Online ISSN: 2682-2628
Print ISSN: 2682-261X

IJCBR

INTERNATIONAL JOURNAL OF CANCER AND BIOMEDICAL RESEARCH

https://jcbr.journals.ekb.eg

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PUBLISHED BY
EACR EGYPTIAN ASSOCIAN FOR CANCER RESEARCH
Since 2014

International Journal of Cancer & Biomedical Research
(IJCBR) https://jcbr.journals.ekb.eg

IJCBR is an Int. journal published by the Egyptian Society of Cancer Research (EACR, established in 2014, http://eacr.tanta.edu.eg) and sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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INTERNATIONAL JOURNAL OF CANCER AND BIOMEDICAL RESEARCH (IJCBR)

RESEARCH ARTICLE

Impact of interleukin-7 on the differentiation and anti-tumor capabilities of CD8$^+$ T cells

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ABSTRACT

Background: Developing optimal strategies for generating T cells capable of effectively controlling tumors is one of the most important prerequisites for the clinical application of adoptive cell therapies in cancer patients. However, the generation of sufficient numbers of tumor-reactive T cells capable of efficient tumor regression and long-term persistence remains a significant impediment to widespread clinical implementation. **Aim**: The main aim of the present study was to evaluate the beneficial anti-tumor effects of a simplified combinatorial approach that involves a short activation of naïve CD8$^+$ T cells with the T cell mitogen concanavalin A (CON-A; 4 ug/mL) and the survival cytokine IL-7 (10 ng/mL), after a single intraperitoneal injection of cyclophosphamide (CTX; 4 mg/mouse) after their adoptive transfer into Ehrlich ascites carcinoma (EAC) tumor-bearing CD1 mice. **Results**: We found that adoptive transfer of in vitro IL-7-conditioned T cells into EAC-bearing (3-day) mice previously treated with a single dose of CTX induced a delay in the progression of EAC, and establishment of long-term immunological memory, which has the efficiency to provide full protection for mice against cancer. Our results indicated that in the presence of IL-7, the short-term T-cell receptor signaling mediates promiscuous qualities in naïve cytotoxic CD8$^+$T cells. **Conclusion**: The data indicate that upon the adoptive transfer of IL-7 conditioned T cells into lymphopenic hosts, they were able to eradicate tumors and also to generate long-term tumor-specific memory.

Editor-in-Chief: Prof. M.L. Salem, PhD - Article DOI: 10.21608/JCBR.2020.23254.1011

ARTICLE INFO

Article history

Received: Jan 31, 2020

Revised: June 5, 2020

Accepted: June 6, 2020

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INTRODUCTION

Cancer is one of the major public health problems and one of the three leading causes of adult mortality in developing countries. Although considerable progress has been made in the diagnosis and treatment of cancer, it remains a public health problem. Given the significant toxicities associated with anti-cancer chemotherapy, development of alternative approaches is of immense importance. So, immunotherapy represents an attractive anti-cancer approach that can target tumor killing with no effect on healthy cells. Development of the optimal strategies for generating T cells that capable of effective control of tumors is one of the most important prerequisites for clinical application of adoptive T cell therapies in cancer patients (June, 2007).

Genetic manipulation of T cells is one of the potential approaches to redirect their antigenic specificity or to enhance their survival (Clay et al., 1999; Duval et al., 2006; Hsu et al., 2005; Perez et al., 2008; Roszkowski et al., 2005). Consequently, the advent of optimized culture systems and gene transfer methods have brought engineered T cells closer to the clinic (Diaz-Montero et al., 2011). However, safety concerns and laborious manufacturing requirements for the generation of T cells limited the application of this potential approach (June et al., 2009). An alternative approach is by generating optimal T cells with *ex vivo* programming before adoptive cell transfer. The programming of activated CD8$^+$ T cells to become memory cells required a wide panel of signals.

The differentiation process could be directed either by signals from T-cell receptors or by the co-stimulatory molecules and or by the cytokine receptors direct the differentiation process (Schluns and Lefrancois, 2003; van Stipdonk et al., 2003; Masopust et al., 2004; Bradley et al., 2005; Janssen et al., 2005; Jung et al., 2010).

Although such mechanisms that control the formation of memory T cells are not known, the strict capability of many cytokines i.e. IL-2, IL-7, and IL-15 to regulate T-cell homeostasis may influence this process (Nanjappa et al., 2008). Consequently, at any given time, a normal pool of $CD8^+$ T cells comprises of different cell subtypes at different stages of development; hence they have diverse phenotypic and functional characteristics (Wherry et al., 2003). It is now well established that the stage of $CD8^+$ T cell differentiation has a profound effect on the *in vivo* activity of transferred cells, with more differentiated $CD8^+$ T cells being less effective at killing tumors *in vivo* (Gattinoni et al., 2005; Paulos et al., 2008). Thus, the immunological approaches that aim to generate early effector populations of $CD8^+$ T, which can establish and maintain in the lymphopenic hosts could represent a new avenue of treatment for cancer.

MATERIAL AND METHODS

Mice

BALB/C mice were purchased from Holding Company for Biological Products & Vaccines (VACSERA), Cairo, Egypt. All mice used were females, 6-8 week-old, and 20 g mean body weight. Mice were housed under specific pathogen-free conditions**.** All procedures were following the protocol of National Animal Care and Use Committee and Guidelines for the Care and Use of Experimental Animals as previously described (Muobarak et al., 2018).

Cytokines

Recombinant Mouse IL-7 (catalog# 407-ML-025) was purchased from R&D Systems (Minneapolis, MN). 100 µg/mL were reconstituted in sterile PBS containing at least 0.1% human or bovine serum albumin (BSA) obtained from Sigma-Aldrich and were used according to the manufacturer's instructions.

Cyclophosphamide (CTX)

Cyclophosphamide (CTX) (catalog# C0768-10G0) was purchased from Sigma-Aldrich (Saint Louis, Missouri). CTX was reconstituted with distilled water and diluted to 4 mg/mL with PBS.

Antibodies

Anti-CD16/CD32 (FC block) and the following fluorescein isothiocyanate (FITC), Phycoerythrin (PE), Allophycocyanin (APC), Pacific Blue (PB), PE-Cy5, PerCP-Cy5.5, V450, Alexa-flour 647, and Cy-Chrome conjugated mAbs were purchased from BD Biosciences, Biolegend and BD Pharmingen (San Diego, California, USA). They were used at concentrations recommended by the manufacturers.

Animal Model

The breast tumor cell line Ehrlich Ascites Carcinoma (EAC) was purchased from the National Cancer Institute, Cairo University, Egypt. Seven days after intraperitoneal (IP) implantation of $2.5x10^5$ EAC cells, 2-3 mice were sacrificed by cervical dislocation and ascetic fluid (EAC cells) was collected from the peritoneal cavity using 10ml plastic syringe containing 5ml of cold saline, washed twice with cold PBS by centrifugation for 5 minutes at 1200 rpm, at 4°C. Harvested cells were diluted with saline 0.9% to the required concentration used in each experiment. The total number of EAC cells was determined with trypan blue dye exclusion assay. The harvested cells were adjusted to $2.5x10^5$ EAC cells in 150µL for subcutaneous (S.C)injection into the normal BALB/c mice of the experimental groups.

In vitro Study

Splenocytes were adjusted to 2×10^6 cells/mL in complete RPMI and activated with CONA and IL-7. Three days after activation in CO_2 incubator, cells were harvested. One day before the ACT, wild type BALB/c mice bearing 9-day-old EAC tumors were conditioned by single IP injection with 4 mg/mouse (200 mg/kg) CTX to induce transient systemic lymphodepletion (Salem et al., 2007; Díaz-Montero et al., 2013). The harvested cells that have been primed were adjusted to $5x10^6$ cells and administered 24 hours after the conditioning of recipient mice by intravenous injection.

Tumor measurements

Tumor volume was measured using a digital caliper every other day by determining the greatest longitudinal diameter (length) and the greatest transverse diameter (width). Tumor volume based on caliper measurements were calculated by the modified ellipsoidal formula {Tumor volume = 1/2(length × width^2)} (Jensen et al., 2008). Mice with tumors larger than 400 mm^2 were euthanized by CO_2 asphyxiation.

Flow cytometry analysis

Evry other day, mice were bled from the orbital sinus to harvest peripheral blood. Erythrocytes were then depleted with ammonium chloride-potassium chloride (ACK buffer) (Lou et al., 2004) and counted using a hemocytometer with trypan blue dye exclusion as described previously (Lutz et al., 1999; Díaz-Montero et al., 2007). Cells were stained with the indicated mAbs and then acquired on a FACS Calibur ™ (BD Biosciences, San Jose, CA) and analyzed using Cell Quest ™ software (BD Biosciences).

Gene expression analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and cDNA was synthesized from 1 mg of RNA using Omniscript RT Kit (Qiagen). Gene expression was measured using quantitative real-time PCR and TaqMan probes (Applied Biosystems, Foster City, CA, USA) in a final reaction volume of 20 ml. Ribosomal 18s RNA was used as the internal standard. RT-PCR was performed on a Step One real-time PCR system (Applied Biosystems). The relative quantification of the target transcripts normalized to the endogenous control was determined by the comparative CT method. Relative changes in gene expression between samples were analyzed using the 2^{-ddCt} method.

Statistical analysis

Statistical analyses were performed using the Student's t-test for determining the significant differences. Log-rank nonparametric analysis using GraphPad Prism (GraphPad Software, Inc. San Diego, CA, USA) was used to graph and analyze survival data of mice bearing tumors. Every experiment was repeated 3 independent times under the same conditions, and all P values were two-sided with $p<0.05$ considered significant (*) (Overholser and Sowinski 2007; Overholser and Sowinski 2008).

RESULTS

Effect of IL-7 on the proliferative response of T cells *in vitro*

First, we determined the impact of IL-7 cytokine on the proliferative response of CD8$^+$ T cells. IL-7 was used to stimulate naïve WT splenocytes cells with or without Con A for 3 days. The culture system was started with 23×10^6 cells on day 0, and then harvested the cells at day 3 and determined the proliferation rates. WT^{IL-7} showed a significant increase in the number of proliferated cells (30.75 × 10^6 cells) at day 3 and when compared to WT + Con A that showed 22×10^6 cells as shown in Figure 1.

Effect of IL-7 on the phenotype and functions of T cells *in vitro*

To assess the activation and functional phenotype of tumor-specific CD8$^+$ T cells primed with IL-7, naïve WT, CD8 T cells were stimulated with Con A in the presence of IL-7 for 3 days. Cells were analyzed by flow cytometry for expression of memory precursor effector cells (MPECs), short-lived effector cells (SLECs), central memory T cells, effector memory T cells. Using the gating strategy (Figure 2A), WT^{IL-7} showed significantly elevated levels ($P < 0.05$) of MPECs (KLRG1^{lo}/CD127^{hi}), stem cell-like phenotype (Sca-1^{hi}/CD44^{lo}), central memory T cells (CD62L^{hi}/CD127^{hi}), when compared with WT cells, activated only with Con A (Figure 2).

Effect of IL-7 on the transcription factors of lymphocyte differentiation

To assess the effect of IL-7 on the transcription factors, that are critical for lymphocyte differentiation and control the expression of Th1 cytokines. The gene expression of the following genes Eomes, T-bet, and TCF-7 were measured in primed naïve CD8 T cells with IL-7 and Con A. There was no significant change in TCF-7 and eomes expression between WT^{IL-7} and WT$^{CON\ A}$. Interestingly, the expression of T-bet was significantly higher in WT^{IL-7} compared with WT$^{CON\ A}$ as shown in Figure 3.

Anti-tumor activity of programmed CD8+ T cells by IL-7

To determine whether priming of tumor-specific CD8+ T cells with different activation strategies would lead to more effective antitumor immunity, WT^{IL-7} were adoptively transferred into C57BL/6 mice on day 7 established EAC tumors (S.C.) preconditioned with a single intraperitoneal injection of 4 mg of cyclophosphamide (CTX) and tumor progression was monitored by measuring the tumor size 3 times a week. Interestingly, WT^{IL-7} cells were significantly delayed the tumor growth of established EAC tumors as compared to tumor-bearing mice or tumor-bearing mice received a single dose of CTX (Figure 4). However, eventually, all tumors progressed, and the animals succumbed to the disease.

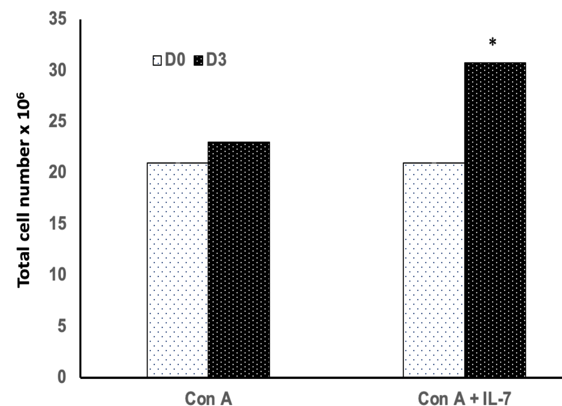

Figure 1. Proliferation of naïve wild type (WT) spleenocytes cells activated *in vitro* with Concanavalin A (Con A) in the presence of IL-7 cytokine for 3 days. Mean±SD. * significant difference versus D0 at P<0.05. n = 3.

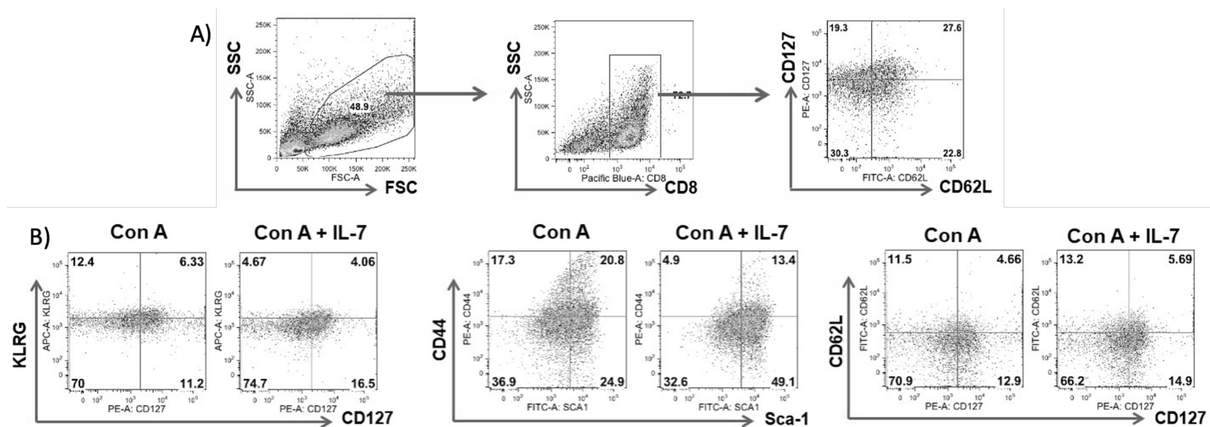

Figure 2. A) The gating strategy to assess the phenotype of naïve WT cells primed with IL-7 in presence of Con A for 3 days. B) Representative flow cytometric analysis show functional phenotype of naïve CD8 T cells primed with IL-7 and Con A.

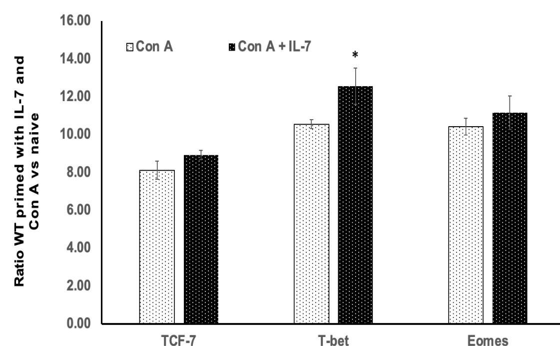

Figure 3. Gene expression analysis by real time PCR (RT-PCR) was performed on mRNA extracted from naïve WT cells primed with IL-7 in the presence of Con A for 3 days. Expression is depicted as the ratio of primed WT cells to naïve WT. Data shown is the average of three independent measurements ± SD. * significant difference versus ConA at P < 0.05.

DISCUSSION

The development of optimal strategies for the ACT is of critical importance to many patients with incurable cancer. CD8+ T cells optimally suited for the anti-tumor ACT should be able to travel to the site of the tumor, recognize the malignant cells, and eradicate them. In addition, some of the transferred CTL should persist as memory CTL lifelong to ensure anti-tumor immunity. This study investigated in detail the impact of priming tumor-specific CD8 T cells using IL-7 cytokine to enhance function and phenotype of these cells *in vitro* and increase their survival and anti-tumor responses *in vivo*.

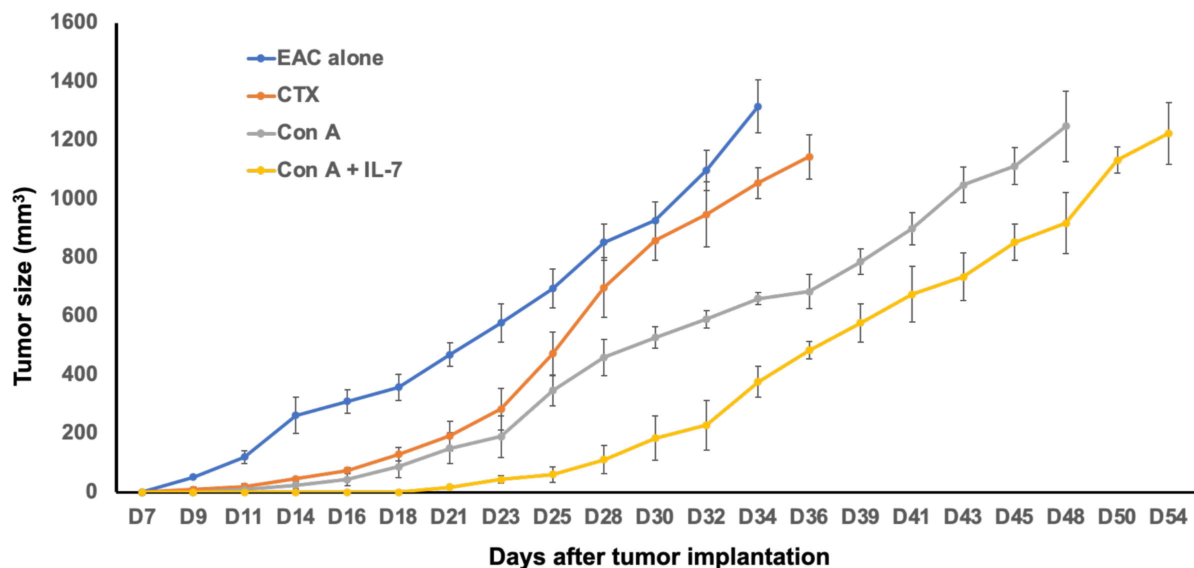

Figure 4. Antitumor activity of CTL primed by Con A and IL-7 in tumor bearing mice preconditioned with single dose of CTX. Tumor progression was monitored in mice receiving primed cells by measuring the tumor size 3 times a week.

Our results demonstrated that cytokines can differentially program an early stage of CTL activation via preconditioning with CTX. This reflects the possibility to benefit from the preconditioned environment of CTX in the hosts in CTL programming and activation. In addition to characterizing the modulation of phenotypic and functional molecules associated with IL-7 priming, we assessed the ability of these cells to migrate, survive, and function in tumor-bearing mice. The functional properties and significance of CD8+ T cell subsets based on KLRG1 and CD127 expression were not fully understood. For example, expression of KLRG1 on CD8+ T cells has been reported to be a marker of cellular senescence and an indicator of the inability to respond to the antigenic challenge (Voehringer et al., 2001). A recent study has been found that forced expression of the IL-7Rα subunit on KLRG1^{hi}CD127^{lo} CD8^{+} T cells failed to restore IL-7–driven proliferation. Taken together, our data suggest that these cells may have inherent deficiencies in cytokine-induced proliferation and that might extend beyond the simple absence of IL-7Rα expression (Hand et al., 2007; Vranjkovic et al., 2007).

Despite such cells exhibit a proliferative defect; others have reported that KLRG1^{hi} CD8^{+} T cells can undergo vigorous proliferation after antigen challenge (Masopust et al., 2006). Relevant to our findings, WT^{IL-7} cells showed significantly elevated levels of MPECs (KLRG1^{lo}/ CD127^{hi}) phenotype. That contains longer-lived memory CD8^{+} T cells as opposed to SLECs (KLRG1^{hi}CD127^{lo}) phenotype. And that was associated with CD8^{+} T cells, which selectively lost over the month when compared with WT^{sham} cells (Kaech et al., 2003; Joshi et al., 2007; ; Sarkar et al., 2008; Salem et al., 2012). Therefore, WTIL-7 cell seems to represent central memory CTL (CD62Lhi/ CD127hi) as reported previously (Diaz-Montero et al., 2008; Lisiero et al., 2011; Salem et al., 2018).

IL-7-primed cells showed elevated expression of the transcription factor, T-bet, as previously reported, (Rao et al., 2010). Up-regulation of T-bet is consistent with the ability of IL-7 to direct CD8+ T cells to a Tc1 phenotype (Szabo et al., 2000). To evaluate the *in vivo* antigen-specific and anti-tumor responses of CD8^{+} T cells primed with IL-7against EAC tumor, we adoptively transferred lymphocytes primed cells into tumor-bearing mice. Strikingly, we found that WT^{IL-7} cells were able to delay EAC tumor growth and failed to eradicate the tumor. The most crucial point of experiments was the determination of optimal condition of WT^{IL-7} that effectively regress EAC tumor.

CONCLUSION

Our results suggest that short-term T-cell receptor signaling in the presence of IL-7 promotes miscues qualities in naïve CTL which upon transfer into lymphopenic hosts are

sufficient to eradicate tumors and generate a life-long tumor-specific memory. These findings have important implications for adoptive cell therapy and provide the scientific rationale for utilizing IL-7 and CON A in *ex vivo* programming of T cells for adoptive transfer.

Conflict of interest

The authors claim no conflict of interest.

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EACR is an NGO society that was declared by the Ministry of Social Solidarity (Egypt) No. 1938 in 19/11/2014 based on the initiative of Prof. Mohamed Labib Salem, the current Chairman of EACR. EACR aims primarily to assist researchers, in particular young researchers in the field of cancer research through workshops, seminars and conferences. Its first international annual conference entitled "Anti-Cancer Drug Discovery" was successfully organized in April 2019 (http://acdd.tanta.edu.eg). Additionally, EACR aims to raise the awareness of the society about the importance of scientific research in the field of cancer research in prediction, early diagnosis and treatment of cancer. EACR is also keen to outreach the scientific community with periodicals and news on cancer research including peer-reviewed scientific journals for the publication of cutting-edge research. The official scientific journal of EACR is "International Journal of Cancer and biomedical Research (IJCBR: https://jcbr.journals.ekb.eg) was successfully issued in 2017 and has been sponsored by the Egyptian Knowledge Bank (EKB: www.ekb.eg).

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International Journal of Cancer & Biomedical Research
(IJCBR) Online ISSN 2682-2628

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