Single-cell transcriptomics identifies pathogenic T-helper 17.1 cells and pro-inflammatory monocytes in immune checkpoint inhibitor-related pneumonitis

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ABSTRACT

Background Immune checkpoint inhibitor (ICI)-related pneumonitis is the most frequent fatal immune-related adverse event associated with programmed cell death protein-1/programmed death ligand-1 blockade. The pathophysiology however remains largely unknown, owing to limited and contradictory findings in existing literature pointing at either T-helper 1 or T-helper 17-mediated autoimmunity. In this study, we aimed to gain novel insights into the mechanisms of ICI-related pneumonitis, thereby identifying potential therapeutic targets.

Methods In this prospective observational study, single-cell RNA and T-cell receptor sequencing was performed on bronchoalveolar lavage fluid of 11 patients with ICI-related pneumonitis and 6 demographically-matched patients with cancer without ICI-related pneumonitis. Single-cell transcriptomic immunophenotyping and cell fate mapping coupled to T-cell receptor repertoire analyses were performed.

Results We observed enrichment of both CD4+ and CD8+ T cells in ICI pneumonitis bronchoalveolar lavage fluid. The CD4+ T-cell compartment showed an increase of pathogenic T-helper 17.1 cells, characterized by high co-expression of TBX21 (encoding T-bet) and RORC (ROR-γ), IFN-γ (IFN-γ), IL-17A, CSF2 (GM-CSF), and cytotoxicity genes. Type 1 regulatory T cells and naive-like CD4+ T cells were also enriched. Within the CD8+ T-cell compartment, mainly effector memory T cells were increased. Correspondingly, myeloid cells in ICI pneumonitis bronchoalveolar lavage fluid were relatively depleted of anti-inflammatory resident alveolar macrophages while pro-inflammatory ‘M1-like’ monocytes (expressing TNF, IL-1β, IL-6, IL-23A, and GM-CSF receptor CSF2RA, CSF2RB) were enriched compared with control samples. Importantly, a feedforward loop, in which GM-CSF production by pathogenic T-helper 17.1 cells promotes tissue inflammation and IL-23 production by pro-inflammatory monocytes and vice versa, has been well characterized in multiple autoimmune disorders but has never been identified in ICI-related pneumonitis.

Conclusions Using single-cell transcriptomics, we identified accumulation of pathogenic T-helper 17.1 cells in ICI pneumonitis bronchoalveolar lavage fluid—a phenotype explaining previous divergent findings on T-helper 1 versus T-helper 17 involvement in ICI pneumonitis—putatively engaging in detrimental crosstalk with pro-inflammatory ‘M1-like’ monocytes. This finding yields several novel potential therapeutic targets for the treatment of ICI-pneumonitis. Most notably repurposing anti-IL-23 merits further research as a potential efficacious and safe treatment for ICI-pneumonitis.

BACKGROUND

Immune checkpoint inhibitors (ICIs) have been approved for the treatment of many tumor types in various disease stages, offering...
more durable responses and better tolerability compared with conventional cytotoxic treatments. However, with the expanding use of ICIs, clinicians are increasingly confronted with immune-related adverse events (irAEs). While most of these side effects present with mild symptoms, some patients experience severe immune-mediated toxicity and require immunosuppressive therapy. ICI-related pneumonitis (ICI-pneumonitis), most often occurring in patients with non-small cell lung cancer (NSCLC) treated with programmed cell death protein-1/programmed death ligand-1 (PD-1/PD-L1) blockade, is an irAE deserving special consideration. Its real-world incidence is up to 19%, and it is the most frequent fatal adverse event in this patient population, responsible for 35% of iatrogenic deaths. Reflecting how difficult it remains to diagnose and effectively treat ICI-pneumonitis, as a result of the scarcity of available pathophysiological knowledge.

Using flow cytometry, Suresh et al analyzed immune perturbations within the ICI-pneumonitis alveolar compartment, comparing bronchoalveolar lavage fluid (BALF) of 12 patients with ICI-pneumonitis to 6 ICI-treated patients without ICI-pneumonitis. They predominantly observed an increase of T cells in BALF. Particularly, CD4+ central memory T cells were increased, which on in vitro stimulation displayed increased tumor necrosis factor alpha (TNF-α) and interferon-gamma (IFN-γ) secretion, suggesting a T-helper 1 (Th1)-mediated immune response to underlie ICI-pneumonitis. Moreover, they observed that regulatory T cells (Treg) had decreased immunoregulatory capacity, leading them to speculate that diminished immunosuppression by Treg might trigger an exuberant Th1 immune response in ICI-induced pneumonitis. Myeloid cells also gained a more inflammatory phenotype in this study, expressing high levels of interleukin-1β (IL-1β) and TNF-α. In contrast, Wang et al profiled by flow cytometry longitudinally-collected peripheral blood mononuclear cells of 13 patients with ICI-pneumonitis and proposed a different mechanism. Particularly, they observed an increase in circulating T-helper 17 cells (TH17), as well as an increase in the levels of IL-17 in plasma and BALF, suggesting an important role of TH17 in mediating ICI-pneumonitis. A recent bulk transcriptomic study on surgical biopsies of eight ICI-pneumonitis lesions on the other hand, reported a predominant increase of CD8+ T cells and B cells.

Finally, two series of case reports in total encompassing five patients with ICI-pneumonitis reported a significant overlap between the T-cell receptor (TCR) repertoire of clonally-expanded BALF and tumor-infiltrating T cells, suggesting that antigens shared by the tumor and lung elicit the autoimmune response in ICI-pneumonitis.

In summary, while the involvement of T cells in ICI-pneumonitis is clear, little is known about the contribution of specific T-cell subsets and the immune cells that modulate the T-cell response. This knowledge gap has direct clinical consequences, resulting in a lack of rational targeted therapies. To gain better insights into the mechanisms underlying ICI-pneumonitis, we performed deep immune profiling using single-cell RNA (scRNA-seq) and T-cell receptor sequencing (scTCR-seq) of ICI-pneumonitis BALF, and comparatively analyzed the bronchoalveolar immune landscape across patients with (lung) cancer with and without ICI-pneumonitis.

**METHODS**

We prospectively collected BALF from 11 patients (10 patients with NSCLC and 1 patient with melanoma) receiving anti-PD-1/PD-L1 treatment and developing ICI-pneumonitis. Diagnosis of ICI-pneumonitis was based on clinical, laboratory, radiographic and (invasive) microbiological findings. Grade was moderate to severe in all patients (grade 2–3 according to Common Terminology Criteria for Adverse Events (V5.0), with only two patients requiring supplemental oxygen, and all patients received first-line treatment according to European Society for Medical Oncology guidelines. Importantly, BALF was collected prior to the start of this treatment. We also analyzed BALF data from six control patients. These patients were demographically matched and underwent bronchoalveolar lavage of an unaffected (contralateral) lobe, solely for research purposes, during bronchoscopy with transbronchial biopsy of a newly diagnosed lung tumor. Detailed inclusion criteria as well as demographic and clinical data of the patient cohort are summarized in table 1, online Supplementary Table S1 and in the Supplementary Methods section.

Immediately after collecting BALF, cells were subjected to single-cell profiling using the 5’ scRNA-seq kit from 10x Genomics. After quality control and filtering (online supplemental methods), we obtained ~775 million unique transcripts from 141,056 cells. Dimensionality reduction and clustering was performed using Seurat V.4.1.0. Main cell types were annotated according to established marker genes panels (figure 1A, B). There was no evidence of clustering bias (ICI-pneumonitis vs control, or across individual patients; online supplemental figure S1).

To refine our subclustering efforts, we used additional samples profiled by scRNA-seq. Specifically, we pooled 3’ scRNA-seq data of eight early-stage NSCLC tissue samples (~238 million unique transcripts from 72,170 cells) and seven normal lung samples (~134 million unique transcripts from 29,616 cells; online supplemental figure S2A). Assignment of main cell types was done in a similar fashion as for the BALF data, without evidence of batch effects (NSCLC vs normal lung, or across individual patients; online supplemental figure S2B–D). Subsequently, data from each main cell type (derived from BALF, NSCLC and normal lung samples) were integrated separately using canonical correlation analysis, as described previously, without signs of batch effects across sequencing technologies, data sets or individual patients (online Supplemental figure S3). After integration, subclustering of main cell types was done based on differential expression of marker genes.
To gain information on clonotype distribution and dynamics, we performed scTCR-seq on these samples and considered all productive TCRs, which we defined as T cells with TCRs that can be joined in a reading frame by V(D)J recombination without premature stop codons, enabling assessment of a complete TCR α or β chain for downstream analysis. Relative clonotype richness was defined as the number of unique TCRs divided by the total number of cells with a unique TCR. Clonotype evenness was assessed by calculating the inverse Simpson index divided by the number of unique clonotypes. Gini coefficient, a summary metric of inequality of clonotype distribution within a repertoire, was calculated using the ‘Gini’ function from the DescTools R package.\(^15\)\(^16\)

We used the CellRank algorithm to functionally characterize CD4+ T-cell state transitions, based on transcriptional similarity between cells and RNA velocity.\(^17\)

A detailed workflow of downstream analyses is provided in the online supplemental methods section.

### RESULTS

#### T cells dominate the bronchoalveolar space in ICI-pneumonitis

After quality control and filtering (online supplemental methods), we obtained 141,056 cells of which 85,481 were derived from ICI-pneumonitis BALF and 55,575 from demographically-matched control BALF. We identified several clusters, which we linked to cell types (figure 1A) based on canonical marker gene expression (figure 1B), and we then evaluated differences in immune cell proportions (figure 1C). T cells made up more than half of all immune cells in ICI-pneumonitis BALF, a striking fourfold increase compared with control BALF. Conversely, we observed a clear relative depletion of monocytes/macrophages in ICI-pneumonitis BALF, while dendritic cells (DCs) were threefold increased. For B cell, neutrophil and mast cell abundance, no statistically significant differences between ICI-pneumonitis and control BALF were observed.

### Table 1 Demographics and characteristics of study cohort

<table>
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<th>ICI-pneumonitis (n=11)</th>
<th>Control (n=6)</th>
<th>P value</th>
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<td>Age, years</td>
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<td>65.7 (53.0–75.9)</td>
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<tr>
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<td>Smoking status</td>
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<td>Never</td>
<td>1 (9.1)</td>
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<tr>
<td>Former</td>
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<td>2 (33.3)</td>
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<td>1 (16.7)</td>
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<tr>
<td>Grade‡</td>
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<td>9 (81.8)</td>
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</tr>
<tr>
<td>3</td>
<td>2 (18.2)</td>
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Data are median (IQR) or n (%). The p values are from Mann-Whitney U test for continuous data and Pearson’s \(\chi^2\) for non-ordered categorical data, all based on a two-sided hypothesis.

*Last administration of chemotherapy at least 4 months prior to sampling.
†Radiotherapy completed at least 3 months prior to sampling.
‡Grading according to Common Terminology Criteria for Adverse Events V5.0.
COPD, chronic obstructive pulmonary disease; ICI, immune checkpoint inhibitor; NSCLC, non-small cell lung cancer.
Altogether, these broad immune cell typing efforts confirm previous reports highlighting prominent T-cell involvement in ICI-pneumonitis, but they fail to point towards neutrophil or B cell involvement.7 9 Furthermore, we describe enrichment of DCs as a novel characteristic of BALF from ICI-induced pneumonitis.

**Pathogenic CD4+ T\textsubscript{H17.1} and effector memory CD8+ T cells in ICI-pneumonitis BALF**

To better characterize the T-cell response, we subclustered all 65,293 T cells and performed an in-depth analysis of T-cell subclusters. Overall, we identified seven CD4+ T-cell subtypes; naïve-like (T\textsubscript{N}, with CCR7, LEF1 and TCF7 as marker genes), effector memory (T\textsubscript{EM}; ANXA1, CXCR4, IL-2), T follicular helper (T\textsubscript{FH}; BCL6, CXCR5, ICA1), two clusters of T\textsubscript{REG} and two clusters of CD4+ T cells with both (T\textsubscript{H17}-like; RORC, IL-23R, CCR6) and (T\textsubscript{H1}-like; TBX21, IFNG, CXCR3) properties, termed T\textsubscript{H17.1} cells (figure 2A,B; online supplemental figure S4A). T\textsubscript{REG} consisted of a FOXP3+ T\textsubscript{REG} cluster (T\textsubscript{FH}; FOXP3, IL-2RA, IL-1R2) and FOXP3-regulatory type 1 T-cell cluster (T\textsubscript{R1}; CRTAM, IL-10, LAG3), which derive from T-helper, CD4+ naïve or memory T cells in inflamed peripheral organs where they induce tolerance, mainly by producing IL-10 and by selective killing of antigen-presenting cells (APCs) through granzyme B and perforin secretion.18 Indeed, the cytotoxic capacity of these CD4+ T cells explains why they cluster in close proximity to CD8+ T cells on the Uniform Manifold Approximation and Projection plot (online supplemental figure S4B). T\textsubscript{H17.1} cells finally constituted a large cluster, termed T\textsubscript{H17.1, RORC} cells, with balanced expression of T\textsubscript{H1}-related and T\textsubscript{H17}-related genes, and a smaller cluster, termed T\textsubscript{H17.1, TBX21} cells, with a pronounced pathogenic phenotype evidenced by retained T\textsubscript{H17}-related gene expression (RORC, CCR6, IL-17A, IL-23R), but...
Figure 2  T-cell phenotypes and relative abundance in ICI-pneumonitis and control bronchoalveolar lavage fluid. (A) Uniform Manifold Approximation and Projection plot of 65,293 T cells, (B) annotated according to canonical marker gene expression of CD4+ (left panel) and CD8+ T cells, MAIT, and NK-cells (right panel). (C) A comparison of relative T-cell subtype abundance, revealed accumulation of CD4+ T-helper 17.1 cells with a pathogenic phenotype (T_{TH17.1,TBX21}) in ICI-pneumonitis, as well as enrichment of CD4+ regulatory type 1 T cells (T_{reg}), CD4+ naïve-like T cells (T_{n}), and CD8+ effector memory (T_{EM}) T cells. Wilcoxon rank-sum test was used; significance is shown as *p<0.05. (D) Volcano plot showing 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 (see online supplemental table S1 for all differentially expressed genes). (E) Differential gene set enrichment analysis (DGSEA) on differentially expressed genes for ICI-pneumonitis versus control T cells using the R package hyperR. 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 (see online supplemental tables S2 and S3 for all differentially expressed gene sets in ICI-pneumonitis and control T cells, respectively). CD4_N, CD4+ naïve-like T cells; CD4_EM, CD4+ effector memory T cells; CD4 TR1, CD4+ regulatory type 1 T cells; CD4_Th17.1_RORC, CD4+ T-helper 17.1 lymphocytes with predominant non-pathogenic features; CD4_Th17.1_TBX21, CD4+ T-helper 17.1 lymphocytes with predominant (pathogenic) T-helper 1-like features; CD4_FH, CD4+ follicular helper T-cells; CD4_REG, CD4+ regulatory T cells; CD8_N, CD8+ naïve-like T cells; CD8_EM, CD8+ effector memory T cells; CD8_RM, CD8+ resident memory T cells; CD8 EMRA, CD8+ recently activated effector memory T cells; CD8_EX, CD8+ exhausted T cells; CD8_gd, CD8+ gamma delta T cells; DGEA, differential gene expression analysis; FDR, false discovery rate; ICI, immune checkpoint inhibitor; MAIT, mucosal associated invariant T cells; NK, natural killer; NK cytotoxic NK-cells; NK infla, inflammatory NK-cells.

Next, we identified seven CD8+ T-cell subtypes, namely (T_{nm}; CCR7, LEF1, TCF7), (T_{EM}; GZMK, GZMM, CXCR4), resident-memory (ZNF683, ITGAE, ITGA1), recently activated effector memory (CX3CR1, FGFBP2, FCGR3A) and experienced (co-expressing effector markers GZMB, PRF1, CTLA4, LAG3) exhaustion-related (GZMB, PRF1, GZMA), cytotoxicity-related (GZMB, PRF1, GZMA), exhaustion-related (PDCD1, CTLA4, LAG3) and monocyte activation-related genes (CSF2, encoding granulocyte-macrophage colony-stimulating factor (GM-CSF)). 10

upregulation of T_{H1}-related (TBX21, IFNG), cytotoxicity-related (GZMB, PRF1, GZMA), exhaustion-related (PDCD1, CTLA4, LAG3) and monocyte activation-related genes (CSF2, encoding granulocyte-macrophage colony-stimulating factor (GM-CSF)).
PRF1, GNLY, IFNG and inhibitory checkpoint molecules HAVCR2, CTLA4, LAG3) CD8+ T cells, as well as the innate-like lymphoid gamma delta T cells (γδ T cells, TRDC, KIR3DL2, KIR3DL4) and mucosal associated invariant T cells (TMAIT; RORC, IL-23R, SL C4A10). Finally, we found two natural killer (NK) cell populations, namely inflammatory and cytotoxic NK-cells marked by immune cell recruitment (NCAM1, NCR1, XCL1, XCL2) and cytotoxic functions (PRF1, FCGRT3A, CX3CR1, FGBP2), respectively.

When comparing the relative proportions of T-cell subtypes in ICI-pneumonitis and control BALF, CD4+ TH17.1 cells were most frequent, accounting for one-third of all immune cells in the latter have been shown to arise during TH17.1- T-mediated inflammation, developing severe autoimmune pneumopathy. 23 Previous observations in a patient with inherited PD-1 deficiency, developing severe autoimmune pneumopathy. 23

CD4+ T N cells were also more abundant in ICI-pneumonitis BALF, as were (IL-10+high) CD4+ TR1 cells. The latter have been shown to arise during TH17.1-mediated inflammation as a negative feedback mechanism, and the abundance of TR1 cells indeed was higher in patients with ICI-pneumonitis showing resolution of radiographic abnormalities after first-line treatment compared with patients with persistent abnormalities (online supplemental figure S4C). 22 Finally, a slight increase in pathogenic (IFNG-high, CSF2-high) TH17.1 cells, TH17.1 cells were increasingly recognized for their role in autoimmune processes, for example, sarcoidosis and inflammatory bowel disease (IBD), in which their capacity for IFN-γ secretion correlates to disease severity. 20,21 More recently, it has become clear that also GM-CSF secretion by these T-helper cells is a critical regulator of auto-inflammation, for example, in multiple sclerosis where IFN-γ 

The above analyses suggested a central role for a pathogenic phenotype shift of T-helper cells. The latter have been shown to arise during TH17.1-mediated inflammation as a negative feedback mechanism, and the abundance of TR1 cells indeed was higher in patients with ICI-pneumonitis showing resolution of radiographic abnormalities after first-line treatment compared with patients with persistent abnormalities (online supplemental figure S4C). 22 Finally, a slight increase in pathogenic (IL-10+high) CD4+ TR1 cells. The latter have been shown to arise during TH17.1-mediated inflammation as a negative feedback mechanism, and the abundance of TR1 cells indeed was higher in patients with ICI-pneumonitis showing resolution of radiographic abnormalities after first-line treatment compared with patients with persistent abnormalities (online supplemental figure S4C). 22 Finally, a slight increase in pathogenic (IL-10+high) CD4+ TR1 cells. The latter have been shown to arise during TH17.1-mediated inflammation as a negative feedback mechanism, and the abundance of TR1 cells indeed was higher in patients with ICI-pneumonitis showing resolution of radiographic abnormalities after first-line treatment compared with patients with persistent abnormalities (online supplemental figure S4C) 22.23
transcription factors, for example, \textit{ZFX} (driving a TH17 self-renewal program) and \textit{FOXO1} (downregulating inflammatory cytokine secretion), coupled to expression of mostly non-pathogenic surface molecules and cytokines; \textit{PROCR} (which negatively regulates pro-inflammatory cytokine secretion), \textit{IL-1RN} (encoding IL-1 receptor antagonist, unexpectedly co-expressed with IL-1 receptor \textit{IL-1R1}), \textit{IL-6R} (which ‘stabilizes’ non-pathogenic TH17-like

\textbf{Figure 3} Cell fate mapping and T-cell receptor repertoire analysis of CD4+ T cells in ICI-pneumonitis and control bronchoalveolar lavage fluid. (A) Uniform Manifold Approximation and Projection plot of 11,570 CD4+ T cells with cell fate trajectories and (B) latent time as calculated by the CellRank algorithm, showing TENEM were connected to the TH17.1_RORC cluster, which branched into either TEM or TR1, as terminal states, or formed a terminal state itself. (C) Continuous gene expression profiling along the TH17.1_TBX21 trajectory. (D) Barplot of relative cell abundance in ICI-pneumonitis and control bronchoalveolar lavage fluid (BALF) along the TH17.1_TBX21 trajectory. (E) Line graph of the Gini coefficient for ICI-pneumonitis and control BALF T-cell receptor repertoire along the TH17.1_TBX21 trajectory, as calculated by the DescTools algorithm. CD4_N, CD4+ naïve-like T cells; CD4_EM, CD4+ effector memory T cells; CD4_TR1, CD4+ regulatory type 1 T cells; CD4_Th17.1_RORC, CD4+ T-helper 17.1 lymphocytes with predominant non-pathogenic features; CD4_Th17.1_TBX21, CD4+ T-helper 17.1 lymphocytes with predominant (pathogenic) T-helper 1-like features; CD4_FH, CD4+ follicular helper T cells; ICI, immune checkpoint inhibitor.
cells), IL-10, and IL-10RA (encoding IL-10 receptor). Further along the trajectory, transcription factors associated with a pathogenic phenotype are upregulated, most notably TBX21 (inducing a.o. IFN-γ secretion) and PRDM1 (encoding BLIMP-1, a transcription factor promoting a.o. GM-CSF secretion). As a result, pro-inflammatory chemokines (CCL4, CCL5), cytotoxicity genes (GZMB, GNLY), and especially key genes mediating T<sub>H17</sub>1 pathogenicity, namely IFNG and CSF2, are progressively upregulated near the end of the T<sub>H17</sub>1 trajectory, where cell density peaks for ICI-pneumonitis BALF samples.<sup>32</sup> In a similar fashion, we comparatively examined T-cell clonality along the T<sub>H17</sub>1 trajectory, obtaining 11,570 CD4<sup>+</sup> T cells with a TCR sequence. To assess clonotype diversity and distribution, we calculated TCR richness and evenness as well as the Gini coefficient (figure 3E; online supplemental figure S4F). While low TCR richness indicates a limited number of T-cell specificities, low evenness marks a skewed distribution of these specificities. The Gini coefficient is a summary metric of inequality of clonotype distribution within a repertoire, such that a high Gini coefficient and low TCR richness and evenness reflect clonal expansion of specific T-cell clones following cognate antigen recognition.<sup>15</sup> We observed a progressive increase of the Gini coefficient, and a decline of TCR richness and evenness, along the T<sub>H17</sub>1 trajectory in ICI-pneumonitis, which was not seen in control BALF. This suggests strong antigen-driven clonal proliferation of T<sub>H17</sub>1 cells, another established feature of pathogenic T<sub>H17</sub>1 cells.<sup>33</sup> Overall, cell fate mapping coupled to single-cell TCR repertoire analysis unraveled the plasticity of T<sub>H17</sub>1 cells in ICI-pneumonitis and control BALF. These cells are skewed towards a pathogenic IFNG<sup>high</sup> CSF2<sup>high</sup> phenotype in ICI-pneumonitis BALF, regulated by transcription factors TBX21 and PRDM1, respectively. Importantly, T-cell clonality analyses showed pronounced antigen-driven clonal expansion of this phenotype, further supporting the notion that these are not just bystander activated T cells but central players in ICI-pneumonitis pathophysiology.<sup>33</sup>

### Cross talk between pathogenic T<sub>H17</sub>1 cells and pro-inflammatory monocytes

We next wondered how innate immune cells are involved in ICI-pneumonitis immunopathology, and subclustered all 63,330 monocytes and macrophages (figure 4A–C). First, monocytes were separated from macrophages based on differential canonical monocyte (FCN1, LILRB2, LILR A5) or macrophage marker gene expression (PPARG, FABP4, MARCO), respectively. Monocytes were then subclustered into classical FCN1<sup>high</sup> (FCN1, S100A8, S100A9) and inflammatory IL-1B<sup>high</sup> monocytes (IL-1B, CCL20, IL-6). Within the macrophages, we identified monocyte-derived (expressing LGMN, CH Tickets) and MERTK) and tissue-resident FABP4<sup>high</sup> alveolar macrophages (FABP4, PPARG, MARCO), which formed a non-proliferating and a proliferating subcluster (MKI67, TOP2A, CDKI). We observed a significant decrease of anti-inflammatory alveolar macrophages in ICI-pneumonitis BALF, and a corresponding increase of monocyte-derived macrophages and pro-inflammatory IL-1B<sup>high</sup> monocytes (figure 4D).

Besides IL-1B, these pro-inflammatory monocytes show high expression of other pro-inflammatory genes, most notably TNF (encoding TNF-α), IL-6, IL-23A, CSF2RA, CSF2RB, CCL20 and the interferon-induced chemokines CXCL9/10, CXCL9/10 and CCL20 are potent chemotactants of CXCR3<sup>+</sup> and CCR6<sup>+</sup> cells, respectively, with presence of both chemokine receptors defining T<sub>H17</sub>1 cells.<sup>34</sup> IL-1 and IL-23 moreover are critical cytokines inducing pathogenic features (notably GM-CSF secretion) in T<sub>H17</sub>1 cells.<sup>19</sup> Reciprocally, GM-CSF signaling through the GM-CSF receptor (CSF2RA, CSF2RB) is known to induce this pro-inflammatory monocyte phenotype.<sup>19</sup> DGEA and DGSEA across all monocytes/macrophages comparing ICI-pneumonitis and control BALF, confirmed that pro-inflammatory IL-1B<sup>high</sup> monocytes are involved in the innate immune response in ICI-pneumonitis (figure 4E,F). Specifically, expression of pro-inflammatory ‘M1-like’ genes (CCL3, CCL4, IL1B, TNF, NFKBIA) was upregulated in ICI-pneumonitis monocytes/macrophages, while anti-inflammatory ‘M2-like’ genes (IGF1, MARCO) were relatively enriched in control monocytes/macrophages.

We also analyzed the DC compartment in a similar fashion, as these cells have a central role in directing T-cell phenotypes. Subclustering of DCs revealed classical type I DCs (CLEC9A, XCR1, GPNE), type II DCs (CLEC10A, FCGR2B, FCER1G), plasmacytoid DCs (LILRA4, CLEC4C, IRF7), and migratory DCs (CCR7, LAMP3, FSCN1) (figure 5A,B). Although DCs were overall enriched in ICI-pneumonitis BALF, we observed no significant differences for DC subtype abundance between ICI-pneumonitis and control BALF (figure 5C). Hence, the role of DCs in shaping the ICI-pneumonitis immune response remains unclear. Our data do suggest, however, that myeloid cells, specifically pro-inflammatory monocytes, might not only directly mediate tissue damage in ICI-pneumonitis but also sustain tissue inflammation by recruiting and shaping pathogenic T<sub>H17</sub>1 cells under the influence of GM-CSF.<sup>16</sup>

### DISCUSSION

Here, we shed light on the mechanisms underlying pneumonitis occurring in patients with cancer during anti-PD-1/PD-L1 therapy. These findings are important, as they are a necessary first step to identifying immunomodulatory treatments effectively targeting the root cause of ICI-pneumonitis. These are currently lacking, making ICI-pneumonitis the most frequent fatal anti-PD-1/PD-L1-related adverse event.<sup>4</sup> For the first time, we present a single-cell transcriptomic atlas of the bronchoalveolar immune landscape in ICI-pneumonitis, comparing the relative abundance of immune cell types as well as their precise phenotype and clonotype distribution to those in BALF of demographically-matched controls.
Figure 4  Macrophage and monocyte phenotypes and relative abundance in ICI-pneumonitis and control bronchoalveolar lavage fluid. (A) Uniform Manifold Approximation and Projection plot of 63,330 macrophages and monocytes, (B) annotated raw text
corresponds to a fourfold increase of the ratio of $T_{H17.1}$ cells over all immune cells in ICI-pneumonitis. More importantly we show, both by subclustering and by fate mapping of $T_{H17.1}$ cells coupled to TCR repertoire analysis, that they display a more pathogenic phenotype evidenced by higher expression of $IFNG$, $CSF2$ (encoding GM-CSF) and cytotoxicity genes and by strong antigen-driven clonal proliferation. This cell type is increasingly being recognized in autoimmune processes, such as IBD, multiple sclerosis and sarcoidosis, previously thought to have been mediated by $T_{H1}$ cells. The type 1 immune response conveyed by $T_{H17.1}$ cells is probably strengthened by $CD8^+$ effector memory T cells, which were more abundant in ICI-pneumonitis BALF. Earlier studies on

Figure 5: Dendritic cell phenotypes and relative abundance in ICI-pneumonitis and control bronchoalveolar lavage fluid. (A) Uniform Manifold Approximation and Projection plot of 2642 dendritic cells, (B) annotated according to canonical marker gene expression. (C) A comparison of relative dendritic cell subtype abundance showed no statistically significant differences between ICI-pneumonitis and control bronchoalveolar lavage fluid. Wilcoxon rank-sum test was used. cDC1, classical type I dendritic cell; cDC2, classical type II dendritic cell; ICI, immune checkpoint inhibitor; migDC, migratory dendritic cell; pDC, plasmacytoid dendritic cell.
ICI-pneumonitis also observed prominent lymphocytosis, with higher abundance of TNF-α^{high} CD8^+ T cells, but contradictory findings were reported on T_{H1} versus T_{H10} involvement. We argue this might be due to limited flow cytometry panels used in these studies (compared with whole-transcriptome scRNA-seq data) that did not include the canonical T_{H1} transcription factor antibody anti-RORγ, hence precluding the identification of a hybrid T_{H17} phenotype.\(^7\)\(^8\)

Second and somewhat unexpectedly, we observed a higher abundance of FOXP3\(^{+}\) IL-10\(^{high}\) T_{R1} in ICI-pneumonitis BALF. In preclinical models, it has been shown that in pro-inflammatory conditions, in the presence of TGF-β/IL-6/IL-23, T_{H17} cells are skewed towards a pathogenic T_{H17,1} phenotype but that a small proportion of these T_{H17,1} cells gives rise to T_{R1} cells, in line with our findings.\(^2\)\(^2\) Indeed, while IL-10 is the prototypical anti-inflammatory IL, increased serum IL-10 levels have been reported during ICI-pneumonitis.\(^2\)\(^5\)\(^2\)\(^6\)

How innate immune cells interact with T_{H17,1} cells in ICI-pneumonitis remains speculative, but our scRNA-seq data provide a solid basis for hypothesis generation. First, we show that anti-inflammatary phagocytosing alveolar resident macrophages are depleted, while monocyte-derived macrophages and pro-inflammatory IL-1^{high} monocytes are relatively enriched in ICI-pneumonitis BALF. A detrimental feedforward loop in which pathogenic T-helper cell-derived GM-CSF instructs GM-CSF receptor expressing monocytes to orchestrate tissue inflammation (TNF-α, IL-6) and tissue damage (reactive oxygen species) while sustaining pathogenic T-helper cells (through IL-23 secretion, intensified by IL-1β secretion), is a well-established mechanism of auto-inflammation, best characterized in multiple sclerosis but also discerned in one patient with inherited PD-1 deficiency.\(^1\)\(^9\)\(^2\)\(^3\)\(^6\)\(^2\)\(^3\)\(^6\) In line with this mechanism, is a prior study in which elevated levels of GM-CSF at baseline and early during ICI treatment were found to predict severe irAEs.\(^3\)\(^7\) In addition, the pro-inflammatory monocytes might recruit CXCR3- and CCR6-expressing T_{H17,1} cells through expression of the chemoattractants CXCL9/CXCL10 and CCL20, respectively.\(^3\)\(^8\)\(^3\)\(^9\) Notably, an increase of serum CXCL9/10 concentrations after anti-PD-1/PD-L1 therapy, was shown to predict occurrence of irAEs.\(^4\)\(^0\)

Lastly, we expected a role for DCs in instructing pathogenic T_{H17,1} cells, based on their known role as orchestrators of adaptive immunity and their increased abundance in ICI-pneumonitis BALF. However, subclustering analysis did not reveal a clear phenotypic shift of DCs. As such, our data do not provide insights into the role of DCs in ICI-pneumonitis pathophysiology. Possibly, profiling of regional lymph nodes is necessary to reveal relevant DC/T-cell interactions, as was the case for IL-23-producing DCs residing in mesenteric lymph nodes of patients with IBD.\(^4\)\(^1\)

Our findings bear important potential to improve the care of patients with anti-PD-1/PD-L1 treated cancer experiencing ICI-pneumonitis. Although 70–80% of symptomatic ICI-pneumonitis cases resolve with corticosteroid treatment,\(^4\)\(^2\) it cannot be formally excluded that corticosteroids negatively impact tumor control.\(^4\)\(^3\) Moreover, second-line treatment for steroid-refractory ICI-pneumonitis lacks a clear scientific rationale and success rates are therefore modest.\(^4\)\(^2\) Our findings advocate clinical trials with agents targeting crosstalk between pathogenic T_{H17,1} cells and pro-inflammatory monocytes. Although GM-CSF emerges as the master regulator, it has important homeostatic functions in the lungs and its role in antitumor immunity is ambiguous.\(^4\)\(^4\) Anti-IL-6 and anti-TNF-α therapy tackles monocyte-mediated tissue inflammation, and has indeed shown efficacy and safety as second-line treatment for steroid-refractory irAEs in limited case series,\(^4\)\(^5\) but does not disrupt the interplay between pathogenic T_{H17,1} cells and pro-inflammatory monocytes. Based on our data, we speculate that repurposing anti-IL-1 and especially anti-IL-23 risankizumab might improve response rates without negatively affecting tumor control.\(^4\)\(^5\)

A potential limitation of the study is that all recruited patients with ICI-pneumonitis had limited grade pneumonitis and did not require second-line immunosuppressive therapy. As such, we cannot exclude that distinct disease processes take place in high grade or steroid-refractory ICI-pneumonitis. Second, while the control cohort of patients was demographically matched to the study cohort (also with regards to smoking status, lung disease and the presence of a thoracic malignancy, importantly), these patients did not receive prior anti-PD-1/PD-L1 treatment. All BALF samples were taken during routine bronchoscopy so we could minimize patients’ exposure to the risks of this invasive procedure, knowing that anti-PD-1/PD-L1 does not seem to induce changes in the immune compartment of non-inflamed organs.\(^7\)\(^3\)\(^6\) While scRNA-seq and scTCR-seq data allowed us to put forward several interesting mechanistic hypotheses, further research is needed to validate these hypotheses. Finally, our data cannot provide an answer to the question why some patients develop ICI-pneumonitis and others do not. Indeed, while we provide data on the TCR repertoire in ICI-pneumonitis BALF, larger studies combining in-silico TCR repertoire with in vitro TCR reactivity data (examining (cross-)reactivity of T cells in ICI-pneumonitis lesions) are presumably key to identify the substrate of the immune reaction in ICI-pneumonitis. In parallel, genomic studies (eg, examining IL-23R or IL-10R polymorphisms) will help to uncover susceptibility factors to the development of irAEs.\(^4\)\(^7\)

In conclusion, we use scRNA-seq and scTCR-seq to perform a deep immune profiling effort and characterize the immune response in ICI-pneumonitis, thereby yielding novel pathophysiological insights. We offer a clear rationale for a novel targeted treatment approach for ICI-pneumonitis to improve outcome of this potentially fatal irAE while maintaining tumor control.
REFERENCES


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 12th 2020 and December 13th 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, 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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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.  

Figure S1
Figure S4