Chapter 5

Experiences of using the cotton rat model for influenza vaccine evaluation

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ABSTRACT

Cotton rats have successfully been infected with clinical strains of influenza viruses without pre-adaptation of the virus like required for mice. Disease symptoms and course of the infection have been studied in detail. Cotton rats have also been used to evaluate influenza vaccines. However, information on advantages and disadvantages of using this model and on points to consider while exploiting this model for influenza vaccine evaluation is limited. Here we show with whole inactivated virus (WIV) influenza vaccine that a single intramuscular immunization with a dose of ≥0.5 µg was immunogenic in cotton rats. Administration of a booster dose significantly increased the humoral immune response. To evaluate protective efficacy, cotton rats were challenged with a clinical isolate of H1N1pdm virus. Non-vaccinated animals were highly susceptible for the infection, presented with high lung virus load on day one and three post challenge and half of the animals succumbed quickly without showing signs of sickness reliably predicting the rapid death. In contrast, vaccinated cotton rats had low or undetectable lung virus titers and two out of four animals of the 0.5 µg group survived the challenge. In spite of low lung virus titers and less pronounced induction of IFNα upon challenge, most vaccinated animals showed increase in breathing frequency though in some cases this increase was of shorter duration than in non-vaccinated animals. Our data suggests that cotton rats are a suitable animal model for influenza infection and vaccine evaluation but careful fine tuning of the experimental parameters is required.

Key words: Cotton rats, whole inactivated virus (WIV) influenza vaccine, humoral immune response, virus challenge, lung viral load, breathing frequency.
Cotton rat model for influenza vaccine evaluation

INTRODUCTION

Cotton rats (Sigmodon hispidus) have been used as a small animal model for respiratory virus infections such as human parainfluenza virus, respiratory syncytial virus (RSV), measles virus, human metapneumovirus and as well as for influenza virus infection and pathogenesis studies. All these respiratory viruses can easily replicate in cotton rats and induce pathogenesis similar to that in humans. Unlike mice, cotton rats are susceptible to clinical isolates of influenza virus without prior adaptation of the virus. Indeed, cotton rats have been infected successfully with a broad range of clinical influenza virus strains. Upon infection, cotton rats show easily quantifiable disease symptoms like increased breathing frequency, reduced body weight and decreased body temperature.

Despite the positive results with cotton rats as a model for influenza infection, there is so far limited information on the performance of influenza vaccines in cotton rats. Early studies evaluated the effect of infection-induced immunity on subsequent challenge with homologous or heterologous virus strains. Later the studies were extended to UV-inactivated virus, seasonal trivalent split vaccine and more recently recombinant adenovirus vaccines and AS03-adjuvanted A/H1N1pdm09 pandemic influenza vaccine. Some of these studies report on reduced increase in breathing frequency (BF) in immunized versus naïve cotton rats upon challenge. Some studies investigated additional parameters like lung virus titers and lung pathology and/or determined induction of antibodies. However, none of the studies gives a complete description of clinical symptoms like changes in weight and temperature. Moreover, none of the studies describes evaluation of vaccines against H1N1pdm virus, a clinically highly relevant virus which is still in circulation.

The aim of this study was to obtain more detailed insight in the advantages and disadvantages of cotton rats for the evaluation of influenza vaccine candidates against a clinically relevant virus. To this end, we immunized cotton rats once or twice with different doses of a whole inactivated virus (WIV) influenza vaccine derived from a H1N1pdm vaccine strain and challenged the animals with a clinical isolate of H1N1pdm. Antibody titers in immunized animals correlated with vaccine dose and were boosted by a second immunization. Upon challenge, some animals of the non-immunized control group and the low dose immunization group succumbed quickly without prior overt signs of their poor condition. In the surviving immunized animals, lung virus titers on day one post challenge were strongly reduced as compared to titers in control animals. Yet, immunization had limited effects on clinical symptoms like tachypnea and disease parameters or on induction of cytokines and Mx1 in the lungs. We conclude that cotton rats are useful for evaluating protective effects of influenza vaccines but experimental conditions have to be fine-tuned and readout parameters have to be chosen carefully.
**MATERIALS AND METHODS**

**Vaccine and virus**

NIBRG-121, a vaccine strain produced from A/California/7/2009 virus, was obtained from NIBSC (Potters Bay, UK) and grown in embryonated chicken eggs. The virus was inactivated by overnight treatment with 0.1% β-propiolactone (Acros Organics, Geel, Belgium) in citrate buffer (125 mM sodium citrate, 150 mM sodium chloride, pH 8.2) at 4°C to produce whole inactivated virus (WIV) vaccine. Inactivation was followed by dialysis against HNE buffer (145 mM NaCl, 5 mM Hepes, 1 mM EDTA, pH 7.4, sterilized by autoclaving). Inactivation of the virus was verified by inoculation of MDCK cells and the amount of protein was determined by micro-Lowry assay.

A clinical isolate of H1N1pdm (isolate E9-6714) was provided by the Department of Clinical Virology, UMCG. The virus was diagnosed by qPCR as being similar to A/California/7/2009 virus. This virus will be called A/Cal/Gro in the following. The virus was further amplified on MDCK cells, titrated in cotton rats and was used as challenge virus. Virus titer was determined by TCID50 titration. Whole inactivated A/PR/8/34 (H1N1) and X-31 (H3N2, reassortant strain of A/Aichi/68 and A/PR/8/34 viruses) used for determination of cross-reactive IgG ELISA were kindly provided by NIBSC, UK.

**Cotton rats and immunization**

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (IACUC-RuG), The Netherlands. Outbred female cotton rats at an age of 10-12 weeks were purchased from Harlan Laboratories, USA. The animals were housed in individually ventilated cages with two cotton rats per cage. All the cotton rats were injected with implantable electronic ID transponders (Bio Medic Data Systems Inc (BMDS), Delaware, USA) subcutaneously (s.c.) for individual identification and temperature measurement. The weight of the cotton rats ranged between 120 and 150 grams during the challenge phase. A sample size of 4 animals per group was based on the literature.

The study comprised two independent experiments the details of which are described in Table 1.

Cotton rats were vaccinated via the intramuscular (i.m.) route with 100 µl vaccine distributed over the hind limbs. Four weeks after the single immunization in Experiment 1 or the 2nd immunization in Experiment 2, cotton rats were challenged with A/Cal/Gro, 5\(^{10}\) TCID50 in Experiment 1 and 1\(^{10}\) TCID50 in Experiment 2. Inoculation doses were chosen on basis of the literature (Blanco et al., 2013) and were tested in cotton rats prior to the immunization/challenge experiments. The virus in a volume of 100 µl was distributed over both the nostrils using a pipette. Both vaccination and challenge were...
cotton rats were sacrificed. For Experiment 1, various clinical symptoms were assessed and the animals were followed for ten days for assessment of clinical symptoms.

Assessment of clinical symptoms and sample collection
After challenge, cotton rats were followed daily for determination of changes in weight, temperature and breathing frequency for three days in the 1st experiment and ten days in the 2nd experiment. Animals were weighed by catching them into a pre-weighed cardboard roll. Weight loss of 10% in one day or 15% from the day of challenge was considered as criteria for the humane endpoint. Ten days post challenge the remaining cotton rats were sacrificed.

Breathing frequency (BF) was measured by plethysmography as described previously. Briefly, the animal was placed in a 1,500 ml air-tight but transparent tube of a whole-body plethysmograph which was connected to a pressure transducer. The frequency of pressure changes inside the tube was recorded and displayed as breaths per minute (bpm). A mean BF of an animal was then calculated from a minimum of four steady regions lasting for at least 15 seconds. For many of the animals, if they breathed at a constant rate we even recorded breathing for a minute or more consecutively. Maximal variation between the readings was +/- 5 to 10 breaths/minute, thus about 1-3%. Temperature was measured while the animal was restrained in the cardboard container using a DAS-7008/9 detector for s.c. injected electronic ID transponders (BMDS, Seaford, USA).

Blood was drawn on the day(s) of immunization, the day of challenge and the days of sacrifice. Serum was separated and stored at -20°C until assessment of IgG, neutralizing (MN) and hemagglutination inhibition (HI) antibodies. A small part of the same lung was stored in RNAlater (QIAGEN, The Netherlands) for cytokine profiling by quantitative real time polymerase chain reaction (qRT-PCR).

Lung virus titration
Equally sized parts of the lungs were collected in 1 ml complete EPISERF medium (100 U/ml penicillin, 100 mg/ml streptomycin, 1 M HEPES, 7.5% sodium bicarbonate, all Life Technologies BV, Bleiswijk, The Netherlands) and were homogenized, centrifuged and the supernatants were used for determination of viral load in the lungs as described previously. Virus amounts are represented as log_{10} titer per ml of medium.

Carried out under 5% isoflurane/O2 anaesthesia. The two non-treated control animals in Experiment 2 received neither vaccination nor challenge. For Experiment 1, various clinical symptoms were assessed and the animals were sacrificed three days post challenge. For Experiment 2, one day after the challenge half of the cotton rats from the vaccinated and non-vaccinated groups were sacrificed for the assessment of lung virus titers and systemic immune responses. The remaining animals were followed for ten days for assessment of clinical symptoms.

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Limit of detection (LoD) was calculated using the 1st dilution made; negative samples were assigned a value corresponding to half of the LoD value for calculation purposes.

**IgG and IgA ELISA**

IgG ELISA was performed by coating ELISA plates (Greiner Bio One, Alphen a/d Rijn, The Netherlands) with 0.3 μg/well of A/PR/8, H1N1pdm or X-31 WIV in coating buffer (17.8 mM Na₂CO₃, 22.5 mM NaHCO₃, pH9.6) overnight at 37°C. ELISA was done as described previously. IgG titers were calculated as log₂ of the reciprocal of the sample dilution corresponding to an absorbance of 0.2 at the wavelength of 492 nm. Limit of detection (LoD) was calculated using the 1st dilution made and the negative samples were given half the value of the LoD value.

**Microneutralization assay (MN) and Haemagglutination inhibition (HI)**

Serum samples taken on the day of the 2nd immunization, the day of challenge and ten days post challenge were assessed for MN and HI antibodies against A/California/2009 virus using a previously described protocol respectively. Titers are presented as log₂ HI titers for individual cotton rats. Limit of detection (LoD) was calculated using the 1st dilution made and the negative samples were given half the value of the LoD value.

**Cytokine measurement by qRT-PCR**

For determination of cytokine expression in the lungs, lungs stored in RNAlater were homogenized using a pestle and RNA was extracted with the help of the QIAGEN RNeasy extraction mini kit (Qiagen, Hilden, Germany). cDNA was synthesized with PrimeScript™ RT-PCR Kit (Takara, Westburg, Leusden, Netherlands) using 500 ng RNA. qPCR was run with specific anti-cotton rat primers for Mx-1, Mx-2, IFNγ, IFNα, IL1β, IL-4, IL-6 and IL12 (primer sequences, see Supplementary Table 1). Primers were designed with help of the program Primer Blast, using cotton rat sequences from NCBI BLAST®. The specificity of the primers was validated by checking if there was a single melt curve for all samples tested. GAPDH was used as a house keeping gene. PCR cycling conditions were set as 10 min 95 °C followed by 40 cycles of 15 sec 95°C and 1 min 60°C on an Applied Biosystems’ StepOnePlus real time PCR system. SYBR green ROX-mix used was from Westburg (Leusden, Netherlands).

For analysis, mean CT values of GAPDH per sample was subtracted from the mean CT values of the cytokine for the same animal to calculate delta CT values. Delta delta CT values were then calculated by subtracting delta CT of the non-treated cotton rats from delta CT of the vaccinated and non-vaccinated cotton rats that were challenged.
Cotton rat model for influenza vaccine evaluation

and killed on day one and day ten post challenge. The fold change was then calculated. Cytokines are represented as log fold change in vaccinated and non-vaccinated cotton rats with respect to non-treated cotton rat.

Statistics
The statistical analysis was performed using GraphPad Prism 5 software with which the graphs were plotted as well. Non-parametric Mann-Whitney U test was used to test if the differences between two groups i.e. vaccinated and non-vaccinated cotton rats, with respect to different parameters were significant. A p value of less than 0.05 was considered significant.

RESULTS
Effect of a single immunization on clinical symptoms after virus challenge.
In order to assess the effect of vaccination on disease symptoms and lung virus growth, in a first experiment cotton rats were i.m. injected with a single dose of either PBS, 0.5 µg, 1 µg or 5 µg WIV followed by homologous challenge with a dose of 5x10^5 TCID_{50} H1N1pdm 30 days later. Upon challenge clinical symptoms like weight loss, breathing frequency and temperature were assessed for three days in vaccinated and non-vaccinated cotton rats.

Two animals from the PBS control group were found dead on day three post challenge. The two remaining cotton rats from the PBS group did not show considerable weight loss on day one post challenge; however their weight reduced over the next two days (Fig. 1A). Two cotton rats from the 0.5 µg WIV group were found dead on day two post challenge, the other animals from this group did not show much weight loss (Fig. 1B). Animals from the 1 µg and 5 µg WIV groups lost no or little weight (Fig. 1C and 1D respectively).

The two remaining animals from the PBS control group showed a slight drop in temperature (Fig. 1E). Temperature was not affected by the virus infection in the animals from the 0.5 µg, 1 µg and 5 µg WIV groups (Fig. 1F, 1G and 1H respectively). All four cotton rats from the PBS control group presented with a significant increase in breathing frequency (BF) on day two post challenge and then BF started to normalize (Fig. 1I). Also, all the animals in the 0.5 µg WIV group showed increase in breathing frequency on day two (Figure 1J). While two of the animals in the 1 µg WIV group demonstrated a marked and sustained increase in BF post challenge, the remaining two animals presented with only moderately increased BF which returned to normal values by day three (Figure 1K). Cotton rats in the 5 µg WIV group also showed increased
breathing on day 1 post challenge. However, on day two, BF in these animals started to decrease and was significantly lower than the BF in the mock-immunized control animals on day two (*p = 0.0286). For 3 of the 4 animals the BF came to the baseline on day three (Figure 1L). Hence, breathing frequency was the most sensitive parameter of infection in the used cotton rat model.

In conclusion, immunization with a sufficiently high dose of WIV prevented infection-induced death of the cotton rats but provided limited protection against clinical symptoms. Some of the control animals and of the 0.5 µg WIV group died rapidly without prior overt signs of distress.

Effect of a single immunization on lung virus titers.
To assess the effect of the immunization on virus growth, immunized and PBS treated animals were sacrificed three days post challenge to evaluate lung virus titers. Since two animals from each the PBS treated and the 0.5 µg WIV groups were found dead before day three virus titers could not be retrieved from them. The two surviving cotton rats from the PBS group showed titers of $10^{1.2}$/ml and $10^{0.5}$/ml, respectively. Of the surviving animals from the 0.5 µg WIV group one had a low virus titer of $10^{0.3}$/ml while the other had no detectable virus in the lung. Animals vaccinated with 1 µg or 5 µg WIV were all free of virus in the lungs three days post infection (Fig. 2). No virus was found in the nose of any of the animals. Thus, even a single vaccination resulted in restricted virus growth in the lungs upon challenge.

Systemic immune response after a single vaccination.
To assess the systemic immune response induced by WIV, serum IgG titers were evaluated by ELISA on the day of immunization and the day of challenge. On the day of immunization all cotton rats were sero-negative for influenza (data not shown). On the day of challenge, all vaccinated cotton rats had developed antibodies with a titer of $10^3$ or higher. Compared to IgG titers induced by 0.5 µg WIV, significantly higher IgG titers were induced by 1 and 5 µg WIV (Fig. 3). Yet, the differences were rather small (about 2.5 fold). Thus, all vaccine doses induced robust serum IgG responses upon a single vaccination.

Effects of prime/boost vaccination on clinical symptoms post challenge.
Since a single immunization protected from virus growth but not from clinical symptoms we next assessed whether the protection provided by WIV could be improved by giving a booster vaccination. For this purpose, in the 2nd experiment cotton rats were

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vaccinated twice with 1 µg NIBRG-121 WIV with a 21-day interval. 30 days after the 2nd vaccination, the cotton rats were challenged with 1x10^5 TCID_50 of homologous A/Cal/Gro. The lower challenge dose compared to the 1st experiment was chosen in order to slow down the disease process. Upon challenge, animals were followed daily for ten days for changes in weight, temperature and breathing frequency.

Despite of the five-fold lower virus challenge dose, two of the four mock-vaccinated cotton rats were found dead in the cage on day three and four post challenge. The remaining two cotton rats from the control group displayed minor weight loss but survived until the end of the follow-up period (Fig.4A). Cotton rats vaccinated twice with 1 µg WIV lost some weight on day two post challenge (Fig.4B) and thereafter the weights were stable till sacrifice. Interestingly, two control cotton rats which were neither vaccinated nor challenged also lost also some weight over time (Fig.4 C) and did not regain it, which might suggest that daily handling caused a stress response affecting eating or drinking behavior. Temperature was not affected in the infected animals except for one of the mock-vaccinated cotton rats which showed a decline in temperature one day before death (Fig. 4D-F).

All of the mock-vaccinated cotton rats showed a significant increase in BF between the day of challenge and day two post challenge, indicating successful infection (Fig. 4G). One of the four cotton rats reached a BF of around 460 and was found dead two days later. Cotton rats vaccinated with 1 µg WIV also presented with significantly higher BF on day two post challenge as compared to the day of challenge (Fig. 4H). There was no statistically significant difference in BF between mock-vaccinated and vaccinated animals in this experiment (p=1.0000). After day two, BF started to return to normal. Non-treated cotton rats did not show much change in the breathing frequency compared to their baseline breathing frequency (Fig.4I).

**Effect of prime/boost vaccination on lung virus titers after challenge.**

One day post challenge, half of the animals from the vaccinated and the mock-vaccinated group were sacrificed to evaluate the viral load in the lungs. Lungs of the sacrificed cotton rats were homogenized and the supernatants were used for determination of the TCID_50 in MDCK cells. All cotton rats from the mock-vaccinated group had virus in their lungs, with a mean titer of 10^3.9/ml (Fig.5). In contrast, out of four immunized cotton rats, two did not show detectable virus and the remaining two showed reduced titers (10^2 and 10^3) as compared to the virus titers in non-vaccinated cotton rats (lowest titer: 10^4). Thus, vaccination provided significant protection (p=0.0294) from virus growth in the lungs as observed in the previous experiment.

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IgG responses after prime/boost vaccination and challenge.

To measure the systemic immune response induced by immunization and/or infection, IgG ELISA was performed on the serum samples taken on the days of immunization (d0, d21), day of challenge (d50) and ten days post challenge (d61) from vaccinated as well as mock-vaccinated cotton rats. IgG titers were determined against homologous NIBRG-121. None of the animals showed any IgG titers in the beginning of the experiment (data not shown). Cotton rats from the 1 µg WIV group developed IgG titers of around 10^4 (Fig. 6A) after the 1st immunization on day 21 which increased significantly after the 2nd vaccination. Interestingly, IgG titers further increased significantly upon challenge indicating a booster by the infection itself. For non-vaccinated cotton rats, there was no serum IgG on the day of challenge (Fig. 6B), but ten days post challenge virus-specific IgG was readily detectable in the two surviving animals confirming successful infection.

To assess the cross-reactive potential of these IgG antibodies, ELISA was performed against heterologous A/PR/8 H1N1 and heterosubtypic X-31 H3N2. All four cotton rats vaccinated with 1 µg WIV showed IgG responses to A/PR/8 but titers were about 1 log lower than those against the homologous virus (Fig. 6C). Two of the four vaccinated cotton rats showed also IgG to X-31 (Fig. 6D). Yet, responses to this heterosubtypic virus were much lower than responses to the homologous virus or were even below the detection limit.

Assessment of functional potential of systemic antibodies by HI and MN upon prime/boost vaccination.

To assess the functional potential of the serum IgG, hemagglutination inhibition (HI) and micro-neutralization (MN) assays were performed using homologus NIBRG-121. In line with the IgG antibody responses, HI (Fig. 7A) and MN (Fig. 7B) antibodies were induced after two vaccinations with 1 µg WIV and increased further after challenge. For non-vaccinated cotton rats, HI (Fig. 7C) and MN (Fig. 7D) antibodies were undetectable before challenge but were present 10 days post challenge although at lower levels than in vaccinated animals. We also checked for the cross-neutralizing potential of serum antibodies against A/PR/8 H1N1 and X-31 H3N2 virus. However, despite the presence of cross-reacting antibodies detected by ELISA these antibodies could not neutralize the heterologous and heterosubtypic viruses (results not shown).

Effect of prime/boost vaccination on infection-related expression of Mx and cytokine genes in the lung.

To evaluate the effects of vaccination on expression of infection-related genes in lungs upon challenge, qRT-PCR was performed on mRNA isolated from lung tissue, mRNA derived from lungs of non-treated cotton rats was used to set the baseline expression.
Mx proteins can inhibit virus replication and have been described to be strongly induced in the lungs of influenza-infected cotton rats\textsuperscript{24, 25}. Infection clearly led to increased expression of Mx1 1 day post challenge in both vaccinated and non-vaccinated cotton rats which had returned to normal or even less than normal levels by day 10 (Fig. 8 Mx1). On the contrary, Mx2 was strongly downregulated (around 5-fold) in cotton rats from both groups on day 1 post challenge (Fig. 8 Mx2), but reached baseline levels again by day 10. There was no difference in expression of Mx1 and Mx2 between non-vaccinated and vaccinated animals.

Another response to influenza infection in cotton rats is the upregulation of cytokine expression\textsuperscript{19}. We observed that infection led to increased expression levels, particularly of IFNα, IFNγ, IL1β and IL6 on day 1 post challenge. By day 10 expression of these cytokines had declined again though not in all cases to baseline (Fig. 8 IFNα, IFNγ, IL1β, IL6). While expression of above mentioned cytokines clearly peaked shortly after infection and then declined again, expression of IL4 and IL12 did not show such a trend (Fig. 8 IL4, IL12). Vaccination did not have significant effects on cytokine expression. Only for IFNα there was a trend to lower expression in vaccinated animals and IL4 expression was higher in 3 out of 4 vaccinated animals as compared to non-vaccinated animals on day one post challenge.

DISCUSSION

In this study, we set out to get more insight into the pros and cons of using the cotton rat model for influenza vaccine evaluation, especially by closely monitoring the clinical symptoms in vaccinated cotton rats upon challenge. To this end, we immunized the animals with WIV derived from an H1N1pdm vaccine strain and subsequently infected them with a clinical isolate of H1N1pdm. We demonstrate that all immunized animals developed a humoral immune response against the virus strain. Above a dose of 0.5 µg antigen, a single immunization as well as prime/boost immunization had clear-cut effects on lung virus titer and survival but rather moderate effects on clinical symptoms upon challenge. Among the clinical parameters studied (weight loss, temperature, breathing frequency), we found that breathing frequency was the most sensitive parameter of infection.

In contrast to previous studies, we used outbred cotton rats and found variation among the animals acceptable\textsuperscript{6,11,12,14,26}. Moreover, the cotton rats used in our studies were fully grown when challenged (age at challenge ~20 weeks) as indicated by the fact that even non-infected animals did not gain weight during the study period. In previous studies, the age of the animals is often not well indicated but the fact that non-infected or protected animals gain about 30 g of weight during a 14-day study.
period indicates that the animals used were still juvenile 25. We observed that young animals are much more susceptible to influenza infection than older ones (unpublished observations), thus age is an important parameter to consider in experimental design.

We could readily detect virus in the lungs of the animals 1 or 3 days after virus inoculation confirming that the infection was successful. Some of the infected animals were found dead without having shown severe clinical symptoms during the last check-up. Autopsy revealed that these animals had massive inflammation of the lungs. Obviously, the animals can cope rather well even with severe infection but at a given moment deteriorated very quickly. From our observations, we did not get the impression that the sudden deterioration observed in some animals was related to the infection dose as it was observed for different virus doses. A possible explanation could be the virulence of this particular virus strain compared to other H1N1 viruses. Although, the challenge dose was reduced in the 2nd experiment we still saw this rapid deterioration indicating the higher virulence.

We observed no or very little effect of the infection on weight and temperature in our experiments. This is in line with Blanco et al who previously reported that infection with H1N1pdm does not cause overt weight loss and/or drop in temperature in cotton rats, in contrast to infection with other influenza virus strains like H5N1, H3N2, and H9N2 25.

H1N1pdm infection led, however, to a marked increase in the breathing frequency of the animals, which had not been reported earlier for this virus strain. The peak of increased BF was on day 2 post challenge which correlates with the peak of lung histopathology on day 2 as reported by Blanco et al 25. Increased BF as prominent disease symptom in cotton rats has also been described for other influenza virus strains and is thought to be caused by damage of epithelial cell layers and the resulting difficulties in breathing might cause death 15.

When investigating gene expression in the lungs of infected cotton rats we found among others an upregulation in the IFNγ and Mx1 mRNA indicating induction of an antiviral response. Increased expression of IFNγ and Mx1 has been reported earlier 24,25,27. IFNγ mediates antiviral responses against influenza by induction of Mx genes and other response modifiers 28. In cotton rats, IFNγ expression was found to correlate with replicating virus in the lungs 24. Interestingly we observed a strong down-regulation in Mx2 mRNA on day 1 post challenge in both vaccinated and non-vaccinated animals. It is known that Mx2 plays an important antiviral role in the response to infection by hanta virus, vesicular stomatitis virus (VSV) and La Cross virus, but does not contribute to antiviral responses against influenza in cotton rats and mice 27,29,30. Down-regulation of Mx2 in our study might be the result of the general shut down of host gene expression observed in influenza-infected cells 31,32. In agreement with the results of Ottolini et al we also observed induction of IFNa, IL6, and IL1β on day 1, coinciding with virus infection dose as it was observed for different virus doses. A possible explanation could be the virulence of this particular virus strain compared to other H1N1 viruses. Although, the challenge dose was reduced in the 2nd experiment we still saw this rapid deterioration indicating the higher virulence.

We observed no or very little effect of the infection on weight and temperature in our experiments. This is in line with Blanco et al who previously reported that infection with H1N1pdm does not cause overt weight loss and/or drop in temperature in cotton rats, in contrast to infection with other influenza virus strains like H5N1, H3N2, and H9N2 25.

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replication in the lungs. IFNα is a hallmark of early virus infection and known to play an important role in controlling virus replication in the lungs. IL1β does not play a direct role in influencing influenza infected cells but it can recruit CD4+ T cells to the site of infection, while IL-6 is thought to be necessary for the resolution of influenza infections.

With respect to vaccination, we observed that a single injection of WIV was sufficient to induce a humoral immune response. A booster vaccination did increase the serum antibody titers significantly, yet, the increase in titers was moderate. Upon infection, the vaccinated animals showed a significantly reduced lung virus titer as compared to control animals. Moreover, all animals vaccinated with 1 or 5 µg WIV survived the challenge while half of the control animals died. Thus, immunization with WIV was successful in inducing protective immune responses in cotton rats as was earlier reported for immunization with trivalent split and adenovirus-based vaccines. Despite the fact that 0.5, 1 and 5 µg WIV induced comparable antibody titers, half of the animals from the 0.5 µg WIV group died. This likely indicates that, along with the antibodies, T cells are playing a role in protection of these animals. There is one previous publication showing evidence that memory T cells can contribute to protection. Other potential mechanisms include NA (Neuraminidase) inhibiting antibodies or HA and NA specific antibodies at the mucosal surfaces.

Despite the overt effects of vaccination on lung virus titers several observations indicate that vaccination did not result in sterilizing immunity: (i) the breathing frequency in immunized and challenged cotton rats was only slightly lower than in challenged control animals, (ii) infection-induced cytokine production in the lungs was hardly affected, and (iii) the virus infection boosted the vaccine-induced antibody responses. Possibly, virus was still replicating in the nose of immunized and challenged cotton rats and caused enhanced breathing and cytokine induction and provided antigen for B cell stimulation. It has been shown earlier that lung virus titers are more readily controlled by vaccination than nose virus titers. We conclude that under the conditions used in our experiments complete protection was not achieved indicating that the vaccine dose was suboptimal. Increase of vaccine dose and/or use of a different administration route might further improve vaccination outcome. Our results confirm that cotton rats are a suitable model for influenza infection and can be useful for the evaluation of vaccine candidates. However, the model has clear limitations and to use it successfully a number of aspects have to be considered. (i) The age of the animals is very important. During previous studies we observed that young animals (about 10 weeks) are highly susceptible to influenza virus and their weight drops rapidly upon infection. In contrast, older animals (more than 20 weeks) are rather resistant to infection (unpublished data). (ii) Disease progression in cotton
rats is very rapid as compared to mice which deteriorate more gradually. Cotton rats, upon infection show either little symptoms or die rapidly. In both experiments described in this paper, some non-vaccinated cotton rats were found dead by day 3 or 4, sometimes without showing severe symptoms before. The virus dose used for challenge should therefore be chosen carefully and frequent monitoring of infected animals is indicated to prevent unnecessary suffering. (iii) The choice of virus strain is also very critical as it determines the clinical symptoms elicited and thus the readout parameters to be used. As mentioned above, H1N1pdm infection does not affect weight and temperature of cotton rats in contrast to other strains like H5N1, H3N2, and H9N2. (iv) Even if the experimental conditions are controlled as carefully as possible there might be some variation in disease symptoms among the animals. This could be particularly the case for outbred animals which we chose intentionally as a model closer to the human situation.

In conclusion, use of cotton rats for determination of vaccine efficacy with respect to amelioration of clinical symptoms requires careful fine-tuning of the model as mentioned above. Nonetheless, being small, affordable and susceptible to clinical influenza virus isolates cotton rats have their place among the clinically less relevant inbred mouse model and the expensive ferret model for the evaluation of influenza vaccine candidates.

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CONFLICT OF INTEREST

The authors have no conflict of interest.
Fig. 1. Effect of a single immunization on clinical symptoms after virus challenge. Cotton rats mock-immunized with PBS or immunized once with 0.5 µg WIV, 1 µg WIV or 5 µg WIV and challenged on day 30 with $5 \times 10^7$ TCID$_{50}$ of homologous A/Cal/Gro were followed for three days and weight (A-D), temperature (E-H) and breathing frequency (I-L) were recorded. Discontinued line with X symbol indicates dead animal.

Fig. 2. Effect of a single immunization on lung virus titers. The surviving cotton rats from the experiment described in the legend to Fig. 1 were sacrificed three days post challenge and lung virus titers were determined. Virus titers for individual cotton rats and the mean titers per experimental group are depicted. LoD for the virus titers is indicated at 0.3 with a dashed line and negative samples are assigned a value corresponding to half of the LoD.
**Fig. 3.** Systemic immune response after a single vaccination. Serum IgG responses were evaluated 30 days after a single immunization with the indicated amounts of WIV. IgG titers are presented as log_{10} titers for individual cotton rats with means per group (n=4). LoD is indicated at 2 with a dashed line and significance is represented as *p<0.05.

**Fig. 4.** Effects of prime/boost vaccination on clinical symptoms post challenge. Cotton rats were injected with PBS, immunized twice with 1 µg WIV, or were left untreated. 30 days after the 2nd immunization immunized and mock-immunized animals were challenged with 1*10^7 TCID_{50} of homologous virus, non-treated animals were again left untreated. After challenge, animals were followed for weight loss (A-C), change in temperature (D-F) and breathing frequency (G-I). Discontinued line with X symbol indicates dead animals.
Fig. 5. Effect of prime/boost vaccination on lung virus titers after challenge. 30 days after the 2nd vaccination cotton rats were challenged with $10^7$ TCID$_{50}$ homologous A/Cal/Gro by i.n. administration. One day post challenge, lung virus titers were determined by TCID$_{50}$ Virus titers of individual animals and the mean virus titer per experimental group are depicted. Lung virus titers from the vaccinated cotton rats are compared with the lung virus titers from mock vaccinated animals and significance is represented as *p<0.05. LoD for the virus titers is indicated at 0.3 with a dashed line.

Fig. 6. IgG responses after prime/boost vaccination and challenge. Serum samples collected on day 0, after the 1st vaccination (d21), on the day of challenge (d50) and 10 days post challenge (d61) were used for determination of IgG titers. IgG ELISA was performed for sera of (A) vaccinated cotton rats and (B) non-vaccinated cotton rats. Cross reactive IgG against A/PR/8 H1N1 (C) and
against X-31 H3N2 (D) was measured in d50 serum samples. IgG titers from the day of challenge are compared with the IgG titers on day 10 post challenge (also with day 0 for the vaccinated cotton rats). Titers are represented as log_{10} titers with significance *p<0.05 and **p<0.01. LoD for the IgG titers is indicated at 2 with a dashed line.

**Fig. 7.** Assessment of the functional potential of systemic antibodies by HI and MN assays

Serum samples were collected at the 2nd vaccination (d21), day of challenge (d50), and 10 days post homologous challenge (d61) and functionality of the IgG was measured by HI and MN for vaccinated (A,B) and non-vaccinated cotton rats (C,D). HI and MN antibody titers against NIBRG-121 virus are represented as log_{2} titers. HI and MN titers from the day of challenge are mainly compared with the HI and MN titers on day 10 post challenge and the significance as *p<0.05, **p<0.01. LoD for the MN titers is indicated at 4.32 and LoD for the HI titers is indicated at 2.58, both indicated with a dashed line.
Fig 8. Effect of prime/boost vaccination on gene expression in the lungs upon infection. Cotton rats were (mock-)immunized, boosted and challenged as previously described. On day 1 and day 10 after virus challenge cytokine mRNA expression in lungs of the animals was assessed by qRT-PCR with gene specific primers using the housekeeping gene GAPDH as reference.
REFERENCES


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