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Cancer-Initiating Cells from Colorectal Cancer Patients Escape from T Cell–Mediated Immunosurveillance In Vitro through Membrane-Bound IL-4

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Cancer-initiating cells (CICs) that are responsible for tumor initiation, propagation, and resistance to standard therapies have been isolated from human solid tumors, including colorectal cancer (CRC). The aim of this study was to obtain an immunological profile of CRC-derived CICs and to identify CIC-associated target molecules for T cell immunotherapy. We have isolated cells with CIC properties along with their putative non-CIC autologous counterparts from human primary CRC tissues. These CICs have been shown to display "tumor-initiating/stemness" properties, including the expression of CIC-associated markers (e.g., CD44, CD24, ALDH-1, EpCAM, Lgr5), multipotency, and tumorigenicity following injection in immunodeficient mice. The immune profile of these cells was assessed by phenotype analysis and by in vitro stimulation of PBMCs with CICs as a source of Ags. CICs, compared with non-CIC counterparts, showed weak immunogenicity. This feature correlated with the expression of high levels of immunomodulatory molecules, such as IL-4, and with CIC-mediated inhibitory activity for anti-tumor T cell responses. CIC-associated IL-4 was found to be responsible for this negative function, which requires cell-to-cell contact with T lymphocytes and which is impaired by blocking IL-4 signaling. In addition, the CRC-associated Ag COA-1 was found to be expressed by CICs and to represent, in an autologous setting, a target molecule for anti-tumor T cells. Our study provides relevant information that may contribute to designing new immunotherapy protocols to target CICs in CRC patients. *The Journal of Immunology*, 2014, 192: 523–532.

R ecent studies have shown that human solid tumors, including colorectal cancer (CRC), include, among the heterogeneous cell populations, a small subset of cells defined as cancer-initiating cells (CICs) (1–3). This cell subpopulation can self-renew; can generate cells that exhibit diverse degrees of differentiation; and, upon transplantation into immunodeficient mice, can give rise to tumors that resemble those of origin. CICs are believed to be responsible for tumor initiation,

progression, and resistance to therapeutic agents (4–6); therefore, treatments aimed at blocking/destroying CICs should be pursued.

Despite the advent of new diagnostic tools and targeted molecule agents, most CRC patients die of metastatic disease and almost half of patients receive chemotherapy without clinical benefit. Along this line, immunotherapy based on antitumor-specific T cells may represent a promising treatment, as it may induce the killing of CICs that remain after surgery and that are resistant to standard therapies.

The majority of immunotherapy protocols carried out in CRC patients are based on vaccination with defined Ags loaded or not on dendritic cells that, despite early promising results, has led to limited clinical benefit (7). Thus an improvement in this strategy is urgently needed. A possible explanation for these limited clinical results may lie in the failure to target CICs, a hypothesis corroborated by the evidence that an increase in "stemness" profile was obtained in postvaccinated glioblastoma patients and following antitumor T cell activity (8, 9). We have previously characterized the immune profile of CICs isolated from glioblastoma multiforme (GBM) tissues and found low immunogenicity (10). Moreover, a preferential induction in vitro of Th2-type responses occurred following the coculture of CICs with autologous PBMCs (10). Cancer-testis Ags have been found to be expressed by mesenchymal stem cells and to regulate the epithelial-to-mesenchymal transition, a process involved in development of metastases; thus these Ags may possibly have a role in the stemness properties of cancer cells and could represent target molecules for immunotherapy (11). Nevertheless, antitumor immunity could be attenuated by tumor-related immunosuppressive mechanisms that have been described in association with CICs isolated from melanoma or GBM (12–14). These findings suggest

that a detailed immunological characterization of CICs isolated from different human tumors is a relevant issue to design new CIC-targeted immunotherapeutic interventions. Of interest, we found that the CRC self Ag COA-1, encoded by the UBXD5 gene, was expressed at high levels by both CICs and FBS tumor cells. The peculiarity of this Ag is that it is recognized by T cells preferentially on tumor cells and not on normal cells, suggesting that a differential processing of this Ag can occur in these cells (15, 16). Although biological characterization of this Ag still needs to be accomplished, our group demonstrated that COA-1 can elicit specific T cell responses in cancer patients and not in healthy donors (16). Indeed, among different tumor Ags tested, COA-1 was found to represent a candidate target molecule for CRC CIC-targeted immunotherapy studies.

Materials and Methods

Tissues and cells

Tumor samples and PBMCs were obtained from patients with a diagnosis of primary CRC admitted for surgery at the San Raffaele Hospital, Milan, Italy. The clinical characteristics of these patients are reported in Supplemental Table I. The Institutional Review Board and the Ethics Committee of the San Raffaele Hospital approved this study and an informed consent was obtained from the participating subjects.

The MHC class I and class II typing of the patients was performed on their PBMCs by single-stranded oligonucleotide probe–PCR typing (10).

CICs were isolated in vitro by the mechanical processing of primary tumor tissues (#1076, 1247, 111011, and 14583) and were cultured in the form of spheres (colon-spheres) in the presence of stem cell–permissive medium (DMEM/F12) containing 20 ng/ml epidermal growth factor and 50 ng/ml fibroblast growth factor 2 (PeproTech, Rocky Hill, NJ), as previously described (17). Part of the primary cell dissociation of CRC tissues was cultured in the presence of RPMI 1640 supplemented with 10% FBS (Biowittaker, Lonza, Treviglio, Italy), hereafter denominated FBS tumor cells (#1076, 1247, 111011, 1039, and 20299), and representing the differentiated tumor cell counterparts, as previously described (10, 17, 18); these cell lines were used in parallel with CICs. Additional CIC lines (#1, 2, and 3) isolated from primary CRC patients have been previously characterized (19). Other cell lines used in this study were as follows: the CRC 1869 col, 1872 col (15), and SW480 (American Type Culture Collection); and the 1869 B cells immortalized with EBV (1869 EBV-B) (15). These cell lines were cultured in vitro with RPMI 1640 plus 10% FBS.

Monoclonal Abs used for immunofluorescence analysis

The expression of CIC- or CRC-associated markers has been evaluated by immunofluorescence (IF) and cytofluorimetric analysis using the following Abs: anti-EpCAM VU-1D9 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-HCAM MEM-85 (Santa Cruz Biotechnology); anti-CD24 SN3 (Novus Biological, Littleton, CO) or 528807 (R&D Systems, Minneapolis, MN); anti-Myc 3C118 (Santa Cruz Biotechnology); anti-CD133 293C3 (Miltenyi Biotec, Bergisch Gladbach, Germany); anti-CEA Col1 (Santa Cruz Biotechnology); anti-SOX2 245610 (R&D Systems); anti-S100A4 1F12-1G7, anti-S100A6 6D1 (Abnova, Taipei City, Taiwan); anti-Oct-3/4 40/Oct3 (Becton Dickinson Pharmingen, Palo Alto, CA); anti-Nanog M55-312 (BD Pharmingen) and anti-Lgr5 4D11F8 (BD Pharmingen).

MHC class I and class II HLA expression by CIC and FBS tumor cell lines was determined by IF and flow cytometry analysis using the mAbs W6/32 and L243 (BD, Pharmingen), respectively. The expression of Ag-processing machinery (APM) and MICA, MICB, and ULBPs molecules by these cell lines was determined using the previously described purified Abs provided by one of us (S.F.) and by Amgen (Thousand Oaks, CA), respectively (10). The expression of immunoregulatory molecules or cytokines and their receptors was assessed using the following Abs: anti–IL-4 3007 and anti–IL-4R 25463 (R&D Systems). Furthermore, tumor-associated Ag (TAA) expression by both CICs and FBS tumor cells was determined by the use of anti–NY-ESO-1 E978 (Zymed Laboratories, San Francisco, CA), anti–IL-13R (2 B-D13; Santa Cruz Biotechnology) mAbs, and anti-MAGE 57B and 6C1 kindly provided by Dr. G. Spagnoli (Institute of Surgical Research and Hospital Management, Department of Biomedicine, University Hospital of Basel, Basel, Switzerland). The PE-conjugated goat anti-mouse IgGs (DAKO, Glostrup, Denmark) were used for fluorochrome staining of the Abs used. The flow cytometry analysis was performed by the FACSCanto HTS instrument (Becton Dickinson).

The ALDEFLUOR staining kit (STEMCELL Technologies, Vancouver, BC, Canada) was used for aldehyde dehydrogenase (ALDH-1) detection; briefly, the fluorescent aldefluor reagent, which is a substrate for ALDH-1, is used for staining of cells and diffuses into cells. The amount of fluorescent ALDH-1 reaction product accumulated in the cells directly correlates to the ALDH-1 activity and was determined by standard cytofluorimetric analysis.

The phenotype characterization of T cell lines was carried out by multicolor IF and cytofluorimetric analysis (LSR II Fortessa; BD) with fluorochrome-conjugated anti-CD3, -CD4, -CD8, CD16, -CD56, -CD57, -CD28, -CD27, -CD45RA, -CD45RO, -CCR7, -CD25, -CD127, -CD134, -CD137 (BD, Pharmingen), and –NKG2D 1D11 (eBioscience, San Diego, CA) mAbs. Results are expressed as mean ratio of fluorescence intensity (MRFI), representing the ratio between the mean fluorescence intensity of cells stained with the selected mAb and that of cells stained with the negative Ab control (10) or, for the multiparametric lymphocyte phenotype analysis, as percentage of positive cells.

In vitro differentiation assay

CICs cultured in vitro as serum-free–derived spheres, following mechanical dissociation of the spheres to single cells by extensive pipetting (17), were plated in six-well culture plates in the presence of DMEM plus 5% of FBS, representing the differentiating agent, as previously described by Vermeulen and coworkers (20) for 7–10 d to induce the cells to acquire the adherence-dependent growth and the differentiated phenotype.

Confocal microscopy analysis

CRC-derived CICs and FBS tumor cells were cultured ($3–5 \times 10^4$ cells per well) onto cover glasses precoated with Matrigel in 24-well plates in culture medium. Cells were fixed onto glass either with methanol for 10 min at $-20°C$ or with 4% paraformaldehyde for 20 min at room temperature, washed with PBS, and then treated with PBS plus 0.1% Triton for 10 min. Afterward, cells were incubated for 30 min at $25°C$ with the following primary Abs: anti-Survivin 8E2 (Thermo Scientific, Fremont, CA); anti-MAGE 57B and 6C1, kindly provided by Dr. G. Spagnoli (Institute of Surgical Research and Hospital Management, Department of Biomedicine, University Hospital of Basel, Switzerland); and anti–COA-1 polyclonal Ab (ProteinExpert, Grenoble, France). The bound Abs were visualized with goat anti-mouse IgG Alexa Fluor 594 (Molecular Probes, Eugene, OR) or the Alexa Fluor 488–conjugated goat anti-mouse IgG F(ab')$_2$ fragment (Molecular Probes). Glasses were extensively rinsed with PBS and then mounted on microscope slides with VECTASHIELD antifade medium containing DAPI (Vector Laboratories, Burlingame, CA). Confocal laser-scanning microscopy (Leica Microsystems, Milan, Italy) analysis was then carried out. Multiple cells were analyzed for each staining condition, with $\times20$, $\times60$, and $\times100$ magnifications.

In vivo tumorigenicity

Tumorigenicity of CICs was determined by s.c. transplantation of cells into adult (8-wk-old) NOD/SCID CB-17 mice (Charles River Laboratories, Calco, Italy). Mice were maintained according to the animal care committee guidelines of the San Raffaele Foundation Centre. A total of 1×10^5 cells of either CICs or FBS tumor cells suspended in 100 µl physiological solution mixed at 1:1 ratio with Matrigel-containing medium (DMEM with 1% penicillin/streptomycin) were injected s.c. into the flank of NOD/SCID CB-17 mice. Mice were monitored every 3 d, and tumor formation was determined by caliper measurement. After 3–6 wk, visible tumors arose and their volumes (V) were determined using the following formula: $V = (a \times b^2)/2$, with a = length and b = width. When the tumor size reached 1.5–2 cm^3, the mice were sacrificed and tumor tissues were processed either for morphological and immunohistochemical analysis or for in vitro culture.

For serial dilution experiments, 1×10^5, 1×10^4, 1×10^3, or 1×10^2 cells of either CICs or FBS tumor cell suspensions were injected into NOD/SCID CB-17 mice, as described above.

Xenograft tissues obtained by the injection of 1×10^5 cells of either CICs or FBS tumor cells were mechanically processed and the cell suspensions cultured in vitro with the permissive CIC growth medium or RPMI 1640 plus 10% FBS, respectively. Then, serial cell transplantations were performed by injecting 1×10^5 cells into secondary NOD/SCID CB-17 mice. Tumor volumes were monitored weekly and compared with those of the first cell transplantation. Three mice for each group were used for the in vivo tumorigenic assays, and each experiment was repeated three times.

Statistical analysis of differences between tumor volumes was performed using the two-tailed t test ($p < 0.05$).

Detection of cytokine and soluble factors released by CRC cells

CRC CICs and FBS tumor cell lines (2×10^5/ml) were cultured in DMEM (Biowittaker, Lonza, Basel, Switzerland), without growth factors or FBS, for 48 h at 37°C; then supernatants were collected, centrifuged at 3000 rpm for 15 min, and stored at −80°C. TGFβ-1 and TGFβ-2 released by CRC cells were detected by ELISA (DRG Instruments, Marburg, Germany). PGE$_2$ detection in these supernatants was performed using the specific EIA Kit (Cayman Chemical, Ann Arbor, MI). Moreover, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and TNF-α were determined in the supernatants by SearchLight Array Technology (Thermo Fisher Scientific Pierce Protein, Rockford, IL). Data were represented as picograms per milliliter, and the significance of the differences of soluble factor release by CRC cell lines was determined by the two-tailed *t* test ($p < 0.05$).

Cell division analysis by CFSE staining

PBMCs (1×10^6 cells) isolated from CRC patients or from healthy donors were labeled with 1 μM CFSE (Molecular Probes, Invitrogen) for 8 min at 37°C. The labeled cells were stimulated with 10 μg/ml PHA plus Con A (Sigma-Aldrich, Milan, Italy) in the presence or absence of irradiated (300 Gy) autologous CICs or FBS tumor cells with either anti–IL-4 or anti–IL-4R mAbs or their combination. The homeostatic cytokine recombinant human (rh) IL-7 (5 ng/ml) (PeproTech) was added to all different cultures. Mixed lymphocyte tumor cell (MLTC)–derived T cells were also assessed for cell division after coculture or not with autologous CICs or FBS tumor cells and in the presence or not of anti–IL-4 or –IL-4R mAb. After 72 h of stimulation, cells were stained with CD3-Pacific Blue (Becton Dickinson) and were acquired with the LSR II Fortessa cytofluorimeter (Becton Dickinson); data for CD3$^+$ gated cells were analyzed with FCS Express 4 software (De Novo Software, Los Angeles, CA).

For the Transwell experiments, 24-well Transwell plates with a 0.4-μm pore membrane (Costar, Corning, NY) were used to separate the PBMCs from the CRC CICs. PBMCs stained with CFSE were plated with PHA/Con A into the lower chamber at 1×10^6 cells per well, and CICs (1×10^5 cells per well) were cultured in the upper chamber of the Transwell insert in the presence or not of anti–IL-4 mAb. After 3 d of coculture, the PBMCs were analyzed for proliferation, using flow cytometry as described above. CFSE-stained PBMCs, with or without PHA/Con A, were used as positive or negative controls, respectively. Data represent the mean of the proliferation index, that is, the total number of divisions divided by the number of cells that went into division, for each tumor cell line from three independent experiments or, where indicated, the percentage of divided cells. The efficiency of the neutralization of IL-4 and IL-4R by mAbs has been determined by evaluating the proliferation rate of PBMCs cultured in vitro for 5 d in the presence of 100 IU IL-2 and 10 ng/ml IL-4 (PeproTech) (Table II). Then PBMCs were washed, and a 3-d CFSE staining assay was performed as described above.

Isolation of CRC-reactive T lymphocytes

PBMCs from the peripheral blood of CRC patients (#1076, 1247, and 14583) were cultured in vitro in the presence of autologous irradiated (300 Gy) colorectal CICs or, when available, FBS tumor cells, pretreated or not with rhIFN-γ (1000 IU/ml; PeproTech), with 100 IU/ml rhIL-2 (Chiron Corporation, Emeryville, CA) and 10 ng/ml rhIL-7 (PeproTech) in X-VIVO-15 (Cambrex, Lonza, Basel, Switzerland) plus 5% human serum. Moreover, in some of these cultures 10 μg/ml of either the neutralizing anti–IL-4 3007 mAb or the anti-IL-4R 25463 mAbs (R&D Systems) was added simultaneously with the stimulating tumor cells. Cell cultures were restimulated weekly with irradiated autologous CICs or FBS tumor cells in the presence or not of anti–IL-4 or anti-IL-4R mAbs; starting from the third week of culture, the reactivity of the T lymphocytes against colorectal CIC or FBS tumor cell lines was determined by IFN-γ secretion, measured by ELISPOT assay as previously described (10). The T cells ($1–4 \times 10^4$ cells per well) were incubated in flat-bottom 96-well plates in the presence of 1.7×10^4 cells per well of CRC cell lines or K562 or EBV-B cells. The specificity of T lymphocyte recognition was assessed by inhibition of cytokine release after preincubation of the target cells with 10 μg/ml each of the anti-HLA class I mAb W6/32 and the anti-HLA class II (DR) mAb L243. T lymphocytes incubated with mitogens, such as Con A or PHA, were the positive controls. Unstimulated T lymphocytes represented the negative control. Results represent averages of triplicates with SD ≤ 10%; statistical analysis of differences between means for cytokine release assays was performed using the two-tailed *t* test ($p < 0.05$). These experiments have been repeated three times, showing consistency of results.

Results

Isolation and biological characterization of CICs from CRC tissues

Spheroid cell cultures, known to be enriched for CICs (1–3), were isolated and propagated in vitro by sphere-forming assay from seven primary CRC surgical specimens (10, 19). In three of seven cases, adherent growing tumor cells were also established in the presence of FBS (FBS tumor cells). This allowed us to isolate two major subpopulations of tumor cells: those with cancer-initiating/stemness properties (Supplemental Fig. 1A) and the bulk of differentiated tumor counterparts (Supplemental Fig. 1C), as previously described by a few groups, including ours, in the GBM model (10, 18, 20).

Initially, by analyzing the expression of Nanog, OCT-4, Sox2, ALDH-1/Aldefluor, CD133, CD44, CD24, Ep-CAM and CEA, we determined that the phenotype of these spheroid cell lines was concordant with that previously described for CRC-derived CICs (1–3, 21–23), as shown by the representative results of the #1076 CICs and FBS tumor cell pairs (Supplemental Fig. 2A). Indeed, we demonstrated that CRC-derived CIC-associated markers, such as CD44, CD24, EpCAM, and CEA, were significantly (*t* test $p < 0.01$) upregulated in all CICs versus FBS tumor cells (Supplemental Fig. 2A). By cell sorting we could select from both CICs and FBS tumors the cell subpopulations with different levels, although the range percentage of positive cells was 82–99% of MRFI of ALDH-1 expression (representative data of #1076 cell lines are shown in Supplemental Fig. 2B). ALDH-1-high and ALDH-1-low cell populations were isolated from CICs (MRFI = 12 and 7, respectively; Supplemental Fig. 2B), whereas from FBS tumor cells only ALDH-1–low and ALDH-1-negative cells (MRFI = 3.7 and 1, Fig. 1B) could be obtained. Moreover, high levels of CD24, CD44, CD133, Ep-CAM, and CEA were found in association with ALDH-1 expression (Supplemental Fig. 2B). Thus, we confirmed that ALDH-1 expression is upregulated and correlated with the expression of CIC-associated markers in our spheroid cell cultures compared with FBS tumor cells. Moreover, the sphere-forming cells homogeneously expressed (85–98% of positive cells; MRFI: 5–9; representative results from #1076 and 1247 are shown in Supplemental Fig. 1D, 1E) the colon stem cell–specific marker Lgr5 (24), whereas FBS tumor cells showed weak or negative expression, with 11–13% of positive cells that did not show statistically significant differences in the mean fluorescence intensity (MRFI = 1.4–1.6), compared with the negative control (Supplemental Fig. 1F, 1G). Thus the results of MRFI showed a clear overexpression of Lgr5 by CICs compared with FBS tumor cells.

In addition, the sphere-forming cells displayed differentiation ability following the culture in vitro for 7 d in the presence of FBS. These cells lost the ability for growth in suspension (see Supplemental Fig. 1A) and acquired the single-cell adherence–dependent growth (Supplemental Fig. 1B), similarly to the FBS tumor cells (Supplemental Fig. 1C).

The tumorigenic ability of these spheroid cell cultures and of their FBS tumor cell pairs was assessed by s.c. transplantation of different numbers of cells (1×10^5, 1×10^4, 1×10^3, 1×10^2, and 1×10 cells) in immunodeficient NOD/SCID CB-17 mice and by the weekly monitoring of tumor formation. Fig. 1A and 1B shows representative results from patient #1076. Notably, cells obtained from spheroid cell cultures formed tumor nodules more efficiently than did FBS cells (Fig. 1A versus 1B; $p < 0.05$). In fact, spheroid cells showed detectable tumors from day +17, reaching 1.87, 1.2, and 0.87 cm^3 of volume following injections of 1×10^5, 1×10^4, and 1×10^3 cells, respectively (Fig. 1A). Of note, tumor formation was obtained also following injections of 1×10^2 CICs (Fig. 1A),
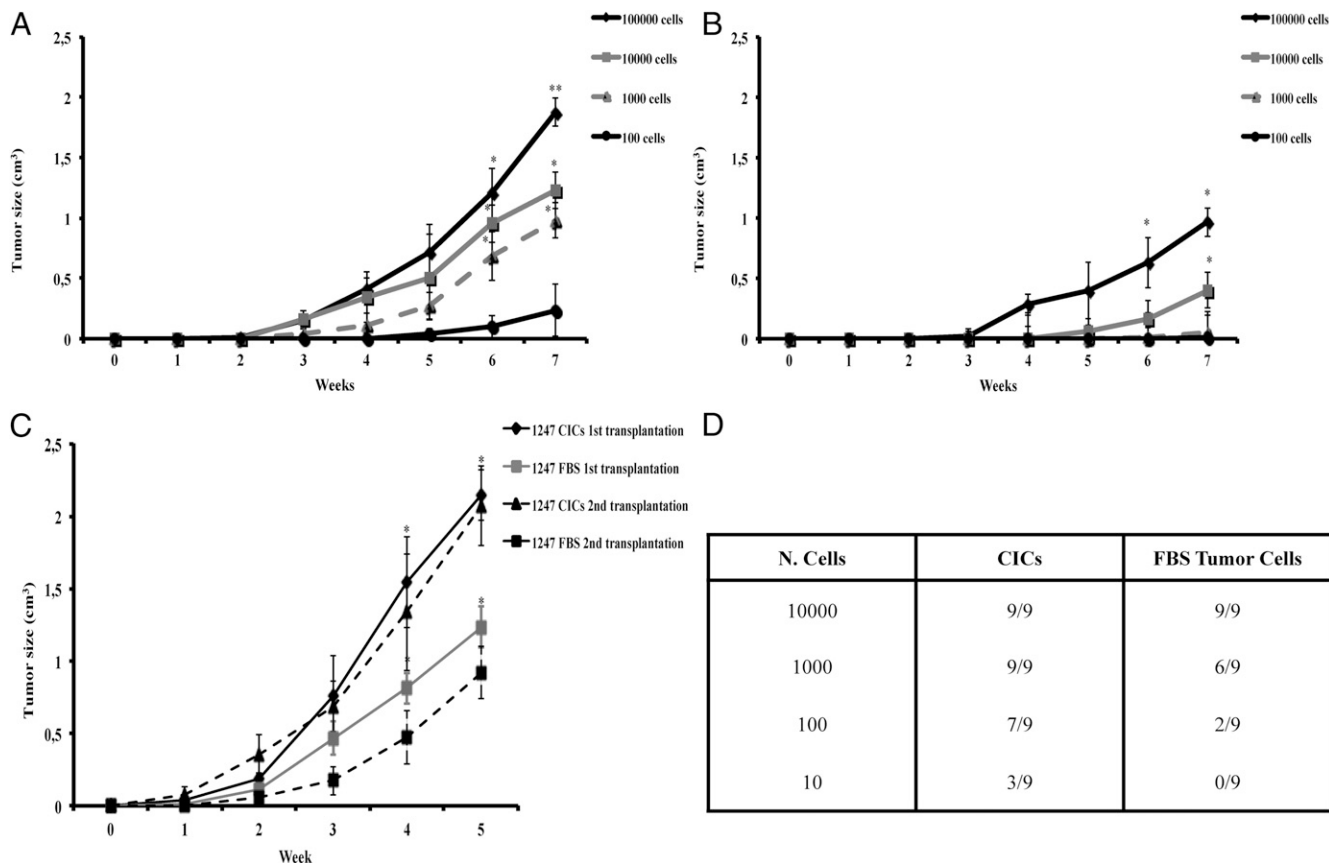
FIGURE 1. Characterization of CIC properties in CRC sphere-forming cell cultures. The tumorigenic properties of CICs compared with autologous FBS tumor cells (**A**, **B**) were evaluated by the s.c. injection of 1×10^5, 1×10^4, 1×10^3, 1×10^2, and 10 cells into immunodeficient NOD/SCID CB17 mice (see *Materials and Methods*). Data are indicated as the means ± SD of tumor volumes deriving from three independent experiments. Three mice per treatment were used for each experiment. *$p < 0.05$, **$p < 0.01$ (99% *t* test). (**C**) Either CICs or FBS tumor cells (1×10^5 cells) were mixed at a 1:1 ratio in Matrigel-containing medium (DMEM with 1% penicillin/streptomycin; 100 µl of final volume) and transplanted into immunodeficient NOD/SCID CB17 mice. Tumor formation was monitored weekly, and when tumor sizes reached 1.5 cm^3 the mice were sacrificed and tumor tissues were removed and mechanically processed to isolate single cells. These cells, derived from either CICs or FBS xenograft tumors, were cultured in vitro and subsequently transplanted into secondary mice and tumor formation was monitored weekly as well. (**D**) The table indicates the number of mice who developed tumors following the injection of either CICs or FBS tumor cells (patient #1076); the results are from the use of three mice for each condition for three experiments (no. of mice per condition = 9).

whereas FBS tumor cells generated tumors with small dimensions (0.9 and 0.4 cm^3) after transplantation of 1×10^5, 1×10^4 cells and failed to generate tumors after injection of 1×10^3 or 1×10^2 cells (Fig. 1B). Of mice injected with 100 cells of CICs, 78% developed tumors, whereas the same number of FBS cells induced tumors in only 22% of tumor-bearing mice (Fig. 1D). Of note, 33% of mice developed detectable tumors after inoculation of 10 CICs but not of 10 FBS cells (Fig. 1 D). The morphological characterization of CRC tissues derived from surgical specimens and from xenografts, studied by H&E staining, revealed that the sphere-forming cells following transplantation in mice can more efficiently give rise to neoplastic tissues that resemble the primary tumors of origin, compared with the FBS tumor cells (representative results are shown in Supplemental Fig. 1H–M). Moreover, serial transplantation into immunodeficient mice showed that efficiency in tumor formation by CICs was similar between the first inoculation and the second round of injection of xenograft-derived cells. On the contrary, half of tumor size was recovered following the second round of in vivo injection of FBS tumor cells (Fig. 1C). Therefore, on the basis of available tools to isolate and define CICs, it appears that our sphere-forming cells indeed constitute a homogeneous cell population that displays CIC properties. FBS tumor cells showed phenotypical and functional characteristics distinct from those of CICs, but we cannot exclude that a rare subpopulation of cells with

CIC properties could still be present in these cell lines (11–13% of Lgr5^{low}-expressing cells) or that these cells can have high grade of plasticity and thus modulate their functional state.

Taken together, these results indicate that the sphere-forming cells isolated in vitro from CRC tissues displayed the phenotypic characteristics and high tumorigenicity that resemble these features in CICs, and therefore they can represent bona fide CICs.

Immunological profile of CRC-derived CICs versus FBS tumor cells

The immunological profile of CICs compared with their non-CIC counterparts was determined by IF and cytofluorimetric analysis by evaluating the expression of MHC class I and II; NKG2D ligands; and, at the intracellular level, an array of molecules belonging to the APM, including MHC class I molecules and their heavy chains (e.g., HLA-HC), β$_2$-microglobulin, constitutive proteasome subunits (Δ, MB1, and Z), immunoproteasome (LMP2, 7, and 10), transporter molecules (TAPs), and chaperon molecules (tapasin, calnexin, calreticulin, and ERp57). Of interest, a frequent downmodulation of MHC class I was found in CICs, compared with their autologous FBS tumor cells, whereas a failure in the expression of MHC class II molecules was evident in both CICs and FBS tumor cells (Supplemental Fig. 2C). All the APM molecules were downmodulated or absent in CICs compared with the FBS tumor cells (representative

results of some APM molecules from patients #1247, 1076, and 111011 are shown in Supplemental Fig. 2D), whereas NKG2D ligands were expressed commonly in all the CRC cell lines and with upregulation, although to a variable extent in CICs compared with their FBS tumor cell pairs (Supplemental Fig. 2C). In addition, no or low upregulation of these molecules was observed by treatment with IFN-α or -γ (data not shown). These results suggest that a low efficiency or impairment of Ag processing and presentation can occur preferentially in association with CICs.

In addition, we found that IL-4 was detectable at the membrane level in all the CICs (seven of seven cell lines with 66–82% of positive cells) and was overexpressed ($p < 0.01$) in these cells compared with their FBS pairs, although autologous FBS tumor cells were available for only three patients (Fig. 2A). This cytokine was commonly found in the cytoplasm of both types of tumor cell lines. We have then analyzed three additional FBS tumor cells available in our laboratories (#1039, 20299, and 1869), confirming the previously obtained results (Fig. 2A). IL-4R was detected in six of seven CICs both at the membrane and, with higher levels, in the cytoplasm, whereas it was almost negative in FBS tumor cells (Fig. 2 A). The secretion of IL-4 was found to be preferentially associated with CRC-derived CICs, with low or negative secretion by FBS tumor cells (#1076 and 1247, respectively; Fig. 2B). In addition, the pretreatment of cells with IFN-γ increased the levels of cytokine release, with statistically significant differences ($p < 0.005$) between CICs and their FBS tumor cell counterparts (Fig. 2B). These results suggest that, as previously documented (19, 25), IL-4 and IL-4R, both membrane associated and as soluble factors, are preferentially expressed by CICs isolated from CRC and correlated with their low immunogenic profile.

The neutralization of CIC-associated IL-4 can rescue the proliferative activity of T lymphocytes

To determine the role of CIC-associated IL-4 on T cell–mediated immune responses, these cells were cocultured with autologous (#1076, 1247, and 14583 CRC patients) or with allogeneic healthy donors (patients #1, 2, and 3) PBMCs for 72 h with PHA/Con A. These cocultures were set up with or without neutralizing Abs directed to IL-4 or IL-4R or both. As negative control, FBS tumor cells (#1076 and 1247) were used as stimulators. The proliferative activity of CD3+ T cells was determined by CSFE staining and FACS analysis. As shown in Fig. 3, the efficiency of proliferation (indicated as proliferation index) of T cells was reduced (1.5–2.6 times, $p < 0.001$) by culture with CICs, compared with stimulation with mitogens alone (PHA and Con A). Of interest, recovery of T cell proliferation was obtained when IL-4–specific mAbs were present in the cocultures, achieving values for the proliferation index similar to those of the positive controls (PHA/Con A) for all the six CICs isolated from CRC patients (Fig. 3). Of note, the coculture of PBMCs with autologous CICs preincubated with anti-IL-4R mAb (#1076, 1247, 14583, 1, 2, and 3) did not affect the T cell proliferation (Fig. 3). Moreover, the pretreatment of CICs with the combination of anti–IL-4 and –IL-4R mAbs confirmed that the blocking of IL-4 is sufficient to lead to the rescue of T cell proliferation. These data indicate that CIC-associated IL-4 is responsible for the CIC-mediated inhibition of T lymphocyte proliferation, possibly by direct interaction with the IL-4R expressed on T cells and not by the activation of an autocrine pathway on CICs.

No inhibition of T cell proliferation by coculture, in the presence or not of anti–IL-4 mAb, with autologous FBS tumor cells (#1076 and 1247; Fig. 3) was detected. Representative CFSE staining histograms from patient #1247 are represented in Supplemental Fig. 3. Of interest, we found that cell-to-cell contact was necessary for CICs to mediate the negative immunomodulatory activity on T cells. T cells from CRC patients were cultured with CRC-derived CICs in Transwell plates, thus preventing direct contact between the two types of cells. We found that T cell proliferation was not affected by CICs, compared with the positive control (representative results from patients #1247 and 1076 are shown in Table I). T cell proliferation was partially inhibited by coculturing T lymphocytes in Transwell plates with CICs pretreated
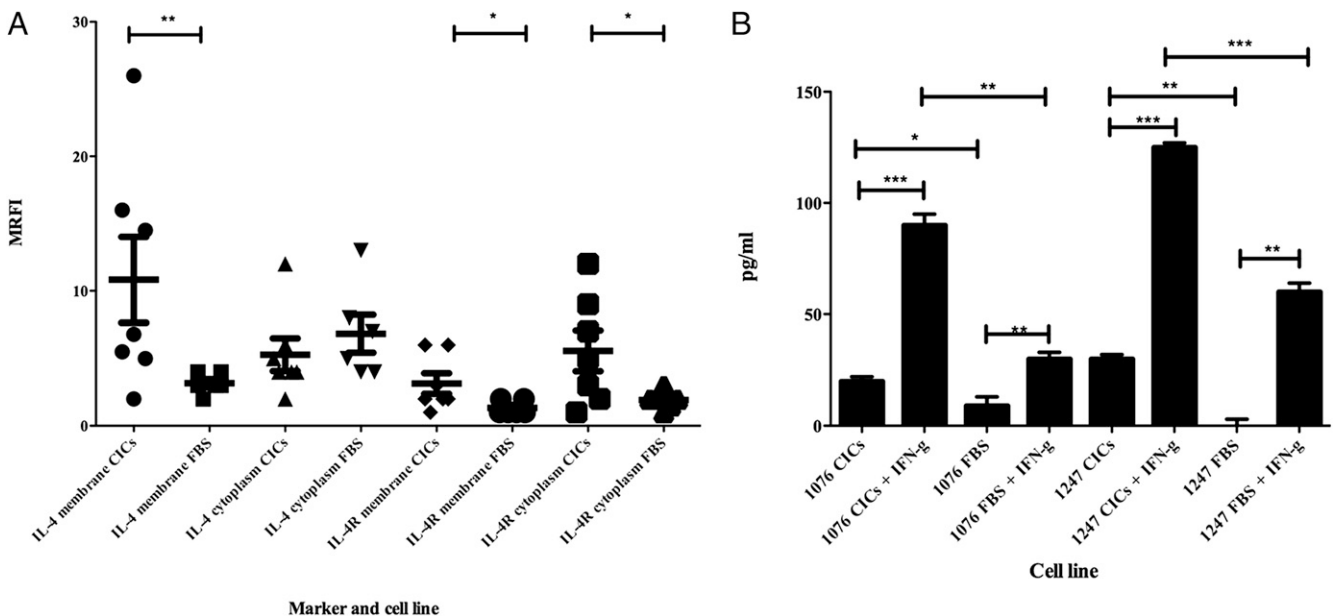
FIGURE 2. Expression or secretion of IL-4 and IL-4R by CICs and FBS tumor cell lines isolated from CRC patients. (**A**) IL-4 and IL-4R expression on CICs and FBS tumor cells [#1076, 1247, 111011, 14583, 1, 2, 3, 1039, 20299 and 1869 (A)] was evaluated by membrane or intracellular IF. For the mAbs used, see *Materials and Methods*. Data are represented as MRFI; significant values are MRFI ≥ 2. The results are the mean of three independent experiments (SD < 5). (**B**) Evaluation of soluble IL-4 in cell culture supernatants of CRC CICs or FBS tumor cells pretreated or not with IFN-γ (1000 IU/ml for 48 h) was performed by SearchLight Array Technology (see *Materials and Methods*). The data are expressed as picograms per milliliter. Results represent averages of duplicates with SD ≤ 10%. *$p < 0.5$, **$p < 0.05$, ***$p < 0.001$.
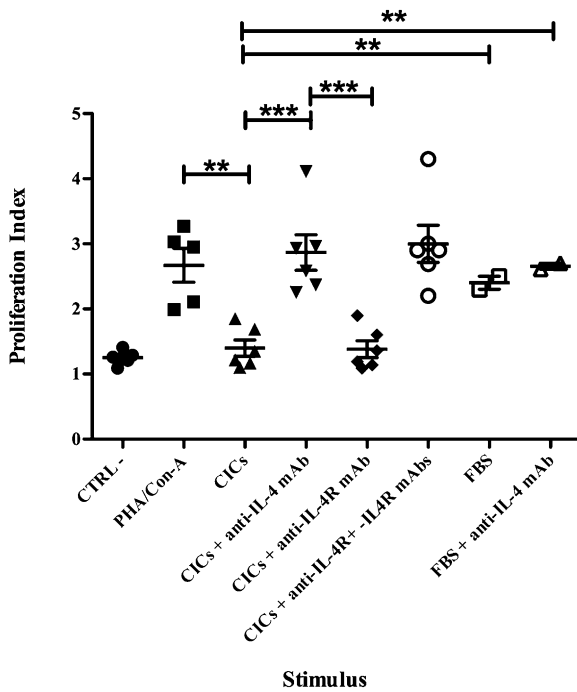
FIGURE 3. Restoration of T cell proliferation in vitro by blocking CIC-associated IL-4. PBMCs (1×10^7) from CRC patients (patients #1076, 1247, and 14583) or healthy donors (for patients #1, 2, and 3) were stained with 1 μM CSFE and stimulated with PHA/Con A in the presence or not of 3-d cultured CICs or FBS tumor cell lines. The coculture of tumor cells and autologous or allogeneic PBMCs was performed in the presence or not of anti–IL-4 mAb, anti–IL-4R mAb, or both the mAbs. Then, cells were harvested and the CFSE profile was assessed by flow cytometry. Data are referred to CD3$^+$ gated cells. Data represent the proliferation index that is the total number of divisions divided by the number of cells that went into division. Data represent the mean of the proliferation index for each tumor cell line from three independent experiments. $**p < 0.05$, $***p < 0.001$.

with IFN-γ, thus upregulating the secretion of IL-4 (Fig. 2B), and indeed the blocking of the secreted IL-4 by the specific mAb could restore the efficiency of T cell proliferation (Table I). This finding indicates that the IFN-γ–induced secretion of IL-4 by CICs can play a partial role in inhibiting T cell proliferation, whereas most of the immunomodulatory activity by CICs is due to the membrane-

associated cytokine and, thus, to cell-to-cell contact between CICs and T lymphocytes. In addition, the evidence that Transwell co-culture with CICs in the presence or not of IL-4 blocking did not affect T cell proliferation indicates that this phenomenon is not dependent on the possibly reduced viability of CICs following treatment with anti–IL-4 mAb.

These results demonstrate that IL-4, which is expressed at high levels by CRC CICs, is one key mediator in the immunomodulatory activity of these cells. In addition, we showed that this CIC-associated inhibitory role is mainly dependent on cell-to-cell contact with T cells.

The blocking of CIC-associated IL-4 augments the efficiency of anti-CRC Th1-type tumor-specific reactivity in autologous settings

PBMCs from CRC patients (#1076, 1247, and 14583) were stimulated in vitro with autologous irradiated CICs or, when available (#1076 and 1247), with FBS tumor cells (MLTC culture). The pattern of reactivity of T cells deriving from these MLTCs against autologous tumor cell lines was determined by IFN-γ release assay (ELISPOT). As shown in Fig. 4, tumor-reactive T cells could be isolated from PBMCs stimulated in vitro with autologous CICs (Fig. 4A–C) from patients #1247, 14,583, and 1076, respectively. The reactivity of these T cells against CICs was HLA class I restricted (50–52% of reduction of cytokine secretion, in the presence of the anti-HLA class I mAb W6/32, $p < 0.05$) for two of three patients (#1247 and 1076; Fig. 4A, 4C), whereas 50% of reduction ($p < 0.05$) of IFN-γ release after stimulation with CICs was achieved with anti-HLA class II mAb (L243) only for patient 1076 (Fig. 4C). MLTCs stimulated in vitro with autologous CICs showed higher reactivity against FBS tumor cells, when used as target (Fig. 4A, 4C), although all tumor cells to be used as target cells were pre-treated with IFN-γ to optimize the Ag processing and presentation. These findings were in line with the low immunogenicity of CICs and with the reduced activity of IFN-γ treatment in upregulating APM in these cells compared with FBS tumor cells (as discussed previously). The stimulation of lymphocytes with PHA and Con A represented the positive controls of the assays.

The phenotype of the CIC-stimulated, MLTC-derived T cells resulted in a preferential selection of CD4$^+$ T cells (73% of positive cells; data not shown) and a lower frequency of CD8$^+$ T cells (27% of positive cells; data not shown). On the contrary, the

**Table I.** The CIC-associated inhibition of T cell proliferation is dependent on cell-to-cell contact

Patient	Mitogen	Tumor Cells in Transwell Plate	% Divided Cells[a]	Proliferation Index[b]
1076	No	No	0.34	1.20
	PHA/Con A	No	**69.20**	**2.93**
	PHA/Con A	CICs	**60.38**	**3.20**
	PHA/Con A	CICs + anti–IL-4	**63.11**	**2.82**
	PHA/Con A	CICs + IFN-γ	42.60	1.80
	PHA/Con A	CICs + IFN-γ + anti–IL-4	**58.40**	**2.30**
1247	No	No	0.45	1.14
	PHA/Con A	No	**70.20**	**2.84**
	PHA/Con A	CICs	**73.96**	**2.66**
	PHA/Con A	CICs + anti–IL-4	**70.01**	**2.70**
	PHA/Con A	CICs + IFN-γ	37.26	1.47
	PHA/Con A	CICs + IFN-γ + anti–IL-4	**65.20**	**2.70**

PBMCs from CRC patients were stained with 1 μM CSFE and plated with PHA/Con A in the bottom of Transwell plates. In the 0.8-μm membrane insert well, CICs pretreated or not for 48 h with 1000 IU/ml of IFN-γ, with or without anti–IL-4 mAb, were cultured. After 3 d, T cells were harvested and the CFSE profile was assessed by flow cytometry. Data refer to CD3$^+$ gated cells.

[a]The % Divided Cells is the percentage of cells of the original sample that divided.

[b]The Proliferation Index is the total number of divisions divided by the number of cells that went into division. Bold data represent the percent of divided cells or the proliferation index of the positive controls or of rescued values following the neutralization of IL-4.
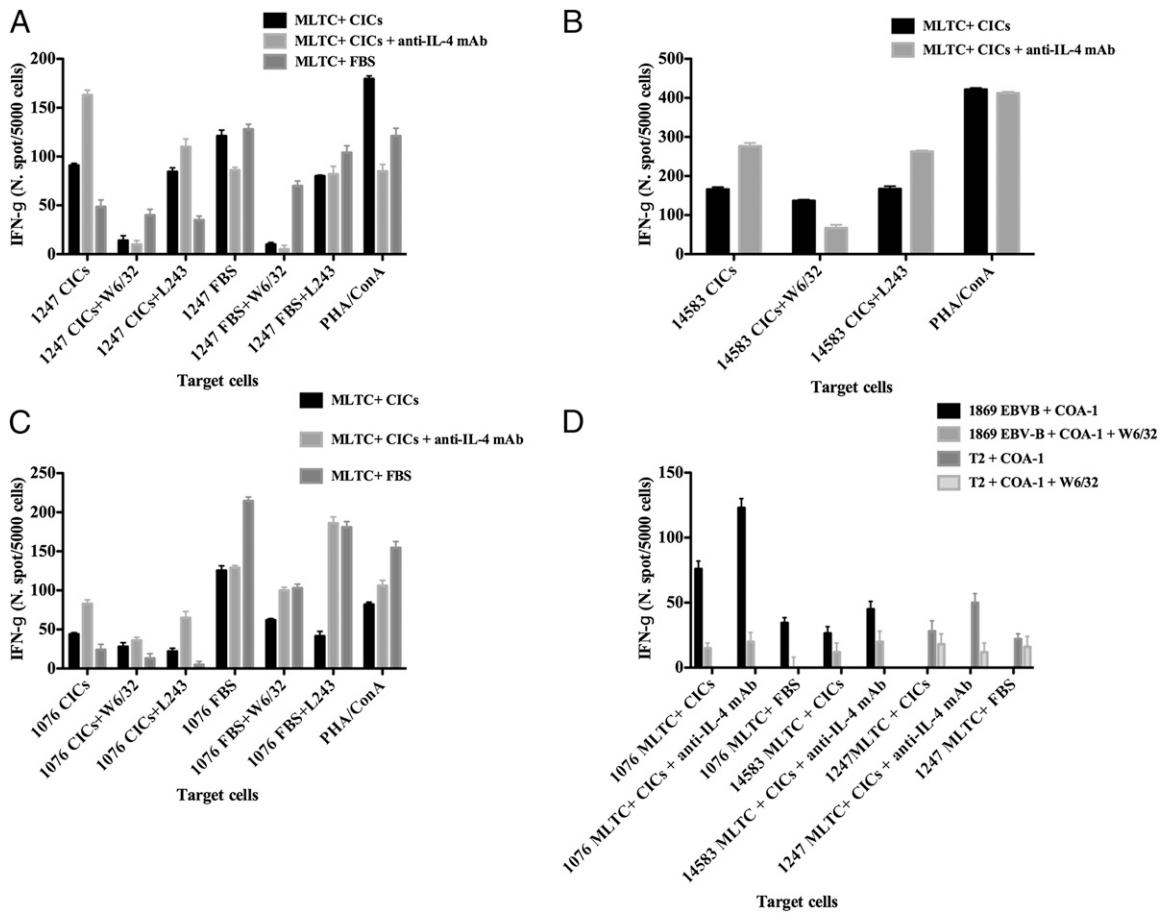
FIGURE 4. Specific tumor reactivity of T cells from MLTCs of CRC patients. PBMCs isolated from CRC patients 1076, 1247, and 14583 were stimulated in vitro in the presence or not of the neutralizing IL-4 mAb with autologous irradiated CICs (**A–C**) or, when available, FBS tumor cells (A, C). Following 3 wk of culture, the reactivity against autologous tumor cell lines was assessed by IFN-γ release (ELISPOT assay). Data are expressed as no. of spots per 5×10^4 cells and are subtracted from the background of IFN-γ release of T cells alone. (**D**) The reactivity of CIC- or FBS-stimulated MLTCs against allogeneic HLA-matched EBV-B cells (1869) pulsed with the HLA-A24 epitope (SLQDLSELV, Ref. 16) or T2 cells loaded with the HLA-A2–restricted epitope (FMTRKLWDL, Ref. 16) from COA-1. The positive controls of T cell stimulation are PHA/Con A. Data are expressed as no. of spots per 5×10^4 cells and subtracted from the background of IFN-γ release by T cells alone or stimulated with allogeneic target cells. Results represent averages of triplicates with SD ≤ 10%; statistical analysis of differences between means of IFN-γ released by T cells was performed by two-tailed *t* test. Each experiment has been repeated three times, showing consistency of the results.

phenotype analysis of MLTC-derived T cells stimulated with FBS cells revealed an enrichment (75% of positive cells) of CD3^{+}CD8^{+}CD45RO^{+} T cells and a minor population (25% of positive cells) of CD3^{+}CD4^{+}CD45RO^{+} T lymphocytes (data not shown). Moreover, the phenotype characterization of the #1247 MLTC with CICs showed 26% of CD3^{+}CD8^{+}CD45RO^{+}, 40% of CD3^{+}CD4^{+}CD45RO^{+} T cells, and 34% of CD16^{+}CD56^{+}CD57^{+}CCR7^{-} NK cells (data not shown). Of note, no and low levels of Il-4 and IL-5 release, respectively, were detected by these T cells after stimulation with tumor cell lines (data not shown).

We have then set up MLTCs with autologous CICs in the presence of the neutralizing anti–IL-4 mAb, and as represented in Fig. 4A–C, the pattern of tumor reactivity of these cells showed an augmentation (1.7–1.9 times increase) of CIC recognition, compared with the recognition of these cells by MLTCs without the blocking of IL-4. This reactivity was specifically inhibited by W6/32 mAb (Fig. 4A–C), whereas their recognition of autologous FBS tumor cells was not affected (Fig. 4C) or even slightly reduced (Fig. 4A) by the anti–IL-4 mAb. The phenotype characterization of the MLTCs with the anti–IL-4 mAb showed an enrichment of CD3^{+}CD8^{+}CD45RO^{+} (90%), whereas for patient #1247 NK cells were no longer detectable (data not shown). The neutralization of IL-4 can affect the pattern of reactivity of MLTC possibly by modifying the frequency

and/or the avidity of Ag-specific T cells. This may lead to the increase of specific reactivity against CICs (Fig. 4A–C) and either to the reduction of recognition of FBS tumor cells (Fig. 4A) or to the decrease of this specific reactivity (decrease of the inhibition of FBS tumor cell reactivity by blocking with MHC class I mAb) (Fig. 4C).

MLTCs stimulated in vitro with autologous FBS tumor cells recognized specifically (inhibition of IFN-γ release by W6/32 and/or L343 mAbs) the autologous tumor cells, with higher cytokine release when FBS tumor cells were used as target cells, compared with CICs (Fig. 4A, 4C). Moreover, no modification of the pattern of reactivity was observed by adding the anti–IL-4 mAb to these MLTCs (data not shown), in line with the low expression and secretion of IL-4 by 1076 or 1247 FBS tumor cells (Fig. 2A, 2B). In addition, the phenotype of this MLTC comprised 60% of CD3^{+}CD4^{+}CD45RO^{+} and 37% of CD3^{+}CD8^{+}CD45RO^{+}, whereas no NK cells were detected (data not shown).

The proliferative ability of MLTCs from patients 1076, 1247, and 14583 were also assessed by CFSE staining and cytofluorimetric analysis. The results, reported in Table II, confirmed that the 72-h incubation of MLTC-derived cells with autologous CICs reduced the proliferation of these T cells. A recovery of the rate of proliferation was achieved when these T cells were cultured with anti–IL-4 mAb (Table II). Furthermore, the supply of the anti–IL-4 mAb to

the MLTCs concomitantly with tumor cell stimulation led to higher levels of restoration of T cell proliferation. The proliferative rate of MLTCs stimulated with FBS tumor cells was not affected either by these cells or by the blocking of IL-4 signaling (data not shown); these results were similar to those obtained for fresh PBMC proliferation (Fig. 3).

Taken together, these data indicate that T cell responses against CICs can be obtained from the peripheral blood of CRC patients, although with lower efficiency, compared with FBS tumor cells. Of interest, the neutralization of IL-4 along with the stimulation of T cells with autologous CICs can lead to the augmentation of both antitumor Th1 responses and T cell proliferation.

COA-1 recognition by MLTC–derived lymphocytes stimulated with autologous CICs

The reactivity of T cells from MLTCs of three CRC patients (#1076, 1247, and 14583) stimulated in vitro with autologous CICs has been assessed against a panel of TAAs (SVV-1, NY-ESO-1, IL13Rα2, Ep-CAM, and COA-1) (15, 26). These T cells, which recognized in an HLA class I–restricted manner the autologous CICs (Fig. 4A–C), displayed a recognition of the COA-1–derived epitopes (Fig. 4D). Of note, for patients #1076 and 14583 (both HLA-A24 positive) an efficient reactivity was found against the HLA-A24–binding COA-1 peptide loaded on HLA-matched allogeneic 1869 EBV-B cells (15, 16). Moreover, MLTCs from HLA-A2$^+$ patient #1247 specifically (HLA class I restricted) recognized the HLA-matched T2 cells preincubated with the HLA-A*0201–binding COA-1 epitope (16). Notably, the COA-1 reactivity of these MLTCs was increased by blocking IL-4 with the neutralizing mAb (Fig. 4D). In addition, higher levels of COA-1 reactivity were observed with MLTCs stimulated in vitro with CICs than with MLTCs stimulated with FBS tumor cells.

These results indicate that CICs can act as stimulators when the expression of MHC and APM is upregulated, although not at optimal levels, by IFN-γ treatment and can efficiently activate COA-1–specific T cells. The extent of COA-1 recognition by these T cells was heterogeneous in the three patients studied, perhaps depending on the patient's efficiency of Ag processing and/or on

the TCR avidity of T cells. Recognition of other CRC-associated Ags, such as SVV-1, NY-ESO-1, IL13Rα2, and Ep-CAM, by T cells was not observed (data not shown).

The IF and cytofluorimetric or confocal microscopy analyses of a panel of TAAs previously reported as expressed by CRC (e.g., MAGE, NY-ESO-1, Gp100, SVV-1, COA-1, and IL-13Rα2) (15–26) revealed that all CICs and FBS tumor cells from patients 1076, 1247, 111011 and 14583 were negative for MAGE and Gp100 and expressed low levels (MRFI = 2–4) of NY-ESO-1 and IL-13Rα2 (data not shown). Sox2, Ep-CAM, and CEA, which in addition to their role as markers associated with CICs or CRC-derived CICs (Supplemental Fig. 2A) represent target molecules of T cell responses (26), were found to be upregulated in CICs compared with FBS tumor cells. Of interest, COA-1 (Supplemental Fig. 1N, 1O) and, to a lesser extent, SVV-1 (Supplemental Fig. 1 P–Q) were clearly expressed with both nuclear and cytoplasmic localizations in 100% of the evaluated CICs (Supplemental Fig. 1N, and 1P, respectively) and FBS tumor cells (Supplemental Fig. 1O, 1Q, respectively), with the SVV-1 expressed more homogeneously by CICs than by FBS tumor cells.

Therefore, for the first time, to our knowledge, we could identify a novel candidate TAA (COA-1) that can be targeted by T cell responses directed against CRC CICs.

Discussion

The existence within human CRCs of a subpopulation of cancer cells with tumor intiating/stem-like properties has been widely documented (1–3, 20–24). However, the detailed characterization and identification of the CIC antigenic/immunogenic profile have not yet been accomplished. In the present work we have isolated from seven primary CRCs a subpopulation of cells growing as spheres and displaying CIC properties, as shown by the overexpression of CRC- and CIC-associated markers and by the in vivo high tumorigenicity. Moreover, for three CRC patients also the autologous differentiated tumor cell counterparts of the tumor (i.e., FBS cells) were isolated in vitro. This strategy of isolating two different subpopulations of tumor cells from the same clinical sample has been previously reported in the GBM model (10, 17, 18).

Table II.   IL-4 neutralization can augment the proliferation of MLTCs stimulated with autologous CICs

T Cells	Mitogen	Tumor Cells	Proliferation Index[a]
MLTC	No	No	1.24 (0.29)
	PHA/Con A	No	**2.25** (0.23)
	PHA/Con A	CICs	1.45 (0.59)
		CICs + anti–IL-4	**2.50** (0.34)
MLTC + anti-IL-4	No	No	1.40 (0.30)
	PHA/Con A	No	**2.40** (0.56)
	PHA/Con A	CICs	1.90 (0.26)
		CICs + anti–IL-4	**3.08** (0.49)

T Cells	Mitogen	Treatment	Proliferation Index[a]
IL-4– + IL-2–dependent growth	No	No	1.2
	PHA/Con A	No	**4**
	PHA/Con A	+ Anti–IL-4	**2.4**
		+ Anti–IL-4R	**2.7**
		+ Anti–IL-4 + anti–Il-4R	**1.5**

A total of 1×10^7 T cells from MLTCs of CRC patients #1076, 1247, and 14583 were stained with 1 μM CSFE and stimulated with PHA/Con A in the presence or not of 3-d cultured CICs pretreated or not with 10 μg/ml of anti–IL-4 mAb. Then, cells were harvested and the CFSE profile was assessed by flow cytometry. Data are referred to CD3$^+$ gated cells and represent the mean of the proliferation index of the three patients; the number in parentheses is the SD.

^{a}The Proliferation Index is the total number of divisions divided by the number of cells that went into division. The efficiency of neutralization of IL-4 and IL-4R by the specific mAbs was determined in performing CFSE staining and proliferation assay on PBMCs from healthy donors that were cultured in vitro for 5 d in the presence of 100 IU of IL-2 and 10 ng/ml of IL-4. Bold data represent the percent of divided cells or the proliferation index of the positive controls or of rescued values following the neutralization of IL-4.

A high degree of heterogeneity in CIC phenotype can be found likely owing to modulation by different factors, including genetic background, epigenetic modifications, and tumor–microenvironment interactions (27, 28). Thus far, a failure in the identification of CIC-specific markers precluded the use of a unique method for ex vivo isolation and characterization of these cells. Surrogate markers associated with stem cell properties, such as CD133, CD24, CD44, and ALDH-1, have been commonly used for CRC CIC detection, although controversial results with the stem cell properties of these cells have been reported (1, 2, 21–23, 29). More recently for the first time a potential stem cell–specific marker, Lgr5, has been identified for both normal and tumor colorectal tissues (24). Of interest, our sphere-forming cells were homogeneously positive for the expression of this molecule, thus indicating that indeed these cells displayed CIC properties. Conversely, FBS tumor cells were almost negative for this marker and showed lower tumorigenic properties than did CICs, although on the basis of the heterogeneity and plasticity of tumor cells we cannot rule out that these cells can modulate in vivo their phenotypic and functional state.

A detailed immune profile characterization of CRC-derived CICs and FBS tumor cells was carried out to assess whether these cells can be targeted by immunotherapy. We found a weak immunogenicity of CICs compared with autologous FBS tumor cells because MHC and APM molecules were preferentially downmodulated in the CICs. Moreover, IFN (α or γ) and the demethylating agent (5-Aza CdR) were not or only weakly effective as modulators of these molecules (data not shown). Along with the low immunogenicity of the CRC-derived CICs, we found that these cells can play a role in the modulation of T cell–mediated responses. In fact, proliferation of T cells was inhibited by coculture with autologous or allogeneic CICs, but not with FBS tumor cells. CIC-associated factors, such as TGF-β, IL-10, IL-13, galectin, and PDGE2, which can play a negative regulatory role in the induction of tumor reactive T cells, have been described (12–14) and, notably, shown to be shared with normal stem cells as well (30). The complexity of this issue is demonstrated by the evidence that none of the molecules described above have been found to be specifically expressed or upregulated in CICs that we have isolated in vitro (data not shown), in line with the results that have been previously published by our group for GBM (10).

Along this line, we found that the membrane expression of IL-4 and, to a lesser extent, of IL-4R, was preferentially associated with CICs rather than with FBS tumor cells. Studies on the expression of this molecule by CRC CICs have been previously published, demonstrating that these cells, by the autocrine production of IL-4, protect themselves from apoptosis and acquire resistance to chemotherapy (19, 25). The novelty of our findings is that CIC-associated IL-4 mediates the inhibition of T cell proliferation in the coculture of PBMCs from either CRC patients or healthy donors with autologous or allogeneic CICs (6/6 CRC patients). Of note, this phenomenon was shown to be dependent on cell-to-cell contact between CICs and T cells. In fact, coculture without cellular contact of T lymphocytes with the CICs or with CIC-derived cell culture supernatants did not affect their proliferation (Table I and data not shown). Moreover, we demonstrated that the proliferation of T cells was only partially affected by the CIC-secreted IL-4.

The negative immunomodulatory activity of CICs is not mediated by the autocrine engagement of IL-4 signaling, contrary to the chemotherapy resistance pathway of these cells (19, 25), because the neutralization of IL-4R on CICs failed to restore T cell proliferation. Thus this CIC function is mostly dependent on the engagement of the IL-4R on T cells by the CIC-associated specific ligand. Of note, the phenotype of MLTC-derived T cells, showing

a skew toward the preferential enrichment of Th1-type CD8$^+$ T cells, was also affected by IL-4 blocking. A total of 34% of NK cells could be detected in the MLTC from one CRC patient stimulated with CICs. This evidence is in line with the reported observation that NK cell susceptibility was higher in CRC CICs than in FBS tumor cells, correlating with higher expression and lower levels by CICs of NK receptor ligands and MHC class I molecules, respectively (31). Of interest, by the neutralization of IL-4 signaling, the MLTC showed enrichment of CD8$^+$ T cells, indicating that the blocking of IL-4 can prevent one of the immunosuppressive mechanisms associated with CICs and can switch the tumor–lymphocyte interaction supporting activation and proliferation of T rather than NK cells. Thus, IL-4 represents one of the key molecules that regulate CIC-associated immunomodulatory activity, and therapeutic approaches based on the neutralization of this molecule should be sought. Additional mechanisms of immunosuppression associated with CICs need to be investigated in the future to obtain a more detailed immunological profile of these cells. IL-4 signaling blockade in vivo has been shown to reprogram tumor-associated macrophages and to inhibit their proangiogenic and tumorigenic activities (32). IL-4/IL-13 and their receptors have been shown to be expressed by epithelial tumors and to represent potential players in tumor behavior and in its responsiveness to standard therapeutic interventions (33). Nevertheless, these molecules are critical regulators of immune responses. On the basis of our results, the blocking of IL-4 signaling affects T cell–mediated tumor reactivity, leading to the skew toward T cell effector responses and to the induction of efficient anti-CIC immune responses. This issue needs to be further investigated in vivo by immunotherapy-based experiments developed in mouse models; however, interventions aimed at blocking IL-4 signaling may result in novel immune-based therapeutic targeting of CRC CICs.

Another key observation of this study is the identification of a target molecule recognized by CIC-specific T cells. We found that COA-1 (15, 16) was recognized in vitro by T cells from three CRC patients (#1076, 1247, and 14573), isolated by MLTCs whose autologous CICs represented the source of Ags. We failed to detect T cell responses against T cell epitopes deriving from CRC-associated SVV-1, NY-ESO-1, and IL-13Rα2 (data not shown). Surprisingly, although FBS tumor cells also expressed COA-1 (Supplemental Fig. 1O), CICs could elicit anti–COA-1 T cell responses more efficiently than could FBS tumor cells. This finding suggests that IFN-γ treatment of CICs can induce the secretion of immunomodulatory factors such as IL-4 (Fig. 2B) but can also activate the APM to induce T cell responses against highly expressed TAAs endowed with strong immunogenic potency, such as COA-1 (16). The limiting factor of this type of experiment is the need for an autologous setting and thus the availability from the same patients of CICs, PBMCs. and, possibly, FBS tumor cells. The limited availability of these biological samples from all CRC patients prevented us from investigating a larger number of subjects. Nevertheless, our results suggest that COA-1 may represent a target molecule for T cell responses against CRC CICs. In future studies it would be worthwhile to investigate the mechanisms that can lead to differential COA-1 processing and presentation in CICs compared with FBS tumor cells. Of note, the COA-1–mediated reactivity associated with CICs inversely correlated with the levels of expression and secretion of IL-4 (see results in Fig. 2B, Fig. 4) and was augmented in the presence of neutralizing IL-4 mAb.

COA-1 has not been extensively studied for clinical treatments of CRC patients; however, circulating T cells, both CD4$^+$ and CD8$^+$, directed to this Ag could be isolated from CRC patients, indicating its relevance as a target molecule for immunotherapy for this disease. Thus, the results provided by the present work may have

relevant implications for the design of new combined immunotherapy for CRC, based on active vaccination plus an immunomodulatory agent to target CRC CICs.

Acknowledgments

Disclosures

The authors have no financial conflicts of interest.

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