Table I. HSP105-derived peptides conserved between human and mouse HSP105 predicted to bind to HLA-A2 or HLA-A24.

Peptides	Position	Subsequent residue listing	HLA-A2 binding score
HSP105 A2-4	120-128	MLLTKLKET	107
HSP105 A2-5	141-149	VISVPSFFT	55
HSP105 A2-6	155-163	SVLDAAQIV	37
HSP105 A2-7	169-177	RLMNDMTAV	591
HSP105 A2-9	202-210	DMGHSAFQV	21
HSP105 A2-10	222-230	VLGTAFDPFL	759
HSP105 A2-12	275-284	KLMSSNSTDL	276
HSP105 A2-13	276-284	LMSSNSTDL	26
HSP105 A2-14	300-309	KMNRSQFEEL	50
HSP105 A2-15	304-313	SQFEELCAEL	32
HSP105 A2-16	313-321	LLQKIEVPL	36
HSP105 A2-19	434-442	FLRRGPFEL	43
HSP105 A2-20	458-467	KIGRFVVQNT	76
HSP105 A2-25	668-676	LLTETEDWL	401
HSP105 A2-26	675-684	WLYEEGEDQA	146
HSP105 A2-29	757-765	EVMEWMNNV	15

Peptides	Position	Subsequent residue listing	HLA-A24 binding score
HSP105 A24-1	180-188	NYGIYKQDL	240
HSP105 A24-2	214-223	AFNKGKLKVL	30
HSP105 A24-3	251-260	KYKLDAKSKI	110
HSP105 A24-4	305-313	QFEELCAEL	47
HSP105 A24-5	433-442	TFLRRGPFEL	33
HSP105 A24-6	613-622	MYIETEGKMI	90
HSP105 A24-7	640-649	EYVYEFRDKL	330
HSP105 A24-8	725-733	HYAKIAADF	140
HSP105 A24-9	739-748	KYNHIDESEM	82

The binding scores were estimated by using BIMAS software: http//bimas.dcrt.nih.gov/cgi-bin/molbio/ken_parker_comboform.

BM-DCs pulsed with the HSP105 A2-4 peptide. A similarly strong response was observed for the HSP105 A24-7 peptide (Fig. 1C). $CD4^-$ spleen cells ($2x10^4$/well) showed 79.5±27.6 spot counts/well in response to the BM-DCs pulsed with the HSP105 A24-7 peptide, whereas they showed 20.5±14.8 spot counts/well in the presence of BM-DCs with the HSP A24-6 peptide. These assays were performed twice with similar results and they suggest that the HSP105 A2-7 and A24-7 peptides are potential CTL epitope peptides in both HLA Tgm and humans.

Identification of a CTL epitope in BALB/c mice and CTLs that are cytotoxic against C26 tumors in mice. There were similar structural motifs within the peptides that bound to human HLA-A24 and mice K^d. We selected those peptides with binding motifs for both HLA-A24 and K^d molecules and prepared 9 different synthetic peptides (HSP105-1-9). When we tested these peptides for their potential to induce *in vitro* tumor reactive CTLs in spleen cells derived from BALB/c mice immunized with the HSP105 peptides, only the HSP105 24-1 peptide-induced CTLs showed specific cytotoxicity against C26 tumors ($HSP105^+$, $H\text{-}2K^d$) (Fig. 2). The cytotoxicity against C26 was attenuated by HSP105 siRNA. These findings indicate that the HSP105 A24-1 peptide has the capacity to induce tumor reactive CTLs and that peptide vaccination-primed CTLs are reactive to this peptide *in vivo*. We would expect this HSP105 A24-1 (NYGIYKQDL) peptide to also be an epitope for human CTLs.

HSP105-reactive CTLs from PBMCs of HLA-A2-positive colorectal cancer patients and CTLs induce cytotoxicity against HSP105-expressing cancer cells. We generated a CTL line from PBMCs of colorectal patients by stimulation with the HSP105 A2-12 peptide. As shown by ^{51}Cr release assays, the resulting CTLs showed HSP105-specific cytotoxicity against SW620 cells ($HSP105^{+++}$, HLA-A2) and against T2 cells pulsed with the HSP105 A2-12 peptide ($HSP105^-$, HLA-A2), but not against HepG2 cells ($HSP105^{\pm}$, HLA-A2) or T2 cells pulsed with an irrelevant peptide (Fig. 3A). HSP105 siRNA decreased the cytotoxicity against SW620 cells. We investigated the effects of the HSP105 A2-12 peptide-reactive CTL lines on the mice implanted with the SW620 cells. Fourteen days after inoculation of HSP105 A2-12 peptide-reactive CTLs, there was an apparent reduction in tumor size in the SW620 compared to that in untreated mice (Fig. 3B). These results clearly indicate the efficacy of HSP105 A2-12 (KLMSSNSTDL) peptide-reactive CTL injection therapy for $HSP105^+$ tumors in mice.

Detection of HSP105-specific CTLs in peripheral blood of pre-surgical patients with colorectal cancer. Our results suggest that the four peptides, HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGIYK QDL) and HSP105 A24-7 (EYVYEFRDKL), are HSP105-derived, HLA-A2, or HLA-A24-restricted CTL epitopes. To determine the frequencies of the HSP105-derived T cells specific for these peptide in pre-surgical colorectal cancer patients, we analyzed the PBMC responses for each peptide using the ELISPOT assay. HSP105 expression was detected in 20 of 21 (95%) patients, consistent with previous studies (4). HSP105-specific T cells secreting IFN-γ were detected in patients stimulated with the HSP105 A2-7 (4 patients), HSP105 A2-12 (6 patients), HSP105 A24-1 (2 patients) and HSP105 A24-7 (6 patients) peptides (Table II). ELISPOT assay detected positive IFN-γ responses to at least one of the HSP105-derived peptides in PBMCs in 14 of the 21 patients. In contrast to the results for colorectal cancer patients, the 4 peptides were not recognized by PBMCs from healthy donors. Both the ratio of normal donors who showed positive T-cell responses to CMV-derived peptides and the frequencies of the specific T cells were identical to those of the colorectal cancer patients (data not shown).

HSP105-derived peptide immunization does not induce autoimmunity in HLA-A2 Tgm. HSP105 in normal adult mice is expressed in only certain tissues, and expression in these tissues is less than that in C26 tumor cells, suggesting a low

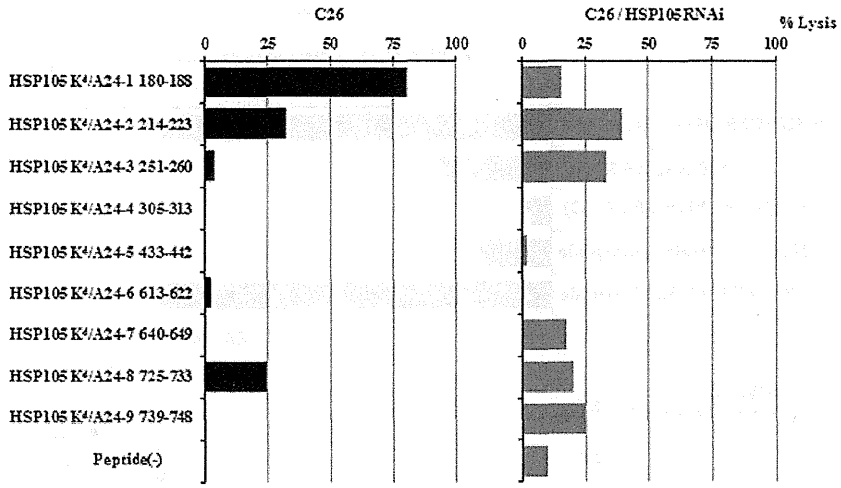

Figure 2. Identification of an HSP105-derived HLA-A24 and K^d-restricted CTL epitope. BALB/c mice were immunized with 9 HSP105 peptides. Using the ^{51}Cr release assay, sensitized spleen cells that had been stimulated *in vitro* with each HSP105 peptide (10 μmol/l) and cultured for 5 days with 100 U/ml interleukin-2 were examined for CTL activity against C26 cells and C26 cells transfected with HSP105 siRNA (C26/HSP105 RNAi). Values represent the percentage of specific cell lysis, based on the mean values from triplicate assays.

Table II. Expression of HSP105 in colorectal cancer tissue and quantification of HSP-specific CTLs in colorectal cancer patients.

						[c]Spot number of peptide-specific CTLs				
HLA-A2-positive patients	Age (yrs.)	Gender	HLA	Stage[a] of tumor	HSP105 expression[b]	HSP105 A2-7		HSP105 A2-12		CMV
1	62	M	0201/2601	IIIB	++	27	+	126	+	160
5	79	M	0207/1101	IIIB	++	0	-	2	-	10
6	51	M	0201/0206	I	+	0	-	49	+	136
8	55	M	0206/2402	I	±	0	-	0	-	66
11	69	M	0206/2402	IIIC	+	143	+	0	-	0
12	61	M	0201/3303	I	±	2	-	45	+	367
13	64	F	0201/2601	IIIC	±	0	-	2	-	254
14	66	M	0206/2402	IIIC	-	13	+	0	-	58
15	78	M	0201/1101	IIA	+	0	-	5	+	57
16	51	F	0206/2601	IV	±	31	+	7	+	15
17	63	F	0206/1101	IIA	++	0	-	25	+	96
HLA-A2402-positive patients						HSP105 A24-1		HSP105 A24-7		CMV
2	64	F	2402	IV	++	2	-	44	+	6
3	60	M	2402/3101	IIIC	++	0	-	0	-	11
4	71	F	2402/3101	IIA	++	25	+	51	+	12
7	47	M	2402/3101	IIIA	++	4	-	6	+	3
9	66	M	2402	IV	++	8	+	6	+	7
10	60	M	2402/3101	I	++	1	-	19	+	26
18	64	M	1101/2402	IV	+	0	-	2	-	40
20	46	F	1101/2402	IIIB	++	4	-	7	+	5
21	66	F	2402	I	++	3	-	0	-	38

F, female; M, male. [a]Stage, staging was performed according to the TNM classification (Union for International Cancer Control; UICC). [b]HSP105 expression, staining intensity of tumor cells was scored on a scale according to the following four grades: -, absent; ±, weak; +, moderate; ++, strong. [c]Spot number indicates the number of peptide-specific CTLs calculated by subtracting the spot number in a well of no peptide. -, Spot number <5; +, Spot number ≥5.

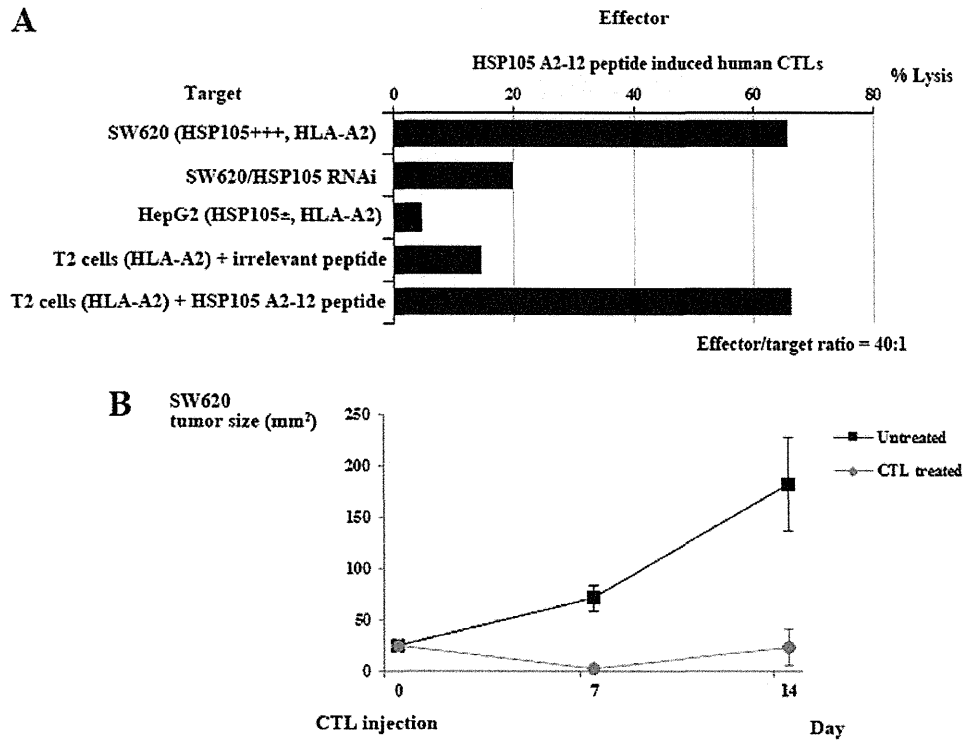

Figure 3. CTL induction from PBMCs of HLA-A2-positive cancer patients. (A) HSP105 peptide-reactive CTLs were generated from $CD8^+$ T cells of $HLA\text{-}A2^+$ colorectal cancer patients. After three or four stimulations with autologous monocyte-derived DCs pulsed with the HSP105 A2-12 peptides, the CTLs were subjected to a standard ^{51}Cr release assay at the indicated effector/target ratio (40/1). Their cytotoxicity against SW620 cells ($HSP105^{+++}$, HLA-A2), SW620 cells transfected with HSP105 siRNA ($HSP105^-$), HepG2 cells ($HSP105^{\pm}$, HLA-A2), T2 cells pulsed with an irrelevant peptide ($HSP105^-$, HLA-A2) and T2 cells pulsed with the HSP105 A2-12 epitope peptide were all examined by ^{51}Cr release assay. Values represent the percentage of specific cell lysis, based on the mean values from triplicate assays. (B) There was marked growth inhibition of SW620 cells ($HSP105^+$) engrafted into nude mice after intratumoral injection of human CTLs induced by the HSP105 peptides. When tumor size reached 25 mm^2 on day 9 after s.c. tumor implantation, human CTLs ($3x10^6$) reactive to the HLA-A2-restricted HSP105 peptide, generated from an $HLA\text{-}A2^+$ donor, were i.t. inoculated. Tumor sizes in nude mice administered the HSP105 epitope peptide-induced CTL lines (n=3), or no treatment (n=3), are shown. The mean tumor size (mm^2) for each group of mice was expressed, and bars represent SD.

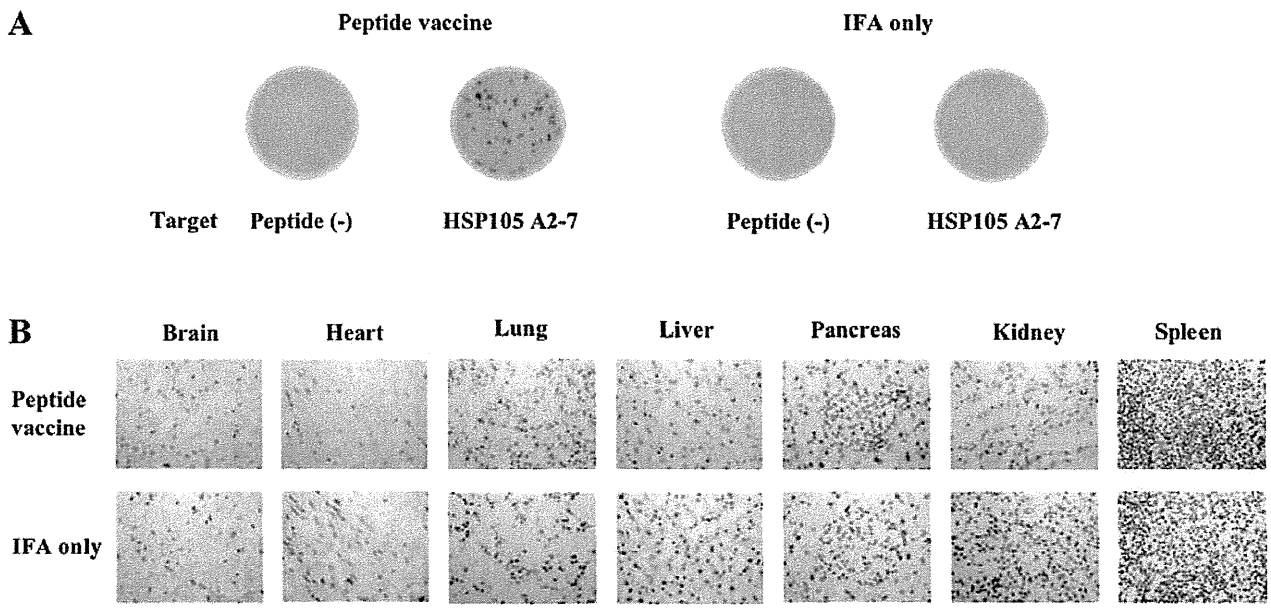

Figure 4. The HSP105 peptide vaccine induces HSP105 peptide-specific CTLs, while CD8 T cells do not infiltrate into normal tissues. (A) HSP105 peptide-specific CTLs were induced in the spleen cells of immunized mice with the HSP105 peptide vaccine. IFN-γ ELISPOT assays were performed using BM-DCs pulsed with HSP105 A2-7 and non-pulsed BM-DCs as target cells. The representative data are shown (n=6). (B) Immunohistochemical staining with anti-CD8 mAb was performed in tissue specimens of HLA-A2 Tgm immunized with the HSP105 A2-7 and A2-12 peptides. The tissue specimens were removed and analyzed 7 days after the second vaccination (original magnification, x400). The representative data are shown (n=3).

risk of damage to normal tissues posed by HSP105 antigen-induced immune responses (6). To investigate whether immunization of the mice with HSP105-derived peptides causes autoimmunity, HLA-A2 Tgm were immunized with the HSP105 A2-7 and A2-12 peptides emulsified in incomplete Freund's adjuvant at 7-day intervals and then sacrificed 7 days after the second vaccination. Using the IFN-γ ELISPOT assay, we confirmed the induction of HSP105 peptide-specific CTLs in the spleen cells of immunized mice (Fig. 4A). We did not detect any pathological changes, such as $CD8^+$ lymphocyte infiltration or tissue destruction/repair, in the brain, heart, lung, liver, pancreas, or kidney of HLA-A2 Tgm (Fig. 4B). These results indicate that the HSP105 peptide-reactive $CD8^+$ CTLs did not attack the healthy tissue specimens that we evaluated.

Discussion

Heat shock proteins (HSPs) have essential functions in the regulation of protein folding, conformation, assembly and sorting. They function as molecular chaperones to maintain the native conformational states of proteins, preventing protein aggregation (18). HSPs are classified into several families based on their molecular weight, including HSP105/110, HSP90, HSP70, HSP60, HSP40 and HSP27 (19). HSP105 is a stress protein within the HSP105/110 family that we previously reported to be overexpressed in a variety of human cancers but with little to no expression in normal tissues, aside from the testis. Thus, HSP105 presents a promising candidate for a target antigen in cancer immunotherapy (3-7). In particular, HSP105 is specifically overexpressed in colorectal cancer (83%) (4). Furthermore, HSP105 is expressed in highly metastatic colon cancer cell lines and its expression is correlated with advanced clinical cancer stages and positive lymph node involvement (20). When considering immunogenic target molecules for cancer immunotherapy, it is important to select a tumor antigen that does not run the risk of becoming lost during immunoediting (21). We reported previously that siRNA-mediated suppression of HSP105 protein expression induced apoptosis in various types of cancer cells, but not in fibroblasts (12). Therefore, it is possible that tumor cells do not lose HSP105 expression, allowing for continued growth.

Advances in molecular biology and tumor immunology have paved the way for identification of a large number of tumor-associated antigens (TAAs) and antigenic peptides recognized by tumor reactive CTLs; hence, peptide-based cancer immunotherapy has become an intensely studied field (22,23). Several HSPs, including HSP70, HSP90 and gp96, bind and deliver (through receptor-mediated endocytosis of HSP) antigenic peptides to the antigen-processing pathway of antigen-presenting cells (APCs) and these peptides are then presented on major histocompatibility complex (MHC) class I molecules. This HSP-mediated pathway has been demonstrated to evoke potent antiviral and antitumor immune responses (24). On the other hand, many researchers have identified MHC class I-presenting peptide epitopes derived from HSP (25). Furthermore, HSP105 itself may induce $CD8^+$ T cells to become reactive towards tumor cells that express HSP105, using HSP105-DNA and HSP105-pulsed DC vaccines in mice (6-8).

We found 4 peptides [HSP105 A2-7 (RLMNDMTAV), HSP105 A2-12 (KLMSSNSTDL), HSP105 A24-1 (NYGI YKQDL) and HSP105 A24-7 (EYVYEFRDKL)] to be potential HSP105-derived, HLA-A2 or A24-restricted CTL epitopes. There was a discrepancy between the expected HSP105 CTL epitopes in Tgm and in PBMCs of colorectal cancer patient. To identify the HSP105-derived CTL epitope peptides, we analyzed the PBMC responses to each of the 4 peptides in colorectal cancer patients using the *ex vivo* IFN-γ ELISPOT assay.

In this study, we used an *ex vivo* assay to detect HSP105-specific IFN-γ-secreting T cells in PBMCs from 14 of 21 pre-surgical patients with colorectal cancer. Generally, CTLs specific for tumor antigens cannot be detected directly *ex vivo*; rather only after expansion by repeated *in vitro* stimulation with the antigenic peptide in the appropriate antigen-presenting cells. This is attributed to assay sensitivity and the low frequency of tumor antigen-specific CTLs (26). HSP105-specific CTLs in PBMCs, which can be detected directly *ex vivo* without *in vitro* stimulation, provide strong immunological evidence of HSP105-derived CTL epitopes, which we were able to identify in this study. However, because the prognosis of the pre-surgical patients was affected by various factors, it was difficult to evaluate the correlation between a positive CTL response before surgery and clinical improvement at the present stage; an increase in the number of patients at each stage and further analyses of this relationship are necessary.

Although the SEREX method facilitated the identification of tumor antigens that could be recognized by antibodies and $CD4^+$ T cells, few of their T-cell epitopes have been determined (27). We previously reported in mice that HSP105-DNA and HSP105-pulsed DC vaccines induced a reaction in $CD4^+$ T cells and $CD8^+$ T cells towards tumor cells expressing HSP105 (6-8). HSP105 was identified by SEREX (3) and thus, HSP105-specific $CD4^+$ T cell reactions may be induced by HSP105 immunization. It was shown that antigen-specific $CD4^+$ T cells are required to activate memory $CD8^+$ T cells into fully functional effector killer cells (28). We are now preparing a clinical trial to investigate HSP105-based immunotherapy for HSP105-expressing tumors, including those from colorectal cancer. We plan to use the HSP105 epitope peptides identified in this study as an initial attempt. We expect that HSP105-based immunotherapy will be a novel treatment strategy for colorectal cancer patients.

Acknowledgements

This study was supported by MEXT KAKENHI grant numbers 12213111, 17015035 and the National Cancer Center Research and Development Fund (25-A-7), as well as Health and Labor Science Research Grants for Research on Hepatitis and Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor and Welfare, Japan. Y.S. would like to thank the Foundation for Promotion of Cancer Research (Japan) for the Third-Term Comprehensive Control Research for Cancer for the award of a research resident fellowship. T.N. is supported by funding from MEDINET Co., Ltd.

References

1. Weitz J, Koch M, Debus J, Höhler T, Galle PR and Büchler MW: Colorectal cancer. Lancet 365: 153-165, 2005.

2. Mlecnik B, Tosolini M, Kirilovsky A, *et al*: Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol 29: 610-618, 2011.
3. Nakatsura T, Senju S, Yamada K, Jotsuka T, Ogawa M and Nishimura Y: Gene cloning of immunogenic antigens overexpressed in pancreatic cancer. Biochem Biophys Res Commun 281: 936-944, 2001.
4. Kai M, Nakatsura T, Egami H, Senju S, Nishimura Y and Ogawa M: Heat shock protein 105 is overexpressed in a variety of human tumors. Oncol Rep 10: 1777-1782, 2003.
5. Hatayama T, Yasuda K and Nishiyama E: Characterization of high-molecular-mass heat shock proteins and 42 degrees C-specific heat shock proteins of murine cells. Biochem Biophys Res Commun 204: 357-365, 1994.
6. Miyazaki M, Nakatsura T, Yokomine K, Senju S, Monji M, Hosaka S, *et al*: DNA vaccination of HSP105 leads to tumor rejection of colorectal cancer and melanoma in mice through activation of both CD4 T-cells and CD8 T-cells. Cancer Sci 96: 695-705, 2005.
7. Yokomine K, Nakatsura T, Minohara M, *et al*: Immunization with heat shock protein 105-pulsed dendritic cells leads to tumor rejection in mice. Biochem Biophys Res Commun 343: 269-278, 2006.
8. Yokomine K, Nakatsura T, Senju S, Nakagata N and Minohara M: Regression of intestinal adenomas by vaccination with heat shock protein 105-pulsed bone marrow-derived dendritic cells in Apc(Min/+) mice. Cancer Sci 98: 1930-1935, 2007.
9. Browning M and Krausa P: Genetic diversity of HLA-A2: evolutionary and functional significance. Immunol Today 17: 165-170, 1996.
10. Pascolo S, Bervas N, Ure JM, Smith AG, Lemonnier FA and Pérarnau B: HLA-A2.1-restricted education and cytolytic activity of CD8(+) T lymphocytes from beta2 microglobulin (beta2m) HLA-A2.1 monochain transgenic H-2Db beta2m double knockout mice. J Exp Med 185: 2043-2051, 1997.
11. Firat H, Garcia-Pons F, Tourdot S, *et al*: H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumor immunotherapeutic strategies. Eur J Immunol 29: 3112-3121, 1999.
12. Hosaka S, Nakatsura T, Tsukamoto H, Hatayama T, Baba H and Nishimura Y: Synthetic small interfering RNA targeting heat shock protein 105 induces apoptosis of various cancer cells both in vitro and in vivo. Cancer Sci 97: 623-632, 2006.
13. Komori H, Nakatsura T, Senju S, *et al*: Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 12: 2689-2697, 2006.
14. Nakatsura T, Komori H, Kubo T, *et al*: Mouse homologue of a novel human oncofetal antigen, glypican-3, evokes T-cell-mediated tumor rejection without autoimmune reactions in mice. Clin Cancer Res 10: 8630-8640, 2004.
15. Nakatsura T, Senju S, Ito M, Nishimura Y and Itoh K: Cellular and humoral immune responses to a human pancreatic cancer antigen, coactosin-like protein, originally defined by the SEREX method. Eur J Immunol 32: 826-836, 2002.
16. Yoshitake Y, Nakatsura T, Monji M, *et al*: Proliferation potential-related protein, an ideal esophageal cancer antigen for immunotherapy, identified using complementary DNA microarray analysis. Clin Cancer Res 10: 6437-6448, 2004.
17. Monji M, Nakatsura T, Senju S, *et al*: Identification of a novel human cancer/testis antigen, KM-HN-1, recognized by cellular and humoral immune responses. Clin Cancer Res 10: 6047-6057, 2004.
18. Feder ME and Hofmann GE: Heat-shock proteins, molecular chaperones and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61: 243-282, 1999.
19. Craig EA, Weissman JS and Horwich AL: Heat shock proteins and molecular chaperones: mediators of protein conformation and turnover in the cell. Cell 78: 365-372, 1994.
20. Hwang TS, Han HS, Choi HK, *et al*: Differential, stage-dependent expression of Hsp70, Hsp110 and Bcl-2 in colorectal cancer. J Gastroenterol Hepatol 18: 690-700, 2003.
21. Kawakami Y and Rosenberg SA: Human tumor antigens recognized by T-cells. Immunol Res 16: 313-339, 1997.
22. van der Bruggen P, Traversari C, Chomez P, *et al*: A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254: 1643-1647, 1991.
23. Kawakami Y, Eliyahu S, Delgado CH, *et al*: Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. Proc Natl Acad Sci USA 91: 6458-6462, 1994.
24. Srivastava P: Interaction of heat shock proteins with peptides and antigen presenting cells: chaperoning of the innate and adaptive immune responses. Annu Rev Immunol 20: 395-425, 2002.
25. Hickman-Miller HD and Hildebrand WH: The immune response under stress: the role of HSP-derived peptides. Trends Immunol 25: 427-433, 2004.
26. Romero P, Cerottini JC and Speiser DE: Monitoring tumor antigen specific T-cell responses in cancer patients and phase I clinical trials of peptide-based vaccination. Cancer Immunol Immunother 53: 249-255, 2004.
27. Jäger E, Chen YT, Drijfhout JW, *et al*: Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. J Exp Med 187: 265-270, 1998.
28. Gao FG, Khammanivong V, Liu WJ, Leggatt GR, Frazer IH and Fernando GJ: Antigen-specific $CD4^+$ T-cell help is required to activate a memory $CD8^+$ T-cell to a fully functional tumor killer cell. Cancer Res 62: 6438-6441, 2002.

Human Vaccines & Immunotherapeutics 10:2, 1–8; February 2014; © 2014 Landes Bioscience

Significant clinical response of progressive recurrent ovarian clear cell carcinoma to glypican-3-derived peptide vaccine therapy

Two case reports

Shiro Suzuki[1,*], Kiyosumi Shibata[1], Fumitaka Kikkawa[1], and Tetsuya Nakatsura[2]

[1]Department of Obstetrics and Gynecology; Nagoya University Graduate School of Medicine; Showa-ku, Nagoya Japan; [2]Division of Cancer Immunotherapy; Exploratory Oncology Research and Clinical Trial Center; National Cancer Center; Kashiwa, Chiba Japan

Keywords: Glypican-3, peptide vaccine, refractory disease, ovarian clear cell carcinoma, clinical response

Abbreviations: HLA, human leukocyte antigen; UMIN-CTR, University Hospital Medical Information Network Clinical Trials Registry; CT, computed tomography; GMP, Good Manufacturing Practice; RECIST, Response Evaluation Criteria in Solid Tumors; PR, partial response; ^{18}F-FDG PET, Fluorine-18-fluorodeoxyglucose positron emission tomography; IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell

Carcinoembryonic antigen glypican-3 (GPC3) is expressed by > 40% of ovarian clear cell carcinoma (CCC) and is a promising immunotherapeutic target. We previously reported the safety of and immunological and clinical responses to a GPC3-derived peptide vaccine in a phase I clinical trial of patients with advanced hepatocellular carcinoma (HCC). Although the efficacy of the GPC3-derived peptide vaccine against HCC patients was evaluated, other GPC3-positive cancer patients have not yet been investigated. Therefore, we conducted a phase II trial to evaluate the clinical outcome of ovarian CCC patients treated with a GPC3-derived peptide vaccine. The GPC3 peptide was administered at a dose of 3 mg per body. Patients received an intradermal injection of the GPC3 peptide emulsified with incomplete Freund's adjuvant. Vaccinations were performed biweekly from the first until the 6th injection and were then repeated at 6-week intervals after the 7th injection. Treatment continued until disease progression. We herein present two patients with chemotherapy-refractory ovarian CCC who achieved a significant clinical response in an ongoing trial of a GPC3 peptide vaccine. Case 1, a 42-year-old patient with advanced recurrent ovarian CCC with liver and retroperitoneal lymph node metastases, received the HLA-A24-restricted GPC3 peptide vaccine. Contrast-enhanced CT at week 10 revealed a partial response (PR) using RECIST criteria. Case 2 was a 67-year-old female with multiple lymph node metastases. She was injected with the HLA-A2-restricted GPC3 peptide vaccine. According to RECIST, PR was achieved at week 37. The stabilization of their diseases over one year provided us with the first clinical evidence to demonstrate that GPC3 peptide-based immunotherapy may significantly prolong the overall survival of patients with refractory ovarian CCC.

Introduction

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecological malignancy. Ovarian clear cell carcinoma (CCC) accounts for 5–25% of all EOC, depending on the geographic location. It accounts for <10% of all EOC diagnosed in the USA.[1] In contrast, the incidence of CCC is reportedly >15% of EOC in Japan.[2] Compared with other EOC subtypes, CCC is associated with a poorer prognosis and increased chemoresistance.[2,3] In particular, the response rate of recurrent CCC to salvage chemotherapy was reported to be less than 10%.[4] Progression-free survival was also less than 6 mo, even in patients who achieved a response when treated with conventional anti-cancer cytotoxic agents.[5] The long-term clinical outcome of patients with recurrent CCC is extremely poor.[6] Therefore, new treatment modalities are urgently required for patients with CCC refractory to chemotherapy.

Immunotherapy is a potentially attractive option for EOC. Glypican-3 (GPC3) is useful not only as a novel tumor marker, but also as an oncofetal antigen for immunotherapy. It is specifically overexpressed in hepatocellular carcinoma (HCC).[7] Previous studies demonstrated that GPC3 was also overexpressed in several malignant tumors, including ovarian CCC.[8-12]

We previously identified the HLA-A24-restricted $GPC3_{298-306}$ (EYILSLEEL) and HLA-A2-restricted $GPC3_{144-152}$ (FVGEFFTDV) peptides, both of which can induce GPC3-reactive cytotoxic T cells (CTLs).[13] We recently reported the safety of and immunological and clinical responses to a

*Correspondence to: Shiro Suzuki; Email: shiro-s@med.nagoya-u.ac.jp
Submitted: 09/11/2013; Revised: 10/26/2013; Accepted: 11/15/2013
http://dx.doi.org/10.4161/hv.27217

GPC3-derived peptide vaccine in a phase I trial for advanced HCC patients.[14] We are currently conducting a phase II trial with a GPC3-derived peptide vaccine in ovarian CCC patients (UMIN-CTR: 000003696).

This study presents, for the first time, two patients with refractory ovarian CCC who achieved a significant clinical response in an ongoing trial of a GPC3 peptide vaccine.

Materials and Methods

Patient eligibility

This study describes two patients from our GPC3 peptide vaccination trial. This clinical trial was approved and monitored by the Institutional Review Board at Nagoya University School of Medicine. Patients with progressive metastatic ovarian CCC were enrolled after providing written, informed consent. The following eligibility criteria were used: diagnosis of ovarian CCC on the basis of histological examinations; no expectation of a response to other therapies; an Eastern Cooperative Oncology Group performance status of 0–2; age between 20 and 80 y; HLA-A24- or HLA-A2-positive status as determined using commercially available genomic DNA typing tests; and adequate organ function (white blood cell count ≥2000/mm^3, platelets ≥50000/mm^3, serum creatinine ≤2.1 mg/dl, total bilirubin ≤3.6 mg/dl, aspartate aminotransferase ≤165 IU/L, alkaline phosphatase ≤1795 IU/L). The following exclusion criteria were applied: other active malignancies; clinically serious infection; active gastrointestinal bleeding; severe cardiac insufficiency; severe interstitial pneumonitis; massive ascites and/or hydrothorax; concurrent treatment with steroids or immunosuppressive agents; and unsuitability for the trial based on a clinical judgment.

Immunohistochemical analysis

Surgical specimens were stained with hematoxylin and eosin or monoclonal antibodies against GPC3 (clone 1G12; dilution 1:300; BioMosaics), CD8 (clone 1A5; dilution 1:80; Novocastra), and HLA class I (clone EMR8/5; dilution 1:1000; Hokudo), according to the manufacturers' directions.

Ex vivo IFN-γ enzyme-linked immunospot assay

An ex vivo IFN-γ enzyme-linked immunospot (ELISPOT) assay was conducted to measure the antigen-specific CTL response, as described previously.[14] Non-cultured PBMCs were added to plates in the presence of peptide antigens (10 μg/mL) and incubated for 20 h at 37 °C in 5% CO_2. The numbers of PBMCs plated per well for case 1 and case 2 were 5×10^5 and 2.5×10^5, respectively.

GPC3 double-determinant ELISA

Double-determinant (sandwich) ELISA of GPC3 was performed as described previously.[14] The serum-soluble protein GPC3 was detected by indirect ELISA using an anti-human GPC3 monoclonal antibody (clone 1G12), anti-human GPC3 sheep polyclonal antibody (R&D Systems), and recombinant human GPC3 (R&D Systems).

Case 1

A 42-y-old nulligravid Japanese female was referred to us in November 2011. She had been diagnosed with an ovarian tumor when she presented with bilateral lower extremity deep vein thrombosis. She had undergone laparotomy including total abdominal hysterectomy, bilateral salpingo-oophorectomy, and sampling of the pelvic lymph nodes at a nearby hospital in August 2010. There were residual tumors of peritoneal dissemination and enlarged retroperitoneal lymph nodes. Histopathology revealed ovarian CCC and the presence of pelvic lymph node metastases. Therefore, her initial clinical stage was IIIC. She was treated postoperatively with six cycles of paclitaxel and carboplatin (TC) chemotherapy. After TC chemotherapy, she once again underwent laparotomy including omentectomy and pelvic and para-aortic systemic lymphadenectomy in March 2011, and a complete response was achieved. The histology of the resected tumor revealed retroperitoneal lymph node metastases, and no pathological chemotherapeutic effects were observed. Thus, she received four cycles of postoperative chemotherapy with irinotecan and cisplatin. CT scans revealed the enlargement of the retroperitoneal lymph nodes and calyx of the right kidney in August 2011. The multiple metastases rapidly progressed, and oral opioids were administered to relieve back pain.

After confirming her HLA type as HLA- A*24:02, she was enrolled in a phase II trial of the GPC3 peptide vaccination. She began receiving intradermal injections of 3 mg of HLA-A24-restricted $GPC3_{298-306}$ GMP grade peptide emulsified with incomplete Freund's adjuvant in November 2011. Vaccinations were performed biweekly from the first until the 6th injection and were repeated at 6-wk intervals after the 7th injection according to the trial schedule.

Pretreatment tumor markers were as follows: CA125, 405.4 U/ml and CA72-4, 264.1 U/ml. The serum levels of these tumor markers decreased after the initiation of treatment (**Fig. 1A**). The pretreatment serum GPC3 protein (17.3 ng/ml) was detectable, but changes in the serum levels of GPC3 unlike other tumor markers had been broadly flat while there was an increase or decrease (data not shown). Liver and para-aortic lymph node metastases grew during the first few weeks before tumor regression. According to RECIST, PR was achieved at week 10. Oral opioids were discontinued. Right hydronephrosis, pleural effusion, and ascites disappeared. Liver and para-aortic lymph node metastases were no longer visible on contrast-enhanced CT after 23 wk of exposure to the vaccine (**Fig. 1B**). However, we

Figure 1 (See opposite page). (**A**) Clinical course from the beginning of the GPC3 peptide vaccination. Serum levels of CA125 and CA72–4 decreased after the initiation of therapy. Black arrows indicate vaccinations. The asterisk indicates right inguinal lymph node resection biopsy. The double asterisk indicates bilateral inguinal lymphadenectomy. (**B**) Contrast-enhanced CT scan showing liver (white, red, blue, and orange arrows) and paraaortic lymph node (yellow arrows) metastases. The size of metastases increased immediately following the initiation of the GPC3 peptide vaccination; however, tumor sizes decreased markedly within three months. (**C, D**) Pathological findings of primary ovarian carcinoma (**C**) and right inguinal lymph node biopsy specimens (**D**). A microscopy image of a hematoxylin and eosin (H&E)-stained section shows CCC (**a, i**). Immunohistochemical staining for GPC3 and HLA class I showed positivity in the primary ovarian carcinoma, respectively (**b, c**). Most CCC cells in the resected right inguinal lymph node metastasis appeared to lack GPC3 expression and a reduction in the expression of HLA class I (**ii, iii**). Immunohistochemical analysis showed a few CD8-positive T cells in the primary ovarian CCC tissue (**d**), whereas there was little infiltration of CD8-positive T cells in the resected right inguinal lymph node metastasis (**iv**). Original magnification, x200.

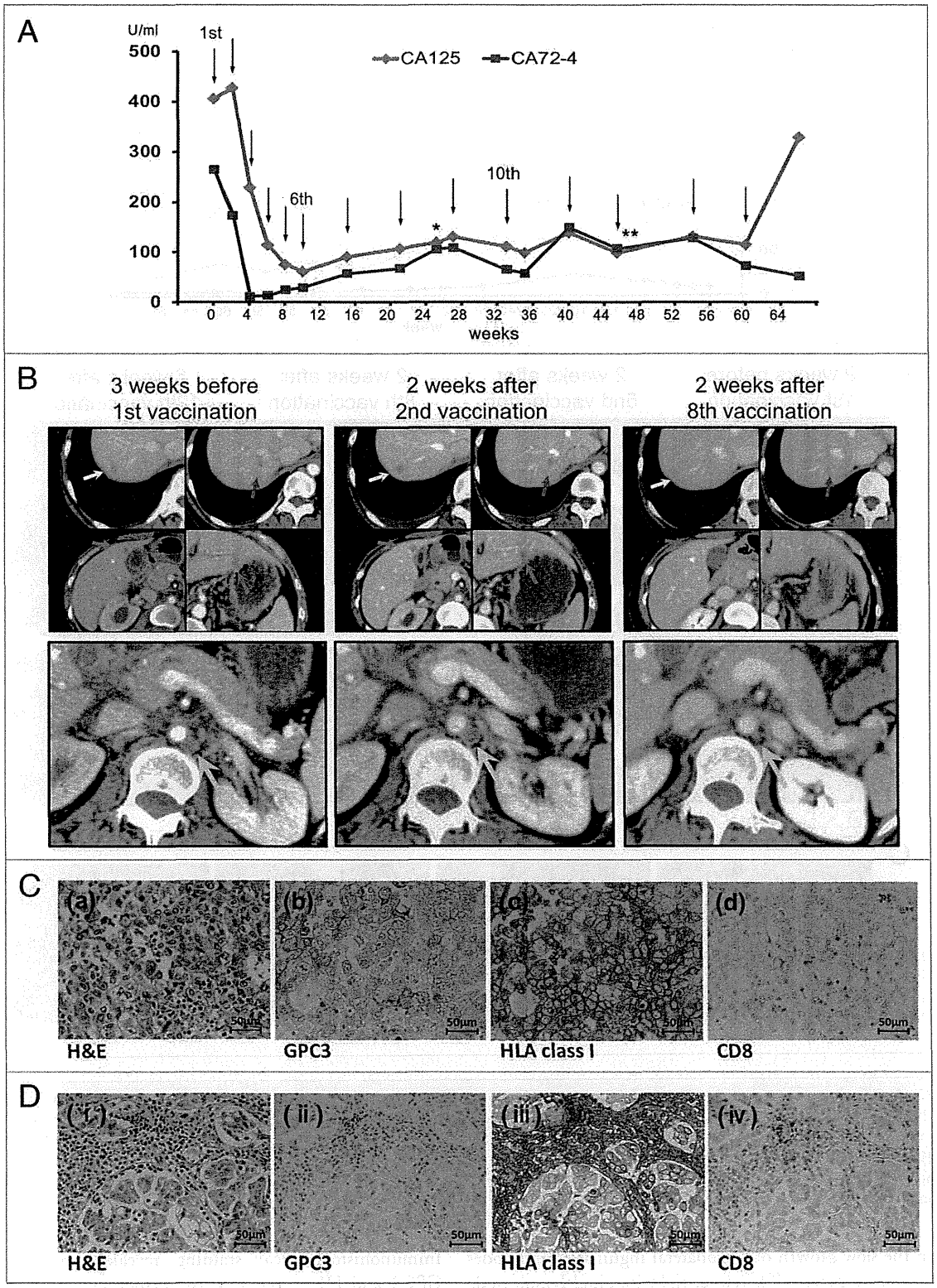

Figure 1. See page 2 for legend.

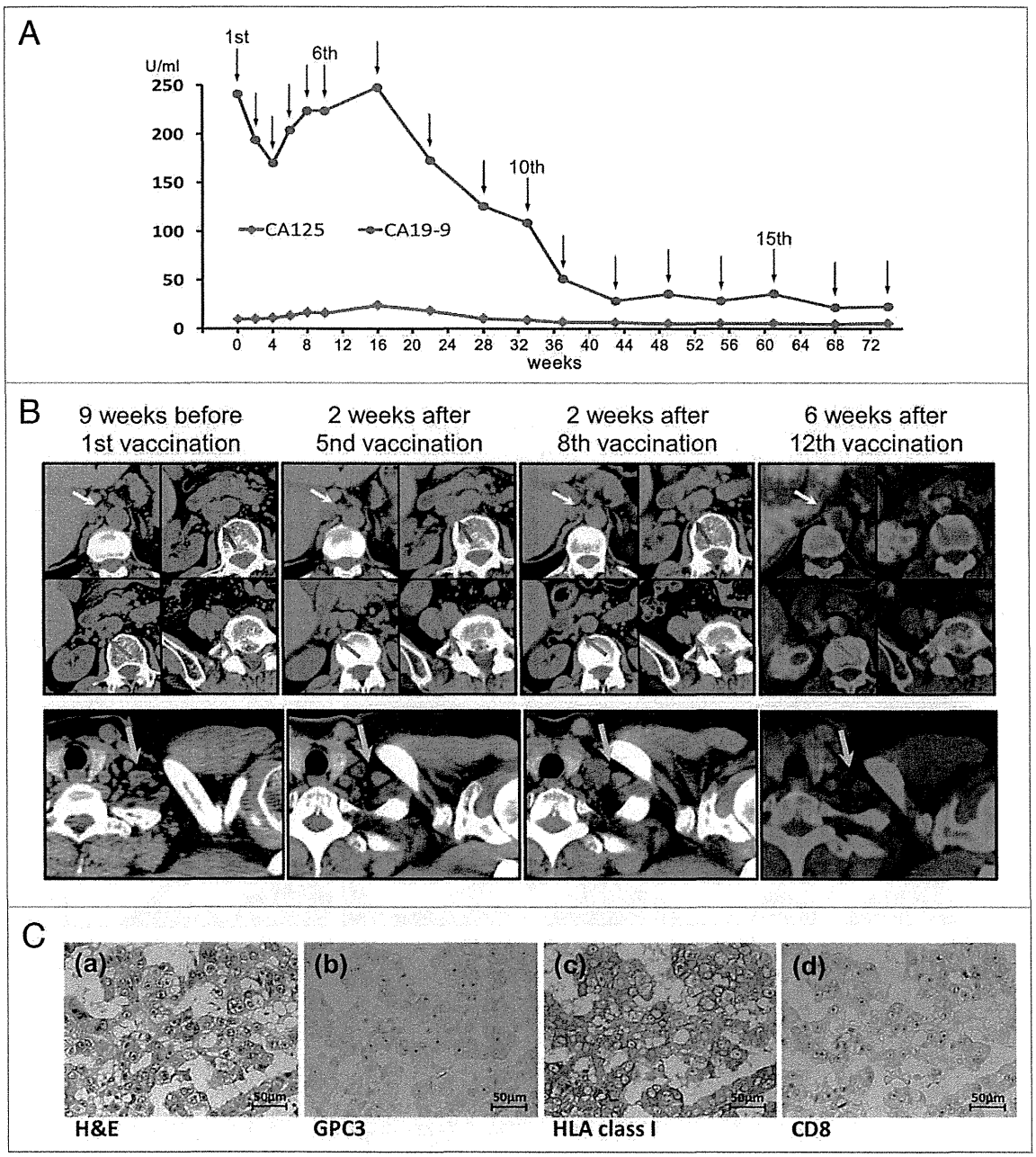

Figure 2. (**A**) Clinical course from the beginning of the GPC3 peptide vaccination. Serum levels of CA19–9 and CA125 decreased after the 7th vaccination. The CA19–9 level decreased to within the normal range. Black arrows indicate vaccinations. (**B**) Plain CT and ^{18}F-FDG PET/CT scans showing retroperitoneal lymph node (white, red, blue and orange arrows) and Virchow's node (yellow arrows) metastases. These metastases were negative on ^{18}F-FDG PET/CT at week 49. (**C**) Pathological findings of primary ovarian carcinoma. A microscopy image of a hematoxylin and eosin (H&E)-stained section shows CCC (**a**). Immunohistochemical staining was performed for GPC3, HLA class I, and CD8. (**b, c, d**). The expression of HLA class I was positive, while that of GPC3 was not, and there was no infiltration of CD8-positive T cells. Original magnification, x200.

observed the slow growth of the bilateral inguinal lymph nodes during the same period. Therefore, right inguinal lymph node resection biopsy was performed at week 25. A histological examination of the biopsied specimen revealed the metastasis of CCC. Immunohistochemical staining was performed with monoclonal antibodies against GPC3, HLA class I, and CD8. Immunohistochemical staining revealed the expression of GPC3 and HLA class I in the cytoplasm and membranes of carcinoma cells and a few CD8-positive T cells in the primary ovarian CCC tissue (Fig. 1C), whereas most CCC cells in the resected right inguinal lymph node metastasis appeared to lack GPC3 expression, showed a reduction in the expression of HLA

class I, and there was little infiltration of CD8-positive T cells (Fig. 1D).

Because the metastases that had disappeared remained absent, followed by stable disease, we continued to administer the vaccine. The treatment was eventually discontinued due to the development of lower abdominal subcutaneous metastases and progressive disease after the 14th vaccination.

Case 2

A 67-y-old parous Japanese female was referred to our hospital in March 2012. She had undergone laparotomy including total abdominal hysterectomy, bilateral salpingo-oophorectomy, omentectomy, and sampling of the retroperitoneal lymph nodes in September 2010. Residual enlarged right common iliac and para-aortic lymph nodes were noted. A histological examination showed ovarian CCC. Her initial clinical stage was IIIC due to retroperitoneal lymph node metastases. She was treated postoperatively with nine cycles of weekly TC chemotherapy. Pelvic and para-aortic lymph nodes persisted following weekly TC chemotherapy and increased in size. Two subsequent regimens yielded no response: three cycles of second-line chemotherapy with irinotecan and nedaplatin, and one cycle with gemcitabine and docetaxel as third-line chemotherapy.

She had the HLA-A*02:01 genotype, and began receiving 3 mg of the HLA-A2-restricted $GPC3_{144-152}$ peptide vaccine in April 2012. Pretreatment tumor markers were as follows: CA19-9, 241 U/ml and CA125, 9.8 U/ml. The serum levels of these tumor markers decreased after the 7th vaccination. CA19-9 levels had decreased to within the normal range by week 43 (Fig. 2A). The serum GPC3 protein was undetectable.

She achieved stable disease except for Virchow's node by week 24. However, she showed PR at week 37 (Fig. 2B). The response resulted in almost the complete resolution of all measurable lesions on plain CT. Unfortunately, she had a past history of allergies to CT contrast media; therefore, we performed ^{18}F-FDG PET/CT to improve the diagnostic accuracy of residual tumors at week 49. ^{18}F-FDG PET/CT showed mild positive FDG uptake in four masses (smaller than 1 cm in size) in the right common iliac lymph node and intraperitoneal dissemination. Although ^{18}F-FDG PET did not always reflect malignancy or detect metastatic lesions, the presence of residual tumors was still suspected.

Because she remained progression free at week 74, she is still receiving the trial treatment. The expression of HLA class I was positive, while that of GPC3 was not positive in the primary ovarian CCC tissue, and there was no infiltration of CD8-positive T cells (Fig. 2C).

No adverse effects of the vaccination were observed in either case, except for a local inflammatory response with erythema at the injection site and low-grade fever.

Discussion

Most gynecological oncologists are aware that recurrent or persistent ovarian CCC has a chemoresistant phenotype. Although the different histological types of EOC may represent different diseases with unique clinical and molecular characteristics, ovarian CCC is still currently being treated in the same manner as other EOCs because of its low rate of incidence among EOCs in western countries. Novel treatment approaches should be adopted for ovarian CCC, especially in cases that are recurrent or refractory to previous therapies. Between 5 and 10% of all currently open clinical trials for ovarian cancer patients evaluate approaches using immune-based therapies. Although most immunotherapeutic strategies for ovarian cancer treatment investigated so far are capable of inducing antigen-specific immunity, the unequivocal clinical benefit for these patients has not yet been demonstrated.[15] To date, we have confirmed that a HLA-A2-restricted $GPC3_{144-152}$ peptide-specific CTL clone can recognize and kill HLA-A2-positive and GPC3-positive ovarian CCC cell lines.[16]

Based on these conditions, we conducted a trial to assess the clinical outcome of ovarian CCC patients treated with a GPC3-derived peptide vaccine. In the ongoing clinical trial, 20 refractory patients were enrolled until the end of August 2013. Ten of these patients were vaccinated at least six times, and a significant clinical response was achieved in two patients (2/10, 20%) who received the HLA-A24 or A2-restricted GPC3 peptide vaccine. In spite of resistance to multiple chemotherapeutic drugs, the stabilization of their diseases over one year suggests the efficacy of the GPC3 peptide vaccination.

Two patterns of responses, fast and slow, after the initial increase in the total tumor burden were observed in this study. In case 1, a fast response was preceded by an apparent early enlargement in liver and para-aortic lymph node metastases. Metastases were stable for several months in case 2 after beginning the vaccination treatment, except for Virchow's node, which showed radiographical progression; however, all radiographically measurable metastases thereafter almost completely regressed. We hypothesize that the immune response elicited during the first several weeks of vaccination, similarly to ipilimumab,[17] may be mistaken for progressive disease: CTL infiltration and immune-mediated inflammation may not be radiographically distinguishable from a growing tumor. The initial tumor enlargement was suspected to be caused by inflammation. Meanwhile, bilateral inguinal lymph node metastases grew without reductions within the same patient in case 1. Disease progression may occur as part of a "mixed response", i.e., the regression of some lesions and apparent progression of others.

The difference in effectiveness may have been caused by the heterogeneity associated with immune-escape mechanisms, including the downregulation of cancer-specific antigens and/or HLA class I in tumor cells. The intratumor heterogeneity of GPC3 expression was observed at different levels in our preliminary study depending on the locations and timing of biopsies. Although no correlation was observed between the degree of GPC3 expression and $GPC3_{298-306}$ peptide-specific CTL response, GPC3 expression in bilateral inguinal lymph node metastasis in case 1 may be associated with the clinical benefits of GPC3 peptide vaccine therapy. On the other hand, we were unable to perform biopsies of retroperitoneal lymph node metastases in case 2. Therefore, further pathological analysis was limited. Although GPC3 immunohistological expression was

negative in part of the primary ovarian CCC tissue, its expression may be positive in other parts of the tumor. It may be difficult to predict the clinical response against metastatic tumors based on the strength of GPC3 expression in the primary tumor.

In a phase I trial of GPC3-derived peptide vaccination, while GPC3 immunohistological expression was detected in more than 80% of advanced HCC patients, pretreatment serum GPC3 protein was detectable in only approximately half of those patients. Unlike case 1, the serum GPC3 protein in case 2 was undetectable by ELISA using a monoclonal antibody (clone 1G12) and sheep polyclonal antibody. Delayed clinical response may have been due to lower expression of GPC3 than the detection limit of assays using clone 1G12 in metastatic sites.

Ex vivo IFN-γ ELISPOT analysis in these two cases revealed vaccine-induced immune reactivity against the GPC3 peptide (data not shown). Although we were unable to discuss whether there were any differences in the quantity or quality of the responses between the two cases because the number of PBMCs plated per well did not correspond, there were more GPC3 peptide-specific CTL spots and less non-specific background spots in case 2 than in case 1.

It is difficult to confirm whether tumor regression was actually induced by peptide-specific CTLs or other mechanisms. As one of several possibilities, antigen spreading may have occurred following the GPC3 peptide-specific CTL response after the vaccination and contributed decisively to tumor regression.

Although many clinical trials have been conducted with cancer peptide vaccines, none of these have succeeded in Phase 3. The development of a biomarker to select potential responders would contribute significantly to potential success in a Phase 3 trial. In an effort to identify such biomarkers, we consider it possible to predict a response based on HLA class I/peptide complex expression on the cell surface in prevaccine biopsy specimens. Thus, we have attempted to prepare monoclonal antibodies against the HLA-A24/GPC3$_{298\text{-}306}$ peptide complex and HLA-A2/GPC3$_{144\text{-}152}$ peptide complex. Further analyses are needed in the future.

These results provide the first clinical evidence to demonstrate that GPC3 peptide-based immunotherapy is a promising treatment for patients with ovarian CCC. The complexity of the immune response and impact of each individual patient's status on the immune system create challenges for predicting the time course of the response. Ongoing and future trials will yield information on the best clinical use of this vaccine and the most appropriate method for assessing the response.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Kennedy AW, Biscotti CV, Hart WR, Webster KD. Ovarian clear cell adenocarcinoma. Gynecol Oncol 1989; 32:342-9; PMID:2920955; http://dx.doi.org/10.1016/0090-8258(89)90637-9
2. Sugiyama T, Kamura T, Kigawa J, Terakawa N, Kikuchi Y, Kita T, Suzuki M, Sato I, Taguchi K. Clinical characteristics of clear cell carcinoma of the ovary: a distinct histologic type with poor prognosis and resistance to platinum-based chemotherapy. Cancer 2000; 88:2584-9; PMID:10861437; http://dx.doi.org/10.1002/1097-0142(20000601)88:11<2584::AID-CNCR22>3.0.CO;2-5
3. Chan JK, Teoh D, Hu JM, Shin JY, Osann K, Kapp DS. Do clear cell ovarian carcinomas have poorer prognosis compared to other epithelial cell types? A study of 1411 clear cell ovarian cancers. Gynecol Oncol 2008; 109:370-6; PMID:18395777; http://dx.doi.org/10.1016/j.ygyno.2008.02.006
4. Crotzer DR, Sun CC, Coleman RL, Wolf JK, Levenback CF, Gershenson DM. Lack of effective systemic therapy for recurrent clear cell carcinoma of the ovary. Gynecol Oncol 2007; 105:404-8; PMID:17292461; http://dx.doi.org/10.1016/j.ygyno.2006.12.024
5. Takano M, Goto T, Kato M, Sasaki N, Miyamoto M, Furuya K. Short response duration even in responders to chemotherapy using conventional cytotoxic agents in recurrent or refractory clear cell carcinomas of the ovary. Int J Clin Oncol 2013; 18:556-7; PMID:22552358; http://dx.doi.org/10.1007/s10147-012-0404-x
6. Kajiyama H, Shibata K, Mizuno M, Yamamoto E, Fujiwara S, Umezu T, Suzuki S, Nakanishi T, Nagasaka T, Kikkawa F. Postrecurrent oncologic outcome of patients with ovarian clear cell carcinoma. Int J Gynecol Cancer 2012; 22:801-6; PMID:22617480; http://dx.doi.org/10.1097/IGC.0b013e3182540145
7. Nakatsura T, Yoshitake Y, Senju S, Monji M, Komori H, Motomura Y, Hosaka S, Beppu T, Ishiko T, Kamohara H, et al. Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker. Biochem Biophys Res Commun 2003; 306:16-25; PMID:12788060; http://dx.doi.org/10.1016/S0006-291X(03)00908-2
8. Nakatsura T, Kageshita T, Ito S, Wakamatsu K, Monji M, Ikuta Y, Senju S, Ono T, Nishimura Y. Identification of glypican-3 as a novel tumor marker for melanoma. Clin Cancer Res 2004; 10:6612-21; PMID:15475451; http://dx.doi.org/10.1158/1078-0432.CCR-04-0348
9. Saikali Z, Sinnett D. Expression of glypican 3 (GPC3) in embryonal tumors. Int J Cancer 2000; 89:418-22; PMID:11008203; http://dx.doi.org/10.1002/1097-0215(20000920)89:5<418::AID-IJC4>3.0.CO;2-I
10. Toretsky JA, Zitomersky NL, Eskenazi AE, Voigt RW, Strauch ED, Sun CC, Huber R, Meltzer SJ, Schlessinger D. Glypican-3 expression in Wilms tumor and hepatoblastoma. J Pediatr Hematol Oncol 2001; 23:496-9; PMID:11878776; http://dx.doi.org/10.1097/00043426-200111000-00006
11. Aviel-Ronen S, Lau SK, Pintilie M, Lau D, Liu N, Tsao MS, Jothy S. Glypican-3 is overexpressed in lung squamous cell carcinoma, but not in adenocarcinoma. Mod Pathol 2008; 21:817-25; PMID:18469798; http://dx.doi.org/10.1038/modpathol.2008.37
12. Maeda D, Ota S, Takazawa Y, Aburatani H, Nakagawa S, Yano T, Taketani Y, Kodama T, Fukayama M. Glypican-3 expression in clear cell adenocarcinoma of the ovary. Mod Pathol 2009; 22:824-32; PMID:19329941
13. Komori H, Nakatsura T, Senju S, Yoshitake Y, Motomura Y, Ikuta Y, Fukuma D, Yokomine K, Harao M, Beppu T, et al. Identification of HLA-A2- or HLA-A24-restricted CTL epitopes possibly useful for glypican-3-specific immunotherapy of hepatocellular carcinoma. Clin Cancer Res 2006; 12:2689-97; PMID:16675560; http://dx.doi.org/10.1158/1078-0432.CCR-05-2267
14. Sawada Y, Yoshikawa T, Nobuoka D, Shirakawa H, Kuronuma T, Motomura Y, Mizuno S, Ishii H, Nakachi K, Konishi M, et al. Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. Clin Cancer Res 2012; 18:3686-96; PMID:22577059; http://dx.doi.org/10.1158/1078-0432.CCR-11-3044
15. Leffers N, Daemen T, Helfrich W, Boezen HM, Cohlen BJ, Melief K, Nijman HW. Antigen-specific active immunotherapy for ovarian cancer. Cochrane Database Syst Rev 2010; 20:CD007287; PMID:20091627
16. Suzuki S, Yoshikawa T, Hirosawa T, Shibata K, Kikkawa F, Akatsuka Y, Nakatsura T. Glypican-3 could be an effective target for immunotherapy combined with chemotherapy against ovarian clear cell carcinoma. Cancer Sci 2011; 102:622-9; PMID:21205085; http://dx.doi.org/10.1111/j.1349-7006.2011.02003.x
17. Weber JS, Kähler KC, Hauschild A. Management of immune-related adverse events and kinetics of response with ipilimumab. J Clin Oncol 2012; 30:2691-7; PMID:22614989; http://dx.doi.org/10.1200/JCO.2012.41.6750

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

Cancer Immunology Research

Enhancement of Tumor-Reactive Cytotoxic CD4$^+$ T-cell Responses after Ipilimumab Treatment in Four Advanced Melanoma Patients

Shigehisa Kitano, Takemasa Tsuji, Caillian Liu, et al.

Cancer Immunol Res 2013;1:235-244. Published OnlineFirst August 12, 2013.

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

Research Article

Cancer Immunology Research

Enhancement of Tumor-Reactive Cytotoxic CD4$^+$ T-cell Responses after Ipilimumab Treatment in Four Advanced Melanoma Patients

Shigehisa Kitano[1], Takemasa Tsuji[6], Caillian Liu[1,3], Daniel Hirschhorn-Cymerman[3], Chrisann Kyi[4], Zhenyu Mu[1], James P. Allison[7], Sacha Gnjatic[5], Jianda D. Yuan[1], and Jedd D. Wolchok[1,2,3,4]

Abstract

CD4$^+$ T cells provide help to enhance and sustain cytotoxic CD8$^+$ T-cell responses. A direct lytic role for this cell population in mouse models further supports the use of tumor-reactive CD4$^+$ T cells for cancer immunotherapy. CTLA-4 blockade has been shown to expand antigen-specific cytotoxic CD4$^+$ T cells in mouse models. We took advantage of spontaneous immunity to the NY-ESO-1 cancer-testis antigen to investigate quantitative and qualitative changes in antigen-specific CD4$^+$ T-cell responses after ipilimumab (anti-CTLA-4 monoclonal antibody) treatment in patients with advanced melanoma. Four patients with NY-ESO-1 seropositive melanoma were chosen upon the availability of suitable blood specimens for characterizing the functions of NY-ESO-1 antigen-specific CD4$^+$ T-cell response by enzyme-linked immunospot (ELISPOT), intracellular cytokine staining (ICS), and cytotoxicity assays. Multiple NY-ESO-1 antigen-specific CD4$^+$ T-cell responses with T_H1 dominance were induced or enhanced after ipilimumab treatment in peripheral blood in all four patients. NY-ESO-1 antigen–specific CD4$^+$ T-cell lines established from all four patients after ipilimumab treatment recognized naturally processed NY-ESO-1 protein in antigen-presenting cells, expressed master transcription factor Eomesodermin (Eomes), and secreted perforin and Granzyme B. Finally, we showed that these NY-ESO-1 antigen-specific CD4$^+$ T-cell lines directly lysed autologous melanoma cell lines expressing NY-ESO-1 in an MHC class II restricted manner. Our results show that antigen-specific cytotoxic CD4$^+$ T-cell responses are induced after ipilimumab therapy in human cancer patients. Ipilimumab may induce the expression of lytic granules on antigen-specific cytotoxic CD4$^+$ T cells via Eomes, revealing a novel consequence of immunologic checkpoint blockade. *Cancer Immunol Res; 1(4); 235–44. ©2013 AACR.*

Introduction

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a coinhibitory molecule expressed on activated T cells and regulatory T cells. CTLA-4 is essential in maintaining immune homeostasis and contributes to immune tolerance by downregulating T-cell activation (1–3). Ipilimumab is a fully humanized immunoglobulin G1 (IgG1) monoclonal antibody (mAb) that blocks CTLA-4. Two pivotal phase III trials in patients with advanced melanoma showed a survival benefit for ipilimumab as monotherapy or when added to chemotherapy (4, 5), leading to its approval by the U.S. Food and Drug Administration in March 2011, marking a milestone in the field of immunotherapy. Immunotherapeutic strategies have also focused on targeting antigens expressed exclusively on melanoma cells, for example, melanoma differentiation antigens (such as gp100 or Melan-A) or cancer/testis (CT) antigens. CT antigens are expressed in a variety of human malignancies but not in normal adult tissues except for the testis and placenta (6). To date, the best characterized CT antigen is NY-ESO-1, which is highly immunogenic and elicits spontaneous antibody and T-cell responses in patients with cancer whose tumors express NY-ESO-1 (7). Prior studies by our group and others have shown that ipilimumab treatment of patients with advanced melanoma induces polyfunctional NY-ESO-1- and Melan-A–specific CD8$^+$ T-cell responses (8, 9). We have also correlated the induction of polyfunctional NY-ESO-1 CD4$^+$ T-cell responses with the development of integrated antibodies and CD8$^+$ T-cell responses in patients, who experience clinical benefit (10).

The essential role of CD4$^+$ T helper (T_H) cells in enhancing and sustaining CD8$^+$ T-cell responses is well established (11). Recent evidence in mouse models, however, suggests more

Authors' Affiliations: [1]Ludwig Center for Cancer Immunotherapy, Immunology Program, [2]Department of Medicine, [3]Swim Across America Laboratory, and Ludwig Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center; [4]Weill Cornell Medical College of Cornell University; and [5]Mount Sinai Icahn School of Medicine, Tisch Cancer Institute, New York; and [6]Center for Immunotherapy, Roswell Park Cancer Institute, Buffalo, New York; [7]Department of Immunology, The University of Texas MD Anderson Cancer Center, Houston, Texas

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

J.D. Yuan and J.D. Wolchok share senior authorship.

Corresponding Author: Jedd D. Wolchok, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Phone: 646-888-2395; Fax: 646-422-0453; E-mail: wolchokj@mskcc.org

doi: 10.1158/2326-6066.CIR-13-0068

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

direct roles for tumor-reactive $CD4^+$ T cells for cancer immunotherapy (12, 13). Specifically, CTLA-4 blockade has the capacity to expand antigen-specific cytotoxic $CD4^+$ T cells *in vivo* in mice (12). Furthermore, adoptive transfer of $CD4^+$ T cells expanded from a single tumor-reactive T-cell clone resulted in a durable complete response in a patient with melanoma (14). However, the cytotoxic function of antigen-specific $CD4^+$ T cells during ipilimumab treatment and its intracellular mechanism have not been characterized. We hypothesized that CTLA-4 blockade could result in expansion and/or enhancement of cytotoxic $CD4^+$ T-cell responses in human cancer patients through the modulation of T_H1 transcription factors. To address this question, we conducted in-depth immune monitoring of 4 NY-ESO-1 seropositive melanoma patients who were treated with ipilimumab and had availability of properly annotated specimens. Peripheral blood mononuclear cells (PBMC) were analyzed by intracellular cytokine staining (ICS) using multiparametric flow cytometry. Samples were analyzed following *in vitro* stimulation with NY-ESO-1 overlapping or single peptides. IFN-γ ELISPOT was conducted to define specific $CD4^+$ T-cell peptide responses. Transcription factors T-bet and Eomesodermin (Eomes) as well as cytotoxic degranulation markers perforin and granzyme B were analyzed on NY-ESO-1–specific $CD4^+$ T cells. NY-ESO-1–specific $CD4^+$ T-cell lines were established to confirm their ability to recognize NY-ESO-1–positive tumor cell lines and to induce tumor lysis.

Materials and Methods

Patients

Blood and tissue samples were analyzed from 4 patients (09-079-1, 09-079-7, 09-079-10, and 09-079-17) treated on a clinical trial at Memorial Sloan-Kettering Cancer Center (MSKCC) evaluating the pharmacokinetics of two different biosynthetic formulations of ipilimumab (CA184-087, NCT00920907). All patients received 4 doses of antibody at a dose of 10 mg/kg i.v. administered every 3 weeks for 4 doses during induction therapy. Patients without dose-limiting toxicity and with evidence of clinical benefit (in this case, 09-079-1, 09-079-10, and 09-079-17) then received maintenance ipilimumab at the same dose every 12 weeks starting at week 24. Responses were adjudicated by the recently proposed immune-related response criteria (15). Toxicity was assessed using National Cancer Institute Common Terminology Criteria for Adverse Events, version 3.0. All patients provided informed consent for the clinical studies and additional consent for the collection of blood and tumor tissue for investigational purposes on a separate MSKCC biospecimen utilization protocol. All studies were approved by the MSKCC Institutional Review Board.

Peptides and cell lines

NY-ESO-1 overlapping peptides (17 peptides with ~20-mer length and 10 aa overlap) (16) and NY-ESO-1_{92-100} peptide (LAMPFATPM), NY-ESO-1_{94-102} peptide (MPFATPMEA), NY-ESO-1_{94-104} peptide (MPFATPMEAEL), NY-ESO-1_{96-104} peptide (FATPMEAEL), and NY-ESO-$1_{157-165}$ peptide (SLLMWITQC) were purchased from JPT Peptide Technologies. Peptides were dissolved in dimethyl sulfoxide at a concentration of 1 mg/mL and stored in aliquots at −80 °C before use. The following autologous or MHC-matched melanoma cell lines were used as target cells: SK-MEL-381 (from patient 09-079-7) and SK-MEL-351 (from patient 09-079-10, NY-ESO-1 negative). Autologous B-lymphoblastoid cell lines (LCL) were generated in our laboratory from the patients' PBMCs, using EBV-containing supernatants and also used as target cells.

Preparation of PHA-stimulated $CD4^+$ T cells (T-APC)

Phytohemagglutinin (PHA)-stimulated $CD4^+$ T cells (T-antigen presenting cells or T-APCs) were prepared as described previously (17–19). $CD4^+$ T cells were separated from PBMCs using Dynabeads (Invitrogen) according to the manufacturer's instruction and seeded into 48-well plates (NUNC) at a density of $1–2 \times 10^6$ cells per well in 1 mL RPMI-1640 medium supplemented with 25 μmol/L HEPES, 10% heat-inactivated human AB serum (Gemini Bio-Products), 2 μmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium). A total of 10 μg/mL of PHA (REMEL) was added to the culture at day 0. Half of the medium was replaced with complete medium containing interleukin (IL)-2 (20 IU/mL, Roche) and IL-7 (40 ng/mL; R&D systems) at day 3, which was then repeated twice weekly. The activated $CD4^+$ T-APCs were pulsed with peptides and used as target cells in various assays.

In vitro sensitization for monitoring T-cell immune responses

In vitro sensitization was conducted as described previously (17–20). After separating $CD8^+$ T cells or $CD4^+$ T cells from PBMCs using Dynabeads, $CD4^+$ T cells (5×10^5 cells/well) were cultured with antigen peptide-pulsed and irradiated (30 Gy) autologous $CD4^-$ $CD8^-$ PBMCs (5×10^5 cells/well) in 96 round bottom-well plates in complete medium. From day 4 onward, half of the medium was replaced with complete medium containing IL-2 and IL-7 twice a week. These $CD4^+$ T cells in the culture wells were used as effector cells in various assays on day 20.

Generation of NY-ESO-1–specific $CD4^+$ T-cell lines

NY-ESO-1–specific $CD4^+$ T cells were isolated by CD154 (CD40L) expression sorting as described with some modification (21, 22). Presensitized $CD4^+$ T cells were restimulated for 6 hours in 500 μL X-VIVO15 (BioWhittaker-Lonza) with T-APCs that were pulsed overnight with NY-ESO-1 peptide (1 μmol/L) and labeled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen-Molecular Probes) in the presence of 20 μL of phycoerythrin (PE)-conjugated anti-CD154 mAb and 0.3 μL GolgiStop (BD Biosciences). $CFSE^-CD154^+$ NY-ESO-1–specific effector T cells were sorted using a FACSAria instrument and FACS Diva software (BD Biosciences). Sorted cells were stimulated with plate-bound anti-CD3 (0.5 μg/mL) and CD28 mAbs (0.5 μg/mL; eBioscience) in the presence of irradiated allogeneic PBMCs and were expanded for about 20 days in the presence of 20 IU/mL of IL-2 and 20 ng/ml of IL-7 in complete medium as described with some modification (23). Reactivity of the $CD4^+$ T-cell lines was tested against autologous melanoma cell lines or autologous LCL pulsed with peptides.

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

ELISPOT assay

The IFN-γ ELISPOT assay was conducted as described previously (18–20). Briefly, 96-well nitrocellulose ELISPOT plates (MAHA S4510; Millipore) were coated overnight at 4°C with 2 μg/mL anti-human IFN-γ mAb (1-D1K) and blocked with 10% human AB serum-containing RPMI-1640 for 2 hours at 37°C. A total of 2×10^4 sensitized $CD4^+$ T cells and 2×10^4 peptide-pulsed T-APCs were placed in each well of the ELISPOT plate at a final volume of 200 μL RPMI-1640 medium without serum. After incubation for 22 hours at 37°C in a CO_2 incubator, the plate was developed using 0.2 μg/mL biotinylated anti-human IFN-γ mAb (7-B6-1, Mabtech), 1 μg/mL streptavidin-alkaline phosphatase conjugate (Roche Diagnostics), and 5-bromo-4-chloro-3-indolyl phosphate/NBT (Sigma-Aldrich). The number of spots was evaluated using a C.T.L. Immunospot analyzer and software (Cellular Technology). Results were shown as the number of spot-forming cells (SFC) without subtracting the number of background spots, as the number of SFC in negative control was less than 3 spots per well in all assays. A positive response with more than 10 spot counts per well as well as spot counts ≥3-fold more than background spots obtained with nonpulsed target cells was considered to be significant.

Intracellular cytokine staining

For surface staining, APC-Cy7-CD8, PE-Cy7-CD3, Pacific Blue-CD3 (BD Bioscience), ECD-CD4, ECD-CD45RA, ECD-CD45RO (Beckman Coulter Inc.), and FITC-CCR7 (R&D Systems) were used. For ICS, *in vitro* stimulated and cultured $CD4^+$ T cells were harvested as effector cells. A total of 2×10^5 effector $CD4^+$ T cells were cocultured for 6 hours in 500 μL 10% human AB serum RPMI in the presence of 0.35 μL GolgiStop and with PE-Cy5-CD107a (20 μL/mL, BD Bioscience) with 2×10^5 autologous T-APCs that had been pulsed overnight with each antigen peptide pool and labeled with CFSE. Cytoplasmic cytokines were stained using a BD Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer's instructions with FITC, Alexa Fluor 647, PE-Cy7, or Horizon-V450-IFN-γ, PE-Macrophage inflammatory protein (MIP)-1β, APC-IL-2, PE or FITC-IL-4, APC-IL-5, Horizon-V450 or APC-IL-13, Alexa Fluor 700-granzyme B, PerCP-Cy5.5-T-bet (BD Bioscience), PE-Cy7-TNF-α, PE-IL-10, Alexa Fluor 647-IL-17A, Alexa Fluor 647-Eomesodermin (Eomes; eBioscience), PE-perforin (Cell sciences).

CFSE-negative effector $CD4^+$ T cells were analyzed using a CyAn flow cytometer with Summit software (Dako Cytomation Inc.) or a LSR Fortessa with FACS Diva software (BD Biosciences). All analyses were carried out using FlowJo software (TreeStar, Inc.). Results were shown as a frequency (%) of antigen-specific cytokine responses and CD107a mobilization (functions$^+$) in $CD4^+$ T cells after subtracting the frequency of background functionpositive cells. A positive response ≥3-fold more than the negative control obtained with nonpulsed target cells (% function-positive cells/sample in all assays) was considered to be significant. All background intracellular responses (function-positive) were <0.1%, except for the transcription factors T-bet and Eomes, which were <0.5%.

Cytotoxicity assay

A cytotoxicity assay was conducted as described previously with some modifications (12). To determine the *in vitro* killing of tumor targets, target cells were labeled with 0.5 μmol/L CFSE, whereas control target cells were labeled with 5 μmol/L CFSE. NY-ESO-1–specific $CD4^+$ T cell lines were cocultured at different ratios (24) with a 50:50 mixture of target cells and control target cells for 6 hours. Then, cells were acquired on a CyAn flow cytometer with Summit software and analyzed by FlowJo software. A total of 20 μg/mL of anti-human HLA-DR, DP, or DQ antibody (BD Bioscience) and mouse IgG2a, κ antibody (BD Bioscience) as a control were used for blocking of MHC class II on target cells. Cytotoxicity was calculated using the following formula: cytotoxicity (%) = $[1 - (\%\text{live cell}_{\text{target cells}} / \%\text{live cell}_{\text{control target cells}})] \times 100$.

Statistical analysis

Data were analyzed using Prism 5.0 (GraphPad Software, Inc.). All the experiments were repeated two or three times. Statistical significance was determined by a Student t test. $P < 0.05$ was considered a statistically significant difference.

Results

Patients

Patient profiles and clinical demographics are summarized in Table 1.

Case 1—Patient 09-079-1

Patient 09-079-1 is a 63-year-old man with metastatic melanoma to soft tissue, liver, lymph nodes, bone, and left adrenal gland. He had experienced progressive disease (PD) on multiple chemotherapy regimens. In August 2009, he initiated ipilimumab therapy. After completing induction therapy, imaging at week 12 revealed PD but repeat imaging at week 24 showed a partial response (PR), although the adrenal lesion was noted to have increased in size. He initiated maintenance ipilimumab and subsequently underwent resection of the adrenal metastasis during week 27, as a presumed immunologic "escape" lesion. Since that time, he has maintained a durable PR.

Case 2—Patient 09-079-7

Patient 09-079-7 is a 49-year-old man who was diagnosed with metastatic melanoma to the lung, lymph nodes, pancreas, retroperitoneum, bone, soft tissue, thoracic nodes, and spleen in April 2009. He received first-line therapy with ipilimumab. Imaging after induction therapy revealed PD. Further imaging at week 16 indicated further significant PD. He was taken off the study protocol, transitioned to supportive care, and died in March 2010.

Case 3—Patient 09-079-10

Patient 09-079-10 is a 39-year-old woman with stage IV melanoma to the lung, liver, lymph nodes, spleen, and bone, with prior PD on a cisplatin/vinblastine/temozolomide. She initiated ipilimumab therapy in September 2009. Imaging after completing induction therapy revealed PD at week 12 but stable disease (SD) at week 24. She then received maintenance

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

Table 1. Patient profiles

Patient ID	09-079-1	09-079-7	09-079-10	09-079-17
Age (years)	63	49	39	54
Gender	Male	Male	Female	Female
PS (Karnofsky)	90	90	90	90
Stage	IV	IV	IV	IV
Metastatic lesions	Adrenal gland, liver, peritoneal	Lung, lymph nodes, pancreas, retroperitoneum, bone, soft tissue, thoracic nodes, spleen	Lung, thoracic nodes, spleen, liver	Skin, axillary lymph node and mesenteric mass
WBC ($\times 10^3/mm^2$)	7.0	8.2	3.9	7.7
ALC ($\times 10^3/mm^2$)	1.6	1.1	1.2	1.8
Adverse event (grade)	Fatigue (G1) Neuropathy (G1) Tinnitus (G1) Myalgia (G1)	Diarrhea (G1) Rash (G1)	Fatigue (G2) Fatigue (G1) Rash (G1)	Pruritus (G1) Rash (G1)
Prior therapy	Temozolomide IMC-1121B+DTIC CVT	None	CVT	Gp100 DNA, High-dose IL-2, Oncovex
Total # of doses	12	3	11	12

Abbreviations: CVT, cisplatin + vinblastine + temozolomide; DTIC, dacarbazine; G, toxicity grade; IMC-1121B, ramucirumab; PS, performance status.

ipilimumab, with her last treatment in July 2011. She continues to have SD.

Case 4—Patient 09-079-17

Patient 09-079-17 is a 54-year-old woman with recurrent melanoma to the back, abdomen, and pelvis. This patient has a history of melanoma dating back to 1990, when she had a 2.4-mm melanoma with deep dermal invasion removed from her left lower back. She was disease free for approximately 15 years. She was noted to have a recurrence in 2005 and underwent multiple resections, including dermal, axillary node, and mesenteric masses. She previously experienced progression of disease on temozolomide, a gp100 DNA vaccine, talimogene laherparepvec (recombinant attenuated herpes simplex virus expressing GM-CSF) and high-dose IL-2. She then initiated protocol therapy with ipilimumab in October 2009. Computed tomographic imaging revealed PD at week 12 and SD at week 24. She received maintenance ipilimumab, and approximately 2.5 years into this treatment, she continues to have SD.

Ipilimumab therapy induces T_H1-dominant $CD4^+$ T-cell responses against multiple NY-ESO-1–specific epitopes in all four patients

Sera collected from these 4 patients were analyzed for NY-ESO-1 antibody responses. All four patients were seropositive at baseline (Patients 09-079-7, -10 and -17) or following ipilimumab treatment (Patient 09-079-1). NY-ESO-1 antibody titers increased in all 4 patients, especially in patient 09-079-1, with subsequent ipilimumab treatments (Supplementary Table S1) as we have reported previously (10). We confirmed baseline NY-ESO-1 antigen expression in tumor tissue from all 4 patients by immunohistochemistry and reverse-transcriptase PCR.

Purified $CD4^+$ T cells isolated from PBMCs were stimulated *in vitro* with NY-ESO-1 overlapping peptides and cultured for 20 days. Reactivity against NY-ESO-1 overlapping peptides and the individual NY-ESO-1 peptides was then determined for these cultured $CD4^+$ T cells by ICS and IFN-γ ELISPOT assay. In the 3 patients, who were seropositive at baseline (Patients 09-079-7, -10 and -17), pretreatment NY-ESO-1–specific $CD4^+$ T-cell responses were detected (TNF-α in patient 09-079-7; IFN-γ and TNF-α in patient 09-079-10; IFN-γ, TNF-α, IL-2, MIP-1β, and CD107a in patient 09-079-17). In the patient seronegative at baseline (09-079-1), baseline NY-ESO-1–specific $CD4^+$ T-cell responses were not detected. After ipilimumab treatment, NY-ESO-1–specific $CD4^+$ T-cell responses (IFN-γ, TNF-α, and CD107a with/without IL-2) were detected in all 4 patients. These cells did not secrete T_H2 cytokines (IL-4, IL-5, or IL-13) or T_H17 cytokine (IL-17A) by ICS, suggesting that that they are T_H1 cells. (Fig. 1A)

Because of the low frequency of NY-ESO-1 antigen-specific $CD4^+$ T cells in peripheral blood in these 4 patients, we did not detect NY-ESO-1 antigen-specific $CD4^+$ T-cell response using *ex vivo* ICS staining. After 20 days of *in vitro* cell culture, we were able to obtain approximately 5×10^6 $CD4^+$ T cells with an

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

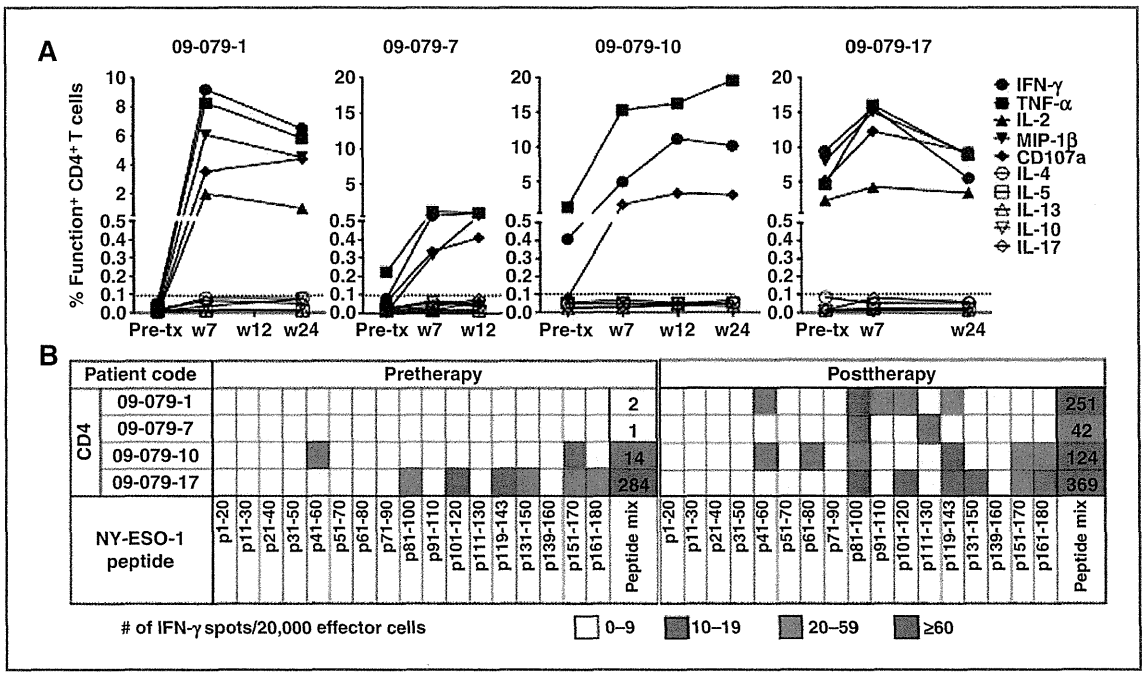

Figure 1. Broad T_H1-dominant NY-ESO-1 antigen-specific $CD4^+$ T-cell responses were detected in peripheral blood from melanoma patients treated with ipilimumab. Purified $CD4^+$ T cells from patients were cultured for 20 days after presensitization with NY-ESO-1 overlapping peptides. A, NY-ESO-1 antigen-specific cytokine secretion and CD107a mobilization of $CD4^+$ T cells were assessed against NY-ESO-1 overlapping peptides by ICS assay. Autologous T-APCs were used as target cells for ICS assay. Representative data from two or three independent experiments for each patient are shown. B, the specificity of $CD4^+$ T cells was tested using autologous T-APCs pulsed with each individual peptide or overlapping peptides by IFN-γ ELISPOT assay. Experiments were repeated twice to confirm the peptide reactivity. *, Peptide mix; all of NY-ESO-1 overlapping peptides mixture, with number of spots indicated.

approximately 1 log expansion of $CD4^+$ T cells from the initial 5×10^5 $CD4^+$ T cells per well. The yield of detectable NY-ESO-1 peptide-specific $CD4^+$ T cell ranged from 2,500 to 92,250 per well. Using these parameters, a peptide-specific $CD4^+$ T-cell response with 10 IFN-γ spots per 20,000 effector cells in an ELISPOT assay after *in vitro* stimulation is estimated to yield 2,500 NY-ESO-1–specific $CD4^+$ T cells after one 20-day *in vitro* culture. We studied the breadth and frequency of each peptide-specific T-cell response by IFN-γ ELISPOT. Baseline spontaneous $CD4^+$ T-cell responses to two individual peptides (10–19 spots, 2,500–4,750 cells/well after stimulation) were detected in patient 09-079-10 and to 6 peptides: 2 peptides, >60 spots (>15,000 cells/well after stimulation); 4 peptides, 20–59 spots (5,000–14,750 cells/well after stimulation) in patient 09-079-17. Following ipilimumab therapy, a broader spectrum of $CD4^+$ T-cell peptide responses was observed with new peptides recognized by 3 patients (all except 09-079-17). The number of peptide-specific responses did not change following ipilimumab therapy (6 peptides) in patient 09-079-17; however, 5 (p81–100, p101–120, p119–143, p131–150, p151–170) of 6 individual T-cell peptides (which were positive at baseline) were increased significantly in IFN-γ spot number ($P < 0.05$; Fig. 1B).

After ipilimumab treatment, the majority of peptides recognized by $CD4^+$ T cells included sequences located in four immunodominant distinct regions of the protein, corresponding to peptides NY-ESO-$1_{41–60}$ (2/4 patients), NY-ESO-$1_{81–100}$ (4/4 patients), NY-ESO-$1_{119–143}$ (3/4 patients), and NY-ESO-$1_{151–170}$ (2/4 patients) as previously reported in patients with spontaneous responses as well as those immunized with NY-ESO-1 vaccines (refs. 25–29; Fig. 1B). In summary, polyfunctional NY-ESO-1 $CD4^+$ T-cell responses were detected at baseline or posttherapy samples in all 4 patients.

High-avidity $CD4^+$ T-cell responses with cytotoxicity to naturally processed antigen are detected in peripheral blood after ipilimumab treatment

We sought to determine whether the NY-ESO-1–specific $CD4^+$ T cells detected after ipilimumab treatment had high-affinity T-cell receptors capable of recognizing naturally processed tumor antigen. NY-ESO-1 single peptide-specific $CD4^+$ T-cell lines were generated from PBMCs from patients 09-079-1 and -17 before and after the treatment at weeks 7 and 24 or from patients 09-079-7 and -10 at weeks 12 and 24 by a CD154 (CD40L) sorting method (Supplementary Fig. S1A) after stimulation with individual, dominant NY-ESO-1 peptides. We confirmed that there were no contaminating $CD8^+$ T cells in CD40L-sorted NY-ESO-1–specific $CD4^+$ T cells (Supplementary Fig. S1B). These cells were then cocultured with

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

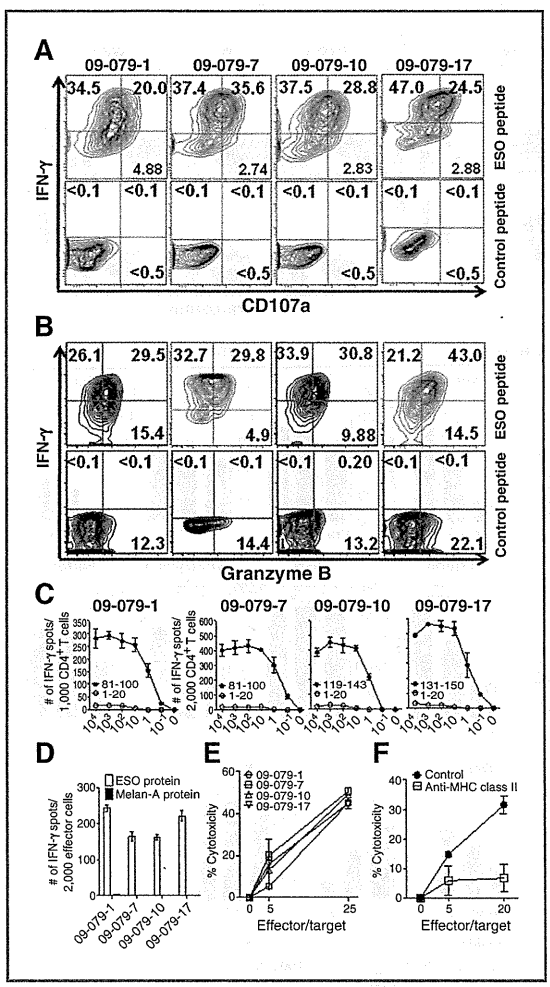

Figure 2. High-avidity NY-ESO-1 antigen-specific CD4$^+$ T-cell responses with naturally processed protein recognition were detected in PBMCs after ipilimumab treatment. Purified CD4$^+$ T cells from patients after ipilimumab treatment (week 7; 09-079-1, -17, week 12; 09-079-7, -10) were cultured for 20 days after presensitization with NY-ESO-1 overlapping peptides. NY-ESO-1 single epitope-specific CD4$^+$ T-cell lines were isolated by CD40L sorting after 6-hour *in vitro* restimulation. After expansion, these cell lines were tested by ICS assay using autologous APCs pulsed with low-concentration NY-ESO-1 peptide or control peptide (10 nmol/L). A and B, intracellular IFN-γ and surface CD107a (A), and IFN-γ and granzyme B (B) were analyzed in ICS assay. Representative plots are shown from two independent experiments; therefore the gate for patient 09-079-17 was different from the other 3 patients. Nonspecific staining by isotype control antibodies was less than 0.1% in all samples (data not shown). C, avidity of these CD4$^+$ T-cell lines in the recognition of the cognate peptide was tested by ELISPOT assay using autologous APCs pulsed with graded doses of peptide in ELISPOT assay. D, recognition of recombinant NY-ESO-1 protein or control Melan-A protein naturally processed in autologous APCs (LCLs) by CD4$^+$ T-cell line was tested by ELISPOT assay. E, cytotoxicity against autologous LCL pulsed with each cognate peptide (100 nmol/L) by CD4$^+$ T-cell line was determined at 5:1 and 25:1 Effector:Target ratio. F, NY-ESO-1_{81-100}–specific CD4$^+$ T-cell line from 09-079-7 was cocultured for 6 hours at different ratios with target cells (labeled with 0.5 μmol/L CFSE) and control target cells (5 μmol/L CFSE) in the presence or absence of MHC class II blocking antibodies. Target cell (T): autologous melanoma cell line (SK-MEL-381; NY-ESO-1 positive) treated with IFN-γ (10 ng/mL) for 24 hours prior to cytotoxic assay. Control target cell: melanoma cell line (SK-MEL-351 from patient 09-079-10; NY-ESO-1 negative). Cytotoxicity (%) was calculated with the formula as described in Materials and Methods. Each experiment was repeated independently twice or three times with similar results. Data are presented as mean ± SD.

autologous melanoma cell lines and autologous APC (EBV-infected B cell lines; LCLs) with each corresponding peptide or MAGE-A4 overlapping peptides as the negative control. The NY-ESO-1–specific CD4$^+$ T-cell lines established from 4 patients' expressed intracellular IFN-γ, granzyme B, and surface CD107a, as compared with the CD4$^+$ T-cell response to the negative control (Fig. 2A and B).

All NY-ESO-1–specific CD4$^+$ T-cell lines before and after the treatment were of high avidity to recognize 10 to 100 nmol/L peptide (data not shown). Furthermore, they recognized recombinant NY-ESO-1 protein naturally processed in autologous APCs (LCLs) by ELISPOT assay (Fig. 2C) and lysed autologous LCL pulsed with corresponding NY-ESO-1 protein (100 nmol/L; Fig. 2D) using the cytotoxicity assay described above. NY-ESO-1–specific CD4$^+$ T-cell lines lysed the autologous LCL pulsed with each cognate peptides (100 nmol/L) at 5:1 and 25:1 effector:target ratio (Fig. 2E). Among the autologous melanoma cell lines derived from these patients, SK-MEL-381 (from patient 09-079-7) expresses both NY-ESO-1 and MHC class II. The NY-ESO-1_{81-100}–specific CD4$^+$ T-cell line from patient 09-079-7 lysed the autologous tumor cell line (SK-MEL-381), and cytotoxicity was inhibited by MHC class II antibody blocking of the target cells (Fig. 2F). The NY-ESO-1–specific CD4$^+$ T-cell lines do not coexpress CD8 markers before and after restimulation in the ICS assay (Supplementary Fig. S1C).

Serial changes in cytotoxic NY-ESO-1 antigen-specific CD4$^+$ T cells after ipilimumab treatment

From patient 09-079-17, we were able to establish NY-ESO-$1_{131-150}$ peptide-specific CD4$^+$ T-cell lines at the pretreatment time point as well as at weeks 7 and 24 by the CD154 (CD40L) sorting method and subsequently analyzed serial changes in cytotoxic function. These cell lines recognized autologous APCs (LCLs) pulsed with NY-ESO-$1_{131-150}$ peptide (100 nmol/L) in the ICS assay. In contrast with IFN-γ, which was consistently produced before and after the therapy, accumulation of granzyme B and perforin was observed only after ipilimumab treatment (Fig. 3A–C). In addition, *in vitro* peptide restimulation induced granzyme B synthesis only in CD4$^+$ T cells after the therapy (Fig. 3C). In accordance with the expression of cytotoxic molecules, higher expression of the transcription factor Eomes previously reported as the master regulator of cytotoxic CD8$^+$ T cell (30) was observed after ipilimumab treatment at weeks 7 and 24 when compared with expression levels before treatment (Fig. 3D; *, $P < 0.05$). In contrast, expression of T-bet was not increased (Fig. 3D). Expression of PD-1 became lower at week 24 after treatment when compared with pretreatment and at week 7 (Fig. 3D; *, $P < 0.05$).

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

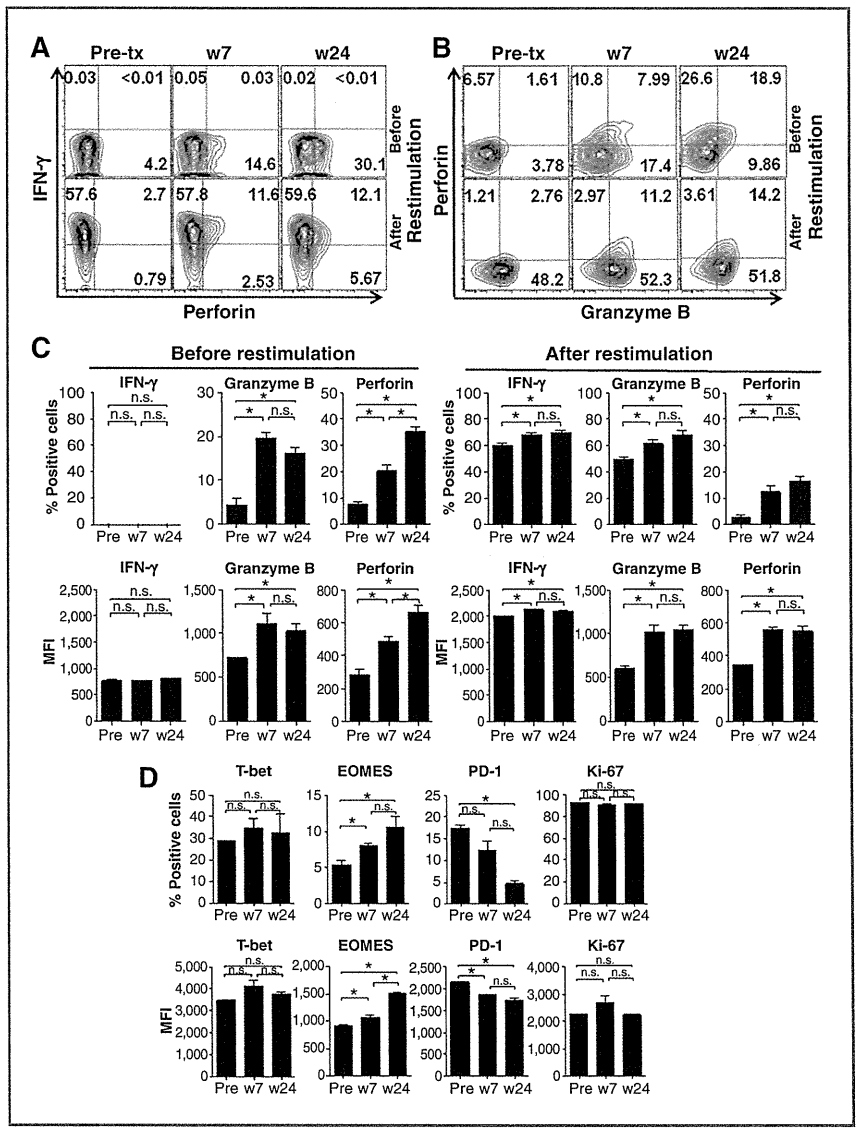

Figure 3. NY-ESO-1 antigen-specific cytotoxic CD4+ T-cell responses were enhanced during ipilimumab treatment in patient 09-079-17. Purified CD4+ T cells from patient 09-079-17 before and after treatment (tx) with ipilimumab at weeks 7 (w 7) and 24 (w 24) were cultured for 20 days after presensitization with NY-ESO-1 overlapping peptides. NY-ESO-$1_{131-150}$ epitope-specific CD4+ T cells from each time point were isolated by CD40L sorting after 6-hour *in vitro* restimulation with NY-ESO-$1_{131-150}$ peptide and expanded. A and B, IFN-γ, perforin, and Granzyme B expression of these cell lines were tested after 6-hour restimulation with autologous APCs (LCLs) pulsed with NY-ESO-$1_{131-150}$ peptide (10 nmol/L) or without restimulation in ICS assay. Nonspecific staining by isotype control antibodies was less than 0.1% in all samples (data not shown). C and D, expression of cytotoxic molecules (C), transcription factors, and activation and exhaustion makers (D) in CD4+ T-cell line from patient 09-079-17 at pretreatment, at weeks 7 and 24 are shown (*, $P < 0.05$; n.s., not significant).

We next assessed whether the ipilimumab-related changes in expression of lytic markers described above in the NY-ESO-$1_{131-150}$–specific CD4+ T-cell lines from patient 09-079-17 affected their ability to lyse autologous LCL pulsed with NY-ESO-$1_{131-150}$ peptides (100 nmol/L) at 5:1 and 25:1 E/T ratio. The degree of cytotoxicity (%) was significantly increased after ipilimumab treatment (peak at week 7), when compared with that of cells obtained pretreatment (*, $P < 0.05$). This cytotoxicity was inhibited with the use of an MHC class II blocking antibody on target cells (Fig. 4).

Discussion

In this study, we conducted in-depth immune monitoring on four NY-ESO-1–seropositive melanoma patients treated with ipilimumab, based upon the availability of suitable PBMCs. We found that NY-ESO-1 antigen-specific T_H1 responses against multiple epitopes were induced or expanded after ipilimumab treatment in all 4 patients. NY-ESO-1 antigen-specific CD4+ T-cell lines established from all 4 patients were of high avidity and recognized naturally processed NY-ESO-1 protein in APCs, as indicated by the production of perforin, granzyme B, and the upregulation of CD107a. Finally, we showed that one of these NY-ESO-1 antigen-specific CD4+ T-cell lines directly lysed autologous melanoma cells in an MHC class II-restricted manner. We believe this is the first demonstration of the induction or enhancement of tumor-reactive cytotoxic CD4+ T cells after ipilimumab treatment and is therefore a novel property of CTLA-4 blockade.

Published OnlineFirst August 12, 2013; DOI: 10.1158/2326-6066.CIR-13-0068

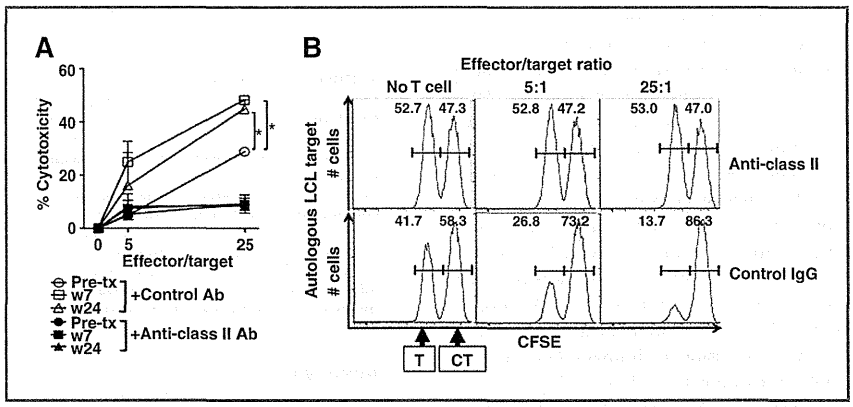

Figure 4. Enhancement of cytotoxicity of NY-ESO-1–specific CD4$^+$ T-cell lines during ipilimumab treatment in patient 09-079-17. The NY-ESO-$1_{131-150}$–specific CD4$^+$ T-cell lines from patient 09-079-17 were cocultured for 6 hours at different ratio with target cells (labeled with 0.5 μmol/L CFSE) and control target cells (5 μmol/L CFSE). Target cell (T): autologous APCs (LCLs) pulsed with NY-ESO-$1_{131-150}$ peptide. Control target cell (CT): autologous LCL pulsed with MAGE-A1 peptide mix as a control antigen. A, percentage of cytotoxicity was calculated with the formula described in Materials and Methods. To determine MHC class II restriction of the cytotoxicity, anti-MHC class II antibodies or control IgG was added during the assay (*, $P < 0.05$). Each experiment was repeated twice with similar results. Data are expressed as mean ± SD. B, representative histograms of NY-ESO-1–specific killing are shown at different effector/target ratio.

Induction of antigen-specific T cells against NY-ESO-1 has previously been accomplished in many ways. NY-ESO-1–specific CD8$^+$ T cells could be induced in HLA-A2$^+$ patients with cancer vaccinated with NY-ESO-1 peptides p157–165/p157–167 (31). These T cells were highly reactive with the peptides used for vaccination, but only rarely recognized HLA-matched, NY-ESO-1–expressing tumor cell lines. Vaccine-induced antigen-specific T cells were heterogeneous in functional activity, especially in terms of natural tumor recognition. The frequency of antigen-specific T cells does not always equate with functional tumor reactivity. Therefore, precise and multiparametric immune monitoring assays are critical to identify the proportion of tumor-reactive T cells within the population of vaccine-induced antigen-specific effector cells. An NY-ESO-1 helper peptide vaccine has been reported to induce NY-ESO-1 antigen-specific CD4$^+$ T cells with lower avidity (>1 μmol/L) compared with NY-ESO-1–specific preexisting naïve CD4$^+$ CD25$^-$ T-cell precursors or spontaneously induced CD4$^+$ T-cell effectors in NY-ESO-1–seropositive cancer patients. These cells were only able to recognize NY-ESO-1 helper peptide, but not naturally processed NY-ESO-1 protein in APCs from patients with NY-ESO-1–expressing epithelial ovarian cancer (32). Our analysis showed that most of the NY-ESO-1–specific CD4$^+$ T-cell lines generated after ipilimumab treatment were of high avidity and recognized naturally processed NY-ESO-1 protein in APCs in specimens of the 4 patients analyzed, distinguishing these results from those of exogenous vaccination strategies. Our observation in this study was based upon the results collected from either *in vitro* culture of CD4$^+$ T cells or CD40L sorted NY-ESO-1–specific CD4$^+$ T-cell lines. Because of the limited PBMC availability and low frequency of NY-ESO-1 antigen-specific CD4$^+$ T cells in peripheral blood from these patients, we were not able to characterize directly these NY-ESO-1 antigen-specific CD4$^+$ T cells in *ex vivo* assays.

The optimal T-cell receptor (TCR) affinity threshold for natural tumor recognition and maximal antitumor T-cell response has been measured using human CD8$^+$ T cells transduced with TCR variants (33). Thus, the relative lack of high affinity/avidity TCRs for CD8$^+$ T cells is considered to be one reason why immune responses toward self-tumor antigens have not been protective. It is possible that the cytotoxic capacity of CD4$^+$ T cells might be subject to an optimal TCR affinity threshold as well. In this study, NY-ESO-1–specific CD4$^+$ T-cell lines were directly sorted from bulk CD4$^+$ T-cell cultures after one round of *in vitro* presensitization. Therefore, these CD4$^+$ T-cell lines, not "clones," reflect a spectrum of the NY-ESO-1–specific CD4$^+$ T-cell population in the immune repertoire induced after ipilimumab treatment. The clinical activity of CTLA-4 blockade may mechanistically involve expansion of naturally occurring antigen-specific T cells (NY-ESO-1 cells, as an example) with high avidity. The ability to recognize antigenic epitopes naturally processed endogenously in tumor cells and to lyse tumors are logical characteristics to expect from an effective T-cell response.

Cytotoxic effector cells kill tumor cells through FAS, TRAIL, or granzyme-perforin-dependent mechanisms (34). The T-box transcription factor Eomes is critical for inducing the expression of granzyme B and other lytic granules (30). Dual costimulation of OX40 plus 4-1BB was shown to induce Eomes in HA-specific CD4$^+$ T cells toward cytotoxic T_H1 differentiation (35). Recently our group showed that OX40 agonist antibody in combination with cyclophosphamide treatment and adoptive transfer of tumor-specific CD4$^+$ T cells could produce cytotoxic CD4$^+$ T cells through Eomes and T-bet (36). We analyzed Eomes and T-bet by flow cytometric staining and found higher expression of Eomes on NY-ESO-1 antigen-specific CD4$^+$ T cells after ipilimumab