Silent NK/T cell reactions to dasatinib during sustained deep molecular response before cessation are associated with longer treatment-free remission

Takashi Kumagai1 | Chiaki Nakaseko2 | Kaichi Nishiwaki3 | Chikashi Yoshida4 | Kazuteru Ohashi5 | Naoki Takezako6 | Hina Takano7 | Yasuji Kouzai8 | Tadashi Murase9 | Kosei Matsue10 | Satoshi Morita11 | Junichi Sakamoto12 | Hisashi Wakita13 | Hisashi Sakamaki5 | Koiti Inokuchi14 | the Kanto CML, Shimousa Hematology Study Groups

Abstract
This study presents the final report of the multicenter, prospective tyrosine kinase inhibitor discontinuation study, D-STOP, after a 3-year follow-up of 54 patients with chronic CML who discontinued dasatinib after a sustained deep molecular response (DMR) for ≥2 years with dasatinib treatment. Estimated treatment-free remission (TFR) rates at 12 and 36 months were 63.0% [95% confidence interval (CI): 48.7-74.3] and 59.3% (95% CI: 45.0-71.0), respectively. CD3−CD56+ NK, CD16+CD56+ NK, and CD57+CD56+ NK large granular lymphocyte (NK-LGL), CD8+CD4− cytotoxic T cell, and CD57+CD3+ T-LGL cell numbers were relatively elevated throughout the 24-month consolidation only in failed patients who molecularly relapsed within 12 months. In successful patients, these subsets elevated transiently after 12 months, but returned to basal levels after 24-month consolidation. Therefore, smaller changes in NK/T, particularly the NK subset throughout consolidation, reflected higher TFR rates. TFR rates of those patients exhibiting elevation in CD3−CD56+ NK >376 cells/μL, CD16+CD56+ NK > 241 cells/μL, or CD57+CD56+ NK-LGL >242 cells/μL during consolidation compared with others were 26.7% (8.3%-49.6%) vs 78.3% (55.4%-90.3%), HR 0.032 (0.0027-0.38; P = .0064), 31.2% (11.4%-53.6%) vs 85.0% (60.4%-94.9%), HR 0.039 (0.0031-0.48; P = .011), or 36.8% (16.5%-57.5%) vs 77.3% (53.7%-89.8%), HR 0.21 (0.065-0.69; P = .010), respectively. Therefore, silent responses of T/NK subsets to dasatinib throughout consolidation were significant for longer TFR. Elevated NK/T, particularly NK lymphocytes responsive to dasatinib, may be immunologically insufficient to maintain TFR. Their decline, subsequently replaced by altered lymphocyte population with less response to dasatinib during sustained DMR, might be immunologically significant. (D-STOP, NCT01627132).
INTRODUCTION

Tyrosine kinase inhibitors (TKIs) markedly enhance the prognosis of patients with chronic myelogenous leukemia (CML), potentially enabling the attainment of a deep molecular response (DMR). The life expectancy of patients with CML has become closer to that of the general population. Subsequently, the discontinuation of TKI became imperative to evade adverse events and the financial burden of TKI therapy that many patients with CML experience.

In several studies, beginning with STIM1, nearly 40%-60% of patients with chronic CML who sustained long DMR could discontinue TKIs and attain long-term treatment-free remission (TFR). In addition, these studies proposed several predictive factors for successful discontinuation, including deeper molecular response, prolonged DMR duration, lack of prior TKI resistance, and higher numbers of natural killer (NK) cells before discontinuation.

In the Japanese multicenter prospective D-STOP trial, dasatinib was discontinued following a 2-year consolidation to sustain DMR in chronic CML to assess the TFR rate. Markedly, initial dasatinib specifically induces the elevation in lymphocytes, including large granular lymphocytes (LGL), NK cells, and cytotoxic T cells, as well as the decline in regulatory T cells (Tregs) in the early phase of treatment, related to early clinical responses. A recent study has reported a correlation between the larger NK cell count before cessation and successful TFR. Nevertheless, lymphocyte variations by dasatinib during sustained DMR before cessation is an area of growing interest. Previously, we reported that the more NK cells increased during dasatinib consolidation before discontinuation, the less successful would be TFR.

This study aims to present the final results of the D-STOP trial, including the peripheral NK/T cell change during dasatinib consolidation associated with successful TFR.

MATERIALS AND METHODS

Study design and patients

The D-STOP trial (NCT01627132) is a multicenter, single-arm, phase 2 study conducted at 22 centers in Japan. In this trial, patients diagnosed with chronic-phase CML who attained DMR after receiving any TKI were eligible for 2-year dasatinib consolidation therapy to sustain DMR. The inclusion criteria were as follows: age ≥ 15 years; performance status [Eastern Cooperative Oncology Group score] of 0-2; and no severe primary organ dysfunction involving the liver, kidneys, or lungs. Of note, all prior treatments were permitted, except for allogeneic hematopoietic stem cell transplantation. The exclusion criteria were as follows: other chromosomal abnormalities besides the Philadelphia chromosome, a history of BCR-ABL1 mutation, and/or other active malignant disorders. The D-STOP trial was approved by the Ethics Committees of all participating institutes. We obtained written informed consent from all participants per the Declaration of Helsinki guidelines. Between February 1, 2012, and January 31, 2014, 60 patients with a confirmed DMR were enrolled in the dasatinib consolidation phase.

Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

During the dasatinib consolidation, we performed real-time quantitative RT-PCR (RQ-PCR) every 3 months in the central laboratory [Bio Medical Laboratories (BML), Tokyo, Japan] to assess molecular responses based on the BCR-ABL1 International Scale (IS) and the laboratory’s conversion factor, as described previously. Briefly, we used ABL1 as an internal control, and the cutoff corresponded to BCR-ABL1 of 0.0069% IS or molecular response of 4.0 (a detectable disease with a BCR-ABL1 < 0.01% IS or undetectable disease in cDNA with > 10 000 ABL1 transcripts). Subsequently, patients with DMR confirmation every 3 months during 2-years consolidation entered the discontinuation phase. Following dasatinib cessation, DMRs were monitored by RQ-PCR every month for the first year (clinical cutoff), followed by every 3 months for the remaining 2 years (a total of 3 years).

An additional RQ-PCR was performed within 1 month if BCR-ABL1 > 0.0069% IS was detected at any point during the consolidation or discontinuation phase. Two successive BCR-ABL1-positive results established a molecular relapse. For relapses during the discontinuation phase, dasatinib was restarted at the previously effective dose. After dasatinib recommencement, we assessed the...
response by RQ-PCR after 1, 3, 6, and 12 months. Of note, dasatinib dose reduction was allowed at any time in response to adverse events at the physicians’ discretion.

2.3 | Flow cytometric analysis

We established the whole peripheral blood lymphocyte profiles by the central laboratory (BML) before and after 3, 6, 12, and 24 months of dasatinib consolidation. In addition, blood samples were collected more than a few hours after dasatinib intake. We measured peripheral white blood cell counts using an automated cell count analyzer. Flow cytometry methods are detailed elsewhere.\textsuperscript{13,15} Briefly, the lymphocyte fraction was ascertained using forward-scatter vs side-scatter gating (Figure S1B).\textsuperscript{13,15} and immunophenotypic examinations were performed using two-color or three-color flow cytometry on a FACSCalibur System with CellQuest software, version 3.3 (Becton Dickinson, Franklin Lakes, NJ). All antibodies used in this study were purchased from Becton Dickinson. The defined lymphocyte subsets comprised NK cells (CD3\textsuperscript{−}CD56\textsuperscript{+} and CD16\textsuperscript{−}CD56\textsuperscript{+} cells), NK-LGL (CD57\textsuperscript{−}CD56\textsuperscript{+}), helper T cells (CD4\textsuperscript{+}CD8\textsuperscript{−}), cytotoxic T cells (CD4\textsuperscript{−}CD8\textsuperscript{+}), T-LGL (CD57\textsuperscript{−}CD3\textsuperscript{+}), and Tregs (CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{low})\textsuperscript{17} (Figure S1B).

2.4 | Endpoints

In this study, the primary endpoint was the TFR rate at 12 months, defined as the percentage of patients with no dasatinib treatment without molecular relapse. To establish the TFR-related predictive factors, we assessed patients by sex, age at discontinuation, Sokal risk score at diagnosis, duration of BCR-ABL1 transcript negativity before consolidation, TKI therapy duration before consolidation, total dasatinib dose, and type of TKI used when DMR was attained. In addition, we assessed the above-described lymphocyte subsets before and after 3, 6, 12, and 24 months of dasatinib consolidation while in treatment-free survival (TFS). Furthermore, safety was assessed throughout the consolidation period, and adverse events were classified using the Common Terminology Criteria for Adverse Events, version 4.0.

2.5 | Statistical analysis

In this study, we determined a sample size of, at least, 50 patients to illustrate that patients who discontinued dasatinib remained in TFS at a power > 80%, when compared with data from a prior study.\textsuperscript{6} We separated each continuous variable into 2 groups using the cutoff points evaluated by the concordance index.

Using Kaplan-Meier analysis, we calculated the proportion of patients in TFR; a log-rank test was used to statistically compare the stratified groups (2 or more). In addition, Cox proportional hazards analysis of significant predictors in the univariate analysis was used to evaluate the factors contributing to successful discontinuation. Strongly correlated explanatory variables independently entered into the Cox regression model. Furthermore, factors significant in, at least, one of the tested models were considered possible independent predictors of relapse risk.

We generated receiver-operating characteristic (ROC) curves to evaluate the cutoff values of the cell number change in each lymphocyte subset and clinical data for the Kaplan-Meier analysis. Optimal thresholds along the ROC curves were ascertained by searching for plausible values where the sum of the sensitivity and specificity were maximized. We considered a P-value < .05 as statistically significant. All statistical analyses in this study were performed using the statistical software EZR on R commander (R Project for Statistical Computing, Vienna, Austria).\textsuperscript{18}

3 | RESULTS

3.1 | Patients’ characteristics

All patients in this study were followed up for ≥ 3 years after dasatinib discontinuation. Of 60 patients, 6 were excluded during consolidation because of consent withdrawal or variations in the BCR-ABL1 transcript levels suggestive of molecular relapse. The safety analyses revealed no severe (grade ≥ 3) treatment-related toxic effects during the consolidation phase. Overall, 54 patients (32 males and 22 females) were enrolled in dasatinib discontinuation (STOP) phase as described earlier.\textsuperscript{15} At treatment discontinuation, the median age of patients was 56 years (range: 27-84 years). In addition, the median duration of TKI treatment was 92 months (36-177 months), and the median duration of BCR-ABL1 negativity before treatment cessation was 51 months (24-173 months). All patients were followed up for 36 months after discontinuation. Overall, 34, 19, and 1 patient(s) used imatinib, dasatinib, and an unknown agent, respectively, when attaining DMR before consolidation. Notably, no patient received interferon-α.

3.2 | TFS based on the patients’ characteristics

The estimated overall probabilities of TFS at 12 and 24 months were 63.0% [95% confidence interval (CI): 48.7-74.3] and 59.3% [95% CI: 45.0-71.0], respectively (Figure 1). Overall, 22 patients experienced relapses during discontinuation, and 20 patients relapsed within 7 months, except for 2 who relapsed 18 and 21 months after discontinuation. We observed the loss of major molecular response (MMR) in 20% of relapsed patients. All 22 relapsed patients responded to dasatinib retreatment within 3 months, thereby encompassing DMR achievement within 3, 6, 9, and 12 months in 13, 5, 2, and 2 patients, respectively.
In addition, we analyzed the clinical factors affecting molecular relapse during discontinuation (Table 1). We observed no significant correlation between the 12-month TFS rate and patients’ characteristics such as sex, Sokal score at diagnosis, BCR-ABL1 mRNA-negative duration, TKI treatment duration, age at discontinuation, total dasatinib dose, and type of TKI used when DMR was attained by the univariate analysis.

3.3 | Clinical data during D-STOP study

Adverse effect during consolidation has been already described.\textsuperscript{15} No patients withdrew from the study because of adverse effects before discontinuation. During the discontinuation phase, the adverse events ≥ G3 included gastrointestinal bleeding from colon diverticulum with anemia (G3) in 1 patient. Two arthralgia of extremities (G2) occurred in 2 patients, which may be considered TKI withdrawal syndrome.\textsuperscript{19}

3.4 | Lymphocyte changes during consolidation therapy

We assessed the lymphocyte changes conducted by dasatinib during consolidation. We defined TFR-successful (S) or TFR-failed (F) patients as those who had kept TFR > 12 months or not > 12 months. While the lymphocyte counts did not differ after consolidation between the S and F groups (P = .24), the relative lymphocyte counts were significantly increased only in the F group (P = .0026\textsuperscript{*}; Figure 2A).

Accordingly, we further assessed cell number changes in CD3\textsuperset{+} CD56\textsuperset{+} NK, CD16\textsuperset{−} CD56\textsuperset{−} NK, CD57\textsuperset{−} CD56\textsuperset{−} NK-LGL, CD8\textsuperset{−} CD4\textsuperset{+} cytotoxic T, CD4\textsuperset{−} CD8\textsuperset{−} helper T, CD57\textsuperset{−} CD3\textsuperset{+} T-LGL, and CD4\textsuperset{−} CD25\textsuperset{−} CD127\textsuperset{low} Treg cells during consolidation.

3.5 | Cell number changes in various T/NK cell populations during consolidation

In this study, the cell number of CD3 CD56\textsuperset{+} NK cells after 24-month consolidation was significantly higher than the baseline before consolidation only in failed (F) patients (P = .0056\textsuperscript{*}) but not successful (S) patients (P = .98). However, the number after 12-month consolidation in successful patients was higher than the baseline (P = .0094\textsuperscript{*}; Figure 2B). We observed similar tendencies in CD16\textsuperset{−} CD56\textsuperset{−} NK and NK-LGL cells (Figure 2C and D).

In addition, the cell number of CD8\textsuperset{−} CD4\textsuperset{−} cytotoxic T cells after 24-month consolidation was higher than the baseline before consolidation only in failed (F) patients (P = .00024\textsuperscript{*}) but not successful (S) patients (P = .41), however the number after 12-month consolidation in successful patients was higher than the baseline (P = .0014\textsuperscript{*}; Figure 2E). Notably, the results were similar in CD57\textsuperset{−} CD3\textsuperset{+} T-LGL cells (Figure 2F). Furthermore, CD4\textsuperset{−} CD8\textsuperset{−} helper T cells did not change during consolidation in both S and F patients (Figure 2G). The changes in numbers of CD4\textsuperset{−} CD25\textsuperset{−} CD127\textsuperset{low} Tregs tended to decline in both groups, although the decline in the S group was significant. (Figure 2H). In summary, CD3\textsuperset{−} CD56\textsuperset{−} NK cells, CD16\textsuperset{−} CD56\textsuperset{−} NK, and NK-LGL, as well as cytotoxic T and T-LGL cells, were relatively increased throughout 24-month consolidation only in F patients. In S patients; this relative increment transiently occurred after 12 months, but returned to the basal level after 24-month consolidation (Figure 2B–F).

Moreover, patients with a higher elevation in CD3\textsuperset{−} CD56\textsuperset{−} NK > +376 (\mu L), CD16\textsuperset{−} CD56\textsuperset{−} NK > +241 (\mu L), and NK-LGL cell counts > +242 (\mu L) after consolidation exhibited a significantly higher TFR rate after 12 months than others (P = .00096*, 0.00054*, and 0.00014*, respectively) by univariate analysis (Table 1). These cutoff values were evaluated by ROC analysis. Likewise, those with the higher cell number elevation in CD8\textsuperset{−} CD4\textsuperset{−} cells > +212 (\mu L) had higher TFR than others (P = .033\textsuperscript{*}).

Multivariate analysis using factors of BCR-ABL1-negative duration (>50 months), elevation in cytotoxic T cells during consolidation (> +212), and increase in NK cell subsets during consolidation (CD3\textsuperset{−} CD56\textsuperset{−} NK > +376, CD16\textsuperset{−} CD56\textsuperset{−} NK > +241, or NK-LGL > +242) were subsequently performed. A higher elevation in each NK cell subset correspondingly exhibited significantly higher TFR rates (P = .0064\textsuperscript{*}, .011\textsuperscript{*}, and .010\textsuperscript{*}, respectively; Table 1 and Figure 3A-C). Higher elevation in cytotoxic T cells tentatively correlated with longer TFR, but not significantly (Figure 3D).

4 | DISCUSSION

For successful TKI discontinuation after sustained DMR in CML, some clinical factors, such as deeper molecular response, lengthier consolidation, and no prior resistance to TKI, are reported favorably.\textsuperscript{8,12} However such clinical factors were not always the same among studies because of their different clinical settings. The background conditions, such as immunological and genetic factors in CML, and...
**TABLE 1**  Statistical analysis for factors affecting treatment-free remission rates

| Factors                              | TFR (%) (at 12 mo) | 95% CI       | Univariate analysis | Multivariate analysis |
|--------------------------------------|--------------------|--------------|---------------------|-----------------------|
|                                      |                    |              | HR 95% CI           | P-value  | HR 95% CI | P-value |
| Clinical factors                     |                    |              |                     |          |           |         |
| Gender                               | M                  | 56.2         | 37.6-71.3           | 1.83     | 0.70-4.77 | .19     |
|                                      | F                  | 72.7         | 49.1-86.7           | 1.00     |            |         |
| Age at discontinuation               | >56 y              | 62.1         | 42.1-76.9           | 1.00     | .85       |         |
|                                      | <56 y              | 64.0         | 42.2-79.4           | 0.92     | 0.38-2.22 |         |
| Social score                         | Low                | 66.7         | 44.3-81.7           | 1.00     | .69       |         |
|                                      | Intermediate or High| 58.8       | 32.5-77.8           | 1.22     | 0.44-3.36 |         |
| Duration of TKI treatment            | >100 mo            | 78.9         | 53.2-91.5           | 1.00     | .18       |         |
|                                      | ≤100 mo            | 60.7         | 40.4-76.0           | 2.07     | 0.66-6.51 |         |
| Total dose of dasatinib              | ≤72 540 mg         | 69.6         | 46.6-84.2           | 0.74     | 0.28-1.96 | .525    |
|                                      | >72 540 mg         | 63.0         | 42.1-78.1           | 1.00     |           |         |
| BCR-ABL1 mRNA-negative duration      | >50 mo             | 77.8         | 51.1-91.0           | 1.00     | .122      | 1       |
|                                      | ≤50 mo             | 55.9         | 37.8-70.6           | 2.23     | 0.74-6.73 | 2.35    |
| Type of TKI used at achieving DMR    | Imatinib           | 61.8         | 43.4-75.7           | 1.09     | 0.43-2.73 | .85     |
|                                      | Dasatinib          | 63.2         | 37.9-80.4           | 1.00     |           |         |
| Increase in the cell number of the lymphocyte subset during consolidation (cells/µL) | | | | | | |
| CD3 CD56⁺NK cells                    | >+376              | 26.7         | 8.3-49.6            | 1.00     | .00096*   | 1       |
|                                      | <=+376             | 78.3         | 55.4-90.3           | 0.22     | 0.074-0.63| 0.032   |
| CD16 CD56⁺NK cells                   | >+241              | 31.2         | 11.4-53.6           | 1.00     | .00054*   | 1       |
|                                      | <=+241             | 85.0         | 60.4-94.9           | 0.15     | 0.04194-0.5515| 0.039 |
| CD57 CD56⁺NK-LGL cells               | >+242              | 36.8         | 16.5-57.5           | 1.00     | .0053*    | 1       |
|                                      | <=+242             | 77.3         | 53.7-89.8           | 0.26     | 0.092-0.76| 0.21    |
| CD8 CD4⁺CTL cells                    | >+212              | 41.2         | 18.6-62.6           | 1.00     | .033*     | 1       |
|                                      | <=+212             | 76.5         | 48.8-90.4           | 0.32     | 0.10-1.03 | 0.1759 |
| CD57 CD8⁺ T-LGL cells                | >+141              | 41.7         | 15.2-66.5           | 1.00     | .153      |         |
|                                      | <=+141             | 70.8         | 48.4-84.9           | 0.49     | 0.17-1.40 |         |
| CD4⁺CD8⁻ helper T cells              | >-47               | 63.2         | 37.9-80.4           | 1.00     | .785      |         |
|                                      | <=-47              | 58.8         | 32.5-77.8           | 1.15     | 0.40-3.28 |         |
surrounding cells should support these clinical factors. Recent studies have reported that immune effector recoveries, including NK and T cells, during sustained DMR under TKI are critical for TFR, as it might be used for immune surveillance against molecular relapse.20,21

Regarding lymphocyte responses to dasatinib, this study characterized the differences between those with or without successful TFR (>12 months). In F patients with TFR ≤ 12 months, NK subsets, including CD3−CD56+ NK, CD16+CD56+ NK, and CD57+CD56+ NK-LGL, and T subsets, including CD8+CD4− cytotoxic T cells and CD57+CD3− T-LGL, increased relatively during the 24-month consolidation, although those in S patients exhibited no significant changes after consolidation. Markedly, even in S patients, those subsets also increased transiently after 12-month consolidation (Table 2).

Previously, we have reported the relative elevation in lymphocyte subsets, including CD14−CD56+ NK, CD57+CD56+ NK-LGL, CD8+CD4− cytotoxic T cells, and CD57+CD3− T-LGL, before attaining
DMR after initial dasatinib treatment as the second-line therapy after imatinib. In the D-STOP trial, the lymphocyte changes in F patients corroborated those patients in the report with large reactions to dasatinib (Table 2). The NK/T lymphocytes at diagnosis reportedly expanded clonally by initial dasatinib.

To date, the antileukemic functions of NK/T lymphocyte subsets remain partially elucidated. Perhaps, these proliferated lymphocytes might have a strong immunological effect on leukemic cells because early lymphocytosis correlated with an early clinical response such as attainment of DMR.

This study raised the question whether such elevated NK/T lymphocytes were really significant to maintain TFR. Even in patients with sustained DMR after TKI discontinuation, BCR-ABL1 DNA was detected, which may harbor minimal residual leukemia. Most primitive quiescent CML cells appear to be inherently resistant to TKI, and leukemic cell progressively remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche that impairs normal hematopoiesis and favors leukemic stem cell function. Furthermore, immune effector responses were downregulated in CML patients at diagnosis. Failure to eradicate primitive quiescent CML cells may result in reinitiating malignancy after a period of latency. However, the immune system is not permanently compromised in CML. Effector responses had been restored to normal levels in CML patients who achieved MMR and DMR on TKIs compared with those at diagnosis. This suggested that there are staged recoveries of some immune responses in CML patients on TKI that may be linked to restrain quiescent CML cells.

The NK/T cells that expanded in response to dasatinib from diagnosis reportedly had antileukemic effects against CML cells, but had not been proven to have antileukemic effects against primitive quiescent CML cells. Sufficient restoration of normal immune effectors that are capable of suppressing primitive quiescent CML cells during DMR may be required for successful TFR. Dasatinib-responsive NK/T cells that still remained during DMR may indicate that such immune reconstitution has not been completed in failed patients.

Perhaps, lymphocytosis by initial dasatinib occurs because of its possible off-target effects. These were only in parallel with its direct action to BCR-ABL1 and early lymphocytosis and could suggest an early response to dasatinib, which has been already reported as good prognosis for other TKIs. In addition, lymphocytes in S patients had different, more silent, reactions to dasatinib after the 24-month consolidation from those of F patients. This lymphocyte population should work functionally to suppress the primitive leukemic cell expansion, preventing molecular relapse after discontinuation. Markedly, even in S patients, lymphocyte reactions to dasatinib after 12-month consolidation were much higher, corroborating those of F patients (Table 2). This might suggest that, during consolidation, the lymphocyte population with the higher response to dasatinib changed to give a lower response to dasatinib as observed in S patients after 24-month consolidation. Reportedly, such immunological reconstitution was needed for successful TFR and, thus, TFR tended to be more successful with the adequately longer period of sustained DMR.

In this study, some patients had already taken dasatinib before consolidation whereas others had taken imatinib. This difference might affect lymphocytic reactions to dasatinib during consolidation. To evaluate this, we presented lymphocyte counts (Figure 2A and their fractions including CD3+CD56−, CD57+CD56− NK, and CD8+CD4− T cells from both TFR-successful (TFR > 12M) and TFR-failed (TFR ≤ 12M) patients (Figure S2). After consolidation, CD3+CD56− and CD57+CD56− NK fractions were significantly higher in failed patients whereas the CD8+CD4+ T cell fraction was not. These results were consistent with this study.

In D-STOP, patients with ≤ +242 CD3+CD56− NK cells or ≤ +376 NK-LGL during consolidation had a significantly higher TFR (12M) rate compared with other patients (78.3% vs. 26.7% [P = .0064] and 77.3% vs 36.8% [P = .010], respectively) (Table 1).

Patients who had taken imatinib (imatinib → dasatinib) or dasatinib (dasatinib → dasatinib) before consolidation with ≤ +242 NK-LGL during consolidation had a higher TFR (12M) rate than those patients with > +242 NK-LGL (91.3% vs. 33.3% [P = .00643] and 72.7% vs NA [P = .001], respectively) (Figure 4A and B). Patients who had taken imatinib or dasatinib before consolidation with ≤ +376 NK-LGL during consolidation tended to have a higher TFR rate than those with > +376 NK-LGL (84.6% vs. 38.5% [P = .06] and 80.0% vs. 33.3% [P = .07], respectively) (Figure 4C, D). The results in each population were comparable with this study. Therefore, the type of TKI (imatinib or dasatinib) before consolidation did not affect our results.

We considered here the reasons for differences in D-STOP from findings in other discontinuation studies.

In this study, CD56− NK cells were rapidly downregulated after dasatinib discontinuation, as seen in the DADI study (Imagawa,
personal communication), and NK cells in successful patients seemed to become higher than those in failed patients without dasatinib treatment. Therefore, after elimination of dasatinib-induced elevated NK cells after cessation, NK cell states seemed similar to that found for imatinib discontinuation.

Of note, most NK cells expanded by dasatinib exhibited CD56 bright expression on their cell surfaces (Figure S1), this result differs from the findings for immunologically powerful CD56 dim NK cells, as reported in imatinib discontinuation. An imatinib discontinuation study reported higher NK cells just before discontinuation.
in S patients, but only higher CD56<sup>dim</sup> functional NK cells resulted in a higher TFR rate, and, in contrast, higher immature CD56<sup>bright</sup> NK cells correlated with a lower TFR rate. Therefore, we strongly suspected that the number of CD56<sup>dim</sup> NK cells could be higher in successful patients than in failed patients.
To explore the difference between D-STOP and DADI, we analyzed patients who achieved DMR by dasatinib after imatinib intolerance or resistance before consolidation in D-STOP, because this population was similar to that of DADI. Interestingly, CD56+ NK cell count tended to be higher during consolidation in successful patients than failed patients in this population, consistent with DADI. This was not significantly probable because of the small number of patients (Figure 4E). In contrast, increase in CD56+ NK cell during consolidation was clear only in failed patients in the population consistent with D-STOP (Figure 4F). These results could explain the association between the 2 studies.

In dasatinib discontinuation, significance of NK cell count before cessation should be interpreted more carefully than imatinib discontinuation, because dasatinib elevated lymphocytes, particularly in NK cells, even after achieving DMR which at the small level was not regarded as lymphocytosis and NK cells rapidly downregulated after dasatinib cessation.10,15 NK cell count before cessation might vary in situations such as prior imatinib intolerance or resistance and timing of dasatinib treatment. Actually, higher NK cells before dasatinib cessation led to successful TFR in DADI, but not First-Line DADI.10,34

Regarding Tregs, dasatinib tended to downregulate these cells in both groups, especially in S patients. We previously demonstrated that first-line dasatinib decreased Tregs not in the early period, but at later than 12 months.35 Thus, Tregs had different response patterns from other NK/T subsets, whose role for TRF seemingly warrants further investigation.

In this study, Treg, reported as negative regulators for NK/CD8+ T cells,36 tended to decrease during consolidation, consequently NK/CD8+ T cells increased during consolidation in F patients. However, these cells decreased, in contrast, during the latter half of consolidation in successful patients. One reason for this was that S patients probably gained more adopted NK/CD8+ T cells specific for leukemia. Actually, such adopted NK cells are reportedly more resistant to Treg regulation than canonical cells.37

We previously showed NK cell differentiation that was associated with decrease in regulatory T cells.38 However, this relationship occurred until DMR was achieved. After DMR, we considered that altered immune reconstitution occurred in successful patients as described. Therefore, after DMR, another NK cell population such as CD56dim NK cells that suppressed primitive quiescent CML cells for TFR might be responding to regulatory T cells, but not CD56bright NK cells that we chiefly measured in this study.

Reportedly, immune reconstitution during sustained DMR is crucial, and included an elevation in mature NK cells and functional dendritic cells, and decline in Treg, PD1+ T cells and myeloid-derived suppressor cells in patients with CML during DMR.20,21 In addition, the recovery of the marrow environment conducted by myeloproliferative disorders to normal marrow when keeping DMR could be crucial for TFR.26,39 These studies support the significance of adequate, longer consolidation before discontinuation.

In conclusion, this study suggests that the silent reactions of NK/T peripheral lymphocyte subsets to dasatinib, especially NK subsets, during sustained DMR before discontinuation is vital for attaining longer TFR, perhaps because of more established immune surveillance in silent cells. Nevertheless, further investigation on its mechanism is warranted for more successful TFR.

**ACKNOWLEDGMENTS**

This study was supported by the Epidemiological and Clinical Research Information Network (RCRIN). This research was conducted as Investigator Sponsored Research with financial support by Bristol-Myers Squibb KK We thank Yumi Miyashita and Keiko Arai at ECRIN for collecting the data, and Narutaka Sakurada and Yoshinori Yamamoto at BML for performing flow cytometry and RQ-PCR. We thank Ishida Tadao (Department of Gastroenterology, Rheumatology and Clinical Immunology, Sapporo Medical University, Sapporo), Hiroyuki Sugawara (Department of Hematology, Sumitomo Hospital, Osaka), Sakaie Tanosaki (Department of Hematology, Tokyo Doai Memorial Hospital, Tokyo), Masayuki Koizumi (Department of Hematology, Asahi Chuo Hospital, Chiba), Megumi Akiyama (Department of Hematology, Yokosuka Kyosai Hospital, Kanagawa), Hiroshi Inoue (Department of Hematology, Inoue Memorial Hospital, Chiba), Iwai Kazuya (Department of Hematology, Shizuoka City Shizuoka Hospital, Shizuoka) and Akira Ohwada (Department of Hematology, Tokyo Metropolitan Bokutoh Hospital, Tokyo) for participating in this study.

**DISCLOSURE**

TK received honoraria from Bristol-Myers Squibb, Novartis, Pfizer, and Otsuka pharmacology. CN received honoraria from Bristol-Myers Squibb, Pfizer, and Novartis, and grants from Bristol-Myers Squibb and Pfizer. KN received the research funding from Novartis. CY received honoraria and Speakers Bureau from Bristol-Myers Squibb, Pfizer, and honoraria, Speakers Bureau, and grants from Otsuka. KM received honoraria from Celgene. SM received honoraria from Bristol-Myers Squibb. JS received remuneration from Yakult Honsha Co. Ltd. IK received research grants from Bristol-Myers Squibb, and honoraria from Bristol-Myers Squibb, Novartis, Celgene, and Pfizer.

**ORCID**

Takashi Kumagai https://orcid.org/0000-0001-6850-4545
Koseli Matsue https://orcid.org/0000-0002-8669-9865

**REFERENCES**

1. Druker BJ, Guilhot F, O’Brien SG, et al. Five-year follow-up of patients receiving imatinib for chronic myeloid leukemia. *N Engl J Med*. 2006;355:2408-2417.
2. Cortes JE, Saglio G, Kantarjian HM, et al. Final 5-year study results of DASISION: The dasatinib versus imatinib study in treatment-naive chronic myeloid leukemia patients trial. *J Clin Oncol*. 2016;34:2333-2340.
3. Bower H, Björkholm M, Dickman PW, Höglund M, Lambert PC, Andersson TM. Life expectancy of patients with chronic myeloid leukemia approaches the life expectancy of the general population. *J Clin Oncol*. 2016;34:2851-2857.
4. Douxfils J, Huguet H, Mullier F, Chatelain C, Graux C, Dogné JM. Association between bcr-abl tyrosine kinase inhibitors for chronic myeloid leukemia and cardiovascular events, major molecular response, and overall survival: A systematic review and meta-analysis. JAMA Oncol. 2016;2:625-632.

5. Padula WV, Larson RA, Dusetzina SB, et al. Cost-effectiveness of tyrosine kinase inhibitor treatment strategies for chronic myeloid leukemia in chronic phase after generic entry of imatinib in the United States. J Natl Cancer Inst. 2016;108:Djw003.

6. Mahon F-X, Réa D, Guilhot J, et al. Discontinuation of imatinib in patients with chronic myeloid leukemia who have maintained complete molecular remission for at least 2 y: the prospective, multicentre Stop Imatinib (STIM) trial. Lancet Oncol. 2010;11:1029-1035.

7. Etienne G, Guilhot J, Réa D, et al. Long-Term follow-up of the french stop imatinib (STIM1) study in patients with chronic myeloid leukemia. J Clin Oncol. 2017;35:298-305.

8. Takahashi N, Tauchi T, Kitamura K, et al. Deeper molecular relapse after treatment discontinuation: the KID study. Front Immunol. 2017;8:469.

9. Saussele S, Richter J, Guilhot J, et al. EURO-SKI investigators. Discontinuation of imatinib discontinuation in patients with chronic phase chronic myeloid leukemia: the JALSG-STIM213 study. Int J Hematol. 2018;107:185-193.

10. Rea D, Nicolini FE, Tulliez M, et al. Intergroupe des Leucémies Myéloïdes Chroniques. Discontinuation of dasatinib or nilotinib in chronic myeloid leukemia: interim analysis of the STOP 2G-TKI study. Blood. 2017;129:846-854.

11. Ilander M, Olsson-Strömberg U, Schlums H, et al. Increased proportion of mature NK cells is associated with successful imatinib discontinuation in chronic myeloid leukemia. Leukemia. 2016;31:1108-1116.

12. Kumagai T, Matsuki E, Inokuchi K, et al. Relative increase in lymphocytes from as early as 1 month predicts improved response to dasatinib in chronic-phase chronic myelogenous leukemia. Int J Hematol. 2014;99:41-52.

13. Kreutzman A, Juvenon V, Kairisto V, et al. Mono/oligoclonal T and NK cells are common in chronic myeloid leukemia patients at diagnosis and expand during dasatinib therapy. Blood. 2010;116:772-782.

14. Kumagai T, Nakaseko C, Nishikawa K, et al. Dasatinib cessation after deep molecular response exceeding 2 y and natural killer cell transi- tion during dasatinib consolidation. Cancer Sci. 2018;109:182-192.

15. Yoshida C, Fletcher L, Ohashi K, et al. Harmonization of molecular monitoring of chronic myeloid leukemia therapy in Japan. Int J Clin Oncol. 2012;17:584-589.

16. Liu W, Putnam AL, Xu-yu Z, et al. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med. 2006;203:1701-1711.

17. Kanda Y. Investigation of the freely available easy-to-use software ‘EZR’ for medical statistics. Bone Marrow Transplant. 2013;48:452-458.

18. Lee S-E, Choi SY, Song H-Y, et al. Imatinib withdrawal syndrome and longer duration of imatinib have a close association with a lower molecular relapse after treatment discontinuation: the KID study. Haematologica. 2016;101:717-723.

19. Hughes A, Yong ASM. Immune effector recovery in chronic myeloid leukemia and treatment-free remission. Front Immunol. 2017;8:469.

20. Hughes A, Clarson J, Tang C, et al. CML patients with deep molecular responses to TKI have restored immune effectors and decreased PD-1 and immune suppressors. Blood. 2017;129:1166-1176.

21. Watanabe N, Takaku T, Takeda K, et al. Dasatinib-induced anti-leu- kemia cellular immunity through a novel subset of CD57 positive helper/cytotoxic CD4+ T cells in chronic myelogenous leukemia pa- tients. Int J Hematol. 2018;108:588-597.

22. Schiffer CA, Cortes JE, Hochhaus A, et al. Lymphocytosis after treatment with dasatinib in chronic myeloid leukemia: Effects on response and toxicity. Cancer. 2016;122:1398-1407.

23. Ross DM, Branford S, Seymour JF, et al. Patients with chronic myeloid leukemia who maintain a complete molecular response after stopping imatinib treatment have evidence of persistent leukemia by DNA PCR. Leukemia. 2010;24(10):1719-1724.

24. Copland M, Hamilton A, Elrick LJ, et al. Dasatinib (BMS-354825) targets an earlier progenitor population than imatinib in primary CML but does not eliminate the quiescent fraction. Blood. 2006;107(11):4532-4539.

25. Schepers K, Pietras EM, Reynaud D, et al. Cell death-related myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. Stem Cell. 2013;13:285-299.

26. Takeishi S, Matsumoto A, Onoyama I, Naka K, Hirao A, Nakayama KI. Ablation of Fbxw7 eliminates leukemia-initiating cells by pre- vening quiescence. Cancer Cell. 2013;23:347-361.

27. Iriyama N, Takahashi H, Miura K, et al. Enhanced perforin expres- sion associated with dasatinib therapy in natural killer cells. Leuk Res. 2018;68:1-8.

28. Chang MC, Cheng HI, Hsu K, et al. NKG2A down-regulation by dasatinib enhances natural killer cytotoxicity and accelerates effective treatment responses in patients with chronic myeloid leukemia. Front Immunol. 2019;9:3152.

29. Damele L, Montaldo E, Moretta L, Vitale C, Mindari MC. Effect of tyrosin kinase inhibitors on NK cell and ILC3 development and function. Front Immunol. 2018;9:2433.

30. Paydas S. Dasatinib, large granular lymphocytosis, and pleu- ral effusion: useful or adverse effect? Crit Rev Oncol Hematol. 2014;89:242-247.

31. Baccarani M, Cortes J, Pane F, et al. Chronic myeloid leukemia: an update of concepts and management recommendations of European LeukemiaNet. J Clin Oncol. 2009;27:6041-6051.

32. Rea D, Henry G, Khaznadar Z, et al. Natural killer-cell counts are as- sociated with molecular relapse-free survival after imatinib discon- tinuation in chronic myeloid leukemia: the IMMUNOSTIM study. Haematologica. 2017;102:1368-1377.

33. Kimura S, Imagawa J, Murai K, et al. Treatment-free remission after first-line dasatinib discontinuation in patients with chronic myeloid leukemia (first-line DADI trial): a single-arm, multicentre, phase 2 trial lancet. Lancet Haematol. 2020;7(3):e218–e225.

34. Iriyama N, Fujisawa S, Yoshida C, et al. Early cytotoxic lympho- cyte expansion contributes to a deep molecular response to dasa- nitinb in patients with newly diagnosed chronic myeloid leukemia in the chronic phase: results of the D-first study. Am J Hematol. 2015;90:819-824.

35. Nikolova M, Lelievre JD, Carriere M, Bensussan A, Lévy Y. Regulatory T cells differentially modulate the maturation and apop- tosis of human CD8+ T-cell subsets. Blood. 2009;113:4556-4565.

36. Sarhan D, Hippen KL, Lemire A, et al. Adaptive NK cells resist regulatory T-cell suppression driven by IL37. Cancer Immunol Res. 2018;6:766-775.

37. Najima Y, Yoshida C, Iriyama N, et al. Regulatory T cell inhibition by dasatinib is associated with natural killer cell differentiation and a favorable molecular response—the final results of the D-first study. Leuk Res. 2018;66:66-72.
39. Schepers K, Campbell TB, Passegué E. Normal and leukemic stem cell niches: insights and therapeutic opportunities. *Cell Stem Cell*. 2015;16:254-267.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Kumagai T, Nakaseko C, Nishiwaki K, et al; the Kanto CML, Shimousa Hematology Study Groups. Silent NK/T cell reactions to dasatinib during sustained deep molecular response before cessation are associated with longer treatment-free remission. *Cancer Sci*. 2020;111: 2923–2934. [https://doi.org/10.1111/cas.14518](https://doi.org/10.1111/cas.14518)