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The Apoptosis Inhibitor Protein Survivin Induces Tumor-specific CD8$^+$ and CD4$^+$ T Cells in Colorectal Cancer Patients[1]

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ABSTRACT

The identification of tumor-associated antigens expressed by colorectal carcinoma remains one of the major goals for designing novel immunological treatments for this tumor. By using a reverse-immunology approach, we show here that the inhibitor of apoptosis protein, survivin, is immunogenic in colorectal cancer patients. In particular, we found that survivin elicited CD8$^+$ T cell-mediated responses in peripheral blood or in tumor-associated lymphocytes from patients at different disease stage. Colorectal carcinoma cells were recognized by survivin-specific T lymphocytes, and the survivin-specific, class-I HLA-restricted T lymphocytes were fully activated and released interleukin-2 in response to HLA/survivin-peptide complexes expressed by tumor cells. In addition to CD8-mediated responses, survivin specifically stimulated CD4$^+$ T-cell reactivity in peripheral blood lymphocytes from the same patients, thus suggesting that a complete activation of the immune system may occur in response to this antiapoptotic protein. These findings indicate that survivin could be considered a valuable tumor-associated antigen for immune-based clinical approaches in colorectal cancer.

INTRODUCTION

CRC[3] is one of the most common and dreadful malignancies, for which surgery remains the main therapeutic option, although the success of the treatment depends on the disease stage. Unfortunately, the majority of patients present at diagnosis with already advanced local disease with microscopic spread to local lymph nodes; for these patients the life expectancy does not exceed 15 months (1). Although adjuvant systemic chemotherapy or chemoradiation has shown a limited but significant survival advantage, novel and more effective therapies are needed.

CRC has been considered poorly immunogenic and, therefore, refractory to immune-based interventions. However, with the discovery of the molecular nature of TAAs as well as the development of the reverse-immunology approaches, it became clear that CRC cells do express proteins able to induce a T cell-mediated response (2–5). Systemic antitumor T-cell response may occur in CRC patients with advanced metastatic disease (2). Moreover, it has been demonstrated that TILs within cancer/cell nest do have a prognostic value (6). These findings support the conclusion that CRC could be immunogenic *in vivo* and imply that strategies aimed at potentiating immune-responses may lead to a clinical benefit when administered in an adjuvant setting (7, 8).

Most of the immunological interventions in CRC patients were aimed at stimulating the immune system against a specific TAAs defined by reverse-immunology approaches (3, 9, 10). Attention has been focused on mucins, carcinoembryogenic antigen and epithelial cell adhesion molecule (11–13). Although present also in normal colonic mucosa, these proteins are overexpressed in CRC, and immunotherapy approaches exploiting these antigens have led to the *in vivo* expansion of a systemic antigen-specific immunity, although only occasionally associated with objective clinical responses (14–17).

Thus, identification of new TAAs remains one of the major goals for designing a more effective immunological treatment of CRC. Ideal targets for immunotherapy are gene products silenced in normal tissues, overexpressed in cancer cells, and directly involved in tumor survival and progression. Boosting the immune response against this type of proteins can be a strategy that, avoiding or at least limiting the *in vivo* generation of antigen-loss variants, could be more efficient in the control of *in vivo* tumor growth and in affecting the disease prognosis.

IAPs constitute a family of proteins that potentially fulfills the features described above, because their main function is to block apoptosis, thus preventing cells from physiological death and, therefore, extending cell survival (18, 19). Survivin, a recently described member of IAPs (20), shows unique features if compared with other apoptosis regulators. First, it is practically undetectable in normal differentiated tissues but overexpressed in most human cancers (20–26), including CRC (27–29). Second, survivin is endowed with dual functions and, in addition to suppress apoptosis, it is also involved in regulating cell division (30, 31). In proliferating cells, survivin reaches maximal levels in G_2/M phase and becomes down-regulated in G_1 phase. Because of its cell-cycle-regulated expression and tumor specificity, survivin is thought to directly confer a selective growth advantage to tumor cells, allowing their progression through mitosis and leading to resistance to proapoptotic drugs (31, 32). In agreement with these functions, survivin overexpression correlates with tumor progression and appears to be a bad prognostic factor for a variety of solid tumors including CRC (22, 24–27). Therefore, survivin may be operationally considered a potential TAA, provided that such protein encompasses peptide epitopes recognized by T cells of cancer patients.

In the present study, we aimed at assessing such a hypothesis in CRC patients. Therefore, HLA-A2-positive CRC patients at different disease stages have been selected and their T cell-mediated response to survivin evaluated by a reverse-immunology approach. Taking advantage of a HLA-A2 survivin-derived peptide, shown previously to be a CD8$^+$ T-cell epitope (33–35), and using the recombinant protein, our data indicate that CRC patients may develop systemic class I and class II HLA-restricted T-cell responses against survivin; this specific immunity could be detected also in subjects with primary tumor and no metastatic disease. The simultaneous detection of survivin-specific CD8$^+$ and CD4$^+$ T-cell immunity, together with the evidence that CRC cells process and present survivin-derived peptides, support an immunological role of this protein in CRC patients and make survivin an appealing candidate for an immune-based therapy.

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[3] The abbreviations used are: CRC, colorectal cancer; APC, antigen-presenting cell; CTL, cytotoxic T lymphocyte; IAP, inhibitor of apoptosis protein; PE, phycoerythrin; IL, interleukin; LCL, lymphoblastoid cell line; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cell; TAA, tumor-associated antigen; TIL, tumor-infiltrating lymphocyte; ELISPOT, enzyme-linked immunospot.

Table 1 *Clinical features of CRC patients*

Patient number	Sex	Age	Site of tumor	Dukes' stage	TNM	Interval between surgery and blood sample (days)
1	M	53	Rectum	D	pT3N2M1	−6
2	M	55	Colon ascendens	B	pT3N0M0	0
3	M	70	Colon transversum	B	pT3N0M0	0
4	F	51	Colon sigmoideum	B	pT3N0M0	+6
5	F	55	Rectum	A	pT2N0M0	−1
6	F	64	Rectum	D	pT3N1M1	+7
7[a]	M	72	Rectum	C	pT2N1M0	0
8[b]	M	68	Rectum	D	T3N2M1	+10

[a] This patient received presurgical RT.
[b] The resection of the primary tumor and the diagnosis were performed in another Institution and the stage performed at the time of first relapse, 2 years later. The blood sample was obtained after resection of local relapse and the patient was receiving chemotherapy.

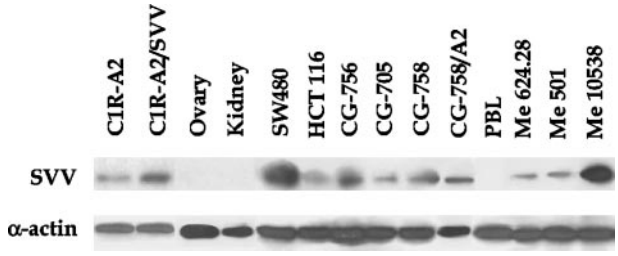

Fig. 1. Survivin expression as assessed by Western blot analysis. C1R-A2, C1R-A2 transfected with survivin (C1R-A2/SVV), colon cancer cell lines (SW480, HCT116, CG-756, CG-758, and its HLA-A2 transfected variant CG-758/A2), and melanoma cell lines (Me624.28, Me501, and Me10538), PBMCs, and normal ovarian and renal epithelial cells were lysed, and 30 μg of proteins were electrophoresed, blotted, and blot hybridized with survivin-specific mAb (*top panel*). As control for protein loading, filters were stripped and hybridized with mAb directed against actin (*bottom panel*).

MATERIALS AND METHODS

Peptide Synthesis. Peptides were purchased from Neosystem (Strasbourg, France); their degree of purification was analyzed by high-performance liquid chromatography. All of the peptides tested showed >95% purity.

MHC Stabilization Assay. Evaluation of HLA-A2 binding affinity was performed by flow cytometric assay based on the use of the HLA-A*0201 TAP-deficient T2 lymphoma cell line as described previously (36). Briefly, 5×10^5 T2 cells/well were seeded in a 48-well plate and incubated overnight at 27°C in 500 μl of RPMI 1640 (Bio-Whittaker Europe, Verviers, Belgium) supplemented with 10% FCS (Bio-Whittaker Europe) and 2.5 μg/ml of β_2-microglobulin (Sigma-Aldrich, Munich, Germany). Peptides were added to each well at concentrations ranging from 10 μM to 0.01 μM. After incubation, cells were washed to remove unbounded peptide and the surface expression of HLA-A2 molecules was evaluated by indirect immunofluorescence using, as primary antibody, the anti-HLA-A2 BB7.2 mAb (37) and a FITC-conjugated goat antimouse immunoglobulin-specific antibody (BioSource International, Camarillo, CA). Fluorescence intensity was evaluated using a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Data are reported as percentage of increase (over control) of BB7.2 mean fluorescence channel number reactivity. Controls are T2 cells cultured with 2.5 μg/ml of β_2-microglobulin but without peptide.

To determine the stability of the peptide/HLA-A2 complex, 2×10^6 T2 cells were incubated overnight at 27°C in 2 ml of RPMI 1640 supplemented with 10% FCS and 2.5 μg/ml of β_2-microglobulin. Peptides were added at a concentration of 10 μM. Cells were then washed to remove the unbounded peptide and incubated at 37°C for 0, 2, 4, 6, or 8 h. Cell surface expression of HLA-A2 at each time of incubation was evaluated by indirect immunofluorescence using the anti-HLA-A2 BB7.2 mAb. Results are expressed as percentage (over control) of the remaining BB7.2 mean fluorescence channel number reactivity. Controls are the BB7.2 mean fluorescence channel number of T2 cells at the onset of incubation at 37°C.

Patient PBMCs. Upon informed consent, blood samples were collected from HLA-2-positive patients with CRC, who underwent curative resection at the Unit of Colorectal Surgery of our Institute. PBMCs were isolated by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation of heparinized blood. Erythrocytes were lysed using ACK hypotonic solution, and then mononuclear cells were washed twice with PBS (Bio-Whittaker Europe), resuspended in RPMI 1640 supplemented with 30% heat-inactivated human serum and 10% DMSO (Fluka, Buchs, Switzerland) and cryopreserved in liquid nitrogen. One ml of fresh blood samples treated previously with ACK [100 μM EDTA, 10 mM $KHCO_3$, and 155 mM NH_4Cl (pH 7.2)] hypotonic solution was used for the analysis of HLA-A2 expression. Cells were stained with the mAb BB7.2 and then with a FITC-conjugated goat antimouse IgG (BioSource International). Fluorescence intensity was analyzed by a FACScan flow cytometer and CellQuest software (Becton Dickinson). This preliminary screening allowed us to select HLA-A2 patients additionally confirmed to be HLA-A*0201 by genotyping. The clinical features of the selected patients are reported in Table 1.

Antigen Stimulation of Peripheral Blood Lymphocytes. On day 0, 5×10^6 cells were seeded in 24-well plates in 2 ml RPMI 1640, supplemented with 10% heat-inactivated HS, 2 mM L-glutamine (Bio-Whittaker Europe), and antibiotics. To induce the generation of $CD8^+$ survivin-specific T cells, PBMCs from patients were first sensitized for 1 week with 10 μg/ml of the

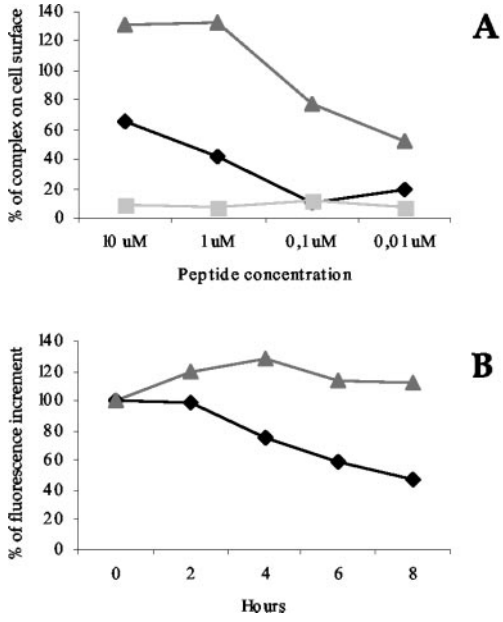

Fig. 2. HLA-A2 binding capacity of SVV-1 peptide, as evaluated by MHC stabilization assay. *A*, T2 cells were incubated overnight at 27°C in RPMI 1640 supplemented with 10% FCS and 2.5 μg/ml β_2-m in the presence of the following peptides at the indicated concentrations: HLA-A2-restricted ELTLGEFLKL SVV-1 (♦), HLA-A2-restricted GILGFVFTL $Flu_{NP58-66}$ (▲), and (as a negative control) HLA-A3-restricted ILRGSVAHK $Flu_{NP265-273}$ (■). The expression of the HLA-A2/peptide complex was evaluated by indirect immunofluorescence using the BB7.2 mAb. Results are expressed as percentage increment in BB7.2 reactivity relative to T2 incubated in the absence of peptide (see "Materials and Methods"). *B*, T2 cells were incubated overnight at 27°C with 10 μg/ml of each peptide, then washed and incubated at 37°C. Cells were collected at the indicated time points and HLA-A2/peptide complex expression evaluated by BB7.2. Results are expressed as percentage (over control) of the remaining BB7.2 mean fluorescence channel number reactivity. Controls are the BB7.2 mean fluorescence channel number of T2 cells at 0 h of incubation at 37°C.

survivin-derived peptide. On day 1, IL-2 (EuroCetus, Amsterdam, the Netherlands) was added at a final concentration of 60 IU/ml; then cells were maintained under the same conditions, only replacing half of the medium and IL-2 every 2 days. Restimulation was performed every 7 days, using autologous PBMCs pulsed with 10 μg/ml of ELTLGEFLKL SVV-1 peptide for 2 h and then γ-irradiated (3000 rad). Free peptide was washed out and APCs added at a stimulator:responder ratio of 5:1.

Patient PBMCs were also cultured *in vitro* for 1 week in the presence of 5 μg/ml of the survivin recombinant protein, and IL-2 was added 2 days later, at a final concentration of 60 IU/ml. Restimulation was performed every week, by adding autologous, γ-irradiated (3000 rad) PBMCs pulsed for 2 h with 5 μg/ml of the recombinant protein. Pulsed APCs were added at stimulator: responder ratio of 5:1. Cells were maintained in culture in the presence of 60 IU/ml of IL-2. Functional characterization of T-cell lines was performed by ELISPOT assay 7 days after the last restimulation. Cell-surface phenotype was also monitored in the growing T cells by analyzing the expression of T cell-specific markers CD3, CD4, and CD8.

Stable Transfection of C1R Cell Line. The C1R cell line is a human plasmacytoid leukemia cell line that does not express endogenous HLA-A or -B. C1R expressing HLA-A2 allele available in our laboratory (C1R-A2) were electroporated with pcDNA3/SVV plasmid, encoding the full-length cDNA of survivin gene. Briefly, 5×10^6 cells in 500 μl of RPMI 1640 without serum were transfected with 10 μg of plasmid DNA at 250 V/950 μF and then cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS and subjected to selection with 0.7 mg/ml of G418 (Sigma-Aldrich). After a selection period of 1 month, survivin expression in transfected cells was tested by reverse transcription-PCR and by Western blot analysis. The parental C1R-A2 cell line showed a basal level of survivin expression that was strongly increased in the survivin-transfected C1R-A2 cells (C1R-A2/SVV) as detected by both reverse transcription-PCR (data not shown) and Western blot (Fig. 1, *Lanes 1* and *2*).

Tumor Cell Lines and Normal Epithelial Cells. The CG-758 colon cancer cell line (kindly provided by Tamás Schweighoffer, Department of NBE Discovery, Boehringer Ingelheim Austria, Wien, Austria) has been established from a primary carcinoma of the colon sigmoideum; although derived from an HLA-A2 patient, CG-758 lacks expression of HLA-A2. CG-758 cells have been induced to express the HLA-A*0201 by gene transfer (kindly provided by Dr. Catia Traversari, MOLMED, Milan, Italy). The following HLA-A2-positive tumor cell lines were also used: SW480, HCT116 (from American Type Culture Collection), CG-756, and CG-705 colon cancer cell lines (the two latter also provided by T. Schweighoffer); Me 624.28, Me 501, and Me 10538 melanoma cell lines were generated in our laboratory from tissues obtained from metastatic melanoma patients.

Normal ovarian cells were obtained by scraping the surface of normal ovaries obtained at surgery for benign or malignant gynecological diseases other then ovarian carcinoma (38), and normal renal epithelial cells were obtained by enzymatic digestion of nephrectomy specimens (39). Human normal cells were maintained in culture for three to five passages in 199-

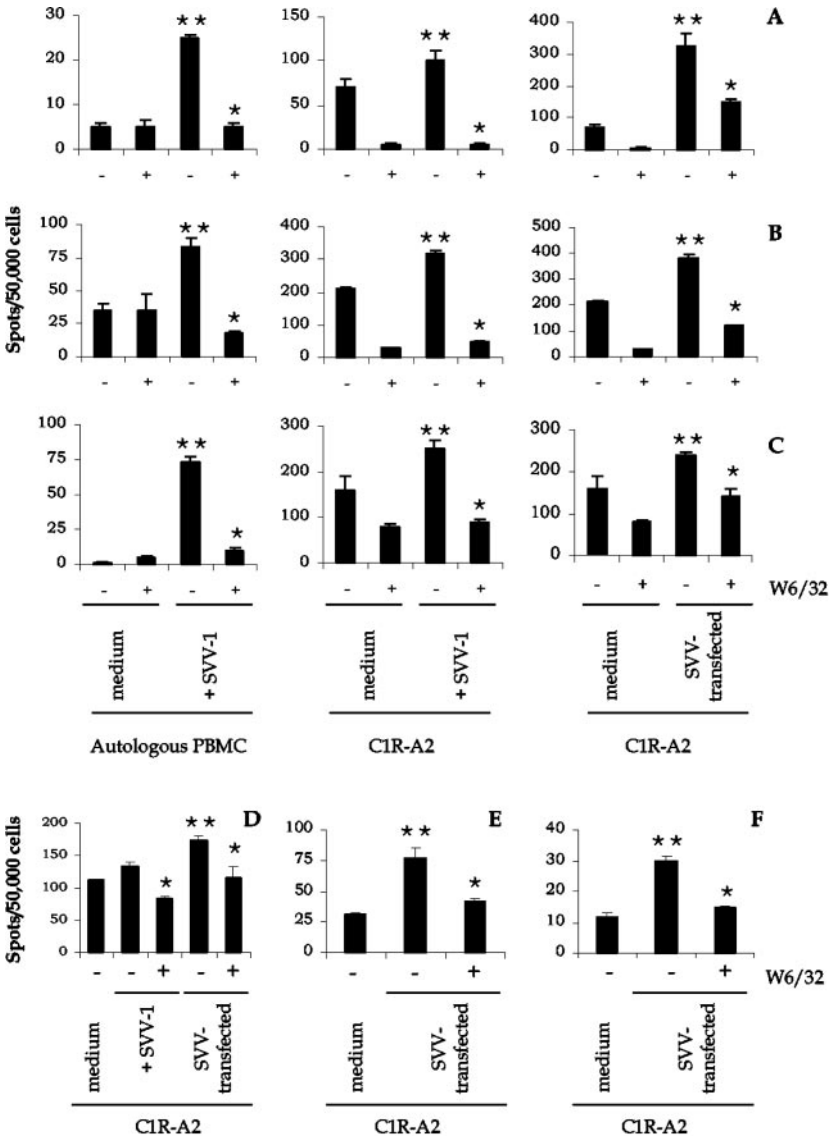

Fig. 3. Frequency of SVV-1 peptide-reactive T cells in PBMCs from CRC patients. PBMCs of patients #1, #7, #3, and #8 (*A, B, C,* and *D,* respectively) cultured *in vitro* for 3 weeks with SVV-1 peptide and PBMCs of patients #5 and #6 cultured for 1 week with SVV-1 peptide (*E* and *F,* respectively) were analyzed in the ELISPOT assay for the presence of IFN-γ-secreting T cells. The *in vitro* sensitized T lymphocytes were stimulated with: autologous PBMCs (2×10^4; *A–C, left panels*) alone or loaded with 2 μg/ml of SVV-1 peptide, C1R-A2 cells (2×10^4; *A–C, middle panels* and *panel D*) alone or loaded with 2 μg/ml of SVV-1 peptide; with survivin gene-transfected C1R-A2 cells (*A–C, right panels,* and *panels E* and *F*). Recognition of each target was evaluated in the presence (+) or in the absence (−) of the W6/32 anti-class I HLA mAb; *bars,* ±SD. *, $P < 0.05$ (evaluated by Student's *t* test for unpaired samples), as compared with the recognition of same target in the absence of anti-HLA mAb. **, $P < 0.05$ (evaluated by Student's *t* test for unpaired samples), as compared with the recognition of same target in the absence of peptide loading or not transfected with survivin gene.

MCDB105 medium (Sigma-Aldrich) or in DMEM (Bio-Whittaker Europe) supplemented with 15% FCS.

Primary cultures of ovarian and renal epithelial cells were obtained selecting adherent cells after enzymatic digestion of normal tissue specimens. The expression of HLA-A2 allele in the engineered CG-758 (CG-758/A2) as well as in all of the other tumor cell lines was assessed by fluorescence-activated cell sorter analysis using the anti-HLA-A2 BB7.2 mAb. All of the cell lines showed >95% of HLA-A2$^+$ cells with a mean fluorescence intensity ranging from 10^2 to 10^3 (data not shown). Ovarian and renal epithelial cells were HLA-A2 negative (data not shown).

Although with different level of intensity, all of the tumor cell lines tested were positive for survivin expression as evaluated by Western blot (Fig. 1). PBMCs, and renal and ovarian epithelial normal cells did not show any survivin-specific band at 16 kDa (Fig. 1).

T-Cell Stimulation Assay. For experiments aimed at evaluating the presence of a CD8$^+$-specific T-cell response, tumor cells, survivin-transfected C1R-A2, autologous PBMCs, and C1R-A2 pulsed for 2 h at 37°C with 2 μg/ml of SVV-1 peptide were used as targets. Inhibition experiments were performed by adding, during the last 30 min of the pulsing period, 1 μg/well of anticlass I HLA W6/32 mAb. Stimulation assays were performed in 96-well plates in a final volume of 150 μl and using an E:T ratio of 1:1. After overnight incubation at 37°C and 5% CO_2, supernatant were collected and the content of cytokines determined with specific ELISA.

For experiments evaluating the presence of a CD4$^+$-specific T-cell response, 5×10^4 autologous LCLs pulsed overnight at 37°C and 5% CO_2 with 2 μg/ml of the recombinant protein (survivin or TT as a control) or with total lysates obtained by four cycles of freezing and thawing of 5×10^4 cells were used as target. Inhibition experiments were performed by adding, during the last 2 h of the pulsing period, 1 μg/well of anti-HLA-DR L243 mAb. T cells were then added and incubation continued for 24 h. Stimulation assays were performed in 96-well plates in a final volume of 200 μl. Culture supernatants were collected, and IFN-γ released was determined by ELISA using a commercial kit (Endogen, Woburn, MA).

ELISPOT Assay. Nitrocellulose plates (96-well; MAIPS 450; Millipore, Bedford, MA) were coated overnight with 50 μl/well of 15 μg/ml antihuman IFN-γ mAb (Mabtech, Nacka, Sweden), washed, and blocked with PBS (BioWhittaker). Peptide-sensitized T cells (5×10^4 or 2×10^4 as indicated in the figures) were then seeded in 100 μl of medium. After incubation at 37°C and 5% CO_2 for 30 min, 100 μl of medium containing 2×10^4 target cells, prepared as described above, were added to each well. After 24 h of incubation at 37°C and 5% CO_2, ELISPOT was then developed according to manufactory instructions. Briefly, plates were washed six times with PBS. Wells were incubated for 2 h at 37°C with 50 μl/well of biotinylated mouse antihuman IFN-γ mAb (Mabtech) at a concentration of 2.5 μg/ml. After washing with PBS, 100 μl of streptavidin-alkaline phosphatase diluted 1:1000 was added for 1 h at room temperature. After another washing step with PBS, 100 μl/well of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Bio-Rad, Hercules, CA) was added to each well for 10–20 min. Color development was stopped by washing under running tap water. After drying at room temperature, IFN-γ-secreting T cells were counted using the automated image analysis system ELISPOT Reader (AID, Strassberg, Germany). Each experiment was performed in triplicate.

ELISA for IL-2. Evaluation of IL-2 release by stimulated lymphocytes was performed using precoated wells (IL-2 ELISA kit; Diaclone Research, Besançon, France). Target cells and lymphocytes were incubated in 96-well plates at stimulator:responder ratio of 1:1 in 96-well plates, at a concentration of 2×10^4 cells/well for each population. Coculture was performed overnight at 37°C and 5% CO_2. Then supernatants were collected and added to the precoated plates.

CTLL-2 Proliferation Assay. Target cells and lymphocytes were incubated in 96-well plates at stimulator:responder ratio of 1:1 in 96-well plates, at a concentration of 2×10^4 cells/well for each population. Coculture was performed overnight at 37°C and 5% CO_2. Supernatants were removed after 24 h for estimation of IL-2 secretion by culturing with the IL-2-dependent T-cell line CTLL-2. Briefly, CTLL-2 cells were seeded in 96-well plates at a concentration of 5000 cells/well, in the presence of 53 μM β-mercaptoethanol; supernatants were added to each well, and culture was performed for 18 h. Proliferation was determined by pulsing with [^{3}H]thymidine (1 μCi/well) for the final 18 h of culture.

Tetramer Staining. To stain survivin-specific CD8$^+$ T cells, soluble tetrameric complexes of HLA-A2/peptide were produced using the SVV-1 decamer. Tetramers were prepared by the tetramer facility at DIBIT (San Raffaele Hospital, Milan, Italy). 5×10^5 stimulated T cells were incubated for 45 min at 4°C with 1 μl of SVV-1/HLA-A*0201 tetramer (PE-conjugated) and

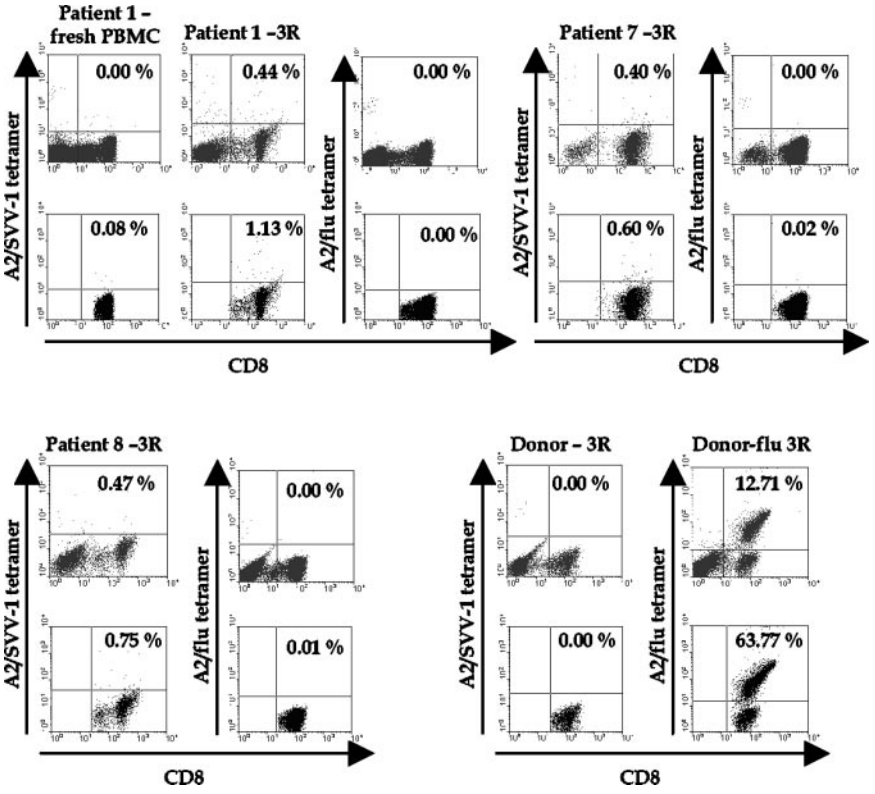

Fig. 4. Tetramer-guided analysis of survivin-specific CD8$^+$ T cells. PBMCs from three CRC patients (*patient 1–3R, patient 7–3R,* and *patient 8–3R*) and from a healthy donor (*donor-3R*) stimulated *in vitro* for 3 weeks with SVV-1 peptide were analyzed for the presence of survivin-specific CD8$^+$ T cells using HLA-A2-survivin tetramers. Unstimulated PBMCs from patient #1 (*patient 1-fresh PBMCs*) and HLA-A2$^+$ PBMCs from a healthy donor stimulated *in vitro* for 3 weeks with Flu nucleoprotein 58–66 peptide were also analyzed (*donor-flu 3R*). Cells were stained with FITC-conjugated anti-CD8 mAb together with PE-conjugated HLA-A2/SVV-1 tetramer or with PE-conjugated HLA-A2/flu $_{58-66}$ tetramer. *Numbers in the top right quadrant* indicate the percentage of CD8$^+$, peptide-specific T cells. *Top panels,* fluorescence analysis on whole T-cell population; *bottom panels:* fluorescence analysis on CD8$^+$-gated T-cell population.

washed three times with PBS 1×. Then cells were incubated with FITC-conjugated anti-CD8 mAb for 30 min at 4°C and washed. Fluorescence intensity was evaluated using a FACScan flow cytometer and analyzed using CellQuest software (Becton Dickinson). The percentage of peptide-specific T cells was evaluated after gating the $CD8^+$ T cells inside the T-cell population. As a negative control, survivin-specific T cells were also stained with Flu_{58-66}/HLA-A*0201 tetramer (ProImmune, Oxford, United Kingdom). As positive control of Flu_{58-66}/HLA-A*0201 tetramer, this same tetramer was used to stain PBMCs from a HLA-A*0201 healthy donor, cultured for 3 weeks in the presence of Flu_{58-66} peptide.

Western Blot Analysis. Cells were pelleted, washed in PBS, and lysed using a buffer containing 1% NP40, 0.1% SDS, 50 mM Tris HCl and 150 mM NaCl plus 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride. After protein quantification performed by using the DC Protein Assay (Bio-Rad), 20 μg of proteins were loaded on each lane and subjected to SDS-PAGE on 15% acrylamide gel. Proteins were then transferred overnight onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech).

Membranes were saturated for 1 h using 5% dry milk in 1× Tris-buffered saline 0.2% Tween and incubated for 2 h with 1 μg/ml antisurvivin antibody (ab7170; AbCam, Cambridge, United Kingdom) or with antiactin antibody (Sigma-Aldrich) diluted 1:1000 in the same solution. The membranes were then incubated for 1 h with a mouse antihuman IgG antibody conjugated to peroxidase (BD Transduction Laboratories, Heidelberg, Germany). The detection followed with the ECL detection system (Amersham Pharmacia Biotech). Blots were washed, and bands were visualized by ECL plus (Amersham Pharmacia Biotech) and autoradiography.

RESULTS

HLA-A2 Binding Capacity of a Survivin-derived Peptide. For the present study, the decamer survivin-derived epitope, ELTLGEFLKL, hereafter named SVV-1, was selected among the survivin-derived peptides described previously (34, 35) because of its highest HLA-A2 binding capacity. In fact, SVV-1 peptide did show an intermediate affinity for the HLA-A2 molecules, when compared with the Flu nucleoprotein 58–66 (GILGFVFTL) in an *in vitro* reconstitution assay using the TAP-deficient T2 cell line (Fig. 2A). Moreover, the HLA-A2/SVV-1 complexes were relatively stable with a low dissociation rate, measured as the time required for 50% of the cell surface HLA-A2 molecules to decay. We found that HLA-A2/SVV-1 complexes had a time required for 50% of the cell surface HLA- A2 molecules to decay >4 h (Fig. 2*B*). Considering the existing correlation between peptide-induced MHC stability and immunogenicity (40), SVV-1 could effectively constitute a T-cell epitope. In keeping with these *in vitro* data, T-cell response against the decamer survivin-derived peptide ELTLGEFLKL has been documented previously in melanoma, breast, and CLL patients (34, 35); however, no extensive data are available concerning a T-cell-mediated response directed against survivin in CRC patients.

Immunogenicity of SVV-1 Peptide in CRC Patients. To explore the immunogenicity of survivin in CRC patients, SVV-1 peptide was used to sensitize *in vitro* PBMCs or T lymphocytes purified from TIL obtained from $HLA\text{-}A2^+$ patients. The clinical status of each patient included in this study is reported in Table 1. To evaluate the specificity of the *in vitro* peptide-activated T cells, IFN-γ ELISPOT assays were performed. Autologous PBMCs loaded with SVV-1 peptide (Fig. 3, *A–C, left panels*), the $HLA\text{-}A2^+$ B lymphoblastoid C1R-A2 cell line expressing a basal level of survivin protein and the C1R-A2/SVV engineered to overexpress the survivin protein by gene transfer (Fig. 1) were used as specific targets (Fig. 3, *A–F*).

After 3 weeks of culture, peptide-specific reactivity was detectable in TIL (Fig. 3*A*) or in PBMCs (Fig. 3, *B–D*) of 4 CRC patients studied. T cells from patients were specifically activated to release cytokine by autologous PBMCs loaded with SVV-1 peptide (Fig. 3, *A–C, left panels*), or by the C1R-A2 cells expressing a basal level of survivin protein (Fig. 3, *A–C, central panels;* Fig. 3*D*). The number of T cells releasing IFN-γ increased when peptide-loaded C1R-A2 cells were used as targets (Fig. 3, *A–C, central panels;* Fig. 3D). Moreover, C1R-A2 overexpressing survivin by gene transfer was the best IFN-γ inducer suggesting that the *in vitro* peptide-stimulated T cells efficiently recognized SVV-1 peptide when endogenously processed and presented (Fig. 3, *A–C, right panels;* Fig. 3, *D–F*). Recognition of peptide-pulsed APCs, C1R-A2, and survivin-transfected C1R-A2 cells was inhibited by the mAb W6/32 showing that this T-cell reactivity was class-I-HLA-dependent and additionally supporting the specificity of our T-cell lines (Fig. 3, *A–F*).

T cells specifically recognizing the naturally processed and presented survivin peptide were earlier detectable in 2 additional patients (Fig. 3, *E* and *F*). T cells activated *in vitro* by peptide stimulation for 1 or 2 weeks were able to release IFN-γ in response to C1R-A2 cell line overexpressing survivin protein. Conversely, no specific reactivity was found in PBMCs of 2 additional patients (patients #2 and #4, see Table 1) and in 2 $HLA\text{-}A2^+$ healthy donors tested (data not shown).

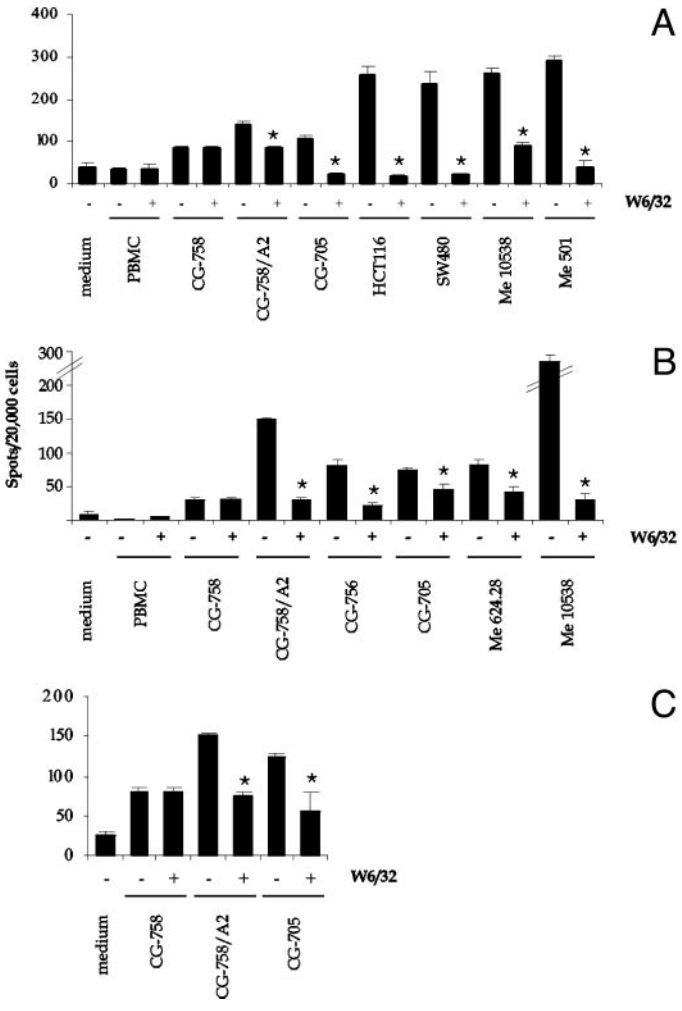

Fig. 5. Survivin-specific T cells recognized HLA-A2-positive, survivin-positive colon cancer and melanoma cell lines in a class I HLA-dependent fashion. The reactivity of peptide-stimulated T lymphocytes obtained from patients #1, #7, and #8, (*A–C*) was assessed by IFN-γ ELISPOT assay. T cells were incubated with medium alone or with the indicated tumor cell lines. Autologous PBMCs from patients were used as negative control. The T cell-specific reactivity against the cell lines was also evaluated in the presence of W6/32 anti-HLA class I mAb. *, $P < 0.05$ (evaluated by Student's *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA class I mAb; *bars*, ±SD.

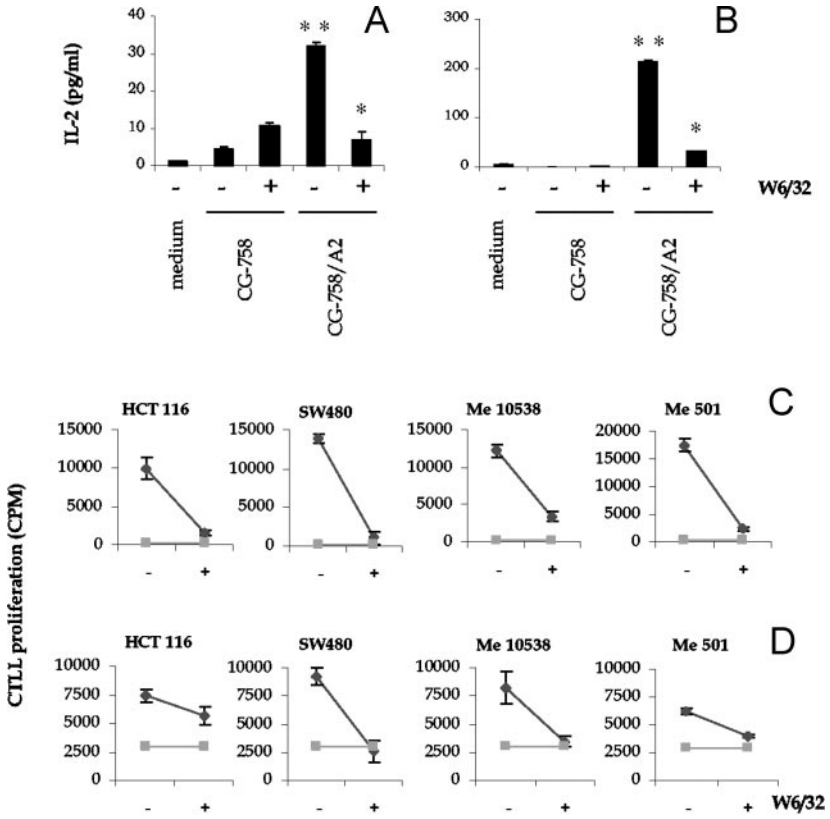

Fig. 6. Survivin-specific T cells released IL-2 when stimulated with survivin-positive cancer cells. *A* and *B*, survivin-specific T lymphocytes (5×10^4) of patients #1 and #7 (*A* and *B*, respectively) were incubated with medium alone or with CG-758 and CG-758/A2 colon cancer cell lines. T cell-specific reactivity was evaluated in the presence (+) or in the absence (−) of anti-class I mAb W6/32. The amount of IL-2 released was evaluated by ELISA. ∗, $P < 0.05$ (evaluated by Student's *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA class I mAb. ∗∗, $P < 0.05$ (evaluated by Student's *t* test for unpaired samples), as compared with the recognition of the HLA-A2 negative target. *C* and *D*, survivin-specific T cells (5×10^4) of patients #1 and #7 (*C* and *D*, respectively) were incubated with medium alone or with the indicated cancer cell lines in the presence (+) or in the absence (−) of anticlass I mAb W6/32. The IL-2 released in the supernatant by T cells was measured as the ability to support the proliferation of the IL-2-dependent T-cell line CTLL-2 (●). CTLL-2 proliferation was measured by [^{3}H]thymidine incorporation (*CPM*). ▨ indicate CTLL-2 proliferation supported by supernatants obtained by incubating survivin-specific T cells with medium alone; *bars*, ±SD.

SVV-1-specific CD8$^+$ T Cells Are Detected by Tetramer Staining. T cells at week 3 of culture obtained from PBMCs of patients #1, #7, and #8 were enough in number to allow additional *in vitro* studies. To additionally confirm their survivin-specificity and their CD8$^+$ phenotype, immunofluorescence staining was performed using SVV-1 specific PE-conjugated tetramer (Fig. 4). Compared with a nonstimulated control (Fig. 4; patient 1, fresh PBMCs), a significant percentage of tetramer-positive T cells was observed in the CD8$^+$ T-cell population for all of the 3 patients tested, with the highest frequency detected in T cells of patient #1 (Fig. 4). Conversely, no SVV-specific tetramer staining was detected in SVV-1-stimulated PBMCs from a healthy donor (donor 3R) that did not developed any survivin-specific reactivity as analyzed by ELISPOT (data not shown). Altogether, these results are, thus, consistent with those obtained by ELISPOT assays.

Survivin-specific T Cells Specifically Recognized Colon Carcinoma Cells. To see whether survivin-specific T cells recognize colon cancer cells, we selected as target a panel of colorectal carcinoma (CG-758 and its HLA-A2 engineered variant CG-758/A2, CG-705, CG-756, HCT116, and SW480, all HLA-A2 positive) and melanoma cell lines (Me 10538, Me 501, and Me 624.28, all HLA-A2 positive). Although with a different intensity, all of the tumor cell lines were found to express the survivin protein as evaluated by Western blot (Fig. 1). All of the melanoma and CRC cell lines were recognized by survivin-specific T cells in a class I-HLA-dependent fashion. The strongest tumor recognition was found for T cells from patient #1 (Fig. 5*A*) that did show the highest percentage of SVV-tetramer-positive T cells (Fig. 4; patient 1–3R).

Stimulation with the HLA-A2-negative CG-758 cell line led to a background release of IFN-γ by patient T cells that, however, was not inhibited by the anticlass I HLA mAb W6/32. Again, as shown in Fig. 2, no recognition of autologous PBMCs was found (Fig. 5, *A* and *B*).

IFN-γ is one of the cytokines released by stimulated T cells. However, optimal T-cell activation also leads to the induction of other cytokines that include IL-2, and, indeed, absence of induction of IL-2 release has been often associated with T-cell anergy (41). T cells of patients #1 and #7 were also evaluated for their capacity to release IL-2 in response to tumor stimulation (Fig. 6, *A* and *B*, respectively). The CG-758/A2 colon cancer cells, but not the HLA-A2-negative parental cell line, specifically induced IL-2 release in a MHC-class I-dependent fashion, as detected by ELISA.

To extend these findings, two additional colon carcinoma and two melanoma cell lines were used to stimulate survivin-specific T cells from patients #1 and #7. Tumor-stimulated T cells released biologically active IL-2 as shown by the ability of conditioned supernatants to support the proliferation of the IL-2-dependent CTLL-2 cell line (Fig. 6, *C* and *D*). Supernatants produced by T cells, cocultured with tumor cells in the presence of anticlass-I HLA W6/32 mAb, significantly inhibited the CTLL-2 proliferation indicating that IL-2 production by patient T cells was specifically induced by the recognition of HLA-A2/peptide complexes on tumor cells.

Altogether these data support the conclusion that survivin-specific T cells retain all their effector functions in response to tumor stimulation and imply that MHC/peptide complexes expressed at the tumor cell surface can lead to a full T-cell triggering.

Survivin-specific Immunogenicity in CRC Patients Includes Class II HLA-restricted T Cells. Taking advantage of the availability of the survivin recombinant protein, the presence of class II HLA-restricted T cell-mediated response against survivin was evaluated in patients #1, #2, and #8 (Fig. 7, *A*, *B*, and *C*, respectively). Patients #1 and #8 showed also a T cell-specific response to SVV-1 peptide (Fig. 3). Patient PBMCs were stimulated *in vitro* with the recombinant protein, and the reactivity of T cells obtained after two rounds of stimulations was evaluated by IFN-γ ELISA. All of the 3 patients showed a class II-restricted recognition of autologous LCLs loaded with survivin protein, whereas no reactivity was detected when

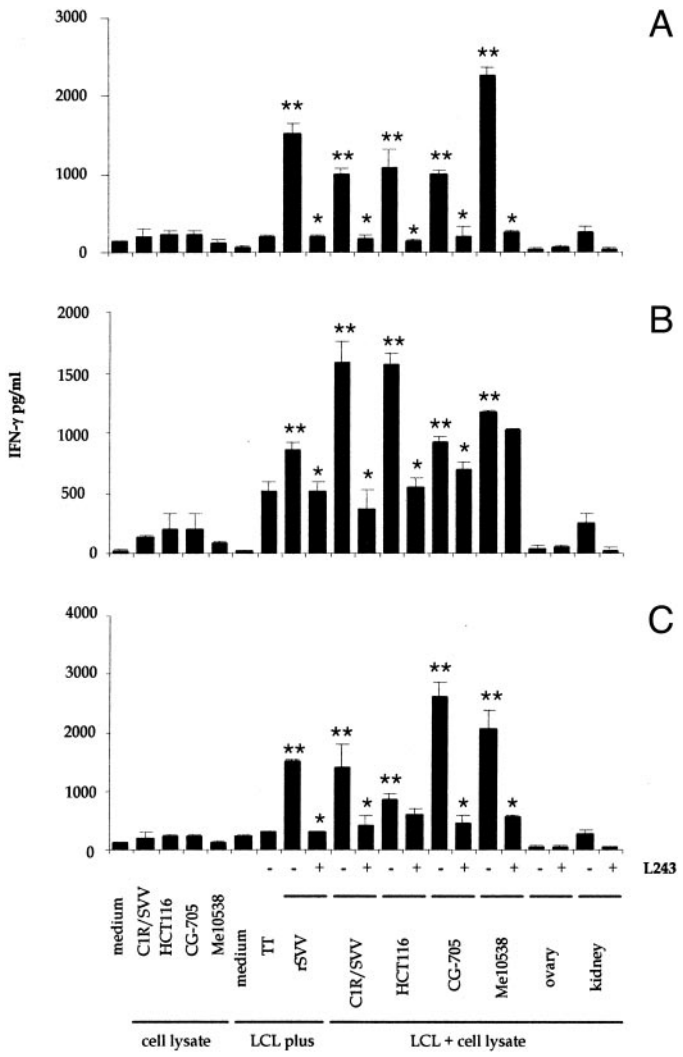

Fig. 7. HLA-DR restricted, survivin-specific immunity in colorectal patients. T cells (1×10^4) from patients #1, #2, and #8 (*A*, *B*, and *C*, respectively) sensitized *in vitro* with survivin recombinant protein for 2 weeks were stimulated with the following targets: autologous LCLs alone, or loaded with 2 μg/ml of the survivin recombinant protein or with 2 μg/ml of tetanus toxoid as a negative control; autologous LCLs pulsed overnight with lysates obtained from 5×10^4 cells from the survivin-positive colon cancer cell lines (*HCT116* and *CG-705*); melanoma cell line (*Me 10538*) or normal epithelial ovarian and renal cells. As control, the stimulation capacity of each cell lysate alone not reprocessed by APCs was also evaluated (*cell lysate*). Data are reported as pg/ml of IFN-γ released evaluated by commercial ELISA. HLA-DR restriction of T cells was assessed by adding anticlass II HLA mAb L243 to the test. *, $P < 0.05$ (evaluated by Student's *t* test for unpaired sample), as compared with the recognition of the same target in the absence of anti-HLA mAb. **, $P < 0.05$ (evaluated by Student's *t* test for unpaired sample), as compared with the recognition of the same target in the absence of protein loading; *bars*, $\pm$SD.

autologous LCLs were loaded with tetanus toxoid, used as negative control (Fig. 7, *A–C*). The extent of the response varied between patients, with the best reactivity detected in the T-cell population of patient #8 (Fig. 7*C*).

To evaluate the ability of the survivin-specific T cells to recognize the endogenous protein expressed by tumor cells, *in vitro* cross-presentation experiments were set up using as source of antigens cell lysates obtained from colon carcinoma (HCT116 and CG-705) and melanoma cell lines (Me 10538). Lysate of survivin-transfected C1R-A2 cell line was also added as positive control. As negative controls, lysates of primary culture of renal and ovarian normal epithelial cells were included. Autologous LCLs pulsed with lysate from tumor cells or from SVV-transfected C1R-A2 induced $CD4^+$ T cells to release IFN-γ (Fig. 7, *A–C*). IFN-γ production was HLA-DR-dependent, because the amount of cytokine was strongly reduced when T cells were stimulated in the presence of the anti-HLA-DR mAb L243. On the contrary, no activation occurred when normal epithelial cells that did not express survivin protein (see Fig. 1) were used as source of antigens.

Altogether these findings indicate the presence of survivin-specific, class-II HLA-restricted T cells in the peripheral blood of CRC patients. These T cells are endowed with the capacity to specifically release IFN-γ in response to cell-derived survivin protein, providing its processing and presentation by autologous APCs.

DISCUSSION

In the present study we show that CRC patients can develop a survivin-specific immunity involving both a $CD8^+$ and $CD4^+$ T cell-mediated response. These class-I or -II HLA-restricted T lymphocytes were able to recognize antigenic determinants derived from the survivin protein naturally processed by tumor cells or by patient APCs. Together with previous findings that showed the presence of survivin-specific antibodies in sera of CRC patients (42, 43), our data indicate the ability of this protein to stimulate both $CD4^+$ and $CD8^+$ T cells, strongly supporting its potential role as TAA for colon carcinoma.

To date very few molecules have been found to be antigenic in CRC patients, the majority of such molecules being expressed by normal mucosal cells (3, 9–12, 44). The finding that T-cell epitopes for $CD8^+$ and $CD4^+$ T cells are present in survivin protein that is down-regulated in normal tissues, but overexpressed in most common cancer types (20, 27) and has a crucial role in tumor progression (29), makes this protein a relevant antigenic candidate also for an immune-based anticancer therapy (45).

The SVV-1 (ELTLGEFLKL) peptide used to asses the immunogenic role of survivin in a class-I HLA restriction has been shown previously to be a T-cell epitope (33) eliciting specific CTLs in melanoma, CLL, and breast cancer patients (34, 35). Here we extend these findings, showing that the majority of CRC patients tested, 6 of 8, do have in their peripheral blood or in their tumor-associated lymphocytes survivin-specific T-cell precursors that, once sensitized *in vitro*, can recognize not only peptide-pulsed target cells, but also endogenously processed proteins expressed by appropriate APCs or by tumor cells. This same protocol of *in vitro* sensitization did not lead to any specific response in healthy donors, indicating that T cells of CRC patients have already recognized these TAAs upon encountering the tumor cells *in vivo*. Notably, the presence of a survivin-specific response was independent from the disease stage, because specific immunity could be detected even in a patient with a Dukes A tumor.

In the absence of the autologous tumor cells, the reactivity of survivin-specific T lymphocytes was tested using a panel of colon cancer cell lines. Our data showed that CRC cells do process and present sufficient HLA/survivin-peptide complexes to elicit a full T-cell response. In fact, tumor cells are efficiently recognized by T cells and, in addition to release IFN-γ when stimulated by colon cancer cells, survivin-specific $CD8^+$ T cells also released a detectable amount of IL-2. This cytokine is a key factor for $CD8^+$ T cells, and impaired IL-2 release is often associated with T-cell anergy (41). Moreover, it has been shown that tumor cells often lead to a suboptimal activation of T-lymphocyte clones that does not include the induction of IL-2 (46). The mechanisms accounting for the full activation achieved by survivin-specific T cells, when stimulated by CRC cells, may include the high TCR avidity/affinity of the specific T cells in conjunction with the high level of survivin expression in tumor cells.

Increasing attention has been focused recently on the role of $CD4^+$

T cells in antitumor immunity (47–49). Stimulation of $CD4^+$ T-cell functions was proved to be crucial for successful tumor eradication, and vaccination with tumor-specific helper epitope was a required step for the induction of a protective immune response (50). Here we show that survivin-specific immunogenicity in CRC patients includes also class-II HLA-restricted T-cell responses, and survivin-specific HLA-DR-restricted T cells were detectable in 3 of 4 patients. Two patients with a survivin-specific $CD4^+$ T cell-mediated response (patients #1 and #8) also developed a specific reactivity to HLA class I-restricted SVV-1 peptide, thus indicating that a complete activation of the different components of the adaptive immune response could occur in a single patient. Survivin-specific $CD4^+$ T cells recognized the protein expressed by tumor cells suggesting a reprocessing of the protein and presentation of its epitopes by autologous APCs. These data suggest a role for these CD4+ T cells in cross-priming events occurring *in vivo*. However, we were unable to detect specific release of IL-2 with our *in vitro* activated T cells in response to recombinant or tumor-derived survivin (data not shown).

Studies aimed at identifying the immunogenic epitope eliciting HLA class-II restricted response are currently in progress. However, comparing the HLA-DR typing of the responding patients, it is likely that different T-cell epitopes might be present in the survivin protein, because no HLA-DR allele sharing was evident among the patients who showed a $CD4^+$ T-cell response to survivin.

In conclusion, we demonstrated the simultaneous presence of T-cell response against survivin protein and survivin-derived class-I HLA-restricted epitope in CRC patients. We additionally showed that CRC cells could be recognized by survivin-specific T cells, whereas autologous PBMCs or normal epithelial cells did not.

Therefore, our results indicate that survivin could be considered an immunogenic TAA for CRC for which few molecularly characterized TAAs are currently available. Its immunological features, together with its pattern of expression, make this IAP protein a suitable target for designing immunotherapy approaches in CRC patients.

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