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Interleukin-15 enhances cytokine induced killer (CIK) cytotoxic potential against epithelial cancer cell lines via an innate pathway

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ABSTRACT

CIK cells are a subset of effector lymphocytes endowed with a non-MHC restricted anti-tumor activity making them an appealing and promising cell population for adoptive immunotherapy. CIK are usually generated *ex-vivo* by initial priming with Interferon-γ (IFN-γ) and monoclonal antibody against CD3 (anti-CD3), followed by culture in medium containing Interleukin-2 (IL-2). Interleukin-15 (IL-15) shares with IL-2 similar biological functions and recently it has been reported to induce CIK with increased anti-leukemic potential.

The aim of the study was to compare the killing efficacy of CIK generated by IL-2 alone or IL-2 and IL-15 toward tumor targets of different origins, leukemic cells and malignant cells from epithelial solid tumors. CIK bulk cultures were examined for cell proliferation, surface phenotype and cytotoxic potential against tumor cell lines K562, HL60, HeLa and MCF-7. The results showed that IL-15 is able to induce a selective expansion of CIK cells, but it is less effective in sustaining CIK cell proliferation compared to IL-2. Conversely, our data confirm and reinforce the feature of IL-15 to induce CIK cells with a potent cytotoxic activity mostly against tumor cells from epithelial solid malignancies via NKG2D-mediated mechanism.

1. Introduction

The immune system is devoted to defensive action of human body against invading pathogens and occurrence of malignant cell transformation by generating prompt innate and adaptive immune responses. Cell immunotherapy is a promising therapeutic approach, which takes advantage of this human body's natural ability to eliminate tumor cells.

Immunotherapy strategies have been focused to select, expand *ex vivo* and induce immune effectors able to recognize and kill malignant cells when reinfused back into the patient. Various approaches have been developed in the field of immunotherapy involving different sets of effector cells, including dendritic cells (DC), peripheral and tumor infiltrating cytotoxic T lymphocytes (CTL), natural killer (NK) cells, lymphokine activated killer cells and,more recently, cytokine induced killer cells (CIK).CIK cells are a subset of *ex-vivo* expanded effector lymphocytes endowed with a non-MHC restricted anti-tumor activity and reduced allo-reactivity potential, which make them an appealing and promising cell population for adoptive immunotherapy either in autologous [1–4] or in allogeneic settings [5–7]. First described and characterized by Schmidt-Wolf and co-workers [8], CIK are T derived cells sharing both NK phenotype and functional properties. CIK cells derived from T cell precursors acquire CD56 molecules during *ex-vivo* expansion resulting in a population of cytotoxic NK-T effector cells [9–11]. The exact mechanism responsible for tumor cell recognition and killing by NK-T cells is not completely understood, but it seems to be mostly related tothe expression of NKG2D molecules on the effector cell surface,which interact with ligands on tumor cells, mainly MIC A/B [12].

Usually CIK are generated from peripheral blood mononuclear cells (PBMCs) after initial *in vitro* priming with Interferon-γ (IFN-γ) and monoclonal antibody against CD3 (mo-Ab anti-CD3), followed by culture in medium containing Interleukin-2 (IL-2). High variability in the range of CIK proliferation, from few to more than 1000 fold expansion, has been reported, suggesting that some

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patient/donor might be classified as "poor CIK expander" [13–15]. Considering that the recruitment of a sufficient number of immune cells capable to be specifically effective against tumor target cells is a crucial issue for adoptive immunotherapy, alternative strategies, based on additional soluble factors such as IL-1, IL-7, IL-12 or on allogeneic stimulation by irradiated PBMC, have been investigated to ameliorate both the number and the anti-tumor cytotoxic potential of CIK [16–18]. Moreover, immunotherapy approaches combining different immune effector cells have also been explored and, the association of DC, CTL and CIK have displayed encouraging results in the treatment of various malignant tumors [19]. Recently, Interleukin 15 (IL-15) has been reported to be valuable in supporting CIK expansion and anti-tumor function particularly against leukemia and rhabdo-myosarcoma cell lines [20,21]. IL-15 is a cytokine which shares with IL-2 similar structure and biological functions. Nevertheless, IL-15 mainly plays a major role in stimulating cytolytic activity and cytokine secretion of NK cells as well as in promoting the induction of T cytotoxic effectors and the survival of memory $CD8^+$ T cells [22,23]. Therefore, the insights of IL-15 based strategies adopted to produce CIK are of great interest to generate immune effectors with enhanced anti-tumor potential, which could be successful candidates for immunotherapy purposes.

In the present study, CIK expansion sustained by IL-2 alone or in combination with IL-15 was evaluated during a period of four weeks of culture. We focused on the killing efficacy of these two effector CIK populations against different tumor target cells, including leukemic cell lines (human myelogenous leukemia and acute promyelocytic leukemia) and epithelial cancer cell lines (cervix and breast adenocarcinoma).

2. Materials and methods

2.1. Cell lines

The following human cell lines, K562 (CCL-243 human myelogenous leukemia), HL-60 (acute promyelocytic leukemia), MCF-7 (mammary gland/breast adenocarcinoma) and HeLa (cervix adenocarcinoma) were purchased from American Type Culture Collection (ATCC Manassas, VA, USA). K562 and HL-60 cells were cultured at 500.000 cells/mL in RPMI 1640 medium (EuroClone, Pero, MI Italy) or Iscove's Modified Dulbecco's medium (EuroClone) respectively, supplemented with 10% Foetal Bovine Serum (FBS, EuroClone). Culture medium (CM) was replaced every 3/4 days. HeLa and MCF-7 cells were cultured in adherence at the concentration of 10.000 cell/cm^2 in D-MEM high glucose (EuroClone) supplemented with 10% FBS. CM was renewed twice a week and when the cells reached the confluence they were detached by Trypsin-EDTA treatment (EuroClone), counted and re-seeded at appropriate concentration for next propagation steps.

2.2. Generation of CIK cells

CIK cells were generated from PBMCs according to two different expansion protocols, the former using the conventional scheme with IFN-γ/anti-CD3 and IL-2 alone and the latter combining IFN-γ/anti-CD3 and IL-2 until day 7, followed by the addition of IL-15 alone up to the end of culture. White blood cells were obtained from blood donations after donor's written informed consent. The study was approved by the Hospital Committee for Blood and Stem Cells Transfusion and Usage, which is the board that has jurisdiction for laboratory use of blood and stem cells in our Institution (approved on June 4, 2015). Seven residual buffy coats (BCs) from blood units obtained from healthy volunteer donors were processed to separate PBMCs by density gradient centrifugation (Lympholyte, Cedarlane, Burlington Ontario USA). After washing, PBMCs were cultured at 2×10^6 cells/mL in serum free medium (X-vivo 10, Lonza, Basel Switzerland), which is suitable for *ex-vivo* cell expansion intended for therapeutic use [24]. Cell cultures were initially supplemented with IFN-γ (1000 IU/mL) (Miltenyi Biotech, Bergisch Gradbach, Germany) and twenty-four hours later with pure anti-CD3 functional grade mo-Ab (50 ng/mL) (Miltenyi Biotech) and IL-2 (300 U/mL) (Miltenyi Biotech). On the 7th day of culture, one fraction of the cell suspension ($CIK_{IL\text{-}15}$) was maintained with IL-15 (50 ng/mL) (Miltenyi Biotech), while the remaining cells ($CIK_{IL\text{-}2}$) were kept in culture with IL-2 (300 U/mL) (Miltenyi Biotech). Culture medium and cytokines were added every 3–4 days and cell density regularly adjusted at 1×10^6 cells/mL throughout the cell culture period of 28 days.

2.3. Cell surface phenotype analysis

Aliquots of cells were analyzed for the expression of various surface markers at baseline (day 0) and at day 7, 14, 21 and 28 of culture. Multi-parameter flow cytometry analysis was performed by incubating the cells with mo-Abs conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein or allophycocyanin. Mouse anti-human mo-Abs were used to stain the following cell surface markers: CD3, CD4, CD8, CD45, CD16, CD56, CD19 (Immunomonitoring Kit, Becton Dickinson BD Bioscience, S. Josè CA), CD314 (NKG2D), CD158a (KIR2DL1), CD158b (KIR2DL2/DL3), CD335 (NKp46), T cell receptor (TCR) αβ and γδ (BD Biosciences). The intracellular expression of FoxP3 transcription factor was detected in fixed/permeabilized cells that were initially labeled with anti-CD4 and anti-CD25 mo-Abs followed by anti-FoxP3 (BD Biosciences). Viability of the cells throughout the culture time was checked by using fluorescent dye 7-amino-actinomycin D (7-AAD, BD Biosciences). Data were acquired on FACSCalibur and analyzed with CellQuest pro software (BD Biosciences).

2.4. Cytotoxic assay

The cytotoxic activity of $CIK_{IL\text{-}15}$ and $CIK_{IL\text{-}2}$ was evaluated at day 21 and 28 of culture against haematological tumor cell lines, K562 and HL60, and epithelial cancer cell lines, HeLa and MCF-7, by Carboxy Fluorescein diacetate Succinimidyl Ester (CFSE, Invitrogen, Waltham Massachusetts USA) based cytotoxic assay. This assay is an alternative to the more common radioactive ^{51}Chromium release assay [3,25]. Briefly, 10×10^6 target cells were labeled with 2 μM CFSE 10 min at 37 °C in the dark. Quench staining was performed on ice for 5 min by adding 5 volumes of ice-cold phosphate buffered saline (PBS, EuroClone) additioned with 20% FBS. Cells were then washed 3 times with cold PBS plus 10% FBS and re-suspended in CM (RPMI 1640 supplemented with 10% FBS) at appropriate concentration. $CIK_{IL\text{-}15}$ and $CIK_{IL\text{-}2}$ effector (E) cells were added to viable labeled target (T) cells at the following E/T ratios 30:1, 10:1, 5:1, 2.5:1 and co-cultured for different time periods (K562 60 min, HL60 20 min, HeLa and MCF-7 120 min). The time of E-T co-incubation had been optimized considering our preliminary findings to provide a valuable kinetic of specific lysis at each E/T ratios. The fluorescent viable dye 7-AAD was added to mark target dead cells. 7-AAD easily penetrates into the damaged permeable membranes of non-viable cells, while vital cells with intact membranes exclude the dye. 7-AAD is able to enter into the cells and stain the nuclear DNA as soon as cell membrane damage occurs. Hence, all compromised target cells are stained immediately.

The percentage of specific lysis was calculated as % of residual viable targets at the different E/T ratios according to the following formula: