Compartmental Analysis of T-cell Clonal Dynamics as a Function of Pathologic Response to Neoadjuvant PD-1 Blockade in Resectable Non–Small Cell Lung Cancer

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ABSTRACT

Purpose: Neoadjuvant PD-1 blockade is a promising treatment for resectable non–small cell lung cancer (NSCLC), yet immunologic mechanisms contributing to tumor regression and biomarkers of response are unknown. Using paired tumor/blood samples from a phase II clinical trial (NCT02259621), we explored whether the peripheral T-cell clonotypic dynamics can serve as a biomarker for response to neoadjuvant PD-1 blockade.

Experimental Design: T-cell receptor (TCR) sequencing was performed on serial peripheral blood, tumor, and normal lung samples from resectable NSCLC patients treated with neoadjuvant PD-1 blockade. We explored the temporal dynamics of the T-cell repertoire in the peripheral and tumoral compartments in response to neoadjuvant PD-1 blockade by using the TCR as a molecular barcode.

Results: Higher intratumoral TCR clonality was associated with reduced percent residual tumor at the time of surgery, and the TCR repertoire of tumors with major pathologic response (MPR; <10% residual tumor after neoadjuvant therapy) had a higher clonality and greater sharing of tumor-infiltrating clonotypes with the peripheral blood relative to tumors without MPR. Additionally, the posttreatment tumor bed of patients with MPR was enriched with T-cell clones that had peripherally expanded between weeks 2 and 4 after anti–PD-1 initiation and the intratumoral space occupied by these clonotypes was inversely correlated with percent residual tumor.

Conclusions: Our study suggests that exchange of T-cell clones between tumor and blood represents a key correlate of pathologic response to neoadjuvant immunotherapy and shows that the periphery may be a previously underappreciated originating compartment for effective antitumor immunity.

See related commentary by Henick, p. 1205

Introduction

PD-1/PD-L1 axis blockade enhances antitumor immunity, induces sustained tumor regression, and extends overall survival in many advanced cancers (1). More recently, neoadjuvant PD-1/PD-L1 pathway blockade in early stage lung cancer has shown clinical efficacy (2, 3) while inducing peripheral expansion of mutation-associated neoantigen-specific T-cell clones (3). Neoadjuvant phase III clinical trials incorporating PD-1 blockade are now active across multiple solid tumors (3–6).

T cells are key determinants of immune response to checkpoint blockade. Blockade of PD-1 signaling with anti–PD-1/PD-L1 antibodies reinvigorates preexisting tumor-specific T-cell clones (7). In addition to PD-L1 expression (8), other immune correlates of clinical response to PD-1 blockade include CD8+ T-cell infiltration (9), the presence of PD-1+CD8+ T cells at the invasive tumor margin (9, 10), high densities of CD45RO+ granzyme T cells (11), IFNγ-associated gene expression (12), the proximity of PD1+ to PD1− cells (13), and CD8/Ki-67 coexpression (9). Peripheral expansion of intratumoral clonotypes (14) and proliferation of peripheral Ki-67+ CD8+ T cells (15) have been linked with clinical benefit from PD-1 blockade in advanced NSCLC using pretreatment tumor specimens, but connecting these changes with the intratumoral immune response after treatment has not been done. Herein we report the coordinated dynamics of the peripheral and tumor-infiltrating T-cell repertoire following neoadjuvant anti–PD-1 treatment in resectable NSCLC. We analyzed specimens collected during a clinical trial evaluating the safety and feasibility of neoadjuvant PD-1 blockade in resectable NSCLC (NCT02259621). The neoadjuvant setting, whereby PD-1 blockade is given before surgical resections, was introduced based on the hypothesis that the "in-place" tumor at the time of...
immunotherapeutic intervention could serve as a large antigen source to drive enhanced systemic antitumor immunity (3). The neoadjuvant format also provides a unique opportunity to monitor the TCR repertoire across time (in serial peripheral blood draws) and space (across different biological compartments) according to pathologic response.

Because the T-cell receptor (TCR) confers unique antigen specificity, we use TCR Vβ CDR3 sequencing (TCR-seq) to track intratumoral clonotypes (ITCs) over time and across biological compartments to show that treatment-induced peripheral TCR repertoire remodeling correlates with increased tumor infiltration of T cells and major pathologic response (MPR). Specifically, peripheral T-cell clonotypic expansion during weeks 2 and 4 after neoadjuvant anti–PD-1 treatment initiation correlated with greater intratumoral clonotype accumulation for patients with MPR. These results indicate that the peripheral blood TCR repertoire is an important compartment for rejuvenating antitumor immunity.

**Materials and Methods**

**Study design**

The biospecimens evaluated in this study were obtained from patients enrolled to a phase II study evaluating the safety and feasibility of preoperative administration of nivolumab in patients with high-risk resectable NSCLC, along with a comprehensive exploratory characterization of the tumor immune milieu and circulating immune cells and soluble factors in these patients (NCT02259621). Specifically, 21 adults with untreated, surgically resectable (stage I, II, or IIIA) NSCLC were enrolled. Two preoperative doses of nivolumab (at a dose of 3 mg per kilogram of body weight) was administered intravenously every 2 weeks, with surgery planned approximately 4 weeks after the first dose (3). Longitudinal peripheral blood samples (pre-, posttreatment, and follow-ups), pre- and posttreatment tumor samples, posttreatment lymph nodes, and normal lung tissues were collected (Fig. 1A; Supplementary Table S2). Normal lung tissues were sampled 10 to 15 cm from tumor margin of surgically resected specimens. Pathologic response and tumor mutational burden for each patient were shown in Supplementary Table S2. This study was approved by the Institutional Review Board (IRB) of the Johns Hopkins University and Memorial Sloan Kettering Cancer Center, and conformed to the Declaration of Helsinki and Good Clinical Practice guidelines. Informed consent was obtained from all patients.

**Translational Relevance**

Neoadjuvant PD-1 blockade has emerged as a promising treatment for resectable NSCLC and is being tested in at least 10 cancer types. It will be critical to investigate mechanisms of action and identify novel biomarkers for robust antitumor immune responses. Here, we used matched tumor, normal lung tissue, and longitudinal peripheral blood samples to examine the quantitative and qualitative changes in the T-cell repertoire in NSCLC patients receiving neoadjuvant anti–PD-1. Our results add to the growing body of evidence that PD-1 blockade can boost antitumor immune responses by reinvigorating peripheral T cells to enter the tumor. Our results indicate that the periphery may be a previously underappreciated compartment for antitumor T cells that could be exploited in biomarker approaches for monitoring the response to immunotherapy.

**TCR sequencing and assessment of the TCR repertoire**

DNA was extracted from posttreatment tumor tissue, normal lung tissue, lymph nodes, and longitudinal pre- and posttreatment peripheral blood using a Qiagen DNA blood mini kit, DNA FFPE kit, or DNA blood kit, respectively (Qiagen). TCR Vβ CDR3 sequencing was performed using the survey (tissues) or deep (PBMC) resolution ImmunoSEQ platforms (ref. 16; Adaptive Biotechnologies). TCR repertoire diversity was assessed by productive clonality, which is a measure of species diversity (17). To normalize between samples that contain different numbers of total CDR3 TCRB sequencing reads, entropy was divided by log2 of the number of unique productive sequences. Nonproductive TCR CDR3 sequences (premature stop or frameshift), sequences with amino acid length less than 5, and sequences not starting with "C" or ending with "F/W" were excluded from the final analyses.

**Fluorescence-activated cell sorting (FACS) for PD-1-positive T cells in PBMCs**

Treatment-induced dynamics of exhausted T cells were assessed by tracking clones with upregulation of PD-1. PBMCs obtained at baseline, immediately prior to the first anti–PD-1 infusion, were washed and incubated with BV786-conjugated anti–CD8 (RPA-T8), BV605-conjugated anti–CD3 (SK7), BV510-conjugated anti–CD4 (SK3), and PE-Cy7-conjugated anti–PD-1 (EH12.1) for 30 minutes at 4C. Cells were washed, resuspended in FACS Buffer, and live CD3+/PD-1 T cells were sorted into four populations: CD4+/PD-1+, CD4+/PD-1−, CD8+/PD-1−, and CD8−/PD-1− using the FACSAria Fusion SORP cell sorter (BD). gDNA extractions were performed on sorted cells and samples were subsequently sent for TCR sequencing as described above.

**Identification of differentially expanded/contracted clones in PBMCs**

Bioinformatic and biostatistical analysis of differentially expanded/contracted clones in PBMC after each dosing of anti–PD-1 monotherapy was performed using Fisher exact test with multiple testing correction by Benjamini–Hochberg procedure, which controls false discovery rate (FDR < 0.05; ref. 18). Differential clonotypes were further analyzed for tissue and longitudinal PBMC representation in MPRs and non-MPRs.

**Statistical analysis**

Statistical analysis was performed using R software. The Mann–Whitney U test was used for comparison of two-group data. For analysis of >2 group data, Kruskal–Wallis H test was used. Spearman rho was used to measure the correlation between two continuous variables. Data were expressed as mean ± SEM unless otherwise indicated, and P < 0.05 was considered significant. ns: P > 0.05; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001.

**Results**

**Intratumoral TCR repertoire is associated with pathologic response to neoadjuvant anti–PD-1**

We previously reported the feasibility, efficacy, and safety of neoadjuvant PD-1 blockade in treatment-naive, surgically resectable (stage I, II, or IIIA) NSCLC (3). Briefly, 21 patients were treated with two preoperative doses of anti–PD-1 with minimal toxicity and no delays to surgery. Among the 20 patients who underwent surgical resection, 9 had ≤10% residual viable tumor upon histopathologic examination of the surgically resected tumor (Supplementary
T-cell Dynamics during Neoadjuvant Anti–PD-1 in NSCLC

An overview of the trial design and biospecimen collection is shown in Fig. 1A and Supplementary Table S2. Because T-cell clonality in the tumor has been associated with clinical outcome in metastatic cancers (9), we first assessed whether clonality of the intratumoral TCR repertoire following neoadjuvant anti–PD-1 may reflect an antitumor immune response. TCR-seq on posttreatment (resection) tumor bed was performed to determine the clonality of the intratumoral repertoire. A clonality value of 0 represents the most diverse repertoire (every T-cell in a sample contains a unique TCR), whereas a value of 1 represents a monoclonal T-cell population. Tumor mutational burden (TMB) was also evaluated as a potential correlate of immunologic response. The methods and results of whole-exome sequencing and TMB have been reported previously (3). TMB positively correlated with intratumoral clonality (Spearman rank correlation, $R = 0.7$, $P = 0.025$; Fig. 1B), suggesting expansion of a small subset of clonotypes in high TMB tumors. An inverse association was observed between intratumoral TCR repertoire clonality and percent residual tumor at the time of surgery (Spearman rank correlation, $R = -0.65$, $P = 0.041$; Fig. 1C).

Table S1). An overview of the trial design and biospecimen collection is shown in Fig. 1A and Supplementary Table S2.

Figure 1. Clonality of the TCR repertoire and association with the antitumor response. A, Flow chart of the phase II clinical trial and biospecimen collection, along with correlative studies performed at each time point. B, Correlation between productive clonality in the tumor bed at the time of resection (after anti–PD-1) with the number of nonsynonymous sequence alterations (Spearman rho, 0.70; $P = 0.025$). Each patient is represented by a black dot ($n = 10$). The blue line indicates the linear regression line, and the gray area indicates the upper and lower boundaries of the 95% confidence interval. C, Correlation between productive clonality in the posttreatment tumor bed and the percent residual tumor ($n = 10$, Spearman rho, $-0.65$; $P = 0.041$). The blue line indicates the linear regression line, and the gray area indicates the upper and lower boundaries of the 95% confidence interval. Productive clonality: Clonality as determined by using the productive amino acid (AA) sequence of the CDR3. D, Productive clonality of the TCR repertoire in the posttreatment tumor bed and normal lung for patients with MPRs (blue) and without MPRs (non-MPR; red). E, Occupied clonal space (the total frequency among all intratumoral T cells) of ITCs according to their percent rank (top 1% ranked ITCs vs. top 1%–2% ranked ITCs vs. top 2%–5% ranked ITCs vs. >5% ranked ITCs) in the posttreatment tumor bed of each patient. F, Comparison of the clonal space occupied by ITCs segregated by frequency ranks between MPRs ($n = 9$) and non-MPRs ($n = 5$).
Figure 2.
Dynamics of ITCs across tissue compartments and in longitudinal peripheral blood. 

A, Proportion of ITCs shared between pretreatment blood and normal lung, comparing non-top 1% ITCs and top 1% ITCs ($n = 14$). 

B, Top 1% ITCs shared between pretreatment blood and resected normal lung, comparing MPRs (blue) and non-MPRs (red; $n = 14$). 

C, Proportion of top 1% ITCs by their shared compartment (pretreatment blood + resected normal lung, pretreatment blood, resected normal lung, and tumor resident only). (Continued on the following page.)
rank correlation, \( R = -0.65, P = 0.041; \text{Fig. 1C} \). No correlation was observed between the total number of reads \((\text{i.e., total number of sequenced cells})\) used for TCR-seq and percent residual tumor or intratumoral clonality \((\text{Supplementary Figs. S1 and S2})\), indicating that differences in sample yield or T-cell number did not bias our analysis. These observations support the hypothesis that high TMB increases the likelihood that one or several mutations can drive a clonally skewed intratumoral T-cell repertoire leading to tumor pathologic regression.

Posttreatment tumor, but not normal lung tissue, from patients with MPR had a significantly higher T-cell clonality relative to patients with non-MPR \((\text{Mann–Whitney U test, } P = 0.0085; \text{Fig. 1D})\). Conversely, patients with MPR had a trend toward lower clonality in the peripheral blood compared with non-MPRs at each longitudinal time point, although this was not statistically significant \((\text{Mann–Whitney U test, all } P > 0.05; \text{Supplementary Fig. S3})\). These findings of increased clonality only inside the tumor suggest that these changes may be directly related to tumor recognition, with the caveat that antigen-specific recognition is not assessable by TCR-seq alone.

We then tested if the most abundant ITCs, rather than all ITCs, were specifically contributing to the difference in T-cell repertoire between MPRs and non-MPRs. ITCs were ranked according to their frequency as the top 1%, top 1% to 2%, 2% to 5%, and >5% most frequent clonotypes in the resected tumor bed. T-cell clonal space was defined and calculated as the summed frequency of clones in each of the four respective groups relative to the total T-cell repertoire. There was no difference for clonotype richness, defined as the total number of unique clonotypes, among the different ranges between MPRs and non-MPRs. However, consistent with the clonality calculations described above, the top 1% most abundant ITCs occupied a significantly greater clonal space in MPRs compared with non-MPRs \((\text{Mann–Whitney U test, median: 31.6% vs. 18.8%, } P = 0.011; \text{Fig. 1E and F})\), suggesting the top frequency-ranked ITCs may drive the antitumor response. Of note, MPR patient MD-01-010 with relatively low clonal space for top 1% ITCs had 100% PD-L1 positivity on pretreatment tumor IHC staining. Of the 788 top 1% ITCs from 16 patients with available tumor TCR data, only 1 clonotype was detected to be shared across patients \((\text{CDR3: CASSLQGAYEQYF, shared between patient NY016-007 and patient NY016-009, both were non-MPR})\), suggesting that the antitumor TCR repertoire was unique to each patient in our cohort.

**Top 1% most abundant ITCs have the highest compartmental dynamics during PD-1 blockade**

Using the TCR Vβ CDR3 as a biological barcode, we next assessed the cross-compartmental (normal lung and pretreatment blood) and temporal (before-treatment, on-treatment, and posttreatment blood) dynamics of ITCs, identified as T-cell clonotypes that were detected in the resected tumor bed, and its association with pathologic response. The top 1% most frequent ITCs had a significantly higher proportion shared between the pretreatment peripheral blood and resected normal lung as compared with less highly represented ITCs—i.e., non-top 1% frequency-ranked clones \((\text{Mann–Whitney U test, median: 61.6% vs. 28.8%, } P = 1.1e–5; 81.4% vs. 24.5%, } P = 1.4e–5, \text{respectively, Fig. 2A})\). Notably, MPRs had a higher proportion of top 1% most frequent ITCs detected in pretreatment blood \((\text{Mann–Whitney U test, median: 85.7% vs. 55.6%, } P = 0.045, \text{Fig. 2B})\) and normal lung \((\text{Mann–Whitney U test, median, 94.3% vs. 70.6%, } P = 0.023, \text{Fig. 2B})\) as compared with non-MPRs. No significant differences were found between MPRs and non-MPRs for non-top 1% frequency-ranked ITCs \((\text{all } P > 0.1; \text{Supplementary Fig. S3})\). The high proportion of ITCs mobilizing across blood and normal lung, and in particular for top 1% frequency-ranked ITCs among MPR patients, suggests an active trafficking of antitumor T cells between the tumor and other biological compartments.

Top 1% ITCs were then categorized into four mutually exclusive subsets: ITCs shared in the pretreatment blood and the resected normal lung; ITCs found only in the pretreatment blood; ITCs found only in the resected normal lung; and tumor-resident ITCs (not found in the resected normal lung or the pretreatment blood). Although the total number of top 1% ITCs was comparable in MPRs and non-MPRs \((\text{Supplementary Fig. S5})\), MPRs had a greater proportion of top 1% ITCs shared with both pretreatment blood and the resected normal lung compartment \((\text{Mann–Whitney U test, median, 81.0% vs. 50.7%, } P = 0.053, \text{Fig. 2C; Supplementary Fig. S6})\). In contrast, a greater proportion of the top 1% tumor-resident ITCs was observed in non-MPRs as compared with MPRs \((\text{Mann–Whitney U test, median, 21.4% vs. 3.6%, } P = 0.013, \text{Fig. 2C; Supplementary Fig. S7})\), suggesting that more migratory T-cell clones correlated with the antitumor response. Supporting this notion, total ITCs shared with both the pretreatment blood and the resected normal lung occupied a higher clonal space in MPRs as compared with non-MPRs \((P = 0.011)\), whereas non-MPRs had a greater clonal space occupied by tumor-resident ITCs \((P = 0.048, \text{Fig. 2D})\). It is conceivable that the normal lung TCR repertoire could be a reflection of the increased vasularization in healthy lung rather than true tissue-resident T cells; however, the normal lung had significantly fewer shared clones with the peripheral blood compared with the level of sharing between blood samples obtained from different time points \((\text{median 18% vs. 53%, Wilcoxon: } P = 1.5e–07)\), thereby demonstrating the presence of a T-cell repertoire specific to the normal lung. No difference was found in the clonal space of ITCs shared with only the pretreatment blood or the resected normal lung between MPRs and non-MPRs \((P > 0.1)\). These observations suggest that the difference of the top clonal space occupied by the top 1% ITCs between MPRs and non-MPRs could be driven by ITCs trafficking through the blood and normal lung, which could mark a consort of T cells associated with pathologic response.
Peripheral “TCR repertoire remodeling” (i.e., fluctuations in the frequency and composition of T-cell clonotypes within the repertoire) has been demonstrated in metastatic melanoma patients treated with anti-CTLA4 (19). In our study here, we characterized the repertoire remodeling in response to PD-1 blockade by evaluating the temporal dynamics of TCR repertoire in blood before, during, and after neoadjuvant treatment and linking these alterations with their tumor-infiltrating status. Between treatment initiation and surgery, the proportion of top 1% ITCs shared with the peripheral TCR repertoire significantly increased at weeks 2 and 4 relative to baseline and declined after tumor resection (one sample t-test, both P < 0.0005; Fig. 2E). Both MPRs and non-MPRs had an increased percentage of top 1% ITCs in the peripheral blood during anti-PD-1 treatment. However, non-MPRs showed an earlier decline of top 1% ITCs compared with MPRs (Fig. 2E). No significant increase in shared TCRs between non-top 1% ITCs and peripheral blood were observed in MPRs or non-MPRs at each time point (all P > 0.05). These findings indicate that top-ranked ITCs are readily detected in peripheral blood before treatment, increase in the periphery subsequent to PD-1 blockade regardless of MPR status, and decrease in the peripheral blood after tumor resection/removal of tumor antigen, consistent with T-cell repertoire turnover in response to treatment. Yet this pattern was not present among non-top 1% ITCs, bolstering the notion that top 1% ITCs were key migratory T cells.

We further systematically evaluated peripheral dynamics of ITCs in all patients at pairwise time points during PD-1 blockade. Clonotypes with a significant increase in abundance compared with the previous time point were defined as expanded clones, whereas those with a significant decrease in abundance were defined as contracted clones.

We quantified the degree of TCR repertoire remodeling by clonotypic fold change between pairwise time points. Peripheral clones were grouped based on their representation in the tumor bed as PBMC-only clonotypes, non-top 1% frequency-ranked ITCs, and top 1% frequency-ranked ITCs. Both non-top 1% ITCs and the top 1% ITCs showed greater fold changes of expansion and contraction as compared with PBMC-only clonotypes during anti-PD-1 treatment (Fig. 2G). Particularly, the top 1% ITCs consistently showed the highest degree of reshaping, regardless of MPR status. To consolidate our hypothesis that perturbations of the peripheral T-cell repertoire were specific to anti-PD-1 treatment and not the result of random fluctuations in the TCR repertoire, we evaluated changes in frequency between two long-term follow-up time points taken 6 months apart, during which a significant amount of peripheral repertoire turnover would be expected relative to time points taken 2 weeks apart, in a patient who did not receive adjuvant chemotherapy and had serial blood samples for >1 year after surgery. Top 1% ITCs detected in the peripheral blood underwent appreciable peripheral expansions and contractions during the 4 weeks of anti-PD-1 treatment (Fig. 2F, time window: pretreatment to week 2; weeks 2 to 4). Strikingly, limited remodeling of the peripheral TCR repertoire was observed during the 6-month follow-up interval relative to on-treatment time points (Fig. 2F, right), suggesting the systematic remodeling in the peripheral repertoire is a direct, early effect of PD-1 pathway blockade.

Clonal expansion of ITCs in peripheral blood correlates with pathologic response

We next sought to identify the kinetic pattern and time window of TCR remodeling that correlates with tumor regression upon PD-1 blockade. We recently detected peripheral expansion of neoantigen-specific peripheral T-cell clonotypes that were also observed in high abundance in the posttreatment tumor in a single patient with MP (3). We therefore hypothesized that peripherally expanded clones may home to the tumor (primary and potentially undetected micrometastatic deposits) to produce the antitumor response. Clonotypes undergoing contraction or expansion were identified in pairwise time points (pretreatment vs. week 2; week 2 vs. week 4) during neoadjuvant PD-1 blockade using methods previously described and controlling for an FDR of 0.05 (18). A total of 689 dynamic clonotypes were found among 17 patients with available serial blood samples (Supplementary Fig. S7). Compared with nondynamic clones, dynamic clones are more likely to be intratumoral clones, regardless of MPR status (Fig. 3A) and clones that contracted in the periphery between pretreatment and week 2 were more likely to be in the tumor bed as compared with those contracted at between weeks 2 and 4; in contrast, clones expanded between weeks 2 and 4 were more likely to infiltrate the tumor bed (Fig. 3B). No difference was observed for the proportion of nondynamic clones in the tumor bed. Furthermore, the intratumoral clonal space of clones expanded between weeks 2 and 4 was inversely correlated with residual tumor (Spearman rank correlation, R = −0.62, P = 0.041; Fig. 3C).

Because peripherally dynamic clones are thought to be trafficking to the tumor, we tested if intratumoral clonal occupancy of clones with differential peripheral patterns differentiates MPRs from non-MPRs. ITCs were stratified as peripherally contracted, expanded, nondynamic, or tumor-resident only based on their different dynamic patterns during treatment. There were no differences in the intratumoral clonal space of peripherally contracted, expanded, or nondynamic clones between pretreatment to week 2 (Mann–Whitney U-test, all P > 0.05, Supplementary Fig. S8). However, ITCs that specifically expanded between 2 and 4 weeks from anti-PD-1 treatment initiation demonstrated a significantly greater clonal space in MPRs compared with non-MPRs (P = 0.018, Mann–Whitney U-test; Fig. 3D), suggesting an enhanced antitumor response with the influx or efflux of peripherally expanded clones. Moreover, no difference was observed in the intratumoral clonal space of tumor-resident-only clones between MPRs and non-MPRs (Fig. 3D), consistent with a previous study suggesting low and variable tumor reactivity of the intrinsic tumor-resident TCR repertoire (20).

To further interrogate the tumor-specific localization of expanded clones, we repeated the analysis on clonotypes found in normal lung and observed no statistically significant difference in representation of differential clonotypes between MPR and non-MPR (Fig. 3E), suggesting an enrichment of peripherally expanded clones that are specifically concentrated within the tumor. In addition, we found the top 1% ITCs of MPRs were preferentially constituted of peripheral clones expanded at weeks 2 to 4 (Fig. 3F) as compared with non-MPRs (median, 17.7% vs. 0.3%, Mann–Whitney U-test, P = 0.012). No differences in clonotype composition among top 1% ITCs were observed for contracted, nondynamic, or tumor-resident only clones between MPRs and non-MPRs (all P > 0.1). Based on the above observations, we proposed the model that T-cell clones trafficking between the tumor and peripheral blood, particularly those differentially expanded at 2 to 4 weeks after PD-1 blockade may be responsible for tumor regression (Fig. 3G).

A complete pathologic responder is characterized by significant expansion of PD-1 + ITCs in the periphery

Our data suggest that PD-1 blockade rejuvenates preexisting peripheral T cells that expand upon treatment and accumulate in tumor tissues. As prior studies have demonstrated PD-1 expression to be reflective of a subset of PD-1-blockade–responsive, tumor-specific T cells, we examined the PD-1 expression status of these expanded clones.
ITCs using TCR-seq of sorted PD-1\(^+\) and PD-1\(^-\) peripheral blood T cells. This analysis focused on two patients with extreme but opposite responses to neoadjuvant PD-1 blockade: a complete pathologic responder, patient MD01-005, with 0% residual primary tumor with minimal tumor involvement of an adjacent lymph node, and nonresponding patient MD01-024, with 100% residual tumor. In the pathologic responder (MD01-005), among the 5 top 1\% ITCs that differentially expanded between 2 and 4 weeks after treatment initiation, all were found to be PD-1\(^+\) in the pretreatment peripheral blood (4 PD-1\(^+\)CD8\(^+\) clones; 1 PD-1\(^+\)CD4\(^+\) clone, Fig. 4A). These clonotypes were additionally found to be top 1\% clonotypes in a tumor-involved lymph node and a tumor-draining lymph node obtained at

**Figure 3.** Differentially dynamic clones and their association with tumor infiltration and tumor accumulation. **A,** The proportion of dynamic clones vs. nondynamic clones that infiltrated tumor bed upon PD-1 blockade in MPR and non-MPR (n = 12). **B,** The total proportion of clones in the tumor is shown for significantly contracted clones, significantly expanded clones, and nondynamic clones at week 2 (dark gray) and week 4 (light gray) after treatment initiation (n = 12). **C,** Association of intratumoral clonal space of peripherally expanded clones and percent residual tumor by time window (pretreatment–W2, Spearman rho = −0.35, W2–W4 Spearman rho = −0.62). Each patient is represented by a black dot. The blue line indicates the robust linear regression line (fitted using R function "rlm" from "MASS" package based on M estimator), and the gray area indicates the upper and lower boundaries of the 95% confidence interval (n = 12). **D,** The clonal space occupied by ITCs with different peripheral dynamic patterns (expanded, contracted, nondynamic or tumor-resident only) at 2–4 weeks after immunotherapy initiation is shown for MPRs and non-MPRs (n = 12). **E,** The clonal space occupied by normal lung T cells with differential patterns in the periphery is shown for MPRs and non-MPRs at 2 to 4 weeks after treatment initiation (n = 12). **F,** The proportion of top 1\% ITCs and their peripheral dynamic pattern at 2 to 4 weeks after treatment initiation for MPRs and non-MPRs (n = 12). **G,** Proposed schema of peripheral activation of the antitumor repertoire homing back to the tumor bed. ns, not significant. *, P ≤ 0.05; ***, P ≤ 0.01; ****, P ≤ 0.001.
the time of surgical resection (Fig. 4A). PD-1 intraclonal positivity, defined as the percent of PD-1+ T cells within a particular clonotype, was markedly high for all 5 clonotypes in MD01-005 (Fig. 4B; median, 99%; range, 98%-100%). By contrast, for the nonresponder (MD01-024), the 7 top 1% ITCs that expanded at 2 to 4 weeks demonstrated low PD-1 intraclonal positivity (median, 0.6%; range, 0.2%-20.6%, Fig. 4C and D). Moreover, these expanded clones in the tumor bed of the nonresponder had a significantly lower proportion in the tumor relative to normal lung (paired Wilcoxon test, P = 0.022, Fig. 4E). These results suggest that peripheral expansion is indicative of activation and migration of PD1+ T cells into the tumor microenvironment, which could subsequently facilitate pathologic response in the tumor bed.

Discussion

We recently identified neoantigen-specific T-cell clones in blood obtained prior to anti–PD-1, at the preoperative visit, and after surgery in a patient with MPR, with a transient expansion of these clones in peripheral blood after treatment initiation (3, 18). Based on this observation, we hypothesized that trafficking of relevant T-cell clones between the periphery, normal tissues, and tumor would be important for effecting pathologic response. We therefore performed a comprehensive assessment of T-cell repertoire and dynamics in the neoadjuvant setting and further sought to determine if specific variables of clonal dynamics may be an early biological correlate of antitumor immunity. As such, we used pathologic response to PD-1 blockade as an outcome. Although longer follow-up will be necessary to determine how pathologic response correlates with clinical outcome in lung cancer, reports in melanoma indicate that early pathologic responses to anti–PD-1 indeed correlate with improved survival (21, 22).

In this study, we use the TCR as a barcode to track clonal sharing and dynamics and observed a positive association between intratumoral T-cell repertoire clonality and pathologic tumor regression at surgery. Although we did not observe significant global changes in clonality of the peripheral TCR repertoire throughout the 4 weeks of treatment, we determined that the most significant parameter that differentiates MPRs from non-MPRs is the clonal space occupied by the most frequent, i.e., top 1% frequency-ranked, ITCs, which demonstrated systematic perturbations in frequency in the periphery upon PD-1 blockade. More interestingly, our results showed that the top 1% ITCs were observed to be highly shared in both peripheral blood and normal lung tissue from the same patient. Furthermore, the level of clonal sharing of ITCs in blood and normal lung was significantly higher in MPR patients relative to non-MPR patients, whereas non-MPRs had more tumor-resident–only ITCs, consistent with the notion that intrinsic tumor-resident T-cell repertoire may likely to be exhausted with low tumor reactivity and that the T-cell response to checkpoint blockade may derive from reinvigorated T cells that recently entered the tumor (23). Whereas the observation that non-MPRs have more clones that are restricted in the tumor bed implicates potential deficiency in replenishment by the peripheral T-cell repertoire.

We additionally systematically characterized the dynamic pattern of ITCs in longitudinal blood. We found that significant dynamic changes in the frequency of shared peripheral clones, regardless of their dynamic nature (contraction or expansion), associated with increased tumor infiltration, suggesting an active compartmental exchange of ITCs induced by neoadjuvant PD-1 blockade. Notably, peripherally expanded ITCs at weeks 2 to 4 from the initiation of anti–PD-1 preferentially occupy a greater clonal space in the tumor bed of MPRs relative to non-MPRs. This observation highlights the importance of effective peripheral activation in promoting pathologic response. On the contrary, we observed that non-MPR tumors do not successfully traffic top 1% ITCs to the tumor bed, possibly due to an intrinsically more exhausted, less migratory T-cell repertoire. These clones, when trapped in the tumor, could experience inhibited reinvigoration upon PD-1 blockade owing to an immunosuppressive tumor microenvironment. Therefore, detection of tumor-infiltrating clones’ expansion in the peripheral blood may be an early biological correlate of antitumor T-cell recognition and the peripheral blood may be a valuable compartment for monitoring early T-cell responses to PD-1 blockade.

Supporting the notion that dynamics of shared clones are relevant to outcomes of PD-1 blockade, we demonstrated that expanded clonotypes in a patient with a complete pathologic response exhibited extremely high intraclonal PD-1 positivity, in contrast to a patient with no pathologic response, whose expanded clonotypes exhibited very low frequencies of PD-1 positivity. Although anti–PD-1 has been reported to reinvigorate peripheral T-cell clones that are also present in the tumor (24), our study further suggests that anti–PD-1 may induce an exchange of reinvigorated effecter PD1+ T cells between the periphery and tumor, where they may contribute to tumor regression. We recently showed that in stage 4 NSCLC treated with anti–PD-1, general expansion in the blood of all clones found in pretreatment tumors correlated with radiographic response and clearance of circulating tumor DNA (14). Here we show in the neoadjuvant setting that peripheral expansion of the most frequent ITCs (top 1% frequency) correlates much more closely with the pathologic response at the time of resection.

Although a limited number of patients have been included in our analysis, we performed an in-depth integration of the intratumoral antitumor repertoire and peripheral T-cell repertoire. Larger cohorts are warranted to evaluate these features as predictive markers for response to neoadjuvant PD-1 blockade and relapse-free survival. However, we acknowledge that the median duration of recurrence-free survival has not been reached in this cohort, and we are therefore unable to assess the association of TCR dynamics with survival. Additionally, although our approach utilizing TCR β chain sequencing is a common approach owing to (i) greater diversity of the β chain relative to the α chain, (ii) stricter allelic exclusion of the β locus, and

Figure 4.

PD-1 sorting revealed differential patterns of intraclonal PD1+ positivity between complete pathologic responder and nonresponder. A, TCR-seq was performed on sorted PD1+–positive and –negative CD4+ and CD8+ peripheral blood T cells obtained from patient MD01-005 (0% residual tumor) prior to treatment (left). The frequency of clonotypes detected in the PD1+ population is shown in serial peripheral blood (center) and biopsied and resected tissues (right) is shown. All 5 differentially expanded ITCs identified in this patient were detected in the PD1+–sorted population. B, Pie charts showing the intraclonal PD1+ positivity (the proportion of total reads of each clone that was detected in the PD1+–vs. PD1+–sorted population) for the 5 peripherally expanded, top 1% ITCs is shown. C, TCR-seq was performed on sorted PD1+–positive and –negative CD4+ and CD8+ peripheral blood T cells obtained from patient MD01-024 (100% residual tumor) prior to treatment (left). The frequency of clonotypes detected in the PD1+ population is shown in serial peripheral blood (center) and resected tissues (right). D, Pie charts showing the intraclonal PD1+ positivity for peripherally expanded, top 1% ITCs. E, The read proportion of top 1% ITCs in the normal lung and tumor bed is shown for patient MD01-005 and patient MD01-024. Comparisons between normal lung and tumor bed were evaluated using the Wilcoxon signed-rank test.
(iii) technical ease of performing TCR-seq on the β chain, we recognize that single-cell paired ββ sequencing approaches should be used in future studies aimed at determining the antigen specificity or function of these T cells. Similarly, a limitation of our study is that we did not differentiate between CD4+ and CD8+ T-cell clones. Although, traditionally, CD8+ T-cell infiltration has been associated with improved prognosis in untreated/pretreatment tumor specimens, it is important to note that our specimens were obtained after neoadjuvant PD-1 blockade and the association of CD4+ versus CD8+ T-cell infiltration with pathologic response or clinical outcome has not been systematically evaluated in this setting. To that end, ascertaining the CD4 versus CD8 identity of these T cells would also be useful in determining function in future studies. Correlation with ‘Immune-Related Pathologic Response Criteria’ (irPRC; ref. 25), a newly proposed pathologic assessment of residual tumor in immunotherapy, should also be explored if validated as a surrogate for recurrence-free and overall survival. Efforts to extrapolate the current findings to patients with metastatic disease should be met with caution, as we are specifically evaluating tumor-infiltrating T cells after PD-1 blockade in patients with resectable disease. However, our results have important implications for the establishment of predictive biomarkers through liquid biopsy approaches. In conclusion, our study shows that TCR profiling holds promise for monitoring antitumor responses in the periphery and will spur future development of biomarkers to predict response to immunotherapy and to guide what additional therapies may be warranted after surgical resection.

Disclosure of Potential Conflicts of Interest

V. Anagnostou reports receiving commercial research grants from Bristol-Myers Squibb. J.E. Chaft is a paid consultant for Bristol-Myers Squibb, AstraZeneca, Merck, and Genentech. D.R. Jones is an employee of Diffusion Pharmaceuticals and is a paid consultant for Merck and AstraZeneca. J. Naidoo is a paid consultant for AstraZeneca, Roche/Genentech, and Bristol-Myers Squibb, and reports receiving commercial research grants from Bristol-Myers Squibb and Akoya Biosciences. V.E. Velculcescu is an employee of Personal Genome Diagnostics and is a paid consultant for Takeda, and holds ownership interest (including patents) in Personal Genome Diagnostics. J.R. Brahmer is a paid consultant for Bristol-Myers Squibb, Merck, AstraZeneca, and Genentech, and reports receiving commercial research grants from Bristol-Myers Squibb, AstraZeneca, Merck, and Akoya Biosciences. V.E. Velculcescu is an employee of Personal Genome Diagnostics and is a paid consultant for Takeda, and holds ownership interest (including patents) in Personal Genome Diagnostics. J.R. Brahmer is a paid consultant for Bristol-Myers Squibb, Merck, AstraZeneca, and Genentech, and reports receiving commercial research grants from Bristol-Myers Squibb, AstraZeneca, Genentech, Bristol-Myers Squibb, Blueprint Medicine, Nektar, Synax, Mirati, Shattuck Labs, and Immumx; reports receiving commercial research grants from Bristol-Myers Squibb; holds ownership interests in Shattuck Labs and Immumx; and is listed as a coinventor on a patent that has been filed by MSK related to the use of tumor mutation burden to predict response to immunotherapy which has received licensing fees from PGDx. P.M. Forde reports receiving commercial research grants from AstraZeneca, Bristol-Myers Squibb, Corvus, Kiyowa, and Novartis, and is an unpaid consultant/advisory board member for AstraZeneca, Bristol-Myers Squibb, Janssen, Merck, Novartis, Lilly, and Boehringer. D.M. Pardoll holds ownership interest (including patents) in Bristol-Myers Squibb. K.N. Smith reports receiving speakers bureau honoraria from Illumina, Inc. and Neon Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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References


## Clinical Cancer Research

**Compartmental Analysis of T-cell Clonal Dynamics as a Function of Pathologic Response to Neoadjuvant PD-1 Blockade in Resectable Non–Small Cell Lung Cancer**

Jiajia Zhang, Zhicheng Ji, Justina X. Caushi, et al.


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