Journal of Neuro-Oncology (2006) 76: 23–30
DOI 10.1007/s11060-005-3280-7

Laboratory Investigation

Identification of EGFRvIII-derived CTL epitopes restricted by HLA A0201 for dendritic cell based immunotherapy of gliomas

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Key words: dendritic cell vaccine, EGFRvIII, glioma, HLA A0201, immunotherapy

Summary

The type III variant of the epidermal growth factor receptor (EGFRvIII) mutation is present in 20–25% of patients with glioblastoma multiforme (GBM). EGFRvIII is not expressed in normal tissue and is therefore a suitable candidate antigen for dendritic cell (DC) based immunotherapy of GBM. To identify the antigenic epitope(s) that may serve as targets for EGFRvIII-specific cytotoxic T lymphocytes (CTLs), the peptide sequence of EGFRvIII was screened with two software programs to predict candidate epitopes restricted by the major histocompatibility complex class I subtype HLA-A0201, which is the predominant subtype in most ethnic groups. Three predicted peptides were constructed and loaded to mature human DCs generated from peripheral blood monocytes. Autologous $CD8^+$ T cells were stimulated *in vitro* with the EGFRvIII peptide-pulsed DCs. One of the three peptides was found to induce EGFRvIII-specific CTLs as demonstrated by IFN-γ production and cytotoxicity against HLA-$A0201^+$ EGFRvIII transfected U87 glioma cells. These results suggest that vaccination with EGFRvIII peptide-pulsed DCs or adoptive transfer of *in vitro* elicited EGFRvIII-specific CTLs by EGFRvIII peptide-pulsed DCs are potential approaches to the treatment of glioma patients.

Introduction

Gliomas are the most common primary tumor of the CNS [1]. Despite advances in conventional treatments such as surgical resection, radiation therapy, and chemotherapy, the prognosis for most patients with malignant gliomas is poor. Consequently, there is a need to develop adjuvant therapies for more effective treatment of this type of cancer. In recent years, numerous attempts have been made to develop cancer immunotherapies, and results supporting the potential efficacy of such approaches have been obtained both *in vitro* and *in vivo* [2–7]. The existence of tumor associated-antigens that can be recognized by cytotoxic T lymphocytes (CTLs) forms the conceptual basis for an anti-cancer immunotherapeutic approach that should, in principle, be applicable to the treatment of malignant gliomas, due to their high expression of proteins such as tenascin, gp240 and EGFRvIII [1]. The latter of these, in particular, should be an ideal CTL target because of its tumor specificity.

EGFRvIII is the most prevalent of several EGFR mutatations found in human gliomas, and is expressed in 20–25% of GBM [8]. The expression of EGFRvIII results from intragene deletion rearrangements that eliminate EGFR exons 2–7, and cause the joining of exons 1 and 8 coding sequences. The resulting receptor lacks amino acids 6–273 of the wild-type EGFR sequence, and additionally contains a glycine residue between amino acids 5 and 274 due to the alteration of a codon at the exon 1–8 splice site [9]. While EGFRvIII drives enhanced tumorigenicity through constitutive and unattenuated activation [10], its expression has not been detected in any normal tissue examined [11], thus making this aberrant receptor a suitable target for glioma immunotherapy.

Several immunotherapy approaches based on EGFRvIII have been evaluated. Heimberger et al. [12] demonstrated that immunization of mice with dendritic cells (DCs) mixed with a 14 amino acid peptide, representing an EGFRvIII fusion sequence, resulted in a prolonged survival of mice with EGFRvIII expressing tumors, and a long lasting, humoral immunity. Moscatello et al. [13] and Heimberger et al. [14] immunized mice with a 14 or 13 amino acid EGFRvIII fusion sequence, respectively, and showed that this peptide immunization protocol prevented tumor formation, enhanced tumor regression, and eliminated tumor cells by antigen-specific CTLs. The involvement of CTLs suggests that 13 and 14 amino acid

EGFRvIII sequences were processed to produce a 9–10 amino acid sequence for presentation in association with MHC class I cell surface molecules. The critical MHC class I binding amino acid sequence(s) from the EGFRvIII mutation, however, have yet to be delineated.

DCs are desirable antigen presenting cells (APCs) because they are capable of activating naïve T cells and initiating primary immune responses. At an early stage of their development, immature DCs in the periphery are specialized for antigen (Ag) capture by endocytosis or macropinocytosis. After being triggered by proinflammatory stimuli such as TNF-α and LPS, mature DCs lose these capabilities, but show significant enhancement for stimulating naïve CD4 and CD8 T cells [15]. With the development of *in vitro* culture systems for the generation of large numbers of human DCs from either $CD34^+$ hematopoietic progenitor cells or peripheral blood monocytes [16–18], the use of DCs activated by exposure to specific tumor antigens has become a promising approach in cancer immunotherapy. DCs have been pulsed/activated with tumor lysates, recombinant protein, or peptides, with the latter being most widely investigated. Studies show that peptide-pulsed DC immunization can generate tumor antigen-specific cytolytic T cell responses against several kinds of human cancers [12, 19–23]. As a result of these and other studies, the identification of tumor-specific antigens has become a major interest in the development of cancer immunotherapies. Most of the tumor-associated peptide epitopes for CTLs that have been identified to date are related to melanoma-specific antigens such as tyrosinase, tyrosinase-related protein 1 and 2, gp100, MAGE-1 and MAGE-3 [22, 24–27]. No glioma specific MHC class I epitopes for CTLs have yet been characterized. Here, by using two software programs, we predicted three candidate EGFRvIII-derived peptides as potential CTL epitopes restricted by MHC class I subtype HLA A0201, and tested their capacity to elicit tumor-specific CTLs; one of these proved to be highly immunogenic.

Material and methods

Cell culture

U87 glioma cells are HLA-A0201 positive. The expression of HLA-A0201 molecules on U87 cells was determined by flow cytometry and PCR-SSP methods in our lab as previously described [28]. The U87 cells were infected with EGFRvIII gene-containing recombinant retroviruses [10], populations of drug-resistant and EGFRvIII-expressing cells were obtained by FACS and grown in DMEM (10% FBS) containing G418 (400 μg/ml). T2 cells were purchased from ATCC and cultured in our lab according to instructions provided by the vendor.

Peptides

Using two computer algorithms, SYFPEITHI and BIMAS, three peptides with an amino acid sequence motif appropriate for binding to HLA-A0201 were predicted (Table 1) in the 14 amino acid sequence (LEEKKGNYVVTDHC) corresponding to the junction area of the EGFRvIII mutation. The peptides were synthesed in the microchemical facility at the University of Minnesota. Each peptide was purified by HPLC and the purity was greater than 95%. The identity of each peptide was confirmed by mass spectrometry. Lyophilized peptides were diluted in ddH_2O to 100 mg/ml and stored in −20 °C.

Table 1. Peptide sequence predicted by SYFPEITHI and BIMAS

Peptide	Amino acid sequence of peptide	Score	
		SYFPEITHI	BIMAS
Peptide 1	LEEKKGNYV	13	0.809
Peptide 2	LEEKKGNYVV	12	0.060
Peptide 3	KKGNYVVTD	10	0.000

Peptide binding assay

The human TAP-defect $HLA\text{-}A0201^+$ T2 cell line was used as a measure of the binding affinity of EGFRvIII peptides to HLA-A0201 molecules in a peptide-binding assay described previously [29]. Briefly, T2 cells resuspended in serum free medium (5×10^5 cells/ml/well of a 24-well plate) were incubated in medium alone or with individual peptides at a concentration of 25 μg/ml plus 3 μg/ml β_2-microglobulin for 18 h at 37 °C. The HLA-A0201 strong binding HIV peptide ILKEPVHGV and the HLA-A0201 poor binding BCR-ABL peptide GFKQSSKAL were used as positive and negative controls in the peptide binding assays. T2 cells with or without peptide pulsing were then harvested, washed twice with FACS buffer, and indirectly stained with an anti-HLA-A0201 monoclonal antibody (BB7.2) followed by FITC-conjugated goat anti-mouse IgG antibodies. Fluorescence intensity and positive cell percentages were measured on a FACS Caliber flow cytometer and data were analyzed using CellQuest software (Becton Dickinson). Peptide binding was calculated as fold increases by comparing the cell surface expression of HLA-A0201 (MFI: mean fluorescence intensity) on peptide-pulsed T2 cells versus that (MFI) of T2 cells in medium alone.

Generation of monocyte-derived dendritic cells

Human DCs were generated from monocytes isolated from PBMC of healthy HLA A0201 donors. Briefly, PBMC were resuspended in X-VIVO-15 serum free medium at 5×10^6 cells/ml, plated in T75 tissue culture flasks in a final volume of 30 ml per flask, and incubated for 2–3 h at 37 °C in a 5% CO_2 humidified incubator. Non-adherent cells were subsequently removed by washing with warmed HBSS. The plastic adherence-purified monocytes were then cultured in X-VIVO-15 medium supplemented with recombinant human GM-CSF (200 ng/ml, Immunex) and IL-4 (30 ng/ml, R&D systems) at 30 ml per flask. On day 5, half of the

medium was removed and replenished with fresh X-VIVO-15 medium containing GM-CSF and IL-4. On day 7, rhTNF-α (10 ng/ml, R&D systems) and PGE2 (10 ng/ml, Cal Biochem) were added for the maturation of DCs. Mature monocyte-derived DCs were harvested on day 9 and used as APCs.

Phenotypic assessment of DCs

At day 7 or after 9 days of maturation, DCs were harvested and stained with PE-conjugated antibodies directed against CD40, CD80, CD83, CD86, HLA-ABC, and HLA-DR antigens (BD Pharmingen). Species and isotype matched monoclonal antibodies were used as controls. Antibodies were diluted 1:6 in FCM buffer ($PBS + 1\%BSA + 0.1\%NaN_3$), 10^5 DCs were resuspended in 60 µl of each of the diluted antibodies and kept on ice for 45 min. Cells were washed twice with PBS containing 0.1% NaN_3, resuspended in 250 µl FCM buffer and analyzed using a Becton–Dickinson FACScan. Ten thousand cells were analyzed after gating on forward and side scatter by a flow cytometer. Data analysis was performed using Cell Quest software.

In vitro generation of EGFRvIII peptide-specific CTLs

CTLs and DCs were obtained from peripheral blood mononuclear cells from the same subjects. EGFRvIII peptide-specific CTLs were generated *in vitro* according to the method described by Lu et al. [30] with slight modification. Briefly, mature DCs were pulsed with 40 µg/ml of individual EGFRvIII peptides in X-VIVO15 media for 4 h at 37 °C. The peptide-pulsed DCs were washed twice in HBSS and irradiated (3500 rad) in a cesium irradiator, then cultured with autologous purified $CD8^+$ T cells at 1:20 ratio in 48 well plates. Each well contained 0.25×10^5 DCs and 5×10^5 $CD8^+$ T cells in 0.5 ml of X-VIVO15 medium. The T cells were individually re-stimulated with autologous DCs pulsed with the priming peptide every 9 days. Starting on day 12, the T cell cultures were fed with fresh X-VIVO15 medium containing 50 U/ml of rh-IL-2 every 3 days. The Elispot assay and CTL assay were performed 7 days after three rounds of *in vitro* stimulation with peptide-pulsed DCs.

Elispot assay

A human IFN-γ Elispot kit was purchased from Cell Sciences Inc. and the Elispot assay was performed according to the manufacture's instruction. Briefly, PVDF-bottomed 96–well plates were coated with capture antibody. In each well of the plates, 5×10^3 *in vitro* stimulated CD8 T cells were mixed with 7.5×10^4 stimulator cells (T2 cells only or peptide-pulsed T2 cells). Cells were incubated at 37 °C in CO_2 for 17 h. After 3 washes with PBS –0.1% tween 20, plates were incubated with detection antibody for 1 h and 30 min at 37 °C, and then incubated with streptavidin alkaline phosphatase for 1 h at 37 °C. After staining with BCIP/NBT, blue spots corresponding to single cytokine secreting T cells appeared. Spots were counted using a stereomicroscope at 40× magnification. Each sample was tested in triplicate and the number of peptide-specific CD8 T cells was calculated as the difference between the number of IFN-γ secreting cells and the negative controls (T2 cells only).

CTL assay

The CTL assay used in this study was described in detail by Piriou et al. [31]. Briefly, EGFRvIII transfected U87 cells (target) were labeled with the green fluorescent membrane dye DIOC18 and adjusted to a concentration of 10^5/ml, and the *in vitro* stimulated $CD8^+$ T cells (effectors) were adjusted to a concentration of 5×10^6/ml. Fifty microliters of target cells were mixed with effector cells at E/T ratios of 3:1, 11:1, 33:1 or 100:1. Cells were then incubated in PI, a red dye that penetrates through the membrane of dying cells. Following PI addition (10 µg/ml), the mixture was centrifuged for 2 min and further incubated as a cell pellet for 4 h at 37 °C in CO_2 in culture medium (RPMI-1640 supplemented with 10% HSA). Spontaneous cell death was determined by incubation at 4 °C for target and effector cells. For maximum lysis, target and effector cells were incubated at 37 °C with 20 µl of saponin at 0.3 mg/ml. For the antibody blocking experiments, effector cells were incubated with 10 µg/ml of anti HLA-A2 monoclonal antibody for 30 min before the assay. Data acquisition and analysis were performed by flow cytometry and the % cytotoxicity was determined by quantifying the number of dying target cells (red and green labeled cells) relative to the total number of target cells (green cells).

Results

Identification of HLA-A0201 restricted EGFRvIII peptides

To identify EGFRvIII-derived, HLA-A0201 restricted CTL epitopes, peptides within the 14 amino acid sequence LEEKKGNYVVTDHC that corresponds to the junction region of the EGFRvIII mutation was screened by two computer algorithms, BIMAS and SYFPEITHI. BIMAS is based on a predicted dissociation time between the predicted peptide and HLA class I molecules. SYFPEITHI is based on a predicted binding strength between a specific peptide and relative MHC molecules. Three peptides (Table 1) were synthesized based on the results of screening with these programs. To evaluate whether the constructed EGFRvIII peptides in fact could bind to HLA-A0201 molecule, a T2 cell-based peptide binding assay was performed. Our results (Figure 1) showed that all three peptides actually bind to HLA-A0201 moderately well, with peptide 1 showing the highest affinity. However, even the addition of a single residue can dramatically affect peptide binding as seen in comparing peptides 1 and 2. In addition to their HLA-A0201 binding, the peptides increased T2 intensity of HLA A0201 fluorescence by stabilizing the surface

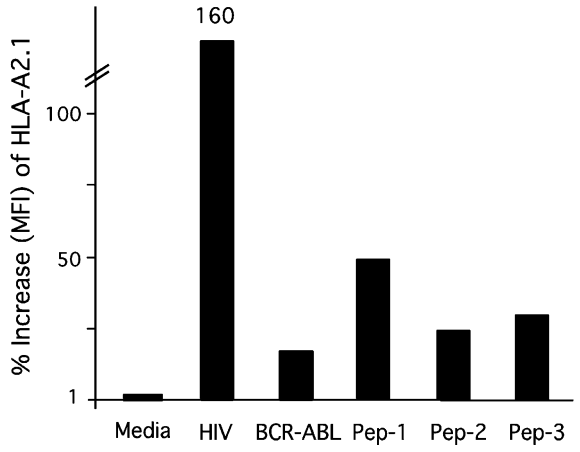

Figure 1. Binding of EGFRvIII peptides to HLA-A0201 molecules on T2 cells. Three EGFRvIII peptides and control peptides (HIV and BCR-ABL) were individually measured for their binding affinity to HLA-A0201 molecules on T2 cells as described in Materials and methods. T2 cells with or without peptide pulsing were incubated for 18 h at 37 °C and subsequently stained for cell surface expression of HLA-A0201. Percent (%) increase of cell surface HLA-A0201 molecules were calculated by dividing the MFI on peptide-pulsed T2 cells versus the MFI of T2 cells in medium alone.

expression of this molecule, and resulted in individual increases between 23 and 48%.

In vitro induction of EGFRvIII peptide-specific CTLs

To determine if the synthetic EGFRvIII peptides could elicit specific CTL responses, HLA-A0201$^+$ monocyte-derived DCs generated from cytokine-driven culture system were pulsed with each one of the three EGFRvIII peptides and used to prime autologous T cells. Our results showed that these monocyte-derived DCs expressed high levels of CD40, CD83, CD86, HLA-ABC, and HLA-DR antigens, which correspond to the typical surface phenotype of mature DCs at day 9 compared to the immature DC phenotype at day 7 (Figure 2). The three EGFRvIII peptides were pulsed individually to DCs under identical conditions and were used to prime autologous CD8$^+$ T cells by *in vitro* stimulation every 9 days. After the third *in vitro* stimulation, functional assays were performed to evaluate the EGFRvIII peptide reactivity of CD8$^+$ T cells stimulated with the peptide-pulsed DCs.

IFN-γ Elispot is a reliable and highly sensitive assay for measuring antigen-reactive T cell frequencies. It allows the quantification of IFN-γ producing CTLs in response to antigen contact. In this experiment, T2 cells with or without pulsing a priming peptide were used as stimulators to the peptide primed CD8$^+$ T cells. As shown in Figure 3, EGFRvIII peptide 1 primed T cells produced the highest number of IFN-γ spots when stimulated with T2 pulsed with peptide 1, which was 10-fold higher than the number of IFN-γ spots produced by the peptide 1 primed T cells stimulated with T2 without peptide. This result indicates that peptide 1 can serve as an EGFRvIII-derived CTL epitope and effectively induce EGFRvIII peptide 1 reactive CTLs. In contrast, EGFRvIII peptide 2 or peptide 3 primed T cells produced low numbers of IFN-γ spots when stimulated with T2 pulsed with the priming peptide, indicating that EGFRvIII peptides 2 and 3 are not as immunogenic as peptide 1.

EGFRvIII peptide-specific CTLs can effectively kill EGFRvIII transfected glioma cells

The appropriateness of any tumor specific protein to serve as a T cell target not only depends upon whether the protein contains an appropriate peptide epitope for class I or class II MHC binding, but also requires that the resultant peptide/MHC molecule complex is present at the cell surface of the target cell or APC in a concentration high enough to stimulate the specific T cell receptor. To determine whether endogenously expressed EGFRvIII protein could be processed and presented by MHC class I to enable CTL recognition of the mutated peptide segment of the protein, HLA-A0201 positive U87 glioma cells expressing EGFRvIII were used as target cells in CTL assays. As shown in Figure 4, EGFRvIII peptide 1 elicited CTLs effectively lysed the EGFRvIII transfected U87 cells in a dose-dependent manner. In contrast, T cells stimulated with DCs pulsed with EGFRvIII peptide 2 or peptide 3 failed to lyse the EGFRvIII-expressing U87 cells. The killing of EGFRvIII-expressing glioma cells by the peptide 1 elicited CTLs reconfirmed that peptide 1 is an EGFRvIII-derived CTL epitope and demonstrated that this peptide segment can be efficiently processed and presented in the context of the HLA A0201 allele. To further demonstrate that the recognition of the EGFRvIII peptide 1 by CTLs is HLA-A2 restricted, blocking experiments using a monoclonal anti-HLA-A2 antibody were performed. As shown in Figure 5, the killing of EGFRvIII-expressing U87 glioma cells by EGFRvIII peptide 1 specific CTL was significantly inhibited by the anti-HLA-A2 blocking antibody. This indicates that EGFRvIII peptide 1 is presented on HLA-A2 molecules and the killing of EGFRvIII-expressing glioma cells by the peptide-specific CTLs is HLA-A0201 restricted.

Discussion

Despite intensive efforts to develop new treatments over the past two decades, the survival rates of patients with GBM have not significantly improved. DC-based immunotherapy offers a promising approach to the treatment of brain tumors, and for improving the outcome of glioma patients. Successful DC-based brain tumor immunotherapy has been reported in animal models and patients by others [3, 6, 12], and such results are consistent with our own experience involving the demonstration of significantly increased survival time of animals receiving glioma implantation following injection of glioma extract-primed DC2.4 dendritic cells [3]. Liau et al. [32] reported that vaccination of rats harboring intracranial 9L gliosarcomas with syngeneic DC, pulsed with 9L derived protein, led to increased CD8$^+$ T cell tumor infiltration and induction of 9L specific CTL response that prolonged rat survival. In human glioma patients, Yu et al. [6], Kikuchi et al. [33], and Yamanaka

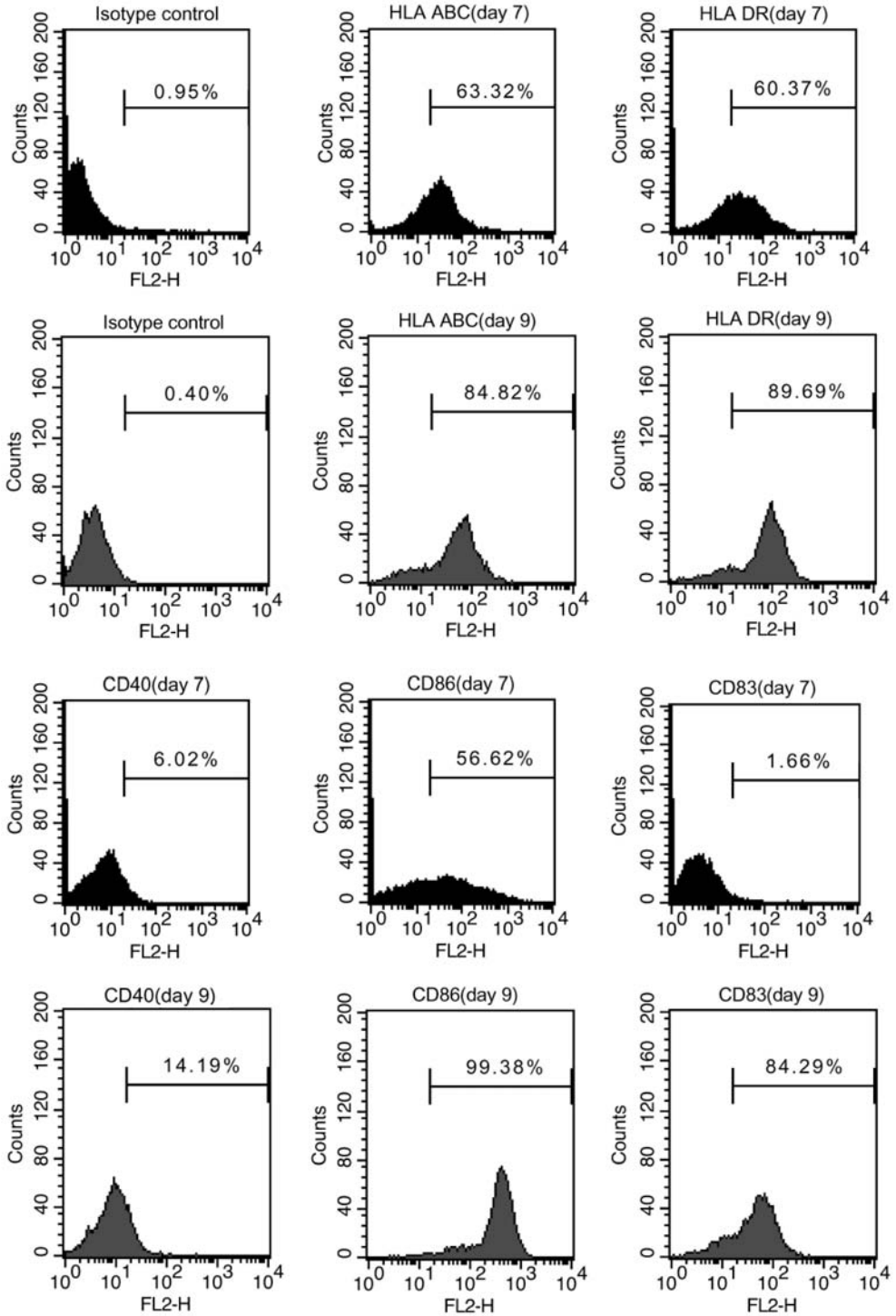

Figure 2. Phenotypic analysis of mature human monocyte-derived DCs. Cells were stained with the PE-conjugated mAbs as described in Materials and methods. The DCs derived from a 9-day cytokine driven culture express the DC maturation marker CD83 and up-regulate the expression of CD40, CD86, MHC class I (HLA ABC) and MHC class II (HLA DR).

et al. [34] found that DC vaccination appears to be safe and not associated with autoimmunity.

DC-based immunotherapy can be approached in one of two ways: direct immunization with antigen (tumor-associated proteins, peptides, or tumor lysates) pulsed DCs, or adoptive transfer of *in vitro* expanded CTLs following stimulations with antigen-pulsed DCs. Since a broad range of immunological defects have been documented for glioma patients, including decreased T cell numbers, impaired T cell responsiveness, and defective signaling after T cell receptor TCR/CD3 stimulation [35], the adoptive transfer of *in vitro* activated and expanded tumor-reactive CTLs would seem the more appropriate choice for treating glioma patients. In the past decade, the development of *in vitro* DC culture systems have allowed the generation of large numbers of human DCs from either $CD34^+$ hematopoietic progenitor cells or peripheral blood monocytes [16–18]. A major challenge for the *in vitro* induction of glioma reactive CTLs for adoptive immunotherapy is

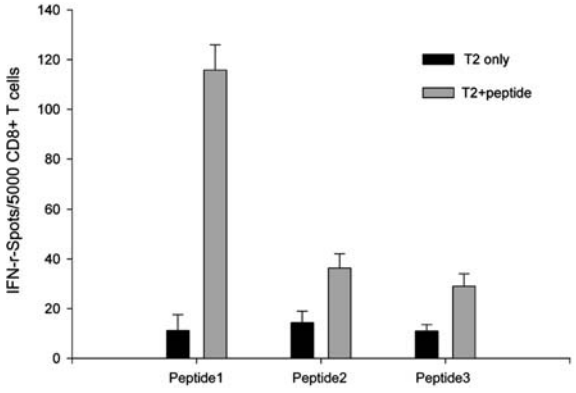

Figure 3. Analysis of $CD8^+$ activation by EGFRvIII-primed DCs. $CD8^+$ T cells obtained from HLA-A0201$^+$ PBMC were stimulated by autologous DCs pulsed with or without individual EGFRvIII peptides. IFN-γ Elispot assays were performed on day 7 after the third *in vitro* stimulation, and the number of IFN-γ producing T cells was determined. Each bar represents the mean of triplicates $\pm$SD.

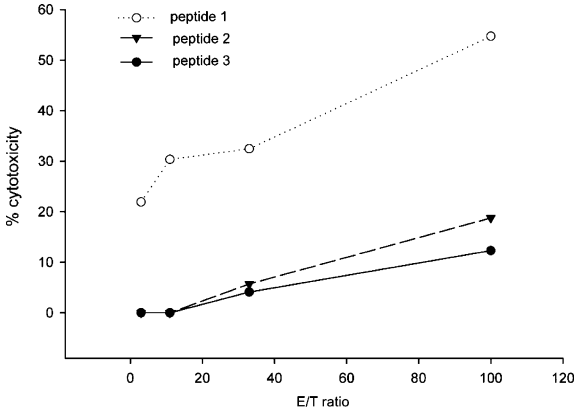

Figure 4. CTL assay of $CD8^+$ T cells activated by EGFRvIII peptide-pulsed DCs. $CD8^+$ T effector cells were stimulated every 9 days as described in Materials and methods. Cytotoxic activity against HLA-A0201$^+$ EGFRvIII-expressing GBM cell line U87 was measured on day 7 after the third *in vitro* stimulation.

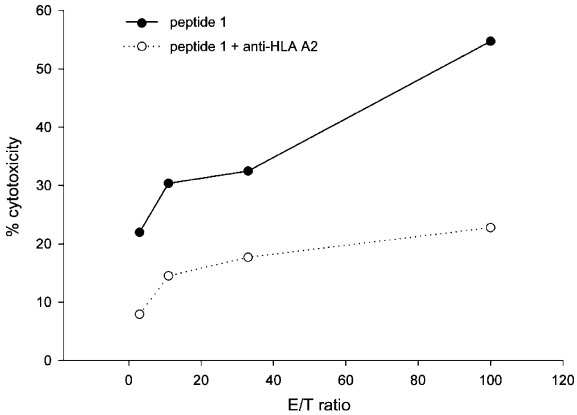

Figure 5. Inhibition of the cytotoxic activity of EGFRvIII-specific CTL by monoclonal antibody specific for MHC class I molecules (w6/32). The target cells were EGFRvIII expressing GBM cell line U87, and the effector cells were activated $CD8^+$ T cells stimulated *in vitro* by peptide 1 pulsed DCs. CTL assay was performed on day 7 after the third *in vitro* stimulation.

the identification of MHC class I restricted CTL epitopes derived from glioma associated antigens.

Malignant transformation in human tumors results from the activation of oncogenes by several mechanisms including gene amplification, somatic DNA mutation, and gene translocation. Each mechanism can cause the expression of proteins having the potential to serve as tumor antigens and thus as targets for T cell therapy [36]. DNA mutation or gene translocation of cancer related genes can result in the expression of altered proteins containing amino acid residue different than normal. The proteins expressed by such mutated oncogenes are in essence cancer specific proteins and thus potential cancer specific antigens to elicit specific T cell responses [37–40]. Moreover, the elevated expression of oncogeneic proteins can result in MHC presentation of high concentrations of pro-immunogenic peptides [28]. EGFRvIII is a sequence variant of EGF receptor that has been shown to be only expressed in tumor cells [41]. This spontaneous alteration is present in 20–25% of human GBMs. The novel amino acid sequence at the fusion junction of EGFRvIII is thought to serve as a potential tumor-specific target for immune-based therapy. In this study, we have demonstrated for the first time that human DCs pulsed with a 9-mer peptide, containing the amino acid fusion site of EGFRvIII protein, effectively induced autologous EGFRvIII peptide-specific CTLs *in vitro*, and that these CTLs effectively kill EGFRvIII-expressing human glioma cells. Our results also demonstrate that this 9-mer EGFRvIII peptide is presented on HLA-A2 molecules, and that the killing of EGFRvIII-expressing glioma cells by peptide-specific CTLs is HLA-A2 restricted. Our new findings presented here extend the previous work of Moscatello et al. [13] by identifying a specific 9-mer EGFRvIII fusion peptide responsible for stimulating autologous CTL anti-tumor response. Our future investigations will focus on the potential use of EGFRvIII peptide 1 in DC-based immunotherapy in rodent models of EGFRvIII expressing brain tumors.

Acknowledgements

This work was supported in part from the Lyle French Fund (to W.C.L.), the Viking Children's Fund (to W.C.), the National Cancer Institute (to W.K.C.) and the National Foundation for Cancer Research (to W.K.C). We thank Kirstin Olmon for administrative assistance and David James for a critical reading of this manuscript.

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