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Chronic Marijuana Use Is Associated with Gene Expression Changes in BAL

To the Editor:

Despite marijuana's increasing national and global consumption (1), its impact on respiratory health remains poorly understood. As marijuana smoke contains immunomodulatory cannabinoids including Δ^9-tetrahydrocannabinol (2), marijuana is hypothesized to increase susceptibility to respiratory infections by altering pulmonary immune cell function. Obstacles to establishing marijuana's effects on immunity include concomitant tobacco use (3). We used RNA sequencing to profile gene expression in BAL from chronic marijuana smokers without concurrent tobacco use, tobacco-only smokers, and nonsmokers. We identified differentially expressed genes (DEGs) between the three groups, focusing on the hypothesis that marijuana smoking is associated with gene expression changes relevant to respiratory immunity.

Participants were recruited from Denver, Colorado, from 2015 to 2017, when marijuana was legal and commercially available. Included were marijuana smokers ($n = 15$) reporting ≥ 20 joint-years consumption (joints per day, multiplied by years smoking marijuana) without current tobacco use, and <1 pack-year tobacco smoking history; tobacco smokers ($n = 16$) reporting daily or near-daily tobacco smoking, no current marijuana smoking, and <1 joint-year marijuana smoking history; and nonsmokers ($n = 10$) reporting no current marijuana or tobacco smoking, with <1 joint-year and <1 pack-year smoking history. Substance use was verified with urinary Δ^9-tetrahydrocannabinol and cotinine screening. This study was approved by the Colorado Multiple Institutional Review Board. Participants provided informed consent.

Median marijuana exposure was 45.0 (interquartile range [IQR], 30.0–52.0) joint-years history with 15.0 (IQR, 12.0–21.0) years of smoking and 5 (IQR, 3.8–8.0) uses daily. Tobacco smokers had 11.5 (IQR, 10.0–23.0) pack-years smoking and were older than other groups (median [IQR], 39.0 [37.2–47.8] years, vs. others 31.0 [30.0–37.0]). Bronchoscopy with BAL were performed (4). The majority cell type was monocytes/macrophages in all participants (median [IQR], 94.3% [92.9–96.8%]), with no significant between-group differences in average cell frequencies.

RNA was extracted from cell pellets (QIAGEN RNeasy) for sequencing (Illumina chemistry) in two batches, with all nonsmokers sequenced in batch 1 and different library prep/sequencers by batch due to logistic limitations. Sequences were converted to counts using Kallisto (5). Principal component analysis (PCA) was used to visualize between-participant variability in overall gene expression

This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

profiles. Average between-group differences in gene expression at a 5% false discovery rate were tested using edgeR (6). Gene counts were regressed on participant group, adjusting for age, sex, obesity (body mass index ≥ 30), batch, and three RUVSeq factors (7). Each set of DEGs was summarized with Gene Ontology (GO) enrichment (overrepresentation analysis (8, 9), false discovery rate < 0.05). In the data supplement, we show that our findings are robust to batch and cell composition effects, demonstrate correction for batch effects, and reproduce existing tobacco versus nonsmoker BAL literature, suggesting external validity.

We found extensive BAL gene expression differences by participant group. In the PCA plot, participants were separated by smoking group, suggesting that the global transcriptome of marijuana smokers is distinct from nonsmokers and tobacco smokers (Figure 1). Of 17,602 genes analyzed, 1,504 were significant DEGs between marijuana smokers and nonsmokers and 3,231 were DEGs between marijuana and tobacco smokers (Figure 2). Moreover, these DEGs may be functionally relevant based on GO enrichment analyses, as both marijuana versus tobacco DEGs and marijuana versus nonsmoker DEGs were significantly enriched in biological processes critical to airway homeostasis (e.g., "response to hypoxia," "cellular response to oxidative stress") and important BAL cell functions (e.g., "apoptotic signaling pathway," "regulation of cytokine production," "response to lipopolysaccharide").

DEGs within immunity-related GO groups were selected for RT-PCR validation, confirming increased average expression of *HIF1A* (hypoxia-inducible factor 1-α, fold-change [FC] > 2.1) and *TLR6* (toll-like receptor 6, FC > 1.6) among marijuana smokers compared with both other groups, together with decreased *CASP1* (caspase 1, FC < 0.76) expression compared with both other groups. *HIF1A* is implicated in lung injury and inflammation, including expression after bacterial infection and hypoxia (10). TLRs play integral roles in initiating innate immune responses to respiratory infection (4). Increased *TLR6* expression was previously noted in airway epithelium from marijuana smokers (4). *CASP1* activates *IL-1β* and *IFN-γ*, and by extension, T-helper cell type 1 inflammatory responses (11). We observed decreased expression of *CASP1* and many of its downstream products, including *IFN-γ*, among marijuana smokers relative to both other groups (FC < 0.43). Our findings thus suggest functional differences in BAL associated with marijuana smoking, including immunomodulatory changes confirmed by RT-PCR.
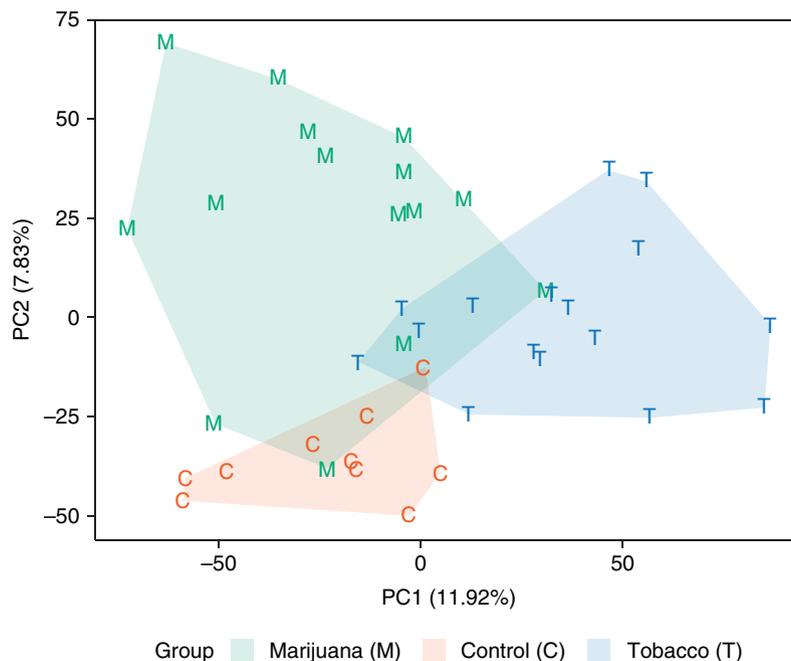
Although distinctions between the marijuana and tobacco groups are evident, there were gene expression signatures of concern common to both sets of smokers. For example, upregulation of *MMP-12* (matrix metalloproteinase-12) is a well-known signature of tobacco smoking that contributes to cigarette smoke–induced emphysema and chronic obstructive pulmonary disease (12). In our data, *MMP-12* was upregulated in not only tobacco smokers (FC = 4.0) but also marijuana smokers (FC = 3.3) compared with nonsmokers. Thus, despite the many differences at the gene level and GO process level between tobacco and marijuana smokers, marijuana smoking may still share some of the deleterious gene expression changes linked to tobacco exposure.

Overall, we demonstrate that pulmonary immune cell functional profiles of chronic marijuana smokers are distinct from both tobacco smokers and nonsmokers. We find previously unreported DEGs and biological processes associated with marijuana smoking that merit additional investigation given their

Figure 1. Principal component analysis (PCA) on covariate-adjusted BAL gene expression profiles. Each point represents a participant, labeled by their smoking group. Principal component 1 (PC1) plotted on the *x*-axis explains 11.92% of the variation in gene expression. Principal component 2 (PC2) plotted on the *y*-axis explains 7.8% of the variation in gene expression. Participants with more similar BAL expression profiles after accounting for covariates appear closer within the PCA plot. Covariate adjustment was performed by refitting edgeR models on all covariates except smoking group (i.e., on age, sex, body mass index, batch, and RUVSeq components), then extracting the resulting model deviance residuals as input into PCA.
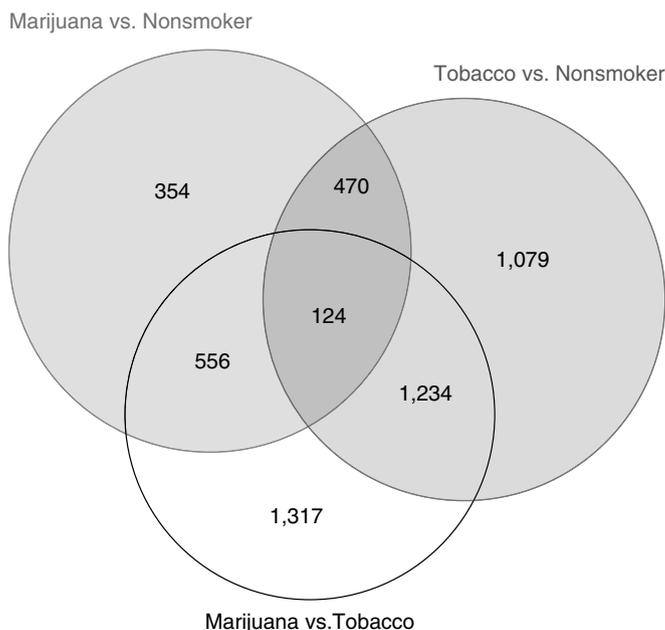
Figure 2. Venn diagram of differentially expressed genes (DEGs) in all between-group comparisons. For example, 354 genes were DEGs between marijuana smokers and nonsmokers but not DEGs in the other two pairwise comparisons. In contrast, 470 genes were DEGs between marijuana smokers and nonsmokers as well as between tobacco smokers and nonsmokers but not between marijuana and tobacco smokers.

potential relevance to respiratory health. Namely, we observed evidence of broad alterations of pulmonary immunity, consistent with prior investigations reporting immunomodulatory impacts of marijuana smoking (13). However, the cumulative effects of BAL DEGs require further functional and mechanistic study, such as alterations in alveolar macrophage phenotypes or host–microbiota interactions among marijuana smokers. Additional limitations include our pilot sample size and inherent challenges of quantifying real-world marijuana exposure (14). Although recruitment of heavy, daily marijuana-only smokers allowed us to isolate the signature of marijuana use, DEGs may not generalize to intermittent smokers or tobacco–marijuana dual smokers. Lastly, BAL is a composite of diverse pulmonary immune cells. Although our results were robust to between-participant cell composition in sensitivity analyses, single-cell experiments may clarify cell-specific pulmonary effects of marijuana smoking. To our knowledge, this is the first RNA sequencing study of respiratory biospecimens from chronic marijuana smokers. The publicly available differential expression data and results (NCBI-GEO accession GSE155213; journal online supplement and https://www.github.com/chooliu/ajrcmb_marijuana_rnaseq) represent novel resources for understanding the impact of marijuana smoking on lung biology. ∎

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