Identification of a Novel HLA-A*0201-restricted, Cytotoxic T Lymphocyte Epitope in a Human Glioma-associated Antigen, Interleukin 13 Receptor α2 Chain[1]

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ABSTRACT

Purpose: **Interleukin 13 receptor α2-chain (IL-13Rα2) has been reported to be abundantly and specifically overexpressed in glioblastoma multiforme. Here we report the identification of a CTL epitope derived from the IL-13Rα2.**

Experimental Design: **Mature dendritic cells (DCs) were pulsed with each of the synthetic peptides that were designed, based on a binding affinity-based prediction and a proteosomal cleavage site prediction system, and used to stimulate autologous CD8+ T cells from an HLA-A2+ healthy donor. After four to six cycles of restimulation, the immunoreactivity of the T cells was analyzed for specific IFN-γ production and CTL reactivity.**

Results: **Of the five peptides tested, IL-13Rα$_{345–354}$ (WLPFGFILI) induced a CD8$^+$ T-cell line that specifically produced IFN-γ in response to HLA-A2+ T2 cells pulsed with the relevant peptide and lysed these cells. Peptide titration assays demonstrated that half-maximal lysis of IL-13Rα$_{345–354}$ peptide-reactive CD8$^+$ T cells required peptide loading concentration of ~5 nM. Perhaps most importantly, this CD8$^+$ T-cell line also displayed lytic activity against the HLA-A2+ human glioma cell lines that express IL-13Rα2.**

Conclusions: **This novel CTL epitope may therefore serve as an attractive component of peptide-based vaccines to treat glioma and as a surrogate marker of T-cell immune responses in patients before and after therapy.**

INTRODUCTION

Although cellular mechanisms underlying the "immunologically privileged" status of the CNS[3] and CNS tumors have been increasingly well-characterized during the past decade, it has also been becoming clearer that this "privileged" status is not absolute, and various new approaches of immunotherapy against CNS tumors have been suggested (1). Recent data from us (2, 3) and others (4) demonstrate that vaccinations with cytokine-gene-modified peripheral tumor cells can elicit therapeutic immune responses against intracranial gliomas. On the basis of our preclinical studies using IL-4-transfected glioma cells as vaccines (2, 3), we have begun Phase I clinical trials to test this strategy (5, 6). With regard to the concern of inducing an autoimmune reaction against normal brain components, no evidence of allergic encephalomyelitis was noted clinically or pathologically in 20 patients peripherally immunized with allogeneic glioma cells (7). A more practical limitation on the use of gene-modified whole cell brain tumor vaccines, however, is the considerable *ex vivo* manipulation of fresh glioma explants that is required to generate clinical grade vaccines. Tumor-specific, antigen-based vaccines, in contrast, would eliminate the potential risk of autoimmune encephalitis and be easily formulated using synthetic peptides and DCs generated from peripheral blood (8). We have reported preclinical efficacy of vaccinations using DCs loaded with synthetic peptides against intracranial tumors (9), and clinical efficacy of vaccinations with CTL epitopes has been demonstrated in other forms of cancers (8, 10). The development of glioma-specific, antigen-based vaccine therapy has awaited identification of glioma-specific, antigen-derived CTL epitopes. It has been well-documented that IL-13Rα2 is a glioma-associated antigen that has a cancer-testes antigen-type expression profile (11, 12). In this study, we assessed whether specific T-cell responses could be induced by IL-13Rα2-derived antigens in HLA-A*0201$^+$, CD8$^+$ T cells. For prediction of HLA-A*0201-restricted epitopes, we used a binding affinity-based prediction, BioInformatics & Molecular Analysis Section, and a proteosomal cleavage site prediction system. Our results indicate that we have isolated a novel HLA-A*0201-restricted CTL epitope in a glioma-associated antigen, IL-13Rα2.

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[3] The abbreviations used are: CNS, central nervous system; IL, interleukin; rhIL, recombinant human IL; IL-13R, IL-13 receptor; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; FBS, fetal bovine serum; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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MATERIALS AND METHODS

Cells and Cell Culture. Healthy volunteers were genetically typed as being *HLA-A0201*$^+$ at the Histocompatibility Center in Children's Hospital (Pittsburgh, PA; University of Pittsburgh). PBMCs from an HLA-A*0201$^+$ healthy donor were isolated using lymphocyte separation medium gradient (Ref. 13; Organon-Teknika, Durham, NC). The *HLA-A*0201*-transfected, transporter associated with antigen processing-deficient (T × B) cell hybrid T2 cell line (T2.A2; provided by Dr. Janice Blum, Indiana University, Indianapolis, IN) was maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FBS (Life Technologies, Inc.), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all reagents from Life Technologies, Inc.).

The T98G and A172 glioma cell lines were purchased from the American Type Culture Collections (Manassas, VA). The U251 and SNB19 glioma cell lines were kindly provided by Drs. M. R. Jadus (University of California, Irvine, CA) and W. C. Welch (University of Pittsburgh, Pittsburgh, PA; Ref. 14), respectively. These glioma cell lines uniformly express HLA-A*0201 and were cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10 mM L-glutamine (all reagents from Life Technologies, Inc.).

Selection and Synthesis of Candidate HLA-A*0201-binding Peptides Derived from IL-13Rα2. The protein sequences of IL-13Rα2 was obtained from GenBank and analyzed for HLA-A*0201 binding motifs using BioInformatics & Molecular Analysis Section and a proteosomal cleavage site prediction system (15), respectively. Peptide sequences that were given high binding scores and predicted proteosomal cleavage sites at the ends of the sequences were chosen. Peptides were synthesized by FMOC chemistry in the University of Pittsburgh Cancer Institute Peptide Synthesis Facility and were >90% pure as indicated by analytical high-performance liquid chromatography. Peptides were dissolved in 100% DMSO at a concentration of 2 mg/ml and stored at −20°C until use.

RNA Isolation and RT-PCR Analysis of Expression of IL-13Rα2. For RT-PCR analysis, reverse transcription was performed using total cellular RNA extracted by the guanidinium thiocyanate/cesium chloride method (16) in the presence of oligo dT and SuperScript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's instructions. The primers used for amplification of human IL-13Rα2 and GAPDH were as follows: IL-13Rα2 (S), 5′-TGGTCAGAAGTGTGCCTGTC-3′; (AS), 5′-TCTGCCCAGGAACTTTGAAC-3′; and GAPDH (S), 5′-ACAGTCAGCCGCATCTTCTT-3′; (AS), 5′-TTGATTTTGGAGGGATCTCG-3′. The PCR reactions using Ex taq DNA polymerase (Takara Co., Ohtsu, Shiga, Japan) were subjected to 25 amplification cycles in a Geneamp PCR System 2700 (Applied Biosystems, Foster, CA); conditions consisted of 94°C denaturation for 30 s, 57°C primer annealing for 30 s, followed by primer extension at 72°C for 1 min.

***In Vitro* Induction of Anti-IL-13Rα2 CTLs Using the Peptide-pulsed Autologous DCs.** DCs were prepared from the donor-derived PBMCs as described previously (17) with minor modifications. Briefly, the plastic adherent cells from PBMCs were cultured in AIM-V medium (Life Technologies, Inc.) supplemented with recombinant human granulocyte/macrophage-colony stimulating factor and recombinant human IL-4 (1000 units/ml each) at 37°C in a humidified CO_2 (5%) incubator. Six days later, the culture medium was removed, and the immature DCs were cultured in AIM-V supplemented with recombinant human granulocyte/macrophage-colony stimulating factor, rhIL-4, rhIL-6 (1000 units/ml each), recombinant human tumor necrosis factor-α, and IL-1β (10 ng/ml each). Mature DCs were harvested on day 8, resuspended in AIM-V medium at 1×10^6 cells/ml with peptide (10 μg/ml), and incubated for 4 h at 37°C. The peptide-pulsed DCs were then irradiated (3000 rad), washed, and resuspended in AIM-V medium supplemented with 10% human AB serum. Populations of autologous CD8$^+$ T cells were enriched from PBMCs using magnetic microbeads (Miltenyi Biotech, Auburn, CA) and were added (1×10^6/well) to the peptide-pulsed DCs (1×10^5/well) in 2 ml of AIM-V medium supplemented with 10% human AB serum, 1000 units/ml rhIL-6, and 10 ng/ml rhIL-12 in each well of 24-well tissue culture plates. On day 7, lymphocytes were restimulated with irradiated autologous DCs pulsed with peptide in AIM-V medium supplemented with 10% human AB serum, rhIL-2, and rhIL-7 (10 units/ml each). One week later and weekly thereafter, responder cells were restimulated with irradiated peptide-pulsed autologous PBMCs in medium supplemented with rhIL-2 and rhIL-7 (10 units/ml each). The CD8$^+$ cultured cells were analyzed for IFN-γ production and CTL activity 7 days after each stimulation.

Screening and Characterization of Reactivity in T Cells for the IL-13Rα2-derived Peptide and Tumor Cells. T2.A2 cells were inoculated at 5×10^4 cells/well in flat-bottomed 96-well culture plates and incubated for 4 h at 37°C in the presence of peptides, at the concentrations indicated in each experiment. CD8$^+$ T cells (5×10^3/well) were added in 100 μl of AIM-V medium supplemented with 10% FBS. Supernatants were harvested after 24 h, and IFN-γ was measured by ELISA. CTL activity of the CD8$^+$ effector cells was assessed in a standard 4-h ^{51}Cr-release assays.

RESULTS AND DISCUSSION

Induction of a T-Cell Line That Specifically Recognized Peptide IL-13Rα2$_{345-354}$ Presented on HLA-A*0201. On the basis of the algorithms, we synthesized the following five peptides: FILILVIFV (350–358), YLQWQPPLSL (50–59), WLPFGFILI (345–354), WQCTNGSEV (111–119), and VIFVTLLL (355–363). DCs derived from a healthy HLA-A*0201$^+$ donor were incubated with each of the five peptides (10 μg/ml) and used to stimulate autologous CD8$^+$ T cells. The individual responder cell cultures were then restimulated on a weekly basis with autologous DCs or PBMCs loaded with the corresponding peptide used in the primary stimulation. Among the five peptides tested, four cycles of stimulation with IL-13Rα2$_{345-354}$ resulted in continuous growth of responder T cells (data not shown). Specific immunoreactivity of the induced T-cell line was first tested with T2.A2 cells loaded with the relevant peptides, irrelevant peptides, or no peptides. As demonstrated in Fig. 1*A*, the T-cell line produced ~400 pg/ml of IFN-γ in response to T2.A2 cells pulsed with IL-13Rα2$_{345-354}$, whereas it produced <50 pg/ml in response to T2.A2 cells alone

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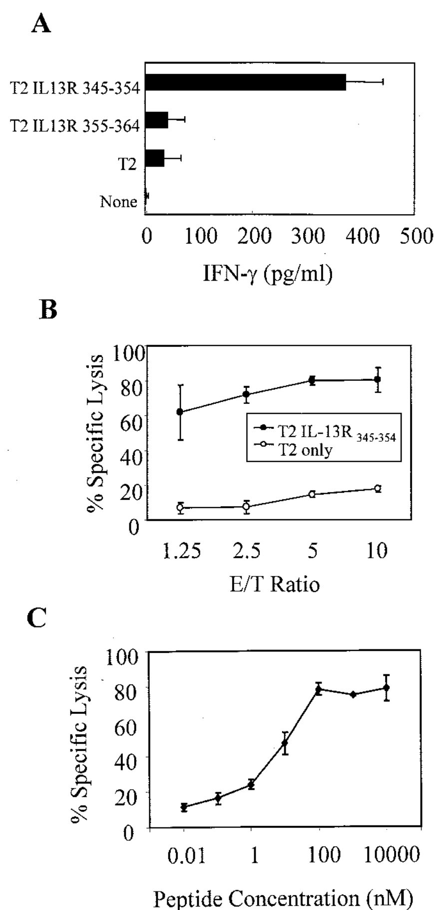

Fig. 1 Stimulation of $CD8^+$ T cells from a HLA-A*0201$^+$ healthy donor with IL-13R$\alpha_{345-354}$ peptide (WLPFGFILI) induced a CTL line that specifically recognized and lysed T2.A2 cells pulsed with the relevant peptide. $CD8^+$ T cells from an HLA-A*0201$^+$ healthy donor were stimulated *in vitro* with autologous DCs pulsed with the IL-13R$\alpha 2_{345-354}$ peptide. *A,* aliquots of 1000 stimulated T cells were incubated in the presence of 5×10^4/well T2 cells pulsed with 10 μg/ml IL-13R$\alpha 2_{345-354}$, an irrelevant IL-13R$\alpha 2_{355-364}$ peptide, or without peptide for 24 h. Supernatant samples were then harvested and analyzed for production of IFN-γ by a specific ELISA. Data from one representative experiment of three performed with similar results are depicted; *bars,* SD. *B,* lysis of T2 cells pulsed with IL-13R$\alpha 2_{345-354}$ peptide (WLPFGFILI) by the induced $CD8^+$ T cells. T2.A2 cells were preincubated with 10 μg/ml IL-13R$\alpha 2_{345-354}$ peptide for 1 h and then labeled with ^{51}Cr for 1 h. Aliquots of 5×10^3 labeled target cells were then incubated with the responder T cells at the indicated ratios for 4 h. Data shown is representative of three experiments with similar results. ●, T2 cells pulsed with IL-13R$\alpha 2_{345-354}$ peptide; ○, T2 cells without peptides. *Bars,* SD. *C,* peptide titration of the CTL line specific for IL-13R$\alpha 2_{345-354}$ peptide. ^{51}Cr-labeled T2 target cells were preincubated with various concentrations of IL-13R$\alpha 2_{345-354}$ peptide. The T8.IL-13R CTLs were then added at an E:T ratio of 10:1. After 4 h of incubation, chromium release was measured. Data represent the average of triplicate cultures; *bars,* SD.

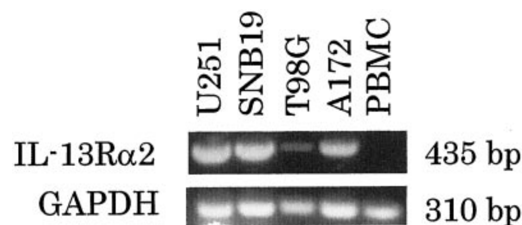

Fig. 2 Glioma cell lines express message for IL-13Rα2. Expression of mRNA encoding IL-13Rα2 was analyzed in four glioma cell lines by RT-PCR. The origin of the RNA is indicated at the *top* of each lane. The amount of the template cDNA was adjusted by the 25-cycle amplification with primers for *GAPDH* as a housekeeping gene, then the adjusted amount of cDNA template was used for a 25-cycle amplification with primers for IL-13Rα2 that were designed to generate the 435-bp product. Data are representative of three independent experiments with similar results.

or T2.A2 cells pulsed with the irrelevant peptide, IL-13R$\alpha 2_{355-364}$, indicating that the response was peptide specific. After two additional cycles of stimulation, the responder cells were tested for cytotoxic activity. As shown in Fig. 1*B,* the T cells efficiently lysed T2 target cells pulsed with the relevant peptide, whereas only low background lysis was observed in the absence of the peptide. These results demonstrated that the CTL line induced with IL-13R$\alpha 2_{345-354}$ recognized the relevant antigen-peptide specifically and was able to lyse cells presenting the peptide. The CTL line was thereby designated as T8.IL-13R.

Subsequently, the minimum stimulatory concentration was determined using the T8.IL-13R CTL line and T2.A2 cells loaded with various concentrations of the IL-13R$\alpha 2_{345-354}$ peptide. T2.A2 cells were pulsed with increasing concentrations of the peptide, and the lysis by T8.IL-13R was determined (Fig. 1*C*). Peptide titration demonstrated that half-maximal lysis by this T-cell line was obtained at IL-13R$\alpha 2_{345-354}$ peptide concentrations between 10 and 100 nM. These results indicate that the T8.IL-13R line recognized IL-13R$\alpha 2_{345-354}$ with a sensitivity comparable with several known HLA-binding epitopes from nonmutated peptides, such as those derived from melanoma antigens (17, 18).

The T8.IL-13R Line Specifically Lysed HLA-A*0201$^+$ Glioma Cells That Express IL-13Rα2. More importantly, we examined whether T8.IL-13R cells were able to recognize and lyse HLA-A*0201$^+$ human glioma cells that endogenously expressed and presented IL-13Rα2-derived epitopes. Human glioma cell lines U251, SNB19, and T98G were determined to express HLA-A*0201 by a flow cytometric analyses, but human glioma cell line A172 did not express HLA-A*0201 (data not shown). Fig. 2 demonstrates the expression of IL-13Rα2 in these glioma cell lines as determined by semiquantitative RT-PCR. The U251, SNB19, and A172 cell lines expressed a high level message for IL-13Rα2, whereas the T98G cell line expressed a low level of message, and the IL-13Rα2-specific message was completely absent in PBMCs.

The lytic ability of T8.IL-13R against the HLA-A*0201$^+$, IL-13R$\alpha 2^+$ glioma cells was examined using 4-h ^{51}Cr-release assays. As illustrated in Fig. 3, the U251 and SNB19 cell lines were highly susceptible to lysis by the CTL line, whereas lower, but significant, lysis was also observed using T98G cells. On the other hand, the HLA-A*0201$^-$, IL-13R$\alpha 2^+$ glioma cell line, A172, was not susceptible to lysis by the CTL line, suggesting that the CTL reaction is HLA-A*0201 restricted. To determine the specificity of the lytic activity, cold target competition experiments were performed by the addition of nonradiolabeled

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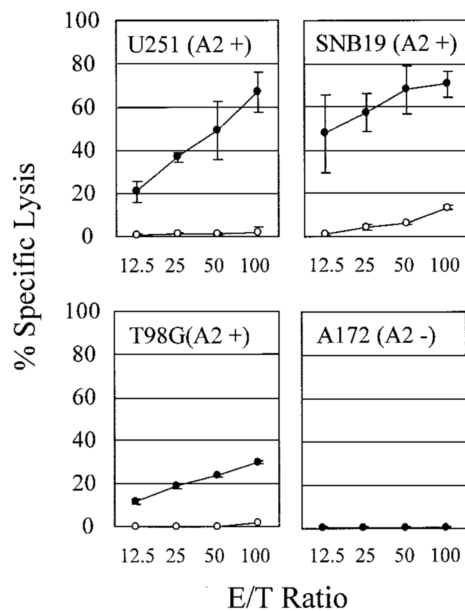

Fig. 3 The CTL line raised against IL-13Rα2$_{345–354}$ peptide specifically lysed HLA-A*0201^{+}, IL-13Rα2^{+} glioma cells. The T8.IL-13R CTLs were incubated for 4 h with ^{51}Cr-labeled human glioma cell lines U251, SNB19, T98G, and A172 at the indicated E:T ratios for evaluation of specific lytic ability (●). For the cold target inhibition assay (○), ^{51}Cr-labeled tumor target cells (1×10^3 cells) and cold T2.A2 cells (1×10^4 cells) pulsed with peptide WLPFGFILI were incubated with the CTLs. *Bars,* SD.

T2.A2 cells pulsed with IL-13Rα2$_{345–354}$ peptide in the 4-h ^{51}Cr-release assay. The lytic activity of the CTL line against these glioma cell lines was almost completely inhibited by the addition of the cold target, demonstrating that the lytic ability was specific for the epitope IL-13Rα2$_{345–354}$.

These data indicated that that the CTL line raised against IL-13Rα2$_{345–354}$ peptide was capable of recognizing and lysing HLA-A*0201^{+} glioma cell lines that endogenously expressed IL-13Rα2, suggesting that the IL-13Rα2$_{345–354}$ peptide might be useful for inducing antiglioma immunoreactivity.

Although information based on RT-PCR is only semiquantitative, U251 and SNB19 cells appeared to express a higher level of message for IL-13Rα2. Accordingly, flow cytometric analyses by Bernard *et al.* (19) have demonstrated high-level expression of IL-13Rα2 protein on the surface of U251 and SNB19 cells, whereas in the same study, T98G cells did not express a detectable level of IL-13Rα2. In another study (19), low levels of expression of IL-13Rα2 on T98G cells was described. In our study, susceptibility of U251, SNB19, and T98G cells against the T8.IL13R CTLs appears to be correlated with the expression level of IL-13Rα2; however, other factors, such as expression level of functional HLA-A*0201, must also be taken into consideration.

Future Applications of the Novel Epitope IL-13Rα$_{345–354}$. IL-13Rα2 has been reported by a significant proportion of malignant gliomas (11), and approximately 40–50% of Caucasians and Asians express the *HLA-A*0201* allele (20). This novel CTL epitope may therefore serve as an attractive component of peptide-based vaccines to treat glioma and as a surrogate marker of T-cell immune responses in patients before and after therapy.

We also recognize that identification and subsequent implementation of one T-cell epitope as a therapeutic target may not be sufficient to improve the dismal prognosis of patients with malignant gliomas because of the marked heterogeneity of the disease. However, our efficient algorithms used for epitope prediction and *in vitro* analysis may soon lead to the identification of additional T-cell epitopes for glioma-derived antigens.

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