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Identification of HLA-A2- and A24-restricted T-cell epitopes derived from SOX6 expressed in glioma stem cells for immunotherapy

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Malignant gliomas are the most aggressive human primary brain tumors and are currently incurable. Immunotherapies have the potential to target glioma and glioma stem cells (GSCs) that are resistant to conventional therapies. We previously identified SOX6 as a human glioma antigen and demonstrated that vaccination with SOX6 DNA induced cytotoxic T lymphocytes (CTLs) specific for glioma, thereby exerting therapeutic antitumor responses in glioma-bearing mice. In this study, we attempted to identify SOX6-derived peptides as specific targets for effective and safe T-cell-mediated immunotherapy targeting SOX6-positive glioma and GSCs. *In vitro* stimulation with human leukocyte antigen (HLA)-A*2402 (A24)-restricted peptides, RFENLGPQL ($SOX6_{504}$) and PYYEEQARL ($SOX6_{628}$) or the HLA-A*0201 (A2)-restricted peptide, ALFGDQDTV ($SOX6_{447}$) was capable of inducing SOX6 peptide-specific CTLs in peripheral blood mononuclear cells derived from healthy donors and glioma patients. These CTLs were able to lyse a majority of glioma cell lines and a GSC line derived from human glioblastoma in an HLA Class I-restricted and an antigen-dependent manner. Furthermore, peptide vaccines of $SOX6_{628}$, which was conserved in the murine SOX6 protein and expected to bind to major histocompatibility complex (MHC) H-2^d, induced CTLs specific for $SOX6_{628}$ in H-2^d mice. Normal autologous cells from mice, in which SOX6-specific immune responses were generated, were not destroyed. These results suggest that these SOX6 peptides are potnetially immunogenic in HLA-A24 or -A2 positive glioma patients and should be considered as a promising strategy for safe and effective T-cell-based immunotherapy of patients with gliomas.

Key words: tumor antigen, SOX6, CTL epitope, immunotherapy, glioma stem cells

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Malignant gliomas, such as glioblastoma multiforme (GB), represent the most common primary brain tumors and patients have a dismal prognosis.[1] Over 12,000 new cases are diagnosed annually in the U.S.[2] with a median survival rate of ~15 months.[3] Development of novel, molecularly targeted, multimodal therapeutic approaches is critical to further improve the outcomes of patients with these deadly tumors. The recent identification of chemotherapy and radiotherapy-resistant glioma stem cells (GSCs) in GBs[4,5] may help explain why conventional therapies are ineffective. Although the exact mechanism of tumor stem cell resistance to conventional therapies remains elusive, their quiescent state and increased capacity to eliminate cytotoxic drugs[5] and repair damaged DNA are thought to be key contributing factors.[4,6–8] Immunotherapy might offer some benefit to GB patients, because immune-mediated killing relies neither on tumor cell proliferation nor the aforementioned cytotoxic pathways. Indeed, dendritic cell-based vaccine therapy in a GSC-bearing mouse model showed an efficient anti-tumor immune response against GSCs derived from malignant glioma cells.[9]

The effectiveness of T-cell-mediated immunotherapy for cancer depends on both an optimal immuno-stimulatory context of the therapy and the proper selection with respect to

quality and quantity of the targeted tumor antigens, and, more precisely, the T-cell epitopes contained in these tumor proteins (reviewed in Ref. 10). We previously identified the developmentally regulated transcription factor, Sry-related high mobility group box-containing gene 6 (SOX6), as a glioma antigen by serological screening using a testis cDNA library.[11] Vaccination with a plasmid DNA encoding murine SOX6 induced cytotoxic T-lymphocytes (CTLs) specific for SOX6-expressing glioma cells and exerted protective and therapeutic antitumor responses in the glioma-bearing mice,[12] suggesting SOX6 to be a potential target for immunotherapy.

Peptide vaccination has the advantages of a defined nature and the ease of synthesizing peptides employing good manufacturing practices, enabling peptide vaccines to be used as prefabricated "off-the-shelf" vaccines. The gene frequency of HLA-A*2402 (A24) is relatively high in Asian populations, especially in the Japanese, whereas being low in Caucasians. In contrast, the gene frequency of HLA-A*0201 (A2) is high in various ethnic groups, including both Asians and Caucasians.[13] These facts indicate that the HLA-A2- and HLA-A24-restricted SOX6-derived CTL epitope peptides may be useful for immunotherapy in many glioma patients. Herein, we demonstrated that HLA-A24- and HLA-A2-restricted SOX6 peptides can induce SOX6-reactive CTLs *in vitro* and *in vivo*. Our results support the development of novel peptide-based immunotherapy for glioma patients.

Material and Methods

Cell lines

CIR, a B cell-lymphoblastic cell line with loss of the expressions of HLA-A and -B molecules, CIR-A24, CIR transfected with the HLA-A24 gene and CIR-A2, CIR transfected with the HLA-A2 gene, were cultured in RPMI 1640 medium (Sigma-Aldrich Corp.) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100 IU/mL penicillin, 100 μg/mL streptomycin and 10 mmol/L L-glutamine. CIR-A24-SOX6, CIR-A24 transfected with the full-length human SOX6 gene, and CIR-A2-SOX6, CIR-A24 transfected with the SOX6 gene, were cultured in the medium supplemented with 500 μg/mL hygromycin B (Calbiochem). Five human-glioma cell lines, SK-MG-1 (HLA-A*0302/2402), T98G (HLA-A0201/-), KNS-42 (HLA-A2402/2601), SF126 (HLA-A0201/2402) and U87 (HLA-A0201/-) were purchased from Health Science Research Resources Bank or American Type Culture Collection. All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich Corp.) containing 10% heat-inactivated FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 10 mmol/L L-glutamine. P815 cells [from a mouse mastocytoma cell line (H-2^d)] were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS, and penicillin-streptomycin. Three GSC lines (X01GB, X02GB and X03AOA) were established and characterized as described previously.[14–16] Tumor spheres were cultured in a medium containing DMEM/F-12 (Invitrogen), penicillin G, streptomycin sulfate, B-27 (Invitrogen), recombinant human FGF-2 (20 ng/mL; R&D Systems, Minneapolis, MN) and recombinant human EGF (20 ng/mL; R&D Systems).[14,16]

Mice

Female and male 6–8-week-old BALB/c mice (major histocompatibility complex (MHC) haplotype H-2^d) were purchased from Japan SLC. The Animal Care and Use Committee of the Keio University School of Medicine approved all animal procedures.

Selection and synthesis of candidate HLA-A*0201- or HLA-A*2402-binding peptides derived from SOX6

The protein sequences of SOX6 were obtained from GenBank and analyzed for HLA-A2- or HLA-A24-binding motifs using the HLA Peptide Binding Predictions Program (http://bimas.dcrt.nih. gov/molbio/hla_bind/) of the BioInformatics and Molecular Analysis Section (BIMAS).[17] Peptide sequences that were given high binding scores and predicted proteasomal cleavage sites at the ends of the sequences were chosen. For proteasomal cleavage prediction, we used an online cleavage prediction program, PAProC (http://www.uni-tuebingen.de/uni/bcm/kuttler/paproc1.html).[18] The synthetic peptides, $SOX6_{504}$ (504–512, RFENLGPQL), $SOX6_{447}$ (447–455, ALFGDQDTV), $SOX6_{628}$ (628–636, PYYEEQARL), cytomegalovirus (CMV) $_{pp65}$ (495–503, QYDPVAALF), Flu-$M1_{58–66}$ (58–66, GILGFVFTL) from the influenza matrix protein and K^d-restricted peptide 533–541 of A/PR/8 hemagglutinin (K^d-HA) (533–541, IYSTVASSL) were synthesized using *N*-(9-fluorenyl) methoxycarbonyl chemistry, by American Peptide Company, and were >95% pure as indicated by analytic high-performance liquid chromatography and mass spectrometric analysis. Peptides were dissolved in DMSO at a concentration of 10 mg/mL and stored at −20°C until use.

Western blot analysis of SOX6 in tumor cell lines

SOX6 expression in tumor cells was tested using a Western blot analysis as described previously.[11,19] Briefly, after the nuclear extracts were isolated from tumor cells, 20 μg of the proteins were blotted onto nitrocellulose membranes and reacted with a rabbit anti-human SOX6 polyclonal antibody (10 μg/mL, CHEMICON International), followed by 1:2000-diluted peroxidase-conjugated mouse anti-rabbit immunoglobulin G (IgG) (Cappel). The proteins were visualized with the help of an ECL Western blot detection system (Amersham Biosciences). 293T cells transfected with the full-length human SOX6 cDNA (293T cell-SOX6), which we had previously prepared,[11] were used as a positive control.

In vitro induction of SOX6-specific CTL

This study was approved by the local ethical review board of Keio University (No.12-21-2). After obtaining their informed consent, peripheral blood mononuclear cells (PBMCs) were obtained from glioma patients managed by the Department of Neurosurgery, Keio University School of Medicine. To generate dendritic cells (DCs), plastic adherent PBMCs in AIM-V

medium (Invitrogen) supplemented with 1,000 units/mL recombinant human granulocyte macrophage colony-stimulating factor (R&D Systems) and 500 units/mL recombinant human IL-4 (rhIL-4; Cell Sciences) were cultured at 37°C in a humidified CO_2 (5%) incubator. Mature DCs were then harvested on Day 7, resuspended in AIM-V medium at 1×10^6 per cells milliliter with peptide (10 μg/mL), and incubated for 2 hr at 37°C. Autologous $CD8^+$ T cells were enriched from PBMCs using anti-human CD8 Particles DM (SK1) (BD Biosciences Pharmingen). $CD8^+$ T cells (2×10^6 per well) were co-cultured with 2×10^5 peptide-pulsed DCs per well in 2 mL/well of AIM-V medium supplemented with 10% heat-inactivated FBS, 10 units/mL rhIL-1α (R&D Systems), 20 units/mL rhIL-2 (R&D Systems), 1 ng/mL rhIL-4 (Cell Sciences), 125 units/mL rhIL-6 (Cell Sciences) and 1 ng/mL rhIL-12 (Cell Sciences) in each well of 24-well tissue culture plates from Multiwell Primaria (Falcon). On Days 14 and 21, $CD8^+$ T cells were restimulated with autologous DCs pulsed with peptide in AIM-V medium supplemented with 10% heat-inactivated FBS, 10 units/mL rhIL-1α, 20 units/mL rhIL-2, 1 ng/mL rhIL-4 and 125 units/mL rhIL-6. On Day 28, the $CD8^+$ cultured cells were analyzed for CTL activity by standard 4-hr ^{51}Cr release assay. Epstein-Barr virus (EBV) transformed B cells (EBV-B) were produced from healthy donors' or patients' PBMCs by infection with supernatant from the EBV producer line B95.8. Then, we generated a SOX6 peptide-specific CTL line derived from the $CD8^+$ T cells by weekly re-stimulation with autologous EBV-B cells which had been irradiated (150 Gy) and pulsed with the SOX6 epitope peptide.

^{51}Cr release cytotoxicity assay

Target cells (1×10^4 cells in 100 μL) labeled with 50 μCi of $Na_2{}^{51}CrO_4$ (^{51}Cr) were added to wells containing varying numbers of effector cells (100 μL) using U-bottomed 96-well plates. After 4 hr incubation at 37°C, cells were centrifuged and 100 μL of supernatant were collected and measured for radioactivity. Percentage of specific lysis (% specific lysis) was calculated using triplicate samples, as follows: percentage lysis = (cpm experimental release-cpm spontaneous release)/(cpm maximal release-cpm spontaneous release) × 100. Cold inhibition assay for the analysis of SOX6 peptide-specific CTL cytotoxicity was determined by measuring cytotoxicity against ^{51}Cr-labeled (hot) $HLA\text{-}A24^+$ $SOX6^+$ SK-MG-1 cells or $HLA\text{-}A2^+$ $SOX6^+$ SF-126 cells in the presence of unlabeled (cold) CIR-A24 cells or CIR-A2 cells pulsed with either SOX6 peptide or irrelevant peptide at the indicated E/T ratios. Hot target cells (1×10^3 per well) and cold target cells (1×10^4 per well) pulsed with or without the $SOX6_{628}$ peptide were incubated with CTL lines at 37°C for 4 hr, and the percentage specific lysis was then measured.

Intracellular IFN-γ staining assays of CTLs raised against the SOX6 peptide

CTLs raised against the SOX6 peptide were incubated with autologous EBV-B cells pulsed with the SOX6 peptide (10 μg/mL) or were stimulated with PMA (10 ng/mL) and ionomycin (25 μmol/L) for 6 hr at 37°C. Brefeldin A (1 μg/mL) was added for the last 2 hr of stimulation. Then, cells were harvested, washed with FACS buffer (0.5% BSA and 0.05% sodium azide in PBS), and stained with PerCP-conjugated anti-CD8 (BD Bioscience). After washing, the cells were treated with FACS lysing solution and then with FACS permeabilizing solution (BD Bioscience) and stained with PE-conjugated anti-IFN-γ antibodies (BD Bioscience). After incubation at room temperature in the dark for 30 min, the cells were washed twice with FACS buffer, fixed with 1% paraformaldehyde in PBS and analyzed by utilizing a FACScan flow cytometer (FACSCalibur).

Induction of $SOX6_{628}$ peptide-specific CTLs *in vivo*

Male ($n = 6$) and female ($n = 6$) BALB/c mice ($H\text{-}2^d$) were given two s.c. injections of 100 μg of $SOX6_{628}$ emulsified in Incomplete Freund's adjuvant (IFA; Difco) at 7-day intervals. Control animals received IFA only. On Day 10 after the second immunization, the animals were sacrificed, and splenocytes and lymphocytes from inguinal lymph nodes were stimulated *in vitro* with P815 cells ($H\text{-}2^{d+}$, $SOX6^-$, data not shown), which had been irradiated and pulsed with the $SOX6_{628}$ or an irrelevant peptide, K^d-HA. After 5 days of culture, the cells were tested for specific cytotoxicity against the P815 cells pulsed with $SOX6_{628}$ or K^d-HA, or control unpulsed P815 cells.

Histology

Immunized mice were perfused, and post-fixed in 4% paraformaldehyde (Sigma). The main organs expressing SOX6 in the embryo, including brain tissue (hippocampus, subventricular zone, cerebral cortex and cerebellum), cartilage tissue in the trachea and testicular tissue were removed from immunized mice, in which $SOX6_{628}$ peptide-specific CTL responses had been induced. Eight-micrometer paraffin sections were deparaffinized, rehydrated and stained with hematoxylin and eosin for histological examination. Histological analysis was performed by independent pathologists from the Sapporo General Pathology Laboratory Co.

Statistical analysis

Data are presented as means and standard deviation (SD). Statistical differences between two groups were evaluated by the unpaired Student's *t*-test using SPSS version 14.0 (SPSS, Chicago, Illinois) and Statcel 2 (OMS Publishing, Saitama, Japan). *p*-values less than 0.05 were considered significant.

Results

Selection of HLA-A24- or HLA-A2-binding peptides

On the basis of the algorithms, nonamer SOX6 peptides at positions 504–512 ($SOX6_{504}$, RFENLGPQL) and 628–636 ($SOX6_{628}$, PYYEEQARL), or a nonamer at position 447–455 ($SOX6_{447}$, ALFGDQDTV), showed high binding affinity to

Table 1. Binding score of SOX6-derived peptides to HLA-A molecules

Restriction element	Peptide	Amino acid position	Amino acid sequence	Binding score
SOX6-derived peptides				
A2402	$SOX6_{504}$	504–512	RFENLGPQL	72.0
A2402	$SOX6_{628}$	628–636	PYYEEQARL	24.0
A0201	$SOX6_{447}$	447–455	ALFGDQDTV	126.1
Known epitope peptides				
A2402	CMV_{pp65}	495–503	QYDPVAALF	168.0
A0201	Flu-$M1_{58-66}$	58–66	GILGFVFTL	550.9

HLA-A24 or HLA-A2, respectively, in the prediction program (Table 1).

Induction of HLA-A24-restricted cytotoxic $CD8^+$ T cells by $SOX6_{504}$ and $SOX6_{628}$ peptides

We examined whether $CD8^+$ T cells primed against $SOX6_{504}$ (CTL-$SOX6_{504}$) or $SOX6_{628}$ (CTL-$SOX6_{628}$) can recognize HLA-$A24^+$ human glioma cells. U87, SK-MG-1, SF-126, KNS-42, CIR-A24-SOX6 and CIR-A2-SOX6 expressed SOX6 (Fig. 1*a*), allowing us to evaluate the antigen-specific lytic activity of CTLs against these tumor cells. As illustrated in Figure 1*b*, these CTLs efficiently lysed SK-MG-1 (HLA-$A24^+$, $SOX6^+$), KNS-42 (HLA-$A24^+$, $SOX6^+$) and CIR-A24-SOX6 (HLA-$A24^+$, $SOX6^+$), whereas CIR-A24 (HLA-$A24^+$, $SOX6^-$), CIR-A2-SOX6 (HLA-$A24^-$, $SOX6^+$) and U87 (HLA-$A24^-$, $SOX6^+$) showed only the background level of lysis. These results suggested both CTL-$SOX6_{504}$ and CTL-$SOX6_{628}$ to have the capacity to lyse relevant HLA-A molecule/SOX6-expressing target cells in an HLA Class I-restricted and antigen-dependent manner.

To confirm that the CTLs recognized SOX6 peptide specifically and lysed cells presenting the peptide, we performed cold target competition experiments by adding non-radiolabeled (cold) CIR-A24 cells pulsed with $SOX6_{504}$ or $SOX6_{628}$ in the 4-hr ^{51}Cr release assay (Fig. 1*c*). The cytolytic activities of CTL-$SOX6_{504}$ or CTL-$SOX6_{628}$ against ^{51}Cr-labeled SK-MG-1 cells in the presence of non-peptide-pulsed or irrelevant peptide (CMV_{pp65})-pulsed cold CIR-A24 cells were significantly inhibited in the presence of CIR-A24 pulsed with the relevant $SOX6_{504}$ or $SOX6_{628}$, respectively, demonstrating a lytic ability and SOX6 epitope-specific inhibition.

Subsequently, the minimum stimulatory concentration was determined using CIR-A24 cells loaded with various concentrations of $SOX6_{504}$ or $SOX6_{628}$. CIR-A24 cells were pulsed with increasing peptide concentrations, and lysis by CTL-$SOX6_{504}$ or CTL-$SOX6_{628}$ was determined (Fig. 1*d*). Peptide titration demonstrated that half-maximal lysis by these CTLs was obtained at SOX6 peptide concentrations between 0.01 and 0.1 nM. CTLs primed with the control CMV peptide (CTL-CMV_{pp65}) showed no specific lytic activity against the relevant SOX6 peptide-pulsed CIR-A24 cells over background control levels. These results indicate that the CTL-$SOX6_{504}$ or CTL-$SOX6_{628}$ recognized $SOX6_{504}$ or $SOX6_{628}$ with a sensitivity comparable with those of several known HLA-binding epitopes from non-mutated peptides, such as those derived from melanoma antigens.[20,21]

To assess specific immunoreactivity of the CTL lines raised against the SOX6 peptide, we also evaluated productions of IFN-γ in CTL-$SOX6_{504}$ and CTL-$SOX6_{628}$ following 6 hr of *in vitro* re-stimulation with autologous EBV-B pulsed with the relevant SOX6 peptide. As shown in Figure 1*e*, both CTL-$SOX6_{504}$ and CTL-$SOX6_{628}$ produced IFN-γ to EBV-B pulsed with the relevant SOX6 peptide, indicating the response to be peptide-specific.

We further evaluated the ability of SOX6 peptides to induce specific CTL responses *in vitro* using PBMCs from HLA-$A24^+$ healthy donors and glioma patients. When using $SOX6_{504}$ for stimulations, six out of nine (66.7%) donors including two glioma patients displayed positive reactivity against SK-MG-1 (HLA-$A24^+$, $SOX6^+$) (Table 2). With regard to $SOX6_{628}$, five out of seven (71.4%) donors including a glioma patient exhibited positive reactivity against SK-MG-1 (Table 3). In these analyses, positive reactivity was defined as the CTLs tested lysing the relevant HLA-A/SOX6 positive target cells at a level exceeding 30% specific lysis at an E/T ratio of 30, but not lysing negative control cells above the background level ($\leq$15%). These data raise the possibility that $SOX6_{504}$ and $SOX6_{628}$ are immunogenic and can induce SOX6-specific CTL responses in glioma patients.

Induction of HLA-A2-restricted cytolytic $CD8^+$ T cells by $SOX6_{447}$ peptide

To investigate the potential of $SOX6_{447}$ as an HLA-A2-restricted CTL epitope, we performed a series of experiments. The $CD8^+$ T cells primed against $SOX6_{447}$ (CTL-$SOX6_{447}$) were first tested for cytolytic activity. As shown in Figure 2*a*, CTL-$SOX6_{447}$ efficiently lysed U87 (HLA-$A2^+$, $SOX6^+$) and CIR-A2-SOX6 (HLA-$A2^+$, $SOX6^+$), whereas lysis of SK-MG-1 (HLA-$A2^-$, $SOX6^+$) and CIR-A2 (HLA-$A2^+$, $SOX6^-$) was at the background level. We next performed cold target competition experiments by addition of non-radiolabeled (cold) CIR-A2 cells pulsed with $SOX6_{447}$ (Fig. 2*b*). The cytolytic activities of CTL-$SOX6_{447}$ to ^{51}Cr-labeled SF-126 cells in the presence of non-peptide-pulsed or irrelevant peptide (Flu-$M1_{58-66}$)-pulsed cold CIR-A2 cells were significantly inhibited in the presence of CIR-A2 pulsed with the relevant $SOX6_{447}$,

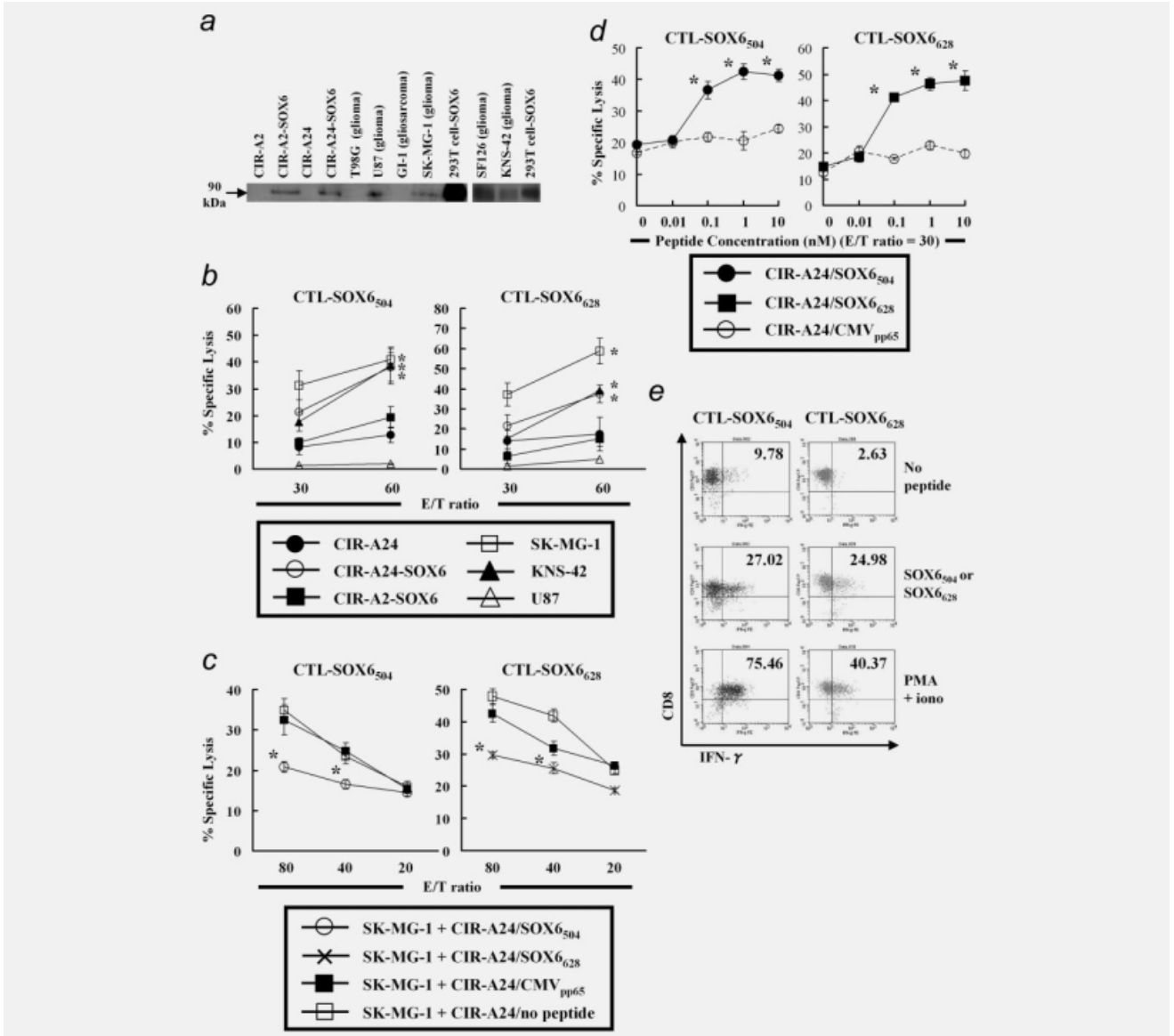

Figure 1. Identification of HLA-A*2402 (A24)-restricted CTL epitopes in SOX6. (*a*) SOX6 expression in glioma cell lines. 293T cells transfected with the full-length human SOX6 gene (293T cell-SOX6) were used as a positive control. (*b*) Cytolytic activity of $CD8^+$ T cells stimulated with $SOX6_{504}$ (CTL-$SOX6_{504}$, *Left panel*) or $SOX6_{628}$ (CTL-$SOX6_{628}$, *Right panel*). The CTLs were tested for their cytolytic abilities against SK-MG-1 (HLA-$A24^+$, $SOX6^+$), KNS-42 (HLA-$A24^+$, $SOX6^+$), U87 (HLA-$A24^-$, $SOX6^+$), CIR-A24-SOX6 (HLA-$A24^+$, $SOX6^+$), CIR-A24 (HLA-$A24^+$, $SOX6^-$) or CIR-A2-SOX6 (HLA-$A24^-$, $SOX6^+$) using 4-hr ^{51}Cr release assays. Values indicate averages of triplicate samples. Bars indicate SD. *, $p < 0.05$ for the specific lysis of SK-MG-1, KNS-42 or CIR-A24-SOX6 cells, compared with the specific lysis of CIR-A24 cells at an E/T ratio of 60 (*Left and Right panels*). One of two representative experiments with similar results is shown. (*c*) Cold target inhibition assay. The CTL-$SOX6_{504}$ (*Left panel*) or CTL-$SOX6_{628}$ (*Right panel*) were incubated for 4 hr with ^{51}Cr-labeled human glioma SK-MG-1 cells (HLA-$A24^+$, $SOX6^+$) (1×10^3 per well) in the presence of cold CIR-A24 cells (1×10^4 per well) pulsed with $SOX6_{504}$, $SOX6_{628}$, or irrelevant peptide CMV_{pp65} at the indicated E/T ratios for evaluation of specific lytic abilities. Values indicate averages of triplicate samples. Bars indicate SD. *, $p < 0.05$ for the specific lysis of SK-MG-1 cells in the presence of CIR-A24 cells pulsed with the relevant $SOX6_{504}$ (*Left panel*) or $SOX6_{628}$ (*Left panel*) peptide, compared with the specific lysis of SK-MG-1 cells in the presence of CIR-A24 cells pulsed with the irrelevant CMV_{pp65} (*Left and Right panels*) peptide at E/T ratios of 80 and 40. (*d*), Peptide concentration-dependent cytolysis of CTL-$SOX6_{504}$ (*Left panel*) or CTL-$SOX6_{628}$ (*Right panel*). $CD8^+$ T cells isolated from HLA-$A24^+$ healthy donors (HD24-1, Tables 2 and 3) were stimulated with autologous DCs loaded with $SOX6_{504}$ or $SOX6_{628}$ three times weekly. The CTLs were then tested for their cytolytic activities against CIR-A24 cells loaded with the indicated concentrations of $SOX6_{504}$, or $SOX6_{628}$, or irrelevant peptide CMV_{pp65} in 4-hr ^{51}Cr release assays at an E/T ratio of 30. Values indicate averages of triplicate samples. Bars indicate SD. *, $p < 0.05$ for the specific lysis of CIR-A24 cells pulsed with $SOX6_{504}$ peptide (*Left panel*) or $SOX6_{628}$ peptide (*Right panel*), compared with the specific lysis of CIR-A24 cells pulsed with irrelevant CMV_{pp65} peptide (*Left and Right panels*). (*e*) Flow cytometric analysis of IFN-γ producing cells in CTL-$SOX6_{504}$ derived from HD24-3 (*Left panels*) or CTL-$SOX6_{628}$ derived from HD24-2 (*Right panels*). The CTLs were double-stained with PerCP-conjugated anti-CD8 mAb and PE-conjugated anti-IFN-γ mAb. Numbers in each histogram indicate the percentage of IFN-γ^+/$CD8^+$ cells among lymphocyte-gated cells. *Top panels*, CTLs stimulated by autologous EBV-B cells pulsed with no peptide. *Middle panels*, CTLs stimulated by autologous EBV-B cells pulsed with $SOX6_{504}$ or $SOX6_{628}$. *Bottom panels*, CTLs treated with PMA and ionomycin.

Table 2. Cytolysis of CTLs raised against $SOX6_{504}$

Case	HLA type	Age	Sex*	% specific lysis to SK-MG-1 (E/T ratio = 30)	% specific lysis to CIR-A*2402 (E/T ratio = 30)
Healthy donors					
HD24-1	2402/-	23	M	49.1 ± 5.3	10.9 ± 2.1
HD24-2	1101/2402	21	M	57.5 ± 8.2	0.3 ± 0.0
HD24-3	1102/2402	22	M	50.4 ± 6.0	2.8 ± 0.1
HD24-4	0206/2402	43	M	24.9 ± 1.8	−0.3
HD24-5	2402/2602	22	M	23.1 ± 3.4	−0.1
HD24-6	2402/3303	22	M	34.4 ± 4.2	14.1 ± 3.0
HD24-7	2402/-	21	M	48.2 ± 4.8	12.7 ± 1.9
Glioma patient					
PT-1	2402/-	25	F	31.2 ± 3.3	8.2 ± 2.7
PT-2	2402/-	31	M	28.7 ± 2.5	11.0 ± 3.5

*M, male; F, female.

Table 3. Cytolysis of CTLS raised against $SOX6_{628}$

Case	HLA type	Age	Sex*	% specific lysis to SK-MG-1 (E/T ratio = 30)	% specific lysis to CIR-A*2402 (E/T ratio = 30)
Healthy donors					
HD24-1	2402/-	23	M	61.9 ± 6.2	12.8 ± 1.8
HD24-2	1101/2402	21	M	45.6 ± 3.7	6.89 ± 0.9
HD24-3	1102/2402	22	M	9.42 ± 1.3	5.94 ± 0.7
HD24-4	0206/2402	43	M	29.3 ± 2.0	−8.02
HD24-5	2402/2602	22	M	36.6 ± 3.5	−1.84
HD24-6	2402/3303	22	M	36.9 ± 4.8	0.46 ± 0.1
Glioma patient					
PT-1	2402/-	25	F	37.2 ± 4.0	14 ± 2.2

*M, male; F, female.

demonstrating a lytic ability and SOX6 epitope-specific inhibition. The minimum peptide concentration for CTL lysis was tested using the CTL-$SOX6_{447}$ and CIR-A2 cells loaded with various concentrations of the $SOX6_{447}$ peptide. Peptide titration showed that half-maximal lysis by CTL-$SOX6_{447}$ was obtained at $SOX6_{447}$ peptide concentrations between 0.01 and 0.1 nM (Fig. 2*c*), indicating $SOX6_{447}$ to potentially be an HLA-A2-restricted and peptide-specific CTL epitope. Furthermore, three out of seven (42.9%) HLA-$A2^+$ healthy donors showed positive responses to U87 cells (HLA-$A2^+$, $SOX6^+$) after stimulation with $SOX6_{447}$ peptide (Table 4). Positive reactivity was defined as the CTLs tested lysing the relevant HLA-A/SOX6 positive target cells at a level exceeding 30% specific lysis at an E/T ratio of 30, but not lysing negative control cells above the background level ($\leq$15%). Taken together, these observations indicate CTLs raised against $SOX6_{447}$ peptide to be capable of recognizing and lysing HLA-$A2^+$/$SOX6^+$ glioma cells, suggesting that the $SOX6_{447}$ peptide may be useful for inducing anti-glioma immunoreactivity.

Induction of specific CTLs by vaccinations with $SOX6_{628}$ in mice

The amino-acid sequence for human $SOX6_{628}$ (PYYEEQARL) capable of inducing HLA-A24-restricted CTLs is conserved in the murine SOX6 protein. As the human MHC Class I molecule HLA-A24 shares similarity with murine MHC Class I molecule K^d in the anchor motif of binding peptides,[22,23] HLA-A24-binding peptides are capable of binding to MHC H-$2K^d$. Indeed, $SOX6_{628}$ was expected to exhibit a high binding affinity to mouse MHC Class I H-$2K^d$ based on a peptide-MHC binding prediction program (BIMAS, data not shown). Moreover, a previous study suggested that immunization with an HLA-A24-binding peptide can induce H-$2K^d$-restricted CTLs in BALB/c (H-2^d) mice.[24] Thus, we next examined whether $SOX6_{628}$ can induce SOX6-specific CTLs in BALB/c mice. Following two s.c. vaccinations with $SOX6_{628}$ emulsified in IFA, the splenocytes were cultured *in vitro*, and then evaluated for specific cytotoxicity against P815 cells (H-2^{d+}, $SOX6^-$) pulsed with the $SOX6_{628}$ or an irrelevant peptide (K^d-HA). Splenocytes of 12 mice immunized with $SOX6_{628}$ exhibited significantly higher

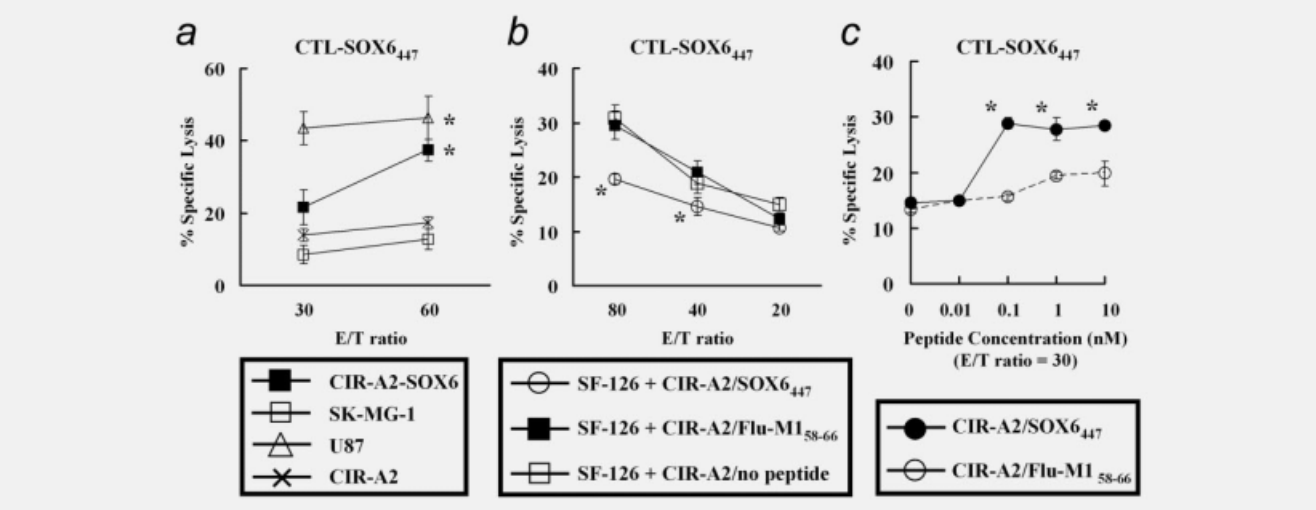

Figure 2. Identification of an HLA-A2-restricted CTL epitope in SOX6. (*a*) Cytolytic activity of $CD8^+$ T-cells stimulated with $SOX6_{447}$ (CTL-$SOX6_{447}$). The CTLs were tested for their cytolytic abilities against U87 (HLA-$A2^+$, $SOX6^+$), SK-MG-1 (HLA-$A2^-$, $SOX6^+$), CIR-A2-SOX6 (HLA-$A2^+$, $SOX6^+$) or CIR-A2 (HLA-$A2^+$, $SOX6^-$) using 4-hr ^{51}Cr release assays. Values indicate averages of triplicate samples. Bars indicate SD. *, $p < 0.05$ for the specific lysis of U87, or CIR-A2-SOX6 cells, compared with the specific lysis of CIR-A2 cells at an E/T ratio of 60 (*Left and Right panels*). One of two representative experiments with similar results is shown. (*b*), Cold target inhibition assay. The CTL-$SOX6_{447}$ were incubated for 4 hr with ^{51}Cr-labeled human glioma SF-126 cells (HLA-$A2^+$, $SOX6^+$) (1×10^3 per well) in the presence of cold CIR-A2 cells (1×10^4 per well) pulsed with $SOX6_{447}$, or irrelevant peptide Flu-$M1_{58-66}$ at the indicated E/T ratios for evaluation of specific lytic abilities. Values indicate averages of triplicate samples. Bars indicate SD. *, $p < 0.05$ for the specific lysis of SF-126 cells in the presence of CIR-A2 cells pulsed with the relevant $SOX6_{447}$ peptide, compared with the specific lysis of SF-126 cells in the presence of CIR-A2 cells pulsed with the irrelevant Flu-$M1_{58-66}$ peptide at E/T ratios of 80 and 40. (*c*) Peptide concentration-dependent cytolysis of CTL-$SOX6_{447}$. $CD8^+$ T cells isolated from HLA-$A2^+$ healthy donors (HD2-7, Table 4) were stimulated with autologous DCs loaded with $SOX6_{447}$ three times weekly. CTLs were then tested for their cytolytic activities against CIR-A2 cells loaded with the indicated concentrations of $SOX6_{447}$ or irrelevant peptide Flu-$M1_{58-66}$ in 4-hr ^{51}Cr release assays at an E/T ratio of 30. Bars indicate SD.

Table 4. Cytolysis of CTLS raised against $SOX6_{447}$

Case	HLA type	Age	Sex*	% specific lysis to U87 (E/T ratio = 30)	% specific lysis to SK-MG-1 (E/T ratio = 30)
HD2-1	0201/2402	22	M	12.2 ± 1.9	33.6 ± 3.9
HD2-2	0201/2402	21	M	33.4 ± 3.0	13.0 ± 1.0
HD2-3	0201/2402	20	F	37.1 ± 3.8	26.1 ± 2.1
HD2-4	0201/2603	21	F	42.0 ± 4.2	14.6 ± 0.8
HD2-5	0201/2402	20	F	6.1 ± 1.0	16.8 ± 1.4
HD2-6	0201/0206	20	F	5.9 ± 0.9	49.4 ± 2.2
HD2-7	0201/-	28	M	46.4 ± 2.3	12.2 ± 1.3

*M, male; F, female.

cytolysis against P815 pulsed with $SOX6_{628}$ than that pulsed with K^d-HA ($p = 0.001$, Table 5). In contrast, control splenocytes of three mice treated with mock vaccines showed no significant cytolysis against P815 pulsed with $SOX6_{628}$ (data not shown). These results suggest vaccination with $SOX6_{628}$ to induce CTLs specific to the SOX6 peptide *in vivo*.

No recognition of normal self-cells by SOX6 peptide-specific CTLs in treated mice

Sox genes exhibit highly dynamic expression patterns during development of diverse tissues and cell types, especially during embryogenesis.[25] To evaluate the potential risk of autoimmune reactions to SOX6 vaccination, we tested SOX6 expression in human normal tissues. Western blot analysis revealed that among all human normal tissues analyzed SOX6 was expressed only in the adult testis, which is an immuno-privileged site (Fig. 3), suggesting that CTLs induced against SOX6 would not damage normal human tissues. We further examined the tissues of immunized mice, which acquired SOX6 peptide-specific CTL responses (Mice 1, 5 and 11, Table 5). As mouse *Sox6* is expressed in the brain[26] and all cartilaginous sites[27] during embryogenesis, the brains,

Table 5. Cytolysis of splenocytes derived from mice treated with SOX6-peptide vaccines (E/T ratio = 80)

	Target cells	
	P815 pulsed with SOX6	P815 pulsed with K^d-HA
Mouse 1	14.5	2.6
Mouse 2	4.0	7.8
Mouse 3	6.2	1.3
Mouse 4	10.0	0.0
Mouse 5	15.2	5.9
Mouse 6	7.0	6.5
Mouse 7	1.7	2.0
Mouse 8	2.5	0.0
Mouse 9	8.5	0.0
Mouse 10	3.2	0.0
Mouse 11	13.7	2.6
Mouse 12	8.5	2.6
Average	7.9	2.6
SD	± 4.7	± 2.7

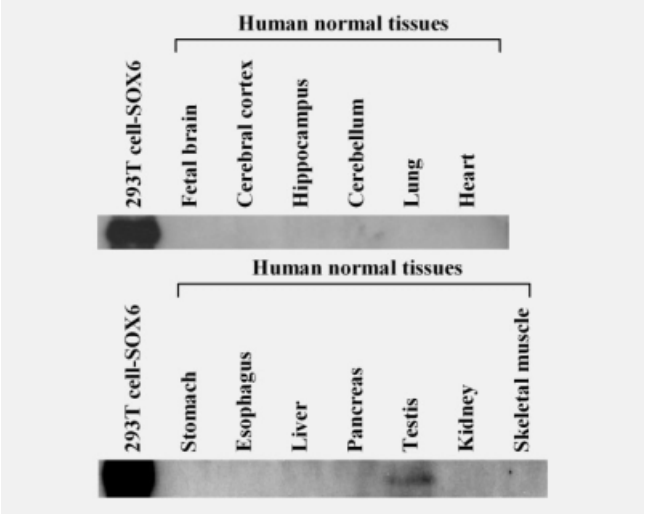

Figure 3. SOX6 expression in human normal tissues. The 293T cells transfected with the full-length human SOX6 gene (293T cell-SOX6) were used as a positive control.

cartilage tissues of the trachea and testes of the three mice were intensively examined. In all three mice, tissues including the hippocampus and subventricular zone of the adult brain and testicular tissues showed normal structures and cellularity and there were no pathological changes caused by immune responses, such as lymphocyte infiltration or tissue destruction and repair (Fig. 4*a–e*). Slightly degenerative changes were observed in tracheal cartilage tissue derived from two out of three $SOX6_{628}$-immunized mice as well as all three mock-vaccinated mice (Fig. 4*f*). These results indicate that CTLs raised against $SOX6_{628}$ do not recognize normal self-cells that have expressed SOX6 during embryogenesis at physiological levels. Taken together, these data suggest that the SOX6-peptide-based vaccine may be a safe and effective strategy targeting glioma.

Killing of GSCs by SOX6 peptide-specific CTLs

We evaluated whether CTLs raised against the SOX6 peptide could kill SOX6-positive GSCs. A western blot analysis showed that SOX6 was expressed in the GSC lines established from three human glioma samples (X01GB and X02GB from two GBs, and X03AOA from an anaplastic oligoastrocytoma)[14,16] (Fig. 5*a*). As shown in Figure 5*b*, in ^{51}Cr-release assays, CTL-$SOX6_{628}$ efficiently lysed CIR-A24 cells pulsed with relevant $SOX6_{628}$ peptide, but not CIR-A24 cells pulsed with irrelevant CMV_{pp65} peptide, demonstrating that the CTLs were SOX6 peptide-specific. These CTL-$SOX6_{628}$ also lysed HLA-$A24^+$/$SOX6^+$ X01GB (A2402/3303) cells. Furthermore, the blockade of HLA Class I with a specific monoclonal antibody (W6/32) inhibited the specific lysis of CTL-$SOX6_{628}$ against X01GB cells. These results suggest that the SOX6 peptide is an immunogenic peptide that is naturally processed and expressed with HLA-A24 on GSCs, indicating that the SOX6 vaccine may be effective for the treatment of GSCs.

Discussion

To date, T-cell epitopes derived from several glioma-associated proteins have been shown to elicit T-cell responses against gliomas.[17,28–36] However, some candidates are expressed in various normal tissues carrying the potential risk of autoimmune reactions, such that candidates suitable for specific immunotherapy against glioma are still limited. Therefore, the identification of additional targets for CTLs is required for glioma immunotherapy. In this study, we showed that SOX6-derived peptides can induce CTLs capable of reacting against the epitope in HLA-$A24^+$ or -$A2^+$ donors and H-2^{d+} mice. Furthermore, these CTLs were able to lyse a GSC line derived from human GB in an HLA Class I-restricted and an antigen-dependent manner. These results indicate that these SOX6 peptide epitopes to be potential immunogenic targets for development of glioma immunotherapy.

It is desirable to develop immunotherapy approaches, which allow broad and non-ethnically biased population coverage. In addition, recent studies indicate that the antitumor T-cell repertoire is directed toward multiple CTL epitopes.[37–43] Therefore, we have endeavored to identify HLA-A2 and HLA-A24-restricted/SOX6-derived epitopes. The most important factor for success in peptide-based immunotherapy is the immunogenicity of peptides which induce cytolytic activity against tumor cells in patients. We have demonstrated that it is possible to induce SOX6-reactive CTLs by

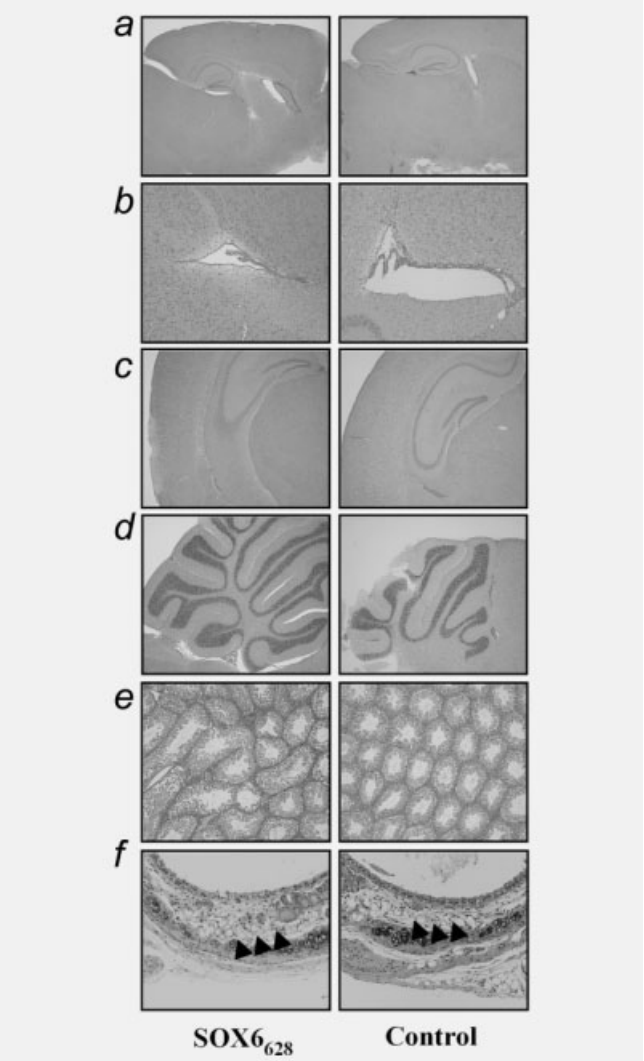

Figure 4. Histological analysis of organs from $SOX6_{628}$-immunized mice (*Left panel*) and IFA-immunized control mice (*Right panel*). Hematoxylin and eosin staining of the cerebral cortex (*a*, original magnification, × 16), subventricular zone of gray matter (*b*, original magnification, × 80), hippocampus (*c*, original magnification, × 32), cerebellum (*d*, original magnification, × 32), testis (*e*, original magnification, × 80), and tracheal cartilage tissue (*f*, original magnification, × 140). Arrow heads in (*f*) indicate slight degeneration of tracheal cartilage tissue.

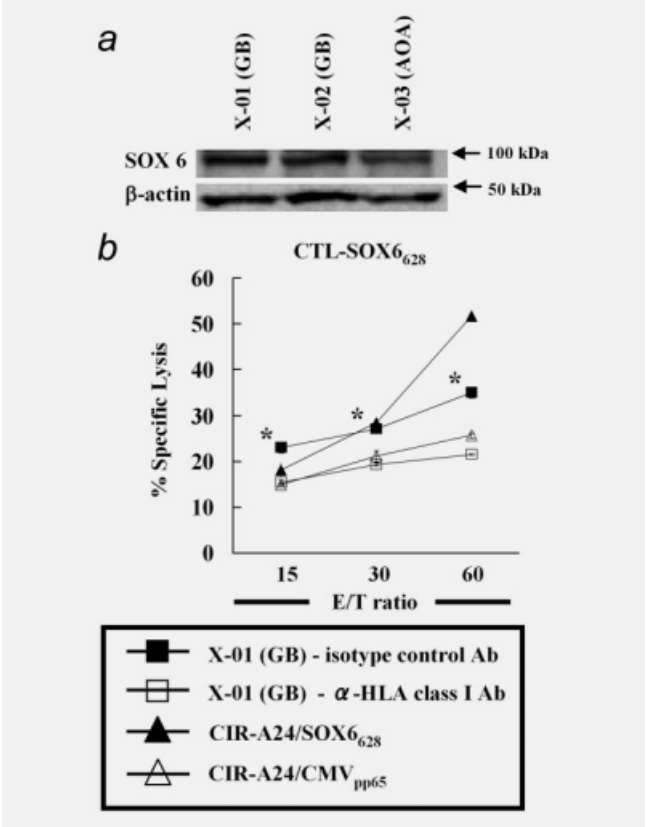

Figure 5. (*a*) SOX6 is expressed in human GSC lines. Western blotting demonstrated SOX6 expression in three human GSC lines, X01GB, X02GB and X03AOA (*Upper*). β-actin was used as an internal control for the amount of protein loading among the lanes (*Lower*). (*b*) CTL primed against $SOX6_{628}$-lysed HLA-$A24^+$/$SOX6^+$ GSCs. CTLs raised against $SOX6_{628}$ were tested for their lytic ability against a human GSC line, XGB01 (solid squares, HLA-$A24^+$, $SOX6^+$) in the presence of isotype IgG, XGB01 cells in the presence of anti-HLA Class I (W6/32) mAb (hollow squares), CIR-A24 cells loaded with $SOX6_{628}$ (solid triangles) or CMV_{pp65} (hollow triangles) using 4-hr ^{51}Cr-release assays. The values indicate the averages of triplicate samples. The bars indicate the SD. *, $p < 0.05$ for the specific lysis of XGB01 cells in the presence of anti-HLA Class I mAb, compared with the specific lysis of XGB01 cells pretreated with isotype IgG antibodies. One of two representative experiments with similar results is shown.

stimulating PBMCs with each HLA-A24-restricted/SOX6-derived epitope *in vitro* in more than 60% of glioma patients and healthy donors or with the HLA-A2-restricted/SOX6-derived epitope in more than 40% of the HLA-$A2^+$ healthy donors (Tables 2–4), although it is necessary to investigate more patients to estimate the probability of a successful induction of SOX6-reactive CTLs in glioma patients. Furthermore, we demonstrated that vaccinations with $SOX6_{628}$, the amino-acid sequence of which is conserved in the murine SOX6 protein, induced CTLs specific for $SOX6_{628}$ in mice. These data suggest the immunogenicity of these peptides to induce cytolytic activity against tumor cells.

Recent studies have indicated that gliomas contain cancer stem cells (CSCs) that may be capable of initiating tumor growth.[44–46] The CSCs are likely responsible for the malignant behavior of tumors because of their acquired resistance to chemotherapy and radiotherapy,[4,5,7,8] thereby contributing to the ineffectiveness of conventional therapies. The CSCs could thus be a novel target for cancer therapy, including vaccines/immunotherapy. Indeed, the strong expression of the HOX

gene cluster, which may be involved in the maintenance of a stem cell-related self-renewal signature, was associated with resistance to combined chemoradiotherapy of concomitant temozolomide (TMZ) and radiotherapy in GBs.[47] Furthermore, although TMZ was reported to preferentially deplete CD133-positive stem cells in primary GBs *in vitro*, this effect was absent in O^6-methylguanine-DNAmethyltransferase (MGMT)-non-methylated tumors, which represent 50–70% of all primary GBs and are associated with a poorer prognosis.[48] The use of TMZ in a randomized prospective clinical trial in patients with GB has thus only resulted in a marginal survival advantage (14.6 *vs.* 12.1 months).[3] These previous studies point to the significance of identifying CTL epitopes that can induce CTL responses against GSCs. In this study, we demonstrated that GSCs derived from human malignant gliomas express SOX6 and are lysed by CTLs primed against a SOX6-derived peptide (Fig. 5). Hence, immune-targeted therapies may eradicate malignant GSCs that are resistant to conventional therapy. Taken together, our studies support the potential use of immunotherapy with HLA-A2 and HLA-A24-restricted/SOX6-derived peptides for the treatment of a large number of glioma patients.

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