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Therapeutic Effect of a T Helper Cell Supported CTL Response Induced by a Survivin Peptide Vaccine against Murine Cerebral Glioma

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Abstract

Survivin is a tumor-associated antigen (TAA) that has significant potential for use as a cancer vaccine target. To identify survivin epitopes that might serve as targets for CTL-mediated, anti-tumor responses, we evaluated a series of survivin peptides with predicted binding to mouse H2-K^b and human HLA-A201 antigens in peptide-loaded dendritic cell (DC) vaccines. H2-K^b-positive, C57BL/6 mice were vaccinated using syngeneic, peptide-loaded DC2.4 cells. Splenocytes from vaccinated mice were screened by flow cytometry for binding of dimeric H2-K^b:Ig to peptide-specific CD8⁺ T cells. Two survivin peptides (SVN₅₇₋₆₄ and SVN₈₂₋₈₉) generated specific CD8⁺ T cells. We chose to focus on the SVN₅₇₋₆₄ peptide because that region of the molecule is 100% homologous to human survivin. A larger peptide (SVN₅₃₋₆₇), containing multiple class I epitopes, and a potential class II ligand, was able to elicit both CD8⁺ CTL and CD4⁺ T cell help. We tested the SVN₅₃₋₆₇ 15-mer peptide in a therapeutic model using a peptide-loaded DC vaccine in C57BL/6 mice with survivin-expressing GL261 cerebral gliomas. This vaccine produced significant CTL responses and helper T cell-associated cytokine production, resulting in a significant prolongation of survival. The SVN₅₃₋₆₇ vaccine was significantly more effective than the SVN₅₇₋₆₄ core epitope as a cancer vaccine, emphasizing the potential benefit of incorporating multiple class I epitopes and associated cytokine support within a single peptide.

Keywords

dendritic cell; glioma; peptide; survivin; tumor antigen; vaccine

Introduction

Even with intensive treatment, malignant gliomas frequently recur as a result of continued growth of residual microscopic disease located beyond surgical resection margins [1,2]. Surgery, radiation therapy and chemotherapy provide definite therapeutic benefit, but long-term survival is uncommon [3]. This situation has stimulated interest in exploring the usefulness of immunotherapy for patients with malignant gliomas.

Survivin is one of the most specific cancer antigens identified to date because it is expressed in a large percentage of tumors and is rarely detectable in normal tissue [4,5]. Survivin expression occurs commonly in malignant gliomas [6-8] where its expression is associated

with a poor prognosis [7,9]. Also, because survivin is expressed by many different cancer types, its use as a tumor vaccine target may have broad implications for cancer vaccine therapy.

Epitopes of intracellular proteins, including those of survivin, appear on the surface of tumor cells in association with MHC I molecules. CD8+ CTLs are activated by tumor-derived peptides coupled to MHC class I antigens. The tumor antigen-MHC class I peptide complex binds to the antigen-specific T cell receptor on CD8+ cells and stimulates T cell activation. Thus, certain survivin epitopes that are correctly presented by tumor cells and are recognized by specific T cells may elicit potent antitumor responses [10]. Therefore, identification of survivin peptides that can bind to MHC class I molecules and elicit strong cellular immune responses is an important goal of anti-survivin cancer immunotherapy.

The generation of an effective and specific cell-mediated immune response may require the activation of both highly specific CD8+ lymphocyte subsets and CD4+ helper T cells. To activate a CD4+ T-cell response, antigens must be presented to CD4+ T cells in conjunction with an MHC class II antigen. Once CD4+ cells have been activated, they proliferate and produce cytokines (e.g. IFN- γ , IL-2, and IL-4) that enhance the immune response. These cytokines are essential to provide a fully activated CD8+ antitumor CTL response.

The current study is based on our preliminary observation that a DNA vaccine against survivin inhibits the growth of malignant glioma in rats and mice [11,12]. We demonstrated that a CTL response could be generated using the entire survivin molecule, as well as with specific domains of survivin, and that the responses generated from these could have a beneficial effect against cerebral gliomas [12]. In our current study, we set out to design a survivin peptide based upon a study of the immunogenicity of smaller peptides with predicted binding to both human and mouse MHC class I molecules. The purpose of this approach was to develop a survivin peptide that would be suitable for clinical application.

Here we hypothesized that a single peptide with multiple epitopes could be capable of eliciting an effective immune response to survivin epitopes presented on tumor cells. We also speculated that incorporation of CD4+ helper cell support of that immune response would promote a more robust antitumor response. We studied a peptide vaccine strategy based upon survivin amino acid sequences that are capable of stimulating CD8+ specific T cells against several different epitopes of survivin. In addition, we investigated a strategy using a larger survivin peptide containing a potential MHC class II ligand that is present in the survivin molecule to stimulate CD4+ cytokine-secreting helper T cells.

Materials and Methods

Cell lines and culture conditions

GL261 murine glioma cells were grown on 100-mm tissue culture plates in complete Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 5,000 units penicillin/streptomycin, 50 μ M 2-mercaptoethanol, 25mM HEPES, and 1 $\times$ non-essential amino acids at 37°C in 5% CO₂ with media changes two to three times per week. The DC2.4 dendritic cell line was provided by Dr. Ken Rock (University of Massachusetts Medical School), and RMA-S cells were provided by Dr. Ron Germain (NIH). DC2.4 and RMA-S were cultured at a density of 2 $\times$ 10⁶ cells/ml in RPMI containing 10% fetal calf serum, 5,000 units penicillin/streptomycin, 50 μ M 2-mercaptoethanol, 25mM HEPES, 1 $\times$ non-essential amino acids and 20ng/ml GM-CSF (PeproTech, Rocky Hill, NJ).

Peptides

Peptide synthesis was performed using Fmoc chemistry and a solid support resin (Sigma-Genosys, The Woodlands, TX). Each peptide was stored at -20°C until use and initially diluted

in DMSO. The following peptide sequences were synthesized SVN₉₋₁₆ AWQPFLKD, SVN₁₈₋₂₅ RISTFKNW, SVN₃₉₋₄₆ AEAGFIHC, SVN₅₃₋₆₇ DLAQCFFCFKKELEGW, SVN₅₅₋₆₄ AQCFFCFKEL, SVN₅₆₋₆₄ QCFFCFKEL, SVN₅₇₋₆₄ CFFCFKEL, SVN₈₂₋₈₉ SGCAFLSV, SVN₉₇₋₁₀₄ TLGEFLKL. A chicken ovalbumin (OVA₂₅₈₋₂₆₅) peptide sequence SIINFELK was used as a control.

Peptide Binding

Computer predictions of peptide binding were determined utilizing the “SYFPEITHI” program [13]. The prediction is based on published motifs (pool sequencing, natural ligands) and takes into consideration the amino acids in the anchor and auxiliary anchor positions, as well as other frequent amino acids. Peptides were chosen for studies based upon strong binding predictions to both H2-K^b and HLA-A*0201. Direct binding of survivin peptides to H2-K^b molecules was quantitated by flow cytometry analysis of RMA-S cells [14]. RMA-S cells (deficient for expression of peptide loaded surface MHC class I) were cultured at 27°C for 24 hrs, after which 6×10^5 /50µl of PBS supplemented with 20% FCS were incubated with 50µl of PBS containing 100µM peptide 27°C for 1 hr and transferred to 37°C for 3 hrs. After washing with PBS, cells were incubated with anti-H2-K^b mAb (AF6-88.5) (Biolegend) followed by goat anti-mouse PE conjugated (Fab')₂ (DAKO). The upregulation of H2-K^b molecules after binding with specific peptides was shown as mean fluorescence representative of increased binding characteristics of SVN peptides. OVA₂₅₈₋₂₆₅ peptide was used as a positive control for H2-K^b binding. A random peptide (RND) without any known anchor motifs was used as a negative binding control.

Immunization of mice with peptide-loaded DCs

DC2.4 cells were maintained via *in vitro* cell culture. Prior to vaccination 1×10^7 cells were pulsed with 100 µg of peptide at 37°C for 2 hours. Cells were washed and re-suspended in PBS. Four days after tumor implantation, 1×10^6 peptide-loaded DCs were injected subcutaneously into C57BL/6 mice. This inoculation was repeated as a booster vaccination 7 and 14 days later.

Splenocyte and T cell isolation

C57BL/6 mice were vaccinated with survivin peptide-loaded DC2.4 cells at weekly intervals for a total of three immunizations. Splenic tissue of vaccinated mice was harvested at endpoint sacrifice. Cells were teased from mouse spleens with sterile forceps and passed through a 70µm filter. (BD Falcon). Splenocytes were cultured in DMEM containing 10% fetal bovine serum, IL-2 (10 units/ml) and IL-7 (1 ng/ml). Medium was replenished on day 2. To isolate T cells, splenocytes were centrifuged for 5 min and the pellet was re-suspended in RBC lysis buffer (R&D Systems). Cells were washed twice and re-suspended in complete RPMI culture medium. Re-stimulation of splenocytes from immunized mice was performed *in vitro* for 5 days in media containing 10 µg/ml peptide.

Dimers

Survivin specific CD8⁺ T cells were detected utilizing BD DimerX Soluble Dimeric Mouse H2-K^b:Ig Fusion Protein (BD Biosciences Pharmingen). H2-K^b:Ig (0.5 µg) were passively loaded with survivin specific peptides at 160 molar excess overnight at 37°C. Peptide loaded H2-K^b:Ig complexes were prepared as a staining cocktail with anti-mouse PE conjugated (Fab')₂ (DAKO). Splenocytes were resuspended in FACS staining buffer (PBS, 1% FCS, 0.1% Sodium Azide) at 1×10^6 cells and treated with 20 µg rat anti-mouse CD16/CD32 FcγIII/II Receptor block (BD Biosciences Pharmingen). Cells were doubly stained for CD8 using a FITC-conjugated rat anti-mouse CD8α mAb (GK1.2) (Biolegend). Flow cytometry with dimers was performed with 3×10^5 cells per sample. Cells were washed in FACS wash buffer

containing 0.2% FBS and 0.5% sodium azide. Cells were incubated in antibodies and complexes for 1 hr at 4°C, washed and prepared for FACS analysis. Final data was analyzed using FCS Express software. Analysis is based upon gating on CD8+ T cells only.

Tumor cell lysis by CTL

Cellular assays for specific T cell lysis of target were performed using the Live/Dead cell mediated cytotoxicity kit (Molecular Probes-Invitrogen). GL261 cells were trypsinized and suspended at 1×10^6 cells/ml followed by addition of 10 μ l DiOC₁₈ label (green fluorescence) for 20 min at 37°C. After labeling, cells were centrifuged, washed in PBS twice, and resuspended at 1×10^6 cells/ml of DMEM. Next, 1×10^4 GL261 DiOC₁₈-labeled target cells were added to individual culture tubes. Splenocytes from vaccinated animals were added to the target cells in ratios ranging from 1:25 to 1:100 for 2 hours at 37°C. Conditions were also set up to assay background levels, spontaneous membrane integrity loss and maximal lysis. Propidium Iodide was added to each tube to label cells with permeated cell membranes red. Cells were pelleted and used for FACS analysis. Percentage cytotoxicity was calculated using the formula: (experimental PI-DiOC₁₈ cells – spontaneous)/(maximum PI-DiOC₁₈ - spontaneous PI-DiOC₁₈) $\times$ 100. Maximum release was determined by ethanol treatment of DiOC₁₈ labeled target cells. Spontaneous release was measured by incubating PI-DiOC₁₈ cells in the absence of effector cells. Final data was analyzed using FCS Express software. Analysis is based upon gating on GL261 cells to eliminate any background from effector cells.

Intracellular Cytokine Staining

Splenocytes from immunized mice were stimulated *in vitro* via survivin peptides. Cells were harvested and surface stained with anti-mouse CD4 PE/Cy5 (GK1.5) (Biolegend) for CD4 surface antigen, then fixed with paraformaldehyde to stabilize the cell membrane and permeabilized with saponin. Anti-mouse IL-2 FITC (JES6-5H4), anti-mouse IFN γ FITC (XMG1.2), anti-mouse IL-4 PE (11B11) (eBiosciences) were used to stain for intracellular cytokines at pre-titrated concentrations. Commercially prepared mouse *in vitro* activated splenocytes known to contain IL-2, IL-3, IL-4, IL-10, GM-CSF, IFN- γ expressing cells (eBiosciences) were used as positive control cells. Briefly, cells were incubate in the dark at room temperature for 20 minutes with mAb combinations, washed in permeabilization buffer, resuspended in staining buffer without saponin and prepared for FACS analysis. Final data was analyzed using FCS Express software. Analysis is based upon gating on CD4+ T cells only.

Intracerebral GL261 tumor cell injection and survival analysis

Male C57BL/6 mice were anesthetized with an intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) and fixed in a stereotactic head frame (David Kopf Instruments, Tujunga, CA). A midline scalp incision was made and the bregma was identified. Stereotactic coordinates were measured (2.0 mm lateral, and 1.2 mm anterior to the bregma) for implantation of cells into the deep frontal white matter. A burr hole was drilled at this point and 1×10^5 GL261 cells suspended in 2.5 μ l of DMEM were injected through a Hamilton syringe with a fixed, 25-gauge needle at a depth of 3.0 mm relative to the dura mater. Injections were performed at 1 μ l/min. The needle was withdrawn and the incision sutured. Kaplan-Meier survival plots were drawn and median survival times were determined for all groups. Survival differences were assessed for significance using the logrank Mantel-Cox method.

Results

H2-K^b binding of survivin epitopes

Table 1 shows predicted binding for each of the survivin peptides using SYFPEITHI analysis. Upregulation of H2-K^b molecules on the surface of murine RMA-S cells (deficient for expression of peptide loaded surface MHC class I) was used to determine actual peptide binding *in vitro*. Mean fluorescence represent increased binding characteristics of SVN peptides. Data shows the binding of 100 μ M peptide at 37°C. The negative control peptide (random) does not contain anchor binding residues. The positive control (OVA₂₅₈₋₂₆₅) peptide is a known immunogenic MHC class I ligand. As predicted by SYFPEITHI analysis and confirmed in actual cell binding assays (Figure 1), SVN₅₇₋₆₄ and SVN₈₂₋₈₉ displayed the highest affinity for MHC-I. We continued to focus upon SVN₅₇₋₆₄ based peptides, as this region of the survivin molecule is 100% homologous to human survivin.

CTL responses against GL261 cells

We tested splenocytes from mice immunized and boosted with peptide-loaded DC2.4 cells for their ability to kill survivin expressing GL261 glioma cells in culture (Figure 2). CTL assays revealed significant cytotoxic effects from effector lymphocytes of SVN₅₇₋₆₄ and SVN₈₂₋₈₉ vaccinated mice against GL261 target cells, but not from lymphocytes of OVA₂₅₈₋₂₆₅ peptide loaded DC vaccinated mice (not shown), consistent with the presence of specific anti-survivin CTL response. Although SVN₈₂₋₈₉ produced a higher cytotoxic effect, we have chosen to focus on SVN₅₇₋₆₄ based peptides, as this region of the survivin molecule is 100% homologous to human survivin and can avoid xenogeneic responses.

Specific CD8+ T cells against naturally processed survivin epitopes

We analyzed splenocytes from mice immunized and boosted with full length survivin cDNA transfected DC2.4 cells for the presence of CD8+ T cells capable of recognizing the indicated peptide epitopes after re-stimulation *in vitro* (Figure 3). FACS analysis utilizing specific peptide-loaded H2-K^b dimer IgG and gated on the CD8+ T cell population detected CD8+ T cells that were specific for SVN₅₇₋₆₄ and SVN₈₂₋₈₉ peptide, but not SVN₁₈₋₂₅, SVN₃₉₋₄₆, SVN₉₋₁₆, SVN₉₇₋₁₀₄ or OVA₂₅₈₋₂₆₅ peptides. OVA peptide is shown as an irrelevant peptide control, splenocytes were not stimulated with OVA *in vitro*. The immune response derived from the survivin cDNA is an indicator of the natural processing of SVN₅₇₋₆₄ and SVN₈₂₋₈₉ peptide epitopes from the full length immunogen that primed this immune response *in vivo*.

Individually, SVN₅₇₋₆₄ and SVN₈₂₋₈₉ are effective immunogens *in vivo*

We analyzed splenocytes from mice immunized and boosted with peptide-loaded DC2.4 cells and re-stimulated with the same peptide *in vitro*, for the presence of CD8+ T cells capable of recognizing their respective immunizing peptide epitopes (Figure 4). FACS analysis utilizing specific peptide-loaded H2-K^b dimer:IgG and gated on the CD8+ T cell population detected CD8+ T cells that were specific for SVN₅₇₋₆₄ and SVN₈₂₋₈₉ peptides from mice immunized with the same peptide, but without reactivity to empty dimers (IgG), or to dimers loaded with OVA₂₅₈₋₂₆₅ peptides. OVA peptide is shown as a negative peptide control since splenocytes were not stimulated with OVA *in vitro*. Mice immunized with SVN₅₇₋₆₄ were not reactive to SVN₈₂₋₈₉ loaded dimers. Similarly, mice immunized with SVN₈₂₋₈₉ were not reactive to SVN₅₇₋₆₄, indicating non-crossreactivity. These results confirm that both SVN₅₇₋₆₄ and SVN₈₂₋₈₉ can effectively stimulate individual CD8+ T cell responses *in vivo*.

Using SVN₅₇₋₆₄ as the basis (core) for the design of a larger peptide capable of stimulating CD4+ T cell support, we designed a peptide with additional amino acids flanking SVN₅₇₋₆₄. As a result, this produced a larger peptide that is predicted to bind human, and possibly murine,

class II molecules, while retaining numerous internal MHC class I ligands in addition to those present in the core SVN₅₇₋₆₄ peptide (Table II). Of note, the 15-amino acid sequence of SVN₅₃₋₆₇ contained in this peptide is also 100% homologous between mouse and human survivin proteins. Thus, SVN₅₃₋₆₇ may be applicable as a peptide vaccine in both mouse and humans.

CTL responses against GL261 cells in response to SVN₅₃₋₆₇ 15-mer peptide vaccine

We tested splenocytes from mice immunized and boosted with SVN₅₃₋₆₇ peptide-loaded DC2.4 cells for their ability to kill survivin expressing GL261 glioma cells in culture (Figure 5). CTL assays revealed significant cytotoxic effects of lymphocytes from SVN₅₃₋₆₇ peptide loaded DC vaccinated mice, but not from OVA₂₅₈₋₂₆₅ loaded DC vaccinated mice. This demonstrates the presence of specific anti-survivin CTL in response to SVN₅₃₋₆₇ based immunization.

Stimulation of CD4+ T cells in response to SVN₅₃₋₆₇ 15 mer

Intracellular cytokine production was measured in CD4+ T cells by FACS following immunization and *in vitro* re-stimulation with survivin peptides (Figure 6). The SVN₅₃₋₆₇ (15-mer) strongly stimulated IL-4 and IFN γ production, but not IL-2 production. In contrast, the smaller survivin peptides, including SVN₅₆₋₆₄, which contained the core SVN₅₇₋₆₄ motif, failed to stimulate either IL-4 or IFN γ . These results indicated that the larger 15-mer peptide (SVN₅₃₋₆₇) was indeed capable of stimulating CD4+ T cell based cytokine support while smaller peptides (SVN₅₆₋₆₄ SVN₅₇₋₆₄) did not.

Survival of C57BL6 mice with intracerebral GL261 glioma implants treated with survivin peptide DC vaccines

C57BL/6 mice were immunized with SVN₅₃₋₆₇, SVN₅₇₋₆₄, SVN₈₂₋₈₉, or OVA₂₅₈₋₂₆₅ peptide loaded DC2.4 cells. Vaccinations began 4 days after tumor cell implantation and were repeated (boosted) every 7 days to simulate a therapeutic setting (Figure 7). While the smaller survivin peptides, SVN₅₇₋₆₄ and SVN₈₂₋₈₉ produced strong CTL responses and specific CD8+ T cells *in vitro*, these peptides were not highly effective *in vivo* against murine survivin expressing GL261 cerebral gliomas (Table III). In contrast, the SVN₅₃₋₆₇ (15-mer) which contains multiple internal MHC class I ligands and at least one MHC class II ligand, was significantly more effective (>200% increase in survival) than any other smaller peptides *in vivo*. This result appears to be associated with the ability of the SVN₅₃₋₆₇ 15-mer peptide to initiate both CD4+ and CD8+ T cell responses.

Discussion

Survivin is an important tumor-associated antigen with restricted expression [4,15,16]. This characteristic makes it an excellent candidate target antigen for antitumor vaccines. MHC class I-restricted cytotoxic T cells directed against survivin peptides have been identified in patients with breast cancer, leukemia and melanoma [17]. Peptide epitopes from tumor-associated antigens (TAA), including survivin, can be recognized by cytotoxic T lymphocytes (CTL) in the context of MHC I molecules [10]. The human survivin protein contains HLA-A*0201 (HLA-A2) binding motifs. Andersen et al. [18] used HLA-A2-restricted peptides to test for specific T-cell reactivity in leukemia and melanoma patients. CTL responses against two survivin epitopes were detected in both patient groups, but no T-cell reactivity was found in healthy control patients. Therefore, self-immunization with the survivin molecule appears to take place in some tumor patients. This raises the possibility that immunization with survivin epitopes could stimulate a memory response with therapeutic benefit for cancer patients.

Schmitz et al. [19] were the first to show that two survivin peptides (ELTLGEFLKL and TLPPAWQPFL) can bind to HLA-A*0201 and elicit CD8⁺ immune responses when presented by DC. Moreover, one of these peptides was shown to result from the natural intracellular processing of survivin. These studies demonstrated that survivin is recognizable by autologous T cells. In addition, the identification of HLA-A1, HLA-A2, HLA-A3, and HLA-A11-restricted survivin epitopes [10] suggests that it might be possible to target survivin-expressing tumor cells using survivin-based cancer vaccines for a large clinical population.

In the current study, we have shown that the survivin protein is processed into additional peptides that elicit a class I immune response capable of being re-stimulated by SVN₅₇₋₆₄ and SVN₈₂₋₈₉ peptides (Figures 3 and 4). This is a further indicator of the natural processing of survivin into potentially immunogenic epitopes, not previously described. The fact that immune response to SVN₁₈₋₂₅, SVN₃₉₋₄₆, SVN₉₋₁₆, SVN₉₇₋₁₀₄ peptides were not detected in these animals could be attributed to one or more of the following: i) suboptimal class I binding by these peptides, ii) lack of adequate T cell receptor interaction with the peptides in the context of class I, and iii) lack of appropriate processing of the survivin protein.

Peptides processed from SVN₅₃₋₆₇ are capable of binding HLA-201 and are predicted to bind many additional common HLA molecules, including: A*03, A*1101, A*2402, A*26, A*6801, B*0702, B*08, B*1402, B*1501(B62), B*1510, B*18, B*2705, B*2709, B*3901, B*4402 and B*5101. Therefore, just as SVN₅₃₋₆₇ is immunogenic in mice, it should be immunogenic in humans expressing the appropriate MHC-I haplotypes.

Immunotherapy has emerged as a potential treatment modality for cerebral gliomas because it is: (i) specific to tumor cells, (ii) has the potential to eradicate infiltrating tumor cells after surgical debulking, and (iii) may be capable of eliciting a long-lasting memory response to prevent tumor recurrence. Both immunological [20-22] and clinical responses [22,23] have been observed in glioma patients immunized with (i) dendritic cells (DC) loaded with peptides eluted from the surface of allogeneic tumor cells [20]; (ii) DC loaded with peptides eluted from autologous tumor cells [21]; (iii) DC pulsed with tumor lysate from autologous tumor cells [22]; and (iv) hybrids generated by fusing DC with autologous tumor cells [23]. In one study [22], patients with recurrent glioblastoma multiforme, who were immunized with tumor lysate-pulsed DCs, had increased survival (median = 133 weeks). Thus, DCs offer significant potential as an immunization vehicle to treat malignant gliomas.

More specifically, Zeis et al. [24] have shown that transfection of dendritic cells (DC) with survivin mRNA leads to resistance to tumor challenge. Xiang et al. [25] showed that a DNA-based survivin vaccine could produce a CTL response against Lewis lung carcinoma with regression of both primary tumor and pulmonary metastases. The mechanism of the antitumor immunity induced by this DNA vaccine involved MHC class I antigen-restricted CD8⁺ T cells. The survivin vaccine triggered tumor cell apoptosis, together with release of IFN γ . Similarly, others have shown that survivin DNA vaccines generate specific antitumor effects with increased lymphocyte infiltration at tumor sites [26,27]. Recently a SVN₈₅₋₉₃ peptide combined with a plasmid encoding murine interleukin-15 (IL-15) was shown to support robust, CTL-mediated, long-term, immunity *in vivo* when used as a tumor vaccine. This vaccine effectively protected BALB/c mice against fatal CT26 tumor challenge [28].

Another distinct survivin derived peptide epitope “SVN₁₀” was used to derive T-cell clones from cancer patients with multiple HLA-DR genotypes capable of recognizing naturally processed antigen [29]. In addition, an anti-DEC205-human survivin protein was able to stimulate CD4⁺ T cell responses better than approaches involving survivin plasmid DNA or survivin peptides with adjuvants, but was unable to induce CD8⁺ T cell immunity [30]. Each of these reports confirms the ability of survivin vaccines to generate CTL responses and the

importance of cytokine support. Our study describes the first defined survivin peptide vaccine that is capable of stimulating a response to multiple CD8+ T cell epitopes and a CD4+ T cell cytokine response from a defined, single-peptide immunogen.

The effectiveness of a peptide vaccine may be enhanced by the capacity of DC to present both class I and class II associated epitopes. SVN₅₃₋₆₇ is processed to expose different epitopes for CD8+ T cell recognition and is capable of stimulating CD4+ T helper cells as well. Thus, SVN₅₃₋₆₇ represents a potential class II ligand in C57BL/6 mice. In our study, the smaller core peptides (SVN₅₇₋₆₄ and SVN₅₆₋₆₄) stimulated CD8+ responses and produced effective CTL *in vitro*. However, these same core peptides only produced modest prolongation of survival of mice with cerebral gliomas. In contrast, the SVN₅₃₋₆₇ 15-mer produced significant prolongation of survival in this model. Thus, the addition of CD4+ support may contribute to a more effective antitumor response *in vivo*.

Our study shows that the contribution of CD4 support may lead to a more effective peptide vaccine strategy. The enhanced survival demonstrated by SVN₅₃₋₆₇ suggests that the addition of CD4+ helper T cell support associated with a specific survivin-derived CD8+ T cell response may lead to a more effective peptide antitumor vaccine. Other studies of ours indicate that SVN₅₃₋₆₇ is capable of re-stimulating an autologous human CTL response capable of lysing tumor cells *ex vivo* (data not shown). In addition, SVN₅₃₋₆₇ is predicted to function as a ligand for HLA-DRB1*0301 (DR17), HLA-DRB1*0401 (DR4Dw4), HLA-DRB1*0701, HLA-DRB1*1501 (DR2b) molecules, making it potentially capable of stimulating CD4+ support in humans. A peptide vaccination strategy combining CTL and CD4+ support provides one additional avenue to investigate for survivin-targeted, immunotherapeutic approaches for cerebral gliomas.

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Figure 1. H2-K^b binding of survivin peptides

Survivin peptide epitopes were used in MHC class I peptide binding assays. Mean fluorescence represents binding of SVN peptides to H2-K^b. Upregulation of H2-K^b molecules on the surface of murine RMA-S cells which are deficient in the expression of surface MHC class I molecules was used to determine actual survivin peptide binding in vitro. Data show the binding of 100μM peptide at 37°C. Positive control peptide (ovalbumin) represents a known immunogenic MHC class I ligand. The negative control is an irrelevant peptide that does not bind MHC class I. Data represent mean fluorescence ± S.E.M. of triplicate samples.

Figure 2. Cytotoxic T cell responses against GL261 glioma cells

CTL assays for specific T cell lysis of target were performed using the Live/Dead cell mediated cytotoxicity method using flow cytometry. Splenocytes from peptide-loaded, DC vaccinated animals were added to the target cells in ratios ranging from 60:1 to 100:1 and cytotoxicity was calculated as described in Materials and Methods. Analysis is based upon gating on labeled GL261 cells to eliminate background from effector cells. Maximal cytotoxicity was simulated using ethanol-treated target cells. Spontaneous cytotoxicity represents GL261 target cells incubated in the absence of effector cells. Data represent mean percent specific lysis $\pm$ S.E.M. of triplicate samples.

Figure 3. Specific CD8+ T cells against naturally processed survivin epitopes

Recombinant dimeric mouse H2-K^b:Ig molecules were loaded *in vitro* with survivin peptides and used to probe splenocytes from DC transfected with an expression vector containing the full length survivin cDNA that had been used to immunize mice. PE-labeled H2-K^b dimers were used to detect survivin-specific CD8+ T cells by flow cytometry. Splenocytes from vaccinated mice were doubly stained for CD8+ using a FITC conjugated rat anti-mouse CD8 α mAb as described. Analysis is based upon gating on total CD8+ T cells. Ovalbumin serves as a control for nonspecific immune responses.

Figure 4. Specific CD8+ T cells against specific survivin peptides

Recombinant dimeric mouse H2-K^b:Ig molecules were loaded *in vitro* with survivin peptides and used to probe splenocytes from SVN₅₇₋₆₄ or SVN₈₂₋₈₉ peptide-loaded, DC-vaccinated mice. PE-labeled H2-K^b dimers were used to detect survivin-specific CD8+ T cells by flow cytometry. Splenocytes from vaccinated mice were doubly stained for CD8+ using a FITC-conjugated rat anti-mouse CD8 α mAb as described. Analysis is based upon gating on total CD8+ T cells. Ovalbumin serves as a control for nonspecific immune responses. Data is representative of three separate experiments.

Figure 5. SVN₅₃₋₆₇ derived CTL responses against GL261

CTL assays for specific T cell lysis of target were performed using the Live/Dead cell-mediated cytotoxicity method using flow cytometry. Splenocytes from peptide-loaded, DC vaccinated animals were added to the target cells in ratios ranging from 5:1 to 40:1 and cytotoxicity was calculated as described in Materials and Methods. Analysis is based upon gating on labeled GL261 cells to eliminate background from effector cells. Maximal cytotoxicity was simulated using ethanol-treated target cells. Spontaneous cytotoxicity represents GL261 target cells incubated in the absence of effector cells. Data represent mean percent specific lysis $\pm$ S.E.M. of triplicate samples.

Figure 6. Stimulation of CD4⁺ cells in response to SVN₅₃₋₆₇ immunization

Splenocytes from immunized mice were stimulated *in vitro* using survivin peptides and intracellular cytokine production was measured in CD4⁺ cells by flow cytometry.

Commercially prepared mouse *in vitro* activated splenocytes known to contain IL-2, IL-4, IFN- γ expressing cells were used as positive controls. Analysis is based upon gating on CD4⁺ T cells only. Data represent mean fluorescence $\pm$ S.E.M. of triplicate samples.

Figure 7. Survival of C57BL/6 mice with intracerebral GL261 glioma implants

Mice were implanted with GL261 cells and were treated with survivin peptide DC vaccines. C57BL/6 mice were immunized with SVN₅₃₋₆₇, SVN₅₇₋₆₄, SVN₈₂₋₈₉, or OVA₂₅₈₋₂₆₅ peptide loaded DC2.4 cells. Vaccinations began 4 days after tumor cell implantation and were repeated (boosted) every 7 days to simulate a therapeutic setting. Survival was plotted according to Kaplan-Meier methods. Long-term survivors were confirmed tumor-free by high field strength MRI (data not shown).

Table I

Selected survivin peptide epitopes predicted by SYFPEITHI to bind MHC class I (H2-K^b) are shown. Positive control peptide (ovalbumin) represents a known immunogenic MHC class I ligand with a score indicating strong potential binding. Underlined amino acid residues represent MHC I anchor positions.

Peptide Position	Epitope 1 2 3 4 5 6 7 8	Predicted Binding Score
OVA ₂₅₈₋₂₆₅	S I I N <u>F</u> E K <u>L</u>	25
SVN ₅₇₋₆₄	C F F C F K E <u>L</u>	20
SVN ₈₂₋₈₉	S G C A <u>F</u> L S <u>V</u>	18
SVN ₉₇₋₁₀₄	T L G E <u>F</u> L K <u>L</u>	22
SVN ₁₈₋₂₅	R I S T F K N W	13
SVN ₃₉₋₄₆	A E A G F I H <u>C</u>	12
SVN ₉₋₁₆	A W Q P <u>F</u> L K <u>D</u>	12

Table II

Amino acid sequence of the survivin peptide vaccine molecule containing potential class II epitope and core class I binding peptides used in vaccination studies. Amino acids 49 through 71 represent a region of 100% homology between mouse and human survivin molecules.

AA49-ENE <u>PD</u> LAQCFFCFKELEGWEPDD-AA71		
DLAQCFFCFKELEGW = SVN ₅₃₋₆₇		Potential MHC class II ligand
AQCFCFKEL = SVN ₅₅₋₆₄ QCFFCFKEL = SVN ₅₆₋₆₄		
CFKEL = SVN ₅₇₋₆₄		Potential MHC class I ligands (contained within SVN ₅₃₋₆₇)

Survival data of C57BL/6 mice with intracerebral GL261 glioma implants as depicted in Figure 7. Mice were implanted with GL261 cells and were treated with survivin peptide DC vaccines. MeST represents median survival time of groups with p values determined by the logrank Mantel-Cox test for significance.

Table III

Immunizing Antigen	MeST	Range	n =	p value (vs. Control DC)	p value (vs. OVA)
Control DC	19.5	18-25	10	-	p = 0.79
OVA ₉₈₋₂₆₅	21	19-22	7	p = 0.79	-
SVN ₈₂₋₈₉	25	21-32	10	p < 0.001	p < 0.001
SVN ₅₇₋₆₄	25	19-35	14	p < 0.001	p < 0.001
SVN ₅₃₋₆₇	57	43-120+	8	p < 0.0001	p < 0.0001