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Role of tissue protection in lethal respiratory viral-bacterial coinfection

Amanda M. Jamieson^{1,3,†}, Lesley Pasman¹, Shuang Yu¹, Pia Gamradt³, Robert J. Homer², Thomas Decker³, and Ruslan Medzhitov^{1,*}

¹Howard Hughes Medical Institute and Department of Immunobiology Yale University School of Medicine, New Haven CT 06520

²Department of Pathology, Yale University School of Medicine, New Haven CT 06520

³Max F. Perutz Laboratories, University of Vienna, Dr. Bohr Gasse 9/4 A-1030 Vienna, Austria

Abstract

Secondary bacterial pneumonia leads to increased morbidity and mortality from influenza virus infections. What causes this increased susceptibility, however, is not well defined. Host defense from infection relies not only on immune resistance mechanisms, but also on the ability to tolerate a given level of pathogen load. Failure of either resistance or tolerance can contribute to disease severity, making it hard to distinguish their relative contribution. We employ a coinfection mouse model of influenza and *Legionella pneumophila* where we can separate resistance and tolerance. We demonstrate that influenza virus can promote susceptibility to lethal bacterial coinfection even when bacterial infection is controlled by the immune system. We propose that this failure of host defense is due to impaired ability to tolerate tissue damage.

Resistance and tolerance are two distinct strategies of host defense from infections: the former is based on pathogen detection and elimination, whereas the latter relies on host adaptation to a given level of pathogen burden (1–4). This distinction is important because infectious disease morbidity and mortality can be due to failed resistance or failed tolerance, which may in turn dictate different therapeutic options. Thus, a lethal outcome of microbial infection is usually ascribed to either high pathogen virulence, or low host resistance (for example caused by immunosuppression or immunodeficiency). Pathogen virulence can be due to direct damage to the host by toxins and virulence factors (intrinsic virulence), or, more commonly, due to excessive inflammatory response with collateral tissue damage (extrinsic virulence). However, insufficient tissue protection and repair could also be an important contributor to infectious disease phenotype (4).

The upper respiratory tract is exposed to numerous pathogens simultaneously, and viral-bacterial coinfection in the lung is a common clinical manifestation (reviewed in (5–8)). Complications from secondary bacterial infection are a leading cause of morbidity and mortality associated with influenza virus infection (5–8). Influenza virus can suppress the immune response to a bacterial infection, and this can lead to increased bacterial load and decreased survival. This has been shown in both clinical studies and in mouse models for multiple bacterial pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Streptococcus pyogenes*, and *Staphylococcus aureus* (reviewed in (5–8)). Bacterial overgrowth in these models complicates the analysis of other possible causes of morbidity

[†]Current address: Department of Molecular Microbiology and Immunology, Brown University, 171 Meeting St. Providence, RI 02912

and mortality. We therefore looked for an alternative model of coinfection that lacks this complication.

L. pneumophila recently has been recognized as a clinically relevant complication of influenza virus infection (9). When mice were infected with a sublethal dose of influenza virus, and then coinfecting with a sublethal dose of *Legionella pneumophila*, 100 percent of coinfecting mice died within a week of coinfection, whereas all mice survived single infections (Fig. 1A). An established viral infection was necessary for lethality because mice infected with influenza at the same time or 3 days after *L. pneumophila* survived coinfection (Fig. 1A). A resolved influenza virus infection no longer affected the ability to survive coinfection with *L. pneumophila*, because mice infected with bacteria 10 or 14 days after viral infection also survived (Fig. 1A). The mice coinfecting with *L. pneumophila* 3 days after influenza virus infection also had other signs of morbidity including decreased body weight and temperature (Fig. 1B,C).

Importantly, despite the dramatic difference in host survival, there was no significant difference in the viral or bacterial pathogen burden after single infections or coinfections (Fig. 1 D,E). Moreover, there was no detectable systemic dissemination of influenza or *L. pneumophila* after infections (Fig. S1). These results indicated that lethal synergy between influenza virus and *L. pneumophila* was not due to impaired resistance to either of these pathogens. This is in contrast to coinfections with influenza virus and opportunistic bacterial pathogens where bacterial overgrowth and systemic dissemination are commonly observed (5–8, 10–13).

To further address the role of pathogen virulence, we used an attenuated mutant strain of *L. pneumophila*, which lacks the Dot/Icm type-IV secretion system and therefore is unable to secrete virulence factors (14). Administration of *dotA* mutant or thymidine auxotroph (LP02) *L. pneumophila*, which are severely attenuated *in vivo*, still resulted in 100 percent mortality of coinfecting mice (Fig. 1F) (Fig S2)(14). These results indicate that bacterial virulence or growth is not essential for lethal synergy of influenza-*L. pneumophila* coinfection. Furthermore, mortality is unlikely to be due to failed immune resistance. However, administration of formalin-inactivated influenza virus did not synergize with the subsequent *L. pneumophila* coinfection (Fig. 1G), indicating that a productive virus infection is necessary to make the host sensitive to secondary bacterial infection. Moreover, treatment of mice with neuraminidase inhibitors (NAi) increased survival and decreased weight loss and hypothermia after coinfection (Fig. S3A–C), presumably because NAi suppressed viral load (Fig. S3D) (15).

We next examined whether mortality of influenza- *L. pneumophila* coinfection was due to excessive inflammatory response. Influenza virus activates three innate immune signaling pathways: Toll like receptor (TLR)7-MyD88, RIG-I-interferon (IFN)- α/β , and Nlrp3-Caspase-1-interleukin (IL)-1 pathways (16) (17). *L. pneumophila* is recognized by the innate immune system via several mechanisms, including the Naip5/Birc1e dependent pathway, which requires an intact Dot/Icm secretion system, and TLRs (18–22). Gene expression analysis of the lungs after single infection and coinfection indicated that some of the inflammatory genes, including tumor necrosis factor α (TNF α), nitric oxide synthase 2 (Nos2) and several chemokines, were expressed at higher levels in coinfecting compared to single infected mice (Fig. S4). TNF α and IL-1 β protein levels were also elevated in the broncho-aveolar lavage fluid (BALF) at day 3 post coinfection (Fig. 2A). Moreover, there was a significant increase in neutrophil infiltration in the lungs of coinfecting mice compared to singly infected controls (Fig. 2B,C). TNF α , IL-1 β , Nos2 and neutrophils are all known to play important roles in immunopathology, including in the context of influenza virus infection (23–25). However, we found that genetic deletions of TNF α , Caspase-1, MyD88,

TLR2/4 and *Nos2*, or antibody mediated depletion of neutrophils or natural killer cells did not rescue coinfecting mice from mortality (Fig. 2D–J). Similarly, *Rag2*^{−/−} mice, which lack an adaptive immune system, also succumbed to lethal coinfection, indicating that lymphocyte-mediated immunopathology is not essential for the lethal outcome of coinfection (Fig. S5A). Virus induced IFN- α/β can interfere with anti-bacterial responses (26). However, IFN- α/β receptor (IFNAR) deficient mice (*Ifnar1*^{−/−}) were still susceptible to coinfection (Fig. S5B). *Rag2* and IFNAR knockout mice were also susceptible to influenza infection alone, *Nos2*^{−/−} mice were susceptible to infection with *L. pneumophila* alone, and *Myd88*^{−/−} mice were susceptible to both single infections. However in all cases the mortality from coinfections was kinetically distinguishable from that of single infections and was similar to the kinetics of mortality of wild type mice (Fig. 2D–J, S5A,B Fig 1A). Finally, systemic treatment of the mice with synthetic glucocorticoid dexamethasone or antioxidant N-acetyl cysteine (NAC) did not rescue them from mortality of coinfection (Fig. S5C,D). Collectively, the elimination of all major immune and inflammatory pathways triggered by either the viral or bacterial infection did not rescue the lethal synergy. These results suggest that the lethal outcome of coinfection in our model was not solely due to excessive inflammatory response or immunopathology.

Because neither bacterial growth or virulence, nor host immune responses were individually required to cause lethality in coinfection, we next combined host immunodeficiency and bacterial attenuation. We used a severely attenuated *L. pneumophila* strain LP02 Δ dotA Δ flaA, which lacks flA and dotA, and is also a thymidine auxotroph (27). This strain lacks flagellin and is unable to replicate and secrete effectors, thus lacking major immunostimulatory factors except for cell wall components detectable by TLR2 and TLR4. Therefore, we used this strain to coinfect TLR2/TLR4-double deficient mice. *Tlr2*^{−/−}/*Tlr4*^{−/−} mice coinfecting with influenza virus and LP02 Δ dotA Δ flaA had a small increase in survival compared to wild type mice infected with the same strain of bacteria (Fig. 3A), however most mice still succumbed to coinfection. *Tlr2*^{−/−}/*Tlr4*^{−/−} coinfecting with LP02 Δ dotA Δ flaA had decreased immune cell infiltrate into the BALF at day 3 after infection, when compared with C57BL/6 mice infected with either LP02 Δ dotA Δ flaA or Jr32 Δ flaA (Fig. 2B,C Fig. 3B,C). Thus, severely attenuated, non-replicating *L. pneumophila* still caused mortality in coinfecting *Tlr2*^{−/−}/*Tlr4*^{−/−} mice despite almost complete lack of immunostimulatory signals.

The lethal outcome of influenza-*L. pneumophila* coinfection despite normal control of pathogen growth suggested that the mortality could be due to a failed tolerance to tissue damage caused by coinfection. Indeed, coinfecting mice had high levels of red blood cells (RBCs) and albumin in the BALF (Fig. 3D,E), indicating a damage to the lung epithelial-capillary barrier (28). The lung epithelial damage was further confirmed by histological analysis (Fig. 3F,G). The principal difference among the singly infected and coinfecting mice was in the degree of airway epithelial necrosis, with the coinfecting lungs having a significant increase in epithelial cell damage. Extensive damage to the airway epithelia, with secondary alveolar collapse (Fig 3D–G) are presumably responsible for the mortality of the coinfection.

Consistent with the histological evidence of lung tissue damage, a gene expression analysis revealed that a cohort of genes involved in tissue protection and repair was specifically downregulated in coinfecting compared to singly infected or uninfected mice (Fig 4A). This cohort included genes that are essential for tissue and cellular repair and development in the lung such as *Mdk*, *Adamts2*, *Timp4*, *Slpi*, *Mmp2*, *Mmp9*, *Vegfc*, *Itgb7*, and *Itga1*, (29) and genes involved in stress response in lung tissue, such as *Gcnt2*, *Hif3a*, *Stra13*, *Hmox1* and *Aifm1* (30) (Fig S6).

We next tested whether the defective expression of the tissue repair program is responsible for mortality of coinfection. The epithelial growth factor family member, amphiregulin (AREG) was recently found to contribute to tissue homeostasis in the lung during influenza infection (31). Although AREG did not have a significant effect in wild type mice, administration of AREG to *Tlr2*^{-/-}/*Tlr4*^{-/-} mice coinfecting with influenza virus and the LP02ΔdotAΔflaA strain of *L. pneumophila* significantly increased survival while decreasing weight loss and hypothermia (Fig 4, A–C). AREG treatment resulted in decreased lung damage as indicated by histopathological analysis, decreased albumin level in the BALF and decreased pulmonary infiltrate (Fig 4, D–G). Importantly, AREG treatment significantly decreased mortality of coinfection (Fig 4A), but did not affect the viral and bacterial burdens (Fig. 4H,I). The reason AREG administration did not rescue wild type mice from coinfection is likely because the disease in this case is too severe and may require a more optimal regimen of AREG administration or perhaps additional methods of promoting tissue protection and repair.

Collectively, these results demonstrate that i) lethal synergy of influenza virus and bacterial coinfection can result from loss of tolerance to infection-induced tissue damage; ii) morbidity and mortality of coinfection can be independent of pathogen burden or excessive inflammatory response; iii) promoting tissue repair can in principle rescue coinfecting animals from morbidity and mortality even without affecting pathogen burden. Finally, our influenza-*L. pneumophila* coinfection model demonstrates the distinction between resistance and tolerance as separate host defense strategies that can both contribute to morbidity and mortality of infectious disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and Notes

1. Råberg L, Sim D, Read AF. Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. *Science*. 2007; 318:812–814. [PubMed: 17975068]
2. Råberg L, Graham AL, Read AF. Decomposing health: tolerance and resistance to parasites in animals. *Philos Trans R Soc Lond, B, Biol Sci*. 2009; 364:37–49. [PubMed: 18926971]
3. Schneider DS, Ayres JS. Two ways to survive infection: what resistance and tolerance can teach us about treating infectious diseases. *Nat Rev Immunol*. 2008; 8:889–895. [PubMed: 18927577]
4. Medzhitov R, Schneider DS, Soares MP. Disease tolerance as a defense strategy. *Science*. 2012; 335:936–941. [PubMed: 22363001]
5. Beadling C, Slifka MK. How do viral infections predispose patients to bacterial infections? *Curr Opin Infect Dis*. 2004; 17:185–191. [PubMed: 15166819]
6. McCullers JA. Insights into the interaction between influenza virus and pneumococcus. *Clin Microbiol Rev*. 2006; 19:571–582. [PubMed: 16847087]

7. Hament JM, Kimpen JL, Fleer A, Wolfs TF. Respiratory viral infection predisposing for bacterial disease: a concise review. *FEMS Immunol Med Microbiol.* 1999; 26:189–195. [PubMed: 10575129]
8. Peltola VT, McCullers JA. Respiratory viruses predisposing to bacterial infections: role of neuraminidase. *Pediatr Infect Dis J.* 2004; 23:S87–97. [PubMed: 14730275]
9. Iannuzzi M, et al. Respiratory failure presenting in H1N1 influenza with Legionnaires disease: two case reports. *J Med Case Rep.* 2011; 5:520. [PubMed: 22018019]
10. Shahangian A, et al. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J Clin Invest.* 2009; 119:1910–1920. [PubMed: 19487810]
11. Iverson AR, et al. Influenza virus primes mice for pneumonia from *Staphylococcus aureus*. *J Infect Dis.* 2011; 203:880–888. [PubMed: 21278211]
12. Sun K, Metzger DW. Inhibition of pulmonary antibacterial defense by interferon-gamma during recovery from influenza infection. *Nat Med.* 2008; 14:558–564. [PubMed: 18438414]
13. Didierlaurent A, et al. Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. *Journal of Experimental Medicine.* 2008; 205:323–329. [PubMed: 18227219]
14. Berger KH, Isberg RR. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. *Mol Microbiol.* 1993; 7:7–19. [PubMed: 8382332]
15. Mendel DB, et al. Oral administration of a prodrug of the influenza virus neuraminidase inhibitor GS 4071 protects mice and ferrets against influenza infection. *Antimicrob Agents Chemother.* 1998; 42:640–646. [PubMed: 9517945]
16. Ichinohe T. Respective roles of TLR, RIG-I and NLRP3 in influenza virus infection and immunity: impact on vaccine design. *Expert Rev Vaccines.* 2010; 9:1315–1324. [PubMed: 21087109]
17. García-Sastre A, Biron CA. Type 1 interferons and the virus-host relationship: a lesson in détente. *Science.* 2006; 312:879–882. [PubMed: 16690858]
18. Ren T, Zamboni DS, Roy CR, Dietrich WF, Vance RE. Flagellin-deficient *Legionella* mutants evade caspase-1- and Naip5-mediated macrophage immunity. *PLoS Pathog.* 2006; 2:e18. [PubMed: 16552444]
19. Molofsky AB, et al. Cytosolic recognition of flagellin by mouse macrophages restricts *Legionella pneumophila* infection. *J Exp Med.* 2006; 203:1093–1104. [PubMed: 16606669]
20. Zamboni DS, et al. The Birc1e cytosolic pattern-recognition receptor contributes to the detection and control of *Legionella pneumophila* infection. *Nat Immunol.* 2006; 7:318–325. [PubMed: 16444259]
21. Archer KA, Roy CR. MyD88-dependent responses involving toll-like receptor 2 are important for protection and clearance of *Legionella pneumophila* in a mouse model of Legionnaires' disease. *Infect Immun.* 2006; 74:3325–3333. [PubMed: 16714560]
22. Spörri R, Joller N, Albers U, Hilbi H, Oxenius A. MyD88-dependent IFN-gamma production by NK cells is key for control of *Legionella pneumophila* infection. *J Immunol.* 2006; 176:6162–6171. [PubMed: 16670325]
23. La Gruta NL, Kedzierska K, Stambas J, Doherty PC. A question of self-preservation: immunopathology in influenza virus infection. *Immunol Cell Biol.* 2007; 85:85–92. [PubMed: 17213831]
24. Peiris JSM, Hui KPY, Yen HL. Host response to influenza virus: protection versus immunopathology. *Curr Opin Immunol.* 2010; 22:475–481. [PubMed: 20594815]
25. Schmitz N, Kurrer M, Bachmann MF, Kopf M. Interleukin-1 is responsible for acute lung immunopathology but increases survival of respiratory influenza virus infection. *J Virol.* 2005; 79:6441–6448. [PubMed: 15858027]
26. Decker T, Müller M, Stockinger S. The yin and yang of type I interferon activity in bacterial infection. *Nat Rev Immunol.* 2005; 5:675–687. [PubMed: 16110316]
27. Fontana MF, Shin S, Vance RE. Activation of host mitogen-activated protein kinases by secreted *Legionella pneumophila* effectors that inhibit host protein translation. *Infect Immun.* 2012; 80:3570–3575. [PubMed: 22851749]

28. Bhalla DK. Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. *J Toxicol Environ Health B Crit Rev.* 1999; 2:31–86. [PubMed: 10081525]
29. Crosby LM, Waters CM. Epithelial repair mechanisms in the lung. *Am J Physiol Lung Cell Mol Physiol.* 2010; 298:L715–31. [PubMed: 20363851]
30. Wong HR, Wispe JR. The stress response and the lung. *Am J Physiol.* 1997; 273:L1–9. [PubMed: 9252533]
31. Monticelli LA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol.* 2011; 12:1045–1054. [PubMed: 21946417]
32. Okuda K, et al. Protective immunity against influenza A virus induced by immunization with DNA plasmid containing influenza M gene. *Vaccine.* 2001; 19:3681–3691. [PubMed: 11395202]
33. Sadosky AB, Wiater LA, Shuman HA. Identification of *Legionella pneumophila* genes required for growth within and killing of human macrophages. *Infect Immun.* 1993; 61:5361–5373. [PubMed: 8225610]

Fig. 1. Decreased tolerance with unchanged resistance of mice coinfecting with influenza virus and *L. pneumophila*

(A) Survival of mice infected intranasally with 300 PFU of influenza virus and 0, 3, 6, 10 or 14 days later with 1×10^6 *L. pneumophila*. To better mirror a human infection, mice were infected with a strain of *L. pneumophila* lacking the *flaA* gene (JR32Δ*flaA*), which encodes flagellin. (B) Weight of mice infected with influenza virus or *L. pneumophila* alone or coinfecting 3 days after influenza virus. (C) Body temperature of mice coinfecting 3 days after influenza virus compared to the singly infected controls. (D) Lung bacterial load in mice coinfecting 3 days after influenza virus or mice infected with *L. pneumophila* alone. Day 0 indicates CFUs in the lung 1 hour after infection. (E) Lung viral load in mice coinfecting 3 days after influenza virus or mice infected in influenza virus alone. (F) Survival of mice coinfecting 3 days after influenza virus infection with LP01Δ*dotA* or the thymidine auxotroph LP02 strains of *L. pneumophila*. (G) Survival of mice coinfecting 3 days after infection with inactivated influenza virus. Data are combined from at least 3 independent experiments with at least 5 mice in each group (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$) Data was analyzed using the logrank test, generalized linear model, or ANOVA. Error bars represent standard deviation from the mean.

Fig. 2. Decreasing inflammatory pathways does not increase survival of coinfecting mice
(A) TNF α and IL-1 β levels in the BALF of mice infected with JR32 Δ fliA 3 days after influenza virus infection. **(B)** Immune cell infiltrate in BALF of mice coinfecting 3 days after influenza virus infection. **(C)** Types of infiltrating cells in the BALF. **(D)** Survival of *Tnf* α ^{-/-} **(E)** *Casp1*^{-/-} **(F)** *Tlr2*^{-/-}*Tlr4*^{-/-} **(G)** *Myd88*^{-/-} **(H)** *Nos2*^{-/-} **(I)** Gr-1 depleted **(J)** NK1.1 depleted mice coinfecting 3 days after infection with influenza virus compared to singly infected controls. Data are combined from at least 3 independent experiments with at least 5 mice in each group (* p 0.05, ** p 0.001, *** p 0.0001) Data was analyzed using the logrank test, t-test, or ANOVA. Error bars represent standard deviation from the mean.

Fig. 3. Increased tissue damage in coinfecting lungs

(A) Survival of *Tlr2*^{-/-}/*Tlr4*^{-/-} and C57BL/6 mice coinfecting with the LP02ΔdotAΔflaA strain of *L. pneumophila*. (B) Types of infiltrating cells in BALF of *Tlr2*^{-/-}/*Tlr4*^{-/-} and C57BL/6 mice coinfecting with strain LP02ΔdotAΔflaA 3 days after influenza virus infection. (C) Amount of infiltrating immune cells in BALF of *Tlr2*^{-/-}/*Tlr4*^{-/-} and C57BL/6 mice coinfecting with strain LP02ΔdotAΔflaA 3 days after influenza virus infection. (D) Red blood cells and (E) Albumin in the BALF day 4 after bacterial infection in C57BL/6 mice infected with influenza virus and 3 days later with the JR32Δfla strain (F) Representative images of airways and (G) Histological damage scoring of lung sections day 4 after bacterial infection from C57BL/6 mice infected with influenza virus and 3 days later with the JR32Δfla strain. Data are combined from at least 3 independent experiments with at least 5 mice in each group (* p 0.05, ** p 0.001, *** p 0.0001) Data was analyzed using the logrank test, t-test, or ANOVA. Error bars represent standard deviation from the mean.

Fig. 4. Targeting tolerance mechanisms increases survival of coinfecting mice

(A) Survival (B) weight loss and (C) temperature of *Tlr2*^{-/-}*Tlr4*^{-/-} and C57BL/6 mice coinfecting with the LP02ΔdotAΔflaA *L. pneumophila* strain and treated with AREG daily. (D) Albumin levels in BALF from coinfecting mice treated with AREG (E) Histological damage scores, (F) Representative samples of lung airway histology and (G) percent area of inflammation per lung of coinfecting lungs from *Tlr2*^{-/-}*Tlr4*^{-/-} and C57BL/6 mice coinfecting with the LP02ΔdotAΔflaA *L. pneumophila* strain and treated with AREG (H) PFUs and (I) CFUs in the lungs of coinfecting *Tlr2*^{-/-}*Tlr4*^{-/-} after treatment with AREG. Data are combined from at least 3 independent experiments with at least 5 mice in each group (* p 0.05, ** p 0.001, *** p 0.0001). Data was analyzed using the logrank test, t-test, or ANOVA. Error bars represent standard deviation from the mean.