Antiviral Chemistry and Chemotherapy
2018, Vol. 26: 1–13

DOI: 10.1177/2040206618770518
journals.sagepub.com/home/avc
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Cotton rat model for testing vaccines and antivirals against respiratory syncytial virus

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Abstract

Respiratory syncytial virus is the leading cause of pneumonia and bronchiolitis in infants and is a serious health risk for elderly and immunocompromised individuals. No vaccine has yet been approved to prevent respiratory syncytial virus infection and the only available treatment is immunoprophylaxis of severe respiratory syncytial virus disease in high-risk infants with Palivizumab (Synagis®). The development of respiratory syncytial virus vaccine has been hampered by the phenomenon of enhanced respiratory syncytial virus disease observed during trials of a formalin-inactivated respiratory syncytial virus in 1960s. A search for effective respiratory syncytial virus therapeutics has been complicated by the fact that some of the most advanced respiratory syncytial virus antivirals, while highly effective in a prophylactic setting, had not demonstrated clinical efficacy when given after infection. A number of respiratory syncytial virus vaccines and antivirals are currently under development, including several vaccines proposed for maternal immunization. The cotton rat *Sigmodon hispidus* is an animal model of respiratory syncytial virus infection with demonstrated translational value. Special cohort scenarios, such as infection under conditions of immunosuppression and maternal immunization have been modeled in the cotton rat and are summarized here. In this review, we focus on the recent use of the cotton rat model for testing respiratory syncytial virus vaccine and therapeutic candidates in preclinical setting, including the use of special cohort models. An overview of published studies spanning the period of the last three years is provided. The emphasis, where possible, is made on candidates in the latest stages of preclinical development or currently in clinical trials.

Keywords

Animal model, respiratory syncytial virus, vaccine, immunotherapy

Date received: 17 November 2017; accepted: 12 March 2018

Introduction

Respiratory syncytial virus (RSV) is the leading cause of pneumonia and bronchiolitis in infants and it is associated with significant morbidity and mortality.[1] RSV is also an important cause of health risk in older adults and immunocompromised individuals.[2–5] No vaccine has yet been approved to prevent RSV infection. The development of such a vaccine encountered the significant hurdle of the phenomenon of enhanced RSV disease (ERD) observed during trials of a formalin-inactivated RSV (FI-RSV) in the 1960s when vaccinated infants naturally infected with RSV developed augmented illness.[6] The only prophylactic treatment against severe RSV disease in high-risk infants is available in the form of the monoclonal antibody Palivizumab (Synagis, MEDIMMUNE®). However, a number of RSV vaccines and antivirals are currently under development, including several vaccines proposed for maternal immunization.[7,8]

Animal models of RSV infection are instrumental for understanding disease pathogenesis and evaluating methods of intervention. Cotton rats, mice, sheep, Syrian hamsters, chinchillas, guinea pigs, ferrets, and nonhuman primates (NHP) have all been used to study RSV (reviewed in Taylor, 2017).[75] Of all the small animal models, the cotton rat (*Sigmodon hispidus*) appears to be the most permissive model for

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RSV replication. Although RSV replicates efficiently in the lungs and noses of infant ferrets, older ferrets are less permissive to RSV replication in the lower respiratory tract.[9,10] Cotton rats are 50–1000 fold more permissive for RSV replication than most common laboratory strains and mice, which display significant strain-to-strain variations in susceptibility to RSV.[11,12] Cotton rats carry a functional set of genes encoding Mx1 and Mx2 proteins, crucial components of human innate antiviral defense system.[13,14] In contrast, most common laboratory strains of mice lack functional Mx system, and murine antiviral defense relies mostly on adaptive immune mechanisms. In spite of its advantages, the cotton rat is not a natural model system of RSV infection and has its own limitations, including semi-permissiveness and requirement for a large-dose inoculum to induce disease. Additionally, the paucity of species-specific reagents and modified strains puts cotton rat at a disadvantage for studies on mechanisms of RSV immunity.

Cotton rats demonstrated translational value for clinical studies by correctly predicting efficacy and dose of immunoglobulin prophylaxis currently used against RSV disease in high-risk infants. Early studies in cotton rats indicated that pups born to RSV-immune mothers are protected from RSV disease even when nursed on nonimmune mothers.[15] Subsequent studies showed that intraperitoneal inoculation of RSV convalescent serum into cotton rats conferred protection without inducing immunopathology.[16] Serum neutralizing antibody (NA) titers of 1:100 were associated with some pulmonary protection, while titers of 1:380 conferred sterilizing immunity in the lung. This was consistent with studies in human infants under two months of age, in which children with maternally acquired antibodies at a titer of 1:400 were less susceptible to RSV disease, while infants with serum antibody titers of 1:100–1:200 still could develop RSV pneumonia and bronchiolitis.[17] The cotton rat model was used to predict the exact dose of immunoprophylaxis with Synagis that worked in human infants: 15 mg/kg. This dose of Synagis resulted in trough levels of about 40 μg/mL and was associated with 55% reduction of hospitalizations due to severe RSV disease in high-risk children.[18] The translational value of the cotton rat model of RSV disease was further confirmed by the demonstration that anti-RSV antibodies given to cotton rats after RSV infection do not reduce pathology in an animal lung[19] and afford no therapeutic benefit to human infants and young children treated after the onset of infection.[20,21]

In this review, we will focus on the recent use of the cotton rat model for testing RSV vaccine and therapeutic candidates in a preclinical setting. An overview of published studies spanning the period of the last three years is provided to show how the model has been used to achieve a defined set of goals. The emphasis, where possible, is made on candidates in the latest stages of preclinical development or currently in clinical trials. The examples provided represent only a small subset of all RSV intervention strategies being developed, with the selection made to emphasize the use of the cotton rat model for testing RSV vaccines and antivirals that has been published.

RSV replicates in the upper and lower respiratory tract of *S. hispidus*, with peak pulmonary viral load on day 4 and viral clearance from the lungs by day 7. Viral replication in the nose is slightly prolonged, with clearance by day 9.[11] A rise in serum NAs is evident by day 6 postinfection and is established in 100% of animals by day 9 postinfection. Disease is primarily inflammatory, with cellular infiltration in the lung peaking on day 5–6 postinfection. Inflammatory cells aggregate around bronchioles (peribronchiolitis), small blood vessels (perivasculitis), within alveolar walls (interstitial pneumonitis), and in the alveolar spaces (alveolitis). Alveolitis and interstitial pneumonia are disproportionately increased in RSV-infected animals previously immunized with FI-RSV, compared to mock-immunized RSV-infected animals or compared to animals that have been infected with RSV twice. Alveolitis serves as a marker of FI-RSV vaccine-enhanced disease in cotton rats and is subject to modulation by a particular adjuvant choice (e.g., monophosphoryl lipid A, MPL).[22–24] Vaccine-enhanced disease in cotton rats is accompanied by a broad dysregulation of cytokine responses, with enhanced expression of both Th1- and Th2-type cytokines, particularly evident within hours of RSV challenge of FI-RSV-immunized animals.[24] Reinfection of cotton rats with RSV results in no detectable virus production, however virus replicates abortively and pulmonary inflammation accompanies the process.[25] Upregulation of interferon response and expression of Mx genes are seen shortly after RSV reinfection.[14]

Basic structure of cotton rat preclinical studies

The protocols for testing RSV vaccines and antivirals in the cotton rat model have become relatively standard. In a typical vaccine study in the cotton rat model, animals are immunized with a candidate vaccine on day 0, boosted four weeks later and infected with RSV three weeks after boosting (Figure 1(a)). Samples are collected on days 4 and 6 postinfection, the time of maximal viral replication and pulmonary pathology, respectively. Day 5 can be selected as the single time point for analysis of both, replication and lung pathology, to minimize the use of animals. End points include viral replication in

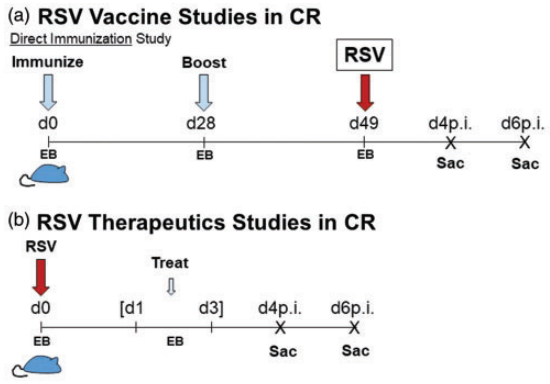

Figure 1. General diagram of RSV vaccine (a) or RSV antiviral (therapeutics) (b) study in the cotton rat model.
CR: cotton rat; EB: eye bleed; sac: sacrifice; p.i.: postinfection.

the lung and nose, pulmonary histopathology, and lung quantitative polymerase chain reaction (qPCR) for virus detection and cytokine gene expression. Serology end points such as serum NAs against RSV and binding IgG against viral antigens and/or RSV are assessed. Blood is collected for these assays at various times during the experiment, including before immunization, boosting, or challenge. Control groups include mock-immunized animals (primary infection control), animals primed via intranasal RSV infection (secondary infection control), and FI-RSV-immunized animals (ERD control).

In a typical RSV therapeutics study, animals are infected with RSV intranasally and the treatment with test article starts on day 1–3 postinfection (Figure 1(b)). The delayed onset of treatment (days vs. hours postinfection) is selected to demonstrate efficacy under conditions that approximate the time when RSV patients may develop symptoms. Treatment can continue for several days thereafter. Samples are collected for analysis on days 4 and 6 (or day 5) postinfection. Several control groups are included in the study, including mock-treated animals, animals treated with a reference antiviral (e.g., Synagis®, MEDIMMUNE), and untreated/uninfected animals. Samples are collected for analysis of viral replication in the lung and noses, pulmonary histopathology, and lung qPCR for virus and cytokines. Serum/plasma is collected before infection and at different times after treatment to assess drug kinetics. Prophylactic treatment study is structurally similar to therapeutics study with one important difference in that the treatment starts prior to rather than after RSV infection.

An overview of RSV vaccine testing in the cotton rat model

An impressive array of RSV vaccine candidates of different varieties is currently under development ("RSV Vaccine and mAb Snapshot," http://www.path.org/publications/detail.php?i=2747). Reports of preclinical testing of many of these candidates in the cotton rat model have been published in the past three years (Table 1). These candidates include live attenuated RSV strains modified by codon-deoptimization and modification/replacement of RSV NS, SH, F, and G genes,[26,27] virus-vectored vaccines expressing RSV F or G protein from the backbone of modified parainfluenza 5,[28,29] measles,[30] adenoviruses 26 and 35[76], or Sendai viruses;[31] DNA-plasmid-vectored vaccines,[32] virus-like particles (VLPs) alone, or in combination with DNA boost,[33–36] adjuvanted subunit vaccines,[37–39] and whole-virus inactivated preparations.[40]

Important findings emerged as a result of these preclinical studies in the cotton rat model. For example, Stobart et al., demonstrated that live attenuated vaccine candidate OE4 generated via codon-deoptimization of NS1 and NS2 (dNS), deletion of SH ΔSH, replacement of wt F with line 19 F, codon-deoptimization of G, and ablation of the secreted form of G has increased thermal stability and immunogenicity in spite of demonstrated significant attenuation in the upper and lower respiratory tracts of cotton rats.[27] Phan et al.[28] used cotton rats to show that subcutaneous administration of PIV5-RSV F vaccine is as efficacious as intranasal administration, emphasizing that this is important as in infants intranasal vaccination may lead to nasal congestion. Smith et al. used surface electroporation device to deliver SynCon DNA-based vaccine encoding RSV F protein to epidermal cells of cotton rats.[32] In this project, aimed at developing a single-dose intradermal DNA vaccine, Syn-Con RSV-F vaccine-induced complete pulmonary protection at a low dose and did not lead to ERD. Wang et al.[29] tested PIV5-vectored vaccines encoding RSV or G protein as a single-dose intranasal administration and found high efficacy and no induction of ERD. Fuentes et al., demonstrated that a recombinant adjuvanted RSV G protein vaccine induces pulmonary and nasal protection against RSV infection in the cotton rat model.[39] This vaccine also induced a NA response directed against not only the homologous RSV-A2 strain but also the heterologous RSV-B1 strain.

Cotton rat model for structural vaccinology studies

The cotton rat model has been used for studies on structural vaccinology in which immunogens are meticulously engineered based on available structural information. The RSV palivizumab epitope in the F protein and its variants have been displayed in the context of

Table 1. The use of the cotton rat model for testing respiratory syncytial virus (RSV) vaccines and antivirals. A selection of studies presenting results of testing RSV intervention strategies in cotton rats published between 2015 and 2017 is shown. Notation of vaccines and antivirals has been modified in some cases to provide a more uniform representation of platforms used. The reader is directed to original source for details on each test article referenced in this review. VLP: virus-like particle.

Use of the cotton rat model for testing RSV vaccines.

Vaccine type	Formulation	Publication
Live attenuated RSV	RSV-A2-dNS-ΔSH-BAF (DB1)	Rostad et al.[26]
	RSV-A2-dNS1-dNS2-ΔSH-dGm-Gsnull-line19F (OE4)	Stobart et al.[27]
Virus vectored	PIV5/F	Phan et al.[28]
	PIV5/F and PIV5/G	Wang et al.[29]
	MeV/F	Sawada and Nakayama[30]
	Ad26/F, Ad35/F	Widjojoatmojo et al.[76]
	SeV/F	Zhan et al.[31]
DNA-vectored	SynCon DNA-based vaccine encoding RSV F	Smith et al.[32]
VLP	VLP containing RSV F, G, and M	Walpita et al.[33]
	Palivizumab epitope in WHcAg-VLP	Schickli et al.[34]
	VLP/F + VLP/G	Cullen et al.[35]
VLP + DNA	F-encoding plasmid +VLP containing F&G	Hwang et al.[36]
Subunit	post-F and pre-F in GLA-SE	Schneider-Ohrum et al.[37]
	post-F in GLA-SE	Lambert et al.[38]
	RSV G in Emulsigen	Fuentes et al.[39]
Whole-virus inactivated	RSV-NE (RSVL19 nanoemulsion-inactivated, adjuvanted)	O'Konek et al.[40]

Use of the cotton rat model for testing RSV antivirals

Antiviral type	Formulation	Publication
Monoclonal antibody	MEDI8897	Zhu et al.[66]
Single-domain antibody	ALX-0171	Detalle et al.[67]
Fusion inhibitor	GS-5806	Mackman et al.[70]
Attachment inhibitor	SRI 29365	Evans et al.[68]
RSV-IVIG	RI-002	Boukhvalova et al.[71]

the Woodchuck hepadnavirus core-based VLP (WHcAg-VLP) to demonstrate efficacy of epitope-focused immunogen design in cotton rats.[34] With the recent discovery of methods to stabilize the F protein of RSV in the pre- *vs.* post-fusion conformation (reviewed in Graham et al.[41]), it became important to assess whether the pre-fusion form of the protein may provide an advantage to the more stable post-fusion conformation of F in defining a better vaccine antigen, and if the use of pre-fusion F may help to avoid ERD phenomenon. Several recent studies demonstrated that pre-fusion F protein adjuvanted with Adjuphos (aluminium phosphate gel) (Krarup et al., 2015)[74] or glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE)[37] can induce more potent NA responses in cotton rats. In the context of PIV5-vectored vaccine, however, the pre-fusion F was not able to induce a better NA response than wild type (mainly post-fusion) F protein.[28] The choice of RSV strain for assessing NA strength appears to make a difference. For example, while pre-F induced a stronger NA response against homologous RSV A strain in cotton rats, cross-NA response against RSV B induced by pre- and post-F was comparable.[37] This is in contrast to mice and NHP where pre-F induced stronger neutralization of both RSV A and B subtypes compared to RSV post-F.[42] Differences in the type of adjuvant used (GLA-SE in Scheider-Ohrum et al.[37] vs. poly(I:C) in McLellan et al.[42]), and differences in RSV B strains tested (RSV B9320 in Schneider-Ohrum et al.[37] vs. RSV 18537 in McLellan et al.[42]) could have contributed to the observed discrepancies. Importantly, neither pre-F nor post-F RSV subunit vaccines appeared to induce ERD in the cotton rat model when adjuvanted with GLA-SE and used at high dose of antigen.[37] At low vaccine dose adjuvanted with either GLA-SE or alum, however, ERD was detected for both protein conformations. These findings emphasize the importance of performing dose-de-escalation studies, as suboptimal dosing of RSV F subunit candidates, irrespective of F protein conformation type, may lead to ERD in target population.

Evaluating strength/duration/mechanisms of immunity

Challenging of cotton rats with 10^5 plaque-forming units (PFU) RSV is generally accepted as a standard dose for evaluating efficacy and safety of vaccines and antivirals. The challenge dose in some cases can be increased to $10^{5.5}$ or 10^6 plaque-forming units (PFU) per animal to evaluate the potency of vaccine or therapy or to increase the extent of the inflammatory changes subject to modulation by treatment. Interestingly enough, some of the recent reports suggest that increasing the challenge dose may not only augment inflammatory changes in the lung, but also lead to some qualitative differences in pathology. Thus, Hwang et al.[36] reported that challenge of FI-RSV-immunized cotton rats with a high-dose RSV A2 (10^6 PFU) may lead to weight loss, airway hyperresponsiveness, and the appearance of mucous plaques in the airways, the features not normally accompanying ERD in FI-RSV-immunized animals challenged with 10^5 PFU of virus.[23,24] Possible strain variations, differences in FI-RSV preparation method and differences in methodologies of infection and/or harvesting used by different laboratories could result in these variations.

For vaccine studies, the period between immunization and challenge normally spans several weeks, but can be increased to several months if duration of immunity is a parameter to be assessed and if delayed hypersensitivity is suspected. Schneider-Ohrum et al. evaluated duration of immunity to the pre-F and post-F protein vaccines and found that the NA responses remained above the protective threshold of 8.5 $\log_2$ in the cotton rat model[15] 380 days after immunization.[37] They also extended the period to challenge to evaluate whether ERD is seen three months after immunization. Animals immunized with RSV F protein, adjuvanted with alum, did develop ERD after the delayed challenge, but only when low dose of antigen (0.05 μg) was used. This is in contrast to earlier findings by Murphy et al.,[43] where immunization with high rather than low dose of antigen led to ERD. The differences in these results could be attributed to differences in the source of the RSV F protein and its purity.

The cotton rat model has also been used to evaluate the potential contribution of antibody-dependent enhancement (ADE) to the severity of RSV disease.[44] One of the proposed contributing factors of FI-RSV ERD in infants has been the low avidity antibodies generated in response to FI-RSV vaccination.[45] ADE of RSV infection has been demonstrated in monocytic cell lines that carry Fc-receptor.[46,47] Recently, Van Erp et al.[44] have analyzed ADE in infants hospitalized with severe RSV disease and in cotton rats immunized with FI-RSV. The effect was compared to ADE in infants with moderate RSV disease and in cotton rats with regular RSV infection. The ADE effect was seen in both mild and severe cases and was concluded not likely to be a contributing factor in severity of RSV disease.

Special cohorts models: Maternal immunization

Maternal vaccination is being proposed as one of the methods for controlling RSV-induced severe disease in infants.[7,48] Although higher levels of RSV-specific maternal antibodies present during the first months of life correlate with reduction in RSV-associated acute lower respiratory infection (ALRI),[49,50] the peak incidence of RSV disease is observed between two and eight months of age.[50–52] This coincides with the time when maternal IgG levels are waning (Chu and Englund, 2014; England et al., 1998).[48] This age group of infants is unlikely to benefit from active immunization, due to the presence of passively transferred maternal immunity that can inhibit the efficacy of vaccination (Englund et al., 1998).[53,54,73] Evaluating candidate vaccines for maternal immunization requires a complex animal model in which efficacy of vaccination is ultimately assessed in a different population than the immediate recipients of vaccination. Thus, numerous parameters need to be measured, including efficacy and safety of vaccination in the mothers, levels of transferred protection to the infants, the duration of the protection, and the safety parameters in the infants. In recent years, we have expanded the earlier cotton rat model of maternal immunity[16] to include all these parameters.[55,56]

The model uses female cotton rats, which are naive (unprimed) or primed via intranasal infection with RSV to mimic preexisting immunity (Figure 2). Using primed animals better approximates real-life scenario in which mothers are seropositive for RSV. Intramuscular inoculation with live RSV is included as one of the immunization approaches to monitor the transfer of immunity and to verify the immunosuppressive effect of preexisting immunity.[55] Primed females are set up for mating with naive males and the resulting pups are challenged with RSV at various times after birth (Figure 2(a)). Significant pulmonary protection against RSV replication is seen in one-week-old pups born to mothers primed with RSV either intranasally (i.n.) or intramuscularly (i.m.) (Figure 2 (b)). This protection is progressively diminishing as pups become older, and is significantly reduced once pups reach four weeks of age. The decline in pulmonary protection is paralleled by the decay of the maternally transferred NA in the serum of the pups (Figure 2

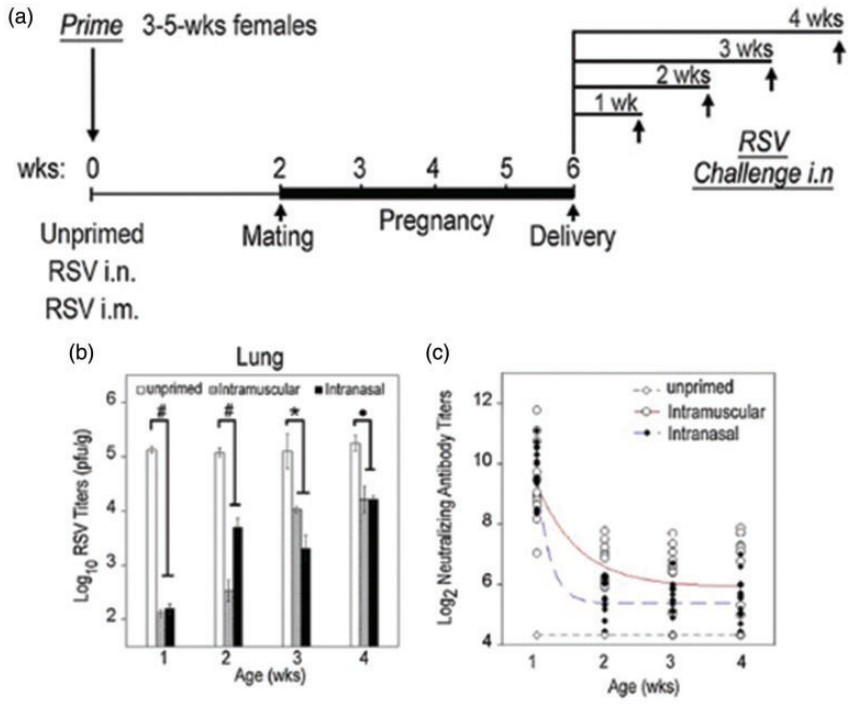

Figure 2. Maternal transfer of immunity in cotton rats. (a) Scheme of the protocol of RSV priming and transfer of immunity by RSV-primed cotton rat mothers. Female animals were primed with live RSV A/long (10^5 PFU/100 μL/animal), given intranasally (i.n.) or intramuscularly (i.m.), or left unprimed. Females were set in mating pairs and four weeks later they gave birth to litters that were subsequently analyzed by RSV challenge at one, two, three, and four weeks after birth. Number of pups in each group was between 12 and 22 animals. (b) Lung RSV titers four days after challenge of litters born to mothers unprimed, primed intramuscularly or intranasally. Bars represent $M \pm SE$. Significance was assessed by one-way analysis of variance followed by Student–Newman–Keuls post hoc test. #$p < 0.0001$; *$p < 0.01$; •$p < 0.05$. (c) Decay of maternal RSV NA titers during the first month of life of infant cotton rats whose mothers were unprimed or primed with RSV intranasally or intramuscularly. Adapted from Blanco et al., Vaccine 2015. Note: i.n.: intranasally; i.m.: intramuscularly; wk(s): week(s).

(c)), and there is a significant correlation between high level of serum NA shortly after birth and strong pulmonary protection at that time. Maternally transferred NA drops abruptly between weeks 1 and 2 in pups born to females infected intranasally, and less abruptly if females received RSV i.m., and stabilize at a low titer (~64) within a month. The rapid decrease in protection correlating with a decline in circulating maternal NA in cotton rats resembles the phenomenon observed in human infants, although it occurs more rapidly in cotton rats. The faster waning of maternal immunity in cotton rats may be due in part to shorter half-life of circulating maternal antibodies in cotton rats compared to humans (7 vs. 20–80 days).[16,57–59]

For studies on the efficacy of immunization during pregnancy, primed cotton rats (i.n. RSV infection) are vaccinated twice with a test vaccine (e.g., live RSV i.m.) administered first before mating and then as a boost during pregnancy (Figure 3(a)).[55] For one-week-old pups, single-time immunization with RSV i.m. before pregnancy does not provide pups with an advantage in protection against RSV or in the amount of NA they received from primed mothers (compare groups B and C in Figure 3(b) and 3(c)). However, boosting of prospective mothers during pregnancy significantly increases the amount of NAs and it results in almost complete protection of lungs in one-week-old pups (compare groups B and D). For four-week-old animals, both immunization before pregnancy or boosting of primed mothers during-pregnancy has a significant impact on serum NA and pulmonary protection, indicating that maternal immunization has an even larger impact at the time of waning immunity.

The safety of RSV vaccines has been of paramount importance since the realization of existence of ERD during unfortunate FI-RSV vaccine trials in the 1960s. FI-RSV immunization has been tested in the cotton rat model of maternal immunity, when FI-RSV is administered to mothers or to infants.[56] In the first scenario, prospective cotton rat mothers, primed via i.n. RSV infection, are vaccinated with FI-RSV twice (before and during pregnancy), and their infants are challenged

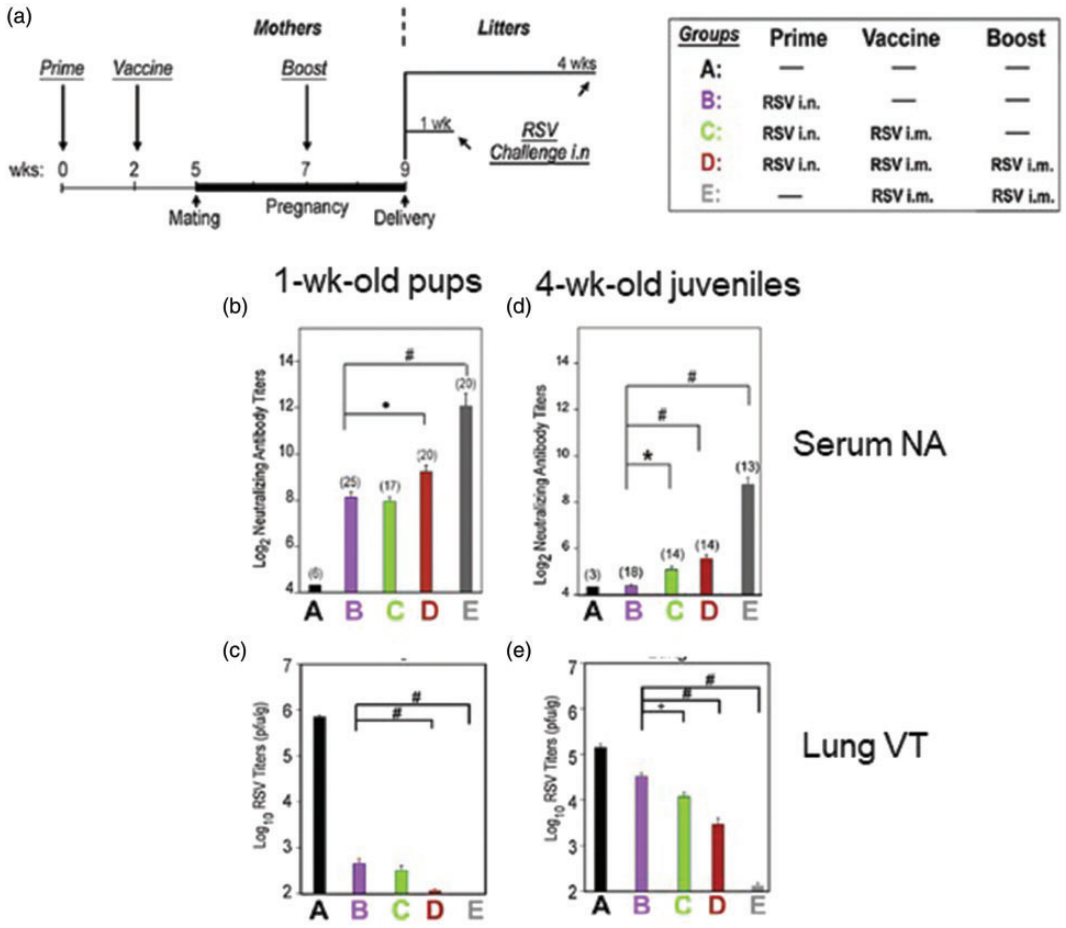

Figure 3. Transfer of RSV immunity after vaccination during pregnancy. (a) Protocol of priming and maternal vaccination against RSV. Female cotton rats in Group A remained naive. Female cotton rats in Groups B to D were infected with RSV i.n. (Prime), whereas animals in Group E were left unprimed. Two weeks after priming, animals in Groups C–E were inoculated with live RSV i.m. (10^5 PFU, vaccine). All females were set in mating pairs five weeks postinfection and those in Groups D and E were boosted with the same dose of live RSV i.m. during pregnancy (week seventh, Boost). Females started deliveries on week 9 after priming. (b, d) NA titers in one-week-old pups and four-week-old juveniles born from group of mothers described in A. Number in parenthesis represents total amount of animals per group. (c, e) Pulmonary protection against RSV in newborn and juvenile cotton rats born from vaccinated mothers. Bar represent the $M \pm SE$. Significance of vaccination during pregnancy was evaluated between Group B (primed only group) and Groups D or E (primed, vaccinated, boosted; or not primed, vaccinated, and boosted, respectively); $N = 13$–25 pups per group. Comparison of responses between pups in Group B and pups in Groups C, D, and E was assessed by one-way analysis of variance followed by Student–Newman–Keuls post hoc test. $\#p < 0.0001$; $+p < 0.005$; $\bullet p < 0.05$. Adapted from Blanco et al., Vaccine 2015. NA: neutralizing antibodies; VT: viral titers.

with RSV at one or four weeks of age for analysis the occurrence of ERD. No enhanced pathology or altered cytokine expression is seen in pups born to FI-RSV-immunized mothers. When pups rather than mothers receive FI-RSV immunization, they develop FI-RSV ERD independent of whether they were born to naive mothers or mothers primed via intranasal RSV infection, reemphasizing that seropositive status of mothers is not likely to protect infants from developing ERD after immunization with an "unsafe" vaccine. Interestingly enough, pups immunized with live RSV i.m., a vaccine generally considered "safe," developed ERD if they were born to primed compared to naive mothers. This suggests that maternally transferred immunity may modify vaccine profile and points to the potential need to evaluate candidate vaccines for infants in the face of preexisting immunity.

Maternal immunity studies in cotton rats revealed an additional interesting phenomenon, namely that the level of vaccination-induced RSV NA abruptly drops in expectant cotton rat mothers shortly before delivery.[56] Primed pregnant animals immunized with various vaccine preparations, including RSV-F + MPL, RSV F alone, or live RVS i.m. lost 70–85% of all

RSV NA antibodies within a week of delivery. This drop occurred over a period of just two weeks. For comparison, the natural decline of RSV NA in infected nonpregnant, or vaccinated nonpregnant female cotton rats occurred slowly and amounted to a gradual 40% decline over a 10-weeklong period. It is not known whether a similar decline in vaccination-induced RSV NA antibody levels may occur in pregnant human females shortly before delivery. However, a decline in influenza HAI titers in pregnant HIV-positive females vaccinated with pH1N1 vaccine during pregnancy has been noted to occur at the time of delivery.[60] If a similar decline occurs in RSV NAs in human females, it may have a deleterious impact on maternal defenses against RSV during the late stage(s) of pregnancy or postpartum.

Use of the cotton rat model for study of RSV antivirals

The cotton rat achieved its current status as the preferred translational small animal model of RSV infection by confirming efficacy and predicting the dose of immunoglobulin RSV antivirals. Palivizumab (Synagis®, MEDIMMUNE), the only market-approved drug against severe RSV disease in high-risk infants, advanced to licensure based on the strength of cotton rat studies alone. The cotton rat model predicted not only the efficacy of antibody immunoprophylaxis but also the exact dose of drug (15 mg/kg) to be effective in human infants. The model was instrumental for predicting that nasal protection would be incomplete in human infants receiving antibody immunoprophylaxis[66] and that therapeutic administration of anti-RSV antibody preparations is not as effective as prophylactic treatment.[20,21,62–64] The cotton rat model demonstrated that therapeutic administration of anti-RSV antibodies reduces viral replication, but has little effect on pulmonary pathology associated with RSV infection. Combating both viral load and inflammation required coadministration of antiviral and anti-inflammatory agent in the model.[19,25]

In contrast to the wide list of RSV vaccine candidates currently undergoing evaluation, the number of RSV antivirals in advanced preclinical/clinical testing appears to be more limited and includes MEDI8897 (MEDIMMUNE), ALX-0171 (Ablynx), GS-5806 (Presaltovir, GILEAD), ALS-8176 (ALIOS), and ALN-RSV01 (ALNYLAM).[65] Subsequently, the number of antivirals with published reports of testing in the cotton rat model in the past three years is smaller than the list of vaccines (Table 1). Devising an effective therapeutic intervention against RSV-induced disease is a challenging task. Several RSV antivirals proven effective in prevention of RSV infection, including IVIG, RSVIg (RespiGam®, MEDIMMUNE), and palivizumab (Synagis®, MEDIMMUNE), did not produce clinically beneficial outcome when given therapeutically.[20,21,62–64] Existing evidence suggests that clinical efficacy by a single antiviral can be obtainable if the antiviral candidate has an anti-inflammatory component in its action and/or, if the immunosuppression is involved (see below). An effective RSV antiviral would also need to control viral infection not only in the lower, but also in the upper respiratory pathways. Animal models show that the task of protecting the lungs is more easily achieved than the task of protecting the nose. MEDI8897 (MEDIMMUNE), one of the RSV antivirals currently in clinical trials, is the humanized monoclonal antibody based on D25 directed against the prefusion-specific antigenic site Ø of the RSV F protein. MEDI8897 was engineered by introducing a modification into Fc region that significantly extends antibody half-life in vivo. MEDI8897 was efficient in protecting both the nose and lungs of cotton rats, as opposed to Palivizumab that did not inhibit RSV replication in the nose at the dose tested (8 mg/kg).[66]

ALX-0171 (Ablynx) is one of the most advanced RSV antivirals currently tested. Detalle et al.[67] evaluated the intranasal delivery of nanobody ALX-0171 in prophylactic and therapeutic modes of treatment using the cotton rat model and found both to be highly effective against RSV replication. ALX-0171 is a trimeric anti-RSV nanobody that has entered Phase IIb clinical trials in infants hospitalized with RSV infections in 2017. ALX-0171 demonstrated acceptable safety and tolerability in Phase I/IIa trial when administered daily via inhalation (https://www.europeanpharmaceuticalreview.com/news/40828/alx-0171-infant-rsv-study/). ALX-0171 demonstrated high antiviral efficacy in cotton rats when administered intranasally after RSV (strain Tracy) challenge (day 2 and/or day 3) or via nebulization 1 h before RSV challenge.[68] The clinical efficacy of ALX-0171 will be evaluated as a part of Phase II studies.

The cotton rat model helps to rank targets for RSV therapeutic intervention. The majority of advanced anti-RSV antivirals target RSV F protein and its fusion ability. Other RSV proteins are being explored as potential targets as well. Benzimidazole analog SRI 29365 (Southern Research) (1-[6–(2-furyl)**[1,2,4]**triazolo**[3,4-b][1,3,4]**thiadiazol-3-yl]methyl-1H-benzimidazole) inhibits attachment of the RSV G protein to target cells. While SRI 29365 effectively inhibited viral replication in vitro, it was not effective in reducing RSV replication or associated disease in the cotton rat model, potentially suggesting that G may be a suboptimal target of RSV antivirals in vivo.[68] Studies in BALB/c mice, however, suggest that antibodies

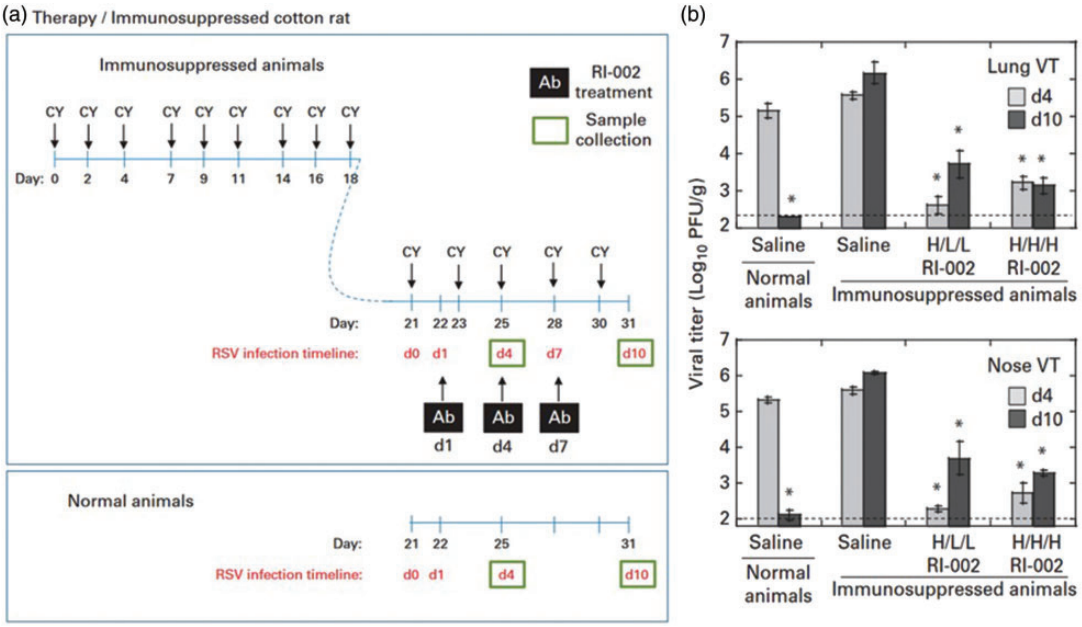

Figure 4. RI-002 therapy of RSV infection in immunosuppressed cotton rats. (a) The diagram of the experiment. Immunosuppression was induced by treating animals with 50 mg/kg of cyclophosphamide (CY) three times a week over the course of 18 days before RSV infection. On day 21, animals were infected with 10^5 PFU of RSV A/Long per animal. On days 1, 4, and 7 postinfection, RI-002 treatment (Ab) was administered. Groups of animals were killed on days 4 and 10 postinfection for sample collection. CY treatment was continued until the end of the study. As an internal control for RSV infection without immunosuppression, age-matched normal animals were infected with RSV, treated with saline and killed on days 4 and 10 postinfection. (b) Effect of RI-002 therapy on RSV replication in immunosuppressed cotton rats. RI-002 treatment was given as a high/low/low (H/L/L) regime of 1500 mg/kg followed by two doses of 750 mg/kg, or as high/high/high (H/H/H) regime of 1500 mg/kg given three times. Control immunosuppressed RSV-infected animals were inoculated i.p. with saline. Experiments included five animals per group; results represent the geomean $\pm$ *SE* Pulmonary and nasal viral titers (VT) were quantified by plaque assay in samples collected from normal and immunosuppressed cotton rats on days 4 and 10 postinfection. Results represent the $M \pm SE$ for five animals per group. *$p < 0.05$ when compared to RSV-infected immunosuppressed animals treated with saline and killed on the same day. Adapted from Boukhvalova et al., BMT 2016. CY: cyclophosphamide.

targeting RSV G protein reduce viral replication and RSV-associated pathology.[69]

GS-5806 (Presaltovir, GILEAD), an oral fusion inhibitor, showed antiviral efficacy in the cotton rat model of RSV infection and subsequently in Phase IIa study in healthy human volunteers experimentally infected with RSV.[70] In human volunteers, GS-5806 also appeared to reduce disease severity compared to placebo. Testing of GS-5806 in hematopoietic cell transplant (HCT) recipients has been completed earlier this year (https://clinicaltrials.gov/ct2/show/NCT02254421). Data on clinical efficacy of GS-5806 treatment in HCT-recipients, when available, would be of particular interest from the standpoint of potentially different mechanisms of RSV-induced pathology in normal and immunosuppressed subjects.

RSV affects between 2% and 17% of all hematopoietic stem cell transplant recipients (HSCT), with infection progressing to lower respiratory tract infection in 17–84% of those cases. The mortality rate of RSV lower respiratory tract infection in HSCT patients, if left untreated, can reach 83% or higher. Studies in patients with hematologic disorders and in recipients of HSCT show that they maintain dramatically prolonged RSV replication, with a reported median duration of 30.5 days. Condition of immunosuppression has been modeled in the cotton rat. Cotton rats imunosuppressed via repeated cyclophosphamide administration have significantly delayed RSV clearance (Figure 4). In animals infected with 10^5 PFU RSV A/Long, $\sim$6 $\log_{10}$ PFU of virus is seen in the lungs and noses of immunosuppressed animals 10-days post-infection, while infection is cleared from naive animals by that time. Moreover, viral dissemination to organs outside of respiratory tract, with RSV detected in both, liver and kidney. This model has been used for testing efficacy of RSV antivirals, such as RSV-IVIG formulation RI-002.[71] RI-002 (ADMA) administered three times on days 1, 4, and 7 postinfection of immunosuppressed animals with RSV inhibited RSV

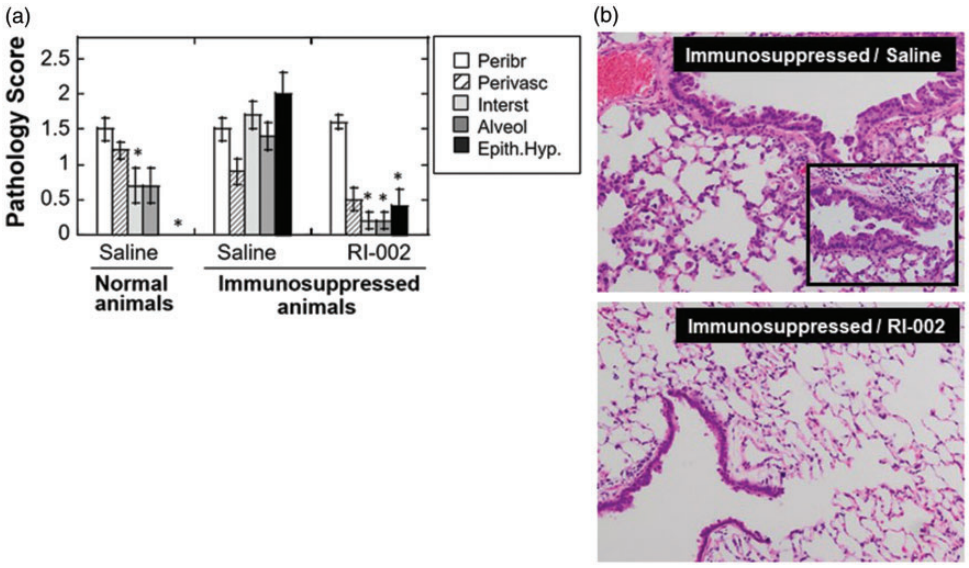

Figure 5. Effect of RI-002 therapy on pulmonary histopathology in RSV-infected immunosuppressed cotton rats. Lung samples were collected from RSV-infected animals on day 10 post infection (details are described in Figure 4 legend). (a) Pulmonary histopathology was evaluated in hematoxylin and eosin (H&E) slides in each of the following categories: peribronchiolitis (Peribr), perivasculitis, (Perivasc), interstitial inflammation (Interst), alveolitis (Alveol) and epithelial hyperplasia (Epith.hyp.). Results represent the $M \pm SE$ for five animals per group. $^*p < 0.05$ when compared with RSV-infected immunosuppressed animals treated with saline. (b) Photomicrographs of lung sections with focus on terminal bronchioles and surrounding parenchyma. The top panel shows the lung of immunosuppressed, RSV-infected animal treated with saline. Alveolar septa is thickened by infiltrates of inflammatory cells (interstitial pneumonia). Exudates into the alveolar air spaces (alveolitis) are visible. Note the hyperplasia of the bronchiolar mucosal epithelium. The inset is a 400× snapshot of a terminal bronchiole that shows that epithelial cells are haphazardly piled into multiple layers. The lower panel shows the lung of an immunosuppressed, RSV-infected animal treated with high-dose regime of antivirals (H/H/H RI-002). Lung damage is significantly reduced. Alveolitis, interstitial pneumonia and epithelial hyperplasia are minimal to absent. H&E stain, 200×. Adapted from Boukhvalova et al., BMT 2016.

replication in the lungs and noses, limited systemic dissemination of virus, and reduced pulmonary pathology (Figures 4 and 5). The anti-inflammatory activity associated with postinfection antiviral treatment alone suggested that excessive viral replication under conditions of immunosuppression may be a driving factor of RSV pathogenesis in immunocompromised individuals. By extension, controlling delayed viral clearance in immunosuppressed subjects may be sufficient for clinical efficacy that is difficult to achieve in normal individuals receiving antiviral RSV therapy alone. Recently completed Phase III study of RI-002 in patients with various forms of primary immunodeficiency disease reports that the treatment was safe and efficacious.[72]

Overall, the cotton rat model has been used to evaluate a wide array of RSV vaccine candidates ranging from attenuated RSV strains, to virus-vectored and VLP vaccines, to whole RSV inactivated virus preparations, and a number of RSV antivirals that include antibodies and small molecule inhibitors. The model has been used to evaluate efficacy and safety of treatments as well as to optimize treatment routes, formulations, and dosing regimens. Structural vaccinology studies have been conducted in the cotton rat model to facilitate structure-driven design of RSV vaccine, and special models have been expanded that include immunosuppressed animals and the model of maternal immunization that includes RSV-primed mothers. Selection of the right type of cotton rat model is crucial for a successful product development. Majority of vaccine studies referenced in this review have been conducted in RSV-naïve cotton rats. These studies yield invaluable information on vaccine safety and immunological quality of the antigen. They are also instrumental for identifying the most promising vaccine candidates to advance to further testing and for highlighting optimal doses/adjuvants/regimes of vaccination. It is important to emphasize, however, that unless a vaccine is intended for RSV-seronegative population of infants/young children and will be tested in such, these studies represent only one step en route to a successful vaccine development. To predict vaccine performance in a real-life scenario where RSV-seropositive subjects are involved, candidate formulations should be further tested in seropositive animal models rendered immune to RSV by prior exposure to the virus.

Acknowledgement

Sigmovir Biosystems, Inc. is a contract research organization specializing in the use of the cotton rat model for testing vaccines and antivirals against human infectious diseases.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the authorship, and/or publication of this article.

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