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New Insights for Development of a Safe and Protective RSV Vaccine

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Abstract

Respiratory Syncytial Virus (RSV) is the leading cause of pneumonia and bronchiolitis in infants and children <1 year old, resulting in significant morbidity and mortality worldwide. There is currently no RSV vaccine. In the 1960's, a formalin-inactivated RSV (FI-RSV) vaccine trial led to exacerbated disease upon natural infection of vaccinees, including two deaths. The causes involved in the disastrous results of these vaccine trials are still unclear but they remain the engine for searching new avenues to develop a safe vaccine that can provide long-term protection against this important pathogen. This article reviews some of the early history of RSV vaccine development, as well as more recent information on the interaction between RSV and the host innate and adaptive immune responses. A safe and efficacious vaccine for RSV will require "re-education" of the host immune response against RSV to prevent vaccine-enhanced or severe RSV disease.

Keywords

RSV vaccine; TLR-4; vaccine enhancement of disease

INTRODUCTION

RSV, the most significant cause of serious lower respiratory tract infection in infants and young children1, results in 75,000–125,000 hospitalizations2 and ~500 deaths yearly in the USA3. Due to the horrific outcome of a failed clinical trial in the mid-1960's in which a formalin-inactivated RSV (FI-RSV) vaccine preparation was evaluated (discussed below), interest in live attenuated vaccines followed. In a recent review, Schickli et al.[4] have provided an in depth analysis of the constraints associated with the development of live attenuated vaccines for RSV. They note that since the most vulnerable population is infants, an ideal RSV vaccine would have to be administered very early in life, provide protection in the presence of maternal antibody, not interfere with the safety or efficacy of other vaccines routinely administered to infants (and vice versa), and elicit protection with minimal reactogenicity. In addition, we believe that any licensed RSV vaccine will have to be readily accepted by parents and would likely need to elicit immunity that is more durable than that provided by natural infection, a unique requirement for a vaccine. All of these constraints apply to subunit vaccines as well. In this chapter, we will focus on the immunological aspects of developing a safe and efficacious

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RSV vaccine, with particular emphasis on the roles of RSV fusion (F) protein and Toll-like receptor 4 (TLR4) in this process.

Respiratory Syncytial Virus (RSV): A long-term challenge to vaccine development

Although severe RSV-induced disease predominates in infants and children, it has more recently been identified as an increasing cause of morbidity and mortality in the elderly[5–7], transplant patients, and in patients with immunodeficiencies[8, 9]. RSV is relatively stable antigenically, yet most adults are re-infected every few years, suggesting that natural immunity is not long-lasting[10]. Why we fail to develop durable immunity after RSV infection is not understood. It is also unclear why children, the elderly, and the immunosuppressed are at a much higher risk for severe disease; however, an immune pathological mechanism has long been suspected in the development of severe RSV disease. In children, severe RSV disease is most often associated with prematurity, bronchopulmonary dysplasia (BPD), or congenital heart disease (CHD)[11]. RSV-specific immunity has been implicated in both protection and the immunopathological mechanism(s) that lead to severe lower respiratory tract disease and long-term changes in the immunological environment of the lung, i.e., RSV infection in early infancy has been correlated with development of allergic and asthmatic symptoms later in life[12]. While there is currently no licensed RSV vaccine, the two antibodies licensed for prophylactic use, RespiGam® and Synagis®, unquestionably provide highly significant passive protection to high-risk infants[13, 14]. However, due to the high cost of antibody prophylaxis, the USA is the only country that administers this drug to the majority of high-risk infants. Therefore, in the absence of a safe and effective vaccine, antibody prophylaxis and parental education, including basic hygiene practices on avoiding RSV, are the only options for reducing infection in healthy infants, children, and adults[15], populations with the majority of RSV-related hospitalizations.

The idea that the immune response plays an adverse role in RSV-induced disease is based largely on the observations from an early clinical trial in which vaccination of infants with formalin-inactivated RSV (FI-RSV) resulted in greatly enhanced susceptibility to develop severe lower respiratory tract involvement upon natural RSV infection. Thus, a better understanding of the immune mechanisms operative in primary, secondary, and vaccine-enhanced infection will be required to identify novel approaches to induce safe, long-lasting immunity to RSV and/or to intervene therapeutically.

RSV, a member of the family *Paramyxoviridae*, has a nonsegmented negative strand genome[11]. The virion consists of a nucleocapsid within a lipid envelope of irregular size and shape. Three glycoproteins are found in the virus envelope. The hydrophobic SH protein is the smallest and its function is relatively unknown. Fusion (F) protein mediates viral penetration and cell to cell spread by fusion of membranes. Fusion of RSV with host cell membranes is required for transfer of the viral ribonucleoprotein into the cell cytoplasm. F protein also promotes syncytia formation due to fusion of infected cell membranes with those of adjacent cells. This phenomenon may have limited relevance, however, since it is often seen *in vitro*, but infrequently *in vivo*. RSV F protein shares structural elements and a low, but significant, level of sequence homology with other paramyxoviruses[16]. RSV F protein contains *N*-linked oligosaccharide groups and is composed of two disulfide-linked protein subunits of 47 (F1) and 20 kD (F2). The G protein mediates virus attachment. Two major antigenic groups, A and B, are distinguished primarily by differences in the G protein, with group A being most prevalent. G and SH proteins are not required for infectivity, as demonstrated by the isolation of a viable RSV mutant (*cp52*) lacking these genes[17]. Conversely, RSV mutants that lack the F protein gene cannot replicate. The F protein is highly conserved among the two antigenic groups of RSV and has long been recognized as a major vaccine candidate as it is an immunodominant target for neutralizing antibodies[18] and for virus-specific cytotoxic T

lymphocytes[19]. A passively administered humanized (IgG1, κ) monoclonal antibody (MAb) (i.e., Synagis®) that completely protected cotton rats (*Sigmodon hispidus*) against RSV challenge[20], significantly reduces disease severity in high-risk infants[14]. The neutralizing epitope that is recognized by Synagis® is contained within the F protein[21].

Failed RSV human vaccine trials

Shortly after RSV isolation, NIH initiated a program to develop a vaccine using an approach that had been successfully applied to polio and influenza. Based on the prior success of other formalin-inactivated virus vaccines, a similar procedure was attempted for RSV. RSV "Lot 100" vaccine was propagated in monkey kidney cells, formalin-inactivated, and concentrated by ultracentrifugation and alum precipitation. Clinical trials began in winter of 1965–1966 in 4 centers in the USA. The results of these trials were entirely unexpected[22–25]: there was *no protection* against RSV in vaccinees whose rate of naturally occurring infection was significantly higher than in controls. Second, RSV infection caused more severe disease in vaccinees, with a 16-fold increase in hospitalizations and two fatalities among the youngest patients, who likely had no previous natural exposure to RSV. The legacy of Lot 100 has had a profoundly negative influence on vaccine development, and no RSV vaccine has since been licensed for any age group.

Analysis of the failed vaccine trials and early epidemiological studies yielded two intriguing correlations. First, those Lot 100 vaccinees who developed enhanced RSV disease had significant levels of serum antibody to RSV at the time of illness. In contrast, parainfluenza-vaccinated or unvaccinated controls experienced relatively mild RSV infection and had much lower titers of anti-RSV antibody[22–25]. Second, severe RSV disease was observed most frequently in infants <6 months old, when maternal antibody is present[26]. The lack of an animal model at the time of the Lot 100 trials precluded experimental corroboration. Nonetheless, these observations led to the hypothesis that antibody, normally considered protective, contributed to RSV disease severity. However, the subsequent finding that prophylactic administration of polyclonal human anti-RSV immunoglobulin or anti-RSV F protein MAb to cotton rats is both safe and protective against primary RSV disease[26–29], diverted attention away from antibodies as mediators of RSV-induced disease and led to the highly successful prophylactic use of polyclonal, and subsequently MAb, anti-RSV antibodies in high-risk infants.

After the failed FI-RSV trial, concerns over safety prompted development of live attenuated vaccines with cold-passaged (*cp*), temperature-sensitive (*ts*) mutations or recombinant virus with deletion mutations (SH, NS1 or NS2) combined with the aforementioned (reviewed in4). Such vaccines exhibited residual virulence, genetic instability, or insufficient immunogenicity in clinical testing30, 31. Subunit vaccines (*e.g.*, chromatographically purified F glycoprotein[32–34], recombinant chimeric F/G glycoprotein[35], recombinant chimeric RSV-F/parainfluenza–HN glycoprotein[36], plasmid DNA encoding F and G glycoproteins[37–41], recombinant G glycoprotein[42, 43], and G protein peptides[44, 45] were also developed. However, since the failed FI-RSV vaccine trial, no non-replicating RSV vaccine has been put into clinical trials in immunologically naïve infants, and it is likely that approval for any future trial will be contingent upon demonstrating a compelling safety profile for candidate vaccines in animal models.

In 1971, the susceptibility of the cotton rat *S. hispidus* to human RSV was described[46]. Human RSV replicates to high titers in the nose and lungs of cotton rats of all ages and they are 50–1000-times more permissive than several inbred mouse strains[47]. Viral antigen can be detected in the nasal, bronchial, and bronchiolar epithelium[48]. Primary RSV infection in *S. hispidus* lasts ~5 days in the lungs and slightly longer in the nose. Lower doses cause mild to moderate

peribronchiolitis (inflammatory cells, primarily lymphocytes, around the small airways), while $\geq 10^6$ plaque-forming units (pfu) also cause interstitial pneumonitis (thickening of alveolar walls accompanied by inflammatory cells) and alveolitis (inflammatory cells in air spaces), compromising pulmonary function.

Passive administration of polyclonal anti-RSV antibody prophylactically was first shown to be protective in cotton rats[49], and then in humans[13, 14, 50]. These findings led to licensure of RespiGam® and, later, the MAb anti-F protein antibody, Synagis®, for prevention of severe RSV disease in high-risk infants. Both products advanced to human clinical trials on the strength of data from cotton rat studies alone. Prince et al. reproduced vaccine-enhanced disease by immunizing cotton rats with the original Lot 100 vaccine, followed by in. RSV challenge[51]. Although most mouse strains are less susceptible to RSV than cotton rats, certain inbred mouse strains[52], or mice that lack genes that encode key inflammatory molecules (*e.g.*, TLR4, TLR2, STAT1, STAT6, IFN-γ, IL-13, and IL-4Rα43, 53–58), have provided important insights into mechanisms of immunity to RSV. Among inbred mouse strains, the BALB/c strain appears to be more sensitive than other commonly used inbred mouse strains, *e.g.*, C57BL/6, although high inocula, compared to cotton rats, are required to elicit severe pathology[59].

Molecular insights into potential mechanisms of enhanced disease

Prince et al.[51] identified 2 peaks of pulmonary disease following RSV challenge of FI-RSV-immunized cotton rats. The timing of the first peak (1 day p.i.) and the predominant infiltrating cell type (neutrophils) were suggestive of antigen-antibody and complement deposition, similar to a type III "Arthus reaction." A second peak of pulmonary disease (4 days p.i.) seen in cotton rats with vaccine-enhanced disease is associated with a predominance of lymphocytes, suggesting a delayed, cell-mediated immune mechanism[51]. Enhanced disease in the cotton rat60 presents with *alveolitis*, consisting primarily of neutrophil infiltrates, and peribronchiolitis, consisting primarily of lymphocyte infiltrates, features that are remarkably similar to the original histopathology described for the two Lot 100 fatalities[61]. The fact that alveolitis is a hallmark of FI-RSV underscores its importance as a histologic marker of vaccine-enhanced disease. When the results of the failed vaccine trial were published and the results of the two autopsies described, Kim et al.[25] commented only briefly on the histopathologic findings, citing “peribronchiolar monocytic infiltration with some excess in eosinophils.” Unfortunately, this description did not accurately reflect the original autopsy reports. Kim et al.’s mention of eosinophils, and failure to mention neutrophils, have led many to conclude incorrectly that eosinophilia is a primary marker of *vaccine-enhanced* RSV disease (*e.g.*, 62–64), whereas *neutrophilia* was, in fact, the predominant histologic finding of both autopsies[61]. Vaccine-enhanced RSV disease in African green monkeys[65] and calves[66] is also characterized by neutrophilic alveolitis, without eosinophils. In contrast, enhanced disease in mice is not accompanied by neutrophils[67], and pulmonary eosinophilia, while predominant in some strains of mice (*e.g.*, BALB/c), is absent in others[52].

Graham et al.[68] first reported that vaccine-enhanced disease was associated with a “Th2 type” response. BALB/c mice immunized with FI-RSV, then challenged with RSV, exhibited a pattern of cytokine mRNA expression characterized by an increased ratio of IL-4/IFN-γ. In contrast, unvaccinated animals showed a “Th1 type” response to RSV infection, with undetectable IL-4[68]. In some mouse strains, this Th2 type response was accompanied by pulmonary eosinophilia and increased $CD4^+$ T cells[56, 62–64, 69]. Although an imbalance in cytokine levels favoring a Th2 “skew” has frequently been put forth to explain FI-RSV-induced vaccine-enhanced disease, it has not been possible to corroborate this in humans since cytokine levels were not measured in the original FI-RSV trial. A number of studies have investigated cytokine profiles in supernatants of peripheral blood mononuclear cells and nasopharyngeal

aspirates of patients with RSV and correlated these findings with disease severity[70–78]. Although a direct comparison of these studies is difficult because of differences in the cohorts used and the endpoints considered, increases in Th2-like responses in infants with severe RSV disease were reported in some studies[70, 71, 74, 75, 77], whereas other similar studies reported induction of both Th1 and Th2 type cytokines[73] or no evidence of a Th1-Th2 cytokine imbalance72, 76, 78. Finally, inflammatory mediators in plasma, inflammatory cells in nasal washings, and virus-specific responses in T cell cultures established from peripheral blood mononuclear cells of infants <6 months were analyzed in relation to clinical severity. IL-6 and IL-8 were found more frequently and at higher levels in plasma samples of more severely ill patients, but no significant differences were found in the levels of cytokines that distinguish Th1 and Th2 responses[79]. These conflicting results raise the possibility that Th2 cytokines may be more readily detected in FI-RSV-enhanced disease than in primary RSV infection. It is also possible that the mechanism(s) underlying FI-RSV-enhanced disease differ(s) from that of severe primary infection, despite the frequent implication in the literature that they are similarly mediated.

Using the *identical* Lot 100 FI-RSV vaccine from the failed clinical trials, we previously established a cotton rat model for enhanced RSV disease that faithfully recapitulated the pathology induced by RSV infection in Lot 100-immunized children51, 61. Therefore, we sought to characterize FI-RSV-enhanced pathology molecularly, again using the original Lot 100 vaccine80. We postulated that FI-RSV vaccine-enhanced disease was due to a failure of formalin-modified RSV F protein to trigger a strong proinflammatory “Th1” response due to a diminished capacity for interaction with TLR4, resulting in a “Th2”-type bias[81]. Using reverse transcription (RT)-PCR, expression of 19 genes (*e.g.*, pro- and anti-inflammatory cytokines, chemokines, and others) was quantified after RSV challenge of animals vaccinated with a mock vaccine *vs.* Lot 100 FI-RSV. We confirmed that FI-RSV-enhancement of RSV-mediated disease was associated with a significant increase in neutrophilic alveolitis, a marker of vaccine-enhanced disease both in cotton rats and infants[61]. The expression patterns of several cytokine/chemokine mRNAs differed dramatically between RSV-challenged, mock-*vs.* FI-RSV-vaccinated cotton rats (Figure. 1). FI-RSV-enhanced disease was associated with a stronger cytokine response than that seen in primary infection controls (mock-vaccinated animals). For example, vaccination with FI-RSV resulted in statistically significant increases in mRNA levels for Th2 markers, IL-4 and IL-5. IL-9, considered to be a key Th2 cytokine involved in asthma predisposition[82], was also highly elevated in FI-RSV-vaccinated animals after RSV infection, supporting previous results in other animal models83.

Surprisingly, the effect of FI-RSV vaccination on gene induction by RSV infection was *not* restricted to an upregulation of Th2 cytokines as has been perpetuated in the literature based on early studies in the murine (BALB/c) model of RSV infection[68]. Chemokine expression was also increased significantly, *e.g.*, Monocyte Chemoattractant Protein-1 (MCP-1) mRNA was dramatically increased only in FI-RSV-vaccinated animals early after infection[80]. In addition, the expression of genes that encode prototypical Th1 cytokines, *e.g.*, IL-2, IFN-γ, and IL-12 p40, were significantly increased in RSV-infected cotton rats that were previously vaccinated with FI-RSV (Fig. 1, Th1). Collectively, our results challenge the long-standing paradigm that a skew toward Th2-type cytokines underlies FI-RSV-induced enhancement of RSV disease. Our data indicate a more complex and generalized dysregulation of the immune response revealed by a significant enhancement of not only Th2 cytokines, but also Th1-type cytokines and chemokines not previously associated with RSV infection of FI-RSV-sensitized animals.

We also found that enhanced disease, normally associated with FI-RSV, could be induced by a vaccination of cotton rats against human metapneumovirus (hMPV), a paramyxovirus related to RSV[84]. Cotton rats vaccinated with formalin-inactivated (FI)-hMPV and challenged with

hMPV exhibit dramatic enhancement of lung pathology. In contrast to RSV, FI-hMPV-enhanced pathology is paralleled by increased IL-4 and a decrease in IFN-γ. Thus, vaccine-enhanced disease induced by different FI-paramyxovirus vaccines cannot be characterized only by changes in Th1/Th2 ratio. Individual components of each vaccine, or the manner in which live virus subsequently engages primed host cells, likely activate the innate and adaptive immune responses to infection in mechanistically distinct ways.

Role of Toll-like receptor 4 (TLR4) in the response to lipopolysaccharide (LPS) and RSV

LPS, an integral outer membrane component of all Gram negative bacteria, is one of the most potent inflammatory stimuli. LPS activates macrophages and other cells to produce TNF-α, IL-1β, and other potent cytokines and chemokines that induce inflammation through receptor-mediated interactions with target cells. LPS signaling is initiated when circulating LPS-binding protein (LBP) transfers LPS to CD14. CD14 exists on the surface of certain cell types, *e.g.*, macrophages, as a glycosyl phosphatidylinositol (GPI)-linked protein, or in a soluble form (sCD14). Both forms of CD14 bind monomeric LPS with high affinity, and transfer it to MD-2, an extracellular protein that binds to the TLR4 ectodomain. CD14 does not signal intracellularly because it has no intracellular portion with which to transmit intracellular signals. $CD14^{-/-}$ mice, and their macrophages, are poorly LPS-responsive[85, 86].

The discovery of TLR4 proved central in our understanding of LPS-induced signaling. TLR4 is one of >10 human homologs of *Drosophila* Toll, a transmembrane, signaling molecule required for innate immunity to fungal and bacterial infection in adult flies. Ligand-mediated dimerization of TLRs leads to intracellular signaling. Purified enterobacterial LPS preparations utilize TLR4 exclusively for signaling. In contrast, TLR2 responds to bacterial lipopeptides but, rather than forming homodimers, TLR2 signaling requires formation of heterodimers between TLR2 and either TLR1 or TLR6. TLR5 senses bacterial flagellin. While TLRs 2/1, 2/6, 4, and 5 are typically associated with detection of extracellular microbes through their N-terminal ectodomains, TLRs 3, 7, 8, and 9 are found in endosomes where they detect viral or bacterial nucleic acids (reviewed in87). Critical to the establishment of TLR4 as the LPS signal transducer was the finding that certain spontaneously derived, LPS-hyporesponsive inbred mouse strains possess TLR4 mutations or deletions88–90, later confirmed in $TLR4^{-/-}$ mice (reviewed in91). Macrophages from mice with tlr4 mutations, or from $TLR4^{-/-}$ mice, fail to respond to LPS with inflammatory gene expression[92]. MD-2, a non-membrane-spanning protein that associates with the TLR4 *ectodomain*, is required for LPS-induced, TLR4 signaling[93]. Close physical proximity of LPS and TLR4 is likely achieved by CD14-mediated transfer of LPS to MD-2[94] or the generation of stable LPS:MD-2 complexes95 that lead to TLR4 dimerization, followed by recruitment of downstream signaling molecules. Recently, the crystal structures of murine and human MD-2 were solved96, 97. The structural basis of LPS recognition by the TLR4- MD-2 complex[98] was recently resolved and revealed that MD-2 binds to the concave surface of the N-terminal and central domains of TLR4 and that the acyl chains of the lipid A region of LPS insert into a deep hydrophobic pocket in MD-2, thereby inducing heterotetramer formation (i.e., $(TLR4/MD\text{-}2)_2$).

Three domains, *i.e.*, a Leucine Rich Region (LRR) in the N-terminal ectodomain, a transmembrane region, and a Toll/IL-1R resistance (TIR) domain in the intracellular region, are structural hallmarks of all known TLRs (reviewed in91). While the overall structure of different TLRs is similar, the pattern of gene expression induced by TLR4 differs substantially from that induced by other TLRs, such as TLR299 or TLR9100. TLR4, but not TLR2, activation results in induction of IFN-β, that, in turn, acts in an autocrine/paracrine fashion to activate STAT199. Many genes not induced by TLR2 agonists (*e.g.*, IP-10, MCP-5, and iNOS) are STAT1-dependent[101]. Differential utilization of 4 TIR-containing adapter molecules

(*i.e.*, MyD88, TIRAP, TRIF, and TRAM) by distinct TLRs leads to activation of distinct downstream signaling pathways, findings based largely on studies in adapter knockout mice. Two major TLR signaling pathways were identified, *i.e.*, one that is MyD88-dependent, and gives rise to strong and early activation of NF-κB, and a TRIF-dependent, MyD88-independent pathway that primarily drives strong activation of another key transacting factor, IRF-3, with delayed NF-κB. The MyD88-dependent pathway results in induction of highly NF-κB-dependent, proinflammatory genes (*e.g.*, TNF-α, IL-1β), while the MyD88-independent pathway leads to IRF-3-dependent gene induction (*e.g.*, IFN-β, RANTES). TLR4 is unique in that it activates both pathways for gene expression because it is the only TLR that uses all 4 adapter proteins[102].

Innate immunity, initiated by the interaction of host cells with pathogens through recognition of conserved microbial structures, is central in the early response to infection and facilitates development of the adaptive immune response. Kurt-Jones and colleagues first reported that the RSV F protein triggers the innate immune response to RSV through mammalian TLR4 and CD1453, 103. Although RSV F protein shares no structural similarity with LPS, it requires CD14, TLR453, [104] and MD-2 (manuscript in preparation) to signal. Importantly, the ability to clear live RSV was impaired in mice with *tlr4* mutations[53, 103], indicating that TLR4 signaling is necessary for control of primary RSV replication. Haynes et al.[103] found that TLR4-deficient mice challenged with RSV also exhibited impaired NK and $CD14^+$ cell pulmonary trafficking, diminished NK cell function, and impaired IL-12 induction, in addition to impaired RSV clearance. Haeberle et al.105 employed a model of alveolar macrophage depletion and the TLR4-defective C3H/HeJ mouse strain to show that the early NF-κB response that occurs in the lung after RSV infection is dependent upon both alveolar macrophages and TLR4. Early NF-κB activation was "consistently reduced compared with TLR4 competent mice[105]." However, NF-κB translocation was observed in the C3H/HeJ mouse at 24 hr p.i., suggesting that TLR4-independent NF-κB inducers are also activated in response to infection. This is not surprising since IL-1β and TNF-α, both NF-κB-inducing cytokines, are induced early in response to RSV infection, and could mediate a second wave of NF-κB. Importantly, other pattern recognition receptors (PRRs) have since been implicated in RSV infection and all activate NF-κB43, 106–108. The role of TLR4 in RSV infection is not without controversy, however, and was challenged by Ehl et al.109. Briefly, the C57BL/10ScCr (TLR4- and IL-12R-deficient) and the C57BL/10ScN (TLR4-deficient) strains were compared after RSV infection. The authors concluded that a transient delay in clearance was attributable to IL-12R, but not TLR4, deficiency. However, these authors stated in their discussion, "The reason for the discrepancy between our study and the two previous studies remains unclear." In a recent paper, the Finberg laboratory has outlined some factors, *i.e.*, differences in baseline and induced TLR expression in different mouse strains, different doses of RSV used for infection, or status of RSV stocks that present substantial changes in infectivity over time, that could highly affect the study outcome[43].

LPS hyporesponsiveness of peripheral blood mononuclear cells (PBMCs) has been identified as a risk factor for intensive care unit hospitalization in infants with RSV bronchiolitis[110]. Inheritance of two single nucleotide polymorphisms (SNPs) that encode point mutations in the TLR4 ectodomain (Asp299Gly and Thr399Ile) has been associated with decreased LPS responsiveness *in vitro* and *in vivo*104, [111]. In HEK293T cells that were transiently transfected to express CD14, MD-2, and either wild-type (WT) or mutated TLR4, we showed that the TLR4 variants that express the SNPs respond less robustly than the WT TLR4 to LPS as well as purified RSV F protein, despite equivalent TLR4 expression104. If both SNPs were simultaneously expressed on the same TLR4, the response to both LPS and purified F protein was reduced even further. Subsequently, we identified a highly significant association between RSV infection in a case series of high-risk infants/children with confirmed RSV and inheritance of these two TLR4 SNPs112. DNA from archived nasal lavage samples of participants with

documented RSV infection of two early, multi-center clinical trials of antibody-mediated prophylaxis13, 14 were genotyped for the TLR4 ectodomain polymorphisms (Asp299Gly and Thr399Ile). Specifically, 94 of 105 samples from high-risk infants with RSV (89.5%) derived from the original clinical trials of passively administered polyclonal or monoclonal anti-RSV antibodies were heterozygous for the Asp299Gly SNP, in contrast to 742 of 7,092 control healthy individuals (10.5%) derived from data pooled from 25 published studies. Similarly, 92 of 105 (87.6%) of these samples were heterozygous for the Thr399Ile SNP, compared to only 144 of 2,213 healthy controls (6.5%). Thus, inheritance of mutations that confer diminished LPS and F protein responsiveness are found at high frequency in high-risk infants with documented RSV infection. Tal et al.[113] also reported that inheritance of these same SNPs correlated with severity of RSV infection in a non-premature cohort.

In addition to TLR4, other TLRs have been implicated in RSV biology. TLR3 has been suggested to participate in cytokine and chemokine production in RSV-infected epithelial cells107, [108]. RSV infection of plasmacytoid dendritic cells (pDCs) blocked TLR7/8 signaling107 that are involved in the detection of viral single-stranded RNA. In addition, several laboratories have shown that TLR3 signaling induced by RSV could enhance later responses to the virus107, 114. Boukhvalova et al.,115 recently demonstrated that the pathology associated with RSV infection was increased in cotton rats treated with poly ICLC, a poly IC (polyriboinosinic-polyribocytidylic acid) stabilized with poly-L-lysine carboxymethyl cellulose, and a strong TLR3 agonist. Augmented lung inflammation was accompanied by an early induction of type I and II interferons, TLRs (*i.e.*, TLR1, 2, 3 and 7), and a stronger chemokine (GRO, MCP-1 and MIP-1α) response. In another recent paper, Murawski et al.[43] reported that TLR2/6 signaling in leukocytes can activate immunity against RSV by promoting TNF-α, IL-6, MCP-1, and RANTES; this signaling was important for controlling viral replication *in vivo*. Furthermore, it was demonstrated that RSV-TLR2 interaction promoted neutrophil migration and dendritic cell activation in the lung. In addition to TLRs, other intracellular PRRs, including retinoic acid-inducible gene I (RIG-I) and Nod2, were found to mediate the early antiviral response to RSV by inducing type I IFN [108, 116]. Collectively, these data support the existence of multiple PRRs that contribute to the host response to RSV infection and, hence, a more comprehensive characterization of the contribution of each to resistance to RSV will ultimately dictate the composition of a successful vaccine against RSV.

As discussed above, TLR4 signaling is distinguished from TLR2 signaling by the capacity to induce IFN-β via the "MyD88-independent pathway" that contributes to a second wave of transcriptional activity through STAT1 activation[99]. STAT1$^{-/-}$ mice exhibit severe illness (weight loss, characteristic pulmonary pathology) following RSV infection compared to IFN-γ$^{-/-}$ or WT BALB/c mice, despite similar viral titers and rates of clearance[55]. These data underscore the important role that type I (α/β) IFNs play in establishing a protective and safe antigen-specific immune response to RSV.

Ancillary TLR4 signaling mitigates pathology and lung cytokine expression in FI-RSV vaccine-enhanced disease

RSV F protein, like LPS, has been reported to be a TLR4 agonist53. We posited that formalin denaturation of F protein would diminish its ability to stimulate TLR4-mediated signaling. Experimental support for this hypothesis was provided by the observation that formaldehyde creates reactive carbonyl groups on RSV proteins that could be associated with vaccine-enhanced disease[117]. Moghaddam et al. showed that formaldehyde-treated RSV, used as a vaccine, reduced viral replication (1000-fold), but produced enhanced disease in the absence of neutralizing antibody titers. Therefore, we hypothesized that co-administration of a TLR4 agonist with FI-RSV might provide a surrogate signal that would compensate for diminished

TLR4 signaling induced by formalin-denatured F protein, and thereby counteract the enhanced pathology and gene expression profile normally induced by FI-RSV vaccination. Since LPS is toxic, even at low doses, due to its ability to elicit a strong proinflammatory response, we utilized monophosphoryl lipid A (MPL), a non-toxic lipid A derivative[118] that has been used for over 25 years as an adjuvant and continues to be extensively explored in human vaccine formuations119. Although MPL retains TLR4 agonist activity, it is considerably less potent than LPS and induces in macrophages diminished IL-12 and IFN-γ and increased IL-10 compared to LPS, perhaps accounting, in part, for its decreased toxicity120. Nearly a decade ago, Prince et al.121 immunized cotton rats with FI-RSV *vs.* FI-RSV + MPL. Upon subsequent RSV infection, cotton rats immunized with the FI-RSV + MPL exhibited greatly mitigated pathology upon subsequent RSV challenge with no protective effect on lung viral replication, despite a slight increase in neutralizing antibody titers. This early observation strongly suggested that protection from the pathologic effects of RSV were dissociable from virus replication (and hence, antibody titers). These findings were confirmed and extended in 2006 by Boukhvalova et al.[80]. Briefly, cotton rats were immunized twice with FI-RSV (FI) alone or together with MPL (FI+ MPL). Control groups included mock-vaccinated cotton rats (control for primary RSV infection), and cotton rats immunized by live RSV infection ("RSV IN") as the control for secondary RSV infection. Three weeks after boosting, all animals were challenged with RSV and lungs collected at various times post-infection for analysis of cytokine mRNA expression, histology, and viral load. As initially reported by Prince et al., co-administration of MPL with FI-RSV reversed pathology associated with vaccine-enhanced disease following RSV challenge80. Moreover, cytokine and chemokine gene expression profiles in lung samples revealed several important effects of MPL (Fig. 280). FI-RSV + MPL strongly inhibited expression of Th2 cytokines in response to RSV challenge (Fig. 2; compare "FI" with "FI+ MPL"): peak IL-4, IL-5, and IL-13, but not IL-10[80], mRNA were reduced significantly in "FI+ MPL"-vaccinated animals (Fig. 2, Th2). However, expression of all Th1 and other pro-inflammatory cytokine genes (*e.g.*, Fig. 4[80], Th1) was also significantly reduced early after RSV challenge compared to animals vaccinated with FI-RSV only. MPL similarly dampened expression of all chemokine genes examined (Fig. 4[80], *e.g.*, GRO). Thus, inclusion of MPL in the FI-RSV vaccine diminishes the host's capacity to respond pathologically when challenged with RSV by blunting the "cytokine storm" that is normally elicited. These findings strongly suggest that the engagement of TLR4 is critical for mitigating or precluding the pathology normally induced by primary RSV infection following vaccination with FI-RSV with essentially no change in viral replication in the lung (*i.e.*, 3.9; 4.4; and 4.2 $\log_{10}$ pfu RSV/gm lung tissue for FI-RSV, FI-RSV + MPL, and mock vaccination, respectively).

Possible role of increased antibody affinity in blunting of vaccine-enhanced disease

More than four decades have passed since the failed RSV-vaccine trials took place, and still the controversy surrounding the mechanism(s) by which vaccination with FI-RSV predisposes to the development of enhanced pathology upon RSV infection, including the two deaths in those trials, remain a topic of heated debate[122, 123]. At the time of the failed clinical trials of Lot 100, it was initially thought that FI-RSV pathology was antibody-mediated25. This view went out of favor since studies in cotton rat and subsequent clinical trials demonstrated conclusively that passively administered IgG was highly efficacious in preventing severe RSV pulmonary disease13, [14]. A role for antibodies in FI-RSV enhanced disease was revisited when Polack and colleagues demonstrated extensive peribronchiolar deposition of the classical (*i.e.*, antibody-mediated) complement cleavage product C4d in lung sections derived from the two infants that died during the FI-RSV vaccine trials in 1967[124]. The authors concluded that the pathology seen in FI-RSV-vaccinated children was the result of immune complex deposition containing either high levels of non-neutralizing antibodies generated by the poorly

immunogenic FI-RSV vaccine, or abundant low avidity antibodies derived from B cells that underwent "altered maturation." More recently, Delgado et al. reported that immunization of mice with FI-RSV or UV-inactivated RSV resulted in enhanced airway disease and lung pathology upon RSV challenge and correlated this with the production of low-avidity anti-RSV antibodies with a decreased IgG2a/IgG1 ratio[122]. Consistent with our earlier publications that the TLR4 agonist, MPL, mitigated enhanced disease induced by RSV infection of cotton rats vaccinated with FI-RSV[80, 121], Delgado et al. also reported that vaccinating mice with UV-RSV plus the potent TLR4 agonist, LPS, reduced enhanced RSV disease[122]. They concluded that inclusion of a TLR4 agonist with the UV-RSV blunted vaccine-enhanced disease by inducing B cell affinity maturation leading to increased antibody avidity. However, their conclusions have been recently challenged by Shaw et al.[123] who pointed out that the data presented by Delgado et al., "do not explain why an RSV vaccine that fails to block infection actually enhances subsequent disease." (*i.e.*, why is the pathology worsened over that seen in primary RSV infection?). Experiments using sensitive animal models vaccinated with the original Lot 100 vaccine and subsequently administered passively transferred antibodies of differing affinities will be required to test directly the correlation between antibody avidity and isotype, viral titers, and more importantly, protection from vaccine-enhanced RSV-induced pathology.

Possible role of alternatively activated macrophages in preventing RSV-induced pathology in primary and secondary infection

"Classically activated" macrophages (CA-Mϕ) differentiate in response to inflammatory stimuli, such as IFN-γ, in combination with TLR activation by microbial stimuli, such as Gram negative LPS. CA-Mϕ kill intracellular pathogens and secrete inflammatory cytokines that amplify Th1 immune responses. CA-Mϕ are also associated with the pathology seen in many inflammatory diseases and produce iNOS, the enzyme that generates nitric oxide (NO) that can damage cells. "Alternatively activated" macrophages (AA-Mϕ) differentiate in response to the Th2 cytokines, IL-4 and IL-13, and are functionally and biochemically distinct from CA-Mϕ. AA-Mϕ produce arginase-1 that competes with iNOS for arginine to produce L-ornithine and urea, rather than NO[125] as well as chitinase enzymes that have been implicated in tissue repair126. Although much work on the response to RSV has focused on whether a "Th1" or "Th2" adaptive immune response is induced, it is surprising that essentially no attention has been paid to RSV-induced differentiation of CA-Mϕ vs. AA-M ϕ, since their cytokine profiles are so similar to that of Th1 and Th2 cells, respectively.

A recent report by Shirey et al.[58] provides new insights into a highly novel mechanism by which RSV-induced lung pathology may be controlled. Specifically, RSV infection of murine lung and peritoneal macrophages, as well as a macrophage cell line, resulted in IL-4 and IL-13 production, IL-4Rα/STAT6-dependent AA-Mϕ differentiation, and led to significantly enhanced inflammation in lungs of IL-4Rα$^{-/-}$ mice. IL-4Rα is required for both IL-4- and IL-13-mediated signaling[127]. Adoptive transfer of highly purified WT macrophages to IL-4Rα$^{-/-}$ mice restored RSV-inducible AA-Mϕ phenotype and diminished lung pathology. RSV-infected TLR4$^{-/-}$ and IFN-β$^{-/-}$ macrophages and TLR4$^{-/-}$ and IFN-β$^{-/-}$ mice also failed to express AA-Mϕ markers, but exhibited sustained proinflammatory cytokine production (*e.g.*, IL-12) *in vitro* and *in vivo* and epithelial damage *in vivo*. TLR4 signaling was found to be required for RSV-induced PPARγ expression, a DNA-binding protein that induces AA-Mϕ genes, while IFN-β regulates IL-4, IL-13, IL-4Rα, and IL-10 expression in response to RSV. Thus, TLR4- and IFN-β-mediated signals contribute to the amelioration of RSV-induced pathology. It is tempting to speculate that the remarkable association between RSV susceptibility and the TLR4 SNPs that we previously reported might reflect a diminished responsiveness to RSV F protein or endogenous TLR4 agonists generated during RSV

infection, thereby leading to a decreased capacity for development of tissue reparative AA-Mϕ.

Secondary infection of cotton rats with RSV results in faster resolution of inflammation and no detectable viral titers[60]. When cotton rats were infected once, IL-4 and IL-13 mRNA were detected in the lungs during primary infection, but these genes, as well as the A-AMϕ marker, arginase-1 mRNA, were more strongly activated upon re-infection 60 days later[58]. This observation suggests that AA-Mϕ development during primary infection facilitates a less robust secondary RSV infection.

Cyclooxygenase 2 (COX-2) was previously shown in cotton rats to contribute to RSV-mediated pathology, as evidenced by the fact that COX-2- or prostaglandin E-specific inhibitors blocked RSV-induced pathology[128]. Treatment of RSV-infected cotton rats with a COX-2-specific inhibitor also increased expression of lung AA-Mϕ. Future studies using FI-RSV-vaccinated mice and cotton rats will be required to determine if manipulation of the macrophage activation state *in vivo* will also protect against vaccine-enhanced disease. Obviously, treating severely ill patients with agents or vaccines that favor development of AA-Mϕ might be expected to predispose the patient to airway hyperreactivity; however, given that our current therapeutic interventions often fail to save patients with severe RSV disease, the critical balance of mitigating severe RSV disease with managing asthma in later life may have to be accepted until a safe and efficacious vaccine can be developed.

Concluding remarks

The genetic and immunological components of the host that led to the disastrous results of vaccine trials of FI-RSV remain an enigma. The delineation of the mechanisms underlying vaccine-enhanced disease will require robust animal models that closely mimic the key components of the disease. The role that antibodies and cell-mediated immune responses play in the overall pathology of FI-RSV and the genetics of RSV sensitivity will continue to be grounds for investigation. However, the interaction of the F protein of RSV and TLR4 has emerged as an important component in the multifactorial host response to RSV infection that should be considered when developing vaccine to achieve safe, long-lasting protection against RSV.

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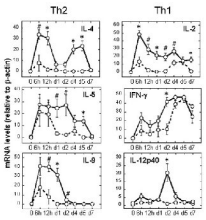

Fig. 1.

Lung cytokine gene expression during FI-RSV vaccine-enhanced disease. Cotton rats were vaccinated with mock- or FI-RSV vaccine (days 0 and 28). Four weeks later, animals were challenged with RSV and sacrificed at the indicated times. mRNA levels of prototype Th2 and Th1 cytokine genes were quantified by RT-PCR, and normalized to β-actin. Results represent the mean ± S .E.M. of 6–8 animals per time point for FI-RSV-vaccinated animals (solid line) and 4–6 animals per time point for mock-vaccinated (dotted line). #, $p < 0.05$; *, $p < 0.01$; (FI-RSV- *vs.* mock-vaccinated animals). This figure represents a subset of 19 genes examined. Adapted from reference[80].

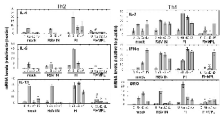

Fig. 2.

MPL blunts the "cytokine storm" associated with FI-RSV vaccine-enhanced disease. Cotton rats were vaccinated i.m. with FI-RSV alone (FI) or in combination with MPL (FI +MPL). Control animals were vaccinated with a mock vaccine (mock) or infected with RSV (RSV IN). After RSV challenge of all groups, animals were sacrificed at the indicated times and lung samples analyzed for expression of Th2 and Th1 cytokine mRNA as described in Fig. 3. Results represent the mean ± S.E.M. for 4 – 6 animals per time point. #, $p < 0.05$; *, $p < 0.01$; X, $p < 0.001$ (FI + MPL *vs.* FI treatment). Adapted from reference[80].