by flow cytometry using thawed PBMC stained with monoclonal antibodies recognizing CD56, CD16, CD3, DNAM-1, KIR2D, NKp46, NKp30, NKG2A, CD94 and CD57 (*Online Supplementary Methods*).

Natural killer-cell functional assays

To assess degranulation, PBMC were thawed, maintained in 10% fetal calf serum-RPMI-1640 overnight at 37°C and incubated with or without the HLA class I-deficient *BCR-ABL1*⁺ leukemia cell line K562 at a 1:1 effector to target ratio for 18 h at 37°C with a CD107a monoclonal antibody. Thereafter, PBMC were stained with monoclonal antibodies recognizing CD137, CD56, CD3 and CD16. CD137⁺ and CD107a⁺ NK cells were detected by flow cytometry (*Online Supplementary Methods*). To study interferon (IFN)- γ production, thawed PBMC were activated overnight in medium supplemented with interleukin (IL)-12 and IL-18 (10 ng/mL each). The following day, brefeldin A was added for 4 h. IFN- γ -producing CD3⁺CD56^{bright}CD16^{low} NK cells were detected by flow cytometry as previously described (*Online Supplementary Methods*).¹⁷

Statistical analyses

A Mann-Whitney U-test, a Kruskal-Wallis test and a Wilcoxon matched-pairs signed ranked test were used to compare, respectively, quantitative variables from two independent groups, more than two groups with a Dunn test for multiple comparisons, and quantitative variables following imatinib discontinuation. Molecular relapse-free survival was estimated with the Kaplan-

Meier method.⁹ Clinical variables and NK cells were assessed as potential prognostic factors for molecular relapse-free survival. Quantitative factors were categorized into two groups with cut-offs set at the median. All variables were assessed by univariate analysis using the Kaplan-Meier method and the two-tailed log-rank test. A backward stepwise multivariate Cox proportional analysis was performed to determine the influence of variables potentially associated with outcome in univariate analysis ($P \leq 0.10$). In the final model, P values < 0.05 were considered statistically significant. Quantitative variables were categorized into two groups with cut-offs set at the median. Hazard ratios and 95% confidence intervals (CI) were estimated from the Cox regression analysis.

Results

Patients' characteristics

Fifty-one of the 100 patients enrolled in the STIM trial who agreed to participate to IMMUNOSTIM and from whom blood samples were received and processed within 48 h of collection were included; their baseline characteristics are detailed in Table 1. Thirty-three patients (64.7%) were females and the Sokal score at diagnosis of CP-CML was low in 28 (54.9%). Single-agent imatinib was given as first-line TKI therapy in all patients, either soon after the diagnosis of CML ($n=24$) or after resistance or intolerance to IFN- α ($n=27$). None of the patients had a history of allogeneic stem cell transplantation. The median duration of imatinib treatment was 57 months (range; 36-94). The median age at imatinib discontinuation was 63 years (range; 39-81) and the median follow-up after imatinib discontinuation was 63 months (range; 47-72). After treatment cessation, 28 patients (54.9%) experienced a protocol-defined molecular relapse and all restarted imatinib treatment. The median *BCR-ABL1/ABL1* IS ratio was 0.015% (range; 0.0004-0.201) at first detection of molecular relapse, 0.056% (range; 0.006-1.54) at confirmation of molecular relapse and 0.165% (range; 0.024-4.49) at imatinib resumption (Figure 1A). The median time from imatinib discontinuation to first detection of molecular relapse was 2 months (range, 1-20) and all molecular relapses but

one occurred before 6 months. The median time until imatinib resumption was 4 months (range, 3-29). At 24 months, molecular relapse-free survival was 45.1% (95% CI: 31.44%-58.75%) (Figure 1B). These findings were consistent with those of the entire STIM study population.¹⁸ The median follow-up for non-relapsing patients was 63 months (range, 48-72).

Leukocyte, T-cell and natural kill-cell counts at imatinib discontinuation

Leukocyte and lymphocyte counts at the time of imatinib discontinuation did not differ significantly between non-relapsing and relapsing patients (*Online Supplementary Table S1*). When CD3⁺ T cells, CD4⁺ and CD8⁺ T cells, naïve, central memory, effector memory CD4⁺ and CD8⁺ subsets and CD4⁺CD25⁺CD127^{low/-} regulatory T cells were analyzed, no significant differences were observed between non-relapsing and relapsing patients (*Online Supplementary Table S1*). In contrast, although CD3⁺CD56⁺ NK cells were within the range established in our laboratory using samples from healthy donors (*data not shown*), non-relapsing patients had significantly higher frequencies

Table 1. Baseline characteristics of the patients (n=51).

Parameters	Results (n=51)*
Median age (range)	63 years (39-81)
Female gender n, (%)	33 (64.7)
Sokal score at diagnosis n, (%)	
Low	28 (54.9)
Intermediate	18 (35.3)
High	4 (7.8)
Unknown	1 (2)
IFN- α intolerance or resistance prior to imatinib n, (%)	27 (52.9)
Median duration of imatinib treatment (range)	57 months (36-94)
Daily dose of imatinib n, (%)	
400 mg	44 (86.3)
<400 mg	4 (7.8)
Unknown	3 (5.9)
Median follow-up (range)	63 months (47-72)

*70 patients were enrolled in IMMUNOSTIM but only 51 were included. The 19 other patients were excluded because of missing baseline samples ($n=4$) or arrival of samples more than 48 h after blood was drawn ($n=15$).

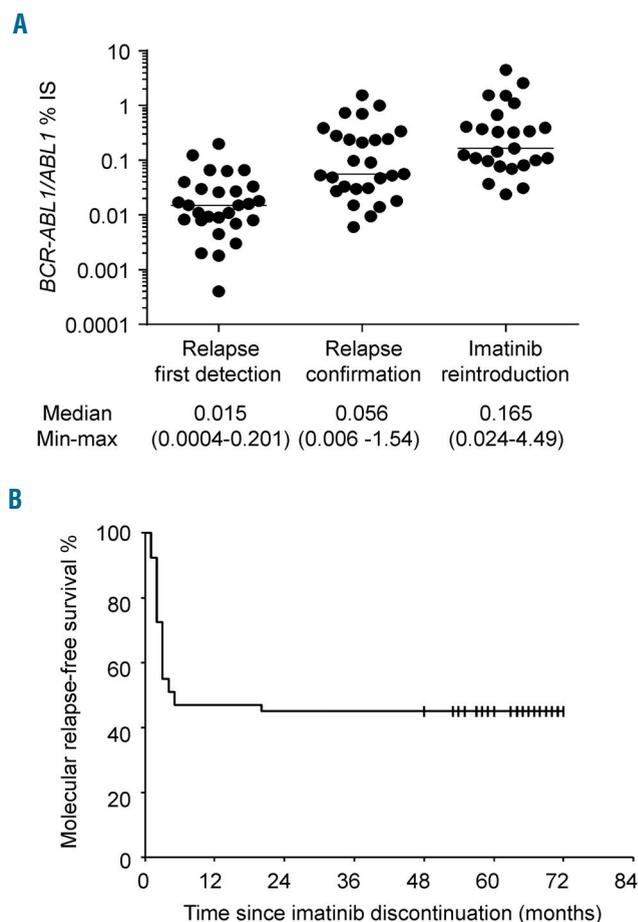


Figure 1. Evolution of *BCR-ABL1* transcripts in relapsing patients and molecular relapse-free survival. (A) Scatter dot plots represent *BCR-ABL1* IS % for each individual relapsing patient at first detection of relapse, relapse confirmation and at imatinib reintroduction; median values (horizontal bars) are also shown. (B) Kaplan-Meier estimate of molecular relapse-free survival defined as the time interval between imatinib discontinuation and first occurrence of molecular relapse or death, whichever came first. Data were censored at last molecular assessment for patients who were alive and had not relapsed.

and counts of CD3CD56⁺ NK cells than had relapsing patients, with a median count of 233/mm³ (range, 70-727) in the former and 145/mm³ (range, 67-450) in the latter ($P=0.014$) (Figure 2A,B). This finding was not explained by differences in age or imatinib doses between the two groups (*data not shown*). In addition, we did not find any association between CD3⁺CD56⁺ NK-cell counts and duration of imatinib therapy (*data not shown*). CD3⁺CD56⁺ NK cells were thus subtyped based upon cell-surface density of the adhesion molecule CD56 and the FcγRIII receptor CD16. Counts of the cytotoxic CD3⁺CD56^{dim}CD16⁺ population (hereafter named CD56^{dim}) were significantly greater in non-relapsing patients [median: 216/mm³ (range, 55-723)] than in relapsing patients [median: 139/mm³ (range, 58-438)] ($P=0.011$) and accounted for higher total NK-cell numbers in the former (Figure 2C). Indeed, counts of the cytokine-secreting CD3⁺CD56^{bright}CD16^{low} fraction (hereafter named CD56^{bright}) did not differ between the two groups of patients (Figure 2D).

Baseline prognostic factors for molecular relapse

In the STIM trial, the Sokal risk group and duration of imatinib treatment were associated with outcome following imatinib discontinuation.^{9,18} Here, clinical and biological variables and NK-cell counts at baseline were analyzed as potential prognostic factors for molecular relapse-free survival using univariate analysis. Patients with a low

Sokal score had a significantly higher molecular relapse-free survival rate than patients with an intermediate or high Sokal score ($P=0.017$) (Figure 3A). There was a trend toward a longer duration of imatinib treatment and higher estimated molecular relapse-free survival rate ($P=0.078$) (Figure 3B). Notably, patients with CD56^{dim} NK-cell counts higher than the median (>162/mm³) at baseline had a significantly higher molecular relapse-free survival rate than those with a lower CD56^{dim} NK-cell count ($P=0.0008$) (Figure 3C). No significant association with age, sex, or prior IFN- α therapy was found (Figure 3D-F). After multivariable analysis, CD56^{dim} NK-cell count was identified as an independent prognostic factor for molecular-relapse-free survival (Table 2). These findings led us to analyze NK cells further in the setting of immunoprofiling and functional experiments.

Natural killer-cell receptors and maturation marker at imatinib discontinuation

NK-cell receptors play a key role in recognizing targets and transducing activating or inhibitory signals upon binding to their ligands, thereby controlling cell function.¹⁹ We thus examined the expression of a large panel of NK-cell receptors and that of the carbohydrate antigen CD57, a marker linked to NK-cell terminal differentiation.^{20,21} No statistically significant differences were found on NK cells between non-relapsing and relapsing patients with respect

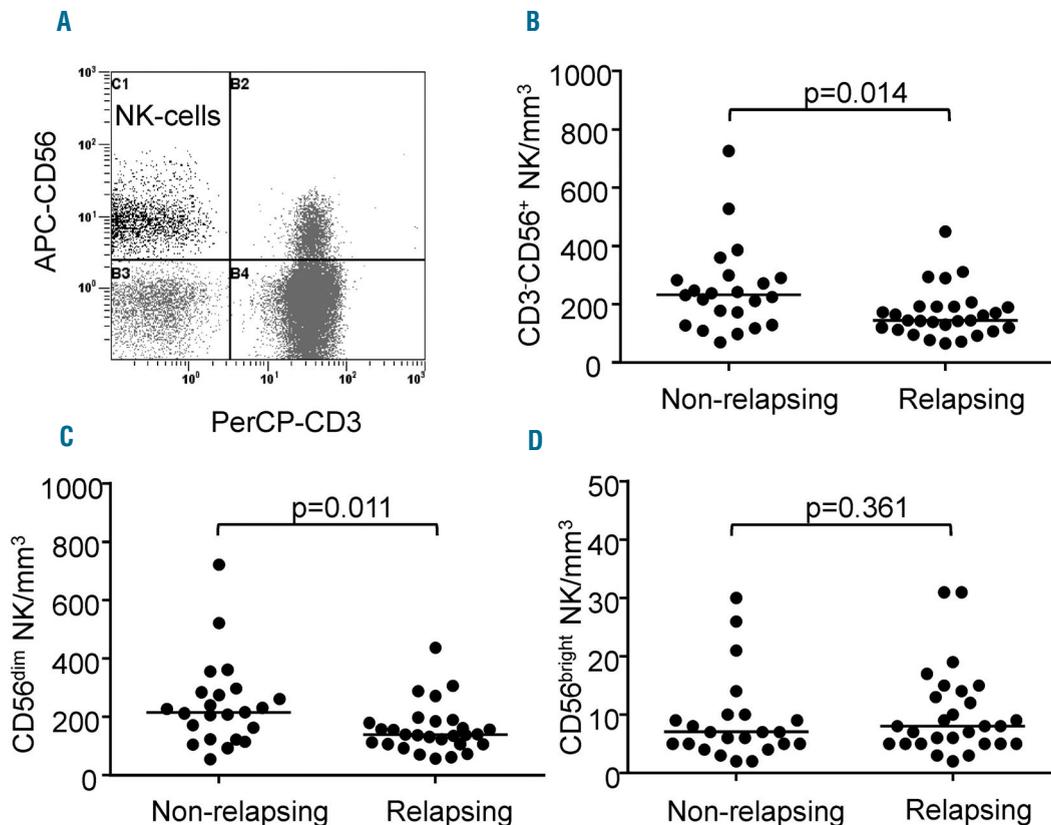


Figure 2. Natural killer cells at imatinib discontinuation in non-relapsing and relapsing patients. (A) Flow cytometry dot plot from a representative patient showing CD3CD56⁺ NK cells in the upper left quadrant after lymphocyte gating using the side and forward scatter display. (B) Scatter dot plots represent CD3CD56⁺ NK-cell counts for each individual non-relapsing and relapsing patient; median values (horizontal bars) are also shown. (C) Scatter dot plots represent CD3CD56^{dim} NK-cell counts for each individual non-relapsing and relapsing patient; median values (horizontal bars) are also shown. (D) Scatter dot plots represent CD3CD56^{bright} NK-cell counts for each individual non-relapsing and relapsing patient; median values (horizontal bars) are also shown. *P* values (by the Mann-Whitney U-test) are indicated for each panel.

to expression of the activating receptors CD16, NKG2D, NKp46, NKp30 and DNAM-1, the maturation marker CD57, the activating and inhibitory KIR2D isoforms and the C-type lectin inhibitory receptor NKG2A/CD94 (*data not shown*). However, when we compared NK-cell receptor expression profile of patients with that of healthy individuals, significant alterations were found. The proportions

of NK cells expressing the activating receptors NKp46 and DNAM-1 were significantly lower in the CD56^{dim} and CD56^{bright} subsets of non-relapsing and relapsing patients (Figure 4). In addition, the CD56^{bright} subset of non-relapsing and relapsing patients showed significantly increased KIR2D and CD57 and decreased NKG2A expression as compared to the expression in healthy donors (Figure 4).

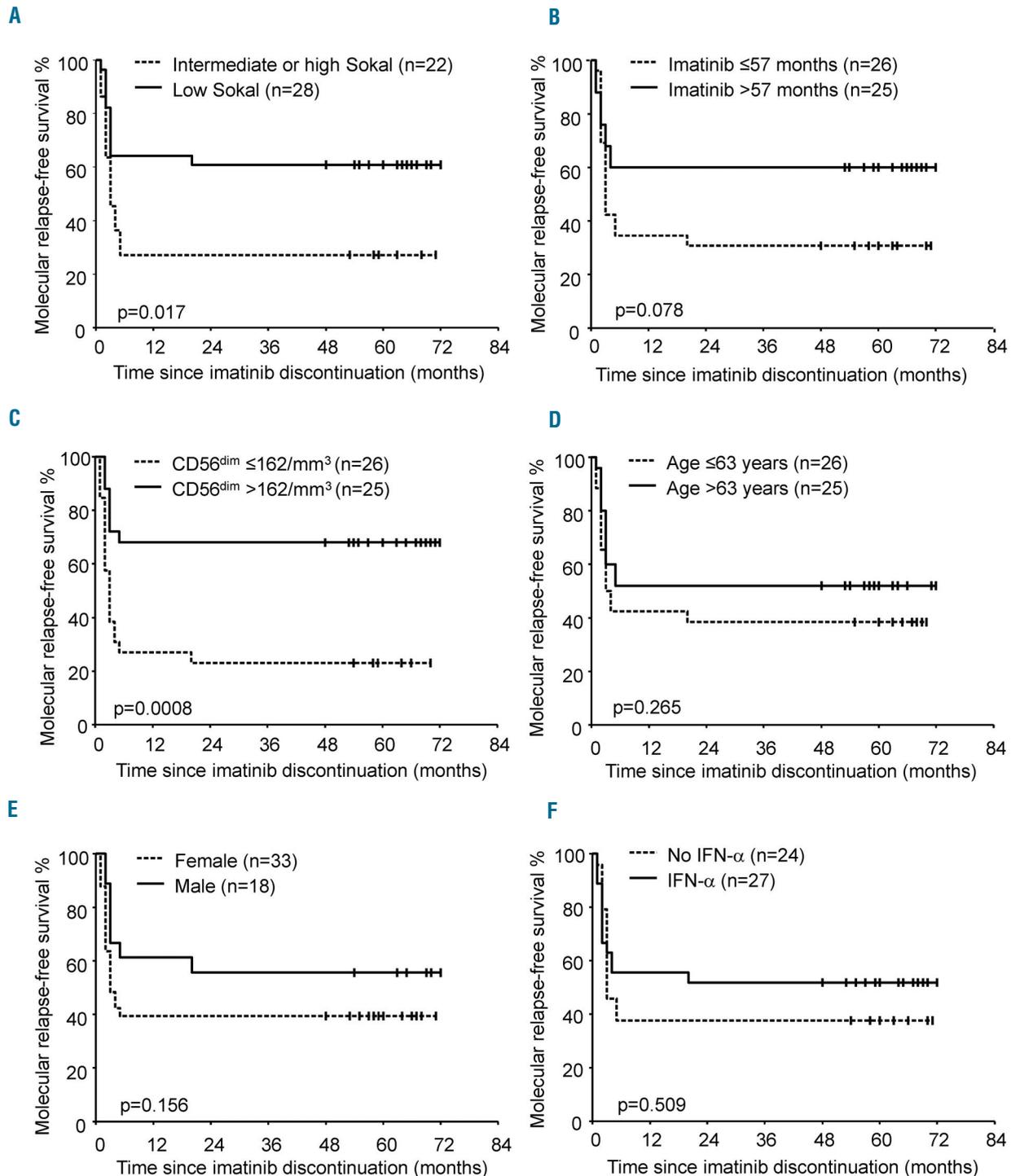


Figure 3. Molecular relapse-free survival after discontinuation of imatinib according to clinico-biological factors. (A) Sokal risk group, (B) imatinib treatment duration, (C) CD56^{dim} NK-cell counts at baseline, (D) age, (E) sex, and (F) prior exposure to IFN-α. For each survival plot, a corresponding log-rank P value is shown.

Natural killer-cell function at imatinib discontinuation

The capacity of NK cells to degranulate cytolytic vesicles was measured through the delocalization of the lysosomal-associated membrane protein-1 CD107a onto the cell surface after stimulation with the NK-sensitive *BCR-ABL1*⁺ cell line K562. This assay is considered a general indicator of NK activity and particularly of tumor cell lysis.²² Raw quantification of CD107a in the presence of K562 indicated a comparable degranulation in non-relapsing and relapsing patients and in healthy donors (Figure 5A). However, the propensity of NK cells for surface CD107a expression in the absence of K562 revealed significantly higher levels of spontaneous degranulation in both groups of patients (Figure 5A). Consequently, K562-specific degranulation of NK cells from non-relapsing and relapsing patients was significantly weaker than that of NK cells from healthy donors (Figure 5B). This finding was corroborated by a lower induction of the tumor necrosis factor receptor CD137 (also known as 4-1BB) upon K562 encounter in patients, a marker upregulated following NK-cell activation (Figure 5C). We also investigated the ability of the CD56^{bright} NK-cell subset to secrete IFN- γ after stimulation by IL-12 and IL-18. We found that IFN- γ production was comparable in non-relapsing and relapsing patients and significantly reduced compared to that in healthy donors (Figure 5D).

Natural killer cells after imatinib discontinuation

Several studies support the notion that TKI exert off-target effects on NK cells and other cells of the immune system.²³⁻²⁶ We thus wondered whether cell counts, receptor expression and functional features assessed at baseline could have been influenced at least in part by imatinib. To address this point, experiments were repeated after cessation of imatinib. Because most relapsing patients resumed imatinib within a short time frame (64.3% within 3 months and 85.7% within 6 months), follow-up samples obtained within 6 months after imatinib discontinuation were used, prior to imatinib reintroduction. After imatinib was stopped, median leukocyte and lymphocyte counts increased in non-relapsing and relapsing patients from 4810/mm³ (range, 2250-7860) to 5950/mm³ (range, 3760-10440) ($P < 0.0001$) and 1310/mm³ (range, 720-2610) to 1520/mm³ (range, 670-3060), respectively ($P < 0.0001$). A rise in NK cells also occurred in both groups of patients, from a median value of 179/mm³ (range, 67-727) at baseline to 205/mm³ (range, 74-736) after imatinib discontinuation ($P = 0.0011$). This rise was observed within both the CD56^{dim} subset [median count 164/mm³ (range, 55-723) at baseline and 195/mm³ (range, 62-723) after imatinib discontinuation ($P = 0.0032$)] and the CD56^{bright} fraction [median count 7/mm³ (range, 2-31) at baseline and 10/mm³ (range, 4-37) after imatinib discontinuation ($P = 0.0008$)]. Of note, CD3⁺ T cells also increased from a median value of 889/mm³ (range, 315-1949) at baseline to 990/mm³ (range, 265-1817) after imatinib discontinuation ($P = 0.0445$). Importantly, the comparison between groups after imatinib discontinuation showed that NK cells of the CD56^{dim} subset remained significantly higher in non-relapsing patients, with a median value of 249/mm³ (range, 85-723), than in relapsing patients, who had a median value of 148/mm³ (range, 62-442) ($P = 0.0179$) (Table 3). Immunophenotypic analyses revealed no significant modification in the expression of NKp46, NKp30, DNAM-1 and KIR2D after imatinib discontinuation in

either group of patients (Online Supplementary Figure S1A-D). A significant reduction in NKG2A⁺ NK cells was observed in non-relapsing patients but not in relapsing patients (Online Supplementary Figure S1E). The proportion of CD57⁺ NK cells was significantly decreased in relapsing patients (Online Supplementary Figure S1F). NK-cell degranulation and activation and IFN- γ production did not show any significant enhancement (Online Supplementary Figure S1G-I).

Discussion

The current principles of CML management rely on the use of life-saving TKI through the induction of an optimal response to prevent progression to blast crisis, followed by maintenance of the optimal response by means of lifelong TKI treatment.⁷ However, recent demonstrations that imatinib can be successfully stopped in a substantial proportion of patients with deep and durable molecular responses is currently modifying this view and avoiding lifelong dependency on TKI treatment may become the ultimate goal.²⁷⁻²⁹ While serial molecular monitoring is essential to detect a relapse rapidly after an attempt to discontinue TKI, in order to trigger medical intervention and avoid any negative impact on the patient's outcome, our ability to predict relapses accurately remains limited and determinants of relapse are unknown.³⁰

Here, we demonstrated that patients free from molecular relapse after imatinib discontinuation within the STIM trial had higher numbers of circulating NK cells than had patients who relapsed. In addition to CML-related variables, such as the Sokal score and imatinib treatment duration, a greater load of CD56^{dim} NK cells at the time of imatinib discontinuation was associated with a greater chance of treatment cessation being successful in univariate analysis. Although IFN- α has the ability to activate NK cells, no association was found between past exposure to IFN- α and molecular relapse-free survival, in agreement with what was previously found in the single-agent imatinib discontinuation studies STIM and TWISTER.^{9,10} Multivariable analysis enabled us to show that the amount of CD56^{dim} NK cells was the only independent prognostic factor for molecular relapse-free survival in this population of patients. Two recent and independent studies are in line with our results. Indeed in the DADI dasatinib discontinuation trial and in the EUROSKI imatinib cessation immunological sub-study, greatest peripheral NK-cell burden at the time TKI therapy was stopped was associated with a higher probability of treatment-free remission.^{31,32} Given the importance of NK cells in immune

Table 2. Potential prognostic factors of molecular relapse-free survival: multivariable Cox proportional analysis.

Variable	Molecular relapse-free survival over time (n=51)	
	Hazard ratio (95% CI)	P value
Sokal score* (n=50)	0.474 (0.219-1.024)	0.0576
Imatinib duration (n=51)	0.573 (0.256-1.286)	0.177
CD56 ^{dim} NK-cell counts (n=51)	0.292 (0.122-0.699)	0.0057

*The Sokal score was categorized as low versus intermediate and high. Quantitative variables were categorized into two groups with cut-offs set at the median. $P < 0.05$ was considered statistically significant.

surveillance against malignancies, these results and ours led us to hypothesize that NK cells and especially the differentiated and cytotoxic CD56^{dim} subset²⁰ might contribute to prevent overt CML relapse originating from residual leukemic cells, in a direct or indirect fashion, after imatinib discontinuation. The involvement of NK cells in immune surveillance against CML is also supported by

other lines of evidence. *BCR-ABL1*⁺ CD34⁺ cells from CML patients express NK activating receptor ligands.³³⁻³⁷ In addition, *BCR-ABL1*⁺ CD34⁺ cells from CML patients are sensitive to NK-cell-mediated lysis *in vitro*. Indeed, Sconocchia and colleagues³⁴ found that IL-2-stimulated NK cells from allogeneic HLA-matched siblings inhibited *BCR-ABL1*⁺ CD34⁺-derived colony-forming unit – granulocyte-

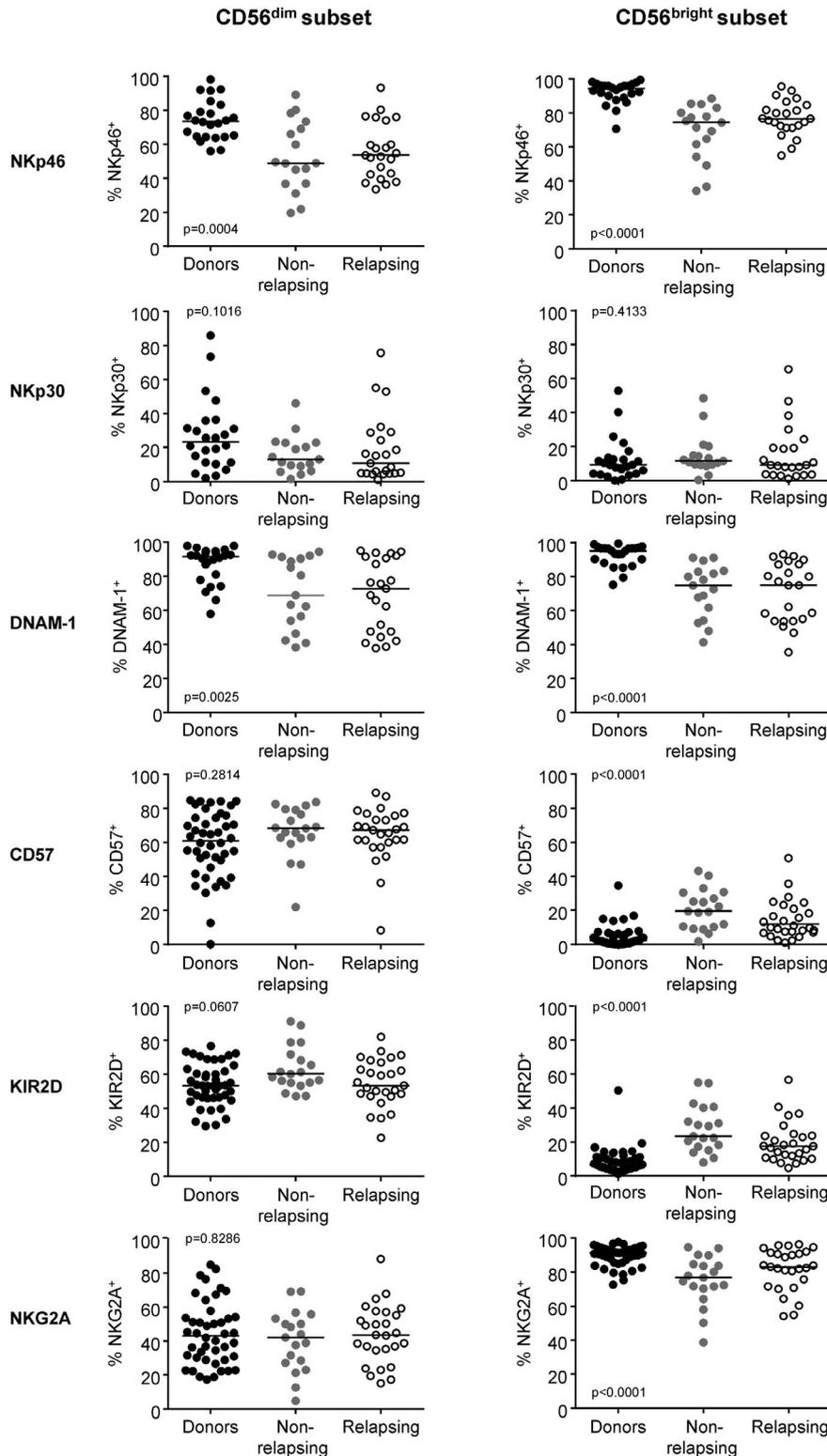


Figure 4. Expression of natural killer cell receptors in CD56^{dim} and CD56^{bright} natural killer cell subsets. Scatter dot plots for healthy donors (black dots, n=44), non-relapsing patients (filled gray dots, n=19) and relapsing patients (empty black dots, n=27) with median values (horizontal bars) are shown. Overall P value (by the Kruskal-Wallis test) is indicated for each panel.

macrophage growth in colony formation assays. Yong and colleagues³⁸ reported that primitive quiescent *BCR-ABL1*⁺ CD34⁺ stem cells were sensitive to lysis by NK cells from HLA-identical siblings, although to a lesser extent than cycling CD34⁺ leukemic cells. Cervantes and colleagues³⁹ observed that IL-2-activated NK cells were capable of suppressing autologous *BCR-ABL1*⁺ CD34⁺ cell-derived colony-forming cells and long-term culture-initiating cells. A further indication of a protective role of NK cells against CML is that early recovery of NK cells was able to predict a positive clinical outcome for patients undergoing T-cell-depleted allogeneic transplantation from HLA-identical

siblings.⁴⁰ Moreover during treatment with the NK-cell-stimulating cytokine, IFN- α , an association between the achievement of a complete cytogenetic response and NK-cell cytolytic activity was described.^{41,42} Finally, cross-talk between NK cells and other immune cells, such as dendritic cells, may potentiate anti-CML adaptive immune responses.^{43,44}

Despite striking quantitative differences, we found that the immunophenotypic and functional features of NK cells from non-relapsing and relapsing patients at the time of imatinib discontinuation were comparable. In addition, NK cells from both groups of patients differed phenotypically and functionally from those of healthy individuals. Expression of the activating receptors NKp46 and DNAM-1 was decreased. Overall degranulation upon encounter of K562 cells was preserved, as found in the immunological EUROSKI sub-study but contrary to EUROSKI, we were able to show lower activation marker induction and higher basal CD107a mobilization in patients, suggesting poorer K562-specific degranulation. These differences between the immunological EUROSKI sub-study and our study remain unexplained. A high level of spontaneous degranulation was described in other pathological settings, such as in human immunodeficiency virus infection, and was linked to chronic inflammation but its significance in well-controlled CML remains unclear.⁴⁵ Unfortunately, it was not possible to assess degranulation toward autologous leukemic cells because it is not routine practice to collect and store CD34⁺ *BCR-ABL1*⁺ cells at CML diagnosis, it is challenging to find and isolate very rare residual leukemic stem cells at imatinib discontinuation and this was not planned in our study. Finally, we found that IFN- γ production in response to cytokine stimulation in the CD56^{bright} subset was lower in patients irrespective of their relapse status. Although NK cells are not derived from the leukemic clone in CP-CML, deficient NK-cell function has been described by several groups.^{25,46,47} In newly diagnosed CP-CML prior to any treatment, NK-cell proliferation in response to the K562 leukemic cell line and IL-2 is reduced, as is degranulation triggered by K562 alone.²⁵

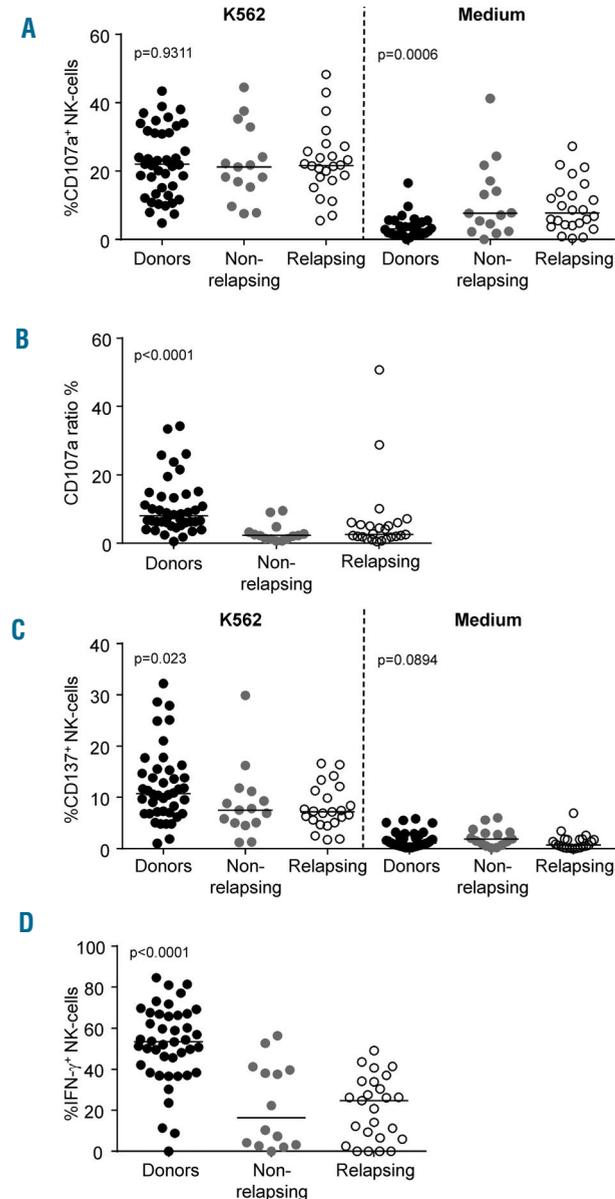


Figure 5. Natural killer-cell function. (A) NK-cell degranulation assay against K562 or medium control. (B) Target-specific degranulation estimated by the CD107a ratio. (C) NK-cell activation estimated by CD137 expression in the presence of K562 or medium control. (D) Production of IFN- γ upon stimulation with IL-12 and IL-18. Scatter dot plots for healthy donors (black dots, n=43), non-relapsing patients (filled gray dots, n=15) and relapsing patients (empty black dots, n=25) at baseline with median values (horizontal bars) are shown. Overall P value (by the Kruskal-Wallis test) is indicated for each panel.

Table 3. Leukocytes, lymphocytes and natural killer-cell subsets after imatinib discontinuation.

	Non-relapsing (n=23)	Relapsing (n=26)	P value*
Leukocytes /mm ³			
Median	6360	5465	0.072
Range	3760-10440	3920-7100	
Lymphocytes /mm ³			
Median	1580	1375	0.0134
Range	1140-3060	670-2500	
CD3 ⁺ CD56 ⁺ NK cells /mm ³			
Median	260	155	0.0205
Range	99-736	74-452	
CD56 ^{dim} NK cells /mm ³			
Median	249	148	0.0179
Range	85-723	62-442	
CD56 ^{bright} NK cells /mm ³			
Median	11	9	0.4455
Range	5-37	4-27	

*The Mann-Whitney U test was used to compare variables from non-relapsing and relapsing patients with a level of significance of 0.05. Median values and range (min-max) are shown.

These alterations are partially restored by imatinib, thereby suggesting that the disease itself has a deleterious impact on NK cells which persists beyond the imatinib-induced remission.²⁵

In our study, imatinib discontinuation was accompanied by a rapid and significant increase in leukocytes and lymphocytes, as also observed by Park and colleagues.⁴⁸ In addition, a significant increase in NK cells occurred. This finding is concordant with the inhibitory impact of imatinib on NK-cell expansion *in vitro*, although such an impact was described at higher concentrations than the trough plasma concentrations usually expected in patients treated with imatinib at a dose of 400 mg daily.²⁵ It is also consistent with the fact that patients in deep molecular response who have successfully stopped imatinib have higher NK-cell counts than those with a comparable level of response but who do not stop the drug.⁴⁹ However, it is important to note that in our study, non-relapsing patients maintained higher counts of NK cells than relapsing patients after imatinib cessation and that the imatinib dose prior to discontinuation was similar in non-relapsing and relapsing patients. Although imatinib plasma levels were not measured prior to discontinuation, this argues against a different drug exposure as the sole explanation for a lower NK-cell burden in relapsing patients. Other factors involved in NK-cell peripheral expansion, such as stromal cell factors or the immunoregulatory cytokine IL-15, need to be explored in future research studies.⁵⁰

In our experimental setting, discontinuation of imatinib did not result in the disappearance of NK-cell differences with healthy individuals, ruling out a major deleterious effect of the drug on NK-cell function. Expression of the activating receptors NKp46 and DNAM-1 remained unchanged. A significant decrease in NKG2A⁺ NK cells in non-relapsing patients was observed but there was no gain in cytolytic and CD56^{bright}-IFN- γ secretion capacities. Our study was not designed to decipher the underlying mechanisms of NK-cell defects in CML but several hypotheses can be made, including a negative impact of residual leukemic stem cells or their microenvironment on immune cell function or a weaker activation of the NK-cell repertoire due to HLA class I/KIR variation as compared to that in healthy individuals.⁵⁰ In the immunological

EUROSKI sub-study, individual KIR genes and haplotypes were not associated with imatinib discontinuation outcome but, unfortunately, comparisons with the general population were not performed.³²

To conclude, we provide evidence that NK cells are associated with outcome after imatinib discontinuation in CP-CML patients in deep molecular response. Beyond functional aspects, larger amounts of cytotoxic CD56^{dim} NK cells delivered to the leukemic reservoir in non-relapsing patients may play an important role in controlling residual CML-initiating cells and their progeny soon after cessation of imatinib treatment while a reduced CD56^{dim} compartment, and thus a lower effector to target cell ratio in relapsing patients, may leave less chance to counterattack in an efficient manner. Of course, our results do not preclude the role of additional biological aspects in dictating patients' outcome, such as the involvement of other immunological factors or functional heterogeneity of the residual *BCR-ABL1*⁺ hematopoietic stem-cell reservoir. Thus integration of NK-cell counts into the TKI discontinuation decision-making process is not reasonable at this stage. In addition, NK-cell alterations in patients suggest that the use of agents that stimulate NK-cell expansion or function, such as the IL-15 cytokine, or antibodies modulating NK-cell receptors may increase the chance of successfully TKI discontinuation, an aspect that may be addressed in future studies.

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