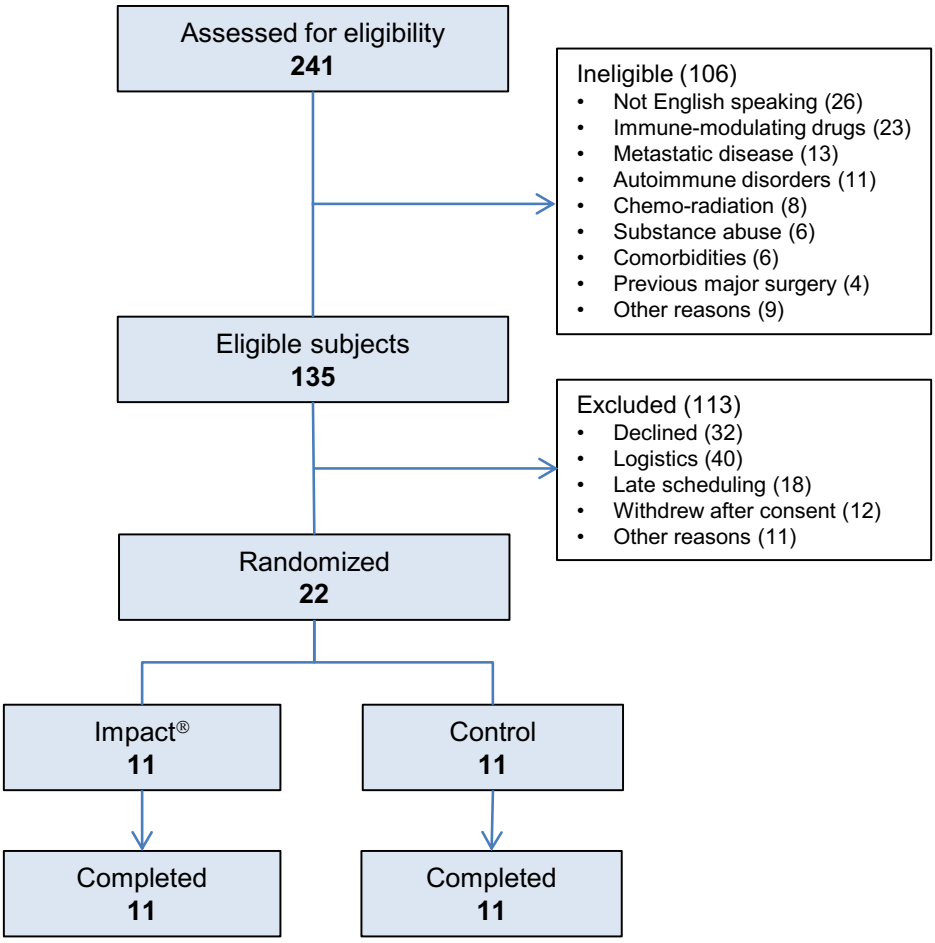
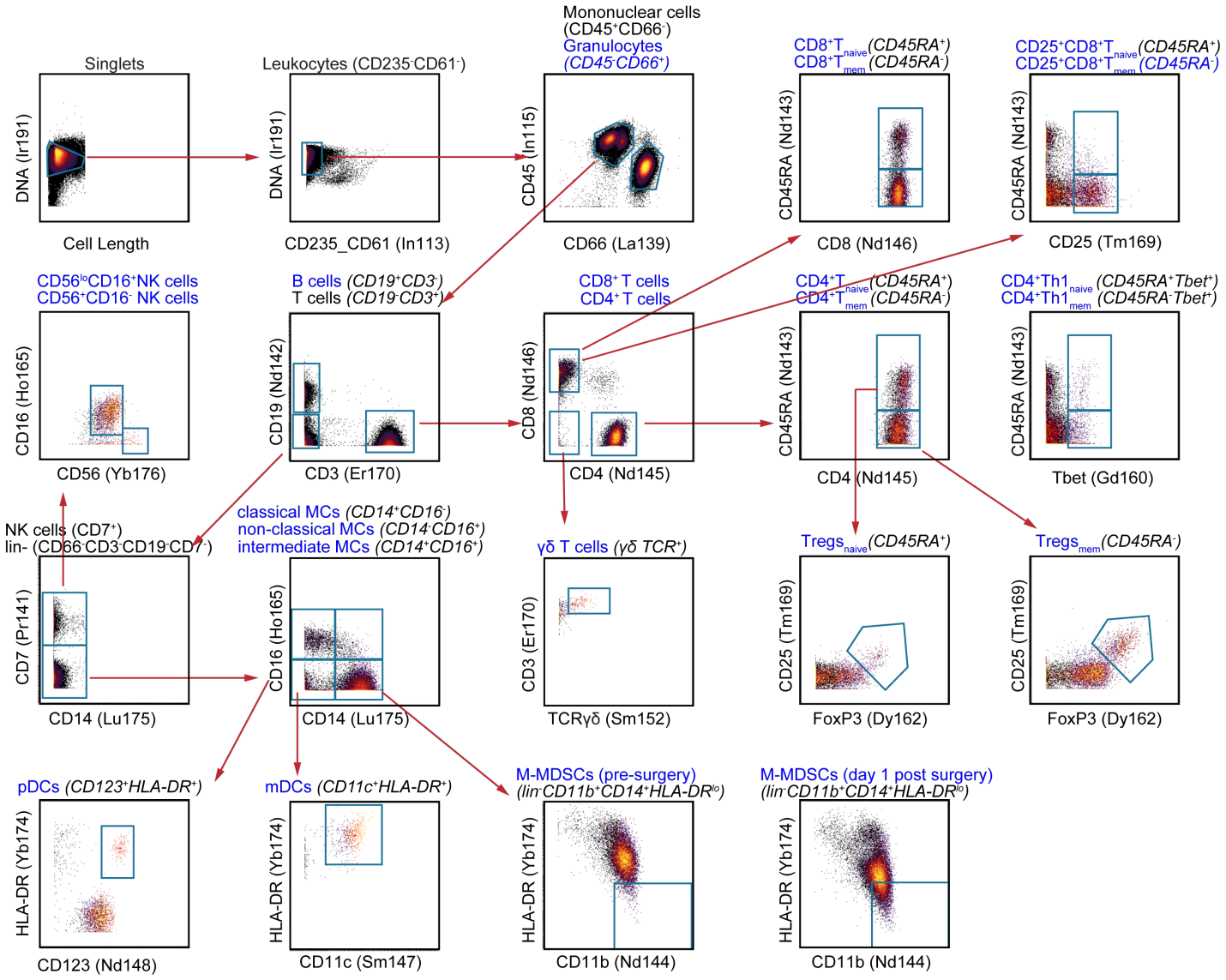
Deep Immune Profiling of an Arginine-Enriched Nutritional Intervention in Patients Undergoing Surgery

SUPPLEMENTARY MATERIALS:

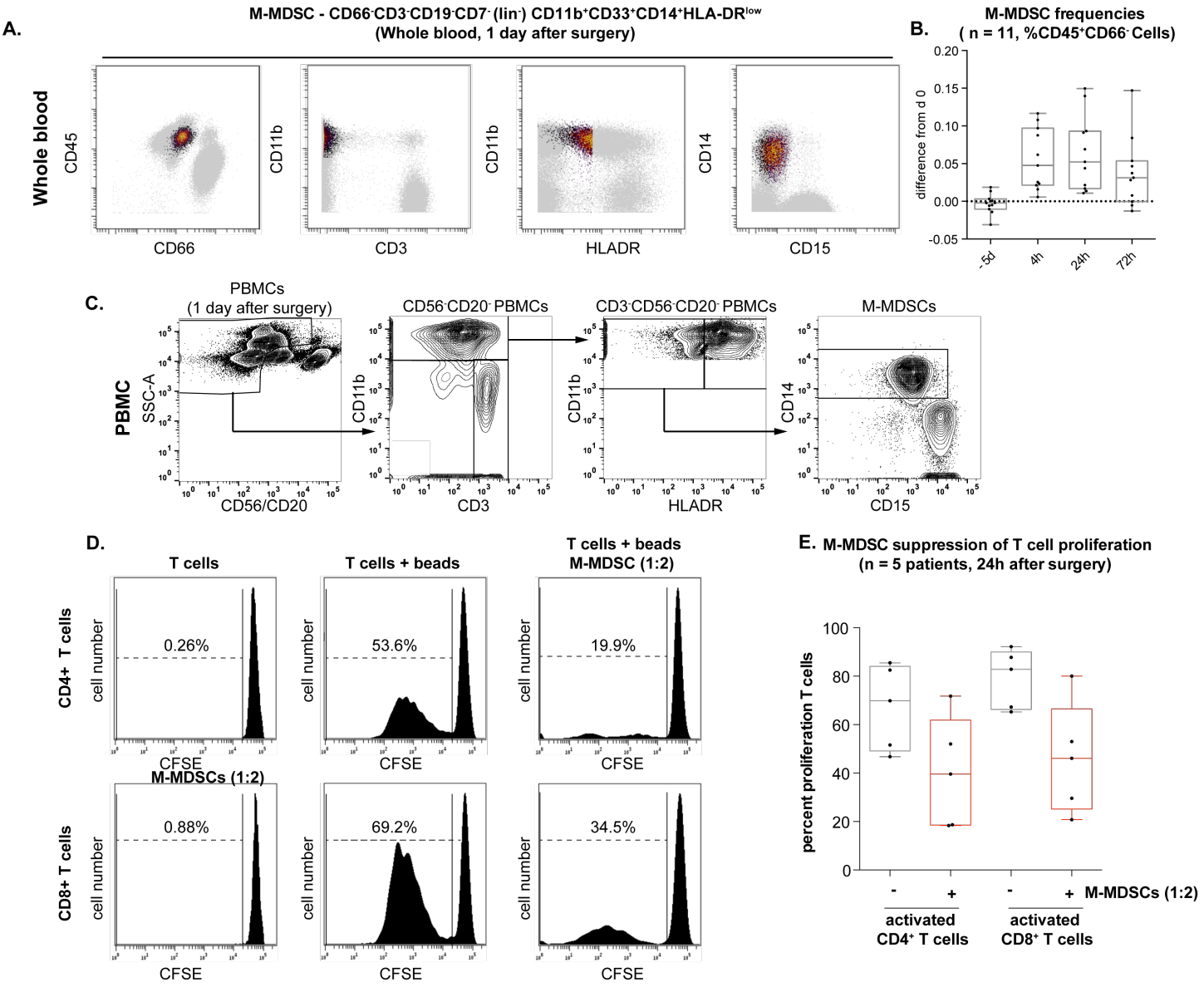


Supplemental Figure 1: Consort chart and inclusion/exclusion criteria. Inclusion criteria were 1) colon surgery, 2) age between 18 and 90 years, and 3) willing and able to sign an informed consent form and Health Insurance and Portability and Accountability Act (HIPAA) authorization, and to comply with study procedures. Changes in inclusion criteria as initially posted on ClinicalTrials.gov included 1) extending indications for colon surgery to non-cancerous conditions, and 2) increasing the maximum age from 65 to 90 years. Exclusion criteria were 1) immune-suppressant therapy within the last month (e.g., azathioprine or cyclosporine, 2) chemo-radiation within the last month, 3) chronic medication with potential immune-modulatory effects (e.g., daily oral morphine-equivalent intake > 30 mg or daily prednisone intake > 10 mg), 4) active infectious disease within 2 months, 5) significant metabolic disease (e.g., diabetes type I), 6) clinically significant organ dysfunction including renal and hepatic dysfunction, 7) significant cardiovascular and respiratory comorbidities resulting in impaired function and frailty, 8) autoimmune disease (e.g., lupus), 9) substance abuse (e.g., alcoholism, drug dependency), 10) undernourished as indicated by a weight loss > 10% during the last 6 months, 11) galactosemia, 12) previous

major abdominal surgery, 13) participation in another clinical trial of an investigational drug or device within the last 30 days that, in the investigator's opinion, would create increased risk to the participant or compromise the integrity of the study, 14) pregnancy, and 15) other conditions compromising a participant's safety or the integrity of the study. Changes in exclusion criteria as initially posted on ClinicalTrials.gov included 1) shortening of the time span between the last intake of an immunosuppressant drug and the time of surgery to 1 month, 2) shortening of the time span between chemo-radiation and the time of surgery to 1 month, 3) allowing daily intake of low-dose steroids (≤ 10 mg prednisone-equivalent), 4) inclusion of patients with metastatic disease if considered curable, 5) inclusion of patients with ulcerative colitis or Crohn’s disease, 6) inclusion of patients having had major abdominal surgery > 6 months ago. Initial inclusion and exclusion criteria were changed because they were highly conservative and restricted patient recruitment.



Supplemental Figure 2: Gating strategy of immune cell subsets. Two-dimensional flow cytometry plots are shown for a representative patient sample. Gating was performed using Cytobank software (www.cytobank.org). Twenty-three manually gated cell subsets (blue font) were included. This analysis included all innate and adaptive cellular immune changes that were previously detected by mass cytometry in patients undergoing surgery (1). Abbreviations are NK: Natural Killer, cMC: classical monocytes, ncMC: non-classical monocytes, intMC: intermediate monocytes, pDC: plasmacytoid dendritic cell, mDC: myeloid dendritic cell, mem: memory, T_{regs}: regulatory T cells, TCR: T cell receptor, and M-MDSC: monocytic MDSCs. The M-MDSC phenotype is based on recent recommendations for the identification of MDSC subsets. The M-MDSC gate is shown for samples from a representative patient before (day 0) and 1 day after surgery (27).



Supplemental Figure 3: Identification and characterization of monocytic myeloid-derived suppressor cells. **(A)** Shown are two-dimensional flow cytometry plots of M-MDSCs gated from whole blood samples 1 day after surgery (see gating strategy Supplemental Figure 2). Gated M-MDSCs (colored dots) are overlaid onto all peripheral leukocytes ($CD235^{-}CD61^{-}$ cells) to visualize their phenotype. M-MDSCs are lineage^{-} ($CD66^{-}CD3^{-}CD7^{-}CD19^{-}$), $CD11b^{+}CD14^{+}$, HLA-DR^{lo}, and $CD15^{-}$. **(B)** M-MDSC frequency changes with respect to day 0 (1h before surgery) at peri-operative time points -5d (before surgery), and 4h, 24h, and 72h after surgery in samples from patients in the control group (n = 11). M-MDSC frequency peaked 1 day after surgery (median fold increase 5.6, standard deviation 4.3, p = 0.001). **(C-E)** T cell suppression assays performed in PBMC samples collected from 5 patients 1 day after surgery. **(C)** Gating of lin-$CD15^{-}CD11b^{+}CD14^{+}$HLA-$DR^{lo}$ M-MDSCs from PBMCs samples. **(D)** Representative histograms of Carboxyfluorescein succinimidyl ester (CFSE) expression in $CD4^{+}$ (upper panels) or $CD8^{+}$ (lower panels) T cells. Activating (anti-CD3/CD128) beads were titrated to obtain 50-80% proliferating T cells after 5 days in culture (dashed line, middle panels). Activated T cells were co-cultured in the absence or presence of M-MDSCs at a 1:2 M-MDSC to T cell ratio (right panels). **(E)** M-MDSCs suppressed both $CD4^{+}$ (p = 0.03, n = 5) and $CD8^{+}$ T cell proliferation (p = 0.03, n = 5). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, Wilcoxon match-pairs sign rank test.

Covariates	Unstandardized Coefficients β	Standard Error	Standardized Coefficients β	t	p-value
y-intercept	-0.341	0.604		-0.565	0.58
AES Treatment	**-1.046**	**0.244**	**-0.838**	**-4.287**	**0.001**
Age	0.007	0.014	0.141	0.517	0.612
Sex	0.385	0.225	0.287	1.712	0.108
Race	-0.078	0.233	-0.062	-0.332	0.744
Diagnosis of malignancy	0.329	0.321	0.235	1.026	0.321
Open vs. laparoscopic surgery	0.208	0.257	0.149	0.81	0.43
(Dependent Variable: Elastic Net Value)					

Supplemental Table 1: Effect of demographic, clinical, and surgical variables on elastic net model association with AES treatment. Multiple linear regression was calculated using SPSS version 20 to determine whether any demographic or surgical variables were confounders for the effect of AES treatment on the EN value. The model used the EN value as the dependent variable and AES treatment, age, race, sex, gender, presence of malignancy, and surgical approach (open or laparoscopic) as covariates. The results indicated that the effect of AES treatment remained highly significant as an independent predictor of the EN value (p-value = 0.001). Multiple linear regression was used to assess whether demographic factors, presence of malignancy, or surgical approach were confounders for the effect of AES treatment on the individual key elastic net model components. The effect of AES treatment remained significant as an independent predictor of the pSTAT1 signal in $CD25^{+}CD8^{+}$ T_{mem} cells (residual p-value = 0.004), the pSTAT3 signal in $CD25^{+}CD8^{+}$ T_{mem} cells (residual p-value = 0.035), and MAPKAPK2 in M-MDSCs (residual p-value = 0.008).

Antigen	Supplier	Symbol	Atomic Mass	Clone	Comment
Barcode 1		Pd	102		Barcode
Barcode 2		Pd	104		Barcode
Barcode 3		Pd	105		Barcode
Barcode 4		Pd	106		Barcode
Barcode 5		Pd	108		Barcode
Barcode 6		Pd	110		Barcode
CD235ab	Biolegend	In	113	HIR2	phenotype
CD61	BD	In	113	VI-PL2	phenotype
CD45	Biolegend	In	115	HI30	phenotype
CD66	BD	La	139	CD66α-	phenotype
CD7	BD	Pr	141	M-T701	phenotype
CD19	Fluidigm	Nd	142	HIB19	phenotype
CD45RA	Fluidigm	Nd	143	HI100	phenotype
CD11b	Fluidigm	Nd	144	ICRF44	phenotype
CD4	Fluidigm	Nd	145	RPA-T4	phenotype
CD8a	Fluidigm	Nd	146	RPA-T8	phenotype
CD11c	Fluidigm	Sm	147	Bu15	phenotype
CD123	Biolegend	Nd	148	6H6	phenotype
pCREB	CST	Sm	149	87G3	function
pSTAT5	Fluidigm	Nd	150	47	function
pP38	CST	Eu	151	36/p38/pT184/pT182	function
TCRγδ	Fluidigm	Sm	152	GL3	phenotype
pSTAT1	Fluidigm	Eu	153	58D6	function
pSTAT3	BD	Sm	154	4/P pY705	function
prpS6	CST	Gd	155	N7-548	function
CD33	Fluidigm	Gd	158	WM53	phenotype
pMAPKAPK2	Fluidigm	Tb	159	27B7	function
Tbet	Fluidigm	Gd	160	4B10	phenotype
FoxP3	Fluidigm	Dy	162	PCH101	phenotype
IκB	Fluidigm	Dy	164	L35A5	function
CD16	Fluidigm	Ho	165	3G8	phenotype
pNFκB	Fluidigm	Er	166	K10-	function
pERK1/2	CST	Er	167	D13.14.4E	function
CD25	Fluidigm	Tm	169	2A3	phenotype
CD3	Fluidigm	Er	170	UCHT1	phenotype
CD15	Fluidigm	Yb	172	W6D3	phenotype
HLA-DR	Fluidigm	Yb	174	L243	phenotype
CD14	Fluidigm	Yb	175	M52E	phenotype
CD56	Fluidigm	Yb	176	NCAM16.2	phenotype
DNA1		Ir	191		
DNA2		Ir	192		

Supplemental Table 2: Antibody panel used for mass cytometry analysis

	Sort Panel			Day 5 Suppression Panel		
Fluorophore	**Antigen**	**Clone**	**Supplier**	**Antigen**	**Clone**	**Supplier**
FITC	CD19/CD56	HIB19/5.1H11	Biolegend	CFSE		Invitrogen
PE	CD3	OKT3	Biolegend	CD25	BC96	Biolegend
APC	CD11b	ICRF44	Biolegend			
APC-H7	CD14	MfP9	BD	CD8	SK1	BD
PE-Cy7	CD11c	B-Ly6	BD	CD279	EH12.2H7	Biolegend
PerCp-Cy5.5				CD45RO	UCHL1	Biolegend
PE-CF594	HLA-DR	L243	Biolegend	CD62L	DREG56	BD
Pac Blue	CD15	SSEA-1	Biolegend			
Aqua	Aqua Live/Dead	BL	Biolegend	Aqua Live/Dead		Biolegend
BV 570				CD4	RPA-T4	Biolegend
BV 605	CD16	3G8	Biolegend	CD127	A019D5	Biolegend
BV 785				CD3	OKT3	Biolegend

Supplemental Table 3: Antibody panels used in MDSC suppression assays