**Airway Prevotella promote TLR2-dependent neutrophil activation and rapid clearance of Streptococcus pneumoniae from the lung**

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This study investigates how specific members of the lung microbiome influence the early immune response to infection. *Prevotella* species are a major component of the endogenous airway microbiota. Increased abundance of *Prevotella melaninogenica* correlates with reduced infection with the bacterial pathogen *Streptococcus pneumoniae*, indicating a potentially beneficial role. Here, we show that *P. melaninogenica* enhances protection against *S. pneumoniae*, resulting in rapid pathogen clearance from the lung and improved survival in a mouse lung co-infection model. This response requires recognition of *P. melaninogenica* lipoproteins by toll-like receptor (TLR)2, the induction of TNFα, and neutrophils, as the loss of any of these factors abrogates *Prevotella*-induced protection. Improved clearance of *S. pneumoniae* is associated with increased serine protease-mediated killing by lung neutrophils and restraint of *P. melaninogenica*-induced inflammation by IL-10 in co-infected mice. Together, these findings highlight innate immune priming by airway *Prevotella* as an important protective feature in the respiratory tract.
The respiratory tract is home to a diverse microbial community that varies in composition by anatomical location and over the course of life. *Prevotella* are frequently identified as one of the top three most abundant bacteria detected in the oral cavity and lungs of healthy adults. While the largest biomass and most stable microbial communities in the respiratory tract are located in the upper airway, the lung is constantly exposed to these bacteria by micro-aspiration. Bacteria from the oral cavity are the dominant source of lung microbiome “seeding.” In the absence of the steep oxygen gradients that influence the early immune response to infection with the bacterial pathogen *Streptococcus pneumoniae* (the pneumococcus), *S. pneumoniae* is the most common cause of community-acquired pneumonia, which has an estimated economic impact of $17 billion in the U.S. alone and is the leading cause of death in children under 5 years old. Pneumococcal vaccines are extremely effective against invasive disease, but are not as protective against pneumonia, and the phenomenon of serotype replacement has increased the number of non-vaccine strains in circulation. Asymptomatic colonization of the nasopharynx with *S. pneumoniae* is a prerequisite for disease development, following invasion to other tissues including the lung. However, it remains unclear why some individuals are more predisposed to lung infection with *S. pneumoniae* compared with others. The microbiome is thought to help protect against bacterial lung infections, which are worsened in antibiotic treated and germ-free mice. *S. pneumoniae* also persists more readily following antibiotic therapy. *Prevotella* are a candidate “protective” member of the airway microbiome in the context of *S. pneumoniae* infection, as several studies indicate an association between increased abundance of *Prevotella* and reduced *S. pneumoniae* in both the upper airway and lung. Similarly, *Prevotella* species including *P. melaninogenica* are more abundant in healthy adults than those with pneumonia. While species-level classification in such analyses is limited, in one report *P. melaninogenica* was ranked as the most discriminative operational taxonomic unit (OTU) differentiating healthy adults from patients with pneumococcal pneumonia. *P. melaninogenica* is an important member of the endogenous airway microbiota, as it is part of the core oral microbiome and selectively enriched in the lung. The factors underlying the potential benefit of airway *Prevotella* on protection against pneumococcal pneumonia are unresolved. In healthy adults, the enrichment of *Prevotella* rRNA gene concentration in bronchoalveolar lavage fluid (BAL) is associated with sub-clinical inflammation, including increased numbers of activated neutrophils. However, *Prevotella*-induced inflammation is subdued compared to that induced by opportunistic bacterial pathogens. For example, lung transplant recipients with *Prevotella*-dominant BAL microbiome profiles had lower innate immune activation profiles than those with *Staphylococcus* or *Pseudomonas*-dominant microbiomes. In mice, lung exposure to *Prevotella melaninogenica* induced less toll-like receptor (TLR) 2-dependent inflammation than the pathogen *Haemophilus influenzae*. Similarly, another *Prevotella* species induced TLR2 signaling but fewer pro-inflammatory cytokines than a cystic fibrosis (CF) pathogen in human CF bronchial epithelial cells. In the context of CF, both positive and negative roles have been attributed to anaerobic bacteria including *P. melaninogenica*, though *Prevotella* abundance correlates with milder CF in some cases. *Prevotella* species are not universally beneficial or benign members of the airway microbiome. Unlike *P. melaninogenica*, *Prevotella intermedia* is frequently isolated from periodontal abscesses, expresses virulence factors including a type 6 secretion system (T6SS), and enhances susceptibility to *S. pneumoniae* by upregulating a critical host adherence receptor. *P. melaninogenica* has been identified in polymicrobial lung abscesses, though it is unclear which species drive abscess formation, and causes increased tissue damage in the context of bleomycin-induced pulmonary fibrosis in mice. These studies suggest that the overall beneficial versus detrimental effects of *Prevotella* on airway health depend on the amplitude and context of *Prevotella*-induced immune responses.

For this study, we developed an animal model to determine how airway *Prevotella* modulate innate immune-mediated protection against *S. pneumoniae*. Using this model, we identify an innate immune response induced by several *Prevotella* species including *P. melaninogenica* associated with rapid clearance of *S. pneumoniae* from the lung, highlighting airway *Prevotella* as an important immune regulatory signature that contributes to respiratory tract health.

**Results**

*P. melaninogenica* enhances clearance of *S. pneumoniae*. While the murine airway microbiome contains both *Prevotella* and *Streptococcus* species, the strains used in these studies are not resident members, allowing us to control exposures to each bacterium. *Prevotella* aspiration was modeled in mice by instilling *P. melaninogenica* intratracheally (i.t.) prior to challenge with *S. pneumoniae* (Fig. 1a). *S. pneumoniae* burdens at 24 h post-infection were not detected in any mice pre-exposed to an equivalent dose of live *P. melaninogenica*, compared to burdens of ~10^5 colony forming units (CFUs) in mice infected with *S. pneumoniae* alone (Fig. 1b). Titration of *P. melaninogenica* revealed that significant protection was maintained with up to a 100-fold lower dose of live *P. melaninogenica* than that of *S. pneumoniae* (Fig. 1b). Burdens of 10^5 CFU/mL *P. melaninogenica* have been reported in human lung lavage fluid, which falls within this protective range. These data suggest that exposure to live *P. melaninogenica* mediates rapid clearance of *S. pneumoniae* from the murine lung.

We next examined the impact of inactivated (heat-killed, HK) *P. melaninogenica* on survival following a lethal dose of *S. pneumoniae*. Exposure to *P. melaninogenica* significantly increased the probability of survival, with 33% of *Prevotella*-exposed mice succumbing to infection compared to 85% of mice infected with *S. pneumoniae* alone (Fig. 1c). Improved survival in mice exposed to *P. melaninogenica* correlated with lower pneumococcal burdens in the lung 3 days post-infection (Supplementary Fig. 1a). The rapid protection induced by *P. melaninogenica* was not dependent on instillation of *Prevotella* into the lung, as intranasal inoculation similarly enhanced *S. pneumoniae* clearance by 24 h (Supplementary Fig. 1b). *P. melaninogenica* was protective against both serotype 2 *S. pneumoniae*, which spreads systemically by 24 h, and serotype 3 *S. pneumoniae*, which is restricted to the lung (Fig. 1c, d). Together, these data indicate that exposure to live or inactivated *P. melaninogenica* protects against *S. pneumoniae* infection.

We next determined whether instillation of any HK bacterium enhances protection against *S. pneumoniae*. Initially, we investigated other HK bacteria which are common members of the upper airway microbiota and are known to mediate direct competition with *S. pneumoniae*, including two *Corynebacterium* species and *Streptococcus salivarius*. In contrast to live strains, two of which reduce *S. pneumoniae* infection in a similar pre-exposure model, none of the HK strains were protective.
(Supplementary Fig. 1e), indicating a unique effect mediated by *P. melaninogenica* HK compared with these airway commensals, which like *S. pneumoniae* are Gram-positive. *P. melaninogenica* is a Gram-negative bacterium, and the lipopolysaccharide (LPS) of Gram-negative bacteria is an abundant TLR4 agonist. We next compared the impact of exposure to Gram-negative *Escherichia coli* HK as well as *E. coli* LPS, which improves protection against *E. coli* LPS (Fig. 2b). The recruitment and activation of innate immune cells in the lung was evaluated by intracellular flow cytometry (Supplementary Fig. 2a). *P. melaninogenica* significantly enhanced the recruitment of myeloid cells including inflammatory monocytes and neutrophils, similar to *E. coli* LPS (Fig. 2c, d and Supplementary Fig. 2b, c). However, unlike *E. coli* LPS, *P. melaninogenica* also induced TNFα production in lung neutrophils (Fig. 2e). Neither stimulus affected the recruitment or TNFα production of CD11b+ dendritic cells (DCs) (Supplementary Fig. 2d, e). These data indicate that exposure to *P. melaninogenica* induces a pro-inflammatory response in the lung associated with increased neutrophil recruitment and activation.

**P. melaninogenica**-exposed mice had fewer alveolar macrophages (AMs) in their lungs than mice exposed to either PBS or *E. coli* LPS (Supplementary Fig. 2f). Others have reported that *E. coli* LPS activates AM production of TNFα, which we confirm, though this response was not induced by *P. melaninogenica* (Supplementary Fig. 2g). These findings indicate distinct lung innate immune cell activation profiles for mice exposed to *E. coli* LPS compared with *P. melaninogenica*, which unlike *E. coli* LPS is protective against *S. pneumoniae*.

**Neutrophils and TNFα are critical for *P. melaninogenica* protection.** Neutrophils are important for *S. pneumoniae* killing at
early time points, prior to the development of specific immunity, which is typically required for clearance. Exposure to *P. melaninogena* HK increased the number of neutrophils recruited to the lungs in response to *S. pneumoniae* infection (Fig. 3a). To address the contribution of neutrophils to pneumococcal clearance in *Prevotella*-exposed mice, we depleted neutrophils prior to *S. pneumoniae* infection. In the absence of neutrophils, *P. melaninogena* was no longer protective against *S. pneumoniae*, as lung burdens in *Prevotella*-exposed mice were similar to those in mice infected with *S. pneumoniae* alone (Fig. 3b). These data indicate that neutrophils are required for *P. melaninogena*-mediated protection against *S. pneumoniae*. *P. melaninogena*-induced TNFα was significantly reduced in the lungs of neutrophil-depleted mice, indicating neutrophils as a major source of TNFα in this setting (Supplementary Fig. 2h). We next considered whether the pro-inflammatory response associated with elevated neutrophil TNFα is required for *P. melaninogena*-mediated protection by depleting TNFα. Neutrophil recruitment and production of TNFα were reduced in TNFα-depleted mice (Fig. 3c, d, and Supplementary Fig. 3a, b). Similar to the effect of neutrophil depletion, TNFα depletion resulted in loss of *P. melaninogena*-mediated protection (Fig. 3e). Inflammatory monocyte recruitment was also reduced in TNFα-depleted mice, though CD11b DCs and AM populations were unchanged (Supplementary Fig. 3c–e). Together, these data indicate a requirement for both neutrophils and TNFα in *P. melaninogena*-mediated protection against *S. pneumoniae*.

**P. melaninogena** is protective in microbiome-depleted mice. While our findings suggest that innate immune priming is important for *P. melaninogena*-mediated protection, it was unclear whether the endogenous microbiome, which regulates immune homeostasis, is required for this protective effect. The contribution of the endogenous microbiome to protection in *Prevotella*-exposed mice was evaluated using antibiotic treated and Germ-free mice. In antibiotic treated mice, live *P. melaninogena* significantly improved *S. pneumoniae* clearance from the lungs by 24 h (Fig. 4a and Supplementary Fig. 4a). Similarly, exposure to live *P. melaninogena* enhanced clearance of *S. pneumoniae* from the lungs of Germ-free mice (Fig. 4b). In Germ-free mice, *Prevotella*-mediated protection against *S. pneumoniae* was associated with increased lung neutrophil recruitment and activation (Fig. 4c and Supplementary Fig. 4b). These data indicate that *P. melaninogena* is sufficient to improve protection against *S. pneumoniae* lung infection in the absence of an intact microbiome.

**P. melaninogena** lipoproteins induce TLR2-dependent neutrophil TNFα. We next investigated the host and bacterial requirements for *P. melaninogena*-induced neutrophil TNFα
in vitro. In neutrophils purified from the bone marrow of naïve mice, exposure to *P. melaninogenica* HK induced TNFα secretion in a dose-dependent manner (Fig. 5a). Small molecule inhibitors of TLR2 (C29) and TLR4 (TAK-242) were used to compare the importance of TLR2 versus TLR4 for *P. melaninogenica*-induced TNFα. Inhibition of TLR2, but not TLR4, resulted in loss of *P. melaninogenica*-induced neutrophil TNFα (Fig. 5b), suggesting that TLR2 signaling is important for this response. In support of this conclusion, *P. melaninogenica*-induced TNFα secretion was absent in neutrophils purified from TLR2-deficient (*Tlr2−/−*) mice, in contrast to neutrophils from wild-type (WT) mice (Fig. 5c). These data indicate that TLR2 is required for *P. melaninogenica* stimulation of neutrophil TNFα secretion.

Lipoproteins, a component of the cell membrane in both Gram-negative and Gram-positive bacteria, are the primary bacterial TLR2 ligand. In contrast, TLR4 is responsive to LPS. We purified lipoproteins and LPS from *P. melaninogenica* and found that *P. melaninogenica* lipoproteins, but not *P. melaninogenica* LPS, induced neutrophil TNFα secretion in a TLR2-dependent manner (Fig. 5c). The digestion of bacterial lipoproteins with lipoprotein lipase abrogates TLR2 activation. We found that lipoprotein lipase digestion of *P. melaninogenica* resulted in significant loss of neutrophil TNFα secretion in vitro (Fig. 5d). Lipoprotein lipase-treated *P. melaninogenica* was also no longer protective against *S. pneumoniae* infection in mice (Fig. 5e). However, lipoprotein-TLR2 signaling was not sufficient for protection, as neither the TLR2 agonist Pam3SK4 nor purified *P. melaninogenica* lipoproteins altered lung burdens of *S. pneumoniae* compared to mice treated with PBS (Fig. 5f).

Together, these findings indicate that *P. melaninogenica* lipoproteins activate TLR2-dependent secretion of TNFα in neutrophils and are required, but not sufficient, for *P. melaninogenica*-mediated protection against *S. pneumoniae*.

**TLR2 is required for *P. melaninogenica*-mediated protection.** We next addressed the importance of TLR2 for *P. melaninogenica*-induced immune activation and protection in vivo. In *Tlr2−/−* mice exposed to *P. melaninogenica* HK alone (uninfected), the recruitment of both neutrophils and inflammatory monocytes was similar to that in WT mice (Fig. 6a and Supplementary Fig. 5c, d). However, *P. melaninogenica*-induced neutrophil TNFα was lost in *Tlr2−/−* mice (Fig. 6b). These findings indicate that TLR2 is required for *P. melaninogenica*-induced neutrophil TNFα, but not *P. melaninogenica*-induced myeloid cell recruitment.

In *Tlr2−/−* mice infected with *S. pneumoniae*, lung burdens were elevated regardless of *Prevotella* exposure, in contrast to WT mice in which *Prevotella* exposure was protective (Fig. 6c). *P. melaninogenica*-induced neutrophil recruitment was lost in *Tlr2−/−* mice infected with *S. pneumoniae*, while the recruitment of inflammatory monocytes was maintained (Fig. 6d, e and Supplementary Fig. 5e, f). These data indicate that *Prevotella*-induced neutrophil recruitment is TLR2-dependent in *S. pneumoniae*-infected mice, a setting which may require TNFα...
feedback. Neutrophils in the lungs of Tlr2−/− infected mice also no longer expressed TNFα in response to P. melaninogenica (Fig. 6f). The proportions of other lung myeloid cells, including CD11b+ DCs and AMs, were similar between WT and Tlr2−/− mice infected with S. pneumoniae (Supplementary Fig. 5g, h). Together, these data indicate a critical role for TLR2 in P. melaninogenica-induced neutrophil activation and protection against S. pneumoniae. The separation between neutrophil expression of TNFα, which was TLR2-dependent, and the recruitment of neutrophils and inflammatory monocytes, which were TLR2-independent in P. melaninogenica-exposed mice, indicate that myeloid cell recruitment is by itself insufficient to improve protection against S. pneumoniae, which requires the activation of cells including neutrophils.

P. melaninogenica enhances serine protease-mediated killing

To address whether neutrophils activated by P. melaninogenica directly contribute to enhanced clearance of S. pneumoniae, neutrophil killing of S. pneumoniae was measured in vitro. Neutrophils purified from the bone marrow of naïve mice were no better at killing S. pneumoniae following pre-incubation with P. melaninogenica HK for up to 6 h (Supplementary Fig. 6a), after which the viability of primary neutrophils declines. This suggests that while direct exposure to P. melaninogenica induces neutrophil secretion of TNFα, it is not sufficient to enhance killing of S. pneumoniae within this timeframe.

We next considered whether additional cells or signals in the lungs of P. melaninogenica-exposed mice promote neutrophil killing of S. pneumoniae. To address this, neutrophils were purified from the lungs of mice exposed to either P. melaninogenica or E. coli LPS for 24 h (Fig. 7a). E. coli LPS was chosen as a non-protective immune stimulus that would still induce neutrophil recruitment. Neutrophils purified from the lungs of Prevotella-exposed mice killed significantly more S. pneumoniae than neutrophils purified from the lungs of mice exposed to E. coli LPS (Fig. 7b). The two primary mechanisms for neutrophil killing of S. pneumoniae are the production of reactive oxygen species (ROS) and activity of serine proteases. Neutrophils from P. melaninogenica-exposed and E. coli LPS-exposed mice produced similar levels of total ROS (Fig. 7c). In contrast, the activity of two serine proteases, cathepsin G and elastase, was significantly higher in neutrophils purified from the lungs of P. melaninogenica-exposed mice, compared to neutrophils from the lungs of mice exposed to E. coli LPS (Fig. 7d). Neutrophils pooled from naïve mice had similarly low serine protease activity as those from E. coli LPS-exposed mice (Supplementary Fig. 6b). The addition of protease inhibitors, but not the ROS inhibitor DPI, significantly reduced S. pneumoniae killing by neutrophils isolated from P. melaninogenica-exposed mice (Fig. 7e), without affecting S. pneumoniae growth (Supplementary Fig. 6c). These data indicate that serine protease activity is important for P. melaninogenica-enhanced killing in lung neutrophils.

The importance of TLR2 signaling for Prevotella-induced neutrophil killing was determined by comparing neutrophils purified from WT versus Tlr2−/− mice. Neutrophils from the lungs of Tlr2−/− mice exposed to P. melaninogenica were less efficient at killing S. pneumoniae compared to those from WT mice (Fig. 7f) and these cells had negligible serine protease activity (Fig. 7g), demonstrating a critical role for TLR2 in P. melaninogenica-enhanced neutrophil killing. Together, these findings suggest that P. melaninogenica exposure increases TLR2-dependent, serine protease-mediated killing of S. pneumoniae by lung neutrophils.

P. melaninogenica protection requires regulation by IL-10

Immune regulation is critical to mitigate the damaging effects of inflammation in the lung such as barrier disruption and reduced oxygen exchange. While TNFα primes several protective immune responses, overproduction causes tissue damage and impairs S. pneumoniae clearance40,41. The anti-inflammatory cytokine IL-10 is a master regulator of pro-inflammatory responses including TNFα. In purified neutrophils, we found that in addition to TNFα, P. melaninogenica HK induced the secretion of IL-10 in a dose-dependent and TLR2-dependent manner (Supplementary Fig. 7a). Further, neutrophil secretion of TNFα was inhibited in cultures exposed to both P. melaninogenica and S. pneumoniae, suggesting regulation of this response (Supplementary Fig. 7b). These findings mirror the systemic TNFα response in Prevotella-exposed mice, which was reduced following S. pneumoniae infection. Additionally, P. melaninogenica-exposed mice infected with S. pneumoniae had significantly reduced levels of several pro-inflammatory cytokines in lung BAL, including TNFα, IL-6, IL-1α, IFNβ, and IFNγ, compared to those infected with S. pneumoniae but not exposed to Prevotella (Fig. 8a). This is in contrast to uninfected mice, where P. melaninogenica exposure increased BAL pro-inflammatory cytokines at 24 h, indicating that by 48 h in co-infected mice, these responses were reduced. These data suggest that exposure to P. melaninogenica regulates S. pneumoniae-induced inflammation in the lung.

To address the potential role of regulatory IL-10 in P. melaninogenica-exposed mice, we compared S. pneumoniae burdens in WT and IL-10 deficient (Il10−/−) mice with and without pre-exposure to P. melaninogenica HK. Unexpectedly, P. melaninogenica-mediated protection against S. pneumoniae was lost in Il10−/− mice, which had similarly high burdens regardless of P. melaninogenica exposure (Fig. 8b). In Il10−/− mice, serum
TNFα was elevated following *P. melaninogena* exposure (Fig. 8c). In contrast, WT mice exposed to *P. melaninogena* had reduced serum TNFα and elevated IL-10 (Supplementary Fig. 7c). These results indicate that *P. melaninogena*-induced IL-10 regulates systemic TNFα during *S. pneumoniae* infection and is important for *P. melaninogena*-mediated protection. Analysis of lung myeloid cell TNFα expression revealed that TNFα was significantly elevated in several cell types, including neutrophils, inflammatory monocytes, and AMs, in IL10−/− mice, regardless of strain (*P. mel.* or *P. mel. lipopolysaccharide* exposure) (Fig. 8d–f and Supplementary Fig. 7d–e). Depletion of TNFα was not sufficient to reverse the loss of *P. melaninogena*-mediated protection in IL10−/− mice (Supplementary Fig. 7f), suggesting a broad loss of IL-10-mediated restraint of myeloid cell activation. Together, these findings reveal that IL-10 regulation of lung inflammation is a critical component of *P. melaninogena*-mediated protection against *S. pneumoniae* infection.

**Protection mediated by other airway Prevotella.** While *P. melaninogena* is an abundant *Prevotella* species, it was unclear whether other airway *Prevotella* isolates mediate a similar protective effect. As with *P. melaninogena* strain 25845, used throughout these studies, exposure to live *P. melaninogena* strain D18 significantly improved clearance of *S. pneumoniae* from the lung by 24 h post-infection (Fig. 9a). Three additional live airway *Prevotella* species, including *Prevotella buccae*, *Prevotella tannerae*, and *Prevotella nanceiensis*, also increased *S. pneumoniae* clearance from the lung (Fig. 9a). In contrast, the periodontal pathogen *P. intermedia* was not protective (Fig. 9a). These data suggest that several airway *Prevotella* species are capable of enhancing protection against *S. pneumoniae* infection. Finally, we compared the ability of HK preparations of each *Prevotella* species to activate neutrophils purified from the bone marrow of WT versus Tlr2−/− mice. Similar to *P. melaninogena* strain 25845, strain D18 as well as *P. buccae*, *P. tannerae*, and *P. nanceiensis* induced neutrophil secretion of TNFα and IL-10 in a TLR2-dependent manner (Fig. 9b). In contrast, *P. intermedia* activated neutrophils in a TLR2-independent manner (Fig. 9b). These data are consistent with a critical role for TLR2-dependent neutrophil activation in species of *Prevotella* which are protective against *S. pneumoniae*.
Prior to 24 h, treatment with either PBS (−) or Dunn's reagent from one of four independent experiments (p = 0.9858), one-way ANOVA with Sidak's post hoc test, **p = 0.0026 (WT), **p = 0.009 (Tlr2−/−), one-way ANOVA with Sidak's post hoc test, b **p = 0.0002 (WT), P. melaninogenica (Tlr2−/−), one-way ANOVA with Sidak's post hoc test, and p = 0.0165 (WT), **p = 0.0081 (WT), Kruskal-Wallis with Dunn's post hoc test, c from left to right **p = 0.0017, p > 0.9999, p > 0.9999, Kruskal-Wallis with Dunn's post hoc test, d **p = 0.0494 (WT), P. melaninogenica (Tlr2−/−), one-way ANOVA with Sidak's post hoc test and p = 0.0162 (WT), P. melaninogenica (Tlr2−/−), Kruskal-Wallis with Dunn's post hoc test, e **p = 0.0014 (WT), P. melaninogenica (Tlr2−/−), one-way ANOVA with Sidak's post hoc test and p = 0.0142 (WT), P. melaninogenica (Tlr2−/−), one-way ANOVA with Sidak's post hoc test and p = 0.2056 (WT), P. melaninogenica (Tlr2−/−), Kruskal-Wallis with Dunn's post hoc test. Source data are provided as a Source Data file.

Discussion

Work over the past decade has defined the gut-lung axis, highlighting a critical role for the gut microbiome in lung immune homeostasis. By comparison, our understanding of immune regulation by members of the airway microbiome is underdeveloped. Several individual members of the respiratory tract microbiome, including commensal Streptococcus and Corinebacterium species, produce factors that inhibit growth of S. pneumoniae. However, it is less clear how these and other airway commensals regulate innate immune-mediated protection against lung infection. Brown et al. showed that a compilation of Gram-positive airway bacteria including Lactobacillus crispatus, Staphylococcus aureus, and Staphylococcus epidermidis induced Nod2-dependent protection against S. pneumoniae. In this case, a Nod2-activating bacterial cohort from the gut also induced protection, which required GM-CSF and AMs. As we did not detect a GM-CSF response to Prevotella, this suggests there are at least two distinct microbial-induced immune pathways capable of enhancing protection against S. pneumoniae. Sustained protection against S. pneumoniae infection in antibiotic treated and germ-free mice indicates that P. melaninogenica is sufficient to drive immune-mediated protection without the participation of other members of the endogenous microbiota.

This study focused on the early response to P. melaninogenica in the context of acute lung infection. Recently, a compilation of oral commensals which included P. melaninogenica was shown to protect against S. pneumoniae for as long as 2 weeks. Protection at this timepoint was associated with the induction of a Th17 response, which correlated primarily with the presence of Veillonella rather than Prevotella. However, RNA sequencing of lung tissue 24 h post-commensal aspiration confirmed the induction of TLR signaling and inflammatory responses including TNFα and IL-6, similar to our findings with P. melaninogenica. This indicates that exposure to either P. melaninogenica alone or in combination with other oral commensals enhances immune-mediated protection against S. pneumoniae. Prevotella-induced immune responses may coordinate with those induced by other commensal bacteria, such as Veillonella, for extended protection.

Inflammation itself is not automatically protective against S. pneumoniae infection. For example, virus-induced inflammation supports increased S. pneumoniae growth, and viral-pneumococcal co-infections are associated with higher morbidity and mortality. S. pneumoniae co-infection with H. influenzae involves synergistic inflammation, including the upregulation of TLR2 signaling in the lung and middle ear. S. pneumoniae is well-equipped for immune modulation and evasion, and expresses virulence factors that limit the efficacy of innate immune responses and impede neutrophil-mediated killing. As a result, innate immune cells are typically insufficient for pneumococcal clearance. Our findings highlight a protective role for TLR2-dependent inflammation induced by airway Prevotella exposure, the timing of which may be important for overcoming S. pneumoniae immune evasion mechanisms. This may relate to reduced acquisition of S. pneumoniae in the
lungs of individuals with “optimal” innate immune priming by their airway microbiota.

The concept of trained immunity refers to non-specific beneficial immune priming, primarily mediated by innate immune signaling and directed by the microbiota. The critical cells and signaling pathways in the lung for trained immunity are not clear. Compared with all other tissues including the gut, the lungs have the highest expression of TLR2/49. Immune priming by TLR agonists has been explored as a potential immunotherapy strategy for several infections, including *S. pneumoniae*. Specific combinations of TLR agonists are protective against *S. pneumoniae* infection, such as TLR2 together with TLR9 agonists/50. *S. pneumoniae* lipoproteins are capable of activating TLR2/25, similar to Prevotella, but TLR2 is not sufficient for protection/52, as we confirm. One study, however, found that TLR2 is protective against pneumolysin-deficient *S. pneumoniae*, which no longer activates TLR4/53. Prevotella express penta-acylated LPS, which can behave as a TLR4 agonist, in contrast to hexa-acylated LPS expressed by bacteria such as *E. coli*, which is a strong TLR4-inducer/24. Further investigation is required to dissect the immune priming environment which coordinates with TLR2 to promote *P. melaninogenica*-mediated protection against *S. pneumoniae*, including the potential role of TLR4 antagonism. The protective capacity of TLR4 priming also differs by infection, as TLR4 priming with *E. coli*-derived LPS improves protection against lung infection with *Klebsiella pneumoniae* and influenza A virus/31,32, in contrast to *S. pneumoniae*. Together, these studies highlight the importance of understanding the context and mechanism of protective airway microbiome trained immunity.

While our data indicate an important role for neutrophils, it remains unclear which cells initiate neutrophil recruitment in response to *P. melaninogenica*. AMs are a likely candidate as the first immune cells to encounter inhaled particles and as a major source of neutrophil chemotacticants/33. *P. melaninogenica* outer membrane vesicles induce AM responses including IL-17A and IL-17B/27, which can synergize with pro-inflammatory cytokines including TNFa. As others have shown/35, we find that TNFa is important for neutrophil recruitment during *S. pneumoniae* infection, and TNFa can prime activation of human neutrophils/30. However, TNFa priming in neutrophils is typically associated with stronger NADPH oxidase activation and respiratory burst/37. This contrasts with our findings of increased serine protease activity, which was dependent on additional signals in the *P. melaninogenica*-activated lung. Serine protease activity is essential for neutrophil killing of *S. pneumoniae*/58, and serine protease knockout mice have reduced clearance of *S. pneumoniae* from the lung/59, highlighting the importance of this pathway for infection control.

![Schematic of lung neutrophil purification](image1)

**Fig. 7 P. melaninogenica (P. mel.) enhances serine protease-mediated killing of S. pneumoniae by lung neutrophils in a TLR2-dependent manner.**

a Schematic of lung neutrophil purification. b Percent of type 2 *S. pneumoniae* killed by lung neutrophils purified from mice exposed to either *E. coli* lipopolysaccharide (LPS) or *P. mel.* strain 25845 heat-killed (HK) i.t. for 24 h following 1 h incubation with *S. pneumoniae* opsonized with 3% fresh mouse serum (n = cells isolated from 11 mice/group). c Total reactive oxygen species (ROS) produced in 1 h by lung neutrophils purified from mice exposed to either *E. coli* LPS or *P. mel.* HK i.t. for 24 h detected by luminol, with area under curve (AUC) shown (n = cells isolated from 6 mice/group). d Serine protease activity for cathepsin G and elastase (Prot Inhib) detected by substrate cleavage for lung neutrophils purified from WT mice exposed to either *E. coli* LPS or *P. mel.* HK (n = cells isolated from 6 mice/group). e Percent of *S. pneumoniae* killed by lung neutrophils purified from WT mice exposed to *P. mel.* HK i.t. for 24 h following 1 h incubation with opsonized *S. pneumoniae* in the presence of the ROS inhibitor DPI (ROS), protease inhibitors (Protease), or no inhibitors (none), (n = cells isolated from 9 mice/group). f, g Percent of *S. pneumoniae* killed by lung neutrophils purified from WT or Tlr2/-/- mice exposed to *P. mel.* HK i.t. for 24 h (n = cells isolated from 13 WT mice, 9 Tlr2/-/- mice) (f) or serine protease activity +/− protease inhibitor cocktail (Prot Inhib) (g) Data are pooled from three independent experiments, with cells plated in duplicate or triplicate. Data are displayed as mean ± SEM. For b ***p < 0.0001, two-tailed t-test, c p = 0.9566, two-tailed t-test, d ***p = 0.0004 (Cathepsin G), ***p < 0.0001 (Elastase), two-tailed t-test, e p = 0.9926 (ROS), ***p < 0.0001 (Protease), one-way ANOVA with Dunnett’s post hoc test, f ***p < 0.0001, two-tailed t-test, g ***p = 0.0008 (Cathepsin G), ***p = 0.0002 (Elastase), two-tailed t-test. Source data are provided as a Source Data file.
**Fig. 8** *P. melaninogenica* (*P. mel.*)-mediated protection against *S. pneumoniae* requires restraint of detrimental inflammation by IL-10. **a** Quantification of cytokines and chemokines in lung bronchoalveolar lavage (BAL) following exposure to PBS (-) or *P. mel.* strain 25845 heat-killed (HK) i.t. prior to 24 h type 2 *S. pneumoniae* infection, 5 \( \times 10^6 \) CFU/mouse (n = 9 mice/group). **b**, **c** Lung *S. pneumoniae* burdens (b) and serum TNFα (c) detected in WT or *Il10*−/− mice exposed to either PBS (-) or *P. mel.* HK i.t. prior to 24 h *S. pneumoniae* infection (n = 5 mice/group). **d**-**f** Representative flow cytometry plots and total cell numbers of neutrophil TNFα (d), percentage of inflammatory monocyte TNFα (e), and percentage of AM TNFα (f) detected by intracellular flow cytometry from WT or *Il10*−/− mice treated with either PBS (-) or *P. mel.* HK i.t. prior to 24 h *S. pneumoniae* infection (n = 4 mice/group). LOD = limit of detection. Data are pooled from three independent experiments (a) or representative from one of four independent experiments (b-f). Box boundaries in (a) indicate the 25th and 75th percentiles, with a horizontal line representing the median and whiskers indicating minimum and maximum values. Data in **b-f** are displayed as mean ± SEM. For *a* \( **p < 0.0001, ***p < 0.0001 \), two-way ANOVA with Sidak’s post hoc test, **b** \( *p = 0.0257 \) (WT), \( *p > 0.9999 \) (*Il10*−/−), Kruskal–Wallis with Dunn’s post hoc test, **c** from left to right \( *p = 0.0393, p = 0.9973, **p = 0.0012 \), one-way ANOVA with Tukey’s post hoc test, **d** from left to right \( *p = 0.0116, p = 0.7388 \), one-way ANOVA with Sidak’s post hoc test, **e** from left to right \( *p = 0.0101, p = 0.1698 \), one-way ANOVA with Sidak’s post hoc test, **f** from left to right \( *p = 0.0280, p = 0.9298 \), one-way ANOVA with Sidak’s post hoc test. Source data are provided as a Source Data file.

**Fig. 9** Protection against *S. pneumoniae* is induced by other airway *Prevotella* that activate neutrophils in a TLR2-dependent manner. **a** Lung *S. pneumoniae* burdens in mice treated with PBS (-), live *P. melaninogenica* (*P. mel.*) strain D18, live *P. buccae*, live *P. tannerae*, live *P. nanceiensis*, or live *P. intermedia* i.t. prior to 24 h *S. pneumoniae* infection, 5 \( \times 10^6 \) CFU/mouse (n = 12 mice/group). **b** Supernatant cytokines detected 24 h following incubation of BM neutrophils with *P. mel.* strain D18 heat-killed (HK), *P. buccae* HK, *P. tannerae* HK, *P. nanceiensis* HK, or *P. intermedia* HK (n = 3 independent experiments/group). Data are pooled from three independent experiments, with cells plated in triplicate for in vitro studies, displayed as mean ± SEM. For **a** from left to right \( ***p < 0.0001, **p < 0.0001, ***p < 0.0005, ***p < 0.0001 \), Kruskal–Wallis with Dunn’s post hoc test, **b** from left to right, \( **p < 0.0001, **p < 0.0001, **p = 0.9948 \) (IL-10), two-way ANOVA with Sidak’s post hoc test. Source data are provided as a Source Data file.
Here, we find that Prevotella-mediated protection requires the co-induction of systemic IL-10, which regulates Prevotella-induced inflammation in S. pneumoniae-infected mice. In contrast to this beneficial role, we recently found that systemic IL-10 induced at 3 days post-infection exacerbates S. pneumoniae lung infection. However, this response was delayed relative to the protective IL-10 response we observe in Prevotella-exposed mice. Others have shown that P. melaninogena induces the production of IL-10 by human DCs, and we find that murine neutrophils secrete IL-10 in response to P. melaninogena, suggesting that myeloid cell-derived IL-10 contributes to inflammatory control. These studies highlight the importance of inflammatory fine-tuning in the lung, where IL-10 plays a major role in limiting tissue damage, though at the risk of worsened infection under some circumstances.

Despite the genetic heterogeneity reported among Prevotella species, we identified two different isolates of P. melaninogena and several other airway Prevotella species that enhance protection against S. pneumoniae. P. melaninogena was previously shown to induce similar immune activation profiles, including the production of TNFα and IL-10 by lung CD4+ cells, as P. nanceiensis, one of the strains we identify as protective. Although not as prevalent as P. melaninogena, P. buccae, P. tannerae, and P. nanceiensis are also common in the upper and lower airways of healthy individuals. In contrast, P. intermedia, which was not protective against S. pneumoniae, is a major periodontal pathogen. Others have reported that P. intermedia upregulates a critical adherence receptor used by S. pneumoniae in airway epithelial cells, exacerbating type 4 S. pneumoniae infection. Here, we find that P. intermedia activates neutrophils in a TLR2-independent manner, unlike the other Prevotella strains, which are protective. The critical features of protective airway Prevotella species, including the role of TLR2-dependent neutrophil activation and regulation of the epithelial immune system, is an important area for further investigation. Regardless, these findings suggest that the species-specific composition of airway Prevotella dictates infection outcome following S. pneumoniae exposure.

The immune activation profile we describe is consistent with several observations in humans, including the association between increased Prevotella and sub-clinical inflammation characterized by increased neutrophils, the human neutrophil chemotactic factor IL-8, Mip-1α, and IL-10 in the lungs of healthy adults. Similarly, Prevotella abundance was associated with increased TNFα and IL-10 in the lungs of transplant patients, in whom Prevotella correlated with increased lung function and airway microbiome stability. Finally, in vitro studies demonstrate the capacity of P. melaninogena to induce TLR2 activation and responses including IL-6, TNFα, and IL-10 secretion by human monocytes and DCs. Together, these findings indicate that Prevotella species including P. melaninogena are associated with lung innate immune responses including TLR2 activation and the production of TNFα and IL-10 in both humans and animal models.

Our findings highlight an important role for airway Prevotella in directing immune-mediated protection in the respiratory tract. Further studies will be necessary to determine whether similar protective immune priming is induced by Prevotella aspiration in healthy adults and how such protection may be leveraged to reduce the burden of lung infections in susceptible individuals. Airway immunobiotics, or probiotics designed to enhance protective immunity, may be particularly impactful in individuals who are at increased risk of S. pneumoniae infection, though such use should be tempered by potentially detrimental effects, for example by exacerbating fibrosis in those with chronic lung disease. Alternatively, targeting the critical Prevotella-modified immune pathways could improve clearance of S. pneumoniae regardless of the presence of Prevotella as an immunotherapeutic strategy.

**Methods**

**Animals.** Adult male and female mice aged 6-12 weeks of age were used for these studies. C57BL/6 (WT), B6.129TfiC58<sup>il2-<sup>−/−</sup> Tnfα<sup>−/−</sup></sup> mice, and B6.129il10<sup>−/−</sup> (Il10<sup>−/−</sup>) mice were purchased from The Jackson Laboratory (stocks 000664, 004630, and 002251 respectively). All strains used in these studies (WT, Tnfα<sup>−/−</sup> and Il10<sup>−/−</sup>) are on the C57BL/6 genetic background. Mice were maintained in the University of Colorado College of Laboratory Animal Resources. Housing conditions included a light cycle of 14:10 (light:dark) hours, a temperature of 72 ± 2°F, with 40 ± 10% humidity. Mice are housed in sterilized vinyl film isolators with positive pressure airflow through HEPA filtration. Any items introduced into the isolators are sterilized, with quality control indicators to verify sterilization. The internal isolator environment and housed mice are tested bi-weekly and prior to experimental use for microbiota through culture-dependent methods and by qPCR (see Microbiome Depletion).

**Microbiome depletions.** Antibiotic treated mice were exposed to a broad-spectrum antibiotic cocktail (ampicillin 1 g/L, neomycin 1 g/L, metronidazole 1 g/L, vancomycin 0.5 g/L, MilliporeSigma and McKesson) in drinking water ad libitum for 5 days. Water containing antibiotics was replaced with normal drinking water 48 h prior to live Prevotella exposure. Bacteria were enumerated directly from the Gnotobiotic Core Facility into BSL2 vivarium space. Transferred mice were exposed to inhaled bacteria (see Infections) within 8 h of transfer.

**Bacteria.** Prevotella melaninogena strain ATCC® 25845®. Prevotella tannerae strain ATCC® 51259®. Prevotella intermedia strain ATCC® 25611® were obtained from the American Type Culture Collection, Manassas VA. Prevotella melaninogena strain D18 (catalog #HM-80) and Prevotella buccae strain D17 (catalog #HM-45) were obtained from BEI resources, NIAID, NIH as part of the Human Microbiome project. Prevotella nanceiensis strain PP746 was a kind gift from Dr. Paul Planet, Division of Infectious Diseases at the Children’s Hospital of Philadelphia. Prevotella strains were grown under anaerobic conditions on Brucella agar containing 5% sheep blood, hemin and vitamin K at 37 °C for 72 h (ThermoFisher Scientific). Heat-killed Prevotella were prepared from fresh plates for whole mount immunization in PBS and suspension at 56 °C for 30 min prior to use before and after heat-killing were used to determine CFU equivalents/mL and confirm killing, respectively. Heat-killed Corynebacterium aculeos strain ATCC® 49726® (American Type Culture Collection) and Corynebacterium amycolatum strain SK46, catalog #HM-109 (BEI resources, NIAID, NIH as part of the Human Microbiome project) were prepared following growth in BHI broth cultures supplemented with 1% Tween® 80 (polysorbate, WVR). Heat-killed Streptococcus salivarus strain SK126, catalog #HM-109 (obtained from BEI Resources, NIAID, NIH as part of the Human Microbiome project) was prepared following growth in Todd Hewitt Broth with 5% Yeast Extract (BD Bacto) at 37 °C with 5% CO2 without shaking. Heat-killed Escherichia coli (strain DH5α, ThermoFisher Scientific) was prepared following growth in LB broth (BD Bacto®) at 37 °C with shaking (200 rpm). A streptomycin resistant variant of serotype 2 Streptococcus pneumoniae strain D39 was a kind gift from Dr. Jeffrey N. Weiser (New York University). Serotype 3 S. pneumoniae strain ATCC® 6303® (American Type Culture Collection) was also used. S. pneumoniae was grown in Todd Hewitt Broth with 5% Yeast Extract (BD Bacto®), with 50 µg/mL streptomycin (Sigma), added for type 2 strain D39 only, at 37 °C with 5% CO2 without shaking.

**Infections.** For live P. melaninogena infections, bacterial suspensions from fresh plates were prepared in PBS to an optical density (OD<sub>600</sub>) of 0.3, centrifuged at 22000 x g for 10 min, and resuspended in PBS prior to injection. S. pneumoniae was grown in broth from frozen stocks to mid-log phase and centrifuged at 1000 x g for 10 min following growth in PBS. Lower airway burdens were determined by serial dilution for CFU enumeration. Lungs and spleens collected from infected mice were homogenized using a Bullet Blender...
Flow cytometry. Lungs were harvested following perfusion by transcardiac injection of 10 mL PBS, and single cells were prepared for flow cytometry38. Briefly, lungs were subjected to mechanical (milling) and enzymatic (DNAse 30 μg/mL, Sigma) and type I collagenase 1 mg/mL, Worthington Biochemical Corporation) digestion prior to passage through a 70 μm strainer. Red blood cells were lysed in RBC lysis buffer (0.15 M NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA, pH 7.4). Fc receptors were blocked by incubation in anti-CD16/32 (2.4G2 hybridoma supernatant) prior to staining in FACS buffer (1% BSA, 0.01% Na₂O₃, PBS). For intra-
cellular flow cytometry, cells were incubated with Brefeldin A (BD Biosciences) prior to staining and permeabilized with 1 mg/mL saponin (Sigma) prior to intracellular staining. All cells were fixed in 1% paraformaldehyde. Antibodies used for staining included anti-mouse: Siglec F (BD, catalog #562681, clone E50-2440, lot #B257977), Ly6C (BioLegend, catalog #128012, clone HK1.4, lot #B257614), Ly6G (BioLegend, catalog #180121, clone HK.4, lot #B250462), CD45.2 (BD, catalog #109824), CD11b (BioLegend, catalog #101212, clone M1/70, lot #B290360), CD45.2 (BioLegend, catalog #101212, clone M1/70, lot #B281990), and TNFα (ThermoFisher Scientific, catalog #25-7321-82, clone MP6-XT22, lot #B830792). Antibodies were detected on the LSR Fortessa X-20 in the Immunomicro Flow Cytometry Shared Resource Laboratory at the University of Colorado Anschutz Medical Campus (RRID:SCR_021321). Data analysis was performed using FlowJo™ Software, version 9.9.6 (BD Life Sciences).

Cytokeine and chemokine analysis. BAL cytokines and chemokines with the exception of MCP-2 were measured using a LEGENDplex™ Mouse Inflammation Panel (BioLegend), with analytes detected on the LSR Fortessa X-20 in the Immunomicro Flow Cytometry Shared Resource Laboratory at the University of Colorado Anschutz Medical Campus (RRID:SCR_021321). Data analysis was performed using FlowJo™ Software Analysis Data Suite (BioLegend). BAL MIP-2 was measured using the LEGENDplex™ Mouse CXCL2/MIP-2 kit (R&D Systems Biocytom). serum cytokines were measured using mouse IL-10 and TNFα ELISA kits (BD) and were detected on a Synergy HT Microplate Reader (BioTek). Data analysis was performed using Prism (GraphPad, version 8).

Cell isolations and stimulations. Bone marrow neutrophils were isolated from the femurs of mice by Histopaque density gradient centrifugation71 and purity was confirmed to be >90% Ly6G+ neutrophils by flow cytometry.16 P. melaninogenica lipopolysaccharide (LPS) preparations were prepared by Triton X-114 phase partitioning68 and P. melaninogenica LPS was prepared using an LPS isolation kit (Sigma). Concentrations were determined relative to Pam3CSK4 and E. coli LPS standard curves by running purified lipopolysaccharide and LPS preparations on a 15% sodium dodecyl-sulfate polyacrylamide gel electrophoresis gel prior to detection using a Silver Stain Kit (Bio-Rad Laboratories, Inc) and imaging on a ChemiDoc XRS + Gel Imaging System (Bio-Rad Laboratories, Inc). P. melaninogenica LPS endotoxin activity was confirmed using a Pierce™ Chromogenic Endotoxin Quant Kit (ThermoFisher Scientific). Lipopolysaccharide lipase-treated P. melaninogenica HK was prepared by incubation with 200 μg lipopolysaccharide (Sigma) for 2 h at 37°C. For all cell stimulations, 105 neutrophils in RPM1 media supplemented with penicillin and streptomycin were exposed to P. melaninogenica HK (1:1 ratio), lipase-treated P. melaninogenica HK, P. melaninogenica lipopolysaccharide (10 ng/mL), P. melaninogenica LPS (10 ng/mL), C99 TR2 inhibitor (100–200 μM, Selleck Chemicals), Resovast TK-242 TR4 inhibitor (100–200 μM, Selleck Chemicals), or Pam3CSK4 (10 ng/mL, InvivoGen, San Diego, CA) and incubated for 24 h at 37°C with 5% CO₂ prior to supernatant collection. For cell infection assays, 105 neutrophils were exposed to 10⁵ S. pneumoniae for 1 h prior to washing and incubation for 24 h at 37°C with 5% CO₂ with or without P. melaninogenica HK (1:1 ratio) in media containing gentamicin (10 μg/mL).

Neutrophil functional assays. Lung neutrophils were isolated by positive selection (MojoSort PE-positive selection kit, BioLegend), and purity was confirmed to be >90% Ly6G+ neutrophils by flow cytometry. Similar results were obtained using lung neutrophils isolated by negative isolation (19762, Mouse Neutrophil Enrichment kit, STEMCELL Technologies). Neutrophils were isolated from the lungs of mice following 24 h treatment with PBS, E. coli LPS, or P. melaninogenica HK 1:1 as indicated. For opsonophagocytic killing assays, 10⁵ S. pneumoniae grown to mid-log phase were opsonized for 30 min with 3% fresh mouse sera prior to incubation with 10⁵ neutrophils in Hank’s buffer/0.1% gelatin for 1 h at 37°C under rotation. Killing assays were completed in the presence or absence of 1x Halt® protease inhibitor cocktail (ThermoFisher Scientific) or 10 μM diphenyl-
mononitrobenzene (DNB) (ThermoFisher Scientific), which we found did not impact neutrophil viability over the course of 1 h. CFUs were measured by serial dilution plating, and percent kill was determined relative to reactions without neutrophils. Serine protease activity was determined using substrates specific to elastase (0.85 mM MoO4Sc-Ala-Ala-Pro-Val-pNA, Sigma) and chymotrypsin G (0.1 mM Succinyl-Ala-Ala-Pro-Phe-pNA, Sigma)38. Briefly, 10⁵ purified neutrophils were incubated with or without 1x Halt® protease inhibitor cocktail for 30 min prior to washing and lysis in 0.1% Triton X-100. Substrates were added to neutrophil lysates and incubated in the dark for 45 min at 37°C. Absorbance was determined by reading the OD₄₅₀ on a Synergy™ HT Microplate Reader (BioTek) minus control wells with no neutrophils added. For detection of total ROS, 10⁵ purified neutrophils were resuspended in KR buffer (5 mM glucose, 1 mM CaCl₂, 1 mM MgSO₄, in PBS) and equilibrated for 15 min prior to plating on Greiner Bio-
One LUMITRAC™ plates (Fisherscientific) followed by the addition of 50 μM 
Fluoro (Fluoricient™). Luminescence over 1 h at 37°C was detected using a Synergy™ HT Microplate Reader (BioTek). Area under curve (AUC) was calculated, minus wells with no neutrophils added.

Study approval. These studies were approved by the Animal Care and Use Committee of the University of Colorado School of Medicine (protocol #00927) and by the Institutional Biosafety Committee (protocol #1418).

Statistical analysis. All measurements were analyzed from distinct experiments, with error bars showing standard error of the mean. Specifically, student t-tests were used for all statistical analysis. All data were tested for normality using the Shapiro–Wilks test. For data with normal distributions, two-tailed Student’s t-tests, one-way or two-way ANOVA tests with Dunnett’s, Sidak’s, or Tukey’s post hoc analyses for multiple comparisons were used as specified. Two-tailed Mann–Whitney U-tests and Kruskal–Wallis tests with Dunn’s post hoc analysis for multiple comparisons were used for all data with non-Gaussian distributions. Log-
rank Mantel-Cox test was used for survival group comparison. For all statistical tests, p-values < 0.05 was considered significant. Individual p-values are specified in Figure Legends.

Data availability. All data supporting the findings of this study are available in the paper (and its supplementary files). Source data are provided with this paper.

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S.E.C. conceived and designed the experiments and wrote the original manuscript draft. S.E.C., K.J.H., and M.A.S. performed the experiments, analyzed data, and edited the manuscript draft. Z.G.D. performed experiments and analyzed data.

Competing interests
The authors declare no competing interests.

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