Online Supplement

CD4+ T-Cell Profiles and Peripheral Blood Ex-Vivo Responses to T-Cell Directed Stimulation Delineate COPD Phenotypes

Mustimbo E. P. Roberts, PhD[1] Brandon W. Higgs, PhD[1] Philip Brohawn, BS[1] Fernanda Pilataxi, BA[1] Xiang Guo, PhD[1] Michael Kuziora, PhD[1] Russell P. Bowler, MD, PhD[2] Wendy I. White, PhD[1]

Flow Cytometry. Whole blood from COPD patients and control individuals were used for flow cytometry assays. Complete blood counts (CBC), in combination with flow cytometric analyses, were used to enumerate the numbers of T cells, T-cell subsets and monocytes. Whole blood was aliquoted and 200μLs used for each flow cytometry assay. The following anti-human antibodies were used for whole blood staining of T-cell subsets; anti-CD3 AF 700 (eBiosciences, San Diego, CA), anti-CD4 PE-Cy5 (eBiosciences), anti-CD8 PE-Cy7 (eBiosciences), anti-CD45RA Qdot605 (Life Technologies, Carlsbad, CA), anti-CCR7 BV421 (BioLegend, San Diego, CA), anti-CCR5 A488 (BioLegend), anti-CD146 PE (BD Biosciences, San Jose, CA), and anti-CD19 APCeFluor780 (eBiosciences). The following anti-human antibodies were used for whole blood staining of monocyte subsets; anti-CD14 FITC (eBiosciences), anti-CD16 PE-Cy7 (eBiosciences), anti-CD45 APCeFluor780 (eBiosciences), anti-HLA-DR A700 (eBiosciences), anti-CCR2 PE-Cy5 (Biolegend), anti-CD56 PerCpCy5.5 (BioLegend). After antibody staining, BDFACS lyse (BDBiosciences) was used to remove red blood cells. Cells were washed and fixed with IC fixation buffer (eBiosciences). The fixed samples were analyzed on a 5-laser LSRII flow cytometer (BD, Franklin Lakes, NJ). FlowJo version 9.1 (TreeStar, Ashland, OR) was used for analysis of flow cytometry data.

Cellular Isolations. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Ficoll-Paque (GE Healthcare, Piscataway, New Jersey) density gradient centrifugation. Positive selection was used to purify cells as it results in improved purity over negative selection, and there are no significant consistent differences in expression data between the two methods.[1] Briefly, CD8+ T cells were isolated from PBMC samples by magnetic selection using CD8 microbeads (all microbeads for cellular isolations from Miltenyi Biotech). The CD8+ depleted PBMC fraction was used to isolate CD14+ cells using CD14 microbeads. The remaining PBMC sample (CD8 and CD14 depleted), was used to isolate CD4+ T cells using CD4 microbeads. Cell purity was assessed by flow cytometry. Isolated cell subset pellets were stored in buffer RLT (Qiagen, Valencia, California) plus 1% 2-mercaptoethanol (Sigma Aldrich) at -80^0C.

Table S1. TruCulture Analysis

Analytes	CTRL Median	COPD Median	FC	*P*	
Alpha-1-Antitrypsin (AAT)mg/mL	0.6	0.615	1.03	0.6943	
Alpha-2-Macroglobulin (A2Macro)mg/mL	0.64	0.59	-1.08	0.2809	
Beta-2-Microglobulin (B2M)ug/mL	0.56	0.56	1.00	0.1913	
Brain-Derived Neurotrophic Factor (BDNF)ng/mL	8.3	7.45	-1.11	0.1298	
C-Reactive Protein (CRP)ug/mL	0.74	1.8	2.43	0.2045	
Complement C3 (C3)mg/mL	0.42	0.415	-1.01	0.4818	
Eotaxin-1pg/mL	209	202.5	-1.03	0.4157	
Factor VII ng/mL	219	183	-1.20	0.8298	
Ferritin (FRTN)ng/mL	55	57	1.04	0.5843	
Fibrinogen mg/mL	1.3	1.4	1.08	0.7311	
Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) pg./mL	696	49	-14.20	0.0079	*,#
Haptoglobin mg/mL	0.035	0.035	1.00	0.4163	
Intercellular Adhesion Molecule 1 (ICAM-1)ng/mL	60	59.5	-1.01	0.8187	
Interferon gamma (IFN-gamma) pg/mL	1940	321.5	-6.03	0.0054	*,#
Interleukin-1 alpha (IL-1 alpha)ng/mL	0.0074	0.00405	-1.83	0.0269	*,#
Interleukin-1 beta (IL-1 beta)pg/mL	61	36.5	-1.67	0.2467	
Interleukin-1 receptor antagonist (IL-1ra)pg/mL	1660	2050	1.23	0.7217	
Interleukin-2 (IL-2)pg/mL	639	452	-1.41	0.1659	
Interleukin-3 (IL-3)ng/mL	0.0069	0.0069	1.00	0.3418	
Interleukin-4 (IL-4)pg/mL	523	403	-1.30	0.2146	
Interleukin-5 (IL-5)pg/mL	236	137.5	-1.72	0.3601	
Interleukin-6 (IL-6)pg/mL	81	60.5	-1.34	0.0030	*
Interleukin-7 (IL-7)pg/mL	12	12	1.00	0.4420	
Interleukin-8 (IL-8)pg/mL	8190	8175	-1.00	0.8253	
Interleukin-10 (IL-10)pg/mL	614	423.5	-1.45	0.0442	*
Interleukin-12 Subunit p40 (IL-12p40)ng/mL	0.28	0.28	1.00	0.3355	
Interleukin-12 Subunit p70 (IL-12p70)pg/mL	49	49	1.00	0.6209	
Interleukin-15 (IL-15)ng/mL	0.39	0.39	1.00	0.4917	
Interleukin-17 (IL-17)pg/mL	46	23.5	-1.96	0.1639	
Interleukin-18 (IL-18)pg/mL	154	170	-0.91	0.3691	
Interleukin-23 (IL-23)ng/mL	0.46	0.46	1.00	0.7814	
Macrophage Inflammatory Protein-1 alpha (MIP-1 alpha)pg/mL	8080	5195	-1.56	0.1774	
Macrophage Inflammatory Protein-1 beta (MIP-1 beta)pg/mL	102000	81150	-1.26	0.3780	
Matrix Metalloproteinase-2 (MMP-2)ng/mL	78	79	1.01	0.6379	
Matrix Metalloproteinase-3 (MMP-3)ng/mL	5.4	5.6	1.04	0.8381	
Matrix Metalloproteinase-9 (MMP-9)ng/mL	40	40	1.00	NA	

Monocyte Chemotactic Protein 1 (MCP-1)pg/mL	6110	7550	1.24	0.9794	
Stem Cell Factor (SCF)pg/mL	116	116	1.00	0.4602	
T-Cell-Specific Protein RANTES (RANTES)ng/mL	17	16	-1.06	0.9242	
Tissue Inhibitor of Metalloproteinases 1 (TIMP-1)ng/mL	51	57	1.12	0.7913	
Tumor Necrosis Factor alpha (TNF-alpha)pg/mL	5520	1810	-3.05	0.0071	*,#
Tumor Necrosis Factor beta (TNF-beta)pg/mL	62	25	-2.48	0.0177	*,#
Tumor necrosis factor receptor 2 (TNFR2)ng/mL	2	1.9	-1.05	0.9396	
Vascular Cell Adhesion Molecule-1 (VCAM-1)ng/mL	145	159	1.10	0.8385	
Vascular Endothelial Growth Factor (VEGF)pg/mL	90	75	-1.20	0.3302	
Vitamin D-Binding Protein (VDBP)ug/mL	57	83	1.46	0.5705	
von Willebrand Factor (vWF)ug/mL	21	25	1.19	0.6235	

* = $p<0.05$, # FC >1.5

Table S2. Individual Cytokine Correlation with FEV_1 in COPD Patients

	FEV_1 % Predicted vs. GM-CSF	FEV_1 % Predicted vs. IFN-ɣ	FEV_1 % Predicted vs. IL-1α	FEV_1 % Predicted vs. TNF-α	FEV_1 % Predicted vs. TNF-β
r	0.32	0.29	0.10	0.28	0.26
P value	0.02	0.03	0.50	0.05	0.06

Table S3. Stimulation Index of Individual Cytokines that Constitute the T-Cell Stimulation Score (study population, COPD and smoker controls)

	GM-CSF (pg/ml) Median	IFN-ɣ (pg/ml) Median	IL-1α (ng/ml) Median	TNF-α (pg/ml) Median	TNF-β (pg/ml) Median
α-CD3/ α-28	126.00	370.00	0.0051	2500.00	30.00
null	49.00	1.70	0.0020	12.00	9.10
stim Index	2.57	217.65	2.55	208.33	3.30

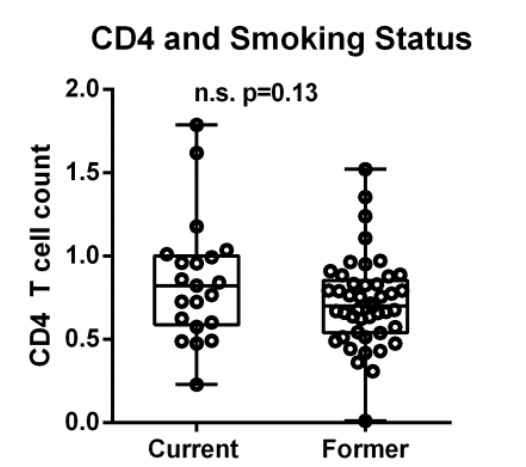

Figure S1. The numbers of CD4+ T cells are not significantly different between current and former smokers in the study cohort. Flow cytometry was used to enumerate CD4+ T cells. The CD4+ T-cell count was compared, using all individuals in the cohort, between current and former smokers. $P < 0.05$ was considered significant using unpaired t-test.

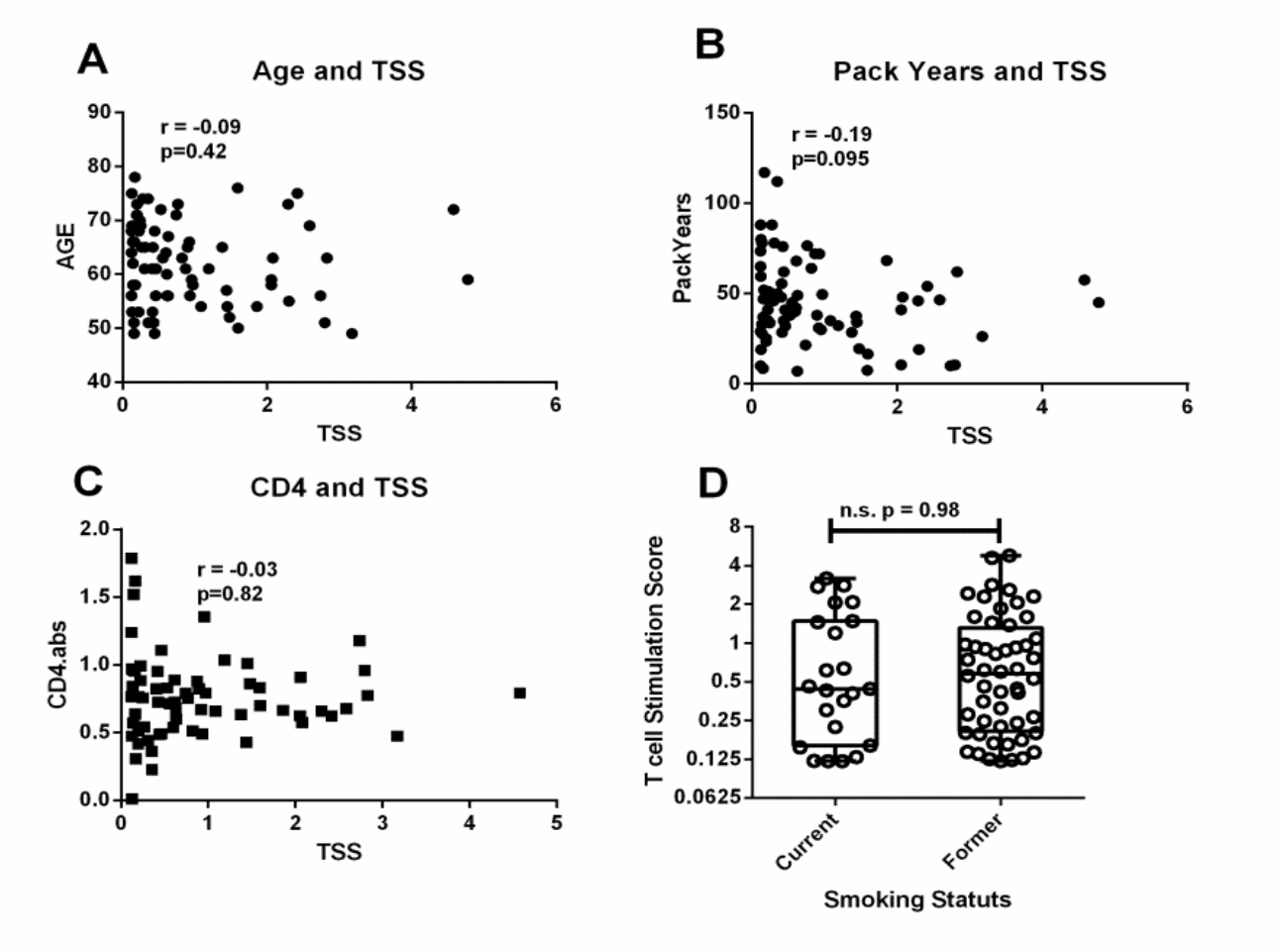

Figure S2. Relationship between T-cell stimulation score (TSS) and age, pack years, CD4+ T-cell number or smoking status is not significant. A.-C. TSS, of the entire cohort, was correlated to age, pack years, and CD4+ T-cell number. $P<0.05$ was considered significant using Pearson's correlation. D. TSS, in the entire cohort, was compared between current and former smokers. $P<0.05$ was considered significant using unpaired t-test.

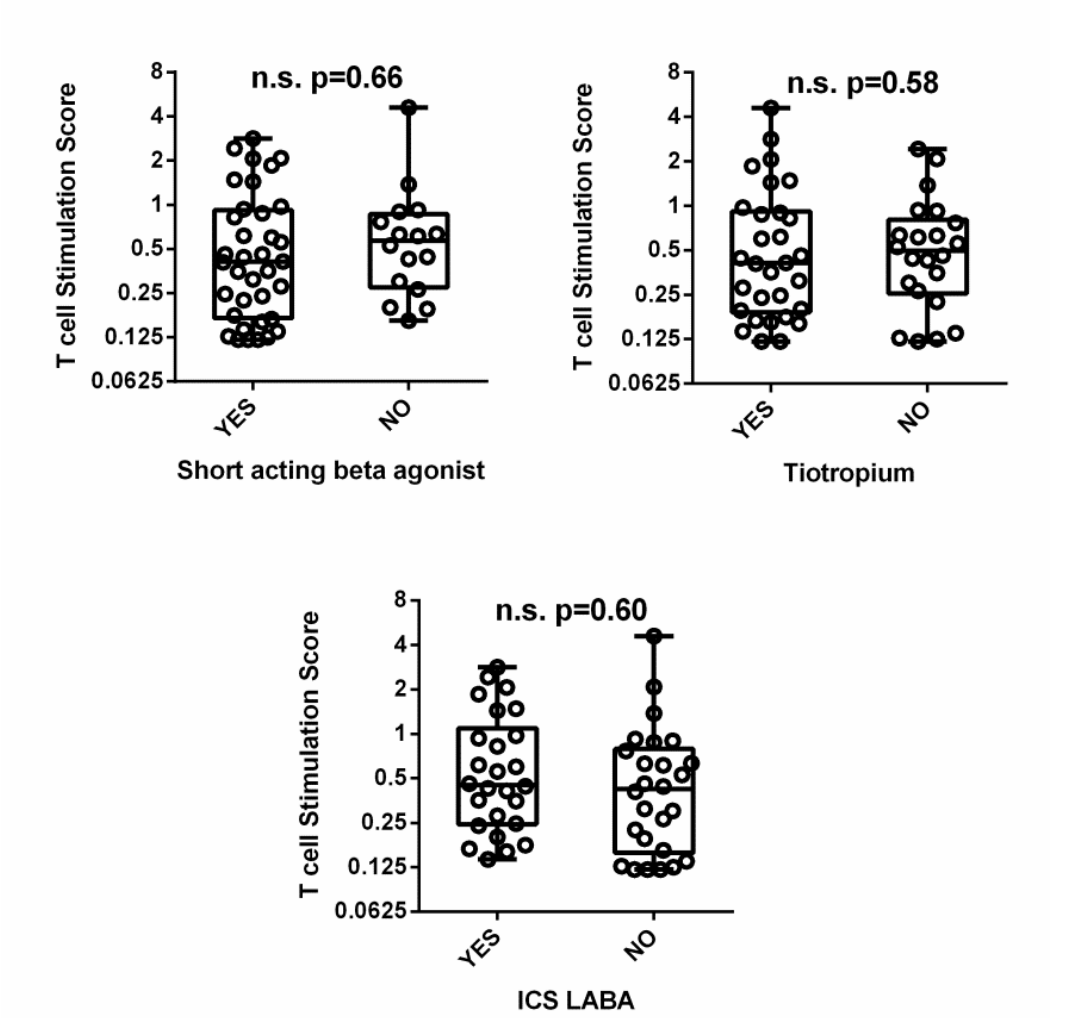

Figure S3. The relationship between T-cell stimulation score (TSS), among patients with COPD, and COPD medication usage was not significant. The TSS was compared between patients with COPD currently using (YES) or not currently using (NO) the indicated medications. $P<0.05$ was considered significant using unpaired t-test.

References

1.Lyons PA, Koukoulaki M, Hatton A, Doggett K, Woffendin HB, Chaudhry AN, Smith KG. Microarray analysis of human leucocyte subsets: The advantages of positive selection and rapid purification. *BMC Genomics.* 2007;8:64.