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Th1/Th2 CD4+ T cell responses against NY-ESO-1 in HLA-DPB1*0401/0402 patients with epithelial ovarian cancer

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The NY-ESO-1 antigen is expressed in a significant proportion of patients with epithelial ovarian cancer (EOC) and appears to be an ideal target for immunotherapy. In order to elucidate the nature of the HLA-DPB1*0401/0402 (DP4+)-restricted CD4+ immune response in patients with NY-ESO-1-expressing EOC, peripheral blood CD4+ T cells from HLA-DP4+ patients were stimulated with the NY-ESO-1 epitope 157-170 and tested for the release of type 1 (IFN-gamma) and type 2 (IL-5) cytokines in enzyme-linked immunospot assays. Of 14 DP4+ EOC patients who tested seronegative for NY-ESO-1, 3 patients had a detectable CD4+ T cell response to NY-ESO-1 epitope 157-170 by IFN-gamma ELISPOT assay. Six of 10 DP4+ EOC patients with serum antibodies to NY-ESO-1 had CD4+ T cell responses to NY-ESO-1 epitope 157-170 by IFN-gamma assay. Six patients had mixed Th1/Th2 CD4+ T cell responses to NY-ESO-1 epitope 157-170 regardless of their antibody response to NY-ESO-1. Four EOC patients had Th1 cells expressing IFN-gamma, but not IL-5. This suggests that the NY-ESO-1 epitope 157-170 stimulates both Th1 and Th2 type CD4+ T cell responses in EOC patients. These data suggest the NY-ESO-1 epitope 157-170 has a key role in the induction of cellular and humoral immune responses against NY-ESO-1-expressing EOC tumors. Our study supports the relevance of cancer vaccine trials with the NY-ESO-1 epitope 157-170 in HLA-DP4+ EOC patients with NY-ESO-1-expressing tumors and strategies to improve Th1-dominated tumor-reactive CD4+ T cell bias.

Introduction

The NY-ESO-1 antigen, initially defined by serological analysis of recombinant cDNA expression libraries (SEREX) in esophageal cancer (1), is particularly immunogenic, eliciting both cellular and humoral immune responses in a high proportion of patients with advanced NY-ESO-1-expressing tumors (2, 3). Subsequently, NY-ESO-1 was shown to encode several class I-restricted peptides capable of stimulating CD8+ T cells (4, 5, 6). In addition, there are reports of multiple NY-ESO-1-derived epitopes presented by HLA-DRB1*0401, HLA-DRB4*0101, and HLA-DP4 capable of stimulating CD4+ T cells (7, 8, 9, 10, 11). These MHC class II epitopes are particularly important because there is increasing evidence from both human and animal studies that CD4+ T cells are required for the generation and maintenance of a cytolytic CD8+ antitumor

immune response (12). In a previous study, we showed that high titers of NY-ESO-1 antibodies were present in a large proportion of patients with EOC, and the majority of antibody-positive patients demonstrated unusually favorable clinical courses (13). These findings suggest that antigen-specific CD4+ T cell responses might also be found in these patients. Understanding the behavior of antigen-specific T cells in EOC could provide us with crucial information toward the rational development of more efficient T cell-directed immunotherapy.

The NY-ESO-1 peptide presented by HLA-DP4, a dominant MHC class II allele expressed in 43-70% of Caucasians, was recently identified by Zeng et al. (11). This peptide, ESO p157-170, containing the core region of the DP4-restricted T cell epitope, was recognized by both CD4+ T cells and HLA-A2-restricted CD8+ T cells (11, 14). Since the ESO p157-170 epitope is a potential candidate for cancer vaccines aimed at generating both CD4+ and CD8+ T cell responses, it is important to understand the precise nature of the antitumor CD4+ T cell responses to this epitope. CD4+ Th cells can be divided into Th1 and Th2 cells, producing type 1 (IFN-gamma, TNF-alpha, and IL-2) and type 2 (IL-4, IL-5, and IL-13) cytokines, respectively. Th1 cells are involved in the induction of cellular immunity, whereas Th2 cells stimulate humoral immunity (15, 16). The objectives of the present study were (i) to determine the frequency of CD4+ T cell responses to NY-ESO-1 in a series of HLA-DP4+ patients with NY-ESO-1-expressing EOC; (ii) to examine the Th1 and Th2 profiles of the antigen-specific CD4+ T cells; and (iii) to determine the relationship between CD4+ T cells and humoral immune responses to NY-ESO-1. Our results demonstrate that rather than simply providing help for CD8+ T cells, NY-ESO-1 spontaneously induces HLA-DP4-restricted CD4+ Th1 and Th2 responses in a significant proportion of patients with EOC. These findings have implications for effective vaccine strategies that can simultaneously marshal cooperative Th1 and Th2 effector mechanisms, leading to efficient tumor killing in EOC.

Results

Study population and tumor expression of *NY-ESO-1/LAGE-1*

A total of 26 HLA-DP4+ patients with cancer (24 with EOC and 2 with other cancers) whose tumors express *NY-ESO-1* by RT-PCR and/or immunohistochemistry (IHC) were included in this study (Table 1). The tumors from four of the patients (patients 7, 11, 21, and 25) were negative by IHC and RT-PCR for *NY-ESO-1*, but positive for *LAGE-1* by RT-PCR. Since *LAGE-1* shows 94% identity to *NY-ESO-1* (17), these four patients were considered along with those with *NY-ESO-1*-expressing tumors. Two HLA-DP4-positive healthy donors without EOC and two additional HLA-DP4-negative patients with EOC whose tumors expressed *NY-ESO-1* by RT-PCR and/or IHC were also included. The patients with schwannoma (patient 27) and melanoma (patient 28) were HLA-DP4+, and their tumors expressed *NY-ESO-1*. The median age of the EOC study population was 59 yr (range, 21-91 yr), and the median duration of follow-up was 28 mo (range, 2-227 mo). As expected, the majority of patients presented with stage IIIc disease (22/24, 92%) and with serous histology (22/24, 92%). All patients were treated uniformly with cytoreductive surgery followed by adjuvant platinum-based chemotherapy. A complete response to the platinum-based therapy was achieved in 18 of the patients (75%), and a partial response was achieved in 5 patients (21%). The median disease-free survival time for all the patients was 26 mo [95% confidence interval (CI), 3-49 months]. The median overall survival time could not be calculated because the number of events in these patients was insufficient.

Antibody response to NY-ESO-1/LAGE-1 in ovarian cancer patients

Serum samples from the 26 patients were analyzed by ELISA for NY-ESO-1/LAGE-1 antibodies. These sera consisted of preoperative and serial specimens obtained during the patients' courses of disease (range, 1-3 yr). Because of the high degree of homology between the NY-ESO-1 and LAGE-1 antigens, the serological assay does not reliably distinguish between them in human sera. Therefore, the antibody responses to both antigens are considered together. Among the 24 HLA-DP4+ EOC patients with NY-ESO-1-expressing tumors, 10 (42%) had evidence of spontaneous antibody response. The remaining 14 patients had no antibody response to NY-ESO-1. The two HLA-DP4-negative EOC patients and the two patients with other cancers (schwannoma and melanoma) with NY-ESO-1-expressing tumors also had a demonstrable antibody response. There was no detectable antibody response in the two healthy donors (Table 1).

Peptide-specific IFN-gamma and IL-5 release by CD4+ T cells in healthy donors

The HLA-DP4-restricted NY-ESO-1 peptide p157-170 (DP4 peptide) was not recognized by freshly isolated CD4+ T cells obtained from normal HLA-DPB1*0401/0402+ donors in both IFN-gamma and IL-5 ELISPOT assays (Figures 1 and 2, Table 1). For all experiments, influenza nucleoprotein 206-229 peptide, previously shown to be immunogenic in the majority of individuals (18), was used as a positive control. A patient was considered to have CD4+ T cell response only if the IFN-gamma or IL-5 spots exceeded the spots from negative controls (irrelevant peptide) by at least 30 cells per 50,000 and there was a ratio of IFN-gamma or IL-5 spots to negative controls greater than 2 (19).

Peptide-specific IFN-gamma release by CD4+ T cells in HLA-DP4+ ovarian cancer patients

IFN-gamma ELISPOT assay was used to discern the Th1 functional bias of patient-derived CD4+ T cells against NY-ESO-1 DP4 peptide. The frequencies of CD4+ T cell responders against the DP4 peptide were evaluated in the 26 HLA-DP4+ cancer patients (including 24 EOC, 1 schwannoma, and 1 melanoma whose tumors expressed NY-ESO-1) (Table 1). The results indicate that 7/12 (58%) of HLA-DP4+ cancer patients with serum antibodies to NY-ESO-1 (patients 1, 2, 3, 4, 18, 25, and 28) had CD4+ T cell responses to NY-ESO-1 epitope 157-170 by IFN-gamma ELISPOT (Figure 1). In contrast, only 3/14 (21%) HLA-DP4+, NY-ESO-1 antibody-negative patients (patients 7, 23, and 24) demonstrated a CD4+ T cell response by IFN-gamma ELISPOT. The two HLA-DP4-negative and NY-ESO-1 antibody-positive EOC patients demonstrated no CD4+ T cell responsiveness by IFN-gamma ELISPOT (Table 1). The specificity of the IFN-gamma ELISPOT assay was confirmed by demonstrating that CD4+ T cells that were reactive against influenza nucleoprotein peptide 206-229 from patients did not produce IFN-gamma in response to the irrelevant DP4 157-170 peptide. Similarly, CD4+ T cells presensitized with the DP4 175-170 peptide specifically produced IFN-gamma in response to 157-170, but not to irrelevant nucleoprotein 206-229 peptide.

Peptide-specific IL-5 release by CD4+ T cells in HLA-DP4+ ovarian cancer patients

In order to evaluate Th2 CD4+ T cell responses against the DP4 peptide, we evaluated two mAbs directed against different determinants of human IL-5. CD4+ T cells from patient 1 were presensitized with NY-ESO-1 p157-170 and tested by IL-5 ELISPOT against autologous T cell APCs pulsed with peptides ESO 157-170 or NP 206-229 (negative control). The results shown are representative of at least two separate reproducible ELISPOT assays, with the control values given in parentheses (Table 2). The optimal Ab dilutions were determined to be 5 μg/ml for the capture Ab and 0.5 μg/ml for the detection Ab. These produced the highest number of ESO 157-170-specific spots, with relatively low numbers of background spots against NP 206-229 when ESO 157-170-specific CD4+ T effector cells were tested.

Two different substrates, AEC (3-amino-9-ethylcarbazole) for peroxidase and BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) for alkaline phosphatase, were compared for color development in IL-5 ELISPOT assays. Whereas red color spots obtained with the AEC substrate were typically pale and difficult to distinguish from the background, those observed when BCIP/NBT was used as the substrate had a strong purple color and were well defined (data not shown). Thus, BCIP/NBT, which allowed for more accurate counting of spots by image analysis, was selected for subsequent experiments. The IL-5 ELISPOT assay required a more prolonged incubation time than the IFN-gamma ELISPOT assay, as IL-5 activity could only be detected after the incubation time was extended to 40 h (data not shown).

The specificity of the IL-5 ELISPOT assay was also assessed by testing CD4+ T cell reactivity against the presensitizing DP4 peptide as compared to reactivity against unrelated peptides. As shown in Figure 3, CD4+ T cells from patient 1, presensitized with NP 206-299, secreted IL-5 against influenza nucleoprotein peptide 206-299, with low background when tested against irrelevant NY-ESO-1 DP4 peptide 157-170. Similarly, CD4+ T

Table 1
HLA-DPB1*0401/0402-positive patients with epithelial ovarian cancer evaluated in this study

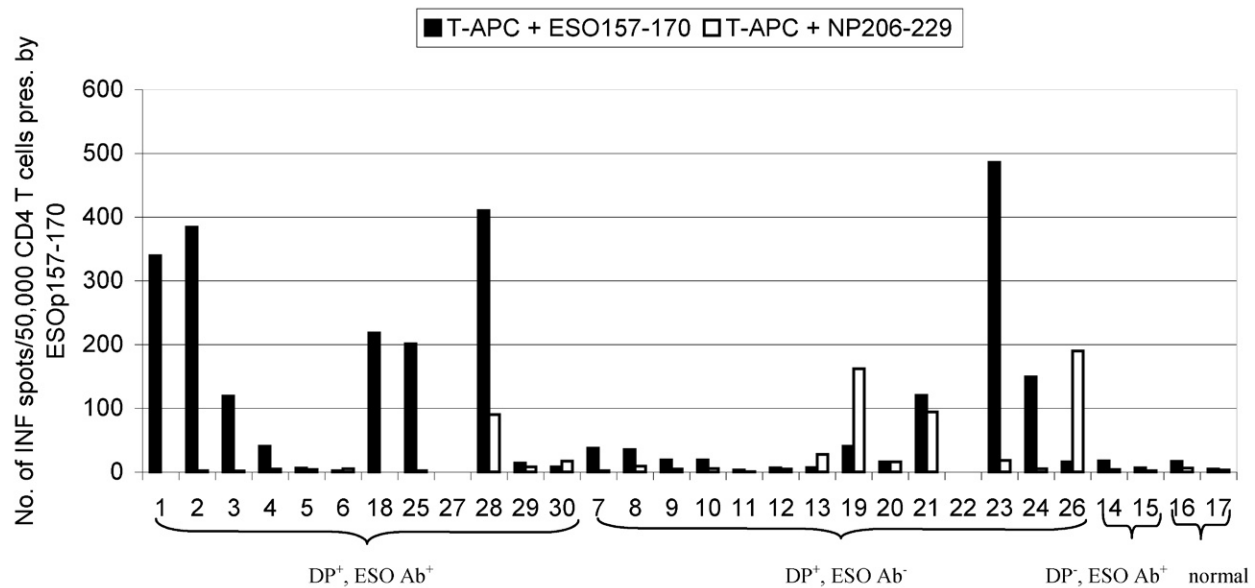| Patients				Clinical			NY-ESO-1			ELISPOT assays[c]	
No.[a]	Age	Stage	Response	Recurrence	Follow-up (mo)	Status[b]	IHC	RT-PCR	ELISA	IFN-γ	IL-5
NY-ESO-1 Ab-positive/HLA-DP4-positive patients											
1	75	IV	PR	persistent disease	40	AWD	+++	+	+	+	+
2	60	IIIc	CR	yes	45	AWD	+	+	+	+	+
3	66	IIIc	CR	yes	40	DOD	+++	+	+	+	-
4	58	IIIc	CR	no	47	ANED	-	+	+	+	+
5	58	IIIc	CR	no	51	ANED	+++	NA	+	-	-
6	43	IIIc	CR	no	13	ANED	+	-	+	-	-
18	75	III	CR	no	6	ANED	++	+	+	+	-
25	91	IIIc	PR	yes	7	AWD	NA	-/+[d]	+	+	+
27	39	NA	CR	yes	18	DOD	+	NA	+	-	-
28	39	3	PR	yes	39	AWD	+	NA	+	+	+
29	76	IIIc	CR	yes	99	AWD	+	NA	+	-	-
30	21	IIIb	PR	yes	18	DOD	+	NA	+	-	-
NY-ESO-1 Ab-negative/HLA-DP4-positive patients											
7	47	IIIc	NA	NA	11	ANED	-	-/+[d]	-	+	-
8	51	IIIc	PR	persistent disease	51	ANED	+++	NA	-	-	-
9	53	IV	CR	yes	36	AWD	+	-	-	-	-
10	52	IIIc	CR	yes	26	AWD	+	-	-	-	-
11	62	IIIc	CR	no	9	ANED	-	-/+[d]	-	-	-
12	75	IIIc	CR	yes	74	ANED	+	+	-	-	-
13	70	IIIc	CR	yes	37	AWD	+	-	-	-	-
19	75	IIIc	CR	no	78	ANED	+	NA	-	-	-
20	73	III	CR	yes	15	AWD	-	+	-	-	-
21	41	IIIc	CR	yes	13	ANED	-	-/+[d]	-	-	-
22	51	IIIc	NA	NA	3	AWD	+	+	-	-	-
23	48	IIIc	CR	yes	106	ANED	++	+	-	+	+
24	79	IIIc	CR	NA	8	ANED	+++	-	-	+	-
26	57	IIIc	NA	NA	5	AWD	NA	+	-	-	-
NY-ESO-1 Ab-positive/HLA-DP4-negative patients											
14	72	II	CR	no	3	AWD	++++	-	+	-	-
15	66	IIIc	CR	yes	33	ANED	+++	NA	+	-	-
Healthy donors											
16	NA	NA	NA	NA	NA	NA	NA	NA	NA	-	-
17	NA	NA	NA	NA	NA	NA	NA	NA	NA	-	-

[a] All patients had EOC, except for 27 (schwannoma), 28 (melanoma), and 16 and 17 (healthy donors).
[b] Abbreviations: AWD, alive with disease; ANED, alive with no evidence of disease; DOD, die of disease; NA, not available.
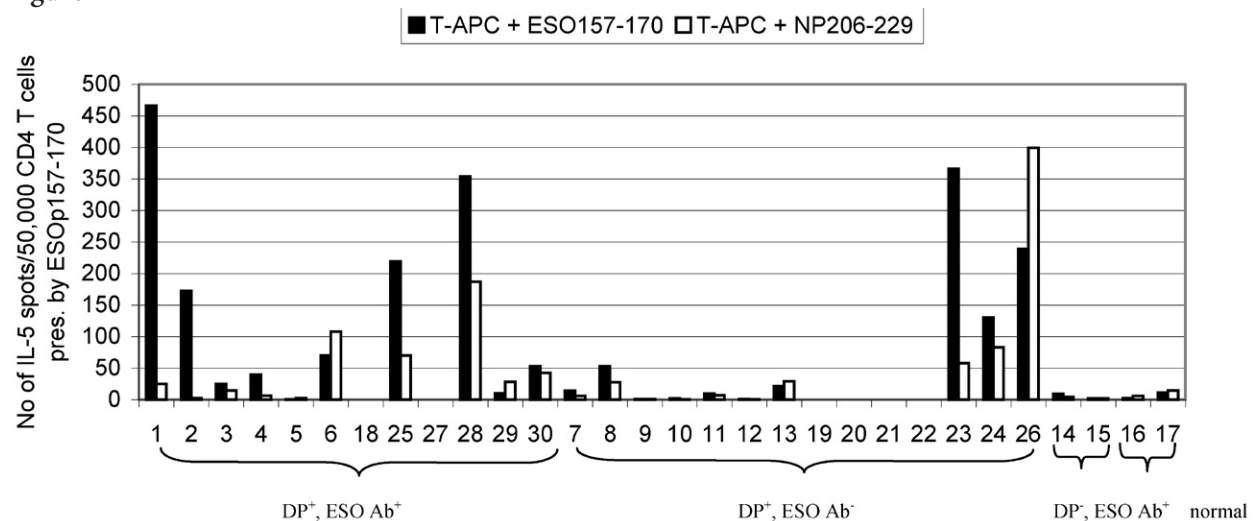[c] Results are representative of at least two separate reproducible ELISPOT assays.
[d] These patients were *NY-ESO-1* negative and *LAGE-1* positive by RT-PCR.

Figure 1

Analysis of the Th1 type CD4+ T cell response to NY-ESO-1 p157-170 in HLA- DPB1*0401/0402 patients with EOC. CD4+ T cells were presensitized with ESO p157-170 peptide and tested by IFN-gamma ELISPOT on d25 against autologous T cell APCs pulsed with peptides NP 206-229 or ESO 157-170

Figure 2

Analysis of the Th2 type CD4+ T cell response to NY-ESO-1 p157-170 in HLA- DPB1*0401/0402 patients with EOC. CD4+ T cells were presensitized with ESO p157-170 peptide and tested by IL-5 ELISPOT at d25 against autologous T cell APCs pulsed with peptides NP 206-229 or ESO 157-170. The results represent the mean number of spots per 50,000 effector CD4+ T cells in duplicate wells

cells from patient 1, presensitized with NY-ESO-1 DP4 peptide 157-170, specifically produced IL-5 in response to 157-170 but not to irrelevant control peptide nucleoprotein 206-229 (Figure 3).

Overall, 6/26 HLA-DP4+ cancer patients with NY-ESO-1-expressing tumors demonstrated CD4+ responses by IL-5 ELISPOT. These included 5/7 patients with demonstrable humoral immunity and CD4+ T cell responses to NY-ESO-1 epitope 157-170 by IFN-gamma assay and 1/3 patients without antibody response but with CD4+ T cells response by IFN-gamma assay, indicating a mixed Th1/Th2 response in these patients. Thus, patients 3, 18, 7, and 24 had Th1 cells expressing IFN-gamma but not IL-5. Also of interest, patients 5, 6, 27, 29, and 30 appeared negative by both IFN-gamma and IL-5 despite an antibody response to NY-ESO-1, suggesting a non-DP4-restricted immune response in these patients (Table 1, Figure 2).

Table 2

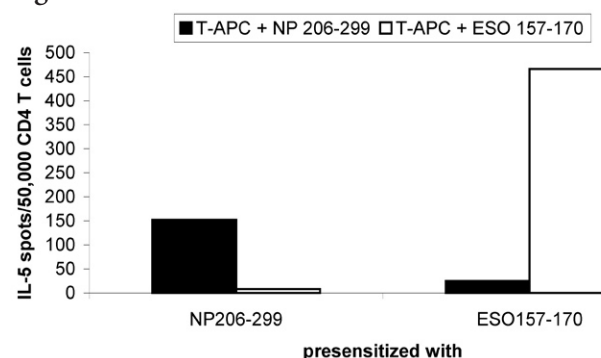**Titration of the capture and detection Abs for use in IL-5 ELISPOT assays**

Detection Ab (µg/ml)	Spots/50,000 CD4+ cells plated per well (control)			
	Capture Ab (µg/ml)			
	0.63	1.25	2.5	5
0.25	7 (1)	47 (22)	344 (30)	307 (21)
0.5	7 (2)	222 (24)	397 (31)	466 (25)
1	117 (19)	256 (19)	384 (43)	357 (35)
2	42 (16)	183 (7)	270 (34)	373 (21)

Figure 3

Specificity controls of CD4+ T cell presensitization from patient 1 in IL-5 ELISPOT assays. CD4+ T cells were presensitized with the indicated peptides and tested by IL-5 ELISPOT at d25 against autologous T cell APCs pulsed with peptides NP 206-229 or ESO 157-170. The results represent the mean number of spots per 50,000 effector CD4+ T cells in duplicate wells.

Humoral immune response, Th1/Th2 CD4+ T cell response to NY-ESO-1 and clinical course of EOC

The median disease-free survival time of the NY-ESO-1 Ab-negative HLA-DP4+ patients was 20 mo (CI, 6-34 mo), while the median disease-free survival time of the NY-ESO-1 Ab-positive HLA-DP4+ patients was 26 mo (CI, 0-57 mo; P = not significant). Similarly, patients with demonstrable CD4+ T cell responses by IFN-gamma ELISPOT had a median disease-free survival time of 34 mo (CI, 0-76 mo), compared with 20 mo (CI, 3-37 mo) for those without a demonstrable IFN-gamma ELISPOT response. This did not reach statistical significance. Finally, when patients with mixed Th1/Th2 responses (n = 5) were compared with patients with only Th1 responses (n = 4), the median disease-free survival times could not be reliably computed because of the small numbers and because comparisons of the curves were not statistically significant.

Discussion

There is increasing evidence that the immune system has the ability to recognize tumor-associated antigens displayed on human malignancies and to direct cytotoxic responses to these targets. In EOC, several studies support the notion that TILs may represent an active immune response of the host specifically directed at the tumor (20, 21, 22). The need to understand the nature of this antitumor immune response in EOC has become even more compelling because of recent evidence demonstrating that the frequency of tumor-infiltrating CD8+ T cells correlates with improved clinical outcome (23). While CD8+ T cells represent the major effector arm of the antitumor immune response, a growing body of evidence suggests that CD4+ T cells play a pivotal role in orchestrating these responses (24, 25, 26). In the current study, we have analyzed the functional nature of the CD4+ T cell response to a unique NY-ESO-1 epitope, ESO 157-170, with dual MHC class I (HLA-A2) and class II (HLA-DP4) specificities. We observed evidence of a NY-ESO-1-specific CD4+ T cell response in a subset of patients, predominantly those with a simultaneous humoral immune response. The short, *in vitro* peptide stimulation step, as well as the absence of NY-ESO-1-specific T cells in healthy donors, strongly suggests that NY-ESO-1-specific CD4+ T cells in EOC patients have been

primed *in vivo*. Intracellular staining showed that IFN-gamma positive cells were CD4+ T cells, and no IFN-gamma positive CD8+ T cells were detected by flow cytometric analysis (27).

Most approaches to the study of CD4+ T cells have relied on the generation of T cell lines after multiple peptide stimulations, which are typically assessed for bulk cytokine secretion or proliferation (10, 11, 12, 14). This lack of reliable and quantitative approaches for analyzing CD4+ T cells at the single-cell level has hindered the detailed analysis of tumor antigen-specific immune responses. Using a recently developed general strategy for monitoring CD4+ responses to NY-ESO-1 in cancer patients (27, 28), we showed that 0/18 patients who tested seronegative for NY-ESO-1 had detectable CD4+ T cell responses. On the contrary, 11/13 cancer patients with serum antibodies to NY-ESO-1 had polyclonal CD4+ T cell responses directed against various known and previously undescribed NY-ESO-1 epitopes. In the present study of ovarian cancer patients, we have again shown that CD4+ reactivity was tightly associated with the NY-ESO-1 antibody status of patients. Taken together with an earlier study by Jäger *et al.* (29) showing that humoral responses to NY-ESO-1 correlated with the presence of NY-ESO-1-specific CD8+ T cells, our data supports the notion that NY-ESO-1 is capable of inducing integrated humoral, CD4+ and CD8+ adaptive immune responses in a subset of patients with NY-ESO-1-expressing tumors.

Among the EOC patients with demonstrable CD4+ T cell responses to NY-ESO-1, we observed mixed Th1/Th2 responses in 5/9 (56.6%) patients. Previous reports on whether or not NY-ESO-1 could induce Th2 type CD4+ T cell responses are conflicting. For example, Zarour *et al.* (9) demonstrated the ability of the promiscuous HLA-DR epitope NY-ESO-1 119-143 peptide to stimulate both Th1 and Th2 type CD4+ T cells. In contrast, another report found no evidence of CD4+ Th2 immune responses to NY-ESO-1 (28). The lack of consistency in these studies might be related to the type and specificity of the Th2 cytokine assay utilized. In the current study, we adapted our previously described approach for CD4+ T cell analysis (27, 28) and made slight modifications for Th2 cytokine analysis. We had previously shown that the presensitization method used here reflects the *in vivo* nature of specific CD4+ T cells (27, 28). In our study, the CD4+ T cell cultures were subjected to strict Th1 or

Th2 culture conditions for IFN-gamma ELISPOT assay. The Th2 culture milieu completely eradicated IFN-gamma production. In addition, the expansion of T cells in a Th1 milieu enhanced the response of these cells in an IFN-gamma ELISPOT read-out assay. Furthermore, the mixture of CD4-/CD8- cells (B cells and monocytes) that we used as APCs are typically poor IL-12 producers and are likely to be Th1/Th2 neutral for the expansion of CD4+ T cells. Our results demonstrate that the IL-5 ELISPOT assay could be carried out with virtually no background, in a quantitative and reproducible manner.

Since NY-ESO-1 elicits both cellular and humoral immune responses that can be detected simultaneously in cancer patients, it is reasonable to expect that the function of CD4+ T cells be polarized toward a Th1 or Th2 type of immune response by different cytokine profiles in the tumor environment. In addition, the frequency and activity of macrophages, DCs, and B lymphocytes functioning as potential APCs in tumor tissues may affect the type of Th response (30). Both Th1 and Th2 cell types have been shown to play an antitumor role *in vivo*. Using adoptive transfer of either Th1 or Th2 T cells from OVA-specific T cell receptor-transgenic mice, Th1 cells have been shown to induce a marked lymphocyte infiltration into the tumor mass and to eradicate the tumor mass via cellular immunity (31). In contrast, Th2 cells induce inflammatory responses at the tumor site and induce tumor necrosis (32). Furthermore, in another report, the administration of Th1 and Th2 cell clones to a tumor antigen (P815 AB) induced tumor rejection, with quantitative rather than qualitative differences in the antitumor activities of both types of cells (32). The rejection of the P815 tumors following adoptive transfer with either type of clone (Th1 or Th2) induced a strong immunological memory. These results would indirectly suggest that both Th1 and Th2 responses directed against NY-ESO-1-derived epitopes could be important in eliciting antitumor responses in vivo. For example, the Th2 cytokines IL-4 and IL-13 could markedly enhance the secretion of IL-12 by macrophages and DCs (33), which in turn would drive the differentiation of naive T cells toward a Th1 phenotype. Although the sample size is relatively small and there were few failures, our clinical data provide indirect support for this observation since there were no significant differences in disease-free and overall survival times when patients with mixed Th1/Th2 responses were compared with patients with only Th1 responses. Clearly, more extensive longitudinal studies will be required to determine the prognostic significance of differential Th1 versus Th2 type responses in the progression, regression, and/or recurrence of EOC.

We have also demonstrated Th1 type CD4+ T cell responses to NY-ESO-1 in three patients with EOC who had no evidence of antibody response. Although the demonstration of CD4+ T cell reactivity to NY-ESO-1 in the absence of humoral immune response is likely to be uncommon (28), our results and those of Rodolfo *et al.* (34) indicate that this may occur in a subset of patients. In the latter report, NY-ESO-1-specific CD4+ T cell response was detected and maintained for a long period of time in the absence of antibody response and tumor burden in patients with neuroblastoma.

In general, Th2 polarization of CD4+ T cells has been shown to be related to disease progression in some human (35) and animal studies (36). In our study, we found evidence of Th2 type CD4+ T responses only in the presence of Th1 response, and no patient demonstrated an exclusive Th2 type CD4+ T cell response. While it is possible that the IFN-gamma and IL-5 secreting CD4+ T cells are T-regulatory 1 cells that may be involved in peripheral tolerance to tumor challenge (37), the fact that the patients in our study did not demonstrate a worse prognosis

would suggest that the NY-ESO-1 DP4 peptide-induced Th2 type tumor-specific CD4+ T cells may be considered productive collaborators with Th1 type CD4+ T cells in mediating antitumor immunity. Given the role of collaborative Th1 and Th2 CD4+ T cells in maintaining CD8+ T cell responses and the potential direct antitumor effector function of these cells (31), the NY-ESO-1 DP4 peptide 157-170 is a reasonable vaccine candidate for the *in vivo* induction of both tumor-reactive CD4+ and CD8+ T cells in patients with EOC.

Abbreviations

CI, confidence interval; EOC, epithelial ovarian cancer; IHC, immunohistochemistry

Acknowledgements

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Materials and methods

Patients and specimens

Flash-frozen tissue specimens were obtained from patients undergoing debulking surgery for EOC at the Roswell Park Cancer Institute, Buffalo, NY, USA. All tissue specimens were collected under a protocol approved by the Institutional Review Board. All pathology specimens were reviewed in our institution, and tumors were classified according to World Health Organization (WHO) criteria (38). For a subset of the patients, serum samples were available over extended periods of time during the course of disease. The medical records of the patients were also reviewed retrospectively under an approved Institutional Review Board protocol. The review included outpatient and in-patient treatment, including surgery and chemotherapy. Study outcomes included overall survival time and time to progression, each measured from the time of definitive surgery. Progression was defined as objective evidence of recurrence, since all therapy was given in the adjuvant setting. The duration of overall survival was the interval between surgery and death. Observation time was the interval between surgery and last contact (death or last follow-up). Data were censored at the last follow-up for patients with no evidence of recurrence, progression, or death. Patients with persistent disease were excluded from the time to progression analyses.

Total tissue RNA isolation

Total tissue RNA was isolated from frozen tumor tissues using the TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA) according to the manufacturer's protocol. Potentially contaminating DNA was removed by treating with RNase-free DNase I (Boehringer-Mannheim, Mannheim, Germany). After phenol treatment and drying, RNA was dissolved in RNase-free H_2O. The resulting RNA concentration was measured spectrophotometrically (GeneQuant, Amersham Pharmacia Biotech, Ltd., Cambridge, UK), and the quality of the RNA was checked by electrophoresis on a 1% agarose gel.

Tumor expression of *NY-ESO-1* by RT-PCR and IHC

RT-PCR for NY-ESO-1 was performed using the primers ESO1A (5'-CACACAGGATCCATGGATGCTGCAGATGCGG-3') and ESO1B (5'-CACACAAAGCTTGGCTTAGCGCCTCTGCCCTG-3') as previously described (13.). In addition, we examined the expression of *LAGE-1*, another CT antigen with 94% homology to *NY-ESO-1* (17). The *LAGE-1* primers were LAGE1A (5'-CTGCGCAGGATGGAAGGTGCCCC-3) and LAGE-1B(5'-CACACAAAGCTTGGCTTAGCGCCTCTGCCCTG-3'). Amplification for both gene products was 1 min at 94°C, 1 min at 60°C, and 1.5 min at 72°C for 35 cycles. These cycles were followed by a 10-min elongation step at 72°C. Testicular tissue was used as a positive control. The PCR products were 341 bp and 338 bp for *NY-ESO-1* and *LAGE-1*, respectively, and were visualized by ethidium bromide staining after separation over a 1.5% agarose gel. IHC was also performed using the mAb to NY-ESO-1 (clone ES121) as previously described (13). Thus, in some cases, both fresh and archived specimens were available, and tumor expression of *NY-ESO-1* was confirmed by RT-PCR and IHC.

ELISA

Recombinant NY-ESO-1 and LAGE-1 truncated proteins (3) at a concentration of 1 μg/ml in coating buffer (15 mM Na_2CO_3, 30 mM $NaHCO_3$, pH 9.6, with 0.02% NaN_3) was adsorbed to TC microwell plates 60 x 10 (Nunc, Roskilde, Denmark) at 10 μl/well overnight at 4°C. Plates were washed with PBS and blocked overnight at 4°C with 10 μl/well of 2% BSA in PBS. After washing, 10 μl/well of serum dilutions in 2% BSA was added and incubated for 2 h at room temperature. Plates were washed, and 10 μl/well diluted secondary antibody in 2% BSA was added (goat antihuman IgG-AP; Southern Biotechnology, Birmingham, AL, USA) and incubated for 1 h at room temperature. Plates were washed, incubated with 10 μl/well of substrate solution (Attophose substrate, JBL Scientific, San Luis Obispo, CA, USA) for 25 min at room temperature, and

immediately read (CytoFluor 2350; Millipore, Bedford, MA, USA). Sera were tested over a range of 4-fold dilutions from 1:100 to 1:100,000, as described previously (3).

Molecular typing of HLA-DP molecules

HLA-DPB typing was performed at the HLA typing laboratory of the Roswell Park Cancer Institute using sequence-specific primer pairs obtained from Genovision (39).

Peptides

The synthetic influenza A nucleoprotein peptide 206-229 (F WRGENGRKTRIAYERMCNILKGK) was obtained from Bio-Synthesis (Lewisville, TX, USA) with a purity of greater than 90%. The HLA-DP4-restricted NY-ESO-1 peptide p157-170 (SLLMWITQCFLPVF) was synthesized by Multiple Peptide Systems (San Diego, CA, USA) and was more than 95% pure, as determined by reverse-phase HPLC.

In vitro stimulation of PBMCs

PBMCs were obtained from 26 EOC patients and 2 healthy subjects with informed consent under an Institutional Review Board approved protocol at Roswell Park Cancer Institute, Buffalo, NY, USA. In addition, PBMCs were obtained from one patient with malignant schwannoma and from another patient with melanoma under an Institutional Review Board approved protocol at Medizinische Klinik, Hämatologie-Onkologie, Krankenhaus Nordwest, Frankfurt, Germany. PBMCs were collected using a Ficoll gradient and were frozen in RPMI containing 10% FCS and 10% DMSO in liquid nitrogen until use. CD4+ T lymphocytes were separated from PBMCs by positive selection using magnetic beads (Dynabeads, Dynal, Oslo, Norway) and seeded into round-bottomed 96-well plates (Corning, Inc., Corning, NY, USA) at a concentration of 5×10^5 cells per well in RPMI medium 1640 supplemented with 10% human AB serum (NABI, Boca Raton, FL, USA), L-glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 1% nonessential amino acids (complete medium). As APCs for antigen-specific presensitization, CD4-/CD8- PBMCs depleted of CD4+ and CD8+ T cells were pulsed with 10 μM peptide overnight at 37°C in 250 μl serum-free medium (X-VIVO-15™, BioWhittaker, Walkersville, Inc., MD, USA). Pulsed CD4-/CD8- APCs were then washed, irradiated, and added to plates containing CD4+ T cells at a concentration of 1×10^6 APCs per well. After 8 h, IL-2 (10 units/ml, Roche Molecular Biochemicals, Indianapolis, IN, USA) and IL-7 (20 ng/ml, R & D Systems, Minneapolis, MN, USA) were added to culture wells, and this was repeated every 3-4 d thereafter.

Generation of target cells

A fraction of CD4+ T cells from the initial separation (see previous section) was seeded into 48-well plates (Corning, Inc., Corning, NY, USA) at a concentration of 1×10^6 cells/ml in complete medium supplemented with 10 μg/ml PHA (HA15, Murex Diagnostics, Dartford, UK). Cells were fed and expanded twice a week with complete medium containing IL-2 (10 units/ml) and IL-7 (20 ng/ml). The activated T cell APCs were typically harvested and used as target cells after 20-30 d of culture. At the end of the incubation period, target cell APCs were washed twice in X-VIVO-15™ medium to remove serum and resuspended in RPMI for testing.

ELISPOT assays

The recognition of T cell APCs pulsed with peptides was assessed by ELISPOT assays specific for hu-IFN-gamma and IL-5. For the ELISPOT assays, flat-bottomed, 96-well multiscreen HA plates

(Millipore, Bedford, MA, USA) were coated with either 4 μg/ml mAb antihuman IFN-gamma (1-D1K, Mabtech, Stockholm, Sweden) or 5 μg/ml of mAb antimouse IL-5 (BD-Pharmingen, San Diego, CA, USA, suitable for use with human IL-5 ELISPOT pair) in 0.1 M NaHCO3 (pH 9.6) overnight at 4°C. After washing with RPMI, plates were blocked with 10% human AB type serum for 1 h at 37°C. CD4+ T cells presensitized with NY-ESO-1 p157-170 (effector cells) and autologous T cell APCs (target cells) pulsed with peptide ESO 157-170 or influenza A nucleoprotein (NP) peptide 206-229 (negative control) were used. Presensitized CD4+ T effector cells (5×10^4 or 1×10^4) and 1×10^5 target cells were added to each well and incubated for 20 h (IFN-gamma) or 40 h (IL-5) in RPMI medium 1640 without serum. Plates were then washed thoroughly with water containing 0.05% Tween 20 to remove cells, and biotinylated IFN-gamma mAb (0.2 μg/ml, 7-B6-1-biotin, Mabtech, Stockholm, Sweden) for IFN-gamma ELISPOT assay or biotinylated mAb antihuman IL-5 (0.5 μg/ml, Pharmingen, San Diego, CA, USA) for IL-5 ELISPOT assay was added to each well. After incubation for 2 h at 37°C, plates were washed and developed for 1 h at room temperature with avidin-peroxidase complex (diluted 1:100, Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) for the IFN-gamma ELISPOT assay or with streptavidin-alkaline phosphatase (1 μg/ml, Mabtech, Stockholm, Sweden) for the IL-5 ELISPOT assay. After washing, substrate (AEC substrate, Sigma-Aldrich, Inc., St. Louis, MO, USA, for the IFN-gamma ELISPOT assay or BCIP/NBT, Sigma, St. Louis, MO, USA, for the IL-5 ELISPOT assay) was added and incubated for 5 min. After final washes, plate membranes displayed red spots (IFN-gamma) or purple spots (IL-5) that were scanned and counted using the Zeiss ELISPOT Reader system with KS 4.3 software (Carl Zeiss, Thornwood, NY, USA). All determinations were performed in triplicate.

For both IFN-gamma and IL-5 ELISPOT assays, a patient was considered to have CD4+ T cell response only if the IFN-gamma or IL-5 spots exceeded the spots from negative controls (irrelevant peptide) by at least 30 cells per 50,000; and if there was a ratio of IFN-gamma or IL-5 spots to negative controls that was greater than 2 (19).

Statistical analysis

All statistical analyses were performed with the SPSS software (40). The distribution of humoral and CD4+ T cell responses to NY-ESO-1 and clinical outcome was analyzed by the chi-squared or Fisher's exact test as appropriate. Estimated survival distributions were calculated by the method of Kaplan and Meier (41). Survival distributions were compared using the log rank statistic.

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