Importin-α7 is Required for Enhanced Influenza A Virus Replication in the Alveolar Epithelium and Severe Lung Damage in Mice

Patricia Resa-Infante*, René Thieme**, Thomas Ernse, Petra C. Arck*, Harald Ittrich*, Rudolph Reimer* and Gülsah Gabriel†

*Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology, Hamburg, Germany; **Laboratory for Experimental Fetomaternal Medicine, Department of Obstetrics and Fetal Medicine, University Medical Center Hamburg, Hamburg, Germany; †Diagnostic and Interventional Radiology Department and Clinic, University Hospital Hamburg-Eppendorf, Hamburg, Germany;

Running title: Importin-α7 & influenza virus alveolar replication

*Address correspondence to Gülsah Gabriel, guelsah.gabriel@hpi.uni-hamburg.de

Abstract: 249 words; Importance: 146

Text: 5355 words
Abstract

Influenza A viruses recruit components of the nuclear import pathway to enter host cell nucleus and promote viral replication. Here, we analyzed the role of the nuclear import factor importin-α7 in H1N1 influenza virus pulmonary tropism using various ex vivo imaging techniques (magnetic resonance imaging, confocal laser-scanning microscopy and correlative light-electron microscopy). We infected importin-α7 gene deficient mice (α7−/−) with a recombinant H1N1 influenza virus and assessed in vivo viral kinetics in comparison to wild type (WT) mice.

In WT mice, influenza virus replication occurred in bronchial and alveolar epithelium already a few days after infection. Accordingly, extensive mononuclear infiltration and alveolar destruction was present in lungs of infected WT mice followed by 100% lethality. Conversely, in α7−/− mice, virus replication was mostly restricted to bronchial epithelium with marginal alveolar infection resulting in significantly reduced lung damage and enhanced animal survival. To investigate the host immune response during alveolar virus replication, we studied the role of primary macrophages in virus propagation and clearance. The ability of macrophages to support or clear virus infection as well as the host cellular immune responses did not significantly differ between WT and α7−/− mice. However, cytokine and chemokine responses were generally elevated in WT mice likely reflective of increased viral replication in the lung.

In summary, these data show that a cellular factor, importin-α7, is required for enhanced virus replication in the alveolar epithelium resulting in elevated cytokine and chemokine levels, extensive mononuclear infiltration and thus, severe pneumonia and enhanced virulence in mice.
Importance

Influenza A viruses are respiratory pathogens which may cause pneumonia in humans. Viral infection and replication in the alveoli of the respiratory tract is believed to be crucial for the development of the acute respiratory distress syndrome associated with fatal outcome in influenza virus infected patients. Here, we report the requirement of a cellular factor, importin-α7, for efficient virus replication in the alveolar epithelium of mice. Using complementary ex vivo imaging approaches, we show that influenza virus replication is restricted to the bronchial epithelium followed by enhanced survival in importin-α7 deficient mice. In contrast, presence of this gene results in enhanced virus replication in the alveoli, elevated cytokine and chemokine responses, mononuclear infiltration, alveolar destruction and 100% lethality in wild type mice. Taken together, we show that importin-α7 is particularly required for virus replication in the alveolar epithelium associated with severe pneumonia and death in mice.
Introduction

Influenza A viruses cause yearly epidemics and recurrent pandemics. The emergence of new influenza virus strains from animal reservoirs further poses a continuous threat to human health (1). Influenza virus transcription and replication occurs in the nucleus of respective host cells. Since cellular components which transport the viral ribonucleoprotein complex into the nucleus are divergent between species, the viral polymerase subunit PB2 and the nucleoprotein (NP) require adaptation to the nuclear import machinery upon interspecies transmission (2). The classical nuclear import pathway consists of an adaptor protein, importin-\(\alpha\), that binds to cargo proteins containing a nuclear localization signal and the transport receptor protein, importin-\(\beta1\), which then promotes nuclear entry (3). Influenza viruses with host adaptive signatures in their ribonucleoprotein complexes, such as PB2 627K or 701N, bind more efficiently to human importin-\(\alpha7\) proteins. It was shown that importin-\(\alpha7\) is a positive cellular factor required for efficient virus polymerase activity and replication in human cells (4, 5). Furthermore, deletion of the importin-\(\alpha7\) gene results in reduced pathogenicity of mammalian influenza viruses in mice (5, 6). These findings highlight the importance of importin-\(\alpha7\) for influenza virus replication and pathogenicity in the mammalian host.

The primary target cells for influenza virus replication are epithelial cells of the respiratory tract. Virus replication in respiratory cells and subsequent modulation of the host immune response may lead to compromised respiratory functions (7). Hereby, influenza virus infection of the alveolar epithelium is particularly crucial for the development of an acute respiratory distress syndrome (ARDS) which often results in fatal outcome in humans (8). Viral replication in the alveolar epithelium can damage the epithelial-endothelial barrier resulting in leakage of
proteinaceous fluid into the alveolar lumen and thus respiratory insufficiency and death (8).

Moreover, infected epithelial cells produce cytokines which then attract leucocytes, such as macrophages and neutrophils stimulating further mononuclear infiltration and respiratory failure (7, 8).

It was previously reported that lung macrophages of WT mice were more abundantly positive for viral RNA compared to those of $\alpha 7^{-/-}$ mice (6). This observation suggests that a potential modulation of the host immune response might be a reason for enhanced pathogenicity in the mammalian host. To shed light on the role of the importin-$\alpha 7$ gene in lung pathology, we used a recombinant influenza virus which expresses a green fluorescent protein (GFP) fused to the viral NS1 gene of the A/Puerto Rico/8/34 PR8 (H1N1) virus (PR8:NS1-GFP) which replicates efficiently in the murine lung and causes severe pathogenicity in mice (9, 10). In both, WT and $\alpha 7^{-/-}$ mice we analyzed the in vivo virus kinetics and the pulmonary tropism. These studies were complemented by ex vivo imaging techniques (magnetic resonance imaging, confocal laser-scanning microscopy and correlative light-electron microscopy) to assess the role of the importin-$\alpha 7$ in viral lung tropism and its potential effect on the host immune response.
Materials and Methods

Ethical Statement

All animal experiments were approved by the relevant German authority (Behörde für Stadtentwicklung und Umwelt Hamburg) and performed according to the national guidelines of the animal protection law (Tierschutzgesetz; project number: 97/11) in Germany.

Cells and viruses

Madin-Darby canine kidney (MDCK) cells were grown in minimal essential medium (PAA, Linz, Austria) supplemented with 10% fetal calf serum (PAA), 1% glutamine (PAA), and 1% penicillin-streptomycin (PAA).

Generation of the PR8:NS1-GFP (H1N1) virus was described previously (9). Briefly, the GFP used in this recombinant virus derived from the pMAXGFP plasmid (Amaxa) which contains the enhanced green fluorescent protein (EGFP) gene from the copepod P. lumata..

Animal experiments

Importin-α7/− (α7−/−) mice (6, 11) and wild type (WT) littermates thereof in the C57BL/6J genetic background were maintained at the animal facility of the Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology in Hamburg, Germany.

Mice were anaesthetized with ketamine-xylazine (70 mg/kg and 7 mg/kg, respectively). MLD50 studies were performed using serial virus dilutions of PR8:NS1-GFP (10^4, 10^5 and 10^6 p.f.u.) in WT and α7−/− mice. In all other studies, WT and α7−/− mice were intranasally infected with 10^6 p.f.u. of the PR8:NS1-GFP virus. Survival and weight loss were monitored for 14 days. Mice
were humanely killed upon >25% weight loss according to the guidelines of the German animal protection law.

For detection of virus replication kinetic and lung tropism, whole lungs of WT and α7−/− mice infected with PR8:NS1-GFP influenza virus were harvested on days 1, 3 and 6 p.i.. The lung of each animal was divided in two parts: one part was processed for immunohistochemical analysis and the other part was used for virus titration and cytokine measurements. The anatomical areas within the lung were chosen randomly to diminish detection bias. For cytokine and virus titer detection, lungs were further homogenized in 1 ml of PBS, centrifuged at 956xg for 5 minutes at 4ºC, and aliquots of supernatants were stored at -80ºC. Virus titers were determined by plaque assays on MDCK cells.

**Cytokine assay**

A mouse cytokine antibody array (RayBiotech, Inc.) was used according to the manufacturer’s instructions to detect cytokines and chemokines in lung homogenates of influenza virus infected mice. Samples were not thawed more than once for this assay. The lung homogenate of 3-5 mice were pooled and measured with one array. Intensity of signals was quantified with the Bioimager Image Quant LAS 4000 at non-saturated levels. The array tests for the following cytokines and chemokines: GCS-F, GM-CSF, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL12 (p40p70), IL12(p70), IL-13, IL-17, IFN-γ, MCP-1, MCP-5, RANTES, stem cell factor, sTNFRI, TNF-α, thrombopoietin and vascular endothelial growth factor.

**Histological analysis**

Lungs were harvested and fixed with 3.7% of paraformaldehyde and embedded in paraffin as described before (12). Briefly, tissue sections were directly visualized by fluorescent microscopy.
or further processed by immunohistochemistry using an anti-FPV-serum and the ZytoChem Plus HRP-DAB Kit Broad Spectrum (Zytomed) according to the manufacturer’s instructions.

**Correlative imaging**

Mice were intranasally infected with $10^6$ p.f.u. of PR8:NS1-GFP or treated with PBS as a control. On day 3 p.i., they were sacrificed and intracardially perfused with 2% PFA. Lungs were removed *en bloc*, degassed in 2% PFA solution overnight and embedded in PBS buffered low melting point agarose (1%). Magnetic resonance imaging (MRI) examinations were performed using a dedicated small animal MRI scanner at 7 T (ClinScan, Bruker BioSpin, Ettlingen Germany) and a circularly polarized bird cage send/receive-coil with an inner diameter of 40 mm (Bruker BioSpin, Ettlingen Germany). MRI is a non-invasive tomographic imaging technique utilizing the sensitivity of the nuclear magnetic resonance of hydrogen to its chemical environment to generate contrast in grey-scale images of biological samples. The generated digital images are discrete and the volume elements of the sample corresponding to the image pixels are named voxel. As the signal intensity, i.e. the grey value of each voxel is given in arbitrary units, it is appropriate to use the signal-to-noise-ratio (SNR) to compare images acquired from different samples.

High resolution images with an isotropic voxel dimensions of 90 μm were acquired with a 3-dimensional fast recovery fast spin echo sequence. Imaging parameters were chosen as follows: Repetition time 1500 ms, echo time 44 ms, turbo factor 8, number of signal averages 2, field of view 35 mm, and acquisition matrix size $384 \times 384 \times 96$. The total acquisition time was 6:43 h. This special sequence generates T2-weighted images, in which high SNR corresponds to a high hydrogen density and motility, e.g. free watery fluid. In contrast, low SNR indicates tissues with low hydrogen density, structure heterogeneities, e.g. caused by fibrotic tissue alterations, the
presence of high protein density or paramagnetic substances as deoxyhemoglobin. Regarding to
the animal models, low SNR values of the lung parenchyma represent regions of inflammatory
lung tissue alterations.
Quantitative MR analyses were performed using ImageJ 1.37 (National Institutes of Health,
Bethesda, USA). Therefore the lung parenchyma was automatically segmented from the agarose
gel background (lower and upper threshold of signal intensities: 400, 1500). Then, the
distributions of the signal intensities within the lungs of all specimens were determined. The SNR
of each lung voxel was calculated measuring the standard deviation of the background noise of
the agar surrounding the lungs. With these data, the mean SNR and normal standard deviation
(SD) of all lung voxels were calculated for each lung separately.
After MRI imaging, the lungs were sectioned into 200 μm sections with a Vibratome (Leica
VT1200 S). Several sections were preselected by fluorescence microscopy at low magnifications
(Nikon AZ100) and imaged by confocal microscopy (Zeiss LSM510 meta and Nikon C2 plus)
after staining with the CellMask Orange Plasma membrane Stain (Life Technologies, Carlsbad,
CA, USA). Additionally, selected sections were stained with 1% osmium tetroxide in PBS and
investigated fully hydrated in the ESEM (Philips XL30ESEM) with the BSE detector. Alternative
selected sections were postfixied with glutaraldehyde (2.5 % in PBS) and processed for TEM as
described before (13).

**Primary macrophage isolation and in vitro activity assays**
Primary macrophages were isolated either by broncho-alveolar lavage (BAL) to obtain alveolar
macrophages or by peritoneal lavage to obtain naive tissue resident macrophages, as described
previously (14). Briefly, cells obtained from peritoneal exudates were harvested from euthanized
naive mice by administration of 5 ml PBS/0.5% EDTA into the peritoneal cavity. BAL was
performed using the same animal. Therefore, the chest cavity was opened and the trachea was
cannulated with a needle and anchored by suturing. The lung was gently infused 10 times with
0.5 ml PBS/0.5% EDTA. Cells from mice \((n = 4-6)\) were pooled and pelleted by centrifugation at
400 x g and 4 °C for 10 min. Cells were resuspended in Macrophage Media SFM (Gibco) and the
phenotype was assessed by flow cytometry (Table 2).

For \textit{in vitro} infection of macrophages, \(10^5\) cells/well were plated into a 24-well cell culture plate
(Nunc, China). Sixteen hours after plating, more than 80% of the remaining cells were
macrophages and were directly infected at a multiplicity of infection of 1 in the presence of
0.25µg/µl TPCK-trypsine (Table 2). Viral titers were determined by plaque assay on MDCK cells
at several time points after infection (0, 6, 24 h p.i.).

Alternatively, isolated macrophages were directly used in virus clearance experiments as
previously described (15). Briefly, MDCK cells \((2 \times 10^5\) cells/well\) were seeded into a 24-well
cell culture plate (Nunc, China) and infected with PR8:NS1-GFP \((\text{MOI} = 0.01)\) 24 h later. After
4 h, cells from BAL or peritoneal exudates were added to the infected MDCK cell cultures. The
ratio between effector and MDCK cells was 1:1 for cells from BAL and 10:1 for cells obtained
from peritoneal lavage. Cell culture media was supplemented with 0.25µg/µl TPCK-trypsine.
Supernatants were collected 7 h later and virus titers \((11\ h\ p.i.)\) were determined on MDCK cells
by plaque assays.

\textbf{Flow cytometry}

Mice were euthanized and exsanguinated at indicated times post infection. BAL was performed
as described above with 500 µl ice cold PBS \((2\ mM\ EDTA)\). Lungs were removed after PBS
perfusion of the right ventricle of the heart and digested using 30 IU DNase I (Sigma-Aldrich,
Taufkirchen, Germany) and 2 mg/ml Collagenase D (Roche, Mannheim, Germany) at 37 °C for
30 minutes. Then, cells were passed through a 40 μM cell strainer and red blood cells were lysed by RCL buffer (eBiosience, Frankfurt a.M., Germany). Unspecific antibody binding was blocked by CD16/CD32 block (TruStain fcX™ BioLegend, San Diego, US) and normal rat serum (Jackson Immuno Research, Suffolk, UK). Multicolor analyses were performed using a LSR II (BD, Heidelberg, Germany) and analyzed with FlowJo software (Tree Star, Ashland, USA). The gating strategy illustrated in Fig. 10 has been adapted from previous studies especially to analyse the effect of viral infection on different macrophage populations that were distinguished as either resident in the lung (CD11c⁻) or as those recruited from the blood stream and infiltrating into the lung (CD11cmid) (16). Monoclonal antibodies specific for CD19 (6D5), NKp46 (29A1.4), CD8 (53-6.7), CD4 (RM4-5), CD11b (M1/70), Gr-1 (RB6-8C5), CD11c (N418), CD3e (145-2C11), F4/80 (CI:A3-1) and CD45 (30-F11) were purchased from BioLegend or eBiosciences. Alexa Fluor 594 carboxylic acid, succinimidyl ester was used to stain and exclude dead cells before fixation with 1% PFA for 15 minutes. Cells were reconstituted in 0.5% BSA / 2 mM EDTA / PBS before acquisition.

Statistical analysis

All data shown are presented as mean ± SEM. Mean, SEM, Student’s t test (unpaired, 2-tailed), ANOVA and Mantel-Cox test were calculated with Prism GraphPad software (GraphPad Software, Inc., La Jolla, USA). p < 0.05 was considered significant.
Results

Influenza virus replication and pathogenesis is reduced in α7-/- mice

First, we have assessed PR8:NS1-GFP (H1N1) influenza virus pathogenesis in WT and α7-/- mice. Infected WT animals showed severe weight loss and pathogenicity resulting in approximately three times more virulence compared to α7-/- mice (Fig. 1A and 1B; Table 1). Analysis of viral lung titers showed similar virus replication properties between WT and α7-/- mice on day 3 p.i., but reduced virus titers in α7-/- mice on day 6 p.i. (Fig. 1C). These findings confirm and extend previous reports (5, 6) showing that importin-α7 is required for enhanced H1N1 influenza virus replication and pathogenicity in mice.

Influenza virus replication is reduced in the alveoli of α7-/- mice

First, we correlated the NS1-GFP expression profile to viral NP-positive cells in the lungs of PR8:NS1-GFP (H1N1) influenza virus infected WT and α7-/- mice by histochemical and immunohistochemical analysis (Fig. 2). NS1-GFP positive pulmonary cells were detected in bronchial epithelium and alveolar tissue already on day 1 p.i. in WT mice (Fig. 2A). At 3 days p.i., viral replication manifested in bronchial epithelial cells with increasing levels of GFP-positive cells in the alveoli. Virus replication further increased in both, bronchial epithelium and alveolar tissue on day 6 p.i. in WT mice. In contrast, virus replication was strongly reduced in the lungs of α7-/- mice. GFP-positive epithelial cells were not detectable in the bronchi until day 3 p.i. and only marginally present in the alveoli on day 6.p.i. (Fig. 2A). Consistently, NP-positive cells were most abundant in bronchial and alveolar cells of WT lungs following the kinetics of GFP expression. Again, in α7-/- mice, virus replication was largely restricted to epithelial cells of the bronchi with single NP-
positive alveolar cells on day 6 p.i. (Fig. 2B). Furthermore, enhanced virus replication in the
alveoli correlated with mononuclear infiltration as early as day 3 p.i. and severe alveolar
destruction on day 6 p.i. in WT mice. In contrast, alveolar damage was strongly reduced in the
lungs of α7−/− mice where first single mononuclear infiltrations were observed on day 6 p.i. (Fig.
2).

These findings show that importin-α7 is required for enhanced virus replication in alveolar cells
of the respiratory tract.

**Lung damage upon influenza virus replication is strongly reduced in α7−/− mice**

Comparable expression patterns of GFP- and NP-positive cells in the lungs of PR8:NS1-GFP
(H1N1) influenza virus infected mice confirmed the feasibility of using GFP expression as a
marker for active virus replication in subsequent *ex vivo* imaging. Since the highest number of
GFP-positive stromal and immune cells as well as the highest virus titers were detected on day 3
p.i. (Fig. 1C and 3), we used this time point of productive virus replication for further imaging of
the lung.

Therefore, we developed an *ex vivo* method to analyze the macroscopic damage of infected lungs
by magnetic resonance imaging (MRI) (Fig. 4 and Videos S1, S2 and S3). The major advantages
of this non-destructive method are the high soft tissue contrast and the relatively rapid
examination of the whole lung pathology in contrast to histological analysis which is restricted to
certain pulmonary areas.

*Ex vivo* MRI of the lungs of uninfected control mice differed from those of WT and α7−/− mice
(Fig. 4). Bronchi, bronchioles, alveoli as well as all pulmonary vessels (pulmonary arteries and
veins) were fluid-filled and therefore the lung tissue appears artificially hyperintense in T2-
weighted MRI. Control mice showed a regular anatomical structure and texture in T2w MRI with
a homogenous tissue pattern. In contrast, WT and $\alpha_7^{-/-}$ mice presented virus induced lung damage with typical inflammatory changes, as septal thickening leading to reticular consolidations and more tissue heterogeneity (Fig. 4A). In consequence, these changes result in a reduced SNR of the lung parenchyma in T2w images which was most prominent in WT mice whereas reduced in $\alpha_7^{-/-}$ mice (Fig. 4B). T2w MR image-based quantitative volumetric SNR analyses of the lungs showed the highest SNR in the lung of control mice (71.5±15.07), followed by $\alpha_7^{-/-}$ (67.99±14.88) and WT mice (59.4±10.37).

These data show that WT lung parenchyma is more damaged in WT mice than in $\alpha_7^{-/-}$ mice.

**Progeny virus was most abundantly detected in areas of infected bronchi**

Next, we analyzed physical sections of the lungs described above by fluorescence light microscopy, environmental scanning electron microscopy (ESEM) and correlative light-electron microscopy in order to assess whether areas of virus infection and replication in the lung correlate with areas of virus production (Figures 5).

Fluorescence microscopy imaging of lungs revealed that virus infection and replication predominantly occurred in the bronchial tubes of $\alpha_7^{-/-}$ lungs in contrast to WT lungs where in addition, alveolar cells were abundantly infected. However, the density of GFP$^+$ cells in the bronchi was higher in $\alpha_7^{-/-}$ than in WT mice (Fig. 5A and 5C). ESEM revealed severely damaged parenchymal areas in WT lungs in contrast to $\alpha_7^{-/-}$ lungs (Fig. 5B and 5D). These findings correlate with the observations made in the histological sections (Fig. 2) and MRI analysis (Fig. 4).

Next, we preselected infected areas using a light microscope and further processed small circular biopsies by transmission electron microscopy (Fig. 5E and 5G). Upon progressive magnification of these areas, we could detect virus particles especially in areas of infected bronchial epithelia.
By inspection of random fields, we found a higher content of virus particles especially around bronchial epithelial cells in both, \(\alpha 7^{-/-}\) and WT mice, compared to the alveoli (Fig. 5F and 5H).

These observations indicate that primary virus production most likely occurs in infected bronchial epithelium. Furthermore, importin-\(\alpha 7\) is particularly required for alveolar replication which seems to be essential for severe lung damage and enhanced pathogenicity in mice.

**Cytokine and chemokine levels are reduced in influenza virus infected \(\alpha 7^{-/-}\) mice**

We next analyzed whether infection of differential respiratory cells in WT and \(\alpha 7^{-/-}\) mice might affect cytokine and chemokine responses which are associated with lung damage upon influenza virus infection. Therefore, we measured the innate immune response in the lungs of PR8:NS1-GFP (H1N1) influenza virus infected mice using an antibody array for 22 different cytokines and chemokines.

Here, we only show the analytes that revealed significant differences between WT and \(\alpha 7^{-/-}\) mice upon influenza virus infection. In general, chemokine and cytokine levels were considerably more elevated during the course of infection in WT than in \(\alpha 7^{-/-}\) mice (Fig. 6). The only cytokines which showed an exception hereof were IL-9 and IL-12 with higher levels in \(\alpha 7^{-/-}\) than WT mice throughout the infection course (Fig. 6H and I).

These data suggest that the presence of importin-\(\alpha 7\) generally correlates with elevated cytokine and chemokine responses in the lung.

**Cellular immune responses are not significantly altered in WT and \(\alpha 7^{-/-}\) mice**

In order to identify infected immune cell subsets, which could potentially contribute to reduced cytokine and chemokine responses in \(\alpha 7^{-/-}\) infected mice, we quantified the cellular immune sub-
populations in the lung upon infection with PR8:NS1-GFP on day 3 p.i. by multicolor flow cytometry (Fig. 7 and 8).

In general, the distribution of immune cell populations in the lung was similar in uninfected WT and α7−/− control mice, except for dendritic cells (DC), identified as CD11b+, CD11chigh, and alveolar macrophages (CD11b−, CD11chigh) populations that were considerably elevated in uninfected α7−/− controls compared to uninfected WT controls (Fig. 7C, 7D and 7I). Upon influenza virus infection, a higher frequency of granulocytes with a CD11b+, CD11c−, F4/80−, Gr-1+ phenotype was present in cell suspensions obtained from α7−/− lungs compared to those of WT mice (Fig. 7A). A similar pattern was seen for lung macrophages, identified as CD11b+, CD11c−, F4/80−, Gr-1+ (Fig. 7B). Moreover, the presence of GFP in lung granulocytes and macrophages was significantly increased in lung cell suspensions obtained from α7−/− infected mice. The frequency of alveolar macrophages (CD11b−, CD11chigh) and DCs (CD11b+, CD11chigh) was overall reduced upon infection in both, WT and α7−/− mice (Fig. 7C and 7D). Moreover, the frequency of other immune cell sub-populations, including Natural Killer (NK-) and T-cells upon influenza virus infection was not significantly different in the lungs of α7−/− compared to WT mice (Fig. 8A, 8C, 8E, 8G and 8I). Flow cytometric analyses of granulocytes, macrophages, alveolar macrophages and DCs in bronchoalveolar lavage (BAL) largely mirrored the findings observed in lung tissues (Fig. 7F, 7G, 7H, 7I and 8).

These data indicate that the cellular immune response upon influenza virus infection is similar in the lungs of WT and α7−/− mice with regard to the frequency of alveolar macrophages and DCs. However, the frequencies of granulocytes and macrophages are elevated in the absence of importin-α7.
In vitro primary macrophage function in promoting virus replication or clearance are not significantly altered in WT and α7⁺/- mice

To assess the role of increased macrophage frequency upon infection of α7⁺/- mice, we performed in vitro macrophage functionality assays. With this approach we addressed whether viral RNA-positive macrophages in alveoli, which were reported before to be predominantly detected in infected WT but not α7⁺/- lungs (6), reflect active virus replication or the remains of virus clearance.

Therefore, we isolated primary macrophages from naive WT and α7⁺/- mice using two methods: BAL, in order to obtain alveolar macrophages as well as peritoneal lavage in order to obtain interstitial macrophages (Table 2). First, we infected these macrophages in vitro at a multiplicity of infection of 1 (MOI = 1) and analyzed virus replication at different time points after infection by plaque assay (Fig. 9A). Although virus titers increased by approximately four times at 6 h p.i., virus replication was far less efficient in primary mouse macrophages compared to epithelial cells consistent with previous reports (17, 18).

Additionally, we examined virus clearance produced in infected epithelial cells (MDCK) by primary macrophages. MDCK cells infected with PR8:NS1-GFP virus (MOI = 0.01) and co-cultured with primary macrophages isolated from BAL or peritoneal lavages at 4 h p.i. Virus replication was then analyzed 7 h later by plaque assay (Fig. 9B). Both, WT and α7⁺/- primary macrophages isolated from BAL and peritoneal lavages, were able to reduce virus titers at early but not at later time points (48 h, 72 h and 96 h) post infection where high virus titers are produced by epithelial cells (data not shown).

Taken together, these data suggest that primary macrophages are not the primary promoters of productive virus replication. Further, primary alveolar and interstitial macrophages did not show any significant differences in their abilities to clear viral infection in vitro.
Discussion

To our knowledge, this is the first report of a cellular factor which contributes to enhanced virus replication in the alveolar epithelium. The pathogenesis of influenza virus induced acute respiratory distress syndrome (ARDS) has been recently reported to be centered on the alveolar epithelium. Infection of the alveoli is considered to be a crucial site which decides whether primary viral pneumonia with ARDS might develop or not (8). In general, influenza virus infection and replication starts in the respiratory tract in areas close to large conducting airways followed by bronchioles and deeper areas of the lung such as alveoli (19, 20). Influenza viruses target a broad range of epithelial cells in the respiratory tract. Viral replication in the alveolar epithelium, consisting of type I and type II pneumocytes, might damage the epithelial-endothelial barrier of the alveoli leading to fluid leakage into the alveolar lumen and thus ARDS (8).

Combining various \textit{ex vivo} imaging techniques (magnetic resonance imaging, confocal laser-scanning microscopy and correlative transmission electron microscopy), we here show that PR8:NS1-GFP influenza virus replication in α7\textsuperscript{-/-} mice is mostly restricted to the bronchial epithelium. In contrast, the bronchial and alveolar epithelium of WT mice is abundantly infected at a time point where overall virus lung titers do not differ (Fig. 1C and 2). Interestingly, GFP signal intensity increased upon multicycle replication in the bronchial epithelium of α7\textsuperscript{-/-} mice without elevated alveolar replication (Fig. 2 and 5). This suggests that influenza virus replication is indeed restricted to the bronchial epithelium where virus positive cells increase with the replication cycle and cannot be explained by delayed virus kinetics only. Conversely, virus replication increases in the presence of importin-α7 in WT mice with increased alveolar replication during the infection course resulting in alveolar mononuclear infiltration and severe...
lung damage. Furthermore, the grade of alveolar destruction and pneumonia, as analyzed in the MRI, correlates with enhanced virulence in WT compared to α7−/− mice (Fig. 4).

In previous studies where reduced pathogenicity in mice lacking the importin-α7 gene was initially reported, *in situ* hybridization analysis further revealed that MAC3-positive lung macrophages were less positive for influenza virus RNA in α7−/− compared to WT mice (6). To analyze whether this finding is due to improved virus replication in macrophages of WT mice or its improved virus clearance properties, we performed *in vitro* macrophage functionality assays. Since it has been reported that monocyte-derived macrophages are not a good model as they do not reflect the findings in primary alveolar macrophages (20), we have performed all of our studies in primary murine macrophages (Table 2). Both, alveolar and interstitial macrophages only supported low virus production at very early time points after infection (Fig. 9A). However, virus titers detected from alveolar or interstitial macrophages did not differ in WT and α7−/− mice suggesting that importin-α7 is not required for promotion of virus replication in primary macrophages in the lung. These data are consistent with previous reports that virus replication in primary macrophages is usually not highly productive (17, 20, 21). Furthermore, viral clearance activity of primary alveolar and interstitial macrophages obtained from WT and α7−/− mice did not significantly differ suggesting that importin-α7 does not affect the phagocytic activity of macrophages (Fig. 9B). However, we cannot exclude that macrophages might behave differently in the *in vivo* setting since the functional assays in this study have been performed *in vitro*.

Electron microscopical analysis further revealed that virus particles could be readily detected budding from the bronchial but far less from the alveolar epithelium (Fig. 5). This suggests that primary virus production most likely occurs in the bronchi of infected mice. Since our *in vitro*
assays in primary alveolar macrophages did not reveal high level virus production, it seems that at least these immune cells are not the primary contributors to virus production in the alveoli. These findings suggest that virus production in bronchial epithelia, which is not affected in α7/−/− mice, is contributing but not sufficient to develop severe lung damage. This further highlights the importance of alveolar virus infection and replication in lung destruction and enhanced virulence.

Importin-α7 has been shown previously to act as a positive cellular factor required for enhanced influenza virus polymerase activity and replication in human cells (5, 6). Human and mammalian influenza viruses seem to have adapted to importin-α7 usage by acquiring host adaptive mutations 627K or 701N in their polymerase subunit PB2. Thereby, viral polymerase activity is promoted either by enhanced nuclear import (in case of PB2 701N) of the polymerase complex or after nuclear entry (in case of PB2 627K) by a yet unknown mechanism (5, 6). However, importin-α proteins have also been described to transport key cytokines or transcription factors thereof into the nucleus. Interferon-γ was reported to be transported into the nucleus in complex with its receptor IFNGR1 by the importin-α/β pathway mediated by the nuclear location signal in IFN-γ (22). TNF-α induced nuclear translocation of NFκB responsible for gene expression of many cytokines was also reported to be mediated by the importin-α/β pathway (23, 24).

In order to address whether importin-α7 might be involved in displaying differential innate immune responses in influenza virus infected mice, we have analyzed a panel of cytokines and chemokines in the lung (Fig. 6). In general, cytokine and chemokine levels were increased to higher levels in infected WT than α7/−/− mice with the exception of IL-9 and IL-12. Since neither virus replication in primary murine macrophages nor the cellular host immune response in general was affected, this observation might be predominantly reflecting enhanced virus
replication kinetics in epithelial cells. For IL-9 and IL-12, which were considerably elevated in the lungs of α7−/− mice, it was reported that they have protective functions in disease outcome. Thus for IL-9, it was shown to play an important role in tissue repair after helminth-induced lung inflammation. The IL-9 receptor is involved in up-regulation of the anti-apoptotic protein BCL-3 in the murine lung. IL-9 receptor deficient mice fail to restore helminth-induced lung damage (25). IL-12 was reported to contribute to early NK-cell dependent IFN-γ production and to inhibit early influenza virus replication in mice (26). It remains unclear why α7−/− mice display elevated IL-9 and IL-12 levels compared to WT during influenza virus replication in the lung. Whether importin-α7 is involved in the nuclear import pathway of these cytokines needs future investigation to understand their potentially protective role upon influenza virus infection in α7−/− mice.

Thus, reduced virus replication in the alveoli of importin-α7 deficient mice as shown in Figures 2 and 5 seems to lead to a generally reduced innate immune response. This is crucial for protective disease outcome since hypercytokinemia is known to be detrimental for clinical outcome as observed especially upon highly pathogenic avian influenza virus infections in humans (18).

We also analyzed a large variety of immune cell populations of the cellular immune response to study whether these might be affected in importin-α7 deficient mice and additionally contribute to importin-α7 mediated lung pathology. In uninfected α7−/− control lungs, DCs and alveolar macrophages showed slightly higher frequencies than in uninfected WT lungs (Fig. 7). DCs are professional antigen presenting cells and are the main contributors of antigen transport from the infected lung to the draining lymph nodes where they activate virus-specific cytotoxic CD8+ T-cells (7). DCs also belong to the main cytokine and chemokine producing immune cells,
especially for IL-12 in response to pulmonary inflammation. This might pose a potential explanation why higher IL-12 levels were detected in α7−/− mice. This would then link the observed differences in the innate immune system to the altered adaptive immune system. Upon infection with highly pathogenic influenza virus strains, macrophages and neutrophils are the prominent cell types associated with severe lung pathology (17, 18, 27). However, infected α7−/− mice exhibited higher levels of granulocytes and macrophages in lungs and BAL. This might suggest a higher recruitment of these immune cells in α7−/− mice or a reduced cellular viability in WT mice. Furthermore, a higher frequency of GFP+ immune cells was detected in α7−/− than WT lungs on day 3 p.i.. However, this experimental setting does not allow us to distinguish whether these GFP+ cells are reflecting active virus replication or are positive due to uptake of infected cells as a consequence of phagocytosis. In general, flow cytometry studies of infected lungs revealed very similar cellular immune responses in WT and α7−/− mice. The solely elevated GFP+ content in granulocytes and macrophages suggests that importin-α7 does not directly affect the infection or phagocytosis capacity of cells of the innate immune response.

Thus, our findings support the concept that importin-α7 is particularly required for enhanced virus replication in the alveolar epithelium without a major involvement of the cellular host response. It has been described before that importin-α isoforms are expressed at differential levels in different cell types (28). Thus, potential differences in importin-α7 expression levels in alveolar and bronchial epithelial cells might explain its role in promoting virus replication in the respiratory tract. Clearly, future studies are needed to measure importin-α7 expression levels in distinct cell types to understand the mechanism by which virus tropism is affected in the mammalian respiratory tract.
In summary, this study reveals first insights into the role of a cellular factor, importin-α7, in promoting virus replication particularly in the alveolar epithelium. As a likely consequence of alveolar virus replication, cytokine and chemokine responses are elevated resulting in alveolar inflammation, pneumonia and fatal outcome in mice.
Acknowledgments

We are grateful to Adolfo García-Sastre (Icahn School of Medicine at Mount Sinai, New York, USA) for kindly providing the PR8:NS1-GFP (H1N1) virus. We are grateful to Enno Hartmann (Institute of Biology, Centre for Structural and Cell Biology, University of Lübeck, Germany) and Michael Bader (Max-Delbrück Centre, Berlin, Germany) for kindly providing the importin-α7−/− mice. We thank Carola Schneider, Gökhan Arman-Kalcek and Gundula Pilnitz-Stolze for excellent technical assistance. The help from Markus Heine with the preparation of the lungs for correlative imaging is gratefully acknowledged. We would also like to thank the Nikon Application Center and the Flow Cytometry Core Facility.

GG is funded by the Emmy-Noether Programme of the German Research Foundation. PRI is funded by the Alexander von Humboldt Foundation. This work was further funded by the Foundation for Research and Science Hamburg (to PA) and grants from the European Union (FLUPHARM) (to GG). The Heinrich-Pette-Institute, Leibniz Institute for Experimental Virology is supported by the Free and Hanseatic City of Hamburg and the Federal Ministry of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.


<table>
<thead>
<tr>
<th>Virus dose (p.f.u.)</th>
<th>Survival (%)</th>
<th>Survival significance between WT and α7⁻/⁻</th>
<th>MLD_{50} (p.f.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>α7⁻/⁻</td>
<td></td>
</tr>
<tr>
<td>10⁷</td>
<td>10</td>
<td>50</td>
<td>p = 0.1018</td>
</tr>
<tr>
<td>10⁶</td>
<td>90</td>
<td>100</td>
<td>p = 0.3173</td>
</tr>
<tr>
<td>10⁵</td>
<td>100</td>
<td>100</td>
<td>p = 1</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>α7⁻/⁻</td>
<td></td>
</tr>
</tbody>
</table>

Table 1

MLD_{50} of PR8:NS1-GFP virus in WT and importin-α7⁻/⁻ mice

Mouse-lethal-dose-50 (MLD_{50}) was calculated as described before (Reed L, Muench H. 1938. American Journal of Epidemiology 23:493-497). The significance of differences in survival between WT and α7⁻/⁻ mice was calculated by Mantel-Cox Test.
The distribution of cell components in bronchoalveolar (BAL) and peritoneal lavages isolated from mice were assessed according to the gating strategy described in Figure 10 by flow cytometry before and after culturing cells in Macrophage SFM media (Gibco) for one day. MΦ: macrophage.

### Table 2

<table>
<thead>
<tr>
<th>Cell type</th>
<th>BAL before</th>
<th>BAL after</th>
<th>Peritoneal lavage before</th>
<th>Peritoneal lavage after</th>
</tr>
</thead>
<tbody>
<tr>
<td>B cells</td>
<td>4.7%</td>
<td>0.08%</td>
<td>17.6%</td>
<td>0.07%</td>
</tr>
<tr>
<td>T cells</td>
<td>0.9%</td>
<td>0.1%</td>
<td>9.7%</td>
<td>0.15%</td>
</tr>
<tr>
<td>NK cells</td>
<td>0.6%</td>
<td>0.14%</td>
<td>2.9</td>
<td>0.04%</td>
</tr>
<tr>
<td>AM</td>
<td>82.6%</td>
<td>80%</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>DCs</td>
<td>7.58%</td>
<td>11%</td>
<td>0.95%</td>
<td>--</td>
</tr>
<tr>
<td>MΦ CD11c^-</td>
<td>0.08%</td>
<td>--</td>
<td>50.8%</td>
<td>85%</td>
</tr>
<tr>
<td>MΦ CD11c^-^-</td>
<td>0.38%</td>
<td>--</td>
<td>2.89%</td>
<td>--</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>0.57%</td>
<td>0.0%</td>
<td>0.12%</td>
<td>0.00%</td>
</tr>
</tbody>
</table>
Figure 1

Pathogenicity of PR8:NS1-GFP (H1N1) influenza virus in WT and importin-α7−/− mice

WT (black square; n = 10) or α7−/− (red open square; n = 10) mice were intranasally inoculated with 10^6 p.f.u. of PR8:NS1-GFP virus. Weight loss (A) and survival (B) were monitored for 14 days. Mice receiving PBS were used as controls (grey circle; n = 6). Data shown represent means ± SEM. (C) Lungs were removed from WT or α7−/− mice at 1h, 3h and 6h p.i. (n = 2) and on day 1, 3 and 6 p.i. (n = 5) and virus titers were determined by plaque assay.

Figure 2

In vivo kinetics of influenza virus replication in lungs of WT and importin-α7−/− mice

WT or α7−/− mice were intranasally inoculated with 10^6 p.f.u. of PR8:NS1-GFP virus. Control mice received PBS. On day 1, 3 and 6 p.i., lungs were harvested, formalin-fixed, paraffin-embedded and visualized in a wide-field fluorescent microscope. (A) Direct visualization of GFP expression in histological tissue sections. (B) Viral nucleoprotein (NP) detection in tissue sections by immunohistochemical staining. Virus antigen-positive cells are red-brown. Representative infected cells are indicated with arrows.

Figure 3

Kinetics of influenza virus infection in live cells from infected WT and importin-α7−/− mice

WT or α7−/− mice were intranasally infected with 10^6 p.f.u. of PR8:NS1-GFP virus. Percentage of GFP+ stromal cells (CD45−) (A and B) or immune cells (CD45+) (C and D) from homogenized
lungs (A and C) or BAL (B and D) were assessed by flow cytometry at day 1, 3 and 6 p.i. ($n = 2$-6). Gating strategy is described in Fig. 10.

**Figure 4**

**Total lung imaging of WT and importin-$\alpha7^{-/}$ mice upon influenza virus infection**

Macroscopical damage of lung tissues was analyzed ex vivo by magnetic resonance imaging (MRI). Control mice treated with PBS were compared to WT and $\alpha7^{-/}$ mice infected with $10^6$ p.f.u. of PR8:NS1-GFP virus. Lungs were processed at day 3 p.i.. (A) T2-weighted MRI presents virus induced lung inflammation and damages with formation of a hypointense reticular pattern within the lung parenchyma, most severe in WT followed by $\alpha7^{-/}$ mice. In contrast, uninfected control mice showed homogenous lung tissues. (B) According to this qualitative rating, the quantitative distribution analysis of the signal-to-noise-ratio (SNR) of each voxel of the lungs demonstrated highest values in control mice (white diamonds) and reduced SNR in $\alpha7^{-/}$ (light grey triangles) and WT mice (dark grey squares). See also Video_S1, Video_S2 and Video_S3.

**Figure 5**

**Correlative imaging of influenza virus infected WT and importin-$\alpha7^{-/}$ lungs**

On day 3 p.i., lungs of PR8:NS1-GFP virus infected WT (A, B, E and F) or $\alpha7^{-/}$ mice (C, D, G and H) were fixed with paraformaldehyde and removed for vibratome sectioning. Without any further treatment, sections of infected lungs were visualized using a wide-field fluorescence microscope (Nikon AZ100) (E and G). Sections of infected lungs were stained with the CellMask Orange Plasma membrane Stain (in red) (Life Technologies, Carlsbad, CA, USA) and visualized using a Zeiss LSM510 meta confocal microscope (A and C) and a Philips XL30 ESEM after staining with 1% OsO$_4$ (B and D). Biopsies were taken in preselected areas (white circles in E...
and G), processed and embedded in epon resin for transmission electron microscopy (F and H) (FEI Tecnai G20 equipped with Eagle 4k CCD camera and operated at 80kV). Virus particles were detected upon consecutive magnification of indicated areas.

**Figure 6**

Chemokine and cytokine responses in lungs of influenza virus infected WT and importin-α7−/− mice

Lungs of WT (black bars) or α7−/− (red bars) mice infected with 10^6 p.f.u. of PR8:NS1-GFP virus were harvested on days 1, 3 and 6 p.i. (n = 3-5). Homogenates of these lungs were assessed for chemokine and cytokine responses with mouse cytokine antibody array membranes (RayBiotech, Inc.). Here, respective cytokines and chemokines are shown which revealed significant differences between WT and α7−/− mice upon influenza virus infection: IL-2 (A), IL-13 (B), TNF-α (C), MCP-1 (D), RANTES (E), IL-6 (F), IL-10 (G), IL-9 (H) and IL-12 (I). Relative expression levels were calculated in relation to the standardized array positive control that was set 100%. Baseline cytokine levels from PBS inoculated control mice are shown as a dashed line in each cytokine graph (black line for WT; red line for α7−/−).

**Figure 7**

Immune cell sub-populations in WT and importin-α7−/− mice upon influenza virus infection

WT (black bars) or α7−/− (red bars) mice were infected with 10^6 p.f.u. of PR8:NS1-GFP (n = 6). Immune cell populations in lung homogenates (A-E) or BAL (F-E) were analyzed at day 3 p.i. using a BD LSR II flow cytometer. Cell frequencies were determined within the total leukocytes (CD45+) (graphs with black/red bars) and within GFP+ live cells (green bars). Frequencies of granulocytes (CD11b+, CD11c−, F4/80−, Gr-1+) (A and F), macrophages (CD11b+, CD11c−,
F4/80+, Gr-1+) (B and G), alveolar macrophages (CD11b⁺, CD11c<sup>high</sup>) (C and H) and dendritic cells (CD11b⁺, CD11c<sup>high</sup>) (D and I) were determined according to the gating strategy shown in Fig. 10. Cell numbers from PBS treated mice are shown in grey columns. Representative dot plots of GFP<sup>+</sup> cells are shown to the right of the corresponding analysis. (E and J) Representative plots of the flow-cytometric analysis. * p < 0.05, ** p < 0.01.

**Figure 8**

**Additional immune cell sub-populations in WT and importin-α7<sup>−/−</sup> mice upon influenza virus infection**

Mice were infected with 10⁶ p.f.u. of PR8:NS1-GFP (n = 6). Immune cell populations were analysed at day 3 p.i. using a BD LSR II flow cytometer. Cell frequencies in lungs (A, C, E, G and I) and BAL (B, D, F, H and J) were determined within the total leukocytes (CD45<sup>+</sup>) (graphs with black/red bars) and within GFP<sup>+</sup> live cells (green bars). Frequencies of macrophages (CD11b⁺, CD11c⁻, F4/80⁺, Gr-1⁻) (A and B), macrophages (CD11b⁺, CD11c<sup>mid</sup>) (C and D), DCs (CD11b⁻, CD11c<sup>mid</sup>) (E and F), NK cells (NKp46<sup>+</sup>, CD3e⁻) (G and H) and T-cells (NKp46⁻, CD3e⁺) (I and J) were determined and gated as shown in Fig. 10. Cell numbers from PBS-treated mice are shown in grey columns. Representative dot plots of GFP<sup>+</sup> cells are shown to the right of the corresponding analysis. * p < 0.05, ** p < 0.01, *** p < 0.001.

**Figure 9**

**Role of primary macrophages in virus influenza virus replication and clearance in vitro**

Primary macrophages were isolated from WT and α7<sup>−/−</sup> mice by bronchioalveolar lavage (AM) or by peritoneal lavage (PM). (A) In vitro macrophage infection: cells were infected 24 h after seeding at MOI 1. (B) Virus clearance: MDCK cells were infected at MOI 0.01. At 4 h.p.i.,
isolated primary macrophages were added. In both experiments, supernatants were harvested at indicated time points for subsequent virus titer determination by plaque assay. The virus titer at 0h p.i. (A) or 4h p.i. (B) was set to 100%. The representative experiments shown are the average of three independent experiments. MΦ: macrophage.

Figure 10

Gating strategy to define immune cell populations in mice infected with influenza virus

Cell suspensions were analyzed by flow cytometry with an antibody panel that allowed distinguishing these sub-populations:

- Granulocytes CD11b⁺, CD11c⁻, F4/80⁻, Gr-1⁺
- Macrophages CD11b⁺, CD11c⁻, F4/80⁺, Gr-1⁺
- Macrophages CD11b⁺, CD11c⁻, F4/80⁺, Gr-1⁻
- Macrophages CD11b⁺, CD11cmid
- Alveolar macrophages CD11b⁻, CD11chigh
- Dendritic cells CD11b⁺, CD11chigh
- Dendritic cells CD11b⁺, CD11cmid
- NK cells NKp46⁺, CD3e⁻
- T-cells NKp46⁻, CD3e⁺

Here, we illustrate the gating strategy in the lung homogenates and the BALs with samples from an uninfected mouse (PBS) and an infected mouse (3 d p.i.).
T cells (NKP46, CD3ε⁺)

NK cells (NKP46⁺, CD3ε⁺)

Dendritic cells (CD11b⁺, CD11c⁻)

Macrophages (CD11b⁺, CD11c⁻)

Macrophages (CD11b⁺, CD11c⁻, F4/80⁺, Gr-1⁻)

Lung

BAL
1 – NK cells (NKp46+; CD3ε+);
2 – T cells (NKp46+, CD3ε+);
3 – Alveolar МΦ (CD11b+, CD11chigh);
4 – DCs (CD11b+, CD11chigh);
5 – DCs (CD11b+, CD11cmid);
6 – МΦ (CD11b+, CD11cmid);
7 – МΦ (CD11b+, CD11c, F4/80+, Gr-1+);
8 – МΦ (CD11b+, CD11c, F4/80+, Gr-1+)
9 – Granulocytes (CD11b+, CD11c, F4/80+, Gr-1+)