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Human NK Cells Selective Targeting of Colon Cancer–Initiating Cells: A Role for Natural Cytotoxicity Receptors and MHC Class I Molecules

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Tumor cell populations have been recently proposed to be composed of two compartments: tumor-initiating cells characterized by a slow and asymmetrical growth, and the “differentiated” cancer cells with a fast and symmetrical growth. Cancer stem cells or cancer-initiating cells (CICs) play a crucial role in tumor recurrence. The resistance of CICs to drugs and irradiation often allows them to survive traditional therapy. NK cells are potent cytotoxic lymphocytes that can recognize tumor cells. In this study, we have analyzed the NK cell recognition of tumor target cells derived from the two cancer cell compartments of colon adenocarcinoma lesions. Our data demonstrate that freshly purified allogeneic NK cells can recognize and kill colorectal carcinoma–derived CICs whereas the non-CIC counterpart of the tumors (differentiated tumor cells), either autologous or allogeneic, is less susceptible to NK cells. This difference in the NK cell susceptibility correlates with higher expression on CICs of ligands for NKp30 and NKp44 in the natural cytotoxicity receptor (NCR) group of activating NK receptors. In contrast, CICs express lower levels of MHC class I, known to inhibit NK recognition, on their surface than do the “differentiated” tumor cells. These data have been validated by confocal microscopy where NCR ligands and MHC class I molecule membrane distribution have been analyzed. Moreover, NK cell receptor blockade in cytotoxicity assays demonstrates that NCRs play a major role in the recognition of CIC targets. This study strengthens the idea that biology-based therapy harnessing NK cells could be an attractive opportunity in solid tumors. The Journal of Immunology, 2013, 190: 000–000.

Cancer-initiating cells (CICs) have been proposed to play a major role in the metastatic process and in the recurrence of tumors (1, 2). Metastasis formation is a complex, multistep process that involves a sequence of events; namely, cancer cells must leave the original tumor anatomical site, migrate through the blood or lymph, move from the circulation into the local tissue, form micrometastases, develop a blood supply, and grow to form macroscopic metastases. It has been estimated that <2% of solitary cells that successfully migrate to a new site are able to initiate growth once there. Moreover, <1% of cells that initiate growth at a secondary site are able to maintain this growth sufficiently to become macroscopic metastases (3). These observations suggest that a small, and most likely specialized, subset of cancer cells drives the spread of disease to distant organs. Recently, CICs have been proposed as responsible for this phenomenon. According to this hypothesis, the metastatic efficiency may reflect the relative amount of CICs present within the tumor population and their interaction with the tumor microenvironments (2). It has been demonstrated that CICs and metastatic cancer cells share several properties that are essential to the metastatic process, including the requirement of a specific microenvironment (or “niche”) to support growth and provide protection (1). Metastatic sites for a given cancer type could therefore represent those tissues that provide or promote the development of a compatible CIC niche, from which CICs could expand through cellular signaling. Initiating cells tend to be quiescent unless activated to divide (3–6). CICs express multidrug resistance genes that make them resistant to the common antineoplastic treatments: chemotherapy and radiotherapy (7, 8). As such, this subpopulation could form the kernel of cells responsible for metastasis and cancer recurrence following treatment and remission. Colorectal carcinoma (CRC) is the second most common cause of death from cancer (9); CICs have been recently isolated...
from CRC tumor biopsies and have been biologically and functionally characterized (10). The CRC-derived CICs have been demonstrated previously to be the key tumor compartment in establishing this neoplastic disease in animal models (11, 12). Although different immunotherapies have been considered in relation to CRC tumor, there is little information available concerning immunologically important properties of the CRC-derived initiating cells. NK cells are large granular lymphocytes that are potent effectors of the innate immune system, with a critical role in early host defense against invading pathogens (13, 14). Historically, the NK cells have been defined for their ability to recognize and kill virus infected and cancer cells, making them appealing effector cells for immune therapy strategies in the treatment of human cancer (15). Human NK cells comprise ~10% of PBLs and are characterized phenotypically by the presence of the cell surface marker CD56 and the lack of CD3. Most (~90%) human NK cells are CD56dim and express high levels of FcγRIII (CD16), whereas a minority (~10%) are CD56bright and CD16dim/neg. Additionally, CD57+CD56dim NK cells were recently identified as the major NK cell cytotoxic subpopulation (16).

NK cells participate in innate immune responses by recognizing, without prior specific sensitization, virus-infected, transformed, and allogeneic cells while sparing autologous healthy cells (17). This capability depends on the integrated balance of input to activating and inhibitory NK cell receptors that scrutinize the surface of potential target cells. Some ligands for activating receptors are cellular stress inducible molecules such as NK2G2 ligands; these include, among others, MICA, MICB, and a group of ULBPs (18). Other triggering receptors include the group of natural cytotoxicity receptors (NCRs): NKP30, NKP44, and NKP46 (19, 20), as well as the DNAX-activating molecule-1 (DNAM-1) (21). The main inhibitory receptors are killer Ig-like receptors, CD94/NKG2A heterodimers, and Ig-like transcript (LIR, CD85) (20–22), most of which recognize classical MHC class I molecules. Increased NK susceptibility can thus be caused by increased expression of activating ligands, decreased expression of MHC class I molecules or other inhibitory ligands, or a combination of these two events (23).

Studies on NK cells function in vivo, mainly in murine models, have shown that they can contribute to control and prevent tumor growth and dissemination (24, 25). The capacity of human NK cells to exert antitumor effects ex vivo has been documented in several reports (26–30). Given the proposed use of NK cells in immunotherapy approaches against cancer (31) and the emerging concept of CICs as discussed above, it appears relevant to ask how NK cells interact with, for example, CRC-initiating cells, particularly because these may be relatively resistant to cytostatic drugs and radiotherapy (32). We therefore set out to test whether NK cells can kill CRC-initiating cells, and to further investigate which molecules may be involved in regulating a possible difference between NK susceptibility of CRC-initiating cells and the complete tumor population.

Materials and Methods

**Cell culture: CRC-initiating cells and CRC cell lines**

The human colon carcinoma cell lines HCT116 and RKO (allogeneic CRC lines) were originally obtained from the American Type Culture Collection and were cultured in complete DMEM (EuroClone) supplemented with 10% FBS (BioWhittaker/Lonza, Treviglio, Italy) and 1% penicillin/streptomycin (Invitrogen).

All the CRC-initiating cells (CRC-derived CICs) (DV29, AV9, AP24, DN08, AG2, CC09, 1247, and 1076) (Supplemental Table I) were obtained by digesting human colon carcinoma specimens from patients undergoing colorectal resection admitted at the University of Palermo (Palermo, Italy) or at the San Raffaele Hospital (Milan, Italy) in accordance with the ethical standards of the institutional committees.

**Tumor diagnosis** was based on the morphologic microscopic features of tumor cells. Tumor tissues were mechanically and enzymatically digested using collagenase (1.5 mg/ml; Life Technologies) and hyaluronidase (20 µg/ml; Sigma-Aldrich).

The tumor digest was divided into three different culture conditions: primary tumor cells, hereafter denominated autologous CRC tumor cells (1247 CRC tumor and 1076 CRC tumor), were obtained using Advanced RPMI 1640 (Lonza) supplemented with 5% heat-inactivated FBS, antibiotics/antimycotic (Invitrogen), penicillin/streptomycin (EuroClone), and l-glutamine (EuroClone); the CICs were selected plating tumor cells on ultralow adherence flasks (Corning, Lowell, MA) in DMEM F12 serum-free medium (Life Technologies) with the addition of epidermal growth factor (20 ng/ml) and basic fibroblast growth factor (10 ng/ml; both from Sigma-Aldrich, St. Louis, MO) to promote the growth of the spheres (33). These sphere cultures were used for the first time to form a xenograft in immunocompromised mice, resembling the parental tumor (10), and for a colorectal CIC phenotype by assessing the expression of CD166, CD44, CD24, CD133, Lgr5, EpCAM, CEA, Nanog, Sox2, and Aldehyde Dehydrogenase 1A1 (ALDH1). Briefly, CD44, CD24, EP-CAM, and ALDH1 were homogeneously (~60–80%) expressed by CIC and overexpressed (2- to 6-fold) as compared with the non-CIC counterparts (A; Volonté, T. Di Tomaso M. Spinelli, F. Samvito, L. Albarelli, M. Bissolati, L. Ghirardelli, E. Orsengio, S. Ferrone, C. Doglioni, P. Dellabona, C. Staudacher, G. Pariani, and C. Maccauli, submitted for publication). To achieve the in vitro differentiation of CRC-derived CICs, dissociated sphere cultures were cultured in DMEM supplemented with 10% FBS in adherent conditions, obtaining three differentiated CRC-derived CICs (AG2D, DN08D, CC09D). Several lineages were passed by trypsine digestion (trypsin 0.05%/EDTA 0.02% in PBS) without calcium, magnesium, and phenol red; EuroClone) every 4 d to avoid reaching confluence, for cells in adhesion, or to avoid the formation of big spheroids, which would lead to the death of the inner cells. For CRC-derived CICs, this treatment requires cell sedimentation, removal of supernatant, and subsequent suspension in an appropriate volume of trypsin solution. The cells were then placed at 37˚C for cycles of 2 min and then subjected to mechanical disruption. The cells were eventually resuspended in their growth medium (inhibition by dilution) and centrifuged at 192 × g; the cell pellet was resuspended in the appropriate growth medium and finally plated.

**CICs stem-like features**

When colon cancer samples from patients were dissociated into single cells and cultured in a serum-free medium containing epidermal growth factor and fibroblast growth factor 2, a sphere-like culture was obtained. The sphere-like aggregates could be expanded for several months in this medium. We tested common stemness markers such as CD133, β-catenin, or Nanog on human colorectal tumor tissues through confocal microscopy analysis. The immunofluorescence was performed on 5-µm-thick paraffin-embedded tumor sections using the following Abs: CD133 (AC133, Becton, Dickinson, Franklin Lakes, NJ), β-catenin, or HIC-34 (anti-HIC, IgG2a; BioLegend, San Diego, CA); clone BAM 195 (anti-H-102, polyclonal rabbit IgG; Santa Cruz Biotechnology), and nanog (N-17, polyclonal rabbit IgG; Santa Cruz Biotechnology).

In single-cell cloning experiments, 1, 2, 4, or 6 cells were seeded in wells from a 96-well plate and their growth was followed. The clonogenic potential was evaluated by extreme limiting dilution analysis (35).

**Tumorgenesis capacity of CICs**

For the in vivo experiments, 5-wk-old NOD/SCID mice from Charles River Laboratories were maintained in accordance with the institutional guidelines of the University of Palermo and San Raffaele Foundation Center Animal Care Committee. Freshly dissociated cells (0.5 × 10⁶/finjection) from CIC lines were reseeded in a 1:3 mixture of growth factor-depleted Matrigel (BD Biosciences, Palo Alto, CA) and medium, for a final volume of 100 µl, and s.c. injected. These CICs display the tumor-initiating ability as reported elsewhere (10).

Serial dilution of CIC and non-CIC counterparts to be inoculated in NOD/SCID mice was performed, resulting in a high rate of efficiency in tumor formation, even by the inoculation of NOD/SCID mice of 10 × 10⁴ and 10 × 10⁵ cells only by CIC (A. Volonté et al., submitted for publication).

**mAbs and immunofluorescence procedures**

The colon cancer-initiating cell lines were analyzed by indirect immunofluorescence and flow cytometry analysis using the following Abs: W6/32 (anti-CD90, HLA class I, IgG2b; BioLegend, San Diego, CA); clone BAM 195 (anti-MICA, IgG1) (36) and mAb 6D4 (anti-MICA/B, IgG1) were provided by Veronika Groh (Fred Hutchinson Cancer Research Center, Seattle, WA); M295 (anti-ULBP1, IgG1), M310 (anti-ULBP2, IgG1),
M550 (anti-ULBP3, IgG3), and M478 (anti-ULBP4, IgG1) were gifted by D. Cosman (Ampgen, Seattle, WA); mAb L95 (anti-PVR, IgG1) and mAb L14 (anti-Nectin-2, IgG2a) were developed and characterized as described in Bottino et al. (21).

After the cell incubation with appropriate primary mAbs, cells were incubated by FITC-conjugated goat anti-mouse secondary Abs (BioLegend). In all experiments, as a first step cells were incubated with human serum for 15 min and isotype-match controls were used to set up the negative values. Samples were analyzed by a FACSVantage (Becton Dickinson, Mountain View, CA). The NK cells were analyzed using the following Abs: anti-CD56, clone B159; anti-CD57, clone HNK1; anti-CD3, clone UCHT1; anti-CD16, clone 3G8; and anti-CCR7, clone 3D12 (Becton Dickinson).

A double flow cytometry staining on CICs was performed to understand the correlation between CD133 and HLA-1 in CICs and primary tumor cells. The coxin cancer-initiating cell lines were analyzed by indirect immunofluorescence and flow cytometry analysis using the following Abs: CD133/2 (293C3, mouse IgG2b; Miltenyi Biotec) and HLA-1 as previously described. CICs and their tumor counterparts were characterized by immunofluorescence and cytofluorimetric analysis for the expression of CD133, CD24 clone ML5, CD44 clone G44-26, Ep-CAM clone EBA-1, SOX2 clone 245610, and CEA clone B1.1/C66 (Becton Dickinson). CICs expressed homogeneously (70–90% of positive cells) these markers (Ref. 10 and 21).

Fluorescence staining of cells by NCR-Fc molecules
To measure NCR ligand expression, immunofluorescence and flow cytometry analysis were performed using NCR-Fc fusion protein: NKp30-Fc, NKp44-Fc, and NKp46-Fc.

Cells (2 × 10⁶) were sequentially incubated with 200 µl heat-inactivated human serum for 15 min at room temperature and then with 2.5 µg/µl NCR-Fc fusion protein for 2 h on ice. Binding of NCR-Fc was revealed by secondary incubation with R-PE–conjugated F(ab\(^\prime\))₂ fragments of goat anti-human Fc secondary Ab (Jackson ImmunoResearch, Baltimore, MD). As control staining for recombinant soluble NCR proteins, secondary Ab alone was used. Cells were washed and analyzed by FACSVantage (Becton Dickinson) and the results were analyzed using FlowJo software version 9.3.1.

**NK cell generation assay**
NK cells preparation was done as described elsewhere (37). Briefly, PBMCs were isolated by Biocoll separating solution (Biochrom AG, Berlin, Germany) density gradient centrifugation. Enriched NK cells were isolated from the separated PBMCs utilizing the NK cell isolation kit and VarioMACS (Miltenyi Biotec) according to the manufacturer’s instructions. The purity of the isolated CD3⁺ CD56⁺ NK cell populations was >95%.

This protocol was also used to isolate NK cells from PBMCs of cancer patients. Freshly enriched NK cells were suspended in RPMI 1640 culture medium (Life Technology, Milan, Italy) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml) and 10% FBS.

**Cytotoxicity assay**
Cytotoxicity assays were performed using the fluorescent 5,6-carboxyfluorescein diacetate (CFDA) NK assay. In CFDA NK assays, cytotoxicity was analyzed by flow cytometry using the protocol described elsewhere (38). Briefly, the target cells were labeled with CFDA-mixed isomers (Invitrogen, Milan, Italy). Target cells were mixed with effector cells at different E:T ratios. The incubation was performed in 96-well U-bottom plates at 37°C in a humidified 5% CO₂ incubator for 3 h. The specific lysis of target cells was calculated as follows: % specific lysis = (CT – TE/CT) × 100, where CT indicates mean number of fluorescent target cells in control tubes and TE indicates mean number of fluorescent cells in target plus effector tubes.

In the receptor blocking experiments, freshly purified NK cells were incubated for 30 min at room temperature with various mAbs before the addition of target cells. To block NKGD2 and NCRs, the mAb clone BAT221 (IgG1) and a combination of anti-NCR mAbs were used: anti-NKp46 clone KL247, anti-NKp44 clone KS38, and anti-NKp30 clone F252 provided by S. Parolini (University of Brescia, Brescia, Italy) (39). To block DNAM-1, mAb F5 (IgM) was used. As isotype control mAb (IB2002) was used, which recognizes a lymphocyte membrane–associated CD57 glycoprotein. All mAbs were used at a final concentration of 10 µg/ml.

**Mixed lymphocyte tumor cell cultures**
PBMCs from the peripheral blood of 1247 CRC patients were cultured in vitro at a 5:1 ratio with autologous irradiated (300 Gy) CRC-derived CICs or CRC tumor cells with 100 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA) and 10 ng/ml recombinant human IL-7 (PepperTech, Rocky Hill, NJ) in X-VIVO 15 (Cambrex/Lonza, Basel, Switzerland) plus 10% human serum. Cell cultures were weekly restimulated with irradiated autologous CRC-derived CICs or CRC tumor cells. Following two rounds of in vitro stimulation the specificity of PBMCs was assessed by IFN-γ secretion ELISPOT assay as previously described (40) (data not shown) or by the determination of the cytotoxic activity. The phenotype analysis of PBMCs was performed using a triple-color flow cytometric analysis (LSRFortessa II; BD Biosciences) using the following Abs: CD3, CD4, CD8, CD16, CD45RA, CD45RO, CD56, CD57, CD27, CD28, CCR7, CD25, CD127, CD134, CD137 (BD Pharmingen), and NKG2D (eBioscience, San Diego, CA).

**Confocal microscopy analysis**
Tumor cells and related initiating cells (2 × 10³) were treated with inactivated human serum (200 µl), washed by addition of 1× PBS, pelleted, and stained with MHC class I W6/32 (anti-HLA class I, IgG2a, 3 µg/ml; ebioscience) revealed by anti-mouse FITC secondary Ab, and with NCR-Fc fusion proteins (NKp30 and NKp44) stained with R-PE–conjugated F(ab\(^\prime\))₂ fragments of goat anti-human Fc secondary Ab (Jackson ImmunoResearch). Stained cells were fixed with fixation solution (Cytosoft/Cytoperm kit; BD Biosciences) and washed twice with permeabilization solution (5× PBS, 0.5% BSA, 5% Triton X-100), with the first wash containing DAPI (1 mg/ml stock, final dilution 1:1000; Molecular Probes).

Stained cells were recovered in mounting medium (ProLong antifade; Molecular Probes) and mounted on a glass coverslip. The images were collected on a Leica TCS SP2 confocal microscope (Leica Microsystems, Wetzlar, Germany) with a ×63 Apo PL A oil immersion objective (numerical aperture, 1.4) and 60-µm aperture. Cells were scanned from the bottom to the top (usually 7–10 horizontal scans) to identify the orthogonal plane for the evaluation of MHC class I, NKp30, and NKp44 distribution; Z-stacks of images were collected using a step increment of 0.2 µm between planes; at least 30 independent fields were scanned for each experimental point. The distribution patterns were analyzed by scanning the fluorescence intensity with ImageJ software 1.45s around the perimeter of the cells.

Human paraffin-embedded tumor tissues and CICs were stained for CD133, NKp30, NKp44, and NKp46. Both of the human tissue sections and cytospins of CICs were immobilized onto polystyrene slides to prevent loosening of the samples during the following step of the staining.

The slides of human tissues were placed in a rack, deparaffinized, and rehydrated according to the following washes: xylene, 10 min; xylene 1.1 with 100% ethanol, 3 min; 100% ethanol, 5 min; 95% ethanol, 5 min; 70% ethanol, 5 min; 50% ethanol, 5 min; and finally running cold tap water to rinse. All slides were kept in PBS buffer solution until ready to perform Ag retrieval.

Cytospins were prepared with freshly dissociated cells, which were washed twice with PBS buffer solution. After spinning, the samples were fixed with 2% paraformaldehyde for 30 min at 37°C. The slides were then washed twice with PBS and maintained in this same buffer until staining. After incubation with the appropriate primary Ab (8 h at 4°C), all samples were incubated with Rhodamine Red–conjugated secondary Ab (goat anti-mouse IgG [H+L]) for CD133 detection, or with Alexa Fluor 488–conjugated secondary Ab (goat anti-human IgG [H+L]) for NCR ligand detection. Both of the secondary Abs were diluted in the appropriate buffer and used for 1 h at 37°C. The nuclei were then counterstained with TOTO-3 iodide (Molecular Probes/Invitrogen). All slides were finally mounted with fluorescent mounting medium (Dako) and covered with slim coverslip. The fluorescent mounting medium increases the display of samples when subjected to observation with a fluorescence microscope. The slides mounted with fluorescent mounting medium were stored in the dark at 2–8°C, allowing them to retain the fluorescent signal for at least 1 mo.

**Micro-Raman spectroscopy measurements and spectra analysis**
Micro-Raman analysis is an unbiased method that allows determination of numerous chemical changes among different biological samples. Our previous study successfully demonstrated that this method could discriminate the difference in MHC class I molecule expression on several kinds of cells (41). Micro-Raman spectra are acquired by means of an inVia Raman microscope from Renishaw, equipped with an 832-nm laser source. All Raman measurements are recorded with a total laser power of ∼10 mW at the sample level and an accumulation time of 20 s, in the range from 800 to 1800 cm⁻¹. All cells were washed three times with PBS to eliminate all medium contaminants and then placed on calcium fluoride substrates.
Principal component analysis (PCA) is carried out on the preprocessed spectra to highlight the spectral differences between CRC-derived CICs (DV29, AV9, AP24, DN08, and AG2) and one allogeneic CRC line (HCT116). PCA is a multivariate statistical tool to handle problems described by a large number of variables. For spectroscopic studies, the total number of variables is given by all of the recorded frequencies (channels), as each one of these could provide useful information regarding the probed sample. PCA works on the data covariance matrix to extract few parameters, the PCs, which account for most information in terms of variance of the recorded spectra. Each PC is a linear combination of the former variables and it retains a certain amount of useful information, usually referred to as latent PCs. Typically only few PCs are needed to describe nearly the entire information hidden in the numerous old variables.

**Results**

**CICs and tumorigenesis**

Because CD133 is considered to be a selective marker for CICs in colon cancer (42), we tested its association with common stemness markers such as β-catenin, or Nanog, on human colorectal tumor tissues by confocal microscopy analysis (Fig. 1A). Their close association demonstrates that CD133 indeed is a suitable stemness marker in our colorectal cancer cell system. Therefore, we measured the percentage of CD133+ cells in our CIC lines. The flow cytometry analysis showed that ~75% of the spheroid cells were expressing this protein on their cell surface. Moreover, the CICs also express the Lgr5, CD166, and CD44 markers (Fig. 1C).

To clarify whether a single colon CIC retains the capability of multilineage differentiation, a single-cell cloning experiment was performed. We have calculated a mean percentage of 28% by extreme limiting dilution analysis. We therefore conclude that CICs represent a subpopulation of cells that contain stem-like features and have the capacity to differentiate in vitro (Fig. 1D).

The derived xenograft confirmed that CICs retain the capacity to initiate and sustain tumor growth in NOD/SCID mice. Histological examination of xenografts derived from CIC cultures showed that they present the same histopathological features as do their related human tumors (Fig. 1E). These data therefore confirm that the CICs within colon carcinoma represent cancer stem-like cells that contain tumorigenic capacity.

**Recognition of CRC-derived CICs by allogeneic and autologous NK cells**

To address whether colon CICs can be recognized by NK cells, we initially performed cytotoxicity assays with allogeneic NK cells.
purified from the blood of different healthy donors and challenged them in vitro with four different colon CIC lines. These had been derived from colon or rectum adenocarcinoma biopsies. As shown in representative experiments in Fig. 2A and 2B, all four of these CIC lines were more susceptible to NK cell lysis than were the adenocarcinoma cell lines included as controls. Fig 2C and 2D summarize the results from five consecutive experiments comparing the NK susceptibility of CIC lines (DV29, AV9, AP24, DN08, AG2, CC09) with conventional adenocarcinoma cell lines (HCT116, RKO). The CIC lines showed significantly increased NK susceptibility compared with adenocarcinoma lines.

To validate these observations and to introduce a more stringently controlled comparison, we next applied the same experimental approach using pairs of cell lines derived from the same tumors. One of the cell lines in each pair was established and maintained in stem cell–specific medium, leading to growth of CICs in the form of spheroids. In parallel, a cell line representing the whole colorectal cancer population from the same biopsy was established in conventional culture medium. This made it possible to directly compare the NK susceptibility of different tumor cells derived from the same neoplastic lesion. The representative experiment in Fig 3A illustrates that there was some variability between patients/tumors, but within each patient/tumor, the CIC line showed a higher susceptibility than did the “complete” CRC tumor. In a series of six comparative experiments, including CICs from two different patients and their autologous complete tumor lines as controls, each of the CICs showed a significant elevation of NK susceptibility (Fig. 3B–E).

In one patient it was possible to analyze the NK cells autologous response to either CICs (Fig. 4B) or related tumor cell stimulation (Fig. 4A). By culturing PBMCs in the presence of autologous CICs, a clear expansion of NK cells was observed (Fig. 4B). The subset of CD16+CD56dim NK cells expands with CIC stimulation (Fig. 4C), and half of these cells have a fully matured phenotype, as shown by CD57 staining (Fig. 4D), whereas there was no staining using an anti-CCR7 Ab (Fig. 4E). The NK cells purified from autologous PBMC/CIC cocultures were used in autologous cytotoxicity assays (Fig. 4F) where they showed a preferential recognition of autologous CICs, confirming the data obtained in the allogeneic experimental setting.

**The molecular dissection of NK cell–mediated recognition of CRC-derived CICs**

To decipher the molecular mechanisms behind NK cell recognition of CICs, we first focused on MHC class I molecules. These are the most potent inhibitory ligands for NK cell recognition, and it was thus conceivable that these molecules might play a role in regulating the recognition of CICs. As shown in Fig. 5, the MHC class I expression was barely detectable on CICs, whereas significantly higher levels were measured on autologous or allogeneic cancer lines representing the whole population (Fig. 5A, 5B). Our data are in agreement with a previous report (40).

We also carried out a double flow cytometric analysis using HLA-I and CD133 Abs to evaluate a possible correlation in the

![FIGURE 2](http://www.jimmunol.org/)  

**FIGURE 2.** NK susceptibility of CRC-derived CICs and CRC tumor cell lines. (A) Representative experiment where one CRC-derived CIC line, AG2 (▴), and two colorectal carcinoma cell lines, HCT116 (○) and RKO (△), were tested for their susceptibility to highly purified peripheral blood NK cells. (B) Representative experiment where three different CRC-derived CIC lines, AP24 (▴), AV9 (△), and DV29 (○), and one CRC cell line, HCT116 (●), were tested for their susceptibility to highly purified peripheral blood NK cells. (C and D) Combined data from five experiments using CRC-derived CICs (DV29, AV9, AP24, DN08, AG2, CC09) and CRC cell lines (RKO and HCT116) as targets for NK cells. There was a statistically significant difference between the two types of target cells at the 12:1 E:T ratio (p = 0.007) as well as at the 6:1 E:T ratio (p = 0.02), *p ≤ 0.05, **p ≤ 0.01.

![FIGURE 3](http://www.jimmunol.org/)  

**FIGURE 3.** Pairwise comparison of NK susceptibility of CRC-derived CICs and complete CRC cell lines derived from the same tumor. (A) Representative experiment where the colon CICs from patient 1076 (▴) and 1247 (●), maintained in stem cell medium as spheroids, and the complete autologous CRC tumor cells derived from the same patients 1076 (△) and 1247 (○) were tested for susceptibility to highly purified peripheral blood NK cells. (B–E) Combined data from six experiments where the NK susceptibility of CRC-derived CICs were compared with complete CRC cell lines from the same tumor (patient 1076 in (B) and (D), patient 1247 in (C) and (E)) at E:T ratios of 12:1 (B, C) and 6:1 (D, E). Using a pairwise Student t test two-tailed analysis on both cell systems there was statistically significant difference between the two types of target cells at an E:T ratio of 12:1 (B, 1076 p = 0.0005; C, 1247 p = 0.005) and at an E:T ratio of 6:1 (D, 1076 p = NS; E, 1247 p = 0.04); *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
expression of these two proteins, both in CIC lines and in their differentiated counterpart.

The results show that CD133 is overexpressed in CICs compared with their differentiated counterparts, whereas HLA-I expression is downregulated in CICs (Fig. 5C). We validated this observation by single-cell analysis of MHC class I expression by Raman microspectroscopy, as previously described (41). This biophysical analysis addresses a large variety of membrane properties without bias (e.g., introduced by Ab). Supplemental Fig. 1A shows that the PCA discriminated well between the colon CICs and the control line HCT116. The PC2 component alone was enough to account for the total spectral differences between these two classes of cells. The spectral composition (i.e., the loadings curve) of the PC2 component (Supplemental Fig. 1B) showed a pronounced peak located in the region 1650–1680 cm\(^{-1}\), thus indicating the mean peak corresponding to detection of MHC class I molecules. Collectively, the Raman analysis indicated that there are some differences between the CICs and the whole tumor populations, and that one of these concerns MHC class I expression.

When looking for molecules that might account for the differential NK susceptibility of CICs, we tested cell lines in our target cell systems for expression of ligands for key activating receptors on NK cells. No major differences correlating with NK susceptibility were observed for the ligands of LFA-1, DNAM-I, and NKG2D (ICAM-1, CD155 and CD112, and MICA/B and ULBP1–4, respectively; Supplemental Fig. 2). ICAM-1 showed a tendency to be more expressed on CICs, but none of these molecules was found to be expressed at statistically significant different levels on CICs and tumor cell lines. We next probed the target cells with Fc fusion proteins with each of the three members of the NCR group of activating receptors: NKp30, NKp44, and NKp46. Fig. 6 shows the FACS plot from one representative experiment (Fig. 6A–C) and a compilation of the total (n = 15) experiments (Fig. 6D–F) measuring binding of each of these fusion proteins to target cells. Ligands for NKp30, NKp44, and NKp46 were readily detectable on the surface of CIC lines, but not on cell lines representing the complete tumor population of either HCT116, RKO, or the autologous tumor. Interestingly, the expression of NKp30 and NKp44 ligands decreased dramatically when the CICs were cultured in differentiation-inducing media (Supplemental Fig. 3). The binding of NKp46-Fc fusion protein to CICs was less impressive, and no significant changes when comparing differentiated cells or complete tumor cell populations were found (Fig. 6C, 6F, Supplemental Fig. 3). Note that the same expression pattern of the NCR ligands and MHC class I molecules, as well as the same NK susceptibility, was found on xenografted explanted CICs (Supplemental Fig. 4).

To better understand whether NCRs ligands could correlate and/or colocalize with CD133 expression, a confocal microscopy analysis with immunofluorescence was performed on primary tumor tissues and CICs/primary tumor cells. The results show that in colon tumor tissues the expression of CD133 and NCR ligands is relatively low and is found preferentially at the very base of the crypts of the colon, often with a strong colocalization (Fig. 7A). As positive control we used the decidual tissue (43).

In the tumor cell cultures the results show that primary tumor cells maintain a very low expression of both CD133 and NCR ligands, whereas CICs expressed both proteins at higher levels (Fig. 7B). We also studied the expression and cell surface distribution of NK receptor ligands on the target cells with confocal microscopy–assisted immunofluorescence. We focused on the NKp30 ligand (NKp30L), NKp44 ligand (NKp44L), and MHC class I, because the expression of these molecules showed interesting differences between target cells in the experiments described so far. CICs and a CRC cell preparation from the same neoplastic lesion were probed using anti-MHC class I mAbs stained with anti-mouse FITC secondary Ab (green) and NCR-Fc fusion proteins (NKp30 and NKp44), revealed by secondary incubation with R-PE–conjugated anti-human Fc secondary Ab (red). As expected from the FACS analysis, the NCR ligand fluorescence intensity of both NKp30

**FIGURE 4.** Expansion and cytolytic activity of autologous NK cells stimulated either with CICs or related tumor. PBMCs from patient 1247 were cultured in the presence of autologous tumor cells (A) and autologous CICs (B). Analysis of NK cells performed gating on CD3 \(^+\)CD56 \(^+\) population. The CD3 \(^+\)CD56 \(^+\) population was analyzed for the expression of CD16 (C), CD57 (D), and CCR7 (E). Autologous NK cells mediated cytotoxicity against CICs and related tumor target cells (F).
(Fig. 8A, 8C) and NKp44 (Fig. 8B, 8D) was more abundant in CICs compared with the complete tumor cell line derived from the same neoplastic lesion. Moreover, the overlay of the two stainings demonstrated that the two molecules tended to colocalize in the same membrane region (Fig. 8A, 8B, yellow staining, and in surface scanning curves of Fig. 8C, 8D). The colocalization data suggest that MHC class I molecules may efficiently inhibit the formation of an activating synapsis between NK cells and CRC by being prelocalized close to areas harboring NKp30 and NKp44 ligands (44), whereas their low expression levels on the CRC-derived CIC cells surface could lead to a dominant activating effect of NKp44 and NKp30 ligand recognition by NCRs.

**NK susceptibility of CICs after Ab blockade of activating receptors**

To test the role of activating receptors more directly, we finally performed NK receptor blockade experiments. Fig. 9A summarizes the data obtained from three independent experi-
ments where CICs from patient DN08 was used as a target for NK cells. Different activating NK receptors were blocked using specific mAbs. The most prominent effect was obtained when an anti-NCR receptor mAbs mixture was added. Similar results were obtained using as target the 1247 CRC-derived CICs (Fig. 9B). These experiments demonstrate an important dominant role for NCRs in driving NK cell killing of CRC-derived CICs. Note that the blockade by the NCR mixture was not complete. The residual lysis may reflect the involvement of additional activating receptors.

FIGURE 7. Confocal microscopy analysis of distribution of CD133 and NCR ligands on CRC-derived CICs and complete tumor cell line derived from the same neoplastic lesion. (A) Confocal microscopy analysis of NKp30, NKp44, and NKp46 (green) on human decidual tissue (positive control, upper panels) and tumor tissue (lower panels). Nuclei were counterstained by TOTO-3 iodide (blue staining). Arrows indicate cells in which a correlation of expression of CD133 (red staining) and NKp30, NKp44, and NKp46 is observed. Original magnification ×40. (B) Confocal microscopy analysis of NKp30, NKp44, and NKp46 (green) on colorectal CICs (upper panels) and primary adherent CRC cells (lower panels). Nuclei were counterstained by TOTO-3 iodide (blue staining). Original magnification ×40. The phase-contrast microscopy images permit observation of cell morphology. Original magnification ×40. Arrows indicate cells in which a correlation of expression of CD133 (red staining) and NKp30, NKp44, and NKp46 is observed. One representative experiment of six different lines is shown.

FIGURE 8. Confocal microscopy analysis of distribution of MHC class I, NKp30, and NKp44 ligands on CRC-derived CICs and complete tumor cell line derived from the same neoplastic lesion. (A and B) The distribution of MHC class I molecule stained with W6/32 mAb (green) and NCR-Fc fusion proteins (red) in CICs and CRC tumor cells from patient 1247 was detected by confocal microscopy. The cell nuclei were stained with DAPI (blue). For each panel, a single plane confocal image shows the central section of the cell. Scale bars, 5 μm. (C and D) Relative fluorescence intensity profiles for MHC class I and NCR-Fc fusion protein channels, along a line scan through a representative cell, are shown. One representative comparison is shown from a series of three experiments.
FIGURE 9. Effect of Ab-mediated receptor blockade on NK cell recognition of CRC-derived CICs. NK cell–mediated killing in the absence and presence of Abs to different activating receptors (NKG2D, DNAM-1, a mixture of Abs to NKP30, NKP44, and NKP46 and isotype control). (A and B) Results from a representative CFDA cytotoxicity assay with CRC-derived CICs from patients DN08 (A) and 1247 (B) at E:T ratio 10:1.

Discussion

We have explored the possibility of targeting colorectal-derived CICs with allogeneic NK cells. Moreover, we have compared the NK cell recognition patterns of the two colon adenocarcinoma cell compartments; that is, the initiating cancer cells (CICs) and the related “differentiated” tumor. Our results reveal that resting allogeneic NK cells show a robust cytotoxicity effect on CRC-derived CICs, whereas killing of related differentiated cancer cells was lower.

Autologous NK cell recognition of CICs derived from patient 1247 has been performed using PBMCs stimulated with autologous CICs. In this coculture a preferential expansion of NK cells was observed, and when they were purified and tested in autologous cytotoxicity assays against CICs and complete tumor, a preferential recognition of the former was observed. The observed expansion of the CD56dimCD57+ NK cell subset is reminiscent of that observed during virus infections (45, 46). To our knowledge, we report in this study for the first time that CICs express NKP30L and NKP44L and that their levels are higher than those in the related differentiated and CRC cell lines. These data define a new biological context where NCR ligand expression occurs, that is, the early stage of tumor formation.

Moreover, MHC class I molecules, known to inhibit NK recognition, showed the reverse expression pattern: low on CICs, high on cells in the complete tumor population. Our confocal microscopy studies of the topographical membrane distribution of NKP30L/NKP44L and MHC class I are in line with the flow cytometry data and, additionally, they suggest that these molecules may colocalize in the membrane prior to binding of NK cells. It is thus conceivable that this high expression of inhibitory ligands and low expression of activating ligands may contribute to the relative resistance of the more differentiated tumor lines. The notion that ligands for the activating receptors of NKP30 and NKP44 are partly responsible for the high NK susceptibility of CICs was verified by NK receptor blockade experiments. Abs directed against NCRs reduced the killing considerably, but not completely, thus leaving the possibility that additional activating receptors may be involved.

Three earlier studies analyzed the NK cell interactions with glioblastoma, melanoma, oral carcinoma, and mesenchymal-initiating cells (47–51). A common pattern emerges from the reports. Melanoma and glioblastoma cancers initiating cells were highly resistant to resting allogeneic NK cells; they became susceptible to NK cytotoxicity only after effector cells had been activated by IL-2. Their phenotypes are characterized by low MHC class I expression and the presence of activating ligands for NK receptors. These cells, although resistant to freshly isolated NK cells, were highly susceptible to lysis mediated by both allogeneic and autologous IL-2 (or IL-15)–activated NK cells. The analyzed glioblastoma-initiating cell culture did not express protective amounts of HLA class I molecules, whereas they did express DNAM-I and NKP46 ligands (39). Probably the killing activity of resting or activated NK cells is modulated by the different derivation compartment of the specific tumor: epithelial in CRC, whereas ectoderm for the others (melanoma and glioblastoma). A direct correlation between NK cell infiltrates of the colon cancer lesion and a better prognosis has been proposed (52). It is tempting to speculate that the main contribution of NK cell infiltrates in the colon adenocarcinoma lesion may be to eliminate the CICs, therefore limiting the disease burden.

Because CICs have been reported to be both drug resistant and radioresistant, encouraging results reported in literature (45–49) indicate that NK cells could target CICs. Todaro et al. (53) have recently shown that bisphosphonate zolendronate treatment of colon CICs induces a high susceptibility to γδT cell killing. This cytotoxic interaction was regulated via TCR and to a lesser extent by NKG2D receptors. Our data add to this observation, indicating that CICs can be targeted also by another effector mechanism, NK cells. It has recently been reported that bisphosphonate zolendronate triggers NK cell activation via dendritic cell maturation in a γδT cell–dependent manner (54). Considering the data from Todaro et al. (53) together with data in the present study, it is interesting to speculate that CICs could be optimal targets for immunotherapy intervention based on activation and/or adoptive transfer of NK and γδT cells.

Numerous innate and adaptive immune effector cells and molecules participate in the recognition and destruction of cancer cells, a process often referred to as cancer immunosurveillance. Cancer may sometimes avoid such immunosurveillance through the outgrowth of nonimmunogenic tumor cell variants (immuno-selection) and through subversion of the immune system (immunosubversion) (55). It will be important to study both of these processes in relationship to the CIC compartment.

Definition of the CIC compartment plays a crucial role in the natural history of the disease and metastatic progression, as well as in its chemo- and radioresistance. This may provide a piece in the puzzle of the frustrating history of anticanter therapy and the limited success of immunotherapy. This calls for new approaches to target the tumor-initiating cells. In this study, we provide evidence that NK cells can eliminate CICs with high efficiency. This study provides a base for further exploration of NK cells as a possible key player in immunotherapy of solid tumors.

Disclosures

The authors have no financial conflicts of interest.

References

NK CELLS RECOGNITION OF COLON CANCER–INITIATING CELLS


