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Lenalidomide improves NKG2D-based CAR-T cell activity against colorectal cancer cells invitro

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ABSTRACT

Introduction: Although CAR-based immunotherapy is viewed as a promising treatment for tumors, particularly hematological malignancies, solid tumors can pose challenges. It has been suggested that the immunomodulatory medication Lenalidomide (LEN) may increase the effectiveness of CAR T cells in the treatment of solid tumors. The purpose of our study was to investigate the effect of NKG2D-based CAR T cell therapy on colorectal cancer cell lines, and then we assessed combinatorial therapy using NKG2D CAR T cells and lenalidomide in vitro.

Methods and results: To prepare NKG2D CAR T cells, a second-generation NKG2D-CAR construct was designed and transfected into the T cells using a lentiviral system. The NKG2D CAR T cells showed significantly higher cytotoxic activity against colorectal cancer cell lines, HCT116 and SW480, compared to untransduced T cells. In addition, our data demonstrated that the cytotoxicity and cytokine secretion of NKG2D CAR T cells significantly increased in the presence of higher doses of lenalidomide.

Conclusions: The study findings suggest that combinational therapy, utilizing NKG2D-based CAR T cells and lenalidomide, has a high potential for effectively eliminating tumor cells in vitro.

1. Introduction

Globally, colorectal cancer (CRC) is considered a significant health concern, with an estimated 147,950 new cases diagnosed and 53,200 deaths from the disease in 2020 [1]. Recent epidemiological studies have shown that CRC is the third most common cancer among men and women (9% and 8%, respectively), as well as the third leading cause of death (10.7%) [2,3]. Approximately one-third of CRC patients are diagnosed with metastatic disease, which still carries a poor prognosis [4]. More than 2.2 million new cases and 1.1 million deaths from colorectal cancer are anticipated worldwide by 2030, representing a 60% increase in the disease's burden [5]. Considering the massive increase in colorectal cancer incidence in the last few decades, it is crucial to develop targeted drugs that are effective in treating this disease.

The emerging field of cancer immunotherapy, which leverages the immune system's ability to target cancer cells, is becoming an increasingly effective strategy to supplement conventional cancer treatments such as surgery, chemotherapy, and radiation. Recent advancements in T-cell engineering with chimeric antigen receptors (CARs) have shown promising results, particularly in the

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treatment of hematological cancers. CAR T cells are genetically altered T cells with artificial receptors on their cell surface that can recognize tumors expressing specific antigens [6–8]. The clinical data currently available describes CD19-specific CAR T cell therapy for patients with B cell malignancies and BCMA-based CAR T cell therapy for patients with Multiple myeloma, which got approval from the FDA [9]. There have already been several CAR T cell therapies approved for different types of hematological cancers, including Kite Pharma's axicabtagene ciloleucel (Yescarta), which was approved for B-cell lymphoma. Janssen developed idecabtagene vicleucel (Abecma) for treating multiple myeloma, which was the first CAR T cell therapy approved by the FDA for the disease [9,10].

The clinical success of CAR T cell therapy for solid tumors has not yet reached comparable levels. This is due, in part, to several limitations that decrease its effectiveness. These limitations include tumor cell heterogeneity, off-target effects, an immunosuppressive microenvironment, poor CAR T cell trafficking to the tumor site, and difficulty accessing the tumor [8,11,12]. However, efforts are being made to improve CAR T cell therapy for solid tumors. Classic CARs, which typically use a single chain fragment variable (scFv) to detect a single antigen on the surface of cancer cells, can be hindered by tumor heterogeneity and antigen escape mechanisms [8]. NKG2D is a natural receptor on the surface of natural killer cells that can recognize multiple ligands on the tumor cells. So, it seems that using NKG2D as a chimeric antigen receptor is a suitable therapeutic target for these tumors. NKG2D ligands express in a wide range of cancer cell lines and primary tumors like colorectal cancer, ovarian carcinoma, pancreatic cancer, Prostate cancer, acute lymphoblastic leukemia, and lymphoma, which indicates it can be used for both hematological and solid tumors [13,14]. The expression of NKG2D ligands in healthy adult tissue is carefully controlled to avoid self-identification and the possibility of autoimmune reactions. While mRNA for NKG2D ligands can be found in healthy adult tissues, it appears that a process called posttranscriptional regulation prevents the translation and subsequent appearance of these proteins on the surface of healthy cells [15].

Developing strategies to prevent CAR T cell exhaustion and dysfunction is essential to effectively treating solid tumors with CAR T cell therapy. Lenalidomide is an immunomodulatory medication used to treat myelodysplastic syndromes and multiple myeloma (MM) [16]. Lenalidomide has been demonstrated to have an effect on cytokine release, T-cell activation, and CAR T-cell activity in hematological malignancies [17,18]. There is still limited research on how lenalidomide affects CAR T cell activity against solid tumor cells [19].

In this study, we aimed to develop NKG2D CAR T cells by creating a CAR construct based on NKG2D. We transfected it into T cells using a lentiviral system. Additionally, we evaluated the impact of lenalidomide on NKG2D CAR T cells functionality. Our results demonstrated that combining lenalidomide with NKG2D CAR T cells had a high potential for effectively removing colorectal cancer cells in vitro.

2. Materials and methods

2.1. Cell lines and reagents

The human colorectal cancer cell lines HCT116 and SW480 were obtained from the National Cell Bank of Iran (NCBI), Pasteur Institute (Tehran, Iran). The 293T cell line was purchased from the Pasteur Institute and used for lentiviral packaging. All cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin in an atmosphere of 5% CO2 and 95% humidity at 37 °C. Primary human peripheral blood mononuclear cells (PBMCs) were taken from healthy voluntary donors and cultured in RPMI 1640 containing 10% FBS. Lenalidomide was purchased from Sobhan Darou (LENASOBTM 10 MG Capsule) Iran, dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, and stored at −80 °C until further usage.

2.2. Design and construction of NKG2D-CAR

The NKG2D CAR constructs are composed of the CD8α signal sequence, the extracellular domain of human NKG2D (amino acids 82–216), the hinge region of CD8α linked to the transmembrane, and the intracellular domain of CD28 followed by the intracellular signaling domain CD3ζ. The complete NKG2D CAR sequence (Biomatic, Canada) was cloned into the BamH1 and *Xho*I restriction sites of the lentiviral PCDH-Cd513B vector backbone, yielding PCDH-CMV-NKG2D-28ζ-F2A-eGFP.

2.3. Lentiviral packaging

The packaging of lentiviruses containing NKG2D-CAR was accomplished using a second-generation packaging system. Briefly, 293T cells were cultured in 10 cm2 Petri dishes and transfected with PEI reagent according to the manufacturer's instructions. PCDH vectors containing the chNKG2D (transfer vector) were co-transfected with psPAX2 (packaging vector) and pMD2G (envelope vector) in a ratio of 2.5:2.5:1 at a total of 18 g plasmid in the presence of 20 μL PEI (transfection reagent) per 10 cm2 Petri dish. After 24 h, the medium was changed to a fresh medium, and the cells were observed for GFP expression using the fluorescent microscope. 48 and 72 h after transfection, the supernatant containing virus particles was obtained and centrifuged for 10 min at 1800 rpm to remove cell debris, then stored at −80 for next use. The Ethics Committee of Golestan University of Medical Sciences (code: IR.GOUMS.REC.1398.019) have approved this study and informed consent was obtained from healthy voluntary donors.

2.4. T cell transduction

Blood samples were taken from healthy voluntary donors, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using endotoxin-free Ficoll-Paque. Magnetic beads coated with anti-CD3/anti-CD28 antibodies

(Invitrogen, USA) were used to activate PBMCs at a 1:1 cell-to-bead ratio. and after 48 h, lentiviral particles were added to the cultures at a multiplicity of infection (MOI) of 10 in the presence of polybrene at a final concentration of 8 μg/ml. Cells were cultured and expanded in RPMI 1640 (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Gibco, Life Technologies) in a 95% air and 5% CO2 atmosphere at 37 °C, and human recombinant interleukin-2 (IL-2; Biolegend, USA) was added every 2 days to a 200 IU/ml final concentration.

2.5. Flow cytometric analysis

Cell phenotypes were analyzed by flow cytometry. NKG2D-CAR expression on transduced T cells was detected by GFP and staining with an anti-NKG2D antibody. 5×10^5 cells were harvested, washed twice with 1 × PBS, and suspended in cold PBS containing 2% FBS and 1% sodium azide. Subsequently, PE-conjugated anti-NKG2D antibodies (biolegend, USA) were added to the cell suspension and incubated for 1 h at 4 °C in the dark. For the NKG2DLs detection, 1×10^6 colorectal cancer cells were stained by an anti-MICA, anti-MICB, or anti-ULBP-1 PE-conjugated antibody (biolegend, USA) for 30 min. Isotype-matched antibodies (PE Mouse IgG1, κ Isotype Ctrl, biolegend, USA) were used in all analyses. Flow cytometry was performed using a BD Accuri cell analyzer (BD Biosciences, USA), and data were analyzed with FlowJo version 10.5.3 software.

2.6. Cytotoxicity assays

Cytotoxic activity and specificity of engineered T cells were measured by a lactate dehydrogenase (LDH) release assay kit (Promega, USA) according to the manufacturer's instructions. First, 1×10^4 colorectal cancer cells per well in 200-μl assay medium were transferred into 96-well plates and incubated for one night. After overnight incubation, T cells or NKG2D CAR T cells were co-cultured for 24 h at different effector-target (E: T) cell ratios of 1:1, 5:1, and 10:1. Then the supernatant was collected by centrifuging the cells at 250 g for 10 min. Following this, transfer 50 μl/well supernatant into corresponding wells of a new optically clear 96-well plate and add 50 μl LDH solution to each well. Then incubate for up to 30 min at room temperature away from the light, and measure the absorbance of all samples at 490–500 nm using a microtiter plate reader. The maximum release of target cells and the spontaneous release of effector or target cells were also determined. The following formula was employed to calculate the percentage of cytotoxicity: Cytotoxicity (%) = (test sample LDH release-spontaneous LDH release)/(maximal LDH release - spontaneous LDH release) × 100. To measure the effect of lenalidomide, NKG2D CAR T cells were co-cultured with colorectal tumor cells (E: T ratios of 5:1) in a 96-well plate, and then lenalidomide (final concentrations of 10, 1, and 0.1 μM, respectively) and the same dilution of the solvent DMSO (10^3, 10^4, and 10^5) were added as different groups, and tumor cells were cultured separately as controls. The comparisons between the lenalidomide and DMSO control groups demonstrate the impact of lenalidomide at various doses on CAR T cell cytotoxicity.

2.7. Cytokine release assays

Untransduced T cells or NKG2D CAR-T cells were co-cultured with target cells (HCT116 and SW480 cells) in RPMI 1640 at an E/T ratio of 5:1 for 24 h. To investigate the effect of lenalidomide on CAR T cells, we added lenalidomide at a concentration of 10 μM, DMSO (10^3 ×), and T-cell culture medium at the same dilution. Cell supernatants were harvested for cytokine measurements of IL-2 and interferon-γ concentrations using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, USA), according to the manufacturer's protocols.

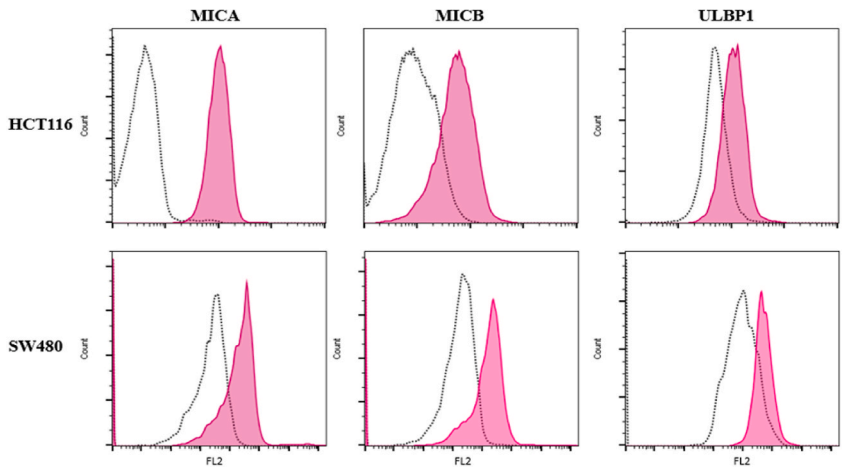

Fig. 1. Main NKG2D ligands expression on colorectal cancer calls. Two colorectal cancer cell lines HCT116 and SW480 were stained with PE-conjugated antibodies that identify MICA, MICB, and ULBP1(filled histogram) or isotype control antibody (PE Mouse IgG1, κ Isotype Ctrl) (open histogram).

2.8. Statistical analysis

To analyze the data, we employed the statistical software GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). Student's t-test and two-way analysis of variance (ANOVA) were utilized to evaluate significant differences in cytokine release and killing effectiveness. The data were presented as the mean ± the standard deviation. The experiments were repeated a minimum of three times. In this study, statistical significance was defined as $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

3. Results

3.1. The expression of NKG2DLs in human colorectal cancer cell lines

Here the main ligands for the NKG2D receptor MICA, MICB, and ULBP1 on the cell surface of two colorectal cancer cell lines, HCT116 and SW480, were identified by flow cytometry (Fig. 1). As expected, flow cytometry data analysis results showed high amounts of NKG2D ligands, especially MICA and MICB, on the surface of these cell lines (Fig. 1). Previous studies showed high and moderate expression of NKG2D ligands in several colorectal cancer cell lines [20,21].

3.2. Construction of NKG2D CAR and surface expression on T cells

CAR expressed on T cells is made up of an extracellular binding portion fused to a transmembrane and intracellular tail that allows antigen/ligand-activated T cells to function. The expression of NKG2DLs in colorectal cancer cells showed that NKG2D-expressing CAR T cells can be a potential therapy for this cancer. Thus, we created a second-generation NKG2D CAR consisting of the extracellular portion of the human NKG2D receptor linked to a CD28 transmembrane and intracellular region, followed by a CD3z signaling motif to

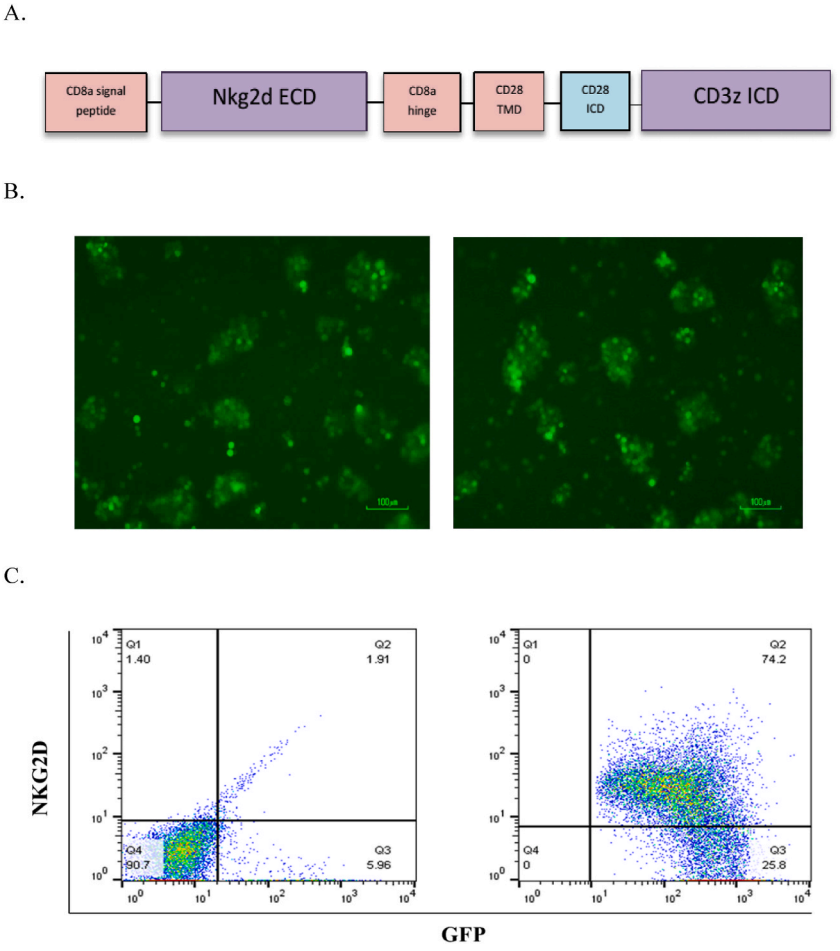

Fig. 2. NKG2D-based CAR construction and expression on the T cell surface. A. Schema of NKG2D CAR construct; B. fluorescent microscopy images 48(left) or 72(right) hours after lentiviral transduction: C. flow cytometry was used to detect GFP and NKG2D-CAR expression on the control T cell (left) and NKG2D-CAR T cell (right) surface.

target human T cells to NKG2DLs on colorectal cancer cells (Fig. 2-A). PBMCs obtained from healthy donors and subsequently activated using CD3/CD28 beads. T cells were identified based on their CD3 expression (Supplementary Fig. 1). Subsequently, NKG2D CAR T cells were produced utilizing a lentiviral transduction system. The NKG2D CAR-T cells were confirmed by dual expression analysis of GFP and staining of cell surface NKG2D (Fig. 2B and C). As shown in Fig. 2-B GFP expression detects 48 or 72 h after transduction using fluorescent microscopy. After 5 days of transduction, flow cytometry was used to detect GFP and NKG2D CAR expression on the transduced T cell surface. Flow cytometry data analysis showed higher expression of NKG2D on the surface of NKG2D CAR T cells than control T cells (Fig. 2C).

3.3. Anti-tumor activity of NKG2D CAR-T cells

In our study, we utilized engineered T cells as effector cells and various colorectal cancer cells as target cells to evaluate the activation and cytotoxicity of NKG2D CAR T cells against tumor cells. To determine the cytotoxic activity, NKG2D CAR T cells after 6 days of transduction, were co-cultured with SW480 or HCT-116 cells at E: T ratios of 1:1, 5:1, and 10:1 for 24 h in 96-well-plate triplicate. Target cell death and cell lysis were analyzed based on the measurement of LDH activity released from the cytosol of damaged cells into the supernatant. The data from the analysis of the supernatant demonstrate that in E: T 1:1 ratio, SW480 cell line unlike HCT116 does not show significant cytotoxicity of NKG2D CAR T cells compared to untransduced T cells. But in higher E: T ratios, NKG2D CAR T cells significantly (p-value <0/05) killed colorectal cancer cells in both cell lines, HCT116 and SW480, compared to untransduced T cells (Fig. 3-A).

Cytokine secretion by CAR-T cells demonstrates the activation and specific cytotoxicity of T cells; thus, we tested the levels of the proinflammatory cytokines INF-γ, and IL-2 when CAR-T cells were incubated with HCT116 and SW480 colorectal cancer cell lines. Our results demonstrate that NKG2D CAR T cells secrete significantly more IL-2 and IFN-γ than untransduced T cells when co-cultured with HCT-116 or SW480 cells (Fig. 3-B).

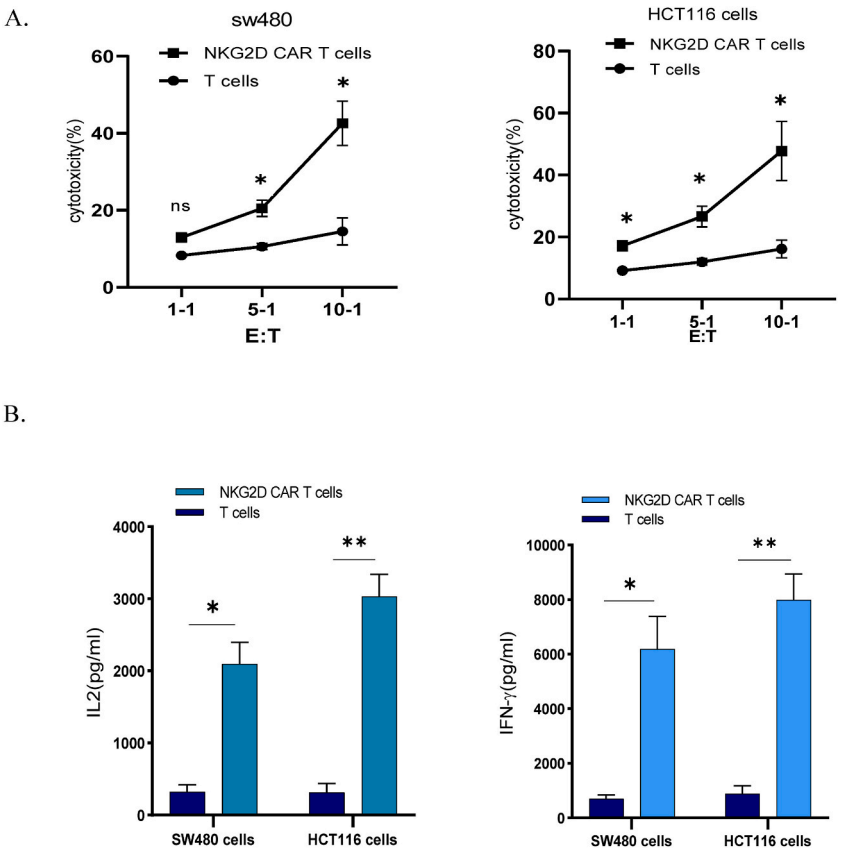

Fig. 3. NKG2D CAR T cells in vitro cytotoxicity against colorectal cancer cells A. Cytotoxic activity of CAR T cells or T cells on SW480 and HCT116 colorectal cancer cell lines. Co-incubate the effector cells with target cells at an E: T ratio of 1:1, 5:1, and 10:1, and analyze cytotoxicity using the LDH release assay kit. B. ELISA was used to test the cytokine secretion of car T cells or T cells on the co-culture with colorectal cancer cells in 24 h at the E: T ratio of 5:1. The levels of IL-2 and IFN-γ were analyzed by an ELISA detection kit. Data are shown as the mean of killing efficiency +standard deviation in three replicates. Differences between the T cells and NKG2D CAR-T cells at each E:T ratio were evaluated using Student's t-test. (*), $p < 0.05$; (**), $p < 0.01$.

3.4. Lenalidomide improves the functions of NKG2D-CAR T cells

It is well known that lenalidomide exhibits both antitumor and immunomodulatory properties, thus enhancing the activity of T and NK cells whether it is used alone or in combination with cancer cells to treat cancer. Studies have demonstrated that lenalidomide induces cytokine modulation and T-cell activation [22]. In this study, we investigate the effect of lenalidomide on the antitumor activity of NKG2D-based CAR T cells. For the cytotoxicity assay, 6 days after transduction, NKG2D CAR T cells were co-cultured with colorectal cancer cell lines in an E: T ratio of 5:1. Lenalidomide at different concentrations (0.1, 1, and 10 μM) was added to the co-culture medium, and DMSO (the solvent of lenalidomide) was used as the control group. After 24 h, the supernatant was collected, and LDH activity was measured to determine the killing efficiency. The results showed that the cytotoxicity of NKG2D CAR T cells significantly increased in the presence of higher doses of lenalidomide. As shown in Fig. 4, when the lenalidomide concentration was 0/1 μM there was no significant difference between the lenalidomide and DMSO groups in both colorectal cancer cell lines SW480 and HCT116, whereas killing efficiency was significantly high in the 1 μM and 10 μM lenalidomide concentration groups. our findings from MTT assay indicated that the use of lenalidomide alone did not have a significant impact on the survival of colorectal cancer cell lines, HCT116 and SW480 (Supplementary Fig. 2).

Next, we investigated the effect of lenalidomide at a final concentration of 10 μM on the cytokine secretion of NKG2D CAR T cells in co-culture with colorectal cancer cells in an E: T ratio of 5:1. To this, lenalidomide was added to the medium, and after 1 day, the supernatant was obtained. The amount of IL-2 and IFN-γ in the supernatant was measured using an ELISA technique. The data indicate that there was no significant difference in the amount of cytokines IL-2 and IFN-γ between the CAR-T cell medium and the DMSO group, but in the lenalidomide (10 μM) group compared with the DMSO group, the secretion of IL-2 and IFN-γ significantly increased in both SW480 and HCT116 colorectal cancer cell lines, as shown in (Fig. 5).

4. Discussion

In light of a deeper understanding of the interplay between tumors and the immune system, a number of novel drugs have been developed, which have had a profound impact on therapeutic approaches to cancer treatment. The use of chimeric antigen receptors (CARs) to improve immune response to certain types of hematological cancer and solid tumors has recently resulted in some highly encouraging results in patients. Genetically modified T cells, also known as CAR T cells, can bind to tumor-specific antigens by expressing artificial receptors on their cell surfaces. Throughout the past decade, CAR T cells have played a crucial role in the fight against cancer and have made some considerable advances [23,24].

The NKG2D receptor, a well-known NK activating receptor, is mainly expressed on NK cells, NK T cells, $CD8^+$ T cells, and subsets of γδT cells [25]. In humans, NKG2D receptors can recognize two families of NKG2D ligands: the MIC family, which includes MICA and MICB, and the ULBP family (ULBP 1–6) [26,27]. Several tumor cell lines and primary tumors from various tissue origins express NKG2DLs, which is not expressed in healthy tissues, suggesting that NKG2DLs are a potential target in solid and hematological tumors [13,28]. It has been shown that tumor heterogeneity is one of the major reasons for immune escape in tumors, which often leads to recurrence and metastasis. However, NKG2D CAR T cells can target multiple ligands expressed by tumor cells. NKG2D CAR T cells are able to overcome the limitations of traditional CAR T cells that only target a single antigen, thus potentially increasing their effectiveness in fighting immune escape [29,30]. Accordingly, we designed and constructed a second-generation chimeric antigen receptor

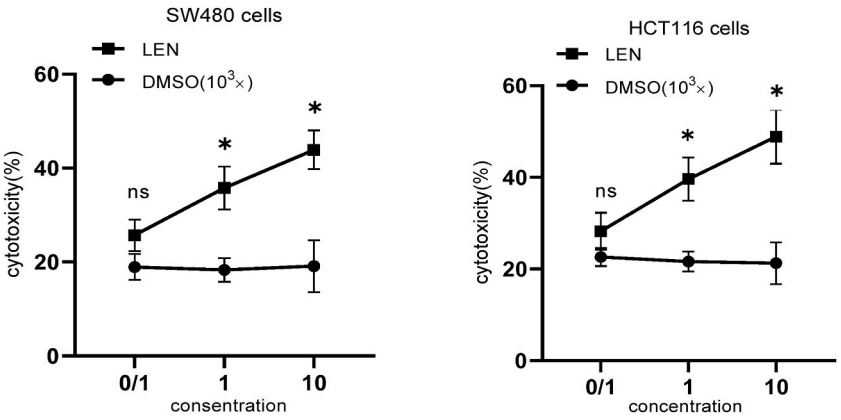

Fig. 4. lenalidomide (LEN) evaluates the cytotoxic activity of NKG2D CAR T cells against colorectal cancer cells dose-dependent. NKG2D CAR T cells co-cultured with HCT116 and SW480 colorectal cancer cell lines to E: T ratio 5-1. LEN was added into the medium in a final concentration of 0.1, 1, 10 μM or the same dilution fold of solvent DMSO (10^3 ×, 10^4 ×, 10^5 ×) as the control groups. After 24h supernatant was obtained, and LDH release assay kit was used to determine the killing efficiency. The formula employed to calculate the percentage of cytotoxicity is; Cytotoxicity (%) = (test sample LDH release - spontaneous LDH release)/(maximal LDH release - spontaneous LDH release) × 100. Data are shown as the mean of cytotoxicity +standard deviation in three replicates. The student's t-test was used to evaluate differences between LEN and DMSO at each concentration. (*), $p < 0.05$; (**), $p < 0.01$.

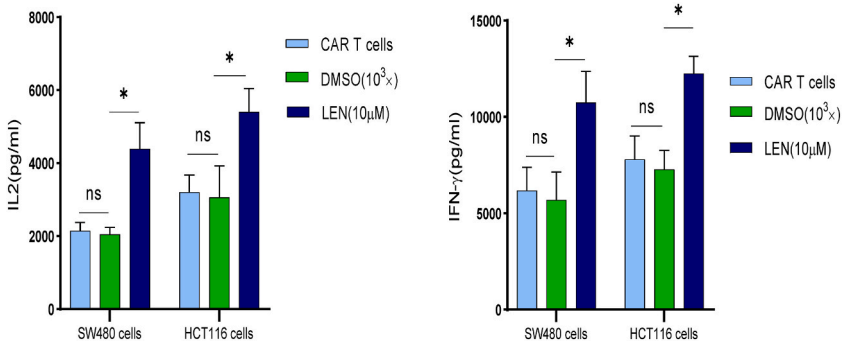

Fig. 5. Lenalidomide (LEN) increases NKG2D-CAR T cells' cytokine secretion. NKG2D CAR T cells were co-incubated with HCT116 and SW480 colorectal cancer cell lines according to the E: T ratio of 5:1. IL-2 and IFN-γ in the supernatant were measured using an ELISA detection kit after 24 h of incubation. Results are expressed as means ± SD of triplicate experiments and two-way ANOVA was used to evaluate differences between groups. (*), $p < 0.05$; (**), $p < 0.01$.

based on NKG2D encoded on a lentiviral vector that consists of the extracellular portion of NKG2D fused to cd28 and cd3z intracellular domains.

In this study, we investigated the effect of NKG2D CAR T cells on two NKG2D-ligand-positive colorectal cancer cell lines, SW480 and HCT 116, in vitro. Based on our findings (as shown in Fig. 3B), NKG2D CAR T cells secreted high levels of IFN-γ and IL2 after stimulation with both NKG2DL + colorectal cancer cell lines. In addition, data (Fig. 3-A) showed NKG2D CAR T cell lysis activity significantly increased in higher E: T ratios (5-1, 10.1), in either colorectal cancer cell line. Although in low E: T ratio [1–1], SW480 cell line unlike HCT116 does not show significant cytotoxicity of NKG2D CAR T cells compared to untransduced T cells. This result can be due to the different expression of NKG2D ligands in two colorectal cancer cell lines. Previous preclinical and clinical studies in this field have shown promising results in terms of its anti-tumor properties. It has been reported by Wang et al. that the use of a non-viral vector for transfection of NKG2D CAR, despite certain limitations, resulted in a stronger cytolytic effect and higher secretion of IL-2 and IFN-γ against LS174T and HCT-116 cells in vitro [19]. In 2018, Kelong et al. demonstrated that cisplatin treatment increases the expression of NKG2D ligands on the surface of tumor cells and sensitizes gastric cancer cell lines to NKG2D-based CAR T cell-mediated killing. They also discovered that NKG2D-CAR-bearing T cells produced high levels of IFN-γ and moderate levels of TNF-α and GM-CSF [31].

Clinical studies investigating the therapeutic potential of CAR T cells based on NKG2D are currently in progress. The CAR NKR-2 is a first-generation NKG2D-based CAR developed by Celyad and involves the infusion of full-length NKG2D into the CD3z cytoplasmic domain. The results of phase I clinical trial examining NKR-2 (NCT02203825) showed it effectively treated seven patients with acute myeloid leukemia and five patients with multiple myeloma without any significant toxicities or adverse effects [32]. The outcomes of our study, along with the support of additional studies, show that NKG2D-based CAR T cells can successfully eradicate NKG2DL + tumor cells in vitro.

We also investigated the effect of lenalidomide in different concentrations on NKG2D CAR T cell activity in vitro. Lenalidomide is an immunomodulatory drug that has known effects on T cell activation and expansion [22]. Our findings indicate that lenalidomide improves the ability of NKG2D-based CAR T cells to kill colorectal tumor cells. To determine the effects of lenalidomide on the cytotoxicity of NKG2D CAR T cells, we first add lenalidomide in various concentrations or DMSO as a control into the co-culture medium of NKG2D CAR T cells and tumor cells, SW480 and HCT116 (E: T ratio of 5-1). The data from the LDH cytotoxicity assay demonstrate that lenalidomide increased the killing ability of NKG2D CAR T cells against both colorectal cancer cell lines in a dose-dependent manner (Fig. 4). We also add 10 μM lenalidomide into the co-culture medium (E: T of 5-1) to test its effects on the cytokine secretion of NKG2D CAR T cells. To detect the amount of cytokine released (IL2, IFN-γ) into the supernatant; we used an ELISA detection kit. Data analysis showed that IL-2 and IFN-γ significantly increased in the lenalidomide (10 μM) group compared with the DMSO group in both colorectal cancer cell lines SW480 and HCT116 (Fig. 5). Related studies indicated the role of lenalidomide in regulating CAR T calls against hematological and solid tumors [19,33,34]. A study published in 2020 showed that lenalidomide could increase the CD133 CAR T cells' ability to kill cancer cells and the release of cytokines like IL-2, TNF-a, and IFN-γ. They also showed that lenalidomide increased cytotoxicity and the release of cytokines like IL-2, TNF-a, and IFN-γ against tumor cells by HER2 CAR T cells. They indicated that lenalidomide can reduce the level of Ikaros and Aiolos in the promoter of the IL-2 gene, which leads to restoring the IL-2 gene transcription and elevating IL-2 secretion [19]. In a recent study, Zhang et al. showed that combining lenalidomide and CAR T cell therapy improved the cytotoxicity and persistence of WT1-CAR T cells against K562-A24 target cells. Moreover, they showed that lenalidomide resulted in CAR T cells expressing more proteins associated with T cell activation in their protein profiles [35].

Although the exact ways in which lenalidomide works are not completely known, recent studies have shown that it can bind to a protein called E3 ubiquitin ligase Cereblon [36]. This binding triggers the process of ubiquitinylation and degradation of transcription factors Aiolos and Ikaros. These factors typically suppress the production of IL2 in T cells. As a result, when Aiolos and Ikaros are broken down, IL2 and other cytokines that regulate the functioning of T cells are elevated. It has also been reported that lenalidomide stimulates ERK phosphorylation following activation through CAR. This effect was attributed to the suppression of transcription factors Ikaros and Aiolos as a result of lenalidomide [37]. By increasing the activity of the transcription factor AP1, which stimulates T

cells, lenalidomide can influence the production of interleukin-2, interferon-γ, and other cytokines derived from T cells [22,38]. Lenalidomide enhanced CAR-T cells' efficiency in a dose-dependent manner and helped CAR-T cells to sustain their functionality even after prolonged exposure to antigens by preventing T-cell exhaustion [18]. Lenalidomide, in conjunction with BCMA-specific CAR-T cells, enhanced in vivo CAR-T cell expansion and considerably prolonged the survival of mice in a disseminated multiple myeloma model [39].

In this study, we show how lenalidomide could support NKG2D-CAR T cells in their battle against colorectal cancer cells in vitro. Elucidating the molecular mechanisms underlying lenalidomide's effects on NKG2D-CAR T cells is crucial for understanding its mode of action. Animal studies are useful for identifying precise molecular pathways that contribute to lenalidomide's ability to enhance CAR T cell functionality and persistence. This can involve characterizing changes in immune cell populations, cytokine production, and immune cell infiltration within the tumor microenvironment. Exploring these aspects through animal studies can broaden our knowledge of the potential benefits of combining lenalidomide with NKG2D-CAR T cell therapy for colorectal cancer. By obtaining such insights, it may be possible to translate these findings into clinical trials, which would ultimately benefit patients in the future.

5. Conclusion

Our findings support earlier research in this area and show that NKG2D-based CAR T cells have a high potential for tumor cell removal in vitro. Additionally, combination therapy using lenalidomide, an immunomodulator drug, and NKG2D-based CAR T cells may enhance the antitumor responses of NKG2D CAR T cells.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Golestan University of Medical Sciences, Gorgan, Iran (code: IR.GOUMS.REC.1398.019).

Authors' contributions

Conceived and designed the experiments; Majid Shahbazi and Mahdi Zarei.
Performed the experiments; Mahdi Zarei.
Analyzed and interpreted the data; Majid Shahbazi, Shahriyar Abdoli, Touraj Farazmandfar and Mahdi Zarei.
Contributed reagents, materials, analysis tools or data; Majid Shahbazi, Shariyar Abdoli.
Wrote the paper; Mahdi Zarei drafted the manuscript and Shahriar Abdoli and Touraj Farazmandfar helped in the writing of the ultimate version. And finally, the paper edited and approved by Majid Shahbazi.

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Availability of data and materials

All data generated or analyzed during this study are included in this publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20460.

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