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Identification of a new peptide recognized by autologous cytolytic T lymphocytes on a human melanoma

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Abstract

Melanoma line LG2-MEL expresses several antigens recognized by autologous CTLs. One of them consists of a peptide derived from tyrosinase and presented by HLA-B*3503. We have identified another antigen of LG2-MEL as a peptide presented by HLA-B*4403 and resulting from a point mutation in gene *OS-9*. This gene is expressed in various normal tissues. It is located on chromosome 12 in the vicinity of the *CDK4* locus and is frequently co-amplified with *CDK4* in human sarcomas. The mutation, a C-to-T transition, changes a proline residue into a leucine at position 446 of the OS-9 protein. Mutated transcripts were found in all the melanoma sublines of LG2-MEL. None of the 184 tumor samples collected from other cancer patients expressed the mutated transcript, indicating that this is a rare mutational event. Interestingly, some of the melanoma sublines of LG2-MEL have lost the wild-type allele of gene *OS-9*. Those sublines appear to grow faster *in vitro* than the sublines that retained the wild-type allele, suggesting that this loss of heterozygosity may favor tumor progression. The mutation we have identified in gene *OS-9* might therefore participate in the oncogenic process by affecting the function of this potential tumor-suppressor gene.

Introduction

Cytolytic T lymphocyte clones that specifically lyse human tumor cells can be obtained after stimulation of blood lymphocytes with autologous irradiated tumor cells. These CTLs recognize specific antigens, which are presented at the tumor cell surface by MHC class I molecules and usually consist of peptides 8 to 11 amino acids in length. The antigens identified so far can be classified according to their expression profiles ([1](#)). A first group is composed of shared tumor-specific antigens which are encoded by genes such as the *MAGE* genes, which are expressed in a large variety of tumors but not in normal tissues, except for testis and placenta ([2](#)). A second group is composed of differentiation antigens such as tyrosinase, which are expressed in melanomas and in normal melanocytes ([3](#)). A third group comprises antigens encoded by genes that show a higher level of expression in tumors, such as *PRAME* ([4](#)) and *HER-2/neu* ([5](#)).

Besides those antigens shared among various tumors, a number of CTLs recognize antigens that derive from mutated genes. Because in many cases the mutation is not shared by distinct tumors, these antigens are often specific to the individual and therefore not convenient for cancer immunotherapy. However, the characterization of such antigens can lead to the identification of genes where the mutation contributes to tumor cell development, such as *CDK4* (6), *beta-catenin* (7), or *caspase-8* (8).

Melanoma line LG2-MEL expresses several antigens recognized by autologous CTLs. We report here the identification of one of these antigens, named LG2-C, which results from a point mutation in gene *OS-9*.

Results

LG2-MEL expresses an HLA-B44-restricted tumor antigen

LG2-MEL is a melanoma cell line derived from a lymph node metastasis of patient LG2. A number of sublines were obtained *in vitro* as indicated in Figure 1. Subline LG2-MEL-5-35 was previously used to stimulate blood lymphocytes *in vitro*. CTL clones anti-LG2-A and anti-LG2-B were obtained (9). The latter was used to select *in vitro* subline LG2-MEL-5-35isc3.1.1, which was resistant to anti-LG2-B but not to anti-LG2-A CTLs (Figure 1) (10). We recently identified LG2-A as a nonapeptide presented by HLA-B35 and derived from tyrosinase (9). Preliminary data indicate that LG2-B is encoded by gp100, another melanoma differentiation antigen (data not shown).

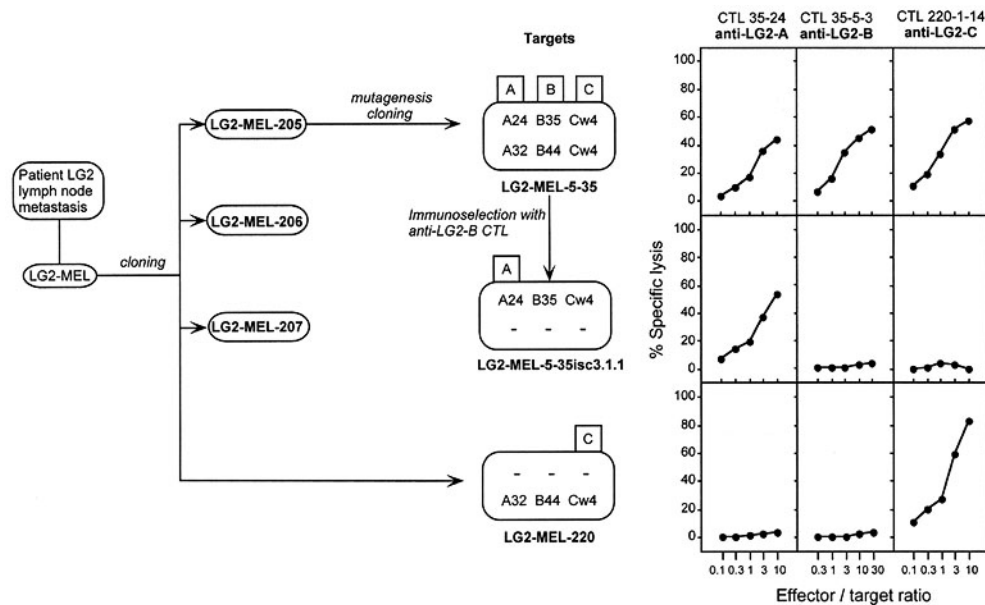


Figure 1. Origin and main features of the various sublines of melanoma LG2-MEL. Sublines LG2-MEL-205, -206, -207, -220 were obtained by cloning of LG2-MEL, a melanoma cell line that was established from a lymph node metastasis of patient LG2. Clonal subline LG2-MEL-5-35 was derived from subline LG2-MEL-205 after two mutagenic treatments, as previously described (10). Subline LG2-MEL-5-35isc3.1.1 is resistant to the anti-LG2-B CTL clones and was obtained by *in vitro* immunoselection of LG2-MEL-5-35 with the anti-LG2-B CTL clones. This subline has lost expression of HLA-A32, -B44 and -Cw4. Subline LG2-MEL-220 was obtained by cloning of LG2-MEL and has lost the HLA-A24, -B35 and -Cw4 alleles. The lysis of the melanoma sublines by the different CTL was measured in a standard chromium-release assay and is shown on the right.

In order to derive additional CTLs with different specificities, we made use of subline LG2-MEL-220, which is resistant to both anti-LG2-A and anti-LG2-B CTLs (Figure 1). Frozen blood lymphocytes from patient LG2 were stimulated with irradiated cells from subline LG2-MEL-220. After two weekly stimulations, the responding population was cloned by limiting dilution and CD8⁺ CTL clone 220-1-14 was isolated. This CTL clone specifically lysed LG2-MEL-220, indicating that it recognizes an antigen different from antigens LG2-A and LG2-B and which was named antigen LG2-C (Figure 1). The CTL clone appeared to be tumor-specific as it did not lyse autologous EBV-transformed B cells (LG2-EBV) and NK-sensitive target K562 cells (Figure 2). Subline LG2-MEL-5-35isc3.1.1, which has lost an HLA haplotype containing HLA-A32, -B44 and -Cw4 (9), was not recognized by this CTL (Figures 1 and 2). The sensitivity of this subline to the anti-LG2-C CTLs was restored after transfection of a cDNA encoding HLA-B*4403, indicating that antigen LG2-C is presented by HLA-B*4403 (Figure 2).

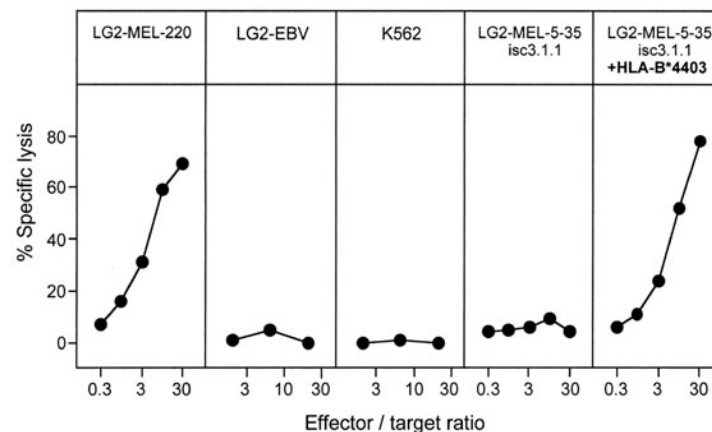


Figure 2. Specificity and HLA restriction of anti-LG2-C CTLs. CTL 220-1-14 was assayed for its ability to lyse the following targets in a standard 4-h chromium release assay: autologous melanoma subline LG2-MEL-220, autologous EBV transformed B cell line LG2-EBV, NK-sensitive line K562, autologous melanoma subline LG2-MEL-5-35isc3.1.1 and the same cell line transfected with a plasmid construct containing the HLA-B*4403 cDNA. ⁵¹Cr release was measured after 4 hours.

Identification of a cDNA encoding antigen LG2-C

In order to identify the cDNA encoding antigen LG2-C, we prepared a cDNA library from autologous tumor cells LG2-MEL-5-35. Pools containing approx. 100 cDNA clones were transfected into COS-7 cells together with a cDNA encoding HLA-B*4403. Transfected cells were tested for their ability to induce TNF release by the anti-LG2-C CTLs. One out of the 624 pools that were tested proved positive, and cDNA clone 68/196 was isolated. It induced TNF production by the anti-LG2-C CTLs when transfected into COS-7 cells along with the cDNA encoding HLA-B*4403 (Figure 3).

The sequence of the positive cDNA was 1870 bp long and corresponded to the terminal part of the cDNA sequence of gene OS-9, which is 2736 bp in length (11). Gene OS-9 is expressed in many normal tissues, and was found to be amplified in several sarcomas (11). The sequence of cDNA 68/196 differed from the OS-9 sequence at position 1422 where a C in the OS-9 sequence is replaced by a T in the 68/196 cDNA. This changes a proline residue into a leucine at position 446 in the OS-9 protein (Figure 4). A second C-to-T transition is located after the open reading frame at position 2668.

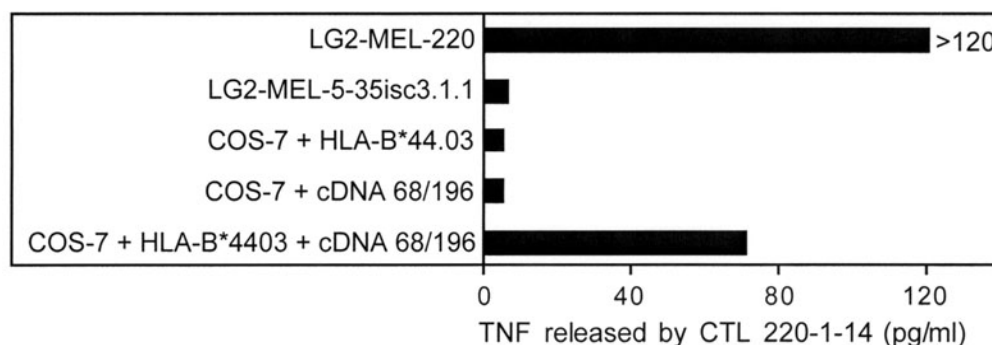


Figure 3. Identification of the cDNA coding for antigen LG2-C. COS-7 cells transfected with the indicated plasmid constructs were tested for their ability to stimulate the release of TNF by anti-LG2-C CTLs. TNF release was measured by testing the toxicity of the supernatants on WEHI 164 cl13 cells.

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1271
cctcagaggggaaccagagaaggaaaggggtgatccagaacggcagagagagatggaagaagaggaggatgaggatgaggatgaggatgaagatgaggatg
P Q R E P E K E R G D P E R Q R E M E E E E D E D E D E D E D E D E
396

aacggcagttactgggagaatttgagaaggaactggaagggatcctgcttctgtcagaccgagaccgggtccggttcggaagtgaaggctggcatggagcgg
R Q L L G E F E K E L E G I L L L S D R D R L R S E V K A G M E R
                                     ...ccg...
                                     P
                                     } Normal sequence
                                     462

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Figure 4. Partial sequence of cDNA 68/196. The mutated nucleotide that induces the replacement of a proline by a leucine in LG2 melanoma cells is indicated in bold. The peptide recognized by the anti-LG2-C CTLs is boxed. The acidic domain of the OS-9 protein is underlined. The complete sequence of gene OS-9 is available (GenBank Accession No. [U41635](http://www.ncbi.nlm.nih.gov/nuccore/U41635)).

In order to determine whether the C-to-T transition at position 1422 is an allelic polymorphism or a mutation that occurred in our tumor cells, we tested autologous B cell line LG2-EBV for the presence of this mutation. We designed an assay involving restriction digestion of PCR products performed with a reverse primer located immediately after the mutation (position 1423-1445) and designed to create an *Hpa*II restriction site CCGG on the wild-type sequence. Digestion of PCR products obtained from the wild-type sequence leads to the production of two fragments of 130 bp and 24 bp, whereas the 154 bp mutated PCR product remains undigested. We observed that PCR products obtained from normal autologous LG2-EBV cells were completely digested by *Hpa*II while PCR products obtained from the various tumor cell lines derived from LG2 melanoma were not or only partially digested (Figure 5). This indicated that the substitution was due to a mutation that occurred in LG2 tumor cells. The mutation was found in all the autologous tumor sublines that were examined.

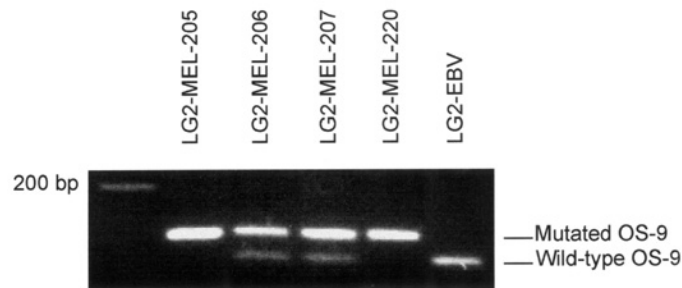


Figure 5. Detection of the C-to-T transition in genomic DNA obtained from various cell lines of patient LG2. DNA obtained from the indicated cell lines was amplified by PCR using a reverse primer located immediately after the mutation (position 1423-1445) and designed to create an *Hpa*II restriction site CCGG on the wild-type sequence. Digestion of PCR products obtained from the wild-type sequence leads to the production of two fragments of 130 bp and 24 bp, whereas the mutated 154 bp product remains undigested. Digested PCR products were loaded on a 3% agarose gel and stained with ethidium bromide.

Identification of the antigenic peptide

Several peptides containing the leucine 446 and corresponding to the HLA-B44 binding motif, which is characterized by a glutamic acid at position 2 and a hydrophobic residue at position 9 (12), were synthesized and tested for their ability to sensitize LG2-EBV cells to lysis by anti-LG2-C CTLs (Figure 6). Of all the peptides tested, peptide KELEGILLL was recognized the best, with half-maximal lysis achieved at a dose of 200 nM. The corresponding wild type peptide KELEGILLP was not recognized.

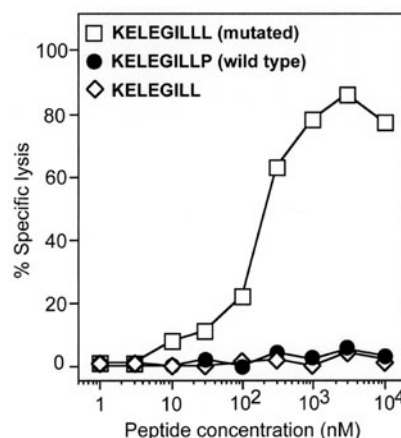


Figure 6. Recognition of peptide KELEGILLL by anti-LG2-C CTLs. ^{51}Cr -labeled LG2-EBV cells were incubated for 30 minutes with various concentrations of the indicated peptides. CTL 220-1-14 was added at an effector to target ratio of 10:1. ^{51}Cr release was measured after 4 hours.

The mutation at position 1422 of gene OS-9 is a rare event

By screening sequence databases for sequences containing the mutation, we identified two ESTs with the same C-to-T substitution at position 1422 in the OS-9 cDNA. These ESTs were derived from uterine and thyroid tumors (GenBank Database, Accession Nos. [AW797189](#) and [AW607948](#)). This suggested that the mutation identified in

LG2 tumor cells was present in other tumors. To test this hypothesis, we searched for the presence of this mutation in RNA samples from 184 allogeneic tumors including 40 melanomas, 19 renal cell carcinomas, 20 sarcomas, 20 non-small cell lung carcinomas, 20 head and neck carcinomas, 20 breast carcinomas, 20 bladder carcinomas, 20 colorectal carcinomas and 5 cervical carcinomas. The screening was performed by *Hpa*II digestion of RT-PCR products as described above. None of these tumors expressed the mutated transcript, indicating that the mutation identified in LG2-MEL is a rare mutational event.

We also made the intriguing observation that melanoma sublines LG2-MEL-220 and LG2-MEL-205 only express the mutated transcript, while both non-mutated and mutated transcripts were found in LG2-MEL-207 and LG2-MEL-206 (data not shown). In addition, analysis of the genomic DNA of those sublines showed that LG2-MEL-220 and LG2-MEL-205 contained only the mutated allele, whereas both alleles were present in the other sublines (Figure 5). This indicates that the wild-type allele was lost during tumor progression in sublines LG2-MEL-205 and LG2-MEL-220 and not in the other two sublines. This loss of heterozygosity, which occurred at a late stage of tumor development, could favor tumor cell proliferation. Consistent with this hypothesis, we observed that both sublines, LG2-MEL-205 and LG2-MEL-220, multiplied more rapidly (doubling times of 35 hours and 38 hours, respectively) than subline LG2-MEL-206 (doubling time of 78 hours) and, to a much lesser extent, than subline LG2-MEL-207 (doubling time of 40 hours).

Discussion

Several tumor antigens recognized by autologous CTLs arise from point mutations in ubiquitously expressed genes. Some of these mutations appear to be related to tumor cell development. For example, the mutation identified in *CDK4* was shown to affect the binding of CDK4 to its inhibitor p16^{INK4a}, thereby favoring uncontrolled cell division (6). The reported *beta-catenin* mutation was found to result in the stabilization of the protein. This leads to the formation of constitutive complexes between beta-catenin and transcription factor LEF-1 which may result in persistent transactivation of unidentified target genes (7). Another example is the mutation identified in *CASP-8*, which was found to decrease cell sensitivity to apoptosis (8).

In this work, we identified a new HLA-B*4403-restricted melanoma peptide which is derived from a point mutation in the previously described OS-9 gene. This gene is located on chromosome 12q13-15 next to the *CDK4* locus and is frequently co-amplified with *CDK4* in human sarcomas (11). The OS-9 cDNA encodes a polypeptide 667 amino acids in length which contains a putative nuclear targeting sequence and an acidic region (11). A similar acidic sequence is found in nucleolin (13), a major nucleolar protein which induces chromatin decondensation by interacting with histone H1 (14). The murine OS-9 protein was shown to interact in a Ca⁺⁺-dependent fashion with the second C2 domain of N-copine, a brain-specific protein that belongs to a family of membrane-binding proteins (15). Such C2 domains have been identified in proteins that are involved in processes such as membrane trafficking, generation of lipid second messengers, GTPase activation, as well as the control of protein phosphorylation (16).

The antigen identified in this work results from a C-to-T transition at a dipyrimidine site, a mutation typically induced by UV exposure and frequently observed in skin cancers (17). This mutation induces the replacement of a proline by a leucine residue at position 446 in the OS-9 protein, a modification that could impair the structure of the protein since proline residues are known to induce protein kinks due to their rigid cyclic lateral chain.

Since the function of the OS-9 protein is unknown, it is difficult to determine whether the mutation we identified in LG2-MEL tumor cells is involved in tumorigenesis. We could not find this mutation in the 184 tumor samples

analyzed, which indicates that this mutation is rare. However, we observed that two tumor sublines derived from melanoma LG2-MEL displayed a loss of heterozygosity affecting the wild-type allele of gene OS-9. Further investigation will be required to define the role of OS-9 in the control of tumor progression and its potential function as a tumor-suppressor gene.

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Materials and methods

Cell lines

All melanoma cell lines used in this work were derived from LG2-MEL, a cell line obtained from a lymph node metastasis of patient LG2 ([10](#)). Antigen-loss variant LG2-MEL-5-35isc3.1.1, which has lost expression of HLA-A32, -B44 and -Cw4, was previously obtained by *in vitro* selection of LG2-MEL-5-35 cells with an anti-B CTL clone ([9](#)). All melanoma cell lines and the COS-7 cells were cultured in Dulbecco's Eagle Medium (Life Technologies, Gaithersburg, MD) supplemented with 5% FCS (Life Technologies). Cell line K562 and the autologous Epstein-Barr virus transformed B cells (LG2-EBV) were grown in Iscove's medium (Life Technologies) containing 5% FCS. Wehi 164 cl13 cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 5% FCS. All culture media were supplemented with L-arginine (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), penicillin (100 U/ml) and streptomycin (100 µg/ml).

CTL clones

CTL clone 220-1-14 was obtained as previously described ([10](#)) from a mixed lymphocyte-tumor culture using LG2 blood lymphocytes and autologous LG2-MEL-220 tumor cells as stimulator cells. CTL clones were stimulated weekly with 3 x 10⁴ irradiated (100 Gy) tumor cells in the presence of 3 x 10⁵ irradiated LG2-EBV

cells as feeder cells, in RPMI medium containing 10% human serum, human recombinant IL-2 (Eurocetus, Amsterdam, the Netherlands) (50 U/ml), and the amino acid supplements and antibiotics listed above. After 4 days, another 50 U/ml IL-2 supplement was added to the culture.

Assay for cytolytic activity

A standard chromium release assay (18) was performed by incubating Na⁵¹CrO₄-labeled target cells with the CTLs in 96-well microplates at various effector-to-target ratios. After 4 hours of incubation, an aliquot of the supernatant was collected and its chromium content was determined using a gamma counter.

Transient transfection and CTL stimulation assay

Transient transfection of COS-7 cells was performed using the DEAE-dextran-chloroquine method (19). COS-7 cells were transfected in duplicate with 120 ng of plasmid. After 24 hours, transfected cells were tested for their ability to stimulate TNF production by CTL 220-1-14. Briefly, 1500 CTLs were added to the transfected cells in 100 µl RPMI medium containing 5% FCS and IL-2 (25 U/ml). After 20 hours, supernatants were collected and their TNF content was estimated by testing their cytotoxic activity on WEHI 164 cl13 cells in a colorimetric assay (20).

Mutation detection by PCR-restriction fragment length polymorphism (PCR-RFLP)

In order to detect the presence of the mutation in genomic DNA or cDNA purified from different tumor samples or normal tissues, we designed a PCR reaction using as forward primer 5'-ATGAGGGGTGATCCAGAACG-3' and as reverse primer 5'-AACGGAGCGGGTCTCGGTCTGCC-3' (located immediately after the mutation at nucleotides 1423-1445). This reverse primer was designed to create an *Hpa*II restriction site CCGG in the 154 bp PCR product obtained from the wild-type sequence. This was done by replacing a T by a G at position 1424 of the OS-9 sequence (i.e. by replacing an A by a C in the corresponding position of the reverse primer). Digestion of PCR products obtained from the wild-type sequence leads to the production of two fragments of 130 bp and 24 bp, whereas digestion of the mutated product yields a single band of 154 bp. These products were separated by electrophoresis on a 3% agarose gel.

Stable transfection of adherent cells

Stable transfectants were obtained by the calcium phosphate precipitation method (21). In brief, 1 x 10⁶ cells were seeded in 20 ml of medium. Twenty-four hours later, cells were transfected with the 40 µg of plasmid pcDNA3 (Invitrogen, San Diego, CA) containing the HLA-B*4403 cDNA and the neomycin resistance gene. On the third day, transfectants were selected with the neomycin analog G418 (1 mg/ml) (Life Technologies). The resulting population was cloned by limiting dilution.

Construction of the cDNA library

Total RNA was extracted from LG2-MEL-5-35 using the guanidine-isothiocyanate procedure. Poly(A)⁺ RNA enriched with an oligodT cellulose column was converted to cDNA using Superscript Choice System (Life Technologies) with an oligodT primer containing a *Not*I restriction site at its 5'-end. *Bst*XI/*Eco*RI adaptors (Invitrogen) were ligated onto the cDNAs. The cDNAs were digested with *Not*I and ligated into the *Bst*XI and *Not*I sites of expression vector pcDNA3 (Invitrogen). *Escherichia coli* TOP10F' (Invitrogen) were transformed by electroporation and selected with ampicillin (50 µg/ml). Pools of about 100 bacteria were amplified for 4 hours and plasmid DNA extracted using the QIAprep 8 extraction kit (Qiagen GmbH, Hilden, Germany).

Peptide synthesis and recognition assay

All peptides used were synthesized on solid phase using Fmoc for transient N-terminal protection and characterized by mass spectrometry. The lyophilized peptides were diluted for a final concentration of 20 mg/ml in DMSO and stored at -80°C. Chromium-labeled LG2-EBV cells were incubated with various concentrations of peptides for 30 min at 37°C before addition of the CTLs and incubated further for 4 hours. The final peptide concentrations, after addition of the CTLs, are indicated.

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