Regulatory T-Cell – Mediated Attenuation of T-Cell Responses to the NY-ESO-1 ISCOMATRIX Vaccine in Patients with Advanced Malignant Melanoma

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Abstract

Purpose: NY-ESO-1 is a highly immunogenic antigen expressed in a variety of malignancies, making it an excellent target for cancer vaccination. We recently developed a vaccine consisting of full-length recombinant NY-ESO-1 protein formulated with ISCOMATRIX adjuvant, which generated strong humoral and T-cell – mediated immune responses and seemed to reduce the risk of disease relapse in patients with fully resected melanoma. This study examines the clinical and immunologic efficacy of the same vaccine in patients with advanced metastatic melanoma.

Experimental Design: Delayed-type hypersensitivity responses, circulating NY-ESO-1 – specific $CD4^+$ and $CD8^+$ Tcells, and proportions of regulatory Tcells (Treg) were assessed in patients.

Results: In contrast to patients with minimal residual disease, advanced melanoma patients showed no clinical responses to vaccination. Although strong antibody responses were mounted, the generation of delayed-type hypersensitivity responses was significantly impaired. The proportion of patients with circulating NY-ESO-1 – specific $CD4^+$ Tcells was also reduced, and although many patients had $CD8^+$ Tcells specific to a broad range of NY-ESO-1 epitopes, the majority of these responses were preexisting. Tregs were enumerated in the blood by flow cytometric detection of cells with a $CD4^+CD25^+FoxP3^+$ and $CD4^+CD25^+CD127^-$ phenotype. Patients with advanced melanoma had a significantly higher proportion of circulating Treg compared with those with minimal residual disease.

Conclusions: Our results point to a tumor-induced systemic immune suppression, showing a clear association between the stage of melanoma progression, the number of Treg in the blood, and the clinical and immunologic efficacy of the NY-ESO-1 ISCOMATRIX cancer vaccine.

The capacity of the immune system to eradicate tumors has been convincingly shown in numerous animal models (1). In response to these promising findings, many vaccines have been developed and trialed in cancer patients with the aim of evoking effective immunity against tumor-associated antigens and thereby eradicating the tumor cells that express these antigens (2). The family of cancer-testis antigens is a particularly promising target in such approaches as it is generally expressed in a wide range of malignancies but not in normal tissues, except for the germ cells of the testis and placental trophoblasts, both of which are immunologically privileged tissues (3). As a result of this highly restricted expression pattern, tolerance to cancer-testis antigens is likely to be limited and immune responses directed toward them should be highly specific for tumor cells.

Among the cancer-testis antigens, NY-ESO-1 has been the focus of our attention due to its exceptional immunogenicity and widespread distribution among many cancer types, including melanoma (4). We recently completed a phase I clinical trial using an experimental vaccine consisting of full-length recombinant NY-ESO-1 protein formulated with ISCOMATRIX adjuvant (CSL Limited), a saponin-based adjuvant that targets full-length proteins to dendritic cells for efficient presentation of both MHC class I – restricted and MHC class II – restricted epitopes (5). This vaccine was used to immunize patients with fully resected NY-ESO-1 – positive melanoma. These patients had minimal residual disease (MRD), that is, undetectable or small volume locoregional disease only, following surgical tumor resection. The vaccine was well tolerated and induced strong anti – NY-ESO-1 immunity, including high-titer antibody responses, strong delayed-type hypersensitivity (DTH) reactions, and circulating $CD4^+$ and

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Translational Relevance

Recombinant NY-ESO-1 protein administered in ISCOMATRIX adjuvant has been found to be highly immunogenic in patients with resected cancer. We show that NY-ESO-1–specific cellular immune responses were attenuated in patients with advanced melanoma when compared with those seen in a previous trial that was done in patients with fully resected disease. These included peptide-specific CD4 and CD8 T-cell responses in blood and in skin as delayed-type hypersensitivity reactions. In these patients, regulatory lymphocytes were increased in blood, reflecting a potentially more immunosuppressive environment *in vivo*. This may partially explain why such patients often respond poorly to immunotherapy. Vaccine strategies optimized for patients without bulky melanoma may have to be modified for the advanced disease setting. Enhancement of immunity by reducing tumor-induced immune suppression may allow better immunization of patients with advanced melanoma and consequently improve the prospect of clinical responses.

$CD8^+$ T cells specific for a broad range of NY-ESO-1 epitopes, including many previously unidentified epitopes (6, 7). Furthermore, although this study was not designed to assess clinical end points, patients vaccinated with NY-ESO-1 ISCOMATRIX vaccine seemed to relapse less frequently than those receiving ineffective vaccination (protein alone without ISCOMATRIX adjuvant, or placebo; ref. 6).[6]

The immunologic activity and safety of this vaccine in MRD patients suggested that this approach has the potential to benefit patients with advanced melanoma. In the present study, we have undertaken a prospective phase II clinical trial using the same vaccine as in the previous study. The major difference between the two trials is that the study population had MRD in the first trial and advanced metastatic disease in the second. We were therefore interested to compare the quality and magnitude of immune responses between the two patient groups and to determine if the vaccine can provide clinical benefit in the advanced disease setting.

Materials and Methods

Trial design. The LUD2002-013 trial was an open-label two-center phase II study of NY-ESO-1 ISCOMATRIX vaccine given by i.m. injection. Safety of the vaccine formulation in this patient population was assessed by observing for dose-limiting toxicity (as defined by the National Cancer Institute Common Terminology Criteria for Adverse Events version 3.0). All patients received three injections of the NY-ESO-1 ISCOMATRIX vaccine at weeks 1, 5, and 9 (cycle 1) and were then evaluated for immunologic and clinical response. At that time, if there was no progressive disease that required treatment by systemic chemotherapy, a second cycle of treatment was offered, consisting of three further injections administered at 4-wk intervals. Patients were reassessed again and, if they had progressive disease, were removed from study. Patients without progressive disease were offered further ongoing therapy, consisting of additional injections administered once every 12 wk, until development of progressive disease or another reason for withdrawal from the study. Accrual continued until a total of 25 patients were entered; however, two additional patients were later entered as replacements for patients who progressed before completion of the first cycle of vaccination. Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors criteria (8). Target (index) lesions were defined before treatment. This study was approved by the Human Research Ethics Committees of Austin Health and the Peter MacCallum Cancer Centre. All patients provided written informed consent. Kendle Australia independently monitored the study.

Patient population. All patients had histologically confirmed stage IV (metastatic) or unresectable stage III malignant melanoma with measurable disease using Response Evaluation Criteria in Solid Tumors. The demographics of the patients are shown in Table 1. Other inclusion criteria were as follows: no other effective therapy was available or appropriate at the time of enrollment; melanoma expressed NY-ESO-1 or LAGE-1 by immunohistochemistry or reverse transcription-PCR, as

Table 1. Patient characteristics at study entry (n = 27)

Characteristic	Value (range)	%
Age at study entry		
Median	61 (36-86)	
Sex		
Male	14	51.9
Female	13	48.1
Days from primary diagnosis to enrollment date		
Median	2,049 (204-9,463)	
Days from primary diagnosis to first relapse		
Median	992 (82-9,019)	
Days from recent relapse to enrollment date		
Median	195 (3-2,017)	
Total days on study		
Median	162 (61-753)	
KPS at study entry		
100%	18	66.7
90%	4	14.8
80%	3	11.1
70%	2	7.4
Previous therapies		
Surgery	27	100
Radiotherapy	17	63
Systemic therapy	13	48.1
Tumor antigen expression (IHC and/or PCR)		
NY-ESO-1 positive (by either IHC or PCR)	26	96.3
LAGE-1 positive (by PCR)	3	11.1
Tumor stage at study entry		
III	3	11.1
IV	24	88.9
No. patients completing study at:		
Discontinued prematurely (<week 11)	2	7.4
Week 11	6	22.2
Week 17	1	3.7
Week 23-25	13	48.1
Week 33	2	7.4
Week 45	1	3.7
Week 69	1	3.7
Week 105	1	3.7

Abbreviations: KPS, Karnofsky performance status; IHC, immunohistochemistry.

[6] In preparation.

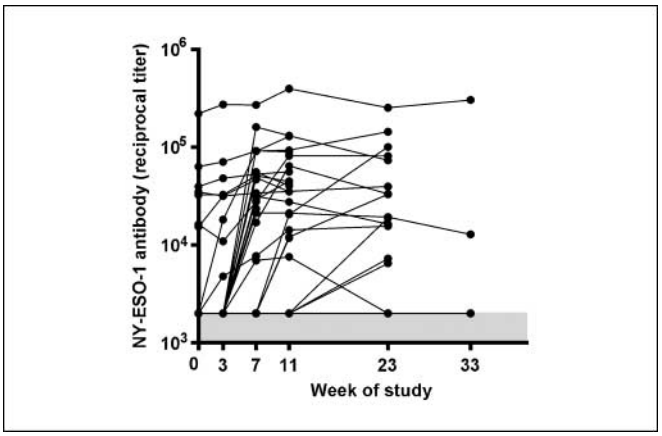

Fig. 1. Anti – NY-ESO-1 antibody titers in patients receiving the NY-ESO-1 ISCOMATRIX vaccine. Antibody responses were assessed by ELISA analysis of serum samples collected at the indicated time points. The lower limits of detection and quantitation were 2,000 and 5,000, respectively, and the shaded area represents the limit of detection. Each curve represents the response of an individual patient. Values represent reciprocal titers.

described (6); life expectancy ≥4 mo; adequate major organ function; and a Karnofsky performance status ≥70%. Exclusion criteria were chemotherapy, radiotherapy, and/or immunotherapy within 4 wk before study week 1; other malignancy within 3 y before study entry; known immunodeficiency; other serious illnesses; pregnancy or lactation; or concomitant systemic treatment with corticosteroids, antihistaminic drugs, or nonsteroidal anti-inflammatory drugs.

NY-ESO-1 ISCOMATRIX vaccine. Recombinant NY-ESO-1 protein was produced in *Escherichia coli* as described (9) The vaccine comprised 200 μg/mL of NY-ESO-1 protein formulated with 240 μg/mL ISCOMATRIX adjuvant and was administered in a 0.5 mL i.m. injection to deliver an intended dose of 100 μg NY-ESO-1 protein and 120 μg ISCOMATRIX adjuvant. This is the same dose as administered to patients in the "100 + ISCOMATRIX adjuvant" cohort of our previous study (6).

Serology. Peripheral blood NY-ESO-1 – specific antibodies were measured using a standardized ELISA method, as described (6), and the level of antibody was expressed as reciprocal titer. The lower limits of detection and quantitation of the assay were 2,000 and 5,000, respectively. Patients with pretreatment titers >5,000 were deemed to have a preexisting response, whereas patients were deemed to have had a positive humoral response to vaccination if they developed a titer >5,000 and had no preexisting response.

DTH testing. DTH reactions to NY-ESO-1 were assessed by i.d. injection of 1 μg of recombinant NY-ESO-1 protein or 30 μg of synthetic peptide corresponding to defined NY-ESO-1 epitopes. The peptides corresponded to the HLA-A2 – restricted epitope NY-ESO-$1_{157\text{-}165}$ (SLLMWITQC) and the HLA-DP4 – restricted epitope NY-ESO-$1_{157\text{-}170}$ (SLLMWITQCFLPVF) and were manufactured by Multiple Peptide Systems to Good Manufacturing Practice specifications. Induration and erythema were measured 48 h after injection. Testing was done before treatment (baseline) and at week 11 for every patient. Testing continued once every 12 wk for patients receiving ongoing treatment. For NY-ESO-1 protein, preexisting reactivity was defined as baseline induration of >5 mm, whereas a positive response to vaccination was recorded if the second DTH reading was >5 mm and at least double the baseline reading. To establish a baseline for NY-ESO-1 peptide cutaneous reactivity, a series of controls was obtained from NY-ESO-1 vaccine-naive patients with MRD who had participated in two other Ludwig Institute – sponsored trials (nine LUD2003-003 participants[7] and six LUD99-008 placebo participants; ref. 6). The cutoff for a positive DTH response was defined as the mean plus two SDs of baseline values for these control participants. Based on these results, a positive response to vaccination with peptide was recorded if the DTH reading was >1 mm induration.

Analysis of T-cell responses. Peripheral blood mononuclear cells (PBMC) were isolated from blood by Ficoll-Paque density gradient centrifugation (Amersham Biosciences) and cryopreserved in 10% DMSO until required. *In vitro* culture followed by intracellular cytokine staining was used to assess T-cell responses within patient PBMC samples and identify the epitopes recognized, as previously described (6, 10). Briefly, two peptide libraries were synthesized; each of which covers the entire sequence of NY-ESO-1 with an overlap of either 12 amino acids (for the 18-mer library) or 11 amino acids (for the 13-mer library). Cryopreserved PBMCs were thawed and pulsed with pools of three to four 18-mer peptides at 10 μmol/L for 1 h at 37°C and then cultured in the presence of 25 units/mL interleukin-2. Cultures were first screened for responses on ~day 11 by restimulating with the same 18-mer peptides used for culture in the presence of 10 μg/mL brefeldin A followed by staining for CD4, CD8, and intracellular IFN-γ. Based on these responses, further examination was done using 13-mer within the relevant 18-mer region. Responses were defined as positive when a clear population of strongly IFN-γ^+ events could be discerned on the flow cytometry dot plot, and this population was at least 0.1% of gated events.

Regulatory T-cell enumeration. The following antibodies were obtained from BD Biosciences: CD4 (clone RPA-T4), CD25 (clone 2A3), and CD127 (clone hIL7R-M21). Antibody to FoxP3 (clone PCH101) was purchased from eBioscience. Cryopreserved PBMCs were thawed and immediately stained using two different approaches. In the first, cells were stained for CD4 and CD25 and then stained for FoxP3 after fixation and permeabilization according to the manufacturer's recommendations. In the second approach, cells were stained with antibodies to CD4, CD25, and CD127 and then fixed using 1% formaldehyde. Flow cytometric analysis was done on a BD FACSCalibur or FACSCanto II, and data were analyzed using FlowJo v4.6, gating on lymphocytes using forward/side scatter. Data are expressed as percent of $CD4^+$ T cells with a regulatory T-cell (Treg; $CD25^+$ $FoxP3^+$ or $CD25^+$ $CD127^-$) phenotype. In every experiment, an aliquot of standard "calibrator" PBMC was stained to control for interassay variability. These cells were also stained with isotype-matched irrelevant antibody controls to determine the level of background staining and aid accurate setting of gates. Prevaccination samples were analyzed from 25 of 27 patients enrolled on the current LUD2002-013 trial and 27 of 46 patients enrolled on the previous LUD99-008 trial. Healthy control PBMCs were obtained from the Australian Red Cross Blood Service.

Statistical analysis. χ^2 Test for independence of nominal data was done using Prism v4.03 software. Fisher's exact test of independence within categories of nominal data was done using the Exactoid online analysis software.[8] Where two-tailed P values of <0.05 were determined, categories were deemed to be statistically independent. Two-tailed Student's t test was done on numerical data using Prism v4.03 software. Values of $P < 0.05$ were considered statistically significant.

Results

Patient characteristics. The demographics of the patients are summarized in Table 1. The majority of patients had stage IV disease at study entry, although three patients had unresectable stage III disease. The tumors of all patients expressed NY-ESO-1 (26 of 27) and/or the highly homologous antigen LAGE-1 (3 of 27).

NY-ESO-1 ISCOMATRIX vaccine is safe in patients with advanced melanoma. There were no serious adverse events deemed to be related to study drug reported for this study and

[7] Unpublished data.

[8] http://www.exactoid.com/fisher/index.php

no grade 3 or 4 toxicities were observed. Only minor toxicities were reported in relation to administration of the NY-ESO-1 ISCOMATRIX vaccine, NY-ESO-1 protein, and peptides. The seven events of grade 2 toxicity included lethargy (3), generalized body ache (1), myalgia (1), sweats (1), and injection site pain (1). This is consistent with previous trials and no areas of concern for safety have arisen from this study.

Clinical responses. As shown in Table 1, all patients except two (who were later replaced) completed the first cycle of vaccination (week 11). Nineteen patients continued on to receive further cycles of vaccination. However, by the end of the second cycle of vaccination (week 23), the majority of the patients had come off study due to progressive disease. Five patients continued to receive further cycles beyond week 23. The median time on study was 162 days. No objective confirmed responses were seen, and stable disease was observed in only one patient who did not progress with a follow-up of 105 weeks. These results were unexpected based on observations from patients in the prior LUD99-008 study of the NY-ESO-1 ISCOMATRIX vaccine in the MRD setting. In the LUD99-008 study, patients receiving effective vaccination had a significantly reduced probability of relapse compared with those who received placebo (6),[6] suggesting that the vaccine may have had clinical efficacy in the setting of MRD.

Seroconversion is frequent after vaccination with NY-ESO-1 ISCOMATRIX vaccine. As shown in Fig. 1, seven patients had preexisting anti–NY-ESO-1 antibodies before vaccination, and in these patients, antibody titers remained relatively stable throughout the monitoring period. Of the remaining 20 patients without preexisting responses, the majority seroconverted following vaccination, with most having detectable anti–NY-ESO-1 antibodies by week 11 (n = 15/20) or weeks 23 to 25 (n = 18 of 20). One patient did not seroconvert until week 57, whereas another failed to seroconvert while on study (last measurement at week 33). The antibody titers observed in the present study are comparable with those observed in our previous trial with MRD patients (6).

Prolonged DTH responses are infrequent after vaccination with NY-ESO-1 ISCOMATRIX vaccine in patients with advanced melanoma. DTH responses to NY-ESO-1 were determined by i.d. injection of full-length NY-ESO-1 protein followed by measurement of induration 48 hours later (Fig. 2). This was done before vaccination to provide a baseline measurement and at the conclusion of each cycle of vaccination. Three of 25 patients (12%) had preexisting DTH responses that were detectable before vaccination, whereas an additional 6 patients (24%) developed a response by the time of the first postvaccination measurement (week 11). However, the remaining patients (64%) failed to exhibit significant DTH responses at any time point during the study. Furthermore, several patients lost responsiveness despite ongoing vaccination. These patients are highlighted in Fig. 2 using open symbols and broken lines. Three of the patients who developed DTH responses at week 11 subsequently lost reactivity by the time of the next testing (week 23), although one regained the response at week 45. In addition, one of the three patients with a preexisting response at baseline lost reactivity at week 11, only to regain it by week 23 and lose it again at week 33. Such patterns may also have been detected more frequently had fewer patients been withdrawn from the study due to disease progression before week 23. These results contrast strikingly with the results of DTH testing in the prior trial using MRD patients, where 10 of 16 patients (63%) developed a positive DTH response following vaccination (ref. 6 and see below).

DTH testing was also conducted using synthetic peptides corresponding to defined $CD4^+$ (NY-ESO-$1_{157\text{-}170}$; HLA-DP4 restricted) and $CD8^+$ (NY-ESO-$1_{157\text{-}165}$; HLA-A2 restricted) epitopes (data not shown). In patients with the appropriate class I or class II haplotype, responses were observed even less frequently than with full-length protein, as only 4 of 13 responded to the HLA-A2 peptide (all of these responses were preexisting) and 5 of 16 responded to the HLA-DP4 peptide (only 2 of which were induced by vaccination). Furthermore, three of these responses were lost with repeated vaccination.

Blood anti–NY-ESO-1 T-cell responses are detected to a range of NY-ESO-1 epitopes but are often preexisting. We next did a detailed analysis of anti–NY-ESO-1 $CD4^+$ and $CD8^+$ T-cell responses in peripheral blood. PBMCs collected at week 11 were cultured with a panel of synthetic 18–amino acid peptides that together cover the entire sequence of the NY-ESO-1 protein (10). The cells were then restimulated with the same peptides used for culture and the proportion of T cells specific for each NY-ESO-1 peptide was determined by intracellular staining for IFN-γ. This analysis revealed that 10 of 25 patients (40%) had a $CD4^+$ T-cell response to at least one NY-ESO-1 epitope, whereas 17 of 25 patients (68%) had at least one $CD8^+$ anti–NY-ESO-1 response. The remaining six patients had no responses within either the $CD4^+$ or $CD8^+$ T-cell compartment.

The positions of the epitopes recognized by responding patients are detailed in Fig. 3. Similar to our previous study with the NY-ESO-1 ISCOMATRIX vaccine (6), patients responded to a wide range of NY-ESO-1 epitopes that tended to cluster within the central and COOH-terminal regions of the NY-ESO-1 sequence (within the region NY-ESO-$1_{60\text{-}160}$). Of note, however, a large proportion of the responses detected in the present study were shown to be preexisting (indicated by open bars on Fig. 3) because PBMCs collected before vaccination also responded to the same epitopes. Furthermore,

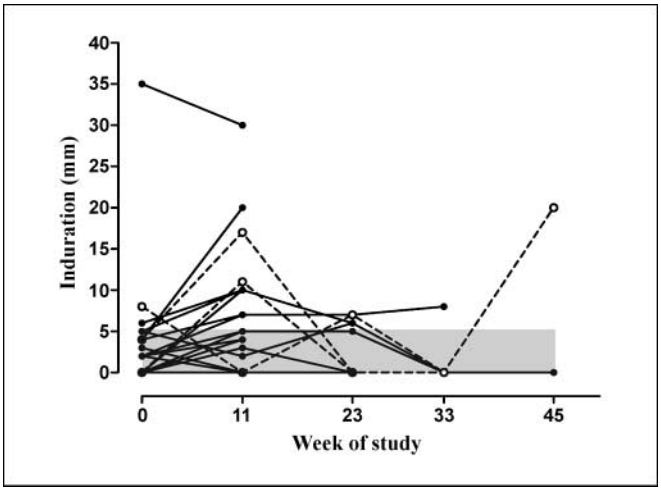

Fig. 2. Cutaneous DTH responses to NY-ESO-1 protein. At the indicated time points, patients received an i.d. injection of 1 μg NY-ESO-1 protein and induration was measured 48 h later. Each curve represents an individual patient. Patients who showed a DTH response and subsequently lost it are highlighted using open symbols and broken lines. The shaded area represents the threshold below which induration was not considered significant.

the responses detected before and after vaccination were generally of a similar magnitude, indicating that preexisting responses were not significantly boosted by vaccination.

T-cell immunity to the NY-ESO-1 ISCOMATRIX vaccine is inferior in patients with advanced melanoma compared with those with MRD. During the course of this study, it became apparent that the extent of anti–NY-ESO-1 T-cell–mediated immunity induced in patients with advanced melanoma was generally inferior to that observed in our previous study with MRD patients. These differences are illustrated in Fig. 4, which summarizes the protein DTH reactions and $CD4^+$ and $CD8^+$ T-cell responses observed for the two groups of patients vaccinated with NY-ESO-1 ISCOMATRIX vaccine. All patient responses were examined for independence; overall, the responses within both the MRD cohort ($\chi^2 = 78.3$, degrees of freedom = 4, $P < 0.001$) and this study ($\chi^2 = 37.7$, degrees of freedom = 4, $P < 0.001$) were found to be independent. In the prior study, 69% of patients showed a DTH response at week 11, and for all patients except one, this response was induced by vaccination. In contrast, the proportion of patients with a DTH response at week 11 in the present study was nearly half that number (36%), and the majority of these responses were preexisting. Similar trends were observed for anti–NY-ESO-1 T-cell responses. Thus, within the MRD cohort, 92% and 67% of patients had detectable $CD4^+$ and $CD8^+$ responses, respectively. Moreover, for the majority of patients for which prevaccination samples were available for testing, these responses could be shown to be induced by vaccination ($P = 0.003$). In the present study, a similar proportion of patients had detectable $CD8^+$ T-cell responses (68%), although only 40% had $CD4^+$ responses. Interestingly, the DTH response observed in both the advanced disease and MRD cohorts was dependent on $CD4^+$ responsiveness ($P = 0.27$ and 0.07, respectively; thus, independence was not found by Fisher's exact test). More importantly, however, the majority of these T-cell responses were entirely preexisting and were not broadened or detectably boosted by vaccination ($P = 0.038$). Therefore, compared with patients with MRD, T-cell reactivity to NY-ESO-1 among the advanced disease patients was less frequent, and the responses that were detected were less likely to be induced by vaccination.

Circulating Treg levels are significantly higher in patients with advanced disease. Our analyses of T-cell and DTH responses suggested that cellular immunity to the NY-ESO-1 ISCOMATRIX vaccine was suppressed in patients with advanced melanoma compared with those with MRD. The observed lack of clinical benefit in the advanced disease setting suggests that

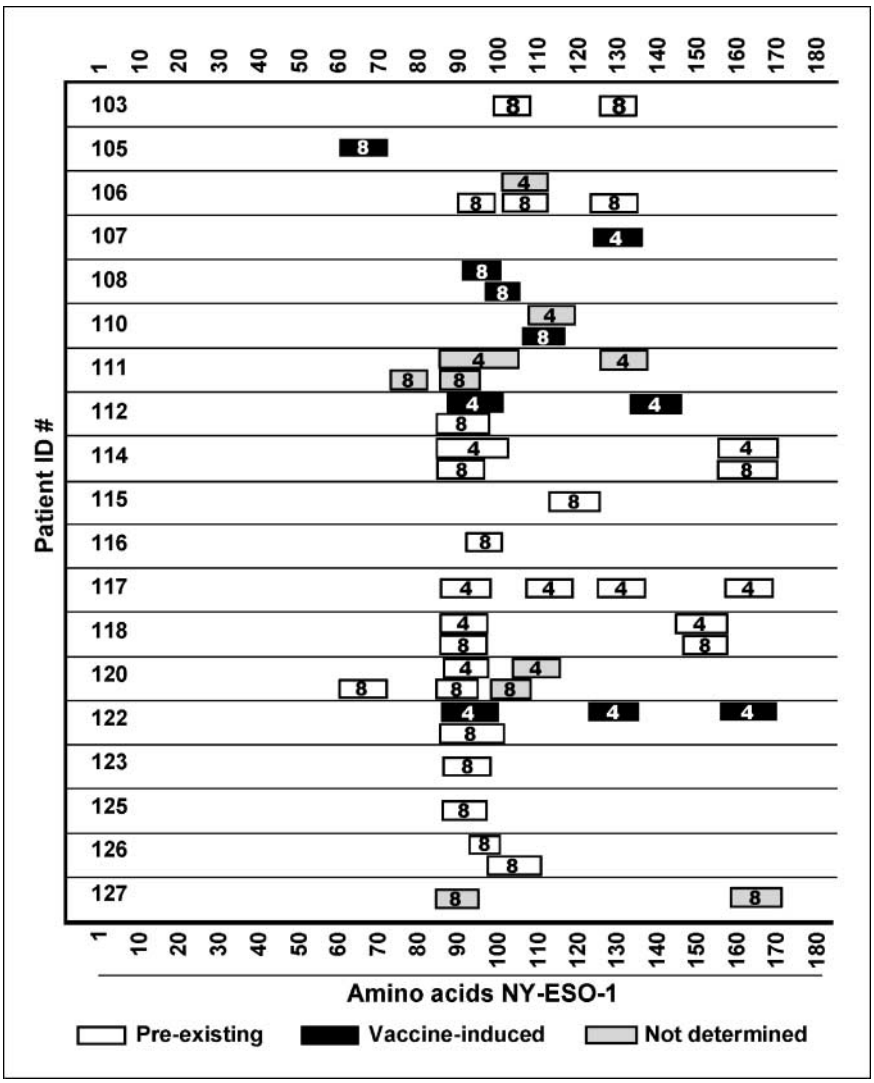

Fig. 3. Epitope map summarizing $CD4^+$ and $CD8^+$ T-cell responses to NY-ESO-1. PBMCs were prepared from blood collected at week 11 after vaccination and screened for T-cell responses using overlapping peptide libraries. For some patients, PBMCs collected before vaccination were also tested to determine if responses detected at week 11 were preexisting. Horizontal numbers represent the amino acids of NY-ESO-1, whereas vertical numbers indicate the patient identification number. Each bar indicates a T-cell response to an epitope within the indicated region of NY-ESO-1; bars labeled with "4" and "8" denote $CD4^+$ and $CD8^+$ T-cell responses, respectively. Open bars represent preexisting responses, solid bars represent vaccine-induced responses, and shaded bars represent responses where it was not possible to determine whether the response was preexisting or vaccine induced.

this relative immune suppression might be of clinical significance. To address this, $CD4^+$ $CD25^+$ Treg cells were enumerated as a surrogate marker using blood samples collected before vaccination from patients on the two trials, as well as a cohort of healthy controls (Fig. 5). For each patient, the proportion of $CD4^+$ T cells with a Treg phenotype was determined using two complimentary methods. The first method (Fig. 5A) is based on coexpression of CD25 and FoxP3 ($CD25^+$ $FoxP3^+$ Treg), whereas the second (Fig. 5B) is based on CD25 expression combined with reduced/negative expression of CD127 ($CD25^+$ $CD127^-$ Treg). Using both methods, a statistically significant increase in the proportion of Treg was detected for patients with advanced disease compared with those with MRD (P = 0.006 and 0.03 using the FoxP3 and CD127 methods, respectively). Advanced disease patients also had significantly more Treg than healthy controls (P = 0.0006 and 0.0021). Interestingly, a small but statistically significant increase in Treg numbers was also apparent when comparing MRD patients with healthy controls (P = 0.04 and 0.025). Thus, the proportion of Treg was slightly elevated above normal even in patients with MRD. However, a much larger increase was apparent in patients with advanced disease, with several individuals having more than twice the normal number of Treg.

In addition to the prevaccination measurement of Treg frequency, 11 patients on the current trial also had a postvaccination measurement (either day 42 or 70) to determine if Treg numbers remained stable over the course of vaccination (data not shown). Although there were small individual fluctuations, there was no statistically significant difference between prevaccination and postvaccination samples (P > 0.05 for all).

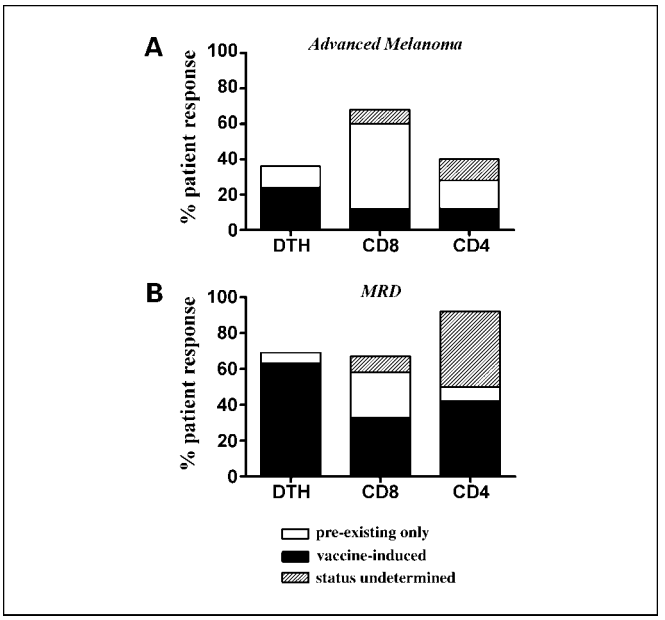

Fig. 4. Comparison of T-cell – mediated immune responses to NY-ESO-1 in patients with advanced melanoma or MRD. Immune response parameters are summarized for advanced disease patients participating in the current study (*A*) and the cohort of MRD patients from the previous trial who received the same dose of vaccine (*B*). Graphs show the percentage of patients with a positive protein DTH response and detectable $CD4^+$ and $CD8^+$ T-cell responses, as a proportion of total patients evaluated. For the current trial, 25 of 25 patients were evaluated for each parameter. For the previous trial, 15 of 15 patients were evaluated for DTH responses and 12 of 15 were evaluated for T-cell responses. For each parameter, the responding patients were categorized as follows: vaccine induced (a response detected after vaccination that either was not detectable before vaccination or was clearly boosted by vaccination; *black*), preexisting (all responses detected after vaccination were also detectable before vaccination; *white*), or status undetermined (unable to determine if response was vaccine induced; *shaded*).

Discussion

Tumor-induced immune suppression is an increasingly well-recognized paradigm that may help explain the failure of many experimental cancer vaccine approaches (11, 12). The results of the present study suggest that, in melanoma, the extent of immune down-regulation and the effect this has on vaccine efficacy may be closely tied to disease progression. First, patients with advanced disease failed to develop clinical responses to the vaccine, whereas vaccination of MRD patients resulted in an apparent clinical benefit. Second, T-cell – mediated immunity (as measured by DTH responses and circulating NY-ESO-1 – specific T cells) was greatly attenuated and sometimes lost in the advanced disease setting compared with MRD. Finally, the proportion of Treg was significantly elevated in patients with advanced disease compared with those with MRD, suggestive of systemic tumor-induced immune suppression.

T-cell responses to the NY-ESO-1 ISCOMATRIX vaccine were evaluated both indirectly (by measuring DTH responses to NY-ESO-1 protein) and directly (by screening for $CD4^+$ and $CD8^+$ NY-ESO-1 – reactive T cells). Both approaches support the concept that patients with advanced melanoma were compromised in their ability to mount T-cell – mediated immune responses to the vaccine. DTH responses occurred much less frequently in this patient cohort compared with MRD patients, and surprisingly, several patients lost their DTH response following repeated vaccination. The proportion of patients with detectable NY-ESO-1 – specific $CD4^+$ T cells in the blood was also greatly reduced in the advanced disease setting compared with MRD. Although the frequency of patients with $CD8^+$ T-cell responses was similar between the two groups, a greater proportion of these responses in advanced disease patients were entirely preexisting (i.e., the range of epitopes recognized was not broadened by vaccination, nor was the magnitude of existing responses detectably increased). The observation of increased preexisting immunity in advanced disease patients was not unexpected, considering that these patients have had prolonged exposure to the antigen. Furthermore, we and others have previously shown that spontaneous T-cell responses to NY-ESO-1 frequently occur in patients with advanced melanoma (13, 14). However, the failure of many of these patients to generate any new responses to the vaccine was suggestive of immune down-regulation at the time of vaccination.

In contrast to T-cell responses, the generation of antibody responses seemed to be largely independent of disease stage. After vaccination, the frequency and magnitude of anti – NY-ESO-1 serum antibody responses were similar in advanced disease and MRD patients, although, unsurprisingly, more preexisting responses were detected in the former group. This observation suggests that tumor-induced immune suppression in advanced disease patients is focused on the cell-mediated arm of the immune response, whereas the humoral response may be spared or may be regulated by other means, such as persistence of antigen (15).

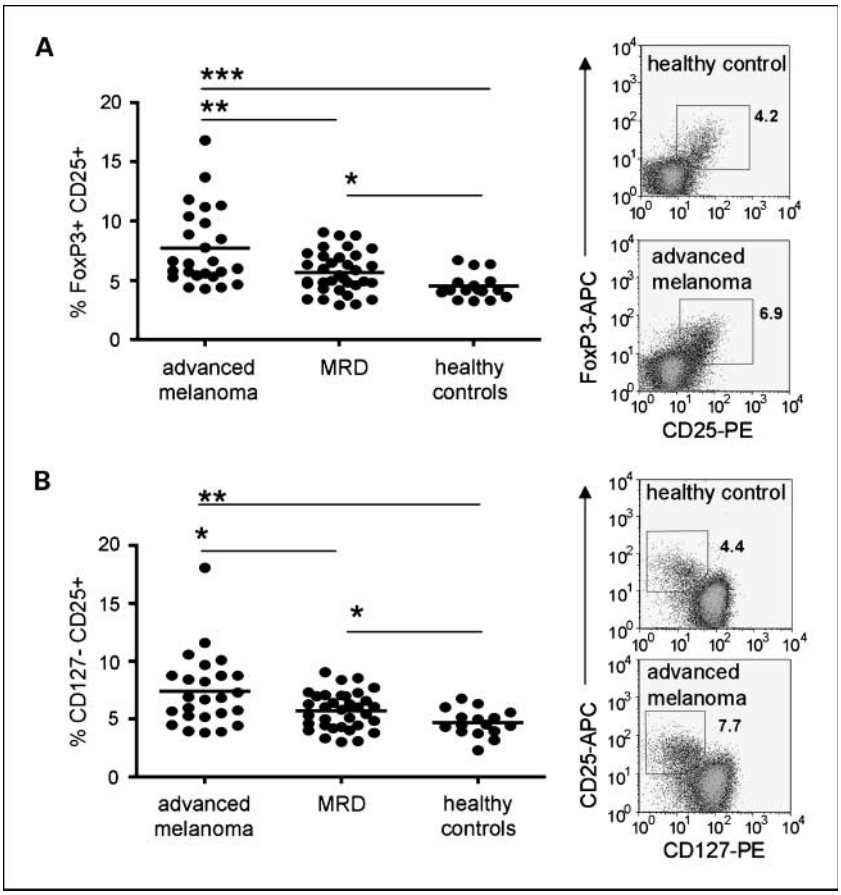

Fig. 5. Treg frequency in peripheral blood of patients with advanced melanoma or MRD compared with healthy controls. PBMCs were obtained before vaccination for patients on the current trial (*advanced melanoma*), patients from the previous trial (*MRD*), or healthy controls. For each patient, the percentage of $CD4^+$ T cells with a Treg phenotype was determined using flow cytometry after staining with antibodies to CD4, CD25, and FoxP3 (*A*) or CD4, CD25, and CD127 (*B*). Scatter plots display individual values for every patient tested and the population mean (*horizontal line*). Flow cytometry density plots illustrate typical staining patterns observed for the indicated populations. Significance between groups is denoted by the following: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

It has recently been recognized that Tregs play a key role in suppression of antitumor T-cell immunity. This subset of $CD4^+$ T cells is characterized by coexpression of CD25 and the FoxP3 transcription factor but lacks expression of CD127, the interleukin-7 receptor α chain (16–18). Numerous studies in animal models have shown that removing or inhibiting Treg dramatically improves tumor clearance and survival (12, 19). Furthermore, in human ovarian cancer, the frequency of Treg infiltrating the tumor has been shown to negatively correlate with survival (20, 21), and systemic depletion of Treg using a recombinant interleukin-2/diphtheria toxin conjugate resulted in enhanced immune responses in patients with metastatic renal cell carcinoma (22). In melanoma, we have observed high proportions of Treg infiltrating metastatic tumor tissue, such that up to 40% of $CD4^+$ T cells within the tissue have a Treg phenotype,[9] which is in keeping with other studies (23, 24). This suggests that tumors can create a local immunosuppressive environment by selective recruitment and/or expansion of Treg. Interestingly, we have also recently shown that tumor cells themselves can express FoxP3 (25), potentially allowing them to adopt some of the immune-regulatory characteristics of Treg and thereby further enhancing the local immunosuppressive environment.

In addition to the high proportion of Treg within tumor tissue, Treg numbers in the peripheral blood have also been shown to be increased in several types of cancer, suggesting that tumor-induced immune suppression may be systemic (12, 19). Our results provide evidence that such a phenomenon also occurs in melanoma and, moreover, that the level of such suppression correlates with the stage of disease. Thus, patients with advanced melanoma had significantly more Treg than MRD patients and, on average, nearly twice as many Treg as healthy controls. Furthermore, even the MRD patients had a small but significant increase in Treg numbers compared with healthy controls, suggesting that some level of systemic immune suppression may be maintained even after resection of all detectable tumor deposits. These patterns were observed using two independent, highly accurate methods of Treg identification, and it is therefore difficult to compare with earlier studies of melanoma in which Tregs were identified simply by expression of CD25 (which is also expressed by activated T cells; refs. 26, 27). However, our observation of increased Treg numbers in advanced melanoma is supported by previous studies in which patients with metastatic melanoma had a substantial increase in $CD25^+$ $FoxP3^+$ Treg frequency in the blood compared with healthy controls (24, 28).

The results of this study show an association between the stage of melanoma progression, the number of Treg in the blood, and the clinical and immunologic efficacy of the NY-ESO-1 ISCOMATRIX cancer vaccine. At this stage, it is not possible to show directly that the increased proportion of Treg in advanced melanoma is responsible for the inferior responses to the vaccine. However, this concept is supported by several studies that have confirmed a role for Treg in suppression of anti–NY-ESO-1 immunity. First, depletion of Treg *in vitro* can

[9] L.M. Ebert, unpublished observations.

unmask "hidden" T-cell responses to NY-ESO-1 in cancer patients and even healthy individuals, suggesting that Treg can efficiently suppress NY-ESO-1–specific T-cell proliferation (29, 30). Furthermore, Tregs specific for NY-ESO-1 have recently been detected in the peripheral blood of patients with metastatic melanoma (31). To address these questions, the LUD2002-013 protocol has been amended to include an additional cohort of patients treated with the NY-ESO-1 ISCOMATRIX vaccine in combination with low-dose cyclophosphamide, which has been reported to have a selective cytotoxic effect on Treg (32, 33). Accrual to the amended protocol is currently under way (clinicaltrials.gov identifier: NCT00518206).

Together, our results support the concept that even highly efficacious vaccine-based therapies are of limited use in patients with advanced cancer due to the overwhelming immunosuppressive networks that have already been established. In such patients, clinical and immunologic responses might be improved by combining the vaccine with approaches to deplete Treg. Alternatively, our findings support a model whereby future efforts at vaccine-based treatment would be better focused on patients that are most likely to receive benefit: those at an earlier stage of disease where the volume of tumor to be eradicated and the extent of immune suppression are both minimized.

Disclosure of Potential Conflicts of Interest

I.D. Davis, W. Chen, J. Cebon, patent holders of ISCOMATRIX vacccine, CSL Limited.

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