PNAS
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Supplementary Information for

An immunometabolic pathomechanism for chronic obstructive pulmonary disease

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Supplementary Material and Methods

Lung function

Forced spirometry was performed using a computer-assisted spirometer (Quark PFT, Cosmed) according to the American Thoracic/European Respiratory Societies acceptability and reproducibility standards (1). COPD patients had to stop inhalation treatment 12 hours before testing. Active smokers were also asked to refrain from smoking for the same time course.

Antibodies and flow cytometry

Immune cell profiling of cells from healthy and COPD subjects was done at the time of blood drawing. Prior to flow cytometry for lymphocyte subsets, whole blood cells were analysed with a clinical-grade hematocytometer to determine absolute lymphocyte numbers in each sample. 100 µl of blood was incubated 30' at room temperature with the specific antibodies combinations. Red blood cells were lysed using BD FACS lysing Solution 2 (BD Bioscience) for 10' and samples subsequently washed and resuspended in 300 µl phosphate buffered saline (PBS). Flow cytometry was carried on cells gated on $CD45^+$ - Side Scatter (SSC). Immunophenotypic analysis was performed with an EPICS XL flow cytometer (Beckman Coulter, Milan, Italy) using the Beckman Coulter software program XL System II. Triple combinations of different human mAbs, e.g., FITC- and phycoerythrin (PE)-anti-CD3, PE- and PE-cyanine (PC) 5-anti-CD4, PC5-anti-CD8, PE-anti-CD16, PC5-anti-CD19, PE-anti-CD25, FITC-anti-CD45, PE-anti-CD56, PE anti-CD45RA, PE anti-CD28, PE anti-HLA-DR and PE anti-CD11b (all from Coulter Immunotech,) were used to identify different cell populations.
For staining of PBMCs, T_{conv} and iT_{reg} cells, combinations of the following mAbs were used– APC–H7 - anti–human CD4 (clone RPA-T4; BD Biosciences)– PE–Cy7 - anti–human CD25 (clone M-A251; BD Biosciences–, BV421 - anti–human PD-1 (clone EH12-1; BD Biosciences). Thereafter cells were washed, fixed and permeabilized (fixation-permeabilization buffer; BD Biosciences) and were stained with the following mAbs: PE – anti-human FoxP3-all (clone 259D/C7; BD Biosciences), PE – anti-human FoxP3-E2 (clone 150D/E4; eBioscien–e), APC - anti–human CD152 (CTLA-4) (clone BN13; BD Biosciences), and BV510 – anti-human Ki67 (clone B56; eBioscience). Analyses were performed with Diva software (BD) and FlowJo (Tree Star).

Inflammatory molecules and cytokine measurement

Plasma were centrifuged and kept at –20°C until use. Levels of leptin, (sCD40L), sICAM-1, MCP-1, MPO, resistin, and sTNFR were analyzed using the bead-based analyte detection system Human Obesity 9plex-kit (Bender MedSystem Inc, Burlingame, CA) (2). GM-CSF, IFN-g, IL-2, IL-8, IL-17A, TNF-a, IP-10, MCP-1, MIG, MIP1-a (Invitrogen, Thermo Fisher Scientific) were measured by Multiplex technology (Luminex 200, Luminex).

Tissue sampling and processing

Tissue samples from lung parenchyma were obtained as far distal to the tumour as possible. Tissue fragments were fixed in 10% buffered formalin for 24 hours, processed and paraffin-embedded (FFPE). Lung tissues were cut into 4-µm-thick sections and stained with haematoxylin-eosin for initial analysis.

Confocal microscopy

Immunofluorescence (IF) was carried out by mean of Opal Multiplex IF Assay (PerkinElmer, Waltham, MA-USA) as suggested by the manufacturer using 4-µm-thick formalin-fixed, FFPE tissue sections from lung. Tissue sections were dewaxed in xylene, hydrated in graded series of alcohol and fixed in 10% neutral buffered formaldehyde for 20 minutes; sections were washed with H_2O and subjected at cycle 1 of multiple staining of three markers with Opal reagent (3): antigen retrieval/MWT (Micro Wave Treatment) was done in AR9 buffer, sections were washed in H_2O and in TBST (TBS: 150mM NaCl, 20mM Tris-HCl (pH 7.5) plus 0,05% Tween 20 and incubated for 10 minutes in blocking solution; primary antibodies anti FoxP3 (Abcam mouse monoclonal 236A/E7, 1:50) were added for 1 hour. Sections were washed in TBST and the Opal Polymer HRP anti Mouse-Rabbit secondary antibody solution was applied for 10 minutes, sections were washed in TBST and incubated with the Opal Fluorophore (Opal 520) for 10 minutes, sections were washed in TBST and MWT was performed in AR9 buffer. Two cycles were performed in the same way using as primary antibody Foxp3 (anti-Exon 2, Thermo Fisher Scientific mouse monoclonal 150DE4, 1:50) and as Opal Fluorophore, Opal 570.After MWT sections were incubated in spectral DAPI and finally coverslips were mounted with ProLong Antifade Mountants (Thermo Fisher Scientific, Fremont, CA, USA.). Experiments were carried out on an inverted and motorized microscope (Axio Observer Z.1) equipped with a 63x/1.4 Plan-Apochromat objective. The attached laser-scanning unit (LSM 700 4x pigtailed laser 405-639, Zeiss, Jena, Germany) enabled confocal imaging. For excitation, 405 nm 488 nm and 555 and 639 lasers were used. Fluorescence emission was revealed by MBS 405/488/555/639 for Opal 520, Opal 570 and DAPI. Triple staining immunofluorescence images were acquired separately in the green red and blue channels at a resolution of 1024x1024 pixels, with the confocal pinhole set to one Airy unit and then saved in TIFF format.

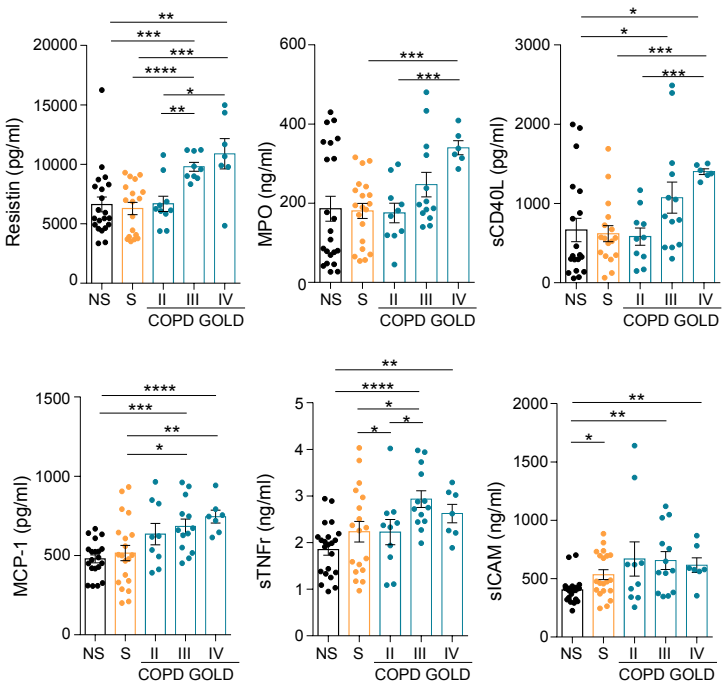
Supplementary References

1. Miller MR, et al. (2005) Standardisation of spirometry. *Eur Respir J* 26:319-338.

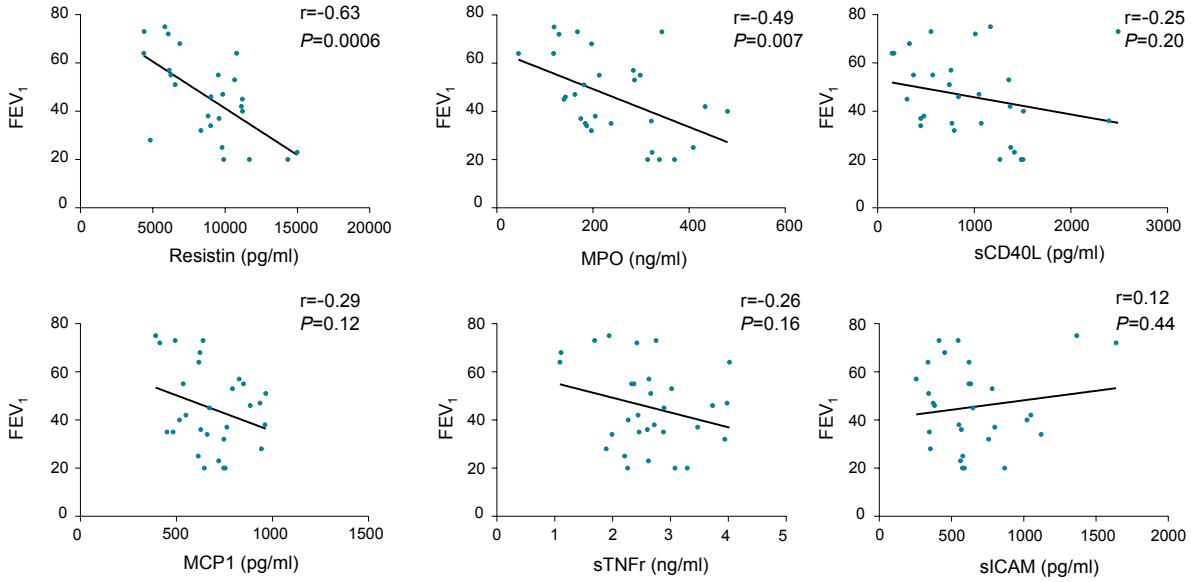
2. Galgani M, et al. (2013) Meta-immunological profiling of children with type 1 diabetes identifies new biomarkers to monitor disease progression. *Diabetes* 62:2481-91.

3. Stack EC, Wang C, Roman KA, Hoyt CC. (2014) Multiplexed immunohistochemistry, imaging, and quantitation: a review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. *Methods* 70:46-58.

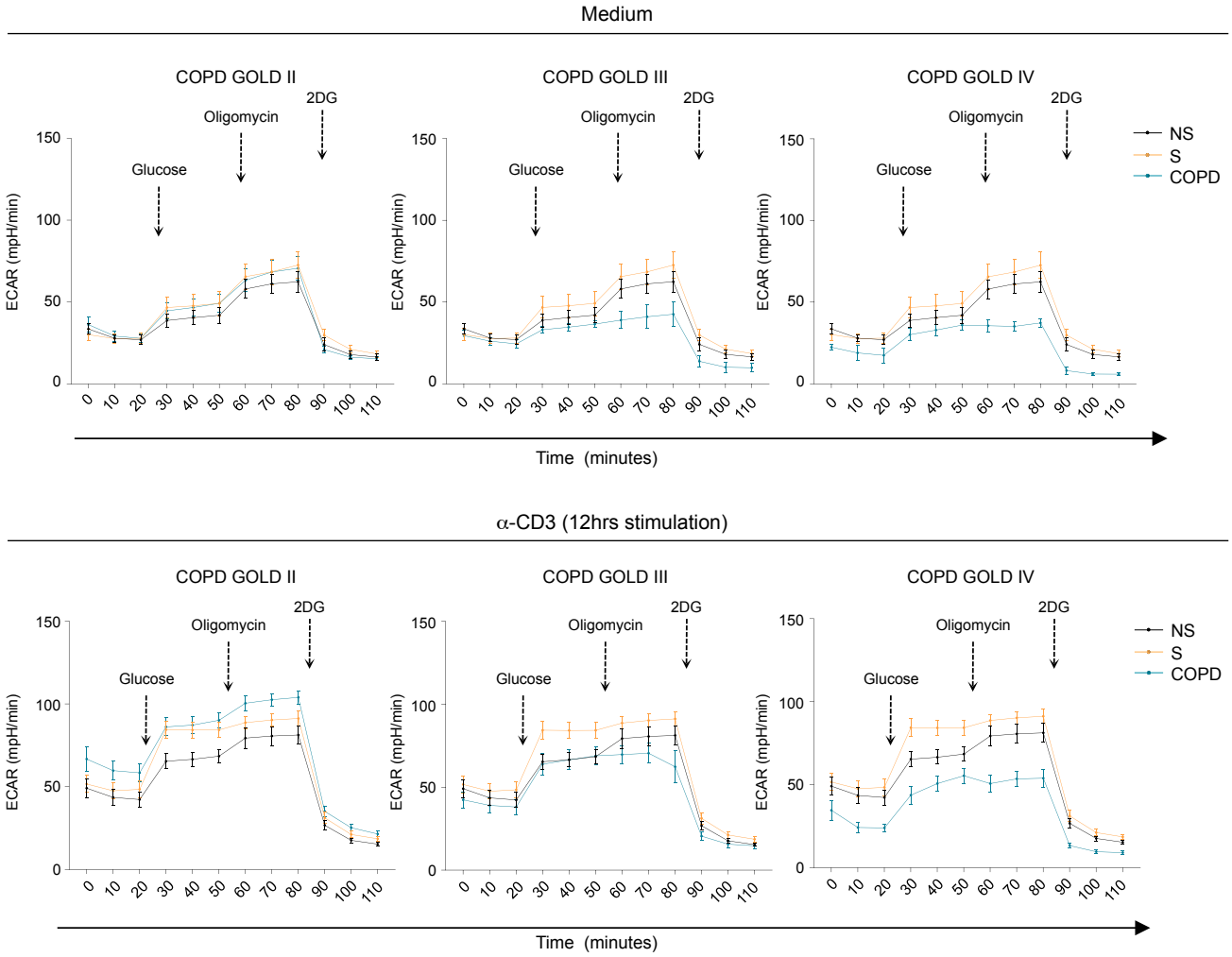
Supplemental Figures



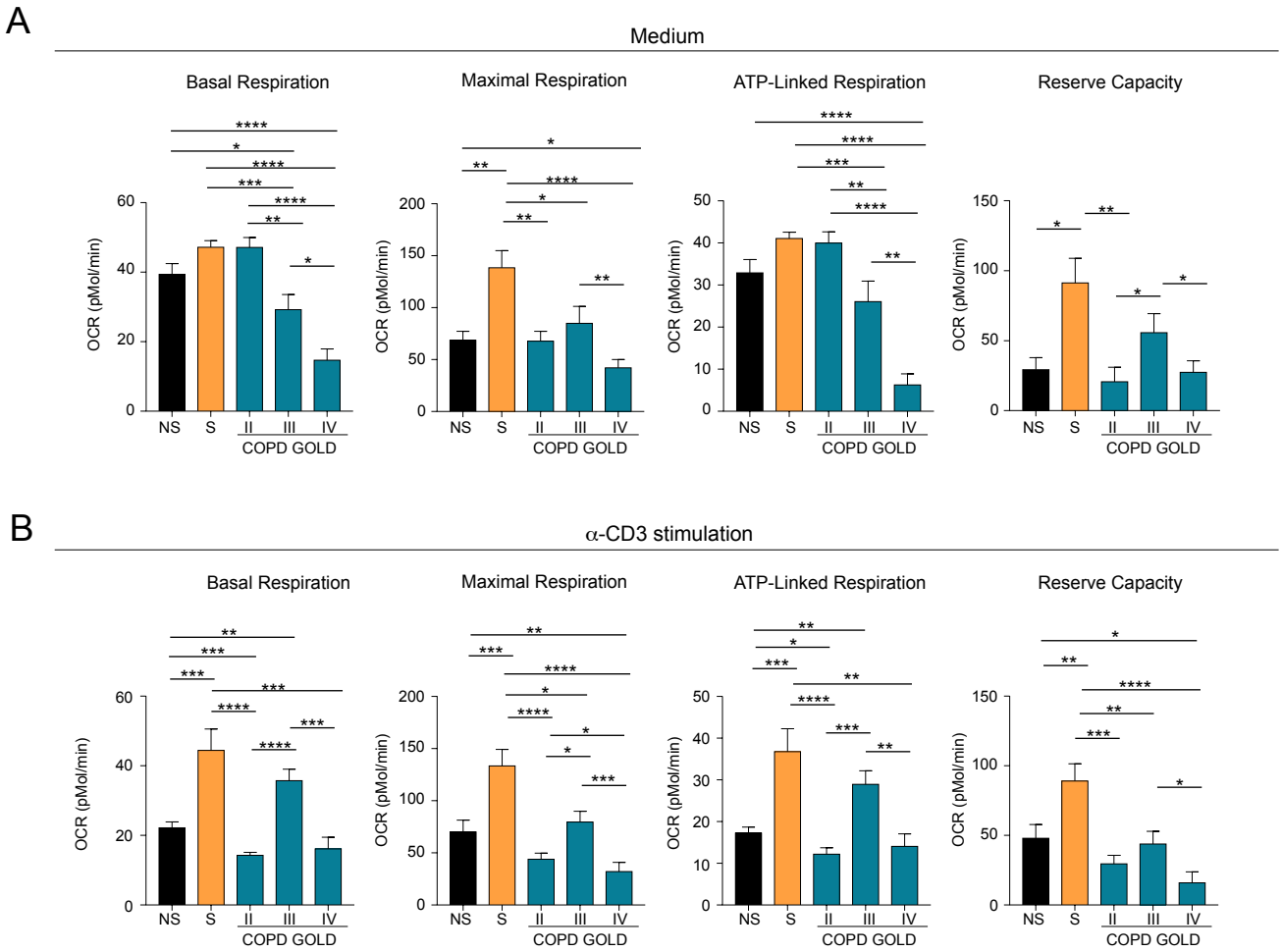
SI Appendix Figure S1. Progression of plasma levels of inflammatory/metabolic molecules in COPD with different GOLD stages.
Plasma circulating levels of resistin, MPO, sCD40L, MCP-1, sTNFr and sICAM-1 in never-smoker (NS), smoker (S) healthy subjects and COPD individuals with different GOLD stages. Data are from at least n=6 subjects. Data are expressed as mean ± SEM. Each symbol represents an individual healthy control or COPD subject as indicated. **P* <0.05; ***P* <0.01; ****P* <0.001; *****P* <0.0001 by two-tailed Mann-Whitney test.



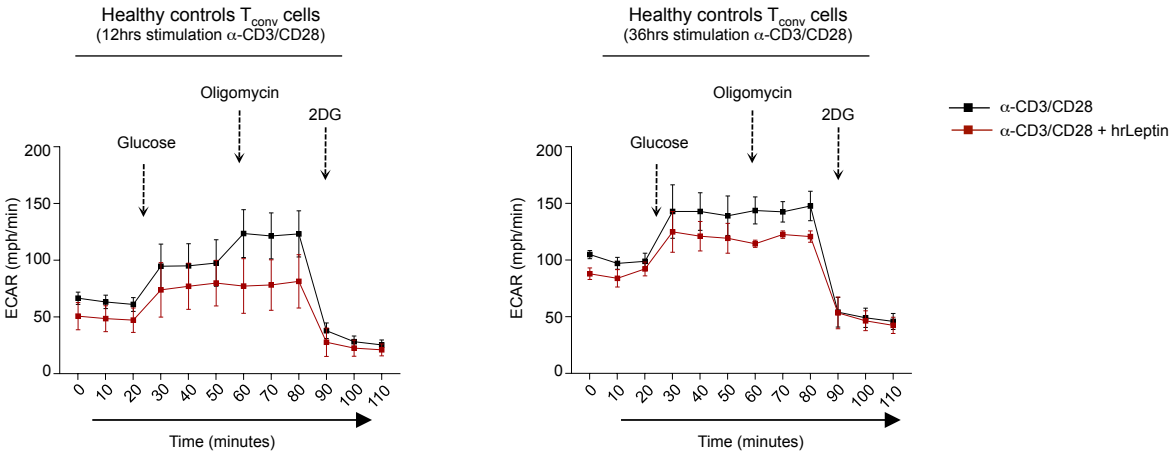
SI Appendix Figure S2. Correlation between plasma levels of inflammatory/metabolic molecules and lung function in COPD subjects with different GOLD stages.
Statistical correlation between Forced Expiratory Volume in 1s (FEV_1), as measure of lung function, and plasma levels of resistin, MPO, sCD40L, MCP-1, sTNFR and sICAM-1, respectively. Data are from at least n=26 COPD subjects. Each symbol represents an individual COPD subject. r=-0.63, *P*=0.0006; r=-0.49, *P*=0.007; r=-0.25, *P*=0.20; r=-0.29, *P*=0.12, r=-0.26, *P*=0.16; r=0.12, *P*=0.44 by Pearson's correlation.



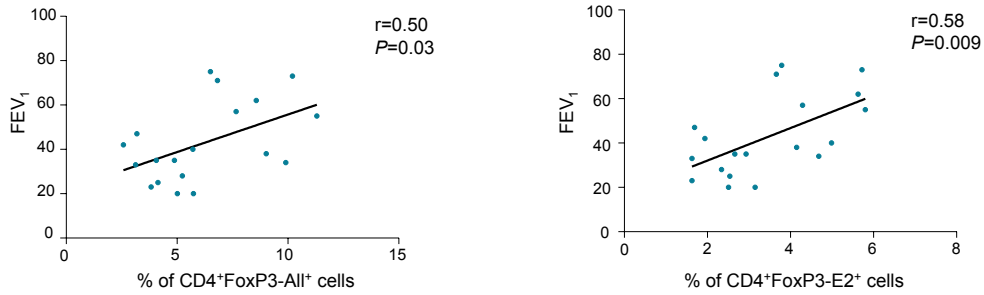
SI Appendix Figure S3. Impaired engagement of glycolysis of T cells from COPD subjects during disease progression. ECAR as indicator of glycolysis of PBMCs unstimulated (upper) or 12 hours α-CD3-stimulated (lower) from never-smoker (NS), smoker (S) healthy subjects and COPD individuals at different disease stages, in the presence of glucose, the ATP-synthase inhibitors oligomycin and 2-deoxy-D-glucose (2DG), as indicated. Data are from n=7 independent experiments (at least n=2 subjects in technical triplicate); data are expressed as mean ± SEM.



SI Appendix Figure S4. Impaired mitochondrial respiration of T cells from COPD subjects. (*A*) Respiratory parameters of the oxygen consumption rate (OCR) in unstimulated PBMCs from never-smoker (NS), smoker (S) and COPD subjects with different GOLD stages. (B) Respiratory parameters of the OCR in PBMCs after 12 hours α-CD3-stimulation from NS, S and COPD subjects with different GOLD stages. OCR parameters (in *A* and *B*) were calculated as follow: basal respiration (calculated as the difference between the mean of the first 3 measurements and the mean of the 3 measurements after rotenone/antimycin A injection), maximal respiration (calculated as the difference between the mean of the 3 measurements after FCCP injection and the mean of the 3 measurements after rotenone/antimycin A injection), ATP-linked respiration (calculated as the difference between the mean of the first 3 measurements and the mean of the 3 measurements after oligomycin injection) and reserve capacity (calculated as the difference between the mean of the first 3 measurements and the mean of the 3 measurements after FCCP injection). For *A* and *B*, data are from at least n=3 independent experiments (at least n=2 subjects); data are expressed as mean ± SEM. *P <0.05; **P <0.01; ***P <0.001; ****P <0.0001 by two-tailed Mann-Whitney test.

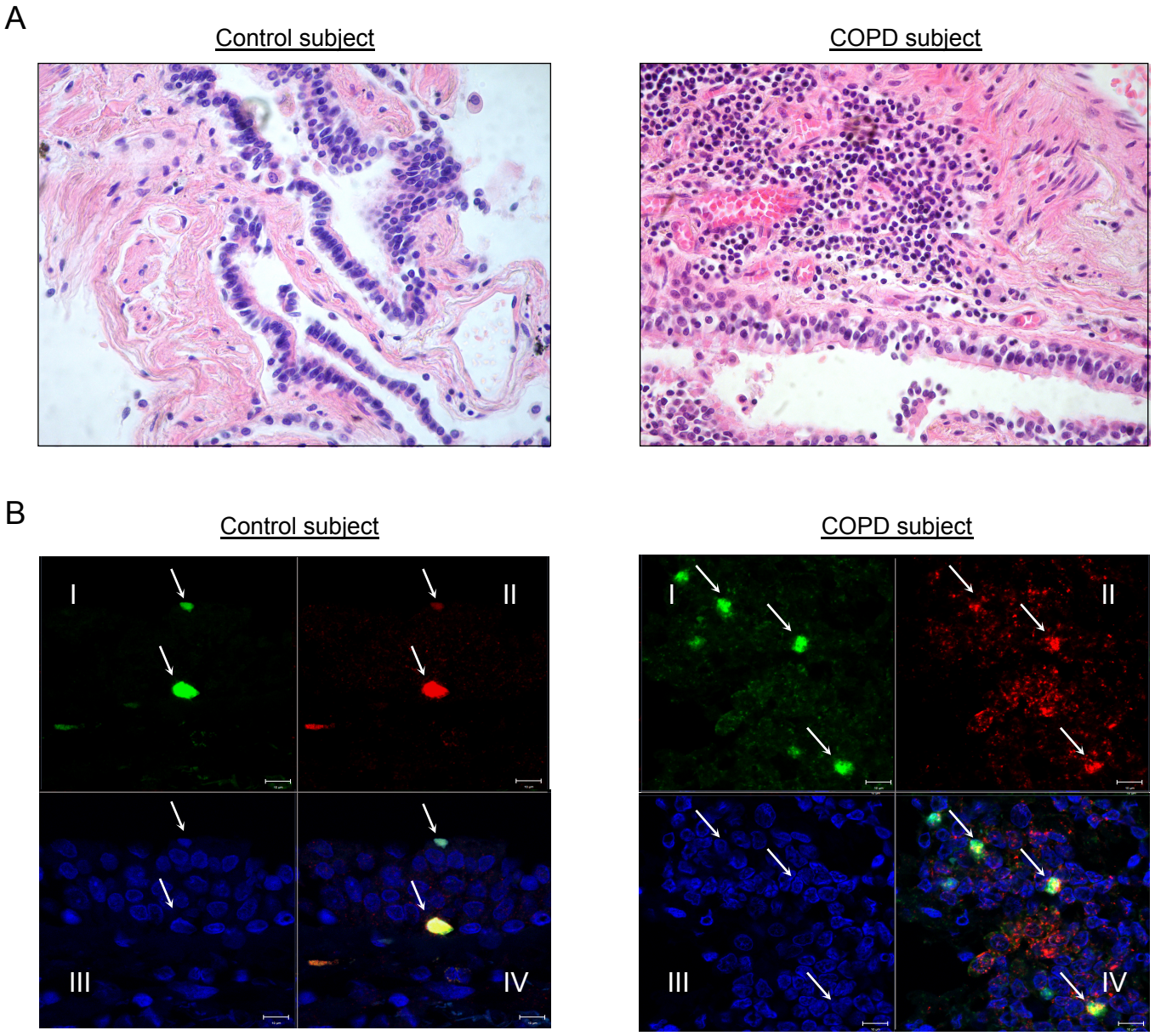


SI Appendix Figure S5. Leptin reduces the glycolysis of T cells in healthy subjects. ECAR as indicator of glycolysis of $CD4^{+}CD25^{-}$ isolated from healthy subjects and stimulated for 12 hours (left) or 36 hours (right) with α-CD3/CD28 alone or in presence of human recombinant (hr) leptin; injection of glucose, the ATP-synthase inhibitors oligomycin and 2-deoxy-D-glucose (2DG), as indicated.

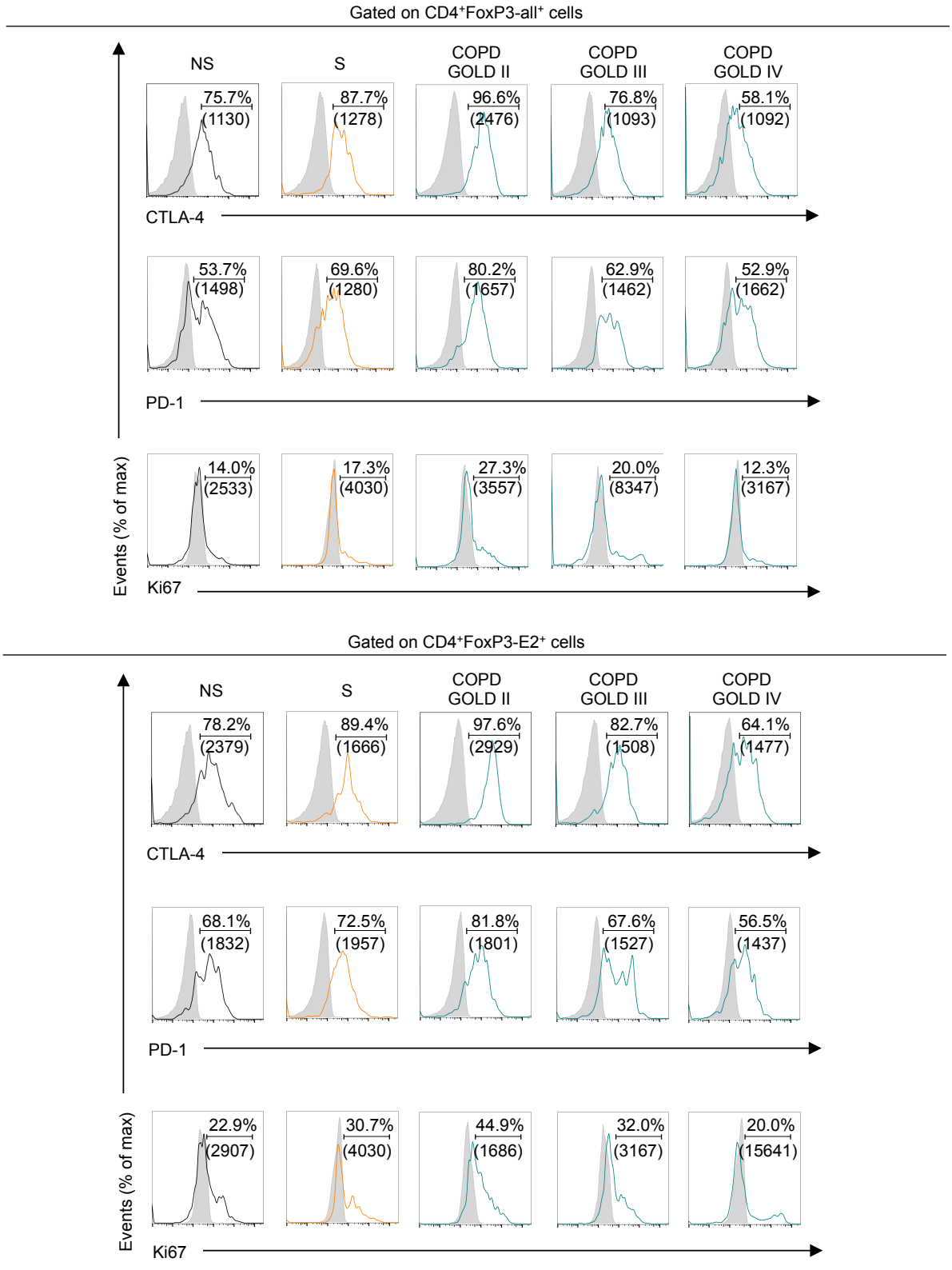


SI Appendix Figure S6. Biological correlation between circulating T_{reg} cells and lung function.

Left, positive statistical correlation between frequency of peripheral $CD4^+FoxP3\text{-}all^+$ cells and between Forced Expiratory Volume in 1s (FEV_1), as measure of lung function. Right, positive statistical correlation between frequency of peripheral $CD4^+FoxP3\text{-}E2^+$ cells and FEV_1. Data are from n=19 COPD subjects. Each symbol represents an individual COPD subject. r= 0.50, *P*=0.03 and r= 0.58, *P*=0.009 by Pearson's correlation.

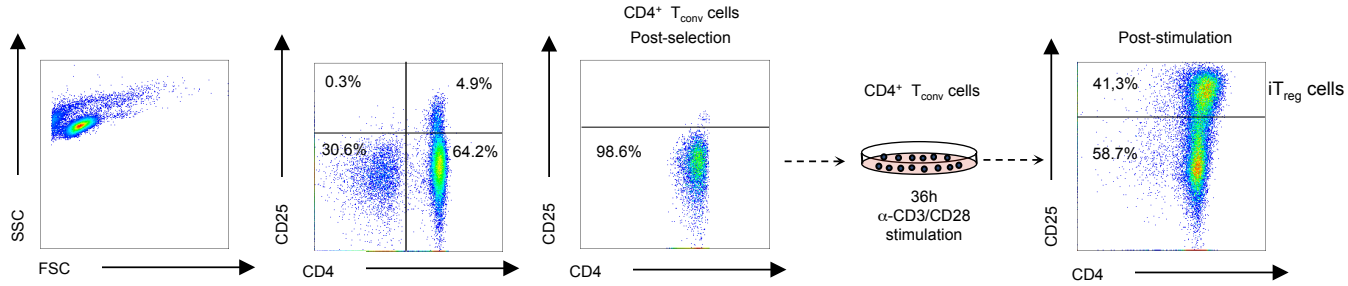


SI Appendix Figure S7. **FoxP3⁺ cells in the lung tissue from healthy and COPD subjects**. (*A*), Left, hematoxylin eosin staining of lung parenchyma from control subject affected by pulmonary hamartoma. Terminal bronchioles with a well preserved covering epithelium with no signs of inflammation. Rare endoalveolar macrophages with moderate anthracosis. Thin wall capillaries with intact endothelium. Right, hematoxylin eosin staining of lung parenchyma of COPD subject with GOLD III. Evidence of inflammation and lymphoplasmacytic intra-epithelial and peri-bronchiolar infiltration. Anthracosis and vascular congestion. Magnification 40x. (*B*) Left, confocal microscopy images show FoxP3-all protein expression (I, green) and FoxP3-E2 protein (II, red) in lung tissue section from control subject. Nuclei were counterstained with DAPI (III, blue); Merge is showed in IV. Right, confocal microscopy images show FoxP3-all protein expression (I, green) and FoxP3-E2 protein (II, red) in lung tissue section from COPD subject. Nuclei were counterstained with DAPI (III, blue). Merge is showed in IV. Bar=10 μm. Staining of FoxP3-all was performed by using mAb clone # 236A/E7 (from eBioscience); staining of FoxP3-E2 was performed by using mAb clone 150D/E4 (from eBioscience).

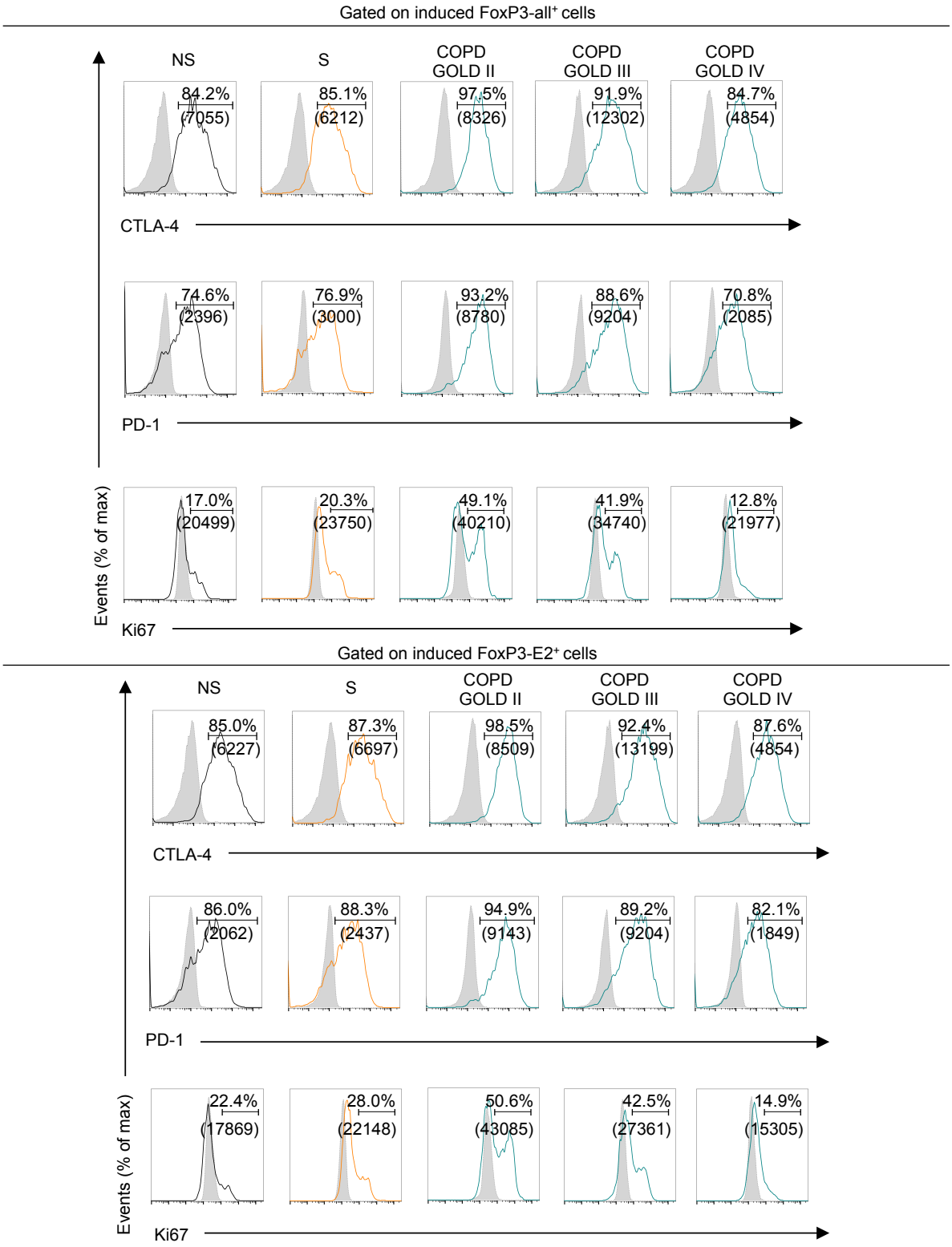


SI Appendix Figure S8. Progressive reduction of T_{reg} cell-specific markers in pT_{reg} of COPD subjects with different GOLD stages.

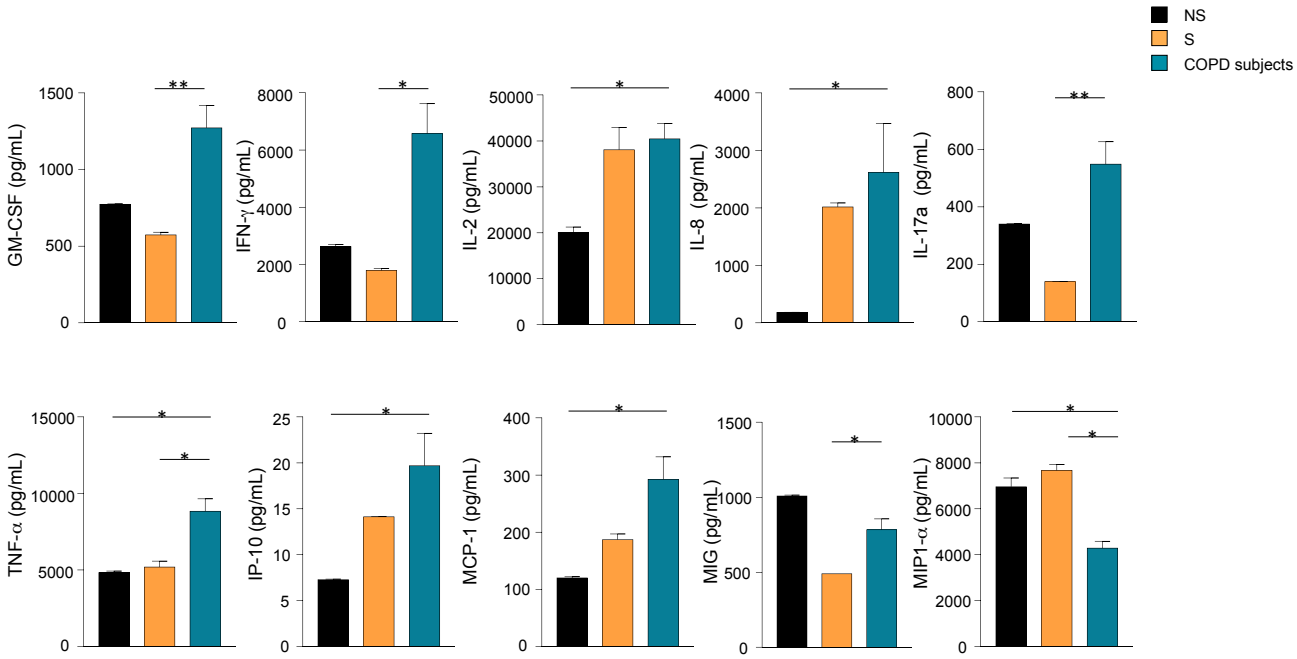
Representative flow cytometry histograms show the expression of T_{reg} cell-specific markers (CTLA-4, PD-1) and Ki-67 in peripheral $CD4^+FoxP3\text{-}all^+$ (upper panels) and $CD4^+FoxP3\text{-}E2^+$ cells (lower panels) in never-smoker (NS), smoker (S) healthy subjects and COPD subjects with GOLD II, GOLD III and GOLD IV stages. Numbers in the plots indicate percent of positive cells and in brackets mean fluorescence intensity (MFI) of the marker; grey fill histograms are the fluorescence minus one (FMO) controls.



SI Appendix Figure S9. Schematic experimental procedure for the *in vitro* generation of human induced (i)T_{reg} cells from activated $CD4^+CD25^-$ (T_{conv}) cells.
T_{conv} cells were isolated from PBMCs of healthy and COPD subjects by negative selection with magnetic beads. T_{conv} cells were then stimulated with α-CD3/CD28 (0.1 bead/cell) for 36 hours to obtain $CD25^+$ iT_{reg} cells, as indicated.

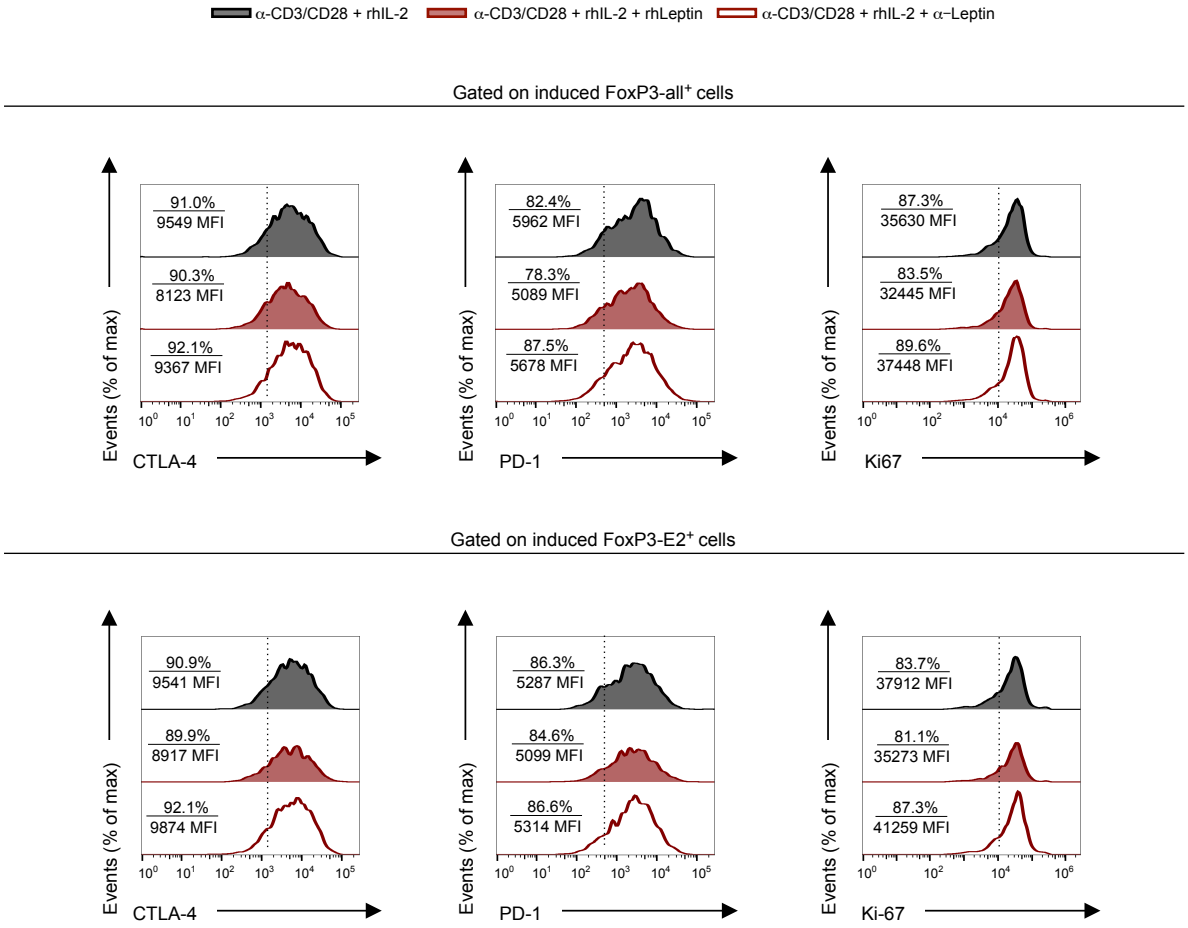


SI Appendix Figure S10. Progressive reduction of T_{reg} cell-specific markers in induced Foxp3$^+$ iT_{reg} cells of COPD subjects with different GOLD stages.
Representative flow cytometry histograms show the expression of T_{reg} cell-specific markers (CTLA-4, PD-1) and Ki-67 in induced FoxP3-all$^+$ (upper panels) cells and induced FoxP3-E2$^+$ cells from never-smoker (NS), smoker (S) healthy subjects and COPD individuals with GOLD stage II, GOLD stage III and GOLD stage IV, as indicated. Numbers in the plots indicate percent of positive cells and in brackets mean fluorescence intensity (MFI) of the marker; grey fill histograms are the fluorescence minus one (FMO) controls.



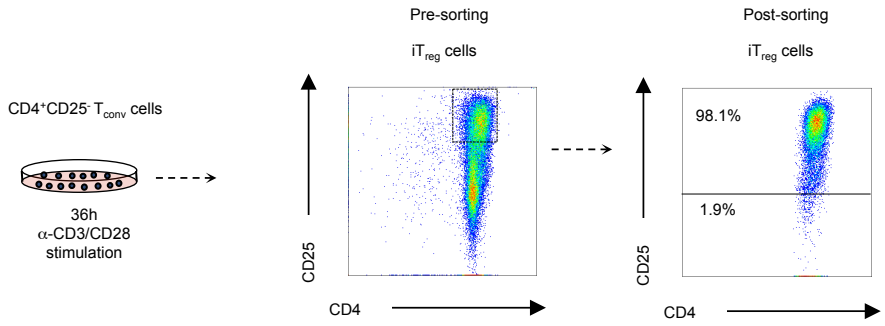
SI Appendix Figure S11. Cytokines and chemokines released from activated CD4$^+$CD25$^-$ (T_{conv}) cells *in vitro* during generation of iT_{reg} cells in healthy and COPD subjects.

Cytokines and chemokines produced during the induction of T_{reg} from T_{conv} cells stimulated with α-CD3/CD28 (0.1 bead/cell) for 36 hours, in never smoker (NS), smoker (S) healthy subjects and COPD individuals. Data are expressed as mean ± SEM. Data are from at least n=3 subjects. **P* <0.05; ***P* <0.01 by two-tailed Mann-Whitney test.



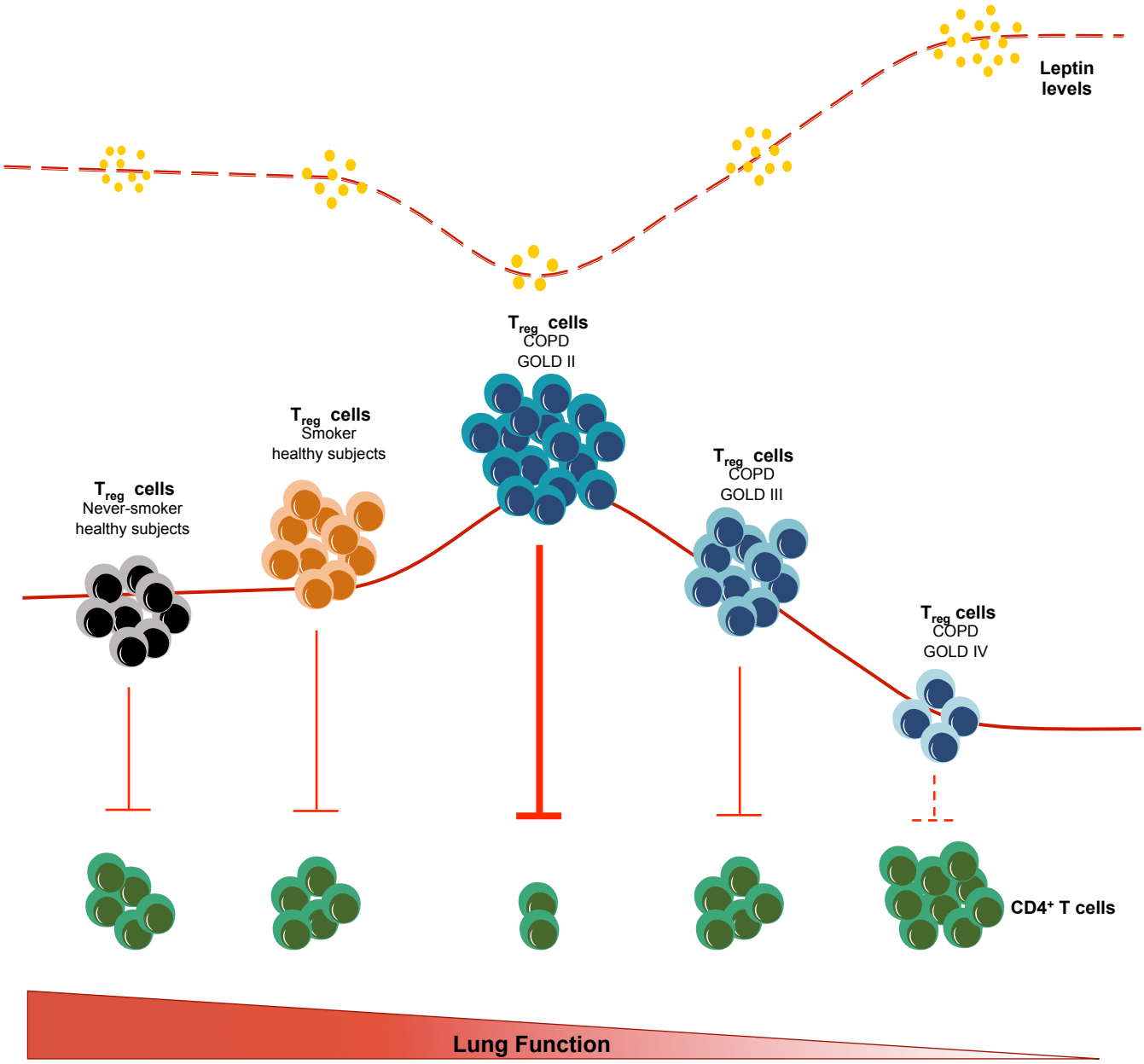
SI Appendix Figure S12. Leptin treatment affects the expression of T_{reg} cell lineage-markers and Ki-67 in iT_{reg} cells.

Representative flow cytometry plots showing the percentage and MFI of the main T_{reg} cell specific-markers (CTLA-4, PD-1) and Ki-67 in induced FoxP3-all$^+$ (upper panels) and FoxP3-E2$^+$ (lower panel) cells, generated from T_{conv} cells of healthy subjects (in autologous 5% plasma) stimulated for 10 days with α-CD3/CD28 plus hrIL-2 (upper panels), in the presence of exogenous chronic hrLeptin (200 ng/ml) (middle panels) or in the presence of neutralizing α-leptin (20ng/ml) monoclonal antibody (lower panels).



SI Appendix Figure S13. Schematic experimental procedure for isolation of iT_{reg} cells.

$CD4^+$ T_{conv} cells were stimulated with α-CD3/CD28 (0.1 bead/cell) for 36 hours to obtain $CD25^+$ iT_{reg} cells. Then, iT_{reg} cells were flow-sorted and used for functional experiments.



SI Appendix Figure S14. Representative model of the immunometabolic pathomechanism at basis of COPD progression.
COPD patients showed a progressive increase of systemic leptin secretion that associated with lower number of regulatory T (T_{reg}) cells. Thus, the loss of T_{reg} cell regulatory activity leads to hyper-activation (dysregulation) of $CD4^+$ T cells that further fuels inflammation and progressive decline of lung function in COPD patients.

Supplemental Tables

Cell populations	Never Smoker healthy subjects (n=9)	Smoker healthy subjects (n=6)	COPD GOLD II subjects (n=18)	COPD GOLD III subjects (n=11)	COPD GOLD IV subjects (n=4)	*P*
Leucocytes	6581.11±2053.94	7360.17±995.75	7000±1877.96	7745±2437.66	5520±718.61	*NS*
Lymphocytes	2014.3±616.69 (31.61±7.56)	2266.16±853.49 (31.33±8.52)	2113.38±751.69 (32.20±7.15)	2196.27±1554.69 (38.8±14.24)	1897±698.39 (33.7±12.31)	*NS*
$CD3^+$	1550.44±501.37 (76.88±6.91)	1717.33±611.16 (76.16±3.06)	1546.27±573.70 (73.05±10.38)	1689.27±1334.26 (74.18±7.99)	1531±575.91 (80.50±5.06)	*NS*
$CD4^+$	937.44±261.37 (47.11±6.35)	1806.00±502.38 (47.0±4.64)	936.83±341.07 (44.33±6.77)	1009.36±788.56 (45.09±12.33)	936.75±549.90 (46.50±13.91)	*NS*
$CD8^+$	546.33±259.53 (26.55±5.54)	548.50±149.14 (25.50±7.45)	546.94±239.58 (25.72±6.85)	605.54±540.28 (25.54±9.04)	472.50±285.50 (26.50±12.79)	*NS*
Natural Killer	200.56±136.83 (9.66±4.82)	182.83±60.76 (8.83±4.07)	277.94±161.54 (12.61±5.11)	279.63±159.59 (14.09.±7.35)	135.75±34.80 (8.25±4.34)	*NS*
B lymphocytes	211.88±85.24 (10.77±3.70)	284.00±201.35 (11.66±3.77)	262.55±254.54 (11.88±10.08)	199.72±130.36 (10.09.±5.16)	171.00±133.65 (7.75±5.12)	*NS*
$CD3^+CD56^+$	99.67±89.34 (4.88±4.28)	82.88±32,92 (4.0±2.09)	95.03±64.99 (4.50±2.28)	75.82±120.90 (2.72±2.19)	87.33±91.87 (4.50±3.87)	*NS*
$CD4^+CD8^+$	18.21±12.46 (0.88 ±0.60)	18.93±16.39 (0.83±0.75)	21.98±28.20 (0.88±0.83)	32.03±38.74 (1.72±2.41)	38.49±51.16 (3.50±5.68)	*NS*
$CD3^+CD45RA^+$	649.47±257.51 (32.66±9.0)	851.67±305.79 (38.0 ±4.69)	637.11±326.76 (30.05±9.52)	631.09±446.76 (28.36±9.14)	609.25±469.61 (30.25±16.82)	*NS*
$CD3^+CD45RO^+$	898.96±366.11 (44.22±7.94)	858.81±324.41 (38.16±2.85)	906.94±364.24 (42.9.±11.03)	1053.10±942.85 (45.81±10.21)	921.86±515.22 (50.25±21.15)	*NS*
$CD4^+CD28^+$	735.66±324.01 (44.5±6.48)	1025.26±501.61 (44.33±6.15)	892.62±333.92 (42.11±12.44)	982.77±785.59 (43.63±10.21)	883.28±506.51 (44.00±11.74)	*NS*
$CD4^+DR^+$	7.34±8.75 (0.50 ±0.53)	12.83±16.10 (0.50±0.54)	16.85±16.89 (0.83±0.85)	24.57±34.39 (1.00±0.77)	25.96±7.86 (1.5±0.57)	*NS*
$CD4^+CD45RA^+$	438.59±177.61 (22.2±7.03)	569.92±274.08 (24.83±6.33)	382.70±215.16 (18.22±8.19)	399.24±238.23 (18.90±6.39)	415.82±413.34 (20.00±15.97)	*NS*
$CD4^+CD45RO^+$	517.38±257.52 (25.37±9.19)	512.43±278.49 (22.16±6.36)	565.01±243.76 (26.11±7.64)	609.86±635.63 (26.18±12.96)	520.99±270.01 (26.50±6.55)	*NS*
$CD4^+CD25^+$	20.45±14.18 (1.2±0.7)	21.8±14.4 (1.0±0.63)	28.17±46.45 (1.28±1.9)	16.41± 16.5 (1.64±1.80)	17.15±16.0 (1.0±0.8)	*NS*
$CD8^+CD45RA^+$	211.18±93.21 (10.44±2.83)	282.98±112.95 (13.17±5.67)	216.49±174.4 (10.65±5.68)	239.38±281.94 (9.73±6.07)	193.42±69.9 (10.25±1.26)	*NS*
$CD8^+CD45RO^+$	688.74±324.15 (21.67±7.47)	348.43±97.83 (16.0±4.43)	300.30±202.6 (15.15±8.30)	446.9±391.86 (19.64±8.72)	400.75±355.47 (23.75±17.84)	*NS*
$CD8DR^+$	4.11±8.17 (0.25±0.46)	6.16±9.55 (0.33±0.51)	13.94±12.96 (0.72±0.76)	10.47±11.98 (0.72±0.78)	15.8±20.95 (1.00±1.15)	*NS*
$CD8^+11b^+$	60.20±28.01 (3.11±1.45)	64.45±40.11 (3.00±2.19)	109.25±83.11 (5.50±3.41)	120.70±138.51 (4.90±3.59)	98.50±72.14 (4.77±3.00)	*NS*
$CD3^-CD8^+$	63.53±64.00 (2.88±2.31)	42.22±27.15 (2.00±1.26)	126.53±92.88 (5.72±3.87)	85.37±85.58 (4.72±5.46)	50.34±28.78 (2.50±1.29)	*NS*

SI Appendix Table S1. Absolute numbers and percentages of circulating immune cells in never-smokers, smokers healthy subjects and COPD subjects with different GOLD stages (percentages in brackets).

Data are expressed as mean ± SD. The absolute number *per* mm^3 of each cell populations was calculated as follows: percent of a given cell population multiplied by the number of lymphocytes/100. COPD, chronic obstructive pulmonary disease. *NS*= not significant by non parametric Kruskal-Wallis test.

Inflammatory and metabolic molecules in plasma	Never-smoker healthy subjects	Smoker healthy subjects	COPD GOLD II subjects	COPD GOLD III subjects	COPD GOLD IV subjects
Leptin, ng/ml	14.28 ± 1.70$^{§,\#}$ (n=21)	11.75 ± 1.58$^{\$,\#\#}$ (n=18)	8.42 ± 1.21$^{*,\$\$,\#\#\#}$ (n=10)	18.56 ± 2.43†,§§ (n=14)	25.45 ± 3.39*,††,§§§ (n=7)
Resistin, pg/ml	6626 ±627.3$^{\$\$\$,\#\#}$ (n=21)	6286 ±512.7$^{\$\$\$\$,\#\#\#}$ (n=18)	6683 ±641.1$^{\$\$,\#}$ (n=10)	9794 ±374.5***,††††,§§ (n=9)	10884 ±1274**,†††,§ (n=7)
MPO, ng/ml	186 ±31.4 (n=22)	180.4 ±18.9$^{\#\#\#}$ (n=20)	175.4 ±24.6$^{\#\#\#}$ (n=10)	246.9 ±31.1 (n=13)	340.1 ±17.75†††,§§§ (n=6)
sCD40L, pg/ml	665 ±147.9$^{\$,\#}$ (n=19)	619.2 ±100.9$^{\#\#\#}$ (n=17)	582.1 ±108.1$^{\#\#\#}$ (n=10)	1074 ±195.9* (n=13)	1402 ±36.01*,†††,§§§ (n=6)
MCP-1, pg/ml	479.4 ±25.0$^{\$\$\$,\#\#\#\#}$ (n=20)	515.8 ±48.6$^{\$,\#\#}$ (n=20)	635.2 ±67.8 (n=9)	683.8 ±46.5***,† (n=13)	745.1 ±40.6****,†† (n=7)
sTNFr, ng/ml	1.8 ±0.1$^{\$\$\$\$,\#\#}$ (n=22)	2.2 ±0.2$^{§,\$}$ (n=18)	2.2 ±0.3$^{†,\$}$ (n=10)	2.9 ±0.2****,†,§ (n=13)	2.6 ±0.2** (n=7)
sICAM, ng/ml	403.8 ±23.5$^{†,\ \$\$,\#\#}$ (n=22)	534.1 ±42.6* (n=20)	668.4 ±146.6 (n=10)	655.4 ±76.2** (n=13)	616 ±62.9** (n=7)

SI Appendix Table S2. Inflammatory and metabolic molecules in plasma samples from healthy and COPD individuals with different GOLD stages.

Data show the plasma circulating levels of leptin, resistin, MPO, sCD40L, MCP-1, sTNFr and sICAM-1 in never smoker, smoker healthy subjects and COPD individuals with different GOLD stages. Data are expressed as mean ± SEM. Data are from at least n=6 subjects. *, †, §, $, # *P* <0.05; **, ††, §§, $$, ## *P* <0.01; ***, †††, §§§, $$$, ### *P* <0.001; ****, ††††, §§§§, $$$$, #### *P* <0.0001 by Mann-Whitney test. * *versus* NS; † *versus* S; § *versus* COPD GOLD II; $ *versus* COPD GOLD III; # *versus* COPD GOLD IV

Inflammatory and metabolic molecules in plasma	% of $CD4^+FoxP3\text{-}all^+$ pT_{reg} cells		% of $CD4^+FoxP3\text{-}E2^+$ pT_{reg} cells	
	r	*P*	r	*P*
Leptin, ng/ml	-0.64	0.01	-0.75	0.001
Resistin, pg/ml	-0.46	*NS*	-0.42	*NS*
MPO, ng/ml	-0.06	*NS*	0.18	*NS*
sCD40L, pg/ml	-0.38	*NS*	-0.09	*NS*
MCP-1, pg/ml	-0.16	*NS*	-0.41	*NS*
sTNFr, ng/ml	-0.48	*NS*	-0.52	0.05
sICAM, ng/ml	0.16	*NS*	0.22	*NS*

SI Appendix Table S3. Statistical correlation between frequency of circulating $CD4^+FoxP3\text{-}all^+$ or $CD4^+FoxP3\text{-}E2^+$ pT_{reg} cells and circulating levels of inflammatory/ metabolic molecules, respectively. r values were calculated by Pearson's correlation; NS=not significant.

Inflammatory and metabolic molecules in plasma	% of $CD4^+FoxP3\text{-}all^+$ iT_{reg} cells		% of $CD4^+FoxP3\text{-}E2^+$ iT_{reg} cells	
	r	*P*	r	*P*
Leptin, ng/ml	-0.72	0.002	-0.76	0.001
Resistin, pg/ml	-0.52	*NS*	-0.69	0.009
MPO, ng/ml	-0.20	*NS*	-0.43	*NS*
sCD40L, pg/ml	0.16	*NS*	-0.05	*NS*
MCP-1, pg/ml	-0.64	0.01	-0.63	0.01
sTNFr, ng/ml	-0.24	*NS*	-0.32	*NS*
sICAM, ng/ml	0.58	0.02	0.52	0.05

SI Appendix Table S4. Statistical correlation between frequency of circulating $CD4^+FoxP3\text{-}all^+$ or $CD4^+FoxP3\text{-}E2^+$ iT_{reg} cells and circulating levels of inflammatory/ metabolic molecules, respectively. r values were calculated by Pearson's correlation; NS=not significant.

Parameters	Never-smoker healthy subjects	Smoker healthy subjects	COPD GOLD II subjects	COPD GOLD III subjects	COPD GOLD IV subjects	*P*
Number of subjects	22	20	30	22	11	-
Age,yr	57±12	55±9	68±7	67±7	66±8	*NS*
Sex:						
Male	16	10	23	16	8	-
Female	6	10	7	6	3	-
BMI, Kg/m^2	27±2	26±5	30±6	29 ±7	31 ±7	*NS*
Smoking status:						-
Current Smokers	0	15	10	4	4	-
Ex Smokers	0	5	20	17	7	-
Never Smokers	22	0	0	1	0	-
Pack-years of smoking	-	40±19	50±27	43 ±24	68 ±49	*NS*
Lung function:						
FEV_1, % pred	98±12	96±11	63 ±9*	39 ±6*	24 ±4*	**P*<0.05
FEV_1/FVC, %	82±11	85±9	62 ±7*	52 ±7*	46 ±5*	**P*<0.05
Disease duration, yr	-	-	3±3	5±2	5±4	-
COPD assessment test (CAT) score	-	-	16±8	19±6	22 ±8	-
N exacerbations/last yr	-	-	1.4±0.8	1.7±0.9	1.7±1	-
ABCD grading:						
A	-	-	0	0	0	-
B	-	-	17	7	2	-
C	-	-	6	0	0	-
D	-	-	7	15	9	-

SI Appendix Table S5. Baseline clinical characteristics of healthy and COPD subjects enrolled in the study.

Data are expressed as mean ± SD; Body mass index (BMI) is the weight in kilograms divided by the square of the height in meters. COPD, chronic obstructive pulmonary disease; FEV_1, Forced Expiratory Volume in 1s; FVC, Forced Vital Capacity; GOLD, Global Initiative for Chronic Obstructive Lung Disease. *NS*= not significant. **P* <0.05 by non parametric Kruskal-Wallis test. **versus* healthy subjects.