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**Benefits of immune protection versus immunopathology costs:  
a synthesis from cytokine *KO* models**

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12 **Abstract**

13 The inflammatory response can produce damage to host tissues and in several infectious diseases  
14 the most severe symptoms are due to immunopathology rather than a direct effect of pathogen  
15 multiplication. One hypothesis for the persistence of inflammatory damage posits that the benefits  
16 of protection towards infection outweigh the costs. We used data on knocked-out (*KO*) cytokine  
17 models [and the corresponding wild-type (*WT*) controls] to test this hypothesis. We computed  
18 differences in pathogen load and host survival between *WT* and *KO* and divided them by the *WT*  
19 values. Using this ratio provides an internal control for variation in pathogen species, host strain,  
20 pathogen dose, and inoculation route. We predicted that i) if mortality is essentially due to  
21 immunopathology, there should be a loose association between pathogen load and host survival; ii)  
22 if mortality is essentially due to pathogen proliferation, we expect a tight association between  
23 pathogen load and host survival. The results provide strong support to this latter hypothesis. In 85%  
24 of *WT – KO* comparisons ( $n = 126$ ), an increase in pathogen load was associated with an increase in  
25 host mortality, and a decrease in pathogen load was associated with a decrease in host mortality.  
26 Overall, these findings are in agreement with the idea that immunopathology persists because  
27 immune protection confers immediate benefits in terms of infection clearance.

28

29 *Key-words:* cytokine, immunopathology, inflammation, pathogen load, host survival, virulence

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## 33 **1. Introduction**

34 By definition pathogens exploit resources provided by an unrelated organism, called host (Combes,  
35 2001). Pathogen exploitation diverts resources from host functions and as such is considered costly  
36 for the host. Indeed, infection is usually associated with impaired host fitness, either in terms of  
37 increased mortality or decreased reproductive output (Schmid-Hempel, 2008). Such infection-  
38 associated costs define virulence (Read, 1994). The evolution of virulence has been a puzzling  
39 question for evolutionary biologists for decades. Indeed, why should a pathogen that relies on host  
40 survival for its own survival kill its host? A solution to this paradox was provided a few decades ago  
41 when it was suggested that pathogen fitness does not depend on its survival but on its transmission  
42 to other susceptible hosts (Anderson and May, 1982; Ewald, 1983). The model, coined the trade-off  
43 model of virulence, assumes that pathogen transmission increases with pathogen multiplication  
44 within the host, and that host mortality increases with multiplication (Alizon et al., 2009). While  
45 pathogen multiplication (host exploitation) improves transmission rate, it can also induce host death.  
46 Pathogen fitness is therefore maximized at intermediate levels of multiplication (exploitation) (see  
47 for instance de Roode et al., 2008; Alizon and Michalakis, 2015). This model establishes a direct link  
48 between cost of infection and pathogen multiplication. However, the severity of disease is often  
49 unrelated to pathogen load (see Graham et al., 2005 for a review). In this case, the severity of disease  
50 symptoms is rather associated with an overreacting immune response. Immune costs, incurred  
51 following an infection, are usually referred to as immunopathology (Graham et al., 2005).

52 While theoretical work has shown that taking into account immune costs does not invalidate  
53 the trade-off model of the evolution of virulence (Day et al., 2007; Best et al., 2012), a pending  
54 question is why immunopathology persists despite the associated fitness costs for the host (Graham  
55 et al., 2005 ). Immunopathology might be an unavoidable consequence of immune activation and  
56 even though it potentially causes damage to the host, a lack of immune activation upon infection  
57 would produce even more dramatic fitness consequences for the host due to pathogen proliferation  
58 (Okin and Medzhitov 2012). In addition to this, while failure to keep pathogen proliferation under

59 control incurs immediate costs (including mortality), several inflammatory diseases (but not all) have  
60 late-life onset (occur at relatively old ages), and early benefits are expected to outweigh late costs  
61 (Okin and Medzhitov 2012). While sound, this hypothesis has proved difficult to test over a large  
62 spectrum of host-pathogen systems. Here, we took advantage of published information on cytokine-  
63 knocked out mouse models to test the idea that benefits of immune protection outweigh the  
64 immunopathology costs.

65 Cytokines are signaling molecules produced by different immune cells playing a central role  
66 in the immune response (Opal and DePalo, 2000; Scapigliati et al., 2006; Banchereau et al., 2012).  
67 Cytokines exhibit interesting properties such as synergy, redundancy, or inhibitory effects (Paludan,  
68 1998; Elenkov et al. 2005). Based on the immune cells that produce them and on their action on the  
69 immune response, cytokines have been grouped and classified as having pro- or anti-inflammatory  
70 properties or skewing the immune response towards a Th1, Th2, or Th17 type. It is worth noting  
71 however, that not all cytokines clearly fall into one of these categories, and their anti- or pro-  
72 inflammatory role can also be context-dependent (Cavaillon, 2001).

73 As signaling molecules, cytokines play a central role in the process of resistance to infectious  
74 diseases, and they have received a lot of attention to identify how they promote clearance of  
75 infection (Guidotti and Chisari, 2000; Cooper et al., 2011; Beltra and Decaluwe, 2016). One useful  
76 tool to study the effect of cytokines on pathogen resistance has been to produce mouse strains  
77 where the gene that codes for a given cytokine has been inactivated or disrupted. Traits of interest of  
78 these knock out (*KO*) mouse models are then compared to those of the corresponding wild type (*WT*)  
79 strains, in response to an infectious threat. This offers the opportunity to explore whether an  
80 intrinsically up or down-regulated immune response affects both pathogen proliferation and host  
81 fitness (i.e., survival).

82 We compiled a database comprising 19 *KO* cytokines (or cytokine receptors) from 86  
83 published articles. This gives 126 *WT* – *KO* comparisons for differences in host survival and pathogen  
84 load, involving 21% of viruses, 52% bacteria, 6% fungi and 22% protozoa. Based on the observation

85 that disease severity may not be correlated with pathogen load (Gazzinelli et al., 1996; Kullberg et al.,  
86 1998; Long et al., 2006; Long et al., 2008a,b; Long and Graham, 2011), we predicted that if cost of  
87 infection is due to immunopathology there should be no clear association between differences in  
88 host survival and differences in pathogen load. On the contrary, if cost of infection is essentially due  
89 to pathogen proliferation, we should expect a strong association between differences in host survival  
90 and differences in pathogen load.

91

## 92 **2. Material and methods**

### 93 *2.1 Data acquisition*

94 We searched the literature indexed in the Web of Science, using a list of key words (including  
95 'KO', 'cytokine', 'infection', 'survival', 'mortality'). For each article, we checked that there was a  
96 cytokine *KO* model (either the protein or the receptor), an infectious model (a viral, bacterial, fungal,  
97 or protozoan infection), a measure of pathogen load in both *KO* and *WT* hosts, the assessment of  
98 survival in *KO* and *WT* hosts. Some articles also reported the production of cytokines in *KO* and *WT*  
99 hosts in response to the infection (evidently not the cytokine that was knocked out).

100 Data on pathogen load, host survival and when available cytokine production, were extracted  
101 from published figures using the freely available software ImageJ and webplotdigitizer  
102 (<http://arohatgi.info/WebPlotDigitizer/app/>). For survival data, we computed the areas below the  
103 survival curves for *WT* and *KO* individuals. For pathogen load, when means were reported at different  
104 time points, these values were summed up to have a cumulative load. Similarly, when pathogen load  
105 was reported for several organs, we summed up the means to obtain an overall value per group (*WT*  
106 and *KO*). Table 1 reports the list of cytokines, their role (pro- or anti-inflammatory), and the number  
107 of articles used to extract the data. The full dataset is available as an online supplementary material.

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## 112 2.2 Statistical analyses

113 For each trait (host survival, pathogen load and cytokine production), we computed the  
114 difference between *WT* and *KO* and divided it by the *WT* value ( $\frac{WT-KO}{WT}$ ). Even though computing this  
115 ratio controls for the differences in scale between measurements (which is a concern for  
116 measurements of pathogen load), we decided to avoid using linear models to analyze the data.  
117 Instead, we preferred using a semi-quantitative approach. These ratios have signs indicating whether  
118 *WT* had more pathogens than *KO* or suffered more mortality than *KO*. Signs are obviously totally  
119 independent from the measurement scale used in the original data. The ratios for host survival and  
120 pathogen load were plotted one against the other. This gives a four quadrants plot. Each of these  
121 quadrants refers to a particular case. Moving in a clockwise direction, quadrant 1 refers to the case  
122 where *WT* have better survival and lower pathogen load than *KO*, quadrant 2 refers to the case  
123 where *WT* have better survival and higher pathogen load than *KO*, quadrant 3 refers to the case  
124 where *WT* have lower survival and higher pathogen load than *KO*, and quadrant 4 refers to the case  
125 where *WT* have lower survival and lower pathogen load than *KO*. Figure 1 reports a schematic  
126 illustration of the four quadrants and their meaning. We then tested the distribution of these ratios  
127 across the different quadrants using a  $\chi^2$  test. If host survival and pathogen load are uncoupled, we  
128 expect to observe an homogenous distribution of points across the different quadrants (25% of  
129 points in each quadrant); if pathogen load and host survival are negatively associated we expect to  
130 find that the majority of points lies in the diagonal across quadrants 1 and 3.

131

## 132 3. Results

133 In the vast majority of cases, suppressing pro-inflammatory cytokines increased pathogen load,  
134 since 83% of the ratios were negative ( $\chi^2_1 = 48.00$ ,  $p < 0.0001$ ,  $n = 108$ , fig. 2A). Sixty one percent of  
135 the pathogen load ratios were positive when anti-inflammatory cytokines were suppressed.  
136 However, this was not significantly different from the random distribution (50% positive, 50%  
137 negative) ( $\chi^2_1 = 0.89$ ,  $p = 0.346$ ,  $n = 18$ , fig. 2B). The difference in distribution of ratio signs between

138 pro- and anti-inflammatory cytokines was, however, highly significant ( $\chi^2_1 = 17.20$ ,  $p < 0.0001$ ,  $n =$   
139 126).

140 We found that the distribution of points across the four quadrants was not random ( $\chi^2_3 =$   
141 137.05,  $p < 0.0001$ ,  $n = 126$ , fig. 3A). In agreement with the hypothesis that host survival is tightly  
142 negatively associated with pathogen load, we found that 85% of the data points laid in the quadrants  
143 along the diagonal (quadrants 1 and 3). This pattern was even stronger when computing mean values  
144 per cytokine, since 95% of mean values were in quadrants 1 and 3 ( $\chi^2_1 = 15.21$ ,  $p < 0.0001$ ,  $n = 19$ , fig.  
145 3B).

146 The distribution of ratio values across the four quadrants differed between pro- and anti-  
147 inflammatory cytokines ( $\chi^2_3 = 18.41$ ,  $p = 0.0004$ ,  $n = 126$ , fig. 3A). While 75% of points involving pro-  
148 inflammatory cytokines laid in quadrant 1 vs. 39% for anti-inflammatory cytokines, only 10% of pro-  
149 inflammatory cytokines were in quadrant 3 vs. 44% for anti-inflammatory cytokines (fig. 3A). This  
150 reflects the general trend that silencing pro-inflammatory cytokines enhanced pathogen load and  
151 mortality in *KO* individuals.

152 The distribution of ratio values across the four quadrants was remarkably consistent when  
153 comparing cytokine vs. receptor *KO* models, or over the three major pathogen taxonomic groups  
154 (viruses, bacteria and protozoa) (see online supplementary material, figures S1 and S2).

155 We also explored the possible compensatory effects induced by knocking out pro-  
156 inflammatory cytokines. Here, two possible predictions can be made. First, failure in producing a  
157 given pro-inflammatory cytokine might induce the over-expression of other cytokines with  
158 redundant functions, therefore producing a compensatory effect. Alternatively, knocking out pro-  
159 inflammatory cytokine genes might have pleiotropic effects on other signaling molecules. We did not  
160 find support for any of these hypotheses since in 55% of the cases silencing a pro-inflammatory  
161 cytokine induced the up-regulation of other pro-inflammatory cytokines and in 45% it produced a  
162 down-regulation ( $\chi^2_1 = 1.17$ ,  $p = 0.278$ ,  $n = 103$ ). Silencing an anti-inflammatory cytokine almost

163 always produced an up-regulation of pro-inflammatory cytokines (in 92% of cases,  $\chi^2_1 = 9.31$ ,  $p =$   
164 0.0023,  $n = 13$ ).

165

#### 166 **4. Discussion**

167 Using a compiled database from published literature we show that, by far, changes in  
168 pathogen load are the most important determinant of cost of infection, assessed as host mortality, in  
169 cytokine *KO* mouse models. This finding shows that immediate benefits of infection clearance  
170 outweigh the, possibly delayed, immunopathology costs. This strongly suggests that  
171 immunopathology costs persist in face of natural selection because immune protection confers  
172 immediate benefits in terms of survival.

173 Traditionally, evolutionary biologists saw virulence (host mortality or impaired health) as the  
174 unavoidable consequence of within-host pathogen multiplication, and within-host multiplication as a  
175 trait that increases pathogen transmission rate (Alizon and Michalakis, 2015). This view therefore  
176 establishes a direct link between pathogen load and host mortality risk (or health impairment). On  
177 the contrary, biomedical sciences have, since long, stressed the idea that mounting an immune  
178 response against an infectious organism can produce devastating collateral effects, possibly more  
179 severe than the direct damage due to resource exploitation by the pathogen (Lambrecht and  
180 Hammad 2012; Sharma and Thomas, 2014; Newton et al., 2016; Shin et al., 2016). Text book  
181 examples of such infection-induced immunopathology costs involve cerebral malaria or respiratory  
182 distress during influenza (Peiris et al., 2010; Schmidt et al., 2011; Damjanovic et al., 2012). Since the  
183 seminal paper published by Graham and coworkers in 2005 (Graham et al., 2005), evolutionary  
184 biologists have started to take into consideration how immunopathology might affect the evolution  
185 of parasite virulence and host defenses (Sadd and Siva-Jothy 2006; Belloni et al., 2010; Cressler et al.,  
186 2015). While theoretical work has overall shown that the general conclusions of the trade-off model  
187 of parasite virulence hold even when host death is due to immune-mediated diseases (Day et al.,  
188 2007; Best et al., 2012), we still have little insights on the evolutionary forces that maintain

189 immunopathology. Pioneering work conducted on rodent malaria has provided a detailed  
190 assessment of the relative role of immunopathology and pathogen load as drivers of disease severity.  
191 Using a combination of experimental infections with different clones of the protozoan *Plasmodium*  
192 *chabaudii* and the blockade of pro- (TNF- $\alpha$ ) or anti-inflammatory (IL-10) effectors in infected mice,  
193 Long and coworkers were able to drive virulence (infection-induced mortality) up and down  
194 (depending on the immune treatment used), independently of pathogen load (Long et al., 2008a,b).  
195 These examples show that immune activation contributes to the cost of infection and that this cost  
196 can be decoupled from pathogen load.

197         Although the idea that immunopathology is the unavoidable consequence of immune  
198 protection makes perfectly sense (Okin and Medzhitov, 2012), to our knowledge no quantitative  
199 study, on a broad range of infectious organisms, has been produced to support or invalidate this  
200 idea. Here, we show that inactivating pro-inflammatory cytokine genes increases parasite load and  
201 host mortality over a large spectrum of disease models. This produces a tight association between  
202 pathogen load and host mortality, in agreement with the hypothesis that uncontrolled pathogen  
203 proliferation induces immediate and potentially devastating costs for the host. Inflammatory  
204 diseases are often associated with up-regulation of pro-inflammatory cytokines and failure to shut-  
205 down the production of inflammatory effectors (Wallace et al., 2014). Therefore, immunopathology  
206 might be removed by down-regulating pro-inflammatory cytokines (Gottschalk et al., 2016; Liu et al.,  
207 2016); however, it is likely that the associated overwhelming pathogen proliferation weakens the  
208 adaptive value of reducing the expression of pro-inflammatory effectors.

209         Although the strength of our study is that it involves different immune effectors and  
210 pathogens, this also raises a series of challenges. Obviously the first challenge is that it is difficult to  
211 compare studies that used different pathogenic organisms that have been inoculated to different  
212 mouse strains, at different doses, using different inoculation modes, monitored over different  
213 periods of time, etc. We got rid of all these confounding effects by using *WT* individuals as the  
214 internal control. Even if studies differ in many aspects, within studies, *KO* and *WT* mice were always

215 exposed to the same inoculum size, monitored over the same period of time and so on. Therefore,  
216 computing the differences between *WT* and *KO* and dividing them by the *WT* values, gives an  
217 objective estimate of the relative effect of knocking out a given cytokine on host survival and  
218 pathogen load. To be even more conservative, we decided to refrain from using linear models to  
219 explore the relationship between pathogen load and host mortality, because of the possible effect of  
220 different scales used to assess pathogen load across infectious organisms. The method that we  
221 adopted, based on the distribution of the signed ratios, does not allow us to quantitatively estimate  
222 the fraction of variance in host survival accounted by pathogen load; nevertheless, it unambiguously  
223 shows that variation in pathogen load (up or down, depending on the type of cytokine knocked out)  
224 is associated with variation in host mortality. It should also be made clear that the computation of  
225 these ratios does not take into account whether the original study reported a statistically significant  
226 difference or not. Actually, in several studies pathogen load or host survival were indeed not  
227 significantly different between *WT* and *KO*. However, if the signed ratios were mostly due to random  
228 variation around a zero mean, we should have found a homogenous distribution of the data points  
229 across the four quadrants.

230 A potential problem for any study based on published results is publication bias. To address  
231 this issue, we investigated whether there was a temporal trend in the data by looking at the variation  
232 in the ratios among years and we did not find any consistent trend (see online supplementary  
233 material, figures S3). In addition to this, the reported association is so strong that the publication bias  
234 should be very substantial to mask the observed pattern, which seems rather unlikely.

235 While our finding suggests that host survival is overall tightly associated with variation in  
236 pathogen load, we do not discard the role of immunopathology as an important component of  
237 infection cost. In particular, to stick with the definition of virulence (impairment of host fitness), we  
238 focused on studies dealing with pathogens that do induce host mortality (almost no studies in the  
239 context of *KO* infected models have investigated reproductive output) and therefore are amenable  
240 for the study of the immediate cost of infection. Several pathogen species are not immediately lethal

241 and instead have more diffuse and long term costs. Further work should include such infectious  
242 models to have an even more comprehensive view on the benefits of immune protection vs. the  
243 costs of immunopathology.

244 Cytokine signaling is characterized by properties such as redundancy, synergy, pleiotropy,  
245 inhibition (Elenkov et al., 2005; Cavaillon, 2001). Silencing genes coding for a given pro-inflammatory  
246 cytokine might therefore induce a compensatory effect involving other cytokines with redundant  
247 properties. The finding that mice with *KO* pro-inflammatory cytokines overall suffered from increased  
248 pathogen load and mortality suggests that, if any, the compensatory effect is far from being perfect.  
249 To go further in this direction, we investigated whether *KO* individuals had an up-regulated  
250 expression of some of the main pro-inflammatory cytokines (TNF- $\alpha$ , IFN- $\gamma$ , IL-1 $\beta$ , IL-17). We found no  
251 consistent pattern since in half of the cases *KO* individuals had down-regulated expression of pro-  
252 inflammatory cytokines, and the other half an up-regulated expression. On the contrary, knocking  
253 out anti-inflammatory cytokines consistently produced an up-regulation of pro-inflammatory  
254 cytokines. This latter finding is in agreement with the prominent role that anti-inflammatory  
255 cytokines play in the process of resolution of the inflammatory response.

256 To conclude, we provide evidence, based on a diverse set of infectious organisms that even  
257 though suppressing pro-inflammatory signaling reduces infection-associated immunopathology,  
258 pathogen proliferation incurs an immediate major fitness cost in terms of mortality risk. The balance  
259 between immediate cost of pathogen proliferation and delayed cost of immunopathology therefore  
260 provides an evolutionary explanation for the persistence of immunopathology (Okin and Medzhitov,  
261 2012).

262

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266

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359 **Table 1.**

360 List of *KO* cytokines considered in the study, their role (pro- or anti-inflammatory), and the number of  
361 articles from which data were extracted.

362

<i>KO</i> cytokine	Cytokine type	Number of articles
<i>IL-1</i>	Pro-inflammatory	3
<i>IL-1</i> receptor	Pro-inflammatory	8
<i>IL-3</i>	Pro-inflammatory	2
<i>IL-4</i>	Anti-inflammatory	5
<i>IL-4</i> receptor	Anti-inflammatory	1
<i>IL-5</i>	Anti-inflammatory	1
<i>IL-6</i>	Pro-inflammatory	7
<i>IL-10</i>	Anti-inflammatory	10
<i>IL-12</i>	Pro-inflammatory	6
<i>IL-15</i>	Pro-inflammatory	2
<i>IL-17</i>	Pro-inflammatory	1
<i>IL-17</i> receptor	Pro-inflammatory	5
<i>IL-18</i>	Pro-inflammatory	7
<i>IL-18</i> receptor	Pro-inflammatory	1
<i>IL-21</i>	Pro-inflammatory	2
<i>IL-21</i> receptor	Pro-inflammatory	1
<i>IL-22</i>	Pro-inflammatory	2
<i>IL-23</i>	Pro-inflammatory	5
<i>IL-27</i> receptor	Pro-inflammatory	2
<i>IL-33</i> receptor	Pro-inflammatory	1
<i>IL-36</i> receptor	Pro-inflammatory	1
<i>GM-CSF</i>	Pro-inflammatory	2
<i>IFN</i>	Pro-inflammatory	17
<i>IFN</i> receptor	Pro-inflammatory	7
<i>TNF</i>	Pro-inflammatory	10
<i>TNF</i> receptor	Pro-inflammatory	7

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365 **Figure legends**

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367 Figure 1. Schematic illustration of the method used to assess the association between differences in  
368 pathogen load and differences in host survival in *WT* and *KO* mice. Plotting the differences between  
369 *WT* and *KO* (divided by the *WT* values) defines a four quadrant space, with each quadrant indicating a  
370 particular combination of differences between the two variables (pathogen load and host survival). In  
371 the absence of association between pathogen load and host survival, the distribution of data points  
372 should be equal across the four quadrants. This random distribution represents the null hypothesis  
373 ( $H_0$ ) against which the observed distribution of data points is tested.

374

375 Figure 2. Difference in pathogen load between *WT* and *KO* mice divided by *WT* pathogen load. A)  
376 genes coding for pro-inflammatory cytokines were inactivated; B) genes coding for anti-inflammatory  
377 cytokines were inactivated.

378

379 Figure 3. Association between difference in host survival and pathogen load in *WT* and *KO* divided by  
380 *WT* values. A) Empty and full dots refer to pro- and anti-inflammatory *KO* cytokines, respectively. B)  
381 Dots refer to the mean values per *KO* cytokine ( $\pm$  SE).

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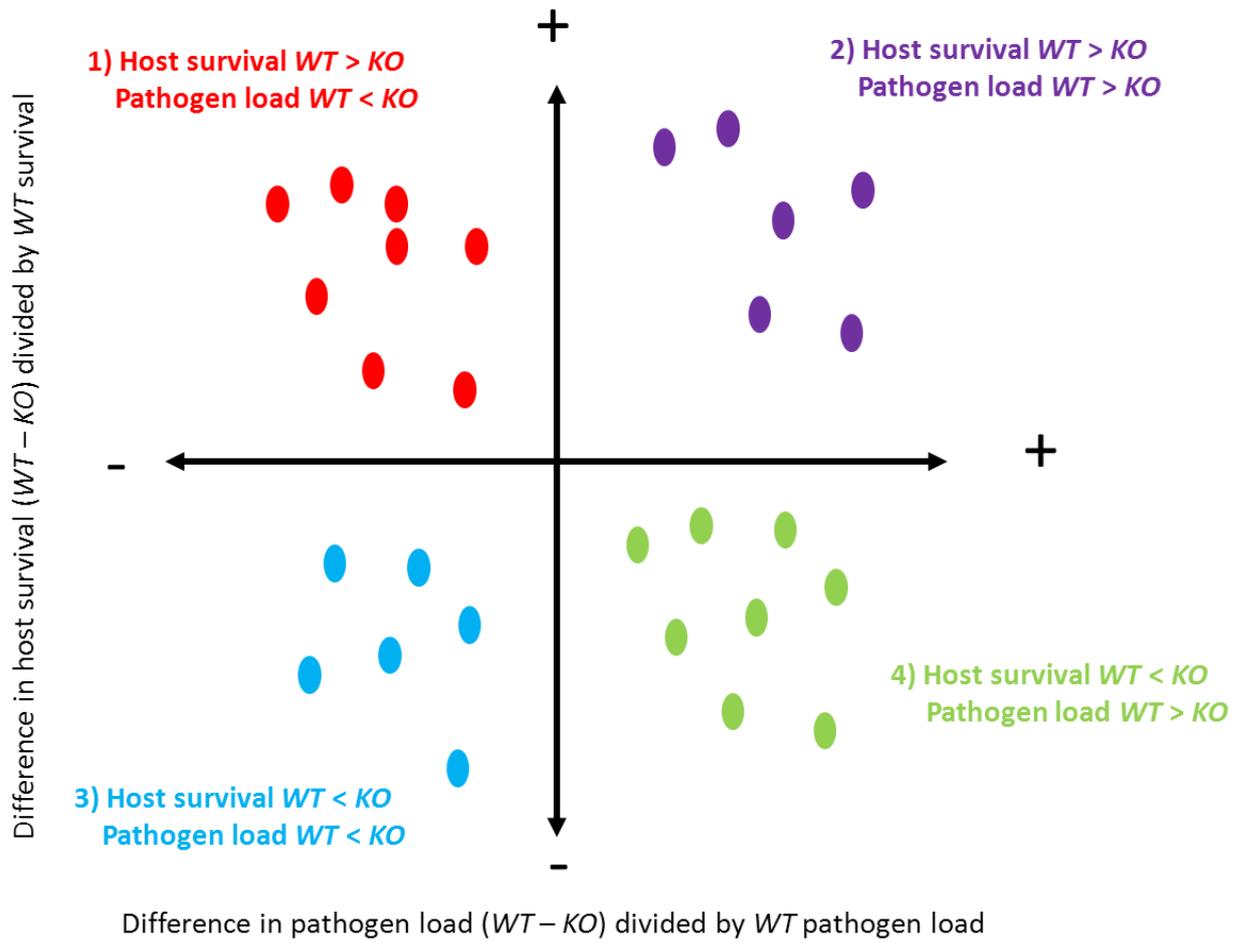
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386 **Figure 1**

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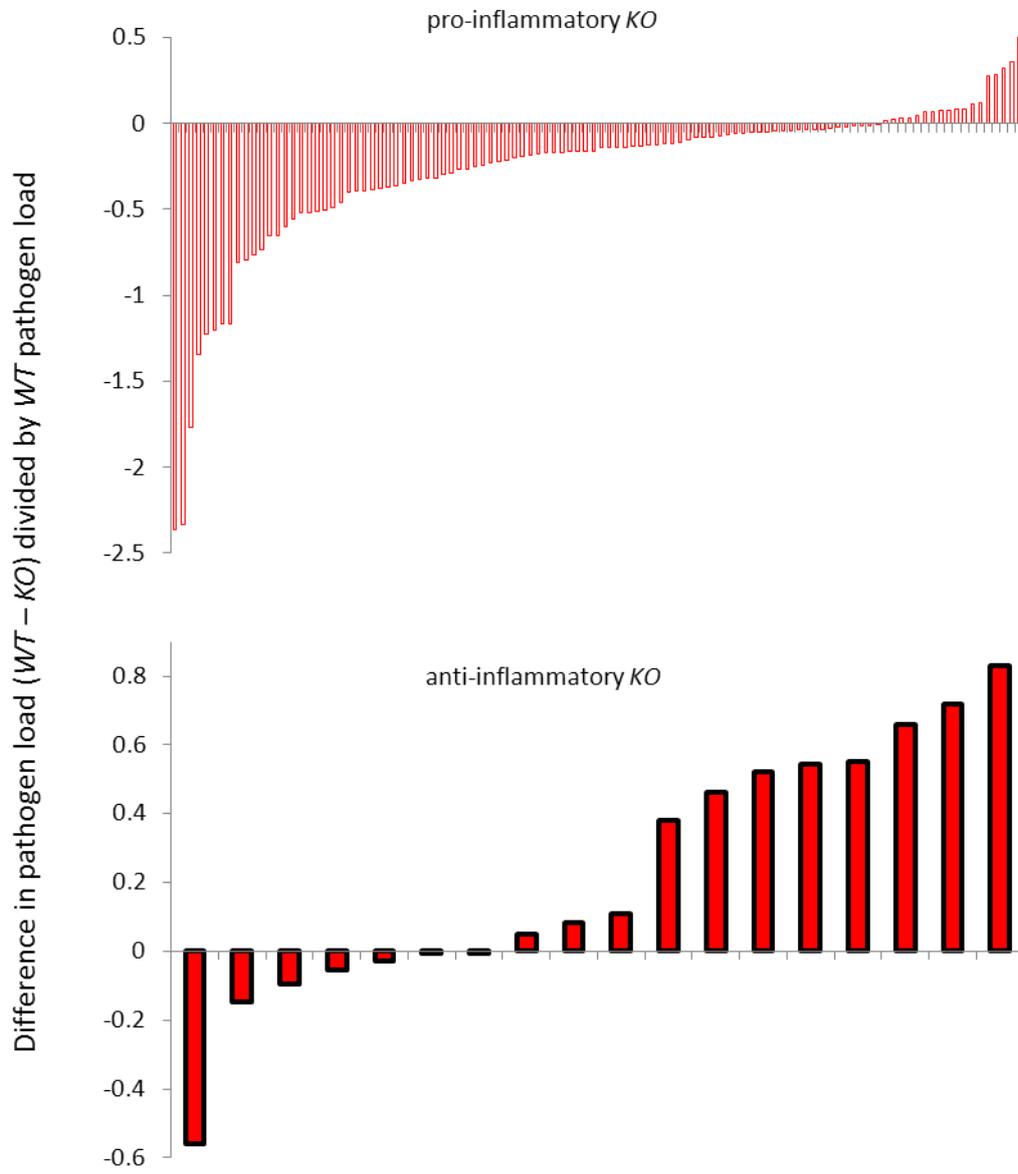
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397 **Figure 2**

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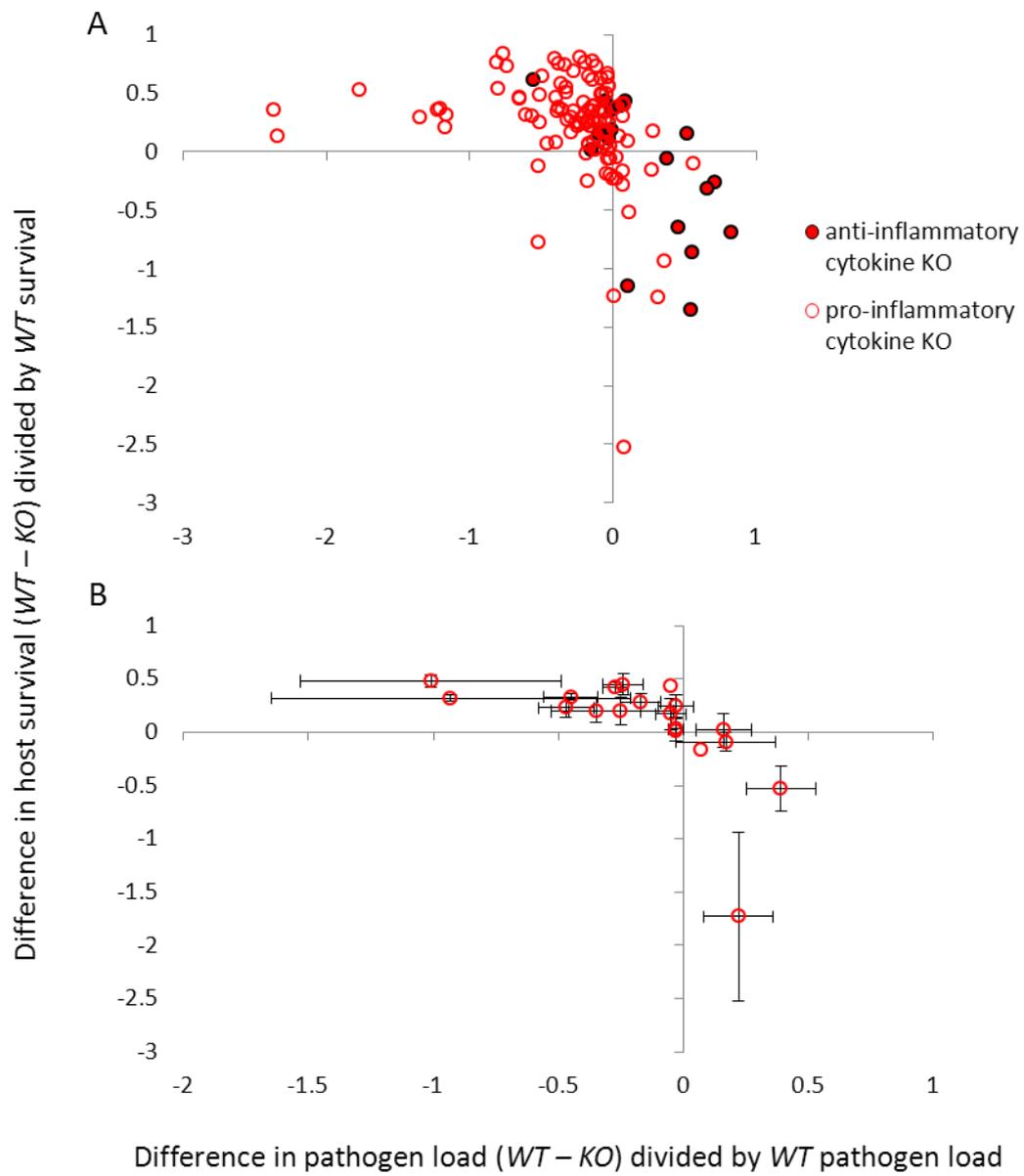
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407 **Figure 3**

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