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Graphical abstract

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The alveolar immune cell landscape is dysregulated in checkpoint inhibitor pneumonitis

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Abstract

Background: Checkpoint inhibitor pneumonitis (CIP) is a highly morbid complication of immune checkpoint immunotherapy (ICI), one which precludes the continuation of ICI. Yet, the mechanistic underpinnings of CIP are unknown.

Methods: To better understand the mechanism of lung injury in CIP, we prospectively collected bronchoalveolar lavage (BAL) samples in ICI-treated patients with (n=12) and without CIP (n=6), prior to initiation of first-line therapy for CIP (high dose corticosteroids). We analyzed BAL immune cell populations using a combination of traditional multicolor flow cytometry gating, unsupervised clustering analysis and BAL supernatant cytokine measurements.

Results: We found increased BAL lymphocytosis, predominantly CD4+ T cells, in CIP. Specifically, we observed increased numbers of BAL central memory T-cells (Tcm), evidence of Type I polarization, and decreased expression of CTLA-4 and PD-1 in BAL Tregs, suggesting both activation of pro-inflammatory subsets and an attenuated suppressive phenotype. CIP BAL myeloid immune populations displayed enhanced expression of IL-1β and decreased expression of counter-regulatory IL-1RA. We observed increased levels of T cell chemoattractants in the BAL supernatant, consistent with our pro-inflammatory, lymphocytic cellular landscape.

Conclusion: We observe several immune cell subpopulations that are dysregulated in CIP, which may represent possible targets that could lead to therapeutics for this morbid immune related adverse event.

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Introduction

With recent clinical trials demonstrating clear efficacy for immunotherapy in locally advanced and advanced stage non-small cell lung cancer (NSCLC) as well as other tumors, the use of immune checkpoint inhibitors (ICI) for NSCLC has rapidly increased\(^1\)\(^–\)\(^3\), becoming standard of care. However, ICIs are associated with a constellation of toxicities termed immune related adverse events (irAEs). These toxicities include arthritis, colitis, endocrinopathies, and lung injury, the latter termed checkpoint inhibitor pneumonitis (CIP)\(^4\),\(^5\). Clinically, CIP presents with acute to subacute onset of dyspnea, hypoxemia and pulmonary infiltrates similar to seen in patients with lung injury from acute respiratory distress syndrome (ARDS)\(^6\). Although CIP can result in high morbidity, it was previously thought to an uncommon complication ICI therapy, with an incidence of around 3-5\%\(^7\),\(^8\) based on clinical trial data. However, recent evidence from our group and others suggest that CIP incidence may be higher in real-world settings\(^9\),\(^10\). For instance, using a multi-disciplinary, standardized approach\(^11\), we recently observed an incidence of 19% in a cohort of 205 ICI-treated NSCLC patients\(^12\). In addition, we also observed an association between CIP development and increased mortality in ICI-treated NSCLC patients\(^13\).

Despite rising incidence of CIP and its association with increased mortality, the current paradigms for diagnosis and treatment of CIP are largely based on anecdotal evidence, primarily because fundamental knowledge of the pathobiology of CIP is lacking\(^14\). CIP is diagnosed by the presence of compatible symptoms (shortness of breath, hypoxia, cough), new radiographic infiltrates (which can be either unilateral or bilateral\(^15\),\(^16\), typically with ground glass and consolidative components – Figure 1) and the exclusion of infectious etiologies (with sputum cultures or bronchoalveolar lavage). There are currently no diagnostic biomarkers for CIP, so the diagnosis remains largely one of exclusion. Once diagnosed, clinical severity is used to determine CIP grade (Supplementary table 1). For CIP Grade 2 (i.e. symptomatic patients with compatible radiographic infiltrates) and higher, ICI therapy is immediately discontinued and empiric high-
steroids are initiated. More targeted, disease-specific therapy is not instituted as first-line treatment for CIP in part because there is currently no available data on the mechanism of lung injury in CIP. Due to the lack of diagnostic and therapeutic options, patients diagnosed with CIP are typically also not eligible for further ICI; this is particularly disadvantageous in patients with ongoing tumor response.

As part of a multi-disciplinary immune-related toxicity (irTox) team(11) engaged in diagnosis, management and study of irAEs following ICI therapy, we prospectively collected bronchoalveolar lavage fluid (BALF) specimens from ICI-treated patients with no evidence of CIP as well as those with suspected CIP. Clinical, laboratory and radiographic data of patients suspected of having CIP were subsequently reviewed by the irTox team and a determination was made as to whether the presenting symptoms were due to CIP or another etiology. Using these specimens, we performed multi-parametric flow cytometric analysis on BALF samples to better understand the landscape of immune dysregulation in CIP. In part due to the lack of available data on the biology of lung injury in CIP, we utilized unbiased clustering analytic techniques to examine our flow cytometric results. Such approaches have the advantage of detecting changes in small cell populations that may otherwise be excluded with manual gating. Importantly, the control group comprised patients who also received ICI but did not exhibit any clinical evidence of CIP at time of bronchoscopy.

**Results**

**BAL lymphocytosis is a hallmark for CIP**

Study design as well as baseline clinical characteristics for the patients enrolled in this observational study are shown in Figure 2 and Table 1, respectively. Clinical grade, management and outcomes data for the 12 patients with CIP are presented in Supplementary Table 2. We first manually counted BAL cell differentials in a subset of control and CIP samples. We found a
relative increase in lymphocytes with a concomitant decrease in monocytes in CIP (Supplemental Figure 2) compared to patients without CIP. Notably, BAL neutrophils were not abundant in CIP patients. To further characterize subsets of BAL immune cells, we performed multi-parametric flow cytometric analysis using optimized T-cell and monocyte panels (Supplemental Table 3). We initially analyzed these data using traditional gating methods, and similar to our manual cell differentials, found an increase in % T-lymphocytes in CIP (Figure 3). Specifically we found an increase in CD3⁺CD4⁺ cells (Figure 3A; p=0.04) and a possible association with increased CD3⁺CD8 cells (Figure 3B; p=0.073). We also noted a decrease in monocytes, specifically CD3⁻CD19⁻CD14⁺ cells (3C; p=0.04). We did not observe any differences in %Tregs (CD3⁺CD4⁺CD127loCD25⁺Foxp3⁺) among CIP+ and CIP- patients (data not shown).

Unsupervised clustering reveals differential T-cell subpopulations in CIP

To understand immune cell subpopulations in our samples in more granular detail, we next turned to unsupervised clustering analysis. The total numbers of cells per condition used for our unsupervised analyses are shown in Supplemental Table 4. We represent the results of our clustering analysis using star charts. As shown in Supplemental Figure 1, in this graphical representation, groups of cells that share similar cytokine profiles are identified as a node, and represented by a circle, whose diameter reflects the number of cells present within that subpopulation. The cell surface or intracellular MFI for each fluorophore is expressed as a wedge within the circle; the radius of the wedge segment represents the expression level of that particular marker. For instance, in Supplemental Figure 1B, a node with very high PD-1, CD45RA and CD127 expression is shown. Topologically, nodes are arranged by similarity to each other in a cluster map (Supplemental Figure 1C). Cell subsets occupy distinct areas within a map; for instance, in the T-cell cluster map, as expected, CD4⁺ and CD8⁺ cells are clustered together in opposite ends, since they are very distinct from each other (Supplemental Figure 1D-E).
In control, unstimulated T-cells, we observed clustering around two cell populations – CD4+ cells with high PD-1 expression (bottom left, Figure 4A) and CD8+ cells with moderate PD-1 expression (top right, Figure 4A). Unstimulated CIP samples exhibited increased CD8+ cell populations compared to unstimulated controls (middle right, Figure 4B) as well as a local shift in CD4+Treg+ populations (bottom left, Figure 4B), as discussed in more detail below.

To better understand the specific T-cell subsets that were up/downregulated in CIP, we examined the differential cluster map of T-cell subsets, which highlights only clusters where the magnitude of difference between groups was > 95%. As shown in Figure 5, in CIP, we observed a significant increase in a CD4+CD45RA+CD25- cells that also expressed CD62L. Since the cytokine profile of resembled that of central memory T cells (Tcm), a non-Treg (i.e. conventional) T cell subpopulation characterized by high CD62L and low CD45RA expression, we performed manual gating for Treg and non-Treg subpopulations (Supplemental Figure 3) and observed a significantly higher percentage of Tcm in CIP samples (p=0.01). As mentioned earlier, we observed a shift in CD4+FoxP3+ cells between unstimulated control and CIP cluster maps. Closer examination of these clusters revealed that while clusters of PD-1lo CTLA-4lo Tregs were similarly expressed in both CIP and controls, a subpopulation of Tregs with high PD-1 and CTLA-4 expression was only seen in controls and these effector molecules were downregulated in alveolar Tregs in CIP (Figure 5). Compared to controls, multiple CD8+TNF-αhi subpopulations were upregulated at baseline in CIP (Figure 5). Ex vivo stimulation of CIP samples polarized T cells towards a Type 1 phenotype with increased TNF-α and IFN-γ production across multiple cell subsets with varying degrees of CD8 expression; these cell populations were not increased in control cells following stimulation (Supplemental Figure 4).

In summary, these findings suggest multiple dysregulated T-cell subsets in CIP. At baseline, we observe, in CIP: a) increased Tcm, b) loss of PD-1hi/CTLA-4hi CD4+ Tregs and c) upregulation of pro-inflammatory (i.e. TNF-αhi, IFN-γhi) CD8+ cells. With stimulation, we observe increased in
numbers of CD8⁺ TNF-α^hi subsets and amount of TNF-α expression in stimulated CIP samples compared to controls.

**Upregulation of IL-1β^hi monocytes in CIP and IL-1RA expressing B-cells in controls**

Next, we sought to examine population differences in non-T (i.e. CD3⁻) cells. Similar to our T-cell analyses, we represented the results in cluster maps, where the distinct cell populations (e.g. CD14⁺ monocytes, CD16⁺ monocytes, B-cells) occupy various regions within the map (Supplemental Figure 5). We observed clear differences between unstimulated control and CIP samples (Figure 6). As shown in Figure 6A and 6B, and in closer detail in Figure 7, two reciprocal populations were upregulated in controls and CIP, respectively. In controls, we observed a large increase in several clusters corresponding to IL-1RA expressing CD86⁺ B-cells (CD19⁺). While this cluster was downregulated in CIP, a different cluster of IL-1β^hi, TNF-α^hi CD-11b^hi myeloid cells (CD19neg, CD14int/CD16int) was significantly upregulated in CIP. Similar to our T-cell analysis, we confirmed the presence of a TNF-α^hi, IL-1β^hi, CD11b^hi population in CIP samples with manual gating (Supplementary Figure 6) Unlike T cells, we did not observe significant differences in cluster profiles between unstimulated and stimulated cells either in the control or CIP condition (Supplemental Figure 7)

We also compared to the subpopulations identified above as being significantly different in controls or CIP to the results of a meta-clustering analysis, to determine whether the subpopulations selected to be differentially upregulated in our prior analyses were also identified as distinct populations using an “auto-gating” strategy. As shown in Supplemental Figure 8, meta-clustering identified the clusters previously examined in our T-cell and monocyte/B-cell cluster maps (Figures 4,6) as distinct subpopulations.

**Upregulation of lymphocyte chemoattractants in the BALF of CIP patients**
To determine whether BALF cytokines were promoting the cellular phenotypes observed in our flow-cytometry data, we measured key cytokines in the cell-free BAL supernatant (Figure 8A-C, Supplementary Table 5). Surprisingly, despite observing an increased number of IL-1β<sup>hi</sup> cells in our flow analysis, we observed decreased levels of IL-1β in CIP BAL supernatants. We observed no differences in TNF-α levels, but observed increased levels of the Type 1 skewing cytokine IL-12p40. We also measured levels of cytokines involved in recruitment of inflammatory cells to the alveolus. We observed lower levels of IL-8, the classical neutrophil chemoattractant, in CIP. While no differences were observed in levels of monocyte chemoattractant proteins 1 or 4, we observed lower levels of macrophage inflammatory protein-3 alpha (MIP-3α), a significant increase in levels of the interferon gamma-induced protein 10 (IP-10, or CXCL-10) and a trend towards increased levels of T-cell chemoattractant protein TARC (CCL17; p=0.06).

**Discussion**

In this study, we describe multiple baseline and functional abnormalities in both lymphoid and myeloid alveolar cell types in patients who developed CIP. These abnormalities involve both upregulation of pro-inflammatory subsets and downregulation of counter-regulatory anti-inflammatory process in both T-cells and myeloid cells (Figure 9). In normal adults, the BAL is composed primarily of macrophages (>85%) and lymphocytes (10%)(17). This is similar to the pattern seen in our control samples (i.e. patients who received ICI but did not have CIP at the time of bronchoscopy), suggesting that ICI therapy alone does not appear to significantly alter the alveolar immune cell pattern. In contrast, we observed a lymphocytosis of > 20% in most of our CIP+ BAL samples. BAL lymphocytosis has been reported in other conditions such as sarcoidosis, hypersensitivity pneumonitis, cryptogenic organizing pneumonia, non-specific interstitial pneumonia (NSIP) and radiation pneumonitis. Our finding of lymphocytosis in the BALF of CIP patients argues for the use of BAL cell count differentials and flow cytometry for CD4+/CD8+ cells as part of the clinical evaluation scheme during BAL in patients with suspected
CIP. As no biomarker currently exists for this disease, this represents a translational application of our current findings.

Our unbiased clustering approach identified several subpopulations of T cells that are likely to be playing key roles in the pathobiology of CIP. First, CD4⁺ central memory subsets (Tcm, CD4⁺CD45RA⁻ CD62L⁺) were increased in CIP. Tcms have been shown to be more resistant to steroid-induced apoptosis than other conventional T cells, such as effector memory (Tem) T-cells. Moreover, CD62L⁺ cells play an important role in adhesion to inflammatory sites and can perpetuate injury(18). Increased Tcm in CIP might explain why some patients fail high dose steroid therapy (we recently reported steroid-refractory disease in up to 40% of CIP patients in our cohort(10)); from a lung injury standpoint, this feature of CIP is unique compared to other lymphocytic pneumonitides, which generally tend to be steroid responsive. The incidence of CIP is significantly higher in patients with underlying non-small cell lung cancer than other cancers, and we have shown(5) that within NSCLC patients, tumor histology further stratifies CIP incidence and risk. These findings, coupled with our current data, suggest that Tcm could be responding to tumor-specific antigens. T-cell receptor (TCR) sequencing of the T-cells subsets in CIP samples will be useful in this regard. Second, a subpopulation of CD4⁺ cells skewed towards a Type I phenotype with high IFN-γ and TNF-α production is upregulated in CIP. Type I lymphocytes have been linked to several lung diseases including sarcoidosis, hypersensitivity pneumonitis and lung allograft rejection(19–21). Thus, the combination of “sticky” lung CD4+ T cells (i.e. CD62L+ CD4 cells) and Type I skewing may be synergistically contributing to lung injury seen in CIP patients.

Third, we observed decreased CTLA-4 and PD-1 expression within Treg (i.e. FoxP3⁺) populations suggesting an attenuated Treg suppressive phenotype. One explanation for our findings is that, in CIP, loss of Treg suppression may be promoting exuberant Th1 T cell responses. We have shown that alveolar Tregs play a pivotal role orchestrating resolution of lung inflammation and are present in humans with lung injury(22), while others have shown that PD-1⁺Tregs are more
suppressive to control CD8+ T cells(23). In addition to PD-1, the lack of CTLA-4 may further impair Treg ability to control conventional T-cell (such as Tcm) and macrophage pro-inflammatory responses(24). Overall, our findings suggest a highly activated alveolar T cells with loss of a regulatory, anti-inflammatory Treg suppressive phenotype contributing to unchecked immune dysregulation seen in CIP.

Interestingly, while we observed decreased numbers of CD14+ monocytes based on traditional gating methods, our clustering data show additional dramatic shifts in myeloid populations between controls and CIP, such as a significant increase in CD11bhi IL-1βhi, myeloid cells with varying degrees of CD14/CD16 expression. This is accompanied by a loss, in CIP, of IL-1RA+ CD19+ cells, reflected in the cluster maps as a relative upregulation of these cells in controls. These findings suggest that an imbalance in IL-1 signaling, along with overexuberant TNF-α signaling may be contributing to the pathobiology of lung injury in CIP. The concomitant presence of increased Tcms, as discussed earlier, may also serve to augment T-cell and monocyte inflammation.

Our BALF cytokine results also point towards a pro-inflammatory, chemoattractant cytokine milieu. Interestingly, we observed a decreased in soluble IL-1β while an increase in IL-1β expressing monocyte subsets was observed in flow cytometry. However, the dynamics of IL-1β production and release is complex and thought to be related to the strength of the inflammatory stimulus(25). Thus, one possibility is that, in CIP, the underlying source of inflammation promotes IL-1β translation and endosomal storage, but not membrane release. Another possibility is that soluble IL-1β release occurs earlier in injury, and is decreased by the time our samples are obtained (generally 2-3d at a minimum, after onset of symptoms). This lack of time resolution in our BALF data may also explain why TNF-α levels were not significantly different; another explanation is that, while our controls did not have CIP, they underwent bronchoscopy prior to tumor sampling/resection; this bias may be skewing our control IL-1β results. Despite this, our IL-
12p40 and CXCL-10 (IP-10) data further implicate CD4 cells in the pathobiology of CIP. IL-12 is a known orchestrator of tissue inflammation and Type I polarization. IL-12p40 can form heterodimers with IL-12p70 and IL-23(26); however neither of these cytokines were elevated in the BALF of CIP subjects (Supplementary Table 5). Thus, we postulate the increased IL-12p40 observed in CIP constitutes the monomeric form. This secreted form has been reported to be 10-20 fold in excess compared to IL-12p70 in stimulated human peripheral blood cells(27), and has been elevated in asthmatics during airway inflammation(28). Additionally CXCL-10 (IP-10) are known to guide T_{cm} lymphocytes (a T cell subset seen to be upregulated in our flow cytometry data) to their destination within lymph nodes(29). Therapeutically, antibody-mediated blockade of IL-12p40 and CXCL10 have been used to treat inflammatory diseases(30, 31). Our chemotactic cytokine data collectively reflect a lack of neutrophil chemoattraction to the lung (decreased IL-8).

Similarly, MIP-3α, which is decreased in CIP BALF, has been previously observed in the context of airway infections(32), is thought to have antimicrobial properties. This observation, along with our IL8 data and lack of significant neutrophil predominance in our BAL cell differentials (Supplementary Figure 2) further support the notion that CIP may not be a bacterial infection-triggered phenomenon. Lastly, our finding of increased CCL17 (TARC) levels correlates with our flow cytometric finding of increased CD11b^{hi} populations of myeloid cells; CD11b^{+} cells have been previously identified as a key source of the CCL17 honing chemokine in the lung(33).

Our findings suggest several targets for therapeutic consideration in steroid-refractory CIP. We note upregulation of several TNF-α^{hi} subsets (lymphoid and myeloid) at baseline in CIP; this finding provides some tissue-specific rationale for the use of infliximab for steroid-refractory CIP, although our BAL cytokine data suggests that timing of TNF-α inhibition may need to further explored. Importantly, our data also identifies several novel populations upregulated in CIP (such as CD62L^{hi} Tcms and IL-1β expressing monocytes) that could be targeted using existing therapies. Anti-CD62L antibodies or small molecule inhibitors have been used to attenuate
models of lung injury(34)(35), although these inhibitors are not currently approved for any clinical indication. Biologicals against IL-1β (e.g. anakinra or canakinumab) are currently either in trials or in use, and thus, further validation of these results could provide the rationale for testing these therapies either as first line adjuncts or as salvage therapies for high grade CIP. It is known that transient expression of IL-1β can induce lung inflammation, increase TNF-α and contribute to progressive tissue fibrosis(36); hence targeting IL-1β could represent an attractive target in CIP.

CCL-17 (TARC) is the ligand for CCR4 is usually considered a selective chemoattractant for Type 2 cells, although it has been shown to be elevated in sarcoidosis, a classical Type I–mediated lung disease(37). Blocking TARC or its receptor CCR4 could decrease T cell infiltration into the inflamed CIP lungs. Alternatively, transiently enhancing Treg suppressive function could lead to multiple beneficial effects, such as improving control of exuberant Type I responses and limiting proliferation, abrogating macrophage pro-inflammatory responses and ultimately orchestrating lung repair(22)(38). For instance, we have previously shown that short course administration of DNA methyl transferase inhibitor decitabine can potently augment endogenous Tregs and mediate resolution of lung inflammation and promote lung repair(39). Analysis of CIP rates in ongoing trials utilizing ICI/DNA methyltransferase inhibitor combinations could provide further insight into a potential beneficial effect for these agents from a CIP standpoint.

While our data provides new insight into potential pathobiologic mechanisms in CIP, CIP is unique in comparison to other irAEs with regards to incidence (across cancer types)(40) and relationship to OS. CIP is much more common in lung cancers compared to other cancers, and while other irAEs have been associated with improved OS, we did not observe a similar association with CIP(13). Thus, we do not believe that our results are necessarily generalizable to other irAEs.

There are several limitations to this study. First, due to the logistical challenges associated with identifying and promptly performing lavage in patients with suspected CIP before antibiotic or steroid administration, our samples sizes are low, and thus preclude adjustment for clinical
comorbidities (such as COPD) that may confound our results. Second, while only patients with negative infectious work-up were included in the CIP cohort, it is possible that BAL cultures did not identify a focus of infection in patients thought to have CIP. Third, while BAL of CIP infiltrates were performed in areas not previously affected by tumor, it is possible that presence of malignancy in the nearby airways could have influenced our results.

In conclusion, our data provide several hypothesis-generating insights into the dysregulated alveolar immune dysregulation in CIP. In the absence of a pre-clinical model for CIP, our finding provide the first rigorous report of immunological mechanisms underlying CIP. In addition to validation in larger clinical cohorts, these data could inform design of pre-clinical and translational studies aimed at further understanding the mechanistic basis of CIP, so that targeted therapies can be developed for this morbid complication of immunotherapy.

Methods

Study Population: Patients were enrolled in this prospective observational study if they were a) diagnosed with NSCLC and b) treated with immune checkpoint inhibitors. Patients who received neo-adjuvant ICI underwent bronchoscopy with collection of BALF prior to surgery. Otherwise, BALF was collected whenever patients underwent bronchoscopy. If CIP was suspected, the BALF sampled was categorized as “CIP” if i) the sample was obtained prior to initiation of steroids and antibiotics and ii) a clinical diagnosis of CIP was adjudicated by the multi-disciplinary irTox team (see below). Following adjudication, patients with CIP were treated with high-dose steroids (1mg/kg Prednisone). Second line agents (Infliximab, IVIG or mycophenolate mofetil) were added at the discretion of the treating team if no improvement was noted after 72 hours, as described previously(12).

CIP Diagnosis: CIP was defined as a) the presence of shortness of breath, decreased exercise tolerance, exertional desaturation, and/or cough along with b) presence of new radiographic
infiltrates and c) lack of evidence of infection (negative cultures on BAL, negative respiratory viral swab) or alternate etiologies (DAH, heart failure). Radiographic assessment was performed based on RECIST criteria; cases where the new infiltrates were deemed to represent progression of tumor were excluded from both control and CIP groups. A diagnosis of CIP was adjudicated following review and discussion of the pertinent microbiologic and radiographic(11, 12) data by the primary oncologist, a second oncologist (JN), a pulmonologist (KS, SD) and a radiologist (CTL), with additional input from other members of the immune-related toxicity team(11) (such as radiation oncology or infectious disease), as needed. Patients in whom clinical equipoise regarding infection was present (for example, clinical presence of fever, purulent sputum, sick contacts, elevated bands on CBC differential) were not adjudicated as CIP even if the BAL cultures were negative.

**Bronchoalveolar Lavage:** In control patients, the middle lobe was lavaged. In patients with CIP, an area with new infiltrates not previously known to be associated with tumor was lavaged. The volume of instilled and returned saline was abstracted from the BAL procedure note. BAL specimens were processed with ammonium–chloride–potassium (ACK) lysis solution. Cells were then counted following Trypan blue staining to exclude dead cells.

**Manual Cell Differentials:** BALF cells were stained with Diff quik and equal numbers of total cells (n=500) were counted per specimen by two investigators blinded to the sample group classification as previously described(22).

**BAL Cytokine measurements:** BAL supernatant was collected following centrifugation of the cellular components and stored at -80 degrees until further processing. Cytokine measurements were performed using the Mesoscale Discovery platform (Gaithersburg, MD). Values were normalized to total volume of BAL fluid returned, as noted during bronchoscopy.
Flow Cytometry: After thawing samples at 37°C cells were stained for flow cytometry. Approximately $1 \times 10^6$ cells per sample were stained with violet Live/Dead (Invitrogen, Carlsbad, CA). Cells were Fc incubated with human IgG (Rockland) to block Fc receptors. Cells were then surface stained with BD Biosciences-Pharmigen antibodies: BV510–conjugated anti-CD3 (UCHT1), BUV395-conjugated anti-CD4 (RPA-T4), allophycocyanin-Cy7–conjugated anti-CD25 (M-A251; BD Biosciences), BUV737-conjugated anti-CD8 (SK1), PE-CF594-conjugated anti-CD62L (DREG-56) BV421-conjugated anti-CD127 (HIL-7R-M21), BV650-conjugated anti-CD45RA (HI100), PE-Cy7-conjugated anti CD14 (MoP9); BV711-conjugated anti-PD-1 (EH12), BB700-conjugated anti-CD19 (SJ25C1), PE-Cy7-conjugated CD80 (L307), APC-Cy7-conjugated anti HLD-DR (G46-6), BV650-conjugated anti-CD11b (M1/70), BV711-conjugated anti-C86 (2331-FUN-1), APC-R700-conjugated anti- CD274 (MIH1), BV786-conjugated anti-CD206 (19.2), PE-conjugated anti-IL-1RA (AS-17), BUV395 conjugated anti-CD16 (3G8) and intracellularly stained with allophycocyanin-conjugated anti-Foxp3 (PCH101; eBioscience) and the following intracellular antibodies from BD Biosciences: Alexa-488-conjugated anti-IL-4 (8D4-8), PE-conjugated anti-IL-10 (JES3-9D7), APC-R700-conjugated anti-IL-17A (N49-653), BV605-conjugated anti-IFN-γ (B27), BV750-conjugated anti-TNF-α (Mab11), BV786-conjugated anti-CTLA-4 (BNI3), PE-CF594-conjugated anti-TGF-β (TW4-9E7), BV510-conjugated anti IL-8 (G265-8) and BV421-conjugated anti-IL-1β (H1b-98; BioLegend). A UV excitable live dead discrimination assay (Invitrogen) was applied. Cells were then analyzed on a FACSAria (BD Biosciences). Data were analyzed using either FlowJo (TreeStar, Inc) for traditional gating analyses or R/Bioconductor for unsupervised clustering, as detailed below.

BAL cells ex vivo stimulation: Cells were resuspended in a 96-well U-bottom plate using Iscove’s DME-based CTL (10%HIFBS, 1% sodium pyruvate, 1% HEPES, 2mM glutamax 100U/ml Penicillin/streptomycin and 50 μM β-mercaptoethanol). For lymphocyte stimulation, cells were stimulated with PMA (40ng/ml), ionomycin (500ng/ml) for a total for 4 hours and golgistop and
golgiplug added the last 3 hours. For myeloid stimulation, cells were stimulated with LPS (1 µg/ml) and IFN-γ (100 ng/ml) for a total of 4 hours with golgistop/golgiplug (BD Biosciences) added for the last 3 hours.

**BAL biomarker measurements using Vplex immunoassays:** BAL supernatants were used to measure CRP, Eotaxin, Eotaxin-3, FGF (basic), GM-CSF, ICAM-1, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-8 (HA), IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-16, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31, IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, MIP-3α, PIGF, SAA, TARC, Tie-2, TNF-α, TNF-β, VCAM-1, VEGF-A, VEGF-C, VEGF-D, VEGFR-1/Flt-1 using Vplex immunoassays (Meso-Scale Discovery, Rockville, Maryland, USA), according to manufacturer’s instructions. Samples were run in duplicates. All BAL supernatants were diluted equivalently, and the cytokine results were normalized for amount of BALF recovered, and the results are thus expressed as micrograms per mL of recovered BALF, as per guidelines for measurement of acellular BALF components(17, 41).

**Statistical Analysis:** Unsupervised clustering analysis was conducted using the *FlowSOM* and *flowCore* packages in R/Bioconductor(42). Briefly, scaled, transformed MFIs for each cell are used as the coordinates for a data point in n-dimensional space, where n is the number of fluorophores. A self-organizing map of nodes in this space was to maximize similarity within each node. The distances between nodes reflects the degree of similarity between groups of cells. Importantly, mean fluorescence intensity (MFI) is treated as a continuous variable, thus allowing visualization and analysis of cell subsets where a surface marker expression may be intermediate. Further, since this method of analyzing flow cytometry data incorporates the MFIs for each fluorophore for each cell, it allows for greater resolution of differences in cytokine expression in a multiparametric flow cytometric dataset. A graphical abstract of the algorithm is provided in Supplemental Figure 1A. All MFI values are compensated, scaled and transformed as previously described (43, 44). For both T-cells (i.e. singlet, live, CD3+ cells) and
monocytes/B-cells (i.e. singlet, live, CD3−) cells, the cluster map was first constructed on a concatenated dataset composed of unstimulated control, stimulated control, unstimulated CIP and stimulated CIP samples. This concatenated set represents the sum total of biological replicates (n=18; 6 controls and 12 CIP cases). Next, group comparisons between controls and CIP as well as unstimulated and stimulated samples were made by generating group-specific cluster maps as well as a differential cluster map, where a node was considered to be upregulated if there was a >95% difference between groups. As the initial conditions for the clustering algorithm is randomly chosen, the map shape can differ slightly with each run; each analysis was re-run 5 times to ensure that the same clusters were upregulated across multiple runs (map stability). Lastly, a meta-clustering analysis was performed. This represents an “auto-gating” strategy where the algorithm attempts to classify cell populations across clusters based on their cytokine profile. The code used to generate the clustering analysis and high resolution copies of cluster maps are provided in Supplemental Data 1. Scaled, centered values were used to generate the heatmap for BALF cytokine data (gplots package, R). Cytokine profiles were hierarchically clustered (using complete linkage clustering and euclidean distance). Individual cytokine comparisons were plotted and compared using GraphPad Prism. Two tailed non-parametric tests (Mann-Whitney) were used to compare mean differences between control and CIP cytokine values. A p value of < 0.05 was accepted as significant.

**Study approval:** IRB and ethical approval as well as consent was obtained for all participants in this study. All human work was approved by an Institutional Review Board at the Johns Hopkins Hospital.

**Author Contributions:**

Co-first authorship: KS – Flow cytometric and multiplex experimental design, data processing and statistical analysis, manuscript writing and review, figure preparation; JN – Study design, IRB and clinical database design/administration, clinical data analysis, manuscript writing and
review. All other authors: study design (KS, FD, JN, JB, SD, MS, PF, JM,MF, LC, VA), conducting experiments/adjudication of patient data/data acquisition (KS, FD, QZ, YX,TP, AB, JM,MF,LC, VA, DE, KM,RK,CH,BL, JF, CL, DFK, AL, HL, MS, LY, EL ), data analysis (QZ, YX,TP,KS,FD,AB), writing the manuscript (KS,FD,JN, SD), reviewing/editing the manuscript (all authors).

Acknowledgements

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Table 1: Baseline characteristics

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<th>No CIP (n=6)</th>
<th>CIP (n=12)</th>
<th>p</th>
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<td>Median Age – yr</td>
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<td>71 (65-77)</td>
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<tr>
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<tr>
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<td>Never</td>
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<tr>
<td>Tumor histology - no. (%)</td>
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<tr>
<td>Combination ICI</td>
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<tr>
<td>Pulmonary embolism</td>
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a Basal cell carcinoma (skin) b Durvalumab. NSCLC: Non-small cell lung cancer; ICI: Immune checkpoint inhibitor; COPD: Chronic Obstructive Pulmonary Disease; ILD: Interstitial Lung Disease

Figure Legends

Figure 1: Radiographic presentation of CIP. Representative computed tomography images of an immune checkpoint inhibitor (ICI)-treated non-small cell lung cancer (NSCLC) patient a) prior to development of checkpoint inhibitor pneumonitis (CIP), b) at time of CIP diagnosis and c) after
3 weeks of steroid treatment. * denotes area of pre-existing post-XRT changes that were stable prior to initiation of ICI

Figure 2: Study design and participating patients. Consort diagram showing study enrollment and adjudication of patients into control and CIP groups

Figure 3: BAL lymphocytosis in CIP patients. Scatter plots showing number of A) CD4+, B) CD8+, C) CD14+ and D) CD16+ T-cells (A-B) and monocytes (C-D) respectively in control and CIP samples. N=6 (CIP-), 12 (CIP+). Comparisons between groups performed using Mann-Whitney test.

Figure 4: T-cell populations in CIP. Unsupervised clustering of T cells in BALF samples of patients without (Control, n=6) and with CIP (n=12). Cluster maps showing distribution of T cell subpopulations in A) unstimulated controls, and B) unstimulated CIP. Within each cluster map, larger cell populations distinct to that particular condition are highlighted (square boxes).

Figure 5: Abnormal T-cell subsets in CIP. Differential cluster map (center) shows clusters where the number of cells within the cluster were increased by 95% in controls (red, n=6) or CIP (cyan, n=12). Cytokine profile (inset) and scatter plot of relevant cytokines showing MFI in the selected clusters (red) compared to MFI across all clusters (black) in (counterclockwise): 1) CD4+ FoxP3lo CD25- CD62Lhi CD45RAlo cluster increased in CIP 2) PD-1hi CTLA-4hi clusters of Tregs increased in controls; scatter plot showing PD-1/CTLA-4 MFI in selected clusters 3) Similar (i.e. < 95% difference) expression of PD-1lo CTLA-4lo Treg clusters in CIP and controls; scatter plot showing PD-1/CTLA-4 MFI in selected clusters 4) A CD3+ CD4lo CD8- TNFαhi population increased in CIP; scatter plot showing CD4/TNF-alpha MFI in selected clusters 5) CD8+ TNF-αhi PD-1hi clusters increased in CIP; scatter plot showing CD8/TNFα MFI in selected clusters 6) A second set of CD8+ TNFαhi clusters increased in CIP

Figure 6: Monocyte populations in CIP. Unsupervised clustering of non-T cells (singlet, live, CD3-) in BALF samples of patients without (Control, n=6) and with CIP(n=12). Cluster maps showing distribution of myeloid subpopulations in A) unstimulated controls and  B) unstimulated CIP.

Figure 7: Abnormal monocyte subsets in CIP. Differential cluster map of myeloid cells showing clusters that are increased by at least 95% between unstimulated controls(n=6) and unstimulated CIP (n=12) samples. Cytokine profiles and scatter plot of relevant cytokines showing MFI in the selected clusters (red) compared to MFI across all clusters (black) showing (counterclockwise): 1) Population of IL-1RAhi B cells (CD19+) increased in controls and 2) Large population of related clusters of IL-10hi IL-1βhi myeloid cells (CD14lo CD16lo CD19+) increased in CIP.

Figure 8: BALF cytokine analysis. A) Heatmap showing expression of various cytokines in control and CIP BAL supernatant samples. Cytokines are scaled, centered and hierarchically clustered. B) Box-and-whisker plots showing median, min and max with individual data point overlay (dots) for select cytokines involved in alveolar inflammation and immune cell skewing (B) or inflammatory cell recruitment/chemotaxis (C). * denotes significant difference from control BALF samples (Mann-Whitney, p < 0.05).

Figure 9: Summary of dysregulated immune cell phenotypes in CIP
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ICI: immune checkpoint inhibitor; VATS: Video-assisted thoracic surgery; CIP: Checkpoint inhibitor pneumonitis

* Pertinent clinical, radiographic, laboratory and microbiologic (including BAL culture when available) data were reviewed by the immune related toxicity (irTox) team before and (in cases of suspected CIP) after bronchoscopy. At both time points, patients with suspected CIP with an alternative etiology for symptoms were excluded from the CIP group (n=2).

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Figure 9: Summary of dysregulated immune cell phenotypes in CIP

- **T-cells**
  - Increased Tcm (expressing CCR6, CD4, CD8, and Th1/Th2 cytokines)
  - Increased PD-1/CTLA-4 expression in Tregs

- **Myeloid Cells**
  - Increased CD11b^hi IL-1β^hi populations
  - Decreased IL-1Ra^hi cell populations
  - Increased in pro-inflammatory cell subsets with increased expression of endothelial adhesion markers
  - Loss of counter-regulatory suppressive cell phenotypes