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COMPARATIVE ANALYSIS OF IMPACT OF TUMOUR ANTIGEN PREPARATION METHODS ON HUMAN DENDRITIC CELLS PRIMING AND EFFICIENT CYTOKINE-INDUCED KILLER CELLS ACTIVATION *IN VITRO*

Adaptive cell immunotherapy namely combination of dendritic cells (DC) and cytokine-induced killer (CIK) cells is a promising tool in treating various types of cancer and choosing the appropriate technique of tumour antigens obtaining is a challenging issue. CIK cells also called NKT cells are an in vitro propagated population of cells characterized by hybrid NK cells and T cells phenotype. DCs are highly specialized antigen-presenting cells, which uptake, process and present antigens to effector cells of the immune system. In this study, we compared the effect of tumour antigens obtained by different methods on dendritic cells maturation and their ability to activate CIK cells. Cytotoxicity of CIK cells, generated in two different conditions (whole blood or peripheral blood mononuclear cells), was accessed using SW620 cells as a target by MTT assay. According to obtained results, CIK cells expanded from whole blood showed significantly strong anti-tumoral activity compared to CIK cells generated from peripheral blood mononuclear cells isolated by Histopaque-1077 density gradient centrifugation. Also, we determined that all examined methods of antigen preparation can be used, but only in the case of antigen-loaded DCs cocultivation with CIK cells.

Key words: Cytokine-induced killer cell, dendritic cell, immunogenic cell death, damage-associated molecular pattern, TNF-alpha.

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Ісік антигендерін дайындаудың әртүрлі әдістерінің адам дендриттік жасушаларын праймирленуі мен цитокин белсендірілген киллер жасушаларды активтенуіне әсерін салыстырмалы *in vitro* талдау

Адаптивті жасушалық иммунотерапия, атап айтқанда дендритті жасушалар (ДЖ) мен цитокин белсендірілген киллер (ЦБК) жасушаларды бірге әр түрлі қатерлі ісік түрлерін емдеуде қолдану перспективті құрал болып табылады және ісік антигендерін дайындаудың сәйкес техникасын таңдау күрделі мәселе болып табылады. ЦБК жасушалары – НКТ жасушалар деп аталады және НК және Т жасушаларының гибритті фенотипімен сипатталатын in vitro көбейтілген жасушалар популяциясы. ДЖ антигенді қабылдап, процессингтен өткізіп, иммундық жүйенің эффектор жасушаларына таныстыратын жоғары мамандандырылған антиген презентациялаушы жасушалар. Бұл зерттеуде біз әртүрлі әдістермен алынған ісік антигендерінің дендритті жасушалардың жетілуіне және олардың ЦБК жасушаларын белсендіру қабілетіне әсерін салыстырдық. Екі түрлі жағдайда (жалпы қан немесе перифериялық қанның мононуклеарлы жасушалары) пайда болған ЦБК жасушаларының цитотоксикалық әсеріне SW620 жасушаларымен MTT талдауы арқылы қол жеткізілді. Алынған нәтижелерге сәйкес, толық қаннан алынған ЦБК жасушалары Histopaque-1077 тығыздық градиентінде центрифугалаумен перифериялық қанның мононуклеарлы жасушаларынан оқшауланған ЦБК жасушаларымен салыстырғанда, ісікке қарсы айтарлықтай белсенділікті көрсетті. Сондай-ақ, біз антиген жүктелген ДЖ мен ЦБК жасушаларын бірге өсірген жағдайда антигенді дайындаудың барлық зерттелген әдістерін қолдануға болатындығын анықтадық.

Түйін сөздер: цитокин белсендірілген киллер жасушалар, иммуногендік жасуша өлімі, зақымданумен байланысты молекулалық фрагмент, TNF-альфа.

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Сравнительный анализ влияния методов подготовки антигенов опухоли на праймирование дендритных клеток человека и эффективное активирование цитокин-индуцированных клеток *in vitro*

Адаптивная клеточная иммунотерапия, а именно комбинация дендритных клеток (ДК) и цитокин-индуцированных киллерных (ЦИК) клеток, является многообещающим подходом в лечении различных типов рака, и выбор подходящей методики получения опухолевых антигенов является сложной задачей. Клетки ЦИК, также называемые НКТ-клетками, являются in vitro размноженной клеточной популяцией, для которой характерен смешанный НК клеточный и Т-клеточный фенотип. ДК – это высокоспециализированные антиген-презентирующие клетки, поглощающие, перерабатывающие и предоставляющие антигены эффекторным клеткам иммунной системы. В этом исследовании мы сравнили влияние опухолевых антигенов, полученных разными методами, на созревание дендритных клеток и их способность активировать ЦИК-клетки. Цитотоксичность клеток ЦИК, полученных двумя различными способами (цельная кровь или мононуклеарные клетки периферической крови – МКПК), оценивали с использованием клеток SW620 в качестве мишени анализом МТТ. Согласно полученным результатам, ЦИК-клетки, размноженные из цельной крови, показали значительно сильную противоопухолевую активность по сравнению с клетками ЦИК из МКПК, выделенных центрифугированием в градиенте плотности Histopaque-1077. Также мы определили, что могут быть использованы все проверенные методики получения антигена с условием, что антиген-нагруженные ДК будут культивироваться совместно с ЦИК-клетками, полученными из цельной крови.

Ключевые слова: цитокин-индуцированная киллерная клетка, дендритная клетка, иммуногенная гибель клеток, молекулярный фрагмент ассоциированный с повреждением, ФНО-альфа.

Introduction

Colorectal cancer is the second leading cause of cancer-related deaths worldwide with 4789635 prevalent incidences during 5 years. According to information obtained by the International Agency for Research on Cancer, there were 19,7 of new patients with colorectal cancer (4th place) and mortality cases 8.9 (3rd place) per 100000 population registered in 2018 worldwide. In South Central Asia Kazakhstan ranks first as a country with a high number of new cases and mortality caused by colorectal cancer [1].

Immunotherapy in contrast to conventional cancer treatment approaches such as chemotherapy, hormone therapy, radiation and surgery has a crucial aim to encourage host immunity against cancer, but at the same time immunotherapy can be used in combination with each of the mentioned above therapies. Adoptive cell therapy is one of the prospective types of immunotherapy, which essentially involves *ex vivo* stimulation or genetic modifications of isolated host cells and infusion of final cellular products (dendritic cells, cytokine-induced killer cells, lymphokine-activated killer cells, tumour-infiltrating lymphocytes, cytotoxic T-lymphocytes, chimeric antigen receptor T cells, T-cell receptor engineered T-cells) to cancer patients [2].

Dendritic cells (DCs) are the most proficient antigen-presenting cells linking both innate and adaptive immune systems [3, 4]. In cancer settings after engulfment of tumour-derived antigen immature DC undergoes maturation which results in CD80 and CD86 upregulation, IL-12 secretion with sequential translocation to lymph nodes to prime effector T-cells [5].

CIK cells are *ex vivo* expanded heterogeneous cell subset which shares properties of T/NK-cells with MHC-unrestricted cytotoxicity [6, 7]. Cultivation of human CIK cells generally includes stimulation with IFN-gamma, antibodies against CD3, IL-2, but also there can be used additional inducers such as IL-15 and IL-21[8–10]. A recent study discovered correlations between phenotypes and lytic activity of CIK cells populations in patients with hepatocellular carcinoma, where $CD3^{+/-}CD56^{+}$ cells defined high cytotoxic ability, but not $CD3^{+}CD4^{+}$ cells [11]. A number of studies showed an elevated anticancer effect of DCs and CIK cells combination in the case of solid tumours and haematological malignancies [12, 13].

It was shown that cancer cells subjected to immunogenic cell death (ICD) expose damage-associated molecular patterns (DAMPs) which act as immunogenic signals to dendritic cells and thereby facilitate their maturation [14]. Among DAMPs calreticulin, high-mobility group box 1 (HMGB1), adenosine triphosphate (ATP) and heat shock proteins (HSP) have been well studied [15].

There is, however, no consensus in choosing source and way of antigen obtaining to most effective DCs priming, which in turn provides sufficient T-cells activation, in our case – CIK cells. There are number of antigen sources, namely whole tumour lysate obtained after freeze-thaw, total tumour RNA, heat and drug-stressed cancer cells and their supernatants, intact cancer cells etc [16]. Therefore, the aim of this study was to compare the most frequently used techniques of tumour antigen preparation, their impact on DCs maturation and further CIK cells activation. In particular, we evaluated cytotoxic activity of CIK cells, expanded from whole blood samples and peripheral blood mononuclear cells, against cancer cells by co-cultivation with DCs that are pulsed with antigens obtained by mild heat-shock treatment (≤42°C), expose to chemotherapy drugs (oxaliplatin, 5-fluorouracil, staurosporine, methotrexate), freeze-thawing and total tumour RNA isolation. Obtained results showed that CIK cells isolated from unpurified whole blood have strongest anti-tumour properties compared to CIK cells isolated from PBMCs. Also, we determined that all examined techniques of antigen preparation can be utilized in combination with wbCIK cells.

Materials and methods

Preparation of CIK cells and colorectal cancer cells

CIK cells were expanded from peripheral blood mononuclear cells (PBMCs) or whole blood. Blood was received from the volunteer's peripheral blood sample (20 ml). Isolation of PBMCs was performed using Histopaque-1077 density gradient centrifugation. PBMCs and whole blood cells were seeded into 6-well plate at a density 1-3*10^6 cells per mL and cultured in Biotarget™ medium (Biological Industries Ltd., Haemek, Israel) containing 1000 IU/mL of IFN-gamma in a humidified atmosphere with 5% CO_2 at 37 °C. The next day, cells in medium containing 500 IU/mL IL-2 were plated to anti-CD3 mAb precoated Petri dish with the subsequent transfer to the new vial after 4 days and every 2 or 3 days medium change for 14-21 days. Cell sorting was performed on BD Biosciences Imagnet Cell Separation Magnet using CD56 biotinylated mAb.

The human colon cancer cell line SW620 was purchased from ECACC (UK) and cultivated in DMEM/F12 (Life Technologies Limited, Paisley, UK) containing 10% fetal bovine serum (FBS) 100 U/mL penicillin, 100 µg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C.

Dendritic cells cultivation and tumour antigen obtaining

DCs were obtained in two different ways: monocytes isolation by Histopaque-1077 density centrifugation and further 6-7 days cultivation or $CD14^+$ DCs sorting from whole blood. Isolated monocytes were cultivated in Biotarget™ medium (Biological Industries Ltd., Haemek, Israel) supplemented with 20 ng/mL GM-CSF and 20 ng/mL IL-4. The incubation lasted 6 days at the end of which cells were enriched using $CD11C^+$ separation.

All types of tumour antigens were obtained from SW620 cancer cell line. SW620 cells were subjected to subsequential 2 h of heat-shock, 24 h of chemo drug treatment with 500 µg/mL oxaliplatin alone or in combination with 1000 µg/mL 5-fluorouracil, 10 µM staurosporine, 100 µM MTX. Freeze-thaw lysates were received as a result of the repeated freeze-thawing of SW620, total tumour RNA was extracted from SW620 after heat-shock according to manufacturer's protocol.

In vitro antitumor activity analysis

The cytotoxic activity of CIK cells against SW620 tumour cells was determined by MTT-assay. Briefly, prepared antigens were combined with negative enriched BC or $CD14^+$DCs to the antigen fusion. After 24 h DCs were stimulated with 50 ng/mL TNF-alpha for 2 h. On the next step, to activate $CD56^+$CIK cells DCs were added at a ratio 5:1 respectively for 2 days. The human colon cancer SW620 cells were used as target cells, CIK cells used as effector cells. The safety of CIK cells, DCs and their combination for normal human cells were verified using HDFn (human dermal fibroblast neonatal, ATCC, PCS-201-010 cells). Target cells in the quantity of 5x10^6 cells per well were seeded to 96-well plate day before CIK cells addition. The groups that consisted of CIK cells activated by DCs loaded with antigens were the experimental groups, while the control group contained only CIK cells without DCs activation. MTT-assay was performed to assess cell viability by reading optical density at 580 nm using Bio-Rad 680 spectrophotometer.

Results and discussion

The safeness of adaptive immunotherapy was shown in numerous clinical studies. In our research CIK cells and DCs were checked regarding their safety, HDFn (human dermal fibroblast neonatal) cells were used as a target (Figure 1). Obtained data confirms statements concerning harmlessness of CIK cells, DCs and their combination toward normal cells. The typical antitumor effect of CIK cells resulted in significantly decreased viability of SW620 cells more than three times compared to HDFn (P-value). Briefly, HDFn cells after addition of expanded from whole blood CIK cells showed not significant falling in viability whereas SW620 cells were notably affected by CIK cells, there were 93.3% and 35 % live cells respectively ($p<0.0001$). CIK cells cultivated from PBMCs isolated by the Histopaque-1077 method demonstrated strong lytic activity towards cancer cells and its absence in case of normal cells (99.4% and 28.8%, $p<0.0001$). Further safety of CIK-DC combination was validated (96.3% – HDFn live cells and 19.8%- SW620 cells, $p<0.0001$). Kornacker M. and colleagues' findings also demonstrated the killing ability of CIK cells against autologous chronic lymphocytic leukaemia (CLL) cells and showed no lytic activity towards non-malignant mononuclear cells [17]. As previously reported by Liu Y., there were no serious side-effects after infusion of DC-CIK as adjuvant therapy to patients 65 years and older with solid tumours and hematological malignancies [18]. *In vivo* experiments revealed the role of IFN-gamma in mild graft-versus-host disease (GVHD) induced by infusion of allogeneic CIK cells compared to lethal cases caused by splenocytes injection [19]. *In vitro* study concerning the comparative analysis of IL-15 stimulated CIK cells also confirmed the poor alloreactivity of CIK cells against allogeneic PBMCs and fibroblasts [20].

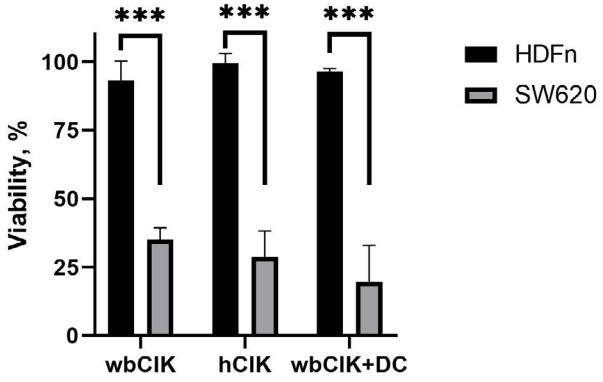

Figure 1 – The cytotoxicity of CIK cells against HDFn and SW620. CIK cells obtained by two different techniques (wbCIK- CIK cells expanded from whole blood, hCIK – CIK cells cultured from PBMCs, isolated by Histopaque-1077 density gradient centrifugation) were co-cultured with target cells (HDFn or SW620) at a ratio of 10:1 (E: T) for 48 h. The cell viability was measured by MTT-assay, $P \leq 0.001$

Comparative analysis of CIK cells expanded from whole blood and PBMCs detected the first method is more efficient and suitable for CIKs *ex vivo* cultivation. DCs were pulsed with tumour antigens received by different techniques preceding co-cultivation with CIK cells. Cancer cells were exposed to heat and various chemo drugs treatment, including oxaliplatin, 5-fluorouracil, staurosporine, MTX, repeated freezing-thawing cycling, also intact SW620 cells were used. In addition, we have tested the impact of TNF-alpha on DC maturation which defines effective CIK priming and their potent killing activity against malignant cells.

Cytolytic activity of wbCIK cells activated by TNF-alpha matured DCs loaded with antigens of heat and oxaliplatin treated SW620 cells had a significantly high level of cytotoxicity against target cells and reached 95% while without TNF-alpha addition cytotoxicity was 78% ($P=0.003$). Interestingly, there were no important differences between the killing activity of CIK cells expanded from PBMCs which completed almost 80% in both cases (Figure

2A). In a recent study concerning immunogenic cell death induction, a combination of oxaliplatin and one of STAT3 inhibitory molecules (stattic) were used to assess levels of DAMPs. A significant increase in the levels of calreticulin, HMGB1 and HSP70 in CT26 cells treated with oxaliplatin alone or with stattic compare to the control group were detected. Also, they measured the production of IL-12 by bone marrow originated DCs after expose to conditioned media. According to results, high levels of IL-12 were found in DCs groups cultured in conditioned media of CT26 cells treated with oxaliplatin and/or stattic (with no significant differences between both variants) [21].

To compare monotherapy with oxaliplatin and its frequently used combination with 5-fluorouracil [22] we combined CIK cells (from whole blood and PBMCs) with DCs loaded with heat and chemically (5FU+Ox) stressed SW620 cells and measured the cytotoxic activity of CIK cells (Figure 2B). Before addition to effector cells, DCs also were treated with TNF-alpha or not. Obtained results showed that activated wbCIK cells have a slightly significant killing ability as opposed to PBMCs-obtained CIK cells (87%, 75% – without TNF-alpha, 99%, 88 % -with TNF-alpha). Combination of oxaliplatin with 5-fluorouracil as oxaliplatin alone was characterized by potent ability to induce maturation of DCs followed by efficient CIK cells activation resulted in successful tumour elimination. In a recent study, the pivotal role of TLR-4 as DAMPs receptor has been proved. Fang H. and colleagues showed that colorectal cancer cells (SW480) undergoing immunogenic cell death induced by oxaliplatin and/or 5-fluorouracil treatment realise high concentrations of HMGB1 and HSP70. Also, HLA-DR, CD80 and CD86 overexpression were detected after preconditioning of DCs with supernatant of chemically-stressed tumour cells are hallmarks of mature DCs [23].

One of the most successful combinations in tumour-killing ability was wbCIK cells co-cultured with TNF-alpha matured and heat-stressed, staurosporine-treated SW620 cells loaded DCs (Figure 2C). A series of studies confirmed the ability of staurosporine to induce DAMPs formation. The highest level of extracellular HMGB1 was released after treatment of two cancer cell lines (U2OC-human bone osteosarcoma epithelial cells, MCA205-mouse fibrosarcoma cells) with staurosporine in comparison to methotrexate, azacitidine, decitabine, oxaliplatin and etc. [24]. There linear correlation between the amount of ATP, ADP and AMP extracellular mix and time to staurosporine exposition was revealed [25]. A relatively new conception developed by Yoon S. and colleagues concerning caspase-dependent regulated necrosis which shares features of apoptotic and necrotic ways of cell death was also prompted by staurosporine. DNA attached to histone H1 and HMGB1, Hsp90, ERp57 in the conditioned medium of chemically-stressed cells were detected [26].

Cancer cells treated with MTX also were a very prospective loading agent for DCs maturation and further CIK cells activation (Figure 2D). *In vitro* study indicated high concentrations of DAMPs hallmarks such as calreticulin, ATP and HMGB1 in MTX-treated prostate cancer cell lines (LNCaP and 22RV1) conditioned media, *in vivo* experiments showed that injection of chemically stressed cancer cells provoked in abundant mature DCs. As a result, MTX-triggered immunogenic cell death via p53/PERK and GCN2 upregulation – eIF2α S51 phosphorylation – endoplasmic reticulum stress axis was revealed [27, 28].

Similar results were observed in experiments using DCs loaded with freeze-thawed whole tumour lysates, heat-stressed cancer cells and total tumour RNA (Figure 2E, F, H). Series of studies indicated strong immunogenic responses toward cancer cells subjected to hyperthermia. Daniel Rojas-Sepúlveda and colleagues investigated the effect of combined lysates of a few heat-stressed cancer cell lines on antigen-presenting and effector cells in the case of melanoma (TRIMEL) and gallbladder cancer (M2). Both types of lysates were able to induce maturation of moDC, that resulted in upregulation of HLA-DR, CD80 and CD86, while lysates of a single heat-stressed cancer cell lines failed to induce DCs maturation. Additionally, the contribution of DCs loaded with a mixture of heat-stressed lysates resulted in significant upregulation of CXCR3, CXCR4, CD25 and CD69 on CD4 and CD8 subsets of T cells [29, 30].

DCs fused with intact cancer cells and stimulated by TNF-alpha showed high activating capacity towards wbCIK cells but not hCIK cells. Also noticeable relatively strong cytotoxicity of CIK cells in absence of DCs activation (Figure 2G).

Conclusion

According to obtained results, CIK cells expanded from whole blood showed significantly strong anti-tumoral activity compared to CIK cells generated from peripheral blood mononuclear cells isolated by Histopaque-1077 density gradient centrifugation. Also, we determined that all examined methods of antigen preparation can be used, but only in the case of antigen-loaded DCs cocultivation with CIK cells.

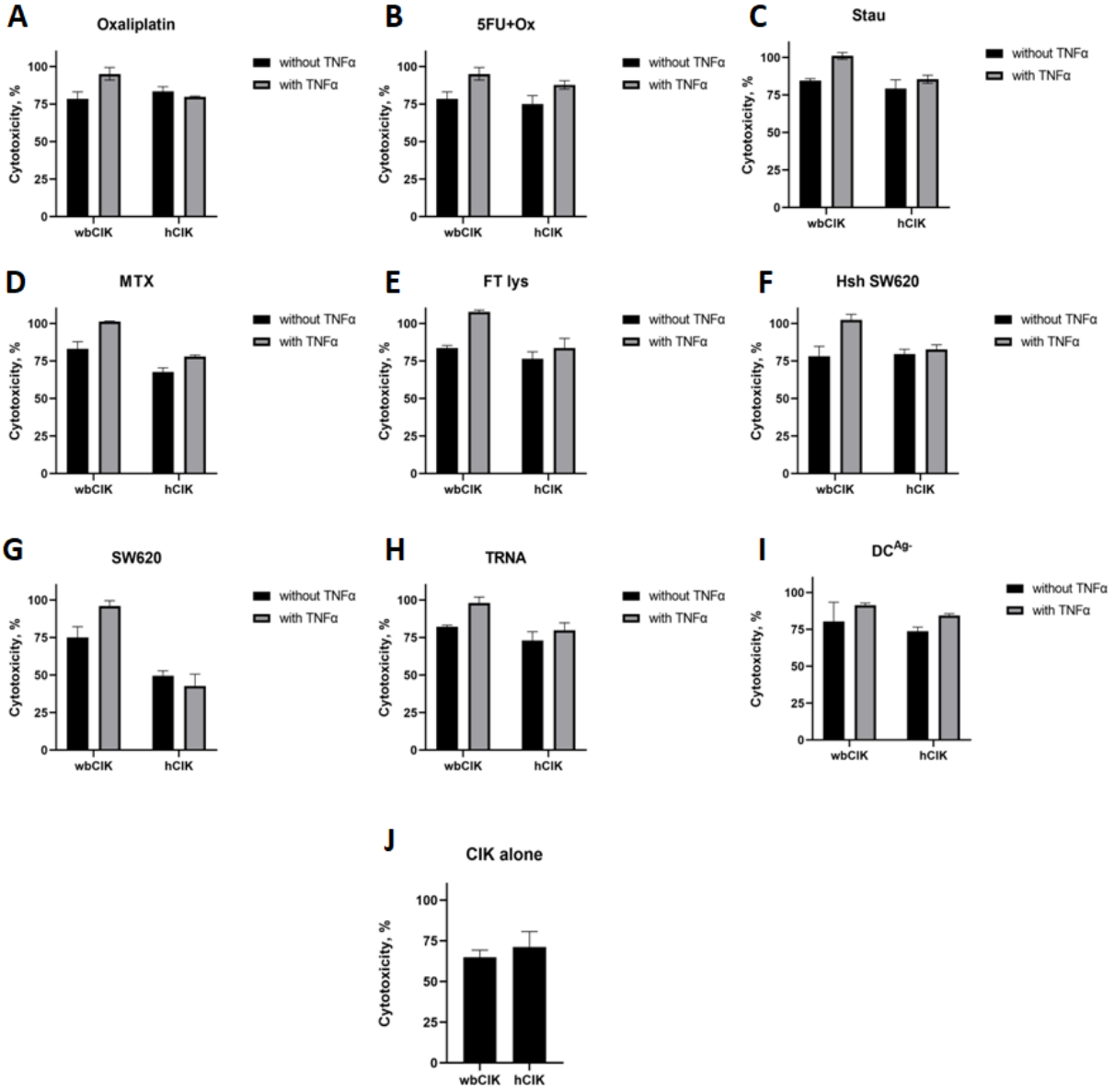

Figure 2 – The cytotoxic activity of CIK cells activated by DCs against SW620 cancer cells. A-CIK cells cultivated with DCs loaded with antigens of heat-stressed and oxaliplatin treated cancer cells, B-CIK cells cultivated with DCs loaded with antigens of heat-stressed and 5-fluorouracil plus oxaliplatin treated cancer cells, C-CIK cells cultivated with DCs loaded with antigens of heat-stressed and staurosporine (Stau) treated cancer cells, D-CIK cells cultivated with DCs loaded with antigens of heat-stressed and mitoxantrone (MTX) treated cancer cells, E -CIK cells cultivated with DCs loaded with antigens of heat-stressed and freeze-thawed cancer cells, F-CIK cells cultivated with DCs loaded with antigens of heat-shocked (Hsh SW620) cancer cells, G-CIK cells cultivated with DCs fused with intact cancer cells (SW620), H-CIK cells cultivated with DCs loaded with total tumour RNA of cancer cells(TRNA), I-CIK cells cultivated with DCs without tumour antigens (DC^{Ag-}), J-CIK cells without DCs activation. WbCIK- CIK cells expanded from whole blood, hCIK – CIK cells cultured from PBMCs, isolated by Histopaque-1077 density gradient centrifugation

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