Title

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Journal

Annals of Surgical Oncology, 27(11)

ISSN

1068-9265

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Publication Date

2020-10-01

DOI

10.1245/s10434-020-08648-7

Peer reviewed

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Journal:	*Annals of Surgical Oncology*
Manuscript ID	ASO-2020-04-1048
Manuscript Type:	Original Article
Date Submitted by the Author:	22-Apr-2020
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Tumor immune profiling based neoadjuvant immunotherapy for locally advanced melanoma

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Running title (45 characters including spaces MAX):
Neoadjuvant immunotherapy for melanoma (40)

Conflict of Interest and Sources of Funding:
A.P.A discloses institutional research funding from Novartis, Merck, BMS OncoSec, Acerta, AstraZeneca, MedImmune, Tessa, Celldex, and Celgene, consulting with stock options for Oncosec and Valitor, paid advisory board positions for Array and Regeneron, and an unpaid advisory role with Sensei Biotherapeutics. A.I.D discloses research funding from the Amoroso and Cook fund, Parker Institute for Cancer Immunotherapy, Novartis, Merck, BMS, Incyte, AbbVie, OncoSec, Xencor, Pfizer, Roche/Genentech and Exelixis, Advisory Board participation from Amgen, Array, Roche/Genentech and stock ownership in Trex Bio and Pionyr Immunotherapeutics. M.F.K. is the founder and board member of Pionyr Immunotherapeutics. L.S.L has received research funding from the Conquer Cancer Foundation CA-0122026. K.M.M. received support by the NIH T32 training grant in Gastrointestinal Surgery T32DK007573, M.D.R is a consultant with equity ownership for Trex Bio and holds equity in Sitryx Bio and has received funding on grants NIH DP2-AR068130, K08-AR062064, AR066821 Burroughs Wellcome CAMS-1010934. M.H.S. discloses research funding from Genentech/Roche, Pfizer, Valitor Inc., and Bristol-Myers Squibb, and is a paid consultant for Five Prime Therapeutics, Ono Pharmaceutical, and January, Inc. K.K.T. discloses institutional research funding from BMS, Parker Institute for Cancer Immunotherapy, Oncosec and Regeneron. The remaining authors have declared that no conflict of interest exists.

Synopsis:

In this retrospective study, patients with locally advanced Stage III melanoma underwent biopsy for flow cytometric analysis of exhausted tumor-infiltrating lymphocytes (Tex). Baseline Tex frequency was highly predictive of response to neoadjuvant single-agent or combination checkpoint blockade. Immune profile directed neoadjuvant immunotherapy resulted in favorable outcomes with minimal adverse events.

Structured Abstract:

Background: The frequency of "exhausted" or checkpoint-positive (PD-1$^+$CTLA-4$^+$) cytotoxic lymphocytes (Tex) in the tumor microenvironment (TME) is associated with response to anti–PD-1 therapy in metastatic melanoma. In the current study, we determined if pretreatment Tex cells in locally advanced melanoma predicted response to neoadjuvant anti-PD-1 blockade.

Methods: Pretreatment tumor samples from 17 patients with locally advanced melanoma underwent flow cytometric analysis for pretreatment Tex and regulatory T cell (Treg) frequency. Patients who met criteria for neoadjuvant checkpoint blockade were treated with either PD-1 monotherapy or PD-1/CTLA-4 combination therapy. Best Overall Response (BOR) was evaluated by RECIST v1.1, RFS was calculated by Kaplan Meier test and incidence and severity of adverse events were tabulated by clinicians using the NCI CTCAE v4.

Results: Of the neoadjuvant treated patients, 10 patients received anti-PD-1 monotherapy and seven received anti-CTLA-4/PD-1 combination therapy. Twelve of 17 achieved a complete response, four achieved partial responses, and one patient exhibited stable disease. Eleven of 17 underwent

subsequent surgery and eight attained a complete pathologic response. Median RFS and OS were not reached. Immune-related adverse events (irAEs) included four grade 3 or 4 events, including pneumonitis, transaminitis, and anaphylaxis.

Discussion: Our results show a high rate of objective response, relapse free and overall survival in patients undergoing immune profile directed neoadjuvant immunotherapy in locally advanced melanoma. Furthermore, treatment stratification based upon Tex frequency can potentially limit the adverse events associated with combination immunotherapy. These data merit further investigation with a larger validation study.

Introduction:

Immunotherapy by both CTLA-4 and PD-1 blockade induces durable antitumor immune responses and has recently revolutionized the treatment of melanoma and other malignancies. This has translated to a dramatic improvement in survival in the metastatic setting (1–6), prompting clinical trials of adjuvant immunotherapy for regionally metastatic melanoma. The benefit of adjuvant checkpoint blockade has been confirmed in two prospective clinical trials with significant prolongation of recurrence free survival (7,8). Neoadjuvant immune and targeted therapy for melanoma have been explored recently in clinical trials (9) and offer several advantages over surgery followed by adjuvant therapy. These include reduced surgical morbidity, better evaluation of outcome, as well as reduced duration of treatment and consequently reduced side effects from systemic therapy.

A randomized neoadjuvant phase II study examining the use of anti-PD-1 alone or anti-PD-1/CTLA-4 combination therapy for high risk resectable Stage III melanoma further indicated the feasibility of this approach, with anti-PD-1 monotherapy eliciting modest responses with an overall response rate (ORR) of 25% and rate of a complete pathologic response (pCR) of 25%. Toxicity was low with 8% of patients having immune-related adverse effects (irAEs) (10). While combined ipilimumab and nivolumab augmented clinical responses (ORR 73%, pCR 45% by RECIST), this was at the expense of substantial toxicity, with 73% of patients experiencing grade 3 irAEs (10). Another prospective study comparing combined CTLA-4 and PD-1 blockade in the neoadjuvant and adjuvant setting reported similar findings, with pCR observed in 78% of patients in the neoadjuvant arm (11). However, 90% of patients in both cohorts experienced one or more grade 3 or 4 irAEs.

Given the modest response rates with PD-1 monotherapy and substantial toxicity associated with combination checkpoint blockade, a genuine concern lies in delaying surgery to pursue systemic treatment in the setting of potentially curable regional disease. Several biomarkers have been

evaluated as predictors of immune response (12–15). Recent research has shown that the presence of exhausted T lymphocytes (Tex), which are T cells characterized by a dysfunctional state along with high expression of inhibitory receptors such as PD-1, correlates with response to anti-PD-1 mono- or combination therapy (16–18). Prior functional analyses indicate that Tex cells can be reinvigorated by immune checkpoint blockade (16,19–21). In this study, we examine the efficacy of neoadjuvant immunotherapy guided by immune-profiling and T cell analysis.

Methods

Study design and tumor sample acquisition

We performed a retrospective study of patients with locally advanced stage III or stage IV oligometastatic melanoma that received neoadjuvant immunotherapy prior to intended surgical resection of their disease. To be included in our study, patients were required to have a pre-treatment biopsy of their primary or locoregional disease within 30 days prior to the initiation of treatment. Samples were obtained after patients provided written consent under the UCSF Committee on Human Research Protocol 138510. Specimens were procured via core biopsy with a 16- or 18-gauge needle or punch biopsy with a 4mm punch tool under sterile precautions. Fresh tumor samples were immediately placed on ice and transported to the laboratory for dissociation and analysis. Between April 2013 and August 2018, 18 patients with locally advanced melanoma who received immunotherapy with neoadjuvant intent underwent pretreatment biopsy for immune-profiling and Tex frequency determination as discussed in the flow cytometric analysis methods section below. One patient was excluded on the basis of inadequate numbers of cells for analysis, defined as fewer than 200 $CD8^+$ T cells within the live $CD3^+CD45^+$ gate. Of the patients with interpretable tumor infiltrating lymphocyte profiling, nine patients received neoadjuvant treatment with anti-PD-1 Ab monotherapy

(nivolumab or pembrolizumab), one patient underwent treatment with anti-PD-L1 Ab, and 7 patients were treated with nivolumab in combination with ipilimumab. Treatment disposition was primarily determined by Tex frequency, with exceptions relating to patient preference or anticipated intolerance of combination immunotherapy.

Treatment outcome groups, efficacy, and adverse events analysis

Efficacy and immunological data available as of August 2018 were included in all the analyses. The efficacy analysis was limited to best overall response (BOR), defined as the best tumor response according to RECIST v1.1 criteria from the start of treatment to the time of disease progression or death. Recurrence free survival (RFS) was defined as the interval between the date of definitive surgery or complete response and the date of progression or death or the last clinic visit date for which the patient was known not to have had radiographic or clinical progression. Overall survival (OS) was calculated as the time from the date of enrollment to the time of death or to the last known date that the patient was known to be alive. Immune-related adverse events (irAE) of any grade that occurred after initiation of neoadjuvant immunotherapy were extracted by retrospective chart review. The investigators determined the relatedness of an adverse event to treatment. The severity of adverse events was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 4.0.

Flow cytometric analysis

Multiparameter flow cytometry was performed and analyzed on pretreatment samples obtained from metastatic tumors as previously described (17,18). Freshly isolated samples were minced and digested overnight with buffer consisting of collagenase type 4; (4188 Worthington Biochemical Corp.), DNAse (SDN25-1G; Sigma-Aldrich), 10% FBS, 1% HEPES, and 1% penicillin-streptavidin in

RPMI medium. Single-cell suspensions were double-filtered, centrifuged, and counted. Approximately 2 x 10^6 cells were stained with multiple fluorochrome-conjugated mAbs. The following Abs were used all from eBioscience, unless otherwise stated: anti-human CD3 (anti-hCD3) (UCHT1) ; anti-hCD8 (RPA-T8); anti-hCD45 (HI30); anti-CD4 (SK3); anti-FOXP3 (PCH101); anti–hCTLA4 (14D3); anti–PD-1 (EH12.2H7; BioLegend); and LIVE/DEAD Fixable Aqua Dead Cell Stain Life Technologies, Thermo Fisher Scientific. Data were acquired by an LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc.). All the samples were fresh and acquired by the LSRFortessa at different time points. Sphero Ultra Rainbow Beads (Spherotech) were used to calibrate and normalize to baseline intensity voltages as samples were obtained at different time points. Gating strategy was determined using both isotype control Ab staining and an internal negative control cell population i.e., PD-1 and CTLA-4 expression on CD3- cells. The frequency of exhausted T lymphocytes (Tex) within pretreatment samples was determined by calculating the percentage of $CD8^+$ T cells that expressed both inhibitory receptors PD-1 and CTLA-4 within the total intratumoral $CD8^+$ T cell population that was present. T regulatory cells were defined as $CD4^+CD25^+Foxp3^+$ T cells, and their proportion was calculated by comparing their frequency to the total intratumoral $CD4^+$ T cells population.

Statistics

Waterfall and spider plots were constructed with Prism (Graphpad), version 8. Patients had radiographic measurement of their target lesions evaluated at baseline and throughout their treatment. Change in tumor burden is defined as the percentage decrease in the sum of the reference diameters of the target lesion from baseline to nadir, observed up until the date of progression, as assessed by the investigator per RECIST version 1.1, the date of subsequent anticancer therapy (including tumor-directed surgery) or death, whichever occurred first. RFS and OS curves were constructed with the Kaplan-Meier method using Prism (Graphpad), version 8. Progression was

recorded as the date of scans showing progression or on the date clinical progression or death was noted. All tests were two-sided, with *P* values of less than 0.05 considered statistically significant.

Results:

Patient Characteristics and disposition

A total of 17 patients with locally advanced melanoma met inclusion criteria for analysis. All patients received neoadjuvant immunotherapy. Baseline characteristics and demographics are reported in Table 1. In the neoadjuvant cohort, 88.2% of the patients were classified as stage III disease, and 82.4% originated from a cutaneous primary. The two patients with stage IV disease who underwent neoadjuvant therapy had limited metastatic disease and were considered potentially operable. Seventy-one percent of the patients were immunotherapy-naïve, and 17.6% of patients had previously received anti-CTLA-4 therapy with ipilimumab. Patient disposition was primarily determined by the intratumoral frequency of exhausted T cells (Tex), with a value greater than 20% predictive of a response to anti-PD-1 monotherapy and greater than 3% for combination anti-CTLA-4 and PD-1 therapy as previously reported (17,18). In keeping with these previously defined parameters, 10 patients were treated with either anti-PD-1 or anti-PD-L1 monotherapy, while the remaining seven patients received combination immunotherapy with anit-CTLA-4 and anti-PD-1 (Figure 1). T regulatory cell (Treg) abundance was also measured in pretreatment samples, and we sought to determine whether Tex and Treg frequencies were predictive of treatment response.

Other factors that influenced treatment disposition included patient preference and expected tolerance of immunotherapy. In one case, a 55-year-old man with Stage III V600E BRAF WT melanoma and HIV with a Tex frequency of 18.6% (Table S1, Patient 11) was felt to be high risk for immune-related adverse effects due to his HIV history. As his Tex frequency was marginal and risk of

adverse events was high, he received PD-1 monotherapy and achieved a complete response. Relatedly, two patients in our cohort received treatment with combination PD-1/CTLA-4 therapy due to patient and provider preference. The first patient was a 44-year-old man (Table S1, Patient 13) with Stage IIIC V600E BRAF WT melanoma who presented with a painful, rapidly enlarging axillary mass. Given the rapid progression of his disease and severity of his symptoms, combination PD-1/CTLA-4 therapy was initiated at the discretion of his treating physician per his preference in spite of a Tex frequency of 42.3%. Similarly, a 52-year-old woman with Stage IV V600E BRAF mutant melanoma with a Tex frequency of 34.1% (Table S1, Patient 12) presented with a solitary pulmonary metastasis and elected to pursue combination immunotherapy due to her circumstances. She elected to pursue the FDA-approved treatment with the highest reported efficacy, fully aware of the increased risk of immune-related adverse effects.

Patient outcomes with immune profile directed neoadjuvant immunotherapy

We have previously reported that the relative abundance of tumor infiltrating Tex cells correlates with response to anti-PD-1 antibodies both as monotherapy and in combination with ipilimumab in the metastatic setting. However, whether this metric is capable of predicting response in the context of advanced locoregional disease remains unknown. We therefore quantified Tex frequency and Best Overall Response (BOR) by RECIST v1.1 criteria to neoadjuvant immunotherapy either as anti-PD-1 monotherapy (*n*= 10) or in combination anti-CTLA-4 (*n*=7) (Table 2). Patients who received neoadjuvant immunotherapy were enriched for high Tex with a mean frequency of 25.7%. The overall response rate (ORR), determined by BOR, was 94.1% in comparison to the previously reported ORR of 42% for pembrolizumab, 40% for nivolumab, and 58% for combination therapy (8,9). Of the 16 patients who responded, 12 achieved a complete response (CR) and four patients were partial responders (PR). Six complete responders underwent subsequent resection of the initially

involved regional lymph node basin with no evidence of malignancy, confirming pathologic CR. An additional patient with a stable oligometastatic adrenal mass that was no longer FDG-avid also underwent resection of the residual mass, which demonstrated no evidence of viable tumor consistent with pathologic CR. The remaining five patients opted against surgery given a negative on-treatment biopsy pathologic result and a complete radiographic response. Of the four partial responders, three underwent consolidative resection while the remaining patient opted to pursue brachytherapy. In the illustrative example shown in Figure 2, a patient with Tex fraction of 6.54% responded to combination immunotherapy (Figure 2a), while a patient with a Tex fraction of 34.4% responded to PD-1 monotherapy (Figure 2b), confirming our previously reported observation that a lower Tex threshold is required to mount an effective antitumor immune response to combination therapy. The solitary patient that did not respond to neoadjuvant immunotherapy initially presented with locally advanced V600E BRAF mutant Stage IIIC disease and involvement of the right inguinal lymph node basin (Figure S1). He was begun on neoadjuvant PD-1 monotherapy for a pretreatment Tex of 33.1% and had a BOR of stable disease by RECIST v1.1 criteria. He therefore underwent surgical salvage of the involved lymph node basin, and surgical pathology was consistent with viable tumor. He relapsed locally one year later with the development of subcutaneous lesions of the right thigh. Subsequently, he was salvaged with the targeted MEK/BRAF inhibitors trametinib and dabrafenib and has remained disease free. While his suboptimal response to PD-1 immunotherapy remains unclear, it is notable that his pretreatment tumor biopsy had a markedly elevated Treg fraction at 41.1%.

Overall, the vast majority of patients experienced deep responses to treatment with reduction in tumor burden of over 50% from baseline (Figure 3a-b). Median RFS (defined as time of systemic treatment initiation to date of relapse or last known follow-up) was not reached (Figure 3c). All

patients receiving neoadjuvant immunotherapy were alive at time of last follow up (Figure 3d). Notably, in agreement with our previous data in metastatic patients, Treg frequency was not independently predictive of response to immunotherapy (data not shown).

Safety profile

As with any medical intervention, analysis of the risks as well as the benefits of the proposed approach must be carefully considered. Indeed, these calculations become even more critical when faced with the option of proceeding directly to definitive resection and potential cure in the absence of any further systemic therapy. We therefore assessed the toxicity profile of neoadjuvant immunotherapy in patients with advanced locoregional disease. Twelve of 17 (70.6%) patients experienced adverse effects while on systemic therapy (Table 3). However, clinically severe (grade 3 or 4) events were uncommon and observed in only three (17.6%) of patients on treatment, predominantly among those receiving combination immunotherapy. These clinically severe events include an infusion reaction to anti-PD-L1 monotherapy, and autoimmune mediated transaminitis and pneumonitis from the PD-1/CTLA-4 combination therapy. The most common grade 1 or 2 adverse effects reported included diarrhea (23.5%), arthralgias (17.6%) and pruritus (17.6%). Neoadjuvant treatment, especially anti-PD-1 antibody monotherapy, was generally well tolerated with an adverse event profile comparable to that of previously reported large phase III clinical studies (1-7).

Discussion:

The increasing utilization of immune checkpoint inhibitors has drastically changed the treatment algorithm for advanced melanoma. These gains were initially established in the metastatic setting and have now demonstrated great benefit in the adjuvant setting (1-4). As with any

intervention offered in a potentially curative setting, the benefit of improved survival must be carefully weighed against the risks of significant toxicity associated with these agents, and disease progression on treatment which could preclude surgical resection. Therefore, patient selection utilizing reliable predictive biomarkers will be imperative for the success of this strategy, as tumor PD-L1 expression level by immunohistochemical staining has not been validated in the neoadjuvant setting and has low resolution (3,4) in the context of metastatic disease.

Recent reports have shown that the frequency of Tex in freshly isolated melanoma tumors strongly correlated with response to both single agent and combination checkpoint blockade in advanced melanoma with a positive predictive value of 82% and negative predictive value of 100% (17,18). Indeed, patients with high Tex frequency receiving neoadjuvant immunotherapy achieved response rates in excess of those generally reported. Notably, five patients who achieved complete radiographic responses by RECIST 1.1 criteria declined surgery, and all remain disease free at median follow up of 27.6 months. Therefore, in our dataset, pCR and radiographic response by RECIST criteria appeared to serve as equally predictive markers of recurrence free response.

Neoadjuvant anti-PD-1 therapy was generally well tolerated, however irAEs of grade 3 severity or higher were noted in two out of the seven patients receiving neoadjuvant combination immunotherapy. While this toxicity profile may be acceptable in the metastatic setting, the risks of combined CTLA-4 and PD-1 blockade may not outweigh the survival benefits of this approach in the curative setting. Moreover, given the efficacy of combination immunotherapy in the context of metastatic disease (1-4), the risk of ipilimumab exposure with lower disease burden, particularly with locoregional disease, may not be justified. Overall, our data suggest that if Tex frequency is greater than 20%, PD-1 inhibitor monotherapy may be sufficient. However, a Tex frequency of less than 3% requires careful consideration of patient specific factors in the decision of

whether to pursue combination checkpoint blockade or proceed directly to surgical resection. Further investigation with a larger prospective cohort validation study will be required to address this question.

Recent work indicates that rapid, durable responses to even a single dose of nivolumab are associated with the early expansion of a Tex subset in the peripheral blood. At three weeks, these expanded cells have a similar immunophenotype to pre-treatment baseline cells, which is highly suggestive of systemic reinvigoration of a pre-existing Tex cell pool (16). This observation is also in agreement with the increased T cell diversity and higher clonality of responders to single-agent PD-1 blockade, suggestive of a more diverse T cell repertoire compared to the more heterogenous pattern of clonality displayed by responders to combination immunotherapy (12). However, the complex interplay between these adaptive subsets with one another and the tissue-resident antigen presenting cells in the tumor microenvironment to coordinate effective tumor rejection remains incompletely understood, and further studies in other tumor types and animal models will be necessary to further define these relationships. Moreover, a comprehensive understanding of the global immune dynamics and anatomic sites driving human anti-tumor immunity has yet to be established, and future preclinical and translational efforts will be necessary to address these fundamental questions and further improve patient outcomes.

Neoadjuvant immunotherapy has gained increasing traction in the treatment of locally advanced melanoma, with several early phase studies indicating the efficacy of this approach (9, 10, 21,22). However, when performed without stratification, these studies have uniformly demonstrated the toxicity of combination checkpoint blockade and poor response rates of PD-1 inhibitor monotherapy in this context. This is in contrast to certain patients for whom this approach has proven quite effective with responses observed to even a single dose of treatment. Our approach permits an

upfront pre-treatment stratification option that may enhance response and limit toxicity if applied in future studies.

Author Contributions:

A.I.D., M.D.R., M.F.K. and M.A. designed the current study and tissue banking studies. A.I.D. K.K.T, and A.O. administered the tissue bank study. M.A., A.I.D., K.K.T, A.P.A., L.S.L, J.C.L, acquired patient specimens. K.M.M. and M.P. performed flow cytometry experiments and tabulated Tex percentages. M.D.R. supervised laboratory studies. C.W., L.S.L., and A.O. performed chart review and tabulated patient data. L.S.L performed RECIST analysis, adverse event grading, and survival analysis. D.M.M. analyzed pathology specimens. A.I.D. supervised clinical analyses. L.S.L, K.M.M., C.W., and D.M.M., prepared figures. L.S.L, C.W., K.M.M., and A.I.D. wrote the manuscript. K.K.T., A.P.A., M.H.S., and J.C.L. reviewed the manuscript and made important intellectual contributions to the interpretation of the data. All authors reviewed the manuscript prior to submission.

Acknowledgements:

We gratefully acknowledge the patients who participated in this study. This work was supported by M.D.R grants: NIH DP2-AR068130, K08-AR062064, AR066821 Burroughs Wellcome CAMS-1010934, A.I.D. grants: Amoroso and Cook fund, Parker Institute for Cancer Immunotherapy L.S.L. grants: Conquer Cancer Foundation Young Investigator Award CA-0122026. K.M.M. as supported by NIH T32 training grant in Gastrointestinal Surgery T32DK007573.

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Figure Legends

Figure 1. Patient neoadjuvant immunotherapy disposition.

Eighteen patients with locally advanced melanoma underwent pretreatment biopsy for immune-profiling and Tex frequency determination. One patient was excluded for a pretreatment biopsy with inadequate number of cells for analysis. Patients were stratified to a neoadjuvant immunotherapy group determined by their Tex frequency; patients with Tex > 20% received PD-1 monotherapy and those with Tex <20% received anti-PD-1/CTLA-4 combination therapy. Three patients had neoadjuvant regimens not aligned with their Tex frequency due to patient preference or anticipated poor tolerance to the risk of irAE with combination immunotherapy. Nine patients received neoadjuvant treatment with anti-PD-1 monotherapy, one patient received anti-PD-L1 monotherapy (grouped with anti-PD-1 for simplification), and seven patients received anti-PD-1/CTLA-4 combination therapy.

Figure 2. Illustrative examples of treatment response for two responders treated with neoadjuvant immunotherapy.

Panel 2a depicts a patient with inguinal lymph node disease and a Tex frequency of 6.5% (as shown in the flow cytometry plot) who responded to anti-PD-1/CTLA-4 combination immunotherapy. **Panel 2b** depicts a patient with axillary lymph node disease and Tex 34.4% who responded to anti-PD-1 monotherapy. In both panels, the pre-treatment and post-treatment imaging (computed tomography) and histology are shown and demonstrate marked radiographic and histologic responses. Red arrows mark the radiographic response.

Figure 3. Reduction in tumor burden and survival benefit in patients receiving neoadjuvant immunotherapy.

Panel 3a shows a waterfall plot depicting the maximum percentage change in tumor size with neoadjuvant immunotherapy compared to the pre-treatment baseline for each patient. The x-axis represents the individual patients in our study. **Panel 3b** shows spider plots depicting percentage change in tumor burden per RECIST version 1.1 over each determined assessment period. Each line represents an individual patient. **Panel 3c** depicts Kaplan-Meier estimates of relapse free survival among patients receiving neoadjuvant immunotherapy. The median RFS was not reached. **Panel 3d** shows Kaplan-Meier estimates of overall survival among patients with receiving neoadjuvant immunotherapy compared the adjuvant cohort. The median overall survival was not reached.

Variable	*Neoadjuvant Cohort (n=17)*
Age, years	
Average	53
Range	23-80
Sex, n (%)	
Male	9 (52.9)
Female	8 (47.1)
Primary Site, n (%)	
Cutaneous	14 (82.4)
Mucosal	1 (5.8)
Unknown	2 (11.8)
Pre-treatment stage, n (%)	
III	15 (88.2)
IV	2 (11.8)
Regional/distant disease timing, n (%)	
Synchronous	5 (29.4)
Metachronous	12 (70.6)
*LDH, U/l	
Average	239
Range	120-1452
BRAF Status, n (%)	
Wildtype	13 (76.5)
†Mutant	4 (23.5)
Neoadjuvant Therapy, n (%)	
PD-1 or PD-L1	10 (58.8)
PD-1/CTLA-4	7 (41.2)
Prior Therapy, n (%)	
Targeted Therapy	
BRAF/MEK	3 (17.6)
Immunotherapy	
PD-1	1 (5.8)
CTLA-4	3 (17.6)
Intralesional IL-12	1 (5.8)
None	9 (52.9)

*Serum lactate dehydrogenase, normal range 105-333 units/liter
†Refers to V600E or V600K mutation

Table 1. Baseline clinical characteristics of patients receiving neoadjuvant immunotherapy.

	Tex Percentage (Average ± SD)	Treg Percentage (Average ± SD)	ORR Patient n (%)
Neoadjuvant Cohort	25.7 ±14.5	18.4 ±11.2	16 (94.1)

Tex: Exhausted T lymphocytes, $CD8^+PD\text{-}1^+CTLA\text{-}4^+$; Treg: regulatory T lymphocytes, $CD4^+CD25^+Foxp3^+$; ORR: Overall response rate to treatment as evaluated by RECIST v1.1

Table 2. Pretreatment immune profiles and treatment response of patients receiving neoadjuvant immunotherapy.

irAE	*Any (%)*	*Grade 3 or 4 (%)*
Arthralgia	3 (17.6)	0 (0)
Abdominal pain	1 (5.8)	0 (0)
Rash	2 (11.8)	0 (0)
Pruritus	3 (17.6)	0 (0)
Fatigue	2 (11.8)	0 (0)
Diarrhea	3 (17.6)	0 (0)
Hypothyroidism	2 (11.8)	0 (0)
Pneumonitis	1 (5.8)	1 (5.8)
Transaminitis	2 (11.8)	1 (5.8)
Infusion Reaction	1 (5.8)	1 (5.8)
Any event leading to delay in therapy	1 (5.8)	1 (5.8)

irAE: immune-related adverse event as graded by CTACE v4.0

Table 3. Immune-related adverse events (irAEs) associated with neoadjuvant immunotherapy.

Supplementary Materials for

Tumor immune profiling based neoadjuvant immunotherapy for locally advanced melanoma

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Supplementary figure legends:

Figure S1. Illustrative example of a nonresponder to neoadjuvant immunotherapy.

This figure depicts a patient with locally advanced Stage IIIC disease with inguinal lymph node involvement and a Tex frequency of 33.1% who did not respond to anti-PD-1 monotherapy. Pre-treatment and post-treatment imaging (computed tomography) and histology are shown, which demonstrate a lack of response to therapy. Red arrows mark the involved right inguinal lymph node basin. Flow plots are included which display the Tex frequency as well as the markedly elevated Treg frequency.

ID	*Sex*	*Age*	*Stage*	*Primary Site*	*BRAF Status*	*LDH*	*†Neoadjuvant Group*	*%Tex*	*%Treg*	*ORR*	*irAE*
1	M	71	III	neck	wildtype	156	Mono	23.6	14.0	CR	G1 abdominal pain G1 rash
2	F	66	III	arm	wildtype	169	Mono	47.5	10.0	CR	none
3	F	34	III	leg	wildtype	148	Mono	37.2	33.3	CR	G3 Infusion reaction G1 fatigue
4	F	80	III	scalp	wildtype	139	Mono	40.1	13.4	CR	none
5	M	48	III	neck	wildtype	174	Mono	27.0	34.6	CR	G2 transaminitis G2 arthralgia G2 pruritis
6	M	63	III	thigh	wildtype	120	Mono	33.1	34.7	SD	G2 hypothyroidism
7	F	49	III	thigh	wildtype	156	Combo	6.5	7.6	CR	G2 hypothyroidism G2 diarrhea
8	M	69	III	back	mutant	1452	Combo	0.62	19.7	CR	G2 diarrhea G1 pneumonitis
9	M	77	III	back	mutant	154	Mono	27.5	13	CR	G2 diarrhea
10	M	57	III	scalp	wildtype	135	Combo	11.4	6.8	CR	G2 transaminitis G2 pruritus G1 arthralgia
11	M	55	III	back	wildtype	128	Mono	18.6	23.2	CR	G1 fatigue
12	F	52	IV	unknown	wildtype	133	Combo	34.1	10.1	PR	none
13	M	44	III	unknown	mutant	178	Combo	42.3	20.3	PR	G1 rash G1 arthralgia G1 pruritus
14	M	55	III	cheek	wildtype	128	Combo	10.2	12.3	CR	none
15	F	24	III	vagina	wildtype	151	Combo	5.0	38.3	PR	G3 transaminitis
16	F	29	III	chest	wildtype	127	Mono	34.4	3.69	PR	none
17	F	23	IV	cheek	mutant	413	Mono	35.5	NA	CR	none

Tex: Exhausted T lymphocytes, $CD8^+PD\text{-}1^+CTLA\text{-}4^+$; Treg: regulatory T lymphocytes, $CD4^+CD25^+Foxp3^+$; ORR: Overall response rate to treatment as evaluated by RECIST v1.1; irAE: immune-related adverse event as graded by CTACE v4.0; NA: not available

†Neoadjuvant group: mono refers to anti-PD-1 monotherapy (except for patient 4 who received anti-PD-L1 monotherapy) while combo refers to anti-PD-1/CTLA-4 combination therapy

Table S1: Demographics, clinical characteristics, and immune profiles of patients receiving neoadjuvant immunotherapy.

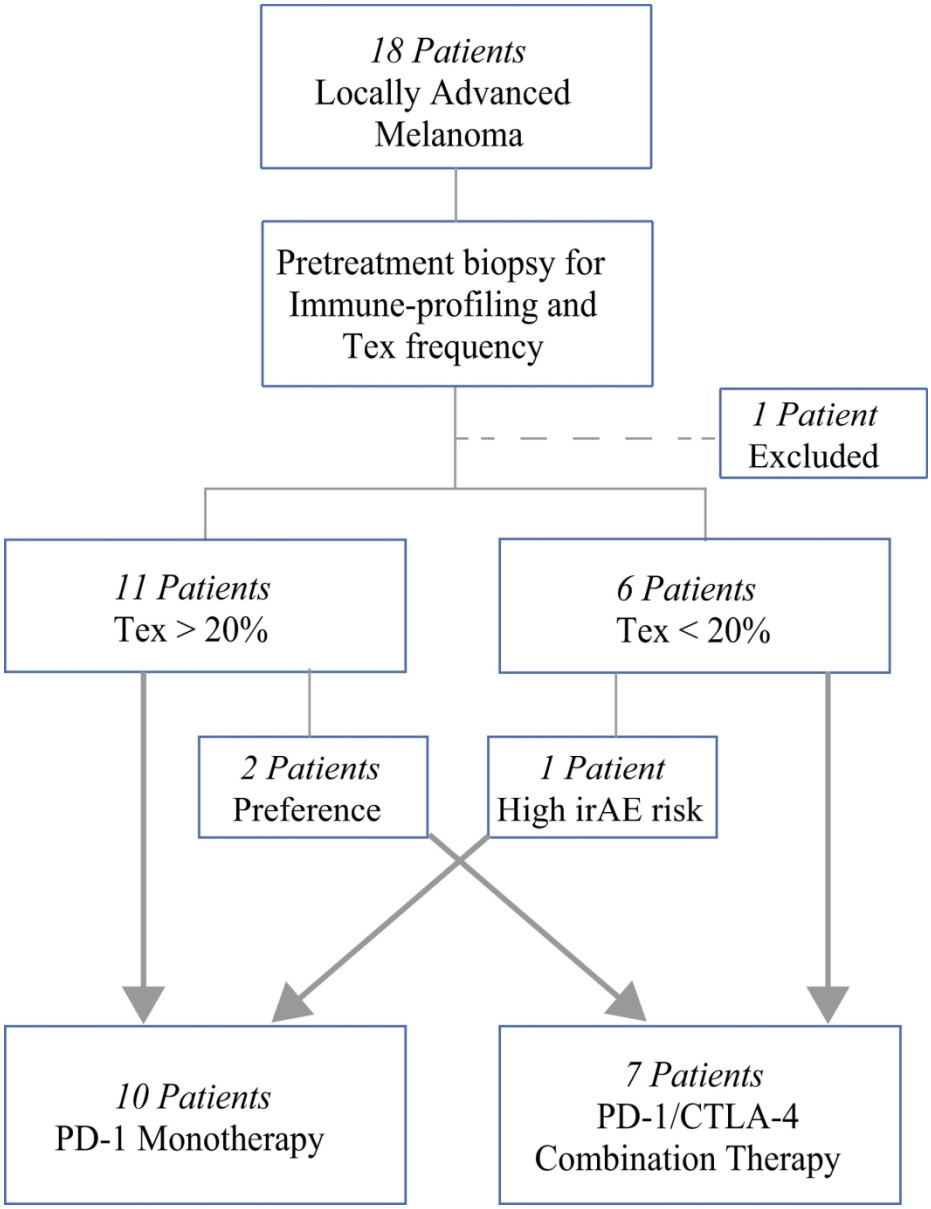

Figure 1. Patient neoadjuvant immunotherapy disposition.
Eighteen patients with locally advanced melanoma underwent pretreatment biopsy for immune-profiling and Tex frequency determination. One patient was excluded for a pretreatment biopsy with inadequate number of cells for analysis. Patients were stratified to a neoadjuvant immunotherapy group determined by their Tex frequency; patients with Tex > 20% received PD-1 monotherapy and those with Tex <20% received anti-PD-1/CTLA-4 combination therapy. Three patients had neoadjuvant regimens not aligned with their Tex frequency due to patient preference or anticipated poor tolerance to the risk of irAE with combination immunotherapy. Nine patients received neoadjuvant treatment with anti-PD-1 monotherapy, one patient received anti-PD-L1 monotherapy (grouped with anti-PD-1 for simplification), and seven patients received anti-PD-1/CTLA-4 combination therapy.

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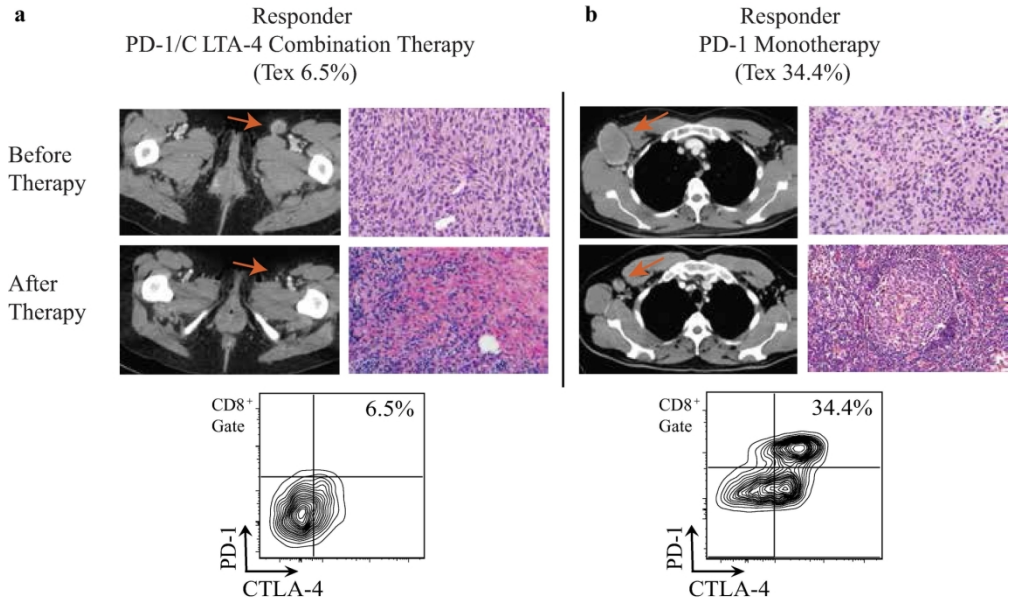

Figure 2. Illustrative examples of treatment response for two responders treated with neoadjuvant immunotherapy.
Panel 2a depicts a patient with inguinal lymph node disease and a Tex frequency of 6.5% (as shown in the flow cytometry plot) who responded to anti-PD-1/CTLA-4 combination immunotherapy. Panel 2b depicts a patient with axillary lymph node disease and Tex 34.4% who responded to anti-PD-1 monotherapy. In both panels, the pre-treatment and post-treatment imaging (computed tomography) and histology are shown and demonstrate marked radiographic and histologic responses. Red arrows mark the radiographic response.

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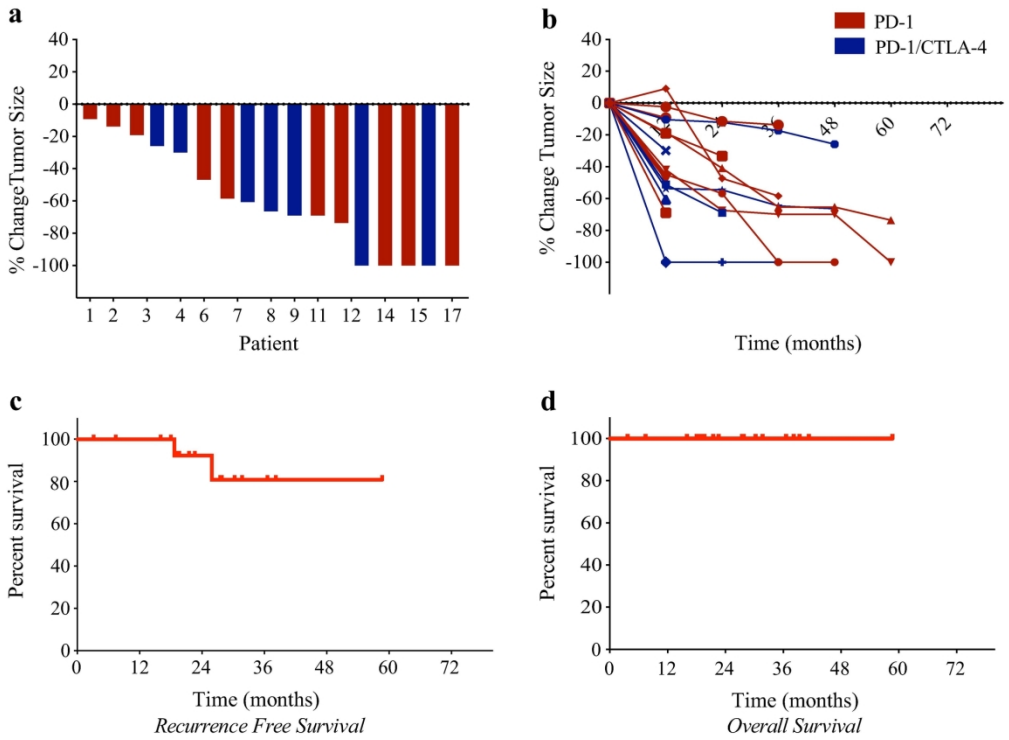

Figure 3. Reduction in tumor burden and survival benefit in patients receiving neoadjuvant immunotherapy. Panel 3a shows a waterfall plot depicting the maximum percentage change in tumor size with neoadjuvant immunotherapy compared to the pre-treatment baseline for each patient. The x-axis represents the individual patients in our study. Panel 3b shows spider plots depicting percentage change in tumor burden per RECIST version 1.1 over each determined assessment period. Each line represents an individual patient. Panel 3c depicts Kaplan-Meier estimates of relapse free survival among patients receiving neoadjuvant immunotherapy. The median RFS was not reached. Panel 3d shows Kaplan-Meier estimates of overall survival among patients with receiving neoadjuvant immunotherapy compared the adjuvant cohort. The median overall survival was not reached.

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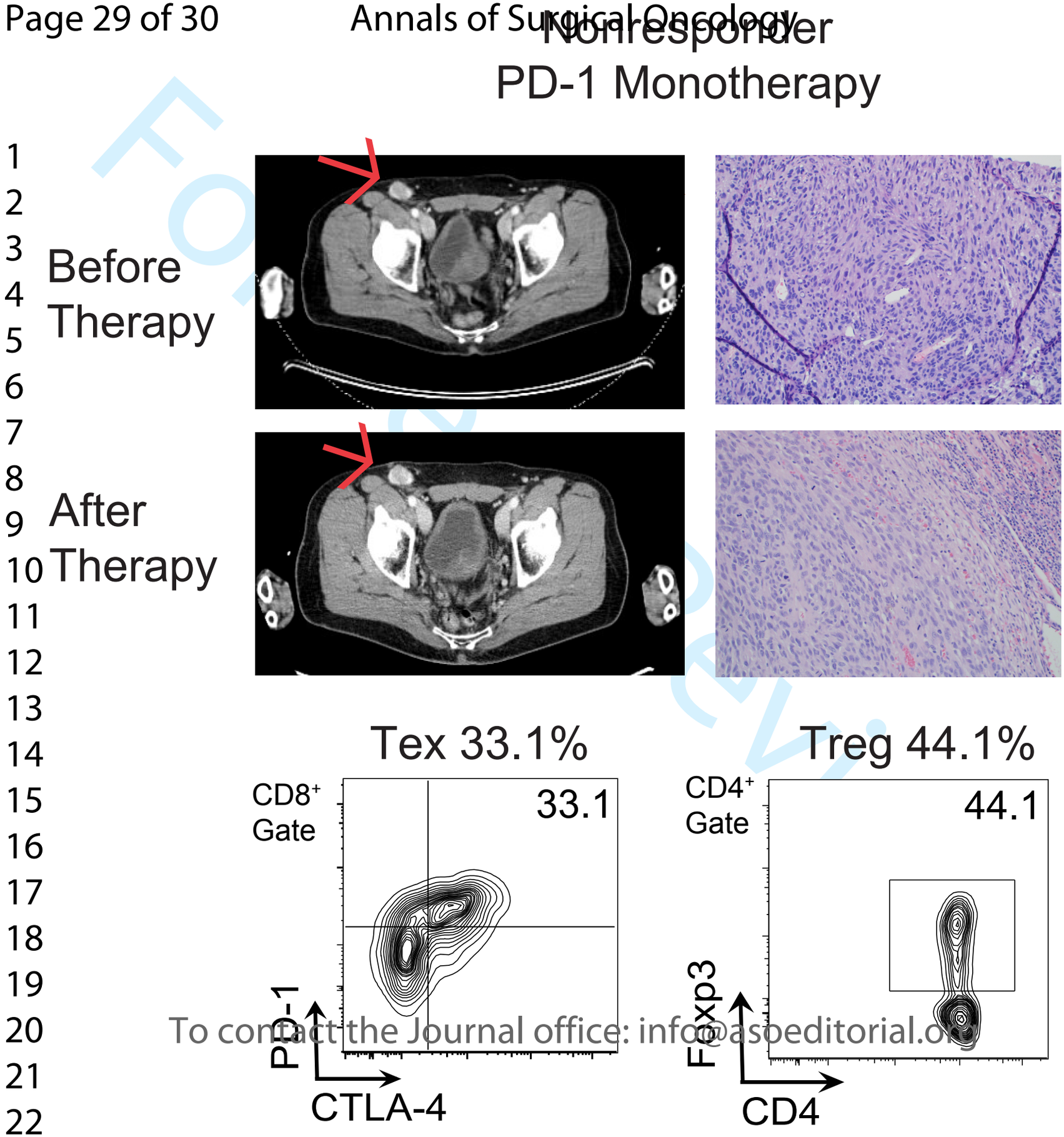

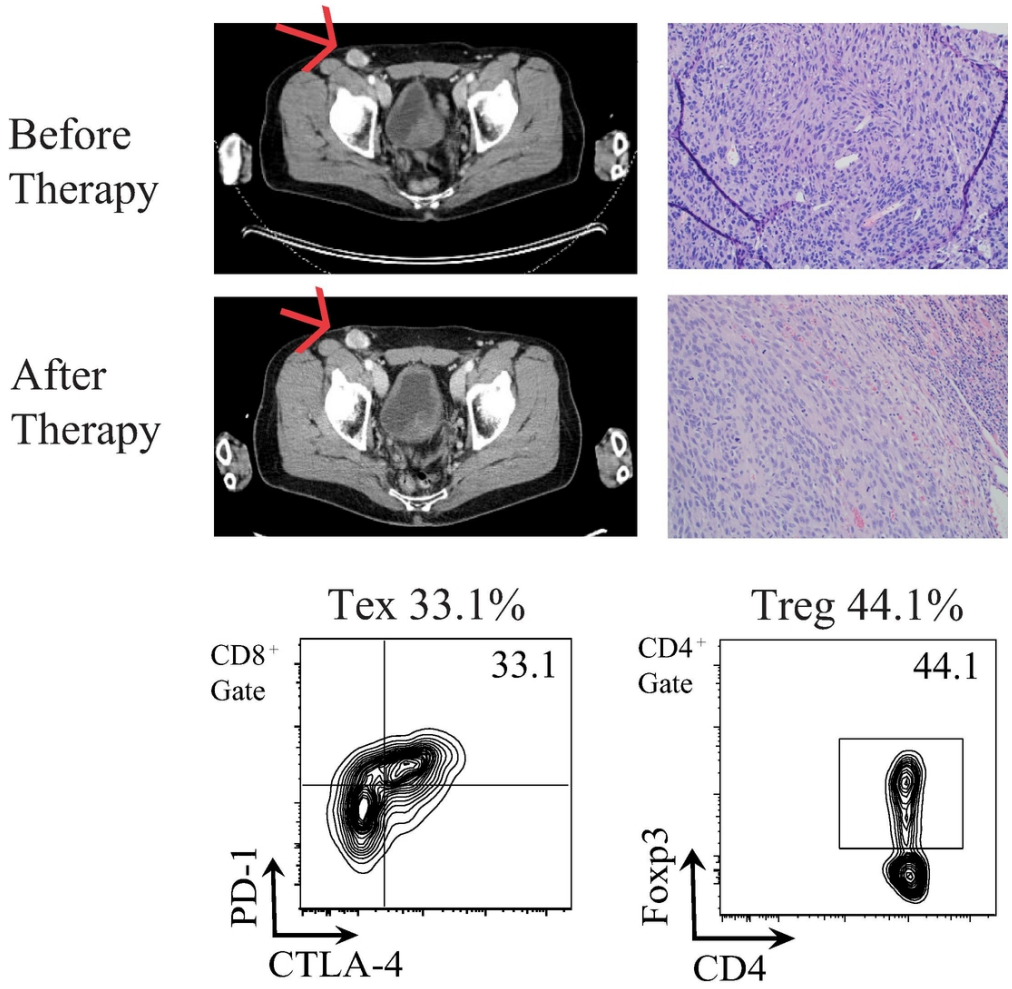

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