Naturally Acquired MAGE-A10- and SSX-2-Specific CD8$^+$ T Cell Responses in Patients with Hepatocellular Carcinoma[1]

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Immunotherapy is being proposed to treat patients with hepatocellular carcinoma (HCC). However, more detailed knowledge on tumor Ag expression and specific immune cells is required for the preparation of highly targeted vaccines. HCC express a variety of tumor-specific Ags, raising the question whether CTL specific for such Ags exist in HCC patients. Indeed, a recent study revealed CTLs specific for two cancer-testis (CT) Ags (MAGE-A1 and MAGE-A3) in tumor infiltrating lymphocytes of HCC patients. Here we assessed the presence of T cells specific for additional CT Ags: MAGE-A10, SSX-2, NY-ESO-1, and LAGE-1, which are naturally immunogenic as demonstrated in HLA-A2$^+$ melanoma patients. In two of six HLA-A2$^+$ HCC patients, we found that MAGE-A10- and/or SSX-2-specific CD8$^+$ T cells naturally responded to the disease, because they were enriched in tumor lesions but not in nontumoral liver. Isolated T cells specifically and strongly killed tumor cells in vitro, providing evidence that these CTL were selected in vivo for high avidity Ag recognition. Therefore, besides melanoma, HCC is the second solid human tumor with clear evidence for in vivo tumor recognition by T cells, providing the rational for specific immunotherapy, based on immunization with CT Ags such as MAGE-A10 and SSX-2. *The Journal of Immunology,* **2005, 174: 1709–1716.**

Although hepatocellular carcinoma (HCC)[4] can be treated by surgery, 5 year survival remains relatively low (15–30%), and only 10–30% of patients are eligible for curative surgery. Other treatment modalities such as chemotherapy and irradiation have not yet demonstrated significant improvement of clinical outcome (1, 2). Immunotherapy of HCC has been attempted with IFN-α/IFN-β (3, 4), autologous tumor-pulsed dendritic cells (5), or in vitro-expanded and adoptively transferred lymphocytes (6). The limited clinical experience suggests that tumor growth and recurrences may have been delayed in some patients.

A clinico-pathological study demonstrated that some (11/162) patients with HCC had strong intratumoral lymphocyte infiltration, which correlated with lower disease recurrence and better survival as compared with patients with weak lymphocyte infiltration (7). This suggests that antitumor immune responses can occur naturally in HCC and are associated with better prognosis. Similar observations have been made in melanoma (8) and ovarian carcinoma (9).

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[4] Abbreviations used in this paper: HCC, hepatocellular carcinoma; CT, cancer-testis; TIL, tumor-infiltrating lymphocyte; IHL, intrahepatic lymphocyte; rh, recombinant human; HCV, hepatitis C virus.

It is well accepted that CD8$^+$ T lymphocytes play a central role in immune defense against cancer. To do so, T cells must be activated through TCR-mediated, specific recognition of peptide Ags presented by MHC class I molecules (i.e., HLA-A, -B, -C). A recent report demonstrated the presence of tumor Ag-specific CD8$^+$ T cells (specific for MAGE-A1 and MAGE-A3) in the tumor of HCC patients (10). However, TCR avidity was not assessed in this study (10). In the remaining studies of immunity against HCC, Ag-specific T cells were not investigated (5, 6). Thus, it remains a major challenge to identify dominant tumor Ag-specific T cell responses in HCC.

The so-called cancer-testis (CT) Ags constitute a highly interesting group of tumor Ags (11, 12), as they are expressed in variable proportions of melanomas and carcinomas originating from various tissues. The genes encoding CT Ags are not expressed by normal tissues, with the exception of testis germline cells which are MHC (HLA) class I negative and thus not recognized by T cells (11, 13). Thus, the expression of T cell-defined CT Ags is highly tumor specific, making them ideal targets for tumor immunity and immunotherapy (14). Importantly, CT gene expression has been reported to be a frequent event in HCC (15–18). For example, many members of the MAGE-A gene family were found to be expressed in up to 70% of HCC (17). Moreover, the NY-ESO-1 and SSX-2 genes have also been reported to be expressed in HCC (15, 16).

During recent years, a crucial finding was that tumor Ag-specific T cell responses can naturally arise in human melanoma. Interestingly, these cells were found to accumulate at relatively high frequencies, as detected by ex vivo analyses of tumor-infiltrated-lymph node cells (19). This indicates that tumor Ag-specific T cells react in vivo, confirming that tumor cells are indeed recognized and immunogenic, excluding the possibility that such T cells are in vitro artifacts. Considering the frequent expression of CT Ags (15–18), the conserved expression of TAP and MHC class I molecules in HCC (20–22) and the frequent natural recognition by CD8$^+$ cytolytic T lymphocytes (CTL) in melanoma, these Ags are

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prime candidates for clinical investigation of tumor-specific immunity in HCC patients.

In this study we focused on the tumor Ags NY-ESO-1, CAMEL, MAGE-A10, and SSX-2. All four proteins generate peptides that bind to HLA-A2 and are efficiently recognized by specific $CD8^+$ T cells from melanoma patients (23–25). NY-ESO-1 and CAMEL epitopes can be generated by both *NY-ESO-1/LAGE-2 and LAGE-1* gene products (26). We analyzed six HLA-$A2^+$ HCC patients and investigated whether their tumors expressed CT Ags, and whether CT Ag-specific T cells were present in tumor, distant nontumoral liver tissue, and PBMC.

Materials and Methods

Patients and sampling

Six HLA-$A2^+$ patients with primary HCC treated with partial hepatectomy at the Centre Hospitalier Universitaire Vaudois (Lausanne, Switzerland) were included in this study upon written informed consent. The study protocol was accepted by the ethical committee of Lausanne University (Lausanne, Switzerland) and conformed to the 1975 Declaration of Helsinki. Fresh HCC tissue was recovered from the center of nonnecrotic tumor tissue and nontumoral liver tissue distant from the tumor. A portion of each was snap frozen for subsequent immunohistochemical analysis and RNA extraction. Lymphocyte and RT-PCR analyses were performed with material derived from the largest tumor. Other HCC lesions from patients LAU 674 and LAU 766 were sampled for RT-PCR analysis only (LAU 674 tumor 2 and LAU 766 tumor 2; Fig. 1). Nontumoral liver tissue and blood from patient LAU 766 were not available.

PCR analysis

RNA from tissue (10–20 mg) was prepared using a Nucleospin RNA II kit (Macherey-Nagel) and a Fast-Prep device (Bio 101 Savant; Savant Instruments). Reverse transcription was performed using M-MLVRT enzyme and buffers (Invitrogen Life Technologies) with 1–2 μg of RNA. PCR amplification of β-actin, NY-ESO-1, LAGE-1, SSX-2, and MAGE-A10 cDNA transcripts was performed as described (26–29). RNA from SK-MEL37 cells was used as a positive control for expression of CT Ags and RNA from NA8-MEL cells was used as a negative control. As reported previously (26, 27), analysis of LAGE-1 or MAGE-A10 expression revealed two splice-variants (Fig. 1, *lower* and *upper bands*). β-Actin and SSX-2 gene expression levels were confirmed by quantitative PCR with the same cDNA samples.

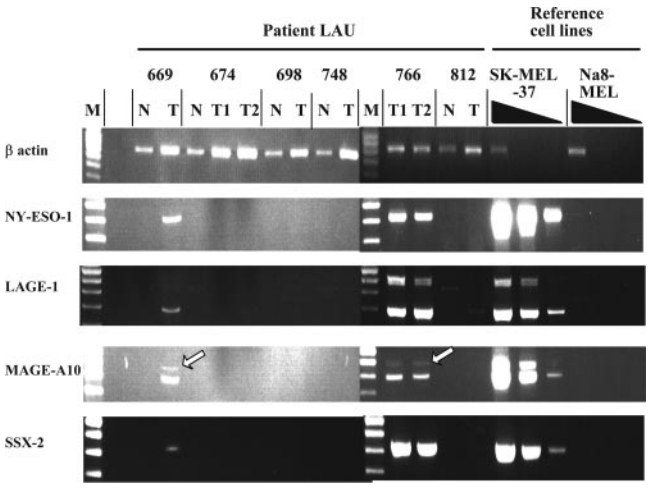

FIGURE 1. Expression of CT Ags in HCC but not normal liver tissue. cDNA was prepared by reverse-transcription using 1–2 μg of total RNA extracted from tumor (T) and nontumoral (N) liver tissue. In parallel, serially diluted (1/10) RNA from SK-MEL-37 (CT Ag-expressing line, positive control) and Na8-MEL (CT Ag nonexpressing line, negative control) melanoma cell lines were used. PCR was performed with equivalent amounts of cDNA to detect expression of the CT Ags NY-ESO-1, LAGE-1, MAGE-A10, and SSX-2, and β-actin as internal control. Open arrows indicate the weakly detectable long splice variant of MAGE-A10 in tumors of patients LAU 669 and LAU 766.

Histology and immunohistochemistry

Serial sections of formalin-fixed, paraffin-embedded tissue samples were obtained, and HE stainings were performed according to standard procedures. All the tumors were microscopically examined by the same pathologist (H.B.). The tumors were classified according to the World Health Organization classification of tumors of the digestive system and staged according to the TNM classification (Tumor/Node/Metastasis classification). Tissue sections were immunostained with anti-CD3 (clone F7.2.38; DakoCytomation), anti-CD8 (clone C8/144B; DakoCytomation), and anti-CD4 (clone 1F6; Novocastra) Abs using the Vectastain avidin-biotin peroxidase system (Vector Laboratories).

Isolation of lymphocytes and cell lines

To obtain tumor-infiltrating lymphocytes (TIL), tumor samples from HCC patients were minced and placed in IMDM (Invitrogen Life Technologies) supplemented with 8% heat-inactivated pooled human serum (CTL medium), 150 IU/ml recombinant human (rh)IL-2 (GlaxoSmithKline), and 10 ng/ml rhIL-7 (Biosource International) for 3–4 wk. To obtain intrahepatic lymphocytes (IHL), samples from nontumoral liver tissues were finely minced in CTL medium and pressed through a 100-μm cell strainer. The cellular suspension was pelleted and resuspended at $100-400 \times 10^6$ cells in 2 ml of 63% PBS equilibrated Percoll. Percoll gradient was prepared in 15-ml Falcon tubes over the 63% Percoll cell suspension by the addition of 2-ml layers of 57, 49, and 27% Percoll, then centrifuged for 30 min at $300 \times g$. The cell layers at 57%/49% and 49%/27% Percoll interfaces were collected, pooled, and used as IHL. TIL and IHL cells were stimulated with the lymphocyte mitogen PHA (1 μg/ml) in the presence of irradiated PBMC in CTL medium and rhIL-2 at 150 IU/ml, in the absence of synthetic peptides. The melanoma cell line Me 275 was established at the Ludwig Institute for Cancer Research, Lausanne Branch, from a surgically excised melanoma lesion from patient LAU 50. The SK-MEL37 cell line is a gift from Y. T. Chen (Ludwig Institute for Cancer Research, New York branch). The NA8-MEL cell line was kindly provided by F. Jotereau (Institute National de la Santé et de la Recherche Médical Unité 463, Nantes, France). T2, Me 275, SK-MEL37, and NA8-MEL cell lines were maintained in RPMI 1640 medium supplemented with 10% FCS. HCC cell line Hep-G2 cells were maintained in DMEM medium supplemented with 10% FCS.

Peptide stimulation of PBMC

PBMC were recovered from peripheral blood on the day of surgery, isolated by standard centrifugation on a Ficoll layer, and frozen until use. Unfortunately, no blood was available from patient LAU 766. After thawing, $CD8^+$ lymphocytes were positively selected from PBMC of HCC patients using a miniMACS device (Miltenyi Biotec). Cells from the $CD8^-$ fraction were irradiated (3000 rad) and used as APCs (APC, 100×10^3/well). $CD8^+$ lymphocytes ($50-100 \times 10^3$/well) were stimulated using peptide-pulsed (10 μM) irradiated autologous APC in CTL medium. rhIL-2 (50 IU/ml) and rhIL-7 (10 ng/ml) were added 2 days after stimulation. Cells were restimulated at days 10–14 with irradiated (10,000 rad) T2 cells after pulsing with peptide (10 μM) and washing three times. Peptide sequences were as follows: $CAMEL_{1-11}$, MLMAQEALAFL; $SSX\text{-}2_{41-49}$, KASEKIFYV; and $MAGE\text{-}A10_{254-262}$, GLYDGMEHL. $NY\text{-}ESO\text{-}1_{157-165(C165A)}$ peptide analog SLLMWITQA was used because of its increased HLA-A2 binding and its largely identical antigenicity compared with the parental peptide $NY\text{-}ESO\text{-}1_{157-165}$ (30).

Multimer staining, isolation of MAGE-$A10_{254-262}$- and SSX-2_{41-49}-specific $CD8^+$ T cells, and chromium-release assays

HLA-A*0201/peptide multimeric complexes (also known as tetramers) were synthesized (31) and cells were stained as described (25). MAGE-A10- and SSX-2-specific $CD8^+$ T cells were isolated by multimer guided cell sorting from TIL and/or peptide-stimulated PBMC. Sorted cells were stimulated with PHA and irradiated PBMC to obtain polyclonal monospecific T cells. To generate T cell clones, cultures were established by limiting dilution. T cells were maintained in culture by restimulation every 2–3 wk with PHA and irradiated allogenic PBMC, followed by medium exchange every 2 days. Chromium-release assays were performed as described.

Results

Six HLA-$A2^+$ patients were recruited for this study; their clinical and pathological characteristics are depicted in Table I.

Table I. *HCC patient characteristics*

Patient Code	Age/Sex	Number of Tumor Lesions, Size	Histology[a]	Serology	HLA-A, -B[b]
LAU 669	70/male	Six lesions, diameters 18, 5.5, 2, 1.8, 1.6, and 0.8 cm; infiltration of neighbouring organs	Low differentiation; moderate lymphocytic infiltration	HBs negative, anti-HBc negative, HCV negative, anti-HCV negative	A*0201, A24(9), B62(15), Bx
LAU 674	68/male	Three lesions, largest lesion $3 \times 2 \times 2$ cm	Moderate differentiation; slight lymphocytic inifiltration; liver cirrhosis.	HBs negative, anti-HBc negative, anti-HCV positive	A*0201[c], A3, B35, B51(5)
LAU 698	75/male	Two lesions, diameters 5 and 3.5 cm	Moderate differentiation; slight lymphocytic infiltration	HBs negative, anti-HBc negative, HCV negative, anti-HCV negative	A1, A*0201, B7, B37
LAU 748	81/female	One lesion, diameter $13 \times 6 \times 5$ cm	Low differentiation; no lymphocytic infiltration	HBs negative, anti-HBc negative, anti-HCV negative	A*0201, A28, B18, B27
LAU 766	70/male	Multiple lesions, diameters between 1 and 2 cm	Moderate differentiation; no lymphocytic infiltration; liver cirrhosis	HBs negative, anti-HBc negative, anti-HCV positive	A*0201, A*31[d]
LAU 812	68/female	One lesion, diameter $2 \times 2 \times 2$ cm	Well differentiated; strong lymphocytic infiltration	HBs negative, anti-HBc negative, anti-HCV negative	A*0201, A3, B13, B62(15)

[a] Tumor differentiation and cirrhosis was analyzed with H&E sections. Intratumoral T cell infiltration was analyzed with sections stained with CD3-specific Ab.
[b] HLA typing for HLA-A,-B was done by serology, and for A2 subtypes with HLA allele-sequence-specific primers.
[c] The rare alleles A*0259/60/64 cannot be excluded.
[d] The rare heterozygous combinations A*0258/59/60/64 and A*3109 cannot be excluded.

Expression of CT Ags by HCC

To guide the search for evidence of tumor Ag-specific T cell immunity in HCC, we investigated tumor Ag expression. We focused particularly on the CT Ags NY-ESO-1, LAGE-1, MAGE-A10, and SSX-2, because these Ags are highly relevant for tumor-specific immune responses in melanoma (23–25). To test CT Ag expression at the mRNA level, total RNA was extracted from HCC tissues that were snap frozen after hepatic resection. Nontumoral liver was also tested in parallel to assess whether Ag expression was tumor specific.

To allow a semiquantitative analysis, decreasing amounts of RNA prepared from well-characterized melanoma cell lines were tested in parallel as a reference. As a positive control, we used RNA from SK-MEL-37, a CT Ag-expressing melanoma cell line (NY-ESO-1^+, LAGE-1^+, MAGE-$A10^+$, and SSX-2^+). Similarly, RNA prepared from Na8-MEL, a melanoma cell line that does not express these CT Ags, was used as a negative control (Ref. 27 and Fig. 1). Two, 0.2, and 0.02 μg RNA (serial dilution at 1/10) from both control cell lines, as well as 1–2 μg of tissue-derived RNA were reverse-transcribed in the same experiment.

All CT Ags tested were found to be expressed at the mRNA level in the tumor(s) of patients LAU 669 and LAU 766 (Fig. 1). LAU 669 tumor showed a weak expression of the four CT Ags, equivalent to a 1/100 dilution of SK-MEL-37-derived RNA. Both tumor samples from LAU 766 (Fig. 1, T1 and T2) expressed CT Ags more strongly, comparable to undiluted RNA derived from SK-MEL-37, with the exception of MAGE-A10 that was expressed weakly at a comparable level as in patient LAU 669. Interestingly, all four CT Ags tested were expressed in tumors from patients LAU 669 and LAU 766. No CT Ag expression was detected in nontumoral liver tissue from patient LAU 669. Unfortunately, we did not have access to LAU 766 nontumoral liver. Tumor and nontumoral liver from the remaining four patients (LAU 674, 698, 748, and 812) had no detectable CT Ag expression. Thus, CT Ag expression was found in tumor(s) from two of six HLA-$A2^+$ patients (LAU 669 and LAU 766). To extend our analysis of CT Ag expression, we performed RT-PCR analysis in 10 additional HLA-A2-negative HCC patients (data not shown). NY-ESO-1 was expressed in 12.5%, LAGE-1 in 25%, MAGE-A10 in 19%, and SSX-2 in 25% of HCC from all 16 patients analyzed.

Tumor infiltration by T lymphocytes

Histological analyses revealed slight to strong intratumoral infiltration with lymphoid cells. T cell infiltration was investigated by immunohistochemical staining with CD3, CD4, and CD8 Abs in tumors and nontumoral livers. A moderate to marked intratumoral lymphocytic infiltration was observed in patients LAU 669 and LAU 812, respectively. This lymphoid infiltrate was concentrated at the margin of the tumor of LAU 812 (Fig. 2) or dispersed through the tumor of LAU 669 (not shown). In these two cases, a moderate to slight peritumoral lymphocytic infiltration was also noticed. The lymphoid infiltrate in tumors was constituted of a majority of $CD3^+/CD8^+$ cells, with a moderate proportion of $CD4^+$ T cells. Patients LAU 748 (Fig. 2) LAU 674, LAU 698, and LAU 766 (not shown) displayed absent to slight intratumoral infiltration with $CD4^+$ and $CD8^+$ lymphocytes scattered in the HCC. In parenchymal livers sampled at distance from the tumors, the density of the lymphoid reaction was correlated with the basic liver pathology (Table I) and was predominantly constituted of $CD4^+$ T cells. In patients LAU 669 (not shown) and LAU 812 (Fig. 2), few $CD8^+$ and $CD4^+$ T cells were observed in the portal fields. In conclusion, a significant intratumoral T cell infiltration was found in two of six patients.

Enrichment of tumor Ag-specific $CD8^+$ T cells in HCC tissue

Human metastatic melanomas frequently contain enriched populations of tumor Ag-specific T cells, demonstrating that T cells can specifically react to tumor cells in cancer patients. Our aim was to determine whether this may also be the case in primary HCC tissue. Therefore, we searched for the presence of CT Ag-specific T cells in TIL and in IHL of all six HLA-$A2^+$ patients. To identify tumor Ag-specific T cells, we prepared fluorescent HLA-A*0201 multimers with the peptides NY-ESO-$1_{157-165(C165A)}$, $CAMEL_{1-11}$, MAGE-$A10_{254-262}$, or SSX-2_{41-49}. TIL and IHL were prepared and stained with these multimers and CD8-specific Ab, and were analyzed by flow cytometry. MAGE-A10- and SSX-2-specific T cells were detected in TIL but not in IHL of patient LAU 669. These cells accounted for 0.23% and 0.09% of $CD8^+$ cells, respectively (Fig. 3*A*). No NY-ESO-1- or CAMEL-specific cells were detectable in the same TIL and IHL (data not shown). Interestingly, we also found MAGE-A10-specific cells at the level of 0.22% of $CD8^+$ TIL but not IHL derived from patient LAU 812 (Fig. 3*B*). Thus, MAGE-A10- and/or SSX-2-specific T cells were detectable in TIL from patients LAU 669 and LAU 812, without prior specific stimulation with antigenic peptides. Specificity of tetramer staining was validated using CT Ag-specific clones with the corresponding specificity (not shown) and using a CTL clone restricted by Flu $matrix_{58-66}$/HLA-A*0201 to assess nonspecific staining (Fig. 3*C*).

No CT Ag-specific cells were detectable in TIL and IHL from LAU 674, 698, and 748. We also did not find Ag-specific T cells

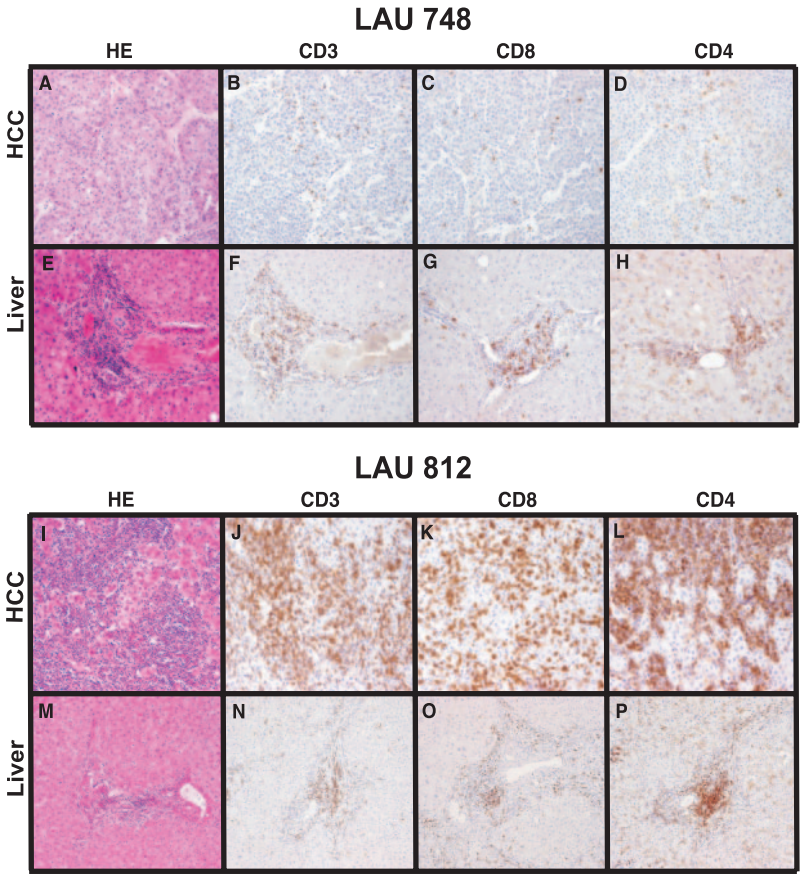

FIGURE 2. Immunohistochemical detection of $CD3^+$, $CD8^+$, and $CD4^+$ cells in HCC and nontumoral liver tissue. Immunohistochemical analysis of HCC from patient LAU 748 (*A* to *D*) and patient LAU 812 (*I* to *L*) and nontumoral liver from the same patients (*E* to *H* and *M* to *P*, respectively). Samples were analyzed by HE staining (*A*, *E*, *I*, and *M*), CD3 staining (*B*, *F*, *J*, and *N*), CD8 staining (*C*, *G*, *K*, and *O*) and CD4 staining (*D*, *H*, *L*, and *P*). In HE-stained sections, infiltrating cells appear in dark blue. Cells labeled with Abs specific for CD3/CD4/CD8 are stained in brown. Note that the CD4-specific Ab stains $CD4^+$ T and Kupffer cells because of their CD4 expression. Patient LAU 748 shows low tumor infiltration by lymphocytes in contrast to patient LAU 812 with strong intratumoral infiltration by lymphocytes.

in TIL from both LAU 766 tumor subtypes, despite a strong mRNA expression of the corresponding Ags.

Circulating CT Ag-specific T cells in PBMC

We next analyzed whether CT Ag-specific $CD8^+$ T cells were present in peripheral blood withdrawn at the day of surgery. PBMC were stimulated twice with peptide before flow cytometric analysis with fluorescent HLA-A2/peptide multimers and CD8-specific Ab. SSX-2- and MAGE-A10-specific T cells were detected in peptide-stimulated PBMC of patient LAU 669. These cells accounted for 0.19 and 0.11% of $CD8^+$ cells, respectively (Fig. 4). Two distinct SSX-2-specific cell populations displayed a low and a high multimer labeling intensity. No NY-ESO-1- or CAMEL-specific cells were detectable in blood from the same patient.

Also, no NY-ESO-1-, CAMEL-, SSX-2-, or MAGE-A10-specific T cells were detectable in peptide-stimulated PBMC from patient LAU 812 (Fig. 4) or from patients LAU 674, 698, and 748 (data not shown). Unfortunately, no blood was available from patient LAU 766. Taken together, MAGE-A10- and/or SSX-2-specific $CD8^+$ T cells were detectable in TIL but not IHL from patients LAU 669 and LAU 812. After peptide stimulation, MAGE-A10- and SSX-2-specific $CD8^+$ T cells could also be detected in the blood of patient LAU 669.

Specific recognition and lysis by CT Ag-specific $CD8^+$ T cells

$CD8^+$/multimer$^+$ T cells identified above were sorted and expanded to establish T cell lines and clones for functional analysis. MAGE-$A10_{254-262}$-specific polyclonal T cells from peptide-stimulated PBMC (line 4), polyclonal T cells (line 3) and a clone (1F8) were generated from TIL of patient LAU 669. We failed to generate SSX-2_{41-49}-specific T cells using tumor and blood samples obtained at the day of surgery. However, we were able to generate a polyclonal SSX-2-specific CTL line (line A) derived from peptide-stimulated PBMC obtained 14 mo after surgery of patient LAU 669. All expanded T cell populations stained with fluorescent multimers in a highly specific manner for MAGE-$A10_{254-262}$ or SSX-$2_{41-49,}$ despite a partial down-regulation of CD8 expression for the polyclonal TIL line 3 (Fig. 5*A*). Efficiency of Ag recognition by the CT Ag-specific T cell polyclonal and monoclonal cell lines was assessed in 4-h chromium-release assays using T2 cells loaded with titrated amounts of peptide. This method allows evaluation of the avidity/efficiency of CTL recognition of cognate Ag. MAGE-$A10_{254-262}$- and SSX-2_{41-49}-specific CTLs recognized the antigenic peptide with high efficiency (Fig. 5*B*), with half-maximal lysis at peptide concentrations around 0.1 nM (10^{-10} M). MAGE-$A10_{254-262}$-specific TIL line 3 cells showed half-maximal lysis at ~50 pM, clone 1F8 at 100 pM; and PBMC line 4 at 50 pM. SSX-2_{41-49}-specific PBMC-derived line A showed half-maximal lysis around 5 pM. Marginal lytic activity was found either in the absence of the antigenic peptide or in the presence of an irrelevant Flu matrix$_{58-66}$ peptide. Thus, CTLs had T cell receptors with high avidity to cognate Ag. Avidity was similar to protective virus-specific $CD8^+$ T cells, suggesting efficient tumor recognition in vivo and selection of high avidity cells in cancer patients, as described in melanoma patients (25).

We next tested Ag-dependent recognition and killing of melanoma and HCC cell lines. MAGE-$A10_{254-262}$- as well as SSX-2_{41-49}-specific CTL lines displayed strong killing of CT Ag- and HLA-A2-expressing melanoma cell line Me 275 (Fig. 6, □). This high cytotoxic activity was found without addition of exogenous peptide, and even at a low E:T cell ratio. The cytotoxic activity was not increased when the same target cells were pulsed with MAGE-$A10_{254-262}$ or SSX-2_{41-49} antigenic peptides at 10 μM (Fig. 6, ■), indicating that endogenous processing and presentation

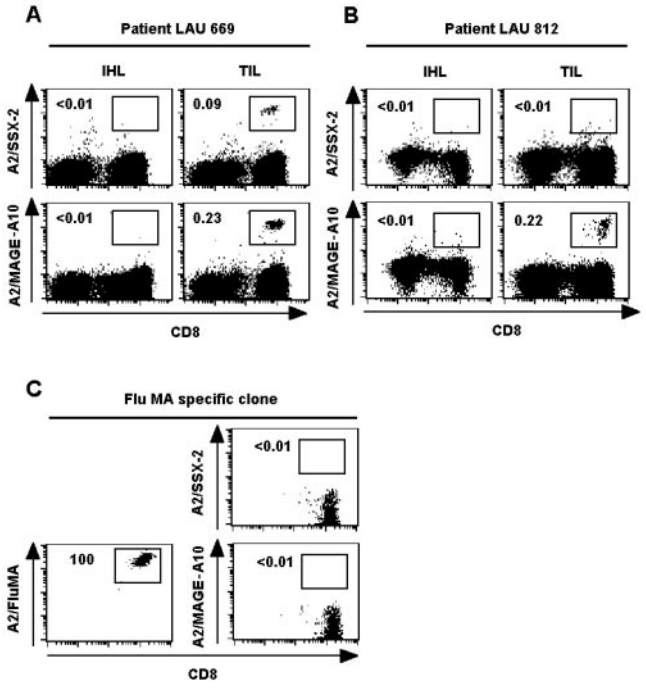

FIGURE 3. $CD8^+$ T cells specific for CT Ags in tumor but not normal liver tissue. Flow cytometric analysis of CT Ag-specific $CD8^+$ T cells using MAGE-$A10_{254-262}$ or SSX-2_{41-49} peptide-loaded HLA-A*0201 fluorescent multimers. Results using IHL and TIL derived from patients LAU 669 (*A*) and LAU 812 (*B*) are shown. TIL were generated by incubation of thin tumor fragments in IL-2- and IL-7-supplemented medium during 3–4 wk and an additional 2-wk PHA stimulation. IHL cells were recovered after Percoll gradient separation of mechanically dissociated liver tissue, and were also stimulated with PHA before analysis. Percentage of $tetramer^+$ cells among $CD8^+$ cells is indicated. *C*, In addition a Flu $matrix_{58-66}$/HLA-A*0201-specific CTL clone was used for negative control stainings.

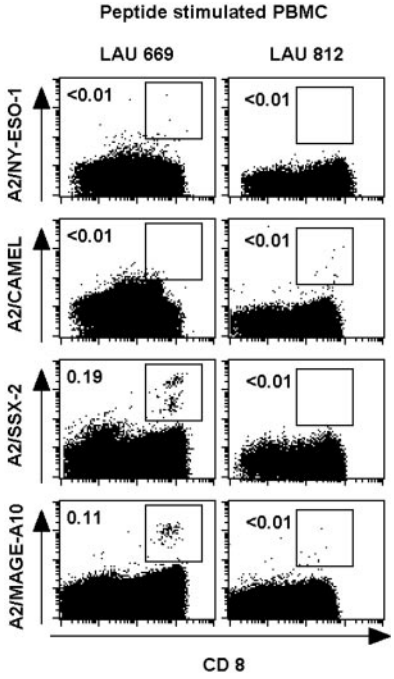

FIGURE 4. $CD8^+$ T cells specific for CT Ags in PBMC from HCC patients. Flow cytometric analysis of CT Ag-specific $CD8^+$ T cells using NY-ESO-$1_{157-165(C165A)}$, $CAMEL_{1-11}$, SSX-2_{41-49}, or MAGE-$A10_{254-262}$ peptide-loaded HLA-A*0201 multimers. Results with peptide-stimulated PBMC derived from patients LAU 669 (*left panel*) and LAU 812 (*right panel*) are shown. $CD8^+$ PBMC were stimulated with specific peptide at 10 μM using autologous CD8 depleted cells, restimulated 2 wk later using peptide-pulsed T2 cells and analyzed 2 wk later. Percentage of $tetramer^+$ cells among $CD8^+$ cells is indicated.

of MAGE-$A10_{254-262}$ or SSX-2_{41-49} CT Ags by Me 275 cells were sufficient to trigger maximal lytic activity.

We also tested recognition and killing of a HCC cell line. The well characterized HLA-$A2^+$ Hep G2 cell line was investigated for CT Ag expression by RT-PCR and no MAGE-A10 and SSX-2 mRNAs were found to be expressed (data not shown). Not surprisingly, Hep G2 cells were not recognized by specific T cells from HCC patients in absence of exogenous peptide Ag (Fig. 6, ○). On the contrary, Hep G2 HCC cells incubated with antigenic peptides were killed to the same extent as Me 275 cells (Fig. 6, ●). This demonstrated strong specific recognition and killing of Hep G2 HCC target cells by TIL- and PBMC-derived T cells specific for MAGE-$A10_{254-262}$ or SSX-2_{41-49}. Similar results were obtained with the HLA-$A2^+$ melanoma cell line Na8-MEL that does not express MAGE-A10 and SSX-2. In conclusion, all MAGE-$A10_{254-262}$- and SSX-2_{41-49}-specific T cell lines tested displayed high avidity for Ag and efficiently recognized and killed melanoma or HCC cells.

Discussion

Analysis of CT Ag expression revealed that the frequency of positive tumors from the 16 analyzed HCC patients was 12.5–25% (depending on CT Ag) and thus lower (15–17) or higher (16) compared with previously reported frequencies. Such discrepancies may be due to technical reasons (use of different primer sets and/or different PCR cycle numbers) or may be due to patient selection. Interestingly, in tumors from patients LAU 669 and LAU 766, we found simultaneous expression of the four CT Ags analyzed. It has been reported that MAGE-A1 expression is primarily regulated by promoter methylation (32), and other CT genes also contain methylated CpG-rich promoters. This suggests that CT Ag coexpression may be linked to genome-wide hypomethylation that occurs during tumorigenesis. In any case, simultaneous expression of multiple CT Ags is highly interesting because this opens the possibility to simultaneously target multiple Ags in future immunotherapy approaches.

Two of six HLA-$A2^+$ patients displayed CT Ag-specific $CD8^+$ T cells which were spontaneously enriched in TIL but not in IHL, and were readily detectable without the need for in vitro stimulation with synthetic peptide. These results suggest that tumor Ag-specific T cells were primed in vivo, expanded, and able to specifically localize to the tumor site.

Patient LAU 669 had detectable MAGE-A10 and SSX-2 mRNA expression and corresponding MAGE-$A10_{254-262}$- and SSX-2_{41-49}-specific $CD8^+$ T cells in tumor tissue. After peptide stimulation, MAGE-A10- and SSX-2-specific $CD8^+$ T cells could also be detected in the blood of this patient. HCC tissue from patient LAU 812 also contained MAGE-$A10_{254-262}$-specific $CD8^+$ T cells despite that MAGE-A10 mRNA remained undetectable. Interestingly, tumors from these two patients displayed remarkable intratumoral infiltration by T lymphocytes, containing tumor Ag-specific $CD8^+$ T cells. This suggests a correlation between strong T cell infiltration and recruitment of specific T cells at the tumor site. T cell infiltration in the tumor is an important prerequisite for protective immunity. Although patient LAU 812 had clear evidence for a naturally acquired T cell response against MAGE-A10, no CT Ag expression was detected in her tumor sample. It is

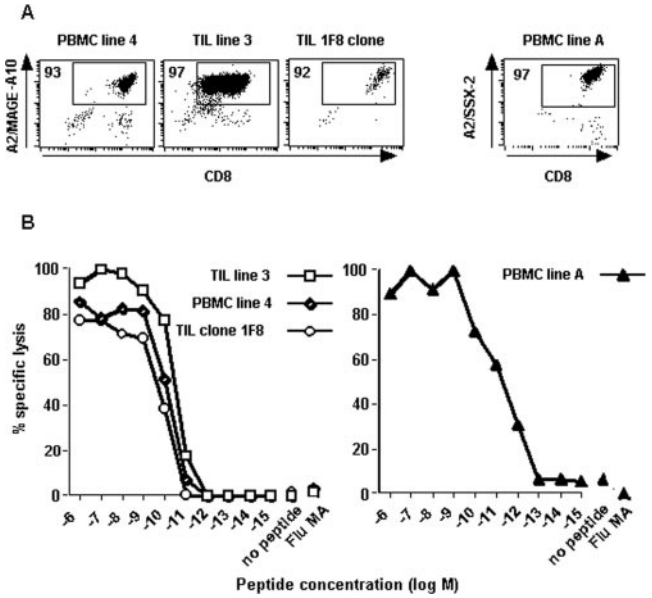

FIGURE 5. MAGE-A10$_{254-262}$- and SSX-2$_{41-49}$-specific CD8$^+$ T cell lines and clones recognize tumor Ag with high avidity. *A*, Flow cytometric analysis of CD8$^+$/multimer$^+$ sorted cells after expansion with PHA. Multimer$^+$ MAGE-A10$_{254-262}$-specific polyclonal CTL were derived from peptide-stimulated PBMC (PBMC line 4) or from TIL (TIL line 3). MAGE-A10$_{254-262}$-specific clone 1F8 was isolated by limiting dilution from multimer$^+$ sorted TIL. Multimer$^+$ SSX-2$_{41-49}$-specific polyclonal CTL was derived from peptide-stimulated PBMC (line A). The percentage of tetramer/CD8 double-positive cells is indicated. *B*, Avidity of Ag recognition of MAGE-A10$_{254-262}$ (*left panel*)- or SSX-2$_{41-49}$ (*right panel*)-specific CD8$^+$ T cells was assessed in a 4-h chromium-release assay, using T2 cells pulsed with various peptide concentrations ranging from 10^{-6} to 10^{-15} M (-6 to -15) and controls without peptide (no peptide) or with 10^{-6} M of the irrelevant Flu matrix$_{58-66}$ peptide (Flu MA). T2 cells were pulsed with MAGE-A10$_{254-262}$ peptide (□, ○, and ◇, *left panel*) or with SSX-2$_{41-49}$ (▲, *right panel*). E:T cell ratio was 30:1.

known that CT Ag expression can be heterogeneous (33–35). Even though we used closely adjacent tumor tissue for the analysis of T cells and tumor Ags, it remains possible that MAGE-A10 expression was selectively absent in the fraction of tissue used to analyze tumor Ags expression. To address these questions, analysis of CT Ag protein expression is under investigation, by immunohistochemistry (33–35) and Western blotting (36).

No CT Ag-specific T cells were detected in IHL and/or TIL from patients LAU 674, 698, 748, and 766. Patient LAU 766 was intriguing as the tumor strongly expressed CT Ags mRNA. It is possible that this patient had no lymphocytes with T cell receptors specific for the four HLA-A2-restricted epitopes studied here. Alternatively, such T cells may have not been identified by our techniques. Particularly strongly activated T cells may undergo apoptosis upon in vitro manipulations with multimers or in cell cultures (37), and this was apparently the case for some other CT Ag-specific CTL recently reported in HCC (10).

As HCC cells can support hepatitis C virus (HCV) replication (38), we also raised the question whether HCV-positive patients generated T cell responses against HCV. However, using HLA-A*0201 multimers loaded with Core$_{178-187}$, Core$_{729-737}$, NS3$_{1073-1081}$, NS3$_{1406-1415}$, and NS5$_{2594-2602}$ HCV-derived peptides, we could not identify HCV-specific T cells among TIL and IHL from patients LAU 766 and LAU 674 (M. Knuchel, data not shown). For both HCC and HCV, the patients may have had T cells specific for unknown epitopes. In general, it is necessary to develop laboratory reagents that allow to screen for T cell activities with alternative specificities, to know precisely whether individual patients have Ag-specific T cell responses.

To examine TCR fine specificity, we generated T cell lines and clones from patient LAU 669. We found that the T cells had high avidity to cognate Ag, because half-maximal lysis of peptide-pulsed T2 cells was achieved at low peptide concentrations (5–100 pM). Consequently, cytolytic activity was efficient against tumor cells endogenously expressing MAGE-A10 or SSX-2 protein. In addition to melanoma cells, we also found efficient killing of HCC cells. In all instances, target cells were recognized and killed even at a low E:T cell ratio. Unfortunately, we failed to generate autologous tumor cell lines. Only a very small number of HCC cell lines exist worldwide, reflecting the fact that the generation of such cells remains a great challenge. Despite that it was not possible to test CTL reactivity against autologous tumor cells, our data demonstrate efficient Ag-specific tumor cell recognition and lysis.

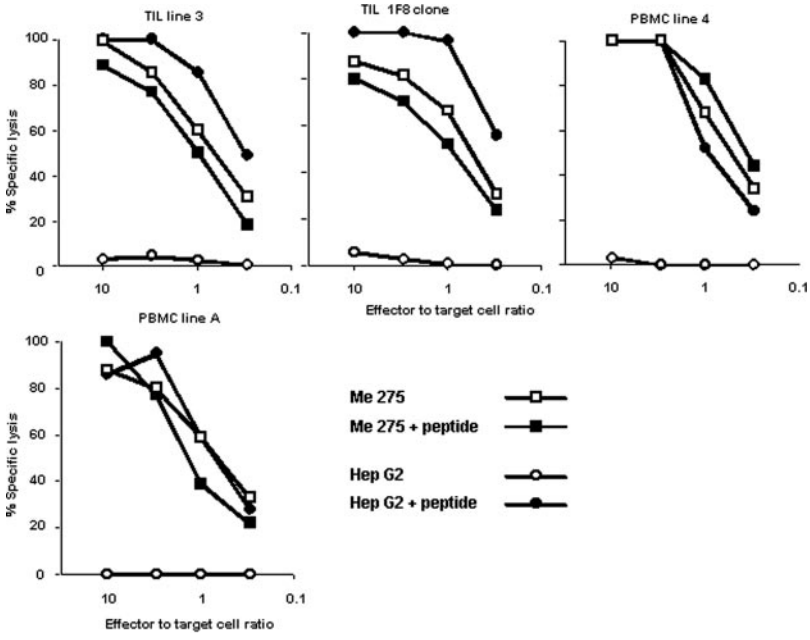

FIGURE 6. MAGE-A10$_{254-262}$- and SSX-2$_{41-49}$-specific CD8$^+$ T cells efficiently kill melanoma and HCC cell lines. Functional lytic activity of MAGE-A10$_{254-262}$ (TIL line 3, clone 1F8 and PBMC line 4)- or SSX-2$_{41-49}$ (PBMC line A)-specific cells against two tumor cell lines in absence (○ and □) or presence (● and ■) of synthetic peptide. Tumor cells were melanoma cell line Me 275 (■ and □; HLA-A2$^+$, MAGE-A10$^+$, SSX-2$^+$) and HCC cell line Hep-G2 (● and ○; HLA-A2$^+$, MAGE-A10$^-$, SSX-2$^-$).

The finding that $CD8^+$ CTLs were of high avidity is important, because low avidity T cells are unlikely to exert protective immune responses in vivo (39). Heterogeneous MAGE-A10-specific T cell responses have been reported in melanoma patients, whereby only high but not low avidity T cells were capable to lyse tumor cells (40). Interestingly, the low avidity T cells were obtained from PBMC after in vitro peptide stimulation, whereas T cells derived from tumor lesions primarily contained high avidity T cells (40). Our study provides comparable evidence in HCC patients, because MAGE-A10-specific T cells derived from TIL killed tumor cells with high avidity and we did not detect cells with low avidity.

Staining with multimers revealed two distinct SSX-2-specific T cell populations in peptide-stimulated PBMC from patient LAU 669: One population with low intensity multimer staining and the other one with high intensity staining (Fig. 4). Interestingly only one population, with high multimer intensity staining, could be found in tumor-derived T cells (Fig. 3*A*). This also suggests that SSX-2-specific T cells may be selectively recruited in HCC lesions, resulting in T cells enriched for high avidity Ag recognition, again similarly as in melanoma patients (25). However, multimer staining intensity does not always tightly correlate with TCR avidity (41). Although, we could not generate stable SSX-2-specific T cell lines and clones from TIL or PBMC of the day of surgical intervention, we generated an SSX-2-specific T cell line from peptide-stimulated PBMC obtained 14 mo after surgery. This line displayed high multimer labeling intensity (Fig. 5*A*), high avidity of Ag recognition and efficient tumor cell killing. It may well be that high avidity T cells were selected during time and became dominant among SSX-2-specific T cells in the blood, again as observed in melanoma patients (25).

At present it remains impossible to determine whether the observed T cell activity contributes to tumor control. Nevertheless, it is of note that patient LAU 669 still remains without tumor recurrence after a follow up of 30 mo despite the fact that his disease was very advanced at the time of surgery. Future studies are required to determine whether natural and immunotherapy-induced activation of tumor Ag-specific T cells can improve clinical outcome. This hypothesis can be tested, particularly in studies where patients are immunized with molecularly defined Ags. Using defined Ags in immunotherapy has the central advantage that induced immune responses can be measured precisely, a central issue in modern vaccine development (42).

In summary, our results show that MAGE-A10- and SSX-2-specific T cells were spontaneously activated in vivo, extending the natural immunogenicity of these Ags to HCC in addition to melanoma. Our findings contribute to the development of specific immunotherapy against HCC, by proposing to develop cancer vaccines based on CT Ags such as MAGE-A10 or SSX-2 in HLA-$A2^+$ patients. Such cancer vaccines could also be applied in adjuvant therapy to prevent disease recurrence after surgery. Additionally, tumor-specific T cells could also be expanded in vitro and adoptively transferred to patients, particularly because T cells have been found to be enriched in liver after i.v. infusion (43–45). Finally, CT Ag-derived epitopes, restricted by HLA molecules other than HLA-A2 (e.g., HLA-A1 and HLA-A3) have been described to be immunogenic in melanoma. Thus, immunotherapeutic strategies could be extended to patients with other HLA alleles and other CT Ags, allowing the treatment of a significant proportion of HCC patients.

Acknowledgments

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