ONLINE DATA SUPPLEMENT

Peripheral blood myeloid-derived suppressor cells reflect disease status in IPF

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MATERIAL AND METHODS.

Cytospin preparation

Cytospins were obtained by centrifuging 1-3 x 10^4 cells at 250 x g for 5 minutes at room temperature onto microscope slides, and stained with May-Grünwald-Giemsa dye for morphological determination. For immunofluorescent staining, cytospins were dried, stored at -20°C, and processed for immunofluorescent staining, as previously published (1). A polyclonal antibody against human CD33 and a monoclonal antibody against Human ITGAM (Atlas Antibodies-Sigma-Aldrich; St Louis, MO, USA), was used at 1:50, and 1:250 dilution, respectively, in 5% BSA/0.2% Tween/DPBS. Cells were washed and mounted in fluorescent mounting medium (Dako; Hamburg, Germany). Fluorescent microscopy was performed using an Axiovert II (Carl Zeiss AG; Oberkochen, Germany). Images were processed using ZEN 2010 software (Carl Zeiss AG).

Immunofluorescent stainings of tissue sections

Explanted lungs from three unused donors and three fibrotic patients (one EAA, two IPF), who were previously recruited and subjected to MDSC measurement in whole blood, were used. All participants gave written informed consent and the study was approved by the local ethics committee. Tissue was embedded in paraffin and Immunofluorescence was performed as previously described (2). Briefly, slides were stained with anti-human CD33 and CD11b. For antigen retrieval, slides were immersed in citrate buffer pH 6,0 and heated in a Decloaking

Chamber 30 seconds at 125°C, followed by 10 seconds at 90°C. Then, 1:250 dilution of each secondary antibody was applied (Alexa Fluor 568 goat anti-rabbit, and Alexa Fluor 488 goat anti-mouse). Slides were counterstained with DAPI (Sigma-Aldrich; St Louis, MO, USA. 1:2000) for 1 min. Slides were rinsed with Tris buffer and imaged as outlined above.

Gene expression in lymphocytes

For RNA extraction from frozen PBMC, the RNeasy Mini Plus Kit (Quiagen; Venlo, Holland) was used as previously reported (1). RNA concentration was determined using the NanoDropTM 1000 spectrophotometer (NanoDrop Tech. Inc; Wilmington, DE, USA) at 260 nm. For analysis of mRNA expression, total RNA was reverse transcribed using MuLV reverse transcriptase (Applied Biosystems; Carlsbad, CA, USA) and random hexamer primers (Applied Biosystems). The qRT-PCR was performed in 96-well format using a Light Cycler LC480II instrument (Roche; Penzberg, Germany) and LightCycler® 480 DNA SYBR Green I Master (Roche). GAPDH was used as a housekeeping gene for standardization of relative mRNA expression. All qRT-PCR reactions were performed in technical triplicates and non-template controls were used for quality controls.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Table E1. Antibody used for flow cytometric analysis. Antibodies used for myeloid-derived suppressor cell strategy, and lymphocyte gating. For each antibody the antigen, fluorophore, clone, manufacturer, and isotype are depicted.

Supplemental Figure E1 . Lymphocyte gating strategy in PBMCs. Representative dot-plot of peripheral blood mononuclear cells flow cytometric analysis. Cells were stained with a panel of mAb, and gated on $CD3^+$ and $CD4^+$, for determining $CD4^+CD25^+$ cells, which includes T regulatory cells. T helper was gated as $CD3^+CD4^+$: effector ($CCR7^-$) and non-effector ($CCR7^+$). T cytotoxic was gated as $CD3^+CD8^+$: effector ($CCR7^-$) and non-effector ($CCR7^+$). Abnormal T cells were gated as $CD3^+CD4^+CD8^+$.

Supplemental Figure E2 . Gender specific correlation of MDSC and VCmax. The percentages of gated MDSCs and VCmax (VCmax %predicted) was discriminated by gender, and correlated in all IPF patients. *p* values were determined by Student's t distribution for Pearson correlation.

Supplemental Figure E3. T cell lymphocyte profiling in chronic lung diseases. Scatter dot plot diagrams from flow cytometric analysis of lymphocytes in controls (n= 22), IPF (n= 69), non-IPF ILD (n= 56), and COPD (n= 23). Gated percentage of T helper $CD4^+$ (A), $CD8^+$ (D), and $CD4^+CD8^+$ (G) cells from total $CD3^+$ cells, as 100%. T helper effector ($CD4^+CCR7^-$), and non-effector

($CD4^+CCR7^+$) gated as $CD4^+$ as 100% (B-C). T cytotoxic effector ($CD8^+CCR7^-$), and non-effector ($CD8^+CCR7^+$) gated as $CD8^+$ as 100% (E-F). For statistical analysis one-way analysis of variance was used with the non-parametric Kruskal-Wallis test, followed by Dunnnett's multiple comparison test. *p* *<0.05, **<0.01, versus healthy controls.

REFERENCES

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