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Co-infection subverts mucosal immunity in the upper respiratory tract

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Abstract

Polymicrobial interactions on mucosal surfaces can influence inflammation, immunity, and disease outcome. Here, we review how host responses to colonization in the upper respiratory tract with the bacterial pathogen *Streptococcus pneumoniae* can be altered by co-infection. Recent advances provide a mechanistic understanding of how mucosal immunity can be subverted at distinct immunological time-points during pneumococcal colonization by other pathogens such as *Haemophilus influenzae*, influenza type A and *Staphylococcus aureus*. These examples use animal models of co-infection to highlight how otherwise effective host responses can be rendered ineffective by co-infection, and vice versa. The complex microbial ecology of mucosal sites must be considered to fully understand how immune responses in a natural setting influence the outcome of host-pathogen interactions.

Introduction

Our understanding of host-pathogen interactions has been achieved mainly by a reductionist approach. The power of reductionism is that it allows us to gain a precise, mechanistic understanding of how a specific pathogen interacts with a particular host response at the molecular level. Such insight can be especially valuable when studying how the host responds to invasive pathogens found in otherwise sterile sites, where they are the only pathogen and the host response is specifically directed against them. However, the reductionist approach can fall short in the context of host-pathogen interactions at non-sterile mucosal surfaces, where a given pathogen is just one member in a complex community of microbes. The microbiota of humans contains thousands of species, including many opportunistic pathogens, that can compete or cooperate to establish a niche in the host environment. Host responses elicited by a given pathogen can have far-reaching effects on other members of the mucosal community, including other pathogens. These off-target effects can be just as influential in the outcome of host-pathogen interactions as the pathogen-specific responses that are traditionally studied using a reductionist approach. There is a growing appreciation for the dynamic interactions among pathogens and how inflammation and immunity at mucosal surfaces can be influenced by polymicrobial interactions. This review highlights how co-infection with multiple pathogens can subvert mucosal immune responses and can alter disease outcome. As a model for these interactions,

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we focus on the bacterial pathogen *Streptococcus pneumoniae* and its primary ecological niche, the upper respiratory tract.

Modeling host-pathogen-pathogen interactions with *S. pneumoniae* in the upper respiratory tract

In the upper respiratory tract, the line between pathogen and commensal is often blurred, as many important human pathogens such as *S. pneumoniae*, *Haemophilus influenzae*, and *Staphylococcus aureus* can be found colonizing an average of 20–50% of healthy individuals [1–3]. Sequential and/or simultaneous colonization with more than one of these pathogens is common, especially during childhood. Colonization is the first step and a prerequisite for the development of disease [1]. Experimental human carriage studies have demonstrated that the acquisition of colonization is associated with mild inflammatory symptoms, including pharyngitis and rhinorrhea [4] (M. Apicella, unpublished observations), but that these are insufficient to clear colonization which may last for weeks to months. A murine model of nasopharyngeal colonization with *S. pneumoniae* recapitulates these colonization dynamics and allows us to better characterize the time course of host responses [5] (Figure 1). Thirty minutes following atraumatic inoculation of pneumococci to the nares of mice, bacteria can be detected in the lumen of the nasal cavity. By one day post-inoculation, pneumococci have traversed the mucus layer and reached the epithelial surface of the nasopharynx where they establish colonization (Figure 1A). Bacterial contact with epithelial cells induces a TLR2-dependent signaling cascade that results in acute inflammation by day three, characterized by an influx of neutrophils [6,7]. However, these cells are insufficient to fully clear the pneumococcus which instead requires a gradual Th17-dependent monocyte/macrophage infiltrate over the course of weeks [6] (Figure 1B). During this time, a robust adaptive immune response is mounted to a diversity of pneumococcal antigens (Figure 1C), including antibody against the immunodominant capsular polysaccharide which contributes to protection against secondary exposure by promoting opsonophagocytic killing [8].

At critical points along the time-course of pneumococcal colonization, co-colonization or co-infection with other pathogen species can have dramatic consequences on disease progression and outcome. Here, we provide three mechanistic examples of subversion of mucosal immunity at distinct immunological stages during pneumococcal colonization. Specifically, we review how 1) ***H. influenzae* co-colonization with *S. pneumoniae*** enhances the acute neutrophil response, leading to enhanced clearance of *S. pneumoniae* and a selection for virulent serotypes; 2) **influenza A co-infection with *S. pneumoniae*** inhibits the late-stage macrophage influx via the expression of type I interferons, leading to increased pneumococcal colonization density and risk of invasive disease; and 3) ***S. aureus* co-colonization with *S. pneumoniae*** is inhibited in immunocompetent, but not immunocompromised, hosts, potentially via the generation of cross-reactive pneumococcal antibody.

Effect of co-colonization with *H. influenzae*

S. pneumoniae and *H. influenzae* share the nasopharynx as the site of colonization in humans [1]. Significantly high carriage rates (>50%) of both of these organisms in children suggest that co-colonization is common [9]. Competition between these organisms is likely due to the overlap in their site and frequency of colonization. *S. pneumoniae* possesses multiple mechanisms that provide a fitness advantage over *H. influenzae* during competition *in vitro* [10,11]. However, the outcome of competition is reversed during *in vivo* colonization in mice: co-colonization with *H. influenzae* results in a rapid depletion of pneumococci from the nasopharynx within three days post-inoculation and complete

clearance by two weeks [12]. This drastic enhancement in clearance is in stark contrast to mono-colonization with *S. pneumoniae* which remains stable for over a month.

The effects of co-colonization with *H. influenzae* occur rapidly, are only observed *in vivo*, and thus implicate a modulation of the acute inflammatory response. Indeed, simultaneous colonization with *S. pneumoniae* and *H. influenzae* results in a synergistic proinflammatory response [13]. Similar responses are noted during co-stimulation of human airway epithelial cells, which results in a log₁₀ increase in the production of the proinflammatory chemokine IL-8 over single-species stimulation with either organism [13]. The observed synergistic IL-8 response is independent of cell surface pattern recognition receptors such as TLR2 and TLR4 but dependent on NF-κB and the pneumococcal pore-forming toxin, pneumolysin [13]. Pneumolysin acts in two ways to enhance proinflammatory responses in this setting: 1) pore-formation activates p38 MAP kinase phosphorylation, which then stabilizes chemokine mRNA [14] and 2) the pore facilitates the diffusion of extracellular bacterial products into the intracellular milieu for recognition by cytoplasmic pattern recognition receptors [15]. Lysenko and colleagues demonstrate that peptidoglycan from *H. influenzae* but not *S. pneumoniae* is sufficient to induce enhanced pneumococcal clearance and that this stimulation requires the intracellular pattern recognition receptor Nod1 [12,16]. Nod1 recognizes the minimal structure γ-D-glutamyl-*meso*-diaminopimelic acid (*meso*-DAP) common in peptidoglycan from gram-negative but not gram-positive bacteria [17]. Synthetic *meso*-DAP is sufficient to recapitulate the immunomodulatory effects of co-colonization with live *H. influenzae* in a Nod1-dependent manner [16]. Taken together, these data describe a proinflammatory signaling cascade that is initiated by the pore-forming toxin pneumolysin, which allows peptidoglycan fragments from gram-negative bacteria to access their cytosolic receptor Nod1, thereby activating NF-κB-mediated expression of chemokines such as IL-8.

IL-8 in humans and the equivalent in the mouse, MIP-2, are known to be potent inducers of neutrophil recruitment. In mice, a synergistic amplification of MIP-2 production can be measured following co-colonization with *S. pneumoniae* and *H. influenzae* and correlates with a robust migration of polymorphonuclear cells into the lumen of the nasopharynx one day following inoculation [12,13]. These cells are essential effectors of the observed immunomodulation since their depletion completely abrogates the inhibitory effect of *H. influenzae* on pneumococcal colonization regardless of similarly elevated MIP-2 levels [12]. A similar requirement for active complement in this system suggests that the mechanism of enhanced pneumococcal clearance is by neutrophil-mediated opsonophagocytic killing [12]. At this early time-point during primary colonization, antibody is neither present nor required for opsonization of pneumococci, which instead occurs via the alternative pathway of complement activation and recognition by complement receptor 3 (CR3; also known as Mac-1 or CD11b/CD18) [16].

Immunomodulation by co-colonization with *H. influenzae* results in a complete reversal in the outcome of early-stage interactions between the host and *S. pneumoniae*. To do so, *H. influenzae* induces a host-mediated inflammatory response to which itself is resistant that clears its niche of susceptible competitors. To survive this additional selective pressure, successful bacteria must evolve ways of resisting opsonophagocytic killing in order to establish colonization in the presence of competing *H. influenzae*. In this way, within-host colonization competition between microbes drives the selection for innate immune evasion mechanisms that in other settings contribute to virulence and allow for invasive disease. Pneumococci defend themselves against neutrophil-mediated killing by expressing an anti-opsonic capsular polysaccharide [5]. Over 90 distinct capsule types have been identified and each contributes to opsonophagocytic resistance in varying degrees [18]. Resistance to opsonophagocytic killing by capsule is the major virulence mechanism in the pathogenesis

of *S. pneumoniae* disease. Recently, it was demonstrated that capsule type dictates whether *S. pneumoniae* are sensitive or resistant to the immunomodulation of *H. influenzae* colonization [19]. This effect was independent of the genetic background of pneumococcal strains, since mutant strains expressing heterologous capsule types maintained the phenotype of the capsule regardless of strain genotype [19]. Therefore, when pneumococci of different capsule types compete for niche-space within the nasopharynx, innate immune pressure by co-colonizing *H. influenzae* selects for colonization by more opsonophagocytosis-resistant, and thus more virulent strains. In this way, immunomodulation by *H. influenzae* not only influences the outcome of pneumococcal disease progression within a host but also influences the outcome of future host-pathogen interactions by promoting the evolution of virulence traits in an otherwise commensal organism.

Effect of co-infection with influenza A

Co-morbidity of influenza and *S. pneumoniae* infection has been reported widely throughout the past century and has been recently reviewed elsewhere [20]. The majority of deaths during influenza pandemics are due to secondary bacterial infections from prevalent upper-respiratory tract bacteria, such as *S. pneumoniae* [21]. Seasonal influenza has also been temporally associated with an elevated incidence of invasive pneumococcal disease [22]. Many studies have described mechanisms of synergistic virulence based on mouse models wherein influenza and *S. pneumoniae* are simultaneously inoculated directly into the otherwise sterile lower respiratory tract, the ultimate site of fatality-inducing pathology [20]. However, it may be equally important to understand the initial stages of co-infection in the more physiologically-relevant setting of the upper respiratory tract, where the influenza virus first interacts with the pneumococcus in its natural reservoir.

During the natural course of influenza infection, the virus replicates in the mucosal epithelial cells of the upper respiratory tract [23,24] and is temporally associated with significant increases in the numbers of colonizing pneumococci in children [25,26] and in mice [24]. Elevated bacterial load in the nasopharynx due to viral co-infection significantly increases the risk of transmission of pneumococci into normally sterile anatomical sites, resulting in increased rates of invasive disease and death [24]. Similarly, greater densities of pneumococcal colonization increases the risk of transmission from host to host, resulting in increased spread within human populations [26] and animal models [27]. Thus, infection with influenza during upper respiratory tract colonization with pneumococci dramatically alters the outcome of pneumococcal host-pathogen interactions, independent of the effects of co-infection in the lung.

The influenza-induced increase in pneumococcal colonization density in mice occurs one week following inoculation, co-incident with the peak macrophage influx normally required to clear the pneumococcus during mono-inoculation [6]. Indeed, co-infection in the nasopharynx results in an inhibition of macrophage recruitment at this time point, correlating with a synergistic increase in type I interferon (IFN) expression and a resulting decrease in expression of the macrophage recruitment cytokine CCL2 [24]. These immunomodulatory effects of co-infection are abrogated during co-infection of mice with a targeted deletion in the type I IFN receptor (*Ifnar^{-/-}*), suggesting that type I IFN signaling is required for the observed increases in pneumococcal colonization density [24]. Moreover, boosting type I IFN expression by the synthetic TLR3 ligand poly-ICLC during mono-inoculation of pneumococci recapitulates the synergistic effects of co-infection with influenza, indicating that type I IFN signaling is necessary and sufficient to increase pneumococcal burden in the upper respiratory tract [24]. Synergistic increases in type I IFN expression were dependent on the bacterial toxin, pneumolysin, and the cytosolic pattern recognition receptor for peptidoglycan, Nod2, as bacteria and mice deficient for these

factors, respectively, no longer mounted elevated type I IFN levels in response to co-infection [24]. Together, these data support a mechanism wherein co-induction of type I IFN by influenza and pneumolysin-expressing pneumococci signaling through Nod2 attenuates the CCL2 response and blocks the macrophage recruitment that is necessary to clear pneumococcal colonization. Although it is well established that type I IFNs are central to the antiviral host response [28], the study of immunomodulation during co-infection reveals how type I IFN expression in response to viral co-infection can negatively impact anti-pneumococcal host defenses in the upper respiratory tract.

Effect of co-colonization with *S. aureus*

The primary ecological niche for the opportunistic pathogen *Staphylococcus aureus* is the anterior squamous epithelium of the upper respiratory tract [3]. Nasal colonization is a major risk factor for disease since approximately 80% of *S. aureus* infections are due to the host's colonizing strain [29,30]. Very little is known about what determines whether a human host will be permissive for nasal carriage. However, epidemiological studies have revealed that one subpopulation with a significantly reduced risk of *S. aureus* carriage are children who are colonized with *S. pneumoniae* [31-39]. These nine independent cohorts, which include over 10,000 subjects across 5 continents, have reproducibly demonstrated that immunocompetent pneumococcal carriers are approximately 50% less likely to be colonized with *S. aureus* than non-pneumococcal carriers. Conversely, a decline in pneumococcal colonization due to vaccination has been associated with an increased incidence of *S. aureus*-induced otitis media in children [40].

The mechanism underlying the observed protection against *S. aureus* by *S. pneumoniae* colonization is unknown. Similar to the studies of *H. influenzae*/*S. pneumoniae* antagonism described above, *in vitro* co-culture experiments implicated hydrogen peroxide-mediated competition between these two species [41-44]. However, neither hydrogen peroxide secretion by *S. pneumoniae* nor hydrogen peroxide sensitivity of *S. aureus* is predictive of co-colonization patterns in children [45,46] or animal models [47], suggesting that this mechanism may not be relevant *in vivo*. Moreover, since *S. aureus* and *S. pneumoniae* do not share the same microniche in the upper respiratory tract [48,49], a direct competitive mechanism is unlikely.

Rather, we and others [50] hypothesize that an immunological mechanism may be involved because the inhibitory effect of pneumococcal colonization on *S. aureus* carriage is abolished in HIV-positive immunocompromised hosts [33,34,51]. Generally, antibody functions to block the natural acquisition of bacterial pathogens in the upper respiratory tract [52]. During pneumococcal colonization in immunocompetent hosts, a robust antibody response is mounted to a diversity of proteins, however this is ineffective against the pneumococcus itself due to its anti-opsonic polysaccharide capsule. In contrast, anti-pneumococcal antibody responses and opsonophagocytosis are significantly ablated in HIV-positive individuals [53,54], who have impaired opsonic function of IgG isolated from their respiratory mucosa [55]. Data from co-colonization in animal models in our lab suggest that antibody elicited by pneumococcal colonization cross-reacts with the surface of *S. aureus* and is necessary and sufficient to inhibit the acquisition of *S. aureus* nasal colonization (RS Lijek *et al.*, submitted). If this antibody response provides cross-protection against the acquisition of *S. aureus* carriage in children, then its absence in hosts lacking a well-orchestrated adaptive response could explain the observed reduced incidence of *S. aureus* carriage in HIV-negative but not HIV-positive children.

Interspecies cross-protection by antibody is not novel. For example, cross-reactive antibody is the basis for the development of natural immunity to *H. influenzae* type b [56]. Just as this

cross-reactivity led to an understanding of immune protection against *H. influenzae* type b, a mechanistic understanding of the immunological inhibition of *S. aureus* carriage by *S. pneumoniae* colonization may inform future vaccine development against *S. aureus*, a public health priority.

Conclusions

The outcome of host-pathogen interactions at mucosal sites is a composite of the complex microbial ecology in those sites and the immunological pressures exerted by the host in response to colonization and infection. We describe the model of *S. pneumoniae* colonization in the upper respiratory tract and how the introduction of even one additional player – eg. *H. influenzae*, influenza A or *S. aureus* – can drastically influence disease progression. These examples highlight that otherwise effective host responses can be rendered ineffective by co-infection, and vice versa. While it is widely understood that successful pathogens are resistant to the immune responses they induce, pathogens are not necessarily resistant to immune responses induced by other organisms. “Off-target” immune responses can alter the microbial landscape by removing competing species from a host niche, driving the acquisition of virulence characteristics, enhancing or inhibiting pathogen clearance, and preventing pathogen acquisition. This can occur at myriad points along the time-course of host-pathogen interactions and can include components of both the innate and adaptive immune response. An important conclusion from these examples is that the triad of pathogen-host-pathogen interactions is not the sum of its individual pairings. Indeed, results from *in vitro* bacterial competition experiments that lack the host or *in vivo* analyses with a single pathogen may be misleading. The inclusion of additional species and variables into controlled experiments can be challenging [57]. However, understanding how co-infection subverts mucosal immunity may better inform us of the underlying immunological mechanisms in a natural setting.

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Highlights

Host responses elicited by a pathogen affect other microbes including other pathogens

Effective host responses can be rendered ineffective by co-infection, and vice versa

H. influenzae enhances PMN clearance of *S. pneumoniae* colonization but promotes virulence

Influenza increases pneumococcal carriage levels and risk of invasive disease via IFN

S. pneumoniae may reduce the risk of *S. aureus* carriage via adaptive immunity

Figure 1. Co-infection alters immune responses to *S. pneumoniae* colonization in the upper respiratory tract

(A) *S. pneumoniae* establishes colonization on the mucosal epithelium of the upper respiratory tract at day 1 post inoculation (p.i.) into the nares. Pneumococcal colonization elicits a neutrophil response that peaks around day 3 p.i. but is insufficient to clear the pneumococcus. However, co-colonization with *H. influenzae* results in a synergistic amplification of the neutrophil response that leads to elimination of pneumococci from the nasopharynx at this time-point. These enhanced host responses also drive the evolution of opsonophagocytosis-resistance determinants in the pneumococcus that contribute to this organism's pathogenicity. (B) During mono-inoculation with *S. pneumoniae*, a gradual monocyte/macrophage influx peaks around day 7 p.i. and is responsible for pneumococcal clearance. However, co-infection with influenza A results in a synergistic induction of type I interferon expression which inhibits macrophage recruitment and results in an increase in pneumococcal colonization density. Influenza-induced increases in *S. pneumoniae* in the nasopharynx lead to an increased risk of invasive pneumococcal disease and pneumococcal transmission. (C). A robust antibody response to a diversity of antigens is mounted by two weeks following *S. pneumoniae* colonization. Cross-reactive antibody could explain why *S. aureus* carriage is negatively associated with *S. pneumoniae* colonization in immunocompetent, but not immunocompromised, hosts. Immunofluorescent microscopy images generated and kindly shared by Dr. Aoife Roche (Univ. of Pennsylvania) and adapted from Ref.#5. Host cells, blue (DAPI); *S. pneumoniae* cells, red.