1 # CD4+T cells in aging-associated interstitial lung abnormalities show evidence of pro-inflammatory phenotypic and functional profile

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7 Running title: Pro-inflammatory CD4+ T cells in ILA

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Supporting information

Methods

Study populations

Respiratory asymptomatic volunteers aged 60 or older have been invited to participate in our “Lung Aging Program”, initiated in Mexico City in March 2015. From this cohort we studied 15 subjects with diagnosis of Interstitial Lung Abnormalities based in high resolution computed tomography (HRCT) findings. Twenty-one age-matched subjects without abnormalities in HRCT and 28 young individuals (<30 years old) were evaluated as controls. It is important to note that because of feasibility issues, the number of samples processed for experiments varied; the number processed by technique is indicated in each figure.

All individuals signed a consent letter to participate in this research and procedures were performed in accordance with the 1964 Helsinki Declaration as well as with the ethical standards of the Institutional Ethic Committee (protocol numbers: C39-14 and B16-19).

Multiparametric cytometry analysis

PBMCs were obtained and prepared to flow cytometry analysis. The following monoclonal antibodies (mAbs) were used: CD3, CD8a, CD4, CD197 (CCR7), CD45RA, CD57, CD25, CD27, CD28, KLRG1 and CD279 (PD-1), more information of the specific fluorochrome used is indicated in **figure S1**. PBMCs were incubated with mAbs for 20 minutes at 4ºC, washed and resuspended in staining buffer (BioLegend, San Jose, CA, USA). Multiparametric flow cytometry was performed using a FACS Aria II flow cytometer (Becton Dickinson, San Jose, CA, USA). The side scatter/forward gating strategy was used to exclude dead cells. Fluorescence minus one (FMO) controls were employed to set the gates for specific immune cell subpopulations. We acquired at least 100,000 events per

52 sample. Compensation set up and calculation of the frequency of specific cell
53 subsets were made using Flow Jo (Flow Jo, LLC, Ashland, OR, USA) (**figure S2**).

54 Preparation of peripheral blood mononuclear cells (PBMCs)

55 PBMCs were collected from peripheral blood samples via ficoll density gradient
56 (Lymphoprep™ Axis-Shield. Oslo, Norway). PBMCs were collected, counted and
57 preserved in fetal bovine serum with 10% of DMSO and stored in liquid nitrogen
58 until used. Plasma was stored at -80ºC until use.

59 **Total T and CD4+ T cells enrichment**

60 Total T cells were isolated from PBMCs by negative selection, using a MACS
61 system and following the provider instructions (Pan T isolation kit Miltenyi Biotech,
62 Bergisch Gladbach, Germany). The negative fraction represented the total T cells,
63 and the efficiency of the enrichment of the total T cells fraction was routinely >90%,
64 as analysed by flow cytometry. As control, we evaluated the positive fraction (total
65 T cells depleted and a minimum percentage of CD3+ cells still in this fraction
66 (**figure S3A and B, respectively**). Total T cells were used to develop the
67 polyclonal stimulus culture as is indicated below.

68 For other experiments, enriched total T cells were incubated with CD4+ T Cell
69 Biotin Antibody Cocktail from CD4+ T Cell Isolation kit. We obtained two cellular
70 fractions, one of enriched CD4+ T cells and a second with CD4- T cells (which are
71 mainly CD8+ T cells); both CD4+ and CD4- T cells fractions were used to evaluate
72 multimeric complexes of KLRG1.

73 **Western Blot**

74 CD4+ and CD4- T cells were lysed, and suspended in Laemmli buffer. SDS-PAGE
75 under non-reducing conditions was performed, and proteins were transferred to a
76 0.2 µm-pore-size polyvinylidene difluoride (PDFV) membrane (Biorad, Hercules,
77 CA, USA). Western blot was performed using the following antibodies anti-: KLRG1
78 (dilution 1:2000), Glyceraldehyde 3-phospate dehydrogenase (GAPDH, dilution
79 1:1000), Mouse- and Rabbit-IgG horseradish peroxidase-labeled (both used
80 1:2000) (R&D Systems, Minneapolis, MN, USA). Protein bands were detected by

81 incubation with enhanced chemiluminiscence reagent (Thermo Scientific, Pierce
82 Biotech., Rockford, IL), and visualised with the Imaging System from Bio-Rad
83 (ChemiDoc^{TM} XRS+System). Band densities were analysed by densitometry using
84 online IMAGEJ 1.39c software provided by NIH. Each sample was normalized
85 using GAPDH as a loading control. More information of the antibodies used is
86 indicated in **figure S1**.

87 **Polyclonal stimulation of T cells**

88 Total T cells were cultured at 2×10^5 cells/well in 96-well plates (Costar, Ontario,
89 Canada). Polyclonal stimulation was induced with mAbs anti-CD3 (fixed in plate)
90 and CD28 (soluble), both mAbs at 1µg/ml; and with phorbol myristate acetate
91 (PMA) 50nM– ionomycin (IO) 2ng/µL (both provided by Sigma Aldrich, USA),
92 during 48 h at 37 °C in a humidified atmosphere containing 5% CO2. As negative
93 controls, T cells in media or with isotype control (1µg/mL) were included.
94 Supernatants were collected for cytokine measurements, and cells for proliferation
95 assay using KI-67 (Biolegend, San Jose, CA, USA) (**figure S3C**). More information
96 of antibodies used is indicated in **figure S1**.

97 In order to evaluate the activation and proliferation of T cells by flow cytometry,
98 cells were harvested and washed, and then surface molecules (CD4, CD8 and
99 CD25) were stained as described above. Cell pellet was suspended in
100 fixation/permeabilization solution (eBioscience, Beverly, MA, USA) at 4°C, washed
101 with permeabilization buffer (eBioscience), and then stained with KI-67 for 30 min
102 at 4°C and analysed by flow cytometry (**figure S3C**). Flow cytometry was
103 performed using a FACS Aria II flow cytometer (Becton Dickinson, San Jose, CA,
104 USA). The side scatter/forward gating strategy was used to exclude dead cells and
105 fluorescence minus one (FMO) controls were employed to set the gates.
106 Frequencies of CD25+ and Ki-67+ T cells from the younger group were normalized
107 as the 100%. Culture supernatant was stored to evaluate cytokines and
108 chemokines levels by ELISA or multiplex cytokine assay.

109 **ELISA determinations in culture supernatants and plasma samples**

110 The culture supernatant from T cells and plasma from the three study groups were
111 stored at -80ºC until use. Following the manufacturer recommendations, we used
112 the standard sandwich ELISA for the cytokine IFN-γ (Cat. 430104), IL-2 (Cat.
113 431804), IL-6 (Cat. 430504) and IL-10 (Cat. 430604), provided by Biolegend, and
114 for the proteins E-Cadherin (Cat. DY648) and Granzyme B (Cat. DY2906-05)
115 provided by R&D Systems.

116 Multiple cytokine assay in supernatant samples

117 A Bio-Plex Pro Human cytokine 48-Plex Screening Panel (Cat. 12007283, Bio-Rad
118 Labs, Hercules, CA, USA) was used following the manufacturer's instructions. The
119 data were acquired by means of a Bio-Plex 200 System and analysed using Bio-
120 Plex Manager 6.1 software.

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122 # Supplementary figures

Anti-	Conjugate to	Use to	Reference number	Company
CD3	PE/Cy7	Flow cytometry	317334	BioLegend
CD3	Pacific Blue	Flow cytometry	300330	BioLegend
CD8a	PerCP/Cy5.5	Flow cytometry	300924	BioLegend
CD8a	APC	Flow cytometry	344722	BioLegend
CD4	Brilliant Violet 510	Flow cytometry	35742	BioLegend
CD197 (CCR7)	PE	Flow cytometry	353204	BioLegend
CD45RA	FITC	Flow cytometry	304106	BioLegend
CD57	PE/Cy7	Flow cytometry	359624	BioLegend
CD27	PerCP/Cy5.5	Flow cytometry	356408	BioLegend
KLRG1	APC/Cy7	Flow cytometry	138426	BioLegend
CD28	APC	Flow cytometry	302912	BioLegend
CD25	Brilliant Violet 421	Flow cytometry	302630	BioLegend
CD279 (PD1)	Pacific Blue	Flow cytometry	329916	BioLegend
KI-67	Pacific Blue	Flow cytometry	350512	BioLegend
KLRG1	None	Western blot	MAB70291	R&D Systems
GAPDH	None	Western blot	5174S	Cell signaling
Rabbit IgG	HRP	Western blot	HAF008	R&D Systems
Mouse IgG	HRP	Western blot	HAF007	R&D Systems
CD3	Purified	Activation of T cells	317315	BioLegend
CD28	Purified	Activation of T cells	302934	BioLegend
Mouse IgG1	Purified	Isotype control	553447	BD Pharmingen
Mouse IgG2a	Purified	Isotype control	554645	BD Pharmingen

Supplementary 1

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124 **Figure S1.** Information of the monoclonal antibodies used in this study.

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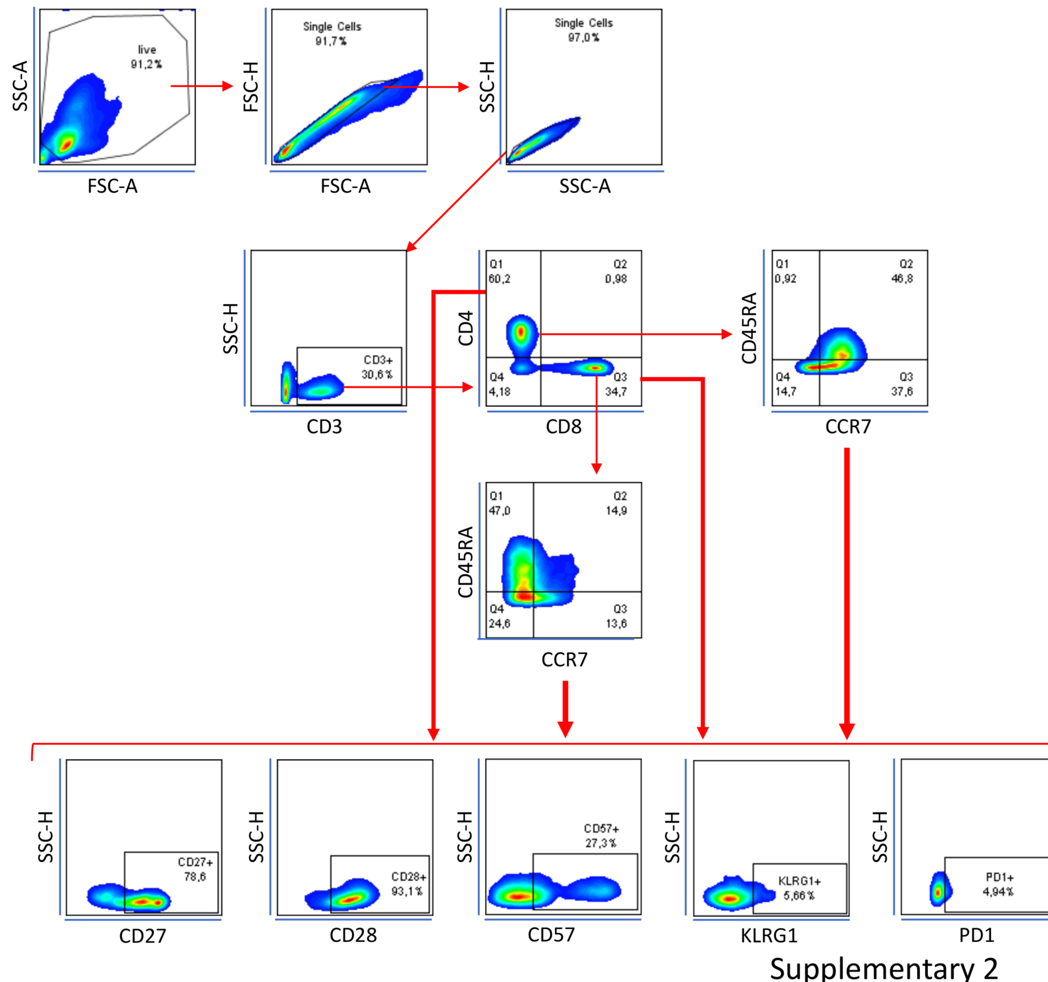

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127 **Figure S2. Representative analysis of peripheral blood mononuclear cells**
128 **(PBMC) by flow cytometry.** Single cells were identified using forward scatter
129 (FSC) and side scatter (SSC), first was limited FSC-H/FSC-A and then SSC-
130 H/SSC-A. Next, from gate of CD3+ cells, CD4+ and CD8+ cells were evaluated.
131 Inside gate CD3+CD4+ and CD3+CD8+ the expression of CD45RA and CCR7
132 was used to report the Naïve (CD45RA+/CCR7+), central memory (TCM,
133 CD45RA-/CCR7+), effector memory (TEM, CD45RA-/CCR7-), and effector
134 memory RA (TEMRA, CD45RA+/CCR7-) cells. In a second analysis performed
135 inside gate CD3+CD4+ and CD3+CD8+, the expression of CD27, CD28, CD57,
136 KLRG1 and PD1 was examined as percentage and fluorescence mean intensity.
137 Representative figure of one patient.

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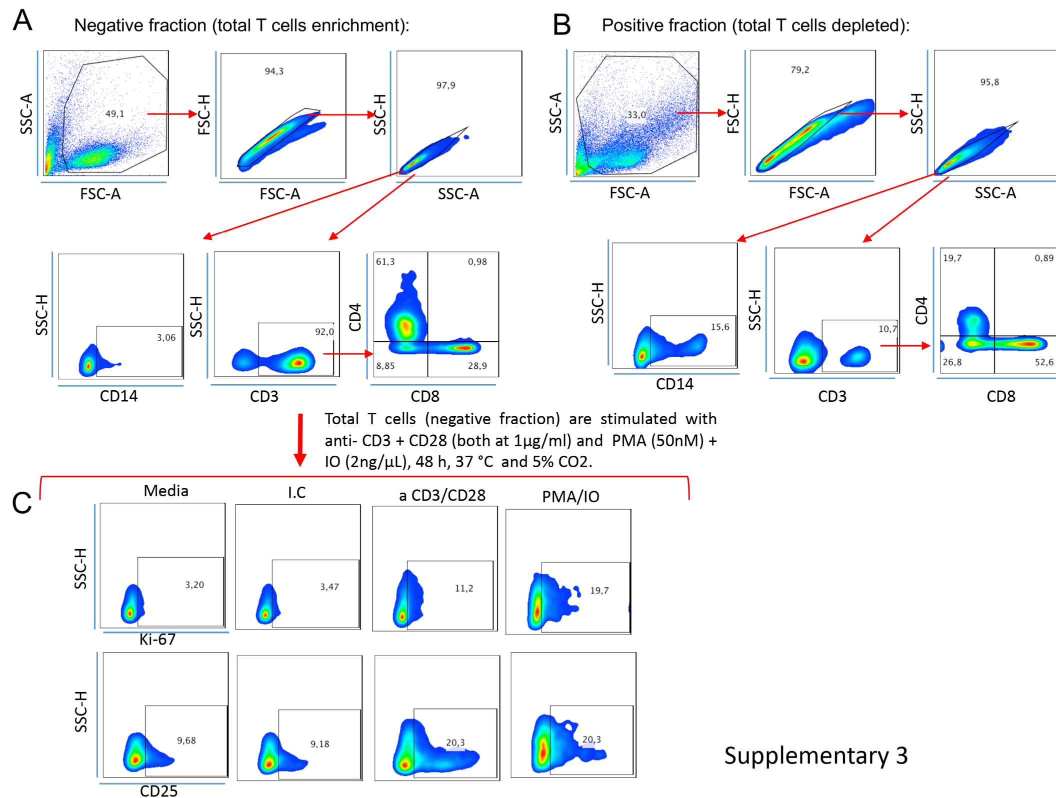

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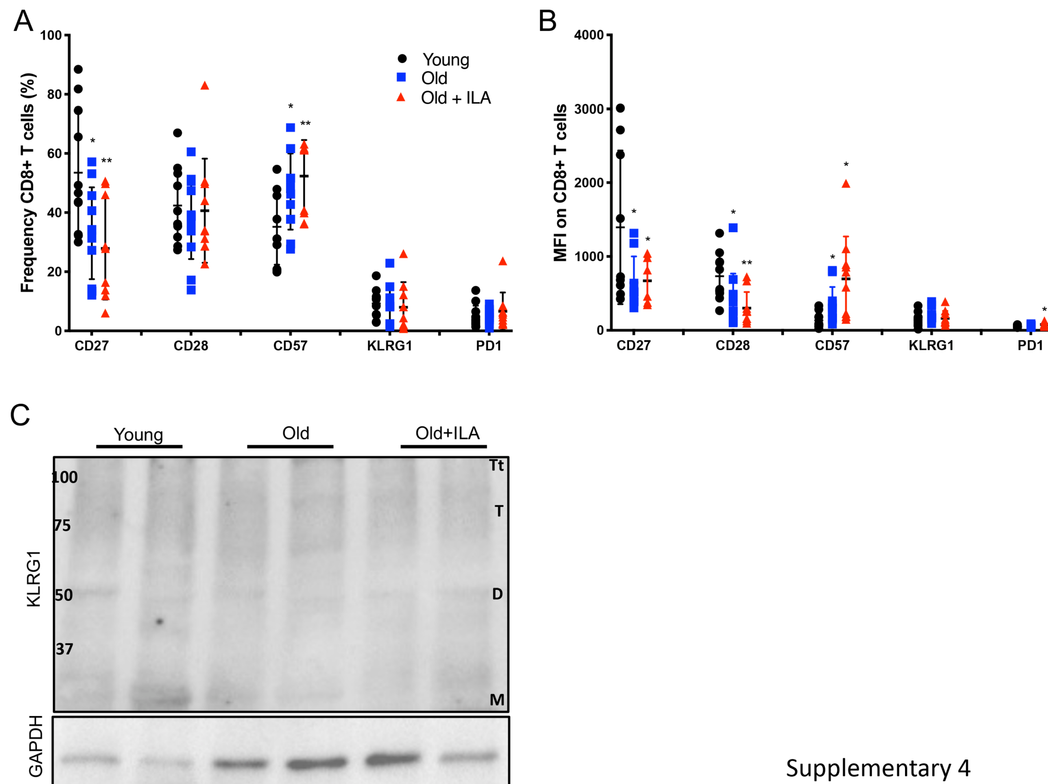
140 **Figure S3. Evaluation of the total T cells enrichment and proliferation**
141 **analysis.** Peripheral mononuclear cells were obtained, and then total T cells were
142 obtained by negative selection using a MACS system. Purity was >90% and a
143 <5% of other cells such as CD14+ cells were present in the negative fraction
144 (panel A). As control, the positive fraction (CD3+ cells depleted) was also
145 evaluated by flow cytometry (panel B). Total T cells enrichment was cultured 48
146 hours using either monoclonal antibodies anti-CD3 and CD28 (both at 1µg/ml) or
147 phorbol myristate acetate (PMA) 50nM– ionomycin (IO) 2ng/µL, as negative
148 controls, T cells in media or with isotype control (1µg/mL) were included.
149 Posteriorly, T cells were recovered and prepared to flow cytometry using adequate
150 controls, first a gate of CD3+CD4+ cells were delimited and then the expression of
151 CD25 (to activation) and Ki-67 (to proliferation) was evaluated (panel C).
152 Representative figure of one patient.



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155 **Figure S4. CD8+ T cells from individuals with interstitial lung abnormalities**
156 **(ILA) loss CD27 and gain CD57 expression similar to the older control group.**
157 Peripheral mononuclear cells were prepared for flow cytometry and western blot.
158 First, live cells were identified, the gate of CD3+CD8+ was delimited, and then the
159 expression of CD27, CD28, CD57, KLRG1 and PD1 was evaluated both in
160 percentage and mean fluorescence intensity (MFI) (panel A and B, respectively).
161 (panel C) Total T cells were obtained using a Pan T kit, and then enrichment of
162 CD4+ T cells was performed. In the fraction of CD4- T cells the KLRG1 expression
163 was evaluated by western blot under non-reducing condition, due KLRG1 is
164 absence, the densitometry analysis was not performed. To flow cytometry analysis,
165 graphs show individual value mean ± SD: Young: n=12, Old: n=13, Old+ILA: n=10,
166 and to western blot: n=2 per group. *p<0.05 **p<0.01. Asterisk means compared to

167 young controls. Multiple T-test correction for multiple comparisons was performed
168 using the Holm-Sidak method.

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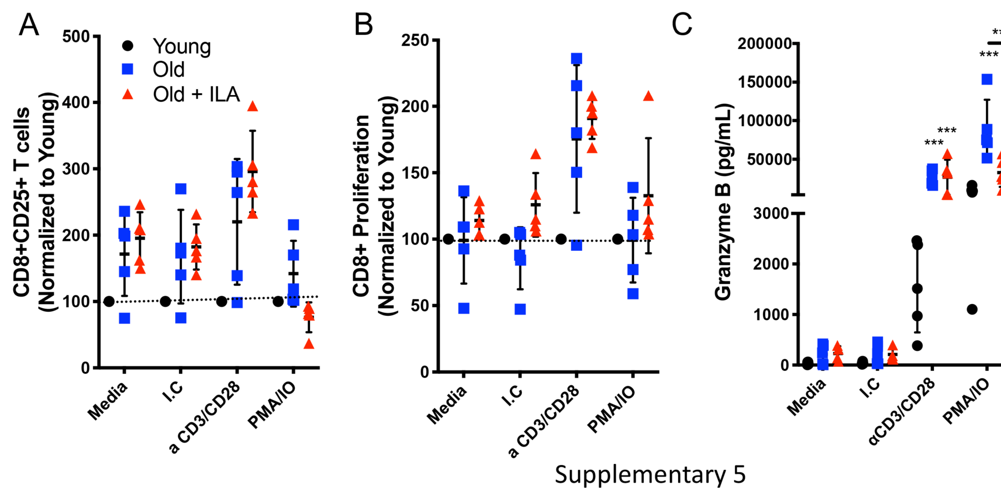

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171 **Figure S5. CD8+ T cells from individuals with interstitial lung abnormalities**
172 **(ILA) show similar proliferative ability compared to old group, but produce**
173 **less Granzyme B.** After enrichment T cells were cultured 48 hours with polyclonal
174 stimuli using either monoclonal antibodies anti- CD3 and CD28 (both at 1μg/ml) or
175 phorbol myristate acetate (PMA) 50nM– ionomycin (IO) 2ng/μL. Cells were
176 recovered and prepared to flow cytometry; culture supernatant was used to
177 Granzyme B by ELISA. By flow cytometry, CD3+CD8+ cells were delimited and the
178 expression of CD25 (to activation) and Ki-67 (to proliferation) was evaluated (panel
179 A and B, respectively); the level of activation and proliferation displayed by CD8+ T
180 cells from the young group was considered as 100% (black dot line. (panel C)
181 Granzyme B released by stimulated T cells was measured by ELISA in the culture'
182 supernatant. Graphs show individual value mean ± SD (n=5 per group). Asterisks
183 indicate comparison of the three groups. When the asterisk is on a line, indicates,
184 the comparison between old control group and old+ILA. *p<0.05 **p<0.01, One-
185 Way ANOVA test and Dunnett as post hoc analysis.

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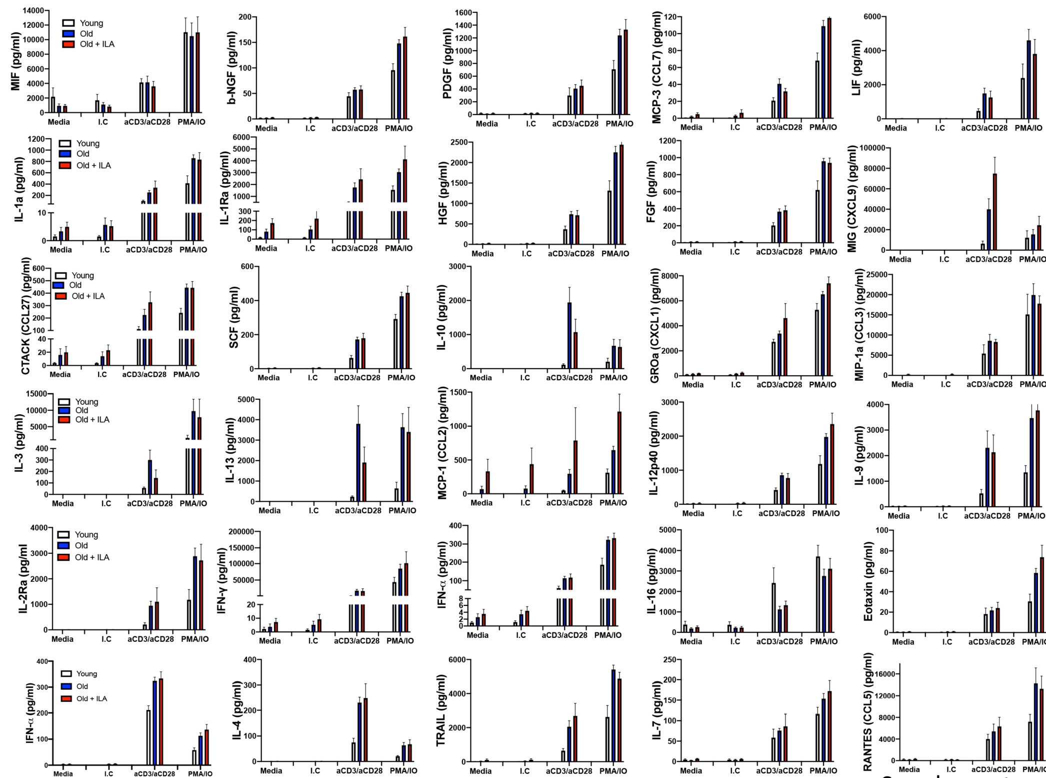

Figure S6. Under polyclonal stimuli the T cells from persons with interstitial lung abnormalities (ILA) secrete this group of soluble proteins similar to old control group. Enriched T cells were cultured 48 hours with polyclonal stimuli either with monoclonal antibodies anti- CD3 and CD28 (both at 1µg/ml) or phorbol myristate acetate (PMA) 50nM– ionomycin (IO) 2ng/µL; culture medium (Media) and isotype control (I.C) to monoclonal antibodies were used as negative controls. Culture supernatant was used to evaluate a panel of 48 soluble molecules by Multiplex. We did not observed differences in the profile of 30 soluble proteins evaluated between ILA and old control group.