Circulating bcr-abl-specific CD8+ T cells in chronic myeloid leukemia patients and healthy subjects

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Background and Objectives. The fusion oncoprotein bcr-abl that characterizes chronic myeloid leukemia (CML) is a leukemia-specific antigen, which may be immunogenic *in vivo*. KQSSKALQR and GFKQSSKAL, peptide sequences spanning the b3a2 bcr-abl junction, have affinity for HLA-A3 and HLA-B8, respectively, and we have shown the presence of KQSSKALQR on the surface of CML cells. We analyzed the existence of bcr-abl-specific T cells *in vivo* and correlated their presence to contemporary disease burden.

Design and Methods. We investigated circulating CD8+ T lymphocytes directed against the bcr-abl junction, using fluorochrome-labeled tetramers of HLA-A3 with KQSSKALQR and of HLA-B8 with GFKQSSKAL, and flow cytometry analysis. Using chromium-release assays and interferon-γ ELISPOT assays, we also studied the functionality of these expanded T cells.

Results. Eight of 12 b3a2+ HLA-A3+ and/or HLA-B8+ CML patients studied serially on at least three occasions had bcr-abl junction-specific CD8+ T cells. Specific T cells were more likely to be found in patients with a low leukemic burden (p=0.03). Three of 18 HLA-A3+ and/or HLA-B8+ healthy donors had bcr-abl junction-specific T cells, though these were not detected in any of 13 subjects who were HLA-A3− and HLA-B8−. Bcr-abl-specific T cells were expandable *in vitro* in three of seven healthy donors and five of seven CML patients.

Interpretations and Conclusions. Bcr-abl-specific T cells are detectable in CML patients, and might contribute to leukemic control. The occurrence of specific CD8+ T cells in some healthy donors might represent an immune response to occult *BCR-ABL* rearrangements.

Key words: leukemia, CML, BCR-ABL, cytotoxic T lymphocytes, tetramers.

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Chronic myeloid leukemia (CML) is characterized by the Philadelphia chromosomal translocation. This juxtaposes the *ABL* gene on chromosome 9q34 to the *BCR* gene on chromosome 22q11, creating the *BCR-ABL* fusion oncogene, which appears to be central to the pathogenesis of early chronic phase disease. There are two common resultant mRNA transcripts, b3a2 and b2a2 (also known as e13a2 and e14a2)[1] which have different junctional sequences.

The bcr-abl protein is specific to leukemic cells, and the peptide sequences that span the bcr-abl fusion junction are therefore also leukemia-specific. In an investigation of the binding affinity of bcr-abl junctional sequences of 8-11 amino acids to common HLA class I molecules, the b3a2 junctional sequence KQSSKALQR was found to bind well to HLA-A3, while GFKQSSKAL bound to HLA-B8, albeit with somewhat lower affinity. In contrast, no sequences from the b2a2 junction bound well to any of the common HLA class I molecules.[2] These sequence associations between b3a2 junctional peptides and class I HLA were confirmed in cellular studies.[3] HLA-A2 has been associated with the sequence SSKALQRPV,[4] although this association has not been confirmed in other studies.[2,3,5] Several studies have indirectly suggested that CML cells may express bcr-abl junctional peptides on the cell surface.[6-8] Recently, *in vitro* proteosomal digestion of a 27-mer bcr-abl junctional peptide showed that KQSSKALQR peptide is generated by both the household-proteasome and the immuno-proteasome. However, the authors were not able to detect cleavage for the GFKQSSKAL peptide.[9] Using mass spectroscopy on peptides eluted from surface HLA molecules, we have shown that HLA-A3-positive CML primary cells from patients express bcr-abl junctional peptides in association with surface class I HLA.[10] This suggests that CML

cells can present bcr-abl junctional peptides in a manner that may be recognizable by T lymphocytes.

Soluble tetramers of peptide-MHC complexes can be used to analyze T cells specific for infectious agents, tumors, and autoantigens.[11] Fluorochrome-labeled tetramers can be used in flow cytometry, to identify T lymphocytes that carry the T-cell receptor recognizing that particular peptide-MHC complex. Tetramer staining has been used to identify cytomegalovirus (CMV) reactive T cells after allogeneic stem cell transplantation (SCT),[12] and can be adapted to purify reactive T cells prior to *ex vivo* expansion and re-infusion, as a means of treating CMV infection after allogeneic SCT.[13] Using tetramer staining, we showed that circulating $CD8^+$ T cells recognizing KQSSKALQR were present in two of three HLA-A3-positive b3a2-positive CML patients.[10] However, these patients were all studied at a time of active disease, and on a single occasion. We now report the use of tetramer staining for serial monitoring of a total of 14 HLA-A3 and/or HLA-B8 positive b3a2-positive cases. We show that the majority of these cases have low levels of circulating bcr-abl-specific $CD8^+$ T cells, and that their presence may be inversely related to the contemporary leukemic burden. We also report the presence of bcr-abl-specific $CD8^+$ T cells in three of 18 normal subjects, and show that bcr-abl-specific cytotoxic T lymphocytes (CTL) may be expanded from both CML and normal donor samples.

Design and Methods

Patients and normal subjects

The study was approved by the Liverpool Research Ethics Committee and all subjects gave informed consent. The group of patients comprised 14 b3a2-positive HLA-A3 and/or HLA-B8 positive CML patients (Table 1). All but two were studied serially on at least three occasions, at minimum intervals of 1 month. The healthy donor group comprised 18 HLA-A3 and/or HLA-B8 positive healthy subjects (ten HLA-A3 positive; ten HLA-B8 positive; two subjects being both HLA-A3 and HLA-B8 positive). All were studied serially as defined above. The negative control group consisted of 11 subjects: ten were negative for HLA-A3 and HLA-B8 [(six healthy subjects, four CML patients in first chronic phase (two with b3a2; two with b2a2)]; the remaining case was an HLA-A3-positive and b2a2-positive CML patient. Treatment details for the five CML negative control patients are given in Table 1. Each subject was studied on a single occasion only.

Table 1. Clinical details of the patient test group and additional patient controls.

UPN	Age/Sex	Serial tetramer	Treatment details	Disease status	Transcript status	BCR-ABL	HLA type
Patient Test Group							
1	52/M	Pos	IFN; auto SCT; IM 18 months	CCR	Low	b3a2	A3+ B8+
2	37/F	Pos	IM 18 months	CCR	Changing	b3a2	A3+ B8+
3	62/M	Pos	IFN; IM 24 months	CHR; NR	High	b3a2	A3+ B8+
4	51/M	Pos	Auto SCT then IM 26 months	CHR; NR	Changing	b3a2	A3+
5	42/M	Pos	AlloSCT & DLI	CCR	Low	b3a2	A3+
6	43/M	Pos	IM 6 months (since diagnosis)	CHR: PR	Low	b3a2	A3+
7	66/F	Neg	ALL therapy; IM 6 months	AP	High	b3a2	A3+
8	22/M	Neg	Allo SCT then IM 15 months	CCR	High	b3a2	A3+
9	36/M	Neg	IFN 6 months (since diagnosis)	CHR; NR	High	b3a2	A3+
10	29/F	–	Allo SCT	CCR	Low	b3a2	A3+
11	38/M	–	IM 5 months (since diagnosis)	NR	High	b3a2	A3+
12	41/M	Pos	IFN; IM 10 months	CCR	Low	b3a2	B8+
13	53/F	Pos	IFN 28 months (since diagnosis)	CCR	Low	b3a2	B8+
14	48/M	Neg	Allo SCT & DLI	CCR	Low	b3a2	B8+
Additional patient control cases							
15	60/M	Neg	IFN; IM 13 months	CCR		b3a2	A3 - B8 -
16	40/M	Neg	IM 21 months (since diagnosis)	CCR		b2a2	A3 -B8 -
17	39/F	Neg	IFN; IM 13 months	CCR		b2a2	A3 - B8 -
18	36/M	Neg	Allo SCT & DLI; IM 23 months	CCR		b3a2	A3 - B8 -
19	83/F	Neg	IFN; IM 18 months	CHR; NR		b2a2	A3+ B8 -

SCT: stem cell transplantation; DLI: donor leukocyte infusion; IM: imatinib; IFN: a-interferon; ALL: acute lymphoblastic leukemia; AP: accelerated phase; CCR: complete cytogenetic remission; PR: partial cytogenetic remission; NR: no cytogenetic response; CHR: complete hematologic response; Pos: positive; Neg: negative. Patients were considered tetramer-positive when at least one sample during the serial testing was positive as defined in the Design and Methods section.

Tetramer staining

Peripheral blood mononuclear cells (PBMC) or cultured effectors (10^6) were stained with 1 µg of fluorochrome-labeled tetramer (HLA-A3-KQSSKALQR; HLA-B8-GFKQSSKAL; both from ProImmune, Oxford, UK) and with anti-CD8 (Sigma). Briefly, cells were washed twice in phosphate-buffered saline (PBS) + 0.1% bovine serum albumin (BSA) + 0.02% sodium azide and incubated for 20 min at 37°C with tetramer. Cells were then washed twice in PBS + 0.1% BSA + 0.02% sodium azide and incubated with anti-CD8-fluorescein isothiocyanate-conjugated antibody for 20 min on ice. After washing, cells were examined on an EPICS XL-MCL™ flow cytometer. This two-step protocol was optimized for these particular tetramers, since it was observed that the simultaneous addition of anti-CD8 antibody and tetramer to the sample reduced the number of tetramer-positive events measured. This suggests that, as observed by others,[9] the binding of low affinity tetramers requires CD8 stabilization of the T-cell receptor-tetramer complex. Analysis was performed on tightly gated lymphocytes to exclude dead cells and debris. In some cases, cells were counterstained with propidium iodide to exclude dead cells. Tetramer events were only considered positive when their fluorescence was at least 2 log above the mean fluorescence intensity in the tetramer channel. Moreover, samples were defined as positive only if at least 0.05% of $CD8^{bright}$ cells showed tetramer staining in accordance with the manufacturer's recommendation.

Expansion of bcr-abl-specific CTL

In vitro expansion of bcr-abl-specific T cells was performed in seven healthy donors (five HLA-A3-positive; two HLA-B8-positive) and four CML patients (UPN 1, 3, 4 and 13 in Table 1). The peptides KQSSKALQR and GFKQSSKAL were used for experiments in HLA-A3 and HLA-B8 subjects, respectively. For expansion, dendritic cells were used as antigen-presenting cells at a 1:30 ratio. Dendritic cells were prepared, using a modification of the technique described by Yi *et al.*,[14] by culturing adherent PBMC with 1000 IU/mL recombinant human granulocyte-macrophage colony stimulating factor (Peprotech EC Ltd., London UK) and 500 IU/mL interleukin-4 (Sigma) on days 0, 2 and 4. Tumor necrosis factor-α (1000 IU/mL) was added on day 6. Dendritic cells were peptide-pulsed (50 µg/mL) on days 3, 5 and 8.

Effectors were cultured and used in a standard ^{51}Cr release assay (CTL assay), as previously described,[8,10] against HLA-matched B-lymphoblastoid cells as targets. Wells were considered positive when the ^{51}Cr release in the well was more than three standard deviations above the mean background ^{51}Cr release. CTL expansion was considered positive when a statistically significant lysis was observed with peptide-pulsed versus unpulsed targets.[8,10] Effectors were also analyzed by tetramers.

Expansion of bcr-abl-specific T cells for the enzyme-linked immunospot (ELISPOT) assay

PBMC (2×10^6/well) from five patients (UPN 1-3, 6 and 12) were stimulated in 24-well plates with irradiated autologous KQSSKALQR or GFKQSSKAL peptide-pulsed PBMC (1×10^6/well), together with 1 ng/mL of interleukin-7 and 1 ng/mL of interleukin-12. On day 2, 50 IU/mL of interleukin-2 was added to the culture. Cultures were restimulated as above on day 7, and interleukin-2 was added on day 9. On day 15, cultures were tested for the presence of interferon-γ-producing cells by ELISPOT assay, and the remaining cells were stained with KQSSKALQR-A3 tetramer as described before. Briefly, 5×10^4 effectors were co-cultured overnight with $2\text{-}3\times10^4$ irradiated autologous PBMC either pulsed or unpulsed with the peptide. ELISPOT assays were performed as described by the manufacturer's protocol (Diaclone, France). Results are presented as an average of triplicate or quadruplicate cultures.

Molecular studies for BCR-ABL transcript type and quantitative real time polymerase chain reaction (Q-RT-PCR)

The BCR-ABL transcript type and *BCR-ABL* mRNA quantification were performed as described elsewhere.[15]

Statistical analysis

The levels of *BCR-ABL* transcripts, as assessed by Q-RT-PCR, were compared between tetramer-negative and tetramer-positive subjects using the Mann-Whitney log rank test. The comparison of tetramer positivity between patients and healthy donors was made by Fisher's exact test. In the CTL expansion experiments, the proportion of wells positive for specific lysis of pulsed versus unpulsed targets was compared using Fisher's exact test. In the ELISPOT assay, the proportion of interferon-γ-producing cells was compared using the unpaired Student's t test. In all cases, a p value less than 0.05 was taken to be a statistically significant result.

Results

Specificity of tetramer staining

Figure 1A illustrates the gating of events during analysis of tetramer-stained PBMC. Analysis was performed on tightly gated lymphocytes such that propidium iodide-positive events were excluded, and only $CD8^{bright}$ events were considered. Examples of cultures in which antigen-specific T cells were expanded are given in Figure 1B. No tetramer positive cells were

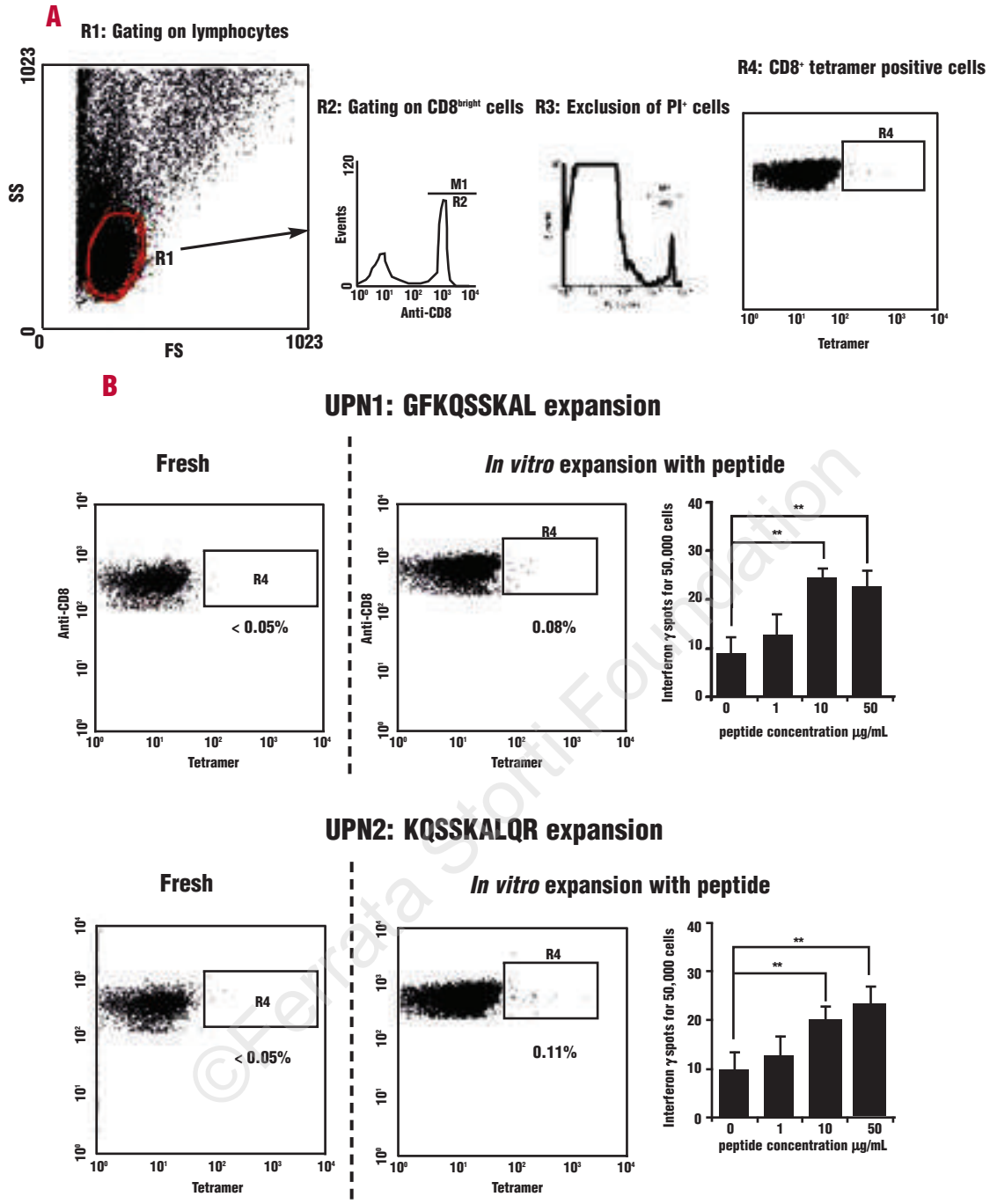

Figure 1. Specificity of tetramer staining. A. Events were tightly gated on lymphocytes and dead cells were excluded by propidium iodide (PI) staining. Only CD8^{bright} events were considered. B. No staining was observed on fresh PBMC. However upon peptide stimulation, tetramer-positive cells could be detected. The presence of tetramer-positive cells in cultures correlated with the presence of interferon-γ-producing cells in ELISPOT assays suggesting that the tetramer staining observed is specific for the peptide-bound tetramers.

observed in the fresh PBMC; however upon stimulation with peptide for 2 weeks a population of tetramer-positive cells became detectable. Moreover, the presence of tetramer-positive events correlated with the presence of interferon-γ producing cells in these cultures. Taken together these data illustrate the specificity of the observed tetramer staining.

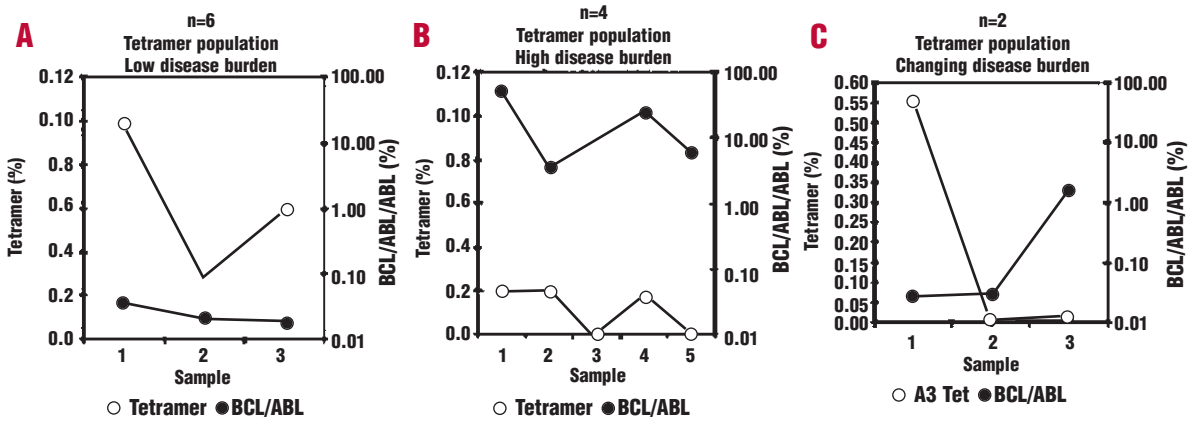

Figure 2. Serial tetramer analysis at a minimum interval of one month. Panels A and B give representative plots for patients with consistently low and high *BCR-ABL/ABL* ratios, respectively. Panel C shows serial tetramer analysis for a patient with a rising *BCR-ABL/ABL* ratio. The number of cases in each group is indicated in the top left corner of each plot.

Serial tetramer analysis on CML patients and healthy donors

The 14 patients were studied on a total of 92 occasions (3-10 times per patient) and serial data were available for 12 cases (Table 1). Eight of the 12 cases (UPN 1-6 and UPN 12 and 13) had 0.05-0.55% tetramer-positive CD8^{bright} cells detected on at least one occasion (6/9 HLA-A3 patients; 5/6 HLA-B8 patients). No obvious correlation was observed between the presence of tetramer positivity and time from diagnosis, or the pattern or duration of previous therapy. In comparison, 1/10 HLA-A3 and 2/10 HLA-B8 healthy donors were tetramer-positive (range 0.05-0.37%) on serial testing. These frequencies are significantly lower than those seen in the patients (p=0.008 overall; p=0.017 and 0.024 for HLA-A3 and HLA-B8, respectively). None of the 13 HLA-A3 and HLA-B8 negative control subjects were tetramer-positive.

Relationship of tetramer with the contemporary disease burden

Figure 2 gives the serial data for representative cases with a low disease burden (*BCR-ABL/ABL* ratio less than 0.1%), poorly controlled disease, or changing disease burden. Most patients with well-controlled disease (Figure 2A) were tetramer-positive on some occasions whilst, in contrast, patients with poorly controlled disease (Figure 2B) were invariably tetramer-negative on serial testing. Interestingly, one case had a rise in disease burden that was preceded by a loss of tetramer-positive cells (Figure 2C). A further case with poorly controlled disease was consistently tetramer-negative (*data not shown*), but 2 years earlier, at the time of a pilot study, had had 0.3% circulating tetramer-positive cells, as previously reported.[10]

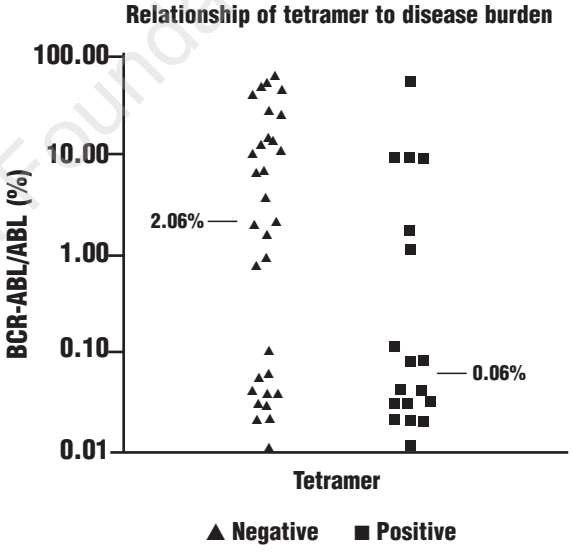

Figure 3. Comparison of the intercurrent *BCR-ABL/ABL* ratio between tetramer-positive and negative patients. The bars and digits denote median levels of *BCR-ABL/ABL* for each population. The tetramer staining (as defined in the Design and Methods section) for each patient at each time point was correlated with its concomitant *BCR-ABL/ABL* ratio. Results are presented in two groups: tetramer-positive samples (■) and tetramer-negative samples (▲) which are correlated with *BCR-ABL/ABL* ratio at the time of the assay.

Figure 3 shows the pattern of tetramer positivity stratified by disease burden as assessed by the *BCR-ABL/ABL* ratio measured by Q-RT-PCR. Tetramer-negative cases had a median *BCR-ABL/ABL* ratio of 2.06% whereas tetramer-positive cases had a median BCR-ABL/ABL ratio of 0.06%. Tetramer-positive cases had a significantly lower *BCR-ABL/ABL* ratio (p=0.031; Mann-Whitney). We have previously shown that a *BCR-ABL/ABL* ratio of 2% or more is

Table 2. Results of CTL expansion experiments with BCR-ABL peptides in CML patients and healthy donors. UPN details for patients correspond to the clinical details in Table 1. The bcr-abl junctional peptides KQSSKALQR and GFKQSSKAL were used to pulse HLA-A3-positive and HLA-B8-positive subjects, respectively.

	Cytotoxicity		p value	Tetramer Pre-Expansion (%)	Tetramer Post-Expansion (%)
	Positive wells/Total wells Unpulsed[a]	Pulsed[b]			
	Control:EBV expansion				
A2	27/53	53/53	p<0.0001	0.39	36
	CML patients				
UPN 1: A3	33/60	11/60	*p*=NS	<0.05	<0.05
UPN 3: A3	3/57	25/60	*p*<0.0001	<0.05	0.05
UPN 4: A3	4/60	4/60	*p*=NS	<0.05	0.05
UPN 13: B8	0/30	0/30	*p*=NS	<0.05	<0.05
	Healthy donors				
A3	6/50	0/50	*p*=NS	<0.05	<0.05
A3	15/60	9/60	*p*=NS	<0.05	0.07
A3	4/60	8/60	*p*=NS	<0.05	<0.05
A3	13/60	51/60	*p*<0.0001	<0.05	<0.05
A3	29/60	58/60	*p*<0.0001	<0.05	0.05
B8	3/30	1/30	*p*=NS	<0.05	0.06
B8	0/20	0/20	*p*=NS	<0.05	<0.05

a: expanded cells tested against unpulsed HLA-A3+ / B8+ B-LCL cells;
b: expanded cells tested against peptide-pulsed HLA-A3+ / B8+ B-LCL cells;
NS: not significant.

associated with Philadelphia chromosome positivity on marrow cytogenetic analysis, whereas a ratio of less than 1% correlates with complete cytogenetic remission.[15]

Bcr-abl-specific T-cell expansion in CML patients and healthy donors

CTL responses to bcr-abl peptides were assessed in patients and healthy subjects following *in vitro* expansion (Table 2). All subjects were tetramer-negative prior to expansion. Bcr-abl-specific CTL expansion was detected by the CTL assay in 2/7 healthy donors and in 1/4 CML patients, all of whom were positive for HLA-A3. Expansion, as assessed by tetramer analysis, was observed in 3/7 normal subjects (2 positive for HLA-A3, 1 for HLA-B8) and in 2/4 patients (both HLA-A3-positive). This suggests that tetramer analysis may be a more sensitive assay.

However, tetramer-positive populations were demonstrated in only two of the three cases in whom expansion was observed by the CTL assay. Since tetramer analysis does not measure T-cell function, it is plausible that a CTL population carrying a low-affinity T-cell receptor was expanded *in vitro* but failed to be detected by tetramer staining. Alternatively, a potent cytotoxic population may have been expanded, but due to cell activation (and therefore T-cell receptor down-regulation) tetramer constructs may have failed to bind to the T cells at a detectable level.

In contrast, a better correlation between cell function and tetramer staining was observed when interferon-γ production was measured by an ELISPOT assay (Table 3). Expanded cells from patients UPN 2 and 3 secreted interferon-γ in the presence of KQSSKALQR peptide, which correlated with the presence of KQSSKALQR-A3 tetramer-positive cells in the culture. Similarly, expanded cells from patients UPN 1 and 2 secreted interferon-γ in the presence of GFKQSSKAL peptide and this correlated with the presence of GFKQSSKAL-B8 tetramer-positive cells in the culture. On the other hand, expanded cells from patients UPN 6 and 12 failed to produce interferon-γ specifically. This correlated with the presence of fewer tetramer-positive $CD8^+$ T cells (i.e. average: 0.35% for patient UPN 3 vs 0.08% for patient UPN 6). These data indicate that tetramer staining likely detects lower numbers of antigen-specific cells than do ^{51}Cr release cytotoxicity assays.

Discussion

Several clinical observations have established the importance of the immune system in conferring cure of CML after allogeneic transplantation. For example, recipients of T-cell-depleted grafts have a higher incidence of relapse,[16] and many post-transplant relapses may be restored to durable molecular remission by infusions of DLI.[17] Several antigens have been identified as possible targets in this immune response. Bcr-abl has received particular attention, because of its unique occurrence in leukemic cells. CTL directed against bcr-abl have been elicited from samples from both normal subjects and CML patients.[18] However, very little is known about whether bcr-abl-specific T cells exist and circulate *in vivo*. We previously reported the presence of CTL recognizing the bcr-abl junction in two of three HLA-A3 positive patients with poorly controlled b3a2-positive CML.[10]

In the present report, we extend this initial observation to a larger population of patients, whom we have now studied serially, and to a similarly sized control population. We also extend our observations to HLA-B8 positive subjects. Two-thirds of HLA-A3 and HLA-B8-positive patients with b3a2-positive CML had evidence of bcr-abl-specific T cells. We also show that ^{51}Cr release assays can only detect the expansion of bcr-abl-specific CTL in a proportion of both CML patients and normal subjects, suggesting

Table 3. Bcr-abl peptide expansion in patients tested by interferon-γ ELISPOT assay. PBMC were stimulated for 2 weeks *in vitro* with KQSSKALQR peptide and tested for interferon-γ production by ELISPOT in the presence or absence of peptide. The remaining expanded cells were stained using KQSSKALQR-A3 tetramer.

	Experiment	IFN-γ producing cells to		p value	Tetramer-positive cells pre-expansion	Tetramer-positive cells post-expansion
		Control[a]	Peptide[b]			
		Expansions with HLA-A3 peptide				
UPN 2	1	10.3±0.8	**23.3**±4.0[c]	$p<0.05$	<0.05%	**0.11%**
UPN 3	1	14.3±1.4	**28.7**±5.1	$p<0.05$	<0.05%	**0.33%**
	2	24.5±0.7	**41.3**±4.1	$p<0.05$	<0.05%	**0.29%**
	3	20.8±3.5	**30**±0.5	$p<0.05$	<0.05%	**0.44%**
UPN 6	1	9.7±9.3	21.3±9.1	p=NS	<0.05%	**0.07%**
	2	21.3±9.6	27±5.0	p=NS	<0.05%	**0.12%**
	3	17.3±2.6	20.3±2.0	p=NS	<0.05%	<0.05%
		Expansions with HLA-B8 peptide				
UPN 1	1	8.5±3.0	**24.0**±2.8	$p<0.05$	<0.05%	**0.08%**
UPN 2	1	5.8±1.3	**17.3**±2.5	$p<0.05$	<0.05%	**0.10%**
UPN 12	1	0.4±0.2	0.8±0.4	p=NS	<0.05%	<0.05%

[a]: control: expanded cells tested against autologous irradiated unpulsed PBMC; [b]: peptide: expanded cells tested against autologous irradiated peptide-pulsed PBMC
[c]: results are average of triplicates or quadruplicates. Significant numbers of IFN-γ producing cells in cultures with peptide are indicated in bold; NS: not significant.

that this assay may not be sensitive enough when working with rarely occurring T cells. Indeed, after expansion, tetramer analysis detected a greater proportion of positive cells than did the specific lysis assays. However, tetramer status correlated well with the interferon-γ ELISPOT assays, suggesting that tetramer analysis may mirror more sensitive specific functional assays. Despite our efforts, we could not attempt T-cell expansions from a tetramer-positive sample. For future work, it could be of interest to investigate the relationship between the presence of functional bcr-abl-specific T cells and the presence of tetramer-positive cells in the original sample. Taken together, the data provide further evidence for the existence of circulating T cells directed against bcr-abl. Where present, these T-cells were detectable on multiple serial occasions.

We also show that the presence and level of these cells alter in inverse relation to the leukemic burden as assessed by Q-RT-PCR, demonstrating a biological relevance for our findings. In two patients, the specific T-cell level had decreased at a time of changing disease burden, in one case preceding a rise in disease burden. It is noteworthy that Westermann and co-workers could detect the presence of bcr-abl-specific T cells in two patients in complete cytogenic remission but not in three patients with 100% of Philadelphia-positive metaphases in a bone marrow specimen.[19] Moreover, Molldrem and co-workers were able to correlate the presence of specific T cells against the potential CML antigen proteinase 3 with clinical responses after interferon-α and allogeneic bone marrow transplant.[20] Recently, Gannagé *et al.* also observed a large expansion in CML-specific tetramer-positive cells in two patients during severe episodes of graft-versus-host disease.[21] It is interesting to speculate that these cells contribute to disease control; however, the low disease burden in some tetramer-negative individuals suggests additional mechanisms for disease control. It is unclear whether alterations in the T-cell level are responsible for the change in disease control, or whether the T-cell level merely mirrors the contemporary disease.

Previous studies have not addressed the relationship of specific T-cell level to the control of CML, nor has the serial pattern of the CTL activity been previously reported. A recent study identified T cells with specificity for the b3a2 bcr-abl junctional peptide sequence GFKQSSKAL in HLA-A2-positive CML patients.[22] Another report described the presence of anti-bcr-abl T cells specific for extrajunctional peptides in HLA-A2-positive CML patients in approximately 60% of cases.[21] To the best of our knowledge, these are the only two studies to look for circulating bcr-abl-specific T cells in CML patients, and their findings are broadly in line with the present results.

We also demonstrated the presence of bcr-abl-specific T cells in three of 18 normal subjects. This proportion is significantly lower than that in CML patients. Using nested PCR and stringent conditions

to avoid cross-contamination, *BCR-ABL* transcripts have been reported in up to one third of healthy subjects.[23,24] It is therefore plausible that *BCR-ABL* may be far more prevalent than the incidence of CML, but that in the vast majority of occasions, the *BCR-ABL* clone may be eliminated. We were unable to test the three healthy individuals for the presence of *BCR-ABL* transcripts, as this was felt to be unethical. It is interesting to speculate that bcr-abl-directed CTL may have a role in the elimination of *BCR-ABL*-bearing clones. The corollary of this hypothesis is that clinical CML requires not only the presence of bcr-abl but also the failure of an anti-bcr-abl immune response.

It is of note that in contrast to our present findings in HLA-A3 and HLA-B8 positive subjects, Rezvani *et al.*[22] did not detect T cells with specificity for the junctional sequence GFKQSSKAL in HLA-A2-positive normal subjects. This may be because the response against bcr-abl varies according to HLA type, or because b3a2 peptide binding is weaker in HLA-A2 subjects. In contrast to the mass spectrometry findings with HLA-A3,[10] it is not currently known whether HLA-A2-positive CML cells express a peptide derived from the bcr-abl junction on the cell surface in association with HLA. Interestingly, in a recent report, $CD8^+$ responses were not detected in any of nine HLA-A2-positive patients immunized with SSKALQRPV, unlike the findings in HLA-A3-positive patients vaccinated with other junctional sequences.[25]

It is not clear to what extent an anti-bcr-abl immune response may contribute to the overall immune response against CML *in vivo*. It is of concern that a $CD4^+$ T-cell clone isolated after DLI was found to recognize bcr-abl breakpoint peptides but not CML cells,[26] although CML cells may be deficient as antigen-presenting cells or target cells. It is not known whether a $CD4^+$ proliferative response against bcr-abl exists *in vivo*, though $CD4^+$ proliferative responses to bcr-abl appear to be defective in CML in comparison to normal subjects.[27-29] It is therefore possible that a CTL response against bcr-abl is ineffective in eradicating CML because of lack of concurrent $CD4^+$ help. Vaccination of CML patients with bcr-abl peptides may elicit a $CD4^+$ proliferative response against bcr-abl,[25,30,31] and this approach deserves further study.

NMB and JMR contributed equally to the work; NMB, JMR, SEC and REC designed the study; NMB, JMR, and HMA-E collected the immunological data; LW collected the molecular data; NMB, JMR, SEC and REC analyzed the data; NMB, JMR and REC drafted the manuscript; NMB and JMR created the figures and tables. All authors revised the manuscript and gave their approval for publication. All authors also declare they have no potential conflict of interest.

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References

1. Deininger MWN, Goldman JM, Melo JV. The molecular biology of chronic myeloid leukemia. Blood 2000; 96: 3343-56.
2. Bocchia M, Wentworth PA, Southwood S, Sidney J, McGraw K, Scheinberg DA, et al. Specific binding of leukemia oncogene fusion protein peptides to HLA class I molecules. Blood 1995;85:2680-4.
3. Bocchia M, Korontsvit T, Xu Q, Mackinnon S, Yang SY, Sette A, et al. Specific human cellular immunity to bcr-abl oncogene-derived peptides. Blood 1996;87:3587-92.
4. Yotnda P, Firat H, Garcia-Pons F, Garcia Z, Gourru G, Vernant JP, et al. Cytotoxic T-cell response against the chimeric p210 BCR-ABL protein in patients with chronic myelogenous leukemia. J Clin Invest 1998; 101: 2290-6.
5. Buzyn A, Ostankovitch M, Zerbib A, Kemula M, Connan F, Varet B, et al. Peptides derived from the whole sequence of BCR-ABL bind to several class I molecules allowing specific induction of human cytotoxic T lymphocytes. Eur J Immunol 1997; 27: 2066-72.
6. Choudhury A, Gajewski JL, Liang JC, Popat U, Claxton DF, Kliche KO, et al. Use of leukemic dendritic cells for the generation of antileukemic cellular cytotoxicity against Philadelphia chromosome-positive chronic myelogenous leukemia. Blood 1997; 89:1133-42.
7. Osman Y, Takahashi M, Zheng Z, Koike T, Toba K, Liu A, et al. Generation of bcr-abl specific cytotoxic T-lymphocytes by using dendritic cells pulsed with bcr-abl (b3a2) peptide: its applicability for donor leukocyte transfusions in marrow grafted CML patients. Leukemia 1999;13:166-74.
8. Norbury LC, Clark RE, Christmas S.E. b3a2 BCR-ABL fusion peptides as targets for cytotoxic T cells in chronic myeloid leukaemia. Br J Haematol 2000;109:616-21.
9. Posthuma EF, Van Bergen CA, Kester MG, De Paus RA, Van Veelen PA, De Ru AH, et al. Proteosomal degradation of BCR/ABL protein can generate an HLA-A*0301-restricted peptide, but high-avidity T cells recognizing this leukemia-specific antigen were not demonstrated. Haematologica 2004; 89:1062-71.
10. Clark RE, Dodi IA, Hill SC, Lill JR, Aubert G, Macintyre AR, et al. Direct evidence that leukaemic cells present HLA-associated immunogenic peptides derived from the BCR-ABL b3a2 fusion protein. Blood 2001; 98:2887-93.
11. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, et al. Phenotypic analysis of antigen-specific T lymphocytes. Science 1996;274:94-6.
12. Keenan RD, Ainsworth J, Khan N, Bruton R, Cobbold M, Assenmacher M, et al. Purification of cytomegalovirus-specific CD8 T cells from peripheral blood using HLA-peptide tetramers. Br J Haematol 2001; 115: 428-34.
13. Szmania S, Galloway A, Bruorton M, Musk P, Aubert G, Arthur A, et al. Isolation and expansion of cytomegalovirus-specific cytotoxic T lymphocytes to clinical scale from a single blood draw using dendritic cells and HLA-tetramers. Blood 2001; 98:505-12.
14. Yi Q, Ekman M, Anton D, Bergenbrant S, Osterborg A, Georgii-Hemming P, et al. Blood dendritic cells from myeloma patients are not infected with Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8). Blood 1998; 92:402-4.
15. Wang L, Pearson K, Pillitteri L, Ferguson JE, Clark RE. Serial monitoring of BCR-ABL by peripheral blood real time polymerase chain reaction predicts the marrow cytogenetic response to imatinib mesylate in chronic myeloid leukaemia. Br J Haematol 2001; 118:771-7.
16. Apperley JF, Jones L, Hale G, Waldmann H, Hows J, Rombos Y, et al. Bone marrow transplantation for patients with chronic myeloid leukaemia: T-cell depletion with cam-

path-1 reduces the incidence of graft-versus-host disease but may increase the risk of leukaemic relapse. Bone Marrow Transplant 1986; 1:53-66.
17. Kolb HJ, Schattenberg A, Goldman JM, Hertenstein B, Jacobsen N, Arcese W, et al. Graft-versus-leukemia effect of donor lymphocyte transfusions in marrow grafted patients. Blood 1995; 86:2041-50.
18. Clark RE, Christmas SE. BCR-ABL fusion peptides and cytotoxic T cells in chronic myeloid leukaemia. Leuk Lymphoma 2001;42:871-80.
19. Westermann J, Schlimper C, Richter G, Mohm J, Dorken B, Pezzutto A. T cell recognition of bcr/abl in healthy donors and in patients with chronic myeloid leukaemia. Br J Haematol 2004; 125:213-6.
20. Molldrem JJ, Lee PP, Wang C, Felio K, Kantarjian HM, Champlin RE et al. Evidence that specific T lymphocytes may participate in the elimination of chronic myelogenous leukemia. Nat Med 2000; 6:1018-23.
21. Gannage M, Abel M, Michallet AS, Delluc S, Lambert M, Giraudier S, et al. Ex vivo characterization of multi-epitopic tumor-specific CD8 T cells in patients with chronic myeloid leukemia: implications for vaccine development and adoptive cellular immunotherapy. J Immunol 2005; 174:8210-8.
22. Rezvani K, Grube M, Brenchley JM, Sconocchia G, Fujiwara H, Price DA, et al. Functional leukemia-associated antigen-specific memory CD8+ T cells exist in healthy individuals and in patients with chronic myelogenous leukemia before and after stem cell transplantation. Blood 2003; 102:2892-900.
23. Biernaux C, Loos M, Sels A, Huez G, Stryckmans P. Detection of major bcr-abl gene expression at a very low level in blood cells of some healthy individuals. Blood 1995; 86:3118-22.
24. Bose S, Deininger M, Gora-Tybor J, Goldman JM, Melo JV. The presence of typical and atypical BCR-ABL fusion genes in leukocytes of normal individuals: biologic significance and implications for the assessment of minimal residual disease. Blood 1998; 92:3362-7.
25. Cathcart K, Pinilla-Ibarz J, Korontsvit T, Schwartz J, Zakhaleva V, Papadopoulos EB et al. A multivalent bcr-abl fusion peptide vaccination trial in patients with chronic myeloid leukemia. Blood 2004;103:1037-42.
26. Zorn E, Orsini E, Wu C, Stein B, Chillemi A, Canning C, et al. A CD4+ T cell clone selected from a CML patient after donor lymphocyte infusion recognizes BCR-ABL breakpoint peptides but not tumor cells. Transplantation 2001: 71:1131-7.
27. Pawelec G, Max H, Halder T, Bruserud O, Merl A, da Silva P, et al. BCR/ABL leukemia oncogene fusion peptides selectively bind to certain HLA-DR alleles and can be recognized by T cells found at low frequency in the repertoire of normal donors. Blood 1996; 88:2118-24.
28. Bertazzoli C, Marchesi E, Passoni L, Barni R, Ravagnani F, Lombardo C, et al. Differential recognition of a BCR/ABL peptide by lymphocytes from normal donors and chronic myeloid leukaemia patients. Clin Cancer Res 2000;6:1931-5.
29. Abu-Eisha H, Butt NM, Rojas JM, Christmas SE, Clark RE. The CD4+ T helper response to b3a2 BCR-ABL is deficient in chronic myeloid leukaemia (CML) and normal subjects. Br J Haematol 2004;125(suppl. 1):14-5.
30. Pinilla-Ibarz J, Cathcart K, Korontsvit T, Soignet S, Bocchia M, Caggiano J, et al. Vaccination of patients with chronic myelogenous leukaemia with bcr-abl oncogene breakpoint fusion peptides generates specific immune responses. Blood 2000;95:1781-7.
31. Bocchia M, Gentili S, Abruzzese E, Fanelli A, Iuliano F, Tabilio A, et al. Effect of a p210 multipeptide vaccine associated with imatinib or interferon in patients with chronic myeloid leukaemia and persistent residual disease: a multicentre observational trial. Lancet 2005;365:657-6.