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IFN γ enhances cytotoxic efficiency of the cytotoxic T lymphocytes against human glioma cells

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ABSTRACT

Cytotoxic T lymphocytes (CTLs) are a key player in cancer immunotherapies, and MHC class I molecules on the cell surface are crucial for cellular recognition. However, the aberrant expression of MHC class I molecules is frequently found in various malignancies. IFN γ has dual functions in cancer progression, and its effect on tumor immunity is controversial. To investigate whether IFN γ can enhance cytotoxic efficiency of the tumor antigen-specific CTLs, we generated the CTLs using modified human dendritic cells as antigen presenting cells, then studied the activities of CTLs on human leukocyte antigen (HLA)-A2 positive glioma cells treated with, or without IFN γ . The results from both ELISpot and cytotoxicity assays demonstrated that the CTLs recognized and eliminated the HLA-A2 positive glioma cells treated with IFN γ more effectively when compared to the glioma cells deprived of IFN γ treatment. In addition, *in vitro* experiments showed that the levels of MHC class I molecules were upregulated in all of the HLA-A2 positive glioma cells. Using the publicly accessed TCGA data of low-grade glioma, we found significantly positive associations between IFN γ and both MHC class I molecules and CD8⁺ T cell activation score ($p < 0.0001$). Furthermore, we found a significantly reduced risk of death in the glioma patients with high T cell activation score in comparison to those with low score ($p = 0.022$). These findings suggest that a clinical application of IFN γ treatment may have potential benefits.

1. Introduction

Recent progress in immunotherapy research has been crucial for the development of new cancer therapies. In view of the preeminent role of T cells in cellular immunity and therapeutics, there is an enormous interest in directing cytotoxic T lymphocytes (CTLs) to cancer cells. CTLs are a key player in current immunotherapies, which promote apoptotic death of target cancer cells by using not only granule (perforin/granzyme)-mediated, but also receptor (Fas/tumor necrosis factor)-mediated mechanisms [1]. In addition, CTLs have an exquisite specificity for recognizing tumor antigens via a clonally unique T-cell receptor (TCR).

Major histocompatibility complex (MHC) class I is one of two primary classes of MHC molecules. We successfully delivered tumor antigenic peptides to CD8⁺ T cells for better antigen presentation via

poly lactic-co-glycolic acid (PLGA) nanoparticles (NPs) in our previous studies [2,3]. The peptides that were originally encapsulated in the PLGA-NPs were transported into the endoplasmic reticulum (ER), loaded onto MHC class I molecules and presented on the cell surface in a sustained manner. Thus, MHC class I molecules continuously display the intracellular peptides to CTLs. Peptide loading results in stabilization of the MHC class I molecules, and these molecules were transported to the cell surface where the formed peptide-MHC class I complexes can be recognized by the circulating CD8⁺ CTLs.

Survivin, a member of the inhibitor of apoptosis (IAP) family, is aberrantly expressed in most solid cancer types [4] and hematopoietic malignancies [5], but is undetectable in terminally differentiated normal adult tissues [6] with the exception of the testis, thymus, and placenta [7]. The significant difference in survivin expression between normal and malignant tissues makes survivin a perfect target for cancer

Abbreviations: CI, confidence interval; CTL, cytotoxic T lymphocyte; DC, dendritic cell; GM-CSF, granulocyte-macrophage colony-stimulating factor; gp100, glycoprotein 100; HLA, human histocompatibility leukocyte antigen; IFN γ , Interferon gamma; HLA, human leukocyte antigen; HR, hazards ratio; imDC, immature dendritic cell; LPS, lipopolysaccharides; MHC, major histocompatibility complex; NP, nanoparticle; PBMC, peripheral blood mononuclear cell; PLGA, Poly Lactic-co-Glycolic Acid; SV95, survivin_{95–104}; TIL, tumor-infiltrating lymphocyte.

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therapies.

Interferon gamma (IFN γ) is a dimerized soluble cytokine that is the only member of the type II class of interferon, and is produced predominantly by activated lymphocytes, such as natural killer (NK) and natural killer T (NKT) cells, CD4 $^{+}$ Th1 and CD8 $^{+}$ effector CTLs, once antigen-specific immunity develops. IFN γ plays a pivotal role in antitumor immunity by inducing Th1 polarization, CTL activation, and the tumoricidal activity of dendritic cells (DCs), under both natural and therapeutic conditions [8,9].

Down-regulation of MHC class I expression in tumor cells is a common mechanism by which tumor cells escape immune surveillance and can be associated with coordinated silencing of antigen-presenting machinery genes. Nevertheless, the expression of these genes can be restored by IFN γ [10]. However, IFN γ has dual functions. For example, IFN γ is known to induce immunosuppressive programmed death-ligand-1 (PD-L1) in cancer cells [11,12]. But under certain conditions, IFN γ acts to induce tumor progression, and IFN γ treatment has negatively affected patient outcomes in some clinical trials [13]. IFN γ insensitivity and the down-regulation of the MHC complex are proposed explanations for this discrepancy [13]. In addition, the role of IFN γ signaling in tumor cells in the context of a checkpoint blockade by CTLA-4 remains unknown. A recent study demonstrated that cancer patients who failed anti-CTLA-4 (ipilimumab) treatment had tumors with genomic defects in IFN γ pathway genes [14].

In this study, in order to examine whether IFN γ renders human glioma cells more susceptible to tumor antigen-specific CTLs, we incubated HLA-A2 positive glioma cells with IFN γ , then investigated the cytotoxic efficiency of the CTLs that we generated by using modified human DCs as antigen presenting cells. We also use publicly accessed TCGA data of human low-grade glioma to examine the associations between IFN γ and both MHC class I molecules and CD8 $^{+}$ T cell activation score.

2. Materials and methods

2.1. Peptides and PLGA polymer

Peptides of survivin_{95–104} (SV95, ELTLGEFLKL) [15] and gp100_{154–162} (gp100, KTWGQYWQV) [16] were synthesized by GenScript Corporation (Piscataway, NJ, USA). The purity and identity of the peptide was determined by HPLC and mass spectrometry analysis. PLGA (MW 23,000, copolymer ratio 50:50) was purchased from Birmingham Polymers (Birmingham, AL, USA).

2.2. Cytokines, IFN γ mAb and cytotoxicity assay kit

Interleukin 2 (IL-2), interleukin 7 (IL-7) and IFN γ were purchased from R & D Systems (Minneapolis, MN, USA). The IFN γ mAb used for the ELISpot assay was purchased from Mabtech (Cincinnati, OH, USA). CytoTox 96® Non-Radioactive Cytotoxicity Assay (G1780) is a colorimetric alternative to ^{51}Cr release cytotoxicity assay, which measures lactate dehydrogenase (LDH), and was purchased from Promega Corporation (Madison, WI, USA).

2.3. Human cell lines

HLA-A2 positive cancer cell lines T2, LN-18, T-98 G, U-251 MG, and U-373 MG, and HLA-A2 negative LN-229 cell line were purchased from ATCC (Manassas, VA, USA). TIL771 was kindly provided by Dr. John Wunderlich (NIH/NCI, Bethesda, MD, USA). RPMI 1640 medium was supplemented with either 10% fetal bovine serum for the cancer cell lines, or 10% human AB serum (Omega Scientific, Tarzana, CA, USA), IL-2 (20 U/ml) and IL-7 (30 U/ml) (R & D, Minneapolis, MN, USA) for the TIL771 cell line.

2.4. Isolation of CD8 $^{+}$ cells from PBMC and preparation of PLGA-NPs encapsulated SV95 peptide

According to the protocol, peripheral blood from HLA-A2 positive healthy donors, purchased from Scripps Green Hospital (San Diego, CA, USA), was used to isolate CD8 $^{+}$ cells using CD8 MicroBeads (Miltenyi Biotec, Auburn, CA, USA).

We used the double emulsion-solvent evaporation technique to make the PLGA-NPs encapsulated SV95 peptide [17]. The particle size was analyzed using a Zetasizer® Nano ZS90 (Malvern Instruments, Worcestershire, UK), and the zeta potential of the PLGA-NPs was measured using ZetaPlus™ (Brookhaven Instruments Corporation, Holtsville, NY, USA).

2.5. Generation of human immature DCs (imDCs)

Human imDCs were generated from HLA-A2 positive peripheral blood mononuclear cells (PBMC) as previously described. Briefly, complete RPMI medium, supplemented with recombinant human GM-CSF at 1000 U/ml and recombinant human IL-4 at 400 U/ml (R & D, Minneapolis, MN, USA), was added to the adherent cell population (enriched monocytes) and the cells were incubated at 37 °C and 5% CO $_2$. After 3 days, the same dose of IL-4 and GM-CSF was added to the culture again. On day 5 of the culture, aggregates of loosely adherent imDCs were apparent, which were used for either the maturation or the generation of modified human DCs.

2.6. Generation of modified human DCs and tumor antigen-specific CTLs

We generated the modified human DCs by using human imDCs loaded with PLGA-NPs encapsulated SV95 peptide (PLGA-NPs-SV95), followed by maturation with the addition of lipopolysaccharides (LPS) (Sigma-Aldrich, St. Louis, MO, USA) at 100 ng/ml for an additional 2 days. More detailed procedures have been described in our previous reports [2,3].

Human imDCs loaded with PLGA-NPs-SV95 were used as the modified human DCs to induce the SV95-specific CTLs. The modified human DCs were added to the isolated HLA-A2 positive CD8 $^{+}$ T cells at a ratio of 1 to 10 in the CTL medium (RPMI 1640 with 10% human AB serum). IL-2 (20 U/ml) and IL-7 (30 U/ml) were added to the culture medium after day 3. Restimulation was performed on day 7 using the irradiated autologous CD8 $^{+}$ cells as the stimulator cells. The second restimulation was repeated on day 14 with the same procedures. The SV95-specific CTLs were eventually harvested and examined on day 21 by using both ELISpot and cytotoxicity assays.

2.7. ELISpot assay

An ELISpot assay was completed according to the protocol, as previously described [3]. gp100 peptide-pulsed T2 cells with the addition of TIL771 cells were used as the system positive control. SV95 peptide-pulsed T2 cells with the addition of SV95-CTLs were used as the internal positive control. Five glioma cell lines including the HLA-A2 negative cell line LN-229 and the HLA-A2 positive cell lines LN-18, U-251 MG, U-373 MG and T-98 G, which naturally express survivin, were used as the target cells after incubation with, or without IFN γ at 100 ng/ml for 48 h.

For the ELISpot assays, house-coated 96-well nitrocellulose anti-IFN γ plates were used. T2 cells (2.0×10^4) were added to each well, with an addition of SV95 peptide at the concentration of 0.5 $\mu\text{g}/\text{ml}$, and then incubated for 1 h. Human glioma cells including LN-18, U-251 MG, U-373 MG, and T-98 G were added as the targets to each well. After incubating the plates for 24 h at 37 °C, the plates were washed with PBS to remove the cells. The biotinylated secondary antibody for IFN γ was then added in each well. After incubation for 2 h at 37 °C, the avidin-biotinylated horseradish peroxidase complex was added and

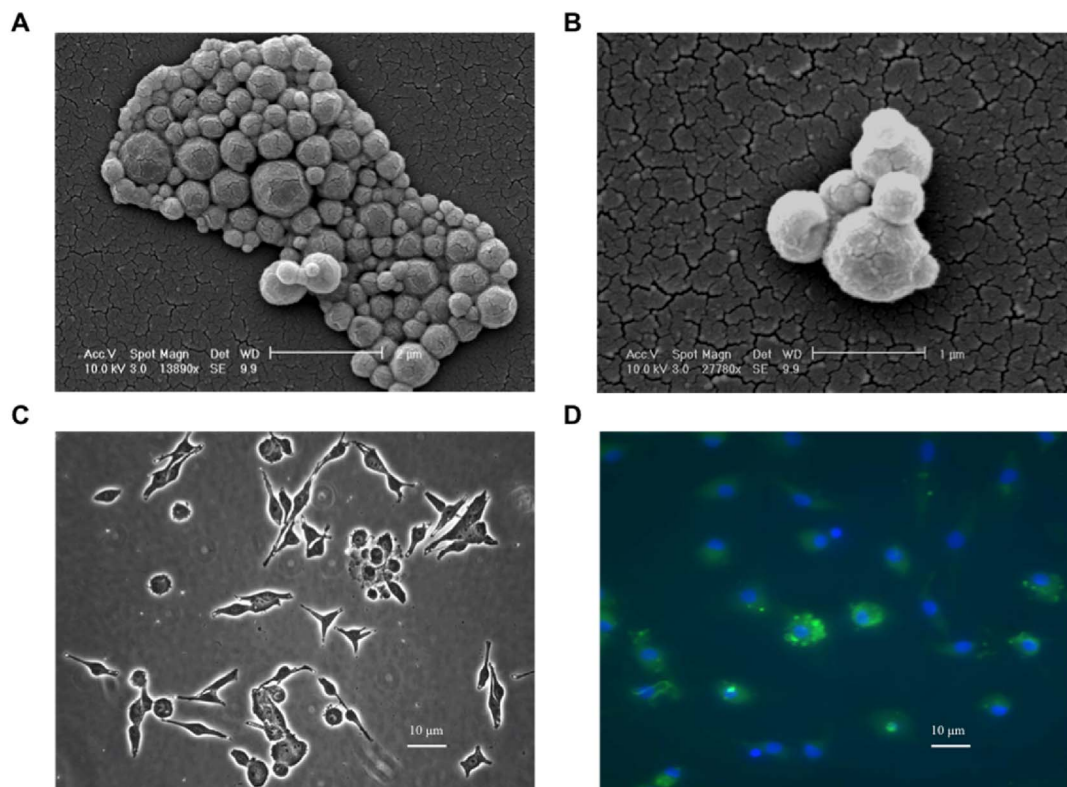


Fig. 1. Characterization of PLGA-NPs encapsulated SV95 peptide and generation of the modified human DCs. To investigate the PLGA-NPs under a SEM, PLGA-NPs loaded with SV95 peptide were sputter coated with Au/Pd using a Cressington 108 auto sputter coater (Cressington, Watford, UK) for 100 s. The images were taken under a SEM (Phillips XL 30, FEI, Hillsboro, OR, USA) at 10 kV. (A) SEM image of PLGA-NPs (Magnification 13,890 ×, scale bar = 2 μm); (B) SEM image of PLGA-NPs (Magnification 27,780 ×, scale bar = 1 μm). To generate the modified human DCs, human imDCs were incubated with PLGA-NPs loaded with SV95 peptide for 45 min, then washed twice with PBS. (C) Image of human imDCs was taken under an Olympus microscope (10 ×, bright field); (D) Image of human imDCs was taken under a fluorescent microscope (40 ×, merged, DAPI stained nuclei (blue), coumarin 6 labeled PLGA-NPs (green)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

incubated for 1 h at room temperature. After washing with PBS, the substrate was added and incubated for 3 min. The plates were loaded onto the ELISpot reader system (CTL Analyzers LLC, Cleveland, OH, USA) for spot counting.

2.8. Cytotoxicity assay

T2 cells, SV95 peptide-pulsed T2 cells, human glioma cell lines including the HLA-A2 positive cell lines LN-18, U-251 MG, U-373 MG and T-98 G, and the HLA-A2 negative cell line LN-229, which were incubated with or without IFN γ at 100 ng/ml for 48 h, were all used as the target cells when analyzing the potency and selectivity of the SV95-specific CTLs. The assays were performed following the manufacturer's instructions (CytoTox 96 $^{\circ}$ Non-Radioactive Cytotoxicity Assay, Promega Corp. Madison, WI, USA) and our protocols. The plates were read at a wavelength of 490 nm, and the percent cytotoxicity was calculated using the formula: Lysis specific (%) = $100 \times (\text{Experimental release} - \text{Effector spontaneous release} - \text{Target spontaneous release}) / (\text{Target maximum release} - \text{Target spontaneous release})$ [3].

2.9. Flow cytometry analysis

Five untreated human glioma cell lines stained with mouse anti-human isotype (IgG2b, κ , BD Pharmingen, La Jolla, CA, USA) were used as the isotype controls. The five human glioma cell lines stained with mouse anti-human HLA-A2 mAb (clone BB7.2, BD Pharmingen, La Jolla, CA, USA) were used as internal controls. To prepare the samples, these five human glioma cell lines were harvested at 80% confluence, and incubated with IFN γ at 100 ng/ml for 48 h. The cells were then stained with the mouse anti-human HLA-A2 mAb in the dark for 30 min on ice, washed twice with PBS and subjected to flow cytometry analysis

for the expression of MHC class I molecules.

2.10. Gene expression and clinicopathologic data

Upper quartile normalized RNA-seq V2 RSEM data were retrieved from a TCGA low-grade glioma dataset publicly available at TCGA provisional (www.cbioportal.org) for the expression levels of *NKG7*, *CCL4*, *CST7*, *PRF1*, *GZMA*, *GZMB*, *IFNG*, *CCL3*, *PD-1*, *PD-L1*, *TIGIT*, *LAG3*, *TIM3* and *CTLA4* genes, which are indicated in association with effector CD8 $^{+}$ T cell activation and exhaustion [18–20]. Clinicopathologic data on these patients were also downloaded and were combined with gene expressions for further data analyses.

2.11. Statistical analyses

One-way ANOVA was performed to compare (1) the means of recognition and killing of the SV95-CTLs between the groups of the SV95-peptide pulsed T2 cells and non-pulsed T2 cells; the groups of glioma cell lines treated with and without IFN γ ; (2) Cytotoxic activities of the SV95-CTLs when targeting T2 cells, glioma cells with and without IFN γ incubation.

T cell activation score was calculated as a weighted average of gene expression, where the absolute value of the weight is the reciprocal of variance of the expression level of a gene. Each of gene expression values plus one were performed before the log-transformation in data analyses. We assigned the values of T cell activation-related genes to be positive, and the values of T cell exhaustion-related genes to be negative. Thus, a positive correlation is expected between T cell activation score and the capacity of effector CD8 $^{+}$ T cells to kill tumors.

Multivariate Cox proportional hazards regression models were used to assess the association of T cell activation score with patient survival

by estimating hazards ratio (HR) and its 95% confidence interval (95% CI), in which T cell activation score was classified into three categories, low, medium and high, based on its tertile distribution. The survival times for overall and disease free survival were calculated as time from surgery until death or relapse, or the last follow-up, respectively. Spearman correlation was used to evaluate correlations between the expressions of IFN γ and MHC class I molecules. A two-side p value < 0.05 was considered statistically significant. The data analyses were carried out using SAS version 9.4.

3. Results

3.1. Generation of the modified human DCs

To generate the modified human DCs, we prepared PLGA-NPs encapsulated SV95 peptide as previously described [3]. Fig. 1 shows the scanning electron microscope (SEM) images of the PLGA-NPs, which were taken at 10 kV. The size distribution of the unfractionated PLGA-NPs sample range from 70 nm to 795 nm, with 80% of the PLGA-NPs ranging from 150 nm to 500 nm in diameter.

When the cultured human imDCs were ready on day 5, images were taken under a bright field using an Olympus microscope. We observed that the monocytes developed imDCs morphology after culturing with GM-CSF and IL-4 cytokines (Fig. 1C). After the incubation of human imDCs with the PLGA-NPs encapsulated with both SV95 peptide and coumarin 6 for 45 min, the human imDCs were washed twice with PBS. The images were then taken under a fluorescent microscope. We observed that the scattered fluorescent granules (PLGA-NPs loaded with coumarin 6) were taken up in the cytoplasm of human imDCs, the nuclei were blue, which were stained with DAPI (Fig. 1D).

3.2. IFN γ renders human glioma cells more susceptible to the CTLs

The ELISpot assay results demonstrated that TIL771 cells recognized and killed gp100 peptide-pulsed T2 cells. As an internal system positive control, SV95-CTLs significantly killed SV95 peptide-pulsed T2 cells ($p < 0.0001$, Fig. 2A). In addition, all of the HLA-A2 positive human glioma cell lines treated with IFN γ were significantly eliminated by the SV95-CTLs ($p < 0.0001$, $p < 0.0001$, and $p = 0.002$, respectively, Fig. 2B). However, as a negative control, the HLA-A2 negative human glioma cell line LN-229 was not recognized and killed

by the same SV95-CTLs after IFN γ treatment with the same procedures (Fig. 2B).

We also used cytotoxicity assays to investigate the susceptibility of the five human glioma cell lines to the SV95-CTLs. The result showed that SV95 peptide-pulsed T2 cells were significantly eliminated by SV95-CTLs ($p < 0.01$, Fig. 3A). As a negative control, the HLA-A2 negative human glioma cell line LN-229 was not recognized and killed by the SV95-CTLs (Fig. 3B). In contrast, all of the HLA-A2 positive human glioma cell lines pre-treated with IFN γ were greatly eliminated by the SV95-CTLs when compared to the cell lines without IFN γ treatment (Fig. 3C, D, E, F), of which U-373 MG cell line treated with IFN γ was significantly recognized and killed by SV95-CTLs ($p < 0.05$, Fig. 3E).

3.3. IFN γ upregulates the expression of MHC class I molecules on HLA-A2 positive glioma cells

The LN-229 glioma cell line is HLA-A2 negative. The flow cytometry results indicated that the level of MHC class I molecules was not changed after IFN γ treatment (Fig. 4A). In contrast, the MHC class I molecule levels were increased in all of the HLA-A2 positive human glioma cell lines including LN-18, U-251 MG, U-373 MG and T-98 G following treatment with IFN γ (Fig. 4B, C, D, E).

3.4. Correlation of IFN γ with MHC class I molecules

To validate the *in vitro* finding as described above, we retrieved gene expression data from publicly accessed TCGA dataset of low-grade glioma, which includes 530 patients for RNA-seq analyses, and performed spearman correlation analyses (Table 1). The results show that IFN γ levels significantly positively associate with the levels of MHC class I molecules (HLA-A, HLA-B and HLA-C) ($p < 0.0001$). Their correlation coefficients were 0.32 (95% CI: 0.24–0.39) for HLA-A, 0.31 (95% CI: 0.23–0.38) for HLA-B, and 0.28 (95% CI: 0.20–0.35) for HLA-C, respectively.

3.5. Association of T cell activation score with the risk of death and relapse in glioma

IFN γ has dual functions in upregulating not only MHC class I molecules, which present antigens to effector CD8⁺ T cells, but also T

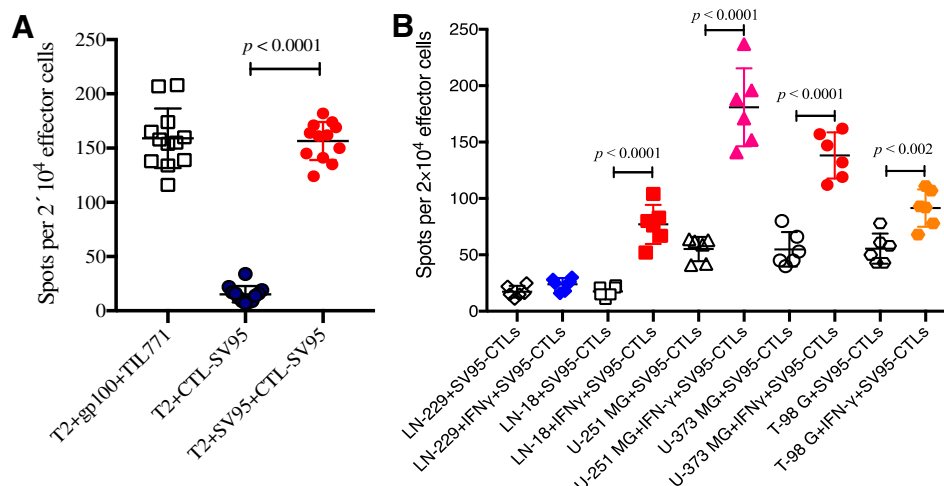


Fig. 2. IFN γ renders human glioma cells more susceptible to SV95-specific CTLs (ELISpot assay). To evaluate whether IFN γ cytokine targets the human glioma cells to the CTLs we generated with the modified human DCs, an ELISpot assay was conducted. The results showed that T2 cells pulsed with gp100 peptide can be killed by TIL771 cells, which were used as the system positive control (Fig. 2A). T2 cells pulsed with SV95 peptide can be recognized and killed by SV95-CTLs (Fig. 2A), which were used as a positive control for testing the potency of the SV95-CTLs that we generated. The HLA-A2 negative cell line LN-229 cannot be recognized and killed by the SV95-CTLs (Fig. 2B). The HLA-A2 positive cell lines LN-18, U-251 MG, U-373 MG and T-98 G can be recognized and killed by the SV95-CTLs. Importantly, all HLA-A2 positive glioma cell lines can be significantly killed by the SV95-CTLs after IFN γ treatment ($p < 0.0001$ is for LN-18, U-251 MG and U-373 MG; $p = 0.002$ is for T-98 G, Fig. 2B).

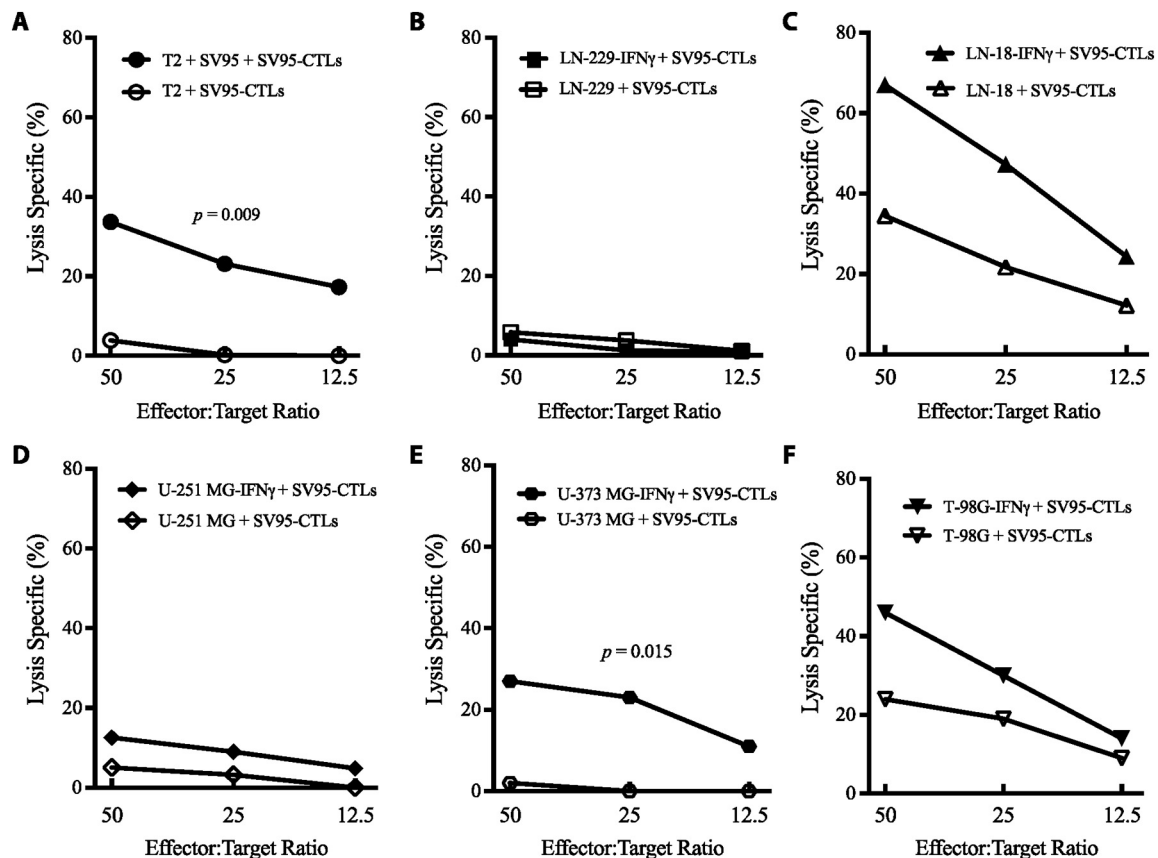


Fig. 3. IFN γ renders human glioma cells more susceptible to SV95-specific CTLs (cytotoxicity assay). We also used a cytotoxicity assay to examine whether IFN γ cytokine renders human glioma cells more susceptible to the SV95-specific CTLs. SV95 peptide-pulsed T2 cells cultured with SV95-CTLs were used as the positive control. The results indicated that T2 cells pulsed with SV95 peptide were significantly killed by SV95-CTLs (Fig. 3A, $p < 0.01$). The HLA-A2 negative cell line LN-229, a negative control, was not recognized and killed by the SV95-CTLs (Fig. 3B). All the HLA-A2 positive human glioma cell lines were killed by the SV95-CTLs in a consistent manner after being treated with IFN γ (Fig. 3C, D, E, F). Of which, the U-373 MG cell line treated with IFN γ was significantly recognized and killed by SV95-CTLs ($p < 0.05$, Fig. 3E).

cell inhibitory molecules of PD-1/PD-L1. Thus, we use T cell activation score based on a group of T cell activation- and exhaustion-related gene expressions to evaluate the status of effector CD8⁺ T cells. Spearman correlation result shows that a significantly positive correlation exists between IFN γ expression and T cell activation score ($p < 0.0001$), and the correlation coefficient is 0.57 (95% CI: 0.51–0.63) (Table 1).

To further examine the association of T cell activation score with the risk of death and relapse in glioma, we performed multivariate Cox proportional hazards regression models. No violation was shown for the proportional hazards assumption in the Cox model. The multivariate Cox regression results are shown in Table 2. Compared to those with low T cell activation score, patients with high score had a significantly reduced risk of mortality with the adjustment of patient age at surgery, tumor grade, tumor histologic type and patient with seizure. The adjusted HRs were 0.94 (95% CI: 0.61–1.44) for medium score ($p = 0.763$) and 0.57 (95% CI: 0.36–0.92) for high score ($p = 0.022$), respectively. The trend of reduced mortality risks was statistically significant ($p_{\text{trend}} = 0.019$). The reduced risk of relapse was also observed for T cell activation score. However, it is not statistically significant. The adjusted HRs for the relapsed patients were 0.84 (95% CI: 0.59–1.20) for medium ($p = 0.341$), and 0.69 (95% CI: 0.47–1.02) for high ($p = 0.062$), respectively. The trend was not significant ($p_{\text{trend}} = 0.062$).

4. Discussion and conclusions

Cancer immunotherapy research has made a significant breakthrough in recent years because of the developments of T cell therapies, and the use of checkpoint inhibitors including cytotoxic T-lymphocyte-

associated protein 4 (CTLA-4, also known as CD152) and programmed cell death 1 (PD-1). However, the key players in these therapies are CTLs, which are a subset of T cells that are able to recognize and kill tumor cells [21].

Most CTLs express TCRs that can recognize a specific tumor antigen. A tumor neoantigen, often produced by cancer cells, is able to stimulate an immune response. Antigenic peptides inside a cell are bound to MHC class I molecules, and can be transported by the MHC class I molecules complex to the cell surface, where they can be recognized as foreign antigens by T cells. Through the binding of TCRs to the antigen-MHC complex, CTLs are activated and differentiated, thereby executing their cytotoxic functions [22]. Therefore, both the presentation of tumor antigenic peptides and the abundance of MHC class I molecules are important in determining the response magnitude and differentiation of the tumor antigen-specific CD8⁺ T cells.

In this study, we generated modified human DCs as antigen-presenting cells by using human imDCs loaded with PLGA-NPs encapsulated survivin peptides, which controls the release of the antigen in the cells. This strategy allows the survivin peptides to be continuously delivered by MHC class I molecules to the cell surface, facilitating the recognition by CTLs.

The expression levels of MHC class I molecules at the cell surface are of crucial importance for cellular recognition. However, abnormalities in MHC class I antigen surface expression are frequently found in malignancies. These abnormalities are often associated with reduced recognition by MHC class I antigen-restricted, tumor antigen-specific CTLs and disease progression. It has been shown that IFN γ has dual functions; the regulation of MHC class I molecules is tumor type-specific while the up-regulation of PD-L1 is consistent. This is one possible

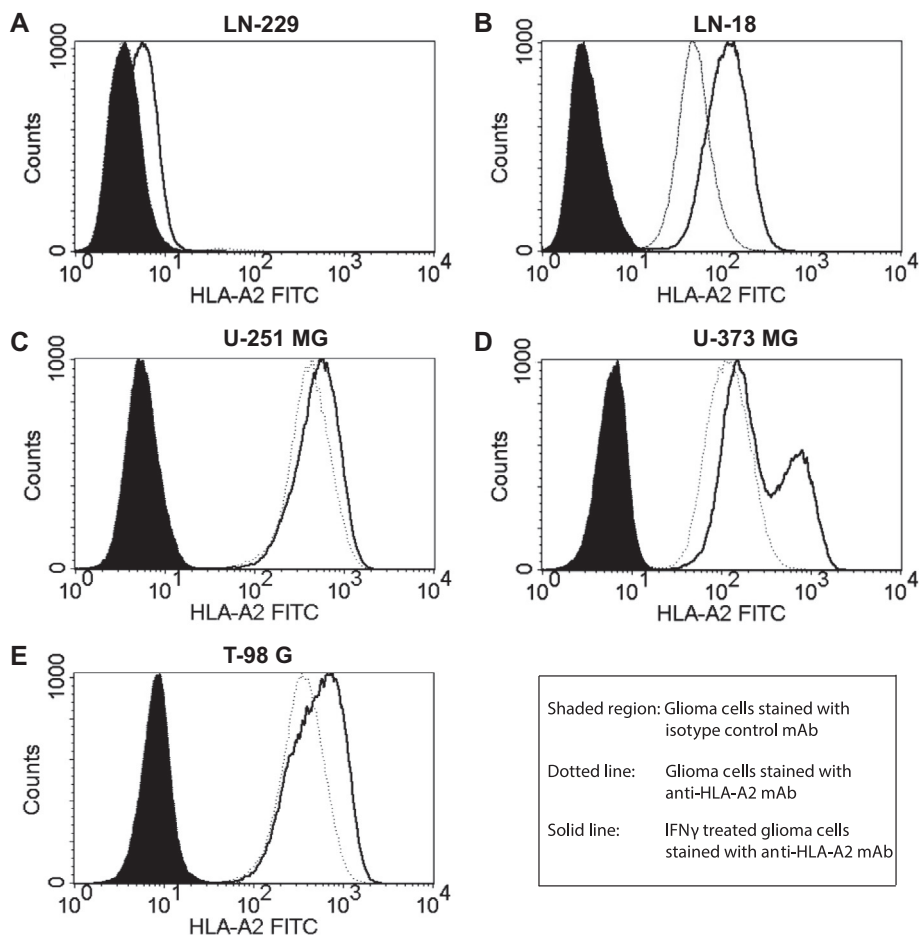


Fig. 4. IFN γ upregulates the expression of MHC class I molecules on HLA-A2 positive glioma cell lines. The five human glioma cell lines were analyzed for the expression of MHC class I molecules by flow cytometry. Human glioma cell lines stained with isotype control mAb (IgG2b-FITC, BD Pharmingen, San Diego, CA, USA) are in the shaded region. Human glioma cell lines stained with anti-HLA-A2-FITC are in the dotted line region. Human glioma cell lines treated with IFN γ for 48 h and stained with anti-HLA-A2-FITC mAb are in the solid line region. The flow cytometry results showed that the level of MHC class I molecules on the HLA-A2 negative LN-229 glioma cell line was not changed after IFN γ treatment (Fig. 4A). Distinctly, the MHC class I molecule levels were increased in the solid line region in a consistent manner among all of the HLA-A2 positive human glioma cell lines including LN-18, U-251 MG, U-373 MG and T-98 G (Fig. 4B, C, D, E).

Table 1
Spearman correlation of IFN γ with MHC class I molecule and T cell activation score ($n = 530$).

Gene	Correlation coefficient	95% CI ¹	<i>p</i> value
HLA-A	0.32	0.24–0.39	< 0.0001
HLA-B	0.31	0.23–0.38	< 0.0001
HLA-C	0.28	0.20–0.35	< 0.0001
T cell activation score	0.57	0.51–0.63	< 0.0001

1. CI: Confidence interval

explanation as to why IFN γ negatively affects patient outcomes in some clinical trials [13], but positively in other studies; IFN γ activates macrophages and induces the expression of MHC class I molecules, thereby enhancing the immune response [23–26].

In this study, we demonstrated that SV95-CTLs recognized and eliminated the HLA-A2 positive human glioma cells including LN-18, U-251 MG, U-373 MG, and T-98 G when treated with IFN γ *in vitro* (Fig. 2B, Fig. 3E). One explanation for these results is that IFN γ remarkably upregulates the expression of MHC class I molecules on the HLA-A2 positive human glioma cell lines (Fig. 4). Accordingly, These results indicate that IFN γ rendered HLA-A2 positive human glioma cells more susceptible to the SV95-CTLs. These results are consistent with other previous reports [27–30].

In agreement with the *in vitro* findings, we found that IFN γ was

positively associated with MHC class I molecules and effector T cell activation score using publicly accessed TCGA low-grade glioma dataset that includes 530 patients. We also found that high T cell activation score led to over 40% reduction in mortality risk and approximately 30% reduction in relapse risk in low-grade glioma. Taken together, these findings suggest that IFN γ may enhance MHC class I molecules, favoring the presentation of tumor antigens to activate effector CD8⁺ T cells and thereby resulting in favorable prognosis of glioma patients. Thus, IFN γ treatment may have potential benefits before vaccination with either modified human DCs, or other adoptive immunotherapies comprising of tumor antigen-specific CTLs.

In conclusion, IFN γ cytokine makes human glioma cells more susceptible to the tumor antigen-specific CTLs *via* the upregulation of MHC class I molecules, and consequently enhancing the abilities of effector CD8⁺ T cells. Thus, IFN γ may benefit patients with glioma in a clinical setting. Further investigations in both basic medicine and translational medical research are warranted.

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Table 2
Association of T cell activation score with the risk of death and relapse in glioma in multivariate Cox regression models.

Variable	Death			Relapse		
	HR ¹	95% CI ²	p value	HR	95% CI	p value
Age (per 5 yrs)	1.37	1.27–1.48	< 0.0001	1.14	1.07–1.22	< 0.0001
<u>Histologic type</u>						
Astrocytoma	1.00			1.00		
Oligoastrocytoma	0.78	0.48–1.26	0.311	0.69	0.46–1.04	0.073
Oligodendroglioma	0.54	0.35–0.82	0.005	0.65	0.46–0.94	0.020
Grade	2.76	1.83–4.17	< 0.0001	1.34	0.97–1.85	0.074
Seizure	0.94	0.65–1.34	0.714	0.92	0.67–1.24	0.571
<u>T cell activation score</u>						
Low	1.00			1.00		
Medium	0.94	0.61–1.44	0.763	0.84	0.59–1.20	0.341
High	0.57	0.36–0.92	0.022	0.69	0.47–1.02	0.062
p value for Trend		0.019			0.062	

1. HR: hazards ratio, which was obtained from multivariate cox proportional hazards regression model
2. CI: confidence interval

Potential conflict of interest

The authors declare that they have no conflict of interest.

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