SUPPLEMENTARY MATERIALS

Methods

Isolation of mononuclear cells and flow cytometric analysis

Mononuclear cells from peripheral blood (PB) and BM specimens were obtained by Ficoll-Hypaque density gradient centrifugation, then cryopreserved and stored in liquid nitrogen until use. For flow cytometric analysis, specimens were stained for multicolor panels including viability-, surface- and intracellular-markers, according to the manufacturer's instructions and based on previous experience.[1-3] Human FC receptor block (BioLegend) to prevent nonspecific binding and all antibodies used are listed in **Supplemental Table 1.** The stained specimens were analyzed on a BD LSRII Flow Cytometer (Becton Dickinson Immunocytometry Systems). Data were analyzed post-collection using either BD FACSDiva V8.0.1 (Becton Dickinson, Franklin Lakes, NJ) or FlowJo (Tree Star) software packages. Data are presented as frequencies/percentage expression or as total numbers, which were calculated as described previously (Kanakry et al., Blood 2011, PMID: 20935254);

Cell Sorting

Mononuclear cells were stained with the following surface antibodies CD45 – PE (BD), CD56 – PECF594 (eBioscience), CD3 –APC (eBioscience), CD4 – BV605 (BioLegend), CD8 – PerCP-Cy5.5 (eBioscience). Sorting of CD4+ and CD8+ T cells (CD45hi, SSClo, CD56-, CD3+, CD4+/-, CD8+/-) was performed on a Becton Dickinson FACSAria II (BSL-2). A purity of >90% was achieved in all specimens consistent with previous work.[1-3]

T-cell receptor sequencing and sequence analysis

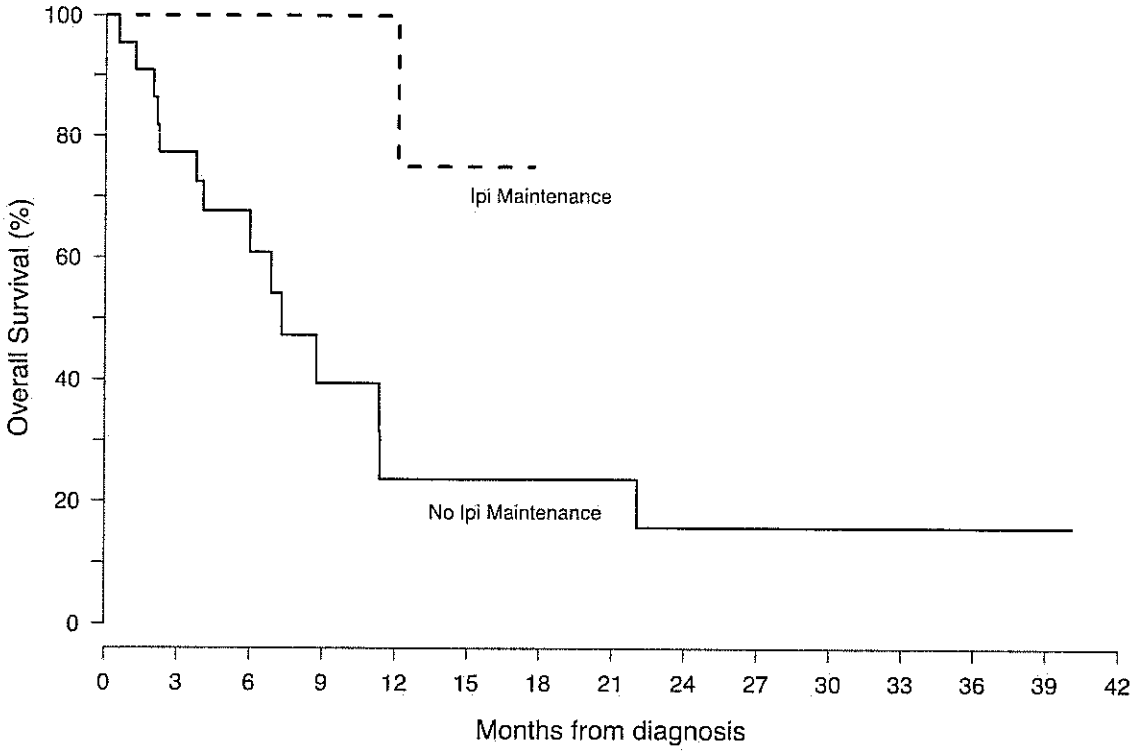
Sorted $CD4^+$ and $CD8^+$ T cells were subjected to standard DNA extraction and analysis as described previously.[1] PBMC were sorted for the CD4+ and CD8+ T cell populations prior to isolating genomic DNA using the DNEasy Blood and Tissue Kit (Qiagen, Valencia, CA). Sequencing of *TRB* loci was performed at Adaptive Biotechnologies (Seattle, WA) using the immunoSEQ assay at survey level resolution or at the FHCRC using the hsTCRB kit (Adaptive Biotechnologies).[1,4-6] Initial analysis of sequence reads was performed using the immunoSEQ suite of software tools (Adaptive Biotechnologies). Repertoire analyses and longitudinal tracking of sequence frequency over time were performed using a package of software tools (LymphoSeq; available for download from http://www.bioconductor.org) created by D.G. Coffey and implemented in the R statistical computing environment. The raw sequence data will be made publicly available for download upon manuscript acceptance.

Clonality was calculated as the reciprocal of normalized Shannon's entropy as reported by our group previously. Clonality values range from 0 to 1, with lower values representing more diverse populations. The Gini coefficient is calculated as the ratio of area between the line of equality and the Lorenz curve to the area under the line of equality. Values range from 0 to 1, with 0 indicating equal frequency of all clonotypes, and 1 indicating a monoclonal sample. Mean clonality was compared using two-sample t-tests.[1] Two-sided p-values < 0.05 were considered statistically significant, and no adjustment was made for multiple comparisons.

Finally, we aligned the sequences identified from TCRβ sequencing against a database at the Fred Hutchinson Cancer Research Center containing over 205 million TCR sequences from healthy controls, patients with a variety of solid tumors, or patients with chronic infections such as HIV to determine if there were unique sequences in multiple patients in our study that may be directed

towards specific myeloid tumor antigens. Using a cutoff of finding the CDR3 sequence in at least 5 samples from our patients and with zero prevalence in the database, we identified 20 unique CDR3 sequences as potential candidates for MDS-specific T cells **[Supplemental Figure 9 and Supplemental Table 3].** If we relaxed the criteria and searched for sequences found in > 5 samples from the study with a prevalence less than or equal to 40, we were able to identify a total of 42 unique CDR3 sequences **[Supplemental Table 4]**

Supplementary Figures



No. at risk															
No Ipi Maintenance	22	17	9	5	3	3	3	3	2	2	2	1	1	1	0
Ipi Maintenance	7	7	7	5	4	2	0	0	0	0	0	0	0	0	0

Figure S1. Continuing therapy through maintenance phase is positively correlated with survival. Overall survival of the study participants as depicted by Kaplan-Meir curves stratified by those who received maintenance therapy (N=7) and those who did not receive maintenance (N=22).

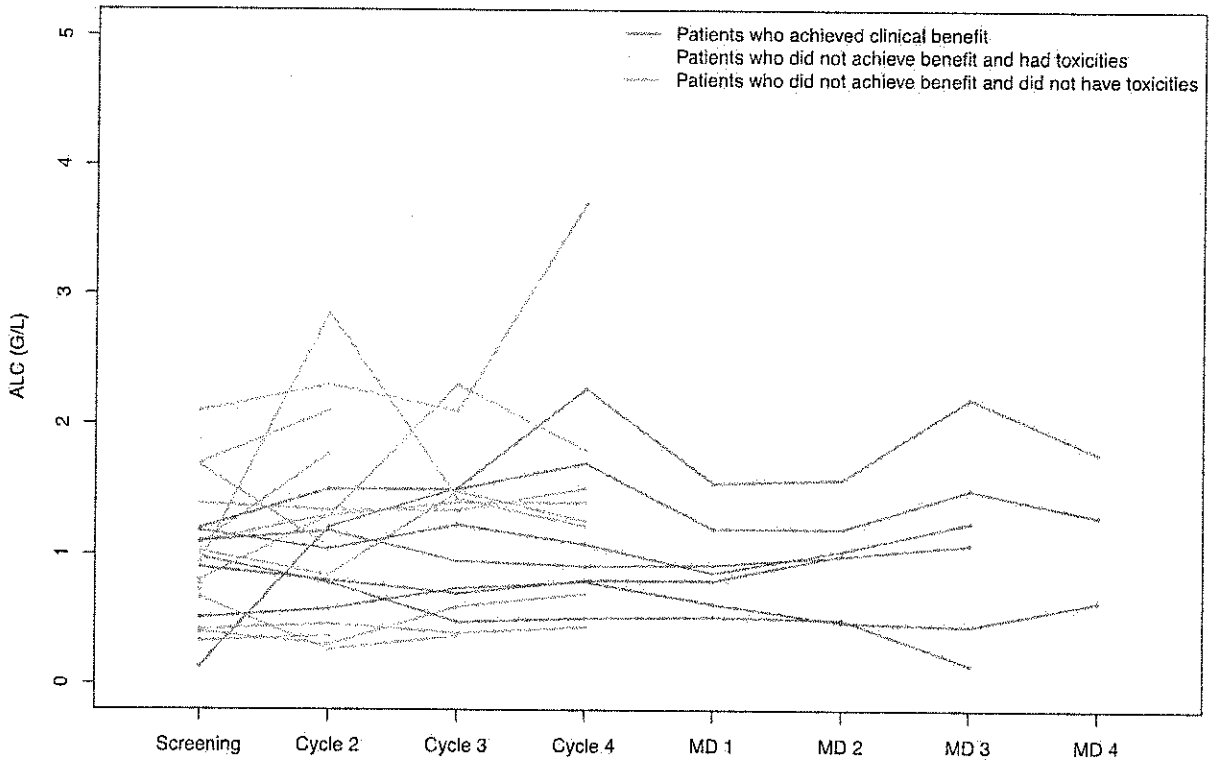


Figure S2. Serial changes in the absolute lymphocyte count (ALC).

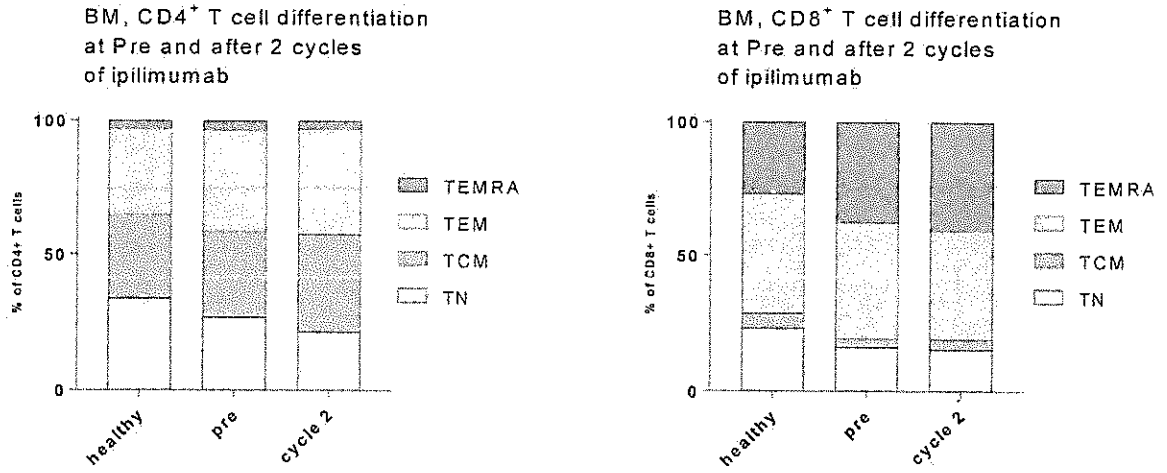


Figure S3. Differentiation status of BM T-cells from MDS patients. Freshly frozen BMMCs prospectively collected from MDS patients before and after 2 cycles of ipilimumab treatment (n=9) and healthy donors (n=8) were immuno-phenotyped. Percentages of four major T cell differentiation subsets, naïve T cells (T_N, $CCR7^+CD45RA^+$), central memory T cells (T_{CM}, $CCR7^+CD45RA^-$), effector memory T cells (T_{EM}, $CCR7^-CD45RA^-$), and terminally differentiated effector cells (T_{EMRA}, $CCR7^-CD45RA^+$). $CD4^+$ T cells are presented on the left, $CD8^+$ T cells are shown on the right. Data are presented as the mean percentages of the different T cell subsets.

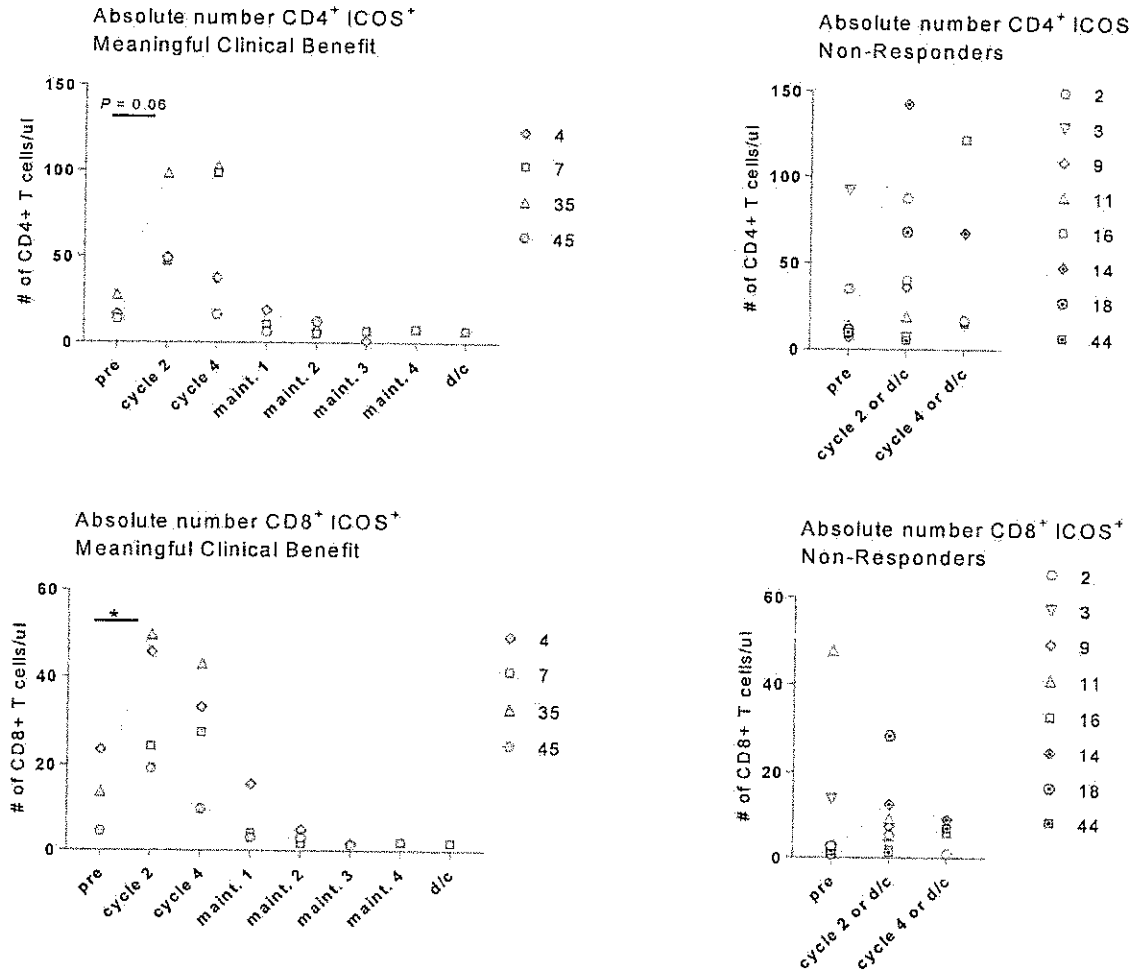


Figure S4. Absolute numbers of ICOS+ T cells before and after ipilimumab treatment. Absolute numbers of $CD4^+ICOS^+$ (top) and $CD8^+ICOS^+$ (bottom) per microliter peripheral blood after treatment with 3 or 10 mg/kg/dose of anti–CTLA-4. Data are presented for 4 patients who achieved Meaningful Clinical Benefit (MCB, left; UPIN 04/07 = prolonged SD and 35/45 = mCR) and 6 non-responding patients (right; UPIN 14/18/44 = PD and UPIN 02/03/08 = PD with IRAE). Dotted lines indicate patients with IRAEs/toxicity. Statistical comparisons were calculated in R as described in Materials and Methods with * $P \leq 0.05$.

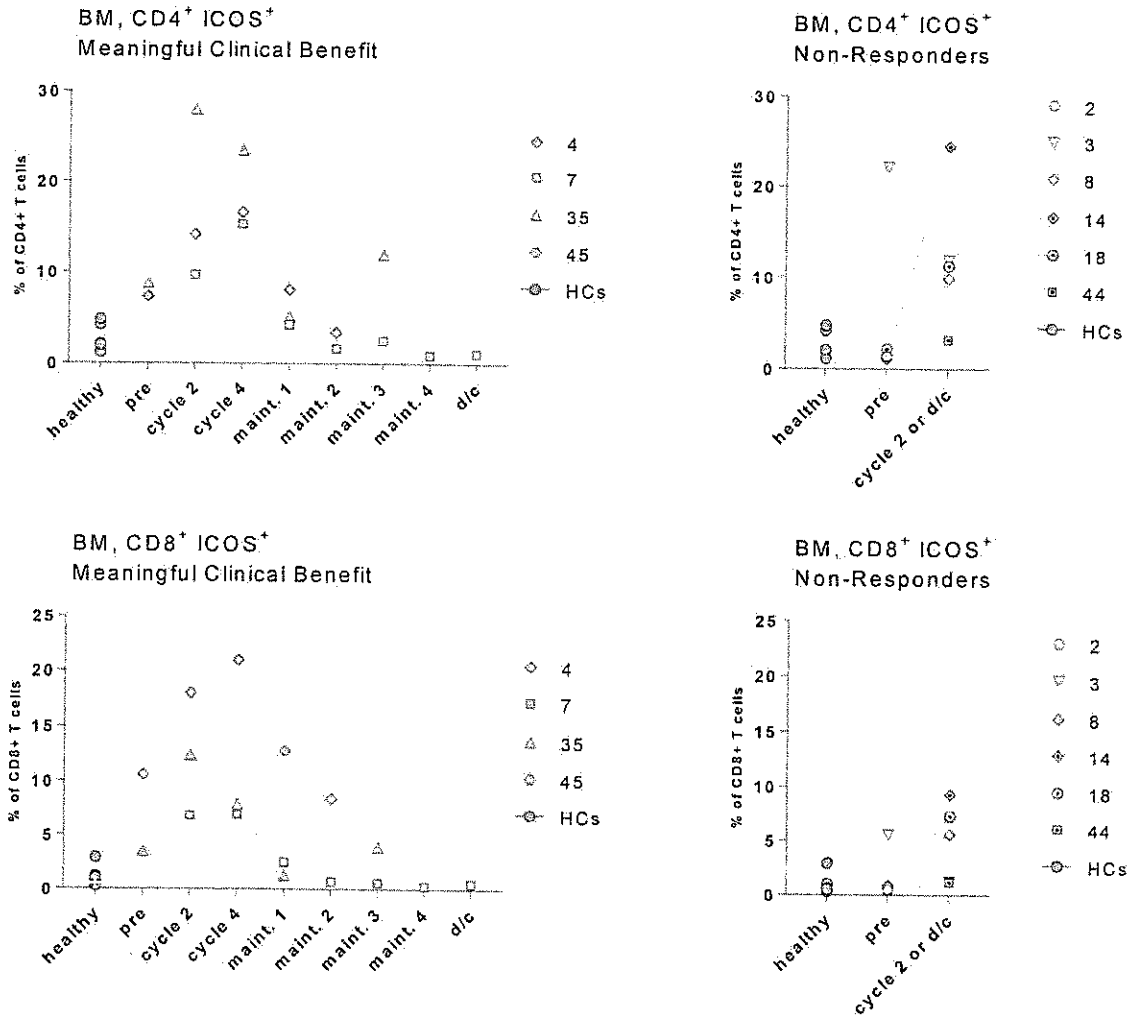


Figure S5. Frequencies of ICOS expression in bone marrow samples before and after ipilimumab treatment. Immunophenotyping by flow cytometry was performed on BMMC samples collected from HR-MDS patients (n=10) at multiple timepoints before and after ipilimumab treatment as well as on HC BMMC samples (n=6). $CD4^+ICOS^+$ (top) and $CD8^+ICOS^+$ (bottom) PB T-cells after treatment with 3 or 10 mg/kg/dose of anti–CTLA-4. Data are presented for 4 patients who achieved Meaningful Clinical Benefit (MCB, left; UPIN 04/07 = prolonged SD and 35/45 = mCR) and 6 non-responding patients (right; UPIN 14/18/44 = PD and UPIN 02/03/08 = PD with IRAE). Dotted lines indicate patients with IRAEs/toxicity.

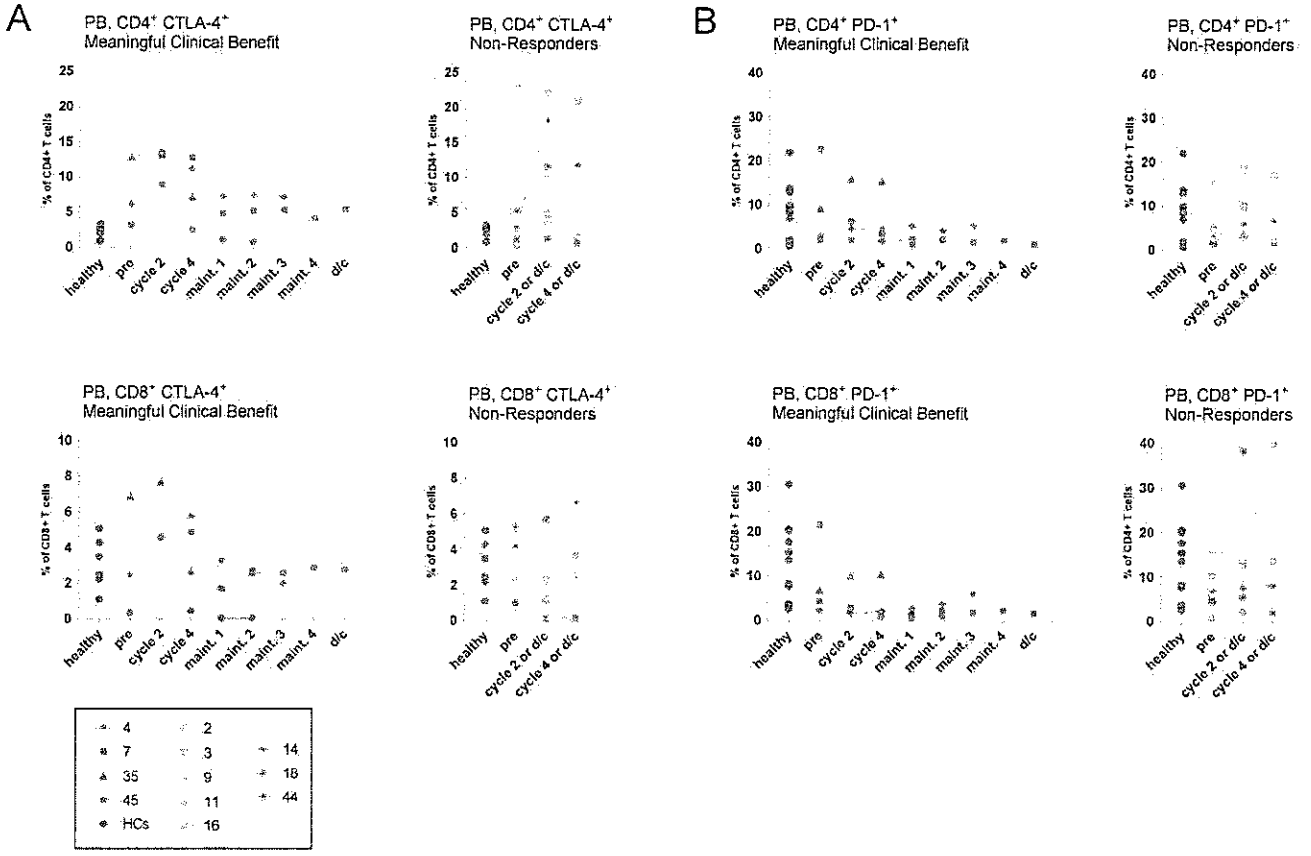


Figure S6. Ipilimumab treatment is associated with increased CTLA-4 expression among patients who achieved clinical benefit but doesn't lead to up-regulation of alternate checkpoint molecules like PD-1. Immunophenotyping by flow cytometry was performed on PBMC samples collected from MDS patients (n=12) at multiple timepoints before and after ipilimumab treatment as well as on HC PBMC samples (n=12). $CD4^+$ and $CD8^+$ T cells expressing **(A)** CTLA-4, and **(B)** PD-1 are presented for 4 patients who achieved MCB left; UPIN 04/07 = prolonged SD and 35/45 = mCR) and 8 non-responding patients (UPIN 14/18/44 = PD and UPIN 02/03/09/11/16 = PD with IRAE). Dotted lines indicate IRAEs/toxicity.

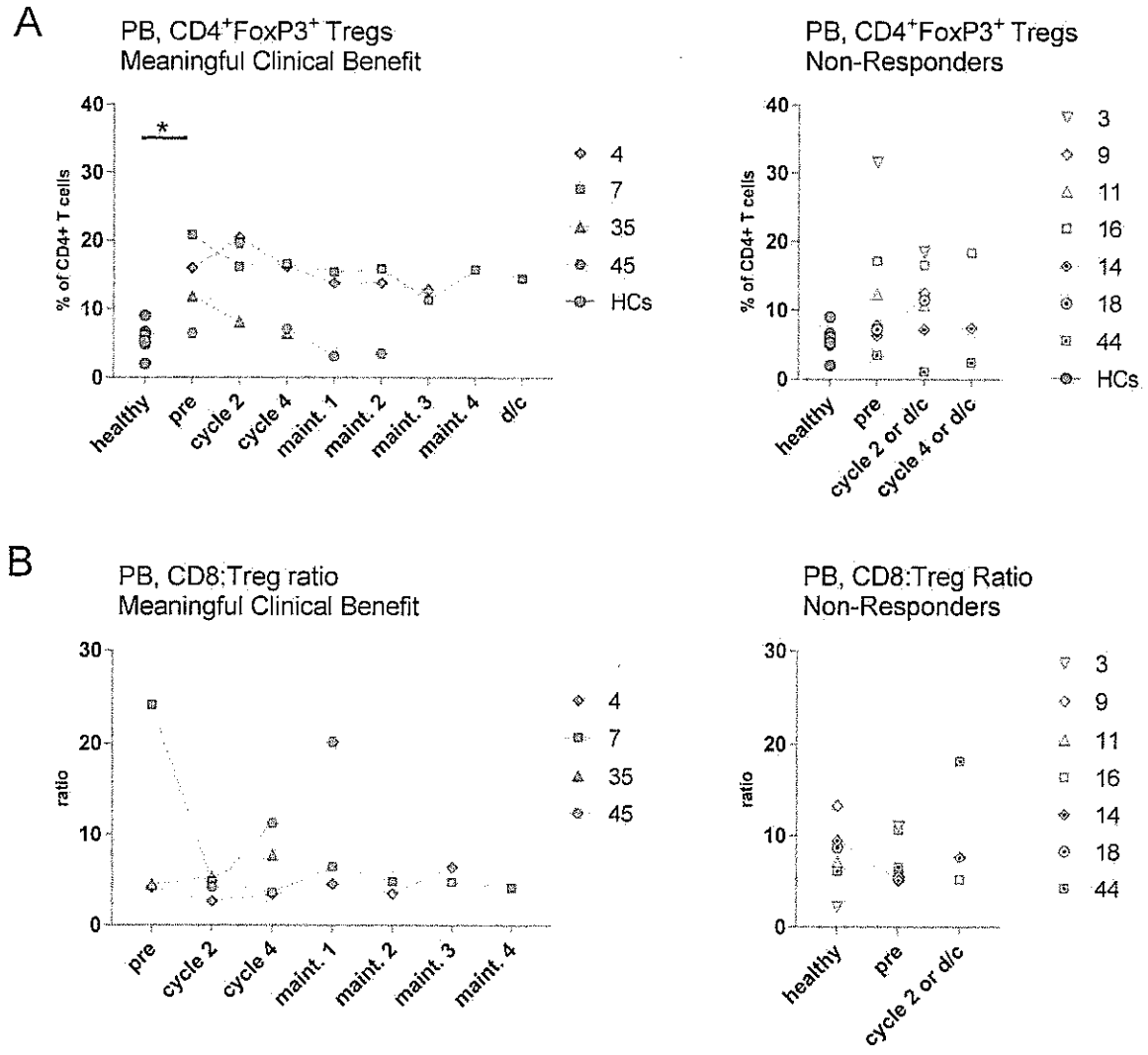


Figure S7. Tregs persist at elevated levels throughout all time points and are not affected by ipilimumab treatment. Immunophenotyping by flow cytometry was performed on PBMC samples collected from MDS patients (n=11) at multiple timepoints before and after ipilimumab treatment as well as on HC PBMC samples (n=11). **(A)** Percentages of $CD4^+FoxP3^+$ T cells, and **(B)** CD8:Treg ratios are presented for 4 patients who achieved MCB, left; UPIN 04/07 = prolonged SD and 35/45 = mCR) and 8 non-responding patients (right; UPIN 14/18/44 = PD and UPIN 03/09/11/16 = PD with IRAE). Dotted lines indicate IRAEs/toxicity. Statistical comparisons were calculated in R as described in Materials and Methods with * $P \leq 0.05$.

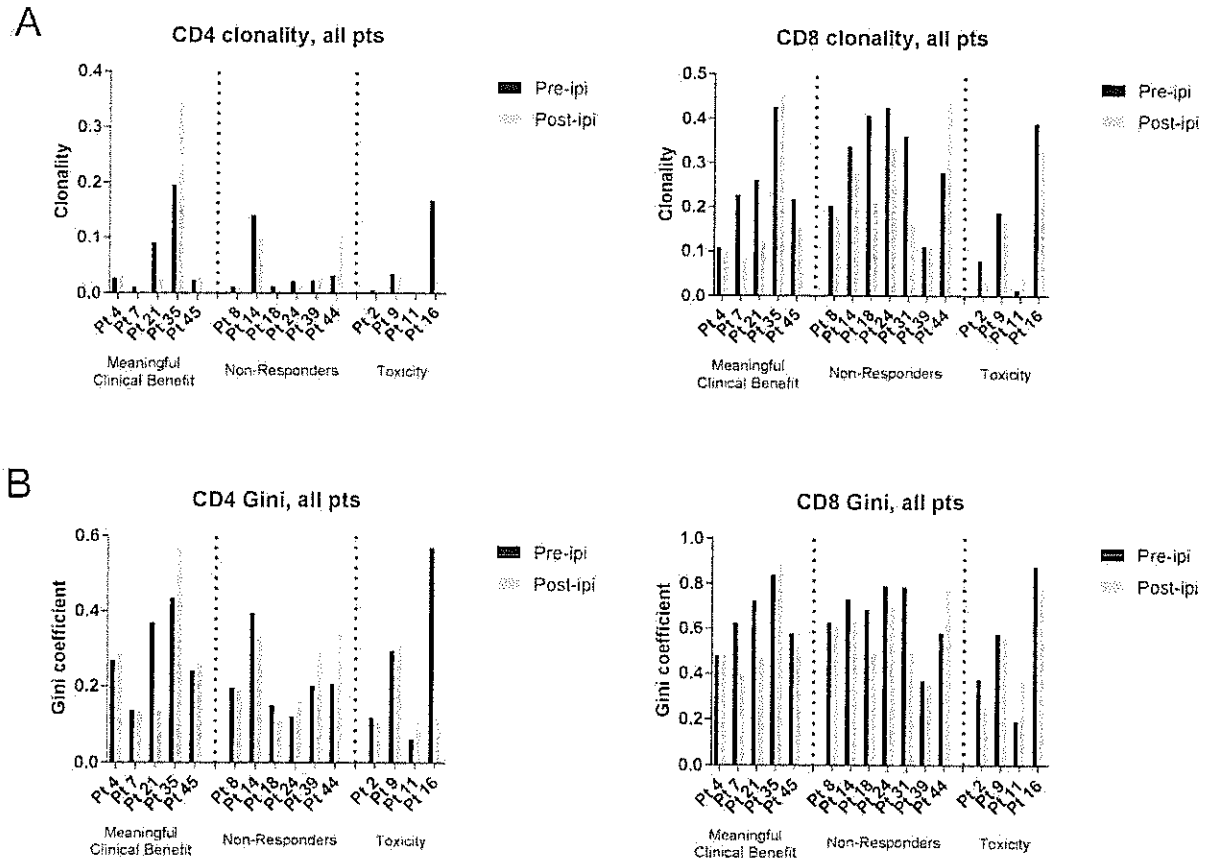


Figure S8. Ipilimumab treatment increases peripheral T cell receptor diversity but does not correlate with clinical outcomes. PBMC samples from 16 patients, both at baseline and after 2 cycles of ipilimumab treatment, were sorted into $CD4^+$ and $CD8^+$ T cells and subjected to survey level next-generation high throughput sequencing of the TCR Vβ CDR3 region. Patients were chosen to represent 3 subgroups: 1) Patients who achieved MCB (n=5); 2) patients who progressed/did not achieve MCB (n=7), and 3) patients who developed IRAEs to ipilimumab treatment and had to be taken off study (n=4). TCR diversity for both CD4+ (left) and CD8+ T cells (right) was measured by calculating **(A)** Clonality (top) and **(B)** Gini Coefficient (bottom) as described in the supplemental methods. Changes in clonality from individual patients shown in these graphs were averaged and results are shown in figure 3.

A

B

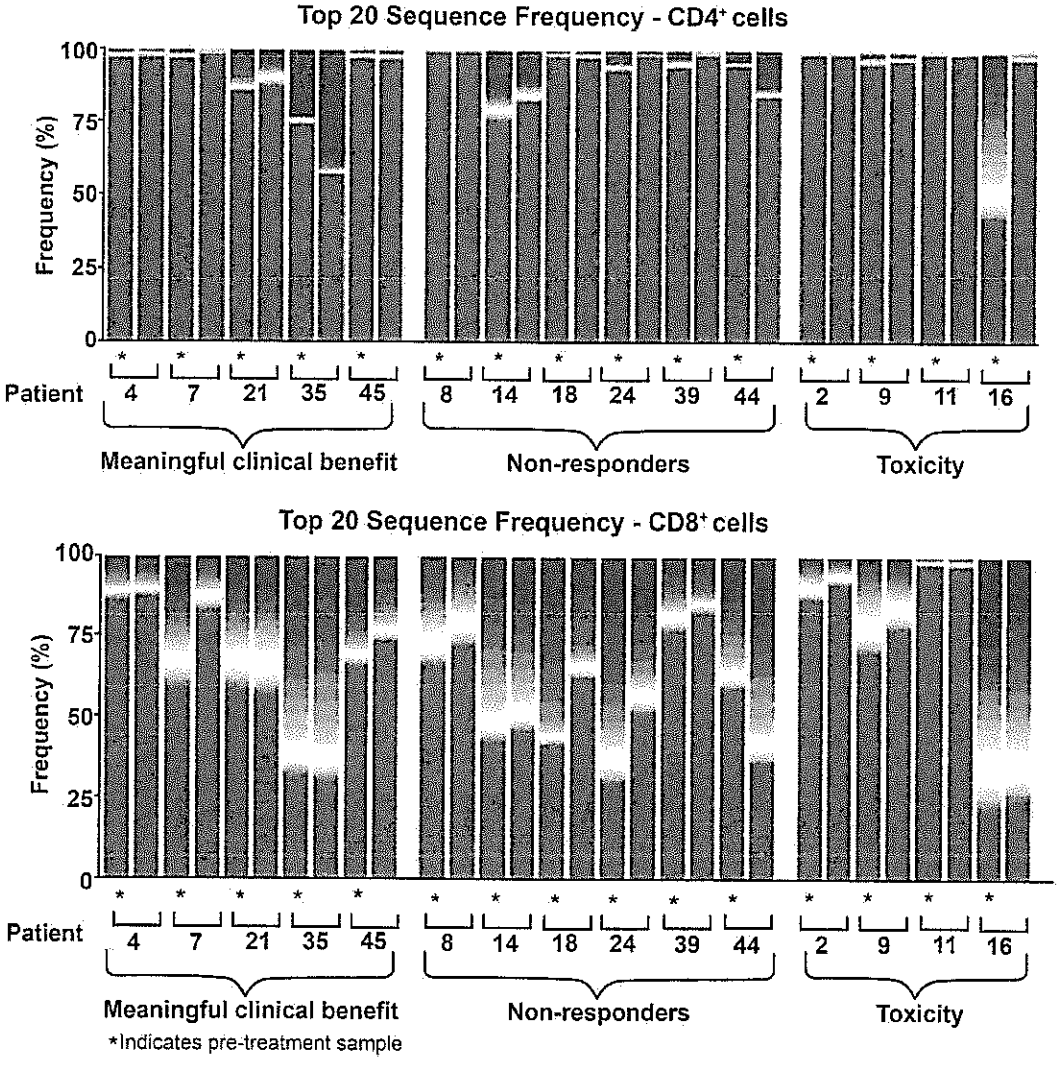

Top 20 Sequence Frequency - CD4+ cells
Frequency (%)
100
75
50
25
0
Patient
4 7 21 35 45 8 14 18 24 39 44 2 9 11 16
Meaningful clinical benefit
Non-responders
Toxicity
Top 20 Sequence Frequency - CD8+ cells
Frequency (%)
100
75
50
25
0
Patient
4 7 21 35 45 8 14 18 24 39 44 2 9 11 16
Meaningful clinical benefit
Non-responders
Toxicity
*Indicates pre-treatment sample

Figure S9. Top 20 most frequent T cell clones identified by T cell receptor sequencing. (A-B) Top 20 most frequent T cell clones for **(A)** $CD4^+$ T-cells and **(B)** $CD8^+$ T-cells. The 20 most frequent unique *TRB* sequences are indicated by the colored horizontal bars at the top of each column (each clone is presented as different color); the violet area at the bottom of each column represents the fraction of all reads comprised by all of the remaining sequences in that dataset. Two columns are presented for each patient with the first one representing pretreatment a sample, and the second column representing a sample after 2 cycles of ipilimumab treatment.

Table S1. Antibodies used for correlative studies

Antigen	Flourochrome	Clone	Company	Isotype
CD45	PE	HI30	BD	Mouse IgG_1, κ
CD3	AF700	HIT3a	BioLegend	Mouse IgG2a, κ
CD3	evolve 605	OKT3	eBioscience	Mouse IgG2a, κ
CD4	BV605	OKT4	Biolegend	Mouse IgG2b, κ
CD8	PerCP-Cy5.5	RPA-T8	eBioscience	Mouse IgG_1, κ
CD45RA	APC-Cy7	HI100	BioLegend	Mouse IgG2b, κ
CCR7	BV650	G043H7	BioLegend	Mouse IgG2a, κ
CD56	PE-Cy7	HCD56	BioLegend	Mouse IgG_1, κ
CD56	PECF594	CMSSB	eBioscience	Mouse IgG_1, κ
PD-1 (CD279)	APC	MIH4	BD	Mouse IgG_1, κ
FoxP3	pacblue	PCH101	eBioscience	Mouse IgG2a, κ
CTLA-4 (CD152)	PE-Cy7	L3D10	Biolegend	Mouse IgG2a, κ
ICOS (CD278)	FITC	C398.4A	Biolegend	Hamster IgG

Table S2. Immune related adverse events detailed per patient

Patient UPIN	Dose level	Toxicities
02	3 mg/kg	G2 colitis, G4 transaminitis
03	3 mg/kg	G2 Pneumonitis, G2 Myositis
09	3 mg/kg	G3 colitis
11	10 mg/kg	G3 dermatitis
13	10 mg/kg	G2 Bell's palsy, G1 rash
14	10 mg/kg	G1 dermatitis
16 *	10 mg/kg	G3 transaminitis, G2 dermatitis, G3 cGVHD

*underwent alloSCT before enrollment on trial

References

1. Kanakry CG, Coffey DG, Towlerton AM, et al. Origin and evolution of the T cell repertoire after posttransplantation cyclophosphamide. JCI Insight. 2016;1(5):e86252. Epub 2016 Apr 21.

2. Kanakry CG, Ganguly S, Zahurak M, et al. Aldehyde dehydrogenase expression drives human regulatory T cell resistance to posttransplantation cyclophosphamide. Sci Transl Med. 2013;5(211):211ra157.

3. Kanakry CG, Hess AD, Gocke CD, et al. Early lymphocyte recovery after intensive timed sequential chemotherapy for acute myelogenous leukemia: peripheral oligoclonal expansion of regulatory T cells. Blood. 2011;117(2):608-617.

4. Robins HS, Campregher PV, Srivastava SK, et al. Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. *Blood.* 2009;114(19):4099-107

5. Robins HS, Srivastava SK, Campregher PV, et al. Overlap and effective size of the human CD8+ T cell receptor repertoire. *Sci Transl Med.* 2010;2(47):47ra64

6. Warren EH, Matsen FAt, and Chou J. High-throughput sequencing of B- and T lymphocyte antigen receptors in hematology. *Blood.* 2013;122(1):19-22