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The generation of cytotoxic T cell epitopes and their generation for cancer immunotherapy

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Citation

Kessler, J. (2009, October 27). *The generation of cytotoxic T cell epitopes and their generation for cancer immunotherapy*. Retrieved from https://hdl.handle.net/1887/14260

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

CHAPTER 5

Chapter 2

Published in
Leukemia 20:1738-1750, 2006

BCR-ABL fusion regions as a source of multiple leukemia-specific CD8+ T-cell epitopes

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For immunotherapy of residual disease in patients with Philadelphia-positive leukemias, the BCR-ABL fusion regions are attractive disease-specific T-cell targets. We analyzed these regions for the prevalence of cytotoxic T lymphocyte (CTL) epitopes by an advanced reverse immunology procedure. Seventeen novel BCR-ABL fusion peptides were identified to bind efficiently to the human lymphocyte antigen (HLA)-A68, HLA-B51, HLA-B61 or HLA-Cw4 HLA class I molecules. Comprehensive enzymatic digestion analysis showed that 10 out of the 28 HLA class I binding fusion peptides were efficiently excised after their C-terminus by the proteasome, which is an essential requirement for efficient cell surface expression. Therefore, these peptides are prime vaccine candidates. The other peptides either completely lacked C-terminal liberation or were only inefficiently excised by the proteasome, rendering them inappropriate or less suitable for inclusion in a vaccine. CTL raised against the properly processed HLA-B61 epitope AEALQRPVA from the BCR-ABL e1a2 fusion region, expressed in acute lymphoblastic leukemia (ALL), specifically recognized ALL tumor cells, proving cell surface presentation of this epitope, its applicability for immunotherapy and underlining the accuracy of our epitope identification strategy. Our study provides a reliable basis for the selection of optimal peptides to be included in immunotherapeutic BCR-ABL vaccines against leukemia.

Leukemia (2006) **20,** 1738–1750. doi:10.1038/sj.leu.2404354; published online 24 August 2006

Keywords: BCR-ABL fusion peptides; HLA class I peptide binding; proteasome; antigen processing; cytotoxic T lymphocyte epitopes

Introduction

Transfusions of donor lymphocytes (DLT) in relapsed chronic myeloid leukemia (CML) after allogeneic stem cell transplantation (SCT) induces lasting remissions in the majority of patients.[1] Proposed antigens involved in this graft-versus-leukemia effect include minor histocompatibility antigens,[2] BCR-ABL fusion proteins, proteinase 3,[3,4] Wilm's tumor protein 1[3] and the telomerase catalytic subunit (hTERT).[3] Treatment of CML with defined vaccines or T cells would enable bypassing SCT, which is often accompanied with serious graft-versus-host reactions. In the search for leukemia-specific antigens particularly the BCR-ABL fusion proteins have attracted much attention. The (9;22) chromosome translocation gives rise to the Philadelphia (Ph) chromosome and the BCR-ABL chimeric gene.[5] In CML, either the second or the third exon of the BCR gene is spliced to the second exon of the ABL gene creating b2a2 and b3a2 RNA messages, respectively. In Ph+ acute lymphoblastic leukemia (ALL) a third BCR-ABL mRNA form is transcribed: the e1a2 variant.[6] The b3a2, b2a2 and e1a2 fusion proteins have abnormal tyrosine kinase activity and are critical to the leukemogenic process. The tyrosine-kinase inhibitor imatinib (Gleevec, Novartis, East Hanover, NJ, USA) can induce complete molecular remissions in chronic phase CML patients.[7–9] However, its effect in blast crisis CML and Ph+ ALL is limited and resistance to this inhibitor owing to BCR-ABL kinase domain mutations is frequent.[9–11] For the eradication of residual disease, potent and specific immunotherapy is therefore still a worthy goal.

Peptides spanning the BCR-ABL fusion regions are strictly leukemia-specific antigens. Several studies have reported CD4+ T-helper cell responses directed to BCR-ABL fusion peptides.[12,13] Although the role of CD4+ T cells, either by directly targeting leukemic cells or in providing help for CD8+ T cells via dendritic cell maturation, should not be underestimated, for massive and direct killing of leukemic cells, robust CD8+ cytotoxic T lymphocyte (CTL) responses are required. Therefore, BCR-ABL fusion regions have been scrutinized as targets for CTL-mediated immunotherapy. Fusion peptides from the b3a2 and b2a2 regions were found to bind to the human lymphocyte antigen (HLA)-A3/A11,[14,15] HLA-B7,[16] HLA-B8,[14,17] HLA-B27,[16] HLA-B35[16] and HLA-B44[17] HLA class I molecules. Binding of fusion peptides to HLA-A2, by far the most prevalent molecule, is subject of conflicting data. Most studies[14,15,17–19] did not find binding of fusion peptides in HLA-A2, however, two others reported significant binding[16,20] (summarized in Table 2).

In vitro CTL responses were demonstrated against several of these HLA class I binding fusion peptides. Peptide specificity of CTL raised *in vitro* against $b3a2^{HSATGFKQSSK}$ and $b3a2^{KQSSKALQR}$ loaded in HLA-A3 was achieved in several studies[15,20–22] and CTL recognition of CML cells suggested their natural presentation.[20,22] HLA-B8 binding peptide $b3a2^{GFKQSSKAL}$ was proven immunogenic *in vitro* and data supporting endogenous presentation of this peptide have been reported.[20,22] Finally, CTL raised against $b3a2^{SSKALQRPV}$ in HLA-A2-killed HLA-matched CML cells,[20] although this peptide was not detected in peptides eluted from HLA-A2+ CML cells.[23] To date, results – either biochemical or functional – unequivocally demonstrating natural HLA class I presented cell surface expression of BCR-ABL fusion peptides is mainly lacking. Only for $b3a2^{KQSSKALQR}$ the natural expression was biochemically established because it was detected in peptides eluted from HLA-A3 expressed on CML cells.[24]

Based on these studies, phase 1 and 2 trials with a peptide vaccine containing five of the HLA class I binding b3a2 fusion peptides were nevertheless initiated by the group of Scheinberg.[25,26] In some patients CD8+ T-cell reactivity was observed against two of the HLA-A3/A11 binding b3a2 peptides.

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Received 31 May 2006; accepted 6 July 2006; published online 24 August 2006

Table 1 Newly identified HLA class I binding BCR-ABL fusion peptides

BCR-ABL peptide sequences[a]																		*Binding capacity (IC_{50})*[b]			
																		A68[c]	*B51*	*B61*	*Cw4*
b3a2																					
H	S	A	T	G	F	K	Q	S	S	**K**								**2**	—	—	—
	S	A	T	G	F	K	Q	S	S	**K**								**2.5**	—	—	—
		A	T	G	F	K	Q	S	S	**K**								**7**	—	—	—
			T	G	F	K	Q	S	S	**K**								32	—	—	—
		A	T	G	F	K	Q	S	S	**K**	A							> 100	—	—	—
			T	G	F	K	Q	S	S	**K**	A							—	> 100	—	—
		A	T	G	F	K	Q	S	S	**K**	A	L						> 100	—	—	> 100
			T	G	F	K	Q	S	S	**K**	A	L						—	> 100	—	> 100
				G	F	K	Q	S	S	**K**	A	L						—	—	—	95
					F	K	Q	S	S	**K**	A	L	Q	R				30	—	—	—
						K	Q	S	S	**K**	A	L	Q	R				98	—	—	—
							Q	S	S	**K**	A	L	Q	R	P	V		—	> 100	—	—
b3b4 (BCR)																					
H	S	A	T	G	F	K	Q	S	S	N								> 100	—	—	—
b2a2																					
		I	P	L	T	I	N	K	E	**E**	A							—	> 100	—	—
				L	T	I	N	K	E	**E**	A	L	Q	R				**3.5**	—	—	—
					T	I	N	K	E	**E**	A	L	Q	R				**6.5**	—	—	—
						I	N	K	E	**E**	A	L	Q	R	P	V		—	—	**10**	—
							N	K	E	**E**	A	L	Q	R	P	V		—	—	21	—
								K	E	**E**	A	L	Q	R	P	V		—	—	< **1**	—
							N	K	E	**E**	A	L	Q	R	P	V	A	—	—	22	—
								K	E	**E**	A	L	Q	R	P	V	A	—	—	< **1**	—
									E	**E**	A	L	Q	R	P	V	A[d]	—	—	< **1**	
										E	A	L	Q	R	P	V	A[d]	—	—	**11.5**	—
e1a2																					
			G	A	F	H	G	D	A	**E**	A							—	> 100	—	—
				A	F	H	G	D	A	**E**	A							—	—	—	> 100
		E	G	A	F	H	G	D	A	**E**	A	L						—	—	—	> 100
			G	A	F	H	G	D	A	**E**	A	L						—	> 100	—	> 100
				A	F	H	G	D	A	**E**	A	L						—	—	—	**15**
					F	H	G	D	A	**E**	A	L						—	—	—	> 100
						H	G	D	A	**E**	A	L	Q	R	P	V		—	—	**3**	—
							G	D	A	**E**	A	L	Q	R	P	V		—	> 100	**1**	—
								D	A	**E**	A	L	Q	R	P	V		—	**15**	**2.5**	—
									A	**E**	A	L	Q	R	P	V		—	—	< **1**	—
							G	D	A	**E**	A	L	Q	R	P	V	A	—	—	17	—
								D	A	**E**	A	L	Q	R	P	V	A	—	85	23.5	—
									A	**E**	A	L	Q	R	P	V	A	—	—	< **1**	—
										E	A	L	Q	R	P	V	A[d]	—	—	**11.5**	—
a1a2 (ABL)																					
								L	E	E	A	L	Q	R	P	V	A	—	—	< **1**	—

HLA, human lymphocyte antigen.
[a]The aa. encoded by the BCR-ABL fusion DNA-triplet is printed bold and underlined.
[b]Binding affinity classified according to the following cutoffs:
High affinity (bold printed): $IC_{50} \leqslant 5\,\mu M$; intermediate affinity (bold printed): $5\,\mu M < IC_{50} \leqslant 5\,\mu M$; low affinity: $15\,\mu M < IC_{50} \leqslant 100\,\mu M$; no binding $IC_{50} > 100\,\mu M$. Dash, not tested.
[c]The HLA class I molecule that was tested.
[d]BCR-ABL fusion-peptides that are identical to ABL a1a2-derived peptides.

Recently, a similar peptide vaccination trial was conducted by Bocchia *et al.*[27] in patients who were treated with either imatinib or interferon-α and resulted in several complete cytogenetic remissions.

A systematic study of the proteasomal processing of BCR-ABL breakpoint peptides binding to HLA class I molecules has not been reported although our previous work[28,29] and that of others[30–32] have unequivocally shown that C-terminal precise excision by the proteasome is a major requirement for cell surface representation of any CTL epitope (reviewed by Kloetzel and Ossendorp[33]), whereas N-terminal extentions of the epitope can be trimmed by aminopeptidases.[34] As a consequence of inappropriate processing, on average, only less than one third of all HLA class I binding peptides is generated by the intracellular processing machinery.[35]

The current study, therefore, evaluates the BCR-ABL fusion regions as source for naturally expressed CTL targets by a systematic reverse immunology approach involving both HLA binding and proteasomal processing studies.[28] The ability to be processed and thereby appear at the cell surface of BCR-ABL fusion peptides has not been comprehensively tested before. Furthermore, many HLA class I molecules have not yet been tested for their capacity to harbor fusion peptides and binding of the e1a2 fusion region derived peptides to HLA class I was neglected until now. We first searched for BCR-ABL fusion peptides binding in novel HLA class I molecules to enable a

BCR-ABL-targeted immunotherapy covering all haplotypes. Subsequently, the C-terminal generation of both the already reported and the newly identified HLA class I binding fusion peptides was assessed by *in vitro* proteasome digestions. In our previous work,[28] and thereafter in studies of other groups,[36–38] this combination of HLA class I binding studies with proteasome digestion analysis resulted in the efficient and accurate identification of CTL epitopes. Here, the analysis of the enzymatic generation of all HLA class I binding BCR-ABL fusion peptides led to the identification of multiple novel predicted BCR-ABL CTL epitopes. Against one HLA-B61 presented epitope functional T cells were generated that recognized leukemic cells expressing the relevant fusion protein, thereby underscoring the reliability of our approach. Importantly, we show that processing constraints preclude the efficient endogenous generation of more than half of HLA class I binding fusion peptides, including two peptides that have previously been considered suitable as vaccine peptides. Our study strongly facilitates the selection of appropriate vaccine peptides for improved BCR-ABL breakpoint-directed CML and ALL immunotherapy.

Materials and methods

Cell lines

For HLA-A2, HLA-A68, HLA-B51, HLA-B61 and HLA-Cw4 binding assays, respectively Epstein–Barr virus transformed B-lymphoblastoid cell lines (B-LCL) JY (A*0201, B*0702, CW*0702), A68HI (A*6801, B*4402, Cw*0704), C1R-B*5101 (C1R transfected with HLA-B*5101[39]), Sweig007 (A*2902, B*4002 (B61), Cw*0202) and the C1R cell line (HLA-Cw*0401 positive), were chosen. For T-cell recognition the ALL cell line TOM1 (HLA type: HLA-A*11, -A*26, -B*46, -B*54, -Cw*01) was used and stably transduced with either plasmid pcDNA3.1-HLA-B*4002 (encoding HLA-B61) or, as control, the empty vector. The neomycin resistant population was enriched for high HLA-B61 expression by flow cytometric sorting using human Moab JOK3H5 against HLA-B61. Cell lines were cultured in complete culture medium Iscove's modified Dulbecco's medium ((IMDM); Biowithaker, Verviers, Belgium) supplemented with 8% fetal calf serum ((FCS); Gibco BRL, Carlsbad, CA, USA) and 2 mM L-glutamine.

Peptides

Short 8–11-mer peptides and long 27-mer polypeptides were synthesized as described before.[28]

HLA class I peptide-binding prediction and HLA class I molecule selection

The b3a2, b2a2 and e1a2 fusion regions were screened with HLA peptide-binding prediction software of BIMAS (NIH, USA; http://bimas.cit.nih.gov/molbio/hla_bind/)[40] and SYFPHEITI (University of Tuebingen, Germany; www.syfpeithi.de).[41] For prediction of peptide binding in HLA-A*0201 the MOTIF (MTF) algorithm was used as well.[42] Octameric, nonameric and decameric fusion peptides harboring two primary anchor residues, which for that reason scored high in peptide-binding prediction, were selected. HLA class I molecules were selected for which such peptides were found and that were not studied before. For these molecules, the set of test peptides was extended with the length and shift variant peptides that harbored only one major anchor residue.

Cellular binding assays for HLA-A2, HLA-A68, HLA-B51, HLA-B61 and HLA-Cw4

Affinity of peptides for HLA-A2 (A*0201), HLA-A68 (A*6801), HLA-B51 (B*5101), HLA-B61 (B*4002) and HLA-Cw4 (Cw*0401) was measured using competition-based cellular peptide-binding assays developed at our department as described.[43,44] The capacity of test peptides to compete with the cell surface expressed HLA class I binding of a fluorescein (Fl)-labeled reference peptide that is known to bind with high affinity in the HLA molecule of interest was tested. As Fl-labeled reference peptides were used: FLPSDC(Fl)FPSV (derived from hepatitis B virus (HBV) nucleocapsid 18–27) and YMLDLC(Fl)-PETT (human papilloma virus (HPV) E7 11–20) for HLA-A*0201, KTGGPIC(Fl)KR for HLA-A*6801, TGYLNTC(Fl)TV[45] for HLA-B*5101, GEFGGC(Fl)GSV for HLA-B61 (B*4002) and SFNC(Fl) GGEFF[46] for HLA-Cw*0401.[43,44] The percentage inhibition of Fl-labeled reference peptide binding by the competitor test peptide was calculated using the following formula:

$$(1 - (MF_{\text{reference and competitor peptide}} - MF_{\text{no reference peptide}})/(MF_{\text{reference peptide}} - MF_{\text{no reference peptide}})) \times 100\%.$$

The binding affinity of competitor peptide is expressed as the concentration needed to inhibit 50% binding of the Fl-labeled reference peptide (IC_{50}). An $IC_{50} \leqslant 5\,\mu M$ was considered high-affinity binding, $5\,\mu M < IC_{50} \leqslant 15\,\mu M$ was considered intermediate-affinity binding, $15\,\mu M < IC_{50} \leqslant 100\,\mu M$ was judged low-affinity binding and $IC_{50} > 100\,\mu M$ was considered as absence of binding.

HLA-A2-peptide assembly and dissociation assay

Stability of peptides in HLA-A*0201 was measured as described.[47] In short, JY cells (HLA-A*0201$^+$) were treated with 10^{-4} M emetine (Sigma, St Louis, MO, USA) for 1 h at 37°C to stop synthesis of major histocompatibility complex (MHC) class I molecules. Naturally bound peptides in HLA-A*0201 were removed by acid treatment and reconstituted with the peptide of interest at 200 μM in 2% FCS/IMDM containing 2 μg/ml human β_2-microglobulin for 1.5 h at room temperature. At this time point HLA-A2 – peptide assembly was determined by staining with the Moab BB7.2 and GAM-Fitc followed by flow cytometric analysis. Peptide assembly is expressed as fluorescence index which is calculated as

$$(MF_{\text{sample}} - MF_{\text{background}})/MF_{\text{background}}.$$

Remaining cells were washed twice to totally remove free peptide and incubated at 37°C for another 2 h and subsequently the residual HLA-A2 – peptide complexes were again stained and analyzed to determine the peptide dissociation rate. The HLA-A*0201-peptide stability (dissociation) is expressed as percentage of remaining complexes after 2 h relative to the amount of assembled complexes at 0 h. As positive control the HBV nucleocapsid 18–27 epitope was used.

Subunit composition of 20S proteasome preparations

Proteasomes (20S) were purified as described[48] from HeLa cells, a B-LCL cell line and CML cells that were isolated from a patient in blast crisis. B-LCL are known to contain mainly immunoproteasomes[49] and HeLa mainly the constitutive proteasome subunits. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis to separate proteins and Western blotting was

performed according to standard protocols. For staining of the immunosubunits polyclonal antibodies raised against human LMP2 and MECL-1 peptides were used[50] and the constitutive subunits were stained with antibody MCP421 against human Delta and antibody P93250 for human MB1.[51,52]

In vitro *proteasome-mediated digestions and mass spectrometrical analysis*

In vitro 20S proteasome-mediated digestions of 27-mer BCR-ABL polypeptides were performed as described.[28] Briefly, peptides (3 nmol) were incubated with 1 μg of purified proteasome at 37°C for 1, 4 and 24 h in 300 μl proteasome digestion buffer. Trifluoroacetic acid was added to stop the digestion and samples were stored at −20°C. Electrospray ionization mass spectrometry was performed on a hybrid quadrupole time-of-flight mass (Q-TOF) spectrometer (Micromass) as described.[28] The peaks in the mass spectrum were searched in the digested precursor peptide using the Biolynx software (Micromass). The intensity of the peaks was used to establish the relative amounts of peptides digested. The relative amounts of the digestion fragments are given as the percentage of the total mass-peak intensities of digested 27-mer at 1 h incubation time, because this time point best reflects physiologically relevant enzymatic activity.[28]

In vitro *CTL response inductions against putative HLA-A68 and HLA-B61 CTL epitopes*

In vitro CD8$^+$ CTL inductions against synthetic peptides were performed as described.[28] Peripheral blood mononuclear cells of an HLA-B61-positive healthy donor (for inductions against b2a2$^{12\text{-}21}$ and e1a2$^{13\text{-}21}$) and an HLA-A68-positive (for induction against b2a2$^{8\text{-}18}$) healthy donor were used for the generation of antigen presenting cells and autologous CD8$^+$ T cells. Peptide specificity of bulk CTL cultures was tested at day 28 in a standard ^{51}Cr cytotoxicity assay. The highly peptide-specific T-cell culture against e1a2$^{13\text{-}21}$ (AEALQRPVA) was cloned by limiting dilution at day 29. Recognition of tumor cells by peptide-specific CTL clones was tested in an interferon-γ enzyme-linked immunosorbent assay (ELISA): 20.000 CTL/well were coincubated with 20 000 tumor cells/well in a 96-well plate and supernatants were harvested after 14 h.

Results

Identification of novel HLA class I binding BCR-ABL fusion peptides

To date, 12 BCR-ABL fusion peptides have been reported to bind to several HLA class I molecules (HLA-A2, -A3/A11, -B7, -B8, -B27, -B35 or HLA-B44) with a least intermediate affinity (listed in Table 3). To expand this set, enabling immunotherapy for more haplotypes thereby covering a greater percentage of the population, the BCR-ABL fusion regions were screened, with the help of two freely accessible HLA class I peptide-binding predictions algorithms, for the presence of binding motifs of all HLA class I molecules that were not evaluated before. Fusion peptides containing two primary anchors, a prerequisite for efficient binding, were found for HLA-A68, HLA-B51, HLA-B61 and HLA-Cw4, motifs for all other HLA class molecules were not found. In general, HLA class I binding predictions are reliably selecting peptides with actual binding capacity,[43] but false negative predictions cannot be completely excluded. Therefore, we extended the set of fusion peptides that were predicted to have binding capacity with length and shift variants containing only one primary anchor, because in particular these peptides may have binding capacity despite not predicted to bind strongly. These peptides were synthesized and tested for their actual binding capacity in competition-based cellular-binding assays which we developed for these HLA molecules.[43,44]

Ten peptides from the b3a2 and b2a2 breakpoint regions that contained an anchor residue at position 2 (Val, Thr or Ser) and/or the C-terminal anchor (Arg or Lys)[41] were tested for binding to HLA-A68 (A*6801). Five peptides bound with high ($IC_{50} \leqslant 5$ μM) or intermediate affinity (5 μM $< IC_{50} \leqslant 15$ μM). The other peptides displayed low (15 μM $< IC_{50} \leqslant 100$ μM) or undetectable binding capacity ($IC_{50} > 100$ μM). Because the three high-affinity binding b3a2 peptides differ from the non-leukemic BCR b3b4 region only at their C-terminal lysine (encoded by the fusion-triplet codon), we additionally tested the BCR b3b4 peptide HSATGFKQSSN. Importantly, this peptide, lacking the C-terminal HLA-A68 anchor, did not display any binding in HLA-A68 (Table 1), indicating that the binding of the b3a2 peptides is strictly BCR-ABL-specific and cross-reactivity of CD8$^+$ T cells against the b3a2 peptides with normal BCR peptides will not occur.

Ten peptides harboring at least one anchor complying with the HLA-B51 (B*5101) binding motif (Ala, Pro or Gly at P2; Phe, Ile or Val at the C-terminus)[41] were tested. Only e1a2$^{\text{DAEALQRPV}}$, which is the peptide that scored best in binding prediction, showed a significant binding affinity (IC_{50} 15 μM).

Fourteen b2a2 and e1a2 fusion peptides were tested for binding to HLA-B61 (B*4002). Three b2a2 peptides and two e1a2 peptides that contained anchors at both the P2 (Glu) and the C-terminal position (Val, Ala or Ile)[53] displayed high-binding affinity ($IC_{50} \leqslant 5$ μM). Furthermore, three e1a2 peptides (HGDAEALQRPV, GDAEALQRPV and DAEALQRPV) that harbored only one anchor bound with high affinity as well. For GDAEALQRPV this can be explained by the presence of the glycine at P1 and the glutamine at P4, which at these positions are preferred binding residues for HLA-B61. The other six peptides displayed intermediate or low-binding capacity for HLA-B61 ($IC_{50} > 5$ μM). It is important to note that, although harboring the fusion DNA triplet encoded aa., HLA-B61 binding b2a2$^{\text{EEALQRPVA}}$ and b2a2/e1a2$^{\text{EALQRPVA}}$ are identical to normal ABL a1a2 sequences. Furthermore, b2a2$^{\text{KEEALQRPV(A)}}$ and e1a2$^{\text{AEALQRPV(A)}}$ differ only in one residue from their corresponding ABL a1a2 peptides ((L)EEALQRPVA) which displayed equally high-binding capacity ($IC_{50} < 1$ μM). This implicates that cross-reactivity of T cells specific for those b2a2 and e1a2 peptides may occur towards normal a1a2 ABL peptides.

Finally, eight fusion peptides from b3a2 and e1a2 that contained at least one anchor residue for HLA-Cw4 were tested for binding. Only e1a2$^{\text{AFHGDAEAL}}$ that contained both anchors (Tyr, Pro or Phe at P2; Leu, Phe or Met at the C-terminus)[41] bound efficiently (IC_{50} 15 μM).

In summary, we identified five fusion peptides for HLA-A68, one for HLA-B51, 10 fusion peptides for HLA-B61 and one for HLA-Cw0401 with high or intermediate class I binding capacity ($IC_{50} \leqslant 15$) (Table 1).

*Reevaluation of binding and stability of fusion peptides in HLA-A*0201*

Binding of fusion peptides in HLA-A2, with a frequency of 44% in the Caucasian population, is subject of conflicting data (see Table 2). Most studies[14,15,17–19] did not find significant binding of BCR-ABL fusion peptides in HLA-A2. In contrast, four

Table 2 Binding and stability of BCR-ABL fusion peptides in HLA-A2 (A*0201)

BCR-ABL fusion-peptide sequences[a] *and positive control peptide*		*Competition assay*			*Stabilization assay*		*Published binding*[f]	
		Pred.[b]		*IC₅₀*[c]	*Assembly*[d]	*Dissociation*[e]	*Efficient Refs.*	*No bind. Refs.*
	Length	*MTF*	*SYFP.*					
F L P S D F F P S V (HBV 18–27)	10	67	24	<1	1.53	74%		
B3A2								
A T G F K Q S S **K** A L	11	27	—	>100	—	—	—	14;16
T G F K Q S S **K** A L	10	27	11	>100	—	—	—	16
G F K Q S S **K** A L	9	27	10	nt	—	—	—	18;19;15;16
K Q S S **K** A L Q R P V	11	32	—	17.5	0.37	3%	20	—
Q S S **K** A L Q R P V	10	27	10	>100	—	—	20	—
S S **K** A L Q R P V	9	31	12	>100	0.01	0%	20	18;19;15
S **K** A L Q R P V	8	30	0	nt	—	—	20	—
B2A2								
P L T I N K E **E** A	9	49	12	>100	—	—	—	18;19;14;15
I P L T I N K E **E** A L	11	29	—	>100	—	—	—	—
P L T I N K E **E** A L	10	62	17	>100	—	—	—	—
L T I N K E **E** A L	9	47	20	29	0.45	3.1%	16	18;19;14;15
T I N K E **E** A L	8	52	0	>100	—	—	—	14
P L T I N K E **E** A L Q	11	36	—	>100	—	—	—	—
K E **E** A L Q R P V	9	35	11	nt	—	—	—	18;19;15
E1A2								
E G A F H G D A **E** A L	11	30	—	>100	—	—	—	—
G A F H G D A **E** A L	10	30	17	79	0.22	2.9%	—	—
H G D A **E** A L Q R P V	11	34	—	>100	—	—	—	—
G D A **E** A L Q R P V	10	30	15	>100	—	—	—	—
D A **E** A L Q R P V	9	26	13	>100	—	—	—	—
A **E** A L Q R P V	8	30	0	>100	—	—	—	—

HBV, hepatitis B virus; HLA, human lymphocyte antigen; MTF, MOTIF algorithm; Pred., predicts; Refs., references.
[a]All fusion peptides containing at least one canonical HLA-A*0201 anchor are listed. The BCR-ABL fusion aa is printed bold; primary anchors for HLA-A*0201 are underlined.
[b]Peptide binding prediction by MTF- and SYFPEITHI algorithms (see Materials and methods). A higher score predicts better binding.
[c]IC_{50} in μM; nt, not tested. Fl-labeled HPV E7 11–20 was used as reference peptide.
[d]Assembly expressed as fluorescence index (see Materials and methods) after 1.5 h loading with 200 μM peptide. Dash, not tested.
[e]Dissociation: % complexes left after 2 h incubation in peptide-free condition. Dash, not tested.
[f]Overview of published HLA-A2 binding capacities of the listed peptides. Dash, no publications.

HLA-A2 binding b3a2 peptides have been identified by Yotnda *et al.*[20] and b2a2^{LTINKEEAL} has been found by Berke *et al.*[16] to bind to HLA-A2. Therefore, we re-evaluated the binding to HLA-A2 (A*0201). All fusion peptides harboring at least one canonical HLA-A2 binding anchor (Leu or Met at P2; Val or Leu at the C-terminus)[41] were tested. As data obtained with our competition assay are relative to the binding capacity of the reference peptide, we performed the assay with two reference peptides.

Using the high-affinity binding Fl-labeled HBV cAg 18–27 peptide we did not observe binding of the selected fusion peptides (data not shown). In contrast, using Fl-labeled HPV E7 11–20 as reference peptide, which has lower affinity for HLA-A2 than HBV cAg 18–27,[44] two BCR-ABL peptides displayed significant binding for HLA-A2 (Table 2). The 11-mer b3a2^{KQSSKALQRPV} bound with IC_{50} of 17.5 μM, confirming the binding reported by Yotnda *et al.*[20] In contrast, nonamer b3a2^{SSKALQRPV} that has been observed in the latter study to bind in HLA-A2 as well and was suggested to be naturally presented,[20] showed hardly any binding (Table 2). It only inhibited the binding of the Fl-labeled reference peptide for 6% at the highest concentration of 100 uM tested (data not shown), which is in line with its absence of HLA-A2 binding in other studies.[15,18,19] Nonamer b2a2^{LTINKEEAL} was the second peptide that bound with moderate capacity (Table 2, IC_{50} of 29 μM). This confirmed its binding in HLA-A2 as reported by Berke *et al.*,[16] but is in contrast to earlier studies reporting lack of binding.[14,15,18,19] The only peptide containing both the genuine P2 and C-terminal anchors for HLA-A2, namely decamer b2a2^{PLTINKEEAL}, did not bind (IC_{50} > 100 μM) (Table 2), which has also been reported in other studies.[14,16] This can be attributed to a deleterious effect of Pro at position 1 for binding capacity.[40] Of the e1a2 fusion peptides, only decamer GAFHGDAEAL bound with low affinity (IC_{50} > 79 μM) (Table 2).

To more precisely define binding characteristics of the four peptides of primary interest, HLA-A2 – peptide complex stability was assessed by first measuring assembly after 1.5 h peptide loading and subsequently determining the dissociation rate of HLA-A2 – peptide complexes (off-rate of the peptide) after 2 h incubation at 37°C in peptide-free condition[47] (Table 2). In this assay as well, peptide b3a2^{SSKALQRPV} did not show appreciable assembly of HLA-A2 molecules. The three peptides that exhibited binding capacity in our competition assay also induced HLA-A2 assembly. However, this binding was very unstable because these peptides showed high dissociation rates from HLA-A2: <5% of HLA-A2-peptide complexes were detectable after 2 h of incubation. We conclude that stable binding of BCR-ABL junctional peptides in HLA-A2 did not occur.

In vitro *proteasome-mediated digestions of 27-mer BCR-ABL fusion regions*

In vitro proteasome digestions are known to reliably yield MHC class I ligands from polypeptides[54–57] and thus cleavage pattern analysis has been applied for the successful identification of CTL

Table 3 Proteasome digestions of BCR-ABL fusion regions in relation to HLA class I binding fusion peptides.

A **Digestion b3a2 fusion region**[a]

HLA class I binding b3a2 peptides[b]

Peptide	C-term[c]	HLA[d]	Ref.[e]
H S A T G F K Q S S **K**	**Minor**	A3, A11, A68	14; Curr.
S A T G F K Q S S **K**	**Minor**	A68	Curr.
A T G F K Q S S **K**	**Minor**	A3, A11, A68	14; Curr.
A T G F K Q S S **K** A L	**No**	B7	16
G F K Q S S **K** A L	**No**	B8	14
F K Q S S **K** A L Q	**No**	B8	17
F K Q S S **K** A L Q R	**Major**	B27	16
K Q S S **K** A L Q R	**Major**	A3, A11	14
K Q S S **K** A L Q R P V	**No**	A2	Curr.
S S **K** A L Q R P V	**No**	A2	20

Digest. sites[f]

27-mer V I V H S A T G F K Q S S **K** A L Q R P V A S D F E P Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fragments[g]

Fragment[g] Start	End	Intensity, 1 h (%)[k] B-LCL	Hela	CML
1	18	9.9	9.7	9.5
1	21	15.9	32.2	41.6
5	21	11.5	0,0	0,0
5	27	14.9	4.1	15.9
6	27	3.7	3.5	0,0
7	27	5.6	20.7	6.2
10	27	5.8	0,0	0,0
15	27	1.4	1.6	4.8
19	27	14.4	10.4	7.9

B **Digestion b2a2 fusion region**

HLA class I binding b2a2 peptides

Peptide	C-term	HLA	Ref.
I P L T I N K E **E** A L	**Minor**	B7, B35	16
P L T I N K E **E** A L	**Minor**	B7	16
L T I N K E **E** A L	**Minor**	B8, B7, A2	17; 16; Curr.
L T I N K E **E** A L Q R	**Major**	A68	Curr.
T I N K E **E** A L Q R	**Major**	A68	Curr.
I N K E **E** A L Q R P V	**No**	B61	Curr.
K E **E** A L Q R P V	**No**	B44, B61	17; Curr.
K E **E** A L Q R P V A	**Major**	B61	Curr.
E **E** A L Q R P V A	**Major**	B61	Curr.
E A L Q R P V A	**Major**	B61	Curr.

Digest. sites

27-mer Q T V H S I P L T I N K E **E** A L Q R P V A S D F E P Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fragments

Fragment Start	End	Intensity, 1 h (%) B-LCL	Hela	CML
9	10	4,0	4.1	3.5
1	13	4.5	10.9	4.8
1	16	5.3	4.1	4.9
1	18	12.5	12.8	12.3
1	21	32.1	30.9	32.1
5	21	6.2	3.6	5.1
7	21	5,0	7.3	11.5
8	25	6.1	5.7	5.9
11	27	5.1	2.6	4.2
19	27	7.7	5.6	4.8

C **Digestion e1a2 fusion region**

HLA class I binding e1a2 peptides

Peptide	C-term	HLA	Ref.
A F H G D A **E** A L	**Minor**	C4	Curr.
H G D A **E** A L Q R P V	**No**	B61	Curr.
G D A **E** A L Q R P V	**No**	B61	Curr.
D A **E** A L Q R P V	**No**	B61, B51	Curr.
A **E** A L Q R P V	**No**	B61	Curr.
G D A **E** A L Q R P V A	**Major**	B61	Curr.
D A **E** A L Q R P V A	**Major**	B61	Curr.
A **E** A L Q R P V A	**Major**	B61	Curr.
E A L Q R P V A	**Major**	B61	Curr.

Digest. sites

27-mer W P N D G E G A F H G D A **E** A L Q R P V A S D F E P Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fragments

Fragment Start	End	Intensity, 1 h (%) B-LCL	Hela	CML
1	11	4.2	5.5	6.6
1	12	8.3	17.4	8.6
1	16	2.9	3.9	3.5
11	17	9.5	7.4	12.0
1	18	6.7	8.3	8.2
1	21	42.1	21.8	27.4
5	21	3.5	7.2	2.8
13	27	9.3	9.5	11.7
17	27	2.8	4.8	3.4
19	27	5.5	10.1	7.1

D **Digestion ABL, a1a2 region**

Digest. sites

27-mer K K G L S S S S S C Y L E **E** A L Q R P V A S D F E P Q

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Fragments

Fragment Start	End	Intensity, 1 h (%) B-LCL	Hela	CML
1	12	26.0	10.9	25.1
1	21	8.2	26.2	14.5
11	21	2.5	2.3	0.0
13	21	4.2	2.6	0.0
13	27	18.4	5.3	18.9
14	27	6.0	7.4	16.6
17	27	4.5	6.9	0.0
15	27	7.8	15.3	24.9
19	27	0.0	9.9	0.0

a For digestions, 27-mer peptides were used with the breakpoint aa. at position 14 (printed bold)
b All efficiently class I binding fusion peptides (from literature and this study) are listed (breakpoint aa. bold)
c Liberation of C-terminal aa. of HLA class I binding peptide by minor or major cleavage site. No; no cleavage after C-term
d HLA class I molecule to which the indicated fusion peptide can bind efficiently.
e Literature reference (first publication) of indicated peptide with respect to HLA class I binding. Curr.; current study.
f Major site: bold arrow: fragments containing as C-terminus the residue N-terminal from the cleavage, or complements, are present for ≥ 5% at 1 h incubation for all proteasome types. Minor site: thin arrow: fragments containing as C-terminus the residue N-terminal from the cleavage, or complements, are present for < 5% at 1 h incubation.
g Digestion fragments are ordered according to their C-terminus. Some low abundant irrelevant fragments have been ommited
k Intensity is expressed as % of total summed mass-peak intensities of digested 27-mer at 1 h digestion.

Table 4 Predicted cell surface expressed HLA class I – BCR-ABL fusion peptide complexes

	BCR-ABL fusion peptide[a]																		*C-terminal processing*[b]	*HLA class I binding*			*Candidate CTL epitope*[f]
																				HLA[c]	*IC_{50}*[d]	*Ref.*[e]	
b3a2	H	S	A	T	G	F	K	Q	S	S	**K**								Minor	A3/A11	int./high	14	+
	H	S	A	T	G	F	K	Q	S	S	**K**								Minor	A68	2	Curr.	+
		S	A	T	G	F	K	Q	S	S	**K**								Minor	A68	2.5	Curr.	+
			A	T	G	F	K	Q	S	S	**K**								Minor	A3/A11	int./high	14	+
			A	T	G	F	K	Q	S	S	**K**								Minor	A68	7	Curr.	+
						F	K	Q	S	S	**K**	A	L	Q	R				Major	B27	high	16	++
							K	Q	S	S	**K**	A	L	Q	R				Major	A3/A11	high	14	++
b2a2			I	P	L	T	I	N	K	E	**E**	A	L						Minor	B7	high	16	+
			I	P	L	T	I	N	K	E	**E**	A	L						Minor	B35	high	16	+
				P	L	T	I	N	K	E	**E**	A	L						Minor	B7	int.	16	+
					L	T	I	N	K	E	**E**	A	L						Minor	B8	Int.	17	+
					L	T	I	N	K	E	**E**	A	L						Minor	B7	high	16	+
					L	T	I	N	K	E	**E**	A	L						Minor	A2	29	Curr.	+
					L	T	I	N	K	E	**E**	A	L	Q	R				Major	A68	3.5	Curr.	++
						T	I	N	K	E	**E**	A	L	Q	R				Major	A68	6.5	Curr.	++
									K	E	**E**	A	L	Q	R	P	V	A	Major	B61	< 1	Curr.	++
										E	**E**	A	L	Q	R	P	V	A[g]	Major	B61	< 1	Curr.	++
e1a2					A	F	H	G	D	A	**E**	A	L						Minor	C4	15	Curr.	+
								G	D	A	**E**	A	L	Q	R	P	V	A	Major	B61	17	Curr.	+
									D	A	**E**	A	L	Q	R	P	V	A	Major	B61	23,5	Curr.	+
										A	**E**	A	L	Q	R	P	V	A	Major	B61	< 1	Curr.	++
											E	A	L	Q	R	P	V	A[g]	Major	B61	11,5	Curr.	++

Curr.; current study; CTL, cytotoxic T lymphocyte; HLA, human lymphocyte antigen; ref., reference.
[a]Fusion peptides with signficicant HLA class I binding capacity that are excised after their C-term are listed (breakpoint aa. bold and underlined).
[b]Efficiency of C-terminal liberation: see by major site: fragments containing this C-terminus together with their complements are present for $\geqslant$5%. By minor site: fragments containing this C-terminus together with their complements are present for $<$5% (at 1 h digestion).
[c]HLA class I molecule for which the indicated fusion peptide has efficient binding capacity.
[d]Binding capacity in IC_{50} (μM) from current study or according to literature (classified in: intermediate (int.) or high-affinity binding).
[e]Literature reference (first publication) of indicated peptide with respect to HLA class I binding.
[f]Epitope prediction. Classification: (++) high-density epitope: major digestion site and high-affinity binding; (+) low abundant epitope: minor site and/or intermediate-affinity binding.
[g]BCR-ABL fusion-peptides that are identical to ABL a1a2-derived peptides (the EALQRPVA peptide is both in e1a2 and b2a2).

epitopes among HLA class I binding peptides.[28,36–38,58] We digested polypeptides encompassing the b3a2, b2a2 and e1a2 fusion regions *in vitro* with purified proteasome complexes to assess the C-terminal liberation of HLA class I binding fusion peptides, which is considered to be mediated solely by the proteasome. The digestion pattern after 1 h incubation was analyzed because this best reveals physiological cleavages.[28]

The proteasome complex can exist mainly in two forms: constitutive proteasomes expressing β1 (delta), β2 (Z) and β5 (MB1) catalytic subunits and immunoproteasomes in which these units are replaced for β1i (LMP2), β2i (MECL-1) and β5i (LMP7), respectively.[50] Both types of proteasomes are expressed dependent on differentiation by interferons and source tissue[59] and have overlapping but qualitatively and/or quantitatively different enzymatic activity.[60,61] Thus for immunotherapy it is essential to know which proteasome type is expressed in the leukemic cells.[62] Western blotting with subunit-specific antibodies revealed that purified 20S proteasomes isolated from blast crisis CML cells consisted exclusively of the immunotype (Figure 1).

The leukemic proteasomes, proteasomes derived from HeLa cells, known to express mainly constitutive proteasomes, and proteasomes from B-LCL, containing preferentially immunoproteasomes[49] were incubated with fusion region spanning 27-mers and digestion mixtures were analyzed by mass spectrometry.

Table 3A shows the digestions of the 27-mer encompassing the b3a2 fusion region, containing 10 HLA class I binding fusion peptides (reported in literature or identified in this study as indicated). Abundant cleavage sites were present after Arg-18 and especially after Ala-21. This indicates that HLA-B27 binding b3a2$^{9-18}$ and HLA-A3 binding b3a2$^{10-18}$ (KQSSKALQR), which share Arg-18 as C-terminus, are likely efficiently produced by the proteasome *in vivo*. Cleavages after Leu-16, Gln-17 and Val-20 were not observed, indicating that peptides with these C-termini, which include HLA-B8 binding b3a2$^{8-16}$ (ref. 19) and HLA-A2 binding b3a2$^{10-20}$ (this study) and b3a2$^{12-20}$ (SSKALQRPV),[20] are very unlikely to be naturally presented.

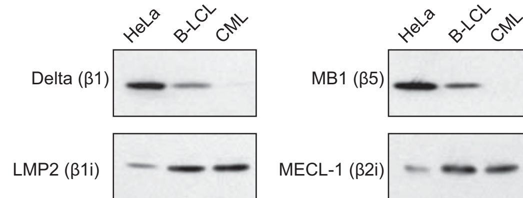

Figure 1 Western blot analysis of proteasome composition. Expression of constitutive proteasome subunits (Delta and MB1 proteins) versus immunoproteasome subunits (LMP2 and MECL-1 proteins) was analyzed by Western blotting. Proteasomes were isolated from HeLa, B-LCL and CML cells from two patients in blast phase, here we show a representative example.

The HLA class I binding peptides with Lys-14 as C-terminus, including two HLA-A3/A11 binding peptides ((HS)ATGFKQ-SSK),[14] are not expected to be cell surface presented at a density needed for immunogenicity because only a low abundant complementary digestion fragment (aa 15–27) was found.

Table 3B depicts digestions of the b2a2 fusion region containing nine HLA class I binding peptides. Abundant cleavage sites were again produced after Arg-18 and Ala-21, indicating that both HLA-A68 peptides ($b2a2^{8/9-18}$) and three HLA-B61 binding peptides ($b2a2^{12/13/14-21}$), which contain these residues as C-terminal anchors respectively, are expected to be efficiently liberated C-terminally and expressed on living cells. A much less abundant cleavage site was observed after Leu-16, rendering it possible that the HLA class I binding peptides ending at Leu-16 will be produced although in low abundance. Cleavage after Val-20 did not take place, which precludes intracellular generation of 11-mer $b2a2^{10-20}$ (HLA-B61) and nonamer $b2a2^{12-20}$ (HLA-B44, -B61).

Table 3C shows the digestions of the e1a2 fusion region containing nine HLA class I binding fusion peptides. The C-terminal Ala-21 of four HLA-B61 binding N-terminal length variants was efficiently liberated. Therefore, these peptides are expected to be efficiently liberated and cell surface expressed. Because an abundant cleavage site was also observed after Asp-12, which will generate the N-terminus, this points to especially $e1a2^{AEALQRPVA}$ as a putative abundantly expressed CTL epitope. In contrast, the four HLA-B61 binding peptides and one HLA-B51 binding peptide that share Val-20 as C-terminus are not predicted to be endogenously produced, because of the absence of cleavage after Val-20. HLA-Cw4 binding peptide $e1a2^{8-16}$ may be naturally presented, however, probably in low abundance, because only a minor cleavage was observed after Leu-16.

We observed abundant cleavages after Arg-18 and Ala-21 in both the b3a2, b2a2 and e1a2 polypeptides (Table 3A–C). This similar cleavage pattern in the ABL-derived a2 region, irrespective of the joining BCR region, fits with the current knowledge that proteasome specificity is mainly dependent on residues directly adjacent to the cleavage site. However, residues further up- or downstream may influence the specificity as well.[56] Because two HLA-B61 binding fusion peptides ($b2a2^{13-21}$ and $b2a2/e1a2^{14-21}$) equaled non-leukemic ABL peptides and four others differed only at their N-terminus (Tables 1 and 3), we studied the digestion of the non-leukemic ABL a1a2 polypeptide. As shown in Table 3D, analysis of cleavages in the a2 region showed a different pattern when contained in the a1a2 polypeptide that was proteasome type dependent. Digested with CML or B-LCL-derived immunoproteasomes, the cleavage after Arg-18 was absent and the cleavage efficiency after Ala-21 was strongly reduced (e.g., only 14.5% of the digested a1a2 polypeptide in the CML digestion) when compared to this cleavage in the BCR-ABL fusion regions (41.6, 48.7 and 30.3% of the digested b3a2, b2a2 and e1a2 polypeptides, respectively, in the CML digestion). The reduced cleavage efficiencies in a1a2 after Arg-18 and Ala-21 by immunoproteasome preparations were accompanied by an abundant cleavage after Leu-12 upon digestion by this proteasome type: fragment 1–12 and its complements added up to 48.6 and 44% for B-LCL and CML proteasome digestions respectively. In contrast, in the a1a2 HeLa digestion those fragments added up to only 18.8%. Enhanced cleavage efficiency by immunoproteasomes after leucine residues is reported in the literature.[60] In the digestion with (constitutive) HeLa proteasomes, the less abundant cleavage after Leu-12 correlated with a quantitative recurrence of the cleavages after Arg-18 and Ala-21 (Table 3D). These results indicate that $b2a2^{13-21}$ and $b2a2/e1a2^{14-21}$ will be much less abundantly generated from the normal ABL a1a2 region in professional antigen presenting cells (containing immunoproteasomes).

CTL response against HLA-B61 binding e1a2 peptide AEALQRPVA

The combined strength of our reverse immunology approach enabled the identification of six novel predicted CTL epitopes expected to be abundantly expressed at the cells surface. These include two HLA-A68 binding fusion peptides and two HLA-B61 binding peptides from breakpoint region b2a2, and from e1a2 two HLA-B61 peptides. All of these peptides have both high binding capacity and are efficiently liberated after their C-terminus by the proteasome.

To demonstrate the accuracy of our approach and to prove natural presentation of these novel predicted CTL epitopes, $CD8^+$ T cells from healthy donors were induced against synthetic peptides. Specific responses against HLA-B61 binding peptides $b2a2^{12-21}$ and $e1a2^{13-21}$ and HLA-A68 binding peptide $b2a2^{8-18}$ were generated, indicating their immunogenicity (data not shown). The T-cell culture against $e1a2^{13-21}$ (AEALQRPVA) was highly peptide-specific and was cloned by limiting dilution. Several CTL clones efficiently lyzed peptide-pulsed $HLA\text{-}B61^+$ B-LCL Sweig007 (Figure 2a). To test endogenous production and natural presentation of $e1a2^{AEALQRPVA}$ in HLA-B61, cell line TOM1, derived from ALL and expressing e1a2, was transfected with HLA-B61 (TOM1-B61). The peptide specific CTL clones recognized TOM1-B61 efficiently, whereas recognition of TOM1 expressing the empty vector was only at background levels (Figure 2b), indicating that $e1a2^{AEALQRPVA}$ is endogenously produced and expressed on the surface of leukemic cells and underscoring the relevance of our epitope prediction procedure.

Because $e1a2^{AEALQRPVA}$ differs only at its N-terminus from normal ABL a1a2 sequence EEALQRPVA, which may be expressed on normal cells, the recognition of the latter peptide, loaded at the same concentration, was tested. All CTL clones displayed only very low cross-reactivity with this peptide (Figure 2a), implying the e1a2 specificity of the CTL response. Absent recognition of B-LCL Sweig007 ($HLA\text{-}B61^+$ and expressing normal ABL), either non-pulsed or pulsed with an irrelevant peptide (Figure 2a and b), also confirmed the lack of an ABL-a1a2-directed CTL response. Thus the applicability of $e1a2^{AEALQRPVA}$ as immunotherapeutic target in $HLA\text{-}B61^+$ ALL patients is warranted.

Discussion

This comprehensive analysis of the BCR-ABL fusion regions as a source of CTL epitopes yielded 17 b3a2, b2a2 and e1a2 fusion peptides that were shown for the first time to bind efficiently in HLA-A68, HLA-B51, HLA-B61 or HLA-Cw4 (Table 1, IC_{50} $<15\ \mu M$), thereby more than doubling the 12 fusion peptides shown previously to bind in other HLA class I molecules.[14–17,20] Motif-based peptide-binding predictions, which are needed to limit the amount of peptides to be tested, are adequately préselecting peptides with binding capacity.[3,43] Despite that, binding affinities have to be determined experimentally because the ranking of prediction scores mostly differs substantially from the ranking of measured values as we have shown previously.[43] Predicted absence of binding is only occasionaly contradicted by actual positive binding measurements.[43] Despite that, by

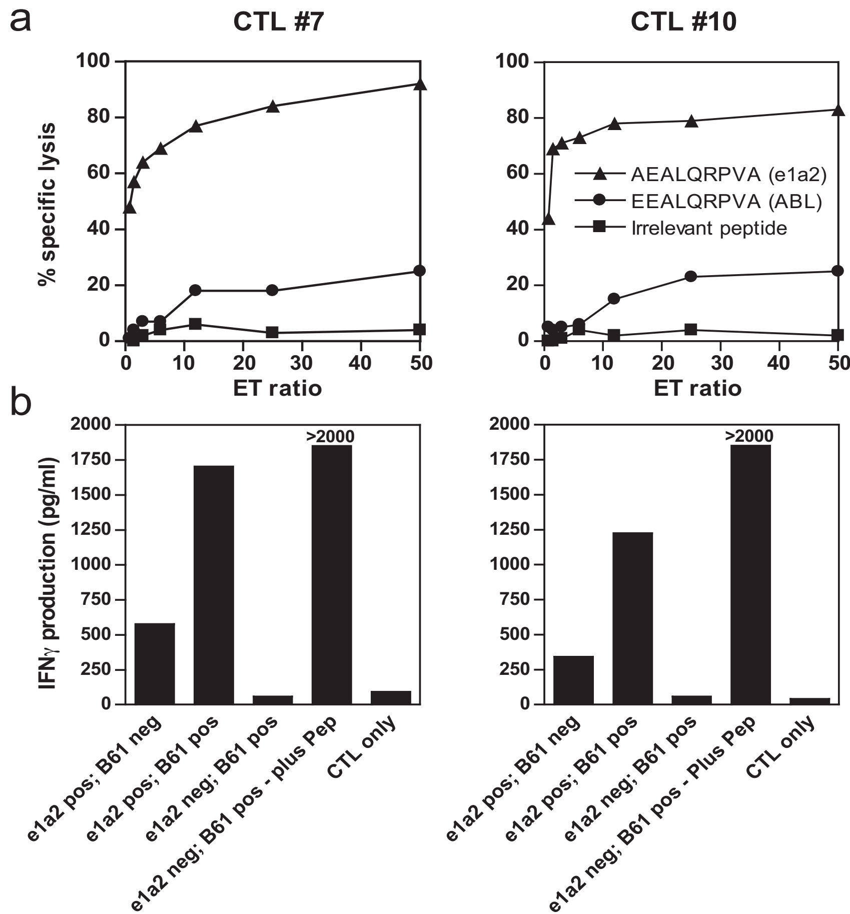

Figure 2 HLA-B61 restricted recognition of the endogenously processed e1a2 epitope AEALQRPVA on ALL cell line TOM1 by two CTL clones. Panel (**a**): lysis by CTL clones #7 and #10 anti-e1a2[AEALQRPVA] of ^{51}Cr-labeled B-LCL Sweig007 (homozygously expressing HLA-B61) loaded with 5 μM of either the relevant peptide (▲), an irrelevant HLA-AB61 binding peptide (■) or ABL a1a2 peptide EEALQRPVA (●) at ET ratios ranging from 50 to 0.75. Panel (**b**): recognition by CTL clones #7 and #10 anti-e1a2[AEALQRPVA] of ALL-derived TOM1 cell line expressing HLA-B61 (e1a2 pos; HLA-B61 pos), TOM1 transfected with the empty vector (e1a2 pos; HLA-B61 neg), and B-LCL Sweig007 (e1a2 neg; HLA-B61 pos) without peptide or Sweig007 loaded with EEALQRPVA. Readout is interferon-γ production by CTL as measured with ELISA. Results of one representative experiment out of three performed are shown.

including negatively scoring length- and shift variants of predicted binders that harbor only one anchor residue in our assays, we reduced the risk of missing fusion peptides with binding capacity. Therefore, and because we screened the fusion regions for binding motifs of all HLA class I molecules, it is unlikely that a significant number of class I binding BCR-ABL fusion peptides remains to be identified.

Apart from HLA class I binding and TAP translocation, of which the latter is less restrictive,[63] the third and foremost important event defining a CTL epitope is its intracellular enzymatic generation. As discussed in recent reviews, N-terminal extensions of epitope precursors can be trimmed by a diversity of aminopeptidases, but, owing to the absence of carboxypeptidases in the cytosol and endoplasmic reticulum, the precise C-terminal excision by the proteasome is an established sine qua non for the far majority of CTL epitopes.[33,34,64] To date, only one defined exception to this rule has been reported.[65] Our proteasome digestion analysis enabled an appraisal of the endogenous generation of all already published and newly identified HLA class I binding BCR-ABL fusion peptides and showed that the 28 different peptides fall into three major groups (Tables 3 and 4).

The first group consisted of 11 peptides that lacked enzymatic C-terminal excision by the proteasome, which precludes their natural presentation.[28,33] Among these peptides are b3a2[SSKALQRPV] (in HLA-A2)[20] and b3a2[GFKQSSKAL] (in HLA-B8)[22] that were already included in the BCR-ABL peptide vaccines.[25–27] The absence of proteasomal excision of these peptides, however, is hard to reconcile with their natural presentation and, therefore, we score them as inappropriate for immunotherapeutic targeting.

The second group included seven fusion peptides that are excised after their C-terminus by a minor cleavage site. Therefore, although likely produced intracellularly, their cell

surface density is expected to be low (Table 4). In this category especially the b3a2 peptides HSATGFKQSSK and ATGFKQSSK, binding in HLA-A3/A11[14] and now also demonstrated to bind to HLA-A68, are important because they were included in published vaccines.[25–27] Efficient induction and full activation of CTL responses in patients vaccinated with these peptides may enable subsequent lysis of leukemic HLA-A3/A11/A68$^+$ target cells expressing those peptides at low densities, because for the elicitation of CTL effector functions a much lower epitope density (100- to 1000-fold lower) than for full T-cell activation and proliferation is required.[66–68] A distinct disadvantage, however, of immunotherapeutic targeting of low-density epitopes is that either lower cytotoxic levels are reached[69] or that for equally efficient killing a higher T-cell receptor affinity is required than for targeting abundantly expressed epitopes,[70] which limits the availability of T cells with a sufficient affinity.

The third group consisted of the remaining 10 peptides (34%) that were efficiently liberated from their C-terminal flanking region, because major cleavage sites were observed after their C-terminal residues Arg-18 or Ala-21 (Table 3). Consequently, these 10 peptides are expected to be abundantly produced in living cells. In this group, especially the eight peptides with high or intermediate HLA class I binding affinity, namely $b3a2^{10\text{-}20}$ (KQSSKALQR), binding in HLA-A3[14,24] and $b3a2^{9\text{-}18}$ binding in HLA-B27[16] and six of the newly identified HLA-A68 and HLA-B61 binding peptides (from b2a2 and e1a2), are predicted to be high-density cell surface expressed CTL epitopes (Table 4). For that reason, these eight peptides are to date the most favored vaccine candidates.

Unequivocal evidence for the natural cell surface presentation of BCR-ABL fusion peptides until now has only been reported for $b3a2^{KQSSKALQR}$, because it has been identified by Clark *et al.*[24] in peptides eluted from HLA-A3 expressed on CML cells. This is in line with the abundant proteasome-mediated cleavage after its C-terminus (Arg-18; Table 3) and warrants its application in vaccine trials.[25–27] We now demonstrate by CTL recognition of leukemic cells that $e1a2^{AEALQRPVA}$ is a second proven BCR-ABL fusion region derived CTL epitope presented in HLA-B61 (Figure 2).

Future biochemical and T-cell studies have to confirm cell surface expression and immunogenicity of the other predicted CTL epitopes (Table 4). This will help to further establish the suitability of these epitopes for peptide vaccines. Importantly, in our previous work, using the same strategy, four out of four predicted CTL epitopes that were C-terminally excised by a major proteasomal cleavage were found to be both immunogenic and cell surface expressed.[28] The reliability of the current extended reverse immunology strategy has thereafter been further validated by other groups;[36–38] for example, six out of six predicted CTL epitopes from human proinsulin were proved to be immunogenic and cell surface expressed,[37] implying a high degree of accuracy in epitope prediction.

We ré-evaluated binding of BCR-ABL fusion peptides in HLA-A*0201 (Table 2). The near absence of binding capacity of $b3a2^{SSKALQRPV}$ in HLA-A2 in our hands was in concordance with earlier studies[15,18,19] but in contradiction with Yotnda *et al.*[20] The latter study used the same competitor peptide as we did (HBV cAg 18–27), but tested $b3a2^{SSKALQRPV}$ at 20 mM, whereas our highest concentration was 100 μM, which may explain the discrepancy. We only found intermediate, but unstable, binding capacity of $b3a2^{10\text{-}20}$ and $b2a2^{8\text{-}16}$ for HLA-A2 and low affinity, unstable binding of $e1a2^{GAFHGDAEAL}$. Recently, Pinilla-Ibarz *et al.*[71] designed analogue HLA-A2 binding BCR-ABL fusion peptides with improved binding capacity that were found to efficiently induce CTL cross-reacting with the native peptide. According to our proteasome digestions, showing a cleavage site after Leu-16, the b2a2 analog YLINKEEAL may induce T cells that recognize the naturally expressed native $b2a2^{8\text{-}16}$ sequence (LTINKEEAL, IC_{50} 29 μM; Table 2) which is therefore a candidate vaccine peptide. The unstable binding of LTINKEEAL (Table 2) in conjunction with its C-terminal excision by only a minor proteasomal cleavage site (Table 3), will certainly negatively influence its cell surface density, but it still may be a cell surface expressed epitope that can be used for immunotherapeutic targeting. Unstable binding in principle may not preclude elicitation of T-cell cytotoxic effector functions, because killing can already be triggered by short time engagement with the target cell.[66,68] In contrast, full T-cell activation and proliferation is only induced after sustained interactions with epitopes at higher density,[68] and, therefore, unstable low-affinity binding peptides are not suitable as vaccine peptides; analogues with improved binding capacity indeed might serve as the solution for this problem.[71]

The cleavage site in b3a2, b2a2 and e1a2 that predominates occurs after Ala-21. Therefore, the five fusion peptides from b2a2 and e1a2 that bind avidly in HLA-B61 and share Ala-21 as their C-terminus are expected to be efficiently expressed on leukemic cells (Table 4). However, in this group, $b2a2^{EEALQRPVA\text{-}21}$ and $b2a2/e1a2^{EALQRPVA\text{-}21}$ equal ABL a1a2 sequences, and the other peptides ($b2a2^{12\text{-}21}$, $e1a2^{11\text{-}21}$ and $e1a2^{13\text{-}21}$) differ only N-terminally from normal ABL peptides that have equally good binding capacities (Table 1). Thus, immunotargeting of these peptides requires either absence of cross-reactivity of reactive T cells towards the corresponding ABL peptides or, alternatively, differential antigen processing leading to preferential presentation of these peptides when excised from b2a2 and e1a2. The CTL induced against $e1a2^{13\text{-}21}$ (AEALQRPVA) showed near absence of cross-reaction with ABL a1a2 sequence EEALQRPVA (Figure 2a). Importantly, our study also revealed a differential processing effect that was dependent on the proteasome subtype (Table 3). In the a1a2 (ABL) region, the cleavage after Arg-18 was absent and the cleavage after Ala-21 was strongly reduced, when digested with leukemic immunoproteasomes. This will lead to a leukemia specific high-density presentation of the HLA-B61 binding peptides when excised from b2a2 and e1a2 and thus their applicability in immunotherapy. Moreover, as discussed by Sun *et al.*,[72] in leukemic cells the BCR-ABL protein is expressed in addition to the ABL and BCR products, which will also contribute to the preferential expression of these peptides on leukemia cells.

We are the first to define HLA class I binding of peptides in the e1a2 region. Results of DLT after SCT in ALL are in general disappointing.[1] However, the specific targeting of CD8$^+$ T-cell epitopes in the e1a2 fusion region may contribute to successful immunotherapy of ALL (e.g. by vaccination or adoptive T-cell transfer). The identification of $e1a2^{AEALQRPVA}$ as an HLA-B61 presented CTL epitope now enables such endeavors.

Negative associations of HLA-A3[73] and recently also HLA-A68 and HLA-B61 (HLA-B*40)[74] with BCR-ABL transcripts have been reported. This suggests natural immunity and CTL-mediated immunosurveillance against BCR-ABL peptides presented in these HLA class I molecules. The reported natural presentation of $b3a2^{KQSSKALQR}$ in HLA-A3[24] is in line with the protection effect of HLA-A3. Importantly, our current data identifying $e1a2^{AEALQRPVA}$ as an HLA-B61 presented CTL epitope (Figure 2) and several other b2a2 and e1a2 fusion peptides as predicted HLA-B61 and HLA-A68 presented epitopes with high cell surface density (Tables 1, 3 and 4) are

now also providing a basis for the protection against Ph$^+$ leukemia conferred by HLA-A68 and HLA-B61.[74]

In the last decade, numerous studies have extensively elaborated immunity against BCR-ABL fusion regions. This previous work in conjunction with the current study, applying a reliable and systematic extended reverse immunology strategy, renders it unlikely that a significant number of candidate CTL epitopes in the BCR-ABL fusion regions still remains to be identified, particularly when taking into account their short length with only two major proteasomal cleavage sites in the a2-exon encoded region.

We conclude that the patient population coverage of (predicted) CTL epitopes in BCR-ABL fusion regions is not complete owing to the only moderate cumulative prevalence of the presenting HLA class I molecules in combination with the different BCR-ABL variants (Table 4). The relevance of our study lies, therefore, not only in the identification of the best candidate vaccine peptides among the combined set of old and novel HLA class I binding BCR-ABL fusion peptides, but also in the conclusion that a defined and broadly applicable immunotherapeutic vaccine for Ph$^+$ leukemia will benefit from the identification of CTL epitopes in other leukemia specific antigens, next to those identified in BCR-ABL fusion regions.

Acknowledgements

We thank Dr M Groettrup for kindly providing antibodies of the immunoproteasome subunits, Dr KB Hendil for the antibodies to the constitutive proteasome subunits and Dr M Takiguchi for providing the C1R-B51 cell line. This work was supported by Grant UL-1994-870 from the Dutch Cancer Society (Amsterdam, The Netherlands). Furthermore, PvV and AdR are supported by the Centre for Medical Systems Biology (a center of excellence approved by the Netherlands Genomics Initiative).

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