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Human Tumor-Derived Heat Shock Protein 96 Mediates In Vitro Activation and In Vivo Expansion of Melanoma- and Colon Carcinoma-Specific T Cells[1]

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Heat shock proteins (hsp) 96 play an essential role in protein metabolism and exert stimulatory activities on innate and adaptive immunity. Vaccination with tumor-derived hsp96 induces $CD8^+$ T cell-mediated tumor regressions in different animal models. In this study, we show that hsp96 purified from human melanoma or colon carcinoma activate tumor- and Ag-specific T cells in vitro and expand them in vivo. HLA-A*0201-restricted $CD8^+$ T cells recognizing Ags expressed in human melanoma (melanoma Ag recognized by T cell-1 (MART-1)/melanoma Ag A (Melan-A)) or colon carcinoma (carcinoembryonic Ag (CEA)/epithelial cell adhesion molecule (EpCAM)) were triggered to release IFN-γ and to mediate cytotoxic activity by HLA-A*0201-matched APCs pulsed with hsp96 purified from tumor cells expressing the relevant Ag. Such activation occurred in class I HLA-restricted fashion and appeared to be significantly higher than that achieved by direct peptide loading. Immunization with autologous tumor-derived hsp96 induced a significant increase in the recognition of MART-1/Melan-$A_{27–35}$ in three of five HLA-A*0201 melanoma patients, and of $CEA_{571–579}$ and $EpCAM_{263–271}$ in two of five HLA-A*0201 colon carcinoma patients, respectively, as detected by ELISPOT and HLA/tetramer staining. These increments in Ag-specific T cell responses were associated with a favorable disease course after hsp96 vaccination. Altogether, these data provide evidence that hsp96 derived from human tumors can present antigenic peptides to $CD8^+$ T cells and activate them both in vitro and in vivo, thus representing an important tool for vaccination in cancer patients. ***The Journal of Immunology,*** **2003, 171: 3467–3474.**

Heat shock protein (hsp)[3] gp96 (hsp96), or GRP94, is located in the endoplasmic reticulum, where it exerts protective functions during cellular stress and plays an important role in the maintenance of protein homeostasis (1). The housekeeping activity of this protein is ascribed to its ability to chaperone nascent or aberrantly folded proteins (1). Recent studies have shown that hsp96 can additionally exert potent stimulatory activity on both innate and adaptive immunity (2, 3). In mice, vaccination with tumor-derived hsp96 protects against tumor challenge and cures established tumors (2). The basis for the immunological activity of this protein is known to reflect two intrinsic functions of hsp96: 1) the ability of chaperoning tumor Ag-derived immunogenic peptides and delivering them to APCs for specific T cell stimulation (2, 3), and 2) the activity as natural adjuvant, promoting maturation and activation of APCs (such as macrophages and dendritic cells), and eliciting secretion of proinflammatory cytokines and chemokines (4, 5).

The evidence that the immunogenicity of tumor-derived hsp96 could stem from the ability of this protein to chaperone antigenic epitopes comes from both structural and immunological studies. Peptide-binding activity of hsp96 or other hsp family members (such as hsp70 or hsp90) has been directly demonstrated in vitro by using labeled peptide probes and by crystallographic studies, which allowed the identification of a possible hydrophobic binding pocket within the hsp96 molecule (6–9). Additional direct evidence of this activity could come from the structural scrutiny of hsp96-associated peptides by mass spectroscopy. However, for technical difficulties related to hsp96 structure (2), efforts in identifying these peptides by biochemical means have been to date successful only in few cases (10–13).

Nevertheless, the vast majority of data supporting the association of antigenic peptides with hsp96 derives from immunological studies in murine models, which demonstrated that preparations of these proteins purified either from virus-infected or from tumor cells could trigger Ag-specific T cell recognition when pulsed on appropriate MHC-matched APC, through a mechanism known as cross-priming (2, 14, 15). Recently, similar data have been obtained by our group in human melanoma-derived hsp70, which was found to be associated with antigenic peptides from the melanoma Ags gp100 and MART-1/Melan-A (hereafter MART-1) (16). However, no direct evidence on the chaperone properties of

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[3] Abbreviations used in this paper: hsp, heat shock protein; CEA, carcinoembryonic Ag; EpCAM, epithelial cell adhesion molecule; TIL, tumor-infiltrating lymphocyte; MART-1, melanoma Ag recognized by T cell-1; Melan-A, melanoma Ag A.

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human tumor-derived hsp96, which belongs to a different hsp family and displays a diverse intracellular localization as compared with hsp70, is presently available. Because immunization with autologous tumor-derived hsp96 has recently started to be evaluated in clinical setting (2, 17–19), it is essential to prove that hsp96 purified from human cancer cells can actually transport immunogenic peptides for cross-presentation to tumor and Ag-specific T cells.

Thus, the purpose of the present study was to investigate whether hsp96 purified from melanoma or liver metastases of colon carcinoma cells did indeed contain immunogenic peptides derived from tumor Ags. To this aim, we used an immunological approach to evaluate the ability of tumor-derived hsp96 to reconstitute the epitopes recognized by $CD8^+$ T cells specific for highly expressed melanoma or colon carcinoma Ags. Additionally, we investigated whether immunization with tumor-derived hsp96 could result in expansion of such specific T cells in the peripheral blood of patients with metastatic melanoma or liver metastases from colon carcinoma. In this study, we provide the first evidence that hsp96 derived from human tumors can present antigenic peptides to $CD8^+$ T cells and activate them both in vitro and in vivo.

Materials and Methods

Ag-specific T cell clones and lines, PBMCs, and in vitro peptide sensitization

As Ag-specific T cells, we used: the HLA-A*0201-restricted MART-1-specific $CD8^+$ T cell clone A42, autologous to 501mel (20) and recognizing the immunodominant epitope MART-$1_{27–35}$, and the $CD8^+$ tumor-infiltrating lymphocyte (TIL) line (GDN-TIL), an oligoclonal T cell line recognizing MART-$1_{27–35}$ epitope in a HLA-A*0201-restricted fashion (20). PBMCs from HLA-A*0201^+ melanoma or colon carcinoma patients were purified from peripheral blood by Ficoll gradient, aliquoted, and stored in liquid nitrogen. MART-1-, carcinoembryonic Ag (CEA)-, and epithelial cell adhesion molecule (EpCAM)-specific T cells were generated from PBMCs by in vitro peptide sensitization, as previously described (21). T cell cloning was performed by limiting dilution in the presence of OKT3 mAb. The anti-CEA T cell line 2708 was generated from PBMC of an HLA-A*0201^+ healthy donor by in vitro peptide sensitization, and contained >90% of $CD8^+$HLA-A*0201/$CEA_{571–579}$ tetramer$^+$ cells.

Melanoma, colon cancer cells, and other targets

The following targets of specific T cell recognition were used in the ELISPOT assay: 501mel, a melanoma line expressing both HLA-A*0201 and MART-1; the colon carcinoma line Colo 206, expressing HLA-A*0201, CEA, and EpCAM (22); and the TAP-deficient lymphoblastoid cell line T2, known to efficiently bind exogenously supplied peptides in the context of HLA-A*0201. The melanoma line Me 2658, expressing significant MART-1 levels, was used for hsp96 purification. Ag (i.e., MART-1, CEA, and Ep-CAM) expression was also evaluated in all tumor specimens (from melanoma or liver metastases from colon carcinoma) used for hsp96 purification. Immunohistochemistry on Formalin-fixed specimens was performed, as previously described (18), using the anti-MART-1 mAb (clone A103; Novocastra Labs, Newcastle upon Tyne, U.K.), the anti-CEA Ab (DAKO, Glostrup, Denmark), and the anti-EpCAM mAb (clone Ber-EP4; DAKO). MART-1 and EpCAM were highly expressed only in a fraction of melanoma (18) and colon carcinoma lesions (data not shown), respectively, including those from the patients developing anti-MART-1 and EpCAM T cell responses upon vaccination (i.e., melanoma patients 003, 007, and 020, and colon carcinoma patients 038 and 045). CEA was overexpressed in the totality of liver metastases from colon carcinoma (data not shown).

Hsp96 purification and vaccine treatment

Hsp96 was purified from tumor cells by affinity columns, as previously described (23). Purification was perfomed by Antigenics (Woburn, MA), starting from at least 3 g of nonnecrotic frozen tumor tissue or from a 3-g pellet of melanoma cells (501mel and Me 2658). Purification of hsp96 for clinical use was obtained under current Good Manufacturing Practice procedures; thus, all preparations contain no detectable level of endotoxin. For the immunization treatment, patients affected by stage IV melanoma (partially or radically resected for s.c. or visceral metastases) or patients with colon carcinoma (curatively resected for liver metastases) gave written informed consent to be vaccinated and fulfilled inclusion criteria required by two different clinical protocols, approved by the Internal Review Board and by the Independent Ethics Committee of the Istituto Nazionale Tumori of Milan (18, 19). Detailed vaccine schedule is reported elsewhere (18, 19). Briefly, patients received four weekly s.c. injections of autologous tumor-derived hsp96 (at 25 μg/injection), followed 1 or 2 mo later by a second cycle of four injections at 2-wk intervals.

Peptides

Peptides were synthesized using an Applied Biosystems 432 A peptide synthesizer (Foster City, CA), following a standard protocol, as previously described (22). The following HLA-A*0201-binding peptides were used: MART-1 p27–35 (AAGIGILTV), EpCAM p263–271 (GLKAGVIAV), and CEA p571–579 (YLSGANLNL, CAP-1). The peptide derived from Flu A matrix protein M1 (GILGFVFTL) was used as control Ag.

IFN-γ ELISPOT and ^{51}Cr release assays

IFN-γ ELISPOT was performed, as previously described (18, 19), and evaluated by a computer-assisted ELISPOT reader (Bioline, AID, Torino, Italy). HLA-blocking experiments were conducted by preincubating target cells with the anti-class I HLA (-A,-B,-C) mAb W6.32 or the anti-HLA-DR mAb L243 (18). For detecting the presence of antigenic peptides in tumor-derived hsp96, monocytes were purified by adherence from PBMCs of HLA-A*0201^+ donors and incubated in the presence of melanoma- or colon carcinoma-derived hsp96 (10 μg/ml), or with 5 μM peptide. After 2-h incubation, monocytes were transferred into ELISPOT plates (at 1×10^5 cells/well) containing specific T cell effectors at the appropriate concentration. For statistical evaluation, a *t* test for unpaired samples was used to compare pre- and postvaccine spots of the same patient, or to evaluate statistical significance of HLA-blocking experiments. Values of $p < 0.05$ were considered as statistically significant. Cytotoxicity was evaluated by a standard ^{51}Cr release assay, as previously described (21). Monocytes, enriched by adherence from PBMC of HLA-A*0201^+ or HLA-A*0201^- healthy donors, were incubated for 2 h at 37°C with hsp96 from 501mel (at 10 μg/ml), labeled with ^{51}Cr, washed, and used as targets (at 1000 cells/well) in a 6-h assay. For competition experiments, monocytes were pretreated with a 2-fold excess of hsp96 from colon carcinoma, or of BSA as negative control, washed several times, incubated with hsp96 from 501mel (at 10 μg/ml), and used as targets.

IFN-γ secretion assay

Monocytes, purified by adherence from PBMCs of HLA-A*0201^+ donors, were incubated overnight at 37°C with hsp96 from colon carcinoma or melanoma cells (at 10 μg/ml), or with MART-$1_{27–35}$ or $CEA_{571–579}$ peptides (10 μM). T cells specific for MART-1 (A42 T cell clone) or CEAs (oligoclonal T cell lines, composed by >90% CEA-specific $CD8^+$ T cells, as assessed by HLA/tetramer staining) were then added. After 3 h at 37°C, nonadherent cells were collected and analyzed for IFN-γ secretion by the Miltenyi IFN-γ cytokine secretion assay (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's procedures. Data were analyzed by FACSCalibur and CellQuest software (BD Biosciences, Milan, Italy).

Staining with HLA/peptide tetramers

HLA-A*0201/MART-$1_{26–35/2L}$ tetramers, used for detecting anti-MART-1-specific T cells, were kindly provided by C. Giachino (Fondazione Maugeri, Pavia, Italy). HLA-A*0201/$CEA_{571–579}$ tetramers and $EpCAM_{263–271}$, used for staining colon carcinoma-reactive T cells, were produced by Beckman Coulter (San Diego, CA). Tetramer binding was evaluated after staining with the iMASC Gating Kit (Beckman Coulter), containing FITC anti-CD8 mAb, together with PC5 anti-CD4, CD13, and CD19 mAbs. Data are reported as percentage of tetramer$^+CD8^+$, CD4/CD13/$CD19^-$ cells. As negative control, we used iTAgTM HLA class I human negative tetramers streptavidin-PE (Beckman Coulter), developed for assessing the level of background PE fluorescence. They are loaded with a peptide that has been shown to tightly bind to HLA-A2 and has been proven not to be recognized by any T cells from HLA-$A2^+$ individuals. Cells were analyzed using FACSCalibur and CellQuest software (BD Biosciences).

IFN-γ intracellular staining

IFN-γ intracellular staining was performed, as previously described (22). Briefly, PBMCs were stimulated with MART-1 peptide; then brefeldin A (Sigma-Aldrich, Deisenhofen, Germany) was added to block cytokine secretion. PBMCs were stained with PerCP anti-CD3, APC anti-CD8, PE anti-CD45RA, and PE anti-CCR7 mAbs (BD Biosciences). After lysing and permeabilizing the cells, IFN-γ was stained by incubation with FITC anti-IFN-γ mAb (BD Biosciences). Data acquisition was performed on FACSCalibur and analyzed using CellQuest software (BD Biosciences).

Results

T cells specific for known tumor Ags recognize hsp96 derived from human cancer cells

Ag-specific T cells were used for detecting immunogenic epitopes in hsp96 extracted either from autologous or HLA-matched tumor cells. To this aim, we focused on three tumor Ags containing HLA-A*0201-restricted epitopes: 1) MART-1 (epitope 27–35), highly expressed by melanoma cells (20, 21); 2) CEA (epitope 571–579), overexpressed in several epithelial tumors, including colon carcinoma; and 3) EpCAM (epitope 263–271), an adhesion molecule overexpressed in colon carcinoma cells. The two latter peptides were recently reported to be target of spontaneous $CD8^+$ T cell-mediated immune recognition in colon carcinoma patients (22). To show the presence in tumor-derived hsp96 of peptides originated from these Ags, epitope-specific T cells were incubated with monocytes from HLA-A*0201^+ healthy donors, pulsed with hsp96 purified from melanoma or colon carcinoma cells, and their reactivity was measured by IFN-γ ELISPOT and ^{51}Cr release assays. As shown in Fig. 1, both the anti-MART-1 T cell clone A42 (Fig. 1*A*) and the oligoclonal T cell line GDN-TIL (Fig. 1*B*) released considerable amounts of IFN-γ in response to monocytes pulsed with hsp96 from both the MART-1^+ melanomas 501mel (autologous to A42) and Me2658. Such reaction was significantly blocked by the anti-HLA-A,-B,-C mAb W6.32, but not by the anti-HLA-DR mAb L243, indicating class I HLA-restricted T cell recognition and TCR involvement. A certain degree of cross-reactivity was observed in the presence of hsp96 purified from colon carcinoma cells, but such response was significantly weaker than that mediated by melanoma-derived hsp96 and could not be blocked by anti-HLA class I or II mAbs (Fig. 1). No cross-presentation was detected when HLA-A*0201-negative monocytes were used as APC (data not shown). To confirm that the oligoclonal anti-MART-1 T cell line GDN-TIL was recognizing HLA-A2/MART-1 complex on monocytes loaded with 501mel-derived hsp96, a cold target inhibition assay using T2 cells pulsed with MART-$1_{27–35}$ peptide or with $CEA_{571–579}$, as negative control, was performed (Fig. 1*C*). A significant inhibition of the lysis of monocytes loaded with 501mel-derived hsp96 was achieved when T2 + MART-$1_{27–35}$, but not T2 + $CEA_{571–579}$, was added to the assay at 20-fold excess. The killing by the GDN-TIL of HLA-A*0201^+ monocytes pulsed with 501mel-derived hsp96 was reduced by the addition of irrelevant hsp96 (i.e., derived from MART-1-negative colon carcinoma cells), in a dose-dependent manner (Fig. 1*D*). In contrast, no competition was observed when a 2-fold excess of BSA was used as control (data not shown). These latter data suggest that colon carcinoma- and melanoma-derived hsp96 competed for the binding to monocytes through the same receptor, but only hsp96 from MART-1^+ melanoma cells contain the relevant T cell epitope (i.e., MART-$1_{27–35}$).

Chaperone activity of hsp96 was then analyzed in human colon carcinoma cells. For such experiments, T cell clones and bulk cultures recognizing HLA-A*0201-binding peptides derived from CEA and EpCAM were used. As shown in Fig. 2, *A* and *B*, both the anti-$CEA_{571–579}$ T cell line and the $CD8^+$ T cell clone produced IFN-γ in response to HLA-A*0201^+ monocytes pulsed with hsp96 purified from autologous colon carcinoma cells, and not from allogeneic melanoma. Such recognition was significantly blocked by anti-HLA class I, but not by anti-HLA class II mAb. Overlapping results were obtained with $EpCAM_{263–271}$-specific T cell line and $CD8^+$ T cell clone (Fig. 2, *C* and *D*, respectively). Even in this case, HLA class I-restricted recognition of HLA-A*0201^+ monocytes occurred only after pulsing with hsp96 from colon carcinoma cells. The amounts of IFN-γ released by anti-CEA and anti-EpCAM effectors were remarkably higher when APCs were pulsed with colon carcinoma-derived hsp96 as compared with the relevant peptide provided exogenously (Fig. 2).

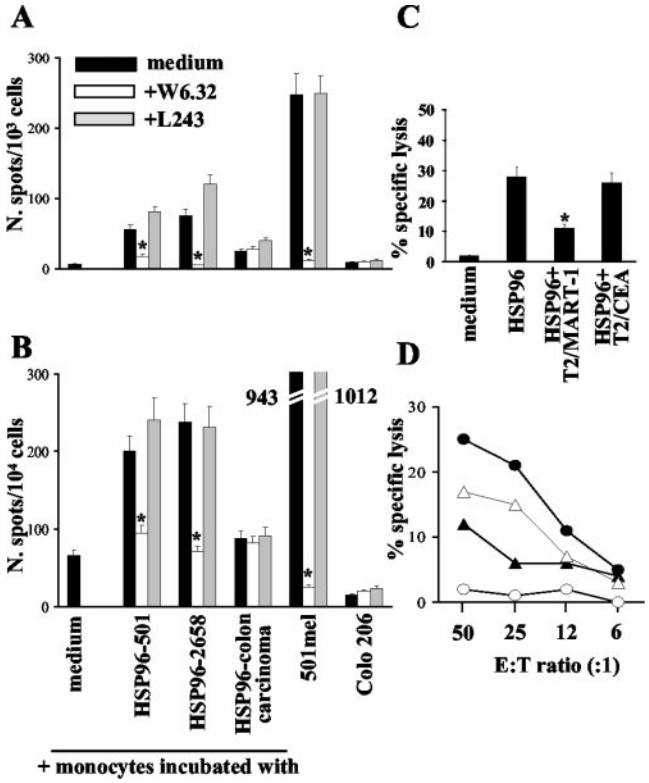

FIGURE 1. Recognition of melanoma-derived hsp96 by anti-MART-1 T cells. The $CD8^+$ T cell clone A42 (*A*) and the oligoclonal TIL $CD8^+$ T cell bulk culture GDN-TIL (*B*), both specifically recognizing MART-$1_{27–35}$ in the context of HLA-A*0201, were cultured in the presence of HLA-A*0201^+ monocytes incubated with medium, with hsp96 derived from two different HLA-A*0201^+/MART-1^+ melanoma cell lines (501 and 2658), or with hsp96 derived from HLA-A*0201^+ colon carcinoma cells. Reactivity was measured by IFN-γ ELISPOT. Whole melanoma (501mel) and colon carcinoma (Colo 206) cells were also included as positive and negative controls, respectively. Targets were pretreated or not with anti-HLA class I (W6.32) or class II (L243) mAbs to prove TCR involvement. *, $p < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA mAb. *C*, Cold target-inhibition assay on recognition of HLA-A*0201^+ monocytes pulsed with melanoma-derived hsp96, by the anti-MART-1 GDN-TIL. Effector cells were incubated with ^{51}Cr-labeled monocytes loaded either with medium or with 501mel-derived hsp96 (10 μg/ml), in the absence or presence of cold T2 cells pulsed with MART-$1_{27–35}$ or with $CEA_{571–579}$ peptide (at hot:cold ratio of 1:20). Only cold T2 + MART-1 peptide inhibited recognition of 501mel-pulsed monocytes by GDN-TIL. *, $p < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of 501mel-hsp96-pulsed monocytes in the absence of cold targets. *D*, Competition assay of the lysis of HLA-A*0201^+ monocytes loaded with melanoma-derived hsp96 by GDN-TIL. Lytic activity of the anti-MART-1 GDN-TIL line against HLA-A*0201^+ monocytes pulsed with hsp96 from 501mel (10 μg/ml) (●) could be competed with excess of irrelevant hsp96 (from colon carcinoma), in a dose-dependent manner (▲, 2-fold excess; △, 1-fold excess), but not with the same excess of BSA (data not shown). GDN-TIL did not lyse HLA-A*0201^- monocytes pulsed with 501mel-hsp96 (○). Results are representative of three independent experiments.

To further confirm the presence of antigenic peptides in tumor-derived hsp96, a cytokine secretion assay combined to cytofluorimetric analysis was performed after 3-h incubation of Ag-specific T cells with HLA-A*0201^+ monocytes pulsed either with hsp96 derived from melanoma or colon carcinoma cells, or with

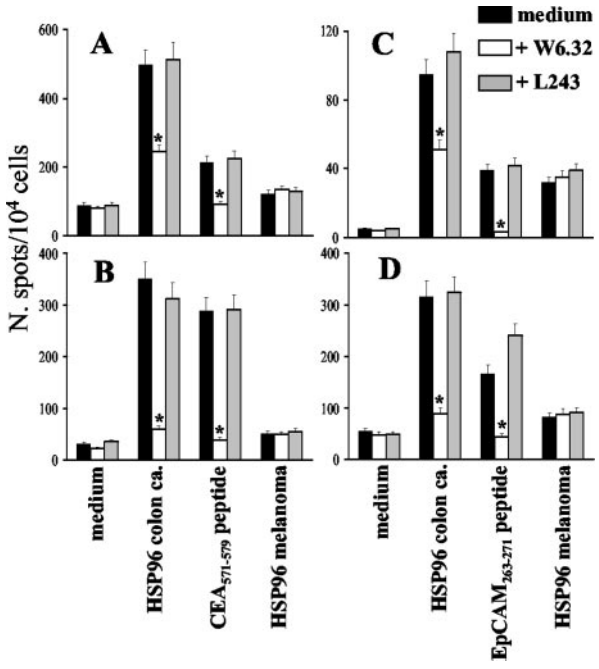

FIGURE 2. Recognition of colon carcinoma-derived hsp96 by anti-CEA and anti-EpCAM T cells. Anti-CEA bulk $CD8^+$ T cell line (*A*) and $CD8^+$ T cell clone (*B*), and anti-EpCAM bulk $CD8^+$ T cell line (*C*) and $CD8^+$ T cell clone (*D*) were incubated with HLA-A*0201^+ monocytes pulsed with medium, with hsp96 derived from HLA-A*0201^+ colon carcinoma cells or with hsp96 from the melanoma line 501mel. Reactivity was measured by IFN-γ ELISPOT. Peptide-pulsed monocytes were also included as positive control. Targets were pretreated or not with anti-HLA class I (W6.32) or class II (L243) mAb to prove TCR involvement. Effectors were generated from PBMCs of an HLA-A*0201^+ colon carcinoma patient (autologous to the colon carcinoma-derived hsp96) by in vitro peptide sensitization (bulk $CD8^+$ T cell line) and subsequent cloning by limiting dilution in the presence of OKT3 mAb. *, $p < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA mAb. Results are representative of three independent experiments.

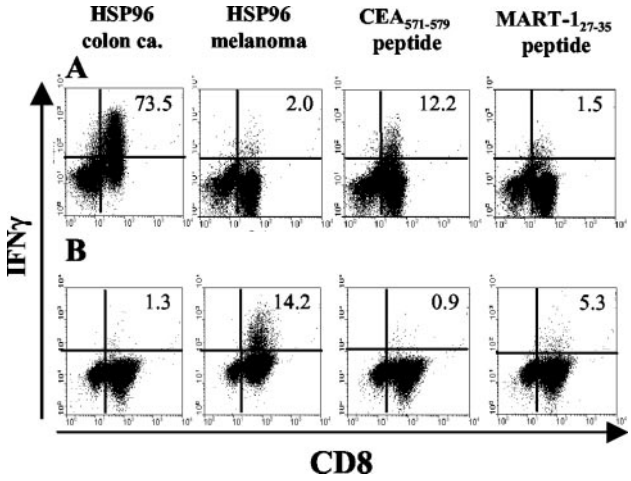

FIGURE 3. Ag specificity of cytokine secretion by anti-MART-1 and anti-CEA T cells in response to monocytes pulsed with tumor-derived hsp96. HLA-A*0201^+ monocytes, pulsed overnight with hsp96 derived from colon carcinoma or melanoma, or with control peptides (10 μM $CEA_{571-579}$ and MART-1_{27-35}, respectively), were incubated with the oligoclonal CEA-specific T cell line 2708 (*A*) or the anti-MART-1 T cell clone A42 (*B*). Staining for IFN-γ was performed by the Miltenyi secretion assay, and analyzed by FACSCalibur and CellQuest. Numbers represent the percentage of IFN-γ^+CD8^+ cells. Results are representative of three independent experiments.

the relevant synthetic peptide. As reported in Fig. 3*A*, hsp96 from colon carcinoma cells elicited IFN-γ secretion in 73% of $CEA_{571-579}$-specific T cells, while no effect (2%) was seen in the presence of hsp96 from melanoma. The triggering of IFN-γ production mediated by colon carcinoma-derived hsp96 was significantly higher than that observed in the presence of the synthetic $CEA_{571-579}$ peptide at the saturating dose of 10 μM (mediating cytokine secretion in only 12% T cells), and it was selectively detected in CEA-specific T cells, but not in the anti-MART-1 T cell clone. The more efficient pathway of presentation by hsp96 as compared with purified synthetic peptides was also evident with anti-MART-1 T cells (Fig. 3*B*), only 5% of which could be triggered to secrete IFN-γ by 10 μM MART-1_{27-35} peptide, whereas hsp96 derived from the autologous melanoma cells mediated cytokine secretion in 14% cells. Hsp96-induced IFN-γ secretion by both anti-MART-1 and anti-CEA T cells was significantly blocked by anti-class I HLA mAb and could not be detected in the presence of HLA-A*0201-negative monocytes (data not shown).

*Hsp96 vaccine increases in vivo frequency of anti-MART-1 melanoma-specific T cells in HLA-A*0201 patients*

Two phase I-II clinical trials based on vaccination with autologous tumor-derived hsp96 were recently performed in our Institute. In the first trial, 39 stage IV melanoma patients were treated according to a protocol described elsewhere in details (18) and summarized in *Materials and Methods*. The immunological monitoring, performed by IFN-γ ELISPOT, showed a significant increase of HLA class I-restricted T cell recognition of either autologous or allogeneic HLA-A-matched tumor cells in approximately half of the tested patients, as recently reported elsewhere (18).

Because our in vitro data suggested that tumor-derived hsp96 contained antigenic peptides, we evaluated whether T cells specific for MART-1 could have been expanded in vivo in HLA-A*0201^+ patients vaccinated with autologous melanoma-derived hsp96. Indeed, among the five HLA-A*0201^+ patients displaying increased CD8-mediated melanoma recognition after hsp96 vaccination (18), three showed a significant and specific increment of IFN-γ release in response to MART-1_{27-35} peptide (Fig. 4), with unchanged T cell responses to the influenza-derived peptide M1Flu, used as irrelevant control. In contrast, in the remaining seven HLA-A*0201^+ melanoma patients, which did not show any enhancement of CD8-mediated melanoma recognition after hsp96 vaccination (18), no sign of increased anti-MART-1 reactivity was detected (data not shown). The enhancement of IFN-γ release in response to MART-1_{27-35} peptide occurred with different kinetics in these three cases, being detectable since the fourth week of treatment (i.e., at V5) in patients 007 and 020, and 1 mo after the end of the first vaccine cycle (V6) in patient 003 (Fig. 4). A significant boost of the IFN-γ release in response to the HLA-A*0201^+ MART-1 melanoma cell line 501mel was additionally observed in all three patients (Fig. 4). Of note, patients 003 and 007 were the only 2 patients among 28 who responded clinically to the vaccine with a complete tumor regression (18).

PBMCs obtained before and during treatment were additionally evaluated for staining with HLA-A*0201/MART-1 tetramers. A significant increment of $CD8^+$tetramer$^+$ cells could be detected in fresh PBMCs from all the three patients, with kinetics comparable to that observed by ELISPOT (Table I). In contrast, no modification of PBMC staining with negative tetramers was observed (Table I). Fresh PBMCs from patient 003 were also cultured for 1 wk in the presence of MART-1_{27-35} peptide and IL-2 to enrich for

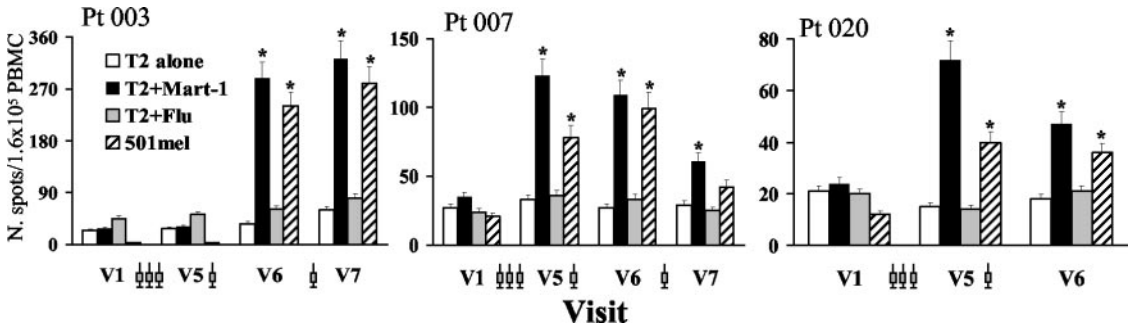

FIGURE 4. In vivo induction of anti-MART-1 reactivity in melanoma patients vaccinated with autologous tumor-derived hsp96. Fresh PBMCs were obtained from three different HLA-A*0201$^+$ melanoma patients before (Visit, V1) and at different times during vaccination with hsp96 (V5, 1 wk after the third injection and before the fourth vaccine; V6, 1 mo after the end of the first cycle; V7, V8, and V9, 2 wk after the first, second, and third injection of the second cycle, respectively). Syringes indicate injection times. T cell reactivity was measured by IFN-γ ELISPOT in response to T2 cells either alone or pulsed with MART-$1_{27–35}$ and M1Flu peptides, or to the HLA-A*0201$^+$/MART-1$^+$ melanoma cell line 501mel. *, $p < 0.05$ (evaluated by t test for unpaired samples), as compared with reactivity in PBMCs obtained before vaccination (V1). Results are representative of two independent experiments.

Ag-specific activity. The percentage of CD8$^+$ HLA-A*0201/MART-$1_{27–35}$ tetramer$^+$ cells (Fig. 5*A*), but not of CD8$^+$ HLA-A*0201-negative tetramer$^+$ (Fig. 5*B*) cells, rose notably during vaccination, reaching a 4.4% in PBMCs obtained at V7. The rise of MART-1 tetramer$^+$ T cells was paralleled by the increase of specific IFN-γ release in response to both MART-1 peptide pulsed on T2 cells and MART-1$^+$ HLA-A*0201$^+$ melanoma cell line 501mel (Fig. 5*C*). Cytofluorimetric analysis for IFN-γ intracellular staining combined to phenotypic analysis was additionally performed to evaluate the phenotypic features of MART-1-specific T cells in patient 003. As reported in Fig. 5, *D* and *F*, T cells releasing cytokine in response to MART-1, and expanded in vivo by hsp96 vaccine (V6), showed a CD3$^+$ CD45RA$^+$ CCR7$^-$ phenotype, recently reported to be associated to lytic effector cells (24, 25). On the contrary, no IFN-γ production in response to MART-$1_{27–35}$ peptide could be detected in CD45RA-negative T cell subpopulations. A similar phenotypic pattern of MART-1-reactive CD8$^+$ T cells was also observed in PBMC from patient 007 (data not shown).

*Hsp96 vaccine increases the in vivo frequency of Ag-specific T cells in HLA-A*0201-positive colon carcinoma patients*

A second clinical protocol of hsp96 vaccination was conducted in our Institute on 29 colon carcinoma patients surgically resected for liver metastases (19). On the basis of the data obtained in melanoma patients, we analyzed whether treatment with autologous tumor-derived hsp96 could lead to in vivo expansion of T cells recognizing the same CEA and EpCAM epitopes detected in vitro as bound to hsp96. Among the 5 HLA-A*0201$^+$ patients displaying increased CD8-mediated colon carcinoma recognition after hsp96 vaccination (19), 2 developed a significant enhancement of $CEA_{571–579}$ and $EpCAM_{263–271}$ peptide recognition, paralleled by increase in the specific reactivity against HLA-A*0201$^+$ CEA$^+$ EpCAM$^+$ colon carcinoma cells, as detected by IFN-γ ELISPOT (Fig. 6). On the contrary, no changes in the T cell reactivity against the influenza virus-derived epitope M1Flu were observed (data not shown). In the remaining four HLA-A*0201$^+$ colon carcinoma patients, which did not show any enhancement of CD8-mediated tumor recognition after hsp96 vaccination (19), no sign of increased anti-CEA and anti-EpCAM reactivity was detected (data not shown).

HLA-A*0201 tetramers containing $CEA_{571–579}$ and $EpCAM_{263–271}$ peptides were then used for tracing such reactivity in peripheral blood of these patients. PBMCs from patients 038 and 045, obtained before (at V1) and after the first cycle of vaccination with autologous HSP96 (V5 or V6), were enriched for peptide specificity by 1 or 2 wk in vitro culture in the presence of peptide and exogenous IL-2. In both patients, a significant increase of CD8$^+$HLA/$CEA_{571–579}$ tetramer$^+$ cells was detected in PBMCs obtained after hsp96 vaccine as compared with PBMCs from V1 (Fig. 7, *A* and *B*). No increment of staining with HLA-A*0201-negative tetramers was observed (Fig. 7, *C* and *D*). The increase in CEA-tetramer labeling was associated with the appearance of class I HLA-restricted $CEA_{571–579}$ peptide-specific T cell recognition and with the induction of reactivity toward the HLA-A*0201$^+$ CEA$^+$ colon carcinoma line Colo 206 (Fig. 7, *E* and *F*). Staining with HLA/$EpCAM_{263–271}$ tetramers (Fig. 8, *A* and *B*), but not with negative tetramers (Fig. 8, *C* and *D*), showed a significant rise in PBMCs from postvaccine samples, again associated with a boost of $EpCAM_{263–271}$-specific HLA class I-restricted IFN-γ release (Fig. 8, *E* and *F*). The relatively low tetramer-staining intensity observed in anti-CEA and anti-EpCAM T cells, as compared with that achieved with anti-MART-1 T cell cultures (Fig. 5*A*), could be a sign of limited TCR affinity and reduced peripheral frequency of these T cells, as already reported by others (26). In keeping with

Table I. *Increased frequency of HLA-A*0201/MART-1 tetramer$^+$ CD8$^+$ T cells in PBMCs of HLA-A*0201$^+$ melanoma patients vaccinated with autologous tumor-derived hsp96*

Patient Number	Visit (V)	Day[a]	% CD8$^+$ HLA-A2/MART-$1_{26-35/2L}$ Tetramer[b]	% CD8$^+$ HLA-A2-Negative Tetramer[c]
003	V1	0	0.08	0.06
	V5	21	0.16	0.01
	V6	49	0.96	0.05
	V7	63	1.17	0.07
007	V1	0	0.16	0.04
	V5	21	0.98	0.02
	V6	49	1.45	0.05
	V7	63	0.47	0.04
020	V1	0	0.12	0.01
	V5	21	0.98	0.02
	V6	49	0.86	0.01

[a] PBMCs from melanoma patients were obtained at day 0 and at different days during vaccination as indicated.

[b] PBMCs were stained with FITC-CD8 (positive gate), PC5-CD4-CD13-CD19 (negative gate), as well as with PE-HLA-A*0201/MART-$1_{26-35/2L}$ tetramers, and analyzed as three-color flow cytometry by FACSCalibur and CellQuest. Numbers represent the percentage of CD8$^+$ HLA-A*0201/MART-$1_{27-35}$$^+$ cells within the CD8$^+$ population. Results are representative of two independent experiments.

[c] PBMCs were stained with FITC-CD8 (positive gate), PC5-CD4-CD13-CD19 (negative gate), as well as with PE-HLA-A*0201-negative tetramers. Results are representative of two independent experiments.

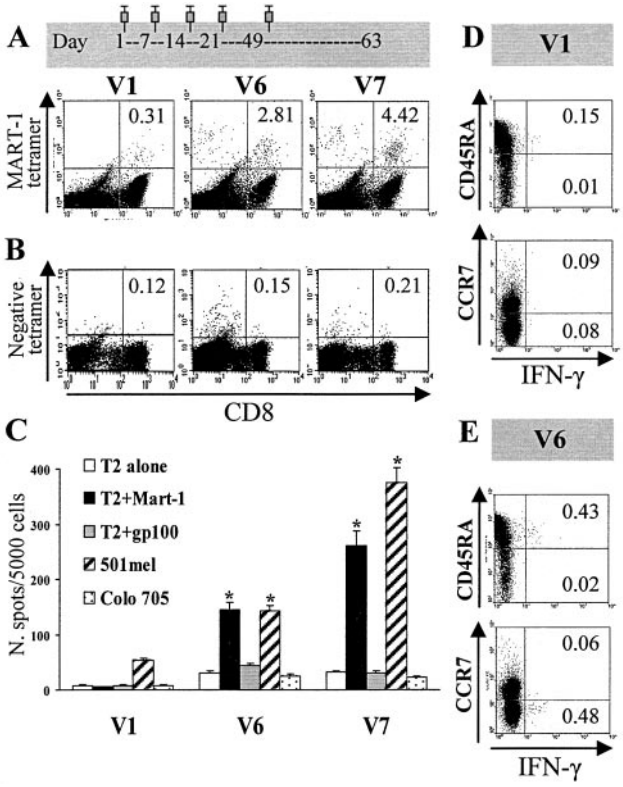

FIGURE 5. In vivo expansion of MART-1-reactive effector T cells in melanoma patients vaccinated with autologous tumor-derived hsp96. Fresh PBMCs obtained from patient 003 before or at different times during vaccination with autologous tumor-derived hsp96 were cultured for 1 wk in the presence of MART-1_{27-35} peptide and IL-2 (60 IU/ml). Cells were stained with FITC-CD8 (positive gate); PC5-CD4, -CD13, -CD19 (negative gate); as well as with PE-HLA-A*0201/MART-$1_{26-35/2L}$ tetramers, and analyzed as three-color flow cytometry by FACSCalibur and CellQuest. Numbers represent the percentage of $CD8^+$ HLA-A*0201/MART-$1_{26-35/2L}{}^+$ cells within the $CD8^+$ population (*A*). PBMC cultures were additionally stained with HLA-A*0201-negative tetramers (*B*). PBMCs were obtained before treatment (V1), 1 mo after the end of the first cycle (V6) or 2 wk after the first vaccination of the second cycle (V7). Syringes indicate injection times. Simultaneously, IFN-γ ELISPOT was performed for detecting functional anti-MART-1 reactivity (*C*). As targets, T2 cells pulsed with MART-1_{27-25} or $gp100_{209-217}$ peptides, the MART-1^+ HLA-A*0201^+ melanoma line 501mel, and the HLA-A*0201^+ colon carcinoma line Colo 705 were used. *, $p < 0.05$ (evaluated by *t* test for unpaired samples), as compared with reactivity in cultures derived from PBMCs obtained before vaccination (V1). To assess phenotypic features of anti-MART-1 T cells present in fresh PBMCs before (V1, *D*) and after (V6, *E*) vaccination, IFN-γ intracellular staining in response to MART-1 was combined with staining in the presence of CD45RA and CCR7 mAb. Results are representative of two independent experiments.

this hypothesis, we could not detect any reproducible increment of $CEA_{571-579}$ and $EpCAM_{263-271}$/tetramer staining in fresh PBMCs of these patients (data not shown). The observed lower frequency of T cells specific for $CEA_{571-579}$ and $EpCAM_{263-271}$, as compared with anti-MART-1 T cells, is to be ascribed to the strong variability in the peptide immunogenicity, MART-1_{27-35} peptide being one of the most immunogenic T cell epitopes in cancer patients (21).

In colon carcinoma patients, vaccination with hsp96 was given in an adjuvant setting, i.e., for prevention of tumor recurrence after radical resection of liver metastases, and, therefore, tumor regression data were not available. However, the in vivo expansion of anti-CEA and EpCAM T cells upon hsp96 vaccination appears to be associated with a better prognosis, because both colon carcinoma patients are still free of recurrence after 30 mo of follow-up (19).

Discussion

Hsp96 is known to induce specific immune responses against murine tumor and viral Ags (2, 3). In the present work, we report that hsp96 purified from human cancer cells can activate tumor- and Ag-specific T lymphocytes both in vitro and in vivo. Our data show for the first time that hsp96 purified from human cancer cells and pulsed on appropriate APCs can reconstitute class I HLA-restricted recognition by Ag-specific $CD8^+$ T cells, through a mechanism of cross-presentation. These results, as those previously described by our group with hsp70 (16), provide evidence, albeit indirect, of the ability of tumor-derived hsp96 to bind and transport peptides derived from immunogenic Ags such as MART-1, CEA, and EpCAM.

The antigenic transfer to APC mediated by hsp96 appeared to be superior to that achieved with exogenous peptide loading. This enhanced presentation was Ag dependent, i.e., it could be selectively detected when anti-MART-1 and anti-CEA or EpCAM T cells were activated by hsp96 derived from tumor cells expressing the relevant Ag (i.e., melanoma and colon carcinoma cells, respectively), and it could be specifically competed by excess of unrelated hsp96. This phenomenon could be ascribed to different and

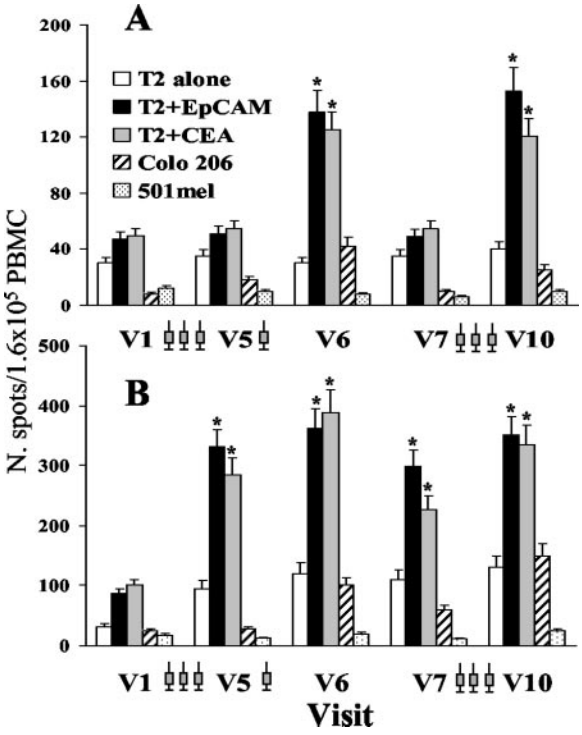

FIGURE 6. In vivo increase of anti-CEA and EpCAM reactivity in colon carcinoma patients vaccinated with autologous tumor-derived hsp96. Fresh PBMCs were obtained from two different HLA-A*0201^+ colon carcinoma patients (*A*, patient 038; *B*, patient 045) before (V1) and at different times during vaccination with hsp96 (V5, 1 wk after the third injection and before the fourth vaccine; V6, 1 mo after the end of the first cycle; V7, 2 mo after the end of the first cycle; V10, 2 wk after the third injection of the second cycle). Syringes indicate injection times. T cell reactivity was measured by IFN-γ ELISPOT in response to T2 cells either alone or pulsed with $CEA_{571-579}$ or $EpCAM_{263-271}$ peptides, or to the HLA-A*0201^+/CEA^+/$EpCAM^+$ colon carcinoma cell line Colo 206. The HLA-A*0201^+ melanoma line 501mel was also used as negative control. *, $p < 0.05$ (evaluated by *t* test for unpaired samples), as compared with reactivity in PBMCs obtained before vaccination (V1). Results are representative of three independent experiments.

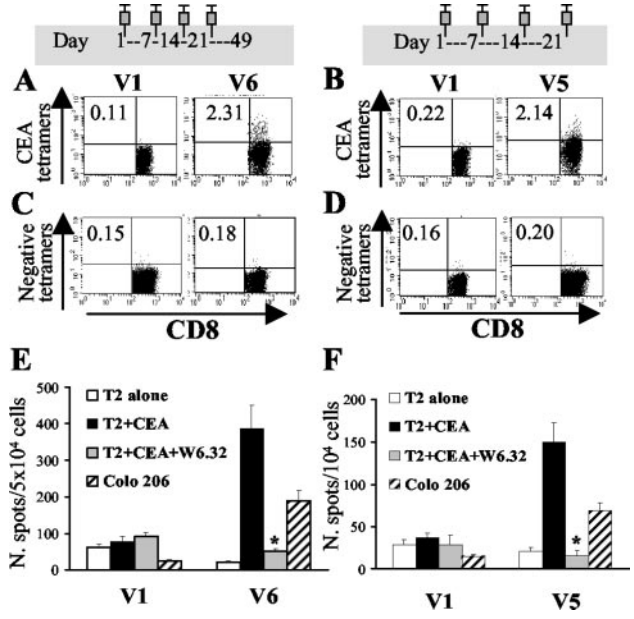

FIGURE 7. In vivo expansion of $CEA_{571–579}$ peptide-reactive T cells in colon carcinoma patients vaccinated with autologous tumor-derived hsp96. Fresh PBMCs obtained before (V1) or during vaccination (at V6, i.e., 1 mo after the end of the first vaccine cycle in patient 038 and at V5, i.e., 2 wk after the third vaccine injection in patient 045) were cultured for 1–2 wk in the presence of $CEA_{571–579}$ peptide and exogenous IL-2 (60 IU/ml). Cells were stained with FITC-CD8 (positive gate), PC5-CD4-CD13-CD19 (negative gate), as well as with PE-HLA-A*0201/$CEA_{571–579}$ tetramers, and analyzed as three-color flow cytometry by FACSCalibur and CellQuest. Numbers represent the percentage of $CD8^+$ HLA-A*0201/$CEA_{571–579}{}^+$ cells within the $CD8^+$ population (*A*, patient 038; *B*, patient 045). PBMC cultures were additionally stained with HLA-A*0201-negative tetramers (*C*, patient 038; *D*, patient 045). Simultaneously, IFN-γ ELISPOT was performed for detecting functional anti-$CEA_{571–579}$ reactivity (*E*, patient 038; *F*, patient 045). PBMCs were tested against T2 either alone, or pulsed with $CEA_{571–579}$ peptide. To prove TCR involvement, pulsed T2 cells were pretreated with the anti-HLA class I mAb W6.32. The HLA-A*0201^+ CEA^+ colon carcinoma cell line Colo 206 was also included as target. *, $p < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA mAb. Results are representative of two independent experiments.

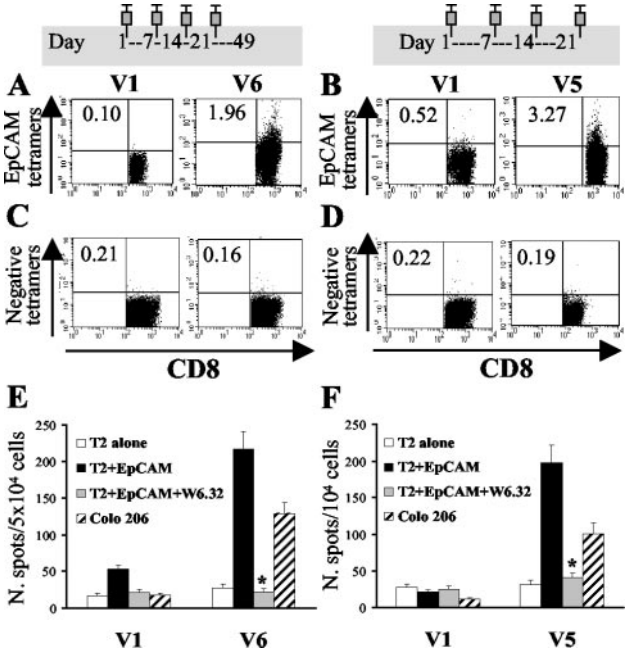

FIGURE 8. In vivo expansion of $EpCAM_{263–271}$ peptide-reactive T cells in colon carcinoma patients vaccinated with autologous tumor-derived hsp96. Fresh PBMCs obtained before (V1) or during vaccination (at V6, i.e., 1 mo after the end of the first vaccine cycle in patient 038, and at V5, i.e., after the third vaccine injection in patient 045) were cultured for 1–2 wk in the presence of $EpCAM_{263–271}$ peptide and exogenous IL-2 (60 IU/ml). Cells were stained with FITC-CD8 (positive gate), PC5-CD4-CD13-CD19 (negative gate), as well as with PE-HLA-A*0201/$EpCAM_{263–271}$ tetramers, and analyzed as three-color flow cytometry by FACSCalibur and CellQuest. Numbers represent the percentage of $CD8^+$ HLA-A*0201/$EpCAM_{263–271}{}^+$ cells within the $CD8^+$ population (*A*, patient 038; *B*, patient 045). PBMC cultures were additionally stained with HLA-A*0201-negative tetramers (*C*, patient 038; *D*, patient 045). Simultaneously, IFN-γ ELISPOT was performed for detecting functional anti-$EpCAM_{263–271}$ reactivity (*E*, patient 038; *F*, patient 045). PBMCs were tested against T2 either alone, or pulsed with $EpCAM_{263–271}$ peptide. To prove TCR involvement, pulsed T2 cells were pretreated with the anti-HLA class I mAb W6.32. The HLA-A*0201^+ $EpCAM^+$ colon carcinoma cell line Colo 206 was also included as target. *, $p < 0.05$ (evaluated by *t* test for unpaired samples), as compared with the recognition of the same target in the absence of anti-HLA mAb. Results are representative of two independent experiments.

nonmutually exclusive mechanisms. An improved processing and HLA peptide loading mediated by hsp96, as compared with free peptide, can stem from the ability of hsp96 to access class I HLA presentation pathway through receptor-mediated uptake (2, 3), a mechanism that, at variance with nonspecific phagocytosis, operates with high efficiency even at low Ag concentrations (14). Furthermore, endogenous processing of exogenously provided epitopes is per se associated to improved CTL induction, as recently shown by Zwaveling et al. (27), who reported that vaccination with long peptides resulted in markedly enhanced T cell induction in comparison with the minimal CTL epitope vaccine, as a consequence of a preferable endocytosis, processing, and presentation by professional APC. Finally, the activatory functions that hsp96 plays on APCs (4) could eventually contribute in providing a more favorable environment for T cell activation.

Hence, hsp96 may represent an efficient and well-characterized tool for inducing class I HLA cross-presentation of tumor Ags from exogenous protein sources in human setting. Such presentation pathway, which is considered to play a key role in immune responses to tumors and viruses (28), seems to operate with low efficiency and through poorly understood mechanisms when exogenous Ags are given as tumor lysate or apoptotic bodies (29). The ability of hsp96 to simultaneously deliver antigenic peptides and appropriate activatory stimuli to APCs could provide an additional advantage upon the other cell-derived tumor Ag sources that, on the contrary, have been shown to potentially mediate suppressive effects on APC differentiation and maturation (30, 31).

We further demonstrated that hsp96 can activate and expand specific T lymphocytes in vivo. Among patients developing increased CD8 T cell-mediated recognition of autologous or HLA-matched allogeneic tumor cells upon vaccination with tumor-derived hsp96 (18, 19), expansion of T cells recognizing the same Ags detected in vitro in the hsp96 preparations was observed in a significant number of cases. Indeed, three of five melanoma patients displayed increased MART-1 activity, while enhanced T cell recognition of CEA/EpCAM Ags was seen in two of five colon carcinoma patients. Such reactivities, assessed by IFN-γ ELISPOT and HLA tetramer staining, were undetectable in PBMCs before vaccination, and involved T cells displaying a $CD45RA^+$ $CCR7^-$ phenotype, recently proposed to characterize cytolytic effector T

cells able to migrate into inflamed peripheral tissues (24, 25). Interestingly, hsp96-mediated T cell triggering appeared to be associated to in vivo tumor growth control, because four of the five patients showing expansion of Ag-specific T cells after hsp96 vaccination seemed to benefit from the treatment, with two complete responses in melanoma and two long-term disease-free survivals in colon carcinoma (18, 19). Ag-specific T cell effectors boosted by hsp96 immunization could thus be directly responsible for the in vivo tumor cell destruction, or may be alternatively just a marker of a potent activation of antitumor immune responses induced by hsp96 and possibly involving other unknown T cell reactivities. Indeed, given the physiological role of hsp96 as chaperone of the whole antigenic repertoire of the cell (2–4), it is conceivable that immunization with tumor-derived hsp96 may activate T cells directed to multiple antigenic determinants, including those derived from other shared as well as unique tumor Ags. Because the major rationale to the usage of autologous tumor-derived hsp96 consists in the possibility to immunize against a broad and patient-tailored Ag array, an extensive monitoring of different T cell reactivities directed toward immunogenic epitopes derived from shared tumor Ags and restricted by common HLA alleles would provide important information about the immunogenic potential of hsp96 vaccine. Preliminary experiments at this regard indeed show that increased $CD8^+$ T cell responses directed against other common Ags (such as for instance the gp100-HLA-A*0301 epitope) (32) can be detected in hsp96-vaccinated melanoma patients (data not shown).

For its combined features of peptide vehicle and APC maturation promoter, hsp96 is deemed to be an appealing tool for immunization against tumor and infectious diseases. The results of the present study extend previous work in murine setting and provide strong evidence to the efficient chaperone activity of human tumor-derived hsp96, which could be exploited in the development of new and more effective immunotherapeutic strategies in cancer patients.

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