TITLE

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

AUTHORS

Phillip A. Erice[1,2], Xinyan Huang[2,7], Matthew J. Seasock[1,2], Matthew J. Robertson[3], Hui-Ying, Tung[4], Melissa A. Perez-Negron[2], Shivani L. Lotlikar[2], David B Corry[2,4,6], Farrah Kheradmand[4,5,6], Antony Rodriguez[2,6*]

[1]Immunology Graduate Program, Baylor College of Medicine, Houston, TX, 77030.

[2]Department of Medicine, Immunology & Allergy Rheumatology, Baylor College of Medicine Houston TX, 77030.

[3]Dan Duncan Comprehensive Cancer Center, Baylor College of Medicine Houston, TX, 77030.

[4]Department of Pathology and Immunology, Baylor College of Medicine Houston, TX, 77030.

[5]Department of Medicine, Section of Pulmonary and Critical Care, Baylor College of Medicine. Houston, TX, 77030.

[6]Center for Translational Research on Inflammatory Diseases, Michael E. Debakey, Baylor College of Medicine, Houston, TX, 77030.

[7]Current address, Department of Pulmonary and Critical Care Medicine, The First Affiliated Hospital of Sun Yat-sen University. Guangzhou, Guangdong Province, P.R. China

Author Correspondence: antony.rodriguez@bcm.edu

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.

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

126 emphysema samples relative to controls (Figure 1C). Because *Let-7* has been shown to
127 participate in IL-17^{+} T cell responses (Angelou et al., 2020; Guan et al., 2013; Newcomb et al.,
128 2015), we next sought to determine if the expression pattern of *Mirlet7ahg* and *Mirlet7bhg*-derived
129 *Let-7* members are impaired in purified CD4^{+} T cells from emphysematous lungs. In support of
130 our original hypothesis, the CD4^{+} T cell expression of *Let-7a*, *Let-7b*, *Let-7d*, and *Let-7f* were all
131 inversely correlated with more severe emphysema distribution in the lungs as determined by CT
132 scan (Figure 1D).

133 Next, we elucidated *let-7a1/let-7f1/let-7d-* and *let-7b/let-7c2*-clusters expression (herein
134 referred to as *let-7afd* and *let-7bc2* respectively) in murine models of CS- or nCB-induced
135 emphysema respectively (Figure 1E). Paralleling our observations in human COPD and
136 emphysema, mice with CS- or nCB-induced emphysema exhibited reduced expression levels of
137 *pri-let7a1/f1/d* and *pri-let7b/c2* transcripts in the lung and from isolated lung CD4^{+} and CD8^{+} T
138 cells (Figure 1F-H). Collectively, our expression results indicate suppression of *let-7afd* and *let-*
139 *7bc2*-clusters in the lung and T cells in human and pre-clinical models of emphysema.

140

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

143 To investigate the *in vivo* requirement of the *let-7bc2*-cluster within T cells, we generated
144 conditional ready floxed mice (*let-7bc2*$^{flox/flox}$). We then crossed *let-7bc2*$^{flox/flox}$ mice with *CD4-Cre*
145 mice to generate *let-7bc2*$^{flox/flox}$; *CD4-Cre* LOF mice (denoted as *let-7bc2*^{LOF} mice hereafter)
146 (Figure 2A). This approach allowed us to conditionally delete the *let-7bc2*-cluster in all T cells
147 derived from the CD4^{+}CD8^{+} double-positive (DP) stage (P. Lee et al., 2001; Shi & Petrie, 2012).
148 We confirmed that *let-7bc2*^{LOF} mice exhibit robust conditional deletion of the *let-7bc2*-cluster in
149 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.

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

198 inflammation in emphysema, the loss of *let-7bc2*-cluster is intrinsic for the potentiation of T cells
199 towards Tc17 differentiation.

200

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

203 We utilized the TargetScan predictive algorithm to identify putative *let-7* miRNA targets
204 that are known to control the IL-17-mediated T cell response (Agarwal et al., 2015). This analysis
205 revealed that the 3'UTR region of *Rorc*, encoding RORγt, contains an evolutionarily conserved
206 and complementary motif for the *let-7* miRNA family (Figure 4A). Thus, we examined if *let-7bc2*-
207 cluster loss in T cells would stimulate and enhance RORγt. Initially, we carried out flow cytometric
208 quantification for RORγt in thymocyte, splenic, and lung T cells of naïve control and *let-7bc2*^{LOF}
209 mice up to 6-months of age. Our interrogation of RORγt mean fluorescent intensity (MFI) by flow
210 cytometry showed induction of RORγt in single-positive CD8^{+} and CD4^{+} thymocytes, as well as
211 peripheral splenic CD8^{+} and CD4^{+} T cells (Figure 4B). However, RORγt levels appeared
212 unchanged in purified lung CD8^{+} T cells and CD4^{+} T cells of naive *let-7bc2*^{LOF} mice, alluding to a
213 compensatory effect in homeostatic lung T cells (Figure 4B). Since we and others have shown
214 that miRNAs are frequently associated with stress-dependent phenotypes, we posited that
215 emphysematous *let-7bc2*^{LOF} T cells are poised towards induction of RORγt and production of IL-
216 17^{+} subsets after challenge with nCB. Indeed, nCB-emphysematous *let-7bc2*^{LOF} mice exhibited
217 enhanced RORγt protein levels in both CD8^{+} and CD4^{+} T cells relative to control mice with
218 emphysema (Figure 4C).

219 Because we had found that the *let-7afd*-cluster is downregulated in T cells isolated from
220 COPD lungs in human and mice, and that the *let-7* family operates with some functional
221 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 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

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

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

343 It is also important to note that *let-7* has been reported to exert potent effects by titrating
344 the levels of multiple gene targets in mechanisms that contribute to Th17 inflammatory response
345 and influence diverse set of processes including T cell activation, proliferation, differentiation, and
346 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

424 embedding and tissue sectioning and stained with hematoxylin and eosin. Mean linear intercept
425 (MLI) measurement of mouse lung morphometry were done as previously described (Shan et al.,
426 2014; Morales-Mantilla et al., 2020). Briefly, this was done in a blinded fashion to mice genotypes
427 from ten randomly selected fields of lung parenchyma sections. Paralleled lines were placed on
428 serial lung sections and MLI was calculated by multiplying the length and the number of lines per
429 field, divided by the number of intercepts (Morales-Mantilla et al., 2020).

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

434

435 **Cell isolation from murine lung tissue**

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

446

447 ***In vitro* polarization of $CD8^+$ T cells**

448 $CD8^+$ naïve T cells were isolated from spleen using Mojosort Mouse CD8 Naïve T cell isolation
449 Kit (Biolegend) and adjusted to a concentration of $1.0x10^6$ 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).

476 **RNA Isolation and Quantitative RT-PCR**

477 RNA was isolated using miRNeasy (Qiagen) or RNeasy Mini Kit (Qiagen) in conjunction with the
478 RNase-Free DNase (Qiagen) according to the manufacturer's instructions. cDNA of miRNAs and
479 mRNAs were synthesized using TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher)
480 and High-Capacity cDNA Reverse Transcription Kit Real-Time PCR system (Applied Biosystems).
481 18S and snoRNA-202 were used to normalize mRNA and miRNA expression respectively (W. Lu
482 et al., 2015). Quantitative RT-PCR data were acquired on 7500 Real-Time PCR System or
483 StepOne Real-Time PCR System (Applied Biosystems) with the following TaqMan probes: *hsa-*
484 *let-7a-5p* [478575_mir], *hsa-let-7b-5p* [478576_mir], *hsa-let-7d* [478439_mir], *hsa-let-7f*
485 [478578_mir], *pri-let7a1/f1/d* [44411114, arfvmhy], *pri-let7b/c2* [4441114, areptx2], *Mmp9*
486 [Mm00442991], *Mmp12* [Mm00500554].

487

488 **Luciferase reporter assays**

489 Genomic fragment containing the murine *Rorc* 3'UTR was cloned into psiCHECK2 luciferase
490 reporter plasmid (Promega). This construct was also used to generate the *let*-7 'seed' deletion
491 mutant derivative using the QuikChange Multi Site Mutagenesis Kit (catalog 200514-5,
492 Stratagene). 3T3 mouse embryonic fibroblasts (MEFs) were transfected using Oligofectamine
493 (Invitrogen) with 100 ng of psiCheck-2 plasmid containing wild-type or mutant 3'UTR, along with
494 the miRNA control or *let-7b* duplex (Dharmacon) at a final concentration of 6 nM (Gurha et al.,
495 2012) (Supplementary Table 1). Reporter activity was detected with the Dual-Luciferase Reporter
496 Assay System (Promega).

497

498 **Statistical analysis**

499 Statistical analyses were performed using GraphPad Prism 10.0.1 software. Statistical
500 comparison between groups was performed using the unpaired Student's t-test, two-way analysis
501 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.

ACKNOWLEDGEMENTS

We thank Jason Heaney and Denise Lanza at BCM Genetically Engineered Rodents Core funded in part by NIH P30 CA125123; Patricia Castro at Tissue Acquisition and Pathology Core funded in part by P30 CA125123; and Joel M. Sederstrom at the BCM and Cell Sorting Core with funding from the CPRIT Core Facility Support Award (CPRIT-RP180672) and NIH (CA125123 and RR024574). This work was supported by grants from the NHLBI (R01HL140398 to AR), the Gilson Longenbaugh Foundation (to A.R.), and NIEHS (T32 ES027801 to PE).

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.

REFERENCES

Adeloye, D., Song, P., Zhu, Y., Campbell, H., Sheikh, A., & Rudan, I. (2022). Global, regional, and national prevalence of, and risk factors for, chronic obstructive pulmonary disease (COPD) in 2019: A systematic review and modelling analysis. *The Lancet Respiratory Medicine*, *10*(5), 447–458. https://doi.org/10.1016/S2213-2600(21)00511-7

Agarwal, V., Bell, G. W., Nam, J.-W., & Bartel, D. P. (2015). Predicting effective microRNA target sites in mammalian mRNAs. *eLife*, *4*, e05005. https://doi.org/10.7554/eLife.05005

Angelou, C. C., Wells, A. C., Vijayaraghavan, J., Dougan, C. E., Lawlor, R., Iverson, E., Lazarevic, V., Kimura, M. Y., Peyton, S. R., Minter, L. M., Osborne, B. A., Pobezinskaya, E. L., & Pobezinsky, L. A. (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

Anthonisen, N. R., Connett, J. E., & Murray, R. P. (2002). Smoking and Lung Function of Lung Health Study Participants after 11 Years. *American Journal of Respiratory and Critical Care Medicine*, *166*(5), 675–679. https://doi.org/10.1164/rccm.2112096

Barnes, P. J. (2016). Inflammatory mechanisms in patients with chronic obstructive pulmonary disease. *Journal of Allergy and Clinical Immunology*, *138*(1), 16–27. https://doi.org/10.1016/j.jaci.2016.05.011

Beachy, S. H., Onozawa, M., Chung, Y. J., Slape, C., Bilke, S., Francis, P., Pineda, M., Walker, R. L., Meltzer, P., & Aplan, P. D. (2012). Enforced expression of Lin28b leads to impaired T-cell development, release of inflammatory cytokines, and peripheral T-cell lymphoma. *Blood*, *120*(5), 1048–1059. https://doi.org/10.1182/blood-2012-01-401760

Belteki, G., Haigh, J., Kabacs, N., Haigh, K., Sison, K., Costantini, F., Whitsett, J., Quaggin, S. E., & Nagy, A. (2005). Conditional and inducible transgene expression in mice through the combinatorial use of Cre-mediated recombination and tetracycline induction. *Nucleic Acids Research*, *33*(5), e51. https://doi.org/10.1093/nar/gni051

549 Beringer, A., Noack, M., & Miossec, P. (2016). IL-17 in Chronic Inflammation: From Discovery to
550 Targeting. *Trends in Molecular Medicine*, *22*(3), 230–241.
551 https://doi.org/10.1016/j.molmed.2016.01.001
552 Bhavani, S., Tsai, C.-L., Perusich, S., Hesselbacher, S., Coxson, H., Pandit, L., Corry, D. B., &
553 Kheradmand, F. (2015). Clinical and Immunological Factors in Emphysema Progression. Five-
554 Year Prospective Longitudinal Exacerbation Study of Chronic Obstructive Pulmonary Disease
555 (LES-COPD). *American Journal of Respiratory and Critical Care Medicine*, *192*(10), 1171–
556 1178. https://doi.org/10.1164/rccm.201504-0736OC
557 Bronevetsky, Y., Burt, T. D., & McCune, J. M. (2016). Lin28b regulates fetal Treg differentiation
558 through modulation of TGF-β signaling. *Journal of Immunology (Baltimore, Md. : 1950)*,
559 *197*(11), 4344. https://doi.org/10.4049/jimmunol.1601070
560 Chang, C.-Y., You, R., Armstrong, D., Bandi, A., Cheng, Y.-T., Burkhardt, P. M., Becerra-Dominguez,
561 L., Madison, M. C., Tung, H.-Y., Zeng, Z., Wu, Y., Song, L., Phillips, P. E., Porter, P., Knight, J.
562 M., Putluri, N., Yuan, X., Marcano, D. C., McHugh, E. A., … Kheradmand, F. (2022). Chronic
563 exposure to carbon black ultrafine particles reprograms macrophage metabolism and accelerates
564 lung cancer. *Science Advances*, *8*(46), eabq0615. https://doi.org/10.1126/sciadv.abq0615
565 Chang, Y., Nadigel, J., Boulais, N., Bourbeau, J., Maltais, F., Eidelman, D. H., & Hamid, Q. (2011). CD8
566 positive T cells express IL-17 in patients with chronic obstructive pulmonary disease. *Respiratory*
567 *Research*, *12*(1), 43. https://doi.org/10.1186/1465-9921-12-43
568 Chen, J., Wang, X., Schmalen, A., Haines, S., Wolff, M., Ma, H., Zhang, H., Stoleriu, M. G., Nowak, J.,
569 Nakayama, M., Bueno, M., Brands, J., Mora, A. L., Lee, J. S., Krauss-Etschmann, S., Dmitrieva,
570 A., Frankenberger, M., Hofer, T. P., Noessner, E., … Meiners, S. (2023). Antiviral CD8+ T-cell
571 immune responses are impaired by cigarette smoke and in COPD. *European Respiratory Journal*,
572 *62*(2). https://doi.org/10.1183/13993003.01374-2022
573 Christenson, S. A., Brandsma, C.-A., Campbell, J. D., Knight, D. A., Pechkovsky, D. V., Hogg, J. C.,
574 Timens, W., Postma, D. S., Lenburg, M., & Spira, A. (2013a). miR-638 regulates gene expression

networks associated with emphysematous lung destruction. *Genome Medicine*, *5*(12), 114. https://doi.org/10.1186/gm519

Christenson, S. A., Brandsma, C.-A., Campbell, J. D., Knight, D. A., Pechkovsky, D. V., Hogg, J. C., Timens, W., Postma, D. S., Lenburg, M., & Spira, A. (2013b). miR-638 regulates gene expression networks associated with emphysematous lung destruction. *Genome Medicine*, *5*(12), 114. https://doi.org/10.1186/gm519

Chu, S., Zhong, X., Zhang, J., Lao, Q., He, Z., & Bai, J. (2011). The expression of Foxp3 and ROR gamma t in lung tissues from normal smokers and chronic obstructive pulmonary disease patients. *International Immunopharmacology*, *11*(11), 1780–1788. https://doi.org/10.1016/j.intimp.2011.06.010

Ciofani, M., Madar, A., Galan, C., Sellars, M., Mace, K., Pauli, F., Agarwal, A., Huang, W., Parkurst, C. N., Muratet, M., Newberry, K. M., Meadows, S., Greenfield, A., Yang, Y., Jain, P., Kirigin, F. K., Birchmeier, C., Wagner, E. F., Murphy, K. M., … Littman, D. R. (2012). A Validated Regulatory Network for Th17 Cell Specification. *Cell*, *151*(2), 289–303. https://doi.org/10.1016/j.cell.2012.09.016

Conickx, G., Avila Cobos, F., van den Berge, M., Faiz, A., Timens, W., Hiemstra, P. S., Joos, G. F., Brusselle, G. G., Mestdagh, P., & Bracke, K. R. (2017). microRNA profiling in lung tissue and bronchoalveolar lavage of cigarette smoke-exposed mice and in COPD patients: A translational approach. *Scientific Reports*, *7*(1), Article 1. https://doi.org/10.1038/s41598-017-13265-8

Corgnac, S., Malenica, I., Mezquita, L., Auclin, E., Voilin, E., Kacher, J., Halse, H., Grynszpan, L., Signolle, N., Dayris, T., Leclerc, M., Droin, N., de Montpréville, V., Mercier, O., Validire, P., Scoazec, J.-Y., Massard, C., Chouaib, S., Planchard, D., … Mami-Chouaib, F. (2020). CD103+CD8+ TRM Cells Accumulate in Tumors of Anti-PD-1-Responder Lung Cancer Patients and Are Tumor-Reactive Lymphocytes Enriched with Tc17. *Cell Reports Medicine*, *1*(7), 100127. https://doi.org/10.1016/j.xcrm.2020.100127

600 Donate, P. B., Fornari, T. A., Macedo, C., Cunha, T. M., Nascimento, D. C. B., Sakamoto-Hojo, E. T.,
601 Donadi, E. A., Cunha, F. Q., & Passos, G. A. (2013). T cell post-transcriptional miRNA-mRNA
602 interaction networks identify targets associated with susceptibility/resistance to collagen-induced
603 arthritis. *PloS One*, *8*(1), e54803. https://doi.org/10.1371/journal.pone.0054803
604 Duan, M.-C., Tang, H.-J., Zhong, X.-N., & Huang, Y. (2013). Persistence of Th17/Tc17 Cell Expression
605 upon Smoking Cessation in Mice with Cigarette Smoke-Induced Emphysema. *Journal of*
606 *Immunology Research*, *2013*, e350727. https://doi.org/10.1155/2013/350727
607 Duan, M.-C., Zhang, J.-Q., Liang, Y., Liu, G.-N., Xiao, J., Tang, H.-J., & Liang, Y. (2016). Infiltration of
608 IL-17-Producing T Cells and Treg Cells in a Mouse Model of Smoke-Induced Emphysema.
609 *Inflammation*, *39*(4), 1334–1344. https://doi.org/10.1007/s10753-016-0365-8
610 Eisner, M. D., Anthonisen, N., Coultas, D., Kuenzli, N., Perez-Padilla, R., Postma, D., Romieu, I.,
611 Silverman, E. K., & Balmes, J. R. (2010). An Official American Thoracic Society Public Policy
612 Statement: Novel Risk Factors and the Global Burden of Chronic Obstructive Pulmonary
613 Disease. *American Journal of Respiratory and Critical Care Medicine*, *182*(5), 693–718.
614 https://doi.org/10.1164/rccm.200811-1757ST
615 *Findings from the Global Burden of Disease Study 2017*. (2019, January 4). Institute for Health Metrics
616 and Evaluation. https://www.healthdata.org/policy-report/findings-global-burden-disease-study-
617 2017
618 Flores-Santibáñez, F., Cuadra, B., Fernández, D., Rosemblatt, M. V., Núñez, S., Cruz, P., Gálvez-
619 Cancino, F., Cárdenas, J. C., Lladser, A., Rosemblatt, M., Bono, M. R., & Sauma, D. (2018). In
620 Vitro-Generated Tc17 Cells Present a Memory Phenotype and Serve As a Reservoir of Tc1 Cells
621 In Vivo. *Frontiers in Immunology*, *9*.
622 https://www.frontiersin.org/articles/10.3389/fimmu.2018.00209
623 Globig, A.-M., Hipp, A. V., Otto-Mora, P., Heeg, M., Mayer, L. S., Ehl, S., Schwacha, H., Bewtra, M.,
624 Tomov, V., Thimme, R., Hasselblatt, P., & Bengsch, B. (2022). High-dimensional profiling

625 reveals Tc17 cell enrichment in active Crohn's disease and identifies a potentially targetable
626 signature. *Nature Communications*, *13*(1), Article 1. https://doi.org/10.1038/s41467-022-31229-z
627 Grumelli, S., Corry, D. B., Song, L.-Z., Song, L., Green, L., Huh, J., Hacken, J., Espada, R., Bag, R.,
628 Lewis, D. E., & Kheradmand, F. (2004). An Immune Basis for Lung Parenchymal Destruction in
629 Chronic Obstructive Pulmonary Disease and Emphysema. *PLOS Medicine*, *1*(1), e8.
630 https://doi.org/10.1371/journal.pmed.0010008
631 Guan, H., Fan, D., Mrelashvili, D., Hao, H., Singh, N. P., Singh, U. P., Nagarkatti, P. S., & Nagarkatti, M.
632 (2013). MicroRNA let-7e is associated with the pathogenesis of experimental autoimmune
633 encephalomyelitis. *European Journal of Immunology*, *43*(1), 104–114.
634 https://doi.org/10.1002/eji.201242702
635 Gurha, P., Abreu-Goodger, C., Wang, T., Ramirez, M. O., Drumond, A. L., van Dongen, S., Chen, Y.,
636 Bartonicek, N., Enright, A. J., Lee, B., Kelm, R. J., Reddy, A. K., Taffet, G. E., Bradley, A.,
637 Wehrens, X. H., Entman, M. L., & Rodriguez, A. (2012). Targeted Deletion of MicroRNA-22
638 Promotes Stress-Induced Cardiac Dilation and Contractile Dysfunction. *Circulation*, *125*(22),
639 2751–2761. https://doi.org/10.1161/CIRCULATIONAHA.111.044354
640 Hamada, H., Garcia-Hernandez, M. de la L., Reome, J. B., Misra, S. K., Strutt, T. M., McKinstry, K. K.,
641 Cooper, A. M., Swain, S. L., & Dutton, R. W. (2009). Tc17, a Unique Subset of CD8 T Cells
642 That Can Protect against Lethal Influenza Challenge1. *The Journal of Immunology*, *182*(6),
643 3469–3481. https://doi.org/10.4049/jimmunol.0801814
644 Hewitt, R., Farne, H., Ritchie, A., Luke, E., Johnston, S. L., & Mallia, P. (2016). The role of viral
645 infections in exacerbations of chronic obstructive pulmonary disease and asthma. *Therapeutic*
646 *Advances in Respiratory Disease*, *10*(2), 158–174. https://doi.org/10.1177/1753465815618113
647 Hoenderdos, K., & Condliffe, A. (2013). The Neutrophil in Chronic Obstructive Pulmonary Disease. Too
648 Little, Too Late or Too Much, Too Soon? *American Journal of Respiratory Cell and Molecular*
649 *Biology*, *48*(5), 531–539. https://doi.org/10.1165/rcmb.2012-0492TR

650 Hogg, J. C., Chu, F., Utokaparch, S., Woods, R., Elliott, W. M., Buzatu, L., Cherniack, R. M., Rogers, R.
651 M., Sciurba, F. C., Coxson, H. O., & Paré, P. D. (2004). The Nature of Small-Airway Obstruction
652 in Chronic Obstructive Pulmonary Disease. *New England Journal of Medicine*, *350*(26), 2645–
653 2653. https://doi.org/10.1056/NEJMoa032158
654 Hu, G., Zhou, Y., Tian, J., Yao, W., Li, J., Li, B., & Ran, P. (2010). Risk of COPD From Exposure to
655 Biomass Smoke: A Metaanalysis. *CHEST*, *138*(1), 20–31. https://doi.org/10.1378/chest.08-2114
656 Huber, M., Heink, S., Pagenstecher, A., Reinhard, K., Ritter, J., Visekruna, A., Guralnik, A., Bollig, N.,
657 Jeltsch, K., Heinemann, C., Wittmann, E., Buch, T., Costa, O. P. da, Brüstle, A., Brenner, D.,
658 Mak, T. W., Mittrücker, H.-W., Tackenberg, B., Kamradt, T., & Lohoff, M. (2013). IL-17A
659 secretion by $CD8^+$ T cells supports Th17-mediated autoimmune encephalomyelitis. *The Journal*
660 *of Clinical Investigation*, *123*(1), 247–260. https://doi.org/10.1172/JCI63681
661 Ivanov, I. I., McKenzie, B. S., Zhou, L., Tadokoro, C. E., Lepelley, A., Lafaille, J. J., Cua, D. J., &
662 Littman, D. R. (2006). The orphan nuclear receptor RORgammat directs the differentiation
663 program of proinflammatory IL-17+ T helper cells. *Cell*, *126*(6), 1121–1133.
664 https://doi.org/10.1016/j.cell.2006.07.035
665 Ivanov, I. I., Zhou, L., & Littman, D. R. (2007). Transcriptional Regulation of Th17 Cell Differentiation.
666 *Seminars in Immunology*, *19*(6), 409–417. https://doi.org/10.1016/j.smim.2007.10.011
667 Izzotti, A., Calin, G. A., Arrigo, P., Steele, V. E., Croce, C. M., & De Flora, S. (2009). Downregulation of
668 microRNA expression in the lungs of rats exposed to cigarette smoke. *The FASEB Journal*, *23*(3),
669 806–812. https://doi.org/10.1096/fj.08-121384
670 Jin, Y., Wan, Y., Chen, G., Chen, L., Zhang, M.-Q., Deng, L., Zhang, J.-C., Xiong, X.-Z., & Xin, J.-B.
671 (2014). Treg/IL-17 Ratio and Treg Differentiation in Patients with COPD. *PLOS ONE*, *9*(10),
672 e111044. https://doi.org/10.1371/journal.pone.0111044
673 Kheradmand, F., Zhang, Y., & Corry, D. B. (2023). Contribution of adaptive immunity to human COPD
674 and experimental models of emphysema. *Physiological Reviews*, *103*(2), 1059–1093.
675 https://doi.org/10.1152/physrev.00036.2021

676 Kim, W. J., Lim, J. H., Lee, J. S., Lee, S.-D., Kim, J. H., & Oh, Y.-M. (2015). Comprehensive Analysis
677 of Transcriptome Sequencing Data in the Lung Tissues of COPD Subjects. *International Journal*
678 *of Genomics*, *2015*, 206937. https://doi.org/10.1155/2015/206937
679 Laniado-Laborín, R. (2009). Smoking and Chronic Obstructive Pulmonary Disease (COPD). Parallel
680 Epidemics of the 21st Century. *International Journal of Environmental Research and Public*
681 *Health*, *6*(1), Article 1. https://doi.org/10.3390/ijerph6010209
682 Lee, P. P., Fitzpatrick, D. R., Beard, C., Jessup, H. K., Lehar, S., Makar, K. W., Pérez-Melgosa, M.,
683 Sweetser, M. T., Schlissel, M. S., Nguyen, S., Cherry, S. R., Tsai, J. H., Tucker, S. M., Weaver,
684 W. M., Kelso, A., Jaenisch, R., & Wilson, C. B. (2001). A Critical Role for Dnmt1 and DNA
685 Methylation in T Cell Development, Function, and Survival. *Immunity*, *15*(5), 763–774.
686 https://doi.org/10.1016/S1074-7613(01)00227-8
687 Lee, S.-H., Goswami, S., Grudo, A., Song, L.-Z., Bandi, V., Goodnight-White, S., Green, L., Hacken-
688 Bitar, J., Huh, J., Bakaeen, F., Coxson, H. O., Cogswell, S., Storness-Bliss, C., Corry, D. B., &
689 Kheradmand, F. (2007). Antielastin autoimmunity in tobacco smoking-induced emphysema.
690 *Nature Medicine*, *13*(5), 567–569. https://doi.org/10.1038/nm1583
691 Li, H., Liu, Q., Jiang, Y., Zhang, Y., Xiao, W., & Zhang, Y. (2015). Disruption of Th17/Treg Balance in
692 the Sputum of Patients With Chronic Obstructive Pulmonary Disease. *The American Journal of*
693 *the Medical Sciences*, *349*(5), 392–397. https://doi.org/10.1097/MAJ.0000000000000447
694 Lu, L., Qi, H., Luo, F., Xu, H., Ling, M., Qin, Y., Yang, P., Liu, X., Yang, Q., Xue, J., Chen, C., Lu, J.,
695 Xiang, Q., Liu, Q., & Bian, Q. (2017). Feedback circuitry via let-7c between lncRNA CCAT1
696 and c-Myc is involved in cigarette smoke extract-induced malignant transformation of HBE cells.
697 *Oncotarget*, *8*(12), 19285–19297. https://doi.org/10.18632/oncotarget.15195
698 Lu, W., You, R., Yuan, X., Yang, T., Samuel, E. L. G., Marcano, D. C., Sikkema, W. K. A., Tour, J. M.,
699 Rodriguez, A., Kheradmand, F., & Corry, D. B. (2015). MicroRNA-22 Inhibits Histone
700 Deacetylase 4 to Promote T Helper-17 Cell-Dependent Emphysema. *Nature Immunology*, *16*(11),
701 1185–1194. https://doi.org/10.1038/ni.3292

Maeno, T., Houghton, A. M., Quintero, P. A., Grumelli, S., Owen, C. A., & Shapiro, S. D. (2007). CD8+ T Cells Are Required for Inflammation and Destruction in Cigarette Smoke-Induced Emphysema in Mice1. *The Journal of Immunology*, *178*(12), 8090–8096. https://doi.org/10.4049/jimmunol.178.12.8090

Mai, J., Virtue, A., Maley, E., Tran, T., Yin, Y., Meng, S., Pansuria, M., Jiang, X., Wang, H., & Yang, X.-F. (2012). MicroRNAs and other mechanisms regulate interleukin-17 cytokines and receptors. *Frontiers in Bioscience (Elite Edition)*, *4*, 1478–1495.

Medvedev, A., Chistokhina, A., Hirose, T., & Jetten, A. M. (1997). Genomic Structure and Chromosomal Mapping of the Nuclear Orphan Receptor RORγ (RORC) Gene. *Genomics*, *46*(1), 93–102. https://doi.org/10.1006/geno.1997.4980

Morales-Mantilla, D. E., Huang, X., Erice, P., Porter, P., Zhang, Y., Figueroa, M., Chandra, J., King, K. Y., Kheradmand, F., & Rodríguez, A. (2020). Cigarette Smoke Exposure in Mice using a Whole-Body Inhalation System. *JoVE (Journal of Visualized Experiments)*, *164*, e61793. https://doi.org/10.3791/61793

Newcomb, D. C., Cephus, J. Y., Boswell, M. G., Fahrenholz, J. M., Langley, E. W., Feldman, A. S., Zhou, W., Dulek, D. E., Goleniewska, K., Woodward, K. B., Sevin, C. M., Hamilton, R. G., Kolls, J. K., & Peebles, R. S. (2015). Estrogen and progesterone decrease let-7f microRNA expression and increase IL-23/IL-23 receptor signaling and IL-17A production in patients with severe asthma. *Journal of Allergy and Clinical Immunology*, *136*(4), 1025-1034.e11. https://doi.org/10.1016/j.jaci.2015.05.046

Pauwels, R. A., Buist, A. S., Calverley, P. M. A., Jenkins, C. R., & Hurd, S. S. (2001). Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine*, *163*(5), 1256–1276. https://doi.org/10.1164/ajrccm.163.5.2101039

726 Peleman, R. A., Rytila, P. H., Kips, J. C., Joos, G. F., & Pauwels, R. A. (1999). The cellular composition
727 of induced sputum in chronic obstructive pulmonary disease. *European Respiratory Journal*,
728 *13*(4), 839–843. https://doi.org/10.1034/j.1399-3003.1999.13d24.x
729 Piskounova, E., Polytarchou, C., Thornton, J. E., LaPierre, R. J., Pothoulakis, C., Hagan, J. P., Iliopoulos,
730 D., & Gregory, R. I. (2011). Lin28A and Lin28B Inhibit let-7 MicroRNA Biogenesis by Distinct
731 Mechanisms. *Cell*, *147*(5), 1066–1079. https://doi.org/10.1016/j.cell.2011.10.039
732 Pobezinskaya, E. L., Wells, A. C., Angelou, C. C., Fagerberg, E., Aral, E., Iverson, E., Kimura, M. Y., &
733 Pobezinsky, L. A. (2019). Survival of Naïve T Cells Requires the Expression of Let-7 miRNAs.
734 *Frontiers in Immunology*, *10*. https://doi.org/10.3389/fimmu.2019.00955
735 Pobezinsky, L. A., Etzensperger, R., Jeurling, S., Alag, A., Kadakia, T., McCaughtry, T. M., Kimura, M.
736 Y., Sharrow, S. O., Guinter, T. I., Feigenbaum, L., & Singer, A. (2015). Let-7 microRNAs target
737 the lineage-specific transcription factor PLZF to regulate terminal NKT cell differentiation and
738 effector function. *Nature Immunology*, *16*(5), 517–524. https://doi.org/10.1038/ni.3146
739 Polikepahad, S., Knight, J. M., Naghavi, A. O., Oplt, T., Creighton, C. J., Shaw, C., Benham, A. L., Kim,
740 J., Soibam, B., Harris, R. A., Coarfa, C., Zariff, A., Milosavljevic, A., Batts, L. M., Kheradmand,
741 F., Gunaratne, P. H., & Corry, D. B. (2010). Proinflammatory Role for let-7 MicroRNAS in
742 Experimental Asthma. *Journal of Biological Chemistry*, *285*(39), 30139–30149.
743 https://doi.org/10.1074/jbc.M110.145698
744 Pottelberge, G. R. V., Mestdagh, P., Bracke, K. R., Thas, O., Durme, Y. M. T. A. van, Joos, G. F.,
745 Vandesompele, J., & Brusselle, G. G. (2011). MicroRNA Expression in Induced Sputum of
746 Smokers and Patients with Chronic Obstructive Pulmonary Disease. *American Journal of*
747 *Respiratory and Critical Care Medicine*, *183*(7), 898–906. https://doi.org/10.1164/rccm.201002-
748 0304OC
749 Prevalence and attributable health burden of chronic respiratory diseases, 1990–2017: A systematic
750 analysis for the Global Burden of Disease Study 2017. (2020). *The Lancet. Respiratory Medicine*,
751 *8*(6), 585–596. https://doi.org/10.1016/S2213-2600(20)30105-3

Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L., & Bradley, A. (2004). Identification of Mammalian microRNA Host Genes and Transcription Units. *Genome Research*, *14*(10a), 1902–1910. https://doi.org/10.1101/gr.2722704

Schembri, F., Sridhar, S., Perdomo, C., Gustafson, A. M., Zhang, X., Ergun, A., Lu, J., Liu, G., Zhang, X., Bowers, J., Vaziri, C., Ott, K., Sensinger, K., Collins, J. J., Brody, J. S., Getts, R., Lenburg, M. E., & Spira, A. (2009). MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proceedings of the National Academy of Sciences*, *106*(7), 2319–2324. https://doi.org/10.1073/pnas.0806383106

Senior, R. M., & Anthonisen, N. R. (1998). Chronic Obstructive Pulmonary Disease (COPD). *American Journal of Respiratory and Critical Care Medicine*, *157*(4), S139–S147. https://doi.org/10.1164/ajrccm.157.4.nhlbi-12

Shan, M., Cheng, H.-F., Song, L., Roberts, L., Green, L., Hacken-Bitar, J., Huh, J., Bakaeen, F., Coxson, H. O., Storness-Bliss, C., Ramchandani, M., Lee, S.-H., Corry, D. B., & Kheradmand, F. (2009). Lung Myeloid Dendritic Cells Coordinately Induce TH1 and TH17 Responses in Human Emphysema. *Science Translational Medicine*, *1*(4), 4ra10-4ra10. https://doi.org/10.1126/scitranlsmed.3000154

Shan, M., You, R., Yuan, X., Frazier, M. V., Porter, P., Seryshev, A., Hong, J.-S., Song, L., Zhang, Y., Hilsenbeck, S., Whitehead, L., Zarinkamar, N., Perusich, S., Corry, D. B., & Kheradmand, F. (2014). Agonistic induction of PPARγ reverses cigarette smoke–induced emphysema. *The Journal of Clinical Investigation*, *124*(3), 1371–1381. https://doi.org/10.1172/JCI70587

Shan, M., Yuan, X., Song, L., Roberts, L., Zarinkamar, N., Seryshev, A., Zhang, Y., Hilsenbeck, S., Chang, S.-H., Dong, C., Corry, D. B., & Kheradmand, F. (2012). Cigarette Smoke Induction of Osteopontin (SPP1) Mediates TH17 Inflammation in Human and Experimental Emphysema. *Science Translational Medicine*, *4*(117), 117ra9-117ra9. https://doi.org/10.1126/scitranslmed.3003041

777 Shi, J., & Petrie, H. T. (2012). Activation Kinetics and Off-Target Effects of Thymus-Initiated Cre
778 Transgenes. *PLoS ONE*, *7*(10), e46590. https://doi.org/10.1371/journal.pone.0046590
779 Srenathan, U., Steel, K., & Taams, L. S. (2016). IL-17+ CD8+ T cells: Differentiation, phenotype and
780 role in inflammatory disease. *Immunology Letters*, *178*, 20–26.
781 https://doi.org/10.1016/j.imlet.2016.05.001
782 Tan, F. E., Sathe, S., Wheeler, E. C., Nussbacher, J. K., Peter, S., & Yeo, G. W. (2019). A Transcriptome-
783 wide Translational Program Defined by LIN28B Expression Level. *Molecular Cell*, *73*(2), 304-
784 313.e3. https://doi.org/10.1016/j.molcel.2018.10.041
785 Veldhoen, M. (2017). Interleukin 17 is a chief orchestrator of immunity. *Nature Immunology*, *18*(6),
786 Article 6. https://doi.org/10.1038/ni.3742
787 Viswanathan, S. R., Daley, G. Q., & Gregory, R. I. (2008). Selective blockade of microRNA processing
788 by Lin-28. *Science (New York, N.Y.)*, *320*(5872), 97–100.
789 https://doi.org/10.1126/science.1154040
790 Wedzicha, J. A. (2004). Role of Viruses in Exacerbations of Chronic Obstructive Pulmonary Disease.
791 *Proceedings of the American Thoracic Society*, *1*(2), 115–120.
792 https://doi.org/10.1513/pats.2306030
793 Wells, A. C., Daniels, K. A., Angelou, C. C., Fagerberg, E., Burnside, A. S., Markstein, M., Alfandari,
794 D., Welsh, R. M., Pobezinskaya, E. L., & Pobezinsky, L. A. (2017). Modulation of let-7 miRNAs
795 controls the differentiation of effector CD8 T cells. *eLife*, *6*, e26398.
796 https://doi.org/10.7554/eLife.26398
797 Wells, A. C., Hioki, K. A., Angelou, C. C., Lynch, A. C., Liang, X., Ryan, D. J., Thesmar, I.,
798 Zhanybekova, S., Zuklys, S., Ullom, J., Cheong, A., Mager, J., Hollander, G. A., Pobezinskaya,
799 E. L., & Pobezinsky, L. A. (2023). Let-7 enhances murine anti-tumor CD8 T cell responses by
800 promoting memory and antagonizing terminal differentiation. *Nature Communications*, *14*(1),
801 Article 1. https://doi.org/10.1038/s41467-023-40959-7

802 Williams, M., Todd, I., & Fairclough, L. C. (2021). The role of CD8 + T lymphocytes in chronic
803 obstructive pulmonary disease: A systematic review. *Inflammation Research*, *70*(1), 11–18.
804 https://doi.org/10.1007/s00011-020-01408-z
805 Xu, C., Hesselbacher, S., Tsai, C.-L., Shan, M., Spitz, M., Scheurer, M., Roberts, L., Perusich, S.,
806 Zarinkamar, N., Coxson, H., Krowchuk, N., Corry, D., & Kheradmand, F. (2012). Autoreactive T
807 Cells in Human Smokers is Predictive of Clinical Outcome. *Frontiers in Immunology*, *3*.
808 https://www.frontiersin.org/articles/10.3389/fimmu.2012.00267
809 Yeh, N., Glosson, N. L., Wang, N., Guindon, L., McKinley, C., Hamada, H., Li, Q., Dutton, R. W.,
810 Shrikant, P., Zhou, B., Brutkiewicz, R. R., Blum, J. S., & Kaplan, M. H. (2010). Tc17 Cells Are
811 Capable of Mediating Immunity to Vaccinia Virus by Acquisition of a Cytotoxic Phenotype. *The*
812 *Journal of Immunology*, *185*(4), 2089–2098. https://doi.org/10.4049/jimmunol.1000818
813 You, R., Lu, W., Shan, M., Berlin, J. M., Samuel, E. L., Marcano, D. C., Sun, Z., Sikkema, W. K., Yuan,
814 X., Song, L., Hendrix, A. Y., Tour, J. M., Corry, D. B., & Kheradmand, F. (2015).
815 Nanoparticulate carbon black in cigarette smoke induces DNA cleavage and Th17-mediated
816 emphysema. *eLife*, *4*, e09623. https://doi.org/10.7554/eLife.09623
817 Yuan, X., Chang, C.-Y., You, R., Shan, M., Gu, B. H., Madison, M. C., Diehl, G., Perusich, S., Song, L.-
818 Z., Cornwell, L., Rossen, R. D., Wetsel, R., Kimal, R., Coarfa, C., Eltzschig, H. K., Corry, D. B.,
819 & Kheradmand, F. (2020). Cigarette smoke–induced reduction of C1q promotes emphysema. *JCI*
820 *Insight*, *4*(13). https://doi.org/10.1172/jci.insight.124317
821 Zhang, H., Zhou, X., Chen, X., Lin, Y., Qiu, S., Zhao, Y., Tang, Q., Liang, Y., & Zhong, X. (2019).
822 Rapamycin attenuates Tc1 and Tc17 cell responses in cigarette smoke-induced emphysema in
823 mice. *Inflammation Research*, *68*(11), 957–968. https://doi.org/10.1007/s00011-019-01278-0
824 Zhou, J.-S., Li, Z.-Y., Xu, X.-C., Zhao, Y., Wang, Y., Chen, H.-P., Zhang, M., Wu, Y.-F., Lai, T.-W., Di,
825 C.-H., Dong, L.-L., Liu, J., Xuan, N.-X., Zhu, C., Wu, Y.-P., Huang, H.-Q., Yan, F.-G., Hua, W.,
826 Wang, Y., … Shen, H.-H. (2020). Cigarette smoke-initiated autoimmunity facilitates sensitisation

827 to elastin-induced COPD-like pathologies in mice. *European Respiratory Journal*, *56*(3).
828 https://doi.org/10.1183/13993003.00404-2020
829 Zhu, H., Shyh-Chang, N., Segrè, A. V., Shinoda, G., Shah, S. P., Einhorn, W. S., Takeuchi, A., Engreitz,
830 J. M., Hagan, J. P., Kharas, M. G., Urbach, A., Thornton, J. E., Triboulet, R., Gregory, R. I.,
831 Altshuler, D., & Daley, G. Q. (2011). The Lin28/let-7 Axis Regulates Glucose Metabolism. *Cell*,
832 *147*(1), 81–94. https://doi.org/10.1016/j.cell.2011.08.033
833

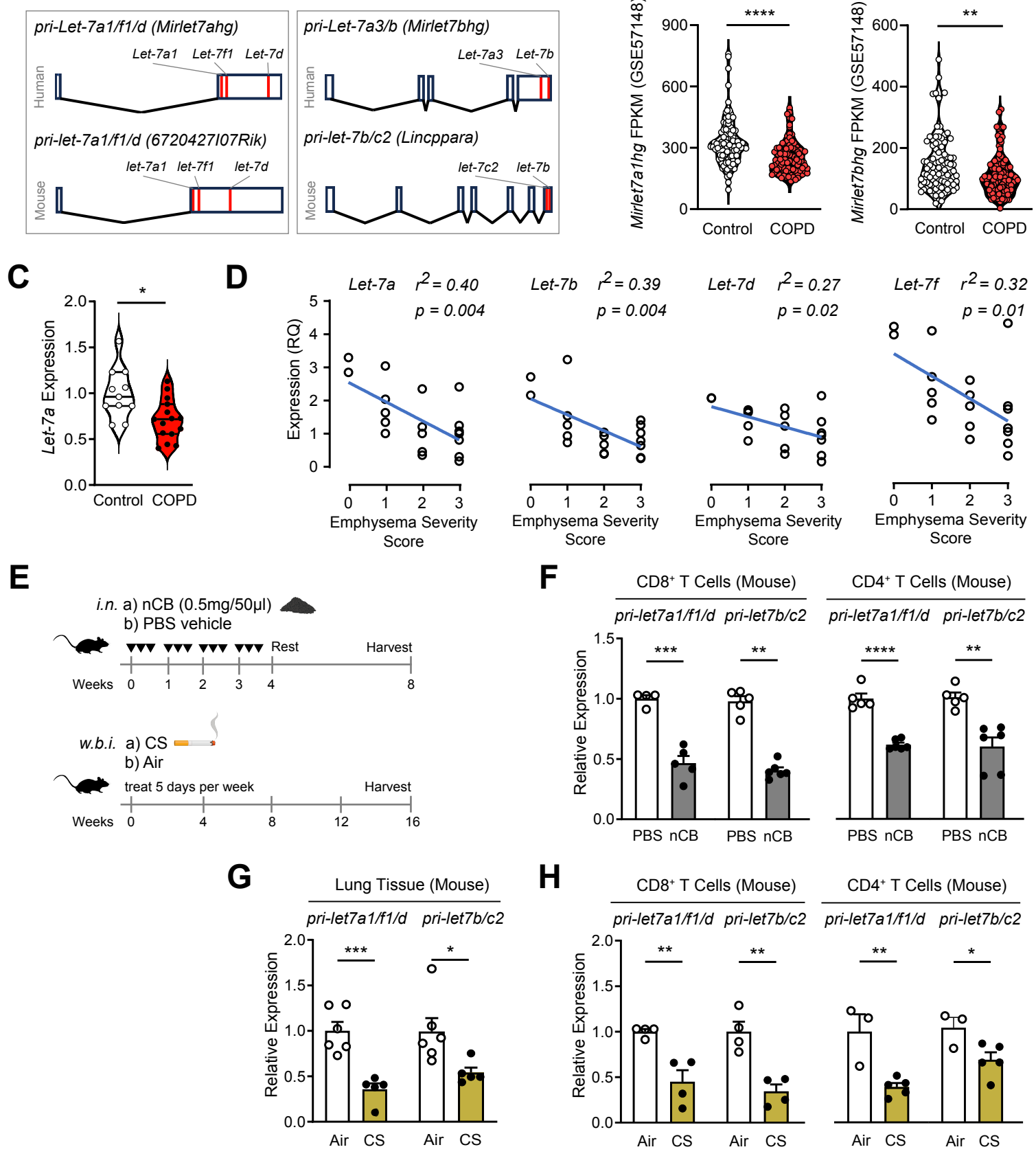

Figure 1. Repression of *Let-7* miRNA gene clusters in lung T cells from COPD patients and murine models of emphysema. (A) Schematic representation of the polycistronic transcripts for the *Let-7a1/Let-7f1/Let-7d-* and *Let-7b/Let-7a3*-clusters in humans and *let-7a1/let-7f1/let-7/d-* and l*et-7b/let-7c2*-clusters 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.

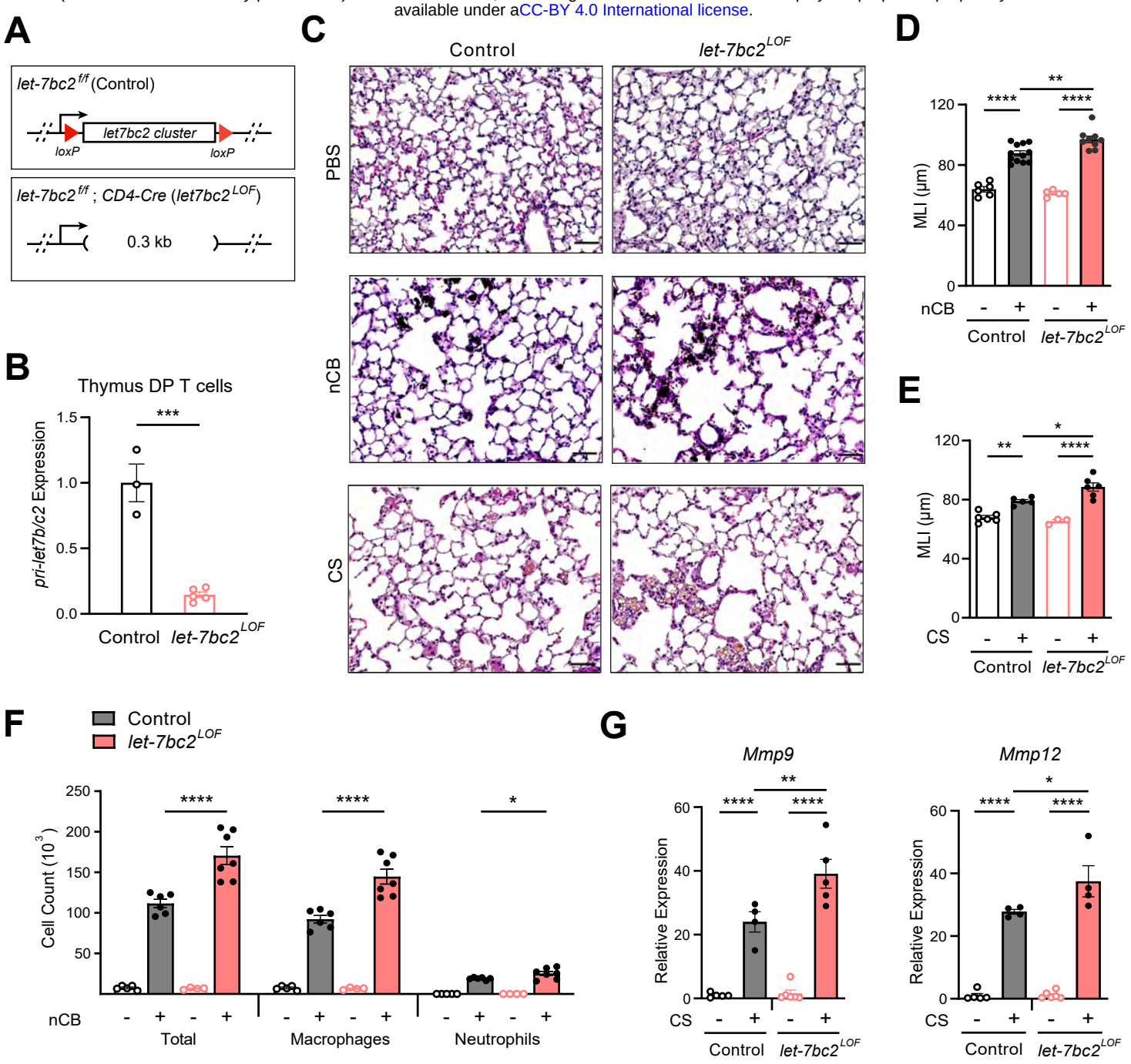

Figure 2. Deletion of the *let-7bc2* cluster in T cells enhances nCB- or CS-triggered emphysema. (A) Schematic representation of CD4-Cre (*let-7bc2*^{LOF}) or *let-7bc2*$^{f/f}$ (Control) mice. (B) QPCR analysis of *pri-let-7b/c2* from flow-sorted live,TCRβ^{+}, CD4^{+}CD8^{+} double-positive (DP) thymocytes 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.

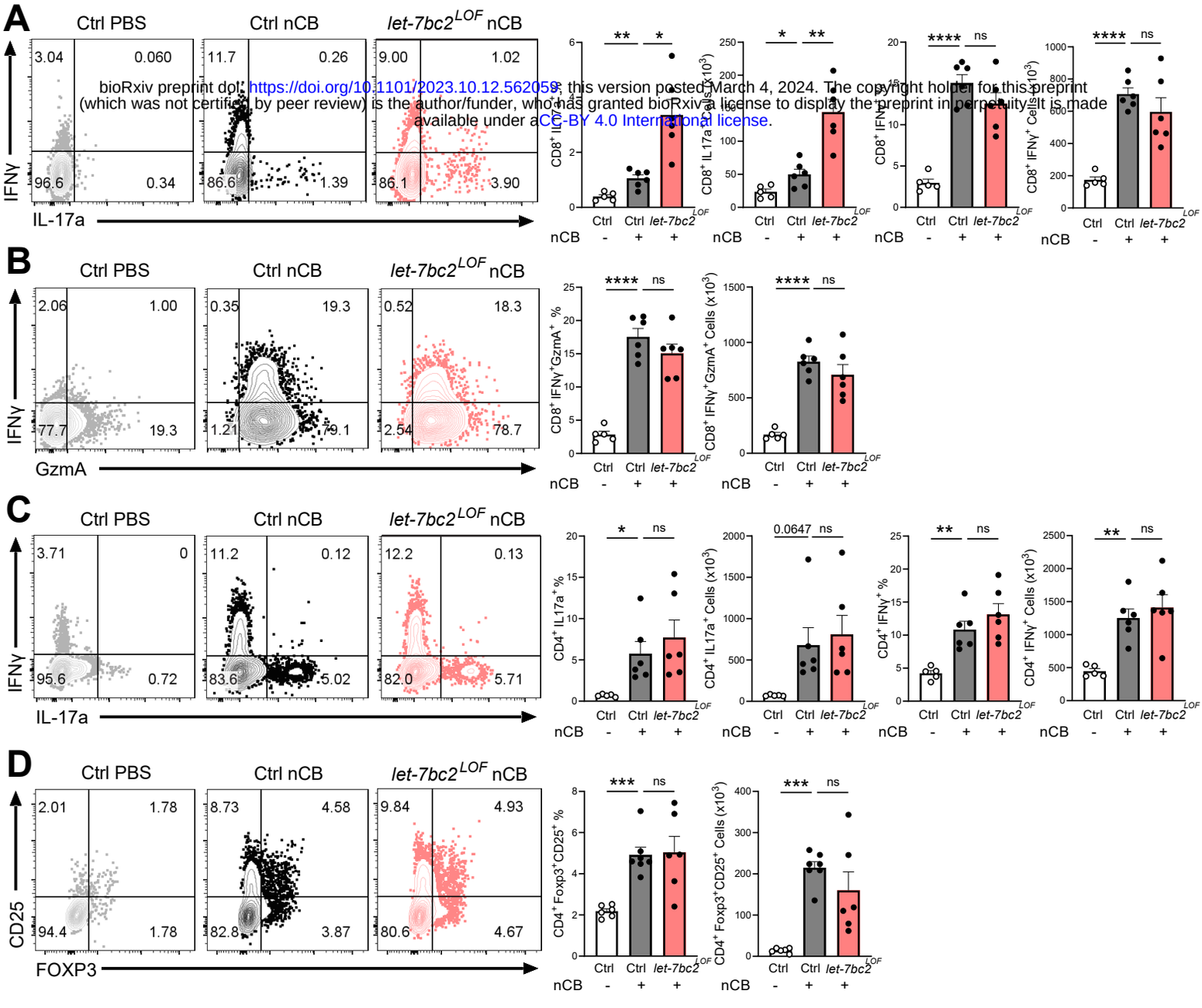

Figure 3. *In vivo* T cell ablation of the *let-7bc2*-cluster enhances Tc17 inflammatory response to nCB-emphysema. Representative flow plots with percentage and counts of live TCRβ⁺ (A) CD8⁺IL-17a⁺ and CD8⁺IFNγ⁺, (B) CD8⁺IFNγ⁺GzmA⁺, (C) CD4⁺IL-17a⁺ and CD4⁺IFNγ⁺, and (D) CD4⁺ Foxp3⁺CD25⁺ cells from 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.

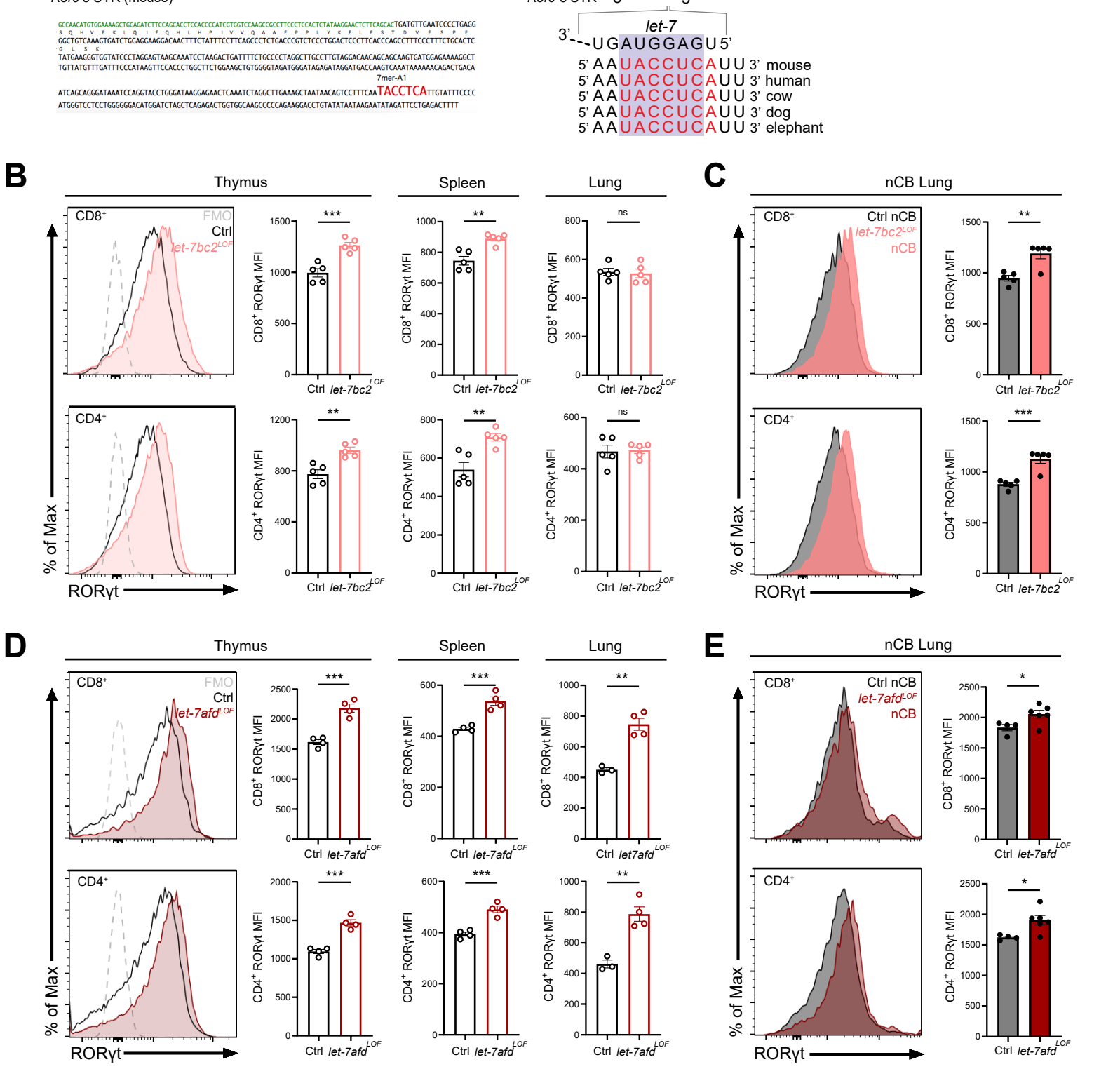

Figure 4. Deletion of either the *let7bc2*- or *let7afd*-cluster in T cells enhances RORγt expression *in vivo*. (A) Left: Schematic representation of the murine *Rorc* 3'UTR with *let-7* miRNA binding sites as identified by TargetScan. Right: Schematic of a conserved *let-7* miRNA target sequence in 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.

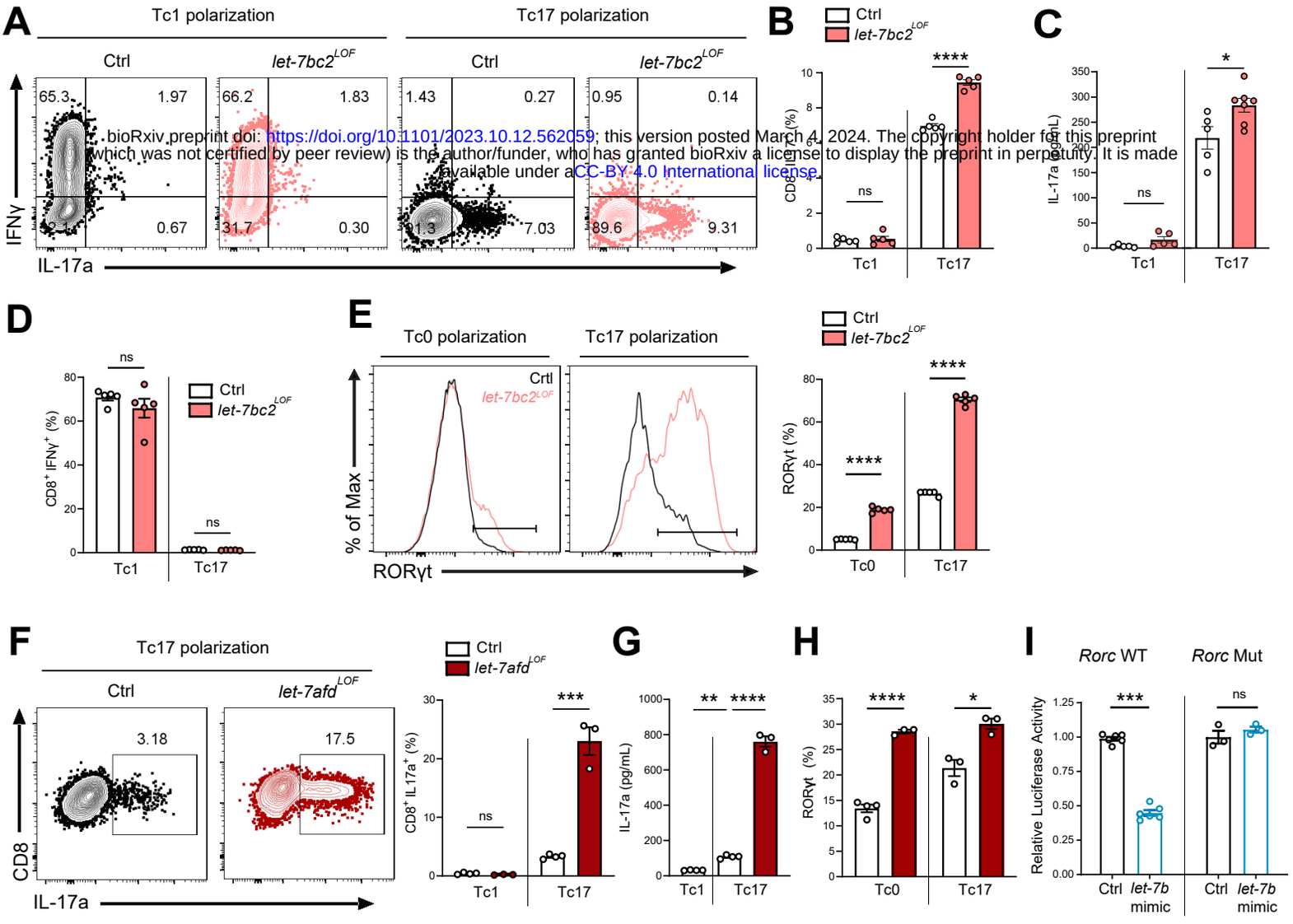

Figure 5. *Let-7* restricts Tc17 *in vitro* differentiation in part via direct targeting of *Rorc* mRNA. (A) Representative flow plots of live TCRβ$^+$ CD8$^+$, IL-17a$^+$ and IFNγ$^+$ populations from Tc1 and Tc17 polarized naïve splenic CD8$^+$ cells from control and *let-7bc2*^{LOF} mice and (B) quantification 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.

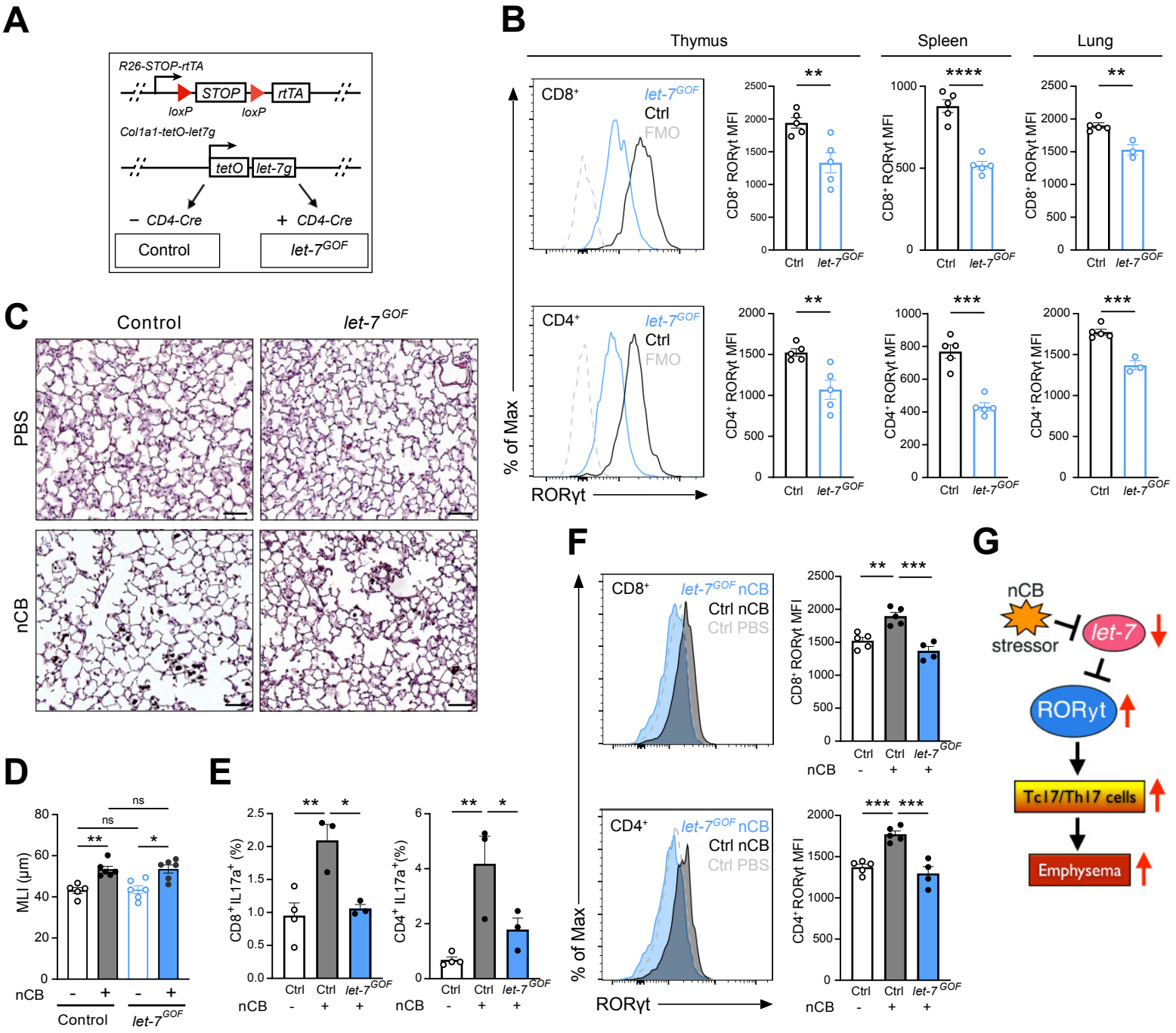

Figure 6. Enforced *let-7* expression in T cells restrains induction of RORγt and Tc17/Th17 inflammation in lungs of nCB-exposed mice. (A) Schematic outlining our T cell-inducible *let-7g* mouse model (*let-7*^{GOF}). (B) Flow analysis of RORγt expression in live, TCRβ$^+$CD8$^+$ or CD4$^+$ T cells from (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.