Low-doses of sequential-kinetic-activated interferon-γ enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study.

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Low-doses of sequential-kinetic-activated interferon-γ enhance the ex vivo cytotoxicity of peripheral blood natural killer cells from patients with early-stage colorectal cancer. A preliminary study

Elisabetta Radice[a], Vincenzo Miranda[b], Graziella Bellone[c, ,]

Abstract

Natural killer (NK) cells are innate immune-system lymphocytes capable of killing tumor cells. They secrete cytokines, including interferon (IFN)-γ, which participate in shaping the initial inflammatory and downstream adaptive immune responses. Its potent immunoregulatory action means that IFN-γ might be beneficial in cases of tumor rejection, but its severe side-effects limit clinical applications. This pilot study compared low-dose IFN-γ prepared by sequential-kinetic-activation (SKA), with standard-dose recombinant (r) IFN-γ, in terms of ex-vivo cytotoxic activity of peripheral blood (PB)-NK cells from colorectal carcinoma (CRC) patients. This was tested against the NK-sensitive K562 cell line and the less-sensitive human CRC Caco-2 and HT-29 cell lines. Twenty primitive non-metastatic CRC patients, five metastatic CRC patients, and thirteen healthy donors were enrolled. PB lymphocytes (PBL) were exposed to medium alone, SKA-IFN-γ (0.25 fg/ml) or rIFN-γ (1 ng/ml). NK-cell cytolytic activity was examined via short-term ^{51}Cr-release. Pretreatment of PBL from non-metastatic patients with SKA-IFN-γ caused a significant increase in NK-cell cytotoxicity, compared to those from normal donors, although less markedly than pretreatment with rIFN-γ against all three cell lines. In contrast, PBL from metastatic CRC patients displayed significantly decreased NK-cell activity and responsiveness to both rIFN-γ and SKA-IFN-γ treatments. These results demonstrate in principle the immunomodulatory capacity of low-dose SKA-IFN-γ, and might open the door to the possibility of generating a novel, safe, and feasible approach to enhancing NK-cell antitumor activity in early-stage CRC patients.

Abbreviations

- NK cell, natural killer cell;
- SKA, sequential-kinetic-activation;
- CRC, colorectal cancer;
- IFN, interferon;
- r, recombinant;
- PBL, peripheral blood lymphocyte;
- LU, lytic unit

Keywords

- Interferon-γ;
- Low-dose cytokines;
- Natural killer cells;

- Cancer immunotherapy;
- Colorectal cancer

1. Introduction

In vivo and in vitro studies have shown that NK cells, by eliminating malignant cells without the classical MHC restriction, play a crucial role in tumor immunosurveillance [1]. They also collaborate with antigen-presenting cells to amplify the immune response and induce T-cell-mediated antitumor immunity [2] and [3]. Interferon (IFN)-γ, produced as a result of initial tumor recognition by NK cells, chemoattracts innate immune effector cells. By producing these cells in turn activate the cytotoxic functions ,additional IFN-γ of tumor infiltrating NK cells and macrophages, leading to enhanced production of immunomodulatory cytokines, e.g. IL-12 and IL-18. The end result is the presence of sufficient numbers of dead tumor cells to activate the antigen-presenting machinery of the adaptive immune response. The IFN-γ- and IL-12-rich tumor environment inhibits T helper (h) 2 cell development, while promoting a Th1 anti-tumor response. This eventually gives rise to a specific cytotoxic $CD8^+$ anti-tumor cell response; it also induces MHC class I pathway protein expression in tumor cells, enhancing their immunogenicity [4]. The production of substantial levels of IFN-γ also indirectly enhances angiostasis, and directly causes tumor-cell anti-proliferative and pro-apoptotic responses [5] and [6]. However, this integrated and necessary link between innate and adaptive immune responses, whose goal is to elicit specific tumor rejection, is not usually efficient in controlling large solid tumors, because of the insufficient immune response and/or the active strategies employed by the tumor cells to escape immune attack [7].

Interest has been increasing, over the past three decades, in harnessing the immune system to eradicate cancer; this has been accompanied by heightened efforts to characterize cytokines and exploit their vast signaling networks, in the hope of developing cancer treatments [8]. Due to the extensive immunomodulatory functions in both innate and acquired immunity [9], and the proven pivotal role of endogenously-produced IFN-γ in animal models of antitumor immunity, IFN-γ quickly inspired clinical applications in a variety of disease conditions, including cancer. However, the clinical use of high-dose IFN-γ as conventional chemotherapy against different types of tumor, including melanoma [10], glioma [11], small-cell lung [12], bladder [13], and breast [14] cancers, and in hematological [15], ovarian [16], renal [17], and gastrointestinal [18] and [19] malignancies, has had limited success, with severe dose-dependent toxicity. This suggests that in-vivo doses route, and/or administration schedule may be inappropriate in vivo. If the natural rapid, but transient production of IFN-γ is not reproduced therapeutically, a potentially-effective action on the functional organization of the immune responses to the tumor could be converted into a substantially negative one. Further studies are thus necessary to clarify the optimal clinical use of this cytokine, and to develop treatments affording potent, specific, and durable anti-tumor immunity, with limited adverse consequences.

Colorectal cancer (CRC), one of the leading causes of morbidity and mortality worldwide [20], is a highly treatable and often curable disease when localized to the bowel. Surgery is the primary form of treatment, and leads to a cure in approximately 50% of patients. Recurrence following surgery is a major problem, and is often the ultimate cause of death, although considerable progress has been made in treatment. An increasing body of evidence supports the hypothesis that the visibility of CRC to immune attack is substantial, and that it potentially could limit disease progression [21]. Despite some preclinical evidence of efficacy, clinical trials using IFN-γ have been generally disappointing, and the treatment is poorly tolerated [19] and [22].

Several lines of evidence suggest that low-doses of cytokines are adequate in many different models [23]. A recent in vitro study showed that low-doses of IL-12 modulate functional activities of T cell sub-populations from non small cell lung cancer patients [24]. In particular, Gariboldi et al. [25] employed a murine model of allergic asthma to demonstrate that cytokines activated by the pharmaceutical preparation process known as "sequential kinetic activation" (SKA) retain their functional activities even at physiological low-dose concentrations.

These results encouraged us to investigate whether this specific method might render cytokines at low-doses as active in the treatment of the human diseases as the high concentrations normally used in clinical pharmacology, but without the side effects typical of high doses.

This explorative study utilized peripheral blood (PB)-NK effector cells, from healthy donors and from non-metastatic and metastatic CRC patients, to assess whether ex-vivo exposure to a very low-dose of SKA-IFN-γ might enhance these cells' antitumoral activity, compared to the normally-administered conventional dose of r-IFN-γ.

2. Materials and methods

2.1. Reagents

SKA-IFN-γ was prepared by GUNA Laboratories (GUNA S.p.a, Milan, Italy) using the standardized method. IFN-γ underwent a shaking process (vertical shaking; 10 cm motion range; shaking speed corresponding to 100 oscillations over 10 s), sequentially diluted in saline solution (serial dilution 1:100) and kinetically energized by a mechanically-applied force [25]. The preparation was supplied at a concentration of $10^{-8}$was obtained from PeproTech Inc. (Rocky Hill, NJ, USA). μg/ml. Recombinant human IFN-γ

2.2. Patients

The study group comprised 25 patients who had received a diagnosis of CRC (15 males and 10 females, median age 70.5 years, range 57–84) from the Department of Surgical Medical Sciences at "Città della Salute e della Scienza" Hospital, Turin (Italy) between April 2011 and September 2013. Twenty patients had histopathologically-confirmed primary CRC and were staged by Dukes' system, revised by Astler and Coller (8 Dukes' A and 12 Dukes' B) [26]. Entry criteria were: primary CRC indicative of surgery with no preoperative evidence of distant metastasis. Five patients had histopathologically-confirmed metastatic CRC (3 Dukes' C with lymph node metastasis, and 2 Dukes' D with hepatic metastasis). None of the patients had undergone surgical or other anticancer treatment at the time of blood sampling. All patients provided their informed consent prior to entering the study. The study procedures complied with the Helsinki Declaration. A group of thirteen healthy donors were used as controls (7 males and 6 females, median age 68, range 48–90).

2.3. Cell isolation and treatment

Peripheral blood (PB) samples (15 ml) were collected in anticoagulant-coated tubes from CRC patients and healthy donors. PB mononuclear cells (PBMC) were isolated using Ficoll–Hypaque density gradient centrifugation. PB lymphocytes (PBL) were obtained from PBMC depleted of $CD14^{+}$ cells by means of immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). PBL were cultured at 1×10^{6}/ml for 24 h in 5 ml of RPMI 1640 medium, containing 10% fetal calf

serum (FCS) (complete medium) (Sigma Aldrich, St. Louis, MO, USA), in the absence or presence of the previously-determined optimal dose of SKA-IFN-γ (0.25 fg/ml) or of rIFN-γ (1 ng/ml).

2.4. Cytotoxicity assays

NK cell activity against the NK-sensitive human myelogenous leukemia cell line K562 (ATCC, Manassas, VA) and against two CRC cell lines, Caco-2 [27] and HT-29 (ATCC) [28], or SKA-IFN-γ-treated PBL was tested in a of untreated- and rIFN-γ standard short-term (4-h) in vitro cytotoxic assay. Briefly, tumor targets were incubated with 100 μCi of sodium [^{51}Cr] chromate (PerkinElmer, Waltham, MA, USA) for 60 min at 37 °C, and washed three times to remove excess isotope. A quantity amounting to 5×10^3 target cells/well was added to different numbers of effector PBL in triplicate, in 96-well U-bottomed microtiter plates (Costar, Cambridge MA, USA), to assess effector-to-target (E:T) final ratios of 40:1, 20:1, 10:1 and 5:1. After 4 h incubation at 37 °C in a CO_2 incubator, 100 μl of supernatant was removed from each well for isotope counting in a γ-counter (Packard, Downers Grove, IL, USA). Spontaneous and maximum release values were determined, respectively, by incubating targets in medium alone, or in medium plus 1% Triton-X-100. Spontaneous release in no case exceeded 15% of maximum release. In all cases, cultures were set up in triplicate and the % specific target cell lysis was calculated from the following formula: $(E - S) / (M - S) \times 100$, where E is the mean cpm release in the presence of effector cells, S is the mean cpm released spontaneously by target cells incubated with medium alone, and M is the cpm release of 100 μl of Triton-X-100-treated cells. NK-cell activity was expressed in terms of the number of lytic units 30% (LU_{30}) per 1×10^6 cells for all groups. LU_{30} was defined as the number of effectors required to produce 30% specific cytotoxicity of 5×10^3 target cells [29]. This method, which uses 4 E:T ratios, is the preferred method for establishing the lytic potential of effector cells, and is preferable to calculating a single percent cytotoxicity value, as it relies on the slope of the graph of E:T ratio versus radioactivity released into the supernatant at the end of incubation.

2.5. Statistical analysis

For matched pairs, the paired Student's t-test was applied. For intergroup comparisons, variables were analyzed by means of one-way ANOVA followed by the Student–Newman–Keuls method, using Sigmastat 3.1 software (Jandel Scientific, San Rafael, CA, USA). Significance was set at $p < 0.05$.

3. Results

3.1. Basal PB-NK cell activity status in healthy donors, patients with non-metastatic or metastatic CRC, against the NK-sensitive K562 cell line, and the two CRC cell lines Caco-2 and HT-29

The characteristic function of NK-cells is their spontaneous cytotoxicity, which can be analyzed by means of a [^{51}Cr]-release assay against the human NK-sensitive K-562 cells. We first assessed whether PB-NK cells from non-metastatic (n = 20) and metastatic (n = 5) CRC patients retained a tumor-cell killing ability comparable to that of their healthy-donor (n = 13) counterparts.

The results are in Fig. 1A–C as % of specific cytotoxicity at the different ratios (left panel), and as LU_{30} (the most common means of expressing cytolytic potential in cell-mediated cytotoxicity assays) (right panel). PB-NK cells from non-metastatic CRC patients were capable of mounting a cytolytic response against K562 cells comparable to that of cells from normal donors (mean ± SE LU_{30}: 52.41 ± 6.83 vs. 59.13 ± 7.09, $p = 0.531$), while those from patients with metastatic tumor

exerted significantly decreased cytolytic activity compared both to cells of normal subjects (mean ± SE LU_{30}: 28.68 ± 6.05 vs. 59.13 ± 7.09, $p = 0.012$) and those of non-metastatic CRC patients (mean ± SE LU_{30}: 28.68 ± 6.05 vs. 52.41 ± 6.83, $p = 0.031$) (Fig. 1A, right panel).

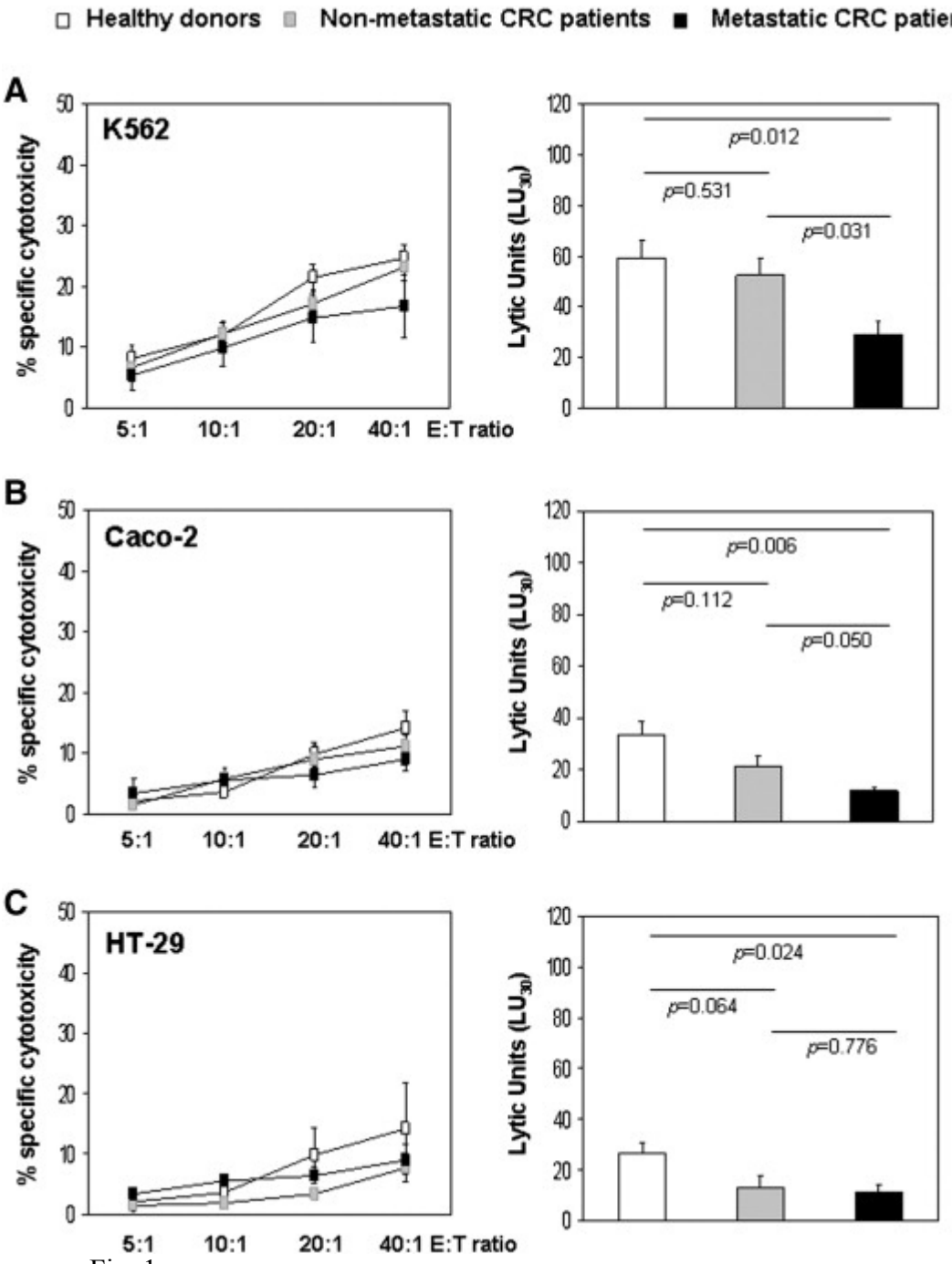

Fig. 1.

Comparison of spontaneous activity in the 4-h [^{51}Cr]-release assay against the NK-sensitive K562 cell line (A) of PB-NK cells from thirteen healthy donors, twenty non-metastatic CRC patients and five metastatic CRC patients, and against the human CRC cell lines Caco-2 (B) and HT-29 (C) from six healthy donors, five non-metastatic and five metastatic CRC patients. Results are expressed as the mean percentage ± SE of specific cytotoxicity at different E:T ratios (left panel) and of means ± SE of the relative cytolytic potential expressed as LU_{30} (right panel). Percentages of specific cytotoxicity and LU_{30} were calculated as described in the Materials and methods section. Statistical differences between

groups were calculated by means of one-way ANOVA followed by the Student–Newman–Keuls method.

The specific cytotoxicity of PB-NK cells was also tested, using the two human CRC cell lines Caco-2 and HT-29 as targets. PB-NK cells from normal donors (n = 6) displayed less killing activity against both Caco-2 and HT-29 cells than they did against K562 cells (mean ± SE LU_{30} Caco-2 vs. K562: 33.64 ± 5.33 vs. 62.69 ± 7.25, $p = 0.002$; mean ± SE LU_{30} HT-29 vs. K562: 26.54 ± 4.38 vs. 62.69 ± 7.25, $p = 0.001$) (Fig. 1A–C right panels, white boxes).

When the spontaneous cytotoxic responses of PB-NK cells from non-metastatic and from metastatic CRC patients, against Caco-2 and HT-29 cells, were compared with that of PB-NK cells from healthy subjects (Fig. 1B and C right panels), PB-NK cells from the non-metastatic patients (n = 5) showed near-normal killing activity (mean ± SE LU_{30} against Caco-2: 21.454 ± 4.01 vs. 33.64 ± 5.33, $p = 0.112$, respectively; mean ± SE LU_{30} against HT-29: 13.01 ± 4.64 vs. 26.54 ± 4.38, $p = 0.064$, respectively), while PB-NK cells from metastatic CRC patients (n = 5) displayed a significantly lower cytolytic capacity (mean ± SE LU_{30} against Caco-2: 12.29 ± 0.95 vs. 33.64 ± 5.33, $p = 0.006$, respectively; mean ± SE LU_{30} against HT-29: 12.37 ± 3.08 vs. 26.54 ± 4.38, $p = 0.024$, respectively). However, comparison between the killing activity of PB-NK cells from the two groups of patients (non-metastatic patients' cells vs. those of metastatic patients) showed higher activity against Caco-2 cells (mean ± SE LU_{30}: 21.45 ± 4.01 vs. 12.29 ± 0.95, $p = 0.05$), and comparable activity against HT-29 cells (mean ± SE LU_{30}:13.01 ± 4.64 vs. 12.37 ± 3.08, $p = 0.776$) (Fig. 1C and B right panels, gray boxes and black boxes, respectively).

These results suggest that the cytolytic effector machinery of PB-NK cells is almost entirely preserved in the early stages of CRC, but that it tends to decline with disease progression.

3.2. Effect of low-dose SKA-IFN-γ versus standard-dose rIFN-γ on the NK-cell cytotoxic activity of patients with non-metastatic and metastatic CRC, against the NK-sensitive K562 cell line, and the two CRC cell lines, Caco-2 and HT-29

Given the critical roles of NK cells in first-line defense against malignancies and in controlling metastasis, the therapeutic use of NK cell activators, such as IL-2, IL-12, IL-15, IL-18, IL-21 and IFNs, in human cancer immunotherapy has been proposed, and applied in a clinical context. However, only modest clinical success has been achieved thus far, as many patients experienced severe life-threatening toxic side effects [30].

An explorative study was thus run to investigate whether low-dose SKA-IFN-γ might be a promising approach to manipulate NK cells, with the ultimate goal of treating cancer patients; it was compared with standard-dose rIFN-γ. The efficacy of low-dose SKA-IFN-γ treatment on the cytotoxic activity of PB-NK cells from healthy donors (n = 13), non-metastatic (n = 20) and metastatic CRC (n = 5) patients, was initially evaluated against the NK-sensitive target K562 cells. Fig. 2A–C shows the mean percentage ± SE of specific cytotoxicity observed in the various experimental conditions and at different E:T ratios (left panels), and the relative cytolytic potential expressed as LU_{30} (right panels). When LU_{30} values were compared, significant enhancement of the lytic activity by both rIFN-γ and SKA-IFN-γ treatment occurred in PB-NK cells from healthy subjects (mean ± SE LU_{30}: basal 59.13 ± 7.09 vs. IFN-γ 87.97 ± 6.91, $p < 0.001$; vs. SKA-IFN-γ 78.13 ± 5.77, $p = 0.001$) (Fig. 2A), as well as in those from non-metastatic CRC patients (mean ± SE LU_{30}: basal 52.41 ± 6.83 vs. IFN-γ 85.01 ± 7.86, $p = 0.012$; vs. SKA-IFN-γ 68.25 ± 5.87, $p = 0.031$) (Fig. 2B right panel) and metastatic CRC patients (mean ± SE LU_{30}: basal 28.68 ± 6.05 vs. IFN-γ 44.74 ± 7.89, $p = 0.005$; vs. SKA-IFN-γ 32.98 ± 5.99, $p = 0.046$) (Fig. 2C right panel). However, SKA-IFN-γ-treated PB-NK cells exhibited a slightly, but statistically-

significantly, lower cytotoxic activity than rIFN-γ-treated PB-NK cells from normal donors as well as in specimens from the two patient groups (mean ± SE LU_{30} normal donors: 78.13 ± 5.77 vs. 87.97 ± 6.91, $p = 0.018$; mean ± SE LU_{30} non-metastatic CRC patients: 68.25 ± 5.87 vs. 85.01 ± 7.86, $p = 0.031$; mean ± SE LU_{30} metastatic CRC patients: 32.98 ± 5.99 vs. 44.74 ± 7.89, $p = 0.004$).

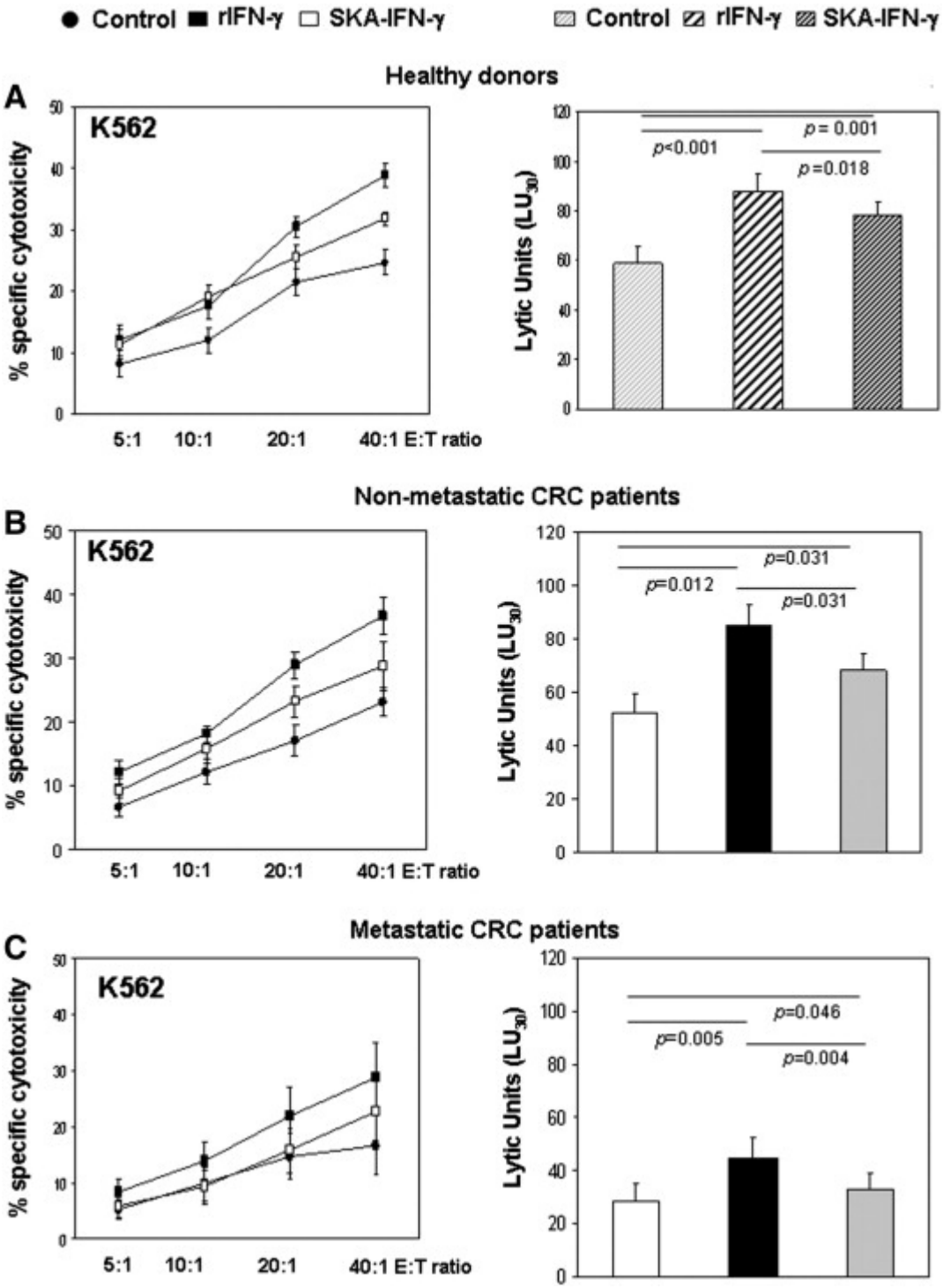

Fig. 2.

Effects of standard-dose rIFN-γ and of low-dose SKA-IFN-γ on cytotoxic activity against NK-sensitive K562 cells of PB-NK cells from normal donors (n = 13) (A), non-metastatic CRC patients (n = 20) (B), and metastatic CRC patients (n = 5). PBL were untreated or pretreated with rIFN-γ (1 ng/ml) or SKA-IFN-γ (0.25 fg/ml) for 24 h. After washing, NK-

cell cytotoxic activity was tested at different E:T ratios by means of the 4-h ^{51}Cr-release assay. The figure shows the mean percentages ± SE of specific cytotoxicity at different E:T ratios (left panel) and the means ± SE of relative cytolytic potential expressed as LU_{30} (right panel). Percentages of specific cytotoxicity and LU_{30} were calculated as specified in the Materials and methods section.

p = statistical significance (Student's t test for paired data).

Having thus determined that PB-NK cells from healthy donors, and from both groups of CRC patients, were effective in spontaneously destroying CRC Caco-2 and HT-29 cells (albeit to a lesser extent than their destructive effect on K562 cells), we then examined whether low-dose-SKA-IFN-γ-stimulated PB-NK cells from normal donors (n = 6), non-metastatic patients (n = 5) and metastatic patients (n = 5) might have stronger cytotoxic activity against CRC cell targets than resting or rIFN-γ-activated PB-NK cells. As shown in Figs. 3A–C and 4A–C, low-dose-SKA-IFN-γ-stimulated PB-NK cells from normal donors and from non-metastatic CRC patients displayed a significant increase in spontaneous cytolytic activity against Caco-2 (mean ± SE LU_{30} normal donors: 38.10 ± 5.10 vs. 33.64 ± 5.33, $p = 0.040$; mean ± SE LU_{30} non-metastatic CRC patients: 36.98 ± 4.68 vs. 21.45 ± 4.01, $p = 0.031$) (Fig. 3A and B right panels) and against HT-29 cells (mean ± SE LU_{30} normal donors: 39.00 ± 5.15 vs. 26.54 ± 4.38, $p = 0.029$; mean ± SE LU_{30} non-metastatic CRC patients: 21.05 ± 9.15 vs.13.01 ± 4.64, $p = 0.014$) (Fig. 4A and B right panels). However, low-dose-SKA-IFN-γ-stimulated PB-NK cells from normal donors and from non-metastatic patients were less effective in destroying Caco-2 target cells than were rIFN-γ-treated PB-NK cells (mean ± SE LU_{30} normal donors: 38.10 ± 5.10 vs. 51.05 ± 5.51, $p = 0.048$; mean ± SE LU_{30} non-metastatic CRC patients: 21.05 ± 9.15 vs. 28.75 ± 7.85, $p = 0.031$) (Fig. 3A and B right panels), while they displayed similar cytotoxic activity against HT-29 cells (mean ± SE LU_{30} normal donors: 39.00 ± 5.15 vs. 46.30 ± 7.63, $p = 0.107$; mean ± SE LU_{30} non-metastatic CRC patients: 21.05 ± 9.15 vs. 28.75 ± 7.85, $p = 0.158$) (Fig. 4A and B right panels). In contrast, the killing activity against Caco-2 cells of low-dose-SKA-IFN-γ-stimulated PB-NK cells from metastatic patients (Fig. 3C right panel) displayed non-significant or minimal changes compared to that of resting and rIFN-γ-stimulated-PB-NK cells (mean ± SE LU_{30}: SKA-IFN-γ 16.03 ± 1.98 vs. basal 12.29 ± 0.95, $p = 0.142$; vs. IFN-γ; 18.11 ± 1.85, $p = 0.063$). Moreover, and interestingly, the increase of PB-NK cell cytotoxic activity induced by rIFN-γ was at the limit of statistical significance in comparison to spontaneous killing (mean ± SE LU_{30}: 18.11 ± 1.85 vs. 12.29 ± 0.95, $p = 0.05$). When HT-29 cells were used as targets (Fig. 4 panel C), low-dose SKA-IFN-γ-activated PB-NK cells from metastatic patients showed no, and only a slight, increase vs. resting and IFN-γ-activated PB-NK cells, respectively (mean ± SE LU_{30}: SKA-IFN-γ 16.03 ± 1.98 vs. basal 11.37 ± 3.08, $p = 0.059$; vs. IFN-γ 18.71 ± 4.44, $p = 0.049$), while rIFN-γ-stimulated PB-NK cells effectively targeted CRC cells compared to resting effector cells (mean ± SE LU_{30}: SKA-IFN-γ 16.03 ± 1.98 vs. basal 11.37 ± 3.08, $p = 0.007$; IFN-γ 18.71 ± 4.44 vs. 11.37 ± 3.08, $p = 0.049$).

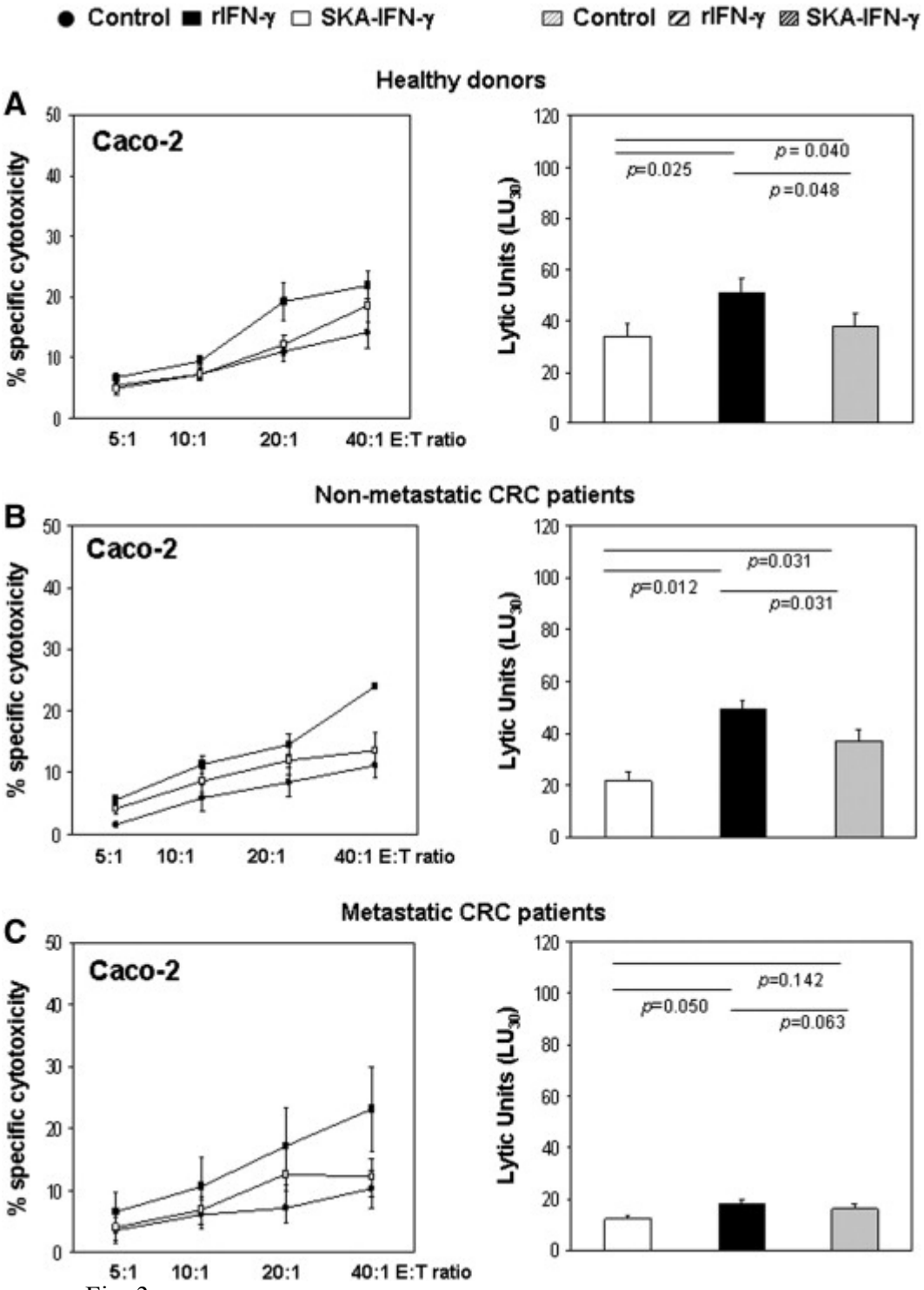

Fig. 3.

Effects of standard-dose rIFN-γ and low-dose SKA-IFN-γ on cytotoxic activity against Caco-2 cells of PB-NK cells from normal donors (n = 6) (A), from non-metastatic CRC patients (n = 5) (B), and from metastatic CRC patients (n = 5). PBL were untreated or pretreated with rIFN-γ (1 ng/ml) or SKA-IFN-γ (0.25 fg/ml) for 24 h. After washing, NK cytotoxic activity was tested at different E:T ratios by means of the 4-h ^{51}Cr-release assay. The figure shows the mean percentages ± SE of specific cytotoxicity at different E:T ratios (left panel) and the means ± SE of relative cytolytic potential expressed as LU_{30} (right panel). Percentage of specific cytotoxicity and LU_{30} were calculated as specified in the Materials and methods section.

p = statistical significance (Student's t test for paired data).

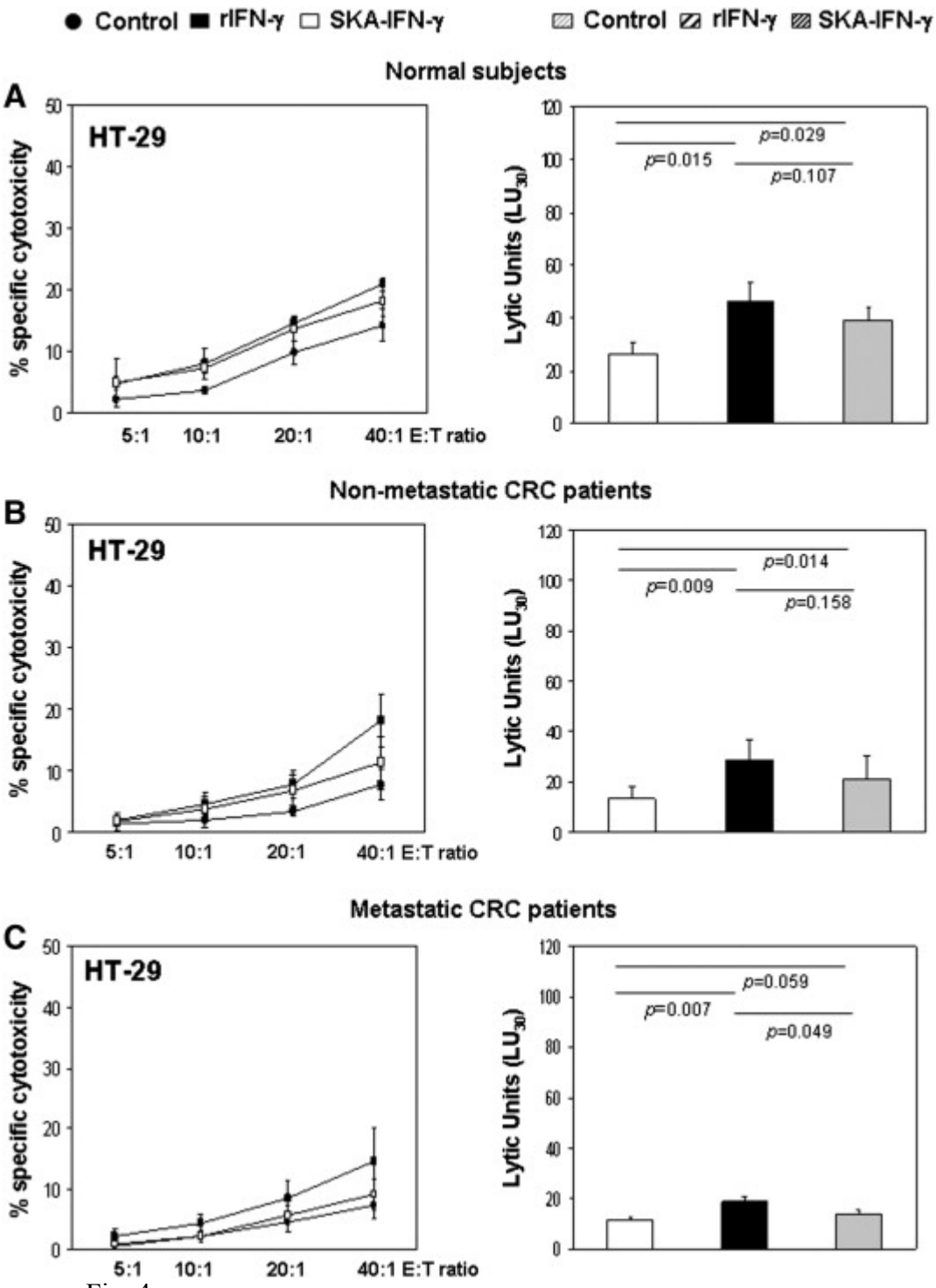

Fig. 4.

Effects of standard-dose rIFN-γ and low-dose SKA-IFN-γ on cytotoxic activity against HT-29 cells, of PB-NK cells from normal donors (n = 6) (A), from non-metastatic CRC patients (n = 5) (B), and from metastatic CRC patients (n = 5). PBL were untreated or pretreated with rIFN-γ (1 ng/ml) or SKA-IFN-γ (0.25 fg/ml) for 24 h. After washing, NK-cell cytotoxic activity was tested at different E:T ratios by means of the 4-h ^{51}Cr-release assay. The figure shows the mean percentages ± SE of specific cytotoxicity at different E:T ratios (left panel) and the means ± SE of relative cytolytic potential expressed as LU_{30} (right

panel). Percentages of specific cytotoxicity and LU_{30} were calculated as specified in the Materials and methods section.

p = statistical significance (Student's t test for paired data).

These results suggest that, at least in the early-stages of CRC, SKA-IFN-γ can help achieve a sustained systemic and/or in situ activation of NK cells, which may prove to be effective in controlling cancer growth.

4. Discussion

The generation of potent, specific, and durable anti-tumor immunity requires fine-tuned regulation of important functions, relating to the balance between tumor rejection by antigen-specific effector cells, on the one hand, and suppressive mechanisms that allow tumors to escape immunological detection, on the other hand. IFN-γ is a central pro-inflammatory cytokine, crucial for both innate and adaptive antitumor immune responses; however, increasing evidence indicates that, in local, unlimited, and irregular inflammatory conditions, it is also involved in the development of tumor immune escape, tolerance induction, and impaired immune functions. It exercises these latter activities by activating negative feedback loops. An example is the development of tolerance-inducing T-regulatory cells [31] and indoleamine 2,3-dioxygenase (IDO)-dependent suppression of CTL [32].

Conventional immunotherapy includes high-dose pulses of cytokines, to attract and activate cytotoxic effector cells. However, the toxicity inherent in systemic cytokine administration and cytokine-activated NK-cell apoptosis are two significant limitations upon cytokine-mediated (and NK-adoptive) immunotherapy for cancer treatment [33]. In particular, IFN-γ-based immunotherapy in CRC has shown very limited clinical utility, as well as severe dose-dependent side-effects that bear some resemblance to a state of overwhelming inflammation [19], [22] and [34] Moreover, a steady flow of reports has suggested that it may also have protumorigenic activities depending on the cellular, microenvironmental, and/or molecular context [35].

These disappointing antitumor effects are possibly attributable to the fact that, in clinical trials, the cytokine has been administered at doses and in schedules that come very close to the maximum tolerated dose. Therefore, additional investigations will be needed to fine-tune and select optimal IFN-γ preparation or dosage schemes.

In this explorative ex vivo study, we found that: i) unlike patients with more advanced disease, early-stage CRC patients preserve both PB-NK cell functional activity and IFN-γ responsiveness intact, and ii) of particular note, very low-doses of SKA-IFN-γ can increase the level of activation of PB-NK cells, significantly enhancing their ability to destroy not only the NK-sensitive K562 cells, but also the less susceptible CRC cells, Caco-2 and HT-29.

Since the potential tumor cell clearance capacity of NK cells is part of the mechanism of action of IFN-γ-based immunotherapy, the basal functional state of NK cells may critically influence both response to treatment and clinical outcome. Therefore, the finding that CRC patients who were free of metastatic spread, unlike metastatic CRC patients [36], displayed normal levels of NK cell activity might lead to these cells' being reconsidered as an interesting therapeutic target in these patients. Overall evidence suggests that basal levels of NK-cell cytotoxicity in cancer patients, including CRC patients, are significantly related to overall survival, progression-free survival, and response rate [37] and [38].

Since the [^{51}Cr]-labeled myelogenous leukemia cells K562 are the standard targets for studying NK cell-mediated cytotoxicity in vitro, in order to closely simulate the in-vivo tumoricidal activity of PB-NK cells from CRC patients, the two CRC cell lines, Caco-2 and HT-29, were also tested. In comparison to the K562 cell line, the susceptibility of both CRC cell lines to undergo spontaneous NK cell-induced killing (not only by NK cells from CRC patients, but also by those from normal donors) was significantly lower. Tumor cell recognition by NK cells is mediated by a fine-tuned balance of power between diverse activating and inhibitory receptors [39]. NK cells are efficient at eliminating cancer cells; these lack expression of either some or all self MHC class I molecules, which mediate inhibitory signals, or else they overexpress the natural killer cell group 2D (NKG2D) ligands, which engage with the activating immunoreceptor NKG2D. However, tumor cells, including CRC cells, can also display elaborate adaptations enabling them to evade and overcome NKG2D-mediated immune responses These adaptations include shedding NKG2D ligands, and secreting immunosuppressive cytokines, such as transforming growth factor (TGF)-β, in order to downregulate NKG2D expression [40]. CRC overexpress TGF-β in a tumor-associated manner [41]. It has been shown that neutralization of TGF-β enhances the antitumor response of NK cells, by causing an accumulation of IFN-γ producing NK cells with IFN-γ preservation of surface NKG2D [42].

Upon cytokine stimulation, NK cells become lymphokine-activated killer (LAK) cells. These produce cytokines and up-regulate effector molecules, such as adhesion molecules, NKp44, perforin, granzymes, Fas ligand (FasL), and TRAIL, leading to an enhanced and broader killing activity against tumor cells [43]. The two CRC cell lines tested in this study displayed decreased susceptibility to PB-NK cells, but not to a biologically-irrelevant extent, inasmuch as boosting the natural killing activity by rIFN-γ, at least in CRC patients free of metastasis, induced increased levels of cytotoxicity. PB-NK cells from metastatic CRC patients were little responsive to rIFN-γ activation, which is in line with the finding that PB-NK cells from late-stage CRC patients exhibited an altered phenotype, and showed profound defects in their ability to activate degranulation and IFN-γ production [44]. Reduced expression of NKG2D on the NK and T cells of cancer patients has also been reported [45].

In this connection, it should be considered that the modest clinical efficacy of activated NK cells in cancer therapy could be due to the fact that most NK-cell-based immunotherapy trials have enrolled heavily-tumor-burdened patients, for whom conventional therapies were ineffective [46].

The effectiveness of low-doses of cytokines has recently been investigated. The study found that very low-doses of solutions of SKA-IL-12 and -IFN-γ, co-delivered by the oral route to experimental asthmatic mice, can revert their pathological condition, restoring a normal balance between Th1 and Th2 cytokines and returning the animals to a healthy condition [25].

Interestingly, we found here that the ex-vivo administration of a low-dose of SKA-IFN-γ induced a significant increase in the cytotoxicity of PB-NK cells from non-metastatic, untreated CRC patients. This increase was only slightly lower than that observed with a standard dose of rIFN-γ, and the cytotoxicity occurred not only against the NK-sensitive K562 cells, but also against the less susceptible CRC cells Caco-2 and HT-29. Our data suggest that administration of IFN-γ at doses in the low-physiological range may be not only effective, but also likely safe, and that early-stage CRC patients might be promising candidates for this form of immunotherapy.

Increasing evidence demonstrates that pharmacological induction of antitumor immunity is rapidly counteracted by homeostatic regulation, and that repeated stimulation results in a progressive loss of therapeutic efficacy, due to increased suppressor activity and eventual immune exhaustion [47]. Because NK cells experience functional anergy or exhaustion, after serially killing of tumor cells,

high-dose cytokine administration to cancer patients, rather than stimulating their immune cells to more effectively kill tumor cells, may have the opposite effect, driving the lytic machinery into burnout and thus helping to explain the negative clinical results of IFN-γ-based immunotherapy [48]. Moreover, recent data have redefined the concept of chronic inflammation as a critical component in tumor progression. IFN-γ is one of the crucial factors involved in sustaining the inflammatory circuit [49].

It which can be administered,may be assumed that low-dose SKA-IFN-γ chronically over long periods, presumably without any deleterious side effects, can keep tumor growth under control by restoring and maintaining an effective immune response against tumor cells.

In vaccination and adoptive-cell strategies against cancer, cytokines play a critical role either ex vivo, to generate the cell populations used in vaccines and adoptive cell therapy, or in vivo, as adjuvants to these therapies, to augment the potency and duration of the anti-tumor response. Moreover, the use of low-dose cytokines in combination with an association of conventional cancer treatments may offer new therapeutic possibilities to counteract malignancies.

In conclusion, although the significance of the present ex vivo study is somewhat limited due to the small number of donor patients, nevertheless SKA-IFN-γ, by virtue of its biological activity at low-physiological-range doses, most certainly deserves further investigation, in view of its possible future in-vivo use as an innovative and safe cancer immunotherapeutic approach.

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