

CTLA-4 blockade increases antigen-specific CD8⁺ T cells in prevaccinated patients with melanoma: three cases

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Received: 9 February 2011 / Accepted: 14 March 2011 / Published online: 5 April 2011
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Abstract

Background Anti-cytotoxic T-lymphocyte antigen-4 (CTLA-4) antibodies, such as ipilimumab, have generated measurable immune responses to Melan-A, NY-ESO-1, and gp100 antigens in metastatic melanoma. Vaccination

This paper is a Focussed Research Review based on a presentation given at the Tenth International Conference on Progress in Vaccination against Cancer (PIVAC 10), held in St. Catharine's College, Cambridge, UK, September 27th–30th, 2010. It is part of a CII series of Focussed Research Reviews and meeting report.

Electronic supplementary material The online version of this article (doi:10.1007/s00262-011-1011-9) contains supplementary material, which is available to authorized users.

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against such targets has potential for immunogenicity and may produce an effector-memory T-cell response.

Methods To determine the effect of CTLA-4 blockade on antigen-specific responses following vaccination, in-depth immune monitoring was performed on three ipilimumab-treated patients prevaccinated with gp100 DNA (IMF-24), gp100^{209–217} and tyrosinase peptides plus GM-CSF DNA (IMF-32), or NY-ESO-1 protein plus imiquimod (IMF-11); peripheral blood mononuclear cells were analyzed by tetramer and/or intracellular cytokine staining following 10-day culture with HLA-A*0201-restricted gp100^{209–217} (ITDQVPFSV), tyrosinase^{369–377} (YMDGTMSQV), or 20-mer NY-ESO-1 overlapping peptides, respectively. Tumors from IMF-32 were analyzed by immunohistochemistry to help elucidate mechanism(s) underlying tumor escape.

Results Following vaccination, patients generated weak to no CD4⁺ or CD8⁺ T-cell response specific to the vaccine antigen but demonstrated increases in effector-memory (CCR7^{lo}CD45RA^{lo}) tetramer⁺CD8⁺ T cells. After ipilimumab induction, patients experienced a robust, although sometimes transient, antigen-specific response for gp100 (IMF-32 and IMF-24) or NY-ESO-1 (IMF-11) and produced polyfunctional intracellular cytokines. Primary and metastatic tumors expressed tyrosinase but not gp100 or class I/II MHC molecules.

Conclusion Vaccination induced a measurable antigen-specific T-cell response that increased following CTLA-4 blockade, potentially “boosting” the vaccine-primed response. Tumor escape may be related to antigen loss or lack of MHC expression necessary for immune activity. These results in a limited number of patients support the need for further research into combining vaccination with ipilimumab and provide insight into mechanisms underlying tumor escape.

Keywords Melanoma · Cytotoxic T-lymphocyte antigen-4 · Ipilimumab · T-cell response · Vaccines · PIVAC 10

Abbreviations

ALC	Absolute lymphocyte count
CTLA-4	Cytotoxic T-lymphocyte antigen-4
GM-CSF	Granulocyte macrophage colony-stimulating factor
H&E	Hematoxylin and eosin
ICOS	Inducible co-stimulator
ICS	Intracellular cytokine staining
IHC	Immunohistochemistry
PBMC	Peripheral blood mononuclear cell
WBC	White blood cell

Introduction

The incidence of melanoma has risen in the past several decades [1]. Unfortunately, this increase has not been accompanied by improved treatments for advanced disease; rather, conventional treatments remain toxic and relatively ineffective [2–4]. Most trials of melanoma therapies have reported objective responses in <15% of patients and these tend to be of short duration. In fact, a clear survival benefit for chemotherapy has yet to be demonstrated. The notable exception is high-dose interleukin-2 (IL-2), which has a low response rate and serious toxicity, but does lead to durable complete responses (CRs) in a small subset of patients [5]. Because of the marginal benefits of conventional therapy, there has been considerable interest in the use of vaccination strategies to stimulate the immune system. Such immune approaches are attractive because of the rare but durable responses noted with IL-2 therapy and anecdotal reports of complete tumor regression, both of which are thought to have an immune-based mechanism.

Vaccine strategies evaluated in pre-clinical and small clinical trials have been very heterogeneous with results demonstrating immunogenic variability and clinical uncertainty. Targets for vaccination include differentiation antigens expressed selectively on melanoma cells (e.g., gp100 and Melan-A/MART-1) and cancer-testis antigens expressed only on normal testes, placenta, and certain tumor tissues (e.g., NY-ESO-1). The vaccine constructs have variably consisted of peptide fragments, whole proteins, or DNA plasmids administered alone or with immune adjuvants (e.g., granulocyte macrophage colony-stimulating factor [GM-CSF]) [6–8] or viral vectors [9, 10]. Clinical benefits noted in these trials have been generally modest, even though many of these constructs have been shown to elicit specific immune responses against the

antigen of interest. One possible explanation for the failure of vaccine strategies to date is the co-induction of suppressive regulatory T cells (T_{regs}), a hypothesis supported by pre-clinical models [11] and the observation that advanced melanoma itself induces an increase in peripheral blood T_{regs} [12].

Another target of interest is cytotoxic T-lymphocyte antigen-4 (CTLA-4), a co-inhibitory molecule expressed on activated T cells and T_{regs} . CTLA-4 is essential to maintenance of immune homeostasis [13–16] and contributes to tolerance to self-antigens by down-regulating T-cell response and proliferation [17–20]. Ipilimumab, a fully human IgG₁ anti-CTLA-4 monoclonal antibody, has been found to induce objective tumor responses in phase I and II trials of patients with advanced melanoma [21, 22] and other tumor types [23, 24]. Additionally, ipilimumab monotherapy has been shown to induce polyfunctional NY-ESO-1 antigen-specific T cells and Melan-A antigen-specific CD8⁺ T cells in melanoma patients [25, 26]. Significant improvement in overall survival was demonstrated in MDX010-20, a phase III trial of ipilimumab as second-line therapy for advanced melanoma. In this trial, patients received one of three therapies: ipilimumab alone, a gp100 vaccine alone, or a combination of both agents. Clinical response was demonstrated in both ipilimumab-containing arms, but not with gp100 vaccine monotherapy; in fact, gp100 appeared to attenuate ipilimumab activity somewhat in the combination arm [27].

Given the positive results seen with anti-CTLA-4 monotherapy, investigators have sought to combine various vaccine strategies with CTLA-4 blockade and results have shown promise. Also, combination treatment with GM-CSF and ipilimumab has been shown to induce activation of T cells, including NY-ESO-1 specific CD8⁺ T-cell responses [28]. Furthermore, autologous dendritic cells pulsed with MART-1 peptides together with the anti-CTLA-4 antibody tremelimumab have shown a measurable tumor response, although there was no difference in MART-1 antigen specific response between patients with an objective tumor response and those without a response [29]. Based on these observations, patients treated with ipilimumab in clinical trials at Memorial Sloan-Kettering Cancer Center (MSKCC) who received prior cancer vaccinations were examined to determine whether a measurable antigen-specific immune response was generated, and if so, the response was characterized. If anti-CTLA-4 immunotherapy does in fact enhance antigen-specific responses after prior vaccination, this may help define why some melanoma patients experience a clinical response to CTLA-4 blockade whereas others do not. In turn, measurably enhanced immunogenicity would promote further research into the use of vaccines combined with other immunomodulatory therapies.

Methodology

Patients

The patients described in this report were treated with ipilimumab at MSKCC in a previously described clinical trial [25, 30]. Pathology was confirmed at MSKCC and all patients provided informed consent. Seventy patients with advanced melanoma received ipilimumab at either 0.3 mg/kg ($n = 1$), 3 mg/kg ($n = 4$), or 10 mg/kg ($n = 65$) every 3 weeks for four treatments. Those without dose-limiting toxicity and with evidence of clinical benefit (defined as an objective response or stable disease at week 24) could continue to receive ipilimumab at the original dose or 10 mg/kg every 12 weeks until disease progression, toxicity, withdrawal of consent, or death occurred. Responses were adjudicated by recently proposed immune-related response criteria [31]. Of the 70 ipilimumab-treated patients, 4 received prior cancer vaccines, of which 3 (IMF-11, -24, and -32) had specimens available for in-depth immune monitoring.

T-cell stimulation in vitro

Thawed peripheral blood mononuclear cells (PBMCs) were incubated at a 1:1 ratio with irradiated autologous PBMCs pulsed with one of the following HLA-A*0201-restricted peptides at 10 μ g/ml: gp100^{209–217} (ITDQVPFSV), tyrosinase^{369–377} (YMDGTMSQV), or 20-mer NY-ESO-1 overlapping peptides (JPT Peptide Technologies, Berlin, Germany). T cells were cultured in vitro for 10 days with the cytokines IL-2 (10 IU/ml) and IL-15 (10 ng/ml). The culture medium was changed every 2–3 days during in vitro stimulation. The cells were harvested at day 10 and analyzed immediately for polyfunctionality by tetramer staining and intracellular cytokine staining (ICS). Details of this analysis were previously described [32].

Tetramer staining and ICS

HLA-A*0201-PE-labeled tetramers loaded with gp100^{209–217} (ITDQVPFSV) and tyrosinase^{369–377} (YMDGTMSQV) were provided by the Tetramer Core, Ludwig Institute for Cancer Research, Lausanne, Switzerland. Tetramer staining and ICS were performed on cells obtained after 10-day T-cell stimulation as previously described [33]. T-cell responses at post-vaccination or post-ipilimumab time points were considered positive if these were ≥ 3 standard deviations greater than the mean value at baseline and had an absolute value $>0.1\%$.

Tumor sample processing

Tissue sections (5 μ m) were prepared from formalin-fixed, paraffin-embedded material and collected on Superfrost®/plus microscope slides (Fisher Scientific, Fair Lawn, NJ). After deparaffinization and rehydration, the slides were boiled in 50 mM citrate buffer (pH 6) for 30 min to retrieve the antigens. After the slides were allowed to cool to room temperature, immunohistochemistry (IHC) was performed using the avidin-biotinylated enzyme complex (ABC) method. Before applying primary antibodies, the sections were blocked with 10% rabbit and horse normal serum (Santa Cruz Biotechnology, Santa Cruz, CA) for HLA DR and HLA class I, respectively. Rat anti-human monoclonal antibody against HLA DR (1:200 dilution, clone YE2/36HLK, Abcam Biotechnology, Cambridge, MA) and mouse anti-human monoclonal antibody against HLA class I (1:200 dilution, clone A4, eBioscience, San Diego, CA) were then applied at 4°C overnight. Biotinylated rabbit anti-rat and horse anti-mouse secondary antibodies (Vectastain® Elite ABC kit, Vector Labs, Burlingame, CA) were added on the second day and incubated for 30 min at room temperature. A tertiary reagent was applied according to the manufacturer's instructions. Antigen detection was performed by a color reaction with 3,3-diaminobenzidine (DAB + chromogen, DakoCytomation, Hamburg, Germany). The sections were counterstained with hematoxylin and mounted with Permount™ media (Fisher Scientific, Fair Lawn, NJ). The slides were scanned with Mirax® scanner (Carl Zeiss, Chester, VA), and images were acquired with Mirax® viewer 1.11 software. IHC detection of gp100 and tyrosinase was performed using monoclonal antibodies HMB45 and T311, respectively, as previously described [33].

Case studies

Characteristics, treatment history, and immune responses for the three patients are summarized in Table 1.

Case 1—Patient IMF-32

In June 2005, this 74-year-old man underwent resection of a left thigh melanoma at MSKCC. He received post-operative experimental therapy in a clinical trial with an HLA-A*0201-specific gp100^{209–217} and tyrosinase^{369–377} peptide vaccine administered together with GM-CSF DNA. Since tumors can be heterogeneous within a patient, this trial did not require included patients to have a tumor biopsy demonstrating gp100 expression; it was learned later that his tumor was gp100 negative. In February 2006, 8 months after his initial surgery, he developed recurrent

Table 1 Patient characteristics, treatment history, and immune response

Patient identifier	IMF-32	IMF-24	IMF-11
Age (years)	74	43	62
Gender	Male	Male	Female
Vaccine setting	Adjuvant	Adjuvant	Adjuvant
Vaccine antigen	gp100 + tyrosinase peptides	gp100 DNA	NY-ESO-1 protein
Vaccine-induced immune response	CD8 (gp100 only)	CD8 (gp100 tetramer)	CD4
Prior therapy	Temozolomide, CVT	Temozolomide, RT, IL-2	TKI, sorafenib, temozolomide, anti-integrin
Time from vaccination to ipilimumab treatment	2.5 years	1 year	10 months
Ipilimumab-induced immune response	CD8 (gp100 only), polyfunctional T cells	CD8 gp100, polyfunctional T cells	CD4 and CD8, polyfunctional T cells
Tumor/metastasis antigen expression	Tyrosinase positive, gp100 negative	NA	NA

CVT cisplatin + vinblastine + temozolomide, IL-2 interleukin-2, NA not available, RT radiation therapy, TKI tyrosine kinase inhibitor

disease in the left groin that was surgically resected, followed by treatment with temozolomide. In April 2007, 6 months after completing chemotherapy, he developed further recurrence in the bilateral lungs with pathology confirmed by biopsy. He was treated with the CVT (cisplatin/vinblastine/temozolomide) regimen. In November 2007, following eight cycles of therapy, the patient underwent resection of left lung nodules that revealed viable tumor. Two months later, he developed further recurrence/enlargement of right lung nodules. In January 2008, he began therapy with ipilimumab (Supplemental Fig. 1), receiving induction doses of 10 mg/kg IV every 3 weeks for four doses. A computed tomography (CT) scan at week 12 demonstrated stability of the lung lesions, but recurrence was detected in the left groin. In April 2008, surgical resection of a left inguinal mass confirmed metastatic melanoma. Changes in these lesions are shown in Supplemental Fig. 1A. Ongoing clinical benefit at week 24 supported the decision to initiate maintenance therapy with ipilimumab administered every 12 weeks. Since that time, he has continued to receive maintenance ipilimumab. CT scans revealed a CR in the lung lesions without further recurrence. He remains without evidence of disease more than 3 years after initiation of ipilimumab.

Although Patient IMF-32 received both gp100 and tyrosinase peptides (along with GM-CSF DNA), he developed gp100-specific, but not tyrosinase-specific, tetramer-reactive CD8⁺ cells in the peripheral blood immediately following vaccination [8]. As such, we were interested in quantifying gp100- and tyrosinase-specific T-cell responses following ipilimumab therapy (Supplemental Fig. 1E). Tetramer staining did not reveal a significant change in low levels of tyrosinase-specific tetramer-reactive CD8⁺ cells (below the defined threshold of $\geq 0.1\%$ for tetramer positivity) with ipilimumab therapy

(Fig. 1a). A significant increase in gp100-specific tetramer-reactive CD8⁺ cells was detected after ipilimumab administration (week 12) and peaked after the week 34 dose of ipilimumab therapy (the first maintenance treatment) and declined by week 48 (after the second maintenance treatment). He experienced rapid increases in ALC above 1,000/mm³ and CD4⁺, CD8⁺, and CD4⁺ICOS^{hi} T cells (Supplemental Fig. 1B,C,D), which confirmed previous observations [30, 34].

The gp100-specific tetramer-reactive CD8⁺ cells were analyzed at week 34 by characterizing CCR7 and CD45RA expression in these cells (Fig. 1b). The tetramer-reactive CD8⁺ cells were CCR7^{lo}CD45RA^{lo}, consistent with an effector-memory phenotype. The CCR7⁻CD45RA⁻ cells from this patient were also characterized as EM2 (CD27⁺CD28⁻) effector cells [35]. In addition to tetramer reactivity, the cultured cells were also analyzed for the expression of interferon gamma (IFN- γ). As with the previous tetramer response, Patient IMF-32 did not exhibit any increase in tyrosinase-specific CD8⁺IFN- γ ⁺ cells following vaccination and subsequent ipilimumab therapy (Fig. 2a); however, there was a slight increase in gp100-specific CD8⁺IFN- γ ⁺ cells after vaccination and a robust response following ipilimumab therapy, again peaking at week 34.

Although a single parameter, most commonly IFN- γ , has previously been used to assess the effector function of T cells, increasing evidence suggests that polyfunctional responses are associated with improved control of viral infections in preclinical models [36, 37], in patients infected with human immunodeficiency virus [38], and those immunized with vaccinia constructs [39]. As such, the week 34 gp100-specific CD8⁺ IFN- γ ⁺ cells were also analyzed for, and found to express, a combination of tumor necrosis factor (TNF)- α , macrophage inflammatory protein

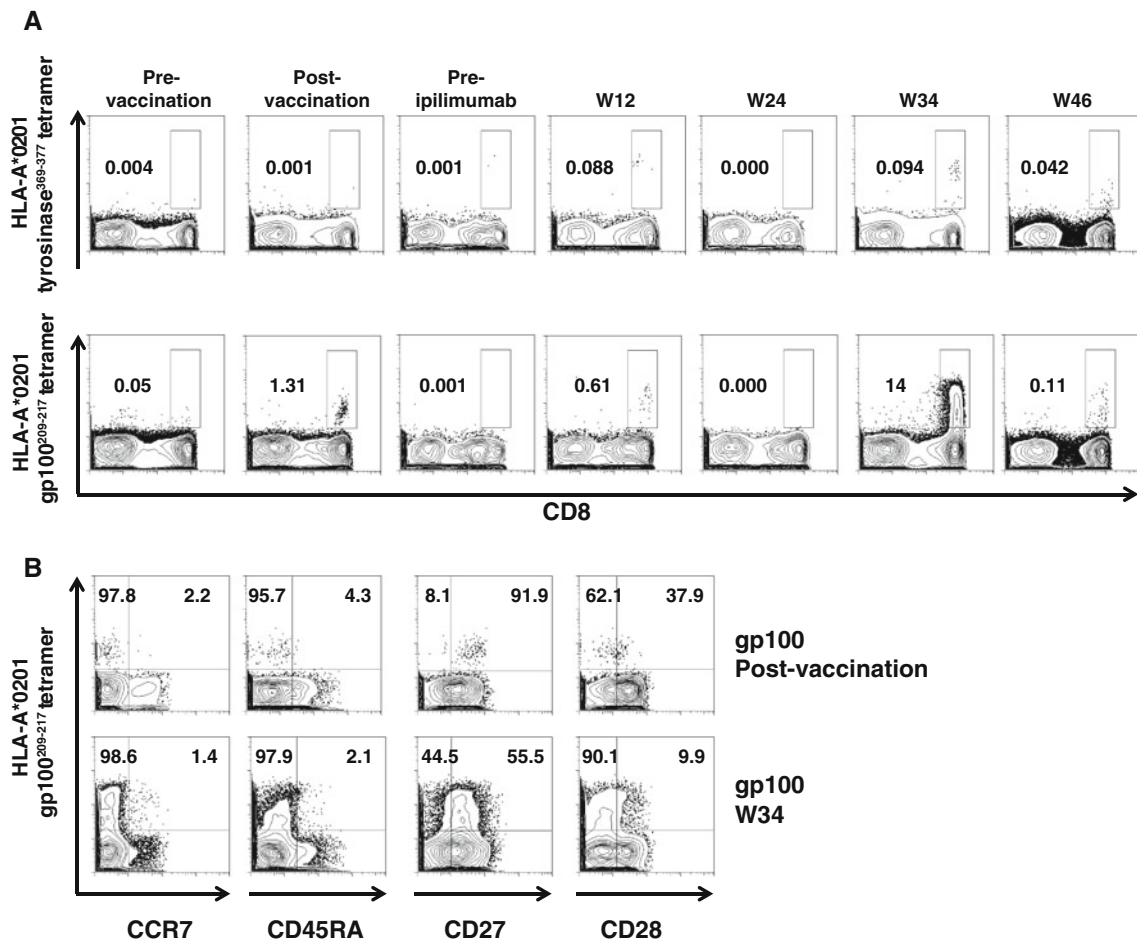


Fig. 1 Changes in gp100- and tyrosinase-specific tetramer-reactive CD8⁺ T cells following ipilimumab therapy in Patient IMF-32. Thawed PBMCs from various time points were cultured for 10 days with either HLA-A*0201-restricted peptides gp100^{209–217} (IT-DQVPFSV) or HLA-A*0201 tyrosinase^{369–377} (YMDGTMSQV) before undergoing tetramer staining. *Panel A* representative dot plots

reveal a significant increase in gp100-specific but not tyrosinase-specific tetramer-reactive CD8⁺ T cells with ipilimumab therapy. *Panel B* further characterization of the week 34 gp100-specific tetramer-reactive CD8⁺ T cells reveals that most of these are CCR7[–]CD45RA[–], consistent with an effector phenotype

(MIP)-1β, and the degranulation surface marker CD107a (Fig. 2b).

Tissue from the primary tumor resected in June 2005 was previously analyzed by IHC for expression of the melanoma differentiation antigens gp100 and tyrosinase. In addition, metastatic tumor tissue resected from left groin metastases in February 2006 and April 2008 and lung metastases in April and November 2007 were analyzed by IHC for gp100 and tyrosinase expression, as well as expression of class I and II major histocompatibility complex (MHC) molecules. The primary and metastatic tumors were found to express tyrosinase but not gp100. All of the metastatic tumors were also found to have low to no expression of class I and II MHC molecules. Immunohistochemical analyses are summarized in Table 2, and images are provided in Supplemental Figs. 2 and 3.

Case 2—Patient IMF-24

In June 2003, this 43-year-old man presented with melanoma of the left temporal region with satellite sites and a positive sentinel lymph node biopsy. Following excision of a left cheek cutaneous metastasis, he was treated with 6 months of temozolomide (75 mg/m² daily) beginning in December 2003. In December 2004, he had a left neck lymph node dissection revealing 5/69 positive nodes and was given radiation therapy to his left temporal region, face, and neck. In July 2005, he was enrolled in a phase I clinical trial of a gp100 DNA vaccine at MSKCC. He received 3 doses of 500 μg human gp100 DNA followed by 3 doses of mouse gp100 DNA via intramuscular injection, with each dose separated by 3 weeks. He developed HLA-A*0201 gp100^{280–288} tetramer-reactive,

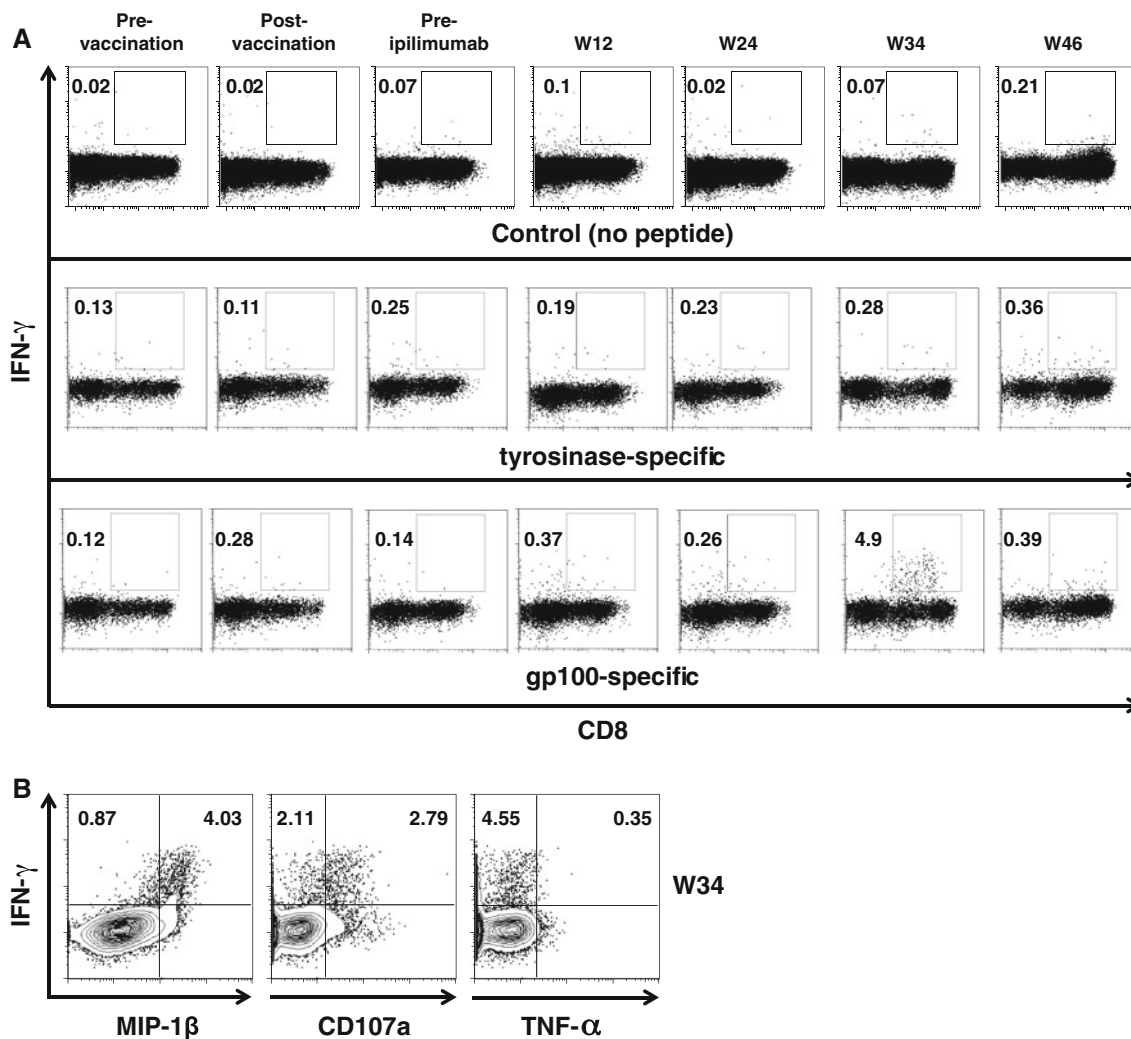


Fig. 2 Changes in gp100- and tyrosinase-specific CD8⁺IFN- γ ⁺ T cells and evidence of polyfunctional responses in Patient IMF-32. Thawed PBMCs from various time points were cultured for 10 days with either HLA-A*0201-restricted gp100^{209–217} (ITDQVPFSV) or HLA-A*0201 tyrosinase^{369–377} (YMDGTMSQV) peptides and were then restimulated with the corresponding peptides for 6 h prior to

ICS. *Panel A* representative dot plots are provided for control [no peptide added] (*top row*) and show an increase in gp100-specific T cells (*bottom row*) but not tyrosinase-specific CD8⁺IFN- γ ⁺ T cells (*middle row*) following ipilimumab therapy. *Panel B* representative dot plots show evidence of polyfunctionality wherein the CD8⁺ T cells express MIP-1 β and CD107a

Table 2 Summary of immunohistochemistry data—Patient IMF-32

Tumor	gp100	Tyrosinase	MHC class I	MHC class II
Primary tumor	–	+	N/A	N/A
Inguinal lymph node	–	+	Low	Low
Lung	–	+	Low	Low

but not HLA-A*0201gp100^{209–217} tetramer-reactive, CD8⁺ T cells in the peripheral blood immediately following vaccination [32 (Patient 8)].

In November 2005, a PET/CT scan revealed new mesenteric lymphadenopathy. He was treated with 6 weeks of chronic low-dose temozolomide but continued to

demonstrate disease progression with the appearance of new retroperitoneal and hepatic metastases. In February 2006, the patient was treated with 5 weeks of high-dose IL-2 therapy at the National Cancer Institute. In August 2006, showing signs of stable disease, the patient initiated ipilimumab therapy at MSKCC in an effort to produce an objective response. He received the same ipilimumab induction regimen as Patient IMF-32. At week 24, ipilimumab maintenance therapy was initiated due to clinical benefit. In June 2007, the patient developed multiple small visceral metastases and a right occipital brain metastasis, the latter of which was resected in July 2007 followed by boost radiotherapy in October 2007. His disease remained stable until January 2009 when he was hospitalized for profound leukocytosis

secondary to rapid disease progression and consequently died in March 2009.

Contrary to Patient IMF-32, Patient IMF-24 received only gp100 DNA prior to receiving immunotherapy and had no measurable immune response to HLA-A*0201 gp100^{209–217} following vaccination; however, on week 56 following ipilimumab initiation, the patient had a robust and durable gp100 antigen-specific tetramer response (Supplemental Fig. 4A). As with Patient IMF-32, these tetramer-positive cells were of the CCR7^{lo}CD45RA^{lo} effector-memory phenotype (Supplemental Fig. 4B). Samples from time points immediately prior to and following ipilimumab treatment were not available for analysis.

Patterns of gp100-specific CD8⁺ tetramer⁺ and gp100-specific CD8⁺ IFN- γ ⁺ T-cell production were similar in this patient, with both showing no significant increase following vaccination, but a strong and durable response after ipilimumab therapy (Supplemental Figs. 4A and 5, middle row). As with Patient IMF-32, the T cells cultured from Patient IMF-24 were also shown to express MIP-1 β and CD107a, respectively (Supplemental Fig. 5, middle and bottom row). Together with the phenotypic characterization of the gp100-specific tetramer-reactive cells, the polyfunctionality of these cells suggests that they possess significant effector function.

Case 3—Patient IMF-11

In April 2005, this 62-year-old woman presented with a 0.49 mm nodular malignant melanoma of the left calf with a positive sentinel lymph node biopsy. She entered a vaccine trial at New York University Langone Medical Center of recombinant human NY-ESO-1 protein (100 μ g; intradermal injection into 5% imiquimod cream pretreated skin) every 3 weeks for a total of four injections. In January 2006, the patient developed in-transit metastases on her leg. Despite treatment with sorafenib followed by two cycles of anti-integrin antibody therapy and then temozolomide chemotherapy, she continued to progress. In October 2006, a PET/CT scan demonstrated increasing size of her leg tumors along with an enlarged left external iliac lymph node.

In November 2006, with unresectable stage III cutaneous melanoma, she was initiated on ipilimumab therapy with the same dose regimen as the previous two patients. After only one treatment, she began to show a profound response evidenced by shrinking tumors, loss of associated pain, and improvement in overall quality of life. By February 2007, all tumors on both her leg and lymph node completely disappeared and her disease remains in remission for more than 3.5 years.

Tissue was biopsied from one of the cutaneous lesions that responded to ipilimumab therapy. Hematoxylin and

eosin (H&E) staining indicated a tumorous melanin-pigmented nodule infiltrated by macrophages and lymphocytes, but with no evidence of tumor cells or melanocytes. Immunostaining demonstrated that the lymphocytes were predominantly of the CD8⁺ subtype, with few cells staining positive for CD4; the macrophages were also weakly CD4 positive (Supplemental Fig. 6). The patient developed NY-ESO-1 antigen-specific antibodies and a CD4⁺ T-cell response after vaccination with NY-ESO-1 protein. An NY-ESO-1-specific CD8⁺ T-cell response was not detectable utilizing different in vitro cell culture methodology [40 (Patient 6)] [40]. During the course of anti-CTLA-4 antibody treatment, Patient IMF-11 did not have an NY-ESO-1 antibody response to recombinant NY-ESO-1 protein; however, a low-titer antibody response to NY-ESO-1 peptides was detected (data not shown). Intracellular cytokine staining of PBMCs revealed an increase in NY-ESO-1-specific CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺ T cells at week 30 following ipilimumab therapy (Fig. 3). Within these populations, there was also an increase in IFN- γ ⁺MIP-1 β ⁺, but not IFN- γ ⁺TNF- α ⁺, T cells.

Discussion

Unique insights into the antigen-specific immune effects of ipilimumab were provided by intensive monitoring of patients with metastatic melanoma who received prior vaccination against melanoma-specific antigens and subsequently received ipilimumab therapy. This comprehensive

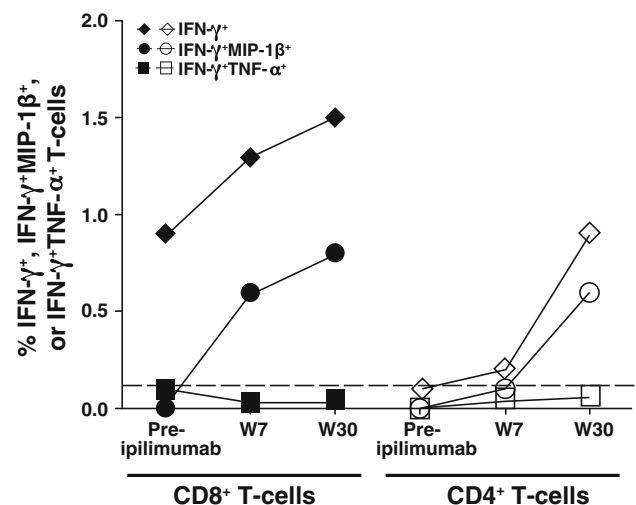


Fig. 3 Ipilimumab induced NY-ESO-1 antigen-specific CD4⁺ and CD8⁺ T-cell response in Patient IMF-11. NY-ESO-1-specific CD8⁺ and CD4⁺ T cells secrete IFN- γ and MIP-1 β , or IFN- γ and TNF- α , after 10-day culture and restimulation with 20-mer NY-ESO-1 overlapping peptides; CD8⁺IFN- γ ⁺, CD8⁺IFN- γ ⁺MIP-1 β ⁺, CD4⁺IFN- γ ⁺, and CD4⁺IFN- γ ⁺MIP-1 β ⁺ T cells increased after CTLA-4 blockade in Patient IMF-11

analysis highlights several novel observations and raises interesting questions. Since the patients received prior vaccination with either gp100 peptide or DNA or NY-ESO-1 protein with or without tyrosinase peptide, we were also able to determine the antigen-specific immunogenicity of these vaccinations and whether ipilimumab therapy affected immune responses to these tumor antigens. Both Patients IMF-32 and IMF-24 did not develop any tyrosinase-specific tetramer-reactive or IFN- γ ⁺ CD8⁺ responses following vaccination or at any time during ipilimumab therapy. In contrast, gp100-specific tetramer-reactive CD8⁺ cells, which were mildly detectable (Patient IMF-32; Patient IMF-24 [gp100^{280–288}]) or absent (Patient IMF-24 [gp100^{209–217}]) after vaccination, increased robustly during ipilimumab therapy, peaking at week 34 in Patient IMF-32 and remaining durable in Patient IMF-24 from week 56 to the most recent time point at week 123. This pattern was mirrored by a similar increase in CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺ responses, which was also true for the NY-ESO-1 antigen-specific T-cell response of IMF-11. The CD8⁺tetramer⁺ populations for the phenotypic markers CCR7, CD45RA, CD27, and CD28 were analyzed after a short-term (10-day) *in vitro* culture stimulation to avoid the expansion of CD8⁺CCR7⁺CD45RA[−] effector T cells that would result from use of a longer culture period [41]. Results suggest that these cells represent an effector-memory population, an observation further substantiated by the polyfunctional nature of these cells as evidenced by the expression of CD107a, TNF- α , and/or the chemokine MIP-1 β .

Patient IMF-32's primary tumor expressed tyrosinase, but not gp100, by IHC staining. When he was treated in the adjuvant setting with a gp100 and tyrosinase peptide vaccine (administered with GM-CSF DNA), he developed gp100-reactive, but not tyrosinase tetramer-reactive, CD8⁺ T cells. The absence of a tyrosinase-specific immune response to vaccination may be related directly to the moderate immunogenicity of the tyrosinase antigen. For example, in our tyrosinase trial, 7/18 patients (39%) produced an antigen-specific T-cell response [7]. An intriguing but speculative possibility is that tyrosinase-specific T_{regs} induced by tyrosinase expression on the primary tumor may exist that dampened any immune response to the tyrosinase peptide vaccine. In contrast, since gp100 was not expressed on the primary melanoma tissue, gp100-specific T_{regs} may not have been present, thereby enabling an immune response to the gp100 peptide vaccination. In support of this hypothesis, recent evidence suggests that antigen-specific T_{regs} occur in patients with melanoma [42]. Regardless of the cause, the absence of an immune response against tyrosinase or a moderate response against gp100 is consistent with the recurrence of disease in Patient IMF-32 8 months after vaccination. IHC of tumor tissue

from this and three subsequent recurrences confirmed persistent expression of tyrosinase, but not gp100. Another possible explanation for tumor escape from immune surveillance is the lack of expression of class I and II MHC molecules on the tumor tissue that would prevent effective antigen presentation to T cells.

Since none of the metastatic tumors analyzed expressed gp100, it is unclear why gp100-specific immune responses were only transiently enhanced by ipilimumab therapy. One possibility is that IHC was not sufficiently sensitive to detect the presence of low-level gp100 expression; real-time polymerase chain reaction, a more sensitive methodology, may have detected evidence of gp100 expression at the mRNA level. Another possibility is that regressing lesions, such as those seen in the lung, expressed gp100 which sustained the immune response whereas progressing lesions lost gp100, resulting in a dampened response when antigen was no longer being presented. A third possibility is that responses to gp100, normally expressed on melanocytes [43], may have been “primed” by the initial peptide vaccination and subsequently “boosted” by ipilimumab therapy. In this case, the induction of gp100-specific immune responses does not represent an anti-tumor mechanism per se, but merely reflects a non-specific activation of the immune system when CTLA-4 is inhibited. Whereas tumors from Patients IMF-24 and IMF-11 were not analyzed by IHC, the increase in T-cell response specific for the vaccine-targeted antigens seen following ipilimumab therapy may also lend support to the “prime-boost” hypothesis.

Currently, overall survival is the only validated endpoint for ipilimumab therapy. The clinical significance of the antigen-specific responses seen in this study is uncertain given the variability of outcomes in these three patients. Furthermore, although not tested, it is possible that the increase in cellular immune response was due to tumor shrinkage and loss of tumor-induced regulatory T cells rather than being a direct result of ipilimumab. Results of this study should also be interpreted with caution given the limited number of patients evaluated; however, the findings support the need for additional research to determine potential clinical implications of inducing immune responses by vaccination prior to administration of ipilimumab for treatment of advanced melanoma.

Although a gp100 vaccine did not demonstrate activity as monotherapy and did not enhance clinical response to ipilimumab in MDX010-20 [27], clinical trials evaluating different temporal combinations of ipilimumab with various vaccines are planned or ongoing in the adjuvant and metastatic setting in melanoma and prostate and pancreatic cancers [44]. Hopefully, these trials will shed light on if, how, and when ipilimumab should be combined with vaccine therapy.

Acknowledgments We thank Dr. Immanuel Luescher from Tetramer Core, Lausanne Branch, Ludwig Institute of Cancer Research, for providing the tetramers; Cora Mariano from the MSKCC Departments of Pathology for the collection of tumor tissues; and Hematology for the lymphocyte counts. This work was supported by Swim Across America, the Experimental Therapeutics Center of MSKCC and Ludwig Foundation. JDW was supported by a Damon Runyon-Lilly Clinical Investigator Award. JY was supported by MSKCC Pilot Grant of Grant number P50AT002779 from the National Center for Complementary and Alternative Medicine (NCCAM) and the Office of Dietary Supplements (ODS). Editorial and writing assistance was provided by StemScientific with funding from Bristol-Myers Squibb Company.

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