

Molecular and immunological evaluation of the expression of cancer/testis gene products in human colorectal cancer

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Abstract Tumor-specific gene products, such as cancer/testis (CT) antigens, constitute promising targets for the development of T cell vaccines. Whereas CT antigens are frequently expressed in melanoma, their expression in colorectal cancers (CRC) remains poorly characterized. Here, we have studied the expression of the CT antigens MAGE-A3, MAGE-A4, MAGE-A10, NY-ESO-1 and SSX2 in CRC because of the presence of well-described HLA-A2-restricted epitopes in their sequences. Our analyses of 41 primary CRC and 14

metastatic liver lesions confirmed the low frequency of expression of these CT antigens. No increased expression frequencies were observed in metastatic tumors compared to primary tumors. Histological analyses of CRC samples revealed heterogeneous expression of individual CT antigens. Finally, evidence of a naturally acquired CT antigen-specific CD8⁺ T cell response could be demonstrated. These results show that the expression of CT antigens in a subset of CRC patients induces readily detectable T cell responses.

Keywords Colorectal cancer · Cancer-testis antigen · T cell response

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Abbreviations

CT	Cancer/testis
CRC	Colorectal cancer
PBMC	Peripheral blood mononuclear cell
HLA-A2	HLA-A*0201

Introduction

Colon cancer is one of the most frequent cancers in affluent countries. Its prognosis remains poor, with 5-year survival rates approximating 40% [1], because the diagnosis is frequently made at an advanced stage of the disease. Although 80% of colorectal cancer (CRC) patients undergo a complete macroscopic removal of their malignant lesions by surgical resection, a significant proportion of them recur, usually as a result of already disseminated micrometastases at the time of surgery. Among the new approaches targeting these disseminated tumor cells, the use of tumor-specific cytolytic T lymphocytes (CTL) are of

particular interest. In that context, the presence of tumor-infiltrating T lymphocytes has been found to be associated with prolonged survival [2–4]. Even if the specificities of these T lymphocytes have not yet been elucidated, *in vivo* stimulation of CRC-specific T lymphocytes could be envisaged as adjuvant therapy to tumor resection.

Over the last 15 years, many tumor-associated antigens recognized specifically by CD8⁺ T lymphocytes have been identified and characterized. These antigens are commonly classified into four categories: cancer/testis (CT) antigens (aka cancer/germline or shared tumor-specific antigens), differentiation antigens, overexpressed antigens and antigens resulting from patient-specific point mutations. In CRC, CT antigens are particularly interesting targets because they are predicted to be expressed specifically by the tumor cells and not by the adjacent normal epithelial cells. Such specificity is not expected for antigens of the second and third category and may therefore reduce their usefulness in the immunotherapeutic treatment of CRC because of the side effects associated with the potential destruction of normal colon epithelia. Unfortunately, contrary to metastatic melanoma in which CT genes are expressed at frequencies ranging from 20 to 70%, CRC is generally considered as a poor CT antigen expresser since less than 10% of all analyzed CT genes are expressed at frequencies exceeding 20% [5]. Despite the rare expression of most CT genes, it remains unclear if the expression of one or the other CT antigen can elicit spontaneous T cell responses in CRC patients, as is the case for melanoma patients [6–9].

In this work, we monitored the expression of CT antigens in primary lesions and liver metastases of 51 CRC patients. We focused our analysis on CT antigens for which HLA-A2-restricted peptides had been previously identified. Expression was analyzed by semi-quantitative PCR and, in selected patients, by immunohistochemical staining of paraffin-embedded tumor sections. Our results indicate that CT antigens are rarely expressed in primary and metastatic colorectal tumors. However, in those patients that showed CT antigen expression in the primary CRC, the expression was maintained in the metastases. Finally, two patients bearing NY-ESO-1- and MAGE-A3-positive tumors (detected by RT-PCR and immunohistochemistry), respectively, were found to have detectable frequencies of circulating CD8⁺ T lymphocytes reactive against these antigens. Taken together, our results indicate that spontaneous T cell responses can be elicited by the expression of CT antigens in CRC lesions.

Materials and methods

Patients

Peripheral blood mononuclear cell (PBMC) and tumor samples of CRC patients were obtained after informed consent. The study protocol was approved by the Ludwig Institute for Cancer Research ethical review committee, as well as by the medical and ethical committees of the University Hospital (Lausanne, Switzerland). The patients' HLA haplotype was determined by serological typing. All patients were operated according to standard procedures and none of them was treated with preoperative neoadjuvant therapy. There were 4 stage I (T1–T2, N0, M0), 9 stage II (T3–T4, N0, M0), 4 stage III (any T, N1, M0) and 37 stage IV (any T, any N, M1) patients according to the TNM classification, sixth edition (2002).

Tissue preparation

Tissue samples from normal colonic mucosa, normal liver, colon cancer and metastatic colon cancer were obtained at the time of surgical resection. Tissue fragments were cut into small fragments and immediately frozen in liquid nitrogen. Samples were stored at -80°C until the time of mRNA extraction. In parallel, tissue samples were also embedded in paraffin and used for pathological analysis (TNM, sixth edition 2002) and tumor staging.

Isolation of mRNA and synthesis of cDNA

Frozen fragments were processed using Qiagen RNeasy MiniKit (Qiagen, Hilden, Germany). In brief, frozen material was weighed and mechanically dissociated by Polytron (Kinematica AG, Newark, NY, USA) in RLT buffer following the manufacturer's instructions. The samples were then treated with DNase I (Qiagen) to remove genomic DNA. Purified mRNA was quantified by spectrophotometry and stored at -80°C . mRNA was reverse-transcribed to cDNA using Qiagen RT-PCR Kit (Qiagen), starting from 4 μg mRNA for each sample.

Semi-quantitative PCR

The sequences of primer pairs used to amplify each CT antigen have been described previously [10–12]. Inter-sample mRNA content was evaluated by quantitative real-time PCR on the house-keeping gene β -actin (primers: 5'-GGCATCGTGATGGACTCCG-3'; 5'-GCTGGAAGGTGGACAGCGA-3') associated with

SYBR Green I dye (Roche Applied Science, Rotkreuz, Switzerland). Reactions were performed in a LightCycler 1.5 Instrument following manufacturer's instructions (Roche Applied Science). Relative abundance of β -actin mRNA did not vary by more than threefold among all samples tested (data not shown). CT genes were analyzed by semi-quantitative PCR on serial fivefold dilutions of cDNA. HotStarTaq DNA Polymerase (Qiagen) was used in all reactions following the manufacturer's instructions, with the exception of NY-ESO-1, which was performed using Taq DNA polymerase and the addition of Q solution (Qiagen). Thermocycling conditions were the following: MAGE-A3 (annealing temp 66°C, 31 cycles); MAGE-A4 (annealing temp 68°C, 33 cycles); MAGE-A10 (annealing temp 55°C, 39 cycles); NY-ESO-1 (annealing temp 60°C, 37 cycles); SSX2 (annealing temp 62°C, 40 cycles). All reactions were performed in a T3 thermocycler (Biometa, Göttingen, Germany). Expression was scored positive when a band was visible on agarose gel stained with ethidium bromide at any of the three dilutions. Absence of detectable band was scored as negative.

Immunohistochemistry

Tumor samples were fixed in 4% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin. Additional sections were studied by immunohistochemistry with two pan-MAGE-A monoclonal antibodies (clones 57B and 6C1) and the monoclonal anti-NY-ESO-1 antibody (clone D8.38) [13, 14]. Paraffin-embedded slides of testicular seminoma were used as positive controls. Immunohistochemistry slides were developed using Ultrateck-Peroxidase kit (Beckman Coulter, Fullerton, USA).

Isolation and expansion of peripheral blood lymphocytes

Blood samples from patients LAU846 and LAU911 were obtained before surgery. Ficoll-Paque centrifuged PBMC were cryopreserved in RPMI 1640 (Invitrogen, Basel, Switzerland) supplemented with 40% FCS, and 10% DMSO. In CTL induction assays, PBMC were thawed and plated in 96 U-bottom plates at 2×10^5 cells/well in the presence of 10 μ M peptide and 1 μ g/ml β 2-microglobulin (Sigma, Oakville, Canada), in RPMI 1640 supplemented with 8% pooled human serum, Penicillin/Streptomycin, HEPES and 2-mercaptoethanol (culture media), as described elsewhere [15]. Seventy-two hours later, 1,000 IU/ml IL-2 was added to cultures and the culture media was

replaced every second day until the day of functional analysis (day 10).

Tetramer analysis

Cytolytic T lymphocytes generated from PBMC were stained with Phycoerythrin-labeled HLA-A2/peptide tetramers. Cells (10^6) were incubated with tetramers (10 μ g/ml, 60 min at room temperature) and then with anti-human CD8 α mAb (30 min, 4°C). Dead cells were electronically excluded by propidium-iodide staining. CD8 $^+$ T cells (5×10^5 cells per sample) were analyzed by flow cytometry on a FACScan machine using CellQuest software (BD Biosciences, San Jose, CA).

IFN- γ ELISPOT assay

Human IFN- γ ELISPOT PVDF-Enzymatic kit (Diaclone, Besançon, France) was used according to the manufacturer's instructions. Human IFN- γ -specific capture mAb were adsorbed on MultiScreen-HA Filter 96 well Plates (Millipore, Billerica, MA, USA) for 16 h at 4°C and blocked with RPMI supplemented with 10% FCS for 60 min at 37°C. PBMC stimulated in vitro with 10 μ M peptide and IL-2 (1,000 IU/ml) for 9 days were seeded in each well (2×10^5 /well) in six replicates and stimulated with 10 μ M peptide. As positive control, an aliquot of the cells was stimulated with 1 μ g/ml PMA (Sigma) and 0.25 μ g/ml Ionomycin (Sigma). Plates were incubated for 18 h at 37°C in 5% CO $_2$, washed, and incubated with biotinylated anti-IFN- γ detection mAb followed by alkaline phosphatase-conjugated streptavidin. Spots were developed by adding peroxidase substrates (5-bromo-4,3-indolyl phosphate and nitroblue tetrazolium) and counted using the automated image analysis system Bioreader 2000 (BIO-SYS, Karben, Germany).

Results

CT gene expression profile in patients with primary colon cancer

On the basis of a recent survey, it was found that most CT antigens that are encoded by genes located on chromosome X were rarely expressed in colon cancers whereas they were frequently expressed in metastatic melanomas [5, 16]. Because it is not known if the sporadic expression of CT antigens in CRC could nevertheless elicit antigen-specific T cell responses we screened a series of CRC patients for the expression of CT antigens. We focused our analysis on MAGE-A3,

MAGE-A4, MAGE-A10, SSX2 and NY-ESO-1 because each contained at least one well-defined HLA-A2-restricted T cell epitope.

Samples from 41 patients with primary CRC and 14 patients with metastatic liver lesions were collected. For 30 of the 41 patients, we obtained fresh tissue from the tumor and from the normal adjacent colonic mucosa. Moreover, for 6 of these patients, we were able to collect tissue both from the primary and hepatic metastatic tumors. The median age of the patients was 66 years, ranging from 43 to 91 years. None of them received chemotherapy or radiotherapy prior to surgery.

RNA was extracted from each individual sample and reverse-transcribed. The expression of CT genes was assessed by semi-quantitative PCR. cDNA from testis and the CT-gene positive melanoma cell line SK-MEL-37 were used as positive controls. Normal colon tissue was used as negative control. To evaluate the amount of RNA extracted from each sample, we performed quantitative PCR on β -actin (data not shown).

As shown in Table 1, the expression of the different CT genes in primary CRC varied between 2% for *MAGE-A10* and 15% for *MAGE-A4*. The frequency of CRC patients expressing *MAGE-A3* was 13 and 4% for *NY-ESO-1* and *SSX2*. The majority of tumors did not express any CT gene, confirming the previous findings that colon cancer is a poor CT antigen expresser [5, 16]. However, co-expression of several CT genes was observed in patients LAU846, LAU888 and LAU911 (Fig. 1). Contrary to bladder carcinoma [17], we did not detect any correlation between the neo-expression of the CT genes and

Table 1 CT gene expression frequencies in colon cancer primary tumors

Antigen	<i>n</i>	Events	Frequency (%)
<i>MAGE-A3</i>	45	6	13
<i>MAGE-A4</i>	48	7	15
<i>MAGE-A10</i>	48	1	2
<i>NY-ESO-1</i>	48	2	4
<i>SSX2</i>	45	2	4

tumor differentiation, although the number of positive tumors was probably too small to draw firm conclusions.

Comparison of the CT gene expression profile between primary and metastatic tumors

Previous reports suggested that, in melanoma, the frequency of expressed genes was increased during progression from primary to metastatic disease [18]. Thus, the low frequency of CT antigen expression in CRC could be due to the fact that, contrary to melanoma, the majority of CRC tumor specimens are isolated from primary lesions [5]. To test this hypothesis, we screened samples obtained from liver metastases of 14 CRC patients. Importantly, for six of those specimens, we also had access to the corresponding primary tumor, allowing a comparison of the expression pattern within the same patient. The results obtained in patient LAU846 (Fig. 1) are representative of what was observed with the samples of the other five patients. Overall, the expression of *MAGE-A3*, -*A4* and -*A10*, *NY-ESO-1* and *SSX2* was not more frequent in the hepatic metastatic lesions

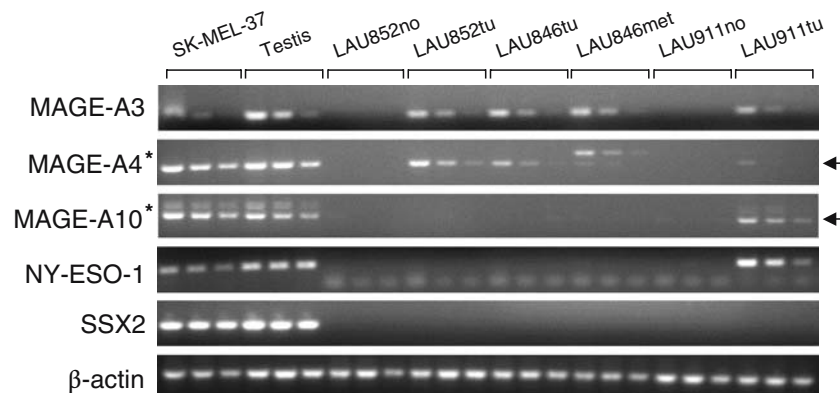


Fig. 1 Analysis of CT gene expression by semi-quantitative RT-PCR. Expression of MAGE-A3, MAGE-A4, MAGE-A10, NY-ESO-1, SSX2 and the housekeeping gene β -actin in normal and tumor samples of LAU852 and LAU911 as well as tumor and metastatic samples of LAU846 was analyzed by RT-PCR. For

each tissue sample, PCR was performed on serial fivefold dilutions of the cDNA. The cDNA prepared from human testis and the melanoma cell line SK-MEL-37 were used as positive controls. Arrows indicate the specific product and stars genomic contamination

than in the primary tumors (Table 2). We therefore conclude that the low expression of CT gene in CRC cannot be ascribed to the stage of the tumor but rather reveal intrinsic differences between melanoma and CRC.

Immunohistochemical analysis of CT antigens in CRC lesions

For some of the patients that were found to express one or several CT genes by RT-PCR, we performed immunohistochemical analysis of their tumor specimens. Paraffin-embedded histological slides of tumor samples located next to the ones used for RT-PCR were stained with monoclonal antibodies raised against MAGE-A3 (clone 57B), MAGE-A1 (clone

6C1) and NY-ESO-1 (clone D8.38). These antibodies have been extensively characterized earlier and cross-reactivity of the two anti-MAGE-A clones against other members of the MAGE-A family has been described [13].

Patient LAU846, LAU852 and LAU911 were found to express MAGE-A3 and MAGE-A4 in their tumors by RT-PCR. Immunostaining of the primary tumor specimens of these patients with mAb 57B confirmed the expression of MAGE-A3 detected by PCR (Fig. 2). A strong staining was observed for MAGE-A3 in testicular seminoma, whereas the intensity of the staining was lower in CRC, suggesting that the antigen was expressed at low levels. Staining with mAb 6C1 showed similar results for patients LAU846 and 852. Expression appeared much stronger in the samples from patient LAU911, possibly due to the strong expression of MAGE-A10, against which this mAb cross-reacts [13]. With both mAb, the staining was heterogeneous in the carcinomatous glands and predominantly in the nucleus of the tumor cells. Positive NY-ESO-1 staining was also found for patient LAU911. This result is in agreement with the expression detected by RT-PCR.

Table 2 CT gene expression frequencies in colon cancer metastases

Antigen	<i>n</i>	Events	Frequency (%)
<i>MAGE-A3</i>	12	1	8
<i>MAGE-A4</i>	14	2	14
<i>MAGE-A10</i>	14	0	0
<i>NY-ESO-1</i>	10	1	10
<i>SSX2</i>	10	0	0

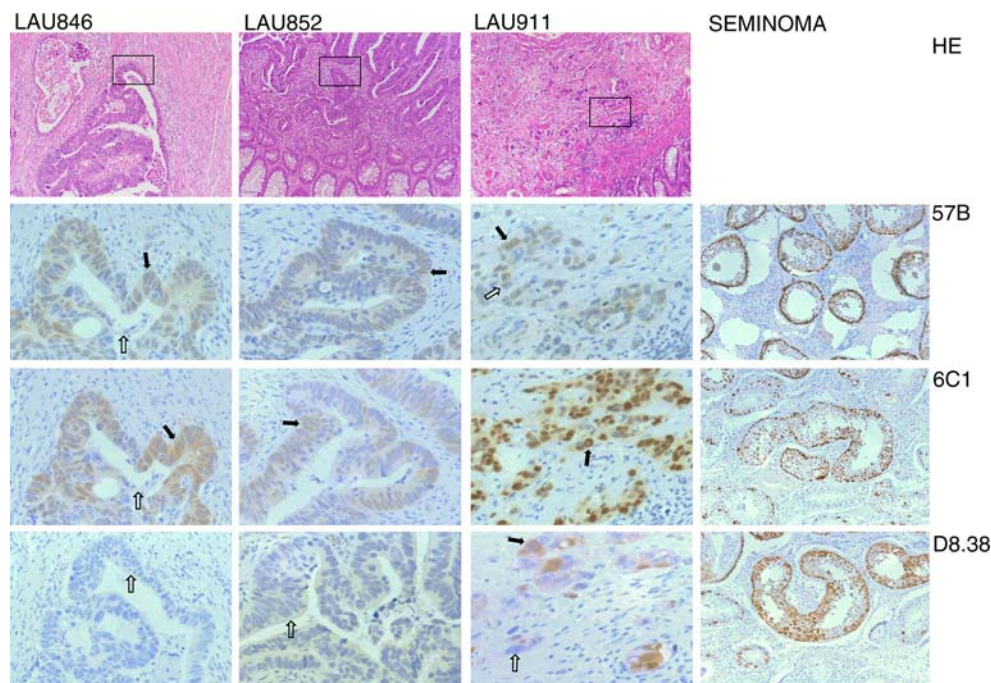


Fig. 2 Detection of CT antigen expression in CRC by immunohistochemistry. Sections of the primary CRC tumors isolated from the CT antigen-positive patients LAU846, LAU852 and LAU911 were stained with mAb anti-MAGE (57B and 6C1) and anti-NY-ESO-1 (D8.38). As positive control, sections from CT antigen-positive testicular seminoma were stained with the same

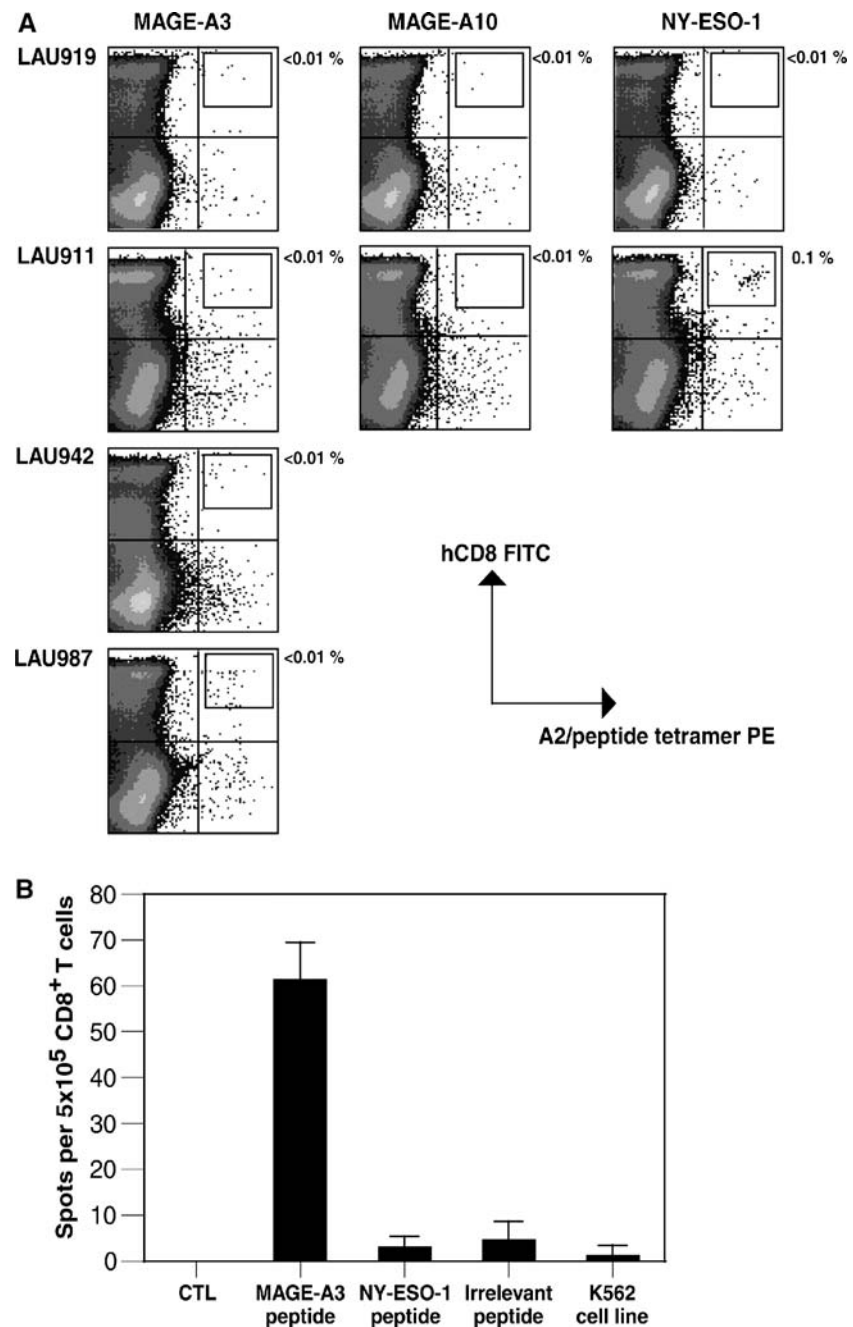
set of antibodies. Hematoxylin/eosin staining of the individual sections is shown at lower magnification. *Filled arrows* indicate an example of positive stained tumor cell. *Empty arrows* indicate an example of negative stained tumor cell. The region shown at higher magnification after mAb staining is *boxed*

Detection of T cell responses against CT antigens

Having demonstrated expression of MAGE-A3, MAGE-A10 and NY-ESO-1 in patients LAU846 and LAU911, we determined if these patients displayed evidence of naturally developed CD8⁺ T cell responses against any of the three CT antigens. PBMC from patient LAU911 (HLA-A1, -A2, -B51, -B57) were stimulated once with the HLA-A2-restricted NY-ESO-1_{157–165} [19], MAGE-A3_{112–120} [20] or MAGE-A10_{254–262} peptides [21] and enumerated, after 10 days of culture,

with HLA-A2 tetramers incorporating the corresponding peptides. As shown in Fig. 3a, 0.1% specific CD8⁺ T cells were detected by the A2^{NY-ESO-1} tetramer in patient LAU911. MAGE-A3_{112–120}⁺ and MAGE-A10_{254–262}⁺ reactive CD8⁺ T cells remained undetectable in patients LAU919, LAU911, LAU942, LAU987 and LAU919, LAU911, respectively. As negative control, we performed the same experiment using PBL obtained from patient LAU919 (HLA-A2⁺ MAGE-A3[−] MAGE-A10[−] NY-ESO-1[−]) but we did not detect any tetramer staining.

Fig. 3 T cell responses in CT antigen-positive CRC patients. **a** Detection of CT antigen-specific T cells in HLA-A2 patients by A2 tetramers. PBMC from patients LAU919 (MAGE-A3[−] MAGE-A10[−] NY-ESO-1[−]), LAU911 (MAGE-A3⁺ MAGE-A10⁺ NY-ESO-1⁺), LAU942 and 987 (MAGE-A3⁺) were stimulated individually in vitro with peptides MAGE-A3_{112–120}, MAGE-A10_{254–262} and NY-ESO-1_{157–165} (LAU919 and LAU911) or only MAGE-A3_{112–120} (LAU942 and LAU987). At day 10, peptide-specific CD8⁺ T cells were detected by flow cytometry with A2 tetramers incorporating the corresponding peptides. Dead cells were excluded by PI staining. **b** IFN- γ ELISPOT assay of in vitro stimulated PBMC of patient LAU846. PBMC were stimulated once in vitro with peptides NY-ESO-1-ORF2_{46–54} and MAGE-A3_{167–176} and analyzed at day 10. Autologous cells were used as antigen-presenting cells in the presence of peptides. Quantification of spot-forming T cells was done after 16 h of incubation



Peripheral blood mononuclear cell isolated from patient LAU846 (HLA-A*2501, -A29, -B*0705, -B18) were stimulated once in vitro with peptides derived from MAGE-A3 and NY-ESO-1. Because LAU846 was HLA-A2-negative, peptides NY-ESO-1-ORF2_{46–54} and MAGE-A3_{167–176} were selected. These two peptides were previously shown to be naturally processed and presented by HLA-B7 and HLA-B18, respectively [22, 23]. Since B7 and B18 tetramers are not available, stimulated T cells were tested in IFN- γ ELISPOT assays, using as targets, peptide-pulsed autologous cells. As shown in Fig. 3b, a significant proportion of T cells stimulated with MAGE-A3_{167–176} released IFN- γ upon incubation with peptide-pulsed target cells. In contrast, no response was detected after stimulation of T cells with NY-ESO-1-ORF2_{46–54} or an irrelevant peptide. On the basis of this assay, the frequency of MAGE-A3-specific T cells was estimated to be approximately 1 in 10⁴ CD8⁺ T cells.

Discussion

Our study shows that expression of CT genes, such as MAGE-A3, -A4, -A10, SSX2 and NY-ESO-1, is infrequent in human CRC. In spite of this fact, evidence of naturally acquired T cell responses could be demonstrated in some of the patients expressing one or the other CT antigens in their tumors.

A recent survey across various cancers revealed a large disparity in the frequencies of CT gene expression. While melanoma, non-small cell lung carcinoma, liver and bladder cancers frequently express products of the MAGE family or NY-ESO-1, CRC, leukemias and renal cancers rarely express these genes ([5] and references therein). Our results confirm these observations with respect to CRC. Interestingly, we found more frequent expression of *MAGE-A3* and *MAGE-A4* (13 and 15%, respectively) than any of the other CT genes. A similar observation was also made by Li et al. [16], who analyzed more than 100 CRC tissues for the expression of CT genes. Despite this slightly more frequent expression of *MAGE-A3* and -A4, most CRC lesions did not express any of the five CT genes tested here. A possibility for this infrequent CT gene expression in CRC could be due to the stage of the tumor at the time of resection. However, our analysis was performed on tumors of different TNM stages, ranging from stage I to stage IV, and, on the basis of our sampling, no correlation between the tumor stages and the profile of CT gene expression could be established. Moreover, no increased frequency of CT genes expression was found among the 14 patients' metastatic

tumors. Taken together, our results indicate that CT gene neo-expression in CRC is unlikely to be caused by epigenetic alterations during cancer progression such as general demethylation [24, 25].

Immunohistochemical analyses of CT antigen-positive CRC samples revealed weak expression of MAGE-A in LAU846 and LAU852. By comparison, strong staining of MAGE-A was detected in testicular seminoma samples. This result suggests that the expression level of MAGE-A gene products in CRC is modest. However, it is possible that the weak staining might be caused by the fact that the affinity of mAb 57B and 6C1 against different members of the MAGE-A family varies significantly [13]. Moreover, the pattern of expression of the antigens within the lesions was heterogeneous. While some tumor regions were heavily stained, other regions were stained less or not at all. This expression pattern is not unusual for CT antigens and underscores the value of immunohistochemical analyses as complement of the commonly used PCR analyses [26, 27]. Indeed, RT-PCR being generally performed on RNA extracted from heterogeneous tissue samples, detection of CT gene expression by PCR might be misleading as the particular CT antigen could be highly expressed in a small subset of cells or, inversely, expressed at low levels in the majority of cells. Such distinctions, which are all too often disregarded, are of critical importance for the development of T cell vaccines against cancer.

Induction of spontaneous anti-tumor T cell responses has been documented for a small number of CT antigen-derived epitopes. Among those tested here, NY-ESO-1 has been shown to be highly immunogenic and to elicit spontaneous T cell responses in HLA-A2⁺ NY-ESO-1⁺ melanoma and breast cancer patients [9, 28]. Despite the very limited number of NY-ESO-1⁺ HLA-A2⁺ CRC patients identified in our study, we nevertheless found a significant frequency of NY-ESO-1-specific T cells in the PBL of such patient. This result shows for the first time, to our knowledge, that the expression of NY-ESO-1 in CRC can induce a specific CD8⁺ T cell response. Unfortunately, further phenotypic analyses of the specific T cells could not be undertaken because the patient had chosen not to continue to participate in the study. Thus, no additional blood sample was obtained. T cell response elicited by peptide MAGE-A3_{167–176} was analyzed in a second patient. Because of the HLA haplotype of this patient, we selected a recently defined MAGE-A3 epitope restricted by HLA-B18 [23]. HLA-B18 is expressed in approximately 11% of the Caucasian population [29]. In this case again, we could show that specific T cells could be stimulated by peptide MAGE-A3_{167–176} to

produce IFN- γ . The frequency of specific T cells remained low but suggested that appropriate vaccination strategies could be used to boost this response.

Taken together, our results indicate that CRC expressing CT antigens can elicit antigen-specific T cell responses, which could be boosted by active vaccination in a small subset of CRC patients. However, priority should be given to the search of frequently and highly expressed CRC-associated antigens.

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