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Reviewer A

The authors studied the development of a new NY-ESO-1 peptide fused with Hsp70 protein for cytotoxic effects on one glioma cell line through the induction of T-cells because glioma cells do not express NY-ESO-1 protein highly. Therefore, they treated 5-AZA first to induce NY-ESO-1 protein in cells, and then NY-ESO-1/Hsp70 protein treatment showed significantly increased T-cell differentiation from mature DC cells, resulting in the cytotoxicity of one glioma cell line. However, the results do not show significance among four different groups because of no statistical analysis, so it is too hard to believe. Also, I do not see markedly improved cytotoxicity of target cells in the Hsp70/NY-ESO treated group compared to the NY-ESO single treatment group. This manuscript does not have enough information and evidence to explain their study. Therefore, many aspects of the current study are described poorly or in a contradictory way, and greater detail and precision in language are needed.

Reply: Thank you very much for your comments. We have revised the manuscript to make it more complete and convincing.

1. They should have mentioned the meaning of the CTL.

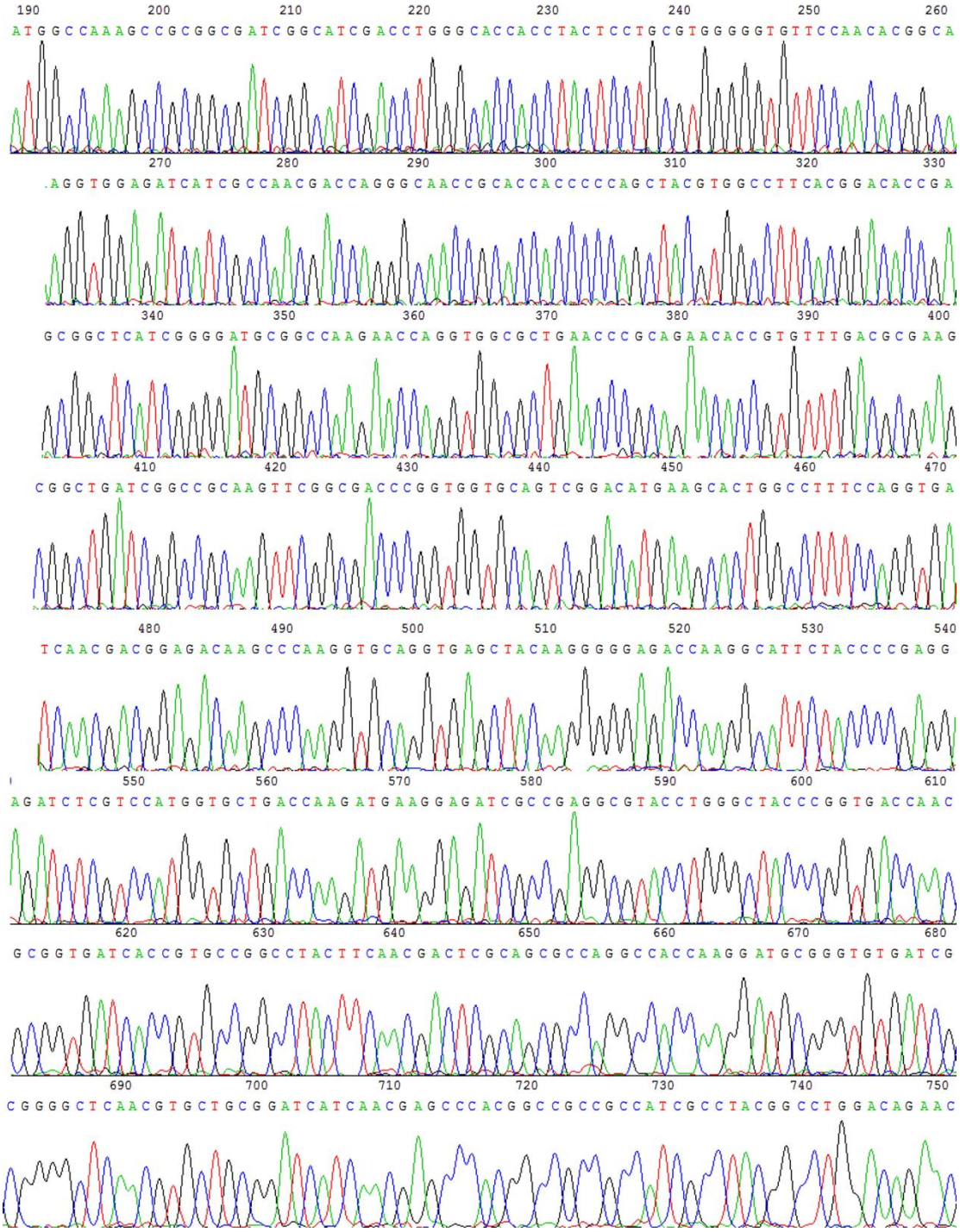
Reply: We have modified our text as advised (see Page 6, line 13-15).

2. The authors do not show that their glioma cell lines express low or no protein levels of NY-ESO-1. Providing western blot results of that would make the manuscript stronger.

Reply: I am sorry that this part was not clear in the manuscript. We confirmed that the human glioma cell line U251 does not express NY-ESO-1 by RT-PCR and immunocytochemistry. we have modified our text (see Page 14, line 1-6; Figure 4).

3. Figure 1 is not good evidence for bacterial Hsp70 protein. They should use Hsp70 antibody or His antibody to detect Hsp70. Only the Coomassie blue staining results are not evidence for Hsp70 they isolated.

Reply: Thank you very much for your comment. The pET-30a-HSP70 vector was comfirmed to be successfully constructed by double enzyme digestion (Figure 1B) followed by DNA sequencing (see Page 12, line 17-21; the figure below).



Sequencing confirmation of the recombinant HSP70. The sequence from base 189 "A" to base 751 "C" is the front part of HSP70 gene.

4. Figure 3 has no information on the CD83, CD86, and HLA-DR population percentages. Figure legend must have it as well as in the manuscript.

Reply: We have modified Figure 3 and its legend as advised (see Figure 3).

5. Statistical significance should be contained in Figure 4 and 5.

Reply: We have modified Figure 4, Figure 5 and the legends as advised (see Figure 4 and Figure 5). We also add the comparison results of the lysis percentages of NY-ESO-1^+ U251 cells and NY-ESO-1^- U251 cells (see Figure 5B).

6. Many English sentences made me hard to understand, making these results weaker.

Reply: We have modified the expressions of some sentences (see the revisions in the manuscript).

7. In Figure 4, Negative control results should be needed.

Reply: Thank you very much for your comment. We confirmed that the human glioma cell line U251 does not express NY-ESO-1 protein by Immunohistochemistry (IHC), and we have added the negative IHC result to Figure 4 (see Figure 4).

Reviewer B

The authors show that stimulation of T cells by mature dendritic cells loaded with recombinant HSP70/NY-ESO1 antigen leads to increased cytotoxic activity against U251 cells treated with Azacitidine compared to mature DCs loaded with NY-ESO1 antigen only.

Much of the work has already been published:

It has been shown previously that Azacitidine and decitabine treatment of glioblastoma cell lines can increase expression of NY-ESO1 and many other CTAs (PMID: 35468205, 26330563).

As mentioned in the introduction, it is well recognised that heat shock proteins enhance antigen cross-presentation in dendritic cells. It has also been shown that a recombinant HSP70/NY-ESO1 antigen leads to increased antigen presentation by DC and T cell activation (PMID: 17991294)

Reply: Thank you for your valuable comments. We have carefully read the excellent articles you mentioned, which provided us with new ideas for experimental design and made us realize our own shortcomings.

Glioma is the most common primary intracranial malignant tumor with poor prognosis. Immunotherapy is a promising treatment strategy for glioma. NY-ESO-1 has strong antigenicity and immunogenicity, and it is one of the most valuable antigens for tumor immunotherapy. Some related studies have been conducted in several tumors, but there are few studies in glioma. Therefore, further studies are warranted.

We used HSP70/NY-ESO-1 peptide to induce immune response against glioma. Antigen peptide vaccines have many advantages compared with protein vaccines, such as easy preparation, stable properties, strong specificity, and high safety. And as immune adjuvant of antigen peptides, HSP70 greatly improves the anti-tumor efficacy of peptide vaccines. The antigen peptide NY-ESO-1 p86-94, which has not been mentioned in previous studies, could be used as a new immunotherapy target.

The authors build on the above to show that T cells stimulated with DC pulsed with this recombinant peptide leads to great cytotoxic activity of T cells against Azacitidine treat U251.

It would be beneficial for the authors to show that this greater cytotoxic activity is indeed due to T cell activation - Is there an increase in T cell activation as determined by flow-cytometry?

Reply: Thank you for your advice. The cytotoxic activity of T cells can be measured by flow cytometry or lactate dehydrogenase (LDH) release assays. We used the latter method.

Is this indeed a NY-ESO p86-94 specific effect - are these effects seen using a NY-ESO p86-94 specific T cell clone? Are they able to isolate the activated T cells in the above population and show it is specific to NY-ESO p86-94.

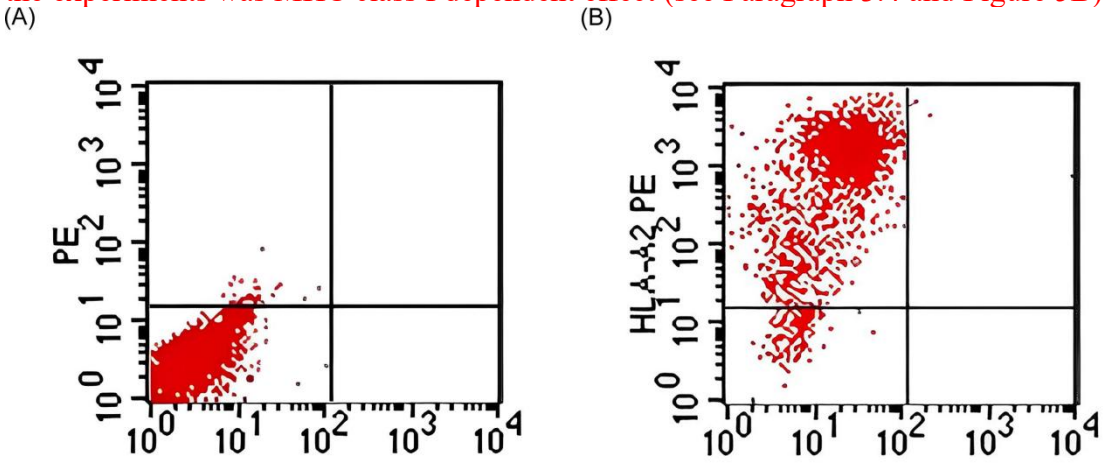
Reply: Yes, this is indeed a NY-ESO p86-94 specific effect. Because the immune response stimulated by a DC-based vaccine of HSP70/NY-ESO-1 p86-94 fusion protein was significantly enhanced compared with that induced by NY-ESO-1 alone.

It is unclear from the methods how long T cells were left following stimulation by mDCs and PHA to prevent non-specific activation and effector activity from PHA.

Reply: Thank you for your problem. We should set a control group to eliminate the effect of PHA on the activation of T cells.

Is this a MHC Class I dependent effect - can this cytotoxic activity by abrogated by blocking HLA-A2 or pan MHC class I?

Reply: Due to the high proportion of HLA-A2 phenotype in Chinese population, we selected NY-ESO-1 p86-94 peptide, which is a HLA-A2-restricted peptide. HLA-A2 expression in mononuclear cells isolated from peripheral blood was detected by flow cytometry (see Paragraph 3.2 and the figure below). We collected HLA-A2-positive PBMCs to obtain DCs. LDH release assays showed that the percentages of lysis of NY-ESO-1^{+} U251 target cells by NY-ESO-1-stimulated and HSP70/NY-ESO-1 p86-94-stimulated T cells were higher than those of NY-ESO-1^{-} U251 target cells ($P<0.01$). Therefore, the immune response induced by NY-ESO-1 p86-94 in the experiments was MHC class I dependent effect (see Paragraph 3.4 and Figure 5B).



A: isotype control; B: HLA-A2-positive PBMCs

It would be useful to see if this effect is present for other glioblastoma cells lines, including primary cell lines and/or in-vivo.

We will conduct experiments to further verify this conclusion, including other glioma cell lines and in vivo.