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ORIGINAL ARTICLE

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A new LAGE-1 peptide recognized by cytolytic T lymphocytes on HLA-A68 tumors

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Abstract Antigens encoded by genes of the *LAGE* family, including LAGE-1 and NY-ESO-1, are of interest for cancer immunotherapy because they are tumor-specific and shared by tumors of different histological types. Several clinical trials are in progress with NY-ESO-1 peptides, protein, recombinant poxviruses, and dendritic cells pulsed with peptides. In this study, CD8 T lymphocytes from an individual without cancer were stimulated with dendritic cells infected with a recombinant avian poxvirus encoding a complete LAGE-1 protein. A CTL clone was isolated that recognized a new LAGE-1 peptide, ELVRRILSR, which corresponds to position 103–111 of the protein sequence. It is presented by HLA-A6801 molecules. When tumor cells expressing LAGE-1 were transfected with HLA-A68, they were lysed by the CTL clone, indicating that the peptide is processed in tumor cells. These results indicate that the LAGE-1.A68 peptide can be used for antitumoral vaccination. We observed also that specific T cells could be detected in a blood sample with a high sensitivity by using an A68/LAGE-1 fluorescent multimer.

Keywords HLA-A68 · LAGE-1 · Peptide · Poxvirus · SNP · Tumor

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Introduction

LAGE-1 and *NY-ESO-1/LAGE-2* belong to the "cancer-germline" gene families, like the MAGE, BAGE, and GAGE genes. These "cancer-germline" genes are expressed in many tumors and in male germline cells but are silent in all other normal tissues [5, 11, 12, 32]. Male germline cells do not express MHC class I and class II molecules and are therefore incapable of presenting antigens to T cells [17]. "Cancer-germline" genes therefore encode tumor-specific shared antigens, which have been used in therapeutic vaccination trials of cancer patients [7, 8, 10, 23].

Two *LAGE-1* transcripts have been described, namely, *LAGE-1a* and *LAGE-1b* (Fig. 1). *LAGE-1b* is incompletely spliced and codes for a putative protein of 210 aa, whereas the *LAGE-1a* gene product contains 180 aa [25]. An alternative reading frame is translated in both transcripts, producing a putative protein of 109 amino acids named CAMEL [1]. Expression of LAGE-1 was observed in many surgical tumor samples: 44% of bladder carcinomas, 33% of non-small-cell lung carcinomas, 29% of melanomas, 27% of head and neck cancers, and 25% of prostate adenocarcinomas [25]. LAGE-1 and NY-ESO-1/LAGE-2 are highly homologous proteins with up to 84% identity [9, 25].

Several peptides recognized by $CD8^+$ CTL have been identified in NY-ESO-1/LAGE-2 and CAMEL proteins (Table 1). The specific CTL have been derived in vitro by stimulating lymphocytes of cancer patients either with autologous tumor cells or with antigen-presenting cells, which were pulsed with peptide or infected with an adenovirus containing a NY-ESO-1/LAGE-2-coding sequence. A few of these antigenic peptides are also encoded by LAGE-1 [1, 27].

The identification of an additional set of antigenic peptides could be useful for the development of new vaccines to be made available for a large cohort of patients. It will also facilitate the design of vaccines comprising several antigens. To identify a new LAGE-1-

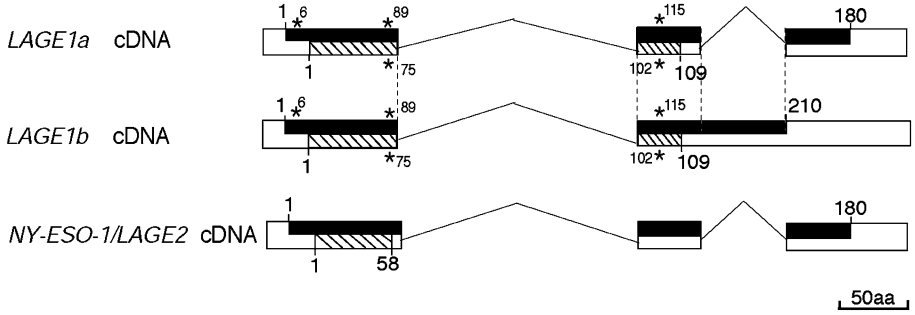

Fig. 1 Structure of the LAGE transcripts. Exons are represented as *white boxes* and spliced introns as *broken lines*. Open reading frames are represented as *dark boxes* for the major reading frame (ORF1), or as *striped boxes* for the second reading frame (ORF2). Amino acids are indicated with *numbers*. The protein encoded by ORF2 of LAGE-1 was named CAMEL [1]. A second reading frame was also reported for NY-ESO-1/LAGE-2 [27, 33]. The polymorphism of gene LAGE-1 was analyzed on RNA extracted from 17 tumor samples and 14 tumor cell lines obtained from both males ($n = 13$) and females ($n = 16$). The ORF contain three single nucleotide polymorphisms indicated by *stars*, namely Q6R (cag > cgg), Q89E (cag > gag) (F75L [ttc > ttg] in CAMEL), and P115P (cct > ccg) (L102R [ctc > cgc] in CAMEL). The three polymorphisms are linked with about 2/3 of the allele corresponding to the basic sequence (accession numbers AJ223023 and AJ223040). No polymorphism was observed in our series for NY-ESO-1/LAGE-2

derived peptide recognized by CTL on tumor cells, $CD8^+$ T lymphocytes from a blood donor were stimulated with autologous dendritic cells (DC) infected with an avian poxvirus containing a complete LAGE-1b-coding sequence. As this requires the processing of the antigen by the DC, we surmised that the peptide that would be identified would also be processed in tumors that express *LAGE-1*.

Material and methods

Cell lines, recombinant viruses, cDNA clones, and reagents

The Epstein–Barr virus-transformed B (EBV-B) cell lines and tumor cell lines were cultured in IMDM (Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (Invitrogen). WEHI-164 clone 13 was cultured in DMEM (Invitrogen) supplemented with 5% fetal calf serum. COS-7 cells were maintained in H16 supplemented with 10% fetal calf serum and Hepes (10 mM). All the media were supplemented with 0.24 mM of L-asparagine, 0.55 mM of L-arginine, 1.5 mM of L-glutamine (AAG), 100 U/ml of penicillin and 100 mg/ml of streptomycin. Geneticin was purchased from Gibco BRL (Gaithersburg, MD, USA). Clonal line LB831-BLC/A68/c14 was obtained by transfecting the LB831-BLC cells with vector pcDNA3 carrying an HLA-A*68012 sequence using calcium phosphate. The transfectants were selected with 1 mg/ml of geneticin, and clones were derived from the geneticin-resistant cells. Aventis Pasteur (Lyon, France) provided the recombinant canarypoxvirus ALVAC- *LAGE-1b*, the vaccinia-*LAGE-1b*, and the parental vaccinia viruses. Retroviral vector *LAGE-1b*-CSM codes for a full-length

Table 1 Antigenic peptides encoded by LAGE genes and recognized by $CD8^+$ T cells

Gene	HLA	HLA frequency (%)[a]		Peptide	Position	Lymphocyte stimulation method	References
		Caucasians	Orientals				
NY-ESO-1/LAGE-2	A2	44	47	SLLMWITQC	157–165	Autologous tumor cells	[6, 21, 27]
	A2	44	47	MLMAQEALAFL	ORF2, 1–11	Autologous tumor cells	[1]
	A31	5	9	ASGPGGGAPR	53–62	Autologous tumor cells	[33]
	A31	5	9	LAAQERRVPR	ORF2, 18–27	Autologous tumor cells	[33]
	B7	17	7	APRGVRMAV	ORF2, 46–54	Adenovirus-APC	[30]
	B35	20	10	MPFATPMEA	94–102	Autologous tumor cells	[4]
	B51	12	13	MPFATPMEA	94–102	Adenovirus-APC	[22]
	Cw3	24	37	LAMPFATPM	92–100	Adenovirus-PBMC[b]	[15]
	Cw6	16	13	ARGPESRLL	80–88	Adenovirus-PBMC	[15]
LAGE-1	A2	44	47	MLMAQEALAFL	ORF2, 1–11	Autologous tumor cells[c]	[1]
	A2	44	47	SLLMWITQC	157–165	Peptide[d]	[27]
	A31	5	9	LAAQERRVPR	ORF2, 18–27	Autologous tumor cells	[33]

[a] Based on Marsh et al. [26].
[b] Recombinant adenoviruses carrying the entire gene were used to infect the dendritic cells or PBMC that were subsequently used as stimulators.
[c] Antitumor CTL were obtained after repeated stimulation of blood lymphocytes with autologous tumor cells.
[d] Peptides chosen on the basis of consensus anchor residues were used to stimulate blood lymphocytes.

LAGE-1b protein and a truncated form of the human low affinity nerve growth factor receptor (LNGFr). EBV-B cells transduced with *LAGE-1b*-CSM (LB1965-EBV/LAGE-1b) were obtained as described [34]. cDNA clones carrying truncated *LAGE-1b* sequences were inserted into vector pcDNA1/Amp (Invitrogen), cDNA encoding HLA-A*68012 and A*01 into pcDNA3 (Invitrogen), cDNA encoding HLA-Cw*0602 into pET3D–tag (Invitrogen), and cDNA encoding HLA-B*40012, B*5701 and Cw*0304 into pcDNA3.1/V5-His-TOPO (Invitrogen). Dr. M. Takenoyama, Department of Surgery, School of Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan kindly provided the latter construct. Human recombinant IL-2 was purchased from Eurocetus (Amsterdam, The Netherlands), IL-7 from Genzyme (Cambridge, MA, USA), and GM-CSF from Schering-Plough (Brinny, Ireland). Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory.

Isolation of anti-LAGE-1 CTL

Peripheral blood mononuclear cells (PBMC) from HLA-A*6801 hemochromatosis patient LB1965 were isolated by Lymphoprep (Axis-shield PoCAS, Oslo, Norway) density gradient centrifugation. DC were obtained from adherent (60 min, 37°C) cells cultured for 6 days in RPMI 1640 supplemented with 10% FBS, 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine, antibiotics (complete RPMI medium), IL-4 (200 U/ml), and GM-CSF (70 ng/ml) [28, 29]. $CD8^+$ cells were purified from the non-adherent cells using magnetic microbeads coated with anti-CD8 antibodies (Miltenyi Biotech, Bergisch Gladbach, Germany), and the Auto-Macs separator (Miltenyi Biotech). DC were incubated for 2 h at 37°C with ALVAC-LAGE-1b at a multiplicity of infection (MOI) of 30 and were washed. $CD8^+$ T cells (1.5×10^5 cells/well) and infected DC (3×10^4 cells/well) were mixed in U-bottomed microwells in 200 µl of complete Iscove's medium with IL-6 (1,000 U/ml) and IL-12 (10 ng/ml). On days 8 and 14, cultures were restimulated with infected DC in a medium supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml). An aliquot of each microculture was tested for the presence of T cells with specific cytotoxicity against autologous EBV-B cells infected with vaccinia-*LAGE-1b*. Infection of autologous EBV-B cells was performed on 2×10^6 target cells for 2 h at an MOI of 20 in 200 µl of complete RPMI medium after virus sonication. Infected cells were washed, labeled with 100 μCi of $Na(^{51}Cr)O_4$ during 1 h and washed again. Responder T cells were added at an E:T ratio of 40:1 in V-bottomed wells. Unlabeled K562 cells were also added (5×10^4 cells/well) to block natural killer activity. Individual microcultures were tested on each target in duplicate. The chromium release was measured after a 4 h incubation at 37°C. Positive microcultures were cloned by limiting dilution and stimulated with irradiated autologous EBV-B cells transduced with a retrovirus encoding *LAGE-1b* (10,000 cells/well), irradiated allogeneic LG2-EBV cells (20,000 cells/well) as feeder cells, IL-2 (50 U/ml), and IL-7 (5 ng/ml). The anti-LAGE-1b.A68 CTL clone LB1965-CTL-s10.E8, referred to below as clone E8, was kept in the culture and restimulated every 10 days with peptide-pulsed EBV-B cells, feeder cells, and cytokines.

Identification of the HLA presenting molecules

COS-7 cells (1.5×10^4) were distributed in flat-bottomed microwells and co-transfected using 1.5 µl of Lipofectamine (Invitrogen) with a *LAGE-1b* cDNA and with each of the sequences coding for the HLA molecules corresponding to the haplotype of the blood donor. Transfected cells were incubated for 24 h at 37°C and 8% CO_2. A total of 2,000 CTL were added in the microwells containing the COS-7 transfectants in a total volume of 200 µl of complete IMDM supplemented with 25 U/ml of IL-2. After 20 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells in a MTT colorimetric assay [14, 18, 31].

Identification of the region containing the antigenic peptide

The *LAGE-1b* cDNA (clone 2) was previously retrieved from a cDNA library of LB373-MEL [25]. Different *LAGE-1b* fragments were produced by PCR that correspond to amino acids 1–210, 1–150, 1–118, and 1–108. The upper primer, 5′-CTCTCTgCCTCCgCATCC-3′, was located immediately downstream to a major transcription start of the *LAGE-1* mRNA; the lower primers were 5′-gATCCACATCAACAagggAA-3′, 5′-CCCAACCCACCACCCTCA-3′, 5′-CCCTggTCgggggAgA-3′,and 5′-gATCCTgCggACCAgCTC-3′. The fragments were cloned into pcDNA3.1/V5-His-TOPO (Invitrogen). The HLA-A*68012 cDNA (80 ng) was transfected into COS-7 cells (1.5×10^4) together with each of the *LAGE-1b* cDNA fragments (80 ng). CTL (2,000 cells) were added to the transfected cells. The IFN-γ production was measured by ELISA after 20 h of coculture.

Peptide recognition assay

Peptides were synthesized on a solid phase using Fmoc for transient NH2-terminal protection and were characterized using mass spectrometry. All the peptides were 90% pure as indicated by analytic HPLC. Lyophilized peptides were dissolved at 2 mg/ml in 10 mM of acetic acid and 10% DMSO, and were stored at −20°C. The first screening by chromium release assay was performed with autologous EBV-B cells incubated with 16 amino acid long peptides at a concentration of 4–5 µM. Pep-

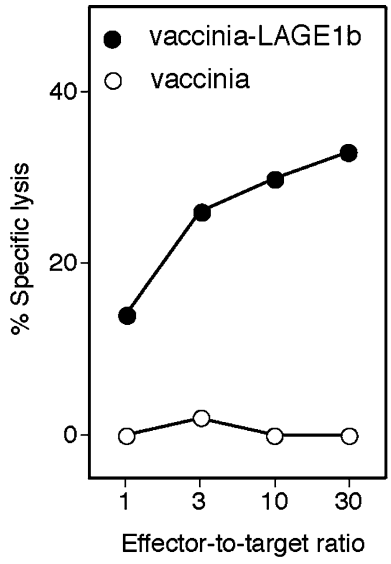

Fig. 2 Lysis of EBV-B cells expressing LAGE-1b by CTL clone E8. LB1965-EBV-B cells were infected for 2 h with the indicated vaccinia vectors at multiplicity of infection of 20 and labeled for 1 h with ^{51}Cr. The targets were incubated with the autologous CTL at the indicated effector-to-target ratios and the chromium release was measured after 4 h. The TCRβ sequence of clone E8 is Vβ29-1*01, CSVE DG SNOPQHFG, Jβ1-5*01

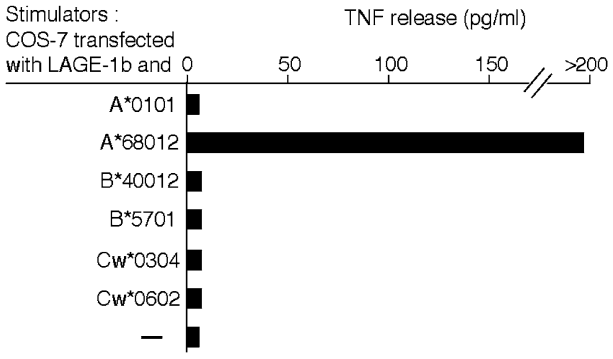

Fig. 3 The LAGE-1 peptide is presented to CTL E8 by HLA-A68 molecules. Monkey COS-7 cells were transiently transfected with 80 ng of pcDNA1/Amp-LAGE-1b and 80 ng of expression vectors encoding each of the 6 HLA class I molecules of donor LB1965. After 24 h, 2,000 CTL were added to the transfected cells; 20 h later, TNF production was estimated by measuring the cytotoxicity of the supernatants on the TNF-sensitive WEHI-164 clone 13 cells. Experiments were performed in triplicate

tide-pulsed targets were tested for recognition by CTL at an E:T ratio of 5:1.

HLA-peptide fluorescent multimers

Recombinant HLA-A6801 molecules were folded in vitro with β2-microglobulin and peptide ELVRRILSR from LAGE-1 or MUM-3 peptide EAFIQPITR [3]. They were purified by gel filtration, biotinylated, and mixed as described [2] with Streptavidin-PE (Sigma, St. Louis, MT, USA) for the HLA-A68/ELVRRILSR multimer or streptavidin-APC (BD Biosciences) for the A68/EAFIQPITR control multimer [3]. For staining and sorting, cells from A*6801 melanoma patient DDHK2 were washed, resuspended at 10^7 cells/ml in Hank's solution modified for flow cytometry [24] with 1% HS and incubated for 15 min at room temperature with HLA-A68 multimers loaded with LAGE-1 peptide (20 nM) or MUM-3 peptide (5 nM). Multimer-labeled cells (10^7 cells/80 µl) were incubated at 4°C with anti-PE microbeads (20 µl) according to the instructions of the manufacturer (Miltenyi Biotec), washed, and sorted through a separation column inserted to a magnet in an AUTOMACS at 0.5 ml/min (Miltenyi Biotec). The selected cells were distributed at 8,000 cells per well in U-bottomed microwells and cultured in 200 µl of IMDM supplemented with AAG, 10% human serum, IL-2 (100 U/ml), and IL-7 (5 ng/ml). They were stimulated on days 0 and 7 with irradiated (100 Gy) autologous cells incubated for 1 h with 4 µg/ml of peptide and washed. The autologous stimulator cells were obtained from the negative fraction of the magnetic sorting on day 0 and, on day 7, from cells amplified with 0.5 µg/ml of PHA and 100 U/ml of IL-2. Approximately 10^5 cells from each microculture were stained with multimer and analyzed by flow cytometry with a FACSCalibur (BD Biosciences) using the Cellquest software (BD Biosciences).

TCR analysis

Total RNA from T cell clones was extracted with the Tripure reagent (Roche Diagnostics). RNA was primed with oligo-dT and reverse-transcribed with MMLV-RT for 1 h at 42°C in 20 µl. cDNA (1/10) served as a template for PCR amplifications using panels of Vβ-specific upstream primers and one downstream Cβ primer. PCR products were purified and sequenced to obtain a complete identification of the CDR3 region.

Results and discussion

Isolation of an anti-LAGE-1 CTL clone

Monocyte-derived immature DC were obtained from a hemochromatosis patient without any evidence of cancer. DC were infected with an avian poxvirus carrying a *LAGE-1b* coding sequence (ALVAC-LAGE-1b) and used to stimulate autologous CD8$^+$ T cells. Ninety-six microcultures were set up with 30,000 stimulator DC and 150,000 responder CD8$^+$ T cells. Responder cells were restimulated once a week with autologous DC infected with ALVAC-LAGE-1b. Responder cells from each microculture were tested on day 21 for their lytic activity on autologous EBV-B cells infected with a vaccinia virus encoding LAGE-1b (vaccinia-LAGE-1b). An excess of unlabeled K562 target cells were added to

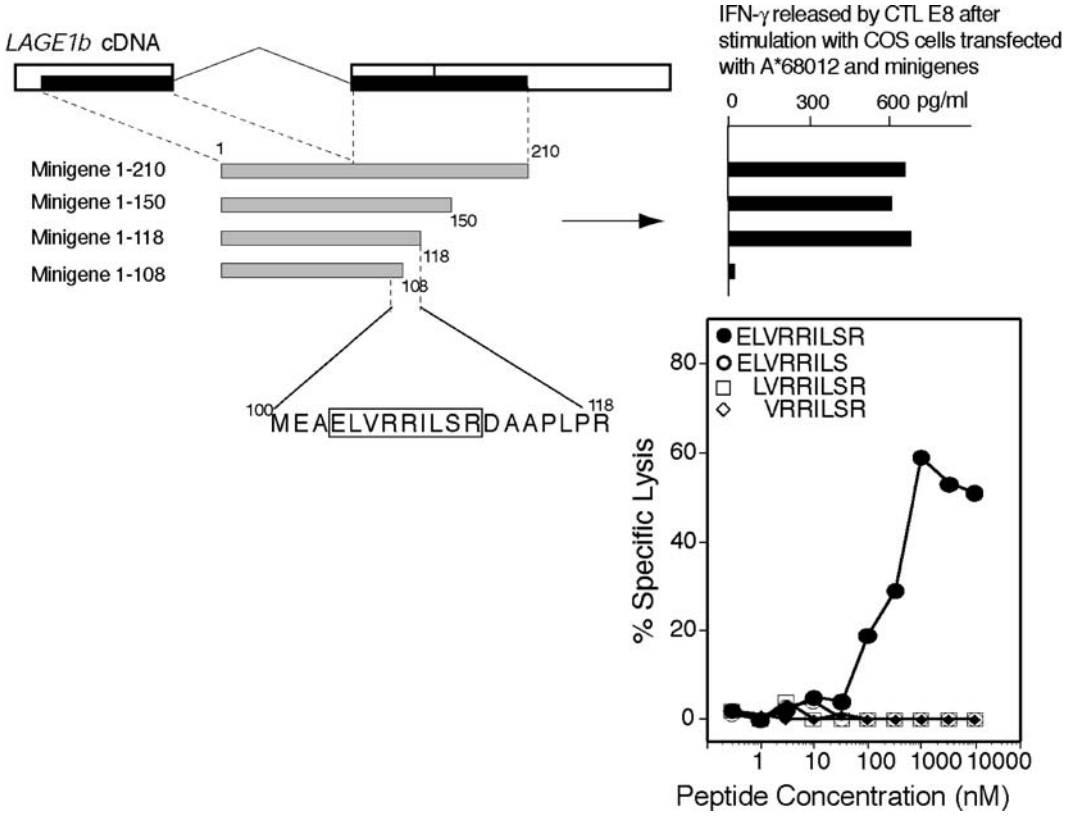

Fig. 4 Identification of the LAGE-1 antigenic peptide. The *LAGE-1b* cDNA is shown in the upper part of the figure with exons appearing as *open boxes*, the spliced intron as *kink*, and the open reading frame as *black boxes*. Truncated *LAGE-1b* cDNA were co-transfected into COS-7 cells together with an HLA-A*68012 cDNA. The transfected cells were tested for recognition by the CTL. Several peptides encoded by this region were tested for recognition by CTL E8: HLA-A68$^+$ chromium-labeled EBV-B cells were incubated for 15 min with each peptide at different concentrations, before addition of clone E8 at an E:T ratio of 5:1. Chromium release was measured after 4 h. The concentrations indicated in the figure are the concentrations during the 4 h of incubation

quench the lytic activity of NK-like effectors. One microculture contained lymphocytes displaying anti-LAGE-1 reactivity. The lymphocytes were cloned by limiting dilution and stimulated with autologous EBV-B cells transduced with a retrovirus encoding LAGE-1b. CTL clones were obtained that specifically lysed EBV-B cells infected with vaccinia-LAGE-1b. Data obtained with representative clone E8 are shown in Fig. 2.

The antigen recognized by CTL E8 is presented by HLA-A*6801 molecules

The blood donor was typed HLA-A*0101, A*68012, B*4014, B*5701, Cw*0304, and Cw*0602. To identify the HLA molecules that present the LAGE-1 peptide to CTL E8, COS-7 cells were co-transfected with a LAGE-1b cDNA and with each of the sequences coding for the HLA molecules corresponding to the haplotype of the blood donor. The transfected cells were tested for recognition by the CTL. Only the cells transfected with *LAGE-1b* and A*68012 stimulated the CTL to produce TNF (Fig. 3). COS-7 cells co-transfected with A68 and *LAGE-1a*, but not *NY-ESO-1*/*LAGE-2*, were recognized by the CTL (data not shown).

Identification of the LAGE-1 antigenic peptide

To determine the region encoding the antigenic peptide, truncated cDNA fragments of *LAGE-1b* were cloned into an expression vector and co-transfected with an HLA-A68 construct into COS-7 cells (Fig. 4). Transfected cells were tested for their ability to stimulate CTL E8. The results indicated that CTL E8 recognized a peptide encoded by the region coding for amino acids 100–118 of open reading frame 1. This part of the protein does not contain any peptide carrying the two consensus anchor residues for HLA-A68, namely, V or T at position 2 and R or K at the C-terminus [16]. Several peptides with a R at the C-terminus were tested and ELVRRILSR appeared to be the optimal one (Fig. 4). Half maximal lysis of the EBV-B cells was obtained with 300 nM of the peptide. Peptide ELVRRILSR corresponds to position 103–111 of the LAGE-1a and LAGE-1b protein sequence. It is encoded by the different LAGE-1 alleles (Fig. 1) but not by the *NY-ESO-1*/*LAGE-2* gene.

Before the construction of truncated cDNAs, we first tested CTL E8 for lysis and cytokine release upon contact with autologous EBV-B cells each incubated with a set of 74 peptides of 16 amino acids overlapping by 12

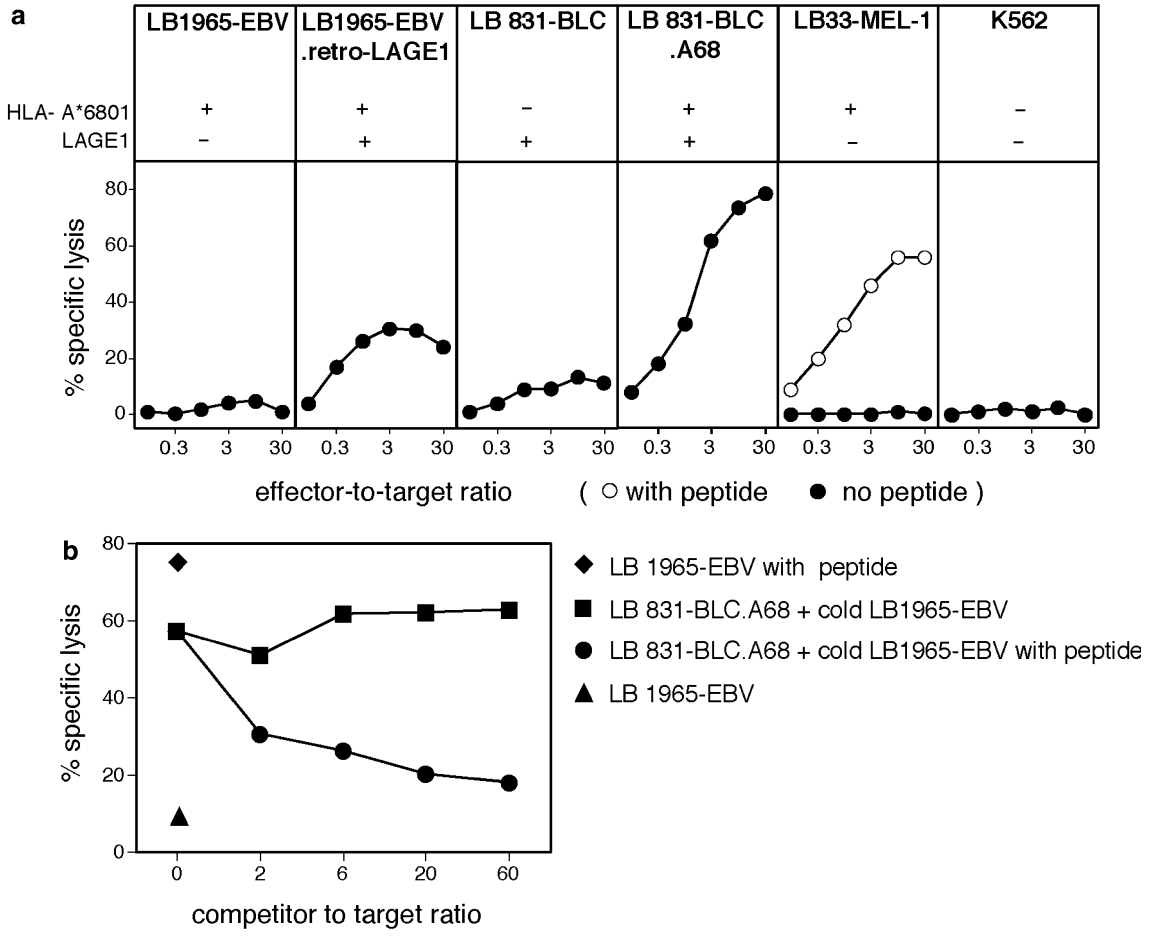

Fig. 5 Lysis of tumor cell lines by CTL clone E8. **a** Cell line LB1965-EBV.retroLAGE-1b has been obtained by transduction of EBV-B cells with a retrovirus carrying a complete LAGE-1 coding sequence. LB831-BLC is a bladder carcinoma cell line. The A68-transfected cell line is a clonal population of cells stably transfected with an HLA-A*68012 cDNA. Target cells were ^{51}Cr-labeled for 1 h, if indicated, pulsed for 15 min with 5 μM of peptide ELVRRILSR and incubated with CTL at various E:T ratios. The chromium release was measured after 4 h. **b** Unlabeled competitors were added before adding CTL at an E:T ratio of 5

amino acids and covering the complete LAGE-1b protein sequence and the protein encoded by the alternative open reading frame. None of them was recognized by the CTL, including peptides SSPMEAELVRRILSRD and EAELVRRILSRDAAPL, both of which contained antigenic peptide ELVRRILSR (data not shown). We do not have any explanation but it has to be noted that the strategy of screening a peptide library was successful for all the antigenic peptides we have identified so far, with the exception of the MAGE-4.A2 peptide [13]. For the identification of the MAGE-4 peptide we also had to construct truncated cDNAs.

Lysis of HLA-A68 tumor cells expressing LAGE-1

As we used DC expressing LAGE-1 to activate CTL E8, it was important to verify that tumor cells also process the LAGE-1.A68 antigen efficiently. As we had no appropriate tumor cell line, we transfected a LAGE-1$^+$ bladder carcinoma line, LB831-BLC, with a vector carrying the A*6801 coding sequence and a sequence coding for the resistance to geneticin. Clonal populations were derived from the geneticin-resistant transfected cells. The A68$^+$ cells were lysed by CTL clone E8, indicating that they processed the LAGE-$1_{103–111}$ peptide (Fig. 5a). This lysis of LB831-BLC.A68 cells was quenched in the presence of an excess of unlabeled A68 EBV-B cells pulsed with peptide ELVRRILSR (Fig. 5b).

Isolation of anti-LAGE-1 CTL in a cancer patient using an A68/LAGE-1 fluorescent multimer

The identification of an antigenic peptide allows the use of HLA-peptide multimers to detect T cell responses in cancer patients. A fluorescent multimer was constructed by folding HLA-A6801 molecules with peptide ELVRRILSR. Sixteen million nonadherent blood cells (±2.5 million CD8 T cells) from an A*6801 melanoma patient, bearing a melanoma expressing LAGE-1, were incubated with A68/LAGE-1 multimers conjugated to phycoerythrin (PE) and anti-PE antibodies coupled to magnetic beads (Fig. 6). The multimer-positive cells se-

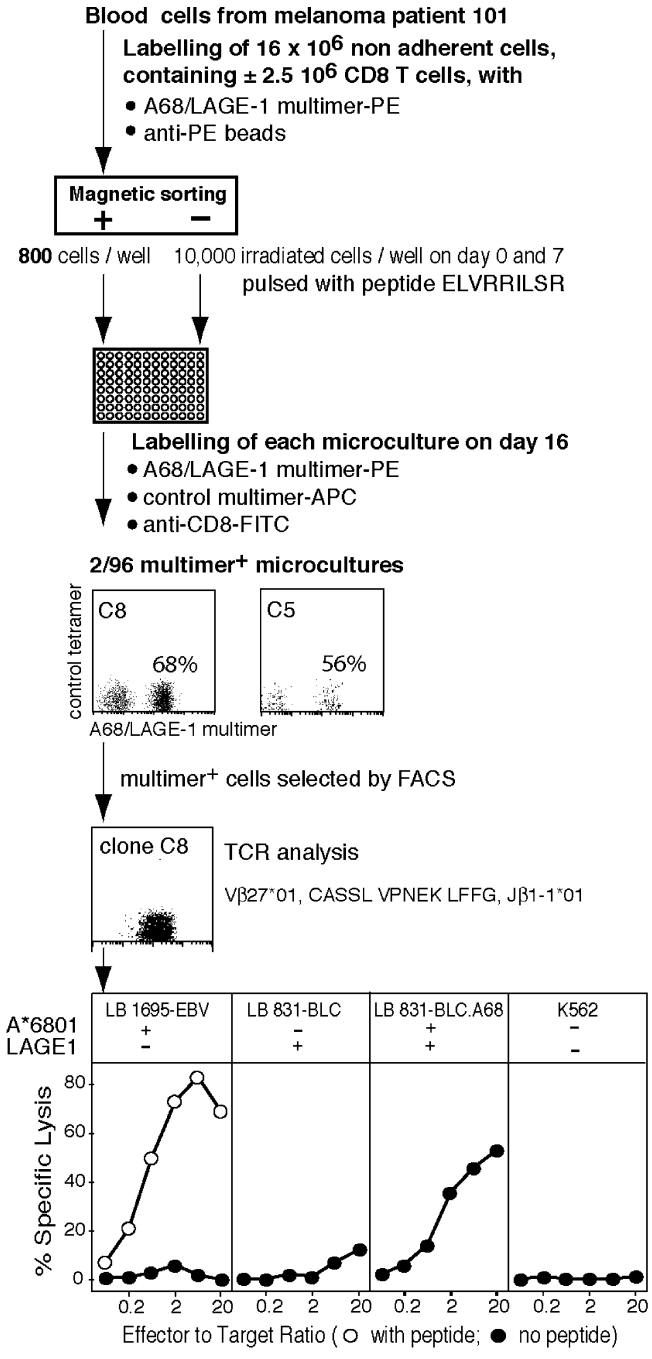

Fig. 6 Overview of the procedure to isolate anti-LAGE-1.A68 CTL with a fluorescent multimer from a blood sample of an A68 patient bearing a melanoma expressing *LAGE-1*. The clinical evolution of patient 101 was described in Zhang et al. [35]

lected by magnetic sorting were distributed in microwells and stimulated on days 0 and 7 with peptide-pulsed autologous cells. The microcultures were screened on day 16 for the presence of cells specifically labeled with multimers and two microcultures were positive, suggesting a frequency of CTL precursors of approximately 1 per million CD8 T cells (Fig. 6). We cannot exclude that the CTL precursors have been primed during the in vitro culture. A clonal population was obtained from one microculture. It lysed A68 cells pulsed with peptide ELVRRILSR and the bladder carcinoma cell line expressing LAGE-1 after transfection with HLA-A68.

This result validated the A68/LAGE-1 multimer as a tool for the monitoring of specific T cell responses in cancer patients. It also demonstrated that anti-LAGE-1.A68 CTL exist in different individuals with enough avidity to lyse tumor cells expressing LAGE-1. Therefore, we concluded that the new antigenic peptide, ELVRRILSR, could be used as target for therapeutic antitumoral vaccination of A68 patients carrying a tumor expressing LAGE-1. HLA-A68 molecules are expressed in 18% of blacks, 8% of Caucasians, and 3% of Orientals [26]. It has to be noted that 11 different HLA-A68 alleles have been identified encoding 9 different HLA molecules [26]. The binding specificity of the LAGE-1 peptide to the different alleles should be further analyzed; however, it is likely that most of the A68 molecules have an equivalent peptide anchor motif. Monitoring of T cell responses of cancer patients vaccinated with peptides should ideally be analyzed with tetramers corresponding precisely to their allele, because the contact sites between the T cell receptor and the HLA molecules could differ between different HLA alleles that can present the same peptide. Such a case has been encountered with B44: we isolated from a B*4402 individual an anti-MAGE-A3 CTL strictly restricted by B*4402 and from a B*4403 individual a CTL strictly restricted by B*4403 that recognized the same peptide [19, 20].

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