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INDUCIBLE Hsp70 AS TARGET OF ANTICANCER IMMUNOTHERAPY: IDENTIFICATION OF HLA-A*0201-RESTRICTED EPITOPES

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The design of a broad application tumor vaccine requires the identification of tumor antigens expressed in a majority of tumors of various origins. We questioned whether the major stress-inducible heat shock protein Hsp70 (also known as Hsp72), a protein frequently overexpressed in human tumors of various histological origins, but not in most physiological normal tissues, constitutes a tumor antigen. We selected the p391 and p393 peptides from the sequence of the human inducible Hsp70 that had a high affinity for HLA-A*0201. These peptides were able to trigger a CTL response *in vivo* in HLA-A*0201-transgenic HHD mice and *in vitro* in HLA-A*0201+ healthy donors. p391- and p393-specific human and murine CTL recognized human tumor cells overexpressing Hsp70 in a HLA-A*0201-restricted manner. Tetramer analysis of TILs showed that these Hsp70 epitopes are targets of an immune response in many HLA-A*0201+ breast cancer patients. Hsp70 is a tumor antigen and the Hsp70-derived peptides p391 and p393 could be used to raise a cytotoxic response against tumors of various origins.

Key words: *tumor antigen; cytotoxic T lymphocytes; immunotherapy; heat shock protein (Hsp) 70; HLA-A*0201-restricted epitope*

Immunotherapy is a strategy of promising efficiency in cancer patients.[1] Several tumor antigens and the corresponding epitopes recognized by specific cytotoxic T lymphocytes (CTL) have been identified, some of them are currently tested in cancer clinical trials. Most of these antigens, however, are only expressed by certain types of tumors. To design a universal cancer vaccine, one has to identify tumor antigens that are expressed by a large panel of tumors, for instance the telomerase reverse transcriptase (TERT), the apoptosis inhibitor survivin or the pan-MAGE epitope we have described recently.[2–4]

The major stress-inducible heat shock protein Hsp70 (also known as Hsp72) is overexpressed frequently in human tumors of various origins, such as breast (40–90%), lung (50–90%), colorectal (80%), cervical carcinomas (50–75%) and osteosarcoma (40%).[5–14]. The inducible Hsp70 is a member of the highly conserved Hsp70 family of proteins that, unlike the homologous heat shock constitutive protein Hsc70 (also known as Hsp73), displays the particularity to be expressed only at a very low or even undetectable level in most normal tissues, that could be of interest in the perspective of breaking tolerance. Hsp70 also protects cells from a wide range of apoptotic, necrotic and hypoxic stimuli, thus conferring survival advantage to tumor cells.[15–17] Hsp70 proteins have been described as potent adjuvants in vaccination strategies designed to mount CD8+ T cell responses against their associated antigenic tumor peptides.[18]

The profile cited above led us to address whether the inducible Hsp70 *per se* constitutes a universal tumor antigen to be used to stimulate antitumor CTL and target a broad spectrum of tumors overexpressing Hsp70. We identified 2 epitopes from the sequence of the human Hsp70, p391 and p393, which exhibit a high affinity for HLA-A*0201 and are immunogenic *in vivo* in the HLA-A*0201 transgenic (HHD) mouse model and *in vitro* in humans. p391- and p393-specific CTL recognized human tumor cells overexpressing Hsp70. Moreover, p391- and p393-specific CD8+ cells are detected in TILs of breast cancer patients, demonstrating that Hsp70-derived peptides are the target of naturally-occurring responses *in vivo*. Thus, Hsp70-derived peptides such as p391 and p393 represent valuable targets for CTL in broad spectrum cancer immunotherapy.

MATERIAL AND METHODS

Animals

HLA-A*0201 transgenic HHD mice were described previously.[22]

Cells

Murine RMA-S HHD cells have been described previously.[22] The HLA-A*0201-expressing human tumor cells were: T2 (TAP1/2 deficient), SAOS (sarcoma), MCF-7 (breast cancer), Caco-2 (colon carcinoma), M44 and M113 (melanoma); SEG (bladder carcinoma) kindly provided by Dr. D. Zeliszewsli (Paris, France). Cells were grown in RPMI 1640 or DMEM medium supplemented with 10% FCS. TILs were grown from dissociated tumor tissue in the presence of 150 IU/ml IL-2. Between Day 7 and 14 TILs were harvested and stained for CD4/CD8 (Immunotech, Marseilles, France). Populations displaying <90% CD8+ were magnetically enriched in CD8+ cells (Miltenyi Biotec, Auburn, CA). CD8+ sorted cells were amplified in the presence of PHA-L (Sigma, Oakville, Canada) and allogeneic irradiated PBMC and EBV-B cells. TILs were assayed at Day 14 after restimulation.

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Plasmids and peptides

Peptides were synthesized by Synt:em (Nîmes, France). The plasmid pCMVHsp70 containing the cDNA coding for the human inducible Hsp70 was kindly provided by Dr. M. Ladjimi (Paris, France).[23] The HHD construct was described previously.[22]

*Measurement of peptide relative affinity for HLA-A*0201*

The protocol used to measure relative affinity has been described previously.[24] Briefly, T2 cells were incubated with various concentrations of peptides (0.1–100 μM) for 16 hr and then stained with the mAb BB7.2 to quantify the surface expression of HLA-A*0201. For each peptide concentration, the HLA-A*0201-specific staining was calculated as the percentage of the staining obtained with 100 μM of the reference peptide $HIVpol_{589}$ (IVGAETFYV). The relative affinity (RA) is determined as: concentration of each peptide/concentration of the reference peptide that induces 20% of HLA-A*0201 expression.

Generation of CTL in HHD mice

HHD mice were injected subcutaneously with 100 μg of peptide emulsified in incomplete Freund adjuvant (IFA) in the presence of 150 μg of the $I\text{-}A^b$ restricted $HBVcore_{128}$ T-helper epitope. After 11 days, spleen cells (5×10^7 cells in 10 ml) were stimulated *in vitro* with peptide (10 μM) in RPMI1640 +10%FCS. The CTL lines were established by weekly restimulation *in vitro* with irradiated spleen cells in the presence of decreasing doses of peptide (1–0.1 μM) and 50 U/ml IL-2 (Proleukin; Chiron Corp., Emeryville, CA).

Cytotoxic assay

Murine RMA-S HHD or human T2 cells were used as targets for cytotoxicity as described.[22] Briefly, 2.5×10^3 ^{51}Cr-labeled targets were pulsed with peptides at 37°C for 60 min. Effector cells (10^5) in 100 μl were then added and incubated at 37°C for 4 hr. After incubation, 100 μl of supernatant was collected and radioactivity was measured in a γ-counter. Percentage of specific lysis was determined as: lysis = (experimental release − spontaneous release)/(maximal release − spontaneous release).

Peptide processing assay on COS-transfected cells

Simian COS-7 cells (2.2×10^4) were plated in flat-bottomed 96-well plates in DMEM + 10% FCS, in triplicate for each condition. Eighteen hours later, the cells were transfected with 100 ng of each DNA plasmid with DEAE Dextran. After 4 hr, PBS + 10% DMSO was added for 2 min. Transfected COS cells were incubated in DMEM + 10% FCS for 40 hr and then used to stimulate murine CTL in a TNF-α secretion assay.

TNF-α secretion assay

Transfected COS-7 cells at Day 4 and human tumor cells (10^5 cells/well) suspended in 50 μl of RPMI + 10% FCS were used as stimulating cells. When necessary, they were incubated with 10 μM peptide for 2 hr. T cells (5×10^4) were then added in 50 μl RPMI 10% FCS and incubated for 6 hr. Each condition was tested in triplicate. Fifty microliters of the supernatant was collected to measure TNFα as described previously.[25]

Western blot analysis of inducible Hsp70 expression by tumor cells

Cellular samples were rinsed in PBS, then lysed 30 min at 4°C in 125 mM Tris/HCl pH 6.8 containing 3 mM EDTA, 10 mM NaF and 0.1% sulfobetain 14. After centrifugation (13,000 rpm, 10 min, 4°C), supernatants were quantified for their protein content using BCA assay (Pierce, Rockford, IL). Proteins (30 μg) were analyzed using anti-inducible Hsp70 mAb (clone SPA-810, dilution 1:1,000, StressGen, Victoria, Canada), anti-actin mAb (dilution 1:2,000, Chemicon, Temecula, CA), a peroxidase-conjugated anti mouse Ig (Sigma), and the ECL kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Relative protein expression was quantitated with the Photo-Capt software (Vilber-Lourmat, Marne-la-Vallée, France).

Generation of CTL from human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were collected by leukapheresis from healthy HLA-A*0201 volunteers. DC were produced from adherent cells cultured for 7 days (2×10^6 cells/ml) in the presence of 500 IU/ml GM-CSF (Leucomax, Schering-Plough, Kenilworth, NJ) and 500 IU/ml IL-4 (R&D Systems, Minneapolis, MN) in complete medium (RPMI-1640 supplemented with 10% heat inactivated human AB serum, 2 μM L-glutamine and antibiotics). On Day 7 maturation agents polyI:C (Sigma) at 100 ng/ml and anti-CD40 mAb (clone G28-5, ATCC, Manassas, VA) at 2 μg/ml were added in the culture for 24 hr. Mature DC were pulsed with 10 μM peptide in the presence of 5 μg/ml β2-m for 2 hr at 37°C and then irradiated (3,500 Rad). CD8+ cells were purified by positive selection with CD8 Microbeads (Miltenyi Biotec) according to the manufacturer's instructions. CD8+ cells (2×10^5) were stimulated with 2×10^4 peptide-pulsed DC in complete culture medium supplemented with 1,000 IU/ml IL-6 and 5IU/ml IL-12 (R&D Systems) in round-bottomed 96-well plates. From Day 7, cultures were weekly restimulated with 2×10^4 peptide-pulsed DC per well in the presence of 20I U/ml IL-2 (Proleukin, Chiron Corp.) and 10 ng/ml IL-7 (R&D Systems). After the third *in vitro* restimulation, CD8+ cells were tested for peptide-specific activation in an intracellular IFN-γ production assay.

Intracellular IFN-γ staining

T cells (10^5) were incubated with 2×10^5 stimulating peptide-pulsed T2 or tumor cells in the presence of 10 μg/ml brefeldin-A (Sigma). Six hours later, they were washed, stained with r-phycoerythrin-conjugated anti-CD8 antibody (Caltag Laboratories, Burlingame, CA) in PBS for 25 min at 4°C, washed and fixed with 4% PFA. Then, cells were permeabilized with PBS 0.5% BSA 0.2% saponin (Sigma), and stained with allophycocyanin-conjugated anti-IFNγ mAb (Pharmingen, Mississauga, Canada) for 25 min at 4°C. Cells were acquired on FACSCalibur (Becton Dickinson, Mountain View, CA).

Tetramer analysis of the frequency of Hsp70 epitopes-specific CD8+ cells

Tetramers were synthesized by ProImmune (Oxford, UK). PBMC (10^6) from healthy volunteers or tumor-infiltrating lymphocytes (TILs) from breast cancer patients were stained with 1 μl of the tetramers-PE in 20 μl PBS 0.5% human AB serum for 1 hr at room temperature. Cells were washed and incubated with allophycocyanin-conjugated anti-CD8 and Fitc-conjugated anti-CD3 antibodies (Caltag) for 30 min at 4°C. Cells were acquired on FACSCalibur (Becton Dickinson), displaying the frequency of tHLA+ cells among CD8+ cells when gated on CD3+ cells.

RESULTS

*Selection of HLA-A*0201-binding peptides derived from the human inducible Hsp70*

The sequence of the human inducible Hsp70 protein was screened for nonamer peptides possessing the HLA-A*0201 specific motif (L/M at position 2 and L/V at position 9). Peptides were also chosen to be shared with the murine sequence of the inducible Hsp70, so that a relevant study of tolerance breaking against this self protein could be assessed *in vivo* in a murine model, thus mimicking the situation of vaccination in humans. Seven candidates were assayed for their predicted affinity for HLA-A*0201 with the Bimas program.[26] We selected 5 peptides with the highest Bimas predicted score and experimentally tested them for their capacity to bind to HLA-A*0201 molecules (relative affinity, RA) (Table I). p391 and p393 displayed the highest affinity, and were, therefore, selected for further studies. Interestingly, both p391 and p393 were not shared with the highly homologous constitutive

TABLE I – AFFINITY OF HSP70-DERIVED PEPTIDES FOR HLA-A*0201

Peptide	Sequence	Bimas score[26]	Relative affinity[1]
p86	DMKHWPFQV	<1	
p313	TLEPVEKAL	1.4	
p10	DLGTTYSCV	7.8	>20
p393	LLDVAPLSL	15	0.5
p199	DLGGGTFDV	30.2	>20
p380	LMGDKSENV	171.5	1.7
p391	LLLLDVAPL	309.1	0.5

[1]Relative affinity: concentration of each peptide/concentration of the reference peptide that induce 20% of HLA-A*0201 expression obtained by 100 μM of the reference peptide. Result of 3 or more independent experiments.

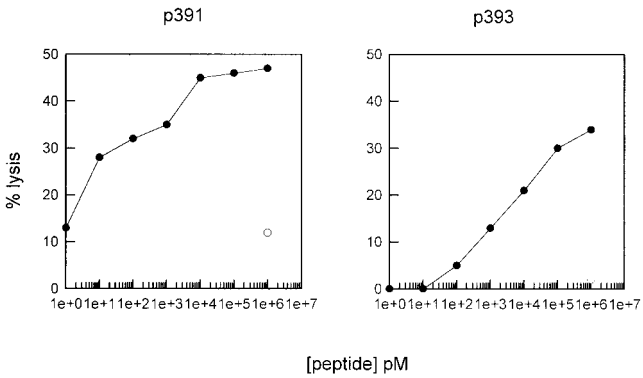

FIGURE 1 – p391 and p393 recognition by mCTL391 and mCTL393 cell lines. mCTL391 and mCTL393 cell lines were established as described in Material and Methods. They were tested against RMA-S HHD targets in the presence of various concentrations of cognate peptide (●) or with 1 μM of the irrelevant $HIVpol_{589}$ (○) at a E/T ratio of 40/1.

Hsc70 protein that is likely to be involved in tolerization of Hsp70-specific T cell repertoires.

In vivo *immunogenicity of Hsp70 peptides in HLA-A*0201 transgenic mice*

We assessed the capacity of p391 and p393 to trigger CTL in HHD mice. Peptide specific CTL were generated in 5 and 3 of 5 mice tested, respectively (data not shown). CTL lines, hereafter referred to as mCTL391 and mCTL393, were obtained from spleen cells of responding mice after multiple *in vitro* restimulations with decreasing doses of peptide. mCTL391 and mCTL393 exhibited a high functional T cell avidity because half maximal lysis of RMA-S HHD targets were obtained when pulsed with <10 nM of peptide (Fig. 1). No lysis was observed against target cells pulsed with 1 μM of the irrelevant peptide $HIVpol_{589}$.

Processing of Hsp70 peptides by Hsp70-overexpressing tumor cells

We next addressed whether p391 and p393 are naturally processed by cells expressing Hsp70 endogenously. We first stimulated mCTL391 and mCTL393 with COS cells cotransfected with the HHD (HLA-A*0201 transgene) and Hsp70 expression plasmids, or with COS cells transfected with either the HHD or the Hsp70 plasmids. mCTL activation was evaluated by TNF-α secretion. mCTL391 and mCTL393 responded to COS cells cotransfected with both HHD and Hsp70 plasmids, but not to COS cells transfected only with either the HHD or the Hsp70 plasmids (Fig. 2*a*). This demonstrates that p391 and p393 are processed from the endogenous Hsp70 protein and presented by HLA-A*0201.

We further studied whether the natural overexpression of Hsp70 in human tumor cell lines results in an efficient presentation of p391 and p393. Various HLA-A*0201+ and one HLA-A*0201− tumor cells were screened for their Hsp70 expression by Western blot (Fig. 2*b*). MCF-7, SAOS, Caco-2 and SEG expressed the Hsp70 protein at least 7–25 times more than M44 and M113 did.

mCTL391 and mCTL393 were activated by the HLA-A*0201+ Hsp70+ MCF-7 cells but neither by the HLA-A*0201+ Hsp70- M113 nor by the HLA-A*0201- Hsp70+ DU-145 cells (Fig. 2*c*) suggesting that mCTL391 and mCTL393 recognize Hsp70-expressing tumors in an HLA-A*0201 restricted manner. This is further confirmed for the mCTL393 in an HLA blocking experiment. Recognition of MCF-7 by mCTL393 was blocked by the HLA-specific W6/32 but not by the irrelevant anti-NKTa mAb. Interestingly, mCTL391 and mCTL393 were activated by different tumor cells over-expressing Hsp70.

These data establish that the Hsp70 epitopes p391 and p393 are presented efficiently by Hsp70 over-expressing tumors of various histological origins and can, therefore, be considered as targets of antitumor CTL immune responses.

Recruitment of p391- and p393-specific human anti-tumor CTLs

To investigate if human CTL can be induced against p391 and p393, we stimulated purified human CD8+ cells from HLA-A*0201 healthy donors with peptide-loaded autologous dendritic cells. After the third stimulation, the generation of Hsp70 peptide-specific CTL was evaluated in a chromium release assay and intracellular IFN-γ staining against T2 target cells pulsed with an irrelevant or the cognate peptide. CTL specific for each peptide p391 and p393 could be observed in multiple donors, and showed specific lysis of the target cells pulsed with the cognate peptide as shown for one donor (Fig. 3*a*). Significant frequencies of IFN-γ producing cells (>1%) were detected in cultures of 2 of 4 donors for both peptides. Cell lines established from responding donors cultures were tested for their capacity to recognize Hsp70-overexpressing tumor cells. Results from one donor in Figure 3*b* show that 5.3% and 9.5% of CD8+ cells were specific for respectively the p391 and the p393 epitope. Interestingly, the p391-specific CD8+ cell line recognized the Hsp70+ MCF-7 and SAOS cells but not the Hsp70- M44 and M113 cells (Fig. 3*c*). Likewise, the p393-specific CD8+ cell line recognized the Hsp70+ MCF-7 but not the Hsp70- M44 and M113 cells. These results demonstrate that p391 and p393 can stimulate Hsp70-specific anti-tumor CTL in humans.

Naturally-occurring CD8+ response against Hsp70 Epitopes in cancer patients

We investigated if p391 and p393 can be targets of anti-tumor CTL *in vivo* by studying the occurrence of an anti-tumor CD8+ response in patients with tumors that frequently overexpress Hsp70. We evaluated the frequency of CD8+ cells specific for p391 or p393 using the appropriate HLA-A*0201/p391 and HLA-A*0201/p393 tetramers (tHLA) in tumor infiltrating lymphocytes (TILs) of breast cancer patients. The specificity of the tetramers was first checked on PBMC stimulated with the appropriate peptides (Fig. 4*a*). An amplification correlating with the presence of tumor was considered when the frequency of peptide-specific CD8+ cells was above the cut-off defined as the mean frequency of tHLA+ cells of HLA-A*0201+ donors + 3 times the standard deviation (SD). The cut-off was 0.12% for p391 and 0.13% for p393. 9 (64.3%) and 11 (78.6%) patients of 14 displayed a frequency of $tHLA/p391^+$ and $tHLA/p393^+$ cells respectively above the cut-off (Fig 4*b*). Interestingly, the frequency of CD8+ cells specific for p391 and p393 were highly correlated in TIL samples (linear regression coefficient $r^2 = 0.77$). To illustrate the responses observed in some patients, representations of the CD8/tHLA staining of two patients are shown in Figure 4*c*. Patient S84 displays tHLA-positive populations in the TILs both from the tumor site and the draining lymph nodes, and for both peptides. Specificity of the response to Hsp70 peptides is demonstrated by an absence of

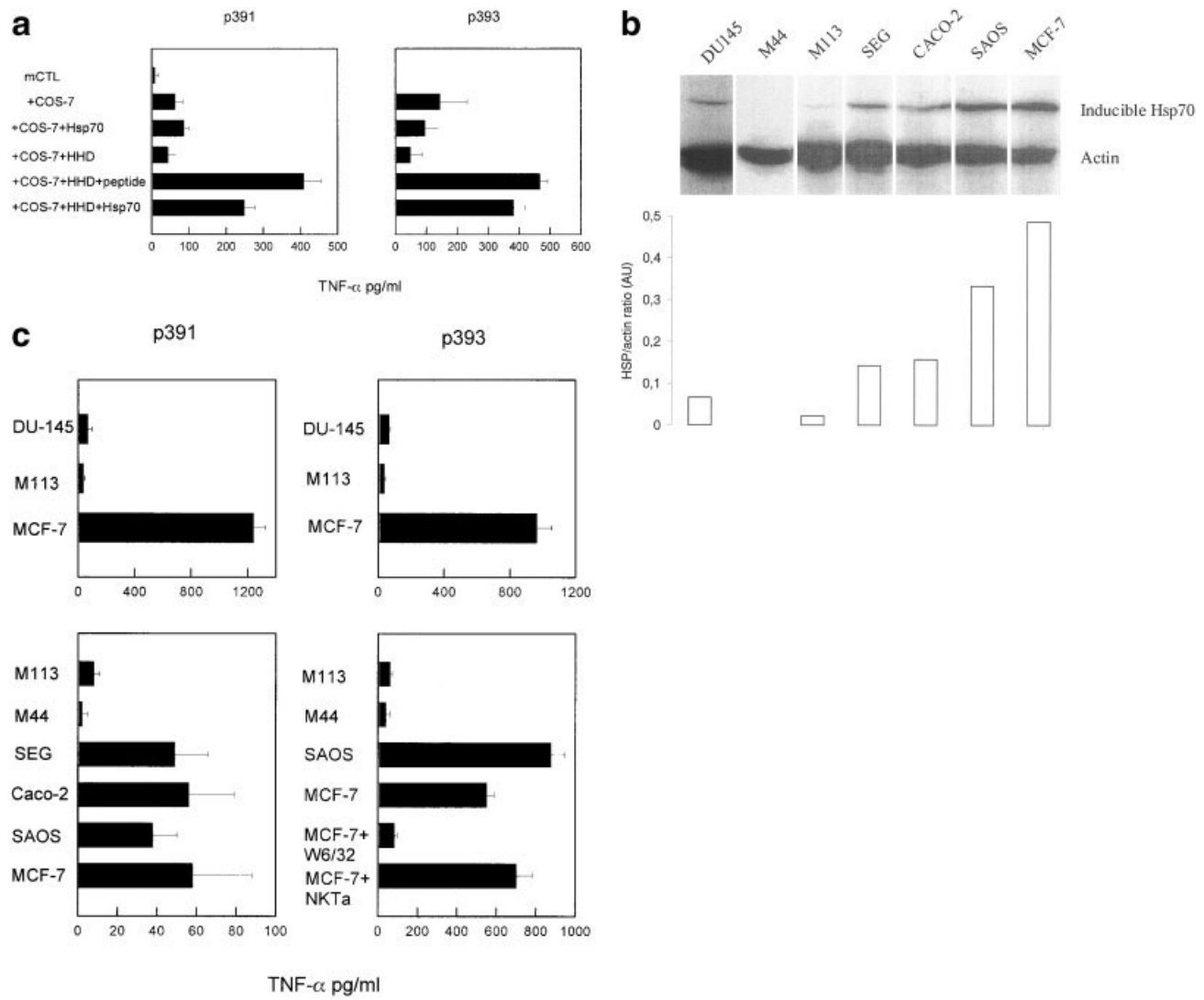

FIGURE 2 – Stimulation of mCTL391 and mCTL393 by tumor cells expressing endogenous Hsp70. (*a*) mCTL391 and mCTL393 cells were stimulated with COS-7 cells expressing HHD or Hsp70 as indicated. In a positive control, mCTL391 and mCTL393 cells were stimulated with HHD expressing COS-7 cells incubated with the peptide (10 μM). mCTL391 and mCTL393 activation was evaluated by measure of secreted TNF-α using the TNF-α sensitive WEHI cell line. Results represent the mean ± SD of triplicates. Results were confirmed in 3 independent experiments. (*b*) Inducible Hsp70 expression by human tumor cells assessed by Western blot as described in Material and Methods. (*c*) mCTL391 and mCTL393 cells were stimulated with the Hsp70 positive MCF-7 cells, by the M113 cells that express barely detectable levels of Hsp70, and by the HLA-A*0201- DU145 cells. mCTL391 and mCTL393 were further tested against the Hsp70 positive SEG, Caco-2, SAOS and MCF-7 cells. Stimulation of mCTL393 with MCF-7 cells was carried out in the presence of the W6/32 mAb and the irrelevant NKTa mAb, as indicated. mCTL activation was evaluated by measure of secreted TNF-α as described in Figure 2*a*. Results were confirmed in 3 independent experiments.

tHLA+ cells when using a non-cognate tetramer. By contrast, patient S101 was clearly negative for such an Hsp70 response, as no tHLA+ population could be isolated for either peptide. These data confirm *in vivo* that the Hsp70 epitopes identified are targets of CD8+ anti-tumor responses, and reinforces the interest in triggering or boosting an immune response against the tumor antigen Hsp70.

DISCUSSION

To propose an immunotherapeutic strategy applicable for a wide variety of tumors, we identified the inducible Hsp70 as a new broad-application tumor antigen. From the sequence of the inducible Hsp70, we selected the p391 and p393 peptides that exhibit a high affinity for the HLA-A*0201 molecule. These peptides were shown to be immunogenic *in vivo* in the HLA-A*0201 transgenic HHD mice and *in vitro* in humans. Importantly, both human and murine CTL recognized Hsp70 overexpressing tumor cells. Tetramer analysis of TILs showed that these Hsp70 epitopes were the targets of an immune response in many HLA-A*0201+ breast cancer patients.

Overexpression of the inducible Hsp70 protein is a frequent and common phenomenon in tumors of various origins such as breast, lung, colo-rectal, cervical carcinomas and osteosarcoma.[5–14] Furthermore, it has been reported that tumor cells escaping chemotherapy such as 5-FluoroUracile or cisplatin display an upregulation of Hsp70.[27,28] Hypoxia can also induce Hsp70 overexpression.[29] Hsp70-derived epitopes could therefore be used for broad-spectrum immunotherapy of cancer, potentially in synergy

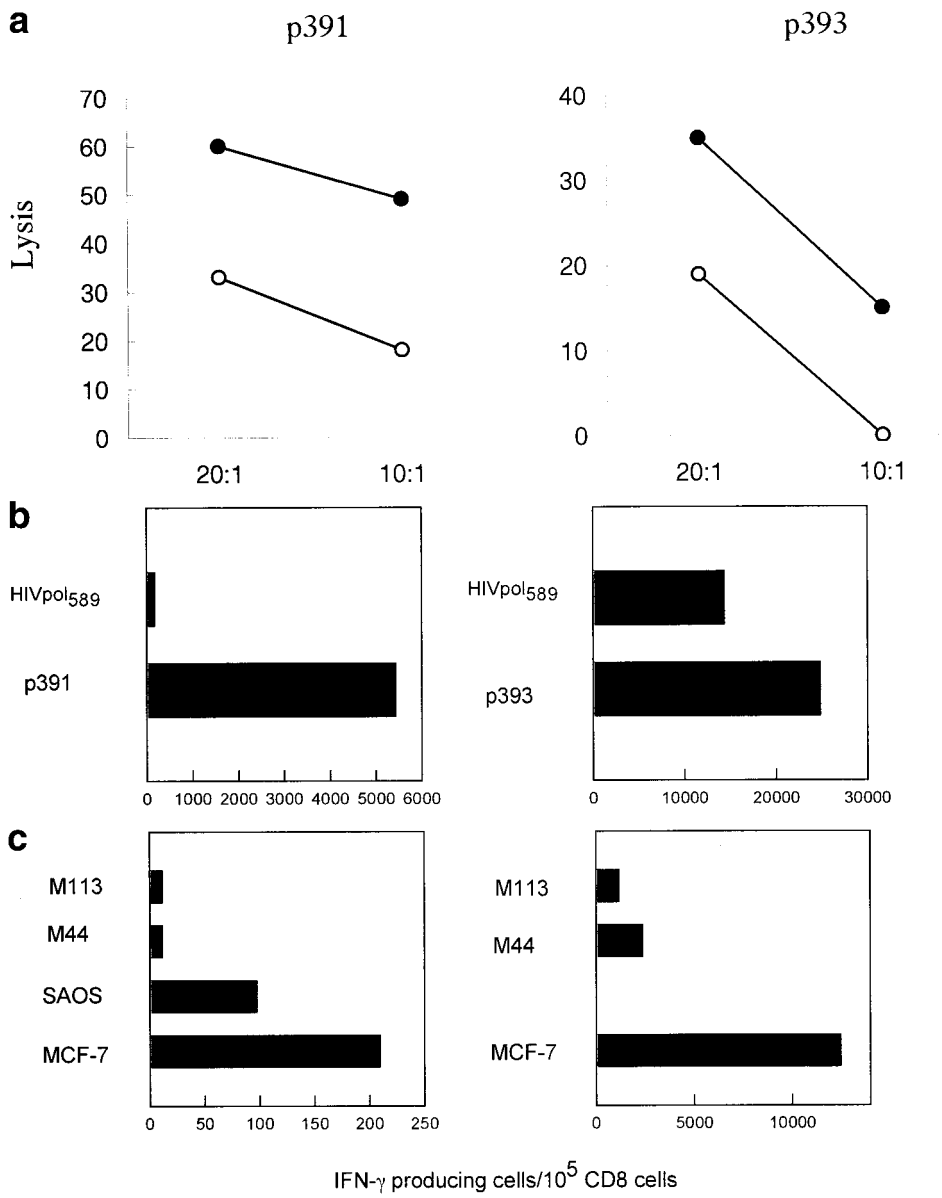

FIGURE 3 – Induction by the p391 and p393 peptides of specific human CTL able to recognize tumor cells over-expressing Hsp70. The induced human CTL were tested for their capacity to lyze T2 cells loaded with the cognate (●) or the irrelevant $HIVpol_{589}$ peptides (○) (*a*) One representative donor shown from multiple donors. p391 and p393-specific human CTL were tested for their capacity to respond to T2 target cells loaded with the cognate peptide or the irrelevant $HIVpol_{589}$ (*b*) and to the Hsp70 positive MCF-7 and SAOS, and the Hsp70 negative M44 and M113 tumor cells (*c*). CTL response was evaluated by measure of IFN-γ producing CD8+ cells upon activation, as assessed by staining cells for CD8 and intracellular IFN-γ.

with chemotherapy or hypoxia-inducing anti-angiogenic treatments.

Hsp70 presents the rare profile of global overexpression in cancer cells and an implication in tumorigenicity as a necessary event in the progression of the tumor.[15] It thus belongs to the category of the few antigens that play a major role in tumor survival and progression and are therefore very interesting to target, such as HER-2/neu, survivin and TERT. Indeed, variant tumor clones that do not overexpress Hsp70 and therefore escape the immune response would present a less tumorigenic or even an apoptotic profile.[16,30]

Gaudin *et al.*[31] described recently a CTL clone specific for an epitope derived from a mutated sequence of the inducible Hsp70. This clone, however, did not efficiently recognize tumor cells not bearing the mutation.[31] In addition, the mutation was shown to be restricted to the patient from whom the TIL clone was derived. Considering the usefulness of non-mutated Hsp70-derived peptides in broad-spectrum tumor immunotherapy, we focused on identifying, by the reverse immunology method, epitopes able to raise a CTL response against native Hsp70-overexpressing tumors. p391 and p393 described in our study present such a profile.

Identification of the p391 and p393 epitope was first achieved in the HHD transgenic mouse model. The use of HHD mice in tumor epitope identification offers 2 major advantages. First, it enables to quickly raise a T cell response against a peptide-candidate and to obtain specific CTL lines with high avidity by *in vitro* stimulations with decreasing doses of peptide as described by Zeh *et al.*.[32] Second, by using human and mouse shared peptides, such as p391 and p393, we can evaluate whether tolerance toward Hsp70 can be broken, CTL responses against overexpressed non-mutated self proteins can be induced *in vivo* and whether such responses result in deleterious auto-immune aggression. For the Hsp70-derived p391 and p393 epitopes described in our study, several CTL lines established from responding mice show an avidity (<10 nM) similar to that of CTL against a non-self viral peptide generated in the HHD mice (unpublished data). This suggests that at least some high avidity p391- and p393-specific CTL able to recognize Hsp70 expressing tumors have escaped self-tolerance. It must be noted, however, that both epitopes are not shared by the murine and

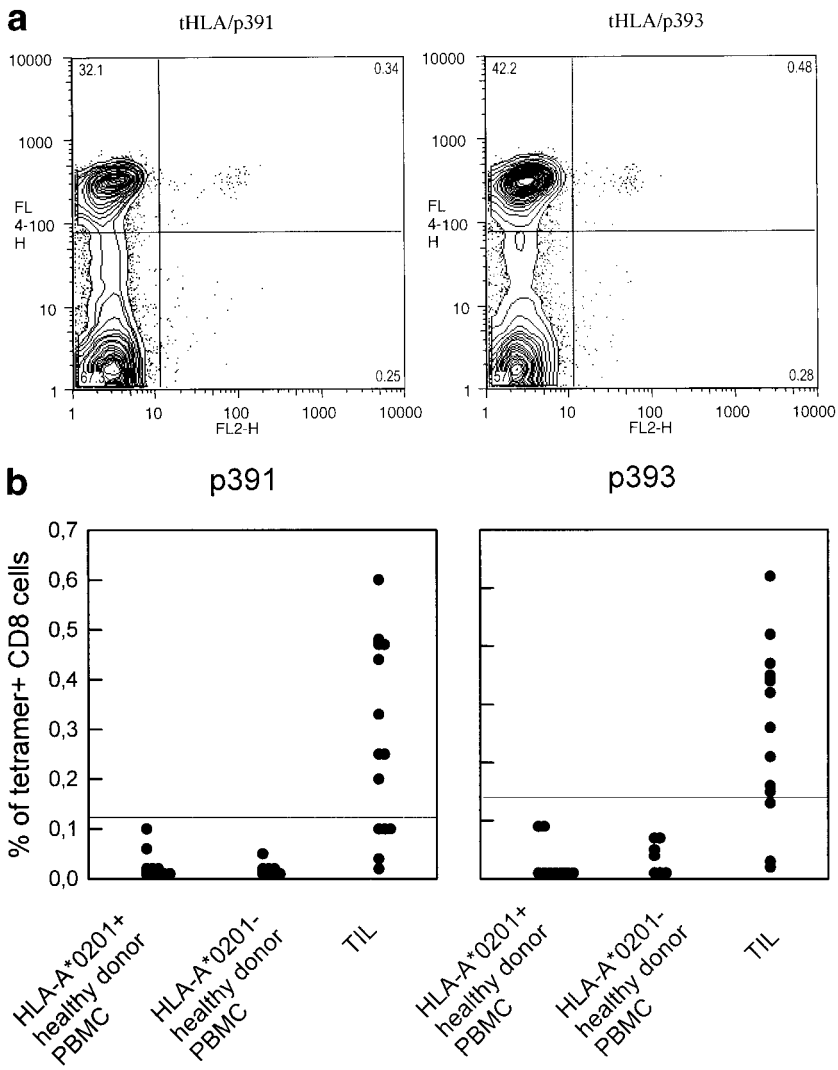

FIGURE 4 – Natural amplification of CD8+ cells specific for Hsp70 epitopes in cancer patients. (*a*) Specificity of the p391 and p393 tetramers was assessed by staining of PBMC stimulated with the appropriate peptides. Frequency of tHLA+ CD8+ cells was correlated with the frequency of IFNγ-secreting CD8+ cells. (*b*) The frequency of CD8+ cells specific for p391 or p393 was evaluated using the appropriate tetramers (tHLA) in tumor infiltrating lymphocytes (TILs) of breast cancer patients. Background level was assessed on HLA-A*0201+ and HLA-A*0201− healthy donors. An amplification correlating with the presence of tumor was considered when the frequency of peptide-specific CTLs was above the cut-off defined as the mean frequency of tHLA+ cells of HLA-A*0201+ donors + 3 times the standard deviation (line). (*c*) FACS dot plot of CD3+ cells from TILs derived from lymph node (LN) or tumor site (Tumor) of 2 breast cancer patients (S84 and S101).

human sequence of the constitutively expressed Hsc70 that is more likely to be involved in the self tolerance process than the inducible Hsp70. This raises the question of whether the choice of Hsp70-derived peptides with a vaccine potential should be limited to those that, like p391 and p393, do not belong to the Hsc70 sequence. We are currently addressing this question in the HHD mouse model. This model represents also an exclusive opportunity to investigate a potential transient auto-immunity against stressed tissue triggered by vaccination. It is documented that hyperthermia or inflammatory conditions *in vivo* can induce the expression of Hsp70.[33,34]

Break of tolerance against tumor antigens that are proteins from the self overexpressed in tumors has been widely documented. Many observations indeed report natural occurrence of amplified T cell repertoires against such antigens in cancer patients, such as Her2/neu and p53.[35–37] Boosting the immune response against self overexpressed tumor antigens was shown to be an efficient strategy against tumors as shown in preclinical models.[38,39] This demonstrates the usefulness of this family of tumor antigens in anti-tumor immunotherapy. Break of tolerance against the inducible Hsp70 has been observed with the report by Trieb *et al.*[40] of the presence of at least a natural CD4+ response to the inducible Hsp70 in 2 sarcoma patients whose tumor overexpressed Hsp70. This further suggests the usefulness of a Hsp70-targeting anti-tumor response and its relative inocuity.

We have described the generation of an anti-Hsp70 immune response *in vivo*. Srivastava *et al.*[19] has shown that vaccination with the whole protein Hsp70 extracted from tumor cells induces an anti-tumor immune response that is directed against the tumor epitopes loaded onto Hsp70 but not against the Hsp70 itself at the level of a humoral response.[20,21] This is not surprising because immunization with whole self proteins (*e.g.*, Hsp70) is unlikely to generate an efficient immune response because of the self-tolerance that principally concerns their immunodominant epitopes. HEL transgenic mice do not, for instance, respond to whole HEL but they respond to non-dominant HEL epitopes,[41,42] β-gal transgenic mice do not respond to whole β-gal,[43,44] flu NP and Ins-HA transgenic mice respond very weakly to NP and HA, respectively.[45,46] This is not limited to transgenic models but, interestingly, also concerns tumor antigens because normal rats do not respond to the rat HER-2/neu,[47] but respond to fragments of HER-2/neu containing non-dominant epitopes, and normal mice do not respond to murine gp100,[39,48] tyrosinase[48,49] and Melan-A,[48] but they respond to their epitopes.[39,50] In accordance with this literature, our results show that humans and mice, who may not respond to the whole protein Hsp70, can however develop a CTL response against the p391 and p393 epitopes.

In conclusion, our results demonstrate that the inducible Hsp70 constitutes a tumor antigen with a potential usefulness in cancer

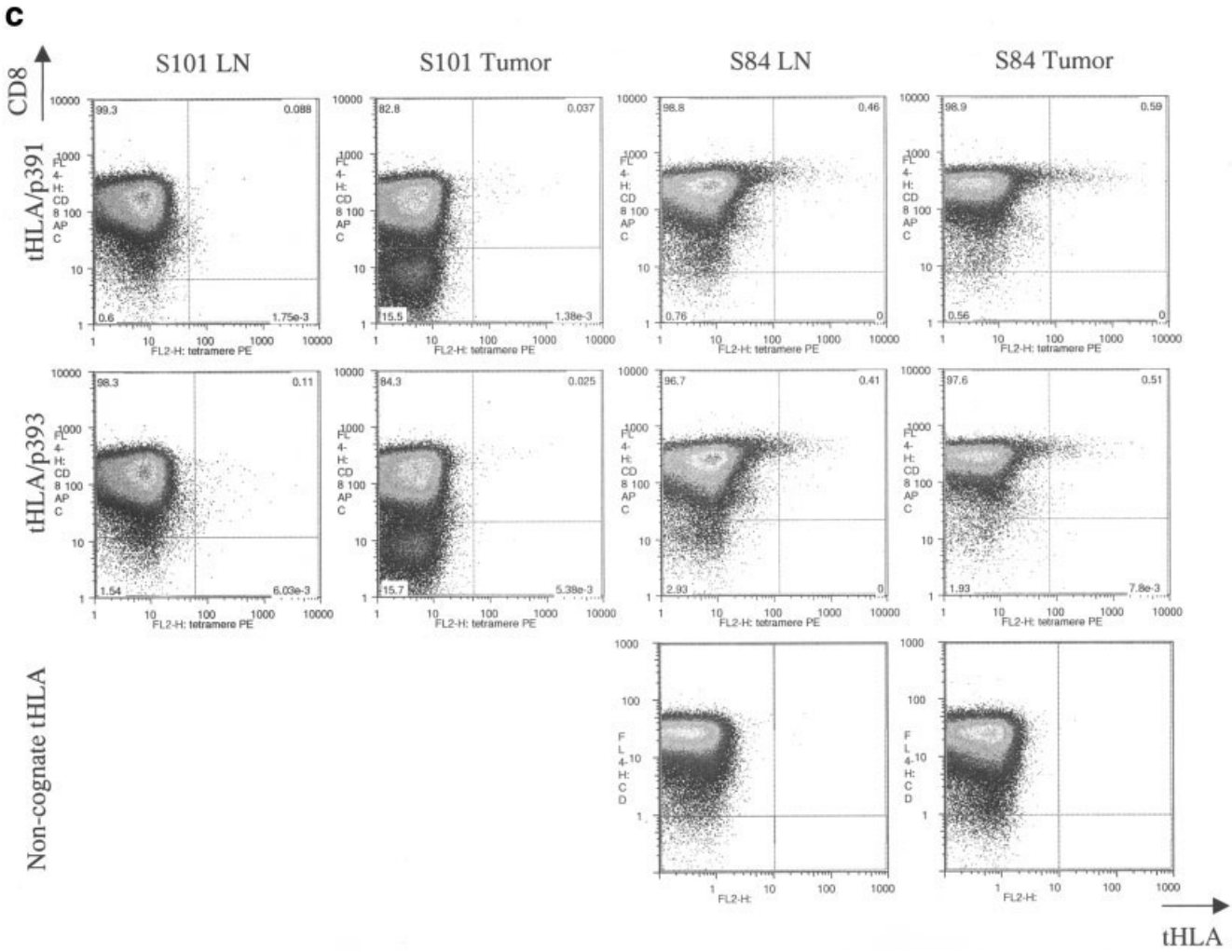

FIGURE 4 – CONTINUED

immunotherapy. Hsp70 is indeed one of the few tumor antigens displaying the interesting profile of broad overexpression in tumors and with a major role in tumorigenicity. We propose the immunogenic HLA-A*0201-restricted epitopes p391 and p393 for induction of antitumor CTL able to target tumors of various origins overexpressing Hsp70.

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