ORIGINAL ARTICLE

Rapid and sustained increase of large granular lymphocytes and rare cytomegalovirus reactivation during dasatinib treatment in chronic myelogenous leukemia patients

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Abstract We retrospectively investigated increases in large granular lymphocytes (LGL) in peripheral blood during dasatinib treatment in 25 chronic myelogenous leukemia patients. Fifteen of 25 patients (60 %) showed an increase in LGL. All 15 of these patients also showed an increase in NK cells, and 11 showed an increase in CD8$^+$ T cells. High frequencies of clonal rearrangements of TCR-β, -γ, and -δ genes were observed in LGL (+) patients, and at lower frequencies in LGL (−) patients as well. Clinical responses were favorable for all. With respect to their newly obtained complete molecular response after dasatinib treatment, LGL (+) patients showed higher response rates than did LGL (−) patients. In contrast, pleural effusions were more commonly observed in LGL (+) patients (60 %) than in LGL (−) patients (20 %). LGL counts significantly increased at 2 h after oral intake of dasatinib in all 25 patients. This was not observed in treatment with imatinib or nilotinib. Cytomegalovirus (CMV) C7-HRP tests were negative in all patients. Serum CMV-IgM antibodies were positive in only 2 of 25 patients without symptom of infection. Thus, LGL lymphocytosis during dasatinib treatment may be correlated with favorable molecular response, and with increased incidence of pleural effusions. In the clinical setting, CMV reactivation appears uncommon.

Keywords CML · LGL · Dasatinib · TCR · CMV

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Introduction

ABL-tyrosine kinase inhibitors (ABL-TKIs), such as imatinib, nilotinib, and dasatinib have dramatically changed the prognosis of chronic myelogenous leukemia (CML) patients. There is a growing evidence of supporting as yet uncharacterized role of ABL-TKIs on the immune system in CML patients. Immunosuppressive effects on T cells and NK cells have been described, in vitro, for dasatinib [1–3], for imatinib [4], and for nilotinib [5] as well. In the clinical setting, however, it seems to be a different story. No increase of infection rates due to immunosuppression have been observed in the large clinical trial of dasatinib study [6], though a single center study reported atypical infections in CML patients treated with high dose of dasatinib at 140 mg daily [7].

Large granular lymphocytes (LGLs) normally account for only 10–15 % of circulating mononuclear cells (MNCs) in peripheral blood (PB). Recently, proliferation of lymphocytes with LGL morphologic features has been reported in a subset of patients with CML or Philadelphia chromosome-positive acute lymphocytic leukemia (Ph$^+$ALL) treated with dasatinib [8–11]. Regarding phenotypes of the increasing LGLs, both NK-cell and CD8$^+$ T-cell phenotypes were observed, and these cell populations exhibited late differentiated (CD27$^-$ CD57$^+$) phenotype [12]. Furthermore, clonality in these cells has been demonstrated through analysis of T-cell receptor (TCR) gene rearrangements [8, 13]. These monoclonal or oligoclonal CD8$^+$ T cell or NK cells already existed in CML patients even at the time of diagnosis, and that half of the dasatinib-treated patients showed marked expansion of their clone during therapy whereas imatinib-treated patients did not [14, 15]. Patients developing LGL lymphocytosis showed higher incidence of auto-immune-like symptoms such as fevers,

colitis, and pleural effusions, which had been reported in dasatinib-treated patients [8, 10, 16]. On the other hand, however, LGL lymphocytosis during dasatinib treatment was associated with enhanced favorable therapeutic responses [8, 9, 11], implicating that these LGL lymphocytes may have potential to exert immune-associated anti-leukemia effects in vivo.

Regarding cytomegalovirus (CMV) infection, it was recently reported that CML and Ph+ALL patients with LGL lymphocytosis treated by dasatinib showed appearances of CMV-specific CD8+ T cells, and that approximately one-third of the patients with LGL lymphocytosis showed symptomatic CMV reactivation during dasatinib treatments [12].

To further elucidate the clinical implications of dasatinib in CML patients from the viewpoints of LGL proliferation, clinical responses, adverse effects, and CMV reactivation, we retrospectively analyzed a series of 25 consecutive CML patients in our single institution who were treated with dasatinib as second-line treatments following preceding imatinib treatments. Moreover, in order to explore the off-target effects of dasatinib, we investigated the lymphocyte counts and LGL counts in PB as short as 2 h after the oral intake of dasatinib, imatinib, and nilotinib as well. These results disclosed unique activities of dasatinib.

Patients and methods

Patients

A total of 25 CML patients in our institution who were treated by dasatinib were studied. The analysis included patients on therapy from April 2009 to August 2011. 17 were males and 8 were females. Median age was 64 years (range 20–84). All 25 patients had prior imatinib treatments before dasatinib treatments, and 6 of the 25 patients had histories of prior interferon alpha (IFNα) treatments before the imatinib treatments. At the time of dasatinib treatments, 21 were in chronic phase (CP), 3 were in accelerated phase (AP), and 1 was in second CP achieved by imatinib treatment after AP. CML-blast phase (BP) or Ph+ALL patients were not included in this study. The definitions of resistance and intolerance were in accordance with the one proposed by Kantarjian et al. [17], though recently proposed definition of intolerance is less stricter than before [18]. Most common types of intolerances in our study were peripheral edema, nausea, gastrointestinal disorder, skin eruption, neutropenia, and thrombocytopenia. Regarding initial doses of dasatinib, 22 patients were 100 mg QD (reduced to 50 mg QD later, in 6 patients), 2 patients were 140 mg QD (reduced to 100 mg QD later, in the 2 patients), and 1 patient was 50 mg QD. Written informed consents were obtained from all the patients. This study was conducted in accordance with the principles of the Helsinki declaration and was approved by the institutional review board of Hiroshima City Asa Hospital.

Counting of LGL in peripheral blood

PB smears were examined with light microscope by two certificated hematology technicians. The technicians were informed of the start of dasatinib treatments, but were not informed of the present status of the patients even when the treatments were discontinued. Lymphocyte having three or more large granules in one cell was defined as LGL. Regarding the PB smears during dasatinib treatments, 200 lymphocytes were counted per each smear for the 10 consecutive PB sampling after the start of the dasatinib treatment. Thereafter, 200 white blood cells per smear were counted every time when PB samples were collected from the patients. Regarding the smears during imatinib treatments, the smears were retrospectively examined for the latest 5 consecutive PB smears during imatinib treatment before dasatinib treatments, in which 200 lymphocytes per smear were counted. Absolute number of LGL in PB was then calculated based on the total lymphocyte counts reported by an automated cell counter. Definition of "Increase of LGL" in this study was set such that: (1) both absolute lymphocyte count is equal or more than 3,000/μL, and absolute LGL count is equal or more than 1,500/μL; and (2) this appears at least on one occasion during the course of the dasatinib treatment. Then, according to this definition, the patients were grouped into "LGL (+) patients" and "LGL (−) patients". Interval between routine intake of dasatinib and blood test was not fixed on every outpatient visits. Examination of the increase of lymphocyte and LGL counts 2 h after ABL-TKIs intake was performed as a separate study, in which the interval of intake and blood sampling was almost fixed to 2 h.

Assessment of molecular response

Complete molecular response (CMR) was examined by Amp-CML® (Mitsubishi Chemical Medience Corp., Tokyo, Japan) as a first step. When it was less than the detection limit, which means BCR-ABL mRNA was less than 5 copies/0.5 μg RNA (approximately 4-log reduction), then we further confirmed the results by reverse-transcription quantitative polymerase chain reaction (RQ-PCR) with international scale (IS) by BML Co. Ltd. (Tokyo Japan), and the results were further confirmed by nested RT-PCR methods to ascertain that BCR-ABL mRNA was undetectable. Major molecular response (MMR) was temporarily defined either when Amp-CML® test was less than 50 copies/0.5 μg RNA.

Flow cytometry

Phenotypic examinations were performed by two-color flow cytometry (FCM) by FACSAria® II flow cytometer (BD Biosciences) at BML Co. Ltd. (Tokyo, Japan) using peripheral blood mononuclear cells (PBMNCs) collected both during (in all 25 patients) and before (in 17 of 25 patients) the dasatinib therapy. The monoclonal antibodies used were anti-CD2, CD3, CD4, CD8, CD16, CD56, CD25, TCRα, TCRβ, TCRγ, TCRδ, and CD57.

T-cell receptor (TCR) gene rearrangement

Analysis of clonal TCR gene rearrangements were performed by PCR-based method using PBMNCs collected during dasatinib treatments, at Mitsubishi Chemical Medience Corp. (Tokyo, Japan), using the test kits "TCRB Gene Clonality Assay®", "TCRG Gene Clonality Assay®", and "TCRD Gene Clonality Assay®" manufactured by InVivoScribe Technologies (San Diego, USA) [19].

Assessment of cytomegalovirus (CMV) infection status

CMV-infection status during dasatinib treatments was assessed by serum CMV-IgG levels, serum CMV-IgM levels, and "CMV antigen test TEIJIN C7-HRP®" (TFB Inc., Tokyo, Japan), which can detect CMV pp65 antigen in leukocytes [20, 21].

Statistical analysis

Statistical analysis was performed by Student's *t* test by Statview® ver.5.0 (SAS Institute Inc.).

Results

Rapid and sustained increase of LGL by dasatinib treatment

The median follow-up time of dasatinib treatment was 11 months (range 3–28). Table 1 summarizes the characteristics of the patients and the results of LGL lymphocytosis, clinical responses, adverse events, and results of CMV-infection assessments. Patients were grouped into 2 groups, i.e., LGL (+) patients and LGL (−) patients. Table 2 shows the summary of comparison of the 2 groups. Fifteen out of 25 patients (60 %) showed increase of LGL during dasatinib treatments, which had not been observed during the preceding imatinib treatments. Regarding the 15 LGL (+) patients, median time that LGL count reached 1,500/μL was 11 weeks (range 6–44) after dasatinib was first administered. Median of the peak LGL count for each

patient was 3,030/μL (range 1,614–6,010). Figure 1 shows the time courses of lymphocyte and LGL counts both before (meaning during imatinib treatments) and during the first 12 weeks after dasatinib treatments were started. Representative 6 cases [3 LGL (+) patients (A), and 3 LGL (−) patients (B)] are shown. Figure 2 shows time courses of 2 representative LGL (+) patients with longitudinal follow-up. Even though some fluctuations were found, high LGL counts were sustained throughout the treatment courses as long as the dasatinib treatments were continued. However, cessation of dasatinib treatments induced rapid decline of the LGL counts almost to the baseline levels.

Phenotype of peripheral blood mononuclear cells by flow cytometry

All 25 patients were analyzed for immunophenotyping by flow cytometry using PBMNCs during dasatinib treatment. In 17 of the 25 patients, flow cytometry analyses were also performed before the dasatinib treatments. Regarding the 15 patients who showed LGL-increase, all patients showed increase of CD56$^+$, CD16$^+$, CD3$^-$ NK cells, and 11 of the 15 patients also showed increase of CD3$^+$, CD8$^+$, CD4$^-$ T cells (Table 1). CD57, which is known to be expressed on the surface of NK cells or T cells with LGL morphology, was positive in all 10 patients examined. We could not find any increase of γδ-T cells in all 25 patients. Figure 3 shows one of the representative patients who was analyzed both before and after the dasatinib treatments. In this patient, NK cells were 37.2 % during imatinib treatment, which then increased to 48.2 and 52.2 %, at 14 weeks and 25 weeks after the dasatinib treatment, respectively. CD8$^+$ T cells were 48.0 % during imatinib treatment, which then moderately increased to 48.4 and 54.3 %, at 14 weeks and 25 weeks after the dasatinib treatment, respectively. Regarding the 10 LGL (−) patients, they also showed that NK cells increased in 5 of 10 patients, and that CD8$^+$ T cells increased in 1 of 10 patients after dasatinib treatments as compared to those before the treatments (Table 1).

Clonal T cell receptor (TCR) gene rearrangements

All 25 patients were analyzed for clonal TCR rearrangements during dasatinib treatment (Tables 1, 2). Regarding the 15 LGL (+) patients, clonal rearrangements of TCR-β gene were observed in 13 of 15 (87 %), and TCR-γ in 12 (80 %), and TCR-δ in 9 (60 %) patients. All the 15 patients showed rearrangements of either one of the three TCR genes. On the other hand, regarding the 10 LGL (−) patients, clonal rearrangements of TCR-β gene were observed in 4 of 10 (40 %), and TCR-γ in 3 (30 %), and TCR-δ in 4 (40 %) patients, where 3 of the 10 patients did

Table 1 Characteristics of patients and summary of results

ID	Age	Stage	M/F	IFN	R/I	Duration (week)	LGL	FCM				TCR rearr.			Dasatinib effects		CMV			PE	Other AEs
								NK	CD8+T	γδ-T	CD57	β	γ	δ	Before	After	C7-HRP	IgM	IgG		
1	61	CP	M	○	I	25	○	○	○	X	○	○	○	○	CCyR	CMR	X	X	○	X	Eruption
2	43	CP	M	X	I	28	○	○	X	X	ND	○	○	○	MMR	MMR	X	X	○	○	Eruption, conj. hemorr.
3	68	CP	F	○	R	27	○	○	○	X	○	○	○	○	NoCyR	CMR	X	X	○	○	Eruption
4	71	2nd CP	M	○	R	28	○	○	X	X	ND	○	○	X	PCyR	CMR	X	X	○	○	Conj.hemorr
5	71	CP	M	X	I	18	○	○	X	X	ND	○	○	○	MMR	MMR	X	X	○	○	Conj.hemorr
6	76	CP	F	○	I	14	○	○	○	X	○	○	X	X	CMR	CMR	X	X	○	○	Eruption
7	65	CP	M	X	I	11	○	○	○	X	○	○	○	X	CMR	CMR	X	X	○	○	
8	76	CP	M	X	I	11	○	○	○	X	○	○	○	○	CMR	CMR	X	X	○	○	
9	54	CP	M	X	I	11	○	○	○	X	ND	X	○	○	CCyR	CMR	X	X	○	○	Eruption, IP
10	40	CP	F	X	I	9	○	○	○	X	○	○	○	○	CMR	CMR	X	X	X	X	
11	40	CP	M	X	I	10	○	○	X	X	ND	○	○	X	CMR	CMR	X	X	○	○	Eruption
12	84	AP	M	X	R	7	○	○	○	X	ND	○	○	○	CCyR	CCyR	X	X	○	X	Fatigue
13	64	AP	F	X	R	5	○	○	○	X	ND	X	○	○	CCyR	MMR	X	X	○	X	
14	34	CP	M	X	I	4	○	○	○	X	○	○	X	X	CMR	CMR	X	X	○	X	
15	83	CP	M	X	I	5	○	○	○	X	ND	○	X	X	CMR	CMR	X	X	○	X	
16	71	AP	F	X	R	28	X	X	X	X	ND	X	X	○	PCyR	MMR	X	X	○	○	Nausea
17	20	CP	M	X	R	28	X	○	○	X	ND	○	X	○	CCyR	MMR	X	X	○	X	
18	34	CP	F	X	I	22	X	X	X	X	ND	X	X	X	CCyR	MMR	X	X	○	X	Eruption, conj. hemorr.
19	64	CP	M	○	I	15	X	○	X	X	○	X	X	X	MMR	MMR	X	X	○	X	Muscle pain
20	54	CP	F	X	I	14	X	X	X	X	ND	○	X	○	CMR	CMR	X	X	○	○	Conj. hemorr., stomatitis
21	76	CP	M	X	I	9	X	○	X	X	ND	X	○	X	CMR	CMR	X	X	○	X	
22	20	CP	M	X	R	8	X	X	X	X	ND	X	X	X	CCyR	CCyR	X	X	X	X	
23	49	CP	F	X	I	7	X	X	X	X	ND	X	X	○	CMR	CMR	X	○	○	X	Eruption
24	36	CP	M	X	I	7	X	X	X	X	○	○	○	X	CMR	CMR	X	X	○	X	
25	65	CP	M	○	I	3	X	○	X	X	○	○	○	X	CMR	CMR	X	X	○	X	

IFN history of interferon treatment, *R* imatinib-resistant, *I* imatinib-intolerant, *duration* duration after the starts of dasatinib treatments (months), *FCM* flow cytometry, *TCR rearr.* TCR gene rearrangement, *PE* pleural effusion, *AE* adverse event, *conj. hemorr.* conjunctival hemorrhage, *eruption* skin eruption, *IP* interstitial pneumonia, *CCyR* complete cytogenetic response, *PCyR* partial cytogenetic response, *NoCyR* no cytogenetic response, *MMR* major molecular response, *CMR* complete molecular response, *ND* not done

not show any rearrangements for all three TCR genes. Thus, clonal TCR gene rearrangements were commonly observed in both groups, and the incidence was higher in the LGL (+) patients than in the LGL (−) patients.

Table 2 Comparison of LGL (+) vs. LGL (−) patients

	LGL increase	
	(+) (n = 15)	(−) (n = 10)
Type of increasing cells		
NK cell	15/15 (100 %)	5/10 (50 %)
CD8$^+$ T cell	11/15 (73 %)	1/10 (10 %)
$\gamma\delta$ T cell	0/15 (0 %)	0/10 (0 %)
Clonal rearrangement of TCR genes		
TCR β	13/15 (87 %)	4/10 (40 %)
TCR γ	12/15 (80 %)	3/10 (30 %)
TCR δ	9/15 (60 %)	4/10 (40 %)
Clinical responses after dasatinib treatments		
Improvement after dasatinib	5/15 (33 %)	3/10 (30 %)
Achievement of CMR among these	4/5 (80 %)	0/3 (0 %)
Sustained former responses	10/15 (67 %)	7/10 (70 %)
Adverse events		
Pleural effusion	9/15 (60 %)	2/10 (20 %)
Skin eruption	6/15 (40 %)	2/10 (20 %)

Favorable molecular response and higher incidence of pleural effusion in the patients with LGL-increase

As shown in Tables 1 and 2, clinical responses were fairly good for both LGL (+) and LGL (−) patients, as 10 of 15 (67 %) and 7 of 10 (70 %) patients sustained the responses to the preceding imatinib treatments in the LGL (+) patients and LGL (−) patients, respectively. Improvement of the responses after dasatinib were observed in 5 of 15 (33 %) of the LGL (+) patients, and 3 of 10 (30 %) of the LGL (−) patients. Newly obtained CMR after dasatinib treatment were observed in 4 of 5 LGL (+) patients (80 %) and 0 of 3 LGL (−) patients (0 %). These results suggest that the LGL (+) patients may achieve more favorable molecular responses, as compared to the LGL (−) patients.

Regarding adverse events, pleural effusions were observed in 9 of 15 LGL (+) patients (60 %), and 2 of 10 LGL (−) patients (20 %). Skin eruptions were observed in 6 of 15 LGL (+) patients (40 %) and 2 of 10 LGL (−) patients (20 %). One LGL (+) patient underwent interstitial pneumonia (IP) and pleural effusion at the time of LGL-increase, then dasatinib treatment was discontinued and steroid treatment was started. As for other adverse events, conjunctival hemorrhage caused by allergic response occurred for both LGL (+) and LGL (−) patients. However, these adverse events mentioned above were not
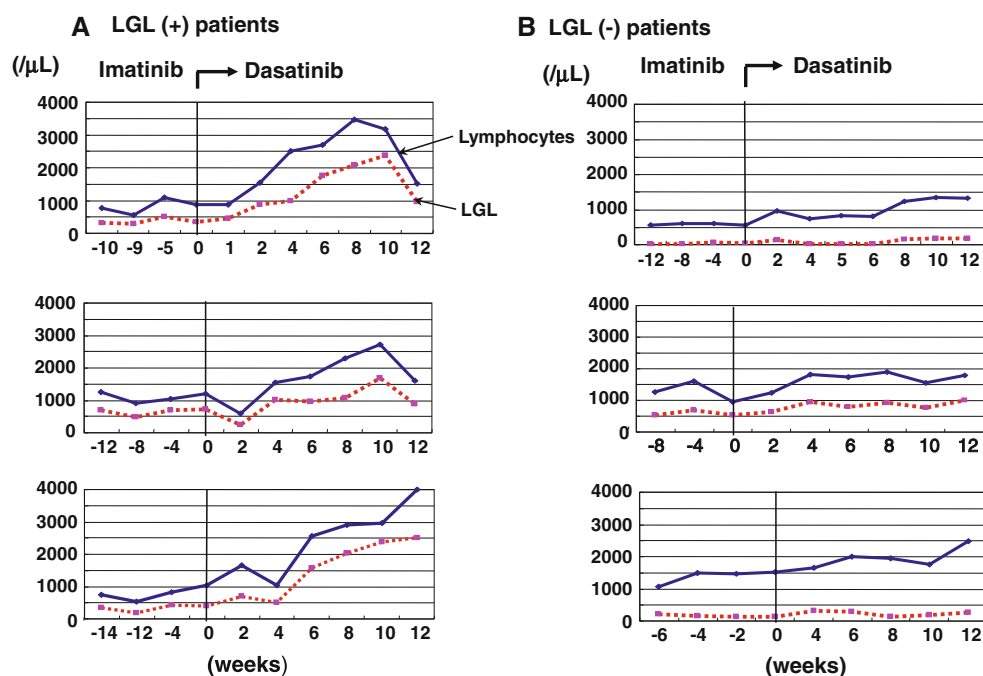
Fig. 1 Lymphocyte and LGL counts in PB both before and during dasatinib treatments for the first 12 weeks. *Solid lines* indicate lymphocyte counts and *dotted lines* indicate LGL counts. *Solid vertical lines* indicate the time points when imatinib were changed to dasatinib. **a** LGL (+) patients (ID #3, #4, #2), and **b** LGL (−) patients (ID #16, #17, #18)

Fig. 2 Persistent increase of lymphocyte and LGL counts in PB during dasatinib treatments for over 60 months of observations in LGL (+) patients. Two representative LGL (+) patients (ID #3, #2) are shown. *Solid lines* indicate lymphocyte counts and *dotted lines* indicate LGL counts. *Solid vertical lines* indicate the time points when imatinib were changed to dasatinib. *Horizontal lines* with *Stop* indicate discontinuation periods that the patients did not take dasatinib during these periods
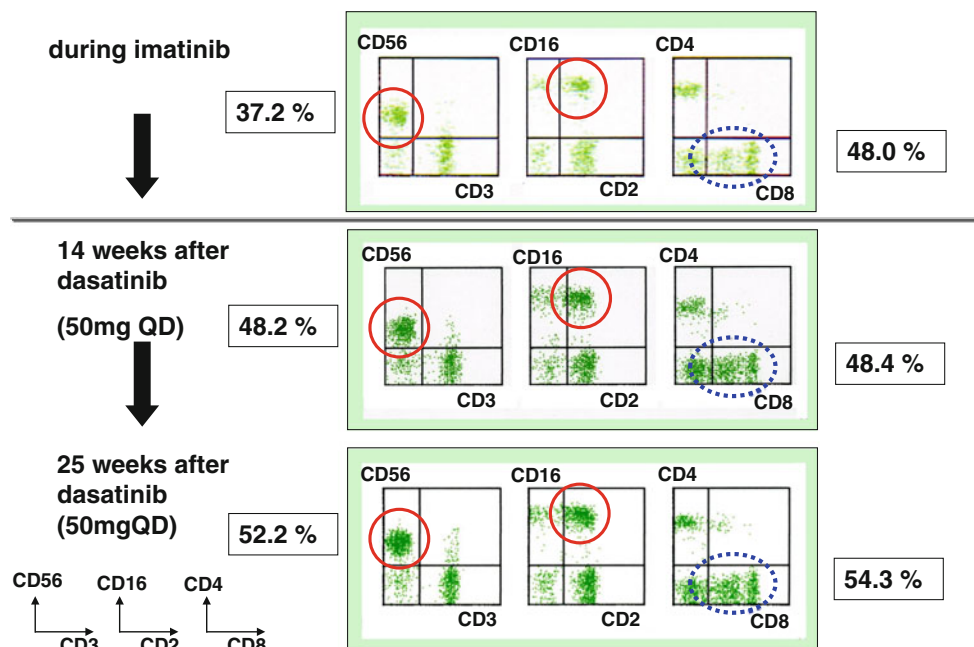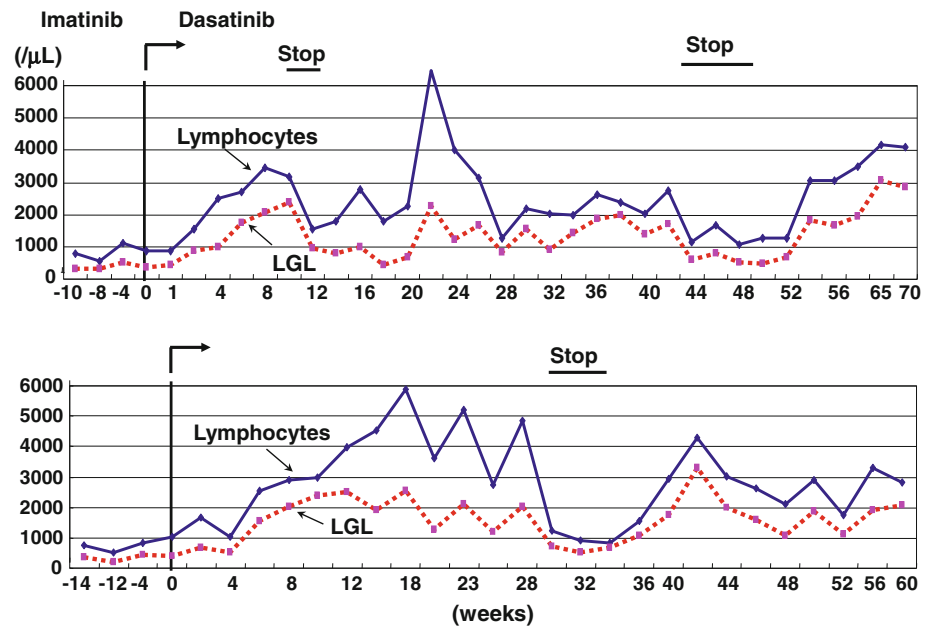
Fig. 3 Two-color analysis of flow cytometry before and after dasatinib treatment. PB samples were collected before dasatinib (meaning during imatinib treatment) and after dasatinib treatment. A representative patient (ID #12) is shown. MNCs were subjected to flow cytometry with antibodies as indicated (*vertical/horizontal*; *left-side* CD56/CD3, center CD16/CD2, *right-side* CD4/CD8). *Solid circles* in the *left-side figures* and *center figures* indicate CD56+ and CD16+ NK cells. Dotted circles in the right-side figures indicate CD8+ T cells. The *numbers in the left rectangular boxes* indicate the percent of the NK cells. The *numbers in the right rectangular boxes* indicate the percent of the CD8+ T cells. *QD* once-a-day oral intake of dasatinib

severe, i.e., within CTCAE grade 1 or 2 for all the adverse events. These results suggest that LGL (+) patients may have higher incidences of pleural effusion and probably skin eruption as well. We could not observe evident enterocolitis or low-grade fever in these patients.

LGL count increases two hours after the oral intake of dasatinib

We examined the lymphocytes and LGL counts in PB before and 2 h after the oral intake of dasatinib. The

interval between the intake of dasatinib and blood test was almost fixed to 2 h. The mean interval was 2 h 1 min (SD ± 8.7 min). Figure 4 shows results of all 25 patients. Total lymphocyte counts were 1,849 ± 1,045/μL (mean ± SD) (before) versus 3,541 ± 2,572/μL (after), indicating 1.9 times (range 1.1–3.9) increase ($P < 0.001$). LGL counts were 1,105 ± 901/μL (before) versus 2,293 ± 1,936/μL (after), indicating 2.1 times (range 1.1–6.1) increase ($P < 0.001$). Thus, both lymphocytes counts and LGL counts significantly increased 2 h after the oral dasatinib intake as compared to those before intake, in all patients.

We then separately analyzed the LGL counts in the LGL (+) patients group ($n = 15$) or in the LGL (−) patients group ($n = 10$) to see if there is any difference of the short-term up-regulation of LGL between these two groups (Fig. 5). As for the 15 LGL (+) patients, LGL counts were 1,349 ± 918/μL (before) versus 2,895 ± 2,240/μL (after), indicating 2.1 times increase ($P = 0.006$). Total lymphocyte counts were 2,153 ± 1,129/μL (before) versus 4,456 ± 3,219/μL (after), indicating 2.1 times increase ($P = 0.01$) (figure not shown). On the other hand, as for the 10 LGL (−) patients, LGL counts were 422 ± 384/μL (before) versus 805 ± 419/μL (after), indicating 1.9 times increase ($P = 0.02$). Total lymphocyte counts were 1,094 ± 556/μL (before) versus 1,859 ± 899/μL (after), indicating 1.7 times increase ($P = 0.03$) (figure not shown). These results indicate that, even though the absolute cell number of the increased LGL was higher in

LGL (+) patients as compared to LGL (−) patients, LGL-increase at 2 h after the intake of dasatinib was seen even in LGL (−) patients as well as LGL (+) patients, and that relative fold-increases of LGL were similar, i.e., approximately 2 times increase, for the two groups.

We next investigated whether these short-term increases of lymphocytes and LGL occur by other ABL-TKIs such as imatinib or nilotinib in the patients who have been treated at the daily basis. The interval between the intake of imatinib or nilotinib and blood test was almost fixed to 2 h, where the mean interval was 2 h 6 min (SD ± 21 min). Figure 6 shows the results of total 14 patients studied (11 nilotinib, 3 imatinib). Total lymphocyte counts were 1,160 ± 471/μL (before) versus 1,210 ± 521/μL (after) (no significant change). LGL counts were 427 ± 206/μL (before) versus 442 ± 250/μL (after) (no significant change). Thus, both lymphocyte and LGL stayed at the basal levels at 2 h after the oral intakes of imatinib or nilotinib. Taken together, the phenomenon of the rapid increase of LGL in the PB occurs exclusively by dasatinib intake, but not by imatinib or nilotinib intakes.
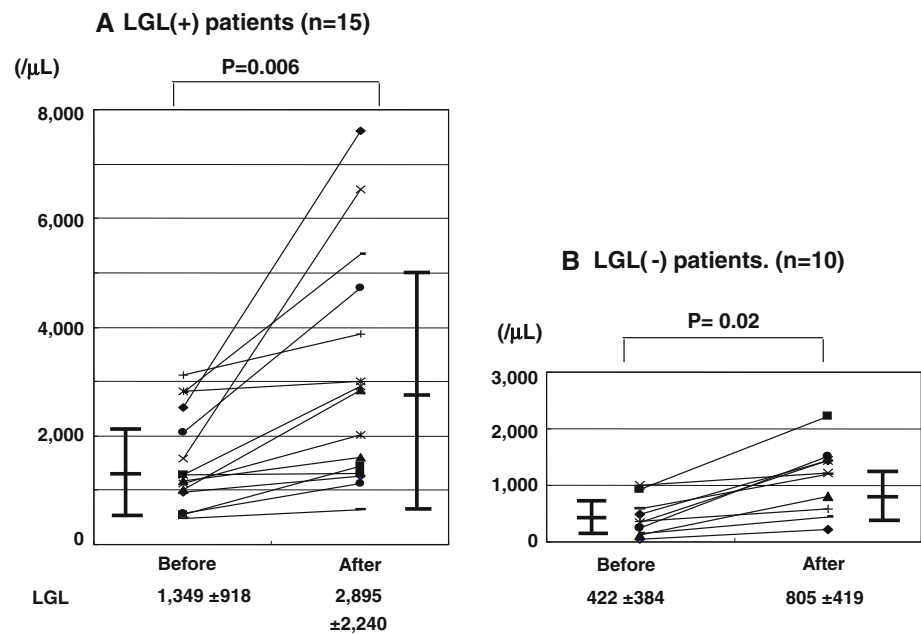
CMV-infection status during dasatinib treatment

We examined CMV-infection status during dasatinib treatments (Table 1). We assessed the status multiple times during dasatinib treatments in all 25 patients except 1
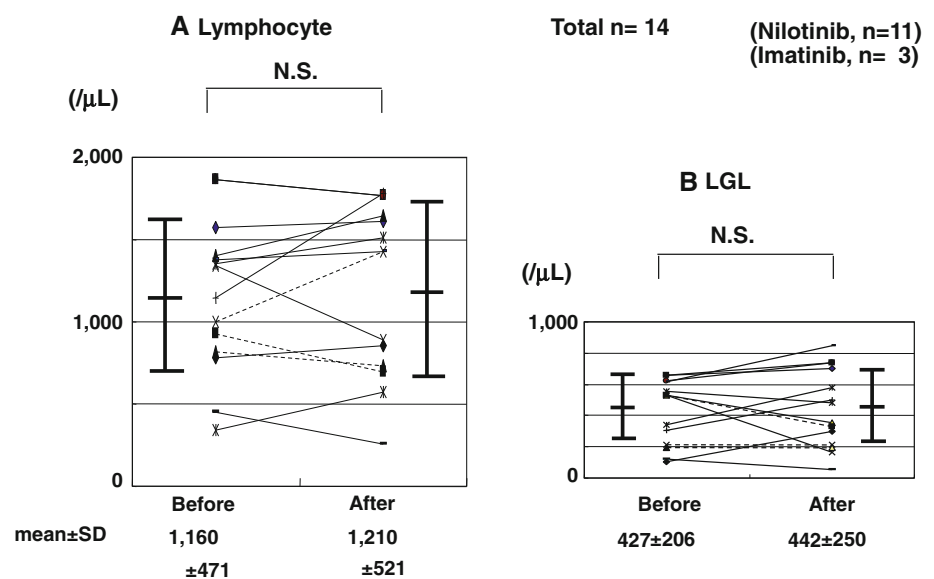

Fig. 4 Lymphocyte and LGL counts in PB before and at 2 h after oral intake of dasatinib. Both lymphocyte (a) and LGL (b) in PB were counted before and after intake of dasatinib in all 25 patients. Vertical lines indicate mean ± SD
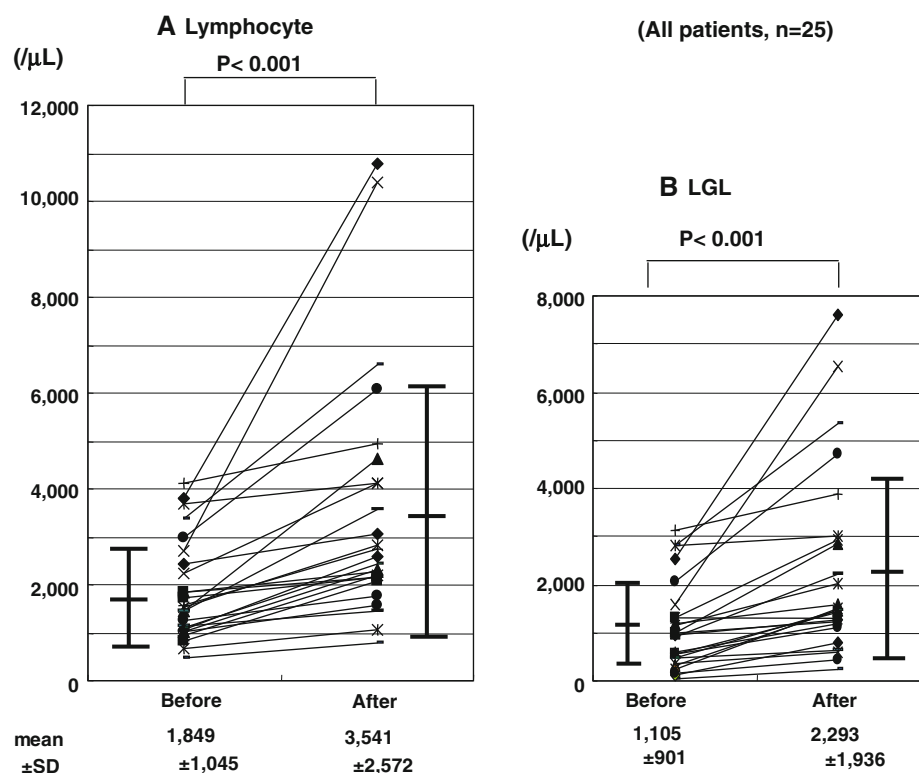
Fig. 5 LGL counts in PB before and at 2 h after oral intake of dasatinib, separately analyzed by the LGL (+) patient group or LGL (−) patients group. **a** LGL (+) patient group (*n* = 15), and **b** LGL (−) patients group (*n* = 10). *Vertical lines* indicate mean ± SD

A LGL(+) patients (n=15)

P=0.006

	Before	After
LGL	1,349 ±918	2,895 ±2,240

B LGL(-) patients. (n=10)

P= 0.02

	Before	After
	422 ±384	805 ±419

Fig. 6 Lymphocyte and LGL counts in PB before and at 2 h after oral intake of nilotinib or imatinib. Both lymphocyte (**a**) and LGL (**b**) in PB were counted before and after intake of nilotinib (*solid lines*, *n* = 11) or imatinib (*dotted lines*, *n* = 3) in the patients who have been taking these drugs at the daily basis. *Vertical lines* indicate mean ± SD. *N.S.* not significant

A Lymphocyte

N.S.

Total n= 14

(Nilotinib, n=11)
(Imatinib, n= 3)

mean±SD	Before	After
	1,160 ±471	1,210 ±521

B LGL

N.S.

	Before	After
	427±206	442±250

patient (ID #13). The frequency of C7-HRP test was 3 times by median (once for 1 patient, 2 times for 9 patients, 3 times for 12 patients, and 4 times for 3 patients), with at least 2 months intervals. Examinations by CMV C7-HRP tests revealed that they were negative in all 25 patients. More precisely speaking, in 1 patient (ID #14 CP patient), only 1 cell was detected out of approximately 60,000 cells examined. This patient showed negative in later examination, i.e., 0 cell in 50,000 cells examined. And in another 1 patient (ID #13 AP patient), only 2 cells were detected out of approximately 60,000 cells examined. This patient was later referred to another hospital, thus re-examination was not available. These 2 patients were considered to be

"negative for CMV infection", because cut-off point for the significant positivity is defined as "over 10 positive cells per 50,000 cells" in this detection system [21].

The frequency of both CMV-IgG antibody and CMV-IgM antibody tests were 3 times by median (once for 1 patient, 2 times for 8 patients, 3 times for 9 patients, 4 times for 5 patients, and 5 times for 2 patients), with at least 2 months intervals. Serum CMV-IgM antibodies were positive only in 2 of the 25 patients (8 %) without any symptoms of CMV infection. One of these 2 patients (ID #8) still showed positive in the later examination. And the other patient (ID #23) showed a reduction of the serum antibody level to the borderline, in the later examination.

Serum CMV-IgG antibodies were positive in 23 of the 25 patients (92 %). These results indicate that most of the patients have clinical histories of CMV infection throughout their lives, but it is unlikely that they caught recent infection or recent reactivation of CMV. Taken together, at least for the CML patients in CP or AP examined in this study, CMV reactivation seems uncommon during dasatinib treatment.

Discussion

In this paper, we investigated the LGL-increase during dasatinib treatment from the viewpoints of its incidence, phenotype of the LGL, TCR gene rearrangements, clinical effects, and adverse events. We also investigated LGL-increase 2 h after the oral intake of dasatinib or other ABL-TKIs. And furthermore, we investigated incidence of CMV reactivation during dasatinib treatments.

LGL lymphocytosis were observed in 60 % of the patients according to our criteria in this study, which were observed by median of 11 weeks after the start of dasatinib treatments, and were persistent during the treatments as long as dasatinib treatments were continued. The frequency of LGL lymphocytosis seems to be almost of similar percentage or slightly higher as compared to the previous reports in Western countries, which showed 64 % (16 of 25) of CML and Ph+ ALL patients [12] and 27 % (4 of 15) of CML patients [11], and also in oriental countries which showed 44 % (8 of 18) [9] and 45 % (9 of 20) [10] of CML and Ph+ ALL patients. One of the issues currently unsolved is that "increase of LGL" has not been clearly defined up to now. Actually in this study, if the definition of "increase of LGL" was tentatively changed to that the absolute LGL number is "equal or more than 1,000/μL" from "equal or more than 1,500/μL", the result changes to that as high as 21 out of 25 (84 %) patients were judged as positive for "LGL-increase". Thus, the actual incidence of LGL lymphocytosis during dasatinib treatment is presumably much higher than we have thought, and it may depend on the definition of "LGL-increase". Increasing LGL cells had NK-cell phenotypes in all the 15 LGL (+) patients in our study, and some of the patients (11 of 15) also showed CD8+ T-cell phenotypes. Previous studies also demonstrated that increasing cells were NK cells and CD8+ T cells, and that these cells characteristically display a late or terminal differentiated phenotype (CD45RO+/−, CD27−, CD57+) [8, 12]. In our study, although expressions of CD45RO and CD27 were not examined, expression of CD57 was positive in all the patients examined in LGL (+) patients.

Favorable molecular responses in the patients with LGL lymphocytosis were suggested in this study. This result seems to be compatible with those reported by other researchers previously [8, 9, 11, 12], implicating that dasatinib-induced T cells or NK cells may be exerting anti-tumor immunomodulatory effects on leukemia cells, and resulting in favorable molecular therapeutic responses. Actually, some previous reports disclosed, by in vitro analysis, that LGLs isolated from dasatinib-treated patients showed enhanced cytotoxicity against either K562 cells (CML cell line) [9, 12] or T2 cells (lymphoblastic cell line expressing HLA-A2) [9] or even against CRL2598 cells (endothelial cells) [13]. In line with this, previous studies have suggested that autologously activated NK cells have a potent cytotoxic activity on CML cells [22, 23]. Another possible hypothesis to account for the favorable responses in LGL (+) patients is that dasatinib has potential to reduce the number of CD4+, CD25+, FOXP3+ regulatory T cells (Tregs) in both PB and bone marrow, which are negatively regulating other immune cells, and that this reduction of Tregs were more substantially observed in LGL (+) patients as compared with LGL (−) patients [8, 24]. A more recent study implicated that some of the patients with LGL-increase had CD8+ T cell populations that are specific for the PR1 leukemia-associated peptide derived from proteinase 3 [12], which is reminiscent of the previously observed phenomenon in CML patients with IFNα treatments [25, 26]. Thus, instead of the immunomodulatory action of IFNα, dasatinib may contribute to the elimination of Ph+ leukemia cells with its own immunomodulatory activity.

Clonal rearrangements of the TCR genes during dasatinib treatments were amazingly high in our current study, in which at least one of the three TCR genes for β, γ, and δ were rearranged in 100 % of the LGL (+) patients, and in 70 % of the LGL (−) patients as well. Amazingly enough, it has been reported that these clonal T cell and NK cells without BCR-ABL1 fusion already existed in over 80 % (15 of 18) of patients with CML at the time of diagnosis, and that dasatinib-treated patients, but not imatinib-treated patients, showed expansion of their pre-existing clones during the therapy [14]. Precise mechanism(s) of the existence of these cells in CML patients at the time of diagnosis and mechanism(s) of the proliferation of these clonal cells by dasatinib treatments are still in vague. However, it is postulated that the proliferation of these cells by dasatinib is probably related to the off-target kinase inhibitory effects mediated by dasatinib, which are not so widely seen imatinib or nilotinib. The pre-existing clonal T cells or NK cells probably expand by dasatinib through inhibition of tyrosine kinases such as Fyn, Lck, Btk, Tec, Lyn, Hck, Src, etc. And several subtypes of Src family kinases such as Fyn [27, 28] or Lck [29] are known to be involved in the regulation or activation of NK cells. Up to now, the phenomenon of LGL-increase has not been

reported in patients treated with imatinib or nilotinib which cannot inhibit Src family kinases. Actually in line with this, our results showed that imatinib treatments could not induce LGL proliferation at all, and nilotinib could not either from our clinical observation. In speculation though, the expansion of these cells by dasatinib treatments might also be associated with HLA-A*02 [12]. This might explain the existence of some patients who did not show LGL-increase by dasatinib treatments. Although we did not have chance to examine in this study whether patients with HLA-A*02 are actually associated with LGL lymphocytosis or not, this could be one of the important issues to be clarified, from the viewpoints that racial differences may exist in terms of LGL lymphocytosis and clinical effectiveness by dasatinib treatments.

Contrary to the favorable response, LGL (+) patients showed higher frequency of adverse events of pleural effusion in this study, which seems in line with the results of previous reports [8, 10]. One of the reports also found out that lymphocytes from pleural fluid showed a similar phenotype ($CD3^+$, $CD8^+$, $CD57^+$) and genotype (identical T cell clone) as simultaneously observed in PB [8]. Several variables on the increased risk of development of pleural effusion has been reported such as, hypertension, hypercholesterolemia, and dasatinib dosage >100 mg per day [30], BP of CML, previous history of cardiac diseases, prior skin rash on dasatinib, and previous history of autoimmune disease [31]. Although the precise mechanism(s) by which dasatinib induces a pleural effusion is still unclear, dasatinib-induced pleural effusion is likely to be immune-mediated phenomenon [30]. It has been suggested that inhibition of platelet derived growth factor (PDGF) receptor-β expressed in pericytes, which are surrounding the endothelial cell layers of the capillary network, may be implicated [30, 32]. In line with this, $CD8^+$T cells isolated from patients with LGL expansion by dasatinib treatments had increased cytotoxicity against pulmonary non-transformed endothelial cells, which may play a role in the autoimmune-like adverse events such as pleural effusion [13]. Thus, pleural effusion induced by dasatinib may be probably multifactorial such as preexisting cardiac or pulmonary dysfunctions, inhibition of PDGFR-β, and auto-immunity associated with LGL increase.

In this study, we showed that dasatinib-induced rapid mobilization of LGL in PB as short as 2 h after the oral intake of dasatinib in all patients examined. This phenomenon was observed irrespective of the patients of LGL (+) or LGL (−), though LGL (+) patients showed higher increase of LGLs by their absolute number. During the assessment of this finding, we also have found out that platelet counts decreased and neutrophil counts and monocyte counts increased at 2 h after the oral intake of dasatinib [33]. These unique phenomena were observed exclusively by dasatinib, but not by imatinib or nilotinib. The rapid mobilization of lymphocytes several hours after the oral intake of dasatinib has also been reported [34], where lymphocytes increased 2.15-fold (range 1.05–5.12) at 1 h of intake, which seems almost similar results with ours. They also reported that close correlation between plasma dasatinib concentration and lymphocyte count was observed. In addition, they reported that differential gene expression analysis from $CD8^+$ T cells at 0 versus 1 h showed up-regulation of IFN-γ-inducible genes such as CXCL-11 or CXCL-9 or C1qB which were known to be correlated with $CD8^+$ T cell viral immune responses [34]. In speculation, temporal inhibition by dasatinib of downstream signaling including adhesion molecules on lymphocytes may have some roles. As yet, clinical implication of this novel and unique phenomenon of rapid mobilization of LGL in PB is presently unknown. This rapid mobilization of LGL and the changes of the counts of hematopoietic cells in PB by dasatinib may raise another issue in terms of designing LGL-related clinical studies, because the time point of oral intake before sampling blood from patients may influence the LGL counts or platelets or other cell counts in PB. From this viewpoint, we tried to check the influence of "the time point of routine oral intake of dasatinib before sampling blood" on the long-term results of LGL-increase in this study. We checked, for each patient, when he or she orally takes dasatinib as a routine intake. The exact time of the routine intake is a slightly difficult to check, then the time points were roughly checked as "morning" or "evening". The results were as follows. In the 15 LGL (+) patients, 11 patients routinely take dasatinib in the morning, and 4 take in the evening. In the 10 LGL (−) patients, 6 patients routinely take dasatinib in the morning, and 4 take in the evening. There was no statistical significance between them. Thus, the long-term-lasting LGL-increase may surpass the short-time LGL-increase. As the number of patients in this study was rather small, that much larger studies may be able to show some differences on this issue.

Regarding CMV infection, a recent report demonstrated that 15 of 16 patients with expansion of LGL were CMV-IgG seropositive, whereas only 3 of 9 patients without expansion of LGL (−) were CMV-IgG seropositive [12]. In some patients with expansion of LGL, CMV DNA viral load was also examined by PCR-based assay in plasma samples. They also reported that 5 of 16 (31 %) patients with expansion of LGL, while 0 of 9 (0 %) patients without expansion of LGL, experienced symptomatic CMV reactivations during dasatinib therapy [12]. Of note, they also reported intriguing findings that most patients with LGL-increase also exhibited high frequencies of CMV-specific $CD8^+$ T cells which recognized CMV pp65$_{495-503}$ peptide

antigen (NLVPMVATV), and that significantly elevated levels of IP-10, IL-6, MIG, and IL-2R were present in the plasma of patients with LGL lymphocytosis as compared with healthy controls [12]. The increased serum levels of these soluble factors observed in patients with LGL-increase may also contribute to the improved therapeutic responses, because these cytokines/chemokines direct the chemotaxis of monocytes, T cells, NK cells, and dendritic cells. Actually, chemotactic effects are known to be relevant for leukemia cell control. They proposed a hypothesis that CMV reactivation was first triggered in the patients treated with dasatinib by its immunosuppressive effects, subsequently leading to the preferential expansion of individual $CD8^+$ T cell or NK-cell clones, which in turn induces favorable outcome of the dasatinib treatments [12]. However, the numbers of positivity of CMV reactivation presented by them seem rather high as compared to our results in the current study, in which CMV reactivation was not evident even in the LGL (+) patients in our study. Actually, we repeatedly examined the CMV C7-HRP tests at several points in the same patients throughout the course of dasatinib treatments, but the results were consistently negative. One of the likely explanations for the difference between their results and our results may be that backgrounds of the patients were slightly different, in which most of the patients (22 of 25) were currently in CP and only 3 of 25 were in AP in our study, whereas 7 of total 25 patients were in advanced phase such as BP or Ph^+ALL in their study. Another explanation may be the difference of the methods for detection of CMV activation, in which the method for the detection of CMV load in our study was horse radish peroxidase (HRP) staining-based method, but not PCR-based method. Another point might be that CMV-IgG seropositivity per se does not necessarily represent recent CMV reactivation. The CMV-IgG seropositivity indicates that the person was infected with CMV at some time during their life. Other researchers also previously reported, by retrospective analysis of 1,150 patients, that dasatinib treatments did not increase opportunistic infections including CMV [35]. Thus, although the number of the patients in our study was relatively limited, our results suggest that CMV reactivation during dasatinib treatment was uncommon at least in CML-CP patients. This is one of the issues to be clarified in the future by studies with a large number of patients.

There is a growing but conflicting body of evidence supporting an as yet uncharacterized role of ABL-TKIs in their interaction with the immune system. For the upcoming new era which aims at "complete cure" of CML patients, it is intriguing that dasatinib has unique potential biological actions as immunomodulator. These unique activities of dasatinib also implicate the possibilities of the usage of this drug for other malignancies. There are still a lot of issues to be clarified in the future. If all these findings are confirmed in larger studies, clinical implication for the treatment of patients with Ph^+ leukemia would be significant.

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