Natural Killer and *NK-Like* T-Cell Activation in Colorectal Carcinoma Patients Treated with Autologous Tumor-Derived Heat Shock Protein 96

Lorenzo Pilla,[1] Paola Squarcina,[1] Jorgelina Coppa,[2] Vincenzo Mazzaferro,[2] Veronica Huber,[1] Daniela Pende,[4] Cristina Maccalli,[1] Gloria Sovena,[1] Luigi Mariani,[3] Chiara Castelli,[1] Giorgio Parmiani,[1] and Licia Rivoltini[1]

Units of [1]Immunotherapy of Human Tumors, [2]Hepatobiliary and Gastro-Pancreatic Surgery, and [3]Biomedical Statistics, Istituto Nazionale Tumori, Milan, Italy and [4]Laboratory of Immunology, Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy

Abstract

Heat shock proteins (HSPs) are involved in the activation of both adaptive and innate immune systems. Here, we report that vaccination with autologous tumor-derived HSP96 of colorectal cancer patients, radically resected for liver metastases, induced a significant boost of natural killer (NK) activity detected as cytokine secretion and cytotoxicity in the presence of NK-sensitive targets. Increased NK activity was associated with a raise in $CD3^-CD56^+$ NK and/or $CD3^+CD56^+$ *NK-like* T cells, displaying enhanced expression of NKG2D and/or NKp46 receptors. Up-regulated expression of CD83 and CD40 and increased interleukin-12 release on stimulation were observed in $CD14^+$ cells from post-HSP96 peripheral blood mononuclear cells, suggesting an indirect pathway of NK stimulation by HSP96-activated monocytes. Additionally, $CD3^-CD56^+$ and $CD3^+CD56^+$ lymphocytes were found to undergo functional and phenotypic activation on *in vitro* exposure to HSP96 even in the absence of monocytes, supporting a potential direct activity of HSP96 on these cell subsets. This evidence was confirmed by the specific binding of FITC-conjugated HSP96 to a subset of both $CD3^-CD56^+$ and $CD3^+CD56^+$ cells in peripheral blood mononuclear cells from colorectal cancer patients. Altogether, these findings identify the activation of the NK compartment as an additional immunologic effect of autologous tumor-derived HSP96 administration in cancer patients. (Cancer Res 2005; 65(9): 3942-9)

Introduction

Tumor cells are known to elicit a complex array of immunologic responses that may eventually lead to immune-mediated tumor destruction (1). Tumor rejection is considered to rely mostly on the expression of tumor-associated antigens that can be target of protective $CD4^+$ and $CD8^+$ T-cell-mediated immunity. However, it is becoming clear that other antigen-independent factors, leading to the *in vivo* development of pleiotropic immune reactivities, can significantly contribute to the induction of effective antitumor immunity (2, 3).

The possibility of generating antitumor T-cell responses by vaccination with heat shock protein (HSP) 96 extracted from tumor cells has been recently tested in both experimental and clinical settings (4–6). HSP96 is a highly conserved chaperone protein resident in the endoplasmic reticulum, functioning as a potent activator of cellular immune responses (7). The immunologic properties of this molecule are considered to stem from its ability to transfer, through receptor-mediated binding, immunogenic peptides acquired in the endoplasmic reticulum into the HLA class I pathway of antigen-presenting cells (APC) for cross-presentation to antigen-specific T lymphocytes (8). Simultaneously, HSP96 promotes functional and phenotypic maturation of APCs, thus providing a favorable milieu for effective T-cell costimulation (9, 10).

Besides these effects, HSP family members have been recently found to mediate activation of other cellular blood components, such as platelets and neutrophils (11, 12). Results obtained in animal models also suggested an involvement of natural killer (NK) cells in the pleiotropic immune response triggered by HSP96 (4, 13, 14). In this setting, the boost of NK activity detected in HSP96 immunized mice is attributed to the stimulatory effect that this molecule exerts on APCs, which in turn trigger NK cells possibly through the local production of cytokines and the interaction with costimulatory molecules (9, 10). Activated NK cells could then contribute to the *in vivo* development of effective cytotoxic and memory antitumor $CD8^+$ T-cell responses as shown clearly in other experimental models (13–15).

To test the efficacy of tumor-derived HSP96 as immunization strategy for cancer patients, we recently did two phase I-II clinical trials of vaccination with autologous tumor-derived HSP96 in 39 stage IV melanoma patients (5) and 29 colorectal cancer patients radically resected for liver metastases (6). By monitoring the immunologic responses induced by the HSP96 vaccine, we observed a significant increase of HLA class I–restricted $CD8^+$ T-cell-mediated recognition of tumor cells or tumor-derived antigens in ~50% of treated patients (5, 6, 16).

With the aim of identifying additional immunologic effects of HSP96 vaccination that could contribute to its immune-mediated antitumor activity, we focused on the potential *in vivo* modulation of NK functions in vaccinated colorectal cancer patients (6). Here, we report that HSP96 administration is associated with a significant boost of NK activity in both NK and T cells together with an increased expression of NK and NK-related activating immunoreceptors. Moreover, we show that such modulating effects are likely produced by an indirect mechanism mediated by HSP96-activated monocytes as well as by direct interaction of HSP96 with immune cells of the NK and T-cell compartments.

Note: C. Maccalli is currently at the Department of Cellular Biology and Neurosciences, Istituto Superiore di Sanità, 00161 Rome, Italy. G. Parmiani is a consultant for Antigenics, Inc. (New York, NY).

Requests for reprints: Licia Rivoltini, Unit of Human Tumor Immunotherapy, Istituto Nazionale Tumori, Via Venezian 1, 20133 Milan, Italy. Phone: 39-02-2390-3245; Fax: 39-02-2390-2630; E-mail: licia.rivoltini@istitutotumori.mi.it.

Materials and Methods

Peripheral blood mononuclear cell samples. Peripheral blood mononuclear cells (PBMC) from colorectal cancer patients were purified from peripheral blood by Ficoll gradient, aliquoted, frozen by a computer-assisted freezing system (Microgitcool, IMV Technologies Italia, Piacenza, Italy), and stored in liquid nitrogen. For IFN-γ ELISPOT assay, PBMCs obtained before and at different times during HSP96 vaccination were simultaneously thawed and incubated overnight at 37°C in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium) supplemented with 10% human AB serum (BioWhittaker Europe). PBMC recovery after thawing was ≥60%, whereas viability after overnight incubation was ≥95% as assessed by trypan blue staining.

Heat shock protein 96 purification and vaccine treatment. HSP96 was purified from tumor lesions by affinity columns as described previously (17). Purification was done by Antigenics (Lexington, MA) under current Good Manufacturing Practice procedures, starting from at least 3 g of nonnecrotic frozen tumor tissue. HSP96 preparations contained no detectable level of endotoxin as analyzed by *Limulus* Amebocyte Lysate kit (BioWhittaker, 0.05 EU/μg detection limit) according to the guidelines published by the Food and Drug Administration. Colorectal cancer patients, radically resected for liver metastases (6), gave written informed consent to the treatment and fulfilled inclusion criteria required by the clinical protocol approved by the Internal Review Board and by the Independent Ethics Committee of the Istituto Nazionale Tumori (Milan, Italy). Details on the vaccine schedule are reported elsewhere (6). Briefly, patients received weekly s.c. injections of autologous tumor-derived HSP96 (25 μg/injection) for 4 weeks followed at 2-month interval by a second cycle of four biweekly injections. Clinical characteristics of enrolled patients, including prognostic factors, disease-free survival, and overall survival after a median follow-up of 54 months, are shown in Table 1 (6, 18). Blood samples for PBMC isolation were obtained at the first day of treatment, before HSP96 injection (V1), after 4 weeks and three HSP96 injections (i.e., before the fourth administration; V5), 1 month after the fourth injection and the end of the first cycle (V6), 2 months after the fourth injection (i.e., before the second cycle; V7), and 2 weeks after the eighth injection (V10). Samples obtained 1 month before treatment (V0) were also available in selected cases.

ELISPOT and ^{51}Cr release assays. IFN-γ ELISPOT (MabTech, Nacka, Sweden) was done as described previously (5, 6). Data were evaluated by a computer-assisted ELISPOT reader (Bioline, AID, Turin, Italy). PBMCs (1.67×10^5 per well) were tested in triplicates against the NK-sensitive target K562 cells (1.67×10^4 per well), a human erythroleukemia cell line, and a panel of HLA class I–mismatched colorectal cancer cell lines (6). To assess interassay variability, IFN-γ production by the anti-Melan-A/MART-1_{27-35} HLA-A*0201-restricted T-cell clone A42 (19) in response to T2 cells pulsed with Melan-A/MART-1_{27-35} peptide was included in each test.

Interleukin (IL)-12 secretion by monocytes in resting and activated [i.e., treated with 1 μg/mL lipopolysaccharide (LPS)] conditions was detected by IL-12 ELISPOT according to manufacturer's instructions.

Cytotoxicity was evaluated by a standard 4-hour ^{51}Cr release assay against K562 and SW480, a human colorectal cancer cell line expressing the NKG2D receptors MICA, ULBP2, and ULBP3 (20). Labeled cells (1,000 per well) were incubated at different E:T ratios with PBMCs obtained before (V1) and during (V5) HSP96 administration. Percentage of lysis was calculated as described previously (19).

Flow cytometry. The following mouse anti-human-conjugated monoclonal antibodies (mAb) from BD PharMingen (San Diego, CA) were used: anti-CD3-peridinin chlorophyll protein, CD3-FITC, CD69-FITC, CD56-phycoerythrin (PE), CD56-FITC, CD56-allophycocyanin, CD8-allophycocyanin, CD14-allophycocyanin, CD14-PE, CD4-allophycocyanin, CD25-allophycocyanin, CD16-FITC, and CD16-PE. The mAbs BAT221 (anti-NKG2D, IgG; ref. 21) and BAB281 (anti-NKp46; ref. 22) were used with a PE-purified goat anti-mouse secondary mAb (DAKOCytomation, Glostrup, Denmark). For multiple staining involving direct and indirect fluorescence, indirect staining was first done followed by four washings in PBS supplemented with 1% fetal bovine serum and finally by direct staining. As negative control, isotype-matched IgG antibodies, either directly conjugated to the fluorochrome or in conjunction with the PE-purified goat anti-mouse secondary mAb, were used. To standardize the values of geometric mean reported in Fig. 4*A*, instrument detector setup for FL2 channel was set so that the PE-Calibrite (BD Biosciences, Milan, Italy) bead peak gave ~780 to 800 linear units in log-scale amplification. Data analysis was done by FACSCalibur using CellQuest software (Becton Dickinson, San Jose, CA). Cells were gated according to light scatter properties to exclude cell debris and to select total PBMCs or lymphocyte subpopulations.

***In vitro* stimulation of natural killer activity by heat shock protein 96 and binding assay.** PBMCs obtained from colorectal cancer patients before vaccination were depleted of CD14$^+$ cells by immunomagnetic sorting (Dynabeads M-450 CD14, Dynal, Oslo, Norway). CD56$^+$ cells were then isolated using the magnetic cell sorting selection system (CD56 pure isolation kit, Myltenyi Biotec, Biergisch Gladbach, Germany) according to manufacturer's instructions. The resulting cell population was represented by <1% CD14$^+$ monocytes and >99% CD56$^+$ cells as assessed by flow cytometry. Alternatively, PBMCs were depleted of CD4$^+$, CD14$^+$, and CD19$^+$ cells through incubation with PE anti-human CD4$^+$, CD14$^+$, and CD19$^+$ mAbs (BD PharMingen) and magnetic sorting with anti-PE microbeads (Myltenyi Biotec). The resulting cell population was represented by <1% CD4$^+$ T lymphocytes, <1% CD14$^+$ monocytes, and <1% CD19$^+$ B lymphocytes as assessed by flow cytometry. Immunosorted PBMC subpopulations were incubated in 96-well plates for 3 hours in the presence of different concentrations of colorectal cancer–derived HSP96 before adding K562 target cells (1.67×10^4 per well). HSP96 preparations were denatured by

Table 1. Clinical characteristics of enrolled patients and effects of HSP96 administration on NK activity

Patient no.	Prognostic factors*	Disease-free survival (mo)	Overall survival (mo)	Increased NK activity
2	Negative	7	14	+†
3	Positive	64+	64+	+
6	Positive	64+	64+	+
7	Negative	4	29	—
8	Positive	5	54	—
9	Negative	6	34	—
10	Positive	34	51+	+
11	Negative	11	30	+
12	Negative	7	14	+
13	Positive	25	54+	+
14	Positive	6	10	—
15	Positive	3	3	+
16	Positive	23	44	+
17	Positive	22	32	+
18	Positive	23	60+	+
19	Negative	55+	55+	+
20	Positive	49+	49+	—
21	Negative	2	54+	+
22	Positive	31	44+	+
23	Positive	48	55+	—
24	Positive	53+	53+	+
25	Positive	10	27	+
26	Negative	27	56+	+
27	Negative	5	10	—
28	Negative	8	36	—
29	Positive	47+	47+	+

*Prognostic factors were defined according to clinical Memorial Sloan-Kettering Cancer Center scores (18).

†NK activity was considered increased when a statistically significant enhancement of IFN-γ release (as detected by ELISPOT) in response to K562 cells could be detected in V5 PBMCs (post-vaccine samples) compared with V1 PBMCs (pre-vaccine samples).

20-minute boiling. IFN-γ release was then evaluated by ELISPOT assay as described above. Alternatively, immunosorted PBMCs, incubated for 48 hours with colorectal cancer–derived HSP96 (16.5 μg/mL), LPS (1 μg/mL), or IL-2 (1,200 IU/mL) as positive control, were tested for cytotoxicity by a standard 4-hour ^{51}Cr release assay and for the expression of NKG2D, NKp46, CD69, and CD25 by flow cytometry. The HSP96 binding assay was done by incubating 30 μg/mL FITC-HSP96 (Immatics Biotechnologies, Tubingen, Germany) for 30 minutes at 4°C with PBMCs from colorectal cancer patients. For the competition assay, excess of unlabeled colorectal cancer–derived HSP96 (300 μg/mL) or equivalent amounts of bovine serum albumin (BSA; Sigma-Aldrich, Milan, Italy) were added together with 30 μg/mL FITC-HSP96. Data analysis was done by FACSCalibur and CellQuest.

Statistical analysis. Statistical analysis was done by *t* test for paired samples. IFN-γ ELISPOT data obtained with pre-vaccine (V1) and post-vaccine (V5) PBMCs from the same patient showed a statistically significant increase of IFN-γ release ($P < 0.05$) in 18 of 26 patients, whereas the remaining 8 patients did not experience any significant change. IFN-γ ELISPOT results (mean of the triplicates within each patient) obtained in these two subsets of patients were also compared with baseline data using the Dunnett's test to account for multiple testing. All test results are shown in Fig. 1. $P < 0.05$ was considered as statistically significant. Kolmogorov-Smirnov (K/S) test was used to determine the statistical significance of the difference in the expression of NKG2D and NKp46 receptors between pre-vaccine and post-vaccine samples. To avoid an excessive sensitivity, values were considered as statistically significant only when $D \geq 0.12$ (extent of peak shift) and $D/S\ (n) \geq 13$ (dissimilarity of the curve shape; refs. 23, 24). The K/S statistical test was done directly with the CellQuest software for fluorescence-activated cell sorting analysis.

Results

Enhanced natural killer activity in peripheral blood mononuclear cells of colorectal cancer patients vaccinated with autologous tumor-derived heat shock protein 96. With the aim of determining whether HSP96 administration could result in the

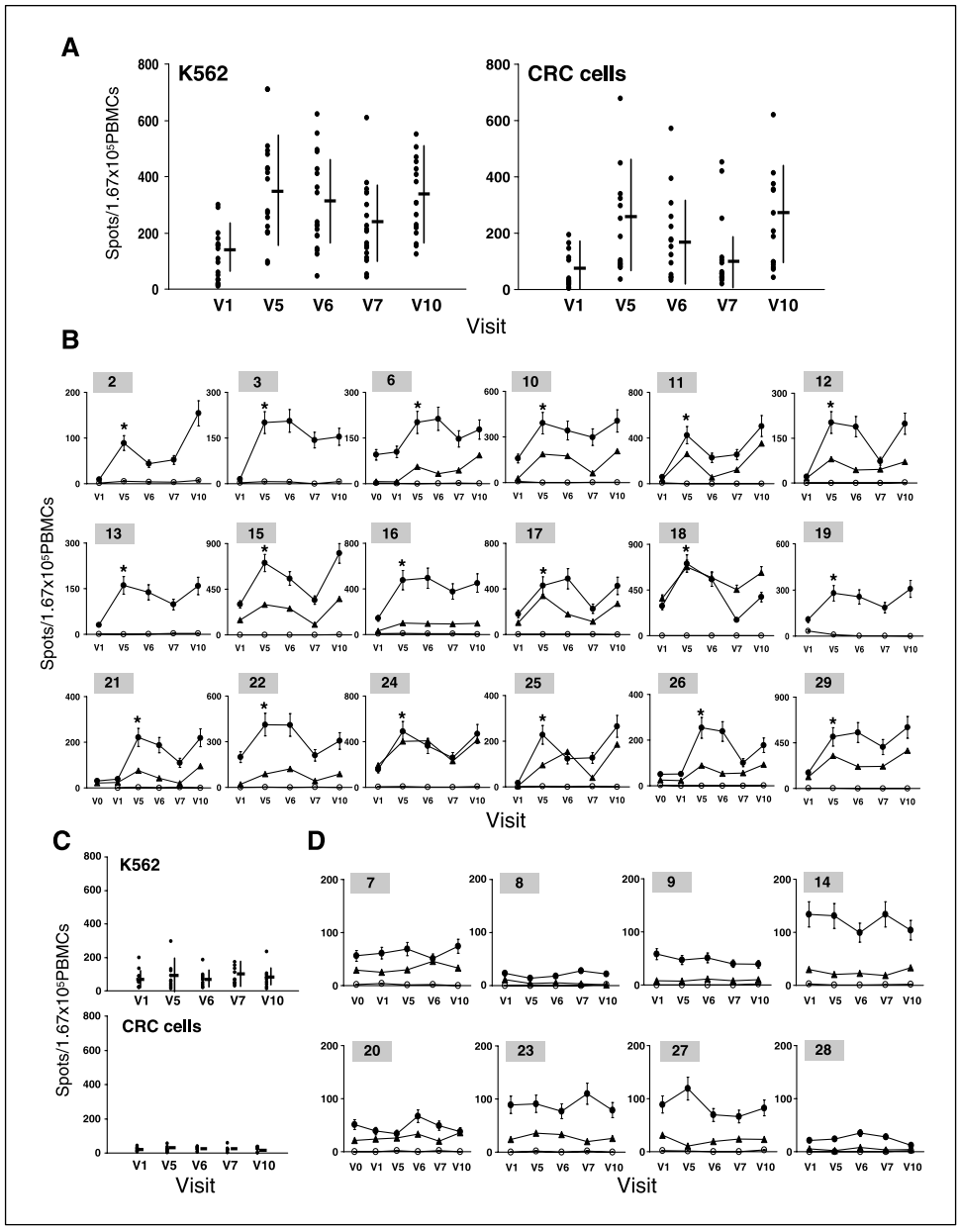

Figure 1. IFN-γ release by PBMCs obtained before and during administration of HSP96 in response to NK-sensitive targets. *A,* IFN-γ secretion as detected by ELISPOT in response to K562 (*left*) and HLA class I–mismatched colorectal cancer (*CRC*) cell lines (*right*) in 18 of the 26 patients tested. In this patients' subset, secretion is significantly increased in V5 (after three HSP96 injections), V6 (1 month after the first cycle), and V10 (end of second cycle; $P < 0.05$, Dunnett's test). *B,* kinetics of IFN-γ secretion at the single patient level (○, medium; ●, K562; ▲, HLA class I–mismatched colorectal cancer cell lines; *, $P < 0.05$, *t* test between V1 and V5 PBMC samples). HLA class I–mismatched colorectal cancer cell lines were not available for patients 2, 3, 13, and 19. *Gray boxes,* patient number. *C,* IFN-γ secretion in response to K562 and HLA class I–mismatched colorectal cancer cell lines in the remaining 8 patients tested showing no significant increment of IFN-γ release. *D,* kinetics of IFN-γ secretion at the single patient level (○, medium; ●, K562; ▲, HLA class I–mismatched colorectal cancer cell lines) showing no increase on HSP96 administration. Representative of two or more independent experiments.

in vivo boost of NK activity, PBMCs collected from colorectal cancer patients before and at different times during vaccination were tested for IFN-γ release in response to the NK-sensitive target K562 cells or to allogeneic HLA class I–mismatched colorectal cancer cells. As depicted in Fig. 1*A*, a statistically significant increase in the recognition of both targets could be detected in post-vaccine (V5, V6, and V10) PBMCs compared with pre-treatment (V1) samples in 18 of the 26 patients tested (ELISPOT data at the single patient level are shown in Fig. 1*B*). The kinetics of the increase in NK activity was characterized by a rapid increment after three HSP96 injections (V5) followed by a decrease at 2 months after treatment interruption (V7) and a subsequent increase during the second vaccine cycle (V10). It is worth noting that in selected cases (patients 6, 21, and 26), where an additional pretreatment sample obtained 1 month before vaccination (V0) could be tested, a rather stable NK activity was observed before the beginning of the treatment, further supporting the association between NK activation and HSP96 vaccination. In the remaining 8 of the 26 patients tested, no relevant change in the recognition of K562 or allogeneic colorectal cancer cells was observed (Fig. 1*C* and *D*).

Table 1 summarizes the clinical characteristics of enrolled patients (including prognostic factors, disease-free survival, and overall survival after a median follow-up of 54 months) together with the increase in NK activity (as detected by IFN-γ ELISPOT) on vaccination. In summary, 5 of 6 patients with favorable course (i.e., alive without recurrence 54 months after vaccination) versus 13 of 20 with unfavorable course (i.e., who recurred at different times after vaccination) developed increased NK activity on vaccination. Moreover, increased NK activity was observed in 11 of the 13 patients who survived at 54 months and in 7 of the 13 patients who died. The limited number of patients enrolled does not allow any definitive conclusion about a possible association between enhanced NK activity and disease course.

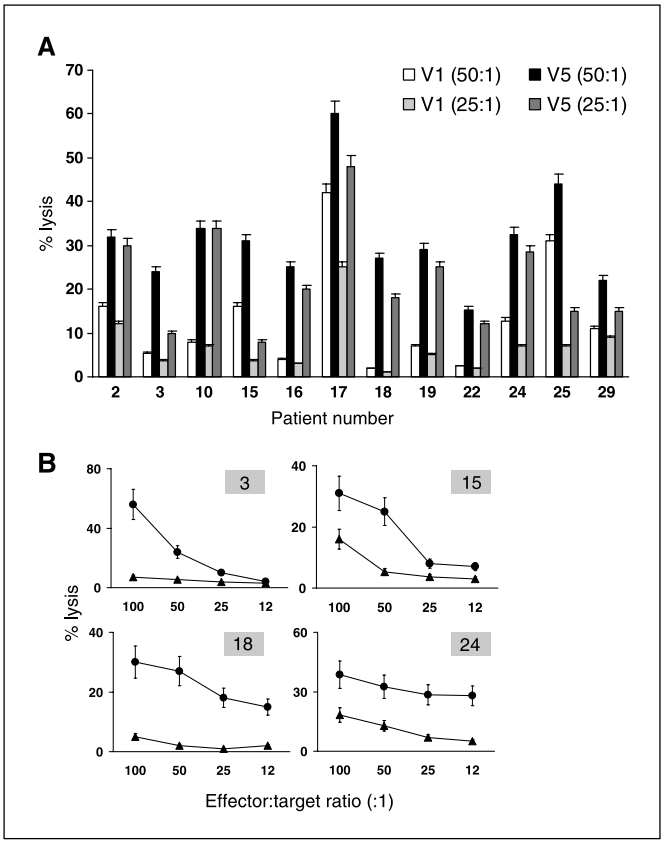

Figure 2. Increase of cytotoxic activity in PBMCs obtained after HSP96 administration. Pre-vaccine (V1) and post-vaccine (V5) PBMCs were evaluated for lytic activity against K562 cells by standard 4-hour ^{51}Cr release assay. *A,* percentage of lysis at 50:1 and 25:1 E:T ratios. Differences between pre-vaccine and post-vaccine samples were statistically significant ($P < 0.05$, *t* test for paired samples) in all patients at 50:1 ratio and in all patients, except patients 3, 15, 22, and 25, at 25:1 E:T ratio. *B,* percentage of lysis at different E:T ratios in four representative cases (▲, V1; ●, V5). *Gray boxes,* patient number. Representative of two or more independent experiments.

Functional and phenotypic characterization of lymphocytes exerting natural killer activity in post-vaccine peripheral blood mononuclear cells. To further characterize the effects of HSP96 administration on NK activity, 12 patients were selected based on PBMC availability from those displaying enhanced K562 recognition. In these cases, cytotoxic activity on NK-sensitive targets and lymphocyte phenotype were assessed in PBMCs obtained before (V1) and after three vaccine injections (V5), when the highest boost of NK activity during vaccination was observed (see Fig. 1*B*). Cytotoxicity evaluation showed that PBMCs obtained at V5 displayed a statistically significant increase in K562 killing compared with pre-vaccine PBMCs in all the cases tested, although to differing extents (Fig. 2*A* and *B*).

These functional properties were associated with a reproducible increase in the frequency of $CD56^+$ lymphocytes belonging to either $CD3^-$ NK or $CD3^+$ T-cell subpopulations (Fig. 3). In particular, 6 of 12 patients showed an increase in $CD3^-CD56^+$ lymphocytes, 2 patients had an expansion of both $CD3^-CD56^+$ and $CD3^+CD56^+$ subpopulations (Fig. 3*A*), whereas 3 patients had a selective increase of $CD3^+CD56^+$ T cells (Fig. 3*B*) as exemplified in Fig. 3*C*.

$CD3^-CD56^+$ and $CD3^+CD56^+$ cell populations expanded in HSP96-vaccinated patients could be identified as NK cells and activated *NK-like* T cells, respectively. To better characterize these populations and to verify whether additional modulations could be detected in their phenotypic profile, we analyzed NKp46 and NKG2D expression and its potential modulation by HSP96 treatment. NKG2D is a triggering receptor expressed in NK and cytotoxic T cells, playing a central role in their effector functions (25–27), whereas NKp46 belongs to a recently identified NK-specific family of natural cytotoxicity receptors, which has been found to trigger NK cell-mediated lysis of virus-infected cells as well as tumor cells (27, 28).

In all patients showing expansion of $CD3^-CD56^+$ cells (further identified as NK cells by the simultaneous expression of CD16; data not shown), NKG2D and NKp46 were up-regulated in post-vaccine PBMCs in terms of surface density (Fig. 4). A similar NKG2D modulation was also detected in the subgroup of patients with expanded $CD3^+CD56^+$ *NK-like* T cells after HSP96 treatment (Fig. 4). In most of the cases, the extent of peak shift and the dissimilarity of the curve shape were statistically significant as evaluated by K/S statistics score. Keeping with previous reports where the enhanced expression of NKG2D (28–30) and NKp46 (22) was associated with an improved cytolytic capacity, these data suggest that the boost of NK activity observed in HSP96-treated patients could be at least in part ascribed to the up-regulated expression of cytotoxicity receptors by $CD3^-CD56^+$ NK and $CD3^+CD56^+$ *NK-like* T-cell subsets.

Potential mechanisms of natural killer activation induced by heat shock protein 96 vaccination. Based on the known immunologic properties of HSP96, the functional and phenotypic activation of NK and T cells observed after HSP96 treatment

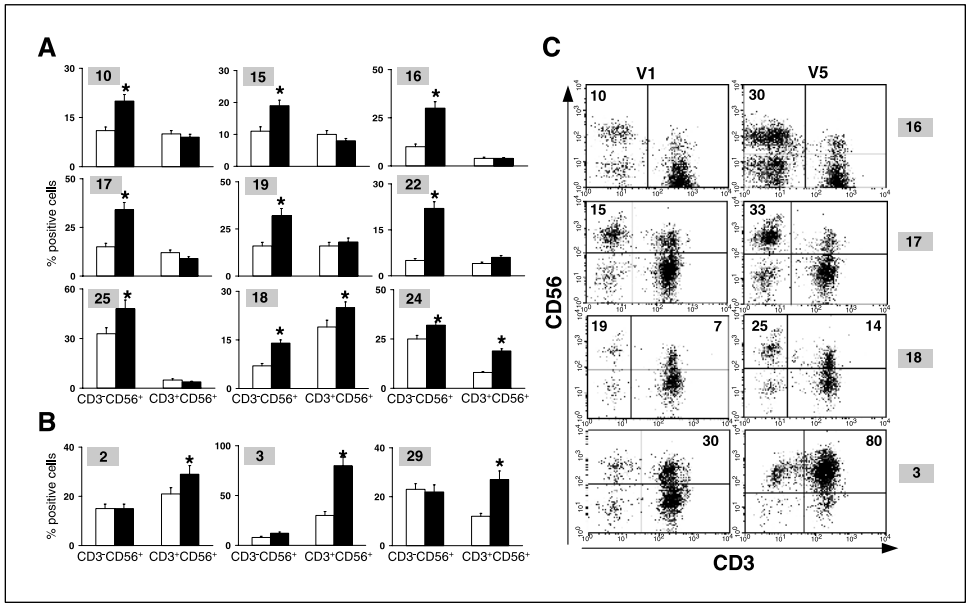

Figure 3. Expansion of $CD3^-CD56^+$ NK and $CD3^+CD56^+$ activated T cells in PBMCs of HSP96-vaccinated patients. Percentage of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells in the lymphocyte-gated region of PBMCs obtained before (V1, *white columns*) and after (V5, *black columns*) HSP96 vaccine as detected by cytofluorimetric analysis. *A,* patients showing expansion in $CD3^-CD56^+$ (patients 10, 15, 16, 17, 19, 22, and 25) or both $CD3^-CD56^+$ and $CD3^+CD56^+$ subpopulations (patients 18 and 24). *B,* patients displaying exclusive increase in $CD3^+CD56^+$ T subpopulation (patients 2, 3, and 29). *C,* expansion of $CD3^-CD56^+$ (patients 16 and 17), both $CD3^-CD56^+$ and $CD3^+CD56^+$ (patient 18), and $CD3^+CD56^+$ (patient 3) cells. *Numbers,* percentage of positive cells; *asterisks,* lymphocyte subpopulation expanded after HSP96 treatment. Representative of two or more independent experiments.

could be an indirect effect of the activating properties that this protein exerts on monocytes and other APCs, including the release of proinflammatory cytokines, such as IL-12 and tumor necrosis factor-α (9, 10). To test this hypothesis, post-HSP96 $CD14^+$ monocytes from patients with increased NK activity were tested for up-regulated expression of costimulatory molecules, such as CD40 and CD83, or enhanced IL-12 release, features that have been shown to be acquired by monocytes under proinflammatory conditions (31–33). As depicted in Fig. 5, no significant expansion of $CD14^+$ cells occurred in PBMCs on vaccination with HSP96. However, in 8 of the 12 patients analyzed, $CD14^+$ cells from post-vaccine samples exhibited a significant increased expression of CD83 and/or CD40 (Fig. 5*A*), associated with enhanced IL-12 production on stimulation (Fig. 5*B*), suggesting the occurrence of *in vivo* monocyte priming mediated by HSP96 administration. No sign of monocyte activation was instead observed in the remaining four patients (Fig. 5*C* and *D*).

The presence of increased NK activity in the absence of detectable monocyte stimulation in a subset of treated patients suggested a potential direct effect of HSP96 on NK and *NK-like* T cells. To address this issue, we evaluated the effects of *in vitro* treatment with tumor-derived HSP96 on the functional properties and phenotypic profile of $CD56^+$ lymphocytes from colorectal cancer patients. PBMCs obtained before HSP96 vaccination were depleted from $CD14^+$ monocytes and enriched for $CD56^+$ cells by immunomagnetic sorting, reaching >99% purity in $CD14^-CD56^+$ cells. The resulting cell population was then incubated with colorectal cancer–derived HSP96 (vaccine preparation), or IL-2 as positive control, and tested for recognition of NK-sensitive target cells. Fig. 6*A* shows that exposure of $CD14^-CD56^+$ cells to HSP96 resulted in a dose-dependent boost of IFN-γ secretion in response to K562 cells. The effect mediated by HSP96 was remarkably higher than that achieved by high-dose LPS and was reduced by protein denaturation (Fig. 6*A*, *right*), suggesting that it could not be attributed to minimal endotoxin contamination, potentially present even in HSP96 preparations produced under Good Manufacturing Practice conditions. *In vitro* treatment with tumor-derived HSP96 was also associated with a trigger of cytolytic function. Indeed, pre-vaccine PBMCs depleted from $CD14^+$, $CD19^+$, and $CD4^+$ cells by immunomagnetic sorting displayed a statistically significant boost in cytotoxic activity

A

Patient number	NKG2D Geo Mean		NKp46 Geo Mean	
	V1	V5	V1	V5
CD3-CD56+				
10	19	37*	54	75*
15	14	53*	36	42*
16	14	30*	9	33*
17	73	98	9	27
18	52	96*	8	25*
19	18	31*	16	36*
22	40	77*	40	52
24	26	32	19	37
25	32	61*	30	43
CD3+CD56+				
2	6	21*		
3	47	82*		
18	26	49*		
24	17	37*		
29	13	16		

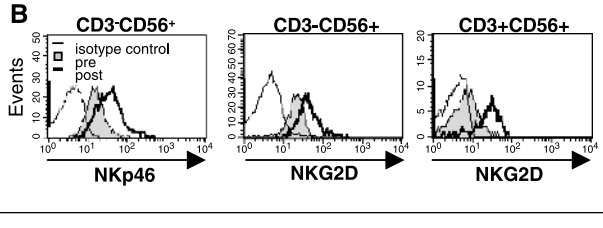

Figure 4. Modulation of lymphocyte NKG2D and NKp46 expression after HSP96 administration. *A,* increase in geometric mean (*Geo Mean*) in arbitrary linear units in pre-vaccine and post-vaccine samples of the 12 evaluated patients. *, $P < 0.01$, statistically significant difference between pre-vaccine and post-vaccine samples ($D > 0.12$, D/S (n) > 13; K/S test; see Materials and Methods). *B,* increase of NKp46 expression in $CD3^-CD56^+$ (*left*, patient 19), NKG2D in $CD3^-CD56^+$ (*middle*, patient 10), and NKG2D in $CD3^+CD56^+$ (*right*, patient 2).

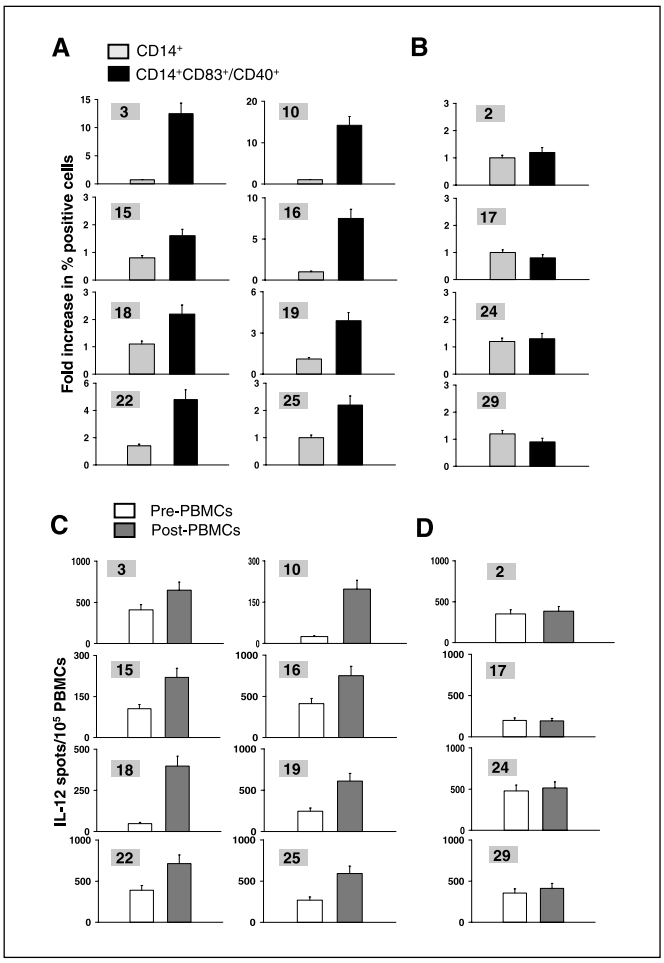

Figure 5. Activation of $CD14^+$ monocytes in PBMCs from HSP96-vaccinated patients. *A,* fold increase in the percentage of $CD14^+$ and $CD14^+CD83^+$ or $CD14^+CD40^+$ cells. A fold increase of 1 means no change. Subgroup of patients showing increased percentage of $CD14^+CD83^+$ or $CD14^+CD40^+$ cells. *B,* subgroup of patients showing no increase in the percentage of $CD14^+CD83^+$ or $CD14^+CD40^+$ cells. *C,* release of IL-12 (detected by ELISPOT) after *in vitro* priming with 1 μg/mL LPS in PBMCs obtained before (V1) and after (V5) HSP96 vaccine. Subgroup of patients showing increased IL-12 release. These patients are the same that displayed enhanced percentage of $CD14^+CD83^+$ or $CD14^+CD40^+$ cells. *D,* subgroup of patients showing no increased IL-12 secretion. These patients are the same that displayed no change in the percentage of $CD14^+CD83^+$ or $CD14^+CD40^+$ cells. $P < 0.05$, statistically significant differences between pre-vaccine and post-vaccine samples (*t* test for paired samples).

against both K562 and SW480 colorectal cancer cells after incubation with tumor-derived HSP96 (Fig. 6*B*). Interestingly, the effect was comparable with that achieved with IL-2. In contrast, the same cells did not display any significant enhancement of cytotoxic activity when cultured with LPS. To determine whether the increase in lytic activity of $CD14^+CD19^+CD4^+$-depleted PBMCs from colorectal cancer patients was associated with the same phenotypic changes observed *in vivo* on HSP96 vaccination, modulation of NKG2D or NKp46 by HSP96 *in vitro* treatment was also evaluated. As reported in Fig. 6*C*, enhanced expression of NKG2D and NKp46 was observed in $CD3^-CD56^+$ cells incubated with HSP96 compared with cells cultured in medium alone. Similarly, $CD3^+CD56^+$ lymphocytes showed a substantial increase of NKG2D expression on HSP96 *in vitro* exposure (*left*). Comparable effects were achieved by culturing with IL-2 but not with LPS (*right*). In addition, both $CD3^-CD56^+$ and $CD3^+CD56^+$ cells showed enhanced expression of activation markers CD25 and CD69 (data not shown).

To further investigate the direct effect of HSP96 on NK and activated T cells, we assessed the ability of FITC-HSP96 to bind to different lymphocyte subsets in PBMCs from colorectal cancer patients. FITC-HSP96, which stained >80% of monocytes from the same PBMC samples (data not shown), bound to a fraction of $CD3^-CD56^+$ and $CD3^+CD56^+$ cells (a representative case is shown in Fig. 6*D*, *left*). HSP96 binding to these cell subsets could be competed by 10-fold excess of unlabeled HSP96 derived from colorectal cancer but not by the same amount of BSA, suggesting the specificity of HSP96 interaction with $CD3^-CD56^+$ and $CD3^+CD56^+$ lymphocyte subpopulations (Fig. 6*D*, *right*).

Discussion

Tumor-derived HSP96 has been recently considered a promising approach for autologous vaccine therapy in cancer patients (5, 6). The main rationale for this strategy is based on the ability of this protein to chaperone antigenic peptides and directly activate APCs, thus providing the favorable environment for T-cell priming (7–10). In the present study, we describe an additional immune effect associated to HSP96 administration, which involves cells of the NK compartment. Indeed, we observed that administration of tumor-derived HSP96 is accompanied with a rapid and substantial boost of NK functions detected as enhanced cytokine release and cytotoxicity against NK-sensitive and colorectal cancer cell targets in the majority of the tested patients. This boost is associated to the *in vivo* expansion of $CD3^-CD56^+$ NK and $CD3^+CD56^+$ *NK-like* T cells in the peripheral circulation, displaying up-regulated expression of receptors involved in cell-mediated cytotoxicity, such as NKp46 and/or NKG2D. In this regard, it is worth mentioning that $CD3^+CD56^+$ *NK-like* T cells expanded on HSP96 administration display a $CD8^+TCR\alpha\beta^+$ phenotype and do not belong to the NKT CD1d-restricted nor to the γδ lymphocytes,[5] which are instead involved in HSP-mediated antitumor activity in some animal models (34). However, further studies are needed to verify whether HSP96-activated $CD3^+CD56^+$ cells may be considered as NK-CTLs, a recently identified subset characterized by HLA-E-restricted cytolytic functions (35).

The *in vivo* up-regulation of the cytotoxicity receptors NKG2D and NKp46 in NK and/or *NK-like* T cells observed after HSP96 administration represents an interesting finding, especially in light of the association with increased cytolytic activity. Indeed, colorectal cancer and other tumor patients are known to display impaired antitumor cytotoxicity related to the down-regulation of NKG2D expression in peripheral lymphocytes and the presence of circulating tumor-derived soluble MICA (29). In this context, the administration of HSP96 to cancer patients could be considered a useful tool for restoring physiologic expression levels of cytotoxicity receptors and thus potentiating lymphocyte cytolytic activity.

In terms of the potential mechanisms responsible for the HSP96-mediated enhancement of NK activity, animal models

[5] L. Pilla, G. Parmiani, and L. Rivoltini, unpublished observation.

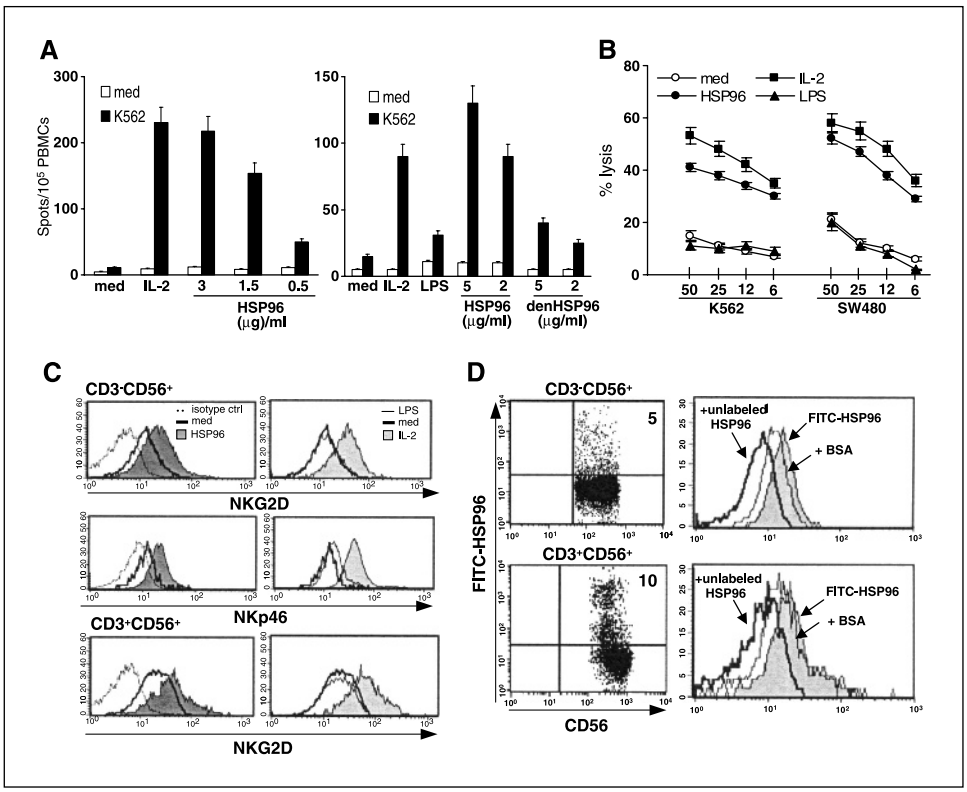

Figure 6. *In vitro* effects of tumor-derived HSP96 on NK activity in PBMCs from colorectal cancer patients. *A,* $CD14^+$-depleted PBMCs from pre-vaccine samples were enriched for $CD56^+$ cells and incubated with medium alone, different doses of colorectal cancer–derived HSP96, or 60 IU/mL IL-2 as a positive control. IFN-γ secretion in response to K562 or medium alone (*med*) was measured by ELISPOT. The same experiment was done using different doses of denatured HSP96 or 1 μg/mL LPS to verify the absence of potential endotoxin contaminants (*right*). *B,* $CD14^+CD19^+CD4^+$-depleted PBMCs from pre-vaccine samples were incubated with medium alone, colorectal cancer–derived HSP96 (16.5 μg/mL), IL-2 (1200 IU/mL), or LPS (1 μg/mL). NK cytotoxic activity was then assessed by ^{51}Cr release assay in the presence of K562 cells or SW480 colorectal cancer cells. *Numbers,* E:T ratios. $P < 0.05$, statistically significant differences between negative controls (medium and LPS), HSP96, and positive control (IL-2; *t* test for paired samples). *C,* same lymphocyte subpopulations were analyzed for NKG2D and NKp46 expression in $CD3^-CD56^+$ and $CD3^+CD56^+$ cells after incubation with medium or HSP96 (*left*). *Dashed line,* staining with isotype-matched IgG control. NKG2D and NKp46 expression was also evaluated after LPS or IL-2 incubation (*right*). Representative of three independent experiments. *D,* binding of FITC-HSP96 to $CD3^-CD56^+$ (*top left*) or $CD3^+CD56^+$ (*bottom left*) cells of colorectal cancer patients. *Numbers,* percentage of positive cells. Competition assay of FITC-HSP96 binding to $CD3^-CD56^+$ FITC-$HSP96^+$ (*top right*) or $CD3^+CD56^+$ FITC-$HSP96^+$ (*bottom right*) cells was done in the absence or presence of a 10-fold excess of unlabeled HSP96 (purified from colorectal cancer cells) or BSA. Representative of two independent experiments.

suggest that NK and T cells may be triggered by the release of activating cytokines, such as IL-12 and tumor necrosis factor-α, which are secreted by monocytes and other APCs on interaction with HSP96 (13, 36). Our data suggest that a similar pathway could also occur in HSP96-vaccinated cancer patients, as increased expression of activation markers and higher IL-12 release capacity were detected in post-HSP96 $CD14^+$ monocytes from patients displaying enhanced NK activity. However, the observation that enhanced NK activity could occur even in the absence of detectable monocyte activation indicates that a direct interaction between HSP96 and NK or T cells could occur as well. In this regard, the phenotypic and functional activation of these cell subsets after HSP96 *in vitro* stimulation, highly resembling the effects observed in HSP96-treated patients, supports a direct involvement of this protein in NK modulation. A further confirmation to this mechanism comes from the ability of this protein to specifically bind a subset of both $CD3^-CD56^+$ NK and $CD3^+CD56^+$ T cells in a manner that could be competed off by cold HSP96 but not by BSA.

The possibility that HSP96 or other members of the HSP family may directly interact with lymphocytes is also supported by recent data reporting the binding of HSP96 and HSP70 to murine $CD4^+$ T lymphocytes (37) or human NK cells (38), respectively. The direct interaction of HSP96 with $CD3^-CD56^+$ and $CD3^+CD56^+$ lymphocytes implies the expression of specific HSP receptors by these cell subsets. The α_2-macroglobulin receptor CD91, playing a potential role in APC activation (39) and HSP96 binding to $CD4^+$ T cells (37), is expressed in a fraction of $CD3^-CD56^+$ and $CD3^+CD56^+$ lymphocytes from colorectal cancer patients, with percentages of positive cells comparable with those showing HSP96 *in vitro* binding.[5] However, other receptors, such as CD94, TLR2, and TLR4, which have been found to mediate HSP70 binding to NK cells (38, 40) or to be up-regulated in $CD56^+$ NK and T cells on virus or parasite infection (41, 42), could also play a role in this phenomenon.

Concerning the clinical benefit of enhanced NK activity in HSP96-treated colorectal cancer patients, our data do not allow any definitive conclusions as the treatment was given in an adjuvant setting (i.e., in the absence of macroscopic disease) and to a relatively small number of patients. We published previously that prolonged disease-free survival and overall survival were observed in patients displaying a significant increase in $CD8^+$ T-cell-mediated antitumor responses (6). As the majority of HSP96-treated patients experienced increased NK functions, it is tempting

to speculate that NK cells may have contributed to the control of *in vivo* tumor growth by favoring T-cell priming as shown recently in experimental models (15).

The evidence that HSP96 can induce a broad array of immune responses, comprising different cellular populations and diverse mechanisms, supports the hypothesis that this protein may act as a pleiotropic activator of antitumor immune responses. As the importance of involving multiple arms of the host defense mechanisms to achieve effective immune-mediated tumor control (3, 4, 13–15) has been highlighted recently, HSP96 may be considered a valid approach for the induction of both adaptive and innate immunity in cancer patients.

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