Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Efficacy and immunologic responses to neoadjuvant anti-PD-1 in clinical stage IIC melanoma are associated with tumor burden.

Huang Alexander

alexander.huang@pennmedicine.upenn.edu

University of Pennsylvania https://orcid.org/0000-0002-0099-0492

Mohammad Farooq

University of Pennsylvania

Xinze Tan

University of Pennsylvania

Anthony Lee

University of Pennsylvania

Anushka Dheer

University of Pennsylvania

Kevin Ma

University of Pennsylvania

Shira Rosenburg

University of Pennsylvania

Guanning Wang

University of Pennsylvania

Yichi Zhang

University of Pennsylvania

Sheryl Mitnick

University of Pennsylvania

Phyllis Gimotty

University of Pennsylvania https://orcid.org/0000-0002-3850-9799

Tara Mitchell

Tara Miller Melanoma Center, Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania https://orcid.org/0000-0002-8186-7284

Ravi Amaravadi

Tara Miller Melanoma Center, Abramson Cancer Center, University of Pennsylvania, Philadelphia, Pennsylvania https://orcid.org/0000-0002-5768-2474

Lynn Schuchter

Lynn.Schuchter@pennmedicine.upenn.edu

Xiaowei Xu
University of Pennsylvania https://orcid.org/0000-0003-4098-7690

John Miura
University of Pennsylvania

Michael Davies
MD Anderson Cancer Center https://orcid.org/0000-0002-0977-0912

Rodabe Amaria
The University of Texas MD Anderson Cancer Center https://orcid.org/0000-0001-6876-278X

Jason Chesney
University of Louisville

Roi Weisner
MD Anderson Cancer Center

Jeffrey Gershenwald
https://orcid.org/0000-0003-4519-5369

Kelly McMasters
Univeristy of Louisville

Giorgos Karakousis
University of Pennsylvania

Article

Keywords:

Posted Date: November 20th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11706754/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: Yes there is potential Competing Interest. M.A.D. is a consultant for Bristol-Myers Squibb. R.N.A. is on the steering committee for Obsidian, KSQ, and Immatics. A.C.H. receives research support from Merck, BMS, and Immunai. M.S.F. has an immediate family member that is an employee for Bayer AG. R.N.A. received research funding from Regeneron, Obsidian, KSQ, Immatics, OnKure, Erasca.Merck Sharp & Dohme LLC, a subsidiary of Merck & Co., Inc., Rahway, NJ, USA, provided drug and financial support for the study.

Efficacy and immunologic responses to neoadjuvant anti-PD-1 in clinical stage IIC melanoma are associated with tumor burden

Mohammad Saad Farooq*[1], Xinze Tan*[2], Anthony Lee*[2], Anushka Dheer[1], Kevin L. Ma[1], Shira G. Rosenberg[2], Guanning Wang[2], Yichi Zhang[2], Sheryl Mitnick[5], Phyllis A. Gimotty[3], Tara C. Mitchell[1,4,5], Ravi K. Amaravadi[1,4,5], Lynn M. Schuchter[1,4,5], Xiaowei Xu[6], John T. Miura[1], Michael A. Davies[7], Rodabe N. Amaria[7], Jason A. Chesney[8], Roi Weiser[9], Jeffrey E. Gershenwald[9], Kelly M. McMasters[10], Alexander C. Huang[#2,4,5,11,12,13], Giorgos C. Karakousis[#1,4,5]

*Contributed equally as co-first authors

#Contributed equally as co-corresponding authors

[1]Department of Surgery, Division of Endocrine and Oncologic Surgery, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

[2]Division of Hematology/Oncology, Department of Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

[3]Department of Biostatistics, Epidemiology and Informatics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

[4]Tara Miller Melanoma Center, Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA, USA.

[5]Abramson Cancer Center, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

[6]Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

[7]Department of Melanoma Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[8]Division of Medical Oncology and Hematology, University of Louisville, Louisville, KY, USA

[9]Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

[10]The Hiram C. Polk, Jr., MD Department of Surgery, University of Louisville School of Medicine, Louisville, KY, USA

[11]Parker Institute for Cancer Immunotherapy, University of Pennsylvania, Philadelphia, PA, USA.

[12]Institute for Immunology and Immune Health, Philadelphia, PA, USA.

[13]Department of Cancer Biology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA.

Abstract

Neoadjuvant immune checkpoint therapy has demonstrated improved event-free survival in patients with resectable clinical stage III/IV melanoma, however its benefit in clinical stage IIB/C melanoma is unknown. Here we present clinical and immunologic data from the first multicenter phase II trial of neoadjuvant pembrolizumab (anti-PD-1) in clinical stage IIB/C melanoma.

Sixty-three patients (29 clinical stage IIB, 34 clinical stage IIC) were included, with the primary outcome of sentinel lymph node (SLN) positivity rate after single dose neoadjuvant pembrolizumab. In the overall cohort, there was no significant difference in SLN positivity rate compared to the projected historical rate; however, on subgroup analysis, neoadjuvant anti-PD-1 was associated with a reduction of SLN positivity in stage IIC disease that was not seen in IIB.

Antigen-specific flow cytometry was performed on blood specimens from 27 trial patients across multiple timepoints. Neoadjuvant PD-1 blockade induced reinvigoration of exhausted CD8 T cells in IIC patients, and not in IIB patients. Employing a combinatorial tetramer system, we detected melanoma-specific CD8 T cells in 71% of IIC patients vs. 23% of IIB patients. Presence of melanoma-specific CD8 T cells was associated with higher recurrence-free survival in IIC patients. Moreover, clinical stage IIC patients had greater tumor burden compared to IIB.

These data indicate that clinical activity and tumor-specific immune responses to neoadjuvant anti-PD-1 are preferentially observed in patients that reach a threshold of tumor antigen load that is sufficient for immune activation and identify circulating melanoma-specific CD8 T cells as candidate biomarkers of response. These findings support prospective evaluation of neoadjuvant anti-PD-1 therapy in clinical stage IIC disease in larger randomized studies.

Introduction

Neoadjuvant immune checkpoint inhibitor (ICI) therapy has emerged as a new treatment paradigm in locally advanced melanoma, including stage III disease[1]. Randomized studies in this setting have demonstrated superior event-free survival compared with adjuvant therapy alone[2,3], and the ability to intercept patients at an even earlier disease stage and prevent metastatic progression holds great promise. However, the safety and efficacy of neoadjuvant programmed cell death protein 1 (PD-1) blockade in high-risk stage II melanoma is unknown.

Clinical stage IIB/C melanoma represents a high-risk form of localized disease that often harbors occult nodal metastases, with recurrence and melanoma-specific mortality approaching or exceeding that of some patients with stage III melanoma[4-7]. Surgical excision of the primary tumor with sentinel lymph node (SLN) biopsy from regional lymph node basins has historically been the standard treatment for these patients, with SLN status providing important prognostic information and guiding postoperative management. More recently, adjuvant PD-1 blockade has demonstrated improvements in recurrence-free survival (RFS) and has become an approved therapy for patients with pathologic stage IIB/C melanoma[8-10]. Nevertheless, only approximately 15% of patients benefit from the adjuvant approach and a substantial proportion experience disease recurrence[8,9], highlighting the need for more effective therapeutic strategies.

Beyond its therapeutic potential, the neoadjuvant setting provides a unique opportunity to interrogate early immune responses that may predict treatment benefit and inform biomarker development. Our previous translational investigations in bulky stage III melanoma have shown that neoadjuvant treatment in the presence of an intact tumor promotes systemic antitumor immunity through the expansion of exhausted and melanoma-specific CD8 T cell populations that correlate with clinical response in advanced melanoma[11-13].

Whether these advantages extend to high-risk stage II melanoma remains unknown, where overall tumor burden is substantially lower than in stage III disease[14] and the determinants of responsiveness to PD-1 blockade remain poorly understood. In addition, although stage IIB and IIC melanoma are grouped together clinically, they often exhibit different clinical outcomes, with overall higher rates of recurrence and SLN positivity in clinical stage IIC[4,15-18]. Whether these subgroups exhibit distinct clinical and immunologic responsiveness to neoadjuvant PD-1 blockade has not been investigated.

Here, we report the results of a multicenter, investigator-initiated phase II trial, the first to prospectively evaluate neoadjuvant pembrolizumab (anti-PD-1) in patients with clinical stage IIB/C melanoma[19,20]. In parallel with prospective assessment of clinical efficacy, we performed longitudinal immune profiling of peripheral blood, including analyses of melanoma-specific CD8 T cells, to define the systemic immune response to neoadjuvant PD-1 blockade. Together, these clinical and translational analyses provide insight into the immunologic determinants of response to neoadjuvant therapy in high-risk stage II melanoma and inform the rationale for development of precise neoadjuvant treatment strategies for this disease.

Results

Trial Cohort Characteristics

To understand the preliminary efficacy and safety of neoadjuvant anti-PD-1 in high-risk stage II melanoma, we conducted a single-arm, multicenter phase II clinical trial of patients with clinical stage IIB/C melanoma who received a single dose of neoadjuvant pembrolizumab (200 mg) 3 weeks prior to surgery, followed by scheduled adjuvant pembrolizumab (200 mg every 3 weeks for 1 year) (**Fig 1A**). From 5 May 2019 to 1 December 2024, 86 patients were screened for eligibility and 64 consented for the study after passing initial screening criteria as follows: resectable clinical stage IIB/IIC melanoma (cT3b-cT4bcN0cM0), age ≥ 18 years, ECOG performance status of 0/1, and adequate organ function (**Supp Fig 2A**). Patients were enrolled from the University of Pennsylvania (UPenn), the University of Louisville, and the University of Texas MD Anderson Cancer Center. One patient failed to achieve SLN localization as they did not undergo SLN biopsy and were deemed unevaluable for the primary study endpoint. The final analyzed cohort included 63 patients for the primary outcome (**Table 1**) and 64 patients for safety/tolerability.

The median age was 61 years (IQR: 54 – 69) and 38 (60%) patients were male. Twenty-nine (46%) were clinical stage IIB (T3b/T4a) and 34 (54%) patients were clinical stage IIC (T4b) based on their primary tumor characteristics at time of definitive surgery (**Fig 1A**; **Table 1**). Four (6.3%) patients were upstaged from IIB (diagnosed on pre-operative biopsy) to IIC (on post-treatment primary tumor surgical specimen). The median tumor Breslow thickness was 5.7 mm (IQR: 3.5 – 8.3), 54 patients (86%) had presence of ulceration, 28 patients (44%) had presence of residual primary tumor on the surgical wide excision specimen, and the median mitotic rate was 9 (IQR: 4 – 14). Overall, the characteristics of the trial population were consistent with higher risk disease based on tumor thickness, ulceration, and mitotic rate. All 63 patients received a single dose of neoadjuvant pembrolizumab and underwent successful wide local excision (WLE) with SLN biopsy. Blood was collected at baseline (on day of first neoadjuvant dose), at time of SLN biopsy/WLE (3 weeks post-neoadjuvant dose), and during adjuvant anti-PD-1 therapy (every 3 weeks for up to 1 year; **Fig 1A**).

Anti-PD-1 Decreases SLN Positivity Rates in Clinical Stage IIC Disease

We tested the hypothesis that neoadjuvant anti-PD-1 could decrease SLN positivity, as SLN status is strongly associated with disease recurrence and prognosis[4,5]. As this was a single-arm trial, we used the National Cancer Database and UPenn's retrospective melanoma database to project the historical rates of SLN positivity in clinical stage IIB and clinical stage IIC patients for statistical comparison. The National Cancer Database contains deidentified patient information from more than 1500 Commission-on-Cancer accredited institutions forming a nationally representative sample. This dataset contained clinical stage IIB/C patients from 2004-2012; the SLN positivity rate for clinical stage IIB was 24% (4,713/19,683 cases) and the rate for clinical stage IIC was 39% (3,340/8,510 cases). In addition, we utilized UPenn's retrospective melanoma database, selecting patients with clinical IIB/C melanoma from 1997 to 2014 as the anticipated trial cohort was expected to be enrolled from UPenn; here the SLN positivity rate for clinical stage IIB was 27.5% (66/240 cases) and the rate for clinical stage IIC was 45% (48/107 cases). For comparative analysis, an integrated estimate of the aforementioned two datasets

projected the SLN positivity rate for treatment-naïve patients as 33.1% for the overall cohort, 25.0% for clinical stage IIB patients, and 40.0% for clinical stage IIC patients prior to the initiation of this trial (**Methods**).

The trial was powered to detect a 50% reduction in the percentage of patients who have a positive SLN by using the mixed SLN positivity rate from the National Cancer Database and UPenn institutional data. The overall SLN positivity rate for the trial was 27% (17 patients); compared to the pre-defined projected historical cohort SLN positivity rate of 33.1%, this difference was not statistically significant (**Fig 1B, C**; p = 0.334). On subgroup analysis of clinical stage IIC patients, the SLN positivity rate was 23.5%, which was significantly lower than the prespecified projected historical benchmark of 40% for clinical stage IIC (p = 0.0499; **Fig 1D**), and comparable to that of clinical stage IIB.

A post-hoc analysis of predicted SLN metastasis risk in our specific trial cohort was also conducted using the Melanoma Institute of Australia (MIA) SLN metastasis nomogram[21], a well-validated prediction model for SLN metastasis risk, by using the matched clinicopathologic characteristics of our trial cohort (**Supp Table 1, Methods**). In this analysis, the predicted SLN metastasis rate was 27% for clinical stage IIB and 42% for clinical stage IIC, which was numerically in line with the projected rates utilized prior to trial initiation (i.e. 25% and 40% respectively). The trial cohort SLN positivity rate of 23.5% for clinical stage IIC was significantly lower than the predicted MIA nomogram rate (p = 0.0357; **Fig 1E, F**).

SLN positivity rate was also assessed using the pre-surgical biopsy staging for the overall cohort (i.e. prior to definitive staging with wide local excision), as 4 patients were upstaged from IIB to IIC. For the 30 patients that were clinical stage IIC using this criteria, SLN positivity rate was 16.7%, which was significantly lower compared to the projected historical benchmark of 40% (p = 0.009, **Supp Fig 1A**) and the predicted MIA nomogram rate of 42% (p = 0.005, **Supp Fig 1B**).

Recurrence-free survival (RFS) and overall survival were key secondary clinical endpoints. The median follow-up time for the overall cohort was 28 months (IQR: 17 – 43). Overall, 11 patients (17%) experienced a recurrence at latest follow-up (**Supp Fig 2B**) with 3 (10%) clinical stage IIB and 8 (24%) clinical stage IIC. Of these, 7 recurrences (64%) were distant, while 4 (36%) were locoregional. Two-year RFS rate for clinical stage IIC was 81.3% (95% CI: 62.8 – 91.1) which was not statistically different than clinical stage IIB, which was 86.9% (95% confidence interval [CI]: 63.9 – 95.7) (**Fig 1G**). Similarly, 2-year OS rates were favorable for both clinical stage IIB (95.5% [95% CI: 71.8 – 99.3]) and clinical stage IIC (90.5% [95% CI: 73.2 – 96.7]; **Supp Fig 2C**). Altogether these data indicate preferential clinical activity of neoadjuvant anti-PD-1 therapy in clinical stage IIC disease, compared to IIB.

Neoadjuvant anti-PD-1 in Clinical Stage IIB/C is Safe and Well Tolerated

Safety and tolerability were additional primary outcomes. In the adjuvant period, the median completed cycles of pembrolizumab were 13; 62 (98%) patients received at least one dose of adjuvant therapy. Through the course of the follow-up period, grade 3/4 treatment-related adverse events (TRAE) attributable to either pembrolizumab or surgery occurred in 16 (25%) unique patients, which was comparable to previous published data for neoadjuvant-adjuvant pembrolizumab[3,22]. Among 22 grade 3/4 TRAE, 6 were surgery-related and 16 were pembrolizumab-related. The most common TRAE were diarrhea/colitis (n = 3), wound infection

(n = 3) and cutaneous rash (n = 2; **Table 2; Supp Fig 2D, E**). Notably, there were no patients who experienced an adverse event that precluded them from undergoing surgery or caused a delay in their surgery.

Exploratory Analyses of Clinicopathologic Factors Associated with Outcomes

With a median follow-up of 28 months in the overall cohort, two-year RFS was assessed stratified by select clinicopathologic factors (**Fig 1H**). As expected SLN- patients demonstrated a higher 2-year RFS compared to SLN+ patients, although this was not statistically significant (log-rank p = 0.10). Female patients had significantly higher 2-year RFS compared to males at 95.8% (95% CI: 73.9 – 99.4) vs. 75.9% (95% CI: 57.2 – 87.1; log-rank p = 0.032). Additionally, the presence of residual primary tumor on the wide excision specimen was found in 28 (44%) patients, which may have been due to remaining tumor following diagnostic biopsy and/or a lack of immunotherapy response. Presence of residual primary tumor was associated with significantly lower 2-year RFS (67.9% [95% CI: 45.4 – 82.7]) vs. no residual primary tumor (96.8% [95% CI: 79.2 – 99.5]; log-rank p = 0.006; **Fig 1H**, **Supp Fig 2F**); importantly, this finding persisted on multivariable Cox analysis controlling for thickness, ulceration, SLN status, and location (adjusted hazard ratio: 6.37, 95% CI: 1.16 – 34.9, p = 0.033). Presence of residual primary tumor at the time of resection was also associated with increased likelihood of SLN metastasis, with 12/28 (42.9%) patients with residual primary tumor having a positive SLN vs. 5/35 (14.3%) (p = 0.021; **Supp Fig 2G**). Interestingly, despite clinical stage IIC tumors being significantly thicker than IIB (7.2 mm vs. 3.4 mm, p < 0.001), residual primary tumor rate did not significantly differ between clinical stage IIB and IIC (**Supp Fig 2H)** and may be explained by the higher clinical efficacy demonstrated in the clinical stage IIC population in this trial.

PD-1 Blockade Reinvigorates Exhausted CD8 T Cells in Clinical Stage IIC Disease

The clinical data indicated that efficacy was largely confined to patients with clinical stage IIC disease. To further explore this observation, we tested whether clinical stage IIC patients had a different immunologic response to neoadjuvant anti-PD-1 compared to clinical stage IIB patients. We used a combinatorial tetramer approach to track 10 melanoma and viral antigen-specific T cell populations simultaneously. The 5 combinatorial tetramer channels were embedded into a 34-parameter spectral cytometry panel to provide phenotypic assessment.

Antigen-specific cytometry was conducted on 27 patients that were HLA A1, A2, A3, or B7, who were representative of the overall cohort and for which we had tetramer reagents. The median follow-up of the immune-profiled cohort was 43 months, and there were no significant differences in clinicopathologic characteristics between the immune-profiled cohort and the non-immune-profiled cohort (**Supp Table 2**). We measured CD8 T cell responses in the blood at baseline prior to the neoadjuvant dose (C1), on the day of surgery (DOS), and during adjuvant doses following surgery (C2-4+). Notably, the pharmacodynamic activity of pembrolizumab was identified only in clinical stage IIC disease, with increases in proliferation in CD8 and exhausted phenotype (PD-1+CD39+) CD8 T cells after a single dose of anti-PD-1 (**Fig 2A-C**). In contrast, there was no significant difference in Ki67 in clinical stage IIB patients.

Anti-PD-1 targets progenitor exhausted CD8 T cells ($Prog_{EX}$), driving their differentiation into intermediate (INT_{EX}) and/or terminal exhausted ($TERM_{EX}$) CD8 T cells[23-25]. The efficacy of PD-1 blockade has been linked to INT_{EX} that retain some degree of effector function[23-25]. We defined exhausted CD8 T cells by PD-1 and CD39, and $Prog_{EX}$ and INT_{EX} by the transcription factors

TCF-1[26-28] and Tbet[23-25] respectively (**Fig 2D**). Indeed, INT_{EX} expressed features of effector-like exhausted CD8 T cells[23-25], including TOX, Perforin, CX3CR1, NKG2A, while $Prog_{EX}$ expressed TOX, CXCR5, and CD28, consistent with published data[26,27,29] (**Fig 2E**). PD-1 blockade enhanced proliferation of INT_{EX} in IIC patients, but not in IIB (**Fig 2F**). The pharmacodynamic effect of anti-PD-1 was seen only in T_{EX} subsets, and not central memory, effector memory, or effector memory RA populations. (**Fig 2G-H**), consistent with the concept that PD-1 blockade reinvigorates T_{EX}. However, this reinvigoration was seen only in IIC patients, and not in IIB. These differences in pharmacodynamic activity between clinical stage IIB and IIC were importantly not due to differences in the CD8 T cell composition, because there were no significant differences in the frequency of exhausted and memory subsets between IIC and IIB at baseline (**Supp Fig 3A-C**).

Greater Frequency of Melanoma-specific CD8 T Cells in Clinical Stage IIC

We next wanted to understand whether the difference in anti-PD-1 activity between clinical stage IIB and IIC was due to differences in the presence of tumor-specific CD8 T cells. Using a combinatorial tetramer system (**Supp Fig 4A**), we tracked melanoma antigen-specific CD8 T cells in the blood across time in the immune-profiled cohort (13 clinical stage IIB and 14 clinical stage IIC patients) and identified melanoma-specific CD8 T cells in 13/27 (48%) of patients (**Supp Fig 4B**). In total, we identified 17 melanoma-specific CD8 T cell populations across 5 melanoma antigens, as well as CD8 T cells against 9 viral antigens (**Supp Fig 4C, D**). Melanoma-specific CD8 T cells were detected in 10 clinical stage IIC (71.4%) versus 3 clinical stage IIB (23%) patients at baseline C1 (p=0.021; **Fig 3A**). On multivariable logistic regression analysis controlling for clinical stage, sex, age, and residual tumor status, clinical stage IIC was associated with increased odds of presence of melanoma-specific CD8 T cells (adjusted odds ratio: 6.06, 95% CI: 1.15 – 31.8, p = 0.033), and the presence of melanoma-specific CD8 T cells was associated with improved 3-year RFS in clinical stage IIC patients (**Fig 3B-D; Supp Fig 4E, F**). Among clinical stage IIC patients, those with detectable melanoma-specific cells had an estimated 3-year RFS of 90.0% (95% CI: 47.3 – 98.5) compared with 25.0% (95% CI: 8.9 – 66.5) among those without detectable melanoma-specific cells (log-rank p = 0.014; **Fig 3C, D**). Other clinicopathologic factors were not significantly associated with 3-year RFS in this cohort (**Fig 3C**)**.** In clinical stage IIC patients, neoadjuvant PD-1 blockade induced proliferation of melanoma-specific CD8 T cells (p = 0.031; **Fig 3E**), and expansion of melanoma-specific CD8 T cells that peaked at time of adjuvant treatment (C2; p = 0.035; median 3 weeks after resection; **Fig 3F, G**).

Clinical Stage IIC Patients Have Greater Primary and SLN Tumor Burden

The presence of tumor antigen has been shown to be important for the efficacy of immune checkpoint blockade[12,30], and recent studies have shown that neoadjuvant followed by adjuvant anti-PD-1 is superior to adjuvant anti-PD-1 alone[3]. Thus, we wondered whether clinical stage IIC patients may have better immunologic responses and priming of tumor-specific CD8 T cells due to a greater tumor antigen load compared to clinical stage IIB.

Indeed, clinical stage IIC patients had significantly thicker primary tumors vs. IIB (median 7.2 mm vs. 3.4 mm, p<0.001; **Fig 3H**). Additionally, we measured SLN metastasis by greatest diameter in a treatment-naïve historical validation cohort of 40 SLN+ patients (IIB n = 30; IIC n = 10). This cohort was derived from a periodically updated retrospective database of UPenn patients with clinical stage IIB/IIC melanoma from 2006 – 2019 who underwent SLN biopsy and had numerically measured SLN tumor metastasis on pathology assessment. Indeed, patients

with clinical stage IIC had a larger median tumor metastasis size (IIC = 6.55 mm vs. IIB = 1.35 mm, p=0.022; **Fig 3I**). Among the 17 patients with SLN+ disease in the trial cohort, median SLN metastasis size was 3.3 mm in clinical stage IIC patients and 0.1 mm in clinical stage IIB patients (p = .106; **Fig 3I, J**) after neoadjuvant anti-PD-1. Altogether, these data support a model in which clinical stage IIC melanoma patients have a greater frequency of melanoma-specific CD8 T cells and a larger magnitude of anti-PD-1 immune response in the setting of greater tumor burden reflected by the size of the primary tumor and occult SLN metastases.

Discussion

In this multicenter phase II trial, the first to assess neoadjuvant PD-1 blockade in clinical stage IIB/C melanoma, we found evidence that these clinically related subgroups differed in their capacity to mount an antitumor immune response following a single dose of pembrolizumab prior to surgical resection. Although the primary endpoint was not met in the overall cohort, clinical stage IIC patients had a reduction in SLN positivity rate. This clinical signal was accompanied by proliferation of T-bet^{+} intermediate-exhausted CD8 T cells, and a higher prevalence of circulating melanoma-specific CD8 T cells. Notably, clinical stage IIC patients also had greater primary and SLN tumor burden than clinical stage IIB patients, providing a potential biological link between disease burden and immune responsiveness. In contrast, these immune responses were not observed in clinical stage IIB disease. Together, these findings suggest that clinical stage IIB and IIC melanoma may reflect tumors with distinct capacities to engage pre-existing tumor-specific immunity following PD-1 blockade. Overall, our study revealed several novel insights.

First, the clinical outcomes in this cohort were encouraging given the known high recurrence risk of clinical stage IIB/C melanoma[4]. Although the prespecified primary endpoint was not met in the overall cohort, exploratory analysis demonstrated a lower-than-expected SLN positivity rate among patients with clinical stage IIC disease, despite historical rates for >4 mm melanomas ranging from 38 to 47%[6,17,18,31-33]. This was additionally corroborated by our post-hoc analysis of predicted SLN positivity rate using the MIA SLN metastasis risk nomogram[21], which predicted a rate of 42% for our matched clinical stage IIC trial population. These findings remained consistent when utilizing biopsy-based staging, which is more applicable in the clinical setting when considering patients for neoadjuvant therapy. Thus, our pre-trial projection using the National Cancer Database/UPenn institutional data, published historical rates, and MIA nomogram prediction all suggest that the rate of SLN metastasis in the clinical stage IIC subgroup in our trial population may represent a clinically meaningful reduction. Additionally, at a median follow-up of 28 months, 2-year RFS was 86.9% for clinical stage IIB and 81.3% for clinical stage IIC, with corresponding OS rates of 95.5% and 90.5%. These survival estimates are numerically favorable compared with outcomes reported with adjuvant pembrolizumab in KEYNOTE-716[34] (2-year RFS 81%) and from the National Cancer Database[35], which focused on a lower risk patient population with pathologic stage IIB/C disease (i.e. SLN-negative); nevertheless cross-trial comparisons are limited by differences in study design, patient populations, and treatment exposure. Together with the clinical stage IIC-specific immune activation observed in our translational analyses, these clinical outcomes provide rationale for larger randomized trials focused on the evaluation of neoadjuvant PD-1 blockade in clinical stage IIC melanoma.

Second, we identified a group of clinical stage IIB patients that have minimal immunologic response to PD-1 blockade. This is striking because reinvigoration of CD8 T cells is generally thought to be a pharmacodynamic property of anti-PD-1 therapy, with the vast majority of patients achieving immunologic responses to PD-1 blockade regardless of clinical outcomes[12,36]. The differences in T cell proliferation provide a potential explanation for the divergent clinical effects between clinical stage IIB and IIC disease. In clinical stage IIC patients, pembrolizumab induced proliferation amongst exhausted-lineage PD-1^{+}CD39^{+} CD8 T cells, with the greatest effect among T-bet^{+} intermediate-exhausted cells. This reinvigoration of T_{EX} is similar to that seen in stage III[13] and IV disease[12,36], showing that the activity of PD-1 blockade is conserved across stage IIC, III, and IV disease, but was not identified in stage IIB. Consistent with this interpretation, melanoma-specific CD8 T cells were detectable at baseline in 71% of IIC patients compared to only 23% of IIB patients. As a comparison, using a similar antigen-specific panel, melanoma-specific CD8 T cells were detected in the blood of only 56% of stage III melanoma patients[13]. Thus, patients with clinical stage IIC disease with relatively low disease burden appear to effectively prime tumor-specific CD8 T cells. PD-1 blockade reinvigorated these melanoma-specific CD8 T cells, resulting in enhanced proliferation and expansion. These melanoma-specific responses are clinically relevant as their presence was also associated with higher RFS estimates. Indeed, clinical stage IIC disease may reflect a window of optimal immunologic activity among patients with clinically localized melanoma, as CD8 T cell responses begin to wane with tumor progression[37]. Importantly, we also demonstrated the feasibility of a blood-based test for detection of melanoma-specific CD8 T cells with rapid turnaround that could identify patients most likely to benefit from neoadjuvant anti-PD-1 in clinical stage IIC patients. Implementation of this potential biomarker assessment tool requires further prospective validation.

Lastly, we demonstrate a link between SLN tumor burden and stage. Historically, clinical stage IIC patients have significantly higher rates of SLN positivity[6]. We found greater SLN tumor burden in IIC patients compared to IIB patients in both our single-institution treatment-naïve cohort and the neoadjuvant trial cohort. Additionally, the concordance of greater tumor burden at both the primary and nodal level was associated with more frequent detection of melanoma-specific CD8 T cells and stronger immune activation following PD-1 blockade, which is consistent with a model in which greater tumor burden may increase antigen exposure and thereby support the establishment of systemic tumor-specific immunity. Prior studies have demonstrated relationships between tumor antigen load and immunotherapy responses[12,30], and recent studies have established the superiority of neoadjuvant-adjuvant immune checkpoint blockade therapy in terms of event-free survival, compared to standard of care adjuvant-only anti-PD-1 therapy[2,3]. The improved efficacy of neoadjuvant therapy is likely due to the importance of having tumor antigen present for T cell receptor stimulation at the time of immune checkpoint administration[30]. In the clinical stage II setting, this tumor antigen burden may come largely from the SLN metastasis burden among patients with tumor-involved SLNs, because all patients in the trial underwent pre-surgical biopsy with partial removal of the primary. The exact contribution, however, of the primary tumor burden is difficult to assess accurately in the neoadjuvant setting because of the pre-surgical biopsy and potential considerable treatment effect from administration of the neoadjuvant dose up until the time of definitive surgical resection, leading to underestimation of overall tumor burden. Nevertheless, the presence of residual primary tumor emerged as a marker of poor pathologic response and shorter RFS in this clinically localized population, possibly acting as a correlate for nodal pathologic response.

This is consistent with our data that patients with residual primaries have a 3-fold higher rate of SLN positivity. Additionally, the rates of residual primary tumor were similar between clinical stage IIB and IIC even though clinical stage IIC tumors were significantly thicker in our trial population. It is indeed possible that a similar rate of residual primary tumor between clinical stage IIB and IIC is a signal of greater efficacy of neoadjuvant anti-PD-1 for clinical stage IIC patients, however, this requires validation of residual primary as an effective post-treatment marker of response.

Several limitations warrant consideration. Stratification of our findings by stage was not the primary endpoint of the study, and our findings in the subgroup of IIC patients inform the need for further prospective study in this group of patients. Additionally, cumulative tumor thickness for definitive staging was based on data from both initial biopsy and at wide excision – this may be subject to error if biopsy and neoadjuvant treatment impacted tumor thickness at time of wide excision; although this would likely have led to underestimation of tumor thickness and therefore an underestimation of anticipated SLN positivity rates, which would strengthen the magnitude of this study's results. Finally, longer follow-up and randomized studies will be needed to determine whether the observed clinical signal translates into improved long-term outcomes.

In summary, our findings suggest that clinical stage IIC melanoma is characterized by greater pre-existing tumor-specific immunity and responsiveness of intermediate-exhausted CD8 T cells to PD-1 blockade than clinical stage IIB disease. These data support prospective evaluation of neoadjuvant PD-1 blockade in clinical stage IIC melanoma and raise the possibility that tumor-specific immunity, together with tumor burden, may provide a biologically informed framework for selecting patients for neoadjuvant immunotherapy.

Methods

Patient Cohort and Study Design

This phase II clinical trial (NCT03757689) was a multi-institution, investigator-initiated study conducted at the University of Pennsylvania, with approval from the Institutional Review Board and written informed consent obtained from all participants. Adults aged 18 years or older with an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1, adequate organ function, and clinical stage IIB/C resectable melanoma were eligible. Patients were enrolled from the University of Pennsylvania, the University of Texas MD Anderson Cancer Center, and the University of Louisville[20]. All research involving trial participants was planned, conducted, and reported in accordance with the Declaration of Helsinki. This work followed Consolidated Standards of Reporting Trials (CONSORT) guidelines for reporting. Individual de-identified patient data and analytical files can be accessed upon reasonable request to the authors.

Treatment and Assessment

After providing informed consent, all patients underwent a baseline pretreatment biopsy. Patients then received a single intravenous dose of pembrolizumab (200 mg) followed by wide excision and SLN biopsy 3 weeks later. Paired blood samples were obtained at pre-treatment time point (C1, 3 weeks before wide local excision), on the day of surgery (DOS) and post-treatment time points (C1 – C16; every 3 weeks for up to 1 year in the adjuvant setting).

Tumor biopsies and resection specimens were processed by the Department of Pathology and Laboratory Medicine at the University of Pennsylvania, where tissues were immediately snap-frozen or formalin-fixed and paraffin-embedded. SLN positivity was determined by a dedicated dermatopathologist at the University of Pennsylvania.

The primary study endpoints of this trial were SLN positivity rate at surgery and safety and tolerability. Secondary endpoints included RFS and OS from date of surgery. Adverse events were graded using the Common Terminology Criteria for Adverse Events (CTCAE) version 4.0[38].

Historical Comparator for SLN Positivity

Two datasets were used to project the historical rates of SLN positivity a priori in stage IIB and stage IIC patients and allow for statistical comparison with the primary endpoint of this trial. The first cohort was clinical stage IIB/C patients from 2004-2012 from the National Cancer Database[39], which contains deidentified patient information from more than 1500 Commission-on-Cancer accredited institutions forming a nationally representative sample; in this dataset the SLN positivity rate for stage IIB was 24% (4,713/19,683 cases) and the rate for stage IIC was 39% (3,340/8,510 cases). In addition, we utilized our institution's retrospective melanoma database as the majority of trial patients were anticipated to be recruited from UPenn; we selected patients with clinical IIB/C melanoma from 1997 to 2014. In this cohort, the SLN positivity rate for stage IIB was 27.5% (66/240 cases) and the rate for stage IIC was 45% (48/107 cases).

We therefore computed power to detect a 50% reduction in the percentage of patients who have a positive SLN, estimating that the historical SLN positivity rate in stage IIB patients is 25% and the SLN positivity rate in stage IIC patients is 40% (mixed SLN positivity rate from the National

Cancer Database and UPenn institutional data using a weighted average). The distribution of stage IIB and stage IIC patients was not controlled in the trial. Therefore, we computed power for a range of possible enrollment distributions by stage. We allowed the percentage of stage IIB patients to vary from 30% to 60%. The overall SLN positivity rates of the null hypothesis based on these varying distributions could vary from 35.5% to 31.0%.

The table below displays the power to detect a 50% reduction in the percentage of treated patients who have a positive SLN at surgery, for a one sample chi-square test based on 63 patients, at a 2-sided 5% type I error rate. With 63 patients, the power for the chi-square test would be acceptable, at a range of 81 – 88%.

Percentage stage IIB patients	Percentage stage IIC patients	SLN positive rate stage IIB patients	SLN positive rate stage IIC patients	Null Hypothesis SLN+ rate	Alternative Hypothesis SLN+ rate	Power chi-square test
30%	70%	25%	40%	35.5%	17.8%	88%
35%	65%	25%	40%	34.8%	17.4%	88%
40%	60%	25%	40%	34.0%	17.0%	86%
45%	55%	25%	40%	33.3%	16.6%	85%
50%	50%	25%	40%	32.5%	16.3%	84%
55%	45%	25%	40%	31.8%	15.9%	83%
60%	40%	25%	40%	31.0%	15.5%	81%

Of note, the historical periods were determined by the availability of the relevant clinical and pathologic variables within each database and were not selected based on patient outcomes.

Post-hoc Melanoma Institute of Australia Nomogram Analysis

A separate post-hoc analysis of predicted SLN positivity rates was conducted using the Melanoma Institute of Australia (MIA) Risk Nomogram[21]. Utilizing the MIA online SLN risk prediction tool, we inputted the specific clinicopathologic characteristics of our trial cohort to estimate expected rates of SLN metastasis stratified by stage. The clinicopathologic characteristics utilized included age, thickness, ulceration status, mitotic rate, and histology; lymphovascular invasion status was set to “unknown” in the online tool. Numerical clinicopathologic characteristics were specified within the MIA online calculator. Subsequently, a weighted average calculation was conducted based on the proportion of patients with a specific histology within our trial cohort to predict SLN positivity rate for the overall cohort, as well as stage IIB and IIC subgroups. If a categorical variable was unknown for a specific patient in this analysis (i.e. histology), the known proportion of the overall group was utilized to derive the weighted average.

Follow-up and Survival Outcomes

As of February 23, 2026, 63 patients had completed protocol-directed treatment and were included in the analysis. The median follow-up duration was calculated from the date of surgery to the date of last clinical follow-up.

RFS was defined as the time from definitive surgery to the first documented recurrence or death from any cause. OS was defined as the time from definitive surgery to death from any cause. Patients without an event were censored at the date of last follow-up.

Nodal Tumor Burden

Patient nodal tumor burden was calculated for patients with positive SLN metastasis in the trial cohort and within a treatment-naïve historical cohort from 2006-2019. For each patient, the largest dimension of each discrete nodal melanoma metastasis was recorded in millimeters. Cumulative nodal tumor burden was defined a priori as the sum of the largest dimension of each discrete nodal metastatic deposit.

Immune Profiling Cohort

Patients included in immune profiling (n = 27) were those with adequate paired pretreatment and day-of-surgery blood specimens available for analysis. There were no significant differences in relevant clinicopathologic characteristics or select clinical outcomes (SLN+ rate and 2-year RFS) between the immune-profiled cohort (n = 27) and the non-profiled cohort (n = 36; **Supp Table 2**); the immune-profiled cohort had significantly longer median follow-up time (43 vs. 23 months, $p<0.001$).

Peptide Reconstitution and Tetramer Preparation

Peptides were reconstituted to a concentration of 10 mg/mL in 100% DMSO at room temperature and mixed thoroughly. Biotinylated monomers were obtained from either BioLegend (Flex-T™ monomer UVX, 0.2 mg/mL) or the NIH Tetramer Core Facility (2 mg/mL) and diluted to 0.1 mg/ml with phosphate-buffered saline (PBS). For UV-exchange, 49.5 µL of Monomer-PBS solution was combined with 0.9 µL of the 10 mg/mL peptide solution in a V-bottom 96-well plate, ensuring thorough mixing. The peptide-monomer-PBS mixture was exposed to UV light (366 nm) for 30 minutes in a cold room without a plate cover. After UV exchange, the plate was incubated at 37°C with a plate cover for an additional 30 minutes. After incubation, the mixture was centrifuged at 2,000 rpm for 2 minutes to separate aggregates. From the top layer of the supernatant, 15 µL of peptide-monomer-PBS (approximately 1.5 µg of monomer) was transferred into each of two PCR tubes. To each tube, 1.5 µL of a different fluorophore-conjugated streptavidin (BioLegend) was added, and the reaction was incubated for at least 1 hour at room temperature or overnight at 4°C. To terminate the reaction, 2.4 µL of 0.4 mM free biotin was added to each tube to block excess streptavidin binding sites. Quality control testing of the tetramers from each batch was performed using HLA-matched, CMV-positive PBMCs from the same healthy donor to ensure consistency. The prepared tetramers were stored at 4°C in the dark and were utilized within three months to maintain optimal performance.

Combinatorial Tetramer Flow Cytometry Staining

Single-cell suspensions prepared from blood and tissue samples were treated with DNase I, washed with FACS staining buffer (PBS supplemented with 2% fetal bovine serum), and aliquoted into U-bottom 96-well plates for staining. Both staining samples and non-staining controls were included.

Tetramer Staining: Tetramers were diluted in FACS staining buffer containing Brilliant Stain Buffer (BD Biosciences; used to reduce spectral overlap) and milk to prevent tetramer aggregation. A total of 50 µL of diluted tetramer solution was added to the staining samples, while 50 µL of tetramer-free buffer was added to the non-staining controls. The plates were incubated for 20 minutes at room temperature in the dark. Following incubation, the samples were washed with 150 µL of FACS staining buffer, centrifuged at 2,000 rpm for 3 minutes, and the supernatant was discarded.

Surface Staining: Surface antibodies and live/dead viability dyes were diluted in FACS staining buffer supplemented with Brilliant Stain Buffer. A total of 50 µL of diluted surface staining solution was added to the staining samples, while 50 µL of surface stain-free buffer was added to the non-staining controls. The samples were incubated for 20 minutes at room temperature in the dark, followed by a wash with 150 µL of FACS staining buffer, centrifugation at 2,000 rpm for 3 minutes, and removal of supernatants. Fixation and Permeabilization: Fixation and permeabilization were performed using commercially available buffer set (Invitrogen™ eBioscience™ Foxp3 / Transcription Factor Staining Buffer Set) prepared according to the manufacturer's instructions. A total of 50 µL of fixation/permeabilization buffer was added to each sample, and the plates were incubated for 20 minutes at room temperature in the dark. The samples were then washed with 150 µL of permeabilization buffer, centrifuged at 2,000 rpm for 3 minutes, and supernatants were removed.

Intracellular Staining: Intracellular antibodies were diluted in permeabilization staining buffer containing Brilliant Stain Buffer. A total of 50 µL of diluted intracellular staining solution was added to the staining samples, while 50 µL of intracellular stain-free buffer was added to the non-staining controls. The plates were incubated overnight at 4°C in the dark. Following incubation, the samples were washed with 150 µL of permeabilization buffer, centrifuged at 2,000 rpm for 3 minutes, and supernatants were removed. Finally, both staining samples and non-staining controls were resuspended in 200 µL of 1% paraformaldehyde solution for fixation before flow cytometric acquisition.

Flow Cytometry Data Acquisition and Basic Analysis

Stained samples and corresponding non-staining controls were acquired using a five-laser Cytek® Aurora spectral flow cytometer at an event rate of less than 10,000 events per second. Autofluorescence subtraction was performed by subtracting the fluorescence intensity of non-staining controls from the staining samples from the same patient. Spectral fluorescence unmixing was conducted using single-color controls according to the manufacturer's instructions. Further compensation and adjustments were applied using SpectroFlo® software.

Basic data analysis, including gating and population quantification, was performed using FlowJo software (BD Biosciences).

Flow Cytometry Analysis

All CD8 T cells were gated from flow cytometry data using FlowJo software. The expression of individual markers on these cells was analyzed for each sample and timepoint.

To obtain CD8 subtypes, $CD45RA^{+}CCR7^{+}$ were identified as naïve, $CD45RA^{-}CCR7^{+}$ cells were identified as central memory, $CD45RA^{+}CCR7^{-}$ were identified as TEMRA, and $CD45RA^{-}CCR7^{-}$ were identified as effector memory. Within non-naïve CD8 cells, exhausted phenotype was defined as $PD\text{-}1^{+}CD39^{+}$. A subsequent TCF1/T-bet quadrant gate was then applied to this exhausted subpopulation, with TCF1+ identified as progenitor exhausted, $TCF1^{-}/T\text{-}bet^{+}$ identified as intermediate exhausted, and $TCF1^{-}/T\text{-}bet^{-}$ identified as terminal exhausted. Proliferating $Ki67^{+}$ subpopulations within each of these CD8 subsets were subsequently identified (**Fig 2D**).

Median fluorescence intensity (MFI) of cells within each CD8 T cell subtype for each patient were retrieved and exported via the statistics and table tab from FlowJo v11 in CSV format and subsequently imported into R 4.4.2 utilizing the Tidyverse ecosystem 2.0.0. Batch files were concatenated, labelled according to patient condition, and the arithmetic mean of MFIs per CD8 subtype were taken for a select list of markers. Subsequently, the expression dataframe was converted into a matrix and raw values were transformed into Z-scores such that the average and standard deviation of average MFI values of each marker was 0 and 1, respectively, to allow for ease of comparability. R packages ComplexHeatMap 2.22.0 and Circlize 0.4.16 were then used to construct a heatmap intersecting cell type and marker MFI expression. Rows and columns were organized according to unsupervised hierarchical clustering as determined by Euclidean distance.

Combinatorial Tetramer Flow Cytometry Analysis

To detect and analyze melanoma-specific CD8 T cells, we employed combinatorial tetramer staining approaches, as previously described. Briefly, peptide-MHC class I tetramers were conjugated to distinct fluorochromes and combined to uniquely label antigen-specific T cell populations. Events were considered tetramer-positive if they displayed specific staining in exactly two combinatorial channels and negative for the other three. A threshold of ≥5 tetramer-positive events in a tight cluster was set to define a sample as tetramer-positive. Additionally, phenotypic analysis of tetramer-positive cells was conducted when ≥10 positive events in a tight cluster were available.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM) unless otherwise specified. Statistical significance was assessed using the Wilcoxon rank-sum test for unpaired comparisons and the Wilcoxon signed-rank test for paired comparisons. Categorical analyses were conducted via Chi-squared tests and Fisher's exact tests as appropriate. A two-sided exact binomial test was utilized to compare historical and trial SLN positivity rates. Kaplan-Meier

analyses were conducted from day of surgery as time 0 and p-values assessed via the log-rank method. Cox proportional hazards analysis was conducted with day of surgery as time 0 for recurrence associated with residual primary tumor; all analyses met the proportional hazards assumption. Firth's logistic regression analysis was conducted for factors associated with presence of melanoma-specific cells. P-values are reported as follows: ns (not significant), *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001. All analyses were conducted using Stata, R, or GraphPad Prism.

Acknowledgements

This work was supported by National Institutes of Health grants R01CA273018 (A.C.H.), P50 CA261608 (A.C.H., G.C.K. X.X., and R.K.A.), and P30 CA016520 (A.C.H.). In addition, A.C.H. was supported by Damon Runyon Clinical Investigator Award, Damon Runyon Continuation Award, Doris Duke Clinical Scientist Development Award, the W. W. Smith Charitable Trust Award, the Institute for Translational Medicine and Therapeutics of the Perelman School of Medicine and the Pew-Stewart.

P.A.G. received funding from Abramson Cancer Center Support Grant, P30-CA016520.

R.N.A. received research funding from Regeneron, Obsidian, KSQ, Immatics, OnKure, Erasca.

Merck Sharp & Dohme LLC, a subsidiary of Merck & Co., Inc., Rahway, NJ, USA, provided drug and financial support for the study.

Author Contributions

M.S.F. conducted flow cytometry on blood samples from UPenn. M.S.F. conducted correlative analyses of clinical outcomes, nodal tumor burden, and flow cytometry data. M.S.F., X.T., A.L., S.G.R. and P.A.G. performed data analyses. G.W., Y.Z., and M.S.F. designed and tested the combinatorial tetramer panel. M.S.F., A.D., and K.L.M. curated correlative clinical and biospecimen data. S.M. curated and confirmed clinicopathologic data for trial patients from all participating institutions. P.A.G. oversaw statistical methodology. X.X. supervised and confirmed pathologic assessment of sentinel nodes. A.C.H. oversaw flow cytometric analyses and translational biospecimen investigation. T.C.M., L.M.S., R.K.A., J.T.M., and G.C.K. oversaw sample collection at UPenn. J.A.C. and K.M.M. oversaw sample collection at the University of Louisville. M.A.D., R.N.A., R.W., and J.E.G. oversaw sample collection at the University of Texas MD Anderson Cancer Center. M.S.F., A.C.H., and G.C.K. wrote the manuscript. A.L., T.C.M., L.M.S., R.K.A., J.T.M., M.A.D., R.N.A., R.W., J.E.G., J.A.C., and K.M.M. reviewed and revised the manuscript. X.T., A.C.H., and M.S.F. designed and drafted the figures/tables. A.C.H. and G.C.K. supervised, interpreted, and oversaw the study. G.C.K. conceived and designed the trial.

Declaration of Interests/Disclosures

M.A.D. is a consultant for Bristol-Myers Squibb. R.N.A. is on the steering committee for Obsidian, KSQ, and Immatics. A.C.H. receives research support from Merck, BMS, and

Immunai. M.S.F. has an immediate family member that is an employee for Bayer AG. R.N.A. received research funding from Regeneron, Obsidian, KSQ, Immatics, OnKure, Erasca.Merck Sharp & Dohme LLC, a subsidiary of Merck & Co., Inc., Rahway, NJ, USA, provided drug and financial support for the study.

References

1. Farooq, M.S., Tidwell, J.C., and Karakousis, G.C. (2025). Neoadjuvant Therapy in Melanoma: Current Evidence and Future Directions of Investigation. Surg Clin North Am *105*, 569-589. 10.1016/j.suc.2024.11.005.
2. Blank, C.U., Lucas, M.W., Scolyer, R.A., van de Wiel, B.A., Menzies, A.M., Lopez-Yurda, M., Hoeijmakers, L.L., Saw, R.P.M., Lijnsvelt, J.M., Maher, N.G., et al. (2024). Neoadjuvant Nivolumab and Ipilimumab in Resectable Stage III Melanoma. N Engl J Med *391*, 1696-1708. 10.1056/NEJMoa2402604.
3. Patel, S.P., Othus, M., Chen, Y., Wright, G.P., Jr., Yost, K.J., Hyngstrom, J.R., Hu-Lieskovan, S., Lao, C.D., Fecher, L.A., Truong, T.G., et al. (2023). Neoadjuvant-Adjuvant or Adjuvant-Only Pembrolizumab in Advanced Melanoma. N Engl J Med *388*, 813-823. 10.1056/NEJMoa2211437.
4. Farooq, M.S., Mattfeld, V., Beniwal, P.C., Vargas, G.M., Shafique, N., Miura, J.T., and Karakousis, G.C. (2026). Recurrence Patterns and Survival Outcomes in Clinical Stage IIB/IIC Melanoma: Can We Stratify Patients for Consideration of Neoadjuvant Immunotherapy? Ann Surg Oncol *33*, 379-388. 10.1245/s10434-025-18263-z.
5. Fonseca, I.B., Lindote, M.V.N., Monteiro, M.R., Doria Filho, E., Pinto, C.A.L., Jafelicci, A.S., de Melo Lobo, M., Calsavara, V.F., Bertolli, E., and Duprat Neto, J.P. (2020). Sentinel Node Status is the Most Important Prognostic Information for Clinical Stage IIB and IIC Melanoma Patients. Ann Surg Oncol *27*, 4133-4140. 10.1245/s10434-020-08959-9.
6. Straker, R.J., 3rd, Sharon, C.E., Chu, E.Y., Miura, J.T., Ming, M.E., and Karakousis, G.C. (2022). Sentinel lymph node biopsy in patients with clinical stage IIB/C cutaneous melanoma: A national cohort study. J Am Acad Dermatol *87*, 754-760. 10.1016/j.jaad.2022.04.025.
7. Bajaj, S., Donnelly, D., Call, M., Johannet, P., Moran, U., Polsky, D., Shapiro, R., Berman, R., Pavlick, A., Weber, J., et al. (2020). Melanoma Prognosis: Accuracy of the American Joint Committee on Cancer Staging Manual Eighth Edition. J Natl Cancer Inst *112*, 921-928. 10.1093/jnci/djaa008.
8. Kirkwood, J.M., Del Vecchio, M., Weber, J., Hoeller, C., Grob, J.J., Mohr, P., Loquai, C., Dutriaux, C., Chiarion-Sileni, V., Mackiewicz, J., et al. (2023). Adjuvant nivolumab in resected stage IIB/C melanoma: primary results from the randomized, phase 3 CheckMate 76K trial. Nat Med *29*, 2835-2843. 10.1038/s41591-023-02583-2.
9. Luke, J.J., Rutkowski, P., Queirolo, P., Del Vecchio, M., Mackiewicz, J., Chiarion-Sileni, V., de la Cruz Merino, L., Khattak, M.A., Schadendorf, D., Long, G.V., et al. (2022). Pembrolizumab versus placebo as adjuvant therapy in completely resected stage IIB or IIC melanoma (KEYNOTE-716): a randomised, double-blind, phase 3 trial. Lancet *399*, 1718-1729. 10.1016/S0140-6736(22)00562-1.

10. NCCN Clinical Practice Guidelines in Oncology (NCCN Guidelines®) for Melanoma: Cutaneous (Version 2.2024). . https://www.nccn.org/professionals/physician_gls/pdf/cutaneous_melanoma.pdf
11. Huang, A.C., Orlowski, R.J., Xu, X., Mick, R., George, S.M., Yan, P.K., Manne, S., Kraya, A.A., Wubbenhorst, B., Dorfman, L., et al. (2019). A single dose of neoadjuvant PD-1 blockade predicts clinical outcomes in resectable melanoma. Nat Med *25*, 454-461. 10.1038/s41591-019-0357-y.
12. Huang, A.C., Postow, M.A., Orlowski, R.J., Mick, R., Bengsch, B., Manne, S., Xu, W., Harmon, S., Giles, J.R., Wenz, B., et al. (2017). T-cell invigoration to tumour burden ratio associated with anti-PD-1 response. Nature *545*, 60-65. 10.1038/nature22079.
13. Wang, G., Yoon, D., Nandi, A., Patel, K., Azar, T., Kim, J., Han, N.A., Nickie, A., Park, S., Wang, K., et al. (2026). Antigen-specific profiling identifies T-bet(+) melanoma-specific CD8(+) T cells associated with response to neoadjuvant PD-1 blockade. Cancer Cell *44*, 221-234 e225. 10.1016/j.ccell.2025.12.004.
14. Gershenwald, J.E., Scolyer, R.A., Hess, K.R., Sondak, V.K., Long, G.V., Ross, M.I., Lazar, A.J., Faries, M.B., Kirkwood, J.M., McArthur, G.A., et al. (2017). Melanoma staging: Evidence-based changes in the American Joint Committee on Cancer eighth edition cancer staging manual. CA Cancer J Clin *67*, 472-492. 10.3322/caac.21409.
15. Garbe, C., Keim, U., Amaral, T., Berking, C., Eigentler, T.K., Flatz, L., Gesierich, A., Leiter, U., Stadler, R., Sunderkotter, C., et al. (2022). Prognosis of Patients With Primary Melanoma Stage I and II According to American Joint Committee on Cancer Version 8 Validated in Two Independent Cohorts: Implications for Adjuvant Treatment. J Clin Oncol *40*, 3741-3749. 10.1200/JCO.22.00202.
16. Helvind, N.M., Brinch-Moller Weitemeyer, M., Chakera, A.H., Hendel, H.W., Ellebaek, E., Svane, I.M., Kjaerskov, M.W., Bojesen, S., Skyum, H., Petersen, S.K., et al. (2023). Stage-Specific Risk of Recurrence and Death From Melanoma in Denmark, 2008-2021: A National Observational Cohort Study of 25 720 Patients With Stage IA to IV Melanoma. JAMA Dermatol *159*, 1213-1222. 10.1001/jamadermatol.2023.3256.
17. White, R.L., Jr., Ayers, G.D., Stell, V.H., Ding, S., Gershenwald, J.E., Salo, J.C., Pockaj, B.A., Essner, R., Faries, M., Charney, K.J., et al. (2011). Factors predictive of the status of sentinel lymph nodes in melanoma patients from a large multicenter database. Ann Surg Oncol *18*, 3593-3600. 10.1245/s10434-011-1826-9.
18. Yamamoto, M., Fisher, K.J., Wong, J.Y., Koscso, J.M., Konstantinovic, M.A., Govsyeyev, N., Messina, J.L., Sarnaik, A.A., Cruse, C.W., Gonzalez, R.J., et al. (2015). Sentinel lymph node biopsy is indicated for patients with thick clinically lymph node-negative melanoma. Cancer *121*, 1628-1636. 10.1002/cncr.29239.
19. Miura, J., Farooq, M.S., Gimotty, P.A., Mitchell, T.C., Amaravadi, R.K., Schuchter, L.M., Mitnick, S., Xu, X., Huang, A.C.C., Davies, M.A., et al. (2025). Neoadjuvant-adjuvant pembrolizumab in clinical stage IIB/C melanoma. Journal of Clinical Oncology *43*, 9502-9502. 10.1200/JCO.2025.43.16_suppl.9502.
20. ClinicalTrials.gov (2025). Neoadjuvant PD-1 Blockade in Patients with Stage IIB/C Melanoma. https://clinicaltrials.gov/study/NCT03757689.
21. Lo, S.N., Ma, J., Scolyer, R.A., Haydu, L.E., Stretch, J.R., Saw, R.P.M., Nieweg, O.E., Shannon, K.F., Spillane, A.J., Ch'ng, S., et al. (2020). Improved Risk Prediction Calculator for Sentinel Node Positivity in Patients With Melanoma: The Melanoma Institute Australia Nomogram. J Clin Oncol *38*, 2719-2727. 10.1200/JCO.19.02362.
22. Shafique, N., Farooq, M.S., Mattfeld, V., Vargas, G.M., Xu, X., Schuchter, L., Amaravadi, R., Mitchell, T., Miura, J.T., and Karakousis, G.C. (2025). Single-Dose Neoadjuvant

Pembrolizumab in Resectable Metastatic Melanoma. Ann Surg Oncol *32*, 8958-8963. 10.1245/s10434-025-18314-5.

23. Beltra, J.C., Manne, S., Abdel-Hakeem, M.S., Kurachi, M., Giles, J.R., Chen, Z., Casella, V., Ngiow, S.F., Khan, O., Huang, Y.J., et al. (2020). Developmental Relationships of Four Exhausted CD8(+) T Cell Subsets Reveals Underlying Transcriptional and Epigenetic Landscape Control Mechanisms. Immunity *52*, 825-841 e828. 10.1016/j.immuni.2020.04.014.
24. Zander, R., Schauder, D., Xin, G., Nguyen, C., Wu, X., Zajac, A., and Cui, W. (2019). CD4(+) T Cell Help Is Required for the Formation of a Cytolytic CD8(+) T Cell Subset that Protects against Chronic Infection and Cancer. Immunity *51*, 1028-1042 e1024. 10.1016/j.immuni.2019.10.009.
25. Hudson, W.H., Gensheimer, J., Hashimoto, M., Wieland, A., Valanparambil, R.M., Li, P., Lin, J.X., Konieczny, B.T., Im, S.J., Freeman, G.J., et al. (2019). Proliferating Transitory T Cells with an Effector-like Transcriptional Signature Emerge from PD-1(+) Stem-like CD8(+) T Cells during Chronic Infection. Immunity *51*, 1043-1058 e1044. 10.1016/j.immuni.2019.11.002.
26. He, R., Hou, S., Liu, C., Zhang, A., Bai, Q., Han, M., Yang, Y., Wei, G., Shen, T., Yang, X., et al. (2016). Follicular CXCR5-expressing CD8+ T cells curtail chronic viral infection. Nature *537*, 412-428. 10.1038/nature19317.
27. Im, S.J., Hashimoto, M., Gerner, M.Y., Lee, J., Kissick, H.T., Burger, M.C., Shan, Q., Hale, J.S., Lee, J., Nasti, T.H., et al. (2016). Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. Nature *537*, 417-421. 10.1038/nature19330.
28. Utzschneider, D.T., Charmoy, M., Chennupati, V., Pousse, L., Ferreira, D.P., Calderon-Copete, S., Danilo, M., Alfei, F., Hofmann, M., Wieland, D., et al. (2016). T Cell Factor 1-Expressing Memory-like CD8(+) T Cells Sustain the Immune Response to Chronic Viral Infections. Immunity *45*, 415-427. 10.1016/j.immuni.2016.07.021.
29. Miller, B.C., Sen, D.R., Al Abosy, R., Bi, K., Virkud, Y.V., LaFleur, M.W., Yates, K.B., Lako, A., Felt, K., Naik, G.S., et al. (2019). Subsets of exhausted CD8(+) T cells differentially mediate tumor control and respond to checkpoint blockade. Nat Immunol *20*, 326-336. 10.1038/s41590-019-0312-6.
30. Liu, J., Blake, S.J., Yong, M.C., Harjunpaa, H., Ngiow, S.F., Takeda, K., Young, A., O'Donnell, J.S., Allen, S., Smyth, M.J., and Teng, M.W. (2016). Improved Efficacy of Neoadjuvant Compared to Adjuvant Immunotherapy to Eradicate Metastatic Disease. Cancer Discov *6*, 1382-1399. 10.1158/2159-8290.CD-16-0577.
31. Gajdos, C., Griffith, K.A., Wong, S.L., Johnson, T.M., Chang, A.E., Cimmino, V.M., Lowe, L., Bradford, C.R., Rees, R.S., and Sabel, M.S. (2009). Is there a benefit to sentinel lymph node biopsy in patients with T4 melanoma? Cancer *115*, 5752-5760. 10.1002/cncr.24660.
32. Scoggins, C.R., Bowen, A.L., Martin, R.C., 2nd, Edwards, M.J., Reintgen, D.S., Ross, M.I., Urist, M.M., Stromberg, A.J., Hagendoorn, L., and McMasters, K.M. (2010). Prognostic information from sentinel lymph node biopsy in patients with thick melanoma. Arch Surg *145*, 622-627. 10.1001/archsurg.2010.115.
33. Mozzillo, N., Pennacchioli, E., Gandini, S., Caraco, C., Crispo, A., Botti, G., Lastoria, S., Barberis, M., Verrecchia, F., and Testori, A. (2013). Sentinel node biopsy in thin and thick melanoma. Ann Surg Oncol *20*, 2780-2786. 10.1245/s10434-012-2826-0.
34. Long, G.V., Luke, J.J., Khattak, M.A., de la Cruz Merino, L., Del Vecchio, M., Rutkowski, P., Spagnolo, F., Mackiewicz, J., Chiarion-Sileni, V., Kirkwood, J.M., et al. (2022). Pembrolizumab versus placebo as adjuvant therapy in resected stage IIB or IIC melanoma (KEYNOTE-716): distant metastasis-free survival results of a multicentre, double-blind, randomised, phase 3 trial. Lancet Oncol *23*, 1378-1388. 10.1016/S1470-2045(22)00559-9.

35. Farooq, M.S., Jona, A., Simons, R.A., Vargas, G.M., Miura, J.T., and Karakousis, G.C. (2026). Adjuvant Immunotherapy for High-Risk Stage II Melanoma: Real-World Utilization and Associated Overall Survival. Ann Surg Oncol. 10.1245/s10434-026-20411-y.
36. Kamphorst, A.O., Pillai, R.N., Yang, S., Nasti, T.H., Akondy, R.S., Wieland, A., Sica, G.L., Yu, K., Koenig, L., Patel, N.T., et al. (2017). Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. Proc Natl Acad Sci U S A *114*, 4993-4998. 10.1073/pnas.1705327114.
37. Mlecnik, B., Tosolini, M., Kirilovsky, A., Berger, A., Bindea, G., Meatchi, T., Bruneval, P., Trajanoski, Z., Fridman, W.H., Pages, F., and Galon, J. (2011). Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. J Clin Oncol *29*, 610-618. 10.1200/JCO.2010.30.5425.
38. Common Terminology Criteria for Adverse Events (CTCAE), Version 4.0. (2009). National Cancer Institute, National Institutes of Health, US Department of Health and Human Services. May 29, 2009, NIH Publication No. 09-7473.
39. Bilimoria, K.Y., Stewart, A.K., Winchester, D.P., and Ko, C.Y. (2008). The National Cancer Data Base: a powerful initiative to improve cancer care in the United States. Ann Surg Oncol *15*, 683-690. 10.1245/s10434-007-9747-3.

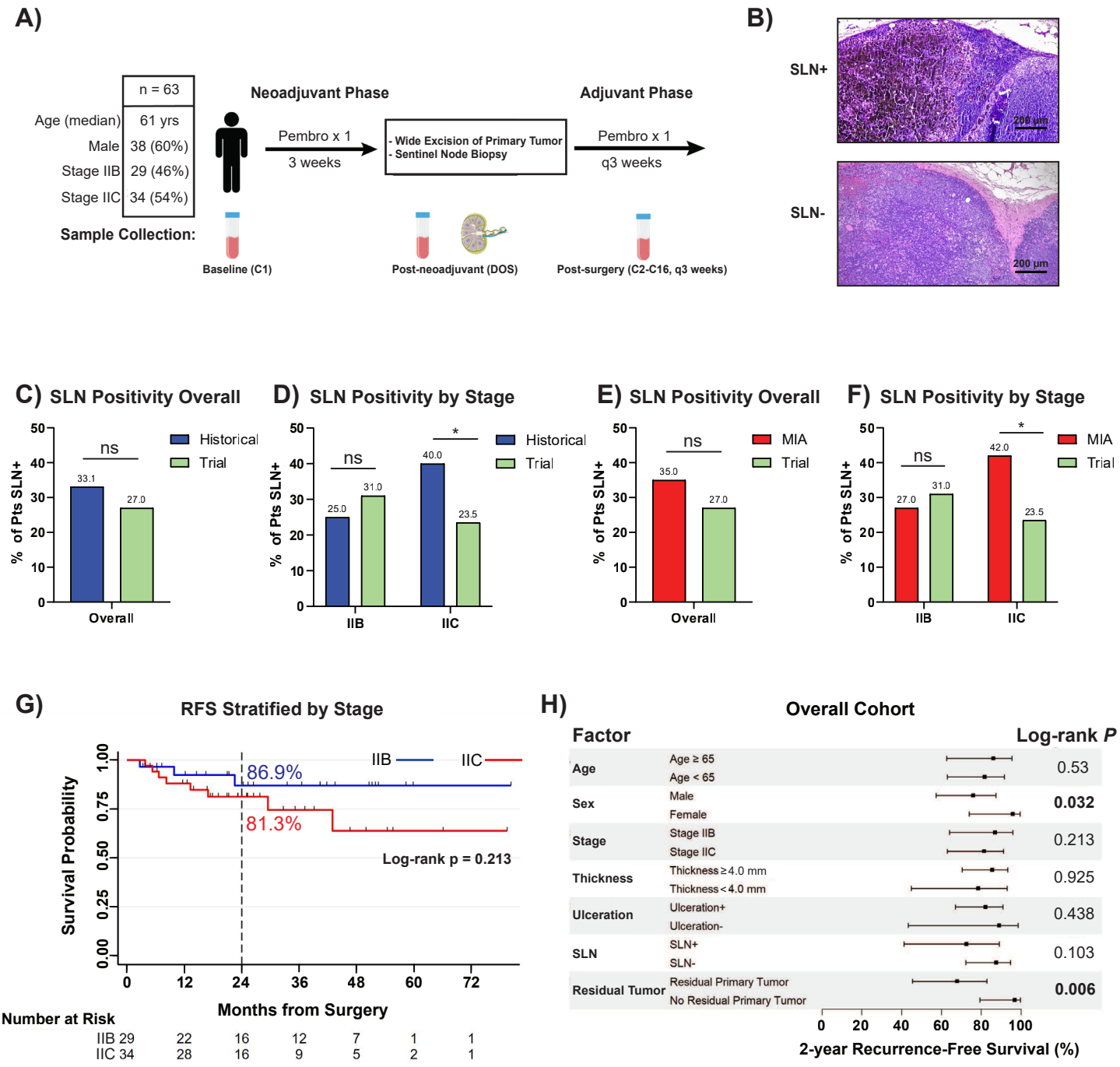


Figure 1: **Neoadjuvant PD-1 blockade reduces SLN positivity rate in clinical stage IIC patients.**

A) Clinical trial schema with select clinicopathologic variables of trial patients; DOS - day of surgery.
B) Hematoxylin and Eosin stained slides of sentinel lymph nodes with (SLN+) and without (SLN-) evidence of metastases; scale bars indicate 200 µm.
C) Primary outcome of sentinel node positivity rate in trial patients versus historical controls in the overall cohort; two-sided exact binomial test.
D) Primary outcome of sentinel node positivity rate in trial patients versus historical controls stratified by stage; *p < 0.05, two-sided exact binomial test.
E) Primary outcome of sentinel node positivity rate in trial patients versus Melanoma Institute of Australia (MIA) Nomogram in the overall cohort; two-sided exact binomial test.
F) Primary outcome of sentinel node positivity rate in trial vs Melanoma Institute of Australia (MIA) Nomogram stratified by stage; *p < 0.05, two-sided exact binomial test.
G) Kaplan-Meier plot of 2-year recurrence-free survival stratified by stage; Log-rank test.
H) Forest plot illustrating 2-year recurrence-free survival estimates stratified by select clinicopathologic variables in the overall cohort. P-values determined using the log-rank method for the full follow-up period.

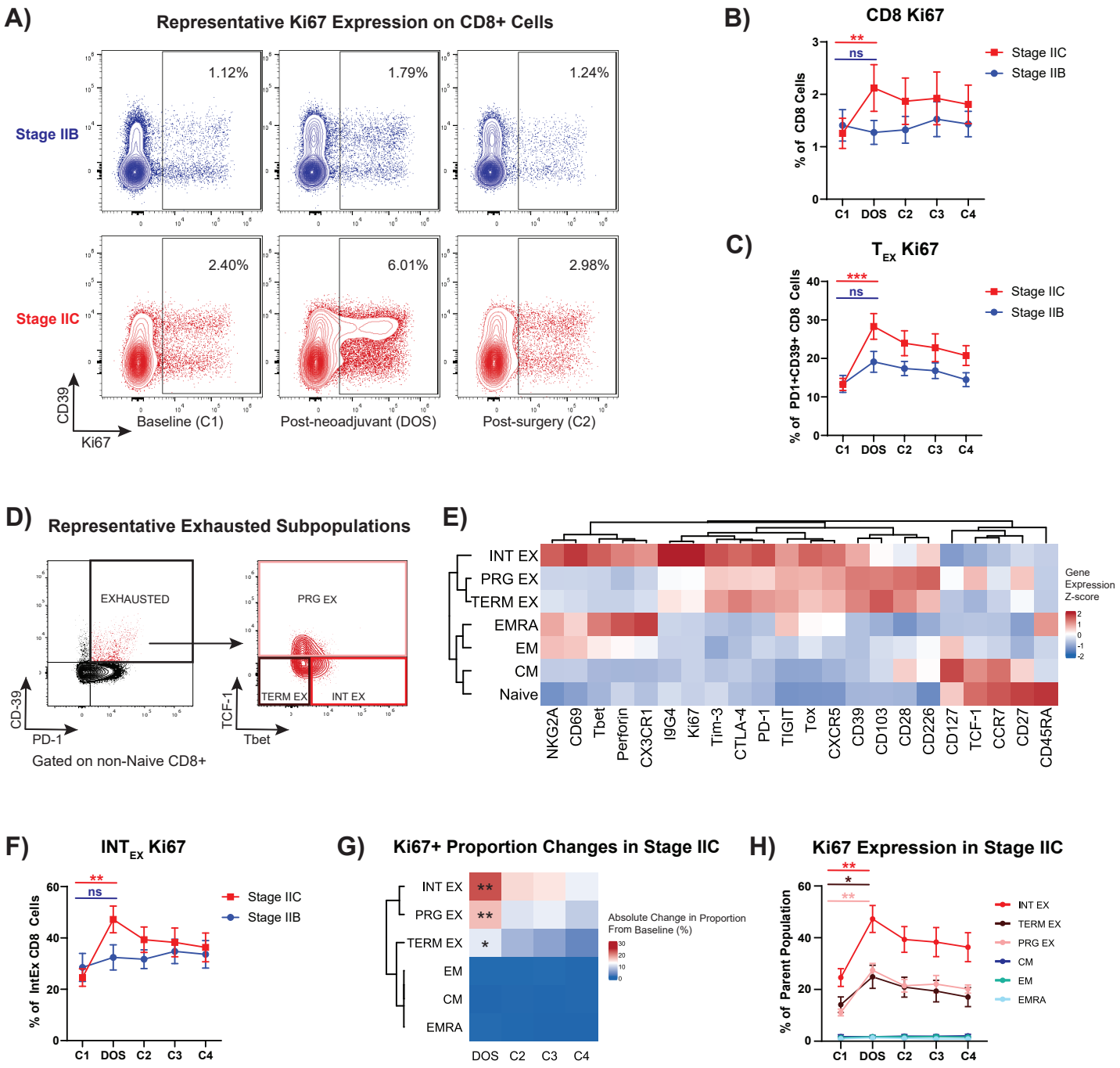


Figure 2: **PD-1 blockade reinvigorates exhausted CD8 T cells in clinical stage IIC patients.**

A) Representative flow plot of Ki67 expression on non-naive CD8 cells in stage IIB and stage IIC patients.
B) Quantified Ki67 expression on CD8 cells stratified by stage across time; **p < 0.01, Wilcoxon signed-rank test.
C) Proliferation of PD1+CD39+ (exhausted) CD8 cells stratified by stage across time; ***p < 0.001, Wilcoxon signed-rank test.
D) Representative CD8 exhausted T cell gating defining progenitor (TCF-1+), intermediate (TCF-1- Tbet+), and terminal (TCF-1- Tbet-) exhausted CD8 T cells.
E) Heatmap of CD8 cell populations defined by flow cytometric markers (INT EX - intermediate-exhausted; PRG EX - progenitor-exhausted; TERM EX - terminal-exhausted; EMRA - terminal effector memory; EM - effector memory; CM - central memory).
F) Proliferation of PD1+CD39+Tbet+ (intermediate-exhausted) CD8 cells stratified by stage across time; **p < 0.01, Wilcoxon signed-rank test.
G) Heatmap of absolute percentile changes of the proportion of Ki67+ cell populations compared to baseline (C1); *p < 0.05, **p < 0.01, Wilcoxon signed-rank test with Benjamini-Hochberg correction.
H) Ki67 expression of exhausted and memory CD8 T cell subsets across time in stage IIC patients; *p < 0.05, **p < 0.01, Wilcoxon signed-rank test with Benjamini-Hochberg correction.

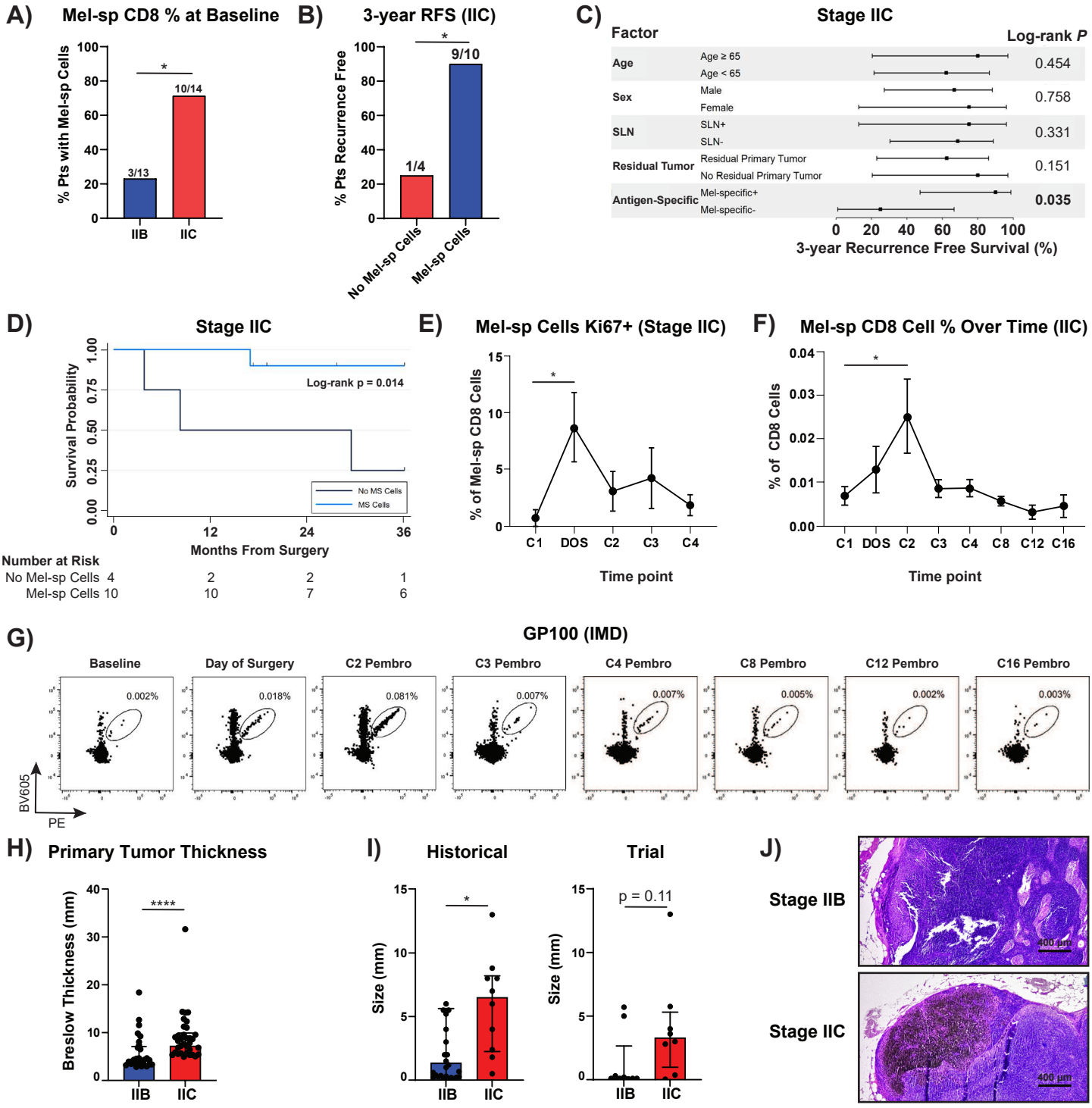


Figure 3: **Greater melanoma-specific T cell responses in clinical stage IIC patients**

A) Baseline frequency of melanoma-specific CD8 cells in stage IIB and stage IIC disease; *p < 0.05, Fisher's exact test.

B) Percentage of patients recurrence-free at 3 years for patients with and without melanoma-specific CD8 T cells in stage IIC disease; *p < 0.05, Fisher's exact test.

C) Forest plot illustrating 3-year recurrence-free survival estimates of stage IIC patients stratified by select clinicopathologic variables. P-values determined using the log-rank method for the full follow-up period.

D) Kaplan-Meier plot of recurrence-free survival stratified by presence of melanoma-specific cells in stage IIC patients; p value calculated for the 36-month time point using the log-rank test.

E) Ki67 expression in melanoma-specific cells in stage IIC over time; *p < 0.05, Wilcoxon signed-rank test.

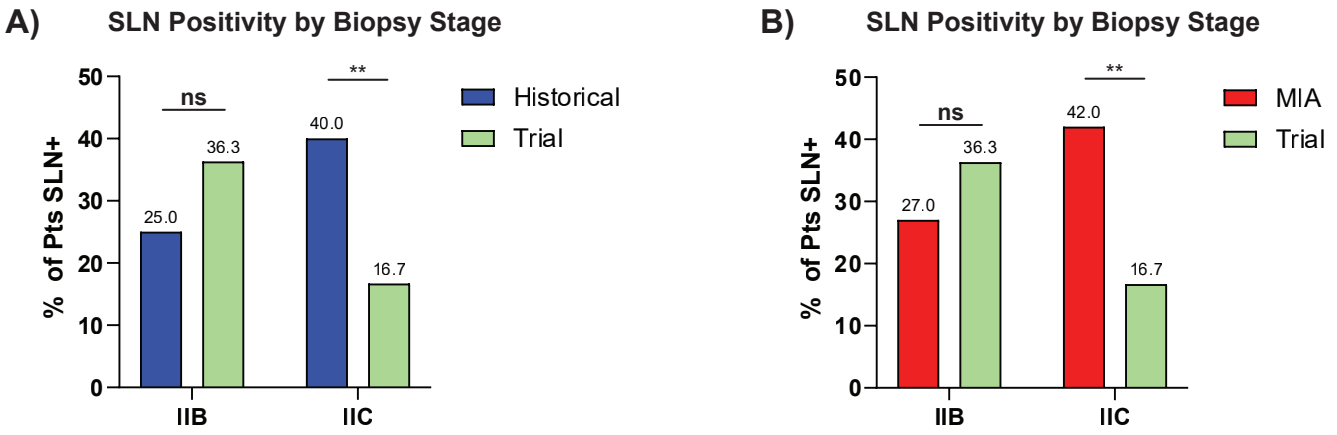
F) Frequency of melanoma-specific cells in stage IIC over time; *p < 0.05, Wilcoxon signed-rank test.

G) Representative tetramer plots gated for melanoma antigen (GP100 (IMD)) specific T cells over time in a stage IIC patient.

H) Median Breslow primary tumor thickness in stage IIB and stage IIC disease; ****p < 0.0001, Wilcoxon signed-rank test.

I) Median SLN tumor metastasis size in stage IIB and stage IIC disease for treatment-naive historical cohort (n=40) and trial cohort (n=17); *p < 0.05, Wilcoxon signed-rank test.

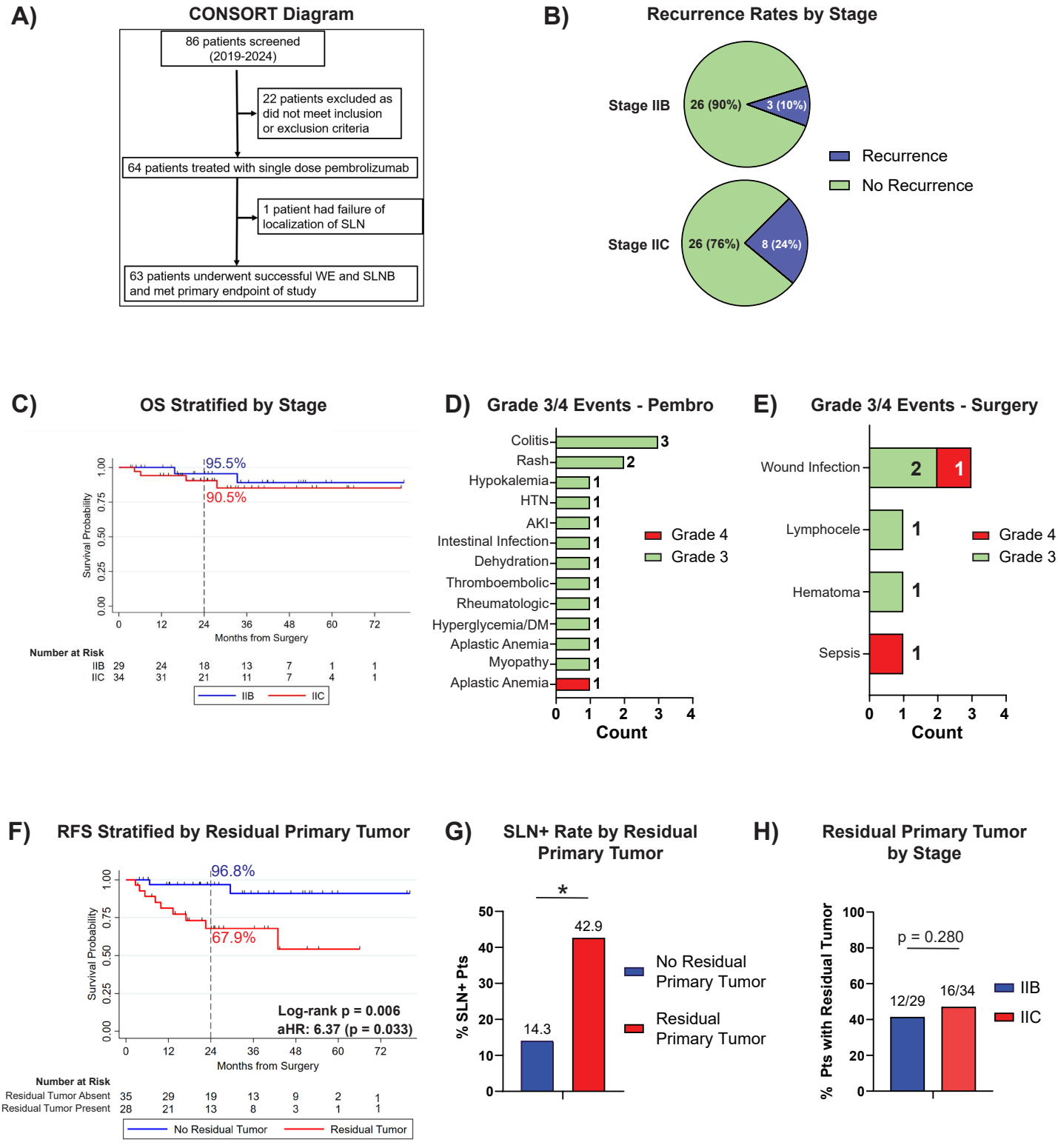
J) Hematoxylin and Eosin stained slides illustrating sentinel lymph node metastases sizes in stage IIB and IIC tumors; scale bars indicate 400 µm.



Supplemental Figure 1: **Primary outcome of SLN positivity rate based on pre-operative biopsy staging.**

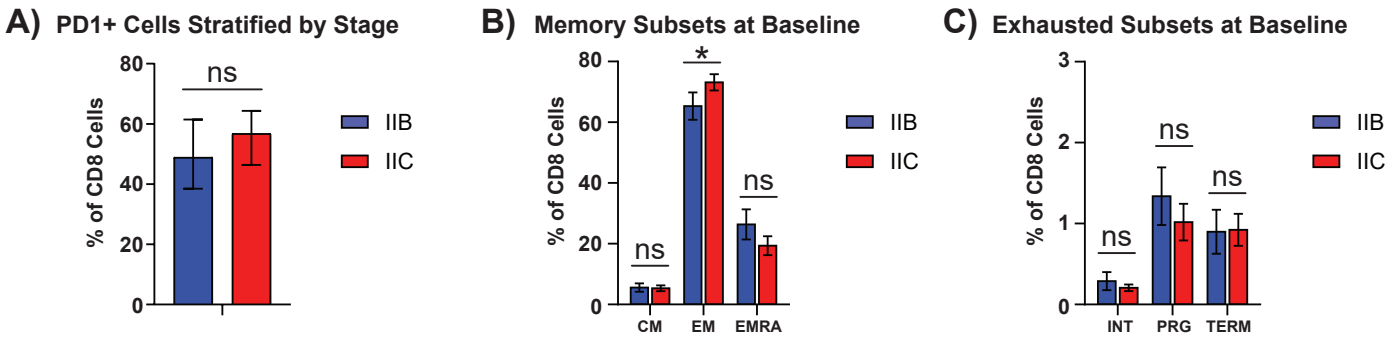
A) Primary outcome of sentinel node positivity rate in trial patients versus historical controls stratified by clinical stage at biopsy; **p < 0.01, two-sided exact binomial test.

B) Primary outcome of sentinel node positivity rate in trial vs Melanoma Institute of Australia (MIA) Nomogram stratified by clinical stage at biopsy; **p < 0.01, two-sided exact binomial test.



Supplemental Figure 2: Clinical trial efficacy and safety profile.

A) CONSORT Plot describing enrollment criteria.
B) Distribution of recurrence rates in patients by disease stage.
C) Kaplan-Meier plot of overall survival stratified by stage.
D) Distribution of pembrolizumab-related grade 3/4 adverse events.
E) Distribution of surgery-related grade 3/4 adverse events.
F) Recurrence-free survival curve stratified by the presence of residual tumor on day of surgery; Log-rank test. Adjusted hazard ratio calculated by multivariable Cox proportional hazards regression.
G) Sentinel lymph node positivity rate stratified by presence of residual primary tumor; *p < 0.05, Chi-Squared test.
H) Proportion of patients with residual primary tumor stratified by stage; Chi-Squared test

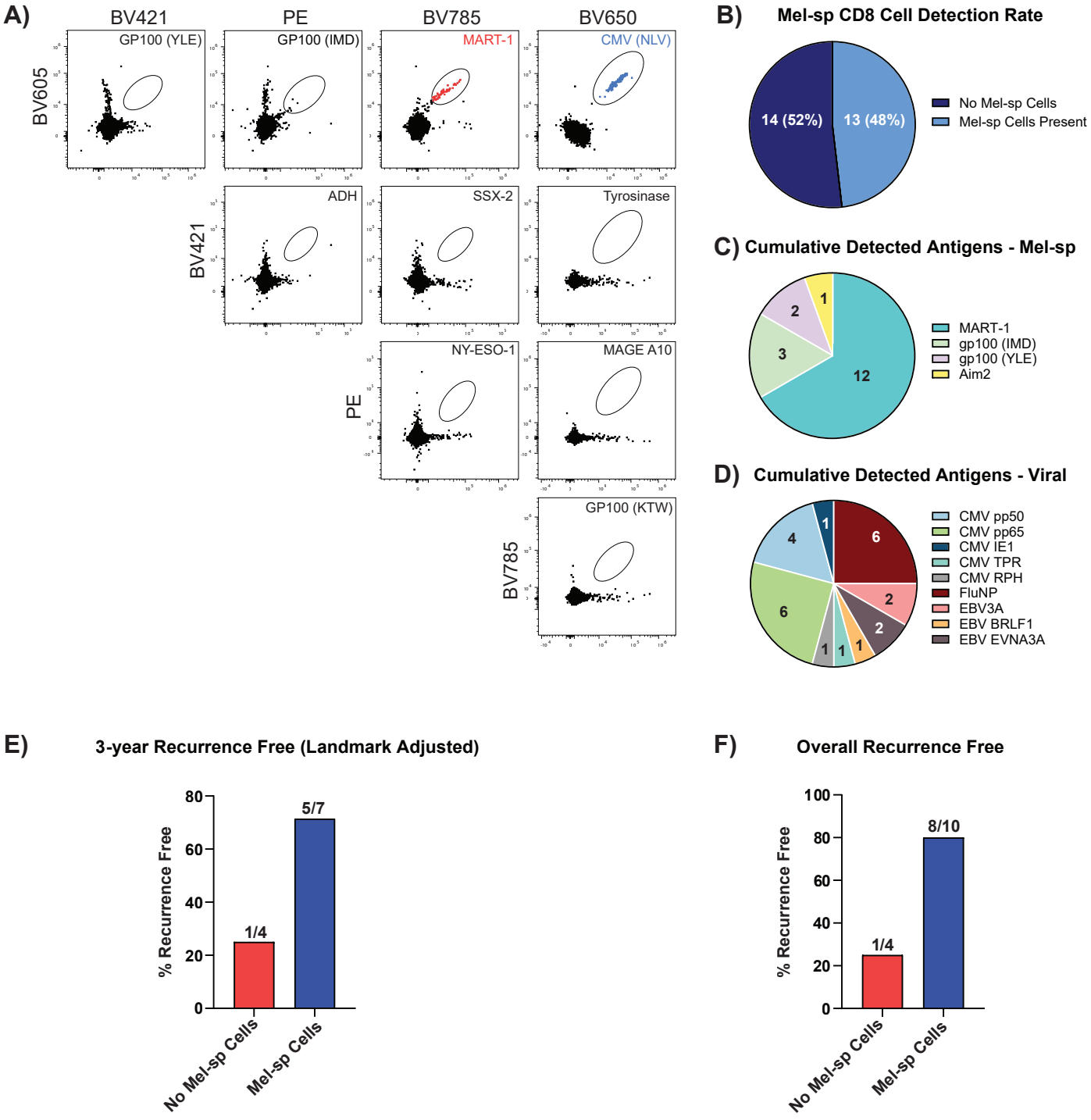


Supplemental Figure 3: CD8 T cell subsets in clinical stage IIB and stage IIC patients at baseline.

A) Proportion of PD1 expressing CD8 T cells, as a fraction of all non-naive CD8 T cells, by stage at baseline (C1); Wilcoxon signed-rank test.

B) Proportion of each memory T cell subset, as a fraction of all non-naive CD8 T cells, at baseline (C1); *$p < 0.05$, Wilcoxon signed-rank test with Benjamini-Hochberg corrections.

C) Proportion of each exhausted T cell subset, as a fraction of all non-naive CD8 T cells, at baseline (C1); Wilcoxon signed-rank test with Benjamini-Hochberg corrections.



Supplemental Figure 4: Antigen-specific T cells identified in trial patients.

A) Representative combinatorial tetramer plots gating for melanoma (red) and viral (blue) specific CD8 T cells.
B) Proportion of patients with detectable melanoma specific cells within the immune-profiled cohort.
C) Distribution of detected melanoma-specific antigens MART-1, gp100 (IMD/YLE), and Aim2.
D) Distribution of detected viral-specific antigens.
E) Percentage of patients recurrence-free at 3 years (landmark adjusted analysis) for patients with and without melanoma specific CD8 T cells in stage IIC disease.
F) Percentage of patients recurrence-free at full follow-up for patients with and without melanoma specific CD8 T cells in stage IIC disease.

Table 1: Clinicopathologic characteristics of overall trial cohort.

Characteristic	
Age, median (IQR), y	61 (54–69)
Sex, No. (%)	
Male	38 (60)
Female	25 (40)
ECOG Performance Status, No. (%)	
0	62 (98)
1	1 (2)
Clinical Stage, No. (%)	
IIB	29 (46)
IIC	34 (54)
Anatomic Site, No. (%)	
Trunk	27 (43)
Extremity	28 (44)
Head and neck	8 (13)
Melanoma Subtype, No. (%)	
Nodular	31 (49)
Superficial spreading	13 (21)
Desmoplastic	1 (1.5)
Lentigo maligna	1 (1.5)
Acral	2 (3)
Unspecified	15 (24)
Tumor Thickness, median (IQR), mm	
Biopsy only	4.3 (2.9–6.1)
Combined biopsy + WLE	5.7 (3.5–8.3)
Ulceration, No. (%)	
Yes	54 (86)
No	9 (14)
Mitotic Rate, median (IQR), mitoses/mm^2	9 (4–14)
Residual Primary Tumor on DOS	
Yes	28 (44)
No	35 (56)
Treatment Center, No. (%)	
University of Pennsylvania	47 (75)
Louisville	13 (20)
MD Anderson	3 (5)

Table 2: Grade 3/4 treatment-related adverse events.

Grade 3/4 Treatment-Related Adverse Events			
Category / Adverse Event	**Grade 3**	**Grade 4**	**TOTAL**
	No.	No.	No.
Blood and Lymphatic System Disorders			
Acquired pure red cell aplasia	0	1 (1.6%)	1(1.6%)
Gastrointestinal			
Diarrhea/Colitis	3 (4.7%)	0	3 (4.7%)
Infections and Infestations			
Sepsis	0	1(1.6%)	1(1.6%)
Small intestine infection	1(1.6%)	0	1(1.6%)
Wound infection[a]	2 (3.1%)	1(1.6%)	3 (4.7%)
Metabolism and Nutrition			
Dehydration	1(1.6%)	0	1(1.6%)
Hyperglycemia	1(1.6%)	0	1(1.6%)
Hypokalemia	1(1.6%)	0	1(1.6%)
Musculoskeletal and Connective Tissue Disorders			
Generalized muscle weakness	1(1.6%)	0	1(1.6%)
Polymyalgia rheumatica- like syndrome	1(1.6%)	0	1(1.6%)
Renal			
Acute kidney injury	1(1.6%)	0	1(1.6%)
Skin and Subcutaneous Tissue			
Pruritis	1(1.6%)	0	1(1.6%)
Rash maculo-papular	2 (3.1%)	0	2 (3.1%)
Vascular			
Hematoma[a]	1(1.6%)	0	1(1.6%)
Hypertension	1(1.6%)	0	1(1.6%)
Lymphocele[a]	1(1.6%)	0	1(1.6%)
Thromboembolic event	1(1.6%)	0	1(1.6%)
TOTAL RELATED ADVERSE EVENTS	**19**	**3**	**22**

[a] Adverse event related to surgery.

Supplemental Table 1: Clinicopathologic characteristics of the trial cohort utilized for weighted average calculation using the Melanoma Institute of Australia (MIA) sentinel lymph node metastasis risk nomogram[a].

Characteristic	Stage IIB	Stage IIC
Age, median (IQR), y	62 (54 – 71)	61 (53 – 68)
Thickness, median (IQR), mm	3.4 (2.6 – 6.0)	7.2 (4.8 – 8.7)
Mitotic Rate, median (IQR), mitoses/mm^2	7 (3 – 11)	9 (5 – 15)
Ulceration Present, No. (%)	20 (68.9%)	34 (100%)
Melanoma Subtype, No. (%)		
Nodular	13 (44.8%)	17 (50.0%)
Superficial spreading	8 (27.6%)	6 (17.6%)
Desmoplastic	1 (3.4%)	0 (0)
Lentigo maligna	1 (3.4%)	0 (0)
Acral	1 (3.4%)	1 (2.9%)
Unspecified	5 (17.2%)	10 (29.4%)

[a] Lymphovascular invasion status was unknown for all patients.

Supplemental Table 2: Clinicopathologic characteristics of immune-profiled cohort (n = 27) vs non-immune profiled cohort (n = 36).

Characteristic	Immune-profiled (n = 27)	Non-profiled (n = 36)	p-value
Age, median (IQR), y	62 (52 – 73)	61 (54 – 68)	0.682
Sex, No. (%)			0.158
Female	8 (29.6%)	17 (47.2%)	
Male	19 (70.4%)	19 (52.8%)	
Thickness, median (IQR), mm	5.7 (3.1 – 8.2)	5.8 (3.6 – 8.6)	0.797
Ulceration Present, No. (%)	23 (85.2%)	31 (86.1%)	0.917
Stage, No. (%)			0.770
IIB	13 (48.1%)	16 (44.4%)	
IIC	14 (51.9%)	20 (55.6%)	
Median Follow-up Duration (IQR), months	43.3 (33.3 – 55.4)	23.0 (14.1 – 30.8)	**<0.001**
SLN+ Rate, No. (%)	8 (29.6%)	9 (25%)	0.682
2-year RFS (95% CI)	76.8% (55.3 – 88.9)	90.1% (72.3 – 96.7)	0.216

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ProtocolSAPnct037576891.pdf
- NATCANCERA27392AntibodyTetramerandGatingStrategy.docx