Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Davids MS, Kim HT, Bachireddy P, et al. Ipilimumab for patients with relapse after allogeneic transplantation. N Engl J Med 2016;375:143-53. DOI: 10.1056/NEJMoa1601202

Supplementary Appendix
Supplement to: Davids MS, Kim HT, Bachireddy P, et al. Ipilimumab for Patients who Relapse after Allogeneic Transplantation.

Contents

Statistical analysis

All patients who received any amount of study drug were included in the safety and efficacy analyses. Baseline characteristics and the safety and efficacy data were reported descriptively. Baseline characteristics were compared between responders and non-responders using Fisher's exact test or the exact Wilcoxon-rank-sum test. Overall survival (OS) and progression-free survival (PFS) were estimated using the Kaplan-Meier method. OS was defined from the time of study entry to death. Patients who were alive were censored at the time last seen alive. PFS was defined from the time of study entry to relapse, disease progression or death whichever occurred first. Patients who were alive without disease relapse or progression were censored at the time last seen alive and progression-free. Exploratory pharmacodynamic correlative studies were analyzed descriptively and graphically, and group comparisons were made using the exact Wilcoxon-rank-sum test. All *p*-values are two-sided at a significance level of 0.05 without consideration of multiplicity. All analyses were performed using SAS 9.3 (SAS Institute Inc, Cary, NC), and R version 2.13.2 (the CRAN project. www.cran.r-project.org). Heatmap was generated and unsupervised hierarchical clustering was performed using GENE-E (http://www.broadinstitute.org/ cancer/software/GENE-E).

RNA Extraction from paraffin-embedded biopsy material

Ten unstained 5μM sections of tissue (or scrolls where applicable) were utilized to scrape tumor region of interest identified from H&E annotated by pathologist (S.R.). The Qiagen AllPrep DNA/RNA FFPE Kit (Qiagen - cat# 80234) following the manual's instructions was utilized for

subsequent RNA extraction. The xylene / ethanol deparaffinization method for upstream processing of the samples was implemented. The scraped paraffin-embedded tissue is dissolved in 99-100% xylene. After precipitation of the sample and removal of the supernatant, residual xylene is removed by washing with 96-100% ethanol. Briefly, once the paraffin has been removed, tissue is lysed with proteinase K digestion. Post-incubation at 4°C and centrifugation, the RNA-containing supernatants and DNA-containing pellet are separated and undergo independent processing. RNA supernatant is incubated at 80°C and then RNA is bound to the membrane of the RNeasy MinElute spin column and treated with DNase, then subjected to a series of wash steps, prior to elution in a 14µl volume of RNase/DNase-free H_2O. The DNA-containing pellet is lysed with proteinase K digestion and incubated at 90°C. DNA is bound to the membrane of the QIAamp MinElute spin column and subjected to a series of wash steps, prior to elution in a 30µl volume of RNase/DNase-free H_2O.

Quantification of RNA from paraffin-embedded biopsy material

RNA isolates were quantified utilizing the Quant-iT RiboGreen assay (Life Technologies – cat# R11490). One ul of RNA is required for quantification. Concentration is measured as ng/ul. For RNA quantification, isolates were excited at 485 ± 10 nm and the fluorescence emission intensity was measured at 530 ± 12 nm using a Victor X3 spectrophotometer (Perkin Elmer cat# 2030-0030). Fluorescence intensity was plotted versus RNA concentration over the calibration range, 0 -100 ng/ul.

Automated RNA-Seq Library Preparation

Removal of ribosomal RNA (rRNA) and human mitochondrial RNA was done using biotinylated, target-specific oligos combined with Ribo-Zero Gold rnRNA removal beads from the TruSeq Stranded Total RNA kit (Illumina - cat # RS-122-2301). The process was automated on a Biomek® FXP Laboratory Automation Workstation (Dual arm system with multichannel pipette and span-8 pipetters, Beckman Coulter - Cat # A31844). The Stranded TruSeq method is a streamlined, time-saving method that proved amenable to automation with limited optimization required. This automated method is suitable for up to ninety-six library constructions in parallel. RNA isolated from archival FFPE tissue is highly degraded due to a combination of fixation conditions, temperature, humidity and age. Therefore the RNA input material for a library construction from FFPE tissue is typically of lower yield and poorer quality than fresh frozen samples. The Stranded TruSeq method comprises ribosomal RNA removal, cDNA synthesis and purification and PCR amplification. The Illumina protocol was adapted to the Biomek Platform without deviations, the exception being for FFPE tissue, the fragmentation step was eliminated for the highly degraded RNA.

Quantification of cDNA libraries

cDNA libraries were quantified utilizing the Quanti-iT PicoGreen assay (Life Technologies – cat# P7589), respectively. One μl of DNA is required for quantification. Concentration is measured as ng/ul. Libraries were excited at 480 nm and the fluorescence emission intensity was measured at 520 nm using a Victor X3 spectrophotometer (Perkin Elmer cat# 2030-0030).

Fluorescence intensity plotted vs. DNA concentration over a low calibration range 0–50 ng/µl.

Sequencing

All libraries were run on a HiSeq 2500 system (Illumina, Carlsbad, San Diego) at the Broad Institute, where 75bp paired-end reads for duplexed samples were sequenced per lane.

RNA sequencing analysis

Bowtie2[1] and RSEM[2] were used to map reads and quantify gene expression across all protein-coding genes in transcripts per million (TPM).

Multiplex protein assays and ELISAs

Cytokine/chemokine assays were performed from plasma stored previously at -80^{o}C. CXCL5 levels were measured using a custom made Multiplex assay (R&D Systems). Fluorescence intensity was measured via the Bio-Plex MAGPIX Multiplex Reader (Biorad Laboratories Inc). Soluble CXCL2 (Cloud-clone Corp.) and sMICA (Abcam, Inc) levels were assessed using specific Human ELISA Kits. All samples were tested in duplicate as experimental repeats against a standard curve of purified protein according to the manufacturer's protocol.

Immunohistochemistry

Double staining of CD8 (CD8; M7103, Dako) and perforin (PRF1; VPP967, Vector Labs) was performed using an automated staining system (BOND-III, Leica Biosystems, Buffalo Grove, IL) following the manufacturer's protocol. Four-µm thick paraffin-embedded sections were pre-baked at 60°C for 1 hour and subsequently loaded onto BOND-III with "Bond Universal

Covertiles" (Leica Biosystems). After slides were dewaxed and rehydrated, heat-induced antigen retrieval was performed using ER2 solution (pH8) (Leica Biosystems) for 20 minutes at 100^{o} C.

PRF1 primary antibody (1:100 dilution of clone 5B10 in Bond Primary Antibody Diluent (Leica Biosystems, Buffalo Grove, IL)) was incubated for a total of 30 minutes at room temperature, followed by 10 minutes of post-primary blocking reagent, 10 minutes of horseradish peroxidase-labeled polymer, 5 minutes of peroxidase block, and 10 minutes of DAB development. All reagents were components of the Bond Polymer Refine detection system and incubated at room temperature (Leica Biosystems).

CD8 immunostaining was subsequently performed. Slides were treated with Dual Enzyme Block (Dako) for 10 minutes. Primary antibody (1:100 dilution of clone C8/144B in Bond Primary Antibody Diluent) was incubated for a total of 30 minutes at room temperature followed by 15 minutes of post-primary AP-blocking reagent, 20 minutes of AP-labeled polymer, and 15 minutes of Red substrate development. Slides were then counterstained with hematoxylin for 5 minutes. All reagents were components of the Bond Polymer Refine Red detection system (Leica Biosystems) and incubated at room temperature. Slides were subsequently dehydrated and cover slipped. Quantitation of percent cellularity for each stain was visually estimated by an expert hematopathologist (S.R.).

Flow cytometry

One and a half ml of whole blood is incubated in 15 ml of 1x BD PharmLyse (Lyse) solution for 15 minutes at room temperature (RT). After red cell lysis, white blood cells are centrifuged at

1700 rpm for 10 minutes at RT. The cell pellet is resuspended in 500 ul Lyse solution and 100 ul is subsequently incubated with the following directly conjugated monoclonal antibodies for 10 minutes at RT in the dark: anti-CD3-V450 (clone UCHT1; BD Horizon), anti-CD4-APC-H7 (clone RPA-T4; BD Biosciences), anti-CD8-P-Orange (clone RPA-T8; Biolegend), anti-CD25-PE-Cy7 (clone M-A251; BD Biosciences), anti-CD127-PE-Cy5 (clone eBioRDR5; eBioscience), anti-CD45RO-FITC (Clone UCHL1; BD Biosciences), and anti-CD62L-APC (Clone DREG-56; BD Bioscience). After incubation, cells are washed in 1 ml Lyse, centrifuged and resuspended with 400 uL Lyse and run on FACSCanto II (BD Biosciences) or 200 uL Lyse and run on BD LSRFortessa. Data is analyzed using FACSDiva (BD Biosciences).

CD4Tcon are defined as $CD3^{+}CD4^{+}CD25^{neg\text{-}low}CD127^{med\text{-}high}$ and CD4Treg are defined as $CD3^{+}CD4^{+}CD25^{med\text{-}high}CD127^{low}$. Within each CD4 population subsets were defined as follows: Naïve cells ($CD45RO^{-}CD62L^{+}$); Central memory (CM) ($CD45RO^{+}CD62L^{+}$); Effector memory (EM) ($CD45RO^{+}CD62L^{-}$).

Mass cytometry

A panel of 33 metal-tagged monoclonal antibodies was used for analysis of cryopreserved patient peripheral blood mononuclear cells (PBMC). A detailed listing of antibodies and corresponding metal tag is provided in Table S1. All pre-conjugated antibodies were purchased from Fluidigm. All other antibodies were purchased in carrier-protein-free PBS and conjugated with the respective metal isotope using the MaxPAR antibody conjugation kit (Fluidigm) according to the manufacturer's recommended protocol. Metal-labeled antibodies were diluted to

0.5 mg/ml in Candor PBS Antibody Stabilization solution (Candor Bioscience GmbH) for long-term storage at 4°C.

PBMC were washed with MaxPar Cell Staining Buffer (Fluidigm) and blocked with Human FcR Blocking Reagent (Miltenyi Biotec) for 10 minutes at room temperature. Cells were then incubated with all antibodies targeting cell surface markers for 30 minutes at room temperature and then washed twice with Cell Staining Buffer. After washing, cells were fixed with Cytofix™ Fixation Buffer (BD Biosciences) and permeabilized with Phosflow™ Perm Buffer III (BD Biosciences) following manufacturer's instructions. Fixed/permeabilized cells were washed twice with Cell Staining Buffer and incubated with all antibodies targeting intracellular antigens for 30 minutes at room temperature. After staining with intracellular antibodies, cells were washed twice with Cell Staining Buffer and incubated with $^{191/193}$Ir DNA intercalator (Fluidigm) following manufacturer's instructions. Prior to mass cytometry analysis, cells were washed twice with Cell Staining Buffer and twice with MaxPar Water (Fluidigm).

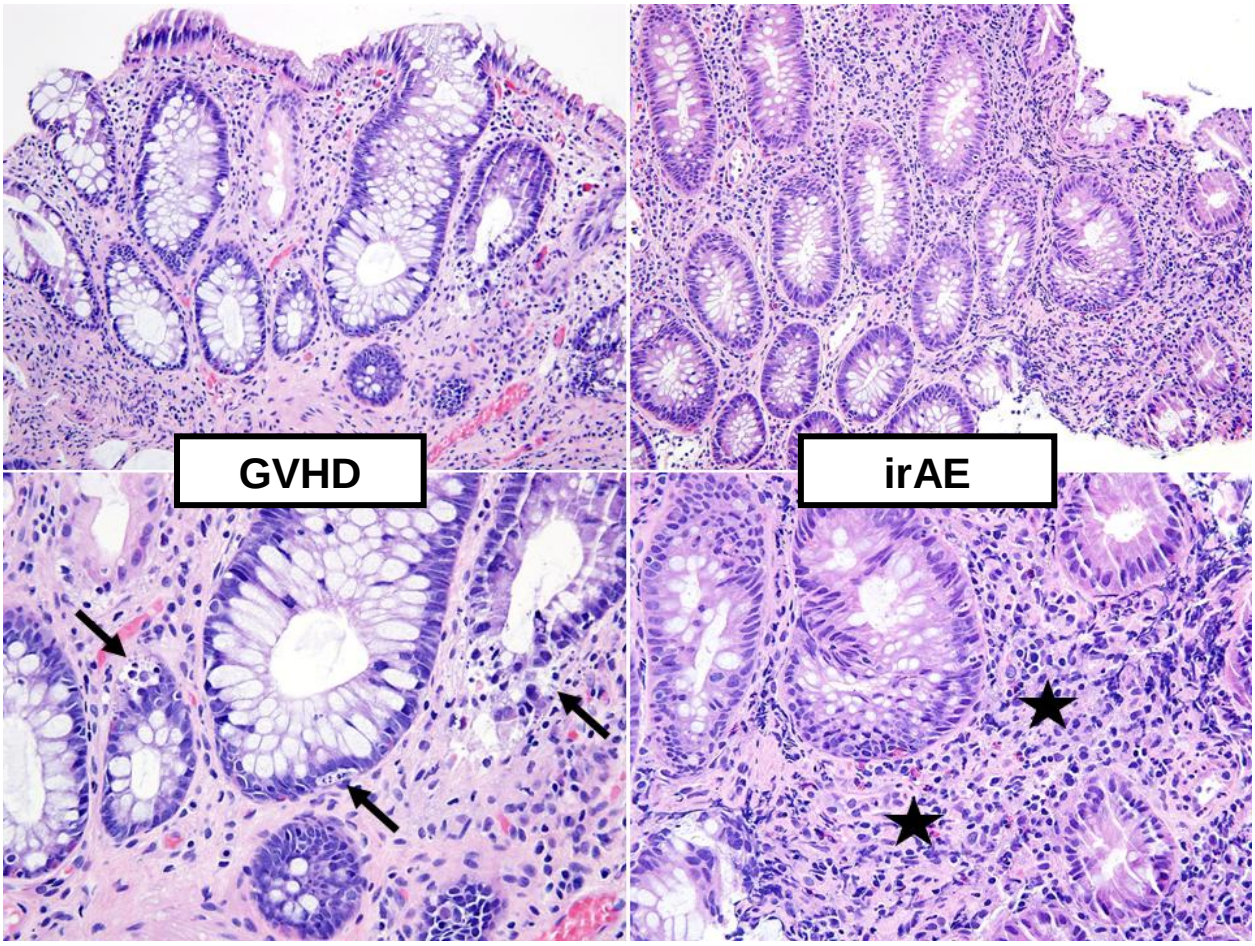

Figure S1. **Histopathologic Differences between GVHD and irAE in the Colon.** Panels A and C represent intermediate and high magnification of a colon biopsy from the patient on study who experienced acute GVHD of the colon. Notable pathologic features typical of GVHD include crypt epithelial cell apoptosis (*arrows*) in the absence of significant inflammation. Panels B and D represent intermediate and high magnification of a colon biopsy from the patient on study who experienced an irAE of ipilimumab colitis. Notable pathologic features typical of ipilimumab-induced colitis include a mixture of chronic inflammation in the lamina propria (*stars*), acute neutrophilic inflammation in the crypts, and less prominent apoptosis.

Figure S2. **PET/CT Scan of a Patient with Extramedullary Relapse of AML in the left breast after 7 and 13 Weeks of Ipilimumab Dosing.** At week 7, the size of the mass and its FDG avidity increased, possibly due to tumor flare, as with continued ipilimumab dosing by week 13 the mass had decreased significantly both in size and FDG avidity.

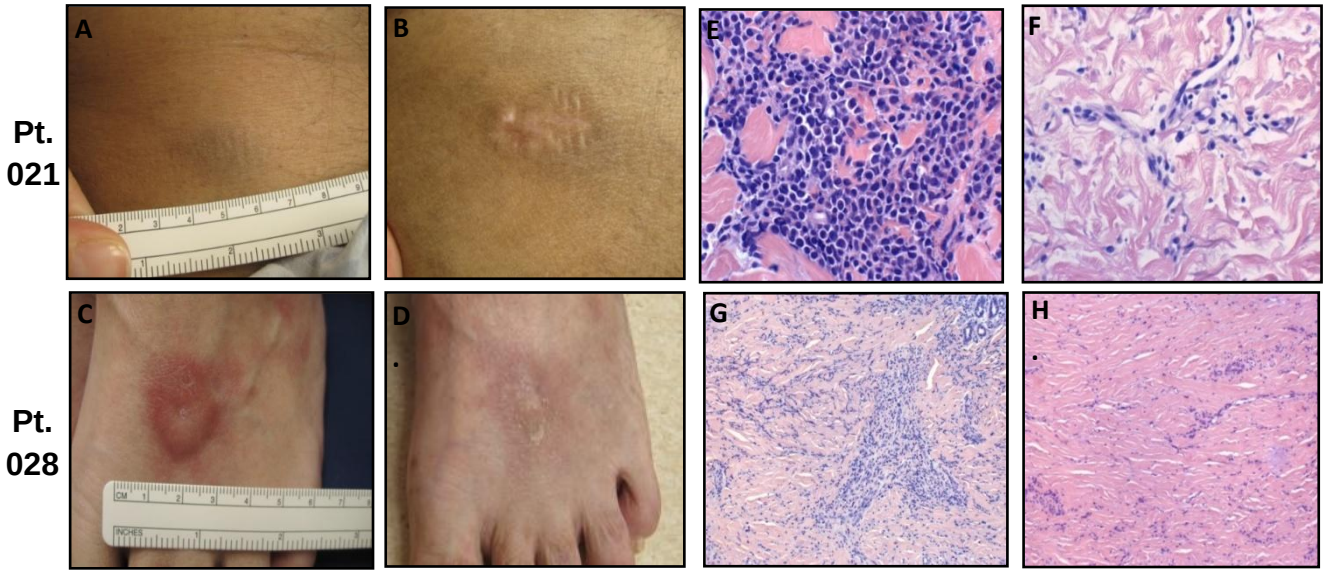

Figure S3. **Clinical and Histopathologic Responses to a Single Dose of Ipilimumab Therapy in 2 Patients with Leukemia Cutis.** Panels A and B show pre- and post-treatment response in the skin in a patient with leukemia cutis, and Panels C and D show the same in a different patient. Panels E and F and Panels G and H show histopathologic response in these same two patients (H & E stain, 400x (E/F) and 100x (G/H)), with sheets of neoplastic cells pre-treatment in Panels E and G, and fibrosis with sparse chronic inflammation without evidence of malignancy post treatment in Panels F and H.

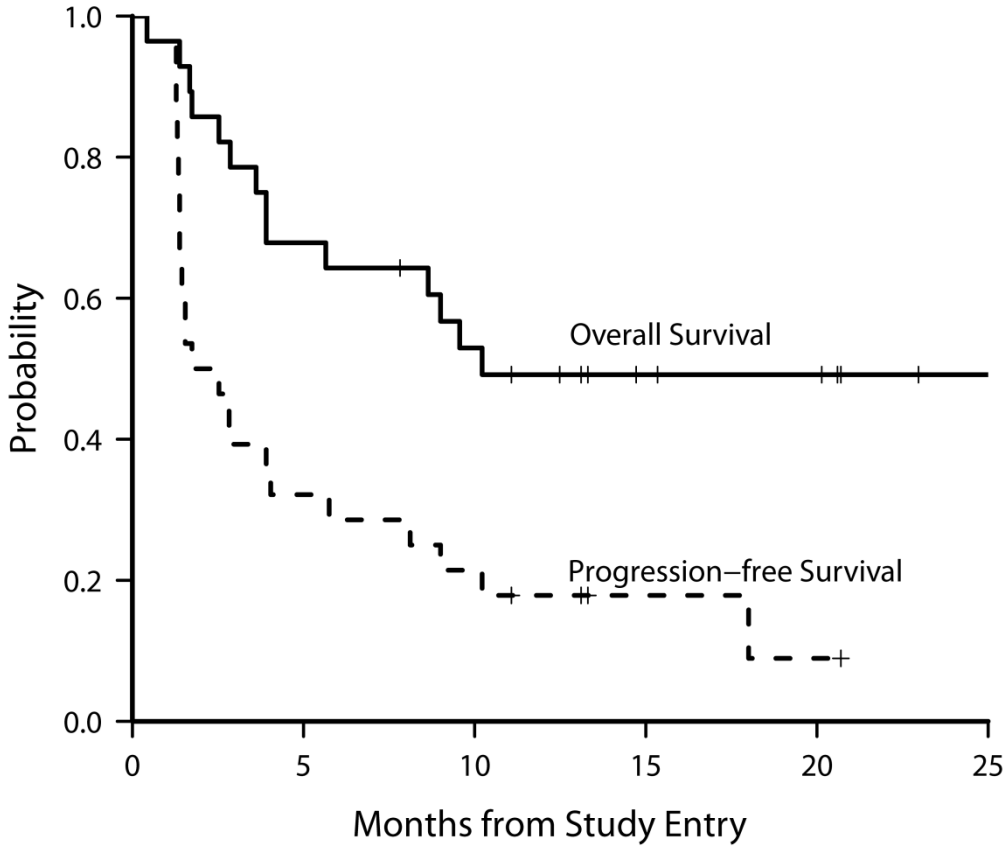

Figure S4. **Progression free and overall survival of all 28 patients with post alloHCT relapse of hematologic malignancies treated with ipilimumab**

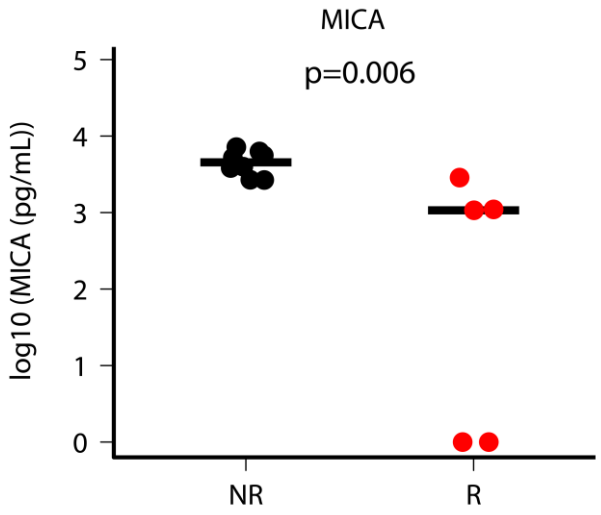

Figure S5. **MICA levels from the plasma of 11 patients at baseline by response status. 'R' denotes responders and NR denotes no responders. 'R' includes 4 CRs and one SD with some anti-tumor activity.**

Table S1 : Mass cytometry panel

	TARGET	SPECIES	CLONE	ISOTOPE	Manufacturer
	Surface				
1	CD45	Human	HI30	154Sm	Fluidigm
2	HLA-DR	Cross	L243	141Pr	Biolegend
3	CD95	Human	DX2	164Dy	Fluidigm
4	CD3	Human	UCHT1	170Er	Fluidigm
5	CD4	Human	SK3	174Yb	Fluidigm
6	CD8	Human	SK1	168Er	Fluidigm
7	CD25	Human	2A3	149Sm	Fluidigm
8	CD127	Human	A019D5	176Yb	Fluidigm
9	CD45RA	Human	HI100	169Tm	Fluidigm
10	CD62L	Human	DREG-56	153Eu	Fluidigm
11	CD31	Human	WM59	145Nd	Fluidigm
12	CD279(PD1)	Human	EH12.2H7	175Lu	Fluidigm
13	CD152(CTLA4)	Human	14D3	163Dy	eBioscience
14	CD19	Human	HIB19	142Nd	Fluidigm
15	IgD	Human	IA6-2	146Nd	Fluidigm
16	CD38	Human	HIT2	144Nd	Fluidigm
17	CD27	Human	L128	155Gd	Fluidigm
18	CD5	Human	UCHT2	143Nd	Fluidigm
19	BAFF-R(CD268)	Human	11C1	160Gd	Biolegend
20	CD56	Human	HCD56	162Dy	Biolegend
21	CD16	Human	3G8	148Nd	Fluidigm
22	NKG2D (CD314)	Human	1D11	161Dy	Fluidigm
	Intracellular				
23	Foxp3	Human	PCH101	165Ho	eBioscience
24	BCL-2	Human	Bcl-2/100	171Yb	BD Bioscience
25	Ki67	Cross	B56	151Eu	BD Bioscience
26	BIM	Cross	Polyclonal	159Tb	BD Bioscience
27	Helios	Cross	22F6	156Gd	Biolegend
	Signaling				
28	pStat5 [Y694]	Cross	47	150Nd	Fluidigm
29	pStat3 [Y705]	Cross	4/P-Stat3	158Gd	Fluidigm
30	pERK 1/2 [T202/Y204]	Cross	D13.14.4E	167Er	Fluidigm
31	pS6 Ribo(S235/236)	Cross	N7-548	172Yb	Fluidigm
32	p-AKT	Cross	S473	152Sm	Fluidigm
	Negative control				
33	HIV1-p24	Human	39/5.4A	166Er	Abcam

Additional Detail on Immunophenotyping Results

To probe the systemic effects of ipilimumab on T-cell immunity, we used flow cytometry to characterize the CD4 regulatory T-cell (T_{reg}) and CD4 conventional T-cell (T_{con}) populations in peripheral blood 8 weeks after starting ipilimumab in 2 patients with progressive disease, 3 with stable disease and 3 with complete response. Based on this phenotypic analysis, unsupervised clustering of T_{reg} and T_{con} subsets grouped the 3 complete responding and 1 stable disease patient together ("CR cluster"), distinguishing them from the 2 progressive disease patients and 2 others with stable disease ("PD cluster") (Fig. 3A). The stable disease patient in the complete response cluster had evidence of disease reduction but did not achieve formal response (Fig. S2, Supplementary Appendix). The complete response cluster is characterized by increased T_{con} and decreased T_{reg} cells 8 weeks after ipilimumab dosing, whereas the progressive disease cluster had an opposite pattern. Further analysis of CD4 T-cell subsets revealed increases in $CD62L^-$ T effector memory (T_{EM}) cells from both T_{con} and T_{reg} subtypes in the complete response cluster, and increased $CD62L^+$ T central memory (T_{CM}) and $T_{naïve}$ subsets in the progressive disease cluster. Changes in peripheral blood CD8+ T-cell subsets by flow cytometry and *in situ* CD4+ T cells by gene expression analyses were not significant (data not shown).

Mass cytometry allows the simultaneous detection of an even larger number of extracellular and intracellular markers in different T-cell subsets. Using viSNE to characterize PBMC from 4 patients (2 complete responders and 2 with progressive disease), we identified activated cells within the $CD4T_{reg}$ population that expressed high levels of FoxP3, CD25, HLA-DR and Helios. Activated T_{reg} were present at a 50% reduced frequency in the 2 responding patients compared to the 2 progressing patients (Fig. 3B).

Supplemental References

1. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nature methods 2012;9:357-9.
2. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC bioinformatics 2011;12:323.