TITLE

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.

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

53 al., 2022; Eisner et al., 2010; Hu et al., 2010). We have previously shown that nano-sized carbon
54 black (nCB), a noxious chemical constituent of $PM_{2.5}$ found in the lungs of smokers, activates
55 macrophages and dendritic cells orchestrating a pathogenic T cell-dependent inflammatory
56 response and emphysema in mice (W. Lu et al., 2015; You et al., 2015; Shan et al., 2009; C.-Y.
57 Chang et al., 2022).

58 Research over the last decade has pointed to the importance of dysfunctional
59 inflammatory T cells in human COPD lung tissue and animal models of emphysema (Grumelli et
60 al., 2004; Xu et al., 2012; Williams et al., 2021). Aberrant T cells are implicated in impaired host
61 defense, exaggerated inflammation, and loss of self-tolerance in COPD (Williams et al., 2021;
62 Chen et al., 2023; Hogg et al., 2004; Maeno et al., 2007; Xu et al., 2012). In this regard, we and
63 others have demonstrated the role and pathogenicity of activated IFN-γ and IL-17-secreting
64 subsets of $CD4^+$ and $CD8^+$ T lymphocytes including Th1, Th17, and Tc1 cells in clinical isolates
65 and in mice with COPD (W. Lu et al., 2015; You et al., 2015; Shan et al., 2009; S.-H. Lee et al.,
66 2007; Kheradmand et al., 2023). The IL-17-secreting Th17 cells are particularly important as they
67 promote the destruction of lung epithelium and recruitment of macrophages and neutrophils which
68 then release proteolytic enzymes such as matrix metalloproteinases (MMPs) involved in the
69 degradation of the lung structural matrix (Barnes, 2016; Hoenderdos & Condliffe, 2013). We
70 previously demonstrated that intranasal inhalation of nCB in mice is sufficient to induce
71 emphysema by stimulating lung T cell activation by dendritic cells and macrophages. Moreover,
72 we found that genetic ablation of IL-17A can attenuate nCB- or cigarette smoke-induced alveolar
73 destruction and airway inflammation (Shan et al., 2012; You et al., 2015). More recently, IL-17A
74 and IL-17F secreting $CD8^+$ T cell (Tc17) subpopulation has been shown to play a critical role in
75 the pathogenesis of several autoimmune and inflammatory disorders (Globig et al., 2022; Huber
76 et al., 2013; Srenathan et al., 2016).

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

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

98 Here we found that *let-7* miRNA, notably the *let-7a3/let-7b* and *let-7a1/let-7f1/let-7d*
99 clusters, are suppressed in the T cells isolated from lungs of emphysema patients. Consistently,
100 the analogous murine *let-7b/let-7c2-* (*let-7bc2*) and *let-7a1/f1/d1-* (*let-7afd*) clusters were similarly
101 downregulated in pre-clinical emphysema models. We engineered mouse models with the

102 specific loss-of-function (LOF) mutations of the *let-7bc2-* or *let-7afd*-clusters (*let-7bc2*^{LOF} and *let-*
103 *7afd*^{LOF}, respectively) in T cells as well as an inducible *let-7g* gain-of-function (GOF) (*let-7*^{GOF})
104 model to determine the T cell-intrinsic role of *let-7* miRNA in emphysema pathogenesis. Deletion
105 of *let-7* miRNA in T cells worsened alveolar damage elicited by inhalation of CS or nCB, and
106 increased infiltration of immune cells in the airways, including IL-17-producing CD8^{+} T (Tc17) cells.
107 Mechanistically, we found that *let-7* controls type 17 differentiation by directly targeting the
108 lineage-determining transcription factor, RORγt. In support of this conclusion, *let-7*^{GOF} mice were
109 resistant to nCB-mediated induction of RORγt and Tc17 responses. Thus, we show a previously
110 unappreciated role for *let-7* miRNA as a repressor of RORγt and a molecular brake to the IL-17-
111 mediated T cell inflammation in emphysema.

112

113 **RESULTS**

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

115 To explore the involvement of *let-7* in emphysema, we scrutinized the genomic locations and
116 transcriptional annotation of *let-7* members frequently downregulated in lung T cells isolated from
117 smoker's lungs as well as mouse models of emphysema. This combined approach showed close
118 linkage and high conservation of two *Let-7* clusters encoded from long intergenic non-coding RNA
119 (linc)-like precursors in humans and mice (Figure 1A). To shed light on whether these *Let-7*
120 clusters are downregulated in patients with COPD, we analyzed a published (GSE57148) lung
121 RNA-seq dataset obtained from COPD (n=98) and control (n=91) subjects (Kim et al., 2015). Our
122 analysis identified significant downregulation of the *Mirlet7ahg* and *Mirlet7bhg* gene cluster
123 transcripts in COPD compared to control subjects (Figure 1B). We carried out quantitative PCR
124 (qPCR) detection of *Let-7a,* which is encoded by both clusters, in lung tissue samples of smokers
125 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

150 were born at the expected Mendelian frequency and did not show any overt histopathologic or
151 inflammatory changes in lungs histopathology up to 1 year of age in comparison to *let-7bc2*$^{f/f}$
152 control mice (Figure 2-figure supplement 1A-C). Furthermore, quantification of major immune
153 populations and T cell subsets by flow cytometry in *let-7bc2*^{LOF} were comparable to control mice
154 under baseline conditions and with moderate aging (Figure 2-figure supplement 1C,D).

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

172

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

174 We sought to identify the T cell-intrinsic mechanisms that underlie the exaggerated
175 inflammation observed in emphysematous *let-7bc2*^{LOF} mice. We focused on the IL-17-mediated
176 T cell response because it promotes neutrophil and macrophage recruitment in the lungs
177 (Beringer et al., 2016; Veldhoen, 2017; Shan et al., 2012). Previously, we established the induction
178 of CD4^{+}IL17^{+} (Th17) cells along with CD4^{+}IFNγ^{+} (Th1) cells in mice with chronic nCB exposure
179 (You et al., 2015), however whether nCB similarly induces CD8^{+}IL17A^{+} T cells (Tc17) or cytotoxic
180 T cells (Tc1) had not been studied. The flow cytometric profiling of lung T cells revealed enriched
181 proportions and counts of Tc1/Tc17 as well as Th1/Th17 cells in wild-type mice upon treatment
182 with nCB (Figure 3A,B). These findings suggests that nCB elicits both the type 17 and type 1 T
183 cell responses, consistent with CS and elastase pre-clinical models of emphysema (Zhang et al.,
184 2019).

185 We next interrogated the regulatory role of the *let-7bc2*-cluster in the type 17 and type 1
186 responses generated from exposure to nCB. Interestingly, *let-7bc2*^{LOF} mice showed increased
187 CD8^{+}IL17A^{+} Tc17 cells relative to nCB control animals. In contrast, CD8^{+}IFNγ^{+} and GZMA^{+} Tc1
188 populations remained unperturbed with absence of the *let-7bc2*-cluster, suggestive of a more
189 refined regulatory role on Tc17 differentiation (Figure 3A,B). There were no significant differences
190 in either Th1 or Th17 cells when comparing nCB-treated *let-7bc2*^{LOF} to wild-type controls,
191 indicating the *let-7bc2*-cluster was dispensable for their generation (Figure 3C). Regulatory T cells
192 form a dynamic axis with Tc17/Th17 cells and act as a counterbalance to lung inflammation in
193 emphysema (Duan et al., 2016; Jin et al., 2014). Therefore, we examined whether Tc17 cell
194 alterations were driven by the *let-7bc2*-cluster acting on regulatory T cells (Tregs). The *let-7bc2*^{LOF}
195 mice showed no significant difference in the Tregs subset relative to controls in our model (Figure
196 3D). Together, our data support the notion that deletion of the *let-7bc2*-cluster is insufficient to
197 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*$^{f/f}$;

222 *CD4-Cre*). The *let-7afd*$^{f/f}$*; CD4-Cre* (*let-7afd*^{LOF}) mice aged up to 6-months did not exhibit overt
223 lung histopathology and inflammatory changes (Figure 4-figure supplement 1A-F). Of particular
224 interest, ablation of the *let-7afd*-cluster enhanced levels of RORγt in thymic and peripheral T cells
225 of mice (Figure 4D). Overall, this indicates that independent *let-7* clusters restrain RORγt
226 expression levels from thymic development to peripheral T cells under homeostatic conditions.
227 Next, we determined whether loss of *let-7afd*-cluster in T cells likewise sensitizes mice towards
228 induction of RORγt in nCB-emphysema. Intranasal administration of nCB provoked increased
229 RORγt expression in lung T cells of *let-7afd*^{LOF} mice compared to control mice (Figure 4E),
230 supporting overlapping functionality between the *let-7bc2-* and *let-7afd*-clusters in repression of
231 RORγt within T cells.

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

247 Next, we focused on *Rorc* as a potential direct target of *let-7*, which could mechanistically
248 mediate enhanced Tc17 differentiation in *let7bc2*^{LOF} mice. Towards this objective, we tested
249 whether *let-7bc2*^{LOF} or *let-7afd*^{LOF} naïve CD8^{+} T cells show elevated RORγt expression under
250 either Tc0 or Tc17 differentiation conditions. In agreement with enhanced Tc17 differentiation,
251 RORγt expression was differentially and significantly upregulated under both Tc0 and Tc17
252 differentiation conditions in *let-7* LOF cells relative to controls (Figure 5E,H). To determine
253 whether *let-7* directly represses *Rorc* mRNA levels we cloned the 3'UTR of *Rorc* into luciferase
254 constructs. These reporter assays with *let-7b* expressing cells independently confirmed that *let-*
255 *7b* represses *Rorc* (Figure 5I, left). Furthermore, deletion of the putative *let-7* binding sequence
256 (Figure 4A) abrogated repression by *let-7b* (Figure 5I, right), thus confirming *Rorc* as a functional
257 target of *let-7* miRNA. Overall, these *in vitro* experiments readily recapitulated an upstream
258 regulatory role of *let-7* in Tc17 differentiation, mediated in part, via direct suppression of RORγt.

259

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

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

272 protection from experimental emphysema, *let-7*^{GOF} and control mice were treated with nCB and
273 then examined for changes in lung pathology and T cell type 17 responses. The *let-7*^{GOF} mice did
274 not exhibit any signs of lung inflammation or pathologic remodeling at baseline (Figure 6C,D and
275 data not shown) Histopathologic analysis revealed a comparable degree of lung alveolar
276 distension via morphometric measurements of MLI in nCB-treated *let-7*^{GOF} mice versus controls
277 suggesting that enforced *let-7* expression is insufficient to protect the lung from emphysema
278 (Figure 6C,D). On the other hand, evaluation of the IL-17^{+} response and RORγt levels in
279 emphysematous lung T cells demonstrated that, in contrast to control nCB-treated mice, *let-7*^{GOF}
280 mice exhibited dampened lung Tc17 and Th17 cell populations and were resistant to the induction
281 of RORγt after nCB-exposure (Figure 6E,F). Taken together, our *let-7* LOF and GOF models
282 demonstrate the necessity and sufficiency of *let-7* miRNA to act as a molecular brake to the type
283 17 T cell response through the direct regulation of RORγt, further our data suggests that nCB- or
284 CS-mediated suppression of this braking mechanism furthers inflammation and exacerbates
285 emphysema severity (Figure 6G).

286

287 **DISCUSSION**

288 MiRNA expression-based studies of COPD patients and mice exposed to CS have
289 reported downregulation of *let-7* miRNA expression in lung tissues (Conickx et al., 2017;
290 Christenson et al., 2013b; Schembri et al., 2009). We and others explored the consequence of
291 loss of *let-7* expression/activity with synthetic oligonucleotides, sponges, lentiviral antisense
292 knockdown, or via ectopic delivery of *Lin28b* (Polikepahad et al., 2010; Viswanathan et al., 2008;
293 Piskounova et al., 2011), but studies pinpointing the role of individual *let-7* clusters as potential
294 drivers of lung inflammation and COPD within T cells remained elusive. In the present study, we
295 established that the *let-7* miRNA family members encoded by the *let-7bc2*- and *let-7afd*-clusters
296 are downregulated in T cells from lungs of emphysema patients and emphysematous mice that

297 were exposed to CS or nCB. Correspondingly, we demonstrated that *in vivo* genetic ablation of
298 *let-7bc2-cluster* further sensitized mice to lung tissue destruction and emphysema upon treatment
299 with nCB or CS. Mechanistically, our studies suggests that *let-7bc2*-cluster prevents the
300 emergence of $CD8^+$ T cell differentiation into Tc17 cells during emphysema in part, by directly
301 silencing of *Rorc*.

302 Tc17 cells are vital for defense against viral, fungal and bacterial infections and they have
303 also been associated with inflammation in various human diseases such as multiple sclerosis,
304 inflammatory bowel disease, and cancer (Huber et al., 2013; Globig et al., 2022; Corgnac et al.,
305 2020). In accordance with the potential pathogenic role of Tc17 cells as drivers of COPD, several
306 studies detected increased cell numbers in airways and tissues of COPD patients as well as lungs
307 of smoke-exposed animal models (Y. Chang et al., 2011; Zhou et al., 2020; Duan et al., 2013).
308 Other researchers also detected increased Tc17 subpopulations in tissues of COPD patients with
309 infectious microbial exacerbations. In our earlier work to define the adaptive T cell immune
310 responses in nCB induced COPD, we predominantly focused on the pathogenic role of Th17 cells,
311 but did not examine Tc17 cells (You et al., 2015). Here we expand upon our prior observations,
312 revealing that chronic exposure to nCB and elicitation of emphysema mice orchestrates the
313 emergence and accumulation of Tc17 cells which may act in parallel with Th17 cells to promote
314 tissue damage.

315 Prior research has shown the importance of both transcriptional and post-transcriptional
316 regulatory control of RORγt expression in T cells (Ciofani et al., 2012; Donate et al., 2013;
317 Medvedev et al., 1997). Altogether, our *in vivo* studies establish *let-7* as a new important link
318 associated with regulation of RORγt and lung Tc17 differentiation in COPD. Our data also showed
319 that *in vivo* conditional genetic ablation of individual *let-7* clusters in T cells stimulates a rise in
320 RORγt protein expression in single-positive thymocytes and peripheral $CD8^+$ and $CD4^+$ T cells
321 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

347 al., 2019; Pobezinsky et al., 2015; Wells et al., 2017). A particular feature of these studies has
348 been the utilization of *Lin28b* transgenic mice to block maturation and activity of entire *let-7* family
349 to promote a *let-7* LOF function phenotype (Angelou et al., 2020; Piskounova et al., 2011;
350 Pobezinskaya et al., 2019; Wells et al., 2023; Zhu et al., 2011). Furthermore, *Lin28b* was recently
351 reported to also influence transcriptome-wide ribosome occupancy and global miRNA biogenesis
352 (Tan et al., 2019). Thus, it is likely that *Lin28b* transgenic overexpression could give rise stronger
353 phenotypes than we observed in our single cluster *let-7*^{LOF} mice. Nonetheless, unbiased omics-
354 based methods will be useful to determine if other gene targets beyond RORγt synergistically
355 potentiate the *in vivo* Tc17-response and emphysema phenotype in context of deletion of *let-7bc2*-
356 cluster.

357 Tc17 cells play a major role in microbial infections, providing a potent anti-viral response
358 (Hamada et al., 2009; Yeh et al., 2010), while viral infection has been an established factor in
359 COPD exacerbations (Hewitt et al., 2016; Wedzicha, 2004). It will be interesting to determine
360 whether loss of *let-7bc2*- or *let-7afd*-cluster activity in the T cell compartment contributes to COPD
361 disease susceptibility in the context of viral exposure. Our experiments with *let-7* GOF were
362 partially successful in limiting the emergence of Tc17 and Th17 in nCB-elicited emphysema. *Let-*
363 *7*^{GOF} mice exhibited a reduction in RORγt expression levels and type 17 responses but were not
364 protected from alveolar remodeling following nCB exposure. A potential limitation of the *let-7*^{GOF}
365 transgenic model is that it expresses only the *let-7g* sequence which may render it less potent
366 than the corresponding two mature forms transcribed from the *let-7bc2*-cluster. Additional studies
367 will be required to ascertain whether other interventions that enhance *let-7* activity in T cells are
368 successful in preventing or reversing COPD.

369

370 **MATERIALS AND METHODS**

371 **Mice**

372 Conditional knockout-ready floxed *let-7bc2* and *let-7afd* mice were generated using CRISPR gene
373 editing in an isogenic C57BL/6 genetic background and were sequence verified for rigor. Mice
374 were PCR genotyped from ear samples with primers flanking loxP sites (Supplementary Table 1).
375 The *let-7bc2*$^{flox/flox}$; *CD4-cre* and *let-7afd*$^{flox/flox}$; *CD4-cre* mice were PCR genotyped. The *R26-*
376 *STOP-rtTA*; *Col1a1-tet0-let-7* (*rtTA-iLet7)* mice were obtained from JAX Jax Stocks 023912 and
377 05670 and then bred to *CD4-Cre* were PCR genotyped with established JAX primers (Belteki et
378 al., 2005; Zhu et al., 2011). Control *rtTA-iLet7* and the *let-7*^{GOF} mice were fed *ad libitum* with
379 200mg/kg of doxycycline-containing chow (Bio-Serv S3888) at weaning age primers (Belteki et
380 al., 2005; Zhu et al., 2011). Syngeneic littermates served as controls for all mouse experiments.
381 All mice were bred in the transgenic animal facility at Baylor College of Medicine. All experimental
382 protocols used in this study were approved by the Institutional Animal Care and Use Committee
383 of Baylor College of Medicine animal protocol (AN-7389) and followed the National Research
384 Council Guide for the Care and Use of Laboratory Animals.
385
386 **Human emphysema tissue samples and T cell isolation**
387 Lung tissues were obtained from a total of 19 non-atopic current or former smokers with significant
388 (>20 pack-years, one pack-year equals to smoking one pack of cigarettes per day each year)
389 history of smoking who were recruited into studies from the chest or surgical clinics at Michael E.
390 DeBakey Houston Veterans Affairs Medical Center hospitals (Supplementary Table 2) (Shan et
391 al., 2009). Emphysema and non-emphysema control patients were diagnosed from CT scans
392 according to the criteria recommended by the National Institutes of Health–World Health
393 Organization workshop summary (Pauwels et al., 2001). Human single-cell suspensions were
394 prepared from surgically resected lungs as previously described (Yuan et al., 2020; Grumelli et
395 al., 2004). Briefly, fresh lung tissue was minced into 0.1 cm pieces in petri dishes and treated with
396 2 mg/mL of collagenase D (Worthington) for 1 hour at 37°C. Digested lung tissue was filtered
397 through a 40-μm cell strainer (BD Falcon) followed by red blood cell lysis using ACK lysis buffer

398 (Sigma-Aldrich) for 3 minutes to yield a single-cell suspension. $CD4^+$ T cells were selected from
399 resultant suspensions by labeling with bead conjugated anti-CD4 for enrichment by autoMACs
400 (Miltenyi Biotec). Studies were approved by the Institutional Review Board at Baylor College of
401 Medicine and informed consent was obtained from all patients.
402
403 **Human lung transcriptome data**
404 A publicly available RNA-seq dataset from a Korean cohort GSE57148 was selected for the
405 analysis (Kim et al., 2015). The raw FASTQ files of paired end reads representing the
406 transcriptome of control and cases were retrieved from the GEO database at the National Centre
407 for Biological Information (NCBI) through accession number GSE57148 and analyzed with R
408 package for differential expression.
409
410 **Cigarette smoke exposure model of pulmonary emphysema**
411 To promote emphysema, mice were exposed to cigarette smoke using our custom designed
412 whole-body inhalation system (Morales-Mantilla et al., 2020). In total, mice were exposed to four
413 cigarettes (Marlboro 100's; Philip Morris USA) per day, five days a week, for four months as
414 previously described (Shan et al., 2012).
415
416 **nCB exposure model of pulmonary emphysema**
417 Nano-sized particulate carbon black was prepared and administered as previously described (You
418 et al., 2015; W. Lu et al., 2015). Dried nCB nanoparticles were resuspended in sterile PBS to a
419 concentration of 10 mg/ml. Fifty µl of reconstituted nCB (0.5 mg) were intranasally delivered to
420 deeply anesthetized mice on a schedule of three times a week for four weeks (total delivered
421 dose of 6 mg). Lung histomorphometry and airway inflammation were assessed four weeks after
422 the final nCB challenge. For histomorphometric analysis, mice lungs were fixed with 10% neutral-
423 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

450 with plate-bound anti-CD3 (1.5μg/mL) and complete RPMI media containing anti-CD28
451 (1.5μg/mL) and β-mercaptoethanol (50nM) for Tc0 polarization, or further supplemented with Tc1
452 [IL-2 (10ng/mL)] or Tc17 [TGFβ (2ng/mL), IL-6 (20ng/mL), anti-IFNγ (10μg/mL), IL-23 (20ng/mL),
453 and IL-1β (5ng/mL)] polarization conditions for 72 hours (Flores-Santibáñez et al., 2018).
454
455 **ELISA**
456 Supernatant was collected from *in vitro* polarized murine $CD8^+$ T cells and centrifuged to remove
457 cellular debris (W. Lu et al., 2015). Cytokine levels of IL-17A and IFNγ were quantified from
458 collected supernatant using Mouse IL-17A Uncoated ELISA and Mouse IFN gamma Uncoated
459 ELISA (Invitrogen) Kits, respectively, per the manufacturer's instructions with colorimetric analysis
460 by the Varioskan LUX microplate reader (ThermoFisher).
461
462 **Flow cytometric analysis**
463 Cells used for *in vitro* or *in vivo* cytokine analysis were stimulated with PMA (20ng/mL; Sigma
464 Aldrich), Ionomycin (1μg/mL; Sigma Aldrich), and Brefeldin A (2μg/mL; Sigma Aldrich) for 4 hours
465 prior to flow staining (W. Lu et al., 2015). For intracellular staining, cells were fixed and
466 permeabilized using the Mouse FOXP3 Buffer Set (BD) per the manufacturer's protocol. The
467 fluorophore-conjugated antibodies used in this study were as follows: Live/Dead Fix Blue
468 (Invitrogen), CD3 PerCPCy5.5 (Biolegend), TCRb PE/Cy7 (Biolegend), CD4 PB (Biolegend), CD4
469 AF700 (Biolegend), CD8 BV650 (Biolegend), CD25 BV421 (Biolegend), FOXP3 AF488
470 (Biolegend), ROR gamma T PE (Invitrogen), TCF1 AF647 (Cell Signaling Technologies), TCF1
471 PE (Biolegend), IFNγ AF647 (Biolegend), IL17A FITC (Biolegend), IL17A PE (ebioscience).
472 Representative flow cytometric gating and quantification strategy for detection of lung Th1/Th17
473 and Tc1/Tc17 cell populations is shown in Supplementary Figure 3. Samples were analyzed using
474 BD LSR II flow cytometer (BD Biosciences) and FlowJo software (TreeStar).
475

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.

502 A P-value less than 0.05 was considered statistically significant; ns indicates not significant.
503 Statistical significance values were set as *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.
504 Data are presented as means ± SEM. P-value and n can be found in the main and supplementary
505 figure legends.
506

507 **COMPETING INTEREST STATEMENT**

508 The authors declare no competing interests.
509

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517

518 **AUTHOR CONTRIBUTIONS**

519 P.E., X.H., D.B.C, F.K., and A.R. conceptualized experiments and interpreted results. P.E., X.H.,
520 M.J.S., H.T., M.A.P., and S.L.L. acquired the data, M.J.R. provided bioinformatics analyses. P.E.
521 and A.R. wrote the manuscript.
522

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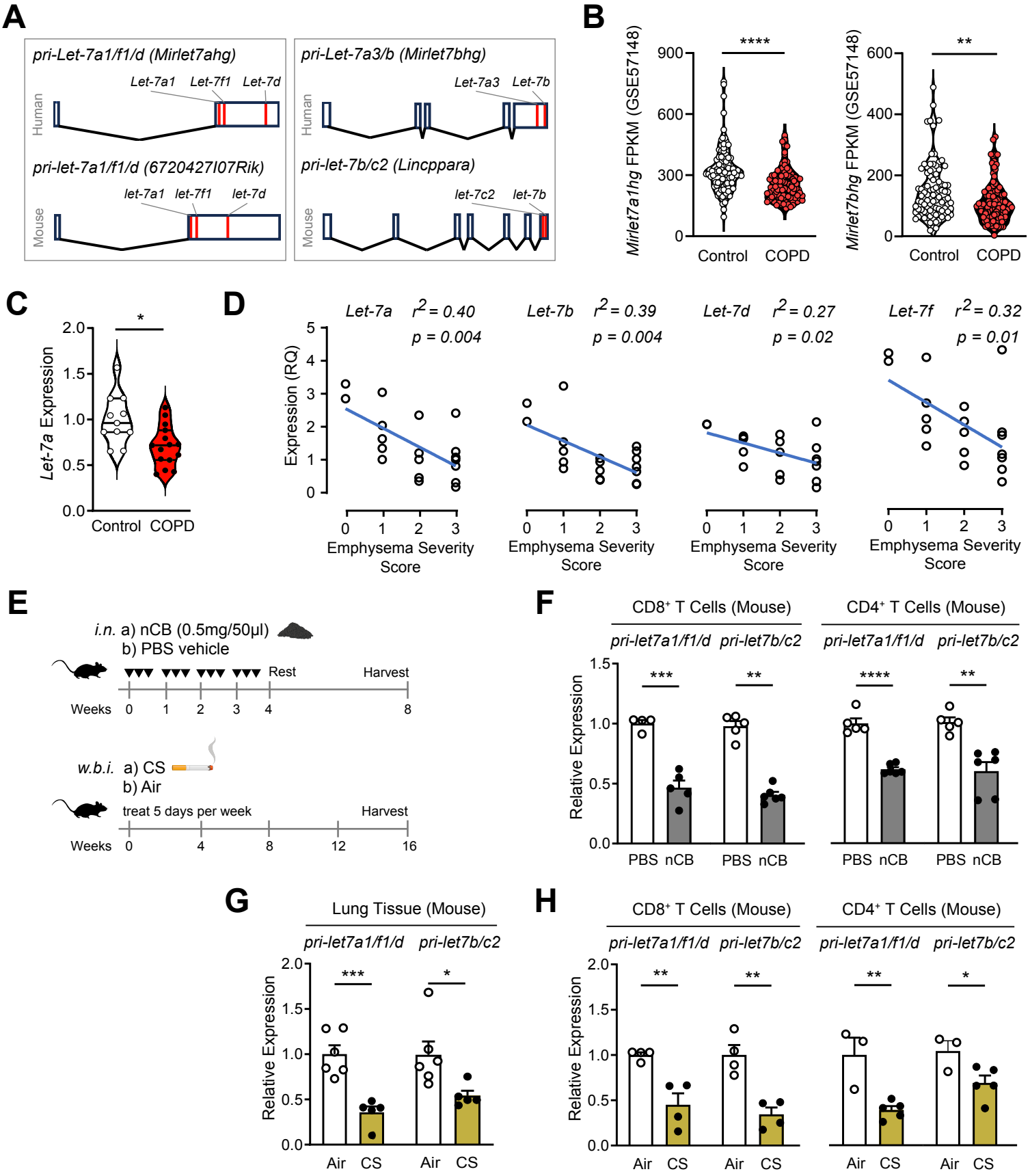

Figure 1. Repression of *Let-7* miRNA gene clusters in lung T cells from COPD patients and murine models of emphysema. (A) Schematic representation of the polycistronic transcripts for the *Let-7a1/Let-7f1/Let-7d-* and *Let-7b/Let-7a3*-clusters in humans and *let-7a1/let-7f1/let-7/d-* and l*et-7b/let-7c2*-clusters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

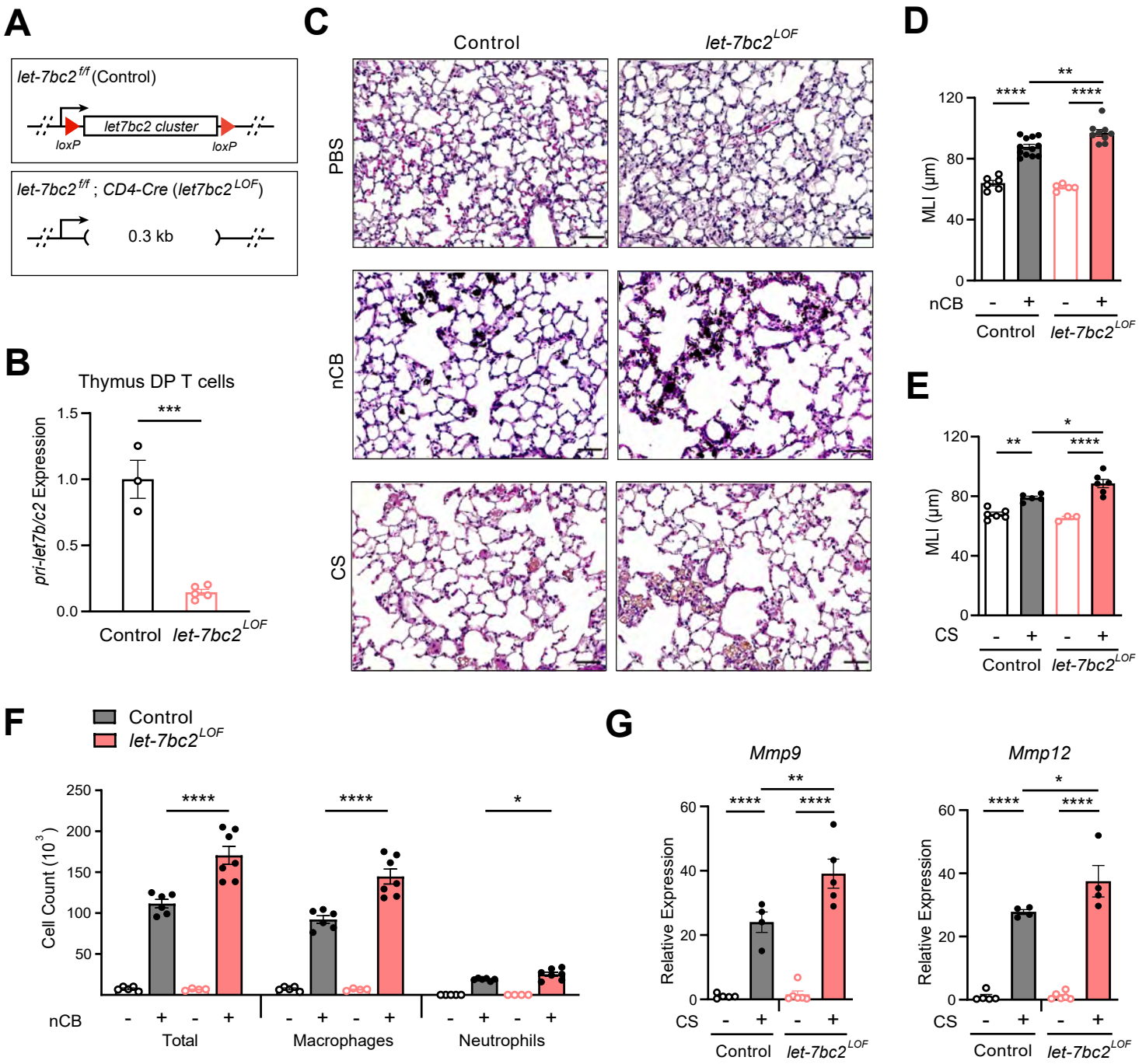

Figure 2. Deletion of the *let-7bc2* cluster in T cells enhances nCB- or CS-triggered emphysema. (A) Schematic representation of CD4-Cre (*let-7bc2*^{LOF}) or *let-7bc2*$^{f/f}$ (Control) mice. (B) QPCR analysis of *pri-let-7b/c2* from flow-sorted live,TCRβ^{+}, CD4^{+}CD8^{+} double-positive (DP) thymocytes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

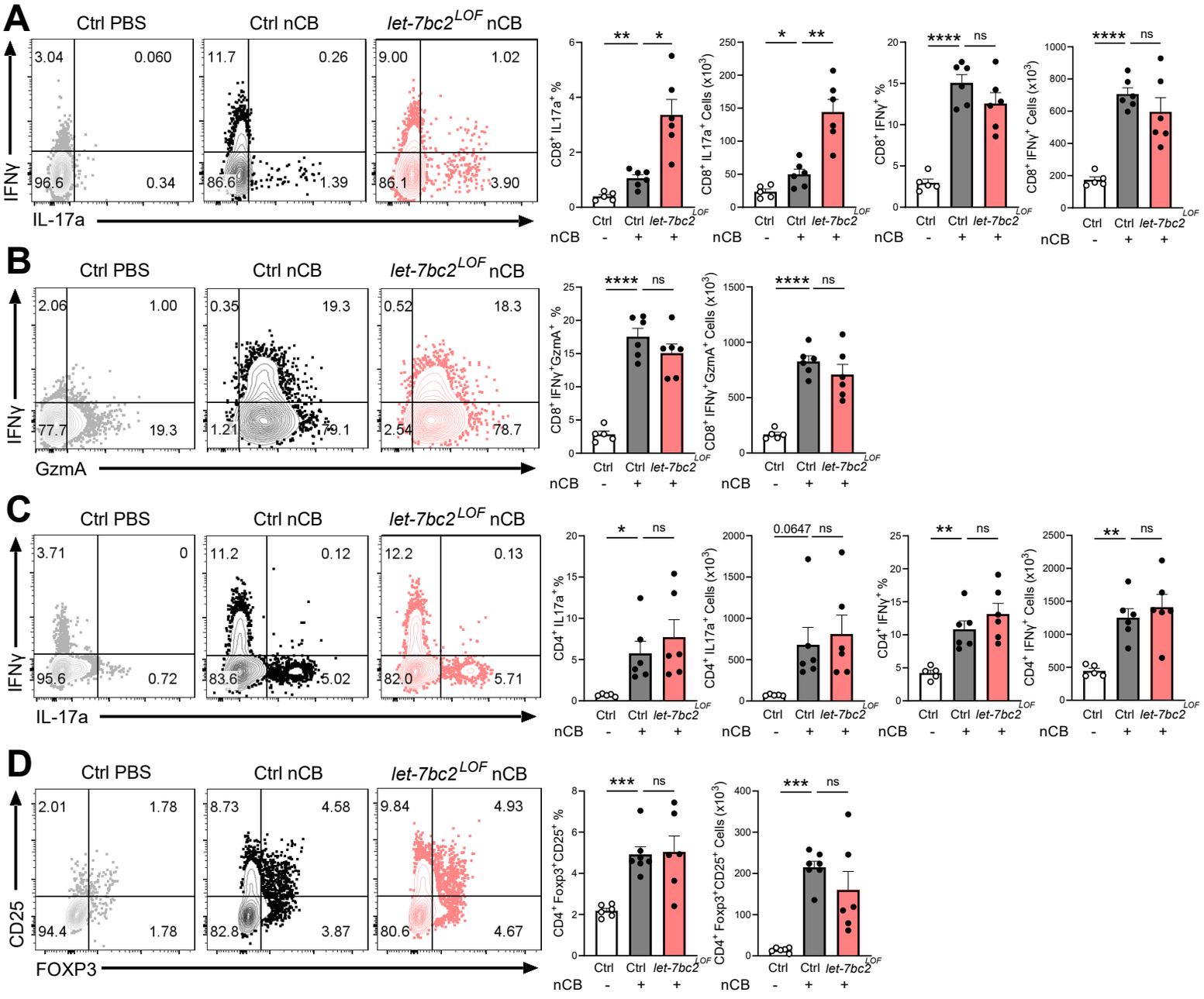

Figure 3. *In vivo* T cell ablation of the *let-7bc2*-cluster enhances Tc17 inflammatory response to nCB-emphysema. Representative flow plots with percentage and counts of live TCRβ⁺ (A) CD8⁺IL-17a⁺ and CD8⁺IFNγ⁺, (B) CD8⁺IFNγ⁺GzmA⁺, (C) CD4⁺IL-17a⁺ and CD4⁺IFNγ⁺, and (D) CD4⁺ Foxp3⁺CD25⁺ cells from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

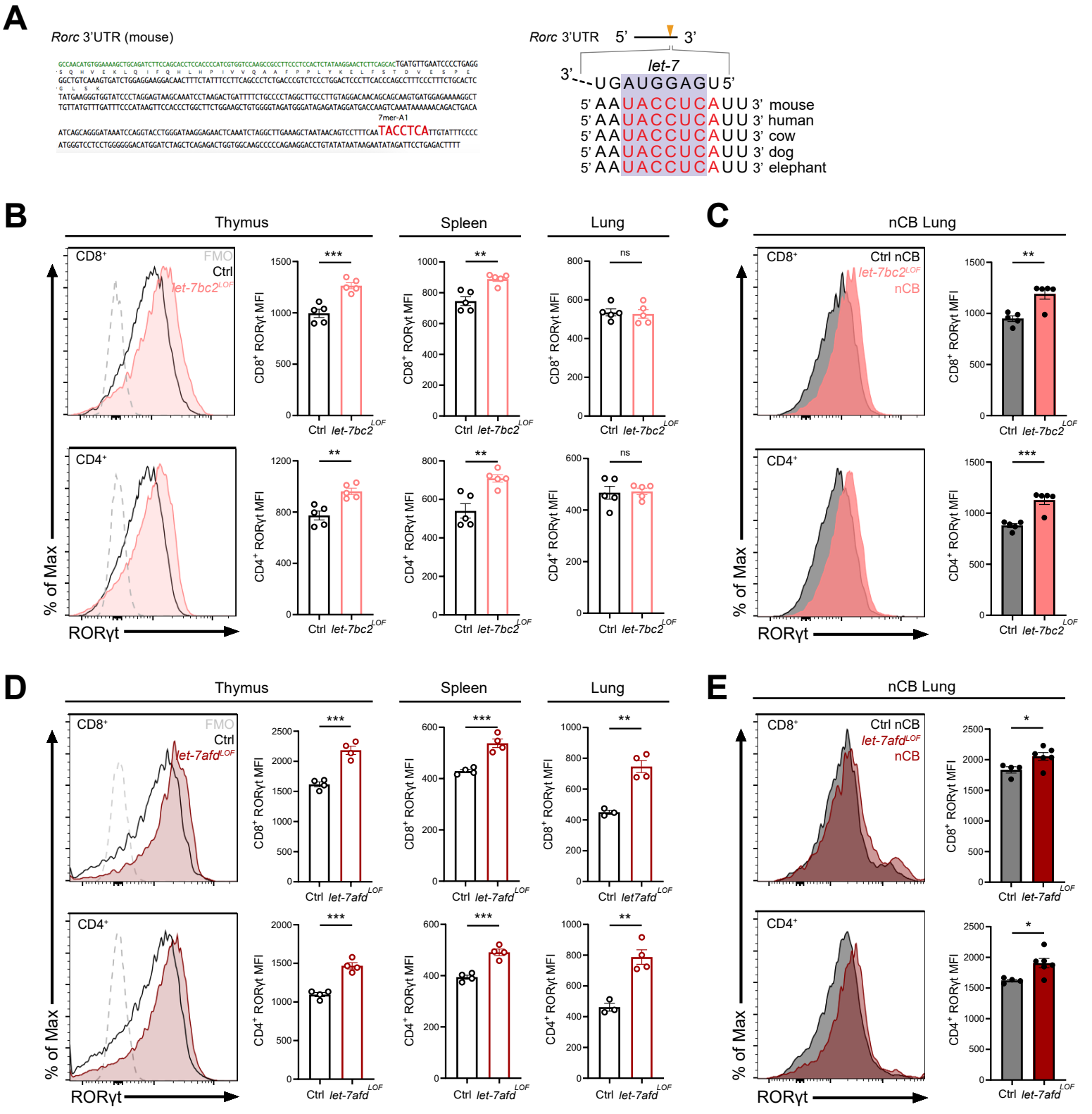

Figure 4. Deletion of either the *let7bc2*- or *let7afd*-cluster in T cells enhances RORγt expression *in vivo*. (A) Left: Schematic representation of the murine *Rorc* 3'UTR with *let-7* miRNA binding sites as identified by TargetScan. Right: Schematic of a conserved *let-7* miRNA target sequence in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

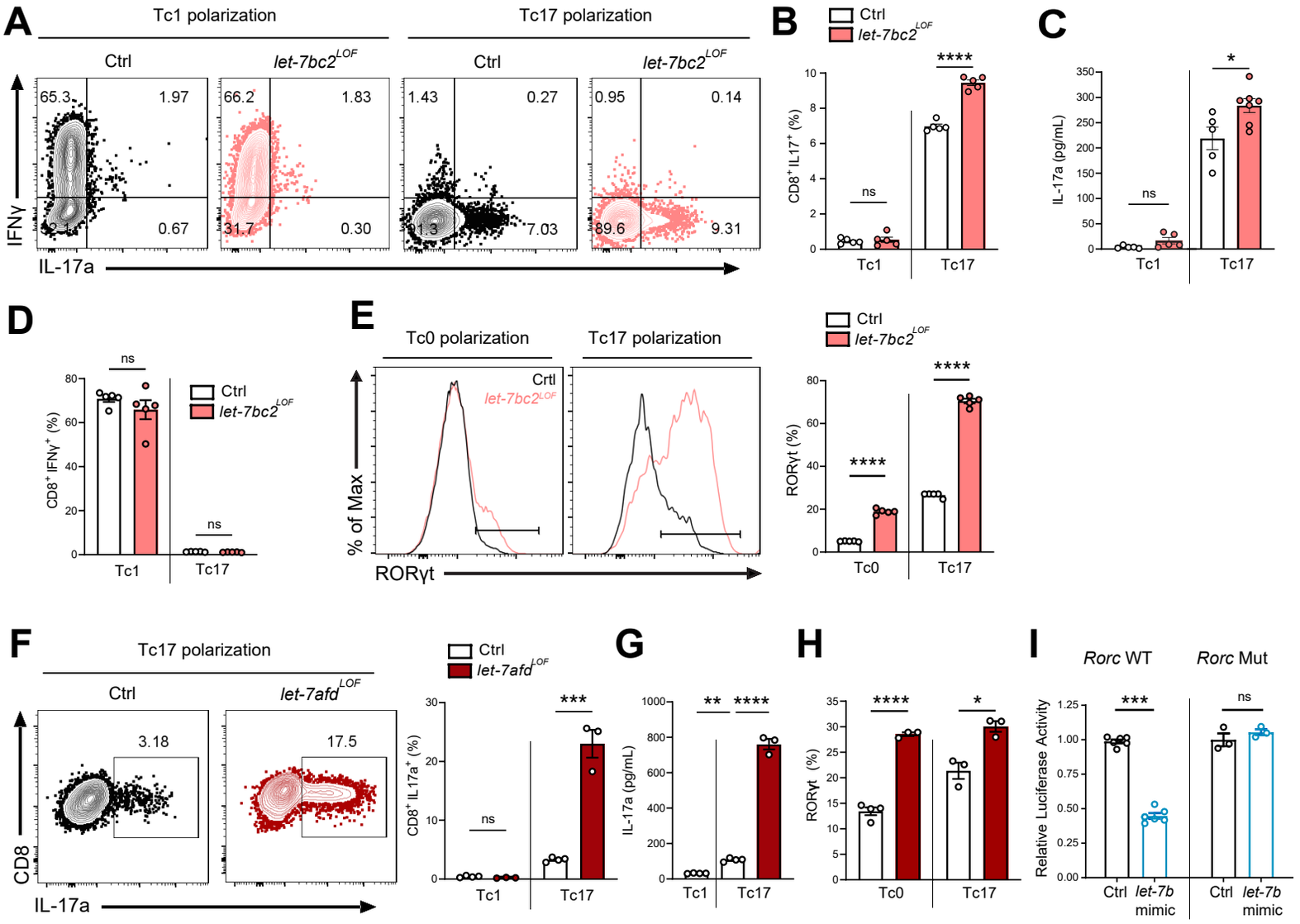

Figure 5. *Let-7* restricts Tc17 *in vitro* differentiation in part via direct targeting of *Rorc* mRNA. (A) Representative flow plots of live TCRβ$^+$ CD8$^+$, IL-17a$^+$ and IFNγ$^+$ populations from Tc1 and Tc17 polarized naïve splenic CD8$^+$ cells from control and *let-7bc2^{LOF}* mice and (B) quantification 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. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$.

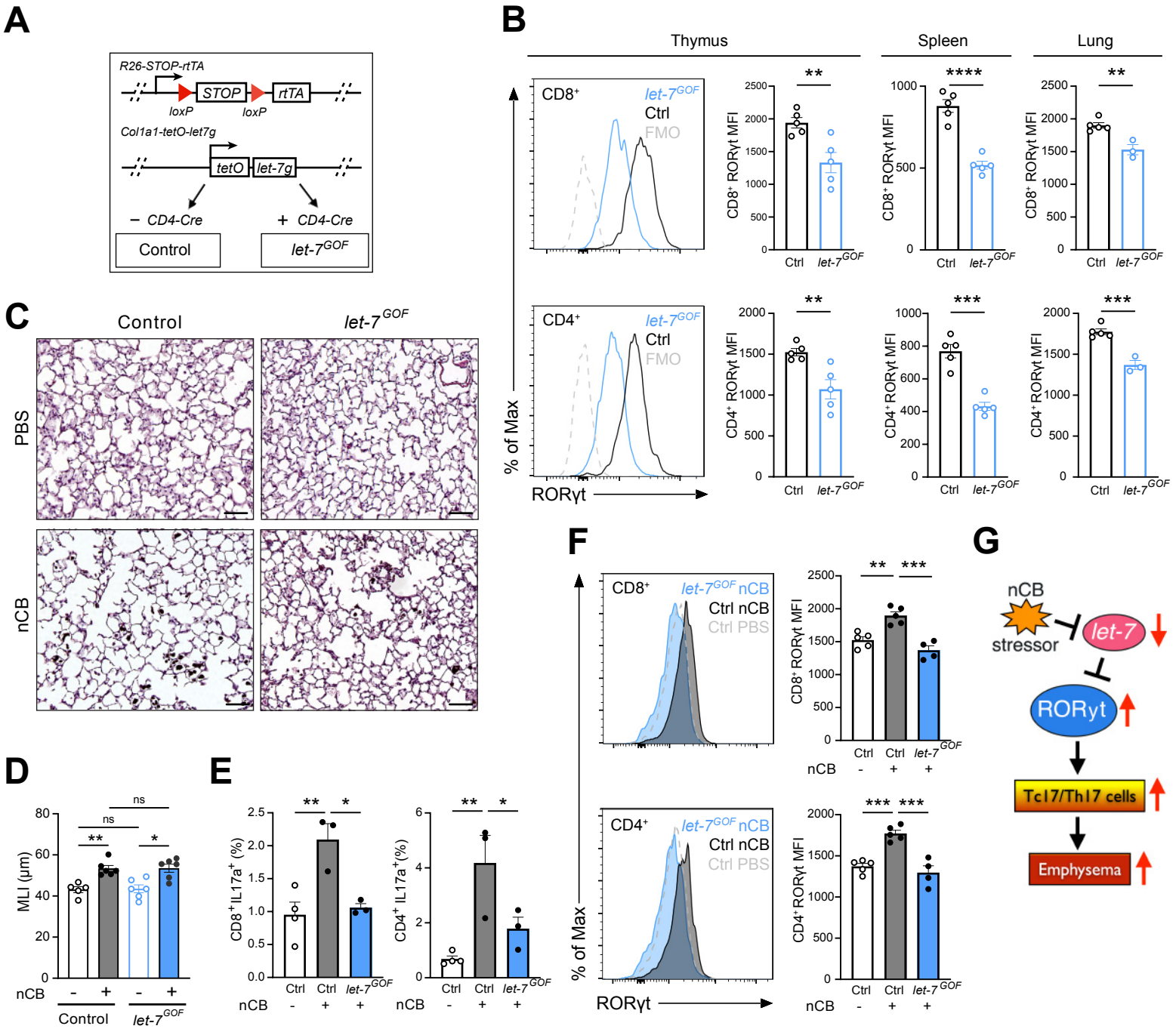

Figure 6. Enforced *let-7* expression in T cells restrains induction of RORγt and Tc17/Th17 inflammation in lungs of nCB-exposed mice. (A) Schematic outlining our T cell-inducible *let-7g* mouse model (*let-7*^{GOF}). (B) Flow analysis of RORγt expression in live, TCRβ$^+$CD8$^+$ or CD4$^+$ T cells from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