Targeting host calpain proteases decreases influenza A virus infection

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Blanc F, Furio L, Moisy D, Yen H, Chignard M, Letavernier E, Naffakh N, Mok CK, Si-Tahar M. Targeting host calpain proteases decreases influenza A virus infection. Am J Physiol Lung Cell Mol Physiol 310: L689–L699, 2016. First published January 8, 2016; doi:10.1152/ajplung.00314.2015.—Influenza A viruses (IAV) trigger contagious acute respiratory diseases. A better understanding of the molecular mechanisms of IAV pathogenesis and host immune response is required for the development of more efficient treatments of severe influenza. Calpains are intracellular proteases that participate in diverse cellular responses, including inflammation. Here, we used in vitro and in vivo approaches to investigate the role of calpain signaling in IAV pathogenesis. Calpain expression and activity were found altered in IAV-infected bronchial epithelial cells. With the use of small-interfering RNA (siRNA) gene silencing, specific synthetic inhibitors of calpains, and mice overexpressing calpastatin, we found that calpain inhibition dampens IAV replication and IAV-triggered secretion of proinflammatory mediators and leukocyte infiltration. Remarkably, calpain inhibition has a protective impact in IAV infection, since it significantly reduced mortality of mice challenged not only by seasonal H3N2- but also by hypervirulent H5N1 IAV strains. Hence, our study suggests that calpains are promising therapeutic targets for treating IAV acute pneumonia.

Influenza is probably one of the most common diseases that we know. It may also be one of the most deadly: the “Spanish flu” (1918–1920), with at least 50 million deaths, has marked the history of major pandemics. The renewal of such a scenario cannot be excluded since the World Health Organization considers that the avian H5N1 influenza virus among other influenza A viruses (IAV) subtypes (H1N1, H3N2, H7N9) are now real threats (20, 32). Although vaccines and antiviral molecules have been developed to control influenza, new treatments are urgently required to circumvent the limitations of currently available drugs. Indeed, high frequencies of resistance to M2 inhibitors and increasing resistance to neuraminidase inhibitors (41) among circulating IAV strains have been reported and have limited efficacy of treatment in severe cases of influenza (12, 16, 18).

We and others (14, 19, 21, 38) have previously demonstrated that IAV triggers an acute inflammatory response, with fatal consequences. In mouse, we observed that IAV induces a severe inflammation with leukocyte recruitment and inflammatory cytokines in bronchoalveolar lavage fluids. We also described early histopathological lung lesions characterized by a destruction of the ciliated epithelium and an inflammatory exudate. In addition, our team has established a close relationship between the respiratory distress and the level of inflammation (14, 21, 38). Interestingly, in humans, abnormal and uncontrolled increase of proinflammatory cytokines during infection by highly pathogenic IAVs are associated with a very high mortality (31, 43).

Calpains (Clan CA, family C02; EC 3.4.22.17) are calcium-activated neutral cysteine proteases. The two major isoforms, calpain 1 (μ-calpain) and calpain 2 (m-calpain), are ubiquitously expressed. Calpains 1 and 2 are 80-kDa catalytic activity subunits forming heterodimers with a 30-kDa regulatory subunit, calpain 4 (calpain small subunit 1). Each calpain isoform is present in the cytosol as an inactive proenzyme (3, 4, 6). Calpain activity is also tightly controlled by calpastatin, a specific endogenous inhibitor (33). Calpastatin overexpression in mice affects the different forms of calpains, the activity of which is blunted rather than suppressed. In addition, this strategy appears to limit calpain activation without interfering with basal calpain activity (24).

Calpains play an important role in inflammatory processes. Accordingly, inhibition of calpain proteases is considered as a therapeutic strategy in several pathological inflammatory disorders, including ischemia-reperfusion injury or atherosclerosis (3, 4, 27). With the use of calpastatin transgenic mice, the role of calpain was evidenced in inflammatory models of glomerulonephritis or endotoxemia (26, 33). Remarkably, very few studies have focused on the role of calpains in the microbial-triggered inflammatory immune response per se. Thus, it was only reported that calpain inhibition protects from endotoxin-induced myocardial and diaphragmatic dysfunctions (26, 40) and virus-induced apoptotic myocardial injury (10) and that calpains are also involved in human immunodeficiency virus (42) and echovirus 1 (45) replication. More recently, calpains were shown to regulate herpes simplex virus intracellular migration (49). Considering that calpains are Ca2+-dependent proteases implicated in the inflammatory process and that IAV triggers calcium mobilization (44) and a potent inflammatory immune response, the present study aimed to study the role of calpains in influenza pathogenesis.

MATERIALS AND METHODS

Viruses and Cells

Influenza viruses A/Scotland/20/74 (H3N2), A/WSN/33 (H1N1), A/Viet Nam/1203/2004 (H5N1), A/Hong Kong/486/1997 (H5N1),
human parainfluenza virus III C (243 strain, ATCC VR-93), and human respiratory syncytial virus (Long strain, ATCC VR-26) were prepared as previously described (11, 14, 25). Human bronchial epithelial BEAS-2B cells (ATCC CRL-9609) and canine kidney epithelial MDCK cells (ATCC CCL-34) were cultured, respectively, in F-12K and minimal essential medium (MEM) supplemented with 2 mM l-glutamine, antibiotic-antimycotic, 10% FBS, and 10 mM HEPES (all from Gibco).

**Cell Infection and Stimulation**

BEAS-2B cells were seeded in complete medium in a four-well cell culture plate at 8 x 10^4 cells·300 µl·well⁻¹. The next day, cells reaching 80–90% of confluence were washed with medium without FBS. Cells were treated with calpain inhibitors [PD-150606 (Calbiochem) or calpain inhibitors I or II (Sigma-Aldrich)] or vehicle at the same dilution (DMSO or ethanol) for 4 h at 37°C. Cells were then infected by IAV at 37°C for 3–24 h. In specific experiments, cells were stimulated at 37°C for 15 h by transfected poly(IC) at a final concentration of 100 ng/ml using FuGENE 6 (Roche). Cell supernatants were collected for cytokine or viral titration assays, and cells were lysed in RIPA and RLT buffers for protein or RNA extractions, respectively.

**Transient Transfection**

For small-interfering RNA (siRNA) transient transfection, BEAS-2B cells were seeded in 24-well cell culture plates at 4 x 10^4 cells·300 µl·well⁻¹. The next day, cells were incubated for 8 h at 37°C with 25 nM of calpain 1, calpain 2, or control siRNA (ON-TARGETplus smart pool siRNA from Dharmacon) mixed with DharmaFECT Duo transfection reagent (Dharmacon). Medium was further replaced by complete medium, and cells were grown for 40 h at 37°C before infection. For plasmid transient transfection, BEAS-2B cells were seeded in 24-well cell culture plates at 8 x 10^4 cells·300 µl·well⁻¹. The next day, cells were incubated with 50 ng of a nuclear factor-kB (NF-kB)- or interferon (IFN)-β-firefly luciferase-reporter plasmid and 5 ng of a CMV-control Renilla luciferase-reporter plasmid mixed with DharmaFECT Duo transfection reagent. After 24 h of incubation at 37°C, cells were further stimulated. Renilla and firefly luciferase activities were measured using the Dual Glo luciferase assay system (Promega).

**Cytokine and Chemokine Assays**

Human CCL5/RANTES, IL-6, CXCL8/IL-8, and IFN-λ2 and mouse IL-6, CXCL1/KC, and CCL5/RANTES concentrations were determined using DuoSet ELISA kits (R&D Systems). IFN-β concentration was determined using a VeriKine ELISA kit (PBL InterferonSource).

**Calpain Activity and Expression**

SDS-PAGE was performed on 7.5 or 12% acrylamide gels loaded with 20 µg of proteins, and proteins were then transferred on PVDF membrane. Membranes were saturated in blocking buffer (TBS containing 1% Tween 20 and 5% nonfat dry milk; namely TBST) for 1 h at room temperature and incubated overnight at 4°C in blocking buffer with anti-human calpain 1, 2, or 4 (clones 15C10, C-19, and P-1; Santa Cruz Biotechnology) at 1:500, 100 or 100 ng/ml and anti-human β-actin as a control (150 ng/ml; Sigma-Aldrich). Calpain activity was revealed by determining the presence of a specific spectrin α II breakdown product by Western blotting using an anti-human spectrin α II antibody (clone C-11, 100 ng/ml; Santa Cruz Biotechnology). After three washes with TBST, corresponding horseradish peroxidase-conjugated secondary antibodies were incubated for 1 h at room temperature in blocking buffer; goat anti-mouse IgG (400 ng/ml; Thermo Fisher) or donkey anti-goat IgG (20 ng/ml; Santa Cruz Biotechnology). After three washes in TBST and three washes in TBS, membranes were revealed with ECL+ substrate (Pierce), and photographic films were developed. Image analysis was performed using ImageJ software.

**IAV Titration by Plaque-Forming Units Assay**

Titration was performed using a plaque assay on MDCK cells, using a method adapted from Matrosovich et al. (28). Briefly, six-well cell culture plates were seeded at 1 x 10⁶ MDCK cells/well. One day later, cells were washed two times with PBS and infected with 0.4 ml of serial dilutions of the sample at 37°C for 1 h. Each well was then covered with 3 ml of a mixture of MEM, 1.2% Avicel (Avicel microcrystalline cellulose NF, FMC Biopolymer), and 1 µg/ml of Trypsine TPCK (Worthington). Plates were then incubated at 37°C with 5% CO₂ for 72 h. Cell layers were finally stained with a solution containing 10% crystal violet oxalate, 10% formaldehyde, and 20% ethanol, and plaques were counted. Titers were finally expressed as plaque-forming units (pfu) per milliliter.

**Quantitative Real-Time RT-PCR**

Total RNA was extracted from BEAS-2B cells or from mice lungs crushed with lysing matrix D (Millipore) and a FastPrep instrument (Bio 101; Qiagen), using the RNeasy Mini Kit (Qiagen). RNA quantity and quality were monitored using a NanoDrop spectrophotometer. RT-PCR was performed on 0.5 µg of RNA using Moloney murine leukemia virus Reverse Transcriptase (Promega) on a PTC-200 Peltier Thermal Cycler apparatus. Quantitative real-time RT-PCR (qRT-PCR) was performed using an Applied Biosystems 7900HT Fast Real-Time PCR System with SYBR green master mix (Thermo Fisher) and the primers described below.

**qRT-PCR Primer Sequences**

**Assessment of poly(I:C) transfection.** BEAS-2B cells were seeded in complete medium in a 24-well cell culture plate at 8 x 10⁴ cells·300 µl·well⁻¹. The next day, cells were washed with medium without FBS and treated by PD-150606 at 100 µM or DMSO (0.5%) for 4 h and further transfected with poly(I:C) labeled with FITC (Invivogen). One hour later, cells were washed, fixed with PBS containing 2% parafomaldehyde, and permeabilized in PBS containing 0.1% Triton, and FITC fluorescence intensity in cells was measured using a CYAN ADP cytomter (Beckman Coulter). The efficiency of poly(I:C) transfection is expressed as RFI corresponding to the ratio of the means of the fluorescence of poly(I:C)-FITC transfected cells vs. nonstimulated cells.

**IAV minigenome assay.** BEAS-2B cells were seeded in 96-well plates at 2 x 10⁴ cells·100 µl·well⁻¹. The next day, cells were pretreated or not by calpain inhibitor PD-150606 (50 µM) for 4 h and further transfected with pdcDNA3.1 plasmids encoding the NP, PB1, PB2, and PA proteins from the A/WSN/33 (H1N1) strain (50, 50, 50, and 100 ng) along with an IAV genome-like Renilla luciferase reporter RNA plasmid and a CMV-control firefly luciferase reporter plasmid (5 ng each) using the FuGENE 6 transfection reagent (Roche). After 24 h of incubation, Renilla and firefly luciferase activities were measured using the Dual Glo luciferase assay system (Promega).

**Calpain inhibition in vivo and mouse infection.** Calpastatin transgenic (CALP TG) mice were previously created using the cDNA clone of rabbit calpastatin inserted on the PCI expression vector, which includes a viral promoter (CMV immediate-early enhancer/promoter region) (33). All experiments with highly pathogenic H5N1 influenza viruses were performed in the biosafety level 3 containment facility at the University of Hong Kong, in compliance with all applicable guidelines. The other in vivo experiments were performed in the Institut Pasteur animal facilities, accredited by the French Ministry of Agriculture to perform experiments on living mice in compliance with the French and European regulations on care and use of laboratory animals.
infection (MOI).

spectrin cleavage only in cells infected by IAV [multiplicity of 150606. inhibited by treatment with the specific calpain inhibitor PD-150606 (3 mg/kg) or vehicle (PBS containing 0.3% DMSO). The first dose was given 4 h before infection with IAV. For infection, mice were anesthetized by intramuscular injection of ketamine (50 mg/kg) and xylazine (10 mg/kg) and challenged intranasally with a lethal amount of influenza A/Scotland/20/74 (H3N2) [380 pfu; i.e., equivalent to 2 lethal dose (LD)50] or influenza A/Hong Kong/486/1997 (H5N1) (1 LD50) virus. Mice were observed daily for signs of morbidity. Alternatively, mice were killed at different time points by intraperitoneal injection of 300 mg/kg pentobarbital sodium. Airways were washed twice with 1 ml of saline, and the bronchoalveolar lavage (BAL) was collected to further determine cell counts using a Coulter counter (Coulter-Electronics). Flow cytometry was further used to determine the percentage of leukocyte subtypes, as previously described (38). Briefly, cells were stained with a Live/Dead fixable near-IR stain kit (Invitrogen) for live/dead discrimination. Cells were then incubated with anti-CD16/32 in PBS containing 1% BSA to block Fc gamma receptors and stained with PerCP-Cy5.5-anti-CD11b (clone M1/70), APC-anti-CD11c (clone HL3), PE-Cy7-anti-Gr-1 (Ly-6G and Ly-6C, clone RB6-8C5) or FITC-anti-CD4 (clone RM4-5), PerCP-Cy5.5-anti-CD8 (clone 53-6.7), PE-Cy7-anti-NK1.1 (clone PK136), and APC-anti-CD3e (clone 145-2C11) (all from BD Biosciences). Cells were fixed overnight with 2% paraformaldehyde and analyzed using a BD FACSCanto flow cytometer and BD FACSDiva software. Distinct aliquots of BAL fluids were stored at −80°C for a subsequent cytokine/chemokine measurement.

Statistical Analyses

Statistical analyses were performed with GraphPad Prism software. Statistical significance between two individual groups was analyzed using the unpaired Student’s t-test and between more than three groups using one-way analysis of variance and Bonferroni’s posttest to compare all groups vs. a control group. Statistical significance is indicated in Figs. 1–9. Survival of mice was compared using Kaplan-Meier analysis and the log-rank test.

RESULTS

IAV Modulates Expression and Activity of Calpains in Human Bronchial Epithelial Cells

By Western blot analysis, we observed a constitutive expression of calpains 1, 2, and 4 in noninfected human bronchial epithelial cells (Fig. 1A). Upon challenge by IAV [A/Scotland/20/74 (H3N2) strain] for 24 h, we observed a dose-dependent decrease in calpain 1, 2, and 4 protein expression that was not associated with a concomitant transcriptional regulation, since there was no significant change of calpain mRNA levels (Fig. 1, A–B). Calpain proteolytic activity was detected by monitoring the cleavage of one of their known cellular substrates, i.e., spectrin α II. As shown in Fig. 1C, we observed a product of spectrin cleavage only in cells infected by IAV [multiplicity of infection (MOI) = 1] for 24 h. The cleavage product is inhibited by treatment with the specific calpain inhibitor PD-150606.  

Role of Calpain-Dependent Signaling in IAV-Triggered Cytokine Secretion

To evaluate the role of calpains in IAV-induced immune responses, we infected bronchial epithelial cells with IAV (MOI = 1) after treatment with increasing concentrations of distinct calpain inhibitors, including PD-150606, calpain in-
Fig. 2. Calpain inhibition reduces IAV-induced cytokine secretion in human bronchial epithelial cells. A: CCL5/RANTES, IL-8, IL-6, interferon (IFN)-α2, and IFN-β cytokines were measured in supernatants of BEAS-2B cells infected by influenza A/Scotland/20/74 (H3N2) (MOI = 1) for 24 h. Before infection, cells were pretreated with increasing concentrations of calpain inhibitors (PD-150606, calpain inhibitor I, or calpain inhibitor II) or vehicle (“0”). Cytokine secretion is expressed as the percentage of secretion of vehicle-treated BEAS-2B samples. Absolute values of cytokine levels in the absence of calpain inhibitors were as follows: CCL5/RANTES (1,391 ± 100 pg/ml), IL-8 (717 ± 223 pg/ml), IL-6 (601 ± 73 pg/ml), IFN-α2 (350 ± 79 pg/ml), and IFN-β (1,079 ± 92 pg/ml). Results are means ± SE of triplicates and are representative of two independent experiments for calpain inhibitor I and II treatments and four independent experiments for PD-150606 treatment (*P < 0.05, **P < 0.01, and ***P < 0.001).

B: expression of CAPN1 or CAPN2 was efficiently knocked down by specific small-interfering RNA (siRNA). BEAS-2B cells were transfected either with a control siRNA or with specific siRNAs. Left, calpain transcriptional level was quantified 48 h later by quantitative real-time (qRT)-PCR. Results are means ± SE from two independent experiments performed in triplicate (*P < 0.05). Right, quantitative representation of calpain protein signals observed in three distinct Western blots, after normalization over β-actin signal (***P < 0.001).

C: silencing of calpain expression reduces IAV-induced cytokines/chemokine secretion. Results are expressed as the percentage of release over the value observed in control small-interfering RNA (siRNA)-treated BEAS-2B cells and are means ± SE of triplicates. Data are representative of two independent experiments for IFN-α2 and IFN-β and four independent experiments for CCL5/RANTES, IL-8, and IL-6 (*P < 0.05, **P < 0.01, and ***P < 0.001). ns, Not significant.
hibitor I, and calpain inhibitor II. After 24 h, we measured secretion of CCL5/RANTES (1,391 ± 100 pg/ml), IL-8 (717 ± 223 pg/ml), IL-6 (601 ± 73 pg/ml), IFN-α2 (350 ± 79 pg/ml), and IFN-β (1,079 ± 92 pg/ml). Interestingly, these cytokine secretions were decreased by all calpain inhibitors although with distinct efficiency levels (Fig. 2A).

To rule out any nonspecific effect related to the pharmacological inhibitors, we further examined the impact of calpains on IAV-induced cytokine secretion using a gene-silencing approach. Reduction of calpain 1 and 2 expression and its specificity were verified at the level of RNA (decrease by ~88%) and protein (decrease by ~65%; Fig. 2B). Interestingly, inhibition of calpain 1 expression reduced significantly CCL5/RANTES, IL-6, IFN-α2, and IFN-β secretions, whereas IL-8 secretion was barely impaired (Fig. 2C). Conversely, inhibition of calpain 2 reduced CCL5/RANTES and IL-6 secretions only moderately and did not affect IFN-α2 and IFN-β release. Remarkably, IL-8 secretion was impaired by calpain 2 silencing treatment.

Cell culture supernatants were further analyzed by an inflammatory protein array to examine whether additional immune mediators were affected by the inhibition of calpain signaling. A total of 36 mediators were measured, which included 20 cytokines, 10 chemokines, 2 growth factors, 1 protease inhibitor, 1 soluble receptor, 1 soluble adhesion molecule, and 1 complement component. In addition to IL-8, IL-6, and CCL5/RANTES secretion, IP-10, MIF, and Serpin E1 releases were found to be upregulated by IAV under these experimental conditions. Interestingly, those cytokine secretions were also impaired by siRNA-mediated calpain inhibition (data not shown). We also found that calpain inhibition also impaired IAV-triggered cytokine secretion in other cell types. Thus, human monocytic THP-1 cells treated with PD-150606 led to a reduced IAV-induced secretion of CCL5/RANTES and IL-8 (data not shown).

**Calpain Inhibition Impairs IAV Replication**

We next examined whether the impact of calpains on IAV-triggered immune response could also involve an effect on IAV replication. For this purpose, we measured the number of infectious viral particles produced in human bronchial epithelial cells, in the presence or absence of the calpain inhibitor PD-150606. We observed a dose-dependent decrease of IAV replication (MOI = 1 for 24 h) after treatment by increasing concentrations of the inhibitor (Fig. 3A, left). This was further confirmed in a multicell experiment using MDCK cells treated with PD-150606 (Fig. 3A) or calpain inhibitor I or II (data not shown) and infected by IAV at a MOI = 0.001 for 48 h. All calpain inhibitors significantly reduced IAV replication, and PD-150606 was the most efficient, since it induced a 2- and 6-log reduction of viral titers at 50 and 100 μM, respectively (Fig. 3A, right). Next, to dissect the stage of the IAV-replicative cycle altered by calpains, we analyzed the effect of PD-150606 on IAV transcription/replication steps using a minigenome assay derived from the A/WSN/33 (H1N1) strain (34). We first checked that calpain inhibition impaired replication of the A/WSN/33 (H1N1) strain, as observed for the A/Scotland/20/74 (H3N2) strain (Fig. 3B). Next, expression plasmids for the NP, PB1, PB2, and PA proteins of A/WSN/33 were cotransfected in bronchial epithelial cells along with an IAV genome-like Renilla luciferase reporter RNA and a firefly luciferase reporter control plasmid. Data are expressed as means ± SE of quadruplicates and are representative of two independent experiments (***P < 0.001).

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with 50 μM PD-150606, indicating that calpain inhibition had no significant effect on viral transcription/replication (Fig. 3C). We next tested whether calpains could impact upstream steps, i.e., from the virus entry up to the start of the transcription/replication phase. To this end, bronchial epithelial cells were treated or not with increasing concentrations of PD-150606 and further infected with IAV (MOI = 3). M2 viral RNA level was then quantified at 1, 4, or 7 h postinfection. As shown in Fig. 3D, PD-150606 decreased the amount of M2 RNA levels, suggesting that calpain inhibition does interfere with at least the earliest steps of the IAV life cycle.

Calpains also Regulate Pattern Recognition Receptor-Dependent Signaling Triggered by IAV

We and others have previously shown that the nucleic acid sensors TLR3 and RIG-I contribute directly to the immune response of respiratory epithelial cells to IAV, in particular through the recognition of viral RNA (14, 22). Thus, we examined whether the aforementioned impact of calpains on IAV-induced immune response could also result from an inhibition of the signaling downstream of nucleic acid recognition. Hence, bronchial epithelial cells were transfected by the viral replicative mimetic poly(I:C), a synthetic double-stranded RNA, with or without PD-150606. By flow cytometry, using FITC-labeled poly(I:C), we first verified that transfection efficiency was not affected by calpain inhibition (Fig. 4A). Next, we measured the secretion of CCL5/RANTES, IL-8, and IL-6 in cells challenged by poly(I:C). Figure 4B shows that PD-150606 preincubation decreased poly(I:C)-induced cytokine secretion in a dose-dependent way. Similar results were observed when considering the activity of the transcriptional factor NF-κB (Fig. 4C, top) and the promoter of the antiviral mediator IFN-β (Fig. 4C, bottom). Remarkably, we did not observe any effect of PD-150606 treatment on tumor necrosis factor (TNF)-α-stimulated cells (Fig. 4C). Thus, calpains are key components in viral nucleic acid-dependent signaling pathways.

Involvement of Calpain Signaling on Distinct Respiratory Virus-Induced Cytokine Secretion

Because the foregoing results suggested that calpains are essential in the immunostimulatory signaling pathways triggered by IAV, we questioned whether these intracellular proteases could modulate the immune responses induced by distinct respiratory virus infection. Thus, human bronchial epithelial cells were treated or not with PD-150606 and infected by a human parainfluenza 3 (HPIV-3) strain. Figure 5A shows a strong inhibition of IL-6 and IL-8 secretions after treatment with PD-150606. Remarkably, this regulatory role of calpains in the virus-triggered immune response seems to be restricted to some viruses. Indeed, whereas PD-150606 drastically reduced (by 95%) IL-8 release triggered by HPIV-3, it only moderately impaired IL-8 secretion induced by respiratory syncytial virus (RSV) (Fig. 5B).

Calpain Inhibition also Regulates the Epithelial Cell Responses to the Highly Pathogenic IAV H5N1 Strain

The highly pathogenic H5N1 IAV strain is highly threatening, since this virus could possibly acquire human-to-human
transmissibility and has a high fatality rate (30). We tested whether calpain inhibition can also have an impact on such hypervirulent IAV infection. Figure 6A shows that PD-150606 reduced the replication of H5N1 IAV strain monitored for 24, 48, and 72 h in human bronchial epithelial cells. Moreover, calpain inhibition also decreased H5N1 IAV strain-triggered cytokine release. IP-10, IL-6, and CCL5/RANTES were significantly decreased in PD-150606-treated bronchial epithelial cells while MCP-1 and IL-8 were not (Fig. 6B).

Calpain inhibition protects from the infection of pathogenic IAV in vivo. All the foregoing in vitro results suggested that calpains could modulate the cell responses to IAV infection. Consequently, we questioned whether inhibiting those proteases could have an impact on IAV infection in vivo. We first assessed the survival of C57Bl6/J mice treated with PD-150606 (ip injections every 2 days, 3 mg/kg) or vehicle (0.3% DMSO) before their infection by either a seasonal H3N2 IAV strain (Fig. 7A) or an hypervirulent H5N1 IAV strain (Fig. 7B). PD-150606-treated mice had a statistically higher survival rate compared with vehicle-treated mice for both the H3N2 IAV strain (33.3 vs. 5.6%) and the H5N1 IAV strain (96 vs. 65.2%).

To further support these pharmacological data, we also infected transgenic mice overexpressing calpastatin, an endogenous inhibitor of calpains, with IAV. This transgenic murine model was previously validated by distinct groups in studies evaluating the pathophysiological role of calpains (24, 26, 33).

As shown in Fig. 8A, CALP TG mice were more resistant to H3N2 IAV virus infection than wild-type mice ($P < 0.001$). This was associated with a reduced viral load in CALP TG mice at day 4 postinfection (Fig. 8B). Regarding the secretion of inflammatory cytokines and chemokines, CCL5/RANTES (Fig. 8C, left) and IL-6 (Fig. 8C, right) amounts were significantly lower at day 4 postinfection in CALP TG compared with wild-type mice. Interestingly, the number of leukocytes was concomitantly lower in CALP TG airspaces than in wild-type animals also at day 4 postinfection ($P < 0.01$; Fig. 8D). When we phenotyped these immune cells at this specific time point, we observed a significantly reduced number of NK cells and CD8+ T lymphocytes in CALP TG mice ($P < 0.01$ and 0.05, respectively). We also observed a trend for a lower amount of

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**Fig. 5.** Distinct role of calpains in respiratory syncytial virus (RSV)- and human parainfluenza 3 (HPIV-3)-triggered activation of human bronchial epithelial cells. BEAS-2B cells were pretreated with PD-150606 at 20 or 50 μM or vehicle as a control (0) and were further infected by HPIV-3 (A) or RSV (B) for 2 h. Next, culture medium was replaced by virus-free culture medium containing PD-150606. IL-8, IL-6, and CCL5/RANTES were quantified in cell supernatants after 48 h for HPIV-3 infection or 40 h for RSV infection. Results are means ± SE of triplicates and are representative of two independent experiments (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

**Fig. 6.** Regulatory role of calpains on H5N1 IAV infection of human bronchial epithelial cells. BEAS-2B cells were pretreated by PD-150606 (50 μM) or vehicle (1:200 DMSO) and were further infected by the influenza A/Viet Nam/1203/2004 (H5N1) strain. A: for IAV replication studies, BEAS-2B cells were infected at MOI 0.01, and the replication of the virus in the supernatant was determined at 24, 48, or 72 h postinfection by TCID50 assay. Results are means ± SE of three independent experiments (*$P < 0.05$ and **$P < 0.01$).

B: for cytokine induction studies, BEAS-2B cells were infected at MOI = 1, and cytokines were quantified from the cell culture supernatants by ELISA at 24 h postinfection. Results are means ± SE of four experiments (***$P < 0.001$).
DISCUSSION

Although vaccines and antiviral drugs to control influenza have been developed, the disease is still not under control. Notably, vaccination has a limited impact in high-risk groups such as the elderly and immunocompromized individuals, and the constant IAV antigenic drift demands annual updating of the vaccine (15). Another major factor that contributes to the limited efficacy of the anti-IAV arsenal includes the inability of antiviral drugs to interfere with immunopathology (12, 46). Accordingly, development of innovative strategies for reducing the severity of IAV infection remains a top global public health priority.

Importantly, viruses are cell obligate parasites. This suggests that antiviral therapy targeting key cellular components rather than viral factors can be advantageous. Indeed, the virus cannot replace pivotal cellular functions by mutation, and viral resistance should then not occur. In that regard, it is noteworthy that calcium homeostasis and calcium-dependent effectors have key pathophysiological roles (8, 35). Among the numerous calcium-dependent molecules, calpains are demonstrably beneficial in animal models of several diseases. Moreover, the value of calpain inhibitors has already been highlighted for many pathologies, including stroke and neurodegenerative dis-
By contrast, knowledge about the regulation and the role of calpains in viral pathogenesis in general, and in IAV infection in particular, is very limited (5, 42, 45, 47). Our new findings now indicate that targeting calpain-dependent signaling pathways might be useful for treating IAV-induced acute pneumonia. Indeed, we first established that calpain expression and activity are altered in IAV-challenged bronchial epithelial cells. These cells are especially important in the pathogenesis of IAV infection as IAV replicates within bronchial epithelial cells, and they are the source of major inflammatory mediators that contribute to IAV-associated lung injury (7, 14, 37). Calpains are involved in a variety of cellular processes, including cell signaling and trafficking. Interestingly, these two biological events are known to be essential for viral replication efficiency (13). Using siRNA gene silencing, specific synthetic inhibitors of calpains, and mice overexpressing calpastatin, we showed that calpain inhibition dampens down IAV replication and IAV-triggered leukocyte infiltration and expression of proinflammatory mediators, including cytokines and chemokines. Importantly, calpain inhibition has a major protective effect in IAV infection, since it also significantly reduced mortality of IAV-infected animal models. Altogether, this part of the study not only provides insight into the role of calpains in IAV pathogenesis but also supports the concept of novel antiviral approaches targeting calpains rather than viral factors.

The immune response to virus infection is initiated when pathogen recognition receptors (PRRs) of the host cell recognize specific nonself motifs within viral products (2). Among these PRRs, we and others have previously demonstrated that viral nucleic acids sensors, including TLR3/TLR7 and RIG-I, are especially important for triggering a type I interferon-dependent antiviral signaling and a proinflammatory response upon activation of several transcription factors, including NF-κB, AP-1, and IRFs (14, 22, 48). We presently show using in vitro and in vivo approaches that calpains contribute to IAV-induced immune signaling. Those proteases may act downstream of PRRs, since calpain inhibition decreased both NF-κB and IFN-β promoter activity and cytokine secretion in cells stimulated directly by the viral PAMP mimetic poly(I:C). Interestingly, we did not observe such regulatory role of calpains in TNF-α-stimulated bronchial epithelial cells, suggesting a rather restricted and specific impact of calpains on the cell signaling machinery.

Besides, the decreased number of CD8+ T lymphocytes and NK cells seen in mice overexpressing calpastatin suggests that those leukocytes may contribute to a calpain-mediated deleterious inflammatory response. Indeed, while the recruitment of NK and CD8+ T cells is essential for protective responses, it is increasingly evident that those immune cells can also be associated with the development of influenza-related immunopathological sequelae (1, 29).

Altogether, our findings suggest that calpain inhibition reduces influenza pneumonia through a mechanism that at least involves a reduction of both viral replication and a detrimental inflammatory response (as shown in Fig. 9), although the precise molecular targets of calpains in this context will require a more specific and comprehensive investigation.

It is of note, however, that our study reveals a differential function of calpain 1 and calpain 2 in IAV-triggered innate immune signaling, since their specific inhibition by siRNA led to a distinct cytokine signature; in particular, the secretion of the archetypical inflammatory cytokine IL-8 is more impaired by calpain 2 than calpain 1 inhibition. This finding underlines the challenge of developing calpain inhibitors that will not only have improved potency and selectivity with respect to calpains vs. other intracellular proteases but that will also have calpain isoform selectivity.

We extended our in vitro findings by demonstrating that inhibition of calpains enhances resistance of animal models to virulent human H3N2 IAV or highly pathogenic avian H5N1 strains. Among the results of various studies that are under way to identify novel classes of anti-inflammatory and antiviral drugs (9, 17), we believe that our investigation is particularly innovative. Besides their antiviral effect, calpain inhibitors may indeed block the inflammatory burst that contributes to the severity of infections by highly virulent influenza virus subtypes. Hence, our study supports the concept that calpain...
targeting may curtail deleterious processes related to acute IAV pneumonia.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


