EVOLVING MECHANISMS OF DISEASE TOLERANCE

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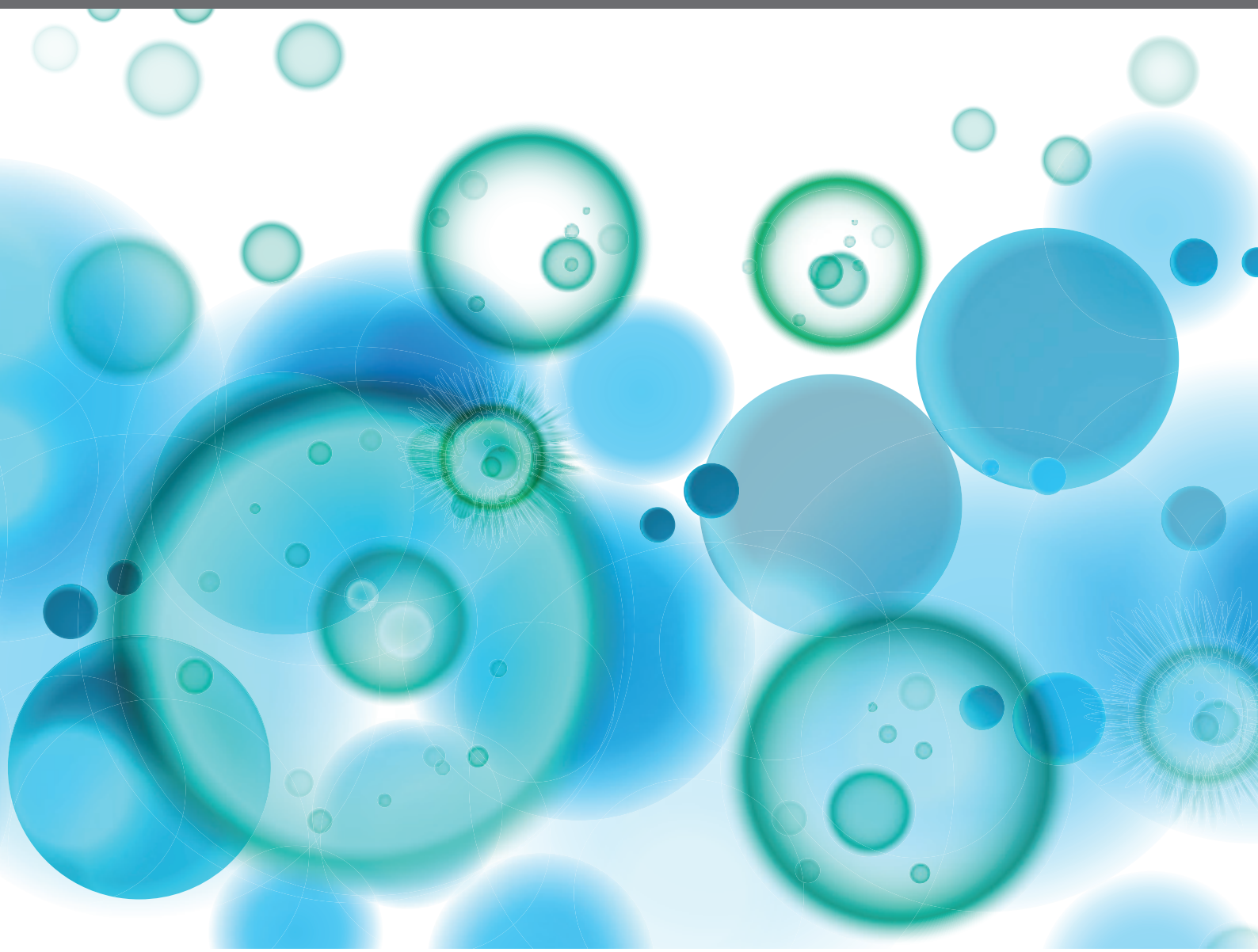

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EVOLVING MECHANISMS OF DISEASE TOLERANCE

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Table of Contents

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EDITORIAL
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Editorial: Evolving Mechanisms of Disease Tolerance

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Editorial on the Research Topic

Evolving Mechanisms of Disease Tolerance

Within the last 150 years, morbidity and mortality due to infectious diseases has drastically decreased worldwide (1). This change in health began with the pioneering cowpox studies of Edward Jenner followed by Pasteur's germ theory and Koch's postulates that eventually led to improved hygiene strategies, the advent of vaccines and discovery of antibiotics (2). These seminal observations about the infectious origins of disease spawned the golden age of Immunology in which investigators such as Elie Metchnikoff and Paul Ehrlich broadly described the cellular and molecular mechanisms of host defense. However, it has now become clear that defense against infection extends beyond host resistance and also includes mechanisms that limit tissue damage independent of changes to pathogen burden (3). This latter strategy is referred to as disease tolerance and involves coordination between immune cells and tissue-specific structural cells to maximize host fitness in the face of disruption to homeostatic conditions (4). It is important that "disease tolerance" is not confused with the equally important concept of "immune tolerance" in which immune reactivity is inhibited by clonal deletion or silencing of antigen-specific lymphocytes (5). However, the possibility that immune tolerance and disease tolerance can operate in a complementary fashion within the same setting of infection or inflammation is certainly not excluded.

The concept of disease tolerance was introduced in 1894 by Nathan Augustus Cobb, an American plant pathologist. From his studies in wheat, he observed the ability of certain strains to yield crop despite the presence of a fungal infection or "rust." He referred to this phenomenon as "rust-enduring" and distinguished this phenotype from "rust-resistant" wheat (6). Following Cobb's seminal observations, plant biologists rebranded the concept of endurance to disease tolerance (7). Although this concept was well-established in plant biology, it was not directly tested in mammals until more than a century later by Lars Råberg and Andrew Read. Specifically, they demonstrated that genetic variation in mice can delineate host resistance vs. disease tolerance following malaria infection (8). Soon after, molecular insights into these observations were provided by Miguel Soares' group demonstrating that tissue protection from the cytotoxic effects of malaria-induced hemolysis in mice is provided by the heme-catabolizing enzyme heme oxygenase-1 (9). In the same year, Ayres and Schneider demonstrated that simple organisms such as the fruit fly *Drosophila melanogaster* can also use disease tolerance as a host defense mechanism in the context of gram-positive and gram-negative bacterial infections (10, 11). Collectively, these studies have provided the impetus for investigating disease tolerance as an alternative and/or complementary form of host defense not only in the context of infection but also in settings of non-communicable diseases such as autoimmunity, asthma, and atherosclerosis.

This Frontiers Research Topic entitled "Evolving mechanisms of disease tolerance" aims to demonstrate how the research and our understanding of this concept is leading to, what we consider, a new golden age of infectious disease research and discovery. Considering the relevance of disease tolerance across the kingdoms of life and throughout the evolution of mammals, we have assembled exciting reviews detailing how this defense strategy is conserved from plants to humans

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against diverse forms of infection. Paudel and Sanfaçon return to the roots of disease tolerance by describing the mechanisms by which plants tolerate viral infection. Budischak and Cressler provide an ecological perspective on how environmental resources contribute to disease tolerance across the evolutionary spectrum. This study dovetails with the reviews of Carlos et al. whom discuss the impact of nutrient metabolism such as iron and glucose on tolerance to diverse pathogen challenge and Harbeson et al. whom examine how early-life metabolic responses impact long-term human health outcomes. Importantly, prokaryotes also utilize disease tolerance to defend against viral (i.e., bacteriophage) infection. Attention is brought to this emerging research field by Chatterjee and Duerkop highlighting the impact of bacteria-phage interactions and how they may contribute to eukaryotic immune responses.

Emphasizing the importance of disease tolerance during respiratory infection, Divangahi et al., Saelens et al., and Olive and Sassetti take aim at *Mycobacterium tuberculosis*. Their reviews emphasize how understanding the mechanisms of disease tolerance to *Mtb* infection may lead to new therapeutic strategies against Tuberculosis, the world's leading infectious killer. Beyond TB, extracellular bacterial and fungal infections of the lung remain important clinical problems, particularly in immunocompromised individuals. Disease tolerance to these pathogens are emphasized by Shourian and Qureshi in their discussion of *Cryptococcus neoformans* infection and Faure et al. whom describe mechanisms of host adaptation to *Pseudomonas aeruginosa* infection and how this response goes awry in patients with cystic fibrosis. Additionally, Allard et al. dedicate an entire article solely to describing how alveolar macrophages regulate disease tolerance in a range of settings from infection to allergy. In a complementary review, Crane et al. discuss the complex scenario in which primary respiratory viral infections can either increase or decrease disease tolerance to secondary bacterial infection depending on the immune status of the host. One important factor in the co-infection scenario are type 1 and type 2 interferons. These cytokines, reviewed by Lee and Ashkar, are rapidly and abundantly produced in response to viral and bacterial infection and have diverse roles in protective and pathogenic immune responses. In addition to highly replicative unicellular pathogens, hosts likely use distinct mechanisms of disease tolerance to protect against non-replicating, multicellular parasites. This form of pathogen challenge is the focus of two reviews in this Research Topic focusing on parasitic worm infection. Specifically, King and Li focus on the diverse mechanisms by which the mammalian host uses disease tolerance to defend as chronic intestinal helminth infection and, in a related article, Yap and Gause explore how helminths shape organ-specific strategies of disease tolerance and the consequences on heterologous infection. Finally, we have included a review by Mandl et al. in which they discuss how diverse species of bats are uniquely tolerant to viruses that are highly pathogenic to humans. Understanding the immune system of this animal will not only provide insight into mammalian mechanisms of disease tolerance, but also inform strategies that limit infection of this important pathogen reservoir. In sum, we hope that this collection will highlight recent developments related to the origins and function of disease tolerance as well as persuade the development of therapeutic strategies targeting this fundamental strategy of host defense against infectious diseases.

AUTHOR CONTRIBUTIONS

IK and MD contributed equally to this editorial and have approved it for publication.

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REVIEW
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Surviving Deadly Lung Infections: Innate Host Tolerance Mechanisms in the Pulmonary System

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Much research on infectious diseases focuses on clearing the pathogen through the use of antimicrobial drugs, the immune response, or a combination of both. Rapid clearance of pathogens allows for a quick return to a healthy state and increased survival. Pathogen-targeted approaches to combating infection have inherent limitations, including their pathogen-specific nature, the potential for antimicrobial resistance, and poor vaccine efficacy, among others. Another way to survive an infection is to tolerate the alterations to homeostasis that occur during a disease state through a process called host tolerance or resilience, which is independent from pathogen burden. Alterations in homeostasis during infection are numerous and include tissue damage, increased inflammation, metabolic changes, temperature changes, and changes in respiration. Given its importance and sensitivity, the lung is a good system for understanding host tolerance to infectious disease. Pneumonia is the leading cause of death for children under five worldwide. One reason for this is because when the pulmonary system is altered dramatically it greatly impacts the overall health and survival of a patient. Targeting host pathways involved in maintenance of pulmonary host tolerance during infection could provide an alternative therapeutic avenue that may be broadly applicable across a variety of pathologies. In this review, we will summarize recent findings on tolerance to host lung infection. We will focus on the involvement of innate immune responses in tolerance and how an initial viral lung infection may alter tolerance mechanisms in leukocytic, epithelial, and endothelial compartments to a subsequent bacterial infection. By understanding tolerance mechanisms in the lung we can better address treatment options for deadly pulmonary infections.

Keywords: host tolerance, pneumonia, lung infections, innate immunity and responses, lung epithelium, lung endothelium, tissue repair and regeneration

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INTRODUCTION

The ultimate goal for a host when responding to an infection is survival and a rapid return to a homeostatic state. This can be accomplished in several non-mutually exclusive ways. One is to quickly and efficiently clear the pathogen, and thus prevent excessive pathogen-induced pathology. The other is to mitigate any damage or changes caused by the infection. The ability to survive an infection is determined by two main factors, pathogen clearance and host tolerance (1–4). Disease tolerance is defined as the ability of the host to tolerate the effects of the pathogens and the potentially damaging effects of the immune response. Problems arise when these strategies are in direct conflict with each other. For example, the immune response in an effort to clear the pathogen often causes

damage, that is detrimental to the host. On the other hand, tolerance processes such as anti-inflammatory responses can cause immunosuppression and decrease pathogen clearance. Normally, however, a balance between these two processes is reached, and the infection resolves.

Changes in host disease tolerance are most obvious when the infection is in an essential organ. This is one reason why lung infections are ideal situations to examine mechanisms of tolerance. In addition to increasing our understanding of this aspect of pulmonary disease it also addresses a clinically relevant need (5). Lung infections are a top cause of disease with high economic and humanitarian costs in the United States and worldwide (6, 7). Community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP) can be caused by a variety of different pathogens, including viral, bacterial, fungal, and polymicrobial infections (7, 8). Bacterial pneumonia is a common complication of respiratory virus infection that leads to increased morbidity and mortality (9). Given the diversity of pathogens that cause pneumonia, treatment is complex and not always effective (10). As detailed in the recent National Heart Lung Blood Institute Working Group Report, future directions for pneumonia treatment should include host-targeted therapeutics, which includes therapeutics directed at host tolerance mechanisms (11). This review will explore the concept of host disease tolerance mechanisms in the context of acute lung infections (see **Figure 1** for a summary).

Public Health Implications of Lung Infections

Pneumonia is an infection of the lung that causes the alveoli, or air sacs, to fill up with fluid or pus (12). There are several risk factors for the development of pneumonia, such as advanced age, being immunocompromised, or having a pre-existing lung disease. Lower respiratory tract infections (LRTIs) cause the most deaths from an infectious disease worldwide (6), and have a large economic and personal burden (6, 13, 14). Pneumonia is the leading cause of death of children under five years of age worldwide (15). This is particularly true in the developing world, where it causes more deaths than either diarrheal disease or malaria. If pneumonia does not resolve it can lead to acute respiratory distress syndrome (ARDS), sepsis, increased risk of cardiovascular disease, and decreased pulmonary function.

There are several viral infections that lead to pneumonia (16). Influenza A virus (IAV) primarily infects the lung epithelium, and can cause viral pneumonia. It leads to an estimated 500,000 deaths annually, in addition to the hospitalizations and loss of productivity from infected people (17). There are also a variety of other viruses that can infect the lower respiratory tract and lead to pneumonia, including respiratory syncytial virus (RSV), parainfluenza, human metapneumonia, and some adenoviruses (14, 18–22). Rhinoviruses and newly described coronaviruses also infect the respiratory tract and cause disease. RSV, in

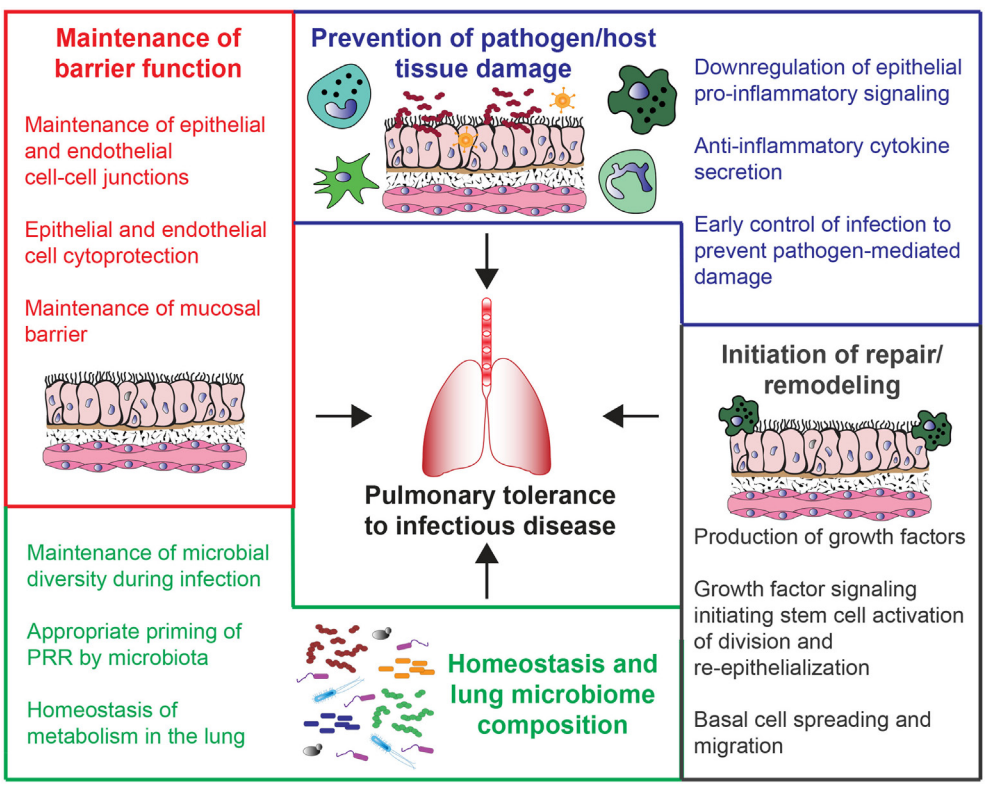

FIGURE 1 | Potential mechanisms of host tolerance to lung infections. These mechanisms are broadly divided into four main categories. Beginning clockwise from the top, they include prevention of pathogen/host tissue damage (blue), initiation of repair/remodeling (gray), changes in lung microbiome composition and homeostasis (green), and maintenance of barrier function (red).

particular, can cause complications in young children and is the leading cause of hospitalization in children less than one year old in the United States (18). Many of these respiratory viruses spread easily from person to person, or can be spread from an animal reservoir (23).

A variety of bacteria can also lead to the development of pneumonia. Bacterial pathogens as well as opportunistic infections (also known as pathobionts) can lead to pneumonia when allowed to infect the lower respiratory region. Bacteria that cause LRTIs naturally colonize the nasopharynx, but can cause disease when allowed to proliferate in the lower respiratory region (24–28). The most common examples of these are *Streptococcus pneumoniae* and *Staphylococcus aureus*. Other bacteria are acquired from the environment, and often these bacteria have specific virulence factors that allow for the adaptation and infection of the lower respiratory tract (29). An example of an environmental pathogen is *Legionella pneumophila*, which is found in freshwater amoebas and is able to proliferate in alveolar macrophages (30–33). Bacterial pneumonia is a common cause of both CAP and HAP (29, 33, 34). Like viruses, bacteria can also spread from person to person through expelled respiratory droplets.

In addition to viral and bacterial pathogens causing pneumonia there are certain fungal infections that can infect the lower respiratory tract. While more rare than viral or bacterial lung infections, fungal infections of the respiratory tract can be severe and cause pneumonia, especially in immunocompromised patients (35). *Aspergillus*, *Cryptococcus*, and *Candida* species have all been shown to cause lung infections in certain populations and in certain circumstances, such as individuals with increased environmental exposure and patients with suppressed immune systems (36–38). As there continues to be an increase in immunocompromised populations due to infections, such as HIV and also organ transplant populations, there has been an increase in overall fungal infections (35). Understanding how fungal colonization and infection influence the respiratory tract is an important area of study.

Polymicrobial Lung Infections

The vast majority of research in infection biology has been devoted to studying the interactions of a single pathogen with a host. In addition to single infections causing pneumonia, a common complication following infection with respiratory viruses is bacterial pneumonia (9, 26, 39–56). Many clinical infections and presumably subclinical infections are often in fact coinfections, in that two (or more) pathogens simultaneously or in close temporal proximity infect a single host (9, 26, 39–59). These infections are termed secondary infection, superinfection, or coinfection. The simultaneous response of two pathogens can manifest in many ways and often results in increased morbidity and mortality. Understanding how an infection with one pathogen can affect the response to another is of paramount importance in the complete understanding of the immune response to infection.

To determine the best treatment options for patients with complex viral/bacterial coinfections increased understanding of the interplay between pathogens and the interaction with the host is necessary. Several viruses and bacteria have been shown to interact to worsen clinical outcomes. It is now believed that most of the deaths associated with the 1918 influenza pandemic were caused by superinfection with bacteria (60, 61). IAV/*S. pneumoniae* coinfection is perhaps the most well-studied example of viral/bacterial coinfection of the lung (62). However, bacterial coinfection also complicates infection with other respiratory viruses, including rhinovirus, metapneumonovirus, RSV, parainfluenza virus, adenovirus, and coronavirus (52, 63–66). Young children are especially vulnerable to bacterial complications following viral infection (44, 62, 67, 68).

There are multiple proposed mechanisms whereby infection with a respiratory virus leads to decreased resistance to bacteria (41–43, 46, 49, 50, 54, 69–73). In most cases examined, initial infection with IAV increases the susceptibility to subsequent bacterial infection (either lung-tropic pathogens or opportunistic commensals), leading to increased bacterial load in the lung and in some cases bacterial dissemination and septicemia. Influenza-induced alterations include a suppression of the pulmonary immune system and changes to the lung epithelium that enable increased bacterial adherence and dissemination. These immunosuppressive mechanisms include neutrophil dysfunction and alterations in expression of essential chemokines and cytokines (41–43, 45, 49, 50, 69–73). Viral neuraminidase alters the lung epithelium causing increased bacterial adhesion (57). While IAV is the best studied and has the clearest causal link to secondary bacterial infections causing pneumonia, several other viruses have also been indicated. RSV has a clear temporal link to causing a secondary pneumonia with *S. pneumoniae* (64, 65). It is likely that most respiratory viruses influence the susceptibility to bacterial infections, either by causing damage or by alteration of the pulmonary immune response.

Bacterial pneumonia secondary to a respiratory virus infection is identified clinically when there is a clear fulminate bacterial overgrowth. This increased pathogen burden correlates with an increased lung pathology, although it is difficult to separate out damage caused by the increased pathogen burden itself from damage caused by the host response. However, there is increasing evidence that alterations in tolerance mechanisms, specifically decreased tissue repair, may play an important role in the pathogenesis of lung infections and this is amplified when the infections are polymicrobial (11, 62, 74–76).

Host Disease Tolerance to Infectious Disease

Tolerance as a defense strategy against infection was first described by researchers studying infectious diseases in plants. It was based on the recognition that plants could survive an infection by limiting tissue damage, despite having a high pathogen load (77). In subsequent years, tolerance has been recognized as an evolutionarily conserved mechanism for hosts of many species to survive infection and has been described in the context of other infectious diseases (4, 78–80). This includes studies regarding tolerance to infection with plasmodium, the causative agent of malaria (81–83). Unfolded protein responses have been shown to be essential in conferring tolerance to *Pseudomonas aeruginosa* infection, and an increase in tissue repair factors can

confer tolerance to lung infections (84, 85). There have also been roles described for the aryl hydrocarbon receptor in controlling the innate immune response that can lead to increased tolerance (86, 87). These and other studies have opened a new line of treatment options for complex infectious diseases.

There are a variety of antimicrobial interventions that have been introduced to combat lung infections. For viruses this includes both preventative vaccines and in some cases antivirals (88–92). There are also a number of antibiotics that target bacterial pathogens and pathobionts that cause infection of the lower respiratory tract. However, despite the increased availability of antibiotics, many bacteria are still associated with pneumonia, including *S. pneumoniae*, *S. aureus*, *Klebsiella* spp, *Haemophilus influenzae*, *Moraxella* spp, and *Legionella* spp (9, 39, 47, 50–52, 56). Often antibiotics are ineffective due to resistance or timing of the intervention. In addition, bacteria that are resistant to all antibiotics are emerging. It is becoming increasingly clear that antimicrobial drugs are not universally effective in treating single infections and especially the more deadly polymicrobial infections of the lung (93, 94). Therefore, novel treatment strategies will be necessary to increase our ability to treat pneumonia. This will be especially relevant as we prepare ourselves for the next IAV pandemic, which will likely include a strong burden of secondary bacterial infection (94–96). It appears that during coinfection the balance between pathogen clearance and host tolerance is disrupted even more than during lung infections with a single pathogen (84, 97). In particular, pathogen-induced damage and damage directly from the immune response may cause a decrease in tissue resilience, making it even more difficult to return to a homeostatic state (24, 98, 99).

This review explores tolerance mechanisms that are affected by infections of the lung, with a specific focus on tolerance mechanisms directed by the innate immune response, the lung epithelium, the lung endothelium, and the lung microbiota. One clear mechanism of decreased tolerance is an excess of inflammation. This can come from innate immune cells as well as from the lung epithelium and endothelium. Decreasing inflammation can increase host tolerance in some cases, but this is complex as the inflammatory response is so closely tied to pathogen resistance (5). Many cells of the innate immune response are also important in tissue repair. These include innate lymphoid cells (ILCs) that produce IL-22 and also the growth factor amphiregulin. Both of these factors primarily act on the lung epithelium to initiate and maintain repair processes. Other important innate immune mediators of repair are alveolar macrophages. Maintaining the barrier functions of the lung epithelium and endothelium is essential for host tolerance in order to regulate the influx of inflammatory mediators during infection. These cells can also themselves become activated during infection and contribute to immunopathogenesis through excessive inflammatory cytokine production. Finally, as is becoming increasingly recognized, not only do the mammalian cells have a role in most aspects of our health, but the microbes that share the body also play important functions. It is likely that host tolerance mechanisms are no exception and microbiota of the lung are able to alter tolerance to pulmonary infections, with loss of homeostasis correlating with microbial dysbiosis. This review will cover these aspects of tolerance to acute lung infections with a specific focus on viral/bacterial coinfections and how tolerance is altered by infection with two distinct pathogens.

INNATE IMMUNE TOLERANCE MECHANISMS

Viral infections of the lung are often characterized by early inflammatory responses from both the lung epithelium and innate immune cells in an attempt to clear the virus, as well as resultant damage done to the tissue by both the virus and the immune response mounted to the virus. Without proper compensatory host mechanisms to return to an anti-inflammatory, homeostatic state, and repair the damage done to the tissue following infection, the host becomes susceptible to secondary bacterial infections which are known to increase morbidity and mortality of the host. It is often challenging to fully separate out how the innate immune response impacts resistance mechanisms from how it affects host disease tolerance. The innate immune response, while necessary to clear the pathogen, can cause damage to the tissue thus decreasing tolerance. However, when the innate immune response is suppressed to prevent immunopathology, this can lead to an increase in pathogen load which in turn can also cause tissue damage. In addition, the acquired immune system plays important roles in both resistance and tolerance to pulmonary infections, but its involvement is beyond the scope of this review, which will focus on innate (or early) tolerance mechanisms. This section of the review will explore the impact of the innate immune system on its contribution to host tolerance to pulmonary infections.

Decrease of Innate Immunity-Induced Damage Can Increase Host Tolerance

Early control of viral replication is mediated by innate immune cells that respond to signals from infected epithelial cells. Among the early-responding cells are natural killer (NK) cells, of which there is a resident population in the lung. NK cells are essential for viral clearance as has been shown in many infectious models, but they have also been implicated in causing severe lung damage. As part of their antiviral response, NK cells produce a great amount of IFN-γ, which contributes to acute lung injury (ALI) and death (100). Studies have shown that either depletion of NK cells, knockout of IL-15 (a cytokine that controls NK cell proliferation), or neutralization of IFN-γ can decrease morbidity and ameliorate the tissue damage done by NK cells during infection with RSV despite an increase in viral burden, indicating that while NK cells are important for control of viral replication, they are also responsible for increased immunopathology in the lung (101, 102). This is not only true for viral infections, but also for bacterial infections (24–28). One example is that in a model of tularemia, mice lacking NKT cells, cells that share properties of both NK and T cells, survive infection better than mice with NKT cells (103). Invariant NKT cells in conjunction with macrophages have also been shown to cause a chronic inflammatory disease following viral lung infection (104) due to persistent activation of the innate immune response. These studies collectively show that, while essential in

responding to lung infections, many innate lymphocyte subsets can cause pathology that decreases tolerance to infection.

Other innate immune cells that are implicated in the pathogenesis of viral infections are inflammatory monocytes and macrophages that infiltrate the lung following infection. Monocytes and monocyte-derived cells, such as macrophages and dendritic cells, are important mediators of the inflammatory response to infection. They are also phagocytes that can help control pathogen burden and remove dead cells and debris that accumulate during infection. However, these cells have also been shown to have roles in contributing to an excessive inflammatory response and resultant damage. In a model of IAV infection, blockage of CCR2, the receptor expressed on monocyte-derived cells that facilitates their entry into the lung, results in decreased inflammatory cell infiltrate, inflammation, tissue damage, and mortality without any effect on viral clearance (105, 106). It has also been shown that failure of these cells to induce programmed cell death during the resolution of infection results in unregulated, prolonged inflammation which in turn decreases tolerance (107–111).

Neutrophils are short-lived polymorphonuclear cells that are potent mediators of the inflammatory response very early during infection that are capable of unleashing powerful antimicrobial defenses at the cost of extreme tissue damage. Although these cells are very important for rapid clearance of pathogens, dampening their inflammatory effects has been shown to be beneficial for improving pulmonary function and survival. In a study of rat coronavirus, depletion of neutrophils results in increased mortality due to delayed viral clearance; however, their absence is also associated with decreased inflammation and breakdown of the epithelium (112). Other studies with IAV infection have shown conflicting protective and pathologic roles for excessive pulmonary neutrophilia. One study has shown that increased neutrophilic recruitment to the lung during IAV infection is associated with increased immunopathology attributed to tissue damage done by neutrophil extracellular traps (113). However, other studies have shown that depletion of neutrophils during early IAV infection not only results in increased viral loads but also increased inflammation and decreased epithelial barrier function (114, 115). Another study showed that depletion of MIP-2/CCL8 results in attenuated neutrophil recruitment into the lung which is associated with decreased pathology without a significant effect on viral burden (116).

Alveolar macrophages are the sentinel cells that patrol the lungs and are often first to encounter pathogenic invaders. These macrophages have very important roles in mediating early defense mechanisms as well as facilitating the return to homeostasis during the resolution of infection. Their roles in fighting against viral infections are as yet controversial and appear to be very virus-specific. For instance, depletion of alveolar macrophages during IAV infection exacerbates inflammation and contributes to decreased epithelial barrier function and vascular leakage (117, 118). Similarly, a model of lung infection with RSV demonstrates increased viral titers, inflammatory cell infiltrate, and resultant inflammation following depletion of alveolar macrophages (119). In contrast, depletion of alveolar macrophages during pulmonary infection with coronavirus is shown to decrease viral titers and increase survival potentially through attenuation of pathogenic T cell responses (120). In addition, depleting alveolar macrophages prior to infection with human metapneumovirus ameliorates disease through significantly decreased viral titers and decreased inflammation (119). Mice with a defect in alveolar macrophages but intact adaptive immunity had normal viral clearance but increased morbidity and lung failure (121). Therefore, the pathogenic or protective contributions of alveolar macrophages appear to depend heavily on the specific viral infection.

Modulating Tolerance Mechanisms to Infections Can Impact Disease Outcomes

There are several mechanisms that the host employs during the resolution of infection to repair lung injury and it has been shown that the absence or impairment of some of these results in worsened disease outcomes and greater susceptibility to secondary infection. Cytokines and growth factors produced by the innate immune response play a crucial role in suppressing inflammation, initiating tissue repair, and returning the pulmonary system to a state of homeostasis after the resolution of the infection. This section describes the innate immune-produced mediators of tolerance in the pulmonary system.

An important example of this is the role of IL-22 in influenza infection. IL-22 is a cytokine that is expressed by a number of immune cell types and acts on the epithelium to induce proliferation and growth, making it an extremely vital player in mediating repair following infection. In models of influenza infection, IL-22$^{-/-}$ mice exhibit increased morbidity and mortality correlative with decreased airway epithelial integrity and increased apoptosis of epithelial cells during the resolution of infection (122, 123). Importantly, influenza-infected IL-22$^{-/-}$ mice show no difference in viral load when compared to wild-type controls indicating that the decreased survival in these animals is due to decreased tolerance and is independent of resistance to the virus. Conventional NK cells were shown to be a major source of IL-22 during influenza infection and adoptive transfer of IL-22-competent conventional NK cells to IL-22$^{-/-}$ mice was shown to rescue epithelial cell regeneration (124). Another mechanism to promote tissue repair following infection is the activity of amphiregulin, which acts on the epithelium to induce cell proliferation much like IL-22. Studies have identified that during influenza infection, amphiregulin is produced by both ILCs and CD4^{+} regulatory T cells (85, 125). These studies have shown that depletion of either of these cell types or inhibition of their ability to produce amphiregulin results in decreased lung function and epithelial barrier integrity without any changes to viral burden. Administration of amphiregulin in either of these cases was shown to ameliorate tissue damage and facilitate tissue homeostasis (85, 125). Collectively, the research done with both IL-22 and amphiregulin provides examples of the importance of host tolerance in maintaining barrier function in the lung and returning to homeostasis in order to promote survival following infection independent from resistance.

The significance of host tolerance during viral infection is especially highlighted by studies that have shown that in its absence, virally infected hosts become more susceptible to secondary bacterial infections. For example, it has been shown that

IL-22$^{-/-}$ mice previously infected with influenza are more susceptible to a secondary bacterial infection with *S. pneumoniae* and exhibit decreased survival and increased bacterial burdens when compared to wild-type coinfected animals (123). In a model of coinfection with IAV and *L. pneumophila*, coinfected mice were shown to have significantly increased morbidity and mortality accompanied by excessive inflammation and tissue damage despite similar viral and bacterial burdens when compared to singly infected animals (84). These effects were abrogated by dampening inflammation through the use of an attenuated bacterial strain combined with administration of amphiregulin, which was able to increase survival and ameliorate damage to the epithelium during coinfection (84). Macrophages also play an important role in regulating tolerance after lung damage (126). Forms of tissue remodeling, such as the remodeling of the extracellular matrix (ECM) during IAV infection, have been shown to critically affect host tolerance (127). Influenza/*S. pneumoniae* coinfections were shown to significantly upregulate MT1-MMP9 expression by macrophages, which contributed to the host-mediated degradation of the ECM and the epithelial cell barrier built upon it. Inhibition of MMP9 by antibody-mediated inactivation was able to significantly limit mortality in mice (127).

Another target for therapy is pattern recognition receptors (PRR). The idea is that by targeting PRR signaling, the damaging aspects of inflammation can be mitigated. Notably, researchers employed the TLR4 inhibitor eritoran in a murine model of lethal influenza infection. Through the first four days of infection and co-treatment with eritoran, viral titers did not notably decrease; however, pro-inflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6, and CXCL1 were mitigated during this early infection and the IAV infection resolved more rapidly (128). Similarly, activation of the inflammasome is crucial for clearance of many lung pathogens; however, delaying the activation of NLRP3 during influenza infection not only decreases inflammation but also decreases bacterial burden with a secondary infection (129).

It is important to note that in some cases factors that modulate tolerance have dual roles that can also play a part in altering the outcome of disease. One example of this is shown in a study by Liu *et al.* which demonstrates the interconnectedness of tolerance and resistance. In this study, IL-27 was administered either during the early or late phase of influenza infection and was shown to have a profoundly different effect depending on the time in which it was given. When IL-27 was administered early in infection, it resulted in impaired viral clearance and worsened disease; however, when administered late in infection, there was decreased pathology, increased survival, and no impact on viral clearance. Other examples of factors that modulate both tolerance and resistance in this kind of reciprocal fashion are TGF-β, IL-10, and interferons, particularly type III (72, 130–143).

These results indicate that although some factors may be able to boost tolerance, they also have the potential to negatively impact resistance and are, therefore, perhaps unsuited for therapeutic use in certain infections. On the other hand, there are some factors that play roles in boosting both tolerance and resistance, making them potentially more attractive for therapeutic use. Examples of these can be found in resolvins which have been shown to decrease inflammation in both long-term and acute bacterial infections as well as viral/bacterial coinfections (144–149). In some cases, these lipid mediators can increase resistance to pathogens as well (147, 149, 150).

Taken together, work done in this field has shown that the early immune response to pulmonary infections can damage host tissue, causing loss of tolerance and potentially increasing susceptibility of the host to secondary infections. Inflammation and tissue damage caused throughout a pulmonary infection without proper compensatory tolerance mechanisms in place to ensure the return to homeostasis is associated with decreased survival and increased vulnerability to bacterial infections, and this phenomenon is seen irrespective of control of pathogen burden. In addition, the innate immune response has many factors as discussed above that act to decrease the inflammatory response and/or repair tissue damage. How these factors contribute to tolerance mechanisms in the lung epithelium will be discussed further in the next section. Recent work has emerged that highlights the previously unappreciated role of host tolerance to infections; however, more research needs to be done in order to fully elucidate further mechanisms of immune-mediated host tolerance and the roles that leukocytes play throughout both single and polymicrobial infections.

LUNG EPITHELIUM TOLERANCE MECHANISMS

Epithelial cells represent critical signaling nodes which are responsible for the orchestration of both intracellular and intercellular immune and tolerance responses throughout all stages of infection in the lung (151). Dysregulation of these processes by epithelial cells during single and polymicrobial infections is a major factor in the loss of pulmonary tolerance during infection. This section will briefly describe the broad-ranging responsibilities of epithelial cells signaling in response to general pulmonary infections, from initial sensing to resolution, before highlighting several common mechanisms through which polymicrobial infection dysregulates or abuses these signaling networks to compromise host tolerance (see **Table 1** for a summary).

Epithelial Cells Modulate the Local Pulmonary Immune Response During Acute Infection

A critical first step in any security system, including the immune response, is to detect the presence of intruders. Airway epithelial cells are responsible for the detection of microbes in the respiratory system *via* the recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (152). Epithelial cells accomplish this through the expression of a diverse repertoire of PRRs, such as toll-like receptors (TLR), C-type lectin receptors, cytoplasmic retinoic acid-inducible gene-I-like receptors, and NOD-like receptors (153). However, modifications in the expression levels of PRRs in response to primary infections can lead to profound diminishments in tolerance for secondary infections. A wide range of viruses upregulate type

TABLE 1 | Summary of epithelial-mediated tolerance responses.

Epithelial-mediated tolerance response	Protein mediators of epithelial cell tolerance response	Pathogens negatively impacting epithelial cell tolerance response
Modulation of pattern recognition and downstream signaling	Toll-like receptors (154), NOD-like receptors (154), RIG-I-like receptor (154)	Respiratory syncytial virus (RSV) (156), influenza A virus (IAV) (156), Sendai virus (156)
Inflammatory upregulation	Type 1 IFN (153), TNF-α (128), IL-1β (128), NF-κB (154), IFN-γ (100)	IAV (128), RSV (100), cytomegalovirus (CMV) (165), Epstein–Barr virus (165), variola virus (165), severe acute respiratory syndrome coronavirus (165)
Barrier function maintenance	Claudin (193), occludin (193), E-cadherin (200), catenin (200)	IAV/*S. pnemoniae* (183), adenovirus (196), coxsackievirus (196), RSV (190, 196), *Haemophilus influenzae* (198), rhinovirus (201), *S. aureus* (198, 202), *P. aeruginosa* (190)
Antimicrobial peptide secretion	Glycoconjugated mucins (161, 186), β-defensins (161, 189), surfactant protein D (161)	IAV, *S. pnemoniae* (187), RSV (177, 189), *H. influenzae* (190), rhinovirus (180)
Immune cell recruitment	Type 1 and 2 IFN (147), CCL5, CCL2, CCL8 (117)	IAV (105, 106, 117, 176), IAV/*S. pneumoniae* (148), *F. tularensis* (103), IAV/*L. pneumophilia* (84)
Resolution of clearance effectors	Resolvins (167, 168), TGF-β (136), IFN-λ, IL-22 (122, 123), IL-10	IAV/*S. pneumoniae* (179), RSV (100, 101)
Increased proliferation, differentiation, and repair	TGF-β (136), AREG (85, 125), IL-22 (122, 182), IFN-λ, Fgfr2b (184), ADAMTS4 (186)	IAV/*S. pneumoniae* (43, 123, 178), IAV (124, 179), RSV (180), *P. aeruginosa* (145)

Potential epithelial-mediated tolerance responses are summarized. The epithelial-derived mediators, and the pathogens that impact these mediators, are described.

I IFN expression in respiratory epithelial cells, which correlates with a significant upregulation of TLRs in many lung resident cells, including respiratory epithelium (154, 155). This dramatic upregulation of TLRs in response to the viral infection has been hypothesized to contribute to the upregulation of cytokine secretion and the initiation of cytokine storm and ARDS. Many therapeutic strategies inhibiting either the activity or signaling downstream of PRRs have been shown to augment host tolerance to secondary bacterial infection through such mechanisms (128, 156).

Upon detection of PAMPs by PRRs, respiratory epithelial cells trigger a broad battery of inflammatory genes and type 1 IFN downstream of NF-κB and the IRF transcription factors, respectively, which has been reviewed extensively elsewhere (152, 157–159). Generally, PRR signaling upregulates cell-autonomous and non-cell-autonomous immune responses to infection. Cell-autonomous functions include the secretion of antimicrobial peptides (AMPs) by epithelial cells, programmed cell death, and other intracellular stress response pathways (145, 160, 161). Non-cell-autonomous signaling primarily works through the initial secretion of cytokines mediating immune cell recruitment (162). However, as has been well-documented in the case of influenza/bacterial coinfection, priming of the immune response by initial influenza infection results in a massive over-recruitment of immune cells by epithelial cells. This occurs due to the cytokine storm generated by epithelial cells, which are primed and actively secreting cytokines to respond to the primary infection, and become hyper-stimulated upon sensing of PAMPs and DAMPs generated by the secondary bacterial infection (163). Similar responses occur with pulmonary infections by cytomegalovirus, Epstein–Barr virus, *Streptococcus* spp, variola virus, severe acute respiratory syndrome coronavirus, and many others (164). Oftentimes, such complications will present as ARDS in the clinic due to diminished pulmonary tolerance when responding to simultaneous infections (164).

Once immune cells are in the pulmonary environment, respiratory epithelial cells further modulate their behavior by signaling through more cytokines, alarmins, and efferocytic signals to augment clearance efforts (165–167). Finally, upon clearing the infection, respiratory epithelial cells direct the resolution of the immune response through the secretion of resolvins to enable cells in the pulmonary space to transition their efforts from clearance to repair and remodeling to restore pulmonary homeostasis (146, 166–169).

Oftentimes, viral respiratory pathogens will take advantage of the proliferative state that epithelial cells enter during remodeling and repair efforts to augment their own proliferation in the cell. For instance, IAV has been observed to induce epithelial cell expression of TGF-β and processing of latent TGF-β precursor into active TGF-β to both suppress the host immune response and to enhance its own replication (136, 170). Add-back therapeutic strategies introducing exogenous resolvins into the respiratory space have also been observed to augment tolerance in certain instances when they do not compromise pathogen clearance (144, 146).

Alteration of the Lung Epithelium During Infection

Respiratory epithelium tissue homeostasis is required to maintain the continuous biomechanical and cellular processes associated with aerobic respiration. However, the respiratory epithelium is also one of the primary tissue types affected by pulmonary infection, with dysfunction and degradation of the epithelial layer being a primary mechanism of pathogenesis. The reason for this is that lung epithelial cells are the primary target for infection in diverse respiratory viral diseases, such as IAV (171), RSV (172), coronavirus (173), rhinovirus (174), parainfluenza virus (175), and respiratory adenovirus (176).

As a target for lung pathogens, the respiratory epithelium plays an important role in pathogen-sensing and orchestrating downstream inflammatory responses (151). The multifaceted immune response of the respiratory epithelium must strike an appropriate balance between pro-inflammatory mechanisms of pathogen clearance that may cause incidental tissue damage and anti-inflammatory mechanisms of cytoprotection and tissue regeneration which can inhibit clearance. While this is true for certain viral, bacterial, and fungal pathogens, it is amplified in coinfection. Current research into respiratory viral/bacterial coinfections indicates that much of the enhanced pathogenicity of these coinfections stems from the inability of respiratory epithelial cells to triage these immune responses to simultaneous respiratory infections while incurring severe damage (177). Fortunately, the pulmonary research community has made significant strides in understanding the immune mechanisms underlying tolerance to respiratory infections by altering components of the respiratory epithelium's response to infection regarding pro-inflammatory and cytoprotective signaling. The following section will review recently identified mechanisms of respiratory epithelial tolerance and their potential significance as therapeutics mitigating the severity of diverse pulmonary infections.

Tissue Repair/Cytoprotection-Mediated Tolerance

Host tolerance is the ability of the host to sustain an ongoing infectious state characterized by high pathogen titers, while maintaining tissue integrity and homeostasis. This allows for proper organ function and the avoidance of pathogen-mediated symptomology, morbidity, and mortality. With most respiratory infections, much initial pathology results from respiratory epithelial cell death. IAV/bacterial coinfections are an excellent case study in this phenomenon. IAV and a range of bacterial coinfections exhibit synergistic lethality resulting from a combination of IAV's initial infection compromising the respiratory epithelium of the host and the subsequent inability to initiate tissue repair due to uncontrolled inflammation and tissue damage incurred while simultaneously combating the secondary bacterial infection (178). IAV initially infects the upper respiratory tract and spreads to the lower respiratory tract within the first several days of infection (178). Infected cells throughout the respiratory epithelium become dysfunctional due to the burdens of intracellular viral replication, resulting in denuding of the respiratory epithelium and exposure of the basement membrane (165). This primes the respiratory environment for the emergence of opportunistic bacterial infections (pathobionts), or infection by bacterial pathogens. Denuding the epithelial layer exposes matrix proteins which contain an array of receptors for bacterial adherence, such as the adherence of *S. pneumoniae* to the tracheal epithelium of IAV-infected mice (166). Bacterial infection of the newly exposed basal layer prevents the initiation of epithelial coordinated tissue repair (178). To clear the bacterial infection, the epithelium recruits immune cells. This response can trigger a severe inflammatory response, further damaging the pulmonary tissue, while worsening the overall progression of the disease state and inhibiting the initiation of the repair process by epithelial cells. In sum, there is a severe loss of host tolerance to IAV/bacterial coinfection resulting from the initial cell death caused by IAV. This allows for the secondary infection and the subsequent over-recruitment of immune cells, which secrete pro-inflammatory cytokines that interfere with the initiation of tissue regeneration and repair.

Augmentation of respiratory epithelial cell cytoprotection and tissue repair has become a central theme in the search for host-directed therapeutic strategies increasing tolerance to pulmonary infection. The role of ILCs targeting tissue repair was described above (85, 125). While pathways inducing cytoprotection or inhibiting cell death are often separate from pathways involved in tissue repair, tolerance is often maximally impacted by inducing both effects simultaneously. Previous research has demonstrated that modulating inflammatory responses by blocking TLR signaling and upregulating tissue repair through amphiregulin treatment significantly increases host survival in a model of IAV and *L. pneumophila* coinfection (84). Inhibition of PRRs and their downstream signaling is capable of significantly suppressing the inflammatory response. However, PRR activation is also a critical trigger initiating bacterial clearance by epithelial and immune cells. Inhibiting the inflammatory response to augment host tolerance is a delicate balance as described in the innate immune section of this review.

It is a general consensus that many respiratory pathogens have evolved strategies to prevent host tissue repair and the return to epithelial homeostasis. This allows them to maintain an environment conducive to pathogen replication (179–181). Many of these tissue repair strategies are started by the innate immune response (as described above), but their effects are upon the lung epithelial cells. Transcriptomics analysis of the 2009 pandemic IAV infection with *S. pneumoniae* coinfection demonstrated that the two pathogens interacted synergistically to significantly downregulate tissue remodeling, epithelial cell proliferation, and cytoprotective transcriptional pathways (43). Many studies have also demonstrated that restoration of critical signal transducers in these repair pathways, such as IL-6 (182), IL-22 (122), Fgf10 (183), and ADAMTS4 (184), are able to restore repair and help to rescue murine models of IAV infection *via* augmented host tolerance. In particular, Barthelemy *et al*. demonstrated that the increase in tissue integrity resulting from IL-22 immunotherapy reduces secondary bacterial systemic invasion (185). Small molecule therapeutics, such as progesterone, which acts on the amphiregulin pathway to initiate tissue repair after IAV infection, have also been investigated with some success in a female murine model (186). Characterizing discrete host tolerance pathways modulating tissue repair and cytoprotection is required to effectively develop tolerance-augmenting therapeutic agents.

Modulation of Respiratory Epithelial Barrier Dynamics

The maintenance of barrier function between the lumen of the lung and the bloodstream is one of the primary functions of respiratory epithelial cells and is critical in tolerance of pulmonary infection. The direct infection of the respiratory epithelium by lung pathogens and commensals is normally prevented by the presence of the mucosal layer containing secreted AMPs, such as β-defensin, MUC5AC, and MUC5B (187). While it has long

been clear that the mucosal layer is critical in regulating tolerance and tissue homeostasis, recent findings have further elucidated mechanisms through which it modulates host tolerance to infection. For instance, mucins and many other AMPs are interspersed with other glycoconjugates in the respiratory mucosa (188). Mucins themselves are also highly sialylated, assisting in the formation of the mucosal barrier (188). However, influenza viral neuraminidase is capable of penetrating through the respiratory mucosal layer and infecting mucus-producing epithelial cells by cleaving sialylated glycoconjugates (189). This results in an inability to maintain the density of the mucosal layer, which promotes the transition of bacteria that are found to colonize healthy individuals to overgrow and become pathogenic (26, 190). It was demonstrated that the impact of influenza infection was modulated by the concentration of sialic acid content in mucins, and increasing the concentration of sialylated substrates in mucins increased the resistance of epithelial cells to influenza infection in a dose-dependent manner (189). Primary RSV infection has also been shown to downregulate the transcriptional expression of β-defensin, which allows for *H. influenzae* to transition from commensal to pathogenic in the upper airway, by inhibiting the microbicidal activity of the mucosal layer (191).

Not only do direct interactions between pathogens and mucosa mitigate the tolerance effects of the mucosal barrier, but indirect effects of primary viral infection on the composition of the respiratory epithelial barrier and behavior of respiratory immune cells also negatively impact mucosal-mediated tolerance. RSV infection has been demonstrated to infect basal epithelial stem cells which control the ratios of ciliated and mucosal cells in the progeny. RSV-infected basal cells produce many more mucosal cells and far fewer ciliated epithelial cells (177). Therapeutic strategies that speed the regeneration of mucus-secreting epithelial cells could serve to augment tolerance in some infections, but also may worsen outcomes in others. This dysregulation of mucociliary function promotes environments more amenable to secondary bacterial infection. Similarly, rhinovirus has been demonstrated to induce neutrophil elastase, which cleaves and inactivates AMPs secreted into the mucosa by respiratory epithelial cells and promotes secondary bacterial infections, thereby inhibiting the steady state tolerance mechanisms in the respiratory epithelium (192). It was proposed that therapeutics downregulating or inactivating neutrophil elastase during rhinovirus infection might help to maintain host tolerance during the infection (192).

Below the respiratory mucosa, the respiratory epithelial cells themselves also operate as a critical barrier preventing pathogenic infections from spreading beyond the respiratory system into a systemic bacteremia. Maintenance of the respiratory epithelial barrier function below the mucosal layer requires the maintenance of a complex network of intercellular junctions linking individual epithelial cell cytoskeletons. The maintenance of this barrier during infection is a critical tolerance mechanism preventing the dire outcomes resulting from respiratory infections transitioning to systemic bacteremia, pulmonary edema, and excess infiltration of immune cells. Epithelial cell–cell junctions bind epithelial cells into the cohesive barrier between the lumen of the epithelium and the parenchyma. Respiratory epithelial junctions have been extensively reviewed elsewhere (193–195). This section serves to summarize their function and relevance in the maintenance of epithelial barrier function and host tolerance to pulmonary infection.

Tight junctions form the separation between the apical and basolateral face of epithelial cells. Integral membrane components of tight junctions are mainly comprised of claudins, occludins, and adhesion molecules (194, 196). However, tight junctions are highly heterotypic with many different constituent components. Tight junctions often contain many entry receptors for pathogens. When components of tight junctions are employed as entry receptors, their ability to effectively maintain barrier function decreases and diminishes the host's ability to tolerate secondary bacterial infections as effectively. This dynamic has been observed in both models of adenovirus and coxsackie virus infection (197). Pathogen modification of gene expression also has the capacity to interfere with tight junction expression. RSV infection was shown to downregulate the expression of *claudin-1* and *occludin* in a mouse model, inhibiting barrier function as mediated by tight junctions (198). Infection with *H. influenzae* was also demonstrated to downregulate host transcription of *e-cadherin* through inhibition of FGF2, mTOR, and Slug (199). IAV infection was also shown to damage respiratory epithelial cell barrier integrity by downregulating the expression of tight junction protein claudin-4 (200). Further research also attributed the loss of tight junction integrity during IAV infection to critical tight junction-associated PDZ proteins (197). Loss of epithelial barrier integrity is a critical component in the migration of bacteria to the bloodstream where they can cause sepsis (188). Influenza-mediated disruption of such tight junctions has been demonstrated to contribute significantly to the onset of ARDS from IAV infection (200).

Adherens junctions are also common targets of microorganisms infecting the lung. Adherens junctions are comprised of E-cadherin and catenin proteins, and serve as critical junctions anchoring epithelial actin cytoskeletons together into a network which generates tensile strength and barrier function, while maintaining the tissue pliability required for the biomechanics of respiration (201). Rhinovirus infection, which is characterized by vascular permeability and associated with bacterial secondary infection (202), has been shown to modify respiratory epithelial cells during infection to lower the transcriptional output of *zo-1*, *occludin*, *claudin*, and *e-cadherin* by over 50% individually (202). Another study validated such findings and demonstrated a significant loss in transepithelial resistance during rhinovirus infection that was not mediated by cell death or apoptosis, but an increase in severity of coinfection (203).

Compounds causing an upregulation in gene expression or assembly of junction proteins on respiratory epithelial cells could be promising tolerance-augmenting therapeutics for use during diverse viral primary infections. Many bacterial and fungal pathogens of the lung, including *S. pneumoniae*, *S. aureus*, *Candida albicans*, and *P. aeruginosa*, employ adhesion junction components, specifically E-cadherin, as an adherence or entry receptor for invasion and colonization (199). The severity of phenotypes observed due to the alpha-toxin protein of *S. aureus* has also been shown to be modulated by the abundance of functional adherens junctions (204). All of these mechanisms dramatically decrease the host's ability to tolerate low level infections by escalating the degree of damage caused by these pathogens with a poorly maintained epithelial barrier.

The maintenance of epithelial barrier function is also reliant on the maintenance of epithelial cell viability. Respiratory epithelial cell death can substantially decrease host tolerance by forming gaps in the mucosal barrier, which enables respiratory pathogens to directly infect the basal layer of epithelial cells (178). Once penetrating to the basal layer of respiratory cells, pathogenic bacteria such as *S. pneumoniae* and *P. aeruginosa* can translocate through the basal membrane to initiate bacteremia that can lead to sepsis (205, 206). However, appropriate modulation of respiratory epithelial cell death can also promote pulmonary tolerance. Epithelial cell induction of apoptosis is canonically regarded as a means through which the host can restrict pathogen replication in infected cells, without loss of membrane integrity and the secretion of DAMPs leading to hyper-inflammatory responses by the immune system (207). Many respiratory pathogens compromise pulmonary tolerance through the inhibition of apoptotic cell death and the upregulation of more inflammatory forms of cell death, such as necrosis, oncosis, or pyroptosis (208–211). The hyper-inflammatory response to such forms of cell death is affected by immune cells sensing DAMPs. A balance between the maintenance of cell viability/barrier function and the need to restrict intracellular pathogens' ability to replicate is required to maximize host pulmonary tolerance.

PULMONARY ENDOTHELIAL CELL TOLERANCE MECHANISMS

Endothelial Barrier Function

The pulmonary endothelium is an important interface between the circulation and the lung tissue and airways. In the homeostatic state, the thin pulmonary endothelium forms a barrier between proteinaceous fluids and leukocytes in the circulation and the lung epithelial layer, which is separated by less than 1 μm in the alveoli. In response to inflammatory stimuli from the lung, as in the event of an infection, this homeostatic state is disrupted when circulating leukocytes are induced to marginate along the vascular endothelium through interactions mediated by adhesion molecules, including selectins and integrins. From there they extravasate into the interstitial space in a process that depends on the loosening of endothelial cell junctions; this modulation of the endothelial barrier function can subsequently tune the magnitude of leukocyte infiltration and, therefore, inflammation in the lung. At the same time, this disruption in the endothelial barrier allows for the movement of protein-rich fluids from the circulation into the lungs, causing edema and, in severe cases, ARDS or ALI (212–215). After infection, a certain degree of vascular permeability is required to facilitate the influx of leukocytes into the lung to allow the inflammatory response to control pathogen elimination; however, if the inflammatory response is too robust, the lung tissue can become severely damaged, as discussed in previous sections. Barrier function is the primary contribution of the endothelial layer to maintaining host tolerance and tissue integrity during pulmonary microbial infection.

Much of what is known about pulmonary endothelial barrier function, and the mechanisms that drive the loss of this function, come from studies of single microbial lung infection. The importance of a functional endothelial barrier was demonstrated in a model of *E. coli* pneumonia, in which blockade of the interaction between integrin αvβ3 and its binding partner IQGAP1 at the endothelial cell–cell junction led to excesses in lung extravascular plasma and water, as well as increased lung weight within just 5 h of infection (216). It has also been shown that influenza infection can lead to vascular leak (217, 218). This loss of barrier integrity stems, at least in part, from active infection of endothelial cells by influenza virus. This has been demonstrated in multiple species, including human, in which the pulmonary microvascular endothelium is permissive to infection with multiple clinical and laboratory strains of influenza (217). Similarly, using a human H1N1 influenza model in ferrets, virus was detected in multiple lung compartments including the vasculature (219). Virus-mediated apoptotic cell death is one way in which infection contributes to loss of endothelial barrier function. Influenza-induced endothelial apoptosis could be ameliorated by inhibition of caspases, thereby restoring barrier function (217). Endothelial apoptosis may be due in part to the induction of TNFR1 receptor expression on the endothelial cell surface by IAV (220). This apoptotic signal was enhanced by the interaction of *S. aureus* protein A and TNFR1 in the event of secondary bacterial infection, leading to eventual development of ARDS (220). This finding illuminates the potential for slight alterations in endothelial cell signaling that are induced during a single infection, such as the induction of caspases or TNFR1, to dramatically reduce the host's ability to maintain homeostasis in the event of a secondary infection.

Apoptosis-independent effects of infection on loss of endothelial barrier function have also been elucidated. Studies simulating viral infection by stimulating human microvascular endothelial cells with poly(I:C) shed light onto the mechanistic link between viral infection and loss of barrier function by showing that signaling through TLR3 and NF-κB induced a loss of claudin-5 expression, a key protein in the formation of endothelial tight junctions (154). A similar effect was demonstrated by infecting human microvascular endothelium with a replication-deficient influenza virus (217). In this study, UV-inactivated virus was still able to induce loss of endothelial barrier function without causing cellular apoptosis. This was driven by the degradation of claudin-5. Interestingly, treatment with the cAMP analog formoterol could restore claudin-5 protein levels and improve endothelial cell barrier function *in vitro* (217). Formoterol's barrier-enhancing effect when administered after influenza infection was corroborated *in vivo* (217), raising the interesting possibility that barrier-enhancing drugs may present a viable therapeutic option to boost tolerance to the tissue-damaging effects of lung infection if they can be shown not to alter host resistance to the pathogen.

Drug repositioning to treat infection-induced ARDS due to loss of endothelial barrier function is an enticing clinical option. This has been probed experimentally using the cancer drug imatinib. This tyrosine kinase inhibitor was originally developed to target the BCR-Abl fusion protein causing the development of chronic myeloid leukemia cells. Imatinib also targets other diverse kinases, such as the platelet-derived growth factor receptor, which suggests that the drug also functions in modulating barrier function. A report by Rizzo *et al*. tested imatinib's function in a model of ALI induced by the combination of LPS and ventilator-induced

lung injury. This work found that administration of imatinib reduced multiple measures of vascular permeability including cellular infiltration and total protein and pro-inflammatory cytokine concentrations in the bronchoalveolar lavage fluid. Imatinib was found to act in this model by reducing NF-κB activity, and reduced the symptoms of ALI, even when administered prophylactically (221). A similar effect of imatinib on edema and neutrophil influx was observed in a rat model of ischemia/reperfusion injury (222). Whether this type of treatment has the potential to be beneficial to the maintenance of host tolerance without compromising resistance to infection will need to be explored.

Virus-infected lungs are also prone to thrombus formation along the endothelium, which has downstream effects on vascular permeability. Autopsy examination of lung histology of IAV-infected patients has shown evidence of microthrombi formation along the endothelium (217). Similarly, thrombi were observed in specimens from the 1918 pandemic influenza outbreak, although they were absent in autopsy examinations from the 2009 pandemic (223), suggesting that there are strain-specific effects on this process. It is possible that this is one mechanism of the pathology caused by highly pathogenic avian influenza strains (224, 225). Experimental evidence has shown that platelets adhere to endothelial cells through interactions between platelet integrin α5β1 and endothelial fibronectin during influenza infection (217). This interaction negatively impacted host tolerance during infection, as platelet inhibition was shown to improve survival (217). Thrombus formation has also been observed in coinfection, with activation of clotting factors, coagulation and tissue factor, as well as neutrophil elastase deposition on endothelial cells; together these events could enhance vascular permeability leading to more severe inflammation in coinfected lungs (223).

Contribution of Endothelial Cells to Cytokine Storm

As has been observed with innate immune cells and the lung epithelium, the endothelium itself can also contribute to loss of host tolerance to infection through excessive induction of cytokines leading to cytokine storm. Influenza infection has been shown in several models to induce upregulation of PRRs and inflammatory cytokine and chemokine production, thereby elevating the risk of inflammation-induced tissue damage. Primary human lung endothelial cells were shown to upregulate transcripts for TLR2 and NOD2 (220). Similarly, infection of ferrets with human H1N1 induced TLR3 expression on endothelial cells (219). Activation of TLR3 on primary human lung microvascular endothelial cells with the synthetic ligand poly(I:C) induced the expression of pro-inflammatory cytokines, including IL-6, IL-8, TNF-α, and IFN-β, leading to the possibility that excessive stimulation could lead to cytokine storm (226). In addition to modulating PRR expression and signaling, influenza infection has been shown to drive cytokine storm through enhanced S1P1 signaling in endothelial cells (226). Cytokine storm following pulmonary infection is detrimental to the host, as limiting its magnitude has been shown to improve survival. For example, limiting H5N1 influenza infection in endothelial cells using microRNAs both reduced cytokine storm and improved survival (218). Similarly, treatment with an S1P1 antagonist during influenza infection reduced mortality in an endothelial cell-specific manner (226). It is reasonable to speculate that induction of inflammatory responses in endothelial cells by a primary viral infection would prime a quicker and potentially more vigorous response to secondary bacterial infection, leading to prolonged cytokine storm with leukocyte infiltration, and ultimately tissue damage and organ failure, and that intervening with these processes could promote tissue protection.

LUNG MICROBIOME IN HOST TOLERANCE

The lung is host to numerous microbiota, and their roles in human health and disease are beginning to be documented (227). Many studies have examined the link between the gut microbiota and pulmonary health; however, that topic is beyond the scope of this review (228, 229). How the commensal microbiota of the lung contribute to host tolerance to infection is not well understood; however, studies are beginning to probe the changes that occur in the lung microbial milieu during active infection, which may shed some light on their roles in tissue homeostasis (28, 230–232). As the previous sections of this review have discussed, the line between what is a commensal bacteria and what is a pathogen in the lung is rather amorphous. This is especially true after preceding viral infections where previously harmless bacteria become pathogenic (233, 234). These opportunistic infections, or pathobionts, comprise the vast majority of secondary bacterial infections following respiratory viral infections.

Regarding the lung microbiome, in a correlative study examining the serial colonization of the nasopharynx of infants in their first year of life, it was found that the onset of viral acute respiratory infections was associated with the transient appearance of *Streptococcus*, *Moraxella*, or *Haemophilus* species. This study also found that the composition of the microbiome was a determinant of whether disease would spread to the lower airways and cause elevated inflammation or asthma (235). The composition of the airway microbiome is not restricted to only bacterial species. In fact, examination of patients admitted to the ICU revealed an overabundance of *Candida* species that was not dependent on the type of pneumonia or whether the patient had been treated with antibiotics (236). Changes in the composition of lung microbiota have also been monitored in patients with viral and bacterial coinfection. In a study that examined bronchoalveolar lavage fluid samples serially drawn from a H7N9 influenza-infected patient, it was found that the microbiota became dominated by *Acinetobacter baumannii*, which eventually became multi-drug resistant and led to secondary bacterial infection (237). This transition was accompanied by increased inflammation, raising the possibility that there could be an associated increase in lung immunopathology (237). Similarly, comparison of the oropharyngeal microbiome revealed distinct differences in composition between healthy patients and those with H7N9 influenza or H7N9 influenza infection with secondary bacterial infection. In particular, the healthy patients had an enrichment of *Haemophilus* and *Bacteroides*

species (238). In contrast, influenza-infected individuals had outgrowths of *Filifactor*, *Megasphaera*, and *Leptotrichia* species, while the addition of secondary bacterial infection led to further dysbiosis, including the enrichment of *Leptotrichia*, *Oribacterium*, *Streptococcus*, *Atopobium*, *Eubacterium*, *Solobacterium*, and *Rothia* species (238).

Documenting the changes in airway microbiota that occur in response to pulmonary infections raises the possibility of using commensal microbes to improve host defense against pathogens. This has been investigated experimentally in a few instances. For example, it has been shown that intranasal administration of *Lactobacillus rhamnosus* can aid in resisting RSV infection in infant mice (239). Another study suggested that this was due to a priming effect, showing that nonviable *L. rhamnosus* or the bacterial cell wall component peptidoglycan can enhance inflammatory responses in a TLR3-dependent manner (240). In a similar manner, TLR3-dependent priming with the upper respiratory tract-resident species *Corynebacterium pseudodiphtheriticum* also improved the outcome of RSV and secondary *S. pneumoniae* infection (241). While these studies emphasized the effect of priming on pulmonary resistance to subsequent infection, it is plausible that these microbiota also influence host tolerance mechanisms. Whether the prevention of commensal dysbiosis during infection can promote pulmonary homeostasis in the face of infectious insult will be an important area for future study.

CONCLUSION

The role that host disease tolerance mechanisms play in the ability to survive a lung infection is an important new area of research. This review focused on the interacting roles that the innate immune response, the lung epithelium, and the lung endothelium play when responding to acute lung infections (as summarized in **Figure 1**). It also demonstrated the complexities that arise in host tolerance to polymicrobial infections, and posed several questions regarding the role of the lung microbiota in tissue protection during infection. An increased understanding of host tolerance to acute lung infections will allow us to not only improve treatments for these deadly diseases but may also open up new treatment options for chronic lung diseases and infections.

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Beyond Bacteria: Bacteriophage-Eukaryotic Host Interactions Reveal Emerging Paradigms of Health and Disease

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For decades, a wealth of information has been acquired to define how host associated microbial communities contribute to health and disease. Within the human microbiota this has largely focused on bacteria, yet there is a myriad of viruses that occupy various tissue sites, the most abundant being bacteriophages that infect bacteria. Animal hosts are colonized with niche specific microbial communities where bacteria are continuously co-evolving with phages. Bacterial growth, metabolic activity, pathogenicity, antibiotic resistance, interspecies competition and evolution can all be influenced by phage infection and the beneficial nature of such interactions suggests that to an extent phages are tolerated by their hosts. With the understanding that phage-specific host–microbe interactions likely contribute to bacterial interactions with their mammalian hosts, phages and their communities may also impact aspects of mammalian health and disease that have gone unrecognized. Here, we review recent progress in understanding how bacteria acquire and tolerate phage in both pure culture and within complex communities. We apply these findings to discuss how intra-body phages interact with bacteria to influence their eukaryotic hosts through potential contributions to microbial homeostasis, mucosal immunity, immune tolerance and autoimmunity.

Keywords: bacteriophage, virome, host–microbe interactions, phage–bacteria interactions, microbiota, microbiome, phage immunity

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INTRODUCTION

The human body hosts a complex and dynamic consortia of microbes consisting of bacteria, archaea, fungi, viruses and protozoa (Zou et al., 2016; Blum, 2017; Chabe et al., 2017; Huseyin et al., 2017; Koskinen et al., 2017; Raymann et al., 2017). Among the core members of the human microbiota, bacteria have garnered significant attention because of their contributions to human physiology and disease (Hooper and Gordon, 2001; Belkaid and Hand, 2014; Byrd et al., 2018; Zhao and Elson, 2018). The emergence of culture-independent approaches and techniques for viral enrichments from complex microbial samples has identified a vast consortium of understudied viruses within host associated microbiotas (Virgin, 2014).

The onset of the "omics" revolution led by 16s rDNA sequencing rapidly advanced our ability to survey the bacterial component of the microbiota in unprecedented detail. Extending from these studies, the implementation of metagenomic DNA sequencing revealed a robust viral component to the microbiota and identified bacteriophages (phages) as dominant members. In humans,

phages populate most surfaces including skin (Foulongne et al., 2012; Oh et al., 2016), the oral cavity (Willner et al., 2011; Pride et al., 2012; Abeles et al., 2014), lungs (Willner et al., 2009; Dickson and Huffnagle, 2015), the intestine (Reyes et al., 2010; Minot et al., 2011; Manrique et al., 2016) and the urinary tract (Santiago-Rodriguez et al., 2015; Miller-Ensminger et al., 2018). Phage–bacteria interactions have been studied in varying detail *in vitro* (Chevallereau et al., 2016; Leskinen et al., 2016; Mojardin and Salas, 2016), however, little work to date has revealed insights into how phages interact with their bacterial hosts in human and animal systems. Body sites are endowed with unique characteristics including microenvironments that can define unique physiologies, thus it is conceivable that in some instances phage–bacteria interactions *in vivo* may be distinct from what has been studied in the laboratory.

Within the human body, phages infect bacterial hosts and undergo lytic replication and phage particle biogenesis to synthesize new infectious phages or integrate into the host bacterial genome as quiescent lysogenic prophages that are propagated vertically during cell division (Weinbauer, 2004; Hobbs and Abedon, 2016). Environmental cues such as nutrients, antibiotics and reactive oxygen species are well documented *in vitro* inducers of prophage excision from bacterial genomes, yet we know very little about the *in vivo* cues that promote prophage excision or those that influence the maintenance of lysogeny (DeMarini and Lawrence, 1992; Duerkop et al., 2012; Matos et al., 2013).

Considering the plethora of lytic and lysogenic phages that associate with humans, these phages are poised to have a significant impact on human physiology during both health and disease. In fact, research using animal models indicate that the intestinal microbiota promotes phage genome evolution allowing phages to infect naïve bacterial species and consequently fostering intra-body persistence (De Sordi et al., 2017). Hence, the cross-talk between resident bacteria and phages potentially contributes to the maintenance of microbial homeostasis within the human body. In this review, we will discuss phage–bacterial interactions within the context of host-associated microbial communities and will explore the underlying reasons for the evolution of phage tolerance in both bacteria and animals.

PHAGE–BACTERIAL COLLABORATION: BENEFITS OF BEFRIENDING THE ENEMY

Predatory lytic phages play crucial roles in maintaining diversity within microbial ecosystems (Maslov and Sneppen, 2017). Lytic phages adsorb to susceptible bacteria and subsequently infect and kill these bacteria. According to the classic "kill-the-winner" model, abundant bacterial species in a population have a greater possibility of encountering virulent phages and consequently face death, thus preventing niche monopoly by a single bacterial species (Thingstad, 2000; Rodriguez-Brito et al., 2010). Coculture studies using two competitive *Pseudomonas* strains demonstrated that phages enable the less competitive bacterial species to persist by infecting the more dominant species at a higher frequency, thus influencing community composition (Brockhurst et al., 2006). The contribution of lytic phages to bacterial diversity and richness in host associated environments is unknown and it is unclear whether bonafide "kill-the-winner" dynamics apply (Reyes et al., 2010; Allen et al., 2011; Abedon, 2012).

Lysogenic phages integrated into host bacterial chromosomes can constitute up to 20% of bacterial genomes (Casjens, 2003), raising the question of why some bacteria tolerate such high burdens of phage DNA? Phage tolerance is likely supported by the potential positive outcomes bestowed upon the bacterium while harboring the viral DNA (**Figure 1**). Specifically, bacteria have co-evolved with their phages to benefit from the inclusion of viral genes within their genomes, which can aid in bacterial fitness, pathogenesis and adaptation to changing environments. Within this context, we will briefly discuss how bacteria benefit from their associated phages and we direct the readers to more recent comprehensive reviews on this subject (Roossinck, 2011; Obeng et al., 2016; Harrison and Brockhurst, 2017; Touchon et al., 2017).

Phages are vehicles for the horizontal transfer of genes which upon acquisition can influence individual and bacterial community phenotypes (reviewed in Touchon et al., 2017). For instance, prophages have been shown to confer pathogenic and antibiotic resistance traits for their bacterial hosts (Matos et al., 2013; Obeng et al., 2016; Lekunberri et al., 2017). Examples include a myriad of toxins which are encoded within prophage elements, the most well studied being the Shiga toxin-encoding prophages of *Escherichia coli* (STEC) which cause fatal gastrointestinal infections in humans (Shaikh and Tarr, 2003). Interestingly, a recent report showed that in addition to toxin production, the carriage of Shiga toxin-encoding prophages enhances antimicrobial tolerance of STEC by modifying the bacterium's metabolism (Holt et al., 2017). Additionally, toxin encoding phages from numerous pathogenic bacteria have been demonstrated to transduce and lysogenize non-pathogenic bacteria, converting them to virulent strains (Faruque et al., 1999; Schmidt et al., 1999; Broudy and Fischetti, 2003).

The induction of lysogenic prophages from bacterial chromosomes has been linked to pathogen fitness and virulence. Production of EfCIV583, a satellite prophage who's DNA is packaged into the capsid of the helper phage vB_EfaS_V583-P1 in *Enterococcus faecalis* strain V583, allows the host bacterium to compete with non-lysogenic peers (Duerkop et al., 2012; Matos et al., 2013). Another example from mixed culture experiments shows that prophage excision and subsequent phage mediated lysis of a subpopulation of host bacteria results in the timed release of bacteriocins that kill bacterial competitors and clear the niche for the phage-harboring bacteria (Nedialkova et al., 2016). Spontaneous prophage induction can also prime a shift in bacterial lifestyle from independently growing bacterial cells to organized cellular aggregates termed biofilms (reviewed in Nanda et al., 2015). The lungs of cystic fibrosis (CF) patients are colonized by sessile communities of *Pseudomonas aeruginosa* whose structural organization resemble

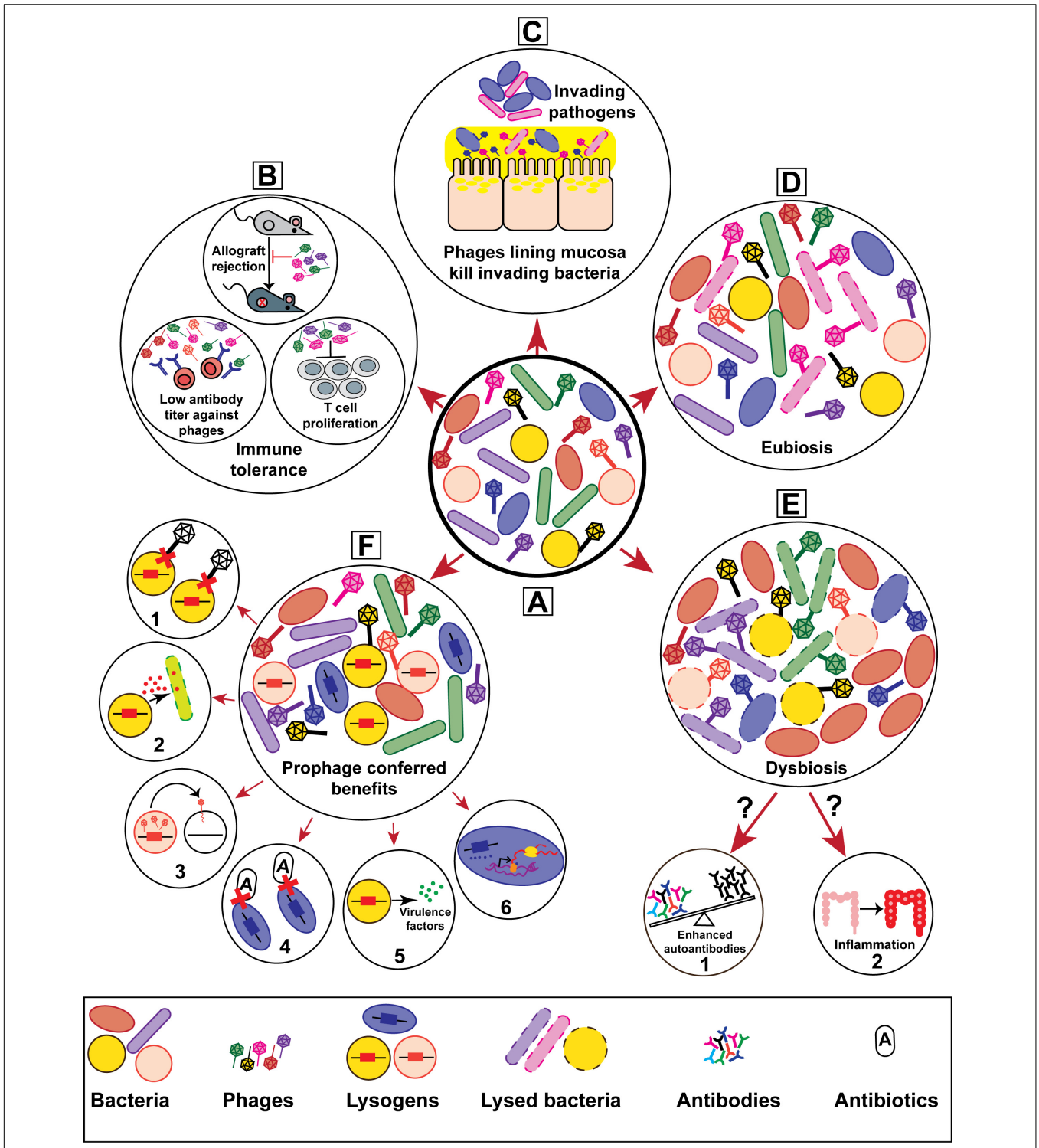

FIGURE 1 | Bacteriophages contribute to the genetic and physiological traits of their hosts thereby influencing host–microbe interactions. **(A)** Schematic representation of a healthy mammalian microbiota consisting of heterogeneous phage and bacterial populations. The prolonged and ubiquitous presence of phages in mammalian microbiotas are hypothesized to have considerable effects on health and disease. Phage-driven impacts on mammalian hosts include **(B)** immune tolerance, **(C)** mucosal immunity, and **(D)** homeostatic eubiosis. Altered phage diversity and richness have been suggested to drive **(E)** bacterial dysbiosis, potentially leading to **(E-1)** autoimmune progression in type I diabetes and **(E-2)** inflammation during inflammatory bowel disease. On the other hand, phage infection endows bacteria with multiple features that alter bacterial interactions with their mammalian hosts. **(F)** Lysogenic conversion via the acquisition of prophages can increase bacterial host fitness. Prophage provided traits include **(F-1)** superinfection immunity, **(F-2)** elimination of bacterial competitors, **(F-3)** horizontal gene transfer, **(F-4)** enhanced antibiotic resistance, **(F-5)** virulence, and **(F-6)** altered gene expression.

biofilms (Bjarnsholt et al., 2013). Within these *P. aeruginosa* biofilms prophage induction occurs resulting in the release of the filamentous phage Pf4 (Secor et al., 2017). Pf4 phages promote biofilm assembly, facilitate persistence of the host bacteria in the lung and modulate inflammatory responses to promote chronic infections (Rice et al., 2009; Secor et al., 2015, 2017). Hence, prophage induction and subsequent phage-driven bacterial cell lysis provides a fitness benefit to the host bacterial community.

The integration of lysogenic phages into bacterial chromosomes can disrupt genes, thus altering phenotypes and in some cases altering bacterial fitness (Coleman et al., 1991; Bernhardt et al., 2000; Brussow et al., 2004). In a phenomenon termed reversible active lysogeny, prophage excision from the bacterial genome re-activates a host gene without activating the phages lytic cycle which promotes host adaptation (Feiner et al., 2015). For example, a *Listeria monocytogenes* prophage integrated within the master regulator of competence gene *comK*, during intracellular growth excises from the *L. monocytogenes* genome to restore the *comK* reading frame. The bacterium represses phage lysis and produces a functional ComK protein to promote immune evasion (Rabinovich et al., 2012). In the case of reversible active lysogeny, prophages provide gain-of-function phenotypes at the cost of the bacterium which must maintain the prophage element both within its dormant integrated form and repress its lytic functions after excision. Such costs are likely mitigated considering maintenance of these phages benefit the bacterium. The mechanisms driving these types of co-evolution are unclear, however, the acquisition and selection of phages within bacterial genomes shows that tolerance of phage lysogeny can promote context dependent attributes that promote bacterial adaptation to diverse environments.

Bacteria have evolved multiple defense strategies to restrict virulent phage predation and prophage acquisition (Labrie et al., 2010). Examples include restriction-modification (RM) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated protein) systems that target invading phage DNA for destruction (Tock and Dryden, 2005; Barrangou et al., 2007; Sorek et al., 2008; Abedon, 2012). The absence of functional CRISPR-Cas has been linked to the emergence of multidrug resistant bacteria (Palmer and Gilmore, 2010; van Belkum et al., 2015), suggesting that the inability of bacteria to protect their genomes from foreign mobile elements including phages promotes bacterial adaptation to defined environmental conditions. Conversely, a recent study demonstrated that a conditional CRISPR/Cas system of *Staphylococcus epidermidis* evolved to favor prophage acquisition promoting a type of selective phage tolerance by degrading lytic phage DNA but allowing phage lysogeny (Goldberg et al., 2014) and RM systems have been shown to advocate prophage acquisition by postponing the onset of viral replication until bacterial population density has reached a point where the probability of lysogenic conversion is high (Pleska et al., 2018). These studies suggest that even in the presence of functional CRISPR-Cas and RM systems there may be preferences for specific DNAs that benefit bacteria in a context dependent manner.

BACTERIOPHAGE-EUKARYOTE INTERACTIONS: THE TIP OF THE ICEBERG

Although metagenomic studies have revealed phages as one of the most abundant components of the human microbiota (Reyes et al., 2010; Minot et al., 2011; Foulongne et al., 2012; Pride et al., 2012; Oh et al., 2014; Dickson and Huffnagle, 2015; Santiago-Rodriguez et al., 2015; Oh et al., 2016), information on the interactions of phages with animal cells and how phages contribute to health and disease is limited. In this section, we will discuss recent literature related to how phages interact with eukaryotic hosts and how these interactions influence host immunity.

Over a decade ago, it was appreciated that intestinal phages breach the physical barrier of the mammalian intestine (Górski et al., 2006b). Compromised intestinal epithelial integrity during inflammation provides phages with access to the bloodstream and consequently their spread to different tissues (Handley et al., 2012). However, in the absence of intestinal barrier distress phages still migrate to host restricted sites such as peripheral blood and organs. It has been proposed that phages are naturally internalized into eukaryotic cells (Duerkop and Hooper, 2013; Tian et al., 2015; Zhang et al., 2017), however, the mechanisms behind phage uptake by eukaryotic cells are just beginning to be explored. A long held theory behind phage internalization by eukaryotic cells is that these events were preceded by the entry of phage-infected bacterial cells that transport phages (Hsia et al., 2000; Johnston, 2002; Duerkop and Hooper, 2013). Recently phages have been shown to gain access to epithelial cells directly through active transcytosis in the absence of their bacterial host (Nguyen et al., 2017). Phages were shown to permeate the apical surface of epithelial cells via endocytosis, become compartmentalized and finally exocytosed through the basal side of the cell (Nguyen et al., 2017). In a separate study, *Escherichia coli* phage PK1A2 was shown to recognize and bind neuroblastoma cells displaying polysialic acids on their cell surface (Lehti et al., 2017). Following adhesion, phage PK1A2 is internalized by the endolysosomal pathway and are eventually degraded in the lysosome (Lehti et al., 2017). It is hypothesized that during these internalization events, phages may escape lysosomal destruction and potentially create opportunities for trans-kingdom genetic exchange or stimulate cellular immunity (Duerkop and Hooper, 2013; Lehti et al., 2017). In another example of potential *trans*-kingdom interactions, it has been proposed that phages act as an additional layer of non-host derived immunity against incoming pathogens at mucosal surfaces by binding to mucin glycoproteins [(Barr et al., 2013a) and discussed later]. Carbohydrate modifications, including sialic acids and various glycosylations are abundant in host derived mucins (Royle et al., 2008). Determining if phage adhesion to the sugar epitopes of host mucins is a common strategy by which phages interact with eukaryotic mucosal surfaces should

reveal possible mechanisms behind the *in vivo* translocation and dispersal of phages within the human body.

As phages are significant reservoirs of genetic diversity and considering phages are capable of entering eukaryotic cells, this raises questions about the possibility of bidirectional trans-kingdom gene exchange between phages and their animal hosts. Multicellular eukaryotes have been reported to harbor phage capsid gene orthologs in their genomes that resemble phages of the obligate intracellular pathogen *Chlamydophila pneumoniae* (Rosenwald et al., 2014). Phages have also been implicated in the dissemination of bacterial aerolysin and lysozyme genes within eukaryotic hosts (Moran et al., 2012; Metcalf et al., 2014). Conversely, metazoan-like gene modules whose functions have yet to be defined have been found in phages of the insect parasite *Wolbachia* (Bordenstein and Bordenstein, 2016). Together, these observations suggest the potential for the genetic interplay between phages and eukaryotes that contribute to trans-kingdom evolution (**Figure 1**).

Commensal bacteria regulate various facets of host immunity, yet there are significant gaps in our understanding of the mechanisms driving microbiota mediated immune regulation (Mazmanian et al., 2005; Ivanov et al., 2008; Arpaia et al., 2013; Fung et al., 2017; Novince et al., 2017; Schnupf et al., 2017). Considering phages influence the assembly of microbial communities and modulate bacterial diversity in various ecosystems (Barr et al., 2013b; Koskella and Meaden, 2013), perhaps phage–bacteria interactions can direct the host immune response. Phages could potentially modulate immune interactions between a eukaryotic host and its microbiota by providing novel traits within subpopulations of bacteria or by causing shifts in the resident bacterial community composition through targeted killing of defined community members (**Figure 1**). Given the prevalence of phages at multiple tissue sites within the human body, it is likely that phages play an unrecognized role in promoting the development and activity of the immune system through interactions with their host bacteria. It is possible that lytic phages could directly stimulate antiviral innate immunity by engaging nucleic acid sensors or inadvertently by killing their bacterial hosts and releasing soluble bacterial antigens that stimulate pattern recognition receptors. If either of these scenarios were true, this would have profound implications for the development of lytic phages as antibacterial therapeutics.

Changes in phage community composition occur during human disease. For example, the diversity and composition of intestinal phages is significantly different between healthy individuals and patients with inflammatory bowel diseases (IBD) (Wagner et al., 2013; Norman et al., 2015). Individuals with IBD have reduced enteric bacterial diversity relative to the healthy individuals. However, alterations in bacterial richness does not always correlate with the dramatic phage expansion associated with IBD (Norman et al., 2015), suggesting signals from the immune system may directly influence phage abundances. Additionally, alterations in enteric phage populations was observed prior to the development of autoantibodies in children who were predisposed to develop Type I diabetes (Zhao et al., 2017). These disease-related shifts in phage community composition suggest a potential role for intestinal phages in the development of bacterial dysbiosis. Although phages have been implicated in diseases associated with bacterial dysbiosis, it is unclear if phages directly contribute to inflammation and autoimmune disease by altering microbial homeostasis (**Figure 1**).

The collaboration between phages and their animal hosts to eliminate deleterious bacteria is opening new avenues for the study of host–microbe interactions during health and disease. In a recent study, researchers revealed a neutrophil-phage alliance that together cleared multi-drug resistant *P. aeruginosa* in a lung infection mouse model (Roach et al., 2017). This study suggests that host innate immunity may be more effective at clearing pathogenic bacteria with help from lytic phages. *In vitro* tissue culture studies suggest that phages protect epithelial mucosal surfaces from invading bacteria (Barr et al., 2013a, 2015). These studies propose that phages with binding affinity for host mucins form a protective antibacterial defense called BAM (bacteriophage adhesion to mucus) which serves as a non-host derived innate immunity at mucosal surfaces. According to the BAM model, adhesion of phages to mucin glycoproteins and subsequent subdiffusive movement through the mucus layer concentrates phages at mucosal surfaces. An enrichment of phages in the mucosa may provide protection against bacterial invaders and limit pathogen colonization (Barr et al., 2013a, 2015). Although, these findings suggest that phages contribute to host defenses (**Figure 1**), their function in promoting mucosal health remain to be explored.

An increasing body of data suggests that phages engage in interactions with mammalian immune cells and modulate different aspects of host immune responses. Phages are weakly immunogenic and the adaptive immune system produces low titers of phage-neutralizing antibodies without mounting an inflammatory response (Dabrowska et al., 2006; Górski et al., 2012; An et al., 2014; Majewska et al., 2015). Knowing that phages are ubiquitous within host associated microbiotas and possibly within the host systemic environment, it is possible that immune tolerance to phages occurs due to the continued exposure of the immune system to phages. Several studies propose a role for phages in promoting immune tolerance by downregulating T cell proliferation, through the reduction of antibody production and in the prevention of allogenic transplant rejection in animal models (Górski et al., 2006a,b, 2007; McVay et al., 2007). For a comprehensive discussion on the effect of phages on immune-modulation readers are directed to a recent review by Górski et al. (2017).

CONCLUDING REMARKS

Phages endow their host bacteria with competitive traits, facilitate adaptation for the colonization of new niches and promote bacterial evolution. It is becoming increasingly clear that the impact of phages extend beyond their bacterial hosts and their potential influences on human health are just beginning to be explored (De Paepe et al., 2014; Norman et al., 2015; Manrique et al., 2016; Wahida et al., 2016). Recent studies

bring to light concepts for how bacteria and animals have co-evolved to tolerate phages through beneficial interactions that may dictate the outcomes of host–microbe associations. Through these interactions phages and their communities hold substantial promise as modulators of human health and disease.

Moving forward, studies that employ modern "omics" technologies to study the microbiota such as metagenomics, transcriptomics and proteomics should by default incorporate analyses of phage communities. Alongside such studies, researchers must make efforts through the use of mouse models and *in vivo* defined microbial communities to move beyond descriptive studies and begin providing mechanistic details into how phages interact with host-associated bacteria and how these interactions influence immunity and physiology. Furthermore, specific attention should be given to the mammalian host responses that drive the assembly of phage community composition within the microbiota and how these signals influence phage interactions with their bacterial hosts. Only after these basic questions are explored can we begin to understand how to harness phages for the manipulation of bacterial communities that promote human health.

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Alveolar Macrophages in the Resolution of Inflammation, Tissue Repair, and Tolerance to Infection

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Pathogen persistence in the respiratory tract is an important preoccupation, and of particular relevance to infectious diseases such as tuberculosis. The equilibrium between elimination of pathogens and the magnitude of the host response is a sword of Damocles for susceptible patients. The alveolar macrophage is the first sentinel of the respiratory tree and constitutes the dominant immune cell in the steady state. This immune cell is a key player in the balance between defense against pathogens and tolerance toward innocuous stimuli. This review focuses on the role of alveolar macrophages in limiting lung tissue damage from potentially innocuous stimuli and from infections, processes that are relevant to appropriate tolerance of potential causes of lung disease. Notably, the different anti-inflammatory strategies employed by alveolar macrophages and lung tissue damage control are explored. These two properties, in addition to macrophage manipulation by pathogens, are discussed to explain how alveolar macrophages may drive pathogen persistence in the airways.

Keywords: disease tolerance, macrophages, tissue damage control, pathogen persistence, lung

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INTRODUCTION

The lung serves the vital function of gas exchange, bringing oxygen to every single cell of the body, and disposing carbon dioxide. We inspire almost 11,000 l of air daily containing countless particles that include antigens, toxins, and microbes. It is remarkable that the lungs maintain a healthy and functional state, permitting in most instances considerable longevity. Ignoring harmless inhaled proteins, adapting to toxicants and limiting immune responses to bacteria and their cellular components are essential forms of adaptation that reduce tissue damage and that may be considered to be aspects of lung tolerance. However, while clearance of nocive inhaled substances is an optimal strategy, in some instances the host defense strategy decreases the host susceptibility to tissue damage but may permit pathogen survival. In other terms, disease tolerance is the result of the magnitude of the host reaction to the organism, which limits tissue damage but in doing so may fails to eliminate the pathogen. Here, we describe two important components leading to limiting of lung disease by alveolar macrophages (Aφs) by (i) repair of tissue damage and (ii) modulation of inflammation.

The Aφ is the first sentinel of the respiratory tree and constitutes the dominant immune cell in the steady state. These innate immune cells, derived from the yolk sac, are present as early as the first week after birth and are regulated in part by granulocyte-macrophage colony-stimulating factor (GM-CSF) (1, 2). Their niche in the alveolar space makes them important guardians of pulmonary homeostasis. Aφs regulate the response to infections and to epithelial damage. These functions

require the engagement of different cellular pathways, one of which is pro-inflammatory and the other trophic, requiring a range of macrophage properties that often lead to a dichotomous classification of the Aφ phenotype.

The term macrophage (from Greek: μακρύς, makros = large and φαγειν, phagein = eater) was introduced by Elie Metchnikoff in 1883, following the description of the fundamental property of phagocytosis (3). A century later, macrophages had been observed in every single organ of the body and were recognized among the first actors of innate immunity. The ultra-structure of Aφs of mouse lung was described by Karrer (4) and the phagocytosis of India ink particles in the alveolar space by Aφs was observed 30 min after intranasal instillation. In the steady state, Karrer also observed a large amount of ferritin within Aφs suggesting that they ingested red blood cells. Sixty years later, the link between erythrocytes and macrophage biology has been established through the role of the heme signaling pathway in the development, differentiation, and function of macrophages (5). The most common function of Aφs phagocytosis is the removal of apoptotic cells to ensure tissue homeostasis. Extensive work by Fadok et al. described different receptors involved in this process (6). Aφs use different receptors such as immunoglobulin receptors and complement receptors to recognize opsonized microorganisms, facilitating their phagocytosis (7). The recognition of damage and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively) by pattern recognition receptors, such as toll-like receptors or C-type lectin receptors, allows them to recognize the presence of pathogens or products of injury, and respond directly to provide optimal host protection (8). For instance, it has been recently shown that CD206 (mannose receptor) is involved in the recognition of *Mycobacterium tuberculosis* and the subsequent signaling (9). Aφs are also responsible for cleaning the epithelial environment by removing "waste materials" such as oxidized lipids using scavenger receptors. Notably, expression of MARCO and class A scavenger receptors (SR-AI/II) on Aφs is augmented so as to decrease pulmonary inflammation after oxidant inhalation (10). Finally, protection offered by pathogen recognition is complemented by enhancing the presentation of antigens to T cells. However, it seems that human Aφs are less efficient in this process due to a reduced expression of B7 costimulatory cell surface molecules (11), perhaps a useful characteristic in the avoidance of an exuberant response to harmless antigens.

MACROPHAGE PHENOTYPES

The opposing properties of Aφs designed to kill pathogens or to promote cellular proliferation and repair of tissues have been associated with supposedly discrete phenotypes termed the M1/kill and M2/repair macrophages (12, 13). Mills based this dichotomy on arginine metabolism: M1 can metabolize arginine to nitric oxide (and citrulline), an inhibitor of proliferation through cyclic guanosine monophosphate-dependent and -independent pathways (14), while M2 produce ornithine (and urea), a promoter of proliferation. Whether macrophages display an M1 or M2 profile is dependent upon the tissue environment as the tissue context may direct macrophages to provide an appropriate response (15). This plasticity results in a large spectrum of macrophage properties. In order to organize a classification of these macrophages, a consortium has published nomenclature and experimental guidelines (16).

Some of our understanding of the physiological functions of Aφs in the lung has resulted from observing the effects of their depletion. For example, the immunosuppressive properties of Aφs in the response to inhaled sensitizing proteins are manifested by prior depletion that results in an enhanced inflammatory response and an increased recruitment of antigen-presenting cells to regional lymph nodes and lung tissues (17). More recent studies confirm Aφ's anti-inflammatory properties from the augmentation of inflammation in allergen challenged animals in which depletion has been induced prior to challenge (18, 19). Adoptive transfer of Aφs from allergen-resistant to allergen-susceptible rats prevents allergen-induced AHR and the inflammatory cytokines interleukin-13 and tumor necrosis factor-α (20). These findings indicate that quiescent Aφs have anti-inflammatory properties. Aφs harvested from allergen challenged animals are less effective in suppressing inflammation following adoptive transfer (18). The epithelial-derived alarmins IL-33 and TSLP promote the differentiation of quiescent Aφs to the M2 phenotype and augment macrophage-dependent allergic inflammation in the mouse (21, 22). Thus, Aφs show plasticity that is dependent on the microenvironment and whereas quiescent Aφs are predominantly immunosuppressive to avoid the development of unnecessary inflammatory responses to the host of inhaled foreign proteins encountered within the airway tree, when activated in the context of allergen challenge, the cells are less effective in their anti-inflammatory role.

ALVEOLAR MACROPHAGES IN TISSUE DAMAGE CONTROL

Whether tissue damage is of infectious or inflammatory origin, Aφs must reduce the inflammation in first instance to limit the extent of injury. To do so, Aφs have been described to develop different anti-inflammatory strategies (**Figure 1**). Aφs are effectors of the resolution of inflammation through phagocytosis of apoptotic cells (efferocytosis), preventing dying cells from releasing pro-inflammatory and toxic contents into the environment while triggering the release of anti-inflammatory and repair factors (23). *In vivo* and *in vitro* studies have shown that apoptotic cell clearance induces the secretion of transforming growth factor β1 (TGF-β1), prostaglandin E_2 (PGE_2), and platelet-activating factor (PAF), with associated suppression of pro-inflammatory cytokines, chemokines, and leukotriene C_4 (24–26). These findings have been confirmed in human. Indeed, defective lipopolysaccharide (LPS)-stimulated uptake of apoptotic cells by Aφs from patients with severe asthma has been associated with failure to induce the synthesis of PGE_2 and 15-hydroxyeicosatetraenoic acid (15-HETE) (27). Moreover, defective phagocytosis has been observed in several respiratory pathologies. In severe asthma in children, macrophage function is abnormal and characterized by reduced phagocytic function and excessive apoptosis (28). In addition to asthma (27, 29), defective phagocytic function has been described in chronic obstructive pulmonary disease (30),

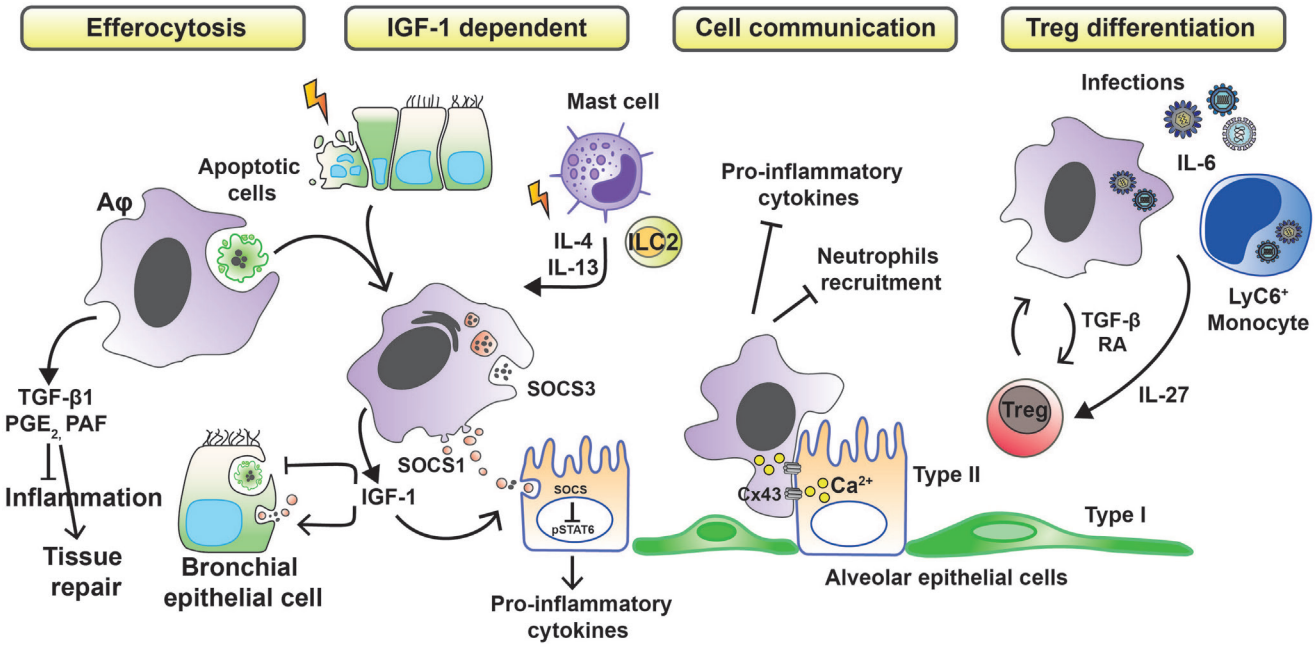

FIGURE 1 | Anti-inflammatory strategies of alveolar macrophages favoring tissue damage control. Removal of apoptotic cells by Aφs (efferocytosis) leads to the secretion of anti-inflammatory mediators, such as transforming growth factor β1 (TGF-β1), prostaglandin E_2 (PGE_2), and platelet-activating factor (PAF), which in turn suppress the synthesis of pro-inflammatory cytokines, chemokines, and leukotriene C_4. During phagocytosis of apoptotic cells or in response to inflammation-associated cytokines, Aφs also release insulin-like growth factor 1 (IGF-1). Binding of IGF-1 to its receptor on epithelial cells changes their phagocytosis pattern. Epithelial cells reduce the clearance of apoptotic cells while increasing the uptake of anti-inflammatory macrophage-derived microvesicles containing suppressor of cytokine signaling proteins (SOCS). Contact-dependent intercellular communication between Aφs and epithelial cells, using connexin 43 (Cx43)-containing gap junction channels, leads to synchronized calcium waves, using the epithelium as the conducting pathway and drives anti-inflammatory actions. Finally, Aφs promote the differentiation of regulatory T cells to further control inflammation.

cystic fibrosis (31, 32), and idiopathic pulmonary fibrosis (33) and also has been attributed a role in sustained/chronic inflammation.

Allergens, such as house dust mite, can cause apoptotic epithelial cell death (34) and trigger the synthesis of IL-4 and IL-13 from mast cells and type-2 innate lymphoid cells (ILC2s). These events lead to the production of insulin-like growth factor 1 from Aφs that enhances the uptake of anti-inflammatory macrophage-derived microvesicles by airway epithelium (35). Bourdonnay et al. report that Aφs can secrete suppressors of cytokine signaling SOCS1 and -3 in exosomes and microparticles, respectively, for uptake by alveolar epithelial cells and subsequent inhibition of STAT activation (36). Notably, airway epithelial cells can use PGE_2 as a signal to evoke SOCS3 release from Aφs to dampen their endogenous inflammatory responses in an LPS inflammation model (37). Contact-dependent communication between Aφs and alveolar epithelium has been described also to modulate immunity through gap junction-like connections and the propagation of calcium waves (38). The consequence of this intercellular communication was immunosuppressive. The binding of CD200R and TGF-βR, expressed by Aφs, with their ligands (CD200 and TGF-β, respectively) present on the cell membrane of epithelial cells is a negative regulator of Aφ activation (15).

An alternative mechanism by which Aφs limit inflammation is through promoting a regulatory T cell (Treg) response. Cancer cell-activated M2-like macrophages induce activated Treg cells from $CD4^+CD25^-$ T cells *in vitro*. Interestingly, the authors also demonstrated a positive-feedback loop in which activated Tregs skewed the differentiation of monocytes toward an M2-like phenotype (39). Lung tissue-resident macrophages (Siglec F^+ $CD11c^+$ AutoFluorescent^{hi}, likely Aφs) isolated from mouse and pulsed with ovalbumin when cocultured with antigen-specific CD4 T cells result in the generation of $Foxp3^+$ Treg cells. Treg cell induction required both TGF-β and retinoic acid. Transfer of the antigen-pulsed tissue macrophages into the airways correspondingly prevented the development of asthmatic lung inflammation upon subsequent challenge with ovalbumin. However, other allergens, such as extracts from *Dermatophagoides pteronyssinus*, *Aspergillus fumigatus*, or cat dander, did not induce Tregs because of protease and TLR-mediated signals (40). Macrophages may also induce Tregs by an indirect pathway. Interleukin-6, a soluble mediator commonly associated with inflammation and elevated in humans with severe respiratory infection, is actually critical in promoting the resolution of the host response to respiratory viral infection and in limiting disease. Early, but not late, IL-6 signaling is required for the resolution of respiratory syncytial virus-induced immunopathology (41). Production of IL-6 after infection induces the production of the regulatory cytokine interleukin-27 by Aφs and recruited $Ly6C^+$ monocytes, which in turn promotes the local maturation of Treg cells.

Since macrophages stand poised to rapidly produce large amounts of inflammatory cytokines in response to danger signals, it is logical that they are also the target of the process of resolution of inflammation. Indeed, several types of molecular controls work to downregulate the inflammatory responses of activated macrophages. These regulatory controls have been exhaustively reviewed by Mosser et al. (42). Regrettably, very few studies focused on Aφs are referenced, suggesting a gap in this field.

Once inflammation is controlled, tissue repair must take place to restore the normal tissue architecture. In the lung, the main cells damaged by infection and inflammation are epithelial cells. As long as the injury persists, pro-inflammatory signals continue, and further damage the epithelium. Thus, the repair process may be considered an integral part of the resolution of inflammation.

Important aspects of tissue repair by macrophages have been reviewed (23). Aφs with an M2 profile are the best candidates to orchestrate the repair of the epithelium since the metabolism of arginine to ornithine leads to cell proliferation and collagen production. Unexpectedly, M1 (or classically activated macrophages) may also participate in the lung repair by producing a large amount of amphiregulin in a mouse with LPS-induced acute lung injury (43). Amphiregulin, a ligand for the epidermal growth factor receptor, as well as other growth factors are necessary to ensure an optimal repair. Aφs produce these growth factors to counteract the epithelial damage induced by infection. For instance, Aφs that phagocytose apoptotic neutrophils produce hepatocyte growth factor (HGF) during bacterial pneumonia in mice (44). HGF is also produced by Aφs to enhance alveolar epithelial proliferation during influenza infection (45). Another major growth factor, also involved in tolerogenic response, is TGF-β1. Interestingly, macrophages that engage in efferocytosis may inhibit the TGF-β1 induced-epithelial–mesenchymal transition in lung alveolar epithelial cells *via* PGE_2, PGD_2, and HGF (46).

In studying the role of Aφs in lung physiology, precautions should be taken since the population of Aφs is heterogeneous. Indeed, monocyte-derived Aφs, recruited from the bone marrow during the inflammatory response, evoke different outcomes than resident Aφs. Monocyte-derived Aφs recruited in response to airway epithelial-derived monocyte chemoattractant protein 1/CCL2, are involved in airway inflammation and remodeling in allergic asthma (47). In a mouse model of lung injury (bleomycin and influenza A virus infection), monocyte-derived Aφs drive lung fibrosis and persist in the lung (48). However, monocyte-derived Aφs recruited after γ-herpesvirus (murid herpesvirus 4) infection may inhibit the development of house dust mite-induced experimental asthma (49). Thus, depending on the trigger for lung tissue damage and repair, Aφs but also monocyte-derived Aφs may have either beneficial or deleterious functions and more studies are required to better delineate the role of these macrophage subtypes in lung diseases.

TISSUE DAMAGE CONTROL MAY DRIVE PATHOGEN PERSISTENCE

The environment created by the tissue damage control may favor the persistence of pathogens in the airways. Indeed, the immunosuppressive properties of Aφs during the process of the control of tissue damage are presumably key in leading to immune evasion. Evasion from immune surveillance is an important parameter leading to the persistence of pathogens (50). The incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) pneumonia in otherwise healthy individuals is increasing (51). These bacteria persist in lower airways by surviving within Aφs. An *in vitro* study found that *S. aureus* persists and replicates inside a murine Aφ cell line (52). Among the mediators used by Aφs to control tissue damage, we previously mentioned that PGE_2 is produced after efferocytosis and exerts anti-inflammatory effects. PGE_2 is known to suppress natural killer cell activity by increasing cellular cyclic adenosine monophosphate (53) and downregulates MHC class II expression on dendritic cells to decrease antigen presentation (54). More recently, it has been shown that the anti-inflammatory action of PGE_2 in the lung is mediated only by the prostaglandin E receptor 4 (EP4) (55). In this way, it seems pathogens can take advantage of PGE_2. Indeed, PGE_2 can inhibit bacterial killing by Aφs by inhibiting NADPH oxidase (56). In macrophages infected by *M. tuberculosis*, PGE_2 generated by TLR2 stimulation/p38 MAPK phosphorylation triggers EP4 to produce increased amounts of PGE_2. Then PGE_2 provides protection against necrosis *via* EP2 (57). Production of PGE_2 by the host is a protective mechanism against *M. tuberculosis* by inhibiting type I IFN (58) as well as inducing apoptosis in macrophages (59, 60). Similarly, Influenza virus induces PGE_2 to suppress type I IFN subverting innate immunity (61). Taken together, it seems that pathogens have developed mechanisms to induce PGE_2 production by macrophages to suppress inflammation and better survive within the host. A recent study by Roquilly et al. shows that dendritic cells and macrophages developing in the lungs after the resolution of a severe infection acquire tolerogenic properties that contribute to persistent immunosuppression and susceptibility to secondary infections (62).

Aφ plasticity associated to the control of tissue damage is an important factor in pathogen persistence. The prevalence of the so-called M2 phenotype has been often associated with a positive outcome because of its ability to control tissue damage. However, M2 macrophages represent a permissive niche for the persistence of many intracellular pathogens (63). Indeed, persistence of bacteria has been described for several human diseases including Legionnaires' disease (64) and tuberculosis (65). Alarmins, such as IL-33, IL-25, and TSLP, play an important role in macrophage polarization during tissue damage (66). The synthesis of IL-33 by epithelial cells, characteristic of the lung environment after birth, triggers the release of IL-13 by ILC2s and induces an anti-inflammatory M2 phenotype. Such an environment has been associated with the delayed response to *Streptococcus pneumoniae* infection in mice (67).

Decreased antimicrobial activity and augmented oxidative metabolism of M2 macrophages compared to glucose-dependent metabolism of M1 cells represent the main factors contributing to pathogen persistence in the host. The decreased production of nitric oxide following IL-4-driven arginase-1 expression facilitates the survival of pathogens sensitive to this reactive species (68) and perhaps explains why *Chlamydia pneumoniae* has been reported to prefer the M2 than M1 macrophage for its proliferation *in vitro*

(69). In this scenario, pathogens not only benefit from but also drive macrophages toward the M2 phenotype that better suits their own requirements, as suggested by recent publications. A mathematical model has been proposed to facilitate the investigation of M1 to M2 switching following infection of macrophages with *M. tuberculosis* (70).

Mycobacterium tuberculosis upregulates the expression of peroxisome proliferator-activated receptor-γ in infected macrophages leading to increased lipid droplet formation, expression of M2 markers and downregulation of the M1 response, including the respiratory burst and nitric oxide production (71). In this way, *M. tuberculosis* not only circumvents the protective host response but may also guarantee the nutrient rich environment required for its growth and survival. Indeed, *M. tuberculosis* secretes a hydrolase to catalyze host lipid hydrolysis (72). This capacity of pathogens to use cell metabolism to persist in the airspaces seems unavoidable. Further, M2 macrophages demonstrate an iron metabolism of benefit for pathogens. M2 macrophages have reduced iron storage and increased iron and heme uptake resulting in a high iron label pool (73), thus favoring the growth and survival of pathogens (63). For instance, *M. tuberculosis* can use macrophages as an iron source and produce siderophores able to sequester iron from host transferrin and lactoferrin, leading to augmentation of iron concentrations in infected macrophages and favoring its growth (74). Other metal metabolism can be "highjacked" by pathogens, such as zinc. Vignesh et al. have shown that IL-4, a well known M2-polarizing signals, alters macrophage zinc homeostasis *via* metallothionein 3 and the zinc transporter SLC30A4, promoting pathogen persistence in M2 macrophages (75).

CONCLUSION

Taken together, these studies demonstrate that Aφs have a central place in lung disease tolerance by (i) involvement in limiting lung tissue damage from potentially innocuous stimuli (ii) decreasing immune surveillance and (iii) by hosting pathogens. Pathogen persistence in the respiratory tract is an important preoccupation, and of particular relevance to conditions such as tuberculosis. Indeed, the equilibrium between the elimination of pathogens and maintenance of tissue integrity is a sword of Damocles for susceptible patients. Better understanding of the mechanisms of disease tolerance and in the appropriate setting breaking this tolerance may provide therapeutic options. An important field requiring further exploration is the discrimination of the role of resident macrophages versus recruited macrophages in the lung environment. How recruited macrophages interfere with various functions of resident Aφs to conserve the lung homeostasis is of great interest.

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AUTHOR CONTRIBUTIONS

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Energy Demands of Early Life Drive a Disease Tolerant Phenotype and Dictate Outcome in Neonatal Bacterial Sepsis

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Bacterial sepsis is one of the leading causes of death in newborns. In the face of growing antibiotic resistance, it is crucial to understand the pathology behind the disease in order to develop effective interventions. Neonatal susceptibility to sepsis can no longer be attributed to simple immune immaturity in the face of mounting evidence that the neonatal immune system is tightly regulated and well controlled. The neonatal immune response is consistent with a "disease tolerance" defense strategy (minimizing harm from immunopathology) whereas adults tend toward a "disease resistance" strategy (minimizing harm from pathogens). One major advantage of disease tolerance is that is less energetically demanding than disease resistance, consistent with the energetic limitations of early life. Immune effector cells enacting disease resistance responses switch to aerobic glycolysis upon TLR stimulation and require steady glycolytic flux to maintain the inflammatory phenotype. Rapid and intense upregulation of glucose uptake by immune cells necessitates an increased reliance on fatty acid metabolism to (a) fuel vital tissue function and (b) produce immunoregulatory intermediates which help control the magnitude of inflammation. Increasing disease resistance requires more energy: while adults have fat and protein stores to catabolize, neonates must reallocate resources away from critical growth and development. This understanding of sepsis pathology helps to explain many of the differences between neonatal and adult immune responses. Taking into account the central role of metabolism in the host response to infection and the severe metabolic demands of early life, it emerges that the striking clinical susceptibility to bacterial infection of the newborn is at its core a problem of metabolism. The evidence supporting this novel hypothesis, which has profound implications for interventions, is presented in this review.

Keywords: neonate, sepsis, disease tolerance, metabolism, inflammation, infection

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INTRODUCTION

Improvements in neonatal mortality have been comparatively slower than in other age groups (1). More than 40% of all under-five deaths occur in the neonatal period and this percentage has been rising over the last few decades (1, 2). Part of the difficulties associated with decreasing newborn mortality stems from an assumption that neonatal immunity is "immature" or a "deficient" version

of its adult counterpart (3–5). Despite much evidence that debunks this myth (i.e., robust immune responses, heightened sensitivity to sterile inflammatory insults such as LPS, ability to tolerate much higher pathogen load than adults, etc.) (3, 4, 6–9), this dogma persists, likely because it presents an easy explanation for the clinically increased susceptibility (10). Ultimately, the inability of this long-held immaturity paradigm to translate into effective immunomodulatory treatments for neonatal sepsis is in itself evidence of a fundamental misunderstanding of newborn imunity (7, 11). The failed interventions aimed at "correcting" immature immune functions, alongside mounting evidence emerging through next generation sequencing technology, indicate that neonatal immunity exists as a tightly regulated, controlled system which is functionally and purposefully distinct from that of adults, not simply lesser (3, 6–9).

To build the conceptual framework for reconcilation of the clinical observation (increased risk to suffer and die from sepsis in newborns vs. adults) with mechanistic insight regarding host defense in early and adult life, it is necessary to consider the range of host responses to infection that are available. Medzhitov et al. in 2012 outlined three distinct strategies of host defense to infection: disease avoidance, disease tolerance, and disease resistance. In disease avoidance, infection is avoided through behavioral adaptations (e.g., our evolved revulsion to the smell of rotting meat). Disease resistance focuses on the reduction of pathogen burden at the risk of host-inflicted damage (immunopathology). Disease tolerance (DT) strives to minimize immunopathological damage, or fitness cost to the host at the potential cost of unchecked pathogen proliferation (it is important to draw the distinction between "disease tolerance" and "immune tolerance," which describes regulatory T cell (Treg)-mediated unresponsiveness to potentially immune-activating agents) (12). Animal models have demonstrated newborns to suffer increased mortality when infected with living bacteria (13), viruses (14, 15), or purified inflammatory agonists (16, 17). DT is a well-established concept in biology, but not yet as readily accepted in the human realm (see this entire special edition of *Frontiers*). Specifically regarding DT in early life: newborns are able to withstand a circulating bacterial load 10–100 times greater than adults (<1 CFU per mL blood has been considered to be the clinical "low" threshold in adults, whereas <50 CFU per mL blood has been considered the "low" neonatal threshold; the same trends are observed in animal models) (3, 18). The juxtaposition of increased sensitivity to infection with an enhanced ability to survive greater pathogen loads is the hallmark characteristic of a "disease tolerance" response (12). Yet, as evidenced by the higher burden of infectious disease in newborns, a host defense strategy relying on DT is likely less effective than the adult focus on disease resistance. Despite this clear clinical disadvantge, the newborn host as an organism across evolution appears programmed to more heavily rely on this apparently less-effective DT strategy (3, 19).

We present here a conceptual framework to resolve this conundrum. Based on data demonstrating important links between metabolic pathways and immune functions, it emerges that the pathology of neonatal sepsis is the result of an energy deficit which renders the host incapable of producing critical metabolic mediators to mainain inflammatory homeostasis. While there is an abundance of literature discussing the relationship between organism-level metabolism and cellular immunometabolism in the context of metabolic diseases such as obesity or diabetes (20, 21), the potential impact of organismal metabolic needs has been largely unexplored in the context of infectious disease. Here we hypothesize that the defense strategies differentially employed between newborns and adults (disease tolerance vs. disease resistance) can simply be attributed to differences in systemic energy supply and demand, manifesting at the cellular level as differences in immunometabolic activity.

METABOLISM IS FUNDAMENTALLY LINKED TO IMMUNITY

Metabolic and immunological functions are intrinsically connected at a level beyond the former simply fueling the latter—metabolic substrates, enzymes, transcription factors, cell receptors, and intermediates have all been shown to have a vast array of immunoregulatory properties. A recent surge in research into this phenomenon ("immunometabolism") has led to the publication of excellent reviews (22–27) which explore the regulatory role of different metabolic pathways on various leukocytes; with this in mind, we only present a brief overview to introduce the key themes of immunometabolic changes focused on bacterial sepsis.

It has long been known that changes in cellular metabolism occur during sepsis, although until recently these changes were considered to be a result of an oxygen-poor microenvironment due to inflammation-induced hypoperfusion (28). However, there is a large body of evidence indicating that both metabolic shifts and tissue damage in sepsis occur independent of oxygen levels (25, 28–31). TLR activation in certain leukocytes has been shown to activate hypoxia-inducible factor 1α (HIF1α), which upregulates glycolytic pathways and downregulates oxidative phosphorylation—a process known as the Warburg effect (27, 32). This "aerobic glycolysis" is critical to the inflammatory immune response (disease resistance) and represents the primary metabolic activity within immune effector cells (granulocytes, M1 macrophages, cytotoxic, and helper T-cells, NK cells, etc.) (23, 27). While the purpose of switching to aerobic glycolysis in lieu of the more energy efficient process (i.e., ATP-producing) of oxidative phosphorylation is still being debated, it is generally thought that effector cells rely on glycolysis because of one or all of the following reasons: (a) glycolytic intermediates are needed for rapid biosynthesis required for an inflammatory response, (b) glycolysis can be rapidly upregulated and thus can provide a burst of energy faster than oxidative phosphorylation, (c) reactive oxygen species (ROS) are produced during glycolysis which are used in an antimicrobial capacity, (d) glycolysis is better suited to hypoxic/normoxic conditions which may arise during inflammation, and/or (e) increased uptake of glucose minimizes the amount of energy available for invasive bacteria (23, 27, 33). Whatever the reason, it is well established that aerobic glycolysis is a critical component of the disease resistance response (27, 34).

Where aerobic glycolysis is enhanced in effector cells involved in disease resistance pathways, the regulatory and longer lasting cells associated with DT (Tregs, M2 macrophages, memory T cells etc.) increase uptake of exogenous fatty acids and sustain high levels of β-oxidation and oxidative phosphorylation during infection and sepsis (23, 27). While lipids in excess have been shown to induce systemic inflammation (i.e., in obesity), many metabolic intermediates of lipid metabolism exert the opposite effect (35). Circulating lipids which are generated through fatty acid metabolic pathways, namely high-density lipoproteins and very low density lipoproteins, are even capable of directly sequestering LPS and dampening the inflammatory response (36, 37). Lipids belonging to the group of omega 3 fatty acids inhibit the production of inflammatory cytokines and upregulate anti-inflammatory cytokines (38). These lipids also act as precursors to a specialized family of lipids identified as "pro-resolving lipid mediators" (including lipoxins, resolvins, and protectins) which are actively produced to tone down the inflammatory immune response produced at the site of infection (39).

Fatty acid metabolism can therefore be considered to be a fundamental part of the DT response; not only do regulatory / immunosuppressive cells rely on exogenous fatty acids to enact their function, but the metabolites themselves reduce the inflammatory immune response. On the other side of the coin, aerobic glycolysis is a fundamental aspect of the disease resistance response. In addition to the aforementioned increase in glycolytic pathways in immune effector cells, multiple enzymes involved in glycolysis have been shown to either inhibit inflammatory pathways or activate immunosuppressive pathways (27). When a TLR ligand induces high glycolytic flux, these enzymes are rendered incapable of maintaining these disease tolerant functions and the disease resistance response is enhanced (27). To summarize, metabolic shifts in sepsis cannot be separated from inflammatory shifts—the two are fundamentally connected.

METABOLISM IN ADULT SEPSIS

Metabolic changes during sepsis in adults have been shown to not only be instrumental in diagnosing the disease, but also highly related to survival. A 2013 study (40) of adult patients with community-acquired sepsis examined changes in the plasma metabolome and proteome at time of enrolment and 24 h later. Comparisons were made between survivors (split into three subgroups: uncomplicated sepsis, day 3 severe sepsis, and day 3 septic shock), non-survivors, and a control group of patients exhibiting symptoms but were later determined to have SIRS for non-infectious reasons (SIRS-positive controls). The plasma metabolome revealed four primary findings: (a) the profile of plasma metabolites during sepsis were distinct and reliably distinguishable from SIRS-positive controls, (b) there were marked differences in plasma metabolites between sepsis survivors and non-survivors, (c) there were no differences between the sepsis-survivor subgroups (varying degrees of severity), and (d) there were no major differences between infections caused by *S. pneumoniae, S. aureus, or E. coli* (40). Plasma proteomics mirrored the trend—significant differences between sepsis vs. SIRS-positive control, significant difference between survivors, and non-survivors, minimal (only one) differences within the survivor subgroups, and no significant differences resulting from infections caused by different bacteria. Alterations in fatty acid metabolism largely separated sepsis survivors from non-survivors—the specific pattern of metabolites which were different "suggest a profound defect in β-oxidation in adult sepsis non-survivors that was absent in sepsis survivors" (40).

The authors indicate the above noted differences were not a result of organ dysfunction or hypoxia, but rather due to defects in the process which transports fatty acids from the cytoplasm into the mitochondrial membrane (the carnitine shuttle), which in turn may be attributed to a decrease in peroxisome proliferator-activated receptor-α (PPARα) expression during sepsis. PPARα is the primary transcription factor responsible for controlling a host of genes associated ketone body synthesis (ketogenesis) and transport, a process which in adults is typically associated with prolonged fasting (41). One explanation for the apparent requirement of ketone body production during sepsis is that ketone bodies act as the alternative to glucose for fueling brain metabolic activity, as they are one of the few energetic substrates which are able to cross the blood-brain barrier (42, 43). An animal model examining the impact of exogenous glucose and 2DG (an unmetabolizable analog of glucose which inhibits glycolysis) on sepsis induced by *Listeria monocytogenes*, LPS, influenza virus, and poly(I:C) showed that 2DG's protective effect in bacterial sepsis was mediated through increase in ketogenic activity (PPARα-dependent), which reduced neuronal cell death independent of bacterial load (44). Exogenous glucose alone worsened outcome acting through the same axis—ketone body production was inhibited, and neuronal cell death increased in bacterial sepsis. Curiously, these effects were reversed in the viral sepsis models (poly(I:C) and influenza)—2DG caused 100% mortality and feeding/glucose caused 100% survival, indicating fundamental differences between metabolism during viral and bacterial sepsis.

Another recent study examining longitudinal changes in serum metabolite concentrations during sepsis in adults found non-survivors had elevated (and increasing) levels of TCA cycle metabolites as well as diminished (and declining) numbers of short and long-chain fatty acids (45)—the same trends have previously been described in animal models (46, 47). Though non-survivors in sepsis have diminished fatty acid levels relative to survivors, sepsis itself is generally associated with an increase in plasma lipids (including free fatty acids) when compared to healthy controls (45, 48). Not only do plasma lipids play a critical role in regulating inflammation and providing energy for the brain, but fatty acid metabolism and ketogenesis has also been shown to fuel metabolic activity in many vital organs during active infection (49–51). An impaired capacity for β-oxidation and/or a depleted fatty acid supply will essentially turn off the disease tolerance pathways—death seems almost inevitable through either uncontrolled inflammation or uncontrolled energy expenditure, leaving vital organ functions without fuel. As with any homeostatic environment, poor

outcomes are more likely if the balance tips too far to the either extreme.

Furthermore, there is mounting evidence that mortality in adult sepsis is less likely associated with excess inflammation, but rather an immunosuppressive or endotoxin tolerant phenotype (M2-macrophage polarization, anti-inflammatory cytokine production without impaired phagocytic capacity) (52–54). One would expect that as organs begin to fail due to insufficient energy, the body would attempt to increase fatty acid metabolic activity (and inevitably anti-inflammatory activity) at all costs. A prolonged disease resistance response is energetically demanding and eventually it is necessary to revert toward DT by necessity. The heightened death observed in this period may therefore not necessarily be caused by DT, but rather the phenotypic switch to DT as a "last-ditch" effort to adapt to an unsustainable metabolic demand. Perhaps it is time to consider these late-phase inflammatory changes in adult septic patients as a reflection of a different biological mechanism—a slow decrease in the energy available to power vital organ functions.

ENERGETIC DIFFERENCES IN NEONATES AND IMPLICATIONS FOR BACTERIAL SEPSIS

The implications of the critical role metabolic pathways play in regulating inflammation and providing energy during infection are enormous for newborns, as the energetic demands of growth and development are intense. After adjusting for body weight, healthy newborns require on average three times as much protein (2.2 vs. 0.8 g/kg/day) and more than three times as much total energy (120 vs. 35 kcal/kg/day) as adults (55). Newborns have a lower reservoir of energy, as demonstrated by the percent bodyweight made up of fat (14 vs. 18%) and protein 11 vs. 18%) in neonates and adults (55). Sustaining a controlled immune response requires not only intense glycolytic flux to fuel the cellular proliferation and biosynthesis of disease resistance, but it also requires substantial fatty acid metabolic flux to regulate the inflammation and provide energy to vital tissues. Adults are able to rely on fat and protein stores to provide enough energy to engage in a robust disease resistance response without pulling resources from critical processes, at least until later in infection (see above). This can be observed as up to a 150% increase in resting energy expenditure during bacterial infections in adults (56). Neonates, however, show either no change or even a decrease in resting energy expenditure during sepsis (57–59). An inability to increase energy expenditure relative to the resting state in neonates suggests that the energy to fuel the immune response must be redirected from processes elsewhere in the body. Clearly these processes (likely growth and development) are important enough to warrant maximum energy expenses at a basal state (part of the explanation for relying more heavily on DT than disease resistance). Adults are able to employ the "expensive" disease resistance response without seriously interfering with other vital survival processes; for neonates, any energy spent on immunity has to be "borrowed" from somewhere else. The increased reliance on DT in the newborn allows for less glycolytic flux and thus a lower risk to incur organ failure through an energy deficit during septic episodes.

The first few postnatal days are likely the most energetically demanding period in all of life (60). Immediately after birth, neonates must transition from reliance on maternal glucose to generating it themselves—this manifests as hormonal activation of both glycogenolytic and gluconeogenic pathways in order to rapidly ramp up glucose production to fuel developing organs, especially the brain (60). Further, the newly born infant faces rapid heat loss in the transition from the warm uterine environment to the (relatively) colder external environment. Heat production and oxygen consumption increase two–three-fold within minutes of birth, through both heightened cellular metabolism and non-shivering thermogenesis (metabolism of brown adipose tissue) (61). The high mortality observed on the first day of life in particular may be related to this sudden inability to rely on maternal metabolic and thermoregulatory processes (61, 62). The more energy siphoned toward mounting an immune response, the more sacrifices must be made to fuel the necessary cell proliferation and antimicrobial activities. One would anticipate evolutionary pressures to naturally equilibrate neonatal immunity toward a balance between immunity and development—hence a heightened reliance on DT in neonatal infection.

Metabolomics of the neonatal population have not been studied in nearly as much detail as adults, though what is available indicates that metabolism is a critical component of neonatal sepsis as well. A transcriptomic comparison of newborns with bacterial sepsis against healthy controls was used to construct a classifier that accurately identified septic neonates; inclusion of only genes which were associated with standard immune functions (inflammation, etc.) resulted in a classifier with 100% sensitivity but less than 30% specificity, but the inclusion of metabolic genes brought the specificity up to 100% (6). Specifically, they showed that bacterial sepsis in neonates is associated with increased expression of genes related to glycolysis (glucose transporter GLUT3, glycolysis activator PFKFB3, and initiating hexokinase HK3), fatty acid metabolism and metabolic homeostasis (principally via regulatory STAT3 and receptor FFAR2). In the validation test set of the classifier, the three instances of viral sepsis clustered with the 6 controls; the viral patients did not show the distinct metabolic profile which was so visible in newbons with bacterial sepsis, further indicating that uncontrolled viral proliferation has a profoundly different impact on the body than uncontrolled bacterial proliferation (6).

As mentioned above, poor outcomes in adult sepsis correlate with an inhibited ability to produce ketone bodies via PPARα. While ketone body metabolism in the adult brain is typically reserved for a starvation response (which perhaps should be updated to "energetically demanding periods" such as sepsis), there is evidence from animal models that neonates rely on ketone bodies as an energy source in the brain independent of starvation (43). Specifically, newborn rats rely on ketone bodies for up to 40% of the energy production in the brain (42) and newborn cynomolgus monkeys exhibited increased expression of blood-brain barrier ketone body transporter protein MCT1, with levels decreasing as a function of age (plateauing in adulthood)

(63). The process of birth necessitates a series of metabolic adaptations from receiving nutrients via the placenta (high-carbohydrate, low-fat) to receiving nutrients via breastmilk (low-carbohydrate, high-fat) (64, 65). One manifestation of these adaptations is the activation of PPARα immediately prior to birth, presumably in anticipation of the new, fat-rich diet (64). Upon the switch to breastmilk, neonates rely on ketone bodies to fuel brain activity which allows glucose (broken down from lactose) to enter the pentose phosphate pathway, producing the nucleic acids and lipids necessary for cerebral growth (43).

Similarly, a metabolomic analysis of urine from septic newborns indicated a substantial increase in acetone ketone bodies (and other byproducts of fatty acid oxidation) relative to healthy controls (66). If more ketone bodies are found outside of the brain but there is little compensatory increase in ketone body production [as indicated by animal models (47), and neonates being "maxed-out" in their energy expenditure at baseline], then one can assume that ketone bodies which are needed in the brain are being deployed elsewhere in the body, and hence the brain is running at an energetic deficit. This is just one example of the type of vital process which may be interrupted by mounting an immune response during bacterial sepsis.

METABOLISM IN VIRAL SEPSIS

As eluded to above, the metabolic signatures specific to bacterial sepsis appear to be absent in viral sepsis; gene signatures from human newborns that predict bacterial sepsis classify viral patients as controls (6) and in an experimental model, inhibiting glucose metabolism led to 100% mortality viral infection and poly(I:C) challenge (44). Hence, metabolic regulation during viral infection is likely distinct from that of bacterial infection. This is not of small consequence, as a substantial proportion of neonatal sepsis may be due to viruses: In a published unit from a Bangladeshi cohort, 36% of suspected neonatal sepsis was viral (67), though it is difficult to estimate the generalizability. Viruses rely on cellular fatty acid synthesis in order to replicate, which is reflected by viral manipulation of cellular metabolism. Human cytolomegavirus has been shown to sequester glucose from the the TCA cycle and redirect it toward virus-induced fatty acid biosynthesis, demonstrating just one example of the potential immunometabolic ramifications of viral infection (68). Inhibiting fatty acid biosynthesis has been shown to slow the growth of viruses; one study showed that treating cells with in inhibitor of fatty acid synthase reduced the proliferation of rotavirus (69). Similarly, AMP-activated kinase is able to decrease Rift Valley virus infection by inhibiting cellular fatty acid synthesis necessary for viral replication (70). Meanwhile, short-chain fatty acids derived from dietary fiber are protective against influenza challenge due to their effect on both innate and adaptive immune cells (71). Thus, strategically manipulating the glucose/fatty acid metabolism has potential in improving outcomes in newborn viral sepsis and need to be studied further in this context. Already, differences in feeding patterns during viral and bacterial infection may hint at the unique nutritional requirements for both types of infections. Poor feeding itself is a hallmark of newborn infection (72). However, it seems to be more prominent during bacterial, compared to viral sepsis: in a cohort of febrile newborns with enterovirus, ∼16% had poor feeding (73) while in another cohort of Ugandan infants with or without culture-positive sepsis, 42% of newborns with culture positive sepsis (i.e., bacterial) were admitted for care with poor feeding as a primary sign, compared to only 17% of infants admitted with a diagnosis of culture negative sepsis (i.e., viral) (74). Clearly more research is warranted into the immunometabolic differences between bacterial and viral sepsis.

NUTRITIONAL THERAPY AND THE MICROBIOME

If mortality in bacterial sepsis can be attributed to an energetic deficiency, then one must be able to explain how nutritional supplementation (a standard practice in any ICU or NICU) does not represent the most effective sepsis treatment. As with everything else in sepsis, the efficacy of feeding as an intervention is limited by its ability to maintain homeostasis. The previously described study by Wang et al. where inhibition of glycolysis led to 100% survival (and feeding led to 100% mortality) in adult, LPS-challenged mice challenged provides an excellent example of nutritional supplementation creating a homeostatic imbalance and leading to negative outcome. Both exogenous glucose and food gavage inhibited ketogenesis which led to an energetic imbalance (glucose being siphoned into the immune response with no ketone bodies to replace it) and an inflammatory imbalance (diminished anti-inflammatory lipid mediators). One also must consider the dangers of overfeeding—overfeeding has been shown to worsen sepsis outcomes in both animal models and human observational studies due to hyperglycemia, elevated inflammatory markers, dysregulated immune responses, and presumably enhanced nutrients for pathogen growth (75–78). Further, this hypothesis poses that the metabolic risk comes from a shift in the proportion of energy expended toward disease resistance pathways over maintaining organ function—the danger is not only tied to the overall capacity, but the utilization of energy present. If a system has reached the point where it is spending 100% of its resources on fighting infection, no amount of exogenous nutrients will make a difference (unless accompanied by a simultaneous change in resource allocation).

Early enteral nutrition (EN) in adult patients with prolonged sepsis has been shown to improve patient outcomes, reduce oxidative stress, improve gut epithelial integrity, and downregulate systemic immune responses (79); correspondingly, negative energy balance has been shown to be associated with worse clinical outcomes (80). EN not only addresses the caloric deficit which is inevitable in prolonged sepsis but seems to modulate immune functions through interfacing with the gut-associated lymphoid tissue and upregulating Th2 cell proliferation. Parenteral nutrition (PN) has been shown to be less effective than EN—a 2013 study by Elke et al. found that rate of death was significantly lower in adult ICU patients with sepsis which received EN rather than PN or EN + PN combined (26.7 vs. 41.3%); in addition to this mortality reduction, duration of mechanical ventilation and rate of secondary infection were also decreased in the EN-alone group (81). Increased

mortality from PN (relative to EN) is thought to be due to a heightening of the inflammatory response associated with hyperglycemia, an effect which exacerbated in PN due to bypassing metabolic regulatory axes associated with the GI tract (79). This has interesting implications for neonates, where the nascent microbiome represents another axis which is distinct from adults (82). The diet of neonates (lactate-heavy breastmilk) results in a colonization pattern of commensal bacteria which fascilitate nutrient absorption and produce a wide array of immunoregulatory metabolites (82, 83). The limited biodiversity present in the neonatal microbiome could result in an impaired ability to incorporate nutrients without altering inflammatory homeostasis—any inability to control the potential energy flux of disease resistance represents another potential explanation for the neonatal reliance on DT.

RECONTEXTUALIZING NEWBORN IMMUNITY

The implications of this hypothesis are broad and may explain other aspects neonatal immunity. Newborns have been described as exhibiting an immunosuppressive phenotype, which has often been considered to be a vestige of time spent *in utero* where active fetal immunity could result in miscarriage (8, 84). This alternative hypothesis to DT fails to explain the persistance of many of these immunosuppressive actors well after the first few days of life. For example, neonatal myeloid-derived suppressor cells and anti-inflammatory $CD5^+$ B cells remain significantly higher than adult levels for more than 6 months and 4 months after birth, respectively (8, 85). Given the high burden of infectious disease in early life, one would anticipate evolutionary pressure to drive the time spend in this "anti-inflammatory phase" to as little as possible. If, however, neonatal immunity is limited by an availability of energy, then it would be critical to maintain some immunosuppressive cells to limit the magnitude of an inflammatory response until the body is able to better sustain it. While the "fetal suppression" hypothesis may in part explain the susceptibility of term infants to bacterial sepsis (86), it seems unlikely that a biological liability of this magnitude (suppressed immune system) would exist and persist if it did not convey some sort of survival advantage (DT).

The extreme susceptibility to infection observed in preterm newborns may be in part due to the extreme energy demands associated with survival and rapid development, but it is more difficult to discount alternative explanations such as immaturity and immune suppression to tolerate maternal antigens. As outlined in a recent review by Collins et al. susceptibility of preterm newborns to infection can be attributed to "comprimised [innate] barriers, inflammatory response elements, and cells"—more research is warranted to elucidate the role metabolic demands play in preterm immunity (87).

SUMMARY

Mortality in sepsis has been attributed to a dysregulated inflammatory response, with the current paradigm indicating that death may be the result of straying too far toward either extreme (88). Here we hypothesize inflammation is only the top layer of this process and that it is an underlying mechanism, namely metabolism, which is the driving force behind mortality in sepsis. Through this paradigm, the neonatal reliance on DT as a host defense strategy is much easier to understand—the newborn response to sepsis can be characterized through the distinct metabolic needs unique to early life. Lack of functional fat stores, resting metabolic rate operating near or at 100% of its potential capacity, and heavy activation of PPARα due to the high fat content of breast milk combine to constrict the magnitude of the neonatal potential for a disease resistance

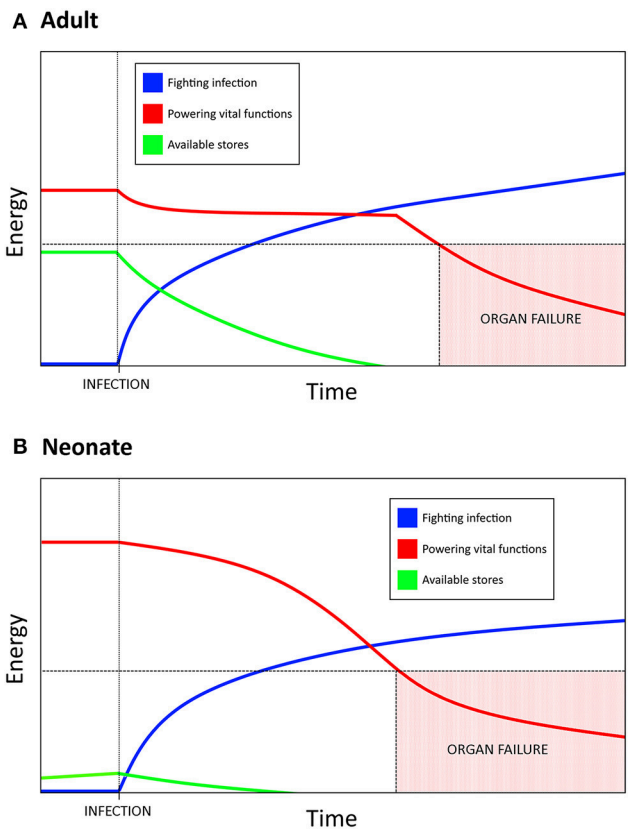

FIGURE 1 | Resource allocation in bacterial sepsis explains the need for disease tolerance as a defense strategy in neonates. Bacterial infection requires a massive, sustained energy input in order to mount an inflammatory, disease resistance response. **(A)** Adults have substantial energy stores and a resting metabolic rate that is not near its max capacity, which allows for a burst of energy to be rapidly allocated toward fighting the infection. If the infection results in a prolonged state of sepsis, eventually the amount of energy required to maintain the inflammatory response necessitates a siphoning from other vital functions. Too much energy siphoned can result in organ failure and lead to mortality. **(B)** Neonates have much higher basal metabolic needs, lower energy stores than adults, and are unable to increase resting energy expenditure during infection. Allocating resources toward fighting infection therefore comes at a greater cost to neonates, which has resulted in an increased reliance on disease tolerance as a defense strategy. Mortality due to sepsis may be a result of the energetic demands of a sustained response, rather than a direct result uncontrolled inflammation.

response (i.e., inflammation). As glycolysis is fundamentally tied to effector/antimicrobial/disease resistance cell functions, it becomes necessary to shift as much glycolytic activity toward fighting infection as possible. The body then transitions into the equivalent of a "starvation state" where ketogenesis is used to support vital organ functions, especially in the brain as there is no other alternative energy source which is able to permeate the blood brain barrier (51). Birth itself overlaps with this "starvation state" in that the process of adapting from *in utero* to *ex utero* life is energetically intense. This creates a situation where neonates are unable to produce the burst of energy associated with aerobic glycolysis which is characteristic of adult immunity—powering a full disease resistance strategy would mean pulling resources from elsewhere in the newborn body. The more intense and prolonged this response is, the more likely it is that a vital organ will fail as there simply is not enough energy devoted to maintaining its functions (**Figure 1**).

Homeostasis in sepsis thus cannot be considered to only be a balance between inflammatory (disease resistance) and immunosuppressive (disease tolerance) processes, but rather a balance of inflammation and aerobic glycolysis (disease resistance) with immunosuppression and fatty acid metabolism (disease tolerance). The less excess energy which exists in the system as a whole, the more an individual must rely on the DT approach. This applies not only to neonates, but also to adults facing prolonged infection. Nutritional supplementation is critical to survival, but it must be provided within the bounds of this homeostatic balance. Excess nutrients or parenteral nutrition would impair ketogenesis which would (a) remove anti-inflammatory lipid substrates/metabolites resulting in excess inflammation, (b) diminish the amount of energy devoted to fuel vital functions (increased inflammation resulting in increased glycolysis which is being used to fight infection rather than support organ functions), and (c) provide resources for the invasive pathogen and not only the host. Impairing glycolysis, on the other hand, runs the risk of impairing the entire disease resistance branch and exposing the host to the danger of uncontrolled pathogen proliferation. It is critial that future interventions which are theoretically focused on managing inflammation also consider the potential impact on energy homeostasis, and interventions aimed at nutritional supplementation (both prenatal and perinatal) must not disrupt inflammatory homeostasis. More research in novel therapeutics which act on both fronts is clearly warranted.

AUTHOR CONTRIBUTIONS

All authors contributed to the conceptual idea and the editing process. DH made the figure and wrote most of the manuscript with writing contributions from NA and FF, as well as guidance from NA and TK. FF and WB provided research assistance.

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REVIEW
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The Dual Nature of Type I and Type II Interferons

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Type I and type II interferons (IFN) are central to both combating virus infection and modulating the antiviral immune response. Indeed, an absence of either the receptor for type I IFNs or IFN-γ have resulted in increased susceptibility to virus infection, including increased virus replication and reduced survival. However, an emerging area of research has shown that there is a dual nature to these cytokines. Recent evidence has demonstrated that both type I and type II IFNs have immunoregulatory functions during infection and type II immune responses. In this review, we address the dual nature of type I and type II interferons and present evidence that both antiviral and immunomodulatory functions are critical during virus infection to not only limit virus replication and initiate an appropriate antiviral immune response, but to also negatively regulate this response to minimize tissue damage. Both the activating and negatively regulatory properties of type I and II IFNs work in concert with each other to create a balanced immune response that combats the infection while minimizing collateral damage.

Keywords: type I interferon, interferon-γ, innate immunity, virus infection, immunoregulation

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INTRODUCTION

Type I and II interferons (IFN) are cytokines produced during virus infection that are integral for regulating the immune response. Type I IFNs are well known for their ability to directly induce an antiviral response within infected and surrounding cells through the upregulation of molecules that can antagonize virus replication (1). As they are produced rather early on during an infection, type I IFNs are also essential for activating the antiviral innate immune response, such as natural killer (NK) cell effector functions (2). Type II IFN, known as IFN-γ, while sharing a similar nomenclature to type I IFN, signals through a different receptor and has effects that are independent from type I IFN. As a part of the innate immune response, they are predominantly produced by natural killer cells during infection (2). IFN-γ, like type I IFN, promotes antiviral immunity through its regulatory effects on the innate immune response and acts as a key link between the innate immune response and activation of the adaptive immune response (3). Beyond their antiviral effects, a growing amount of evidence suggests that type I and type II IFNs have immunoregulatory functions that are critical for dampening immunopathogenic mechanisms and minimizing collateral damage from the infection. Altogether, this review will build a framework and provide evidence demonstrating that these two cytokines are both critical for limiting virus replication and promoting a beneficial virus limiting response, while simultaneously dampening immunopathology. If we consider the world outside of virus infections, however, this fundamental duality of type I and II IFNs can be applied to numerous pathological processes, ranging from allergy to autoimmune diseases.

Type I and II IFN Production and Signaling

Type I IFNs consist of a group of structurally similar cytokines and include 13–14 subtypes of IFN-α along with IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ, IFN-ζ, and IFN-τ (4, 5). As part of the innate immune antiviral response, these cytokines are rapidly produced after pattern-recognition receptor (PRR) stimulation (5). Current research suggests that an initial wave of IFN-β and IFN-α4 is produced and dependent upon IRF3 phosphorylation and NFκb activation (6–8). The initial type I IFN wave subsequently induces IRF7 phosphorylation and results in a positive feedback loop of increasing type I IFN release. Once produced, these cytokines all signal through the same receptor, the type I IFN receptor (IFNAR). IFNAR is composed of two subunits—IFNAR1 and IFNAR2—which when bound to type I IFN are endocytosed and activate their associated tyrosine kinases, Tyk2 and Jak1 (4, 9). The classical signaling cascade results in phosphorylation of STAT2 and STAT1, which forms a complex with IRF9, known as the IFN-stimulated gene factor 3 (ISGF3) (4). ISGF3 then leads to expression of IFN-stimulated genes (4). Beyond ISGF3, type I IFNs can also induce phosphorylation and dimerization of STAT3, STAT4, STAT5, and STAT6 and has been shown to induce activation of Rap1, CrkL, Map kinases, IRS-1 and -2, Vav, RAC1, and PI3-kinase signal transduction pathways (4, 10–14). Interestingly, IFN-β has been shown to additionally signal through the IFNAR1 subunit independent from IFNAR2 and carries through a non-canonical signaling pathway (15).

Type II IFN is predominantly produced by NK cells during the antiviral innate immune response (16). A multitude of evidence has shown that type I IFN, IL-12, IL-15, and IL-18 are all capable of inducing IFN-γ production from NK cells (17). NK cell IFN-γ is dependent upon STAT4 phosphorylation for its production. Once released, IFN-γ signals through the IFN-γ receptor (IFNGR), composed of IFNGR1 and IFNGR2 subunits. In the classical signaling pathway, ligation of IFN-γ to the IFNGR leads to activation of JAK1 and JAK2, resulting in homodimerization and phosphorylation of STAT1 (18). However, like type I IFN, IFN-γ has also been shown to signal through alternative pathways, including STAT4, Erk1/2, Pyk2, and CrkL, among others (18).

Type I IFN: Mastering the Antiviral Response

Type I IFN is one of the first cytokines produced during a virus infection. In the context of HSV-2 infection, for example, there is an initial wave of IFN-β production at 12 h post-infection, followed by both IFN-β and IFN-α production at 48 h post-infection (19, 20). This early production of type I IFN is critical for induction of both an antiviral response within infected and target cells, as well as activation of innate immune cells that will ultimately serve to control virus replication and activate the adaptive immune response to both clear the infection and generate memory to create a rapid response against future infections (21).

Type I IFN is a well-known stimulator of antiviral genes targeted against preventing virus replication from within target cells. When their production is stimulated by virus infection, type I IFN can act in an autocrine, paracrine, or systemic fashion. Their protective role during virus infection is highlighted by the increased mortality observed in mice deficient in the type I IFN receptor (*Ifnar*$^{-/-}$) in comparison to their control counterparts when infected with a virus (22, 23). Upon ligation to its receptor, type I IFN has been shown to induce upwards of 300 ISGs. Of these 300 genes, 51 were found to contribute to host defense, while other genes contributed to inflammation, signaling, transcription, and immunomodulation, among other activities (24, 25). Further, De Veer et al. examined the ability of specific ISGs or combinations of ISGs to inhibit virus replication through overexpression of individual ISGs prior to virus infection (24). They found that many ISGs were capable of inhibiting virus replication, with some acting on a wide range of viruses, while others were only effective against particular viruses (24). Interestingly, they found that select ISGs enhanced virus replication in their experimental system (24). Antiviral ISGs can hinder virus replication through several mechanisms. Protein kinase R, for example, inhibits cellular translational functions (1). 2′5 OAS and RNaseL, on the other hand, degrade RNA (26, 27). Other ISG antiviral activities can prevent virion release, inhibit virus entry, and inhibit virus transcription (28).

Apart from their induction of antiviral ISGs, type I IFNs are key regulators of the innate immune response. Within the type I IFN literature, a theme has emerged wherein acute type I IFN production promotes beneficial antiviral responses, while chronic type I IFN production can have a suppressive and deleterious effect on the immune response. Within this section, we will examine the ability of type I IFN to promote antiviral functions in dendritic cells (DC), monocytes, and NK cells.

Dendritic cells are critical for activation of antiviral T-cells (29). Type I IFN stimulation has been shown to enhance MHC II expression and presentation of antigens as well as upregulate co-stimulatory molecules and promote DC maturation (29–32), Further evidence suggests that type I IFN is able to increase differentiation of plasmacytoid DCs into myeloid-derived DCs to increase T-cell activation (33).

Inflammatory monocytes are rapidly recruited to sites of infection, where they can then stimulate local and migrating immune cell antiviral function, promote inflammation, and differentiate into macrophages and DCs (34). At sites of inflammation, type I IFNs induce production of CCL2 to recruit inflammatory monocytes (2, 34). Type I IFN produced during vaginal HSV-1 infection induces tissue resident macrophages and DCs to produce CCL2 to recruit and initial population of inflammatory monocytes, which then enact a positive feedback loops to produce more CCL2 to attract further inflammatory monocytes (35). A similar phenomenon has been observed during vaginal HSV-2 infection, influenza infection, and inflammatory monocyte recruitment to the brain during LPS-induced systemic inflammation (2, 36, 37), With influenza infection, absence of IFNAR resulted in differentiation of Ly6C intermediate expressing monocytes rather than Ly6C^{hi} inflammatory monocytes, which additionally had a different phenotype (36). Further, Seo et al. demonstrated that *Ifnar*$^{-/-}$ bone marrow had a significantly decreased differentiation of

hematopoietic cells into inflammatory monocytes in the presence of influenza infection (38). In regards to macrophages, Type I IFN has more of a suppressive function and will be discussed below.

Type I IFN and antiviral NK cell functionality are tightly interwoven, where type I IFN has emerged as a key NK cell regulator. Like their monocyte counterparts, type I IFN has been implicated in NK cell recruitment to sites of inflammation. During a vaginal HSV-1 infection, type I IFN was required to induce epithelium production of CCL3, CCL4, and CCL5 to recruit NK cells to the vaginal mucosa (35). Further, type I IFN has been implicated in the activation of NK cell antiviral functions. During an infection, NK cells have several weapons under their belt that they can utilize to combat infection. When activated, they can release IFN-γ, cytotoxic granules, and induce cell death of infected cells. Type I IFN has been implicated in both NK cell cytotoxicity and NK cell IFN-γ production. Mice deficient in STAT1, a key transcription factor downstream of type I IFN receptor, have been shown to have decreased NK cell cytotoxicity and increased virus-induced mortality in comparison to control mice (39). In the context of NK cell IFN-γ production, type I IFN is essential for this process in multiple virus infections, including MCMV, adenovirus, vaccinia virus, and HSV (2, 40–43). Type I IFN has been shown to act directly on NK cells to induce their release of IFN-γ in the context of adenovirus, vaccinia virus, and LCMV infections, whereas other evidence suggests that type I IFN stimulates DCs to trans-present IL-15 to activate NK cells in MCMV infection (2, 40–44). Recently, we have provided evidence demonstrating that NK cell IFN-γ production relies on type I IFN induction of IL-18 from inflammatory monocytes, rather than DCs in a mucosal HSV-2 infection (2). Our differing results may stem from the route of infection, where previous evidence used *in vitro* systems or non-mucosal routes of infection.

Type I IFN Negative Regulation: Beyond Interfering With Infection

As more evidence emerges, there is a greater understanding and appreciation for the suppressive and negative regulatory aspects of type I IFN. Early on, studies had shown that type I IFN exerted anti-proliferative effects on immune cells and cell lines (45, 46). Recently, Thomas et al. elegantly demonstrated that while all type I IFN subtypes were capable of inducing an intracellular antiviral response, the affinity of an individual type I IFN subtype to the type I IFN receptor largely determined the ability of type I IFN to inhibit cellular proliferation (47). The antiproliferation effects of type I IFN required higher binding affinities to IFNAR (47). Beyond proliferation, type I IFN can suppress innate immune cell functions as well.

While an acute infection and upregulation of type I IFN is beneficial for enhancing DC activation of T-cell adaptive functions, a chronic infection with sustained type I IFN production has been shown to dampen DC expansion and induce a suppressive phenotype. In chronic LCMV infection, a persistent type I IFN signature prevented BM differentiation and proliferation of conventional DCs (48, 49). Further, stimulation of splenic DCs with IFN-β, *in vivo*, resulted in a decrease in total CD11c+ cell number. In addition to reducing DC expansion, a chronic type I IFN signature was shown to upregulate PD-L1 expression and IL-10 in both DCs and macrophages (50, 51).

Type I IFN largely has a suppressive effect on macrophages. The literature largely suggests that it downregulates their expression of the IFN-γ receptor, making them less sensitive to IFN-γ stimulation (52). In certain bacterial infections, such as *francisella tularensis* and *mycobacterium tuberculosis*, type I IFN signaling is detrimental to the host (53–56). The ability of type I IFN to downregulate the IFN-γ receptor on macrophages likely contributes to this phenomenon.

As mentioned previously, type I IFN has been shown to be critical for inducing the antiviral functions of NK cells. Conversely, and almost paradoxically, type I IFN has also been shown to suppress the very functions that it enables. During LCMV infection, Teijaro et al. found that blocking the type I IFN receptor rescued IFN-γ production from NK cells (48). Further, persistent type I IFN production can induce expression of PD-L1 ligands, which is a mechanism that can suppress NK cell antiviral function (48). Though administration of pegylated IFN-α2 therapy resulted in an increased NK cell activation, TRAIL, and CD107a receptor expression in HCV-infected individuals, there was a concomitant reduction in IFN-γ+ NK cells within the PBMC compartment (57, 58). This contradictory effect of type I IFN may stem from the timing and magnitude of type I IFN produced or a shift in transcription factor association with the type I IFN receptor. In a *listeria monocytogenes* infection, exogenous IFN-β administered at an earlier time point during infection was able to activate NK cells and promote clearance of the infection, whereas the endogenous IFN-β produced at 24 h post-infection resulted in an impaired NK cell response (59). Further, Marshall et al. found that stimulation of NK cells with supernatants from CpG-stimulated pDCs in addition to IFN-α suppressed IFN-γ release from NK cells (60). In a seminal study from Miyagi et al. they demonstrated that stimulation of NK cells with type I IFN shifted the balance of transcription factors from a STAT4 association with the type I IFN receptor, which upon phosphorylation and nuclear translocation resulted in an initial burst of IFN-γ, to a STAT1 association that subsequently led to inhibition of NK cell IFN-γ production (61). Thus, as increasing amounts of type I IFNs are released during infection, this leads to an increasing shift in association between STAT1 and IFNAR and ultimately inhibition of IFN-γ production from NK cells.

Along with promoting antiviral functions (and later limiting these very same functions), type I IFN has been shown to limit damaging immune responses that can lead to tissue pathology and collateral damage. In a model of influenza infection, absence of the type I IFN receptor resulted in significant virus-induced immunopathology. Duerr et al. demonstrated that this pathology was mediated by an upregulation of type 2 cytokines from unregulated innate lymphoid type 2 cells (ILC2s) (62). Thus, type I IFN suppresses ILC2 function during virus infection. Type I IFN was also found to suppress pro-inflammatory NOS2+ Ly6C^{lo} monocyte function (36). Moreover, type I IFN dampens recruitment of neutrophils by suppressing epithelial CXCL1 and CXCL2 production during virus infection (35, 38, 63). Not only can neutrophils produce a multitude of molecules and

proteases that can promote inflammation and tissue damage, they have been shown to instigate rhinovirus-induced asthma exacerbations in mice (64, 65). A table comparing the effects of type I IFN on the innate immune response is summarized in **Table 1**.

Unweaving the Dual Nature of Type I IFNs

Within the literature, various themes are emerging that provide an explanation for this underlying dual functionality of type I IFN. First, acute virus infections and transient type I IFN production appears to promote antiviral responses from innate immune cells, while chronic infections with persistent type I IFN signatures result in a dampened antiviral response (66). This is particularly evident in the cases of chronic LCMV, which led to deterioration of the lymphoid architecture and T-cell suppression mediated by increased PD-L1 expression on DCs (48, 49). In simian immunodeficiency virus (SIV) infection, early administration of type I IFN resulted in a reduction in viral load, while chronic administration of type I IFN resulted in an increased level of virus and CD4+ T-cell depletion (67, 68). Second, the timing and magnitude of type I IFN produced can result in differing type I IFN responses, as previously discussed.

A growing body of evidence has revealed that individual subtypes of type I IFN can have differing effects, despite signaling through the same receptor. Indeed, stimulation of DCs with different subtypes of type I IFN resulted in varying profiles of receptor expression and cytokine production (69). Additionally, pre-treatment of influenza-infected mice with the same dose of different type I IFN subtypes resulted in varying levels of virus replication, with IFN-α5 and IFN-α6 having the greatest reduction in viral load (70). Their differing affinities for the type I IFN receptor, length of receptor binding, level of type I IFN receptor expression, and innate cellular differences may underlie the ability of these type I IFN subtypes to induce different responses (71). This is outlined in greater and more elegant detail in a review by Gideon Schreiber (71). In the context of virus infection, however, we hypothesize that type I IFN acts to optimize the antiviral response by both activating and enhancing beneficial innate immune cell function, while limiting detrimental and pathological immune responses that can cause unnecessary tissue damage.

Type II IFN: An Antiviral State of Mind

IFN-γ is an important component of the innate antiviral response and is predominantly produced by NK cells or innate lymphoid type 1 cells (2, 72, 73). In the context of HSV-2 infection, absence of IFN-γ production results in increased virus replication and decreased survival (74, 75). Indeed, IFN-γ has been shown to

TABLE 1 | The role of type I IFN in regulating the antiviral innate immune response.

Cell type	Positive Regulation	Negative Regulation
DCs	T-cell activation: – Increases surface expression of CD40, CD80, CD86, OX40L, and MHC II (29, 31) – Stimulation of terminal DCs enhances MHC II and B-7 expression (32) – Sustains Ag processing and MHC II expression (30) Suppressive functions: – Chronic type I IFN stimulation increases expression of IL-10 and PD-L1 (50, 51) Differentiation: – pDC conversion into mDC (33)	Differentiation/proliferation: – Chronic type I IFN stimulation reduces BM-derived cDC differentiation and proliferation (48, 49) – Stimulation during the differentiation process inhibits CD11c, MHC-II, and B-7 expression (32)
Inflammatory monocytes	Recruitment: – Induction of CCL2 for inflammatory monocyte recruitment (2, 34, 36, 37) Differentiation: – Absence of IFNAR leads to decreased Ly6C^{hi} inflammatory monocyte differentiation and results in increased levels of Ly6C^{intermediate} monocytes (36)	Function: – Downregulation of IFNγR expression and subsequently NOS2 expression (36)
Macrophages	Function: – Upregulation of IL-10 and PD-L1 (50, 51)	Function: – Downregulation of IFNγR expression (52)
Neutrophils	No evidence of activation	Recruitment: – Suppresses CXCL1 and CXCL2 production (35, 38, 63)
NK cells	Recruitment: – Induction of CCL3, CCL4, CCL5 for NK cell recruitment (35) Activation: – Implicated in STAT-1-mediated cytotoxicity (39) – Required for IFN-γ production (2, 40–43)	Suppression of IFN-γ due to: – Chronic type I IFN (48, 57, 58) – Increased levels of type I IFN (60) – Timing of type I IFN—early release results in activation, late results in inhibition (59)
ILC2	No evidence of activation	Proliferation: – Reduces ILC2 proliferation (62) Function: – Reduces expression of IL-5, IL-6, and IL-13 (62)

induce NO production, a potent inhibitor of virus replication, from surrounding cells (72, 76). As well, IFN-γ can induce intracellular antiviral programs, including PKR, as a resultant overlap in their gene expression with type I IFNs (77). Beyond that, however, IFN-γ itself has been demonstrated to impact the function of the surrounding innate immune cells, including macrophages and DCs.

The impact of antiviral IFN-γ on antigen presenting cells (APCs) is to enhance stimulation of the adaptive antiviral response to both clear the infection and generate memory as a safe-guard for future infections (78, 79). Thus, it is a critical propellant of the Th1 response. IFN-γ stimulation enhances the antigen presentation process during T-cell priming. It has been shown to increase various aspects of antigen presentation, including efficiency, quantity, quality, and diversity of peptides being loaded into the MHC I receptor (80). Along with MHC I, IFN-γ increases MHC II expression and maturation of DCs (81). Further, it induces the expression of IL-12 and co-stimulatory CD80 in antigen-presenting cells, which is a critical component of Th1 polarization (82–84).

With respect to macrophages, IFN-γ induced NO production from these cells not only inhibits virus replication, but also potently vasodilates blood vessels to decrease blood flow and allow for increased extravasation of recruited immune cells to the site of infection and inflammation (80). Further, IFN-γ has been shown to "prime" macrophages to release reactive oxygen species, through the upregulation of cellular components required for this function (85). IFN-γ also appears to increase macrophage receptor-mediated phagocytosis through the upregulation of complement receptors, though this has been observed more so in bacterial infections, rather than viral (86). Further, IFN-γ promotes polarization of macrophages to an M1 phenotype and primes these cells to produce pro-inflammatory cytokines IL-12, TNF-α, and IL-1β (87, 88).

Type II IFN Negative Regulation: An Emerging Role

IFN-γ has many overlapping features with type I IFNs, including suppression of type 2 immune responses and inhibition of proliferation. In the context of virus infection, however, we believe that IFN-γ released during the innate immune response has more of a supportive role in this respect as it is less potent in its effects in comparison to type I IFNs. Aside from type I IFN, IFN-γ has a number of immunoregulatory functions that serve to optimize the antiviral response and limit overzealous responses that could lead to collateral damage.

An optimal antiviral response involves both activating beneficial immune responses, while simultaneously inhibiting impractical and potentially damaging responses. In the context of virus infection, IFN-γ is a prototypical Th1 promoting cytokine. Further, evidence from Kang et al. demonstrates that IFN-γ plays a critical role in not only polarizing macrophages to an M1 phenotype, but actively suppresses the M2 polarization pathway (87). However, recent evidence has revealed that type I IFN is capable of suppressing type 2 immunity. Independently, both Duerr et al. and Moro et al. demonstrated that, similar to type I IFN, IFN-γ is able to suppress ILC2 proliferation and type 2 cytokine production (62, 89). Indeed, *in vivo* administration of IFN-γ potently suppressed IL-33-induced ILC2 proliferation, which was dependent upon STAT1 signaling (62). In the context of RSV infection, *Stat1*$^{-/-}$ mice, a transcription factor downstream of both type I and type II IFNs, led to increased lung pathology because of increased cytokine production from

TABLE 2 | The role of IFN-γ in regulating the antiviral innate immune response.

Cell type	Positive Regulation	Negative Regulation
APCs	T-cell activation: – Promotes DC maturation (81) – Increases MHC I and MHC II expression (80, 81) – Enhances efficiency, quantity, quality, and diversity of MHC I Ag-loading (30) – Increases expression of IL-2 and CD80 (82–84)	No evidence of negative regulation
Macrophages	Function: – Induces NO production (80) – Primes macrophages for ROS release (85) – Increases phagocytosis (86) – Polarization to M1 phenotype (87, 88)	No evidence of negative regulation
Neutrophils	Function: – Increases PD-L1 expression (93)	No evidence of negative regulation
MDSC	Function: – Upregulation of PD-L1 (92) Differentiation: – Enhances differentiation of MDSCs (92)	No evidence of negative regulation
ILC2	No evidence of activation	Proliferation: – Reduced ILC2 proliferation (62, 89) Function: – Reduced expression of IL-5, IL-6, and IL-13 (62, 89) – Reduced expression of amphiregulin (89)

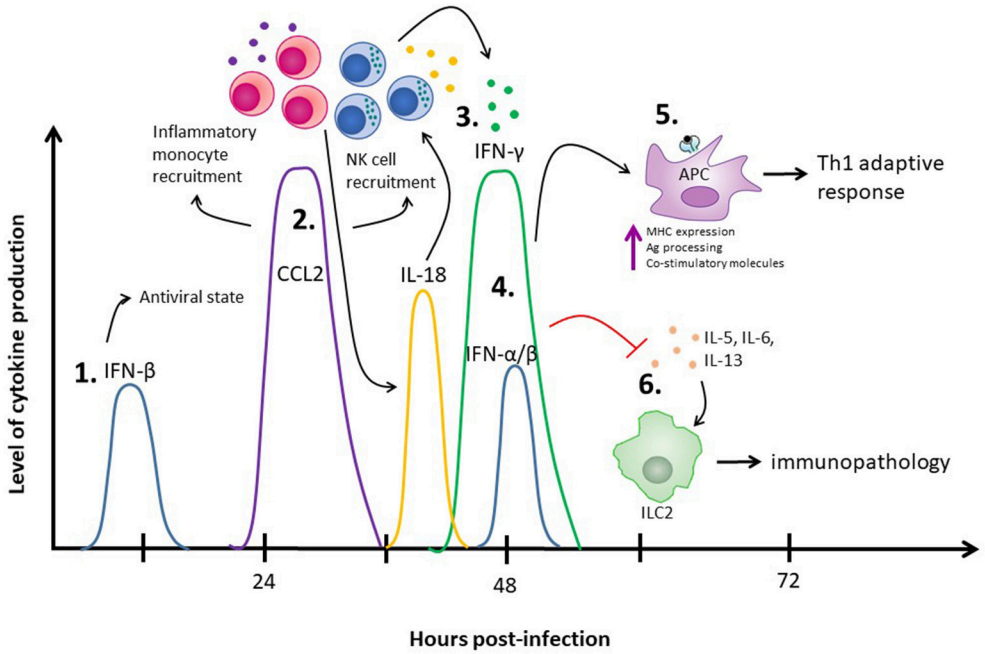

FIGURE 1 | The role of IFNs in the innate immune response to HSV-2 infection. (1) IFN-β is produced at 12 h post-infection and through autocrine and paracrine signaling places surrounding cells into an antiviral state. (2) The IFN-β produced at 12 h post-infection also increases production of CCL2 between days 1 and 2 post-infection, which results in inflammatory monocyte recruitment and has been implicated in NK cell recruitment. (3) The recruited inflammatory monocytes result in release of IL-18, which stimulates NK cells to produce IFN-γ at 48 h post-infection. (4) A second wave of type I IFNs, including both IFN-α and IFN-β, are detected at 48 h post-infection. (5) Both IFN-γ and the type I IFNs produced at 48 h post-infection enhance APC antigen presentation capacities to stimulate a Th1 adaptive immune response. (6) Simultaneously, the type I IFNs at 48 h inhibit ILC2-mediated virus-induced immunopathology. IFN-γ, supporting the negative regulatory effects of type I IFN, also suppresses ILC2-mediated immunopathology.

ILC2s and ILC3s (90). Further, in a mouse model of influenza infection, administration of IFN-γ suppressed ILC2 function while deficiency of IFN-γ led to increased IL-5 and amphiregulin release from ILC2s. These authors ultimately found that the suppressive effects of IFN-γ on ILC2 function led to increased lung pathology (91).

Along with dampening immune responses, there is evidence demonstrating that IFN-γ can indirectly induce immunoregulatory effects through the upregulation of PD-L1 and differentiation of myeloid derived suppressive cells. In conjunction with GM-CSF, IFN-γ was shown to differentiate monocytes into myeloid derived suppressor cells (MDSCs) *in vitro* (92). In a mouse model of endotoxemia, IFN-γ has also been shown to upregulate PD-L1 on neutrophils (93). A table comparing the effects of IFN-γ on the innate immune response is summarized in **Table 2**.

Understanding the Dual Nature of IFN-γ: Unraveling the Paradox

Similar to type I IFNs, IFN-γ has both seemingly paradoxical activating and suppressive functions on the innate antiviral response. These functions can be teased apart if we examine the cell type that IFN-γ is acting upon and bring other cytokines into the picture with IFN-γ. If we consider macrophages, IFN-γ has complementary effects on inducing an antiviral macrophage function. IFN-γ induces NO production, enhances macrophage antigen presenting function, and an overall M1 phenotype while actively suppressing the M2 phenotype (72, 80, 87). Similar to macrophages, IFN-γ predominantly increases antigen presentation function of DCs. Further, IFN-γ has a predominantly suppressive effect on ILC2 cells (62).

IFN-γ as a cytokine rarely acts alone and its effects should be considered in conjunction with other cytokines present in the local microenvironment. The combinatorial effect between IFN-γ and other cytokines likely plays a role in the ultimate outcome of IFN-γ stimulation. Indeed, both IFN-γ and TNF-α have been shown to synergize in the upregulation of iNOS in macrophages. Salim et al. used mathematical modeling to dissect out the roles of each cytokine and found that TNF-α was largely responsible for the timing of iNOS induction by inducing a rapid response, whereas IFN-γ impacted the levels and concentrations of NO production (94). Further, the role of IFN-γ in the *in vitro* differentiation process of MDSCs required an initial priming with GM-CSF. Ribechini et al. found that GM-CSF altered the signaling pathway of IFN-γ allowing it to differentiate monocytes into MDSCs (92). In a recent article by Zha et al. they found that IFN-γ was able to suppress the functions of gp130 cytokines, particularly the ability of OSM, to differentiate mesenchymal stem cells through the upregulation of STAT1, concomitant decrease in STAT3 activation, and internalization of the gp130 receptor (95). Thus, IFN-γ can both be altered by additional cytokine

signaling as well as regulate the signaling pathways of other cytokines.

Putting the Pieces of the Puzzle Together

As we start to put the pieces of this type I and type II IFN puzzle together, we can see that these two cytokines act in concert with one another to limit virus replication and encourage an antiviral adaptive immune response while suppressing detrimental functions of other immune cells to limit tissue pathology. Using vaginal HSV-2 infection as an example, we find that there are multiple waves of type I IFN production, starting with IFN-β at 12 h post-infection (20). This early wave of IFN-β is likely responsible for the induction of MCP-1-mediated inflammatory monocyte recruitment, ultimately leading to IL-18-induced NK cell IFN-γ production (2). From there, we've observed a second wave of type I IFN production, both IFN-α and IFN-β, at 48 h post-infection (19). Along with type I IFN, there's a sharp increase in IFN-γ from NK cells at 48 h post-infection (16). The IFN-γ released from NK cells is also negatively regulated by type I IFN, as NK cells lacking IFNAR have increased IFN-γ production in the context of HSV-2 infection (2). This second wave of type I and II IFNs likely work in concert with each other to promote APC maturation, upregulation of co-stimulatory molecules, and antigen processing and presentation to promote Th1 polarization, while simultaneously suppressing ILC2-mediated immunopathology (**Figure 1**).

Without type I IFN, and potentially type II IFN, there is uncontrolled virus replication coupled with uncontrolled inflammation that work together to cause tissue demise. On the other hand, a chronic type I IFN signature is detrimental as it can result in immunosuppression and increased virus replication. Thus, we believe a *balanced and appropriate* type I IFN response is required to regulate an optimal and advantageous antiviral innate immune response.

Clinical Implications: Going Beyond Infection

While the focus of this review has been on type I and II IFNs and their ability to control the innate immune response, IFNs have been implicated in several non-infectious pathological conditions. Select autoimmune diseases, the most prominent being systemic erythematous lupus (SLE), have high type I IFN signatures associated with their pathology (96). An antibody targeting human IFNAR has recently been developed to block this signature with therapeutic benefit (97). On the other hand, IFN-β therapy has had success in treating multiple sclerosis (98). Indeed, the concepts discussed in this review are relevant in the context of pharmacotherapies targeting the type I and type II IFN pathways. This begs the question: what is the role of type I IFN outside of virus infection? A growing amount of evidence has shown that type I IFN production is not isolated to infectious disease stimuli, it can be produced during any inflammatory insult. Thus, our fundamental understanding of the innate immune response during virus infection has an underlying application to many disease processes, beyond virus infection.

AUTHOR CONTRIBUTIONS

AL generated themes and ideas and wrote the manuscript. AA guided and edited the manuscript.

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Tolerating the Unwelcome Guest; How the Host Withstands Persistent *Mycobacterium tuberculosis*

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Our understanding of the host response to infections has historically focused on "resistance" mechanisms that directly control pathogen replication. However, both pathogen effectors and antimicrobial immune pathways have the capacity to damage host tissue, and the ability to tolerate these insults can also be critical for host survival. These "tolerance" mechanisms may be equally as important as resistance to prevent disease in the context of a persistent infection, such as tuberculosis, when resistance mechanisms are ineffective and the pathogen persists in the tissue for long periods. Host tolerance encompasses a wide range of strategies, many of which involve regulation of the inflammatory response. Here we will examine general strategies used by macrophages and T cells to promote tolerance in the context of tuberculosis, and focus on pathways, such as regulation of inflammasome activation, that are emerging as common mediators of tolerance.

Keywords: *Mycobaterium tuberculosis*, tolerance, inflammasome, immunometabolism, persistent infections

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INTRODUCTION

The ultimate goal of the host response to bacterial pathogens is to survive the infection. Much of the research to understand protective immunity has historically had a singular focus on antimicrobial resistance mechanisms that directly control bacterial replication. In general, these "resistance" mechanisms act by poisoning the pathogen, disrupting the pathogen's niche, or sequestering nutrients in an attempt to restrict growth and spread (1, 2). Classic resistance pathways include the antimicrobial peptide production from epithelial surfaces and the microbicidal functions of phagocytes which are augmented by antigen-specific lymphocyte responses. More recently it has become clear that in addition to these resistance strategies, the host also relies on distinct mechanisms that allow it to withstand infections independently of controlling bacterial growth (3, 4). These "tolerance" mechanisms represent host pathways that modulate diverse aspects of physiology. Both the local control of inflammatory tissue damage and repair, as well as systemic responses such as anorexia, and fever have been shown to promote host survival in a number of infection contexts (5, 6). For many self-resolving infections, resistance mechanisms may be sufficient to restrict bacterial replication and minimize pathology (2). However, some pathogens, like *Mycobacterium tuberculosis* (*Mtb*), are able to resist many of the resistance mechanisms of the host and persist for long periods (7). In these situations, tolerance pathways are critical for preventing the progressive pathology elicited by the persistent presence of the pathogen. Tolerance responses ensure that the locally infected tissues continue to function and that the overall health of the host is maintained (3, 8).

While potential therapies that promote host resistance have received a great deal of interest, promoting tolerance pathways that decrease morbidity and/or mortality in the face of an ongoing chronic infection could represent an equally appealing avenue for intervention (9, 10). In this review, we will discuss the host response to *Mtb* infections from the viewpoint of host tolerance. While tolerance encompasses a potentially large array of host functions, we will consider known and emerging mechanisms that limit lung damage and discuss how distinct cell populations like macrophages and T cells contribute to tolerance by controlling cytokine production and metabolic functions. Ultimately, understanding host tolerance mechanisms will define new pathways of protective immunity to tuberculosis (TB), and could identify new therapeutic strategies.

Tuberculosis Pathogenesis

Mtb infections are transmitted by aerosol (7, 11). Following inhalation of contaminated droplets, *Mtb* is engulfed by alveolar macrophages, where the pathogen replicates and evades the innate antimicrobial mechanisms of this cell (7, 11, 12). After the activation of host adaptive immune responses, bacterial growth is slowed or halted. While evidence from non-human primates (NHP) and human autopsy studies indicate that some infectious foci can be sterilized, the pathogen is able to persist in the face of this adaptive response for long periods. In some individuals, this infection produces the chronic inflammatory disease called, tuberculosis (TB). While any organ in the body can be affected, pulmonary disease promotes transmission of the pathogen, beginning a new infectious cycle.

For most individuals, chronic infection with *Mtb* does not produce symptomatic disease (7, 13). However, a subset of individuals (5–10%) will progress to develop TB after a period of asymptomatic infection that generally lasts for less than 2 years, but can extend for decades in rare cases (14, 15). What drives the heterogeneity of disease progression is not entirely known and is likely a combination of host and bacterial genetic diversity, as well as environmental factors (3, 8, 16). Several distinct aspects of TB pathogenesis could be affected by host tolerance pathways. Most obviously, the risk of developing disease is likely to depend on host tolerance. Most infected individuals never develop symptoms, and the ability to harbor this immunogenic pathogen for long-periods without suffering from progressive pathology likely depends on the ability to control inflammation(10, 17, 18). In fact, the phenomenon of "latent TB infection" (LTBI) could be considered one of the clearer examples of pathogen tolerance in humans. Patients that are cured of TB by antibiotic therapy suffer from reduced respiratory function, indicating that even after bacteria are eradicated, local tissue damage persists (19–21). In fact, multiple rounds of infection and antibiotic therapy are associated with increased erosion of lung function (21). This effect is not simply additive, as rabbits exposed to 5 sequential low dose infections developed significantly more severe cavitary disease than animals exposed to a single large dose of *Mtb* (22). Thus, tolerance mechanisms that control local tissue damage could determine long-term outcome and are influenced by environmental factors such as the frequency of infection. Manifestations of *Mtb* other than pulmonary disease may be even more dependent on host tolerance mechanisms that control inflammation (23). For example, meningeal *Mtb* infection is associated with very high mortality, which is related to the expression of inflammatory cytokines (24, 25). Similarly, TB immune reconstitution inflammatory syndrome (TB-IRIS) is a condition that occurs in HIV/*Mtb* co-infected individuals soon after starting antiretroviral therapy (26). This syndrome still results in almost 40% mortality, and is associated with failed regulation of inflammatory cascades (27–30).

The mechanisms that control TB tolerance are complex because interactions between multiple cell types influence disease progression. Following infection and activation of the host immunity, infected cells are walled off in large structures termed a granuloma (7, 16). Granulomas are thought to be required for the host to tolerate *Mtb* infections, yet their development and progression throughout infection may also drive *Mtb* survival and transmission. Bacterial barcoding and PET-CT studies in non-human primates have shown individual granuloma that are formed from single founder bacteria can have very distinct fates, some contain the pathogen and while others progressively develop into the large cavities that typify pulmonary TB disease (16, 31, 32). As a result, individual lesions are variable in their disease trajectories and transmission potential suggesting complicated dynamics determine the outcome of each lesion (31, 32). Beyond granuloma development, influx of leukocytes such as neutrophils and the expression of proteases such as matrix metalloproteinases (MMPs) can reduce host tolerance by irreversibly damaging tissue (33, 34). As the role of MMPs and neutrophils in modulating immunopathology to *Mtb* have been reviewed elsewhere, we will focus on how macrophages and T cells modulate host tolerance to determine the outcome of *Mtb* infections (35, 36).

Macrophages and Tolerance

Macrophages are an important intracellular niche for *Mtb* to replicate yet they can also restrict *Mtb* growth in an activation dependent manner (12). The balance between *Mtb* replication and control is determined by a diverse array of resistance pathways, including those activated by interferon-γ (IFNγ), granulocyte-macropahge colony stimulating factor (GM-CSF) and interleukin-1β (IL-1β) (37–39). Due to their direct interactions with *Mtb*, macrophages are also central regulators of host tolerance. Several lines of evidence suggest that tolerance mechanisms modulated by macrophages may play a significant role in determining disease progression and controlling the outcome to *Mtb* disease.

Nitric Oxide

One compelling case for the role of tolerance in macrophages during chronic *Mtb* infections is that of inducible nitric oxide synthase (Nos2). For years, it was generally presumed that the protective function of Nos2 could be attributed to the direct antimicrobial activity of nitric oxide (NO) (40). In support of this hypothesis was data that showed that Nos2 deficient mice are extremely susceptible to *Mtb* infection (40, 41). These animals die within 2 months of infection with 10–100 fold more bacteria in lungs than wild type animals as well as a massive infiltration of

tissue-damaging neutrophils. Recent evidence however suggests that the situation is more complex. Mtb expresses a number of defense mechanisms that protect the pathogen from the antimicrobial effects of NO, and recent evidence suggests that the role of Nos2 in regulating inflammatory pathways and host tolerance play a dominant role in protection (**Figure 1**) (42–45).

Disentangling tolerance pathways *in vivo* is a significant challenge due to the interlinked nature of bacterial load and tissue damage; higher bacterial burdens can lead to more inflammation and tissue damage, while higher tissue damage and inflammation may create an environment that drives more bacterial replication (8). The role of each host effector in controlling resistance or tolerance pathways may also be timing and context dependent (46). It is also likely that many pathways control both resistance and tolerance during persistent infection (47). Because of this, distinct *in vivo* models that control either inflammation or bacterial replication are required to break down the mechanisms of a particular "protective" gene like *Nos2*. When these models were applied to Nos2, it became clear in Nos2 deficient animals succumb to *Mtb* infection through hyperinflammatory disease, even when bacterial load is controlled using a conditionally-replicating strain of the pathogen (43, 44). Subsequent mechanistic studies determined that NO nitrosylates the inflammasome component NLRP3, which inhibits the production of bioactive IL-1β and prevents persistent neutrophil recruitment (44). Similarly, Nos2 has also been shown to dampen the inflammatory response by limiting the activation of NF-κβ (48).

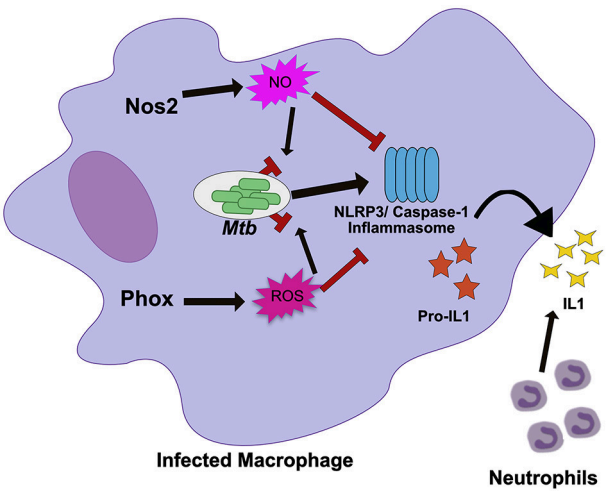

FIGURE 1 | Nos2 and Phox control tolerance during Mtb infection by negatively regulating inflammasome activation. During *Mtb* infection in macrophages, Nos2 and Phox produce NO and ROS respectively. While these molecules are antimicrobial against many pathogens, *Mtb* is mostly resistant. Persistent *Mtb* then activates the NLRP3 inflammasome to produce active IL1β. Prolonged inflammasome activation leads to increased IL1β secretion and neutrophil recruitment that damages the lungs. In order to tolerate persistent infections with *Mtb*, the NO and ROS produced by macrophages also suppresses inflammasome activation to limit the damage caused by recurring neutrophil recruitment. NO directly nitrosylates NLRP3 while the mechanisms of ROS inhibition remain unknown.

Nos2 serves as an important example of the need to understand the mechanisms by which individual immune effectors protect against TB disease progression. While a modest role for Nos2 in modulating *Mtb* replication in macrophages remains possible, the recent evidence strongly suggests the predominant role of NO production in mice during *Mtb* is to control tolerance by dampening inflammatory pathways.

NADPH Phagocyte Oxidase

Many immune mediators have similarly pleiotropic effects as Nos2, raising the possibility that other well-characterized pathways may also play unanticipated roles in regulating tolerance. The NADPH Phagocyte Oxidase Complex (Phox) provides another example. This system is required to produce a burst of reactive oxygen species (ROS) that intoxicate the intracellular bacteria. The importance of Phox in protecting the host during *Mtb* infections is generally considered minimal because Phox deficient animals show no long-term defects in controlling *Mtb* growth and *Mtb* is equipped with many strategies to resist ROS-mediated killing (41, 49–51). However, human studies suggest that mutations in Phox, which leads to the condition known as chronic granulomatous disease, are associated with higher susceptibility to mycobacterial infections including TB (52, 53). In other disease contexts Phox deficiencies have been found cause inflammatory disease, particularly those related to IL-1β activation (54). Recent work shows that Phox is also critical for tolerance to *Mtb* infection (**Figure 1**) (55). Phox-deficient mice have no deficiency in bacterial control, yet Phox-deficient animals accumulate high numbers of neutrophils in an IL-1β dependent fashion, leading to exacerbated disease (55). Similar to the role of Nos2, the ROS produced by Phox control tolerance by inhibiting the activation of the NLRP3 inflammasome which reduces IL-1β production and limits neutrophil influx to the infected lung. The fact that the important tolerance-regulating functions for both Nos2 and Phox were overlooked for some time, suggests that tolerance-regulating roles may still be found for additional host response pathways.

The similar ability of Nos2 and Phox to control inflammasome activation suggests that preventing persistent IL-1β production is a common strategy used by the host to tolerate persistent infections. In support of this, human studies have found that altered IL-1β expression modulates TB disease severity (56). IL-1β alleles that enhance IL-1β expression are associated with increased risk of developing TB disease, more severe pulmonary disease, and poor treatment outcome (56). In addition, inflammasome activation is associated with the development of TB-IRIS and TB meningitis (57, 58). Two recent studies suggest that expression and activation of inflammasome components including NLRP3 and the high expression of IL-1β in plasma and the nervous system are signatures of failed tolerance during antiretroviral treatment and a major risk factor to developing fatal disease (57, 58). The repeated association with inflammasome activity, IL-1β production and more severe TB-related pathology suggests that this pathway could serve as a therapeutic target, particularly for the severe inflammatory syndromes with poor outcomes.

Lysosomal Function and Autophagy

Proper maintenance of cellular organelles is important to tolerate *Mtb* infections (59–62). Loss of critical homeostatic pathways can lead to cellular dysfunction and misregulation of inflammatory cytokines during *Mtb* disease. Mycobacterium infections of zebrafish with mutations in cathepsins leads to loss of granuloma integrity and reduced survival due to improper breakdown in lysosomal contents (59). In humans, this mutation is phenocopied in individuals who smoke tobacco. *Mtb* infected macrophages from smokers accumulate particulates in their lysosomes, inhibiting their function and likely altering tolerance. It is well known that previous smoking history can increase the risk of developing TB disease by over two-fold and it is possible that alterations to lysosomal function are a key aspect to these patients TB susceptibility (63).

Autophagy is another key pathway that maintains the integrity of organelles and regulates a variety of important immune-related processes (64). Recently, the role of autophagy in antimicrobial resistance during *Mtb* has been questioned but the importance of Atg5 in tolerance is undeniable (61, 65, 66). Mice with mutations in most autophagy genes control *Mtb* disease normally (61). However, Atg5-/- mice show a unique susceptibility to TB disease. Infection of Atg5 mice leads to a hyperinflammatory disease state with massive neutrophil migration to the pulmonary tissue and rapid mortality (61). Depletion of neutrophils alone in infected Atg5 deficient mice can reverse the susceptibility and allow long term survival arguing against an inherent defect in antimicrobial control. Exactly how Atg5 controls the inflammatory response, or why loss of Atg5 and not other autophagy components drives neutrophil-mediated disease remains to be understood. But it is clear that altering macrophage homeostasis directly modulates tolerance to *Mtb*.

Macrophage Metabolism

Recent evidence suggests that macrophage metabolic pathways and byproducts can modulate the inflammatory pathways both locally and systemically (1, 2). Similarly, *Mtb* infections are influenced by systemic metabolic dysfunction such as diabetes, which can alter the activation state of macrophages at the site of infection (67). Evidence for how essential local and systemic metabolic networks influence host tolerance to *Mtb* is beginning to emerge.

Central regulators of host cell metabolism are intimately linked with control of inflammatory circuits (68). These pathways, including mammalian target of rapamycin (mTOR), silent mating type information regulation 2 homologs (Sirtuins), and adenosine monophosphate-activated protein kinase (AMPK), are known to regulate cellular functions such as autophagy, NF-kb signaling, and central metabolism. Importantly, many of these networks are disrupted during *Mtb* infection suggesting that they could play a role in regulating the inflammatory milieu that is activated during *Mtb* infection and likely influence host tolerance (69). Because FDA approved modulators of these metabolic networks are available, they represent appealing targets for host directed therapies that may enhance tolerance during *Mtb* infections and improve clinical outcomes (70).

Sirtuin 1 (SIRT1), a known regulator of host stress responses, is downregulated during *Mtb* infection (71). In order to understand how the loss of SIRT1 function impacts *Mtb* disease, Singhal and colleagues treated infected macrophages and animals with a known small molecule SIRT1 activator (**Figure 2**) (71). While activation of SIRT1 resulted in a modest reduction in bacterial growth *in vitro* and *in vivo*, it led to dramatic changes in the inflammatory profile of infected macrophages and immunopathology in mice, indicating that activation of SIRT1 promotes host tolerance during *Mtb* infection. Interestingly, SIRT1 activation during *Mtb* results in similar outcomes to treatment with the AMPK activator metformin, a common treatment for diabetes (72). During *Mtb* infection, metformin treatment leads to subtle decreases in bacterial burden but larger decreases in inflammatory cytokines and tissue damage (**Figure 2**). A retrospective study of diabetic TB patients indicates that metformin may improve outcomes. SIRT1 can also influence AMPK signaling, suggesting that the SIRT1/AMPK signaling axis may be a critical regulator of tolerance during *Mtb* infection. It is also intriguing that diabetes treatments such as metformin, are so effective against treating *Mtb* disease. Diabetes increases *Mtb* risk in humans (73, 74). In a mouse model of hyperglycemia, there was a profound effect on neutrophil accumulation during *Mtb* infection which worsened disease outcome (75). Thus, while it likely the complex effect of diabetes on immunity could include resistance defects, in the mouse model tolerance defects appear to dominate.

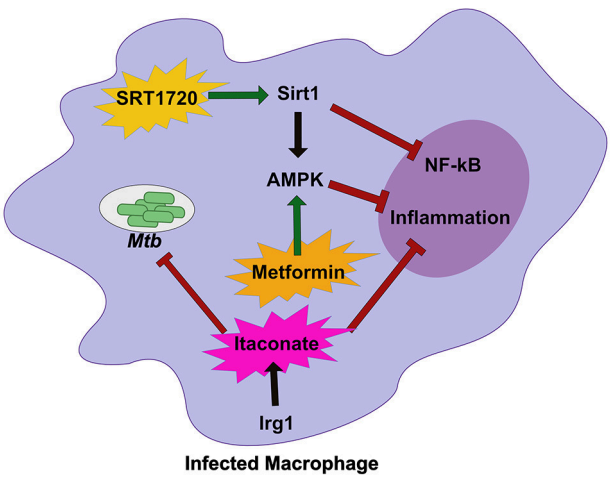

FIGURE 2 | Host metabolic networks modulate tolerance to Mtb infections. Distinct metabolic networks control the inflammatory response during *Mtb* infection. Small molecule activation of Sirt1 with SRT1720 inhibits NF-κβ signaling and activates AMPK and promote tolerance to *Mtb*. This is similar to treatment with the diabetes drug Metformin that activates AMPK to inhibit inflammation and allow the host to better tolerate persistent *Mtb* infections. An alternative metabolic network activated by Irg1, produces the metabolite Itaconate. Itaconate can directly restrict *Mtb* replication, but *in vivo* robustly controls tolerance by modulating the inflammatory response to persistent infection. Together these metabolic networks directly and indirectly control tolerance to *Mtb* infection.

Another important metabolic pathway that modulates tolerance to *Mtb* is mediated by the mitochondrial enzyme immune responsive gene 1 (Irg1) (76). Irg1 produces the metabolite itaconate that recently was shown to dampen cytokine production and reduce damaging ROS during *Mtb* infection (**Figure 2**) (77). Loss of Irg1 *in vivo* leads to rapid mortality that is driven by hyper-inflammation and neutrophil-mediated disease. Itaconate alone is sufficient to reverse the increase in pro-inflammatory gene expression in infected Irg1 deficient macrophages suggesting this metabolite is a robust regulator of tolerance to *Mtb*. While itaconate can directly inhibit bacterial growth, *in vivo* studies indicate that its immunomodulatory function may play a dominant role (76, 77). Future studies will need to carefully dissect the role of Irg1 in both controlling resistance and tolerance to fully understand its pleiotropic functions during *Mtb* infection.

T Cells and Tolerance

T cells are critical for resistance to *Mtb* (7, 78). In addition, it is clear that Th1 cells that produce IFNγ promote tolerance by activating the production of NO and by directly inhibiting the recruitment of neutrophils (44, 79). This profound effect on *Mtb* protection suggested that more robust activation of Th1 cells would lead to improved disease outcomes. In reality, the situation is much more complicated and recent evidence suggests that activating enhanced Th1 responses to *Mtb* leads to increased susceptibility through failed tolerance.

IFNγ. The cytokine IFNγ is produced by activated T cells during *Mtb* infection and is essential for protection of the host. During chronic infections, the levels of IFNγ produced by individual T cells can wain due to persistent antigen and T cell exhaustion (80). Targeting inhibitory receptors on T cells might drive enhanced cytokine responses and lead to more robust *Mtb* control. As a proof of principle of this concept mice lacking the T cell inhibitory receptor PD1 were infected with *Mtb* (81). Surprisingly, rather controlling *Mtb* infection better, PD1 deficient animals had decreased tolerance that was characterized by increased susceptibility and immunopathology. This counterintuitive result suggested that more robust T cell responses might be detrimental to long term *Mtb* protection. What is driving the decrease in tolerance in these animals? One recent study began to examine the mechanisms modulating the tolerance defect in PD1 deficient mice and showed that increased IFNγ production is responsible (82). When PD1 deficient T cells no longer make IFNγ, the defect in tolerance is reversed. In addition, $CD4^+$ T cells that produce more IFNγ on a per cell basis do not control *Mtb* growth more effectively in the lungs, but rather cause tissue damage and more rapid mortality. Similarly, T cells with mutations in the Calcium channel ORAI1 activating protein Stim1 are unable to undergo apoptosis following infection leading to a significant increase in IFNγ in the lungs (83). This increase in T cell survival and IFNγ makes infected mice susceptible to infection by decreasing tolerance. Therefore, during *Mtb* infection pushing the expression of IFNγ beyond a protective threshold leads to failed tolerance.

T cell Metabolism. While it is possible that results with PD1 are an outlier additional evidence suggests that other alterations to T cell activation may have deleterious effects on tolerating *Mtb*. One recent study found an important role for Cyclophilin D in modulating tolerance to *Mtb* in a T cell dependent manner (84). CyclophilinD (CypD) is a mitochondrial protein that modulates cell death mechanisms such as necrosis (85). Inhibition of CypD in macrophages prevents necrosis and limits *Mtb* replication (86, 87). On this basis, Divangahi and colleagues infected CypD mice, and found that they were highly susceptible to infection (84). However, these mice succumbed to disease with identical burdens of bacteria compared to wild type animals suggesting loss of CypD decreases tolerance to persistent *Mtb* infections. Importantly, the defect in tolerance was not related to differences in cell death and control of *Mtb* replication. Instead CypD was found to regulate a metabolic switch between oxidative phosphorylation and glycolysis in T cells. In the absence of CypD, T cells produced more ROS that drove glycolytic flux, leading to enhanced activation and cytokine production. This critical change in the central metabolism of T cells dramatically reduced the tolerance CypD animals to persistent *Mtb* infection.

Taken together the findings that increasing T cell numbers and enhancing their function in the lungs of *Mtb* infected animals reduces tolerance is compelling. We can no longer pursue the development of therapeutics or vaccines that simply drive more activated T cells and more IFNγ production without considering the very real possibility of deleterious effects. Mammalian hosts have clearly evolved an important balance between antimicrobial resistance strategies and tolerance mechanisms to survive persistent infections that must be more adequately evaluated in our research as we pursue more effective *Mtb* treatment strategies.

Outlook

The studies discussed above suggest a critical role for the regulation of inflammatory cascades in tolerance to persistent Mtb infection, and highlights a number of well-studied pathways in this process. It seems clear that macrophages integrate metabolic and innate immune signals with those derived from T cells to control the extent of inflammatory tissue damage. While these pathways are important determinants of disease progression, they likely represent a small fraction of the mechanisms that contribute to tolerance. Our current understanding of TB tolerance is focused largely on immunological factors with an already appreciated protective role in the mouse model of TB. However, in simpler model systems, it is clear that a wide variety of functions involved in tissue repair, systemic metabolism, and energy utilization also play an important role. Furthermore, it is clear that bacterial factors interact with the immune system to regulate tolerance, and a number of Mtb genes have been found to alter immunopathology without affecting bacterial fitness (88, 89). Developing models for TB where these diverse tolerance pathways can be observed and dissected represents a major challenge for the future.

While our understanding of tolerance generally lags far behind our knowledge of resistance mechanisms, the examples

described above highlight the importance of continued research. While antibiotics are generally effective for uncomplicated Mtb infections, several particularly serious and/or long-term sequelae of Mtb infection can be attributed to defects in tolerance. These complications include acute failures of tolerance, such as meningitis and TB-IRIS, as well as the long-term tissue damage and decreased lung function that generally follows infection. Understanding the processes involved in damage and repair will likely produce more effective therapies.

AUTHOR CONTRIBUTIONS

AO and CS conceived of and wrote the article together.

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REVIEW
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Going to Bat(s) for Studies of Disease Tolerance

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A majority of viruses that have caused recent epidemics with high lethality rates in people, are zoonoses originating from wildlife. Among them are filoviruses (e.g., Marburg, Ebola), coronaviruses (e.g., SARS, MERS), henipaviruses (e.g., Hendra, Nipah) which share the common features that they are all RNA viruses, and that a dysregulated immune response is an important contributor to the tissue damage and hence pathogenicity that results from infection in humans. Intriguingly, these viruses also all originate from bat reservoirs. Bats have been shown to have a greater mean viral richness than predicted by their phylogenetic distance from humans, their geographic range, or their presence in urban areas, suggesting other traits must explain why bats harbor a greater number of zoonotic viruses than other mammals. Bats are highly unusual among mammals in other ways as well. Not only are they the only mammals capable of powered flight, they have extraordinarily long life spans, with little detectable increases in mortality or senescence until high ages. Their physiology likely impacted their history of pathogen exposure and necessitated adaptations that may have also affected immune signaling pathways. Do our life history traits make us susceptible to generating damaging immune responses to RNA viruses or does the physiology of bats make them particularly tolerant or resistant? Understanding what immune mechanisms enable bats to coexist with RNA viruses may provide critical fundamental insights into how to achieve greater resilience in humans.

Keywords: bats (Chiroptera), viral immunology, host pathogen interaction, disease tolerance, comparative genome analyses, innate immunity

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INTRODUCTION

An estimated ~60% of emerging infectious diseases are caused by pathogens which originate from a non-human animal source, referred to as zoonoses (1–3). Moreover, the frequency of outbreaks caused by zoonotic pathogens has been increasing over time in the human population, with viruses being the most successful at crossing the species barrier (2–4). Given the impact of viral zoonoses on global public health, considerable resources have been invested into better understanding patterns in their emergence to improve predictions of where they might arise. One key variable in such predictions is to determine the animal reservoir populations within which these novel viruses can be maintained indefinitely (with or without disease) and which therefore act as sources for transmission to humans (5). In some instances, epidemiological associations may provide clues to identifying a reservoir host species, and the detection of natural infection through seroconversion

or the virus itself provides further evidence. Recently, phylogenetic analyses have also been used to investigate viral origins—with a presence of greater diversity and of strains ancestral to those in humans being indicative of a virus circulating within a particular natural host population (6).

Once identified, viral reservoirs have historically been critical levers through which to reduce human cases (5). However, reservoir hosts may also provide us with fundamental insights into host-pathogen interactions and are a rich opportunity to examine the immunological processes that contribute to patterns governing which pathogens cross into humans, cause disease and why (7, 8). This can be particularly informative as in many instances, the zoonotic viruses that are so pathogenic in humans do not cause disease in the reservoirs with which they coexist.

BATS ARE THE RESERVOIRS FOR MANY HUMAN VIRUSES

Bats have been confirmed as reservoir hosts for many viruses, several of which are associated with fatality rates as high as 90% among diagnosed human cases. It has long been appreciated that rabies and other lyssaviruses causing lethal encephalitis can be transmitted from numerous bat species (9, 10). Live Marburg virus (MARV) has been isolated from *Rousettus aegyptiacus* fruit bats which, jointly with epidemiologic evidence and detection of viral RNA, strongly suggests that *R. aegyptiacus* is a reservoir host of this filovirus (11). The related ebolavirus (EBOV) likely also circulates in African fruit bats, with a few species having been implicated so far—the mobility of which accounts for the sudden appearance of Ebola in West Africa during the 2014 outbreak, a region where ebolavirus had not previously been detected (12, 13). The highly pathogenic henipaviruses, of which Hendra virus emerged in Australia and Nipah virus in South-east Asia via horse and pig intermediate hosts respectively, have been shown to be transmitted from *Pteropus* bats (14, 15). In China, horseshoe *Rhinolophus* bats have been identified as the reservoirs for SARS coronavirus via palm civet intermediate hosts, the cause of a large outbreak of atypical pneumonia across several countries that began in 2002 in China (16–18). More recently, MERS coronavirus that has caused lethal respiratory infections mostly in Saudi Arabia, likely transmitted via dromedary camels, was shown to be closely related to several bat coronaviruses, including those sequenced from *Neoromicia capensis*, *Pipistrellus abramus,* and *Vespertilio superans* bats (19, 20). Moreover, additional viruses may continue to emerge from bats, as in the single case of sosuga virus infection in a wildlife biologist collecting bats in South Sudan (21).

In addition to these emerging zoonotic viruses, bats may be the source of a number of viruses with which humans have older evolutionary associations. For instance, bats harbor viruses closely related to both mumps (rubula virus) and measles (morbilli virus) and have likely been donors of these viruses to other mammalian groups, possibly including humans (6, 22). Furthermore, both Old and New World bats carry diverse hepadnaviruses, some of which are related to hepatitis B virus and can infect human hepatocytes (23). Hepaciviruses that are related to hepatitis C virus and pegiviruses that are related to human GB viruses were detected in the sera of many different bat species, and given the basal position of these bat viruses in phylogenetic trees, may also represent strains ancestral to those found in humans (24, 25).

The preponderance of links between bat and human pathogens has led to a debate about whether bats disproportionately contribute to emerging viral infections crossing the species barrier into humans (26–30). Given the diversity of the *Chiroptera* order (**Figure 1**), we may simply see more bat viruses because there are so many (>1,300) species of bats (31). However, even when accounting for the fact that they make up ∼20% of extant terrestrial mammals, bats are overrepresented as reservoir hosts of pathogens with a high potential for spilling into human populations (32, 33). In fact, no known predictors that have been described to impact the likelihood of crossing the species barrier, including reservoir host ecology, phylogenetic relatedness to humans or frequency of reservoir-human contact, explain this pattern (32). Thus, why bats are such a frequent source of pathogenic human viruses remains a tantalizing mystery.

Among viruses, those that have genomes encoded by RNA generally jump across species boundaries more frequently, presumably due to their inherently greater mutation rates that facilitate the rapid adaptation to replicating within new hosts (34). Interestingly, all pathogenic viruses that have made the jump to humans for which bat species may be reservoirs share the common feature that they have single-stranded RNA genomes (with the exception of hepadnaviruses which have a DNA genome but replicate via an RNA intermediate). So far, available evidence suggests that bats remain disease-free when infected with the RNA viruses they carry—even those highly pathogenic to humans—and are able to coexist with them without detectable fitness costs using measures such as changes in temperature, loss of body weight, or overt signs of inflammation (35). Indeed, so far only one RNA virus studied which circulates in a bat population has been shown to consistently cause significant morbidity and mortality: tacaribe virus in the Jamaican fruit bat (*Artibeus jamaicensis*), which recent evidence suggests is not a reservoir host for this virus (36). Data from experimental rabies and lyssavirus infections suggests that rhabdoviruses may also cause disease in bats, although experimental infection outcome is very dependent on the infection route. Intracerebral infection with different strains and in different bat species invariably led to death (37, 38). In contrast, intramuscular infection led to muscle weakness, paralysis and visible histological CNS lesions in 30% of experimentally infected flying foxes (*Pteropus poliocephalus*) (39). Similarly, a subset of vampire bats (*Desmodus rotundus*) experimentally infected intramuscularly with a high dose of rabies virus remained healthy despite viral shedding in the saliva and survived (40). Naturally infected bats are thought to either die or remain healthy and seroconvert, but transmission in free-ranging populations remains incompletely understood (41).

While bats seem to be frequent hosts for RNA viruses, current available data indicates that primates and humans disproportionately harbor DNA viruses such as herpesviruses (32). Interestingly, it is these DNA viruses that can persist in an individual which can also be found in isolated, small indigenous groups—perhaps suggestive of humans having a more ancient

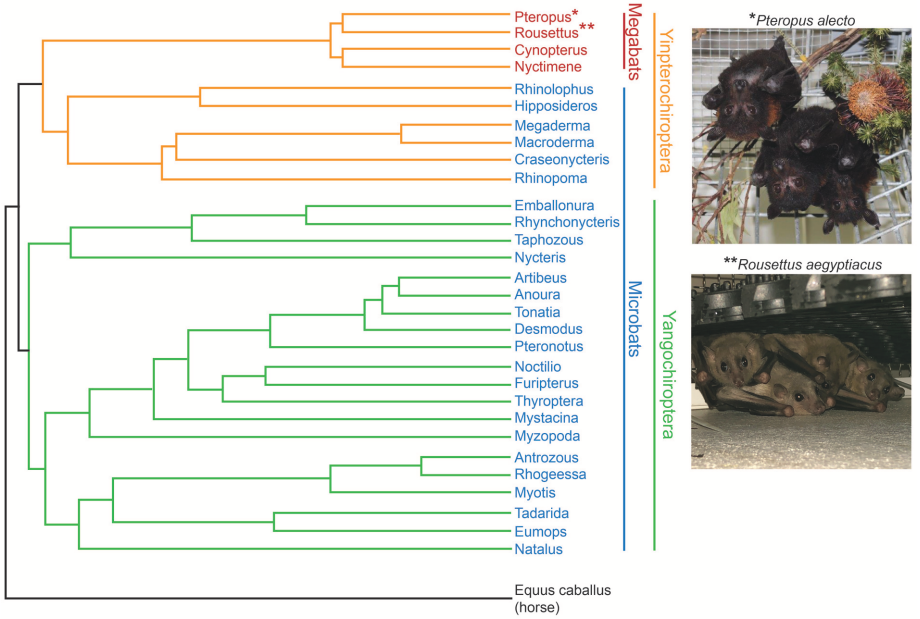

FIGURE 1 | Phylogeny of bat genera [modified from Teeling et al. (31), note that branch lengths are not to scale] indicating the two most studied bat species so far, *Pteropus alecto* and *Rousettus aegyptiacus*. Photos provided by Susanne Wilson, CSIRO, Australia (Pteropus); Anne Balkema-Buschmann and Nils Kiley, Friedrich-Loeffler-Institut, Germany (Rousettus).

relationship with such DNA viruses (42). It may even be the case that persistent DNA viruses in humans impact immune responses specifically to RNA viruses, but this has not yet been examined. It is likely that differences in evolutionary history of pathogen exposure between bats and humans have led to distinct adaptations in anti-viral immune responses and the ability to tolerate certain infections without disease while being susceptible to others. Importantly, bats differ in many aspects of their physiology and behavior from humans that may have direct or indirect effects on immune function.

BAT LIFE HISTORY TRAITS

Bats are a monophyletic mammalian group traditionally divided by morphological data into two suborders, the megabats and microbats, which more recent molecular data has revised into the Yinpterochiroptera and Yangochiroptera suborders (**Figure 1**). Bats possess a suite of traits that make them distinct from other mammals in a number of ways. These unique life history traits may play a role in understanding which pathogens bats have evolved to coexist with and why. In particular, such traits may explain the ability of bat populations to maintain particular viral pathogens indefinitely, and may have effects on immune function through specific energetic or evolutionary trade-offs we have yet to better define.

Longevity, Metabolic Rate, and Hibernation

Despite the diversity of viruses carried by bats, they are not typically known to cause mass bat die-offs or reduce bats' remarkable longevity. In this respect, bats represent a potential opportunity for long-term persistence of viruses within a population and across generations. Bats live significantly longer than similarly-sized terrestrial mammals and, despite their small size, are characterized as "slow" mammals in the slow-fast continuum (43, 44). Although their weights range from 2 grams to 2 kilograms, with respect to longevity bats group with large mammals such as humans and non-human primates (45). Aerial living has an obvious advantage in avoiding predation, but bats outlive even birds. For example, the Brandt's bat (*Myotis brandtii*) lives up to 41 years, compared to *Selasphorus platycercus*, a bird species of similar size that lives for ∼14 years (45, 46). Thus, flight can only partially account for their extraordinarily long lives. Initially, the longevity of some bats was attributed to seasonal hibernation, as temperate-zone species enter continuous torpor of up to 75 days, with a dramatic drop in metabolic rate such that small fat reserves can sustain them throughout the entire hibernating season (43). However, even non-hibernating bat species live three times longer, on average, than predicted by their size, and heterothermy is not an accurate predictor of lifespan in other mammalian orders, suggesting that the driving force behind their surprising longevity is intrinsic to bats as a group (47–49).

Like other "slow" mammals, bat females typically only have one offspring per year, perhaps because the volant lifestyles of bats make it difficult to rear more than one offspring, as pregnant females and those with recent births must navigate and forage with added weight; on average, neonatal bat pups are ¼ of their mother's weight (50). The physical and energetic constraints of rearing multiple offspring may necessitate small litters, which would in turn require prolonged reproductive capability and

enhanced longevity to ensure maintenance of the population over generations. Thus, in bats, the dependence of colony survival as a whole may depend upon enhanced individual survival and delayed senescence (51). Genetic analyses of several bat species have shown differences in the growth hormone (GH)/insulin-like growth factor 1 (IGF1) axis which in humans is associated with aging, resistance to diabetes and cancer (52).

The determinants of adult survival in bats have been historically difficult to identify, as this requires tracking individuals over many years, and until recently longitudinal studies of bat mortality were conducted using tagged bats, of which only a fraction were recovered (53). Recently, a 19-year study of a colony of Bechstein's bats demonstrated that unlike terrestrial mammals, survival could not be predicted by common indicators such as season, age, and body size. Instead, the only accurate predictor of mortality was a single cataclysmic weather event that affected multiple countries in north-central Europe. Additionally, even the oldest female bats were reproductively capable, indicating that bat survival is primarily affected by catastrophic natural events rather than factors that normally dictate an individual's fitness (45).

Echolocation

Molecular phylogenetic studies of bats suggest that there are massive gaps in bat fossil records. As bats are the second most diverse order of mammals, outnumbered only by rodents, the number of species unrepresented in the fossil records is staggering. Over half of microbat and nearly all of megabat fossil histories are missing (31, 54). The enormous incompleteness of the fossil records has made it difficult to identify when specific morphological traits of bats arose. As molecular phylogeny groups two echolocation-reliant microbat species with megabats (also called Old World bats or pteropodids), which do not rely on echolocation, there is some debate as to whether echolocation first arose in the common ancestor of bats and was subsequently lost in megabats, or whether it arose twice, independently (31). Pteropodids have adaptations that enhance visual acuity at night (55), and they do not require echolocation for foraging (56). There are multiple types of echolocation that can be partially delineated by species, but are more clearly categorized by the type of environment. Divergent species that inhabit the same type of environment, such as those that hunt in large, open spaces, often use the same form of echolocation, suggesting that habitat has a greater influence on echolocation than phylogeny (31). Importantly, echolocation can result in the production of droplets or small-particle aerosols of oropharyngeal fluids, mucus, or saliva, thus facilitating transmission of viruses between individuals in close proximity (57, 58). The unique navigation tactic of many bat species may inadvertently facilitate virus transmission among bats in the same habitat.

Flight

Bats are the only mammal capable of powered flight, which likely evolved ∼65 million years ago alongside birds following radical ecological changes that resulted in the extinction of the dinosaurs (54, 59). During flight, bats consume approximately four times as much oxygen, and they have a markedly higher concentration of red blood cells compared to small terrestrial mammals (60). Bat flight is markedly different from that of birds and insects, whose wing surfaces are typically composed of inflexible material, such as feathers or chitin. Bat wings are constructed from live skin stretched across elongated arm and finger bones, making them extraordinarily malleable and sensitive to environmental cues (59). The plasticity of bats' wings allows them to navigate and inhabit diverse ecospheres, contributing to their extensive speciation. Moreover, the capability of powered flight can allow the efficient spread of viruses and thus the introduction of pathogens to which colonies may otherwise have remained naïve.

As flight is extremely metabolically demanding, in addition to evolving the physical mechanisms required for flight, bats have also evolved necessary underlying molecular mechanisms. The mitochondrial respiratory chain accounts for nearly all ATP required for mobility in eukaryotes, and genetic analysis of both micro- and megabat species revealed an enrichment of genes specific to the oxidative phosphorylation (OXPHOS) pathway. Specifically, 4.9% of nuclear-encoded and 23% of mitochondrial OXPHOS genes have evidence of positive selection in bats, which is markedly higher than the expected 2% of orthologous genes in previous genome-wide studies that show evidence of positive selection (61). Genomic analysis of *Pteropus alecto* and *M. davidii* suggests positive selection for the DNA damage checkpoint pathway and changes in overlapping aspects of this pathway with the innate immune system, indicating that evolutionary adaptations important for flight may have secondarily affected bat immunity (62).

Social Interaction and Communities

As a group, bats exhibit the greatest diversity of social systems in mammals. Tropical species are primarily responsible for this diversity, as temperate species are more restricted in their social behavior. Generally, however, bats are extremely social creatures that tend to form dense roosting colonies (63), and almost all temperate-zone species live in closed societies with very little infiltration of foreign bats into established roosts (63, 64). In particular, female bats form maternity colonies in which males do not take part. As bats are capable of long-distance flight, dispersal barriers cannot explain the philopatry of females. Instead, benefits such as knowledge of foraging areas and social thermoregulation likely selected for these colony types. Additionally, there is evidence that forming closed societies limits the potential invasion of new pathogens, thereby protecting colony members that would otherwise be vulnerable to infection. For example, *Pseudogymnaoscus destructans* has decimated North American bat populations that do not live in the type of closed societies observed elsewhere (64). DNA analysis of a closed society of Bechstein's bats revealed extraordinarily high conservation of mitochondrial DNA and relatively low conservation of nuclear DNA, suggesting stable maternal populations within colonies and gene flow between colonies via promiscuous mating with males. It is possible that the mating patterns of temperate-zone species may allow transmission of pathogens between colonies via traveling males while the

more insular females may allow viruses to persist throughout generations within a colony.

ANTI-VIRAL IMMUNE RESPONSES OF BATS

An important commonality among pathogenic RNA viruses in humans presenting with disease is that the host response is an important contributor to the disease process, with dysregulated and excessive innate immune responses being particularly important drivers of tissue damage during infection (8). Given the general absence of clinical signs of disease in bats infected with the same viruses that are so lethal in humans or other non-natural hosts infected experimentally, a critical question has been to understand whether bats might establish effective disease tolerance, thus maintaining fitness despite pathogen replication, or whether bats are more resistant to infection through more successful control of pathogen replication and what the contribution of the immune response is (65, 66). The lack of many fundamental immunological tools enabling the probing of bat immune responses has meant that truly mechanistic studies of bat immunity have been very limited, although recently there has been some progress in establishing approaches such as flow cytometry to identify distinct bat immune cell populations (67, 68). So far, studies of bat immunity have primarily taken one of three approaches, whereby each comes with important strengths and weaknesses that have to be kept in mind: (i) comparative genome studies, (ii) *in vitro* cell culture assays, and (iii) experimental infections.

Comparative genome studies have confirmed that the critical components of the innate and adaptive immune system are conserved in bats at the gene level and that bats have the machinery for innate responses to pathogen-associated molecular patterns (PAMPs), the production of anti-viral effector molecules such as type I interferons (IFN), T cell responses (variable T cell receptors, MHCI and MHCII), and B cell responses [reviewed in (35)]. Interestingly, based on the 10 bat genomes sequenced so far, the only family of genes lost entirely in all of them are PYHIN genes (69). Members of the PYHIN family are DNA sensors capable of recognizing foreign DNA, including DNA viruses and damaged self DNA which can be generated by RNA viral infection. Recognition of DNA results in production of IFN through interaction with stimulator of interferon genes (STING). The PYHIN family also encode the only identified class of DNA sensors capable of activating the inflammasome. It has been hypothesized that the absence of the PYHIN family may allow bats to limit activation of the innate immune response to damaged self-DNA generated by RNA viral infection, thus avoiding excessive inflammation (69, 70). Genome comparisons highlighting contractions or expansions of specific gene families, specific genes under positive selection, or non-conserved sequence differences in critical protein domains can thus provide the basis for hypotheses worth testing further. However, it is important to note that much can be missed in absence of data on gene regulation, especially during infection when gene expression kinetics can make a critical difference to the infection outcome. Moreover, the absence of a gene or gene family does not rule out that other proteins have evolved to compensate for their loss of function. Thus, while whole genome analyses can provide a context for specific questions or be hypothesis-generating, on their own they cannot distinguish tolerance from resistance mechanisms. The repeated identification of signatures of positive selection in innate immune genes in particular, does however lend credence to the idea that bats have specific adaptations as a result of a long co-evolutionary history with viruses.

Cell culture assays with bat cell lines, or, in some instances, primary bat cells, have been used to assess whether bats are permissive for viral replication and to determine whether particular immune receptor signaling pathways are intact. As discussed below, such studies have probed the type I IFN pathway in particular, revealing some possible species-specific differences among bats (71–83). However, it is important to note that in some instances immortalized cells can behave differently from primary cells and that such cultures may miss additional differences imposed by changes in cell localization, cell recruitment or cell-cell interactions in a whole animal. Careful experiments measuring the quality, magnitude, and kinetics of immune responses in bats during infection and upon administration with defined stimuli for which we have comparative information from humans remain to be done to provide additional evidence that specific innate immune pathways are wired differently.

Experimental infections come with the enormous challenge of having to house and/or breed colonies of bats and to have biosafety-level 4 facilities in place to perform infections with viruses lethal to humans. Moreover, some trial and error is involved in determining which route and dose leads to viral replication, establishing a source of the virus (human-adapted strains tend to replicate less well in bats than strains obtained from naturally infected bats), and amplifying this viral stock without extensive tissue culture passaging. Studies to date have examined the kinetics of viral replication by quantifying the extent of viremia and dissemination to other tissues, and assessing changes in white blood cell counts, body mass, and temperature. Given the generally low levels of viral shedding and short infectious periods observed so far it remains poorly understood how transmission occurs in the wild to sufficient levels that cross-species jumps occur. Some infection experiments have also provided evidence that a particular bat species is unlikely to be a reservoir despite epidemiological evidence, for example for *R. aegyptiacus* and ebolavirus. Certainly, once good experimental infection models are established, such studies have the potential to be hugely informative with regard to anti-viral immune responses elicited using, for instance, comparative transcriptome analyses. One drawback may be that experimental infections do not mimic the impact of chronic stress arising from the disruption of wildlife populations, which bats are particularly sensitive to Jones et al. (84). Comparison of either cave-roosting or foliage-roosting species in areas of Malaysian Borneo designated as actively logged forest, recovering forest, or fragmented forest revealed varying impacts of habitat disturbance on stress and circulating white blood cells (85).

Overall, the limited studies of bat immunity that have been done have focused largely on 2 species: *P. alecto* and *R. aegyptiacus*. We summarize this work below, but comparisons of observations made across species suggest that although a number of species appear to be capable of avoiding the pathological effects of RNA virus infection, each bat species may have achieved this through distinct pathways, possibly involving changes to both increase pathogen replication control and to mitigate any immunopathology through decreased inflammatory responses and hence increased disease tolerance.

Pteropid Bats

The most well studied bat species with regard to antiviral immune responses is the Australian black flying fox (*P. alecto*). This interest has stemmed from the fact that pteropid bats have been identified as the natural reservoirs for the deadly Hendra and Nipah viruses (86), which continue to cause outbreaks [such as most recently in India in May 2018 (87)]. To date, several studies have examined the kinetics of viral infection in Pteropus bats and the nature of transmission and replication in other susceptible species (88–91). In Australia, all four species of pteropid bats (*P. alecto*, *P. poliocephalus*, *P. scapulatus,* and *P. conspicillatus*) have antibodies to Hendra virus but only *P. alecto* and *P. conspicillatus* are considered to be the primary reservoir hosts (14, 92, 93). In South East Asia, both pteropus spp. occurring in Malaysia have been found to be seropositive for Nipah virus neutralizing antibodies, and the virus has been isolated from *P. hypomelanus* and *P. vampyrus* (15, 94).

Experimental infections of pteroid bats with Hendra or Nipah virus result in sub-clinical infection with short periods of virus replication and shedding, and low antibody titres (88–91). Upon subcutaneous infection of *P. poliocephalus* with Hendra virus, viral antigen was detected by immunohistochemistry at 10 dpi in blood vessels of spleen, kidney and placenta (89). Similarly, oronasal Hendra virus infection of *P. alecto* led to the presence of viral genome in lung, spleen, liver and kidney 3 weeks later, but virus isolation was unsuccessful at this timepoint (89, 91). The Malaysian flying fox, *P. vampyrus* and the Australian species, *P. poliocephalus* demonstrate similarly short periods of viremia upon infection with Nipah virus. In subcutaneously infected *P. poliocephalus*, virus was isolated from the kidney and uterus of bats euthanized at 7dpi, but no virus was isolated at any of the other timepoints examined (3, 5, 10, 12, or 14 dpi) and there was no evidence of antigen in any tissue by immunohistochemistry, including tissues collected at 7 dpi. In this study, low neutralizing antibodies were detected in all bats with the exception of one individual that developed a significant neutralizing antibody titre — possibly reflecting the fact that *P. poliocephalus* is not the natural host for Nipah virus (90). In *P. vampyrus* challenged by oronasal Nipah inoculation, viral genome was detected in a throat swab at 4 dpi and a rectal swab of the same individual at 8 dpi but virus was undetectable in tissues collected at post-mortem from all individuals (49, 50, or 51dpi), consistent with a short period of viremia. Similar to previous studies, antibody titres were low in all *P. vampyrus* bats (91). Overall, these results are consistent with bats controlling replication rapidly, at least following experimental infections which involve higher doses of virus compared to what bats would likely be naturally exposed to in the wild. The absence of a robust antibody response also appears to be typical of all experimental Hendra and Nipah virus infections performed to date. Since antibody responses are the only immune parameter that has been measured during experimental infections of bats so far, it is difficult to speculate on the mechanisms responsible for control of viral infections *in vivo*.

Pteropus alecto was among the first bat species to have its genome described in detail. Genomic studies provided initial clues for possible differences in the innate immune system of bats, with evidence for selection of key innate immune genes and the expansion or contraction of specific immune gene families (62, 68, 95). The MHCI region is contracted (96), as is the type I IFN locus, which in *P. alecto* contains fewer IFN genes than any other mammalian species sequenced, with only three functional IFN-α loci (68). In contrast, pteropid bats have the largest and most diverse family of APOBEC (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) proteins identified in any mammal (95). APOBECs interfere with the replication of retroviruses by deaminating cytosine residues in nascent retroviral DNA. This is notable, as bats are an important source of mammalian retroviruses, many of which have been transmitted to other mammals (97, 98). APOBEC diversification may therefore have occurred to counteract the effect of retroviruses and possibly other viruses, as APOBECs have been shown to restrict the replication of other virus families including hepadnaviruses, and parvoviruses (99, 100). Members of the APOBECA3 protein family exhibit direct antiviral activity through DNA cytosine deamination which results in hypermutation of the nascent retroviral DNA which is then degraded or rendered non-functional (101). The mechanism of antiviral activity against non-retroviruses remains largely unknown. For parvovirus adeno-associated virus, APOBEC meditated inhibition has been speculated to involve direct interaction with the viral DNA or the replication machinery (102). Whether the expanded family of ABOBECs in bats have evolved other mechanisms to control DNA and RNA viruses remains to be determined. As APOBECs can be induced by even low levels of type I IFN (103), one hypothesis to be tested is that bats, through their multiple APOBECs, are able to restrict viral replication without causing inflammation. *Pteropus alecto* is the only bat species to date in which APOBEC genes have been mapped, and whether the expansion of this gene family extends to other bat species remains to be determined.

In addition to the identification of putative immune pathways distinct in *P. alecto* through genome studies, differences have been identified in the activation of innate immune effectors in *P. alecto* from studies performed *in vitro*, primarily using cell lines derived from tissues including the kidney and lung. IFNs are the first line of defense following viral infection and unsurprisingly, because of this, they have been the most extensively studied group of genes in bats. Both type I (IFNA and IFNB) and III (IFNL) IFNs are detectable in bat cells. Curiously, a unique characteristic of pteropid bats is the constitutive expression of mRNA for IFNA and the signaling molecule, IFN regulatory factor 7 (IRF7) in unstimulated tissues and cells [75, 68a]. Constitutively expressed IFNA and IRF7 may allow bats to

respond more rapidly to infection, thus avoiding the lag time between pathogen detection and response. Furthermore, viral infection or stimulation with synthetic ligands result in little IFNA induction in pteropid bat cells (68). The constitutive expression of IFNA has been described in two species of pteropid bats (*P. alecto* and *Cynopterus brachyotis*) and is a first for any species. IFNB and IFNL are activated following stimulation of cells from *P. alecto* and *P. vampyrus* with synthetic ligands such as polyIC (71–74). Moreover, bat IFNs demonstrate antiviral activity (68, 71–74, 104). However, viral infection of *P. alecto* splenocytes results in induction of IFNL but not IFNB, hinting at differences in the function of type I and III IFNs (74). In humans and mice, IFNL has recently been demonstrated to have a role not only in controlling virus replication, but also in dampening damage-inducing neutrophil functions and in modulating tissue-damaging, transcription-independent responses such as production of ROS (77, 80). A hypothesis yet to be tested is whether upregulation of IFNL rather than IFNB has a similar function in bats.

The endoplasmic reticulum (ER) membrane protein, STING, is involved in induction of type I IFN by cytosolic DNA (105). Stimulation of bat splenocytes with GMP-AMP, which is produced following sensing of cytosolic DNA by cGAS, results in little induction of IFN compared to responses observed in mouse splenocytes (83). Bat STING contains an amino acid substitution of the highly conserved and functionally important serine residue S358 which may be responsible for dampening STING-dependent IFN activation in bat cells in response to DNA. However, comparable levels of IFN induction in mouse and bat cells in response to the RNA viral mimic polyIC indicate that STING-associated inhibition of the IFN response does not extend to RNA viruses (83), thus the relevance to RNA viruses in bats remains unknown.

Downstream of the induction of IFNs, novel subsets of IFN stimulated genes (ISGs) have been detected in unstimulated and stimulated pteropid bat cells indicative of a response that is less damaging to the host. Furthermore, the ISG response is elevated for a shorter period of time in *P. alecto* compared to human cell lines which again may be a strategy to avoid tissue damage (78, 81). The less inflammatory profile of ISGs may be the key to the ability of bats to tolerate higher IFN expression without adverse consequences. The balance between resistance and tolerance may therefore be achieved through careful selection of the pathways that are activated and shorter periods of activation or limited activation to prevent inflammation. In this regard, studies of the regulation of IFN signaling in bats is likely to provide important additional insights.

Rousettus Bats

A second bat species whose host responses to viral infections has been studied more recently is the Egyptian fruit bat (*R. aegyptiacus*). Marburg virus (MARV) has been repeatedly isolated from this species with demonstrated seasonal pulses of active MARV replication in juvenile bats living in caves in Uganda (11, 106). Moreover, *R. aegyptiacus* were a suspected reservoir for ebolavirus (EBOV) based on epidemiological evidence and detected seroreactivity to EBOV, but no infectious virus has been isolated thus far from wild rousettus bats (107). Indeed, while cell lines from *R. aegyptiacus* are equally susceptible to MARV and EBOV (79, 108), experimental infections of *R. aegyptiacus* seem to confirm that it is a reservoir for MARV, but is unlikely to be the source of EBOV spillover to humans. Subcutaneous EBOV infection results in very low viral replication, no viremia, little dissemination to other tissues, and no viral shedding, although some animals seroconvert, suggesting that *R. aegyptiacus* are unlikely to perpetuate EBOV in the wild (109, 110). In contrast, experimental MARV infection of *R. aegyptiacus* resulted in acute viremia that peaked on days 5–6 post-infection (although generally at lower levels than in humans), oral shedding that peaked on days 7–8 post-infection, and dissemination to other tissues including spleen, liver, kidney and salivary glands (109, 111–113). Interestingly, viral replication was not associated with increases in white blood cell counts, any clinical signs of infection such as changes in body temperature or body weight, and infected tissues showed little evidence of inflammatory infiltrates (109). In all experiments, viremia was cleared by day 13 and oral shedding ceased by day 19. Intriguingly, a cohousing experiment resulted in MARV transmissions to uninfected bats 4–7 months after experimental infection, raising the question of whether persistent infection with intermittent shedding is possible or whether very long latent periods without detectable viral replication could follow exposure (114). Upon secondary challenge of previously MARV-infected bats, none showed any detectable viral replication or shedding, providing evidence that protective immunity is established (115).

Unlike for pteropus bats, no constitutive expression of type I IFNs has been detected in *R. aegyptiacus* (79), but type I IFNs are induced in *R. aegyptiacus* cell lines upon stimulation with Sendai virus as seen in other mammals (82). Furthermore, in *R. aegyptiacus* the type I IFN genes are expanded, again in contrast to *P. alecto* (82), but like for *P. alecto* a number of genes in the type I IFN pathway or involved in innate immune recognition of PAMPs show signs of having been under positive selection (82). Whether positive selection of genes in either bat species is associated with tolerance remains to be determined, especially given that innate immune genes in humans have also been under positive selection (116). A transcriptome study which generated 20 RNA sequencing libraries from 11 tissues taken from 1 female and 1 male *R. aegyptiacus* found a reduced coverage of NK cell related genes compared to other mammals, but confirmed that in these bats the predominant T cells had an αβ T cell receptor, and showed that IgE, IgG, IgM, and IgA, as well as a number of pro- and anti-inflammatory cytokines, were all detectable (117). The recently sequenced *R. aegyptiacus* genome revealed substantial differences in the repertoire of NK cell receptors, with this bat species entirely lacking functional killer cell immunoglobulin receptors (KIRS) and with all killer lectin-like receptors (KLRs) encoding either activating and inhibitory interaction motifs, or inhibitory interaction motifs only (82). NK cells are important immune cell players in an antiviral response but without assessment of the consequences of these genomic differences it is difficult to draw any specific conclusions with regard to viral control or the magnitude of inflammation elicited upon infection with viruses like MARV. Nonetheless,

these genomic data provide some interesting hypotheses to be tested in the future.

Other Bat Species

Some additional studies probing the induction of cytokines upon stimulation of bat cells with defined innate immune stimuli provides some evidence that innate immune recognition of viruses may be altered, leading to a reduction in pro-inflammatory responses. Stimulation of kidney and myeloid cells from the big brown bat (*Eptesicus fuscus*) with polyinosinic-polycytidylic acid (polyI:C) resulted in only limited activation of the inflammatory cytokine, tumor necrosis factor alpha (TNFα) compared to human cells which display a robust TNFα response. Induction of TNFα is controlled by transcription factors, including the NF-kappa B (NF-κB) family which consists of five members, [RelA (p65), RelB, c-Rel, NFκB-1 (p50), and NFκB-2 (p52)] which form homo- or hetero-dimers that are bound by molecules of the inhibitor of NFκB (IκB) family and retained in the cytoplasm of the cell in an inactivated state (118). In *E. fuscus,* a potential repressor (c-Rel) binding motif was identified in the TNFα promoter region which may explain the difference in induction of TNFα in *E. fuscus* cells. Consistent with this hypothesis, partial knockdown of c-Rel transcripts significantly increased basal levels of TNFα transcripts in *E. fuscus* cells (104). The transcription factor, c-Rel has also undergone positive selection in the bat ancestor which may indicate that this mechanism is common to other species of bats (62). Of note, low levels of TNFα induction have also been associated with tolerance in European bank voles which are a natural reservoir for Puumala hantavirus (PUUV) (119).

Stimulation of macrophages from the greater mouse eared bat (*Myotis myotis*) suggested that this species may have also evolved mechanisms to avoid excessive inflammation caused by cytokines. While high levels of TNFα, IL1β, and IFNβ were produced in response to *in vitro* challenge with lipopolysaccharides (LPS) and PolyI:C, there was also a sustained, high-level transcription of the anti-inflammatory cytokine IL-10, which was not observed in mouse macrophages (120). Furthermore, unlike in the mouse, *M. myotis* macrophages did not produce the proinflammatory and cytotoxic mediator, nitric oxide, in response to LPS. The same study also showed evidence of bat specific adaptations in genes involved in antiviral and pro-inflammatory signaling pathways through comparison with other mammalian taxa, including RIG-I, IL1b, IL-18, NLRP3, STING, and CASP1, further supporting the evolution of adaptations associated with reducing inflammatory responses in bats (120).

BAT IMMUNE RESPONSES TO NON-VIRAL PATHOGENS

Even less is known about immune responses of bats to non-viral pathogens than to viral pathogens, but it is clear that while anti-inflammatory responses may be characteristic of anti-viral responses in bats, they are susceptible to disease upon infection with particular pathogens—in some instances due to dysregulated and damaging immune responses. One particular example of this is the emerging infectious disease, white nose syndrome (WNS), that has decimated North American bat populations beginning in 2006, in what will likely rank as one of the most devastating wildlife diseases in history (121–123). For reasons that remain poorly understood, the psychrophilic fungus *Pseudogymnoascus destructans* (formerly *Geomyces destructans*) causes no mass mortality in European bats despite being abundantly detected (124, 125). Indeed, evidence suggests that a single *P. destructans* genotype was introduced to North American bat species from Europe (125). In North America, *P. destructans* infection is not specific to a particular bat genus, replicating in many different bat species during hibernation and targeting the furless skin of the wings, ears, and muzzle (126). Distinct hypotheses have been proposed for why *P. destructans* is so deadly in North American bats, ascribing the impaired tolerance to infection compared to European bat counterparts to either physiological or immunological factors. On the one hand, more frequent arousal, electrolyte depletion, and dehydration are thought to contribute to mortality following infection (127, 128). The destruction of wing tissue in WNS results in a marked electrolyte imbalance, as the wings play a critical role in maintaining water levels, especially during hibernation, during which bats are particularly vulnerable to dehydration (129, 130). Dehydration catalyzes arousal in hibernating bats, which is extraordinarily metabolically costly and rapidly depletes the fat reserves necessary to survive until spring (127). An alternative hypothesis posits that the restoration of the immune system following emergence from hibernation induces the fatal pathology of WNS. During hibernation, destruction of cutaneous tissue is limited and infiltrating immune cells are entirely absent, yet in the weeks following arousal, infected bats exhibit overt wing damage and corresponding neutrophilic and lymphocytic infiltration (131). Hibernation does not preclude a localized immune response to *P. destructans* at the site of infection and transcriptomic analysis of infected tissue showed upregulation of some acute inflammatory genes in infected tissue (132, 133). However, the observed immune responses likely occur during arousal periods, which are more common in infected bats. Ultimately, immunosuppression during torpor allows *P. destructans* to colonize infected bats relatively unchecked (124), and upon emergence from hibernation, the exuberant immune response may result in deadly immunopathology during WNS (131).

In addition to general studies of immune cell recruitment and transcriptional responses during WNS, body mass and white blood cell counts were examined following LPS administration in four bat species (134–137). Subcutaneous LPS challenge in of Pallas's mastiff bats (*Molossus molossus*) led to a loss of body mass of ∼7% within the first day, but did not result in changes in circulating white blood cell counts or body temperature (135). Seba's short-tailed fruit bat (*Carollia perspicillata*) also showed a decrease in body mass following LPS challenge, but this was associated with increases in white blood cell counts as well as increases in derivatives of reactive oxidative metabolites (dROM) (134). Subdermal LPS challenge of fish-eating Myotis (*Myotis vivesi*) led to body mass decreases, increased resting metabolic rate and

skin temperature (136), while intraperitoneal LPS challenge of wrinkle-lipped bats (*Chaerephon plicatus*) caused an increase in circulating leukocytes, but did not result in a reduction in body mass compared to controls (137). The differential responses to LPS challenge suggest that the immune response to bacterial infection varies across species. Of note, post-mortem examinations of ∼500 dead bats comprising 19 species from Germany revealed inflammatory lesions, many of which had evidence of underlying bacterial or parasitic infections, particularly in the lung (138).

CONCLUSIONS

Bats have an array of unique life history characteristics that not only allow them to be particularly good reservoirs for viruses that are highly pathogenic in other species, but also appear to have shaped their immune systems. Although research on bat antiviral immunity has focused on only a few species to date, at the genomic level, selection on genes is concentrated on the innate immune system across both suborders of bats. However, while these studies have provided a rich source of hypotheses, the majority remain to be tested at the functional level and many questions remain that cannot be answered from comparative genome studies. Experimental studies to date have demonstrated some functional differences between bat species, with the common emerging theme that the overall antiviral response appears to converge on a lower inflammatory profile, with tight regulation of the cytokine and inflammatory response key to clearing viral infection without the pathological outcomes typically associated with infection. However, whether this is due to specific tolerance mechanisms that are at play or increased resistance to RNA virus replication still remains unclear. Fewer studies have examined the adaptive immune system than those probing innate immune pathways, but experimental infections with bat borne viruses have demonstrated that bats generate low or absent antibody responses which often wane rapidly. This is reminiscent of the response of another reservoir host, the sooty mangabey which is the natural reservoir for simian immunodeficiency virus (SIV) and for yellow fever virus. Sooty mangabeys given an attenuated yellow fever virus vaccine strain generate much lower, transient antibody responses as compared to humans or rhesus macaques. Changes to innate immune responses are also evident in sooty mangabeys (139). Thus, intriguingly, different reservoir hosts may have arrived at similar solutions to avoid the pathological consequences that follow viral infection in non-natural hosts.

Despite the ability of bats to avoid disease associated with viral infection, this trait does not extend to all pathogens, as evidenced by the severe consequences associated with infection of North American bats with the fungus that causes WNS. Thus, the pathways associated with the control of other pathogens have not been under the same selection pressures as those responsible for controlling infections with RNA viruses—or there are immunological trade offs involved which lead to greater susceptibilities to some pathogens than others. Overall, it is clear that studying host-pathogen interactions in reservoir hosts has considerable potential to provide novel insights into host tolerance mechanisms that eventually could assist in the treatment of diseases in humans and other susceptible hosts and may also offer solutions for the treatment of diseases that are a conservation threat to bats themselves.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Host–Parasite Interactions Promote Disease Tolerance to Intestinal Helminth Infection

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Parasitic helminths are among the most pervasive pathogens of the animal kingdom. To complete their life cycle, these intestinal worms migrate through host tissues causing significant damage in their wake. As a result, infection can lead to malnutrition, anemia and increased susceptibility to co-infection. Despite repeated deworming treatment, individuals living in endemic regions remain highly susceptible to re-infection by helminths, but rarely succumb to excessive tissue damage. The chronicity of infection and inability to resist numerous species of parasitic helminths that have co-evolved with their hosts over millenia suggests that mammals have developed mechanisms to tolerate this infectious disease. Distinct from resistance where the goal is to destroy and eliminate the pathogen, disease tolerance is an active process whereby immune and structural cells restrict tissue damage to maintain host fitness without directly affecting pathogen burden. Although disease tolerance is evolutionary conserved and has been well-described in plant systems, only recently has this mode of host defense, in its strictest sense, begun to be explored in mammals. In this review, we will examine the inter- and intracellular networks that support disease tolerance during enteric stages of parasitic helminth infection and why this alternative host defense strategy may have evolved to endure the presence of non-replicating pathogens and maintain the essential functions of the intestine.

Keywords: helminth, infection, immunity, intestine, disease tolerance

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> *"Generalising about the Nematoda is extremely hazardous. The often cryptic diversity is such that it will frustrate the best of intentions."*
>
> -WC Clark

INTRODUCTION

Parasitic helminths include a diverse group of intestinal worms that are one of the most successful pathogens of the animal kingdom. Current estimates indicate that over 1.5 billion people and many other agricultural and wild mammalian species are infected with at least one species of intestinal helminth (1). The incredible prevalence of these parasites is largely due to their chronicity of infection—many species can live for years in the host intestine—and the inability of the host to prevent reinfection (2). Although helminth infection is associated with important co-morbidities such as anemia, growth-stunting and digestive disease, infection-induced mortality is relatively rare (<1 per 20,000 individuals) compared to other infectious diseases prevalent in the developing world such as Tuberculosis (~1 in 10) and Malaria (~1 in 100) (3). This low mortality rate is

surprising given the fact that the host must accommodate a large (ranging from 1 mm to several meters in length, depending on the species), tissue-invading parasite. The physical characteristics of helminths, their general ability to induce a tissue-healing rather than tissue-destructive immune response and, in some cases, their long-lasting relationship to the host collectively indicate that mammals have evolved to tolerate these parasites.

Tolerance to infection, also called disease tolerance, is a defense strategy by which the host activates intra- and inter-cellular networks to limit the damage incurred by the infectious agent or the immune response without affecting pathogen load (4). Although appreciated in plant biology for decades, the concept of disease tolerance has only recently gained traction as an important mammalian host defense strategy against bacterial, viral and parasitic microorganisms that can occur in combination with or independent of resistance and derive from immune as well as non-immune pathways (5). Disease tolerance is also conceptually distinct from immunological tolerance which involves the unresponsiveness to self or foreign antigens (6). Here we provide a rationale for why disease tolerance is an important defense strategy against helminth infection and include recent data that adds complexity yet excitement to this rapidly evolving research field. Given the diversity of parasitic helminth species, life cycles and susceptible hosts—as eloquently stated by WC Clark (7)—we limit our discussion to the intestinal stage of invasion and/or colonization by nematode species that have co-evolved with rodents and humans and, although they cause significant inflammation and tissue damage during the invasive stage of infection, lead to chronic infection. In addition, we will consider how the parasite itself may promote disease tolerance to ensure its survival and continuation of its life cycle. Finally, we will discuss mechanisms of disease tolerance within the intestine that extend beyond tissue repair programs associated with helminth infection and how they may maintain host fitness in the face of these ancient tissue invaders.

TYPE 2 IMMUNE-MEDIATED DAMAGE CONTROL

The most common intestinal parasitic nematodes of humans include the roundworm *Ascaris lumbricoides*, the whipworm *Trichuris trichiura* and the hookworm *Necator americanus*. To propagate their species, these enteric worms have developed mechanisms to invade the host via the skin and/or ensure their survival passage through the oral cavity and stomach until they arrive within the intestinal tissue where they produce eggs that are shed via host feces (8). However, the presence of these large, motile foreign bodies within the epithelial and submucosal layers of the gut disrupts the intestinal architecture and requires tissue remodeling to minimize organ damage and maintain host fitness. These adaptations to infection rely, in large part, on the induction of a type 2 immune response (9).

Studies using naturally-occurring rodent parasites such as *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis, Trichinella spiralis,* and *Trichuris muris* in a laboratory setting have demonstrated that upon entry into the intestine, epithelial cells (IECs) are critical for initiating a type 2 immune response. IECs release damage-associated molecules such as ATP as well as the cytokines interleukin (IL)-25 and thymic stromal lymphopoietin that, in combination with diverse sources of IL-33, stimulate tissue-resident type 2 innate lymphoid cells (ILC2s) to produce IL-4, IL-5, and IL-13 (10–14). These quintessential type 2 cytokines rapidly recruit eosinophils and alternatively activated macrophages (AAMacs) with tissue-reparative properties to the site of infection that feedback on to the epithelium to fortify the intestinal barrier by stimulating the production of mucus and anti-microbial peptides as well as enhancing the shedding of dead enterocytes (10). Although the mechanisms by which IECs detect helminth infection remain largely undefined, recent studies demonstrated that succinate, derived from the metabolism of dietary fibers by intestinal *protist* spp., is detected by a specialized subset of IL-25 producing chemosensory IECs called tuft cells. Succinate stimulated tuft cell proliferation (and therefore increased amounts of intestinal IL-25) in a succinate receptor (Sucnr1)-dependent manner. Increased IL-25 stimulated the proliferation of IL-13 producing ILC2s that, in turn, induced goblet cell hyperplasia, intestinal remodeling, and enhanced immunity to subsequent *N. brasiliensis* infection (15–17). Importantly, succinate signals were not required for worm expulsion. These results support the exciting possibility that metabolic signals, while not necessarily critical for host resistance, provide an important pathway used by the host to promote tissue repair and disease tolerance to *N. brasiliensis* infection.

In parallel to ILC2 activation, *T. muris* has been shown to stimulate production of thymic stromal lymphopoietin by IECs that condition intestinal dendritic cells (DCs) en route to the draining lymph nodes to polarize CD4+ T cells into Th2 cells that home to the intestine and amplify the ongoing type 2 response (18). DCs have also been shown during *H. polygyrus* infection to initiate the differentiation of T follicular helper cells that migrate to the B cell follicles and drive a humoral immune response skewed toward the generation of IgG1 and IgE antibody-secreting plasma cells (19, 20). This antibody response enhances the effector functions of macrophages, mast cells and basophil populations through Fc-mediated clearance of cellular debris and release of histamines and eicosanoids that maintain or enhance gut contractility and intestinal blood flow (21–23). Helminth-specific immunoglobulins have also been shown to directly bind and limit parasite motility (21, 24), the latter being necessary for parasite survival.

The importance of the type 2 immunity in response to tissue injury is underscored by a seminal study by Loke and Allen demonstrating that incision of the peritoneal cavity of mice was sufficient to induce transient IL-4Rα-dependent AAMac polarization (25). This work has been recently supported and expanded upon in human vascular disease (26), a zebrafish model of tissue regeneration (27) and mouse models of acute skin (28), liver (29), and muscle injuries (30) where IL-4/IL-13 signals promote clearance of cellular debris and tissue healing by structural cells and AAMacs. Collectively, these results suggest that type 2 immunity is part of a conserved tissue repair program co-opted to limit tissue damage and support barrier integrity

during helminth infection. For an in-depth examination of type 2 immunity in tissue repair, we refer you to recent reviews (31, 32).

It is important to note, however, that innate responses to the tissue invasive stages of helminth infection may not be exclusively type 2 immune-driven. For example, Klein and colleagues recently demonstrated that following *H. polygyrus* larvae invasion into the duodenal mucosa, production of a quintessential type 1 cytokine, IFNγ, was important for initiating intestinal crypt remodeling and repair of epithelial barrier integrity (33). Additionally, Bradley and colleagues have described substantial variability in response to TLR2 and TLR4 stimulation of blood monocytes isolated from children infected with *A. lumbricoides*, *T. trichiura* or hookworms (34). Nevertheless, fecal egg counts positively correlated with production of "pro-inflammatory" cytokine such as TNFα and IL-1β (34). Thus, early responses to helminth infection may simultaneously involve components of a type 1 and type 2 immune response that not only limit microbial invasion during a helminth-induced barrier breach but also promote tissue repair/regeneration and limit tissue damage, yet have minimal effect on parasite burden.

DISEASE TOLERANCE AS A DEFENSE STRATEGY AGAINST HELMINTHS

The germ theory, posited by Girolamo Fracastoro in the Sixteenth century and proven by Louis Pasteur three hundred years later, stated that microorganisms were the cause of communicable diseases. Although this work led to incredible advancements in our understanding of immunity to infection and the development of antibiotics that have saved millions of lives, it underestimated the diverse functions of microbes in relation to their hosts. It is now well-accepted that mammals have evolved to live in symbiosis with hundreds, if not thousands, of diverse species of bacteria, viruses and fungi (35). Epidemiological data from endemic regions of the world suggest that humans have also developed a mutualistic relationship with helminths. Despite the extraordinarily high prevalence of helminth infection world-wide, the low mortality rate indicates that humans have developed effective strategies, including type 2 immunity, to defend themselves against these parasites. For example, infection with *A. lumbricoides* and *T. trichiura* fail to elicit clinical signs of illness during the intestinal stage of infection except in cases of heavy parasite loads where symptoms likely result from physical obstruction rather than inflammation-induced tissue damage (2). Tolerance to infection is also likely at play in wild rodents as Behnke et al found that at least one of the roundworms *T. spiralis*, *H. polygyrus* and *T. muris* were present in 90% of wild mice (36). Follow up studies found that trickle infection (repeated administration of <40 larvae) of laboratory mice with *H. polygyrus*, the most common helminth of wild mice, led to asymptomatic chronic infection (37).

Additional evidence that tolerance is an important form of defense against helminth infection are epidemiological studies of "dewormed" human populations (2). Although anthelmintics are very effective at eliminating the primary infection, resistance to re-infection has been rarely observed (2). However, these results have not borne out in laboratory studies of mice as protective immunity to re-infection by the same or heterologous helminth infection can be readily achieved (15, 38). Although the reasons for these disparate results are not entirely clear, one explanation may be the much higher infectious dose typically used in the laboratory setting (>200 larvae or eggs) compared to a lower and repeated trickle infection scenario that occurs in nature. In support of this suggestion, a primary high dose challenge with *T. muris* eggs and *H. polygyrus* larvae promotes worm expulsion whereas lower doses lead to stable or chronic infection (37, 39).

The failure to develop protective immunity to helminths, at least in natural settings of infection, are in part due to the life cycle of the parasite. First, although the specific cell types that are damaged during helminth migration through the intestine are not well-characterized, increased apoptosis of intestinal epithelial cells has been reported during the tissue invasion stage of *H. polygyrus* and *T. muris* infection (11, 40), a process that may simultaneously promote chronic infection and amplify the tissue repair program (discussed in more detail below). Second, type 1 cytokine production including IL-1β and IFNγ that occurs during both *H. polygyrus* and low dose *T. muris* infection (presumably occurring as a result of inflammation-induced cell death or induction of toll-like receptor signals by microbial antigens following a breach in the intestinal barrier) promotes chronic helminth infection by limiting early induction of a protective type 2 response via inhibition of ILC2s and/or Th2 cell activation (39, 41). This work calls for further use of established *in vitro* protocols (42) or the development of new models such as organoid cultures or "tissue-on-a-chip" methodology to allow for more detailed studies on the types of cell stress and/or death that helminths impose on stromal cells and leukocytes. Third, most pathogenic microorganisms including bacteria, fungi, viruses and protozoa possess virulence factors that have direct cytotoxic effects to mediate replication and, ultimately, dissemination. By contrast, intestinal helminths (with the exception of some *Strongyloides spp.*) do not replicate within the host to propagate their species. However, they must reside in the intestine long enough to mature to an egg-laying stage to continue their life cycle. Identifying the fundamental processes of cell and tissue stress that structural components of the intestine undergo to promote an environment hospitable for worm growth will provide a foundation for a more in-depth understanding of disease tolerance to helminth infection.

Several lines of evidence indicate that the benefits of tolerating helminth infection may outweigh the costs in terms of host fitness. For example, type 2 cytokine-mediated goblet cell hyperplasia and expansion of *Clostridia* species in mice infected with *T. muris* can protect susceptible hosts against intestinal inflammation and immunopathology driven by pathobiotic species of *Bacteroides* (43). The same study found that deworming of humans living in regions endemic with helminth infection was associated with an increased *Bacteroides/Clostridia* ratio. Furthermore, in a study of tolerance to macroparasites in a wild vole population, *Gata3* expression—encoding for a transcription factor required for Th2 cell differentiation and ILC2 development and maintenance (44)—by splenocytes and circulating cells was positively associated with parasite burden,

animal size and lifespan in older male animals (34). Because the study population was infected with multiple species of macroparasites (e.g., mites, worms, etc), these results cannot causally link helminth infection *per se* to the observed effects on host fitness. Nevertheless, they provide evidence in a natural setting for type 2 immunity in disease tolerance and point to the considerable ecological importance of this defense strategy in wild mammals (45). These results are part and parcel to the concept of concomitant immunity in which the prevention of sterile immunity to one parasite prevents subsequent infection by the same or heterologous pathogens, a phenomenon observed for helminthiasis and other parasitic infections (46, 47). It will be important to determine whether helminths regulate concomitant immunity to other micro- or macroorganisms that impact host health.

In addition to the impact of helminths on intestinal health, their effects extend beyond the gut. Examining a population of Soay sheep in northern Scotland, Hayward et al demonstrated that the amount of weight loss in response to *Strongyloides* burden (as determined by fecal egg counts) was negatively associated with lifetime breeding success (48). These studies suggest that helminth infection may promote the selection of "fitness traits" in mammals. Although the mechanism behind these observations are unclear, helminth infection has important effects on systemic metabolism that may have direct or indirect effects on fecundity. For example, infection of mice with *N. brasiliensis* stimulates the recruitment of IL-4 producing eosinophils to adipose tissue that promote insulin sensitivity and tolerance to glucose (49). Similarly, infection with soil-transmitted helminths including *A. lumbricoides*, *T. trichiura,* and *N. americanus* has been associated with increased insulin sensitivity (50). As type 2 cytokine production by ILC2s has also been shown to sustain adipose tissue macrophages that regulate thermogenesis and beige fat production (51, 52), it is possible that tolerance to helminth infection evolved to complement physiological mechanisms of core body temperature and metabolic stability in times of variable food abundance and changing seasons in migratory animal species.

MEASURING DISEASE TOLERANCE DURING HELMINTH INFECTION

The statistical framework for analysis of disease tolerance was initially established in plants in which a "reaction norm" to infection was developed (53). This approach has been more recently supported by Råberg and colleagues as a method to assess disease tolerance in animals (54, 55). A reaction norm in disease tolerance is defined as the health of an animal (or group of animals) across different environments (i.e., pathogen loads) (54). The results can then be plotted with an increasing slope being interpreted as a decrease in tolerance (**Figure 1A**). This methodology distinguishes tolerance from what Råberg et al refers as "general vigor" or any differences in baseline fitness that may be masked when examining pathogen burden at one point in time (**Figure 1B**). These strict measures of disease tolerance are difficult to quantify in humans but can be carefully examined in experimental systems in which the response to infection can be assessed over time and infection intensity. As most parasitic worms do not replicate in their definitive hosts, experimental models in which a given number of infectious eggs or larvae are administered results in the same number of adult worms. This relatively stable parasite number provides an excellent opportunity to study changes in tolerance to infection. Nevertheless, it remains important to verify parasite load at different time points or at various infectious doses as persistence of infection can vary depending on the genetic background and environment of the host species (8). A current obstacle in studying host fitness to helminthiasis is that most infections do not elicit such robust clinical phenotypes commonly used as research outcomes such as weight loss, lethargy, or death. In future studies it will be important to expand the breadth and depth of fitness measures (e.g., intestinal and peripheral organ function, serum metabolites, behaviorial abnormalities, etc) in laboratory or natural settings of infection to more effectively define changes to host physiology and better model the co-morbidities associated with human helminth infection.

PARASITE-DERIVED MECHANISMS THAT PROMOTE TOLERANCE

An important feature of helminths is their potent excretory/secretory (ES) system that not only promotes tissue invasion and acceptance by the host of a large foreign body, but also enhances wound repair, tissue remodeling and evasion or blunting of the inflammatory response (56). These strategies range from inhibition of immune cell signaling (57) to blockade of antigen presenting cell migration (58, 59) to protease secretion that degrade parasite-trapping antibodies (60). Perhaps the most studied mechanism of immunomodulation by helminths is their ability to alter the stimulatory capacity of DCs while enhancing the generation and function of CD4+ T cells with regulatory function (Foxp3$^+$ Tregs or IL-10-producing Foxp3$^-$ Tr1 cells) (61–63). For example, DCs conditioned with ES products from diverse types of parasitic helminths can promote Th2 responses and limit Th1 responses while also promoting anti-inflammatory IL-10 producing T cells and Tregs (62, 64, 65). Importantly, Treg cells have been shown to promote tissue repair independent of their immunosuppressive abilities via production of amphiregulin, a member of the epidermal growth factor family of cytokines also produced by ILC2s and Th2 cells during helminth infection (66–68). Consistent with these results, several groups have demonstrated that expansion of Treg cells in *T. muris*-infected mice protects from intestinal pathology (69, 70). Moreover, depletion of Foxp3$^+$ Treg cells in *H. polygyrus*-infected animals either did not affect or, in some cases, increased adult worm burden and led to increased morbidity and mortality (70, 71). Collectively, these data support a key role for helminth-induced Treg cells in disease tolerance during helminth infection. It has been additionally determined that helminths can promote Treg expansion directly or indirectly through inducing the production of the regulatory cytokine TGFβ (63). TGFβ is a critical component of the wound repair

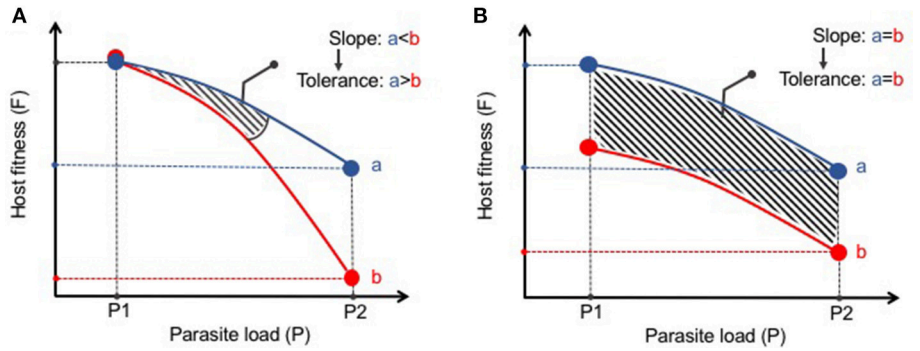

FIGURE 1 | Distinguishing disease tolerance from general vigor during helminth infection. **(A)** Graphical representation of a reaction norm (slope of the curve) where, despite similar starting points (P1), Host B has a greater loss in fitness (i.e., increased morbidity) with increasing parasite load than Host A. Thus, Host A displays greater disease tolerance to infection than Host B. **(B)** Despite differences in host fitness across various parasite loads, the reaction norm between Hosts A and B remains the same. Thus, there is no differences in disease tolerance between A and B, only a difference in general vigor.

program and promotes the generation of non-stimulatory, tolerogenic DCs (72, 73). Interestingly, *H. polygyrus* secretes a TGFβ mimic that uses canonical SMAD-dependent signaling to promote Treg differentiation while simultaneously enhancing parasite colonization (63). The combined effects of TGFβ signaling on DC function, Treg induction and tissue repair may place TGFβ at a critical nexus of tolerance to helminth infection. Along with TGFβ, another cytokine generally associated with dampening inflammation, IL-10, has been shown to be increased in the context of many helminth infections and limit immunopathology (74, 75). Therefore, helminths have evolved various immune regulatory pathways which have drawn increasing interest for their potential as novel therapies for the treatment of autoimmune and other chronic inflammatory diseases (76).

HELMINTH-MICROBIOTA CROSSTALK AMPLIFY THE IMMUNOREGULATORY RESPONSE IN THE INTESTINE

As intestinal helminths co-habitate with the most abundant and diverse microbial community in the host, important interactions occur between these organisms that reside in the same niche (77). Although commensal bacteria and multicellular helminths occupy very different taxonomic space, they have both responded to evolutionary forces by developing strategies of host immunomodulation. Moreover, it is apparent that these different kingdoms of life have developed a surprising degree of dialogue with a common agenda of establishing a new homeostasis in the host intestinal tract (78). For example, *T. muris* migrates to the proximal colon, the site of greatest bacterial abundance in mammals, where they exploit commensal bacteria for egg hatching and adult worm development (79). In turn, *T. muris* infection alters the gut microbiota and promotes resistance against pathogenic bacteria, an effect dependent on the induction of a type 2 immune response (43). However, initial reports investigating the impact of human *T. trichiura* infection on the composition and function of the gut microbiota have provided mixed results (80, 81). Fricke et al. also reported that a type 2 immune response following *N. brasiliensis* infection in mice reduced abundance of segmented filamentous bacteria (SFB) in the small intestine compared to uninfected controls (82). SFB is a potent inducer of IL-17 production by murine T cells, an immune pathway shown to exacerbate tissue damage at the expense of limiting worm burden (82). In complementary studies, Walk et al. found that *H. polygyrus* infection increased the abundance of *Lactobacillaceae*, a family of lactic-acid producing bacteria with established anti-inflammatory and immune suppressive effects (83). Additionally, helminths could also mediate metabolic changes of the commensal bacteria that promote immunoregulatory functions. Indeed, Zaiss et al. demonstrated that *H. polygyrus* infection enhanced the production of short chain fatty acids (SCFAs) by the intestinal bacteria that have potent ability to amplify Treg cell differentiation (84). In summary, experimental models indicate that helminths and the microbiota influence each other's ability to persist in the mammalian intestinal tract and potentially dampen unwanted inflammatory responses in the intestine. Although studies are emerging that support an impact of helminth on the human gut microbiota, more studies are needed to provide a causal relationship and its impact on tolerance to homologous or heterologous co-infection.

INTESTINAL PHYSIOLOGY SHAPES DISEASE TOLERANCE TO INTESTINAL HELMINTHS

The induction of a type 2 immune response to repair tissue damage can require days to take action. However, intestinal helminths can invade host tissues within the first hours of infection. Thus, the intestine must have intrinsic properties that protect its vital functions prior to a robust immune response. An examination of intestinal physiology may help understand mechanisms by which these organisms parasitize their host

niches and inform us about how hosts evolved to tolerate infection.

Phylogenetic studies indicate that parasitic nematodes diverged from their free-living ancestors at least five times during the course of evolution (85). A parasitic lifestyle may have been exploited by helminths during evolution to avoid predators, obtain a consistent source of nutrients and increase fecundity. The conservation of larval developmental stages and the stimuli that promote maturation of diverse nematode species supports this proposition. For example, *in vitro* studies have demonstrated that cholesterol derivatives such as 3-keto bile acid-like steroids (e.g., dafachronic acid) inhibit a state of dormancy (referred to as the dauer stage) at the L3 larval stage in both free-living (*Caenorhabditis elegans*) and parasitic nematode species (e.g., *Strongyloides spp.*) and promote maturation to an adult egg-laying stage (86). Similar bile acid components secreted in the duodenum and re-absorbed in the ileum may provide important cues for larval development *in vivo* (87) while simultaneously possessing immunomodulatory properties. Human intestinal macrophages express the g-protein coupled bile acid receptor TGR5 (i.e. GPR131), expression of which can be enhanced by inflammatory cues such as IFNγ (88). Complementary studies in mice have found that, upon ligand binding, TGR5 activates an AKT-mTOR-dependent pathway that limits toll-like receptor signals and promotes an anti-inflammatory phenotype characterized by increased secretion of IL-10 and decreased production of TNFα (89). Whether a bile acid-macrophage axis contributes to disease tolerance during helminth infection is unknown. In addition, many adult worms feed on host tissue and the rapid turnover of epithelial cells, which is further enhanced during inflammation and infection, provides a rich source of food without directly compromising the integrity of the intestinal barrier. Interestingly, artificially increasing the rate of intestinal epithelial cell death in the absence of overt infection leads to a downregulation of pattern recognition receptors by mononuclear phagocytes and amplifies an anti-inflammatory transcriptional profile of efferocytosing CD64+ gut macrophages including upregulation of TAM family members *Axl* and *Mer* (90). TAM members are not only involved in apoptotic cell sensing but, in the presence of the type 2 cytokines IL-4 or IL-13, enhanced the tissue repair response during pulmonary *N. brasiliensis* infection and experimental colitis (91). Increased IEC apoptosis also increased the ability of CD103+ dendritic cells to induce CD4+ T regulatory cells, a population shown to expand during *H. polygyrus* infection and limit tissue damage without affecting worm burden as mentioned above (91, 92).

Although nutrient availability has been shown to play an important role in tolerance and resistance to bacterial and viral infections, how host or parasite-derived nutrients impacts tolerance to helminthiasis is only beginning to be understood. However, the sharing of (or competition for) metabolites between the host and parasite is not without precedent as iron metabolism by macrophages promotes a "tissue-healing" phenotype during infection whereas blood feeding is an important energy source for the hookworms *N. americanus* and *Ancylostoma duodenale* (93). Whether nutritional immunity contributes to disease tolerance to helminth infection is not well understood. Although competition for nutrients between host cells and the parasite may, in most cases, promote symbiosis, heavy worm burdens may shift the balance toward pathology that lead to co-morbidities associated with helminth infection including malnutrition-induced growth-stunting or cognitive dysfunction. Conversely, an increased consumption of nutrients such as arginine by local infiltrating immune cells that double as worm growth factors, could limit parasite survival. Thus, a metabolic tug-of-war may be a critical mediator of host-parasite co-evolution that has promoted host tolerance to parasitism.

Another key component of intestinal physiology is the mucus barrier that lines the length of the intestine. This viscous sheet of glycoproteins covers the epithelium and contains a multitude of viruses (e.g., bacteriophages) and anti-microbial peptides derived from IECs that are toxic to invading bacteria and the commensal microbiota (94, 95). In addition, one of the most abundant mucus proteins is a gel-forming mucin called Muc2 (96). Consistent with the tolerant phenotype of the intestine, resident DCs proximal to the mucus barrier of the small intestine constitutively sample Muc2 through a Galectin-3–Dectin-1–FcγRIIB receptor complex (96). Signaling via this receptor complex inhibits IL-12 production, increases IL-10 and TGFβ production and enhances retinoic acid metabolism by DCs. As a result, Muc2 was able to limit inflammation in a model of experimental colitis as well as promote oral tolerance via induction of Treg cells (96). Although the type 2 cytokine IL-13 produced by ILC2s and Th2 cells is a potent inducer of mucus production by goblet cells and facilitates the "weep and sweep" that can contribute to worm expulsion, the inherent properties of mucus may act as a first line of defense to not only limit bacterial invasion but play an active role in immunomodulation during helminth infection. Collectively, these results suggest that the unique physiology of the intestine complements a type 2 immune response that together provides a highly tolerant ecosystem for host-parasite mutualism and disease tolerance to helminth infection (**Figure 2**).

CONCLUDING REMARKS

Historically, parasitism has been thought to be solely detrimental: the parasite benefits at the expense of host health, with only one "winner" emerging from this interaction. Therefore, developing resistance to these invaders was the conceptual framework that led to great advances in understanding type 2 immunity and its relation to anti-helminth immunity. However, adapting concepts of host defense from studies in plants to a rodent model of malaria infection, Råberg et al. demonstrated that genetic variation in mice can dictate susceptibility to infection without appreciable effects on parasite burden (55). This demonstration of disease tolerance in mammals has now set the stage for investigating the relevance of disease tolerance in other settings of infection. Given that helminth infection almost universally activates type 2 immune pathways yet does not necessarily lead to resistance or protective immunity to re-infection suggests that tolerance is an important, mode of host defense to this unique class of parasitic infection. Although the global morbidity resulting from parasitic helminth infection cannot

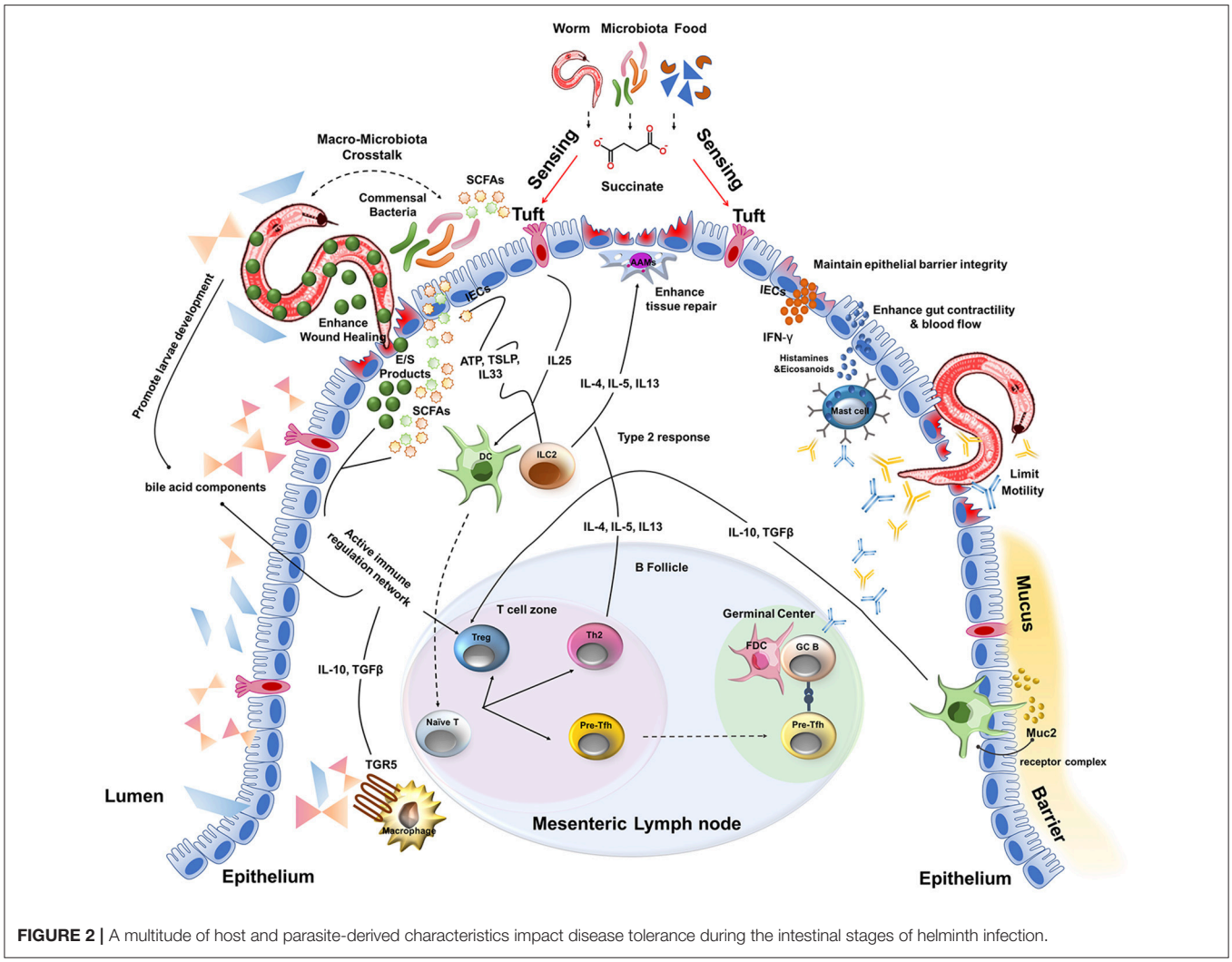

FIGURE 2 | A multitude of host and parasite-derived characteristics impact disease tolerance during the intestinal stages of helminth infection.

be understated, increasing evidence suggests that, under certain conditions, helminths may provide a benefit to host fitness. Combining the potential advantages of helminth infection for both host and parasite with the observation that type 2 immunity is a fundamental component of the mammalian response to tissue injury provides a rich example of adaptation between host and parasite that maximizes the survival of both species. Indeed, it is suggested that helminths have interacted with the vertebrate immune system for hundreds of millions of years thus likely shaping the characteristics of both (7, 97). As opposed to other cytokine signaling networks, no individuals have been identified that possess loss-of-function mutations in IL-4Rα or STAT6, the common receptor subunit and downstream transcription factor required for IL-4 and IL-13-induced gene expression. These observations make it tempting to speculate that trait selection is based, in part, on adaptation to helminth infection.

Tolerance to helminth infection also corresponds well with the "hygiene hypothesis" (and the expanded "old friend's hypothesis") suggesting that diminished exposure to infections or decreased diversity of commensal microorganisms has led to an increased prevalence of allergic (and potentially autoimmune) disease because of defective regulation of the immune system in early life (98). Going forward, a more complete picture of helminth–microbiota interactions and their effects on the host will certainly yield new approaches for the treatment of these "diseases of the developed world". It will also be important to identify the specific types of tissue damage and cell stress imposed by intestinal helminth infection to understand how the host limits tissue damage and initiates a repair process that is critical for tolerance. Moreover, further investigations will be required to understand the role of intestinal physiology on susceptibility to helminth invasion and its ability to simultaneously minimize immune-driven pathology in the context of tissue damage, a body of knowledge that could be applied to diverse settings of tissue infection and injury. Given the pleiotropic effects that helminths have on the immune system and on host health, tolerance to these parasites may have evolved to provide a unique form of "physiological inflammation" so elegantly conceptualized by Mechnikov over 100 years ago (99).

AUTHOR CONTRIBUTIONS

YL constructed the figures and wrote the manuscript. ILK conceived of the topic and wrote the manuscript.

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REVIEW
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Helminth Infections Induce Tissue Tolerance Mitigating Immunopathology but Enhancing Microbial Pathogen Susceptibility

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Helminths are ubiquitous and have chronically infected vertebrates throughout their evolution. As such helminths have likely exerted considerable selection pressure on our immune systems. The large size of multicellular helminths and their limited replicative capacity in the host necessarily elicits different host protective mechanisms than the immune response evoked by microbial pathogens such as bacteria, viruses and intracellular parasites. The cellular damage resulting from helminth migration through tissues is a major trigger of the type 2 and regulatory immune responses, which activates wound repair mechanisms that increases tissue tolerance to injury and resistance mechanisms that enhance resistance to further colonization with larval stages. While these wound healing and anti-inflammatory responses may be beneficial to the helminth infected host, they may also compromise the host's ability to mount protective immune responses to microbial pathogens. In this review we will first describe helminth-induced tolerance mechanisms that develop in specific organs including the lung and the intestine, and how adaptive immunity may contribute to these responses through differential activation of T cells in the secondary lymphoid organs. We will then integrate studies that have examined how the immune response is modulated in these specific tissues during coinfection of helminths with viruses, protozoa, and bacteria.

Keywords: tolerance, helminth, resistance, confection, immune, injury, microbes

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INTRODUCTION

Helminths are ubiquitous and have chronically infected vertebrates throughout their evolution. A number of studies have shown that they can severely impact wild vertebrate populations affecting their body weight, fecundity and their ability to survive hardship in the winter (1–3). In humans, low-level infections can be asymptomatic, but more heavily infected individuals are adversely affected, exhibiting morbidity in adults and impaired physical and cognitive development in children (4–6). As such helminths have likely exerted considerable selection pressure on our immune systems. The large size of multicellular helminths necessarily requires different host protective mechanisms than the immune response evoked by microbial pathogens such as bacteria and viruses. Also, the immune response to microbial pathogens includes mechanisms that limit reproduction and associated dissemination of the microbe. Such controls are unnecessary with

many helminths, as they need to leave the mammalian host to complete their life cycles. Instead, components of the host protective responses against helminths include coopted wound repair mechanisms, which mitigate the considerable tissue damage these parasites may cause as they traffic through vital organs such as the lung and liver (7, 8). These innate wound healing responses contribute to the type 2 immune response evoked by helminths, and provide a critical springboard for the subsequent adaptive immune response including antigen-specific effector T and B lymphocytes. This cellular damage resulting from helminth migration through tissues is a major trigger of the type 2 immune response, as danger associated molecular patterns (DAMPs) are released that induce the cytokine alarmins, IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), that help drive the response. In contrast, the type 1 immune response may be more dependent on pathogen associated molecular patterns (PAMPS), where microbial structures, such as endotoxin, bind toll like receptors (TLRs) that help drive the initiation of the response, resulting in IL-12 production by myeloid cells, which in turn drives IFN-γ production by innate lymphoid cells (ILCs) and T cells. The overall protective type 2 immune response that ensues includes both resistance and tolerance mechanisms. Resistance immune mechanisms specifically impact the parasite and when effective reduce the parasite burden. Tolerance mechanisms reduce host tissue damage without affecting the parasite burden (3, 9).

The helminth induced type 2 immune response includes characteristic activation of immune cells. Although these activated immune cell lineages share stimulation of common signaling pathways, they also exhibit lineage specific activation states which support their characteristic effector functions. This type 2 immune cell activation motif was originally described in CD4+ Th2 cells, but it is now clear that this characteristic activation, also referred to as alternative activation, occurs in other T cells and also B cells, innate lymphocytes, mast cells, macrophages, basophils, eosinophils, and neutrophils (10–12). Unraveling how helminth infection differentially affects these innate and adaptive immune cells is as yet little understood and likely involves various epigenetic regulatory mechanisms. In many of these cells alternative activation is associated with the production of type 2 cytokines, including IL-4, IL-5, and IL-13, with different cell lineages preferentially expressing one or more of these cytokines. A range of other molecules are also associated with this alternative activation state including: arginase, Relmalpha, YM-1, IL-33, and several chemokines. In contrast, immune cells activated by microbial pathogens express chemokines and cytokines associated with type 1 and type 17 immunity including: IL-12, IFN-γ, IL-17, NOS2, and TNF-α. High levels of either type 1 or type 17 cytokines can result in harmful inflammation leading to tissue damage. Helminth induced immune responses also have immune regulatory components that include activation of FOXP3+ T regulatory cell responses, which can function to control harmful inflammation through their production of IL-10 (13). Although not specific to type 2 immune responses, IL-10 upregulation during helminth infection appears to have an important role in downregulating both type 1 and type 2 immunity. IL-10 independent immune regulatory effects have also been identified, which are not yet well defined (14, 15). Although the type 2 immune response has important wound healing characteristics, chronic type 2 inflammation can also be harmful, leading to fibrosis and associated tissue damage (16). It should be noted that immune regulatory cells activated during helminth infection have also been shown to inhibit chronic type 2 responses, including allergy-associated inflammation (17).

The helminth-induced type 2 immune response thus has important wound healing and anti-inflammatory properties. However, this beneficial response that helps to mediate tolerance by mitigating tissue damage during infection with these large multicellular parasites can have a dark side as well. Many properties of this immune response can potentially reduce the effectiveness of the protective response against many microbial pathogens. As coinfection with helminths and microbes affects much of the world's population, this as yet little studied area of research has considerable real world significance. In this review we will first describe helminth-induced tolerance mechanisms that develop in specific organs including the lung and the intestine, and how adaptive imunity may contribute to these responses through differential activation of T cells in the lymph nodes. With this background, we will review studies that have examined how the immune response is modulated in these specific tissues during coinfection of helminths with viruses, protozoa, and bacteria.

Helminth-Induced Immune and Tissue Responses in the Lung

Extensive and specific remodeling of tissues and associated organs can occur following invasion by specific pathogens. In turn, subsequent or even coincident coinfection by a different pathogen can markedly alter the course of the response in some cases compromising resistance and tolerance mechanisms directed against either pathogen. Recent studies have begun to unravel the mechanisms through which the intestinal nematode parasite, *Nippostrongylus brasiliensis*, influences lung tissue. As with several other intestinal nematode parasites, including human hookworms, *N. brasiliensis* larvae invade the host through skin penetration, migrate through the circulation to the lung, where they are coughed up and swallowed. Once in the intestine they mature to adults, breed and produce eggs. Following *N. brasiliensis* primary infection, the parasites enter the lung between 12 and 48 h after inoculation, and usually exit the lung 48 h later. Thus by 3–4 days after inoculation all the parasites have left the lung. This 2 day time interval in the lung triggers a cascade of immune responses that initially triggers acute lung injury (ALI), followed by rapid mitigation of lung damage, and finally subsequent chronic lung remodeling associated with fibrosis and emphysema. Understanding the immune components of this lung remodeling response has elucidated a number of tolerance mechanisms associated with the type 2 immune response.

As early as 1–2 days after *N. brasiliensis* inoculation, a pronounced increase in IL-17 triggers massive recruitment of

neutrophils to the lung peaking at about 1×10^6 total cells by day 2 (7). Further studies have shown that the source of IL-17 is γ/δ T cells, which are activated by chitinase-like proteins (CLPs) released by lung epithelial cells damaged by the invading larval parasites (18, 19). Thus, the CLPs are essentially acting as DAMPs triggering the initial inflammatory response. The infiltrating neutrophils contribute to ALI associated with hemorrhaging, inflammation, and impaired lung function. Mechanical damage by the helminth itself is also a factor contributing to ALI, which is pronounced by about 3 days after inoculation. The type 2 immune response, characteristic of helminth infections, becomes pronounced by day 4 and its increase coincides with a decrease in IL-17 and ALI. Blocking IL-4R signaling inhibits the development of type 2 immunity and results in sustained IL-17 elevations, neutrophil inflammation, and associated ALI (7). These studies thus demonstrated that IL-4R signaling can play an essential role in mitigating tissue damage during helminth infections.

An essential myeloid cell type activated at early stages of the type 2 immune response is the alternatively activated macrophage (AAM). As IL10 is not elevated at early stages of the response, IL-4R signaling is the major trigger and the helminth-activated macrophage also does not produce IL-10 (7, 20). However, macrophages activated through helminth infection do express a number of factors important in both control of inflammation and in directly enhancing the wound healing process. These include: insulin-like growth factor (IGF-1), Resistin-like molecule α (RELMα), and arginase 1 (Arg.1), all of which are IL-4R dependent (7, 21). RELMα and Arg. 1 have pleiotropic effects, the former being capable of both downmodulating type 2 immune responses (22, 23) and directly enhancing wound healing (24). Arg1, in addition to catalyzing arginine metabolism which results in the production of ornithine and polyamines, also can downmodulate type 1 inflammation by depleting local arginine concentrations (25). In the lung, besides IL-4Rα signaling, AAM activation and proliferation is also dependent on other factors characteristic of the lung microenvironment and the specific infectious agent. In particular, recent studies have shown that the infiltrating alternatively activated (N2) neutrophils interact with the macrophages to drive their alternatively activated phenotype. This includes both their production of IL-13 (11) and their apoptotic state which is recognized by AXL/Mertk, apoptotic sensors expressed by the macrophage (26). In addition, surfactant protein A (SPA) expressed by lung epithelial cells also drives AAM activation and proliferation (27). Thus, both myeloid cell crosstalk and the local tissue microenvironment provide critical cues driving AAM activation in the lung during helminth infection. Other myeloid and also innate lymphoid cell (ILC) populations also likely play an important role in orchestrating initiation of the type 2 immune response and mitigation of ALI. In particular, at early stages of the response ILC2 cells may provide an initial source of IL-13 and potentially other factors that drives the development of infiltrating N2 neutrophils and other components of the innate type 2 response.

The above model (see **Figure 1**) describes helminth induced tolerance mechanisms that mitigate ALI. As a result ALI is largely resolved by 5–7 days after inoculation. However, despite the presence of the parasite in the lung for only 48 h, chronic tissue remodeling also occurs that can persist for weeks after *N. brasiliensis* inoculation. Previous studies have shown that M2 macrophages persist in the lung for at least 45 days after inoculation, and this persistent macrophage phenotype is capable of mediating acquired resistance resulting in accelerated parasite destruction upon secondary challenge (11). Also emphysema develops by day 30 after inoculation and significant fibrosis is also observed. Emphysema apparently requires infection with live parasites, as *N. brasiliensis* excretory/secretory (ES) products, which still induce type 2 responses, can drive fibrosis and associated impaired lung function, but not emphysema (28). Few studies have yet examined mechanisms contributing to emphysema following helminth infection, but secretion of proteases and elastases by myeloid cells may be causal in other emphysema models (29, 30). Intriguingly, IL-17 is also implicated in emphysema development (31, 32), raising the possibility that early elevations in IL-17 in the response to *N. brasiliensis* may trigger this specific lung tissue remodeling pathology, perhaps in part by its recruitment of neutrophils to the lung.

The type 2 immune signaling pathways activated by helminth infection in the lung may influence responses to other pathogens. The persistence of this immune milieu, as indicated by the long-lived AAM phenotype, may potentially

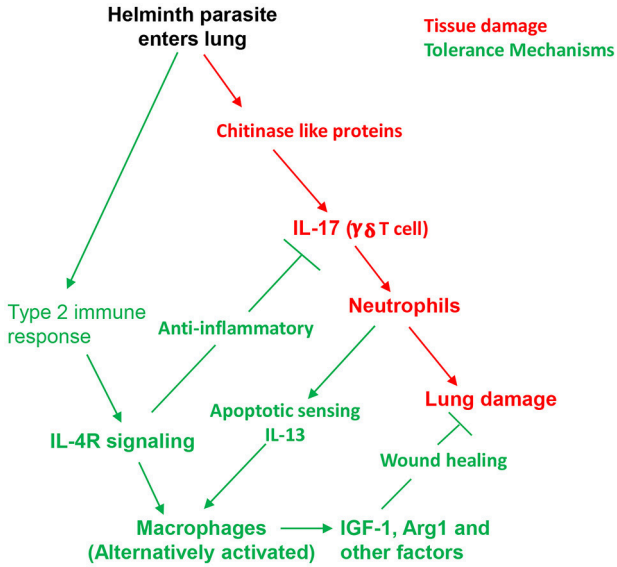

FIGURE 1 | Host tolerance mechanisms contribute to protective helminth-induced type 2 response by controlling lung damage. Invasion of the lung by *N. brasiliensis* L3 triggers release of chitinase-like proteins which stimulate IL-17 production by γδ T cells and consequent recruitment of neutrophils. Inflammation and physical damage of cells by migrating helminths result in acute lung injury. Within several days a potent type 2 immune response is also induced and tolerance mechanisms dependent on IL-4R signaling inhibit IL-17. Combined signals from neutrophils, lung surfactant protein A (SPA), and direct IL-4R signaling drives alternative macrophage activation, which contributes to both anti-inflammatory and direct wound repair processes.

delay or attenuate development of a type 1 immune response important in resistance against many microbial pathogens. Studies where *N. brasiliensis* inoculated mice were coinfected with *Mycobacterium tuberculosis* showed generally increased susceptibility to this intracellular bacteria, which was IL-4R dependent and transfer of WT macrophages into IL-4R$^{-/-}$ mice restored helminth-induced susceptibility. Interestingly, however, the protective Mtb-specific Th1 cellular response was not impaired, although marked increases in AAMs were observed. Apparently, the presence of AAMs compromised effective elimination of bacteria, possibly as a result of their impaired Mtb killing and potential function as an Mtb reservoir (33). The observation that Th1 cells still developed in response to Mtb raises the intriguing possibility that type 1 and type 2 pulmonary immune responses simultaneously develop in coinfected mice and that helminth infection cannot completely override the type 1 immune response triggered by MTb, even though in this model helminth infection preceded Mtb infection by 5 days. More studies are needed to ascertain whether this apparent plasticity within the lung microenvironment is due to different separable microenvironments supporting polarized type 1 and type 2 responses or whether both polarized immune cell populations coexist in granulomas and immune cell infiltrates. Analysis of individual cells using techniques such as single cell RNAseq may also potentially reveal mixed response heterogeneity in individual myeloid cell populations.

Similar results have also recently been obtained following coinfection with the malarial parasite, *Plasmodium berghei*, and *N. brasiliensis*. In these experiments a sequential protocol was also used where *N. brasiliensis* infection preceded malarial infection by about 2 weeks. Although helminth infection blunted the protective type 1 immune response, it still was sufficiently strong to mediate effective resistance against the malarial parasite (34). In another study where mice were infected simultaneously with *N. brasiliensis* and *P. chabaudi*, type 1 immunity was not affected while type 2 cytokines were attenuated (35). However, as *N. brasiliensis* is an acute infection, with parasites residing in the host for only about 9 days, sequential or simultaneous malarial infection may not as readily modify the response as a chronic infection where the parasite persists and provides ongoing stimulation in the host. This may be in part due to plasticity in the T cell compartment, with recent studies indicating that malarial infection can rewire helminth induced Th2 cells, downmodulating their production of type 2 cytokines with a concomitant upregulation of IFN-γ (36). Also, as discussed above the initial immune response to *N. brasiliensis* is complex and includes pronounced IL-17 elevations, which may indeed be exacerbated by *Plasmodium* infections and may thus impact resistance and tolerance mechanisms. Chronic infections with other parasitic helminths including *Heligmosomoides polygyrus*, *Litosomoides sigmondontis*, or *Schistosoma mansoni* eventually result in a more polarized and potent type 2 immune response. In coinfection studies with these parasites, type 1 immunity and associated resistance is generally reduced, while tissue damage is mitigated (37). Thus, chronic coinfection of helminths and malarial parasites may at least in some cases impair resistance but at the same time enhance tolerance mechanisms.

Helminth-Induced Immune and Tissue Responses in the Gut

Initiation of the Response

The type 2 immune response triggered in the intestine by helminth infection (see **Figure 2**) is characterized by many of the same immune cell populations observed in lung mucosal tissues, including AAMs, differentially activated granulocytes, ILC-2s, and Th2 cells. Of course many intestinal helminths invade the skin and transit through the lungs on route to the intestine. What actually triggers the type 2 immune response in the gut is still not well understood, but it appears that it is partly triggered by endogenous danger associated molecular patterns (DAMPS) induced by tissue damage resulting from these large multicellular parasites interacting with the intestinal barrier surface.

Recent studies indicate that after infection with the murine intestinal nematode parasite *Heligmosomoides polygyrus*, adenosine interacting with the A2B adenosine receptor (A2BAR) is required for upregulation of IL-33 and the corresponding downstream type 2 immune response (38). It should be noted that the mechanism through which IL-33 works may be complex as it both binds cell surface ST2 (suppressor of tumorigenicity 2), a component of the IL-33 receptor, and also enters the nucleus as a regulatory protein (39). However, recent studies indicate that blockade of ST2 can inhibit the type 2 immune response to *H. polygyrus* (40) and that mast cells are an important source of IL-33 (41). Presumably tissue damage results in ATP release from stressed cells. The extracellular ATP is then degraded by cell surface ectonucleotidases to adenosine, which then locally accumulates extracellularly, binds cell surface A2BAR, and contributes to initiation of type 2 immunity. As such, adenosine functions as a DAMP during intestinal helminth infection alerting the host to tissue damage associated with the helminth infection (38). An epithelial cell derived molecule, trefoil factor 2 (TFF2), which can mediate tissue repair functions, has also been shown to act as a helminth-induced DAMP capable of driving initiation of the type 2 immune response through stimulation

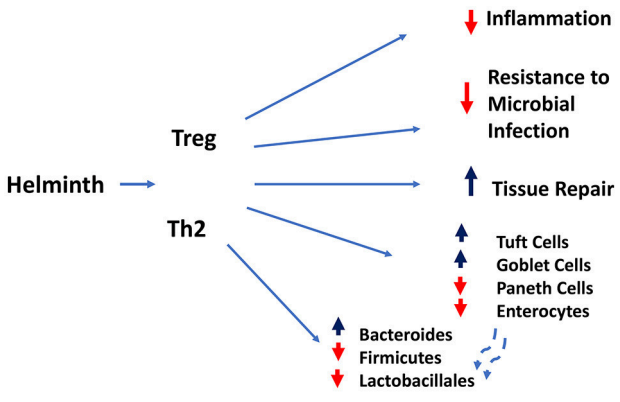

FIGURE 2 | Regulatory and type 2 immune responses (mediated by T lymphocytes and other cell types) induced by helminth infection mediate dampening of inflammatory responses and compromise resistance to microbial infection, while increasing goblet and tuft cell hyperplasia and intestinal tissue repair.

of IL-33 release (42). It will be important in future studies to investigate potential interactions and/or associations of TFF2 with A2BAR signaling as both seem to play a critical role in driving type 2 response to helminths.

Tuft cells, specialized intestinal epithelial cells, appear to recognize helminth infection through chemosensory receptor signaling. perhaps providing a mechanism for how ES products may contribute to initiation of the type 2 immune response Tuft cells are the sole producers of IL-25 in the intestine and IL-25 is essential for the type 2 immune response to H. polygyrus (43–45). Together IL-33 and IL-25 likely support the development of the innate type 2 immune response to helminths with one or the other playing a more predominant role in the development of the protective response to specific helminths. IL-33 and IL-25 can be considered cytokine alarmins, as they are the initial cytokines that signal invasion by helminths and trigger the appropriate host response. A third cytokine alarmin associated with type 2 immunity is thymic stromal lymphopoietin (TSLP), also produced by intestinal epithelial cells. In the context of helminth infection, rather than triggering type 2 immunity, TSLP appears to downregulate type 1 responses, which is particularly important in the colonic immune response to *Trichuris muris* where an underlying type 1 response, likely elicited by bacteria associated with *T. muris* invasion, is controlled by TSLP (46). In the mouse response to Schistosome infection, IL-33, IL-25, and TSLP can play partially redundant roles, with one essentially substituting for the other (47).

Helminth excretory/secretory (ES) molecules likely also contribute as inoculation with ES supernatants alone can promote a type 2 immune response (28, 48), though not as potent as a live helminth infection. The ES material derived from parasite cultures is a heterogeneous mixture composed of many bioactive molecules, ranging from small to complex glycoproteins, which are produced by the parasite to modulate the host response. They are likely a product of the dynamic relationship resulting from millions of years of vertebrate/helminth coevolution. A number of specific molecules have now been isolated from helminths and many of these can downregulate host immune responses. For example TGF-β mimic, derived from *Heligmosomoides polygyrus*, binds the TGFβ receptor and can upregulate FOXP3^{+} Treg cells through binding the TGFβ receptor (40). Also, ES62, a filarial glycoprotein, interferes with myd88 signaling, thereby inhibiting TLR (49) and IL-33 (50) signaling. Isolation of these ES products remains at a very early stage, but already potential candidates that control harmful inflammation have been identified raising the possibility that ES derived molecules could provide a rich source of future immunomodulatory therapeutics.

Thus helminth infection in both the lung and in the small intestine triggers type 2 immunity in part through tissue damage, and associated release of DAMPS, and is then further modulated through the release of helminth ES products. The type 2 immune response has many components shared with wound healing responses raising the possibility that the innate type 2 immune response may have originated from a conventional wound healing response, coopted by the immune system to mitigate tissue damage during helminth infection. The development and overlay of the adaptive type 2 immune response over the innate response may have in part evolved to incorporate antigen specificity to enhance resistance against helminths (8). Together the innate and adaptive type 2 immune response thereby mediate both tolerance and resistance mechanisms that together enhance host protection against helminths.

Helminth-Induced Immunomodulation

A number of components of the helminth-induced response also directly inhibit both type 1 and type 17 responses. T regulatory (Treg) cells are expanded in response to many helminths and have been shown to downregulate harmful type 1 responses and also type 2 immunity associated with allergic responses (13). In many cases IL-10 mediates these responses, though other molecules have also been implicated, including ES products such as TGM (40). Overall, T reg cells contribute an essential component in mediating tolerance mechanisms that control inflammatory responses that would otherwise contribute to tissue damage.

The potency of the immune response evoked by helminths to downregulate harmful inflammation as well as directly promote tissue repair provides two potent and complementary tolerance mechanisms. This has direct implications on how the intestinal tissue responds to other infectious and inflammatory insults. A clear example of this concept is the finding that the gastrointestinal nematode *H. polygyrus* is able to exert potent immunomodulatory effects and inhibit intestinal inflammation induced by IL-10 deficiency (51), by TNBS hapten administration (52), and by dietary antigen challenge. As expected, multiple mechanisms appear to be induced by helminth infection including: suppression of the IL-17 response (53), activation of regulatory Foxp3+ T cells and their regulatory cytokine production (54) and the induction of "tolerogenic dendritic cells" that prevent induction of antigen specific gut T cell responses (55). Unlike the nearly uniform protective effects of helminths in inflammatory bowel disease models induced by non-viable insults, the picture that emerges from studies involving bacterial and parasitic challenges is more complex. For example, *H.polygyrus* infection exacerbates intestinal inflammation caused by *Salmonella typhymurium* infection by dampening CXCL2 chemoattraction of neutrophils, resulting in defective control of bacterial growth. In the case of challenge with the enteropathogen C*itrobacter*, where it was previously shown that bacterial burden and tissue pathology was exacerbated by H. polygyrus infection (56, 57), helminth infection induced changes in the microbiota, with increased abundance of Bacteroides and decreased representation in Firmicutes and Lactobacillales. Interestingly, gut microbiota transfer from helminth infected wildtype, but not STAT-6 deficient donors caused significant worsening of the *Cibrobacter*-induced intestinal inflammation, demonstrating an involvement of the host Th2 responses in precipitating the alterations in gut microbiota that exacerbated intestinal inflammation (58).

Strictly enteric infection with helminths can also modulate systemic immune responses. Oral Inoculation with *H. polygyrus* can control allergic responses in the lung, in part through activation of T regulatory cells (17, 59). Other immunoregulatory

cell populations include B cells and macrophages, in some cases acting independently of IL-10 (48, 60). Also, oral helminth infections can control type 1 diabetes through mechanisms that involve both CD4 T cell production of IL-4 and IL-10 acting in an independent and redundant manner (61). Intriguingly excretory/secretory products derived from helminths can also have potent anti-inflammatory effects and recent studies have begun to isolate these immune modulators from a variety of helminth parasites (48, 62). Helminth infection may also perturb colonization by the intestinal microbiota thereby influencing its composition which in turn can affect immune regulation and control of harmful inflammation (63, 64). Transfer of helminth modified intestinal microbiota can protect against allergic asthma through their production of short fatty acids (SFCA) (65). In terms of coinfection, recent studies showed that *H. polygyrus* infected mice had markedly reduced pulmonary lung damage and viral load following intranasal infection with respiratory syncytial virus. The response was independent of adaptive immune responses but protection was lost in germ-free mice, indicating a role for intestinal microbiota (66). In contrast, *H. polygyrus* infection did not affect immunity or progression of disease following coinfection of mice with *Mycobacterium tuberculosis* (67). These studies indicate that although strictly enteric helminth infection may have potent systemic immunoregulatory effects, in some cases it has little effect. Understanding the conditions under which helminths can preferentially modulate a concomitant immune response will likely provide important insights into development of future therapies based on helminth treatments or on specific immune modulators purified from helminths.

Future work is needed to take into account how helminth-induced remodeling of the epithelium niches contributes to the altered tolerance of the intestine to microbial and inflammatory challenges. While it is clear that helminths induce hyperplasia of the Tuft cell and goblet cell compartments, how it impacts the absorptive epithelial and antimicrobial Paneth cell compartments remain unexplored. For example, the shift in the production of Tuft cells and Goblet cells may come at the expense of the Paneth cell niche or their ability to produce antimicrobial peptides required to maintain the normal microbiome and resist microbial challenges in the intestine. Similarly, it is not clear whether expansion of the secretory Tuft and Goblet cell niches is accompanied by a compensatory hyperplasia of the absorptive epithelial cell compartment. Future studies should also take advantage and take into account that ability of single cell (scRNASeq) technologies to resolve shifts in differentiation trajectories of intestinal stem cell and transit amplifying cells caused by helminth infection and the inherent intraniche heterogeneity and functional specialization of the Tuft cells and Goblet Cells compartments during helminth infection. A recent scRNA Seq study has already pinpointed an interesting dichotomy within the Tuft cell niche, which was previously characterized as having both neuronal and inflammatory gene expression programs. It appears that these two functional modules may be embodies in two distinct subpopulations, both of which express IL-25, IL-25 receptor (IL17rb) and receptors for IL4 and IL-13. Nevertheless, only one subset expressed high levels of TSLP and interestingly expressed CD45, a pan-marker of hemopoietic cells (68).

Helminths Alter the Systemic Immune Landscape

As alluded to above, helminth induction of alternative immune activation and regulatory mechanisms that promote tolerance may be propagated through systemic changes in the innate and adaptive immune landscape of secondary and primary lymphoid organs. This is perhaps best exemplified by recent studies indicating that helminths induce profound shifts in the migratory behavior of both group 2 innate lymphoid cells (ILC2s) and naïve T lymphocytes (69, 70). Upon *N. brasiliensis* infection, resting ILC2s residing in the intestinal lamina propria rapidly acquire an activated KLRG1+ phenotype and become mobilized to seed the lung and liver. IL-25 appears to mediate this effect, as it is sufficient to cause activation and redistribution of activated ILCs without any apparent proliferative step. Activated ILC2s gain entry into the lymphatics and blood circulation and accumulated at distal tissue sites, including the lung, based on the well-known sphingosine-1-phosphate-mediated chemotactic mechanism used by T lymphocytes to egress from lymphoid organs. In the lung, relocalization of recently activated ILC2s promoted tissue repair and prevention of acute lung injury (69).

In contrast to the behavior of ILC2s, the naïve T and B cell pools become depleted from non-involved lymphoid organs and accumulated in the T helper 2-reactive mesenteric lymph node during helminth (*H. polygyrus*) infection in mice. This systemic redistribution of non-activated lymphocytes persists into the chronic stage of infection and requires the participation of the lymphoxin-beta receptor signaling. IL4 secretion by Th2 and Tfh cells during helminth infection likely promotes LTβ expression by follicular B cells which then expands the stromal cell compartment to reorganize lymph node architecture. Alternatively, expression of another alternate LTβR ligand, LIGHT by T cells and DCs in the reactive lymph node may be responsible for expansion of the stromal cell compartment to further promote humoral responses to the helminth parasite. Nevertheless, the relative depletion of the naïve lymphocyte pool at other lymphoid sites result in impaired responsive to heterologous immunization or infections with other unrelated micro-organisms (70).

In addition to systemic shifts in lymphocyte migration, helminth infections induces dramatic alterations in the cell type distribution and functional attributes of dendritic cells in the secondary lymphoid organs. During *N. braziliensis* infection, dermal dendritic cells acquire parasite material and migrate to the draining lymph nodes to prime CD4 T cells capable of making IL-4 (71). These dermal derived dendritic cells exhibit a unique $CD11c^{dull}$ $MHCII^{hi}$ phenotype and expressed Th2 promoting factors including PDL2, IRF4 and OX40L as well as CD301b (72). However, the induction of Th2-priming or Th2 associated dendritic cell types may not be sufficient for dampening opposing Th1 or Th17 responses. Recent studies indicate that IL4 exposure of dendritic cells, although resulting in the expression of a wide range of alternative activation markers, could also drive higher

levels of bioactive IL-12 production and consequently promoting Th1 type responses. Nevertheless, RELM-alpha expression by DCs downstream of IL4 signaling further promotes IL-10 and IL-13 production, suggesting a more complex and potentially antagonistic relationship between IL-4 induced factors produced by dendritic cells (73).

In keeping with the prevailing theme that helminths induce both Th2 and regulatory immune mechanisms, chronic gastrointestinal helminth infection has also been shown to promote the development of a $CD11c^{lo}CD103^{-}$ dendritic cell population that may be important for the expansion of Treg cells during chronic helminth infection (74). Interestingly, these helminth-expanded $CD11c^{lo}$ DCs exhibited poor responsiveness to TLR activation and consequently deficient T cell activating potencies. Instead, naïve T cells stimulated by these $CD11c^{lo}$ DCs were more likely to become Foxp3-positive Tregs. Consistent with a model where distinct DC subsets mediate helminth induction of Th2 and Treg responses, depletion of $CD11c^{hi}$ DCs abrogated Th2 effector responses, while sparing Treg expansion.

The ability of helminths and helminth products to dampen the antigen-presenting and costimulatory functions of dendritic cells and the induction of Tregs may not be the sole mechanism for how these parasites modulate proinflammatory Th1 and Th17 responses. The production of type 2 cytokines by ILC2s, eosinophils, neutrophils and basophils instruct the formation of alternatively activated macrophages, not only at affected tissue sites but also within secondary lymphoid organs. A key difference between these alternatively activated macrophages and their classically activated counterparts is in their alternative metabolism of the amino acid arginine. IL4 induces arginase 1 which results in the formation of ornithine and urea. The elaboration of arginase can result in depletion of this essential amino acid and restrain the activation and function of T cells. T lymphocytes subjected to arginine depletion become blocked in the G1 stage of the cell cycle and subsequently downregulated mTORC1 activity, while mTORC2 mediated cell cycle arrest in these starved T cells (75). In relation to this, it is interesting to note that mTORC1 is required for the generation of Th1 cells and CD8 effector T cells, while mTORC2 is involved in the formation of Th2 cells and CD8 memory T cells (76, 77). Thus, it is likely that alternative macrophage induction, through arginase-modulation of T cell metabolism, may explain why helminths can potently inhibit the generation of heterologous effector Th1 and CD8 CTLs, and instead favor Th2 and memory CD8 T cell responses. In addition, arginine-depletion could also modulate the intrinsic ability of T lymphocytes to signal through the T cell receptor (TCR) by impairing expression of the CD3 zeta chain (78). Thus, by downmodulating the assembly and signaling potency of the TCR, alterations in lymphocyte metabolic pathways and the lack of expression of Th1 promoting chemokines and costimulatory molecules, alternative macrophages can effect, through both lymphocyte intrinsic and extrinsic mechanisms, impose a regime that inhibits proinflammatory effector cell generation and favor Th2 and Treg responses. AAM conversion of both pre-existing tissue-resident macrophages and newly arrived monocyte-derived migrants provide a mechanism to initiate and perpetuate this immunological regime (79, 80) The ability of helminth infection to simultaneously exert immunoregulatory activities on both DCs and macrophages may provide an explanation for how it potently suppresses both differentiation and functional maturation of type 1 effector T cells in the context of *Toxoplasma* coinfection (81). Recent publications have highlighted a requirement for sequential engagement by

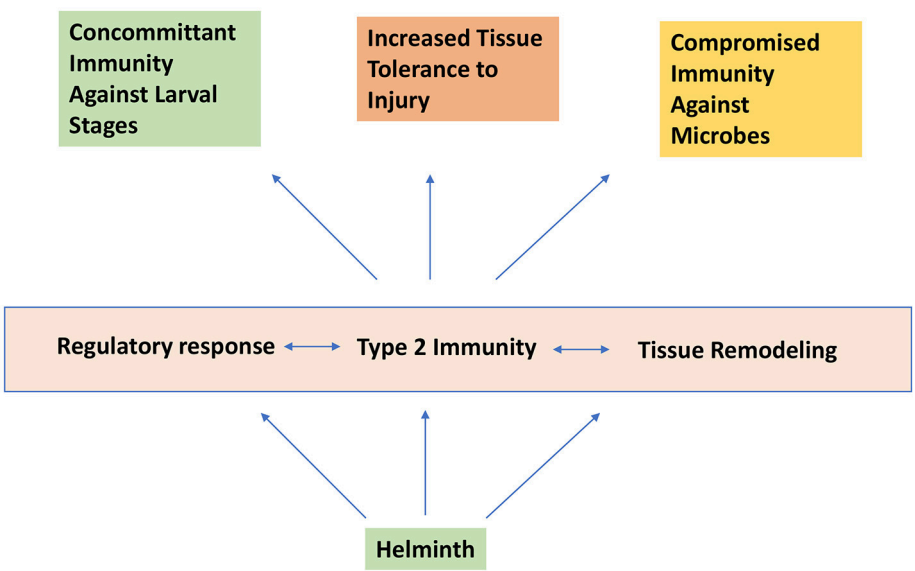

FIGURE 3 | Helminth infection induces concomitant immunity and increased tissue tolerance to injury while promoting compromised immunity to certain microbial agents.

dendritic cells and macrophages and their production of IL-12 and IFN-γ induced chemokines for optimal type 1 effector cell differentiation (82–84). Thus, helminth immunodulation of the innate immune landscape in both the T cell and the extrafollicular areas of lymphoid organs provides a powerful mechanism to thwart type 1 effector responses. Furthermore, this immunosuppressive mechanism could act dominantly to thwart vaccine-induced protective immunity, despite higher frequencies of memory cells, because the transition from central memory to effector memory or effector cells require costimulatory signals from innate accessory cells (83, 84).

Perspectives and Concluding Remarks

From a teleological perspective, helminth parasites may have coopted the type 2 and Treg response to "optimize" their species-specific host niche (see **Figure 3**). Besides preventing extensive tissue damage and excessive and overt inflammatory responses, an important consideration would be to limit the overall parasite load that could result in host morbidity and mortality. Because most helminths do not proliferate within the host, a state of concomitant immunity, where the presence of adult worms induces and preserves resistance mechanisms that prevent further infestation by larval stages of the same or even a different helminth organism maybe a useful lens to view the various manifestations of helminth infections. Thus, besides promoting host viability for effective reproduction, tissue remodeling and enhanced immune resistance in the lung and the gastrointestinal tract could be also viewed as a tactic to prevent further colonization. Similarly, alterations in the innate and adaptive immunological landscape in lymphoid tissues may represent a mechanism to perpetuate the changes enforced within host tissues. A striking example of this concept is the recent demonstration that *Trichuris muris* coopts the host microbiota to increase its own fitness and alters the microbiome in such a way that inhibits subsequent rounds of infection (85). It is likely that helminth infection exerts multiple collateral changes in other host tissue systems (e.g., the hemopoietic and neuro-endocrine systems), which may have a profound impact on resistance and/or tolerance to other infectious agents.

In particular, these immune regulatory mechanisms, including products directly produced by the parasite, can modulate the immune response in some cases impairing effective type 1 immunity against microbial pathogens. On the other hand these same tolerance mechanisms, including factors directly enhancing wound healing, may mitigate severity of tissue damage associated with microbial infections, raising the possibility that eradication of helminths may not only enhance resistance but also deleterious effects of type 1 inflammation leading to increased severity of disease. Understanding the multiple mechanisms through which helminths modulate immune responses and promote tissue repair may lead to new and effective targeted treatments to control harmful inflammation associated with microbial pathogens as well as noncommunicable inflammatory diseases.

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Pseudomonas aeruginosa in Chronic Lung Infections: How to Adapt Within the Host?

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Bacteria that readily adapt to different natural environments, can also exploit this versatility upon infection of the host to persist. *Pseudomonas aeruginosa*, a ubiquitous Gram-negative bacterium, is harmless to healthy individuals, and yet a formidable opportunistic pathogen in compromised hosts. When pathogenic, *P. aeruginosa* causes invasive and highly lethal disease in certain compromised hosts. In others, such as individuals with the genetic disease cystic fibrosis, this pathogen causes chronic lung infections which persist for decades. During chronic lung infections, *P. aeruginosa* adapts to the host environment by evolving toward a state of reduced bacterial invasiveness that favors bacterial persistence without causing overwhelming host injury. Host responses to chronic *P. aeruginosa* infections are complex and dynamic, ranging from vigorous activation of innate immune responses that are ineffective at eradicating the infecting bacteria, to relative host tolerance and dampened activation of host immunity. This review will examine how *P. aeruginosa* subverts host defenses and modulates immune and inflammatory responses during chronic infection. This dynamic interplay between host and pathogen is a major determinant in the pathogenesis of chronic *P. aeruginosa* lung infections.

Keywords: *Pseudomonas aeruginosa*, cystic fibrosis, immune evasion, chronic lung infection, host evasion, bacterial adaptation

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INTRODUCTION

Bacterial pathogens are most commonly studied for their ability to invade and injure the host, causing acute and invasive infections. In contrast, chronic infections present a distinct paradigm in infection pathogenesis which may challenge conventional notions of bacterial virulence and host defenses. To healthy individuals, *Pseudomonas aeruginosa* (PA) is a ubiquitous Gram-negative bacterium commonly encountered in the environment and readily cleared by host defenses. However, PA is also a formidable opportunistic pathogen that can cause invasive and fulminant infections, such as acute pneumonia or bloodstream infections, in immune compromised hosts. Remarkably, the same pathogen also causes chronic infections that persist for months to decades, such as the chronic lung infection in individuals with the genetic disease cystic fibrosis (CF). Chronic PA infections thus result from a dynamic and complex interplay between pathogen and host, where bacteria persist without causing overwhelming host injury, and where host defenses fail to eradicate the pathogen.

PA has a large genome (>6 Mb) that encodes many regulatory genes involved in sensing environmental signals, controlling expression of virulence factors, metabolism and resistance mechanisms. PA thus readily adapts to a wide range of environments and can exploit this versatility to enhance its long-term survival and persistence in the host. Importantly, host-pathogen interactions evolve over time and anatomical space, with the balance fluctuating between host recognition and vigorous activation of defense mechanisms, and immune evasion and tolerance by the host.

Chronic PA lung infections in individuals with CF persist for decades and provide a unique opportunity to examine how a bacterial pathogen can adapt to its host, modulate host responses and shift between different infection phenotypes. It is widely recognized that CF disease is associated with several intrinsic host defects, including impaired mucociliary clearance, and immune and inflammatory dysregulation. The implications of these host defects to the development of CF lung disease are beyond the scope of this review but may be found in excellent other ones (1–3). In this review, we will examine how PA defines the interactions central to the host immune and inflammatory response, and the bacterial adaptive strategies that promote bacterial persistence, and allow evasion and tolerance by the host during chronic infection. Specifically, we will highlight bacterial factors that undergo host-adaptation during chronic infections.

BACTERIAL FACTORS INVOLVED IN HOST INTERACTIONS AND RECOGNITION

Flagellin and Flagellar Motility

PA possesses a single polar flagellum composed of polymerized flagellin, its major structural protein, and attached to a transmembrane motor complex. The flagellar-host interaction plays a major role in defining the immune and inflammatory outcomes of PA infection, as the flagellar complex interacts with immune and non-immune cells through its structural components and as well as motility function.

The flagellar-host interactions have been extensively characterized at the cellular and molecular level. Flagellin is best known as a pathogen-associated molecular pattern that binds to the extracellular Toll like receptor TLR5 (4) and intracellular NOD-like receptor (NLR) neuronal apoptosis-inhibitory protein (NAIP) (5), in human (6), leading to activation of the pro-inflammatory MyD88 pathway and the NLRC4-inflammasome, respectively (7). TLR5 mediates a major component of the epithelial cytokine and chemokine responses leading to neutrophil recruitment in PA lung infection (8–10), and contributes to the production of pro-IL-1ß in monocytes and macrophages (11). Flagellin is also translocated by the Type-3 secretion system (T3SS) in the cytoplasm of mammalian cells, thereby activating the NAIP-NLRC4-inflammasome and inducing mature IL-1ß secretion (12, 13). Notably, IL-1ß promotes phagocytosis through its autocrine and paracrine effects (11, 14). Interestingly both flagellin and a motile flagellum are required to activate the NAIP-NLRC4-inflammasome (5, 15–17), but how host cells sense flagellar motility remains unclear. Beyond its ability to activate host cell signaling pathways, the flagellum also promotes adherence and colonization of host surfaces, and various specific targets have been identified including MUC1 mucin (18), heparin sulfate (19), surfactant protein A (20), and asialoGM1 (21).

During chronic infection, PA uses multiple strategies to evade flagellum-mediated host recognition. Flagellin expression is under the complex regulation by several global transcriptional regulators (22–25). It is repressed in mucoid variants which over-produce the exopolysaccharide alginate (26), during biofilm growth (27), upon as well as in response to the host nutritional and inflammatory environment. Notably, flagellin is repressed in the presence of CF sputum and airway fluid (28) as well as neutrophil elastase released at sites of inflammation (29). PA also expresses the secreted bacterial proteases AprA and LasB which cleave extracellular flagellin, suggesting an intrinsic mechanism to shut down flagellin-mediated immune recognition (30). Finally, loss of flagellar motility is common in host-adapted PA strains from CF lung infections and is associated with increased bacterial burden and disease severity (31). Genome sequencing studies of longitudinal PA strains have revealed evidence of convergent evolution and genetic mutations in regulatory genes such as *rpoN* and *fleQ* which lead to downregulation of flagellar expression and motility (32, 33). In fact, PA isolates recovered from chronic CF lung infections fail to activate the inflammasome due to reduced expression of flagellin and T3SS (34).

Type 3 Secretion System (T3SS)

The type III secretion system (T3SS) is a complex needle-like secretion machinery found in gram-negative bacteria that allows the translocation of bacterial effectors directly into the cytoplasm of host cells, causing cytotoxicity, or subversion of host defenses (35). The T3SS causes tissue injury, promotes bacterial dissemination and has been implicated in the pathogenesis of acute and invasive infections, including pneumonia (36–38). Four T3SS-dependent effectors have been identified in PA, namely ExoS, ExoT, ExoY, and ExoU, and have been recently reviewed elsewhere (35). The T3SS effectors cause disruption of host cell cytoskeleton (ExoS, T, and U) and cleavage of phospholipases (ExoU), leading to cell death, a breach of epithelial and endothelial barriers and killing of phagocytes (39–41). ExoS also dampens phagocytosis by interfering with lysosome signaling in macrophages (42, 43).

Beyond its role in cytotoxicity, the T3SS activates innate immune responses through secretion of IL-1ß (44). The T3SS apparatus itself, independently of exotoxin, can activate the NLRC4-inflammasome through NAIP recognition (44–46), leading to pyroptotic cell death and the secretion of mature IL-1ß and IL-18. Whether inflammasome activation contributes to the effective immune response to control bacteria, or to the immunopathology associated with PA lung infections

Abbreviations: PA, Pseudomonas aeruginosa; CF, cystic fibrosis; cyclic di-GMP, cyclic diguanylate; EPS, exopolysaccharide; IL, interleukin; LPS, lipopolysaccharide; NAIP, neuronal apoptosis-inhibitory protein; ROS, reactive oxygen species; T3SS, Type-3 secretion system; T4P, Type 4 pili; TLR, Toll like receptor.

remains incompletely understood. On one hand, inflammasome activation and IL-1R signaling may be protective at early stages of infection (47, 48). On the other hand, NLRC4 activation is associated with reduced alveolar macrophages, reduced PA clearance and increased neutrophil recruitment, leading to greater lung immunopathology and mortality in a murine model of acute lung infection (49, 50).

Chronic infections appear to select against T3SS-expressing PA. Although many CF patients carry antibodies against T3SS effector proteins (51), suggesting that these effector proteins were secreted at some stage of the infection, most PA strains isolated from chronic infection are T3SS-negative (34, 52, 53). Loss of T3SS results in dampened inflammasome activation and lesser pyroptotic cell death in macrophages and neutrophils (34). CF isolates are rarely ExoU+ (54), also consistent with the notion that acute cytotoxicity, particularly when conferred by ExoU, is less compatible with chronic infection. As discussed later in this review, several mechanisms contribute to the loss of T3SS in CF-adapted PA strains.

Secreted Proteases

PA produces several secreted proteases, which include LasB (also known as PA elastase or pseudolysin), LasA, AprA, and protease IV. Secreted PA proteases interact with a wide range of host molecules, leading to diverse outcomes, from degradation of structural components to modulation of inflammatory responses. The PA proteases are most studied for their ability to cause direct tissue damage, and they are primarily known as virulence factors involved in the pathogenesis of acute infections. LasB, a broad specificity metallo-protease, degrades elastin (55), disrupts epithelial tight-junctions (56), and reduce endothelial barrier integrity (57, 58). As a consequence, LasB mutants are attenuated in virulence in experimental models of bacteremia (59), acute pneumonia (60), or burn wound model (61).

PA proteases also alter host responses by degrading secreted mediators, leading to a dampening of inflammatory and immune responses, which likely contributes to its ability to evade host defenses. *In vitro* studies have shown that PA proteases potently degrades secreted mediators such as cytokines (e.g., INF-γ, IL-6), chemokines (e.g., IL-8/CXCL1, MCP-1, CXCL-5, RANTES/CCL5) (62–66), host defense components such as immunoglobulins (67, 68), antimicrobial peptides (e.g., LL-37) (69), and membrane receptors (e.g., protease-activated receptor PAR-1,2 and 4) (70, 71). LasB helps PA subvert alveolar macrophage activity by down-regulating the oxidative burst and production of complement factors (72). LasB mediated degradation of surfactant proteins SP-A and SP-D also leads to phagocytosis resistance (73, 74). Proteolysis of thrombin by LasB releases an anti-inflammatory thrombin-derived peptide FYT21, which inhibits the activation of the transcription factors NF-κB and AP-1 (75). Finally, AprA and LasB can degrade flagellin monomers, and thus blunt TLR5-mediated responses (30) and inflammasome activation (76). Interestingly, the inflammasome activation is also dampened due to proteolytic degradation of extracellular inflammasome components by PA proteases (76).

Although most PA isolates recovered from environmental sources or acute infections produce secreted proteases, protease-deficient PA isolates are commonly isolated from patients with CF and chronic obstructive pulmonary disease (COPD) chronically colonized with PA (77, 78). In fact, loss of secreted protease activity occurs as part of the genetic adaptation of PA to the host environment (see section below) and is associated with chronic and more advanced lung disease (32, 79). As secreted proteases dampen inflammation, loss of protease activity in CF-adapted PA variants conversely can promote exaggerated inflammation and lung immunopathology, as observed *in vitro*, *in vivo* in murine models of chronic PA lung infections and in CF patients (80). The impact of secreted PA proteases on host responses and pathology thus varies in different infection settings, such as acute vs. chronic, invasive vs. localized, as the presence or loss of proteases promote disease through different mechanisms of host interactions.

Exopolysaccharides (EPS)

PA produces three extracellular polysaccharides (or exopolysaccharides), namely alginate, Psl, and Pel. They provide many protective properties and confer surface and self-adherence. They are constituents of the biofilm matrix, are involved in surface colonization and promote host immune evasion. A detailed review of these EPS and their distinct functions can be found elsewhere (81).

Mucoid PA overproduces the exopolysaccharide alginate and these strains are commonly associated with chronic CF lung infections and other chronic lung diseases (79, 82, 83). Alginate over-production (mucoidy) impairs host defenses and promotes bacterial persistence through several mechanisms. Alginate overproduction interferes with opsonophagocytosis and complement activation, scavenges ROS and inhibits phagocytic killing (82, 84, 85). It also confers resistance to host antimicrobials such as LL-37 and reactive oxygen species H_2O_2 (86). Whether mucoidy dampens host detection remains unclear. Mucoidy represses flagellar biosynthesis due to the co-regulation of flagellin and alginate (26), leading to reduced TLR5-dependent activation. However, mucoidy is linked with increases bacterial lipoproteins expression (87), which activates TLR2 in host airway epithelial cells (88), and is associated to greater resistance to the anti-inflammatory effects of corticosteroids (89).

Psl and Pel are exopolysaccharides which confer structural and aggregative properties to the biofilm matrix and contribute to the biofilm antibiotic tolerance (90, 91). Psl interferes with complement deposition and hinders neutrophil opsonophagocytosis and oxidative killing (92). Although its interactions with host cells are less well-characterized, Pel likely also contributes to resistance against neutrophil killing (93). PA genetic variants that overproduce Psl and/or Pel are found in chronic CF infections (94) and are associated with increased bacterial burden and host immune evasion (95).

Lipopolysaccharides (LPS)

LPS (also known as endotoxin) is a major component of the outer membrane of Gram negative bacteria. LPS is composed

of three components: the lipid A and core oligosaccharides that form the outer leaflet of the bacterial outer membrane, and the O-antigen polysaccharide which interacts with the extracellular environment. LPS is recognized by the Toll like receptor 4 and myeloid differentiation factor 2 complex (TLR4-MD2). The O-antigen consists of highly variable and immunogenic oligosaccharide repeats which elicit a strong humoral response (96).

During chronic infection, the LPS undergoes important adaptive changes at the level of its synthesis and structure, leading to modification of the lipid A structure and loss of O antigen which likely promote immune evasion. Lipid A acylation patterns or addition of positively charged components, renders the outer membrane more resistant to host antimicrobial peptides (97–99), modulates TLR4-MD2 receptor recognition and dampens host inflammation (100). PA isolates from chronic infection commonly express little or no O-antigen (101, 102). Mutations in LPS and O-antigen biosynthesis are common (32, 103, 104) and appear to be a hotspot of genetic variation and adaptation during chronic CF infections (105). Finally, O-antigen biosynthesis is also modulated by cyclic-di-GMP, a second messenger involved in the switch from motile to adherent lifestyle of PA (106). A summary of the bacterial factors/complex involved in the host adaptation during chronic PA infections is provided in **Table 1**.

PA PHENOTYPIC AND GENETIC ADAPTION TO HOST ENVIRONMENTS

During the process of chronic infection, PA adapts to the host environment and undergoes changes which promote bacterial survival and evasion of host defenses. Certain adaptive processes occur at the phenotypic and regulatory level, while others occur through genetic mutations and evolution. We will review here the key regulatory and genetic adaptive processes that PA undergoes during chronic PA infection.

Biofilm Lifestyle

In contrast to the free-living bacterial lifestyle termed planktonic, PA can also grow in a multicellular and sessile form, termed biofilms. Biofilms are formed by self-aggregated or surface-adherent bacteria encased within an extracellular matrix. Biofilms cause many chronic and non-invasive human infections such as medical device associated infections, chronic CF lung infection and chronic wound infections. Our understanding of *in vivo* host responses to PA biofilms is limited by the lack of animal infection models that mimic human biofilm infections. Our insights are thus primarily drawn from *in vitro* studies that examine the response of various cell types to biofilm bacteria. Biofilm formation and its role in disease pathogenesis have been the subject of recent reviews (81, 107), and only aspects relevant to host-biofilm interactions are outlined here.

Host responses to PA biofilms are complex, as biofilms may both stimulate or suppress the immune system. Biofilms may be less immune-stimulatory than their free-living planktonic counterparts. For example, the expression of flagellin and T3SS is down-regulated (108, 109), and the complement system is less activated (110) during biofilm growth. Furthermore, bacterial factors involved in host interactions may be embedded within the biofilm matrix and not readily accessible for host recognition. Conversely, biofilms can induce a robust neutrophilic response where neutrophils are activated, undergo oxidative burst and degranulate, but are immobilized (111–113). Biofilm PA can also trigger necrotic cell death in neutrophils (113), leading to further inflammation and collateral tissue damage.

Importantly, innate immune responses are less effective against biofilm than planktonic PA. As described above, exopolysaccharides constitute the major components of the biofilm matrix and contribute to biofilm resistance against host antimicrobials defenses and phagocytic killing. Biofilm infections are thus associated with a smoldering immune response that is ineffective at clearing bacteria but remains active enough to cause tissue damage over long periods of time.

Regulatory Control to Switch Bacterial Lifestyle and Infection Strategy

PA is capable of phenotypically switching between its motile planktonic lifestyle and the sessile biofilm lifestyle through multiple and overlapping regulatory networks which include the RetS/GacS sensor pathway. Through the opposing functions of RetS and GacS and their signaling cascades, the RetS/GacS pathway converge on the regulator RsmA and is linked to the second messenger cyclic di-GMP. It coordinately controls the expression of motility, Pel and Psl exopolysaccharides, T3SS and Type VI secretion system (T6SS) -related gene (114, 115). Chronic infection is thus favored as PA represses its T3SS, motility and produces the exopolysaccharides that form the biofilm matrix. Interestingly, analysis of host-adapted PA strains from chronic CF infections identified genetic mutations in the RetS/GacS pathway, with the possibility that *retS* mutations promote a chronic infection state (116). Conversely, dysregulation of RetS/GacS pathway due to mutations in *gacS* or its regulator *ladS* can also cause excessive T3SS activity and cytotoxicity, leading to hyper-virulent PA strains that cause fulminant infections (117) or exacerbations during chronic CF infection (118).

Cyclic di-GMP is an intracellular bacterial secondary messenger that regulates multiple bacterial behaviors, most notably those involved in biofilm formation. The cellular level of c-di-GMP are modulated in response to environmental and intracellular signals, and affect expression of genes involved in flagellar and type IV pilus mediated motility, exopolysaccharide production and surface adhesion (115). Genetic variants that overproduce cyclic di-GMP display an auto-aggregative phenotype caused by the overproduction of Psl and Pel, have been recovered from chronic CF lung infections (94).

The RetS/GacS and sensor pathway, cyclic di-GMP signaling and other global regulators (e.g., quorum sensing, two component sensor regulators) allow PA to coordinately regulate numerous factors that define distinct bacterial infection strategies, namely acute and invasive disease, or chronic and localized disease. It is plausible that the ability of PA to

TABLE 1 | Bacterial factors/complex involved in host-adaptation during chronic PA infections.

Bacterial factor/complex	Bacterial function	Host interactions	Adaptation in chronic infection
Flagellum	- Macromolecular motility appendage which confers motility in low viscosity liquids through rotational movement - Flagellin is the principal structural component of the flagellar filament - Mediates biotic and abiotic surface adhesion	- Flagellin binds and activates TLR5 and intracellular Naip5 protein, leading to activation of MyD88 and NLRC4—dependent inflammatory pathways respectively - Promotes surface attachment and colonization by adhering to mucins, surfactant protein A, host surface molecules (e.g., heparin sulfate proteoglycans, AsialoGM1)	- Reduced flagellar motility and/or flagellin synthesis in response to mucin, neutrophil elastase and airway fluid, during biofilm growth, and due to genetic mutations in biogenesis or regulatory genes (e.g., *rpoN*, *fleQ*) - Dampened host recognition, phagocytic uptake and downstream activation of MyD88 and NLRC4—dependent pathways
Type IV pili (T4P)	- Macromolecular motility appendage which confers surface motility through extension, attachment, and retraction movement - Mediates sensing and adhesion to biotic and abiotic surfaces - Promotes biofilm formation (*in vitro*) - DNA uptake	- Binds host surface molecules (e.g., heparin sulfate proteoglycans and N-glycans) and promotes surface colonization - Promotes direct bacterial-host cell membrane contact and thus T3SS-dependent toxicity	- Reduced pilus-mediated motility due to regulatory control (e.g., cAMP and cyclic-di-GMP pathways) or due genetic mutations in biogenesis or regulatory genes - Reduced colonization and invasion of host tissues
Type 3 secretion system (T3SS)	- Needle-like structure that injects and translocates bacterial effector proteins across cellular membranes into the host cell cytoplasm	- Translocation of effectors proteins (ExoU, ExoY, ExoS, ExoT, flagellin) which interact with the eukaryotic cytoskeleton and immune responses in phagocytes and non-phagocytic cells - Translocation of flagellin and other flagellar components into host cytosol, leading to inflammasome activation	- Repressed expression due to regulatory control or mutations of regulatory genes (e.g., RetS/GacS, cyclic-di-GMP pathways) - Reduced host cell cytotoxicity and inflammasome activation
Type 6 secretion system (T6SS)	- Secretion/injection system that delivers effector proteins into prokaryotic and eukaryotic target cells - Involved in bacterial competition	- The effectors PldA and PldB activate the PI3K/Akt pathway, and VgrG2b interacts with microtubules, which promote bacterial internalization in non-phagocytic cells (*in vitro*)	- Expression potentially induced due to regulatory control or mutations of regulatory genes (e.g., RetS/GacS, cyclic-di-GMP pathways)
Exopolysaccharides	- Alginate scavenges reactive oxygen species and is overproduced in mucoid variants - Psl and Pel have aggregative properties that confer cell-cell and surface adherence - Major structural component of biofilm matrix, which contribute to biofilm antibiotic resistance	- Pel and Psl promotes adherence to host cell surface - Interferes with opsono-phagocytosis, phagocyte oxidative burst and killing	- EPS overproduction due to mutations or environment control in regulatory genes (e.g., *mucA*, cyclic-d-GMP pathway) - Co-regulation of EPS with other bacterial factors through common pathways (e.g., AlgT, cyclic-di-GMP) leads to repression of flagellar biosynthesis and T3SS activity, increased expression of bacterial lipoproteins (TLR2 agonists) in EPS over-expressing strains. - Impaired bacterial clearance
Lipolysaccharides (LPS)	- Lipid A component is embedded in the outer membrane - O-antigen is composed of highly variable oligosaccharide repeats exposed at the bacterial surface	- Lipid A binds TLR4-MD2 - O-antigen is a common antibody epitope - Confers resistance to complement killing and cationic antimicrobial peptides	- Different lipid A modifications with varying impact: enhanced or dampened TLR4 activation, leading to immune evasion or enhanced immune-stimulation - Loss of O-antigen due to mutations in biosynthetic genes, leading to immune evasion
Secreted proteases (LasA, LasB, AprA, Protease IV)	- Proteolytic degradation of extracellular peptides	- Degrades elastin, thrombin, fibrinogen, surfactant proteins A and D, complements proteins, immunoglobulins, cytokines, and other extracellular mediators - Degrades flagellin - Disrupts epithelial tight-junctions and reduces barrier integrity	- Loss of secreted protease activity due to genetic mutations in regulatory genes (e.g., LasR quorum sensing) - Reduced host tissue destruction and invasion - Dampened immune recognition - Increased accumulation of mediators and inflammation

phenotypically switch between acute and chronic virulence modes contributes to the complex disease phenotype it causes: the natural history of chronic PA lung infections is characterized by slowly progressive tissue pathology, but is also interrupted by periods of acute and more fulminant disease termed acute exacerbations. It is possible to speculate that exacerbation episodes may be caused in part by a phenotypic switch to acute virulence.

Genetic Adaptation During Chronic Infection

The bacterial genetic adaptation to host environments is a common theme during chronic infection. For PA, this has been best documented in chronic CF lung infection, and we suggest several excellent recent reviews (33, 116, 119) for a detailed discussion of the topic. In CF, factors that contribute to the mutagenesis of PA include the presence of hypermutator strains (120), and the pro-inflammatory environment of the CF lung rich in oxidative and nitrosative stresses (33).

During its long residence in the CF lung, PA populations show both genetic diversification as well as convergent evolution. On one hand, PA undergoes significant genetic and phenotypic diversification during chronic CF infection, a process likely attributable to the divergent evolution of clonally related PA inhabiting different regions and micro-environments of the lung (121). On the other hand, numerous studies have shown evidence of convergent evolution when comparing the PA genomes within patients over time, and across different patients (122). Genome sequence analyses show a strong positive selection for non-synonymous mutations in genes encoding or regulating virulence factors (e.g., T3SS, exotoxin A, quorum sensing), immunogenicity factors (e.g., O-antigen), motility (flagellar and T4P mediated motility), drug resistance (e.g., multidrug efflux pumps), and metabolism (e.g., iron uptake). Importantly, many of these mutations confer loss of function or secretion of extracellular factors (e.g., proteases, T3SS) and promote immune evasion (32, 123). For example, LasR quorum sensing and protease-deficient variants are observed in over a third of CF patients with chronic PA infections. This suggests that the host environment likely confers strong selective forces that shape host-pathogen interactions and drive the genetic adaptation of PA toward a state that promote bacterial survival and persistence in the face of host defenses.

ADVANCES AND CHALLENGES IN THE DEVELOPMENT OF ALTERNATIVE OR ADJUVANT THERAPIES FOR CHRONIC PA INFECTIONS

Alternative or adjuvant therapies that minimize direct bacterial damage to the host, that enhance protective host responses or subvert pathological ones, can improve infection outcomes (124). Such therapies are particularly needed in light of the alarming rise in drug resistance, and for drug tolerant chronic infections (125). The latter refers to the phenotypic state of slow growing and biofilm bacteria which are refractory to antibacterial killing even in the absence of drug resistance. Unfortunately, despite intense research efforts and many candidates in pre-clinical studies, the development of novel therapies in chronic PA infections has been arduous and met with very limited success so far.

Anti-virulence therapies target bacterial virulence without disrupting bacterial growth or viability. Although numerous PA targets (e.g., quorum sensing signaling, biofilm exopolysaccharides, T3SS complex, and effectors) and inhibitor molecules have been studied, very few have progressed past pre-clinical studies (126). Anti-virulence therapies face unique challenges due to the bacterial phenotypic heterogeneity and complex host interactions characteristic of chronic PA infections. First, many PA strains isolated from chronic infections do not express functional factors such as flagellum and T3SS, suggesting that these factors may not play as important a role in virulence during chronic infections as during acute PA infections. Furthermore, the genetic and phenotypic adaptation of PA to the host during chronic infection lead to extraordinary heterogeneity between different patients, as well as at different stages or anatomically distinct foci of disease within the same patient. Anti-virulence therapies may thus need to be tailored to specific patients and/or infection states (e.g., early infection or acute exacerbation) based on a more comprehensive microbiological profiling than currently available in the clinic.

Antibacterial antibodies can neutralize bacterial virulence factors, induce complement mediated lysis and enhance opsonophagocytic uptake and killing (127). Advances in antibody engineering and screening have accelerated antibody therapeutics, and a few anti-PA antibodies have reached clinical trials. Polyclonal anti-PA antibodies (PsAer-IgY) (128) are currently in Phase 3 clinical trials (NCT01455675) for the prevention of recurrent PA infections in CF patients. Monoclonal antibodies that target the exopolysaccharides alginate (AR-105, Aridis Pharmaceuticals) and Psl (129), the T3SS needle protein PcrV [MEDI3902, MedImmune (130); KB001 (131)], O11 serotype LPS [AR-101/KBPA101, Aridis Pharmaceuticals (132)], or combinations [e.g., bispecific anti Psl/PcrV MEDI3902, MedImmune (130)] are currently tested for the prevention or treatment of acute PA pneumonia but their utility in preventing or treating chronic infections remains to be determined (133).

Considering the intractable nature of chronic PA infection, an important strategy is also to prevent infection through approaches such as vaccine, antibody, enzyme or antibiotic-based treatments. Although several anti-PA vaccine targeting antigens such as LPS O-antigen, alginate, outer membrane or flagellar proteins showed promise in pre-clinical trials, their clinical efficacy in reducing the risk of chronic PA infection in susceptible individuals (such as CF patients) has been overall disappointing to date (134, 135).

CONCLUSION

Chronic PA infection illustrates a paradigm of chronic bacterial infections where pathogens dampen host defenses, adapt and evolve within the host to persist. Understanding the pathogenesis of chronic PA infection thus requires an intricate assessment of bacteria, host responses, and their interactions over time. Host-PA interactions are exceptionally complex in chronic infections, as they involve numerous host cell types and bacterial factors. These interactions are further complicated by the common co-existence of other pathogens or polymicrobial communities that interact with both host and PA, and by the potential changes in the host due to factors such as aging or environmental exposures. While decades of research have provided us with vast mechanistic data on host-PA interactions, integrating these

mechanistic insights into a whole system understanding of chronic infection and translating this knowledge into effective treatments remain a major challenge. The development of better *in vivo* models of chronic PA infection and tools to simultaneously probe host and pathogen over time is critical in order to gain a more integrated understanding of chronic infections.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Cross-Talk Between Iron and Glucose Metabolism in the Establishment of Disease Tolerance

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Infectious diseases are associated with disruption of host homeostasis. This can be triggered directly by pathogens or indirectly by host immune-driven resistance mechanisms. Disease tolerance is a defense strategy against infection that sustains host homeostasis, without exerting a direct negative impact on pathogens. The mechanisms governing disease tolerance encompass host metabolic responses that maintain vital homeostatic parameters within a range compatible with survival. Central to this defense strategy is the host's ability to sense and adapt to variations in nutrients, such as iron and glucose. Here we address how host responses regulating iron and glucose metabolism interact to establish disease tolerance and possibly modulate resistance to infection.

Keywords: iron metabolism, glucose metabolism, anorexia of infection, disease tolerance, nutritional immunity

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INTRODUCTION

Avoidance, resistance, and disease tolerance are evolutionarily conserved defense strategies that limit the negative impact of pathogens on host health and fitness (1). Avoidance limits exposure to exogenous pathogens and resistance expels, neutralizes or destroys invading pathogens, while disease tolerance acts without interfering directly with pathogens (1, 2) (**Figure 1**).

Disease tolerance relies on stress and damage responses that confer tissue damage control (3), that is, support the functional output of host tissues as a means to maintain vital homeostatic parameters within a range compatible with survival to infection (2, 4, 5). Stress and damage responses sense and react to variations in environmental cues or to damage imposed to cellular macromolecules and organelles, respectively (3). These are essential to provide metabolic adaptation to the stress and damage imposed directly by pathogens or indirectly by immune driven resistance mechanisms (3, 4).

Infections can impose a distinctive host behavioral pattern referred to as sickness behavior (6, 7). This encompasses anorexia, characterized by a reduction of food intake, possibly aimed at limiting nutrient availability to invading pathogens (8, 9) (**Figure 2**). While protective against some classes of pathogens (10–12), anorexia of infection carries a high evolutionary trade-off in that nutrient deprivation can compromise host homeostasis. For example, reduced iron intake in response to infection can lead to anemia of chronic disease (13), while reduced glucose intake can lead to hypoglycemia (10, 14, 15). Here we explore how regulation of host iron and glucose metabolisms impact on the establishment of disease tolerance and possibly on resistance to infection.

IRON METABOLISM AND DISEASE TOLERANCE

Iron is the most abundant transition metal present on Earth and perhaps for this reason was co-opted early in evolution to catalyze vital redox-based reactions in most living organisms, from prokaryotes to eukaryotes (16). Like other divalent metals, iron can shift between reduced (ferrous; Fe^{2+}) and oxidized (ferric; Fe^{3+}) or even higher oxidation states (ferryl; Fe^{4+}), via reversible exchange of electrons with electrophilic or nucleophilic molecules, respectively. In doing so, iron is at the center stage of a variety of vital biological processes, including the transport and storage of gaseous molecules, energy production, as well as other components of cellular metabolism (17, 18). Probably due to its essential role in supporting these vital functions, microbial pathogens evolved multiple strategies to acquire iron from their hosts, while infected hosts co-evolved to limit iron availability to pathogens (18–22). This evolutionarily conserved defense strategy against infection is referred to as nutritional immunity (23).

Regulation of Host Iron Metabolism in Response to Infection

Nutritional immunity is directed at inhibiting pathogens growth, via opposing mechanisms that limit nutrients' availability to intracellular or extracellular pathogens (18–22). Defense strategies limiting iron availability to intracellular pathogens rely on systemic inhibition of iron cellular import and can lead to hyperferremia (18–22). In contrast, defense strategies limiting iron availability to extracellular pathogens rely on cellular iron import mechanisms that promote cellular iron overload and hypoferremia (18–22). If uncontrolled, this can lead to the production of reactive oxygen species (ROS) via the Haber–Weiss–Fenton sequence (24), oxidizing and eventually damaging cellular macromolecules and organelles (22). In support of this notion, patients with genetic disorders characterized by cellular iron overload, such as hereditary hemochromatosis, are highly susceptible to a range of infections (25).

Regulation of Iron Metabolism Confers Tissue Damage Control

Disruption of host iron homeostasis is a hallmark of many infectious diseases (18, 22), as illustrated for example in malaria, the disease caused by *Plasmodium* spp. infection (26–28), polymicrobial sepsis (14, 29), tuberculosis caused by *Mycobacterium tuberculosis* (30, 31) or acquired immune deficiency syndrome, caused by human immunodeficiency virus (HIV) infection (31). Regulation of host iron metabolism is critical to confer tissue damage control, and in doing so, establishes disease tolerance to infection, as demonstrated for example for malaria (32) or polymicrobial sepsis (14).

The majority of the iron present in mammals exists in the form of heme (17, 33, 34), a tetrapyrrole ring that binds a central iron atom through different nitrogen atoms (34, 35). Heme is used essentially as a prosthetic group of hemoproteins, such as hemoglobin, myoglobin, or cytochrome c, where iron is deployed to exchange and store gaseous molecules or to transport electrons, respectively (33, 34). The largest pool of heme in mammals is found within hemoglobin in red blood cells (RBC), a prime target for invading pathogens in their search for iron (22, 33). As such, RBC lysis is a recurrent event associated with infection leading to the release of hemoglobin into plasma (17, 22, 36–38). Extracellular hemoglobin disassembles and auto-oxidizes, releasing its non-covalently bound prosthetic heme groups (33, 38) (**Figure 3**). This can lead to the generation of labile heme, that is, heme loosely bound to plasma acceptor proteins, macromolecules or low molecular weight ligands that fail to control its redox activity (36, 39). As it becomes bioavailable, a fraction of the labile heme in plasma acts in a pathogenic manner, compromising the establishment of disease tolerance to infection, as illustrated for malaria (38, 40, 41) or polymicrobial sepsis (14, 29).

Labile heme can also compromise resistance to infection via mechanisms inhibiting macrophage phagocytosis and impairing bacterial clearance (42) or mechanisms inducing macrophages to undergo programmed cell death (43). Moreover, labile heme can also be scavenged directly by bacterial pathogens, as demonstrated in the case of *Staphylococcus aureus* (44) or *Citrobacter rodentium* (45), promoting pathogen growth and compromising host resistance to infection (21, 46).

The pathological effects of labile heme are countered by host defense mechanisms that converge at the level of heme catabolism and storage of the iron extracted from heme (33, 34, 47). Under physiological conditions heme is catabolized by heme oxygenase-1 and -2 (HO-1 and HO-2), which cleave the tetrapyrrole ring, generating equimolar amounts of iron, carbon monoxide, and biliverdin (48). Upon infection, the stress-responsive HO-1 becomes the rate limiting enzyme in heme catabolism (33), playing a critical role in the establishment of disease tolerance to systemic infections, as illustrated for malaria (40, 41, 49) or polymicrobial sepsis (29).

The iron extracted via heme catabolism by heme oxygenases, integrates the cellular labile iron pool (LIP), becoming available to pathogens while catalyzing the production of ROS via the Haber–Weiss–Fenton sequence (24) (**Figure 3**). The pro-oxidant effects associated with excess heme catabolism and LIP overload are countered via the induction of cellular iron export by the solute carrier family 40 member 1 (SLC40A1), also known as ferroportin 1 (FPN1) (17, 22). Once excreted, iron is captured in plasma by transferrin (17, 22, 50) and delivered, via the transferrin receptor, to erythropoietic precursors where iron is required to support heme and hemoglobin synthesis (17, 22).

To prevent overt accumulation of extracellular iron, ferroportin expression and activity are downregulated by hepcidin, an acute-phase 25-amino acid peptide encoded by the *HAMP* gene (51, 52). In support of this notion, hepcidin accumulates in plasma in response to infection, inhibiting ferroportin expression/activity and impairing cellular iron export (51, 52). This can lead to cellular LIP accumulation, a potentially deleterious effect countered via iron storage and neutralization by ferritin (47, 53, 54).

Ferritin is a multimeric complex composed of ferritin heavy (heart) chain (FTH) and light (liver) chain (FTL) (47, 53,

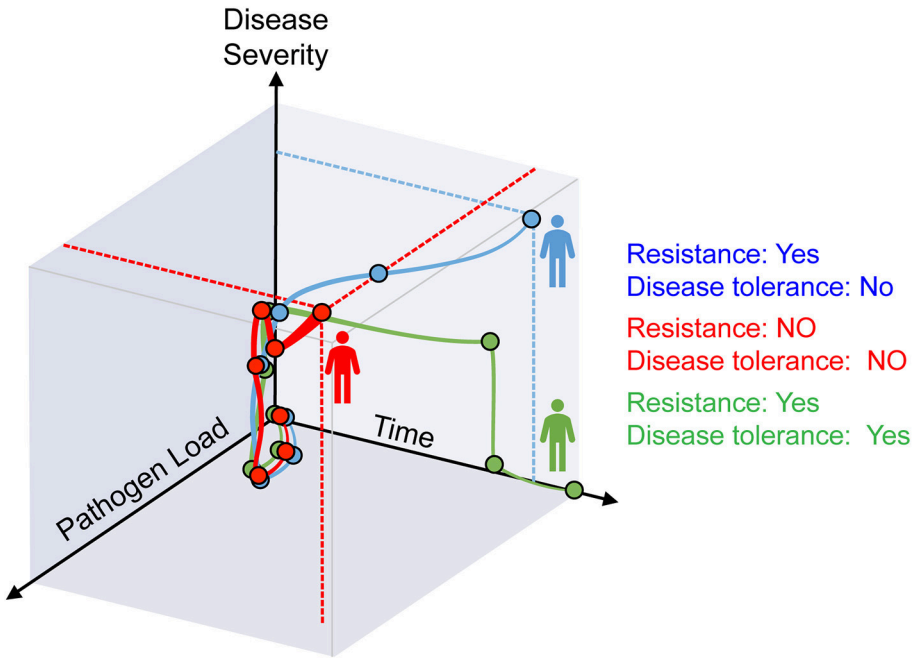

FIGURE 1 | Resistance and disease tolerance to infection. As host pathogen load increases during infection, disease symptoms become apparent and give rise to the clinical signs of infectious diseases. After an initial phase where both pathogen load and disease severity increase, the three possible outcomes are: (i) host homeostasis prevails based on resistance and disease tolerance mechanisms that eliminate pathogens and sustain vital metabolic outputs (green), (ii) resistance mechanisms reduce pathogen load but tissue damage control mechanisms fail to establish disease tolerance, compromising host homeostasis (blue); (iii) resistance mechanisms fail to control pathogen burden and tissue damage control mechanisms fail to establish diseases tolerance, compromising host homeostasis (red).

54) (**Figure 3**). The ferroxidase activity of FTH converts pro-oxidant Fe^{2+} into nucleated Fe^{3+} (47, 53, 54), preventing LIP from participating in Haber–Weiss–Fenton sequence (24), limiting ROS generation and avoiding oxidative damage (17, 33). Supporting this notion, ferritin is essential to enforce tissue damage control and to establish disease tolerance to malaria (32) and to polymicrobial sepsis (14) (**Figure 3**). This protective effect depends on the ferroxidase activity of FTH, suggesting that iron conversion to its oxidized form (Fe^{3+}) and subsequent incorporation into ferritin, are critical to establish disease tolerance to infection.

A significant proportion of ferritin is secreted (55), suggesting that the protective effects of ferritin are not restricted to its intracellular functions. In keeping with this notion, soluble ferritin is protective against *Escherichia coli* infection (56), and acts therapeutically to establish disease tolerance to polymicrobial sepsis (14). This argues for a role of extracellular ferritin as soluble iron chelator/transporter enforcing the establishment of disease tolerance to infection. Unexpectedly, the protective effects of ferritin extends beyond its antioxidant role, in that ferritin also controls glucose metabolism (14).

GLUCOSE METABOLISM AND DISEASE TOLERANCE

Glucose is a key nutrient for most living organisms, acting both as a metabolic fuel for ATP production via glycolysis or mitochondrial electron transport and as a biosynthetic intermediate for amino acid, lipid, and nucleic acid synthesis (57). While glucose intake from food allows for systemic delivery, glucose can also be synthesized endogenously from glucose precursors via gluconeogenesis or glycogenolysis in the liver, kidneys, or intestine (58). Glucose uptake from diet and its endogenous synthesis are tightly regulated to maintain blood glucose levels within a homeostatic range (5, 59). Enforcing this homeostatic range is particularly challenging during an infection (5), given that pathogens and their hosts often compete for this nutrient. Similar to iron, the infected host evolved strategies to limit glucose availability to pathogens, while maintaining glucose levels within a range compatible with survival. One of the strategies limiting glucose availability to pathogens relies on reducing glucose and glucose precursors intake from diet, via anorexia of infection. This is probably a component of nutritional immunity conferring resistance against pathogens (8–12, 60) (**Figure 2**).

Glucose Availability in Response to Infection

The impact of anorexia of infection on the outcome of infectious diseases varies widely depending on the host and pathogen species (9–12, 61). In fruit flies, anorexia of infection promotes the establishment of disease tolerance to *Salmonella* Typhimurium infection, while compromising resistance to *Listeria monocytogenes* infection (11). In mice, anorexia of infection is protective against *L. monocytogenes* (10, 12), but deleterious against influenza virus infections (10, 62). Anorexia of

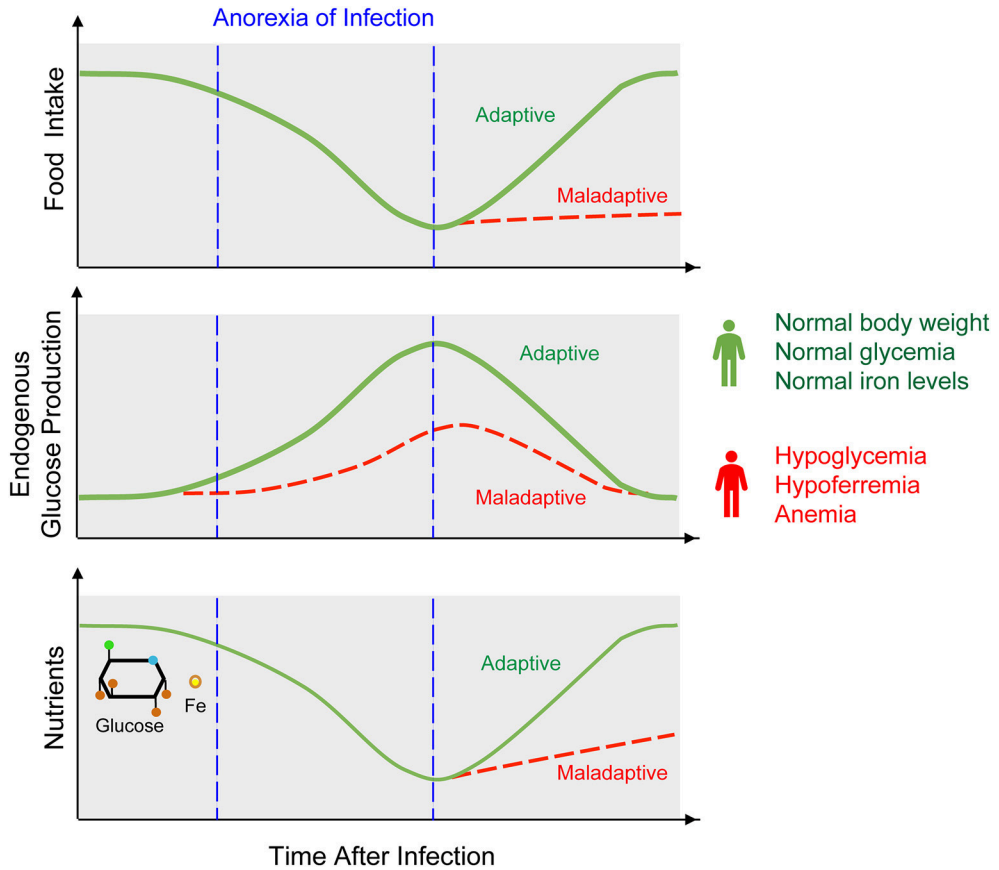

FIGURE 2 | Anorexia of infection, metabolic adaptation, and outcome of infection. Anorexia is a hallmark of sickness behavior that consists on a transient reduction of food intake. Anorexia of infection probably limits pathogens from accessing nutrients, such as glucose or iron. This defense strategy however, cannot be sustained indefinitely as to avoid the development of hypoglycemia, hypoferremia, and anemia, eventually culminating in death of the infected host. Therefore, anorexia of infection must be coupled to a host metabolic response that regulates endogenous production of nutrients, such as illustrated for example for hepatic glucose production. This metabolic response is essential to establish disease tolerance to infection and may also impact on resistance to infection.

infection also impacts on the outcome of gastrointestinal parasitic infections (60, 61), reducing body weight upon *Nippostrongylus brasiliensis* infection (63), while increasing immunopathology in response to *Trichostrongylus colubriformis* infection (64).

Mechanisms regulating anorexia of infection are not clearly established (9, 10, 61), but certainly encompass pathogen sensing via host pattern recognition receptors (PRR) (9). Signaling downstream of PRR elicits the production of interleukins (IL), such as IL-1, IL-6, IL-8, or tumor necrosis factor (TNF), which signal systemically to induce anorexia of infection as well as to regulate glucose metabolism (9). One of the mechanisms via which this occurs involves the secretion of leptin by adipose tissue (9, 65), an hormone that signals in the central nervous system (CNS) to reduce food intake and regulate energy consumption (66).

Pathogens can modulate anorexia of infection directly to promote their survival and/or transmission (9, 10, 61, 67). For example, *S.* Typhimurium inhibits PRR activation/signaling, reducing IL-1β secretion in the gut and increasing food consumption, as well as blood glucose levels (67). This reduces *S.* Typhimurium virulence and promotes host disease tolerance, while increasing *Salmonella* transmission (67), most likely as an evolutionary trade-off. The nematode *N. brasiliensis* also induces anorexia, via the regulation CNS signaling (68, 69), even though the exact mechanism by which this occurs has not been established.

Anorexia of infection is also associated with reduction in caloric intake, i.e., caloric restriction, which can *per se* modulate the outcome of infection (70). For example, caloric restriction increases susceptibility to polymicrobial (71) and viral infections (10, 72), while reducing *Plasmodium* virulence and promoting survival to malaria (73).

Although protective against bacterial infections (8, 9) mechanisms reducing blood glucose levels must be tightly regulated to prevent the development of lethal hypoglycemia. In support of this notion, inhibition of hepatic glucose production in mice carrying a liver-specific deletion of glucose 6 phosphatase 1 (*g6pc1*) compromises disease tolerance to polymicrobial infections (14). This suggests that while reducing blood glucose levels can be protective against bacterial infections

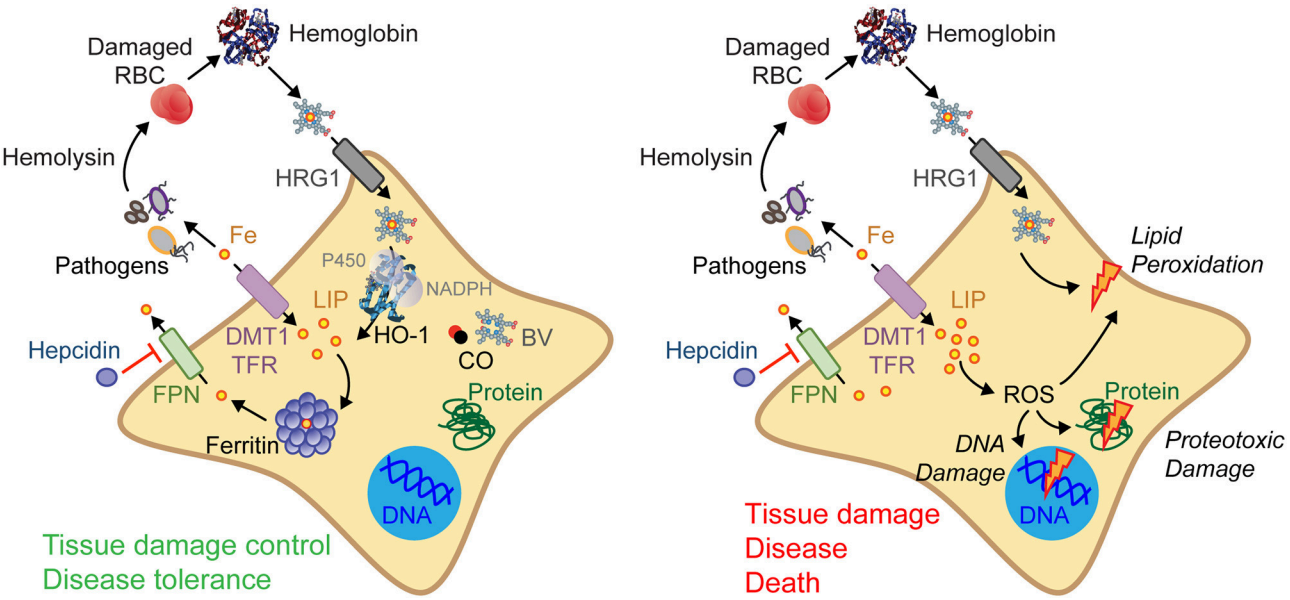

FIGURE 3 | Regulation of cellular iron metabolism in response to infection. Several resistance mechanisms may be used to restrict extracellular pathogens from accessing iron. For example, host cells can import heme/iron via heme transporters, such as the heme responsive gene 1 (HRG1), or via iron transporters, such as the divalent metal transporter-1 (DMT1) or the transferrin (TF)-transferrin receptor (TFR) that uptakes iron-TF complexes. Intracellular heme is catabolized by HO-1, generating iron, biliverdin (BV), and carbon monoxide (CO) **(left)**. Hepcidin prevents cellular iron export via ferroportin (FPN) and as such LIP arising from heme catabolism must be stored by ferritin. These mechanisms are essential to confer tissue damage control and establish disease tolerance to systemic infections **(left)**. When these protective mechanisms fail **(right)** intracellular heme and LIP increases promoting the generation of ROS, damaging DNA, proteins, and lipids. Ultimately this can compromise tissue damage control and the establishment of disease tolerance to infection **(right)**.

(8, 9), endogenous glucose production is required to prevent the development of lethal hypoglycemia and establish disease tolerance to polymicrobial sepsis (14).

Impact of Metabolic Diseases on Infection

The impact of glucose metabolism on the outcome of infectious diseases is illustrated by the effect of metabolic diseases, such as obesity or diabetes, on the outcome of infections. For example, hyperglycemia in diabetic rodents is associated with increased susceptibility to polymicrobial sepsis (74, 75) as well to *L. monocytogenes* (76) or *M. tuberculosis* (77) infections. Moreover, hyperglycemia promotes intestinal permeability and increases susceptibility to bacterial infection in mice (78). This pathogenic effect is mediated via glucose import by intestinal epithelial cells, disrupting the functional integrity of the gut epithelium via a mechanism that interferes with epithelial tight and adherens junctions (78). Despite this experimental evidence, whether deregulation of glucose homeostasis impacts on the outcome of bacterial infections in humans remains unclear. For example, clinical evidence suggests that diabetes mellitus is not a major risk factor for sepsis severity (79), while both hyperglycemia and hypoglycemia are major risk factors for sepsis mortality (80, 81). Of note, rodents develop hypoglycemia rather than hyperglycemia in response to bacterial infections (14, 15, 82, 83). In some cases, hypoglycemia is preceded by a transient state of hyperglycemia, but whether this is triggered by infection and/or other associated experimental procedure is not clear (14, 15).

Glucose Control of Innate and Adaptive Immune Function

Regulation of host glucose metabolism can impact on pathogens directly or indirectly, via modulation of immune-driven resistance mechanisms (84–86). Proliferation, differentiation, and effector function of immune cells is regulated by two major metabolic programs, namely, oxidative phosphorylation, and aerobic glycolysis (84–86). Signaling via PRR in macrophages or dendritic cells shifts metabolic flux from oxidative phosphorylation to aerobic glycolysis, a phenomenon known as the Warburg effect (87). Despite being less energetically effective, glycolysis generates pyruvate, nicotinamide adenine dinucleotide (NADH), and other metabolic intermediates used by major biosynthetic pathways (84, 86). This metabolic shift also promotes the pentose phosphate pathway, generating nicotinamide adenine dinucleotide phosphate (NADPH), a critical component of the NADPH oxidase (NOX) enzyme complexes, generating ROS involved in pathogen killing (84, 86, 88). In contrast to their microbicidal effector functions, other macrophage effector functions promoting tissue healing and regeneration rely primarily on oxidative phosphorylation (86, 88).

A marked increase in aerobic glycolysis is also a hallmark of T cell activation, together with a more modest induction of oxidative phosphorylation (86, 89), presumably accommodating the reduction in oxygen availability that arises during infections (85, 90). This metabolic reprogramming is orchestrated by a complex mechanism involving the store-operated Ca^{2+} entry

(SOCE), a key regulator of cellular calcium signaling (91), the hypoxia-inducible factor 1α (HIF1α), a transcriptional master regulator of hypoxia, as well as the mammalian target of rapamycin complex 1 (mTORC1), a master regulator of cell growth (84, 86, 92). Of note, mTORC1 controls the expression of glycolytic genes in innate and adaptive immune cells via a mechanism involving HIF1α (93–95). The relative impact of these metabolic pathways on the outcome of infections can be illustrated in the context of *M. tuberculosis* infection, where myeloid HIF1α plays a critical role to induce the Warburg effect (96), supporting resistance to *M. tuberculosis* (97). Similarly, mice lacking HIF1α in the myeloid compartment also fail to shift to aerobic glycolysis, succumbing to bacterial sepsis (98).

In contrast to effector T cells, memory T cells rely on oxidative phosphorylation to produce energy, using fatty acids to produce acetyl coenzyme A (acetyl-CoA) and fuel the Krebs cycle, via a mechanism known as fatty acid oxidation (FAO) (84–86). Moreover, recent work has shown that FAO in memory T cells, can occur not only via carnitine palmitoyltransferase IA (CPT IA)-dependent, but also independent mechanisms (99), suggesting that memory T cells are able to use a wide range of fatty acids in order to obtain energy. The switch between aerobic glycolysis and oxidative phosphorylation relies on a mechanism involving the transcription repressor Bcl-6 (100), which downregulates glycolytic genes and promotes the T and B cell differentiation toward the memory compartment (101–103).

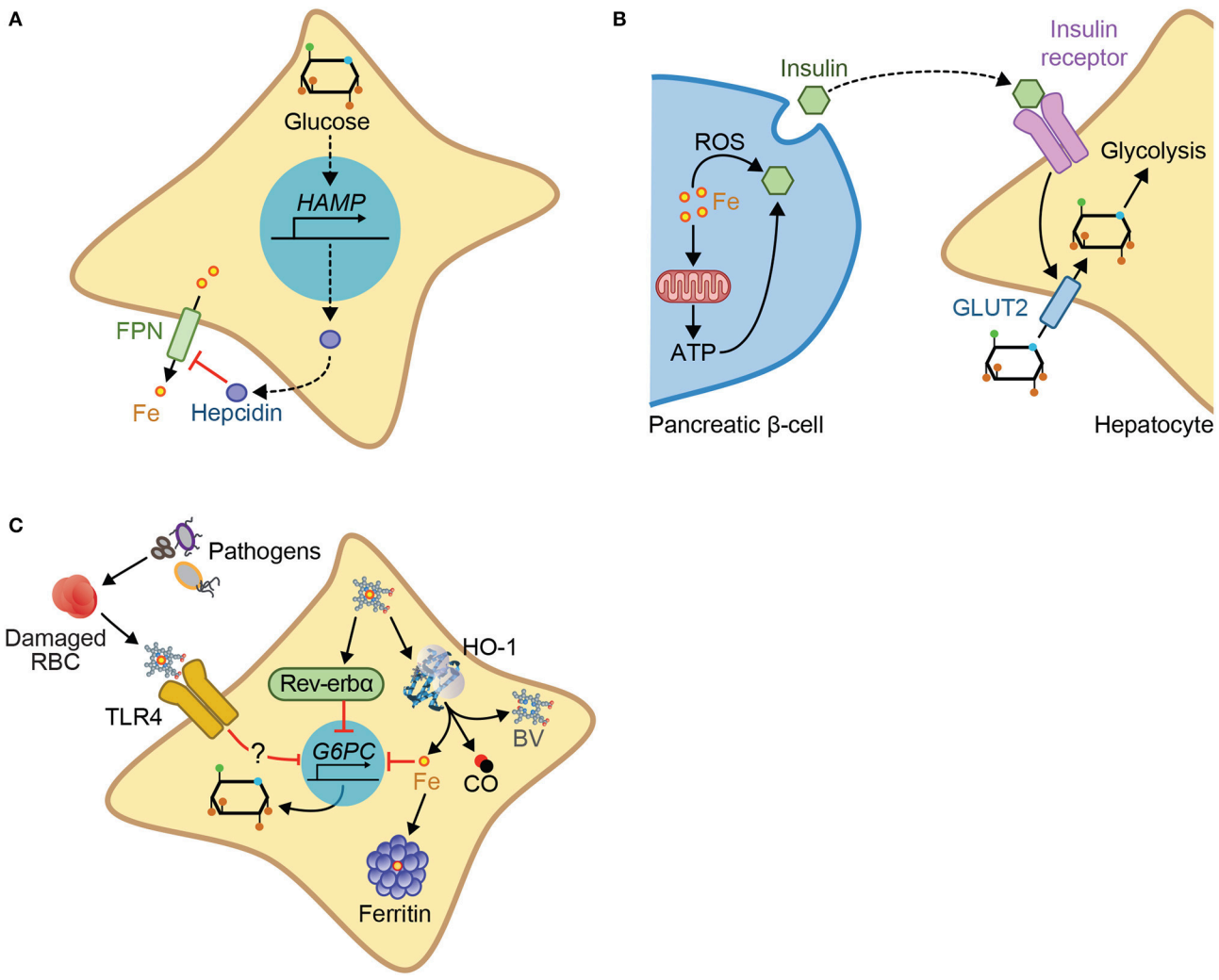

FIGURE 4 | Mechanisms of iron-glucose metabolism cross-talk. Iron and glucose can cross-talk via different mechanisms: **(A)** Glucose increases expression of hepcidin, inhibiting cellular iron export via ferroportin. **(B)** Iron acts via the production of ROS or via mitochondrial respiration and subsequent ATP production, to promote insulin exocytosis by the pancreatic β-cells. Insulin binding to the insulin receptor in target cells, e.g., hepatocytes, promotes cellular glucose import via the glucose transporter 2 (GLUT2), and glycolysis. **(C)** In the context of infection, glucose metabolism can be regulated by heme via a pathway that involves TLR4, but which has not yet been fully described. Heme interaction with the nuclear receptor Rev-erbα, downregulates the transcription of gluconeogenic genes including *G6PC*, the enzyme catalyzing the last step of gluconeogenesis. *G6PC* is also downregulated by iron produced via heme catabolism by HO-1, an inhibitory effect countered by ferritin.

Presumably, the combined effect of reduced glycemia and Bcl-6 expression are likely to promote effector to memory T cell transition in response to infection. Whether glucose availability impacts on immune-driven resistance mechanisms remains, to the best of our knowledge, to be determined.

CROSS-TALK BETWEEN IRON AND GLUCOSE METABOLISM IN RESPONSE TO INFECTION

A functional interplay between iron and glucose metabolism has been established primarily in the context of metabolic diseases, such as porphyria (104, 105) or diabetes (106–108). For example, hepatic glucose production induces hepcidin expression (109, 110) (**Figure 4A**) and inhibits cellular iron export by ferroportin, leading to cellular iron overload and hypoferremia (110). Conversely, cellular iron overload regulates insulin production in pancreatic β-cells (106, 111) and is thought to contribute critically to impair glucose metabolism in diabetic patients (112, 113) (**Figure 4B**). This interplay is probably operational in other pathologic conditions such as atherosclerosis (107) or β-thalassemia major (114).

More recently a crosstalk between iron and glucose metabolism has also been established in the context of infections (14, 115, 116). Namely, iron intake from diet leads to decreased pathogen virulence, without interfering with pathogen burden, favoring asymptomatic infection with the enteric pathogen *C. rodentium* (116). This occurs through a mechanism via which iron intake promotes insulin resistance, reducing glucose uptake by the intestine, and thus promoting glucose availability in the gut, leading to the suppression of virulence factors (116). Deregulation of host iron metabolism in response to polymicrobial infection compromises the establishment of disease tolerance to sepsis, via a mechanism that deregulates glucose metabolism (14, 117), thus also illustrating the crosstalk between iron and glucose. This pathologic mechanism is driven by labile heme, which plays a central role in the pathogenesis of sepsis (29). Namely, labile heme inhibits hepatic G6pase and consequently glucose production leading to hypoglycemia (14) (**Figure 4C**). This pathogenic effect has been linked functionally to a transcriptional repression of *g6pc1* gene (14). In support of this notion, mice lacking hepatic *g6pc1* develop lethal hypoglycemia in response to polymicrobial sepsis or heme administration (14). This suggests that hepatic glucose production is required to counter the hypoglycemia induced by labile heme (14). This is also consistent with the notion that deregulation of glucose metabolism plays a central role in pathogenesis of infectious diseases, including sepsis (10, 14, 80, 81, 118). This occurs via a mechanism that is not associated with modulation of host pathogen load (10, 14, 117), demonstrating that regulation of glucose metabolism controls the establishment of disease tolerance to infection (10, 14, 117).

The molecular mechanism via which labile heme induces hypoglycemia is not entirely clear but has been linked to signaling via Toll-like receptor 4 (TLR4) (14) (**Figure 4C**), a PRR that senses labile heme (119). This is consistent with the induction of hypoglycemia by TLR4 ligands, such as LPS (120). Whether heme sensing by TLR4 mediates the development of hypoglycemia during polymicrobial sepsis was not established. The pathway through which heme represses *g6pc1* transcription (14, 121) is likely to involve the heme sensor and transcriptional repressor Rev-erbα (121) (**Figure 4C**). Whether this mechanism is operational *in vivo* to repress hepatic glucose production and elicit hypoglycemia in response to infection remains to be established. It is possible as well that TLR4 and Rev-erbα synergize to repress *g6pc1* transcription in hepatocytes.

Iron sequestration by ferritin counters heme-driven repression of *g6pc1* transcription, suggesting that heme represses *g6pc1* transcription via a mechanism involving iron (14). In keeping with this notion, polymicrobial infections in mice are associated with the induction of ferritin in the liver, which is essential to sustain hepatic glucose-6-phosphatase (G6Pase) expression and counter the development of lethal hypoglycemia (14, 122) (**Figure 4C**). Whether iron accumulation in hepatocytes synergizes with TLR4 and Rev-erbα to repress *g6pc1* transcription remains to be established.

Regulation of hepatic glucose production by ferritin may be part of an adaptive response promoting the development of insulin resistance, presumably countering unfettered cellular glucose utilization in host tissues and allowing to restore normal blood glucose levels (123). This effect of ferritin should also contribute to prevent the development of hypoglycemia in response to infections.

CONCLUSIONS AND FUTURE PERPECTIVES

Resistance to infection is generally perceived as the predominant host defense strategy against infection. This dogma has been challenged by the recurrent observation that the severity of infectious diseases can at times be dissociated from host pathogen burden. In the last few years these observations have been interpreted as revealing disease tolerance as a critical host defense strategy against infection. This defense strategy relies on tissue damage control mechanisms controlling the metabolic output of host tissue and maintaining vital homeostatic parameters within a range compatible with host survival. This is illustrated for mechanisms regulating iron and glucose metabolism, which cross-talk to establish disease tolerance to infection. To what extent these tissue damage control mechanisms may be targeted therapeutically remains to be established.

AUTHOR CONTRIBUTIONS

ARC wrote the manuscript with SW and MPS. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Fueling Defense: Effects of Resources on the Ecology and Evolution of Tolerance to Parasite Infection

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Resource availability is a key environmental constraint affecting the ecology and evolution of species. Resources have strong effects on disease resistance, but they can also affect the other main parasite defense strategy, tolerance. A small but growing number of animal studies are beginning to investigate the effects of resources on tolerance phenotypes. Here, we review how resources affect tolerance strategies across animal taxa ranging from fruit flies to frogs to mice. Surprisingly, resources (quality and quantity) can increase or reduce tolerance, dependent upon the particular host-parasite system. To explore this seeming contradiction, we recast predictions of models of sterility tolerance and mortality tolerance in a resource-dependent context. Doing so reveals that resources can have very different epidemiological and evolutionary effects, depending on what aspects of the tolerance phenotype are affected. Thus, it is critical to consider both sterility and mortality in future empirical studies of how behavioral and environmental resource availability affect tolerance to infection.

Keywords: tolerance, resistance, resources, foraging, parasite infection, defense strategy

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INTRODUCTION

Parasite-infected hosts have two, non-exclusive options for mitigating the fitness costs of parasite infection. Resistance describes an individual's ability to reduce its parasite load, while tolerance is a measure of an individual's ability to mitigate the fitness costs of parasite infection without reducing parasite load (1–4). Thus, a more tolerant individual attains higher fitness than others with the same parasite burden. Tolerance can be quantified as the slope of the relationship between parasite load and fitness with a less steep slope indicating higher tolerance [(1) but see (5) for a criticism of this approach]. While the ecological and evolutionary drivers of variation in resistance have been elucidated by decades of studies, variation in tolerance is less well-understood (6–9). In plants, where tolerance in response to damage (e.g., herbivory, infection) has long been studied, the important ecological and evolutionary implications of tolerance have been demonstrated and provide useful parallels for understanding animal host-parasite interactions (10).

Notably, plant tolerance to herbivory depends on environmental resource availability (11). The hypothesis that animal tolerance may also be resource-dependent is supported on general evolutionary grounds; resource availability is a principal selective pressure shaping the evolution of species, as evidenced by decades of studies on resource partitioning and character displacement

(12, 13). Moreover, the often strong effects of resources on the ecology and evolution of disease resistance are well-established from both theoretical (14, 15) and empirical (16–19) perspectives. A growing number of animal disease studies suggest that host tolerance to parasites might also be affected by resources [**Table 1**; (8)]. However, as we review, existing studies often come to mixed conclusions as to the effect of resources on tolerance, suggesting that a theoretical framework is needed to guide hypothesis development and to draw general conclusions.

To date, there have not been any theoretical studies that directly address the question of how resources affect host investment in tolerance to infection (9), where this investment reduces the fitness cost of infection at some cost to the host. We distinguish this theory from other work that has examined how resource-dependent effects on mortality or transmission affect ecological dynamics (15, 33). However, existing theory exploring the implications of investment in tolerance for the ecological and evolutionary dynamics of host-parasite systems does provide indirect insights into how resources might affect tolerance investment. Here we review the empirical studies of resources on tolerance, explore key predictions of existing theory, and discuss how combining theoretical and empirical approaches could further understanding of the effects of resources on the ecology and evolution of tolerance.

DIRECT EFFECTS OF ENVIRONMENTAL RESOURCES ON TOLERANCE

Resources Can Increase Tolerance

Intuitively, tolerance should require host investment of potentially limiting resources to compensate for parasite-induced reductions in host fitness, for example by repairing tissue damage. Support for that intuitive prediction comes from both observational and experimental studies showing that organisms with increased resource consumption have higher tolerance, and that organisms with reduced ingestion have compromised tolerance (**Table 1**). This evidence comes from studies investigating resource limitation (e.g., low resources vs. "normal"), studies on resource supplementation (e.g., "normal" resources vs. high), or studies of two resource levels, but with no reference to which (if either) is normal for that host in the wild. Notably, the shape of the reaction norm between resources and tolerance cannot be determined from only two resource levels. A such, results from resource limitation studies should not be extrapolated to high-resource conditions, or vice versa. Determining the shape of such reaction norms by quantifying tolerance across a range of resource levels ranging from scarce to over-abundant is a key area for future research.

Numerous observational studies indicate that increasing resource consumption can be a behavioral mechanism of tolerance (21–23). For example, Knutie et al. (23) used a parasite removal experiment to determine that parasitized Galapagos mockingbird nestlings beg more for food and receive increased provisioning from their parents in comparison to their non-parasitized counterparts. The additional resources they received allowed parasitized nestlings to compensate for some of the costs of infection; fledging success was not affected by parasite load. Notably, in the same experiments, the medium ground finch did not increase provisioning to infected nestlings, which resulted in a negative relationship between parasite load and fledging success. Thus, the resource supplementation behavior of mockingbirds makes them more tolerant than the medium ground finch (23). Interspecific variation in tolerance to a generalist parasite could alter transmission dynamics and competition between species, as the tolerant species will support a higher parasite population, fueling spillover infections that drive down the population size of the intolerant host, analogous to the P* concept in apparent competition theory (34). Thus, interspecific variation in tolerance has the potential to affect the ecology and evolution of host communities. Similar forms of "parental compensation" by increasing resource provisioning to parasite-infected nestlings has been observed in other focal bird species (21, 22). Interestingly, although initially broadly accepted, the parallel theory for plant-herbivore-resource interactions, termed the "compensatory continuum hypothesis," a metaanalysis found little support for the theory (11, 35). For host-parasite interactions, further studies and expanding beyond avian systems may prove useful in determining whether, how commonly, and under what conditions resources and foraging behavior can be used to fuel tolerance.

Moreover, severely malnourished hosts often have diminished investment in both resistance and tolerance defenses (16–19). Resource limitation thus has the potential to result in higher parasite loads and higher fitness costs per parasite. Indeed, Cuban tree frogs show both reduced resistance and tolerance to infection with a parasitic nematode when food abundance is limited (25). If hosts are less able to either resist or tolerate infection, the resulting effects on parasite transmission and host population dynamics may be complex. Individual hosts will have higher load due to reduced resistance, but lower survival and/or reproduction due to reduced tolerance. At the population level, these effects could translate to increased transmission due to higher shedding rates or reduced transmission due to parasite-induced mortality, lower population density, and reduced birth rate of new susceptibles (15).

Even when tolerance responds positively to increasing resource quality and quantity, resistance may not respond similarly. For example, when infected with a bacterial pathogen, the crustacean *Daphnia magna* has increased survival (i.e., higher tolerance) when given high food levels compared to low food levels, despite having higher parasite loads (i.e., lower resistance) at high food levels (32). Likewise, a low-protein diet has been shown to increase resistance but reduce the ability of lab mice to tolerate gastrointestinal nematode infection, when tolerance is measured as a function of weight gain (20) and intestinal barrier function (7). However, the effect of resources on tolerance to nematode infection can vary with host genotype (7); there was no effect of diet on tolerance to infection in a strain of lab mice that better maintained their intestinal barrier during infection. Conversely, genotype did not affect the morality tolerance of bacteria-infected *D. magna* (32). Alternatively, resistance may respond positively to resource quality while tolerance does not; food-limited crickets show reduced resistance but equal tolerance

TABLE 1 | Studies of the effects of resources on tolerance show varied outcomes (red, resources reduce tolerance; yellow, resources have no effect on tolerance; green, resources increase tolerance; white, resources affect tolerance).

Host	Parasite	Study design	Effect of resources on tolerance	Tolerance metric	Source
BALB/c and CBA lab mice (*Mus musculus*)	*Heligmosomoides polygyrus* (nematode)	Resource quality (low vs. high-protein diet) crossed with infection status	low quality resources reduce tolerance, but only for BALB/c mice	Fitness proxies (weight gain, intestinal permeability)	(7)
BALB/c lab mice (*Mus musculus*)	*Heligmosomoides polygyrus* (nematode)	Resource quality (low vs. high-protein diet) crossed with single and co-infection status	No effect of resource quality on tolerance	Fitness proxy (weight gain)	(20)
BALB/c lab mice (*Mus musculus*)	*Nippostrongylus brasiliensis* (nematode)	Resource quality (low vs. high-protein diet) crossed with single and co-infection status	Low quality resources reduce tolerance	Fitness proxy (weight gain)	(20)
Blue tits (*Parus caeruleus*)	*Ceratophyllus gallinae* (flea)	Resource acquisition behavior–Flea removal and addition to nests	Behavioral resource supplementation facilitated tolerance	Sterility (offspring quantity and quality)	(21)
Great tits (*Parus major*)	*Ceratophyllus gallinae* (flea)	Resource acquisition behavior–Flea removal and addition to nests	Behavioral resource supplementation facilitated tolerance	Sterility (offspring number and condition, but reduced body size)	(22)
Galápagos mockingbird (Mimus parvulus)	*Philornis downsi (invasive nest fly)*	Resource acquisition behavior–Fly removal from nests	Behavioral resource supplementation facilitated tolerance	Sterility (offspring quantity and quality)	(23)
medium ground finches (Geospiza fortis)	*Philornis downsi (invasive nest fly)*	Resource acquisition behavior–Fly removal from nests	Without behavioral resource supplementation, tolerance was lower	Sterility (offspring quantity and quality)	(23)
Domestic canaries (*Serinus canaria*)	*Plasmodium relictum* (avian malaria)	Resource supplementation crossed with infection	Resource supplementation reduces tolerance	Fitness proxy (hematocrit)	(24)
Cuban tree frog (*Osteopilus septentrionalis*)	*Aplectana* sp. (nematode)	Resource quantity (# crickets) crossed with infection status	Low quantity of resources reduces tolerance	Fitness proxy (weight change)	(25)
Monarch butterflies (*Danaus plexippus*)	*Ophryocystis elektroscirrha* (protozoa)	Resource variation (12 milkweed food plant species) crossed with infection status	Tolerance varies by milkweed species and increases with cardenolide conc.	Mortality (longevity)	(26)
Texas field crickets (*Gryllus texensis*)	*Serratia marcescens* (bacteria)	Resource limitation crossed with infection and wounding	No effect of resource limitation on tolerance	Sterility (egg output) and immune mechanism (glutathione)	(27)
Fruit fliy (*Drosophila melanogaster*)	*Providencia rettgeri* (bacteria)	Resource quality (low vs. high-sugar diet) crossed with infection status and genotype	Lower mortality tolerance on high-sugar diet, but no effect on sterility tolerance	Sterility (# adult offspring produced) and mortality (survival)	(28)
Fruit fliy (*Drosophila melanogaster*)	*Salmonella typhimurim* (bacteria)	Resource quantity (dilute media) crossed with infection status	Resource limitation increases tolerance	Mortality (longevity)	(29)
Fruit fliy (*Drosophila melanogaster*)	*Lysteria monocytogenes* (bacteria)	Resource quantity (dilute media) crossed with infection status	No effect of resource limitation on tolerance	Mortality (longevity)	(29)
Fruit fliy (*Drosophila melanogaster*)	*Escherichia coli* (bacteria)	Resource quality (low vs. high-protein diet) crossed with infection status	Resource limitation increases tolerance, but only during early infection	Sterility (# adult offspring produced)	(30)
Fruit fliy (*Drosophila melanogaster*)	*Lactococcus lactis* (bacteria)	Resource quality (low vs. high-protein diet) crossed with infection status	No effect of resource quality on tolerance	Sterility (# adult offspring produced)	(30)
Fruit fliy (*Drosophila melanogaster*)	*Lactococcus lactis* (bacteria)	Resource quality (low vs. high-protein diet) crossed with infection status	No effect of resource quality on tolerance	Sterility (# adult offspring produced)	(31)
Daphnia magna	*Pastura ramosea* (bacteria)	Resource quantity (low vs. high) crossed with infection status and genotype	Low quantity of resources reduces tolerance	Mortality (longevity)	(32)

to *ad libitum* fed individuals (27). Taken together, these results indicate that understanding the population-level consequences of resource limitation for disease dynamics will likely require considering the complex interactions among genotype, tolerance, and resistance.

Resources Can Reduce Tolerance

Reduced resource ingestion is a ubiquitous response to infection across the animal kingdom (36). While initially thought to be a maladaptive side-effect of infection, studies increasingly suggest that illness-induced anorexia may carry benefits for the host (37, 38). For example, fruit flies on a limited (dilute) diet are more tolerant of *Salmonella typhimurim* infections, exhibiting increased fecundity relative to parasite load compared to infected individuals on a standard diet (29). Notably, this beneficial effect of resource limitation on tolerance is infection-specific; diet restriction did not affect tolerance to another bacteria, *Listeria monocytogenes* (29). Similarly, a low-protein diet increases sterility tolerance to *Escherichia coli* infection, but not *Lactococcus lactis* infection in fruit flies (30). A low-sugar diet also increases fruit fly tolerance with respect to mortality due to the bacterial pathogen *Providencia rettgeri*. Interestingly, dietary sugar content does not affect fruit fly fecundity relative to parasite load (i.e., sterility tolerance) (28). Tolerance benefits of a low resource diet are not limited to fruit fly-bacteria pathogen interactions; canaries infected with avian malaria (*Plasmodium relictum*) exhibit higher hematocrit relative to parasite load when on a standard rather than supplemented diet (24). Nonetheless, most studies of infection-induced anorexia have primarily focused on it as a parasite avoidance strategy or a side-effect of resistance responses, leaving anorexia-tolerance relationship a topic warranting further empirical and theoretical attention (39).

THEORETICAL PREDICTIONS FOR EFFECTS OF RESOURCES ON TOLERANCE

Modeling the Evolution of Tolerance

Given the limited number of empirical studies on the effects of resources on tolerance to infection, theory may help us understand the implications of these studies and guide hypotheses and design of future empirical research. No prior studies have directly modeled the effects of resources on host investment in tolerance, but existing theory regarding the ecological and evolutionary implications of tolerance investment can be adapted to provide useful, although indirect, insights. In the **Appendix** in Supplementary Material, we extend existing theory to explicitly account for resources. Analysis of this model shows how the shapes of the relationships between tolerance investment, resources, and host life history can drive the evolutionary response of tolerance to resources.

Here, however, we focus on reviewing existing theory. From a theoretical perspective, tolerance is modeled by assuming that some model parameters (such as virulence) are under the control of both the parasite and the host (40). We will use the following simple model to illustrate many of the conclusions of theory (41, 42):

$$\frac{dS}{dt} = a\left(S + fI\right) - qN\left(S + fI\right) - mS - \beta SI + \gamma I$$
$$\frac{dI}{dt} = \beta SI - (\alpha + m + \gamma)\, I$$

In this model, a is the intrinsic birth rate of the host, f is the reduction in intrinsic birth rate due to infection, q is the host susceptibility to crowding, m is the background mortality rate of the host, β is the transmission rate, α is the virulence (infection-induced mortality rate), and γ is the recovery rate. In this simple model, infection may reduce host fitness by reducing host birth rate (f) or increasing mortality rate (α). These two parameters, therefore, depend on both host-specific traits (parameters) and parasite-specific traits. That is, f and α are both functions, $f(h_f, p_f)$ and $\alpha(h_\alpha, p_\alpha)$, where h_i and p_i are host and parasite traits, respectively. In a host-centric analysis, p_f and p_α are assumed to be constant. Finally, investment in tolerance by the host (increasing h_f or h_α) must come at some cost to other aspects of host fitness (otherwise, infinite investment will always be favored). Typically, theory assumes that investment in *mortality tolerance* (h_α) reduces intrinsic birth rate (a is a decreasing function of h_α, $a(h_\alpha)$), whereas investment in *sterility tolerance* (h_r) increases background mortality rate (m is an increasing function of h_r, $m(h_r)$). Importantly, sterility tolerance has no effect on parasite fitness, whereas mortality tolerance increases parasite fitness (43, 44). This distinction has important consequences for both ecological and evolutionary dynamics.

This sets up the basic model for studying the ecological and evolutionary consequences of tolerance. There is also a significant body of research studying "resistance" strategies of host defense (43), such as avoidance (host traits affecting β) or recovery (host traits affecting γ). In these models, there will be trade-offs between host investment in resistance and host intrinsic birth rate.

There are several models that explicitly consider how investment in resistance and tolerance change simultaneously (40, 45) including models that assume a trade-off in investment (44). We will also discuss models that consider the coevolution of hosts and parasites. In these models, parasite traits also vary and are involved in parasite fitness trade-offs (e.g., increasing p_α increases both infection-induced mortality α and transmission rate β).

Existing theory typically studies the *evolution* of tolerance using evolutionary invasion analysis (46). This framework conceptualizes evolution as a series of mutation events, where "mutant" hosts with new trait values attempt to invade a population of "resident" hosts at their epidemiological equilibrium. If the mutant can invade, it does and the trait composition of the population changes. Ultimately, the theory is seeking to find evolutionarily stable traits; such a trait is a fitness maxima and a host population with that trait cannot be invaded. Other interesting outcomes are possible, such as evolutionary bistability (the existence of multiple evolutionarily stable trait values, only one of which will be attained) and evolutionary

branching (evolution of polymorphism in trait values) (46). However, though these predictions tend to be evolutionary, we can also use them to infer how tolerance will change plastically in response to host, parasite, or environmental factors, such as resources. Perfect adaptive plasticity should adjust investment in tolerance in response to changes in the environment such that the population remains at a fitness maximum. Thus, we will assume that predictions for the evolution of tolerance can guide predictions about plastic changes in tolerance as well.

Implications of Mortality vs. Sterility Tolerance

Before delving into specific predictions of theory, and their potential implications for the effect of resources on tolerance, there is an important distinction to be made between mortality tolerance and sterility tolerance. Studies of mortality tolerance (42, 44, 47–54) vastly outnumber studies of sterility tolerance (40, 44, 55, 56). Mortality tolerance will increase parasite fitness by increasing the host lifespan while infected. As such, investment in tolerance increases parasite fitness, thereby increasing parasite prevalence and hence, the selection for investment in tolerance, driving tolerance to fixation via positive frequency dependence (44, 49). This is in contrast to defense mechanisms that directly reduce parasite fitness: investment in such resistance mechanisms reduces parasite fitness, thereby reducing infection prevalence and, hence, selection for investment in resistance. This negative frequency dependence can lead to other evolutionary outcomes, such as polymorphism in resistance investment (44, 56). Such polymorphism is, in general, impossible in models of mortality tolerance (54). Sterility tolerance, however, can generate such negative frequency dependence because parasite fitness is reduced via the trade-off between sterility tolerance and host background mortality rate. As such, polymorphism is possible, meaning that hosts with both high and low investment in tolerance can coexist in both ecological and evolutionary time.

Effects of Resources on Tolerance

There are two ways to that resources could modify host investment in tolerance. The most direct is if tolerance is itself resource-dependent, for example if increasing resources increases tolerance by making it "cheaper" to invest in tolerance. Existing theory is insufficient to guide predictions here. We show in the **Appendix**, using the simple model above, that the response of tolerance investment to increased resources is highly sensitive to the shapes of the functions relating resources to tolerance, and tolerance to host fitness (57).

On the other hand, resources can also alter aspects of host physiology or the environment, including by directly changing virulence. These changes will indirectly modify the optimal investment in tolerance. As existing theory typically explores how tolerance changes across gradients of epidemiologically relevant factors, we can use it to understand these indirect effects of resources on tolerance. In particular, we will consider the influence of transmission rate, host lifespan, and host reproduction on tolerance investment. For all of these, theory makes clear predictions and the influence of resources can be inferred straightforwardly.

One of the most commonly explored gradients is transmission rate, β. A universal finding (47, 51, 53, 56, 58) is that, as transmission rate increases, so does investment in either sterility or mortality tolerance. This increased investment in tolerance occurs even as investment in resistance decreases across this gradient (40, 45). These results are entirely intuitive: as transmission rate increases, hosts spend more of their life infected, and thus compensating for the deleterious effects of infection on fitness becomes more important. Resources are likely to affect the transmission rate of many parasites. If parasites are encountered during foraging, either incidentally, as is the case for many parasites in aquatic systems (59), or via intentional ingestion, as is the case for trophically transmitted parasites (60), then transmission rate will be directly related to host foraging rate and thus will be resource-dependent. If increasing resources causes hosts to forage more (or less), theory would predict that investment in tolerance should increase (or decrease). Alternatively, if abundant resources promote host aggregation or reduced host movement, they can also increase transmission via higher contact rates between individuals and/or infected environments (61–63), and hence, increase investment in tolerance.

Increasing host lifespan (either by decreasing the background mortality rate, m, or parasite virulence, v) is also predicted to increase investment in mortality tolerance (40, 45, 47, 50–53). For sterility tolerance, the results are more complicated, indicting either a unimodal or strictly increasing response of tolerance to host lifespan, depending on the virulence of the parasite (56). Given that increasing resources is likely to reduce the mortality rate from other factors by improving host body condition (64, 65), increasing resources will often increase the investment in tolerance.

The consequences of increasing fecundity on tolerance investment has received only limited theoretical exploration (66). That study varied the birth rate of infected hosts relative to uninfected hosts, f, to study how investment in mortality tolerance and other defense strategies varied. They showed that, as the birth rate of infected hosts increased, so did the investment in tolerance, even when increased investment in tolerance compromised investment in resistance mechanisms (42). Again, increased resources is likely to increase investment in tolerance, as infected hosts are more likely to reproduce at near-normal levels when resources are abundant (67). As we show in the **Appendix** in Supplementary Material, a model incorporating an explicit effect of resources on birth rate would also make the same prediction: if increasing resources increases birth rate, that will also increase investment in tolerance.

The importance of understanding how tolerance will respond to increased resources is magnified by the fact that the evolution of tolerance is often very sensitive to the initial level of tolerance in the population. For example, Miller et al. (53) found that, at an intermediate host lifespan, the host can evolve toward either high tolerance or complete intolerance, depending on the initial level of tolerance in the population. Such bistability between high tolerance and low tolerance strategies is actually a very common finding in studies of tolerance (40, 48, 51, 56), indicating that it is fairly general across a wide range of epidemiological

conditions. The implication of such bistability for predicting how resources affect tolerance investment is therefore two-fold. First, if resources are abundant, they may increase the likelihood that tolerance "wins out" over intolerance, as in models showing contingent competition between tolerant and intolerant host populations (51). Second, evolutionary bistability is often characterized by hysteresis, where small changes in the environment can trigger massive changes in the system state. Thus, were a system to start in a bistable region of parameter space where the fitness-maximizing investment in tolerance was very low, an increase in resources could cause the system to pass into a region where the fitness-maximizing investment in tolerance was very high, leading to a sudden jump in investment. Because of the hysteresis, however, a reduction in resources wouldn't necessarily lead to a sudden drop in investment (53).

MERGING EMPIRICAL AND THEORETICAL INFERENCES

As we discuss below, existing theory has three major implications for empirical studies of tolerance. Existing empirical studies have focused on how resources can directly affect host tolerance. Our review of theory suggests that resources may also indirectly affect tolerance by changing the ecological context of host-parasite interactions (e.g., by altering contact rates and, hence, the benefits of investment in tolerance). Human activities are altering the quality, quantity, and distribution of resources available to hosts in the environment (68, 69). This ubiquitous feeding of wildlife by humans, whether intentional or incidental, has a multitude of consequences for wildlife disease (9, 15, 62). The cross-scale effects of anthropogenic resource subsidies are well-described in a recent theme issue of Philosophical Transactions of the Royal Society B (33), but the effects of resources on tolerance (in contrast to effects on resistance) are only discussed in one review (70) and noted as warranting further research in another (9). In particular, a number of studies have documented how anthropogenic resources can promote host aggregation and limit host movement in ways that will increase transmission, and theoretically, investment in tolerance (61–63). Clearly, the study of resource provisioning on other aspects of infection defense (71) are ahead of research on tolerance. Yet, changes in tolerance in response to anthropogenic resource supplementation could have important implications for disease dynamics.

The prediction that mortality tolerance and sterility tolerance can have very different epidemiological and evolutionary trajectories indicates that a critical empirical consideration in studies of tolerance is to carefully diagnose the benefits and costs of tolerance. This is particularly relevant for understanding the influence of resources, as food intake will influence all aspects of an organism's life history, including traits involved in reproduction and survival. Thus, changes in resources may be very likely to influence both mortality and sterility tolerance and, whenever possible, empirical studies should try to quantify both.

In some cases, the measure of tolerance can be cleanly related to either sterility tolerance [e.g., parental provisioning in birds (21–23)] or mortality tolerance [e.g., lifespan of fruit flies (29)], but in many cases, host tolerance is measured via a fitness proxy like body weight that is more challenging to relate to theory (1, 44). There is also the unique issue that there is no universally agreed-upon way to quantify tolerance. A common approach is to quantify some host trait across varying parasite loads, with tolerance quantified as the slope of a regression of trait against load (1, 2), an approach that has attracted criticism (5). However, this means that empirical measures of tolerance have units of things like "body weight per parasite." Theory, on the other, tends to ignore parasite load, assuming all hosts have equal loads, and measure tolerance as a scalar multiplier on some other trait. Of course, this is a generic problem when trying to relate theory to data, as theoreticians often do not consider how traits are actually measured empirically, and empiricists often do not (or cannot) measure the parameters of a theoretical model. One possible middle ground would be for theory to make more use of models that can account for load, such as classic macroparasite models (72), or nested models (73), and for empiricists to report known relationships between fitness proxies and reproduction and mortality (e.g., if tolerance is measured by body weight, what is the relationship between body weight and reproduction and mortality?).

A further general implication of theory is that tolerance may be difficult to measure (50, 52). For example, if hosts and parasites simultaneously adjust their investment in mortality tolerance (h_α) and virulence (p_α), either coevolutionarily or plastically, infection-induced mortality α may remain constant across environments. This is because increased host investment in mortality tolerance will be countered by increased parasite investment in virulence traits. As hosts increase investment in tolerance, infection-induced mortality decreases; this allows parasites to increase their investment in virulence traits (which typically carry a benefit of increasing transmission, e.g., $\alpha'\left(p_\alpha\right) > 0$ and $\beta'\left(p_\alpha\right) > 0$) without actually increasing infection-induced mortality. Resources may be quite likely to provoke a similar effect; for example, if increasing resources improves investment in mortality tolerance but simultaneously increases parasite abundance within the host (14), the overall change in observed mortality may be negligible. Thus, quantifying or experimentally manipulating parasite abundance will be central to empirically testing the effects of resources on tolerance. Additionally, new tools such as immune gene expression markers of tolerance (74–76) may offer ways to quantify investment in tolerance that are independent of parasite virulence. Finally, a examining tolerance across a range of resource levels ranging from scarce, to normal, to super-abundant will provide much needed insight into the resource-tolerance relationship.

However, it is clear that more theory is needed as well. As our empirical review indicates, increasing resources can either increase or decrease tolerance; our theory review, on the other hand, seems to suggest that the effects of resources on host life history and environment will tend to lead to increasing resources increasing tolerance. The model developed in the **Appendix** in Supplementary Material is much more nuanced, indicating that this prediction is not nearly so straightforward, especially if resources can directly affect tolerance. However, the model also indicates that predictions will be highly sensitive to the shapes

of the functions relating host life history to both tolerance and resources. We hope that the model laid out in the **Appendix** in Supplementary Material will provide researchers with a jumping-off point for future theoretical work.

As recognition of the importance and frequency of tolerance as a defense strategy grows, a critical next step is to understand variation in tolerance. The studies reviewed here show that resources can affect intra-individual, intraspecific, and interspecific variation in tolerance. They also reveal both the taxa-specific investigations of tolerance (e.g., provisioning behavior in birds, anorexia in flies) and cross-taxa trends that supersede them. For example, in both birds and fruit flies, a low resource diet can improve tolerance (24, 29, 30). Adding resources into existing evolutionary models supports the context-dependent empirical results and provides mechanisms and hypotheses warranting further empirical study. Moreover, these models illustrate the need to quantify tolerance in relation to both mortality and sterility to make accurate ecological and evolutionary predictions. Indeed, now that the effects of resources on tolerance have broadly demonstrated, investigating the ecological and evolutionary consequences of resource-dependent tolerance is a critical next step.

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Exploring the Diversity of Mechanisms Associated With Plant Tolerance to Virus Infection

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Tolerance is defined as an interaction in which viruses accumulate to some degree without causing significant loss of vigor or fitness to their hosts. Tolerance can be described as a stable equilibrium between the virus and its host, an interaction in which each partner not only accommodate trade-offs for survival but also receive some benefits (e.g., protection of the plant against super-infection by virulent viruses; virus invasion of meristem tissues allowing vertical transmission). This equilibrium, which would be associated with little selective pressure for the emergence of severe viral strains, is common in wild ecosystems and has important implications for the management of viral diseases in the field. Plant viruses are obligatory intracellular parasites that divert the host cellular machinery to complete their infection cycle. Highjacking/modification of plant factors can affect plant vigor and fitness. In addition, the toxic effects of viral proteins and the deployment of plant defense responses contribute to the induction of symptoms ranging in severity from tissue discoloration to malformation or tissue necrosis. The impact of viral infection is also influenced by the virulence of the specific virus strain (or strains for mixed infections), the host genotype and environmental conditions. Although plant resistance mechanisms that restrict virus accumulation or movement have received much attention, molecular mechanisms associated with tolerance are less well-understood. We review the experimental evidence that supports the concept that tolerance can be achieved by reaching the proper balance between plant defense responses and virus counter-defenses. We also discuss plant translation repression mechanisms, plant protein degradation or modification pathways and viral self-attenuation strategies that regulate the accumulation or activity of viral proteins to mitigate their impact on the host. Finally, we discuss current progress and future opportunities toward the application of various tolerance mechanisms in the field.

Keywords: plant–virus interactions, antiviral defenses, disease tolerance, RNA silencing, salicylic acid

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INTRODUCTION

Tolerance to biotic stresses caused by pathogens, including viruses, is well-documented in plants (Rausher, 2001; Pagan and Garcia-Arenal, 2018). Tolerance has been defined as a mitigation of the impact of virus infection irrespective of the pathogen load (Cooper and Jones, 1983). Although a significant virus load is sustained, the plant growth, yield or reproduction attributes are only minimally affected and visible symptoms are either absent or mild. Tolerance can be explained

as reaching equilibrium to allow acceptable compromises in host and virus fitness for long-term co-existence (**Figure 1**). Because viruses are intracellular obligate parasites, they require host resources to complete their infection cycle (Culver and Padmanabhan, 2007; Nagy and Pogany, 2012; Wang, 2015). Therefore, high virus fitness is at the expense of the host in symptomatic susceptible interactions. In resistant interactions, the plant fitness is preserved by preventing virus accumulation or systemic movement. In tolerant interactions, virus fitness is reduced by preventing over-accumulation of viral RNAs or by minimizing the concentration or activity of viral proteins that play a role in virulence. In turn, this limits the damage to the host. Because of their absolute dependence on their host, maintaining host fitness is also beneficial to viruses.

Plant viruses should not only be viewed as pathogens. In fact, experimental evidence documenting the beneficial impact of accommodating long-term virus infection is accumulating, especially in natural environments (Roossinck, 2011; Roossinck and Bazan, 2017). Indeed, virus infection can improve the plant resilience in sub-optimal environmental conditions, for example tolerance to drought. Virus-induced drought tolerance is associated with global reprogramming of plant gene expression, changes in hormone signaling and increased accumulation of metabolites and antioxidants (Xu et al., 2008; Westwood et al., 2013; Aguilar et al., 2017; Dastogeer et al., 2018). Interestingly, recent studies suggested that the benefits of increased drought resistance can be offset by increased virus virulence (Aguilar et al., 2017; Berges et al., 2018). Maintaining persistent virus infection can also improve the plant resistance to biotic stress including non-vector herbivory insects, other viruses, or unrelated pathogens (van Molken et al., 2012; Shapiro et al., 2013; Mascia and Gallitelli, 2016; Syller and Grupa, 2016). Thus, tolerance to virus infection does not only mitigate the impact on the host as shown in **Figure 1**, but under additional abiotic or biotic stress, it can actually enhance the host fitness. In agricultural settings, tolerance is often effective against a larger spectrum of isolates compared to resistance (Korbecka-Glinka et al., 2017). Because viruses are allowed to persist, the selection pressure for emergence of virulent strains is also reduced in tolerant cultivars compared to resistant cultivars (Rausher, 2001; Pagan and Garcia-Arenal, 2018). Thus, tolerance can be considered as an evolutionary stable defense response.

While many plant antiviral resistance genes (R genes) have been characterized (de Ronde et al., 2014; Miyashita and Takahashi, 2015; Sanfacon, 2015; Hashimoto et al., 2016), the genetic basis of tolerance is much less well-understood. However, tolerance and resistance are not necessarily mutually exclusive in the field and mechanisms that govern both outcomes can overlap significantly (Pagan and Garcia-Arenal, 2018). In fact, many defense responses genes that are activated by dominant R genes are also induced in tolerant interactions (Bengyella et al., 2015). As will be detailed below, tolerance is often explained by the balance between plant antiviral mechanisms and viral counter-defense responses.

A recent review focused on plant–pathogen co-evolution in tolerant interactions (Pagan and Garcia-Arenal, 2018). In this review, we explore the molecular mechanisms that are associated with plant tolerance to virus infection. This review is not meant as an encyclopedic list of all known aspects of plant–virus interactions, rather we have selected examples that illustrate the variety of mechanisms that help attain long-term tolerance to virus infection. We also discuss current knowledge gaps as well as progress and future opportunities toward applications in the field.

PLANT ANTIVIRAL DEFENSE AND VIRUS COUNTER-DEFENSES

The majority of plant viruses are considered generalists as they can infect a large variety of plant hosts (García-Arenal and Fraile, 2013). However, this does not mean that plants are passive in their interactions with viral pathogens. Indeed, although plants do not possess an equivalent to the animal adaptive immune system, they deploy a number of protein- and RNA-mediated defense mechanisms against viruses (Zvereva and Pooggin, 2012; Mandadi and Scholthof, 2013; Moon and Park, 2016; Gouveia et al., 2017; Nicaise, 2017; Carr et al., 2018). In turn, viruses have developed sophisticated counter-defenses to allow systemic infection of plants. The balance between plant defense responses and viral counter-defenses is finely tuned, often allowing the virus to persist without causing too much damage to its host.

Antiviral RNA Silencing

RNA silencing is often considered the most important basal adaptive plant antiviral defense response (Moon and Park, 2016). RNA silencing is a ubiquitous gene regulation mechanism, which is based on the generation of small RNAs that guide the silencing machinery to complementary nucleic acids for transcriptional gene silencing (TGS) or post-transcriptional gene

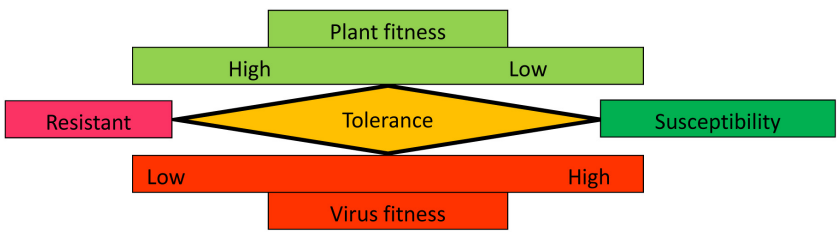

FIGURE 1 | Graphical representation of plant and virus fitness in resistant, tolerant, or susceptible interactions. See text for details.

silencing (PTGS) (Martinez de Alba et al., 2013). TGS results in the methylation and transcription repression of target DNAs, while PTGS operates by slicing target RNAs or repressing their translation. Plant DICER-like (DCL) proteins recognize double-stranded RNA (dsRNA) structures and process them into 21–25 nucleotides small RNA duplexes (Borges and Martienssen, 2015). One of the small RNA strands, the guiding strand, is loaded into ARGONAUTE (AGO) protein-containing RNA-induced silencing complexes (RISC) or RNA-induced transcriptional silencing complexes (RITSs) and directs these complexes to target nucleic acids in a sequence-specific manner for PTGS or TGS, respectively. In the context of antiviral RNA silencing, DCL enzymes recognize dsRNA structures present in replication intermediates produced during the replication of RNA viruses, in hairpin structures of viral RNAs, or in aberrant viral dsRNAs amplified by plant RNA-dependent RNA polymerases to produce viral-derived small interfering RNAs (vsiRNA), which are incorporated in RISC or RITS complexes (Raja et al., 2010; Martinez de Alba et al., 2013; Csorba et al., 2015; Ghoshal and Sanfacon, 2015; Zhang C. et al., 2015; Ramesh et al., 2017). Plant microRNAs (miRNAs) are produced after processing of folded endogenous plant mRNAs derived from miRNA genes by DCL enzymes and are also highly relevant to plant–virus interactions (Martinez de Alba et al., 2013; Cui et al., 2017). As will be described below, specific miRNAs regulate the expression of genes coding for RNA silencing enzymes or other defense proteins.

Most viruses encode a viral suppressor of silencing (VSR) to counteract the plant antiviral RNA silencing. Characterized VSRs show tremendous diversity in their protein sequence and mode of actions (Csorba et al., 2015). VSRs can block RNA silencing by inhibiting the initiation/spread of RNA silencing (e.g., by binding small RNAs and sequestering them away from the silencing complexes), by affecting the assembly/stability/function of silencing complexes (e.g., by destabilizing or inhibiting AGO proteins) or by redirecting silencing complexes in the regulation of host defense genes (e.g., by inducing the transcription of endogenous miRNAs that down-regulate key plant silencing factor genes) (Csorba et al., 2015; Wieczorek and Obrepalska-Steplowska, 2015). VSRs can specifically disrupt PTGS or TGS or can simultaneously affect both. Interestingly, some VSRs function by interacting with endogenous plant suppressors of silencing and/or by activating their transcription (Trinks et al., 2005; Endres et al., 2010; Yong Chung et al., 2014). Finally, it should be noted that some viruses encode more than one VSR (Lu et al., 2004) and that some VSRs can target multiple steps of RNA silencing (Csorba et al., 2015).

Salicylic Acid-Mediated Defense Responses

Salicylic acid (SA) is a key signal molecule in plants that mediates defense responses associated with basal innate immunity and with inducible immunity directed by antiviral dominant R genes (Mandadi and Scholthof, 2013; Gouveia et al., 2017; Carr et al., 2018). Basal innate immunity associated with bacterial and fungal infection depends on surface-associated receptors that recognize conserved microbe/pathogen-associated molecular patterns (M/PAMPs) and induce a cascade of events leading to PAMP- triggered immunity (PTI) (Jones and Dangl, 2006). In the case of virus infection, the presence of intracellular dsRNAs has been shown to trigger the PTI response in plants independently of the RNA silencing pathway (Niehl et al., 2016). PTI is accompanied with SA accumulation, and triggers a cascade of events, including an oxidative burst, activation of mitogen-activated kinases and induced expression of defense genes (e.g., pathogenesis-related or PR proteins) (Bigeard et al., 2015).

The second line of SA-mediated defense responses is often referred to as the effector-triggered defense (ETI). ETI requires the recognition of pathogen avirulent proteins, also termed effectors, by plant intracellular receptors, which are encoded by dominant R genes (Jones and Dangl, 2006). Most known antiviral dominant R genes encode proteins with nucleotide-binding leucine-rich repeats (NB-LRR) that share similarities with R genes directed at fungal and bacterial pathogens (Moon and Park, 2016; Gouveia et al., 2017). The ETI defense response is similar to PTI in its nature, but is more acute. ETI is generally associated with a local hypersensitive reaction (HR), which causes rapid cell death and the formation of visible necrotic lesions on inoculated leaves, and with the subsequent establishment of systemic acquired resistance (Moon and Park, 2016; Gouveia et al., 2017).

Several plant viruses have been shown to suppress the oxidative burst and the expression of defense genes associated with PTI or ETI (Hussain et al., 2007; Mubin et al., 2010; Zvereva et al., 2016; Nicaise and Candresse, 2017). A replicase protein of tobacco mosaic virus promotes the degradation of ATF2, a plant NAC transcription factor, which regulates the expression of PTI-responsive genes (Wang X. et al., 2009). Similarly, interaction between the turnip crinkle virus coat protein (CP) and TIP, another NAC transcription factor was correlated with the inhibition of innate immune defense responses (Donze et al., 2014). Finally, the P6 protein from cauliflower mosaic virus (CaMV) suppresses SA-signaling in part by modulating the expression and sub-cellular localization of NPR1 (NON-EXPRESSOR OF PATHOGENESIS-RELATED1), a transcriptional activator of downstream SA-responsive genes (Love et al., 2012).

Dominant or Recessive Antiviral Resistance Genes That Do Not Depend on SA Signaling

Some characterized dominant R genes do not encode proteins with signature NB-LRR sequences and do not induce ETI-like defense responses (Gouveia et al., 2017). These R genes limit virus infection using different mechanisms. For example, a protein encoded by the tomato *Tm-1* gene binds to the tomato mosaic virus replication proteins and inhibits viral RNA replication (Ishibashi and Ishikawa, 2014). Finally, there are many characterized plant recessive resistance genes that correspond to mutations of plant factors that are essential to the virus infection cycle, most often translation factors, such as

eIF4E or eIF4G (Truniger and Aranda, 2009; Sanfacon, 2015; Hashimoto et al., 2016).

Role of Plant Hormones in Antiviral Defenses and Cross-Talks Between Defense Mechanisms

In addition to RNA silencing and SA-mediated defenses, signaling pathways controlled by various plant hormones influence plant antiviral responses (reviewed in Robert-Seilaniantz et al., 2011; Mandadi and Scholthof, 2013; Alazem and Lin, 2015; Carr et al., 2018). Jasmonic acid (JA) and ethylene (Et) are normally associated with defense mechanisms that operate against necrotrophic pathogens (JA and Et) and insect pests (JA) and have antagonistic effects on SA signaling and associated defense responses. Abscisic acid (ABA) regulates plant development and modulates the response to environmental stresses. ABA also has antagonistic effects on the SA- and JA/Et-pathways. Multiple levels of cross-talk among the SA-, JA-, ABA-signaling pathways and RNA silencing highlight complex regulatory mechanisms of host defense responses that are manipulated by viruses to their advantage. For example, some VSRs interfere not only with antiviral RNA silencing but also with the SA-, JA- or Et-pathways, in some cases down-regulating plant defense responses to promote their transmission by insect vectors (Ji and Ding, 2001; Geri et al., 2004; Lozano-Duran et al., 2011; Love et al., 2012; Westwood et al., 2014; Zvereva et al., 2016; Wu et al., 2017; Poque et al., 2018). SA was also recently shown to regulate cross-talks between gibberellin synthesis/signaling (involved in plant development) and the induction of miRNAs targeting plant defense genes (Kriznik et al., 2017). Finally, primary plant metabolism pathways (synthesis of carbohydrates, lipids, or amino acids) have been shown to impact plant defense responses (Bolton, 2009; Rojas et al., 2014). For example, sugars are both essential energy resources for the activation of defense responses and regulators of these responses (Bolouri Moghaddam and Van den Ende, 2012).

SYMPTOM DETERMINANTS IN PLANT–VIRUS INTERACTIONS

Fitness Costs of Activating the Plant ETI or PTI Defense Responses

Expression of defense genes during ETI or PTI is associated with fitness costs. As mentioned above, mounting the defense response requires energy resources, which are diverted at the expense of plant growth and development. Indeed, constitutive overexpression of R genes or other defense genes often causes pleiotropic effects on plant development (Heil and Baldwin, 2002; Tian et al., 2003; Yang and Hua, 2004; Yi and Richards, 2007). Induction of defense hormones can also result in reduced plant growth (Huot et al., 2014; Havko et al., 2016; Guo et al., 2018). Thus, the activation of SA-dependent defense responses is likely one of the factors contributing to the dwarfing phenotypes observed in many plant–virus interactions.

Plants down-regulate the expression of R genes or associated defense genes in the absence of pathogen pressure using either dedicated repressor genes or miRNA-mediated RNA silencing. For example, the *Arabidopsis thaliana BONZAI1* (*BON1*) gene down-regulates the expression of the R-like gene *SNC1* (Yang and Hua, 2004). Plant miRNAs have been identified that target characterized R genes or R-like genes with signature NB-LRR sequences (He et al., 2008; Zhai et al., 2011; Li et al., 2012; Shivaprasad et al., 2012; Deng et al., 2018). These miRNAs often target conserved regions of R or R-like genes resulting in the production of secondary siRNAs, which in turn down-regulate a larger number of related genes based on sequence similarities (Zhai et al., 2011; Li et al., 2012; Shivaprasad et al., 2012; Boccara et al., 2014). Following virus infection, the miRNA-mediated repression of R and R-like genes is released and the plant defense is upregulated (Shivaprasad et al., 2012). This may be an indirect consequence of the inhibition of plant RNA silencing by VSRs. Indeed, elevated expression of the R-like gene *SNC1* is observed in plants expressing VSRs (Yi and Richards, 2007). Similarly, tobacco plants expressing the potyvirus HC-Pro VSR display enhanced resistance to various pathogens, including several viruses (Pruss et al., 2004; Jovel et al., 2011). As a counter-defense, some plant viruses regulate the expression of specific miRNAs that target R or R-like genes (e.g., miR1885 induced by turnip mosaic virus) (He et al., 2008), or other defense genes (miR164a that targets NAC transcription factors implicated in regulating cell death) (Bazzini et al., 2009).

Necrotic responses associated with HR are generally thought to play a role in restricting virus movement. However, HR is not always efficient at restricting viruses and cells outside of the cell death zone of local necrotic lesions can harbor infectious virus (Lukan et al., 2018). In some pathosystems, induction of HR is either weak or delayed and does not prevent the systemic spread of viruses. Instead, this can result in runaway HR leading to systemic lethal necrosis (Moffett, 2009; Pallas and Garcia, 2011; Mandadi and Scholthof, 2013; Künstler et al., 2016).

Impact of Viral Infection on Plant Organelles

In susceptible plants, virus infection can cause profound reorganization of host cells, by altering the structure and integrity of intracellular membranes and organelles (Laliberte and Sanfacon, 2010). A common symptom of virus infection is chlorosis, often expressed as yellow mosaic symptoms on the leaves. Chlorotic symptoms have been correlated with virus-induced changes in the number or size of chloroplasts, or with structural alterations: invaginations of chloroplast membranes, formation of tubular stromules, changes in the number or appearance of grana or starch grains (Li et al., 2016; Zhao et al., 2016; Bhattacharyya and Chakraborty, 2017). In addition, biotic stress including viral infection has been reported to cause global repression of plant photosynthetic genes (Bilgin et al., 2010). The chloroplast is a key player in the deployment of plant defense responses with SA, JA,

and reactive oxygen species being produced in the chloroplast (Dempsey et al., 2011; Kangasjarvi et al., 2012; Li et al., 2016; Zhao et al., 2016; Bhattacharyya and Chakraborty, 2017). It was recently shown that ETI-dependent activation of MPK3/MPK6 (mitogen-activated kinases) inhibits photosynthesis which in turn leads to the accumulation of reactive oxygen species required for the HR (Su et al., 2018). Specific interactions between viral and chloroplast proteins can also interfere with the normal functioning of the chloroplast (Zhao et al., 2016).

Replication of RNA viruses requires association with and extensive modification of intracellular membranes derived most often from the endoplasmic reticulum (ER), but also from chloroplasts, peroxisomes or vacuoles, depending on the specific plant–virus interaction (Laliberte and Sanfacon, 2010; Jin et al., 2018). Cell-to-cell movement of some viruses also require modification of ER membranes. The ER is an important organelle that orchestrates post-translational modifications and folding of cellular proteins destined to the secretory system. Alterations of the ER structure caused by virus infection and the vigorous ER-associated synthesis of viral proteins can cause severe ER stress, which if not mitigated, can lead to programmed cell death (Zhang and Wang, 2012; Verchot, 2016a). Most often, viral integral membrane proteins are responsible for the ER modifications. In some cases, these viral proteins act as viroporins, creating aqueous pores in the membranes and affecting their integrity (Nieva et al., 2012; Sanfacon, 2013). In addition, viral movement proteins (MPs) interact with and modify the plasmodesmata that connect plant cells to promote virus cell-to-cell movement, a process which can disrupt the natural movement of nutrients and signal molecules between cells (Harries and Ding, 2011). Alterations of the actin and tubulin intracellular transport networks are also common consequences of plant virus infection (Niehl et al., 2013; Pitzalis and Heinlein, 2017).

Toxic Effects of Viral Proteins

In addition to the gross alterations in sub-cellular structures described above, a large network of interactions between plant and virus proteins has been characterized (Wang, 2015; Nagy, 2016). In fact, hub viral proteins may interact with a large number of host proteins. For example, the tombusvirus p33 replication protein has more than 100 known plant protein interaction partners (Nagy, 2016). Although, it is beyond the scope of this review to describe each known protein-protein interaction, it is important to note that many of these interactions affect the host physiology profoundly, which can lead to visual symptoms and/or impact the host general fitness (reviewed in Culver and Padmanabhan, 2007; Mandadi and Scholthof, 2013).

Although many viral proteins contribute to virulence, VSRs are often virulence factors and symptom determinants. VSRs were first discovered in the context of synergistic interactions between two plant viruses. The potyvirus HC-Pro protein was shown to assist a potexvirus with counter-defense responses to the plant antiviral RNA silencing, resulting in increased symptom severity (Anandalakshmi et al., 1998). The virulence properties of VSRs may be partly due to the increased virus accumulation that follows the inhibition of the plant antiviral RNA silencing. However, symptom severity is not always correlated with the level of genomic viral RNA accumulation (Pagan et al., 2007). For example, a chimeric potato virus X expressing the potyvirus HC-Pro VSR accumulates to lower levels than the native virus in infected plants but causes more severe symptoms (Aguilar et al., 2015). Several VSRs are also recognized as elicitors of dominant R genes and trigger necrotic defense responses (Li et al., 1999; Wang et al., 2015). Because RNA silencing is a ubiquitous gene regulation mechanism in plants, VSRs may disturb not only antiviral RNA silencing pathways but also other aspects of the plant metabolism and development that are regulated by RNA silencing. As mentioned above, VSRs can impact the expression, stability or activity of AGO proteins, in particular AGO1 which is required for miRNA-mediated regulation of plant gene expression. Thus, ectopic expression of VSRs in transgenic lines can cause phenotypic changes, similar to symptoms induced during virus infection or to those observed in AGO1-deficient mutants (Zhang et al., 2006; Bortolamiol et al., 2007; Varallyay and Havelda, 2013). Similarly, many VSRs such as the tombusvirus p19 protein have been shown to sequester not only vsiRNAs but also plant siRNAs or miRNAs (Chapman et al., 2004; Wu et al., 2010; Pertermann et al., 2018). Interestingly, recent reports suggest that p19 sequesters vsiRNAs more efficiently than miRNAs and that miRNA binding may only occur early in infection when the concentration of vsiRNAs is still low (Kontra et al., 2016; Pertermann et al., 2018). Thus, the regulation of this VSR activity is fine-tuned during virus infection perhaps to mitigate its impact on the host physiology.

Viral siRNAs Directed at Plant Genes

Reports on how viruses use vsiRNAs to modulate the expression of plant genes are emerging. *In silico* analysis, and in some cases further functional validation, revealed many plant mRNA targets of vsiRNA in several plant–virus interactions (Qi et al., 2009; Miozzi et al., 2013; Stare et al., 2015; Wang et al., 2016a; Moyo et al., 2017; Xu and Zhou, 2017). Perhaps not surprisingly, several targeted transcripts encode proteins related to host stress responses and signal transduction. For example, vsiRNA of cotton leaf curl Multan virus were shown to target a gene encoding a MYB transcription factor that restrict virus accumulation (Wang et al., 2016a). Targeting of plant genes by vsiRNAs can also cause visual symptoms. Infection of cucumber mosaic virus together with the associated satellite Y RNA causes yellowing of leaves in *Nicotiana tabacum*. This was correlated with the down-regulation of a gene involved in chlorophyll biosynthesis (*ChlI*) which is targeted by small RNAs derived from the satellite RNA (Shimura et al., 2011; Smith et al., 2011). Similarly, downregulation of *Nicotiana benthamiana* eukaryotic translation initiation factor (NbeIF4A) was shown to be associated with the stunting phenotype of *N. benthamiana* plants infected with rice stripe virus (Shi et al., 2015).

PREVALENCE OF TOLERANCE IN WILD ECOSYSTEMS AND IMPACT OF ENVIRONMENTAL FACTORS

Long-Term Mutually Beneficial Co-existence Defines Many Plant–Virus Interactions in Natural Environments

Plant viruses were first discovered because of their impact on economically important crops and as a consequence they have been described as pathogens for many years. However, plant–virus interactions are much more complex in natural environments. Metagenomic studies have revealed that virus infection is common in natural ecological settings with 60–70% of plants infected with one or several viruses (Roossinck et al., 2015). Interestingly, virus-infected plants are normally asymptomatic in wild environments (Roossinck, 2014). In fact, the point has been made that large-scale crop monocultures in agriculture settings and the consequent loss of biodiversity has contributed to the emergence of severe plant virus diseases (Roossinck, 2015; Roossinck and Garcia-Arenal, 2015). In natural settings, generalist viruses would be favored. Accommodating a wider host range usually results in reduced virulence, in part because of selection pressures to evade or counteract multiple defense responses that vary in their intensity from host to host (Miyashita et al., 2016). In the wild, plants and viruses are exposed to long-term ongoing selection pressures from multiple biotic and abiotic stresses (McLeish et al., 2018). Mixed virus infections are common in plants and can result in synergistic or antagonistic interactions (Mascia and Gallitelli, 2016) that also influence virus evolution and adaptation to new hosts (McLeish et al., 2018). The strict requirement of many viruses for vector transmission (most often insects) also drives virus evolution and virulence (Hily et al., 2014; Roossinck, 2015; Blanc and Michalakis, 2016; Hamelin et al., 2017). While viruses may afford to kill or damage their hosts in agricultural settings because of the prevalence of specialized insect vectors adapted to specific crops, extending the lifespan and fitness of the host is a more viable option in natural environments. Finally, it should also be noted that in nature many persistent viruses do not depend on vector transmission (Roossinck, 2014; Roossinck and Bazan, 2017). Rather, they are strictly vertically transmitted through seeds and must ensure successful reproduction of their host. While the prevalence of tolerant and often mutually beneficial interactions in the wild is well-documented, the molecular mechanisms that govern these interactions have not yet been characterized. This will likely become a focus of future research.

Age-Dependent Tolerance to Virus Infection

Plants exhibit more tolerance to disease as they age. The maintenance of TGS and PTGS can differ in plants that are in vegetative or reproductive stages and some VSRs are not active in older plants at the reproductive stage (Jackel et al., 2015). For example, mature plants show decreased concentration of the small RNAs that regulate the expression of a tobacco R gene directed at tobacco mosaic virus (the *N* gene) (Deng et al., 2018). Furthermore, plant pre-exposed to other diseases also shows increased tolerance to new infecting viruses, a phenomenon referred to as priming (Jung et al., 2009). In natural environments where multiple pathogens are present and mixed infections are prevalent, plant priming could be a common occurrence.

Impact of Environmental Conditions on Symptom Severity

Environmental conditions such as temperature, light duration and intensity, water availability and concentration of CO_2 affect viral symptom expression (Hily et al., 2016; Berges et al., 2018). Attenuation of virus-induced symptoms on tobacco plants at extreme temperatures (>36°C or <11°C), called temperature masking, was described almost a century ago (Johnson, 1921; Grainger, 1936). Although, detailed molecular studies in such extreme environments are lacking, the effect of temperature on symptom severity is well-documented in the permissive range (15–30°C). In many cases, temperature-dependent symptom attenuation has been correlated with the regulation of antiviral RNA silencing, as evidenced by the increased accumulation of vsiRNAs at higher temperatures (Szittya et al., 2003). Indeed, plants that are deficient in silencing factors show increased susceptibility to viral infection at higher temperatures (Qu et al., 2005; Zhang et al., 2012; Ghoshal and Sanfacon, 2014). On the other hand, viruses that are deficient in VSR activity can only successfully infect plants at lower temperatures (Szittya et al., 2003). However, the effect of temperature on RNA silencing efficiency can vary with the plant species. SiRNAs are abundantly detected in grapevine plants grown at a range of temperature from 4 to 26°C, but they are not detected in *A. thaliana* plants grown at 4°C (Romon et al., 2013). Indirect effects of temperature on the induction of RNA silencing have also been proposed. Higher temperatures allow more efficient viral RNA replication (Zhang et al., 2012) and this is often associated with earlier symptom development (Obrêpalska-Stêplowska et al., 2015). At lower temperatures, although the initial viral titer is lower, viruses accumulate to higher levels later on and consequently, more severe symptoms can develop at late stages of infection (Szittya et al., 2003; Chellappan et al., 2005; Qu et al., 2005; Ghoshal and Sanfacon, 2014; Xu et al., 2016; Paudel et al., 2018). It is possible that the onset of antiviral RNA silencing, which is triggered when the viral RNA concentration reaches a critical level, occurs earlier at higher temperatures as a consequence of the enhanced virus replication. The efficiency of PTI or ETI is also affected by the growth temperature. In several plant–virus interactions, HR or HR-like responses are slower when the temperature is elevated from 21–22 to 27–28°C and are even prevented at temperatures above 30°C (Whitham et al., 1996; Wang Y. et al., 2009; Jovel et al., 2011). Although increased RNA silencing activity would contribute to temper the expression of defense genes at

higher temperatures, it was also shown that the activity and nuclear localization of two R genes (including the *N* gene) are temperature-sensitive directly affecting the defense response (Zhu et al., 2010).

Light intensity also modulates the outcome of plant virus infection. This is not surprising considering that the chloroplast is a major player in plant–virus interactions (Li et al., 2016; Zhao et al., 2016; Bhattacharyya and Chakraborty, 2017). Plants growing under high light conditions show enhanced PTI and ETI responses to various pathogens, including viruses (Chandra-Shekara et al., 2006; Manfre et al., 2011). High light intensity has also been shown to increase localized RNA silencing but reduce the systemic movement of RNA silencing due to shifts in the sink and source status of the leaves (Patil and Fauquet, 2015). Transgenic *N. benthamiana* plants expressing GFP show increased frequency of silencing at higher light intensity and this was correlated with the increased expression of several silencing genes (e.g., *DCL*) (Kotakis et al., 2010, 2011). Consistently, the promoter regions of *DCL* genes contain a light responsive element (Liu et al., 2009).

The level of CO_2 is another factor that influences plant defenses to pathogen infection (Noctor and Mhamdi, 2017). Growth under high CO_2 levels triggers the synthesis of SA and primes plant defense responses (Mhamdi and Noctor, 2016). In the context of virus infection, CO_2 levels have also been shown to influence symptom development (Aguilar et al., 2015; Del Toro et al., 2015; Del Toro et al., 2017). Increased levels of CO_2 generally result in larger leaf size and can attenuate the impact of virus infection in a virus-specific manner. Higher CO_2 exposure alleviated some of the negative effects of potato virus Y infection allowing increased accumulation of biomass, nitrogen content and soluble protein but decreased carbon/nitrogen ratio (Ye et al., 2010). Finally, water availability can also impact virus virulence and/or transmission by insect vectors (van Munster et al., 2017; Berges et al., 2018).

The studies described above were conducted in the controlled conditions of experimental greenhouses or growth chambers. However, it is more difficult to predict the impact of the seasonal and diurnal fluctuations of environmental conditions (Sanfacon, 2017; McLeish et al., 2018). Clearly, more studies are warranted to examine plant–virus interactions under field conditions and determine how fluctuating environmental conditions could influence the effectiveness or durability of tolerance.

SYMPTOM RECOVERY AS AN INDUCIBLE FORM OF TOLERANCE

Symptom recovery is a typical outcome of some plant–virus interactions, in which plants initially displaying systemic symptoms later recover from infection as exemplified by the emergence of young asymptomatic leaves (Ghoshal and Sanfacon, 2015) (**Figure 2**). Although the level of viral nucleic acid accumulation is often reduced in recovered leaves (Covey et al., 1997; Szittya et al., 2003; Chellappan et al., 2005; Santovito et al., 2014; Korner et al., 2018), this is not a strict requirement. For example, in the interaction between tomato ringspot virus and *N. benthamiana*, early onset of recovery is not accompanied with a significant reduction of viral RNA levels, although the concentration of viral proteins is reduced (Jovel et al., 2007; Ghoshal and Sanfacon, 2014). Viruses present in recovered tissues maintain their infectivity and protect the plant against secondary infection in a sequence-specific manner (Ratcliff et al., 1997, 1999; Jovel et al., 2007; Santovito et al., 2014; Paudel et al., 2018). This has been attributed to the induction of antiviral RNA silencing during the symptomatic phase of infection (Santovito et al., 2014). Depending on the specific virus, PTGS (viral RNA slicing and/or translation repression), TGS (DNA methylation) or a combination of PTGS and TGS is associated with symptom recovery (Ghoshal and Sanfacon, 2015; Korner et al., 2018). In all cases, the accumulation of viral proteins is reduced to a level below the threshold required for symptom induction. Because young tissues are symptom-free, the host is able to produce seeds. Interestingly, many viruses associated with recovery phenotypes are seed-transmitted. They apparently escape host surveillance mechanisms to invade meristem tissues, at least transiently (reviewed in Ghoshal and Sanfacon, 2015). Thus, symptom recovery can be viewed as an inducible form of tolerance. This makes it an ideal model

FIGURE 2 | Symptom recovery in *Nicotiana benthamiana* plants infected with tomato ringspot virus. Symptoms are shown during the symptomatic phase of infection as they appear on inoculated leaves **(left)** and systemically infected leaves **(center)**. **(right)** Shows a plant after symptom recovery with asymptomatic young leaves emerging above older symptomatic leaves. Reproduced with permission from Jovel et al. (2007).

system for the study of molecular mechanisms associated with tolerance.

INSIGHTS IN THE COMPLEXITY OF TOLERANT PLANT–VIRUS INTERACTIONS DERIVED FROM GENETIC AND TRANSCRIPTOMIC STUDIES

Field Tolerance to Virus Infection in Agricultural Crops: Mapping and (Limited) Characterization of Associated Genes

Although tolerance to virus infection is a well-known phenotype in the context of agriculture, the genetic basis for field tolerance is still poorly understood. Genetic crosses and mapping studies have identified a number of quantitative traits loci (QTL) or genes that are associated with tolerance. For example, several genes and QTLs have been linked to tolerance to barley yellow dwarf virus in barley, oat, and wheat (McKenzie et al., 1985; Singh et al., 1993; Jin et al., 1998; Riedel et al., 2011; Del Blanco et al., 2014; Foresman et al., 2016). While in some cases the tolerance was mapped to a single gene, in many cases a combination of major and minor loci were shown to contribute to tolerance and segregation analysis only indicated partial dominance of the major loci. In maize, one to four QTLs were found to be associated with tolerance to maize chlorotic mottle virus in different maize populations (Jones et al., 2017). The QTLs differed with the population, revealing a variety of natural sources for tolerance. In okra, tolerance to yellow vein mosaic virus was mapped to a single dominant gene in two different tolerant cultivars, although other factors were also involved (Senjam et al., 2018). As above, the dominant gene proved to be different in the two cultivars. Tolerance to tomato yellow leaf curl virus is also associated with single dominant genes in wild tomato species and was successfully introgressed into cultivated tomato (Zamir et al., 1994; Vidavsky and Czosnek, 1998). In peach, tolerance to plum pox virus (a potyvirus) was mapped to three loci (Cirilli et al., 2017). One of these loci included a candidate gene with similarities to the *A. thaliana RTM-2* gene, which is implicated in the restriction of the systemic movement of other potyviruses (Cirilli et al., 2017). However, functional validation will be required to confirm whether the *RTM-2*-like gene is indeed responsible for the tolerance. In summary, the variety of dominant, semi-dominant, or recessive tolerance genes found in agricultural crops and the common requirement for multiple loci suggests that molecular mechanisms associated with field tolerance are numerous and complex.

Host Resource Reallocation in Some but Not All Tolerant Plant–Virus Interactions

Plants can respond to pathogen infection by reallocating resources from vegetative growth to reproduction (i.e., production of seeds). In the *A. thaliana*-cucumber mosaic virus interaction, plants with longer vegetative growth cycles (i.e., longer life spans) are more tolerant to infection (Pagan et al., 2008; Hily et al., 2014; Shukla et al., 2018). Tolerance is also associated with increased seed yield and a shortened reproduction period, reducing the time span between the production of reproductive structures and seed production (Pagan et al., 2008). However, *A. thaliana* that were tolerant to cucumber mosaic virus did not show similar resource reallocation in response to more virulent viruses, suggesting that this response is virus specific (Shukla et al., 2018). In addition, tolerant plants with extended vegetative growth resulting from resource allocation were less competitive in the context of dense plant populations (Pagan et al., 2009). Additional studies using a variety of tolerant plant–virus interactions grown under various environmental conditions should shed more light on the biological relevance of resource allocation. Little is known regarding underlying molecular mechanism associated with resource reallocation. However, it is likely that they would require multiple genetic determinants affecting various regulatory mechanisms that control plant growth and development.

Reprogramming of the Plant Transcriptome in Tolerant Interactions Affecting Defense Pathways, Primary Metabolism, and Hormone Signaling

Virus infection induces global changes in the plant transcriptome and proteome in both susceptible and resistant interactions (Palukaitis et al., 2008; Llave, 2016). To date, only a limited number of transcriptomic studies have focussed on tolerant interactions (reviewed in Bengyella et al., 2015). Transcriptome changes have been characterized at different stages of virus infection in a tolerant interaction (Stare et al., 2015). Time-course studies have also allowed monitoring symptomatic and asymptomatic phases of infection associated with symptom recovery or with delayed symptom induction (Allie et al., 2014; Madronero et al., 2018). Finally, transcriptomes or proteomes have been compared in susceptible, resistant or tolerant cultivars infected with the same virus strain (Allie et al., 2014; Wang et al., 2016b) or in plants infected with virulent or mild virus strains (Kogovsek et al., 2016; Geng et al., 2017). Not surprisingly, these studies have highlighted both similarities and differences in the transcriptome changes induced by viruses in susceptible, tolerant, and resistant interactions. In many cases, similar plant pathways are affected in the different types of interactions but to different extents or with different dynamics. Pathways commonly impacted by virus infection include defense responses (e.g., R-like genes and PR proteins), primary metabolism, photosynthesis, and hormone signaling.

In the interaction between potato virus Y and the tolerant potato cultivar Désirée, photosynthesis genes were shown to be transiently induced at early stages of infection but then rapidly repressed at the onset of virus multiplication (Stare et al., 2015). It was suggested that the early induction of photosynthesis (and other primary metabolism associated genes) helps promote the induction of defense responses. Transgenic

Désirée, transformed with the *NahG* gene that down-regulates SA signaling, showed more severe symptoms upon virus infection and a diminished induction of photosynthesis genes at early stages of infection (Stare et al., 2015). Analysis of small RNA signaling in the potato virus Y-potato cv. Désirée interaction revealed induction of miRNAs known to down-regulate R-like genes and the presence of vsiRNAs that target plant stress signaling response genes. Plant small RNAs that down-regulate the gibberellin synthesis were also induced and this affected the levels of miR482f, a key regulator of R-like gene expression (Kriznik et al., 2017). This complex regulation of small RNA pathways was shown to be dependent on SA signaling.

Other studies have also shown increased induction of SA signaling, defense response proteins or R-like genes in tolerant cultivars or in asymptomatic phases of infection compared to corresponding symptomatic interactions (Sahu et al., 2012; Allie et al., 2014; Louis and Rey, 2015; Wang et al., 2016b; Madronero et al., 2018). Many of these studies also noted altered primary metabolism. In some cases, increased expression of antiviral RNA silencing genes was also observed in tolerant interactions (Sahu et al., 2012; Allie et al., 2014). The impact of JA and Et signaling pathways is less clear. Delayed symptom induction in the interaction between papaya and the papaya meleira virus complex is associated with concomitant induction of both SA-defense responses and the antagonistic JA pathway (Madronero et al., 2018). Similarly, although susceptible cassava cultivars show reduced JA and Et signaling after infection with South African cassava mosaic virus, a tolerant cultivar does not (Allie et al., 2014). Taken together these studies highlight the complex regulatory networks between various plant hormone signaling pathways and defense responses.

Although the analysis of global transcriptome changes provides useful insights in the intricacy of plant–virus interactions, it is not always clear whether these changes are the cause or consequence of tolerance. Also, since transcriptomic studies do not examine post-transcriptional changes in gene expression, it is not known whether changes in the transcriptome are also reflected in the plant proteome. In fact, a recent study highlighted major discrepancies between transcriptomic and proteomic data that may be of biological significance (Stare et al., 2017). In addition, environmental factors are also predicted to impact the outcome of transcriptome studies. Indeed, transcriptomics analysis of plants exposed under combination of three different stresses exhibit significant differences in their gene expression compared to plants exposed under single stress (Prasch and Sonnewald, 2013). These issues are exemplified in a recent analysis of the expression of AGO2 in plants grown at two temperatures and infected with two tomato ringspot virus isolates of varying virulence (Paudel et al., 2018). Although AGO2 mRNAs were transiently induced to similar levels under all conditions, the accumulation of the AGO2 protein was influenced by the isolate and the growth temperature. Plants that later recovered from infection showed increased accumulation of AGO2 protein at early stages of infection. However, mutation of AGO2 did not prevent the symptom recovery suggesting that other factors influence the outcome of infection.

MOLECULAR MECHANISMS ASSOCIATED WITH ACHIEVING A BALANCE BETWEEN ANTIVIRAL RNA SILENCING AND VIRUS COUNTER-DEFENSE RESPONSES

As described above, symptom recovery, an inducible form of tolerance, is associated with the induction of antiviral RNA silencing. Thus, it could be assumed that viruses that are associated with symptom recovery phenotypes do not suppress silencing efficiently. In fact, mutation of potent VSRs from virulent viruses can lead to symptom recovery (reviewed in Ghoshal and Sanfacon, 2015). On the other hand, ectopic expression of potent VSRs (e.g., the potyvirus HC-Pro) can prevent symptom recovery in nepovirus-infected plants (Siddiqui et al., 2008; Santovito et al., 2014). However, viruses that encode strong VSRs can also be associated with symptom recovery, as long as the activity of these VSRs is reduced in recovered leaves as recently shown in *A. thaliana* plants infected with oilseed rape mosaic virus (Korner et al., 2018). Thus, suppression of antiviral RNA silencing occurs during the initial stages to allow systemic viral infection, and symptom recovery depends on achieving a balance between antiviral RNA silencing and VSR activity during the recovery stage.

Some viruses deploy self-attenuation mechanisms to achieve this balance. Indeed, some viral proteins function to attenuate the accumulation and/or activity of VSRs. Symptom recovery is the normal outcome of the interaction between an isolate of cucumber mosaic virus and *A. thaliana*. However, symptoms were exacerbated by mutation of an Arg-rich region of the CP (Zhang et al., 2017). The wild-type CP was shown to attenuate the silencing suppression activity of the VSR (the 2b protein). This is probably achieved by inhibiting the translation of 2b, via the RNA-binding activity of the CP (Zhang et al., 2017). It was also proposed that binding of the CP to the viral RNA may protect it from degradation and allow enhanced production of vsiRNAs (Zhang et al., 2017), although this will need to be confirmed experimentally.

Another example of viral self-attenuation is provided by the plum pox virus-*N. benthamiana* pathosystem. Plum pox virus proteins are initially expressed as a single large polyprotein (Revers and Garcia, 2015). The P1 protease is the N-terminal protein domain in the polyprotein. Cleavage by P1 contributes to the release of the VSR (HC-Pro, the second protein domain) from the polyprotein. Because the HC-Pro silencing suppression activity is impaired by fusion to P1, the efficiency of the P1 proteolytic cleavage directly affects the activity of HC-Pro (Pasin et al., 2014). Deletion of the N-terminal region of P1 accelerated the release of HC-Pro from the polyprotein, enhanced its VSR activity, stimulated initial accumulation of the virus and enhanced the induction of the HR necrotic response, contributing to the enhanced symptomatology

(Pasin et al., 2014). It was suggested that the N-terminal region of P1 interacts with a host factor to down-regulate the P1 proteolytic activity. The N-terminal region of the P1 protein is highly variable and later work confirmed that it is involved in host adaptation (Shan et al., 2015, 2017). It was hypothesized that the N-terminal region of the P1 protein, although dispensable, is maintained to prevent virus over-accumulation (Pasin et al., 2014).

Strikingly, a viral protein was also shown to enhance the plant antiviral RNA silencing. Viral RNAs move cell-to-cell by modifying the natural channels between plant cells (the plasmodesmata), creating a virus front that invades naïve cells (reviewed in Harries and Ding, 2011; Heinlein, 2015). The vsiRNAs follow a similar route, moving through the plasmodesmata. Intriguingly, the tobacco mosaic virus MP was shown to facilitate the movement of vsiRNAs, thus functioning in a manner opposite to that of many characterized VSRs that hinder vsiRNAs movement (Vogler et al., 2008). Thus, while tobacco mosaic virus encodes a potent VSR to suppress anti-viral silencing, this activity is apparently counter-balanced by that of the MP. Since the MP is only expressed transiently early in infection, this self-attenuation effect would likely also only be effective in the critical initial stages of infection, i.e., at the front of infection (Vogler et al., 2008; Amari et al., 2012). On the other hand, enhancing vsiRNAs movement may also render naïve cells more susceptible to the incoming virus by down-regulating specific plant genes that are targeted by these vsiRNAs (Amari et al., 2012).

Defective-interfering RNAs (diRNAs) are associated with several viruses and have been shown to attenuate symptoms induced by the parent virus. The diRNAs contain non-contiguous segments from the parent viral RNA and are produced by template-switching of the viral RNA-dependent RNA polymerase (RdRp) during viral RNA replication (Simon et al., 2004; Pathak and Nagy, 2009). They contain all the *cis*-acting elements necessary for their continued replication by the viral RdRp and can accumulate *de novo* to very high levels. They interfere with the replication of the parent viral RNAs and prevent over-accumulation of viral products. The mechanisms of diRNA interference are not completely understood. While the *cis*-acting elements present on diRNAs may out-compete the viral RNAs for the viral RdRp and for host factors, other mechanisms likely also play a role, including the enhancement of antiviral RNA silencing (Simon et al., 2004; Pathak and Nagy, 2009). In tombusvirus infected-plants, diRNAs are recognized by DCL enzymes, leading to the enhanced synthesis of siRNAs that share sequences with the parent viral RNAs (Havelda et al., 2005). As described above, the tombusvirus p19 VSR functions by binding to vsiRNAs and sequestering them away from RISC complexes (Scholthof, 2006). However, the binding capacity of p19 was shown to be saturated in the presence of diRNAs leading to increased antiviral RNA silencing against the parental virus (Havelda et al., 2005). Interestingly, a second silencing suppression activity of p19 is not affected by the presence of diRNAs, suggesting that the VSR and the diRNAs act in an antagonistic manner to regulate the levels of virus accumulation in infected plants (Varallyay et al., 2014). Indeed, p19 induces the synthesis of miR168, which down-regulates the expression of AGO1, one of the main effectors of antiviral RNA silencing. The induction of miR168 by p19 was found to be similar in the presence or absence of diRNAs (Varallyay et al., 2014).

Additional evidence for antagonistic interactions between VSRs and diRNAs is documented for the interaction between a crinivirus and *N. benthamiana* (Lukhovitskaya et al., 2013). The 8K viral protein is a weak VSR that enhances virus accumulation. Interestingly, the coding region for the 8K protein was implicated in the template-switching mechanism that produces the diRNAs. It was suggested that diRNAs are essential regulatory molecules that minimize the impact of crinivirus infection on their hosts (Lukhovitskaya et al., 2013). While the role of diRNAs in symptom attenuation is well-established in model hosts under laboratory conditions, their impact on infections in the field or in natural environments is not well-studied and clearly deserves further investigation.

MOLECULAR MECHANISMS AIMED AT LIMITING THE ACCUMULATION OR ACTIVITY OF VIRAL PROTEINS

Plants may be able to accommodate substantial levels of viral nucleic acid accumulation without significant damage, as long as they manage the concentration or activities of viral proteins that orchestrate interactions with plant factors and act as virulence factors (Culver and Padmanabhan, 2007). As will be described below, this can be achieved by repressing the translation of viral RNAs, by destabilizing viral proteins or by modulating their activity.

Repression of Viral Genome Translation

Translation repression has emerged as a common mechanism of RNA silencing-mediated gene regulation in plants (Brodersen et al., 2008; Iwakawa and Tomari, 2013) and has also been suggested to operate against plant viruses in association with tolerance or with symptom recovery phenotypes. In *N. benthamiana* plants infected with tomato ringspot virus, the initial stages of symptom recovery are associated with a drastic reduction in viral protein levels but not with a concomitant reduction in viral RNA concentration (Jovel et al., 2007; Ghoshal and Sanfacon, 2014). Translation of viral RNA2 was shown to be repressed at the onset of symptom recovery and silencing of AGO1 prevented both the translation repression and the symptom recovery (Ghoshal and Sanfacon, 2014). Similarly, recovery of *A. thaliana* from oilseed rape mosaic virus was shown to be dependent on AGO1 and was associated with translation repression preventing over-accumulation of the VSR (Korner et al., 2018). Finally, the reduction of viral titers in late stages of the asymptomatic infection of *A. thaliana* plants with tobacco rattle virus was concomitant with a decrease in ribosome-associated viral RNAs and an increase in the number of processing bodies (Ma et al., 2015), which are RNA granules often associated with translation repression mechanism (Makinen et al., 2017). Although these studies suggest a role for antiviral RNA silencing translation repression mechanisms in tolerant interactions, a direct role for AGO-containing RISC

complexes in the translation repression of viral RNAs has not been experimentally confirmed.

A distinct translation repression mechanism is directed by a transmembrane receptor, NIK1 (NSP-interacting kinase), which is related to leucine-rich repeat receptor-like kinases implicated in the innate immune PTI response (Machado et al., 2015). NIK1 was first identified as an interactor of begomovirus NSP1 protein. NIK1 also interacts with and phosphorylates ribosomal protein RPL10A, redirecting this protein to the nucleus (Carvalho et al., 2008). Once in the nucleus, RPL10A interacts with L10-INTERACTING MYB DOMAIN CONTAINING PROTEIN (LIMYB), a transcription factor that regulates the expression of ribosomal genes (Zorzatto et al., 2015). The RLP10A-LIMYB interaction causes massive down-regulation of ribosomal genes and global translation repression, which also impairs virus translation. Importantly, the translation repression is specifically induced upon virus infection and depends on the autophosphorylation of NIK1 at tyrosine 474. Knock-out of the *NIK1*, *RPL10A,* or *LIMYB* genes exacerbates symptoms and enhances virus accumulation, confirming the importance of the translation repression mechanism in limiting virus-induced damage to the plant (Carvalho et al., 2008; Zorzatto et al., 2015). As a counter-defense, the viral NSP protein suppresses the activity of NIK1 preventing its autophosphorylation (Fontes et al., 2004). Interestingly, ectopic expression of a phosphomimic mutant of AtNIK1 with a mutation of tyrosine 474 to aspartic acid, bypassed the counter-defense and provided broad-spectrum tolerance to begomoviruses in tomato, with minimal impact on plant growth in non-infected plants (Brustolini et al., 2015).

In addition to the plant responses described above, viruses minimize the accumulation of viral virulence factors (e.g., VSRs, RdRps) using sub-optimal translation initiation codons or inefficient frameshift or read-through translation mechanisms (reviewed in Miras et al., 2017). These are highly conserved features of viral genomes, highlighting their importance for viral self-attenuation mechanisms.

Using Cellular Protein Degradation Pathways to Prevent Over-Accumulation of Viral Proteins and to Regulate Plant Defense Responses

Cellular protein degradation mechanisms, in particular the ubiquitin/26S proteasome system (UPS) and the autophagy pathway are key regulators of plant–virus interactions (Alcaide-Loridan and Jupin, 2012; Verchot, 2016b; Clavel et al., 2017; Ustun et al., 2017). By controlling the accumulation of viral and/or plant proteins, they modulate plant defense responses, regulate viral counter-defense responses, control the viral infection cycle and mitigate symptoms. It could be argued that both partners in the interaction benefit from manipulating protein degradation pathways. Indeed, that viral proteins maintain conserved signature sequences for recognition by plant degradation pathways could be viewed as evidence for virus self-attenuation.

Protein substrates targeted by the UPS are ubiquitinated at lysine residues by cellular E3 ubiquitin-ligases, a large family of plant proteins (1400 genes in *A. thaliana*). Depending on the nature of the ubiquitination (mono- or poly-ubiquitination), proteins are selectively targeted to the 26S proteasome for degradation. Cellular E3 ubiquitin ligases are common interactors of plant virus proteins, including, MPs and RdRps, many of which are destabilized by the UPS (Alcaide-Loridan and Jupin, 2012; Verchot, 2016b). Turnip yellow mosaic virus RdRp contains a highly-conserved PEST sequence, which is recognized as a degradation trigger (Camborde et al., 2010). Interestingly, the viral protease acts as a deubiquitinase to protect the RdRp from UPS degradation (Chenon et al., 2012). These results suggest that a delicate cross-talk between viral enzymes and the plant UPS regulates the accumulation of the viral RdRp.

Direct evidence for a role for the UPS in facilitating tolerance is exemplified in the interaction between *N. benthamiana* and tomato yellow leaf curl China virus (TYLCCV) (Shen et al., 2016). The TYLCCV-associated betasatellite DNA encodes βC1, a symptom determinant and a VSR. βC1 interacts with NtRFP1, a plant RING E3 ligase and is targeted to degradation by the 26S proteasome. βC1 induces severe stunting and leaf curling symptoms when over-expressed in transgenic lines (Yang et al., 2008). However, in natural infection it only accumulates to low levels, and symptoms are milder. Symptoms are further attenuated in plants overexpressing NtRFP1, while plants knocked-down for NtRFP1 develop more severe symptoms (Shen et al., 2016). Importantly, viral DNA accumulation is not affected by manipulation of NtRFP1 expression. Thus, this study demonstrates how the destabilization of a viral pathogenicity factor by the UPS can mitigate symptom expression while allowing systemic virus infection. A separate study demonstrated an interaction between cotton leaf curl Multan virus (CLCuMuV) βC1 protein and a distinct E3 ligase complex (the SCF complex) (Jia et al., 2016). However, the CLCuMuV βC1 protein was shown to inhibit the SCF E3 ligase, allowing enhanced virus accumulation and more severe symptoms. These apparently conflicting results are not necessarily mutually exclusive. Rather, they highlight the complexity of the interactions between plant viruses and various branches of the UPS pathway.

Autophagy is another highly conserved protein degradation pathway implicated in many aspects of plant–pathogens interactions including the regulation of programmed cell death (Ustun et al., 2017). Proteins targeted by the autophagy pathway are directed to double-membrane vesicles, autophagosomes, before they are finally released in the vacuoles for degradation. There are extensive cross-talks between autophagy and the UPS degradation pathways. For example the AUTOPHAGY-RELATED GENE 6 (ATG6) protein is ubiquitinated by SINAT E3 ligases and degraded by the 26S proteasome (Qi et al., 2017). Therefore, it is perhaps not surprising that the CLCuMuV βC1 protein is not only interacting with UPS components, but it is also targeted for degradation by the autophagy pathway following its interaction with ATG8 (Haxim et al., 2017). Preventing the interaction between βC1 and ATG8 exacerbated symptoms and enhanced virus accumulation. Similarly, silencing of ATG5 and ATG7 increased the plant susceptibility to three geminiviruses. These results highlight a role for autophagy in mitigating the impact of geminivirus

infection. Similarly, other VSRs are also degraded through the autophagy pathway, notably the potyvirus HC-Pro protein and the cucumovirus 2b protein (Nakahara et al., 2012). This requires an interaction between the VSRs and rgsCaM, a calmudolin-like protein and an endogenous suppressor of silencing, which is itself destined to autophagic degradation. Interestingly, rgsCaM is also a component of the SA-mediated systemic acquired resistance (Jeon et al., 2017).

Another interesting example of regulated autophagic protein degradation comes from the interaction between cauliflower mosaic virus and *A. thaliana* (Hafren et al., 2017). The viral CP interacts with NEIGHBOR OF BRCA1 (NBR1), an autophagy receptor and is targeted to autophagic degradation. This limits virus accumulation early in infection. Later on, virus particles accumulate in inclusion bodies, where they are protected from autophagy (Hafren et al., 2017). The CaMV P6 protein, which represses SA-mediated autophagy, may also help relieve the CP degradation (Zvereva et al., 2016). Similarly, NBR1 is required for the autophagic degradation of the turnip mosaic virus HC-Pro but this is counteracted by two other viral proteins (Hafren et al., 2018). Thus, viral proteins have evolved to be susceptible to degradation by the autophagy pathway and protected from this degradation at different stages of infection. In addition, induction of the autophagy prevented early cell death in these two pathosystems. Indeed, *A. thaliana* mutants deficient in the autophagy pathway display more severe symptoms than wild-type plants after infection with either turnip mosaic virus or cauliflower mosaic virus in a manner that is independent of the level of viral accumulation (Hafren et al., 2017, 2018). Inhibition of SA-mediated autophagy by the CaMV P6 protein also contributes to symptom severity. P6 activates the TOR (target of rapamycin) kinase, a down-regulator of autophagy and exacerbates symptoms, which are normally mitigated by the autophagy pathway (Zvereva et al., 2016). Transgenic lines that express the P6 protein from severe CaMV isolates display chlorotic and dwarfing symptoms, while those expressing the P6 protein from a mild isolate do not (Yu et al., 2003). Interestingly, the P6 protein from this mild isolate is unable to activate TOR or disrupt SA-mediated autophagy (Zvereva et al., 2016).

Finally, the UPS and autophagy pathways are usurped by viruses to target plant defense proteins. A case in point is the ability of several VSRs to target plant RNA silencing factors (notably AGO proteins) to degradation (Csorba et al., 2015). Thus, plant protein degradation pathways modulate both the plant antiviral defenses and the virus counter-defenses.

Regulating the Activity of Viral Proteins With Post-translational Modifications

Another approach to mitigate the impact of toxic viral proteins is to control their activity. This can be achieved by post-translational modification. For example, phosphorylation of the βC1 protein from the betasatellite DNA of TYLCCV by the SNF1-related protein kinase 1 (SnRK1) reduces its silencing suppression activity and diminishes symptom severity (Zhong et al., 2017). Similarly, phosphorylation of the cabbage leaf curl virus VSR (the AL2 protein) delays the symptom formation in *A. thaliana* (Shen et al., 2014). In the case of the turnip yellow mosaic virus RdRp, phosphorylation of the conserved PEST sequence is a prerequisite for its subsequent destabilization by the plant proteasome degradation pathway (Jakubiec et al., 2006). On the other hand, phosphorylation has also been shown to be required for the function of viral CPs, MPs, or RdRps (Stork et al., 2005; Champagne et al., 2007; Kleinow et al., 2009). While the role of protein modification in the regulation of plant–virus interactions is still poorly understood, especially in the context of tolerance, its importance cannot be underestimated.

MOLECULAR MECHANISMS DEPLOYED TO RELIEVE VIRUS-INDUCED STRESS OF THE PLANT ENDOPLASMIC RETICULUM

As mentioned above, virus infection commonly causes ER stress, which needs to be relieved to prevent cell death (Zhang and Wang, 2012; Verchot, 2016a). In plants, ER stress is sensed by transmembrane proteins [e.g., the inositol requiring enzyme (IRE1) and the Bax inhibitor 1 (BI-1) proteins] that induce the unfolded protein response (UPR) to restore proper protein folding in the ER and prevent aggregation. Activation of IRE1 causes splicing of the bZIP60 transcription factor transcript and production of a truncated form of the transcription factor, which translocate to the nucleus to induce the expression of UPR-related genes, including calcium-dependent protein chaperones (e.g., Bip, calmudolin, calreticulin). The ER-associated degradation (ERAD) pathway is also activated as part of the UPR. ERAD functions by translocating unfolded or misfolded proteins back into the cytoplasm where they are degraded by the cytosolic UPS or autophagic pathways. Evidence for the importance of the UPR in mitigating the consequences of virus-induced ER stress is accumulating. Expression of viral integral membrane proteins has been reported to induce the UPR (Ye et al., 2011, 2013; Zhang L. et al., 2015; Gaguancela et al., 2016). For example, the expression of IRE1 and BI-1 is induced by the potexvirus TGB3 or potyvirus 6K2 integral membrane proteins (Gaguancela et al., 2016). Down-regulation of BI-1 or bZIP60 in *N. benthamiana* allowed increased systemic accumulation of potato virus X and potato virus Y and exacerbated systemic necrosis symptoms indicating that the UPR is induced to release ER stress, control virus accumulation, and prevent cell death (Gaguancela et al., 2016). Consistently, overexpression of the ER Bip chaperone suppresses TGB3-induced cell death in *N. benthamiana* infected with potato virus X (Ye et al., 2011, 2013). Intriguingly, down-regulation of IRE1/bZIP60 has also been shown to hinder accumulation of turnip mosaic virus, *in A. thaliana* and to ameliorate non-necrotic virus-induced symptoms, suggesting that in this interaction the UPR actually promotes virus infection, possibly also by mitigating the consequences of ER stress (Zhang L. et al., 2015). Other plant–virus interactions will need to be examined before we can obtain a more complete understanding of the role of the UPR in facilitating tolerant plant–virus interactions.

Finally, how the ERAD and downstream protein degradation pathways contribute to mitigating virus-induced ER stress is also not well-characterized.

TOLERANCE CONFERRED BY MUTATION OF AN INEFFECTIVE R-LIKE GENE TO PREVENT SYSTEMIC LETHAL NECROSIS

The *A. thaliana TTR1* semi-dominant locus was shown to determine symptom expression following infection with tobacco ringspot virus (Lee et al., 1996). Screening of 97 *A. thaliana* lines revealed that although the virus accumulated to similar levels, the intensity of symptoms varied greatly. Systemic necrosis killed the most susceptible lines while tolerant plants were either asymptomatic or only displayed mild symptoms. The *TTR1* gene present in susceptible lines was later shown to correspond to a R-like gene (Nam et al., 2011). An HR-like response was activated in plants with the *TTR1* gene, but the replication and movement of the virus were not restricted and systemic acquired resistance was not established. It was suggested that the systemic lethal necrosis phenotype was caused by a runaway HR response. Interestingly, transfer of the *TTR1* gene to *N. benthamiana* also caused lethal systemic necrosis. The tolerant phenotype in *A. thaliana* accessions was found to be associated with mutations of the *TTR1* gene which prevented the establishment of the systemic HR response (Nam et al., 2011).

ENGINEERING TOLERANCE TO VIRUS DISEASE FOR FIELD APPLICATION: CURRENT PROGRESS AND FUTURE OPPORTUNITIES

As highlighted above, tolerance is a complex genetic trait that involves multiple molecular mechanisms operating simultaneously, many of which are yet to be discovered. The benefits of tolerance compared to resistance have also been described in terms of reduced selection pressure for the emergence of virulent isolates, increased breadth and stability of the phenotype and potential benefits to the host (as exemplified in natural environments). Although natural sources of tolerance are available for some economically important crops, they are generally poorly characterized and have been of limited use. The next question becomes: is it feasible to engineer tolerance for practical field applications?

Only a few examples of engineered tolerance to virus diseases can be found in the literature. Most relate to the identification and manipulation of plant genes involved in signal transduction pathways associated with basal innate immune defense responses. Some are broad-spectrum and also provide tolerance to abiotic stress, in part because some of the signaling pathways are overlapping. We have already discussed how the ectopic expression of a phosphomimic mutant of AtNIK1, an immune receptor kinase, conferred broad-spectrum tolerance to begomovirus infection in tomato (Brustolini et al., 2015). Other kinases implicated in defense signal transduction pathways have also been manipulated to mitigate viral symptoms. Overexpression of SlMAPK3, a MAP kinase, showed enhanced expression of defense genes associated with SA- and JA-signaling, lower accumulation of reactive oxygen species, increased accumulation of antioxidant enzymes, and stronger tolerance to tomato yellow leaf curl virus infection as expressed by a 2-week delay in symptom induction which was sufficient to allow plant flowering (Li et al., 2017). Similarly, overexpression of OsCIPK30, a kinase involved in calcium signaling, in rice provided enhance tolerance to rice stripe virus, that was associated with delayed and milder symptoms and enhanced expression of PR genes (Liu et al., 2017).

Overexpression of a transcription factor, the soybean GmERF3 gene, in tobacco also conferred increased tolerance to tobacco mosaic virus (Zhang et al., 2009). This transcription factor is induced in response to various stresses and up-regulates the expression of many defense genes, including PR proteins. Thus, overexpression of this gene activated the plant basal immunity, achieving a result similar to the plant transcriptome reprogramming observed in several natural tolerant interactions. The tolerance level was modest resulting in delay in the establishment of symptoms rather than long-term symptom attenuation. Increased tolerance to salt, drought, and fungal diseases was also achieved after overexpression of this gene (Zhang et al., 2009).

As discussed above, manipulation of plant genes implicated in protein degradation pathways or the UPR response may also provide novel avenues to engineer tolerance. Examples include the overexpression of the NtRFP1 RING E3 ligase to promote tolerance in *N. benthamiana* plants infected with a begomovirus (Shen et al., 2016), or of the ER Bip chaperone to suppress cell death associated with potexvirus infection of *N. benthamiana* (Ye et al., 2013). Down-regulation of genes associated with the UPR response has been shown to reduce symptom expression in other plant–virus interactions (Zhang L. et al., 2015).

The study of highly symptomatic interactions can help identify novel sources of tolerance. For example, a transcriptomic study of a systemic symptomatic infection associated with runaway HR necrosis conferred by a soybean R gene in response to a virulent isolate of soybean mosaic virus identified eIF5A as a highly induced gene (Chen et al., 2017). eIF5A is a translation factor previously implicated in symptom development in the interaction between *A. thaliana* and the bacterium *Pseudomonas syringae*. Interestingly, silencing of this gene diminished the systemic necrosis and reduced virus accumulation (Chen et al., 2017).

Although tolerance can been enhanced by manipulating plant signaling pathways in herbaceous hosts under controlled environmental conditions, the feasibility of field applications needs to be examined. Indeed, modifying vital plant signaling pathways is likely to have pleiotropic effects that could vary

depending on each plant–virus interaction and could also impact tolerance to other biotic or abiotic stresses. In addition, even if experiments conducted with herbaceous hosts under limited time periods show minimal impact on the plant growth and development, plants with longer lifespans (for example trees) could be affected differently. Further research aimed at elucidating the molecular mechanisms associated with tolerance, in particular in wild plant–virus interactions, may identify novel targets for engineering tolerance or assist in the development of improved agriculture practices.

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REVIEW
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Beyond Killing *Mycobacterium tuberculosis*: Disease Tolerance

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Host defense strategies against infectious diseases are comprised of both host resistance and disease tolerance. Resistance is the ability of the host to prevent invasion or to eliminate the pathogen, while disease tolerance is defined by limiting the collateral tissue damage caused by the pathogen and/or the immune response without exerting direct effects on pathogen growth. Our incomplete understanding of host immunity against tuberculosis (TB) is predominately rooted in our bias toward investigating host resistance. Thus, we must refocus our efforts to understand the entire spectrum of immunity against *M. tuberculosis* to control TB.

Keywords: host defense against pathogenic bacteria, disease tolerance, tuberculosis, innate immunity, adaptive immunity

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INTRODUCTION

Tissue homeostasis is essential for optimal physiological function and overall host fitness for survival (1). Thus, we have evolved with a complex tissue adaptation that involves cellular stress responses and, paradoxically, inflammation to maintain integrity, and functional capacity of an organ despite constant endogenous or exogenous insults, including infections. Historically, the dogma of host defense against infection was unilaterally aimed at eliminating the root of disease (i.e., the pathogen) and ultimately led to the discovery of antimicrobial drugs. While the discovery of antibiotics to directly restrict the growth of pathogens was a "revolution" in medicine, this accelerated drug-induced natural selection leading to the spread of drug-resistant pathogens. Today, the persistence of infectious diseases, the lack of vaccines for major chronic infections (e.g., tuberculosis, malaria, and HIV), as well as the decline in new antibacterial drugs in the pipeline are all indications for the urgent need of novel therapies that require a better fundamental understanding of host defense against infections.

Now, it is increasingly understood that host defense strategies against infectious diseases are comprised of both host resistance and disease tolerance. Host resistance is the ability of the host to prevent invasion or to eliminate the pathogen (2), while disease tolerance is defined by limiting the tissue damage caused by the pathogen and/or the immune response (3). Unlike resistance, disease tolerance does not necessarily exert direct effects on pathogen growth. For this reason, host resistance was considered as the central arm of host defense against infections. In fact, our inconsistency in understanding immunity against infectious diseases might be in part due to our bias toward host resistance to infections. However, this dogma has been recently challenged as we are gaining more fundamental knowledge from simple organisms such as the plant host defense mechanisms (4–7).

As plants are stationary, they have evolved many sophisticated host defense mechanisms to endure severe diseases caused by a large variety of pathogens, including fungi, bacteria, and viruses. In the late 1950s to early 1970s, it was initially observed that plants can tolerate an infection with

normal yield without affecting the pathogen load, which was termed "disease tolerance" (2, 8, 9). Most recently, Medzhitov, Schneider, and Soares broadened this concept (10), which has led to a growing appreciation for the crucial role of disease tolerance in invertebrates and vertebrates against infectious diseases (11, 12).

Mycobacterium tuberculosis (*Mtb*) has coevolved with humans for 70,000 years (13, 14) and achieved an evolutionary trade-off that infrequently compromises host survival. This trade-off has been conventionally considered to be dependent on host resistance for limiting the growth of *Mtb*. However, our understanding of natural immunity in 90 to 95% of infected individuals who become disease-free is extremely limited. As this latter population constitutes approximately a quarter of the world population (15), it is imperative that we delineate the mechanisms underlying host resistance vs. host tolerance during TB. In this Mini-Review, we focus on recent studies that shed light on the cellular and molecular mechanisms of disease tolerance to *Mtb* and aim to fill this gap in knowledge of immunity against TB.

TUBERCULOSIS

Exposure to *Mtb* either results in direct elimination of the pathogen, most likely by the innate immune system, or infection, and containment that requires both innate and adaptive immunity to form the granuloma (**Figure 1**). In 90–95% of individuals infected with *Mtb,* the bacteria are either eliminated or contained and remain in a latent state, termed latent tuberculosis infection (LTBI). These individuals are asymptomatic and do not transmit the disease (16). Both human and non-human primate (NHP) studies indicate that these asymptomatic LTBI individuals have a spectrum of infection that ranges from sterilized and well-contained infections to a small frequency of individuals who are at higher risk for reactivation (17–19). Although the mechanism(s) of host susceptibility to progressive disease is not well understood and is multifactorial, several genetic polymorphisms have been associated with risk of active TB. For instance, a type I IFN signature appears to be linked to development of active TB in NHP (20), and has been described as a marker of active TB in humans as well (21). This ultimately led to the discovery of extensive cellular and molecular mechanisms that were thought to be only engaged in host resistance to TB. However, recent studies indicate that some of these mechanisms, as detailed below, that were thought to be central to host resistance may also play an essential role in disease tolerance against *Mtb*.

GRANULOMA IS THE SIGNATURE OF DISEASE TOLERANCE IN TB

Following the invasion of infectious agents (e.g., bacteria, fungi, and parasites), if the innate immune response is not able to destroy or expel the agent, the host will initiate an adaptive immune response. If the combination of both innate and adaptive immune responses fail to eliminate a pathogen, the host is then required to form granuloma—a mixture of both innate and adaptive effector cells—to "wall off" an agent and prevent dissemination. From that moment on, the host is forced to tolerate the agent. At the same time, this leads to a new set-point of immune responses with different magnitude as well as duration that must be carefully regulated to prevent immunopathology and maintain host fitness.

Granulomas are the hallmark of TB. However, they are a double-edged sword required for controlling and containment of *Mtb*, but also contribute to persistence of the bacteria (22–24). TB granulomas are particularly heterogeneous, but the basic granuloma architecture is composed of a central necrotic core (caseum), which is surrounded by mainly macrophages that are at different activation stages, and a cuff of T and B cells. Monocytes, neutrophils, DCs, and NK cells can also be found in the granulomas. The inflammatory state of granulomas can alter the ratio of its cellular composition, which becomes critical in determining granuloma fates and outcome of infection. Remarkably, despite being a critical step for the initial formation of the granuloma, it is still unclear if granuloma formation driven by the host or *Mtb*? While this fundamental question remains to be answered, tremendous advances in understanding the dynamics of granulomas in TB have recently been made.

While the induction of inflammatory mediators within a granuloma is required for preventing *Mtb* dissemination, overly intense pro-inflammatory responses lead to the destruction of granulomas via necrosis, enhanced lung parenchymal damage, lung cavitation, and transmission that results in the onset of active disease (25–27). Studies in animal models of TB as well as in humans have elegantly demonstrated that inflammatory signaling is highly organized in the granuloma as pro-inflammatory signaling is mainly found at the core of the granuloma, while anti-inflammatory signaling is located in the periphery (28). This spatial compartmentalization of pro- and anti-inflammatory signaling determines the granuloma's function in controlling bacterial dissemination. Thus, the host is better off with a balanced inflammatory and anti-inflammatory signaling that leads to the regulation of inflammation within and around the granuloma and reduced frequency of active disease (29).

MECHANISMS THAT UNDERLAY THE "SWITCH" FROM HOST RESISTANCE TO DISEASE TOLERANCE

The central question that remains to be addressed is how and when host defense strategies switch from resistance to tolerance. While the exact cellular and molecular mechanisms of this phenomenon are still under investigation, we envision that three key pathways contribute to this transition.

1. Pathogen Recognition Signaling

During the early stage of infection, the vast majority of signaling in the host results from the detection of the pathogen

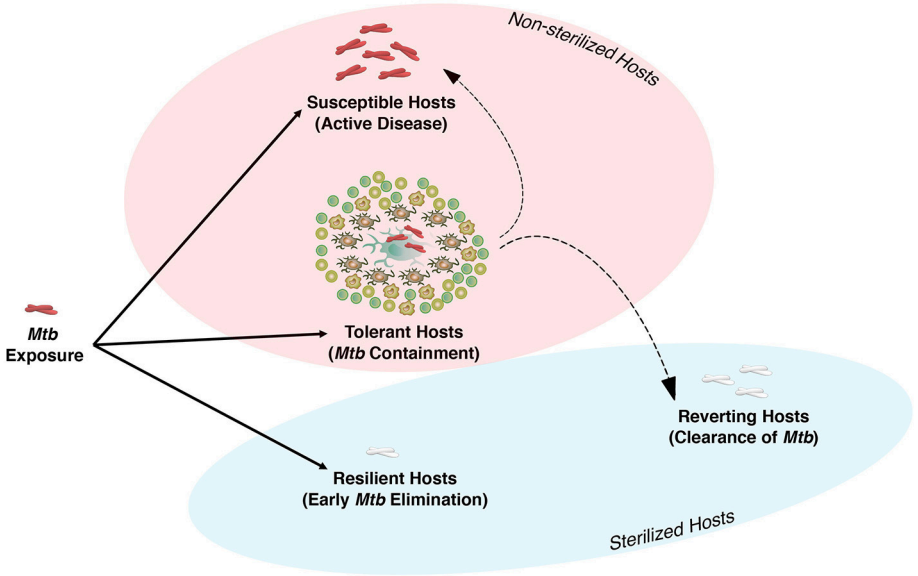

FIGURE 1 | The spectrum of *Mycobacterium tuberculosis* infection in humans**.** Humans and *Mtb* have co-evolved to reach a dynamic equilibrium. There are three major outcomes following exposure to *Mtb*. (1) Resilient Host: These individuals are able to eliminate the bacteria at the early stage of infection via host defense mechanisms of the upper or lower airways. (2) Tolerance Host: if innate immunity is unable to eliminate *Mtb*, the host initiates adaptive immunity and granuloma formation, which is the beginning of the chronic phase of infection and disease tolerance to contain or ultimately eliminate *Mtb* (reverting host). Conditions associated with immunocompromised host may result in loss of *Mtb* containment and active disease in tolerant host. Although 90–95% of individuals are considered to be tolerant hosts, the exact number of these individuals who are able to clear *Mtb* or succumb to disease is still unknown. (3) Susceptible Host: individuals with impaired natural immunity to *Mtb* who progress to active disease and transmit the infection.

that initiates predominantly anti-microbial host resistance to infection. Recognition of *Mtb* or mycobacterial products by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), C-type lectin receptors (CLRs), and scavenger receptors initiates a cascade of events including production of cytokines, nitric oxide, reactive oxygen species, autophagy, and phagolysosome fusion to reduce the growth of *Mtb* and thus enhance host resistance (30). However, this initial host resistance to an infection comes with substantial tissue damage that needs to be repaired especially in a vital organ such as the lung. Additionally, in the context of a persistent infection like *Mtb*, in which innate immunity is often unable to eliminate the bacteria, controlling the magnitude of the inflammatory response becomes essential for host survival. Thus, as the infection persists, the host receives signals from damaged tissue to self-limit inflammation and preserve tissue integrity. For example, Mantovani's group has identified Toll/IL-1R (TIR) 8 receptor, a member of IL1R family, also known as single Ig IL-1-related receptor (SIGIRR), as a negative regulator of TLR/IL-1R signaling. TIR-8 signaling contributes to dampening inflammation and limiting tissue damage in *Mtb* infection (31). Mice deficient in *TIR-8* succumb to *Mtb* infection due to excessive inflammatory responses despite their ability to efficiently control bacterial growth (31). Further investigation is certainly required to dissect the pathways involved in regulating the inflammation to preserve tissue integrity and the maintenance of disease tolerance.

2. Host Immune Signaling

While the production of pro-inflammatory cytokines such as IL-1β and TNF-α are critical in anti-mycobacterial immunity predominantly during the early phase of *Mtb* infection, the constant production of these cytokines promotes inflammation-mediated tissue damage. Thus, their production needs to be tightly regulated. Sassetti's group has elegantly demonstrated that nitric oxide (NO) inhibits NLRP3 inflammasome-mediated IL-1β production to prevent neutrophil-dependent pulmonary tissue damage (32). Most recently, the same group has shown that the role of NO in host resistance to *Mtb* acts via the recruitment of neutrophils, which are permissive to *Mtb* growth (33). Importantly, this immunoregulatory function of NO is coordinated with the initial recruitment of IFN-γ-producing T cells into the lung, which leads to granuloma formation and perhaps the transition from host resistance to disease tolerance (please see the review from Sassetti-group in this special issue) (34).

The identification of mutations in the IL-12/IFN-γ/STAT1 axis that lead to disseminated mycobacterial infections, termed Mendelian Susceptibility to Mycobacterial Disease (MSMD), along with the susceptibility of T cell-deficient hosts to mycobacterial infections established the dogma that IFN-γ-producing T cells play a crucial role in host resistance against TB. However, there is no direct evidence of T cells/IFN-γ in protection against *Mtb*, but rather in the containment of infection (35–37) via regulation of the inflammatory response.

For instance, extrapulmonary TB is associated with individuals having lower measurable Tuberculin Skin Test (TST) responses (38), as well as with HIV-positive individuals with very low $CD4^+$ T cell counts (35). In addition, IFN-γ has been shown to inhibit pulmonary neutrophilic inflammation to prevent lung tissue damage during the chronic phase of *Mtb* infection (39, 40). High levels of neutrophils generate a strong inflammatory response that results in increased pulmonary pathology and mortality. Importantly, neutrophil depletion in $IFN\gamma R^{-/-}$ mice prolonged their survival during *Mtb* infection. (39). The contribution of neutrophils to immunopathology during *Mtb* infection has been well established in mice (41), NHP (42, 43), and humans (21). These studies collectively indicate that the IFN pathway is critical in the regulation of inflammatory signals and disease tolerance rather than host resistance.

Furthermore, dysregulated T cell responses appeared to be detrimental for the host by inducing overt immunopathology. It has been well documented that during chronic viral infection, constant exposure of T cells to antigens and inflammatory cytokines lead to loss of T cell function, a process termed "T cell exhaustion" (44). One of the well-defined pathways in T cell exhaustion is programmed cell death (PD1). The interaction between PD1, which is expressed on antigen-experienced T cells, and its ligands PDL-1 and PDL-2 prevents T cell proliferation and cytokine production. Thus, it was thought that the inhibition of PD1 signaling should promote protection via "reviving" T cell-mediated immunity to chronic *Mtb* infection. However, while disruption of PD1 signaling either genetically or via neutralizing antibodies significantly enhanced T cell-mediated immunity to *Mtb* infection, this was associated with increased bacterial growth, massive pulmonary immunopathology, and reduced survival (45, 46). Thus, the regulatory mechanisms involved in the expansion and contraction of T cell responses become a critical determinant of the outcome of TB infection. While the surface expression of some of these markers (e.g., PD1 or KLRG) on T cells appears to be critical for dictating their functional role during infection, the intrinsic immunoregulatory mechanisms of T cells are poorly understood.

Mitochondria are central platforms that critically regulate cell proliferation and differentiation. To meet the metabolic demands of active cells, mitochondria can rapidly switch from a state of catabolism to anabolism to provide the biosynthetic intermediates that are pivotal for cellular function. Naïve T cells have a low rate of metabolic activity, characterized by minimal nutrient uptake and biosynthesis. These cells procure cellular energy in the form of adenosine triphosphate (ATP) from the energetically efficient processes oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) (47). Upon TCR activation, dramatic metabolic reprogramming occurs to generate the increased energy needed for T cell proliferation, differentiation and cytokine production. To ensure adequate metabolic resources are available, activated T cells increase nutrient uptake and switch from OXPHOS and FAO to aerobic glycolysis (47). While energetically inefficient, glycolysis enables the cells to rapidly produce ATP and other biosynthetic precursors essential for cell growth and proliferation. This switch from predominantly OXPHOS to aerobic glycolysis, despite the presence of abundant oxygen, is known as the "Warburg Effect." Metabolic shift from OXPHOS to glycolysis or vice-versa is also highly associated with the inflammatory and anti-inflammatory function of immune cells (48). For example, inflammatory cells such as activated macrophages exhibit higher glycolysis, by contrast anti-inflammatory cells such as M2 macrophages acquire higher OXPHOS than glycolysis (49). A recent study in non-human primates (NHP) suggests that the relative proportion of inflammatory or anti-inflammatory macrophages is important in deciding the outcome of *Mtb* infection (50). The metabolic status of a cell is also important to regulate immune cell polarization (51). Th17 cell differentiation relies on glycolysis, whereas blocking glycolysis inhibits Th17 development and promotes regulatory T cell (Treg) differentiation. Th17 cells are important in host resistance to *Mtb* but uncontrolled production of IL-17 induces inflammation via recruitment of neutrophils and increases the mortality of *Mtb*-infected mice (39). Higher susceptibility of TLR-2-KO mice to *Mtb* has been linked to reduced accumulation of Treg cells and concomitant increased inflammation (52). These findings suggest that the metabolic state determines the fate of immune cells which is critical in promoting or dampening inflammation.

An equally important function of mitochondria is their role in the cell death program. Cyclophilin D (CypD), a member of the cyclophilin protein family, is a conserved protein located in the mitochondrial matrix (53). It has been previously shown that CypD plays a key role in necrosis by regulating the mitochondrial permeability transition pore (MPTP), which allows the passage of solutes and water from the cytoplasm into the mitochondria (54, 55). Necrosis of macrophages is an exit mechanism for *Mtb* (56–59). Remold and colleagues initially demonstrated that the pharmacological inhibition of CypD in human macrophages lead to the inhibition of necrosis and reduction of *Mtb* growth *in vitro* (60). This observation has been recently extended to the zebrafish and mouse models of tuberculosis where the genetic blockade of CypD prevented macrophage necrosis and enhanced their anti-mycobacterial capacity (61, 62). Based on the role of CypD in macrophage immunity to *Mtb* infection, we initially hypothesized that CypD-deficient mice ($CypD^{-/-}$) are resistant to *Mtb* infection. Surprisingly, $CypD^{-/-}$ mice were highly susceptible to *Mtb* infection compared with control animals, despite similar numbers of bacteria in both groups. We further identified that this susceptibility was related to an enhanced T cell response that promoted lung immunopathology independent of host resistance. We have determined that CypD intrinsically regulates T cell metabolism and critically regulates disease tolerance in TB (63). Similarly, the C3HeB/FeJ mouse strain that generates a profound T cell response to *Mtb* infection quickly succumbs to death due to the overgrowth of necrotic granulomas (64, 65). Although we still don't know why the functional role of CypD is different in macrophages vs. T cells, we envision that as T cells are intrinsically programmed to proliferate, the functional role of CypD in these cells may

be wired to regulate the metabolism and proliferation rather than cell death. Collectively, these data indicate that, similar to granulomas, T cells are a double-edged sword: while they are crucial to initiate granuloma formation during the early phase of *Mtb* infection and prevent the dissemination of disease, they also play an important role in transmission of *Mtb* by promoting granuloma necrosis during the active phase of the disease (66). Thus, the function and location of these effector cells are critical determinants of disease tolerance and host survival in TB.

3. Lung-Stromal Signaling

The term "tissue remodeling" refers to irreversible anatomical and structural changes. The lung injury caused by *Mtb* infection and subsequent granuloma formation results in distortion of the lung architecture. This requires effective and coordinated repair mechanisms to limit the extent of the granulomas and preserve lung function while ensuring pathogen containment. For instance, matrix metalloproteinases (MMPs), which are a family of zinc-dependent proteases, play an important role in extracellular matrix remodeling by degrading collagens. Several MMPs have been associated with active TB and cavitation (67), which reflects the importance of lung tissue repair in generating a preventive granuloma in TB. Furthermore, some of the mechanisms that are engaged in tissue healing, like fibrosis, also play a key role in the formation of fibrosis in the periphery of the granuloma to effectively prevent bacterial dissemination. Therefore, it is not surprising that the presence of type 2 immune responses, which are essential for controlling tissue damage, has commonly been observed in TB (68–71). While type 1 immune responses are crucial for the formation of an effective granuloma to control the infection, type 2 immunity is required at the same time to control lung tissue damages caused by both immune responses and *Mtb*. While the role of type 2 cytokines (e.g., IL-4 and IL-13) in stimulating TGF-β-dependent granulomatous inflammation and fibrosis is well established in parasitic infections, little is known about the exact role of these cytokines in tissue healing and repair in TB. During parasite infections both IL-4 and IL-13 are the major drivers of STAT6 translocation. STAT6-deficient mice are impaired in forming granulomatous fibrosis (72), and IL-13 increases TGF-β activation (73). Interestingly, using a heterologous mouse model of *Nippostrongylus brasiliensis* (Nb) and *Mtb* infection, Salgame's group has shown that the growth of bacteria was increased only at 4 weeks after *Mtb* infection, while there were no differences at two or seven weeks post infection. Despite this early increase in bacterial growth, there was no difference in lung histopathology or granuloma formation (74). Thus, while the type 2 immune bias transiently compromises early host resistance to *Mtb*, it may promote disease tolerance at later timepoints and ultimately control the infection. It therefore becomes important to identify the location of both innate and adaptive immune cells that are responsible for spatial production of type 1 and type 2 cytokines and extracellular matrix (ECM) remodeling in the granuloma.

Additionally, the expression of virulence factors from *Mtb* adds another layer of complexity for the maintenance of this delicate balance between host and *Mtb* in the granuloma. For instance, early secretory antigen-6 (ESAT6) appears to lyse lung epithelial cells and facilitate local dissemination (75). However, ESAT6 also induces MMP9 from epithelial cells, which was associated with the recruitment of monocytes/macrophages and granuloma maturation (76). In contrast to MMP9, it has been shown that MMP1 was significantly upregulated in individuals with active TB. MMP1 specifically degrades type I collagen and increases pulmonary tissue destruction in TB. Additionally, transgenic mice expressing human MMP1 showed extensive tissue damage despite similar levels of bacterial burden in the lungs (77). Interestingly, a recent study has reported that a selective MMP7 inhibitor (cipemastat) has a detrimental impact on pulmonary granulomas by increasing cavitation in a mouse model of TB (78). An elegant study by Tobin's group has also demonstrated extensive angiogenesis within the granuloma, whereas inhibition of vascular endothelial growth factor (VEGF) signaling reduced vascular leakage and bacterial dissemination in a zebrafish model of TB (79). Further studies also suggested that increased angiogenesis in the area that has restricted access to the blood supply may increase the access of immune cells and anti-TB drugs to the bacteria (80). Collectively these studies indicate that the location and balance in the signaling of type 1 and 2 immune responses that regulate lung extracellular matrix (ECM) remodeling via collagen deposition/degradation/angiogenesis define an effective granuloma in TB (please see the review from Tobin in this special issue).

CONCLUSION AND REMARKS

A prolonged co-evolutionary interaction between humans and *Mtb* has almost reached its perfect balance with 90–95% of infected individuals being resilient or "tolerating" the presence of *Mtb* without any disease symptoms (**Figure 1**). This can be interpreted as 9 out of 10 people having a protective natural immunity against TB which renders them asymptomatic and non-infectious and may further explain why humans are the only known host for *Mtb* (14). This epidemiological data also suggests that, through a long evolutionary process, an equilibrium is reached that supports both host fitness and *Mtb* survival. Interestingly, in NHP which are the natural host for SIV, as well as in HIV-viremic pediatric and adult humans, it has been long recognized that viral replication is not the major cause of disease progression but rather immune cell activation (81–83). Similarly, reactivation of latent *Mtb* in a NHP coinfection model of SIV/*Mtb* was directly linked to over-activation of the immune response (84). Thus, it can be argued that the transition from HIV to AIDS, or LTBI to active TB may not depend on the pathogen load but rather on dysregulated immunity to infections.

While for an obvious reason we have been focusing on 5–10% of infected individuals who progress to active disease, we disproportionally have biased our scientific view as well as investigative approach toward resistance and the elimination of *Mtb*. Because of this bias, we incompletely understand the full spectrum of immunity to TB including the mechanisms of disease tolerance and thus fall short in developing an effective

vaccine. Furthermore, any medical intervention targeting host resistance may potentially break disease tolerance which can have catastrophic consequences. While assessments of host resistance, in particular bacterial burden, is the gold standard for the evaluation of an effective therapy or vaccine, we propose that measurements of disease tolerance, such as immunopathology, are also important criteria to be considered in parallel to host resistance.

The new studies that shed light on disease tolerance may yield clinical benefit in designing host-targeted vaccines that minimize tissue damage, prevent granuloma cavitation and disease transmission, and ultimately reduce the global burden of TB disease.

AUTHOR CONTRIBUTIONS

MD wrote the manuscript with contributions from NK and EK.

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REVIEW
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Resistance and Tolerance to Cryptococcal Infection: An Intricate Balance That Controls the Development of Disease

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Cryptococcus neoformans is a ubiquitous environmental yeast and a leading cause of invasive fungal infection in humans. The most recent estimate of global disease burden includes over 200,000 cases of cryptococcal meningitis each year. *Cryptococcus neoformans* expresses several virulence factors that may have originally evolved to protect against environmental threats, and human infection may be an unintended consequence of these acquired defenses. Traditionally, *C. neoformans* has been viewed as a purely opportunistic pathogen that targets severely immune compromised hosts; however, during the past decade the spectrum of susceptible individuals has grown considerably. In addition, the closely related strain *Cryptococcus gattii* has recently emerged in North America and preferentially targets individuals with intact immunity. In parallel to the changing epidemiology of cryptococcosis, an increasing role for host immunity in the pathogenesis of severe disease has been elucidated. Initially, the HIV/AIDS epidemic revealed the capacity of *C. neoformans* to cause host damage in the absence of adaptive immunity. Subsequently, the development and clinical implementation of highly active antiretroviral treatment (HAART) led to recognition of an immune reconstitution inflammatory syndrome (IRIS) in a subset of HIV+ individuals, demonstrating the pathological role of host immunity in disease. A post-infectious inflammatory syndrome (PIIRS) characterized by abnormal T cell-macrophage activation has also been documented in HIV-negative individuals following antifungal therapy. These novel clinical conditions illustrate the highly complex host-pathogen relationship that underlies severe cryptococcal disease and the intricate balance between tolerance and resistance that is necessary for effective resolution. In this article, we will review current knowledge of the interactions between cryptococci and mammalian hosts that result in a tolerant phenotype. Future investigations in this area have potential for translation into improved therapies for affected individuals.

Keywords: *Cryptococcus*, asymptomatic infection, damage response framework, disease tolerance, immunoregulation, host-pathogen interaction

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INTRODUCTION

The incidence of invasive fungal diseases has increased in recent decades and is associated with 1.5 million deaths annually. Much of this increase is attributable to the rising number of people with weakened or dysfunctional immune systems who are at high risk for the development of serious fungal infections (1–3).Major risk factors for invasive mycoses include HIV infection, stem cell, and solid organ transplantation, prolonged immunosuppressive therapy, invasive medical procedures, hematological malignancies, advanced age, and prematurity (4, 5). More than 90% of all reported fungal-related deaths result from species that belong to four genera: *Cryptococcus*, *Candida*, *Aspergillus*, and *Pneumocystis* (4). In addition to delays in diagnosis, similarities between eukaryotic fungi and humans render treatment of fungal infections more difficult compared to bacterial and viral infections. Relatively few antifungal drugs are currently available and their efficacy is limited by toxicity, a narrow spectrum of activity, detrimental drug interactions, the development of resistance, and, in some cases, high cost (6, 7).

The genus *Cryptococcus* contains at least 37 species; however, *C. neoformans* and *Cryptococcus gattii* are the main causes of human disease (8, 9). *Cryptococcus neoformans* classically targets immunosuppressed individuals including those with advanced HIV-AIDS, various T cell deficiencies, pregnancy, chronic lung, renal, or liver diseases, cancer, and patients receiving immunosuppressive therapy, while *C. gattii* has a predilection for immunocompetent individuals (10–12). The initial exposure to cryptococci occurs through inhalation of spores or small desiccated yeast cells that enter the lower respiratory tract. A seroprevalence study in New York demonstrated that 70% of samples from children over the age of 5 years had reactive antibodies against *C. neoformans* antigens, suggesting that exposure is widespread despite a low incidence of disease (13). Although definitive human studies are lacking, circumstantial evidence indicates that asymptomatic colonization of the airways or latent cryptococcal infection of the lungs and associated structures may also be common (14, 15). For example, autopsy studies identified *C. neoformans* infection in subpleural or parenchymal lung nodules where yeasts were contained inside macrophages and multinucleated giant cells in association with a granulomatous response (16–18). On the other hand, the most devastating clinical consequence of cryptococcal infection is meningoencephalitis that can occur following a primary lung infection or by reactivation and dissemination of latent pulmonary infection upon subsequent immunosuppression (19–21). The development of severe cryptococcal disease may occur years or even decades after the initial infection, indicating that humans are able to tolerate the presence of viable cryptococci for extended periods of time (22).

A recent study of the global burden of cryptococcal disease estimated that 278,000 individuals have a positive cryptococcal antigen test that is indicative of infection and 223,100 patients develop cryptococcal meningitis, with 73% of the cases occurring in Sub-Saharan Africa (23). Worldwide, cryptococcal meningitis account for 181,100 deaths annually, including 15% of AIDS-related deaths. These figures indicate that the proportion of AIDS-related mortality has not changed compared to the previous estimate in 2008 (24) with cryptococcosis remaining the second most common cause of AIDS-related death after tuberculosis (23). Notably, up to 20% of cases of cryptococcosis occur in phenotypically "normal" or apparently immunocompetent patients without any known risk factors for infection susceptibility (25). Almost 50% of patients with cryptococcal meningitis die in the year after infection mainly because of unsuccessful therapy (26). A better understanding of the key mechanisms of host immunity to *Cryptococcus* will be important for future development of new and more effective approaches to preventing and treating cryptococcal diseases. The mechanisms of host resistance in *Cryptococcus* infection has been extensively studied and reviewed elsewhere (20, 21, 27, 28). In this article, we will discuss the mechanisms of tolerance that characterize the host-cryptococcal interaction.

OVERVIEW OF TOLERANCE AND RESISTANCE

The concept of disease tolerance was originally described in plants and arose from observations of variation in disease severity at a population level without a direct correlation to pathogen load (29–31). Compared to resistance, which is defined as the ability to reduce pathogen burden to preserve homeostasis, tolerance is the ability to limit the extent of damage and dysfunction to host tissues during infection. Disease tolerance pathways that attempt to maintain host fitness without exerting direct negative effects on pathogen burden may lead to microbial survival and persistence (32–34).

Two types of tissue damage may occur during infection; one is directly caused by the pathogen through toxin production and virulence factor expression, and can be limited by reduction of the microbial load through host resistance mechanisms. The second type of tissue damage is an indirect consequence of infection that results from a vigorous host immune response and manifests as immunopathology despite control of pathogen burden (33). Certain host resistance mechanisms have potentially damaging effects on host fitness; for example, production of reactive oxygen species (ROS), proteases, and growth factors by neutrophils and macrophages may cause cellular destruction, abnormal collagen deposition, and tissue fibrosis (35). Even if overt organ damage is not evident, host resistance mechanisms are usually associated with some degree of subclinical tissue dysfunction; for example, inflammation that is effective in combating lung infection can alter both the integrity and permeability of the pulmonary vascular endothelium and airway epithelium and may culminate in reduced respiratory function (31).

In general, disease tolerance is characterized by stress responses and damage control mechanisms that maintain homeostasis and functional integrity of host tissues in response to environmental changes. When physiological parameters change beyond a certain threshold, stress responses initiate signal transduction pathways to provide metabolic adaptation in host

cells (32). Some of the best known signaling mechanisms involved in the cellular stress responses include transcription factors such as HIF-1alpha (hypoxia-inducible factor 1 alpha) triggered by hypoxia, NRF2 (nuclear factor-erythroid 2-related factor 2) triggered by oxidative stress, and AhR (Aryl hydrocarbon Receptor) triggered by xenobiotic stress (36–38). Other stress response mediators include AMPK (AMP-activated protein kinase) triggered by ATP depletion, and the NLR (Nod-like receptor) protein family that responds to stress caused by microbial toxins and endogenous danger signals (33, 38). In a similar manner, tissue damage control can also occur through various mechanisms that (1) enforce barrier function of epithelial cells and prevent pathogen access to host tissue, (2) neutralize pathogen toxins and virulence factors, (3) regulate the intensity and duration of the host immune and inflammatory responses and (4) enhance resistance against inflammatory damage by promoting parenchymal cell regeneration (32, 33, 39).

Mechanisms of host resistance and disease tolerance function in a pathogen class-specific manner (33). In some cases, the pathogen itself may contribute and/or augment the host's capacity for tolerance to enhance its own survival and transmission. If the host can sustain a high level of tolerance that is sufficient to prevent major disruption of physiological functions, a state of persistent and/or asymptomatic infection will be established. Conversely, if host resistance mechanisms cause significant tissue damage or major alterations of host physiology, various pathological outcomes of infection will occur (31). Ultimately, an ideal immune response is defined by the balance between host resistance and tolerance that facilitates efficient pathogen clearance with an acceptable degree of immunopathology **Figure 1** (32).

DISEASE TOLERANCE AND THE DAMAGE RESPONSE FRAMEWORK IN HOST-*CRYPTOCOCCUS* INTERACTION

Based on serological and epidemiological studies, natural exposure to *Cryptococcus sp*. is common. Yet, despite the observation that a high percentage of children and healthy individuals in certain geographic areas develop cryptococcal antibodies, overt clinical manifestations of disease are rare (13, 22, 40–42). In an immunocompetent host, infectious propagules of *Cryptococcus sp*. are completely cleared from the respiratory tract or may establish a latent asymptomatic infection in pulmonary granulomas or thoracic lymph nodes (15, 16). Following immunosuppression, the fungus can proliferate and disseminate to other parts of the body, including the central nervous system. Given the lack of an inflammatory response during latent infection, symptoms of disease reactivation will not develop until the fungal cell burden causes tissue dysfunction and damage to infected organs (8, 22, 43). Depending on host factors, cryptococci may cause progressive granulomatous inflammation or form discrete fungal masses (termed cryptococcomas) in primary target organs such as the lungs and brain. Each of these vital organ systems has a relatively low tolerance and repair capacity and is highly susceptible to damage; therefore, severe and/or progressive infection of the lower respiratory tract or central nervous system is poorly tolerated and life-threatening (31, 44). Indeed, latent asymptomatic cryptococcal infection, but not clearance, may be considered as a host tolerance mechanism to prevent or limit lung or brain damage (45).

The indispensable role of the host response to the outcome of microbial pathogenesis is a central tenet of the Damage Response Framework (DRF) proposed by Pirofski and Casadevall (46, 47). The DRF integrates the contribution of microbial and host factors that may produce a net benefit or cause disease that is reflected by host damage. Importantly, microbial virulence traits interact with either a weak or strong immune response to cause disease that exhibits a parabolic distribution. In addition to disease, the highly dynamic interaction between microbe and host may also lead to different disease outcomes including colonization, latency, and commensalism. From the viewpoint of the DRF, progressive asymptomatic cryptococcal infection will continue until the damage resulting from host-pathogen interactions over time exceeds a threshold amount that is sufficient to create clinical symptoms (47, 48). *Cryptococcus neoformans* has been classified as a class 2 pathogen that causes disease exclusively in hosts with weak or defective immune responses through expression of virulence traits. However, the emergence of *C. gattii* in apparently healthy individuals in Pacific Northwest and development of immune reconstitution inflammatory syndrome (IRIS)-associated cryptococcosis in HIV/AIDS after antiretroviral therapy, suggests that cryptococci may be class 4 pathogens that cause disease at the extremes of weak and robust immunity. Thus, the pathogenesis of cryptococcal disease and associated host damage is attributable to the interaction of fungal virulence with dysregulated host immune responses (47–49).

As reviewed elsewhere, protection against cryptococcal infection is mainly associated with secretion of pro-inflammatory cytokines, generation of effective Th1/Th17 adaptive immune responses, and classical activation of macrophages that mediate fungal clearance (20, 21, 27, 28, 50–53). Although resistance mechanisms are required for sterilizing immunity, excessive inflammation can be detrimental to the host and culminate in severe tissue damage and immunopathology. In fact, an ideal immune response to cryptococcal infection necessitates a tightly regulated balance between Th1, Th17, and Th2 responses that control fungal growth while preventing excessive tissue damage and immunopathology **(Figure 1)** (19, 21). The pathological consequences of excessive inflammation during cryptococcal infection are clearly exemplified by the problem of IRIS. Development of cryptococcal IRIS is mainly associated with HIV+ patients, solid organ transplant recipients, and pregnancy and is caused by recovery of specific immune responses resulting in exaggerated host inflammation and local organ damage (54). There are two types of cryptococcal IRIS in HIV+ patients: (1) Paradoxical cryptococcal IRIS that occurs after starting ART and presents as a deterioration or recurrence of clinical symptoms in the same or new site even with successful antifungal therapy, and (2) Unmasking cryptococcal IRIS that begins shortly after initiation of ART in patients with no prior diagnosis of cryptococcosis and may be its first manifestation (55–57). A paradoxical immune response, known as post-infectious

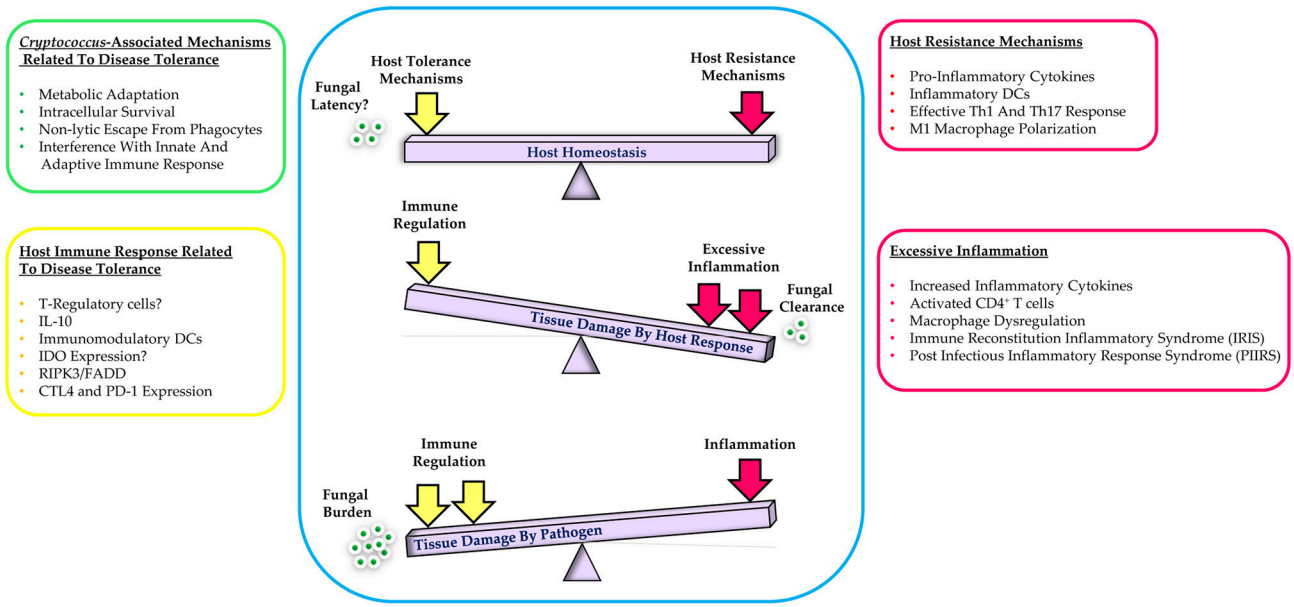

FIGURE 1 | Schematic representation of the balance between host resistance and tolerance to cryptococcal infection. Effective control of infection requires a balanced response between host tolerance and resistance mechanisms while excessive host inflammation or immune regulation leads to tissue damage. Additional details are provided in the text.

inflammatory response syndrome (PIIRS), can also occur in non-HIV patients with cryptococcal meningitis following reduction of immunosuppressive therapy and is associated with severe neurological disease (58, 59).

In the context of the damage response framework, cryptococcal meningitis can be classified in 3 groups (44, 55): (1) In HIV+ patients who have not started highly active antiretroviral therapy, host damage is mainly pathogen-mediated and is characterized by a high fungal burden. Even after initiation of effective antifungal therapy, pathogen virulence is believed to be a major determinant of mortality. Low levels of Th1-associated cytokines including IFN-gamma and TNF-alpha in these patients suggest that immune-associated damage is not a major factor in disease pathogenesis (60, 61). These observations are also consistent with a lack of significant improvement in disease outcomes with adjunctive corticosteroid therapy (62). (2) In HIV+ patients that develop cryptococcal IRIS after starting the antiviral therapy, damage is associated with a vigorous Th1 type host immune response that is characterized by increased inflammatory cytokines IFN-γ and IL-6, activated macrophages/monocytes, and recruitment of $CD4^+$ T cells. Induction of cerebral edema, neurotoxic effects of activated macrophages, and metabolic programming of neurons by adjacent inflammatory signals are some of the mechanisms of immune-mediated damage in the brain (63–65). (3) In non-HIV patients, tissue damage is mainly associated with a robust intrathecal Th1 type cellular immune response that is associated with alternative macrophage activation, high IL-10 and low TNF-α levels. The discordant activation of lymphocytes and macrophages results in persistent expression of cryptococcal antigen that perpetuates local inflammation (44, 55, 59). To maintain homeostasis and prevent unnecessary tissue damage, host tolerance mechanisms regulate the degree and duration of the immune response; therefore, the development of IRIS, a condition that is characterized by excessive and dysregulated immunity, could signify a failure of tolerance during cryptococcal infection (30, 66).

Excessive inflammation and immune-mediated host damage have also been shown in experimental mouse models of cryptococcal IRIS. Following $CD4^+$ T cell transfer into $RAG^{-/-}$ mice on the C57BL/6 or BALB/c genetic background, severe inflammatory disease was established in lungs, brain, and liver without affecting fungal clearance. Compared to controls, heightened systemic inflammation characterized by Th1-type cytokines and activated $CD4^+$ T cells as well as granulomatous inflammation of the liver was observed in reconstituted $RAG^{-/-}$ mice (67). In another model, C57BL/6 mice infected intravenously with 10^6 *C. neoformans* 52D developed lethal neurological dysfunction 3 to 4 weeks post-infection despite fungal clearance in the central nervous system. Activated microglia and antigen-specific IFN-γ producing $CD4^+$ T cells were identified in the brains of infected mice. Depletion of $CD4^+$ T cells reduced CNS inflammation and prevented mortality, although fungal clearance was also decreased (68). Interestingly, despite an extremely high fungal burden at day 7 and 14 post-infection, the presence of central nervous system infection remained relatively asymptomatic. One explanation for this observation could be host tolerance to infection that was ultimately subverted by a vigorous immune response and the development of extensive tissue damage.

CRYPTOCOCCUS-ASSOCIATED MECHANISMS RELATED TO DISEASE TOLERANCE

Microbial pathogens employ a variety of mechanisms to trigger host damage including intracellular and/or extracellular replication, production, and release of toxic substances, disruption of organ homeostasis, and modulation of host immune responses (47). *Cryptococcus sp.* express several virulence factors that facilitate pathogen survival, proliferation, and dissemination in mammalian hosts (69–71). The mechanisms by which *C. neoformans* mediates host damage have been extensively reviewed by Casadevall et al. (72). At the molecular level, *C. neoformans* produces several degradative enzymes such as proteases, urease, phospholipase, and nuclease that degrade host molecules (73–77). Mechanisms of cellular damage include: (1) interference with phagolysosome maturation (78), (2) increased permeability of the phagosome membrane (79, 80), (3) disrupted organelle function; for example, the ability to impair protein synthesis by mitochondria (81, 82), (4) cytoskeletal alterations (83), (5) non-lytic exocytosis and cytoplasmic vacuolation (84–86), and (6) lytic exocytosis resulting in host cell death (87). In addition, *C. neoformans* has several direct and indirect mechanisms that interfere with host immune cell function and damage endothelial cells in the brain vasculature (72).

In contrast to the virulence factors and microbial mechanisms that trigger cell and tissue damage as part of disease pathogenesis, *Cryptococcus sp.* has evolved several unique strategies that facilitate survival and persistence in the host without causing apparent pathology. Remarkably, the persistence of a chronic, low-grade *C. neoformans* infection does not prevent the generation a protective cell-mediated immune response upon secondary infection (47, 88). Some of the main strategies that contribute to latent cyptococcal infection and prevent complete clearance include acquisition of stress tolerance mechanisms against high temperature, reactive oxygen species, and reactive nitrogen species, capacity for facultative intracellular residence, regulation of host cell expulsion mechanisms, and evasion or interference with innate and adaptive immunity (19, 45, 89, 90). Below, several important characteristics associated with long-term or persistent cryptococcal infection are summarized; additional details may be found in previous reviews (45, 89–91).

1) Metabolic Adaptation to Physiological Host Conditions

The fact that environmental cryptococci can infect many vertebrate and invertebrate hosts reflects its capacity to adapt to a variety of different conditions. Metabolic adaptation is a major requirement for fungal persistence in the mammalian host, and many genes and pathways that are essential for stress resistance and high temperature growth have been identified (90, 91). For example, the thermotolerant phenotype of *Cryptococcus sp.* is mediated by Ras1/Ras2 signaling pathways (92, 93) and functional calcineurin A, a Ca^{2+}-calmodulin-regulated protein phosphatase that is activated by stress responses and stimulates the expression of genes required for growth and survival at 37°C as well as during oxidative stress (43, 89, 91, 94).

2) Evasion and Interference With the Innate Immune Response

Cryptococcus sp. express several factors that have been shown to interfere with host immune response (72). For example, the extracellular capsule is a key virulence attribute that is composed of glucuronoxylomannan (GXM) and two minor components, galactoxylomannan (GalXMs), and mannoprotein (MP). The capsule conceals cell wall antigens, inhibits antibody binding to the fungal cell wall, activates and depletes complement, suppresses T lymphocyte proliferation, modulates cytokine production, and induces host cell apoptosis (95–97). Capsular enlargement during infection and formation of giant "Titan cells" that range in size from 50–100 μm is a powerful anti-phagocytic mechanism used by *Cryptococcus sp.* (98–100). Release of capsular GXM causes L-selectin shedding from neutrophils and limits their migration, adhesion to endothelial cells, and tissue extravasation (101). Cryptococcal capsular components also have anti-inflammatory properties that inhibit the maturation and activation of DCs, macrophages, and neutrophils (102–104). Capsule-independent mechanisms including the App1 protein and GATA family of transcription factors have also been implicated in evasion of phagocytosis and immune recognition (105, 106).

Several studies have shown long term survival of cryptococci within macrophages and endothelial cells during asymptomatic infection, suggesting that fungi may persist without causing tissue damage (72, 89). To survive within the harsh phagosomal environment, *Cryptococcus sp.* express several enzymes involved in nitric oxide detoxification and oxidative damage repair such as catalases, superoxide dismutases, glutathione peroxidases, thioredoxin proteins, the inositol phosphosphingolipid-phospholipase C1 (Isc1) and the protein kinase C (Pkc1) and utilize host lipid components for production of cryptococcal eicosanoids (107). Additional factors that promote intracellular survival and persistence include melanin, laccase, urease, phospholipase (PLB1) and heat shock protein 70 homolog Ssa1 (108–110).

The ability to exit the phagocytic cells without killing and triggering an immediate immune response is one of the most important mechanisms associated with survival and long-term persistence of *Cryptococcus sp.* (10, 89). Non-lytic escape from phagocytes, also termed vomocytosis or phagosome extrusion, occurs by merging of the phagosome and plasma membranes followed by release of the organism to the surrounding environment or lateral transfer between host cells. Escape from phagocytes without triggering host cell death and inflammation is beneficial for latency and persistence of cryptococcal infection (84, 111). Finally, there is evidence that *Cryptococcus sp.* disseminates to the CNS from the bloodstream within macrophages using a Trojan Horse mechanism and is subsequently released by non-lytic

extrusion after it has crossed the BBB (84, 111–114). Taken together, intracellular survival and non-lytic exocytosis are beneficial adaptations for both host and pathogen in the context of tolerance hypothesis (45).

3) Interference With the Adaptive Immune Response

In addition to subversion of innate immunity, interference with the adaptive immune response is also essential for cryptococcal persistence and latent infection (89). *Cryptococcus sp.* use various mechanisms to regulate T-cell proliferation, differentiation, and survival (20, 115, 116). For example, expression of cryptococcal urease induces a non-protective Th2 immune response through recruitment of immature DCs to the lung-associated lymph nodes (117). Cleavage of fungal chitin by host chiotriosidase also initiates Th2 cell differentiation by $CD11b^+$ conventional dendritic cells in pulmonary cryptococcal infection (118). Production of PGE2 by *C. neoformans* specifically inhibits IL-17 expression during Th17 cell differentiation in an IRF4-dependent manner (119). Inhibition of the Th17 response has been implicated as a potential mechanism that facilitates latent infection (89). Finally, persistent pulmonary *C. neoformans* infection also interferes with humoral immunity by selectively reducing antibody responses to exogenous cryptococcal polysaccharide (120).

HOST IMMUNE RESPONSE ASSOCIATED WITH DISEASE TOLERANCE IN CRYPTOCOCCAL INFECTION

Host resistance during cryptococcal infection is characterized by the expression of pro-inflammatory cytokines, recruitment of inflammatory DCs, and generation of Th1/Th17 immune responses that is followed by classical activation of macrophages (50, 51, 119, 121–126). However, excessive inflammation and robust Th1/Th17 responses that provide sterilizing immunity can induce severe pathology and damage to the host (59, 127–133). It has been proposed that a tightly regulated combination of pro-inflammatory and anti-inflammatory stimuli is crucial for effective control of fungal infection (134–136). In fact, immunoregulatory mechanisms that control the intensity and duration of the host response are one of the main strategies that may provide tolerance to infection and maintain host fitness and homeostasis (32, 33, 39). Below we describe cellular and molecular mechanisms that could mediate host tolerance during infection with *Cryptococcus sp.*

T-regulatory cells (Treg): Mutations in the Treg-associated transcription factor forkhead box protein P3 (FOXP3) are associated with development of severe immunopathology in both mice and humans, indicating that Tregs control tissue damage and contribute to disease tolerance (32). During fungal infection, activation of Treg cells is one of the critical mechanisms for reducing collateral damage to host tissues and restoring a homeostatic environment (66). Treg function is associated with production of the anti-inflammatory cytokines IL-10 and TGF-β that suppress the immune response (66, 135). In BALB/c mice, pulmonary $CD4^+$ $FoxP3^+$ Tregs increased during the first 4 weeks of infection with *C. neoformans* 1841. Conditional depletion of Tregs during the second week of infection, while both Th1 and Th2 responses were in progress, enhanced the Th2 response and suggested that Tregs limit Th2 cell proliferation and function in this model of infection (137). Another study demonstrated that the accumulation of antigen-specific Tregs in the *Cryptococcus*-infected lungs and their co-localization with Th2 effector cells occurs through expression of CCR5 and IFN regulatory factor 4 (IRF4) (138). In both reports, the immunoregulatory function of Tregs during acute cryptococcal infection was associated with reduced pathological Th2 responses; however, the possibility that long-term persistence of cryptococcal infection is also associated with an increase in Treg function remains to be investigated (66, 89).

IL-10 signaling: IL-10 is an anti-inflammatory cytokine expressed by Tregs and DCs that prevents excessive inflammation by limiting the production of IL-1, IL-6, IL-23, IFN-γ, and TNF-α during fungal infections (66, 135, 139). Early and sustained IL-10 production by lung leukocytes was demonstrated in a mouse model of persistent lung infection with *C. neoformans* 52D (140). C57BL/6 mice with genetically engineered IL-10 deficiency that were infected with *C. neoformans* demonstrated improved fungal clearance from the lung in association with reduced tissue eosinophilia, decreased expression of Th2 (IL-4, IL-5, and IL-13) and increased expression of Th1 (IL-12 and TNF-alpha) cytokines by lung leukocytes (141). Early or late interruption of IL-10 signaling after establishment of cryptococcal infection reduced fungal burden and dissemination to the brain and was associated with enhanced Th1/Th17 responses and increased activation and recruitment of $CD11b^+$ DCs and exudate macrophages (140). In HIV+ patients with *C. neoformans* infection, a high level of IL-10 in the peripheral blood correlated with fungemia and dissemination (142). Therefore, the development of persistent or progressive cryptococcal infection appears to correlate with excessive IL-10 production while experimental IL-10 deficiency results in an enhanced inflammatory response (66).

DCs: Dendritic Cells (DCs) are the most efficient lineage for presentation of cryptococcal antigen to T cells and their activation is critical for activation of adaptive immunity that confers host protection. The role of DCs during cryptococcal infection has been recently reviewed (143, 144). The recruitment and maturation of DCs, as well as their ability to activate T cells, is affected by fungal characteristics as well as the local cytokine, chemokine, and scavenger receptor expression. Several soluble mediators including IL-4, IL-10, IL-17, and GM-CSF have been implicated in the recruitment, differentiation, and activation of DCs in different models of cryptococcal infection. Protection against *C. neoformans* is associated with recruitment and classical activation of monocyte-derived DCs (moDCs) resulting in secretion of pro-inflammatory cytokines and effective Th1/Th17 immune responses (145). Yet, moDCS are highly adaptable cells that can display inflammatory or immunoregulatory functions depending on the local cytokine microenvironment within infected tissues (66). For example, immunomodulatory or "tolerogenic" DCs can play an important role in regulation of inflammation and immunopathology through secretion of anti-inflammatory cytokines, induction of

hyporesponsiveness, and promotion of peripheral or induced Treg cells (146). Human and murine monocytes and DCs that were stimulated *in vitro* with *C. neoformans* antigen produced a significant amount of the immunomodulatory cytokine IL-10 (102, 147). In addition, development of immunomodulatory DCs in a murine model of persistent *C. neoformans* infection was associated with Th1 and Th17 suppression, reduced macrophage activation, and impaired fungal clearance (66, 140, 141).

Tryptophan pathway: Indoleamine 2,3-dioxygenase (IDO), a metabolic enzyme involved in tryptophan degradation and production of kynurenines, plays an important role in the balance between Tregs and Th1/Th17 cells (148, 149). Expression of IDO by DCs results in a tolerogenic phenotype that is associated with immune homeostasis, suppression of inflammation and effector T cells, induction of Tregs, and enhanced tolerance to fungal infection at mucosal surfaces (134, 135, 150, 151). The expression of IDO by host cells following cryptococcal infection has not been reported and could be a potential mechanism of disease tolerance.

Fas-associated death domain (FADD) and receptor interacting protein kinase 3 (RIPK3): The FADD protein is a key mediator of death receptor-triggered extrinsic apoptosis, which plays a crucial immune regulatory role at the site of infection and prevents excessive inflammation (127). Deletion of RIPK3 in combination with FADD led to a robust Th1-biased response with M1-biased macrophage activation, yet this host response was deleterious in a mouse model of cryptococcal infection. The excessive mortality in RIPK3 or RIPK3/FADD knockout mice was associated with significant pulmonary damage due to neutrophil-dominant infiltration with marked upregulation of pro-inflammatory cytokines. These findings demonstrate the role of both molecules in protection of the host by limiting excessive inflammation and conferring tolerance during cryptococcal infection (127).

T cell exhaustion: The loss of proliferation and limited effector function of T cells during states of chronic infection could be viewed as a tolerance-associated mechanism (152). Multiple pathways may mediate a state of T cell exhaustion; for example, binding of Cytotoxic T Lymphocyte-Associated Protein 4 (CTLA4) to co-stimulatory molecules CD80 and CD86 blocks CD28-mediated T cell co-stimulation and inhibits T cell activation and function (151). *C. neoformans* has been shown to rapidly induce CTLA-4 upregulation on murine $CD4^+$ T cells (153). Blockade of CTLA-4 on *C. neoformans*-stimulated $CD4^+$ T cells resulted in enhanced proliferation and IL-2/IFN-γ cytokine production. In addition, differential CTLA-4 upregulation was observed when cells were stimulated with an encapsulated strain of *C. neoformans*. In another study CTLA-4 blockade enhanced fungal control and survival of mice that were subsequently infected with highly virulent *C. neoformans* (154). These results indicate that the induction of CTLA-4 could be a mechanism used by cryptococci to diminish the immune response and facilitate persistent infection (66). Similarly, the contribution of the programmed cell death protein-1 (PD-1) during cryptococcal infection in C57BL/6 mice has been investigated (155). The results demonstrated an association between persistent infection and increased and sustained expression of PD-1 on $CD4^+$ T cells as well as upregulation of PD-1 ligands on specific subsets of resident and recruited DCs and macrophages. Furthermore, PD-1 blockade significantly improved pulmonary fungal clearance. Based on current data, the role of CTLA-4 and PD-1 as potential mediators of disease tolerance could be further studied, for example, in the context of cryptococcal IRIS. In conclusion, protective tolerance during persistent cryptococcal infection has been associated with the development of immunomodulatory/tolerogenic DCs and expression of IL-10, IDO, CTL4 and PD-1 (66).

CONCLUSION AND FUTURE DIRECTIONS

In states of persistent cryptococcal infection, a tightly-regulated balance between resistance and tolerance mechanisms is required to maintain host fitness and homeostasis. Several lines of evidence indicate that *C. neoformans* plays an important role in maintaining host tolerance to favor their own survival. The ability to survive within mammalian cells and to subvert or evade the host immune response without causing damage may be the inadvertent consequences of a long evolutionary path taken by this free environmental yeast to adapt to ecological selection pressures. Within the context of the damage response framework, infection of the host by a microbe is not a major concern in the absence of significant damage. Therefore, in latent cryptococcal infection one might postulate that the fungus is no longer considered to be a pathogen by the host immune system (47, 66).

Morbidity and mortality in cryptococcal infection can result from defective host resistance in advanced states of immunodeficiency, or a failure of tolerance mechanisms as observed during cIRIS. As the spectrum of hosts with cryptococcal disease expands, the ability to understand and distinguish tolerance-associated mechanisms from failures of host resistance will have important therapeutic implications. For example, bolstering immunity to further reduce pathogen burden may be unsuccessful in cases of defective tolerance with significant tissue and/or organ damage, while immunomodulation may be beneficial (31, 52, 156). Thus, a comprehensive therapeutic strategy that takes host resistance and tolerance mechanisms into account could have potential to significantly improve disease outcomes (157–159).

AUTHOR CONTRIBUTIONS

MS wrote and edited the manuscript. SQ edited the manuscript and revised it critically for important intellectual content.

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REVIEW
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Mycobacterial Evolution Intersects With Host Tolerance

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Over the past 200 years, tuberculosis (TB) has caused more deaths than any other infectious disease, likely infecting more people than it has at any other time in human history. *Mycobacterium tuberculosis* (*Mtb*), the etiologic agent of TB, is an obligate human pathogen that has evolved through the millennia to become an archetypal human-adapted pathogen. This review focuses on the evolutionary framework by which *Mtb* emerged as a specialized human pathogen and applies this perspective to the emergence of specific lineages that drive global TB burden. We consider how evolutionary pressures, including transmission dynamics, host tolerance, and human population patterns, may have shaped the evolution of diverse mycobacterial genomes.

Keywords: *Mycobacterium tuberculosis*, evolution, host tolerance, clinical phenotypes, mycobacteria, mycobacterial genomes

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INTRODUCTION

Tuberculosis (TB) is a critical health crisis in our modern world. TB is one of the top ten causes of death worldwide, killing an estimated 1.7 million people in 2017 (1). Despite years of coordinated global efforts to reduce the burden of TB, it is estimated that around 10 million new infections developed around the world in 2017 (1).

Mycobacterium tuberculosis (*Mtb*), the etiologic agent of TB, has evolved through the millennia to become a highly specialized obligate human pathogen. Indeed, some consider *Mtb* as the archetypal human-adapted pathogen (2). Unlike the non-pathogenic soil-dwellers and the opportunistically pathogenic species of mycobacteria, *Mtb* has no known environmental reservoir and does not survive outside of its human host. For its survival, *Mtb* has evolved to subvert and co-opt the very mechanisms the human immune system deploys to clear bacterial infections for its own advantage. However, the host is capable of limiting mycobacterial growth and, in some cases, inducing latency (3, 4), or sterilizing the infection (5, 6). Latent or subclinical disease provides mechanisms whereby *Mtb* can remain in the host and reactivate following immune suppression, transmitting to new hosts (7), although our previous understanding of the nature and significance of latent disease is now being rethought (8, 9). Nonetheless, this balance between host and pathogen is central to the evolutionary survival strategy of *Mtb* as an obligate human pathogen. Indeed, it is estimated that 90% of people that are infected by *Mtb* either contain or clear the infection (10). Yet the 10% of patients who develop active disease transmit *Mtb* to such a degree that one quarter of the world's population is estimated to have mounted an immune response to the pathogen (11). TB has caused over 1 billion deaths in the past 200 years, surpassing all other infectious diseases (12). In this review, we discuss the features of *Mtb* that were central to its emergence as a human pathogen and how genetic diversity among strains contributes to phenotypic diversity in disease presentation, with a focus on the evolutionary interplay between pathogen and host. Bacterial factors that engage the host promote bacterial growth, survival, and transmission in human populations. Yet, overall,

an evolutionary balance has been reached in which host mechanisms of containment and tolerance counteract many of these bacterial features.

The Origins of *Mtb*

The timing of events that contributed to *Mtb*'s specialized adaptation to human hosts remains a matter of debate. Some point to an early origin of *Mtb* ∼70,000 years ago (13, 14), while others have more conservative estimates of 35,000 years ago (15). Other studies suggest a more recent emergence of ∼6,000 years ago is most likely (16). These estimates are based on different assumptions and study materials, and have therefore led to a wide dispersion of estimates.

Most studies have employed inference methods based on DNA sequence among extant strains of *Mtb*. This method relies on the calibration of a molecular clock, which uses genetic distance as a measure of time since divergence (17). *Mtb* demonstrates a clonal population structure that can be divided into seven major lineages (**Figure 1**), and the divergence between these lineages and the other members of the *Mycobacterium tuberculosis* complex (MTBC) has been used by some to calibrate the molecular clock for *Mtb* (25).

Prior to the advent of widespread accessibility to whole genome sequencing, *Mtb*'s molecular clock was estimated using variable numbers of tandem-repeats (VNTR) in microsatellite-like loci (26). This method proposed an origin of the MTBC approximately 40,000 years ago, and highlighted the likelihood of *Mtb* dispersing throughout Africa and Eurasia via human migration (27). However, the use of VNTR in constructing phylogenies can lead to phylogenetic arrangements incongruent with known genetic relationships due to convergent evolution at these loci (28). Therefore, the current gold standard for calibrating a molecular clock is genome sequencing. However, as demonstrated below, the method by which *Mtb's* molecular clock is calibrated will have a significant impact on the resulting estimates.

Multiple studies have employed genome sequencing to determine the molecular clock of *Mtb* and have arrived at vastly different estimates for the age of *Mtb*. The calibration of the molecular clock underlies these differences. Comas et al. estimate *Mtb*'s origins as far back as 70,000 years ago (13). This estimate is based on the parallels of mitochondrial DNA (mtDNA) haplogroups and the lineages of *Mtb* that are most commonly found among the corresponding human populations, and then calibrating the molecular clock using key events in human evolution reflected by mtDNA. This generated an estimated mutation rate in *Mtb* of 2.58×10^{-9} substitutions/site/year, which is low compared to estimates derived from contemporary outbreaks (1.1×10^{-7} substitutions/site/year) (29). However, their estimates produced multiple time points for *Mtb*'s emergence, and 70,000 years was chosen as the most likely. The researchers who put forth this hypothesis on the origin of *Mtb* had previously published work proposing the dispersal of *Mtb* via human migration out of Africa (14). While the phylogeographic distribution of the major lineages of *Mtb* coincide with concordant patterns in human migration (25), calibrating *Mtb's* molecular clock based on these patterns to determine when *Mtb* emerged presupposes its own hypothesis that *Mtb* emerged with modern humans.

Others have challenged this hypothesis and proposed a much later time frame for *Mtb*'s emergence (30). Instead of mtDNA, Pepperell et al. based their estimates on historical samples of MTBC strains and determined that the emergence of the most basal species of *Mtb*, *M. africanum*, occurred approximately 2,200 years ago. The most recently evolved strains of *Mtb,* those among the so-called "modern" lineages, are estimated to have arisen ∼1,300 years ago. The estimated mutation rate of *Mtb* from this study (1.3×10^{-7} substitutions/site/year) was significantly higher than that of Comas et al. Furthermore, based on this early estimate for the origin of *Mtb*, Pepperell et al. propose the estimates for human population divergence do not correlate with the divergence of the *Mtb* lineages, and therefore did not disperse concurrently (30). Another study has put forth an origin estimate similar to that of Pepperell et al. The mummified remains of human samples from Peru dated between AD 1028 and AD 1280 demonstrated skeletal lesions indicative of TB (31–33). Sequenced ancient DNA (aDNA) from these samples revealed disease was caused by *M. pinipedii*, a member of the *Mycobacterium tuberculosis* complex (MTBC) that primarily infects seals (16). Comparing the aDNA against a current strain of *M. pinipedii* generated an estimate of MTBC's emergence occurring 6,000 years ago, with a mutation rate intermediate to the estimates of Comas et al. and Pepperell et al. (4.6×10^{-8} substitutions/site/year). However, the reliance on aDNA comes with the caveat that post-mortem DNA decays due to physical and chemical damage, leading to strand breakage and the hydrolytic deamination of cytosine to uracil (34). Therefore, additional bioinformatic corrections must be implemented to sort out decay artifacts, leading to the possibility of erroneous or missed variant calls in aDNA samples.

The variety of conclusions from these studies demonstrates that the calibration of the molecular clock is critical to the resulting estimates, and raises the question as to how well-suited *Mtb* is for molecular clock estimations. The application of molecular clocks relies on satisfying certain assumptions that could be problematic when applied to *Mtb*: namely, a constant mutation rate through time and the broad applicability of this rate across lineages (17). It is not at all clear that the mutation rate of *Mtb* is stable over evolutionary time, as no study has been able to collect longitudinal data from historical samples. Additionally, the health status of human hosts across space and time is highly variable, creating different pressures on the infecting strains. Furthermore, even among the extant lineages of *Mtb*, which are much more closely related to each other than they are to other members of the MTBC, variable mutation rates have been observed (35). A recent analysis highlights the complexities, uncertainties, and limitations of different methods used to calibrate an *Mtb* molecular clock (36).

The earliest claim of mycobacterial disease comes from a 500,000 year old fossil of *Homo erectus*, which demonstrated lesions characteristic of mycobacterial infection (37). As no ancient DNA (aDNA) was recovered from this sample, it is impossible to determine what species of mycobacteria might have caused the lesions. Using lipid profiles unique to pathogenic

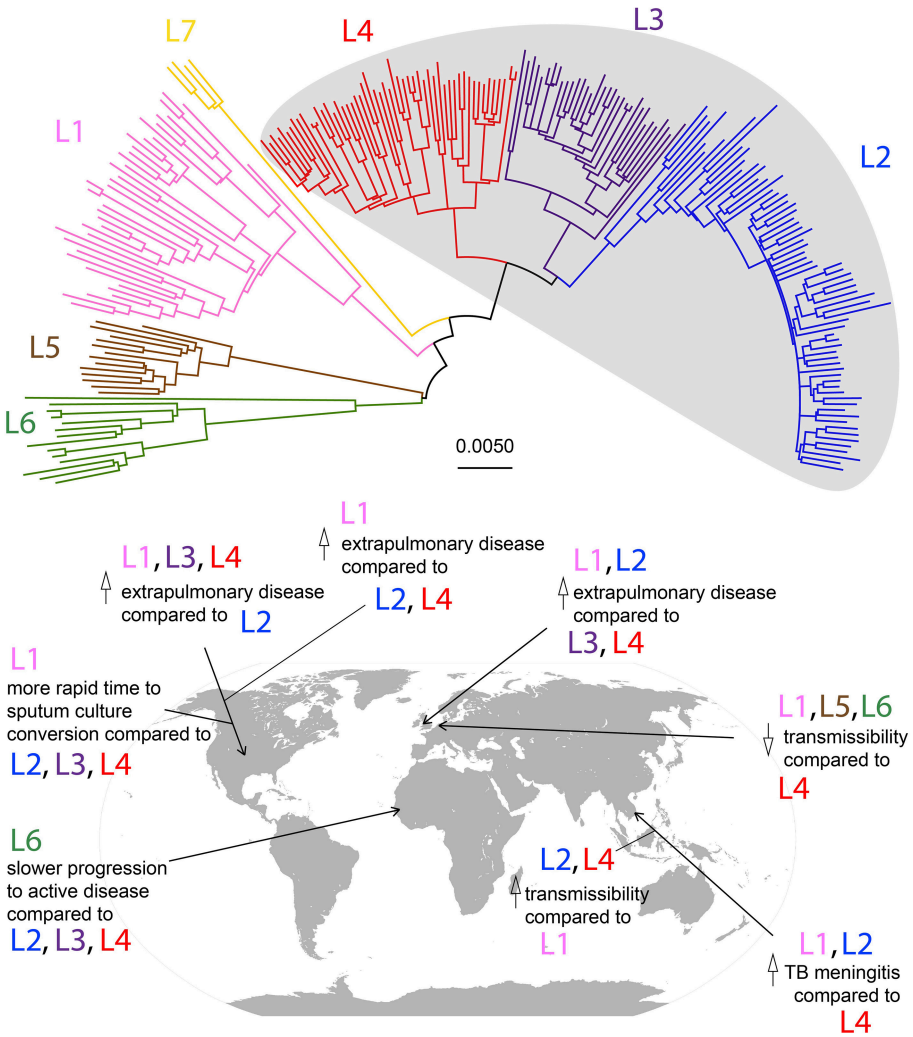

FIGURE 1 | Phylogeny of *Mtb* lineages and geographic associations of disease characteristics. Neighbor-joining phylogeny based on 35,787 SNPs among 225 strains from Comas et al. (13). Lineages are color-coded according to the scheme described in Bos et al. (16), and modern lineages are shaded in gray. Scale bar represents relative number of substitutions per known variant. Disease characteristic associations with *Mtb* lineages in geographic locations by studies described in **Table 1** are marked on a world map.

mycobacteria and the *IS6110* insertion element (38), a feature found only in members of the MTBC (39), the oldest confirmed sample of mycobacterial disease was found in bovid fossils in North America, dating back approximately 17,000 years (40, 41). The earliest known association of the MTBC with humans comes from Atlit-Yam, Israel, dating back 9,000 years (42). Interestingly, this sample bears the TbD1 marker, a genomic deletion found exclusively in the evolutionarily "modern" lineages of *Mtb* (43). Linking definitive archaeological findings with aDNA sequencing will provide the most compelling evidence to settle the divergent estimates. As the techniques for collecting and sequencing aDNA continue to advance, our insight into *Mtb*'s origins will similarly improve, and we may better understand the evolutionary forces and constraints leading to modern *Mtb* and the nature of its interactions with its hosts.

The Evolution of *Mycobacterium tuberculosis* as a Specialized Pathogen

Mycobacteria range from environmental, non-pathogenic species, to opportunistic pathogens that infect immune-compromised hosts, to professional pathogens. The vast majority of *Mycobacteria* are non-pathogenic in nature. Comparative genomic studies have revealed the evolutionary trajectory to pathogenicity, in which environmental mycobacteria acquired virulence loci and became opportunists, and opportunists adapted to their host environments to become professional pathogens. The pathogenic species include but are not limited to: *Mycobacterium ulcerans* (the agent of Buruli ulcer), *Mycobacterium leprae* (leprosy), *Mycobacterium marinum*, *Mycobacterium canetti,* and the range of species that make up the MTBC. The MTBC contains the closely related species of

pathogenic mycobacteria that, together, cause the vast majority of TB. Several of these species are animal-adapted strains that cause disease across a range of mammalian species. These include *Mycobacterium bovis* (infecting cows), *Mycobacterium caprae* (goats and sheep), *Mycobacterium pinipedii* (seals and sea lions), *Mycobacterium microti* (voles), and *Mycobacterium orygis* (oryxes) (44, 45). *Mtb* and *Mycobacterium africanum* cause the majority of disease in humans. Among all of these pathogenic mycobacteria, *M. tuberculosis sensu stricto* has emerged as the most prevalent mycobacterial species and one of the most historically successful human pathogens. The key features and events that underlie the adaptation of mycobacteria into a specialized pathogen are discussed below and have also been highlighted in previous reviews [e.g., (2)].

From the Environment to New Hosts

The soil-dwelling mycobacteria *Mycobacterium kansasii* is an environmental, opportunistic mycobacterial pathogen closely related to the MTBC. This genetic relationship provides insight into the late-stage events conferring *Mtb*'s specialized adaptation that allowed it to expand and persist as an obligate human pathogen. Unlike the nonpathogenic mycobacterial species, *M. kansasii* contains an array of virulence determinants for host adaptation. There are five ESX loci in *Mtb*, and all five are present in *M. kansasii* (46). Furthermore, *M. kansasii* has expanded its set of PE/PPE proteins and, in fact, encodes a greater number of PE/PPE proteins than *Mtb* and other members of the MTBC. Despite these similarities *M. kansasii* is only rarely found in patients, whereas *Mtb* infection in humans is prevalent (47, 48). Therefore, the ESX secretion systems and its effectors are not sufficient to explain the pathogenicity of *Mtb*. Given the shared virulence features of *M. kansasii* with *Mtb* but their vastly different impact on global health, what other features separate *Mtb* from *M. kansasii*?

The enhanced virulence of *Mtb* may have been the result of acquiring pathogenicity islands via horizontal gene transfer (HGT) (49–52). Comparative genomics reveals the presence of 55 genes in *Mtb* absent from *M. kansasii* (51). The majority of these genes contain an unusual GC content for mycobacteria and appear in clusters flanked by the vehicles that provide mechanisms for HGT (mycobacteriophage genes, transposons, and toxin-antitoxin systems). Notably, some of these HGT-acquired genes, encoding factors responsible for cell adhesion (53), arresting phagosome maturation (54, 55), the production of PGLs that function in oxidative stress resistance (56) and modulation of the host immune system (57) have been implicated in *Mtb*'s adaptation to survival within a host (55, 58).

Mycobacterial species comprising the "smooth tubercle bacilli" (STB) are thought to be an evolutionary bridge between the environmental opportunistic species, such as *M. kansasii,* to the pathogenic MTBC (46). Unlike the MTBC, genome sequencing reveals that *M. canetti* demonstrates a non-clonal population structure with >60,000 SNPs separating some strains (50). While the environmental reservoir of *M. canetti* remains unknown, cases are highly geographically restricted and arise predominantly in patients who have some form of contact with East-Africa (59). Like *M. kansasii, M. canetti* harbors compelling signatures of HGT in its genome (60, 61). Boritsch et al. offered conclusive experimental evidence that HGT occurs in *M. canetti,* finding the transfer of DNA fragments as large as 117.6 kilobase pairs (kbp) (62). Like *M. canetti*, the most basal lineages in the MTBC, including L5, L6, and L7, are also strongly geographically restricted to Africa (14, 63, 64). These observations and experiments support a scenario in which an *M. canetti*-like species of mycobacteria in Africa acquired virulence loci via HGT, thus giving rise to the pathogenic progenitor of the MTBC.

The role of ongoing HGT in *Mtb*, however, remains controversial. Most evidence suggests that *Mtb* demonstrates clonal evolution without ongoing recombination events. In the same experiments in which HGT was detected in *M. canetti,* HGT could not be detected among MTBC species (62). The lack of ongoing HGT in the MTBC is supported by the congruence of phylogenetic trees based on a variety of molecular markers (65–67), stable G+C content across the majority of the genome (68), a low frequency of homoplasic mutations (14, 28), and that all known drug-resistance factors arise via *de novo* mutation (69). The mechanism by which *Mtb* lost capacity for ongoing genetic recombination, however, remains unknown. Together, this evidence provides strong support for the role of HGT as a critical component in the emergence of *Mtb,* and that subsequently *Mtb* appears to have lost significant capacity for genetic recombination and evolved in a clonal fashion.

Genetic and Phenotypic Diversity in *Mtb*

Mtb is an obligate human pathogen and has no known environmental reservoir. As such, its population structure is largely isomorphic to its human host population. Despite the clonal evolution of *Mtb*, significant genetic variation exists and based on this it is divided into seven major lineages. These lineages can be grouped into evolutionarily "ancient" and "modern" lineages, with the TbD1 deletion serving as a genetic marker separating the two groups (43). The ancient lineages (L1, L5, L6, L7) demonstrate a high degree of geographic constraint (14, 63, 64), whereas the more recently evolved modern lineages (L2, L3, L4) are found more broadly throughout the world (70). L1 predominantly circulates in Southeast Asia, L5 and L6 in West Africa, and L7 in the Horn of Africa. L2 is strongly associated with an East Asian origin (71), but also causes significant disease burden in Eurasia, South Africa, and Peru. Over the past 200 years, the population size of L2 strains has dramatically increased, and can be found in most countries throughout the world (72). L3 strains circulate mostly in India and Central Asia. L4 strains cause the most global disease and are the most widely distributed among the *Mtb* lineages (73). Interestingly, discrete sublineages within L4 differ in their geographic distribution, suggesting that some L4 strains are more capable of spreading to new host populations (74).

The genetic lineages of *Mtb* were first defined by lineage-specific deletions, referred to as large sequence polymorphisms (LSPs) (25). Due to the extreme rarity of ongoing horizontal gene transfer (HGT) among species of the MTBC, these markers are thought to be largely irreversible and well-suited to lineage

classification (73). Single nucleotide polymorphisms (SNPs) are also phylogenetically informative in *Mtb* due to the lack of ongoing HGT, and help to increase the resolution of relationships among strains within a lineage (75–77). From the application of these markers in constructing the phylogenetic relationships among *Mtb* lineages, it has become clear that the ancestral lineages separate into distinct phylogenetic groups, and are thus paraphyletic in nature. The modern lineages, conversely, are more closely related and share a more recent common ancestor (i.e., monophyletic) than the ancient lineages are with one another. These lineages have evolved independently in separate human populations, resulting in distinct induction of inflammatory phenotypes (78, 79) and differential modulation of innate immune signaling (80). Furthermore, the variable geographic distribution and disease burden of the different lineages raises the question as to how the existing variation among *Mtb* strains contributes to disease phenotype, and whether this variation explains the uneven distribution of *Mtb*'s lineages.

Phenotypic Diversity Among *Mtb* Lineages

Strain variation in disease severity, transmission potential, and resistance to drug therapy is of significant interest to global health. Identifying virulent and/or drug-resistant clones informs current and future treatment. Numerous studies have investigated the phenotypes associated with the different lineages and strains of *Mtb*. By the mid-20th century, TB research had begun to investigate virulence traits among clinical and reference strains of *Mtb* (81, 82). The first attempts to correlate virulence with strain background via typing techniques, however, did not occur until 1978 (83). In a landmark study, Valway et al. utilized *IS6110* typing patterns to identify a strain associated with a particularly virulent outbreak (84). The outbreak was characterized by extensive transmission among patients, and the researchers correlated a significant increase in *in vivo* replication as a potential underlying cause using a mouse infection model. Following the adoption of the restriction fragment length polymorphism (RFLP) typing technique (85) to describe the population structure of *Mtb*, strains originating in China and Mongolia, the so-called "Beijing" strains (now known as L2), demonstrated increased replication in cell culture and mouse models in addition to increased mortality *in vivo* (86, 87). In a rabbit infection model, L2 strains rapidly disseminated to extrapulmonary sites resulting in severe meningeal disease presentation (88). However, we should exercise caution when applying strain-specific characteristics broadly across its genetic lineage, as infection phenotypes can vary widely among strains from the same lineage (79, 89). Correspondingly, L2 strains demonstrate variable virulence patterns. The most recently evolved L2 strains, those comprising the so-called "modern Beijing" sublineage, exhibit increased virulence compared to the ancestral strains (90). These and earlier studies (91, 92) focused attention on the apparent increased virulence of the L2 strains, and their impact on the immune response was identified as an avenue of future research.

Mtb Lineages and Disease Presentation

Transmission of *Mtb* depends on disease within pulmonary tissue in human hosts. Given its status as an obligate human pathogen, there are no environmental reservoirs for *Mtb* to transmit from, and extrapulmonary sites do not afford transmission. This leads to the question: Do particular *Mtb* lineages demonstrate variable disease presentations? Are more transmissible strains less often be associated with non-transmissible disease sites, i.e., extrapulmonary tissues? In a marmoset model of infection, a strain from the ancient L6 group was found to develop lower bacterial load in pulmonary tissue compared to modern strains from L2 and L4, but disseminated to extrapulmonary sites more compared to L4 (93). Interestingly, the L2 strain demonstrated the highest burden in all organs assayed, effectively replicating within the lung and disseminating to extrapulmonary sites. This study suggests the modern strains are more capable of transmitting by establishing pulmonary disease, but L2 also spreads effectively to extrapulmonary sites. Based on the characteristics of infection, it is possible that the L2 and L6 strains disseminated to other tissues by different mechanisms, where L2's dissemination was a byproduct of increased overall virulence as described in the preceding sections. While this study offers novel visualization methods to assess disease progression of diverse tuberculosis lineages in a primate infection model, it is not clear how generalizable these phenotypes are across these lineages.

There are few studies that have compared patterns of disease presentation among a diverse range of strains from more than two lineages in a large sample population (summarized in **Figure 1** and **Table 1**). Even in these, associations between lineage and disease presentation have been variable, and the comparisons differ. In the United States, L1, L3, and L4 strains were more likely to cause extrapulmonary disease compared to strains from L2 (20). In Vietnam, L1 and L2 strains were associated with TB meningitis compared to L4 strains (18). In the UK, L1 and L2 were associated with increased likelihood of exclusively extrapulmonary disease compared to L3 and L4 (22). Aside from site of disease, characteristics such as time to sputum culture conversion and transmissibility differ between lineages as well. In the United States, L1 strains demonstrate a more rapid time to sputum culture conversion compared to strains from the modern lineages (L2, L3, and L4) (21). Additionally, in Gambia, L6 strains progressed to active disease at a significantly lower rate compared to strains from the modern lineages, but displayed no differences in transmissibility (19). However, in the Netherlands, ancient strains (L1, L5, and L6) demonstrated reduced transmissibility compared to L4 strains (23). In Florida, L1 strains were associated with higher rates of extrapulmonary disease compared to L2 and L4 strains (24). Together, these studies indicate that significant differences exist in disease presentation among the different lineages of *Mtb* (particularly between ancient and modern strains), and these patterns can be observed experimentally and in human populations.

Bacterial Determinants of Virulence

Mtb lineages show varying geographic distribution patterns with ancient lineages being geographically restricted in comparison to the modern lineages. Several factors like population density,

TABLE 1 | Studies investigating multiple *Mtb* lineages and their associations with disease characteristics.

Geographic location	Lineages under study	Strain typing method	Lineage associations	References
Vietnam	L1, L2, L4	IS*6110* RFLP, spoligotyping, MIRU-VNTR, & LSP	L1 and L2 cases higher odds of TB meningitis compared to L4	(18)
Gambia	L2, L4, L6	LSP	L6 infections less likely to progress to active disease compared to L2 and L4	(19)
USA	L1, L2, L3, L4	Spoligotyping & MIRU-VNTR	L1, L3, L4 cases higher odds of extrapulmonary tuberculosis compared to L2	(20)
USA	L1, L2, L3, L4	Spoligotyping & MIRU-VNTR	L1 more rapid time to positive sputum culture conversion	(21)
United Kingdom	L1, L2, L3, L4	MIRU-VNTR	L1 and L2 increased likelihood of exclusively extrapulmonary disease compared to L3 and L4	(22)
Netherlands	L1, L2, L3, L4, L5, L6	RFLP and MIRU-VNTR	L1, L5/L6 reduced transmission compared to L4	(23)
USA	L1, L2, L3, L4	Spoligotyping & MIRU-VNTR	L1 higher odds of extrapulmonary disease compared to L2 and L4	(24)

RFLP, Restriction Fragment Length Polymorphism; MIRU-VNTR, Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat; LSP, Large Sequence Polymorphism.

migration pattern, economic and health conditions, and more recently the HIV/AIDS pandemic and emergence of MDR strains could influence this distribution (94). However, bacterial genetic variation within and between each lineage may reflect evolutionary history and pressures.

Cell Envelope-Associated Lipids

As a pathogen, *Mtb* must interface with its host, and the mycobacterial cell envelope makes first contact. The mycobacterial cell envelope is a complex multi-layered structure containing the plasma membrane, cell wall skeleton, mycomembrane and a capsule (95–97). It contains several lipids unique to pathogenic mycobacteria which contributes to their *in vivo* survival by modulating the host immune response, and have been the subject of more comprehensive reviews [e.g. (98)]. These include mannose capped lipoarabinomannan (ManLAM), phenolic glycolipid (PGL) and phthiocerol dimycocerosate (PDIM) (99–101). These features are highlighted in **Figure 2**.

Variations in these components among different strains and lineages may correspond to discrete evolutionary trajectories. For example, variation in ManLAM has been observed in clinical strains leading to altered virulence (102, 103). A subset of lineage 2 strains with truncated and more branched forms of ManLAM exhibited defects in phagocytosis by primary human macrophages when compared to lineage 4 reference strains (103).

Variations in PDIM, PGL and other lipids may also contribute to disease progression. PDIM can neutralize oxidative and nitrosative free radicals and has been proposed to play a role in protecting *Mtb* from these stress causing agents (104, 105). Further, PDIM may also have a role in immune evasion by masking cell wall pathogen-associated molecular patterns (PAMPs) (57), and also is required for proper secretion of ESX-1 substrates (106). Among the modern lineages, L2 strains but not L4 strains produce the phenolic glycolipid PGL, which may play an important role in promoting their virulence and transmission (107). In mycobacterium-infected macrophages, PGL induces the production of chemokine CCL2 which recruits monocytes to the site of infection. This facilitates mycobacterial escape from bactericidal macrophages to permissive monocytes (108). A point mutation in *Rv2952* encoding the S-adenosylmethionine-dependent methyltransferase in Beijing strains resulted in structural variations in PDIM and PGL compared to other lineage strains (109). As noted above, a deletion in the *pks1/15* locus encoding a polyketide synthase in L4 strains leads to defective production of PGL (110). These lipids can also inhibit the production or secretion of proinflammatory cytokines by the host leading to the establishment of infection (105, 107, 111, 112).

The abundant cell wall lipid trehalose dimycolate (TDM) plays multiple roles in pathogenesis (113–118). Specific cyclopropane modifications to the mycolic acids that comprise TDM are associated with pathogenic mycobacteria, but not with non-pathogenic species; PcaA-mediated modification of TDM modulates the host immune response to mycobacterial infection (119, 120). This cyclopropanated TDM plays an important role in inducing or accelerating host angiogenesis around the mycobacterial granuloma, a response that helps to support bacterial growth during early infection (121–123). Thus, intricately structured and complex lipid species provide important host modulatory activities and may be important substrates for evolution. Notably, lineage-specific differences in cytokine induction upon exposure of macrophages to lipid extracts from different lineages have been reported (78).

Type VII Secretion Systems

The ESAT-6 secretion (ESX/Type VII) systems and their secretion substrates are key features that contribute to the pathogenicity of *Mtb* (124). The ESX secretion systems were discovered after genomic analysis of the *M. bovis* BCG vaccine

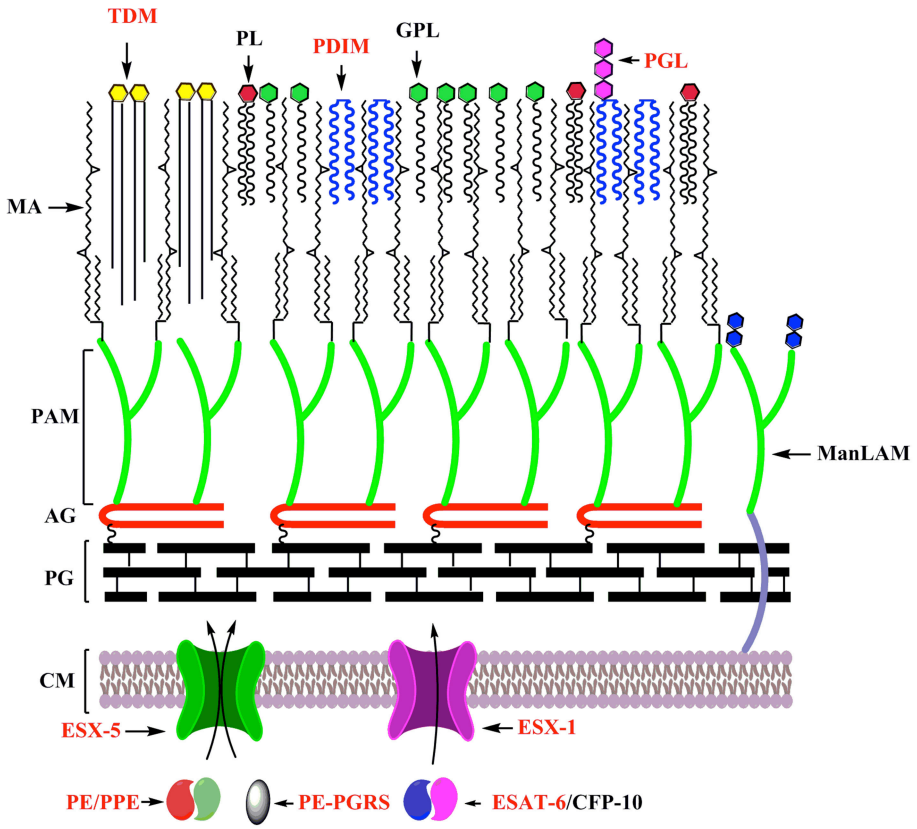

FIGURE 2 | Key features underlying the adaptation of mycobacteria as specialized pathogens: Cell envelope of mycobacteria with factors playing distinct roles in its adaptation as a specialized pathogens labeled in red. CM, Cell membrane; PG, Peptidoglycan; AG, Arabinogalactan; PAM, Penta arabinosyl motif; MA, Mycolic acids; TDM, Trehalose dimycolate; PL, Phospholipids; PDIM, Pthiocerol dimycocerosate; GPL, Glycopeptidolipids; PGL, Phenolic glycolipids; ManLAM, Mannose capped lipoarabinomannan.

strain revealed a large deletion [Region of Difference (RD) 1] that interrupted the ESX-1 system (125). This system was lost in *M. bovis* following 11-year serial culture by Calmette and Guerin in the pursuit of a TB vaccine. The absence of this system was subsequently shown to account for a significant share of BCG's attenuation, and much attention has been paid to the role of this and other ESX systems and their secreted substrates on *Mtb*'s virulence (126, 127).

ESX secretion systems are encoded in clusters throughout mycobacterial genomes. *Mtb* contains five ESX loci, which have expanded through gene duplication, diversification, and insertions of the ancestral ESX-4 locus (128). These clusters share six core genes encoding: three ESX conserved-components (EccB, EccC, EccD), a mycosin (MycP), and two small, secreted Esx proteins. Besides the most ancestral ESX-4 locus, the ESX clusters also encode genes for PE, PPE, EccA, EccE, and ESX-1-specific component (Esp) proteins. The *esp* genes are not specific to ESX-1, but they are most abundant in that system. Orthologs of ESX-4 can be found among mycobacterial and non-mycobacterial species in the phylum Actinobacteria (128, 129). ESX-4 is the simplest gene cluster among the ESX secretion systems, containing only seven genes. ESX-4 encodes the FtsK/SpoIIIE protein EccC4, the WXG proteins EsxU and EsxT, the conserved ESX core components EccB4 and EccD4, the mycosin protease MycP4, and the hypothetical valine and alanine rich protein Rv3446c.

The components of the ESX systems can be divided into cytosolic, membrane bound, and secreted proteins. EspG and EccA function in the cytosol. EspG is found in all ESX clusters besides ESX-4, and is thought to function as a specific chaperone for PE and PPE proteins (130–132). EccA is an AAA+ family (ATPase associated with various cellular activities) protein that is thought to form a hexamer and functions in the secretion of Esx and PE-PPE proteins (133–136). The conserved membrane components of ESX secretion systems (EccB, EccC, EccD, EccE, and MycP) are essential for secretion in all of the studied loci (137–141). These proteins contain large hydrophilic domains in either the N- or C-terminus and a range of transmembrane domains. EccB, EccC, EccD, and EccE are thought to form the transport channel through which the ESX substrates are transported across the inner membrane. EccB, EccC, EccD, and EccE form a stable membrane complex of ∼1,500 kDa that can be co-immunoprecipitated (139). MycP, a mycosin, is a subtilisin-like protease containing a C-terminal transmembrane domain that tethers the protein on the cell membrane (142, 143). Its role in secretion remains unknown.

The components described thus far have been localized to the inner membrane. The inner membrane, however, is surrounded by a thick, lipid-rich cell wall (also referred to as the outer membrane or mycomembrane) in addition to another thick capsular layer [reviewed in (144)]. How ESX substrates are exported beyond these structural boundaries has been a mystery. Recently, Lou et al. discovered that EspC forms a long filamentous structure that localizes to the cell membrane, and its expression is required for secretion of EsxA (145).

The conserved secreted effectors of ESX systems are comprised of Esx and PE/PPE proteins (the latter is described in more detail in the following section). The Esx proteins are also referred to as WxG100 proteins due to a conserved tryptophan-X-glycine motif that causes a turn between two helical domains in the ∼100 amino acid proteins (146). The most well-studied Esx proteins are EsxA and EsxB, encoded within the ESX-1 locus. ESX-1, the prototypical ESX secretion system in tuberculosis research, has been demonstrated to be essential for the intracellular survival of *Mtb* due to its critical role in host-pathogen interaction during *Mtb* infection via secretion of its substrates, many of which are secreted in a codependent manner (147). EsxA and EsxB are secreted as antiparallel heterodimers (148, 149) via recognition of an ESX secretion signal on the C-terminus of EsxB (150). EsxA has long-been associated as a cytolytic virulence factor of *Mtb* (126, 135, 151, 152). Experiments demonstrating recombinant EsxA could induce its cytolytic effect in the absence of infection led to the notion that EsxA was primarily responsible for ESX-1's pathogenicity (152, 153). However, recent work has definitively demonstrated that the cytolytic effect of recombinant EsxA was due to a residual detergent in the extract (154). Therefore, the cytolytic effect is dependent on other factors dependent on *Mtb*'s ESX-1 secretion system.

ESX-1 has been ascribed numerous roles in *Mtb*'s pathogenesis. As previously mentioned, ESX-1 is required for membrane disruptions in its host cell, allowing *Mtb* to escape from the phagosome and enter the cytosol whereupon necrosis-like cell death is induced (155–157). While EsxA has been shown to be insufficient to induce membrane disruptions, this process is dependent on its presence and secretion (154). EspB, which is encoded outside of the ESX-1 locus and depends on secretion of EsxA and EsxB for its own secretion, forms a ring-shaped heptamer with a hydrophobic domain, suggesting the possibility that it could be involved in membrane disruption via EsxA and EsxB (158). EsxA has been shown to induce expression of matrix metalloproteinase-9 (MMP9), which recruits additional phagocytes to the site of infection and facilitates its spread to new cells (156). The recurrent recruitment of additional leukocytes to take up the apoptotic debris of the former round of infected macrophages amplifies the bacterial population in successive waves and leads to the formation of the tuberculosis granuloma (159).

The regulation of ESX-1 differs among MTBC species, perhaps contributing to distinct infection phenotypes among lineages. The PhoPR regulon, a two-component regulation system, regulates the production and secretion of, among other things, EsxA and EsxB (160), and is central to the pathogenesis of *Mtb* (161). Strains from L5, L6 and the animal-adapted species all contain a missense mutation in *phoR* that downregulates the PhoPR system when genetically transferred into L2 and L4 strains (162). Intriguingly, Gonzalo-Asensio et al. noted that there were no significant differences in the production of proteins induced by PhoPR in the L5, L6 and animal-adapted species compared to L2 and L4, and that a deletion found only in the former rescued the defect. The authors went on to show that an outbreak of an unusually virulent strain of *M. bovis* that was transmitting among humans was associated with the insertion of an *IS6110* sequence upstream of *phoP*, serving as a promoter to increase the expression of the PhoPR regulon (162).

The pathogenic species of mycobacteria possess two additional ESX secretion systems, ESX-2 and ESX-5, that are not found in the rapid-growing, non-pathogenic mycobacteria (128, 163). The duplication of these systems in pathogenic mycobacteria is linked to the expansion of the PE and PPE gene families (163). The PE and PPE proteins, the other core substrates of ESX secretion systems, and their role in pathogenesis are discussed below.

PE/PPE Family Proteins

Initial sequencing of the *Mtb* genome led to a surprise finding that 10% of its genes code for a unique family of proteins with signature proline-glutamate and proline-proline-glutamate residues conserved at their N-termini, linked to a variable C-terminus. Due to their variable C-termini, initially they were thought to be a source of antigenic variation to evade host immune system (68). The *pe*/*ppe* genes have greatly expanded in the pathogenic species of mycobacteria and have been critical for host adaptation (164, 165). This family of proteins are thought to help in *Mtb* survival and dissemination through diverse modes. This includes upregulation of anti-inflammatory cytokine levels (166), induction of apoptosis in macrophages (167) and increased secretion of chemokine MCP-1 (168). They also interact with TLR-2, leading to macrophage activation, promote apoptosis and necrosis in host cells (164). PE-PGRS a subfamily of PE family is unique to MTBC and related species (165). Mutations in their corresponding genes have been associated with impaired replication and decreased persistence in the host indicating a direct role for this class of genes in virulence (169). The "modern" Beijing strains from L2 have been demonstrated to harbor a deletion affecting *ppe38,* a consequential mutation that increases the virulence of affected strains (170). The authors found that the absence of *ppe38* inhibits the secretion of a large number of PPE_PGRS and PPE_MPTR (major polymorphic tandem repeats) substrates through ESX-5, and postulate that this mutation played a significant role in the global spread of the "modern" Beijing L2 strains. Thus, variation in these gene classes may contribute to the degree of virulence, transmissibility, and evolutionary success for mycobacterial species and strains within discrete hosts and genetic backgrounds.

Mycobacterial Genetic Diversity and Its Intersection With Host Tolerance

Variation in mycobacterial lipids, ESX secretion systems and their effectors among the genetic lineages and sublineages of *Mtb* intersect with the nature of the host response to mycobacterial

infection. Evidence from experimental infection models suggests that different *Mtb* lineages exhibit diverse growth phenotypes and elicit variable host immune responses. Hence in addition to these factors, the role of variable host tolerance among these lineages in shaping their diversity may be important. Some of the first evidence supporting this argument came from aerosol infections in mice with *Mtb* strains CDC1551, HN878, and HN60. CDC1551 belongs to lineage 4 whereas HN878 and HN60 belong to lineage 2. Mice infected with HN878 and HN60 succumbed earlier. This observation correlated with the cytokine profiles of CDC1551 infected mice which showed increased production of pro inflammatory cytokines TNF-α, IL-12, and IFN-γ in comparison to HN878 and HN60 infected mice (91). Moreover, strains from the modern lineages 2, 3, and 4 induced significantly lower levels of pro inflammatory cytokines than ancient lineages in a human monocyte-derived macrophages infection model (79).

Mtb sublineages too exhibit significant differences in virulence and immune modulatory functions. The M—Strain, a highly prevalent strain in Argentina belonging to the Haarlem family of Lineage 4 failed to induce PMN apoptosis and ROS production as opposed to the LAM family of the same lineage (171). Collectively these findings may help explain the emergence and evolutionary success of the modern lineages.

Recent work on tolerance in animal models of TB suggests that specific host factors can contribute differentially to bacterial restriction and host tolerance. For example, Phox-deficient mice are not compromised for resistance to infection but do display tolerance defects (172–174). Similarly, previous work in the zebrafish model of mycobacterial infection suggested that, in addition to overall bacterial load, inflammatory state influences disease outcome (175, 176). Thus, the degree of host tolerance to infection has important consequences to host survival, bacterial burden, and presumably transmission; indeed the majority of humans who do not manifest active disease upon exposure to *Mtb* suggests a high level of tolerance to infection (177). Reciprocally, how variation within distinct bacterial lineages and strains influences inflammation, tolerance, pathogenesis, and ultimately successful transmission, may determine the evolutionary trajectories of both pathogen and host.

A number of examples exist in which bacterial-host interactions appear to be specific to lineage. For example Lineage 2 mediated TB has been shown to be associated with C allele of *TLR-2*—T597C, and *NRAMP1*—D543N polymorphisms (18, 178). The−261TT variant in the Immunity-related GTPase Family M (IRGM) confers defense against pathogens including Lineage 4 *Mtb* which lacks *pks1/15,* but is not associated with *M. africanum* mediated TB. This gene is associated with PGL biosynthesis highlighting a potential role of the lipid in inhibiting IRGM mediated autophagy (179). Lineage 4 contains both ubiquitous (presumed to be generalist) and specialized (geographically restricted) sublineages, suggesting that at least some *Mtb* strains may have specialized to specific host populations (74). More recently, a large study in a Vietnamese population identified increased transmission of Lineage 2 Beijing strains between individuals than endemic strains, consistent with previous studies of transmission of Beijing strains in other regions (180–182). These studies underscore the need for further research that integrates data on *Mtb* strains and lineages with human genotypes to understand how this intersection contributes to the clinical outcome of *Mtb* infection. Ongoing studies with larger cohorts and deeper descriptions of clinical phenotypes should provide additional insight into these interactions.

Mtb genetic diversity and evolution may reflect the genetic arms race between successful pathogen and its host, leading to reciprocal genetic changes. There is newfound appreciation that host tolerance to mycobacterial infection is an important component of this interplay, contributing to disease trajectory and transmission patterns. Thus, genetic variation in aspects of host tolerance—generated through both bacterial and host mechanisms—is another important consideration in understanding the complex interactions between host and pathogen that have evolved during the long association between *Mtb* and its human hosts.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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