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2 ***Pseudomonas aeruginosa lasR* Mutants Resist Phagocytosis and Alter Inflammatory**

3 **Cytokine Production by Cystic Fibrosis Macrophages**

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6 Daniel S. Aridgides[1], Diane L. Mellinger[1], Lorraine L. Gwilt[1], Thomas H. Hampton[2], Dallas L.

7 Mould[3], Deborah A. Hogan[2], and Alix Ashare[1,2*]

8 [1]Section of Pulmonary and Critical Care Medicine, Dartmouth-Hitchcock Medical Center,

9 Lebanon, NH

10 [2]Department of Microbiology and Immunology, Geisel School of Medicine, Hanover, NH

11 [3]Department of Ecology and Evolutionary Biology, Yale University, New Haven, CT

12 * Correspondence: daniel.s.aridgides@hitchcock.org

13

Abstract

Cystic Fibrosis is characterized by chronic muco-obstructive lung disease and infection. People with CF (pwCF) are often colonized with *Pseudomonas aeruginosa* for years to decades, allowing for evolutionary adaptation. In chronic *P. aeruginosa* lung isolates from pwCF, the quorum sensing regulator LasR frequently is nonfunctional, however the factors enabling *lasR* loss-of-function (LOF) mutant selection are incompletely understood. We hypothesized that LOF mutations in *lasR* could allow *P. aeruginosa* to resist the selective pressure of phagocytosis. We found that in multiple strain backgrounds, LasR LOF decreased phagocytosis by both model THP-1 and primary monocyte-derived macrophages. While exogenous administration of the quorum-sensing autoinducer 3-oxo-C12-homoserine-lactone (3OC12HSL) that is made by an enzyme regulated by LasR activity inhibited phagocytosis and mitochondrial respiration, the phagocytosis resistance seen with *lasR* mutants appears to be bacterial cell intrinsic rather than due to secreted factors. Finally, we found that *lasR* LOF mutations altered the inflammatory profile upon infection of CF macrophages, with a shift from IL-1 family cytokine expression towards canonical inflammatory markers including IL-6 and TNFα. Collectively these data provide a potential explanation for both the prevalence of *lasR* mutants in the CF lung as well as their association with worse outcomes.

Importance

Cystic Fibrosis (CF) is a genetically inherited disease that leads to chronic lung infections. *Pseudomonas aeruginosa* is often implicated with worsening of lung disease, and it evolves in the lung over time to resist eradication. One of the most commonly disrupted gene targets identified in *Pseudomonas aeruginosa* isolates from chronically infected CF lungs is *lasR*, which encodes a transcription factor which regulates multiple virulence factors. What contributes to the apparent fitness of *lasR* mutants in the CF lung is not well known. Our study shows that *lasR* loss-of-function (LOF) mutants resist phagocytosis by macrophages, one of the fundamental

39 mechanisms of clearance by the immune system. We identify mechanisms promoting
40 resistance to phagocytosis, and explore the downstream consequences on inflammatory
41 responses. Understanding why *lasR* mutations arise could inform strategies to eradicate them
42 from the CF lung and improve outcomes.

43

44 **Introduction**

45 Cystic Fibrosis (CF) is a multisystem disorder caused by mutations in the Cystic Fibrosis
46 Transmembrane Conductance Regulator (*CFTR*) gene, encoding a chloride and bicarbonate
47 channel (1). In the lungs of people with CF (pwCF), CFTR mutations lead to dehydrated, sticky
48 mucus and subsequent colonization with bacteria often including *Pseudomonas aeruginosa* (2).
49 Novel highly effective modulator compounds which improve trafficking and function of the CFTR
50 protein have markedly improved outcomes for the subset of pwCF harboring CFTR mutations
51 that are responsive to them (3–12), however CFTR modulators cannot restore sterilizing
52 immunity to the lungs, particularly when there is pre-existing structural damage (13, 14). Given
53 their relatively recent availability, the long-term trajectory of lung function in pwCF remains
54 unknown, even for those who are clinically improved on modulators.

55 Lung function over time is impacted by bacterial colonization, with cycles of infection and
56 dysregulated inflammation leading to lung damage (1). The specific composition of the lung
57 microbiota as well as the host response to them play a role in determining whether lung function
58 worsens or remains stable. Low bacterial diversity, *P. aeruginosa* colonization, and detection of
59 *P. aeruginosa* with disruptions to the LasR/I quorum sensing system have all been shown to
60 correlate with accelerated lung function decline in pwCF (15–17).

61 LasR loss-of-function (LOF) mutants are one of the most commonly isolated morphotypes found
62 in CF lungs (18). LasR LOF mutants have also been isolated from environments such as pond
63 water, and will arise de novo when *P. aeruginosa* are cultured in vitro (18–21). Their common
64 emergence across various settings argues for a broad (or common) competitive advantage over
65 LasR intact (LasR+) comparators, however the reasons for selection *in vivo* are not entirely
66 clear. Social cheating whereby *lasR* LOF mutants can utilize secreted products from other *P.*
67 *aeruginosa* isolates without incurring the metabolic cost of producing them has been proposed
68 as a mechanism for *lasR* mutant selection. *lasR* mutation has also been shown increase

69 microoxic fitness and phosphate scavenging (22–24), providing additional possibilities for its
70 evolutionary advantage. Indeed *lasR* LOF mutants are selected for in vitro cultures due to a
71 growth advantage in post exponential phase (25–27) however these experimental systems lack
72 the host component. Interestingly, the phenotype of lack of protease activity has been shown to
73 increase in *P. aeruginosa* isolates that persist in pwCF on highly effective modulators,
74 suggesting *lasR* mutation may also be increased post modulator therapy (28).

75 In the CF lung *P. aeruginosa* must coexist with host immune cells which are attempting to
76 eradicate it, yet the effects of *lasR* LOF mutations on *P. aeruginosa* interactions with host
77 immune cells remain comparatively unstudied. Studies on interaction with airway epithelial cells
78 (29, 30), neutrophils (31, 32), and macrophages (33) have revealed variable effects on
79 inflammatory responses. Macrophages are responsible for orchestrating the host immune
80 response in the lung, phagocytosis of pathogens and debris, and have been shown to have
81 altered inflammatory and effector activity in CF (34). Although CFTR modulators have been
82 shown to improve CF macrophage function (35–37), an improved mechanistic understanding of
83 how modulators affect the host immune response could benefit the majority of pwCF who are
84 now eligible for highly effective modulator treatment and and lead to novel interventions that
85 might reduce bacterial persistence.

86 We hypothesized that loss of LasR function would alter interactions with host macrophages as a
87 mechanism of *lasR* mutant persistence within the CF lung, and that CFTR modulators would
88 further impact host response. We found that *lasR* LOF mutants indeed resist phagocytosis in
89 multiple *P. aeruginosa* backgrounds including laboratory and clinical isolates. Phagocytosis
90 resistance was maintained in mixed cultures with LasR+ relatives. *lasR* mutant resistance to
91 phagocytosis by primary CF monocyte derived macrophages (MDMs) was maintained despite
92 the presence of highly effective CFTR modulators. Finally, *lasR* mutation shifted the
93 inflammatory response of MDMs to *P. aeruginosa* infection away from IL-1 family cytokines

94 towards canonical proinflammatory cytokines IL-6 and TNFα. Collectively these results highlight
95 the importance of understanding *P. aeruginosa lasR* LOF in the context of immune cells and
96 provide potential explanations for its increased prevalence and virulence in CF. Some of these
97 results have previously been presented in the form of abstracts (38–42).

98 **Results**

99 ***lasR* loss-of-function mutations confer phagocytosis resistance independent of**
100 **background *P. aeruginosa* strain**

101 To test the hypothesis that loss-of-function mutations in *lasR* confer phagocytosis resistance we
102 utilized model THP-1 monocytes which can be differentiated into macrophage-like cells by the
103 addition of the protein kinase C activator phorbol 12-myristate 13-acetate (43). We performed
104 gentamicin-protection assays with three pairs of LasR+ and LasR- *P. aeruginosa* to compare
105 rates of bacterial uptake. We chose a 20 minute timepoint to study phagocytosis as this
106 provides an investigation of uptake alone, whereas longer timepoints may see a mixture of
107 phagocytosis and killing (36). In strain PA14Δ*lasR*-THP-1 co-cultures, fewer cells were
108 phagocytosed then the in the otherwise isogenic wild-type (WT) strain (**Figure 1A**). We then
109 utilized two closely related pairs of LasR+/LasR- clinical isolates for which *lasR* LOF evolved
110 over the course of infection to confirm this effect with *P. aeruginosa* derived from the CF lung.
111 LasR LOF continued to confer phagocytosis resistance in both clinical isolate backgrounds
112 (**Figure 1B,C**) demonstrating that this was not a strain-specific effect.

113 **LasR but not RhlR autoinducer homoserine lactone inhibits phagocytosis**

114 One of the primary signaling molecules regulated by LasR is the autoinducer N-(3-
115 oxododecanoyl)-L-homoserine lactone (3OC12HSL) that binds to the LasR regulator to induce a
116 positive feedback cycle. 3OC12HSL has been shown to have direct effects on eukaryotic
117 metabolism and signaling (44–55), as well as enhancing phagocytosis of yeast (56). We

118 therefore tested whether 3OC12HSL would increase phagocytosis of *P. aeruginosa* as an
119 explanation for the decreased *lasR* mutant phagocytosis. Contrary to this hypothesis, we found
120 that 3OC12HSL inhibited phagocytosis of both LasR+ and LasR- PA14 at concentrations of 50
121 μM or higher in a dose-dependent manner (**Figure 2A**). By comparison, the RhlR autoinducer
122 C4-homoserine lactone (C4HSL) had no appreciable effect (**Figure 2B**). Under all
123 concentrations of HSLs tested there continued to be a reproducible difference between LasR+
124 and LasR- phagocytosis. These results suggest that 3OC12HSL is not responsible for the
125 effects seen in **Figure 1**.

126 **Phagocytosis resistance of LasR- strains is maintained in mixed cultures**

127 We therefore sought to ascertain whether *lasR* mutation effects were due to secreted or cell
128 intrinsic factors. We performed phagocytosis assays with a mixture of LasR+/LasR- PA14
129 derivatives at varying percentages (0/100, 10/90, 30/70, 50/50, 70/30, 90/10 and 100/0). The
130 LasR- competitor in the PA14 background bore a *lacZ* reporter gene and plating onto X-gal
131 plates allowed for post-assay determination of strain identities when mixed with an untagged
132 wild type. While we found bacterial uptake was generally proportional to the percent input for
133 each strain (**Figure 3A**), there was a persistently lower amount of LasR- *P. aeruginosa*
134 phagocytosed. **Figure 3B** displays the same data as in **3A** graphed relative to percent input for
135 each strain such that the decreased phagocytosis of LasR- strains is evident. These data
136 suggest that the effects of *lasR* mutation on phagocytosis is due to cell intrinsic factors.

137 **Primary human macrophages augment phagocytosis with CFTR modulators, but LasR**
138 **phagocytosis resistance is maintained**

139 To determine if the differences between phagocytosis of LasR+ and LasR- cells seen in THP-1
140 cells were also observed when using primary human cells, we isolated peripheral blood derived
141 mononuclear cells (PBMCs) from volunteers and differentiated them into monocyte-derived

142 macrophages (MDMs) in vitro for seven days. We first tested cells from CF volunteers. We and
143 others have demonstrated that CFTR modulators have a positive effect on macrophage and
144 monocyte phagocytosis (36, 37, 57–63), and as a majority of pwCF are now eligible for CFTR
145 modulators it is important to understand whether they may play an additional role in modifying
146 the response to *lasR* mutants. We tested one pair of laboratory isolates and one pair of clinical
147 isolates (DH1137/6) in MDMs that were treated for 48 hours with CFTR modulators or DMSO as
148 a control. The PA14Δ*lasR* (LasR-) strain was less efficiently phagocytosed by primary CF MDMs
149 relative to its LasR+ progenitor (**Figure 4A**). Tezacaftor/ivacaftor (TI) and
150 elexacaftor/tezacaftor/ivacaftor (ETI) improved phagocytosis of both strains in the PA14
151 background (**Figure 4A**) however there was no statistically significant interaction with *lasR*
152 status. We found similar results using paired clinical *P. aeruginosa* isolates, where *lasR* mutants
153 were less efficiently phagocytosed and CFTR modulators improved phagocytosis of both strains
154 with no difference in response to modulators between isolates (**Figure 4B**). We have previously
155 demonstrated that nonCF MDMs respond similarly to CFTR modulators when compared with
156 CF MDMs (37), therefore we tested whether any of the effects on *lasR* mutant phagocytosis
157 might be modified by *CFTR* genotype. NonCF MDMs displayed a similar pattern, with
158 decreased phagocytosis of *lasR* mutants, improved phagocytosis in the presence of CFTR
159 modulators, and no statistically significant interaction between modulators and *lasR* mutation
160 (**Figure 4C**). With the LasR+/LasR- clinical isolates, there continued to be an effect of *lasR*
161 mutation (**Figure 4D**) however double CFTR modulator treatment only displayed a non-
162 significant trend towards enhanced phagocytosis, with triple modulators still proving effective
163 (**Figure 4D**). Collectively these results confirm that *lasR* mutants exhibit reduced phagocytosis
164 by primary human CF and nonCF macrophages, and that while CFTR modulator treatment is
165 still beneficial, it fails to restore phagocytosis to the level of LasR+ strains.

166 ***lasR* mutation abrogates the inhibitory effect of *P. aeruginosa* conditioned medium on**
167 **macrophage mitochondrial respiration**

168 Macrophage metabolism impacts their inflammatory predilection, with activated macrophages
169 relying on glycolysis for ATP generation whereas pro-resolution utilized aerobic respiration (64,
170 65). We have previously demonstrated that secreted products from *P. aeruginosa* inhibit aerobic
171 respiration in MDMs (37), and we hypothesized that *lasR* mutation may impact this effect. In
172 addition, we saw that ETI inhibits aerobic respiration without significantly affecting glycolysis.
173 We therefore performed Seahorse metabolic flux analysis on CF MDMs pre-treated with CFTR
174 modulators and then then conditioned media from either LasR+ or LasR- PA14 strains was
175 added acutely. Oxygen consumption rate (OCR) was measured as a proxy for mitochondrial
176 respiration and extracellular acidification rate (ECAR) as a proxy for lactate
177 generation/glycolysis. **Figure 5A-C** represent example readouts of OCR from a single CF
178 subject pre-treated with DMSO, TI or ETI, respectively and **Figure 5D-F** are the corresponding
179 readouts for ECAR. Addition of oligomycin, FCCP and rotenone/antimycin A allow for
180 measurement of additional parameters related to mitochondrial respiration. In CF MDMs, ETI
181 decreased basal respiration similarly to prior experiments whereas TI had no effect (**Figure 5G**).
182 Upon acute injection of LasR+ conditioned medium, there was a decrease in mitochondrial
183 respiration (**Figure 5H**), however LasR- conditioned medium completely lost this effect. In the
184 setting of low basal mitochondrial respiration with ETI, the effect of *P. aeruginosa* conditioned
185 medium (either LasR+ or LasR-) was lost (**Figure 5H**). Corresponding decreases in ATP-linked
186 respiration were seen with LasR+ conditioned medium and ETI pre-treatment (**Figure 5G**).
187 Basal glycolysis was not affected by CFTR modulators or different prior to injection (**Figure 5J**)
188 however LasR+ but not LasR- conditioned medium caused an acute rise in glycolysis (**Figure**
189 **5K**). This effect was lost upon ETI pre-treatment (**Figure 5K**). OCR/ECAR ratio is a way of
190 gauging relative contributions of each pathway to ATP-generation, and in line with the effects

191 seen on ATP-linked respiration and glycolysis, OCR/ECAR was decreased with LasR+ but not
192 LasR- conditioned media, as well as by ETI (**Figure 5L**). These results collectively demonstrate
193 that *lasR* mutation eliminates the mitochondrial effects of *P. aeruginosa* exoproducts.
194 Additionally, CFTR modulators inhibit basal mitochondrial activity and there is no additive effect
195 of *P. aeruginosa* exoproducts. Both of these observations have potential downstream
196 implications for induction of inflammation.

197 We next examined nonCF MDMs to determine whether there may be any differences in how
198 they respond to *lasR* mutants. Similarly to CF MDMs, we found the ETI inhibits basal respiration
199 (**Figure 5M**) with no effect of TI, in agreement with our prior published work (37). LasR+ but not
200 LasR- conditioned medium inhibited mitochondrial respiration (**Figure 5N**) and decreased ATP-
201 linked respiration (**Figure 5O**). These effects were decreased or eliminated by ETI-pretreatment.
202 Modulators had no effect on basal glycolysis (**Figure 5P**), with increased glycolysis secondary
203 to LasR+ but not LasR- conditioned medium (**Figure 5Q**). In contrast to CF MDMs, differences
204 in OCR/ECAR were not statistically significant (**Figure 5R**).

205 **LasR autoinducer homoserine lactone inhibits macrophage mitochondrial respiration**
206 **and induces glycolysis**

207 The divergence between LasR+ and LasR- conditioned medium effects on MDM metabolism led
208 us to hypothesize that 3OC12HSL might underlie these effects. To test this, we returned to THP-
209 1 derived model macrophages and performed Seahorse metabolic analysis with acute injection
210 of 3OC12HSL, with C4HSL as a control. After first confirming no differences in basal respiration
211 prior to injection (**Figure 6A**), we found a dose responsive effect of 3OC12HSL injection (**Figure**
212 **6B**) causing inhibition of mitochondrial respiration whereas C4-HSL had no effect. This was
213 partially reflected in differences in ATP-linked respiration (**Figure 6C**) although the effects were
214 not statistically significant. Simultaneous measurement of glycolytic rates confirmed no basal
215 differences in glycolysis (**Figure 6D**), with a dose-dependent increase in glycolysis upon

216 3OC12HSL injection (**Figure 6E**) and no effect of C4HSL. The shift in metabolic profile from
217 mitochondrial respiration to glycolysis by 3OC12HSL was confirmed with decreased OCR/ECAR
218 (**Figure 6F**). These results suggest that 3OC12HSL may underlie the effects of LasR+ *P.*
219 *aeruginosa* exoproducts on mitochondrial respiration.

220 ***lasR* mutation shifts inflammatory cytokine profiles of primary human macrophages**
221 **away from IL-1 family cytokines towards IL-6 and TNFα irrespective of CFTR modulators**
222 **and *CFTR* mutation status**

223 Finally, we investigated whether *lasR* mutation impacted inflammatory profiles of MDMs, and the
224 interplay with CFTR modulators. We pretreated MDMs with CFTR modulators for 48 hours, then
225 infected MDMs for two hours with LasR+ or LasR- clinical isolates of *P. aeruginosa* and
226 performed cytokine multiplex analysis on the supernatants. Unsurprisingly we found broad
227 induction of multiple inflammatory cytokines upon *P. aeruginosa* infection of CF MDMs (**Figure**
228 **7A**). There was little appreciable effect of CFTR modulators, consistent with our prior work and
229 others' (37, 66). NonCF MDMs had a similar inflammatory response to CF MDMs, with no
230 statistically significant differences between the groups (**Figure 7B**). We then combined results
231 and tested specifically for differences between LasR+ and LasR- infection. IL-1 family cytokines
232 IL-1α, IL-1β and IL-18 were higher with LasR+ infection, whereas IL-6, IL-10, MCP-1, MIP-1α,
233 and MIP-1β were higher with LasR- infection (**Figure 7C**), demonstrating a specific alteration in
234 the inflammatory profile after macrophage response to *lasR* mutants.

235 **Discussion**

236 Loss-of-function mutations in *lasR* have been demonstrated to be important for *P. aeruginosa*
237 persistence and virulence within the CF lung (15, 18). How this impacts interactions with host
238 immune cells and specifically in the presence of highly effective CFTR modulators is
239 incompletely understood (29–33, 67). We have identified for the first time a robust and

240 reproducible resistance to phagocytosis by macrophages that is hypothesized to contribute to
241 persistence in the CF lung. While the difference was modest (0.3 to 0.5 log depending on the
242 assay), this represents a relative difference of 2-3 fold uptake in 20 minutes, therefore over
243 years this could represent a significant evolutionary pressure. The phagocytosis resistance was
244 present in multiple background strains including a common laboratory strain (PA14) and two
245 pairs of clinical isolates from the lungs of pwCF (**Figure 1**), increasing the generalizability of our
246 findings.

247 We also identified potential factors by which LasR mutants resist phagocytosis. The LasR
248 autoinducer 3OC12HSL inhibited *P. aeruginosa* phagocytosis (**Figure 2**), in contrast to prior
249 studies of yeast phagocytosis (56). If 3OC12HSL were responsible for differences in
250 phagocytosis however, it would be expected that LasR mutants would exhibit *higher*
251 phagocytosis rates, which was the opposite of what was seen. Differences in phagocytosis rates
252 were also maintained between LasR+ and LasR- strains when they were mixed in different
253 ratios (**Figure 3**), arguing that the responsible factors are cell intrinsic rather than secreted.

254 We also explored the interplay between CFTR modulators and *lasR* mutation with phagocytosis
255 and inflammation in primary MDMs. While CFTR modulators were able to enhance
256 phagocytosis of all strains tested consistent with our prior work (37), the decrement with *lasR*
257 mutation was maintained (**Figure 4**). Given the known persistence of *P. aeruginosa* in the lungs
258 of pwCF despite highly effective modulator therapy (14), this is relevant to understanding *P.*
259 *aeruginosa* colonization. We previously saw that *P. aeruginosa* secreted products inhibited
260 mitochondrial respiration in MDMs (37), as did the triple modulator combination ETI. Intriguingly
261 we found that *lasR* mutation essentially eliminated this effect (**Figure 5**). In combination with the
262 effects of exogenous 3OC12HSL (**Figure 6**), this suggests that the acyl-homoserine lactone is
263 the responsible factor, in line with previous studies of epithelial cells and fibroblasts (45, 46, 48–
264 51, 68).

265 Unsurprisingly given the variable rates of phagocytosis and differences in effects on
266 mitochondrial function in MDMs, we found that *lasR* mutation impacted inflammatory cytokine
267 profiles upon infection of CF and nonCF MDMs (**Figure 7**). While infection generally induced a
268 broad inflammatory cytokine profile, there was a shift from IL-1 family cytokines IL-1α, IL-1β, IL-
269 1RA and IL-18 towards more classical inflammatory markers IL-6 and TNFα, as well as
270 macrophage inflammatory protein 1α, 1β and macrophage chemoattractant protein 1. There
271 was a trend towards increased IL-8 for *lasR* mutant infection that was not statistically significant
272 after adjustment for multiple comparisons (**Figure 7C**). A shift towards IL-1 family cytokines may
273 reflect increased inflammasome activation versus direct proteolytic activation of IL-1 family
274 precursor proteins. The LasR-regulated elastase LasB has previously been demonstrated to
275 directly activate pro-IL-1β in mice and human macrophages (69, 70), consistent with our data
276 showing less mature IL-1β for infections with *lasR* loss-of-function mutants (**Figure 7C**). Early
277 CF lung *P. aeruginosa* isolates have been shown to be more potent inflammasome activators
278 when compared to later isolates (71), however LasR functional status was not tested in the
279 study by Phuong et al. Decreased protease expression has been seen in persistent *P.*
280 *aeruginosa* isolates in those on ETI, also suggesting *lasR* mutation may be increased post
281 modulators (28). It is important to note that in our system significant LasR quorum sensing
282 activation would not necessarily be expected, as bacteria are sub-cultured at low density prior to
283 addition to cells, and cells were infected for 2 hours. In contrast to its effects increasing
284 processed IL-1β, LasB can decrease IL-8 levels upon infection of airway epithelial cells via
285 direct digestion of IL-8 (72). Future experiments will help to elucidate the exact mechanism
286 whereby *lasR* mutation alters the inflammatory cytokine milieu in the shorter term. We are also
287 currently conducting transcriptomic studies on macrophages infected in parallel with LasR+ or
288 LasR- isolates which should shed light on whether the effects are transcriptional versus post-
289 transcriptional.

290 Strengths of our work include analysis of *lasR* mutations in multiple backgrounds including a
291 common laboratory strain as well as naturally occurring clinical isolates to improve the
292 generalizability. We began to investigate the mechanisms by which *lasR* loss-of-function
293 mutants may confer phagocytosis resistance although work remains to further elucidate the full
294 mechanism. We confirmed the results found in a human cell line (THP-1 cells) with primary
295 human macrophages from multiple subjects and investigated the interplay with CFTR
296 modulators and genotype (mutant vs wild-type CFTR). Studies performed in parallel on cells
297 from the same subject allowed for a close interrogation of the effects of CFTR modulators and
298 LasR mutation on phagocytosis, metabolism and inflammation.

299 Our work also carries several limitations. MDMs, while they are a commonly used surrogate for
300 primary lung macrophages due to their ease of procurement, do not behave identically to lung
301 macrophages (73). We plan to expand our work with primary lung macrophages isolated via
302 bronchoscopy (74), and compare them with classically generated MDMs as well as the novel
303 differentiation protocol proposed by Pahari et al. Additionally, while our ex vivo studies provided
304 the opportunity to directly compare effects of LasR status and modulators in paired samples
305 from the same subject, they are necessarily reductive and cannot replicate in vivo conditions.
306 Mouse models differ from human CF in chronicity and acuity of lung infection, however there are
307 data suggesting *lasR* mutants are more virulent in mice as well (31, 72). Finally, we found no
308 direct effect of CFTR modulators including ETI on inflammatory responses by MDMs, in contrast
309 to human studies pre- and post-modulator which demonstrate decreased inflammation (14, 60,
310 60, 75–78). In vivo studies however cannot separate direct effects on immune cells from
311 decreased mucus and pathogen burden leading to a systemic decrease in inflammation.

312 In summary, *lasR* mutation confers reproducible resistance to phagocytosis, altered metabolic
313 effects on macrophages, and a change in inflammatory cytokine repertoire upon infection.
314 These effects were independent of background strain of *P. aeruginosa*, as well as CFTR

315 genotype and CFTR modulator therapy. Our work adds to the body of literature on how and why
316 *lasR* mutants are problematic in the lungs of pwCF and will likely continue to be so in the highly
317 effective modulator era.

318

319 **Materials and methods**

320 **Source of key chemicals and bacterial strains**

	Source of materials	**Catalog number**
M-CSF	Miltenyi	130-096-491
Pan monocyte isolation kit	Miltenyi	130-096-537
3-oxo-C12-homoserinelactone (3OC12HSL)	Sigma	O9139
C4-homoserinelactone (C4HSL)	Chemodex	B0267
Ivacaftor	Selleckchem	S1144
Tezacaftor	Selleckchem	S7059
Elexacaftor	Selleckchem	S8851
Seahorse mitostress kit	Agilent	
THP-1 cells	ATCC	TIB-202
PMA	Sigma	P1585
PA14 WT	(79)	Hogan Lab (DH122)
PA14 Δ*lasR*	(80)	Hogan Lab (DH164)
PA14 Δ*lasR* att::P*rhlI-lacZ*	(81)	Hogan Lab
DH1137 (LasR+)	(82)	Hogan Lab (DH1137)
DH1136 (LasR-)	(82)	Hogan Lab (DH1136)

DH2417 (LasR+), NC-AMT0101-1-2 (parent of NC-AMT0101-1)	(82)	Hogan Lab (DH2417)
DH2415 (LasR-), NC-AMT0101-1-1, related to DH2417 with LasR LOF (frameshift) allele	(82)	Hogan Lab (DH2415)

321

322 **THP-1 cell culture**

323 THP-1 cells were cultured in RPMI with 10% FBS, 50 μM β-mercaptoethanol, and 50 μg/mL
324 gentamicin in a 37°C humidity-controlled incubator with 5% CO_2. The initial vial was expanded
325 and frozen down in aliquots which were stored in liquid nitrogen with DMSO. Cultures from
326 individual aliquots were maintained in vitro for ~2 months prior to being discarded. THP-1
327 monocytes were differentiated into macrophages by addition of 50 nM Phorbol 12-myristate 13-
328 acetate (PMA) for 48 hours prior to experiments.

329 **Gentamicin protection phagocytosis assays with THP-1 derived macrophages**

330 THP-1 cells were differentiated at 5 x 10^5/mL in 24-well plates. They were then washed with
331 PBS and medium was replaced with identical medium without antibiotics. Overnight LB cultures
332 of bacteria of indicated strains were sub-cultured 1:10 into LB for 60 minutes, centrifuged at
333 8000 x g for 2 minutes, washed twice with antibiotic-free THP-1 medium, then resuspended and
334 bacterial density was determined by spectrophotometry at 600 nm. OD600 of 1.0 was
335 empirically determined to represent 10^9 bacteria/mL media. After dilution, bacteria were added to
336 THP-1 cells at a multiplicity of infection (MOI) of 10 bacteria per macrophage. Plates were
337 incubated at 37°C for 20 minutes, then cells were washed once with PBS, then THP-1 medium
338 with 10x gentamicin (500 μg/mL) was added for 15 minutes. Cells were then washed twice with
339 PBS at 4°C followed by lysis with 250 μL 0.1% Triton X-100. Lysates were diluted 1:3 in PBS

340 and then 10 μL was then plated overnight onto LB agar plates for colony forming unit (CFU)
341 determination. Colonies were counted the next day and bacteria per 10^6 macrophages was
342 calculated. MOI was also determined empirically from the stock bacterial solutions for each
343 assay as an internal control. Average of at least three technical replicates per assay was then
344 log-transformed and results were combined from at least 5 assays performed on separate days
345 (n for each experiment listed in figure legends).

346 **Phagocytosis competition assay**

347 PA14 Δ*lasR* with a *lacZ* reporter gene integrated at a neutral site on the chromosome was used
348 along with PA14 wild type (WT). Assay was identical to above except fixed percentages of PA14
349 WT and the Δ*lasR* strain were used at 100/0, 90/10, 70/30, 50/50, 30/70, 10/90, and 0/100 such
350 that the total MOI of both strains together was constant at 10. Lysates were plated onto LB agar
351 plates with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) for blue-white colony
352 discrimination. 100/0 samples were entirely white and 0/100 samples were entirely blue,
353 confirming the fidelity of the reporter. Total PA14 WT and Δ*lasR* colonies were counted for each
354 well. Each experiment was performed in triplicate and the means of technical replicates for each
355 assay were log-transformed and combined.

356 **Human subjects**

357 Our blood draw protocol has been approved by the Dartmouth Health Institutional Review
358 Board. Subjects were recruited from our CF clinic population if they were 18-65 years old,
359 F508del homozygous or heterozygous, at clinical baseline without symptoms of exacerbation,
360 and had normal hemoglobin levels. NonCF control subjects were recruited by IRB-approved
361 flyers; smokers or those with respiratory disease, immunologic disorders, or pulmonary
362 medication use were excluded. Phlebotomy was performed (100 mL whole blood isolated) for

363 monocyte isolation by our research nurse and taken directly to the laboratory for downstream
364 processing.

365 **Monocyte derived macrophage (MDM) generation and phagocytosis**

366 MDMs were generated according to previously published protocols (37). Briefly, monocytes
367 were isolated from peripheral blood by Ficoll gradient separation. They were quantified and
368 plated directly onto plates as per intended assay to avoid the need for detachment and re-
369 plating. MDMs were generated by treatment with 100 ng/mL M-CSF for seven days in
370 RPMI/10% FCS with 50 µg/mL gentamicin. Medium was exchanged every 2-3 days. After seven
371 days of differentiation, MDMs were cultured in RPMI/10% FCS + gentamicin, and CFTR
372 modulators were added for 48 hours prior to the experiment. CFTR modulators were replaced
373 with all media exchanges. Gentamicin protection assay was performed as above. Three to four
374 technical replicates per subject were averaged and log-transformed. Data on paired *lasR*
375 mutants were combined with previously published data on *lasR*-WT strains (37).

376 **Seahorse extracellular flux assay**

377 Monocytes were plated initially onto a Seahorse 96-well plate at 50,000 per well and
378 differentiated into MDM directly in the wells for seven days as above. At that point, differentiation
379 medium was removed and CFTR modulators were added for 48 hours. Pa-conditioned medium
380 was prepared in advance by culturing bacteria overnight in Seahorse mitostress assay medium,
381 normalizing cultures to an OD600 of 0.500, centrifuging to pellet bacteria then passing
382 supernatant through a 0.22 µm filter. This was defined as 100% conditioned medium. Aliquots
383 were then frozen at -20° C until use.

384 On the day of assay, cell culture medium was exchanged for mitostress assay medium as per
385 manufacturer's instructions. A mitostress assay with acute injection was run on an Agilent
386 Seahorse XFe96 platform, with 20 µL conditioned medium in injection port A (final concentration

387 10%), plain mitostress medium was included for control wells. Oligomycin, FCCP, and
388 Rotenone/Antimycin A were placed into ports B-D respectively. Four to eight technical replicates
389 per condition were run. Parameters including response to injection, maximal respiration, and
390 others were calculated as per manufacturer's instructions. Data were combined with previously
391 published results with a *lasR*-WT strain only (37).

392 **THP-1 Seahorse extracellular flux assay**

393 THP-1 macrophages were differentiated directly on a Seahorse 96-well plate with 50 nM PMA at
394 50,000 cells/well for 48 h. Seahorse extracellular flux assay was then run with varying
395 concentrations of 3OC12HSL or C4HSL in injection port A. Five to six technical replicates were
396 run per condition for each assay and averaged. Five independent experiments were run.

397 **Cytokine analysis**

398 MDMs were differentiated for seven days as above then treated for 48 hours with CFTR
399 modulators or DMSO. Bacteria were prepared in an identical manner to the phagocytosis
400 assays. After exchange for antibiotic-free media, MDMs were infected with indicated strains at
401 an MOI of 10 for 2 hours, then washed and placed in media with gentamicin for an additional 2
402 hours. Supernatants were then collected, centrifuged at 14,000 x g at 4°C for 10 minutes,
403 transferred to a new tube, and frozen at -20°C until needed. Samples were batched and
404 cytokine multiplex analysis was run by the Dartmouth Immunology and Flow Cytometry Core.
405 One replicate per condition was run. Samples with levels below the limit of the detection of the
406 assay were set to a concentration of 0, and data were log-transformed prior to analysis.

407 **Figure generation and statistical analysis**

408 Figures were generated with GraphPad Prism 10. Statistical analyses were performed
409 separately in R, with mixed model linear analysis using nlme package version 3.1. Models

410 include all variables of interest as fixed variables and assay date (for THP-1 experiments) or
411 subject (for MDM experiments) as random variables.

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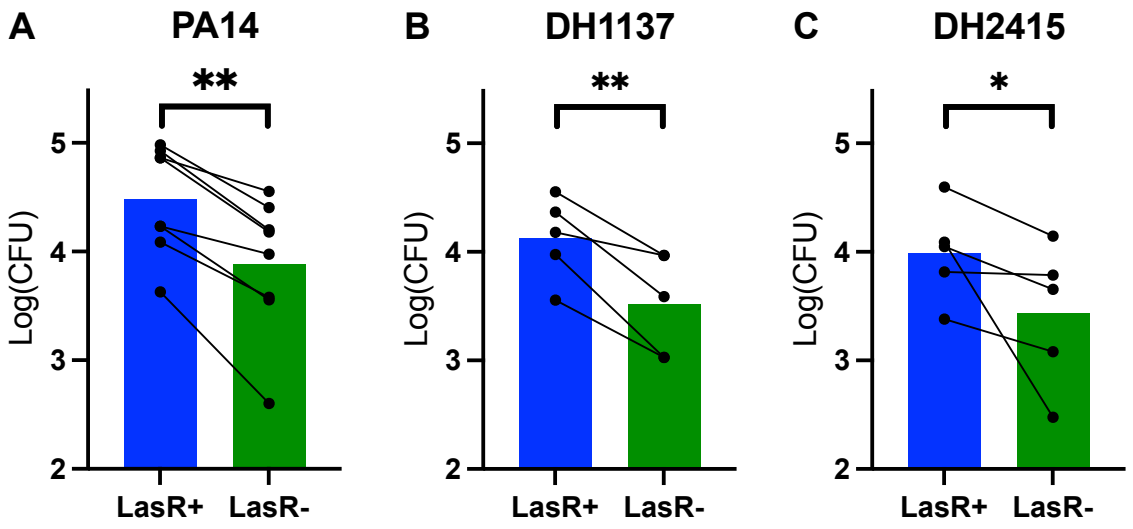

747

748 **Figure 1. *lasR* mutation confers phagocytosis resistance independent of parental strain.**
749 **A)** THP-1 cells were differentiated into macrophages by addition of 50 nM PMA for 48 hours.
750 Phagocytosis was then measured by a gentamicin protection assay at 20 minutes of bacterial
751 uptake. After removal of extracellular bacteria by washing and addition of gentamicin,
752 intracellular CFU were quantified by plating onto LB agar. Phagcoytosis of PA14 wild type and
753 PA14 Δ*lasR* (LasR-) was assessed in eight independent assays, log-transformed means of
754 three technical replicates per assay are displayed. ** $p<0.01$ for PA14 WT vs LasR- by mixed
755 model linear regression. **B)** Identical assays to **A** were run using naturally occurring paired
756 LasR+/- clinical isolates of *P. aeruginosa* derived from the same parental strain DH1137. **
757 $p<0.01$ for PA14WT vs LasR- by mixed model linear regression. **C)** Identical assays to **A** were
758 run using naturally occurring paired LasR+/- clinical isolates of *P. aeruginosa* derived from the
759 same parental strain DH2415. ** $p<0.01$ for PA14WT vs LasR- by mixed model linear
760 regression.

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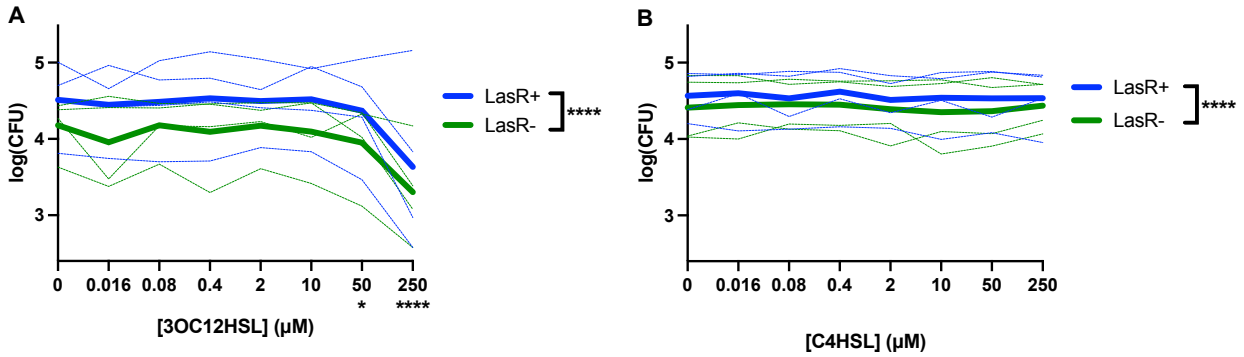

762

763 **Figure 2. LasR-regulated autoinducer inhibits phagocytosis in a dose-dependent manner.**

764 **A)** THP-1 macrophages were incubated with the indicated concentration of 3OC12HSL for 20

765 minutes prior to performing a gentamicin phagocytosis assay with PA14 WT and PA14Δ*lasR*

766 (LasR-) in parallel. Four separate experiments were performed with three technical replicates

767 each. The means of technical replicates from each individual experiment are shown in the light

768 lines, with darker lines at the means of the four experiments. *$p<0.05$, **** $p<0.0001$ for 50 and

769 250 μM 3OC12HSL relative to DMSO respectively, and LasR+ vs LasR- by mixed model linear

770 regression. **B)** Identical assays to **A** were performed with C4HSL in place of 3OC12HSL. ****

771 $p<0.0001$ by mixed model linear regression.

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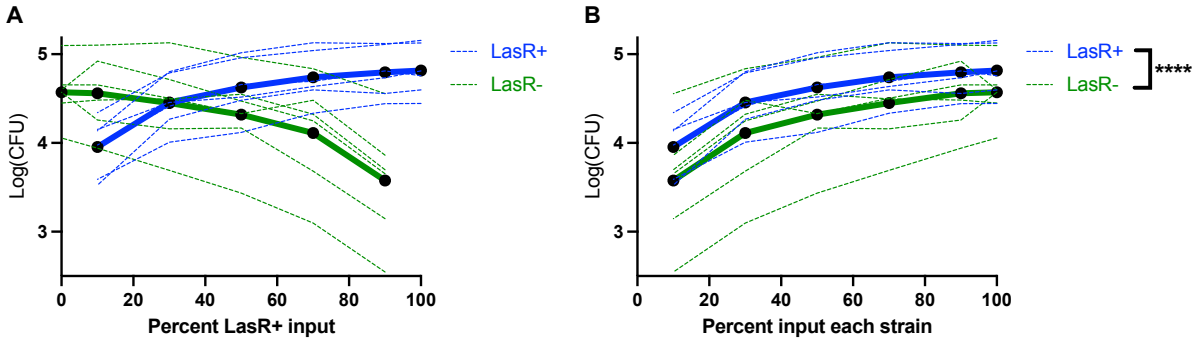

773

774 **Figure 3. *lasR* mutation confers phagocytosis resistance in mixed cultures. A)** A

775 PA14Δ*lasR* strain engineered to express a LacZ reporter was mixed with PA14 wild type at 0%,

776 10%, 30%, 50%, 70%, 90% and 100% with a fixed total MOI of 10 for the two strains and a

777 phagocytosis assay was performed as previously. After lysis, bacteria were plated onto X-gal

778 plates to allow for differentiation of colony genotype. Means of three technical replicates from 6

779 independent experiments are graphed, with a dark line at the mean of the 6 experiments. **B)**

780 The same data as in **A** are graphed relative to the percent of each strain input, rather than

781 percent LasR- to better visualize differences. **** $p<0.0001$ by mixed model linear regression.

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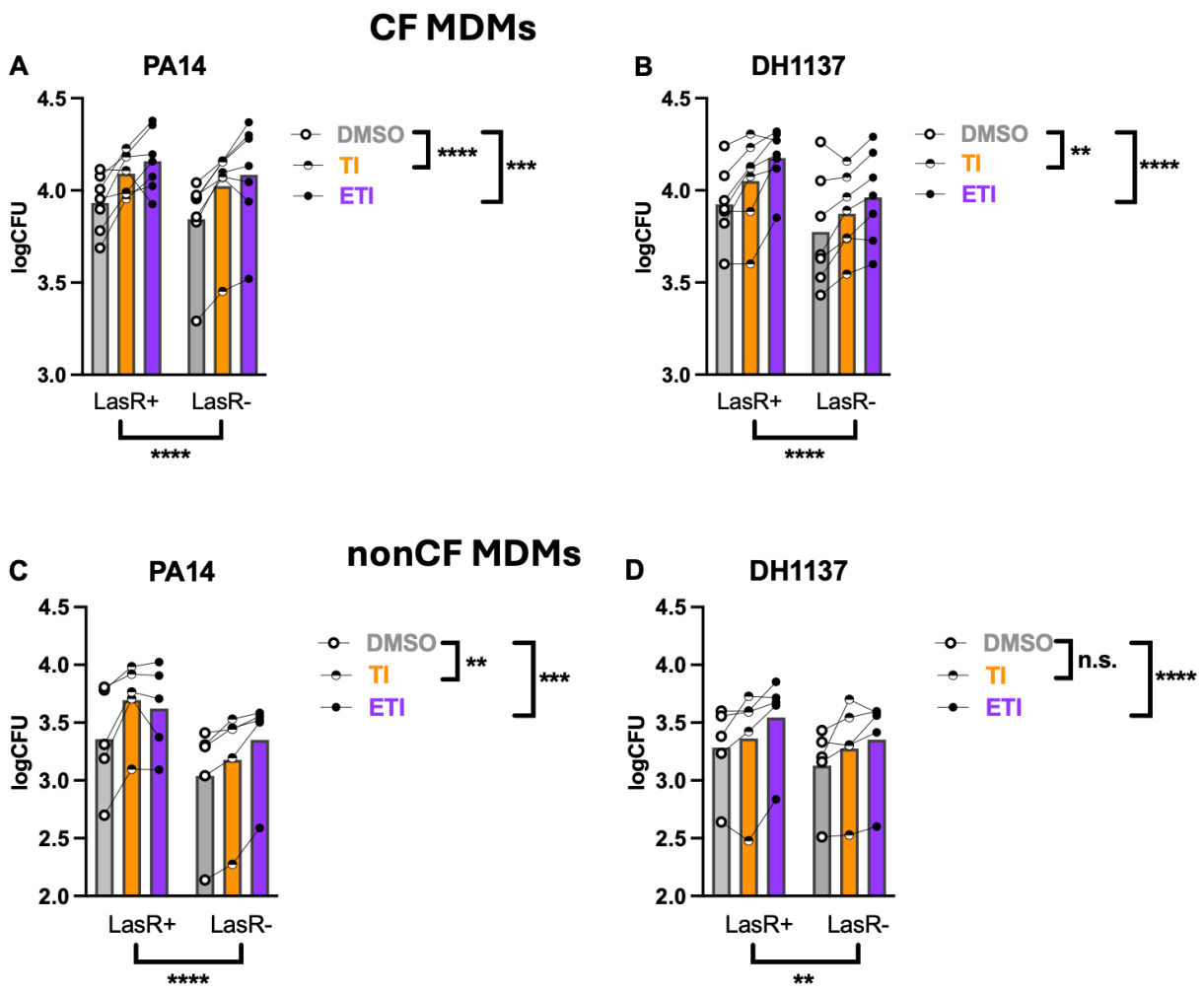

783

784 **Figure 4. *lasR* mutants exhibit reduced phagocytosis by primary MDMs irrespective of**
785 **CFTR genotype or modulators. A)** CF MDMs were treated for 48 hours with CFTR modulators
786 as indicated, then infected with PA14 WT or PA14 Δ*lasR* (LasR-). Lines connecting dots
787 represent data from a single subject (n=7). **B)** As in **A** except the clinical isolate DH1137 and its
788 derivative *lasR* mutant were used. **C)** NonCF MDMs (n=5) were infected with PA14 WT or LasR-
789 derivative as in **A**. D) NonCF MDMs were infected with DH1137 or its *lasR* mutant derivative as
790 in **B**. ** $p<0.01$, ***$p<0.001$, **** $p<0.0001$ for indicated comparisons by mixed model linear
791 regression. Data were combined with previously published data on LasR+ strains only (37).

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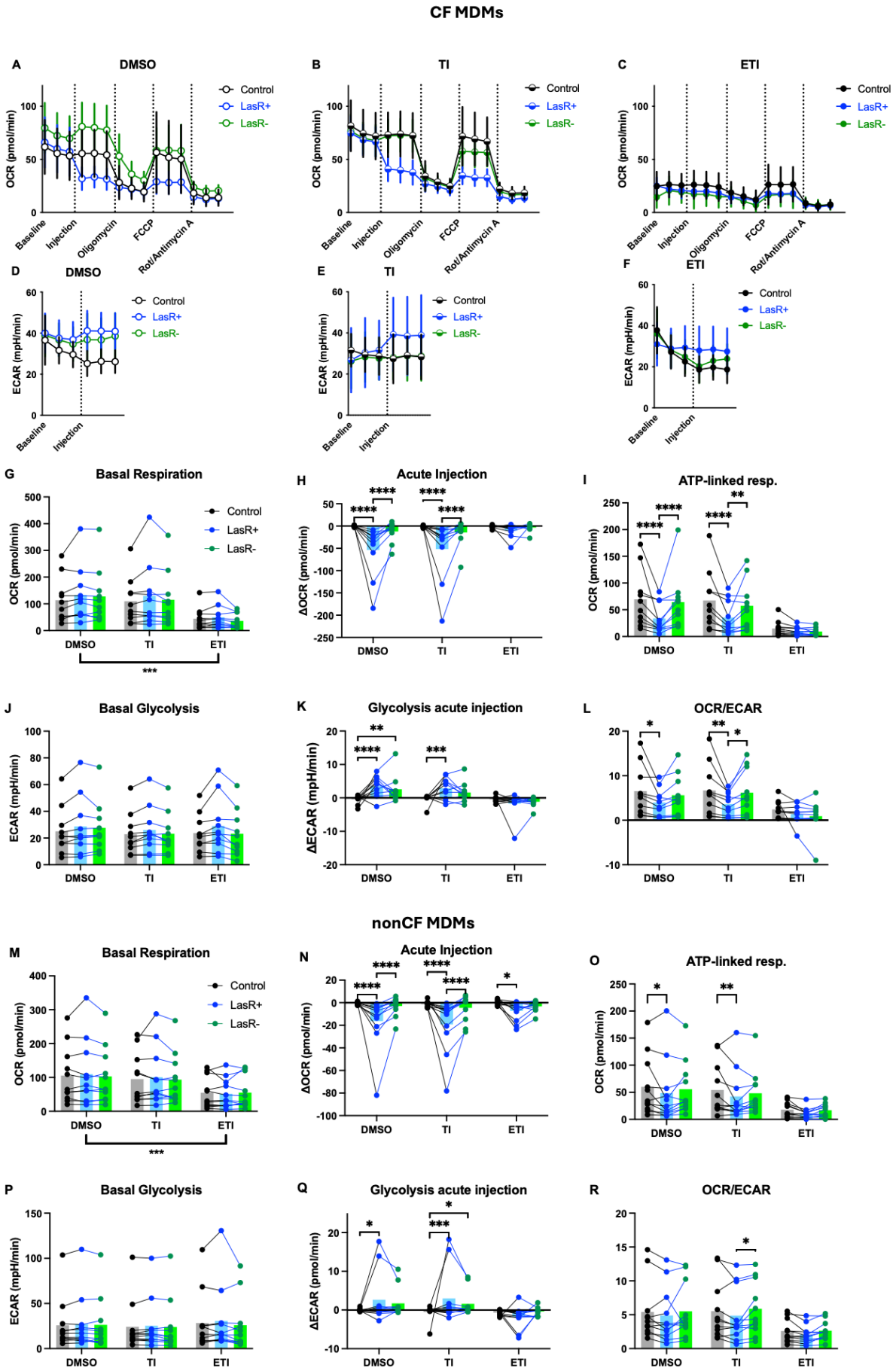
CF MDMs
A
DMSO
B
TI
C
ETI
Control
LasR+
LasR-
OCR (pmol/min)
Baseline
Injection
Oligomycin
FCCP
Rot/Antimycin A
D
E
F
ECAR (mpH/min)
G
Basal Respiration
H
Acute Injection
I
ATP-linked resp.
ΔOCR (pmol/min)
J
Basal Glycolysis
K
Glycolysis acute injection
L
OCR/ECAR
ΔECAR (mpH/min)
nonCF MDMs
M
N
O
P
Q
R
ΔECAR (pmol/min)

794 **Figure 5. Effects of CFTR modulators and LasR+/- secreted products on macrophage**
795 **metabolism.** CF and nonCF MDM were differentiated with M-CSF then treated for 48 hours
796 with CFTR modulators. A Seahorse mitostress assay with acute injection of *P. aeruginosa*
797 conditioned medium was then performed. **A-C**) Representative oxygen consumption rate
798 tracings from a CF subject MDMs pretreated with DMSO, TI, or ETI. **D-F**) Representative
799 extracellular acidification rates from the same subject. **G-L)** Calculated parameters as indicated
800 based on the mitostress assay of CF MDMs (n=10). Each point with connecting lines represents
801 the mean of technical replicates from an individual subject. Bars represent means of all the
802 subjects within the group. **M-R**) Calculated parameters as indicated for nonCF MDMs (n=11).
803 *p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001 for indicated comparisons by mixed model linear
804 regression. Data were combined with previously published data on LasR+ strains only (37).

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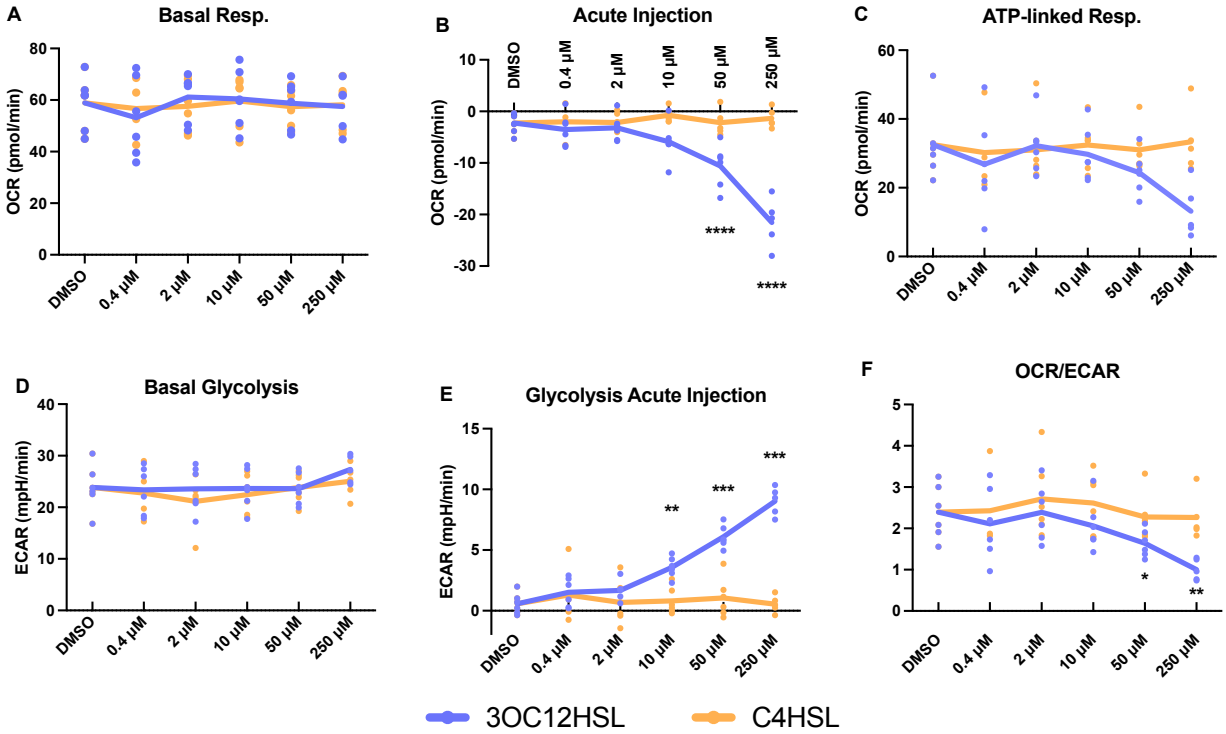
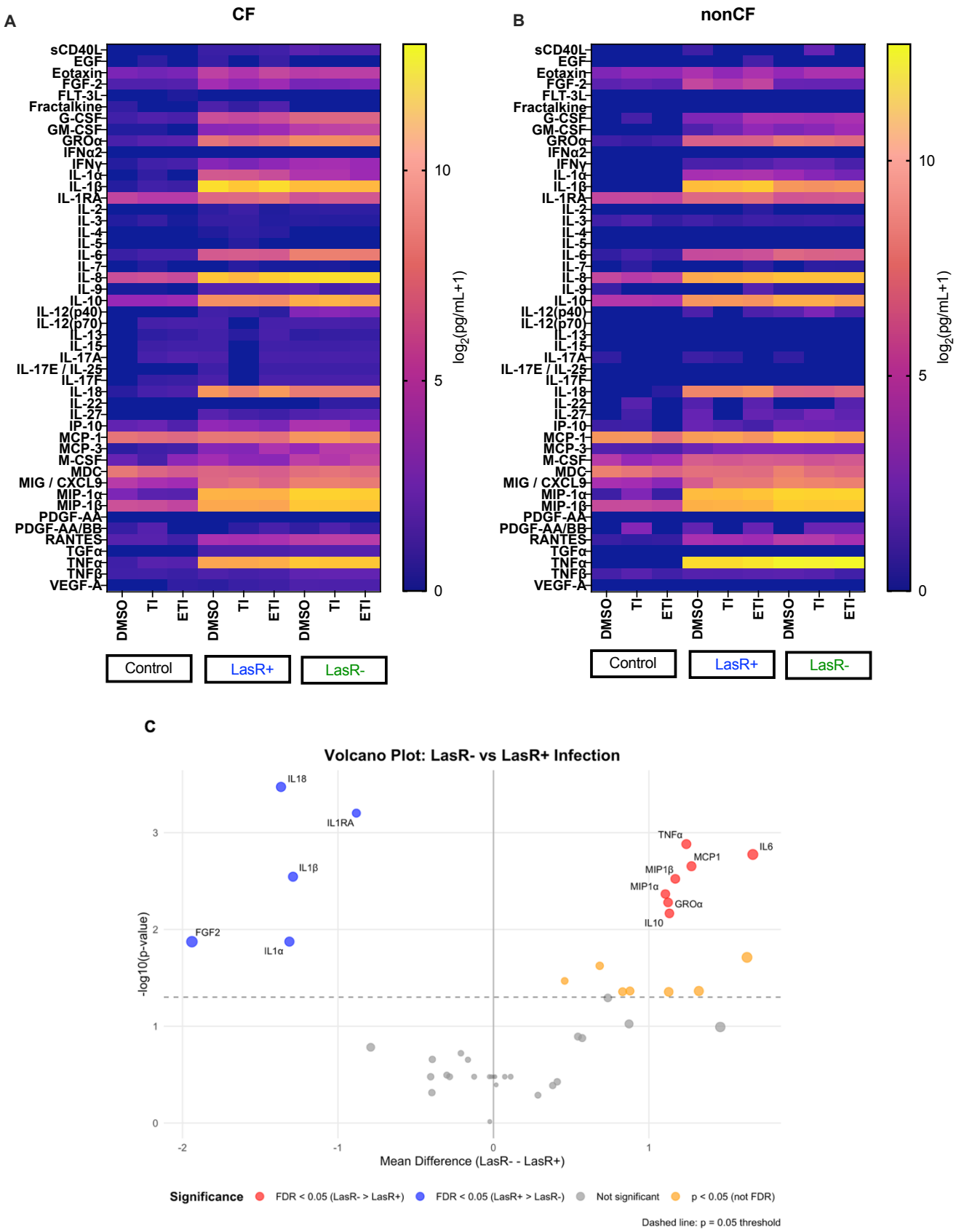


Figure 6. 3OC12HSL inhibits mitochondrial respiration in THP-1 macrophages. A-F) Seahorse mitostress assay was run with acute injection of various concentrations of 3OC12HSL or C4HSL. Parameters were calculated as per standard protocol. Points represent averages of 6-8 technical replicates from 6 individual experiments, with solid lines at the global means. *p<0.05, ** p<0.01, ***p<0.001, **** p<0.0001 for indicated comparisons relative to DMSO treated wells by mixed model linear regression.


A
CF
B
nonCF
sCD40L
EGF
Eotaxin
FGF-2
FLT-3L
Fractalkine
G-CSF
GM-CSF
GROα
IFNα2
IFNγ
IL-1α
IL-1β
IL-1RA
IL-2
IL-3
IL-4
IL-5
IL-6
IL-7
IL-8
IL-9
IL-10
IL-12(p40)
IL-12(p70)
IL-13
IL-15
IL-17A
IL-17E / IL-25
IL-17F
IL-18
IL-22
IL-27
IP-10
MCP-1
MCP-3
M-CSF
MDC
MIG / CXCL9
MIP-1α
MIP-1β
PDGF-AA
PDGF-AA/BB
RANTES
TGFα
TNFα
TNFβ
VEGF-A
DMSO
TI
ETI
Control
LasR+
LasR-
log_2(pg/mL+1)
10
5
0
C
Volcano Plot: LasR- vs LasR+ Infection
IL18
IL1RA
IL1β
FGF2
IL1α
TNFα
IL6
MCP1
MIP1β
MIP1α
GROα
IL10
-log10(p-value)
Mean Difference (LasR- - LasR+)
Significance
FDR < 0.05 (LasR- > LasR+)
FDR < 0.05 (LasR+ > LasR-)
Not significant
p < 0.05 (not FDR)
Dashed line: p = 0.05 threshold

814

815

816 **Figure 7. *lasR* mutation alters inflammatory cytokine expression profile by CF and nonCF**
817 **MDMs. A)** CF MDMs (n=11 subjects) were pre-treated with CFTR modulators and then infected
818 with DH1137 (LasR+) or DH1136 (LasR-) for two hours, then cells were washed and media
819 replaced. Supernatants were collected 2 hours after washing and analyzed by cytokine
820 multiplex assay. Means of log-transformed data are graphed. **B)** nonCF MDMs (n=4) were
821 infected in the same way and cytokines analyzed by multiplex. **C)** Volcano plot of fold difference
822 between LasR+ and LasR- cytokine results, DMSO treated group only.

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