Evaluating the efficacy of a priming dose of cyclophosphamide prior to pembrolizumab to treat metastatic triple negative breast cancer

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ABSTRACT

Purpose Triple negative breast cancer (TNBC) is characterized by the presence of immune cells in the tumor microenvironment, however, the response to single-agent immune checkpoint inhibitor (ICI) therapy is modest. Preclinical models have demonstrated that intratumoral regulatory T cells (Tregs) dampen the antitumor response to ICI. We performed a single-arm phase II trial to evaluate the efficacy of a single low dose of cyclophosphamide (Cy) to deplete Tregs administered before initiating pembrolizumab.

Patients and methods 40 patients with pretreated metastatic TNBC were enrolled. The primary endpoints were progression-free survival (PFS) and change in peripheral blood Tregs after Cy. Secondary endpoints included overall response rate (ORR), duration of response, overall survival, treatment-related adverse events (AEs), and correlative evaluations.

Results Median PFS was 1.8 months, and the ORR was 21%. Tregs were not significantly decreased after Cy prior to ICI (−3.3%, p=0.19), and increased significantly after the first cycle of therapy (+21% between cycles 1 and 2, p=0.005). Immune-related AEs were similar to historical pembrolizumab monotherapy, and were associated with response to therapy (p=0.02). Patients with pretreatment tumors harboring increased expression of B cell gene signatures and increased circulating B cell receptor repertoire diversity were associated with clinical response and immune-related toxicity (IRT).

Conclusions Among patients with heavily pretreated TNBC, Cy prior to pembrolizumab did not significantly deplete Tregs, and in those with decreased numbers there was rapid recovery following therapy. Increased B cell gene expression in baseline samples was associated with clinical response and IRT.

INTRODUCTION

Triple negative breast cancer (TNBC) lacks expression of the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). TNBC is the most aggressive breast cancer subtype and portends a poor prognosis despite initial responses to cytotoxic chemotherapy.1-5 TNBC is associated with early and visceral recurrence, and frequently metastasizes to distant sites, particularly to the lungs and brain.6,7 The median progression-free survival (PFS) for patients with metastatic TNBC (mTNBC) with first-line chemotherapy is <6 months, and overall survival (OS) is typically <2 years from initial metastatic diagnosis.8,9

Previously, our group had shown that TNBC was the breast cancer subtype most heavily infiltrated with immune cells.8 Despite these findings, the activity of immune checkpoint inhibitor (ICI) that inhibit the PD-1/PD-L1 axis as a monotherapy for TNBC has been relatively modest.10 The KEYNOTE-012 study, a phase 1b trial of pembrolizumab in heavily pretreated patients with mTNBC, reported an overall response rate (ORR) of 18.5%, with patients progressing at a mean of 3 months post-therapy.11 Similar studies evaluating the function of antibodies targeting PD-1 or PD-L1 as a monotherapy have shown response rates of 8%-15%.10 More recently, pembrolizumab was approved for patients with mTNBC whose tumors expressed PD-L1 when given with either nanoparticle albumin-bound paclitaxel, gemcitabine or carboplatin. In patients with tumors that express PD-L1 ≥10 by the combined positive score,12 median PFS was 9.7 months in those getting
The exact mechanism underlying the limited response
of patients with TNBC to ICI monotherapy treatment is
not well understood. Our group and others have char-
acterized the presence of immunosuppressive cells such
cas CD25+ FOXP3-expressing regulatory T cells (Treg),
M2-like macrophages and myeloid-derived suppressor
cells in the TNBC tumor microenvironment (TME) that
may contribute to resistance mechanisms.13–18 Previously,
we had shown that the depletion of intratumoral Treg
enhanced the anti-tumor response in genetic murine
models of TNBC treated with dual ICI therapy.19 In these
preclinical studies, treatment with low-dose cyclophos-
phamide (Cy) reduced Treg numbers in tumors, suggesting a
method of sensitizing TNBC to ICI.19

Based on our preclinical observations, we initiated a
multicenter, single-arm phase II study (NCT02768701) to
evaluate the safety and activity of a single low dose of Cy
to deplete Treg prior to pembrolizumab in patients with
previously treated mTNBC. Primary objectives were the
assessment of PFS and the ability of low-dose Cy to deplete
peripheral blood Treg. We also report the ORR, duration of
response (DoR), OS, treatment-related adverse effects
(AEs), and correlative science objectives using RNA-seq,
whole exome sequencing (WES) and adaptive immune
receptor repertoire profiling of tumor and peripheral
blood from patients enrolled in this study to evaluate if
we could confirm a critical role for B cells in the immune
response to anti-PD-1 mAb in patients with breast cancer.

MATERIALS AND METHODS

Patients
Patients were enrolled at five institutions (UNC-Chapel
Hill, University of Pittsburgh, Moses Cone, UNC-Rex
Cancer Center, and George Washington University) from
November, 2016 to February, 2018. Eligible patients had
histologically confirmed mTNBC (ER and PR negative,
<1%; HER2 negative, 0–1 by immunohistochemistry or
non-amplified by fluorescence in situ hybridization).
Eligible patients had received at least one prior cytotoxic
treatment in the metastatic setting; stable brain metast-
sases were allowed. Additional inclusion criteria included
Eastern Cooperative Oncology Group (ECOG) perfor-
ance status of ≤1, adequate organ function, and having
been off corticosteroids for at least 7 days prior to entry.

Patients were excluded if they were pregnant or breast
feeding, had a diagnosis of immunodeficiency or need
for ongoing steroid therapy, receipt of prior immu-
notherapy, a history of HIV or active hepatitis B/C, or
had progressive brain or leptomeningeal metastases. All
patients provided written informed consent.

Study design
This was an open-label, single-arm phase II study. The
primary endpoints were PFS and change in peripheral
blood Treg after a single dose of Cy. Secondary endpoints
were ORR, DoR, OS, treatment-related AEs, and correla-
tive science objectives.

Administration of study treatment
Enrolled patients received a single priming dose of 300
mg/m² Cy intravenously on cycle 1, day 1 (C1D1). The
next day (C1D2), patients received 200 mg pembrol-
zumab intravenously. Pembrolizumab was given every 3
weeks until either progressive disease (PD) was seen or
the patient suffered significant toxicity, at which point
they discontinued protocol-directed therapy.

Efficacy assessments
Serial CT scans of the chest, abdomen, and pelvis, and
a nuclear bone scan to evaluate disease burden were
obtained at 8 weeks, and then every 8–12 weeks thereafter.
Response criteria was evaluated using modified Response
Evaluation Criteria In Solid Tumors V.1.1 (RECIST
V.1.1)20 for the primary objective of PFS, and the best
response was determined for each patient. A complete
response (CR), partial response (PR), stable disease (SD)
and PD were defined as in RECIST V.1.1. SD did not meet
the criteria either PR or PD. ORR was defined as CR +PR.
Clinical benefit (CBR) was defined as CR +PR+SD for
at least 6 months from the best response date. Adverse
events (AEs) were graded according to the NCI Common
Toxicity Criteria for Adverse Events (NCI CTCAE), V.4.0.
DoR was defined as time from duration of treat-
ment response to progression. AEs were assessed every
3 weeks and graded according to the NCI CTCAE v4.0;
a duration of an AE was not required. Immune-related
toxicity (IR) was defined as those AEs in common with
immune-related AEs previously reported with single
agent pembrolizumab, specifically colitis, xerostomia,
fever, influenza-like symptoms, hypothyroidism, immune
system disorders, pruritus, or rash.10 11 21 IR was evaluated
using CTCAE V.4.0, with any grade considered a toxicity
regardless of duration.

Peripheral blood Treg levels
Peripheral blood was collected on C1D1 prior to Cy
administration, C1D2 prior to pembrolizumab, and on
D1 of each subsequent cycle of pembrolizumab. Periph-

eral blood mononuclear cells (PBMCs) were isolated from
peripheral blood via Ficoll gradient and cryopreserved in
10% DMSO and 90% fetal bovine serum. Cryopreserved
PBMCs were subsequently thawed, washed free of DMSO
and exogenous protein with Hanks Balanced Salt Solu-
tion, HBSS, (Gibco) and concentrated to 1–5 × 10⁶/mL.
Cells were first incubated with Live/Dead Aqua (Molec-
ular Probes L34066) for 30 min at room temperature in
the dark before washing with 0.1% sodium azide (Sigma)
and 0.5% Human Serum Albumin (HSA) (CSL Behring).
Purified murine IgG (Sigma) was added to a final concen-
tration of 200 micrograms/ml to block non-specific
binding of the fluorescently labeled murine monoclonal
antibodies. Fluorescently labeled antibodies were added


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to appropriate tubes (including FMO controls) and incubated on ice for 30 min in the dark. The following antibodies were used at predetermined antibody concentrations: CD19-APC-Cy7 (BD 557791) and CD14-APC-Cy7 (BD 561709) were used in a dump channel to exclude B lymphocytes and monocytes respectively from the analyses, CD45-V450 (BD Biosciences 560367), CD4-FITC (BD Pharmingen 561005), CD8-PerCP-Cy5 (BD Biosciences 560662), CD25-PE (BD Biosciences 341010), CD127-PE-Cy7 (BD Biosciences 560822). Cells were incubated with antibodies on ice for 30 min in the dark. After incubation, the cells were washed twice in FACS Staining Buffer (.1% sodium azide, 0.5% HSA in HBSS) and resuspended in FoxP3 buffer A for 10 min at room temperature (Human FoxP3 Buffer set BD Biosciences 560098) to fix the cells. The cells were washed and resuspended in FoxP3 buffer C for 30 min at room temperature to permeabilize the cells. The cells were then washed and resuspended in murine IgG to block nonspecific binding (10 min, room temperature) and then stained with Foxp3-Alexa 647 (BD Pharmingen 560045) for 30 min in the dark at room temperature. Stained cells were washed in FACS staining buffer and fixed in 1% formalin (Poly-sciences) and the entire volume of cells were immediately acquired on the Fortessa cytometer (BD Biosciences). Data were analyzed with FlowJo software.

**Correlative studies**

**Nucleic acid extraction**

Formalin-fixed paraffin-embedded (FFPE) tissue sample slides were reviewed by a pathologist to confirm the presence of tumor tissue when available prior to therapy (please see schema for tissue, online supplemental figure 1); patients did not need to have tissue for correlative evaluations to be enrolled on the study. Nucleic acid isolation was performed on FFPE slides by the UNC Lineberger Comprehensive Cancer Center Translational Genomics Lab using the Maxwell 16 MDx Instrument (Promega AS3000). DNA was extracted using the Maxwell 16 FFPE Plus LEV DNA Purification Kit (Promega AS1135) following the manufacturer’s protocols (Promega TM349). RNA was extracted using the Maxwell 16 LEV simplyRNA Tissue Kit (Promega AS1280) and from FFPE slides using the Maxwell 16 LEV RNA FFPE Kit (Promega AS1260) following the manufacturer’s protocols (Promega TM351 and TM408, respectively). DNA and RNA quality were measured using a Qubit V.3.0 fluorometer (Life Technologies Q33216).

**RNA Sequencing**

Samples of total RNA extracted from FFPE tumor tissue (ROCHE High Pure FFPE kit, Indianapolis, Indiana, USA) were used to prepare Illumina TruSeq RNA Access (Cat. No. 20020189) sequencing libraries. Sequencing was performed in the UNC-Chapel Hill High Throughput Sequencing Facility on an Illumina HiSeq 4000 platform using the Illumina HiSeq SBS 150 Cycles (PE-410-1001) with 2×75 paired end base reads.

**Gene expression analysis**

Paired-end FASTQ files were aligned to an Ensembl transcriptome (release 99, on reference genome GRCh38) using Star (V.2.7.3a) and transcripts quantified using Salmon (V.0.8.2). Quality of FASTQ data and quantified BAMs was verified using FastQC (V0.11.7) and Picard’s (V.1.86) CollectRnaSeqMetrics program, respectively. Expression matrices were imported into R using Bioconductor’s tximport (V.1.22.2) Bioconductor package.22 Immune gene signature expression was calculated by the mean expression of each gene within that signature.

**Adaptive immune receptor repertoire analysis**

Cryopreserved PBMCs were thawed and recovered in complete AIM-V medium with 10% human albumin. Cells were pelleted and lysed for RNA extraction (RNasey Plus Mini kit, Qiagen 74134). Libraries were prepared for TCR and BCR profiling using the SMARTer Human TCR a/b Profiling Kit (Takara Bio USA, Cat. No 63516) and SMARTer Human BCR IgG IgM H/K/L Profiling Kit (Takara Bio USA, Cat. No 634467), respectively. Samples were pooled to a final concentration of 2–4nM before dilution of the pool to 13.5 pM with 10% PhiX control V3 (Illumina, Cat. No FC-110-3001). Sequencing was performed on an Illumina MiSeq sequencer using the 600 cycle MiSeq Reagent Kit v3 (Illumina MS-102–3003) with paired-end 2×300 base pair reads.

To determine immune repertoire changes in peripheral blood during therapy, post-treatment samples were considered as the latest sample following two or more cycles of pembrolizumab. Patients receiving fewer than two cycles pembrolizumab were not assessed for this endpoint. Pretreatment samples were those collected at enrolment, or prior to, the first pembrolizumab treatment. Immune chains were inferred from FASTQ files using MiXCR (V.2.1.9)23 and paired-end reads were subject to alignment in default mode, followed by contig assembly and export. Immune repertoire similarity was calculated from the inferred chains using Horn’s modified Morisita overlap index (44) (online supplemental figures 6 and 7).

**Whole-exome sequencing**

WES was performed on FFPE tumor tissue collected prior to treatment on this trial, with PBMCs collected serving as the matched normal. Library preparation was performed with the TrueSeq DNA, PCR-Free kit (Illumina, San Diego, California, USA) and pooled samples sequenced on the HiSeq4000 platform (Illumina). Somatic and germline WES sequencing files were aligned to Hg38 using bwa (v0.7.17) and sorted, indexed, and duplicates marked.
Breast tumor molecular subtyping was performed on PAM50 Subtyping data were available for 26 patients.

Small indels and substitutions identified by WES, and divided by the mutational burden (TMB) was calculated from small indels and substitutions required a minimum MAF of 10%. Tumor mutations only, Cadabra indel quality >10.5, Mutect2 indel quality >6.8 or single nucleotide variant (SNV) quality >9.2, Strelka2 indel quality >15.2 or SNV quality >19.7. Remaining variants required at least five supporting reads and a minimum read depth of 40, or 10 supporting reads and minimum read depth of 80 if MAF <5%. Variants with a MAF >5% in normal tissue were dropped, as were variants appearing at rates above 1% in any subpopulation in either GnomAD or 1000 Genomes databases. To counter FFPE artifacts, C>T dropped, as were variants appearing at rates above 1% in normal tissue were dropped, as were variants appearing at rates above 1% in any subpopulation in either GnomAD or 1000 Genomes databases.

PAM50 Subtyping

Breast tumor molecular subtyping was performed on tumor RNA-Seq data. The normalized, log2 transformed expression matrices were re-centered based on published methods for comparing FFPE-derived RNA-Seq data against the microarray-derived PAM50 centroids. Samples were assigned to a subtype based on the centroid with the minimum of the absolute Spearman rank correlation coefficient. One patient had tumor available for WES but not RNA-seq, and thus was assigned ‘Unknown’ for PAM50 subtype.

Tumor imputed cell mixture

Tumor immune cell imputation was performed from RNA-Seq data Salmon-quantified transcript counts using CibersortX via the authors’ website at http://cibersortx.stanford.edu (online supplemental figure 8).

PD-L1 Expression

PD-L1 expression was assessed on FFPE tumor samples by Discovery Life Sciences (Newtown, PA) using the mouse anti-PD-L1 antibody clone 22C3 (QualTek) with membrane-specific staining of all cells (tumor and immune cells) estimated by a board-certified pathologist using a discrete scale ranging from 0% to 100%. PD-L1 was considered positive if the mean proportion score was >1% across all cells.

Cytometry by time-of-flight

We used high dimensional multiparameter mass cytometry analysis by cytometry by time-of-flight to phenotype peripheral blood lymphocytes from 16 patients profiled at 43 times across the study. The evaluation included eight responsive and eight non-responders patients after therapy. The assessments were performed longitudinally and grouped as: early (cycles 1–2), middle (cycles 3–5), late (cycles 6+) treatment time points.

Statistical analysis

A sample size of 36 patients was chosen to achieve 80% power to detect a change in median PFS from 1.9 (null) to 2.9 (alternative) months at a 0.05 significance level. Assuming a 10% dropout rate, 40 patients were enrolled. The Kaplan-Meier method was used to estimate time to event overall and for subgroups of interest, and comparisons between these subgroups were made using Fisher’s exact tests to compare nominal categorical data and the Jonckheere-Terpstra method for ordinal data. Both PFS and OS were calculated from the start of treatment (C1D1). Student’s t-test was used to compare effect of Cy on T cell numbers.

Differential gene expression was compared using the default settings (Wald test) in DESeq2. Gene signature expression was compared using two-tailed t-test of the centered LFC values. Where indicated, p values were adjusted for multiple hypothesis testing using the Benjamini-Hochberg method with \( \alpha = 0.20 \). For TMB calculations, samples were classified as both TMB high, intermediate or low (≥20 mutations/MB, ≥5 but<20 mutations/MB, and <5 mutations/MB, respectively) and high or low (≥10 mutations/MB, <10 mutations/MB, respectively) before comparison with the Wilcoxon rank sum test. PAM50 subtype association with clinical outcomes was assessed via Fisher’s exact test. Intergroup comparisons for Morisita-Horn indices and repertoire diversity metrics (abundance, richness) were performed using the two-tailed t-test.

RESULTS

Patient characteristics

Forty patients were consented to this phase II study, of which 40 were evaluable for toxicity and survival, and 39 were evaluable for response. Patient demographics and characteristics are presented in table 1. The majority of patients were white (76%) with stage 0–III disease at diagnosis (75%). Most patients had received prior (neo) adjuvant therapy (n=33, 85%); 29 (83%) underwent a prior mastectomy, 6 (15%) a prior lumpectomy, and 29 (72%) received adjuvant radiation. The median time from initial diagnosis of TNBC to first metastases for this patient population was 2 years (95% CI 0.6 to 3.4 years). Enrolled patients had received a median of 2 prior lines of therapy for the treatment of metastatic disease (range 1–16), with n=21 (53%) receiving 1–3 lines. Sites of metastatic disease included liver (n=12, 30%), lung (n=14, 35%), bone (n=18, 45%), lymph node (n=4, 10%), and brain (n=2, 5%). The majority of patients had an ECOG Performance Status of 0 (n=24, 60%).

Toxicity and treatment discontinuation

All 40 patients were evaluable for toxicity. Cy prior to pembrolizumab was generally well-tolerated (n=40,
The most common AEs were fatigue (45%), nausea (30%), anemia (33%), lymphopenia (20%), edema (17%), and increased alanine aminotransferase (17%). The most common grade 3 or 4 AEs were fatigue (7%), anemia (5%), leukopenia (5%), neutropenia (5%), dyspnea (5%), diarrhea (5%), and increased aspartate aminotransferase (5%).

Disease progression as defined by radiographic imaging was the most common reason for treatment discontinuation (n=25, 63%). Four patients (10%) discontinued treatment due to toxicity (n=1 for each of prolonged grade 2 diarrhea, elevated aspartate aminotransferase levels, pembrolizumab-induced colitis, and unspecified AE). Interruptions in dosing of the pembrolizumab treatment regimen occurred in n=11 patients (28%). The most common cause for interruption of treatment were grade II or greater liver toxicity (n=5), grade 3 leukopenia or neutropenia (n=2) and grade 2 or greater rash (n=2).

**Table 1** Demographics and patient characteristics

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<th>Patient demographics (n=40)</th>
<th>Median age, years (range) 54.5 (33–82 years)</th>
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<tr>
<td>Race (n, %)</td>
<td>White 28 (76)</td>
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<td>Black/other 9 (24)</td>
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<td>Stage at diagnosis (n, %)</td>
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<td>IV 10 (25)</td>
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<td>Median time to first metastases (years, 95% CI)</td>
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<td>No 7 (17)</td>
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<td>Prior surgery (n, %)</td>
<td>Mastectomy 29 (72)</td>
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<td></td>
<td>Lumpectomy 6 (15)</td>
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<td>Prior radiation to breast/chest wall (n, %)</td>
<td>Yes 29 (72)</td>
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<td></td>
<td>No 11 (28)</td>
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<td>Prior metastatic lines of systemic therapy (n, %)</td>
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<td>4 or more 16 (42)</td>
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<td>Sites of metastatic disease (n, %)</td>
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<tr>
<td></td>
<td>0 24 (60)</td>
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<td>1 16 (40)</td>
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**Figure 1** Adverse events and efficacy of treatment on peripheral Tregs. (A) Most common toxicities by grade (1–2 or 3–4). (B) Levels of peripheral CD45+/FOXP3+/CD4+ regulatory T cells from blood collected on C1D1, C1D2, and C2D1, and comparison between time points.

**Efficacy**

Peripheral blood Treg levels

Peripheral blood was drawn for quantification of Treg (CD45+/FOXP3+/CD4+/CD127+/lo) by flow cytometry. An example of data with the gating schema used to enumerate Treg is shown in online supplemental figure 2. There was a non-significant reduction in peripheral Treg levels after a single dose of Cy compared with baseline (C1D1 to C1D2, median change −3.3%, p=0.19, figure 1B). However, there was a significant increase in Treg post-Cy with median Treg.
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numbers increasing between cycles 1 and 2 of pembrolizumab (C1D1 to C2D1, 10% increase \( p = 0.03 \); C1D2 to C2D1, 22% increase \( p = 0.005 \); figure 1B). There was a non-significant improvement in the clinical response of patients whose Tregs decreased after CY compared with those whose Tregs increased after CY (figure 1B; \( p = 0.08 \)).

Progression-free and OS

All 40 patients were evaluable for survival outcomes (PFS and OS). Of the 40 patients, at the time of analysis and with a median of 29 months of follow-up, \( n = 35 \) patients (88%) had died: \( n = 1 \) while on treatment, and \( n = 34 \) following disease progression. The median PFS was 1.8 months (95% CI 1.4 to 2.3, figure 2A). This did not reach the prespecified alternative hypothesis of a PFS of 2.9 months, nor did median PFS significantly differ from the null hypothesis of 1.9 months. The median OS for this patient cohort was 6.3 months (95% CI 2.8 to 9.3, figure 2B).

Response and IRT

Of the 39 patients evaluable for response, the ORR (CR + PR) was 21% (1 CR, 7 PRs, table 2). CBR was 10% (95% CI 2% to 24%). An additional 3 patients had SD as best response, while the remaining 28 patients (72%) had PD. The median DoR was 20.4 months (range 4–34 months) (figure 2C). The one patient with a CR exhibited a durable response for at least 19 months. Of the seven patients with PRs, five had a PR for at least 3 months and up to 9 months. The three patients with SD were stable for at least 3 months, with one patient experiencing stable disease for 18 months. Of the 28 patients experiencing PD, 15 progressed within 3 months while on study, 4 more progressed by 6 months, an additional 6 progressed by 12 months, and the remaining 2 patients progressed by 18 months post-therapy.

Of the 40 patients evaluable for toxicity, 10 patients (25%) experienced any grade IRT (table 2), with all but one of these patients with IRT being on therapy for more than 6 months (figure 2C). IRTs that occurred in more than two patients were rash (\( n = 6 \)), colitis (\( n = 3 \)) hypothyroidism (\( n = 3 \)) and xerostomia (\( n = 3 \)). Interestingly, the presence of an IRT correlated with response to therapy (\( p = 0.02 \)), while the number of prior lines of therapy and changes in Tregs did not correlate with response (\( p > 0.2 \)) (table 2).

Correlative objectives

PD-L1 expression

FFPE tumors from the pretreatment primary (\( n = 13 \)) and metastatic lesions (\( n = 8 \)) were evaluated for PD-L1 expression in a subset of patients. There was an increased response in patients with higher percentage of tumor
PD-L1 positivity (Figure 3A,B) that did not meet the predefined definition of statistical significance using thresholds for positivity of 1% or 10% (Wilcoxon rank sum p>0.37, data not shown).

**Gene expression, WES, and tumor mutational burden**

To evaluate for biomarkers of response to therapy with Cy/pembrolizumab, we performed bulk RNA-seq on tumor tissue prior to therapy. Gene expression differences were observed in all samples from responders (CR+PR) versus non-responders (SD+PD) treated with Cy/pembrolizumab, with ANKRD30A, SYCE1, and TUBB2B being higher in responders while KRT1, NRAP, and KRT2 were higher in non-responders (Figure 3C, online supplemental figure 3).

WES demonstrated that *TP53* was the most frequently mutated gene in pretreatment tumors (n=14/26, 54% of patients) (Figure 3D). The three patients with identified *BRCA2* mutations did not benefit from therapy as they experienced PD as best response (Figure 3D). To assess the intrinsic subtype of the tumors treated in this study, PAM50 molecular subtyping was performed on all samples. The majority of the tumors were the basal-like subtype, with no association between predicted subtype and either response or CBR (Fisher’s exact test p=0.29, Figure 3D). There was no association between TMB and either CB or response (Wilcoxon rank sum test p=0.288, online supplemental figure 4A,B). This may be due to the paucity of patients with high TMB as the patient who had a CR to Cy/pembrolizumab had a high TMB of greater than 20 mutations per sequenced megabase (Figure 3D, data not shown). Similarly, there was no association between tumor PD-L1 expression and TMB (Wilcoxon rank sum test p=0.171) using PD-L1 positivity thresholds of either 1% or 10%. Gene set enrichment analysis and immune gene signatures did not reveal any significant associations with either CB or response (online supplemental figure 5A,B). No somatic mutations were associated with clinical response in this study. The mutation profile of the study cohort did include multiple uncommon mutations that have been associated with immunosuppressive tumor immune microenvironment features, including dampening of interferon responses and decreased T cell infiltration (*ARID1A, PIK3CA, BAP1*) in tumors other than breast cancer.28–30

**Adaptive immune receptor repertoire diversity**

To assess how adaptive immune receptor repertoires may relate to ICI response, we performed T and B cell receptor repertoire profiling of pretreatment tumor samples and PBMCs. Specifically, we investigated several diversity metrics associated with T cell receptor alpha (TRA) and beta (TRB) chains and B cell receptor immunoglobulin lambda (IgL), kappa (IgK) and heavy (IgH) chains. For each sample, we evaluated diversity indices that represent the number of unique clonotypes (eg, richness, Chao1), the relative frequencies of each clonotype (eg, Shannon entropy, evenness), and the total number of all clonotypes (abundance). Overall, there were no significant differences in either the pretreatment tumor, pretreatment peripheral, post-treatment (defined as after two or more cycles of pembrolizumab) peripheral, or pretreatment versus post-treatment peripheral repertoire diversity measures across IgH, IgK, IgL, TRB or TRA chains by either CB or response (data not shown). We noted a small but significant association when examining T cell TRB chain similarity in an intra-group fashion, indicating that patients with CBR or clinical response to therapy were also more likely to have a preserved peripheral TRB repertoire.
chain repertoire after two or more cycles of pembrolizumab (online supplemental figure 4E,F). We noted small differences between tumor IGH chain abundance (raw number of chains, online supplemental figure 4G,H) and richness (online supplemental figure 4I,J) in patients who went on to have CB or response to therapy.

To better explore the relationships between adaptive immune receptor repertoire features and clinical outcomes, univariable Cox regression models were fit with PFS as the response variable and repertoire diversity metrics in pretreatment blood and among those who received at least two cycles pembrolizumab as the predictor variables. We found a significant association (false discovery rate (FDR) $\alpha<0.2$) with multiple B cell diversity features (IGH abundance, IGH Shannon entropy, evenness, and abundance) in post-treatment peripheral blood samples (figure 4A), with directions similar to associations in the pretreatment tumor and
peripheral B cell repertoires (figure 4A). TRA evenness and Chaol in pretreatment tumor samples also demonstrated a less robust (p<0.05) association with clinical outcomes (figure 4B).

Non-nodal tumor tissue subgroup

Gene set enrichment analysis using on RNA sequencing on a subset of tumors (n=22, online supplemental figure 6) showed CBR in patients receiving Cy/pembrolizumab therapy was associated with several immune pathways including the CXCR4, BCR, IL-2, NO2-IL12 pathways and IL-2 signaling. Similarly, immune gene signatures demonstrated upregulation in B cell immune gene signatures correlated with CBR. We hypothesized that B cell gene expression may be biased by samples identified as lymph nodes at the time of surgery, and performed a subgroup analysis that would exclude tumor-containing lymph nodes (n=6).

In this exploratory analysis, we noted several associations between immune features and clinical outcomes in non-nodal tumor tissues (n=25), though none remained significant after multiple testing correction (online supplemental figure 7A). There was a significant correlation between CB (online supplemental figure 7A) and published mRNA signatures for IgG, three B cell signatures and CD56-dim NK cells.

Leucocyte phenotype abundance in the TME

The heterogeneity of the TME can be assessed from RNA-seq data using informatics approaches to estimate the abundance of the immune cells present. Estimated cell mixture abundances using CibersortX did not reveal any significant differences between patient groups by CB or response for the detected cell subpopulations (online supplemental figure 8). Recently our group identified six different immune subtypes present across all TCGA tumors. Using this approach the RNA expression from all tumor samples was strongly representative of the C4 ‘lymphocyte depleted’ immune subtype.

Abundance and phenotype of peripheral lymphocytes

To confirm our tumor findings, we performed mass cytometry on peripheral blood from 8 patients who responded to therapy and compared this to 8 patients who did not respond to treatment. There was no difference in CD45+CD14-, HLA-DR+, CD20+, CD19+, IgD+, CD38+, CD27+ plasmablasts in responders when compared with non-responders only after Cycles 1–2 of treatment (online supplemental figure 9).
found in later cycles of therapy. Although we did not note differences in the frequency of total CD4+ or CD8+T cells in the peripheral blood at any time during treatment, we observed increased expression of PD-1, TIM-3, and CD25 on responder CD8+ T cells when isolated only during cycles 1–2 post-therapy (online supplemental figure 10). Thus, these data confirm our findings regarding activation of antibody-secreting cells after therapy and additionally demonstrate an increase in activation markers in peripheral blood CD8+T cells post-therapy.

**DISCUSSION**

While ICI has changed the landscape of patient care for multiple solid tumors, there is still significant room for improvement to increase response rate and durability of response, particularly in breast cancer. As the presence of Treg cells in tumors has been shown to reduce response to ICI, we hypothesized in this multicenter, phase II trial that a single, low dose of Cy prior to the PD-1 inhibitor pembrolizumab would increase PFS in patients with mTNBC compared with historical controls. The PFS of 1.8 months failed to confirm this hypothesis. The ORR to Cy prior to pembrolizumab was 21%, similar to single agent PD-1 inhibition alone in prior studies. Clinical response did correlate with the presence of immunorelated toxicities. Interestingly, we found that response to Cy/pembrolizumab therapy correlated with enhanced expression of B cell metagene signatures in pretreatment tumors and BCR diversity in peripheral blood, mirroring previous preclinical work that indicated a critical role for antibody production by B cells and T cell help in the response to dual ICI.

Approaches to deplete Treg have been challenging due to the lack of selectable markers that are present on Treg. We chose to use low-dose Cy since this approach illustrated benefit in our prior murine studies, and Cy is a commonly prescribed chemotherapy for breast cancer. The overall impact of low-dose Cy was quite modest with a median decrease of 3.3% in peripheral blood Treg 1 day after administration, which did not meet prespecified thresholds for significance. Cy was not continued after the initial priming dose, as continued exposure to Cy diminished the effector T cell response to breast cancer in animal models. Unfortunately, perhaps due to lymphopenia-driven expansion, there was a marked increase in peripheral blood Treg after discontinuation of Cy with a median 10% increase between receipt of Cy and the initiation of cycle 2 of therapy. The Nivolumab After Induction Treatment in Triple-negative Breast Cancer Patients (TTONIC) study in mTNBC similarly demonstrated that short exposure to Cy does not improve durable response.

Here, we found a correlation with CBR and IRT. While toxicities are commonly associated with anti-PD-1 monoclonal antibody therapy, this has not routinely correlated with response in other studies. In KEYNOTE-012B, 16% of patients had grade III or greater AEs, and this was not correlated with response. KEYNOTE-086 evaluated pembrolizumab monotherapy in a group of 170 patients with mTNBC who had progressed following previous therapy and were enrolled independent of the expression of PD-L1, as was done in the current trial. Treatment-related AEs were seen in 60% of enrolled patients, with 12% having grade III or greater AEs; the presence of significant AEs was not associated with overall outcome. Future studies that target Treg depletion with ICI should determine if clinical efficacy correlates with IRE in this setting.

The initial hypothesis for this study was that depletion of Treg would enhance the antitumor activity of CD8+ T cells in the TME. Thus, we were surprised that increased B cell signature expression prior to therapy best correlated with response. This result was confirmed by using several different B cell signatures. Additionally, there was a correlation between measures of B cell receptor repertoire diversity in the peripheral blood and response to therapy. We assessed multiple indices of diversity in this study, including *species* richness, evenness, and Shannon entropy, an index that combines richness and evenness. A correlation was found with increased abundance and richness of Ig heavy and light chain genes in the peripheral blood, suggesting that B cell population diversity correlated with response. Unfortunately, we were not able to evaluate the TME during therapy to determine if these correlations were also found in the tumor. It is currently not clear what effects anti-PD-1 antibody therapy has on B cell responses and whether these mediate CBR. Activated B cells express PD-1, which limits B cell proliferation and antibody production. Our previous murine data indicated a critical role for antibody generation by B cells in their function after ICI, and it is intriguing to hypothesize that autoantibody generation may be an important part of the immune response to ICI.

Using RNA-seq from tumors prior to therapy, we evaluated if the expression of specific pathways was associated with response to Cy/pembrolizumab. Multiple pathways were associated with CBR including genes associated with caspase activation (apoptosis), the NFAT pathway and C-MYC repression. Granzyme activity, which is upstream of caspase activation and apoptosis and a mechanism of cytotoxicity by NK and T cells, was associated with response. Repression of MYC is associated with the expression of integrins, chemokines and anti-apoptotic proteins that may be critical to the response to Cy/pembrolizumab. Finally, when all tumors including LNs were analyzed, a number of immune relevant pathways were associated with CBR including major histocompatibility complex (MHC) class II antigen expression critical for the activation of CD4+ T cells. However, these analyses were not significant when corrected for multiple testing using an FDR alpha <0.2, but these data may inform future studies.

In summary, we found that low-dose Cy was not effective in depleting Treg prior to anti-PD1 therapy. This combination did not meet the prespecified 1 month increase in PFS that the trial was designed to detect nor
lead to statistically significant decreased peripheral blood T reg. CBR and response were correlated with immune toxicities, which were, in general, modest. Somewhat surprisingly, but in agreement with our preclinical data, we found that CBR was most associated with expression of B cell gene signatures in pretreatment tumor specimens. These data suggest that the B cell response may be important in the immune response to ICI therapy in patients with mTNBC, as previously suggested in preclinical studies. 34

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REFERENCES


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Supplementary Data

Supplementary Figure 1: Study sample collection counts.

Samples listed by data type, optionally further divided by collection time point or clinical attribute.
Supplementary Figure 2: Demonstration of gating strategies for T<sub>reg</sub> flow cytometry analyses.

The first row shows how viable CD45<sup>+</sup> PBMCs were selected from a bulk population (from left to right). The second row demonstrates sorting of CD45<sup>+</sup> PBMCs into CD4<sup>+</sup> and CD8<sup>+</sup> T cells, where CD25<sup>hi</sup>CD127<sup>lo</sup> and FoxP3<sup>+</sup> (2nd row) subsets were subsequently detected within CD4<sup>+</sup> T cells.
Supplementary Figure 3: Tumor differential gene expression, by response to therapy.

Readable gene names and group averages, to accompany Fig 3C (n=31).
Supplementary Figure 4: Tumor mutation burden and diversity metrics

A-B: TMB did not significantly differ between groups by clinical benefit (A) or response (B) by either stratification (low < 10 mut/Mb, high >=10 mut/Mb; or low: <5 mut/Mb, intermediate: 5-20 mut/Mb, high: >=20 mut/Mb; Wilcoxon Rank Sum p > 0.29). C-D: Pre-treatment PBMC-derived T-cell beta chain repertoire similarity (n=29) was lower (p<0.05) in intra-group comparisons by either future clinical benefit (C) or response (D). E-F: T-cell beta chain repertoire similarity (n=15) was higher comparing pre and post treatment samples in patients with either clinical benefit (E) or response (F). G-J: Higher IgH chain abundance and richness was associated (p<0.05) with both clinical benefit (G, I) and response (H, J) in tumor RNA-Seq (n=31).
Supplementary Figure 5: Gene set enrichment

No gene sets were significant by FDR testing in all tumor samples (n=31) by either clinical benefit (A) or response (B). Note the p-value scales have been adjusted to make labels visible in this graphic.
Supplementary Figure 6: Tumor RNASeq Subset Analysis.

Results from a subset of tumor RNASeq samples (n=22). A: Gene set enrichment, B: Immune gene signatures, demonstrating overexpression in B-cell receptor pathways and B-cell signatures in patients without progression of disease.
Supplemental Figure 7: Immune gene features in non-nodal tumor samples

A-B: Immune gene signatures associated (p<0.05) with clinical benefit (CR+PR+SD; A) and response (CR+PR; B) were enhanced after excluding nodal tissue samples.
**Supplementary Figure 8: Imputed tumor immune cell mixture.**

Samples sorted by response status. There were no significant differences in imputed immune cell fractions by response or clinical benefit (two-tailed T-test).
Supplementary Figure 9: CyTOF of peripheral plasmablasts.
Patients whose metastatic TNBC responded to a priming dose of cyclophosphamide prior to pembrolizumab exhibit an increased frequency of plasmablasts in the peripheral blood early during treatment (cycles 1-2). A: Gating strategy used to eliminate metal-spiked beads, doublets, dead cell debris, CD45- cells and CD45+ monocytes. B: The gating strategy described in (A) was further used to identify additional hematopoietic subpopulations of interest, including plasmablasts [HLA-DR (Nd134Di), CD20 (Sm147Di), CD19 (ND124Di), IgD (Eu153Di), and CD27 (Gd158Di)], of peripheral blood plasmablasts in non-responders and responders across early (Cycle 1-2, 6 non-responders, 5 responders), middle (Cycle 3-5, 7 non-responders, 10 responders), or late (Cycle 6+, 2 non-responders, 10 responders) timepoints. An unpaired Student’s t-test was used to determine statistical significance, p = 0.0024. C: Heat map assessment of PD-1, OX40L and TIM-3 expression on IgD- CD217- plasmablasts as gated in B
Supplementary Figure 10: CyTOF of peripheral T-cell subsets
A: Gating strategy used to eliminate metal-spiked beads, doublets, dead cell debris, CD45- cells, CD20+ / CD14+ monocytes, CD3- and CD4-/CD8- cells. B: The gating strategy described in (A) was further used to identify CD4+ and CD8+ T-cell subsets, shown by treatment timepoint [early (Cycle 1-2, 6 non-responders, 5 responders), middle (Cycle 3-5, 7 non-responders, 10 responders), or late (Cycle 6+, 2 non-responders, 10 responders)] and by expression for early (Cycles 1-2) and middle (Cycles 3-5) timepoints.