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Enhancement of the Immunostimulatory Functions of *Ex Vivo*–Generated Dendritic Cells from Early-Stage Colon Cancer Patients by Consecutive Exposure to Low Doses of Sequential-Kinetic-Activated IL-4 and IL-12. A Preliminary Study

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Abstract

Dendritic cells (DCs), specialized antigen-presenting cells bridging innate and adaptive immunity, play a crucial role in determining specific immune response to tumors. Because of their potent immunoregulatory capacities, DCs have been exploited in anticancer vaccination, with limited success thus far. This pilot study compared low-dose interleukin (IL)-4 and IL-12 prepared by sequential kinetic activation (SKA) with standard doses of the same recombinant human cytokines on functional activity of *ex vivo*–generated monocyte-derived (Mo) DCs from colon carcinoma patients and normal subjects. MoDCs were exposed to medium alone, SKA-IL-4 (0.5 fg/ml), or SKA-IL-12 (2 fg/ml), alone or consecutively combined, in parallel with rhIL-4 (50 ng/ml) and rhIL-12 (1 ng/ml). Primary allogeneic one-way mixed lymphocyte reaction (MLR) was the end point to assess *in vitro* T-lymphocyte proliferation in response to MoDCs, and secreted IL-12p70 and interferon-γ in MLR supernatants measured by ELISA to assay for T-helper 1–promoting MoDC phenotype. No single agent enhanced the compromised allostimulatory activity of MoDCs from colon cancer patients, unlike healthy donors. However, MoDCs from nonmetastatic colon cancer patients, after sequential exposure to SKA-IL-4 (48 hours) and SKA-IL-12 (24 hours), displayed increased T-cell stimulatory capacity by MLR and acquired driving T-helper 1 polarization activity, although less markedly than the effects induced by recombinant human cytokines or found in normal subjects. These results point to an immunomodulatory capacity of low-dose SKA-IL-4 and SKA-IL-12 and encourage further investigation to provide clues for the rational development of new and more effective immunotherapeutic strategies against cancer.

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Introduction

Dendritic cells (DCs) were originally considered to be the most potent professional antigen-presenting cells (APCs), which can uptake, process, and present different types of antigens to antigen-specific naïve T cells, linking the innate and adaptive immune systems [1]. However, recent work has established that DCs are a specialized group of APCs with high functional plasticity [2], which express immunostimulating or immunosuppressive potential, or both, depending on the consequence and combination

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of soluble and cellular microenvironmental stimuli affecting their differentiation, maturation, activation, and polarization [3].

Because of their ability to conduct all of the elements of the immune orchestra, DCs have long been considered a fundamental target and tool for cancer immunotherapy. Cancer cells are not immunologically silent: they can express a wide range of common tumor-associate antigens, which raise both $CD4^+$ and $CD8^+$ T cells [4]; DCs, which are found in most human tumors, can sample tumor antigens by capturing dying tumor cells and by "nibbling" live tumor cells [5].

DCs, generated *ex vivo* by culturing hematopoietic progenitor cells or monocytes (MoDCs) with cytokine combinations, have been under test as therapeutic vaccines in cancer patients for more than a decade [6]. Numerous studies, both preclinical experimental models and human clinical trials, have concluded that DC-based vaccines are safe and may induce expansion of circulating $CD4^+$ T cells and $CD8^+$ T cells, which are specific for tumor antigens. The clinical response takes time to build up, but remission can be very long-lasting [7]. However, the overall clinical success of cancer immunotherapy is rather low. Tumors usually return, escaping from the immune system by using a variety of mechanisms, including switching the differentiation of monocytes to macrophages rather than DCs [8]; inhibiting DC maturation by secreting interleukin (IL)-10 [9], which leads to antigen-specific anergy; or inducing "pro–tumor growth" DCs.

In an attempt to improve the efficacy and outcome of DC-based cancer vaccines in human cancer immunotherapy, the therapeutic use of DC cell activators, such as IL-12, IL-15, IL-18, IL-21, and interferons (IFNs), has been also 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 [10].

Despite advances in screening and preventative strategies, colorectal carcinoma (CRC) remains one of the leading causes of cancer-related death in the Western world [11]. Radical surgical resection of the primary colorectal lesions, combined with adjuvant chemotherapy and radiation, when indicated, still remains the mainstay of therapy. However, approximately 30% of patients are diagnosed with metastatic disease at initial presentation, and an additional 25% to 30% of patients will subsequently develop advanced disease, primarily with metastases to the liver and lungs. The median survival for all patients with metastatic CRC is approximately 22 to 24 months, with 5-year survival still <5% [12,13].

As with other tumors, immunotherapy also held great promise in the scenario of potential new approaches to the treatment of CRC refractory to conventional therapies. Although some clinical trials using DC vaccines to elicit antitumor immunity in patients with metastatic CRC were found to be safe and led to positive immunologic end points, clinical response only occurs in a minority of patients [14,15]. It might thus be of interest to investigate the functions of DCs in the tumor bed in the hope of "rewiring" protumor DCs to become antitumor DCs; this might lead to a novel approach to cancer immunotherapy.

Several lines of evidence suggest that low-dose cytokines are adequate for modulating the immune response in many different models [16]. Recent *in vitro* studies have shown that low doses of IL-12 modulate functional activities of T-cell subpopulations from non–small-cell lung cancer patients [17]. In particular, cytokines activated by the pharmaceutical preparation process known as "sequential kinetic activation" (SKA) have been found to retain their functional activities even at physiological low-dose concentrations both in a murine model of allergic asthma [18] and in an *ex vivo* study of the cytotoxicity of natural killer (NK) cells from CRC patients [19].

These findings encouraged us to investigate whether this preparation method might make relevant cytokines as active at low doses, in DC-based treatment of human cancer, as the high concentrations normally used in clinical pharmacology but without the side effects typical of high doses. This explorative study used *ex vivo*–generated MoDCs, from healthy donors and from colon carcinoma patients, to assess *in vitro* whether single or sequentially combined exposure to very low doses of SKA-IL-4 and/or SKA-IL-12 might enhance the DCs' antigen presentation capacity compared with the normally administered conventional dose of recombinant human (rh)IL-4 and rhIL-12.

Materials and Methods

Reagents

SKA-IL-4 and SKA-IL-12 were prepared by GUNA Laboratories (GUNA S.p.a, Milan, Italy) using a standardized method. Cytokines, sequentially diluted in saline solution (serial dilution 1:100), underwent a shaking process (vertical shaking; 10-cm motion range; shaking speed 100 oscillations over 10 seconds, kinetically energized by a mechanically applied force) [18]. The preparation was supplied at a concentration of 10^{-8} μg/ml. rhIL-4 and rhIL-12 were from PeproTech Inc. (Rocky Hill, NJ).

Patients

The study group comprised 16 patients (10 male, 6 female; median age, 73; range, 57-83) who had received a diagnosis of colon carcinoma from the Department of Surgical Medical Sciences at "Città della Salute e della Scienza" Hospital, Turin (Italy), between April 2011 and May 2013. Nine patients had histopathologically confirmed primary colon carcinoma and were staged by Dukes' system, revised by Astler and Coller (two Dukes' A and seven Dukes' B) [20]. Entry criteria were primary colon carcinoma indicative of surgery with no preoperative evidence of distant metastasis. Seven patients had histopathologically confirmed metastatic colon carcinoma (Dukes' C with lymph node metastasis). To avoid pharmacological and operative interferences that might alter DC activity, none of the patients had undergone surgical or other anticancer treatment at the time of blood sampling. A group of 12 healthy donors was used as controls (6 male, 5 female; median age, 64; range, 37-85). All subjects provided their informed consent before entering the study. The study procedures complied with the Helsinki Declaration.

Cell Isolation, DC Generation, and Treatments

Peripheral blood (PB) samples (15 ml) were collected in anticoagulant-coated tubes from colon carcinoma patients and healthy donors. PB mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque density gradient centrifugation. The cells were resuspended in PBS and 1% human albumin and positively selected with anti-CD14 monoclonal antibody–conjugated immunomagnetic beads and MACS Separation columns (Miltenyi Biotec GmbH, Germany) following the manufacturer's instructions. The resulting cells [>95% $CD14^+$ cells, determined by flow cytometric analysis (Coulter Epics XL; Beckman Coulter, Inc., Fullerton, CA)]. To generate MoDCs, $CD14^+$ cells were cultured at a concentration of 5×10^5 cells/ml in a 24-well tissue culture plate (Nunc, Roskilde,

Denmark) in RPMI-1640 medium supplemented with 10% fetal calf serum (Sigma Aldrich, St. Louis, MO). Rh granulocyte-macrophage colony stimulating factor (rhGM-CSF; 50 ng/ml) and rhIL-4 (20 ng/ml) (PeproTech) were added on the initial day of culture. Cultures were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 for 6 days. On day 3, one half of the culture medium was replaced with fresh medium containing growth factors. After differentiation, cells were harvested, washed, and used for subsequent experiments. To induce DC stimulation, two different approaches were used: 1) incubation of MoDCs with the previously determined optimal dose of rhIL-12 (1 ng/ml) (PeproTech) or with increasing concentrations of SKA-IL-12 (from 0.25 to 2 fg/ml) or 2) consecutive addition to MoDC cultures of a high dose of rhIL-4 (50 ng/ml) or SKA-IL-4 (0.5 fg/ml) for 48 hours, and SKA-IL-12 (2 fg/ml) or rhIL-12 (1 ng/ml) for a further 24 hours. After treatment, cells were harvested, washed, and used for subsequent experiments.

Primary Allogeneic One-Way Mixed Lymphocyte Reaction (MLR)

To determine functional activity of MoDCs, MLR was assayed. Briefly, MLR was assayed in 96-well round-bottom microtiter plates by adding graded numbers of irradiated (3000 rad) MoDCs (untreated or treated as reported above) to allogeneic naïve $CD4^+$ T cells (1×10^5) obtained with a $CD4^+$ isolation kit and subsequent negative selection in combination with anti-CD45RO monoclonal antibody plus goat anti-mouse IgG Ab-conjugated immunomagnetic beads (Dynal, Oslo, Norway) at 1:40, 1:20, and 1:10 stimulator (DCs)/responder (T cells) ratio. After 5 days of coculture at 37°C, T-cell proliferation was assessed by the uptake of [^{3}H]-thymidine (TdR) (1.25 μCi per well present for 6 hours; Perkin Elmer, Waltham, MA). The radioactivity incorporated into DNA was measured via β-scintillation counting (cpm = counts per minute). Each MLR culture was performed in triplicate

Generation of Culture Supernatants from MLR and Measurement of IL-12p70 and IFN-γ

MLR culture supernatants were collected before the addition of ^{3}H-TdR, centrifuged at 4°C to eliminate cells, and immediately stored at −80°C. Levels of IL-12p70 and IFN-γ were simultaneously measured using Bio-plex/Luminex technology (Bio-Rad, Veenendaal, the Netherlands). The range of detection sensitivity of the test was between 0.49 and 32.000 pg/ml.

Statistical Analysis

Differences between nonparametric data sets were examined by the Mann-Whitney test. Multiple group means were compared by one-way analysis of variance (ANOVA). The correlation between different parameters was determined with nonparametric correlation Spearman *R* coefficient (Sigmastat 3.1 software; Jandel Scientific, San Rafael, CA). Significance was set at $P < .05$.

Results

Basal MoDC Allostimulatory Activity Status in Patients with Colon Carcinoma and in Healthy Donors

It was first assessed whether MoDC cells from colon carcinoma patients ($n = 16$) retained stimulatory aptitude comparable to that of healthy-donor counterparts ($n = 12$). Notably, as shown in Figure 1*A*, $CD4^+$-naïve T lymphocytes after incubation with allogeneic MoDCs from colon carcinoma patients exhibited a significantly lower proliferative response (indicated by ^{3}H-TdR uptake) (almost 10 times less) than did those stimulated with normal MoDCs ($P \le .001$). Colon carcinoma patients thus demonstrated a marked defect in DC functional activity.

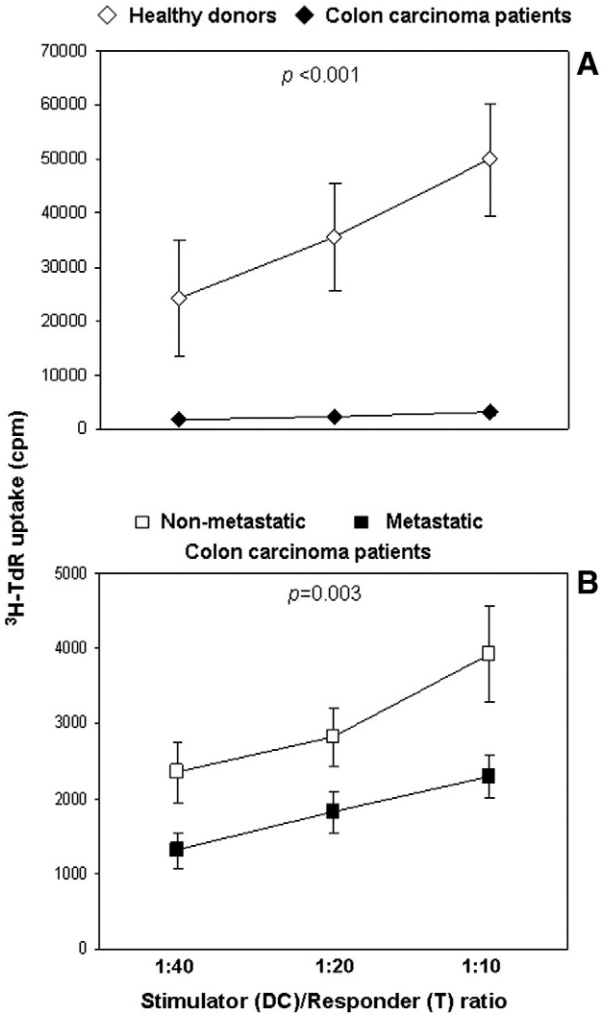

Figure 1. (A) MoDC allostimulatory activity of colon carcinoma patients ($n = 16$) versus healthy donors ($n = 12$). (B) MoDC allostimulatory activity of nonmetastatic ($n = 9$) versus metastatic ($n = 7$) colon carcinoma patients. MoDCs, generated by culturing PB $CD14^+$ cells from tumor and normal subjects in the presence of rhGM-CSF and rhIL-4 for 6 days, were incubated with 1×10^5 allogeneic naïve $CD4^+$ T cells at 1:40, 1:20, and 1:10 ratios for 5 days followed by a 6-hour pulse of ^{3}H-TdR. Results are expressed as mean ± SE cpm of triplicate co-cultures. Statistical significance was determined using one-way ANOVA.

Because a DC functional defect is an important component of the overall inability of the immune system to adequately respond to tumor challenge, patients were categorized by disease stage, and their MoDC allostimulatory capacity was compared and correlated (Figure 1*B*). MoDC-induced T-cell proliferation in the primary MLR assay was significantly lower in colon carcinoma patients with locally advanced disease (Dukes' C, $n = 7$) than it was in patients with early-stage colon carcinoma (Dukes' A + B, $n = 9$) ($P = .003$). Moreover, a significant correlation was found between antigen-presenting activity of DCs and Dukes' stage (at DC/T ratio = 1:40, $R = 0.533$, $P = .033$; at DC/T ratio 1:20, $R = 0.478$, $P = .0584$; at DC/T ratio 1:10, $R = 0.506$, $P = .0442$, Spearman correlation test).

Effect of Low-Dose SKA-IL-12 versus Standard-Dose rhIL-12 on MoDC Allostimulatory Activity in Colon Carcinoma Patients and in Healthy Donors

Inasmuch as immature MoDCs are no longer considered as vaccine candidates because of their low T-cell activation potential [21,22], most recent clinical trials used DCs activated by means of individual or cocktail cytokines associated with inflammation [23].

An exploratory study was thus run to investigate whether low-dose SKA-IL-12 might be a promising approach to manipulate MoDCs to elicit the optimal immune response for cancer therapy; the low dose was compared with standard-dose rhIL-12.

The efficacy of 24-hour treatment with increasing concentrations of SKA-IL-12 (0.25, 0.5, 1, and 2 fg/ml) or with the standard dose of rh-IL-12 (1 ng/ml) on allostimulatory activity of $CD14^+$-derived DCs from nonmetastatic colon carcinoma patients (n = 6) and healthy donors (n = 5) was evaluated preliminarily by the MLR assay. As shown in Figure 2*A*, when MoDCs were pretreated with rhIL-12 (1 ng/ml) before functional assay, the allostimulatory activity increased significantly in nonmetastatic colon carcinoma patients (P = .026), although there was a high variation in interindividual response; none of the concentrations of SKA-IL-12 used affected APC capacity. In contrast, in healthy donors, the highest concentration of SKA-IL-12 evaluated (2 fg/ml) significantly increased the allostimulatory activity of MoDCs in comparison with untreated MoDCs (P = .034) even if the effect of the standard dose of rhIL-12 was more marked (P = .001). These results suggest that MoDCs from nonmetastatic colon carcinoma patients were functionally poorly responsive or unresponsive to low-dose SKA-IL-12–mediated stimulation; this is presumably because of a defective expression of IL-12R complex, which nevertheless is sufficient for pharmacological doses of IL-12 to induce partial correction of MoDC allostimulatory activity.

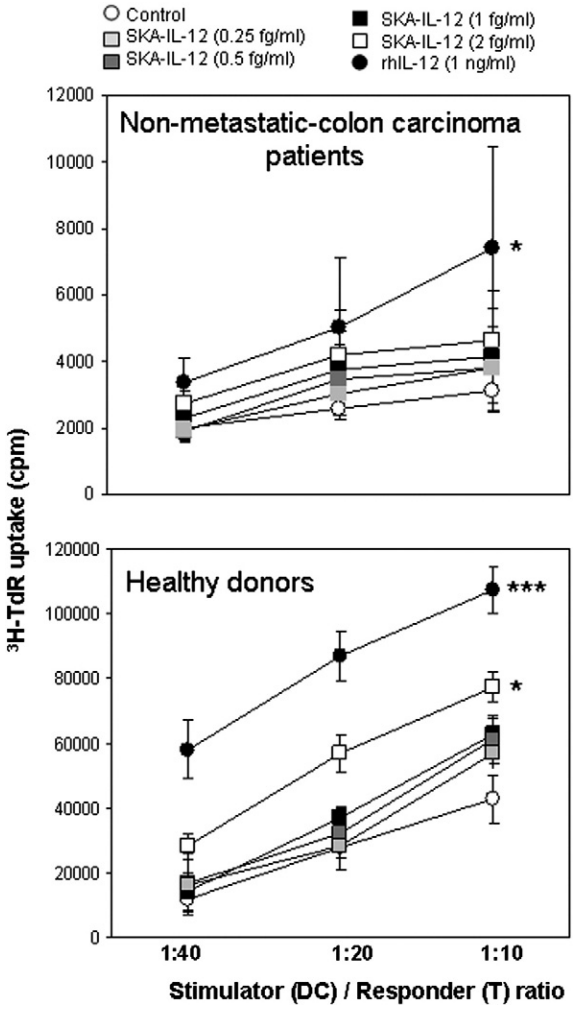

Figure 2. Effect of low-dose SKA-IL-12 versus standard-dose rhIL-12 on the MoDC allostimulatory activity of colon carcinoma patients and healthy donors. MoDCs from nonmetastatic colon carcinoma patients (n = 6) and healthy donors (n = 5) were untreated (control) or 24-hour–treated with increasing concentrations of rhIL-12 or SKA-IL-12 and then co-cultured with allogenic $CD4^+$ naïve cells (responders) at various stimulator-to-responder cell ratios in triplicate. Proliferative response was assessed by ^{3}H-TdR uptake. Results are expressed as mean ± SE cpm. Statistical significance was determined using one-way ANOVA. *P < .05, *P < .05, and ***P = .001.

Effect of SKA-IL-4 Pretreatment Followed by SKA-IL-12 Exposure versus the Standard Dose of rhIL-4 and rhIL-12 on MoDC Allostimulatory Activity in Colon Carcinoma Patients and in Healthy Donors

Consistent with findings that, in the same instance, paradoxically, IL-4 can influence DC differentiation into a DC1 phenotype that produces large amounts of IL-12 [24–27] and, in turn, can autocrinically regulate IL-12R expression in MoDCs [28], it was next examined whether the poor SKA-IL-12 response of DCs from colon carcinoma patients could be overcome by combined sequential treatment with SKA-IL-4 and SKA-IL-12. Therefore, MoDCs, generated in 6-day culture with rhIL-4 and rhGM-CSF from PB $CD14^+$ cells of colon carcinoma patients (n = 13, 6 nonmetastatic and 7 metastatic) and normal subjects (n = 6), were treated for a further 48 hours in the absence or presence of SKA-IL-4 (0.5 fg/ml) or rhIL-4 (50 ng/ml) alone, followed by 24-hour exposure to SKA-IL-12 (2 fg/ml) or rhIL-12 (1 ng/ml); they were then functionally assessed.

Figure 3 shows that, in colon carcinoma patients, following exposure to rhIL-4 or rhIL-12, MoDCs significantly increased their functional activity in comparison with untreated cells (P = .025 and P = .006, respectively). After sequential treatment with rhIL-4 (48 hours) and rhIL-12 (24 hours), MoDCs from tumor subjects became more potent MLR stimulatory cells than did untreated MoDCs (P < .001). By contrast, in MoDCs from patients, treatment with SKA cytokines alone did not significantly enhance their APC activity in MLR in comparison with untreated cells (SKA-IL-4: P = .172; SKA-IL-12: P = .178) even if, after sequential treatment with SKA-IL-4 (48 hours) and SKA-IL-12 (24 hours), MoDCs from tumor subjects became somewhat more potent MLR stimulatory cells than did untreated MoDCs, approaching statistical significance (P = .062).

When patients were subdivided into two groups based on disease stage (Figure 4), significant increases in APC activity in MLR of rhIL-4, rhIL-12, or rhIL-4 and rhIL-12 sequentially combined pretreated MoDCs from early-stage colon carcinoma patients (n = 6, Dukes' A and B) were observed in comparison with untreated cells (P = .034, P = .002, and P = .019, respectively). By contrast, antigen-presenting activity of MoDCs from colon carcinoma patients with metastatic lymph nodes (n = 7, Dukes' C) was enhanced only

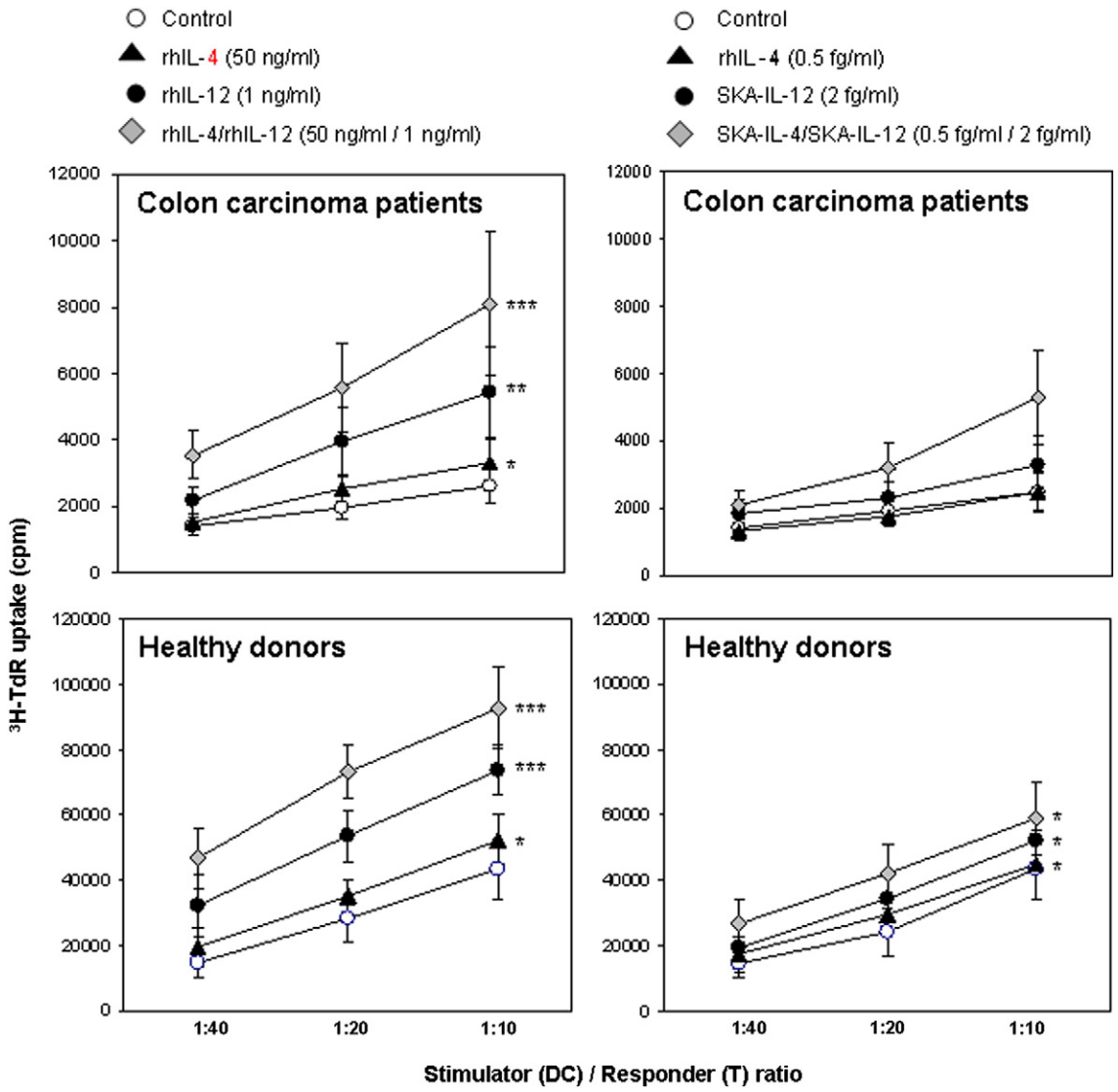

Figure 3. MoDCs sequentially stimulated with SKA-IL4 and SKA-IL-12 or rhIL-4 and rhIL-12 increased their capacity to initiate an allogenic response. MoDCs from colon carcinoma patients (n = 13, 6 nonmetastatic and 7 metastatic) or normal donors (n = 6) were generated from monocytes and exposed in the presence of predetermined concentrations of SKA-IL-4 (48 hours) and SKA-IL-12 (24 hours) as single agents or sequentially in parallel to the rh cytokines, and subjected to MLR with allogeneic naïve T cells in different MoDC-to-T cell ratios. MLR strength was measured by ^{3}H-TdR incorporation of the co-culture. The plot shows the mean ± SE of ^{3}H-TdR incorporation in cpm. Statistical significance versus control was determined using one-way ANOVA.*P < .05, **P < .01, and ***P < .001.

after exposure to rhIL-4 followed by rhIL-12 (P = .011). Moreover, MLR response levels in the presence of MoDCs pretreated with rhIL-4 and rhIL-12 as single agents, or with rhIL-4 and rhIL-12 in subsequent association, were significantly lower in colon carcinoma patients with metastatic lymph nodes than in those with locally extended tumors (Dukes' A + B, n = 6) (P = .003, P = .006, P = .004, and P = .023, respectively).

Interestingly, MoDCs from earlier-stage disease (Dukes' A + B) colon carcinoma patients, but not those from metastatic disease (Dukes' C), when instructed by sequential SKA-IL-4 and SKA-IL-12 exposure, promoted a significant proliferative response in allogeneic MLR in comparison with untreated cells (P < .001 and P = .076, respectively), although their functional recovery did not reach levels of those from normal subjects (P ≤ .001), whereas the same cytokines used singly did not affect functional activity of MoDCs in either group (SKA-IL-4: P = .099 and P = .054; SKA-IL-12: P = .149 and P = .185, respectively). When the two groups of patients were compared, the levels of MLR responses in the presence of untreated MoDCs, MoDCs pretreated with rhIL-4 or with rhIL-12 as single agents, or with rhIL-4 and rhIL-12 in subsequent association were significantly lower in colon carcinoma patients with metastatic lymph nodes compared with those with locally extended tumors (P = .004, P = .001, P = .001, and P < .001, respectively).

In normal donors (Figure 3), both single exposure to SKA-IL-4 (48 hours) and to SKA-IL-12 (24 hours) significantly increased MoDC MLR-stimulating capacity compared with basal condition (P = .035 and P = .026, respectively; right-hand panel), although the effect of the rh cytokines was stronger (approximately double) (P = .021 and P < .001, respectively; left-hand panel). Both SKA-IL-12 and rhIL-12 were more potent stimulators of MoDC functional

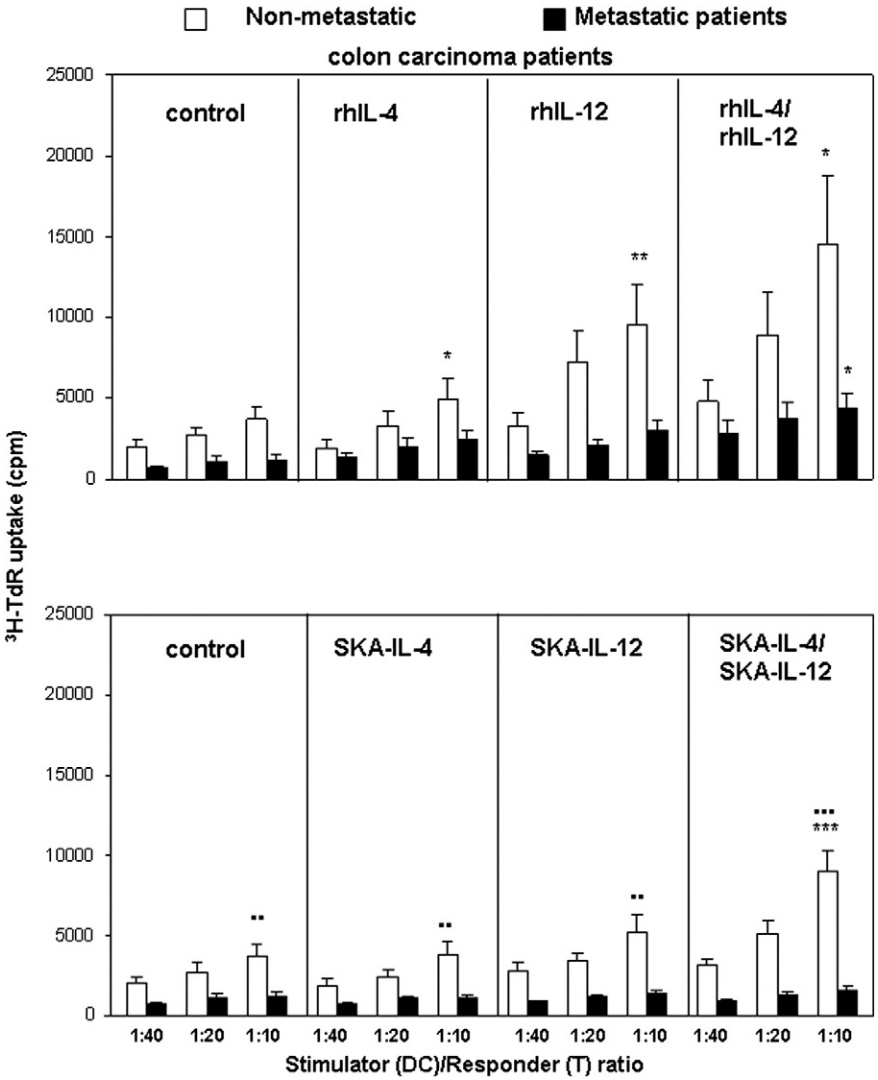

Figure 4. Effects of standard-dose rhIL-4 and/or rhIL-12 and low-dose SKA-IL-4 and/or SKA-IL-12 on APC activity in MLR of MoDCs from nonmetastatic colon carcinoma patients (n = 6) and from metastatic colon carcinoma patients (n = 7). MoDCs were untreated or pretreated with SKA-IL-4 (48 hours) and SKA-IL-12 (24 hours) as single agents or sequentially in parallel to the rh cytokines, and subjected to MLR with allogeneic naïve T cells in different MoDC-to-T cell ratios. The figure shows the mean percentages ± SE of ^{3}H-TdR incorporation in cpm. Statistical significance was determined using one-way ANOVA.Rh/SKA cytokine pretreated nonmetastatic/metastatic colon carcinoma MoDCs versus untreated nonmetastatic/metastatic colon carcinoma MoDCs: *P < .05 and **P < .01.Nonmetastatic colon carcinoma MoDCs versus metastatic colon carcinoma MoDCs: ••P < .01 and •••P < .0001.

activity than were SKA-IL-4 and rhIL-4, respectively (P = .042 and P = .010). When MoDCs from healthy donors were subsequently exposed to SKA-IL-4 (48 hours) and SKA-IL-12 (24 hours), a further significant increase in their APC activity in MLR was observed in comparison to untreated, SKA-IL-4–treated, or SKA-IL-12–treated MoDCs (P = .021, P < .001, and P < .001, respectively). However, sequential treatment with rhIL-4 and rh-IL-12 enhanced MoDC allostimulatory activity more markedly than with untreated or SKA-IL-4–/SKA-IL-12–treated cells (P < .001 and P = .002, respectively).

T Helper (h) 1 Cytokine Production

The question of the extent to which IL-4– and/or IL-12–treated MoDCs can actually induce T-helper (Th) 1 cell polarization was addressed by investigating the profile of cytokines released upon stimulation in allogeneic MLR. It should be stressed that the cytokines used for pretreating MoDCs were no longer present during the MLR assay and that cytokine levels reflect both T-cell and APC cytokine production.

As shown in Figure 5, normal MoDCs (n = 5) cultured in MLR with naïve CD4^{+} T cells did not produce any or at most produced negligible levels of biologically active IL-12p70 (0.313 ± 0.183 pg/ml), whereas rhIL-4–, rhIL-12–, but especially rhIL-4/IL-12–treated MoDCs secreted IL-12p70 in the pg/ml range (47.89 ± 20.82 pg/ml, 61.63 ± 22.39 pg/ml, and 205.39 ± 71.64 pg/ml; P vs untreated MoDCs = .041, .024, and .021, respectively). MoDCs conditioned by SKA-IL-4 slightly increased, though not significantly, IL-12p70 production in the supernatant of MLR culture in comparison with the control (23.46 ± 14.79 pg/ml, P = .111). Conversely, MoDCs treated with SKA-IL-12 alone or

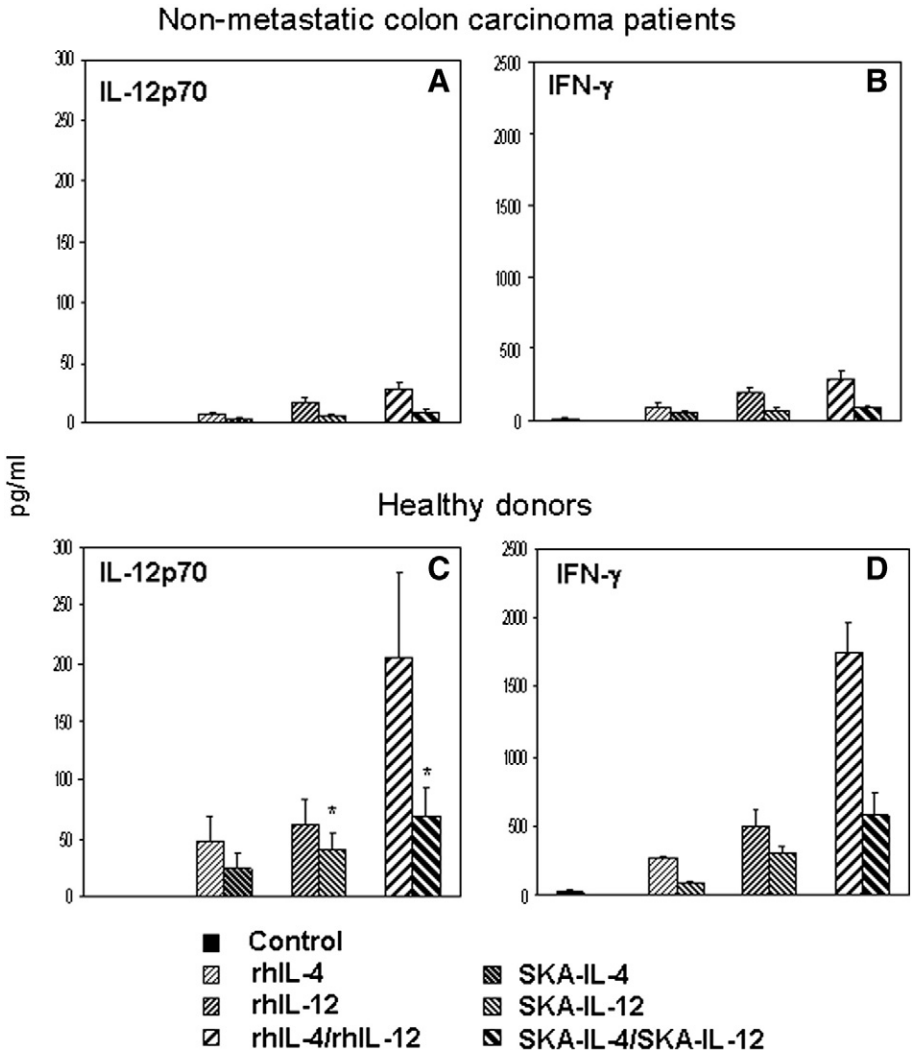

Figure 5. Effects of MoDC treatment with SKA-IL-4 and SKA- IL-12 alone or sequentially combined in priming Th1 response in comparison to rhIL-4 and rhIL-12. MLR was run by co-culturing untreated and SKA- or rh cytokine–treated MoDCs from normal subject ($n = 5$) and from nonmetastatic colon carcinoma patients ($n = 6$) (C and D) with naive allogeneic $CD4^+$ T cells at stimulator (DCs)/responder (allogeneic naive allogeneic $CD4^+$ T cells) ratio of 1:40. IL-12p70 and IFN-γ were measured in MLR supernatants by ELISA after 5 days of co-culture. Data are means ± SE pg/ml of duplicates. Statistical significance was determined using Mann-Whitney test. The *P* values are reported in the text.

sequentially combined SKA-IL-4/SKA-IL-12 enhanced IL-12p70 secretion (40.45 ± 14.85 pg/ml and 69.53 ± 23.59 pg/ml; *P* vs untreated MoDCs = .024 and .029, respectively), but less efficiently than the counterpart rh cytokine-exposed cells (p = 0.046, p = 0.025, respectively).

Though much less markedly than their normal counterparts, MoDCs from nonmetastatic colon carcinoma patients ($n = 5$, sole group examined) when exposed to rhIL-4 and rhIL-12 as single agent or to their sequential combination also acquired an enhanced ability to elaborate IL-12p70 into allogenic MLR compared with untreated cells (7.13 ± 1.85 pg/ml, 16.74 ± 3.68 pg/ml, 27.95 ± 5.16 pg/ml, 0.34 ± 0.22 pg/ml; *P* vs untreated MoDCs (0.183 pg/ml) = .018, .012, and .006, respectively). By contrast, MoDCs from metastasis-free tumor patients only significantly produced IL-12p70 after SKA-IL-4/SKA-IL-12 sequential treatment (8.80 ± 2.35 pg/ml; *P* vs untreated MoDCs = .028) because both SKA-IL-4 and SKA-IL-12 exposure induced a slight enhancement in IL-12p70 secretion, without reaching statistical significance (3.036 ± 1.46 pg/ml and 4.98 ± 2.51 pg/ml; *P* vs untreated MoDCs = .152 and .063, respectively).

Regarding the production of IFN-γ, MoDCs from healthy subjects, cultured in MLR with naive $CD4^+$ T cells, were poor inducers of IFN-γ secretion (28.56 ± 8.59 pg/ml). However, MoDCs conditioned by rhIL-4, rhIL-12, or rhIL-4/rhIL-12 induced production of IFN-γ by T cells (266.63 ± 16.11 pg/ml, 492.57 ± 118.01 pg/ml, and 1756.53 ± 204.43 pg/ml; *P* vs untreated MoDCs = .021, .029, and .004, respectively). The same cells exposed to SKA-IL-12 or sequential combination of SKA-IL-4/SKA-IL-12 (but not to SKA-IL-4 alone) were also able to induce T cells to secrete significant quantities of IFN-γ (302.71 ± 54 pg/ml and 578.35 ± 158.36 pg/ml; *P* vs untreated MoDCs = .011 and .044, respectively), although to a lesser extent (*P* vs rhIL-12– or rhIL-4/rhIL-12–treated MoDCs = .048 and .004, respectively).

MoDCs from nonmetastatic colon carcinoma patients recovered only partially, in comparison with normal donors, their capacity to induce IFN-γ production by naïve $CD4^+$ T cells during MLR when preexposed to rhIL-4, rhIL-12, or rhIL-4/rhIL-12 [95.45 ±

24.51 pg/ml, 194.95 ± 36.11 pg/ml, and 291.32 ± 46.71 pg/ml; *P* vs untreated MoDCs (11.52 ± 6.51 pg/ml) = .045, .027, and .003, respectively]. By contrast, exposure of tumor MoDCs to SKA cytokines had a small but statistically significant positive impact on IFN-γ secretion by T cells only in the presence of SKA-IL-12 or double SKA-IL-4/SKA-IL-12 (74.05 ± 12.20 pg/ml and 89.78 ± 15.74 pg/ml; *P* vs untreated MoDCs = .05 and .038, respectively), supporting the finding that MoDCs, when activated by sequential treatment with IL-4 and IL-12, can promote differentiation of naïve Th cells into IFN-γ–producing Th1 cells.

Discussion

The goals of cancer immunotherapy are to activate and expand tumor-specific $CD4^+$ and $CD8^+$ T cells as effective means of augmenting immunity and overcoming mechanisms used by tumors to evade destruction. To induce a robust antitumor immune response, peptides derived from tumor-associated antigens must be presented to T cells by professional APCs, such as DCs, if possible producing IL-12p70 because of their leading role in promoting Th1 cell polarization [29,30], their innate immunity through induction of NK cell proliferation, and release of IFN-γ [31].

Most DC vaccination studies have used either immature or mature DCs, which lack sufficient capacity to secrete biologically active IL-12p70 [32,33]. Conversely, *in vivo* manipulation of DCs by a single administration of IL-12 or by its administration in combination with different immunomodulatory cytokines, despite appearing very promising in some clinical trials [34], has unfortunately been associated with a severe degree of toxicity and with the generation of counterregulatory (i.e., immunosuppressive) measures that limit their overall usefulness as a cancer therapeutic [35].

Substantial debate still surrounds whether DCs can function as tumor therapy based on the outcome of numerous clinical studies on different malignancies, including CRC, the results of which have not been in line with initial expectations [32].

The question remains of how DCs can be functionally conditioned more effectively to express immunostimulatory cytokines (IL-12p70) and co-stimulatory molecules in parallel with antigen presentation to improve antitumor immune response. Additional investigation is thus needed to fine-tune this strategy, selecting optimal DC preparation and/or cytokine dosage schemes.

This explorative *ex vivo* study found 1) impaired *in vitro* generation of fully functional MoDCs from colon carcinoma patients; in particular, this defect was marked in MoCD from patients with more advanced disease, and 2) notably, the capacity of sequential exposure of MoDCs from early-stage colon carcinoma patients to very low doses of SKA-IL-4 and SKA-IL-12 to improve APC activity in allogeneic MLR, inducing slightly enhanced (but of potentially functional significance) IL-12p70 production and also Th1 polarization. These effects were evidenced by IFN-γ release in MRL supernatants.

Because potential tumor-cell clearance by specific cytotoxic $CD8^+$ T lymphocytes, in addition to NK cells, is part of the mechanism of action of DC-based immunotherapy, the basal functional state of DCs may critically influence both response to treatment and clinical outcome. The poor quality found in MoDCs generated *ex vivo* from colon carcinoma patients, further deteriorating at advanced stages of the disease (activity being approximately 1/10 that of MoDCs from normal subjects), suggests several limiting pathways that hamper the capacity of DCs in the tumor microenvironment. Deficiency in expression of co-stimulatory receptors, poor ability to stimulate T-cell responses, and altered cytokine secretion have been reported in DCs from cancer patients [36,37]. Moreover, in CRC patients, the defective function of DCs from blood precursors cannot be overcome by removing the tumor immunosuppressive factors, unlike DCs generated *ex vivo* from breast cancer patients, which were found to be fully functional [38].

Clinical evidence reports that these reduced DC functional basal levels are significantly related to overall survival, progression-free survival, and response rate [39–41]. Accordingly, the present results, showing that colon carcinoma exerts negative effects on DC generation and maturation, suggest a tumor-induced accumulation of immature cells, with inhibitory function and/or inability to deliver tumor antigens in a manner that renders them immunogenic to the host. Such dysfunction has significant implications for both the induction of natural antitumor immune responses and the efficacy of immunotherapeutic strategies that target endogenous DCs *in situ* or that use exogenous DCs as part of anticancer immunization maneuvers, and may explain why, to date, immunotherapy trials in CRC have failed to translate the immune response into an effective therapeutic outcome.

Emerging evidence points to a developmental and microenvironment-dependent plasticity of DCs: this is a heterogeneous cell population in terms of surface phenotype because the precursors themselves are not uniform. Distinct subsets of DCs have intrinsic differences that lead to functional specialization, playing significant roles in both induction of antitumor immunity and support of tumor growth and progression [42].

Because the available clinical data appear to show that stimulated MoDCs may provide greater therapeutic benefits versus immature MoDCs in the cancer setting [6,43–45], to optimize the utility of DCs in immunotherapy approaches, it is critical to determine properties, such as phenotype and function, that are correlated with clinical efficacy and to apply quality control for their presence.

IL-12 is a hallmark inflammatory cytokine capable of eliciting potent Th1 immune responses [29]. However, recent studies have provided strong indications of an autocrine activity of IL-12 on DCs; these have shown the constitutive expression of both IL-12 receptor (R) β1 and β2 chains on these cells [28,46] and a marked increase in this expression following cell activation [12,13]. Binding of IL-12 to its receptor induces a series of intracellular reactions involving the Jak-Stat signaling cascade; these reactions have a direct impact on MoDC functions, including enhanced IL-12 production, upregulation of the co-stimulatory molecule CD80, increased capacity to stimulate T-cell proliferation, and endogenous production of IFN-γ [47–49], which may affect the development of adaptive immunity [17,50]. The present assessment of rhIL-12 ponderal dose-pretreated MoDCs from nonmetastatic colon carcinoma patients' capacity to stimulate T-cell proliferation in MLR showed that there was a significant gain in functional activity, although it was more limited than that occurring in normal donors.

Because it has recently been found that the SKA pharmaceutical technique enables low doses of cytokines to achieve the same biological results as high doses but presumably without the related opposing and/or side effects [17–19], previous work testing the potential regulatory activity of SKA-IL-12 was extended to the APC capacity of MoDCs. MoDCs from nonmetastatic colon carcinoma patients were found to be unresponsive to SKA-IL12 stimulation or, at most, considerably less than MoDCs from

normal donors that, conversely, significantly upmodulated their functional ability after exposure to the highest of the low doses of the SKA cytokine (2 fg/ml) used.

This finding further supports the idea that different origins and environmental signals, produced by neighboring immune cells and by the tumor itself, may contribute to the induction of unique DC phenotypes in cancer patients, which will ultimately shape the nature of the immune response against the tumor [50]. It has been reported that tumor-infiltrating DCs isolated from CRC express reduced levels of co-stimulatory molecules (CD80 and CD86) and do not respond to cytokines (GM-CSF and tumor necrosis factor-α or CD40 ligand) that normally induce robust expression of these molecules [51].

The lack of SKA-IL-12 response in DCs from colon carcinoma patients might be due to insufficient IL-12R. It would be interesting to evaluate the basal and rh or SKA-IL-12–induced phenotype of MoDCs generated from this series of colon carcinoma patients. However, the frequency of DC precursors in the PB was low, and a blood sample of ethically acceptable size did not produce a sufficient number of cells for both phenotypic and functional studies.

It is clear that DC preparation designed to optimize IL-12p70 production (which presumably is associated with the potent Th1-skewing potential of DCs *in vivo*) [52] would be highly desirable to enhance the effectiveness of tumor immunotherapy. To date, the majority of DC vaccine studies have used either immature DCs (GM-CSF/IL-4) or partially matured DCs (GM-CSF/IL-4 plus activation by IL-1β, IL-6, tumor necrosis factor-α, and/or prostaglandin-2), which express co-stimulatory molecules but fail to produce IL-12p70 [53].

IL-4 is widely known for its role in Th2 cell polarization [54], but the regulatory roles of IL-4 in DC function have been studied in much less depth. Opposing effects on the development of DC-mediated immune responses, depending on the time of application and the concentration used, have emerged from recent studies. IL-4 induces DC maturation, upregulating expression of MHC class II molecules, co-stimulatory receptors, and IL-12Rβ1, which forms the functional high-affinity IL-12 receptor together with IL12Rβ2 [28,55]. Moreover, IL-4 can also enhance IL-12 production in DCs [56]: in particular, it has been reported that immature human DCs activated under high-dose IL-4 produce large amounts of IL-12 and small amounts of IL-10, thus preferentially inducing Th1 differentiation [27]. Based on these findings, it was decided to evaluate whether sequential exposure of MoDCs to IL-4 (48 hours) and IL-12 (24 hours) could be a new strategy to develop a predominant Th1 response, of clear interest in tumor therapy.

Upon sequential rhIL-4/rhIL-12 stimulation, MoDCs from normal donors displayed an increased ability (almost additive) not only to induce T-cell proliferation but also to produce higher levels of biologically active IL-12p70 and to promote IFN-γ release, as detected by ELISA in MLR supernatants, used as a surrogate to measure treatment potency and efficacy.

Interestingly, MoDCs from nonmetastatic colon carcinoma patients, consecutively exposed to rhIL-4/rhIL-12 before the phase of cognate APC–T-cell interactions, also significantly increased their allostimulatory and Th1-skewing potential, although to a lesser extent than with healthy subjects. More importantly, the same effects were observed when, instead of rh cytokines, SKA-IL-4 and SKA-IL-12 were used to activate MoDCs from both normal donors and nonmetastatic colon carcinoma patients. As expected, in nonmetastatic colon carcinoma patients, low-dose SKA cytokines were less effective but were still biologically relevant, inasmuch as preexposed MoDCs improved their functional responses in terms both of naïve $CD4^+$ T-cell allostimulation and of Th1 priming. In accordance with other reports [57], the IL-12p70 levels in MLR supernatants were below the threshold of detection in both patients and volunteers. The fact that MoDCs from nonmetastatic CRC patients, after sequential treatment with SKA-IL-4 and SKA-IL-12, can acquire the ability to elaborate IL-12p70 (at levels that are rather low, but active in inducing IFN-γ release) might, however, be clinically relevant because it could avoid DC exhaustion and tolerance while inducing a persistent and prolonged antitumor immune response (Figure 6).

Increasing evidence demonstrates that pharmacological induction of antitumor immunity is rapidly counteracted by homeostatic regulation, resulting in a progressive loss of therapeutic efficacy [58]. *Ex vivo* studies have shown that potent stimulation driving type-1 DC polarization, such as high doses of IL-12, induces high-level secretion of IL-12p70 over a narrow window of time, peaking after 8 to 12 hours and then returning to baseline. This phenomenon, referred to as "exhaustion," leads to the loss of DCs' capacity to prime Th1 immunity and to the generation of Th2-skewed immunity [59]. Moreover, in most patients, repeated injections of standard doses of IL-12, after initial stimulation of massive production of IFN-γ, led to an adaptive response and a progressive decline of IL-12–induced IFN-γ concentration in the blood [60], whereas an objective clinical response or disease stabilization may occur with the sustained production of IFN-γ [61].

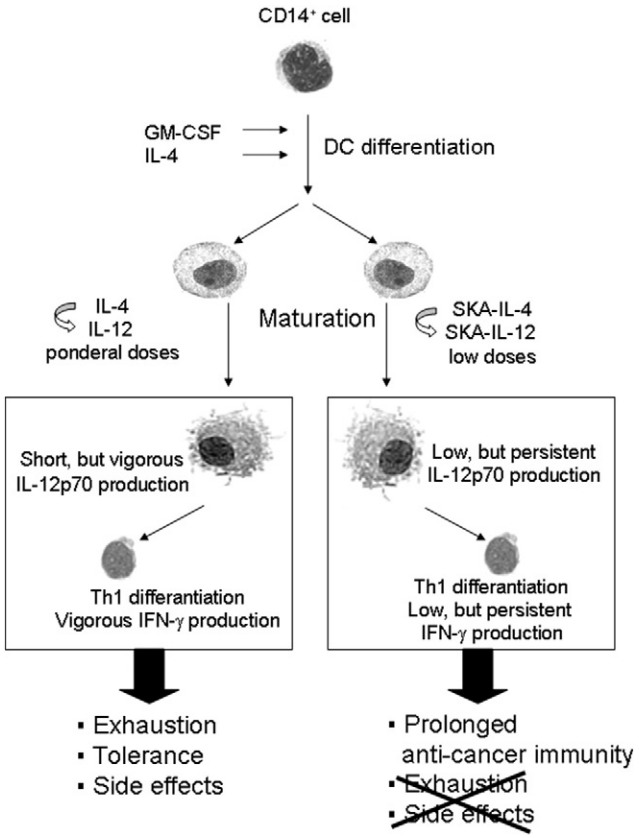

Figure 6. Schematic representation of the hypothetical mode of action and consequences of sequential exposure to low doses of SKA-IL-4 and SKA-IL-12 in comparison with ponderal doses of rhIL-4 and rhIL-12 on DC IL-12p70 secretory activity.

The mechanisms whereby DCs bridge innate and adaptive immunity involve a complex interplay between different cell types, cytokines, and recognition receptors. The two-way interactions between DCs and T cells initiate either an immunogenic or a tolerogenic pathway, both of which can play crucial roles in tumor immunity [62]. Tumors can mimic some of the signaling pathways of the immune system, thus propagating conditions that favor immune tolerance and escaping tumor immunity [63]. It has been shown, here and in other reports, that CRC cells can confer tolerogenic behavior on MoDCs by inducing phenotypic alterations and reducing the ability to stimulate T cells. However, because these defects persist *ex vivo*, this would imply not just a dependency on the local tumor milieu but also recruitment and accumulation by the tumor products of DC subsets in the blood stream; these products selectively promote deleterious mechanisms, such as inducing tolerance to tumor antigens [64] and/or proliferation of regulatory T cells that, in turn, prevent immune responses in a transforming growth factor-β–dependent manner [65].

Immunological or pharmacological therapy that might alter the proportion of conventional immunogenic versus regulatory DCs in the tumor environment could efficiently improve tumor-specific responses in cancer patients. In agreement with these concepts, the present study provides evidence that, by sequential exposure of MoDCs from nonmetastatic colon carcinoma patients to IL-4 and IL-12, it is possible to prime (IL-4) and to boost and maintain (IL-12) an antitumor Th1 response, taking advantage of each cytokines' biological functions. IL-4 plays a key role in instructing DCs to produce less IL-10, thereby favoring Th1 cell differentiation [66]. Bioactive IL-12 and IFN-γ are the critical cytokines initiating the downstream signaling cascade to develop Th1 cells [67].

DC-based immunotherapy is safe and can induce antitumor immunity even in patients with advanced disease. However, clinical responses have been disappointing, with classic objective tumor response rates rarely exceeding 15% [68]. Many clinical protocols using *ex vivo*–generated DC-based vaccines do not consider the fact that DCs administered to patients with cancer might quickly lose their activity in the cancer environment. Moreover, DCs that secrete high levels of IL-12, and thus induce Th1 polarization, are capable of producing IL-12 for only a short time [59], after which they exhaust their ability to produce IL-12 and subsequently activate proliferating T cells toward either a Th2 response or a regulatory T cell response.

Severe side effects associated with the systemic administration of IL-12p70, together with its very narrow therapeutic index, have hindered its wider incorporation into investigational cancer vaccine formulations [69]. Moreover, 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 immune machinery into burnout: this might partially explain the negative clinical results of cytokine-based immunotherapy [58]. Notably, and for the first time, the findings reported here provide evidence that *ex vivo* sequential pretreatment with low-dose SKA-IL-4 and SKA-IL-12 can induce, at least in nonmetastatic colon cancer patients, an improvement of MoDCs' ability to stimulate naïve $CD4^+$ cell proliferation and IFN-γ production in MLR.

In DC-based vaccination against cancer, cytokines play a critical role both *ex vivo*, to generate the cell populations used in vaccines, and *in vivo*, as adjuvants to these therapies, to augment the potency and duration of the antitumor response. It may be assumed that low doses of these SKA cytokines, which can be administered chronically over long periods without any deleterious side effects [70], could keep tumor growth under control by restoring and maintaining an effective immune response against tumor cells.

Although the significance of the present *ex vivo* study is somewhat limited because of the small number of donor patients, it is nevertheless indicative. SKA-IL-4 and SKA-IL-12, by virtue of their biological activities at low-physiological-range doses, most certainly deserve further investigation.

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