Published in final edited form as:

Cancer Immunol Immunother. 2013 August ; 62(8): 1327–1334. doi:10.1007/s00262-013-1433-7.

A Case of Spontaneous Systemic Immunity to Melanoma Associated with Cure after Amputation For Extensive Regional Recurrence

Joshua M. Judge¹, Louis B. Brill II², Kelly T. Smith¹, Donna H. Deacon¹, James W. Patterson³, William W. Grosh⁴, Achim A. Jungbluth⁵, Sacha Gnjatich⁶, and Craig L. Slingluff Jr.¹

¹ Department of Surgery, University of Virginia, Charlottesville, VA, USA

² Dominion Pathology Associates, Roanoke, VA, USA

³ Department of Pathology, University of Virginia, Charlottesville, VA, USA

⁴ Department of Medicine, University of Virginia, Charlottesville, VA, USA

⁵ Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York; NY, USA

⁶ Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA

Abstract

Purpose—Survival after amputation for melanoma is short; however, rare long-term survivors are reported. The mechanism for durable systemic tumor control in patients with regional failure is not known. To explore whether systemic tumor immunity may be implicated, tumor and circulating immune responses were examined in a patient who survived disease-free 14 years after hip disarticulation.

Methods—A 71 year-old female with extensive regional metastases of melanoma in the left lower extremity underwent amputation for palliative reasons. Tumor was collected at surgery, and blood was collected during follow-up. Tumor sections were evaluated for lymphocytic infiltration and NY-ESO-1 expression by immunohistochemistry. Cellular immune responses to defined tumor antigens were evaluated by ELISPOT assay, and antibody responses to a panel of tumor antigens were assayed by ELISA.

Results—The patient's tumor had minimal lymphocytic infiltrate (Immunotype A). NY-ESO-1 was strongly expressed by the melanoma cells. Circulating T cell responses to NY-ESO-1 peptides were observed 6 and 12 years postoperatively, and antibodies to NY-ESO-1 were detected 2-6 years after surgery.

Corresponding author: Craig L. Slingluff, Jr., M.D., Joseph Helms Farrow Professor, Division of Surgical Oncology, Department of Surgery, PO Box 800709, Charlottesville, VA 22908 USA. tel: (434) 924-1730, fax: (434) 243-6844, cls8h@virginia.edu. Joshua Judge: jmj8e@virginia.edu Louis Brill: lbrill@alumni.virginia.edu Kelly Smith: kts4v@virginia.edu Donna Deacon: dnmh9q@virginia.edu James W. Patterson: jwp9e@virginia.edu William W. Grosh: ww9u@virginia.edu Achim A. Jungbluth: jungblua@mskcc.org Sacha Gnjatich: sach.gnjatic@mssm.edu.

Conflict of Interest: The authors have no conflict of interest to disclose.

Conclusion—The patient described in this report experienced relentless regional tumor progression, with intravascular metastases, then 14-year systemic disease-free survival after palliative resection, without evidence of melanoma recurrence before death from other causes. Her immune response to NY-ESO-1 likely failed to control established regional metastases because T cells were unable to infiltrate them. It is possible, however, that among other factors, the host immune response may have contributed to systemic protection.

Keywords

Melanoma; amputation for melanoma; NY-ESO-1; tumor immunity; antibody; T-cell

Introduction

Amputation of a major extremity is rarely performed for management of melanoma. When performed, it is typically with palliative intent, and is associated with short survival [1,2]. However, some long term survivors have been reported [2,3]. The mechanism for failure of loco-regional disease control without progression to distant metastases remains a mystery. Presented here is a case of long-term survival in a patient who underwent palliative amputation with extensive loco-regional metastatic melanoma of the leg including gross blood vessel involvement. Investigation of serum and tumor from this patient demonstrated a systemic response to a melanoma tumor antigen, NY-ESO-1, but a seemingly paradoxical paucity of tumor infiltrating lymphocytes in tumor samples. Though these findings may be coincidental, spontaneous systemic tumor immunity with failure of local immune response represents one possible explanation for the clinical course described. If spontaneous systemic tumor immunity contributed to the long-term survival of the patient described, this phenomenon may have potential implications for cancer immunotherapy and surgical decision-making.

Case Report

In 1983, a 60 year old white female presented with a pigmented lesion on the skin of her anterior tibia. This lesion was biopsied, and histologic examination suggested the lesion was most consistent with an in-transit metastasis. No primary lesion was found. She was initially treated with wide excision of the lesion, but suffered multiple regional recurrences and intransit metastases of the left lower extremity, also treated with resection and inguinal lymph node dissection, with metastases in 4 of 8 femoral lymph nodes. She was subsequently treated with multiple rounds of chemotherapy including DTIC, BCNU, and cisplatin, as well as BCG injections to in-transit metastases.

The patient was first evaluated at our institution in 1994 at age 71, eleven years after initial diagnosis and subsequent to the treatments listed above. On evaluation, she presented with extensive regionally metastatic melanoma of the left lower extremity, with confluent dermal metastases of non-pigmented melanoma covering the vast majority of the skin surface of her left lower extremity, associated with lymphedema and cellulitis. A metastatic work-up was undertaken with computed tomography, magnetic resonance imaging, and bone scan, demonstrating no evidence of disease beyond the left lower extremity. However, magnetic

resonance imaging of the affected extremity revealed many soft tissue nodules in the skin and subcutaneous fat (**Figure 1**). This imaging underestimates the clinical extent of disease, which grossly consisted of a continuous sheet of nodular tumor tissue involving all the skin of the anterior aspect of the left lower extremity up to a level about 3 cm below the inguinal ligament. During this period of evaluation, the patient was admitted for multiple episodes of septic thrombophlebitis, and was maintained on Coumadin therapy. Given the presence of deep venous thrombosis, the extent of disease, the recent infectious complications, and her age, she was not considered a candidate for IL-2 or for isolated limb perfusion.

Following evaluation for possible limb perfusion therapy, the patient continued to require serial hospitalizations for cellulitis of the affected extremity, septic thrombophlebitis, and gastrointestinal bleed related to anti-coagulation therapy. Given these complications and her inability to remain liberated from inpatient care, palliative hip disarticulation was offered to the patient, to remove the affected extremity, which she accepted. Every attempt was made to obtain a clear margin during the operation; however during ligation of the femoral vein, a suspicious lymph node was noted at the proximal resection margin. That node was excised. It was not feasible to re-excite to a higher margin. The patient had an uneventful post-operative recovery. Pathologic evaluation of the extremity demonstrated a confluent tumor mass of melanoma overlying the anterior leg and multiple subcutaneous nodules composed of highly atypical melanocytes, some with epithelioid features. Frequent intranuclear pseudoinclusions and mitotic figures were identified. Perineural invasion was not seen, however; vascular invasion was found present in multiple areas, involving both large and small blood vessels. Representative sections are seen in **Figure 2**, demonstrating vascular invasion with intraluminal tumor (**Figure 2**). Multiple femoral lymph nodes, including the resected lymph node at the proximal margin, were positive for melanoma as well.

The patient was followed by her oncologist (WWG) every three months, with imaging at least yearly. She remained active and clinically free of melanoma recurrence until last follow-up. Ten years after her hip disarticulation, the patient experienced vaginal bleeding and was found to have poorly-differentiated serous papillary carcinoma of the uterus, treated with hysterectomy and bilateral salpingo-oophorectomy. Subsequently, she developed pulmonary nodules, retroperitoneal adenopathy, and supraclavicular adenopathy. The supraclavicular nodes were biopsied and found to contain metastatic serous papillary carcinoma. Thus, she was treated for metastatic uterine cancer, with taxol and carboplatin; however, her disease progressed and she expired of widely metastatic serous papillary uterine cancer at age 85, with no evidence of melanoma recurrence 14 years following her hip disarticulation and 25 years after diagnosis of melanoma of unknown primary origin.

Methods

To identify factors that may have played a role in the long survival of this patient, despite the poor prognosis associated with her presentation, banked peripheral blood mononuclear cells (PBMC) and tumor were evaluated for evidence of tumor immunity and tumor antigen expression.

Immunohistochemical assessment

Paraffin-embedded metastatic tumor samples from the patient's amputation were obtained and assessed for lymphocytic infiltrate and expression of cancer antigens. Representative sections were double stained for CD45 and CD31. Single stains were also performed for CD4, CD8, CD20, CD68, NKp46, and peripheral node addressin (PNAd). Tissue sections were deparaffinized and then rehydrated using xylene and a graded alcohol series. For CD31/CD45 double staining, antigen retrieval was performed in Dako Target Retrieval Solution, pH 9 (Dako North America, Carpinteria, CA) at 100°C for 20 minutes. Sections were then incubated for 60 minutes at room temperature with antibodies to CD31 (1:100, Dako) and CD45 (1:200, Dako). CD31 was detected using an alkaline phosphatase kit (AK-5002, Vector Laboratories, Burlingame, CA) and was visualized with Vector Blue. CD45 was detected using the horseradish peroxidase kit (MP-7402, Vector) and was visualized with VIP, Purple.

Dako Target Retrieval Solution, pH 9, was also used with stains for CD4, CD8, and NKp46. Vector's Target Retrieval Solution, pH 6, was used for CD20, CD68, and PNAd. Following antigen retrieval at 100°C for 20 minutes, sections were incubated 60 min at room temperature with antibodies to CD20 (1:200), CD8 (1:200), and CD68 (1:50) from Dako, CD4 (1:120, Vector), NKp46 (1:60, R&D, Minneapolis, MN) or PNAd (1:25, Pharmingen, San Jose, CA). Vector's ImmPRESS (Peroxidase) kits were used for CD4, CD8, CD20, CD68, and NKp46. The peroxidase was developed by 3-Amino-9-Ethylcarbazole (AEC substrate kit: Vector) or 3,3' Diaminobenzidine (DAB substrate kit: Dako); Vector's Alkaline Phosphatase ABC kit was used for PNAd, with Vector Red substrate. Slides were counterstained with Gill's hematoxylin (Vector). Tissue control slides included lymph nodes and/or tonsils. Negative control slides were obtained by omitting the primary antibodies.

Based on the CD45/CD31 double-stain, the degree of immune cell infiltrate was scored and assigned an immunotype as has been previously described, based on the architectural patterns of those infiltrates relative to intratumoral blood vessels [4]. Additional sections of tumor were stained for NY-ESO-1 expression, using mAb E978, generated in our lab previously. Preceding the primary application, slides underwent antigen-retrieval by heating 30 minutes (98°C) in a buffer solution (hipH solution, DAKO, Carpinteria, CA). E978 was applied overnight at 5°C. As a secondary system, the Powervision kit (Leica Microsystems, Buffalo Grove, IL) was used. Diaminobenzidine (DAB, Biogenex, San Ramon, CA) served as a chromogen. Slides were counterstained with Gill's hematoxylin. Testis with intact spermiogenesis was used as a positive control tissue.

Antigen Recognition and Serology

To investigate the possible role of cell-based systemic tumor immunity, direct interferon-gamma (IFN- γ) ELISpot assays, using pools of peptides, were performed on PBMC isolated from cryopreserved blood collected 6 years and 12 years after amputation. Peptide pools were composed of 15mers, with 11 amino acid overlap derived from 8 immunogenic tumor antigens (gp100, MAGE-A1, MAGE-A3, MAGE-A4, Melan-A/MART1, NY-ESO-1, tyrosinase, and tyrosinase-related protein 2), a positive control (CMV pp65 peptide pool), and one negative control (HIV gag). These pools were purchased from JPT Peptide

Technologies GmbH, Berlin, Germany. A mixture of short viral peptides (CEF peptide pool) was used as another positive control, and HIV gag peptide was used as a negative control. Titration assays demonstrated 4 µg/mL final concentrations to be optimal for detection of response with pools of 15-mer peptides; this concentration was used for the assay data presented. Methods for direct ELISPOT have been reported [5]. Assays were completed in triplicate and averaged for each pool.

Banked samples of plasma and serum from whole blood, stored at -80°C, were available at 3 time points; 1.75, 5.25, and 6 years after amputation (plasma July 1996, and Feb 2000; serum, Oct 2000). A series of full-length recombinant well-defined tumor antigen proteins (including NY-ESO-1, LAGE-1, p53, ATF2, MELAN-A, MAGE-A1, MAGE-A3, MAGEA4, MAGE-A10, SSX1, SSX2, SSX4, SOX2, CT7, CT10, CT45, CT46, CT47, XAGE-1, GAGE2, PASD1, CSAG2, TRAG3, ERG, NXF2, SAGE1, ACTL8, CXorf48, UBQLN2) and a control protein (dihydrofolate reductase [DHFR]) were used to coat plates and to measure specific serum antibody levels in ELISA as described previously [6]. In some experiments, 68-mer synthetic long overlapping peptides from NY-ESO-1 were used as the coating antigen. Plasma/sera were tested in fourfold serial dilutions, and bound IgG reactivity was detected using Alkaline phosphatase (AP)-labeled goat-anti-human IgG (polyclonal antisera; Southern Biotech). A reciprocal titer was estimated from optical density readings of serially diluted plasma/serum samples as described [6]. Negative control sera from healthy individual and positive control sera for each antigen from cancer patients were included in all assays. The plasma samples had been diluted with media to approximately 3x their original volume during initial collection; this was accounted for in testing and calculation of antibody titers. The serum was full concentration. To be considered significant, reciprocal titers had to be > 100. Assays were repeated in at least 3 independent experiments with reproducible results.

Results

Immunohistochemical assessment

The CD45/CD31 double stain revealed a paucity of tumor infiltrating lymphocytes (**Figure 3a,c**); perivascular cuffing was seen only very rarely (**Figure 3b,d**). This was most consistent with immunotype A [4]. Representative sections are displayed in **Figure 3**. Sections of a tumor nodule stained for other immune cell markers revealed no significant infiltrate of CD4⁺ or CD8⁺ T cells, of B cells (CD20) or NK cells (NKp46); CD68 staining highlighted endothelial cells[7], but macrophages were not evident (**Figure 4a**). There was a peritumoral focus of immune cells that included a dense aggregate of B cells, and adjacent T cells (CD4⁺ and CD8⁺), small numbers of NK cells, and a prominent macrophage population; there were prominent vessels in this aggregate (**Figure 4b**), but they did not stain for PNAd (not shown). Each of these cell types were also represented among the rare perivascular cells elsewhere in the tumor, but these were not organized (data not shown).

Staining for NY-ESO-1 demonstrated intense positivity (3+) of 50-75% of the tumor cells, with some areas of heterogeneous staining. Representative sections are displayed in **Figure 5**.

T-cell reactivity to melanoma antigens

In the IFN-gamma ELISpot assays, high T cell reactivity was observed to the NY-ESO-1 peptide pool (65 fold higher than the negative control), but consistent reactivity to the other peptides was not detected (**Figure 5c**). Similar findings were evident in both PBMC samples from about 6 and 12 years after surgery.

Antibodies to melanoma antigens

High antibody titers were identified at all three time points (1.75 – 6 years after surgery) to NY-ESO-1 (titers 501 to 1324) and less strongly to homolog LAGE-1 (titers 22 to 153, **Table 1**). Reactivity was not observed to p53, ATF2, MELAN-A, MAGE-A1, MAGE-A3, MAGE-A4, MAGE-A10, SSX1, SSX2, SSX4, SOX2, CT7, CT10, CT45, CT46, CT47, XAGE-1, GAGE2, PASD1, CSAG2, TRAG3, ERG, NXF2, SAGE1, ACTL8, CXorf48, UBQLN2, or DHFR (data not shown). Reactivity to NY-ESO-1 was further evaluated by testing for antibodies to long peptide fragments of that protein: dominant reactivity was observed to ESO pep1 (amino acid residues 1-68), with low reactivity to ESO pep2 (amino acid residues 57-124).

Discussion

Long-term survival after palliative extremity amputation for melanoma is dismal. Few cases have been reported in the recent literature. In a series of 15 patients undergoing palliative amputation, no patient survived longer than 33 months [2]. In patients undergoing limb amputation for melanoma with curative intent, 5-year survival estimates range from 0 to 37% [1,2,8]. Conversely, there are rare reports of relatively long-term survival, including one patient reported by the Danish Amputation Registry who died 10 years after amputation, of unrelated cancer [3]. Such long-term survivors present a biologic paradox, where failure to combat disease regionally is coincident with unknown mechanisms preventing distant metastasis. This is especially apparent in our patient, in whom the presence of nodal metastasis and extensive local tumor burden confirm aggressive tumor biology, and in whom the degree of vascular invasion almost certainly led to the presence of circulating tumor cells. Despite the extensive presence of intravascular tumor cells, masquerading as deep venous thrombosis for months to years, this patient never developed systemic metastases, and lived more than a decade, to die of another cause at advanced age.

Spontaneous control of systemic dissemination of melanoma naturally raises a question of whether the patient's immune response to melanoma played a role. Surprisingly, there was a striking lack of lymphocyte infiltrate in the regional tumor specimen. Brisk lymphocytic infiltration has been associated with lower rates of metastasis and death from primary melanomas [9,10], and this phenomenon has also been demonstrated in colorectal cancer and ovarian cancer [11-13]. In metastatic melanoma, patients with diffuse lymphocytic infiltrate (Immunotype C) demonstrated improved outcomes, whereas those with few lymphocytes, or limited to perivascular infiltration (immunotypes A and B) had lower rates of long term survival[4]. The CD45/CD31 staining pattern of the tumor in our patient would indicate an immunotype A lesion, further decreasing her expected survival. Another interesting finding is the peritumoral aggregate of immune cells, which contained a B-cell

area and associated T cells. This resembles a lymph node, though the lack of PNAd differentiates it from a true normal lymph node. It may represent a tertiary lymphoid organ. Similar structures have been identified in association with melanoma metastases having good prognosis[14] and in association with lung cancers with improved prognosis[15], though the precise mechanism is not entirely clear.

We postulate that a systemic immune response to melanoma antigens may have prevented distant spread, but may have failed to control disease locally because circulating antitumor effector T cells were unable to infiltrate established metastases. This hypothesis is supported by the finding of T cell and antibody reactivity to NY-ESO-1, which was durable 2-12 years after amputation of the limb and was evident many years before the development of endometrial cancer. NY-ESO-1 is a cancer/testis antigen that is expressed in 32 to 47% of metastatic melanomas including the patient presented here [16,17]. NY-ESO-1 has been investigated as a target in immune therapies and has generated interest due to the findings that NY-ESO-1 antibodies and T cell responses correlate with clinical benefit in patients treated with CTLA4 blockade [18].

Melanoma metastasis and systemic progression are governed by many factors, of which antitumor immunity is just one. The data presented here do not address other factors that may have contributed to the observed clinical course, and these data are limited by their retrospective nature and by the quantity and timing of specimens available. Nevertheless, the findings support speculation that systemic tumor immunity may have played a role in the patient's good outcome. It is possible that spontaneous antitumor immunity may contribute to systemic protection and clinical tumor control after surgery. Further study is warranted, to explore whether spontaneous antitumor immunity may help to identify patients who may benefit from surgical therapy of advanced melanoma.

Acknowledgments

We would like to acknowledge Christine Sedrak, Rita Chiu, Cheryl Murphy Chase, and Nadia Galeassi for excellent technical assistance.

Funding Support: Funding support provided by the University of Virginia Cancer Center, The Cancer Research Institute, and the Ludwig Institute for Cancer Research.

Reference List

1. Kourtesis GJ, McCarthy WH, Milton GW. Major amputations for melanoma. *Aust N Z J Surg.* 1983; 53:241–4. [PubMed: 6192800]
2. Jaques DP, Coit DG, Brennan MF. Major amputation for advanced malignant melanoma. *Surg Gynecol Obstet.* 1989; 169:1–6. [PubMed: 2472674]
3. Ebskov LB. Major amputation for malignant melanoma: an epidemiological study. *J Surg Oncol.* 1993; 52:89–91. [PubMed: 7682264]
4. Erdag G, Schaefer JT, Smolkin ME, Deacon DH, Shea S, Dengel LT, Patterson JW, Slingluff CL Jr. Immunotype and immunohistologic characteristics of tumor infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Res.* 2012; 72:1070–80. 2012. [PubMed: 22266112]
5. Slingluff CL Jr. Petroni GR, Olson WC, Smolkin ME, Ross MI, Haas NB, Grosh WW, Boisvert ME, Kirkwood JM, Chianese-Bullock KA. Effect of GM-CSF on circulating CD8⁺ and CD4⁺ T

- cell responses to a multi-peptide melanoma vaccine: Outcome of a multicenter randomized trial. *Clin Cancer Res.* 2009; 15:7036–44. [PubMed: 19903780]
6. Gnjatic S, Old LJ, Chen YT. Autoantibodies against cancer antigens. *Methods Mol Biol.* 2009; 520:11–9. doi: 10.1007/978-1-60327-811-9_2.11-9. [PubMed: 19381944]
 7. Gottfried E, Kunz-Schughart LA, Weber A, Rehli M, Peuker A, Muller A, Kastenberger M, Brockhoff G, Andreesen R, Kreutz M. Expression of CD68 in non-myeloid cell types. *Scand J Immunol.* 2008; 67:453–63. [PubMed: 18405323]
 8. Cox KR. Survival after amputation for recurrent melanoma. *Surg Gynecol Obstet.* 1974; 139:720–2. 1974. [PubMed: 4428330]
 9. Clark WH Jr, Elder DE, Guerry D4, Braitman LE, Trock BJ, Schultz D, Synnestvedt M, Halpern AC. Model predicting survival in stage I melanoma based on tumor progression. *Journal of the National Cancer Institute.* 1989; 81:1893–904. [PubMed: 2593166]
 10. Clemente CG, Mihm MC Jr, Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. *Cancer.* 1996; 77:1303–10. [PubMed: 8608507]
 11. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P, Zinzindohoue F, Bruneval P, Cugnenc PH, Trajanoski Z, Fridman WH, Pages F. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science.* 2006; 313:1960–4. [PubMed: 17008531]
 12. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN, Rubin SC, Coukos G. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med.* 2003; 348:203–13. [PubMed: 12529460]
 13. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C, Kepner J, Odunsi T, Ritter G, Lele S, Chen YT, Ohtani H, Old LJ, Odunsi K. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A.* 2005; 102:18538–43. [PubMed: 16344461]
 14. Messina JL, Fenstermacher DA, Eschrich S, Qu X, Berglund AE, Lloyd MC, Schell MJ, Sondak VK, Weber JS, Mule JJ. 12-Chemokine gene signature identifies lymph node-like structures in melanoma: potential for patient selection for immunotherapy? *Sci Rep.* 2012; 2:765. doi: 10.1038/srep00765. Epub; 2012 Oct 24.:765. [PubMed: 23097687]
 15. de Chaisemartin L, Goc J, Damotte D, Validire P, Magdeleinat P, Alifano M, Cremer I, Fridman WH, Sautes-Fridman C, Dieu-Nosjean MC. Characterization of Chemokines and Adhesion Molecules Associated with T cell Presence in Tertiary Lymphoid Structures in Human Lung Cancer. *Cancer Res.* 2011; 71:6391–9. [PubMed: 21900403]
 16. Velazquez EF, Jungbluth AA, Yancovitz M, Gnjatic S, Adams S, O'Neill D, Zavilevich K, Albukh T, Christos P, Mazumdar M, Pavlick A, Polsky D, Shapiro R, Berman R, Spira J, Busam K, Osman I, Bhardwaj N. Expression of the cancer/testis antigen NY-ESO-1 in primary and metastatic malignant melanoma (MM)--correlation with prognostic factors. *Cancer Immun.* 2007; 7:11. 11. [PubMed: 17625806]
 17. Goydos JS, Patel M, Shih W. NY-ESO-1 and CTp11 expression may correlate with stage of progression in melanoma. *J Surg Res.* 2001; 98:76–80. [PubMed: 11397121]
 18. Yuan J, Adamow M, Ginsberg BA, Rasalan TS, Ritter E, Gallardo HF, Xu Y, Pogoriler E, Terzulli SL, Kuk D, Panageas KS, Ritter G, Sznol M, Halaban R, Jungbluth AA, Allison JP, Old LJ, Wolchok JD, Gnjatic S. Integrated NY-ESO-1 antibody and CD8+ T-cell responses correlate with clinical benefit in advanced melanoma patients treated with ipilimumab. *Proc Natl Acad Sci U S A.* 2011; 108:16723–8. [PubMed: 21933959]

Precis

We report long term survival in a patient after palliative amputation for melanoma. The patient had immune responses to NY-ESO-1 but immune cells failed to infiltrate tumor. The potential role of the immune response in her outcome is discussed.

Figure 1. Radiologic appearance of extensive regional metastasis of melanoma

MRI image prior to amputation demonstrating marked edema, skin thickening consistent with cutaneous tumor, and tumor nodules throughout the left lower extremity, compared with the normal right lower extremity.

Figure 2. Vascular invasion of metastatic melanoma in the left lower extremity
Representative histologic sections from the amputation specimen demonstrating metastatic melanoma with extensive vascular invasion.

Figure 3. Rare intratumoral immune cells

CD31 (blue) and CD45 (purple) stain of tumor sections for immunotyping at lower (**a,b**) magnification and higher (**c,d**) magnification. Images **a** and **c** are representative of the vast majority of the specimen and are consistent with immunotype A. Images **b** and **d** show small areas of perivascular cuffing consistent with immunotype B, which were rare in this specimen.

a [main tumor mass] **b [peritumoral infiltrate]**

Figure 4. Immunohistochemical evaluation of intratumoral and peritumoral immune cells
 Metastatic melanoma sections were stained for CD4, CD8, CD20, CD45/CD31, NKp46, and CD68: **a**, representative sections of a dominant tumor mass without significant immune cell infiltrate; **b**, sections of a peritumoral immune cell aggregate.

Figure 5. NY-ESO-1 expression by tumor and reactivity by T cells

Immunohistochemical stain of tumor sections demonstrate diffuse NY-ESO-1 expression by the melanoma cells (a,b). ELISpot data from PBMC 6 years after surgery (c); reactivity is based on a direct ELISpot assay, with pools of overlapping 15-mer peptides from each protein listed on the X-axis. Positive controls include reactivity to CEF peptides (63.5 ± 2.5 spots) and CMV pp65 peptide pools (341 ± 2.5 spots). Error bars represent standard deviation among triplicate wells. These data are representative of several repeat experiments.

Table 1

Antibody titers to selected melanoma antigens 2-6 years after surgery

Sample Date	Sample type	Tumor antigen				Control Ag
		NY-ESO-1	ESO pep1 ^a	ESO pep2 ^b	ESO pep3 ^c	
Jul-1996	Plasma	1324	906	236	0	0
Feb-2000	Plasma	501	787	2	0	0
Oct-2000	Serum	553	355	3	0	0

^a ESO pep1 = amino acid residues 1-68 of NY-ESO-1

^b ESO pep2 = amino acid residues 57-124 of NY-ESO-1

^c ESO pep3= amino acid residues 113-180 of NY-ESO-1