Efficacy, Safety, and Biomarkers of Response to Azacitidine and Nivolumab in Relapsed/Refractory Acute Myeloid Leukemia: A Nonrandomized, Open-Label, Phase II Study

Naval Daver[1], Guillermo Garcia-Manero[1], Sreyashi Basu[2], Prajwal C. Boddu[1], Mansour Alfayez[1], Jorge E. Cortes[1], Marina Konopleva[1], Farhad Ravandi-Kashani[1], Elias Jabbour[1], Tapan Kadia[1], Graciela M. Nogueras-Gonzalez[3], Jing Ning[3], Naveen Pemmaraju[1], Courtney D. DiNardo[1], Michael Andreeff[1], Sherry A. Pierce[1], Tauna Gordon[1], Steven M. Kornblau[1], Wilmer Flores[1], Zainab Alhamal[2], Carlos Bueso-Ramos[4], Jeffrey L. Jorgensen[4], Keyur P. Patel[4], Jorge Blando[2], James P. Allison[2], Padmanee Sharma[2,5], and Hagop Kantarjian[1]

ABSTRACT Preclinical models have shown that blocking PD-1/PD-L1 pathways enhances antileukemic responses. Azacitidine upregulates PD-1 and IFNγ signaling. We therefore conducted this single-arm trial, in which patients with relapsed/refractory (R/R) acute myeloid leukemia (AML) were treated with azacitidine 75 mg/m^2 days 1 to 7 intravenously or subcutaneously with nivolumab 3 mg/kg intravenously on days 1 and 14, every 4 to 6 weeks. For the seventy patients who were treated, the median age was 70 years (range, 22–90) and the median number of prior therapies received was 2 (range, 1–7). The overall response rate (ORR) was 33%, including 15 (22%) complete remission/complete remission with insufficient recovery of counts, 1 partial response, and 7 patients with hematologic improvement maintained >6 months. Six patients (9%) had stable disease >6 months. The ORR was 58% and 22%, in hypomethylating agent (HMA)–naïve ($n = 25$) and HMA-pretreated ($n = 45$) patients, respectively. Grade 3 to 4 immune-related adverse events occurred in 8 (11%) patients. Pretherapy bone marrow and peripheral blood CD3 and CD8 were significantly predictive for response on flow cytometry. CTLA4 was significantly upregulated on CD4$^+$ Teff in nonresponders after 2 and 4 doses of nivolumab. Azacitidine and nivolumab therapy produced an encouraging response rate and overall survival in patients with R/R AML, particularly in HMA-naïve and salvage 1 patients. Pretherapy bone marrow aspirate and peripheral blood CD3 percentage may be biomarkers for patient selection.

SIGNIFICANCE: Azacitidine in combination with nivolumab appeared to be a safe and effective therapy in patients with AML who were salvage 1, prior hypomethylator-naïve, or had increased pretherapy CD3$^+$ bone marrow infiltrate by flow cytometry or IHC. Bone marrow CD3 and CD8 are relatively simple assays that should be incorporated to select patients in future trials.

[1]Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, Texas. [2]Department of Immunology, The University of Texas MD Anderson Cancer Center, Houston, Texas. [3]Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas. [4]Department of Hematopathology and Molecular Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas. [5]Department of GU Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas.

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P. Sharma and H. Kantarjian contributed equally to this article.

Corresponding Authors: Naval Daver, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 0428, Houston, TX, 77030. Phone: 713-794-4392; Fax: 713-745-3920; E-mail: ndaver@mdanderson.org; Padmanee Sharma, Phone: 713-792-2830; E-mail: padsharma@mdanderson.org; and Hagop Kantarjian, Phone: 713-792-7026; E-mail: hkantarjian@mdanderson.org

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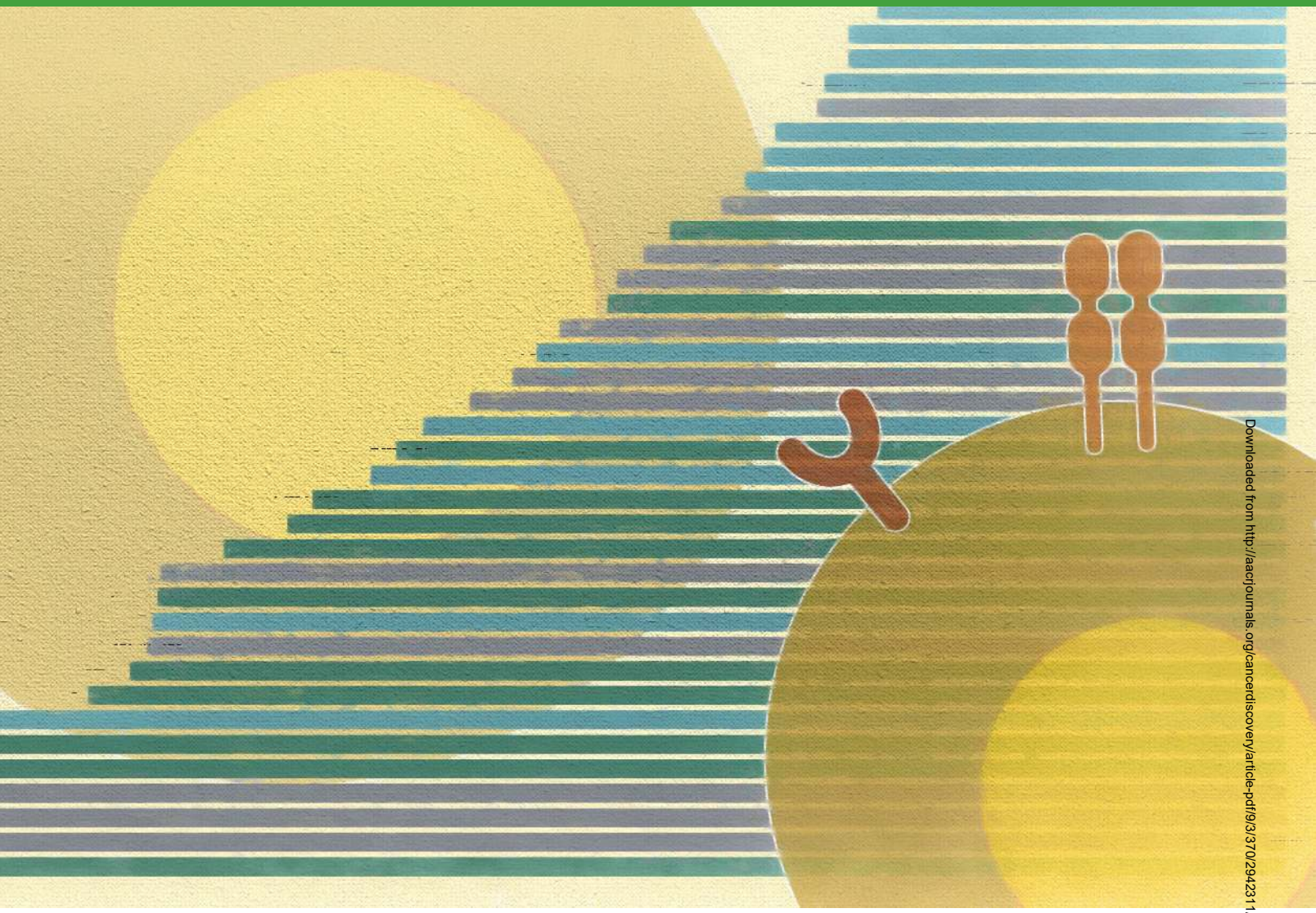

INTRODUCTION

Over the last decade, six PD-1, PD-L1, and CTLA4 antibodies have been approved for more than 25 indications in 10 tumor types in the United States and Europe. High clinical efficacy with single-agent PD-1 inhibition was seen in classic Hodgkin lymphoma (1). In other hematologic malignancies, such as non-Hodgkin lymphoma, the benefits of immune-checkpoint inhibitors (ICPI) were not evident with single-agent CPI therapy (2), but with rationally designed combinations (3).

T-cell population has been shown to be preserved in the bone marrow (BM) and peripheral blood (PB) of patients with acute myeloid leukemia (AML) and comparable with healthy donors (4, 5). In murine models, the progression of AML was associated with increased PD-1 expression on circulating CD8 T cells (6), resulting in decreased CD8 cytotoxic activity. This was partially reversible with murine PD-1 blockade (7). Immune-checkpoint receptors, most strikingly PD-1 and OX-40, were more frequently expressed on CD8 cells from BM aspirates (BMA) in patients with relapsed AML compared with healthy donors (5). Single-agent anti–PD-1 antibodies have demonstrated minimal activity in patients with relapsed AML and high-risk myelodysplastic syndrome (MDS; refs. 2, 8).

Azacitidine is approved in the United States and Europe for patients with MDS, and is approved in Europe and commonly used in the United States to treat older patients with newly diagnosed AML (9). The hypomethylating agents (HMA) azacitidine and decitabine promote antitumor immune signaling by upregulation of IFNγ pathway genes, increased expression of HLA class 1 antigens, and activation of viral defense pathways (10). The HMAs concurrently dampen antitumor immunity by increasing the expression of PD-1 and PD-L1 in solid tumors (11) and in MDS/AML (12). Upregulation of these immune-checkpoint molecules may be a mechanism of resistance to HMAs. This study was designed to assess whether the addition of nivolumab to azacitidine was safe and effective.

RESULTS

Patient Characteristics and Treatments

Seventy patients were treated. All received azacitidine 75 mg/m^2 days 1 to 7 with nivolumab 3 mg/kg days 1 and 14.

Table 1. Patient characteristics for azacitidine + nivolumab patients (*N* = 70) and for historic HMA-based clinical trial control (*N* = 172)

Characteristic	*N* (%); median (range)		*P*
	Azacitidine + nivolumab	Control	
Age, years	70 (22–90)	64 (18–90)	0.004
Age ≥ 60 years	56 (80)	103 (60)	
Diagnosis, *n* (%)			
AML—*de novo*	39 (56)	112 (65)	0.19
Secondary AML	31 (44)	60 (35)	
Prior therapies	2 (1–7)	1 (1–6)	0.76
Prior therapies[a]			
HMA-based	45 (64)	51 (30)	<0.0001
HIDAC	27 (39)	99 (58)	0.0073
IDAC	21 (30)	5 (3)	<0.0001
Targeted therapies[b]	33 (47)	15 (9)	<0.0001
Prior allogeneic SCT	13 (19)	16 (9)	0.0441
BM blast	35 (4–94)	38 (7–98)	<0.0001
White blood cell count ($\times 10^9$/L)	2.7 (0.5–81)	2.4 (0.2–232)	0.9121
Platelets ($\times 10^9$/L)	28 (1–203)	25 (1–816)	0.2379
Cytogenetics			
Diploid	9 (13)	23 (13)	0.9146
Miscellaneous	36 (51)	27 (16)	
Not available	0 (0)	62 (36)	
Del 5/-7/complex	25 (36)	60 (35)	0.9023
Molecular mutational panel	Done on all 70 patients	Positive/total tested	
TP53	16 (23)	18/54 (33)	0.1948
DNMT3A	12 (17)	7/58 (12)	0.4215
TET2	11 (16)	20/32 (63)	<0.0001
ASXL1	11 (16)	13/38 (34)	0.0272
CEBPA	8 (11)	9/81 (11)	0.9509
RAS	9 (13)	8/123 (7)	0.1343
IDH2	9 (13)	5/62 (8)	0.3721
PTPN11	7 (10)	1/27 (4)	0.3123
IDH1	6 (9)	7/82 (9)	0.9939
JAK2	3 (4)	9/62 (15)	0.0413
Treatment group			
HMA single agent	0 (0)	64 (37)	
HMA + immunotherapy	70 (100)	49 (29)	
HMA + others	0 (0)	59 (34)	

Abbreviations: *N*, number; HMA, hypomethylating agent; Ara-C, cytarabine; Del, deletion; SCT, stem cell transplant; HIDAC, high-dose Ara-C–based; IDAC, intermediate dose Ara-C based.

[a]Patients might have received multiple different types of targeted, HMA, HIDC, or IDAC therapy. The number and percentage represent patients, not a percentage from total prior therapy.

[b]This included IDH1/2 and FLT3 inhibitor, BCL2 inhibitor, MEK inhibitor, histone deacetylase inhibitor, JAK2 inhibitor, and GRB2 inhibitor–based therapies.

Patient characteristics are shown in Table 1. The median number of prior therapies for AML was 2 (range, 1–7). Prior exposure to HMA was allowed, and 45 patients (65%) had received prior HMA-based therapy. The median duration on study for all patients was 3.5 months (range, 0.3–26.3). Study discontinuations were due to primary refractory disease ($n = 27$), relapse after initial response ($n = 19$), allogeneic stem cell transplant (ASCT) in complete remission/complete remission with insufficient recovery of counts (CR/CRi; $n = 3$), death on study ($n = 16$; details under Survival section), and patient preference ($n = 3$). No protocol discontinuations were due to myelosuppression or immune toxicities. Two patients died of toxicities possibly related to the CPI, discussed in more detail under "Toxicities."

Table 2. Best response for azacitidine + nivolumab patients (*N* = 70) and for historic HMA-based clinical trial control (*N* = 172)

	N (%); median (range)	
Best response	Azacitidine/nivolumab	Control
Overall response rate	23 (33)	35 (20)
CR	4 (6)	17 (10)
CRi/CRp	11 (16)	15 (9)
PR	1 (1)	1 (1)
HI[a] (6 months+)	7 (10)	2 (1)
Stable disease (6 months+)[b]	6 (9)	NA
Nonresponders	41 (58)	131 (76)
Median cycles to response	2 (1–13)	2 (1–6)
Median follow-up, in months	13.3 (8.2–25.5)	51 (0.1–64.8)

Abbreviations: *N*, number; CR, complete remission; CRi, complete remission with incomplete count recovery; PR, partial response.

[a]Hematologic improvement (HI) in one or more parameter maintained >6 months on study.

[b]Stable disease was defined as the absence of CR, CRi, PR, MLFS, and HI without evidence of clinical deterioration or proliferative disease, maintained >6 months on study (see the text for detailed definition).

The median number of azacitidine and nivolumab cycles received was 3 (range, 1–25). The median number of nivolumab doses received was 6 (range, 1–54). Dose interruptions of nivolumab occurred in 24 of 70 (34%) patients due to pneumonitis/colitis (n = 13), liver enzyme elevation (n = 2), cytokine release syndrome (n = 1), bone pains (n = 1), lung infections (n = 3), hypothyroidism (n = 1), creatinine elevation (n = 2), and febrile neutropenia (n = 1). Nine of 70 patients (13%) discontinued nivolumab and remained on azacitidine alone, due to pneumonitis (n = 7), cytokine release syndrome and immune nephritis (1 each). Overall, 10 of 70 patients (14%) had to hold azacitidine at some point on study, due to cytopenias (n = 7), infection (n = 2), and elevated creatinine (n = 1). Twelve of 70 (17%) patients required dose reductions of azacitidine, all due to cytopenias.

Responses

The overall response rate (ORR) was 33% including 15 CR/CRi (22%; 4 CR and 11 CRi), 1 partial remission (PR), and 7 hematologic improvement (HI; Table 2). The median number of cycles to response was 2 (range, 1–13). Additionally, 6 patients (9%) remained on study with stable disease (SD) >6 months. The remaining 41 patients (58%) were nonresponders (NR). The 4- and 8-week mortalities were 3% and 11%, respectively. Three patients (4%) went to ASCT in CR/CRi. By univariate analysis the factors significantly associated with improved ORR included no prior HMA-based therapy, pretherapy BM blast <20%, circulating white blood cells (WBC) ≤10,000/μL, the presence of an *ASXL1* mutation, and pretherapy BMA $CD3^+$ T cells (Table 3). On multivariate analysis (performed on the 47 patients who

Table 3. Overall response rate (CR, CRi, PR, and HI) by baseline characteristics

	No response (n = 47)		Response (n = 23)		
	N	% (mean)	*N*	% (mean)	*P*
Age					0.79
<60	9	19	5	22	
≥60	38	81	18	78	
Age					0.67
<70	23	49	10	43	
≥70	24	51	13	57	
Salvage status					0.20
S1	19	40	13	57	
>S1	28	60	10	43	
Salvage status					0.12
S1/S2	34	72	21	91	
>S2	13	28	2	9	

(*continued*)

Table 3. Overall response rate (CR, CRi, PR, and HI) by baseline characteristics (*Continued*)

	No response ($n = 47$)		Response ($n = 23$)		
	N	% (mean)	*N*	% (mean)	*P*
Diagnosis					0.54
AML—*de novo*	25	53	14	61	
Secondary AML	22	47	9	39	
Prior ASCT					0.52
Yes	10	21	3	13	
No	37	79	20	87	
Prior HMA					0.01
Yes	35	74	10	57	
No	12	26	13	43	
Cytogenetic					0.13
Diploid	13	28	12	52	
Miscellaneous	16	34	5	22	
Adverse	18	38	6	26	
TP53					0.23
Negative	34	72	20	87	
Positive	13	28	3	13	
IDH1					0.99
Negative	43	91	21	91	
Positive	4	9	2	9	
IDH2					0.46
Negative	42	89	19	83	
Positive	5	11	4	17	
RAS					0.14
Negative	43	91	18	78	
Positive	4	9	5	22	
ASXL1					0.03
Negative	43	91	16	70	
Positive	4	9	7	30	
BM BL ≥30					
No	19	40	12	52	0.35
Yes	28	60	11	48	
BM BL ≥20					0.02
No	8	17	10	43	
Yes	39	83	13	57	
BM BL ≥10					0.10
No	3	6	5	22	
Yes	44	94	18	78	
WBC >10					0.03
No	34	72	22	96	
Yes	13	28	1	4	
PLT >50					0.43
No	33	70	14	61	
Yes	14	30	9	39	
BM pretherapy $CD3^+$	23	(17.56)	19	(32.47)	0.042
PB pretherapy $CD3^+$	22	(21.83)	18	(45.07)	0.0058

Abbreviations: *N*, number; ORR, overall response rate; HMA, hypomethylating agent; ASCT, allogeneic stem cell transplant; BM, bone marrow; PB, peripheral blood.

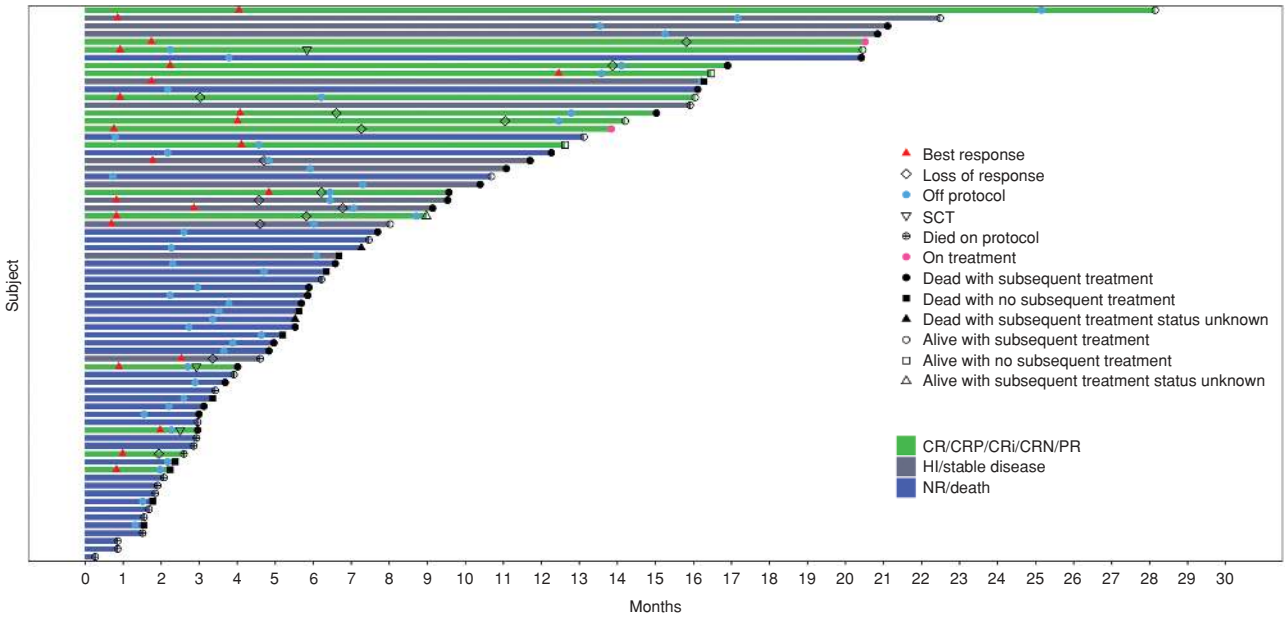

Figure 1. Swimmers plot illustrating the clinical course of study patients ($N = 70$). The best response, on- or off-study status, alive or dead status, and allogeneic stem cell status for the 70 patients enrolled on study are shown in this swimmers plot. CRP, complete remission with incomplete platelet recovery; CRN, complete remission with incomplete neutrophil recovery.

had pretherapy BM CD3$^+$ flow-cytometry data available), no factor was statistically significant, although no prior HMA ($P = 0.059$), higher pretherapy BMA CD3$^+$ ($P = 0.065$), and the presence of *ASXL1* mutation ($P = 0.053$) showed a trend for improved ORR (Supplementary Table S1). A heat map showing the relationship between pretherapy karyotype, mutation profile, and responses is shown in Supplementary Fig. S1.

Survival

With a median follow-up of 21.4 (95% CI, 14.8–not estimated) months, 57 (81%) of the patients have died. Figure 1 is a swimmers plot of the 70 patients enrolled. Sixteen patients died on azacitidine and nivolumab therapy: 8-week mortality ($n = 8$), relapsed/refractory AML ($n = 1$), death in CR/CRi/PR/HI from sepsis ($n = 6$), or hemorrhage ($n = 1$). Forty-one patients died after discontinuation of azacitidine with nivolumab: progressive AML ($n = 8$), pneumonia ($n = 5$), post-ASCT complications ($n = 2$), sepsis ($n = 13$), cardiac arrest ($n = 1$), transition to hospice ($n = 8$), and unknown cause of death ($n = 4$).

The median overall survival (OS) for the 70 patients was 6.3 months (Fig. 2A). The median event-free survival (EFS) among responders/SD ($n = 29$) and duration of response (DOR) among responders ($n = 23$) were 4.5 and 5.2 months, respectively (Fig. 2B and C). Patients who achieved a CR/CRi/PR/HI/SD ($n = 29$; 42%) had significantly improved OS compared with NRs ($n = 41$; 58%), without censoring for ASCT (16.2 vs. 4.1 months; $P < 0.0001$; Fig. 2D) and also after censoring for ASCT ($P < 0{\cdot}001$). OS was not significantly different in patients who achieved CR/CRi/PR versus HI versus SD (17.1 vs. 11.9 vs. 16.2; $P = 0{\cdot}8$; Fig. 2E). By univariate analysis, the factors significantly associated with improved OS were achievement of any response or SD to therapy, salvage 1 status, and the presence of an *ASXL1* mutation (Supplementary Table S2; Supplementary Fig. S2A–S2C).

Three patients proceeded to ASCT in CR/CRi with matched unrelated ($n = 1$) and umbilical cord donors ($n = 2$): Two of the three patients died from post-ASCT infections, after 0.8 and 1.3 months (both in CRi); the third is alive and in remission 6.5 months post-ASCT.

We identified a historical cohort of 172 patients with relapsed/refractory AML treated on HMA-based clinical trials (including single-agent HMA and HMA combinations) at our institution between 2005 and 2017 ($N = 172$; a list of clinical trials is provided in Supplementary Table S3). The baseline characteristics in the study population of azacitidine with nivolumab ($N = 70$) and the historical HMA-based clinical trial controls ($n = 172$) are shown in Table 1. The historical controls were younger ($P = 0.004$), were less frequently exposed to prior HMA-based therapies ($P = <0.0001$), and had a lower frequency of post-ASCT relapses [$P = 0.04$ but with a higher BM blast percentage in the historical controls (38% vs. 35%; $P < 0.001$)]. The ORR with azacitidine and nivolumab was 33% versus 20% with historical controls in the entire population, and 52% versus 22% in the prior HMA-naïve population (Table 2). The median OS with azacitidine with nivolumab ($n = 70$) compared favorably with the historical cohort ($n = 172$) both in the "all salvage" population (6.3 versus 4.6 months; $n = 70$ vs. 172; $P = 0.013$) (Supplementary Fig. S2D), but more prominently in the "first salvage" population (10.6 vs. 5.3 months; $n = 32$ vs. 91; $P = 0.011$; Supplementary Fig. S2A), with and without censoring for ASCT. Similarly, EFS was longer in patients treated with azacitidine and nivolumab than on historical HMA-based clinical trials in the "all salvage" (4.2 months vs. 2.2 months; $P < 0{\cdot}0001$) and in the "first salvage" population (6.8 months vs. 2.7 months, $P < 0.0001$; Supplementary Fig. S3A and S3B).

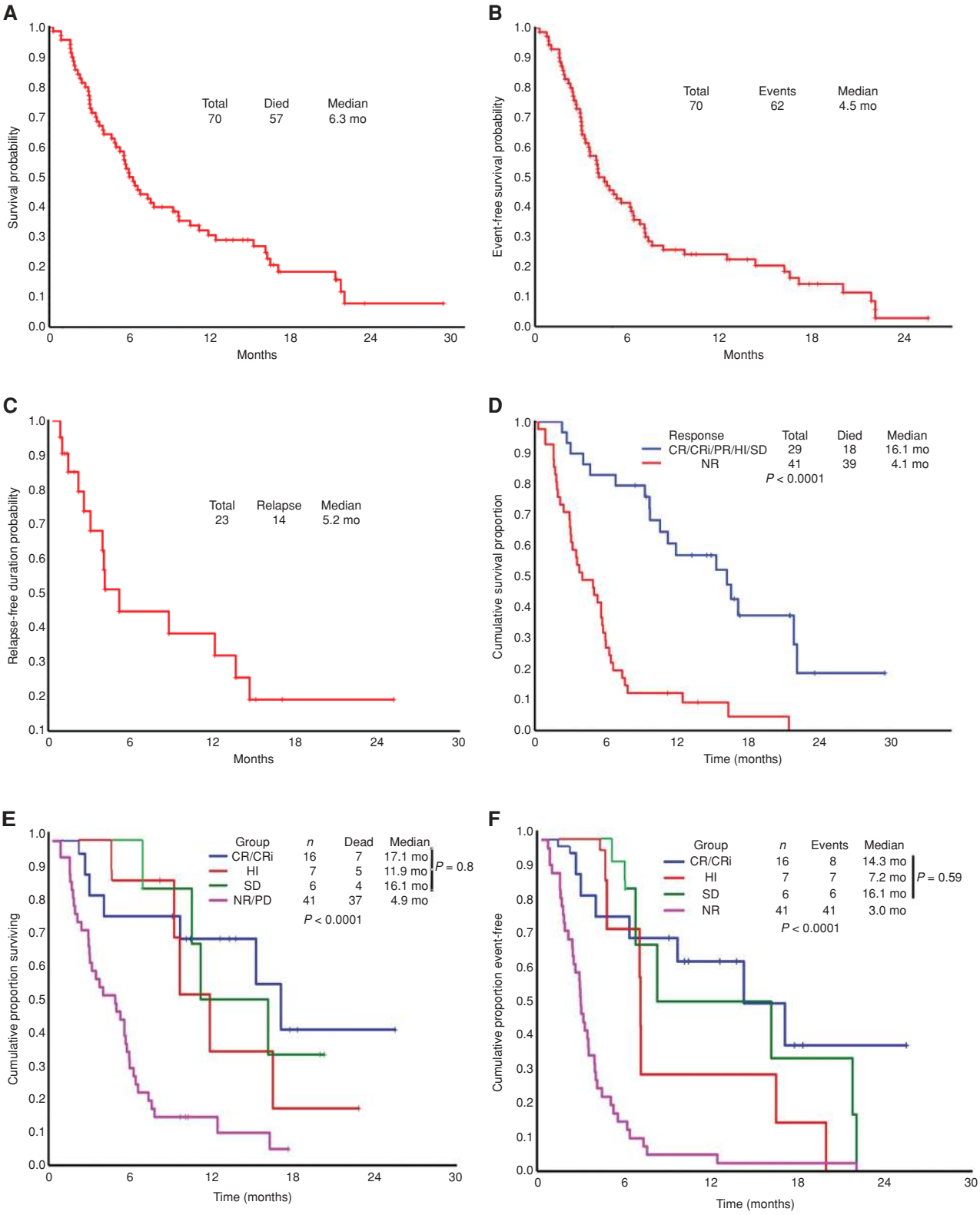

Figure 2. **A,** Overall survival in the 70 patients treated with azacitidine and nivolumab. **B,** Event-free survival in the 70 patients treated with azacitidine and nivolumab. **C,** DOR among the 23 patients with a response (CR, CRi, PR, and HI) on azacitidine with nivolumab. **D,** Overall survival in patients who had response/stable disease (CR, CRi, PR, HI, and SD) versus patients who had no response with azacitidine with nivolumab ($N = 70$). **E,** Overall survival by the best response to therapy ($N = 70$; $P < 0.0001$). **F,** EFS by the best response to therapy ($N = 70$; $P < 0.0001$).

Immune Profiling of Pretherapy and On-Therapy BMAs by Multiparametric Flow Cytometry and CyTOF

Multiparametric flow cytometry (MFC) was performed on pretherapy and post-therapy BMAs, after 2 doses [end of cycle 1 (EOC1)] and 4 doses (EOC2) of nivolumab in 19 of 23 responders (CR/CRi/PR/HI; 83%) and 28 of 41 NRs (68%). Responders had a higher frequency of pretherapy BMA $CD3^+$ cells compared with NRs (32.5% vs. 17.5%; $P = 0.04$; Fig. 3A) and a higher frequency of pretherapy PB $CD3^+$ cells (45% vs. 21.8%, $P = 0.0058$). We also observed a trend toward higher frequency of $CD4^+$ T effector cells (15.6% vs. 9.0%; $P = 0{\cdot}08$), and $CD8^+$ T cells (13.1% vs. 6.9%, $P = 0.09$) in responders compared with NRs in the pretherapy BMAs. The higher frequency of total $CD3^+$T cells and its subsets persisted in EOC1 and EOC2 BMA in responders (Fig. 3A).

A significant increase in the BM $CD4^+$T effector subset expressing CTLA4 was noted in the post-therapy samples, after 4 doses of nivolumab (EOC2), as compared with pretherapy samples (EOC2 20.5% vs. pretherapy 10.4%; $P = 0.03$) in NRs, whereas responders did not demonstrate these changes in post-therapy samples as compared with pretherapy samples (Fig. 3B). Pretherapy PD-L1 on gated AML blasts, $CD3^+$ cells, or combination of AML blasts and $CD3^+$ cells, and pretherapy PD-1 on $CD3^+$ cells did not predict for response. A comprehensive list of comparisons by pretherapy biomarkers for responders versus NRs, and by OS ≤1 versus >1 year is shown in Supplementary Fig. S4 and Supplementary Table S4A.

Optimal cutoffs for predicting responses were identified using the Youden index. $CD3^+$ and $CD8^+$ T cells in pretherapy BMAs were identified to be the best predictors of response, with optimal cutoffs of 13.2% and 4.01%, respectively. The ORR was 56% in patients with pretherapy BM $CD3^+$ T cells ≥13.2% versus 23% in patients with $CD3^+$ T cells <13.2% ($P = 0.020$). The cutoff $CD3^+$ T cells >13.2% in pretherapy BMA had a sensitivity of 74% and a specificity of 65% ($P = 0.029$) for predicting response. The $CD3^+$ T cells were ≥13.2% in 26 of 47 patients (55%) who had an evaluable pretherapy BMA. Twenty-four of the 26 patients (92%) with pretherapy BM $CD3^+$ T cells >13.2% were salvage 1 or 2 status, which may explain the higher response rates and improved OS seen in these patients compared with beyond salvage 2 patients. This suggests that T-lymphocyte depletion, either from progressive AML-related BM and PB T-cell depletion or from exposure to repeated rounds of AML-directed chemotherapy in advanced salvage patients, may abrogate the ability of these patients to achieve response to such therapies. Patients who had $CD8^+$ T cells >4.01% in pretherapy BMA had a sensitivity of 74% and a specificity of 65% for predicting response. Pretherapy PB $CD3^+$ T cells were also predictive for response with optimal cutoff of 20.5%. The ORR was 65% in patients with PB $CD3^+$ T cells ≥20.5% versus 25% in patients with $CD3^+$ T cells <20.5% ($P = 0.024$). A comprehensive list of cutoffs by responders versus NRs and by OS <1 versus >1 year, for pretherapy BM and PB biomarkers, are shown in Supplementary Table S4B and S4C.

We performed 36-parameter CyTOF on pretherapy and post-therapy BMAs after 2 (EOC1), 4 (EOC2), and 8 (EOC4) doses of nivolumab in 5 patients with CR/CRi and 5 NRs. PhenoGraph clustering of all CD3-gated T cells revealed 24 metaclusters of T cells (Fig. 3C), of which 13 were $CD4^+$ T-cell clusters and 9 were $CD8^+$ T-cell clusters. One $CD4^+$ T-cell cluster (cluster C14) coexpressed elevated levels of PD-1 and Ki-67 along with RORγT and ICOS (Fig. 3D), suggesting a Th17-like T-cell population, with significantly different frequencies in the pretherapy BMAs of responders versus NRs (1.5% vs. 4.0%; $P = 0.02$). Previous studies showed that Th17 cells increase in AML, and this negatively correlated with prognosis (13, 14). This appeared to be the case in our analysis, as Th17 was higher in nonresponders compared with responders (Fig. 3D). The frequency of an effector $CD8^+$ T-cell cluster (cluster C2) expressing $CD45RA^+PD1^{lo}Tbet^{hi}Eomes^{lo}$ was significantly higher in the pretherapy BMAs of responders versus NRs (11.2% vs. 2.5%; $P = 0.002$), with a further trend for expansion of this population in responders but not in NRs after 8 doses of nivolumab (EOC4; Fig. 3D).

Immune Profiling of Pretherapy BMs by IHC

We were able to adequately perform IHC on both BM clots and BM biopsies (Supplementary Fig. S5A). On BM IHC, the pretherapy $CD3^+$ T-cell density was higher in patients who achieved CR/CRi/PR compared with NRs ($P = 0.036$). A similar trend was seen for $CD8^+$ cells ($P = 0.08$; Supplementary Fig. S5B). This difference was lost when HI patients were included in the IHC analysis.

Toxicities

Treatment-related nonhematologic toxicities of all grades are shown in Table 4, and all grade toxicities irrespective of attribution are shown in Supplementary Table S5. Grade 3/4 and grade 2 immune-related adverse events (irAE) were observed in 8 (11%) and 8 (11%) patients, respectively. Of the 16 (23%) patients with grade 2 to 4 immune toxicities, 9 episodes were pneumonitis, 6 were nephritis, 3 were immune-related skin rash, and 2 were transaminitis (some patients had more than 1 irAE). Fourteen of the 16 (88%) toxicities responded to steroids, and these 14 patients were safely rechallenged with nivolumab. In our study, a total of 13% had to discontinue nivolumab (all discontinuations were due to grade 3/4 irAEs; no discontinuations due to grade 2 irAEs) and maintained only on azacitidine. irAE-related deaths occurred in 2 (3%) patients; both were refractory to steroids and subsequent infliximab therapy: from progressive pneumonia/pneumonitis (*E. coli* infection with suspicion for a superimposed immune pneumonitis) in one patient, and from hemophagocytosis lymphohistiocytosis in another. The time to onset of irAEs ranged from 4 days after the first dose of nivolumab to 3.5 months after the last dose of nivolumab, with the majority (12 of 16; 75%) of irAEs occurring in the first 8 weeks after nivolumab initiation.

DISCUSSION

Historical studies evaluating single-agent HMA therapy in relapsed/refractory prior HMA-naïve AML have reported ORRs of 10% to 20% with CR/CRi rates of 10% to 16% (15–17). Similarly, the ORR in a historical cohort of 172 patients with relapsed/refractory AML treated on HMA-based salvage clinical trials at our institution was 20%. The combination of azacitidine and nivolumab yielded an ORR of 33% (CR/CRi rate of 22%) with an additional 6 patients (9%) with meaningful SD in the entire study population. Most historical

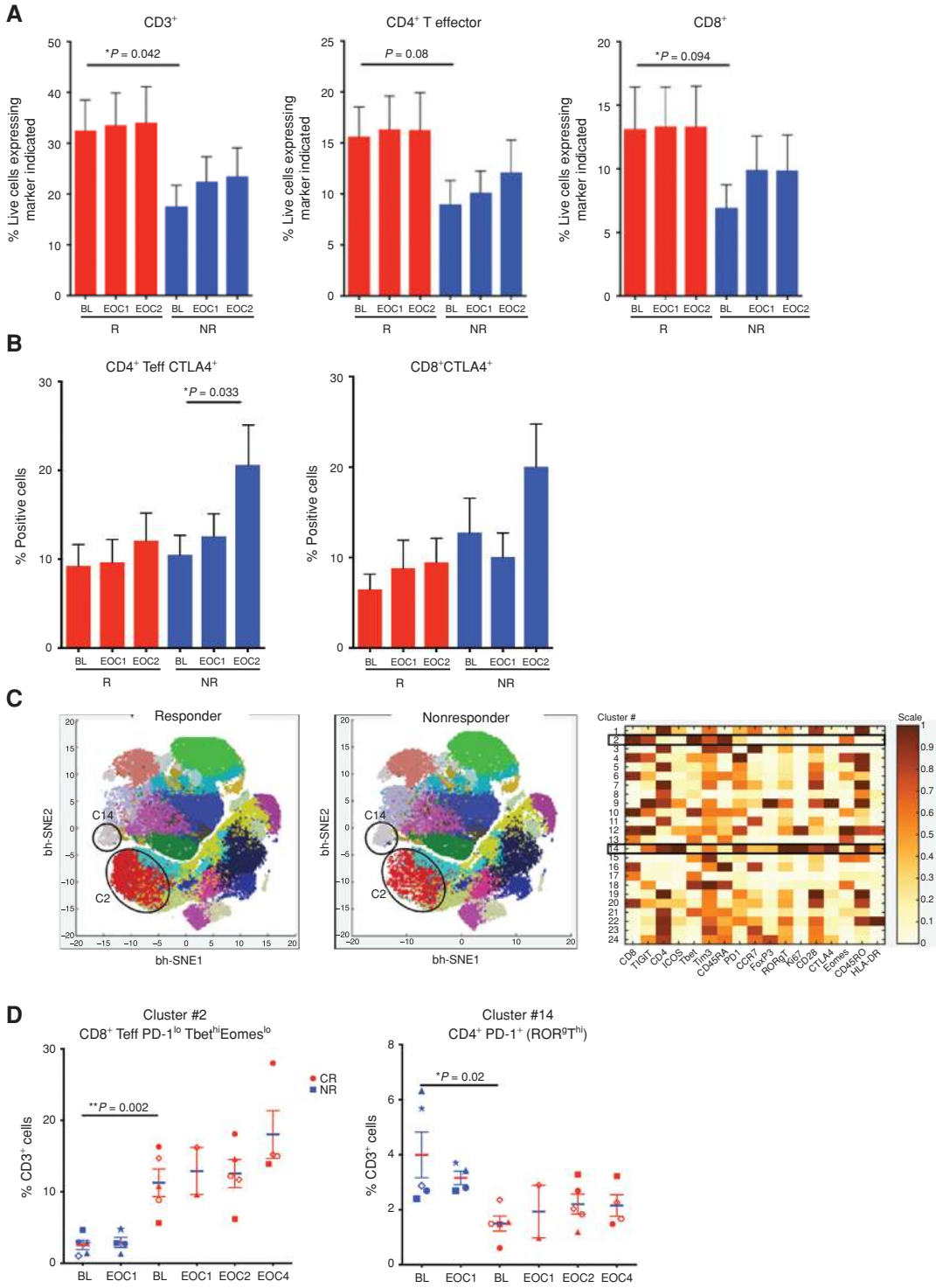

Figure 3. **A** and **B,** Bone marrow T-cell profile and checkpoint expression in responders (R) versus nonresponders (NR). Bar graphs indicating frequency of CD3+, CD4+ T effector, and CD8+ T cells in total live cells (**A**) and CTLA4+ CD4+ T effectors and CTLA4+CD8+ T cells (**B**) in BMA of responders (CR/CRi/PR/HI; $n = 19$) and NRs ($n = 23$) at pretherapy, EOC1, and EOC2 as analyzed by flow cytometry. **C,** Phenograph-based clustering approach of T-cell subsets by mass cytometry (CyTOF). t-SNE map of 10,000 randomly selected CD3+ T cells colored by distinct clusters (1–24) in responders (**C**, left), NRs (**C**, middle), and heat map showing normalized expression of different immune markers on CD3+ metaclusters including cluster 2 (C2) and cluster 14 (C14; **C**, right). **D,** CD45RA + PD1loTbethiEomeslo (C2) cells were significantly higher in the pretherapy BMAs of patients with CR/CRi ($n = 5$) than in NRs ($n = 5$), and with a trend toward expansion in patients with CR/CRi but not in NRs particularly after 8 doses of nivolumab (EOC4), by mass cytometry (**D**, left). By contrast, CD4+PD1+ (RORgThi; C14) cells, which were suggestive of Th17-like T-cell population, are higher in NR compared with responders (4.0% vs. 1.5%; $P = 0.02$). Th17 cells were reported to negatively correlate with prognosis in AML. Each shape/structure in the plot represents an individual patient at baseline and followed over time for this analysis.

Table 4. Nonhematologic treatment-related toxicities (*N* = 70)

	Grade					
Adverse event	G1	G2	G3	G4	G5	Total
Immune system disorders		1 (1)				1 (1)
Alanine/aspartate transaminase elevation			2 (3)			2 (3)
Colitis			1 (1)			1 (1)
Cytokine release syndrome		1 (1)	1 (1)			2 (3)
Autoimmune disorder		1 (1)				1 (1)
Enterocolitis		1 (1)				1 (1)
Erythema multiforme			1 (1)			1 (1)
Elevated bilirubin		1 (1)				1 (1)
Myositis		1 (1)				1 (1)
Rash, acneiform	1 (1)					1 (1)
Rash, maculopapular	1 (1)	4 (6)				5 (8)
Pneumonitis		8 (11)	1 (1)			9 (13)
Pruritus			2 (3)			2 (3)
Chest pain—cardiac	1 (1)					1 (1)
Arthralgia		1 (1)				1 (1)
Confusion	1 (1)					1 (1)
Constipation	15 (21)	3 (4)				18 (26)
Creatinine increased	2 (3)	1 (1)				3 (4)
Diarrhea	14 (20)					14 (20)
Dizziness	1 (1)					1 (1)
Dry skin	3 (4)					3 (4)
Dysphagia	1 (1)					1 (1)
Eye disorders			1 (1)			1 (1)
Fatigue		1 (1)	1 (1)			2 (3)
Gastrointestinal disorders		1 (1)				1 (1)
Generalized muscle weakness		2 (3)				2 (3)
Insomnia	1 (1)					1 (1)
Vomiting	6 (9)					6 (9)
Mucositis oral	1 (1)					1 (1)
Nausea	8 (11)					8 (11)
Sinus bradycardia	4 (6)	1 (1)				5 (8)
Febrile neutropenia			4 (6)			4 (6)
Lung infection			5 (7)		2 (3)	7 (11)
Cough	1 (1)	1 (1)				2 (3)
Skin and subcutaneous tissue disorders	2 (3)	1 (1)	1 (1)			4 (6)
Dyspnea	2 (3)					2 (3)
Sore throat	1 (1)					1 (1)
Total AEs	**62 (89)**	**29 (41)**	**15 (21)**	**0 (0)**	**1 (1)**	**107**
irAEs	**2 (3)**	**8 (11)**	**8 (11)**	**0 (0)**	**0 (0)**	**18 (25)**
All infections	**6 (9)**	**2 (3)**	**6 (9)**	**0 (0)**	**2 (3)**	**15 (23)**

HMA-based studies, including studies at our center, have excluded patients exposed to any prior HMA-based therapies, whereas this study did not. A large proportion (64% of patients) enrolled on this study had received prior HMA-based therapies. The ORR in only the prior HMA-naïve patients on our study was 52%. In our historical controls, the ORR among the prior HMA-naïve patients was 19%. In a recent large multicenter analysis with HMA-based therapies in salvage ($n = 655$) that included only prior HMA-naïve patients, Stahl and colleagues noted an ORR of 25% (17).

The median OS of 10.6 months in the salvage 1 patients treated with azacitidine and nivolumab was significantly better than the median OS of 5.2 months in the historical control salvage 1 patients treated on other HMA-based clinical trials at our institution between 2005 and 2017. This was noteworthy, especially considering that the patients in the historical cohort were younger, more likely to be prior HMA–naïve, and less likely to have relapsed post-ASCT although they did have a slightly higher pretherapy BM blast percentage. Stahl and colleagues noted that salvage 1

patients had a median OS of 6.7 months and 1- and 2-year survival rates of 25% and 15%, respectively (17). All of these patients were HMA-naïve. In salvage 1 patients treated with azacitidine with nivolumab (including 47% who had received prior HMA-based therapies), 1- and 2-year survival rates were 50% and 25%, respectively. Over the last decade, a number of HMA-based combinations have been evaluated. One of the most exciting combinations that has emerged is the combination of HMA with venetoclax, demonstrating CR/CRi rates >70% in first-line elderly patients with AML (18). However, in the salvage setting, the HMA with venetoclax combination had ORR of 25% to 30% and median OS of <5.0 months in two separate analyses (19, 20). Randomized studies are needed to make definitive conclusions, but thus far the response rates and OS with the azacitidine and nivolumab regimen appear encouraging, especially in previously HMA-naïve patients and in salvage 1 patients with AML, respectively. Of note, among prior HMA-exposed patients, the ORR was lower at 22%, but some responses could still be achieved with azacitidine and nivolumab.

Higher response rates were observed among patients who were HMA-naïve, had lower leukemia burden (<20% BM blasts), an *ASXL1* mutation, and higher pretherapy BMA $CD3^+$ infiltrate. In multivariate analysis, no prior HMA, increased pretherapy BM $CD3^+$ T cells, and the presence of ASXL1 mutation had a trend to improved ORR. Patients who were salvage 1, had *ASXL1* mutations, or achieved any response or SD had improved OS. Patients with AML in advanced salvage have depleted BM $CD3^+$, $CD8^+$, and $CD4^+$Teff T-cell populations (5), and this may be one reason they are less likely to benefit from T cell–dependent therapies. This was noted in our analysis wherein patients with higher pretherapy BM $CD3^+$ T cells were more likely to respond, and such patients were more likely to be in the salvage 1 and 2 setting. Lower leukemic burden and early salvage status have similarly been shown to be associated with improved response rates with other T-cell harnessing therapies such as blinatumomab (21, 22) and chimeric antigen receptor (CAR) therapies in patients with acute lymphoblastic leukemia (ALL; ref. 23). These data suggest that in both AML and ALL, the T cell–based therapies may be most effective when introduced early in the course of the disease, and possibly in a lower disease burden setting. Whether T-cell functionality is better preserved in patients who have inherently low-burden disease or whether the disease burden is lower in these patients because they have more functional T cells infiltrating the tumor environment requires further investigation. A recent report based on gene-expression profiling of patients with wild-type or mutated ASXL1 suggested an upregulation of immune response pathways in patients harboring the *ASXL1* mutation (24). It is plausible that the immunogenicity of ASXL1 may have been a driver of better responses and OS seen in patients harboring this mutation in our study, but this observation is based on small numbers and needs validation in a larger set.

Six patients did not achieve an International Working Group measurable response but had SD with a median OS of 16.1 months. The conventional Response Evaluation Criteria in Solid Tumors (RECIST) criteria underestimated the benefit of ICPIs, requiring the development of specific immune response criteria for patients with solid tumors on immunotherapy trials (25). Similarly, the achievement of SD with or without HI with ICPI-based approaches should be independently assessed and collected in ongoing and future ICPI trials in AML and MDS.

The nonimmune toxicities with this combination were similar to other HMA-based salvage therapies (17). Immune-mediated grade 3/4 toxicities were observed in 11% of the patients. Solid tumor and lymphoma studies of single-agent PD-1 inhibitors have demonstrated similar grade 3/4 irAE rates with ICPIs (2, 26). The irAEs frequently occurred within 8 weeks after ICPI initiation, similar to solid tumors (26). All grade 2–4 irAE patients were treated with steroids (26), and most (14 of 16, 88%) responded and could be rechallenged with nivolumab. The grade 2 irAEs in most cases did not result in hospitalization or treatment discontinuation and responded rapidly to steroid therapy.

Patients who achieved a response with azacitidine and nivolumab had higher $CD3^+$, $CD4^+$ Teff, and $CD8^+$ T cells in the pretherapy tumor environment (BMA in this case) compared with NRs. These are well-established biomarkers of response to ICPIs in other tumor types (27, 28). $CD3^+$ T cells in the pretherapy BMAs with a cutoff of 13.2% had a sensitivity of 74% and a specificity of 65% for predicting response. In our study, a sizable proportion (55%) of all evaluable patients (especially salvage 1 and 2) had a pretherapy $CD3^+$ >13.2%. Similar PB CD3 was also predictive for response with optimal cutoff of 20.5%. These are relatively simple biomarkers and, if validated in ongoing/future trials and approval strategies, may be important for selecting patients for future trials. In addition, the frequency of CTLA4-expressing $CD4^+$ T effector and $CD8^+$ T-cell populations increased on therapy in the BMAs of NRs but not in responders, highlighting CTLA4 upregulation as a potential mechanism of resistance to PD-1 blockade in the NRs, as has been in most solid tumors treated with ICPI therapies. Concomitant or sequential blockade of the inhibitory signals mediated by CTLA4 may further enhance T-cell responses. Furthermore, there may be a differential efficacy profile for PD-1 versus CTLA4 inhibition in myeloid malignancies (8, 29). Studies evaluating concomitant PD-1 and CTLA4 inhibition in patients with relapsed AML with or without azacitidine and as a maintenance post-ASCT in high-risk AML are ongoing (NCT02397720 and NCT03600155).

In conclusion, azacitidine with nivolumab produced an encouraging response rate and OS, especially in HMA-naïve and salvage 1 patients, respectively. Immune toxicities should be recognized and treated promptly. A randomized phase III study and a randomized phase II study of azacitidine with or without PD-1 inhibitor in first-line elderly AML (NCT03092674 and NCT02775903) and a randomized trial of PD-1 inhibitor for eradication of minimal residual disease (MRD) in high-risk AML in remission (NCT02275533) have been initiated. Clinical and immune biomarker–enriched trials are likely to yield further improved outcomes with HMA + ICPI therapies in AML and are strongly encouraged.

METHODS

Patient Eligibility

Patients ≥18 years of age who had failed prior therapy for AML (including prior therapy with HMAs) were eligible. Patients were required to have an Eastern Cooperative Oncology Group performance status ≤2; serum creatinine ≤2 × upper limit of normal

range (ULN); serum bilirubin ≤2 × ULN or ≤3 × ULN if the bilirubin elevation was deemed related to leukemic involvement or Gilbert syndrome; serum transaminase ≤2.5 times the ULN or ≤5 times ULN if the transaminase elevation was deemed related to leukemic infiltration. Exclusion criteria included a known history of a systemic autoimmune condition, severe interstitial lung disease or active pneumonitis; prior solid organ allograft; symptomatic central nervous system (CNS) leukemia; and any other uncontrolled disease. Patients with grade 1 or no graft-versus-host disease, requiring ≤10 mg of prednisone without additional immunosuppressive therapies, who had ASCT >3 months prior to study entry were eligible. All patients signed an informed consent form approved by the Institutional Review Board (IRB). The study was conducted in accordance with the Declaration of Helsinki (ClinicalTrials.gov identifier: NCT02397720; full protocol is included in Supplementary Methods).

Study Design and Objectives

This was a single-center, open-label nonrandomized phase II study. The study recruited patients between January 2015 and June 2017. The data cutoff was March 1, 2018. Primary study endpoints were safety and ORR [ORR = CR, CRi, PR, morphologic leukemia–free state (MLFS), ref. 30; durable HI (defined as improvement in one or more parameter of hemoglobin, platelets, neutrophils maintained ≥6 months), ref. 31], captured as the best response achieved on study. Patients who achieved any of these responses were considered responders. SD was defined as the absence of CR, CRi, PR, MLFS, or HI after exposure to treatment for a duration considered sufficiently suitable to achieve a response to therapy (≥6 months), but with no evidence of progressive BM disease (defined as more than 50% increase in BM blast or ≥15% in blasts when blast at baseline <30%), no increase in transfusion requirements and/or hospital admissions, the absence of new or progressive extramedullary or CNS disease, and no clinical deterioration in terms of functional status, weight/appetite, level of energy, or limiting side effects. Patients who did not achieve CR, CRi, PR, HI, or SD were considered NRs. Secondary endpoints included OS, EFS, and the DOR.

Treatment Regimen

Therapy consisted of azacitidine 75 mg/m^2 days 1 to 7 administered intravenously (i.v.) over 60 to 90 minutes or subcutaneously and nivolumab 3 mg/kg administered as a 60- to 90-minute i.v. infusion on days 1 and 14 of each cycle. One cycle was 28 days. The first 6 patients were treated with nivolumab 3 mg/kg and evaluated for dose-limiting toxicities for 28 days. The 3-mg/kg dose of nivolumab was found to be safe and was established as the recommended phase II dose (RP2D) in combination with azacitidine (Supplementary Table S6).

Cycles were repeated every 4 to 6 weeks, depending on count recovery. Required BMAs were done at the end of cycles 1, 2, 4, 7, and 11. Dose reductions or interruptions of azacitidine and/or dose interruptions of nivolumab were allowed as specified in the protocol (Supplementary Table S7). Patients continued on therapy as long as they had evidence of clinical benefit.

Baseline Assessments

Pretreatment evaluations included a complete history and physical examination, complete blood count with differential, comprehensive chemistry panel, pregnancy test, thyroid hormones and cortisol, and BMA for MFC, karyotype, a 28-gene next-generation sequencing (NGS), and immune profiling. MFC for MRD was performed as previously described (32). The NGS-based analysis for the detection of somatic mutations in the coding sequences of 28 genes was performed on DNA extracted from BMA (Supplementary Table S8), as previously described (33).

Immunophenotyping of Lymphocytes and Blasts from BMAs and PB

We performed 17-color flow cytometry on pretherapy and posttherapy BMAs and PB mononuclear cells obtained at protocol-specific time points, to evaluate the expression of inhibitory (PD-1, CTLA4, LAG3, and TIM3) and activating checkpoint receptors (GITR, OX40, 41BB, and ICOS), on the following T-cell subsets: effector CD4$^+$ T cells (Teff) defined as CD3$^+$CD4$^+$CD127$^{lo/+}$FOXP3$^-$; regulatory CD4 T cells (Treg) defined as CD3$^+$CD4$^+$CD127$^-$FOXP3$^+$; and CD8$^+$ T cells (Supplementary Table S9A; Supplementary Fig. S6A). AML blasts were assessed for ligands 41BBL, B7-1, B7-2, ICOSL, PD-L1, PD-L2, and OX40L (Supplementary Fig. S6B). These analyses were performed on BMAs within 12 hours of collection by The University of Texas MD Anderson Cancer Center Department of Immunology, using flow-cytometry panels as previously published (34).

Thirty-six parameter mass cytometry (CyTOF; Supplementary Table S9B) was performed on pretherapy and post-therapy BMAs in a subset of the responders and NRs with available samples. Details of the technique and the time points of BMA and PB collection are given in Supplementary Methods and Supplementary Table S9C.

IHC Staining and Analysis

IHC was performed on formalin-fixed, paraffin-embedded tissue sections. Density (absolute numbers of positive cells/mm^2 area analyzed) calculation was done using Imagescope software (Aperio/Leica Technologies). Details of BM staining are given in Supplementary Methods.

The MFC-based immunophenotyping, mass cytometry, and IHC were performed as a part of correlative research on this study, and willing patients gave a separate IRB-approved informed consent for these analyses.

Toxicity Assessment

Patients were monitored continuously for toxicity. Toxicity was defined as any clinically significant Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 grade 3 or 4 nonhematologic toxic effects or death attributable to the study drug. Per predefined rules, we would stop the trial if there were a more than 95% chance that the toxicity rate would be greater than 30%.

Statistical Methods

Futility and toxicity were monitored simultaneously using the Bayesian approach of Thall and Sung (35). For futility monitoring, we would stop the trial if there was a less than 1% chance that the ORR of azacitidine with nivolumab was greater than the ORR of standard treatment by 15%. OS was calculated from the start of therapy to death from any cause and was censored at last follow-up. DOR was calculated from time of documented response to loss of response or censored at last follow-up or death if response was maintained on the drug combination. EFS was calculated from the start of treatment to disease progression/death or censored at last follow-up while on the drug.

Differences among groups were evaluated by the χ^2 test (or Fisher exact test for cell frequencies <5) for categorical variables and t test or Wilcoxon–Mann–Whitney test for continuous variables. Paired t tests were applied to detect the changes in immune markers between pretherapy and on-therapy. Univariate logistic regression models were fitted to evaluate the relationships between the immune markers and clinical responses, and optimal cutoffs of markers for predicting responses were identified by maximizing the Youden index. Survival distributions were estimated using the Kaplan–Meier method and compared using the log-rank test. All P values were two-sided, and $P < 0.05$ was considered statistically significant. Statistical analyses were carried out using Stata/SE version 13.1 statistical software (Stata Corp. LP) and IBM SPSS Statistics 21 for Windows (SPSS Inc.).

Disclosure of Potential Conflicts of Interest

N. Daver reports receiving commercial research grants from Bristol-Myers Squibb, Pfizer, Incyte, Servier, AbbVie, NOHLA Therapeutics, Glycomimetics, Daiichi-Sankyo, and Kiromics and is a consultant/advisory board member for Bristol-Myers Squibb, AbbVie, Incyte, Agios, Astellas, Daiichi-Sankyo, Pfizer, Novartis, and Jazz. J.E. Cortes is a consultant/advisory board member for Bristol-Myers Squibb. F. Ravandi-Kashani reports receiving a commercial research grant from Bristol-Myers Squibb. T.M. Kadia reports receiving commercial research support from Bristol-Myers Squibb, Pfizer, Celgene, Jazz, and Amgen and is a consultant/advisory board member for Jazz, Novartis, Genentech, Amgen, Takeda, and AbbVie. N. Pemmaraju reports receiving commercial research support from Novartis, Stemline, Cellectis, AbbVie, Daiichi-Sankyo, Plexxikon, and Samus and is a consultant/advisory board member for Celgene, Stemline, and Incyte. C.D. DiNardo is a consultant/advisory board member for AbbVie, Agios, Bayer, Celgene, Karyopharm, MedImmune, Syros, and Jazz. J.P. Allison reports receiving commercial research grants from Astellas Pharma US Inc., Bristol-Myers Squibb Company, and AbbVie; has ownership interest (including stock, patents, etc.) in Jounce Therapeutics, Neon Therapeutics, ImaginAb, Amgen, BioAtla, Apricity, Polaris, Marker Therapeutics, Codiak Biosciences, Forty Seven, Tvardi Therapeutics, and TapImmune; and is a consultant/advisory board member for Jounce Therapeutics, Neon Therapeutics, Marker Therapeutics, Polaris, Amgen, Apricity, BioAtla, Forty Seven, Tvardi Therapeutics, TapImmune, ImaginAb, and Codiak Biosciences. P. Sharma has ownership interest (including stock, patents, etc.) in Jounce Therapeutics, Constellation, Polaris, ImaginAb, Forty-Seven, Apricity Health, and BioAtla and is a consultant/advisory board member for Jounce Therapeutics, Forty-Seven, Pieris, Oncolytics, ImaginAb, Polaris, Bristol-Myers Squibb, and BioAtla. H. Kantarjian has received honoraria from the speakers bureaus of AbbVie, Actinium, Agios, Amgen, Immunogen, Orsinex, Pfizer, and Takeda. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: N. Daver, G. Garcia-Manero, J.E. Cortes, F. Ravandi-Kashani, M. Andreeff, K.P. Patel, H. Kantarjian
Development of methodology: N. Daver, J.E. Cortes, K.P. Patel
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Daver, P.C. Boddu, M. Alfayez, J.E. Cortes, M. Konopleva, F. Ravandi-Kashani, E. Jabbour, T. Kadia, N. Pemmaraju, C.D. DiNardo, T. Gordon, S.M. Kornblau, J.L. Jorgensen, J.P. Allison, P. Sharma
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Daver, G. Garcia-Manero, S. Basu, P.C. Boddu, M. Alfayez, J.E. Cortes, M. Konopleva, G.M. Nogueras-Gonzalez, J. Ning, M. Andreeff, S.A. Pierce, Z. Alhamal, C. Bueso-Ramos, K.P. Patel, J. Blando, J.P. Allison, P. Sharma, H. Kantarjian
Writing, review, and/or revision of the manuscript: N. Daver, G. Garcia-Manero, S. Basu, P.C. Boddu, M. Alfayez, J.E. Cortes, M. Konopleva, F. Ravandi-Kashani, E. Jabbour, T. Kadia, G.M. Nogueras-Gonzalez, J. Ning, N. Pemmaraju, C.D. DiNardo, S.M. Kornblau, K.P. Patel, J.P. Allison, P. Sharma, H. Kantarjian
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Alfayez, J.P. Allison, P. Sharma
Study supervision: N. Daver, T. Gordon, H. Kantarjian
Other (correlative studies): W. Flores

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