HLA Tetramer – Based Artificial Antigen-Presenting Cells Efficiently Stimulate CTLs Specific for Malignant Glioma

Xiaobing Jiang, Xiaoling Lu, Ruen Liu, Fangcheng Zhang, and Hongyang Zhao

Abstract

Purpose: The interleukin-13 receptor α2 (IL-13Rα2) is a glioma-restricted cell-surface epitope not otherwise detected within the central nervous system. Here, we report a novel approach for targeting malignant glioma with IL-13Rα2–specific CTLs.

Experimental Design: Artificial antigen-presenting cells (aAPC) were made by coating human leukocyte antigen (HLA)-A2/pIL-13Rα2345-354 tetrameric complexes, anti-CD28 antibody, and CD83 molecules to cell-sized latex beads, and used to stimulate IL-13Rα2–specific CTLs from the peripheral blood mononuclear cells of HLA-A2+ healthy donors. After multiple stimulations, the induced CTLs were analyzed for tetramer staining, IFN-γ production, and CTL reactivity.

Results: Tetramer staining assay showed that the induced CTLs specifically bound HLA-A2/pIL-13Rα2345-354 tetramers. The CTLs specifically produced IFN-γ in response to the HLA-A2/pIL-13Rα2345-354–aAPCs and exhibited specific lysis against T2 cells pulsed with the peptide pIL-13Rα2345-354 and HLA-A2+ glioma cells expressing IL-13Rα2345-354, whereas HLA-A2+ glioma cell lines that express IL-13Rα2345-354 could not be recognized by the CTLs. The peptide-specific activity was inhibited by anti–HLA class I monoclonal antibody.

Conclusion: The induced CTLs specific for IL-13Rα2345-354 peptide could be a potential target of specific immunotherapy for HLA-A2+ patients with malignant glioma.

Despite significant advances in modern microsurgery, radiotherapy, and chemotherapy, the prognosis for patients with malignant glioma remains poor (1). Therefore, the development of a new treatment modality is needed. Although the central nervous system (CNS), and tumors that arise therein, reside in an "immunologically privileged" site (2), many data have successfully shown that effective anti-CNS tumor immune responses can be induced in preclinical models using syngeneic tumor- and dendritic cell–based vaccines (3–5). In addition, activated, antitumor immune cells have the potential to migrate into the CNS and to selectively destroy malignant cells that have infiltrated normal CNS tissues (6). Therefore, immunotherapy for glioma is an attractive alternative treatment option.

The induction of antigen-specific CTLs has been suggested to be highly efficacious in the prevention and treatment of various types of tumors (7). CTLs recognize “processed” peptides that are derived from endogenous proteins and presented on the cell surface in association with MHC class I molecules (7, 8). Peptides that bind to a given MHC class I molecule have been shown to share common amino acid motifs, which are called major anchor motifs (8). Hence, tumor-specific CTLs can recognize and select the antigenic peptides by scanning peptide sequences, and then kill tumor cells in an antigenic peptide-specific fashion. Interleukin-13 receptor α2 (IL-13Rα2) chain has been reported to be abundantly and specifically overexpressed in malignant glioma and not otherwise presented in the normal brain (9–11). IL-13Rα2–specific CTLs can be generated in vitro using peptide-pulsed autologous dendritic cells as antigen-presenting cells (APC; ref. 12). However, this method is time-consuming and expensive due to the limited availability of donor-derived dendritic cells. To date, several reports indicated effective stimulation of T cells by human leukocyte antigen (HLA)-peptide ligand coating on aAPCs, such as liposomes (13, 14) and microbeads (15–18). The aAPCs simulate the natural APCs (such as dendritic cells, macrophages, and B cells), which are able to provide the dual signals (the antigen-specific and costimulatory signals) for T-cell activation. These aAPCs can be prepared by coating the T-cell receptor ligand (peptide-MHC) and costimulatory molecules (such as B7) on a cell-sized bead, and it provides a practical and convenient approach to generating antigen-specific T cells for adoptive immunotherapy.

In this study, we explored a simple and efficient method to induce IL-13Rα2345-354–specific CTLs from the peripheral blood mononuclear cells (PBMC) of HLA-A2+ normal donors using aAPCs made by coating HLA-A2/pIL-13Rα2345-354.
complexes, anti-CD28 antibody, and CD83 to cell-sized latex microbeads in vitro, in which these CTLs kept their antigen-specific cytolytic properties.

Materials and Methods

Cell line and cell culture. T2 cells were kindly provided by Prof. Nicholas Zavazava (Kiel University, Kiel, Germany). This cell bears the HLA-A*0201 gene, but expresses a very low level of cell-surface HLA-A2.1 molecules and is unable to present endogenous antigens due to a deletion of most of the MHC class II region, including the transporter associated with antigen processing and genes encoding for immunoproteasomal subunits. This cell line was maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and antibiotics. Gloma cell lines U251 (HLA-A2*, IL-13Rα*) and A172 (HLA-A2*, IL-13Rα*) were purchased from the American Type Culture Collection. These gloma cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics.

Synthetic peptides. The following HLA-A2-restricted peptides were used in this study: the IL-13Rα-derived peptide WLPFGFILL (referred to as pIL-13Rα2345-354; 12) and the control peptide HIV-Gag peptide SLYNTVATL (pHIV; ref. 19). Peptides were synthesized by standard solid-phase chemistry and characterized by mass spectrometry. The purity of the synthetic peptides was >95% as indicated by analytical HPLC. Lyophilized peptides were dissolved in DMSO and stored at -80°C after dilution in PBS.

HLA class I typing. HLA class I typing was done with microcytotoxicity by Lambda Antigen Trayce Class I (One Lambda, Inc.).

Detection of IL-13Rα2 gene expression in gloma cell lines by reverse transcription-PCR. The expression of IL-13Rα2 mRNA in gloma cell lines U251 and A172 was detected with reverse transcription-PCR. The primers used for amplification of human IL-13Rα2 and GAPDH were as follows: IL-13Rα2, 5′-TGTGGCAGAAGTGTGCTGTC-3′ (S) and 5′-TCTGCCCCAGGAACTTTGAAC-3′ (AS); GAPDH, 5′-ACAAGTACGCCG-CATCTTCTT-3′ (S) and 5′-TGGATTTTGGAGGGATCTCG-3′ (AS). The PCR reactions using Ex Taq DNA polymerase (Life Technologies) were subjected to 30 amplification cycles; 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.

Preparation of HLA-A2/pIL-13Rα2345-354 tetramers. Synthesis of HLA-A2/pIL-13Rα2345-354 tetramers was carried out according to the protocol of Alman et al. (20). Briefly, plasmids encoding HLA-A*0201 (heavy chain) molecules with a COOH-terminal biotinylation site and human β2m molecule were constructed by insertion of the target genes into pET28a. The heavy chain and β2m molecules were expressed in Escherichia coli, purified from inclusion bodies, and then refolded in the presence of excess pIL-13Rα2345-354 to form the HLA-A2/pIL-13Rα2345-354 monomeric product. The folded product was then subjected to enzymatic biotinylation by BirA enzyme (Avidity) at 25°C for 12 h. This biotinylated HLA-A2/pIL-13Rα2345-354 monomer was used for the preparation of the HLA-A2/pIL-13Rα2345-354 tetramer. The HLA-A2/pIL-13Rα2345-354 tetramer was produced by mixing the purified biotinylated monomer with FITC-labeled streptavidin (Sigma) at a molar ratio of 4:1. HLA-A2/pHLIV tetramer was also prepared according to the manufacturer’s protocol.

Generation of HLA-A2/pIL-13Rα2345-354-aAPCs. The 5-μm sulfamate polystyrene latex (interfacial Dynamics) was incubated sequentially with streptavidin (1 μg/106 beads; Sigma), CD28-specific antibody, and CD83 (1 μg and 1.5 μg/106 beads, respectively; BD PharMingen) for 30 min in 1-mL PBS per 106 beads at 4°C on a rotator. The beads were then incubated with biotinylated HLA-A2/pIL-13Rα2345-354 complexes (2 μg/106 beads) for 1 h at 4°C on a rotator. After each incubation step, the beads were washed with PBS. The HLA-A2/pIL-13Rα2345-354-aAPCs were stored in PBS at 4°C.

Induction of CTL. PBMCs from five healthy HLA-A2* donors were separated using standard Ficoll-Hypaque (Sigma) gradient density centrifugation. The PBMCs were stimulated with HLA-A2/pIL-13Rα2345-354-aAPCs in vitro using the protocol adapted from previous studies (21). Briefly, The PBMCs were used as responder cells and stimulated with HLA-A2/pIL-13Rα2345-354-aAPCs. Responder cells (3 × 105 per well) were cocultured with HLA-A2/pIL-13Rα2345-354-aAPCs (3 × 105 per well) in 24-well plates in RPMI 1640 supplemented with 10% fetal bovine serum (1 ml/well). Interleukin-7 (IL-7; 10 ng/mL, PeproTech EC) was added on day 1. IL-2 (50 units/mL, R&D Systems) was added on day 4. Medium, IL-2, and IL-7 were replenished twice a week. On day 7 and weekly thereafter, responder cells were collected, counted, and replated at 3 × 106 per well, together with 3 × 103 HLA-A2/pIL-13Rα2345-354-aAPCs per well in RPMI 1640 with 10% fetal bovine serum, 10 ng/mL IL-7, and 50 units/mL IL-2. After a total of four rounds of stimulations in vitro, responder cells were tested for tetramer staining and cytotoxicity assay.

Tetramer staining. Tetramer staining was done as previously described (22, 23). In brief, 1 × 106 cells were incubated in 100-μL fluorescence-activated cell sorting staining buffer (PBS supplemented with 1% bovine serum albumin and 0.05% NaN3) with 20 μg/mL HLA-A2/peptide tetramer at 37°C for 30 min. Cells were washed with PBS and subsequently incubated with phycoerythrin-Cy5-labeled anti-CD8 antibody (BD Pharmingen) at 4°C for 30 min. All cells were washed with PBS twice after being stained, and then fixed in 1% formaldehyde. Stained cells were analyzed with FACSCalibur (Becton Dickinson).

ELISA. To determine the frequency of T cells capable of responding to a specific stimulus by secretion of IFN-γ, an enzyme-linked immunosorbent (ELISOT) assay was done. The HLA-A2/pIL-13Rα2345-354-aAPCs were used as stimulating target cells. Beads pulsed without HLA-A2/pIL-13Rα2345-354 complexes were used as the negative control. Responder cells were cocultivated with the target cells at a stimulator-to-responder cell ratio of 1:10 at 37°C for 24 h in 96-well ELISOT plates. The assay was done and developed according to the manufacturer’s instructions (BD Pharmingen).

Cytotoxicity assay. To analyze the cytotoxic activity of the HLA-A2/pIL-13Rα2345-354-aAPCs-induced CTLs against various target cells, cytotoxic assays were done by incubating 11Cr-labeled (500 μCi) target cells with effector cells at various effector/target ratios at 37°C for 4 h. Gloma cell lines U251 (HLA-A2*, IL-13Rα*), and A172 (HLA-A2*, IL-13Rα*), K562, and T2 cells pulsed without a peptide were selected as target cells. Other target cells, T2pIL-13Rα2345-354 (pIL-13Rα2345-354-pulsed T2 cells) and T2PHV (PHV-pulsed T2 cells), were prepared by incubating T2 cells with pIL-13Rα2345-354 (50 μg/mL) and pHIV (50 μg/mL) for 3 h at 37°C. The percentage of 11Cr release was calculated according to the following formula: 100 × (experimental release - spontaneous release) / (maximum release - spontaneous release).

Inhibition of the cytotoxicity with HLA class I-specific monoclonal antibody. T2pIL-13Rα2345-354 and U251 target cells were incubated with anti-HLA class I monoclonal antibody (mAb) W6/32 (American Type Culture Collection; ref. 24) and a control isotypic mAb of irrelevant specificity IgG2 (BD Pharmingen) at a final concentration of 30 μg/mL for 40 min at 4°C before cytotoxicity assay. After incubation, the target cells were mixed with effector cells for the 11Cr release assay.

Statistical analysis. All data in this study were analyzed using SPSS version 11.0 software (SPSS). P < 0.05 was considered as statistically significant.

Results

IL-13Rα2 gene expression and HLA class I typing in gloma cell lines. IL-13Rα2 mRNA expression was detected in U251 and A172 cell lines. The results of HLA class I typing detection

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to a mean expansion of 50- to 60-fold. The phenotype of the expanded cells was measured by flow cytometry. The percentage of CD4+ cells gradually decreased whereas the CD8+ cells increased with the progression of the coculture with HLA-A2/pHIV-pulsed T2 cells. The phenotypes of the CTLs for pIL-13Rα2345–354 were CD8+, CD4+, CD3+, CD16+, and CD56+.

Frequency of pIL-13Rα2345–354–specific CTLs increased by coculture with HLA-A2/pIL-13Rα2345–354-aAPCs as determined by tetramer staining. A flow cytometric analysis of PBMCs was done before or after coculture with the HLA-A2/pIL-13Rα2345–354-aAPCs. Before the four rounds of stimulation using HLA-A2/pIL-13Rα2345–354-aAPCs, the frequency of CD8+ T cells stained with HLA-A2/pIL-13Rα2345–354 tetramers was 0.11%. However, after stimulation, fluorescence-activated cell sorting analysis revealed that 14.7% of CD8+ T cells were stained with HLA-A2/pIL-13Rα2345–354 tetramers, which was not observed when staining with the control tetramers (HLA-A2/pHIV tetramer; Fig. 1). The pIL-13Rα2345–354–specific CTLs expanded by HLA-A2/pIL-13Rα2345–354-aAPCs from the other four normal donors showed similar results (Table 1).

ELISPOT assay. The ELISPOT assay was used to determine the frequency of individual antigen-specific IFN-γ producing T cells. As shown in Fig. 2, priming with HLA-A2/pIL-13Rα2345–354–specific aAPCs resulted in the generation of peptide-specific IFN-γ–producing CD8+ T cells after the stimulation. The average spot number of responding lymphocytes induced by HLA-A2/pIL-13Rα2345–354–specific aAPCs (164 ± 35 peptide-specific spots/10^5 cells) was significant higher than that of responding lymphocytes induced by beads without HLA-A2/pIL-13Rα2345–354 (41 ± 8 peptide-specific spots/10^5 cells; P < 0.05).

Cytotoxicity of pIL-13Rα2345–354–specific CTLs. The cytotoxic activity of the HLA-A2/pIL-13Rα2345–354–specific aAPCs–induced CTLs against various target cells was tested with the 51Cr release assay. The CTLs exhibited ~70% specific lysis against T2pHIV, A172 (HLA-A2–, IL-13Rα2+), K562, and the T2 without a pulsed peptide at the same effector/target ratio of 50:1. However, the CTLs showed an ~10% cytolysis against T2pHIV, A172 (HLA-A2–, IL-13Rα2+), K562, and the T2 without a pulsed peptide at the same effector/target ratio (Fig. 3A). The specific killing activity of the CTLs induced by the HLA-A2/pIL-13Rα2345–354–specific aAPCs against specific target cells was much more obvious than in any other control group (P < 0.05). The specific CTLs for pIL-13Rα2345–354 induced by HLA-A2/pIL-13Rα2345–354–specific aAPCs from the other four normal donors showed similar specific lysis (Table 1). This result shows that the cytotoxicity of the HLA-A2/pIL-13Rα2345–354–specific aAPCs–induced CTL is pIL-13Rα2345–354 specific.

Table 1. Induction of CTLs specific for pIL-13Rα2345–354 in four HLA-A2+ healthy donors

<table>
<thead>
<tr>
<th>Donor</th>
<th>Tetramer staining (%)</th>
<th>Cytotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HLA-A2/pHIV</td>
<td>HLA-A2/pIL-13Rα2345–354</td>
</tr>
<tr>
<td>1</td>
<td>0.14</td>
<td>15.9</td>
</tr>
<tr>
<td>2</td>
<td>0.17</td>
<td>16.4</td>
</tr>
<tr>
<td>3</td>
<td>0.09</td>
<td>14.6</td>
</tr>
<tr>
<td>4</td>
<td>0.08</td>
<td>13.8</td>
</tr>
</tbody>
</table>

NOTE: The CTLs induced by the HLA-A2/pIL-13Rα2345–354–specific aAPCs for 28 d were tested for tetramer staining and cytotoxic activity. Tetramer-staining cells indicate the percentage of viable CD8+ HLA-A2+ with pIL-13Rα2345–354 tetramer–positive or CD8+ HLA-A2+ with pHIV tetramer–positive lymphocytes in the total population of viable lymphocytes; lysis indicates the percent lysis of pIL-13Rα2345–354–pulsed T2 cells (T2pIL-13Rα2345–354), U251 (HLA-A2–, IL-13Rα2+), or pHIV-pulsed T2 cells (T2pHIV). Results are shown for an effector/target ratio of 50:1.
Inhibition of cytotoxicity of HLA-A2/pIL-13R<sub>a</sub>2345-354-aAPCs–induced T cells by HLA class I–specific mAb (W6/32). To determine whether the induced CTLs could recognize the specific target cells in a HLA class I–restricted manner, anti–HLA class I mAb W6/32 was used to block the cytotoxicity of the HLA-A2/pIL-13R<sub>a</sub>2345-354-aAPCs–induced CTLs. The cytotoxic activity against T2pIL-13R<sub>a</sub>2345-354 and U251 was significantly eliminated by W6/32. As shown in Fig. 3B and C, incubation of T2pIL-13R<sub>a</sub>2345-354 and U251 target cells with W6/32 led to the inhibition of the targeted cells lysis, whereas mouse IgG2, used as an isotype control, showed no effect. These results suggested that the HLA-A2/pIL-13R<sub>a</sub>2345-354-aAPCs–induced CTLs lysed the specific targets in a HLA class I–restricted manner.

Discussion

Malignant gliomas represent a significant class of CNS tumors derived from the glial lineage. Despite recent advances in traditional treatment options, the prognosis for these patients has not changed appreciably. The 5-year survival rate for patients harboring the most common class of gliomas, glioblastoma multiforme, is <2% (25). In an effort to improve the outcome of patients with respectable brain tumors, there have been attempts to give adjunctive therapies consisting of radiation with or without chemotherapy. Thus far, research over three decades has failed to provide definitive evidence of improved outcome (i.e., overall survival and disease-free survival) in patients. Clearly, modalities other than radiation and/or chemotherapy must be explored in an adjuvant setting. Immune-based treatments represent a promising new class of therapy designed to harness the immune system to specifically eradicate malignant cells. However, immunotherapy for tumors located in the CNS has generally not achieved the results seen for peripherally located tumors (26–29). These previous findings have historically been ascribed to the “immune privilege” of the CNS (30). In reality, however, the immune privilege of the brain is not absolute but was originally used to describe the observation that
tissue and tumor grafts survived better in the CNS than in other peripheral sites (31). Effective anti-CNS tumor immune responses have been generated by immune-based treatments such as adoptive T-cell transfer (32–37), GAA-pulsed dendritic cells (3–5, 38), and cytotoxic-secretin glioblasts (39–41), lending further credence to the idea that the efficient induction of a cellular antitumor immune response can be targeted to antigens within the CNS.

Tumor-specific CD8-positive CTLs constitute the most important effector cells for antitumor responses (8). It has previously been shown that in vitro specific CTLs can be generated using peptide-pulsed autologous dendritic cells as APCs (42, 43). However, CTL expansion to clinically relevant amounts requires multiple leukophereses to obtain enough autologous dendritic cells. Variability in both quantity and quality of obtained dendritic cells, which presumably relate to underlying diseases and the pretreatment of the patients, also significantly affects the viability of dendritic cell–based therapeutics. For these reasons, use of dendritic cells has been a limiting step in ex vivo expansion of T cells (18). Strategy of aAPC offers a promising way to overcome the disadvantages of natural APCs. CTLs generated by artificial stimulation protocols can kill both peptide-loaded and natural target cells (18, 44, 45). The presented data showed that CTLs specific for pIL-13Rα2345-354 could be induced in vitro with the aAPCs coating HLA-A2/pIL-13Rα2345-354–anti-CD28 antibody, and CD83 molecule to cell-sized latex beads. The HLA-A2+ PBMCs were induced to generate pIL-13Rα2345-354–specific CTLs in vitro by coculture with the HLA-A2/pIL-13Rα2345-354-aAPCs, and specificity of the aAPC-induced CTLs was confirmed by both their binding to HLA-A2/pIL-13Rα2345-354 tetramers and killing activity against HLA-A2/pIL-13Rα2345-354–bearing cells. Following this procedure, we found that the induced CTLs, derived from PBMCs in a HLA-A2+ healthy donor, could kill the HLA-A2+ pIL-13Rα2345-354+ glioma cells when stimulated with HLA-A2/pIL-13Rα2345-354–aAPCs in vitro. The specific killing activity of CTL induced by the HLA-A2/pIL-13Rα2345-354-aAPCs against specific target cells T2pIL-13Rα2345-354 and U251 was much more effective than that of any other control group. The results indicated that the cytotoxicity of the aAPCs-induced CTL is antigen specific (i.e., against the target cells bearing the corresponding HLA-A2/pIL-13Rα2345-354 complexes). In addition, it is possible to increase the percentage of antigen-specific CTL by sorting the cells after staining with tetramer and isolating the positive cells with magnetic beads loaded with anti-FITC antibody (Miltenyi System). Many groups have applied this approach to obtain higher percentages of antigen-specific CTLs that could be further expanded with aAPC or beads coated with anti-CD3/CD28 mAb (46). Therefore, this technique can be extrapolated to expand enough T cells for clinical use.

For clinical studies, HLA tetramer–based aAPCs have several distinct advantages over cellular APCs including dendritic cells. One of these is ease of preparation, which is not required for sterile cell culture and cytokines, thereby reducing both the variability and expense associated with ex vivo expansion. The variability is particularly important when considering therapies for cancer because there have been reported defects in dendritic cells obtained from patients with malignancies (47). Another advantage is the good stability of aAPCs, unlike the biological variability and patient-to-patient variation when producing cellular APCs such as dendritic cells. In addition, aAPCs can present defined combinations of MHC alleles and peptides and be easily adapted using other MHC alleles and/or peptides, so that immunodominant or subdominant epitopes can be expanded preferentially. When cellular APCs are used, an array of MHC molecules is used and a broad but uncontrolled MHC restricted response is generated. The last advantage is the ability to control the combination of costimulatory complexes associated with aAPCs, unlike cellular APCs that may participate in the T-cell-APC interaction in such a way that it promotes tolerance or anergy. For example, on T-cell activation, B7 binding to CTLA-4 drives their antigen-specific expansion; and (c) supporting their long-term survival and function when coupled to aAPCs (49). In contrast to other types of aAPCs previously engineered by others (50), there is no need for additional molecules like intercellular adhesion molecule-1 or lymphocyte function-associated antigen-3. The antigen-specific CTL can be successfully induced with the HLA-A2/pIL-13Rα2345-354-aAPCs in vitro. Further studies need to compare the efficiency of this approach with those of other techniques such as aAPCs with a combination of other costimulatory molecules.

The use of aAPC represents the state-of-the-art generation of antigen-specific CTLs for adoptive immunotherapy. Thus, HLA tetramer–based aAPCs coated with a HLA/peptide complex, anti-CD28 antibody, and CD83 molecule could provide a useful tool for the reproducible expansion of specific CTLs in vitro and significantly advance the field of adoptive immunotherapy. HLA-A2/pIL-13Rα2345-354-aAPCs could be used as a standardized, “off-the-shelf” reagent to enrich pIL-13Rα2345-354–specific CTLs for the treatment of patients with malignant gliomas.

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References

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