A Single Amino Acid Substitution in the Hemagglutinin of H3N2 Subtype Influenza A Viruses Is Associated with Resistance to the Long Pentraxin PTX3 and Enhanced Virulence in Mice

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The long pentraxin, pentraxin 3 (PTX3), can play beneficial or detrimental roles during infection and disease by modulating various aspects of the immune system. There is growing evidence to suggest that PTX3 can mediate antiviral activity in vitro and in vivo. Previous studies demonstrated that PTX3 and the short pentraxin serum amyloid P express sialic acids that are recognized by the hemagglutinin (HA) glycoprotein of certain influenza A viruses (IAV), resulting in virus neutralization and anti-IAV activity. In this study, we demonstrate that specificity of both HA and the viral neuraminidase for particular sialic acid linkages determines the susceptibility of H1N1, H3N2, and H7N9 strains to the antiviral activities of PTX3 and serum amyloid P. Selection of H3N2 virus mutants resistant to PTX3 allowed for identification of amino acid residues in the vicinity of the receptor-binding pocket of HA that are critical determinants of sensitivity to PTX3; this was supported by sequence analysis of a range of H3N2 strains that were sensitive or resistant to PTX3. In a mouse model of infection, the enhanced virulence of PTX3-resistant mutants was associated with increased virus replication and elevated levels of proinflammatory cytokines in the airways, leading to pulmonary inflammation and lung injury. Together, these studies identify determinants in the viral HA that can be associated with sensitivity to the antiviral activities of PTX3 and highlight its importance in the control of IAV infection. The Journal of Immunology, 2014, 192: 000–000.

The first few days of infection. Historically, such proteins have been classified as β- or γ-type inhibitors based on their chemical properties and mechanism of action against IAV (reviewed in Refs. 1, 2). β-type inhibitors are Ca2+-dependent lectins that bind to mannose-rich glycans on the hemagglutinin (HA) and the neuraminidase (NA) glycoproteins of IAV (3) to inhibit hemagglutination, neutralize virus infectivity, aggregate virions, and protect neutrophils from the depressive effects of IAV on respiratory burst responses (reviewed in Refs. 4, 5). It is well established that some members of the collectin superfamily, including human mannose-binding lectin and surfactant protein (SP)-D, act as β-type inhibitors against IAV. In contrast, γ-type inhibitors are soluble sialylated glycoproteins that act as receptor decoys, presenting sialic acids (SIA) that are recognized by the viral HA and resist hydrolysis by NA (6, 7). Of γ-type inhibitors, the antiviral properties of α2-macroglobulin, the major IAV inhibitor in horse serum, have been particularly well characterized (6, 7). More recently, other sialylated glycoproteins, such as the collectin SP-A (8, 9), H-ficolin (10), and the pentraxins, pentraxin 3 (PTX3) (11) and serum amyloid P (SAP) (12), were shown to act as γ-type inhibitors of IAV.

Members of the pentraxin superfamily play diverse roles in physiology, inflammation, and host defense. Pentraxins are a family of evolutionarily conserved proteins characterized by a pentameric structure and by the presence of the pentraxin domain (HxCxS/TWxS, where x represents any amino acid). The short pentraxins C-reactive protein (CRP) and SAP are produced by hepatocytes in response to a range of inflammatory stimuli, in particular to the cytokine IL-6 (reviewed in Ref. 13), and they represent the main acute-phase reactants in humans and mice, respectively. PTX3 protomers are composed of a unique 174-aa N-terminal domain coupled to a C-terminal pentraxin-like domain homologous to SAP and CRP, and protomer subunits assemble into higher-order oligomers stabilized by

Abbreviations used in this article: Auck/09, A/Auckland/1/09; BALF, bronchoalveolar lavage fluid; CRP, C-reactive protein; HA, hemagglutinin; HAU, hemagglutination unit; HI, hemagglutination inhibition; IAV, influenza A virus; MIC, minimum inhibitory concentration; MOL, multiplicity of infection; NA, neuraminidase; NAI, neuraminidase inhibitor; N-Cal/99, A/New Caledonia/2099; NP, nucleoprotein; PCO73, A/Port Chalmers/1/73; PI, propidium iodide; PR8, A/PR/8/34; PTX3, pentraxin 3; PTX3β, pentraxin 3 resistant; PTX3γ, pentraxin 3 sensitive; RG, reverse genetic; SAP, serum amyloid P; Shang/13, A/Shanghai/2/2013; SIA, sialic acid; SP, surfactant protein; WT, wild-type.

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disulfide bonds (14). PTX3 secretion is induced in response to multiple inflammatory stimuli; however, unlike CRP and SAP, it is produced by a variety of innate immune cells (15, 16). PTX3 is also produced by airway epithelial cells of the lung (17) and can be detected in airway fluids from IA V-infected mice (11).

Studies (11, 12) from our laboratory demonstrated that PTX3 acts as a γ-type inhibitor of particular H3N2 subtype IA V in vitro and in vivo. A single site of N-glycosylation is present in the C-terminal domain (Asn→Glu) of PTX3, and this is occupied by a complex-type glycan terminating in α(2,3)-linked Sia [SIAα(2,3)] (18). Accordingly, H3N2 strains that recognized SIAα (2,3) were sensitive to the anti-IA V activities of PTX3 (11). The short pentraxin SAP also mediates anti-IA V activity. Early studies reported SAP to be a β-type inhibitor of IA V (19, 20), whereas we recently demonstrated that sialylated glycans expressed by SAP were critical for its anti-IA V activity (12). However, the γ-type inhibitor activity of SAP was Ca2+-dependent, which may reflect conformational differences in its secondary structure in the presence or absence of Ca2+ (21) that alter the arrangement and/or accessibility of its sialylated glycans to the viral HA. Moreover, SAP expressed SIAα (2,6) and, therefore, inhibited a broader range of human IA V strains than did PTX3.

We previously reported that PTX3 mediated antiviral activity against HKx31 (H3N2), but not PR8 (H1N1), in vitro and that treatment of HKx31-infected, but not PR8-infected, mice with exogenous PTX3 reduced virus growth in the lungs, as well as disease severity (11). However, these strains represent different IA V subtypes and do not provide specific information regarding viral determinants of sensitivity to PTX3. In this study, we selected mutants of H3N2 strains for resistance to PTX3 and identified residues in the viral HA that determine PTX3 sensitivity. PTX3-resistant mutants were more virulent in mice, and this was associated with enhanced virus growth and airway inflammation. Finally, we demonstrate that the activity of either HA or NA can determine the sensitivity of a particular virus strain to inhibition by PTX3.

Materials and Methods

Pentraxin preparations

Human and mouse PTX3 expressed in CHO cells were purified from cell supernatants and biotinylated as described (22). Human SAP purified from human serum (Calbiochem) was biotinylated as described (12).

Viruses

The IA V used in this study were A/PR/8/34 (PR8, H1N1) as well as HKx31 and BJx109, which are high-yielding reassortants of PR8 with A/Aichi/2/68 (H3N2) and A/Beijing/353/89 (H3N2), respectively, and bear the H3N2 surface glycoproteins (23). Other IA V strains used were A/Port Chalmers/1/73 (PC73; H3N2), A/Udom/307/72 (H3N2), A/New Caledonia/20/99 (N.Cal/99, H1N1), and A/Auckland/1/09 (Auck/09; A/H1N1)pdm09, as well as γ-irradiated H5N1 strains A/Vietnam/1194/2004, A/Vietnam/1203/2004, A/Maennchen/1/2006, A/Chicken/Indonesia/Wates/2005, A/Chicken/Indonesia/Magelang1631-5/2007, A/Chicken/Laos/Xthaghan/2006, A/Duck/NgeheAn/NCVD-7/2007, and A/Duck/Vietnam/042B/2004. Viruses were obtained from the World Health Organization Collaborating Centre for Reference and Research on Influenza and from the Commonwealth Scientific and Industrial Research Organization Australian Animal Health Laboratory (Geelong, Australia). A reassortant virus expressing six genes from PR8 in conjunction with the HA and NA of A/Shanghai/212/2013 (Shang/13 H7N9) was kindly provided by The National Institute for Biological Standards and Control (Potters Bar, Hertfordshire, U.K.). IA V were propagated in the allantoic fluid cavity of 10–12 embryonated hens’ eggs by standard procedures (3) and stored at −70°C. Viruses were titrated on MDCK cells, as described (24), and purified from allantoic fluid by rate zonal sedimentation in 15–80% w/v sucrose gradients (3).

PTX3-resistant (PTX3R) mutants of H3N2 subtype IA V were prepared by incubation of virus-containing allantoic fluid with 50 μg recombinant PTX3, in a total volume of 50 μl for 30 min at 37°C, before inoculation into embryonated eggs. The resultant virus was screened for sensitivity to PTX3 by hemagglutination inhibition, and the selection process was repeated two or three times until full resistance to PTX3 had been acquired. The resultant virus was then cloned at high dilution in eggs in the absence of PTX3.

Reverse genetic (RG) viruses used in this study were generated by the eight-plasmid RG technique as described (25). Site-directed mutagenesis was used to introduce nucleic acid mutations to facilitate amino acid substitution Ser→Asn in the HA of HKx31. Viruses generated were 6:2 reassortants consisting of six genes from PR8 in conjunction with NA from HKx31 and either the wild-type (WT) HA (RG-HKx31) or the mutant HA (RG-HKx31-PTX3R).

Hemagglutination and hemagglutination inhibition tests

Hemagglutination tests were performed in round-bottom 96-well microtiter plates at room temperature using 1% (v/v) erythrocytes in TBS (0.05 M Tris-HCl, 0.15 M NaCl [pH 7.2]). In some experiments, turkey erythrocytes were treated with increasing amounts of Vibrio cholerae sialidase (Sigma-Aldrich; 0–60 mU/ml) for 30 min at 37°C, washed, and tested in hemagglutination tests using 4 hemagglutinating units (HAU) of virus (26).

Hemagglutination inhibition (HI) tests were performed by standard procedures in TBS containing 10 mM CaCl2, and results are expressed as the minimum inhibitory concentration (MIC; in μg/ml) of PTX3 or SAP required to inhibit 4 HAU of virus. To inhibit the enzymatic activity of the viral NA, IA V were incubated with a final concentration of 50 nM zanamivir (4-guanidino-2,3-dehydro-N-acetyll neuraminic acid, purchased from GlaxoSmithKline, Melbourne, Australia) for 30 min at room temperature and then added to pentraxin titrations in an HI test.

ELISA

To determine binding of PTX3 and SAP to WT or PTX3R HKx31, wells of microtiter plates were coated overnight with 1 μg/ml purified virus, blocked for 2 h at room temperature with TBS containing 10 mg/ml BSA, and washed with TBST. Wells were then incubated with increasing concentrations of biotin-labeled PTX3 or SAP in TBST containing 5 mg/ml BSA and 5 mM CaCl2. Binding of biotin-labeled pentraxins was detected by addition of streptavidin-conjugated HRP (GE Healthcare).

NA and NA inhibition assays

The ability of PTX3 and SAP to inhibit the activity of the viral NA was measured by ELISA. Biotin-labeled peanut agglutinin (Pierce Biotechnology) was used to detect galactose residues exposed after removal of SIA from fetuin (Sigma-Aldrich). Titrations and inhibition of NA activity by pentraxins were performed as described (11).

Virus neutralization assay

Neutralization of virus infectivity by pentraxins was determined by fluorescent-focus reduction in monolayers of MDCK cells, as described (27). Briefly, dilutions of PTX3 or SAP, prepared in TBS containing 10 mM CaCl2, were incubated at 37°C for 30 min with a standard dilution of virus and then added to MDCK cell monolayer for 1 h at 37°C. Cells were washed, incubated for an additional 6–7 h, and fixed in 80% (v/v) acetone. IA V-infected cells were identified by immunofluorescence microscopy after staining with mAb MP3.1092.1C7, which is specific for the nucleoprotein (NP) of IA V, followed by FITC-conjugated rabbit anti-mouse Ig (Dako). The total number of fluorescent foci in four representative fields was counted and expressed as a percentage of the number of foci in the corresponding area of duplicate control wells infected with virus alone.

Binding of biotin-labeled PTX3 and SAP to IA V-infected cells

MDCK cells detached from plastic flasks using trypsin-versein were mock infected or infected in suspension with IA V at a multiplicity of infection (MOI) of 10 PFU/cell for 1 h at 37°C in serum-free media. Cells were washed, cultured for an additional 5 h in Teflon-coated pots, and then gently resuspended and used in assays to determine binding of biotin-labeled PTX3 or SAP. All experiments were performed on ice with biotinylated PTX3 (2 μg/ml) or SAP (10 μg/ml) in TBS containing 5 mg/ml BSA and supplemented with either 10 mM CaCl2 or 5 mM EDTA. Cells were washed, and bound pentraxins were detected using streptavidin conjugated to allophycocyanin. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) using 10 μg/ml propidium iodide (PI) to exclude dead cells from the analyses. We used a mAb specific for the IA V H3 HA (mAb 514; a kind gift from Prof. Lorena Brown, Department of Microbiology and Immunology, University of Melbourne) to confirm that a similar proportion of IA V-infected cells (80–90%) expressed the HA glycoprotein at the cell surface in each sample analyzed (data not shown).
RNA was extracted from the cell pellets obtained following centrifugation of virus-infected allantoic fluid using an RNAsesy Mini kit (QIAGEN) and converted to cDNA with a QIAGEN Omniscript RT kit using 20 pmol/μl Uni12 primer (5′-AGC AAA AAC AGG-3′). DNA encoding the HA and NA genes was amplified from cDNA, PCR products were separated by agarose gel electrophoresis, and DNA bands of specific size were cut out and extracted (Gel Extraction Kit; MO BIO). Sequencing was performed by Applied Genetics Diagnostics (Department of Pathology, University of Melbourne). H3 numbering (25) was used to align the deduced amino acid sequences.

**Binding and infection of LA-4 and MDCK cells by influenza viruses**

Subconfluent monolayers of mouse LA-4 airway epithelial cells or MDCK cells in eight-well chamber slides (Lab-Tek; Nunc) were incubated with increasing dilutions of IAV for 1 h at 37°C, washed three times, and incubated in serum-free media, as described (28). At 7-8 h postinfection, cells were fixed in 80% (v/v) acetone and stained with anti-NP mAb MP3.1092.ICT, followed by FITC-conjugated rabbit anti-mouse Ig (Dako). Cells were viewed by fluorescence microscopy, using PI (10 μg/ml) staining to determine the total cell number/field in at least four random fields (minimum total cell count = 100/sample) and anti-NP staining to determine the number of IAV-infected cells/field.

The HA/NA balance of different IAV was determined as described (29). Briefly, duplicate tubes of formaldehyde-fixed LA-4 cells (10⁶ cells) were mixed with 128–256 HAU each IA V for 1 h on ice to allow HA-mediated binding of virus to sialylated receptors on the LA-4 cell surface. One sample was held at 4°C (to inhibit the enzymatic activity of the viral NA), whereas the duplicate tube was transferred to 37°C for 30 min (to allow the enzymatic activity of the viral NA). A third tube received no cells and was held on ice throughout. After incubation, cells were pelleted, and virus remaining in the cell supernatant was quantified by hemagglutination assay.

**Infection of mice and processing of murine tissues**

C57BL/6 mice were bred and housed in specific pathogen–free conditions in the Animal Facility at the Department of Microbiology and Immunology, University of Melbourne. Six- to eight-week-old male mice were used in all experiments. Mice were anesthetized and infected with 10³, 10⁴, or 10⁵ PFU of virus via the intranasal route. Control mice were mock infected with PBS alone. Mice were weighed daily and assessed for signs of pneumonia were culled. All research complied with the University of Melbourne. Six- to eight-week-old male mice were used in all experiments. Mice were anesthetized and infected with 10³, 10⁴, or 10⁵ PFU of virus via the intranasal route. Control mice were mock infected with PBS alone. Mice were weighed daily and assessed for signs of clinical disease. Animals that had lost ≥20% of their original body weight and/or displayed evidence of pneumonia were culled. All research complied with the University of Melbourne’s Animal Experimentation Ethics guidelines and policies. At various times postinfection, mice were culled, and samples including blood, bronchoalveolar lavage fluid (BALF), thymus, and lungs were collected as described (28, 30). Pulmonary histopathology

Lungs were perfused, inflated, and fixed in 4% formaldehyde, as described (30), and 4-μm sections were prepared and stained with H&E. Sections were assessed for inflammation using a subjective scale from 0 to 5 (0, no inflammation; 1, very mild; 2, mild; 3, moderate; 4, marked; and 5, severe inflammation). Sections were randomized and blinded, and those corresponding to the least and most severe inflammation were assigned scores of 0 and 5, respectively. Peribronchiolar inflammation and alveolitis were then graded in multiple random fields for each section by three independent readers, as described (28, 30).

**Results**

Role of the viral HA and NA in determining sensitivity of IAV to PTX3

PTX3 and SAP express SIAα(2,3) and SIAα(2,6), respectively, and act as receptor decoys that are recognized by the viral HA, resisting hydrolysis by the viral NA to mediate anti-IA V activity (11, 12). In general terms, mouse-adapted and avian IAV exhibit HA preference for SIAα(2,3), human strains prefer SIAα(2,6), and recent pandemic IAV show dual specificity for SIAα(2,3)/α(2,6). Therefore, we compared a range of IAV strains for sensitivity to PTX3 and SAP in the presence or absence of the NA inhibitor (NAI) zanamivir.

Consistent with previous results (11), an early H3N2 strain [Hkx31, expressing the HA/NA from A/Hongkong/33/1968 with dual HA receptor preference (31)] was inhibited by PTX3 and SAP in the presence or absence of zanamivir (Table I), whereas a later strain [Bx109, expressing the HA/NA from A/Beijing/353/1989 for preference for SIAα(2,6)] was inhibited only by SAP, and NAI did not enhance activity (Table I). All H1N1 IAV, including A(H1N1)pdm09, were resistant to PTX3 (MIC ≥ 10 μg/ml); however, NAI increased the sensitivity of PR8 and A/Co/Vic/1/2002 to PTX3, consistent with HA specificity for SIAα(2,3) (32) and SIAα(2,3)/α(2,6) (33), respectively. Inhibition of the N1 NA also increased the sensitivity of the human H1N1 strains N.Cal/99 and A/Co/Vic/1/2002 to PTX3, expressed by natural PTX3 (from HA/NA-stimulated human fibroblasts) or by recombinant PTX3 expressed by CHO cells (12, 18), and our previous studies confirmed that PTX3 from either source displayed a similar spectrum of antiviral activity against H3N2 IAV (12).

Avian H5N1 show HA receptor preference for SIAα(2,3) (34, 35) and are considered a potential pandemic threat. Therefore, we compared the sensitivity of human and avian H5N1 to inhibition by PTX3 and SAP. All avian and human H5N1 strains tested were largely resistant to PTX3 (MIC ≥ 5 μg/ml) and SAP (MIC > 10 μg/ml), and HI activity was not enhanced in the presence of zanamivir.
Table I. Inhibition of IAV by PTX3 or SAP in the presence or absence of NAI

<table>
<thead>
<tr>
<th>Strain</th>
<th>Subtype</th>
<th>PTX3 (MIC)</th>
<th>SAP (MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HKx31</td>
<td>H3N2</td>
<td>Control</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAI</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>BJx109</td>
<td>H3N2</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>PR8</td>
<td>H1N1</td>
<td>10</td>
<td>0.7 ± 0.5**</td>
</tr>
<tr>
<td>N.Cal/99</td>
<td>H1N1</td>
<td>&gt;10</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Auck/09</td>
<td>H1N1/pdm</td>
<td>&gt;10</td>
<td>2.1 ± 0.7*</td>
</tr>
<tr>
<td>Shang/13</td>
<td>H7N9</td>
<td>1.3 ± 1.1</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>DV/04</td>
<td>H5N1</td>
<td>6.7 ± 2.9</td>
<td>5 ± 0</td>
</tr>
<tr>
<td>CI/05</td>
<td>H5N1</td>
<td>6.7 ± 2.9</td>
<td>5 ± 0</td>
</tr>
<tr>
<td>CI/07</td>
<td>H5N1</td>
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<td>6.7 ± 2.9</td>
</tr>
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<td>H5N1</td>
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<td>4.2 ± 1.5</td>
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<td>H5N1</td>
<td>6.7 ± 2.9</td>
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<tr>
<td>Myan/06</td>
<td>H5N1</td>
<td>6.7 ± 2.9</td>
<td>2.9 ± 4.2</td>
</tr>
</tbody>
</table>

*Data are the mean (± 1 SD) of three independent experiments.

**Addition of zanamivir (to a final concentration of 50 nM) to 4 HAU of virus prior to HI assay.

*p < 0.05, **p < 0.001, versus relevant control samples, Student t test. For MIC > 10 μg/ml, a value of 11 was assigned to assess significance.

A PTX3-resistant mutant of HKx31 remains sensitive to the antiviral activities of SAP

Because both the viral HA and NA can contribute to PTX3 resistance, we investigated molecular changes associated with the acquisition of PTX3 resistance by H3N2 IAV. HKx31 was incubated with purified human PTX3 and inoculated in embryo-
In vitro characterization of WT and PTX3<sup>R</sup> H3N2 IAV

Changes in the HA sequence, particularly those in or around the receptor-binding pocket, can affect the receptor properties of the viral HA and, therefore, modulate interactions between IAV and target cells. We compared the HA receptor properties of WT and PTX3<sup>R</sup> mutants and found that PTX3<sup>R</sup> mutants did not differ in their ability to agglutinate chicken erythrocytes enzymatically altered to express either (2,6)- or (2,3)-linked Neu5Ac, whereas PTX3<sup>R</sup> is MIC 4-fold that against WT.

**FIGURE 1.** Comparison of the antiviral activities of PTX3 and SAP against WT and PTX3<sup>R</sup> HKx31. (A) Binding of human PTX3 or SAP to purified WT (black bars) or PTX3<sup>R</sup> (white bars) HKx31. Biotin-labeled PTX3 or SAP (10 or 1 µg/ml), prepared in TBST containing 5 mg/ml of BSA (BSA-TBST), was applied to wells coated with 5 µg/ml of purified virus, and binding was determined by ELISA. Data are mean ± SD (1 SD) from triplicate wells and are representative of two independent experiments. (B) Inhibition of virus-induced hemagglutination by PTX3 and SAP. Dilutions of PTX3 (human and mouse) or SAP (human) in TBS containing 10 mM CaCl<sub>2</sub> were tested for their ability to inhibit virus-induced hemagglutination. Bars show the mean MIC of each pentraxin (± 1 SD) required to fully inhibit 4 HAU of WT (black bars) or PTX3<sup>R</sup> (white bars) HKx31 from three independent experiments. *MIC against PTX3<sup>R</sup> is >4-fold that against WT. (C) Inhibition of IAV NA activity by PTX3 or SAP. WT (black bars) or PTX3<sup>R</sup> (white bars) HKx31 was incubated with PTX3 or SAP (10 or 1 µg/ml) in TBS containing 5 mg/ml BSA (BSA-TBS), and NA activity was determined as described in Materials and Methods. Data are mean NA activity (± 1 SD) from triplicate wells and are representative of two independent experiments. (D) Neutralization of WT (black bars) or PTX3<sup>R</sup> (white bars) HKx31 by human PTX3 or SAP (10 or 1 µg/ml), as determined by fluorescent-focus reduction assay. Data are mean ± SD from three independent experiments. (E) Binding of PTX3 or SAP to MDCK cells infected with WT or PTX3<sup>R</sup> HKx31. Aliquots of 10<sup>6</sup> MDCK cells infected with WT (shaded graph, gray line) or PTX3<sup>R</sup> (open graph, black line) HKx31 were incubated with biotin-labeled PTX3 (bio-PTX3; 2 µg/ml) (i) or biotin-labeled SAP (bio-SAP; 10 µg/ml) (ii) in BSA-TBS supplemented with 10 mM CaCl<sub>2</sub> (upper panels) or 5 mM EDTA (lower panels). Pentraxin binding was determined by flow cytometry. Data are representative of two independent experiments. *p < 0.05, ***p < 0.001, Student t test.

**FIGURE 2.** Alignment of amino acid sequences of the HA<sub>1</sub> from WT and PTX3<sup>R</sup> H3N2 viruses and human H3N2 strains. Shown is a segment of the HA<sub>1</sub> (residues 129–149), with residue 145 highlighted in bold type. Amino acid sequences of WT/PTX3<sup>R</sup> HKx31 and PC/73 were determined as described in Materials and Methods. The sequences of the entire coding regions of the mature HA gene were aligned with sequences from public databases for a range of human H3N2 subtype viruses that had been tested for sensitivity to human PTX3. PTX3<sup>R</sup> is defined as MIC < 5 µg/ml, whereas PTX3<sup>R</sup> is MIC ≥ 5 µg/ml. GenBank accession numbers can be found at http://www.ncbi.nlm.nih.gov/genbank (HKx31, V01085; HKX31 PTX3R, KF874500; PC/73, CY009348; and PC/73 PTX3R, KF874501). Other H3N2 strains were tested for sensitivity to human PTX3. PTX<sup>S</sup> is defined as MIC > 5 µg/ml, whereas PTX<sup>R</sup> is MIC ≥ 5 µg/ml. GenBank accession numbers can be found at http://www.ncbi.nlm.nih.gov/genbank (HKx31, V01085; HKX31 PTX3R, KF874500; PC/73, CY009348; and PC/73 PTX3R, KF874501).
assessed by incubating IAV with fixed mouse airway epithelial (LA-4) cells at 4°C (when HA binds but the viral NA is inhibited) or at 37°C (when HA binds to SIA and the viral NA is active) before determining levels of virus in cell-free supernatants (Supplemental Fig. 1A). Compared with controls (i.e., virus not incubated with any cells), incubation of virus with cells at 4°C reduced levels of virus in the supernatant; however, essentially all virus was recovered at 37°C, and no differences were noted between WT and PTX3R viruses. Moreover, WT and PTX3R mutants showed no difference in their ability to infect the LA-4 mouse airway epithelial cell line, as assessed by immunofluorescence at 8 h postinfection (Supplemental Fig. 1B). Finally, MDCK cells were infected at a low MOI (0.01 PFU/cell) in the presence of trypsin to allow for multiple cycles of virus replication in cell culture. At various times, cell-free supernatants were removed, and titers of infectious virus released from IAV-infected cells were determined. In these experiments, no significant differences were detected between WT and PTX3R viruses at any time point tested (Supplemental Fig. 1C). Together, these data indicate that resistance to PTX3 did not alter the intrinsic ability of IAV to infect and replicate in mammalian cells.

PTX3R mutants show enhanced virulence in mice

We next compared the virulence of WT and PTX3R mutants of HKx31 and PC/73 in mice. Initial studies examined weight loss (Fig. 3A, 3C) and lethality (Fig. 3B, 3D) of mice following intranasal inoculation with increasing doses of WT or PTX3R viruses. Overall, PTX3R mutants displayed enhanced virulence in mice compared with their respective WT viruses. Mice infected with 10^6 PFU of PTX3R HKx31 lost more weight (day 7, p < 0.01; day 8, p < 0.001, two-way ANOVA) and showed reduced survival (p < 0.01, two-tailed, log-rank Mantel–Cox test) compared with animals infected with an equivalent dose of WT HKx31. PTX3R PC/73 was also more virulent than was WT PC/73, and PTX3R-infected mice showed enhanced weight loss postinfection with 10^5 PFU (day 7–8, p < 0.001; day 9–10, p < 0.05, two-way ANOVA), 10^6 PFU (day 5–7, p < 0.001; day 8, p < 0.01, two-way ANOVA), and 10^7 PFU (day 6, p < 0.05; day 7–9, p < 0.001; day 10, p < 0.01, two-way ANOVA). Moreover, mice infected with 10^7 PFU of PTX3R PC/73 showed reduced survival (p < 0.001, two-tailed, log-rank [Mantel–Cox] test) compared with mice infected with WT PC/73.

To examine viral replication, mice were infected with 10^3 PFU of WT or PTX3R HKx31 or with 10^4 PFU of WT or PTX3R PC/73, and viral titers were determined at day 3 (Fig. 4A) and day 7 (Fig. 4B) postinfection. At day 3, there were no differences between either pair of WT and PTX3R viruses with regard to virus replication in the lungs. At day 7, both PTX3R mutants grew to higher titers in the lungs compared with their respective WT control viruses (Fig 4B, p < 0.001, Student t test).

Enhanced airway inflammation in mice infected with PTX3R PC/73

We characterized the cellular infiltrate and the soluble mediators in BALF recovered from mice 7 d postinfection with 10^5 PFU of either WT or PTX3R PC/73. Numbers of total BALF cells recovered from WT or PTX3R PC/73–infected mice were similar, with no significant differences in the numbers of neutrophils or CD8^+, CD4^+, or B220^+ lymphocytes, although a modest increase in NK cells was observed (Supplemental Fig. 2A).

Because dysregulated cytokine responses are associated with IAV-induced disease, we next determined the levels of proinflammatory chemokines and cytokines in the airways of IAV-infected mice. Levels of IFN-γ, TNF-α, IL-10, and IL-12, p70 were similar in cell-free BALF from mice infected with PTX3-sensitive (PTX3S) or PTX3R viruses (data not shown); however, MCP-1 and IL-6 were significantly higher in BALF from PTX3R–infected mice (Fig. 5A). It is well established that elevated MCP-1 and

**FIGURE 3.** Weight change and survival of mice infected with WT or PTX3R H3N2 viruses. C57BL/6 mice were infected via the intranasal route with 10^5, 10^6, or 10^7 PFU of WT or PTX3R HKx31 (A, B) or WT or PTX3R PC/73 (C, D). Control mice (Mock) received an equivalent volume of the virus diluent PBS. Mice were monitored daily, and any mice that lost >20% of their original body weights were euthanized. Data in (A) and (C) represent the mean percentage (± 1 SD) of weight change over time (n = 10). Data in (B) and (D) show the percentage of survival over time (n = 10). Weight loss over time was analyzed by two-way ANOVA with the Bonferroni correction. Survival was assessed using the log-rank test. *p < 0.05, **p < 0.01, ***p < 0.001.

**FIGURE 4.** Increased viral load in the lungs of mice infected with PTX3R HKx31 and PC/73 at 7 day postinfection. C57BL/6 mice were infected via the intranasal route with 10^5 PFU of WT or PTX3R HKx31 (left panels) or with 10^6 PFU of WT or PTX3R PC/73 (right panels). At day 3 (A) and day 7 (B) postinfection, mice were culled, and virus titers in lung homogenates were determined using a standard plaque assay on MDCK cells. Data for individual mice are shown; the horizontal line represents the mean virus titer (n = 10, pooled from two independent experiments). The dotted line indicates the detection limit for the plaque assay. ***p < 0.001, Student t test.
IL-6 are associated with lung injury and disease in IAV-infected mice (28, 30). Consistent with this, the increased wet/dry ratio of the lungs (Fig. 5B) and enhanced protein levels in cell-free BALF (Fig. 5C) from mice infected with PTX3R PC/73 were indicative of increased vascular leakage and lung injury. Lung sections from WT- and PTX3R-infected mice were also examined for histopathological changes at day 7 postinfection (Fig. 5D). H&E-stained lung sections were blinded, randomized, and scored by three independent readers for peribronchiolar inflammation and alveolitis. Analysis of histological sections indicated that peribronchiolar inflammation and alveolitis were more severe in mice infected with PTX3R PC/73 ($p < 0.05$, Kruskal–Wallis test, Fig. 5E).

In humans and mice, thymic atrophy and leukopenia are systemic markers that have been associated with severe influenza infections (28, 30, 44). Infection of mice with PTX3R PC/73 was associated with reduced cellularity of the thymus, characterized by a marked reduction in the numbers of double-positive, but not single-positive or double-negative, thymocytes (Supplemental Fig. 2B). Total blood leukocyte numbers were also reduced in mice infected with PTX3R PC/73, and this was associated with decreased numbers of neutrophils, B lymphocytes, and CD4+ and CD8+ T cells (Supplemental Fig. 2C). Together, these data demonstrate that infection of mice with PTX3R PC/73 results in more severe airway and systemic disease.

**Increased levels of PTX3 in BALF from PTX3R PC/73–infected mice neutralize IAV infectivity at day 7 postinfection**

Next, we examined the levels of PTX3 in BALF from mice infected with WT or PTX3R PC/73 at days 3 and 7 postinfection (Fig. 6A). At either time point, PTX3 levels from WT- or PTX3R-infected mice were significantly higher than levels in BALF from naive animals. PTX3 levels in BALF from mice infected with WT or PTX3R PC/73 were not different at day 3 postinfection, but PTX3R-infected mice had significantly higher PTX3 levels at day 7 postinfection.

Cell-free BALF collected from mice 7 d after IAV infection was incubated on ice with 10% v/v chicken erythrocytes to remove residual virus and then assessed for its ability to neutralize IAV. BALF from naive or WT PC/73–infected mice did not neutralize WT or PTX3R PC/73 to significant levels (Fig. 6B). In contrast,
BALF from PTX3<sup>R</sup>-infected mice neutralized WT PC/73 more potently than did naive BALF (p < 0.01, one-way ANOVA). Although there was a tendency for BALF from PTX3<sup>R</sup>-infected mice to neutralize PTX3<sup>S</sup> PC/73, this was not significant compared with naive BALF and may reflect sensitivity to other innate proteins, such as SP-D, which are known to increase in BALF following IAV infection (Fig. 6B) (45, 46).

Because airway epithelial cells secrete PTX3 following exposure to a range of stimuli, including LPS and TNF-α (15, 17), we tested whether IAV might also induce PTX3 from human (A549) airway epithelial cells. Although TNF-α induced PTX3 secretion, PTX3<sup>S</sup> (HKx31) or PTX3<sup>R</sup> (HKx31-PTX3<sup>S</sup>) viruses did not (Supplemental Fig. 3), nor did virus inhibit TNF-α–induced PTX3 from epithelial cells. Together, these data indicate that IAV infection of epithelial cells does not induce or inhibit PTX3 production. Instead, the enhanced PTX3 levels in BALF from IAV-infected mice are likely a consequence of the surrounding cytokine milieu.

**Therapeutic treatment of mice with PTX3 ameliorates disease with a PTX3<sup>S</sup> virus but not a PTX3<sup>R</sup> virus**

Therapeutic treatment with recombinant human PTX3 was shown to ameliorate disease severity in a number of murine models, including murine CMV (47), mouse hepatitis virus (48), and Pseudomonas aeruginosa (49). We demonstrated previously that PTX3 treatment reduced virus growth in mice infected with HKx31 (H3N2, PTX3<sup>S</sup>) but not PR8 (H1N1, PTX3<sup>R</sup>) (11). Given the marked differences between these viruses, we refined our studies to determine the effect of therapeutic PTX3 treatment of IAV replication in the airways of mice infected with WT or PTX3<sup>R</sup> PC/73, which only differ by a single amino acid in HA<sub>1</sub>. In these studies, mice were challenged with 10<sup>5</sup> PFU and analyzed at day 5 postinfection. Compared with mock-treated animals infected with IAV, PTX3 treatment significantly reduced viral titers in lungs from mice infected with WT (p < 0.01, one-way ANOVA) but not PTX3<sup>R</sup> PC/73 (Fig. 7A).

**FIGURE 7.** Treatment of mice with human PTX3 reduces replication of WT, but not PTX3<sup>R</sup>, PC/73 in the lungs. C57BL/6 mice were infected via the intranasal route with 10<sup>5</sup> PFU of WT or PTX3<sup>R</sup> PC/73. Recombinant human PTX3 (1 mg/kg) was administered by the i.p. route on the day of infection and at days 1 and 3 postinfection. Control mice received the diluent alone (Mock). At day 5 postinfection, mice were culled, and virus titers were determined in homogenates prepared from the lungs using a standard plaque assay of MDCK cells. Data shown are pooled from two independent experiments (n = 6 mice/group). **p < 0.01, one-way ANOVA, Tukey post test.

**Rescue and amplification of RG viruses expressing the S145N substitution in the viral HA is resistant to PTX3.**

To confirm that a single amino acid substitution in the viral HA (Ser<sup>145</sup>→Asn) was the critical determinant of sensitivity to PTX3, we used RG to generate 6:2 reassortant viruses expressing the HA and NA of HKx31 and all internal components derived from PR8. RG viruses expressing WT HKx31 HA (RG-HKx31) or HKx31 HA containing the S145N substitution (RG-HKx31-PTX3<sup>R</sup>) were rescued, amplified in eggs, and characterized in vitro and in vivo. Compared with RG-HKx31, RG-HKx31-PTX3<sup>R</sup> was resistant to both hemagglutination inhibition (Fig. 8A) and neutralization (Fig. 8B) by human PTX3 in vitro. Furthermore, RG-HKx31-PTX3<sup>R</sup> grew to higher titers than did RG-HKx31 in mouse lung 7 d postinfection with 10<sup>5</sup> PFU of each virus (Fig. 8C). Full genome sequencing confirmed that site 145, and not other unidentified mutations, accounts for increased virulence in the mouse model and resistance to PTX3.

**Discussion**

The current study focused on understanding the viral determinants that contribute to the sensitivity of different IAV to PTX3. First, we demonstrated that the viral HA or NA can be the critical determinant of PTX3 sensitivity. HA-mediated recognition of SIAα(2,3) is an essential feature, but it cannot be used as a predictor of PTX3 sensitivity. For H1N1 IAV, the susceptibility of SIA expressed by PTX3 to the viral NA was also critical in determining sensitivity. Moreover, H5N1 viruses display clear HA preference for SIAα(2,3) (34, 35) yet were resistant to PTX3, even in the presence of NA1. Some avian viruses discriminate between the core structures of oligosaccharides (50), and HA-glycan conformation analysis suggests that the size and shape of SIA-bearing glycans, rather than specific linkage, are important for H5-mediated recognition (51). Therefore, it may be that the type of SIA presented by PTX3 is not recognized by the H5 HA, suggesting further complexity in the interplay between the fine specificity of the viral HA and the particular SIAα(2,3)-rich glycans expressed by PTX3.
H3N2 viruses associated with the 1968 Hong Kong pandemic acquired their HA gene from an avian source (52) and exhibited dual specificity for SIA(2,3)/(2,6), (31), which has been reported for H1N1 (53), H2N2 (54), and A(H1N1)pdm09 (33) viruses associated with pandemics in 1918, 1957, and 2009, respectively. In fact, some early pandemic viruses were reported to retain a preference for SIA(α(2,3)) over SIA(α(2,6)) (54, 55). Early H3N2 IAV were sensitive to PTX3, and A(H1N1)pdm09 viruses were sensitive in the presence of NAI (Table I). Recent H7N9 viruses associated with human disease also displayed HA specificity for both SIA(α(2,3)) and SIA(α(2,6)) (37) and were inhibited by PTX3 (Table I). Together, these data suggest that PTX3 may represent a barrier limiting the introduction of new subtypes into humans and/or limiting initial spread and severity of pandemic IAV.

Early H3N2 viruses were sensitive to PTX3 but rapidly developed resistance as they circulated in humans. To understand the molecular basis underlying resistance to PTX3, H3N2 mutant viruses were selected under experimental conditions in the presence of purified PTX3. Amino acid substitutions at residue 145 of HA1, which forms part of a loop lying adjacent to the receptor-binding site (56), were associated with resistance to PTX3, and similar substitutions were implicated in the natural evolution of human H3N2 strains from PTX3R to PTX3S (Fig. 2). It was suggested that changes at residue 145 can alter the orientation of SIA within the receptor-binding site (57); however, we did not detect any differences in receptor specificity between WT and PTX3R viruses or in their ability to bind, infect, and replicate in MDCK or LA-4 cells in vitro. Together, these data indicate that resistance to PTX3 did not come at a major cost to virus fitness, nor was it associated with the acquisition of compensatory mutations in either HA or NA.

The receptor preference of H3N2 viruses evolved from recognition of SIA(α(2,3)) and SIA(α(2,6)) by early strains to recognition of only SIA(α(2,6)) by strains after 1975 (31). Selective pressures on HA receptor specificity are likely to include the nature of SIA receptors expressed on target cells, as well as soluble extracellular proteins that can neutralize virus infectivity. This concept is not a new one, and the contributions of these two pressures to the selection of virus variants with optimal receptor-binding phenotypes was demonstrated in vitro using inhibitors from a range of animal sera (57–59). In humans, it is conceivable that widespread expression of SIA(α(2,6)) receptors in the airways (60), in concert with selective immune pressure from the SIA(α(2,3))-rich neutralizing inhibitor PTX3, was a factor underlying the evolution of H3N2 receptor specificity in humans. However, because substitutions at residue 145 alone did not result in detectable differences in the HA specificity of PTX3R mutants, it is likely that additional changes in the HA of circulating H3N2 strains also contributed to their evolution toward a preference for SIA(α(2,6)).

SP-D, a major constituent of human airway fluids (45, 61), is a potent inhibitor of highly glycosylated seasonal IAV; however, pandemic viruses are poorly glycosylated and, therefore, are largely resistant to this lectin (27, 45, 62). PTX3 acts as a SIA(α(2,3))-rich “receptor decoy” that is induced following IAV infection, consistent with a role in preventing infection and limiting spread of some pandemic IAV (or avian-origin IAV) in humans. Such a role could be particularly important because lectin-mediated innate defenses against these viruses are limited. In contrast, SAP expresses SIA(α(2,6)) (12, 63) and is likely to be effective against seasonal IAV that have evolved receptor preference for this linkage and cannot evade SIA(α(2,6)) recognition. Other sialylated inhibitors, such as gp340 (64), H-ficolin (10), and SP-A (65), express both SIA(α(2,3)) and SIA(α(2,6)) and, therefore, may contribute to immunity against potential pandemic and/or seasonal strains. Moreover, NAI potentiate the anti-IAV activity of PTX3 and SAP (Table I), as well as other γ-inhibitors, including mucins, SP-A, and H-ficolin in vitro (10, 66). Thus, NAI treatment blocks the ability of the viral NA to inactivate sialylated inhibitors in mucus, saliva, and other airway secretions, thereby enhancing their anti-IAV activity and contributing to virus clearance and recovery.

PTX3 plays a complex multifactorial role in infection and inflammation. Elevated levels of PTX3 in serum and/or BALF were associated with poor outcomes in patients with acute lung injury or acute respiratory distress syndrome (reviewed in Ref. 67). During IAV infection, high levels of endogenous PTX3 in airway fluids from PTX3R PC/73–infected mice were associated with pulmonary inflammation and lung injury (Fig. 6A). However, early treatment with recombinant PTX3 ameliorated disease severity following infection with PTX3R virus (Fig. 7), suggesting that the timing and regulation of PTX3 production are important in determining its role during IAV infection. During murine CMV infection, therapeutic PTX3 treatment induced dendritic cell maturation and enhanced NK cell and T cell activation (47), consistent with an important protective role mediated via its immunomodulatory properties. Therapeutic PTX3 treatment reduced virus replication in the lungs of mice infected with PTX3R virus, but not PTX3S virus, suggesting that the direct antiviral effects of PTX3 were most important in limiting IAV in this model. Although our results demonstrate a clear increase in the virulence of PTX3R IAV in mice, the mechanisms underlying this phenomenon have yet to be fully elucidated. For example, subtle changes in HA preference could enhance the avidity of the PTX3R HA for sialylated receptors in the murine airways, resulting in enhanced virus infection and virulence. In future studies, the use of PTX3-null mice will allow for a clearer understanding of the role that endogenous PTX3 plays in controlling IAV infection in vivo.

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