BMB
Reports

Cross-immunizing potential of tumor MAGE-A epitopes recognized by HLA-A*02:01-restricted cytotoxic T lymphocytes

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Almost all melanoma cells express at least one member of the MAGE-A antigen family, making the cytotoxic T cells (CTLs) epitopes with cross-immunizing potential in this family attractive candidates for the broad spectrum of anti-melanoma immunotherapy. In this study, four highly homologous peptides (P264: FLWGPRALA, P264I9: FLWGPRALI, P264V9: FLWGPRALV, and P264H8: FLWGPRAHA) from the MAGE-A antigens were selected by homologous alignment. All four peptides showed high binding affinity and stability to HLA-A*02:01 molecules, and could prime CTL immune responses in human PBMCs and in HLA-A*02:01/K^b transgenic mice. CTLs elicited by the four epitope peptides could cross-lyse tumor cells expressing the mutual target antigens, except MAGE-A11 which was not tested. However, CTLs induced by P264V9 and P264I9 showed the strongest target cell lysis capabilities, suggesting both peptides may represent the common CTL epitopes shared by the eight MAGE-A antigens, which could induce more potent and broad-spectrum anti-tumor responses in immunotherapy. [BMB Reports 2012; 45(7): 408-413]

INTRODUCTION

Cancer/testis (CT) antigens are considered tumor-specific and ideal targets for developing therapeutic vaccines against tumors. Some of the most well-studied types of CT antigens are the members of the melanoma antigen genes family A (*MAGE-A*). Located in the q28 region of chromosome X, this family consists of 12 closely related genes (*MAGE-A1* to *-A12*), which have 60-98% identity in their coding sequences (1). *MAGE-A*-encoded antigens (known as MAGE-A) are composed of 124-429 amino acids and share 44-96% homology. Similar to other CT antigens, MAGE-A antigens are specifically expressed in tumors and have been detected in a broad range of tumor types (2). Among the MAGE-A antigens, seven (MAGE-A1, -A2, -A3, -A4, -A6, -A10, and -A12) have been demonstrated as appreciably expressed in tumors, but to various extents according to the tumor type (3). Notably, most tumors have been found to express at least one MAGE-A antigen (4), suggesting the possibility of inducing an antitumor immune response that would recognize all or most of the MAGE-A antigens. This comprehensive strategy would significantly widen the target spectrum of anti-cancer immunotherapy against the MAGE-A-expressing cancers.

Because CTLs play a key role in antitumor immune responses, it is important to identify CTL epitopes derived from TAAs. Several of these CTL epitopes have been identified to date, including those from MAGE-A antigens (5, 6); however, most remain limited in their clinical utility because of the restricted expression of TAAs. In a recent study, we demonstrated that the peptide FLWGPRALA is an HLA-A*02:01-restricted CTL epitope derived from MAGE-A4 (7). In subsequent analysis, we found that FLWGPRALA is shared among MAGE-A1, -A4, and -A8 and that it is highly homologous to another HLA-A*02:01-restricted epitope derived from MAGE-A3, FLWGPRALV, which itself is shared with -A12 (8). It has been reported that the P3-P8 segment of the nonapeptide epitope contributes to the peptide's interaction with the T cell receptor (TCR) (9). Therefore, we speculated that the two peptides might both be capable of priming the HLA-A* 02:01-restricted CTLs that recognize and kill tumor cells expressing MAGE-A1, -A3, -A4, -A8, or -A12. It is also possible that additional HLA-A*02:01-restricted CTL epitopes of other MAGE-A family members may exist, which have high sequence homology to FLWGPRALV and FLWGPRALA. Such "super" epitopes would be able to stimulate a range of similar CTLs, thereby enhancing the population of effector cells that can recognize all, or most, of the MAGE-A-expressing tumors and broadening the potential clinical applications of these epitopes in peptide-mediated immunotherapy.

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http://dx.doi.org/10.5483/BMBRep.2012.45.7.068

Received 21 March 2012, Revised 18 April 2012, Accepted 3 May 2012

Keyword: CTL, Epitope, HLA-A*02:01, MAGE-A antigens, Tumor

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Here, we describe two new peptides that are highly homologous to the known FLWGPRALA and FLWGPRALV epitopes from MAGE-A antigens that were identified by protein sequence alignment. All four peptides were characterized by peptide/HLA-A*02:01 binding affinity and complex stability assays, and evaluated for their abilities to induce peptide-specific CTLs from HLA-A*02:01 peripheral blood mononuclear cells (PBMCs) and HLA-A2.1/Kb transgenic mice. Finally, the ability of the peptide-induced CTLs to cross-lyse tumor cells expressing various individual MAGE-A antigens was investigated.

RESULTS

Selection of peptides with high homology to the FLWGPRALA and FLWGPRALV peptides

By aligning the protein sequences of the nine MAGE-A family members, two other homologous peptides were identified and designated according to the position of the first residue in the sequence of MAGE-A1. As shown in supplementary Table 1 (Table S1), P264, P264I9, and P264V9 differ only at the carboxyl-terminus. In addition, P264H8 is different from P264 at the P8 residue. Each peptide is shared only by two or three MAGE-A antigens, but the P1-P7 segment of the four peptides is completely identical and is shared by nine of the MAGE-A antigens.

Affinity of peptides for the HLA-A*02:01 molecule

The binding affinity of these peptides for the HLA-A*02:01 molecule was measured in vitro using a T2-cell-peptide binding test. P264, P264H8, and P264V9 were found to have similar affinities for the HLA-A*02:01 molecule, as compared with the positive control peptide. P264I9, however, had lower affinity, at the indicated peptide concentrations. However, all these peptides had much higher affinity with HLA-A*02:01 molecules in contrast to the negative peptide control $OVA_{257\text{-}264}$, the Kb restricted epitope (Fig. 1).

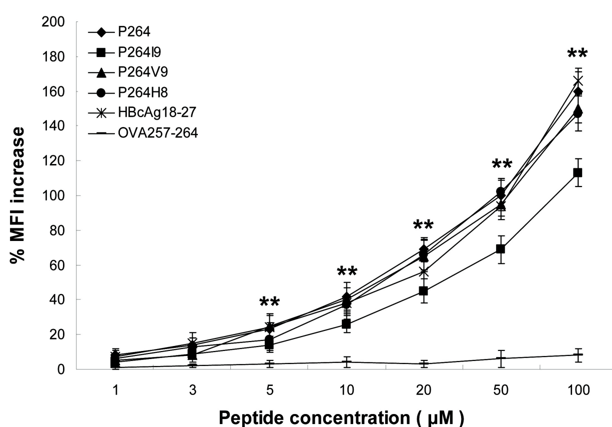

Fig. 1. Binding affinity of peptides for the HLA-A*02:01 molecule. T2 cells were incubated with the indicated concentrations of peptides and human β2m. The cells were then stained with anti-HLA-A2 mAb and FITC-labeled goat anti-mouse IgG, and HLA-A*02:01 expression on the T2 cell surface was determined by using a FACSCalibur flow cytometer. The peptides $HBcAg_{18\text{-}27}$ and $OVA_{257\text{-}264}$ were used as the positive and negative control, respectively. **$P < 0.01$, $HBcAg_{18\text{-}27}$, P264, P264V9, P264H8 or P264I9 vs negative control $OVA_{257\text{-}264}$.

Because a stable peptide-MHC complex is critical for the induction of an antigen-specific CTL immune response, we further assessed the capacity of the selected peptides to stabilize the HLA-A*02:01 molecule. P264, P264I9, and P264V9 were all found to be strong stabilizers of the HLA-A*02:01 molecule ($DC_{50} > 8$ h) (Table S1). While P264H8 exhibited a higher binding affinity than P264I9 (Fig. 1), it had a weaker stabilization capacity (DC_{50} = 2-4 h).

In vitro induction of peptide-specific CTLs

P264 was characterized as an HLA-A*02:01-restricted CTL epitope in our previous study, and P264V9 was demonstrated to be a MAGE-A3 CTL epitope (7, 8). To study whether the other two peptides were also capable of inducing the generation of peptide-specific CTLs *in vitro*, and to compare the immunogenicity of the four peptides, PBMCs isolated from HLA-A*02:01 individuals were stimulated with peptide-pulsed autologous DCs and PBMCs, successively. ELISPOT assay showed that, similar to the P264- and P264V9-primed CTLs, the P264I9- and P264H8-induced CTLs also secreted significant interferon-γ (IFN-γ) upon co-culturing with T2 cells loaded with 20 μM of corresponding peptide. In contrast, these induced CTLs produced little IFN-γ when co-cultured with T2 cells loaded with the irrelevant peptide $HBcAg_{18\text{-}27}$. Moreover, the P264I9- and P264V9-induced CTLs produced appreciably more IFN-γ than the P264- or P264H8-induced CTLs. After blocking the HLA-A*02:01 on T2 cell surfaces and the CD8 molecules on CTLs (with anti-HLA-A2 mAb and anti-CD8 mAb, respectively), the stimulators only marginally activated the effectors, resulting in very little IFN-γ being produced (Fig. 2A). These data indicated that the release of IFN-γ by P264I9- and P264H8-induced CTLs, while recognizing T2 cells pulsed with the corresponding peptide, was HLA-A*02:01-restricted and CD8-dependent.

To address whether the P264I9- and P264H8-induced CTLs could lyse tumor cells, and to compare the cytotoxic activity of CTLs primed by the four peptides, a cytotoxicity assay was performed. The results indicated that, similar to the P264- and P264V9-primed CTLs, both P264I9- and P264H8-induced CTLs were capable of lysing the target LB1751-MEL cells, which expressed HLA-A*02:01 and MAGE-A antigens. In agreement with the results from the IFN-γ ELISPOT assay, P264I9- and P264V9-induced CTLs were also able to lyse a greater amount of target cells than the other two peptides, as evidenced by the E/T ratios (Fig. 2B). Blocking HLA-A*02:01 molecules on the surface of LB1751-MEL cells by exposure to anti-HLA-A2 mAb caused a significant abrogation of P264I9- or P264H8- stimulated PBMCs' ability to lyse these target cells (Fig. 2C).

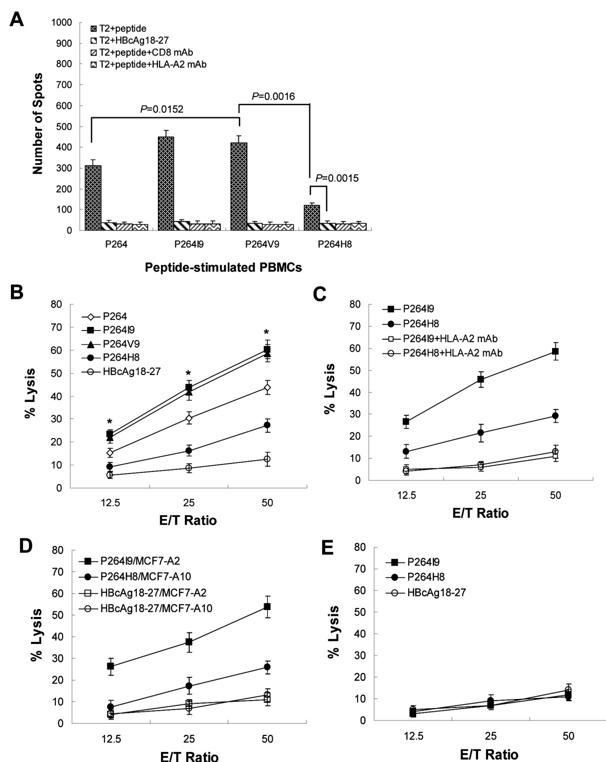

Fig. 2. Functional properties of peptide-specific CTLs induced by selected peptides. PBMCs from healthy HLA-A*02:01-positive donors were stimulated with the reagents described in the *M&M* for three weeks at one week intervals. The $HBcAg_{18-27}$ was used as negative control. After the final stimulation, the IFN-γ production and cytotoxic activity of stimulated PBMCs was evaluated in ELISPOT (A) and cytotoxicity assays (B-E), respectively. (A) Peptide-primed PBMCs were tested against T2 cells pulsed with the corresponding peptide or irrelevant peptide $HBcAg_{18-27}$ with ELISPOT assay. Anti-HLA-A2 and anti-CD8 mAbs were used to determine whether the observed IFN-γ production was HLA-A2- and/or CD8-dependent. (B) P264-, P264I9-, P264V9- and P264H8-stimulated PBMCs-mediated lysis of LB1751-MEL cells. (C) P264I9- and P264H8-stimulated PBMCs-mediated lysis of LB1751-MEL cells or LB1751-MEL cells with anti-HLA-A*02:01 mAb blocked surface HLA-A*02:01 molecules. (D) P264I9- and P264H8-stimulated PBMCs-mediated lysis of MCF7-A2 or MCF7-A10 cells. (E) P264I9- and P264H8-stimulated PBMCs-mediated lysis of MCF-7 cells. *$P < 0.05$, P264I9 or P264V9 vs other peptides.

Moreover, P264I9- or P264H8-primed effector cells were also found to be capable of lysing the MCF7-A2 and MCF7-A10 cells (Fig. 2D), but not the MAGE-A-negative MCF-7 cells (Fig. 2E).

In vivo induction of peptide-specific CTLs in HLA-A*02:01/K^b transgenic mice

To investigate whether P264I9 and P264H8 could elicit CTL immune responses *in vivo*, HLA-A*02:01/K^b transgenic mice were inoculated once with one of the four peptides. Ten days later, the splenocytes were harvested and stimulated *in vitro* with the corresponding peptide. The cytotoxicity assay showed that, similar to splenocytes from P264- or P264V9-immunized mice, splenocytes from P264I9- or P264H8-inoculated mice were capable of lysing LB1751-MEL cells. The splenocytes from IFA-inoculated mice, on the other hand, did not lyse the target cells after *in vitro* stimulation with any of the four peptides. Importantly, splenocytes from the P264I9- or P264V9-inoculated mice killed more target cells than those from the P264- and P264H8-inoculated mice, as evidenced by the E/T ratios (Fig. 3). When anti-HLA-A2 mAb was added, the peptide-induced splenocytes were inhibited from killing the target cells (data not shown).

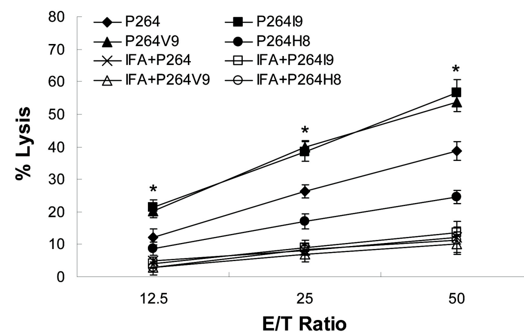

Fig. 3. *In vivo* induction of peptide-specific CTLs. The HLA-A*02:01/K^b transgenic mice were immunized with one of the four peptides prepared in IFA or with IFA alone. The harvested splenocytes from immunized mice were stimulated *in vitro* with the corresponding peptide. A cytotoxicity assay was used to evaluate the ability of peptide-stimulated splenocytes from peptide-immunized mice (P264, P264I9, P264V9, and P264H8) and IFA-immunized mice (IFA+P264, IFA+P264I9, IFA+P264V9, and IFA+P264H8) to lyse the cultured LB1751-MEL cells. *$P < 0.05$, P264I9 or P264V9 vs other peptides.

Cross-recognition of peptide-induced CTLs to MAGE-A antigens

Since the amino acid residues at positions 1-7 of P264, P264I9, P264V9, and P264H8 were found to be identical, it is possible that the four peptides may have the same antigenicity. Therefore, each peptide-primed CTLs was investigated for its ability to cross-recognize the other MAGE-A antigens from which the other peptides were derived. After human PBMCs were stimulated and mice were immunized with peptide, as described in the Materials and Methods section of induction of peptide-specific CTLs *in vitro* and *in vivo*, a cytotoxicity assay was used to evaluate the effector cells-mediated lysis of target cells expressing MAGE-A1, -A2, -A3, -A4, -A6, -A8, -A10, or -A12. To ensure the specificity of the lysis, MCF-7 cells transfected with empty vector was included to exclude the vector effects and NK target K562 cells was included to exclude non-CTL killing effects in the primed bulk T cells. MAGE-A-negative MCF-7 cells was included as negative control. The results demonstrated that all of the *in vitro* peptide-stimulated

PBMCs were capable of lysing all of the tumor cells that expressed each one of the eight MAGE-A antigens at E/T ratio 50:1 (data not shown) and even 12.5:1, but to different extents; in contrast, none of the peptide-stimulated PBMCs lysed the K562 cells, MCF-7 cells transfected with empty vector and MAGE-A-negative MCF-7 cells (Fig. 4A). We also observed that P264I9 and P264V9 peptides primed CTLs could kill the individual targets more efficiently than P264 and P264H8 peptides (Fig. 4A). Similar results were obtained with the splenocytes from peptide-inoculated mice (Fig. 4B).

DISCUSSION

In this study, we identified P264I9 and P264H8 peptides as novel HLA-A*02:01-restricted CTL epitopes derived from MAGE-A, which have high identity to the two previously published MAGE-A CTL epitopes, P264 and P264V9. The P264I9 and P264H8 epitopes were able to efficiently stabilize the peptide/HLA-A*02:01 complex and induce peptide-specific CTLs to produce significant amounts of IFN-γ and to lyse

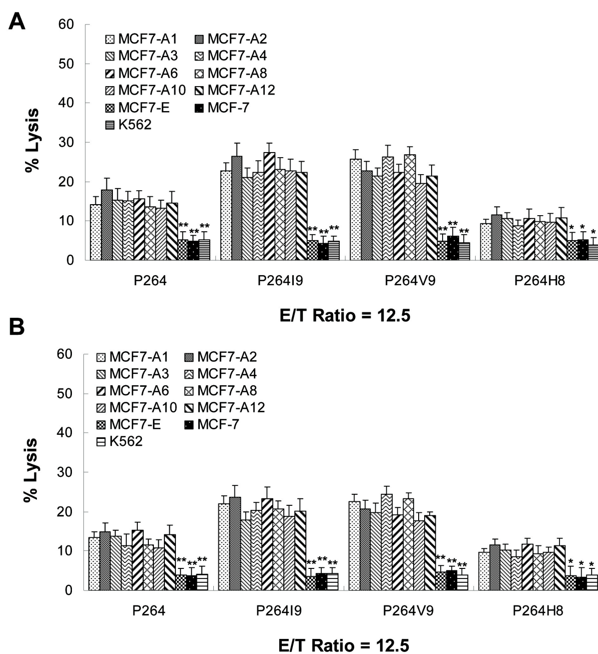

Fig. 4. Cross-recognition of peptide-induced CTLs to MAGE-A antigens. Cytotoxic properties of the isolated PBMCs from healthy HLA-A*02:01 donors that were stimulated *in vitro* with the indicated peptides (A) and harvested splenocytes from peptide-immunized HLA-A*02:01/K^b transgenic mice (B) were tested by cytotoxicity assay. The MCF7-expressing MAGE-A1, -A2, -A3, -A4, -A6, -A8, -A10, or -A12 tumor cells, NK target K562 cells, MCF-7 cells transfected with empty vector (MCF7-E) and MAGE-A-negative MCF-7 cells were used as target. *$P < 0.05$ and **$P < 0.01$, vs MCF-7 cells transfected with the indicated genes of MAGE-A family members.

HLA-A*02:01$^+$MAGE-A$^+$ target cells in a HLA-A*02:01-restricted fashion. At the same time, P264I9 and P264H8- stimulated PBMCs were demonstrated to also be able to lyse HLA-A*02:01-positive MCF7-A2 or MCF7-A10 cells transfected with the MAGE-A2 or MAGE-A10 gene, but had no effect on the MAGE-A-negative MCF-7 cells. Tests of immunogenicity in an animal model revealed that P264I9 and P264H8 could also induce specific CTL immune responses *in vivo*.

Further experiments demonstrated that each of the four peptide-primed CTLs were able to recognize all of the eight MAGE-A antigens (-A1, -A2, -A3, -A4, -A6, -A8, -A10, and -A12), indicating that the four peptides represent common CTL epitopes that are shared by the eight MAGE-A antigens. Unfortunately, in the present study, we failed to amplify the MAGE-A11 gene, so it remains unclear whether any of the four peptide-induced CTLs could recognize or lyse the targets expressing MAGE-A11. Regardless, it has been reported that the P2 and P9 residues of the nonapeptide epitope are responsible for the peptide/MHC binding while the P3-P8 segment contributes to the peptide/TCR interaction (10). Since the P264, P264I9, and P264V9 epitopes have the same TCR ligand sequence, it is reasonable to hypothesize that these epitope-primed CTLs have the cross-immunizing potential to their mutual target cells, although at different cytolytic levels. However, the P264H8 peptide, which has a different P8 residue from other peptides (H→L), can also have the cross-immunizing potential to the other targets, suggesting that the P8 residue in these epitopes might act principally as the minor anchor residue for the MHC molecule, instead of as the key residue for TCR recognition of the peptide. This hypothesis was verified by results from a complex stabilization assay, in which P264 and P264H8, varying only at the position 8, exhibited different peptide/ HLA-A*02:01 complex stabilization capacity.

Among the four epitope peptides, P264I9 and P264V9 induced more secreted IFN-γ from the CTLs and the induced CTLs exhibited more powerful lysis of the target cells. These results suggested that P264I9 and P264V9 are the most potent immunogens of the four peptides, in terms of inducing peptide-specific CTLs. Some previous results have suggested that P264V9 is naturally processed by tumor cells and presented to T cells (8). However, some other studies found that the peptide is not efficiently presented by all tumor cells (11, 12). A recent study offered an explanation for these contradictory results by demonstrating that P264V9 processing requires intermediate proteasomes, which represent between one-third and one-half of the proteasome content of human liver, colon, small intestine, kidney, and are also present in human tumor cells and DCs (13). Likewise, it has been found that MAGE-A antigens are extensively expressed by multiple types of tumors, but at different frequencies, and that most of tumors express at least one MAGE-A antigen (4). Thus, P264I9 and P264V9 may prove beneficial as a specific immunotherapy strategy affecting a large proportion of tumors because the peptide-elicited CTLs can recognize a majority of the MAGE-A antigens. The

cross-immunizing potential of MAGE-A protein derived CTL epitopes might be mediated by cross-reactivity, but such possibility needs to be further confirmed by CTL cloning work in the future.

In conclusion, we have identified P264I9 (FLWGPRALI) and P264H8 (FLWGPRAHA) are novel HLA-A*02:01-restricted CTL epitopes of the MAGE-A antigens. CTLs induced by the previously identified P264 and P264V9 peptides and by the newly identified P264I9 and P264H8 peptides can recognize all of the eight MAGE-A antigens and can lyse tumor cells expressing these antigens. However, P264I9 and P264V9 induce more potent antitumor immunity than the other two epitopes, suggesting that P264I9 and P264V9 have potential as broad-spectrum antitumor immunotherapy agents.

MATERIALS AND METHODS

Cell lines and animals

The T2 human tumor cells (HLA-A*02:01-expressing, and deficient in TAP1 and TAP2 transporters), MCF-7 breast cancer cells (MAGE-A-negative), K562 leukemia cells and BB7.2 hybridoma cells (producing the anti-HLA-A2 monoclonal antibody (mAb)) were purchased from the American Type Culture Collection (Manassas, VA, USA). The LB1751-MEL human melanoma cell line (expressing all the MAGE-A antigens and HLA-A*02:01) was kindly provided by Dr. F. Brasseur (Ludwig Institute for Cancer Research, Brussels, Belgium).

HLA-A*02:01/K^b transgenic mice (6-8 weeks old) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of Third Military Medical University.

Selection and synthesis of peptides

The protein sequences of eleven MAGE-A antigens were aligned. The peptide FLWGPRALA and its highly homologous peptides were selected as candidates and synthesized, along with control peptides (HLA-A2 restricted $HBcAg_{18-27}$ and MHC Kb restricted $OVA_{257-264}$), by Fmoc chemistry (Sangon, China). The products were purified by high-performance liquid chromatography to a purity of $>95\%$.

Affinity measurement of peptide for HLA-A*02:01

The affinity of peptides for HLA-A*02:01 was measured by T2-cell-peptide binding test according to the protocol described previously (14).

Assessment of peptide/HLA-A*02:01 complex stability

The peptide/HLA-A*02:01 complex stability was measured according to the protocol described previously (9).

Plasmid construction and cell transfection

The mammalian expression plasmids pCI-MAGE-A1, -A2, -A3, -A4, -A6, -A8, -A10, and -A12, which each contained the respective coding sequence of a single MAGE-A antigen, were constructed as described below. Total RNA was extracted from LB1751-MEL cells, PCR was performed with the appropriate amplifying primers (Table S1). The amplified products were inserted into the pCI-neo plasmid (Promega, Beijing, China) and the resultant plasmids were identified by sequencing.

To establish a cell line expressing both HLA-A*02:01 and one of the MAGE-A antigens, MCF-7 cells were transfected with the appropriate plasmid by using Lipofectamine 2000 (Invitrogen) and then selected with G418. The expressions of MAGE-A in the established cell lines (designated MCF7-A1, -A2, -A3, -A4, -A6, -A8, -A10, and -A12, respectively) were confirmed by reverse transcription-PCR and Western blotting. MCF-7 cells transfected with the empty vector pCI-neo were used as a control.

Induction of CTLs from human PBMCs

PBMCs were isolated from the buffy coat of heparinized whole blood samples of healthy HLA-A*02:01 donors (15) by means of density gradient centrifugation on Ficoll-Paque PREMIUM (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The effector lymphocytes and dendritic cells (DCs) were prepared by our published method (16). To evaluate the cross-immunizing potential of effector lymphocytes, lymphocytes were stimulated by DCs and PBMCs pulsed with 2 μg peptide. All donors provided written informed consent to participate in the study. This study was approved by the ethics committee of the Third Military Medical University (Chongqing, China).

Enzyme-linked immunosorbent spot (ELISPOT) assay

ELISPOT assays were performed using a commercially available kit (Diaclone, Cedex, France).

Cytotoxicity assay

Three to five days after the final stimulation, the cytotoxic activity of the effector cells was evaluated by a lactate dehydrogenase (LDH) release assay using the Cytotox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega) (17). In brief, 1×10^4 target cells (LB1751-MEL, untransfected MCF-7, transfected MCF-7, or K562) in 50 μl RPMI 1640 containing 5% fetal calf serum (FCS) were placed in the wells of a 96-well round-bottom polyvinylidene fluoride-backed microplates, then 50 μl of various concentrations of effector cells were added at different effector to target (E/T) ratios (50/1, 25/1, and 12.5/1). After 4 h incubation at 37°C, the supernatant was collected to assay LDH release by measuring the optical density (OD) at 490 nm, according to the manufacturer's instructions. Experiments were performed in triplicate, and the percentage of lysis was calculated as: [(experimental LDH release - effector spontaneous LDH release -target spontaneous LDH release)/(target maximum LDH release - target spontaneous LDH release)] $\times$ 100.

Analysis of *in vivo* immunogenicity

HLA-A*02:01/K^b mice were immunized with 100 μg of peptides prepared in incomplete Freund's adjuvant (IFA) or with IFA emulsion alone as a control. After 10 days, mice were sacrificed and splenocytes were cultured for five days with 10 units/ml recombinant murine interleukin-2 (rmIL-2) and 2 μg/ml peptide. Then, effector cells were counted and tested for cytotoxic activity via the cytotoxicity assay described above.

Statistical analysis

Statistical analyses were performed using the variance test and Student's *t*-test. A difference was considered significant at the conventional level of $P < 0.05$.

Acknowledgements

This work was supported by a grant from the Natural Science Foundation of Chongqing (No. 2007BB5009). We would like to thank Dr. Jennifer C. van Velkinburgh, who works for the van Velkinburgh Initiative for Collaboratory BioMedical Research, for substantial writing assistance with this manuscript.

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