

Deep Sequencing of T-cell Receptor DNA as a Biomarker of Clonally Expanded TILs in Breast Cancer after Immunotherapy

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Abstract

In early-stage breast cancer, the degree of tumor-infiltrating lymphocytes (TIL) predicts response to chemotherapy and overall survival. Combination immunotherapy with immune checkpoint antibody plus tumor cryoablation can induce lymphocytic infiltrates and improve survival in mice. We used T-cell receptor (TCR) DNA sequencing to evaluate both the effect of cryoimmunotherapy in humans and the feasibility of TCR sequencing in early-stage breast cancer. In a pilot clinical trial, 18 women with early-stage breast cancer were treated preoperatively with cryoablation, single-dose anti-CTLA-4 (ipilimumab), or cryoablation + ipilimumab. TCRs within serially collected peripheral blood and tumor tissue were sequenced. In baseline tumor tissues, T-cell density as measured by TCR sequencing correlated with TIL scores obtained by hematoxylin and eosin (H&E) staining. However, tumors with

little or no lymphocytes by H&E contained up to 3.6×10^6 TCR DNA sequences, highlighting the sensitivity of the ImmunoSEQ platform. In this dataset, ipilimumab increased intratumoral T-cell density over time, whereas cryoablation \pm ipilimumab diversified and remodeled the intratumoral T-cell clonal repertoire. Compared with monotherapy, cryoablation plus ipilimumab was associated with numerically greater numbers of peripheral blood and intratumoral T-cell clones expanding robustly following therapy. In conclusion, TCR sequencing correlates with H&E lymphocyte scoring and provides additional information on clonal diversity. These findings support further study of the use of TCR sequencing as a biomarker for T-cell responses to therapy and for the study of cryoimmunotherapy in early-stage breast cancer. *Cancer Immunol Res*; 4(10); 835–44. ©2016 AACR.

Introduction

The immune system may play a pivotal role in mediating long-term survival in women diagnosed with early-stage breast cancer. The benefits of conventional breast cancer therapies such as chemotherapy (1), human epidermal growth factor receptor 2 (HER2)-targeted therapy (2–4), and radiotherapy (5) are at least partially dependent on an adaptive immune response against the cancer. The response to chemotherapy and overall survival can be predicted by the quantity of tumor infiltrating lymphocytes (TIL)

in tumor biopsies (6). However, not all breast cancers appear to trigger an antitumor immune response. Some breast tumors are densely infiltrated by lymphocytes, whereas most are devoid of lymphocytes by routine hematoxylin and eosin (H&E) staining. Tumors with abundant lymphocytes grow *despite* immune engagement, whereas tumors with few or no lymphocytes grow in absence of immune engagement. Patients with "TIL-low" tumors, also described as "immunologically ignorant" (7) or "non-inflamed" tumors (8), are more prone to chemotherapy resistance (lower pathologic complete response rates to neoadjuvant therapy) and poor overall survival (6, 9, 10), suggesting that endogenous immune engagement may be a key survival determinant.

Immune therapies have the potential to facilitate *de novo* immune engagement when an endogenous immune response has not occurred. To achieve this, an optimal immune therapy would facilitate four critical components of a *de novo* adaptive response: tumor antigen release, tumor antigen presentation, diminished immune suppression, and tumor antigen-specific T-cell activation. The first components, tumor antigen release and presentation, can be facilitated by tumor cryoablation (cryo, using an image-guided probe to lyse tumor cells). The latter components, T-cell activation and T-regulatory cell depletion, can be induced by a therapeutic antibody against the T-cell coinhibitory molecule CTLA-4 (11, 12). In mice, the combination of these two immunotherapies, coined "cryo-immunotherapy," generated intratumoral T-cell expansion specific to tumor-associated antigens (TAA), systemic tumor regression, and improved overall survival (11).

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During cryo, the tumor is treated with repetitive freeze/thaw cycles, inducing crystallization, mechanical cellular damage, microvascular injury, ischemia, cell death, and massive protein release (13). Freezing is most efficient at the center of the probe, leading to cellular necrosis, release of inflammatory cellular contents, and activation of the innate immune system via activation of Toll-like receptor and other innate receptors. At cryoablation margins (lower isotherms), apoptotic cell death occurs, which is associated with phagocytosis and antigen presentation. Thus, cryotherapy may produce an optimal microenvironment for T-cell activation against a broad array of tumor-associated antigens.

Blocking antibodies to CTLA-4 activate T cells by binding and inhibiting CTLA-4, a T-cell surface protein that is upregulated with TCR signaling and limits T-cell activation. The CTLA-4–blocking antibody ipilimumab improves overall survival in metastatic melanoma (14, 15) and has been approved by the FDA for this condition. CTLA-4 blockade is thought to decrease the threshold required for T-cell activation following exposure to antigen (16), increase the quality and duration of T-cell activation, and deplete suppressive T-regulatory cells within the tumor microenvironment (17). However, immune checkpoint antibody therapy alone may not be sufficient to induce an effective antitumor immune response in all tumors. In metastatic melanomas treated with pembrolizumab, another immune checkpoint antibody against programmed death 1 (PD-1), tumors with low TILs were unlikely to respond to therapy, whereas tumors with robust TILs were more likely to respond (18). Therefore, especially in TIL-low tumors, a combination approach may increase likelihood of mounting an effective antitumor immune response.

In a *post hoc* analysis of a recent study (19) evaluating cryo-immunotherapy in early-stage breast cancer, we sought to determine whether cryoimmunotherapy induced broad and robust T-cell activation and proliferation within the tumor microenvironment. Because breast cancer core biopsies are small and less conducive to comprehensive immunologic profiling by flow cytometry, and because routine histologic or immunohistochemical quantitation of T cells is difficult when tumor microarchitecture is disrupted by cryo, we used a novel high-throughput TCR β (TCRB) DNA sequencing technique capable of quantifying T cells from DNA extracted from a small (25 μ m) tumor sample.

TCRB DNA sequencing can be used to quantify expansion of individual clones, which may serve as a surrogate marker for therapy-associated induction of antigen-specific T-cell activation. During T-cell development in the thymus, the complementarity-determining region 3 (CDR3) region of the TCRB gene undergoes recombination and nucleotide addition/deletion steps to generate a vast pool of T-cell clones, each with a unique TCR conferring antigen specificity. If a T cell binds its cognate antigen and becomes activated, it undergoes clonal proliferation, allowing for an adaptive immune response against the target antigen.

In this study, TCRB DNA sequencing was performed on pre- and post-cryoimmunotherapy human breast cancer specimens to study the effects of cryoimmunotherapy on T cells in the tumor microenvironment. The intent was 2-fold: to evaluate TCRB sequencing as a novel biomarker in early-stage breast cancer, and to provide proof-of-concept that TCRB sequencing may be used to monitor the intratumoral and peripheral blood effects of immunotherapy in a preoperative early-stage breast cancer trial, highlighting analytical limitations as well as opportunities to integrate TCRB sequencing into immunotherapy clinical trials.

Materials and Methods

Clinical trial design

In a pilot study conducted at the Memorial Sloan Kettering Cancer Center (MSKCC) between 2012 and 2013 (MSKCC IRB#11-202, NCT01502592), 18 women with operable early-stage breast cancer who had elected mastectomy were treated with: cryo alone (group A, $n = 6$), single-dose ipilimumab 10 mg/kg i.v. alone (group B, $n = 6$), or combination cryo plus ipilimumab 10 mg/kg i.v. (group C, $n = 6$; Table 1 and Fig. 1). In one subject from group A, a cryo probe malfunction occurred resulting in incomplete tumor freezing; this subject was replaced; however, the baseline core biopsy data (which were unaffected by the probe malfunction) were included in the exploratory biomarker analyses (group A total, $n = 7$).

The treatment schedule was designed to optimize antigen exposure time without disrupting the average 2-week lead time between diagnosis and standard-of-care mastectomy. Following consent, ipilimumab (groups B and C) was administered a median of 2.5 days (range, 1–8) prior to core biopsy, cryo (groups A and C) was administered at the time of core biopsy, and mastectomy was performed a median of 7 days (range, 4–10) following core biopsy. In all subjects, core biopsy and mastectomy specimens were formalin-fixed and paraffin embedded (FFPE), and cryopreserved if additional tumor was available. Peripheral blood mononuclear cells (PBMC) collected on the day of core biopsy, and mastectomy date were cryopreserved.

TCRB CDR3 amplification and sequencing

DNA extraction of PBMCs and FFPE-preserved biopsy/mastectomy tissue or fresh frozen tissue was performed following the DNeasy Blood and Tissue Kit (Qiagen). FFPE was used in all cases when feasible, or fresh frozen in cases where FFPE was not available (itemized in Table 1). After confirmation of the presence of tumor by pathology, a 25- μ m section of core biopsy or tumor-bearing mastectomy block was obtained for DNA extraction. Proteinase K was used for digestion and extraction of DNA following a blood/cell protocol with RNase treatment and using the spin-column method. Samples were analyzed by high-throughput sequencing of the TCRB CDR3 region using the ImmunoSEQ immune profiling system at the deep level (Adaptive Biotechnologies; ref. 20). Sequencing was performed on an Illumina HiSeq system (Illumina). This analysis results in a mean of approximately $5 \times$ sequence coverage for T cells from 3.6 μ g of genomic DNA; in a typical blood sample this equates to $\sim 1,000,000$ sequencing reads from $\sim 200,000$ T cells, depending on the proportion of T cells. The product was sequenced and organized, providing productive and nonproductive sequences (CDR3 regions explicitly encoding a premature stop, and those predicted to put the receptor gene out-of-frame downstream of the CDR3 rearrangement, were considered nonproductive). An algorithm was applied to raw sequencing data for collapsing PCR and sequencing errors, resulting in unique rearrangements of the CDR3 genes. Productive TCRB CDR3 sequences are the object of this study.

Quantification of TILs by staining and immunohistochemistry (IHC)

In addition to TCR sequencing, adjacent tissue from FFPE tumor core biopsies and resection specimens were sectioned with a standard thickness of 5 μ m and visualized by routine H&E

Table 1. Tumor characteristics and TCR deep-sequencing metrics, by patient

Group	ID	Subtype	Diff	Days duration between		H&E TIL Score (%)	Bx T-cell Receptor Deep Sequencing Metrics				
				Ipi→Bx	Bx→Surgery		Sample Source	Absolute # Sequences	# Unique Sequences	Density	Clonality
Cryoablation	1	HR ⁺	mod		10	<5	FFPE	2193838	16895	30	0.11
	2	HR ⁺	mod		8	<5	Frozen	1209573	7909	7	0.20
	3	HR ⁺	poor		8	<5	Frozen	1453469	14020	5	0.12
	4	HR ⁺	mod		8	85	FFPE	867803	49283	24	0.23
	5 ^a	HR ⁺	mod		9	<5	FFPE	652336	2974	5	0.13
	6	HR ⁺	well		7	n/a	Frozen	55054	647	2	0.12
	7	HR ⁺	well		7	<1	FFPE	82343	3467	5	0.12
Ipilimumab	8	HER2 ⁺	poor	8	4	80	FFPE	5278898	67770	19	0.14
	9	TNBC	poor	1	7	<5	FFPE	205971	755	3	0.13
	10	HR ⁺	well	3	7	<5	Frozen	694364	2651	0	0.41
	11	HR ⁺	poor	5	8	n/a	FFPE	164476	697	2	0.17
	12	HER2 ⁺	poor	1	7	40	FFPE	8916191	33101	16	0.23
	13	TNBC	poor	1	8	10	FFPE	368214	13966	15	0.11
Cryoablation + Ipilimumab	14	HR ⁺	mod	3	8	<5	FFPE	2714728	11751	6	0.15
	15	TNBC	poor	1	7	40	FFPE	3479585	14414	10	0.16
	16	HR ⁺	poor	2	6	5	FFPE	235779	9486	13	0.13
	17	HR ⁺	poor	4	7	30	FFPE	429163	12035	7	0.14
	18	HR ⁺	mod	2	10	<5	FFPE	1397054	5412	5	0.12
	19	HR ⁺	mod	5	7	<5	Frozen	583965	7382	4	0.10
Median				2.5	7	<5		781084	10619	6	0.14

NOTE: The reported metrics describe the research core biopsy, obtained at the time of cryoablation (arms A and C) and shortly after ipilimumab (arms B and C). Due to limited specimen availability, experiments were only repeated when sequencing did not meet quality control standards (refer to Materials and Methods). Abbreviations: Diff, differentiation; Ipi, ipilimumab; Bx- biopsy; n/a- not available for analysis.

^aIn patient 5, cryoablation probe malfunction precluded interpretation of therapy effect; however, the core biopsy was still evaluable.

staining. Quantity of stromal TILs was measured by a trained pathologist using the San Antonio 2014 TILs Working Group 2014 guidelines (6).

Analysis of duplicates

Because of the *post hoc* nature of this experiment, sufficient material was not available from all specimens to allow for duplicate analysis. In select specimens where the total DNA content was low and additional tissue was available, the assay was repeated. The larger (greater absolute TCRB sequence count) specimen was used for analysis, except in cases where the absolute sequence count differed by less than a factor of 10, in which case the duplicates were averaged.

Statistical analysis

Analyses were performed using Microsoft Excel, ImmunoSEQ software (Adaptive Biotechnologies), GraphPad prism (GraphPad Software), and the R statistical software. Because of the *post hoc* nature of this study, most analyses are descriptive for the purpose of generating hypotheses. A limited number of findings were evaluated for statistical significance: groups were compared using a nonparametric two-sided Mann-Whitney test, and correlations were evaluated assuming a non-Gaussian distribution (Spearman correlation), unless otherwise indicated.

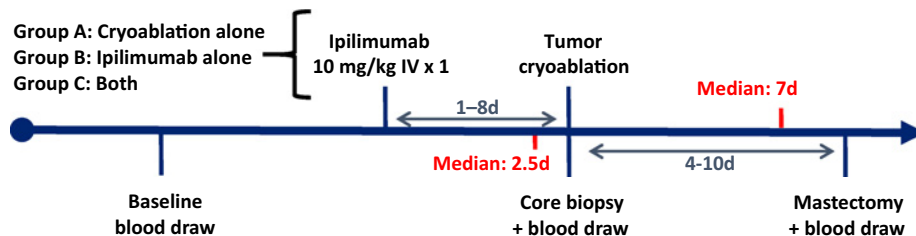
Results

TCRB sequencing measures T-cell infiltration in early-stage breast cancer biopsies

In previous series of early-stage breast cancer patients, TIL counts as measured by routine H&E staining are nonnormally distributed, with most tumors exhibiting sparse or no TILs (as measured by ≤10% of stroma, 69% of triple-negative tumors in a recent report), and the minority of tumors exhibiting dense TILs (comprising upward of 80% of the stromal tissue examined; ref. 21). In our dataset using the standardized H&E TIL scoring methodology proposed by the San Antonio TIL Working group, we found the same nonnormal distribution (Table 1), with the majority of tumors having TIL count ≤10% (6).

As an alternative to H&E, we characterized the degree of T-cell infiltrates in core biopsies using two TCRB sequencing methodologies. The first methodology, absolute T-cell count, is a direct measure of the number of detectable T-cell DNA sequences within the specimen. The second methodology, T-cell density, is a relative measure of T-cell DNA relative to total DNA (including tumor and stromal DNA), which may be more stable to variations in sequencing depth. In our series, both absolute T-cell count and T-cell density exhibited a nonnormal distribution similar to H&E TIL count. Absolute T-cell sequence counts ranged from 5.5×10^4 to 8.9×10^6 sequences per sample (mean 1.98×10^6 ; SD, 2.68×10^6), and T-cell densities ranged from 0 to 30% (mean

Figure 1. Study design.



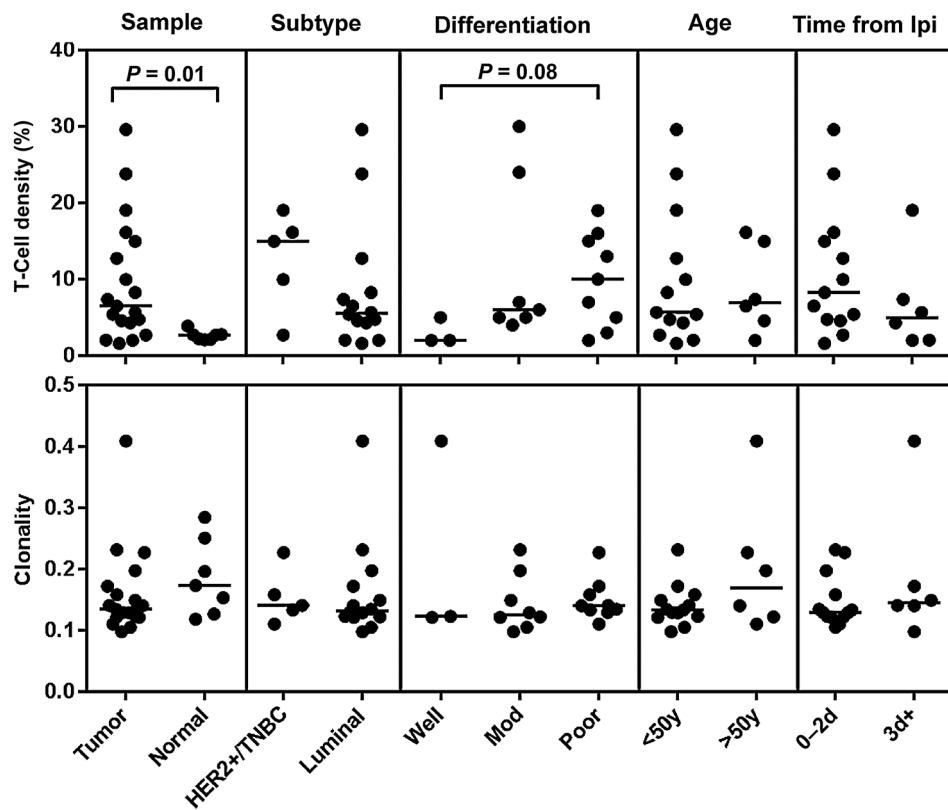


Figure 2.

Influence of patient and tumor characteristics on core biopsy T-cell infiltrates. T-cell density and clonality are assessed by TCR DNA sequencing, with group medians depicted as solid lines. Unless specified, differences across groups are not statistically significant. T-cell density in untreated normal breast tissue was lower compared with tumor; HER2⁺ and TNBC subtypes appear to have denser infiltrates compared with luminal-type (i.e., HR⁺/HER2⁻); tumor grade is associated with T-cell density; age is not associated with T-cell density or clonality; and subjects receiving ipilimumab greater than 3 days preceding biopsy did not have appreciable differences in T-cell density or clonality compared with subjects not receiving ipilimumab, or receiving ipilimumab within 0–2 days. HER2⁺, human epidermal growth factor receptor 2 positive; TNBC, triple-negative breast cancer; mod, moderate; Ipi, ipilimumab.

5.7%; SD 8.1%). T-cell density was significantly correlated with TIL count by H&E ($r = 0.64$, $P = 0.007$). There was a trend toward correlation between absolute T-cell count and H&E ($r = 0.46$, $P = 0.06$).

We conducted TCRB sequencing of normal adjacent breast tissue specimens (at least 3 cm away from tumor) obtained during mastectomy. In subjects who did not receive ipilimumab (i.e., patients undergoing cryo alone, $n = 7$), the T-cell densities and absolute T-cell counts of normal breast tissue were lower and more uniformly distributed compared with tumor (mean/SD T-cell density: tumor, 5.7%/8.1%; normal, 2.3%/1.1%, $P = 0.002$). In subjects who received ipilimumab (i.e., ipilimumab alone and ipilimumab/cryo arms), normal breast tissue appeared to have more dense and less uniformly distributed T-cell infiltrates, similar to the tumoral specimens (mean/SD T-cell density: tumor 7.9%/8.5%). Samples with few or no apparent TILs by H&E ($\leq 5\%$) exhibited a median of 301,603 T-cell sequence counts by TCRB sequencing (range, 42,126 to 3.6×10^6).

The small size of this study precluded definitive assessment of the influence of tumor/patient characteristics on core biopsy T-cell infiltrate quantity. However, Fig. 2 illustrates the potential influence of various factors on baseline T-cell infiltrate quantity. Both tumor differentiation and tumor subtype appeared to influence T-cell infiltrate quantity, with higher grade lesions and TNBC or HER2⁺ lesions having denser infiltrates. Ipilimumab was administered prior to the core biopsy (median 2.5 days prior), and hypothetically this may have influenced T-cell infiltrates in this biopsy. However, subjects who received ipilimumab more than 3 days prior to biopsy ($n = 6$) did not demonstrate appreciable differences in infiltrate quantity compared with subjects not

receiving ipilimumab or receiving ipilimumab within 2 days of biopsy (Fig. 2).

Induction of T-cell polyclonality and clonal repertoire remodeling

TCR sequencing can also assess the clonal nature of infiltrating T cells, i.e., whether the infiltrates are comprised of a few high-frequency clones (oligoclonal) versus many unique low-frequency clones (polyclonal). In our dataset, the number of unique T-cell clonal populations within a sample correlated with the absolute T-cell count ($r = 0.76$, $P = 0.0002$). To assess clonal diversity independent of this observed correlation, we calculated the Shannon entropy of the specimen and normalized it to the number of unique clones within the sample. This previously described "clonality" metric ranges from 0 to 1 and is relatively insensitive to sample size, with 1 representing a purely monoclonal population, versus 0 representing a purely polyclonal population (22). Using this metric, clonality of the core biopsy specimens ranged from 0.10 to 0.41 (mean 0.16; SD 0.07), which was similar in degree and variation to normal breast tissue T-cell clonality (mean 0.18; SD 0.07). In this small dataset, T-cell clonality did not appear to be associated with subject age, tumor pathologic grade, or HR/HER2 subtype (Fig. 2).

To determine the intratumoral effects of cryo and/or single-dose ipilimumab, we conducted T-cell repertoire analysis on matched core biopsies and mastectomies. Effects on absolute count varied widely within treatment groups (data not shown). Cryo decreased T-cell density in the majority (5/6) of subjects conversely ipilimumab alone increased T-cell density in the majority (5/6) of subjects, whereas no trend was observed following combination therapy (Fig. 3). We then evaluated the effect

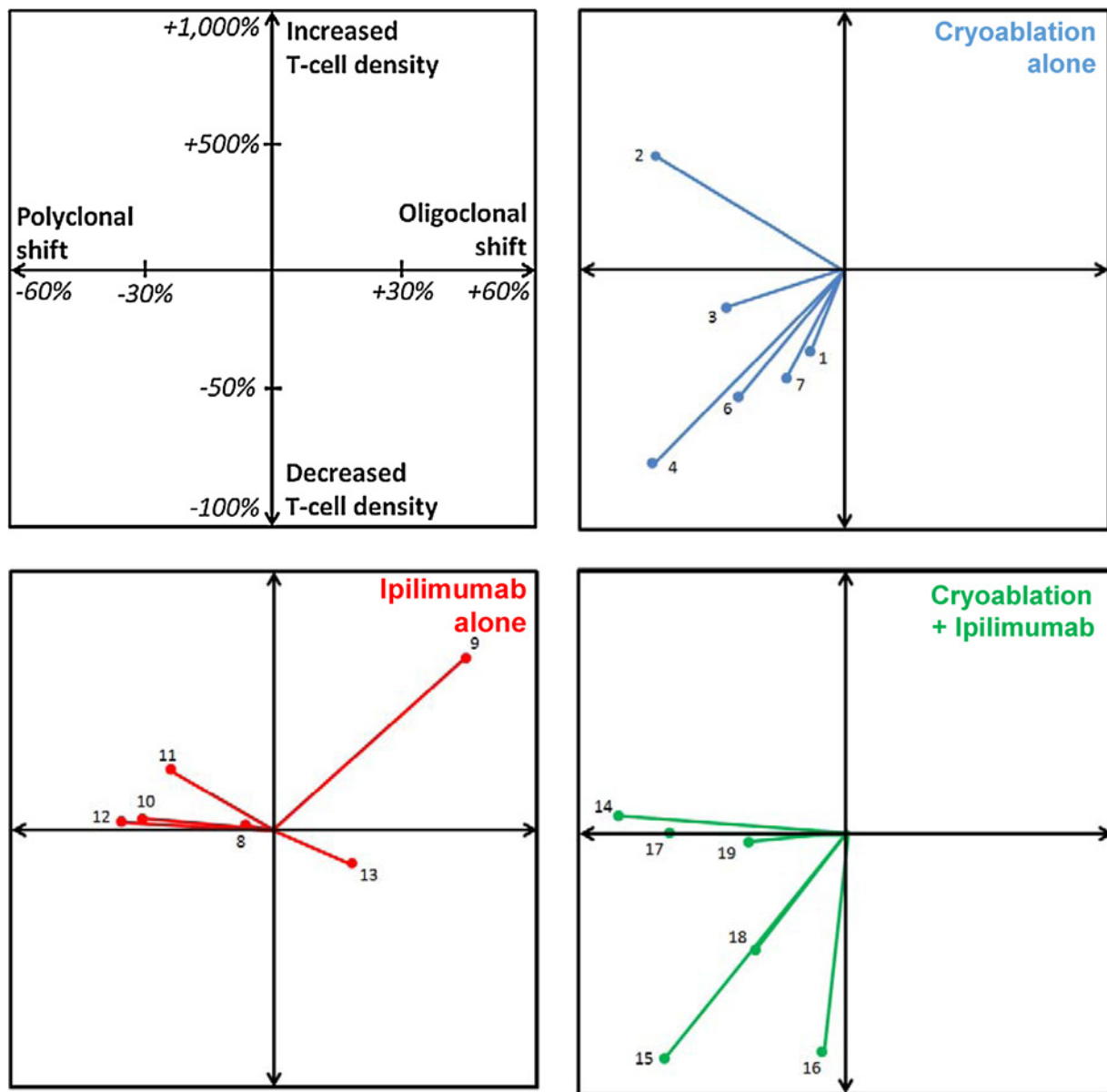


Figure 3. Effect of therapy on intratumoral T-cell density and clonality by TCR deep sequencing. T-cell density and clonality are depicted as fold change, comparing core biopsy to mastectomy. The x-axis depicts fold change in T-cell clonality, with positive values indicating a shift toward oligoclonal T-cell infiltrates; the y-axis depicts fold change in T-cell density, with positive values indicating a shift toward denser T-cell infiltrates. Each line/dot demonstrates an individual patient treated on protocol, and numbers indicate patient identifier (as listed in Table 1).

of therapy on clonality. One hundred percent of cryoablated tumors ($n = 12$), irrespective of ipilimumab, experienced a shift toward polyclonality (median clonality change: -0.04 ; $P = 0.005$), whereas no trend in clonality was observed following ipilimumab alone. These findings are graphically depicted in Fig. 3, which illustrates the change in T-cell clonality (x-axis) and the change in T-cell density (y-axis) for each patient, according to treatment arm (Fig. 3).

Next, we evaluated whether ipilimumab and/or cryo remodels the clonal repertoire, i.e., whether new clones are introduced or the distribution of clones is modified. Morisita's overlap is a

previously described (23) metric that measures the proportion of shared T-cell sequencing reads over two samples, normalizing for biases introduced by sample size. This overlap metric ranges from 0 to 1, with low overlap scores indicating repertoire remodeling and potential influx of novel T-cell clones. Relative to the ipilimumab-alone group, we found a trend of lower Morisita's overlap in cryo-treated groups ($P = 0.08$), suggesting greater remodeling of the intratumoral clonal repertoire following cryo. In summary, these findings suggest that ipilimumab alone expands intratumoral lymphocytes, cryo alone contracts intratumoral lymphocytes and induces polyclonality and repertoire

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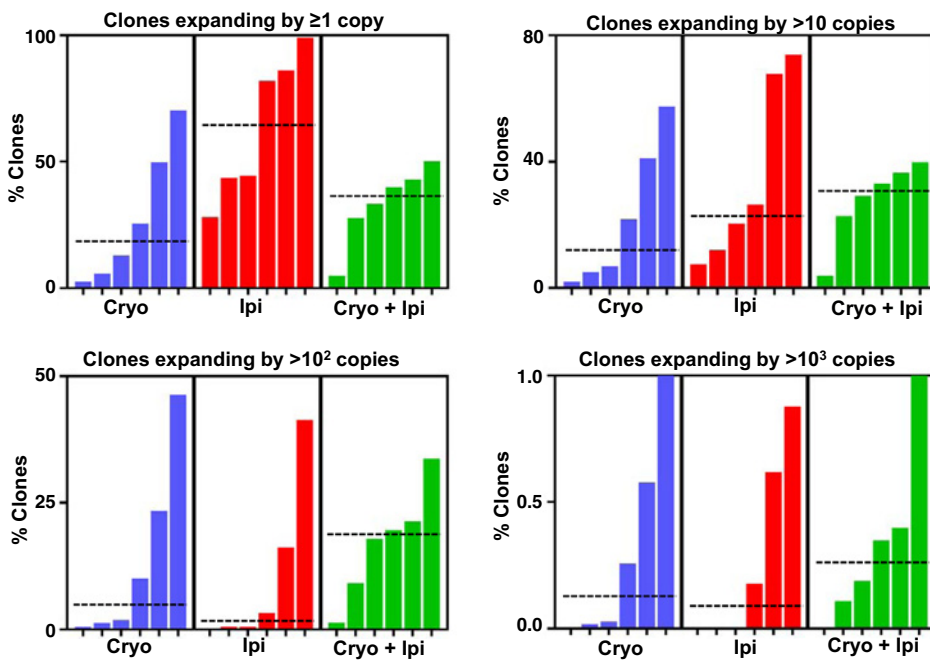


Figure 4.

Effect of therapy on clonal expansion. Each graph depicts the proportion of intratumoral T-cell clones expanding by the specified threshold (i.e., expansion by ≥ 1 copy, >10 copies, >100 copies, or $>1,000$ copies), comparing core biopsy to mastectomy. Each solid line represents an individual patient, with dotted lines representing group medians. Legend: Cryo-cryoablation; Ipi, ipilimumab.

remodeling, and the combination induces polyclonality and repertoire remodeling with less lymphocytic contraction.

Induction of high-magnitude intratumoral T-cell clonal expansions

Certain T-cell clones, when exposed to released antigens following cryo, might expand more robustly with the addition of ipilimumab. Therefore, we calculated the pre-/post-treatment change in abundance of each individual T-cell clone found in either the core biopsy or mastectomy specimen. We then quantified the number of clones that expanded with therapy by certain absolute sequence count thresholds, and normalized this number by the total clone count. Using this technique with the threshold set to ≥ 1 copy (thus measuring the proportion of clones expanding by any magnitude), we found that the greatest percentage of T-cell clones expanded with ipilimumab alone (median % clones expanding: 18% or 4,454 clones, cryo; 62% or 41,399 clones, ipilimumab, 36% or 4,307 clones, cryo + ipilimumab). However, if we repeated this with the threshold set to 10, we found that the greatest percentage of T cells expanded with cryo plus ipilimumab (median % clones expanding: 14% or 2,822 clones, cryo; 23% or 11,322 clones, ipilimumab, 31% or 3,658 clones, cryo + ipilimumab). At higher thresholds (10^2 or 10^3), the differences appear more pronounced (Fig. 4), indicating that more high-magnitude T-cell expansions were observed in the tumors treated with combination ipilimumab plus cryo, relative to monotherapy.

Peripheral blood clonal expansions are less discernible than TIL clonal expansions

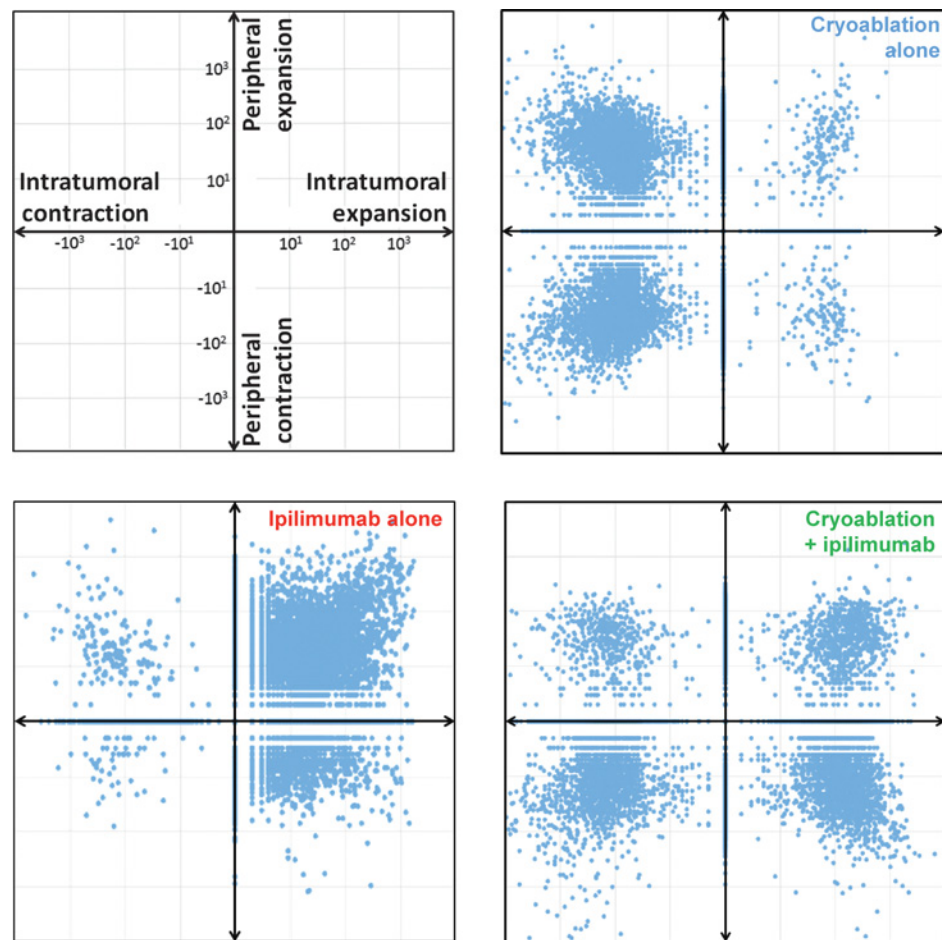
In baseline tumor and matched PBMC samples acquired at time of biopsy, we found a 5-fold greater number of unique clonal T-cell populations (range, -1 - to 232-fold) in matched peripheral blood specimens compared with TILs. Normalizing the clonal diversity of PBMCs for sample size using the clonality metric, PBMCs were only modestly more polyclonal than T cells obtained from biopsy (13/18 subjects, 0.02 median difference in clonality).

We then evaluated the effect of therapy on peripheral repertoire by analyzing serially collected PBMCs. In subjects receiving cryo alone ($n = 6$), two pretreatment time points were analyzed (at screening and within 1 day preceding cryo), allowing for characterization of baseline intrapatient variations in repertoire over time in the absence of intervening therapy. The clonality metric exhibited a low degree of intrapatient variation (median difference between samples 0.02), whereas measures of T-cell quantity (absolute count and T-cell density) were prone to wide intrapatient variation. We then evaluated the effect of therapy over time, comparing baseline to post-treatment peripheral blood obtained during the 30-day safety visit. We found no obvious trends with either of the three arms over time in T-cell clonality, absolute T-cell quantity, or T-cell density (data not shown). We then evaluated the Morisita's overlap of pretreatment versus posttreatment peripheral blood and found no appreciable differences in overlap across the three treatment groups.

Using the same method described for TILs, we then calculated the pretreatment/posttreatment change in abundance of each individual T-cell clone found in the peripheral blood specimens. Peripheral blood concentrations of T-cell clones are likely to fluctuate to some degree over time, in the absence of therapy. To assess the degree of fluctuation of clonotype concentration, we compared the two pretreatment time points of the cryo-treated subjects. In the absence of therapy, roughly half of clones expanded (median 45%) from time point A (screening) to time point B (preceding ablation). However, only $\sim 1\%$ of clones expanded by an absolute count of 10% to 100, $\sim 1\%$ of clones expanded by counts of 100 to 1,000, and virtually no clones expanded by $>1,000$ counts. We then characterized the effect of ipilimumab and/or cryo on peripheral T-cell clone expansion by comparing time points B and C for each of the treatment arms. The degree of clonal expansion was similar to the untreated time points, with the exception of the combined ipilimumab + cryo arm, which exhibited a greater proportion of clones expanding by >100 (median %

Figure 5.

Relationship of intratumoral versus peripheral blood T-cell clonal expansion. Each graph represents the T-clonal repertoire of a representative patient of each of the three treatment groups. Each point represents a unique T-cell clone detectable in the core biopsy, mastectomy, and/or peripheral blood. The x-axis depicts the absolute change in intratumoral clonal frequency count, comparing core biopsy to mastectomy. The y-axis depicts the absolute change in peripheral blood clonal frequency count, comparing pretreatment to post-treatment time points. For example, points in the upper right quadrant depict T-cell clones that have increased in frequency both within the tumor and in the peripheral blood.



of expanding clones: untreated, 1%; cryo, 1%, ipilimumab, 1%; cryo/ipilimumab, 6%). These high-magnitude clonal expansions corresponded to a median absolute number of 1,253 clones per treated patient in the ipilimumab + cryo arm.

Independent intratumoral and peripheral TCR repertoire landscapes

Assuming that TILs are exposed to tumor-associated antigens and therefore enriched for tumor-reactive T-cell clones, the more contracted intratumoral clonal repertoire could be used to identify and monitor tumor-reactive clones within the peripheral blood. We tested the hypothesis that intratumoral T-cell expansions might be mirrored in peripheral blood by categorizing intratumoral clonotypes into groups by degree of intratumoral expansion, using the previously defined thresholds (>10 copies, >100 copies, and >10³ copies). We then evaluated the degree of expansion of each of these clonotype groups within the peripheral blood over time, comparing baseline to 30-day posttreatment follow-up. Most of the clones expanding within the tumor were undetectable in the peripheral blood, either before or after therapy. Regardless of the degree of intratumoral clonal expansion, only a low percentage of clonotypes were also expanding in the peripheral blood, and was not influenced by the degree of intratumoral expansion (median proportion of peripherally expanding clones across all groups: >10 copies, 6%; >100 copies, 7%).

We conducted graphical analyses of intratumoral versus peripheral blood clonotype expansion, plotting the absolute intratumoral expansion/contraction on the x-axis, versus peripheral expansion/contraction on the y-axis (Fig. 5). Using this technique, we found no correlation between intratumoral clonal expansion and peripheral blood clonal expansion; however, we observed several general trends across the three treatment groups. First, cryo alone appeared to induce intratumoral contraction of the majority of T-cell clones, with very little effect on peripheral blood clones (as represented by the asymmetry across the y-axis and symmetry across the x-axis). Next, ipilimumab alone appeared to induce expansion of clonotypes both in the peripheral blood and in the tumor (as represented by a dominance of clones in the double-positive quadrant). Finally, combination ipilimumab/cryo induced a mixed contraction and expansion of intratumoral clones, as represented by symmetry across the y-axis. Furthermore, the median degree of expansion with ipilimumab/cryo was shifted rightward, indicating that a greater proportion of clones experienced high-magnitude expansions, compared with ipilimumab alone.

Conclusion

The two goals of this *post hoc* analysis were to explore the potential utility of TCRB sequencing as an immune-based biomarker in early-stage breast cancer and to characterize the effect of

cryo-immunotherapy on intratumoral and peripheral blood T-cell proliferation. We first compared TCRB sequencing to the current gold-standard immune biomarker in early-stage breast cancer, TIL count by H&E staining. Recently, a standardized method of assessing TILs by H&E has been developed and was validated to be predictive and prognostic in triple-negative breast cancer (6). Despite its utility and simplicity, the H&E TIL score provides only a semiquantitative assessment on immune infiltration and poorly discerns the highest-risk patients (with the highest risk low-TIL group exhibiting relapse rates of only 30% in a recent series; ref. 21). TCRB sequencing is an attractive alternative because it quantifies T cells (the immune cell type most attributable protective immunity) and characterizes T-cell clonal diversity, which might be useful to further stratify risk. In our dataset, we found that the T-cell density, a method of quantifying T cells by TCRB sequencing, correlates with the gold-standard TIL assessment by H&E. Furthermore, the distribution of TIL quantity (with most tumors having low T-cell density), and the association of T-cell density with poorly differentiated and HER2⁺/TNBC tumors, closely resembles the H&E TIL quantity metric (21, 24). One primary difference, however, is that TCR sequencing could quantify up to millions of T-cell DNA sequences even in samples with no TILs or low TILs by H&E. Some of these sequences may arise from non-tumor-bearing breast tissue or vasculature within the sequenced sample; nevertheless, these data suggest that TCR sequencing could be a potentially more sensitive method of measuring intratumoral T-cells. Although not addressed by this dataset, it is conceivable that a more precise quantification of T cells could be helpful in stratifying the highest-risk tumors amongst tumors with low TILs by H&E. The correlation between H&E TIL score and T-cell density was imperfect: discordant scores may be related to intratumoral heterogeneity, or the preponderance of non-T-cell lymphocytes in some specimens, which would result in a higher H&E TIL score but a lower T-cell density.

Our second goal was to demonstrate how TCRB sequencing could be used to characterize the effects of immunotherapy in serially collected intratumoral and PBMC specimens. We used two TCRB sequencing metrics, T-cell density and T-cell clonality, to characterize the effects of cryo and/or ipilimumab over time in tumors. T-cell clonality describes whether T-cell populations are oligo-clonal (i.e., reacting to the one or a few antigens) or polyclonal (reacting to many different antigens). In one trial, melanomas with oligoclonal T-cell infiltrates were more likely to respond to immunotherapy with anti-PD-1 (pembrolizumab; ref. 18), potentially because the dominant T-cell clones were tumor specific and amenable to reinvigoration with anti-PD-1. In our dataset, cryo alone appeared to deplete T cells but induced a polyclonal shift (Fig. 3). The observed decrease in T-cell density is consistent with previous observations that cryo induces thermal necrosis and apoptosis (of both tumors and TILs; ref. 25). The polyclonal shift and the low overlap between pretreatment and posttreatment clones suggest that new T-cell clones are infiltrating the tumor bed and are reactive to a broader variety of antigens compared with the pretreatment resident T cells. The polyclonal shift, however, argues against the presence of dominant, high-magnitude clonal expansions associated with a robust response against the tumor.

In the ipilimumab ± cryo arms, ipilimumab was administered a median of 2.5 days before the research biopsy ± cryo, and surgical mastectomy was performed a median of 7 days thereafter. This provided a unique opportunity to assess serial changes

following ipilimumab ± cryo, with the limitation that early post-ipilimumab changes (i.e., during the short window between ipilimumab and biopsy) would not be captured in this analysis. Ipilimumab appeared to expand tumor-infiltrating T cells, which is consistent with previously described observations that immunologic checkpoint blockade could induce intratumoral T-cell expansion (7). Cryo + ipilimumab had a mixed effect on T-cell density, suggesting that neither ipilimumab nor cryo were dominant in their effects on T-cell density.

We hypothesized that cryo + ipilimumab may generate more T-cell expansions relative to cryo alone; however, as illustrated in Fig. 3, cryo + ipilimumab produced a polyclonal shift similar to cryo alone. Therefore, we more closely evaluated intratumoral clonal T-cell expansion by comparing the concentration of individual T-cell clones before and after therapy. By this approach, we observed that cryo + ipilimumab was associated with a greater number of high-magnitude clonal expansions relative to monotherapy. If confirmed, this finding would suggest that cryo + ipilimumab mediates rapid proliferation of a small subset of clones. This analytic method of clonotype expansion was more useful in this dataset than the clonality metric, for discerning differences across treatment groups. Although clonal TIL expansion does not necessarily indicate an antitumor response, one would expect clonal TIL expansion in the setting of T-cell responses against tumor-associated antigens. Therefore, we argue that this metric may be a candidate biomarker for assessing therapy-associated clonal proliferation in preoperative immunotherapy clinical trials.

We also evaluated the utility of serial TCRB sequencing of PBMCs. In our paired analysis of blood and tumor, we found an increase in the number of peripherally expanding clones associated with combination therapy, but found little evidence in support of an association of intratumoral clonal change with peripheral blood clonal change. One explanation is that intratumorally expanding T-cell clones are diluted in PBMCs by the more diverse and abundant peripheral blood clonal repertoire, and may be difficult to track serially over time in PBMCs in early-stage disease. However, it is conceivable that in metastatic disease, intratumorally expanding T-cell clones expand peripherally after exposure to additional tumor antigen, and therefore may be easier to detect in the blood or in satellite metastases.

Because the analysis was *post hoc* with no explicit statistical analyses or adjustments for multiplicity, because the histologies of the treated tumors varied and were imbalanced across groups, and because the timing of ipilimumab was heterogeneous, we are limited in our ability to generalize our characterization of breast cancer TILs. Also, our observations regarding the clonal effects of ipilimumab ± cryo must be confirmed prospectively in a study with more standardization of variables such as biopsy timing and specimen processing. In such a trial, more information could be derived by characterizing T-cell repertoire changes across various subsets of T cells (for example, in effector T cells, helper T cells, and regulatory T cells, which could be sorted by flow cytometry), or within draining lymph nodes, which might provide additional information regarding priming and expansion of T cells following cryo-immunotherapy.

In metastatic clinical trials, immune checkpoint blockade with anti-PD-1/anti-PD-L1 is modestly effective across all metastatic breast cancer subtypes (26–29). Therefore, we anticipate heightened investigation of novel combination strategies as a means of improving efficacy. We also anticipate increasing utilization of

preoperative clinical trials, on the basis that serial analysis of breast cancer tissue may inform drug development and understanding of underlying mechanism. In conclusion, we have demonstrated that TCRB sequencing is feasible to conduct in early-stage breast cancer using small core biopsy specimens, and that the TCRB T-cell density metric correlates with the current gold-standard immune biomarker, TIL count by H&E. We also illustrated how multi-dimensional data obtained from TCRB sequencing could elucidate the effects of immunotherapy on TILs and PBMCs, and may be informative as a biomarker in preoperative clinical trials.

Disclosure of Potential Conflicts of Interest

D.B. Page reports receiving a commercial research grant from Merck, is a consultant/advisory board member for Peregrine Pharmaceuticals and Celgene, and has received expert testimony from an entity. J. Yuan is Director, Translational Immuno-Oncology Research at Merck & Co Inc. and has ownership interest in the TCR patent with Adaptive Technologies. S.B. Solomon reports receiving a commercial research grant from GE Healthcare and is a consultant/advisory board member for Medtronic. P. Wong is a consultant/advisory board member for Merck. H. Robins is CSO at Adaptive Biotechnologies and has ownership interest (including patents) in the same. J.P. Allison has ownership interest (including patents) in Jounce Therapeutics, Neon, Merck, and Jounce and is a consultant/advisory board member for Kite, Amgen, Jounce, and Neon. J.D. Wolchok reports receiving commercial research grant from Bristol-Myers Squibb, Merck, Medimmune, and Genentech and is a consultant/advisory board member for Bristol-Myers Squibb, Merck, Medimmune, and Genentech. H.L. McArthur reports receiving commercial research grant from Merck, Eli Lilly, and MedImmune/Astra Zenica, other commercial research support from Bristol-Myers Squibb, and is a consultant/advisory board member for Merck. No potential conflicts of interest were disclosed by the other authors.

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