



Immunologic Predictors for Clinical Responses during Immune Checkpoint Blockade in Patients with Myelodysplastic Syndromes

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ABSTRACT

Purpose: The aim of this study is to determine immune-related biomarkers to predict effective antitumor immunity in myelodysplastic syndrome (MDS) during immunotherapy (IMT, α CTLA-4, and/or α PD-1 antibodies) and/or hypomethylating agent (HMA).

Experimental Design: Peripheral blood samples from 55 patients with MDS were assessed for immune subsets, T-cell receptor (TCR) repertoire, mutations in 295 acute myeloid leukemia (AML)/MDS-related genes, and immune-related gene expression profiling before and after the first treatment.

Results: Clinical responders treated with IMT $\pm$ HMA but not HMA alone showed a significant expansion of central

memory (CM) CD8⁺ T cells, diverse TCR β repertoire pretreatment with increased clonality and emergence of novel clones after the initial treatment, and a higher mutation burden pretreatment with subsequent reduction posttreatment. Autophagy, TGF β , and Th1 differentiation pathways were the most downregulated in nonresponders after treatment, while upregulated in responders. Finally, CTLA-4 but not PD-1 blockade attributed to favorable changes in immune landscape.

Conclusions: Analysis of tumor-immune landscape in MDS during immunotherapy provides clinical response biomarkers.

Introduction

Myelodysplastic syndrome (MDS) is a myeloid clonal disorder that mostly occurs in the elderly population, and is characterized by cytopenia, dysplasia, and a propensity to transform into acute myeloid leukemia (AML; ref. 1). Although hypomethylating agents (HMA) are the standard treatment for patients with high-risk MDS, especially elderly patients (2–4), resistance to HMAs occurs in most MDS patients leading to a dismal outcome (5). Therefore, novel therapeutics for high-risk MDS patients are needed to improve clinical outcome.

The aberrant expression of immune checkpoint proteins in the tumor and tumor microenvironment is thought to be the mechanism of immune evasion (6, 7), as blockage of the immune checkpoint pathway has been shown to effectively eradicate certain

solid tumors (8–10) and hematologic malignancies (11, 12). As the overexpression of immune checkpoint proteins was observed in patients with MDS during HMA treatment (6), immune checkpoint blockade can not only resensitize the tumor-immune microenvironment (TIME) in patients with MDS who failed HMAs, but also prolong the duration of response to HMAs in patients with MDS. However, there are limited data on immune biomarkers and novel mechanisms of response or resistance to immune checkpoint blockade in MDS.

In this study, we aimed to determine immune-related biomarkers to predict effective antitumor immunity and uncover the mechanisms of immune resistance in 55 patients with MDS who received immune checkpoint blockade (α CTLA-4 and/or α PD-1 antibody) $\pm$ 5-Azacitidine or guadecitabine alone. As we have previously demonstrated that the immune and genetic landscape of peripheral blood (PB) mirrored those of bone marrow in patients with MDS (13), we utilized PB for a comprehensive immune monitoring strategy, and investigated the evolution of the immune landscape through comprehensive immunophenotypic analysis of T cells, next-generation sequencing (NGS)-based T-cell receptor (TCR) beta repertoire analysis, and immune-related gene expression during therapy. In addition, we evaluated the coevolution of tumor landscape through NGS-based targeted sequencing of 295 genes that are commonly mutated in AML/MDS.

Materials and Methods

Patient cohort

A total of 55 patients with MDS who receive immunotherapy (IMT, α CTLA-4, and/or α PD-1 blockade) $\pm$ HMA, and HMA alone in clinical trials (NCT02530463, NCT03094637, NCT02131597, and NCT02599649) were included in this study (Supplementary Table S1; Supplementary Fig. S1). Patients with chronic myelomonocytic leukemia (CMML) were permitted to

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Translational Relevance

Although currently, immune checkpoint inhibitors are under active investigation for myelodysplastic syndrome (MDS), there are limited data on immune biomarkers and novel mechanisms of response or resistance to immune checkpoint blockade in MDS. For these purposes, we performed a comprehensive immune monitoring strategy in 55 patients with MDS who received immunotherapy (IMT, α CTLA-4, and/or α PD-1 antibodies) and/or hypomethylating agent (HMA). Longitudinal analysis of tumor-immune landscape in MDS during checkpoint blockade supports an essential role of T cells in antitumor immunity. CTLA-4, but not PD-1 blockade, attributes to shaping a favorable immune landscape in MDS. Autophagy inducers and T-cell costimulators, such as ICOS agonists, may play a role in overcoming immune resistance.

enroll clinical trials, and 6 patients with CMML were included in the analysis. Clinical responses were determined according to the International Working Group responses criteria for MDS (14). Written informed consent was obtained from each patient or each patient's guardian before participation in this study. All research was conducted in accordance with the Declaration of Helsinki and The University of Texas MD Anderson Cancer Center Institutional Review Board guidelines.

Blood sample collection and isolation of PB mononuclear cells

A total of 10 cc of PB samples were collected prior to the initiation of treatment, each cycle of treatments up to cycle 8, and at the time of relapse (Supplementary Fig. S2). PB samples were processed within 24 hours of collection. Briefly, white blood cells (WBC) were obtained by lysing red blood cells (RBC) from 1 cc of peripheral blood, snap-frozen, and stored at -80°C until genomic DNA was isolated for TCR β repertoire and mutation analysis. PBMCs were isolated from the remaining blood by standard density centrifugation using Ficoll-Paque (Sigma), portion of PBMCs was used for upfront flow cytometry immune-subset analysis. Remaining PBMCs were cryopreserved in freezing media containing 10% DMSO (Sigma) and 90% FBS (Corning) in N_2 for future use including NanoString gene expression analysis.

Multiparameter flow cytometric analysis

Briefly, freshly prepared PBMCs were stained with the following antibodies: CD56-BUV395, CD16-BUV395, CD3-FITC, CD4-APC, CD8 α -PerCP, HLA-DR-PE, CD45RA-PB, CD62L-BV786, CD161-BV510, CD25-APC-Cy7, CD127-PE-Cy7, and fixable viability stain 620 to exclude dead cells. After fixation with 2% paraformaldehyde, cells were acquired using an LSR Fortessa Cell Analyzer (BD Biosciences), and subsequent analysis was performed using FlowJo version 10.3 (Supplementary Fig. S3). All the antibodies were purchased from either BioLegend or BD Pharmingen. Previously frozen aliquots of PBMCs from healthy donors were included in each MFC analysis as controls for antibody staining, instrumental acquisition, and analysis. Clinical samples containing more than 500 live CD3 $^{+}$ T cells were included in the final analysis, and an average of 18,349 T cells (minimum 544, maximum 184,362) per sample were subjected to further phenotypic analysis.

TCR β sequencing and analysis

Genomic DNA was isolated from previously snap-frozen WBC pellets using the QIAmp DNA Blood Mini Kit (Q), and used for amplification of the CDR3 regions of human TCR β chains and subsequent library preparation using an immunoSEQ hsTCRB Kit (Adaptive Biotechnologies) according to the manufacturer's instructions. The library was sequenced using the NextSeq 500/550 Mid Output V2 (150 cycle) kit (Illumina), and processed by Adaptive Biotechnologies. Subsequent downstream analysis was performed using an Immunoseq Analyzer (Adaptive Biotechnologies).

Mutation analysis of 295 AML/MDS-associated genes

NGS-based targeted sequencing of 295 AML/MDS-associated genes using the SureSelect custom panel (Agilent Technologies) was performed at an average depth of 443 on 50 ng of genomic DNAs extracted from WBC pellets, and high-confidence mutations were selected according to previously reported bioinformatic methodology (15). Subsequent mutation analysis was performed as previously described (16, 17). Briefly, a total number of detectable mutations and sum of variable allelic frequency (VAF) of all detectable mutations per subject or VAFs of individual mutation was compared between two time points—pretreatment and at the time of best response within responders or nonresponders from each treatment groups. Distribution of mutated genes were tabulated according to responders versus nonresponders from each treatment cohort, and changes in VAFs of mutations in gene were represented as “increase (orange)”, “decrease (sky blue)”, “clearance (blue)”, or “new appearance (red)”, “mixed responses (green)”.

NanoString analysis

Total RNAs were isolated from previously cryopreserved PBMCs using the RNeasy Plus Micro Kit (Qiagen), and further amplified using a NanoString Low Input Amplification Kit (NanoString). Up to 100 ng amplified RNAs were hybridized with target-specific, fluorescence-labeled 594 CodeSet and reporter probes from the nCounter Immunology panel (Human v2 – NanoString) for 20 hours, and added to the robotic nCounter Prep Station for automated sample processing for the remaining steps. The cartridge was transferred to an nCounter Digital Analyzer MAX for scanning and data collection. The nSolver software version 4 (NanoString) was used for preprocessing of raw counts, subsequent normalization, and downstream analysis including cell type profiling analysis, gene expression profiling (GEP), and gene set enrichment analysis (GSEA).

Statistical analysis

Nonparametric Wilcoxon matched paired signed rank or Mann-Whitney tests were used to assess statistical differences between two paired values within response groups or unpaired values across response groups, respectively. The Kruskal-Wallis test was used to compare differences among the three values within the group. All statistical analyses were performed using GraphPad Prism 8.0. *P* value less than 0.05 was considered as statistically significant. Only the statistically significant or notable *P* values are shown.

Data availability statement

NanoString data were deposited to the Gene Expression Omnibus (GEO), a public functional genomics data repository at GSE219085.

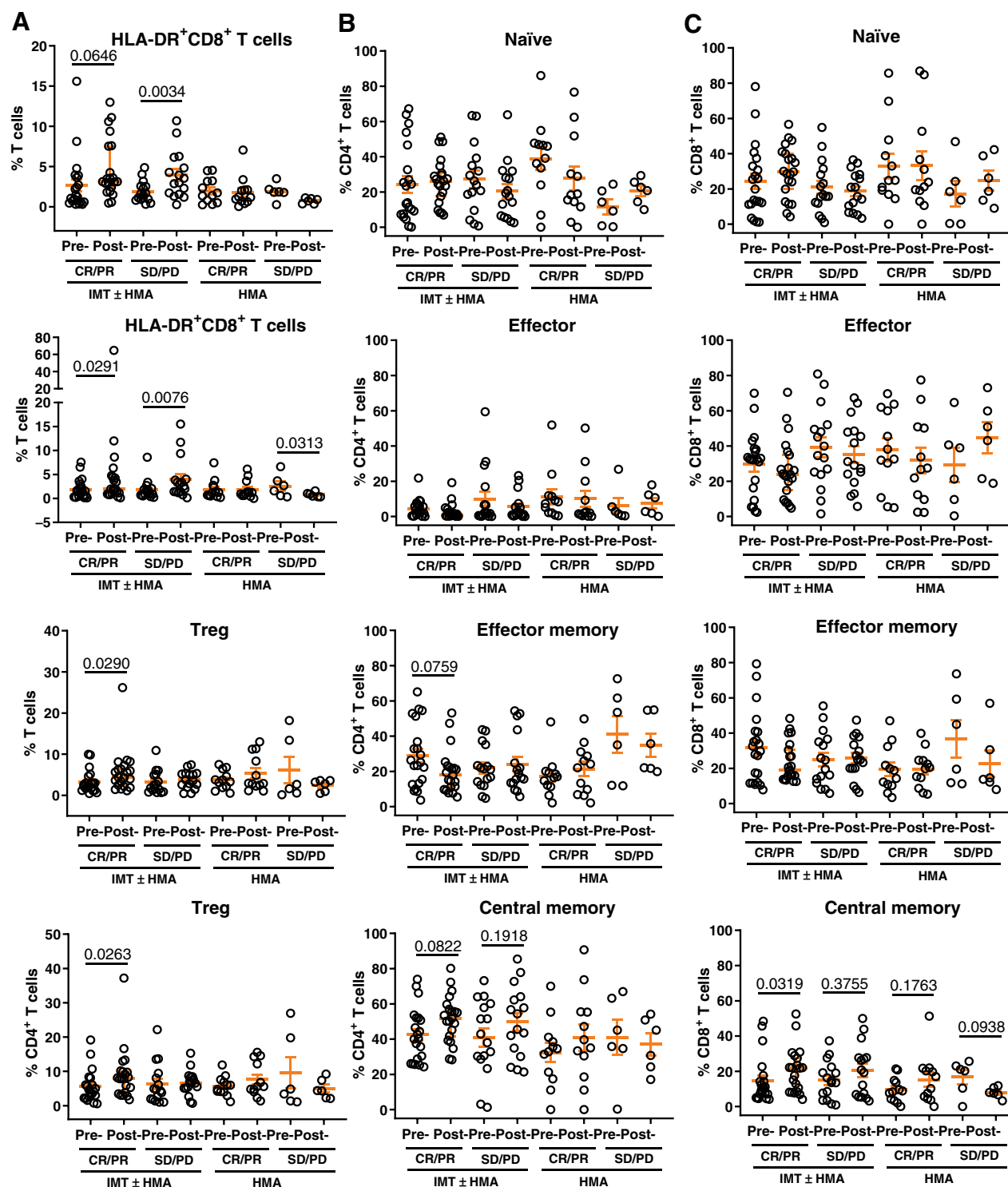
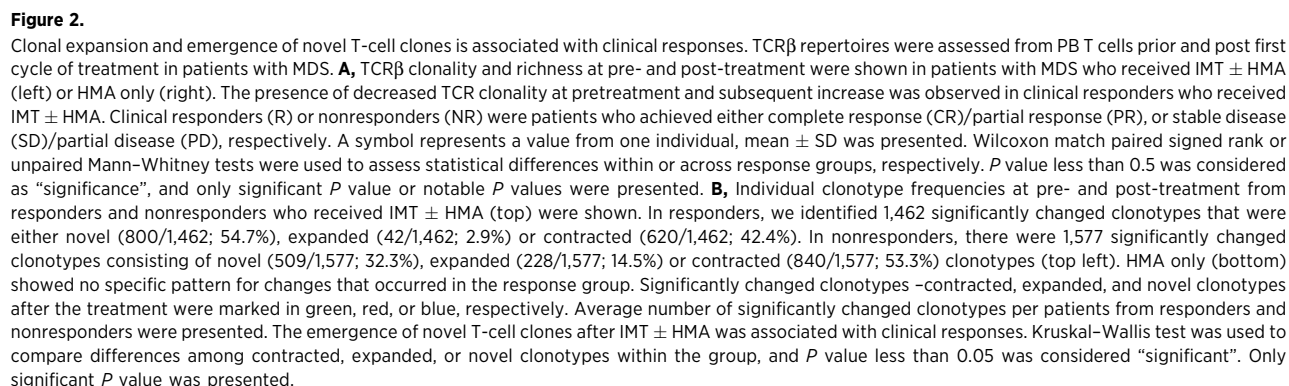


Figure 1.

CM CD8⁺ T cells and Treg expansion are associated with clinical responses in IMT. Multiparameter flow cytometry was used to assess the composition of various immune subsets such as activated T cells (HLA-DR⁺), Treg (CD4⁺CD25⁺CD127^{low}), and naïve (CD45RA⁺CD62L⁺), CM (CD45RA⁺CD62L⁺), effector memory (CD45RA⁺CD62L⁺), and effector (CD45RA⁺CD62L⁺) T cells from PB of patients with MDS before and after the initial treatments - IMT ± HMA or HMA only. Clinical responders (R) or nonresponders (NR) were patients who achieved either complete response (CR)/partial response (PR), or stable disease (SD)/partial disease (PD), respectively. *In vivo* expansion of Treg (A) and central memory CD8⁺ T cells (B and C) after the initial treatment were associated with clinical responses. A symbol represents a value from one subject and mean ± SD was represented. Wilcoxon match paired signed rank or unpaired Mann-Whitney tests were used to assess statistical differences within or across response groups, respectively. *P* value less than 0.5 was considered as "significance", and only significant *P* value or notable *P* values were presented.





Results

Patient characteristics

A total of 55 patients with MDS who had paired samples with adequate quality and quantity peripheral blood lymphocytes, collected at before and after the initial cycle of treatment were included in this study. Patients' characteristics are described in Supplementary Table S1. Thirty-seven patients who received IMT ± HMA consisted of 21 clinical responders [complete response (CR) or partial response (PR)] and 16 nonresponders [stable disease (SD) or progression of disease (PD)]. Eighteen patients who received HMA alone included 12 clinical responders and 6 nonresponders. Detailed information on the treatment regimen and available testing results for the analysis are summarized in Supplementary Figs. S1 and S2.

In vivo expansion of central memory CD8⁺ T cells and Tregs are associated with clinical responses to IMT

The therapeutic efficacy of immune checkpoint blockade may depend on the presence of potential neoantigen-specific, naïve, or memory T cells in the periphery, and their ability to be activated and expanded during treatment. Therefore, we first evaluated the composition of various T-cell subsets at baseline and the subsequent changes after the initial treatment and at the time of best responses.

First, there was no significant difference in the major T-cell subsets such as CD4⁺, CD8⁺, or CD4⁺CD8⁺ T cells between responders versus nonresponders before and average 30.9 days (range: 11–61 days) after the first cycle of treatment (Fig. 1) or at the time of the best responses (Supplementary Fig. S4). There were increased frequencies of DR⁺CD4⁺ or DR⁺CD8⁺ T cells after the first cycle of treatment in both responders and nonresponders after IMT ± HMA, but not after HMA alone, suggesting that the degree of activated T cells after treatment does not predict clinical responses but rather implicate the effects of immune checkpoint blockade (Fig. 1A). Interestingly, significant *in vivo* expansion of Tregs and CM CD8⁺ T cells, and a trend toward increased CM CD4⁺ T cells was observed in clinical responders ($P = 0.0290$, 0.0319 , and 0.0822 , respectively) after the first cycle of IMT ± HMA, but not in nonresponders, or after HMA alone. This change in Tregs and CM CD8⁺ T cells was best observed after the initial treatment, rather than at the time of best responses (Supplementary Fig. S4). While increased Tregs are thought to have a negative impact on prognosis of MDS (18–20), a significant increase in circulating Treg post treatment in clinical responder may reflect a compensatory response during immunotherapy. Our results indicate that *in vivo* expansion of CM CD8⁺ T cells or Treg after the initial

immunotherapy can predict the clinical response in MDS during immunotherapy.

Clonal expansion and emergence of novel T-cell clones is associated with clinical responses

While the CTLA-4:B7 interaction works during the initial activation of naïve T cells, PD-1:PD-L1 interaction modulates the homeostasis of activated T cells (21). Therefore, there might be a qualitative difference in the TCR repertoire between clinical responders and nonresponders during immunotherapy. Here, we performed TCRβ repertoire analysis of peripheral blood T cells at before and after the initial treatment to evaluate whether clinical responses correlate with unique changes in TCRβ repertoire during immunotherapy.

First, we compared the clonality and richness of T cells between responders and nonresponders pre- and post-treatment, as well as the changes from pre- to post-treatment with IMT ± HMA or HMA only (Fig. 2A). The responders had a significantly lower clonality than that of nonresponders ($P = 0.0415$) prior to the treatment, followed by a trend toward increased clonality after treatment with IMT ± HMA ($P = 0.0537$), indicating that clinical responses during immunotherapy were associated with oligoclonal expansion of rare T cells selected from diverse T-cell clonotypes. There was no significant difference in TCRβ richness between responders and nonresponders or after treatment with HMA alone.

Next, we assessed qualitative changes in the TCRβ repertoire during treatment by comparing the number of contracted, expanded, and novel clonotypes that occurred posttreatment in responders and nonresponders after IMT ± HMA or HMA only (Fig. 2B). There was a significantly higher abundance of novel clonotypes compared with expanded or contracted clonotypes in responders ($P = 0.01$), whereas contracted clonotypes were more prevalent in nonresponders ($P = 0.0458$). These findings suggest that the emergence of new clonotypes was associated with responders, while nonresponders had a contracted clonotype repertoire following IMT ± HMA. In contrast, there was no specific pattern for changes that occurred in the response group following HMA only. Our results indicate that clonal expansion of T cells with novel clonotypes selected from diverse clonotypic pools is associated with clinical responses during immunotherapy in MDS.

Evolution of mutation landscape predicts clinical responses during immunotherapy

Considering the therapeutic efficacy of immunotherapy using checkpoint inhibitors relies on the presence of targetable tumor antigens (22), we performed targeted gene sequencing of 295

Figure 3.

Higher individual mutation burden prior to the treatment is associated with clinical responses in patients with MDS during checkpoint blockade ± HMA. NGS-based targeted sequencing of 295 recurring mutated genes in AML/MDS were performed on pre- and matching post-treatment peripheral blood of patients with MDS who received IMT ± HMA or HMA alone, and nonsynonymous somatic mutations were identified. Clinical responders (R) or nonresponders (NR) were patients who achieved either complete response (CR)/partial response (PR), or stable disease (SD)/partial disease (PD), respectively. **A**, The number of somatic mutations per patient (**A**, left), the sum of variant allelic frequencies (VAF) of all detectable mutations per patient (**A**, middle), and VAF of individual mutation of all patients within group – responders versus nonresponders (**A**, right) were shown in left, middle, and right panel, respectively. A symbol represents a value from one individual (**A**, left and middle) or one mutation (**A**, right), and mean ± SD was represented. Wilcoxon match paired signed rank or unpaired Mann-Whitney tests were used to assess statistical differences between two values within or across response groups, respectively. P value less than 0.05 was considered “significant”, and only significant or notable P values were presented. **B**, Changes of VAFs of mutations in individual genes from pretreatment to best-response time-point were shown in patients with MDS: new appearance (red), increased (orange), decreased (sky blue), clearance (blue), mixed response (green, some of subclones decreased, the others increased). **C**, Frequencies of mutation in genes detected prior to the treatment were shown in clinical responders (blue) and nonresponders (orange) who received IMT ± HMA (top) and HMA alone groups (bottom).

recurrently mutated genes in AML/MDS from pre- and matching post-treatment samples after the first cycle, at the best responses and relapse if applicable, to evaluate whether the presence or change of nonsynonymous mutations correlated with the clinical response (Fig. 3).

Although there was not a significant difference in the total number of somatic mutations and the sum of variant allelic frequencies (VAF) of all detectable mutations at pretreatment between responders and nonresponders, higher VAFs of individual mutations prior to the treatment and subsequent reduction were observed in responders among patients with IMT ± HMA than nonresponders ($P = 0.0026$; Fig. 3A). Moreover, 6 of 16 nonresponders (37.5%) showed new mutations while only 1 of 19 responders (5.3%) had the emergence of a new mutation. As expected, more clinical responders (13/19, 68.4%) contained mutations whose VAFs decreased or disappeared after treatment than nonresponders (8/16, 50%; Fig. 3B). Almost all patients who received HMA only had reductions in individual mutation burdens regard-

less of their responses, suggestive of cytoreductive action by HMA (Fig. 3A and B). However, the emergence of new mutations or worsened VAF in nonresponders appeared in 8 of 8 nonresponders in IMT only but also in 6 of 8 nonresponders in IMT+HMA group. Therefore, we cannot make a definitive conclusion that mutation clearance/reduction seen in clinical responders are solely attributed to the addition of HMA, but rather synergistic action between immunotherapy and HMA treatment. Finally, higher frequencies of TP53 mutations were present prior to treatment in responders (6/of 19, 31.6%) than in nonresponders (2/of 16, 12.5%) with IMT ± HMA, suggesting that TP53 mutations may be associated with clinical responders (Fig. 3C).

Thus, the longitudinal mutation analysis of tumor-immune microenvironment demonstrated that a higher mutation burden of individual mutations and subsequent reduction/clearance during immunotherapy and the presence of p53 mutations were associated with clinical responses in patients who received IMT ± HMA but not HMA alone.

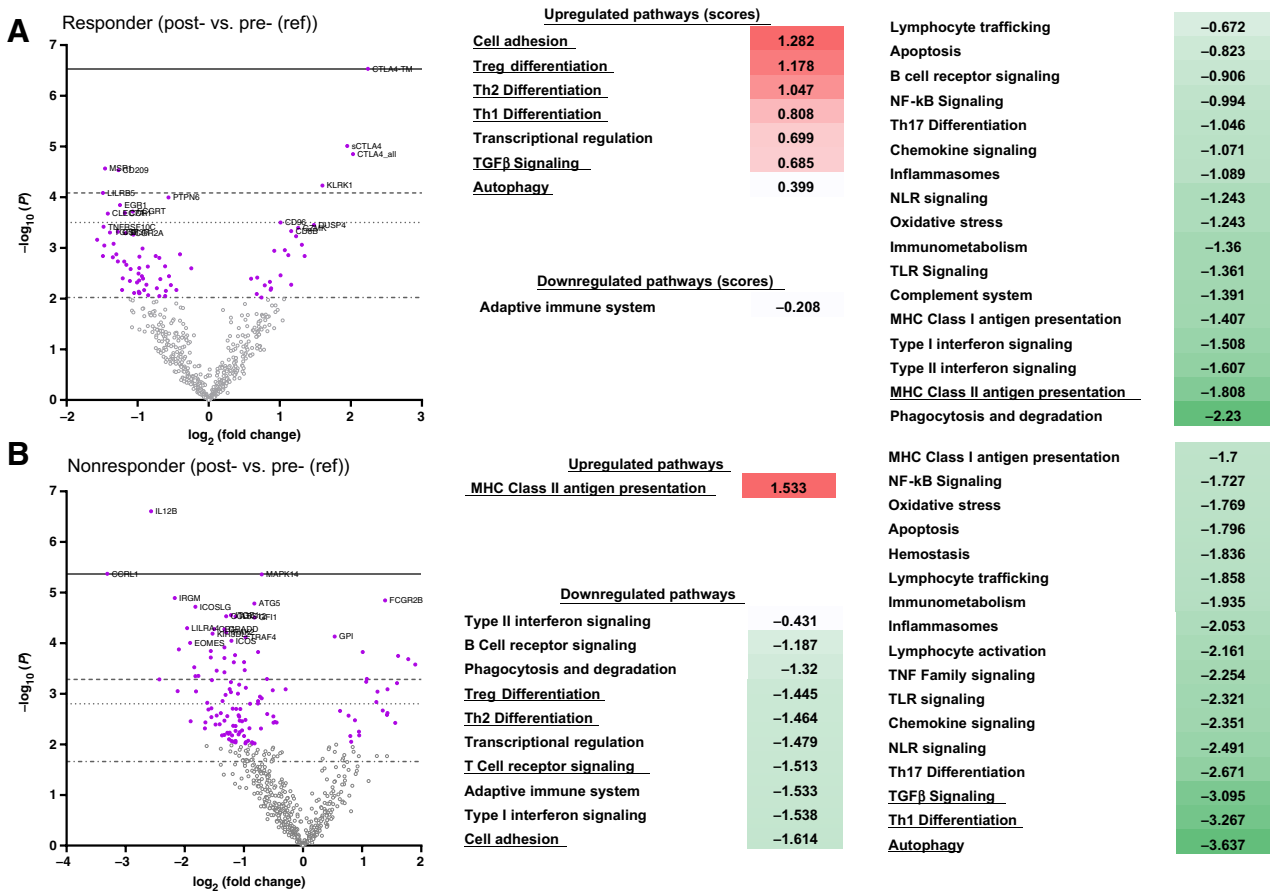
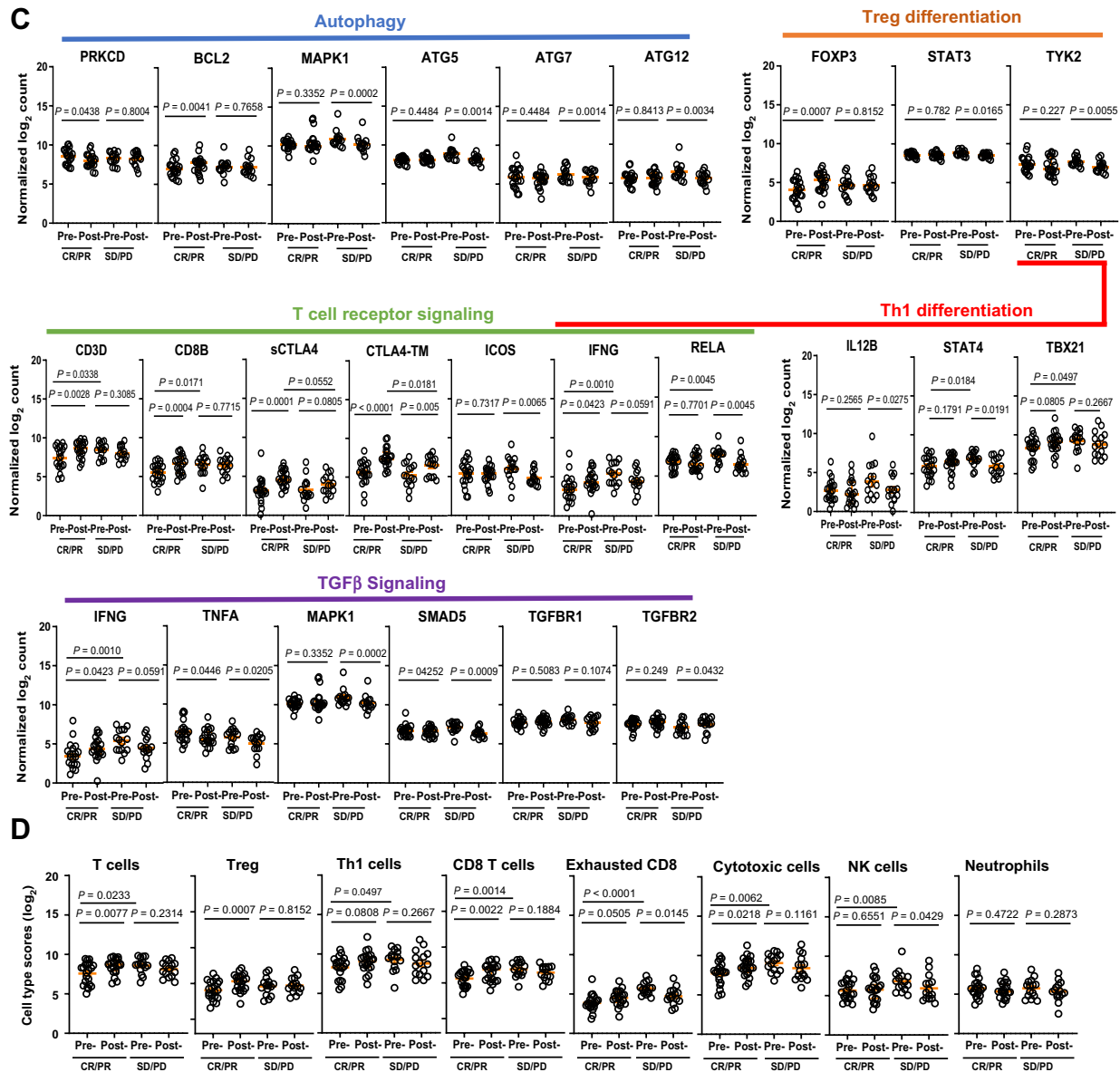


Figure 4. Distinct immune-related gene expression signature and immune cell types are associated with clinical response after IMT ± HMA. The gene expression profiling (GEP) with NanoString nCounter Immunology panel was performed on PBMCs collected from a patient with MDS pre- and post-treatment with IMT ± HMA (36 pairs from 21 responders and 15 nonresponders) and HMA alone (13 pairs from 9 responders and 4 nonresponders). Volcano plot was used to demonstrate the fold change in gene expression between two groups [$\log_2$ (fold change)] against its statistical significance [$\log_{10}$ (unadjusted P value)]. Gene with the FDR-adjusted P value of fold change less than 0.5 were in purple, and top 20 differentially expressed genes between two groups were labeled. Following thresholds represented FDR-adjusted P value less than 0.50 (— · — · —), 0.10 (·····), 0.05 (— — —), and 0.01 (————). Gene Set Enrichment Analysis was performed to investigate significantly upregulated or downregulated immune pathways between groups. The changes in gene expression and immune-pathways after IMT ± HMA compared to pretreatment (reference) were shown for responders in **A**, and nonresponders in **B**. Next, significantly regulated immune-pathways and immune cell types using NanoString gene sets according to the clinical response after IMT ± HMA were evaluated. (Continued on the following page.)

**Figure 4.**

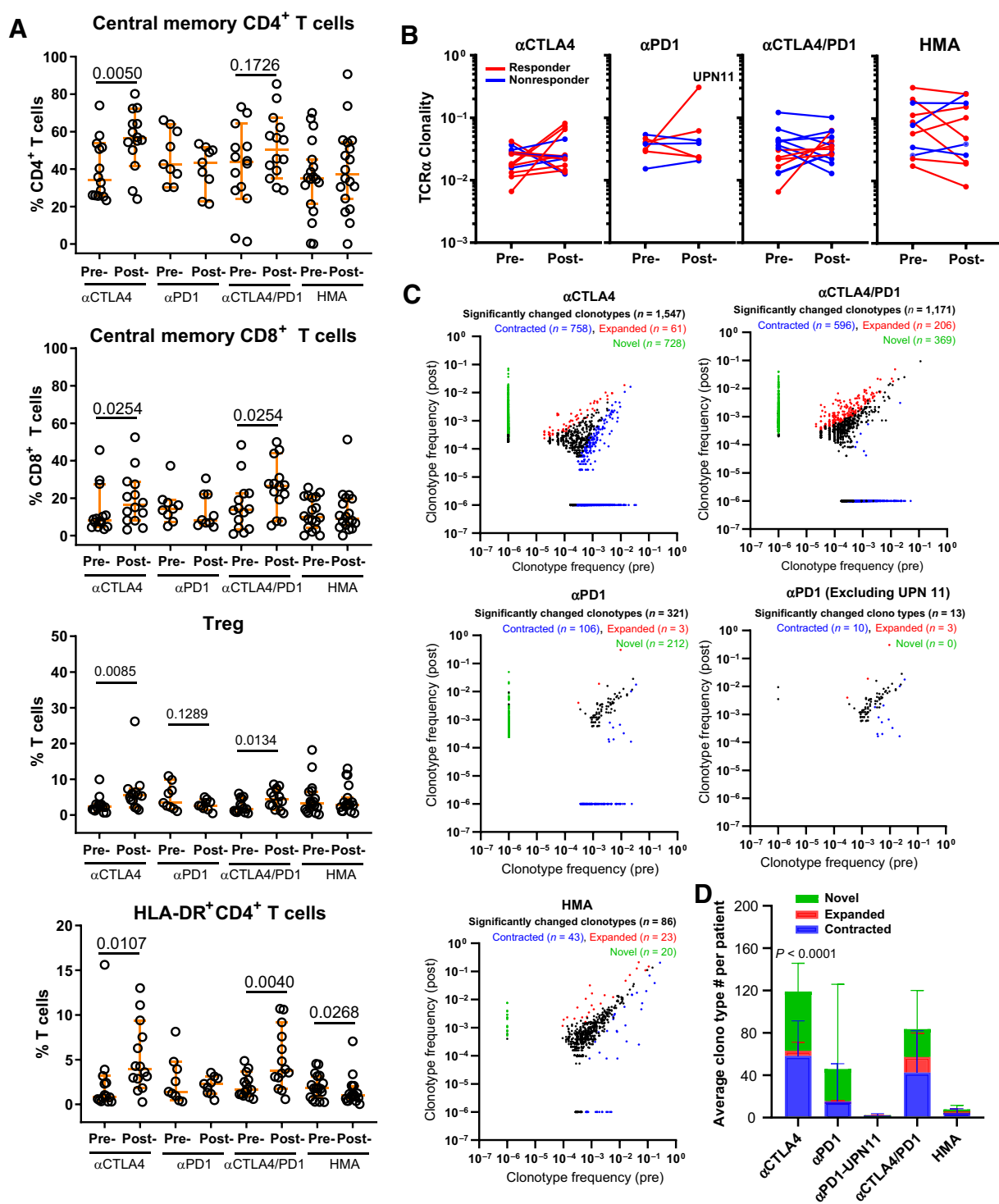
(Continued.) The normalized expression of significantly regulated genes from top upregulated or downregulated immune-pathways after HMA ± HMA in responders or nonresponders, respectively, are shown in **C**. Top upregulated genes in responders after treatment included CTLA-4, CD3D, CD8B, IFN γ , and TBX21 from TCR signaling pathways, and FOXP3 from Treg differentiation, while the majority of these genes except CTLA-4 were either downregulated or showed no significant changes in nonresponders. While responders did not have major changes in gene expression in TGF β signaling pathways except IFN γ , nonresponders downregulated almost all genes involved in TGF β signaling pathways. Major autophagy-related genes such as MAPK1, ATG5, ATG7, and ATG12 were significantly downregulated posttreatment in nonresponders while responders did not have significant changes in these genes except BCL2. Relative abundances of immune cell subpopulations estimated by CellType Analysis were shown in **D**. A symbol represents a value from one individual and mean $\pm$ SD was represented. Wilcoxon match paired signed rank or unpaired Mann-Whitney tests were used to assess statistical differences between two values within or across response groups, respectively. *P* value less than 0.05 was considered “significant”, and only significant or notable *P* values were presented.

Distinct immune-related gene expression signature is associated with clinical response during IMT

The presence of various mutations in epigenetic regulators may contribute to the immune dysregulation in MDS, and influence the therapeutic efficacy of immunotherapy (6). Here, we investigated the immune-related gene expression signature in the tumor-

immune microenvironment (TIME) associated with response or resistance during IMT, by performing targeted GEP and GSEA using a custom NanoString panel composed of 579 immune-related genes (Fig. 4).

First, we compared GEP and immune-related pathways between pre- and post-treatment in both responders and nonresponders to

**Figure 5.**

αCTLA-4 blockade drives the activation of CD4⁺ T cells, expansion of Treg and central memory T cells, and perturbation of TCRβ repertoire. **A**, PB T-cell subsets from MDS patients were assessed at pre- and post-treatments consisting of αCTLA-4 ± HMA, αPD-1 ± HMA, αCTLA-4/αPD-1 ± HMA, or HMA. Immune subset analysis revealed that the frequency of HLA-DR⁺CD4⁺ ($P = 0.0107$) and Treg ($P = 0.0085$ for % T cells) significantly increased in patients who received αCTLA-4 therapy ± HMA. Similarly, patients treated with αCTLA-4 plus αPD-1 ± HMA showed an increased frequency of HLA-DR⁺CD4⁺ T cells ($P = 0.0040$) and Treg ($P = 0.0134$ for % T cells). (Continued on the following page.)

explore the dynamic changes in individual TIME after IMT $\pm$ HMA treatment. Again, the expression of genes related to processes such as cell adhesion, T-cell differentiation (Th1, Th2, Treg), TGF β , and autophagy pathways was significantly upregulated after treatment in responders (Fig. 4A; Supplementary Table S2), while these pathways were reciprocally downregulated in nonresponders (Fig. 4B; Supplementary Table S3). Significantly regulated genes were shown in Fig. 4C and Supplementary Tables S2 and S3. In summary, our immune-related gene expression profiling analysis confirmed the activation and differentiation of T cells in responders, and suggests that downregulation of Th1 differentiation, TGF β , and autophagy pathways may be novel mechanisms of immune resistance that can be targeted to enhance the therapeutic efficacy of immune checkpoint blockade.

Finally, cell type profiling analysis showed that T cells, Th1 cells, CD8 cells, Treg, exhausted CD8 T cells, and cytotoxic cells were increased after treatment in responders, while assessed cell types except exhausted CD8 T cells and NK cells did not show significant changes posttreatment in nonresponders (Fig. 4D). Interestingly, nonresponders had a higher prevalence of T, Th1, and CD8 T cells, exhausted T cells, cytotoxic cells, and NK cells compared with responders in prior treatment, but most of the key immune cells were either downregulated or without significant change after treatment, suggesting that immune cell dysfunction beyond CTLA-4:B7 or PD-1:PD-L1/2 pathways may be present at baseline in nonresponders. In conclusion, T cell-mediated immunity in responders may be suppressed compared with responders prior to the treatment, and subsequently rejuvenated via immune checkpoint blockade.

α CTLA-4 blockade derives a favorable immune landscape in MDS during immunotherapy

As the CTLA-4/B7 or PD-1/PD-L1/2 pathways are involved in the activation of naïve or memory T cells, respectively, the blockade of these immune checkpoint interaction will likely elicit antitumor immunity via distinct mechanisms of action. Our study included cohorts of patients who received a series of immune checkpoint inhibitors, α CTLA-4 versus α PD-1 versus α CTLA-4 plus α PD-1 $\pm$ HMA, which may serve as confounding factor to determine predictive biomarkers for responses versus immune resistance. However, this heterogeneity of treatment in turn provides insights into which immune checkpoint blockade is attributed to favorable changes in the immune landscape. Moreover, we added HMA only group to interpret biologic changes for IMT only and in combination with HMA. First, immune subset analysis revealed that patients who received α CTLA-4 containing treatments showed increased frequencies of DR $^{+}$ CD4 $^{+}$ T cells, Treg, and central memory (CM) T cells (Fig. 5A). These findings suggest that CTLA-4 but not PD-1 blockade likely drove the activation of CD4 $^{+}$ T cells, expansion of Treg, and CM T cells observed in clinical responders.

Although there were no significant changes in TCR β clonality between pre- and post-treatment within each treatment cohort, α CTLA-4 and α CTLA-4/PD-1 $\pm$ HMA treatment resulted in a larger number of significantly changed clonotypes (1547, 1171 respectively) than treatment with α PD-1 $\pm$ HMA (321) or HMA alone (86; Fig. 5B and C). Within the treatment group, α CTLA-4 $\pm$ HMA had a significantly higher abundance of novel (728/1,547; 47.1%), and contracted clonotypes (758/1,547; 49.0%) than expanded clonotypes (61/1,547; 3.9%; $P < 0.0001$), while there were no significant differences in α PD-1 and α CTLA-4 plus α PD-1 groups (Fig. 5D). Considering that one responder (UPN11) in α PD-1 $\pm$ HMA attributed 305 of 321 significantly changed clonotypes, α CTLA-4 blockade likely contributed to the alteration of TCR β repertoire in responders.

Finally, we investigated differential immune-related gene expression signatures and pathways associated with each treatment to determine which immunotherapy elicited changes in the immune landscape favorable for clinical responses (Fig. 6; Supplementary Tables S4–S7). Both α CTLA-4 $\pm$ HMA and α CTLA-4 plus α PD-1 $\pm$ HMA upregulated genes related to pathways of TCR signaling, T-cell differentiation, and lymphocytes activation seen in responders while α PD-1 $\pm$ HMA failed to elicit such changes. There were synergistic effects of α PD-1 and α CTLA-4 treatment as α PD-1 plus α CTLA-4 $\pm$ HMA showed more upregulated genes and pathways compared to α CTLA-4 $\pm$ HMA. Interestingly, HMA alone drastically upregulated multiple immune-inflammation signaling pathways and genes supporting potential immune dysregulation in patients with MDS (Fig. 6D; Supplementary Table S7). Interestingly, TGF β signaling and autophagy pathways were downregulated in all treatment groups but were significantly upregulated in responders (Fig. 4). Thus, GEP analysis confirmed that α CTLA-4 blockade may contribute to shaping a favorable immune landscape to eliminate leukemia in patients with MDS.

Discussion

As immune checkpoint inhibitors are becoming an emerging therapy for hematologic malignancies including AML and MDS (11, 23–28), the investigation of the tumor-immune landscape in TIME in patients with MDS during immunotherapy can provide clinical response determinants and mechanistic insight into immune resistance in MDS during checkpoint blockade and help identify potential targets to overcome therapeutic resistance or synergize to immune checkpoint blockade. Daver and colleagues published the results of a phase II study of nivolumab in combination with 5-Azacitidine in relapsed/refractory AML (29), and showed that clinical responders had higher effector CD4 $^{+}$ T and CD8 $^{+}$ T cells in the bone marrow than nonresponders prior to treatment, and that the increased frequencies of CTLA-4 $^{+}$ CD4 $^{+}$ or

(Continued.) In addition, fractions of CM CD4 $^{+}$ and CD8 $^{+}$ T cells increased significantly in patients who received α CTLA-4 $\pm$ HMA ($P = 0.0050$ and $P = 0.0254$, respectively). A similar pattern was observed in patients treated with α CTLA-4 plus α PD-1 $\pm$ HMA with a significant increase in fraction of CM CD8 $^{+}$ T cells. A symbol represents a value from one subject. Mean $\pm$ SD was represented. Wilcoxon match paired signed rank were used to assess statistical differences within the treatment groups. P value less than 0.5 was considered as “significance”, and only significant P value or notable P values were presented. **B**, Changes in TCR β clonality in patients after following treatment: α CTLA-4 $\pm$ HMA, α PD-1 $\pm$ HMA, α CTLA-4/ α PD-1 $\pm$ HMA, or HMA. A symbol represents a value from one subject. Matching pre and post values were lined. Responders were presented in red symbol and line, and nonresponders were presented in blue symbol and line. Wilcoxon match paired signed rank were used to assess statistical differences within the treatment groups. P value less than 0.5 was considered as “significance”, and only significant P values were presented. **C**, Individual clonotype frequencies at pre- and post-treatment (α CTLA-4 $\pm$ HMA, α PD-1 $\pm$ HMA, α PD-1 $\pm$ HMA excluding UPN11, α CTLA-4/ α PD-1 $\pm$ HMA, or HMA) were plotted. Significantly changed clonotypes—contracted, expanded, and novel clonotypes after the treatment were marked in green, red, or blue, respectively. **D**, Average number of significantly changed clonotypes per patients following treatment (α CTLA-4 $\pm$ HMA, α PD-1 $\pm$ HMA, α PD-1 $\pm$ HMA excluding UPN11, α CTLA-4/ α PD-1 $\pm$ HMA, or HMA) were shown. Kruskal–Wallis test was used to compare differences among contracted, expanded, or novel clonotypes within the group, and P value less than 0.05 was considered “significant”. Only significant P value was presented. Although there was no significant change in overall TCR β clonality following all treatments, α CTLA-4 containing treatment elicited a larger number of clonotypes that were significantly changed (novel, contracted) after the treatment.



CD8⁺ T cells after treatment were associated with nonresponders. Furthermore, paired single-cell RNA analysis and TCR repertoire profiling showed that the emergence of novel CD8⁺ T-cell clonotypes and CD8⁺ T-cell phenotypes with stem-like properties expressing granzyme K were associated with responders (30). We observed significant increases in activated T cells, CM T cells, and Treg within the T-cell compartment after IMT ± HMA, and did not observe significant differences in major T-cell subsets between responders and nonresponders either in pre- or post-treatment (Fig. 1). These dynamic changes were observed only in patients who received αCTLA-4 containing treatment (αCTLA-4 ± αPD-1 ± HMA) but not αPD-1 ± HMA or HMA alone, suggesting that αCTLA-4 blockade may play a critical role in eliciting favorable T-cell responses. These findings reflected on clinical trial results recently reported by Zeidan and colleagues where a phase II study of 5-Azacididine with or without durvalumab for higher-risk MDS did not show significant improvement in clinical outcomes by combination therapy (31). Moreover, we found higher levels of T, Th1, CD8 T cells, cytotoxic T and NK cells in nonresponders prior to treatment, which failed to expand after treatment (Fig. 4D). Finally, we observed an increased expression of soluble and transmembrane CTLA-4 transcripts after treatment, which was significantly higher in responders than in nonresponders (Fig. 4C). The differences in findings between the work by Daver and colleagues and our study are likely due to the different types of immune checkpoint blockade patients received – αPD-1 versus CTLA-4 ± αPD-1, location of TIME analyzed – peripheral blood versus bone marrow, and nature of diseases – robust nature of AML compared with MDS. As we investigated the coevolution of the immune and genetic landscapes in PBMCs as a surrogate TIME to the bone marrow of patients with MDS during immunotherapy, further investigation using bone marrow samples warrant.

T cells are key players in the antitumor response mediated by immunotherapies. Upon recognizing cognate antigens, activated T cells can differentiate and proliferate, a process called “clonal expansion” (32). Therefore, determining T-cell clonality even in peripheral blood can provide information on the degree of tumor antigen-driven T-cell expansion and mechanistic insights into the immune checkpoint blockade (28, 33, 34). In our study, clinical responders had a diverse TCRβ repertoire prior to treatment compared with nonresponders, followed by an increase in the TCRβ clonality with the appearance of novel T-cell clones after treatment (Fig. 3). Interestingly, CTLA-4 rather than PD-1 blockade appears to have driven a favorable perturbation in the TCRβ repertoire, which coincides with the expansion of CM T cells, Treg, and activated T cells observed in patients with CTLA-4 blockade (Fig. 5). Our findings support the association of diverse TCRβ repertoire pretreatment and the appearance of novel TCRβ clones post treatment with a clinical response after immune checkpoint blockade (30, 34). Although HMA treatment may have contributed to the expansion of rare TCR clones in clinical responders (Fig. 2; ref. 35), a larger degree of oligoclonal expansion of novel T-cell clones was observed in responders after IMT ± HMA, especially αCTLA-4 blockade ± HMA, compared with HMA alone (Fig. 2). The reason that we did not observe emergence of novel TCR

clones in clinical responders from HMA alone, differently from previous report (35) may be due to the potential enrichment of tumor specific T cells in bone marrow (35) versus peripheral blood (our study). As clinical responders had a higher tumor mutation burden with subsequent reduction after IMT ± HMA, our findings support the potential antitumor activity of novel T-cell clones in MDS during immunotherapy.

The IFNγ receptor signaling pathway and “T cell inflamed” TIME has been shown to play an essential role in antitumor T-cell responses elicited by immune checkpoint blockade (36–41). More specifically, the increased frequencies of ICOS⁺ CD4⁺ T cells in peripheral blood or TIME after αCTLA-4 or αCTLA-4/αPD-1 therapy were correlated with clinical response in metastatic melanoma and urothelial carcinoma, respectively (42, 43). Our results are in line with previous findings in that type I and II IFN signaling was upregulated in pretreatment peripheral blood of clinical responders, but not supportive of “T-cell inflamed” TIME as several pathways involved in T-cell differentiation, TCR signaling, and T-cell trafficking were downregulated in TIME of clinical responders compared with nonresponders. Nonetheless, these pathways were upregulated in clinical responders after αCTLA-4-containing therapy supporting the role of T cell-mediated antitumor immunity in MDS during checkpoint blockade ± hypomethylation. Interestingly, we observed a significant upregulation in the MHC II antigen presentation pathway in the pretreatment TIME of clinical responders, which was likely driven by concurrent treatment with a hypomethylator. Antigen processing and presentation, one of the major pathways modulated by IFNγ, is a key step to initiate T cell-mediated immune responses and its defects were attributed to acquired immune resistance to immunotherapy (44, 45). Therefore, our findings highlight a critical role of the antigen presentation machinery in eliciting effective antitumor immunity in MDS during checkpoint blockade.

Tumors evade immune surveillance and checkpoint blockade through multiple mechanisms such as defective IFNγ signaling or antigen presenting pathway in tumor impairing the effective T-cell activation (40, 41, 44, 45), and aberrations in Wnt/b-catenin signaling or loss of PTEN or LKB1 in tumor avoiding “T-cell inflamed” TIME by the reduction of intratumoral trafficking of T cells (46–49). In our work, gene expression profiling of peripheral blood TIME revealed that autophagy, Th1 differentiation, and TGFβ signaling pathways were the top three pathways significantly downregulated in nonresponders after checkpoint blockade ± hypomethylation. In addition, the majority, if not all, of the pathways related to T-cell differentiation, signaling, and activation were downregulated in nonresponders. Our findings echo the importance of T cell-mediated antitumor immunity and support the ICOS–ICOSL interaction of the TCR signaling pathway as a feasible target to synergize the efficacy of checkpoint blockade in MDS (Fig. 4C; ref. 50). The differential regulation of the TGFβ signaling pathway along with Th2 and Treg pathways seen in clinical responders (upregulated) and nonresponders (downregulated) may reflect compensatory immune suppression toward T cell-mediated inflammation during immunotherapy, rather than the mechanism of tumor–immune evasion given the suppressive role of

Figure 6.

αCTLA-4 blockade shapes a favorable immune-landscape in MDS during immunotherapy. **A–D**, Differential gene expression between pre- and post-treatment [αCTLA-4 ± HMA (14 pairs), αPD-1 ± HMA (8 pairs), αCTLA-4/αPD-1 ± HMA (14 pairs), or HMA (13 pairs)] were assessed. A volcano plot was used to demonstrate the fold change in gene expression after treatment [$\log_2(\text{fold change})$] against its statistical significance [$\log_{10}(\text{unadjusted } P \text{ value})$]. Genes with a FDR-adjusted *P* value of fold change less than 0.5 were in purple, and the top 40 differentially expressed genes between two groups were labeled. The following thresholds represented FDR-adjusted *P* value less than 0.50 (— · — · —), 0.10 (·····), 0.05 (— — —), and 0.01 (————). GSEA was performed to investigate significantly upregulated or downregulated immune pathways between pre- and post-treatment.

TGF β signaling during inflammation to maintain immune homeostasis (51). Finally, we observed an opposite regulation of autophagy pathways between clinical responders (upregulation) and nonresponders (downregulation) after checkpoint blockade $\pm$ hypomethylation. Autophagy is a cellular process that adapts to metabolic stress through the degradation and recycling of intracellular components to produce energy. While aberrant autophagy pathways are thought to participate in the pathogenesis and prognosis of MDS, autophagy regulation plays a key role in the homeostasis and function of various immune cells (52–54). In T cells, autophagy pathways are activated through TCR-mediated signaling or common gamma chain containing cytokine receptor signaling, and control essential programs in the activation, proliferation, differentiation, metabolism and function of T cells. As such, the downregulation of the autophagy pathway after checkpoint blockade suggests the presence of defective T cell-mediated immunity in nonresponders and supports the concurrent use of autophagy inducers such as mTOR inhibitors with checkpoint blockade to improve T-cell function during immunotherapy in MDS.

Authors' Disclosures

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Authors' Contributions

S. Lee: Conceptualization, investigation, writing—original draft. F. Wang: interpreted results. M. Greife: Writing—review and editing. A. Trujillo-Ocampo: Investigation. W. Ruiz-Vasquez: Investigation. K. Takahashi: Interpreted results. H.A. Abbas: Interpreted results. P. Borges: interpreted results. D.A. Antunes: interpreted results. G. Al-Atrash: Writing—review and editing. N. Daver: Writing—review and editing. J.J. Molldrem: Writing—review and editing. A. Futreal: Writing—review and editing. G. Garcia-Manero: Resources, writing—review and editing. J.S. Im: Conceptualization, writing—original draft.

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