Natural killer or natural killer/T cell lineage large granular lymphocytosis associated with dasatinib therapy for Philadelphia chromosome positive leukemia

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ABSTRACT

Dasatinib, a dual tyrosine kinase inhibitor, is known to modulate or suppress T-cell activation and proliferation. We report a series of 8 patients who developed chronic peripheral lymphocytosis, identified as natural killer cells or natural killer/T-cells based on their large granular lymphocyte morphologies and $CD16^+$, $CD56^+$, $CD3^-$ or $CD3^+$ immunophenotypic profiles, out of 18 patients receiving dasatinib therapy. All cases that developed large granular lymphocyte lymphocytosis achieved optimal molecular response (8/8 in large granular $lymphocyte^+$ patients *vs.* 3/10 in large granular $lymphocyte^-$ patients, p=0.002). A ^{51}Cr release assay demonstrated that natural killer cell cytotoxicity has been enhanced in a case of large granular lymphocyte lymphocytosis compared to normal healthy donors, and that natural killer cell cytotoxicity in dasatinib-responders was superior to that in non-responders. In summary, the present study suggests that natural killer or natural killer/T cell lineage large granular lymphocyte lymphocytosis develops in association with dasatinib therapy and that large granular lymphocyte might have a therapeutic effect on Ph^+ leukemic cells.

Key words: lymphocytosis, large granular lymphocyte, natural killer cells, dasatinib.

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Introduction

Dasatinib is a multi-tyrosine kinase inhibitor targeting BCR/ABL fusion tyrosine kinase and SRC kinases. There is evidence to suggest that the tyrosine kinase inhibitors (TKIs) have immunomodulatory or suppressive effects. The first generation TKI, imatinib mesylate (IM), selectively impairs the expansion of memory cytotoxic T cells.[1] Another study suggested that IM inhibits antigen-specific interferon-α secretion by both $CD4^+$ and $CD8^+$ T-effector cells.[2] Recently, dasatinib has been demonstrated to inhibit the activation and proliferation of T lymphocytes *in vitro*.[3] However, its role in natural killer (NK) cell immunity has not been fully established.

Large granular lymphocytes (LGLs) are a morphologically distinct but an immunophenotypically heterogeneous set of lymphocytes of activated $CD3^+$ T cells or $CD3^-$ NK cells that mediate non-MHC-restricted cytotoxicity. LGLs may increase in response to viral infection or underlying malignant neoplasm. We report a series of patients that developed peripheral lymphocytosis, identified as NK cells or NK/T-cells based on their LGL morphology and $CD16^+CD56^+CD3^-$ or $CD3^+$ immunophenotypic profiles following dasatinib therapy. Interestingly, all cases that developed LGL lymphocytosis achieved optimal molecular responses (8/8 in LGL^+ patients *vs.* 3/10 in LGL^- patients, p=0.002).

Design and Methods

Between March 2005 and October 2007, 18 patients with Ph^+ leukemia [chronic myeloid leukemia (CML), n=17; Ph^+ acute lymphoblastic leukemia (ALL), n=1] received dasatinib at the Princess Margaret Hospital, Toronto, ON, Canada at a starting dose of 70 mg twice daily. The clinical characteristics of the patients are summarized in Table 1. Blood counts were regularly monitored. LGL lymphocytosis has been diagnosed (i) by an increasing number of peripheral blood lymphocyte counts ≥3.0×10^9/L for at least three months, and (ii) by the predominance of LGLs in the peripheral blood

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The online version of this article contains a supplementary appendix.

Table 1. Patients' characteristics and individual clinical courses following dasatinib therapy.

UPN	Stage at dasatinib therapy	Prior response to IM	Mutation	Dose, dasatinib	Onset (mo)	ALCs, peak (x10^9/L)	Immunophenotype	Duration on dasatinib (mo)	Response to dasatinib	Survival
Patients developing LGL lymphocytosis										
1	Ph$^+$ ALL	Resistant	V355A	70 mg bid	1.5	21.0	CD2$^+$/7$^+$/16$^+$/56$^+$/57$^+$/3$^-$	24	CMR	Alive
2	CP1	Intolerant	NA	70 mg bid	8.5	4.7	CD2$^+$/7$^+$/16$^+$/56$^+$/57$^+$/3$^-$	23.5	CMR	Alive
3	CP1	Resistant	M244V	70mg bid	1.5	8.5	CD2$^+$/7$^+$/16$^+$/56$^+$/57$^+$/3$^-$	23.5	CMR	Alive
4	CP1	Intolerant	NA	70 mg bid	5.5	4.5	CD2$^+$/7$^+$/16$^+$/56$^+$/57$^+$/3$^-$	22.5	MMR	Alive
5	CP1	Resistant	ND	70 mg bid	2.0	7.4	CD2$^+$/7$^+$/16$^+$/56$^+$/57$^+$/3$^-$	22.5	CMR	Alive
6	CP1	Resistant	M351T	70 mg bid	2.5	11.6	CD2$^+$/7$^+$/16$^+$/56$^+$/57$^+$/3$^-$	22.0	CMR	Alive
7	CP1	Resistant	G250E	70 mg bid	25.5		CD2$^+$/7$^+$/16$^+$/56$^+$/57$^+$/3$^-$	22.5	CMR	Alive
8	MBC	Resistant	M244V	70 mg bid	14.0	4.1	CD2$^+$/7$^+$/16$^+$/56$^+$/57$^+$/3$^+$	23.5	CMR	Alive
Patients without LGL lymphocytosis										
9	CP1	Resistant	D275G	70 mg bid	–	1.6	–	23.0	CMR	Alive
10	CP1	Resistant	M351T	70 mg bid	–	1.0	–	23.0	No CyR	Alive
11	CP1	Resistant	ND	70 mg bid	–	2.0	–	22.0	CCyR	Alive
12	CP1	Resistant	E355G	70 mg bid	–	2.7	–	23.5	No CyR	Alive
13	CP1	Resistant	T315I	70 mg bid	–	2.5	–	7.5	H-PD	Death[1]
14	CP2	Resistant	ND	70 mg bid	–	0.8	–	21.5	PCyR	Alive
15	CP2	Resistant	M244V	70 mg bid	–	2.0	–	23.5	CCyR	Alive
16	AP	Resistant	ND	70 mg bid	–	0.7	–	5.0	CMR	Death[2]
17	AP	Resistant	M255V and F317L	70 mg bid	–	1.1	–	24.0	MMR	Alive
18	AP	Resistant	ND	70 mg bid	–	2.6	–	25.0	CCyR	Alive

UPN: unit patient number; IM: imatinib mesylate; ALL: acute lymphoblastic leukemia; CP1: first chronic phase; MBC: myeloid blastic crisis; CP2: second chronic phase; AP: accelerated phase; NA: not assessed; ND: not detected; bid, twice daily; MMR: major molecular response; CMR: complete molecular response; CyR: cytogenetic response; H-PD: hematologic progressive disease; CCyR: complete cytogenetic response; PCyR: partial cytogenetic response. [1]due to progressive disease; [2]due to sepsis

smear samples. Immunophenotypic analyses were conducted in cases with LGL lymphocytosis as previously described.[4] The following antibodies were used in relevant combinations and conjugated with fluorescein isothiocyanate, phycoerythrin or phycoerythrin-cyanin 5: CD2, CD3, CD4, CD5, CD7, CD8, CD16, CD56, and CD57.

NK cells were isolated by negative magnetic selection using the StemSep system (StemCell Technologies, Vancouver, British Columbia, Canada) from the mononuclear cells (MNCs) of peripheral blood in patient or donors. Cytolytic activity was determined by performing standard 4-hour ^{51}Cr-release assays using NK-sensitive K562 or T2 target cells. In addition, comparison of NK cell cytotoxicity was performed using MNCs isolated from the patients responding to dasatinib therapy (e.g. responders such as patients achieving major molecular response, n=3) and non-responders (e.g. patients resistant to dasatinib therapy, n=3). The NK cell cytotoxicity testing was repeated three times per each patient, using K562 as target cells. Results were calculated and recorded as percentage of cells killed, as previously described.[5]

Patient demographics and disease characteristics and their responses to dasatinib therapy were compared with respect to the development of LGL lymphocytosis using χ^2, Fisher's exact or Mann-Whitney's U-tests. Cumulative probability of LGL development was estimated using the 1-Kaplan-Meier method. According to the development of LGL lymphocytosis, differences in cytogenetic and molecular response to dasatinib therapy were compared. Statistical analysis was performed using an SPSS software package (SPSS version 13.0, Chicago, IL, USA).

Results and Discussion

With a median of 26 months of dasatinib therapy (range 5-30 months), 8 cases with peripheral blood LGL lymphocytosis were identified among the 18 patients. Median onset and duration of LGL lymphocytosis was four months after the initiation of dasatinib therapy (range 1.5-15 months) and nine months (range 6-24 months) respectively (Figure 1A). The mean peak lymphocyte counts and LGL percentage was 6.7±2.1×10^9/L and 56±8% respectively (mean±standard error). In all 8 patients, no symptoms or signs suggestive of large granular cell leukemia were reported, e.g. lymphadenopathy, splenomegaly, hepatomegaly or persistent fever. The current series met the criteria of chronic LGL lymphocytosis based on LGL morphology (*Online Supplementary Figure S1*) and immunophenotypes as NK cells (n=7) or NK/T cells (n=1) by CD16$^+$CD56$^+$ plus CD3$^-$ or CD3$^+$ immunophenotypic profiles. LGL counts gradually

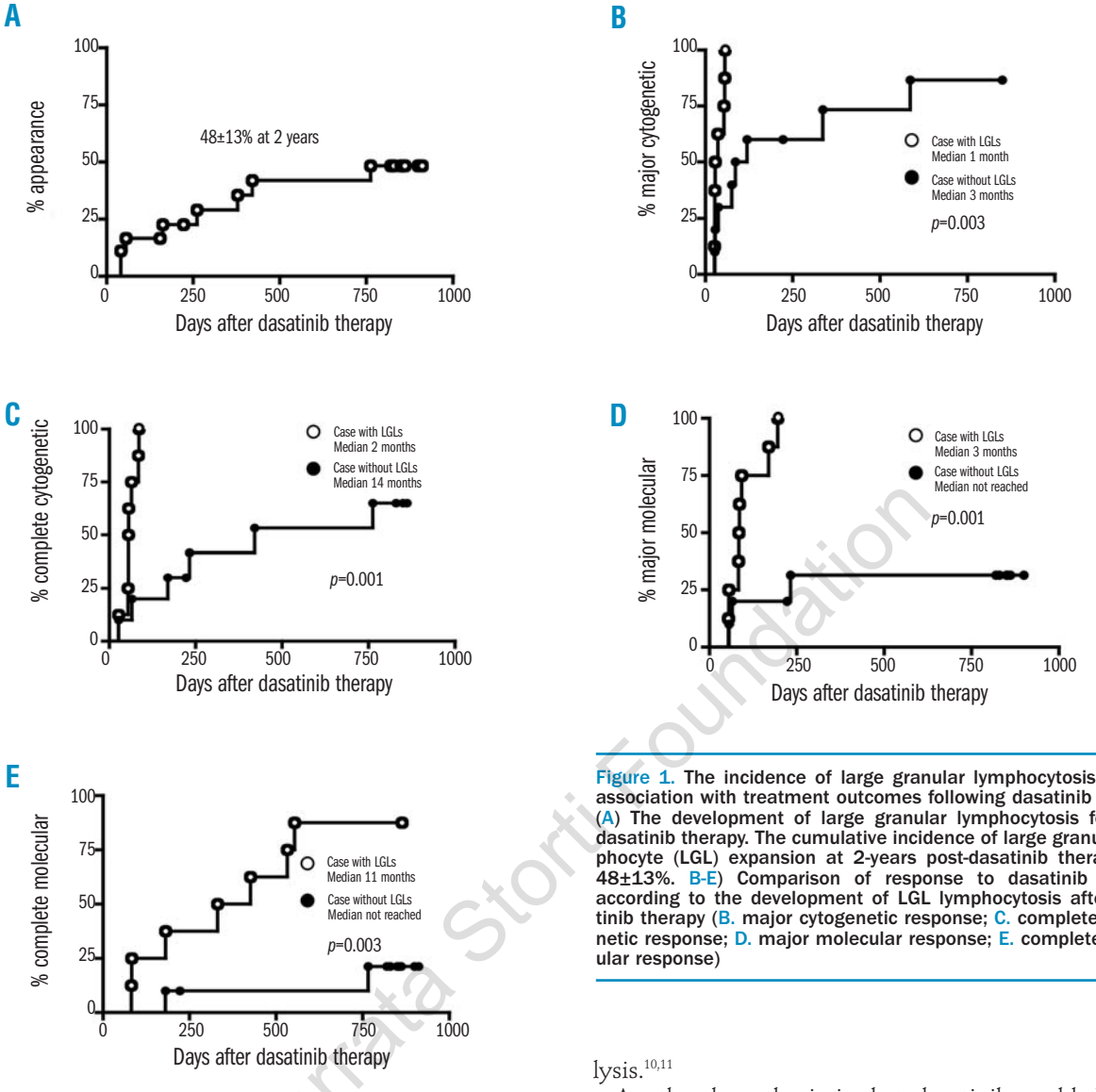

Figure 1. The incidence of large granular lymphocytosis and its association with treatment outcomes following dasatinib therapy. (A) The development of large granular lymphocytosis following dasatinib therapy. The cumulative incidence of large granular lymphocyte (LGL) expansion at 2-years post-dasatinib therapy was 48±13%. B-E) Comparison of response to dasatinib therapy according to the development of LGL lymphocytosis after dasatinib therapy (B. major cytogenetic response; C. complete cytogenetic response; D. major molecular response; E. complete molecular response)

decreased in 4 patients while 4 patients showed persistent LGL lymphocytosis with maximal lymphocyte counts of 21.0×10^9/L. The characteristics of patients with and without LGLs were not significantly different including HLA A2. One potential mechanism for the pathogenesis of LGLs after dasatinib therapy concerns the BCR/ABL targeted TKI therapy-mediated restoration of the functions or proliferative capacities of NK cells. Malignant CML cells have a capacity to produce reactive oxygen species, the main inhibitory mediator toward NK cells.[6] NK cells from CML patients are profoundly defective in NK cell activity[7] and NK cell numbers decrease as the disease progresses to the advanced phase.[8] Nakajima *et al.* concluded that the *BCR/ABL* transgene causes abnormal NK cell differentiation,[9] and thus, it is possible that TKI therapy such as IM or dasatinib could reduce BCR/ABL transcription and restore NK cell numbers and/or functions. It has been recently shown by two different groups that IM can down-regulate NKG2D-L expression on Bcr/abl positive target cells and render them less susceptible to NK cell lysis.[10,11]

Another hypothesis is that dasatinib could directly activate or modulate the proliferation and function of LGLs. Dasatinib is known to affect the development of NK cells as well as T lymphocytes. Several subtypes of SRC kinases are involved in the regulation or activation of NK cells or NK/T cells such as FYN,[12,13] LCK,[14,15] or FYN.[16] A recent study demonstrated that dasatinib inhibits the activation and proliferation of T lymphocytes *in vitro*.[3] However, the current study showed the somewhat paradoxical finding that blockade of SRC kinases by dasatinib could deregulate or modulate NK or NK/T cell activation, and that it enhances NK or NK/T cell proliferation and/or activation, thus modulating LGLs to attack CML stem cells. It still needs to be fully clarified whether the effect of dasatinib on NK or NK/T cell proliferation or activation is mediated via SRC kinase or other unknown potential pathways involved in the proliferation and differentiation of NK or NK/T cells. The development of LGL lymphocytosis in our patients was found to be significantly associated with an improved response to dasatinib therapy in terms of MCyR (p=0.003, hazard ration [HR] 0.17), CCyR (p=0.001, HR 0.10), MMoR (p=0.001, HR 0.11)

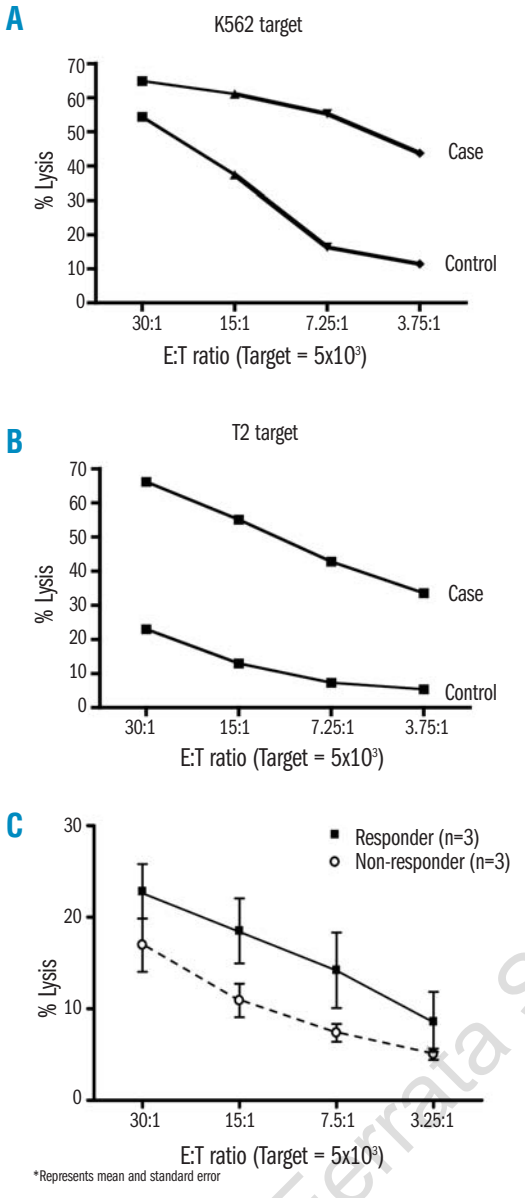

Figure 2. Cytotoxicity of NK cells isolated from the patients developing large granular lymphocytosis following dasatinib therapy. NK cell cytotoxicity was assessed by 51Cr release assays using target cells as K562 (A) and T2 cell line (B) as target cells. (C) The result of 51Cr release assays comparing cytotoxicity of NK cells isolated from patients responding to dasatinib therapy (responder) and not responding (non-responder) following dasatinib therapy using K562 cell line as target cells.

and CMoR (p=0.003, HR 0.12; Figures 1B to E). All 8 patients who developed LGLs achieved MMoR, while only 3 of the 10 patients that did not develop LGLs achieved MMoR. The group with LGLs showed significantly lower levels of the BCR/ABL mRNA transcript compared to those without LGLs at six months (4.1±0.3 *vs.* 1.5±0.6 log reduction, p=0.001), one year (4.0±0.3 *vs.* 1.0±0.5, p=0.001) and two years post-dasatinib therapy (4.3±0.3 *vs.* 1.6±0.5, p=0.001). The LGL appearance was not associated with development of pulmonary abnormalities including pleural effusion or lung parenchyma changes (*data not shown*).

Then what is the clinical relevance of LGLs in terms of Ph$^+$ leukemia disease control following dasatinib therapy? We performed NK cell cytotoxicity testing of ^{51}Cr release assays (Figure 2A and B),[17] using two types of target cells, i.e. K562 cells (a BCR/ABL positive blastic phase cell line) and T2 cells (an acute lymphoblastic cell line expressing HLA-A2), and negatively selected NK cells expressing the CD16$^+$/CD56$^+$/CD3$^-$ phenotypic profiles were used as effector cells. One case (UPN 1 in Table 1) underwent dasatinib therapy (70 mg bid po), and achieved complete cytogenetic response (CCyR) and major molecular response (MMoR) at one and two months respectively. Peripheral lymphocytosis was noted at 1.5 months after starting dasatinib therapy (identified as natural killer cells based on LGL morphology and a CD3$^-$/CD16$^+$/CD56$^+$/CD57$^+$ NK cell immunophenotype). Lymphocyte counts gradually increased to 21.0×10^9/L with continued negative BCR/ABL mRNA by RT PCR. Two weeks after the temporary interruption of dasatinib, her lymphocyte count decreased to 5.5 from 14.1×10^9/L. The patient remained disease free at her 27th month of follow-up on dasatinib at 80mg once daily. NK cells from the case exhibited had moderate to high cytotoxic effects, especially against T2 cell lines, while NK cells from healthy donors had low cytotoxic effect on K562 or T2 cell lines (Figures 2A and B). These results indicate that NK cells from the case that developed LGL lymphocytosis had greater cytotoxic effect than those of normal healthy donors.

An additional study was performed in order to compare the NK cell cytotoxicity between the responders and non-responders. As shown in Figure 2C, the NK cell cytotoxicity in responders was superior to that in non-responders. This result supports the view that enhanced cytotoxicity of NK cells could mediate better disease control of CML, and that the augmented NK cell cytotoxicity might be a potential action mechanism of dasatinib.

It has also been shown that IM treatment can induce NK cell activation which correlates with enhanced response against gastrointestinal stromal tumors (GISTs).[18] Moreover, the same group has shown that IM treatment in mice can induce selective expansion of IFN-producing killer dendritic cells (IKDC).[19] Although it is still debated whether IKDC are simply activated NK cells or cells belonging to a different lineage, it would be of great interest to further characterize LGL cells found in CML patients after dasatinib therapy in order to gain more information on their phenotype and functional activity, and to analyze whether they display a similar phenotype to mouse IKDC.

Previous studies have suggested that autologously activated NK cells have a potent cytotoxic activity on CML cells.[20,21] Moreover, NK cells are potent effector cells of innate immunity due to their ability to eliminate tumor cells in a non-HLA restricted manner. The enhanced cytotoxic effects of NK cells isolated from patients who developed LGL lymphocytosis suggest that LFL development after dasatinib therapy could clear Ph$^+$ leukemic cells and thus mediate the therapeu-

tic effect of dasatinib via an unknown pathway.

In conclusion, the present study suggests that NK or NK/T cell lineage LGL lymphocytosis might develop following dasatinib therapy and that LGL may mediate a therapeutic activity against Ph^+ leukemic cells.

Authorship and Disclosures

DK contributed to the design of this study, the supervision of data collection and of data interpretation, data analysis, and wrote the manuscript. SKR contributed to the BCR/ABL transcript PCR assay and to manuscript revision. HC and RS contributed to the study design, flow cytometry and to manuscript revision. CWJ contributed to the supervision of data interpretation, data analysis and manuscript revision. HJK and JJL contributed to ^{51}Cr release assay, data interpretation, to manuscript revision, and provided critical advice. JHL contributed to the study design, the supervision of data collection, data interpretation, and to manuscript revision. The authors reported no potential conflicts of interest.

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