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Original Article

Comparable Analysis of Mature Antigen-Loaded Dendritic Cells Preparation Methods for Cytokine-induced Killer Cells Activation *in vitro*

Assel Issabekova [1*], Madina Zhunussova [1], Marzhan Zhumabekova [1], Assyl Bari [2], Altynai Adilbayeva [3], Vyacheslav Ogay [1]

[1] Stem Cells Laboratory, National Center for Biotechnology, Korgalzhyn Highway 13/5, Nur-Sultan, Kazakhstan
[2] International School of Medicine, Caspian University, 85A Dostyk Street, Almaty, Kazakhstan
[3] School of Sciences and Humanities, Nazarbayev University, 53 Kabanbay Batyr Avenue, Nur-Sultan, Kazakhstan

Corresponding Author: Assel Issabekova, PhD, Stem Cells Laboratory, National Center for Biotechnology, Korgalzhyn Highway 13/5, Nur-Sultan, Kazakhstan. Tel: +7-7472441436, E-mail: issabekova@biocenter.kz

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Abstract

Introduction: Colorectal cancer remains a leading cause of cancer-related mortality worldwide. Cancer immunotherapy involves autologous tumor-derived antigen-loaded Dendritic Cells (DCs) that activate potent antitumor immunity. Cytokine-Induced Killer (CIK) cells are a heterogeneous population of anti-tumor cytotoxic CD8+ T-cells. Cancer immunotherapy using a combination of CIK cells with DCs vaccines is a promising strategy. We investigated some of the latest developments in the DCs vaccination field, with a special emphasis on strategies to prepare highly immunogenic tumor cell antigens to load and to activate DCs. In this context, we applied the effects of immunogenic treatment modalities (heat shock) and four potent inducers of immunogenic cell death and apoptosis (mitoxantrone, oxaliplatin, 5-fluorouracil, and staurosporine) on DCs biology and their employment in DC-based activation of CIK cells.

Materials and Methods: DCs were generated from bone marrow cells using granulocyte-macrophage colony-stimulating factor and Interleukin (IL)-4. CIK cells were expanded by interferon-gamma (IFN-γ), anti-CD3 monoclonal antibody, and IL-2 stimulation. The cytotoxic activity of CIK cells against CMT-93 cancer cells was assessed by MTT assay. IFN-γ production of CIK cells was examined by ELISA.

Results: Coincubation of untreated DCs with CIK cells significantly induces antitumor immunity of CIK cells. Mature DCs loaded by polyethylene glycol with mitoxantrone-oxaliplatin-induced apoptotic tumor cells stimulate greater cytotoxicity of CIK cells compared to DCs loaded with staurosporine, oxaliplatin- and 5-fluorouracil-inactivated tumor cell antigens, whole tumor lysates, and total tumor RNA in CMT-93 cells.

Conclusions: Heat shock and mitoxantrone-oxaliplatin-treatment are the best approaches for cancer cell antigens preparation for DC-induced CIK cells activation.

Keywords: Dendritic Cell, Cytokine-Induced Killer Cell, Colorectal Cancer, *In Vitro*
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Introduction

Colorectal cancer ranks third among cancer types worldwide, with almost 2 million new cases in 2018.[1] Colorectal cancer rates were 17.5 and mortality was 8.2 per hundred thousand population in 2017, with comparable statistics in the structure of oncopathology in Kazakhstan. Since 2011, colorectal cancer screening is obligatory for individuals aged from 50- to 70-years in Kazakhstan.[2] Despite progression in cancer treatment in most cases, the disease is incurable.

New therapeutic strategies, such as immunotherapy, may benefit the median survival rate. The main goal of antitumor immunotherapy is the generation of tumor specific cytolytic $CD8^+$ T-cells. The CIK cells are a heterogeneous population of non-major histocompatibility complex (MHC)-restricted anti-tumor cytotoxic $CD8^+$ T-cells. CIK cells are generated *ex vivo* by stimulation of peripheral blood mononuclear cells with anti-CD3 monoclonal antibody, interleukin (IL)-2, IL-1, and interferon-gamma (IFN-γ).[3] The major cytotoxic subsets of human CIK cells are Natural Killer T (NKT) cells co-expressing CD3 and CD56 receptors.[4] Mouse NKT-cells are T-cells expressing the NK cell marker NK1.1.[5] In order to stimulate cytotoxic T-lymphocytes, several DC subtypes derived from different sources could be used. DCs are specialized antigen-presenting cells attracting and activating CD8- and CD4-positive T-cells. During cancer, mature DCs migrate into lymph nodes and present antigens of dying tumor cells to Tcells through MHC I. Bone marrow precursors of DCs give a rise to conventional (Pre-DCs) and plasmacytoid DCs. Monocyte-derived Dendritic Cells (Mo-DCs) are distinct

DCs derived from Ly6Chi monocytes with relevant phenotype.[6] DCs express cell surface markers such as MHC I and II; CD11 a, b, and c; CD54, CD58; and co-stimulatory markers, such as CD40, CD80, CD83, and CD86.[7] Mature DCs induce T-cell activation and proliferation by cytokines, such as IL-1, IL-6, IL-8, IL-10, IL-12, and TNF-α. DCs exhibit tumor-associated peptide antigens on MHC class I molecules thereby deliver antigen-specific information to naive T-cells about malignant cells.[8] Several studies have shown anti-cancer potential of CIK cells co-cultured with DCs[9,10] For effective DC-based vaccine, antigen sources, antigen loading strategies and DCs maturation are crucial. Different types of antigens in the form of whole-tumor lysate, apoptotic tumor cells, peptides, proteins, viral vectors, DNA, and RNA have been investigated, however the ideal source has not yet been determined. Cells undergoing immunogenic death have a molecular pattern associated with damage and cause an inflammatory response. Mitoxantrone (MTX) is an anthracenedione antineoplastic agent that causes immunogenic apoptosis called immunogenic cell death and is used in the treatment of multiple sclerosis, leukemia, lymphoma, hormone-refractory prostate cancer, and breast cancer.[11] Several researchers revealed that vaccination with MTX-treated tumor cells resulted in a significant reduction of colorectal liver metastases formation.[12,13] The aim of this research was to compare the influence of MTX-inactivated colorectal tumor cells on DCs maturation and CIK cells activation with other antigen preparation methods. Staurosporine (Stau) is an inducer of cell apoptosis and is used for comparing with MTX. Oxaliplatin (OX) is a conventional chemotherapeutic agent, which induces an immunogenic cell death, and increases antigen processing and cross-presentation.[14] 5-Fluorouracil (5-FU) is a common chemo drug used in combination with OX. Additionally, total tumor RNA, lysates obtained by freeze-thaw cycles, and heat-shock-induced tumor cells were used for antigen preparation. *In vitro* antigen delivery may be accelerated by electrical (electroloading)[15] and chemical (polyethylene glycol; PEG)[16] means.

Here, we investigated the influence of immunogenic and/or apoptotic tumor cells, which were induced by several inducers (MTX, Stau, OX,5-FU) and a modality (heat shock), on DCs antigen loading and maturation, and DC-based activation of CIK cells, and compared them with DCs loaded with tumor lysates. Loading strategies, such as electroloading and PEG (MW 1450), were used to facilitate tumor cell delivery to DCs.

Materials and Methods

Cancer Cell Culture CMT-93

The mouse rectum carcinoma cell culture CMT-93 was purchased from ATCC (Manassas, VA, USA). The CMT-93 cells were cultured in Dulbecco's modified Eagle medium (Life Technologies Limited, Paisley, UK) supplemented with 10% Fetal Bovine Serum (FBS), penicillin (100 µg/ml), and streptomycin (100 µg/ml). Cells were grown in an incubator with 5% CO_2 at 37 °C.

Expansion of Pre-DCs and Mo-DCs

Murine Mo-DCs and Pre-DCs were generated from C57BL/6 mouse bone marrow cells. In order to remove red blood cells, bone marrow cells were incubated for 5 min in BD Pharm Lyse™ 10 buffer (BD Bioscience, San Jose, CA, USA). After 2 h of incubation, adherent cells were separated from suspension cells. Adherent monocytes give rise to Mo-DCs, and suspension cells contain DC precursors and differentiate into Pre-DCs. Both types of cells were cultured in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), and 50 µM 2-mercaptoethanol with the addition of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) (20 ng/ml) (Peprotech, London, UK) and IL-4 (20 ng/ml) (Peprotech, London, UK). On days three and six, half of the medium was replaced with fresh medium. On day seven, the negative fraction of immature DCs was isolated by IMAG DM Set (BD Biosciences, San Jose, CA, USA).

Pulsing DC with Antigens

For antigen preparation, initially all tumor cells in all groups were subjected to heat shock at 42 °C for 2 h and afterwards at 37 °C for an hour. Tumor Freeze-Thawed lysate (FT lys) was prepared by three repeated freeze-thaw cycles in liquid nitrogen, and supernatant was sterilized through 0.22-µm filter membrane. Another four groups of antigens were obtained by stimulation with staurosporine (10 µM, Stau lys), oxaliplatin alone (500 µg/ml, OX lys) and oxaliplatin in combination with mitoxantrone (100 µM, MTX+OX lys) or 5-fluorouracil (1000 µg/ml, 5FU+OX lys) for 24 h. Group six included heat-shocked tumor cells (hshCMT93). RNA from CMT-93 cells as antigen (tRNA) was extracted using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's protocol. The total RNA concentration was measured using NanoDrop and was equal to 5 µg per 10^6 DCs. The last group was without antigens (Ag-). Co-incubation of antigens with DCs was performed for 24 h at 1:2 ratio.

DC Antigen Loading with PEG

To increase DC antigen loading with PEG, Mo-DCs were mixed with tumor cells at a 2:1 ratio in a pre-warmed serum-free RPMI 1640 medium. The mixed cell pellet was gently resuspended in a pre-warmed PEG solution (50%, MW 1450) (1 ml per 1-5×10^6 cells) for 3-5 min at room temperature. Subsequently, the PEG was diluted by slow addition and agitation 1, 2, 4, 8, 16 and 19 ml of pre-warmed serum-free RPMI medium to a final volume of 50 ml. The samples were

centrifuged at 300g, RT. The cell pellet was cultured in complete RPMI 1640 medium with 20 ng/ml GM-CSF and 20 ng/ml IL-4 for one day. Low adherent cells were collected by gently pipetting up and down several times.

DC Antigen Loading with Electroporation

DCs were electroporated on day seven of culturing in EP buffer. DCs were harvested and washed twice in EP buffer. The cell suspension was transferred to a 2-mm gap electroporation cuvette for immediate electroporation using the Gene Pulser Xcell® apparatus (Bio-Rad). Electroporation conditions were compiled as follows: voltage of 110 V, pulse time of 25 ms. After electroporation, cells were immediately diluted in pre-warmed RPMI 1640 medium with 20 ng/ml GM-CSF and 20 ng/ml IL-4, and incubated for a day. In the final experiment, DC maturation was stimulated by Lipopolysaccharides (LPS) (Sigma, Taufkirchen, Germany) (1 µg/ml) and TNF-α (50 ng/ml) for 2 h. Furthermore, eight groups of Mo-DCs and Pre-DCs were co-cultured with $NK1.1^+$, $NK1.1^-$, $NK1.1^-$ $CD8a^+$ or $CD8a^+$ CIK cells at a cell ratio of 1:10 for 24 h.

Expansion of CIK Cells

The C57BL/6 mice were sacrificed by cervical dislocation and placed in 70% ethanol for 3 min. After mouse dissection in sterile conditions, the spleen was isolated and passed through a 70-µm cell strainer. The obtained cell suspension was washed twice in PBS containing 2% FBS, and then red blood cells were depleted by using BD Pharm Lyse™ 10 buffer (BD Bioscience, San Jose, CA, USA). Splenocytes were cultured in RPMI 1640 supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), and 50 µM 2-mercaptoethanol with the addition of 1000 U/ml IFN-γ (Panbiotech, Germany). After 24 h, splenocytes were transferred to a Petri dish coated with 50 ng/ml of the anti-CD3 monoclonal antibody with IL-2 (500 U/ml) (Peprotech, London, UK) supplementation. Fresh medium containing IL-2 was added every 2-3 days. After 12 days, CIK cell selection was performed by positive magnetic separation with NK1.1 biotinylated mAbs (Thermo Fisher, San Diego, CA, USA) and CD8a biotinylated mAbs (BD Bioscience, San Jose, CA, USA). The NK1.1-negative cell fraction was also utilized.

In vitro CIK Cell Cytotoxicity

The cytotoxic activity of combined DCs and CIK cells against CMT-93 cancer cells was assessed by MTT assay. Two combinations of DCs with CIK cells as effector cells were added to a 96-well plate containing earlier seeded CMT-93 cancer cells as target cells at a ratio of 5:1. After the 48-h medium was discarded from each well, 100 µl of MTT reagent was added for 2-4 h following formazan crystal formation. Afterward, the solution was aspirated, and DMSO was added to solubilize crystals. The optical density of each well was read by a spectrophotometer (Bio-Rad 680) at 580 nm.

Cytokine Production of CIK Cells

The supernatant of *ex vivo* expanded CIK cells after co-cultivation with target cells at a ratio of 1:10 in two experiments ($NK1.1^+$ CIK, $NK1.1^-$ CIK, and $CD8a^+$ CIK) was collected and assessed for IFN-γ production using an IFN-γ Mouse Uncoated ELISA Kit with a range of detection of 15-2,000 pg/ml (Invitrogen, Austria). The cell density was 10^6 cells per ml. The optical density of each well was read by a spectrophotometer (Bio-Rad 680) at 450 nm.

Statistical Analysis

Statistical processing of the results was carried out using the Prism Graphpad. The statistical results of experimental data are presented in graphs indicating the value of the standard deviation.

Results

The optimal antigen formulation for DCs loading remains an open issue limiting DC-based vaccines. Our research focused on the effect of DCs pulsed with antigens of the CMT-93 colorectal tumor cell culture on CIK cell cytotoxic activity. DCs were generated from bone marrow plastic adherent monocytes and non-adherent bone marrow precursors of DCs using GM-CSF and IL-4 as previously described. On day six of culturing, CMT-93 tumor antigens were added to the culture for 24 h. Immature CD11c+ DCs (Figure 1A) were pulsed with tumor lysates extracted from the CMT-93 cell culture at a ratio of 2:1 (DCs: tumor cells). Antigens were obtained using distinct methods, including CMT-93 exposure to a variety of chemotherapeutic agents. The generated CIK cells were sorted into $NK1.1^+$, which is one of the main receptor of NK cells, and $NK1.1^-$ subpopulations, representing 20% and 80% of CIK cells, respectively. As previously reported, more than 86% of cells in $NK1.1^+$ subset were $CD8^+CD3^+$, that may be considered as major tumor cytolytic cells.[17,18] After 24 h of antigen-loading, DCs were combined with $NK1.1^+$, and $NK1.1^-$ CIK cells at a ratio of 10:1 for 48 h. After the co-culturing of the DCs and CIK cells, we observed typical cluster formation (Figure 1B). Cytotoxicity of CIK cells was assessed using CMT-93 cells. As shown in Figure 2, the highest lytic activity was detected in the $NK1.1^+$, groups of CIK cells activated by Mo-DC loaded with OX and 5-FU stressed cancer cells (55,8%), total tumor RNA (67,3%) and heat shock-induced CMT-93 cells (66,7%). The treatment of colorectal cancer cells with OX and/or 5-FU leads to the release in culture supernatant high levels of High-Mobility Group Box 1 (HMGB1) and Heat Shock Protein 70 (HSP70). These proteins represent Danger-Associated Molecular Patterns

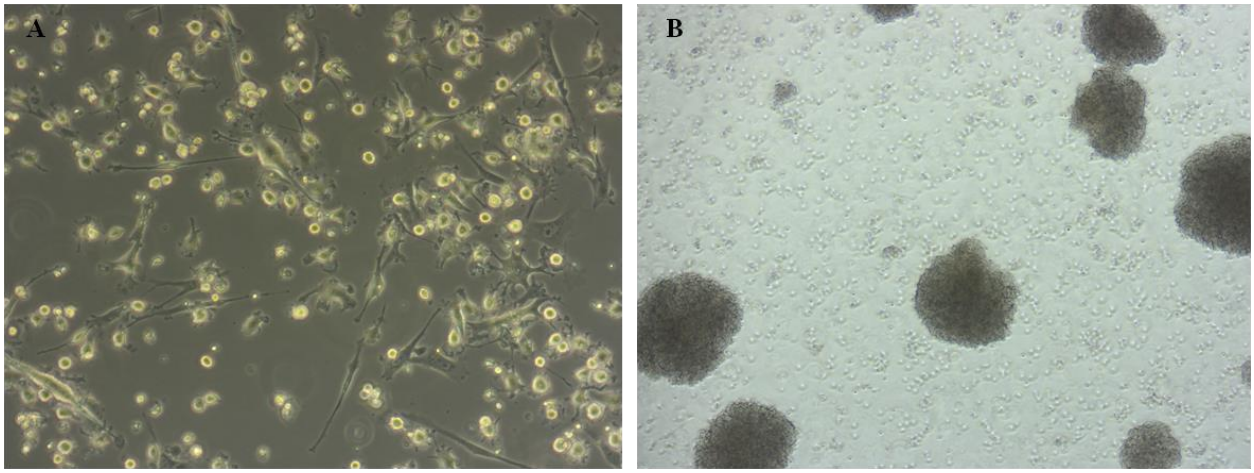

Figure 1. Morphology of DCs and CIK Cells. Magnification 100X, **A)** DCs on 6 day of cultivation, **B)** Clusters of CIK cells after DCs activation.

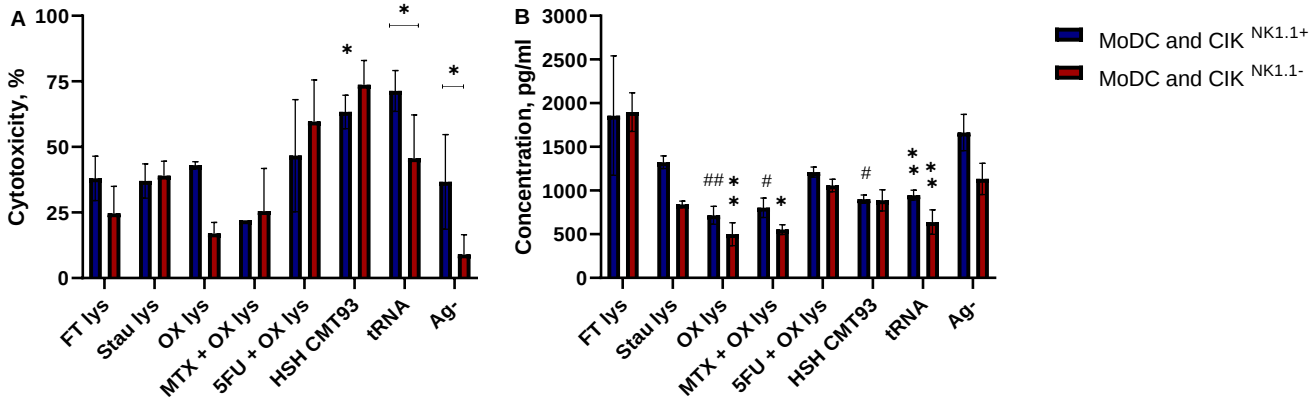

Figure 2. Cytolytic Activity (A) and IFN-γ Production (B) of NK1.1-positive and NK1.1-negative Fractions of CIK Cells after Stimulation with Mo-DCs. *p<0.05, **p<0.005, #$p\leq$ 0.001, ##$p\leq$ 0.0001.

Patterns (DAMPs), which can activate DCs via Toll-Like Receptors (TLRs).[19] However, in this study antigens did not contain proteins from chemically treated cancer cells but were presented in the heat shocked tumor cells supernatants. Supernatant proteins after heat shock contain DAMPs, and had no impact on impairing immunogenicity of antigens. Heat shock (41-43 °C) also stimulates the immunogenic apoptosis of the cells and the production of HSP60, HSP70, and HSP90.[20] In contrast, in the Mo-DC and NK1.1$^-$ groups, the treatment of CMT-93 cells with OX combined with 5-FU (50.4%) and heat shock (68.35%) for 2 h at 42 °C, enhanced cytotoxic ability of CIK cells. Cytokine production level of IFN-γ of expanded CIK cells was measured by ELISA. The supernatant of the two groups was harvested after two days of co-cultivation with Mo-DCs. IFN-γ levels in the Mo-DC-activated NK1.1$^+$, subpopulation exceeded the rates of Mo-DCs pulsed with similar antigens in the NK1.1- group. According to the results of ELISA, the high levels of IFN-γ secretion was observed in the control, freeze-thawed and Stau-treated groups, and relatively low levels in the OX-, 5-FU-, total tumor RNA- and heat-shock-induced CMT-93 cell groups. However, the obtained data do not conform the results from the MTT assay and there is no correlation between killing ability of CIK cell subpopulations and IFN-γ secretion. Despite good results in the FT lys group freeze–thaw effect on the necrosis of cancer cells, therefore this method generates non-immunogenic antigens that impair maturation via TLRs and activity of DCs.[21] To this end, using immunogenic cell death induced by chemicals such as Ox, MTX is more preferable. CD8a$^+$ cytotoxic T lymphocytes may respond to this cytotoxicity in the NK1.1$^-$ fraction. CD8a-positive cells enriched in the NK1.1$^-$ population were also tested to assess their lytic activity against tumor cells (Figure 3 A, B). Analysis of the one-day and five-day antigen loading of DCs exhibited minimal inhibition of response after prolongation of incubation. According to previous research, DCs retain capacity to take up antigens within 20 to 40 h.[22] However prolongation of incubation period until 5 days and loading period of DCs antigens has no influence to the ability of these cells to activate CIK cells. Here, we applied

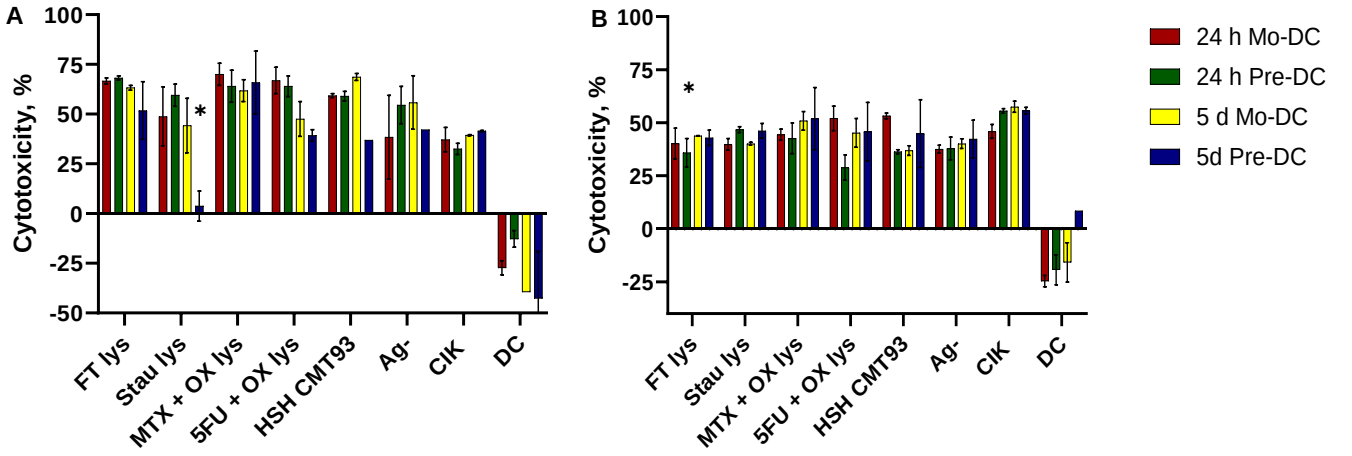

Figure 3. Cytotoxic Activity of NK1.1+ (A) and CD8a+NK1.1- (B) Cell Fractions of CIK Cells. *p<0.05.

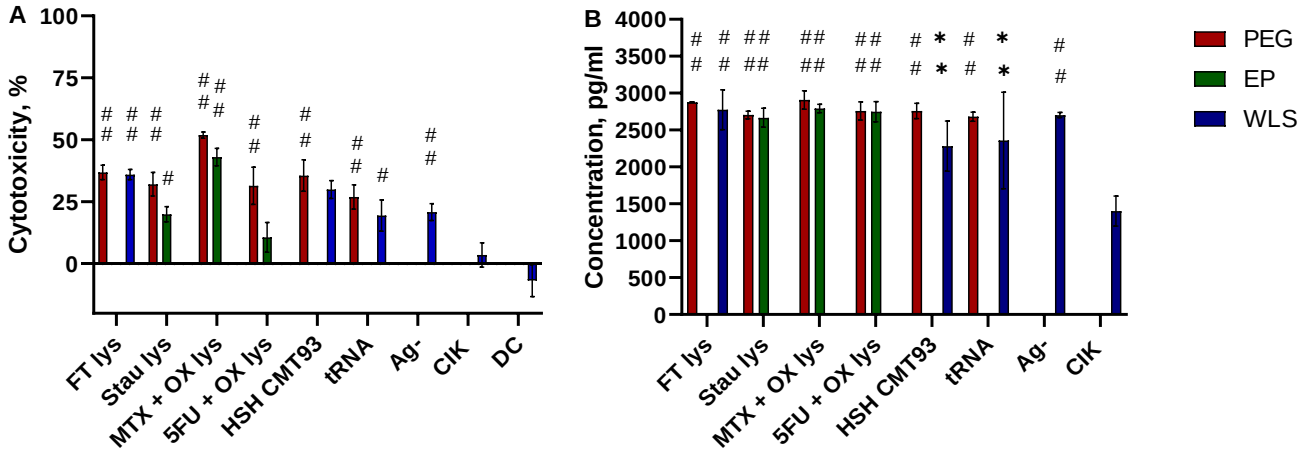

Figure 4: Cytolytic Activity and IFN-γ Production of the CD8a-positive Fraction of CIK Cells After Stimulation with Mo-DCs After Different Methods of Antigen Loading. *p<0.05, **p<0.005, #$p\leq$ 0.001, ##$p\leq$ 0.0001.

applied Mo-DCs and Pre-DCs, and the comparison of the T lymphocyte activation potential of Mo-DCs with that of Pre-DCs revealed a similar capacity. Pre-DCs derived from bone marrow precursors belong to conventional DCs, Mo-DCs differentiate from monocytes and these two populations have differences in antigen cross-presentation. However, this work demonstrates similar efficacy of Mo-DCs and Pre-DCs to activate CIK cells (Figure 3A). The significant differences were undefined within types of antigens. Our data confirms that DCs may activate CIK cells without any antigens. It is contrary to the fact that activated NK cells could lyse autologous immature DCs.[23] Thus generated by us, immature DCs without any antigens and activation were not killed by CIK cells. This evidence may be explained by spontaneous maturation of DCs during *in vitro* manipulation, which has been confirmed by several studies.[24] Next, we isolated CIK cells with a CD8a antibody and evaluated the cytotoxic activity of positive cells against tumor cells. PEG and electroporation were used to stimulate antigen engulfment by DCs. After antigen loading of DCs, 50 ng/ml TNF-α and 1 µg LPS incubation resulted in more effective DCs maturation and immunogenicity than without facilitation. As shown in Figure 4A, CIK cells co-cultured with MTX-OX-treated cancer-loaded DCs exerted significantly increased and more efficient cytotoxic activity against CMT-93 than CIK cells co-incubated with DCs loaded with tumor cells inactivated by other drugs, or unpulsed DCs. This effect also may be caused by additional activation of DCs with TNF-α and LPS. TNF-α also efficiently can activate DCs as IFN-γ. LPS (the active lipid A component) from Gram-negative bacteria is inquired by TLR4 and has been widely used as a method to stimulate the DCs maturation.[22] ELISA revealed an increase in IFN-γ production in all combinations of CIK cells with DCs compared with CIK cells only (Figure 4B). These results showed that IFN-γ expression did not significantly correlate with CIK cells cytotoxicity.

Discussion

CIK cells serve as a prospective pharmacological tool for cancer immunotherapy due to the safety and effective antitumor

activity. Many efforts have been made to improve therapy of CIK cells in combination with other antitumor therapies and CIK cells cytotoxicity. DCs present antigens to naive T-cells and can activate them. Many researches revealed that DCs could stimulate NK cell-dependent antitumor response through a cell-to-cell contact. One of the mechanisms of NK-cells activation during DCs and CIK cells interaction explained by influence of MHC class I-related (MIC) A and B expression on IFN-α-stimulated DCs.[25] NK-cells are also able to induce DCs activation by TNF-α/IFN-γ release and cell-cell contact through NKp30.[23] About a hundred of clinical trials on CIK cells have been registered on Clinical Trials.gov. However, only few investigations have been found to be related to *in vitro* influence of DCs on CIK cells proliferation, cell phenotype and antitumor activity.[26,27] The optimal delivery strategy and type of tumor antigens are one of the most important factors for the development of successful DCs-based anticancer vaccines. Importantly, tumor antigens can be prepared using several methods, and the technique of inducing cell death and cell stress as heat shock could have an impact on the immunogenicity and efficacy of the therapy.[28] Previous *in vitro* investigations, simple maturation modalities as cytokine treatment (TNF-α or certain cancer antigen as Ca 19-9) for the preparation of active DCs have been used. In this research, we activated CIK cells by mature antigen-loaded DCs. As shown by the results, MTX-OX-induced immunogenic apoptotic tumor cell-stimulated DCs exhibit the largest activation potential of CIK cells cytotoxicity. The treatment of CT26 murine colorectal cancer cells by physiological levels of MTX (1 μM) demonstrated that vaccination with these cancer cells resulted in significantly increasing tumor infiltrating leukocytes through the expression of CD69 T cell surface activation marker and decreasing in metastases formation in colorectal liver *in situ*.[12] The obtained data concerning the lytic activity of the $CD8a^+$ CIK fraction stimulated by freeze-thawed DCs exposed to Stau and OX tumor cell antigens correlate with Duewell P. et al.'s results, revealing that the cytotoxicity of $CD8^+$ T lymphocytes towards the tumor was insignificantly increased and associated with inefficient $CD8^+$ DC maturation.[29] Although, apoptotic cancer cells treated with OX alone or in combination with 5-FU induce DC maturation via TLR4 with HLA-DR, CD80 and CD86 were significantly upregulated.[19] Notably, the high antitumoral activity of autologous CIK cells exposed to DCs loaded with total tumor RNA compared to allogeneic CIK-DC-tRNA (1.5-fold increased) has been demonstrated in experiments with osteosarcoma.[30] Tesniere A. et al. previously reported that OX-induced immunogenic death of mouse colon cancer cells resulted in calreticulin exposure and HMGB1 passive emission, which subsequently interacts with TLR4 as DAMP.[31] Therefore, as shown in our research, the optimal method of obtaining tumor antigens is sequential heat shock and the treatment of cancer cells with MTX and OX in combination.

Previously, many strategies of combinational therapy with CIK cells have been developed including additional cytokines, antibodies, check point inhibitors, chemotherapeutic agents, nanoparticles, and chimeric antigen receptor. An alternative method of CIK cell induction can be direct activation. Agonistic glycolipid ligand α-galactosylceramide-activated NKT cells interact with immature DCs, stimulate the maturation of DCs, and mediate long-term antitumor immunity. Another synthesized NKT cell glycolipid ligand, termed RK, confirmed to be superior in anti-tumor responses compared to α-galactosylceramide.[32] Finally, studies on cancer therapy using CIK cells and the exact mechanism of action are need to be further investigated.

Conclusion

Heat shock and treatment with MTX and OX are the best approaches for cancer cell antigens preparation for DCs induced CIK cells activation. This choice is based on the mechanisms of MTX and OX in triggering immunogenic cell death of tumor cells followed by DAMP formation, which constitutes one of the essential factors for DCs maturation, proper DCs licensing by effector cells and their effective cytotoxic activity against target cells.

Authors' Contributions

AI was involved in planning the research, and drafted the manuscript, MZ calculated the experimental data, performed the analysis, and designed the figures. All authors contributed equally in the data acquisition/collection and aided in interpreting the results. All authors participated in the critical revision of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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