SINGLE-CELL TRANSCRIPTOMICS IDENTIFIES PATHOGENIC T-HELPER 17.1 CELLS AND PRO-INFLAMMATORY MONOCYTES IN IMMUNE CHECKPOINT INHIBITOR-RELATED PNEUMONITIS

ONLINE DATA SUPPLEMENT
SUPPLEMENTARY METHODS

Patient cohort, sampling and data collection

Fifteen pneumonitis patients and six control patients were recruited from the University Hospitals Leuven, between February 12\textsuperscript{th} 2020 and December 13\textsuperscript{th} 2021, as part of the observational trial “A single-cell approach to identify biomarkers of pulmonary toxicity for immune checkpoint blockade” (NCT04807127). The demographic and disease characteristics of the prospectively recruited patients studied by scRNA-seq are listed in Table 1. ICI-pneumonitis diagnosis was based on compatible clinical history and radiological findings, in the presence of a negative infectious work-up by bronchoscopy with BAL and after excluding other causes of pneumonitis (including, \textit{e.g.}, progressive disease and radiotherapy-induced pneumonitis), as assessed by the treating pulmonologist and confirmed by pulmonologist and researcher E.W. and dedicated thoracic radiologist W.D.W.. Four pneumonitis patients were excluded; two infectious pneumonia and two radiotherapy-induced pneumonitis cases, such that 11 ICI-pneumonitis patients were included for analyses. The most affected lung lobe/segment (with new infiltrates) was lavaged. Control patients were recruited at the time of bronchoscopy because of a newly clinically diagnosed lung tumor, and underwent bronchoalveolar lavage of an unaffected contralateral lobe in the absence of clinical, radiological or pathological arguments for a (concomitant) infectious or inflammatory process.

Demographic, clinical, treatment and outcome data from patient electronic medical records were obtained through a standardized research form in Research Electronic Data Capture Software (REDCAP, Vanderbilt University).
scRNA-seq and scTCR-seq profiling

BAL samples were processed as previously described.[1] Briefly, BAL fluid was centrifuged and the supernatant was frozen at -80°C for further experiments. The cellular fraction was resuspended in ice-cold PBS and samples were filtered using a 40µm nylon mesh (ThermoFisher Scientific). Following centrifugation, the supernatant was decanted and discarded and the cell pellet was resuspended in red blood cell lysis buffer. Following a 5-min incubation at room temperature, samples were centrifuged and resuspended in PBS containing UltraPure BSA (AM2616, ThermoFisher Scientific) and filtered over Flowmi 40µm cell strainers (VWR) using wide-bore 1 ml low-retention filter tips (Mettler-Toledo). Next, 10 µl of this cell suspension was counted using an automated cell counter to determine the concentration of live cells. The entire procedure was completed in less than 1.5 h.

Single-cell TCR and 5’ gene expression sequencing data for the same set of cells were obtained from the single-cell suspension using the Chromium™ Single Cell 5’ library and Gel Bead & Multiplex Kit with the Single Cell V(D)J Solution from 10x Genomics according to the manufacturer’s instructions. The target number of cells to be loaded on a 10x Genomics cartridge for each sample was 5,000. Cell-barcoded 5’ gene expression libraries were sequenced on an Illumina NovaSeq6000, and mapped to the GRCh38 human reference genome using CellRanger (10x Genomics, GRCh38 v5.0.0). V(D)J enriched libraries were sequenced on an Illumina HiSeq4000 and TCR alignment and annotation was achieved with CellRanger VDJ (10x Genomics, GRCh38 v5.0.0).

Single-cell gene expression analysis

Raw gene expression matrices generated per sample were merged and analyzed with the Seurat package (v4.1.0).[2] Cell matrices were filtered by removing cell barcodes with <301
UMIs, <151 expressed genes or >20% of reads mapping to mitochondrial RNA. We opted for a lenient filtering strategy to preserve the neutrophils, which are transcriptionally less active (lower transcripts and genes detected) as previously described.[1] For samples with high ambient RNA content (>500 UMI counts), evidenced by a high “ambient RNA plateau” when plotting total unique UMI counts per droplet, correction for ambient RNA was performed using the CellBender algorithm.[3] Subsequently, normalization was performed and the 2000 most variable genes were selected to perform a principal component analysis (PCA) after feature scaling and regression for confounding factors: number of UMIs, percentage of mitochondrial RNA, cell cycle (S and G2M phase scores calculated by the CellCycleScoring function in Seurat), interferon response (BROWNE_INTERFERON_RESPONSIVE_GENES in the Molecular Signatures Database or MSigDB v6.2), sample dissociation-induced stress signatures and hypoxia signature. Regressing out the cell cycle genes was particularly important for the T-/NK-cell subclustering. When this was not performed, a proliferating T-cell cluster that contained a mixture of different T-cell phenotypes, including CD4+ and CD8+ T-cells was identified (data not shown).

**scRNA-seq clustering for cell type identification**

For the clustering of the major cell types, we applied PCA to the variable genes in order to reduce dimensionality. The selection of principal components was based on elbow and Jackstraw plots (usually 20-30). Clusters were calculated by the `FindClusters` function in Seurat and visualised using the Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) reduction.[4] Differential gene-expression analysis was performed for clusters generated at various resolutions by both the Wilcoxon rank sum test and Model-based Analysis of Single-cell Transcriptomics (MAST) using the `FindMarkers`
function.[5] A specific resolution was selected when known cell types were identified as a cluster at a given resolution. Annotation of the resulting clusters to cell types was based on the expression of marker genes.

Integration of publicly available datasets and identification of cell subtypes

We additionally reanalyzed scRNA-seq data on NSCLC and on normal lung samples by Lambrechts et al. to enhance subcluster resolution.[6] Specifically, for cell subtype identification, the main cell types identified from multiple datasets were pooled and integrated using canonical correlation analysis (CCA). Each cell type was then further subclustered and annotated using the same Seurat workflow as described earlier. Immunoglobulin genes were excluded from these analyses, except for B-cell and plasma cell subclustering. Finally, doublet clusters were identified, and excluded, based on: 1) expression of marker genes from other cell (sub)clusters, 2) higher average UMIs as compared to other (subclusters), and 3) a higher-than-expected doublets rate (> 20%), as predicted by DoubletFinder v2.[7] To avoid co-clustering of proliferative T-cells, we regressed for cell cycle genes in the T-cell subclustering analysis.

Differential gene expression and gene set enrichment analysis

Differentially-expressed genes (DEGs) were identified using the Model-based Analysis of Single-cell Transcriptomics (MAST) test with FindMarkers and FindAllMarkers functions in Seurat without a threshold for logFC and for expression in a minimum fraction of cells.[5] The R package hypeR was used for GSEA on DEGs.[8] For this analysis the hallmark (referred to as ‘H’) and GO:BP (‘C5’) gene sets were used from the MSigDB v6.2 and were exported using the R package GSEABase. Only significant genes (adjusted p-value < 0.05) and genes with a logFC higher than 0.5 or lower than -0.5 were used.
**Assessing the TCR repertoires**

We only considered productive TCRs, which were assigned by the CellRanger VDJ pipeline. TCR clonotypes were defined as TCRs with the same complementarity-determining region 3 (CDR3) nucleotide sequences. To assess clonotype diversity, we calculated the Gini coefficient using the Gini() function from the DescTools R package, relative clonotype richness (defined as the number of unique TCRs divided by the total number of cells with a TCR) and relative clonotype evenness (defined as the inverse Simpson index divided by the number of unique clonotypes).[9, 10]

**Trajectory inference analysis**

We functionally characterized CD4+ T-cells by performing single-cell trajectory inference based on transcriptional similarity and RNA velocity using the CellRank algorithm.[11] TREG were excluded due to their unique developmental origin. The gene count and RNA velocity matrix are fed to CellRank as inputs, with CellRank then computing a global map of “fate potentials”, assigning each cell the probability of reaching a specific cell state. Based on the inferred potentials, CellRank can chart gene expression dynamics as cells take on different fates. Since CellRank does not uniquely assign cells to a specific trajectory, we used the cell fate probabilities as weighting factors for the density and TCR analyses along each trajectory.

**Quantification and statistical analysis**

Descriptive statistics are presented as median [interquartile range; IQR] and n (%) for continuous and categorical variables, respectively. Statistical analyses were performed using R (version 3.6.3, R Foundation for Statistical Computing, R Core Team, Vienna, Austria).[12]
Statistical analyses were performed with a two-sided alternative hypothesis at the 5% significance level.

REFERENCES

SUPPLEMENTARY FIGURES

Figure S1. Integration of 5-prime end single-cell RNA sequencing datasets (ICI-pneumonitis, control). (A) UMAP plot of 108,476 cells colored for dataset or (B) individual sample, showed adequate correction for batch effects.
Figure S2. Integration and clustering of 3-prime end single-cell RNA sequencing datasets (normal lung, early-stage non-small cell lung cancer (NSCLC)). (A) UMAP plot of 101,786 cells showing major cell type annotation, (B) based on canonical marker gene expression. (C) UMAP plot colored for dataset or (D) individual sample, showed adequate correction for batch effects. DC dendritic cell.
Figure S3. Integration of cell types of interest across sequencing technologies. (A) UMAP plots of all T-cells, (B) myeloid cells and (C) dendritic cells, showing adequate correction for batch effects across sequencing technologies (left panels), datasets (middle panels) and individual samples (right panels). NSCLC non-small cell lung cancer.
Figure S4. NK-/T-cell phenotypes and T-cell receptor repertoire sharing across ICI-pneumonitis and control bronchoalveolar lavage fluid. (A) NK-/T-cell annotation according to canonical marker gene expression. (B) Feature plot showing the average expression level of CD4 in T-cells. (C) Regulatory Type 1 T-cells (T_{R1}) were more abundant in ICI-pneumonitis bronchoalveolar lavage fluid (BALF) of patients showing resolution of radiographic abnormalities after first-line treatment compared to patients with persistent abnormalities. Wilcoxon rank sum test was used; significance is shown as * p < 0.05. T-cell receptor (TCR) repertoire sharing, as calculated by the proportion of TCR sequences shared between CD4+ T-cell subtypes; (D) the proportion of sharing is indicated by the thickness of interconnecting bars between cell clusters or (E) color-coded in a heatmap, with the number of shared clonotypes calculated proportionally per column. (F) Line graph showing TCR richness (left panel) and evenness (right panel) for ICI-pneumonitis and control BALF along the Th17.1_TBX21 trajectory. Limited number of cells may introduce artefacts for TCR richness analysis, as seen at the end of the trajectory. CD4_N CD4+ naïve-like T-cells; CD4_EM CD4+ effector memory T-cells; CD4_TR1 Regulatory Type 1 T-cells; CD4_Th17.1_RORC T-helper 17.1 lymphocytes with predominant non-pathogenic features; CD4_Th17.1_TBX21; T-helper 17.1 lymphocytes with predominant (pathogenic) T-helper 1-like features; CD4_FH follicular helper T-cells; CD4_REG regulatory T-cells; CD8_N CD8+ naïve-like T-cells; CD8_EM CD8+ effector memory T-cells; CD8_RM resident memory T-cells; CD8_EMRA recently activated effector memory T-cells; CD8_EX experienced T-cells; CD8_gd gamma delta T-cells; MAIT mucosal associated invariant T-cells; NK_cyto cytotoxic NK-cells; NK_infla inflammatory NK-cells.
SUPPLEMENTARY TABLES

Table S1. Demographic and clinical data of individual study participants.

Table S2. Differentially expressed genes in T-cells comparing ICI-pneumonitis and control T-cells. P-values were obtained by the model-based analysis of single-cell transcriptomics (MAST) test and Bonferroni-corrected.

Table S3. Gene set enrichment analysis on differentially expressed genes for ICI-pneumonitis T-cells using the R package hypeR. Only significant genes (adjusted p-value < 0.05) and genes with a log-fold change higher than 0.5 or lower than -0.5 were used.

Table S4. Gene set enrichment analysis on differentially expressed genes for control T-cells using the R package hypeR. Only significant genes (adjusted p-value < 0.05) and genes with a log-fold change higher than 0.5 or lower than -0.5 were used.

Table S5. Differentially expressed genes in monocytes/macrophages comparing ICI-pneumonitis and controls. P-values were obtained by the model-based analysis of single-cell transcriptomics (MAST) test and Bonferroni-corrected.

Table S6. Gene set enrichment analysis on differentially expressed genes for ICI-pneumonitis monocytes/macrophages using the R package hypeR. Only significant genes (adjusted p-value < 0.05) and genes with a log-fold change higher than 0.5 or lower than -0.5 were used.

Table S7. Gene set enrichment analysis on differentially expressed genes for control monocytes/macrophages using the R package hypeR. Only significant genes (adjusted p-value < 0.05) and genes with a log-fold change higher than 0.5 or lower than -0.5 were used.
Supplementary Table S1. Demographic and clinical data of individual study participants.

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| Ctrl 5 | 39  | M | Never | n | PLEC st.I | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
|-------|-----|---|-------|---|-----------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Ctrl 6 | 52  | F | Active | y | LUAD st.III | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |

Legend to Supplementary Table S1: 

- According to RECIST v1.1 criteria. 
- Grading according to Common Terminology Criteria for Adverse Events (CTCAE) v5.0. 
- Radiographical response to first-line treatment; no patients required second-line treatment. 
- Two patients’ symptoms improved while awaiting results of bronchoalveolar lavage fluid analysis, so that the planned immunosuppressive therapy was eventually not initiated in these patients. 
- Colitis occurred before pneumonitis and systemic corticosteroids had been tapered completely at the time of pneumonitis. 
- Patient reported dry cough 52 days before sampling, but dyspnoea occurred approximately 1 week before sampling. 
- Multiple irAEs occurred at the same timepoint in this patient. 