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Downregulation of *Let-7* miRNA promotes Tc17 differentiation and emphysema via de-repression of RORγt

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Abstract

Environmental air irritants including nanosized carbon black (nCB) can drive systemic inflammation, promoting chronic obstructive pulmonary disease (COPD) and emphysema development. The *let-7* family of miRNAs is associated with IL-17-driven T cell inflammation, a canonical signature of lung inflammation. Recent evidence suggests the *let-7* family is downregulated in patients with COPD, however, whether this repression conveys a functional consequence on emphysema pathology has not been elucidated. Here we show that overall expression of the *let-7* miRNA clusters, *let-7b/let-7c2* and *let-7a1/let-7f1/let-7d*, are reduced in the lungs and T cells of smokers with emphysema as well as in mice with cigarette smoke (CS)- or nCB-elicited emphysema. We demonstrate that loss of the *let-7b/let-7c2*-cluster in T cells predisposed mice to exaggerated CS- or nCB-elicited emphysema. Furthermore, ablation of the *let-7b/let-7c2*-cluster enhanced $CD8^+IL17a^+$ T cells (Tc17) formation in emphysema development in mice. Additionally, transgenic mice overexpressing *let-7* in T cells are resistant to Tc17 and $CD4^+IL17a^+$ T cells (Th17) development when exposed to nCB. Mechanistically, our findings reveal the master regulator of Tc17/Th17 differentiation, RAR-related orphan receptor gamma t (RORγt), as a direct target of *let-7* miRNA in T cells. Overall, our findings shed light on the *let-7*/RORγt axis with *let-7* acting as a molecular brake in the generation of Tc17 cells and suggests a novel therapeutic approach for tempering the augmented IL-17-mediated response in emphysema.

eLife assessment

This **important** study indicates a significant role for individual *let-7* miRNA clusters in regulating generation of Tc17 CD8 cells and emphysema severity in a mouse model. The authors provide **convincing** evidence for let-7-mediated repression of the transcription factor RORgt and consequent modulation of IL-17-producing CD8 T cells, with correlated data from human emphysema material, though some of the effective *let-7* clusters remain to be tested for the ability to modulate disease. The findings, which substantially advance the understanding of roles that *let-7* miRNA clusters play in modulating both T cell responses and emphysematous lung disease, will be of interest to T cell and lung disease researchers.

Introduction

Chronic obstructive pulmonary disease (COPD) ranks as the third leading cause of mortality and is projected to account for over a billion deaths by the end of the twenty-first century (GBD Chronic Respiratory Disease Collaborators, 2020; *Findings from the Global Burden of Disease Study 2017*, 2019; Laniado-Laborín, 2009). Currently, there are no treatment options to reverse emphysema, the most clinically significant variant of COPD, which often is progressive despite smoking cessation (Bhavani et al., 2015; Anthonisen et al., 2002).

Inhalation of fine particulate matter smaller than 2.5 microns ($PM_{2.5}$) found in outdoor and indoor air pollution as well as tobacco smoke are risk factors for COPD development (Adeloye et al., 2022; Eisner et al., 2010; Hu et al., 2010). We have previously shown that nano-sized carbon black (nCB), a noxious chemical constituent of $PM_{2.5}$ found in the lungs of smokers, activates macrophages and dendritic cells orchestrating a pathogenic T cell-dependent inflammatory response and emphysema in mice (W. Lu et al., 2015; You et al., 2015; Shan et al., 2009; C.-Y. Chang et al., 2022).

Research over the last decade has pointed to the importance of dysfunctional inflammatory T cells in human COPD lung tissue and animal models of emphysema (Grumelli et al., 2004; Xu et al., 2012; Williams et al., 2021). Aberrant T cells are implicated in impaired host defense, exaggerated inflammation, and loss of self-tolerance in COPD (Williams et al., 2021; Chen et al., 2023; Hogg et al., 2004; Maeno et al., 2007; Xu et al., 2012). In this regard, we and others have demonstrated the role and pathogenicity of activated IFN-γ and IL-17-secreting subsets of $CD4^+$ and $CD8^+$ T lymphocytes including Th1, Th17, and Tc1 cells in clinical isolates and in mice with COPD (W. Lu et al., 2015; You et al., 2015; Shan et al., 2009; S.-H. Lee et al., 2007; Kheradmand et al., 2023). The IL-17-secreting Th17 cells are particularly important as they promote the destruction of lung epithelium and recruitment of macrophages and neutrophils which then release proteolytic enzymes such as matrix metalloproteinases (MMPs) involved in the degradation of the lung structural matrix (Barnes, 2016; Hoenderdos & Condliffe, 2013). We previously demonstrated that intranasal inhalation of nCB in mice is sufficient to induce emphysema by stimulating lung T cell activation by dendritic cells and macrophages. Moreover, we found that genetic ablation of IL-17A can attenuate nCB- or cigarette smoke-induced alveolar destruction and airway inflammation (Shan et al., 2012; You et al., 2015). More recently, IL-17A and IL-17F secreting $CD8^+$ T cell (Tc17) subpopulation has been shown to play a critical role in the pathogenesis of several autoimmune and inflammatory disorders (Globig et al., 2022; Huber et al., 2013; Srenathan et al., 2016).

Both Th17 and Tc17, require the fate-deterministic transcription factor RAR-related orphan receptor gamma t (RORγt, encoded by *Rorc*) for differentiation and production of IL-17A (Ivanov et al., 2007). RORγt is the best-studied positive transcriptional regulator of IL-17A and IL-17F (Ivanov et al., 2006). In accordance with the importance of IL-17A transcription, RORγt expression has also been reported to be elevated in COPD patients and in mouse models of COPD (Chu et al., 2011; Li et al., 2015). However, the upstream pathophysiologic mechanisms that contribute to the induction of RORγt and differentiation of Tc17 cells in COPD have not been well elucidated.

We previously reported that *miR-22* inhibits HDAC4, promoting antigen-presenting cell activation (APC) in the lungs and inducing Th17-mediated emphysema in response to CS or nCB in mice (W. Lu et al., 2015). Additional miRNAs that control APC and/or T cell driven IL-17A$^+$ inflammation have been identified by others including the *let-7* miRNA family (Mai et al., 2012; Angelou et al., 2020). MicroRNA expression-based studies have shown frequent downregulation of members of the *let-7* miRNA family, including *let-7a*, *let-7b*, *let-7c*, *let-7d*, l*et-7e*, and *let-7f* in human emphysematous lung tissue and in murine models of emphysema, but the mechanism(s) of action remain ill-defined (Christenson et al., 2013; Pottelberge et al., 2011; Conickx et al., 2017; Izzotti et al., 2009). *Let-7* microRNA genes are encoded across eight loci either as single genes or as polycistronic clusters which have confounded their analysis *in vivo* (Rodriguez et al., 2004). Previous studies used *Lin28b* transgenic overexpression in T cells to block the maturation and processing of the *let-7* miRNA family. They showed an inhibitory role of *let-7* family in Th17-driven response in the murine model of experimental autoimmune encephalomyelitis (EAE) attributed in part to regulation of IL-1 receptor 1 and IL-23 receptor (Angelou et al., 2020).

Here we found that *let-7* miRNA, notably the *let-7a3/let-7b* and *let-7a1/let-7f1/let-7d* clusters, are suppressed in the T cells isolated from lungs of emphysema patients. Consistently, the analogous murine *let-7b/let-7c2-* (*let-7bc2*) and *let-7a1/f1/d1-* (*let-7afd*) clusters were similarly downregulated in pre-clinical emphysema models. We engineered mouse models with the specific loss-of-function (LOF) mutations of the *let-7bc2-* or *let-7afd*-clusters (*let-7bc2*^{LOF} and *let-7afd*^{LOF}, respectively) in T cells as well as an inducible *let-7g* gain-of-function (GOF) (*let-7*^{GOF}) model to determine the T cell-intrinsic role of *let-7* miRNA in emphysema pathogenesis. Deletion of *let-7* miRNA in T cells worsened alveolar damage elicited by inhalation of CS or nCB, and increased infiltration of immune cells in the airways, including IL-17-producing CD8$^+$ T (Tc17) cells. Mechanistically, we found that *let-7* controls type 17 differentiation by directly targeting the lineage-determining transcription factor, RORγt. In support of this conclusion, *let-7*^{GOF} mice were resistant to nCB-mediated induction of RORγt and Tc17 responses. Thus, we show a previously unappreciated role for *let-7* miRNA as a repressor of RORγt and a molecular brake to the IL-17-mediated T cell inflammation in emphysema.

Results

The *let-7bc2-* and *let-7afd-clusters* are downregulated in lungs and T cells in COPD

To explore the involvement of *let-7* in emphysema, we scrutinized the genomic locations and transcriptional annotation of *let-7* members frequently downregulated in lung T cells isolated from smoker's lungs as well as mouse models of emphysema. This combined approach showed close linkage and high conservation of two *Let-7* clusters encoded from long intergenic non-coding RNA (linc)-like precursors in humans and mice (**Figure 1A**). To shed light on whether these *Let-7* clusters are downregulated in patients with COPD, we analyzed a published (GSE57148) lung RNA-seq dataset obtained from COPD (n=98) and control (n=91) subjects (Kim et al., 2015). Our analysis identified significant downregulation of the *Mirlet7ahg* and *Mirlet7bhg* gene cluster transcripts in COPD compared to control subjects (**Figure 1B**). We carried out quantitative PCR

(qPCR) detection of *Let-7a*, which is encoded by both clusters, in lung tissue samples of smokers with emphysema and non-emphysema controls, detecting significant downregulation of *Let-7a* in emphysema samples relative to controls (**Figure 1C**). Because *Let-7* has been shown to participate in IL-17$^+$ T cell responses (Angelou et al., 2020; Guan et al., 2013; Newcomb et al., 2015), we next sought to determine if the expression pattern of *Mirlet7ahg* and *Mirlet7bhg*-derived *Let-7* members are impaired in purified CD4$^+$ T cells from emphysematous lungs. In support of our original hypothesis, the CD4$^+$ T cell expression of *Let-7a*, *Let-7b*, *Let-7d*, and *Let-7f* were all inversely correlated with more severe emphysema distribution in the lungs as determined by CT scan (**Figure 1D**).

Next, we elucidated *let-7a1/let-7f1/let-7d*- and *let-7b/let-7c2*-clusters expression (herein referred to as *let-7afd* and *let-7bc2* respectively) in murine models of CS- or nCB-induced emphysema respectively (**Figure 1E**). Paralleling our observations in human COPD and emphysema, mice with CS- or nCB-induced emphysema exhibited reduced expression levels of *pri-let7a1/f1/d* and *pri-let7b/c2* transcripts in the lung and from isolated lung CD4$^+$ and CD8$^+$ T cells (**Figure 1F-H**). Collectively, our expression results indicate suppression of *let-7afd* and *let-7bc2*-clusters in the lung and T cells in human and pre-clinical models of emphysema.

Conditional deletion of the *let7bc2*-cluster in T cells enhances nCB- or CS-induced emphysema

To investigate the *in vivo* requirement of the *let-7bc2*-cluster within T cells, we generated conditional ready floxed mice (*let-7bc2$^{flox/flox}$*). We then crossed *let-7bc2$^{flox/flox}$* mice with *CD4-Cre* mice to generate *let-7bc2$^{flox/flox}$*; *CD4-Cre* LOF mice (denoted as *let-7bc2^{LOF}* mice hereafter) (**Figure 2A**). This approach allowed us to conditionally delete the *let-7bc2*-cluster in all T cells derived from the CD4$^+$CD8$^+$ double-positive (DP) stage (P. Lee et al., 2001; Shi & Petrie, 2012). We confirmed that *let-7bc2^{LOF}* mice exhibit robust conditional deletion of the *let-7bc2*-cluster in DP thymocytes and peripheral T cells (**Figure 2B** and data not shown). Our *let-7bc2^{LOF}* adult mice were born at the expected Mendelian frequency and did not show any overt histopathologic or inflammatory changes in lungs histopathology up to 1 year of age in comparison to *let-7bc2$^{f/f}$* control mice (Figure 2-figure supplement 1A-C). Furthermore, quantification of major immune populations and T cell subsets by flow cytometry in *let-7bc2^{LOF}* were comparable to control mice under baseline conditions and with moderate aging (Figure 2-figure supplement 1C,D).

We next exposed *let-7bc2^{LOF}* and *let-7bc2$^{f/f}$* control mice to nCB or CS and examined the lungs under the context of experimental emphysema. Histomorphometry measurements of mean linear intercept (MLI) from hematoxylin and eosin (H&E)-stained sections revealed that the enlargement of alveolar spaces sustained from either nCB- or CS-exposure was exaggerated in *let7bc2^{LOF}* mice relative to controls (**Figure 2C-E**). Chronic inflammation in emphysema is characterized by the recruitment of macrophages and neutrophils to the lung tissue and airways (Peleman et al., 1999; Senior & Anthonisen, 1998). Internally consistent with MLI measurements, *let-7bc2^{LOF}* mice treated with nCB showed significantly increased airway infiltration of macrophages and neutrophils in BAL fluid as compared to wild-type control animals (**Figure 2F**). Concomitant with these findings, expression levels of *Mmp9* and *Mmp12*, which are secreted by macrophages and neutrophils to degrade elastin and mediate alveolar damage, were elevated in airways of *let7bc2^{LOF}* mice exposed to CS versus controls (**Figure 2G**). As expected, *let-7bc2^{LOF}* mice treated with nCB exhibit significantly less *pri-let7b/c2* transcript expression in isolated lung T cells relative to wild-type control mice (Figure 2-figure supplement 2A and data not shown). Collectively, our data suggests that the *let-7bc2*-cluster within T cells protects by dampening airway destruction and inflammation because the absence of this cluster worsens the severity of experimental emphysema in mice.

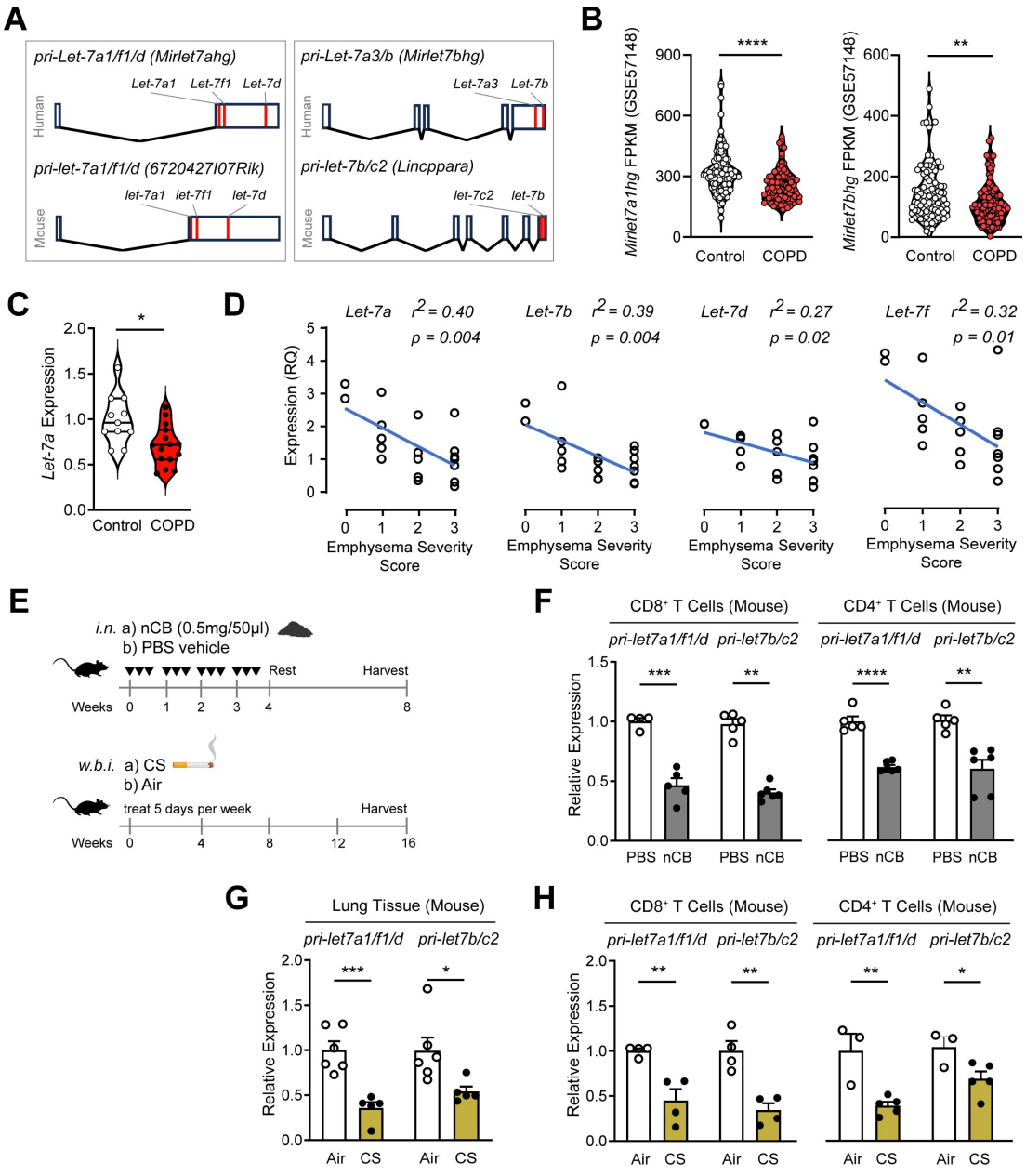

Figure 1.

Repression of *Let-7* miRNA gene clusters in lung T cells from COPD patients and murine models of emphysema.

(A) Schematic representation of the polycistronic transcripts for the *Let-7a1/Let-7f1/Let-7d*- and *Let-7b/Let-7a3*-clusters in humans and *let-7a1/let-7f1/let-7/d*- and l*et-7b/let-7c2*-clusters in mice. (B) *In silico* analysis of *Mirlet7a1hg* and *Mirlet7bhg* from the publicly available lung transcriptome dataset from RNA-seq of COPD and control patients (GEO: GSE57148). (C) Quantitative RT-PCR (qPCR) of mature *Hsa-Let-7a* from resected lung tissue of COPD (n=15) and control subjects (n=11). (D) QPCR and regression analysis of *Hsa-Let-7a, Hsa-Let-7b, Hsa-Let-7d, and Hsa-Let-7f* expression to emphysema severity score based on CT: 0=no, 1=upper lobes only, 2=upper/middle lobes, 3=extensive pan lobular emphysema (n=19). (E) Schematic diagram of experimental emphysema in mice induced by either intranasal (i.n.) instillation of nCB or exposure to CS by whole-body inhalation (w.b.i.). (F-H) QPCR analysis for *pri-let-7a1/f1/d* and *pri-let-7b/c2* from lung tissue or lung-derived $CD8^+$ and $CD4^+$ T cells of mice with emphysema elicited by (F) nCB- or (G-H) CS (n=3-6 per group). Data are representative of three independent experiments displayed as mean±SEM. Mann-Whitney (B,C) or Student's t-test (F,G,H). *p < 0.05, **p < 0.01, ***p <0.001, ****p<0.0001.

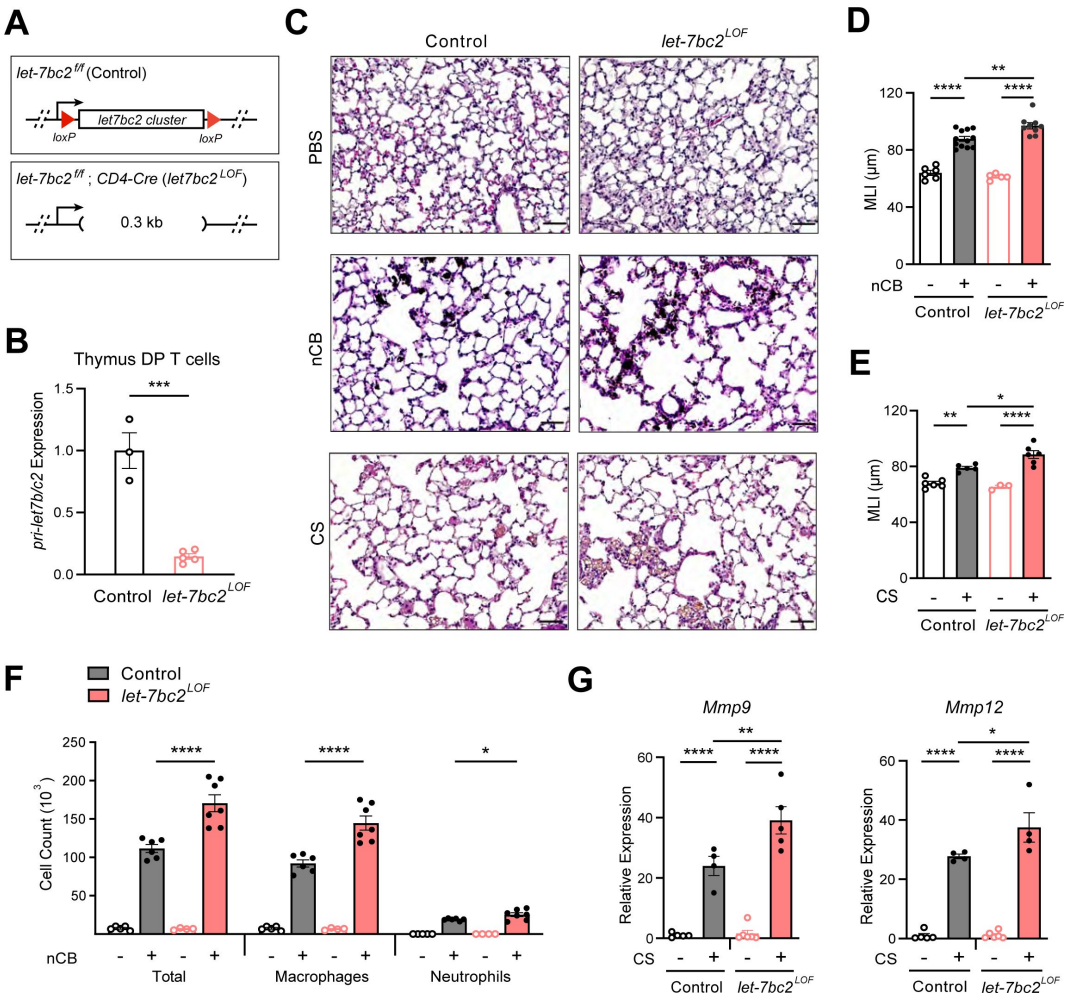

Figure 2.

Deletion of the *let-7bc2* cluster in T cells enhances nCB- or CS-triggered emphysema.

(A) Schematic representation of CD4-Cre (*let-7bc2*^{LOF}) or *let-7bc2*$^{f/f}$ (Control) mice. (B) QPCR analysis of *pri-let-7b/c2* from flow-sorted live,TCRβ^{+}, CD4^{+}CD8^{+} double-positive (DP) thymocytes of control and *let-7bc2*^{LOF} mice (n=3-5 per group). (C-G) Control and *let-7bc2*^{LOF} mice were exposed to vehicle (PBS) or nCB for 4 weeks, or alternatively air or cigarette smoke by whole body inhalation of cigarette smoke (CS) for 16 weeks. (C) Representative H&E stained lung sections from PBS-, nCB-, or CS-exposed mice as indicated on each panel (x20 magnification; scale bars, 50µm). (D-E) Mean linear intercept (MLI) measurement of lung morphometry. (F) Total and differential cell counts from bronchoalveolar lavage (BAL) fluid from controls and nCB-emphysemic mice (n=4-7 per group). (G) *Mmp9* and *Mmp12* mRNA expression from BAL cells of air- and smoke-exposed control and *let-7bc2*^{LOF} mice (n=4-6 per group). Data are representative of at least three independent experiments displayed as mean±SEM using Student's t-test (B) or two-way ANOVA with *post-hoc* Tukey correction (D,E,F,G). *p < 0.05, **p < 0.01, ***p <0.001, ****p<0.0001.

The *let-7bc2* miRNA cluster negatively regulates T_C17 inflammation in emphysema

We sought to identify the T cell-intrinsic mechanisms that underlie the exaggerated inflammation observed in emphysematous *let-7bc2*^{LOF} mice. We focused on the IL-17-mediated T cell response because it promotes neutrophil and macrophage recruitment in the lungs (Beringer et al., 2016; Veldhoen, 2017; Shan et al., 2012). Previously, we established the induction of CD4$^+$IL17$^+$ (Th17) cells along with CD4$^+$IFNγ$^+$ (Th1) cells in mice with chronic nCB exposure (You et al., 2015), however whether nCB similarly induces CD8$^+$IL17A$^+$ T cells (Tc17) or cytotoxic T cells (Tc1) had not been studied. The flow cytometric profiling of lung T cells revealed enriched proportions and counts of Tc1/Tc17 as well as Th1/Th17 cells in wild-type mice upon treatment with nCB (**Figure 3A,B**). These findings suggests that nCB elicits both the type 17 and type 1 T cell responses, consistent with CS and elastase pre-clinical models of emphysema (Zhang et al., 2019).

We next interrogated the regulatory role of the *let-7bc2*-cluster in the type 17 and type 1 responses generated from exposure to nCB. Interestingly, *let-7bc2*^{LOF} mice showed increased CD8$^+$IL17A$^+$ Tc17 cells relative to nCB control animals. In contrast, CD8$^+$IFNγ$^+$ and GZMA$^+$ Tc1 populations remained unperturbed with absence of the *let-7bc2*-cluster, suggestive of a more refined regulatory role on Tc17 differentiation (**Figure 3A,B**). There were no significant differences in either Th1 or Th17 cells when comparing nCB-treated *let-7bc2*^{LOF} to wild-type controls, indicating the *let-7bc2*-cluster was dispensable for their generation (**Figure 3C**). Regulatory T cells form a dynamic axis with Tc17/Th17 cells and act as a counterbalance to lung inflammation in emphysema (Duan et al., 2016; Jin et al., 2014). Therefore, we examined whether Tc17 cell alterations were driven by the *let-7bc2*-cluster acting on regulatory T cells (Tregs). The *let-7bc2*^{LOF} mice showed no significant difference in the Tregs subset relative to controls in our model (**Figure 3D**). Together, our data support the notion that deletion of the *let-7bc2*-cluster is insufficient to provoke Tc17 cell generation under homeostatic conditions. However, under the context of chronic inflammation in emphysema, the loss of *let-7bc2*-cluster is intrinsic for the potentiation of T cells towards Tc17 differentiation.

The *let-7* family directly inhibits RORγt expression governing Tc17 differentiation in emphysema

We utilized the TargetScan predictive algorithm to identify putative *let-7* miRNA targets that are known to control the IL-17-mediated T cell response (Agarwal et al., 2015). This analysis revealed that the 3'UTR region of *Rorc*, encoding RORγt, contains an evolutionarily conserved and complementary motif for the *let-7* miRNA family (**Figure 4A**). Thus, we examined if *let-7bc2*-cluster loss in T cells would stimulate and enhance RORγt. Initially, we carried out flow cytometric quantification for RORγt in thymocyte, splenic, and lung T cells of naïve control and *let-7bc2*^{LOF} mice up to 6-months of age. Our interrogation of RORγt mean fluorescent intensity (MFI) by flow cytometry showed induction of RORγt in single-positive CD8$^+$ and CD4$^+$ thymocytes, as well as peripheral splenic CD8$^+$ and CD4$^+$ T cells (**Figure 4B**). However, RORγt levels appeared unchanged in purified lung CD8$^+$ T cells and CD4$^+$ T cells of naive *let-7bc2*^{LOF} mice, alluding to a compensatory effect in homeostatic lung T cells (**Figure 4B**). Since we and others have shown that miRNAs are frequently associated with stress-dependent phenotypes, we posited that emphysematous *let-7bc2*^{LOF} T cells are poised towards induction of RORγt and production of IL-17$^+$ subsets after challenge with nCB. Indeed, nCB-emphysematous *let-7bc2*^{LOF} mice exhibited enhanced RORγt protein levels in both CD8$^+$ and CD4$^+$ T cells relative to control mice with emphysema (**Figure 4C**).

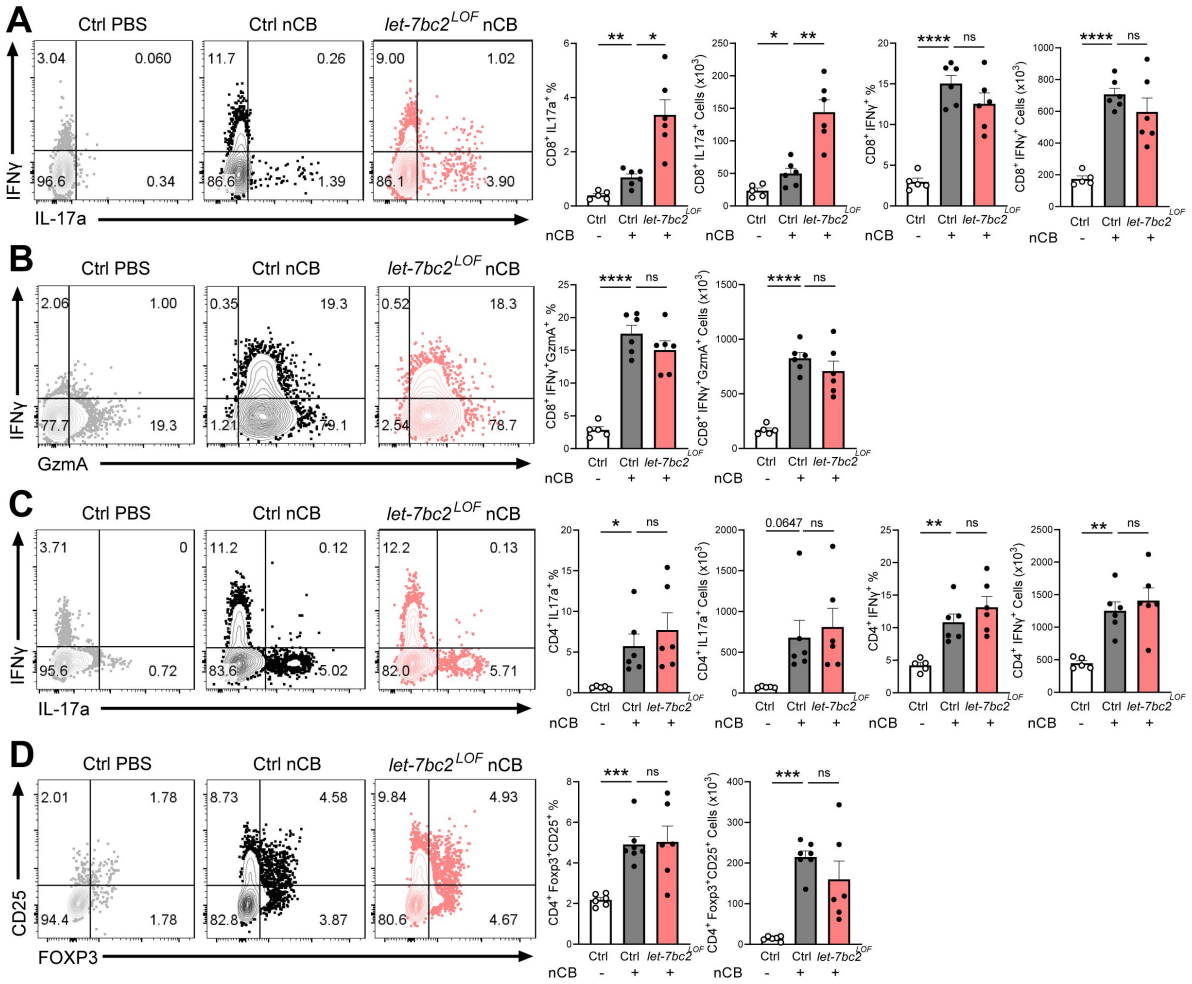

Figure 3.

In vivo T cell ablation of the *let-7bc2*-cluster enhances Tc17 inflammatory response to nCB-emphysema.

Representative flow plots with percentage and counts of live TCRβ$^+$ (A) CD8$^+$IL-17a$^+$ and CD8$^+$IFNγ$^+$, (B) CD8$^+$IFNγ$^+$GzmA$^+$, (C) CD4$^+$IL-17a$^+$ and CD4$^+$IFNγ$^+$, and (D) CD4$^+$ Foxp3$^+$CD25$^+$ cells from the lungs of control (Ctrl) PBS vehicle- (n=5-6), control nCB- (n=6), and *let-7bc2*^{LOF} nCB-exposed mice. Data are representative of three independent experiments displayed as mean±SEM using ANOVA with *post-hoc* Sidak correction. *p < 0.05, **p < 0.01, ***p <0.001, ****p<0.0001.

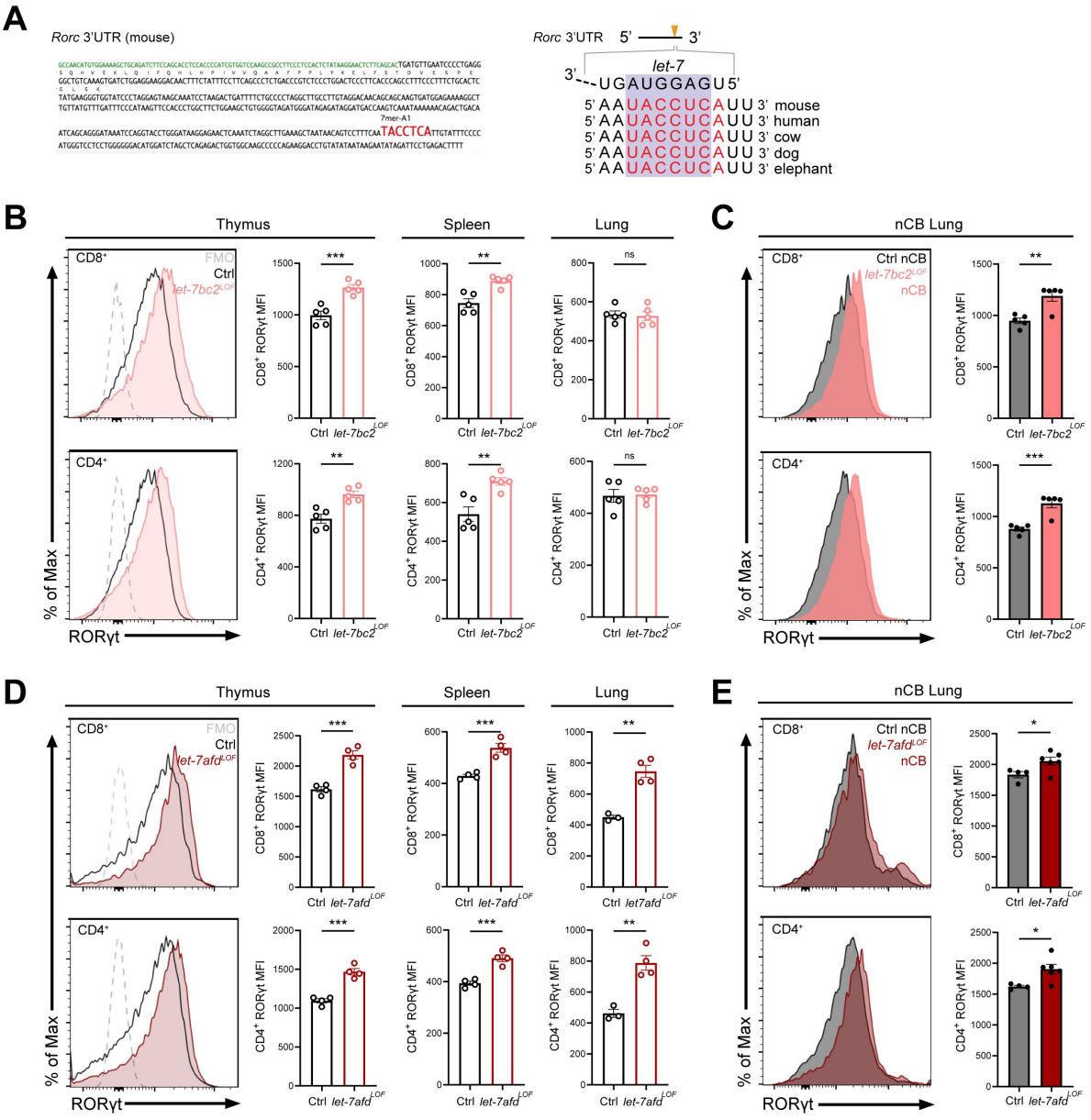

Figure 4.

Deletion of either the *let7bc2*- or *let7afd*-cluster in T cells enhances RORγt expression *in vivo*.

(A) Left: Schematic repre-sentation of the murine *Rorc* 3'UTR with *let-7* miRNA binding sites as identified by TargetScan. Right: Schematic of a conserved *let-7* miRNA target sequence in the 3'UTR of *Rorc*. (B-C) Flow analysis of RORγt expression by MFI quantification in live TCRβ$^+$CD8$^+$ or CD4$^+$ T cells from indicated tissues of (B) naïve control (Ctrl) and *let-7bc2^{LOF}* mice or (C) nCB-treated lungs by representative flow plot and MFI quantification (n=5 per group). (D) RORγt expression by MFI quantification in naïve mice *let-7afd^{LOF}* mice thymus, spleen, and lungs (n=3-4 per group), or (E) nCB-exposed lungs (n=5 per group). Data are representative of at least three independent experiments displayed as mean±SEM using student's t-test. *p < 0.05, **p < 0.01, ***p <0.001, ****p<0.0001.

Because we had found that the *let-7afd*-cluster is downregulated in T cells isolated from COPD lungs in human and mice, and that the *let-7* family operates with some functional redundancy, we generated mice with conditional deletion of the *let7afd*-cluster in T cells (*let-7afd$^{f/f}$; CD4-Cre*). The *let-7afd$^{f/f}$; CD4-Cre* (*let-7afd^{LOF}*) mice aged up to 6-months did not exhibit overt lung histopathology and inflammatory changes (Figure 4-figure supplement 1A-F). Of particular interest, ablation of the *let-7afd*-cluster enhanced levels of RORγt in thymic and peripheral T cells of mice (**Figure 4D**). Overall, this indicates that independent *let-7* clusters restrain RORγt expression levels from thymic development to peripheral T cells under homeostatic conditions. Next, we determined whether loss of *let-7afd*-cluster in T cells likewise sensitizes mice towards induction of RORγt in nCB-emphysema. Intranasal administration of nCB provoked increased RORγt expression in lung T cells of *let-7afd^{LOF}* mice compared to control mice (**Figure 4E**), supporting overlapping functionality between the *let-7bc2*- and *let-7afd*-clusters in repression of RORγt within T cells.

To confirm that the *let-7* family negatively regulates Tc17 cell differentiation, at least in part, cell autonomously in CD8$^+$ T cells, we purified naïve CD8$^+$ T cells from *let-7bc2^{LOF}* and control mice spleens and cultured these cells *in vitro* in the presence of Tc17 polarizing (TGFβ, IL-6, anti-IFNγ, IL-23, and IL-1β) or Tc1 polarizing (IL-2) conditions (Flores-Santibáñez et al., 2018). Our flow cytometric analysis confirmed the enhanced commitment of *let-7bc2*-cluster deficient CD8$^+$ T cells towards Tc17 cells and IL-17A$^+$ production relative to control CD8$^+$ T cells (**Figure 5A,B**). Moreover, enhanced Tc17 cell differentiation mirrored the increased IL-17A detected in the supernatant from *in vitro* polarized cells as quantified by ELISA (**Figure 5C**). Parallel assessment of Tc1 differentiation did not detect a difference in CD8$^+$IFNγ$^+$ cells (**Figure 5A** and **Figure 5D**). Altogether, these data recapitulated our *in vivo* findings that the *let-7bc2*-cluster negatively regulates Tc17 response but is dispensable in Tc1 cells. Finally, to determine whether Tc17 differentiation is likewise controlled by the *let-7afd*-cluster, we cultured naive CD8$^+$ splenocytes from *let-7afd^{LOF}* and controls under Tc17 conditions. As we had observed with *let-7bc2^{LOF}*, absence of the *let-7afd*-cluster in T cells further enhanced differentiation towards Tc17 cells as quantified by flow cytometry and ELISA (**Figure 5F,G**).

Next, we focused on *Rorc* as a potential direct target of *let-7*, which could mechanistically mediate enhanced Tc17 differentiation in *let7bc2^{LOF}* mice. Towards this objective, we tested whether *let-7bc2^{LOF}* or *let-7afd^{LOF}* naïve CD8$^+$ T cells show elevated RORγt expression under either Tc0 or Tc17 differentiation conditions. In agreement with enhanced Tc17 differentiation, RORγt expression was differentially and significantly upregulated under both Tc0 and Tc17 differentiation conditions in *let-7* LOF cells relative to controls (**Figure 5E,H**). To determine whether *let-7* directly represses *Rorc* mRNA levels we cloned the 3'UTR of *Rorc* into luciferase constructs. These reporter assays with *let-7b* expressing cells independently confirmed that *let-7b* represses *Rorc* (**Figure 5I**, left). Furthermore, deletion of the putative *let-7* binding sequence (**Figure 4A**) abrogated repression by *let-7b* (**Figure 5I**, right), thus confirming *Rorc* as a functional target of *let-7* miRNA. Overall, these *in vitro* experiments readily recapitulated an upstream regulatory role of *let-7* in Tc17 differentiation, mediated in part, via direct suppression of RORγt.

Enforced expression of *let-7* tempers RORγt T cell expression levels in experimental emphysema

To explore a potential protective role of *let-7* miRNA in experimentally-induced emphysema, we generated mice which allowed for selective induction of *let-7* activity in T cells using the published *rtTA-iLet7* mice crossed to CD4-Cre (herein referred to as *let-7^{GOF};* **Figure 6A**) (Angelou et al., 2020; Belteki et al., 2005; Pobezinskaya et al., 2019; Wells et al., 2017; Zhu et al., 2011). The rtTA-iLet7 mouse model has been utilized to promote ~2-3-fold rise in total *let-7* activity in T cells (Angelou et al., 2020; Wells et al., 2017, 2023; Angelou et al., 2020). Steady-state *let-7^{GOF}* and control (*rtTA-iLet7*) mice were examined for compromised RORγt protein levels within thymocytes and peripheral T cells. Providing further evidence of *let-7*-dependent regulation of *Rorc*, protein levels of RORγt were suppressed in CD8$^+$ and CD4$^+$ T cells of *let-7^{GOF}* mice relative to

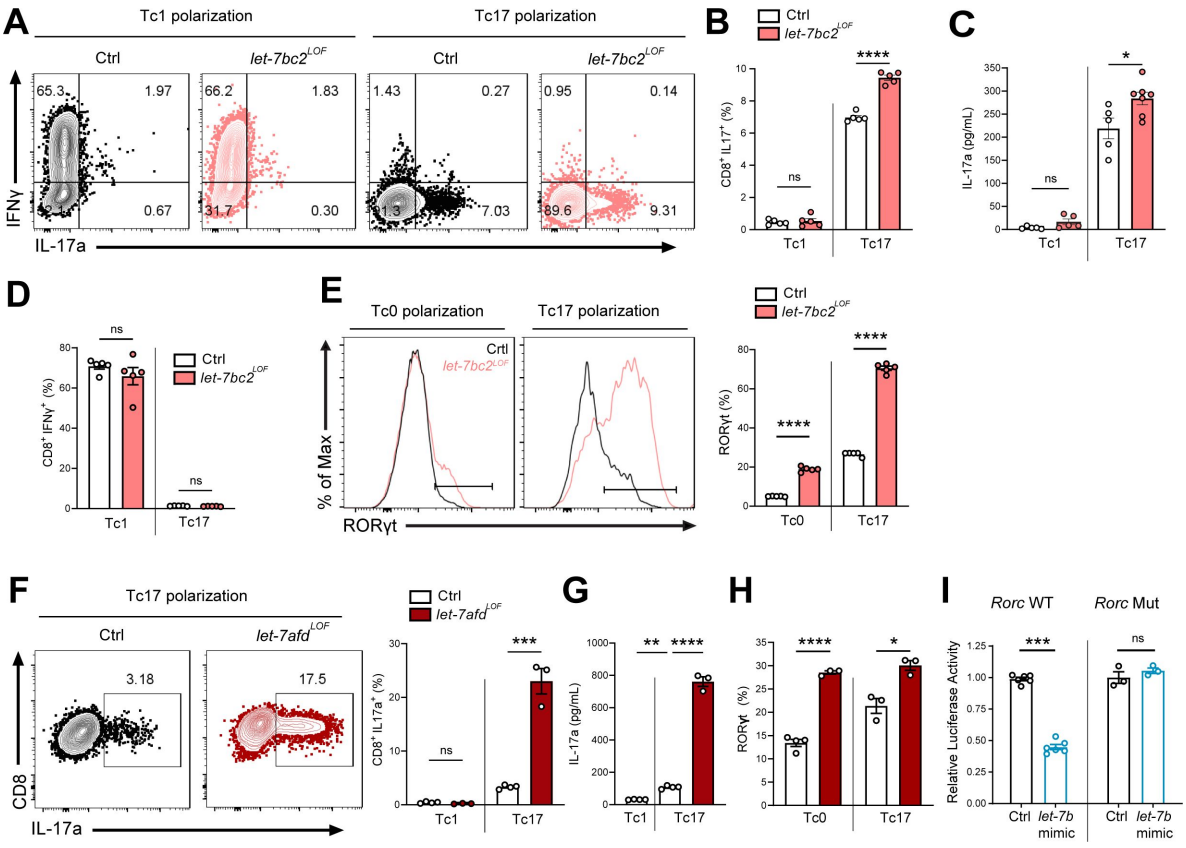

Figure 5.

Let-7 restricts Tc17 *in vitro* differentiation in part via direct targeting of *Rorc* mRNA.

(A) Representative flow plots of live TCRβ$^+$ CD8$^+$, IL-17a$^+$ and IFNγ$^+$ populations from Tc1 and Tc17 polarized naïve splenic CD8$^+$ cells from control and *let-7bc2*^{LOF} mice and (B) quantification of CD8$^+$IL-17a$^+$ cells (n=5 per group). (C) ELISA of IL-17a from the supernatant of Tc1 and Tc17 polarized control and *let-7bc2*^{LOF} cells (n=5-6 per group). (D) Flow quantification of CD8$^+$IFNγ$^+$ populations in Tc1 and Tc17 polarized control and *let-7bc2*^{LOF} cells (n=5 per group). (E) Representative flow plot and quantification of RORγt from Tc0 or Tc17 differentiated naïve splenic CD8$^+$ T cells isolated from control and *let-7bc2*^{LOF} mice (n=5 per group). (F) Representative flow plots of CD8$^+$IL-17a$^+$ population frequency and quantification of Tc17 polarized naive splenic CD8$^+$ cells of indicated mice polarized under Tc1 or Tc17 conditions. (G) ELISA of IL-17a from control, Tc1 (n=4), control Tc17 (n=4), and *let-7afd*^{LOF} Tc17 (n=3) polarized cells. (H) Quantification of RORγt from Tc0 or Tc17 *in vitro* polarized naive CD8$^+$ T cells from control and *let-7afd*^{LOF} mice (n=3-4 per group). (I) Control (*Rorc* WT) or binding site mutant (*Rorc* Mut) 3′ UTRs of *Rorc* were cloned downstream of the renilla luciferase reporter. Plasmids were cotransfected with either a control-miR (black bars) or *let-7b* mimic (blue bars) duplex into cultured cells. Reporter activity was measured 24 hours after transfection and normalized to firefly activity. Data are representative of two (H), three independent experiments (A-G), or carried out in triplicate (I) and displayed as mean±SEM using student's t-test. *p < 0.05, **p < 0.01, ***p <0.001, ****p<0.0001.

controls (**Figure 6B**). To determine whether enforced expression of *let-7* offered protection from experimental emphysema, *let-7*^{GOF} and control mice were treated with nCB and then examined for changes in lung pathology and T cell type 17 responses. The *let-7*^{GOF} mice did not exhibit any signs of lung inflammation or pathologic remodeling at baseline (**Figure 6C,D** and data not shown) Histopathologic analysis revealed a comparable degree of lung alveolar distension via morphometric measurements of MLI in nCB-treated *let-7*^{GOF} mice versus controls suggesting that enforced *let-7* expression is insufficient to protect the lung from emphysema (**Figure 6C,D**). On the other hand, evaluation of the IL-17^{+} response and RORγt levels in emphysematous lung T cells demonstrated that, in contrast to control nCB-treated mice, *let-7*^{GOF} mice exhibited dampened lung Tc17 and Th17 cell populations and were resistant to the induction of RORγt after nCB-exposure (**Figure 6E,F**). Taken together, our *let-7* LOF and GOF models demonstrate the necessity and sufficiency of *let-7* miRNA to act as a molecular brake to the type 17 T cell response through the direct regulation of RORγt, further our data suggests that nCB- or CS-mediated suppression of this braking mechanism furthers inflammation and exacerbates emphysema severity (**Figure 6G**).

Discussion

MiRNA expression-based studies of COPD patients and mice exposed to CS have reported downregulation of *let-7* miRNA expression in lung tissues (Conickx et al., 2017; Christenson et al., 2013b; Schembri et al., 2009). We and others explored the consequence of loss of *let-7* expression/activity with synthetic oligonucleotides, sponges, lentiviral antisense knockdown, or via ectopic delivery of *Lin28b* (Polikepahad et al., 2010; Viswanathan et al., 2008; Piskounova et al., 2011), but studies pinpointing the role of individual *let-7* clusters as potential drivers of lung inflammation and COPD within T cells remained elusive. In the present study, we established that the *let-7* miRNA family members encoded by the *let-7bc2*- and *let-7afd*-clusters are downregulated in T cells from lungs of emphysema patients and emphysematous mice that were exposed to CS or nCB. Correspondingly, we demonstrated that *in vivo* genetic ablation of *let-7bc2-cluster* further sensitized mice to lung tissue destruction and emphysema upon treatment with nCB or CS. Mechanistically, our studies suggests that *let-7bc2*-cluster prevents the emergence of CD8^{+} T cell differentiation into Tc17 cells during emphysema in part, by directly silencing of *Rorc*.

Tc17 cells are vital for defense against viral, fungal and bacterial infections and they have also been associated with inflammation in various human diseases such as multiple sclerosis, inflammatory bowel disease, and cancer (Huber et al., 2013; Globig et al., 2022; Corgnac et al., 2020). In accordance with the potential pathogenic role of Tc17 cells as drivers of COPD, several studies detected increased cell numbers in airways and tissues of COPD patients as well as lungs of smoke-exposed animal models (Y. Chang et al., 2011; Zhou et al., 2020; Duan et al., 2013). Other researchers also detected increased Tc17 subpopulations in tissues of COPD patients with infectious microbial exacerbations. In our earlier work to define the adaptive T cell immune responses in nCB induced COPD, we predominantly focused on the pathogenic role of Th17 cells, but did not examine Tc17 cells (You et al., 2015). Here we expand upon our prior observations, revealing that chronic exposure to nCB and elicitation of emphysema mice orchestrates the emergence and accumulation of Tc17 cells which may act in parallel with Th17 cells to promote tissue damage.

Prior research has shown the importance of both transcriptional and post-transcriptional regulatory control of RORγt expression in T cells (Ciofani et al., 2012; Donate et al., 2013; Medvedev et al., 1997). Altogether, our *in vivo* studies establish *let-7* as a new important link associated with regulation of RORγt and lung Tc17 differentiation in COPD. Our data also showed that *in vivo* conditional genetic ablation of individual *let-7* clusters in T cells stimulates a rise in RORγt protein expression in single-positive thymocytes and peripheral CD8^{+} and CD4^{+} T cells while enforced *let-7* activity leads to partial repression of RORγt in T cells. Despite these alterations in RORγt expression in our *let-7* T cell LOF mice, the mice did not exhibit spontaneous gross

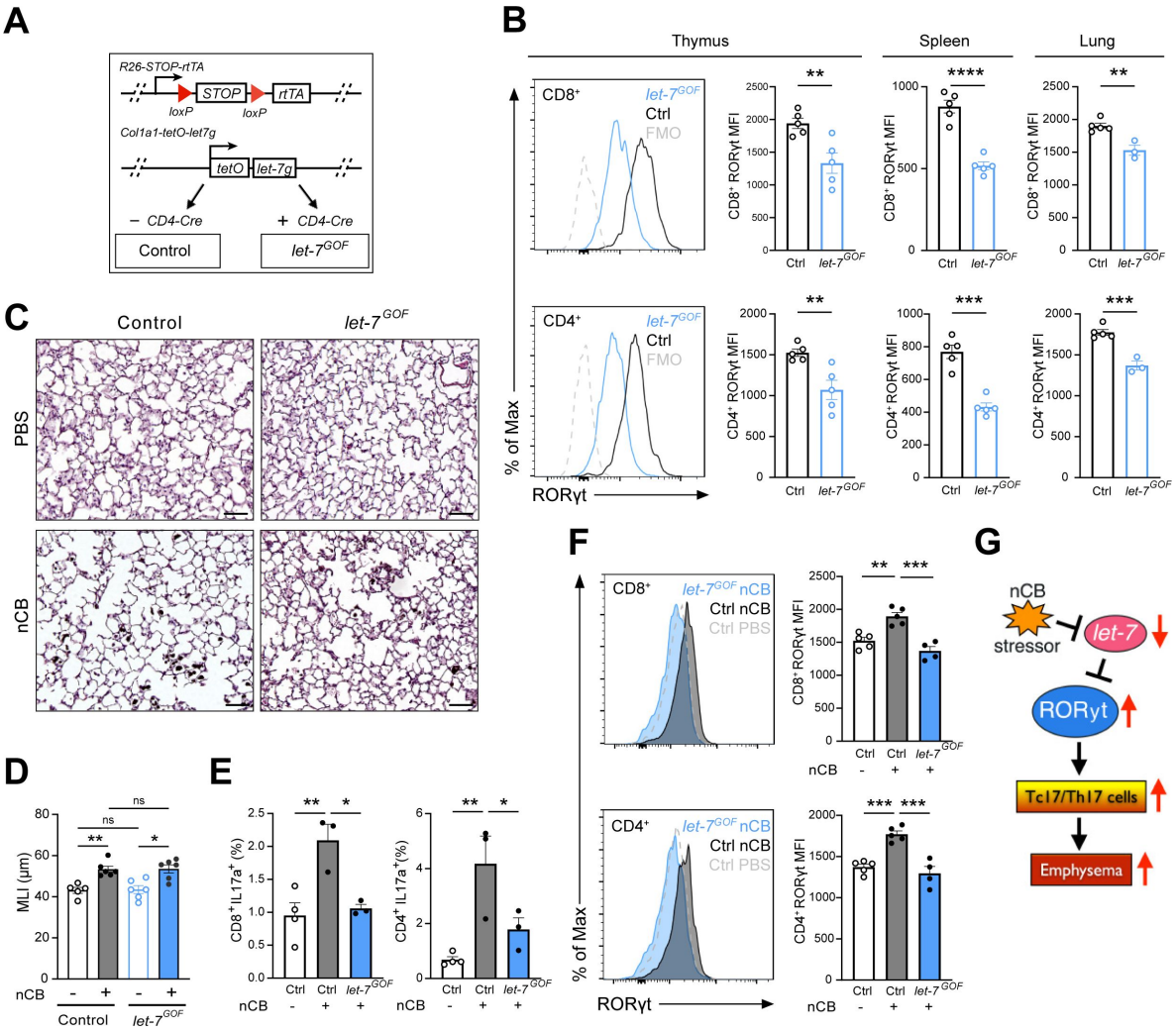

Figure 6.

Enforced *let-7* expression in T cells restrains induction of RORγt and Tc17/Th17 inflammation in lungs of nCB-exposed mice.

(A) Schematic outlining our T cell-inducible *let-7g* mouse model (*let-7*^{GOF}). (B) Flow analysis of RORγt expression in live, TCRβ^{+}CD8^{+} or CD4^{+} T cells from (B) naïve control and *let-7*^{GOF} mice in thymus, spleen, and lungs (n=3-5 per group). (C) Control and *let-7*^{GOF} mice were treated with PBS vehicle or nCB then analyzed. Representative H&E-stained lung sections from PBS- and nCB-exposed mice as indicated on each panel (x20 magnification; scale bars, 50μm) (D) MLI measurements from indicated mice (n=5-6 per group). (E) Flow analysis of lungs gated on live TCRβ^{+} CD8^{+} or CD4^{+} cells for (E) IL-17a^{+} population frequency (n=3-4 per group) or (F) RORγt expression by representative flow plot and MFI quantification (n=4-5 per group). (G) Figure model for *let-7*/RORγt axis in emphysema pathogenesis. Data are representative of two (B) or three (C-F) independent experiments and displayed as mean±SEM using student's t-test (B) or two-way ANOVA with Tukey's multiple correction (D-F). *p < 0.05, **p < 0.01, ***p <0.001, ****p<0.0001.

phenotypes in thymus, spleens, or lungs at baseline nor did they exhibit changes in Tc17/Th17 subpopulations. This may be due to the subtle and modest expression thresholding of RORγt detected in mice and/or residual *let-7* expression in T cells. On the other hand, and in agreement with our Tc17 and experimental emphysema data, we observed enhanced RORγt expression in lungs of *let-7*^{LOF} mice after treatment with nCB. We corroborated the importance of *let-7* activity in Tc17 differentiation of *ex vivo* cultured CD8^{+} T cells, as well as in the direct posttranscriptional control of RORγt, suggesting that this defect, is in part, direct and cell autonomous. We did not ascertain whether deletion of *let-7afd*-cluster is an equally or more effective modulator of experimentally induced emphysema than the *let-7bc2*-cluster. Nonetheless, we predict that under different cell stress contexts, the functions of *let-7* clusters do not fully overlap due to differential thresholding of mRNAs.

Prior studies have elucidated the relative and absolute quantities of individual *let-7* family members in murine thymocytes and peripheral T cells which range from ~2-30% (Pobezinskaya et al., 2019; Pobezinsky et al., 2015). Moreover, the same group reported that all *let-7* miRNAs are coordinately downregulation following antigen stimulation through the T cell receptor (Wells et al., 2017). Another recent study discerned a role for the lncRNA, *CCAT1* (*colon cancer-associated transcript 1*) as a molecular decoy or sponge in human bronchial epithelial cells which drives downregulation of *let-7c* following cigarette smoke extract exposure (L. Lu et al., 2017). Thus, it seems likely that complex synergistic transcriptional and post-transcriptional mechanisms contribute to downregulation of *let-7* activity in emphysematous T cells.

It is also important to note that *let-7* has been reported to exert potent effects by titrating the levels of multiple gene targets in mechanisms that contribute to Th17 inflammatory response and influence diverse set of processes including T cell activation, proliferation, differentiation, and cell homing (Angelou et al., 2020; Beachy et al., 2012; Bronevetsky et al., 2016; Pobezinskaya et al., 2019; Pobezinsky et al., 2015; Wells et al., 2017). A particular feature of these studies has been the utilization of *Lin28b* transgenic mice to block maturation and activity of entire *let-7* family to promote a *let-7* LOF function phenotype (Angelou et al., 2020; Piskounova et al., 2011; Pobezinskaya et al., 2019; Wells et al., 2023; Zhu et al., 2011). Furthermore, *Lin28b* was recently reported to also influence transcriptome-wide ribosome occupancy and global miRNA biogenesis (Tan et al., 2019). Thus, it is likely that *Lin28b* transgenic overexpression could give rise stronger phenotypes than we observed in our single cluster *let-7*^{LOF} mice. Nonetheless, unbiased omics-based methods will be useful to determine if other gene targets beyond RORγt synergistically potentiate the *in vivo* Tc17-response and emphysema phenotype in context of deletion of *let-7bc2*- cluster.

Tc17 cells play a major role in microbial infections, providing a potent anti-viral response (Hamada et al., 2009; Yeh et al., 2010), while viral infection has been an established factor in COPD exacerbations (Hewitt et al., 2016; Wedzicha, 2004). It will be interesting to determine whether loss of *let-7bc2*- or *let-7afd*-cluster activity in the T cell compartment contributes to COPD disease susceptibility in the context of viral exposure. Our experiments with *let-7* GOF were partially successful in limiting the emergence of Tc17 and Th17 in nCB-elicited emphysema. *Let-7*^{GOF} mice exhibited a reduction in RORγt expression levels and type 17 responses but were not protected from alveolar remodeling following nCB exposure. A potential limitation of the *let-7*^{GOF}transgenic model is that it expresses only the *let-7g* sequence which may render it less potent than the corresponding two mature forms transcribed from the *let-7bc2*-cluster. Additional studies will be required to ascertain whether other interventions that enhance *let-7* activity in T cells are successful in preventing or reversing COPD.

Materials and methods

Mice

Conditional knockout-ready floxed *let-7bc2* and *let-7afd* mice were generated using CRISPR gene editing in an isogenic C57BL/6 genetic background and were sequence verified for rigor. Mice were PCR genotyped from ear samples with primers flanking loxP sites (Supplementary Table 1). The *let-7bc2*$^{flox/flox}$; *CD4-cre* and *let-7afd*$^{flox/flox}$; *CD4-cre* mice were PCR genotyped. The *R26-STOP-rtTA*; *Col1a1-tet0-let-7* (*rtTA-iLet7)* mice were obtained from JAX Jax Stocks 023912 and 05670 and then bred to *CD4-Cre* were PCR genotyped with established JAX primers (Belteki et al., 2005; Zhu et al., 2011). Control *rtTA-iLet7* and the *let-7*^{GOF} mice were fed *ad libitum* with 200mg/kg of doxycycline-containing chow (Bio-Serv S3888) at weaning age primers (Belteki et al., 2005; Zhu et al., 2011). Syngeneic littermates served as controls for all mouse experiments. All mice were bred in the transgenic animal facility at Baylor College of Medicine. All experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine animal protocol (AN-7389) and followed the National Research Council Guide for the Care and Use of Laboratory Animals.

Human emphysema tissue samples and T cell isolation

Lung tissues were obtained from a total of 19 non-atopic current or former smokers with significant (>20 pack-years, one pack-year equals to smoking one pack of cigarettes per day each year) history of smoking who were recruited into studies from the chest or surgical clinics at Michael E. DeBakey Houston Veterans Affairs Medical Center hospitals (Supplementary Table 2) (Shan et al., 2009). Emphysema and non-emphysema control patients were diagnosed from CT scans according to the criteria recommended by the National Institutes of Health–World Health Organization workshop summary (Pauwels et al., 2001). Human single-cell suspensions were prepared from surgically resected lungs as previously described (Yuan et al., 2020; Grumelli et al., 2004). Briefly, fresh lung tissue was minced into 0.1 cm pieces in petri dishes and treated with 2 mg/mL of collagenase D (Worthington) for 1 hour at 37°C. Digested lung tissue was filtered through a 40-µm cell strainer (BD Falcon) followed by red blood cell lysis using ACK lysis buffer (Sigma-Aldrich) for 3 minutes to yield a single-cell suspension. $CD4^+$ T cells were selected from resultant suspensions by labeling with bead conjugated anti-CD4 for enrichment by autoMACs (Miltenyi Biotec). Studies were approved by the Institutional Review Board at Baylor College of Medicine and informed consent was obtained from all patients.

Human lung transcriptome data

A publicly available RNA-seq dataset from a Korean cohort GSE57148 was selected for the analysis (Kim et al., 2015). The raw FASTQ files of paired end reads representing the transcriptome of control and cases were retrieved from the GEO database at the National Centre for Biological Information (NCBI) through accession number GSE57148 and analyzed with R package for differential expression.

Cigarette smoke exposure model of pulmonary emphysema

To promote emphysema, mice were exposed to cigarette smoke using our custom designed whole-body inhalation system (Morales-Mantilla et al., 2020). In total, mice were exposed to four cigarettes (Marlboro 100's; Philip Morris USA) per day, five days a week, for four months as previously described (Shan et al., 2012).

nCB exposure model of pulmonary emphysema

Nano-sized particulate carbon black was prepared and administered as previously described (You et al., 2015; W. Lu et al., 2015). Dried nCB nanoparticles were resuspended in sterile PBS to a concentration of 10 mg/ml. Fifty µl of reconstituted nCB (0.5 mg) were intranasally delivered to deeply anesthetized mice on a schedule of three times a week for four weeks (total delivered dose of 6 mg). Lung histomorphometry and airway inflammation were assessed four weeks after the final nCB challenge. For histomorphometric analysis, mice lungs were fixed with 10% neutral-buffered formalin solution via a tracheal cannula at 25-cm H_2O pressure followed by paraffin embedding and tissue sectioning and stained with hematoxylin and eosin. Mean linear intercept (MLI) measurement of mouse lung morphometry were done as previously described (Shan et al., 2014; Morales-Mantilla et al., 2020). Briefly, this was done in a blinded fashion to mice genotypes from ten randomly selected fields of lung parenchyma sections. Paralleled lines were placed on serial lung sections and MLI was calculated by multiplying the length and the number of lines per field, divided by the number of intercepts (Morales-Mantilla et al., 2020).

BALF was collected by instilling and withdrawing 0.8 ml of sterile PBS twice through the trachea. Total and differential cell counts in the BALF were determined with the standard hemocytometer and HEMA3 staining (Biochemical Sciences Inc, Swedesboro, NJ) using 200 µL of BALF for cytospin slide preparation (Morales-Mantilla et al., 2020; W. Lu et al., 2015).

Cell isolation from murine lung tissue

Mouse lung tissue were cut into 2-mm pieces and digested with collagenase type D (2 mg/ml; Worthington) and deoxyribonuclease (DNase) I (0.04 mg/ml; Roche) for 1 hour in a 37°C incubator. Single-cell suspensions from lung digest, spleen, and thymus were prepared by mincing through 40-µm cell strainers then washing and resuspension in complete RPMI media. Mouse lung and spleen single-cell suspensions were additionally overlaid on Lympholyte M cell separation media (Cedarlane) as indicated in the manufacturer's protocol to purify lymphocytes. For murine *let-7* expression studies, lung single-cell suspensions were labeled with anti-$CD4^+$ or anti-$CD8^+$ magnetic beads and separated by autoMACS (Miltenyi Biotec), or $CD4^+CD8^+$ double positive cells purified from thymus single-cell suspensions by flow-cytometric sorting on FACS Aria (BD Biosciences).

In vitro polarization of $CD8^+$ T cells

$CD8^+$ naïve T cells were isolated from spleen using Mojosort Mouse CD8 Naïve T cell isolation Kit (Biolegend) and adjusted to a concentration of 1.0×10^6 cells/mL. Purified cells were activated with plate-bound anti-CD3 (1.5µg/mL) and complete RPMI media containing anti-CD28 (1.5µg/mL) and β-mercaptoethanol (50nM) for Tc0 polarization, or further supplemented with Tc1 [IL-2 (10ng/mL)] or Tc17 [TGFβ (2ng/mL), IL-6 (20ng/mL), anti-IFNγ (10µg/mL), IL-23 (20ng/mL), and IL-1β (5ng/mL)] polarization conditions for 72 hours (Flores-Santibáñez et al., 2018).

Elisa

Supernatant was collected from *in vitro* polarized murine $CD8^+$ T cells and centrifuged to remove cellular debris (W. Lu et al., 2015). Cytokine levels of IL-17A and IFNγ were quantified from collected supernatant using Mouse IL-17A Uncoated ELISA and Mouse IFN gamma Uncoated ELISA (Invitrogen) Kits, respectively, per the manufacturer's instructions with colorimetric analysis by the Varioskan LUX microplate reader (ThermoFisher).

Flow cytometric analysis

Cells used for *in vitro* or *in vivo* cytokine analysis were stimulated with PMA (20ng/mL; Sigma Aldrich), Ionomycin (1µg/mL; Sigma Aldrich), and Brefeldin A (2µg/mL; Sigma Aldrich) for 4 hours prior to flow staining (W. Lu et al., 2015). For intracellular staining, cells were fixed and

permeabilized using the Mouse FOXP3 Buffer Set (BD) per the manufacturer's protocol. The fluorophore-conjugated antibodies used in this study were as follows: Live/Dead Fix Blue (Invitrogen), CD3 PerCPCy5.5 (Biolegend), TCRb PE/Cy7 (Biolegend), CD4 PB (Biolegend), CD4 AF700 (Biolegend), CD8 BV650 (Biolegend), CD25 BV421 (Biolegend), FOXP3 AF488 (Biolegend), ROR gamma T PE (Invitrogen), TCF1 AF647 (Cell Signaling Technologies), TCF1 PE (Biolegend), IFNγ AF647 (Biolegend), IL17A FITC (Biolegend), IL17A PE (ebioscience). Representative flow cytometric gating and quantification strategy for detection of lung Th1/Th17 and Tc1/Tc17 cell populations is shown in Supplementary Figure 3. Samples were analyzed using BD LSR II flow cytometer (BD Biosciences) and FlowJo software (TreeStar).

RNA Isolation and Quantitative RT-PCR

RNA was isolated using miRNeasy (Qiagen) or RNeasy Mini Kit (Qiagen) in conjunction with the RNase-Free DNase (Qiagen) according to the manufacturer's instructions. cDNA of miRNAs and mRNAs were synthesized using TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher) and High-Capacity cDNA Reverse Transcription Kit Real-Time PCR system (Applied Biosystems). 18S and snoRNA-202 were used to normalize mRNA and miRNA expression respectively (W. Lu et al., 2015). Quantitative RT-PCR data were acquired on 7500 Real-Time PCR System or StepOne Real-Time PCR System (Applied Biosystems) with the following TaqMan probes: *hsa-let-7a-5p* [478575_mir], *hsa-let-7b-5p* [478576_mir], *hsa-let-7d* [478439_mir], *hsa-let-7f* [478578_mir], *pri-let7a1/f1/d* [44411114, arfvmhy], *pri-let7b/c2* [4441114, areptx2], *Mmp9* [Mm00442991], *Mmp12* [Mm00500554].

Luciferase reporter assays

Genomic fragment containing the murine *Rorc* 3'UTR was cloned into psiCHECK2 luciferase reporter plasmid (Promega). This construct was also used to generate the *let-7* 'seed' deletion mutant derivative using the QuikChange Multi Site Mutagenesis Kit (catalog 200514-5, Stratagene). 3T3 mouse embryonic fibroblasts (MEFs) were transfected using Oligofectamine (Invitrogen) with 100 ng of psiCheck-2 plasmid containing wild-type or mutant 3'UTR, along with the miRNA control or *let-7b* duplex (Dharmacon) at a final concentration of 6 nM (Gurha et al., 2012) (Supplementary Table 1). Reporter activity was detected with the Dual-Luciferase Reporter Assay System (Promega).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 10.0.1 software. Statistical comparison between groups was performed using the unpaired Student's t-test, two-way analysis of variance (ANOVA) with Tukey's or Sidak's correction, and Mann-Whitney Test when indicated. A P-value less than 0.05 was considered statistically significant; ns indicates not significant. Statistical significance values were set as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001. Data are presented as means ± SEM. P-value and n can be found in the main and supplementary figure legends.

Competing interest statement

The authors declare no competing interests.

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Author contributions

P.E., X.H., D.B.C, F.K., and A.R. conceptualized experiments and interpreted results. P.E., X.H., M.J.S., H.T., M.A.P., and S.L.L. acquired the data, M.J.R. provided bioinformatics analyses. P.E. and A.R. wrote the manuscript.

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Reviewer #1 (Public Review):

Summary:

Inflammatory T cells have been recognized to play an important role in human COPD lung tissue and animal models of emphysema. The authors have previously identified that Th17 cells regulate chronic inflammatory diseases, including in mice exposed to smoke or nanoparticulate carbon black (nCB). Here, the authors interrogate the role of Tc17 cells using similar mouse models. Investigating let-7 miRNA, which induces antigen-presenting cell activation and T cell-mediated Th17a inflammation, they show that the master regulator of Tc17/Th17 differentiation, RAR-related orphan receptor gamma t (RORγt), is a direct target of let-7 miRNA in T cells. Because RORγt expression is elevated in COPD patients and in mouse models of COPD, the authors generate a Let-7 overexpressing mouse in T cells and reduce RORγt expression and Th17 and Tc17 cell recruitment in nCB-exposed mice.

Strengths:

The authors use a previously published RNA-seq dataset (GSE57148) from lungs of control and COPD subjects to explore the involvement of Let-7 in emphysema. They further evaluate Let-7a expression by qPCR in lung tissue samples of smokers with emphysema and non-emphysema controls. Moreover, expression of Let-7a, Let-7b, Let-7d, and Let-7f in purified CD4+ T cells were inversely correlated with emphysema severity lungs. Similar findings were found in their mouse models (CS or nCB) in both lung tissue and isolated lung CD4+ and CD8+ T cells, with reduced let-7afd and let-7bc2 expression.

Using mice harboring a conditional deletion of the let-7bc2 cluster in all T cells (let-7bc2LOF) derived from the CD4+CD8+ double-positive stage, the authors show enhanced emphysema in nCB- or CS-exposed mice with enhanced recruitment of macrophages and neutrophils to the lung. While CD8+IL17a+ Tc17 cells and CD4+ IL17a+ Th17 cells were increased in nCB-exposed control animals, only let-7bc2LOF mice showed an increase in CD8+IL17a+ Tc17 cells. Further, unexposed let-7bc2LOF and let-7afdLOF mice expressed greater RORγt expression in both CD8+ and CD4+ T cells.

Generating a let-7 gain of function mouse with overexpression of let-7g in thymic double-positive-derived T cells, protein levels of RORγt were suppressed in CD8+ and CD4+ T cells of let-7GOF mice relative to controls. Let-7GOF mice treated with nCB showed similar lung alveolar distension as controls suggesting that increased let-7 expression does not protect the lung from emphysema. However, let-7GOF mice showed reduced lung Tc17 and Th17 cell populations and were resistant to the induction of RORγt after nCB exposure.

Weaknesses:

Limited data is shown on the let-7afdLOF mice. Does this mouse respond similarly to nCB as the let-7bc2LOF.
Because the authors validate their findings from a previously published RNA-seq dataset in subjects with and without emphysema, the authors should include patient demographics from the data presented in Figure 1C-D.
To validate their mouse models, the absence of Let-7 or enhanced Let-7 expression needs to be shown in isolated T cells from exposed mice.
In Figure 3, the authors are missing the unexposed let-7bc2LOF group from all panels. This is again an issue in Figure 6 with the let-7GOF.
Because the GOF mouse enhances Let-7g within T cells, the importance of Let-7g should be determined in human subjects. Why did the authors choose to overexpress Let-7g, the

rationale is not clear.
The purity of the CD4+ and CD8+ T cells is not shown and the full gating strategy should be included.
The authors indicate that Tc17 and Th17 T cells were reduced in the GOF mouse, it remains unclear if macrophage or neutrophil recruitment is altered in GOF mice.

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Reviewer #2 (Public Review):

Summary:

This valuable study characterizes the requirement for individual let-7 clusters to limit the generation of IL-17 producing CD8 T cells and the severity of emphysema in mouse models. Mature let-7 family miRNAs originate from multiple loci, several of which have been reported and/or are reported here to be downregulated in emphysematous lung tissue and/or lung T cells. The results provided are convincing but incomplete, as the let-7 cluster with the most convincing effects on T cell cytokine production is not tested for effects on disease pathogenesis.

Let-7 family miRNAs are largely redundant in function and originate from multiple genomic loci ("clusters"). Erice et al demonstrate that two individual clusters (let7afd and let7bc2) in mice regulate the generation of IL-17 producing CD8 T cells in vitro and in vivo in a model of emphysema. These cells also express higher levels of the IL-17-inducing transcription factor RORgt, encoded by Rorc, which the authors demonstrate to be a direct target of let-7. Since multiple let-7 family miRNAs are downregulated in T cells and lung tissue in emphysema, these data support a model in which reduced let-7 allows increased IL-17 production by T cells, contributing to disease pathogenesis.

Strengths:

The inclusion of miRNA and pri-miRNA expression data from sorted human lung T cells as well as mouse T cells from an emphysema model is a strength.

The study includes complementary loss of function and gain of function experimental systems to test the effect of altered let-7 function, though it should be noted that these involved different let-7 family members and did not yield simple, complementary results for all experimental outcomes.

The most important finding is that deletion of just one let-7 cluster ("Let7bc2") is sufficient to exacerbate emphysema in the nCB and CS models.

Weaknesses:

The human miRNA expression data that motivate functional analyses used sorted CD4+ T cells. The authors note that prior work on let-7 showed that it regulates Th17 (CD4) responses, yet this study's functional analyses are all focused on Tc17 (CD8) T cells. Data in this paper show that Tc17 cells are far less numerous than Th17 cells in the nCB and CS models of emphysema.

Compared with Let7bc2 deletion, Let7afd deletion had a much larger effect on IL17 production by CD8 T cells in vitro, and it also had a larger effect on RORgt expression in untreated mice in vivo, especially in the lung. In the revised manuscript, the authors show that let7afdLOF mice have normal numbers of CD4 and CD8 T cells in the thymus and peripheral lymphoid organs and do not exhibit lung histopathology or inflammatory changes at baseline at least up to 6 months of age. As such, they are set up perfectly to test the

requirement for Let7afd in the nCB and/or CS models. These experiments would add strength to the core novelty of this work - demonstration of the functional importance of individual let-7 clusters.

The authors could do more to explain the complexity of the let7 miRNA family and the genomic clusters examined in this study. In particular, it would help to know the relationship between mouse Let7bc2 and corresponding human Let7 clusters. It would also be very helpful to know the relative expression of each mature let-7 family member in Tc17 cells. Are mature miRNAs derived from the Let7afd cluster more or less abundant?

The provided evidence for the effect of Let7GOF has an important caveat that came to light during review. Let7g overexpression caused a marked reduction in Rorgt expression in T cells at baseline and in the setting of nCB challenge, and it reduced the frequency of IL17+ producing CD8 T cells in the lung to baseline levels. Yet there was no change in the MLI measurement of histopathology. However, the responses in the experiment shown in Fig. 6C-D are quite muted compared to those shown in Figure 2. In the response to reviewers, the authors speculate that an anti-inflammatory of doxycycline, required for induction of Let7g in this model, "could account for the differences in the magnitude of emphysemic response".

Although RORgt is a great candidate to have direct effects on IL-17 expression, the mechanistic understanding of let-7 action on T cell differentiation and cytokine production is limited to this single target. As noted in the discussion, others have identified cytokine receptor targets that may play a role, but it is also likely others among the many targets of let-7 also contribute.

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Author response:

The following is the authors' response to the original reviews.

We would like to thank the editors and reviewers for providing feedback and suggestions for our manuscript.

In response to reviewers comments we changed several main Figures and added new tables and supplementary figures. We also made edits to the Discussion.

> ***Reviewer #1 (Public Review):***
>
> *Weaknesses:*
>
> *Limited data is shown on the let-7afdLOF mice. Does this mouse respond similarly to nCB as the let-7bc2LOF.*

In the revised manuscript, we have added a baseline lung phenotypic assessment for the let-7afdLOF mice up to 6-months of age within Figure 4-figure supplement 1. The data supports our original statement and observation that let-7afdLOF mice do not exhibit lung pathology, inflammation, or changes in T cell subsets at baseline. Our view is that current manuscript addresses the importance of let-7bc2-cluster in experimental emphysema and the let-7afd-cluster mice is used to validate Rorc as a direct target of let-7. In the future, new grant funding will make it possible to ascertain whether absence of the let-7afd-cluster also sensitizes mice to experimentally induced emphysema.

> *Because the authors validate their findings from a previously published RNA-seq dataset in subjects with and without emphysema, the authors should include patient demographics from the data presented in Figure 1C-D.*

We thank the reviewers for their recommendation. In address of this, the revised manuscript contains a new Supplementary Table 1 with the human subject demographic information that corresponds with Figure 1D.

> *To validate their mouse models, the absence of Let-7 or enhanced Let-7 expression needs to be shown in isolated T cells from exposed mice.*

In the case of let-7bc2-cluster, we have included Figure 2-figure supplement 2 which shows pri-let7bc2 expression assessed by qPCR from selected CD8+ lung T cells of control and let-7bc2LOF mice exposed to PBS vehicle or nCB. The let-7g GOF model used in our studies has been validated for the induction of let-7g in thymic and peripheral T cells and elicitation of gain-of-function phenotypes (Pobezinskaya et al. 2019; Angelou et al. 2020; Wells et al. 2023).

> *In Figure 3, the authors are missing the unexposed let-7bc2LOF group from all panels.*

We emphasize that our exhaustive characterization of control and let-7bc2LOF mice in absence of challenge showed no phenotype. The baseline data was collectively shown in Figure 2-figure supplement 1.

> *Why did the authors choose to overexpress Let-7g, the rational is not clear?*

We concur that ideal GOF experiments can be carried out with let-7b or let-7c. Unfortunately, let-7b/c2 transgenic mice are not currently available, so we elected to use the well characterized let-7g T cell GOF mouse model (Pobezinskaya et al. 2019; Angelou et al. 2020; Wells et al. 2023). Furthermore, it is worth noting that the binding/seed sequence of let-7g is identical to let-7a/b/c and other members. Nonetheless, we have edited our Discussion section to reflect this as a potential caveat that can confound the utilization of this let-7GOF mouse model.

> *The purity of the CD4+ and CD8+ T cells is not shown and the full gating strategy should be included.*

In the revision, we included the flow gating strategy and display the representative population with purities in Supplementary Figure 1 of the revised manuscript.

> ***Reviewer #2 (Public Review):***
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> *Weaknesses:*
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> *The functional analyses are unusually focused on IL-17 producing CD8 T cells, but it is not made clear whether these cells are an important player in emphysema pathogenesis in the nCB and CS models. The data shown reveal that they are far less numerous than IL-17-producing CD4 T cells. It is also notable that the Figure 1 expression data from human subjects used sorted CD4+ T cells. And as the author mentioned, prior work on let-7 showed that it regulated Th17 (CD4) responses.*

As we showed that the let-7bc2LOF had enhanced the Tc17 cell population without any significant impact on Th17 cells, we elected to focus our analysis on this population. Furthermore, the connection of let-7 with the generation of a Tc17 inflammatory response is a novel finding, which so far remained unappreciated in the field and instigates new lines of inquiry.

> *Compared with Let7bc2 deletion, Let7afd deletion had a much larger effect on IL17 production by CD8 T cells in vitro, and it also had a larger effect on RORgt expression in*

> *untreated mice in vivo, especially in the lung. It would be valuable to more thoroughly characterize the let7afd mice. RORgt expression should be shown in the in vitro assays. In the results, the authors state that let7afdLOF mice "did not exhibit lung histopathology nor inflammatory changes" up to 6 months of age. Similarly, it is stated in the conclusion that "the let-7afdLOF mice ... did not exhibit changes in Tc17/Th17 subpopulations" in vivo. All these data should be shown, and if no baseline changes are apparent, then I also recommend challenging these mice with nCB and/or cigarette smoke.*

We concur that additional phenotypic characterization on the let-7afdLOF mice will contribute valuable information in the future. Reviewer 1 had a similar comment. As described above in response to Reviewer 1, we added comprehensive phenotypic analysis of let-7afdLOF mice within Figure 4-figure supplement 1 in the revised manuscript. The new data indicates that there is no overt lung pathology in the let-7afdLOF mice despite the subtle induction of RORγt expression in T cells. Furthermore, we have now included flow cytometric analysis of RORγt expression from in vitro polarized Tc0 and Tc17 cells from let-7afdLOF mice within revised Figure 5H.

> *This brings up the larger issue of redundancy among the let-7 family members and genomic clusters. This should be discussed, including some explanation of the relative expression of each mature family member in T cells, and how that maps to the clusters studied here (and those that were not investigated). It would also be helpful to explain the relationship between mouse Let7bc2 and human Let7a3b, since Let7bc2 is the primary focus of emphysema experiments in this manuscript. This is especially important because the study of individual let-7 clusters is the core novelty of this body of work, as described in the first paragraph of the discussion. The regulation of let-7 expression has been reported before and its functional role has been investigated with a variety of tools.*

We appreciate the interest and suggestion to expand the discussion on the let-7 family and their expression regulation. To address these points, we included additional references and expanded the Discussion section of the revised manuscript.

> *Let7g overexpression caused a marked reduction in Rorgt expression in T cells at baseline and in the setting of nCB challenge, and it reduced the frequency of IL17+ producing CD8 T cells in the lung to baseline levels. Yet there was no change in the MLI measurement of histopathology. Is this a robust result? The responses in the experiment shown in Fig. 6C-D are quite muted compared to those shown in Figure 2. The latter also shows a larger number of replicates, and it is unclear whether the data in 6D include measurement from all of the mice tested (e.g. pooled from 2 small experiments) or only mice from one experiment.*

We appreciate the reviewer inquiry into the data presented in Figure 6C-D. The data is representative of a single experiment and the number of experiments has been added to the revised Figure 6 legend. We note that all let-7GOF and associated control mice in Figure 6 are exposed to doxycycline as part of the let7g induction model, whereas mice in Figure 2 are not. It has been previously reported that doxycycline, a member of the tetracycline family of molecules, has anti-inflammatory properties (Di Caprio et al. 2015), which we speculate could account for the differences in the magnitude of emphysemic response.

> ***Reviewer #3 (Public Review):***
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> *Weaknesses:*
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> *The authors show no change in frequencies of Treg cells in let-7bc2LOF mice exposed to nCB. Do these Treg cells also express higher levels of RORgt and IL-17? The major question that was not addressed in this study is how let-7 expression is regulated in*

> *emphysema. The other recommendation is that the authors include the sequences of the let-7 mimic oligos used in the luciferase assay.*

We did not have the opportunity to address whether RORγt is in fact also upregulated in Treg cells. It remains unclear what upstream mechanisms drive the downregulation of the let-7 clusters in T cells with exposure to smoke/nCB. However, we agree that this an important question and we therefore updated the Discussion section of manuscript by including several citations that could explain how let-7 clusters become repressed in a coordinated fashion. Regarding the last point, the sequence of the duplex used in luciferase assay corresponds to the canonical mature let-7b in NCBI and has been added to Supplementary Table 3.

> ***Reviewer #2 (Recommendations For The Authors):***
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> *The authors state that "Recent evidence suggests the let-7 family is downregulated in patients with COPD, however, how they cause emphysema remains unclear." This should be reworded. Its downregulation in disease does not necessarily indicate that let-7 causes emphysema. Also, recommend rewording "Overall, our findings shed light on the let-7/RORγt axis as a braking and driving regulatory circuit in the generation of Tc17 cells..." What does it mean to be a "braking and driving" circuit? These terms seem contradictory.*

We recognize that the sentences were not phrased clearly. We have rephrased these statements as “Recent evidence suggests the let-7 miRNA family is downregulated in patients with COPD, however, whether this repression conveys a functional consequence in emphysema pathology has not been elucidated.” and “Overall, our findings shed light on the let-7/RORγt axis with let-7 acting as a molecular brake in the generation of Tc17 cells...”

> *Experimental details are needed for the human miRNA expression studies. Too little information is provided in the methods section, and the article cited there (Yuan et al 2020) is not listed in the bibliography.*

We expanded the Materials and Methods section for the collection, isolation, and qPCR analysis of human subject lung T cells. We have corrected the bibliography and added the missing citation.

> *The claim of novelty for miRNA-mediated silencing of Rorc in the discussion section is unnecessary and incorrect (https://pubmed.ncbi.nlm.nih.gov/23359619).*

Thank you for bringing the publication to our attention. Close inspection of this publication indicates that the authors did not experimentally validate Rorc as a direct target of let-7 itself. Plus the work was limited to immortalized in vitro cell cultures. We amended the sentence in the Discussion section highlighting the novelty of our findings which is the demonstration of Rorc as an in vivo target of let-7 in T cells.

Citations

Angelou, Constance C., Alexandria C. Wells, Jyothi Vijayaraghavan, Carey E. Dougan, Rebecca Lawlor, Elizabeth Iverson, Vanja Lazarevic, et al. 2020. “Differentiation of Pathogenic Th17 Cells Is Negatively Regulated by Let-7 MicroRNAs in a Mouse Model of Multiple Sclerosis.” Frontiers in Immunology 10: 3125. https://doi.org/10.3389/fimmu.2019.03125.

Di Caprio, Roberta, Serena Lembo, Luisa Di Costanzo, Anna Balato, and Giuseppe Monfrecola. 2015. “Anti-Inflammatory Properties of Low and High Doxycycline Doses: An in Vitro Study.” Mediators of Inflammation 2015: 329418. https://doi.org/10.1155/2015/329418.

Pobezinskaya, Elena L., Alexandria C. Wells, Constance C. Angelou, Eric Fagerberg, Esengul Aral, Elizabeth Iverson, Motoko Y. Kimura, and Leonid A. Pobezinsky. 2019. “Survival of Naïve T Cells Requires the Expression of Let-7 miRNAs.” Frontiers in Immunology 10 (May). https://doi.org/10.3389/fimmu.2019.00955.

Wells, Alexandria C., Kaito A. Hioki, Constance C. Angelou, Adam C. Lynch, Xueting Liang, Daniel J. Ryan, Iris Thesmar, et al. 2023. “Let-7 Enhances Murine Anti-Tumor CD8 T Cell Responses by Promoting Memory and Antagonizing Terminal Differentiation.” Nature Communications 14 (1): 5585. https://doi.org/10.1038/s41467-023-40959-7.