1 **Title Page**

2 **Title:** Allergic inflammation hinders synergistic viral-bacterial co-infection in C57BL/6 mice

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4 **Running Title:** Asthma and pulmonary co-infection

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15 **Abstract**

16 Asthma is a chronic airways disease that can be exacerbated during respiratory infections. Our previous

17 findings that the inflammatory state of allergic airways at the time of influenza A virus (IAV) infection

18 in combination with epidemiologic findings that asthmatics were less likely to suffer from severe

19 influenza during the 2009 pandemic suggest that additional complications of influenza, such as

20 increased susceptibility to bacterial superinfection, may be mitigated in the allergic host. To test this

21 hypothesis, we developed a murine model of 'triple-disease' in which mice were first rendered allergic

22 to *Aspergillus fumigatus* and co-infected with IAV and *Streptococcus pneumoniae* seven days apart.

23 Significant alterations to known synergistic effects of co-infection were noted in the allergic mice

24 including reduced morbidity and mortality, bacterial burden, maintenance of alveolar macrophages, and

25 reduced lung inflammation and damage. The lung microbiome of allergic mice differed from that of

26 non-allergic mice during co-infection. To investigate the impact of the microbiome on the pathogenesis

27 of lung disease, we induced a perturbation with a short course of fluoroquinolone antibiotic that is often

28 prescribed for lung infections. A significant change in the microbiome was complemented with

29 alterations to the inflammatory profile and a drastic increase in pro-inflammatory cytokines in allergic

30 mice which were now susceptible to severe disease from IAV and *S. pneumoniae* co-infection. Our data

31 suggest that responses to co-infection in allergic hosts likely depends on the immune and microbiome

32 states and that antibiotics should be used with caution in individuals with underlying chronic lung

33 disease.

34

35 **Author Summary**

36 Asthma is a condition of the lungs that affects millions worldwide. Traditionally, respiratory infections

37 are considered to have a negative impact on asthmatics. However, epidemiological data surrounding the

38 2009 influenza pandemic suggest that asthmatics may be better equipped to counter severe influenza

39   including bacterial pneumonia. Herein, we introduce a novel mouse model system designed to

40   recapitulate an influenza virus and Streptococcal co-infection in a host with fungal asthma. We found

41   that underlying allergic asthma protects against severe disease induced by co-infection. Mice with

42   underlying allergic inflammation had reduced damage to the lungs and did not show signs of respiratory

43   distress. Among the differences noted in the allergic mice that were protected from viral and bacterial

44   co-infection, was the lung microbiome. Allergic mice lost their protection from co-infection after we

45   perturbed their lung microbiome with antibiotics suggesting that the lung microbiome plays a role in

46   host immunity against invading pathogens.

47

48   **Introduction**

49   Lung diseases are a leading cause of morbidity and mortality worldwide. Acute and chronic

50   respiratory diseases, excluding infections, affect greater than 12% of the population in the United States

51   and hundreds of millions worldwide (1). Asthma is the most prevalent of these (2) and has the greatest

52   economic burden (3), in addition to being one of most challenging lung conditions to investigate and treat.

53   While the exact etiology of asthma remains unstipulated, specified phenotypes based on symptoms,

54   immunologic profiles, genes, and environment, are confounded by gender and age. Furthermore, asthma

55   exacerbations can be triggered by respiratory viral infections (4, 5) and some reports suggest that

56   asthmatics are at risk for bacterial pneumonia (6, 7).

57   Over four million deaths every year (predominantly children <5 years) are caused by acute

58   respiratory infections (8). Influenza and Streptococcal pneumonia contribute to approximately one million

59   hospitalizations annually in the U.S. (9, 10). While influenza alone can be fatal, recovering patients have

60   an increased susceptibility to respiratory bacterial infections (11), of which *Streptococcus pneumoniae*

61   (*Spn*), a pathogen associated with community-acquired pneumonia (12), is highly associated with severe

62   disease during influenza infections. The 'Spanish Flu' pandemic exemplified this predilection with the

63   majority of deaths attributed to subsequent bacterial infections (13), as did the 2009 Swine Flu pandemic

64    in which 29-55% of deaths resulted from secondary bacterial pneumonia (14, 15). The occurrence of

65    pulmonary infections in asthmatics is augmented by high disease incidences of each, and seasonal overlap

66    between infectious agents and allergens.

67    Although asthma was a risk factor for hospitalization during the 2009 'Swine Flu' pandemic (16).

68    subsequent studies noted that some asthmatics had less severe outcome (including reduced bacterial

69    pneumonia) compared to non-asthmatics (17-21). Explanations for this unexpected and counterintuitive

70    outcome are sparse, although possibilities include steroid use (22), heightened medical care, and increased

71    likelihood of vaccinations in asthmatics (21). A better understanding of mechanisms that promote

72    protection from pulmonary infection-mediated complications in asthmatics is important and necessary,

73    but currently lacking in the literature.

74    Effective interrogation of host-pathogen interactions during allergic asthma and respiratory

75    infections necessitates a single animal model that can capture the nuances of both morbidities. Our mouse

76    model of fungal asthma and influenza (23) effectively recapitulates epidemiologic findings that some

77    asthmatics had less severe disease during the 2009 influenza A virus (IAV) pandemic (20, 24). To

78    investigate viral-bacterial synergy in the allergic host, we developed a 'triple-disease model' by combining

79    our fungal asthma model (25) with a well-established model of IAV and *Spn* co-infection (26). Our

80    findings suggest that pre-existing allergic asthma protects the host from severe morbidity, as shown by

81    maintenance of weight, and reduced viral-bacterial synergism. Allergic mice also had reduced bacterial

82    burdens, altered inflammatory cell profiles (more eosinophils and macrophages and fewer neutrophils) as

83    well as a distinct lung microbiome compared to those with IAV and *Spn* co-infection alone. Inducing

84    dysbiosis with antibiotics caused a partial reversal of this protective phenotype observed in the allergic

85    mice.

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## Results

Asthmatics are generally considered to have heightened susceptibility to respiratory infections (27). However, exact reasons for this outcome are not well established in humans due to inaccessibility of mucosal tissue samples limiting thorough mechanistic interrogations. Therefore, animal model systems are invaluable in such instances, and yet, those that allow the interrogation into the convergence of immunologically distinct conditions like asthma, influenza, and pneumococcal pneumonia are lacking. Recent work with our animal model of asthma and influenza comorbidity underscored the importance of the state of the allergic airways at the time of viral infection in the pathogenesis of influenza in allergic hosts (23), and identified a novel antiviral function for eosinophils (28). Since viral-bacterial synergy has previously established to cause severe pneumonia and mortality (29), we sought to determine the effect of an allergic microenvironment in the lung on subsequent co-infections with IAV and *Spn*, both alone and in combination.

*Allergic airways inflammation protected mice against severe disease from co-infection.*

Mouse model systems that can simulate complex interactions between allergy and respiratory infections are limited, but important to study disease-disease interactions that may alter host responses. Since respiratory infections with viruses and bacteria are considered triggers for the development of asthma, the previously reported animal models utilized infectious agents prior to allergen provocation (30). While asthma can indeed be triggered by respiratory infections, it can also be exacerbated by the same (31, 32). Herein, our first goal was to develop and characterize a model system in which respiratory infections occurred in established allergic airways disease. Mice were subjected to *A. fumigatus* allergen sensitization and challenge (25, 33), and then infected with IAV (23), followed by *S. pneumoniae* (**Fig 1A**). Naïve mice were used to measure baseline, while asthma-only, influenza-only (Flu Ctr), bacteria-only (Bact Ctr) mice served as single disease controls. Dual condition groups

113    included Asthma+Flu (AF), Asthma+Bact (AB), and Flu+Bact (FB), while Asthma+Flu+Bact (AFB)

114    triple-disease condition served as the experimental group.

115        As previously demonstrated by us (23), IAV infection during peak airways inflammation did not

116    induce weight loss in allergic mice (AF group, **Fig 1B**), whereas the same dose of virus triggered about a

117    12% weight loss in non-allergic mice (F group, **Fig 1B**). Non-allergic mice that were co-infected (FB

118    group) lost ~20% weight at the termination point in this study (**Fig 1B**), and continued to lose weight

119    and succumbed to disease by 6 dpi with *Spn* (data not shown). In stark contrast, allergic mice that were

120    subsequently co-infected (AFB group) did not lose weight and had a comparable weight profile to the

121    AF group (**Fig 1B**) and 83% in the AFB group survived (data not shown). As such, although mortality

122    was not an output of this study, allergy appeared to delay/protect mice from IAV+*Spn*-induced mortality

123    which may provide a time advantage for clinical therapeutics. Infectious virus was absent in the lungs of

124    mice in all groups at 3 dpi (data not shown) which differs from previous studies that have demonstrated

125    a viral rebound after *Spn* co-infection, albeit using the laboratory strain of IAV (34). The bacterial

126    burden in the allergic lungs was not sufficient to visualize by fluorescence like in co-infection alone (**Fig

127    1C**), but conventional enumeration of pneumococci on blood agar showed significant reduced bacterial

128    loads in allergic mice compared to the non-allergic co-infected mice (**Fig 1D**). Bacterial dissemination

129    into the blood may also be delayed/reduced in allergic mice (**Fig 1D**).

130

131    *Allergic mice had a more diverse immune cell signature in the airways although tissue inflammation*

132    *was lower compared to non-allergic mice during co-infection*

133        Inflammation is an important hallmark of both allergic disease and respiratory infections,

134    although the cell types that dominate are different. We measured the number and type of leukocytes in

135    the airways (bronchoalveolar lavage, BAL) as a marker of disease severity. As expected, inflammatory

136    cells were increased significantly over steady state ($3.70 \times 10^4 \pm 1.04 \times 10^4$ live cells) after each trigger

137    (**Fig 2A**). Macrophages were more abundant in single infections with and without allergy, but

138     significantly lower in the FB group (**Fig 2B**) as previously demonstrated (35). Eosinophils, B cells, and

139     CD4^{+} T cells followed a similar pattern of abundance, wherein cell numbers were significantly higher in

140     pathogen infected allergic mice than their non-allergic counterparts (**Fig 2B**). Neutrophil infiltration was

141     markedly higher in the FB group, while CD8^{+} T cell recruitment was similar except for the asthma- and

142     bacteria-only controls which had very low influx into airways (**Fig 2B**).

143         Over exuberant immune responses are considered a mechanism by which synergistic actions of

144     IAV and *Spn* increased host morbidity and mortality (36). Analysis of hematoxylin and eosin stained

145     sections of lung tissue showed that widespread parenchymal inflammation was present in IAV-infected

146     mice (Flu, **Fig 3**) but that *Spn*-infected mice had minimal areas of inflammation (Bacteria, **Fig 3**) most

147     likely due to effective bacterial clearance in otherwise healthy hosts. In contrast, extensive areas of lung

148     parenchyma in FB group mice were consolidated by fluid exudates and inflammatory cells consisting

149     mostly of neutrophils and macrophages (**Fig 3**). As expected, inflammation in the asthma-only control

150     mice mostly surrounded the terminal airways (**Fig 3**), and similar inflammatory foci were observed

151     around the small airways in both the AF (**Fig 3**) and AB groups (**Fig 3**).  Significantly, pulmonary

152     lesions in the AFB group mice (**Fig 3**) were much less severe than those in the FB group (**Fig 3**), and

153     resembled those of AF mice (**Fig 3**). Histopathologic scoring of diffuse alveolar damage markers such as

154     alveolar inflammation and protein/fibrin deposition were all much higher in the FB group than in the

155     AFB. The higher levels of mucus production in all allergic mice, irrespective of the presence or type of

156     infectious agent, correlated with the reduced damage and loss of bronchiolar epithelium in these lungs.

157     These analyses suggest that IAV infection may provide the dominant antigen triggers for the resulting

158     and subsequent inflammation in the lungs.

159

160     *Mucosal microbial abundance in the allergic co-infected mice differed from baseline*

161         The importance of the microbiome in health and disease is increasingly recognized (37). Our

162     understanding of the contribution of endogenous microbes in the gastrointestinal system is more

163     advanced than that of the respiratory system, partially due to the comparatively low biomass and

164     sampling difficulties. Therefore, studies that incorporate the microbiome into disease pathogenesis are

165     important. Herein, we investigated the microbial diversity in the BAL and lungs by comparing the

166     microbiome abundance in each niche of each group in comparison to naïve controls at the genus level

167     (Table S1). Differential abundance changes of microbiota under different conditions although the

168     changes were not significant (p>0.05) after multiple test correction across all comparisons.

169          Comparison of Asthma vs. naïve in BAL, the PCoA ordination based on the abundance profiles

170     did not show a clear separation of sample clusters (**Fig 4A**). *Hyphomicrobiaceae* is enriched in asthma

171     of BAL (p=0.02), while *Methylophilaceae*, *Clostridiaceae*, and *Mycoplana* were decreased in asthma of

172     BAL (p< 0.05). Clear separation of clusters between Asthma and naïve mouse lungs was evident with

173     enrichment of *Exiguobacterium*, *Desulfotomaculum* and S24_7 of *Bacteroidales* (p<0.05) and reduction

174     of *Veillonella* (p=0.01) in Asthma (**Fig. 4A**). Slight overlap between Flu- and Bact- controls occurred

175     with naïve microbiome clusters in both niches (**Fig 4B & C**). *Rhodocyclaceae*, *Lysobacter*, *Skermanella*

176     and *Sphingomonadaceae* were enriched in the BAL of Flu controls while *Mycoplana* and

177     *Cytophagaceae* were depleted (p<0.05). *Lactobacillus*, *Acetobacteraceae*, *Balneimonas*, and *Dietzia*

178     were enriched in the lungs of Flu controls (p<0.05) while *Fusobacterium* and *Veillonella* were

179     particularly reduced (p<0.02) (**Fig 4B**). Differentially, *Clostridiales*, S24_7 of *Bacteroidales*, RF39 of

180     *Mollicutes*, *Capnocytophaga*, *Roseomonas* of *Acetobacteraceae* were enriched while *Nitrospira* and

181     *Micrococcaceae* were reduced in BAL of mice infected with *Spn* (p<0.05). *Skermanella* dominated in

182     the lungs of Bacteria-only control mice while *Rothia* and *Fusobacteria* were reduced (p<0.01) (**Fig 4C**).

183          Clear separation of clusters were identified between AF and naïve mouse BAL samples wherein

184     *Ruminococcus*, *Allobaculum, S24_7* of *Bacteroidales* and *Clostridiales* were enriched in AF (p-value <

185     0.01) and multiple taxa were depleted especially *Ruminococcus* (p=0.005). (**Fig 4D**). Minor overlap was

186     observed between the lung microbiota of AF and naïve mice with *Skermanella*, *Ruminococcaceae* of

187   *Clostridiales*, S24_7 of *Bacteroidales* and *Nitrospira* highly enriched while *Propionibacterium* and

188   *Corynebacterium* were reduced (p=0.01) in AF group (**Fig. 4D**).

189      Microbial clusters in the BAL and lungs of AB mice were slightly separated from naïve with

190   enrichment of *Oscillospira* of *Ruminococcaceae* (p<0.01) and depletion of *Nitrospira* (p<0.01) in the

191   AB group (**Fig 4E**). Tight clusters showed clear separation of lung microbiota between the FB group

192   and naïve mice where *Streptococcus* was highly enriched in the BAL (p<0.01) and >20 taxa were

193   depleted in both BAL and lung (p<0.05) in the FB group (**Fig 4F**). Similarly, clear separation of clusters

194   between the AFB group and naïve animals was observed in both niches (**Fig 4G**). *Bacillaceae*,

195   *Streptococcus*, *Turicibacter*, and *Bacteroides* were enriched in BAL of AFB (p<0.05) while

196   *Streptococcus* was enriched in AFB lungs (p=0.01) and multiple taxa were reduced in both niches of

197   AFB mice.

198

199   *Antibiotic treatment stunted the protection from infection-induced morbidity in allergic mice and*

200   *worsened influenza morbidity while impacting inflammation.*

201      Antibiotic (Abx) overuse is a growing concern with both short- and long-term implications. We

202   hypothesized that a microbiome dysbiosis induced by Abx treatment will increase synergistic

203   pathogenesis of IAV and *Spn* in allergic hosts. Mice were treated daily for two weeks with levofloxacin,

204   a commonly used Abx for respiratory infections, prior to virus infection (**Fig 5A**). Antibiotic-treated

205   IAV-infected mice (Flu ctr) had significantly lower nadir than untreated counterparts (**Fig 5B**). Abx

206   treatment did not alter weight curves in the other groups except the triple-disease state (AFB) in which

207   case allergic co-infected mice treated with antibiotics exhibited weight loss similar to Flu control mice

208   (**Fig 5B**). A concomitant increase in the bacterial burden in the AFB group occurred in all three niches

209   tested while the increase in the FB group did not reach statistical significance in the blood (**Fig 5C**).

210      While the total cell numbers were reduced in all groups after levofloxacin treatment (**Fig 6A**)

211   compared to untreated mice (as shown in Fig 2), the cell types most dramatically altered were

212    macrophages and CD8$^+$ T cells (**Fig 6B**). Most cell types were drastically reduced after Abx treatment,

213    while neutrophils were increased in the Flu control and FB groups while CD4$^+$ T cells and CD8$^+$ T cells

214    were increased in the Flu control group (**Fig 6B**). Interestingly, eosinophils were reduced in all groups,

215    but most notably in the AB and AFB groups (**Fig 6B**). When we normalized the cell types that were

216    measured in each group, the immune profiles were notably of different compositions between groups

217    (**Fig 6C**) and in comparison untreated mice.

218         We investigated the pro-inflammatory cytokine milieu in the BAL and the lungs to better

219    understand the immune pressures influencing infiltrating leukocytes and structural cells. In general, the

220    cytokine backdrop in BAL fluid had very little change compared to baseline in all groups except in the

221    FB group (for most analytes) wherein nearly all markers were significantly greater than the AFB group

222    (**Table 1**). Similar trends were observed in the lung homogenates (**Table 1**). Treatment of mice with

223    Abx affected the cytokine profile in both the BAL and lungs with significant increases occurring in both

224    the FB and AFB groups (**Table 1**). However, even after Abx treatment, most cytokines were

225    significantly more highly expressed in the FB compared to the AFB group (**Table 1**).

226

227    *Mucosal microbiome diversity was reduced by antibiotic treatment.*

228         In order to determine if we were successful in inducing dysbiosis, we analysed the microbiome

229    in the BAL and lungs of mice that were treated with Abx in comparison to untreated mice. The PCoA

230    ordination indicated that microbiota of Abx-treated mice formed independent clusters from untreated

231    mice except in the AFB group (**Fig 7**). Differential abundance analyses indicated that the majority of

232    treated mice had reduced taxa abundance. While *Facklamia*, *Bacillaceae*, and *Enterococcus* (p=0.046)

233    were enriched in BAL of Abx-treated Asthma group, significant depletions in were identified in multiple

234    genera especially *Alphaproteobacteria* and *Actinobacteria* (Table S2). Distinct clusters in the lungs of

235    asthma group (**Fig 7A**) showed significant enrichment for *Anaerococcus* of *Clostridia* and *Lactobacillus*

236    in the Abx-treated group, while numerous genera including *Proteobacteria*, *Firmicutes* and

237    *Actinobacteria*, had decreased abundance after Abx treatment.

238         A majority of taxa in the microbiome of Abx-treated Flu control mice were reduced except for a

239    few genera (*Peptoniphilus*, *Selenomonas*, and *Enterococcus*) that were enriched in the BAL clusters (**Fig**

240    **7B**). *Fusobacterium* enriched the lung microbiome cluster of Abx-treated Flu controls while

241    *Streptococcus* enriched both BAL and lung. All identified taxa in the microbiota of Bacteria-only

242    controls had a significantly reduced abundance after Abx-treatment except for *Streptococcus* that was

243    enriched in the lungs with the clearly separated clusters (**Fig 7C**).

244         Individual clusters were observed between Abx-treated and untreated AF group (**Fig 7D**), with

245    *Streptococcus* (p=0.03) and *Bradyrhizobium* (p=0.04) enrichment in BAL and *Leptotrichia* in the lung

246    (p=0.04) of treated AF mice. Multiple genera including *Proteobacteria*, *Firmicutes* and *Actinobacteria*

247    were reduced in the BAL of Abx-treated AB mice while *Lactococcus* and *Sphingopyxis* were enriched.

248    Similarly separated clusters in the AB group lungs (**Fig 7E**) had dynamic enrichment/depletion in

249    *Firmicutes*, *Fusobacteria* and *Proteobacteria* after Abx-treatment.

250         Clearly separated clusters were evident between the Abx-treated and untreated FB group in both

251    niches (**Fig 7F**). Interestingly, the majority of taxa have increased abundance with *Streptococcus* being

252    the only taxa with decreased abundance after treatment in both BAL and lung in FB group. Overlapping

253    clusters between Abx-treated and untreated mouse microbiota in the BAL and lungs were only observed

254    in the AFB group (**Fig 7G**) wherein most of the identified taxa had reduced abundance; the only

255    exceptions were *Streptococcus* and *Anaerococcus* that were enriched after antibiotic treatment in both

256    niches.

257

258    **Discussion**

259         Susceptibility to and severity of respiratory infections are dictated by a multitude of variables

260    from the perspective of both host and pathogen. Numerous predisposing conditions have been described

261     in having both protective and detrimental roles in the susceptibility of patients to respiratory infections.

262     Such underlying conditions include asthma (27, 38), obesity (39, 40), malnutrition (41), impaired liver

263     function (42), and sickle cell disease(43-45). From the standpoint of respiratory pathogens, the disease

264     strategies for two of the major etiological agents, IAV and *Spn*, are well described in terms of the main

265     virulence factors required during infection of the respiratory tract (46-50). Since virulence strategies

266     utilized by pathogens vary considerably based on the host environment, underlying conditions present

267     unique challenges and opportunities for the invading pathogens. The triple-disease murine model

268     utilized here is clinically relevant as asthmatics are considered at risk for respiratory infections and

269     functions as tool with which to investigate complex host-pathogen interactions within the allergic host.

270     Predisposing host conditions provide a unique environment in terms of nutrient bioavailability,

271     receptor expression, and inflammatory status that can alter the virulence strategies of potential

272     pathogens. Influenza synergy with secondary pneumococcal pneumonia is one of the best characterized

273     exacerbations of respiratory infection, with both clinical data and murine models supporting this viral-

274     mediated superinfection (36). Somewhat counterintuitively, our data show that the well-established

275     detrimental synergy between IAV and *Spn (11, 13, 26, 36, 51)* is reduced in the context of experimental

276     allergic asthma where IAV+ *Spn* co-infection in allergic mice resulted in a dramatically altered

277     inflammatory landscape correlating with less overall lung inflammation and damage, reduced morbidity

278     and enhanced bacterial clearance. It has been previously suggested that a rapidly induced short lasting

279     inflammation may hinder IAV pathogenesis, reduce host pathology (52, 53), and beneficial during IAV-

280     *Spn* co-infection (54). Our data suggest that allergic hosts may hold a similar advantage when infections

281     occur during heightened allergic inflammation.

282     It is becoming increasingly evident that resident bacterial species within the respiratory tract can

283     alter infection susceptibility to exogenous viral and bacterial pathogens (55-58). Perturbations to the

284     respiratory microbiome may occur during disease states as well as during Abx treatments (59, 60). Early

285     life exposures to microbial agents have been demonstrated both as triggers (61) and inhibitors (62) of

286  asthma development based on the agent, atopy, and age (63-65). Early Abx use associated with allergic

287  asthma in young children even when accounting for bias inherent from when Abx are commonly

288  prescribed to treat early symptoms of asthma (66, 67). Analysis of microbial communities in the

289  respiratory tract revealed the asthmatic host to have a distinct microbial signature compared to healthy

290  controls, suggesting these alterations in the respiratory microbiome may contribute to the protective

291  capacity of asthma during IAV+*Spn* co-infection. If this mechanism was operative, we hypothesized that

292  perturbation of the microbial communities would diminish the protective capacity of allergic asthma in

293  the context of influenza-pneumococcal co-infection. This was accomplished by Abx administration,

294  which significantly altered the microbial composition of the respiratory tract. Concurrent with this

295  perturbation of the microbial communities was a significant decrease in the protective capacity allergic

296  asthma engenders against IAV+*Spn* co-infection. Similar reductions in host protection occur after

297  perturbations to gut microbiome with Abx (68, 69). However, since microbiome disruption with Abx

298  also resulted in a modification to the immune cell profile (with a notable reduction in macrophages and

299  increase in pro-inflammatory cytokines), increased susceptibility to co-infection in the allergic mice may

300  have resulted from a decrease in immunity. While this 'chicken or egg' paradox is currently under

301  investigation by our group, it is clear that both the microbiome and immune system play a crucial role in

302  mediating host protection during complicated respiratory infections, and that Abx should be prescribed

303  with caution, especially in patients with chronic underlying conditions as microbiome and immune

304  baselines are likely drifted from an otherwise healthy host.

305       The extensive alterations in the respiratory tract during allergic asthma encompass both

306  immunological and microbiological differences that can have profound impact on susceptibility to

307  infection. These data suggest that allergic asthma may provide a significant advantage in terms of

308  morbidly and mortality against certain types of respiratory infections and that this protection is in partly

309  mediated by alterations in the resident flora and immune milieu. Due to the complex interplay between

310  the microbiota and host immunity, there are likely additional host signaling pathways important for the

311     modulation of the sensitivity of the allergic host to infection. The observation that Abx partially

312     ameliorated this protection also underscores the importance of appropriate Abx use, as prior Abx

313     exposure enhances the susceptibility of mice with allergic asthma to subsequent infection. This is of

314     particular importance to asthmatic patients, as the early symptoms of asthma are similar to respiratory

315     infection, potentially resulting in inappropriate Abx usage (70). These data underscore the complex and

316     interwoven relationship between host immune status and the resident bacterial flora in terms of infection

317     susceptibility.

318

### Methods

*Pathogen strains and growth*

321     A clinical Influenza A Virus isolate recovered during the 2009 influenza A virus pandemic

322     (A/CA/04/2009) was gifted by Dr. Webby (St. Jude Children's Research Hospital, Memphis, TN),

323     propagated in Madin-Darby canine kidney (MDCK) cells (ATCC, Manassas, VA), sequence verified to

324     be void of mutations in HA and NA genes, and stored as single use aliquots at -80°C. The concentration

325     of virus was determined by using the $TCID_{50}$ method in MDCK cells and virus was diluted in sterile

326     PBS to desired concentration for mouse inoculations.

327     *S. pneumoniae* A66.1L is a type 3 strain constitutively expressing luciferase (71), gifted to us by

328     Dr. Jon McCullers (University of Tennessee Health Science Center, Memphis, TN).  A66.1L was

329     cultured in Todd-Hewitt broth (Difco Laboratories, Detroit, MI) supplemented with yeast extract

330     (ACROS Organics, NJ)  to $OD_{620}$ 0.15 corresponding to log growth phase. Cultures were centrifuged at

331     2671 *g*/4°C, re-suspended in fresh Todd-Hewitt Yeast broth supplemented with 30% autoclaved

332     glycerol, then frozen in single use aliquots at -80°C. Aliquot concentration was determined by

333     enumerating colony growth on agar plates with 5% sheep blood (Remel, Thermo Fisher, Lenexa, KS) at

334     the time of freezing, and confirmed by plating at least one week after storage at -80°C. To prepare

335     mouse inocula, A66.1L was thawed at room temperature and serially diluted in PBS in 15 mL tubes,

336     then vortexed.  Inocula concentration was confirmed by colony growth on blood agar plates.

337

338     *Ethics Statement*

339     Animal research described was approved by the University of Tennessee Health Science

340     Center's Institutional Animal Use and Care Committee (IACUC) under protocol 15-003.0 and 18-003.0

341     to be in accordance with the Office of Laboratory Animal Welfare guidelines.

342

343     *Animals and housing conditions*

344     No discernible differences in gender were noted after subjecting mice to the fungal asthma and

345     influenza model, female mice were used in these studies. Six week-old female C57BL/6J mice were

346     purchased from Jackson Laboratories (Bar Harbor, ME) and maintained in sterile microisolator cages on

347     α-dri bedding within the animal biosafety level-2 facilities at SJCRH and University of Tennessee

348     Health Science Center for 1 week prior to being used in experiments. The animal housing facilities were

349     on a 12 h light-dark cycle and all work with animals was done during the light cycle.  Animals were fed

350     autoclaved chow and provided autoclaved water in bottles *ad libitum*.

351

352     *Mouse fungal asthma model*

353     Mice were subjected a previously described and characterized model of *Aspergillus fumigatus*-

354     induced allergic asthma (33, 72). In brief, whole *A. fumigatus* extract (Greer Labs, Lenoir, NC) was used

355     to sensitize mice over a period of five weeks prior to subjecting them to airborne dry conidia in a nose-

356     only inhalation chamber for 10-minutes two weeks apart (25). Allergen sensitized and challenged mice

357     were referred to as the "Asthma Ctr" group.

358

359     *Animal infections*

360    Mice were lightly anesthetized with isoflurane and intranasally infected with 1000 TCID$_{50}$

361    A/CA/04/2009, diluted in 50 µL PBS. Seven days after the viral infection, mice were anesthetized with

362    isoflurane and intranasally infected with 600 colony forming units (CFU) of A66.1L in 100 µL PBS.

363    Each mouse was weighed prior to challenge and every 24 hours after until harvest to monitor weight

364    change. Animals that lost more than 30% of their starting body weight, or exhibited severe signs of

365    morbidity, were euthanized for ethical reasons and recorded as having died on that day. Each group of

366    mice that were infected with a single agent were named after the pathogen as influenza virus (Flu Ctr)

367    and bacteria (Bact Ctr) only. Naïve (N) mice that had no treatments served to determine baseline

368    immune and microbiome information. Allergic mice that were infected with influenza (Flu) virus (23)

369    were referred to as the "Asthma and Influenza" (AF) group and those additionally infected with bacteria

370    were considered the "Asthma, Influenza, and Bacteria" (AFB) group. Allergic mice that were infected

371    with bacteria were referred to as the "Asthma and Bacteria" (AB) group.

372

373    *Antibiotic treatments*

374    Naïve and mice in the various treatment groups were intraperitoneally administered 12.5 mg/kg

375    levofloxacin (Akorn Inc., Lake Forest, IL) daily at the equivalence of one week after the first allergen

376    challenge and ending on the day of viral infection. No visible differences occurred during disease

377    pathogenesis in mice after antibiotic treatment.

378

379    *Bioluminescence measurements*

380    The use of the A66.1L strain allowed for the visualization of bacteria during the active infection

381    in mice. We measured bioluminescence by a Lumina IVIS CCD camera (Perkin Elmer, Waltham, MA).

382    Images were processed with Living Image software, version 4.5.5. Settings for image acquisition were a

383    one second photograph following by one minute luminescence measurement with the bining set at four.

384

385    *Tissue harvest and bacterial enumeration*

386         Tissues were harvested within a class 2A biosafety cabinet with strict adherence to aseptic

387    technique to ensure that samples were not contaminated by exposure to environmental agents.

388    Bronchoalveolar lavage (BAL) was performed using two consecutive infusions of 1 mL sterile PBS.

389    BAL cells were cytospun and stained with Diff-quik (StatLab, McKinney, TX) for visualization. Cells

390    were, then centrifuged at 4°C and BAL fluid (BALF) was stored at -80°C until use. Red blood cells in

391    the cells pellet were removed by lysis with cold water and resultant cells enumerated on a Countess

392    Automated Cell Counter (Invitrogen, Carlsbad, CA) then stained for flow cytometry.

393         Blood was recovered from the chest cavity, and serum was separated by centrifugation and

394    stored at -80°C. Lungs were excised and homogenized in 1 mL PBS containing cOmplete Proteinase

395    Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) then centrifuged at 4°C to remove cell

396    debris. Cell-free supernatant was stored at -80°C until cytokine quantitation. In some experiments,

397    spleen, mediastinal lymph nodes and left lung lobes were perfused with 10% neutral-buffered formalin

398    and processed for hematoxylin and eosin staining.

399         Whole lung homogenate, whole blood, and BAL samples were serially diluted in sterile PBS and

400    10 μL of dilutions were plated on blood agar plates. Plates were incubated at 37°C with 5% $CO_2$ for 12-

401    13 hours, and colony growth was enumerated.

402

403    *Histopathologic analysis*

404         Lungs were first infused and then immersion-fixed in 10% neutral buffered formalin before

405    processing and embedding in paraffin. Tissue sections were stained with hematoxylin and eosin, and the

406    severity and extent of specific pulmonary lesions such as interstitial and alveolar inflammation, alveolar

407    protein exudate, hyaline membrane formation, septal thickening, epithelial hypeplasia, and denuded

408    bronchioles, were assessed and graded in a blinded manner by a veterinary pathologist. Separate severity

409    grades for each type of lesion were assigned as follows: 0, no lesions detected; 1, minimal, rare, barely

410   detectable lesions; 2, mild multifocal, small focal, or widely separated lesions; 3, moderate, multifocal,

411   and prominent lesions; 4, marked, extensive-to-coalescing areas; and 5, severe and extensive lesions

412   with pulmonary consolidation. These severity grades were then converted to weighted semi-quantitative

413   scores as follows: 0 = 0; 1 = 1; 2 = 15; 3 = 40; 4 = 80; and 5 = 100.

414

415   *Flow cytometric analysis*

416   Cells in the airways were identified using flow cytometry. BAL cells were incubated with human

417   γglobulin to prevent nonspecific binding of antibodies to Fc-receptor, then stained with fluorescently

418   tagged antibodies. Samples were fixed with BD Biosciences stabilizing fixative and stored in the dark at

419   4˚C until acquisition on a LSR Fortessa (BD Biosciences, San Jose, CA). Data were analyzed using

420   FlowJo v 10.1r5 (FlowJo LLC, Ashland, OR). Unstained cells, single-color controls and isotype controls

421   were used for instrument settings and compensation. Abs were purchased from BD Biosciences unless

422   specified otherwise. Antibodies used in this study include: CD19-PerCP/Cy5.5 (1D3), CCR3-A647

423   (83103), CD3e-PE/Cy7 (145-2C11), CD4-A700 (RM4-5), CD8a-FITC (53-6.7), Ly6G-V450 (1A8),

424   Mac3-Biotin (ebioABL-93, eBioscience|Thermo Fisher, Waltham, MA), NK1.1-APC/Cy7 (PK136),

425   NP-dextramer-PE (Immudex, Fairfax, VA), Siglec-F-PE-CF594 (E50-2440), Streptavidin-BV605

426   (BioLegend) and matching isotypes control antibodies.

427

428   *Cytokine quantitation*

429   Cytokines present in the airways and interstitial lung tissue were quantitated using magnetic

430   Luminex multiplex assays (R&D) according to the manufacturer's instructions. BALF and cell-free lung

431   homogenates were stored at -80°C in single-use aliquots after harvest until cytokine assay, and used

432   undiluted or diluted ½ in Calibrator Diluent (R&D Systems (Minneapolis, MN), respectively. Beads

433   were read using a Luminex MagPix (R&D Systems). Analyte concentration was determined using

434   MagPix software xPONENT 4.2. Samples which had a reading below the limit of detection were

435     assigned the value of the limit of detection. The mean concentration for the group for each analyte was

436     used to calculate the fold change compared to the mean concentration of the same analyte in the naïve

437     group.

438

439     *16S sequence analysis*

440         BAL and whole lung homogenates were centrifuged at 500 ×*g* at 4°C to remove cell debris, then

441     subsequently centrifuged at 2671 ×*g* at 4°C to pellet bacteria in the samples.  Bacterial pellets were re-

442     suspended in 50 µL of BALF or lung homogenate supernatant and stored at -80°C until DNA extraction.

443     Samples were thawed on ice and DNA was extracted using FastDNA-96 Soil Microbe DNA kit (MP

444     Biomedicals, Santa Ana, CA) according to the manufacturer's instructions, and a FastPrep-96

445     instrument (MP Biomedicals).  Extracted nucleic acid was stored at -80°C until use.

446         The V1-V3 variable region of the bacterial 16S gene was amplified using the Bioo NEXTflex

447     16S amplicon library preparation kit. The multiplexed products were then subsequently utilized for high-

448     depth sequencing on the Illumina Mi-seq platform to obtain comprehensive relative abundance of the

449     bacterial composition at the respective time points with 300 bp paired end read lengths. The quality of

450     the raw 16S rRNA pair-ended reads are initially examined by FastQC.(73) Low quality reads and bases

451     are trimmed by Trim Galore (74). The reads are then merged by PANDAseq(75) and subsequently

452     processed by QIIME (76). The closed reference mapping protocol of QIIME was used for OTU

453     assignment. Specifically, the read sequences were clustered into Operational Taxonomic Units (OTUs)

454     at 97% sequence similarity using the UCLUST algorithm (77). A representative sequence was then

455     selected from each OTU for taxonomic assignment using the Greengenes database (78) as the reference.

456     The alpha diversity estimates were calculated using the R Phyloseq package (79). Kruskal-Wallis non-

457     parametric tests was used to test the significance of the diversity difference. The linear discriminant

458     analysis (LDA) effect size (LEfSe) method was used to test the significant difference of relative

459     abundance of taxa among groups (80).

460

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469

## Author Contributions

470

471      KSL performed infections, harvested tissues, determined bacterial titers, performed flow

472 cytometry and analysis, extracted DNA for 16S sequencing and multiplex for cytokines. ARI performed

473 pilot studies to determine optimum bacterial infections, harvested tissues, determined bacterial burden

474 by IVIS CCD camera, and performed qPCR for BAL and lung samples prior to genomic sequencing for

475 microbiome analyses. T-CC performed data analyses for microbiome. MP subjected mice to the fungal

476 asthma model, helped with tissue harvest, treated mice with antibiotics, and performed viral titrations.

477 PV performed histopathologic analyses. JWR designed the microbiome and antibiotic treatment studies.

478 AES designed and directed the studies, analysed data, and created Figures. KSL, JWR, and AES co-

479 wrote the first draft of the manuscript, and all authors edited and approved the final version.

480

481 **Competing Interests:** The authors have no conflicts of interest to declare.

482

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701    **Supporting Information Legends**

702

703    **Supplemental Table 1.** Bacterial classifications based on 16S sequence analysis for lungs and BAL

704    from A, AF, AB, AFB, F, and B groups.

705

706    **Supplemental Table 2.** Bacterial classifications based on 16S sequence analysis for lungs and BAL

707    from A, AF, AB, AFB, F, and B groups from mice on levofloxacin prophylaxis.

708

709    **Table**

710    **Table 1. Fold change in mucosal cytokines in mice subjected to allergen and infectious agents.**

711    Cytokine levels in the bronchoalveolar lavage fluid (BALF) and lungs were determined for each group

712    and used to determine the change over baseline (naïve) levels in mice that were treated with antibiotics

713    and those that were not treated. Data were analysed by two-way ANOVA with Dunnett's multiple

714    comparisons test.  F and Flu: influenza; B and Bact: bacteria; A: asthma.

715

716    **Figure Legends**

717

718    **Figure 1. Allergic airways disease protects the host from synergistic morbidity from influenza and**

719    **bacterial pneumonia.** Timeline of triple-disease model (A) wherein allergen sensitized and challenged

720    mice are infected with influenza A virus (pH1N1) and *Streptococcus pneumonia* (*Spn*). Weight loss in

721    each group and comparative statistics associated with weight loss (B). Bioluminescence reading for

722    bacteria in mice and harvested lung lobes (C). Bacterial burden in the bronchoalveolar lavage (BAL),

723    lung homogenate, and blood (D). Data are representative of one study from four independent studies.

724    n=5-7 mice per group. Data analysed by one- or two-way ANOVA and *P<0.05, **P<0.01, and

725    ***P<0.001. SQ: subcutaneous; IN: intranasal; IH: inhalation; F and Flu: influenza; B and Bact:

726    bacteria; A: asthma.

727

728   **Figure 2. Inflammatory cell profile in the bronchoalveolar lavage compartment of mice after**

729   **triple-disease model.** Cells purified from the BAL were enumerated (A) and used for flow cytometry to

730   identify changes in cell types (B). The percentage of each cell type was used to normalize data to

731   identify differences in these cell types in the airways during various disease conditions (C). Data are

732   representative of one study from four independent studies. n=5-7 mice per group. Data were analysed by

733   Mann-Whitney test against the direct control group for each experimental condition. *P<0.05 and

734   **P<0.01. Ctr: control; F and Flu: influenza virus; B and Bact: bacteria; A: asthma; N: naïve.

735

736   **Figure 3. Pulmonary tissue inflammation in triple-disease model.** Hematoxylin and eosin stained

737   sections of naïve mice showed no visible inflammation, while mice with asthma showed inflammation

738   around the large airways. Influenza (Flu) virus infected mice had some parenchymal inflammation

739   which was also evident in the asthma and flu (AF) dual disease mice. While no inflammation was

740   evident in the bacteria (Bact) group, allergic mice infected with bacteria (AB) had some inflammatory

741   foci. Co-infection with Flu and Bact (FB) had significant inflammation in the parenchymal spaces in

742   stark contrast to co-infected allergic mice (AFB). While scored criteria of inflammation were reduced in

743   AFB compared to FB, mucus production was maintained at high levels in all allergic mice. Data are

744   representative of one study from two independent studies. n=5-7 mice per group. Data were analysed by

745   Mann-Whitney test against the direct control group for each experimental condition. *P<0.05, **P<0.01,

746   and ***P<0.001.

747

748   **Figure 4. Microbiome profiles of each disease group in comparison with the microbiome profile of**

749   **naïve mice.** Principle coordinate analyses of the microbiome abundance revealed clear separation of

750   clusters in the comparisons of AF in BAL, FB and AFB in both BAL and lung. Data are representative

751   of one study of two independent studies.

752

**Figure 5. The impact of antibiotic treatment on disease pathogenesis of respiratory infections in**

**allergic hosts.** Timeline of model including antibiotic treatments (A). Changes in weight in each group

in antibiotic treated mice in comparison to mock-treated groups (B). Bacterial burden in each disease

group in comparison to antibiotic treated groups (C). Data are representative of one independent study.

n=5-6 mice per group. Data in B were analysed by two-way ANOVA with Sidak's multiple comparisons

test and Data in C were analysed by Mann-Whitney test. *P<0.05, **P<0.01, and ***P<0.001. Ctr:

control; F and Flu: influenza virus; B and Bact: bacteria; A: asthma.

760

**Figure 6. The impact of antibiotic treatment on the immune cell profile in triple-disease model.**
The number of live leukocytes in the bronchoalveolar lavage (BAL) were enumerated in each group (A)

and the various cell populations were identified by flow cytometry (B). Normalized cell populations

were used to identify shifts in cell types in each disease state (C). Data are representative of one study

from four independent studies. n=5-6 mice per group. Data were analysed by Mann-Whitney test against

the direct control group for each experimental condition. *P<0.05 and **P<0.01. Ctr: control; F and Flu:

influenza virus; B and Bact: bacteria; A: asthma; N: naïve.

768

**Figure 7. Microbiome profiles of each disease group with and without antibiotics (Abx).** Principle

component analysis of Abx-treated mice in each group (blue) compared to untreated mice (red) showed

independent clustering of microbiome for all study groups except in the AFB. Data are representative of

one study.

773 **Table 1. Mucosal Cytokines in Mouse Bronchoalveolar Lavage Fluid (BALF) and Lung**
774 **Homogenate.**

775 *Untreated mice*

| BALF | Fold increase over Naïve | | | | | | |
|---|---|---|---|---|---|---|---|
| | Asthma | Flu-ctr | Bact-ctr | AF | AB | FB | AFB |
| CRG2 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $10.75^+$ | $1.86^*$ |
| G-CSF | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $14.19^+$ | $1.58^*$ |
| IFNγ | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $3.89^+$ | $1.03^*$ |
| IL-1α | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.49 | 1.00 |
| IL-6 | 1.00 | 1.04 | 1.00 | 1.00 | 1.00 | $23.66^+$ | $5.68^*$ |
| IL-10 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| KC | 1.00 | 1.23 | 1.00 | 1.00 | 1.00 | $5.10^+$ | $1.38^*$ |
| M-CSF | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.25 | 1.00 |
| MCP-1 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $9.33^+$ | $1.15^*$ |
| MIP-1α | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $18.89^+$ | $0.55^*$ |
| MIP-1β | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $78.25^+$ | $11.57^*$ |
| MIP-2 | 1.00 | 1.02 | 1.00 | 1.00 | 1.00 | $54.25^+$ | $4.40^*$ |
| RANTES | 1.00 | 1.54 | 1.00 | 1.00 | 1.00 | $10.50^+$ | $1.61^*$ |
| TNFα | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $26.33^+$ | $3.88^*$ |

776

777 *Levofloxacin-treated mice*

| BALF | Fold increase over Naïve | | | | | | |
|---|---|---|---|---|---|---|---|
| | Asthma | Flu-ctr | Bact-ctr | AF | AB | FB | AFB |
| CRG2 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $11.85^+$ | $9.39^{+\#}$ |
| G-CSF | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $34.05^+$ | $7.48^{*\#}$ |
| IFNγ | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $2.26^+$ | $1.57^\#$ |
| IL-1α | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $2.74^{+\#}$ | $1.00^*$ |
| IL-6 | 1.00 | 1.74 | 1.00 | 1.00 | 1.00 | $28.34^+$ | $19.97^{+\#}$ |
| IL-10 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $13.07^{+\#}$ | $1.00^*$ |
| KC | 1.00 | 1.14 | 1.00 | 1.00 | 1.00 | $5.67^+$ | $4.47^{+\#}$ |
| M-CSF | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | $1.58^+$ | $1.00^*$ |
| MCP-1 | 1.01 | 1.00 | 1.00 | 1.00 | 1.00 | $11.84^{+\#}$ | $4.85^{+\#*}$ |
| MIP-1α | 1.82 | 1.10 | 1.00 | 1.00 | 1.00 | $85.99^{+\#}$ | $6.53^*$ |
| MIP-1β | 1.57 | 1.00 | 1.00 | 1.00 | 1.00 | $83.89^+$ | $48.14^{+*\#}$ |
| MIP-2 | 7.18 | 2.07 | 1.00 | 2.10 | 1.33 | $144.52^{+\#}$ | $19.94^*$ |
| RANTES | 1.00 | 2.14 | 1.13 | 1.32 | 1.00 | $9.91^{+*}$ | $4.78^{+*\#}$ |
| TNFα | 1.00 | 1.47 | 1.00 | 1.00 | 1.00 | $24.03^+$ | $17.41^{+*\#}$ |

778

779 $^+p<0.05$ compared to Naïve; $^*p<0.05$ AFB compared to FB; $^\#p<0.05$ Abx-treated compared to untreated

780 *Untreated mice*

| Lung tissue | Fold increase over Naïve | | | | | | |
|---|---|---|---|---|---|---|---|
| | Asthma | Flu-ctr | Bact-ctr | AF | AB | FB | AFB |
| CRG2 | 1.00 | 1.52 | 1.00 | 1.00 | 1.00 | 36.30[+] | 3.23[*] |
| G-CSF | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 55.24[+] | 3.19[*] |
| IFNγ | 0.89 | 1.08 | 1.08 | 0.98 | 1.21 | 7.12[+] | 2.38[*] |
| IL-1α | 1.04 | 0.70 | 1.15 | 0.84 | 1.12 | 9.67 | 1.78[*] |
| IL-6 | 0.99 | 0.55 | 1.02 | 0.74 | 0.87 | 11.64 | 2.75 |
| IL-10 | 1.00 | 0.65 | 1.08 | 0.92 | 1.06 | 1.06 | 1.13 |
| KC | 1.06 | 0.56 | 1.11 | 0.82 | 0.95 | 2.07 | 1.1 |
| M-CSF | 0.86 | 0.74 | 1.05 | 0.88 | 0.96 | 2.27 | 0.83 |
| MCP-1 | 1.00 | 3.11 | 1.00 | 1.29 | 1.21 | 38.58[+] | 3.05[*] |
| MIP-1α | 1.88 | 7.11 | 1.00 | 2.71 | 3.13 | 185.27[+] | 10.79[*] |
| MIP-1β | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 209.57 | 18.17 |
| MIP-2 | 1.75 | 4.13 | 1.11 | 2.01 | 2.45 | 994.52[+] | 65.47[*] |
| RANTES | 1.42 | 3.87 | 1.00 | 2.22 | 1.50 | 19.24[+] | 3.03[*] |
| TNFα | 1.01 | 1.20 | 1.14 | 1.16 | 1.19 | 24.78[+] | 3.09[*] |

781

782 *Levofloxacin-treated mice*

| Lung tissue | Fold increase over Naïve | | | | | | |
|---|---|---|---|---|---|---|---|
| | Asthma | Flu-ctr | Bact-ctr | AF | AB | FB | AFB |
| CRG2 | 1.00 | 1.46 | 1.00 | 1.00 | 1.00 | 28.46[+] | 41.95[+*#] |
| G-CSF | 1.00 | 1.00 | 2.32 | 1.00 | 1.00 | OHR | 24.60[+#] |
| IFNγ | 1.00 | 1.00 | 1.00 | 1.00 | 1.33 | 3.41[#] | 9.21[+*#] |
| IL-1α | 1.54 | 1.08 | 1.34 | 1.53 | 2.92 | 25.9[+#] | 24.55[+#] |
| IL-6 | 1.44 | 0.96 | 1.40 | 1.39 | 2.70 | 457.70[+#] | 42.32[*] |
| IL-10 | 1.17 | 0.95 | 1.00 | 1.03 | 2.23 | 46.6[+#] | 1.9[*] |
| KC | 1.22 | 0.67 | 1.27 | 1.11 | 2.69 | 4.75[+#] | 4.02[+#] |
| M-CSF | 1.00 | 1.00 | 1.00 | 1.00 | 1.16 | 76.80[+#] | 1.64[*] |
| MCP-1 | 1.00 | 2.14 | 0.86 | 1.49 | 1.00 | 66.07[+#] | 17.55[*] |
| MIP-1α | 1.02 | 5.32 | 1.00 | 2.03 | 1.03 | OHR | 75.72[+#] |
| MIP-1β | 1.08 | 1.00 | 1.00 | 1.00 | 1.00 | 978.33[+#] | 107.51[+*] |
| MIP-2 | 6.72 | 3.50 | 3.25 | 2.28 | 2.28 | OHR | 576.50[#] |
| RANTES | 1.83 | 6.87 | 1.28 | 5.11 | 2.75 | 18.47[+] | 24.60[+*#] |
| TNFα | 1.03 | 1.00 | 1.00 | 1.00 | 1.68 | 27.42[+] | 17.48[+#] |

783 [+] $p < 0.05$ compared to Naïve
784 [*] $p < 0.05$ AFB compared to FB
785 [#] $p < 0.05$ levofloxacin-treated compared to untreated

# Figure 1

## A



*Aspergillus fumigatus* fungal asthma model

SQ-IP     IN     IN     IN     IH     IH

pH1N1    *Spn*

Weeks:   1     3    4    5    6     8    9    10

Days: 0     3

Endpoint

## B



pH1N1

A66.1L

Change from initial weight (%)

Time after A/CA/04/2009 infection (days)

- ■ Flu Ctr
- □ FB
- ■ AF
- ■ AFB

| Comparison | D5 | D6 | D7 | D8 | D9 | D10 | D11 |
|---|---|---|---|---|---|---|---|
| F vs AF | *** | *** | *** | *** | *** | *** | ** |
| F vs FB | ns | ns | ns | ns | ns | *** | *** |
| F vs AFB | *** | *** | *** | *** | *** | *** | * |
| AF vs FB | *** | *** | *** | *** | *** | *** | *** |
| AF vs AFB | ns | ns | ns | ns | ns | ns | ns |
| FB vs AFB | *** | *** | *** | *** | *** | *** | *** |

## C



AB     FB     AFB

## D



BAL     Lung     Blood

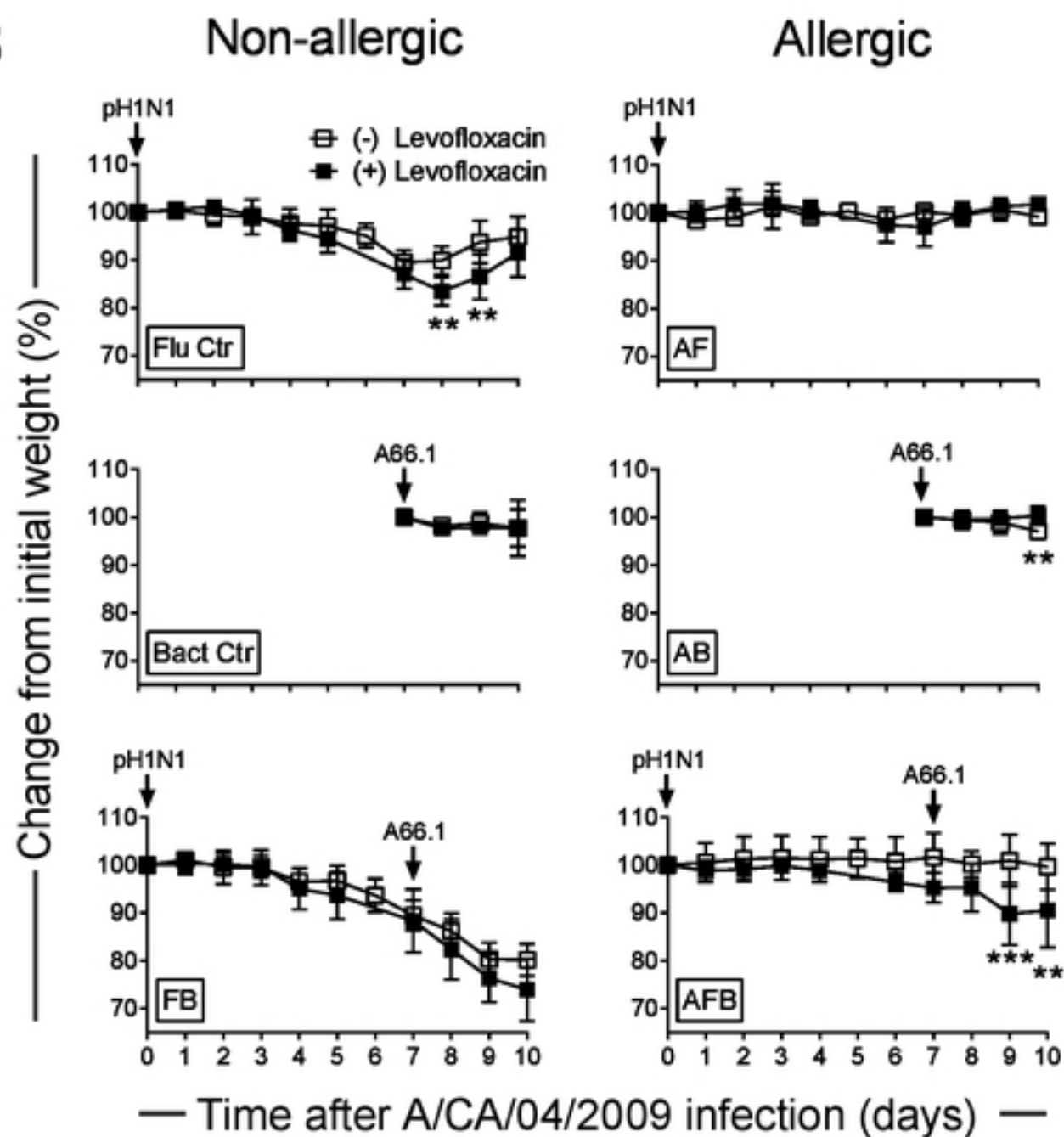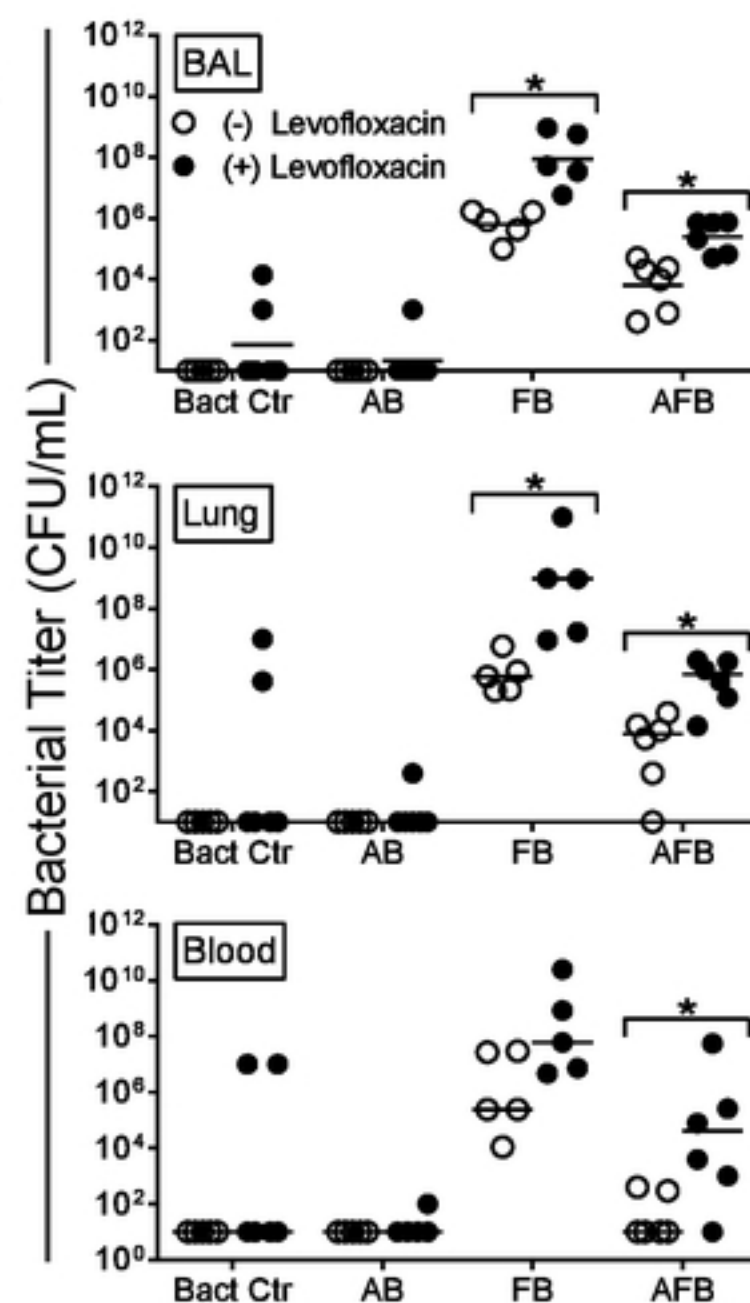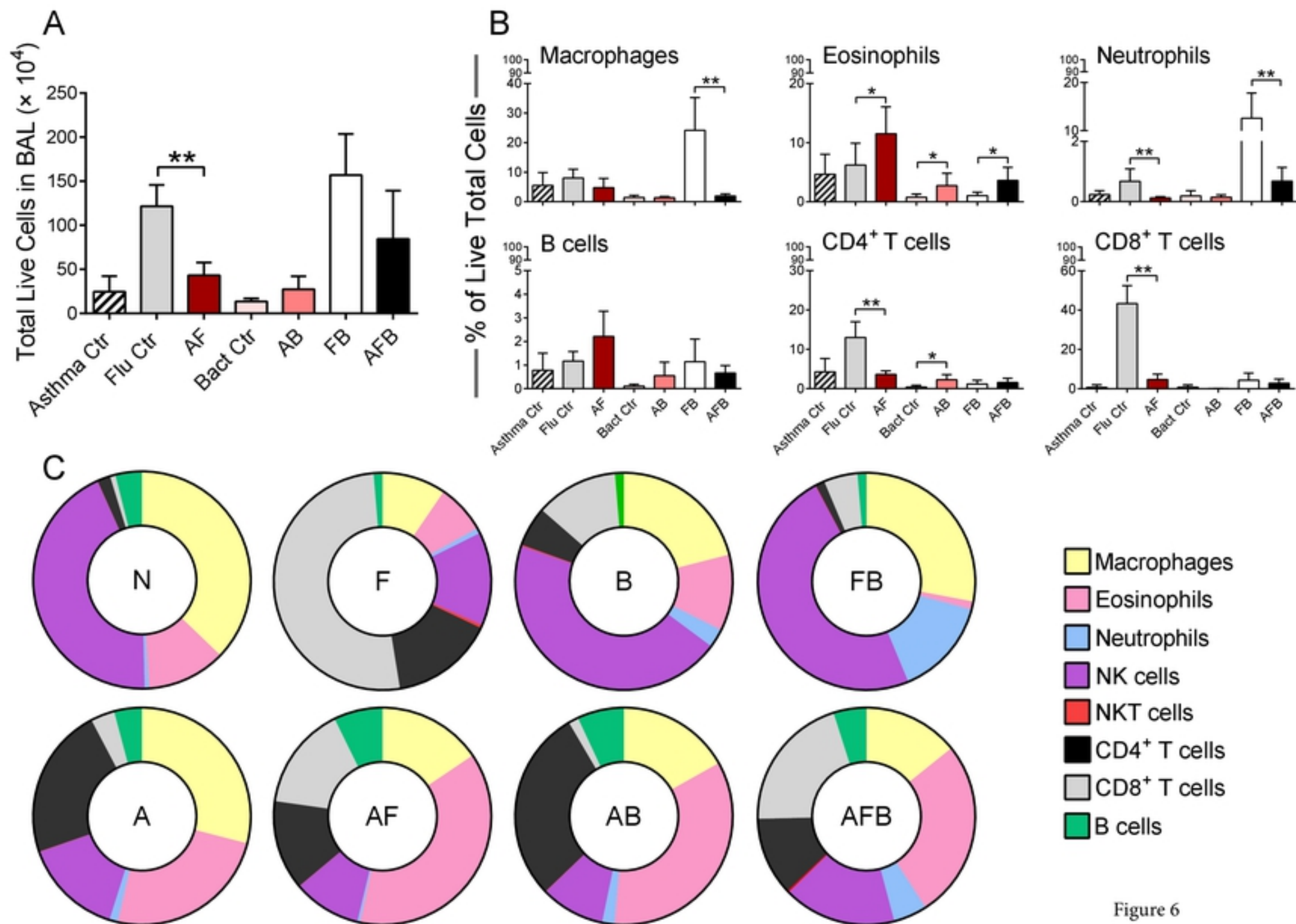Bacterial Titer (CFU/mL)

Bact Ctr   AB   FB   AFB

# Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

# Figure 7