Immunoprofiling bronchoalveolar lavage cells reveals multifaceted smoking-associated immune dysfunction

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Shareable abstract (@ERSpublications)
This study demonstrates the utility of bronchoalveolar lavage leukocytes as a source of airway biomarkers assayable by flow cytometry analysis that are also responsive to patient smoking status – a major clinical determinant of lung cancer risk https://bit.ly/3M2KIJl

Cite this article as: Smith R Jr, Yendamuri S, Vedire Y, *et al*. Immunoprofiling bronchoalveolar lavage cells reveals multifaceted smoking-associated immune dysfunction. *ERJ Open Res* 2023; 9: 00688-2022 [DOI: 10.1183/23120541.00688-2022].

Received: 8 Dec 2022
Accepted: 22 March 2023

Abstract

Background Bronchoalveolar lavage (BAL) is an underutilised tool in the search for pulmonary disease biomarkers. While leukocytes with effector and suppressor function play important roles in airway immunity and tumours, it remains unclear if frequencies and phenotypes of BAL leukocytes can be useful parameters in lung cancer studies and clinical trials. We therefore explored the utility of BAL leukocytes as a source of biomarkers interrogating the impact of smoking, a major lung cancer risk determinant, on pulmonary immunity.

Methods In this "test case" observational study, BAL samples from 119 donors undergoing lung cancer screening and biopsy procedures were evaluated by conventional and spectral flow cytometry to exemplify the comprehensive immune analyses possible with this biospecimen. Proportions of major leukocyte populations and phenotypic markers levels were found. Multivariate linear rank sum analysis considering age, sex, cancer diagnosis and smoking status was performed.

Results Significantly increased frequencies of myeloid-derived suppressor cells and PD-L1-expressing macrophages were found in current and former smokers compared to never-smokers. While cytotoxic CD8 T-cells and conventional CD4 helper T-cell frequencies were significantly reduced in current and former smokers, expression of immune checkpoints PD-1 and LAG-3 as well as Tregs proportions were increased. Lastly, the cellularity, viability and stability of several immune readouts under cryostorage suggested BAL samples are useful for correlative end-points in clinical trials.

Conclusions Smoking is associated with heightened markers of immune dysfunction, readily assayable in BAL, that may reflect a permissive environment for cancer development and progression in the airway.

Introduction

Carcinogens found in cigarette smoke induce oncogenic mutations responsible for most lung cancers [1, 2]. Smoking affects the immune landscape of the lung and is generally associated with chronic airway inflammation [3–6]. This in turn can lead to tissue damage and immune dysfunction resulting from T-cell exhaustion and compensatory engagement of immune suppression mechanisms [7] as seen in COPD [8], sarcoidosis [9] and COVID-19 [10]. Smoking's detrimental effects on the immune defences are also implicated in the development and progression of diverse malignancies including lung cancers [11–16].

In the airway, as in other tissues, specialised leukocyte populations including regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs) are a key mechanism protecting healthy tissue from

autoimmunity and collateral inflammatory damage. Tregs expressing the transcription factor FOXP3 characteristically suppress the activity of cytotoxic CD8 T-cells and non-regulatory (or conventional) CD4 helper cells (Tcon) through several mechanisms [17–19]. MDSCs similarly exert suppressive function through inhibitory receptor signalling, suppressive cytokines (transforming growth factor-β, vascular endothelial growth factor-A, interleukin-10), nitric oxide production, and metabolite depletion *via* arginase (ARG1) and indoleamine 2,3-dioxygenase (IDO) [20, 21]. Immune control in the airway can also be enforced through broad upregulation of checkpoint receptors (*e.g.*, PD-1 and LAG3) as seen in many inflammatory and infectious diseases and malignancies [22–27]. While the role of these suppressive mechanisms in limiting tumour eradication is well appreciated, the factors influencing their presence in the airway and their relationship to meaningful clinical variables remain incompletely defined.

Bronchoalveolar lavage (BAL), which was introduced in the 1960s, can provide a unique snapshot of the cellular and biological processes in the airway, more accurate than blood-based approaches [28–31] and more robust than low-cellular-yield sputum assessments, without resorting to invasive biopsies [32, 33]. Additionally, as cell suspensions, BAL samples readily lend themselves to flow cytometric analysis with minimal processing. Despite this, BAL remains an underutilised tool in the study of airway immunity in clinically relevant patient populations. While studies of smoking's impact on the airway abound [4, 34–37], few have explored effects on the immune landscape using BAL.

In the present study, we used standard and spectral flow cytometry to gain biological insights into the effect of smoking on airway leukocyte populations using patient BAL-derived leukocytes. Elevated suppressor cell frequencies and activation markers were seen in smokers as were significant reductions in T-cell populations. Upregulated PD-1 and LAG3 were also seen across T-cell subsets in smokers. These findings associate smoking with dysfunctional airway immunity, which may provide a niche conducive for developing and progressing tumours. These findings also demonstrate the utility of BAL cells as a source of immune biomarkers relevant to lung cancer that are reliably assayable in cryopreserved samples and thus a useful tool in conducting multicentre clinical trials.

Materials and methods

BAL samples

BAL samples were collected with written consent from 119 patients undergoing bronchoscopy for lung cancer screening and biopsy procedures at Roswell Park Comprehensive Cancer Center from August 2020 to January 2021 under institutional review board-approved protocols. Relevant patient data (summarised in table 1) were managed by an honest broker. COPD diagnosis was established according to the definition supplied by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [38]. BAL was obtained from non-tumour-bearing middle lobes or the lingular bronchus (to avoid middle lobe tumour). Cellular fractions from ∼30 mL/patient were washed, pelleted (350 *g*, 5 min) and resuspended in either staining buffer (2% fetal bovine serum (FBS) and 2 mM EDTA in saline) for immunostaining or cryogenic media (90% FBS and 10% DMSO) for storage at −80°C.

Flow cytometry

3–10×10^6 BAL-recovered cells were resuspended in staining buffer (1×10^6 cells/20 µL) containing antibodies for T-cell (supplementary table S1A), MDSC (supplementary table S1B) or myeloid cell surface markers (supplementary table S1C) after the labelling of non-viable cells with LD-Aqua (Molecular Probes, Eugene, OR, USA). After surface marker staining, cells were fixed/permeabilised using a FOXP3/transcription factor staining kit (eBiosciences) as per manufacturer's protocol for intracellular staining. Data (1–3×10^6 events) were collected on an LSR II cytometer using FACS-DIVA software (BD

TABLE 1 Patient numbers and pertinent clinical data for never-, former and current smokers

	Never	Former	Current
Age years, mean±SD	59±11	68±6	65±10
Female sex n	22	24	22
Male sex n	14	26	11
Cancer at the time of sampling, n/N (%)	19/36 (53)	36/50 (72)	22/33 (66)
Pack-years (range)	NA	41.2 (1–100)	72 (12–140)
COPD/emphysema %	6.2	40	50

NA: not applicable.

Biosciences, Franklin Lakes, NJ, USA). Results were analysed using Flowjo (V10.8) software, and gating was performed as described in supplementary figures S1–S3 to find population frequencies and mean fluorescent intensity (MFI) of phenotypic markers.

For spectral flow cytometry, BAL cells from a subset of never-smokers and current smokers (n=6 each) without cancer or COVID-19 diagnoses were stained as above with a more extensive antibody panel (supplementary table S2). Data collected using a 5-laser Aurora (Cytek, Fremont, CA, USA) instrument was analysed as described elsewhere [39, 40]. Briefly, after gating as shown in supplementary figures S1–S3, samples were equally concatenated (500K events/sample), and tSNE (t-distributed stochastic neighbor embedding) plots were generated using a single file down-sampled to 120K events with 4000 iterations and a perplexity of 40 and fast Fourier transform-accelerated interpolation-based t-SNE (FIt-SNE) interpolation (Flt-SNE) [41]. Population-defining gates [39] were manually applied to determine leukocyte subset frequencies in each group. Phenotypic marker MFIs in each population were expressed by heat map.

Analysis and statistics

Population frequencies and phenotypic marker MFIs were analysed in Graphpad PRISM (V8). Multivariate linear regression analysis was performed in R studio (v1.2). The chi-square test of independence was used to determine significant relationships between age, sex, active cancer in the lungs, COPD and smoking status in R version 1.4.1. This analysis revealed a statistically significant relationship between COPD/emphysema status and smoking status, and this information was used to conduct a multivariate linear rank order regression analysis, as a means of assessing interactions of smoking status, sex, age and cancer diagnosis. Multivariate linear rank sum analysis considering age, sex, cancer diagnosis and smoking status was performed with *post hoc* (Tukey's) assessment. Statistically significant differences were found using one-way ANOVA and multiple comparisons test (Tukey's test with $p<0.05$ being significant). Error bars depict SEM.

Results

BAL-derived cells from 119 patients undergoing lung cancer screening or biopsy procedures were assessed by flow cytometry. Patient demographic and medical history information (see table 1) revealed that 33 (27.7%) were current smokers, 50 (42.0%) self-identified as former smokers and 36 (30.3%) were nonsmokers. COPD diagnosis was present in 50% of current, 40% of former and 6.3% of never-smokers. Cancer was diagnosed in 53% of never-, 72% of former and 66% of current smokers. Chi-squared analysis revealed a statistically significant relationship between COPD/emphysema status and smoking status, and therefore this variable was excluded in further analysis. Multivariate linear rank order regression analysis assessing interactions of smoking status, sex, age and cancer diagnosis at the time of sampling revealed limited interactions among variables that could not be excluded. Ultimately, linear rank sum analysis considering age, sex, cancer diagnosis and smoking status was performed with *post hoc* (Tukey's) assessment.

Cryopreservation of BAL leukocytes did not significantly alter their properties as fresh and frozen/thawed cells of the same sample displayed similar viability, frequencies of bulk leukocytes, select lymphocyte populations ($CD4^+$, Tregs), and both the frequency of $PD\text{-}1^+$ cells and the MFI of PD-1 among Tregs (supplementary figure S4A–F). BAL cellularity varied considerably among patients (figure 1a), and flow cytometric analysis revealed a mean (±SEM) viability of 84±14% for leukocytes ($CD45^+$ cells) which, on average, comprised 38±28.4% of total BAL cells (figure 1b–c). No significant differences were observed in total leukocytes between current, former and never-smokers (figure 1c). However, the cells comprising the $CD45^+$ pool differed markedly by smoking status. $CD4^+$ and $CD8^+$ T-cell frequencies were drastically reduced in current smokers compared with never-smokers. Current smokers displayed lower $CD4^+$ T-cell proportions ($p<0.0001$) relative to never-smokers, and a marginal decrease was seen between former and never-smokers (figure 1d). Current smokers also harboured fewer CD8 T-cells compared to never-smokers ($p=0.0008$) (figure 1d). The viability of CD4 and CD8 T-cell populations was comparable between groups (data not shown).

$CD4^+$ T-cells include both suppressive (Tregs) and effector (Tcon) subpopulations. Within the $CD4^+$ pool, Tcon were significantly reduced in both current and former compared to never-smokers (figure 2a). Tcon with a surface marker profile indicating an activated, effector phenotype or "effector Tcon" ($CD44^+$/$CD45RA^-$) were significantly enhanced in never-smoker BAL compared to both former ($p=0.0199$) and current smokers ($p=0.0285$) (figure 2b). Though no difference was observed in total $CD4^+$ and Tcon viability (data not shown), that of effector Tcon was reduced in current and former smokers (figure 2c).

Immune checkpoint factor expression by T-cells is linked to immune suppression and dysfunction associated with exhaustion or anergy [42, 43]. Never-smokers had significantly lower frequencies of

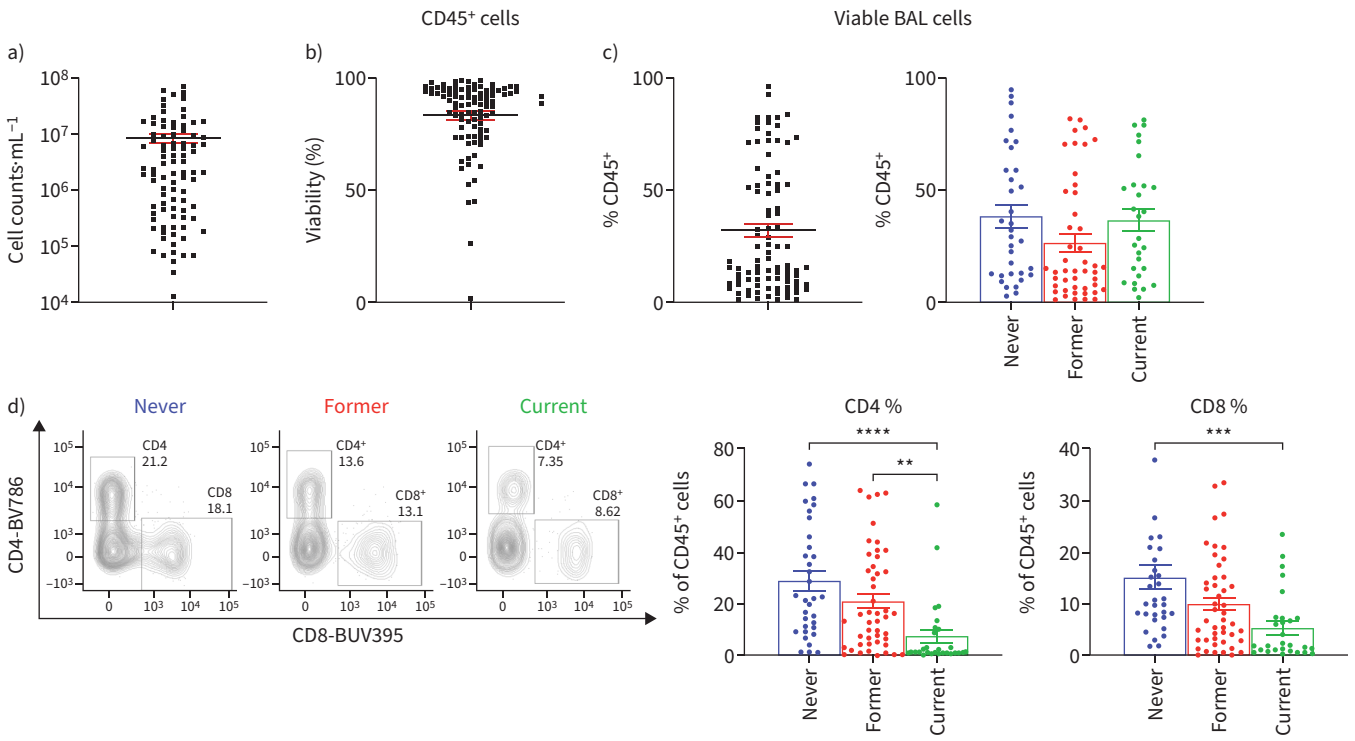


FIGURE 1 Characterizing bronchoalveolar lavage (BAL) cell properties and composition. The spectrum of BAL cellularity and the viability of CD45$^+$ leukocytes across all collected samples were determined based on a) cell counts·mL^{-1} BAL fluid and b) by flow cytometric analysis of Live/Dead (LD)-Aqua staining. c) The proportions of immune cells in general (left) and in the BAL (right) from current (green), former (red) and never-smokers (blue) were determined by flow-based detection of surface CD45. d) The frequencies of CD4$^+$ and CD8$^+$ lymphocytes within the viable CD45$^+$ cell population (total leukocytes) were similarly found by flow cytometry. Mean±SEM values for all samples are shown for a–d, and representative contour plots of CD45$^+$ cells are shown for d. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 using one-way ANOVA and *post hoc* Tukey's multiple comparisons test.

LAG3$^+$ effector Tcon than both former and current smokers (p<0.0001) (figure 2d). BAL samples of never-smokers contained fewer PD-1$^+$ effector Tcon than that of both former and current smokers (p<0.0001, both) (figure 2e). PD-1 expression levels (MFI) were also significantly lower in never- and former than current smokers (figure 2e).

Tregs are important mediators of both immune homeostasis and tumour-associated immune suppression. Yet the impact of smoking on these cells remains unclear [17, 44, 45]. While some prior studies have assessed BAL Tregs in diseases such as sarcoidosis and pulmonary fibrosis, and another explored the impact of smoking on BAL Tregs in the context of COPD, data on BAL Tregs and smoking are relatively scant [46–49]. Like Tcon, the Tregs of current smokers comprised a smaller fraction of CD45$^+$ cells compared to both former (p=0.004) and never-smokers (p=0.002) (figure 3a), and no marked difference in Treg:CD8 was seen between the groups (supplementary figure S5A). However, Tregs were enriched in CD4$^+$ pools of current *versus* never-smokers (p=0.008) and modestly relative to former smokers (figure 3b). Increased Treg viability was also linked to smoking, as significant enhancements were seen between current and former *versus* never-smokers (p<0.0001, both) (figure 3c). Smoking history was also associated with elevated expression of LAG3 and PD-1 by Tregs. Specifically, PD-1 MFI on Tregs (figure 3d) and the frequencies of LAG3$^+$ Tregs (figure 3e) were markedly elevated for current smokers compared to both never-smokers and current smokers.

Activated effector-like Tregs (eTregs) [50, 51] are a subpopulation with high suppressive potency that accumulate in peripheral tissues, including tumours, expressing high levels of checkpoint factors such as PD-1 and LAG3 [52–54]. As with other T-cells, eTregs were reduced as a fraction of the total BAL leukocytes in current compared to both former (p=0.002) and never-smokers (p<0.0001) (figure 3f), and moderately lower eTreg:CD8 was noted in current smokers as well (supplementary figure S5B). However, as with bulk Tregs, eTreg frequencies within the CD4$^+$ compartment were higher (but not significantly

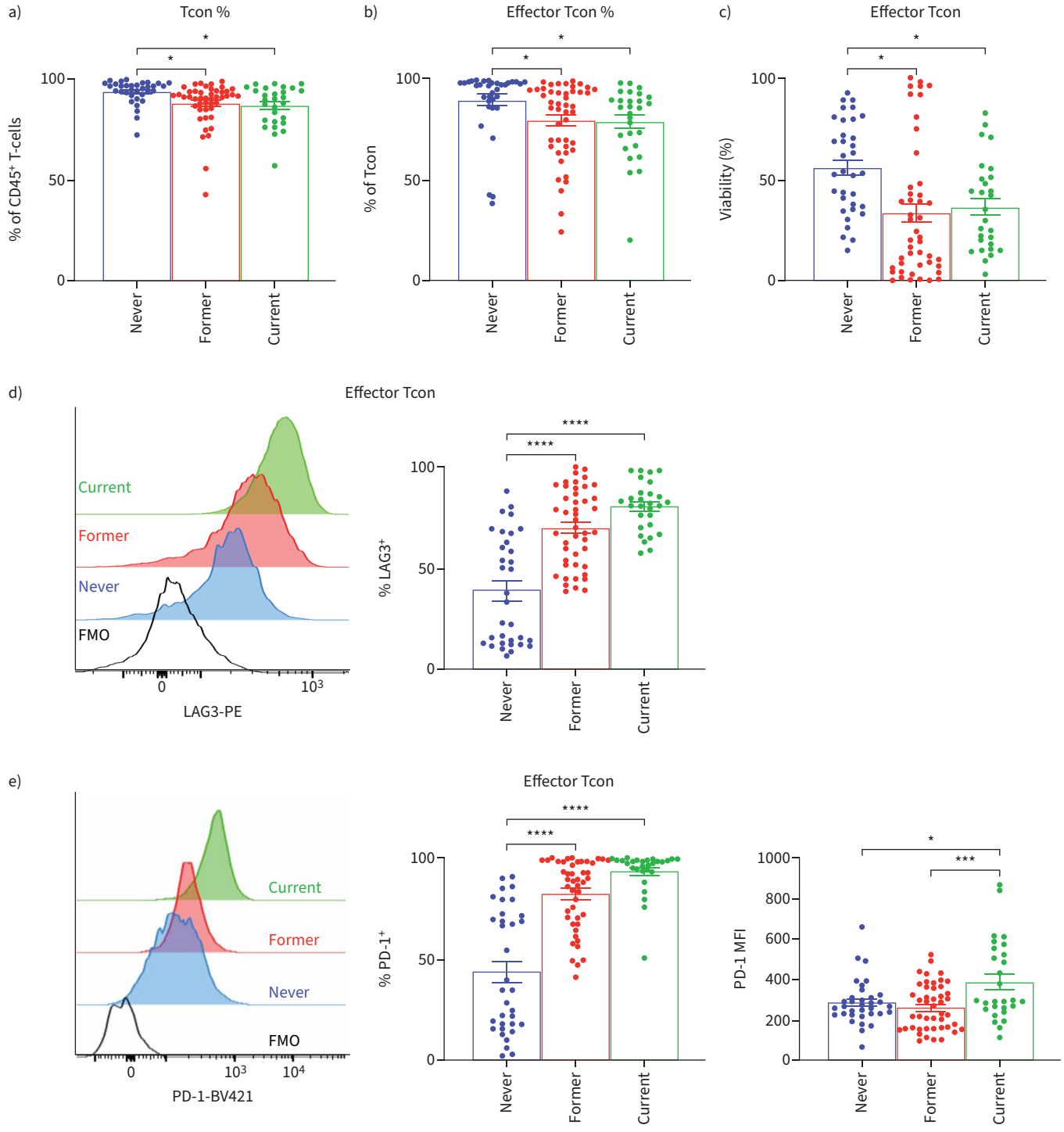


FIGURE 2 Indications of T helper dysfunction in the bronchoalveolar lavage (BAL) of smokers. a) The fraction of the $CD4^+$ T-cell pool displaying a conventional (*i.e.*, non-regulatory) phenotype ($CD45^+/CD4^+/Foxp3^-/CD25^-$; "Tcon") in the never-smoker (blue), former smoker (red) and current smoker (green) BAL was found by flow cytometry as was b) the percentage of these cells bearing an activated effector (e)Tcon cell surface marker profile ($CD44^+/CD45RA^-$), and c) the viability of these eTcon cells was found as in figure 1. d) The frequency of LAG3 and e) PD-1 positive eTcons (left) as well as the expression level of PD-1 (expressed by mean fluorescence intensity (MFI)) (right) were also found by flow cytometry across patients with distinct smoking histories. Values are shown as mean±SEM for all samples analysed per group and representative histogram overlays (panels d and e). *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 using one-way ANOVA and *post hoc* Tukey's multiple comparisons test. FMO: fluorescence minus one.

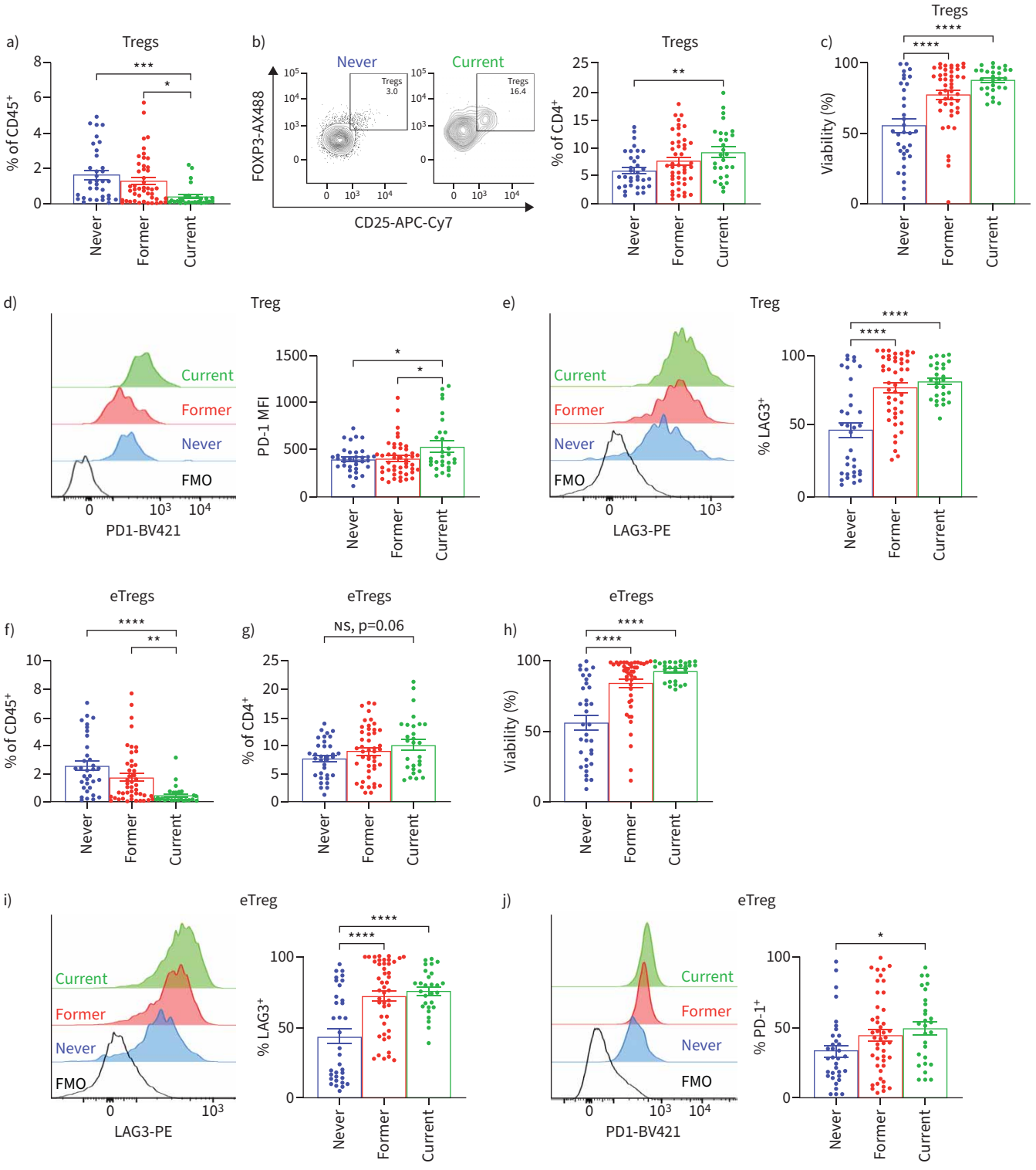


FIGURE 3 Regulatory T-cell frequencies and phenotypes in current, former and never-smoker bronchoalveolar lavage (BAL). a) Proportions of regulatory T-cells (Tregs) ($CD4^+/CD127^-/FOXP3^+/CD25^+$) among $CD45^+$ BAL cells and b) their frequency within the $CD4^+$ T-cell pool were determined for patients of different smoking status by flow cytometry. c) Treg viability, d) the levels of PD-1 expression by Tregs and e) the percentage of Tregs expressing LAG3 were similarly found. f) Effector-like "eTregs" (identifiable as $CD4^+/CD127^-/FOXP3^+/CD45RA^-$) frequencies within the total BAL leukocyte pool, g) proportions of these cells as a fraction of all $CD4^+$ T-cells, h) their viability, and i and j) the frequencies of $LAG3^+$ and $PD\text{-}1^+$ cells in this subpopulation were determined in the BAL of current (green), former (red), never- (blue) smokers (FMO (fluorescence minus one) controls are represented by black histograms). Panels a–j depict the mean±SEM percentage or MFI value. Panels d, e, i and j include representative histogram overlays. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 using one-way ANOVA and *post hoc* Tukey's multiple comparisons test. FMO: fluorescence minus one.

so) in current than never-smokers (p=0.06) (figure 3g). Further, both eTreg viability (figure 3h) and the LAG3 and PD-1 expression observed in these cells were similarly augmented with smoking (figure 3i,j). Thus, smoking was linked to a higher relative presence of Tregs and immune suppression in the airway.

While proportions of effector CD4 Tcon and bulk CD8 T-cells were lower in the BAL leukocytes of smokers, effector frequencies within the CD8 compartment were unchanged by active smoking status (figure 4a). However, as a fraction of $CD45^+$ leukocytes, current smokers had significantly less effector CD8 T-cells than never-smokers (figure 4b). As in other T-cell types, PD-1 expression among these cells was more prevalent in current than never- and former smokers, with that in former smokers being still markedly higher than in never-smokers (p<0.0001) (figure 4c). These results may indicate prevalent exhaustion in the airway CD8 T-cells of smokers.

While smoking negatively impacts the T-cell presence in the airway, we observed a reciprocal accumulation of myeloid cells with suppressive potential including MDSC of the monocytic $CD14^+$ (M-MDSC) and granulocytic $CD15^+/CD66b^+$ (PMN-MDSC) subsets. In current smokers, M-MDSC

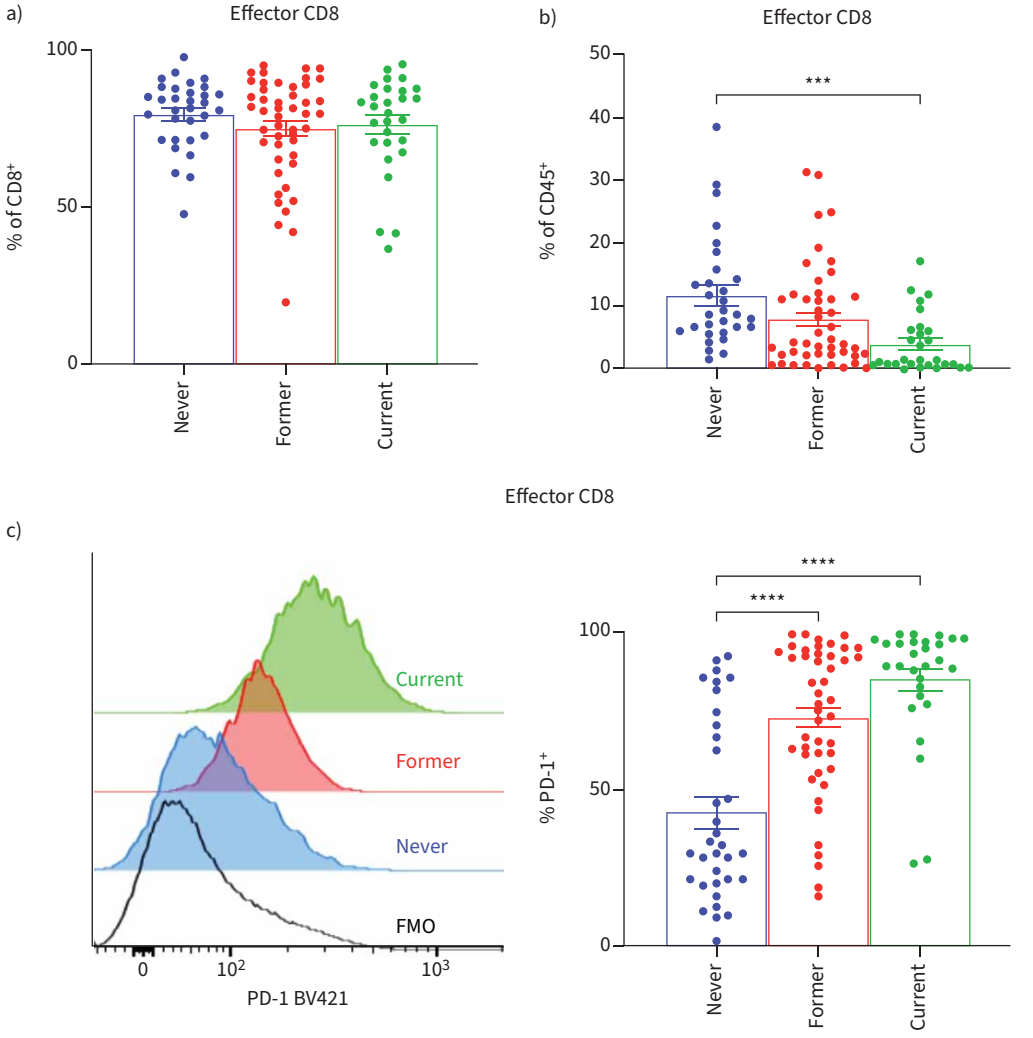


FIGURE 4 Smoking-associated effects on markers of CD8 T-cell dysfunction in bronchoalveolar lavage (BAL). a) The fraction of the $CD8^+$ T-cell pool and b) total BAL leukocyte ($CD45^+$) population displaying an activated, effector phenotype ($CD4^-/CD8^+/CD44^+$ $CD45RA^-$) were found by flow cytometry, as were c) the percentage of these cells expressing PD-1, for current (green), former (red), never- (blue) smokers. Values are shown as mean ±SEM. Panel c (left) is a representative histogram (FMO control in black). ***p<0.001; ****p<0.0001 using one-way ANOVA and *post hoc* Tukey's multiple comparisons test. FMO: fluorescence minus one.

represented a significantly larger proportion of all BAL cells than in both former and never-smokers ($p<0.0001$, both) (figure 5a). While expression of the suppressive mediator ARG1 was significantly higher on the M-MDSCs of only former compared to never- ($p=0.012$) and current smokers ($p=0.006$) (figure 5b), PD-L1 (another well-known mediator of immune suppression) was significantly upregulated in the M-MDSCs of current compared to both former ($p=0.02$) and never-smokers ($p=0.004$) (figure 5c). PMN-MDSCs followed a similar trend as M-MDSCs, except these cells were also elevated in former compared to never-smokers ($p=0.048$) (figure 5d) and ARG1 MFIs were generally more enhanced by smoking (figure 5e). Additionally, the relationship between PD-L1 expression and smoking mirrored that seen in M-MDSCs (figure 5f). Collectively, these findings suggest that smoking enhances MDSC presence and potentially their function in the airway.

In addition to MDSCs, macrophages infiltrate the lungs of smokers [32, 36, 55]. Aside from alveolar macrophages, the cells can be generalised into traditionally activated (M1) or alternatively activated (M2) macrophages and are loosely defined as $CD68^+$ and $CD23^+$ $CD163^-$ or $CD23^{+/-}$ $CD163^+$, respectively [56, 57]. As expected, M1s were more frequent among the current smokers than both former ($p=0.002$) and never-smokers ($p<0.0001$). Former smoker BAL samples contained higher M1 numbers than never-smokers ($p=0.036$) (figure 6a). Interestingly, M1 cells of current smokers upregulated PD-L1 (29.5±6.4%) beyond both former (8±2.3%; $p<0.0001$) and never-smokers (7.9±0.95%; $p<0.0001$) (figure 6b), suggesting the potential subversion of a proinflammatory cell type in the airways of smokers. Like their M1 counterparts, M2 cells were significantly increased in current ($p<0.0001$) and former smoker BAL samples ($p=0.002$) compared to never-smokers (figure 6c). As in other myeloid cells, M2s expressed significantly more PD-L1 in current than both former ($p=0.017$) and never-smokers ($p=0.006$) (figure 6d). Despite important roles for PD-L1 on immune cells in oesophageal [58] and lung cancers [59–61], such an association between macrophage PD-L1 levels and smoking status, independent of cancer diagnosis, has yet to be reported. Antigen-presenting dendritic cells (DCs) are crucial for priming T-cell responses and effective anti-tumour immune surveillance. BAL DCs were enriched in the lungs of current and former smokers ($p=0.003$) compared to never-smokers ($p=0.002$) (figure 6e). However, their viability was substantially compromised in both smoking groups compared to never-smokers ($p<0.0001$) (figure 6e).

To validate and expand upon these findings, BAL from a subset of never- and current smokers (n=6 each) were stained with an expansive (46 parameters) immunophenotyping antibody panel and subjected to spectral flow cytometry and high dimensional analysis [39, 40]. tSNE plots were constructed using collective data (figure 7a) and samples grouped according to smoking status (figure 7b). Overlaying grouped data highlighted differences in cell population frequencies of $CD45^+$ leukocytes between the groups (figure 7b) that were quantified and depicted in figure 7c. Validating our earlier results, bulk $CD4^+$ Tcon and CD8 frequencies were significantly lower in current compared to never-smokers while Treg proportions were elevated. Myeloid populations also mirrored the results described above. Alveolar macrophages (distinguishable here by CD169 expression) and M1s were more frequent in the airways of current smokers. However, M2s were reduced, suggesting major recruitment or expansion of non-resident, inflammation-inducing macrophages in the airway of active smokers. Smoking also enriched potential MDSC subtypes further suggesting an enhancement in this suppressive cell type in current *versus* never-smokers (figure 7c).

We further revealed elevated frequencies of B-cells, plasma cells, natural killer (NK) cells and both plasmacytoid DCs and circulating DCs in current smokers. Conversely, these patients displayed lower frequencies of NK T-cells ($CD3^+$ $CD56^+$), monocytes, endothelial progenitor cells, neutrophils and eosinophils (figure 7c). Few differences were observed in basophils. In addition to PD-1, LAG3, ARG1 and PD-L1, we evaluated a number of additional functional markers associated with immune activation (Ki67, pSTAT5, pMTOR, STUB1, *etc.*) and suppressor cell function (NRP1, HELIOS, NRN1, pSMAD2/3, PD-L2, ICOS, iNOS, IDO, *etc.*) as depicted in supplementary figure S6 and quantified in figure 7d. The resultant heatmap demonstrated the considerable effects of smoking on these markers over an array of cells. As in conventional flow analysis, LAG3, PD-1, PD-L1 and HLA-DR were significantly elevated in current smokers on T-cells and various myeloid cells (figure 7d). These findings illustrate the considerable depth of immune biomarker analysis possible using BAL and high-parameter flow.

Discussion

For decades, BAL has been a tool available for evaluating the lung immune milieu and the leukocytes residing within the airway [62–64]. While used in recent studies of interstitial lung diseases [29, 30, 65] and allergies [66] (most evaluating the effects of COPD) [44, 49, 55, 67–72], the potential value of using BAL-derived biomarkers to advance the study, prevention and treatment of lung cancer has gone largely overlooked. Presently, we explored BAL as a source of immunological biomarkers responsive to clinical

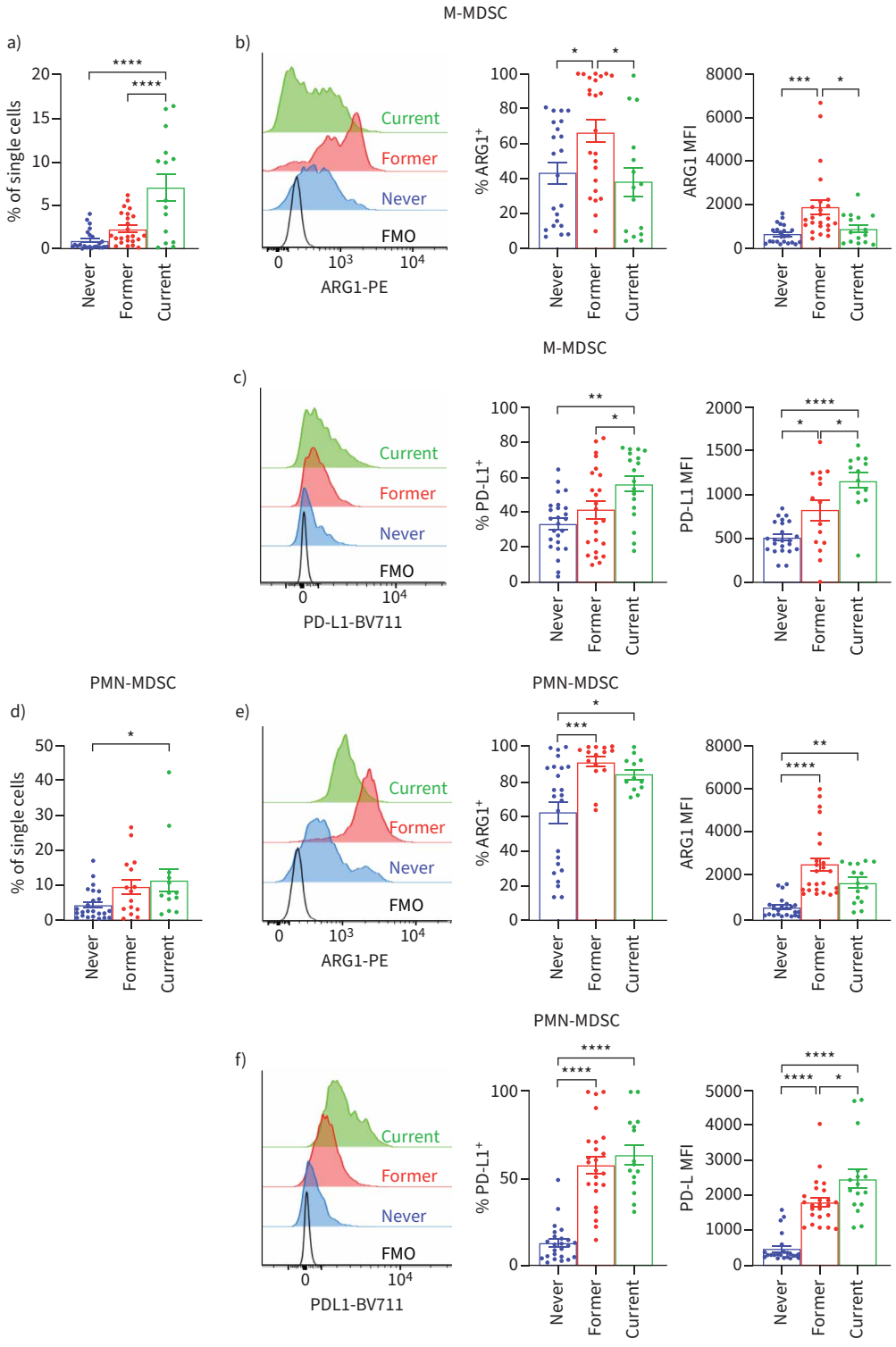


FIGURE 5 The impact of smoking status on myeloid-derived suppressor cell (MDSC) (lineage marker-/HLA-DR-/CD11b$^+$/CD33$^+$) frequencies and function in bronchoalveolar lavage (BAL). a) The frequency of distinct MDSC subsets among the total BAL leukocyte pool, as well as the fraction of cells expressing b) Arg1 and c) PD-L1, and their levels in the CD14$^+$ M-MDSC (a–c) and the CD15$^+$/CD66b$^+$ PMN-MDSC (d–f) populations were found by flow cytometry. Values are shown as mean±SEM. Panels on left-hand side are representative histogram overlays depicting current (green), former (red), never- (blue) smoker BAL samples with FMO (fluorescence minus one) controls shown in black. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 using one-way ANOVA and *post hoc* Tukey's multiple comparisons test. FMO: fluorescence minus one.

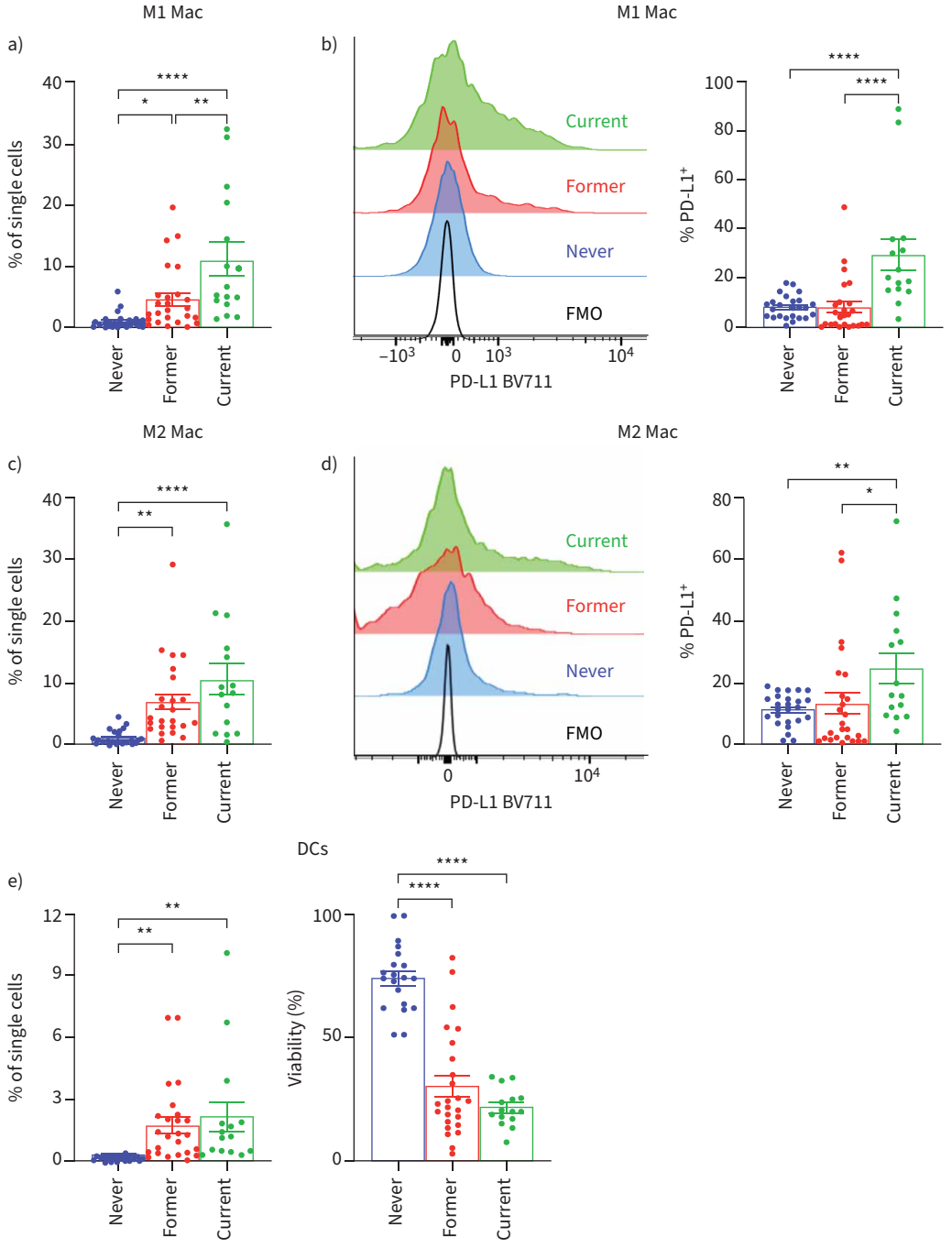
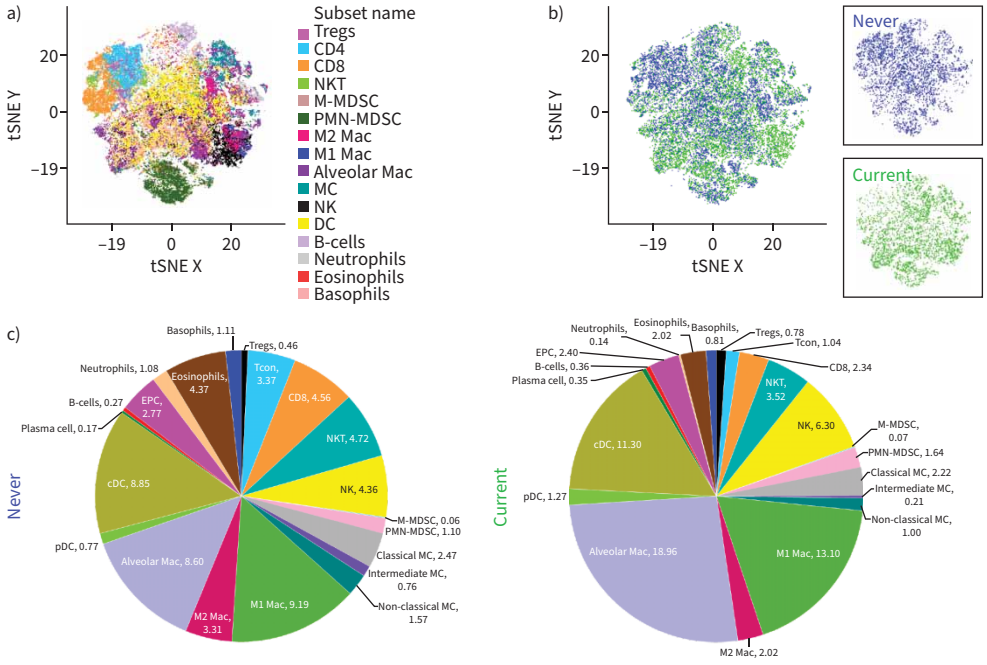


FIGURE 6 Smoking-associated effects on macrophage and dendritic cell (DC) populations. a) The frequency of M1 macrophages (defined as $CD68^+$ and $CD23^+$ $CD163^-$) as well as b) the percentages of PD-L1-expressing M1 macrophages in the bronchoalveolar lavage (BAL) of current (green), former (red), never- (blue) smokers were found by flow cytometry. c) The proportions of M2 macrophages ($CD23^{+/-}$ $CD163^+$) in the BAL were similarly found, as were d) the frequencies of M2s expressing PD-L1. e) DC presence in BAL samples was also determined by quantifying the frequencies of ($CD11c^+$) cells and their viability. Depicted are mean±SEM age and MFIs and representative histogram overlays (FMO (fluorescence minus one) control shown in black). *p<0.05; **p<0.01; ****p<0.0001 using one-way ANOVA and *post hoc* Tukey's multiple comparisons test. FMO: fluorescence minus one.



d)

Marker	CD4		Tcon		Effector Tcon		Treg		Effector Tregs		CD8		Effector CD8		NKT		NK		B-cells	
	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C
KI67	4338	11467	3075	3278	3525	2654	10313	20401	12485	22287	4588	10868	4698	10280	4173	7663	5147	476	4385	14766
LAG3	2208	2670	1409	2293	1896	2652	2051	2661	2224	2667	290	1415	1032	1439	2782	2449	824	1177	10316	14633
PD1	501	1147	490	1345	509	1721	571	971	560	958	544	1742	514	1584	547	1727	782	1039	112	150
PDL1	2615	2994	371	906	603	2129	2125	3841	2256	4232	4577	6255	5763	7691	2935	3624	540	590	5335	5785
PDL2	8836	6539	1894	3874	0	1131	418	3867	383	3823	0	1553	3786	2486	6886	5294	1391	1210	63506	44875
ICOS	6500	13964	4227	4792	3972	3983	19495	23508	209998	25618	6388	12294	6537	11541	7214	10265	8450	9373	15695	27720
IRF4	12630	21206	11050	11392	11641	11187	20632	31331	21996	33331	8566	17328	7491	15176	11018	14874	10489	5742	8342	23733
iNOS	4287	8266	3709	3900	5901	4853	6997	12953	7682	13737	7288	9779	8554	10148	4352	7337	13033	10480	35904	35544
IDO	6854	11276	6334	6533	1244	2257	10865	18189	11657	18198	5329	9385	3408	7091	0	2216	1156	582	18059	23141
ARG1	5634	5134	5664	4859	5752	4785	5396	5275	4823	4878	3235	3056	2121	2004	11819	9644	2191	1927	6231	3764
HLA-DR	3424	2725	3440	2334	2302	1370	3924	3236	3331	3429	3816	3056	3502	2738	4790	4382	3789	3754	11735	9445
CD86	463	396	477	502	807	1431	179	153	122	164	180	212	1315	1183	869	1063	288	226	115	183
VEGFR2	1877	4645	1804	3344	3077	5171	1948	5978	1798	5979	3383	9284	4747	11545	3924	9442	28761	15744	1516	14484
pSTAT5	688	1138	287	0	664	288	1054	609	1197	743	814	25.7	821	196	518	154	1883	1179	4787	4541
pSMAD2-3	157	241	134	171	36.5	32.6	309	349	324	359	224	244	88.9	165	107	204	66.4	181	188	153
pMTOR	3359	3357	1318	673	4194	3399	339	590	137	403	177	122	111	164	2305	2086	942	636	78	172
HELIOS	3787	4997	0	0	0	0	3101	4009	3131	3994	3845	4058	4181	4215	2691	639	6467	4987	4102	3557
NRP1	49.3	309	50	181	36.2	47.7	50.5	354	46.1	519	26.5	70.7	19.4	76.9	45.4	62.7	6.54	10.1	0	49.1
NRN1	98115	93411	98477	81351	56728	32581	101265	115785	101836	114025	142346	113379	131788	95083	111890	97911	220070	27426	1260000	1E+06
STUB1	183768	136903	188777	155478	225775	173456	151558	115497	138250	99691	108624	80798	79788	57545	40186	33849	6523	5986	44478	31553

Marker	M-MDSC		PMN-MDSC		Alv Mac		M1 Mac		M2 Mac		C-MC		IM-MC		NC-MC		DC	
	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C	N	C
KI67	9449	10598	8562	14083	9149	16184	7053	12608	4798	1623	6677	8860	10116	16159	15527	21100	6355	9078
LAG3	410	307	494	409	654	453	478	705	921	1051	740	389	462	278	179	279	312	198
PD1	223	342	200	373	305	840	2165	1856	233	750	327	269	322	559	350	495	198	309
PDL1	2779	4476	6347	7348	948	2016	0	5545	0	4370	1385	2050	3473	3797	6723	7614	0	2617
PDL2	4812	3786	4241	2204	3909	3386	3261	2967	1839	1360	5460	4013	5103	5249	1173	141	2051	1278
CD163	13685	14072	8389	14558	16861	25251	12553	10824	10535	7858	13836	14147	22626	24121	22810	24047	8512	12734
IRF4	18542	21498	10778	18191	17620	27546	14909	2643	9456	5950	16578	25299	21623	32867	25790	29479	11083	15761
iNOS	21280	22290	8755	13050	11990	16778	35472	12693	11088	8958	16989	20341	21908	24522	22275	22018	6084	9345
IDO	9182	8758	5633	8244	7914	11549	4788	216	4863	1385	9024	9730	13067	12839	12999	11750	4615	6857
ARG1	3982	6658	2197	2783	3684	3720	2302	1668	1002	970	2674	4224	2297	4061	6842	5690	1252	1445
HLA-DR	0	0	0	0	6762	13203	13177	48735	10871	51954	19206	20285	11265	11588	1240	3638	10107	9003
CD86	2981	3271	771	809	711	403	0	1341	1097	321	1406	1111	1843	1407	676	1538	502	488
VEGFR2	21744	28932	5718	13861	9882	15710	8181	26677	21354	16901	12051	9994	17113	9142	33023	19697	9406	3482
pSTAT5	2492	2240	2329	2047	1727	761	4247	4694	1135	465	1505	1497	2187	1080	1819	587	1136	742
pSMAD2-3	136	23.5	532	279	610	546	1107	663	494	277	211	112	222	149	378	109	463	327
pMTOR	2571	4265	946	879	3213	2340	5611	133	1924	710	3917	5812	3134	3588	4006	2705	1094	1075
HELIOS	0	0	1916	108	5143	2135	12100	3064	5261	2191	0	0	2201	187	1870	251	3093	1847
NRP1	222	74.5	229	141	1527	948	760	27.2	1097	283	439	550	734	753	659	375	623	674
NRN1	198673	154631	86990	99614	94535	64120	99007	42459	94651	29699	161327	177662	168668	173702	151884	119786	56094	77614
STUB1	123565	151155	53645	47351	36115	23553	89753	889	37477	6974	77636	72989	98741	71992	176183	109155	17435	16882

FIGURE 7 Deep immunoprofiling of bronchoalveolar lavage (BAL) leukocytes with spectral flow cytometry reveals extensive smoking-associated immune dysfunction. A subset of BAL samples from never- and current smokers (n=6 each) were stained with a 46-parameter immunophenotyping

antibody panel (see supplementary Table S2) and subjected to spectral flow cytometry and high dimensional analysis. a) tSNE (t-distributed stochastic neighbor embedding) plots (down-sampled to 120K events with 4000 iterations and a perplexity of 40 and FFT interpolation (Flt-SNE)) were constructed using collective data. b) Overlaying grouped data (blue: never-smokers; and green: current smokers) highlighted differences in cell population frequencies of $CD45^+$ leukocytes between the groups, and c) were quantified. d) Functional marker mean fluorescent intensities (MFIs) on major immune cell populations were quantified and visualised as a heatmap with red indicating high expression and blue indicating low expression. Each heatmap table is independent for colour scale per marker. N: never-smoker; C: current smoker.

covariates. Our findings provide important proof-of-concept that BAL leukocyte readouts can be related to patient smoking history – a major lung cancer risk determinant.

The disposition of the lung immune system can help or hinder lung cancer development [28, 60, 73–75]. As such, the impact of smoking on systemic and airway immune landscapes has been studied previously through collection of sputum [32, 76–78], peripheral blood [79–81] and tissue biopsies [76]. These studies generally link smoking to extensive immune dysfunction, airway inflammation and tissue damage. However, few utilise BAL and many predate recent advances that shape our grasp of the functionally diverse leukocyte subpopulations found in normal and diseased airways [28, 30, 79, 82, 83].

Our characterisation of BAL leukocytes indicated no significant difference in overall leukocyte frequencies in current, former and never-smokers; however, the composition of this population shifts dramatically with smoking. We observed reduced lymphocytes in current and former smokers accompanied by increased myeloid populations. In agreement with earlier studies [28, 79, 83], we found lower BAL $CD4^+$ T-cell frequencies in active and former smokers. Proportions of $CD8^+$ T-cells were also sharply reduced among BAL leukocytes – an observation contrasting with the increased cytotoxic CD8 T-cell frequencies associated with smoking in a previous study with a limited number of patients [83].

Tregs are key regulators of the immune response that characteristically inhibit leukocyte activation and limit inflammatory damage. However, these cells also permit immune evasion by tumours, and in many cancers (including lung) Treg frequency in peripheral blood and biopsied lesions is negatively related to patient survival [84, 85]. Tregs expressing activation markers and the checkpoint factor PD-1 accumulate in both mouse and human tumours [84, 86]. Interestingly, we show that Treg PD-1 expression is significantly higher in smokers. While the negative association between patient survival and the immune checkpoint factors PD-1/PD-L1 is well known [60, 61, 87, 88], the importance of PD-1 expression on Tregs in determining immunotherapy outcomes in lung and other cancers was just recently brought to light [86, 89–91].

Few prior reports have associated smoking with Treg-mediated immune suppression with differing results [46, 49, 71]. Others have shown Tregs from the blood of COPD patients to be enhanced in frequency and *in vitro* suppressive capacity [44]. One study demonstrated an imbalance of BAL Treg subpopulations with smokers having a higher frequency of activated $CD45RA^-$ "eTregs" compared to both nonsmokers and COPD patients [49]. In our study smoking was positively correlated with COPD diagnosis, which was present in 40–50% of former and current smokers and as such did not differentiate these patients. Despite including COPD patients, we also show that Tregs constitute a larger fraction of smoker airway $CD4^+$ compartment. Furthermore, we demonstrate that the Tregs of smokers express significantly more PD-1, LAG3, Ki67, PD-L1 and ICOS than in nonsmokers. These findings link smoking to an activated Treg phenotype associated with accumulation and suppression in tumours. This is aligned with the decreased frequencies, elevated PD-1 and LAG3, and reduced Ki67 expression on effector CD4 and CD8 T-cells in smoker BAL suggesting exhaustion and suppression among the cells responsible for affecting surveillance and elimination of malignant threats. Additional studies that include *ex vivo* assays of Treg function are needed, however, to definitively establish the effects of smoking on the suppressive potency of airway Tregs.

Accumulation of MDSCs, another important mediator of immune suppression, within the lung is associated with tumour development in mice [73, 92], and these cells are elevated in nonsmall cell lung cancer patients and negatively related to survival [93]. Further, smoking is associated with enhanced differentiation of MDSCs to tumour-associated macrophages and tolerogenic DCs, both of which contribute to tumour progression [66]. Yet, MDSC populations in BAL remain relatively unexplored regarding smoking-relevant biomarkers. Similar to prior observations in peripheral blood [85, 92, 94], we find MDSCs are significantly elevated in smoker BAL. MDSC suppressive capacity has been attributed to several mechanisms including the activities of the enzymes iNOS, ARG1, IDO and immune checkpoint PD-L1 [73]. In our spectral flow analysis, BAL MDSCs from smokers express more iNOS, IDO

and PD-L1. Though MDSCs in the blood of smokers reportedly express more ARG1 than those in nonsmokers [69], we saw no difference in this enzyme between current and never-smokers, suggesting effects on its regulation may be tissue-specific.

Smoking-associated myeloid cell accumulation in the lungs is well supported by existing studies [28, 32, 62, 95], and concordantly, we show BAL of smokers to contain significantly elevated macrophages and DCs. Most lung macrophages are alveolar ($CD169^+$), with smaller fractions consisting of both proinflammatory (M1) and anti-inflammatory (M2) macrophages. Future studies aimed specifically at characterising airway macrophages may reveal additional subsets of these populations.

Here we demonstrate that immune biomarkers in BALF from patients are both responsive to clinical variables relevant to lung cancer (smoking history) and readily assayable with high-parameter flow-based approaches. Additionally, several novel observations relating smoking to immune-suppressing cells and pathways in the airway are presented. Few studies have explored smoking's impact on airway immune suppression; however, such an association was implicated by non-BAL biopsy studies in head and neck cancer wherein cytotoxic T-cell infiltration in smokers was reduced [11]. Moreover, in lung epithelium gene analysis, an immune-suppressive signature was found in current smokers [96]. Such dysfunction may lead to enhanced immune evasion by tumours and compromised defence against infection in the airway.

Limitations of our study include its observational nature and the fact that immune cell phenotypes were inferred based on marker profiles rather than functional assays. Also, our findings reflect the impact of smoking without interaction of other variables (*e.g.*, cancer diagnosis or COPD).

To address the potential effects of COPD on the smoking-associated immune changes identified by our analysis, we compared them across patients with and without a COPD diagnosis. While some trends observed between +COPD and −COPD groups were superficially reminiscent of current smokers *versus* former and never-smoker comparisons, the magnitude of the effect due to COPD independent of smoking on these readouts was generally muted and not statistically significant (supplementary Figure S7A). These results suggest that despite the overlap between COPD and smoking, a COPD diagnosis alone is not likely to account for effects on the changes in BAL linked to smoking history in this study.

Interestingly, we did not see an increase in CD8 frequencies in the BAL of patients with COPD in this study that others have reported in blood and sputum as well as BAL. While the reason for the discrepancy is not clear, it may stem from differences in how the CD8 T-cell pool is presented (*i.e.*, as a fraction of all $CD45^+$ immune cells or a fraction of the T-cell compartment); varying representation of COPD subtypes and symptom severities, which may alter the frequency and disposition of immune cells like $CD4^+$ and $CD8^+$ T-cells in patient tissues [97, 98]; or additional, subtle differences in patient age and duration of smoking experience across patient pools.

We also compared several of the immune readouts responsive to smoking status across patients with and without a general cancer diagnosis at sampling. Independent of smoking status, cancer was not associated with discernible changes in any marker examined (supplementary Figure S7B). Also, in our multivariate analysis, while several immune readouts did present significant interactions with clinical variables, cancer was not among them, further suggesting that a cancer diagnosis at the time of BAL sampling is not a major driver of the leukocyte populations and phenotypes we find readily influenced by smoking. Ongoing studies are aimed at uncovering BAL-derived biomarkers associated with cancer and other clinically important variables.

Additional limitations of this study include the fact that scrutiny of potentially useful biomarkers on BAL cellular components beyond immune cells (*e.g.*, epithelial cells) fell outside the scope of our study. Future studies may well apply an approach similar to ours to achieve such an aim. Despite these limitations, our findings provide proof-of-concept that BAL-derived leukocytes offer stable sources of biomarkers for preclinical and clinical studies such as NCT04931017, a recently initiated study using BAL to monitor treatment-associated changes in airway immunity. Results of this trial and our present efforts may inform the design of future lung cancer prevention, development and treatment studies.

Provenance: Submitted article, peer reviewed.

Acknowledgements: S. Yendamuri and J. Barbi assume full responsibility for the study's findings and the content of this manuscript. The authors thank B. Holdaway-Kinney (Roswell Park), who managed clinical data and served

as honest broker of clinical data. Other contributors include the Roswell Park Flow Cytometry and Imaging and Databank and Biorepository Shared Resources.

Support statement: This work was supported by institutional support grant P30-CA016056 to RPCCC from the National Cancer Institute; intramural research funding and CCSG project development funds awarded to S. Yendamuri and J. Barbi; 1R01AI137046 and an American Lung Association Lung Cancer Discovery Award to J. Barbi; and 1R01 CA255515–01A1 to S. Yendamuri, M. Reid and J. Barbi. D. Washington is a CLIMB/IMSD Scholar supported by a grant from the National Institute for General Medical Sciences to the State University of New York at Buffalo. Funding information for this article has been deposited with the Crossref Funder Registry.

Author contributions: R. Smith Jr and R. Zollo performed flow cytometry and BAL processing. R. Smith Jr performed all analysis, figure creation and wrote the manuscript. S. Sass, D. Washington and Y. Vedire assisted in BAL processing and helped prepare the manuscript. S. Rosario conducted statistical analysis. M. Reid directed patient recruitment. N.M. Ivanick provided and analysed clinical data. J. Barbi and S. Yendamuri edited the manuscript and oversaw the project. S. Yendamuri oversaw BAL collection procedures and patient recruitment.

Conflicts of interest: The authors declare that no financial or nonfinancial conflicts of interest exist.

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