A Phase II Clinical Trial of Nivolumab and Temozolomide for Neuroendocrine Neoplasms

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ABSTRACT

Purpose: Treatment options are limited in patients with metastatic neuroendocrine neoplasms (NEN). We present the results for a phase II trial of combination nivolumab and temozolomide in patients with advanced NEN along with results of immune changes in peripheral blood.

Patients and Methods: NCT03728361 is a nonrandomized, phase II study of nivolumab and temozolomide in patients with NEN. The primary endpoint was response rate using RECIST 1.1. Secondary endpoints included progression-free survival (PFS), overall survival (OS), and safety. Immune profiling was performed by mass cytometry to evaluate the effect on peripheral blood immune cell subsets.

Results: Among all 28 patients with NEN, the confirmed response rate was 9/28 [32.1%, 95% confidence interval (CI): 15.9–52.4]. Of 11 patients with lung NEN, the response rate was 64% ($n = 7$); there was a significant difference in responses by primary tumor location (lung vs. others, $P = 0.020$). The median PFS was 8.8 months (95% CI: 3.9–11.1 months), and median OS was 32.3 months (95% CI: 20.7—not reached months). Exploratory blood immune cell profiling revealed an increase in circulating $CD8^+$ T cells (27.9% ± 13.4% vs. 31.7% ± 14.6%, $P = 0.03$) and a decrease in $CD4^+$ T cells (59.6% ± 13.1% vs. 56.5% ± 13.0%, $P = 0.001$) after 2 weeks of treatment. LAG-3–expressing total T cells were lower in patients experiencing a partial response (0.18% ± 0.24% vs. 0.83% ± 0.55%, $P = 0.028$). Myeloid-derived suppressor cell levels increased during the study and did not correlate with response.

Conclusions: Combination nivolumab and temozolomide demonstrated promising activity in NEN.

See related commentary by Velez and Garon, p. 691

Introduction

Neuroendocrine neoplasms (NEN) consist of a diverse group of tumors including low-grade gastroenteropancreatic neuroendocrine tumors (GEP-NET), thymic and lung NET as well as the aggressive, rapidly growing neuroendocrine carcinomas (NEC; ref. 1). NEN can arise as primary tumors in almost any organ system of the body and Ki-67 index is often used to classify NETs into three grades per the World Health Organization (WHO) classification (2). Current treatment options are limited and palliative in nature for patients with all types of metastatic NEN. Systemic therapy with somatostatin analogs (SSA) has been shown to provide control of carcinoid syndrome, improve quality of life as well as progression-free survival (PFS) in patients with NEN; however, tumor response rates are low (3–5). While peptide receptor radionuclide therapy has shown encouraging efficacy in patients with midgut NET [objective response rate (ORR): 18% in combination with octreotide vs. 3% in patients who received octreotide alone], its use is limited to patients with somatostatin-receptor positive, well-differentiated GEP-NET with adequate renal function (6). For patients with metastatic high-grade NEC, there is no confirmed standard therapy, although platinum doublet chemotherapy is often utilized (7). These patients are typically not included in most clinical trials and these tumors are generally somatostatin-receptor negative, making treatment particularly challenging.

Temozolomide (TMZ) is an oral alkylating agent that has been shown to be effective in patients with glioma and melanoma (8, 9). TMZ has been studied in pancreatic NENs in combination with thalidomide (10) and bevacizumab (11) but was associated with significant toxicity, including lymphopenia and serious opportunistic infections in 10% of patients in the thalidomide trial (10). The synergistic activity of 5-fluorouracil and TMZ led to the development of TMZ given in combination with capecitabine (CAPTEM; refs. 12, 13). Synergistic activity was observed to be schedule dependent, requiring TMZ to be given after continuous exposure to CAPTEM (13). Studies have shown a high response rate especially in pancreatic NET of up to 70% (14). CAPTEM is utilized for both pancreatic and non-pancreatic NET (15), but seems to be less efficacious in high-grade tumors including NEC (16–18). One study found that CAPTEM treatment was associated with poorer outcomes for patients with Ki-67% > 5% (19) although another study found that patients with Ki-67% of up to 40% benefited from treatment (20). TMZ has been demonstrated to have immunomodulatory effects on lymphoid cells in patients with melanoma (21, 22) including decreased

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Clin Cancer Res 2023;29:731–41

doi: 10.1158/1078-0432.CCR-22-1552

Translational Relevance

In a phase II trial of combination nivolumab and temozolomide in patients with advanced neuroendocrine tumors (NET) and carcinomas (NEC), we observed a response rate of 32%, including a 64% response rate in patients with lung neuroendocrine neoplasms. Responses were observed in patients with both NET and NEC, but confirmed responses occurred only in patients with lung and pancreatic tumors. Exploratory immune cell profiling revealed an increase in circulating $CD8^+$ T cells and a decrease in $CD4^+$ T cells during treatment. LAG-3–expressing total T cells were lower in patients experiencing a partial response.

$CD4^+$ and regulatory T cell (Treg) lymphocytes, increased $CD8^+$ T cells, and increased T-cell responses against common viral epitopes (23). Although the activity of single-agent immunotherapy has been disappointing in NET, recently combination PD-1 and CTLA-4 inhibition has shown activity in subsets of patients with NET including those with high-grade NEN (response rate of 26%) and lung NET (response rate of 27%; refs. 24, 25). Given the immunomodulatory effects of TMZ, as well as its activity as a single agent and in combination therapies, we conducted a phase II study of combination nivolumab and TMZ in patients with NEN, including an exploratory analysis of immune cell subset changes in the peripheral blood at baseline and during treatment.

Patients and Methods

We conducted a nonrandomized, phase II, multi-cohort, single-center trial of combination nivolumab and TMZ in advanced NENs and recurrent and/or refractory small cell lung cancer (SCLC; NCT03728361, Sponsor: Ohio State University). Here we report results from the neuroendocrine cohort. Eligible patients had metastatic NEN of any WHO grade or primary location, any line of therapy, regardless of PD-L1 expression or histologic differentiation (NET and NEC), with evidence of clinical or biochemical or radiographic progression in the 12 months prior to study registration. The 2019 WHO classification of tumors of the digestive system and the 2021 WHO classification of lung tumors were utilized (26, 27). Patients with SCLC were excluded from this cohort. Prior immunotherapy was not permitted. Patients with brain metastases were permitted if asymptomatic, and previously treated brain metastases were permitted if stable on repeat imaging without the need for increasing dose of steroids. Patients received nivolumab (480 mg as a 30-minute infusion every 4 weeks) and TMZ (150 mg/m^2 orally daily on days 1–5 of a 28-day cycle) until disease progression or unacceptable toxicity. Patients underwent imaging for tumor response every 8 weeks for 12 months, then every 12 weeks thereafter. Treatment with study drug combination therapy was continued until documented disease progression or unacceptable adverse events (AE). Investigators were permitted to discontinue TMZ if 2 years of treatment had passed without evidence of cancer progression. The primary objective was efficacy of nivolumab in combination with TMZ as measured by ORR using RECIST 1.1 (28). Secondary endpoints included PFS—defined as the time from allocation to the first documented disease progression according to RECIST 1.1 or death due to any cause, whichever occurs first—and overall survival (OS) in subjects treated with combination nivolumab and TMZ. Secondary objectives also included safety and tolerability as assessed by CTCAE v5.0. Exploratory objectives included the evaluation of the effect of combination treatment on immunomodulation of T, B, natural killer (NK) cell, and myeloid-derived suppressor cell (MDSC) subsets by mass cytometry analysis of blood. Microsatellite stability (MSS) and tumor mutational burden (TMB) were reported when available from standard-of-care commercial testing: Foundation One CDx $n = 17$; Caris Life Sciences $n = 1$; Guardant360 $n = 1$. This study was reviewed and approved by the OSU Cancer Institutional Review Board (IRB#2018C0149) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was provided by all patients.

Immune cell profiling

Peripheral blood mononuclear cells (PBMC) were collected at screening (baseline) and cycle 1, day 15 (C1D15) of study treatment and analyzed via mass cytometry as described previously (29, 30). Antibodies were purchased and labeled using a custom 37 marker Maxpar Direct Immune Profiling System labeling kit (Fluidigm) summarized in Supplementary Table S1. In brief, cryopreserved PBMCs were Fc receptor blocked with Human TruStain FcX (BioLegend) in cell staining buffer (CSB, Fluidigm) at a concentration of 6×10^7 cells/mL. Cells were then stained and fixed in a fresh 1.6% formaldehyde solution. Next, PBMCs were resuspended in 125 nmol/L Cell-ID Intercalator-Ir (Fluidigm). Following an overnight incubation at 4°C, fixed PBMCs were washed twice with CSB and twice with Maxpar Cell Acquisition Solution (CAS) to remove excess intercalator and resuspended at a final concentration of 1×10^6 cells/mL in CAS containing 0.1X EQ Four Element Calibration beads (Fluidigm). Samples were acquired in CAS containing 0.1X EQ beads on a Helios system with a wide-bore injector, utilizing CyTOF Software version 6.7.1016 and using the Maxpar Direct Immune Profiling Assay template. A total of 500,000 PBMC events were acquired per file at an acquisition rate of 250–450 events/second. Data were normalized using the CyTOF Software v.6.7.1016. Normalized FCS files were gated using Cytobank to establish immune cell populations summarized in Supplementary Table S2.

T-cell proliferation assay

PBMCs at screening and C1D15 of nivolumab and TMZ treatment were labeled with carboxyfluorescein succinimidyl ester (CFSE, Life Technologies) in 0.1% BSA for 20 minutes in a cell culture water bath at 34°C. Following PBMC staining, cells were washed, nonspecifically activated with anti-CD3/CD28 beads (Life Technologies) and cultured for 72 hours. After 3 days, T-cell proliferation was assessed by flow cytometry. APC anti-CD4 and PE-Cy7 anti-CD8 antibodies were used to identify T-cell subsets (BioLegend).

Statistical analysis

ORR for primary analysis was defined as the number of patients achieving a partial or complete response divided by the total number of evaluable patients. For subgroup and correlative analyses, best response included both confirmed and unconfirmed responses. This phase II design required a minimum of 15 and a maximum of 28 evaluable patients using Simon minimax two-stage design with 80% power and a one-sided significance level of 0.05, assuming ORR of 15% or less was not of interest (null hypothesis), and an ORR of 35% or more was considered promising. ORR was calculated with Clopper–Pearson (exact) 95% confidence interval (CI) and compared between groups using Fisher exact test. Summary statistics were calculated for patient demographics and clinical characteristics. Toxicities were also summarized by grade using frequency and percentage. Survival curves (PFS and OS) were estimated using Kaplan–Meier method with median and 95% CI. The

survival curves compared using log-rank test. OS was defined from the start of treatment to death. Patients alive were censored at the last follow-up. PFS was defined from the start of treatment to progression or death, whichever occurred first. Patients without progression or death were censored at the last follow-up. Biomarker levels have been summarized at screening and C1D15, and compared using the paired *t* test. Biomarker levels at screening were also compared between responders and nonresponders using two-sample *t* test. Statistical significance was concluded at $P < 0.05$. *P* values for the *post hoc* and correlative analyses were not adjusted for the multiple comparisons or multiple outcomes considering these findings are exploratory and hypothesis generating. These analyses were conducted in SAS version 9.4 (SAS Institute).

Table 1. Patient characteristics.

Patient characteristics	*N*	%
Total no. of patients	28	100%
Median age (range) years	62 (33–78)	
Gender		
Male	13	46%
Female	15	54%
Ethnicity		
Caucasian	25	89%
African American	3	11%
Ki-67%		
Ki-67 < 3%	3	11%
Ki-67 3%–20%	16	57%
Ki-67 > 20%	9	32%
Ki-67 > 55%	5	18%
Tumor differentiation		
Neuroendocrine tumor (NET)	20	71%
Neuroendocrine carcinoma (NEC)	8	29%
Primary tumor location		
Pancreas	3	11%
Lung	11	39%
Typical	2	
Atypical	7	
Other/unknown	2	
Colorectal	4	14%
Small bowel	6	21%
Ampullary	1	4%
Head and neck	1	4%
Unknown	2	7%
Line of systemic therapy[a]		
First	13	46%
Second	12	43%
More than two	3	11%
Type of prior systemic therapy		
Platinum-based chemotherapy	9	32%
Everolimus	3	11%
Clinical trial	4	14%
Peptide receptor radionuclide therapy	2	7%
Tumor PD-L1 expression		
≥1%	2	7%
≥50%	0	0%
Negative	12	43%
Unknown	14	50%
Tumor microsatellite stability		
Microsatellite stable[b]	19	68%
Unknown	9	32%

[a]Excluding somatostatin receptor therapy, which was required for eligibility.
[b]Determined from commercial standard-of-care tumor ($n = 17$) or blood ($n = 1$) DNA/ctDNA testing.

Data availability

The data generated in this study are not publicly available due to information that could compromise patient privacy but are available upon reasonable request from the corresponding author.

Results

Patient characteristics

Demographics, primary tumor location and WHO grade, and treatment history of the 28 patients with NEN accrued to this study are detailed in **Table 1**. All patients had clinical progression prior to therapy, and all but 1 patient had RECIST radiographic progression within 12 months of study entry; 1 patient had 11% growth of a bowel mass with worsening symptoms and so prior therapy was discontinued and patient was referred for clinical trial. The median age was 62, 54% ($n = 15$) were female, and the most common primary tumor location was lung ($n = 11$, 39%), followed by small bowel ($n = 6$, 21%), colorectal ($n = 4$, 14%), and pancreas ($n = 3$, 11%). The majority of patients had Ki67% between 3% and 20%; 9 patients had Ki67% greater than 20% (range, 30%–90%). Most patients had NET (20/28, 71%) while 8 (29%) patients had NEC. Over half of patients were treated in second or third line of therapy ($n = 15$, 54%; not including SSA therapy). Of the 11 patients with lung NEN, 7 patients had diagnosis of atypical carcinoid, 2 patients had typical carcinoid, 1 patient had NEC, and 1 patient was unknown. In 19 patients tested for MSS, all 19 patients were MSS. Eighteen patients were tested for TMB, with 1 indeterminate result, with only 1 reported as > 10 mut/Mb (16 mut/Mb per Foundation testing), and 16 reported as < 10 mut/Mb.

Efficacy

Among all 28 patients with NEN, the confirmed response rate was 9/28 (32.1%, 95% CI: 15.9–52.4). The confirmed and unconfirmed response rate was 10/28 (35.7%, 95% CI: 18.6–55.9), including 10 patients (36%) who experienced a partial response (PR), 16 (57%) with stable disease (SD), and 2 (7%) with progressive disease (PD) as best response (**Table 2**; **Fig. 1**). The disease control rate (PR + SD) was 93%. Responses occurred in both NET (7/20, 35%) and NEC (3/8, 38%) but all confirmed responses occurred in patients with lung and pancreas primary tumors (Supplementary Fig. S1). Of the 11 patients with lung

Table 2. Response rate in patients with NEN.

Variable	Level	No response ($n = 18$)	Response ($n = 10$)	*P*
Primary location	Lung	4 (36%)	7 (64%)	0.004
	Pancreas	1 (33%)	2 (67%)	
	Others	13 (93%)	1 (7%)	
Lung NEN vs. others	Lung	4 (36%)	7 (64%)	0.020
	Others	14 (82%)	3 (18%)	
Line of therapy	>1	9 (60%)	6 (40%)	0.706
	1	9 (69%)	4 (31%)	
Differentiation	NEC	5 (62%)	3 (38%)	1
	NET	13 (65%)	7 (35%)	
Ki-67	<3	3 (100%)	0 (0%)	0.542
	3–20	10 (62%)	6 (38%)	
	>20	5 (56%)	4 (44%)	

Note: Confirmed response rate was 9/28 (32.1%, 95% CI: 15.9–52.4). Confirmed and unconfirmed response rate for the entire cohort was 10/28 (35.7%, 95% CI: 18.6–55.9). ORR was significantly higher in the patients with the primary location of lung (7/11, 64%) compared with the others (3/17, 18%, $P = 0.02$).

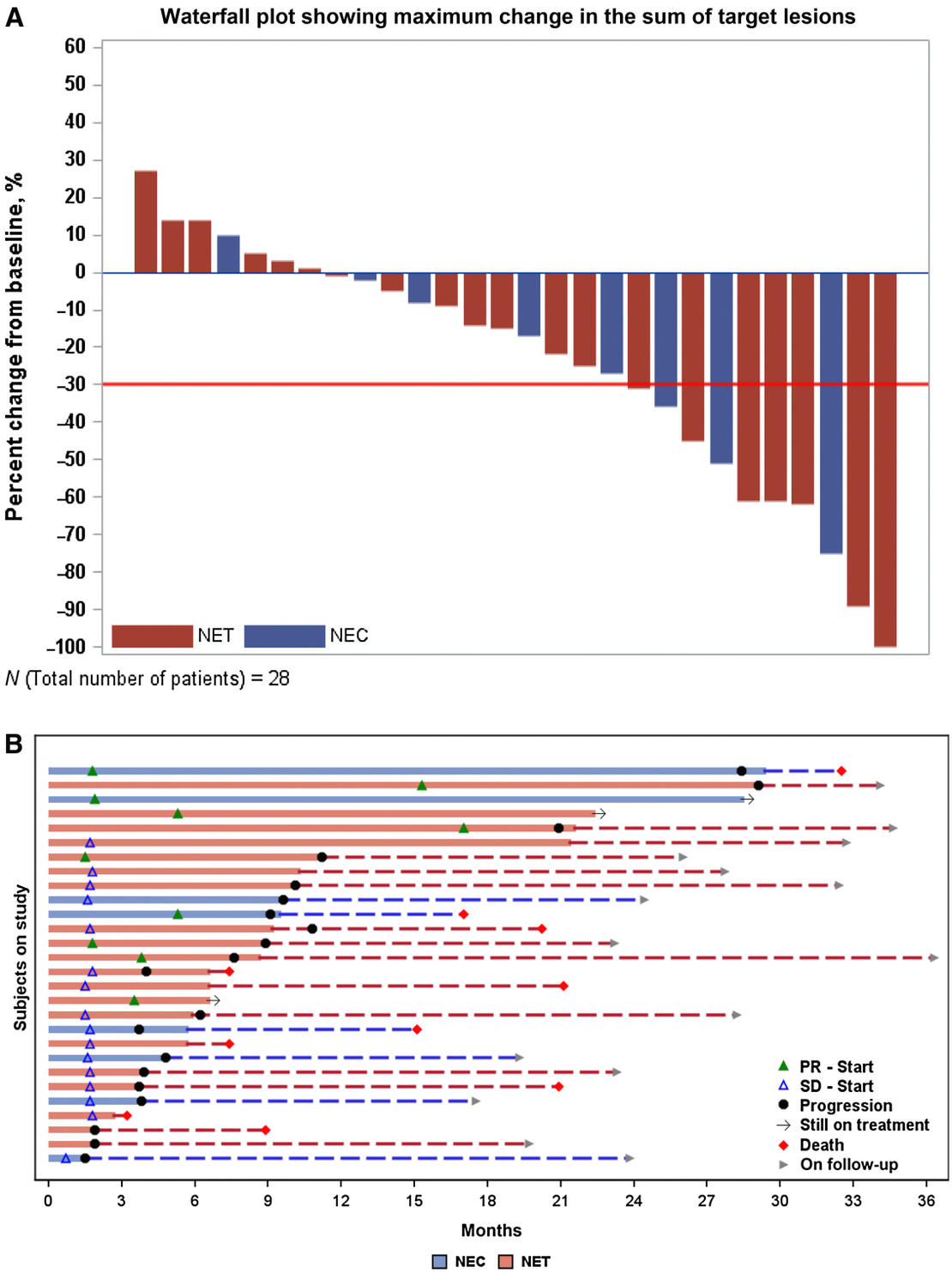

Figure 1.
Waterfall (**A**) and swimmer plots (**B**) of best response. In the swimmer plot, continuous line indicates treatment on study and discontinuous line after study survival.

tumors, the response rate was 64% (7 responses out of 11); the response rate was 67% (2/3) for patients with pancreatic primary. There was a significant difference in responses seen by primary tumor location (lung vs. others, $P = 0.020$). The response rate in patients with Ki-67 3%–20% was 38% (6/16) and was 44% (4/9) in patients with Ki-67 > 20%; no responses were seen in patients with Ki-67 < 3%. There was no

Table 3. Progression-free survival (PFS).

		N	Median (months)	95% CI	*P*
Entire cohort		28	8.8	3.9–11.1	
Primary location	Lung	11	11.1	3.0–29.0	0.210
	Others	17	7.2	3.7–10.0	
	Pancreas	3	28.3	3.8–28.3	0.752
	Others	25	8.8	3.9–11.1	
Line of therapy	>1	15	9.0	3.0–20.8	0.586
	1	13	8.8	3.8–20.8	
Differentiation	NEC	8	6.9	1.4–28.3	0.582
	NET	20	8.8	3.8–20.8	
Ki-67%	<3%	3	10.0	3.8–NR	0.617
	3%–20%	16	7.5	3.6–20.8	
	>20%	9	8.8	1.4–28.3	

Note: PFS is not significantly associated with primary location, line of therapy, and differentiation type.

difference in response in patients treated as first line versus beyond first line excluding SSAs therapy which was required for eligibility, (response rates 31% and 40%, respectively, $P = 0.706$).

The median PFS of the entire cohort was 8.8 months (95% CI: 3.9–11.1 months; **Table 3**; **Fig. 2**). PFS was not significantly associated with primary tumor location, line of therapy, tumor differentiation, or Ki-67% index. The median PFS for patients with lung primaries was 11.1 months (95% CI: 3.0–29.0 months) which was not significantly different compared with all others (7.2 months; 95% CI: 3.7–10.7; $P = 0.210$). There was no difference in PFS between patients with atypical versus typical lung carcinoid ($P = 0.279$). Patients with pancreatic primary NET had median PFS of 28.3 months (95% CI: 3.8–28.3); however, this was not statistically different than non-pancreatic NET (8.8 months; 95% CI: 3.9–11.1 months; $P = 0.480$).

The overall survival for the entire cohort was 32.3 months [95% CI: 20.7–NR (not reached) months]. OS was not significantly associated with primary location, line of therapy, tumor differentiation or Ki-67% (**Table 4**; **Fig. 2**). The OS for patients with lung NET was NR (95% CI: 8.8–NR) compared with 32.3 months for non-lung NET (95% CI: 19.9–NR, $P = 0.602$). There was no difference in OS in patients with atypical versus typical lung carcinoid ($P = 0.260$).

Safety

The most frequent treatment-related AEs (TRAE) of any grade were fatigue (61%), nausea (46%), and thrombocytopenia, anemia, and lymphocytopenia (46% each; Supplementary Table S1). The most frequent grade 3 or 4 TRAE included neutropenia and thrombocytopenia (14% each), and decreased white blood cell and lymphocyte count and (11% each). Treatment-related SAE occurred in 7 patients (Supplementary Table S2). No treatment-related deaths were observed. After the first 13 patients were accrued and treated for at least one cycle at TMZ 200 mg/m^2, it was noted that 4 of these patients incurred the following AEs: grade 3/4 neutropenia ($n = 3$ patients) and grade 3/4 thrombocytopenia ($n = 4$ patients). Only one of these toxicities lasted longer than 1 week (grade 4 neutropenia). None of these patients required hospitalization and no patient had either neutropenic fever or major bleeding. Three of these 4 patients had bone metastases at the time of treatment, and 2 had received prior radiation to bone. All 4 patients required delay of cycle #2 by 2 weeks, and were dose reduced to 150 mg/m^2 as per protocol. Given the historical rates of 10%–16% for grade 3 or 4 thrombocytopenia and 5%–8% grade 3 or 4 neutropenia with TMZ alone in SCLC (31, 32), an amendment was made to start TMZ at 150 mg/m^2 for all patients with resultant improvement in tolerability, as has been done in other combination studies in SCLC (33).

Peripheral immune cell landscape in patients with advanced NET treated with nivolumab and TMZ

Profiling of peripheral immune cell subsets at screening and C1D15 of nivolumab and TMZ treatment revealed changes within the T-cell landscape. Total PBMCs were analyzed by mass cytometry using a panel of 37 cell surface markers (Supplementary Table S3) that permitted the identification of 35 circulating immune cell populations within the CD45$^+$ cell fraction of cryopreserved cells (Supplementary Table S4). CD45$^+$ immune cells were identified in an unbiased manner using viSNE, a visualization tool for high-dimensional single-cell data based on the t-distributed stochastic neighbor embedding (t-SNE; **Fig. 3A** and **B**). Evaluation of the effects of treatment with nivolumab and TMZ compared with screening revealed shifts in circulating immune cell populations (**Fig. 3C**). This included a significant decrease in CD4$^+$ T cells (59.6% $\pm$ 13.1% vs. 56.5% $\pm$ 13.0%, $P = 0.001$) and significant increase in CD8$^+$ T cells (27.9% $\pm$ 13.4% vs. 31.7% $\pm$ 14.6%, $P = 0.03$) from screening to C1D15 within the entire cohort (**Fig. 3D**). Tregs generally increased with study therapy compared with screening (Supplementary Fig. S2A). Finally, the peripheral immune cell landscape was evaluated for predictive markers of response to PD-l blockade. Levels of LAG-3–expressing total T cells at screening were significantly lower (0.18% $\pm$ 0.24% vs. 0.83% $\pm$ 0.55%, $P = 0.028$) in patients that experienced a PR ($n = 5$) compared with patients that experienced a non-PR (SD or PD, $n = 9$; **Fig. 3E**).

Next, CD4$^+$ and CD8$^+$ T cells were further differentiated into subsets based on the expression of CD45RA and CCR7; naïve T cells (CD45RA$^+$ CCR7$^+$), central memory T cells (CD45RA$^-$ CCR7$^+$), effector memory T cells (CD45RA$^-$ CCR7$^-$), and terminal effector memory T cells (CD45RA$^+$ CCR7$^-$) and compared between screening and C1D15 of nivolumab and TMZ treatment (Supplementary Figs. S2B and S3A). Furthermore, the expression of coinhibitory molecules PD-1, LAG-3, TIM3, and KLRG1 were evaluated at screening and C1D15 of the study treatment regimen in both CD4$^+$ and CD8$^+$ T-cell subsets. Expression of PD-1 significantly decreased, while levels of LAG-3, TIM3, and KLRG1 increased at C1D15 of nivolumab and TMZ treatment compared with screening in both T-cell subsets within the entire cohort (Supplementary Figs. S2C and S3B). Next, patients were stratified as experiencing a PR (screening $n = 5$, C1D15 $n = 3$) or a non-PR (SD or PD, screening $n = 9$, C1D15 $n = 7$) and levels of PD-1–, LAG-3–, TIM3-, and KLRG1-expressing CD4$^+$ and CD8$^+$ T cells were evaluated at screening and C1D15 of nivolumab and TMZ treatment. Overall, no differences were observed in PD-1–, TIM3-, and KLRG1-expressing T cells between patients experiencing a PR or non-PR at screening and baseline (Supplementary Fig. S4A, S4C, and S4D). Patients with a higher percent of LAG-3–expressing CD8$^+$ and CD4$^+$ T cells at screening had less propensity to respond to PD-1 blockade ($P = 0.08$). Furthermore, levels of CD8$^+$ LAG-3–expressing T cells were increased in patients that experienced a PR versus non-PR ($P = 0.05$) at C1D15 (Supplementary Fig. S4B). However, screening levels of LAG-3–expressing CD4$^+$ and CD8$^+$ T cells did not correlate with PFS at 6 months ($P = 0.161$ and 0.317, respectively) or with OS at 12 months ($P = 0.186$ and $P = 0.586$, respectively).

MDSC levels correlate with tumor burden and prognosis in several different types of cancer and are affected by treatment with TMZ in preclinical models (34). Therefore, peripheral circulating populations of MDSCs (defined as lineage-negative, CD11b$^+$, CD33$^+$, and

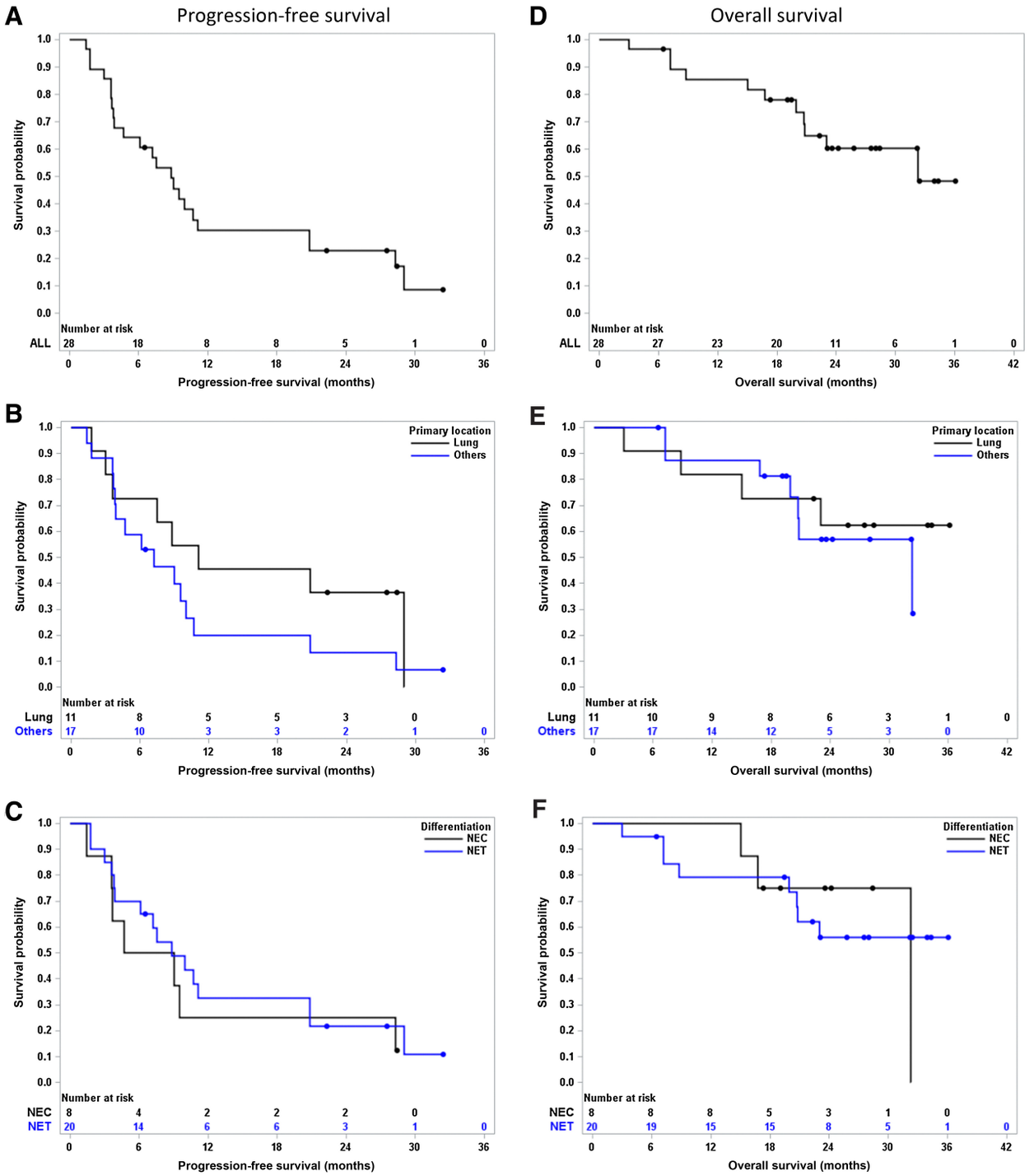

Figure 2.

Progression-free survival (PFS) and overall survival (OS) for patients with NEN treated with nivolumab and TMZ. The median PFS was 8.8 months (95% CI: 3.9–11.1 months) for all patients (**A**) and was not significantly associated with primary tumor location (lung vs. others; **B**) or tumor differentiation (**C**). The OS for the entire cohort was 32.3 months (95% CI: 20.7–NR months; **D**). OS was not significantly associated with primary location (**E**) or tumor differentiation (**F**).

HLA-DR$^{low/neg}$; Supplementary Table S2) were evaluated. Overall, MDSC levels increased at C1D15 of study treatment compared with screening (Supplementary Fig. S5A). Total MDSC were also evaluated by monocytic (M-MDSC; CD14^{+}) and granulocytic (G-MDSC; CD66b^{+}) subsets. M-MDSC decreased and G-MDSC increased following the study treatment regimen compared with screening

Table 4. Overall survival (OS) in patients with NEN treated with nivolumab and TMZ.

		N	Median (months)	95% CI	*P*
Entire cohort		28	32.3	20.7–NR	
Primary location	Lung	11	NR	8.8–NR	0.602
	Others	17	32.3	19.9–NR	
	Pancreas	3	32.3	NR–NR	0.832
	Others	25	NR	19.9–NR	
Line of therapy	>1	15	32.3	15.0–NR	0.815
	1	13	NR	19.9–NR	
Differentiation	NEC	8	32.3	15.0–32.3	0.950
	NET	20	NR	19.9–NR	
Ki-67%	<3%	3	NR	NR–NR	0.353
	3%–20%	16	NR	8.8–NR	
	>20%	9	32.3	16.8–32.3	

Note: OS was not significantly associated with primary location, line of therapy, and tumor differentiation.

(Supplementary Fig. S5B). Baseline MDSC levels did not correlate with best clinical response (Supplementary Fig. S5C).

Nivolumab in combination with TMZ leads to improved T-cell proliferation

Given the immunomodulatory changes within the T-cell compartment following nivolumab and TMZ treatment, it was hypothesized that T-cell function would improve following the combination regimen. The total PBMC population from study patients at screening and C1D15 of treatment were labeled with CFSE and stimulated with anti-CD3/CD28 beads to induce T-cell proliferation. Following 72 hours in culture, the proliferation of $CD4^+$ and $CD8^+$ T-cell populations was measured by flow cytometry. Both T-cell populations demonstrated an increase in proliferation from screening compared to C1D15 of nivolumab and TMZ treatment ($CD4^+$, 34.7% ± 28.9% vs. 46.2% ± 29.2%, $P = 0.055$; $CD8^+$, 32.1% ± 26.6% vs. 45.6% ± 24.2%, $P = 0.087$), but this result did not reach statistical significance (**Fig. 4A** and **B**).

Discussion

Combination nivolumab and TMZ demonstrated broad and promising activity in patients with NEN regardless of tumor differentiation, Ki-67 or line of therapy, with especially high response rates observed in lung NEN. Responses occurred in foregut tumors but not in midgut or hindgut NEN. The toxicity profile observed is similar to that seen with combination immunotherapy and chemotherapy strategies (35).

Few prospective studies have evaluated outcomes for patients with lung NEN treated with TMZ. One of the largest retrospective studies published recently by Al-Toubah and colleagues included 33 patients with lung NEN treated with combination CAPTEM and TMZ and reported 42% (14/33) of patients had decrease in tumors with median PFS of 12 months (95% CI: 5.5–18.5) and median OS of 22 months from treatment initiation (95% CI: 13.9–30.1; ref. 36). The proportion of and outcomes for patients with typical or atypical carcinoid and lung NEC was not available, and importantly the study only assessed decreased tumor size and not standard RECIST. In addition, almost a third (29%) of patients received no prior therapy. In an earlier publication from the same group with a smaller study population, 20 patients with lung NEN of which 14 (70%) were typical carcinoid, a RECIST response rate of 30% (6/20) was observed with median PFS of 13 months (95% CI: 4.4–21.6), and median OS of 68 months from time of first diagnosis (95% CI: 35.3–100.7; ref. 37). However, OS was measured from time of first diagnosis and survival time from starting treatment was not presented. In a *post hoc* analysis from the RADIANT-4 trial which evaluated everolimus in patients with advanced, progressive, well-differentiated (grade 1 or grade 2), non-functional lung or NET, the median PFS by central review for patients with lung NET was 9.2 months (95% CI: 6.8–10.9; ref. 38). Overall, the RADIANT-4 study reported a 2% response rate ($n = 4/184$) and RECIST response was not separately reported for lung NEN (39).

Immunotherapy in patients with NET and NEC has overall shown limited efficacy. A trial using the anti-PD-1 inhibitor spartalizumab demonstrated an overall response rate of 7.4% (95% CI: 3.0–14.6) in patients with NET and an ORR of 4.8% (95% CI: 0.1–23.8) in patients with GEP-NEC; the response rate in lung NET was 16.7% (40). Most recently combination PD-1 and CTLA-4 inhibition therapy demonstrated a response rate of 3 of 11 (27%) patients with lung NEN (all three responses were in patients with atypical carcinoid); however, PFS and OS were not reported separately for these patients (24). In our study, none of the patients with lung NEN experienced PD as best response, and the response rate (64%) and median OS (NR) are among the highest reported to date.

The dynamics of the peripheral immune system are recognized for the ability to correlate with clinical responses in patients with cancer (41, 42) and to provide an immune cell profiling of the peripheral blood signature for response to immune checkpoint therapy in patients with cancer treated with immune checkpoint inhibitors (43). Prior work in NEN has shown that in pancreatic NETs (pNET) $CD3^+$ and $CD4^+$ T-cell and NK-cell percentages in the peripheral blood may reflect the status of distant metastasis and the percentage of peripheral B cells may predict the progression of patients with pNET with or without distant metastasis (44). Furthermore, in gastroenteropancreatic neuroendocrine neoplasms high circulating M-MDSC levels were associated with significantly increased metastases (45).

In this study, comprehensive immune cell profiling of PBMCs via CyTOF was utilized to correlate immune cell populations with clinical response to nivolumab and TMZ therapy in patients with NETs. An increase in circulating $CD8^+$ T cells and decrease in circulating $CD4^+$ T cells was observed in the entire patient cohort with nivolumab and TMZ treatment compared with screening. However, the absolute differences were small, the ranges were wide, and many samples were not available for analysis. A recent study reported that circulating $CD8^+$ T cells, specifically $CX3CR1^+$ $CD8^+$ T cells, are predictive of response to PD-1/PD-L1 therapy in non–small cell lung cancer (ref. 46). Similarly, in the current study, peripheral $CD8^+$ T cells were higher in patients with NEN that experienced a response compared with nonresponders at screening (29.64% ± 18.18% vs. 27.52% ± 11.51%). However, this finding was not statistically significant and at C1D15 of nivolumab and TMZ treatment, patients with a PR to study therapy had fewer peripheral $CD8^+$ T cells than non-PR patients (24.71% ± 20.91% vs. 32.09% ± 12.83%). When evaluating the peripheral immune cell landscape for predictive markers of response to PD-l blockade, the only significant difference was LAG-3–expressing total T cells, in which lower levels of LAG-3 expression on total T cells correlated with a better clinical response. Novel combinations are needed to improve the clinical response to immune checkpoint inhibitors and recently the use of a human LAG-3 blocking antibody relatlimab in combination with nivolumab (anti-PD-1) demonstrated a statistically significant PFS benefit compared with nivolumab monotherapy in patients with advanced melanoma (47). Future studies with a larger sample size will continue to evaluate the significance of

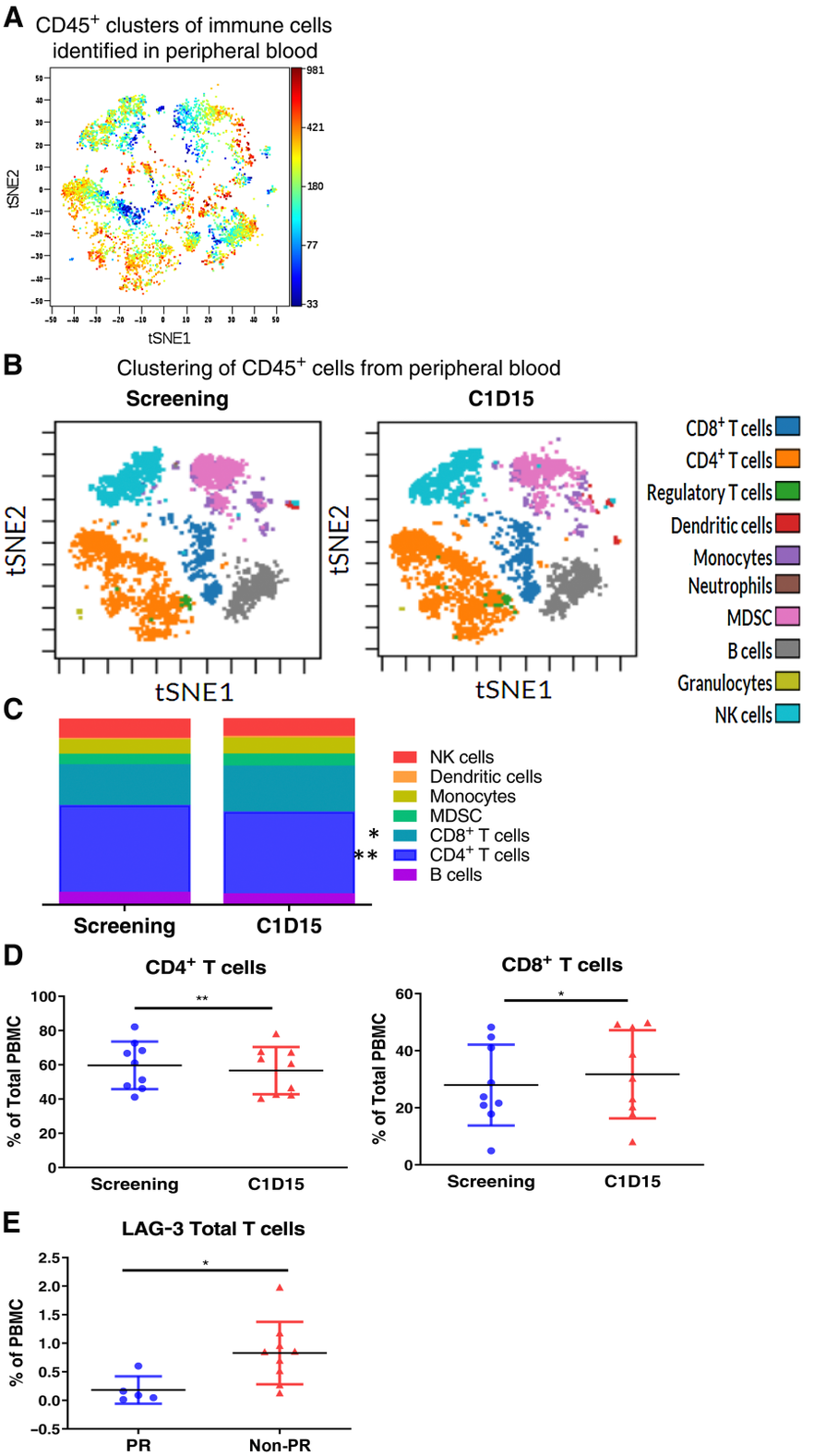

Figure 3.

Peripheral blood immune cell landscape in patients with NETs following treatment with nivolumab and TMZ. Mass cytometry analysis using an immune panel of 37 immune markers analyzed PBMCs from patients at screening and following treatment with nivolumab and TMZ at C1D15 in 9 patients with NETs. **A,** Representative t-SNE plot colored by expression of CD45 in an ungated live, singlet population of PBMCs highlighting the immune cell populations. The live, singlet population was then gated for CD45 positivity to select for immune populations and used to cluster immune cell populations in an unbiased manner from live/CD45$^+$ cells only in t-SNE plots. **B,** Representative t-SNE plots of immune cell population clustering from one patient from study timepoints screening and C1D15. **C,** Bar graph of mean immune cell populations in patients with NETs (n = 9) represents the immune landscape over the duration of the study. **D,** Changes in peripheral CD4$^+$ and CD8$^+$ T-cell populations at C1D15 of nivolumab and TMZ treatment compared with screening in the entire study cohort. **E,** Changes in peripheral LAG-3–expressing total T cells at screening and C1D15 of study treatment in the entire study cohort. Each symbol represents one patient (n = 9). Line indicates mean. *, P <0.05; **, P < 0.01.

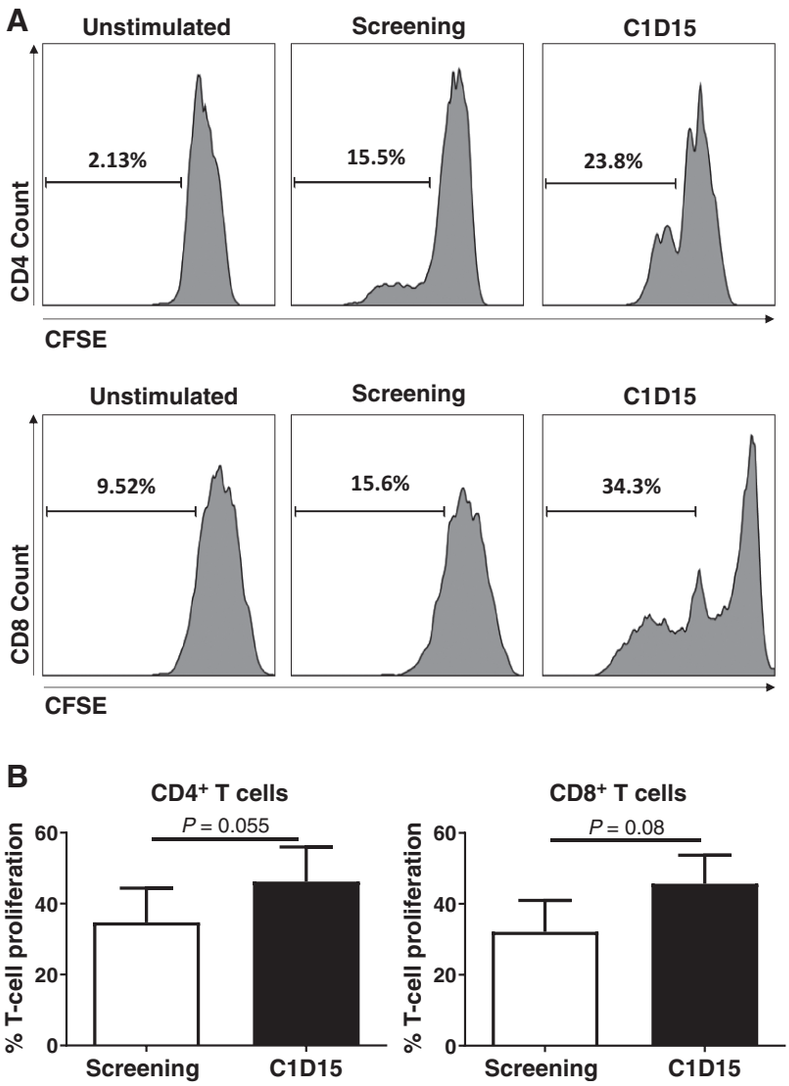

Figure 4.

Nivolumab in combination with TMZ enhances T-cell proliferation. Patient PBMCs were activated with anti-CD3/CD28 beads and labeled with CFSE. After 3 days, cells were collected and stained with anti-CD8 and anti-CD4 antibodies and proliferation was assessed by flow cytometry. **A,** Representative histograms of $CD4^+$ and $CD8^+$ T-cell proliferation in one patient. **B,** Bar graphs display quantification of $CD4^+$ and $CD8^+$ T-cell proliferation from the entire patient cohort ($n = 9$).

circulating $CD8^+$ T cells and the predictive nature of LAG-3–expressing T cells in patients with NEN.

MDSC were also evaluated in this study given that prior work has correlated MDSC levels with tumor burden and prognosis in several different types of cancer (48, 49). We found levels of MDSC were increased with nivolumab and TMZ therapy compared with screening and did not correlate with clinical response. It is not clear what mechanisms contribute to this increase; however, the effect has been previously reported in another study utilizing PD-1 blockade (50). One hypothesis is that circulating levels of MDSC do not mirror levels found within the tumor microenvironment (51). MDSC exert their immunosuppressive effects through multiple mechanisms, including the suppression of T cells. Despite the increase in total MDSC, T-cell function improved at C1D15 of the study regimen compared with screening in both $CD4^+$ and $CD8^+$ T-cell populations, suggesting that MDSC may be less functional, yet a direct assessment of MDSC suppression on T-cell function is needed to confirm this hypothesis.

In summary, the combination of TMZ and nivolumab showed promising activity in NEN, especially in lung and pancreatic NEN. This study included patients with both NET and NEC and activity was seen across the spectrum of differentiation in NEN. We did not observe a difference in circulating MDSCs and response to treatment, although these analyses were limited by relatively limited sample size.

This study has several limitations, including its single-arm, single-institution nature (Supplementary Table S5) with relatively short follow-up. The study population was also heterogeneous, and studies of patient subsets are limited because of small numbers. Biomarker studies were further limited by missing samples predominantly driven by lack of sample collection during the COVID-19 pandemic. The timing of immune analysis at day 15 may also be impacted by myelosuppression of TMZ. Therefore, the immune exploratory analyses should be considered hypothesis generating. Given the single-arm combination nature of the trial, it is not possible to interpret the contribution of each drug to response or synergy of the combination therapy. Although the response rate observed in this study—especially in lung NEN—is higher than expected with either agent given alone, this combination warrants further study though to determine efficacy of TMZ, nivolumab, or the combination.

In conclusion, combination nivolumab and TMZ demonstrated promising efficacy in NENs, especially in patients with lung and pancreas NEN.

Authors' Disclosures

D.H. Owen reports grants from BMS during the conduct of the study as well as grants from Merck, Genentech, Pfizer, Palobiofarma, and Onc.AI outside the submitted work. V. Sukrithan reports institutional support from Eli Lilly & Company and consulting with GE Health. G.A. Otterson reports grants from BMS during the conduct of the study as well as grants from Genentech, Pfizer, AbbVie, Merck, and Elevation Oncology and personal fees from OncLive, Beigene, and Novocure outside the submitted work. M.H. Shah reports grants from Merck during the conduct of the study. B. Konda reports grants from Bristol-Myers Squibb during the conduct of the study as well as grants from Eisai, Merck, Eli Lilly & Co., Xencor, and Bristol-Myers Squibb outside the submitted work. No disclosures were reported by the other authors.

Authors' Contributions

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Acknowledgments

The authors would like to acknowledge BMS for support of this study as well as the OSUCCC Clinical Trials Office. We thank the Biostatistics Shared Resource at The Ohio State University Comprehensive Cancer Center, Columbus, Ohio, for statistical support. D.H. Owen was supported by the OSU K12 Training Grant for Clinical Faculty Investigators (K12 CA133250) and the LUNGevity Career Development Award.

Clinical trial NCT03728361 is an investigator-initiated study supported by a BMS-OSU collaborative research grant. Drug supply of nivolumab was provided by BMS. Support for mass cytometry was provided by R. Wesolowski's internal OSUCCC award and the Carson lab. Research reported in this publication was supported by The Ohio State University Comprehensive Cancer Center and the NIH under grant number P30 CA016058.

The publication costs of this article were defrayed in part by the payment of publication fees. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Note

Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Received June 17, 2022; revised August 25, 2022; accepted October 13, 2022; published first October 18, 2022.

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