

1 **Title**

2 LincRNA-Cox2 functions to regulate inflammation in alveolar macrophages during acute lung  
3 injury.

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16 **Abstract**

17 The respiratory system exists at the interface between our body and the surrounding non-sterile  
18 environment; therefore, it is critical for a state of homeostasis to be maintained through a balance  
19 of pro- and anti- inflammatory cues. An appropriate inflammatory response is vital for  
20 combating pathogens, while an excessive or uncontrolled inflammatory response can lead to the  
21 development of chronic diseases. Recent studies show that actively transcribed noncoding  
22 regions of the genome are emerging as key regulators of biological processes, including  
23 inflammation. LincRNA-Cox2 is one such example of an inflammatory inducible long

24 noncoding RNA functioning to control immune response genes. Here using bulk and single cell  
25 RNA-seq, in addition to fluorescence activated cell sorting, we show that lincRNA-Cox2 is most  
26 highly expressed in the lung, particularly in alveolar macrophages where it functions to control  
27 immune gene expression following acute lung injury. Utilizing a newly generated lincRNA-  
28 Cox2 transgenic overexpressing mouse, we show that it can function in trans to control genes  
29 including Ccl3, 4 and 5. This work greatly expands our understanding of the role for lincRNA-  
30 Cox2 in host defense and sets in place a new layer of regulation in RNA-immune-regulation of  
31 genes within the lung.

## 32 **Introduction**

33 Acute lung injury (ALI) and its more severe form, known as acute respiratory distress  
34 syndrome (ARDS), are caused by dysregulated inflammatory responses resulting from  
35 conditions such as sepsis and trauma (Moldoveanu *et al*, 2009; Mokra & Kosutova, 2015; Wang  
36 *et al*, 2019b; Mowery *et al*, 2020; Butt *et al*, 2016). Fundamentally, the characteristics of ALI  
37 include neutrophilic alveolitis, dysfunction of barrier properties, microvascular thrombosis, the  
38 formation of hyaline membrane, alveolar macrophage dysfunction, as well as indirect systemic  
39 inflammatory responses (Pittet *et al*, 1997; Alluri *et al*, 2017; Gouda & Bhandary, 2019; Fan &  
40 Fan, 2018). Although a variety of anti-inflammatory pharmacotherapy are available, the  
41 morbidity and outcome of ALI/ARDS patients remain poor (Raghavendran *et al*, 2008; Yin &  
42 Bai, 2018; Suo *et al*, 2018; Deng *et al*, 2017). Therefore, obtaining a more complete  
43 understanding of the molecular mechanisms that drive ALI inflammatory dysfunction is of great  
44 importance to improving both the diagnosis and treatment of the condition.

45 Long noncoding RNAs (lncRNAs) are a class of non-coding RNAs that include 18,000 in  
46 human and nearly 14,000 in the mouse genome (Uszczynska-Ratajczak *et al*, 2018; Fang *et al*,

47 2018). Since their discovery, lncRNAs have been shown to be key regulators of inflammation  
48 both *in vitro* and *in vivo* (Robinson *et al*, 2020; Statello *et al*, 2021). Moreover, lncRNAs have  
49 also been characterized to be stable and detectable in body fluids (Quinn & Chang, 2016), and  
50 therefore have enormous potential for biomarker discovery in both diagnosis and prognosis  
51 applications (Aftabi *et al*, 2021; Ma *et al*, 2021; Viereck & Thum, 2017). A number of studies  
52 have been carried out to better understand the gene regulatory network between lncRNAs and  
53 mRNAs during ALI to identify novel biomarkers (Teng *et al*, 2021; Wang *et al*, 2019a). In  
54 addition to searching for biomarkers for ALI there have been studies performed to try and  
55 understand the functional mechanisms for lncRNAs during ALI (Chen *et al*, 2021).  
56 Lipopolysaccharide (LPS)-induced acute lung injury (ALI) is a commonly utilized animal model  
57 of ALI as it mimics the inflammatory induction and polymorphonuclear (PMN) cell infiltration  
58 observed during clinical ALI (Asti *et al*, 2000). One study showed that knocking down  
59 MALAT1, a well-studied lncRNA, exerts a protective role in the LPS induced ALI rat model and  
60 inhibited LPS-induced inflammatory response in murine alveolar epithelial cells and murine  
61 alveolar macrophages cells through sponging miR-146a (Dai *et al*, 2018). Additionally, Xist has  
62 been shown to attenuate LPS-induced ALI by functioning as a sponge of miR-146a-5p to  
63 mediate STAT3 signaling (Li *et al*, 2021).

64 Previous work by ourselves and others identifies long intergenic noncoding RNA-Cox2  
65 (lincRNA-Cox2) as a regulator of immune cell signaling in macrophages (Carpenter *et al*, 2013;  
66 Tong *et al*, 2016; Hu *et al*, 2016; Covarrubias *et al*, 2017; Hu *et al*, 2018; Liao *et al*, 2020; Xue  
67 *et al*, 2019). We have previously characterized multiple mouse models to show that lincRNA-  
68 Cox2 functions *in vivo* to regulate the immune response. We showed that lincRNA-Cox2  
69 knockout mice (Sauvageau *et al*, 2013) have profound defects in the neighboring protein coding

70 gene Ptgs2. We went on to show that lincRNA-Cox2 regulates Ptgs2 in *cis* through an enhancer  
71 RNA mechanism requiring locus specific transcription of the lncRNA (Elling *et al*, 2018). In  
72 order to study the function of lincRNA-Cox2 independently of its role in regulating Ptgs2 in *cis*  
73 we generated a lincRNA-Cox2 mutant mouse using CRISPR to target the splice sites resulting in  
74 significant loss of the RNA allowing us to study the role for the RNA in *trans*. We performed an  
75 LPS-induced endotoxic shock model and confirmed that lincRNA-Cox2 is an important positive  
76 and negative regulator of immune genes in *trans*. We previously showed that lincRNA-Cox2 is  
77 most highly expressed at steady-state in the lung and in this study, we utilize our mutant model  
78 to determine if lincRNA-Cox2 can function in *trans* to regulate gene expression in the lung  
79 (Elling *et al*, 2018). In addition, we characterize a transgenic overexpressing mouse model of  
80 lincRNA-Cox2 and show that the defects in immune gene expression caused by removal of  
81 lincRNA-Cox2 can be rescued by the transgenic overexpression of lincRNA-Cox2 in an LPS-  
82 induced ALI model. Mechanistically, we show through bone marrow chimeric studies that  
83 lincRNA-Cox2 expression is coming from bone marrow derived cells to regulate genes within  
84 the lung. Finally, we show that lincRNA-Cox2 is most highly expressed in alveolar macrophages  
85 where it functions to regulate inflammatory signaling. Collectively we show that lincRNA-Cox2  
86 is a trans-acting lncRNA that functions to regulate immune responses and maintain homeostasis  
87 within the lung at baseline and upon LPS-induced ALI.

## 88 **Results**

### 89 ***Immune gene expression is altered in the lungs of lincRNA-Cox2 deficient mice.***

90 In order to determine the role for lincRNA-Cox2 in the lung we first examined our RNA-  
91 sequencing data to compare gene expression profiles in the lungs of WT versus lincRNA-Cox2  
92 mutant mice (Elling *et al*, 2018, GSE117379) (Figure 1A). We found 85 genes down-regulated

93 and 41 genes up-regulated in the lincRNA-Cox2 mutant lungs compared to WT (Figure 1B).  
94 Gene-ontology analysis showed that both the down and up regulated genes were associated with  
95 the immune system, metabolism, and response to stimulus (Figure 1C-D), which are similar to  
96 pathways that lincRNA-Cox2 has previously been associated with in bone marrow derived  
97 macrophages (BMDMs) (Carpenter *et al*, 2013). To determine if loss of lincRNA-Cox2 impacts  
98 protein expression we performed ELISAs on lung homogenates from WT and lincRNA-Cox2  
99 deficient mice (Figure 1E). While many genes remained unchanged at baseline between the WT  
100 and lincRNA-Cox2 mutant lungs (SFigure 1I-P), we did find that Il-12p40, Cxcl10, Ccl3, Ccl4,  
101 Cxcl2, Ccl5 and Ccl19 are all significantly up-regulated in the mutant lungs at baseline (Figure  
102 1A-H). Interestingly none of these cytokines are up-regulated at the RNA level in our whole lung  
103 tissue RNA-sequencing suggesting that they might be regulated post-transcriptionally or that  
104 they are only regulated in a small subset of cells that cannot be easily captured from the whole  
105 lung lysate RNA-seq data (SFigure 1I-P).

106 Finally, we measured the immune cell repertoire in the bronchiolar lavage fluid (BAL) of  
107 WT and lincRNA-Cox2 deficient mice and found that B cells and dendritic cells, while at very  
108 low expression levels in both strains, are significantly lower at baseline in the mutant mice  
109 (Figure 1M). These findings indicate that lincRNA-Cox2 functions as both a positive and  
110 negative regulator of immune gene expression which can impact the cellular milieu within the  
111 lung at steady state.

### 112 ***LincRNA-Cox2 regulates the pro-inflammatory response during acute lung injury (ALI).***

113 Our data suggest that lincRNA-Cox2 plays a role in regulating immune gene expression  
114 at steady-state, therefore we wanted to determine if loss of lincRNA-Cox2 could impact the  
115 immune response during acute lung injury. We employed an LPS induced acute lung injury

116 (ALI) model as outlined in Figure 2A. We assessed the immune cell repertoire within the BAL  
117 by flow cytometry in WT and lincRNA-Cox2 mutant mice following LPS challenge and found  
118 that the most abundant and critical cell type, neutrophils, were significantly reduced when  
119 lincRNA-Cox2 is removed (Figure 2B). We also assessed the cytokine and chemokine response  
120 both in the BAL and serum of WT and lincRNA-Cox2 mutant mice by ELISA. We found that  
121 Il6, Ccl3 and Ccl4 were downregulated in the serum and the BAL of the lincRNA-Cox2 mutant  
122 mice (Figure 2C-E). In addition, several proteins were specifically affected either in the serum or  
123 BAL, such as Ccl5 and Ccl22 which were upregulated in the serum (Figure 2F-G), while Ifnb1  
124 was upregulated only in the BAL of the mutant mice (Figure 2H). Consistent with our previous  
125 work tnf remains unchanged between WT and lincRNA-Cox2 mutant mice (Figure 2I). These  
126 data suggest that lincRNA-Cox2 exerts different effects within the lung compared to the  
127 periphery and that lincRNA-Cox2 can impact acute inflammation at the protein and cellular  
128 levels within the lung.

129 ***Generation and characterization of a transgenic mouse model overexpressing lincRNA-Cox2.***

130 We have determined that lincRNA-Cox2 is critical in regulating inflammation at  
131 baseline, during a septic shock model (Elling *et al*, 2018) and here during an acute lung injury  
132 model. In order to confirm that lincRNA-Cox2 is functioning in *trans*, we generated a transgenic  
133 lincRNA-Cox2 mouse line using the TARGATT system, which allows for stable integration of  
134 lincRNA-Cox2 into the H11 locus (Tasic *et al*, 2011) (Figure 3A). The inserted cassette is  
135 carrying a CAG promoter, lincRNA-Cox2, and an SV40 polA stop cassette. Mice were bred to  
136 homozygosity (SFigure 2), and lincRNA-Cox2 levels were measured in WT and Transgenic  
137 bone-marrow-derived macrophages (BMDMs) (Figure 3B). As expected, lincRNA-Cox2 is  
138 highly expressed in the transgenic macrophages with no difference in expression following LPS

139 stimulation. Next, we performed a septic shock model of WT and transgenic mice to determine if  
140 overexpression of lincRNA-Cox2 can impact the immune response (Figure 3C). As expected  
141 lincRNA-Cox2 is highly expressed in the lung tissue of the transgenic mice and interestingly we  
142 observe increased levels of Il6 while other lincRNA-Cox2 target genes, such as Ccl5 are not  
143 affected. This suggests that overexpression of lincRNA-Cox2 can have the opposite phenotype to  
144 knocking out the gene to regulate Il6 within the lung (Figure 3D, SFigure 3A-C). We harvested  
145 serum from the mice at steady state and found higher levels of Csf1 and lower levels of Il10  
146 (Figure 3E-F) in the mice overexpressing lincRNA-Cox2. Other inflammatory cytokines  
147 including Il6, Ccl5, Ccl3 and Ccl4 were unaltered in lincRNA-Cox2 transgenic mice serum  
148 (Figure 3G-J). These data suggests that at steady state overexpression of lincRNA-Cox2 does not  
149 have broad impacts on gene expression, however it can impact specific genes including Il6 in the  
150 lung and Il10 and Csf1 in the periphery.

151 ***LincRNA-Cox2 functions in trans to regulate acute inflammation.***

152 To determine if lincRNA-Cox2 can function in *trans* to regulate immune genes following  
153 an *in vivo* challenge with LPS we crossed the mice deficient in lincRNA-Cox2 (Mut) with the  
154 transgenic overexpressing mice (TG) generating mice labeled throughout as MutxTG (Figure  
155 4A). We first performed an intraperitoneal (IP) endotoxic shock model to determine if we could  
156 rescue the lincRNA-Cox2 phenotype identified in our previous study (Elling *et al*, 2018) (Figure  
157 4B). As expected, lincRNA-Cox2 expression is significantly reduced in the lung tissue and BAL  
158 of the deficient mice (Mut) mice, and highly expressed in the MutxTG mouse (Figure 4C-D). We  
159 found that Ccl5 and Cxcl10 are expressed at higher levels in the BAL and serum of lincRNA-  
160 Cox2 mutant mice (Figure 4E-H) and the expression levels can be rescued by trans expression of  
161 lincRNA-Cox2 in the MutxTG mice where the levels of the two proteins return to WT levels.

162 Next, we wanted to determine if transgenic overexpression of lincRNA-Cox2 can reverse  
163 the phenotype observed in the deficient mice during acute lung injury (Figure 5A). We assessed  
164 immune cell recruitment in both the BAL and lung tissue by flow cytometry in WT, mutant and  
165 MutxTG mice. Again, we found that neutrophils are the only immune cell that is significantly  
166 lower in lincRNA-Cox2 mutant mice, while neutrophil recruitment in MutxTG mice return to  
167 WT levels (Figure 5B-C). Next, we performed ELISAs on harvested lung tissue, BAL and serum  
168 to measure the protein concentration of cytokines and chemokines. We found that Il6, Ccl5,  
169 Ccl3, Ccl4, Ccl22 and Ifnb1 are consistently significantly dysregulated in the lincRNA-Cox2  
170 mutant mice and again this phenotype could be rescued back to WT levels by the transgenic  
171 overexpression of lincRNA-Cox2 (MutxTG) (Figure 4D-I). Interestingly, Il6, Ccl3, Ccl4 and  
172 Ifnb1 are all significantly different in the BAL, while Ccl5 and Ccl22 are significantly  
173 dysregulated in the lung tissue only suggesting that lincRNA-Cox2 can impact genes in a cell-  
174 specific manner. We find that Tnf is consistently unaffected between all genotypes (Figure 4J).  
175 From these data we conclude that lincRNA-Cox2 functions in *trans* to regulate the lung immune  
176 response during acute inflammation.

177 ***LincRNA-Cox2 positively and negatively regulates immune genes in primary alveolar  
178 macrophages.***

179 In order to understand how lincRNA-Cox2 could be regulating acute inflammation we  
180 first wanted to determine which cell types lincRNA-Cox2 is most highly expressed within the  
181 lung. First, we utilized publicly available single-cell RNA sequencing (scRNA-seq) data from  
182 two LPS-induced lung injury studies (Riemonyd *et al*, 2019; Mould *et al*, 2019). Overall,  
183 lincRNA-Cox2 was very low in these datasets (SFigure 5A-E). There was a slight increase in  
184 expression in all alveolar epithelial type 2 (ATII) cellular populations (SFigure 5D-E). Due to the

185 expression level limitations of publicly available scRNA-seq datasets, we next performed  
186 fluorescence activated cell sorting (FACS) to isolate all cell-types of interest in the lung at  
187 baseline and following LPS-induced lung injury (Elling *et al*, 2018). Using RT-qPCR we found  
188 that lincRNA-Cox2 was most highly expressed in neutrophils at both baseline and following LPS  
189 stimulation when normalized to cell count (SFigure 6). Interestingly alveolar macrophages  
190 (AMs) were identified as the cell type with the highest induction of lincRNA-Cox2, following  
191 LPS stimulation (Figure 6A).

192 Since lincRNA-Cox2 is most highly induced in AMs we wanted to determine if this was  
193 the cell type contributing to the cytokine and chemokine changes in lincRNA-Cox2 deficient  
194 (Mut) mice following inflammatory challenge. To do this, we harvested BAL fluid from WT and  
195 lincRNA-Cox2 mutant mice to culture primary alveolar macrophages and treated them with LPS  
196 for 24 h (Figure 6B). First, lincRNA-Cox2 induction was validated using RT-qPCR with *in vitro*  
197 LPS stimulated WT AMs while expression as expected is diminished in the lincRNA-Cox2  
198 deficient AMs (Figure 6B-C). Finally, we assessed the level of cytokine and chemokine  
199 expression from primary AMs by ELISA. We confirmed significant dysregulation of Il6, Ccl5,  
200 Ccl3, Ccl4 and Ccl22 in primary alveolar macrophages, which are consistent with the *in vivo*  
201 data (Figure 6D-H). Additionally, we find novel dysregulation of Cxcl2, Cxcl1, Cxcl10 in  
202 alveolar macrophages not detected in our ALI model. Again, Tnf remains consistently  
203 unchanged between WT and mutant in AMs and *in vivo* studies (Figure 6M). These data indicate  
204 that mechanistically lincRNA-Cox2 is functioning to regulate gene expression primarily within  
205 primary alveolar macrophages.

206 ***Peripheral immune cells drive the regulatory role of lincRNA-Cox2 during ALI.***

207 From our *in vivo* mouse models, we can conclude that lincRNA-Cox2 functions in *trans*  
208 to regulate immune genes and cellular milieu within the lung during ALI. To determine if  
209 lincRNA-Cox2 functions through bone-marrow derived immune cells, we performed bone  
210 marrow (BM) transplantation experiments utilizing Ubiquitin C (Ubc)-GFP WT bone marrow to  
211 enable us to easily track chimerism through measurement of GFP. Ubc-GFP WT BM was  
212 transplanted into lincRNA-Cox2 mutant mice and WT mice generating WT→WT and WT→Mut  
213 mice (Figure 7A). First, we determined the reconstitution of HSCs by measuring donor  
214 chimerism (GFP%) in the peripheral blood (PB) for the duration of the 8 weeks. We find that  
215 both the WT→WT and WT→Mut mice have 100% donor reconstitution of granulocytes/  
216 myelomonocytes (GMs) and B cells and ~75% donor T cells in peripheral blood (Figure 7B-D).  
217 While there was a small but significant decrease in T cell reconstitution in WT→Mut mice, we  
218 found there is no significant reconstitution difference of GMs or B cells between WT→WT and  
219 WT→Mut mice.

220 After 8 weeks when the immune system was fully reconstituted, we performed the LPS  
221 ALI model on the chimera WT→WT and WT→Mut mice. We found that the percentage of  
222 donor reconstitution within peripheral blood was 100% indicating a successful BM  
223 transplantation (Figure 7B-D, SFigure 7) (Hashimoto *et al*, 2013). We found that the decrease in  
224 neutrophil recruitment that we identified in the lincRNA-Cox2 mutant mice (Figure 2B,  
225 Figure 5B-C, Figure 7E) were rescued in the WT→Mut model back to similar levels to the  
226 WT→WT mice (Figure 7E). Furthermore, the altered expression of Il6, Ccl3, and Ccl4 found in  
227 the lincRNA-Cox2 mutant mice were also returned to WT levels in the WT→Mut mice (Figure  
228 2C-E, Figure 5D-F, Figure 7F-J). As expected, Tnf acts as a control cytokine showing no  
229 difference across genotypes. (Figure 7K). These data suggest that lincRNA-Cox2 is functioning

230 through an immune cell from the bone marrow, most likely alveolar macrophages to regulate  
231 acute inflammatory responses within the lung.

232 **Discussion**

233 LncRNAs are rapidly emerging as critical regulators of biological responses and in recent  
234 years there have been several studies showing that these genes play key roles in regulating the  
235 immune system (Robinson *et al*, 2020). However very few studies have functionally  
236 characterized lncRNAs using mouse models *in vivo*. We and others have studied the role for  
237 lincRNA-Cox2 in the context of macrophages and shown that it can act as both a positive and  
238 negative regulator of immune genes (Carpenter *et al*, 2013; Tong *et al*, 2016; Hu *et al*, 2016;  
239 Covarrubias *et al*, 2017; Hu *et al*, 2018; Liao *et al*, 2020; Xue *et al*, 2019). We previously  
240 characterized two mouse models of lincRNA-Cox2, a knockout (KO) and an intronless splicing  
241 mutant (Mut). We identified lincRNA-Cox2 as a *cis* acting regulator of its neighboring protein  
242 coding gene Ptgs2 using the KO mouse model. In order to study the role for lincRNA-Cox2  
243 independent of its *cis* role regulating Ptgs2 we generated the splicing mutant (Mut) mouse  
244 model. This model enabled us to show that knocking down transcription of lincRNA-Cox2  
245 impacts a number of immune genes including Il6 and Ccl5 in an LPS induced endotoxic shock  
246 model. In this current study we make use of the mutant mouse model to study the role for  
247 lincRNA-Cox2 in regulating immune responses in the lung, where lincRNA-Cox2 is most highly  
248 expressed, both at steady-state and following LPS-induced acute lung injury (ALI). Both  
249 neutrophil recruitment and chemokine/cytokine induction are hallmarks of acute lung injury  
250 (ALI) (Allen, 2014; Domscheit *et al*, 2020; Ali *et al*, 2020) and here we provide *in vivo* and *in*  
251 *vitro* evidence that lincRNA-Cox2 plays a critical role in these processes.

252 We find that loss of lincRNA-Cox2 at baseline results in the up- and down-regulation of  
253 a number of genes that regulate the immune system and metabolism (Figure 1A-D) indicating  
254 that lincRNA-Cox2 is a key transcriptional regulator of gene expression within lung tissue. In  
255 addition, we measured immune cells and found that at baseline dendritic cells and B cells were  
256 lower in the lincRNA-Cox2 mutant mice. Lung DCs serve as a functional signaling/sensing units  
257 to maintain lung homeostasis by orchestrating host responses to benign and harmful foreign  
258 substances, while B cells are crucial for antibody production, antigen presentation and cytokine  
259 secretion (Wang *et al*, 2019a; Menon *et al*, 2021). Having fewer DC and B cells at steady state  
260 could lead to an increased risk of inflammatory diseases (Seys *et al*, 2015; Cook & MacDonald,  
261 2016). This indicates that lincRNA-Cox2 plays an important role in maintaining lung  
262 homeostasis since gene expression and cellular abundance are altered by the loss of lincRNA-  
263 Cox2.

264 While we identify lincRNA-Cox2 as a crucial element for maintaining lung homeostasis,  
265 we next performed LPS induced acute lung injury (ALI) to assess the importance of lincRNA-  
266 Cox2 during active inflammation. Using a 24 h time point, which shows the maximum influx of  
267 PMNs and cytokine/chemokine expression (Domscheit *et al*, 2020), we find that loss of  
268 lincRNA-Cox2 leads a decrease in neutrophil recruitment and altered cytokine/chemokine  
269 expression in both the BAL and serum (Figure 2). In acute lung injury, neutrophils are crucial for  
270 bacterial clearance during live infection, repair and tissue remodeling after ALI (Blázquez-Prieto  
271 *et al*, 2018; Giacalone *et al*, 2020). Our data suggests that lincRNA-Cox2 plays an important role  
272 in neutrophil recruitment and therefore could also play roles in clearance of live bacteria and  
273 repair of tissue after resolution of infection. We found that Il6 levels are reduced while Ccl5  
274 levels are increased following ALI in the lincRNA-Cox2 mutant mice. These findings are

275 consistent with our previous *in vitro* and *in vivo* studies (Carpenter *et al*, 2013; Elling *et al*,  
276 2018). Newly, we found that Ccl3 and Ccl4 are positively regulated, while Ccl22 and Ifnb1 are  
277 negatively regulated by lincRNA-Cox2 in the lung during ALI. Interestingly, Lee *et al*. reported  
278 in humans that the chemokines, CCL3 and CCL4, promote the local influx of neutrophils (Lee *et*  
279 *al*, 2000). Therefore, the decreased expression of Ccl3 and Ccl4 *in vivo* in the lincRNA-Cox2  
280 mutant mice could explain the significant decrease of neutrophil recruitment seen in the BAL  
281 (Figure 2B).

282 To date there remains only a small number of lncRNAs that have been functionally and  
283 mechanistically characterized *in vivo*. In fact, Pnky, Tug1 and Firre are the only other lncRNA  
284 studies that show that their phenotype can be rescued in *trans in vivo* (Andersen *et al*, 2019;  
285 Lewandowski *et al*, 2019, 2020). From our previous *in vivo* studies, we had concluded that  
286 lincRNA-Cox2 functions in *cis* to regulate Ptgs2 in an eRNA manner while it functions in *trans*  
287 to regulate genes such as Il6 and Ccl5 (Elling *et al*, 2018). In order to prove that indeed  
288 lincRNA-Cox2 can function in *trans* to regulate immune genes we generated a transgenic mouse  
289 overexpressing lincRNA-Cox2 from the H11 locus using the TARGATT system (Tasic *et al*,  
290 2011) (Figure 3A). Simply overexpressing lincRNA-Cox2 has minimal impacts on the immune  
291 response at baseline or following LPS challenge *in vivo* suggesting that locus specific or  
292 inflammatory specific induction of lincRNA-Cox2 is important for its role in regulating immune  
293 genes. However, we do note that Il6 levels are higher in the lungs of lincRNA-Cox2 transgenic  
294 mice and Csf1 and Il10 are lower in serum following endotoxic shock suggesting that simply  
295 overexpressing lincRNA-Cox2 can impact a small number of genes. Our primary goal for  
296 generating the transgenic mouse line overexpressing lincRNA-Cox2 was to determine if crossing  
297 it to our lincRNA-Cox2 mutant (deficient) mouse generating a MutxTG line (Figure 4A) would

298 rescue the observed phenotypes following LPS challenge using either an intraperitoneal delivery  
299 or via oropharyngeal delivery. Interestingly, we find that our MutxTg mice do rescue the  
300 phenotype found in both the endotoxic shock model (Figure 4) and LPS induced ALI model  
301 (Figure 5), showing definitively that lincRNA-Cox2 regulates gene regulation and cellular  
302 recruitment in *trans*. To delve more deeply into exactly how lincRNA-Cox2 is functioning to  
303 regulate immune genes in the lung we focused on determining which cell type lincRNA-Cox2 is  
304 most highly expressed in. Analysis of scRNA-seq indicated that lincRNA-Cox2 was highly  
305 expressed in naive and injured alveolar epithelial type II (AECII) cells (Supplemental Figure 5),  
306 however overall lincRNA-Cox2 was difficult to detect in single-cell data probably due to a  
307 combination of low expression levels and low read depth. Utilizing FACS and qRT-PCR, we  
308 measured the expression of lincRNA-Cox2 in 8 immune cell populations and 4  
309 epithelial/endothelial cell populations and found that lincRNA-Cox2 was most highly expressed  
310 in neutrophils (SFigure 6). However, when assessing induction of lincRNA-Cox2 following LPS  
311 and normalizing to PBS controls we found it to be most highly expressed in alveolar  
312 macrophages with some significant induction also observed in monocytes (Figure 6A). We know  
313 alveolar macrophages are critical effector cells in initiating and maintaining pulmonary  
314 inflammation, as well as termination and resolution of pulmonary inflammation during acute  
315 lung injury (ALI) (Beck-Schimmer *et al*, 2005; Herold *et al*, 2011). Therefore, to determine if  
316 the altered gene expression profiles we observed following ALI in the BAL were due to  
317 lincRNA-Cox2 expression in alveolar macrophages we cultured primary alveolar macrophages  
318 from our WT and lincRNA-Cox2 mutant mice and measured cytokine and chemokine expression  
319 (Machiels *et al*, 2017; Nayak *et al*, 2018; Robinson *et al*, 2021). Excitingly, we found decreased  
320 expression of Il6, Ccl3 and Ccl4 and increased expression of Ccl5 in the lincRNA-Cox2 mutant

321 alveolar macrophages (Figure 6 D-G). Several other chemokines such as Ccl3, Ccl4, Csf3, Cxcl1  
322 and Cxcl2 were significantly lower in the lincRNA-Cox2 deficient alveolar macrophages and  
323 these all are known to play roles in neutrophil influx (Lee *et al*, 2000; Kobayashi, 2008;  
324 Metzemaekers *et al*, 2020). These data suggest that lincRNA-Cox2 functions within alveolar  
325 macrophages to regulate gene expression including key chemokines that can impact neutrophil  
326 infiltration during acute lung injury.

327 During ALI many of the immune cells that infiltrate the lung including some classes of  
328 alveolar macrophages originate from the bone marrow. Therefore, to assess if lincRNA-Cox2 is  
329 functioning through bone marrow (BM) derived immune cells we performed BM transplantation  
330 chimera experiments in WT and lincRNA-Cox2 mutant mice (Figure 7A). We found that  
331 transplanting WT BM into our lincRNA-Cox2 mutant mice completely rescued the neutrophil  
332 and cytokine/chemokine phenotype in the BAL (Figure 7B-K). While we aimed to determine if  
333 lincRNA-Cox2 functions either through resident (SiglecF+) or recruited (Siglec F-) alveolar  
334 macrophages, we found that both populations were composed of >70% donor BM (SFigure 8I-J)  
335 indicating that radiation obliterated resident SiglecF+ alveolar cells which become repopulated  
336 with donor cells from the bone marrow (Guilliams *et al*, 2013; Hashimoto *et al*, 2013; Misharin  
337 *et al*, 2017; Collins *et al*, 2020; Gangwar *et al*, 2020). These experiments enable us to conclude  
338 that lincRNA-Cox2 expression originating from the bone marrow can function to control  
339 immune responses in the lung, since BM derived immune cells transplanted into lincRNA-Cox2  
340 mutant mice are able to rescue the phenotype driven by loss of lincRNA-Cox2 in the lung.

341 In conclusion, in this study we show, through multiple mouse models, that lincRNA-  
342 Cox2 is functioning in *trans* in alveolar macrophages to regulate immune responses within the  
343 lung. This study provides an additional layer of mechanistic understanding highlighting that

344 lncRNAs can contribute to the delicate balance between maintenance of homeostasis and  
345 induction of transient inflammation within the lung microenvironment.

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358 **Author Contribution**

359 E.K.R. designed, performed, and analyzed all molecular biology and *in vivo* experiments for all  
360 figures. A.K.W. and D.M.P. assisted with flow cytometry and sorting experiments, as well as  
361 helped design and perform chimera transplantation experiments. BS set-up and performed  
362 experiments for Figure 3. M.M.S. and H.H. assisted with all *in vivo* experiments. B.M. analyzed  
363 bulk RNA-sequencing experiments. V.S. helped with analyzing RT-qPCR experiments in Figure  
364 5. L.S. analyzed all scRNA-sequencing datasets. E.C.F. helped with the design of BM  
365 transplantation and chimera experiments. E.K.R. and S.C. conceived and coordinated the project.  
366 E.K.R. and S.C. wrote the manuscript with input from all other co-authors.

367 **Competing interests**

368 The authors have no competing financial interests.

369 **Methods**

370 Mice

371 Wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME)  
372 and bred at the University of California, Santa Cruz (UCSC). All mouse strains, including  
373 lincRNA-Cox2 mutant (Mut), transgenic (Tg) and MutxTg mice, were maintained under specific  
374 pathogen-free conditions in the animal facilities of UCSC and protocols performed in accordance  
375 with the guidelines set forth by UCSC and the Institutional Animal Care and Use Committee.

376 Generation of lincRNA-Cox2 Transgenic (Tg) and MutxTg Mice

377 lincRNA-Cox2 transgenic mice were generated by using a site-specific integrase-mediated  
378 approach described previously (Tasic *et al*, 2011). In brief, TARGATT mice in the C57/B6  
379 background contain a CAGG promoter within the Hipp11 (H11) locus expressing the full length  
380 lincRNA-Cox2 (variant 1) as previously cloned (Carpenter *et al*, 2013) generated at the  
381 Gladstone (UCSF). These mice were then genotyped using the same TARGATT approach of  
382 PCR7/8 (PR432:GATATCCTTACGGAATACCACTTGCCACCTATCACC,  
383 SH176:TGGAGGAGGACAAACTGGTCAC, SH178:TTCCCTTCTGCTTCATCTTGC). The  
384 lincRNA-Cox2 transgenic mice were then crossed with the lincRNA-Cox2 mutant mice and bred  
385 to homozygosity to generate MutxTg mice. For genotyping to assess homozygosity of Mutant we  
386 used the primer sets of MutF: ATGCCAGAGACAAAAAGGA and MutR:  
387 GATGGCTGGATTCCCTTGAA, as well as the 3-primer set stated above.

388 ALI model

389 Age- and sex matched WT, lincRNA-Cox2 mutant and lincRNA-Cox2 MutxTg mice were  
390 treated with 3.5mg/kg using the oropharyngeal intratracheal administration technique. The model  
391 of LPS insult via oropharyngeal administration into the lung was previously described in detail  
392 (Allen, 2014; Nielsen *et al*, 2018; Ehrentraut *et al*, 2019). Briefly, mice were sedated using an  
393 isoflurane chamber (3% for induction, 1-2% for maintenance), then 60-75ul of 3.5mg/kg of LPS  
394 (from strain O111:B4) or PBS (control) were administered using a pipette intratracheally. 24 h  
395 after LPS treatment, mice were sacrificed using CO<sub>2</sub> and serum, BALF and lung were harvested  
396 for either cellular assessment by flow cytometry, RNA expression or sent to EVE technologies  
397 for cytokine/chemokine protein analysis.

398 LPS Shock model

399 Age- and sex matched wild-type, lincRNA-Cox2 mutant mice, lincRNA-Cox2 Tg and lincRNA-  
400 Cox2 MutxTg (Elling *et al*, 2018) (8-12 weeks of age) were injected i.p. with 20 mg/kg LPS  
401 (O111:B4). For gene expression analysis and cytokine analysis, mice were euthanized 6 h post  
402 injection. Blood was taken immediately postmortem by cardiac puncture. Statistics were  
403 performed using GraphPad prism.

404 Transplantation reconstitution Assays

405 Reconstitution assays were performed, as previously stated by Poscablo *et al*. (Poscablo *et al*,  
406 2021), by transplanting double-sorted HSCs (200 per recipient) from Ubc-GFP+ whole BM and  
407 transplanting into congenic C57BL/6 WT and lincRNA-Cox2 deficient mice via retro-orbital  
408 intravenous transplant. We also transplanted double-sorted MkPs (22,000 per recipient) from  
409 C57Bl6 into Ubc-GFP+ hosts. Hosts were preconditioned with sub-lethal radiation (~750 rads)  
410 using a Faxitron CP160 X-ray instrument (Precision Instruments).

411 Harvesting Bronchiolar Lavage Fluid (BAL)

412 Bronchoalveolar Lavage Fluid (BALF) was harvested as previously stated by Cloonan *et al.*  
413 (Cloonan *et al*, 2016). 40 mice were euthanized by CO<sub>2</sub> narcosis, the tracheas cannulated, and  
414 the lungs lavaged with 0.5-ml increments of ice-cold PBS eight times (4 ml total), samples were  
415 combined in 50 ml conical tubes. BALF was centrifuged at 500 g for 5 min. 1 ml red blood cell  
416 lysis buffer (Sigma-Aldrich) was added to the cell pellet and left on ice for 5 min followed by  
417 centrifugation at 500 g for 5 min. The cell pellet was resuspended in 500  $\mu$ l PBS, and leukocytes  
418 were counted using a hemocytometer. Specifically, 10  $\mu$ l was removed for cell counting  
419 (performed in triplicate) using a hemocytometer. Cells were plated in sterile 12 well plates at  
420 5e5/well (total of 8 wells) and use complete DMEM with 25 ng/ml supGM-CSF.

421 Lung Tissue Harvesting for cytokine measurement

422 Mice were humanely sacrificed, and their lungs were excised. The whole lungs were snap frozen  
423 and homogenized, and the resulting homogenates were incubated on ice for 30 min and then  
424 centrifuged at 300  $\times$  g for 20 min. The supernatants were harvested, passed through a 0.45- $\mu$ m-  
425 pore-size filter, and used immediately or stored at -70°C, then sent to EVE for measurements of  
426 cytokines/chemokines.

427 Cell culture of Primary AMs

428 24 h post-BALF isolation, media was removed and fresh complete DMEM with 25 ng/ml  
429 supGM-CSF is added (Robinson *et al*, 2021). All cells that adhere to the surface of the plate are  
430 considered alveolar macrophages (AM) as previously determined by Chen *et al*. (Chen *et al*,  
431 1988). After new media is added, AMs are stimulated with 200 ng/ml LPS (Sigma, L2630-  
432 10MG). Harvest supernatant 6 h post-stimulation. Harvested supernatant was sent to Eve  
433 technologies for cytokine analysis. Statistics were performed using GraphPad prism.

434 RNA isolation, cDNA synthesis and RT-qPCR

435 Total RNA was purified from cells or tissues using Direct-zol RNA MiniPrep Kit (Zymo  
436 Research, R2072) and TRIzol reagent (Ambion, T9424) according to the manufacturer's  
437 instructions. RNA was quantified and assessed for purity using a nanodrop spectrometer  
438 (Thermo Fisher). Equal amounts of RNA (500 to 1,000 ng) were reverse transcribed using  
439 iScript Reverse Transcription Supermix (Bio-Rad, 1708841), followed by qPCR using iQ SYBR  
440 Green Supermix reagent (Bio-Rad, 1725122) with the following parameters: 50 °C for 2 min and  
441 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 45 s,  
442 followed by melt-curve analysis to control for nonspecific PCR amplifications. Oligos used in  
443 qPCR analysis were designed using Primer3 Input version 0.4.0 (<https://bioinfo.ut.ee/primer3-0.4.0/>).

445 Gene expression levels were normalized to Gapdh or Hprt as housekeeping genes.

446 *Primers Used:*

447 Gapdh F- CCAATGTGTCCGTCGTGGATC

448 Gapdh R - GTTGAAGTCGCAGGAGACAAC

449 HPRT F - TGCTCGAGATGTCATGAAGG

450 HPRT R - ATGTCCCCGTTGACTGAT

451 lincRNACox2 F - AAGGAAGCTTGGCGTTGTGA

452 lincRNACox2 R - GAGAGGTGAGGAGTCTTATG

453 ELISA

454 The concentration of Il6 and Ccl5 levels in the serum and BAL of WT, lincRNA-Cox2 mutant  
455 mice, lincRNA-Cox2 Tg and lincRNA-Cox2 MutxTg mice were determined using the DuoSet  
456 ELISA kits (R&D, DY1829 and DY478) according to the manufacturer's instructions.

457 Lung Tissue Harvesting for Cellular Analysis

458 Mice were humanely sacrificed, and their lungs were excised. Lung was inflated with a digestion  
459 solution containing 1.5mg/ml of Collagenase A (Roche) and 0.4mg/ml DNaseI (Roche) in HBSS  
460 plus 5% fetal bovine serum and 10mM HEPES. Trachea was tied off with 2.0 sutures. The heart  
461 and mediastinal tissues were carefully removed, and the lung parenchyma placed in 5ml of  
462 digestion solution and incubated at 37°C for 30 minutes with gently vortexing every 8–10  
463 minutes. Upon completion of digestion, 25ml of PBS was added; and the samples were vortexed  
464 at maximal speed for 30 seconds. The resulting cell suspensions were strained through a 70um  
465 cell strainer and treated with ACK RBC lysis solution. Then the cells were stained using the  
466 previously published immune (Yu *et al*, 2016) and epithelial (Nakano *et al*, 2018) cellular  
467 panels.

468 Flow Cytometry Analysis and Sorting

469 After cells were isolated and counted, ~2x10<sup>6</sup> cells per sample were incubated in blocking  
470 solution containing 5% normal mouse serum, 5% normal rat serum, and 1% FcBlock  
471 (eBiosciences, San Diego, CA) in PBS and then stained with a standard panel of  
472 immunophenotyping antibodies (See Supplemental Table for a list of antibodies, clones,  
473 fluorochromes, and manufacturers) for 30 minutes at room temperature (Yu *et al*, 2016). Data  
474 was acquired and compensation was performed on the BD Aria II and Attune NxT (Thermo  
475 Fisher) flow cytometer at the beginning of each experiment. Data was analyzed using FlowJo  
476 v10. Cell sorting was performed on a BD Aria II. The collected cells were harvested for RNA  
477 and RT-qPCR was performed to measure lincRNA-Cox2. Analysis was performed using FlowJo  
478 analysis software (BD Biosciences).

479 RNA-sequencing Analysis

480 Generation of the RNAseq data (GSE117379) and analysis of differential gene expression has  
481 been described previously (Elling *et al*, 2018). RNA-seq 50bp reads were aligned to the mouse  
482 genome (assembly GRCm38/mm10) using TopHat. The Gencode M13 gtf was used as the input  
483 annotation. Differential gene expression specific analyses were conducted with the DESeq2 R  
484 package. Specifically, DESeq2 was used to normalize gene counts, calculate fold change in gene  
485 expression, estimate p-values and adjusted p-values for change in gene expression values, and to  
486 perform a variance stabilized transformation on read counts to make them amenable to plotting.

487 **single cell RNA-sequencing Analysis**

488 Generation of the sc-RNAseq data (Gene Expression Omnibus accession number GSE120000  
489 and GSE113049) and analysis of gene expression has been described previously (Mould *et al*,  
490 2019; Riemondy *et al*, 2019).

491 *Plotting Expression across cells.* For overlaying expression onto the tSNE plot for single genes  
492 or for the average across a single panel of genes, we plotted normalized expression along a  
493 continuous color scale, with the extreme color values being set to the 5th and 95th quantile  
494 expression values. The bubble plot and heatmap show scaled normalized expressions along a  
495 continuous color scale. We produced the heatmap using Heatmap3 (Zhao *et al*, 2014).

496 **Figure Legends**

497 **Figure 1: lincRNA-Cox2 regulates immune signaling within the lung during homeostasis.**  
498 (A) Schematic of RNA-seq analysis of WT and Mutant lungs at baseline. (B) Volcano plot of  
499 differentially expressed genes from WT vs. Mutant lungs. Biological process gene ontology of  
500 (C) up-regulated genes and (D) down-regulated genes. (E) Schematic of cytokine analysis of  
501 lung homogenates from WT and mutant mice. Multiplex cytokine analysis was performed on  
502 lung homogenates for (F) Il-12p40 (G) Cxcl10 (H) Ccl3 (I) Ccl4 (J) Cxcl2 (K) Ccl5 and (L)

503 Ccl19. (M) Flow cytometry analysis of immune cells in the bronchiolar lavage fluid (BAL) at  
504 baseline gated off of CD45+ cells. The student's t-test used to determine the significance  
505 between WT and mutant mice. Asterisks indicate statistical significance (\*=> 0.05, \*\*=>.01,  
506 \*\*\*=> 0.0005).

507 **Figure 2: lincRNA-Cox2 positively regulates the pro-inflammatory response during acute**  
508 **lung injury (ALI).** (A) ALI schematic depicting the oropharyngeal route of 3.5mg/kg LPS  
509 administration in WT and Mutant. Mice were sacrificed after 24 h, followed by harvesting serum  
510 and bronchiolar lavage fluid (BAL). (B) Body temperatures were measured at 0 h, 6 h, 12 h, and  
511 24 h. (C) BAL cells were analyzed by flow cytometry to assess recruitment of immune cells in  
512 WT and immune cells gated off CD45+. Multiplex cytokine analysis was performed on serum  
513 and BAL for (D) Il6, (E) Ccl5, (F) Ccl3, (G) Ccl4, (H) Ccl22, (I) Ifnb-1 and (J) Tnf. The  
514 student's t-test used to determine the significance between WT and mutant mice. Asterisks  
515 indicate statistical significance (\*=> 0.05, \*\*=>.01, \*\*\*=> 0.0005).

516 **Figure 3: Characterization of lincRNA-Cox2 transgenic mouse.** (A) We have generated a  
517 transgenic lincRNA-Cox2 mouse line using the TARGATT system. This approach allows for  
518 stable integration of lincRNA-Cox2 into the H11 locus. Our inserted cassette is carrying a CAG  
519 promoter, lincRNA-Cox2, and an SV40 polA stop cassette. (B) lincRNA-Cox2 levels measured  
520 in WT and Transgenic bone-marrow-derived macrophages with and without LPS for 6 h,  
521 normalized to GapDH. (C) Schematic of 20mg/kg LPS septic shock model of WT and transgenic  
522 mice. (D) lincRNA-Cox2 measured by RT-qPCR in lung tissue. Serum was harvested and  
523 ELISAs were performed to measure (E) Csf1, (F) Il10, (G) Il6, (H) Ccl5, (I) Ccl3 and (J) Ccl4.  
524 The student's t-test used to determine the significance between WT and TG mice. Asterisks  
525 indicate statistical significance (\*=> 0.05, \*\*=>.01, \*\*\*=> 0.0005).

526 **Figure 4: lincRNA-Cox2 functions in *trans* to regulate the innate immune system in a septic  
527 shock model.** (A) Schematic depicting i.p. route of LPS infection in WT, mutant and transgenic  
528 mice. (C) WT, Mutant and TgxMut mice were challenged with 20mg/kg LPS and body  
529 temperatures were measured. Mice were sacrificed after 6h, bronchiolar lavage fluid (BAL),  
530 lungs and cardiac punctures were performed. BAL and Lungs were harvested for gene  
531 expression analysis by RT-qPCR for lincRNA-Cox2 (C-D), Ccl5 (E-F). BAL and isolated serum  
532 were sent for multiplex cytokine analysis (G-H). Each dot represents an individual animal.  
533 Student's t-tests were performed using Graphpad Prism7. Asterisks indicate statistically  
534 significant differences between mouse lines (\*=>0.05, \*\*=>0.01 and \*\*\*=>0.005). One-way  
535 ANOVA used to determine significance between WT, Mut, and MutxTG mice (#=>0.05).

536 **Figure 5: lincRNA-Cox2 regulates the proinflammatory response in the lung in *trans*.** (A)  
537 Generation of lincRNA-Cox2 MutxTG homozygous mouse. (B) ALI schematic depicting the  
538 oropharyngeal route of 3.5mg/kg LPS administration in WT, Mutant, and MutxTG. Mice were  
539 sacrificed after 24 h, followed by harvesting lung tissue, serum, and BAL fluid. (C) Body  
540 temperatures were measured at 0 h, 3 h, 6 h, and 24 h. (D) BAL and (E) Lung cells were  
541 analyzed by flow cytometry to assess recruitment of immune cells in WT and immune cells  
542 gated off CD45+. Multiplex cytokine analysis was performed on serum, BAL, and Lung tissue  
543 for (F) Il6, (G) Ccl5, (H) Ccl3, (I) Ccl4, (J) Ccl22, (K) Ifnb1 and (L) Tnf. Student's t-test used to  
544 determine significance between WT and Mut mice (\*=> 0.05). One-way ANOVA used to  
545 determine significance between WT, Mut, and MutxTG mice (#=>0.05).

546 **Figure 6: lincRNA-Cox2 is inducible and regulates immune genes both positively and  
547 negatively in primary alveolar macrophages.** (A) lincRNA-Cox2 was measured in whole lung  
548 tissue and several sorted immune and epithelial cells from mice treated with PBS and LPS via an

549 oropharyngeal route. Expression was normalized to PBS. Performed in biological triplicates and  
550 student's t-test was performed between whole lung tissue and each sorted cell. (B) The  
551 experimental design is depicted. BALs harvested from 40 WT and 40 Mutant mice, 10 WT or  
552 Mutant mice were pooled per well. Cells were treated with LPS for 24 h. (C) lincRNA-Cox2 was  
553 measured by RT-qPCR in primary alveolar macrophages. Multiplex cytokine analysis was  
554 performed supernatant from primary alveolar macrophages for (D) Il6, (E) Ccl5, (F) Ccl3, (G)  
555 Ccl4, (H) Ccl22, (I) Ccl2, (J) Cxcl10 and (K) Cxcl1. Student's t-test used to determine  
556 significance and asterisks indicate statistical significance (\*=> 0.05, \*\*>=.01, \*\*\*=> 0.0005).

557 **Figure 7: WT Bone marrow transplantation in lincRNA-Cox2 mutant mice rescues the**

558 **ALI phenotype.** (A) WT bone marrow transplantation and ALI experiment schematic.

559 Chimerism was assessed in the peripheral blood by gating for GFP% of (B)  
560 granulocytes/myelomonocytes (GMs), (C) B cells and (D) T cells. (E) Percentage of Ly6G+  
561 neutrophil populations were graphed of WT→WT and WT→Mut mice. Multiplex cytokine  
562 analysis was performed on BAL for (F) Il6, (G) Ccl3, (H) Ccl4 and (K) Tnf. Data of non-bone  
563 marrow transplant (BMT) mice experiments are from Figures 2 and 5. Student's t-test used to  
564 determine significance and asterisks indicate statistical significance (\*=> 0.05, \*\*>=.01, \*\*\*=>  
565 0.0005).

566 **Supplemental Figure 1: Characterization of immune pathways in lincRNA-Cox2 mutant at**

567 **baseline.** (A) Schematic of cytokine analysis of lung homogenates from WT and mutant mice.

568 Multiplex cytokine analysis was performed on lung homogenates for (B) Il16, (C) Timp1, (D)  
569 Ccl22, (E) Ccl11, (F) Vegf, (G) Ifnb1 and (H) Ccl12. (I) Schematic of RNA-seq analysis of WT  
570 and Mutant lungs at baseline. Normalized counts for (J) Il12p40, (K) Cxcl10, (L) Ccl3, (M)

571 Ccl4, (N) Cxcl2, (O) Ccl5 and (P) Ccl19. Student's t-test used to determine significance and  
572 asterisks indicate statistical significance (\*=> 0.05, \*\*=>.01, \*\*\*=> 0.0005).

573 **Supplemental Figure 2: PCR strategy of transgenic lincRNA-Cox2 mouse using the**  
574 **TARGATT system.** Generation of transgenic mouse with the Targatt system. This process  
575 allowed recombination at the H11 locus. Specific primer sets were used to confirm correct  
576 integration of lincRNA-Cox2. The strategy used to generate the lincRNA-Cox2 transgenic mice  
577 was adapted from Tasica *et al*, PNAS 2011 and genotyping (A-D) strategy was utilized to  
578 confirm homozygosity.

579 **Supplemental Figure 3: Overexpression of lincRNA-Cox2 *in vivo* does not regulate acute**  
580 **inflammation.** (A) Schematic of WT and Transgenic septic shock model. RNA was isolated  
581 from lung tissue to measure mRNA expression of (B) Il6 and (C) Ccl5, normalized to GapDH.  
582 Serum was harvested to measure (D) Cxcl10, (E) Il12p40, (F) Ifnb1 and (G) Tnf by ELISA.  
583 Student's t-test used to determine significance and asterisks indicate statistical significance (\*=>  
584 0.05, \*\*=>.01, \*\*\*=> 0.0005).

585 **Supplemental Figure 4: Genotyping strategy for MutxTG lincRNA-Cox2 mouse.** (A)  
586 PCR7/8 TARGATT primers were utilized to check for homozygosity. (B) lincRNA-Cox2  
587 specific primers were utilized to assess homozygosity of mutant mouse allele.

588 **Supplemental Figure 5: scRNA-seq does not significantly identify cell-type specific**  
589 **lincRNA-Cox2 expression in the lung during acute inflammation.** Mould *et al.* generated  
590 scRNA-seq of alveolar macrophages from WT mice pre- and post- LPS induced acute lung  
591 injury. (A) tSNE plots were generated indicating 5 distinct populations. Then tSNE plots were  
592 utilized to examine (B) day of LPS stimulation and (C) lincRNA-Cox2 (ptgs2os2) expression.  
593 Riemondy *et al.* generated scRNA-seq of all CD45- cells from WT mice pre- and post- LPS

594 induced acute lung injury. (D) tSNE plots were generated and clusters were colored based on cell  
595 type and (E) lincRNA-Cox2 (ptgs2os2) expression.

596 **Supplemental Figure 6: lincRNA-Cox2 expression in sorted cells from PBS or LPS treated**  
597 **mice.** lincRNA-Cox2 was measured in whole lung tissue and a number of sorted immune and  
598 epithelial cells from mice treated with (A) PBS and (B) LPS via an oropharyngeal route.  
599 Performed in biological triplicates and student's t-test was performed between whole lung tissue  
600 and each sorted cell.

601 **Supplemental Figure 7: Reconstitution of donor BM in the BAL of WT and Mutant mice.**  
602 Donor (green) and recipient (grey) percentage were assessed for (A) Neutrophils, (B) NK cells,  
603 (C) Eosinophils, (D) Dendritic cells, (E) Monocytes, (F) Eosinophils, (G) Interstitial  
604 macrophages, (H) Alveolar macrophages, (I) Recruited alveolar macrophages and (J) Resident  
605 Alveolar macrophages.

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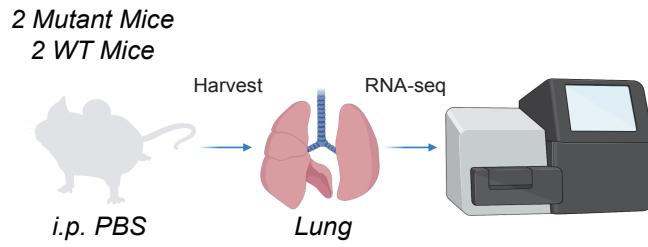
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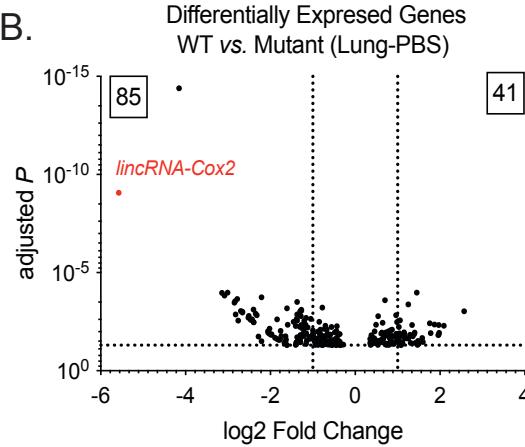
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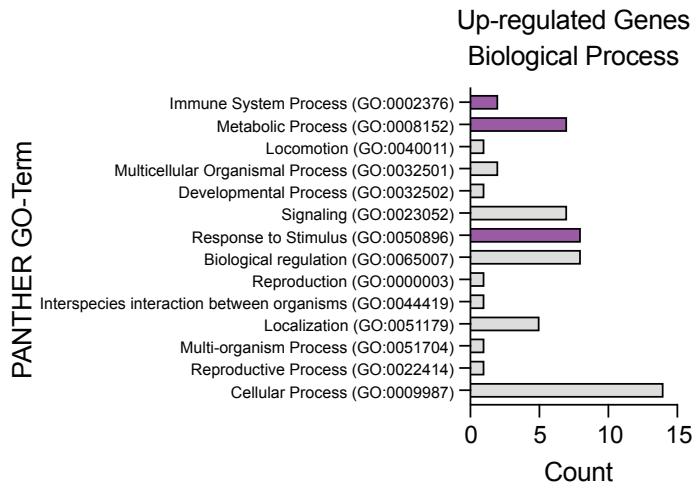
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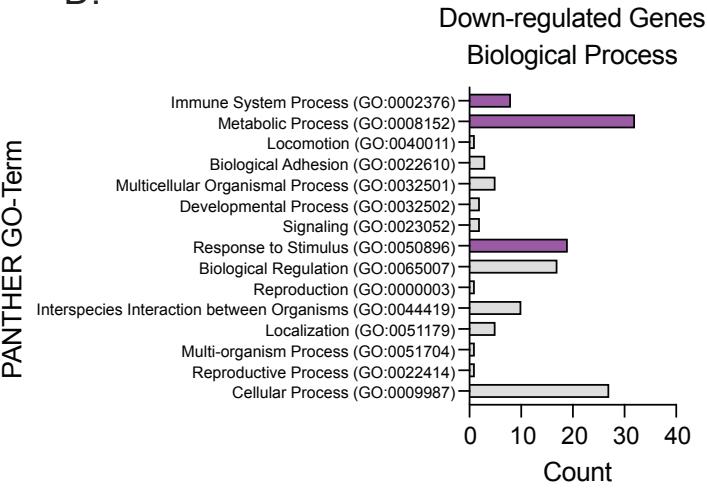
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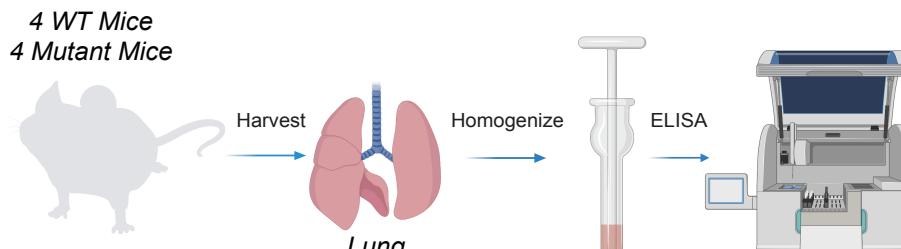
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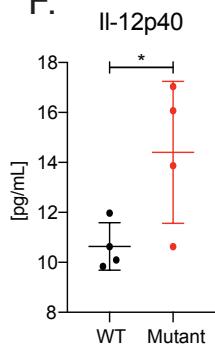
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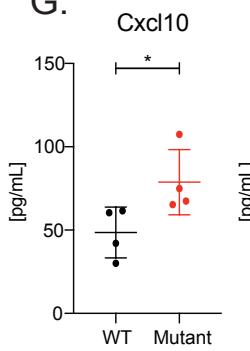
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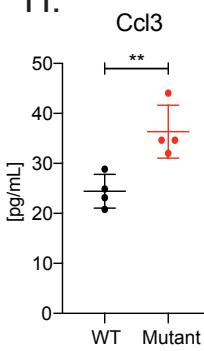
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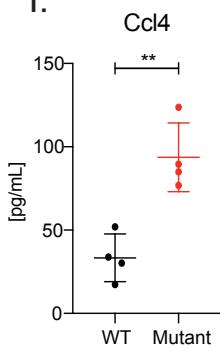
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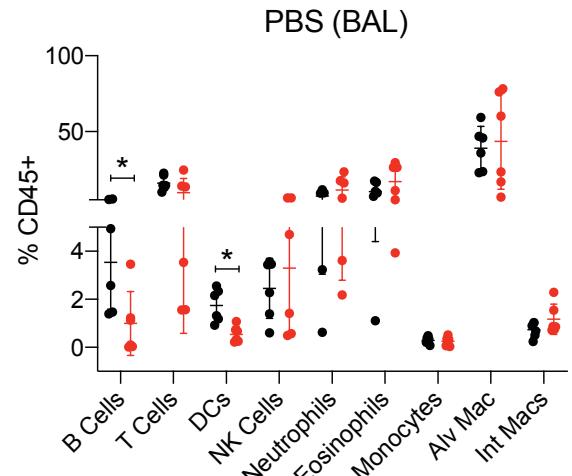
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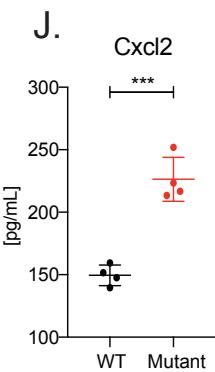
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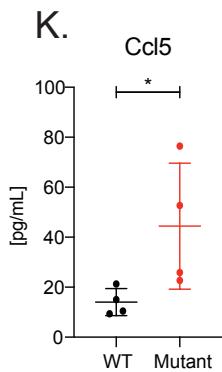
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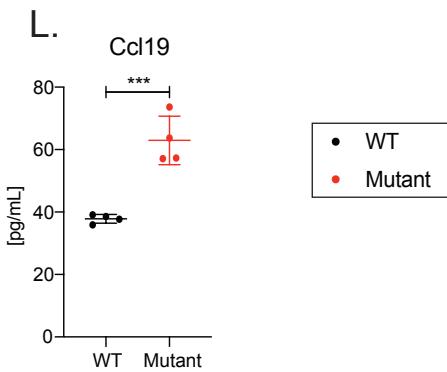
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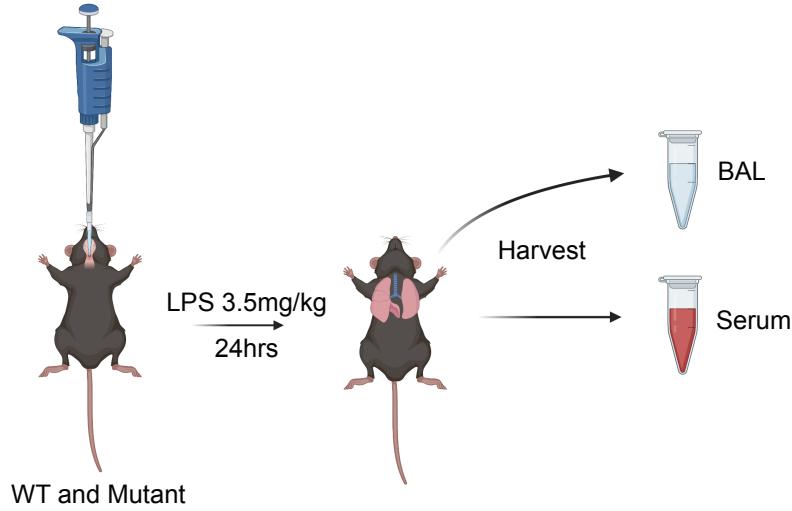


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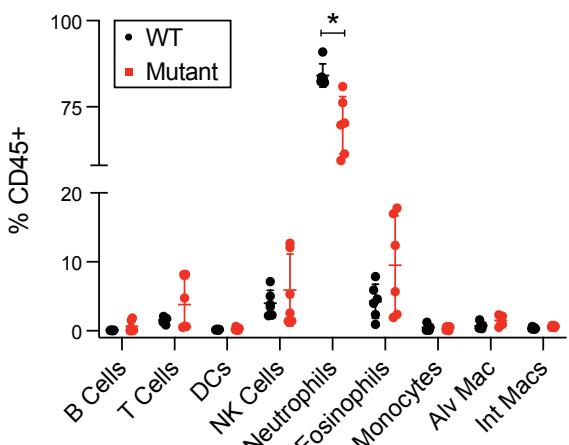
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Oropharyngeal Administration



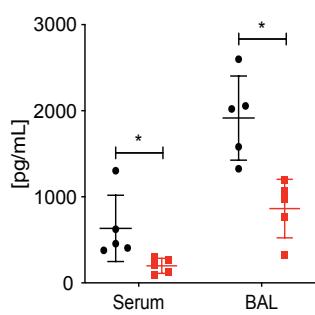
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LPS (BAL)



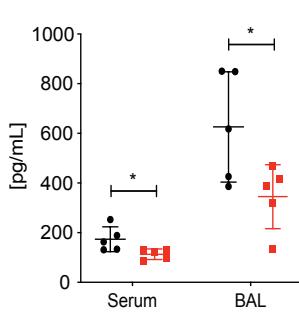
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Il6



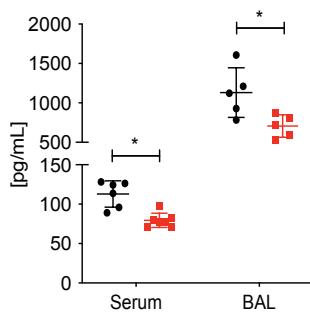
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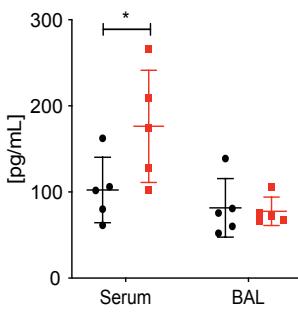
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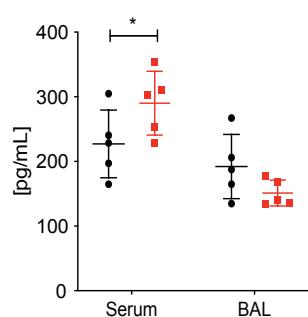
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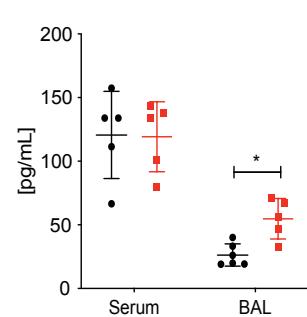
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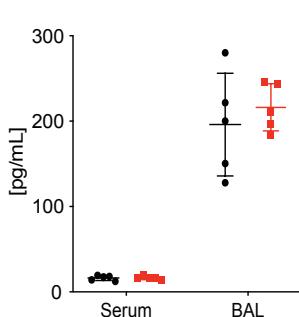
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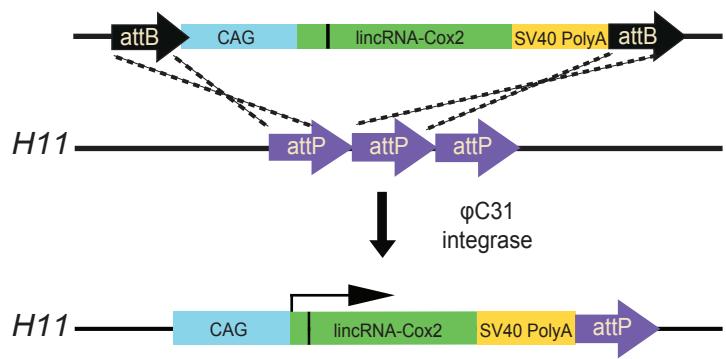
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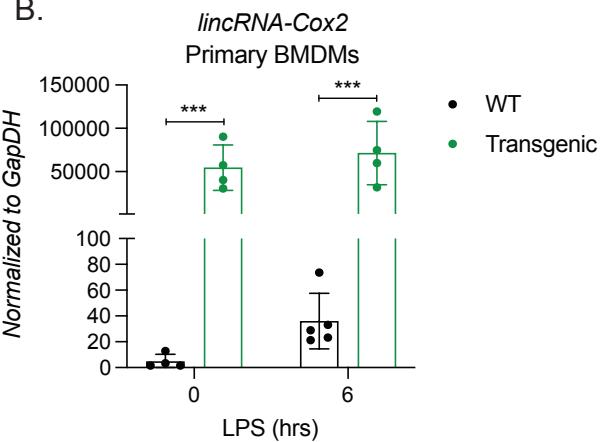


## Figure 3

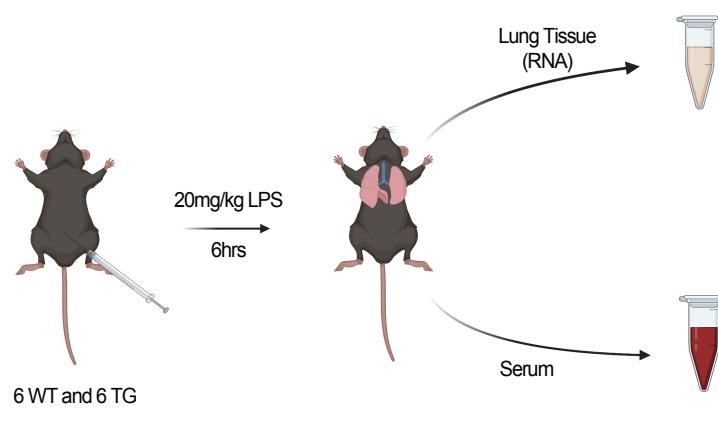
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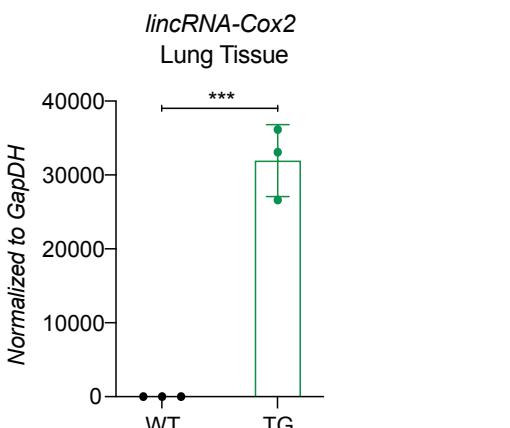
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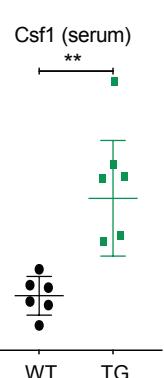
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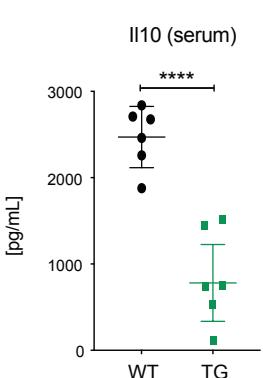
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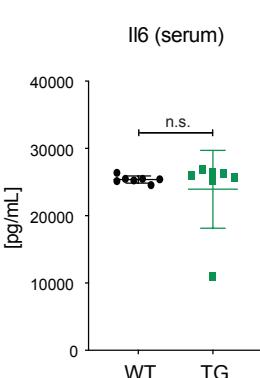
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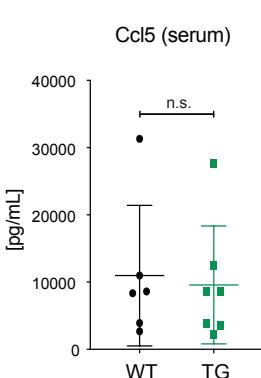
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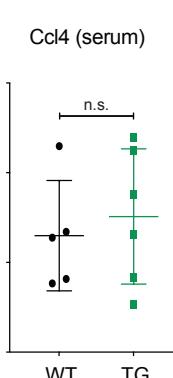
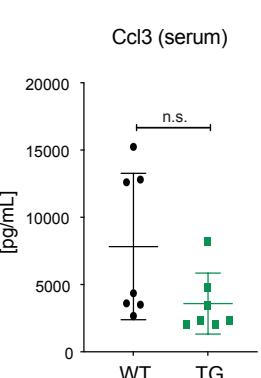
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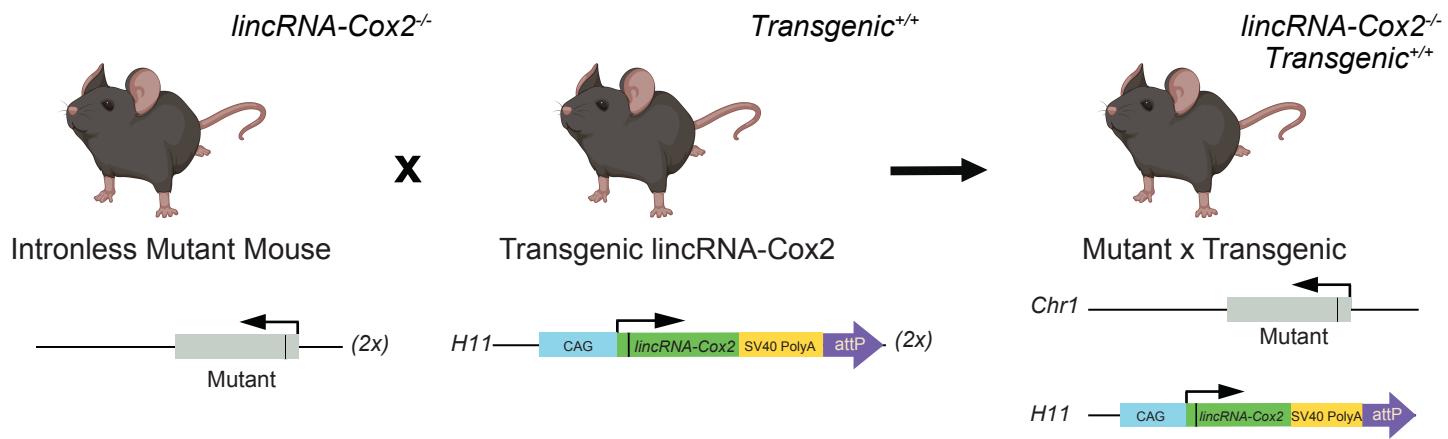


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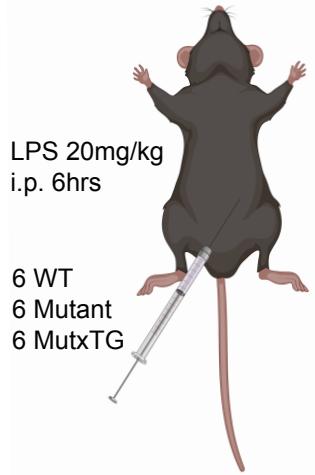


## Figure 4

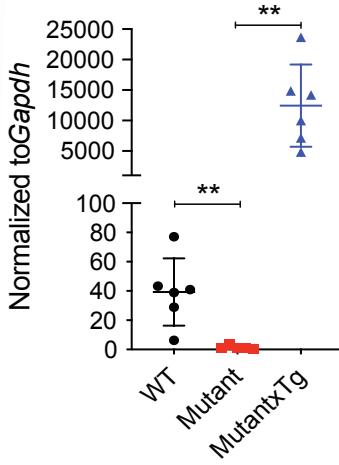
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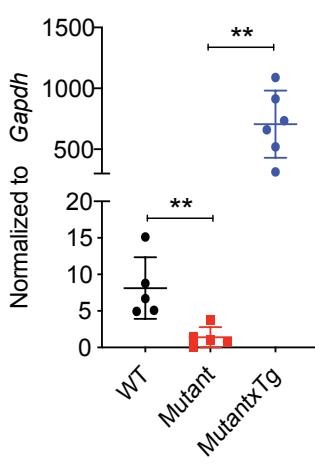
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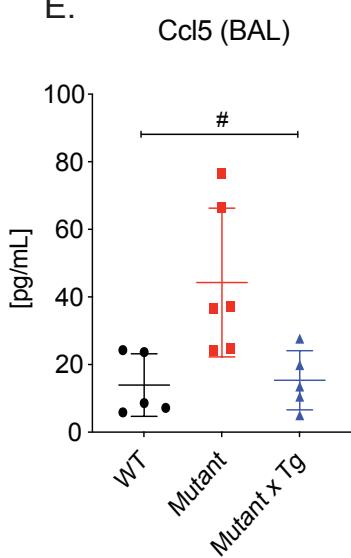
C. *lincRNA-Cox2* (Lung Tissue)



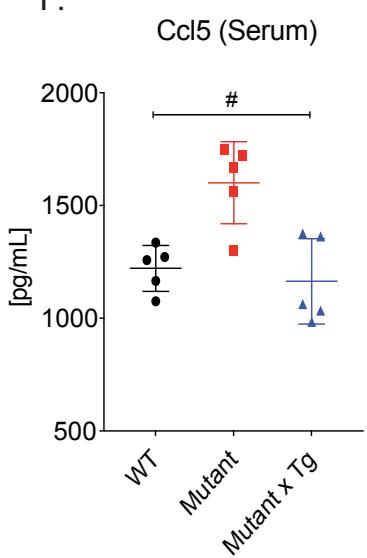
D. *lincRNA-Cox2* (BAL)



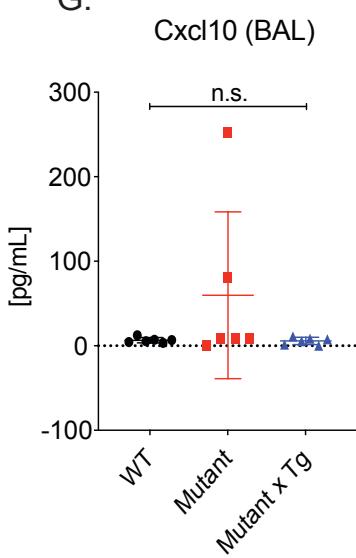
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F.



G.



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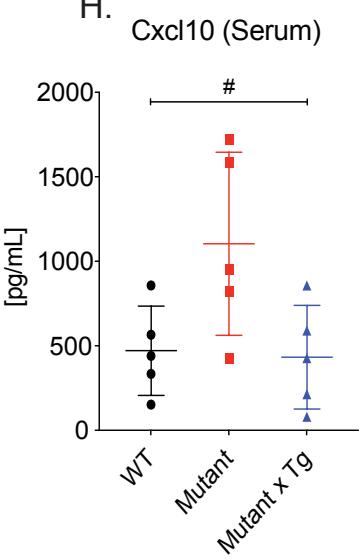
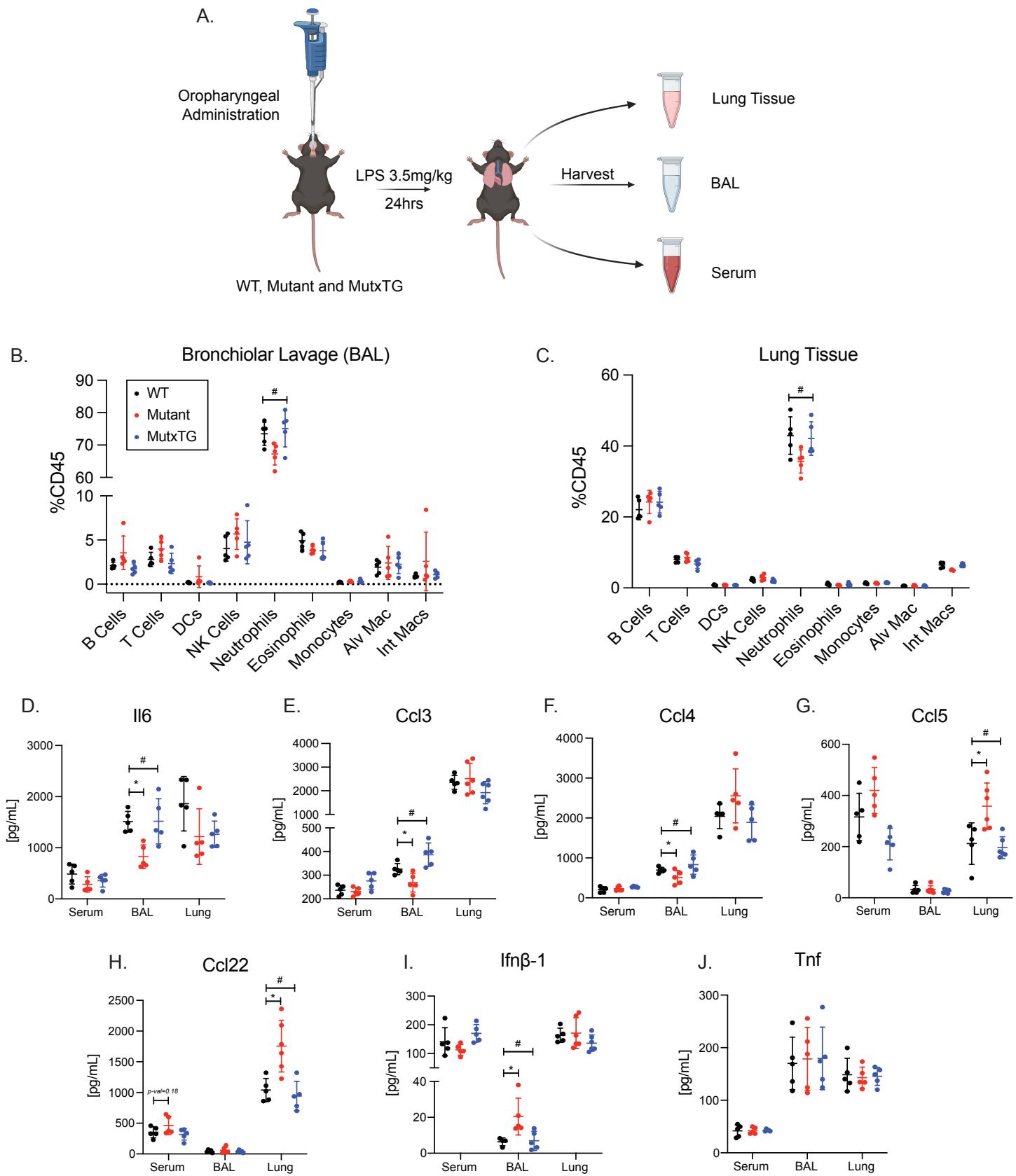
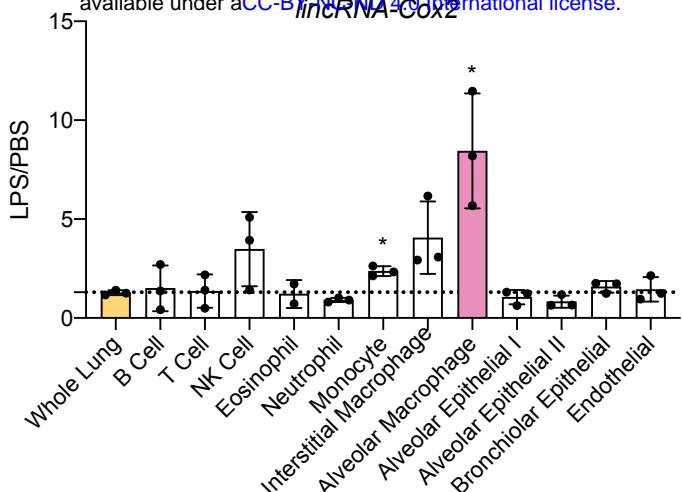


Figure 5

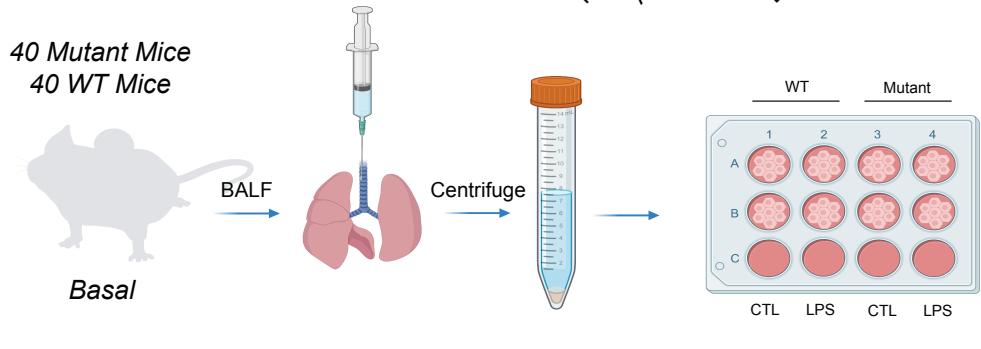


**Figure 6**

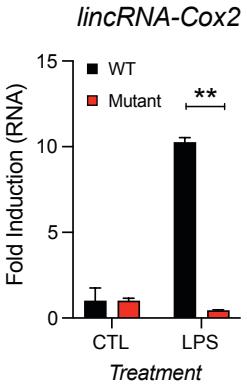
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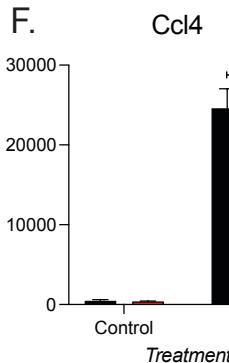
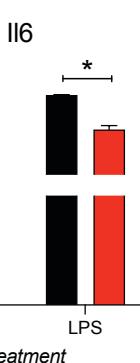
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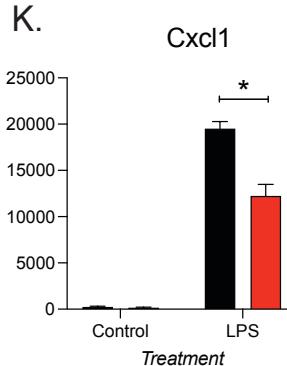
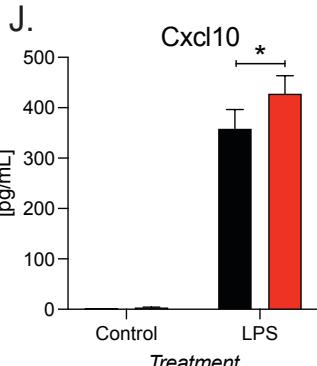
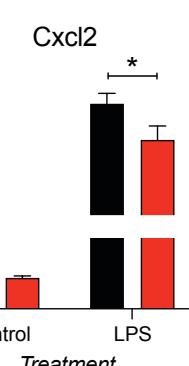
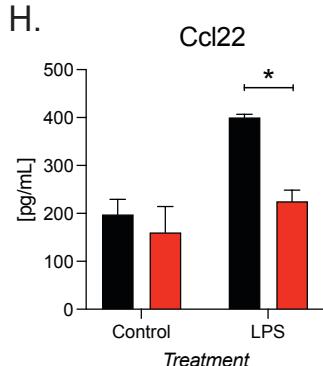
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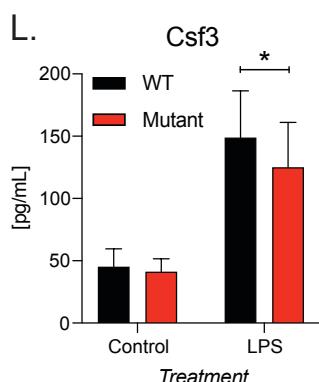
D.



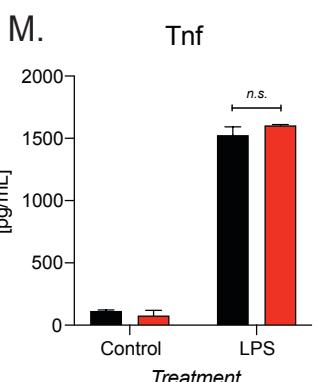
H.



L.

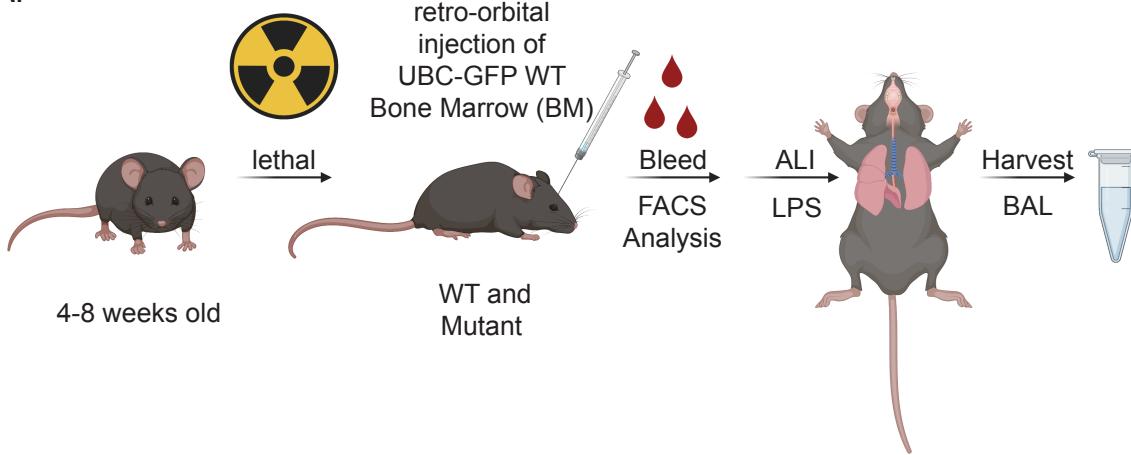


M.



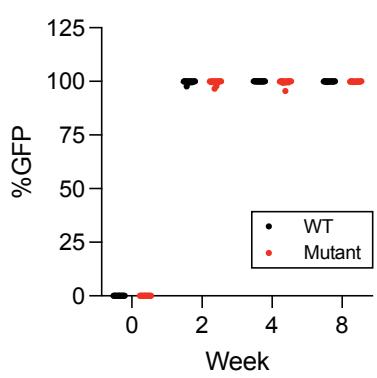
**Figure 7**

**A.**



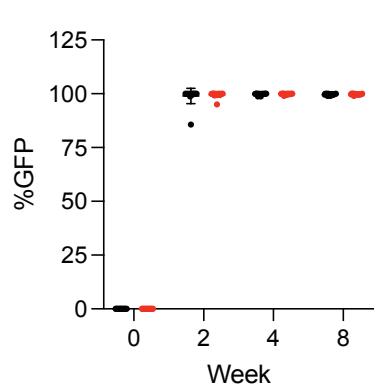
**B.**

GMs



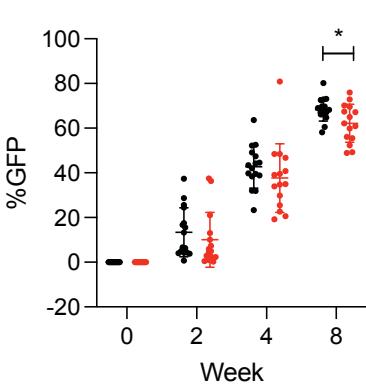
C.

B Cells



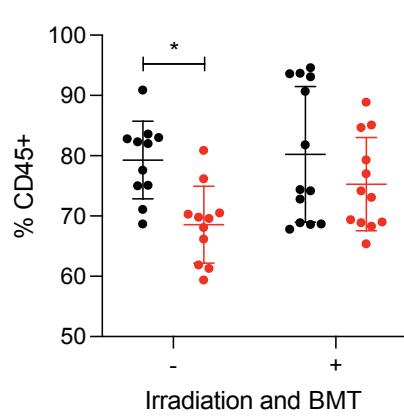
D.

T Cells

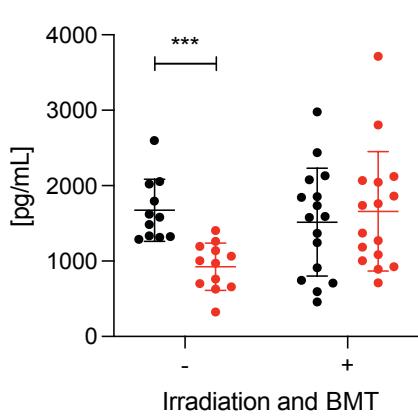


**E.**

Neutrophils (Ly6G+)

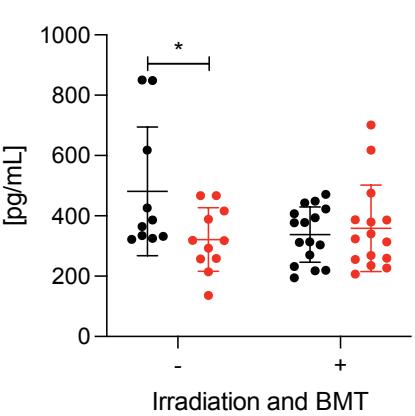


F. II6 (BAL)



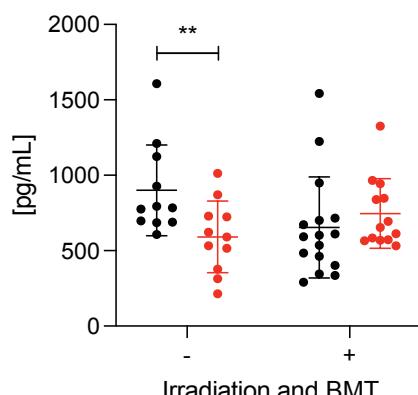
G.

Ccl3 (BAL)



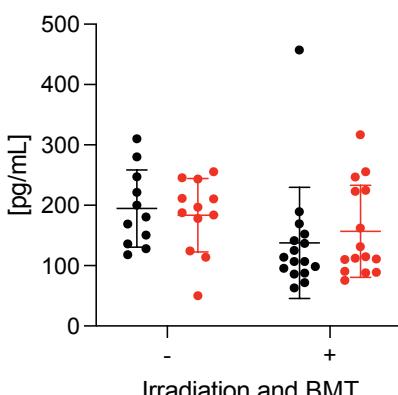
**H.**

Ccl4 (BAL)



**I.**

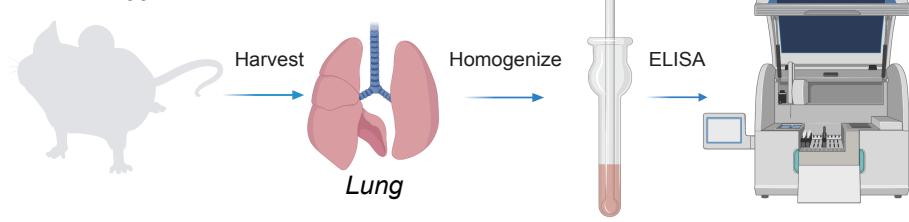
Tnf (BAL)



# Supplemental Figure 1

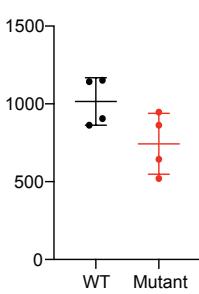
A.

4 Mutant Mice  
4 WT Mice



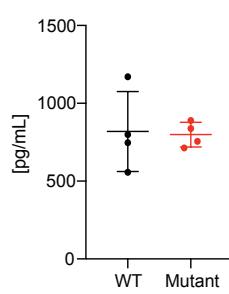
B.

Il16 (Lung)



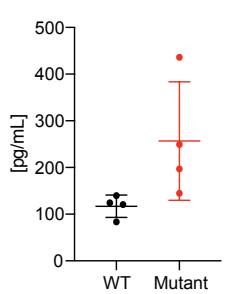
C.

Tim1 (Lung)



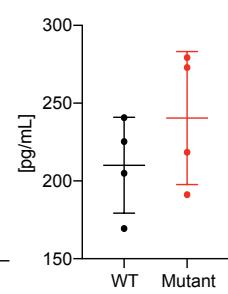
D.

Ccl22 (Lung)



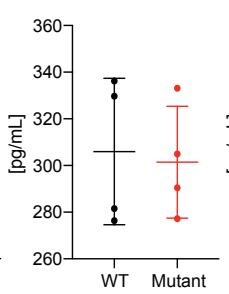
E.

Ccl11 (Lung)



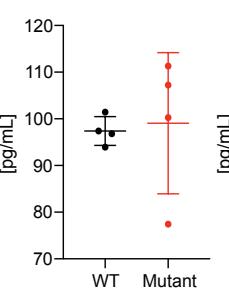
F.

VEGF (Lung)



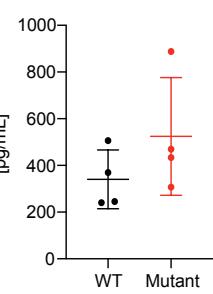
G.

Ifnb1 (Lung)



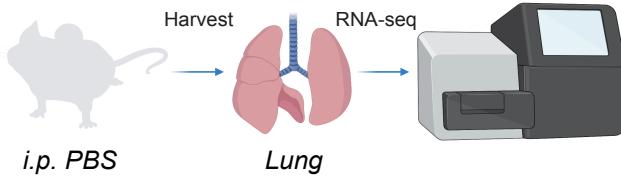
H.

Ccl12 (Lung)



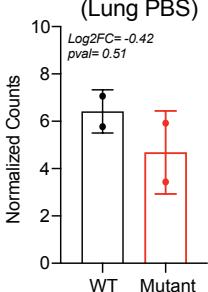
I.

2 Mutant Mice  
2 WT Mice



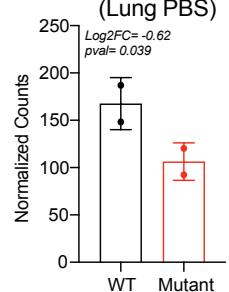
J.

Il12p40 (Lung PBS)



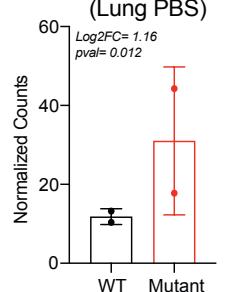
K.

Cxcl10 (Lung PBS)



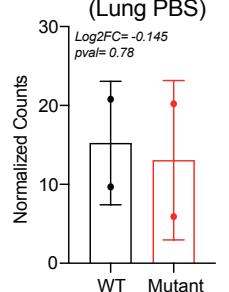
L.

Ccl3 (Lung PBS)



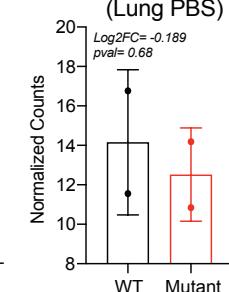
M.

Ccl4 (Lung PBS)



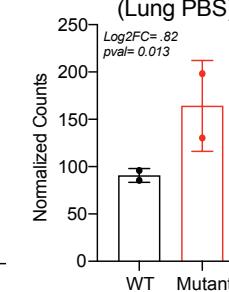
N.

Cxcl2 (Lung PBS)



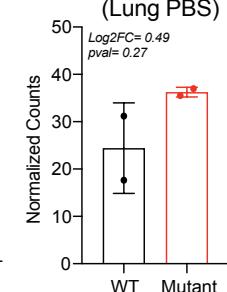
O.

Ccl5 (Lung PBS)



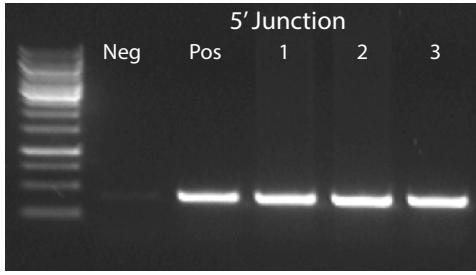
P.

Ccl19 (Lung PBS)

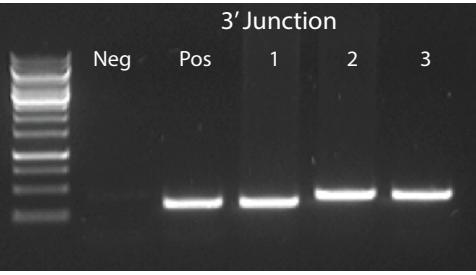


## Supplemental Figure 2

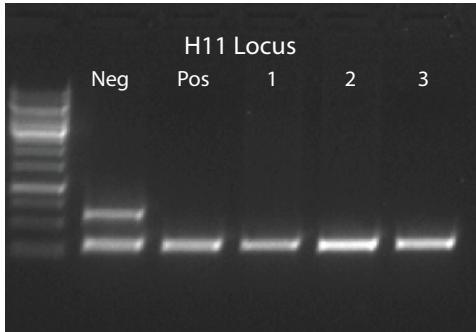
A.



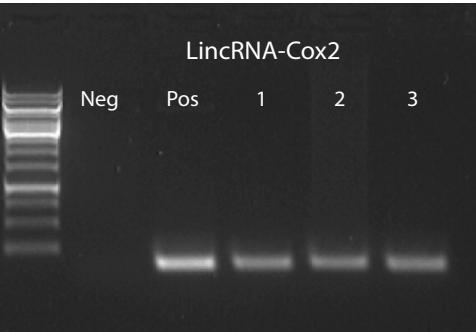
B.



C.

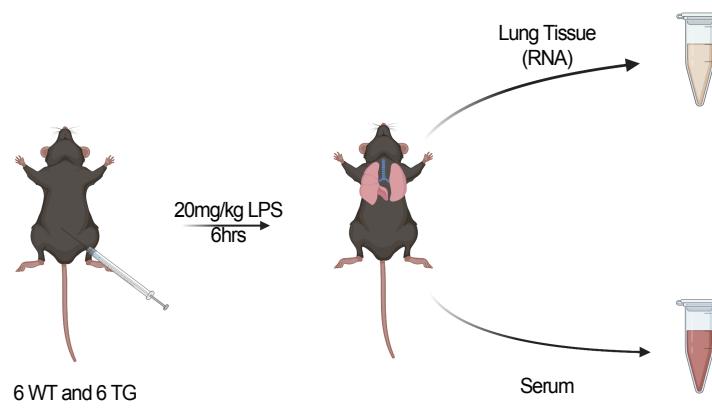


D.

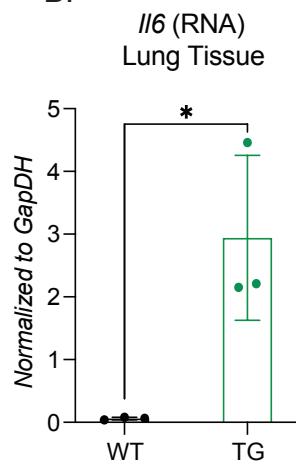


## Supplemental Figure 3

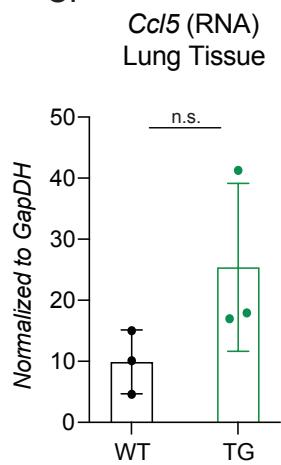
A.



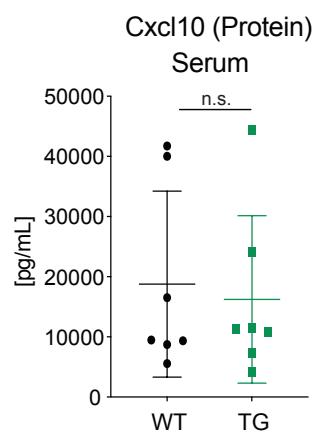
B.



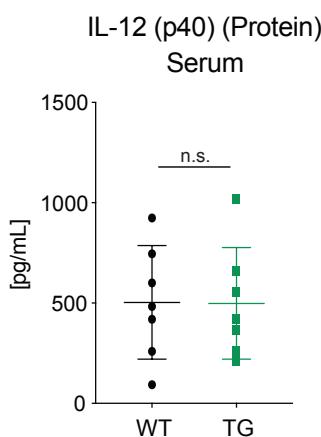
C.



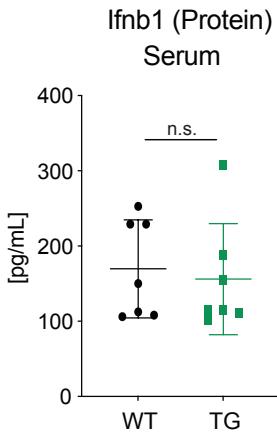
D.



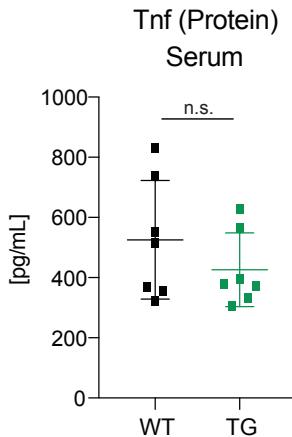
E.



F.



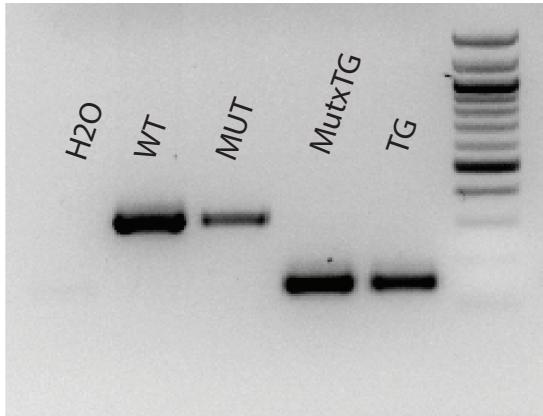
G.



## Supplemental Figure 4

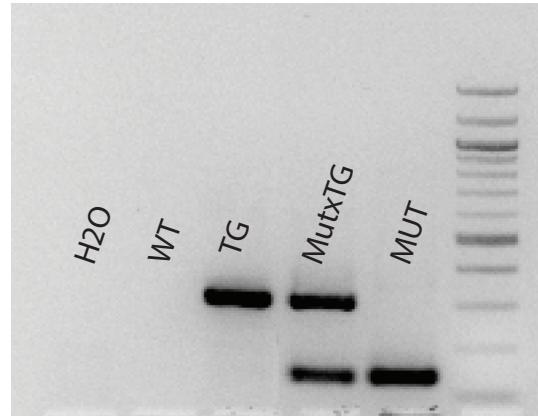
A.

PCR7/8 TARGATT Primers



B.

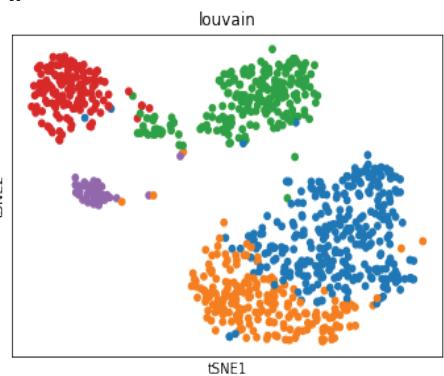
lincRNA-Cox2 F/R Primers



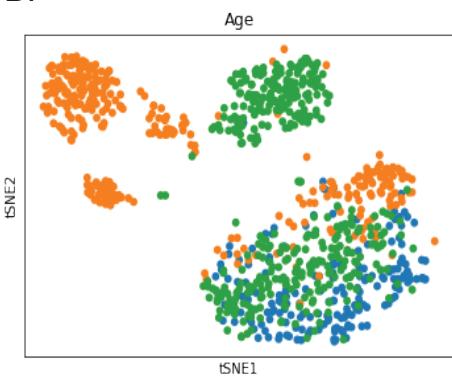
## Supplemental Figure 5

### Single cell RNA sequencing of alveolar macrophage from mice following LPS-induced lung injury

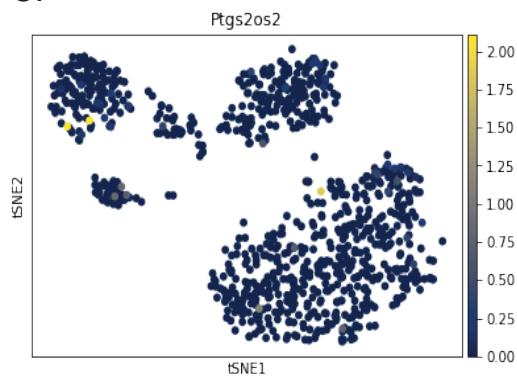
A.



B.

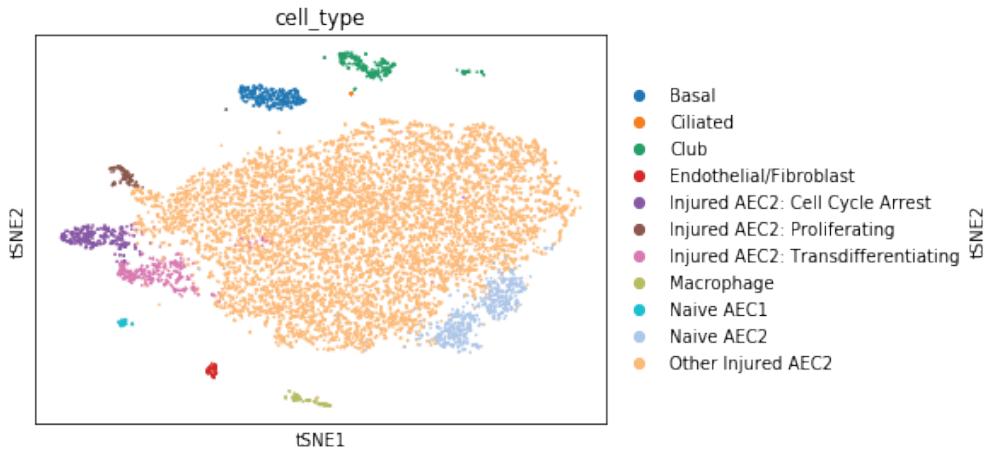


C.

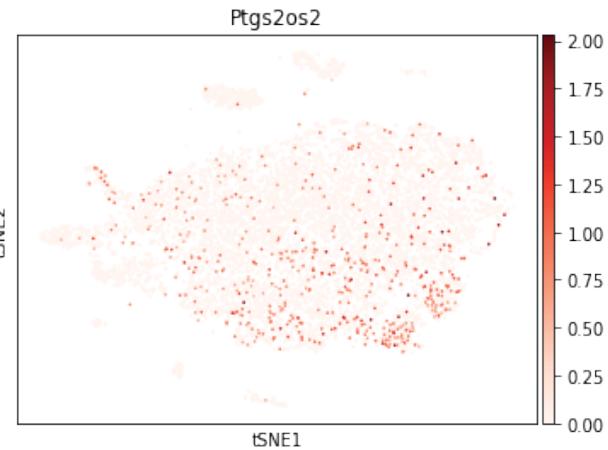


### Single-cell RNA sequencing of CD45- cells from mice lung tissue following LPS-induced lung injury

D.



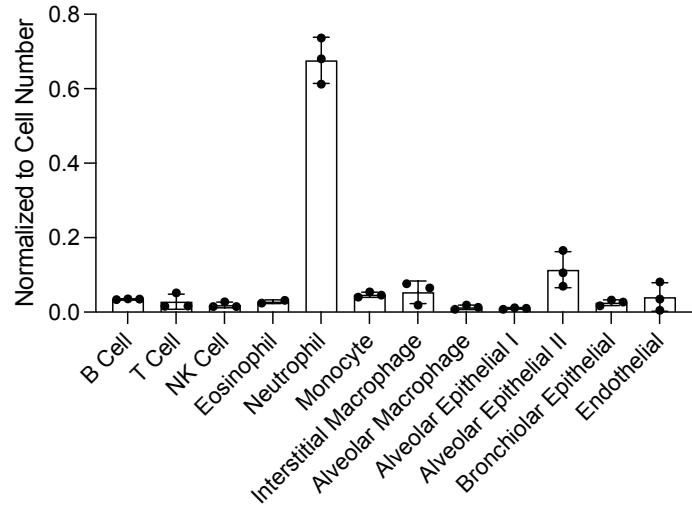
E.



## Supplemental Figure 6

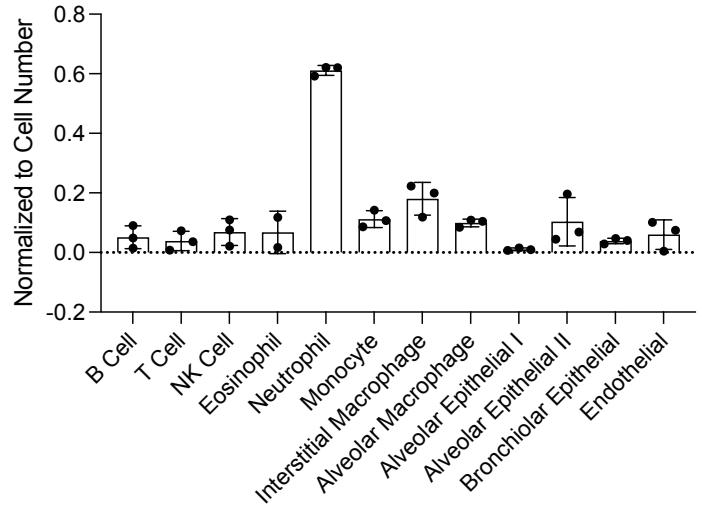
A.

*lincRNA-Cox2 (PBS)*



B.

*lincRNA-Cox2 (LPS)*



## Supplemental Figure 7

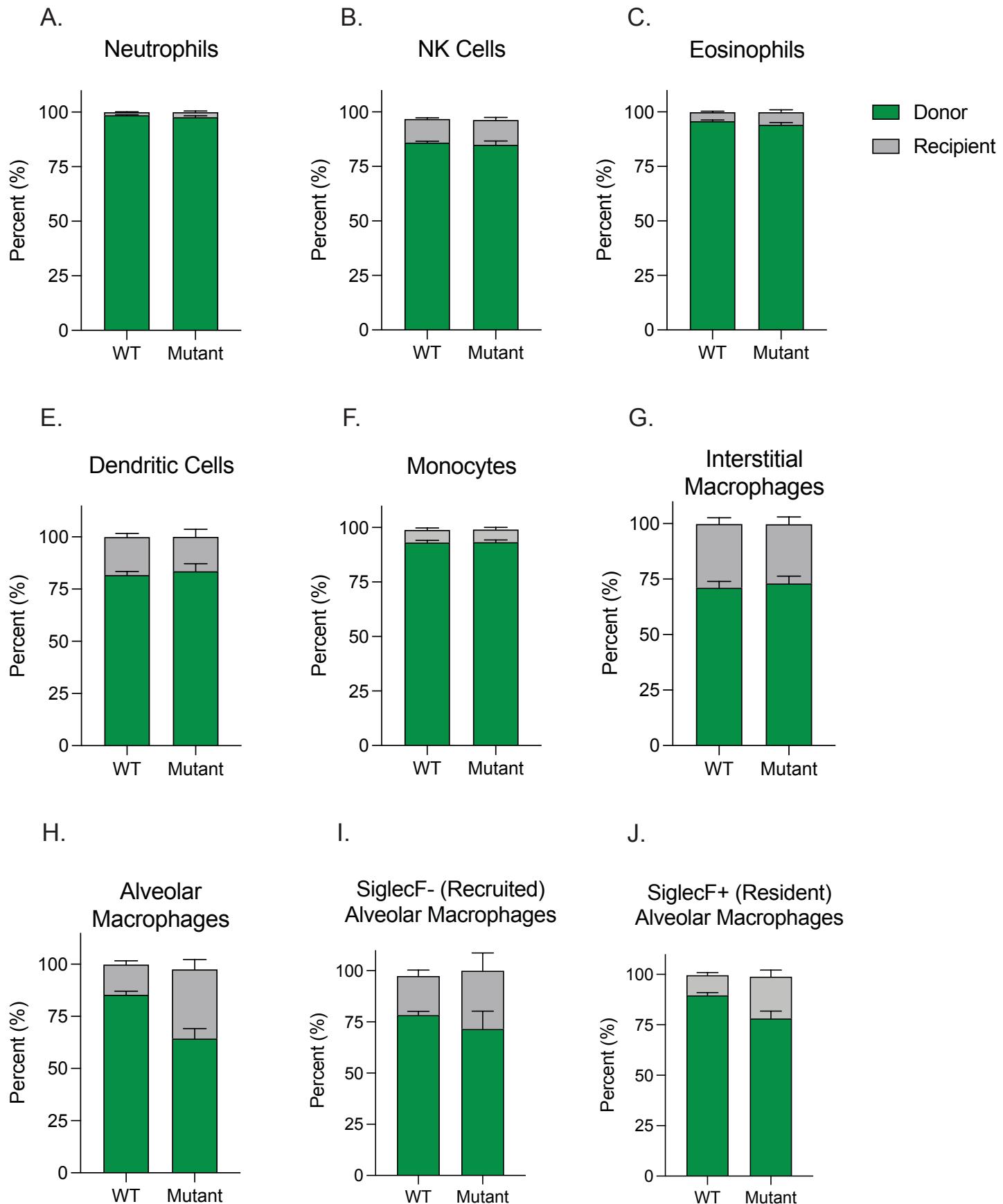


Table 1: Antibodies used in all Non-Chimera Experiments

Marker	Biolegend	Fluorophore	Clone
Live/Dead	420404	7-AAD (695/40)	
Ly6G	127618	BV605	1A8
CD11c	117308	PE	N418
CD11b	101226	BV786	M1/70
IA/IE	107641	BV650	M5/114.15.2
CD64	139306	FITC	X54-5/7.1
CD24	101836	Alexa 700	M1/69
Ly6C	128014	PB	HK1.4
CD45	103140	BV510	30-F11
SiglecF	155504	APC	S17007L
CD90	140310	BV605	53-2.1
CD326	118233	BV711	G8.8
MHC-II I-A	116410	FITC	AF6-120.1
CD24	138504	Alexa 700	30-F1
T1a/Pdpn	127418	APC-Cy7	8.1.1
CD31	102410	APC	390

Table 2: Antibodies used in Chimera Experiments

Marker	Biolegend	Fluorophore	Clone
Live/Dead	420404	7-AAD (695/40)	
Ly6G	127612	PB	1A8
CD11c	117308	PE	N418
CD11b	101226	BV786	M1/70
IA/IE	107628	APC-Cy7	M5/114.15.2
CD64	139314	PE-Cy7	X54-5/7.1
CD24	101827	BV605	M1/69
SiglecF	155504	APC	S17007L
CD45	103128	A700	30-F11