UNIVERSITÉ DE GENÈVE

Archive ouverte UNIGE
https://archive-ouverte.unige.ch

Article scientifique | Article | 2010 | Published version | Open Access

This is the published version of the publication, made available in accordance with the publisher's policy.

PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models

Hai, Rong; Schmolke, Mirco; Varga, Zsuzsanna T; Manicassamy, Balaji; Wang, Taia T; Belser, Jessica A; Pearce, Melissa B; García-Sastre, Adolfo; Tumpey, Terrence M; Palese, Peter

How to cite

HAI, Rong et al. PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models. In: Journal of virology, 2010, vol. 84, n° 9, p. 4442–4450. doi: 10.1128/JVI.02717-09

This publication URL: https://archive-ouverte.unige.ch/unige:40095
Publication DOI: 10.1128/JVI.02717-09

© This document is protected by copyright. Please refer to copyright holder(s) for terms of use.

Journal of Virology

PB1-F2 Expression by the 2009 Pandemic H1N1 Influenza Virus Has Minimal Impact on Virulence in Animal Models

Rong Hai, Mirco Schmolke, Zsuzsanna T. Varga, Balaji Manicassamy, Taia T. Wang, Jessica A. Belser, Melissa B. Pearce, Adolfo García-Sastre, Terrence M. Tumpey and Peter Palese

J. Virol. **2010, 84(9):4442. DOI: 10.1128/JVI.02717-09.**
Published Ahead of Print 24 February 2010.

Updated information and services can be found at:
http://jvi.asm.org/content/84/9/4442

These include:

REFERENCES
This article cites 28 articles, 14 of which can be accessed free at: http://jvi.asm.org/content/84/9/4442#ref-list-1

CONTENT ALERTS
Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: **http://journals.asm.org/site/misc/reprints.xhtml**
To subscribe to to another ASM Journal go to: **http://journals.asm.org/site/subscriptions/**

Journals.ASM.org

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

JOURNAL OF VIROLOGY, May 2010, p. 4442–4450 Vol. 84, No. 9
0022-538X/10/$12.00 doi:10.1128/JVI.02717-09
Copyright © 2010, American Society for Microbiology. All Rights Reserved.

PB1-F2 Expression by the 2009 Pandemic H1N1 Influenza Virus Has Minimal Impact on Virulence in Animal Models▽

Rong Hai,[1] Mirco Schmolke,[1] Zsuzsanna T. Varga,[1] Balaji Manicassamy,[1] Taia T. Wang,[1] Jessica A. Belser,[4] Melissa B. Pearce,[4] Adolfo García-Sastre,[1,2,3] Terrence M. Tumpey,[4] and Peter Palese[1,3]*

Department of Microbiology,[1] Institute of Global Health and Emerging Pathogens,[2] and Department of Medicine, Division of Infectious Diseases,[3] Mount Sinai School of Medicine, New York, New York, and Immunology and Pathogenesis Branch, Influenza Division, CCID, NCIRD, Centers for Disease Control and Prevention, Atlanta, Georgia[4]

Received 24 December 2009/Accepted 5 February 2010

Unlike previous pandemic viruses, the 2009 H1N1 pandemic influenza virus does not code for the virulence factor PB1-F2. The genome of the 2009 H1N1 virus contains three stop codons preventing PB1-F2 expression; however, PB1-F2 production could occur following genetic mutation or reassortment. Thus, it is of great interest to understand the impact that expression of the PB1-F2 protein might have in the context of the 2009 pandemic influenza virus, A/California/04/2009 (Cal/09). We have addressed this question by generating two Cal/09 viruses with productive PB1-F2 open reading frames containing either an asparagine at position 66 of PB1-F2 (66N) or a serine at position 66 (66S): this N66S change has previously been shown to be associated with increased virulence in mice. We used these viruses to investigate the effect on virulence conferred by expression of the 66N or the 66S PB1-F2 protein in both *in vitro* and *in vivo* systems. Our results show enhanced replication of the 66S virus in A549 cells, while studies of BALB/c and DBA/2 mice and ferrets revealed no significant differences in symptoms of infection with wild-type Cal/09 versus the 66N or 66S virus variant. Also, coinfection of mice with *Streptococcus pneumoniae* and the different viruses (recombinant wild-type [rWT] Cal/09 and the 66N and 66S viruses) did not result in significant differences in mortality. Mice infected with either PB1-F2-expressing virus did demonstrate altered protein levels of proinflammatory cytokines; differences were observed to be greater in infection caused by the 66S virus. In summary, our study demonstrates that PB1-F2 expression by the Cal/09 virus modulates the immune response to infection while having a minimal effect on virus virulence in two mammalian models.

Influenza A viruses, members of the family *Orthomyxoviridae*, cause recurrent epidemics and global pandemics (21). Early in 2009, a new H1N1 quadruple reassortant influenza virus of swine origin emerged and spread globally (22). The pandemic 2009 H1N1 virus is generally associated with mild disease and a relatively low mortality rate. In contrast, influenza viruses responsible for the three pandemics of the last century, in 1918 (H1N1), 1957 (H2N2), and 1968 (H3N2), caused millions of deaths worldwide (11, 20). It is speculated that the absence of specific virulence factors, such as expression of the PB1-F2 protein, is responsible for the low virulence associated with the 2009 H1N1 virus. Influenza viruses can acquire virulence factors either through mutation due to the low fidelity of the viral RNA polymerase or through genetic reassortment with other circulating influenza viruses (21). Recent studies have addressed the virulence, pathogenicity, and transmissibility of a prototypic strain of the 2009 H1N1 virus, influenza A/California/04/2009 (Cal/09), in different mammalian models (12, 14, 18). However, a crucial question remains: how might acquisition of additional virulence factors affect the disease caused by the 2009 H1N1 influenza virus? In this study we have sought to address the contribution of PB1-F2 protein production to virulence in order to better understand the potential consequences of genetic changes resulting in the acquisition of a functional PB1-F2 gene by this pandemic virus.

PB1-F2 is a short viral protein of approximately 90 amino acids expressed from a +1 reading frame in the PB1 gene segment (4). Previous studies have shown that the PB1-F2 protein plays an important role in determining the degree of virulence seen in both primary influenza virus infection (5, 27) and secondary bacterial infection (16). In addition, a serine at position 66 in PB1-F2 is associated with increased disease pathology in a mouse model (5). The underlying molecular mechanisms behind the increased virulence associated with PB1-F2 expression are still a subject of discussion and research. PB1-F2 is known to have a proapoptotic function in immune cells as a result of interaction with the mitochondrial-membrane-associated proteins VDAC-1 and ANT-3 (4, 26–27). Additionally, PB1-F2 has been shown to increase influenza virus polymerase activity *in vitro* through binding to the viral polymerase subunit PB1 (15).

The pandemic H1N1 virus encodes a truncated 11-amino-acid form of PB1-F2; the open reading frame (ORF) for PB1-F2 in Cal/09 contains three stop codons preventing expression of the full-length protein. Since PB1-F2 is under the selection pressure of the PB1 segment, the amino acid sequence of the Cal/09 virus PB1-F2 that would be generated if these three stop codons were mutated would be quite different from the PB1-F2 sequences found in the Puerto Rico/8/34 (PR8) virus or the 1918 virus, the prototype viruses used in

* Corresponding author. Mailing address: Department of Microbiology, Mount Sinai School of Medicine, 1 Gustave Levy Place, New York, NY 10029-6574. Phone: (212) 241-7318. Fax: (212) 722-3634. E-mail: peter.palese@mssm.edu.
▽ Published ahead of print on 24 February 2010.

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

prior studies of PB1-F2. The predicted full-length Cal/09 PB1-F2 is most closely related to the PB1-F2 proteins from H3 viruses and contains the basic amphipathic helix in the C terminus. This region has been shown to be responsible for localization to the inner mitochondrial membrane (3, 25) and to mediate the formation of nonspecific pores in synthetic lipid bilayers (3).

In this study we used reverse genetics to rescue Cal/09 viruses coding for both the 66N and 66S forms of the full-length PB1-F2 protein as models of acquisition via genetic mutation. We then evaluated the effect of the introduced PB1-F2 protein on viral virulence in both *in vitro* and *in vivo* systems. We found that viruses possessing a functional PB1-F2 protein displayed altered expression of proinflammatory genes, with enhanced replication in human cells associated with the 66S mutation. However, the PB1-F2 protein did not significantly enhance virus virulence in either the mouse or the ferret model.

MATERIALS AND METHODS

Cells, bacteria, and viruses. A549, 293T, and MDCK cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained either in Dulbecco's minimal essential medium (DMEM) or in MEM (Gibco, Invitrogen) supplemented with 10% fetal calf serum (HyClone; Thermo Scientific) and penicillin-streptomycin (Gibco, Invitrogen). *Streptococcus pneumoniae* ATCC 6303, a serotype 3 strain, was obtained from the ATCC (Manassas, VA) and was grown to mid-logarithmic phase at 37°C in Todd-Hewitt broth (Difco, Detroit, MI) in a 5% CO_2 chamber.

All influenza A/California/04/2009 (Cal/09) recombinant viruses were propagated in MDCK cells for 3 days at 35°C. All experiments involving 2009 H1N1 viruses were conducted under biosafety level 3 (BSL-3) conditions for both *in vitro* work and *in vivo* animal work, in accordance with the guidelines of the World Health Organization (24a).

Construction of plasmids. The eight reverse-genetics plasmids used for the rescue of recombinant influenza A/California/04/2009 (Cal/09) virus—pDZ-Cal04-PB2, pDZ-Cal04-PB1, pDZ-Cal04-PA, pDZ-Cal04-NP, pDZ-Cal04-HA, pDZ-Cal04-NA, pDZ-Cal04-M, and pDZ-Cal04-NS—were constructed by methods described previously (8–9, 19). A genetic tag was inserted into the NS-encoding plasmid by site-specific mutagenesis, using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Nucleotides 263 to 268 (5′-GCTACC-3′) were replaced with 5′-GGTACC-3′, resulting in one silent mutation and the creation of a novel KpnI restriction enzyme site. The mutation was confirmed by sequencing.

The PB1 plasmids coding for complete PB1-F2 proteins—pDZ-Cal04-66N-PB1 and pDZ-Cal04-66S-PB1—were derivatives of the wild-type (WT) PB1 segment. Briefly, the pDZ-Cal04-PB1 plasmid was subjected to site-directed mutagenesis using the Stratagene QuikChange mutagenesis kit. The stop codons at positions 12, 58, and 88 were changed to serine, tryptophan, and tryptophan, respectively. The sequences of each construct were confirmed by automated sequencing performed at the Mount Sinai School of Medicine sequencing core facility.

Rescue of recombinant influenza A viruses. The influenza A Cal/04 viruses were rescued as described previously (9). 293T cells were transfected with eight pDZ vectors containing viral genomic RNA segments. At 12 h posttransfection, the 293T cells were cocultured with MDCK cells. The rescued viruses were further isolated by plaque purification on MDCK cells. The presence of the introduced mutations in the PB1 segments was confirmed by sequencing the reverse transcriptase PCR (RT-PCR) product of viral RNA (vRNA).

Growth curves of recombinant viruses. To analyze viral replication, confluent MDCK and A549 cells were infected at a multiplicity of infection (MOI) of 0.05. After incubation at 33°C in minimal essential medium containing 0.3% bovine albumin and 1 μg of tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin/ml, viral titers in supernatants were determined by plaque assays in MDCK cells at selected time points postinfection (p.i.).

Infections of mice. Eight-week-old female BALB/c mice and DBA/2 mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized with a mixture of ketamine and xylazine administered intraperitoneally and were infected intranasally (i.n.) with different doses of viruses in a volume of 50 μl. To evaluate the pathogenicity of the viruses, mice were inoculated with the indicated doses (see Fig. 3) and were monitored daily for weight loss up to 14 days postinfection. All mice showing more than 25% body weight loss were considered to have reached the experimental end point and were euthanized humanely. For the determination of lung virus titers, histology, and cytokines, mice were euthanized at the indicated day postinfection (see Fig. 3). Lungs were homogenized and resuspended in 1 ml of sterile phosphate-buffered saline (PBS), and viral titers were evaluated on MDCK cells in the presence of 1.5 μg of TPCK-treated trypsin/ml. For viral and bacterial coinfection experiments, recombinant influenza viruses were given intranasally to groups of 10 mice at a dose of 5×10^5 PFU per mouse. At day 7 postinfection, the animals were challenged with 20 CFU of *S. pneumoniae*. Body weights were monitored daily. Bacterial lung titers, cytokine/chemokine expression, and lung histopathology were evaluated at day 4 post-bacterial challenge.

Cytokine/chemokine quantitation. The expression of cytokines/chemokines in the lung was determined by multiplex enzyme-linked immunosorbent assays (ELISAs).

The concentrations of six different cytokines/chemokines (gamma interferon [IFN-γ], interleukin-1β [IL-1β], monocyte chemoattractant protein 1 [MCP-1], macrophage inflammatory protein 1β [MIP-1β], RANTES, and tumor necrosis factor alpha [TNF-α]) in the supernatants of the lung homogenates were investigated. A Beadlyte human multiplex ELISA analysis (Millipore) was performed based on the manufacturer's instructions. Briefly, 100 μl of supernatant samples per well was incubated in a 96-well polyvinylidene difluoride filter (pore size, 1.2 μm) plate especially designed to retain cytokines/chemokines, with a mixture of anti-cytokine IgG-conjugated beads for the different cytokines/chemokines assayed. After overnight incubation at 4°C, the plate was washed three times with assay solution (PBS [pH 7.4] containing 1% BSA, 0.05% Tween 20, and 0.05% sodium azide). The washes were followed by a 1.5-h incubation with biotin-conjugated anti-cytokine IgG at room temperature. After another wash, streptavidin-phycoerythrin (streptavidin-PE) was added, followed by the addition after 30 min of stop solution (0.2% [vol/vol]) formaldehyde in PBS [pH 7.4]). The plate was then filtered, and the contents of each well were resuspended in 125 μl of assay buffer and was read in a Luminex 100 machine.

Histopathologic examination. The mouse lungs were removed immediately following euthanasia, inflated, and fixed with 10% neutral buffered formalin overnight at 4°C. Subsequently, they were embedded in paraffin, sectioned at a thickness of 4 μm, stained with hematoxylin and eosin (H&E), and examined under light microscopy for histopathologic changes. The images were obtained on a Zeiss Axioplan 2IE epifluorescence microscope at a magnification of ×20.

Ferret pathogenesis experiment. Male Fitch ferrets (Triple F Farms, Sayre, PA), 6 to 12 months old and serologically negative for currently circulating influenza viruses by hemagglutination inhibition (HI) assays, were used in this study. Ferrets were housed for the duration of each experiment in a Duo-Flow Bioclean mobile clean room (Lab Products, Inc., Seaford, DE). For each virus, 3 to 5 ferrets were inoculated i.n. with 10^6 PFU. Ferrets were monitored daily for clinical signs as previously described (1). Nasal washes were collected on alternate days p.i. and were titrated by a standard plaque assay for determination of viral titers. Statistical significance for all experiments was determined using Student's *t* test.

Ferret hematologic analyses. On days zero, 3, 7, and 19 p.i., blood was collected in EDTA Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) from 3 to 5 inoculated ferrets from each virus group. Complete blood counts from peripheral blood were determined using a Hemavet HV950FS instrument according to the manufacturer's instructions (Drew Scientific, Inc., Oxford, CT).

RESULTS

Generation of PB1-F2-expressing A/California/04/2009 influenza viruses. All known human pandemic influenza viruses express the virulence factor PB1-F2, except for the current pandemic virus, Cal/09. With stop codons removed, all 2009 pandemic H1 viruses would code for the same PB1-F2 protein (based on available sequences), which is 40% divergent from the 1918 H1N1 PB1-F2 and close to 30% divergent from the 1957 H2 and 1968 H3 virus PB1-F2 proteins (Fig. 1).

In order to restore PB1-F2 expression by Cal/09, we first established a reverse-genetics system for this virus. All eight viral gene segments were cloned into the pDZ rescue plasmid through reverse transcriptase PCR of viral RNA purified from supernatants of MDCK cells infected with A/California/04/

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

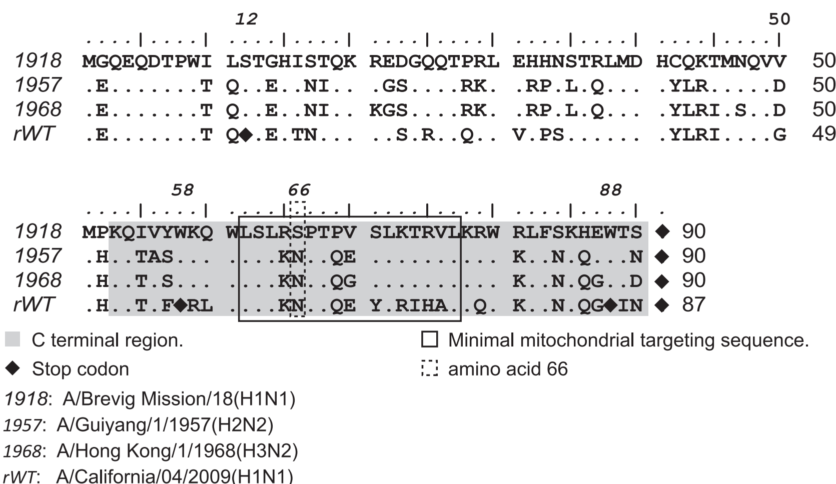

FIG. 1. Alignment of the PB1-F2 open reading frames of pandemic influenza A virus strains. The coding regions for the PB1-F2 amino acid sequences of the wild type Cal/09 virus and of other pandemic influenza viruses (1918 H1N1, 1957 H2N2, and 1968 H3N2) were aligned. The mutations of stop codons necessary for the restoration of the PB1-F2 open reading frame (rWT) coding for the 66N or 66S version of the protein are indicated.

2009 (8, 9). Viruses were rescued by methods described previously (8, 19). The presence of the genetic marker KpnI, located in the viral NS segment, was verified in the rescued viruses (data not shown).

The PB1 segment of the Cal/09 virus contains three stop codons in the PB1-F2 +1 open reading frame (ORF), at amino acid positions 12, 58, and 88, that preclude the expression of the full-length protein (Fig. 1). To mimic a potential genetic change in Cal/09 that would enable PB1-F2 expression, we used our reverse-genetics system to generate recombinant viruses with the desired genetic modifications. We first generated a PB1 segment with a productive PB1-F2 open reading frame by changing the three stop codons to the corresponding conserved codons enabling wild-type PB1-F2 production. This was done without changing the amino acid sequence of the PB1 gene. Additionally, we generated a recombinant Cal/09 virus coding for a previously described point mutation at position 66 in the PB1-F2 ORF that changes the asparagine (66N) to serine (66S) without altering the amino acid sequence of the PB1 gene (Fig. 1). This mutation is associated with enhanced virulence of the 1918 virus in mice (5). The two mutant Cal/09 viruses (Cal/09 66N and Cal/09 66S) were rescued in the same manner as the rWT Cal/09 virus. We could not obtain direct evidence for the expression of the two forms of PB1-F2 under *in vivo* infection conditions due to the lack of a Cal/09 PB1-F2-specific antibody. However, the Cal/09 PB1 segment has all the critical sequences for PB1-F2 translation as proposed by Chen et al. (4), and the Flag-tagged 66S/66N Cal/09 PB1-F2 protein is detected in a Western blot assay under *in vitro* expression conditions (data not shown). This suggests that the PB1-F2 protein is likely expressed in our PB1-F2 mutant viruses. All recombinant viruses were subsequently amplified in MDCK cells. The sequences of the PB1 segments were confirmed through sequencing.

The PB1-F2 66S mutant enhances viral replication in A549 cells. To study the impact of the restored PB1-F2 ORF on the Cal/09 virus, we examined the replication of both the 66N and 66S PB1-F2-expressing viruses compared with that of the rWT Cal/09 virus in both MDCK and A549 cells. All viruses grew to comparable titers in MDCK cells (Fig. 2A). Furthermore, the plaque sizes of the different viruses in MDCK cells were similar (Fig. 2C). In contrast, when we grew these viruses in A549 cells, a human alveolar basal epithelial cell line, the Cal/09 66S virus exhibited higher peak titers than the Cal/09 66N and rWT viruses (Fig. 2B). Following this *in vitro* work, we utilized animal systems to more closely evaluate the effect of PB1-F2 expression on disease.

Expression of PB1-F2 proteins does not have a significant impact on virulence in BALB/c mice. To ascertain if the differences observed in *in vitro* growth kinetics affected virus pathogenicity *in vivo*, we inoculated BALB/c mice with either PB1-F2-expressing or wild-type Cal/09 viruses in order to evaluate weight loss, viral replication in the lungs, proinflammatory gene expression, and lung pathology following infection. In general, mice infected with 5×10^5 PFU of any virus displayed comparable and transient morbidity (as measured by weight loss) (Fig. 3A). Mice infected with the Cal/09 66S virus experienced slightly more weight loss than other infected animals on days 2 and 3 postchallenge. Similar results were obtained following virus challenge with 5×10^3 PFU or 5×10^2 PFU (data not shown).

To better understand the differences in the virulence of disease caused by infection with these viruses, we next examined the kinetics of virus replication in the lungs. Mice were infected intranasally with 5×10^3 PFU of different Cal/09 viruses as indicated in Fig. 3B. On days 3, 6, and 9 postinfection, three mice per group were sacrificed in order to determine viral titers in lung homogenates (Fig. 3B). On both day 3 and day 6 p.i., all three groups showed similar viral lung titers. On day 9 p.i., virus remained in the homogenates of only one out the three animals infected with either PB1-F2-expressing Cal/09 virus.

It has been shown that PB1-F2 affects the immune response to influenza virus infection by enhancing the production of proinflammatory cytokines/chemokines and the recruitment of immune cells (5, 16). We therefore proceeded to evaluate

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

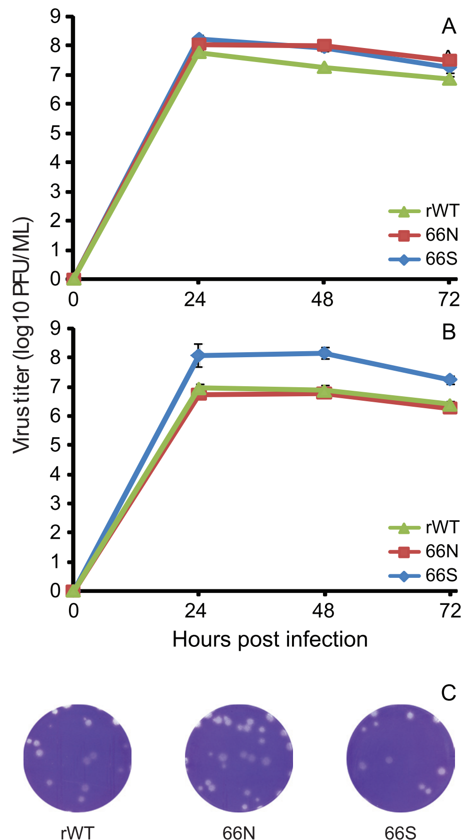

FIG. 2. Characterization of the recombinant Cal/09 viruses *in vitro*. (A and B) MDCK cells (A) and A549 cells (B) were infected with the rWT and PB1-F2-expressing viruses (MOI, 0.05). At the indicated time points after infection, virus titers in the supernatants were determined by plaque assays on MDCK cells. Average titers ± standard deviations are indicated. (C) Plaque phenotypes of the recombinant Cal/09 viruses in MDCK cells.

pathological changes (Fig. 3C) in the lungs of mice infected with the Cal/09 viruses and the induction of proinflammatory genes (Fig. 3D). Histologic examination of lungs taken at 3, 6, and 9 days p.i. revealed degenerative changes with various degrees of neutrophilic infiltrates and diffuse alveolar damage with edema in all infected mice, especially on day 3 p.i. In accordance with the recovery of body weight and decreased proinflammatory gene expression, the degenerative changes were diminished on day 9 postinfection. The production of the cytokines IFN-γ, IL-1β, and TNF-α was similar in all infected animals. However, levels of MCP-1, MIP-1β, and RANTES were significantly higher in mice infected with the Cal/09 66S virus than in mice infected with the Cal/09 rWT virus at day 6 p.i. (Fig. 3D). Together, these data indicate that infection with the Cal/09 66S virus induces higher expression levels of selected cytokines and chemokines at early time points than infection with either of the other two viruses (Fig. 3C and D).

PB1-F2 proteins do not have a significant impact on virulence in DBA/2 mice. It was recently shown that DBA/2 mice are more susceptible to influenza A virus infection than BALB/c mice (2). To better distinguish between potential differences in viral virulence conferred by PB1-F2 expression on the Cal/09 background, we assessed the virulences of these viruses in the DBA/2 model. DBA/2 mice were infected intranasally with the different Cal/09 viruses at either 5×10^5 PFU, 5×10^4 PFU, or 5×10^3 PFU (Fig. 4). Pronounced morbidity and mortality were observed following virus challenge levels as low as 5×10^3 PFU, with all animals succumbing to infection by day 7 p.i. (Fig. 4A). To evaluate viral replication in the lungs of the DBA/2 mice, animals were infected intranasally with recombinant viruses at a dose of 5×10^3 PFU and were sacrificed on either day 2, 5, or 7 p.i. for the determination of viral titers (Fig. 4B). Similar viral titers were recovered from all three groups of infected mice on days 2 and 5 p.i. On day 7 p.i., animals infected with either the Cal/09 66N or the Cal/09 66S virus had a 5-fold increase in titers over those of mice infected with the rWT Cal/09 virus. In summary, all three viruses demonstrated similar virulences in DBA/2 mice with respect to both morbidity and mortality. It should be noted, however, that small differences in virulence may not have been detectable under the conditions used in the experiments.

We next performed an analysis of cytokine/chemokine expression in the lungs during infection (Fig. 4C). Infection with the Cal/09 66S virus was associated with enhanced expression of particular proinflammatory genes, but with a profile different from that of the BALB/c model. On days 2 and 5 postinfection, IL-1β protein could be detected only in samples from mice infected with the Cal/09 66S virus. Protein levels of MIP-1β and RANTES were also significantly increased in the lung samples from mice infected with the Cal/09 66S virus on day 5 postinfection.

PB1-F2 expression does not exacerbate secondary bacterial infection with *Streptococcus pneumoniae*. It has been shown that the PB1-F2 protein promotes bacterial infection secondary to influenza virus infection in mice (16). To determine the impact of the Cal/09 PB1-F2 protein in priming the host for secondary bacterial infection, we conducted coinfection studies of BALB/c mice (Fig. 5). Animals infected with the different Cal/09 viruses were challenged with a sublethal dose of *S. pneumoniae* 7 days post-viral infection. Viral lung titers were determined at the time of bacterial infection. Infection with the Cal/09 66S virus led to a 5-fold increase in the viral lung titer over that resulting from infection with the rWT Cal/09 virus and a 2-fold increase in the titer over that resulting from Cal/09 66N virus infection (Fig. 5A). By day 5 post-bacterial challenge, 2 out of 10 mice in the Cal/09 66S virus infection group, compared with 1 out of 10 mice in each of the other two virus infection groups, had succumbed to infection (Fig. 5B). These data indicate that expression of PB1-F2 in the context of the novel Cal/09 virus does not significantly alter the mortality associated with secondary bacterial infection in mice.

Expression of PB1-F2 does not significantly alter disease caused by infection with Cal/09 in ferrets. Ferrets are an established model in influenza virology for studies of disease pathology and transmission (23, 28). We therefore investigated the impact of PB1-F2 expression on the virulence of the Cal/09 virus in the Fitch ferret model. Animals were inoculated intranasally with 10^6 PFU of the Cal/09 wild-type virus or a variant Cal/09 virus and were observed for 14 days p.i. for clinical signs of infection (Fig. 6A). Ferrets infected with the 66S virus experienced slightly more weight loss than those infected with the 66N or wild-type virus, but as observed in the mouse models, disease associated with 66S infection was minimally

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

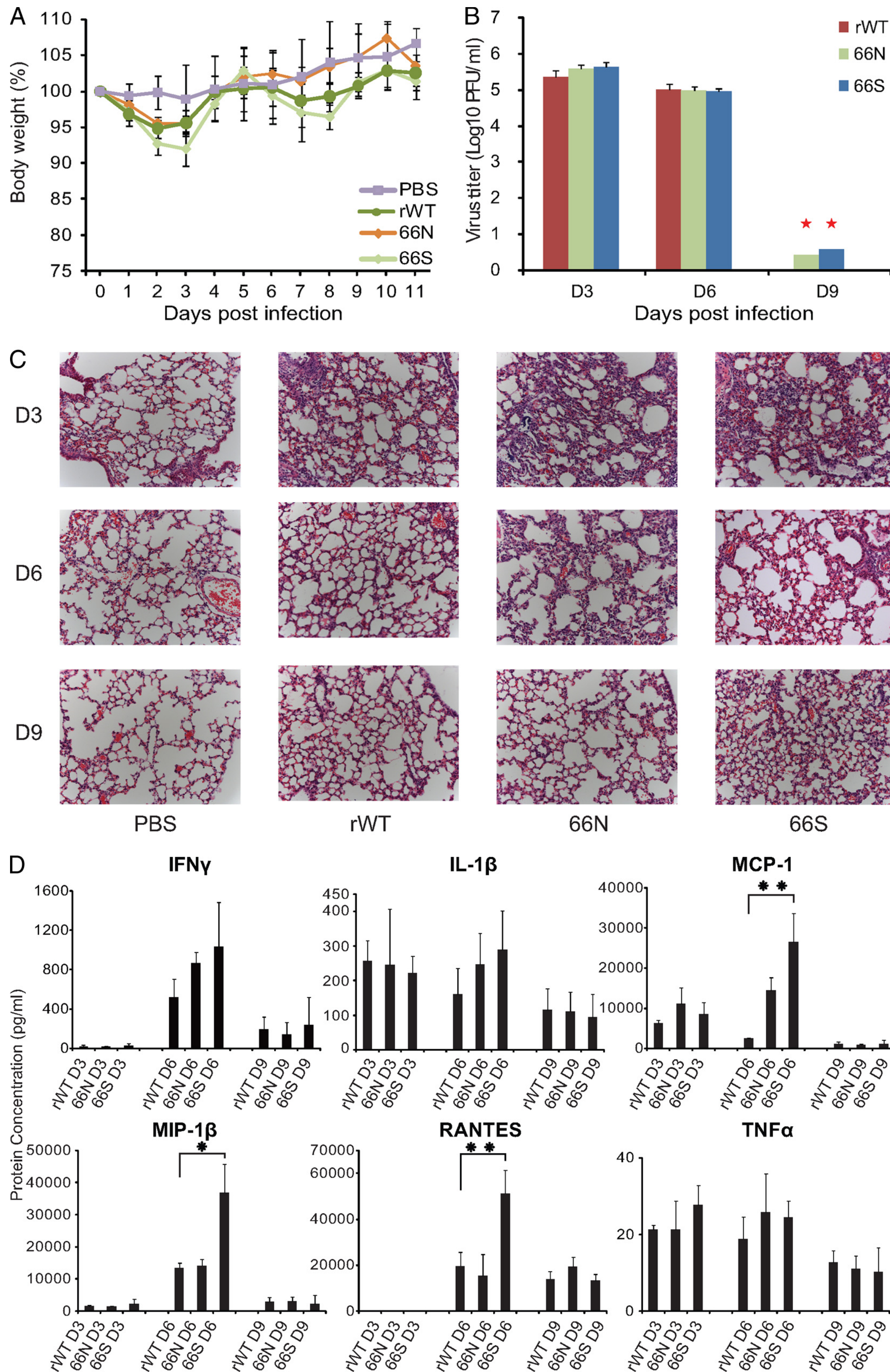

FIG. 3. Disease caused by infection with the wild-type or PB1-F2-expressing Cal/09 viruses in BALB/c mice. Eight-week-old female BALB/c mice (five animals per group) were infected intranasally with 5×10^5 PFU of the indicated rescued rWT or PB1-F2-expressing mutant Cal/09 virus. (A) Following viral infection, mice were weighed daily, and the average body weights ± standard deviations of surviving animals in each group up to day 11 postinfection are shown as percentages of the original body weights. (B) Three mice per group were infected intranasally with 5×10^3

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

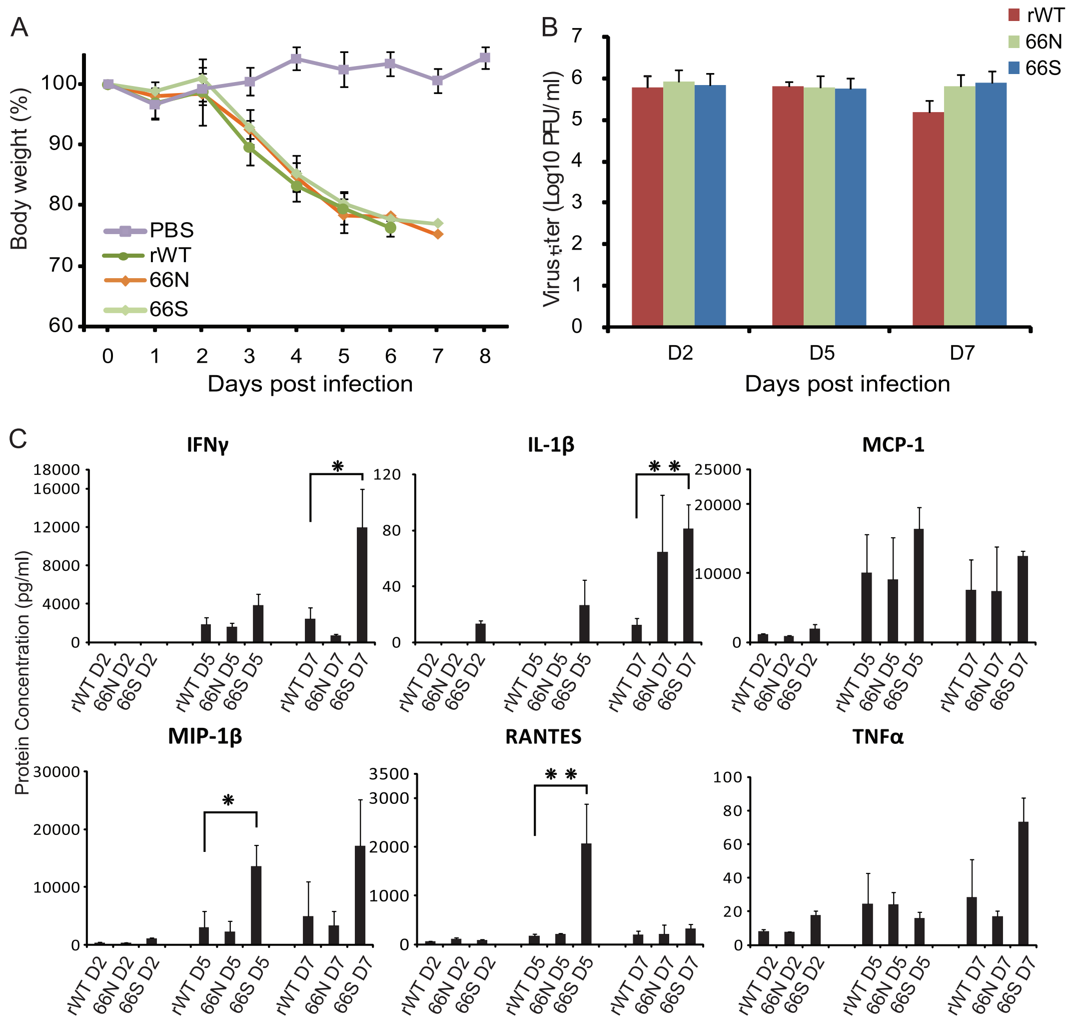

FIG. 4. Disease caused by infection with wild-type or PB1-F2-expressing Cal/09 viruses in DBA/2 mice. Eight-week-old female DBA/2 mice (five animals per group) were infected intranasally with 5×10^3 PFU of the indicated rescued rWT and PB1-F2-expressing mutant Cal/09 viruses. (A) Following viral infection, the body weights of mice were monitored daily, and the average body weights ± standard deviations of surviving animals in each group up to day 8 postinfection are shown as percentages of the original body weights. (B) Nine mice per group were infected intranasally with 5×10^3 PFU of the same set of viruses. On days 2, 5, and 7 postinfection, three mice per group were sacrificed, and virus titers in the lungs were determined. Average lung titers ± standard deviations are shown. The limit of detection was 5 PFU. (C) Changes in cytokine/chemokine expression were detected by multiplex ELISAs at 2, 5, and 7 days postinfection. Average cytokine/chemokine levels ± standard deviations are shown. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) from results for rWT Cal/09 virus-infected mice.

exacerbated over disease caused by wild-type Cal/09 infection. Nasal wash titers on days 1, 3, and 5 p.i. revealed no significant differences in upper respiratory tract growth between any of the Cal/09 viruses (Fig. 6B).

Next we performed a leukocyte analysis on peripheral blood taken 3, 7, and 19 days postinfection. Interestingly, infection with any PB1-F2-expressing virus but not with wild-type virus was correlated with significantly dysregulated white cell counts on days 3 and 7 postinfection (P, <0.05); PB1-F2 expression was associated with both lymphopenia and elevated neutrophil counts (Fig. 7). Lymphopenia in all ferrets was transient, and levels of leukocytes returned to baseline by day 19 p.i.

Since PB1-F2 expression in the Cal/09 virus could occur either by genetic mutation (as has been explored in this study)

PFU of the same set of viruses. On days 3, 6, and 9 postinfection (D3, D6, and D9), three mice per group were sacrificed, and the virus titers in the lungs were determined from the lung homogenate supernatants(★, viruses were recovered from only one out of three animals.). Average lung titers ± standard deviations are shown. The limit of detection was 5 PFU. (C) Six mice per group were infected intranasally with 5×10^3 PFU of the recombinant Cal/09 viruses. On days 3, 6, and 9 postinfection, two mice per group were euthanized. The lungs were extracted for histologic examination. Representative images are shown. (D) Cytokine/chemokine levels in the supernatants of lung homogenates were detected by multiplex ELISAs at 3, 6, and 9 days postinfection. Average cytokine levels ± standard deviations are shown. Asterisks indicate significant differences (*, $P < 0.05$; **, $P < 0.01$) from results for rWT Cal/09 virus-infected mice.

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

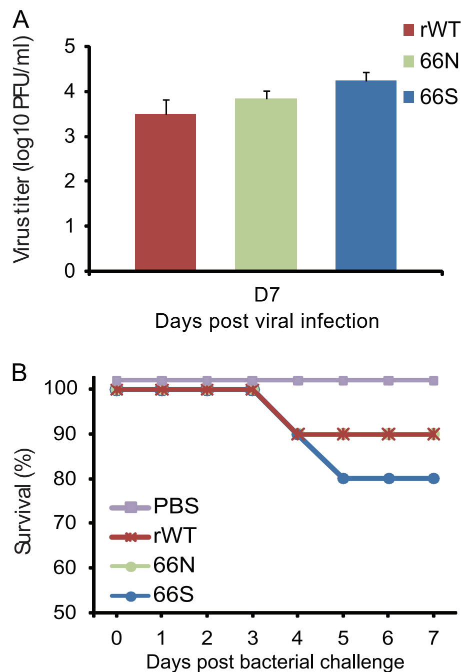

FIG. 5. Characterization of the impact of Cal/09 PB1-F2 on secondary bacterial infection. Groups of 15 8-week-old female BALB/c mice were either treated with PBS or infected intranasally with 5×10^5 PFU of the indicated rescued rWT or PB1-F2-expressing mutant Cal/09 virus. Seven days later, they were challenged with a sublethal dose (20 CFU) of *S. pneumoniae*, serotype 3. (A) On day 7 postinfection, three mice per infection group were sacrificed, and virus titers in the lungs were determined. The limit of detection was 5 PFU. (B) Survival is plotted until day 7 post-bacterial challenge.

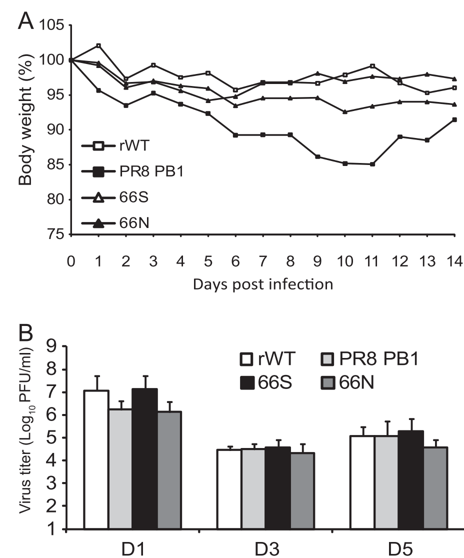

FIG. 6. Weight change of H1N1-infected ferrets and viral replication in the upper respiratory tracts of infected ferrets. (A) Ferrets were inoculated i.n. with 1×10^6 PFU of virus (the rWT, 66S, or 66N Cal/09 virus or the PR8-PB1 reassortant virus). The percentage of weight change was determined daily by comparing the mean weight of animals infected with each virus to the mean preinfection weight. The mean percentage of weight change for each virus is shown. (B) Ferrets were inoculated i.n. with 1×10^6 PFU/ml of the indicated virus. Viral titers were measured in nasal washes collected on the indicated days p.i. Each titer is expressed as the mean $\log_{10}$ PFU/ml plus standard deviation. The limit of virus detection was 10 PFU. Titers on day 7 p.i. were below the limit of detection. D1, day 1; D3, day 3; D5, day 5.

or by genetic reassortment, we generated an additional virus expressing PB1-F2 from a prototype H1 virus, PR/8/34. This model of a Cal/09 reassortant virus, PR8-PB1-F2 Cal/09, was associated with a slightly exacerbated disease phenotype in ferrets compared with disease due to viruses expressing PB1-F2 as a result of genetic mutation in the Cal/09 PB1 gene (66S and 66N viruses) (Fig. 6 and 7).

DISCUSSION

The severity of influenza A virus infections is modulated by the expression of virulence factors such as PB1-F2. In contrast to the PB1-F2-encoding viruses that caused the three pandemics of the 20th century, the H1N1 virus responsible for the 2009 pandemic does not express PB1-F2 and is associated with a relatively mild disease phenotype. In order to better understand the consequences of potential genetic change in the pandemic Cal/09 influenza virus, we conducted experiments designed to characterize the disease phenotype associated with a PB1-F2-expressing version of Cal/09.

By developing a reverse-genetics system for the Cal/09 virus, we were able to rescue three Cal/09 viruses that have identical PB1 protein sequences: the wild-type virus and variants that encode either the 66N or the 66S PB1-F2 protein (Fig. 1). We examined the growth kinetics for all three Cal/09 viruses and observed enhanced replication by the 66S virus in the human epithelial cell line A549, leading to peak titers roughly 1 log unit higher than those for the other Cal/09 viruses. This effect was not observed in the canine MDCK cell line. Since influenza viruses replicate very efficiently in MDCK cells, it is likely that the rather mild contribution to virulence by the PB1-F2 protein in the Cal/09 background is simply not detectable in this cell system.

Our *in vivo* experiments were conducted in two different mouse strains. We first tested the effect of PB1-F2 on the virulence of Cal/09 virus infection in BALB/c mice. Expression of PB1-F2 in the Cal/09 virus had no significant effect on disease pathogenesis (Fig. 3). We observed only transient morbidity, with no mortality, following infection with any of the Cal/09 viruses. In addition, peak viral titers in the lungs were similar for the Cal/09 rWT, 66N, and 66S virus groups. Analysis of cytokine/chemokine levels in the supernatants of lung homogenates revealed increased expression of proinflammatory genes (e.g., MCP-1, MIP-1β, and RANTES), which are essential for the recruitment and activation of immune cells in infected tissues (6, 7, 13, 24). These proinflammatory proteins have also been shown to prevent the apoptosis of alveolar macrophages in the context of influenza virus infection *in vivo* (10). Accordingly, we detected more-severe histopathology in the lung tissues of mice infected with the PB1-F2-expressing viruses, especially the 66S mutant virus, than in those of rWT-infected mice. In summary, the introduction of a functional PB1-F2 ORF did not enhance the mortality associated with infection with the Cal/09 virus in the BALB/c mouse system. PB1-F2 expression did, however, result in a modulated host

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

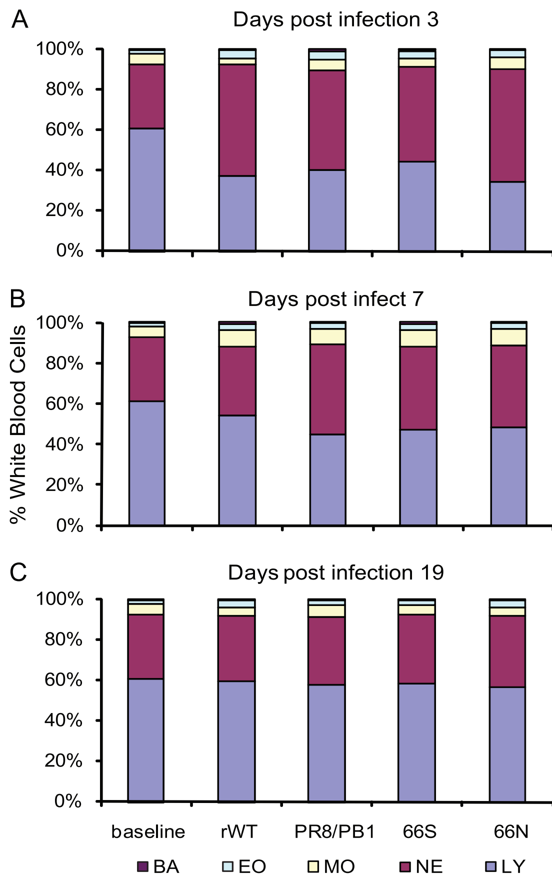

FIG. 7. Analysis of circulating lymphocytes following influenza virus infection. For each virus, three to five ferrets were inoculated i.n. with 1×10^6 PFU. Blood was collected on days 3 (A), 7 (B), and 19 (C) p.i. into EDTA Vacutainer tubes and was analyzed with a hematology scanner. Blood collected immediately prior to inoculation was included as a baseline control (naïve). The average percentages of lymphocytes (LY), neutrophils (NE), monocytes (MO), eosinophils (EO), and basophils (BA) in whole blood are shown.

immune response characterized by increased expression of proinflammatory genes.

The data obtained in our BALB/c mouse study suggested that in a more susceptible mouse strain, such as the DBA/2 mouse (2), we would observe more-pronounced differences between the disease phenotype caused by the PB1-F2-expressing Cal/09 viruses and that caused by the wild-type virus. However, under the experimental conditions used, the results of our studies of DBA/2 mice were very similar to the results of our BALB/c mouse studies. Expression of the 66S PB1-F2 protein was correlated with enhanced expression of the proinflammatory genes IL-1β and RANTES. Additionally, we observed that expression of PB1-F2 did not affect the susceptibility of BALB/c mice to secondary bacterial infections.

Taken together, our mouse studies suggest that a genetic change resulting in the presence of a functional PB1-F2 ORF would not significantly increase the virulence of primary infection caused by the Cal/09 virus or the susceptibility of a host to secondary bacterial infection with *Streptococcus pneumoniae*.

The fact that the expression of the PB1-F2 protein in the Cal/09 virus did not have a significant impact on primary viral infection or secondary bacterial infection in mice is intriguing, given that the prototype PB1-F2 proteins have been shown to affect both viral and bacterial infections (5, 16, 27). As previously mentioned, the sequence of the Cal/09 PB1-F2 protein is unique. Even though Cal/09 PB1-F2 is predicted to contain known functional regions, such as the mitochondrial targeting sequence, the protein seems to have a lesser effect on viral virulence than prototype PB1-F2 proteins, at least in the context of the Cal/09 virus. This supports findings that enhanced viral virulence conferred by PB1-F2 expression is a strain-specific phenomenon (17).

The results of our studies on weight loss and viral replication in ferrets paralleled the results of our mouse studies. Expression of PB1-F2 was not statistically significantly associated with enhanced virulence, but ferrets infected with the 66S variant did demonstrate slightly more weight loss (without mortality) than those infected with the 66N or wild-type Cal/09 virus. PB1-F2 expression did cause significant dysregulation of peripheral leukocyte counts, including lymphopenia and elevated neutrophil levels. Infection with the reassortant PR8-PB1-F2 Cal/09 virus caused the most severe disease phenotype of all viruses studied in ferrets; importantly, however, the PR8 PB1 protein may contribute to the observed phenotype.

In summary, we found that mutations enabling the production of PB1-F2 by the Cal/09 influenza virus do not have a significant impact on the virulence of the virus in mice or in ferrets. These preliminary observations, however, invite further investigations into the virulence of other PB1-F2 mutant viruses and of possible reassortants of the Cal/09 virus with current viruses. The present findings enhance our understanding of PB1-F2 as a virulence factor and provide new insights into the impact that genetic changes may have on the virulence of the 2009 pandemic virus.

ACKNOWLEDGMENTS

We thank Dmitriy Zamarin for helpful discussions and critical review. We also thank Rafael A. Medina and Randy Albrecht for help with the BSL-3 training process, and we express our appreciation to Lily Ngai for excellent technical assistance.

This work was supported by CRIP (Center for Research on Influenza Pathogenesis, NIAID contract HHSN266200700010C) and by grants P01 AI 058113 and U19 AI62623 (Center for Investigating Viral Immunity and Antagonism) from the NIH. T.T.W. was supported by NIH training grant T32 AI007647 and Mount Sinai Medical Scientists Training Grant T32 GM007280.

REFERENCES

1. **Belser, J. A., X. Lu, T. R. Maines, C. Smith, Y. Li, R. O. Donis, J. M. Katz, and T. M. Tumpey.** 2007. Pathogenesis of avian influenza (H7) virus infection in mice and ferrets: enhanced virulence of Eurasian H7N7 viruses isolated from humans. J. Virol. **81:**11139–11147.
2. **Boon, A. C., J. deBeauchamp, A. Hollmann, J. Luke, M. Kotb, S. Rowe, D. Finkelstein, G. Neale, L. Lu, R. W. Williams, and R. J. Webby.** 2009. Host genetic variation affects resistance to infection with a highly pathogenic H5N1 influenza A virus in mice. J. Virol. **83:**10417–10426.
3. **Chanturiya, A. N., G. Basanez, U. Schubert, P. Henklein, J. W. Yewdell, and J. Zimmerberg.** 2004. PB1-F2, an influenza A virus-encoded proapoptotic mitochondrial protein, creates variably sized pores in planar lipid membranes. J. Virol. **78:**6304–6312.
4. **Chen, W., P. A. Calvo, D. Malide, J. Gibbs, U. Schubert, I. Bacik, S. Basta, R. O'Neill, J. Schickli, P. Palese, P. Henklein, J. R. Bennink, and J. W. Yewdell.** 2001. A novel influenza A virus mitochondrial protein that induces cell death. Nat. Med. **7:**1306–1312.
5. **Conenello, G. M., D. Zamarin, L. A. Perrone, T. Tumpey, and P. Palese.** 2007. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog. **3:**1414–1421.
6. **Dawson, T. C., M. A. Beck, W. A. Kuziel, F. Henderson, and N. Maeda.** 2000.

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE

Contrasting effects of CCR5 and CCR2 deficiency in the pulmonary inflammatory response to influenza A virus. Am. J. Pathol. **156:**1951–1959.

7. **Dessing, M. C., K. F. van der Sluijs, S. Florquin, and T. van der Poll.** 2007. Monocyte chemoattractant protein 1 contributes to an adequate immune response in influenza pneumonia. Clin. Immunol. **125:**328–336.
8. **Fodor, E., L. Devenish, O. G. Engelhardt, P. Palese, G. G. Brownlee, and A. Garcia-Sastre.** 1999. Rescue of influenza A virus from recombinant DNA. J. Virol. **73:**9679–9682.
9. **Hai, R., L. Martinez-Sobrido, K. A. Fraser, J. Ayllon, A. Garcia-Sastre, and P. Palese.** 2008. Influenza B virus NS1-truncated mutants: live-attenuated vaccine approach. J. Virol. **82:**10580–10590.
10. **Herold, S., M. Steinmueller, W. von Wulffen, L. Cakarova, R. Pinto, S. Pleschka, M. Mack, W. A. Kuziel, N. Corazza, T. Brunner, W. Seeger, and J. Lohmeyer.** 2008. Lung epithelial apoptosis in influenza virus pneumonia: the role of macrophage-expressed TNF-related apoptosis-inducing ligand. J. Exp. Med. **205:**3065–3077.
11. **Hilleman, M. R.** 2002. Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control. Vaccine **20:**3068–3087.
12. **Itoh, Y., K. Shinya, M. Kiso, T. Watanabe, Y. Sakoda, M. Hatta, Y. Muramoto, D. Tamura, Y. Sakai-Tagawa, T. Noda, S. Sakabe, M. Imai, Y. Hatta, S. Watanabe, C. Li, S. Yamada, K. Fujii, S. Murakami, H. Imai, S. Kakugawa, M. Ito, R. Takano, K. Iwatsuki-Horimoto, M. Shimojima, T. Horimoto, H. Goto, K. Takahashi, A. Makino, H. Ishigaki, M. Nakayama, M. Okamatsu, D. Warshauer, P. A. Shult, R. Saito, H. Suzuki, Y. Furuta, M. Yamashita, K. Mitamura, K. Nakano, M. Nakamura, R. Brockman-Schneider, H. Mitamura, M. Yamazaki, N. Sugaya, M. Suresh, M. Ozawa, G. Neumann, J. Gern, H. Kida, K. Ogasawara, and Y. Kawaoka.** 2009. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature **460:**1021–1025.
13. **Lin, K. L., Y. Suzuki, H. Nakano, E. Ramsburg, and M. D. Gunn.** 2008. CCR2+ monocyte-derived dendritic cells and exudate macrophages produce influenza-induced pulmonary immune pathology and mortality. J. Immunol. **180:**2562–2572.
14. **Maines, T. R., A. Jayaraman, J. A. Belser, D. A. Wadford, C. Pappas, H. Zeng, K. M. Gustin, M. B. Pearce, K. Viswanathan, Z. H. Shriver, R. Raman, N. J. Cox, R. Sasisekharan, J. M. Katz, and T. M. Tumpey.** 2009. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science **325:**484–487.
15. **Mazur, I., D. Anhlan, D. Mitzner, L. Wixler, U. Schubert, and S. Ludwig.** 2008. The proapoptotic influenza A virus protein PB1-F2 regulates viral polymerase activity by interaction with the PB1 protein. Cell. Microbiol. **10:**1140–1152.
16. **McAuley, J. L., F. Hornung, K. L. Boyd, A. M. Smith, R. McKeon, J. Bennink, J. W. Yewdell, and J. A. McCullers.** 2007. Expression of the 1918 influenza A virus PB1-F2 enhances the pathogenesis of viral and secondary bacterial pneumonia. Cell Host Microbe **2:**240–249.
17. **McAuley, J. L., K. Zhang, and J. A. McCullers.** 2010. The effects of influenza A virus PB1-F2 protein on polymerase activity are strain specific and do not impact pathogenesis. J. Virol. **84:**558–564.
18. **Munster, V. J., E. de Wit, J. M. van den Brand, S. Herfst, E. J. Schrauwen, T. M. Bestebroer, D. van de Vijver, C. A. Boucher, M. Koopmans, G. F. Rimmelzwaan, T. Kuiken, A. D. Osterhaus, and R. A. Fouchier.** 2009. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science **325:**481–483.
19. **Neumann, G., T. Watanabe, H. Ito, S. Watanabe, H. Goto, P. Gao, M. Hughes, D. R. Perez, R. Donis, E. Hoffmann, G. Hobom, and Y. Kawaoka.** 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc. Natl. Acad. Sci. U. S. A. **96:**9345–9350.
20. **Palese, P.** 2004. Influenza: old and new threats. Nat. Med. **10:**S82–S87.
21. **Palese, P., and M. L. Shaw.** 2007. *Orthomyxoviridae*: the viruses and their replication, p. 1647–1689. *In* D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 5th ed., vol. 1. Lippincott-Raven Press, Philadelphia, PA.
22. **Smith, G. J., D. Vijaykrishna, J. Bahl, S. J. Lycett, M. Worobey, O. G. Pybus, S. K. Ma, C. L. Cheung, J. Raghwani, S. Bhatt, J. S. Peiris, Y. Guan, and A. Rambaut.** 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature **459:**1122–1125.
23. **Smith, H., and C. Sweet.** 1988. Lessons for human influenza from pathogenicity studies with ferrets. Rev. Infect. Dis. **10:**56–75.
24. **Wareing, M. D., A. B. Lyon, B. Lu, C. Gerard, and S. R. Sarawar.** 2004. Chemokine expression during the development and resolution of a pulmonary leukocyte response to influenza A virus infection in mice. J. Leukoc. Biol. **76:**886–895.

24a. **World Health Organization.** 30 November 2009, revision date. Laboratory biorisk management for laboratories handling human specimens suspected or confirmed to contain pandemic influenza A (H1N1) 2009 virus. World Health Organization, Geneva, Switzerland. https://www.who.int/csr/resources/publications/swineflu/Laboratorybioriskmanagement.pdf.

25. **Yamada, H., R. Chounan, Y. Higashi, N. Kurihara, and H. Kido.** 2004. Mitochondrial targeting sequence of the influenza A virus PB1-F2 protein and its function in mitochondria. FEBS Lett. **578:**331–336.
26. **Zamarin, D., A. Garcia-Sastre, X. Xiao, R. Wang, and P. Palese.** 2005. Influenza virus PB1-F2 protein induces cell death through mitochondrial ANT3 and VDAC1. PLoS Pathog. **1:**e4.
27. **Zamarin, D., M. B. Ortigoza, and P. Palese.** 2006. Influenza A virus PB1-F2 protein contributes to viral pathogenesis in mice. J. Virol. **80:**7976–7983.
28. **Zitzow, L. A., T. Rowe, T. Morken, W. J. Shieh, S. Zaki, and J. M. Katz.** 2002. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J. Virol. **76:**4420–4429.

Downloaded from http://jvi.asm.org/ on September 7, 2014 by UNIVERSITE DE GENEVE