Identification a novel subset of alveolar type 2 cells expanding following pneumonectomy and enriched in PD-L1

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Supplementary methods

Pneumonectomy

Prior to surgery, adult mice (8-12 weeks old) were injected with buprenorphine and anesthetized with 2.5 % isoflurane. The animals were ventilated with 200 µl volume per stroke and 180 strokes/min throughout the pneumonectomy/thoracotomy procedure. The mice were shaved at the left lateral side and the surgical site was sterilized. A 2 cm long incision was made at the 5th intercostal space. The ribs were dissected down using curved forceps and the tip of the curved forceps was used to enter the thoracic cavity. The left lobe was then resected, the ribs and skin closed. A catheter was inserted to evacuate fluid accumulating in the void space and re-establish negative intra-thoracic pressure. Sham control surgeries were performed as thoracotomy procedure without lung removal. Mice remained intubated until spontaneous breathing recovered. Mice were then placed on a warming pad until they awake. The mice were weighed and were observed daily for any signs of distress or changes in behavior. The animals were sacrificed at day 7 post surgery and the lungs

were isolated. The left lobe in the Sham was excluded from the analysis to have the same lobes in both Sham and PNX.

Lung dissociation and FACS

Adult mice were sacrificed and lungs were perfused with 5 ml PBS through the right ventricle. Lungs were inflated via the trachea with dispase and kept in dispase (Coning, NY, USA) and Collagenase Type IV at 37°C for 40 min with frequent agitation. To achieve single cell suspensions, digested tissue was passed serially through 100-, 70- and 40-µm cell strainers (BD Biosciences). Red blood cells (RBC) were eliminated using RBC lysis buffer (Sigma-Aldrich) according to the manufacturer's protocol. Cells were pelleted, resuspended in FACS buffer (0.1% sodium azide, 5% fetal calf serum (FCS), 0,05% in PBS) and stained with antibodies: anti-EpCAM (APC-Cy7-conjugated, Biolegend,1:50), CD49F (APC-conjugated, Biolegend,1:50), anti-PDPN (FITC-conjugated, Biolegend, 1:20) , and anti-CD274 (unconjugated, Thermo Fisher, 1:100) antibodies for 20 minutes on ice in the dark, followed by washing. Then, the cells were stained for goat anti-rabbit secondary antibody Alexa flour 488 (Invitrogen,1:500) for 20 minutes on ice in the dark. Next, cells were washed and stained with SYTOX (Invitrogen) a live/dead cell stain according to the manufacturer’s instructions. Flow cytometry data acquisition and cell sorting were carried out using FACSAria III cell sorter (BD Biosciences, San Jose/CA). Data were analyzed using FlowJo software version X (FlowJo, LLC).

RNA extraction and quantitative real-time PCR

Following lysis of FACS-isolated cells from mouse or human lungs in RLT plus, RNA was extracted using a RNeasy plus Micro kit (Qiagen) and cDNA synthesis was carried

out using QuantiTect reverse transcription kit (Qiagen) according to the instructions provided by supplier. Thereafter, selected primers (Table1,2) were designed via NCBI's primer-BLAST option (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (for primer sequence see supplementary table). Quantitative real-time polymerase chain reaction (qPCR) was performed using PowerUp SYBR Green Master Mix kit according to the manufacturer's protocol (Applied Biosystems) and LightCycler 480 II machine (Roche Applied Science). hypoxantine-guanine phosphoribosyltransferase (Hprt) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as reference genes for mice and human, respectively. Data were presented as mean expression relative to Hprt and GAPDH. Data were assembled using GraphPad Prism software (GraphPad Software, La Jolla/CA). Statistical analyses were performed utilizing two tailed-paired Student t-test. Results were regarded significant when $p < 0.05$.

Immunofluorescent Staining

After lung perfusion with PBS through the right ventricle, isolated lungs were fixed with 4% paraformaldehyde. Afterward, tissues were embedded in paraffin and sectioned at 5 µm thickness. Following deparaffinization, slides were blocked with 3% bovine serum albumin (BSA) (Jackson Immunoresearch Laboratories) in PBS for 1 hour at RT. Immunofluorescent (IF) staining was performed using overnight incubation with polyclonal anti-Prosurfactant Protein C (ProSP-C) (Merck/Millipore/Sigma-Aldrich, 1:500). This was followed by staining with polyclonal secondary antibody Goat anti rabbit Alexa flour 488 (Invitrogen,1:500). Slides were finally mounted with ProLong Gold Antifade Reagent containing DAPI (Molecular Probes). For quantitative analysis, multiple images were acquired and assessed ($n > 8$). For each experiment, sections from at least four independent lungs were analyzed.

Alveolosphere assay

Sorted epithelial cells (Tom^{Low} and Tom^{High}) from [$Sftpc^{CreERT2/+}$; $tdTom^{flox/flox}$] mice and resident mesenchymal cells from C57BL/6J mice ($Epcam^{neg}$, $CD31^{neg}$, $CD45^{neg}$, $Sca1^{pos}$) were centrifuged and resuspended separately in cell culture medium (Dulbecco's Modified Eagle Medium, Life Technologies). To adjust the optimal concentrations, $1x10^3$ epithelial cells in 25 µL media and $2x10^4$ mesenchymal cells in 25 µL media per insert (12 mm cell culture inserts with 0.4 µm membrane Millipore) were used. Mesenchymal and epithelial cell suspensions were mixed, followed by the addition of cold Matrigel® growth factor-reduced (Corning) at a 1:1 dilution resulting in 100 µL final volume per insert. Matrigel cell suspensions were placed on the top of the filter membrane of the insert and incubated at 37°C for 5 min. Next, 350 µL of the medium was transferred to each well. Cells were incubated under air-liquid conditions at 37°C with 5% CO2 for two weeks. Media were changed 3 times per week.

Microarray

Purified total RNA was amplified using the Ovation PicoSL WTA System V2 kit (NuGEN Technologies). Per sample, 2 µg amplified cDNA was Cy5-labeled using the SureTag DNA labeling kit (Agilent). Hybridization to 8x60K 60mer oligonucleotide spotted microarray slides (Human Mouse Genome, Agilent Technologies, design ID 074809) and subsequent washing and drying of the slides was performed following the Agilent hybridization protocol in Agilent hybridization chambers, with following modifications: 3 µg of the labeled cDNA were hybridized for 22 hours at 65°C. The cDNA was not fragmented before hybridization.

The dried slides were scanned at 2 µm/pixel resolution using the InnoScan is900 (Innopsys). Image analysis was performed with Mapix 6.5.0 software, and calculated

values for all spots were saved as GenePix results files. Stored data were evaluated using the R software and the limma package[30] from BioConductor. Log2 mean spot signals were taken for further analysis. Data was background corrected using the NormExp procedure on the negative control spots and quantile-normalized[30,32] before averaging. Log2 signals of replicate spots were averaged, and from several different probes addressing the same gene only the probe with the highest average signal was used. Genes were ranked for differential expression using a moderated t-statistic. Pathway analyses were done using gene set tests on the ranks of the t-values. Pathways were taken from the KEGG database (http://www.genome.jp/kegg/pathway.html).

ATAC-seq

25.000 FACS-sorted cells were collected and used for ATAC Library preparation using Tn5 Transposase from Nextera DNA Sample Preparation Kit (Illumina). Cell pellet was resuspended in 50 µl Lysis/Transposition reaction (12.5 µl THS-TD-Buffer, 2.5 µl Tn5, 5 µl 0.1% Digitonin, and 30 µl water) and incubated at 37°C for 30 min with occasional snap mixing. Following purification of the DNA, fragments were done by Min Elute PCR Purification Kit (Qiagen). Amplification of the Library together with Indexing Primers was performed as described. Libraries were mixed in equimolar ratios and sequenced on the NextSeq500 platform using V2 chemistry. Trimmomatic version 0.38 was employed to trim reads after a quality drop below a mean of Q15 in a window of 5 nucleotides. Only reads longer than 15 nucleotides were cleared for further analyses. Trimmed and filtered reads were aligned versus the mouse genome version mm10 (GRCm38) using STAR 2.6.1d with the parameters “--outFilterMismatchNoverLmax 0.1 --outFilterMatchNmin 20 --alignIntronMax 1 --alignSJDBoverhangMin 999 --outFilterMultimapNmax 1 --alignEndsProtrude 10 ConcordantPair” and retaining

unique alignments to exclude reads of uncertain origin. Reads were further deduplicated using Picard 2.18.16 (Picard: A set of tools (in Java) for working with next-generation sequencing data in the BAM format) to mitigate PCR artefacts leading to multiple copies of the same original fragment. Reads aligning to the mitochondrial chromosome were removed. The Macs2 peak caller version 2.1.2 was employed to accommodate the range of peak widths typically expected for ATAC-seq[37]. Minimum qvalue was set to -4 and FDR was changed to 0.0001. Peaks overlapping ENCODE blacklisted regions (known misassemblies, satellite repeats) were excluded.

In order to be able to compare peaks in different samples to assess reproducibility, the resulting lists of significant peaks were overlapped and unified to represent identical regions. Sample counts for union peaks were produced using bigWigAverageOverBed (UCSC Toolkit) and normalized with DESeq2 1.18.1 to compensate for differences in sequencing depth, library composition, and ATAC-seq efficiency. Peaks were annotated with the promoter of the nearest gene in range (TSS +- 5000 nt) based on reference data of GENCODE vM15.

Table S1:

Mouse primers		
Gene	**Forward primer (5'->3')**	**Reverse primer (5'->3')**
Hprt	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
Fgfr2lllb	TAAATACGGGCCTGATGGGC	CAGCATCCATCTCCGTCACA
Etv5	CAGCCCGCCACGGAG	CCGCTATCACTTTGAAGGGC
Sftpc	GGTCCTGATGGAGAGTCCAC	GATGAGAAGGCGTTTGAGG
Sftpb	GGCTAGACAGGCAAAAGTGTG	GACCGCGTTCTCAGAGGTG
Sftpa1	CAGTGTGATTGGGAGAAACCA	ATGCCAGCAACAACAGTCAA
Cd33	TCTGTCTCGTGTTTCTCATTGTG	GCAGTTGGAGATAGGCAGTGA
Pdl1	AAGTCAATGCCCCATACCGC	TTCTGGATAACCCTCGGCCT
Ki67	CTGCGAGCTTCACCGAGAG	CAATACTCCTTCCAAACAGGCAG
CCNd1	TCAAGTGTGACCCGGACTG	CCACATCTCGCACGTCGG
CCNd2	TACCTGGACCGTTTCTTGGC	TACCAGTTCCCACTCCAGCA
Pecam1	AGCCAACAGCCATTACGGTTA	TCGACCTTCCGGATCTCACT
Ptprc	TGAGCACAACAGAGAATGCCC	AGCGTGGATAACACACCTGGA
Adgre1	TCTGGGGAGCTTACGATGGA	GAATCCCGCAATGATGGCAC
Vim	AACTGCACGATGAAGAGATCCA	CACGCTTTCATACTGCTGGC