Mycobacteria inhibit neutrophil recruitment to avoid neutrophil-assisted killing by macrophages

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Abstract

Neutrophils migrate rapidly to sites of bacterial infection where they mount critical first-line defenses. Puzzlingly, neutrophils are scant at initial sites of tuberculosis infection. Here, in the zebrafish model of tuberculosis, we find that mycobacteria actively inhibit neutrophil recruitment. Upon being phagocytosed by first-responding tissue resident macrophages, mycobacteria use their ESX-1 virulence locus to induce these cells to produce lipoxins, potent inhibitors of neutrophil migration. Inhibition of neutrophils is integral to the mycobacterial strategy to establish infection. While neutrophils are unable to kill the infecting mycobacteria directly, they induce the infected macrophages to do so. Neutrophil secreted myeloperoxidase interacts with macrophage mannose receptors to induce tumor necrosis factor that increases macrophage microbicidal activity. Our work unmasks a neutrophil-macrophage interaction that, when enabled, potentiates mycobacterial eradication. We find that zileuton, an orally active inhibitor of lipoxin synthesis, promotes neutrophil recruitment and early mycobacterial clearance, suggesting its therapeutic potential to prevent tuberculosis.

First Sentence Neutrophils empower macrophages to kill mycobacteria, so mycobacteria co-opt macrophages to prevent neutrophil recruitment.

Introduction

The cornerstone role of neutrophils in immunity is well-known. Primary immunodeficiencies that impair neutrophil development or function and cancer chemotherapy-induced neutrophil depletion result in life-threatening infections (*1*). Not surprisingly, neutrophils are rapidly recruited to infection sites in response to multiple chemoattractants that are generated either by bacteria or by host cells as a result of tissue damage (*1*). Therefore, the long-recognized paucity of neutrophils in early lung tuberculosis is intriguing. Exquisitely detailed human autopsy studies from the pre-antibiotic era revealed that neutrophils were rarely found in the earliest phase of lung tuberculosis, with infecting mycobacteria being present only in macrophages (*2*). Neutrophils were mostly seen in advanced necrotic lesions (*2*).

We study tuberculosis in zebrafish infected with its natural pathogen *Mycobacterium marinum* (Mm), a close genetic relative of *M. tuberculosis* (Mtb) (*3, 4*). The transparent and genetically and pharmacologically tractable zebrafish larva has been especially useful to examine and understand the first mycobacterium-phagocyte interactions at the initial infection site that play a determinative role in either clearing or promoting infection (*5, 6*). Bacteria are infected into the larval hindbrain ventricle (HBV), an epithelium-lined cavity (*3, 5-7*) (Fig. 1A). Zebrafish respond to Gram positive and Gram negative bacteria with recruitment of tissue-resident macrophages (from the adjacent brain) rapidly followed by neutrophils and monocytes (*6*). Mycobacteria elicit resident macrophages followed by monocytes but not neutrophils (*6, 7*). Later, neutrophils are recruited to the tuberculous granuloma in response to signals from dying macrophages (*7*). These findings mirror the observational studies of human tuberculous lesions (*2*) and experimental findings in the mouse, where upon aerosol Mtb infection, neutrophils fail to arrive in the lung until eleven days (*8*); rather late-arriving neutrophils are often associated with pathology (*9*).

76 Here, we sought to understand why neutrophils fail to migrate to the initial site of
77 mycobacterial infection. Our work uncovered a mycobacterium-inhibited neutrophil-
78 macrophage interaction that increases macrophage microbicidal activity and early
79 mycobacterial clearance. This understanding allowed us to identify an approved drug that
80 counters mycobacterial evasion of neutrophils so as to increase clearance.
81
82 **Results**
83 **Mycobacteria inhibit neutrophil recruitment through ESX-1 mediated lipoxin**
84 **production in resident macrophages**
85 Mycobacteria, like other bacteria, produce formyl peptides that are potent neutrophil
86 chemoattractants, and these are found in the supernatants of axenically-grown bacteria (*10,*
87 *11*). We had shown previously that injection of a synthetic N-formylated tripeptide into the
88 zebrafish HBV elicits neutrophils (*7*). We now found that Mm culture supernatants also
89 induced neutrophils, in contrast to live Mm and to culture medium alone (Fig. 1B). These
90 findings suggested that mycobacteria actively inhibit neutrophil recruitment. Mycobacterial
91 infection of cultured human and mouse macrophages induces them to produce lipoxins, lipid
92 mediators that inhibit neutrophils (*12-14*). To test if mycobacterially-induced lipoxins were
93 responsible for the inhibition of neutrophils, we used zebrafish mutant in 5-lipoxygenase
94 (*ALOX5*), the enzyme responsible for lipoxin synthesis (*14*). The zebrafish has four paralogs
95 of human *ALOX5*, with zebrafish *alox5a* being closest to human *ALOX5* (*15*). We therefore
96 used a zebrafish *alox5a* loss of function mutant (*alox5*$^{-/-}$), and first confirmed that mutant
97 larval viability and neutrophil numbers were unaffected (fig. S1, A and B). Upon Mm
98 infection into the HBV, *alox5*$^{-/-}$ mutants recruited neutrophils, in contrast to their wildtype
99 (*alox5*$^{+/+}$) siblings (Fig. 1, A, C and D). Their heterozygote (*alox5*$^{+/-}$) siblings also recruited
100 neutrophils in similar numbers, suggesting a threshold level of lipoxins was required for

101 neutrophil inhibition (Fig. 1D). Consistent with this, while exogenous lipoxins inhibited
102 neutrophil recruitment to Mm in a dose-dependent fashion in both heterozygotes and mutants,
103 the inhibitory concentration was roughly twice higher in mutants (Fig. 1E).

104 We confirmed that macrophages were the source of the lipoxins by showing that
105 zebrafish larvae lacking macrophages (by treatment with an antisense morpholino to the PU.1
106 myeloid transcription factor (*16*)) recruited neutrophils to Mm, whether or not they could
107 synthesize lipoxins; as before, exogenous lipoxins inhibited neutrophils (Fig. 1, F and G). If
108 macrophages are the main source of lipoxins in wildtype animals, their arrival must precede
109 neutrophils in *alox5*$^{-/-}$ mutants. Resident macrophages preceded the contemporaneous arrival
110 of peripheral monocytes and neutrophils, suggesting that they produced the lipoxins (Fig. 1H)
111 (*6*). We confirmed that they were the source of the lipoxins by showing that in Mm infection
112 of *csf1r* mutant zebrafish, which have 75% fewer brain-resident macrophages (*6, 17*),
113 neutrophil recruitment was no longer inhibited (Fig. 1I). We had previously used *csf1r*
114 mutants to show that Mm infection of resident macrophages induces monocyte recruitment
115 (*6*), a finding that was confirmed in this experiment (Fig. 1I). Thus, Mm infection of resident
116 macrophages inhibits neutrophil recruitment while promoting that of monocytes.

117 How might mycobacteria induce lipoxin production in resident macrophages? We
118 found that live bacteria were required to do this (Fig. 1J). Prior work has shown that only the
119 virulent Mtb strain H37Rv induces lipoxins in human macrophages, and not its avirulent
120 counterpart H37Ra (*13*). H37Ra, which is genetically undefined, has been found to be
121 deficient for secretion of components of the ESX-1 virulence secretion system (*18*), so we
122 asked if ESX-1 inhibits neutrophil recruitment. An ESX-1 Mm mutant induced neutrophil
123 recruitment in wildtype animals, albeit less than either wildtype or ESX-1 deficient Mm in
124 *alox5*$^{-/-}$ mutants (Fig. 1K). In sum, these findings suggest that mycobacterial ESX-1 and yet

125 unknown heat-labile factors and/or factors produced only by growing bacteria induce lipoxins
126 in resident macrophages to inhibit neutrophils.
127
128 **Neutrophils are detrimental to mycobacteria by increasing microbicidal activity of**
129 **infected resident macrophages**
130 Do mycobacteria derive benefit from their neutrophil evasion strategy? If so, then
131 *alox5*$^{-/-}$ mutants should be resistant to infection. Indeed, *alox5*$^{-/-}$ mutants had reduced
132 infection after three days (Fig. 2A and fig. S2). To determine if this increased resistance was
133 due to the recruited neutrophils, we used transgenic zebrafish expressing a truncated CXCR4
134 mimicking a mutation causing a severe human immunodeficiency called WHIM syndrome
135 (for Warts, Hypogamaglobulinemia, Infections, and Myelokathexis), in which neutrophils are
136 retained in hematopoietic tissues, and fail to migrate to wound sites and to tuberculous
137 granulomas (*7, 19*). We confirmed that neutrophils no longer migrated to Mm in *alox5*$^{-/-}$
138 WHIM animals (Fig. 2B). *alox5*$^{-/-}$ WHIM animals were not resistant to Mm, confirming that
139 the increased microbicidal capacity of *alox5*$^{-/-}$ mutants was due to the recruited neutrophils,
140 rather than other alterations caused by the mutation, e.g. reduced lipoxin levels in the resident
141 macrophages (Fig. 2C).
142 In the *alox5*$^{-/-}$ mutants, mycobacteria were phagocytosed by the recruited neutrophils,
143 albeit in smaller numbers than resident macrophages (Fig. 2D and fig. S3), likely due to their
144 later arrival (Fig. 1H). However, when we assessed the ability of the neutrophils to kill their
145 phagocytosed Mm we found that they did not kill the bacteria (Fig. 2, E and F). In contrast,
146 neutrophils arriving to, and phagocytosing, *Salmonella enterica* var. Typhimurium (ST),
147 rapidly killed them, whether in wildtype or *alox5*$^{-/-}$ mutants (Fig. 2, E and F). This finding
148 suggested either that neutrophils can kill Mm more slowly, taking longer than the 90 minute
149 observation period, or that their microbicidal activity is indirect. Because later in infection, in

150 the context of the tuberculous granuloma, we had observed neutrophils to kill their ingested
151 Mm within 90 minutes (*7*), we hypothesized that neutrophils were working indirectly by
152 increasing the microbicidal capacity of macrophages. To test this, we turned to the Pu.1
153 morphants that lack macrophages and that recruit neutrophils to Mm both in wildtype and
154 *alox5*$^{-/-}$ animals (Fig. 1F). *alox5*$^{-/-}$ animals that lacked macrophages were no longer resistant
155 to infection (Fig. 2G). Next we monitored over 60 minutes the killing rate of wildtype and
156 *alox5*$^{-/-}$ macrophages which had engulfed similar numbers of Mm (Fig. 2H), and found that
157 *alox5*$^{-/-}$ macrophages were more microbicidal (Fig. 2I). These findings suggested that
158 neutrophils exert their microbicidal activity in early infection indirectly by increasing
159 macrophage microbicidal capacity. To examine this hypothesis more stringently, we infected
160 animals with 1-3 Mm, similar to the infectious dose of Mtb in humans (*5*). >90% of the
161 animals got an inoculum of a single bacterium, with the remaining getting 2-3. Regardless of
162 whether only macrophages (wildtype animals) or both macrophages and neutrophils (*alox5*$^{-/-}$
163 mutants) were recruited, the bacteria were invariably within a single macrophage (Fig. 2J).
164 The propensity for the few bacteria to infect resident macrophages even in the *alox5*$^{-/-}$
165 mutants is because they arrive sooner than the neutrophils even with these small inocula (Fig.
166 1H). In contrast, in PU.1 morphants where only neutrophils are recruited, all of the bacteria
167 were within neutrophils (Fig. 2J). With an infection of 1-3 bacteria, we can monitor the
168 ability of individual animals to clear the infection over the course of five days (*5, 6*). *alox5*$^{-/-}$
169 animals were twice as likely to clear infection (Fig. 2K). This improved clearance was
170 removed in *alox5*$^{-/-}$ WHIM animals showing that neutrophils were required for the enhanced
171 clearance of Mm by macrophages. Thus, neutrophils are unable to kill Mm directly. Rather
172 they increase the ability of macrophages to do so.
173
174 **Mycobacteria evade neutrophil killing by preventing phagosome oxidase activation**

175 The inability of neutrophils to kill mycobacteria early in infection was consistent with
176 the human autopsy studies in which the rare early lesions that were neutrophil-predominant
177 contained the most bacteria (*2*). Yet, our finding was puzzling in light of our previous finding
178 that neutrophils, which are recruited to tuberculous granulomas by signals from dying
179 infected macrophages, can phagocytose Mm-infected macrophages and kill the ingested
180 bacteria by phagosome oxidase (PHOX)-dependent mechanisms - neutrophils arriving at
181 granulomas of PHOX-deficient larvae are unable to kill their ingested Mm (*7*). Why this
182 difference in ability to kill Mm at the two stages of infection? Because neutrophils must be
183 primed in order to activate PHOX (*20, 21*), we considered the possibility that Mm, in contrast
184 to ST, avoided neutrophil priming during initial infection. Neutrophil Toll like receptor
185 (TLR) stimulation by pathogen associated molecular patterns (PAMPs) constitutes one such
186 neutrophil priming stimulus (*20*), and we had previously found that pathogenic mycobacteria
187 used their surface lipid phthiocerol dimycoceroserate (PDIM) to avoid TLR signaling at the
188 site of initial infection (*5*). This avoidance of TLR signaling, likely in epithelial cells,
189 prevents the recruitment of microbicidal monocytes that would be detrimental to the bacteria
190 (*5*). We wondered if mycobacteria likewise use PDIM to prevent neutrophil TLR signaling,
191 and thereby PHOX activation. To test this hypothesis, we used Mm mutant in the PDIM
192 transporter MmpL7 (*5*), and first confirmed that like wildtype Mm, MmpL7 mutant Mm
193 (*ΔmmpL7*) did not recruit neutrophils in wildtype animals, and recruited neutrophils in
194 similar numbers to wildtype bacteria in *alox5*$^{-/-}$ mutants (Fig. 3A). Thus, PDIM is not required
195 for lipoxin production in resident macrophages. Neutrophil TLR activation by infecting
196 bacteria is thought to first promote their phagocytosis (*20*), and we were able to confirm this
197 for Mm. In *alox5*$^{-/-}$ mutants, *ΔmmpL7* Mm were phagocytosed to a greater extent by the
198 recruited neutrophils, and this increase was removed in *myD88* knock-down animals that
199 lacked TLR signaling (*5*) (Fig. 3B). Importantly, the neutrophils were microbicidal to

200 *ΔmmpL7* Mm, as they were to ST (Fig. 3C). Moreover, this killing, like that of ST, was
201 dependent on PHOX activity - *phox* morphant neutrophils could kill neither PDIM-deficient
202 Mm nor ST (Fig. 3, D and E). Finally, in MyD88 morphants, neutrophils could not kill
203 *ΔmmpL7* Mm or ST (Fig. 3F), confirming that PHOX-dependent neutrophil killing requires
204 TLR-mediated priming.
205
206 **Neutrophil myeloperoxidase increases microbicidal activity of resident macrophages via**
207 **interactions with Mannose Receptor**
208 How might neutrophils increase macrophage microbicidal capacity? It is increasingly
209 appreciated that neutrophils can regulate other immune cells in numerous ways (*1*)
210 Particularly tantalizing were the reports that human neutrophil myeloperoxidase (MPO), an
211 abundant secreted enzyme can rapidly induce macrophage tumor necrosis factor (TNF), a
212 cytokine that increases macrophage microbicidal activity against mycobacteria, including in
213 the zebrafish (*22, 23*). Moreover, in a rat arthritis model, exogenous MPO administered into
214 the joint, exacerbated disease in a TNF-dependent fashion (*24*). MPO, a mannosylated
215 protein has been shown to be internalized by macrophages through mannose receptor (MR)
216 (*25*). Consistent with MPO's exacerbation of arthritis being through its interactions with MR,
217 the MPO-induced exacerbations were prevented by blocking MR activity with mannan (*24,*
218 *26*). Finally, exogenous MPO as well mannosylated BSA were found to increase reactive
219 oxygen speicies in murine peritoneal macrophages that increased the killing of *Escherichia*
220 *coli* and *Candida albicans* (*27, 28*).
221 We asked if exogenously administered MPO could decrease bacterial burdens in
222 wildtype animals where neutrophils are not recruited. It did, and this effect was negated by
223 mannan (Fig. 4A). MPO is rapidly inactivated upon secretion, however inactivated MPO is
224 an even more potent inducer of TNF in cultured macrophages, and like MPO exacerbates

225 arthritis in rats in a MR- and TNF-dependent fashion (*24*). We too found that inactivated
226 MPO caused a similar mannan-inhibitable reduction in bacterial burdens as MPO (Fig. 4A).
227 MPO did not decrease bacterial burdens in PU.1 morphants which lack macrophages,
228 showing it acted specifically on macrophages (Fig. 4B). We showed that MPO increased the
229 ability of macrophages to kill similar burdens of phagocytosed Mm, and this was inhibited by
230 mannan (Fig. 4, C and D).

231 To determine if endogenous MPO concentrations could increase macrophage
232 microbicidal capacity, we created zebrafish mutants that were doubly deficient in 5-
233 lipoxygenase (*alox5*$^{-/-}$) and MPO (*mpx*$^{-/-}$) (*29*). In *mpx*$^{-/-}$ *alox5*$^{-/-}$ animals, neutrophils were
234 recruited to Mm as in MPO-competent, 5-lipoxygenase-deficient animals (Fig. S4); however,
235 their presence was no longer associated with reduced bacterial burdens (Fig. 4E). Similarly,
236 their effect on reducing the infectivity of inocula with 1-3 bacteria was lost (Fig. 4F). We
237 then used a MR zebrafish mutant in *mrc1b*, the functional zebrafish ortholog of *MRC1*, that
238 encodes human MR (*30*). *mrc1b* mutants did not reduce bacterial burdens or infectivity in
239 response to exogenous MPO, confirming the specificity of the MPO-MR interaction (Fig. 4,
240 G and H).

241 Next, to determine if MPO-MR interactions increase TNF, we asked if the presence of
242 neutrophils increased *tnf* RNA expression early after Mm infection. Mm infection increased
243 *tnf* expression in wildtype animals as expected, and there was a further increase in the 5-
244 lipoxygenase mutants (Fig. 4I). However, this increase might be at least in part from the
245 removal of lipoxins which are potent inhibitors of *tnf* transcription, including in the zebrafish
246 (*31, 32*). To determine if any of the increased *tnf* was specifically due to MPO-MMR
247 interactions, we assessed *tnf* levels in 5-lipoxygenase mutants that were also mutant in MPO
248 (*alox5*$^{-/-}$*,mpx*$^{-/-}$) or MMR (*alox5*$^{-/-}$*,mrc1b*$^{-/-}$). These two mutant combinations had only a small
249 part of the increased *tnf* seen in the *alox5*$^{-/-}$ mutants alone, showing that most of the increased

250 *tnf* was attributable to MPO-MMR interactions (Fig. 4I). Consistent with this, exogenous
251 MPO increased *tnf* in wildtype animals (Fig. 4I). To determine if this increased TNF was
252 responsible for the increased microbicidal capacity, we assessed bacterial burdens in MPO-
253 treated fish deficient in TNF receptor 1 (TNFR1) through which TNF mediates its
254 macrophage microbicidal activity, including in the zebrafish (*23*). MPO administration failed
255 to reduce bacterial burdens in TNFR1 morphants (Fig. 4J). Furthermore, TNF morphants had
256 increased bacterial burdens over wildtype as expected and this was unchanged with
257 exogenous MPO (Fig. 4J). Finally, we asked if exogenous TNF could increase macrophage
258 microbicidal activity. We found that TNF was sufficient to reduce bacterial burdens in a
259 dose-dependent fashion. That the lower of the two concentrations did not effect a change in
260 bacterial burdens, provided an explanation for why 5-lipoxygenase mutants showed no
261 reduction in bacterial burdens in the absence of MPO (Fig. 4E) - i.e. the increase in *tnf*
262 expression from the loss of lipoxin-mediated inhibition in these mutants (Fig. 4I) was
263 insufficient to reduce bacterial burdens. Much higher TNF concentrations would be expected
264 to increase bacterial burdens by promoting mycobacterium growth-promoting macrophage
265 necrosis (*33*) and we were able to confirm this (Fig. 4K). In sum, the MPO-MR induced
266 reduction in bacterial burdens is mostly, if not entirely, due to increased TNF.
267

268 **Pharmacological inhibition of 5-lipoxygenase recruits neutrophils and increases**
269 **bacterial clearance in Mm and Mtb**

270 We asked if zileuton, an orally administered selective 5-lipoxygenase inhibitor used to
271 treat asthma would increase mycobacterial clearing, similar to genetic 5-lipoxygenase
272 deficiency (*34, 35*). Zileuton promoted neutrophil recruitment in a dose-dependent manner
273 (Fig. 5A) while having no effect on larval viability or overall neutrophil numbers (fig. S5, A
274 and B). We confirmed that the effects of zileuton were specific, similar to 5-lipoxygenase

genetic mutation: its increase in neutrophil migration was inhibited by exogenous lipoxins in a dose-dependent manner (Fig. 5B) and in PU.1-morphants lacking macrophages (Fig. 5C). Moreover, zileuton reduced bacterial burdens and infectivity (Fig. 5, D and E), and both effects were negated in MPO-deficient animals (Fig. 5, F and G), showing they were dependent on MPO-MMR interactions as expected.

These findings, suggesting the utility of zileuton as a drug for the prevention of TB, provided the impetus to determine if the mechanisms we had uncovered for Mm were relevant for Mtb. Virulent Mtb is a biosafety level 3 pathogen that we cannot use in our biosafety level 2 zebrafish facilities. So we used an isogenic leucine and pantothenate auxotroph of the commonly studied Mtb H37Rv strain, a biosafety level 2 pathogen (*36*). H37Rv Mtb also failed to recruit neutrophils in wildtype but not 5-lipoxygenase mutant animals (Fig. 5H). To ask if Mtb-induced neutrophil recruitment was partially ESX-1 dependent, we tested the Mtb H37Ra strain that is deficient for ESX-1 secretion and induces less lipoxin production than virulent Mtb (*13, 18*). Like the Mm ESX-1 mutant, it showed a partial reduction in neutrophil recruitment - 50% reduction versus 64% reduction for the Mm ESX-1 mutant (Fig. 5H, compare to Fig. 1K). Corresponding to neutrophil recruitment, Mtb H37Rv burdens and its infectivity were reduced in 5-lipoxygenase mutants (Fig. 5, I and J). Finally, both were also reduced by zileuton administration (Fig. 5, K and L).

Conclusions

This work provides mechanistic understanding of seminal observations of lung tuberculous lesions from the pre-antibiotic era - that neutrophils were rarely found in early lesions and on the rare occasions that they were, they failed to kill the bacteria (*2*). Mycobacteria, through their ESX-1 virulence locus, reprogram resident macrophages to produce lipoxins that inhibit neutrophils (Fig. 6). If there is a paucity of resident

300 macrophages at the site of initial infection, they are unable to avoid neutrophil recruitment, so
301 they have a fail-safe mechanism to avoid getting killed by these most microbicidal cells.
302 They use their PDIM cell surface lipid to inhibit neutrophil priming so as to diminish their
303 phagocytic capacity and impair their strongest microbicidal mechanism, PHOX (Fig. 6).

304 The need for this dual strategy is best understood when contextualized to the human
305 lung where TB initiates. Mtb enters the distal alveoli in small numbers in fine aerosol
306 particles. It will likely first encounter the alveolar macrophage, the lung resident-macrophage
307 (*37*), which like other tissue resident macrophages, express high levels of MR (*38*). However,
308 in the human lung, the number of alveolar macrophages is limiting so that not every alveolus
309 has one (*39, 40*). If the bacterium lands in an alveolus lacking a macrophage, a neutrophil
310 might reach it first, responding to its formyl peptides, necessitating the PDIM-mediated
311 inhibition of priming as a complementary bacterial survival strategy. Neutrophils, being
312 short-lived cells, would die, affording the released bacteria another opportunity to be
313 engulfed by, and co-opt, a late-arriving alveolar macrophage from a neighboring alveolus
314 (*41, 42*).

315 We previously showed that mycobacteria induce in zebrafish brain-resident
316 macrophages and primary human alveolar macrophages the monocyte chemokine CCL2 that
317 recruits permissive CCR2-positive monocytes for the bacteria to infect (*6*). This is
318 accomplished through a mycobacterial cell surface phenolic glycolipid that is related to
319 PDIM but with distinct functions (*6*). This work shows yet another way in which
320 mycobacteria manipulate the gene expression programs of tissue-resident macrophages.
321 Given that the alveolar macrophage is equipped to kill bacteria (*37, 43*), it is unsurprising that
322 mycobacteria have evolved to multiply reprogram these cells so as to reduce their
323 microbicidal potential while they move into recruited monocytes and macrophages (Fig. 7).

MPO remains an enigmatic enzyme. It is the most abundant protein in neutrophil granules and working in conjunction with PHOX to convert hydrogen peroxide into hypochlorous acid, which is highly microbicidal in vitro (*44*). Yet genetic MPO deficiency, in sharp contrast to genetic PHOX deficiency, produces at best a modest susceptibility to infection; rather it is increasingly thought to play a deleterious pro-inflammatory role in inflammatory diseases through binding to MR (*22, 24, 44-46*). Our work shows why its deficiency is irrelevant in tuberculosis. Mycobacteria have simply evolved to avoid neutrophil recruitment. We can now negate this mycobacterial neutrophil evasion mechanism to enhance early clearance by pharmacological blockade of 5-lipoxygenase. Zileuton, an orally available 5-lipoxygenase inhibitor was licensed to treat asthma 25 years ago. In a mouse model of TB, genetic 5 lipoxygenase blockade as well as zileuton were shown to reduce acute mortality by manipulating the balance of different eicosanoids so as to ameliorate disease exacerbation associated with the expression of Type 1 interferons, suggesting the use of zileuton as an adjunctive TB treatment together with antibiotics (*12, 47*). Now, by uncovering the mechanism by which mycobacteria avoid neutrophil recruitment that is deleterious to them, we identify a potential role for zileuton as a TB preventive treatment. Its utility may be greatest as a way to prevent infection in areas with a high burden of extensively drug resistant tuberculosis which requires long treatment with toxic drugs and carries a very high mortality (*48*) nonetheless .

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490

491

492 **Figure Legends**

493 **Fig. 1. Mycobacteria inhibit neutrophil recruitment through ESX-1-induced lipoxin**
494 **production in resident macrophages** (**A**) Cartoon showing HBV infection site, and timing
495 of morpholino and bacterial injections, and of assays used throughout study. dpf, days post-
496 fertilization; MO, morpholino; hpi, hours post-infection. (**B**) Number of neutrophils recruited
497 into HBV of WT larvae 3 hours after injection of 80 wildtype (WT) Mm, an equivalent
498 volume of WT Mm culture supernatant or culture medium alone (mock). n= 20 per group;
499 red bars indicate medians. (**C**) Confocal images of head (lateral view) of larva at 3 hpi.
500 Yellow dashed lines demarcate HBV. N, green-fluorescent neutrophils. Scale bar, 100 µm.
501 (**D**) Neutrophils recruited in WT, *alox5*$^{+/-}$ or *alox5*$^{-/-}$ larvae 3 hours after injection of culture
502 medium or infection with 80 WT Mm. n= 24, 39, 41, 103, 23, 42, respectively; red bars,
503 medians. (**E**) Number of neutrophils recruited 3 hpi of 80 WT Mm in WT, *alox5*$^{+/-}$ or *alox5*$^{-/-}$
504 larvae, injected with indicated LXA_4 concentrations. n= 11, 10, 13, 12, 22, 24, 24, 25, 14, 12,
505 10, 11, respectively. Red bars, medians (**F**) Number of neutrophils recruited in WT or Pu.1
506 morphant siblings, on a WT or *alox5*$^{-/-}$ background, 3 hpi of 80 WT Mm. n= 22, 21, 23, 20,
507 respectively. Red bars, medians (**G**) Neutrophils recruited in WT or Pu.1 morphant siblings,
508 +/- injection of $3.6x10^{-14}$ mol/fish LXA_4, 3 hpi with 80 WT Mm. n= 25 per group. Red bars,
509 medians. (**H**) Median number of neutrophils (N), resident macrophages (RM), and peripheral
510 monocytes (PM) recruited in WT or *alox5*$^{-/-}$ larvae from 5 to 180 minutes after infection with
511 80 WT Mm. (**I**) Number of resident macrophages (RM), peripheral monocytes (PM), and
512 neutrophils (N) recruited in WT or *csf1r*$^{-/-}$ larvae 1 hpi with 80 WT Mm. n= 14, 15, 14, 15,
513 14, 15, respectively. Red bars, medians. (**J**) Number of neutrophils recruited in WT or *alox5*$^{-/-}$
514 larvae 3 hours post-injection of 80 live or heat-killed (HK) WT Mm. n= 23, 20, 22, 23,
515 respectively. Red bars, medians. (**K**) Number of neutrophils recruited in WT or *alox5*$^{-/-}$ larvae
516 3 hpi with 80 WT or ESX-1-deficient (ΔESX-1) Mm. n= 18, 20, 18, 18, respectively. Red

517 bars, medians. **p<0.01; ***p<0.001 [one-way ANOVA with Tukey's post-test in (B), (D)
518 to (G) and (I) to (K)]. Each panel representative of at least 3 independent experiments.

519

520 **Fig. 2. 5-lipoxygenase deficiency restores neutrophil recrutiment and reduces bacterial**
521 **burdens by increasing macrophage microbicidal capacity.** (**A**) Bacterial burden in WT or
522 *alox5*$^{-/-}$ larvae 3 dpi with 80 WT Mm. n= 15, 13, respectively. Red bars, means**.** (**B**) Number
523 of neutrophils recruited in WT or *alox5*$^{-/-}$ larvae, on WT (Control) or WHIM background, 3
524 hpi with 80 WT Mm. n= 14, 14, 13, 12, respectively. Red bars, medians. (**C**) Bacterial
525 volume in WT or *alox5*$^{-/-}$ larvae, on WT (Control) or WHIM background, 3 dpi with 80 WT
526 Mm. n= 14, 14, 13, 12, respectively. Red bars, means. (**D**) Percentage of recruited phagocytes
527 that became infected in WT or *alox5*$^{-/-}$ larvae, 3 hpi with 80 WT Mm. M, macrophages; N,
528 neutrophils. Red bars, means. N = 14, 13, 15, 12, respectively. (**E**) Representative frames of
529 time-lapse laser confocal images of individual *alox5*$^{-/-}$ green-fluorescent neutrophils (N)
530 starting 3 hpi with 80 WT Mm (upper panel) or 80 WT ST (lower panel). Elapsed minutes in
531 upper right corner. Scale bars, 5 µm. (**F**) Mean (±SEM) bacterial volume over time in
532 individual neutrophils in WT or *alox5*$^{-/-}$ larvae starting 3 hpi with 80 WT Mm or WT ST.
533 Infected neutrophils were sampled from 5 different infected larvae for each group. (**G**)
534 Bacterial volume in WT or Pu.1 morphant siblings, on WT or *alox5*$^{-/-}$ background, 3 dpi with
535 80 WT Mm. n = 17, 16, 12, 12, respectively. Red bars, means. (**H**) Bacterial volume in
536 individual macrophages in WT or *alox5*$^{-/-}$ larvae 3 hpi with 80 WT Mm. Red bars, means.
537 Infected macrophages (n) were sampled from five different infected fish for each group. n=
538 15 per group. (**I**) Killing rate of WT Mm by individual macrophages from (**H**) (decrease in
539 bacterial volume in individual infected macrophages over 60 minutes). Red bars, means. (**J**)
540 Percentage of WT or *alox5*$^{-/-}$ larvae, on WT (Control), WHIM, or Pu.1-deficient (Pu.1-MO)
541 background, in which 3hpi the 1-3 infecting Mm are in a macrophage (black) or a neutrophil

542 (grey) (**K**) Percentage of infected (black) or uninfected (grey) WT or *alox5*$^{-/-}$ fish, on WT
543 (Control) or WHIM background, 5 dpi with 1-3 WT Mm. *p<0.05; **p<0.01; ***p<0.001
544 Student's two-tailed t-test in (A) and (I); one-way ANOVA with Tukey's post-test in (B), (C)
545 and (G); Fisher's exact test in (D), (J) and (K)]. Panel (F) representative of 2 independent
546 experiments; all other panels representative of at least 3 independent experiments.

547

548 **Fig. 3. Mycobacteria use PDIM to prevent PHOX-mediated neutrophil killing.** (**A**)
549 Number of neutrophils recruited into WT or *alox5*$^{-/-}$ larvae 3 hpi with 80 WT or PDIM-
550 deficient (*ΔmmpL7*) Mm. n= 25 per group. Red bars, medians. (**B**) Percentage of recruited
551 phagocytes that became infected in *alox5*$^{-/-}$ animals that are MyD88 competent or deficient, 3
552 hpi with 80 WT or *ΔmmpL7* Mm. M, macrophages, N, neutrophils. n= 12 per group. Red
553 bars, means. (**C**) Mean (±SEM) bacterial volume over time in individual neutrophils in *alox5*$^{-}$
554 $^{/-}$ larvae starting at 3 hpi with 80 WT or *ΔmmpL7* Mm, or WT ST. Infected neutrophils (n)
555 were sampled from 5 different infected larvae for each group. (**D**) Mean (±SEM) bacterial
556 volume over time in individual neutrophils in WT or Phox morphant siblings, on *alox5*$^{-/-}$
557 background, starting 3 hpi with 80 WT or *ΔmmpL7* Mm. Infected neutrophils (n) were
558 sampled from 5 different infected larvae for each group. (**E**) Mean (±SEM) bacterial volume
559 over time in individual neutrophils in WT or Phox morphant siblings, on WT or *alox5*$^{-/-}$
560 background, from 3 hpi with 80 WT ST. Infected neutrophils (n) were sampled from 5
561 different infected larvae for each group. (**F**) Mean (±SEM) bacterial volume over time in
562 individual neutrophils in WT or MyD88 morphant siblings, on *alox5*$^{-/-}$ background, starting 3
563 hpi with 80 *ΔmmpL7* Mm or WT ST. Infected neutrophils (n) were sampled from 5 different
564 infected larvae for each group. ***p<0.001 [one-way ANOVA with Tukey's post-test in (A)
565 and (B)]. Panels (A), (B) and (F) representative of 2 independent experiments; all other
566 panels representative of at least 3 independent experiments.

567

568 **Fig. 4. MPO interacts with macrophage MR to increase macrophage microbicidal**
569 **activity.** (**A**) Bacterial volume in WT injected or not with active (MPO) or inactive (iMPO)
570 myeloperoxidase with or without mannan co-injection, 3 dpi with 80 WT Mm. n= 13, 11, 12,
571 12, 12, 12, respectively. Red bars, means. (**B**) Bacterial volume in WT or Pu.1 morphant
572 siblings, injected or not with MPO, 3 dpi with 80 WT Mm. n= 10, 12, 11, 10, respectively.
573 Red bars, means. (**C**) Bacterial volume in individual macrophages from WT injected with
574 MPO and/or mannan or left uninjected, 3 hpi with 80 wild-type Mm. Infected macrophages
575 (n) were sampled from 5 different infected fish for each group. n= 12, 11, 12, respectively.
576 Red bars, means. (**D**) Killing rate of WT Mm by individual macrophages from (**C**) (decrease
577 in bacterial volume in individual infected macrophages over 60 minutes). Red bars, means.
578 (**E**) Bacterial volume in WT or *alox5*$^{-/-}$ larvae, on WT or *mpx*$^{-/-}$ background, 3 dpi with 80
579 WT Mm. n= 12, 14, 11, 13, respectively. Red bars, means. (**F**) Percentage of infected (black)
580 or uninfected (grey) WT or *alox5*$^{-/-}$ larvae, on WT or *mpx*$^{-/-}$ background, 5 dpi with 1-3 WT
581 Mm. (**G**) Bacterial volume in WT or *mrc1b*$^{-/-}$ larvae, injected or not with MPO, 3 dpi with 80
582 WT Mm. n= 16 per group. Red bars, means. (**H**) Percentage of infected (black) or uninfected
583 (grey) WT or *mrc1b*$^{-/-}$ larvae, injected or not with MPO, 5 dpi with 1-3 WT Mm. (**I**) *tnfa*
584 RNA levels (mean ± SEM of two replicates) in WT larvae 24 hours after injection of culture
585 medium (Mock), and in WT, *alox5*$^{-/-}$, *alox5*$^{-/-}$*;mpx*$^{-/-}$, *alox5*$^{-/-}$*;mrc1b*$^{-/-}$ or MPO-injected larvae
586 24 hpi with 80 WT Mm. n= 25 per group. (**J**) Bacterial volume in WT or *tnfr1* morphant
587 siblings, injected or not with MPO, 3 dpi with 80 WT Mm. n= 10, 12, 10, 11, respectively.
588 Red bars,means. (**K**) Bacterial volume in WT larvae, injected at 3 hpi with the indicated
589 concentrations of recombinant zebrafish TNF, 3 dpi with 80 WT Mm. n= 15, 14, 15, 14,
590 respectively. Red bars,means. *$p<0.05$; **$p<0.01$; ***$p<0.001$ [one-way ANOVA with
591 Tukey's post-test in (A), (B), (D), (E), (G), and (I) to (K); Fisher's exact test in (F) and (H)].

592 Panels (A), (I) and (K) representative of 2 independent experiments; all other panels
593 representative of at least 3 independent experiments.

594

595 **Fig. 5. Zileuton increases neutrophil recruitment to Mm and Mtb and increases their**
596 **killing.** (**A**) Number of neutrophils recruited in WT larvae, treated with the indicated
597 concentrations of zileuton, 3 hours after injection of culture medium (Mock) or infection with
598 80 WT Mm. n= 23, 24, 23, 22, 29, 24, 25, respectively. Red bars, medians. (**B**) Number of
599 neutrophils recruited in WT larvae, treated or not with zileuton and injected with the
600 indicated concentrations of LXA_4, 3 hpi with 80 WT Mm. n= 23, 23, 23, 22, 23, 24,
601 respectively. Red bars, medians. (**C**) Number of neutrophils recruited in WT or Pu.1
602 morphant siblings, treated or not with zileuton, 3 hpi with 80 WT Mm. n= 20 per group. Red
603 bars, medians. (**D**) Bacterial volume in WT larvae, treated or not with zileuton, 3 dpi with 80
604 WT Mm. n= 18, 21, respectively. Red bars, means. (**E**) Percentage of infected (black) or
605 uninfected (grey) WT larvae, treated or not with zileuton, 5 dpi with 1-3 WT Mm. (**F**)
606 Bacterial volume in WT or *mpx*$^{-/-}$ larvae, treated or not with zileuton, 3 dpi with 80 WT Mm.
607 n= 15, 15, 14, 16, respectively. Red bars, means. (**G**) Percentage of infected (black) or
608 uninfected (grey) WT or *mpx*$^{-/-}$ larvae, treated or not with zileuton, 5 dpi with 1-3 WT Mm.
609 (**H**) Number of neutrophils recruited in WT or *alox5*$^{-/-}$ larvae 3 hpi with 80 WT Mm, Mtb
610 H37Rv, or Mtb H37Ra. n= 16, 17, 16, 17, 17, 18, respectively. Red bars, medians. (**I**)
611 Bacterial volume in WT, *alox5*$^{+/-}$ or *alox5*$^{-/-}$ larvae 3 dpf with 80 Mtb H37Rv. n= 12, 25, 14,
612 respectively. Red bars, means. (**J**) Percentage of infected (black) or uninfected (grey) WT,
613 *alox5*$^{+/-}$ or *alox5*$^{-/-}$ larvae 5 dpi with 1-3 Mtb H37Rv. (**K**) Bacterial volume in WT larvae,
614 treated or not with zileuton, 3 dpi with 80 Mtb H37Rv. n= 17, 16, respectively. Red bars,
615 means. (**L**) Percentage of infected (black) or uninfected (grey) WT larvae, treated or not with
616 zileuton, 5 dpi with 1-3 Mtb H37Rv. **$p<0.01$; ***$p<0.001$ [one-way ANOVA with Tukey's

post-test in (A) to (C), (F), (H) and (I); Student's two-tailed test in (D) and (K); Fisher's exact test in (E), (G), (J) and (L)]. Panel (H) representative of 2 independent experiments; all other panels representative of at least 3 independent experiments.

Fig. 6. Schematic of fate of infecting mycobacteria when first phagocytosed by either a resident macrophage or a neutrophil.

Fig. 7. Schematic of two ways in which mycobacteria manipulate gene expression programs of the first-responding resident macrophage.

Figure 1

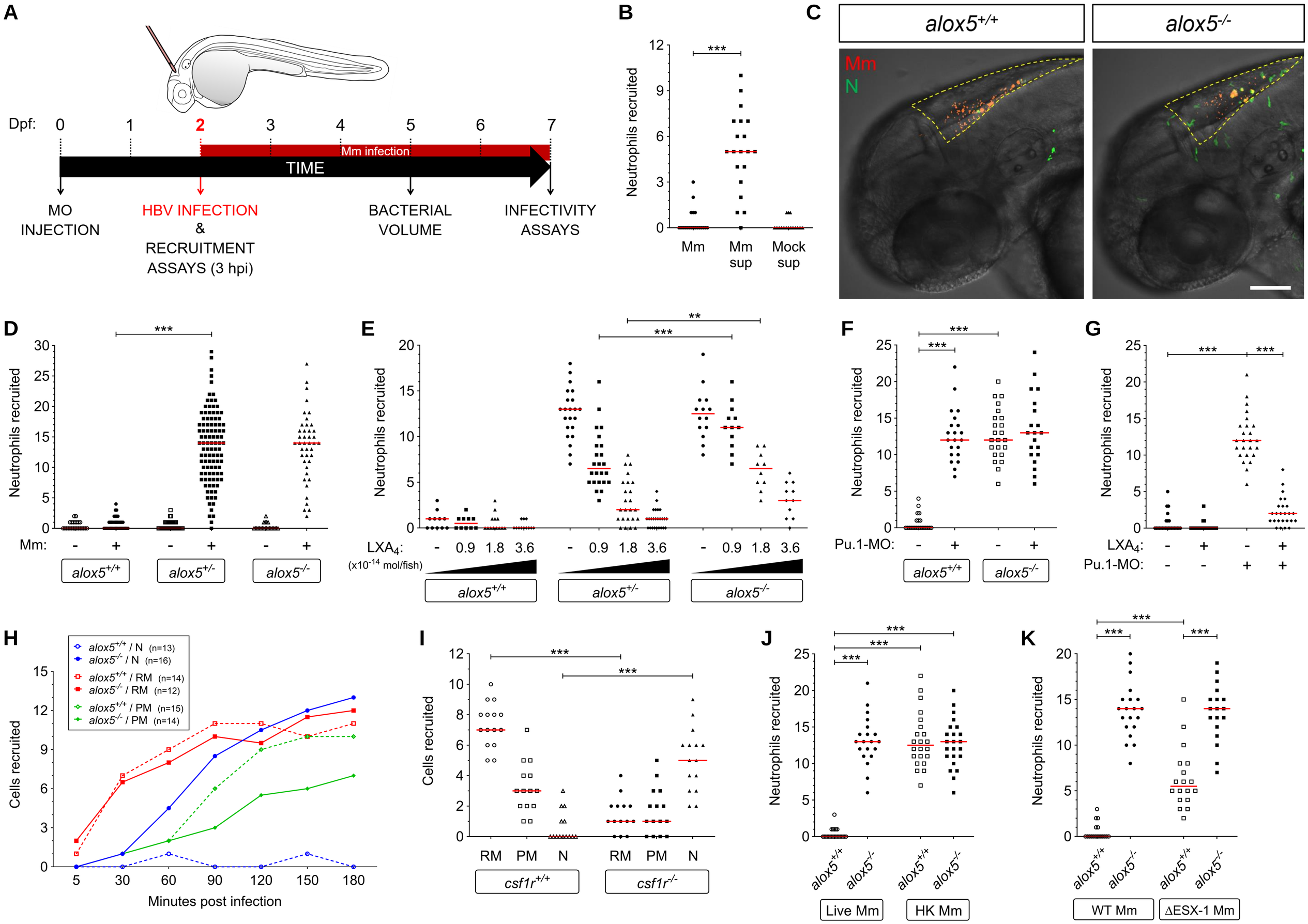

A
Dpf: 0 1 2 3 4 5 6 7
Mm infection
TIME
MO INJECTION
HBV INFECTION & RECRUITMENT ASSAYS (3 hpi)
BACTERIAL VOLUME
INFECTIVITY ASSAYS
B
Neutrophils recruited
Mm
Mm sup
Mock sup
C
alox5+/+
alox5-/-
Mm
N
D
Neutrophils recruited
Mm:
alox5+/+
alox5+/-
alox5-/-
E
Neutrophils recruited
LXA4:
(x10-14 mol/fish)
F
Neutrophils recruited
Pu.1-MO:
G
Neutrophils recruited
LXA4:
Pu.1-MO:
H
alox5+/+ / N (n=13)
alox5-/- / N (n=16)
alox5+/+ / RM (n=14)
alox5-/- / RM (n=12)
alox5+/+ / PM (n=15)
alox5-/- / PM (n=14)
Cells recruited
Minutes post infection
I
Cells recruited
RM PM N RM PM N
csf1r+/+
csf1r-/-
J
Neutrophils recruited
Live Mm
HK Mm
K
Neutrophils recruited
WT Mm
ΔESX-1 Mm

Figure 2

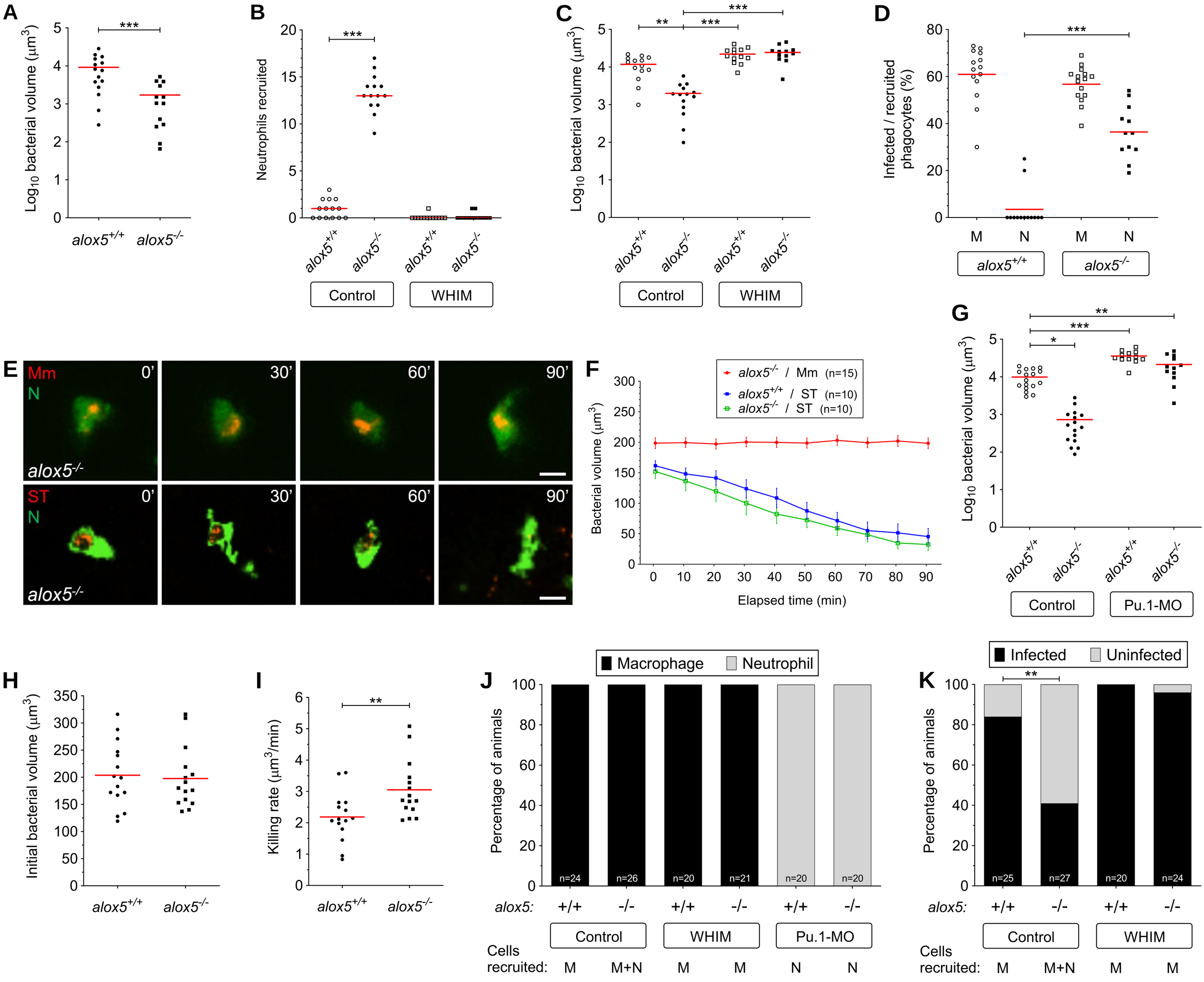

A
Log10 bacterial volume (μm3)
***
alox5+/+
alox5-/-
B
Neutrophils recruited
***
alox5+/+
alox5-/-
alox5+/+
alox5-/-
Control
WHIM
C
Log10 bacterial volume (μm3)
**
***
***
alox5+/+
alox5-/-
alox5+/+
alox5-/-
Control
WHIM
D
Infected / recruited phagocytes (%)
***
M
N
M
N
alox5+/+
alox5-/-
E
Mm
N
0'
30'
60'
90'
alox5-/-
ST
N
0'
30'
60'
90'
alox5-/-
F
alox5-/- / Mm (n=15)
alox5+/+ / ST (n=10)
alox5-/- / ST (n=10)
Bacterial volume (μm3)
Elapsed time (min)
G
Log10 bacterial volume (μm3)
**
***
*
alox5+/+
alox5-/-
alox5+/+
alox5-/-
Control
Pu.1-MO
H
Initial bacterial volume (μm3)
alox5+/+
alox5-/-
I
Killing rate (μm3/min)
**
alox5+/+
alox5-/-
J
Macrophage
Neutrophil
Percentage of animals
n=24
n=26
n=20
n=21
n=20
n=20
alox5:
+/+
-/-
+/+
-/-
+/+
-/-
Control
WHIM
Pu.1-MO
Cells recruited:
M
M+N
M
M
N
N
K
Infected
Uninfected
Percentage of animals
**
n=25
n=27
n=20
n=24
alox5:
+/+
-/-
+/+
-/-
Control
WHIM
Cells recruited:
M
M+N
M
M

Figure 3

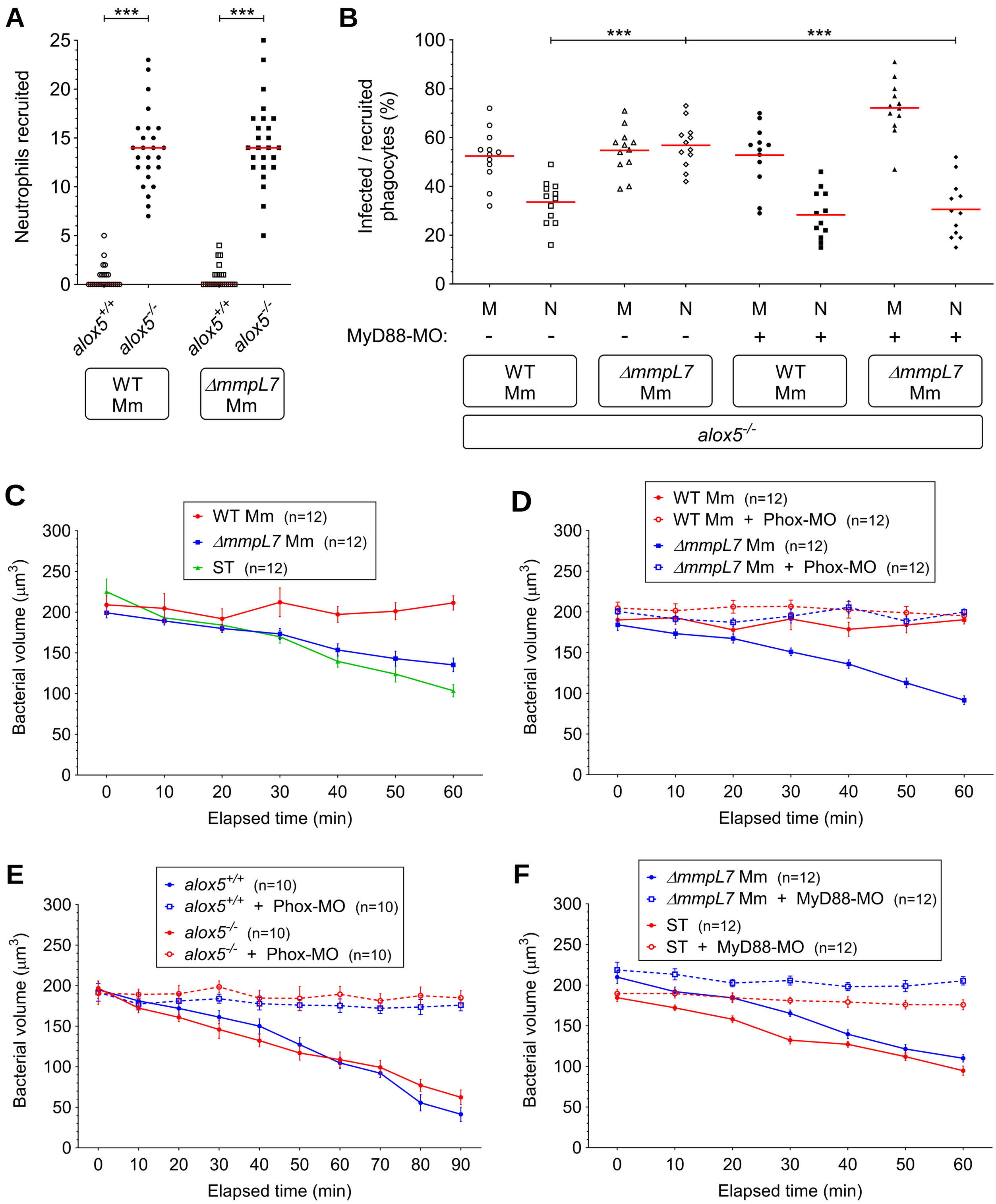

A
***
***
Neutrophils recruited
alox5+/+
alox5-/-
alox5+/+
alox5-/-
WT Mm
ΔmmpL7 Mm
B
***
***
Infected / recruited phagocytes (%)
M N M N M N M N
MyD88-MO: - - - - + + + +
WT Mm
ΔmmpL7 Mm
WT Mm
ΔmmpL7 Mm
alox5-/-
C
WT Mm (n=12)
ΔmmpL7 Mm (n=12)
ST (n=12)
Bacterial volume (µm3)
Elapsed time (min)
D
WT Mm (n=12)
WT Mm + Phox-MO (n=12)
ΔmmpL7 Mm (n=12)
ΔmmpL7 Mm + Phox-MO (n=12)
Bacterial volume (µm3)
Elapsed time (min)
E
alox5+/+ (n=10)
alox5+/+ + Phox-MO (n=10)
alox5-/- (n=10)
alox5-/- + Phox-MO (n=10)
Bacterial volume (µm3)
Elapsed time (min)
F
ΔmmpL7 Mm (n=12)
ΔmmpL7 Mm + MyD88-MO (n=12)
ST (n=12)
ST + MyD88-MO (n=12)
Bacterial volume (µm3)
Elapsed time (min)

Figure 4

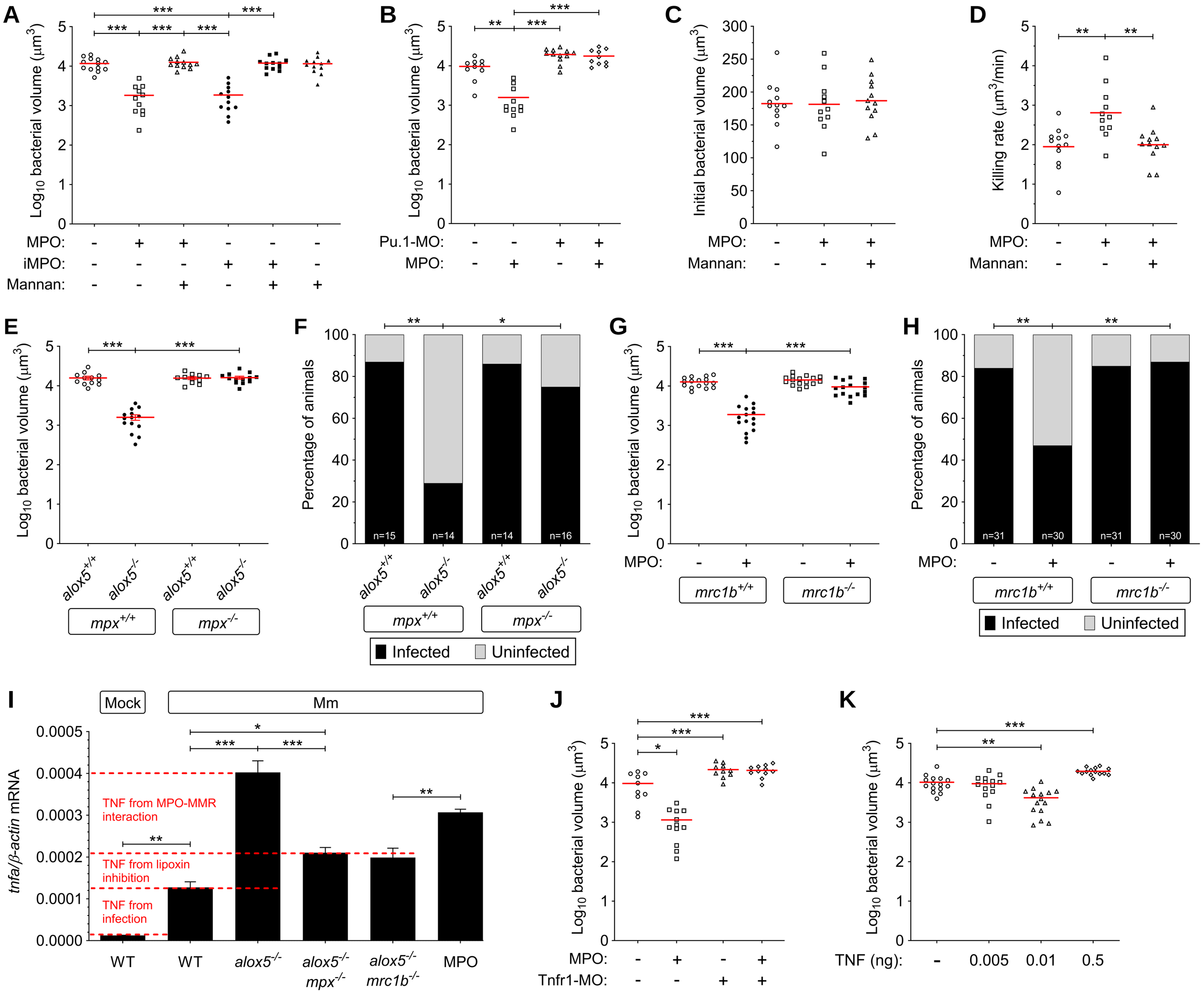

A
Log10 bacterial volume (μm3)
MPO:
iMPO:
Mannan:
B
Log10 bacterial volume (μm3)
Pu.1-MO:
MPO:
C
Initial bacterial volume (μm3)
MPO:
Mannan:
D
Killing rate (μm3/min)
MPO:
Mannan:
E
Log10 bacterial volume (μm3)
alox5+/+
alox5-/-
mpx+/+
mpx-/-
F
Percentage of animals
n=15
n=14
n=14
n=16
Infected
Uninfected
G
Log10 bacterial volume (μm3)
MPO:
mrc1b+/+
mrc1b-/-
H
Percentage of animals
n=31
n=30
n=31
n=30
Infected
Uninfected
I
Mock
Mm
tnfa/β-actin mRNA
TNF from MPO-MMR interaction
TNF from lipoxin inhibition
TNF from infection
WT
WT
alox5-/-
alox5-/- mpx-/-
alox5-/- mrc1b-/-
MPO
J
Log10 bacterial volume (μm3)
MPO:
Tnfr1-MO:
K
Log10 bacterial volume (μm3)
TNF (ng):
0.005
0.01
0.5

Figure 5

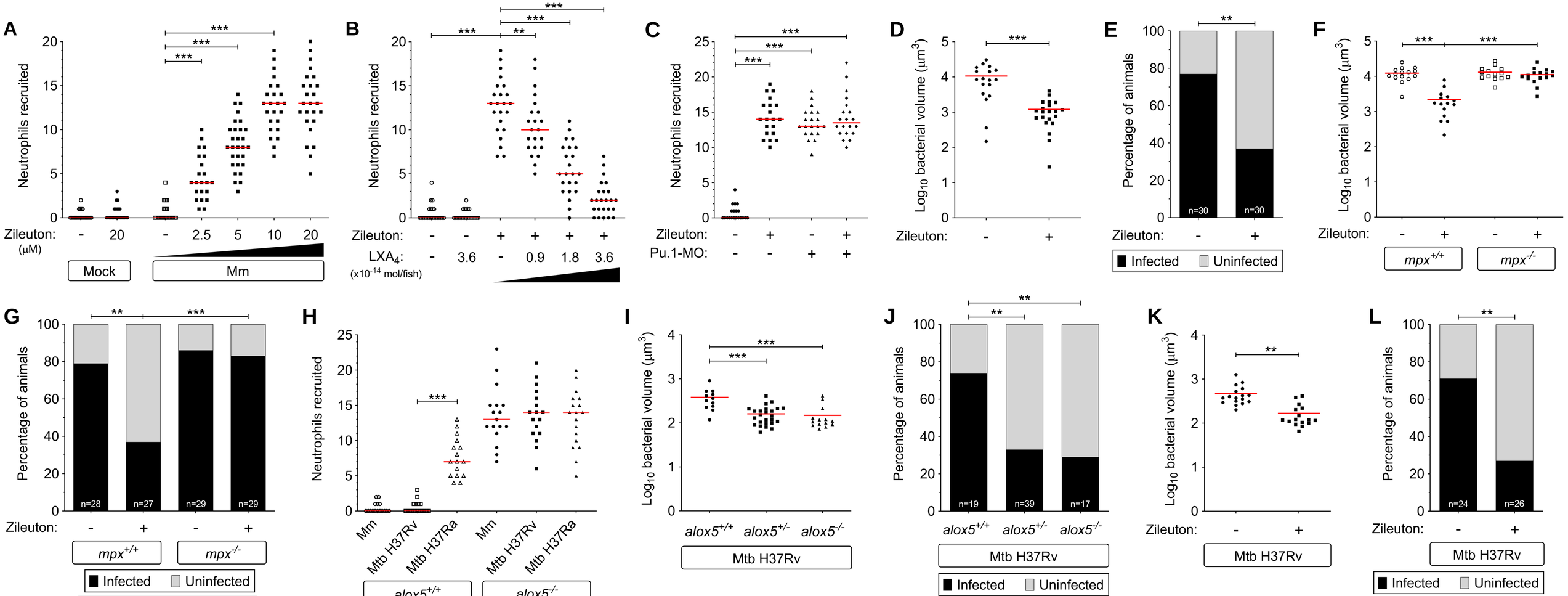

A
Neutrophils recruited
Zileuton: (μM)
- 20 - 2.5 5 10 20
Mock
Mm
B
Neutrophils recruited
Zileuton: - - + + + +
LXA4: (x10-14 mol/fish) - 3.6 - 0.9 1.8 3.6
C
Neutrophils recruited
Zileuton: - + - +
Pu.1-MO: - - + +
D
Log10 bacterial volume (μm3)
Zileuton: - +
E
Percentage of animals
n=30 n=30
Zileuton: - +
Infected Uninfected
F
Log10 bacterial volume (μm3)
Zileuton: - + - +
mpx+/+ mpx-/-
G
Percentage of animals
n=28 n=27 n=29 n=29
Zileuton: - + - +
mpx+/+ mpx-/-
Infected Uninfected
H
Neutrophils recruited
Mm Mtb H37Rv Mtb H37Ra Mm Mtb H37Rv Mtb H37Ra
alox5+/+ alox5-/-
I
Log10 bacterial volume (μm3)
alox5+/+ alox5+/- alox5-/-
Mtb H37Rv
J
Percentage of animals
n=19 n=39 n=17
alox5+/+ alox5+/- alox5-/-
Mtb H37Rv
Infected Uninfected
K
Log10 bacterial volume (μm3)
Zileuton: - +
Mtb H37Rv
L
Percentage of animals
n=24 n=26
Zileuton: - +
Mtb H37Rv
Infected Uninfected

Figure 6

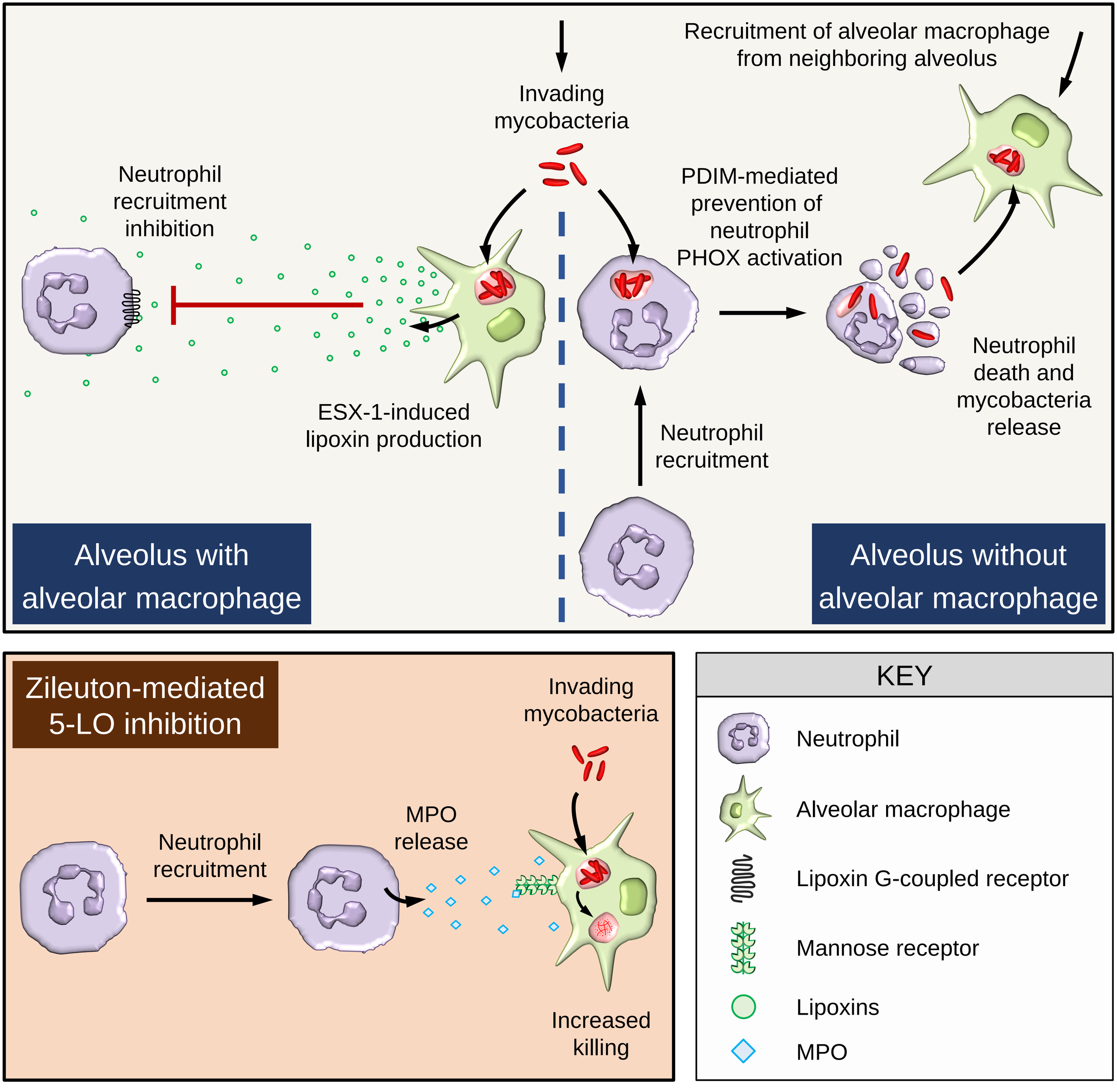
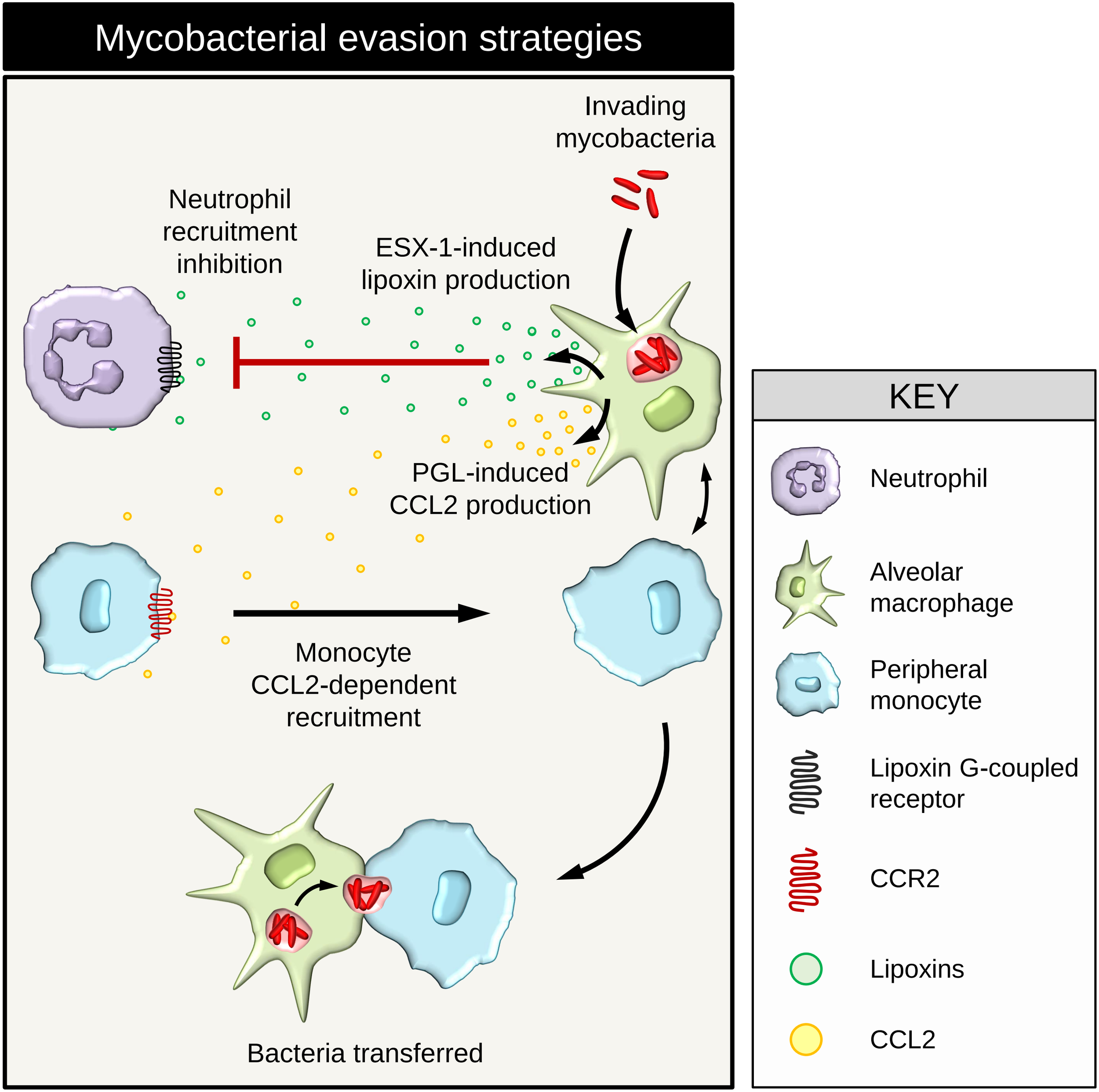

Recruitment of alveolar macrophage from neighboring alveolus
Invading mycobacteria
Neutrophil recruitment inhibition
PDIM-mediated prevention of neutrophil PHOX activation
ESX-1-induced lipoxin production
Neutrophil death and mycobacteria release
Neutrophil recruitment
Alveolus with alveolar macrophage
Alveolus without alveolar macrophage
Zileuton-mediated 5-LO inhibition
Invading mycobacteria
Neutrophil recruitment
MPO release
Increased killing
KEY
Neutrophil
Alveolar macrophage
Lipoxin G-coupled receptor
Mannose receptor
Lipoxins
MPO

Figure 7


Mycobacterial evasion strategies
Invading mycobacteria
Neutrophil recruitment inhibition
ESX-1-induced lipoxin production
PGL-induced CCL2 production
Monocyte CCL2-dependent recruitment
Bacteria transferred
KEY
Neutrophil
Alveolar macrophage
Peripheral monocyte
Lipoxin G-coupled receptor
CCR2
Lipoxins
CCL2

Supplementary Figures

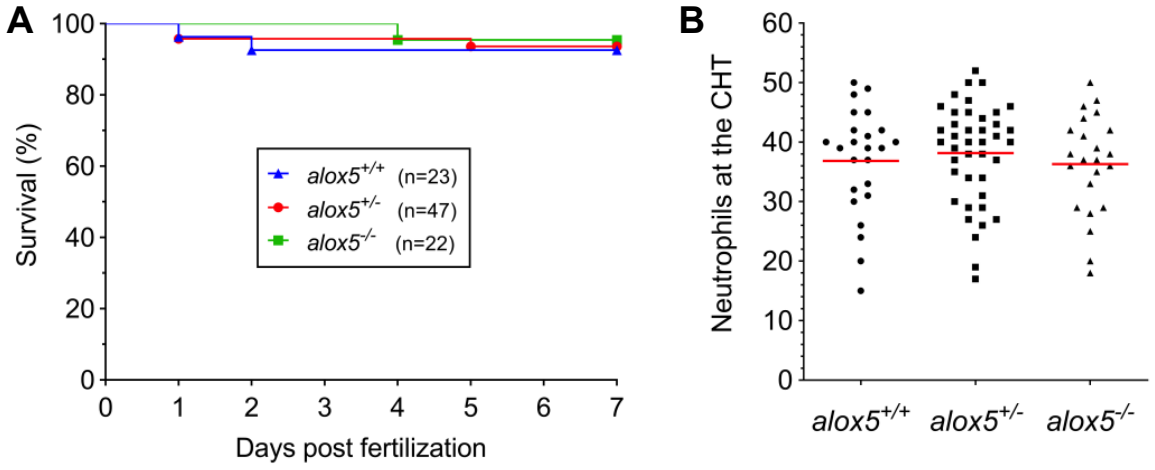


Fig. S1. Viability and neutrophil numbers are not affected in *alox5* mutants. (**A**) Survival of WT, *alox5*$^{+/-}$ or *alox5*$^{-/-}$ larvae. (**B**) Number of neutrophils in caudal hematopoietic tissue (CHT) of WT, *alox5*$^{+/-}$ or *alox5*$^{-/-}$ larvae at 2 dpf, where most neutrophils (~90%) are located at this time point. n= 24, 41, 23, respectively. Red bars, medians. Each panel representative of 3 independent experiments.

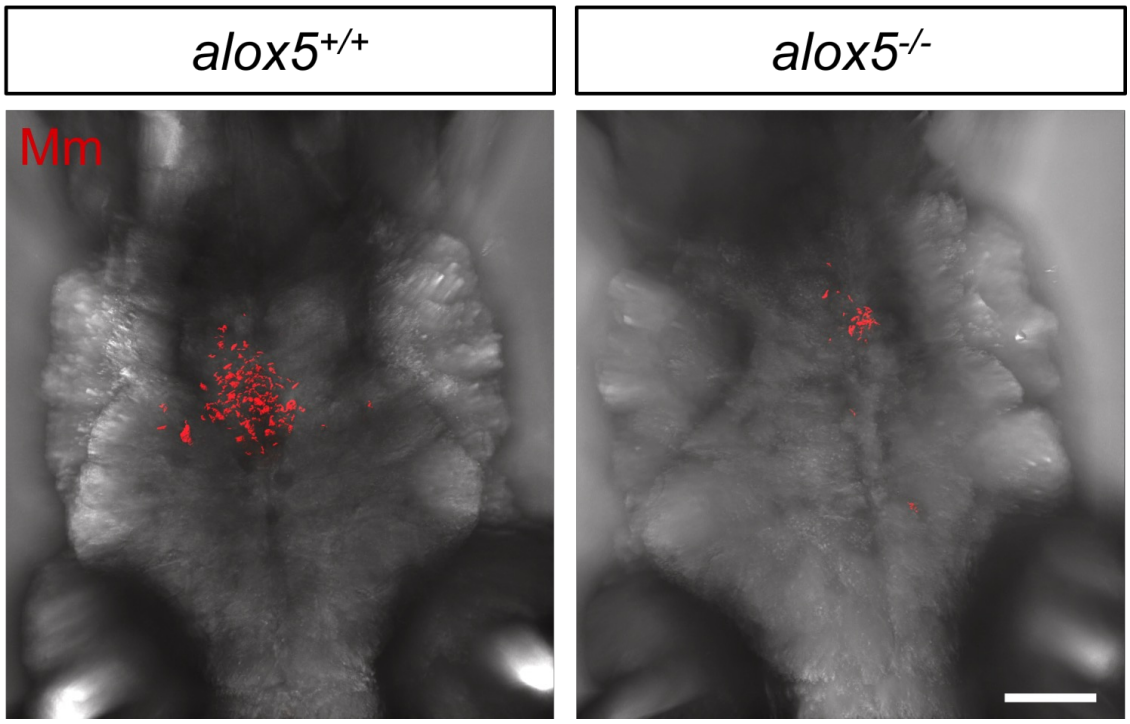


Fig. S2. Mycobacterial infection is attenuated in *alox5* mutants. Confocal images of the head (dorsal view) of WT or *alox5*$^{-/-}$ larvae 3 dpi with 80 WT Mm. Scale bar, 100 µm.

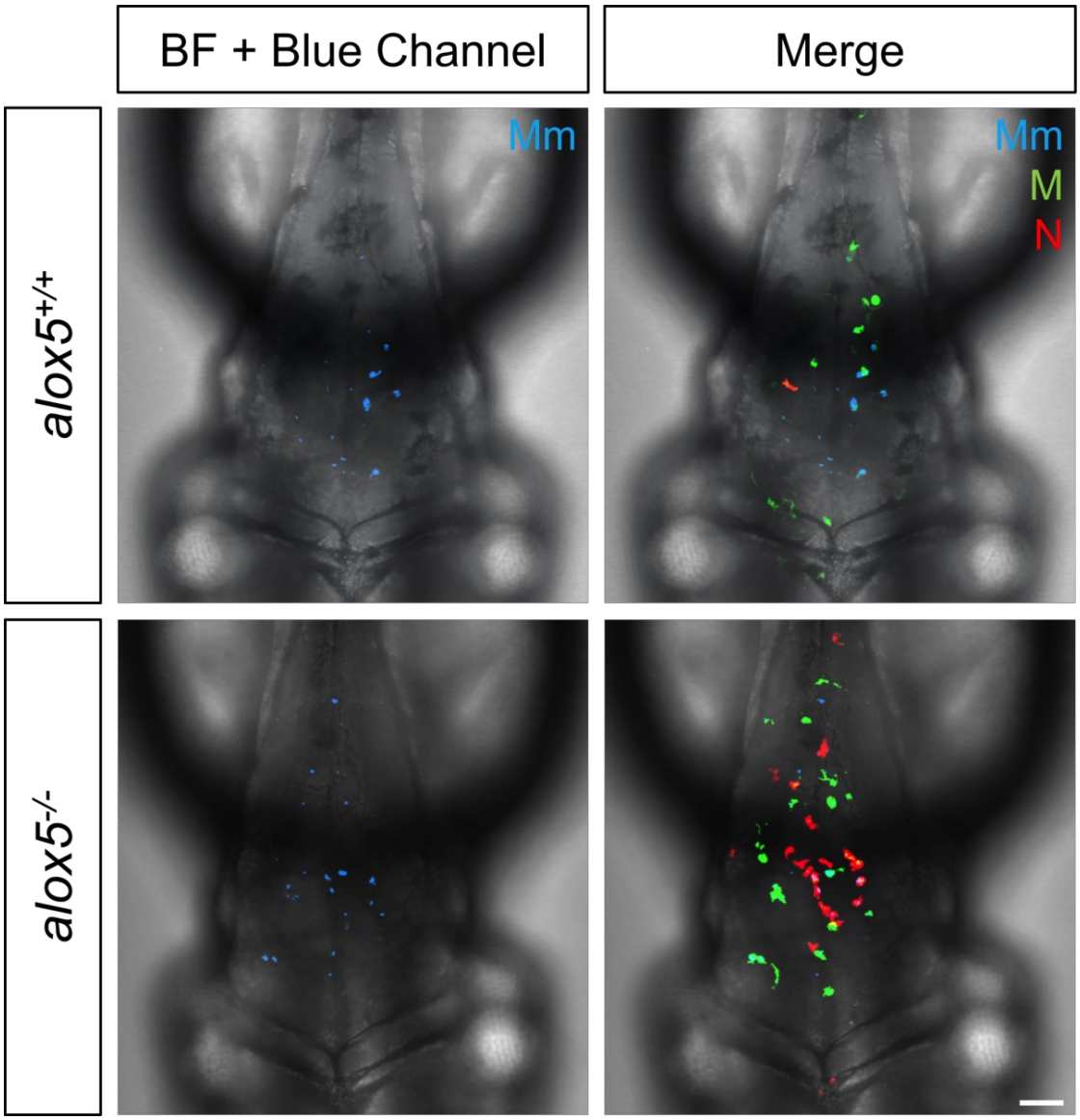


Fig. S3. Neutrophils recruited in *alox5* mutants phagocytose mycobacteria. Confocal images of the head (dorsal view) of WT or *alox5*$^{-/-}$ larvae 3 hpi with 80 WT Mm. M, macrophages; N, neutrophils; BF, brightfield. Scale bar, 50 µm.

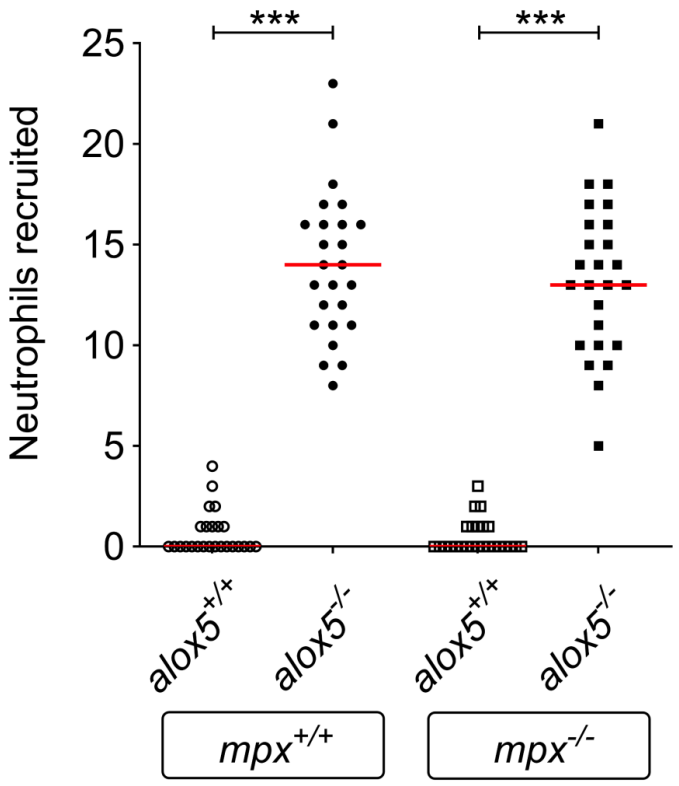


Fig. S4. Neutrophil recruitment to mycobacteria is not affected in *mpx*$^{-/-}$ mutants. Number of neutrophils recruited in WT or mpx$^{-/-}$ larvae, on WT or *alox5*$^{-/-}$ background, 3 hpi with 80 WT Mm. n= 25 per group. Red bars, medians. ***p<0.001 [one-way ANOVA with Tukey's post-test]. Representative of 2 independent experiments.

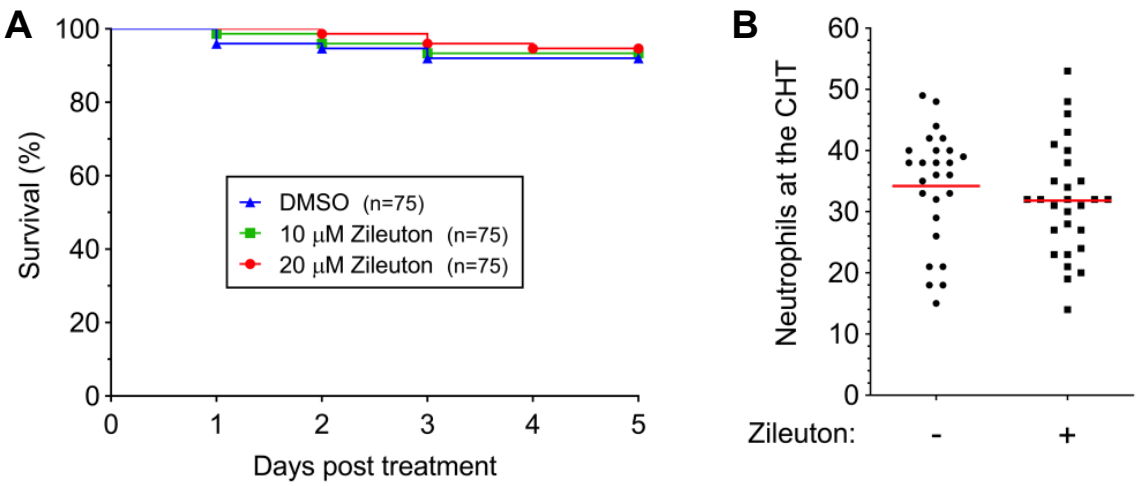


Fig. S5. Viability and neutrophil numbers are not affected in zileuton-treated animals. (**A**) Survival of WT larvae treated with the indicated concentrations of zileuton. (**B**) Number of neutrophils present in CHT of WT larvae, treated or not with zileuton, at 2 dpf. n= 26, 28, respectively. Red bars, medians. Representative of more than 3 independent experiments.

Methods

Zebrafish husbandry and infections

Zebrafish husbandry and experiments were conducted in compliance with guidelines from the UK Home Office. Fish were fed twice daily a combination of dry feed and brine shrimp and were exposed to a 14 h light, 10 h dark cycle to maintain proper circadian conditions. Zebrafish embryos were housed in fish water (reverse osmosis water containing 0.18 g/l Instant Ocean) at 28.5°C. Embryos were maintained in 0.25 mg/ml methylene blue from collection to 1-day post-fertilization (dpf). 0.003% PTU (1-phenyl-2-thiourea, Sigma) was added from 24 hours post-fertilization (hpf) on to prevent pigmentation. Larvae (of undetermined sex given the early developmental stages used) were anesthetized in fish water containing 0.025% tricaine (Sigma) and infected at 48 hpf into the HBV using single-cell suspensions of Mm, Mtb or ST of known titer (*1, 2*). Larvae were randomly allotted to the different experimental conditions. Number of larvae to be used for each experiment was guided by pilot experiments.

Zebrafish AB wild-type strain (Zebrafish International Resource Center) and transgenic or mutant lines in the AB or TLF backgrounds were used, including Tg(*lyz:EFGP*)^{nz117} and Tg(*lyz:DsRED2*)^{nz50} (with green and red fluorescent neutrophils, respectively) (*3*), Tg(*mpeg1:YFP*)^{w200} (with green fluorescent macrophages) (*4*), Tg(-*8mpx:cxcr4b-EGFP*)$^{\mathrm{uwm3}}$ (WHIM) (*5*), *csf1ra*^{j4blue} (*csf1r* mutants) (*6*), *drf*^{gl8} (*mpx* mutants) (*7*), *alox5a*^{sa1464} (*alox5* mutants) (this work), and *mrc1b*$^{\wedge sa18640}$ (mannose receptor mutants) (this work). WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) fish express a mutant CXCR4 receptor under control of the neutrophil-expressed mpx promoter leading to neutrophil retention in the hematopoietic tissues and chronic neutropenia (*5*). *alox5a*^{sa1464} and *mrc1b*$^{\wedge sa18640}$ mutant lines were obtained from the Zebrafish Mutation Project (ZMP) - Wellcome Sanger Institute (*8*). The *alox5a*^{sa1464} mutant line has a nonsense mutation

in an early exon (5 out of 14) of *alox5a*, the gene encoding the enzyme 5-LO which is essential for the synthesis of lipoxins. The *mrc1b*$^{\wedge sa18640}$ mutant line has a nonsense mutation in an early exon (4 out of 31) of *mrc1b*, the gene encoding the mannose receptor in zebrafish. The zebrafish genome involves two paralogs of human *MRC1*, *mrc1a* and *mrc1b*, with *mrc1b* encoding the functional zebrafish ortholog of human *MRC1* (*9*). The *alox5a*^{sa1464} line was genotyped by high-resolution melt analysis (HRM) (*10*) of PCR products (forward primer, 5'-TAGGATCGAGAATCTGTATGTGAACC-3'; reverse primer, 5'-CTCACAAATATCCGCTCAAAGTCTGC-3'), and the *mrc1b*$^{\wedge sa18640}$ line by kompetitive allele specific PCR (KASP) (*11*) (Allele-Specific Primer 1 (Asp1), 5'-GAAGGTGACCAAGTTCATGCTGCATACCTGCTATGAAGGATTGTTC-3'; Allele-Specific Primer 2 (Asp2), 5'-GAAGGTCGGAGTCAACGGATTCGCATACCTGCTATGAAGGATTGTTA-3'; Common Primer (Cp), 5'-CAAGGCGCTGAATTGCTGAGC-3'), both on a CFX Connect thermocycler (BioRad).

Bacterial strains

WT Mm strain M (ATCC #BAA-535) and its derivative mutants *ΔmmpL7* and *Δesx-1* expressing either TdTomato, EBFP2, or mWasabi under the *msp12* promoter (*2, 12*) were grown under hygromycin B (Formedium) selection in 7H9 Middlebrook medium (Difco) supplemented with oleic acid, albumin, dextrose, and Tween-80 (Sigma) (*2*). To prepare heat-killed Mm, bacteria were incubated at 80°C for 20 minutes. To prepare bacterial supernatants, bacteria were grown to an OD600 of 0.6, pelleted and the supernatant was then filtered twice through a 0.2 µm filter.

M. tuberculosis H37Rv *ΔleuD ΔpanCD* double auxotroph expressing mCherry under control of the *msp12* promoter were grown under hygromycin B (Formedium) and kanamycin

selection in Middlebrook 7H9 broth (Difco) supplemented with oleic acid, albumin, dextrose, catalase, Tween-80 (Sigma), 0.05 mg/ml L-leucine and 0.024 mg/ml calcium pantothenate (Sigma) (*13, 14*). *M. tuberculosis H37Ra* (ATCC 25177) were grown in 7H9 Middlebrook medium (Difco) supplemented with oleic acid, albumin, dextrose, and Tween-80 (Sigma) (*15*).

Salmonella enterica serovar Typhimurium transformed with pACYC184-Plac:TdTomato were grown in broth medium (Sigma) at 37°C. Overnight cultures were diluted 1/50 in LB, incubated at 37°C until 1.5 optical density at 600 nm was reached, and finally diluted in PBS.

Drug administration to zebrafish larvae

Equivalently infected sibling larvae were held at 28.5°C until they were then randomly allocated to the drug-treated or control groups. DMSO (Sigma) was kept at 0.5% in all conditions. All drugs were dissolved in DMSO (Sigma) or water and kept in small aliquots at -20°C. The rationale for the doses used in this work was based on previous studies or pilot experiments, using the minimum effective concentration non-toxic to the animals. Drugs were administered by adding them to the fish water (zileuton) or by injecting them into the HBV (LXA_4, MPO, mannan and TNF). Zileuton (10 µM, unless other concentrations are specified in the figures) (LKT Laboratories) was administered by bath at ~30 hpf, ~18 hours before infection, and renewed daily until the end of the experiment. 15-epi lipoxin A_4 (LXA_4) (3.6×10^{-14} mol/fish, unless other concentrations are specified in the figures) (Calbiochem) was injected as previously detailed (*16, 17*). Human myeloperoxidase (MPO) (1 µM) (Sigma-Aldrich) was injected at the infection time, together with the mycobacteria or alone in uninfected controls. When needed, MPO was inactivated by leaving it at room temperature for 24 hours. Mannan from *Saccharomyces cerevisiae* (1 mg/ml) (Sigma-Aldrich) was

injected at the infection time, together with the mycobacteria or alone in uninfected controls. Recombinant zebrafish soluble TNF (*4*) was injected in the concentrations indicated in the figures at 3 hpi.

Morpholino knockdown

Pu.1 (component 1, 5'- CCTCCATTCTGTACGGATGCAGCAT-3'; component 2, 5'-GGTCTTTCTCCTTACCATGCTCTCC-3') (*12, 18*), Myd88 (5'-GTTAAACACTGACCCTGTGGATCAT-3') (*19*), Phox (Gp91^{phox}, 5'-CATAATCCCGATAGCTTACGATAAC-3'; Gp22^{phox}, 5'-ATCATAGCATGTAAGGATACATCCC-3') (*20, 21*), and Tnfr1 (5'-CTGCATTGTGACTTACTTATCGCAC-3') (*4*) morpholinos (Gene Tools) previously described were diluted in injection solution and approximately 2-4 nL were injected into the yolk of one- to two-cell-stage embryos (*22*).

Infectivity and fish survival assays

For infectivity assays, 2 dpf larvae were infected via the hindbrain ventricle with an average of 0.8 bacteria per injection as previously described (*12*). Fish harboring 1-3 bacteria were identified at 3 h post infection by confocal microscopy. These infected fish were then evaluated at 5 dpi and scored as infected or uninfected, based on the presence or absence of fluorescent bacteria. For survival assays, larvae with the genetic background and/or treated with the drugs specified in the figures were monitored every 12 hours following infection for mortality over 5 or 7 days.

Phagocyte recruitment assays

As previously described (*1*), 2 dpf zebrafish were injected into the HBV with the bacterial strain and/or the reagent tested. Number of phagocytes in the HBV was quantified using fluorescence microscopy at 3 hours post injection (unless differently specified). Resident macrophages were distinguished from monocytes by injecting 200 mg/ml Hoechst 33342 (ThermoFisher) into the caudal vein 2 hours prior to infection into the HBV, as previously described (*23*). Differential interference contrast and fluorescent imaging was done every 30 or 60 minutes to identify fluorescent neutrophils, resident macrophages (Hoechst negative) and monocytes (Hoechst positive).

Microscopy

Quantification of infection was performed as described (*2*). A Nikon Eclipse E600 fitted with 20x Plan Fluor 0.5 NA and 40x Plan Fluor 0.74 NA objectives was used for the hindbrain phagocyte recruitment assays. Laser scanning confocal microscopy was used to quantify bacterial burdens in the HBV or within individual phagocytes, and to assess phagocytosis. Anesthetized larvae were embedded in 1.5% low-melting-point agarose on optical bottom plates (MatTek Corporation) and fish water containing 0.007% Tricaine was added to each well. A Nikon A1R confocal microscope with a 20x Plan Apo 0.75 NA objective was used to generate 35-40 µm z stacks consisting of 0.3-2 µm optical sections. The galvano scanner was used for all static imaging and for time-lapse imaging of the HBV. Time-lapse images were taken at different intervals for up to 6 hours. Data were acquired with NIS Elements (Nikon). A heating chamber (Okolab) adapted to the microscope was used to maintain temperature constantly at 28.5°C during imaging. Bacterial burdens were determined by using the 3D surface-rendering feature of Imaris (Bitplane Scientific Software) (*21*). Confocal images are pseudocolored to facilitate visualization.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from pools of 20-30 larvae as previously described (*18*) and described herein, using TRIzol Reagent (Life Technologies), followed by chloroform precipitation. Isolated RNA was used to synthesize cDNA with Superscript III reverse transcriptase and oligo DT primers (ThermoFisher Scientific). RNA levels were quantified by using SYBR green PCR Master Mix (Applied Biosystems) on an ABI Prism 7300 Real-Time PCR System (Applied Biosystems) and data were normalized to *β-actin* for ΔΔCt analysis. Oligonucleotides used were forward primer 5'-ACCTGACAGACTACCTGATG-3' and reverse primer 5'-TGAAGGTGGTCTCATGGATAC-3' for beta actin (*24*), and forward primer 5'-GCGCTTTTCTGAATCCTACG-3' and reverse primer 5'-TGCCCAGTCTGTCTCCTTCT-3' for *tnfa* (*4*).

Statistical analysis

The following statistical analyses were performed using Prism 8.2.1 (GraphPad): Student's unpaired two-tailed t test, one-way ANOVA with Tukey's post-test, and Fisher's exact test. Post-test p values are as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. All bacterial burdens were log transformed prior to statistical analysis. The statistical test used for each panel can be found in the corresponding figure legend. The number of animals used are indicated in the figures or in the corresponding figure legends.

Software Used

The following software was used: NIS-Elements (Nikon) for image acquisition in wide-field and confocal microscopy, Imaris (Bitplane) for image analysis of bacterial burdens, Prism (GraphPad) for data graph and statistical analysis, and PowerPoint (Microsoft) for figure preparation.

Materials and data availability

Materials and Data will be made available upon request.

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