

Can animal models really teach us anything about pneumonia? - Pro

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1. Introduction.

Despite highly effective antibiotics and intensive care support, the mortality associated with pneumonia has not substantially decreased since the 1960's (1). Hence, there remains a major requirement for improved treatment and preventative strategies, which will need new knowledge on the pathogenesis of pneumonia. Animal models have obvious high value when investigating the molecular mechanisms involved in pneumonia pathogenesis, but they are also directly relevant for clinically orientated research into new therapies and vaccines, complications of pneumonia, and identifying high risk groups. In this article we describe how research using animal models will be essential if we are to reduce the immense morbidity and mortality associated with pneumonia.

2. Background on animal models.

Animal models are low cost, broadly available, and can be used for invasive protocols, facilitating detailed mechanistic studies to inform the clinical approach. Issues involving work with animals are summarized below and addressed in detail by Mizgerd and Skerrett (2) and in a European Respiratory Society statement 'Optimising experimental research in respiratory diseases' (3). A wide variety of animal models of pneumonia exist, and which model is the most appropriate will depend on the research question(s) being addressed. Non-mammalian species (insects, roundworms, and zebra fish) are inexpensive and powerful tools that recapitulate many aspects of human innate immunity and host cell signalling. They have been used to

identify virulence determinants (4-9), and their lack of high-order sentience makes them attractive from an ethical perspective. However, they are limited by the lack of a mammalian respiratory system or an adaptive immune response. Non-human primates are the opposite extreme, closely recapitulating human physiology, immunology, and pathology, and generally susceptible to human pathogens, but their use raises major ethical concerns. Hence, non-human primates models are generally restricted to testing the pharmacokinetics, toxicology and efficacy of vaccines or potential therapeutics (10) prior to testing in humans, or the validation of key results obtained with other animal models (11, 12). Rodent models are a compromise that accurately model most aspects of human innate and adaptive immunity, and are also susceptible to the majority of human pneumonia pathogens (13-16). There are some important anatomical differences between rodents and humans (17, 18), and animal models of pneumonia frequently require forced aspiration of high numbers of bacteria (often $>10^6$) (19) rather than inhalation (20, 21) or aspiration from the upper airway (Figure 1A). Additionally, most investigators use healthy young adult rodents, whereas in humans, pneumonia largely affects infants, the immunodeficient, or the elderly. Despite these issues the overlap in anatomy, physiology, immunology and cell biology means animal models still replicate many important parameters of human infection.

The major advantage of animal models is the number of ways they can be manipulated to answer very precise research questions. Infection models can be combined with genetic manipulation of the pathogen to define the specific microbial processes required for pneumonia to develop (22, 23). Comparing different strains of the same pathogen can elucidate why some strains dominate the clinical picture (24, 25), and dual infection models provide important insights into why respiratory viral infection often leads to a secondary bacterial infection (26-28). The host can be manipulated using therapeutic or genetic depletion of immune effectors (29), including tissue specific targeting to precisely define how a host factor influences disease development (30). Host and pathogen responses in infection models can be characterised using all the 'omic' techniques (including single cell sequencing and dual-species RNA seq), flow

cytometry, and *in vivo* imaging, collectively providing a detailed level of information that is impossible for human studies to replicate and at considerable less cost. Hence, animal models are of immense benefit for therapeutic testing of immune modulators, antibiotics, ventilation/oxygenation regimens, vaccination, or countering immunosenescence (**Figure 1B**). Thus, animal models can identify whether a new intervention is likely to succeed in a much shorter period of time, at a considerably lower cost and with a reduced risk than human studies.

3. Alternatives to animal models. What about the experimental alternatives to animal models such as tissue culture models, organoids, *ex vivo* human material models, and pathogen challenge of human volunteers? Can these replace animal models? The short answer is no; pneumonia develops through multiple stages of infection involving a complex interplay between lung-resident and recruited immune cells, a rapidly changing microbial population, different anatomical compartments, and requiring extensive cross-talk between cell types. This is far too complex to be adequately replaced by tissue culture systems or even an organoid model. *Ex vivo* models such as precision-cut lung slice (PCLS) or human whole lung perfusion / ventilation models (31) are limited by their expense and the small number of biological replicates possible. Human models of infection are largely restricted to investigating mucosal host / pathogen interactions during milder infections (32, 33) (34), rather than the mechanisms involved during alveolar infection.

4. Research areas where animal models have made important contributions

Specific areas where animal models have produced important data on pneumonia that could not have readily been obtained using other methodologies are discussed below (examples are listed in **Table 1** and illustrated in **Figure 2**).

4.1 Defining who is at risk of pneumonia. A clear understanding of who is at risk of pneumonia is required, but can only be obtained by epidemiology studies if the risk factor is common

(e.g. age, smoking, comorbidities) or has particularly strong effects (e.g. complement deficiencies). In contrast, animal studies can identify weaker or less common predispositions to pneumonia, and also define the underlying molecular mechanisms involved. For example, the exponential increased risk of *S. pneumoniae* pneumonia in the elderly (35, 36) has multifactorial causes which are hard to define using epidemiology alone. Animal model research has linked age-related changes in DNA integrity and the gut microbiome to increased background inflammation that impairs immunity to *S. pneumoniae* by increasing expression of epithelial ligands for bacterial adhesion, impairing monocyte function, and reducing TLR2 expression (37-39). Without animal models we would not have identified these mechanisms and the potential for modulating the gut microbiome to prevent pneumonia. Animal model data can identify mechanistic links between different subgroups susceptible to pneumonia; for example, exposure to welding fumes, cigarette smoke, air pollution, and aging all increase the risk of *S. pneumoniae* pneumonia partly through increased epithelial expression of Platelet Activating Factor receptor (PAFr) (40-42). Animal model data can also predict groups that might have increased susceptibility to particular infections that can then be looked for in clinical practice, e.g. tyrosine kinase inhibitors were shown to impair immunity to *Aspergillus fumigatus* in mice, and clinical data have confirmed this is the case in patients (43, 44).

4.2 Defining mechanisms underlying differences in pathogen virulence potential. As pathogen and host can both be clonal, animal model research allows investigators to exclude unselected pathogen and host variability in order to identify molecular explanations for disease phenotypes. For example, using deletion mutants site-specific roles were identified for pneumococcal virulence determinants, which helps explain why pneumococcal strains vary in their ability to cause disease at different anatomical sites (45, 46). Similarly, dual infection animal models have characterised how host and pathogen factors affect influenza-induced transmission of *S. pneumoniae* and the efficacy of vaccination in blocking this key event (47, 48).

4.3 Pathogenesis of the complications of pneumonia. The complications of pneumonia such as empyema, bacteraemia, the impact of sepsis on airway immunity, spread of infection to the heart or central nervous system, and inflammation-mediated lung damage including alveolar:capillary barrier breakdown all involve multiple cell types interacting with progressive states of bacterial invasion, and are influenced by multiple host factors including sex, age, underlying comorbidities, genetics and environmental exposures. Animal models have helped our understanding why many of these complications develop, including the identification of *S. pneumoniae* myocardial invasion in pneumonia (49), how bacteria translocates through mesothelial cells to cause empyema (50), and why *Staphylococcus aureus* induces an excessive inflammatory response to cause a destructive pneumonia (51).

4.4 Identifying new therapeutic approaches. The lung inflammatory response to microbial challenge are similar in animal models and humans, including the pattern of early recruited neutrophils for pathogen elimination followed by exudate macrophage recruitment to facilitate resolution of lung inflammation. Growth factors such as GM-CSF, G-CSF and M-CSF also exhibit a high degree of structural and functional similarities between rodents and humans (52, 53). This allows the roles of these common molecular traits to be characterized in animal pneumonia models to inform on adjuvant therapies for pneumonia. For example, GM-CSF regulates terminal macrophage differentiation (54, 55) and protects against pneumonia in mouse models (56, 57), whereas GM-CSF deficiency is a critical risk factor for bacterial pneumonia (58). Intrapulmonary overexpression of GM-CSF provided a high degree of protection to mice from *S. pneumoniae* pneumonia, suggesting GM-CSF could be a future adjuvant therapy. Indeed, inhaled recombinant GM-CSF (Sargramostim, Leukine®) improved oxygenation and outcome in patients with pneumonia-associated acute respiratory distress syndrome (ARDS) (59), demonstrating that preclinical animal models aid the development of adjuvant therapies against infectious lung diseases. Human pluripotent stem cells (iPSC) -derived macrophages prevent early *P. aeruginosa* respiratory tract infections in mice (60); this and other examples (61, 62) may help develop antibiotic-independent cellular immunotherapies for use

in humans. Antibiotic compound screening in animal models can identify novel antibacterial therapies for human pneumonia, and newer techniques such as bioluminescence and biophotonic imaging of bacterial provide powerful tools for monitoring real-time progression of pneumonia and assessing drug efficacy (63-68).

4.5 Novel preventative approaches. There are no vaccines available for the majority of respiratory pathogens including most respiratory viruses, *S. aureus*, the Gram negative pneumonia pathogens, *Pneumocystis jirovecii*, and *Aspergillus fumigatus*. Furthermore, the existing vaccines all have major limitations; the *H. influenzae* vaccine does not protect against the non-typeable strains that cause adult infections, and the existing influenza and *S. pneumoniae* vaccines have restricted strain coverage and reduced efficacy in the elderly, precisely the population they are most needed for. Hence new vaccines against pneumonia pathogens are needed, yet inducing protective pulmonary immunity is harder to achieve than immunity to bacteraemia (69, 70). Animal models will be essential for defining the adaptive immune mechanisms that prevent lung infection, identifying the most protective antigens, and improving adjuvants and methods of antigen delivery (71). Animal models identified the key role for CD4 Th17 responses in protecting the respiratory mucosa from pathogens such as *K. pneumoniae* and *S. pneumoniae* (70, 72) that is dependent on specific dendritic cell subsets (73), and an unexpected role for Tregs in protecting against *S. pneumoniae* (74). These data suggest vaccine approaches against extracellular pathogens could induce cellular rather than humoral immunity. Novel vaccine delivery systems and adjuvants developed using animal models are now reaching clinical use (75), and should hopefully improve targeting of vaccine-induced immunity to the lung in the future. Animal models can also identify new vaccine antigen candidates by screening for genes highly expressed during infection, or identifying antigens recognized by naturally acquired protective immune responses (76, 77). Overall, it is difficult to see how pre-clinical studies of new vaccines can be performed without using animal models.

4.6 Strategies for reducing weaknesses of animal model data. Despite their strengths, animal models do not fully recapitulate human conditions. This can be partially alleviated by combining the data obtained with tissue culture, organoids, or *ex vivo* lung experiments data. How accurately murine responses to inflammatory stimuli reflect human responses is debated (78-80), but exposure of laboratory mice to petshop or wild mice ensures the mouse inflammatory response more closely mimics human responses (81, 82). This provides a potentially simple method for improving the utility of mouse models of infection. Genetic modification of the animal model can also improve their utility. For example deleting either cytidine-monophosphate-N acetylneuraminic hydroxylase (CMAH, converts N-acetylneuraminic acid to N-glycylneuraminic acid on airway epithelium sialylated glyconjugates) or ApoB-100 lipoprotein (a potent inhibitor of the *S. pneumoniae* toxin pneumolysin) increased mouse susceptibility to *S. pneumoniae* infection (83, 84) .

4. Concluding remarks

Only animal models intrinsically allow the study of complex multicellular systems in anatomical context over time. The tractability of the mouse to genetic manipulation, combined with the application of new 'omics technologies and *in vivo* imaging has increased our ability to determine the impact of host or pathogen factors on pneumonia susceptibility, pathogenesis, and resolution. The animal model remains the only method for testing the efficacy of novel vaccines or antimicrobial approaches. Thus, animal models are likely to remain essential for the successful development of novel therapeutic advances for pneumonia.

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Legends:

Figure 1: (A) Main differences in anatomical and biochemical characteristics between human and mouse lungs that influence interpretation and the use of animal models of pneumonia including the mode of infection, the horizontal position of mouse lungs, lung anatomy, and distinct cell surface glyconjugates due to mutation in the *cmah* gene in humans. **(B)** The different roles which animal models of pneumonia can be used for when developing novel preventative and therapeutic interventions against pneumonia in humans. NHP, non-human primate.

Figure 2: Examples of how recent discoveries using animal models could lead to new therapeutic and preventative strategies in the near future.

Table

Table 1: Specific research areas where animal models have been / are important for the answer with selected exemplar studies:

Category	Research areas	Selected example with potential clinical relevance	Reference
Pathogen	Identifying pathogen mechanisms of pathogenesis	Demonstration of the importance of bacterial iron acquisition for the development of <i>Acinetobacter baumannii</i> pneumonia	(85)
	Comparative virulence of pathogen strains	Characterisation of the additional effects of Panton-Valentine leukocidin toxin during <i>S. aureus</i> pneumonia	(86)
	Investigating the effects of dual infection	Demonstration that prior influenza infection impairs TLR mediated innate responses to subsequent <i>S. pneumoniae</i> pneumonia	(87)
Host	Characterising natural mechanisms of innate immunity	Identification of the importance of the classical complement pathway for innate immunity to <i>S. pneumoniae</i>	(88)
	Characterising natural mechanisms of adaptive immunity	Demonstration that Th17 CD4 cells are required for lung immunity	(72)
		Demonstration that human natural adaptive immunity to <i>S. pneumoniae</i> is dependent on antibody to protein antigens	(77)
	Defining effects of age or comorbidity on pathogenesis	Demonstration that cellular senescence increases expression of host ligands for bacterial adhesins in the lungs	(40)
	Characterising effects of environmental exposures	Welding fumes increases expression of PAFr, resulting in increased <i>S. pneumoniae</i> adhesion to respiratory epithelium	(42)
	Defining protective inflammatory responses	Identification of an important role for CXCL1 mediated crosstalk between macrophages and neutrophils for immunity to <i>P. aeruginosa</i>	(89)
	Defining harmful inflammatory responses	Identification that <i>S. aureus</i> stimulate the TNF receptor to cause destructive pneumonia	(51)
	Identifying complications and their pathogenesis	Identification of foci of <i>S. pneumoniae</i> in the myocardium in pneumonia	(90)
Therapies	Identifying and testing novel vaccine approaches and antigens	Identification of Th17 antigens that protect against <i>S. pneumoniae</i>	(91)
		Confirmation that vaccination with a recombinant glycoconjugate is as efficacious as Prevnar vaccination against <i>S. pneumoniae</i>	(92)
	Assessing efficacy of immunomodulation	Demonstration that intrapulmonary overexpression of GM-CSF protects mice against <i>S. pneumoniae</i> pneumonia	(56, 57)
	Assessing efficacy of antibiotic therapies	Multiple studies (routine pharmaceutical company practice)	
	Pharmacokinetics and toxicology	Multiple studies (routine pharmaceutical company practice)	