Enhanced survival of Rifampicin and Streptomycin double resistant *E. coli* inside macrophages

Running Title: Double resistance enhances survival in macrophages

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ABSTRACT

Evolution of multiple antibiotic resistant bacteria is an increasing global problem. Even though mutations causing resistance usually incur a fitness cost in the absence of antibiotics, the magnitude of such costs varies across environments and genomic backgrounds. Here we have studied how the combination of mutations that confer resistance to rifampicin (Rif^R) and streptomycin (Str^R) affects the fitness of *E. coli* when it interacts with cells from the immune system - macrophages (MΦs). We found that 13 Rif^RStr^R double resistant genotypes, out of 16 tested, show a survival advantage inside MΦs indicating that double resistance can be highly beneficial in this environment. Our results suggest that there are multiple paths to acquire multiple-drug resistance in this context, *i.e.* if a clone carrying Rif^R alleles H526 or S531 acquires a second mutation conferring Str^R, the resulting double mutant has a high probability of showing increased survival inside MΦs. On the other hand, we have found two cases of sign epistasis between mutations leading to a significant decrease in bacteria survival. Remarkably, infection of MΦs with one of these combinations, K88R+H526Y, resulted in an altered pattern of gene expression in the infected MΦs. This indicates that the fitness effects of resistance may depend on the pattern of gene expression of infected host cells. Notwithstanding the benefits of resistance found inside MΦs, the Rif^RStr^R mutants have massive fitness costs when the bacteria divide outside MΦs, indicating that the maintenance of double resistance may depend on the time spent within and outside phagocytic cells.

30 **INTRODUCTION**

31 Antibiotic resistance in many pathogens has become a worldwide problem, incurring both
32 loss of human lives and economic costs (1). Bacteria can acquire antibiotic resistance as a
33 result of transfer and acquisition of new genetic material between individuals of the same or
34 different species but also by chromosomal mutations, which alter existing proteins. For
35 instance, resistance to rifampicin (Rif^R, a rifamicin) occurs due to mutations in the gene *rpoB*
36 coding for the β-subunit of RNA polymerase and resistance to streptomycin (Str^R, an
37 aminoglycosidase) occurs due to mutations in the gene *rpsL* coding for a ribosomal protein
38 (2). These genetic targets for resistance are common across a wide range of bacteria species
39 including *Escherichia coli*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* (2-4).
40 Mutations causing antibiotic resistance usually incur a fitness cost in the absence of
41 antibiotics (5-7). However the magnitude of such costs is known to vary with the environment
42 (8,9). Even though most resistances are deleterious in the absence of antibiotics, some can be
43 beneficial. Remarkably, rifampicin resistance can even be selected for in populations evolving
44 without antibiotics (10). Furthermore, evidence is mounting that epistasis is widespread
45 among resistance mutations (2,11,12) and the level of epistasis is also dependent on the
46 environment (13). Given the strong effect of Genotype by Environment interactions on the
47 fitness of both single and double resistances, it is important to determine the effects of
48 resistance in environments that are relevant in the context of infection.
49 In here, we have studied the fitness effects of double resistance mutations conferring Rif^R and
50 Str^R, when *E. coli* encounters macrophages (MΦs), as will happen in an infection. MΦs are
51 key players of the host's innate immune system by recognizing, engulfing and killing
52 microorganisms, and thus, an important selective pressure in the context of infection. *E. coli*
53 is both a commensal and a versatile pathogen, acting as a major cause of morbidity and
54 mortality worldwide (14) and there is evidence that some pathogenic *E. coli* evolved from
55 commensal strains (15,16). *E. coli* colonizes the infant gastrointestinal tract within hours after

56 birth and typically builds a mutualistic relation with its host. However, it can become
57 pathogenic when the gastrointestinal barrier is disrupted as well as in immunosuppressed
58 hosts (17-19). Non-pathogenic *E. coli* does not replicate inside MΦs but different mutants
59 may have different abilities to persist inside these phagocytic cells (20). In a previous study
60 we found that *E. coli* clones with single point mutations in the *rpsL* gene, conferring Str^R,
61 exhibited a survival advantage over non-resistant *E. coli* in the intracellular niche of MΦs
62 (20). To determine if such advantage would be altered in the presence of other resistances, we
63 studied double resistant clones. We combined Str^R mutations - K43N, K43T, K43R and K88R
64 - with mutations that confer resistance to Rif^R, and measured the competitive fitness of the
65 double resistance bacteria, against a sensitive strain, inside and outside MΦs. The chosen
66 *rpoB* mutations conferring Rif^R - S512F, S531F, H526Y and I572F – were shown to exhibit
67 variable effects in competition against sensitive clones (20). S512F and I572F showed a
68 survival advantage inside the MΦs, S531F was neutral and H526Y phenotype was time-
69 dependent being neutral at 2h and beneficial at 24h post-infection (20). Previous work
70 (2,11,13,21-23) has found strong epistatic interactions between alleles that confer rifampicin
71 and streptomycin resistance in different species and in different environments, a result with
72 important consequences for understanding the possible evolutionary paths towards the
73 acquisition of multi-antibiotic resistance. Thus, we ask the following questions: What are the
74 fitness effects of double RifStr resistance when bacteria face the pressure imposed by MΦs?
75 Does the survival advantage conferred by a single Str^R mutation depend on the presence of a
76 Rif^R allele? And, do MΦs show alterations in gene expression when infected with Rif^RStr^R
77 mutants?

78

79 **MATERIAL AND METHODS**

80 **Strains and Media**

81 The RAW 264.7 murine macrophage cell line was maintained in an atmosphere containing
82 5% CO2 at 37ºC in RPMI 1640 (Gibco) supplemented with 2mM L-glutamine (Gibco), 1mM
83 sodium pyruvate (Gibco), 10mM HEPES (Gibco), 50µM 2-mercaptoethanol solution (Gibco),
84 with 10% heat-inactivated FBS (fetal bovine serum, Gibco). Bacterial strains were grown and
85 competed in antibiotic-free RPMI medium in an atmosphere containing 5% CO_2 at 37ºC.
86
87 **Construction of strains**
88 We used susceptible strains *E. coli* K-12 MG1655 ΔlacIZYA galK::CFP/YFP, and a
89 collection of single Str^R and Rif^R mutants (also ΔlacIZYA galK::CFP/YFP) previously
90 studied (2,20). To construct the double Rif^RStr^R mutants, Rif^R and Str^R mutants were
91 transferred into the background of each of the single Str^R and Rif^R mutants (ΔlacIZYA
92 galK::CFP/YFP) by general transduction using P1 bacteriophage (24). To confirm the double
93 mutations, each antibiotic resistance target gene was amplified by PCR and then sequenced.
94 Each confirmed double resistant clone was grown from a single colony in LB medium
95 supplemented with the respective antibiotics and stored in 15% glycerol at -80°C.
96
97 **Survival assays inside the MΦs**
98 To estimate the effect of double resistance on bacterial survival inside phagocytic cells, MΦs
99 were first seeded in plates for 24h for acclimatization and then activated with 2 µg of CpG-
100 ODN 1826 (5’ -TCCATGACGTTCCTGACGTT- 3’)/ml for 24 h (see **Figure 1**). Afterwards,
101 the cells were washed from the remaining CpG-ODN, fresh antibiotic-free RPMI medium
102 was added, and MΦs were infected with $5x10^6$ bacteria (at a 1:1 ratio of double resistant to
103 susceptible strain), and centrifuged at 203 x *g* (1,000 rpm) for 5 min to enhance bacterial
104 internalization. The initial ratios of resistant and susceptible strains were determined by flow
105 cytometry (see below). At 1 h of infection, the MΦs were washed from the extracellular
106 bacteria, and fresh cell culture medium containing 100 µg of gentamicin/ml was added to kill

107 the remaining extracellular bacteria. To determine the number of intracellular bacteria after 2
108 and 24 h of incubation, infected MΦs were washed with phosphate-buffered saline (PBS), and
109 0.1% Triton-X was added for 10 min at 37°C in order to lyse the MΦs. The MΦs were then
110 centrifuged at 10,600 x *g* (10,000 rpm) for 5 min and washed in PBS, and the overall number
111 of bacteria was counted by plating them on LB agar plates. Survival inside the MΦs was
112 estimated as the change in frequency (ΔX), measured as differences in viable cell counts, of
113 the resistant strain, calculated as follows: $\Delta X = Nf_b/(Nf_a + Nf_b) - Ni_b/(Ni_a + Ni_b)$, where Nf_a
114 and Nf_b are the numbers of resistant (*b*) and susceptible (*a*) bacteria after competition, and Ni_a
115 and Ni_b are the initial numbers of resistant (*b*) and susceptible (*a*) bacteria before the
116 competition. Significance was determined using the Wilcoxon signed rank test.
117
118 **RNA Extraction, Reverse Transcription and Quantitative Real Time PCR (RT-qPCR)**
119 To determine changes in macrophage gene expression after infection with bacteria, MΦs
120 ($5x10^6$) were seeded per 6-well plate and infected independently (not in competition) with the
121 chosen bacterial strain. MΦs were treated as described above for the survival assays inside the
122 MΦs. At 2h post-infection MΦs were repeatedly washed with warm (37ºC) RPMI prior to
123 RNA extraction. RNA extraction was performed using the Direct-Zol RNA MiniPrep Kit
124 (Zymo research) according to manufactures specifications. RNA was treated with RQ1 DNase
125 (Promega) according to manufactures protocol. Reverse transcriptase reaction was performed
126 with M-MLV RT (Promega) using random primers (Promega) according to manufactures
127 protocol.
128 qPCR was executed in BioRad CFX 384 with iTaq Universal SYBR Green Supermix
129 (BioRad). MΦ cDNA was diluted 10-fold before being used for qPCR. The cycling
130 conditions were as follows: one step of 5 min at 95ºC and then 40 cycles of 30 sec at 95ºC, 30
131 sec at 60ºC and finally 30 sec at 72ºC, primers used are listed in **Supplemental Table 1**.

132 Melting curve analysis was performed to verify product homogeneity. All reactions included
133 at least three biological replicates for each sample.
134 For analysis, data was normalized by the Pfaffl method (25) using *actinB* housekeeping gene
135 as reference for murine cDNA. When comparing the antibiotic resistance strains against the
136 susceptible strain, significance in differences of expression levels were determined by a t-test
137 on the fold change values. Multiple t-tests were performed when comparing directly the
138 double mutants K88R+H526Y and K88R+I572F.
139

140 **Competitive fitness in the presence and absence of MΦs**

141 The double resistant mutants constructed in the MG1655-CFP strain were competed against a
142 susceptible MG1655-YFP strain in an antibiotic-free environment at a ratio of 1:1 in two
143 different conditions - in the presence or absence of MΦs. Before the competitions, resistant
144 and susceptible strains were grown separately in antibiotic-free RPMI medium for 48 h (with
145 a dilution of 1:100 after 24 h) for acclimatization at 37°C with 5% CO_2. For competitions in
146 the presence of the MΦs, 10^6 MΦs were seeded in the wells. Competitions in presence or
147 absence of MΦs were then performed in 24-well cell culture tissue plates (containing 500 µl
148 of RPMI culture medium in each well), by inoculating a mix of 2.5×10^4 of each bacteria
149 strain. The initial ratios of resistant and susceptible strains were determined by flow
150 cytometry (see below). To determine the number of extracellular bacteria after 24 h of
151 incubation, supernatant RPMI was diluted in PBS, and the overall number of bacteria was
152 counted by plating them on LB agar plates. Competitive fitness outside the MΦs was
153 estimated as the change in relative frequency (ΔX), calculated as described above.
154 Significance for the competitive assays was determined using the Wilcoxon signed rank test.
155 Wilcoxon sum rank test was performed to analyse the behaviour of the mutants in the
156 presence and absence of MΦs during the competitive fitness. To test for a possible trade-off
157 between competitive fitness in RPMI and survival inside MΦs a sign-test was used.

158

159 **Flow Cytometry**

160 To determine the initial ratios of resistant and susceptible strains in the survival and
161 competition assays, bacteria were quantified prior to infection with a LSR Fortessa Flow
162 Cytometer using a 96 well plate auto sampler. Samples were always run in the presence of
163 SPHERO (AccuCount 2.0µm blank particles) in order to accurately quantify bacterial
164 numbers in the cultures. Briefly, flow cytometry samples consisted of 180µl of PBS, 10µl of
165 SPHERO beads and 10µl of a 100-fold dilution of the bacteria culture in PBS. Bacterial
166 concentration was calculated based on the known number of beads added. CFP was excited
167 with a 442 nm laser and measured with a 470/20nm pass filter. YFP was excited using a
168 488nm laser and measured using a 530/30nm pass filter.

169

170 **RESULTS**

171 **Survival advantage of double resistance strains when competing inside MΦs**

172 Non-pathogenic *E. coli* K12 does not replicate inside MΦs, so survival is an important fitness
173 component in this niche (20,26). Survival inside the MΦs was estimated as the change in
174 frequency (ΔX), measured as differences in viable cell counts. We measured the relative
175 survival ability of 16 *E. coli* K-12 strains carrying resistance to two antibiotics inside RAW
176 264.7 murine MΦs. After growing double resistant and susceptible strains separately, we
177 infected activated MΦs in antibiotic-free medium with a co-culture of bacteria. This co-
178 culture was obtained by mixing the appropriate volumes of resistant and susceptible strains so
179 that they start competing at equal densities (1 double resistant cell to 1 susceptible cell) in the
180 co-culture (**see Figure 1**). After 1 h of infection, gentamicin was added to kill the remaining
181 extracellular bacteria, which is sensitive to this drug. To control for the efficacy of gentamicin
182 treatment, we plated the supernatant with bacteria which were exposed 1h to gentamicin and
183 detected a residual number of colonies < 10^3 CFUs/mL, which corresponds to < 1% of the

184 total numbers of bacteria found inside the MΦs at the same time point (> 10^5 CFUs/mL). To
185 determine the relative numbers of resistant versus susceptible intracellular bacteria, infection
186 was halted after 2 and 24 h of incubation and the content of MΦs was plated on LB plates. We
187 found that 13 out of 16 double mutants showed a survival advantage inside MΦs either at 2 or
188 at 24h post-infection (**Figure 2**). At 2h post-infection, 62.5% of the double mutants displayed
189 a significant increase in survival inside MΦs and this percentage increased to 81.3% at 24h
190 post-infection. These results indicate that the combination of $Str^R Rif^R$ double resistance is
191 generally beneficial inside MΦs, in the absence of antibiotics. All but one of the $Rif^R Str^R$
192 double mutants resulting from combining any single (beneficial) Str^R mutation with beneficial
193 Rif^R (S512F or I572F) showed increased survival inside the MΦs when compared to a
194 susceptible strain. Thus, the combination of two resistances which individually are beneficial
195 often results in an overall benefit for the double mutant. Two interesting cases of the opposite
196 scenario were found. In the K43R+H526Y and K88R+H526Y combinations of double
197 resistance decreased survival was observed even though each mutation alone does not confer
198 a survival cost – examples of sign epistasis. By combining these results of the fitness effects
199 of double resistance with the previously measured for single resistances (20), it follows as an
200 outcome that single Rif^R mutations can acquire increased survival inside the macrophages by
201 acquiring a Str^R mutation in 50% of the cases (see **Supplemental Figure 1**). For instance, the
202 clinically common Rif^R S531F mutation, which is neutral when alone, may hitchhike with
203 beneficial Str^R mutations suggesting a path towards acquired double antibiotic resistance in
204 the context of infection in the absence of antibiotics. To further corroborate this hypothesis,
205 we performed competitions between $Rif^R Str^R$ double mutant K43T+S531F against the single
206 S531F (Rif^R) and found that the double mutant outcompeted the single inside the MΦs ($\Delta X =$
207 0.02 ± 0.01, p-value < 0.05). On the other hand, single Str^R mutations can acquire increased
208 survival inside the macrophages by acquiring a Rif^R mutation in 4 out 16 (25%) of the cases

209 (**Supplemental Figure 1**). Those 4 combinations are K43N+S512F, K43T+S531F,
210 K43R+S531F and K88R+I572F.
211
212 **Double resistance showing sign epistasis prompts an altered inflammatory response**
213 Macrophages undergo changes in gene expression after the phagocytosis of bacteria (27).
214 Given the differential survival of the double resistant strains, we hypothesized that MΦs gene
215 expression could differ between the $Rif^{R}Str^{R}$ mutants and the susceptible strain. We selected 7
216 macrophage transcripts (*ccl5, ifit1, ifnβ, il1a, il10, nlrp3*, and *stx11*) previously identified as
217 important in the context of bacterial infection (27) and tested their expression by RT-qPCR.
218 In a previous work, we have adapted *E. coli* to MΦs by propagating bacterial populations for
219 30 days when facing MΦs, while inhabiting both the intracellular and extracellular
220 environments (28). Infection of MΦs with these *E. coli* previously adapted to MΦs also lead
221 to the alteration in the expression of the tested genes (unpublished data from our lab). To
222 confirm that all macrophage genes tested were significantly upregulated when bacterial
223 infection occurs, we infected MΦs with a susceptible strain and compared the transcription
224 levels of the above mentioned genes to a mock (uninfected MΦs) experiment (**Figure 3A**).
225 Having found that these genes were induced upon infection with the susceptible strain, we
226 used the same set of genes to compare the transcriptional response by RT-qPCR of MΦs
227 infected by a susceptible strain or by several resistance strains. The MΦs were infected
228 independently but in parallel with a similar number of bacteria of: a) the double $Rif^{R}Str^{R}$
229 mutants K88R+H526Y (which showed sign epistasis which resulted in decreased survival
230 inside the MΦs) or K88R+I572F (increased survival inside the MΦs); b) the susceptible
231 strain; c) a single resistant $RpsL^{K88R}$ mutant - Str^{R}; d) a $RpoB^{H526Y}$ and a $RpoB^{I572F}$ mutant,
232 each conferring Rif^{R}. **Figure 3B** shows that, at 2h post-infection, the expression of tested
233 genes was altered in all but one of the resistance strains. Interestingly, for the infection with
234 the K88R+H526Y mutant, which showed a decreased survival, three transcripts were

235 significantly upregulated (**Figure 3B**) whereas for the other mutants less changes were
236 detected. The infection with mutant K88R+H526Y resulted in a significant increase in *ifit1*
237 expression ($p = 0.026$, one sample t-test), *il-10* ($p = 0.0005$) and *nlrp3* ($p = 0.009$) relative to
238 the infection with a susceptible strain. Comparing the transcript expression levels between
239 K88R+H562Y and K88R+I572F infections, we found significant differences for *ifit1* ($p =$
240 0.022, multiple t-test), *il1-α* ($p = 0.014$) and *il-10* ($p = 0.012$). Differences in the level of *ifn-β*
241 transcripts ($p = 0.062$) and *stx11* ($p = 0.056$) between the double mutants were marginally
242 significant ($0.05 < p < 0.1$).

243

244 **Trade-off between survival and competitive fitness outside the MΦs**

245 To determine the fitness effects of double resistance mutations when bacteria can grow
246 outside macrophages, we performed competition assays (29) in two different environments: in
247 RPMI medium alone (absence of MΦs) or in RPMI medium with the presence of MΦs (to
248 which we did not apply gentamicin to allow for bacterial growth). **Figure 4** shows that in
249 most cases double resistance incurs a strong decrease in competitive fitness in both
250 environments. Remarkable exceptions are detected for the K43R+S512F, K43R+H526Y and
251 K43R+S531F double mutants which show no competitive disadvantage when grown in the
252 presence of MΦs. The K43R+S512F is a particular worrisome combination of alleles, given
253 that it results in a double resistant clone with no fitness costs for survival inside MΦs and a
254 competitive growth advantage in the presence of MΦs. However, a clear cost is measured
255 when MΦs where absent ($p < 0.0001$, Wilcoxon sum rank test) which suggests that MΦs are
256 altering the medium to a more beneficial environment for this mutant. We have also found
257 that K43R+H526Y is the only mutant that did not show a decreased competitive fitness when
258 growing in RPMI, irrespective of the presence or absence of MΦs (**Figure 4**). This double
259 mutant was actually one of the three exceptions that did not show increased survival inside

260 the MΦs at any of the time points measured. We noticed that the massive fitness costs
261 observed for the $Str^R Rif^R$ double mutants when bacteria are allowed to divide seemed to
262 correlate with the substantial fitness benefits when bacteria are inside the MΦs. Thus, we used
263 our data for the $Str^R Rif^R$ double mutants plus the available data from previous results for the
264 single Str^R and Rif^R mutants (20) to test this hypothesis. We found a trade-off between
265 survival inside the MΦs and competitive fitness in RPMI both in the presence and absence of
266 MΦs ($p < 0.01$ in both cases, sign-test).
267 The observed loss in competitive ability of the double resistance bacteria could be associated
268 with a reduced nutritional competence (30,31). To test for this, we have analysed the growth
269 rates of the double $Rif^R Str^R$ mutants by performing growth curves in RPMI in microaerobic
270 conditions (without shaking). For all the mutants, the growth curves displayed a biphasic
271 behaviour with two distinct growth rates separated by a short plateau (at OD_{600nm} of ≈0.4): an
272 initial, faster growth rate (ε_{r1}) presumably due to the presence of oxygen in low amounts in
273 the RPMI medium, followed by a second slower growth rate (ε_{r2}), presumably in the absence
274 of oxygen (**Table I**).
275

276 **DISCUSSION**

277 Multidrug-resistant bacteria pose a significant threat to human health, and it is important to
278 understand what are the fitness effects of such bacteria during infection. Both single Str^R and
279 Rif^R isolates have been identified in many important pathogens, such as *M. tuberculosis*, *S.*
280 *flexneri*, *V. cholerae*, *P. aeruginosa*, and even in commensal *E. coli* sampled from healthy
281 individuals (32-35). In this study, we have tested 16 $Rif^R Str^R$ double mutants of *E. coli* for
282 their ability to survive in the presence of MΦs. This viability is an important fitness trait
283 because numerous pathogens, which have evolved different mechanisms to survive inside the
284 MΦs, are rapidly acquiring multidrug resistance to these drugs. For instance, *M. tuberculosis*
285 owes its success as pathogen to its ability to interfere with the normally effective

286 antimicrobial properties of the macrophage and is frequently found to be resistant both to Str^R
287 and Rif^R (36-39). We found that most $Rif^R Str^R$ mutants in *E. coli* had increased survival
288 inside MΦs after 24h post-infection and a similar effect was also observed at 2h post-
289 infection. It would be important to test if similar effects are found for the combinations of the
290 highly frequent $rpoB^{H526Y}$ and $rpoB^{S531L}$ mutations in natural pathogens, such as *M.*
291 *tuberculosis* (4,38,39). In fact our *E. coli* results suggest that such pathogens could benefit
292 from the combination of these Rif^R alleles with certain Str^R alelles and suggests a possible
293 path to acquire multidrug resistance in the context of infection and in the absence of
294 antibiotics. This finding suggests that streptomycin treatment should be avoided in patients
295 infected with rifampicin resistant mutants.

296 Our results of fitness benefits of $Rif^R Str^R$ resistance mutations in the absence of antibiotics
297 add to the cases recently found for other resistances. For instance, it has been shown that
298 knock-outs of the *oprD* and *glpT* genes, resulting in antibiotic resistance to carbapenem and
299 fosfomycin, provided an *in vivo* fitness advantage during infection of *P. aeruginosa* in the
300 absence of drugs (40,41). In this same organism the loss of genes such as *ampC* (encoding a
301 cephalosporinase conferring resistance to amoxicillin-clavulanic acid), *aph* (encoding an
302 aminoglycoside phosphotransferase conferring resistance to kanamycin) and the *mexAB-oprM*
303 operon (encoding an efflux pump conferring resistance to both nalidixic acid and
304 trimethoprim-sulfonamide) bears a fitness cost in the absence of antibiotics, indicating that
305 these genes are important fitness determinants for both gastrointestinal colonization and lung
306 infection (40) in the absence of antibiotics. Another study has shown that *Staphylococcus*
307 *aureus* can acquire intermediate levels of resistance to vancomycin in the absence of
308 antibiotic and during *in vivo* infection in a mouse model, solely due to competition between
309 coevolving bacterial strains (42). Overall, our results add to a growing body of evidence
310 suggesting that a reduction in antibiotic use, which a priori should lead to a drop of

311 (multi)drug-resistant strains, might result in an unfortunate outcome, a finding which
312 contrasts to the currently prevailing view that increased antibiotic resistance has a negative
313 fitness cost.

314 In our sample of double resistance, we found two cases of sign epistasis for survival of the
315 bacteria inside the MΦs, where each single resistance is either beneficial or neutral but the
316 combination is deleterious. When we compared the expression level of genes in MΦs infected
317 with a double resistant mutant exhibiting sign epistasis, K88R+H526Y, we found several
318 genes to be upregulated. The significant upregulation of NLRP3 and IFIT1 (and IL1-α when
319 compared directly with the results obtained for the K88R+I572F) point to an exacerbated pro-
320 inflammatory response from the MΦs when in presence of K88R+H526Y. Indeed, NLRP3 is
321 activated in response to a variety of pathogen-associated and danger-associated molecular
322 patterns and the active NLRP3 inflammasome leads to the secretion of potent pro-
323 inflammatory cytokines. *E. coli* has previously been shown to induce NLRP3 activation in
324 MΦs (43,44) and enterohemorragic *E. coli* (EHEC) are able to target NLRP3 inflamosome
325 activation and block IL-1β cytokine production (45). It would be interesting to study the
326 fitness effects of these resistances in this pathogenic strain. IFIT1 is induced upon treatment
327 with interferon (in particular, by IFN-α/β) and is better characterized in the context of a viral
328 infection (46). IFN-β is also involved in the regulation of NLRP3 inflammasome (47,48). The
329 observed upregulation of IL-1α, a protein involved in various immune responses and
330 inflammatory processes, is also in agreement with a pro-inflammatory response from the
331 MΦs. These cytokines are produced by MΦs in response to cell injury and are involved in the
332 inflammatory response with many interactions with other cytokines, ultimately inducing
333 apoptosis (49). On the other hand, we also see a significant upregulation of *il-10* (0.55 log2
334 fold change) in the presence of this double mutant. The protein encoded by *il-10* is a cytokine
335 produced primarily by monocytes with pleiotropic effects involved in limiting the

336 inflammatory response (50). Together, our results suggest that K88R+H526Y mutant may be
337 able to modify the inflammatory response by the MΦs when compared to the susceptible
338 strain, in the specific experimental conditions that we tested. In a real infection both bacterial
339 numbers and macrophage number are likely to be variable, so this effect may be dependent on
340 the context. It is noteworthy to compare our results with those from a previous study by
341 Mavromatis and colleagues (51) where a co-transcriptomics analysis was performed in MΦs
342 infected with two phenotypically different uropathogenic *E. coli* strains, one able to survive
343 and another unable to survive within MΦs. Mavromatis and colleagues did not detect
344 significant host gene expression differences following infection with the different bacteria
345 strains at 2 and 4 hours post infection. Only one gene (*Slc7a11*) coding for a
346 cysteine/glutamate exchanger was found to be upregulated at 24 h post-infection for the strain
347 that was able to survive inside the MΦs (51). In our bacterial strains, which only differ in the
348 mutations conferring resistance to antibiotics, several MΦs genes were found to be differently
349 upregulated, especially in the double mutant that displayed sign epistasis.

350 Our results also suggest that the increased survival inside the MΦs conferred by the double
351 resistance is associated to a substantial loss of competitive fitness in RPMI. The results
352 displayed in **Table I** also show that $Rif^R Str^R$ double resistance incurs a strong cost in the
353 initial growth rate (ε_{r1}) but this cost is reduced along growth. This is in agreement with the
354 notion that the $Rif^R Str^R$ mutants have a decreased ability in competing for the resources
355 present in RPMI and is consistent with the observed decreased competitive fitness (**Figure 4**).
356 Slower growth rates and increased survival suggest that antibiotic resistance mutations might
357 be tilting the so-called SPANC balance (standing for self-preservation and nutritional
358 competence) to an increased general stress response and starvation survival at the expense of
359 a decreased nutritional ability (30,31). Mutations in the *rpsL* gene, conferring Str^R, have been
360 described to improve the accuracy of ribosomes but also to slow down the translation process

(52,53) and slower ribosomes could explain the observed slower growth rates in RPMI. Concurrently, while fast ribosomes are required in actively dividing cells, hyper-accurate ribosomes are advantageous in non-dividing cells, because they lower the fraction of misfolded proteins which are known to be more prone to protein oxidation during growth arrest (54). This should be extremely relevant upon entry to the MΦs, where *E. coli* undergoes growth arrest and nutrient starvation. Importantly, the trade-off between survival and competitive fitness seems to be strong enough to prevent the dissemination of multi-antibiotic resistance. However, while the *E. coli* K-12 strain used for this study is not able to replicate in the phagolysosome, many intracellular pathogens can replicate inside the macrophages (55). For pathogens which are mainly intracellular, it remains an open question how strong the described trade-off will be.

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534 **TABLES**

535

536 **Table I – Relative growth rates (ε_r) normalized to the susceptible strain.**

ε_{r1}	**S512F**	**H526Y**	**S531F**	**I572F**
K43N	0.240 ± 0.026	0.201 ± 0.008	0.114 ± 0.010	0.528 ± 0.221
K43T	0.187 ± 0.007	0.191 ± 0.002	0.241 ± 0.047	0.202 ± 0.005
K43R	0.203 ± 0.007	0.194 ± 0.003	0.203 ± 0.006	0.227 ± 0.005
K88R	0.194 ± 0.003	0.204 ± 0.021	0.157 ± 0.009	0.202 ± 0.009

537

ε_{r2}	**S512F**	**H526Y**	**S531F**	**I572F**
K43N	0.772 ± 0.441	0.770 ± 0.053	0.666 ± 0.090	0.432 ± 0.027
K43T	0.420 ± 0.052	1.046 ± 0.232	1.008 ± 0.626	0.355 ± 0.020
K43R	0.843 ± 0.138	0.803 ± 0.133	0.758 ± 0.139	0.589 ± 0.039
K88R	0.643 ± 0.054	0.836 ± 0.146	0.579 ± 0.101	0.634 ± 0.118

538
539

540

541 **FIGURE LEGENDS**

542 **Fig. 1 – The experimental setup.** Bacteria and macrophages were acclimatized
543 independently for a total of 48h. Macrophages were activated with CpG for 24h during the
544 period of acclimatization. After the period of acclimatization, $1x10^6$ macrophages were
545 infected with $5x10^6$ bacteria (in a ratio of 1:1, resistant versus susceptible strain) labelled
546 either with YFP or CFP. After 1 h of infection, the MΦs were washed from the extracellular
547 bacteria, and fresh RPMI cell culture medium containing 100 µg of gentamicin/ml was added
548 to kill the remaining extracellular bacteria. To determine the number of intracellular bacteria
549 after 2 and 24 h of incubation, infected MΦs were washed with phosphate-buffered saline
550 (PBS) with 0.1% Triton-X in order to lyse the MΦs. The overall number of bacteria was
551 counted by plating on LB agar plates. Survival inside the MΦs was estimated as the change in
552 relative frequency (ΔX), calculated as described in the Methods section.

553

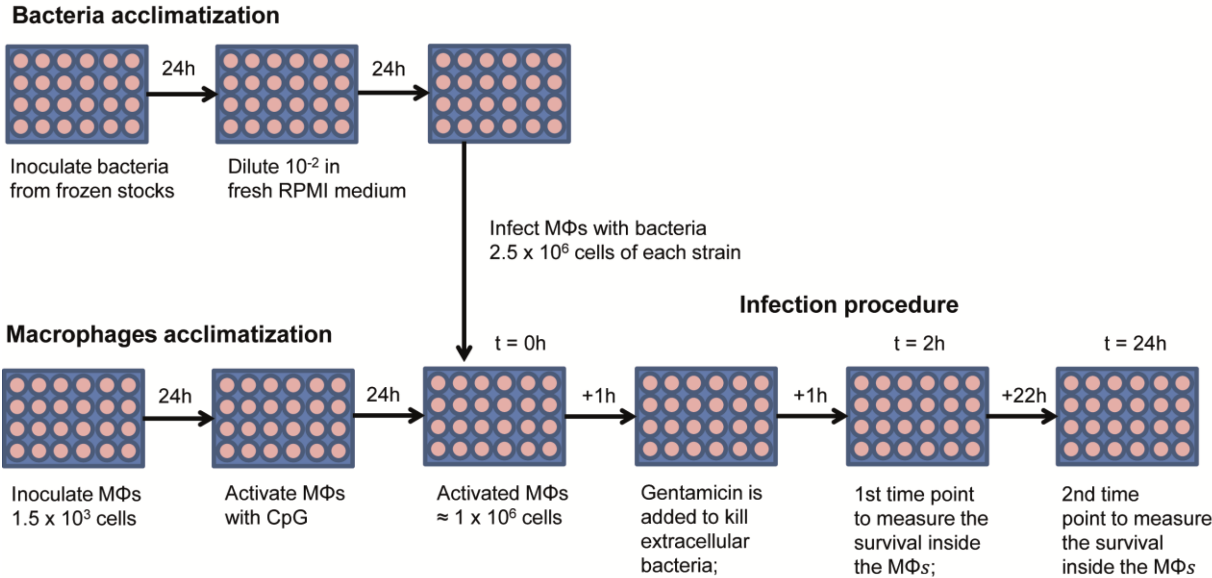
554 **Fig. 2 – Rif^RStr^R double mutants have an increased survival inside the MΦs.** The panel
555 shows the fitness effects of double antibiotic resistance on survival inside MΦs at 2h (black
556 bars) and 24 h (grey bars) post-infection. All fitness effects were estimated using competition
557 assays against a susceptible strain. At least 5 biological replicates were made for each
558 measurement. All mutants showed statistical significance increases in frequency ($p < 0.05$,
559 Wilcoxon signed rank test) when compared to the susceptible strain except for K43R+S512F
560 (both 2 and 24h post-infection), K88R+S512F (at 24h post-infection), K43N+H526Y (at 2h
561 post-infection). Results show that most Rif^RStr^R double mutants have an increased survival
562 inside the MΦs. The opposite scenario occurs for two combinations which display sign
563 epistasis – K43R+H526Y and K88R+H526Y.

564

565 **Fig. 3 – Double resistance with sign epistasis is associated with an enhanced pro-**
566 **inflammatory response. A)** Relative amount of murine transcripts of macrophages infected

567 with *E. coli* MG1655 susceptible to antibiotics relative to transcript levels of uninfected
568 macrophages (mock). The significant higher transcript levels of all the tested genes after
569 infection evidence their role in this context ($p < 0.01$, one sample t-test). **B)** Overall analysis
570 by RT-qPCR of macrophages transcripts infected with different *E. coli* antibiotic resistant
571 mutants. The coloured boxes show the survival effect (ΔX) of the mutants at 24h post-
572 infection. Data was normalized against a susceptible strain and is shown in log 2 fold change.
573 At least 3 biological replicas were made for each measurement.
574
575 **Fig. 4 – Trade-off between survival and competitive fitness outside the MΦs.** Competitive
576 fitness of $Rif^R Str^R$ double mutants were measured in RPMI medium both in the absence
577 (black bars) and in the presence (grey bars) of MΦs. All fitness effects were estimated after
578 24h using competition assays against a susceptible strain. At least 3 biological replicas were
579 made for each measurement. All mutants showed statistical significance decrease in
580 frequency ($p < 0.05$, Wilcoxon signed rank test) when compared to the susceptible strain
581 except for K43R+S512F (in the presence of MΦs), K43R+H526Y (both in the presence and
582 absence of MΦs) and K43R+S531F (in the presence of MΦs).
583

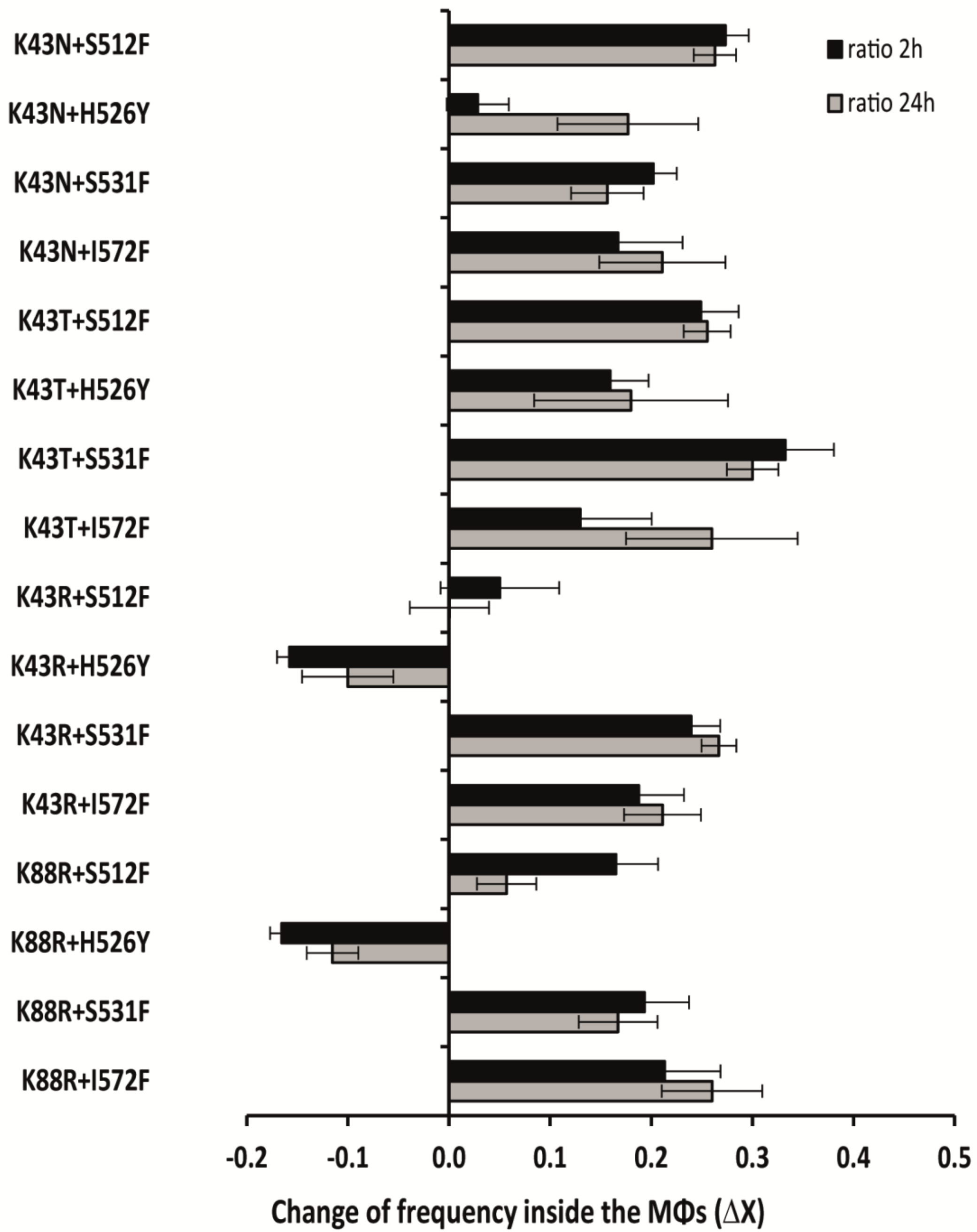
Figure 1



584

585

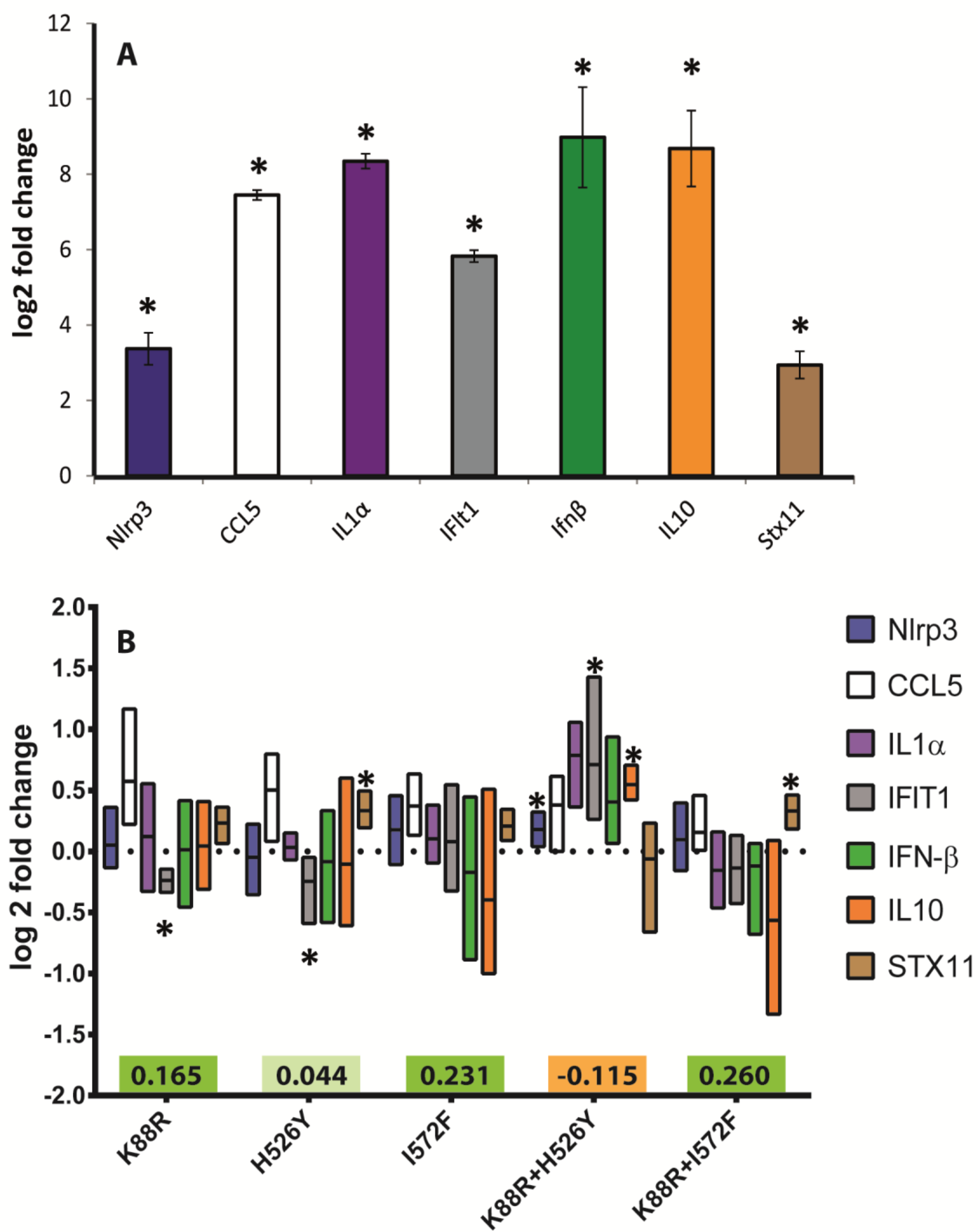
Figure 2



586

587

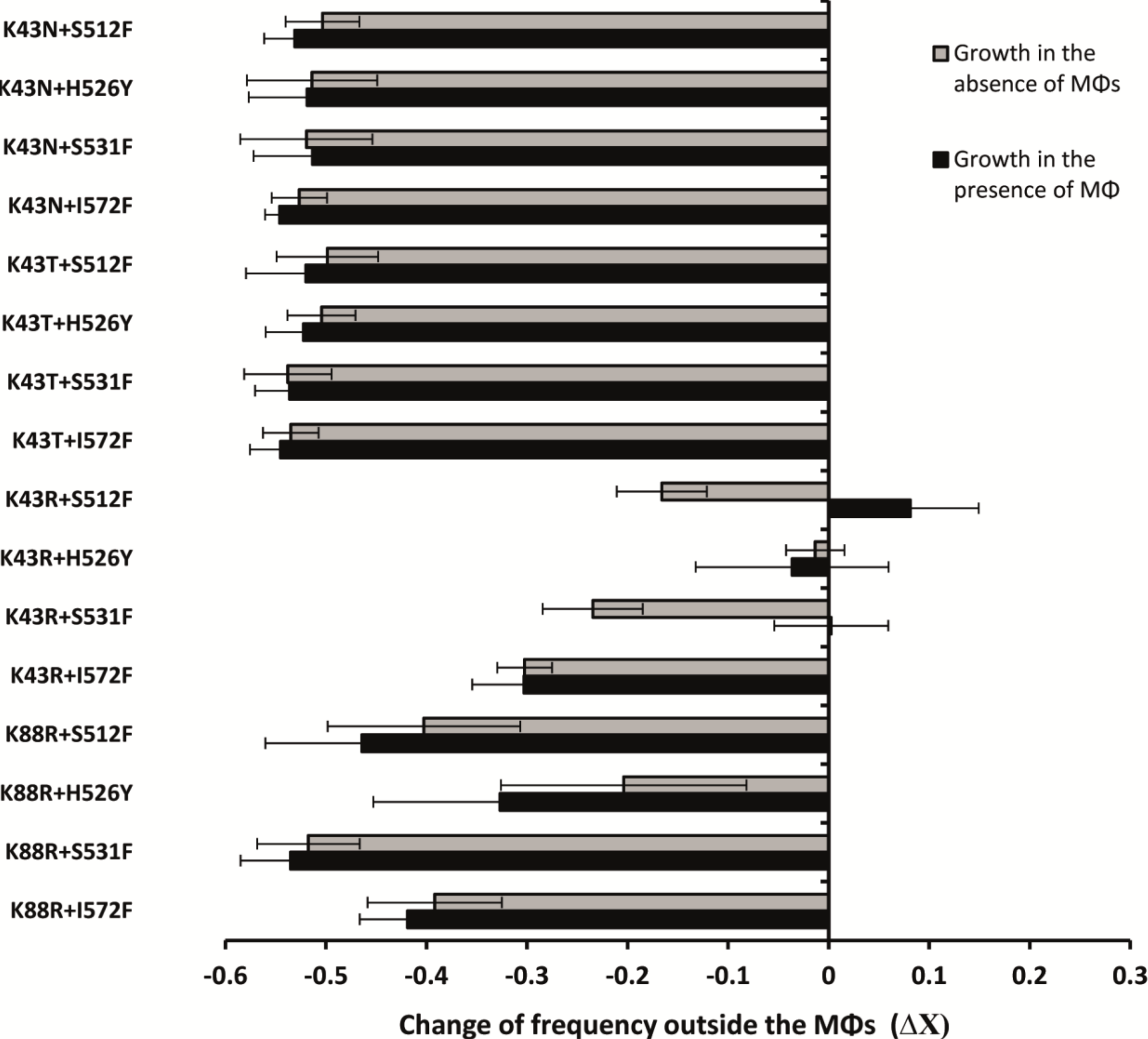
Figure 3



588

589

Figure 4



590