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Severe pandemic 2009 H1N1 influenza disease due to pathogenic immune complexes

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Abstract

Pandemic influenza viruses often cause severe disease in middle-aged adults without preexistent co-morbidities. The mechanism of illness associated with severe disease in this age group is not well understood1[−10]. Here, we demonstrate preexisting serum antibody that cross-reacts with, but does not protect against 2009 H1N1 influenza virus in middle-aged adults. Non-protective antibody is associated with immune complex(IC)-mediated disease after infection. High titers of serum antibody of low avidity for H1-2009 antigen, and low avidity pulmonary ICs against the same protein were detected in severely ill patients. Moreover, C4d deposition - a sensitive marker of complement activation mediated by ICs- was present in lung sections of fatal cases. Archived lung sections from adults with confirmed fatal influenza 1957 H2N2 infection revealed a similar mechanism of illness. These observations provide a novel biological mechanism for the unusual age distribution of severe cases during influenza pandemics.

Pandemic viruses may promote bacterial infections1, injure the lungs2[−4], decrease type I interferon (IFN) levels[5], promote a cytokine storm, and induce apoptosis6. Although all attractive hypotheses, the explanation for the enhanced severity of cases in middle-aged adults during pandemics7[−]10 remains unclear.

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In 2009, a novel H1N1 influenza A virus caused severe disease in naïve middle-aged individuals with preexisting immunity against seasonal strains[11–13]. In contrast to seasonal disease, the elderly were relatively spared and young children had milder disease than middle-aged subjects[11–16]. Preexisting neutralizing cross-reactive antibodies elicited by an H1N1 virus circulating before 1957 protected the elderly[11,12]. Adults had been exposed repeatedly to seasonal influenza viruses leading to antibody production, while young children often lacked previous exposures[12]. An antibody repertoire in adults shaped by seasonal infections may recognize, but fail to neutralize the new pandemic strain, leading to IC-mediated disease[17,18]. In this manuscript, we characterized the pathogenesis of severe pandemic respiratory disease in middle-aged adults with no preexisting co-morbidities.

Tracheal (TA) and nasopharyngeal (NP) aspirates, and serum samples reflecting different disease severities in adult outpatients (n=21) and inpatients (n=54) infected with 2009 H1N1 were obtained. Median age of patients was 39 years (range=17–57). Twenty-three subjects died; 16 (69%) of refractory hypoxemia. Fifteen survivors required intensive care.

In addition, NP secretions from adults hospitalized with seasonal influenza A viruses (2007/08), and from infants and young children infected with 2009 H1N1 were analyzed. In Argentina, universal immunization against influenza in children was not recommended until 2010.

Lung sections of patients with fatal 2009 H1N1 showed widened inter-alveolar septa, interstitial hemorrhages, abundant intra-alveolar edema with deposition of hyaline membranes, and an infiltrate of mononuclear cells (Fig. 1a)[10,19]. Lungs evidenced hyperplasia and detachment of type II pneumocytes into the lumen. Fatal cases of seasonal H1N1 influenza also revealed interstitial edema, desquamation of type II pneumocytes, and mononuclear cell infiltration (Fig. 1a). Influenza A 2009 H1N1 was detected mainly in epithelial cells of bronchioles; seasonal H1N1 was occasionally detected in respiratory epithelial cells from pre-exposed elders (Fig. 1b).

2009 H1N1 RNA (vRNA) expression was similar in outpatients, and inpatients requiring ICU or not surviving (Fig. 1c; p=0.9). However, vRNA levels correlated with days of symptoms (p=0.027; Fig. 1d), and patients with severe disease (ICU+fatal) were sampled later than outpatients (median= 6 vs. 3 days; p= 0.02). Adjusting the relationship between vRNA levels and severity for days of symptoms did not reach statistical significance (p=0.3).

Analysis of type I IFN production showed lower TA than NP levels of IFN-α, with similar levels in 2009 H1N1 vs. seasonal influenza infections (p= NS; Suppl. Fig 1). IFN-β was universally low (p=0.6 pandemic vs. seasonal; Suppl. Fig 1).

Certain pandemic hemagglutinins (HA) are thought to cause a cytokine storm[20]. NP secretions in 2009 H1N1 and seasonal infections evidenced similar levels of TNF-α, IL-6, IL-1β, IL-10, and IL-12 (Fig. 2b–d, Suppl. Fig. 2). IL-8 levels were higher in pandemic patients (p=0.01; Fig. 2a).

The effect of H1-2009, H1-1918 and H1-1999 proteins on human monocyte production of inflammatory cytokines was similar (Fig. 2e–h). Higher levels of inflammatory cytokines were detected with avian H5[21,22]. Surprisingly, the inflammatory response against the human metapneumovirus fusion protein (hMPV-F) was higher than against influenza HAs. In fact, hMPV-F was a vigorous TLR2 and TLR4 agonist, while H1-2009 and H1-1918 were weak TLR2 and TLR4 agonists, respectively (Fig. 2i). Seasonal H1-1999 activated TLR4. No HA protein activated other TLRs.

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Patients with severe pandemic influenza presented profound lymphopenia (Fig. 3a)[10]. Both $CD4^+$T lymphocyte and $CD8^+$T lymphocyte counts were below normal ranges (Fig. 3b,c). Lymphopenia associated with a lung T lymphocytosis (Fig. 3d), likely explained by pandemic virus conservation of numerous T cell epitopes from seasonal strains[23,24]. In fact, many $CD8^+$ T lymphocytes were observed (Fig. 3e)[19]. No significant T lymphocyte apoptosis in the lungs was detected (Suppl. Fig. 3).

We then asked whether lung lymphocytosis was associated with Th2-immunopathogenesis, a mechanism of immune-mediated viral respiratory illnesses[25]. Analysis of IFN-γ (Th1), IL-4(Th2) and IL-17(Th17) showed few cytokine-positive cells in lung sections and low cytokine levels in secretions in pandemic patients(not shown).

2009 H1N1 virus shares 17% of its B cell epitopes against HA and NA with seasonal influenza A viruses[24]. Therefore, we examined whether cross-reactive, non-protective antibodies against 2009 H1N1 were present in sera of naïve adults (Fig. 4a). IgG against HA antigens was absent in infants, but detected in naïve adults and elderly (Fig. 4a). However, antibody avidity for H1-2009 was lower in adults than in older patients ($p<0.05$; Fig. 4b). In fact, adults had higher avidity for H1-1999 than for H1-2009 ($p=0.03$; Suppl. Fig. 4). Moreover, unlike elderly subjects, middle-aged adults lacked protective titers of neutralizing antibody against 2009 H1N1 (Fig. 4c)[11,12].

IgG against H1-2009 and H1-1999 was present in adults <10 days after 2009 H1N1 infection (Suppl. Fig. 5). Interestingly, anti-H1-2009 IgG titers were higher in severe vs. mildly ill adults (Fig. 4d; $p=0.02$). Moreover, IgG avidity remained lower for H1-2009 than for H1-1999 in pandemic patients ($p=0.03$; Suppl. Fig. 5), and severely ill patients had antibody of lower avidity for H1-2009 than mildly ill outpatients ($p<0.05$; Fig. 4e). Importantly, severe cases also had anti-H1-2009 IgG of lower avidity than mild cases in respiratory IC ($p<0.05$; Fig. 4f).

Non-protective antibody responses of low avidity have been associated with IC disease in other respiratory infections[17,27]. We therefore stained lung sections for complement cleavage product C4d[26]. Extensive C4d deposition was detected in bronchioles of patients infected with 2009 H1N1 (Fig. 4g), matching distribution in IC-mediated diseases due to other viruses[27]. Conversely, trace deposition of C4d was found in lung sections from patients infected with seasonal influenza.

ICs were detected using an anti-C1q assay in secretions of individuals infected with 2009 H1N1, but rarely in samples from patients infected with seasonal influenza ($p=0.003$ comparing floor admissions; Suppl. Fig. 6). Furthermore, higher levels of ICs were detected in secretions of adults admitted with 2009 H1N1 to the ICU compared to those admitted to regular floors (Suppl. Fig. 6).

Confirming our observations, most adults admitted to the ICU with pandemic influenza had low serum C3 levels, while C3 levels were higher in moderately ill subjects. Infants infected with 2009 H1N1, adults infected with seasonal influenza, and patients with other pulmonary diseases often had normal C3 levels ($p=0.036$; Fig. 4h).

Finally, we retrieved archived lung sections from adult patients who died during the 1957 H2N2 pandemic in Tennessee. Sections of sufficient quality to be processed and stained showed extensive C4d peribronchiolar deposition (Fig. 4i). Presence of influenza A vRNA was confirmed by real time-PCR. A control archived lung section from an individual with no pulmonary infection evidenced no C4d deposition.

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Taken together, these observations demonstrate that 2009 H1N1 influenza virus leads to IC-mediated disease in adults through high titers of low avidity non-protective antibody and IC-mediated complement activation in the respiratory tract. IC-mediated lung disease also contributed to fatal cases caused by 1957 H2N2 pandemic influenza. We speculate that this phenomenon contributes to severe symptoms in the adult population during all pandemics[8].

Young infants and children -as in previous pandemics-had high rates of infection with comparatively low mortality[7,11]. This paradox is explained by absence of protective and pathogenic immunity in pediatric patients[11–13], particularly in countries where immunization of children against seasonal viruses was not recommended. Therefore, severe pediatric 2009 H1N1 illness associates with widespread infection in a naive population.

This study addresses several attractive hypotheses advanced to explain the pathogenesis of influenza viruses[1–6]. While increased severity of 1918 and 2009 H1N1 pandemic viruses was presumptively associated with higher pulmonary virus titers[2–4], a dose-dependent effect on mortality was never described. Similarly, type I IFN modulation appears to play an important role in severe cases of influenza[28], but its role in the unusual age distribution of severe cases during pandemic flu remains to be determined.

Secondary bacterial infections were responsible for most deaths in 1918[1]. During 2009, most fatal cases were primary infections with refractory hypoxemia[10,12], and neutrophil lung infiltration was minimal[19]. Interestingly, depletion of inflammatory cytokines or pretreatment with steroids did not affect mortality in a murine model of fatal H5N1 influenza infection[29]. Moreover, the main neutralizing antigen of milder hMPV elicits significantly more inflammation *in vitro* than influenza HAs.

However, other roles for innate immunity may be at play in pathogenesis. For example, *S.pneumoniae* nasopharyngeal carriage may also affect illness severity during 2009 H1N1 infection[30]. Indeed, several factors likely contribute to severe pandemic disease in adults and explain different outcomes in individuals of similar ages and backgrounds.

Certain limitations are inherent to a study of these characteristics. For instance, since we lacked determinations of vRNA levels over time, disregarding a role for viral injury in severity is not possible. However, the impact of pandemic flu in adults compared to frail infants and elderly argue against a preponderant pathogenic role for viral injury. Also, we rapidly identified a cross-reactive antibody against 2009 H1N1, known to recognize seasonal H1N1. But whether reactivity against both viruses is identical in our descriptive slides is unknown.

In summary, our study provides a novel biological explanation for the unusual age distribution of severe cases during pandemic influenza. The association of severe pandemic disease in adults with high titers of low avidity, non-protective antibody and complement activation by pulmonary IC opens a new paradigm for future therapeutic interventions.

Methods

Subjects and Samples

Archived tracheal aspirates (TA), nasopharyngeal (NP) secretions, and serum samples were obtained from hospitals in Buenos Aires participating in the National Surveillance Network. 2009 H1N1 infection was confirmed by real time-RT-PCR using the CDC protocol (http://www.cdc.gov/h1n1flu/casedef.htm). No samples were obtained from patients with presumptive (clinical diagnosis) or confirmed (blood, pleural or other sterile site positive culture) bacterial super-infections.

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Lung sections from cases of fatal primary 2009 H1N1 influenza (n=9) were also obtained. Seasonal influenza A viruses was confirmed by RT-PCR and/or culture. Study was approved by the Institutional Review Board of participating institutions.

Histopathology and immunohistochemistry

For immunohistochemistry, 4–5 micron sections were stained with a polyclonal goat anti-H1N1 antibody that cross-reacts with seasonal H1N1 and 2009 H1N1(Biological Swampscott), anti-CD3 (Santa Cruz Biotechnology), anti-CD8 and anti-C4d (Abcam Inc) antibodies.

Protein synthesis and purification

Synthetic, sequence-optimized hemagglutinin constructs were obtained from GeneArt (Regensburg, Germany) or GenScript (Piscataway) and cloned into pcDNA3.1 (Invitrogen). The plasmids were transformed into DH5alpha for EndoFree Plasmid Maxi preparation (Qiagen). The DNA was transiently transfected into HEK 293F cells (Invitrogen). The supernatant was harvested on day seven and purified on nickel columns. Endotoxin contamination was ruled out in HA, hMPV F and control proteins after purification using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (GenScript; sensitivity=0.005 to 1 EU/ml), according to the manufacturer's instructions.

Toll-Like Receptor (TLR) Ligand Screening

TLR stimulation was tested by assessing NF-κB activation in HEK293 cells expressing a given TLR using a reporter gene. No ligand was used as negative control.

Immunoassays

Best-fit titration curves were calculated by non-linear regression to a sigmoidal function using the GraphPad package (Prism). End point titer was defined as the reciprocal of the highest dilution of sera that had a reading above the cut-off, after subtraction of background in all samples. The cut-off was defined as twice the background signal. Avidity was determined by incubating samples with urea 6M, 7M, 8M and 9M for 10 minutes before washing and incubation with secondary antibody.

Type I IFN determinations

IFN-α, IFN-β and inflammatory cytokines were detected using commercial detection kits (pbl interferon source and BD Biosciences, respectively).

Microneutralization assay

One hundred $TCID_{50}$ units of influenza H1N1/A/Arg/17/09 were preincubated with dilutions of serum and then used to infect MDCK cells in 96-well plates, as previously described[12,13]. Six replicate wells were used for each antibody dilution. Neutralizing antibody concentrations were determined and were defined as the reciprocal of the highest dilution of serum where 50% of wells were infected, as calculated by the method of Reed and Muench[12,13].

Immune-complex dissociation and avidity determinations

Extraction of immune-complexes from respiratory secretions was performed by adding 50 ul of 7% PEG in phosphate-buffered saline to every sample (50 ul). Immune complexes were dissociated by adding 1.5 M glycine hydrochloride (pH 1.8). Control samples were treated with 1.5 M glycine hydrochloride (pH 7.2). Samples were neutralized with 50 ul of 5 mM

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Tris-hydrochloric acid (pH 7.2). Avidity against H1 2009 was determined as described above.

Monocyte cytokine assays

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque (GE Healthcare Life Sciences) from anonymous healthy donors. Monocytes were isolated using the Monocyte Isolation Kit II (Miltenyi Biotec) as described. Remaining cells were >95% monocytes by anti-CD14 staining and forward-and side-light scatter analysis using flow cytometry (Becton Dickinson). Purified monocytes (10^5 cells/well) were stimulated for 18 h at 37°C with the respective HAs in triplicates. Inflammatory cytokines were detected in supernatant fluids of treated and control untreated monocytes using the cytokine bead array kit (BD Biosciences) following the manufacturer's instructions.

TLR ligand screening

TLR agonism was tested by assessing NF-κB activation in HEK- 293 cells expressing individual human TLRs. (Invivogen) Hemagglutinins were tested at a dose of 5 μg. All tests were performed in duplicate.

RNA extraction and RT-PCR

Formalin-fixed, paraffin-embedded autopsy lung tissue specimens were retrieved. Ten-μm sections were deparaffinized, digested, and RNA extracted using the RecoverAll Total Nucleic Acid Isolation Kit (Ambion) according to the manufacturer's instructions. Extracted RNA was tested by real-time RT-PCR for human RNAse P and for influenza A and B using the CDC real-time RT-PCR assay to detect seasonal influenza. Matrix gene sequences for 32 1957 H2N2 strains were retrieved from the NIH Influenza Virus Resource and aligned using MacVector 11.0 (MacVector, Inc.). Primer and probe sequences used in the CDC influenza A assay were highly conserved with the 1957 sequences (data not shown). Specimens were considered positive for influenza if they had C(t) <60 in two separate reactions.

Statistical analysis

Data was analyzed using STATA 10.1. Kruskal-Wallis and Mann Whitney U tests were used where appropriate. Correlation between RNA levels and days of symptoms was explored using the Spearman's rank correlation coefficient test. The impact of RNA levels on disease severity was adjusted by days of flu symptoms using a logistic regression analysis. A $p<0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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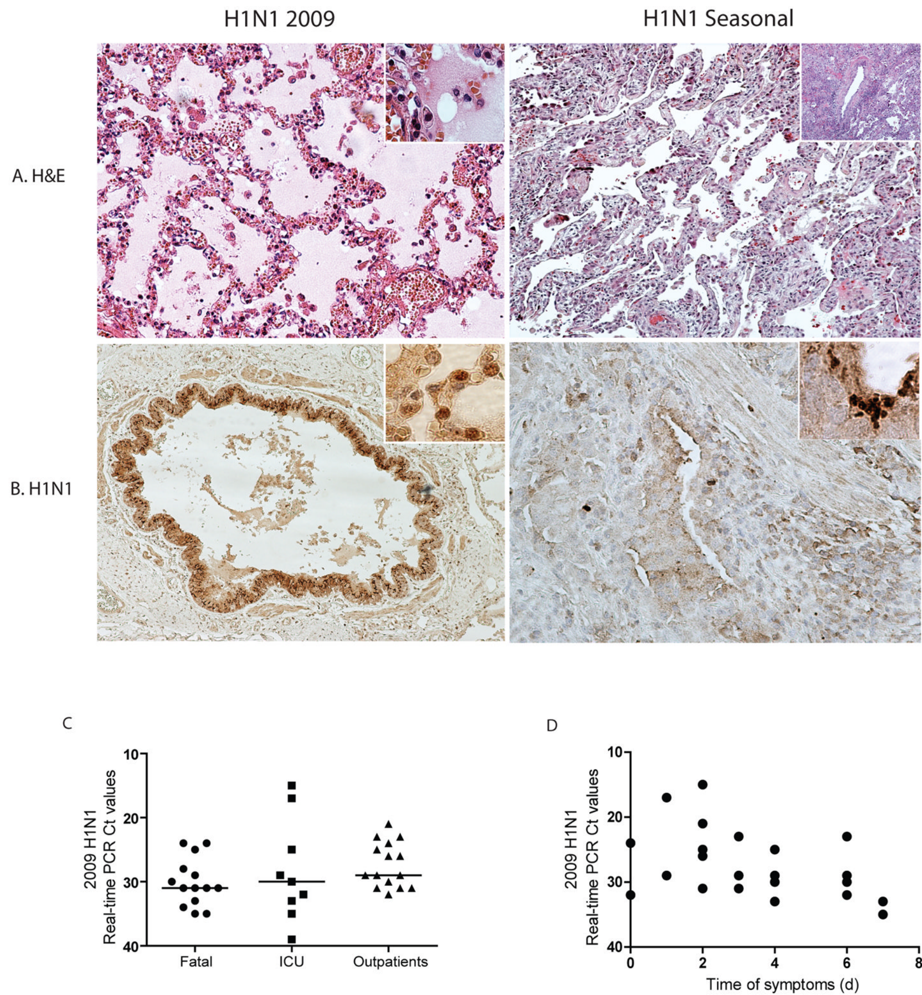

Figure 1. Histopathology and virus titers in 2009 H1N1 disease

(a) Pulmonary histopathology in representative lung sections of fatal 2009 H1N1 and seasonal H1N1 virus infected patients (H&E). (b) Detection of 2009 H1N1 and seasonal H1N1 influenza viruses. Scale, 100μm. The boxes are details of pulmonary edema (2009 H1N1, H&E), peribronchiolar mononuclear cell infiltration (seasonal H1N1, H&E), and virus-infected cells (anti-H1N1 stains). (c) 2009 H1N1 RT-PCR C(t) values in NP secretions of fatal (black circles), ICU (black squares) and ambulatory (black triangles) patients; p=NS. (d) 2009 H1N1 RT-PCR C(t) values by days of symptoms before NP sampling.

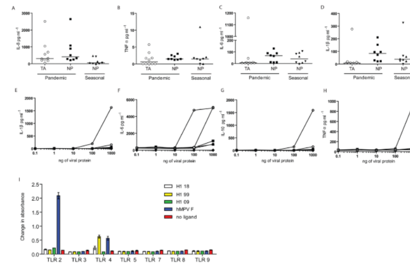

Figure 2. Inflammation in influenza A 2009 H1N1 disease

IL-8(a), TNF-α (b), IL-6(c) and IL-1β (d) responses in respiratory secretions of patients infected with 2009 H1N1 influenza virus (tracheal aspirates, white circles; NP secretions, black squares) or seasonal influenza viruses (NP secretions of: H1N1, black up-pointing triangles; H3N2, black down-pointing triangles). Comparisons for NP secretions: IL-8, $p=0.01$; all other cytokines, p=NS. IL-1β (e), IL-6(f), IL-10 (g), and TNF-α (h) production by human monocytes incubated with a dose range of recombinant protein H1-1918 (black squares), H1-1999 (black triangles), H1-2009 (black circles), avian H5 (gray circles), hMPV F (white circles), and a control monoclonal human IgG against the Sa antigenic site of influenza HA (2D1; black diamond). Representative of three independent experiments. (i) Individual TLR activation in HEK293 cells. Dose: 5μg of H1-1918 (white bars), H1-1999 (yellow bars), H1-2009 (green bars), hMPV F (blue bars), and no ligand (red bars). Representative of two independent experiments.

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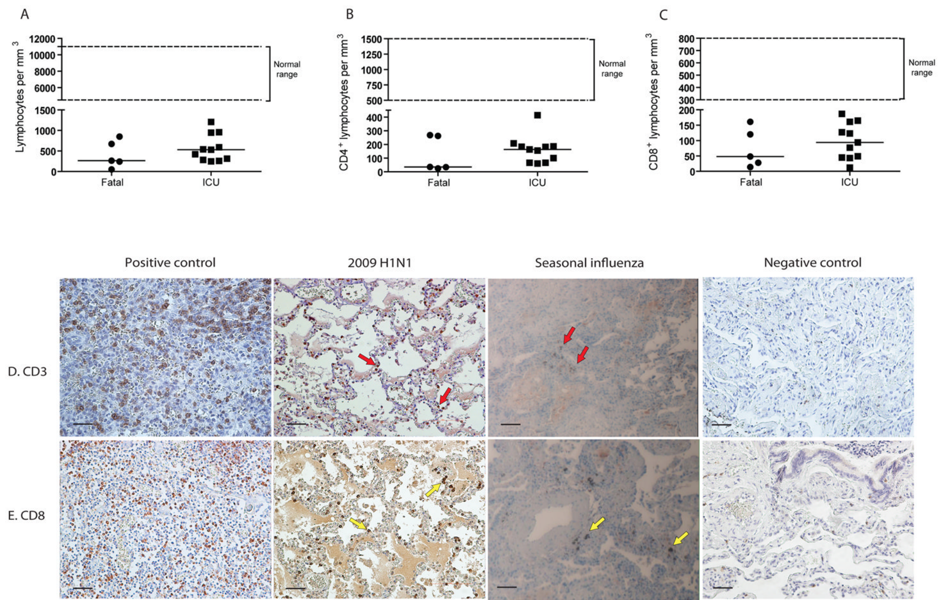

Figure 3. Lymphopenia in influenza A 2009 H1N1 disease

(a)$CD3^+$, (b) $CD4^+$ and (c) $CD8^+$ T lymphocyte counts in fatal and ICU patients infected with 2009 H1N1 influenza A virus on admission. Immunohistochemistry for (d)$CD3^+$ T lymphocytes (red arrows), (e)$CD8^+$ T lymphocytes(yellow arrows) in representative lung sections of fatal influenza 2009 H1N1 and seasonal infection. Positive controls are archived sections from different organs in unrelated subjects provided for every antibody. Negative controls are lung sections from a patient who died due to a non-pulmonary disease (scale, 100μm).

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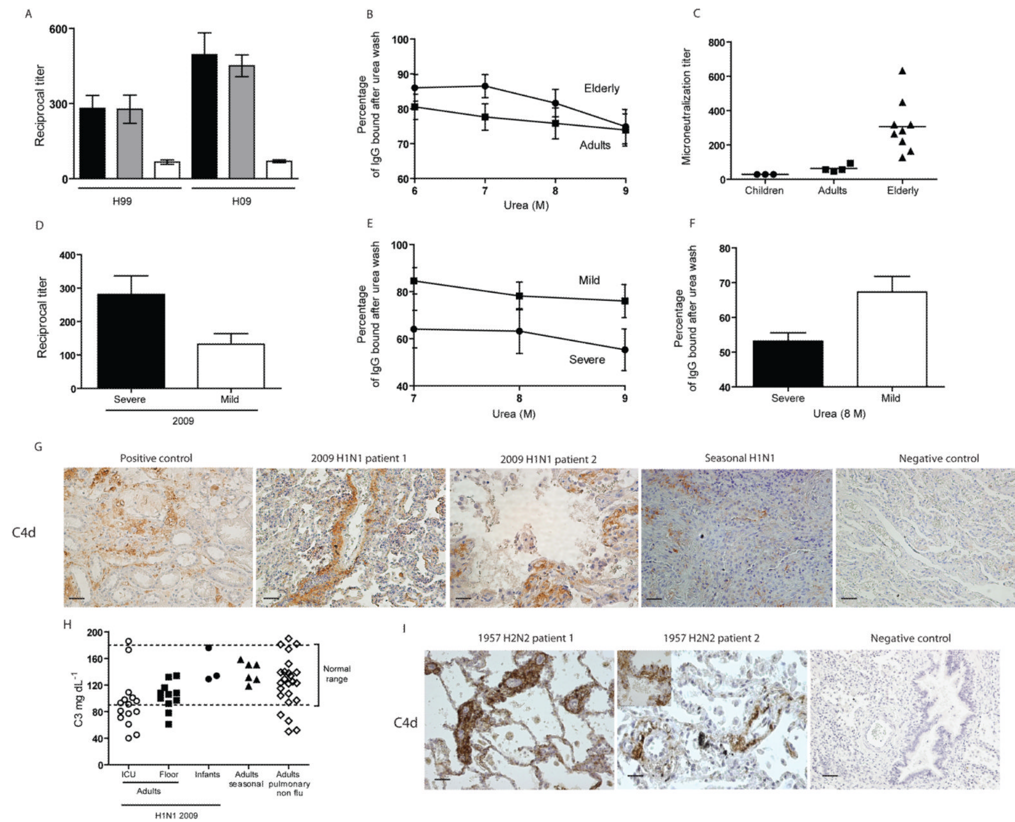

Figure 4. IC-mediated disease in 2009 H1N1 influenza infection

(a) Serum IgG endpoint titers against HA proteins by immunoassay in infants [mean age(range)=7.6 mo(6.1–11.8); n=10; white bars], middle-aged adults (n=16; gray bars), and elderly (n=12; black bars). (b) Protein-specific avidity of IgG after 6–9M urea wash in naïve adults (age range=25–43 year old; black squares) and elderly (age range= 75–97 years old; black diamonds); p<0.05. (c) Microneutralization titers for infants (black circles), adults (black squares) and elderly (black triangles); p value for elderly vs. both groups <0.05. (d) End point titer of serum IgG against H1-2009 antigens by immunoassay in adults with severe (n=12) vs mild (n=11) disease, p<0.05. (e) H1-2009-specific avidity of IgG after 7–9M urea wash in infected adults with severe (n=12) vs. mild (n=11) pandemic disease, p<0.05. (f) H1-2009-specific avidity of IgG from immune complexes after 8M urea wash in infected adults with severe (n=14) vs. mild (n=11) pandemic disease, p<0.05. (g) C4d detection in representative slides from lung sections of 2 of 6 fatal 2009 H1N1 infected middle-aged patients showing extensive peribronchiolar IC-mediated complement activation. Trace C4d deposition in representative lung section of fatal seasonal H1N1 virus from an elderly woman. Positive control: C4d deposition in a kidney from an adult patient with IC-mediated glomerulonephritis; negative control: lung section from an adult patient with solid tumor (scale, 100 μm). (h) Serum complement C3 levels in ICU adults (white circles), floor adults(black squares) and in infants (black circles) infected with H1N1 2009 influenza A virus, adults infected with seasonal influenza virus (black triangles) and adult patients with pulmonary diseases other than influenza in ICU and floor (white diamonds); p=0.036 (i) C4d detection in representative slides from lung sections of two fatal 1957 H2N2 influenza-infected patients showing extensive peribronchiolar deposition of complement cleavage product. Negative control from an archived sample with no infectious pulmonary disease. The box in the microphotograph is a detail of lung complement deposition.