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The Cotton Rat Model of Respiratory Viral Infections Pathogenesis and Immunity

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Abstract

Development of successful vaccines against human infectious diseases depends on using appropriate animal models for testing vaccine efficacy and safety. For some viral infections the task is further complicated by the frequently changing genetic make-up of the virus, as in the case of influenza, or by the existence of the little-understood phenomenon of vaccine-enhanced disease, as in the case of respiratory syncytial virus (RSV). The cotton rat *S.hispidus* has been used for years as an excellent small animal model of the RSV vaccine-enhanced disease. Recently, using cotton rats, we have demonstrated that vaccination against another paramyxovirus, human metapneumovirus (hMPV), can also lead to vaccine-enhanced disease. In addition to the study of paramyxoviruses, *S.hispidus* presents important advantages for the study of orthomyxoviruses such as influenza. The cotton rat is succeptible to infection with unadapted human influenza strains, and heterosubtypic immunity to influenza can be evoked in *S.hispidus*. The mechanisms of influenza, RSV, and hMPV pathogenesis and immunity can now be investigated in the cotton rat with the development of species-specific reagents for this animal model.

Keywords

cotton rat; respiratory virus; RSV; hMPV; influenza

Introduction

The cotton rat *S.hispidus* is a small rodent susceptible to a surprisingly large variety of human pathogens (for review see (1). This model is best known, however, for its use in research related to respiratory viruses. Permissiveness of cotton rats to infection with human respiratory syncytial virus (RSV) surpasses that of mice by more than 100-fold. Predictive quality of this model is so high that the only available prophylactic treatment for severe RSV disease (antibodies RespiGam and Synagis) advanced to clinical trials based on the results of efficacy and safety studies in cotton rats, bypassing the need for testing in primates. The cotton rat model also accurately predicted the dose of the drug currently being used in human infants. The application of cotton rats to biomedical research has been limited until recently because of the lack of species-specific reagents to study mechanisms of disease pathogenesis and immunity in this model. The situation has changed in the past decade with our endeavor to

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develop cotton rat-specific reagents. Over 200 cotton rat genes encoding cytokines, chemokines, cell surface markers and regulatory molecules have been cloned and reagents to many of these were derived through a collaboration with R&D Systems, Inc. (Table 1). Molecular level analysis reveals important features of the model that make *S.hispidus* stand out in comparison to other small animal models. For example, the cotton rat carries functional set of Mx genes encoding antiviral proteins Mx1 and Mx2 (2). Human Mx protein is a crucial component of innate antiviral defense system which, together with the adaptive immune mechanisms, facilitates clearance of viral infections. In contrast, most common laboratory strains of mice lack functional Mx system, and murine antiviral defense relies mostly on adaptive immune mechanisms. This often results in prolonged replication of viruses in murine models compared to what is seen in humans, ferrets and cotton rats.

Although having achieved its reputation in the past primarily as a RSV model, the cotton rat turns out to be a superior model of other respiratory viral diseases as well. For example, the model of recently discovered human metapneumovirus (hMP) was recently established in the cotton rat (3). Available data indicates that the model will be useful for not only elucidating mechanisms of hMPV pathogenesis, but also for developing a safe vaccine against hMPV disease (4). Moreover, *S.hispidus* appears to be a uniquely suitable small animal model for research on influenza pathogenesis and immunity. Unlike mice, cotton rats can be infected with unadapted influenza virus strains and dynamics of viral replication in the cotton rat model resemble those reported in humans. This review will focus on recent advances in the field of respiratory virus research in the cotton rat model and will cover issues related to RSV, hMPV and influenza viruses pathogenesis and immunity.

Human Respiratory Syncytial Virus (hRSV) Infection in the Cotton Rat Model

Respiratory syncytial virus (RSV) is the major cause of severe respiratory infections in infants and young children. It is also a serious health threat in the immunocompromized and the elderly. The quest for a successful RSV vaccine, spanning over four decades, has been hampered by the lack of understanding of RSV pathogenesis and by the failed trials in the 1960s of a formalin-inactivated RSV (FI-RSV) vaccine. Rather than providing protection, immunization with FI-RSV led to a more severe, and in some cases, fatal disease in children upon natural RSV infection. Since then, several candidate subunit RSV vaccines and live attenuated RSV vaccines have been proposed; each, however, is associated with complications.

The cotton rat model of RSV disease was established three decades ago (5). RSV infects both upper and lower respiratory tract of *S.hispidus*, with peak pulmonary replication seen on day 4 and virus clearing from the lungs by day 7. Disease is primarily inflammatory and is accompanied by cellular infiltration in the lung with the peak on day 5–6 post-infection. Inflammatory cells (primarily lymphocytes and neutrophils) aggregate around bronchioles (peribronchiolitis), small blood vessels (perivasculitis) and within the alveolar walls (interstitial penumonitis). FI-RSV disease has been reproduced in the cotton rats vaccinated twice with FI-RSV and then challenged with live virus (6). Compared to the primary RSV infection, FI-RSV vaccine enhanced disease is accompanied by reduced viral replication, but increased pulmonary inflammation. The primary feature of this response is alveolitis, or infiltration of cells (primarily neutrophils) into alveolar space, which peaks on day 5 after RSV infection of FI-RSV-vaccinated animals. Alveolitis serves as a reliable marker of FI-RSV vaccine-enhanced disease and also as a predictor of the potential safety problems of various anti-RSV vaccine candidates. Inclusion of the adjuvant monophosphoryl lipid A (MPL) in the FI-RSV formulation reverses pathology of vaccine-enhanced disease (7,8).

Vaccine-enhanced disease in the cotton rat model is accompanied by the augmentation of the Th2-type response (8). Levels of pulmonary expression of a number of Th2-type cytokines,

including IL-4, IL-5, IL-9 and IL-13 are increased during FI-RSV vaccine-enhanced disease. However, levels of several Th1-type cytokines (e.g., IL-2, IL-12p40) and chemokines (e.g., MIP-1a, GRO) are also increased. Inclusion of MPL in the FI-RSV formulation inhibits FI-RSV-induced augmentation of Th2-, Th1-type and chemotactic cytokine responses (Fig.1), simultaneously with reducing pathology of vaccine-enhanced disease. Thus, a broad disregulation of cytokine responses, rather than a simple Th2-type response augmentation, may contribute to the development of vaccine-enhanced disease.

Re-infection of cotton rats with RSV results in no detectable virus production, and yet pulmonary inflammation is increased during secondary compared to the primary infection. Our recent studies indicate that even though no virus can be detected during secondary RSV infection in the cotton rat, abortive viral replication takes place (9). Viral genome/antigenome is replicated and viral genes are expressed after secondary RSV challenge, albeit production of infectious virus is impaired. These findings indicate that human re-infection with RSV might occur more frequently than currently appreciated. The abortive RSV replication in cotton rats is accompanied by upregulation of interferon response and expression of Mx genes shortly after infection (10). Therefore, even in the absence of infectious virus production, abortive replication of RSV may trigger inflammatory response in the lung and lead to exacerbation of existing respiratory conditions.

Severity of RSV disease is increased in human elderly. We found that responses of cotton rats to RSV also differ with respect to the age of infected animals. The cotton rats display signs of immunosenescence from the 6 months of age, with the age-proportional delay in viral clearance seen in 6–16 months old *S.hispidus* (11). The delay is accompanied by skewed pulmonary expression of cytokines and overexpression of chemokines (12). This altered cytokine and chemokine responses in aged cotton rats might be associated with an age-related defect in innate immune response to RSV. Functions of macrophages, NK cells and neutrophils decline with age and increased severity of RSV disease in the elderly may be associated with deregulation of cytokine/chemokine responses responsible for mediating innate immune defense and for linking antiviral innate and adaptive immune responses.

Human Metapneumovirus (hPMV) Infection in the Cotton Rat Model

hMPV is a newly-described member of the paramyxovirus family, closely related in sequence to RSV. Since its original discovery in 2001 in young Dutch children with acute respiratory tract infections, worldwide distribution of hMPV has been described and many features of hMPV-caused disease were found to resemble the disease caused by RSV. Similar to RSV, hMPV infects virtually all children by the age of 5 and can re-infect humans repeatedly throughout life. Like RSV, hMPV causes more severe disease in the infants, elderly and immunocompromised individuals and is found in children hospitalized with severe cases of acute respiratory tract infections, children with wheezing and with asthma exacerbation.

Only a limited number of animal models of hMPV infection have been described so far. hMPV replicates in the respiratory tract of experimentally infected chimpanzees and monkeys (cynomolgus monkeys, rhesus macaques, and African green monkeys), hamsters, and BALB/c mice. We have recently established a cotton rat model of hMPV infection (Hamelin, Yim et al. 2005). Inoculation of young adult cotton rats *S.hispidus* with 10^8 $TCID_{50}$ of hMPV results in significant viral replication in the lower respiratory tract of animals. Peak viral titer is detected in the lungs of infected cotton rats on day 5 post-infection and no virus is recovered from the lungs by day 21. Infection is accompanied by a pronounced inflammatory response in the lungs characterized by interstitial inflammation, peribronchiolitis, and alveolitis. Peak pulmonary inflammatory response is seen on day 5 post-infection and coincides with the peak hMPV replication in the lung. hMPV infection causes upregulation of a number of cytokines

in the lungs of hMPV-infected cotton rats, including IFN-g, RANTES, MIP-1a, and IL-2 mRNA with the peak on day 5 post-infection (3). Expression of chemokine MCP-1 is also upregulated by hMPV infection, but peaks earlier (day 1 post-infection). Similar to hRSV, however, no clinical manifestations of hMPV infection (such as weight loss, breathing problems, decreased activity) is seen in hMPV-infected cotton rats.

For several members of the paramyxovirus family, including RSV, parainfluenza, and measles, vaccination with formalin-inactivated (FI) virus leads to exacerbation of disease following viral challenge. We have recently demonstrated that the same is true for hMPV infection (4). Cotton rats were vaccinated twice (on days 0 and 28) with various dilutions of FI-hMPV and challenged with 10^7 $TCID_{50}$ homologous hMPV strain on day 49. Pulmonary lesions were significantly increased in cotton rats challenged with hMPV following vaccination with FI-RSV, compared to mock-vaccinated animals challenged with hMPV (primary infection), or to animals challenged with live hMPV twice (secondary infection) (Fig.2). Vaccine-enhancement was especially evident in exacerbation of interstitial pneumonitis and alveolitis, as in the case with FI-RSV vaccine-enhanced disease. In spite of significantly aggravated pulmonary pathology, no viral replication was detected in the lungs of cotton rats challenged with hMPV following FI-RSV vaccination. hMPV vaccine-enhanced disease in cotton rats is accompanied by skewed expression of Th1- and Th2-type cytokines in the lungs of cotton rats (Fig.3). An increase in the expression of Th2-type cytokine IL-4 mRNA and a decrease in the expression of Th1-type cytokine IFN-g mRNA was seen in hMPV-infected animals vaccinated with FI-hMPV when compared to animals with primary or secondary hMPV infection. This finding suggests that an imbalanced immune response is involved in the enhancement of the hMPV disease, similar to what has been shown for RSV infection.

Influenza Infection in the Cotton Rat Model

A number of animal models have been developed for influenza infection, but each one is plagued with problems. Primates get infected with influenza when inoculated intranasally or intracheally, but their limited availability, expense, and outbred nature limits primates' use in influenza research. Ferrets present a promising small animal model of influenza infection, as these animals are succeptible to infection with unadapted influenza strains and upon infection exhibit fever, lethargy, and weight loss. The ferrets, however, are outbred and reagents are not available for studying mechanisms of influenza pathogenesis and immunity in the ferret model. Nevertheless, data from the ferret model is widely used by manufacturers and regulatory agencies in the assessment of influenza vaccines each year when seasonal influenza vaccines are considered for approval. Influenza infection can be established in mice, but virus has to be mouse-adapted by extensive passaging to replicate in this animal. Moreover, laboratory strains of mice lack functional antiviral Mx system, a part of the innate immune response that contributes to early inhibition of viral replication. As a result, influenza replication is prolonged in mice compared to ferrets and man. Influenza viral clearance in mice is more dependent of the cellular immune mechanisms rather than the innate immune response.

The uniqueness of the cotton rat as a small animal model of influenza lies in the fact that *S.hispidus* can be infected with unadapted human influenza strains. Both influenza A and B strains replicate in the upper and the lower respiratory tract of cotton rats (Table 2) (13). Cotton rats can also be infected with and replicate avian influenza H3N2 and H9N2 viruses (data not shown). As is the case with influenza infection in humans and monkeys, infection of cotton rats depends on the dose of administered viral inoculum. Intranasal infection of cotton rats with 10^7 $TCID_{50}$ A/Wuhan/395/95 is accompanied by rapid replication of virus in the lungs and nose of infected animals. Viral titers peak in the lungs at 7–24 hrs after infection and reach peak level in the nose on day 2–3 post-infection (Fig.4). This is accompanied by a transient weight loss on days 1–3 (Fig.4B). Mx response is rapidly unregulated in the lungs of influenza-

infected cotton rats. Expression of Mx genes is initiated as early as 6 hrs after influenza challenge (Fig.4C), with ensuing expression of Mx proteins that peak on day 4 post-infection (data not shown). Significant airway epithelial damage and pronounced pulmonary inflammatory response are seen in influenza-infected cotton rats. Columnar epithelium in the large airways in the lung was sloughed off by day 2 post-infection, but is regenerated by day 5 post-infection. In contrast to the epithelial cells damage that peakes on day 2 post-infection, inflammatory response in the lung is maximal on day 4–6 post-infection and is characterized predominantly by interstitial pneumonitis, alveolitis, and peribronchiolitis. Inflammatory response takes over 4 weeks to resolve.

Influenza infection causes strong upregulation of cytokine/chemokine gene expression in the lungs of infected cotton rats (13). Expression of mRNA of some cytokines, such as IL-1b, IFN-a, IL-6, TNF-a, GRO, and MIP-1b parallels dynamics of influenza replication in the lung in that it increases from 6 hrs post-infection and reduces after day 3 post-infection. Expression of other cytokines, including RANTES, IFN-g, IL-4, IL-10 and IL-12p40, is biphasic, with a one peak overlapping peak of viral replication and a second peak corresponding to the time of maximal cellular inflammatory response. The biphasic nature of cytokine expression during influenza infection may indicate different phases of immune response. Thus, early upregulation of IFN-g mRNA may reflect activation of pulmonary NK cells, while later peak may coincide with the time of antigen-speicific T lymphocyte infiltration in the lung.

Phenomenon of heterosubtypic (or cross-protective) immunity has been addressed in the cotton rat model. Heterosubptypic immunity is seen when infection or immunization with influenza virus with one subtype of hemagglutinin (HA) and neuraminidase (NA) proteins (e.g., H3N2) provides protection from disease caused by infection with influenza virus with a different HA and NA subtype (e.g., H1N1). To address the phenomenon of crossprotection to influenza infection, cotton rats were immunized with A/PR/8/34 (H1N1) virus and 4 weeks later challenged with A/Wuhan/359/95 (H3N2) virus. These animals were compared to the group immunized with Wuhan and then challenged with the same type of virus. A control group of animals did not receive any immunization, but was challenged with Wuhan virus along with the other two groups. Lung and nose viral titers were determined 1, 2, 4, and 7 days following Wuhan infection. As results shown in Figure 5 indicate, priming of cotton rats with H1N1 influenza reduced replication of H3N2 influenza in both noses and lungs of cotton rats (14). This protection was associated with reduced tachypnea and protection from epithelial damage (data not shown). As broadly-acting vaccines to influenza A are needed under the pressure of rapidly emerging highly pathogenic influenza strains, the cotton rat model may provide an very helpful tool for characterization of new vaccine candidates.

Conclusions

The cotton rat is a small animal model that was originally selected for respiratory viral research not because of the availability of reagents and tools, but because of its ability to faithfully reproduce human infectious diseases and to accurately predict efficacy and safety of vaccines and therapeutics. *S.hispidus* model has undergone significant transformation in recent years with the development of species-specific reagents. From a model amenable only to basic virology, serology, and histopathology tests it became a focus of research delving into the molecular mechanisms of disease and immunity. These studies are already providing some answers (e.g., preservation of Mx antiviral system) to the previously unexplained advantages of cotton rats over other small animal models. They are also contributing to our understanding of the mechanisms of virally-induced inflammation, vaccine-enhanced disease, and immunosenescence. In the years to come the cotton rat model will likely yield many more important and relevant discoveries in the area of respiratory viral diseases.

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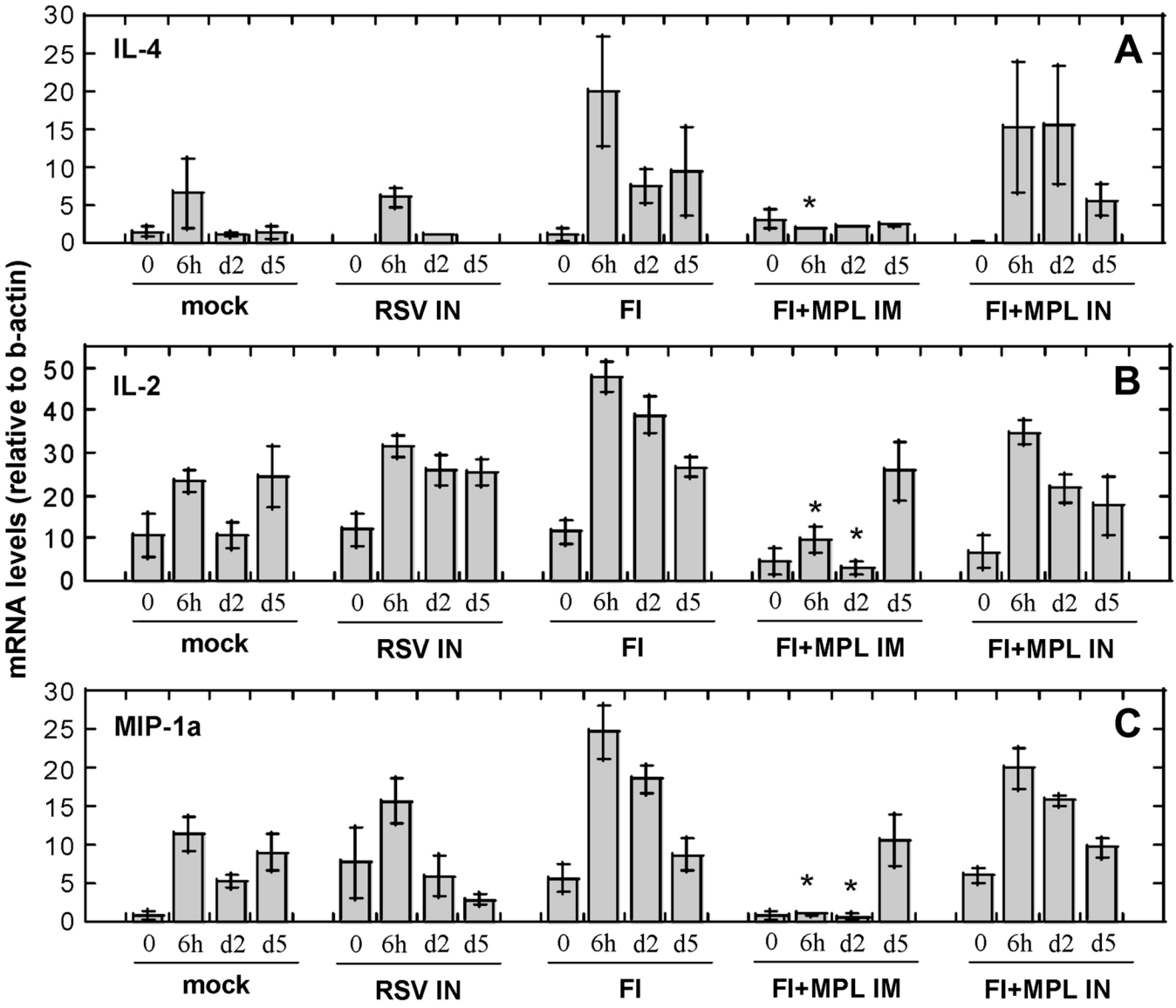

Figure 1.

Effect of MPL on cytokine and chemokine expression in FI-RSV vaccine-enhanced disease. Cotton rats were vaccinated twice with FI-RSV alone (FI) or in combination with MPL administered intramuscularly (FI + MPL IM) or intranasally (FI + MPL IN). Control animals were vaccinated with either mock preparation of the vaccine (mock) or by infection with live RSV (RSV IN). Following RSV challenge of all groups, animals were sacrificed at the indicated times post-infection and lung samples were used for molecular analysis of mRNA expression levels of (A) Th2-type cytokines, (B) Th1-type cytokines, and (C) chemokines (one example for each category is shown). Relative mRNA levels for each gene were measured and normalized to β-actin. The results represent the mean ± SEM for 4–6 animals per time point. *, $p<0.05$ (when FI + MPL IM and FI + MPL IN treatments are each compared to FI treatment). (Adapted from [8])

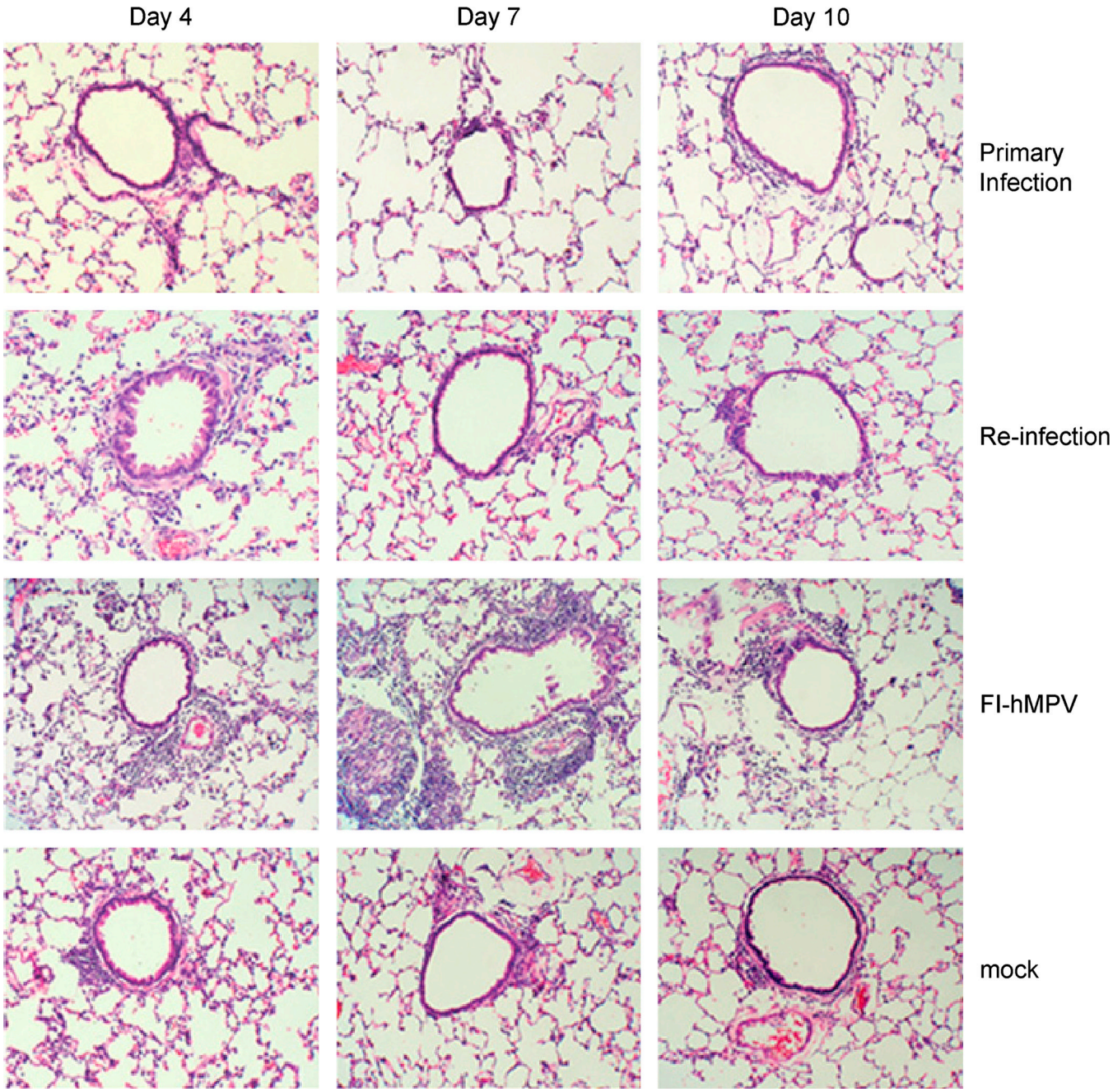

Figure 2.

Enhanced pulmonary disease in cotton rats vaccinated with formalin-inactivated hMPV vaccine (FI-hMPV). Cotton rats *S.hispidus* were immunized intramuscularly with FI-hMPV vaccine (FI-hMPV) or with the mock preparation of vaccine (mock). Immunization was repeated on day 28. On day 49 animals were inoculated intranasally with hMPV A strain C-85473(CAN97-83, NL/00-1) (10^7 $TCID_{50}$ per animal). Animals were sacrificed on various days after infection and lungs were collected for histopathology analysis. Control animals included naïve cotton rats challenged with hMPV (primary infection), and animals challanged with hMPV twice with an interval of 28 days (re-infection, timing of sacrifice corresponds to the time after the second hMPV challenge). H&E stain (Adapted from [4]).

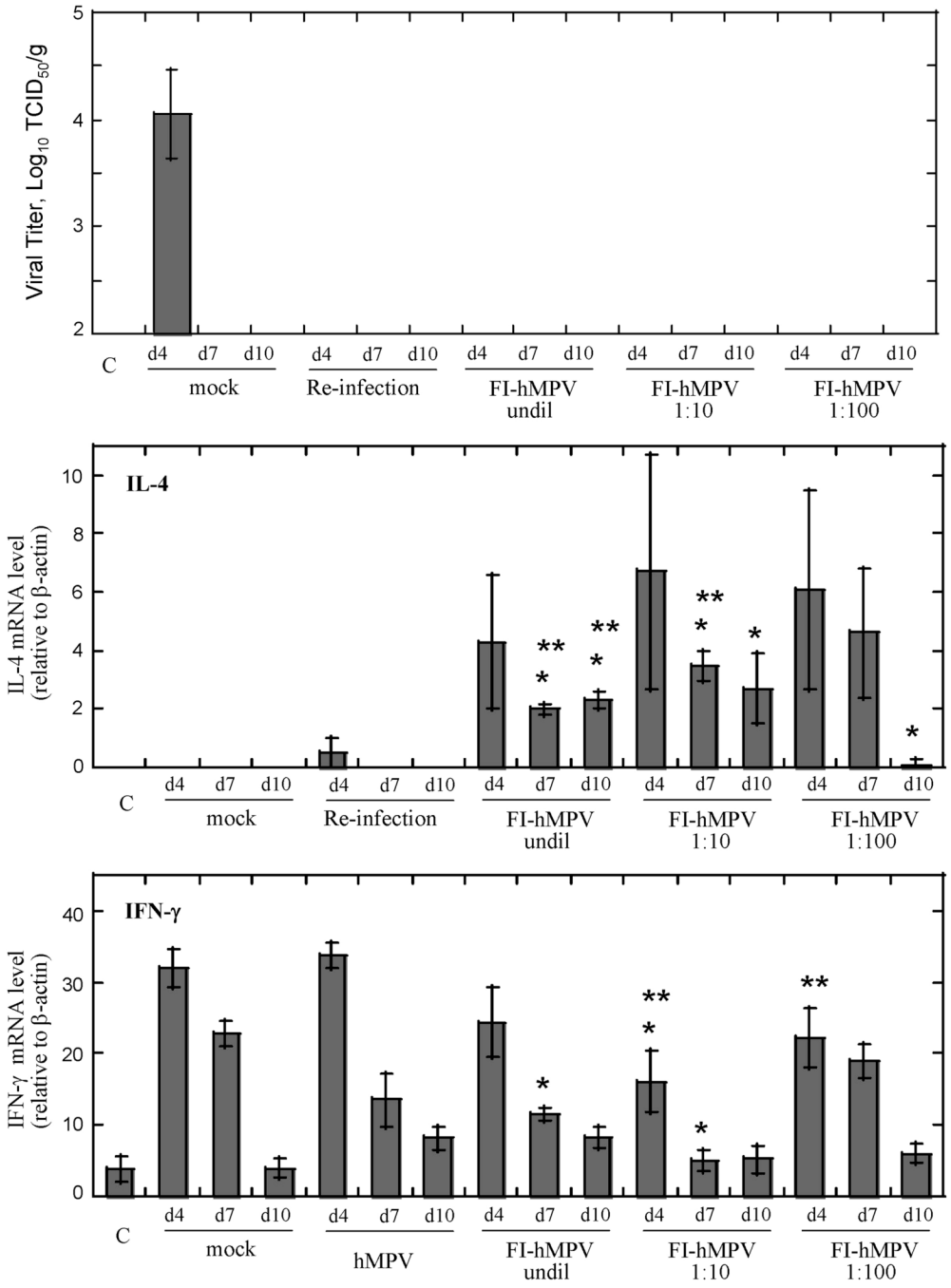

Figure 3.

Viral replication and cytokine expression during FI-hMPV vaccine-enhanced disease in cotton rats. Animals were immunized with FI-hMPV vaccine (prepared by formalin inactivation of hMPV A strain C-85473), undiluted and diluted 1:10 and 1:100, and challenged as described in the previous figure's legend. Lungs were collected for viral titer analysis or for cytokine expression analysis by RT-PCR. The results represent the mean ±SEM for 5 animals per group. (Adapted from [4]).

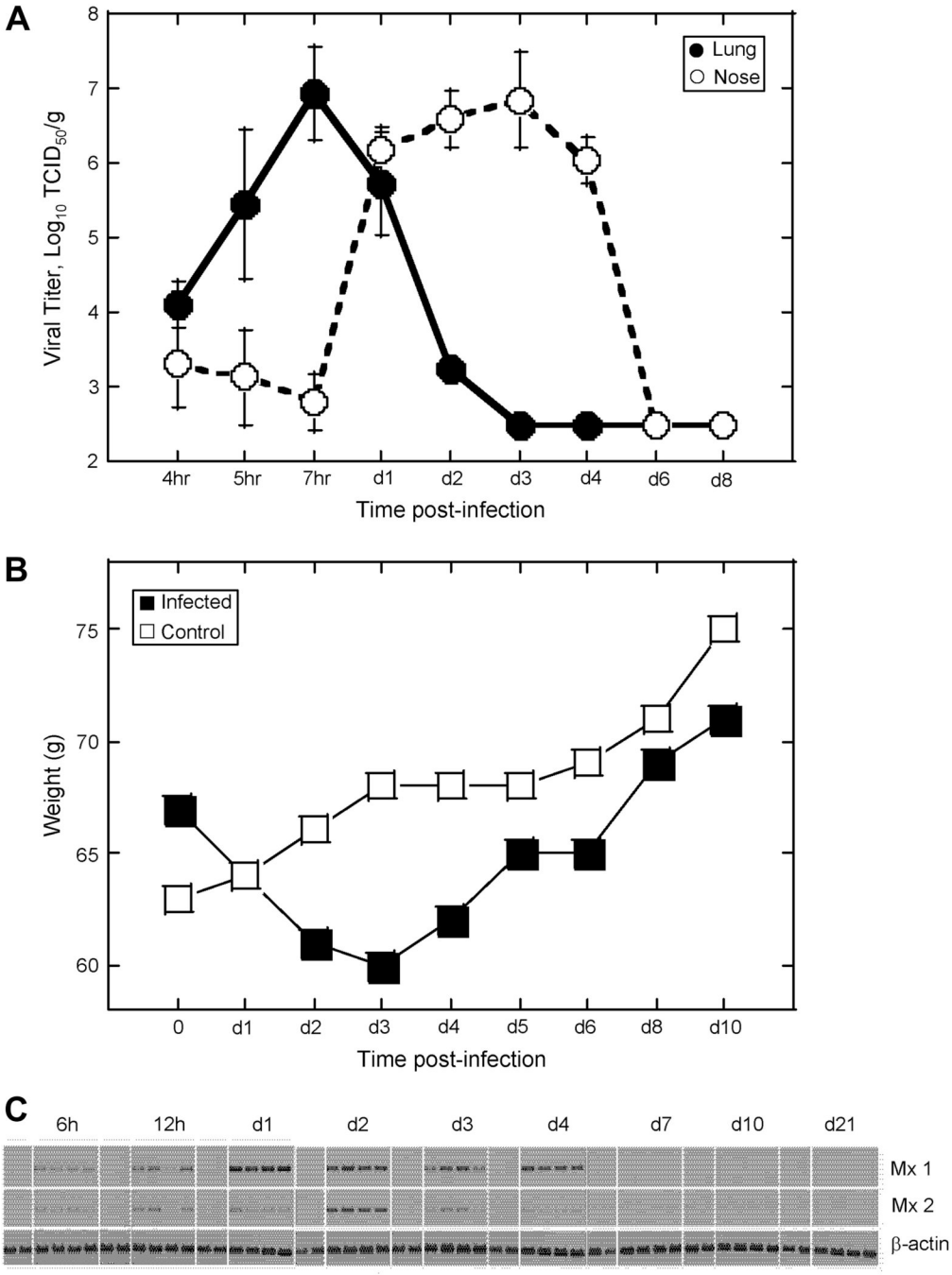

Figure 4.

Influenza virus infection in the cotton rat *S.hispidus* model. Cotton rats were inoculated intranasally with A/Wuhan/359/95 (H3N2) (10^7 $TCID_{50}$ per animal) and sacrificed at various time points after infection (hr, hours; d, days). (A) Viral titers in the lung (filled circles) and nose (open circles) of infected animals were measured and expressed as the geometric mean SEM titre for ≥8 animals for each time point. (B) Serial measurements of weight were made on days 0–10 after infection of animals with A/Wuhan/359/95 (filled squares) and in age-matched uninfected control animals (open squares). (C) Pulmonary expression of Mx 1 and Mx 2mRNA was measured by RT-PCR on various days after influenza infection and is shown against expression of housekeeping gene β-actin mRNA . (Adapted from [2, 13]).

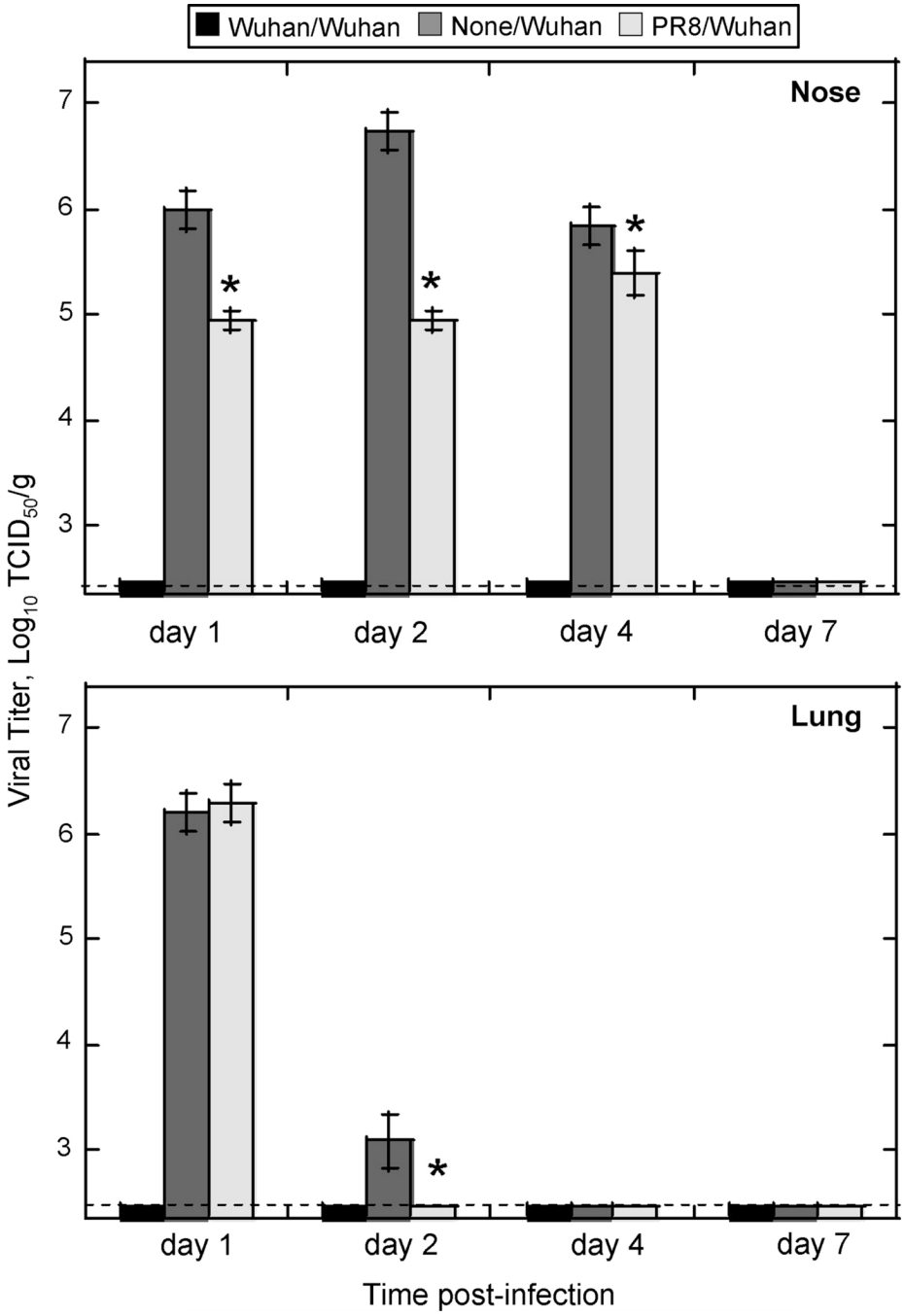

Figure 5.

Immunization with heterosubtypic influenza virus enhances viral clearance in the cotton rat. Cotton rats were immunized (by i.n. inoculation with live virus) with A/Wuhan/359/95 (H3N2) (black bars), A/PR/8/34 (H1N1) (light grey bars), or left untreated (dark grey bars). Four weeks later animals were challenged with 10^7 $TCID_{50}$ A/Wuhan/359/95 virus and sacrificed at various times post-infection for analysis of lung and nose Wuhan viral titers. The results represent the mean±SE for 5 animals per group. *p<0.05, when compared to animals that had not previously been immunized (None/Wuhan). (Adapted from [14]).

Table 1

Cotton rat genes cloned and reagents/assays available

Cytokines:	**Chemokines:**	**Cell surface molecules:**
IFN-γ (A, B, C, D)	MCP-5 analog	
IFN-α (A,B)	MIP-1α (A, B, C, D)	CCR5
IFN-β	MIP-1β (A, B, C)	CD3
IL-1α (A, B, C)	RANTES (A, B)	CD4 (C)
IL-1β (A, B)	IP-10 (A, B)	CD8alpha (C)
IL-2 (A, B, C, D)	GRO/IL-8 (A, C, D)	CD11b
IL-4 (A, B, C, D)	MIP-2/IL-8 (A, C).	CD14
IL-5	MCP-1/JE.(A,C)	CD16
IL-6 (A, B, C)		CD18
IL-9	**Housekeeping genes:**	CD25
IL-10 (A, B, C)		CD45/B220
IL-12p40	β-actin	CD62L (L-selectin)
IL-12-p35	GAPDH	CD74(MHC II)
IL-13	18S rRNA	CD83(HB15)
IL-18		CD86(B7-2)
TNFα (A, B, C, D)	**Other genes:**	Ly-6
TNFβ		MHC I
TGFβ1	IRF-2	MHC II A
GM-CSF	IRF-8 (ICSBP)	MHC II E
	Cox-2	TLR-2
	Hsp70	β-2 microglobulin
	Mx1 and Mx2	

A: recombinant protein; **B**: Polyclonal antibody; **C**: Monoclonal antibody; **D**: ELISA

Table 2a

Replication of influenza viruses in cotton rats[b]

Type (Subtype)	Adaptation	Strain	Lung Titer[c]	Nose Titer[c]
B		B/HK/73	3.6	6.3
		B/Sichuan/379/99	5.2	6.5
		B/HK/330/01	5.5	5.2
A (H1N1)	Mouse	A/PR/8/34	N/D[d]	4.7
	Tissue culture	A/PR/8/34	6.3	5.0
		A/Malaya/302/54	5.2	6.3
A (H3N2)		X-31	4.6	6.2
		A/Wuhan/359/95	3.3	6.4

[a] Addapted from [13].

[b] Groups of 6–12 week old cotton rats *S.hispidus* were inoculated intranasally with 0.1ml of the indicated virus preparation containing ~10^7 $TCID_{50}$/ml. Two days after infection the cotton rats were sacrificed and lungs and noses were collected for viral titrations.

[c] $Log_{10}TCID_{50}$/g of tissue. The results are the geometric man titer for four animals per group.

[d] Not detected