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ORIGINAL RESEARCH

Exploratory Research for HIF-1α Overexpression Tumor Antigen in the Activation of Dendritic Cells and the Potent Anti-Tumor Immune Response

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Background: Tumor-specific antigens play an important role in dendritic cell (DC)-based immunotherapy. The acquisition of tumor-specific antigens, which are essential for DC-based immunotherapy, poses a significant challenge. This study aimed to explore the efficacy of hypoxia inducible factor-1α (HIF-1α) overexpression tumor antigens in DC-based immunotherapy.

Methods: An HIF-1α over-expression cell line was constructed to prepare HIF-1α overexpression tumor antigens. The expression of CD14, CD40, CD80, CD86, and HLA-DR on the surface of dendritic cells derived from monocytes was assessed using flow cytometry after stimulation with tumor antigens enriched in HIF-1α. T cell proliferation was analyzed by CFSE division following incubation with mature DCs. The apoptotic tumor cells were detected through annexin V/PI staining following coculture with dendritic cells (DCs) stimulated by HIF-1α enriched antigens. The detection of damage-associated molecular pattern molecules (DAMPs) HMGB1 and calreticulin (CALR) was performed using Western blotting.

Results: The results demonstrated that HIF-1α-enriched tumor antigens significantly upregulated the expression of CD40, CD80, CD86, and HLA-DR in DCs compared to normal tumor antigens. Furthermore, co-incubation with HIF-1α-enriched tumor antigen-activated DCs enhanced T cell proliferation and stimulated the T cell-mediated cytotoxicity. Notably, the expression of DAMPs, such as HMGB1 and CALR, was elevated in HIF-1α-enriched tumor antigens.

Conclusion: Our findings demonstrate that tumor antigens enriched with HIF-1α may encompass tumor-specific antigens capable of stimulating DC activation, thereby enhancing T cell proliferation and cytotoxicity. These results provide support for the further advancement of HIF-1α enriched tumor antigens in preclinical and clinical investigations pertaining to tumor treatment.

Keywords: dendritic cells, T cell activation, HIF-1α, HMGB1, calreticulin

Introduction

Triple-negative breast cancer (TNBC) is difficult to treat, and often relapse after treatment. Currently, surgical treatment, radiotherapy, and chemotherapy are the main treatments for breast cancer, and the patient's survival period has been greatly extended, breast cancer survival varies substantially by stage at diagnosis. Triple-negative breast cancers have a poorer prognosis than other subtypes, partly because they are also diagnosed at an advanced stage.[1] Immunotherapy is a new treatment for cancer, and since PD-1/PD-L1 has achieved significant clinical effects,[2] it has received sufficient attention again.[3]

Cancer immunotherapy, also known as immuno-oncology, encompasses a range of therapeutic approaches that harness the innate capabilities of the immune system to prevent, control, and eradicate cancer. The current clinical immunotherapy approaches including cytokine therapies, monoclonals antibodies (mAbs), antibody-drug conjugates

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(ADCs), immune checkpoint inhibitors (ICIs), cancer vaccines, adoptive cell therapies (ACTs) and oncolytic virus therapy (OV). Each of these strategies offers a unique mechanism to boost antitumor immunity.[4]

In the process of tumor immunity $CD8^+$T cells mediate host resistance to tumors by recognizing antigens that are localized in the cytoplasm of target cells, and processed and presented as peptide complexes with MHC class I molecules.[5] Tumor infiltrating lymphocytes (TILs), especially T cells and dendritic cell (DCs) are known to be related to tumor immunotherapy.[6]

In TNBC, as in many other cancer types, tumors rich in $CD8^+$ T-lymphocytes are associated with a better prognosis. $CD8^+$ infiltrates are seen in 60% of TNBC.[6] Some evidence suggests that the effect of $CD8^+$ T-cells is more powerful in hormone receptor-negative breast cancer.[7] $CD4^+$ T cells can differentiate into a variety of subtypes upon activation, and their functions are to modulate the activity and differentiation of the immune system through modulation of, eg, B-cells, $CD8^+$-T cells and macrophages. The main subgroups that have been investigated are T-helper cells (TH1), follicular T helper cells and regulator T-lymphocytes. TH1 are the principal source of interferon-γ, and follicular T-helper cells (Tfh) are a relatively newly described subgroup of $CD4^+$ T-cells. Both subgroups have shown improved survival in some hormone receptor-positive breast cancers, but, as yet, not in TNBC.[8]

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that participate in the activation of adaptive immune cells, eg, T cells. However, tumor infiltrating DCs often show an aberrant phenotype with lower expression of costimulatory molecules, blunted antigen cross representation and upregulation of regulatory molecules, pointing towards factors in the tumor environment blunting the stimulatory effect of DCs, turning them towards a protumor genic effect.[9–11] During this process, effective tumor antigens induce DC maturation, and co-stimulatory molecules such as CD40, CD80, and CD86 are upregulated to stimulate T cell proliferation and activation.[12–15] Activated T cells have a strong ability to kill tumor cells, which is the basis for anti-tumor immune response.[16] Tumor cell lysate is the most commonly used tumor antigen, but its anti-tumor effect is limited.[17] To obtain a powerful anti-tumor immune response tumor-specific antigen (TSA) is needed.[18]

In solid tumors, the expression of TSA in outer tumor cells decreases to escape the immune system.[19] These tumor cells, which only express non-specific tumor antigens, cannot induce effective tumor immune response, and ultimately results in the survival of these tumor cells.[20,21] Hypoxia is a common occurrence in the center of the tumor, where the expression of hypoxia-inducible factor 1α (HIF-1α) increases, and hypoxic tumor cells show different physiological characteristics from normal tumor cells.[22–24] Hypoxic tumor cells are chemotherapy-tolerant and exhibit some characteristics of tumor stem cells.[25,26] The findings of Samanta's study demonstrated that chemotherapy induces the enrichment of $CD47^+CD73^+PDL1^+$ cells in TNBC through HIF-1α, thereby enabling immune evasion.[27] Antigens from chemotherapy drug-resistant cells or tumor stem cells can induce a more potent anti-tumor immune response.[28]

Tumor antigens from the central part of the tumor are difficult to obtain, which limits their use in clinical and basic research.[29] Overexpression of a gene by lentivirus transfection in tumor cells is a common research method.[30] Therefore, we speculate that if HIF-1α is upregulated in tumor cells, HIF-1α enriched tumor antigen could contain some TSA, which could induce more DC activation and stimulate T cell proliferation. Therefore, this HIF-1α enriched tumor antigen could be a good candidate for tumor antigen.

In this study, we transfected HIF-1α over-expression lentivirus into MDA-MB-231 cells, extracted related tumor antigens, and detected DC maturation, T cell proliferation, and killing effects.

Materials and Methods

Cell Culture

The triple-negative breast cancer cell-line MDA-MB-231 was purchased from the National Collection of Authenticated Cell Cultures (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM; Corning, USA) supplemented with 10% fetal bovine serum (FBS; FB25015; Clark, USA). Cells were maintained at 37°C in a 5% CO_2 incubator (Thermo Fisher Scientific).

Lentivirus Transduction

HIF-1α lentiviral *phblv-HIF1A-3flag-zsgreen-Puro* and control lentiviral *phblv-zsgreen-Puro* were provided by Hanbio (Shanghai, China). To obtain the HIF-1α high-expression cell line, MDA-MB-231 cells were transfected with lentiviral *phblv-HIF1A-3flag-zsgreen-Puro* at an MOI of 10, 48 hours after transfection, the cells were treated with puromycin (2 μg/mL) (Sigma, USA) for at last 14 days, and the control lentiviral vector was transfected in the same way.

Western Blot

MDA-MB-231-GFP and MDA-MB-231-HIF-1A cells were grown to 90% confluence in a cell culture dish. RIPA (Beyotime Biotechnology, Shanghai, China) containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail was added to the dish, and cell lysates were collected and centrifuged at 4°C at 12000 g for 15 min. A bicinchoninic acid assay kit (Beyotime Biotechnology) was used to measure protein concentrations. A 30μg total protein denaturized using sample loading buffer and high temperature was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).[31] Proteins were transferred to polyvinylidene fluoride (PVDF) membranes and inoculated with 5% non-fat milk at room temperature for 2h. After inoculation at 4°C for 16 h with the following primary antibody: HIF-1α (1:1000, Cell Signaling Technology, USA), the PVDF membrane was washed with TBST. Horseradish peroxidase-labeled secondary antibodies (1:5000, Proteintech Group, Wuhan, China) were added for 2 h at room temperature and washed with TBST. The protein bands were colored and recorded using enhanced chemiluminescence (WBKLS0500, Millipore) with a chemiluminescence imager (CLINX5600, GE, USA). Protein expression was analyzed using ImageJ software.

Induction and Maturation of Peripheral Blood Derived Dendritic Cells

The study included a total of 9 volunteers who underwent pre-collection investigations to exclude malignancies, immunological diseases, and infections. All the volunteers provided informed consent and signed the consent form to participate in the experiment. Peripheral blood was obtained from healthy female volunteers aged 20 to 50 year, after separated by human peripheral blood lymphocyte isolation buffer, the cells were resuspended in RPMI 1640 medium and inoculated in cell culture dish for adherent growth. Two hours later, non-adherent cells were removed, and a culture medium containing 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech, Suzhou, China) and 20 ng/mL interleukin-4 (IL-4) (PeproTech, Suzhou, China) was used to induce dendritic cell induction.[32,33] New 1640 medium was replaced once every three days, and on the fifth day HIF-1α enriched tumor antigen or control tumor antigen was added to the DC. The next day, a DC-activated cytokine cocktail (IL-1β (10ng/mL), IL-6 (1000U/mL), TNF-α (10ng/mL), PEG-2 (1μg/mL)) was used to inducing DC maturation.[32–36] CD40, CD80, CD86, and HLA-DR have been used to detect DC maturation.[5,10]

In vitro T Cell Proliferation Assay

Human T cells were isolated from peripheral blood using human peripheral blood lymphocyte isolation buffer, labeled with carboxyfluorescein succinimidyl ester (CFSE, Biolegend, USA), then were plated in 48-well plates. Two hours later, mature DCs were added at a ratio of DCs to T cells of 1:100.[37] Seven days later, the cells were collected and washed with PBS, and single-cell suspensions were stained with APC-CD4 or APC-CD8 anti-human antibodies. T cell proliferation was assayed by CFSE division.

T Cell Killing Effect

Human T cells were stained with CSFE, stimulated using methods 2.5 were used for co-culture with MDA-MB-231 cells; the ratio of T cells to tumor cells was 1:1. 24 hours later, the cells were collected for annexin V/PI staining, and the results were collected by flow cytometry.

Statistical Analysis

Experimental data were obtained from at least three independent experiments. The results are reported as the mean ± SEM. All statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software). One-way ANOVA was used to determine statistical significance between and among the groups. *P* was set at $p < 0.05$.

Results

Construction and Characterization of HIF-1α Expression Cell Line and the Extraction of Tumor Antigen Rich in HIF-1α

Lentivirus transfection is commonly used to construct stable cell lines. Here, HIF-1α lentiviral *phblv-HIF1A-3flag-zsgreen-Puro* and control lentiviral *phblv-zsgreen-Puro* were transfected into MDA-MB-231 cells. Forty-eight hours after transfection, the transduction efficiency was about 80%, after 15 days treatment with puromycin (2 μg/mL), HIF-1α expression cell-line MDA-MB-231-HIF-1A and control cell-line MDA-MB-231-GFP was constructed, as shown in Figure 1A, there was more than 99% of the cells were GFP positive. For HIF-1α detection Western blot was used, results showed that compared with control cells, there was more HIF-1α expression in MDA-MB-231-HIF-1A cells (Figure 1B and C). These results indicated that a tumor antigen rich in HIF-1α was successfully produced.

Induction and Maturation of DCs

DCs are powerful antigen-presenting cells. To obtain sufficient DCs, peripheral blood mononuclear cells (PBMCs) were separated and induced using granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4). The

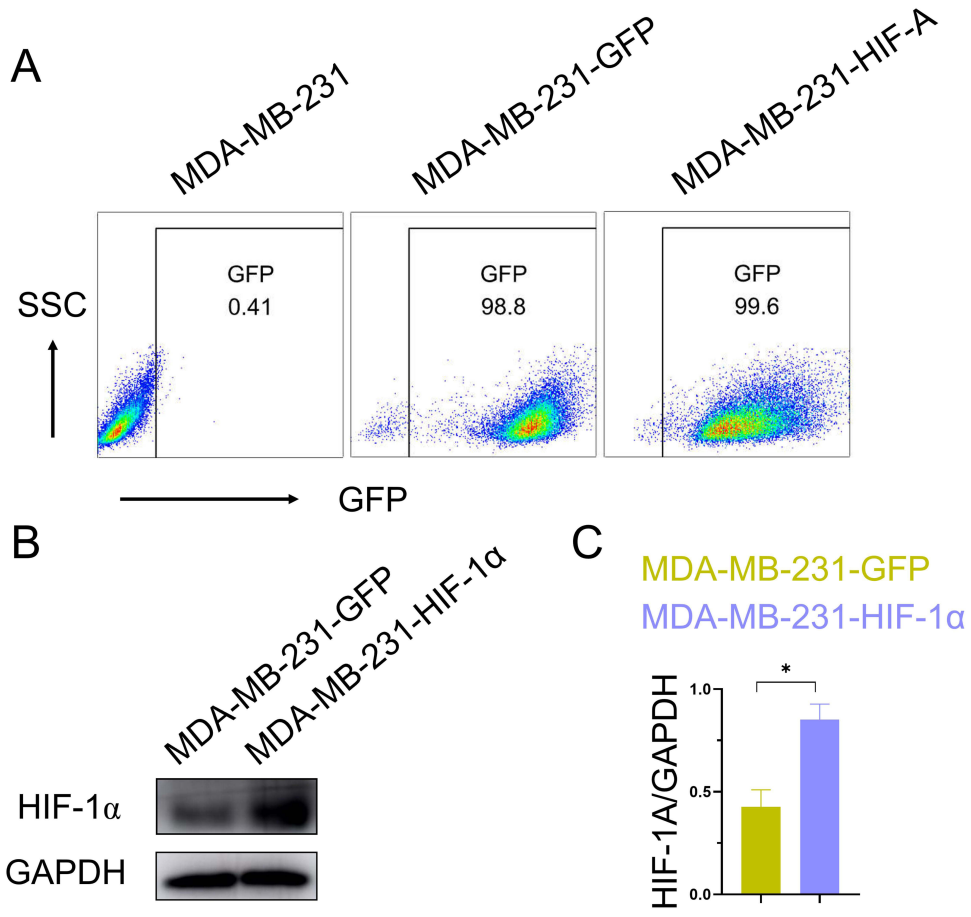

Figure 1 HIF-1α High expression MDA-MB-231 cell line was successfully constructed. (**A**) Flow cytometry results of cells after lentivirus transfection; (**B**) The level of HIF-1α was measured by Western blot; (**C**) statistical analysis of protein expression of HIF-1α. The data are expressed as the *mean* ± *SDs*. Significant differences are shown by * *P*< 0.05, ** *P*<0.01, *** *P*<0.001 by unpaired Student's t-tests comparing the normal control and HIF-1α high expression group.

percentage of DCs after induction was determined by flow cytometry using FITC-CD14 anti-human antibody. As shown in Figure 2A and B, CD14 positive cells in the HIF-1α rich tumor antigen-stimulated group (MDA-MB-231-HIF-1A) than in the control group (MDA-MB-231-GFP). The expression of surface activation molecular markers on DCs is responsible for transmitting activation signals to T cells and stimulating their proliferation. In this study, CD40, CD80, CD83, CD86, and HLA-DR levels were tested using flow cytometry after DC maturation.[38,39] It was shown that, the expression of CD40 (Figure 2A and C), CD80 (Figure 2A and D), CD86 (Figure 2A and E) and HLA-DR (Figure 2A and F) was higher in HIF-1α enriched tumor antigen group as compared with the control group. However, there was no difference in the expression of CD83 between the two groups (data not shown). These results demonstrated that HIF-1α enriched tumor antigen induced the maturation of DCs to a great extent.

3.3 in vitro T Cell Proliferation Assay

T cells are the main tumor-killing cells in the body. T cells proliferate when they are stimulated by mature DCs in vivo, and anti-tumor effects can be detected by the proliferation of T cells. As shown in Figure 3, DCs matured by HIF-1α

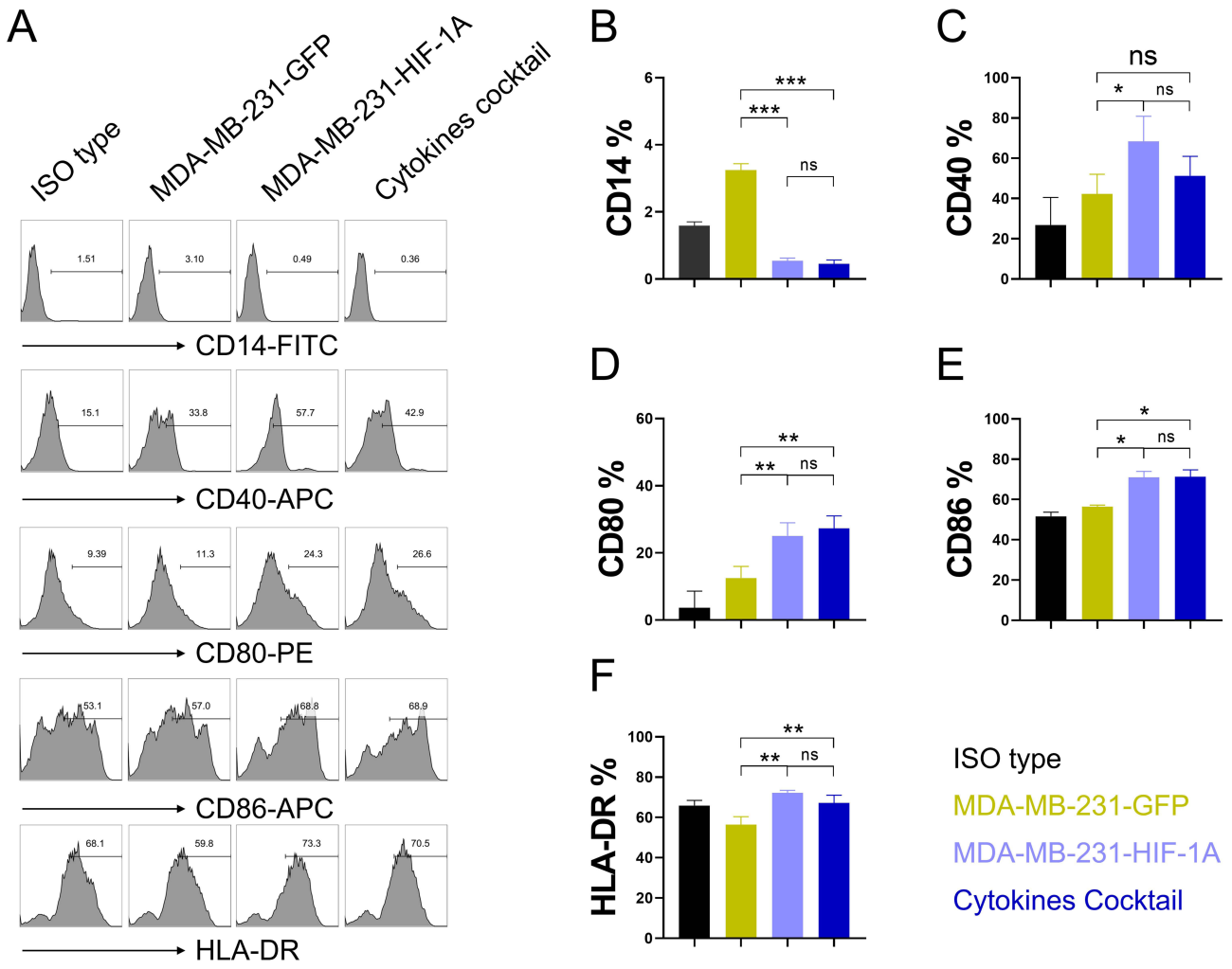

Figure 2 The overexpression of HIF-1α tumor antigens induced the maturation of dendritic cells. (**A**) After stimulated with GM-CSF and IL-4 for 5 days, the cells were treated with culture medium (ISO type), tumor cell antigen extracted from MDA-MB-231-GFP cells (MDA-MB-231-GFP), tumor cell antigen extracted from MDA-MB-231-HIF-1A cells (MDA-MB-231-GFP-HIF-1A) or cytokines containing IL-1α (10ng/mL), IL-6 (1000U/mL), TNF-α (10ng/mL), PEG-2 (1μg/mL) (Cytokines cocktail) for 1 day, the expression of CD14, CD40, CD80, CD86, HLA-DR on the surface of DC cells were detected by flow cytometry. Statistical analysis of the expression of CD14 (**B**), CD40 (**C**), CD80 (**D**), CD86 (**E**), HLA-DR (**F**) on DC cells. The data are expressed as the *mean* ± *SDs*. Significant differences are shown by * *P*< 0.05, ***P*<0.01, *** *P*<0.001 by unpaired Student's t-tests.

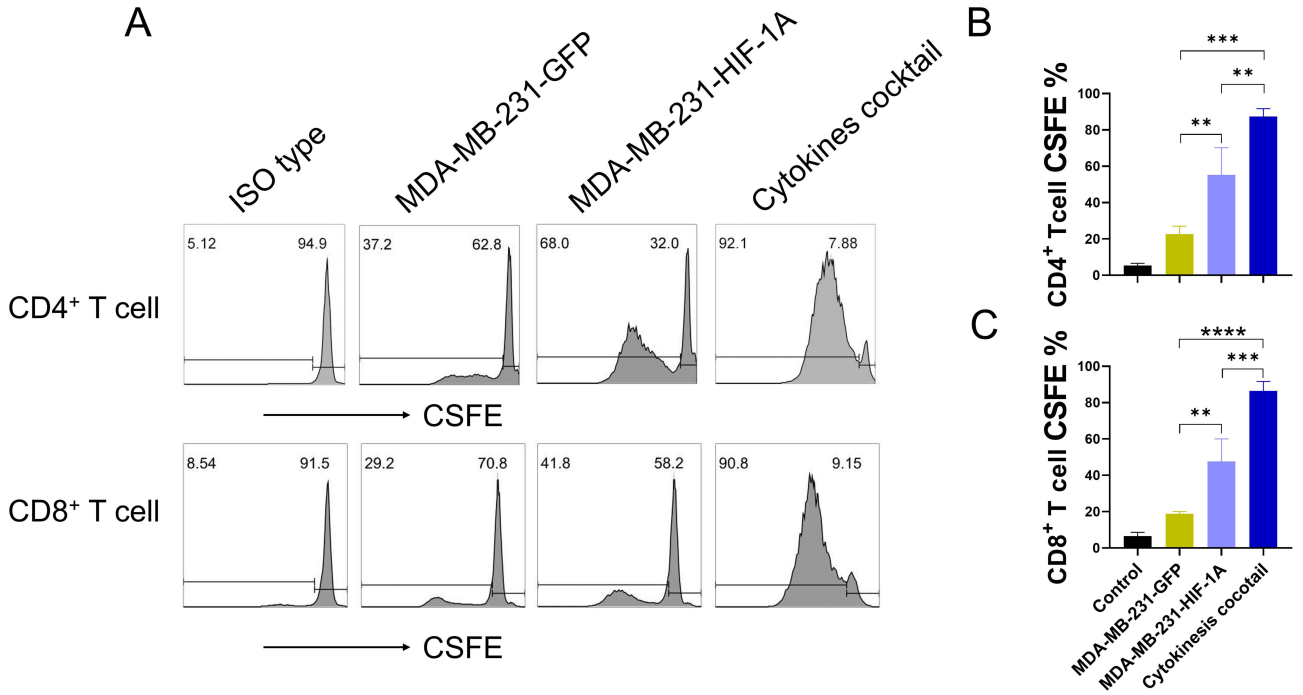

Figure 3 The overexpression of HIF-1α tumor antigens enhances the capacity of DCs to stimulate T cell proliferation. (**A**) DCs maturation by culture medium (ISO type), tumor cell antigen extracted from MDA-MB-231-GFP cells (MDA-MB-231-GFP), tumor cell antigen extracted from MDA-MB-231-HIF-1A cells (MDA-MB-231-GFP-HIF-1A) or cytokines containing IL-1β (10ng/mL), IL-6 (1000U/mL), TNF-α (10ng/mL), PEG-2 (1μg/mL) (Cytokines cocktail) were coculture with T cells for 7 days, $CD4^+$ and $CD8^+$ T cells proliferation was measured by CSFE division. Statistical analysis of $CD4^+$ (**B**) and $CD8^+$ (**C**) T cells proliferation. The data are expressed as the *mean* ± *SDs*. Significant differences are shown by * $P< 0.05$, ** $P<0.01$, *** $P<0.001$ by unpaired Student's t-tests.

enriched tumor antigen stimulated more $CD4^+$ and $CD8^+$ T cell division than DCs matured by control antigen. These results suggested that HIF-1α enriched tumor antigen could induce a more effective anti-tumor effect.

3.4 in vitro T Cell Killing Effect

After MDA-MB-231 cell co-culture with different DC-activated T cells, apoptosis of the tumor cells was detected by flow cytometry. As shown in Figure 4, more apoptotic cells were observed in the MDA-MB-231-HIF-1A group than in the MDA-MB-231-GFP group. This result indicated that T cells activated by HIF-1α enriched tumor antigen stimulated DCs had a stronger tumor-killing effect.

DAMPs Were High Expression in HIF-1α Enriched Tumor Antigens

To explore why HIF-1α enriched tumor antigens induced more potent anti-tumor immune reactions, damage-associated molecular pattern (DAMP), including HMGB1 and calreticulin (CALR), was detected by Western blotting. As predicted, there was more HMGB1 and CALR in HIF-1α enriched tumor antigens (Figure 5A–C). These results indicate that HIF-1α upregulates HMGB1 and CALR in tumor cells, which may be the mechanism by HIF-1α enriched tumor antigens induce a more potent anti-tumor immune reaction.

Discussion

Malignant tumors are the leading cause of morbidity and mortality worldwide.[40] Owing to the significant clinical efficacy of PD1/PDL1, tumor immunotherapy, which stimulates the immune system to combat tumors, is recognized as an indispensable treatment.[41] In the anti-tumor immune response, tumor antigens are recognized by antigen presenting cells (APCs); then, tumor-specific T cells are activated, the activated T cells proliferate rapidly, and secrete cytokines that kill tumor cells.[17] During this process, "signals 1 and 2" play vital roles.

Tumor antigen loaded onto the peptide-binding groove of MHC class I molecules are then transported to the cell surface, where they can be recognized by T cells, via the T-cell receptor (TCR), thus providing what is currently called

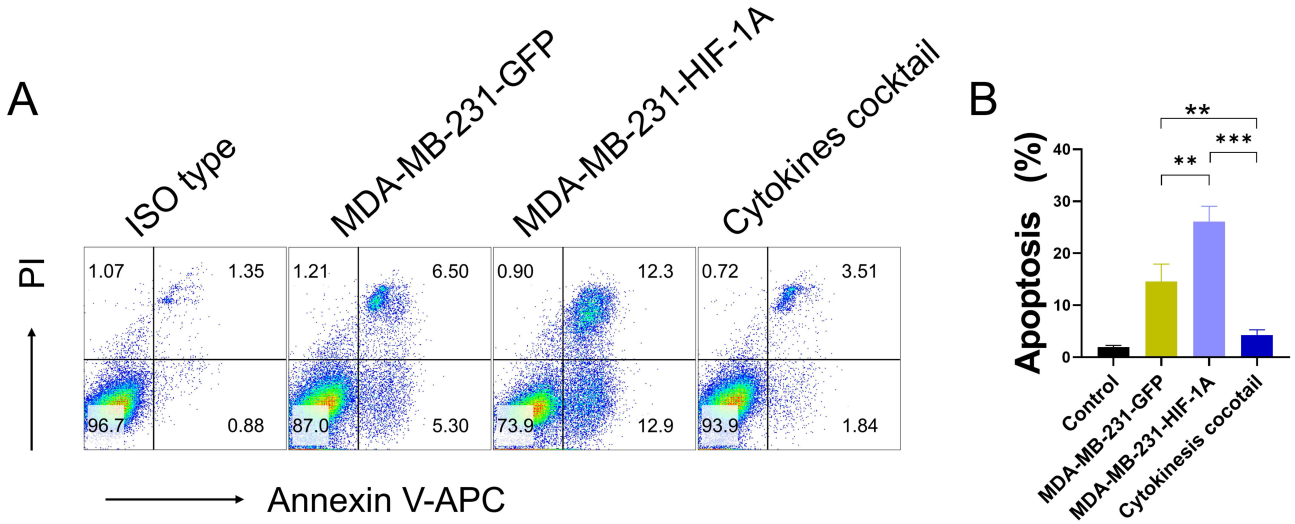

Figure 4 The tumoricidal effect of T cells activated by dendritic cells stimulated with HIF-1α overexpression tumor antigens is enhanced. (**A**) T cells, activated by DCs matured with culture medium (ISO type), tumor cell antigens extracted from MDA-MB-231-GFP cells (MDA-MB-231-GFP), tumor cell antigens extracted from MDA-MB -231-HIF-1A cells (MDA-MB-231-GFP-HIF-1A), or a cytokine cocktail containing IL-1β (10ng/mL), IL-6 (1000U/mL), TNF-α (10ng/mL), and PEG-2 (1μg/mL), were co-cultured with tumor cells for 1 day. Apoptotic tumor cells were detected using flow cytometry; (**B**) Statistical analysis of tumor cells killed by activated T-cells. Data are expressed as *mean* ± *SDs*. Significant differences are indicated by * *P*< 0.05, ***P*<0.01, and *** *P*<0.001 using unpaired Student's *t*-test.

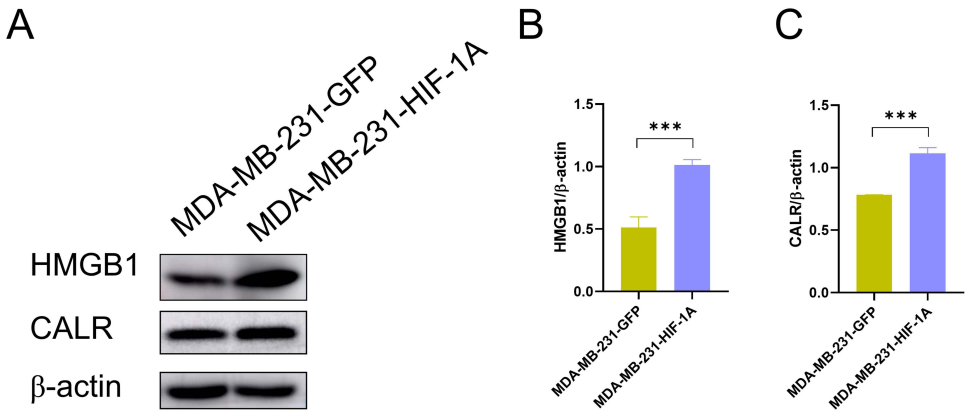

Figure 5 Expression of HMGB1 and CALR in experimental group and control group. (**A**) the level of HMGB1 and CALR was measured by Western blot; statistical analysis of protein expression of HMGB1 (**B**) and CALR (**C**). The data are expressed as the *mean* ± *SDs*. Significant differences are shown by * *P*< 0.05, ***P*<0.01, *** *P*<0.001 by unpaired Student's t-tests comparing the normal control and HIF-1α high expression group.

"signal 1" for T-cell activation. Tumor antigen, especially tumor-specific antigen (TSA), are the first step in initiating anti-tumor immunity; normally, tumors can escape T cell killing because there is not enough tumor-specific antigen.[34] TSA is upregulated in cancer stem cells and other chemotherapy-resistant cells. In this study, TSA was not detected until now, and we did not know which protein could be used as TSA. HIF-1α enriched tumor antigen may contain TSAs, because our previous data and literature have reported that HIF-1α could induce chemotherapy-resistant cells and cancer stem cells, which contain enough TSAs to trigger tumor immunity. As shown in Figure 2, more HLA-DR was detected in HIF-1α enriched tumor antigen treated moDCs, indicating that more TSAs were induced in MDA-MB-231-HIF-1A cells.

"Signal 1" is insufficient for the initiation of T-cell responses, additional signals, called "2" and "3" consisting of co-stimulatory molecules and cytokines, respectively, are indispensable to the development of effective T-cell responses.[42] The delivery of signals 2 and 3 by DCs requires their activation by innate signaling through pattern recognition receptors (PRR).[43] PRRs recognize conserved motifs in microbes or danger signals and are referred to as pathogen- or damage-associated molecular pattern molecules (PAMPs and DAMPs).[5] In this study, DAMPs, including HMGB1 and CALR,

were detected by Western blotting, as shown in Figure 5, and more DAMPs were detected in HIF-1α enriched tumor antigens.

A large number of experiments have confirmed that if there is not enough co-stimulatory signal provided by "signal 2",[6] T cells cannot be activated, and they will be in an anergic state, even undergoing apoptosis. "Signal 2" contains CD40, CD80, and CD86.[13,32,36,44] CD40 regulates $CD8^+$ T cell activation through CD40L is well known,[14,36] recent reports also show that CD40 could upregulate the expression of BCL2 in cDCs, which sustains cDC1 survival during priming of the anti-tumor response.[45] CD80 and CD86 compete with CD28 for CTLA4[13,46] and promote T cell proliferation.[47] Our results (Figure 2) demonstrated that HIF-1α enriched tumor antigen stimulated the expression of CD40, CD80, and CD86 on moDCs, which could activate T-cells.

In our study, the DC activation markers CD40, CD80, and CD86 were detected and exciting results were obtained. The methods commonly employed for assessing T cell activation include T cell proliferation and T cell cytotoxicity assays. HIF-1α enriched tumor antigen stimulated $CD4^+$ and $CD8^+$ T cell proliferation, as detected by CFSE division, and there were more apoptotic tumor cells in the MDA-MB-231-HIF-1A group. These results indicated HIF-1α enriched tumor antigen can be used as candidate tumor antigens.

The identification of a specific antigen that plays a crucial role in the enrichment of HIF-1α in tumor antigens remains unresolved until now, and this aspect may be addressed in our future research. The release of cytokines (eg, IL-12 and IFN-γ) by moDCs and activated T cells[46], along with the expression of T cells activation markers such as CD107a and CD137, which were used to validate that T cells are really activated, were not detected in this study. These are the shortcomings of our research, but we believe that they do not affect the conclusion of the study. Extraction of antigens from cell lines offers the benefits of time efficiency and cost reduction, which are crucial for clinical applications.

Conclusions

In this study, HIF-1α-enriched tumor antigens were obtained from MDA-MB-231-HIF-1A cells generated using our methodology. Subsequently, the stimulation of dendritic cells (DCs) with these enriched tumor antigens resulted in upregulation of CD40, CD80, CD86, and HLA-DR expression. Furthermore, enhanced T cell proliferation and augmented cytotoxicity were observed. Consequently, it can be concluded that our method facilitates the easy acquisition of HIF-1α-enriched tumor antigens which induce robust DC maturation and activation of T cells.

Data Sharing Statement

All data generated or analyzed during this study are available via corresponding author on reasonable request.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of The First Affiliated Hospital of Xinxiang Medical University, with Institution Review Board Number of 2019197. This study was performed in accordance with Declaration of Helsinki. All peripheral blood was collected and applied with the consent and authorization of the volunteers.

Disclosure

The authors declare that they have no competing interests in this work.

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