Identification of Interleukin-13 Receptor α2 Peptide Analogues Capable of Inducing Improved Antiglioma CTL Responses

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Abstract

Restricted and high-level expression of interleukin-13 receptor α2 (IL-13Rα2) in a majority of human malignant gliomas makes this protein an attractive vaccine target. We have previously described the identification of the IL-13Rα2345-353 peptide as a human leukocyte antigen-A2 (HLA-A2)–restricted CTL epitope. However, as it remains unclear how efficiently peptide-based vaccines can induce specific CTLs in patients with malignant gliomas, we have examined whether analogue epitopes could elicit heteroclitic antitumor T-cell responses versus wild-type peptides. We have created three IL-13Rα2 analogue peptides by substitutions of the COOH-terminal isoleucine (I) for valine (V) and the NH2-terminal tryptophan (W) for either alanine (A), glutamic acid (E), or nonsubstituted (W; designated as 1A9V, 1E9V, and 9V, respectively). In comparison with the native IL-13Rα2 epitope, the analogue peptides 9V and 1A9V displayed higher levels of binding affinity and stability in HLA-A2 complexes and yielded an improved stimulatory index for patient-derived, specific CTLs against the native epitope expressed by HLA-A2+ glioma cells. In HLA-A2-transgenic HHD mice, immunization with the peptides 9V and 1A9V induced enhanced levels of CTL reactivity and protective immunity against an intracranial challenge with IL-13Rα2-expressing syngeneic tumors when compared with vaccines containing the native IL-13Rα2 epitope. These findings indicate highly immunogenic IL-13Rα2 peptide analogues may be useful for the development of vaccines capable of effectively expanding IL-13Rα2-specific, tumor-reactive CTLs in glioma patients. (Cancer Res 2006; 66(11): 5883-91)

Introduction

Despite significant advances in modern microsurgery, radiotherapy, and chemotherapy, the prognosis for patients with malignant glioma remains poor (1). Immunotherapy for glioma is an attractive alternative treatment option because activated, antitumor immune cells have the potential to migrate into the central nervous system (CNS) and to selectively destroy malignant cells that have infiltrated normal CNS tissues (2). Although the CNS, and tumors that arise therein, reside in an "immunologically privileged" site (reviewed in refs. 3, 4), we and others have successfully shown that effective anti-CNS tumor immune responses can be induced in preclinical models using syngeneic tumor- and dendritic cell–based vaccines (5–7). Based on our own preclinical data (8, 9), we have previously initiated phase I glioma vaccine trials using interleukin-4 (IL-4) gene–transduced autologous glioma cells or fibroblasts (10) and vaccination regimens using dendritic cells loaded with whole glioma cell lysates (11).

Although vaccinations of glioma patients with autologous dendritic cell loaded with bulk glioma antigens have not yet evoked a single case of autoimmune encephalopathy in treated glioma patients (12–15), glioma-associated antigen (GAA)–specific approaches would be expected to be more effective and to reduce the potential risk of autoimmune encephalitis even further. In addition, these vaccines can be generated in a shorter period of time compared with whole glioma cell approaches, owing to the feasibility of obtaining an "off-the-shelf" preparation of synthetic GAA peptides.

With regard to the human leukocyte antigen-A2.1 (HLA-A2.1)–restricted GAA-derived epitopes available for the induction of CTL responses, our report describing a CD8+ T-cell epitope derived from the GAA IL-13Rα2 (16) is among the first citations in the literature. IL-13Rα2 is expressed at high levels in most human gliomas but not in the normal brain (17, 18).

The concern for the clinical use of nonmutated, autoantigens in vaccine formulations is that high-affinity T cells recognizing such epitopes have been selectively eliminated due to normal tolerance mechanisms, leaving a low-affinity repertoire that may prove ineffective in recognizing the naturally processed and HLA-presented peptides on the surface of tumor cells in situ. To overcome this issue, it has been previously shown that by incorporating certain amino acid substitutions in the natural peptide sequence, one may enhance peptide-HLA complex stability, resulting in the induction of stronger and/or heteroclitic antitumor CTL responses (19–21). In particular, analogue peptides with increased HLA-binding capacity have been obtained by modifying the NH2-terminal (22, 23) and COOH-terminal (24, 25) peptide positions.

In an attempt to increase the efficiency of CTL induction against the natural IL-13Rα2345-353 peptide, we created three IL-13Rα2 analogue peptides (designated as 1A9V, 1E9V, and 9V) that incorporate NH2-terminal and/or COOH-terminal amino acid substitutions. Here, we show that the IL-13Rα2 analogue peptides 9V and 1A9V are superior to the natural IL-13Rα2345-353 epitope in their capacity to induce CTL responses from patient-derived HLA-A2+ peripheral blood mononuclear cells (PBMC) in vitro and HLA-A2-transgenic HHD mice. This most likely reflects the enhanced longevity of analogue peptide presentation to specific CTL precursors, allowing for increased clonotypic proliferation.
Materials and Methods

Peptides. The synthetic peptides IL-13Rα2 (345-353, WLPGFGFLIL 345-9W, WLPGFGFLIL; 345-19W, ALPGFGFLIL; 345-19E, ELPGFGFLIL), MART-1 (27-35, AAGIGILTV), Influenza M1 (58-66, GILGFVFTL), and HBVcore128 (TPAYPRPNAPIL) were synthesized by X-9-fluorenylmethoxycarbonyl chemistry in the University of Pittsburgh Peptide Synthesis Facility and were >95% pure as indicated by analytic high-performance liquid chromatography and mass spectrometric analysis. Peptides were dissolved in PBS/10% DMSO at a concentration of 2 mg/mL and stored at −20°C until use.

Cells and cell culture. The U251 and SNB19 glioma cell lines were generously provided from Drs. Martin R. Jaudis (University of California, Irvine) and William C. Welch (University of Pittsburgh), respectively, and cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10 mmol/L L-glutamine (all reagents from Invitrogen, Carlsbad, CA).

PBMCs were obtained from glial patients and healthy donors under an Institutional Review Board–approved protocol. HLA-A2 expression on the PBMC was validated using the monoclonal antibodies (mAb) MA21 (against HLA A2, B17) and BB7.2 (against HLA A2, Aw66: both from the American Type Culture Collection, Manassas, VA) in indirect immunofluorescence assays monitored by flow cytometry.

The murine EL4-HHD [DpX/p2 microglobulin (β2M) null and transfected for modified HLA-A2-β2 microglobulin single chain (HHD gene)] and control EL-4S-3B cells (DpX/p2 β2M null) were the generous gifts of Dr. François A. Lemmonier (Paster Institute, Paris; ref. 26). The transporter associated with antigen processing–deficient, H-2L−/− T2 cell line, EL4-HHD and EL4-S3-β2 cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS and 100 IU/mL penicillin, 100 µg/mL streptomycin, and 10 mmol/L L-glutamine (all reagents from Invitrogen).

HHD mice. HHD mice were obtained from Dr. François A. Lemmonier through Dr. Pravin T.P. Kaumaya (The Ohio State University, Columbus, OH). HHD mice are DpX/p2 (β2M) null and transfected for modified HLA-A*0201-β2 microglobulin single chain (HHD gene; refs. 26, 27).

Creation of an HHD-syngeneic tumor cell line that expresses IL-13Rα2. The full-length IL-13Rα2 cDNA (National Center for Biotechnology Information accession no. NM_000640) fragment was generated by reverse transcription–PCR using the forward (AGTATGGCTTTCTTTG TGTGC) and reverse (TACGGAGCTCGGTAACTACG) primers and U251 glioma-derived total RNA. The IL-13Rα2 cDNA was then cloned into the expression plasmid pEF6/V5-His-TOPO vector (Invitrogen) to generate the pEF6/V5-IL-13Rα2. EL4-HHD cells were then transfected with the pEF6/V5-IL-13Rα2 using Cell Line Nucleofector kit T (Axamma, Gaithersburg, MD), and a blastocidin-resistant clone that stably expressed the highest level of IL-13Rα2 based on flow-cytometry using anti-IL-13Rα2 mAb (Miltenyi Biotech, Auburn, CA). CD8+ T cells (2 × 10^6 per well) were cocultured with 2 × 10^6 per well peptide-pulsed dendritic cells in 2 mL/well of AIM-V medium supplemented with 5% human AB serum, 10 units/mL rhIL-2 (R&D Systems, Minneapolis, MN), and 10 units/mL rhIL-7 (Cell Sciences) in each well of 24-well tissue culture plates. On day 15, lymphocytes were restimulated with autologous dendritic cells pulsed with peptide in AIM-V medium supplemented with 5% human AB serum, rhIL-2, and rhIL-7 (10 units/mL each). On day 20, the CD8+ cultured cells were analyzed for CTL activity by standard 4-hour 51Cr release assay.

Vaccination of HHD mice and i.c. tumor challenge. HHD mice received (on days 0 and 7) s.c. injections of 100 µg of IL-13Rα2 cDNA or each of the peptide analogues emulsified in Incomplete Freund’s adjuvant (IFA; Difco, Detroit, MI) in the presence of 140 µg of the I-Aβ-restricted HBVcore128 T-helper epitope, which stimulates a CD4+ helper T-cell response. Control animals received IFA containing HBV helper-peptide only. On day 11 after the second immunization, the animals were sacrificed, and 5 × 10^6 splenocytes were stimulated in vitro with the same peptide that was used for in vivo stimulation (10 µmol/L). On day 6 of culture, the bulk populations were tested for specific cytotoxicity against the EL4-HHD cells pulsed with IL-13Rα2 or unpuised EL4-HHD, or EL4-HHD-IL13Rα2 cells.

To assess systemic protective immunity against i.c. tumor challenge, day 7 after the second immunization, HHD mice received an i.c. inoculation of EL4-HHD-IL13Rα2 cells, as previously described (6). Briefly, 5 × 10^4 EL4-HHD-IL13Rα2 cells were stereotactically injected through an entry site at the bregma 2 mm to the right of the sagittal suture and 3 mm below the surface of the skull of anesthetized mice using a stereotactic frame. The animals were monitored daily after treatment for the manifestation of any pathologic signs. All animal experiments were done under an Institutional Animal Care and Use Committee–approved protocol.

Isolation of brain-infiltrating lymphocytes. Mice bearing i.c. EL4-HHD-IL13Rα2 tumors received immunizations on days 14 and 21 after the tumor inoculation, were sacrificed by CO2 asphyxia on day 28, and perfused through the left cardiac ventricle with PBS. Brains were enzymatically digested (28, 29), and cells from each brain were resuspended in 70% Percoll (Sigma, Saint Louis, MO), overlaid with 37% and 30% Percoll and centrifuged for 20 minutes at 500 × g. Enriched brain-infiltrating lymphocyte (BIL) populations were recovered at the 70% to 37% Percoll interface.

Statistical analyses. Survival data were compared using a log-rank test. Comparative numbers of T-cell responses were analyzed by Student’s t test for two samples with unequal variances. Statistical significance was determined at the p < 0.05 level. In addition, positive response was defined as follows: the specific lysis by the responder cells against antigen-positive target cells is at least 15% and 2-fold higher than lytic levels by corresponding control conditions in at least two effector/target (E/T) ratios. The correlation among clinical factors, including age, tumor type and use of temozolomide therapy, and T-cell responses, was assessed by a χ2 test.
Results

**IL-13Ro2** analogue peptides 9V and 1A9V exhibit enhanced binding affinities for HLA-A2. We synthesized three IL-13Ro2 analogue peptides that incorporated a COOH-terminal I for V substitution based on previous studies (24, 25), and either no NH2-terminal substitution or a W for either A or E NH2-terminal substitution designated as 9V, 1A9V, and 1E9V, respectively. The NH2-terminal substitutions were designed to improve steric fit (via replacement of W with the small A residue) and solubility (by replacing W with E).

Because stable binding of HLA-A2 with peptide epitopes further stabilizes the surface expression of HLA-A2 (30, 31), quantitative expression levels of HLA-A2, which is indicated by MFI in Table 1, correlate with the binding affinity of the peptide epitopes that are coincubated with the T2 cells. The MFI values for T2 cells with no peptide indicate the baseline HLA-A2 expression level. The analogue peptides 9V and 1A9V but not 1E9V possess a higher binding affinity to HLA-A2 and higher stabilization capacity as shown by the prolonged DC50 when compared with the native IL-13Ro2 analogue peptides. This suggested the possibility that 9V and 1A9V peptides might prove more immunogenic than the wild-type peptide when incorporated into vaccines.

**Peptide analogues 9V and 1A9V were more immunogenic than wild-type IL-13Ro2** peptide in promoting the in vitro activation of antitumor CTL. We initially sought to show that the CD8+ T cells that were stimulated with the peptide analogues could still recognize the native IL-13Ro2 analogue peptide presented in the context of HLA-A2. CD8+ T cells that had been stimulated with either the wild-type or analogue peptides efficiently lysed T2 target cells loaded with escalating concentrations of the natural IL-13Ro2 peptide (Fig. 1A). T cells that had been stimulated with the 9V or 1A9V peptides showed higher levels of lytic activity against T2 cells loaded with low concentrations (0.1-10 nmol/L) of IL-13Ro2 wild-type peptide compared with T cells stimulated with other peptides. Only low background lysis of T2 cells was observed for any of the CTL lines in the absence of specific peptide. T cells that had been stimulated with the control Influenza M1 peptide or no peptide did not show any specific lytic activity against the IL-13Ro2-pulsed T2 cells over background control levels.

We next examined whether the CTLs developed in response to the 9V and 1A9V analogue peptides exhibited improved recognition of HLA-A2+ human glioma cells that endogenously expressed and presented IL-13Ro2-derived epitopes. Human glioma cell lines U251 and SNB19 express both the HLA-A2 and IL-13Ro2 proteins, whereas the human glioma cell line A172 expresses IL-13Ro2 but not HLA-A2 (16). Therefore, we used U251 and SNB19 as relevant target glioma cells, whereas A172 served as a negative control line in 4-hour 51Cr release assays. As illustrated in Fig. 1B, the U-251 and SNB19 cell lines were highly susceptible to cytotoxic activity mediated by CTL lines that had been induced with IL-13Ro2 or with analogue peptides. A172 cells, in contrast, were not lysed above a background level (<15%) at E/T ratios of ≤40, by any of the CTL lines tested, suggesting that the IL-13Ro2 analogue peptide-induced CTL lines lysed SNB19 and U-251 glioma cells in an HLA-A2-restricted and antigen-dependent manner. In contrast, the T cells stimulated with no peptide showed only background lysis (<20%) against the SNB19 and U-251 glioma cell lines at E/T ratios of ≤40. Although there was a trend for an E/T ratio-dependent increase of lysis in these control effector cells, we believe it was non–antigen-specific killer activity of the T cells based on experiments discussed in Fig. 1D, in which neither addition of cold peptide-loaded T2 cells or neutralizing antibody against MHC-class I (W6/32) significantly reduced the cytolysis of the control effector cells.

As shown in Fig. 1C, peptide analogues 9V and 1A9V induced higher levels of CTL reactivity against the SNB19 and U-251 target cells at all E/T ratios when compared with CTLs primed against the wild-type IL-13Ro2 analogue peptide. These data further confirm that these peptide analogues consistently induce anti-IL-13Ro2 CTL more efficiently than does the native IL-13Ro2 analogue peptide.

To corroborate the specificity of CTL activity, cold target competition experiments were done by addition of nonradiolabeled (cold) T2 cells pulsed with IL-13Ro2 analogue peptide in the 4-hour 51Cr release assay (Fig. 1D). The anti-SNB19 glioma cell lytic activities mediated by CTL lines induced by the wild-type IL-13Ro2 analogue or its peptide analogues were almost completely inhibited by the addition of the cold T2 cells pulsed with IL-13Ro2 analogue peptide. The CTL activities, however, were not inhibited by the addition of non–peptide-pulsed cold T2 cells, showing that the lytic ability and inhibition were specifically linked to the IL-13Ro2 analogue peptide. Furthermore, addition of an anti-MHC class I antibody (W6/32) inhibited the CTL-mediated lysis.

Table 1. Enhanced binding ability and stability of peptide analogues 9V and 1A9V to HLA-A2

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>T2 binding (MFI*)</th>
<th>DC50 (h) †</th>
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<tbody>
<tr>
<td>IL-13Ro2 345-353</td>
<td>WLPFGFILV</td>
<td>24.4</td>
<td>3.1</td>
</tr>
<tr>
<td>IL-13Ro2 345-9V</td>
<td>WLPFGFILV</td>
<td>39.1</td>
<td>6.1</td>
</tr>
<tr>
<td>IL-13Ro2 345-1A9V</td>
<td>ALPFGFILV</td>
<td>44.6</td>
<td>&gt;6</td>
</tr>
<tr>
<td>IL-13Ro2 345-1E9V</td>
<td>ELPFGFILV</td>
<td>25.2</td>
<td>3.4</td>
</tr>
<tr>
<td>Influenza M1</td>
<td>GLGFVFTL</td>
<td>53.4</td>
<td>&gt;6</td>
</tr>
<tr>
<td>No peptide (control)</td>
<td>9.4 ‡</td>
<td>8.9 ‡</td>
<td></td>
</tr>
</tbody>
</table>

*Peptide binding was evaluated by MFI.
†DC50 was defined as the time required for the loss of 50% of the HLA-A*0201 peptide complexes stabilized at t = 0.
‡MFI values for control, non–peptide-loaded cells indicate baseline expression levels when T2 cells were treated with solvent (DMSO in PBS) only.
Figure 1. IL-13R\(\alpha\)2345-353 analogue peptides 9V and 1A9V induced enhanced levels of anti-IL-13R\(\alpha\)2345-353 CTL activity in vitro. A, CD8+ T cells isolated from an HLA-A2+ glioma patient were stimulated with autologous dendritic cells loaded with either wild-type IL-13R\(\alpha\)2345-353 (●), 9V (○), 1A9V (▲), 1E9V (▼), Influenza M158-66 (▲), or no (●) peptide for 10 days. Responder T cells were then tested for their lytic activity against T2 cells loaded with indicated concentrations of IL-13R\(\alpha\)2345-353 peptide in 4-hour \(^{51}\)Cr release assays at an E/T ratio of 12.5. Points, mean % specific lysis; bars, SD. \(P < 0.01\) for 9V versus wild-type IL-13R\(\alpha\)2345-353 peptide and 1A9V versus wild-type IL-13R\(\alpha\)2345-353 peptide at 0.1 and 1 nmol/L by two-tailed Student’s \(t\) test. From one representative experiments of three done for donors exhibiting a positive CTL response against the wild-type IL-13R\(\alpha\)2345-353 epitope. B and C, CD8+ T cells derived from an HLA-A2+ glioma patient were stimulated with autologous dendritic cells loaded with no peptide, the wild-type IL-13R\(\alpha\)2345-353 peptide, or the 9V, 1A9V or 1E9V analogue peptides. On day 19 after the primary stimulation, responder T cells were tested for their lytic ability against human glioma cells SNB19, U-251 (both are IL-13R\(\alpha\)+/HLA-A2+), or A172 (IL-13R\(\alpha\)+/HLA-A2-), using 4-hour \(^{51}\)Cr release assays. Points, mean % specific lysis; bars, SD. Some SD values were smaller than the point marks. B, \(P < 0.05\) at all E/T ratios for SNB19 versus A172 and for U251 versus A172 by two-tailed Student’s \(t\) tests (except for the control group). C, against SNB19 glioma cells (\(P < 0.05\)) at all E/T ratios for 9V versus wild-type IL-13R\(\alpha\)2345-353 peptide and 1A9V versus wild-type IL-13R\(\alpha\)2345-353 peptide by two-tailed Student’s \(t\) tests. Against U251 glioma cells (\(P < 0.05\)) at E/T ratios of 10 and 40 for 9V versus wild-type IL-13R\(\alpha\)2345-353 and 1A9V versus wild-type IL-13R\(\alpha\)2345-353 peptide by two-tailed Student’s \(t\) tests. Stimulation with the 1E9V analogue peptide did not improve the CTL reactivity when compared to stimulation using the wild-type peptide. D, addition of “cold” T2 cells pulsed with the IL-13R\(\alpha\)2345-353 peptide or anti-HLA-class I antibody inhibited CTL activity, supporting the antigen specificity and the HLA-class I restriction of the CTL lines, respectively. The CTL lines induced with each peptide were incubated for 4 hours with \(^{51}\)Cr-labeled human glioma cells SNB19 cells at the indicated E/T ratios for evaluation of specific lytic ability (●). For the cold target inhibition assay, \(^{51}\)Cr-labeled target SNB19 cells (1 \(\times\) 10^5 per well) and cold T2 cells (1 \(\times\) 10^5 per well) pulsed with (▲) or without (●) the IL-13R\(\alpha\)2345-353 peptide were incubated with the CTLs. To block the HLA class I-mediated recognition by the T cells, the pan-class I mAb W6/32 (10 µg/mL) was added before standard 4-hour \(^{51}\)Cr release assay (●). Points, mean % specific lysis; bars, SD. In IL-13R\(\alpha\)2 peptide groups, \(P < 0.05\) at all E/T ratios for SNB19 versus cold target or mAb-treated target by two-tailed Student’s \(t\) tests. From one of the three experiments done yielding similar results using PMBCs isolated from three independent donors that displayed a positive response against the IL-13R\(\alpha\)2345-353 wild-type epitope.
confirming that the antiglioma CTL reactivity induced by these peptides was HLA class I restricted (Fig. 1D).

**In vitro stimulation with the peptide analogues 9V and 1A9V promotes the enhanced development of antigen-specific CD8+ T cells.** To determine whether the enhanced CTL activities induced by the 9V and 1A9V analogue peptides are attributable to the efficient induction of the CTL proliferation (quantity), CD8+ T cells from a HLA-A2+ healthy donor were stimulated with dendritic cells loaded with either the native or peptide analogues. Control groups were stimulated with negative control MART-127-35 peptide or with no peptide. The cells were then evaluated for the number of CD8+ T cells capable of binding to HLA-A2/IL-13Rα2 tetramer by flow cytometry (Fig. 2A). T cells stimulated with the 9V or 1A9V peptides induced higher numbers of tetramer-positive cells (6.86% and 6.80%, respectively) when compared with the naive peptide (4.60%). T cells that had been stimulated with the MART-1 peptide or no peptide had only background levels of tetramer-positive events.

Although we initially synthesized an HLA-A2-tetramer using the native IL-13Rα2345-353 peptide, we were unable to optimize the staining conditions, most likely due to the poor stability of the HLA-A2/IL-13Rα2345-353 peptide complex (data not shown). To examine whether a tetramer made using the 1A9V peptide would allow us to evaluate CTLs that recognize the native IL-13Rα245-353 peptide in the context of HLA-A2, as shown in Fig. 2B, we next showed that the CTLs induced by the native peptide recognized T2 cells loaded with the 1A9V or the wild-type IL-13Rα245-353 peptide, and reciprocally, that CTLs induced by the peptide analogues recognized both native and analogue peptides, suggesting a mutual cross-reactivity of the modified and the native peptides. Therefore, we consider that an evaluation using the HLA-A2/IL-13Rα2 (1A9V) tetramer allows us to evaluate CTL recognition of both native and analogue peptides.

**In vivo immunization of HHD mice with the 9V or 1A9V peptides results in enhanced frequencies of specific CTL reactive against target cells naturally expressing IL-13Rα2345-353.** To examine whether immunization with IL-13Rα245-353 and/or its peptide analogues can elicit CTL responses in vivo, we immunized HLA-A2 transgenic HHD mice twice with the wild-type, 9V, or 1A9V peptides emulsified in IFA in the presence of a heterologous HBVcore128 T-helper peptide. After a 6-day in vitro stimulation, the splenocytes derived from vaccinated HHD mice were able to lyse EL4-HHD cells loaded with the native IL-13Rα2345-353 peptide (Fig. 3A) and EL4-HHD cells transfected to express the IL-13Rα2 (EL4-HHD-IL-13Rα2) gene product (Fig. 3B). Control nonpulsed EL4-HHD cells were not lysed by the splenocytes beyond

![Figure 2](image-url)

**Figure 2.** In vitro stimulation of PBMC with the 9V or 1A9V analogue peptides promotes enhanced proliferation of antigen-specific CD8+ CTLs. CD8+ T cells isolated from a healthy HLA-A2+ donor were stimulated with autologous dendritic cells loaded with either no peptide, MART-127-35, peptide, wild-type IL-13Rα2345-353 peptide, or the analogue peptides 9V, 1A9V, or 1E9V for 2 weeks. A, responder cells were then incubated with FITC-conjugated anti-human CD8 antibody and pycoerythrin-labeled HLA-A2/IL-13Rα2-tetramer for 15 minutes at room temperature. After washing, the cells were evaluated by flow cytometry for the percentage of CD8+ tetramer+ in the lymphocyte-gated population (numbers in the parenthesis). B, the same responder cells were evaluated for their lytic activity against T2 cells loaded with either the wild-type IL-13Rα2345-353 (△), analogue 1A9V (●), or MART-1 (∗) peptides in 4-hour 51Cr release assays. Points, mean % specific lysis; bars, SD.
To determine whether immunizations with the 9V or 1A9V peptides induced the proliferation of IL-13Rα2345-353-loaded EL4-HHD and EL4-HHD-IL13Rα2 cells when compared with the CTL induced by the wild-type peptide (Fig. 3C).

To examine the comparative in vivo anti-intracranial tumor efficacy of peptide-based vaccination, HHD mice were preimmunized twice with the IL-13Rα2345-353 wild-type or analogue peptides before being challenged i.c. with EL4-HHD-IL13R2 tumor cells, which typically form solid and lethal tumor lesions in the brains of nonimmunized syngenic animals (Fig. 4A). As shown in Fig. 4B, all HHD mice treated with control vaccines succumbed due to progressive tumor growth by day 70. In contrast, preimmunizations with the 9V or 1A9V analogue peptides resulted in extended, long-term survival (longer than 100 days) in three of six mice treated in each group. The control EL4-HHD-IL13R2 cells that constitutively express the IL-13Rα2 tetramer, when compared with the wild-type IL-13Rα2345-353 peptide (A); or EL4-HHD-IL13R2 cells that constitutively express the IL-13Rα2 tetramer versus control EL4-HHD cells (B). Points, mean % specific lysis; bars, SD. C, the CTL activities against peptide-pulsed (left) or IL-13Rα2-expressing (right) EL4-HHD cells are combined for the comparison of relative CTL activities induced by either the wild-type IL-13Rα2345-353 peptide or its analogues. Against peptide-pulsed EL4-HHD (P < 0.05) at all E:T ratios for 9V versus wild-type peptide and 1A9V versus wild-type peptide by two-tailed Student’s t-tests. Against IL-13Rα2-expressing EL4-HHD (P < 0.05) at the E:T ratio of 10 and 40 for 9V versus wild-type peptide and 1A9V versus wild-type peptide by two-tailed Student’s t-tests. D, freshly isolated cells isolated from draining lymph nodes were stained with FITC-conjugated anti-mouse CD8 antibody and pyocyanin-labeled HLA-A2/IL-13R2 tetramers. Numbers in each histogram indicate the percentage of CD8α-heterotetramer T cells within the lymphocyte-gated population.

9V and 1A9V analogue peptide-based vaccines elicit superior protective immunity against lethal i.c. challenge dose of EL4-HHD-IL13R2 tumor cells in HHD mice. To examine the comparative in vivo anti-intracranial tumor efficacy of peptide-based vaccination, HHD mice were preimmunized twice with the IL-13Rα2345-353 wild-type or analogue peptides before being challenged i.c. with EL4-HHD-IL13R2 tumor cells, which typically form solid and lethal tumor lesions in the brains of nonimmunized syngenic animals (Fig. 4A). As shown in Fig. 4B, all HHD mice treated with control vaccines succumbed due to progressive tumor growth by day 70. In contrast, preimmunizations with the 9V or 1A9V analogue peptides resulted in extended, long-term survival (longer than 100 days) in three of six mice treated in each group and showed statistically significant prolongations of survival when compared with the control group (P = 0.0031 and 0.0016, respectively). Although one of the six mice preimmunized with the wild-type IL-13Rα2345-353 peptide survived for longer than 100 days, the mean difference with the control group did not reach statistical significance (P = 0.0533). These results indicate that preimmunization with the 9V or 1A9V peptide induces effective anti-brain tumor responses in HHD mice that are superior to those promoted by the wild-type IL-13Rα2-derived epitope.

Furthermore, splenocytes isolated from long-term survivor animals following immunizations with the 9V or 1A9V peptides exhibited specific lytic reactivity against both EL4-HHD cells pulsed with the wild-type IL-13Rα2345-353 peptide and EL4-HHD-IL13R2 cells in vitro (Fig. 4C). In contrast, control splenocytes isolated from nonimmunized mice did not show antigen-specific lytic ability...
following 6-day in vitro restimulation with the 9V or 1A9V peptides, suggesting that the observed lytic activities in immunized mice reflect memory T-cell responses in these long-term survivors.

Peripheral immunizations with the IL-13Ra2-derived peptides also induced the infiltration of antigen-specific CD8+ T cells into the i.c. EL4-HHD-IL-13R tumor lesions, as shown by elevated numbers of IL-13Ra2-tetramer+/CD8+ T cells in BILs isolated from immunized versus control nonimmunized mice (Fig. 4D).

To determine whether any autoimmune status is induced, we evaluated lungs, livers, and brains from the immunized mice for any excessive lymphocyte infiltration or tissue damage using H&E staining. In addition, brain sections were evaluated by Luxol Fast Blue staining (32) for possible demyelination. Although we are aware that vaccine-induced atypical autoimmune encephalitis may present differently than typical demyelination, we found no evidence of autoimmune response against these normal organs.

**In vitro induction of anti-IL-13Ra2345-353 CTL responses from PBMCs isolated from HLA-A2+ glioma patients.** We have evaluated the ability of wild-type or analogue IL-13Ra2 peptides to stimulate specific CTL responses in vitro from 42 HLA-A2+ glioma patients. When using the 1A9V peptide for stimulations, 18 of 42 (42.1%) patients displayed positive cross-reactivity against the wild-type IL-13Ra2345-353 peptide loaded onto T2 cells or HLA-A2+ glioma cell lines that naturally process and present this epitope (Table 2). As we found that the 1A9V was more immunogenic than the wild type in the first several donors (Fig. 1), we did not test the wild-type peptide in all subsequent donors. Among 19 cases that were stimulated with wild-type peptide, 8 (42.1%) cases displayed positive reactivity. Although the positive response rate was not significantly different than that was observed with 1A9V, the magnitude of positive response in each case was constantly improved by the use of the 1A9V peptide compared with the wild-type peptide in accordance with the data in Fig. 1 (data not shown).

We also evaluated whether positive responsiveness was related to clinical variables, such as patient age (>50 or <50), type of glioma (glioblastoma multiforme versus others), and prior treatment (i.e., chemotherapy). We did not discern any statistically meaningful correlations among any of these indices.

**Discussion**

In the present study, we have shown that the IL-13Ra2345-353 peptide analogues 9V and 1A9V exhibit a superior capacity (versus the wild-type peptide) to induce CTLs capable of (cross-)reacting against the wild-type IL-13Ra2345-353 epitope. This was true in both HLA-A2+ patients with glioma and HLA-A2-transgenic HHD mice.

HLA-A2-binding and stability assays suggest that the improved immunogenicity of the 9V and 1A9V peptides was at least partially attributable to the higher binding/stability of these analogue peptides in HLA-A2 complexes that are required for specific CTL recognition. CTL assays analyzing reactivity versus peptide dose titration on T2 target cells indicated that the CTLs developed using the 9V or 1A9V analogue peptides possessed a higher functional avidity than those primed with wild-type peptide. Alternatively or additionally, our data could indicate that these peptide analogues promoted superior proliferation of specific CTL precursors owing to their improved binding/stability in HLA-A2 complexes.

As to whether the analogue-induced CTLs possess higher avidity, it has been shown that the affinity (33) or stability (34) between TCRs and peptide-MHC complexes correlates with the avidity of T cell-target interactions and the antitumor responsiveness of T cells among the total lymphocyte-gated population.

**Figure 4.** Vaccination with the IL-13Ra2345-353 analogue peptides 9V or 1A9V promote enhanced protective immunity in HHD mice against a normally lethal intracranial challenge with IL13Ra2-expressing syngeneic tumors. A to C, HHD mice were immunized twice in the same manner as described for experiments in Fig. 3 (n = 6 per group). On day 7 after the last vaccination, mice received stereotactic injections of 5 × 10^5 EL4-HHD-IL13Ra2 cells per mouse into the right basal ganglia. A, H&E staining of the tumor from a control, nonimmunized mouse. Original magnification, ×4. B, disease-free survival was monitored for each vaccination group. C, splenocytes were harvested from mice that survived for 100 days after the tumor challenge and stimulated in vitro for 6 days with the same peptide used for in vivo immunization (right). Splenocytes isolated from naive mice were also stimulated in vitro with the 9V or 1A9V peptide as controls (left). Splenocytes were tested for their lytic activity against peptide-pulsed EL4-HHD cells and EL4-HHD-IL-13Ra2 in 4 hours 51Cr release assays. D, HHD mice received 1 × 10^5 EL4-HHD-IL13Ra2 cells per mouse into the right basal ganglia on day 0 and were vaccinated with 9V or 1A9V peptides on days 14 and 21 (n = 5 per group). BILs were harvested on day 28, pooled within the same treatment group, stained with pycoerythrin-conjugated HLA-A2/IL13Ra2-tetramer and FITC-conjugated anti-CD8 mAb, and analyzed by flow cytometry. Numbers in each histogram indicate the percentage of CD8+ T cells among the total lymphocyte-gated population.
T cells. In these published studies, the intensity (33), or stability (34) of specific T-cell staining with HLA tetramers, and threshold of positive staining using titrating doses of tetramers (35) are purported to be indicative of the relative avidity of specific T cells. Using these methods, however, we were unable to detect any differences at the single cell level, suggesting that the functional avidity of specific CTLs was comparable regardless of the priming peptide used (data not shown).

In contrast, based on tetramer staining results obtained from both human HLA-A2+ donor- and HHD mouse–derived CTLs (splenocytes), the 9V and 1A9V analogue peptides seem to induce higher frequencies of specific CTLs when compared with stimulations using the wild-type peptide. Hence, we believe that the improved CTL-inducing capacity of the analogue peptides is likely attributable to the stabilization of specific antigen presentation to T-cell precursors, as has been shown by others (26).

As a surrogate marker for CTL responses, cytokine responses, such as IFN-γ (36, 37) and IL-2 (38), are often monitored. In our current study, we also evaluated IFN-γ and IL-2 secretion levels from CTL cultures stimulated with native or analogue peptides using cytokine-specific ELISA and IFN-γ enzyme-linked immunosorbent assays. However, our results failed to show any correlation between the levels of specific cytolyis and cytokine secretion by responder CD8+ T cells (data not shown). These suggest that more appropriate laboratory surrogate assays for CTL responses may involve measurements of perforin (39) and/or granzyme B (40) release.

Our HHD mouse models support the improved in vivo efficacy of protective vaccines incorporating the IL-13Rα2 analogue peptides. For BIL analyses, as we needed a large volume of tumors to obtain sufficient numbers of BILs, we used established tumors for immunizations. This may explain the relatively low frequency of IL-13Rα2-tetramer+/CD8+ T cells observed in BILs; nevertheless, our data suggest that vaccinations with the 9V or 1A9V peptides can yield systemic antigen-specific immunity but also the effective trafficking of vaccine-induced T effector cells into tumor lesions in concert with therapeutic efficacy.

In addition to these murine responders, we have also evaluated a total of 42 HLA-A2+ glioma patients for their in vitro responsiveness against wild-type and analogue IL-13Rα2(245-335) peptides. Nearly half of our patients developed specific CTLs capable of recognizing the wild-type IL-13Rα2 peptide after stimulation with the 1A9V analogue peptide (i.e., 42.9%, 18 of 42). These CTL recognized peptide-pulsed T2 cells or HLA-A2+ glioma cell lines that constitutionally express the IL-13Rα2 protein. Interestingly, various background factors, such as age, tumor type (glioblastoma multiforme versus other gliomas), and prior treatment with chemotherapy, did not correlate with the patient’s ability to respond preferentially to the analogue peptides. Although further analyses are clearly warranted to identify patient populations most suitable for vaccine trials implementing IL-13Rα2 wild-type and/or analogue peptides, these preliminary data suggest that conditions, such as prior application of chemotherapy by itself, should not serve as an exclusion criteria for participation in such prospective vaccine trials. Combination strategies using chemotherapy and vaccination may also be appropriate but require further characterization of the effects of chemotherapy on the tumor-bearing host immune response (41).

As the wild-type IL-13Rα2(245-335) epitope and its analogue peptides were originally characterized in the context of presentation by the HLA-A*0201 allele (16), it is possible that our flow cytometry–based assessment of HLA-A2+ PBMC may have allowed us to include patients bearing non-HLA-A*0201 A2 subtypes. This could have influenced our overall observed response rates, as previously observed for other CTL epitope peptides (42). In this regard, we recognize that further characterization of the analogue peptides for their ability to bind to a variety of A2 subtypes is warranted. It has been shown that >70% of the peptides that bind HLA-A*0201 with high affinity also bind at least two other A2 supertype molecules (42); thus, identification of those analogue peptides exhibiting high HLA-A*0201 binding affinity may portend to the use of these peptides in patients bearing a variety of HLA-A2 subtypes.

We have also noted that donor-derived PBMCs that did not respond to IL-13Rα2-1A9V can often react well against other GAAAs, such as EphA2_{883-891} (43) and GP100 (44). In addition, tumor cells may undergo “immunoediting” in vivo, selecting for heterogeneous progenitor lesions that may lose specific antigen or HLA allele expression (45). Given such variability, we believe that an optimal glioma vaccine design should include multiple GAA-derived T-cell epitopes (43, 44). Our current study strongly supports the inclusion of peptides, such as the IL-13Rα2-9V and/or -1A9V analogues, in such formulations designed to expanding GAA-specific CTL in glioma patients.

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**Table 2. Characteristics of the glioma patients evaluated in this study**

<table>
<thead>
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<th>Characteristic</th>
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<th>Nonresponder (n = 24)</th>
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</tr>
<tr>
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<td>10</td>
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<tr>
<td>&lt;50</td>
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<td>Tumor type</td>
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</table>

Abbreviations: GBM, glioblastoma multiforme; TMZ, temozolomide; CPT11, irinotecan.
References

Identification of Interleukin-13 Receptor \( \alpha_2 \) Peptide Analogues Capable of Inducing Improved Antiglioma CTL Responses

Junichi Eguchi, Manabu Hatano, Fumihiko Nishimura, et al.


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