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Differential Host Determinants Contribute to the Pathogenesis of 2009 Pandemic H1N1 and Human H5N1 Influenza A Viruses in Experimental Mouse Models

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Influenza viruses are responsible for high morbidities in humans and may, eventually, cause pandemics. Herein, we compared the pathogenesis and host innate immune responses of a seasonal H1N1, two 2009 pandemic H1N1, and a human H5N1 influenza virus in experimental BALB/c and C57BL/6J mouse models. We found that both 2009 pandemic H1N1 isolates studied (A/Hamburg/05/09 and A/Hamburg/NY1580/09) were low pathogenic in BALB/c mice [log mouse lethal dose 50 (MLD_{50}) >6 plaque-forming units (PFU)] but displayed remarkable differences in virulence in C57BL/6J mice. A/Hamburg/NY1580/09 was more virulent ($logMLD_{50}$ = 3.5 PFU) than A/Hamburg/05/09 ($logMLD_{50}$ = 5.2 PFU) in C57BL/6J mice. In contrast, the H5N1 influenza virus was more virulent in BALB/c mice ($logMLD_{50}$ = 0.3 PFU) than in C57BL/6J mice ($logMLD_{50}$ = 1.8 PFU). Seasonal H1N1 influenza revealed marginal pathogenicity in BALB/c or C57BL/6J mice ($logMLD_{50}$ >6 PFU). Enhanced susceptibility of C57BL/6J mice to pandemic H1N1 correlated with a depressed cytokine response. In contrast, enhanced H5N1 virulence in BALB/c mice correlated with an elevated proinflammatory cytokine response. These findings highlight that host determinants responsible for the pathogenesis of 2009 pandemic H1N1 influenza viruses are different from those contributing to H5N1 pathogenesis. Our results show, for the first time to our knowledge, that the C57BL/6J mouse strain is more appropriate for the evaluation and identification of intrinsic pathogenicity markers of 2009 pandemic H1N1 influenza viruses that are "masked" in BALB/c mice. *(Am J Pathol 2011, 179:230–239; DOI: 10.1016/j.ajpath.2011.03.041)*

Influenza A viruses are a continuous threat to humans because of their ability to cross species barriers and adapt to new hosts. Zoonotic H5N1 influenza viruses have repeatedly crossed species barriers and infected humans, with high fatality rates (>50%; *http://www.who.int/csr/don/2006_06_30/en*, last accessed May 13, 2011).[1,2] Emerging influenza viruses with an antigenically new subtype may acquire the potential to cause pandemics in the human population.[3] The first pandemic of the 21st century was announced in June 2009 by the World Health Organization in response to the emergence of a novel H1N1 influenza A virus (pH1N1). This 2009 pandemic virus derives six genes from triple-reassortant North American swine virus lineages and two genes (encoding neuraminidase and matrix protein) from Eurasian swine virus lineages.[4,5] The 2009 pH1N1 influenza has resulted in >18,000 deaths in >200 countries worldwide (*http://www.who.int/csr/disease/swineflu/updates/en/index.html*, last accessed August 6, 2010). Most people infected with 2009 pH1N1 experienced mild disease, with upper respiratory illness similar to seasonal influenza virus infection. However, gastrointestinal symptoms (including nausea, vomiting, and diarrhea) occurred more commonly in patients infected with pH1N1 than with sea-

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sonal influenza.[6,7] In addition, in contrast to seasonal influenza, most of the serious illnesses caused by the 2009 pH1N1 influenza occurred among children and nonelderly adults, with approximately 90% of deaths occurring in those younger than 65 years.[8] Approximately 9% to 31% of hospitalized patients were admitted to intensive care units, and 14% to 46% of these patients died.[9–13]

Circulating 2009 pH1N1 influenza viruses possess high sequence homologies, although variants differing from the consensus sequence have been described.[5] In animal models, such as mice, ferrets, pigs, and macaques, pH1N1 isolates have caused more disease than seasonal H1N1 influenza viruses.[14–16] Previously described markers predictive of host adaptation of H5N1 and 1918 pandemic influenza viruses were not present in 2009 pH1N1 viruses. This suggests that unrecognized novel determinants are responsible for the sustained human-to-human transmission and the disease severity among nonelderly adults, indicating a higher pathogenic potential of this newly emerged virus.[5]

Small animals, such as mice, are convenient models to rapidly assess infection and the pathogenesis of newly emerged influenza viruses. BALB/c and C57BL/6J mice are commonly used inbred strains to assess influenza virus pathogenicity. BALB/c mice infected with pH1N1 influenza viruses presented increased pathogenicity compared with seasonal influenza, but lethality has not been observed and no remarkable differences in virulence among circulating 2009 pH1N1 viruses have been reported for this strain.[14,15]

To identify the role of host genetic determinants on influenza pathogenicity, we performed a comparative analysis of the pathogenesis and host innate immune responses to seasonal H1N1, 2009 pH1N1, and human H5N1 influenza viruses in experimental BALB/c and C57BL/6J mouse models. In this study, we report, for the first time to our knowledge, a marked difference in infection dynamics of 2009 pH1N1 influenza in C57BL/6J mice with severe and prolonged infection in this strain.

Materials and Methods

Cells and Viruses

Madin-Darby canine kidney cells were grown in minimal essential medium (PAA, Linz, Austria) supplemented with 10% fetal calf serum (PAA), 1% glutamine (PAA), and 1% penicillin-streptomycin (PAA). A549 (human lung carcinoma) cells were grown in Dulbecco's modified Eagle's medium (PAA) supplemented with 10% fetal calf serum (PAA), 1% glutamine (PAA), and 1% penicillin-streptomycin (PAA).

The 2009 pH1N1 viruses A/Hamburg/05/09 (HH05) and A/Hamburg/NY1580/09 (HH15) were isolated from pharyngeal swabs of patients before oseltamivir treatment. HH05, HH15, A/Solomon Islands/3/06-like (H1N1) (seasonal H1N1), and A/Thailand/KAN-1/04 (H5N1) (human H5N1; isolated from a fatal human case[17]) were grown on Madin-Darby canine kidney cells. All virus strains were passaged only one to two times in Madin-Darby canine kidney cells for virus stock generation and sequenced thereafter to exclude potential mutations upon multiple passaging. The seasonal H1N1 isolate was provided by Armin Baillot (Niedersächsisches Landesgesundheitsamt, Hannover, Germany), and the H5N1 isolate was provided by Hans-Dieter Klenk (Institute of Virology, Philipps University of Marburg, Marburg, Germany). pH1N1 clinical samples tested negative for human herpes viruses (ie, herpes simplex virus types 1 and 2, varicella zoster virus, cytomegalovirus, and Epstein-Barr virus), human enteroviruses, influenza B virus, and human parechovirus by PCR. All experiments with the pH1N1 and H5N1 influenza viruses were performed at a biosafety level 3 facility at the Heinrich-Pette-Institute, Leibniz Institute of Experimental Virology, Hamburg, Germany.

Clinical Information from 2009 Pandemic H1N1 Influenza Viruses Isolated from Patients

HH05 (pH1N1) was isolated from a 22-year-old female patient who returned from Mexico in March 2009. The patient was hospitalized for 6 days and received oseltamivir treatment immediately after admission to the University Hospital Eppendorf, Hamburg. HH15 (pH1N1) was isolated from a 29-year-old male patient who was a visitor from the United States in June 2009. The patient was hospitalized for 5 days and received oseltamivir treatment immediately after admission to Asklepios Clinics, Hamburg.

Both patients presented general flulike symptoms, such as headache, cough, sore throat, myalgia, malaise, and fever (maximum temperature, 39.8°C), and showed high levels of complement-reactive protein.

Animal Experiments

The animal experiments were performed according to the guidelines of the German animal protection law. All animal protocols were approved by the relevant German authorities. BALB/c and C57BL/6J mice were bred and housed at the institutional facilities under specific pathogen-free conditions or obtained from Charles River Laboratories (Sulzfeld, Germany). Mice (aged 4 to 8 weeks) were anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively) and inoculated intranasally with 50 μL of virus diluted in PBS. Mice were infected with 10^5 plaque-forming units (PFU) of seasonal H1N1, 10^5 PFU of HH05 (pH1N1), 10^5 PFU of HH15 (pH1N1), or 10^2 PFU of human H5N1 isolate. Control groups received PBS. Animals were observed for 14 days for weight loss and survival. On days 3 and 6 postinfection (p.i.), three animals per time point were sacrificed, organs (ie, lung, brain, and gut) were removed, and virus titers were determined by plaque assays, as previously described.[18]

Mouse lethal dose 50 (MLD_{50}) was assessed by infecting mice with serial 10-fold virus dilutions and calculated as previously described.[19]

Automated Blood Cell Counts

Blood samples from three infected animals per time point were collected, and automated blood cell counts on whole blood were analyzed with pocH 100iV Diff (Sysmex, Norderstedt, Germany). Frequencies of white blood cells, red blood cells, Hb, hematocrit, platelets, lymphocytes, and granulocytes were measured. Recommended settings and calibrations for mouse strain–specific hematology were set according to the manufacturer's protocol.

IHC Stainings

The lungs and guts of three infected animals at 6 days p.i. were processed for immunohistochemistry (IHC). For IHC stainings, deparaffinized tissues were pretreated with 0.1 mol/L citrate buffer (pH 6.0) and incubated with a polyclonal ferret antibody raised against A/Vic/3/75 influenza virus (H3N2) (1:500; provided by the World Health Organization). The primary antibody cross-reacts with the nucleoprotein of several influenza virus subtypes, as previously described.[20,21] As a secondary antibody, a biotinylated anti-ferret antibody (1:200; Rockland, Gilbertsville, PA) was used, followed by the application of the Zytochem-Plus HRP kit and AEC as substrate (Zytomed, Berlin, Germany) under the conditions described by the manufacturer. Controls using normal rat serum were run to exclude nonspecific staining. Tissues were counterstained with hematoxylin, as previously described.[18]

Cytokine Assays

Cytokine levels were measured from pooled samples (three animals for lung homogenates and four to seven animals for serum specimens) by enzyme-linked immunosorbent assay for macrophage chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-4, IL-6, and IL-10 using the mouse Quantikine assay (R&D Systems, Minneapolis, MN) or the mouse Legend Max (BioLegend, San Diego, CA), according to the manufacturer's instructions. The mean titers of two to four experiments are presented. Cytokine detection limits were as follows: 2 pg/mL for MCP-1, 1.5 pg/mL for TNF-α, 8 pg/mL for IFN-γ, 0.5 pg/mL for IL-4, 1.6 or 2 pg/mL for IL-6, and 4 or 28.8 pg/mL for IL-10.

Sequencing of HH05 and HH15

The gene segments of the 2009 pH1N1 isolates (ie, HH05 and HH15) were sequenced as previously described[22] and deposited in GenBank as follows: HH05, accession no. HQ111361 (PB2), HQ111362 (PB1), HQ111363 (PA), HQ111365 (NP), HQ111364 (HA), HQ111366 (NA), HQ111367 (M), and HQ111368 (NS); and HH15, accession no. GU480807 (PB2), HQ104924 (PB1), HQ104925 (PA), HM598305 (NP), HQ104926 (HA), HQ104927 (NA), HQ104928 (M), and HQ104929 (NS).

Growth Curves

A549 cells were infected with seasonal H1N1, HH05, and HH15 (pH1N1) at a multiplicity of infection of 0.01 and human H5N1 at a multiplicity of infection of 0.001. At 24, 48, 72, and 96 hours p.i., supernatants were collected and plaque titers were determined on Madin-Darby canine kidney cells.[18] The growth curves shown are the average result of two independent experiments.

Results

Pathogenicity of Seasonal H1N1, 2009 Pandemic H1N1, and Human H5N1 Influenza A Viruses in BALB/c and C57BL/6J Mice

BALB/c mice infected with seasonal H1N1 influenza virus had marginal weight loss, and all animals survived (Figure 1, A and B). Infection with HH05 and HH15 led to 10% to 15% weight loss, and 93% survived (Figure 1, A and B). In contrast, all animals infected with human H5N1 influenza succumbed to infection, with severe weight loss (Figure 1, A and B).

Similar to the BALB/c model, all C57BL/6J mice infected with seasonal H1N1 influenza survived, with no significant weight loss (Figure 1, C and D). In contrast to BALB/c, infection of C57BL/6J mice with HH05 and HH15 displayed remarkable differences in pathogenicity. HH05-infected animals experienced 66% survival, whereas HH15 infection was 100% lethal (Figure 1C). HH15-infected animals lost more weight than HH05-infected mice (Figure 1D). All H5N1-infected animals succumbed to infection, with severe weight loss (Figure 1, C and D).

To assess the virulence of the influenza strains in both experimental mouse models, we determined the MLD_{50} in BALB/c and C57BL/6J mice (Table 1). Seasonal H1N1 is not lethal in BALB/c ($logMLD_{50}$ >6 PFU) and C57BL/6J ($logMLD_{50}$ >6 PFU) mice. HH15 is low pathogenic in BALB/c mice ($logMLD_{50}$ >6 PFU) but highly virulent in C57BL/6J mice ($logMLD_{50}$ = 3.5 PFU). HH15 is more virulent than HH05 ($logMLD_{50}$ = 5.2 PFU) in C57BL/6J mice. Both HH05 and HH15 are low pathogenic in BALB/c mice ($logMLD_{50}$ >6 PFU). H5N1 influenza is even more virulent for BALB/c mice ($logMLD_{50}$ = 0.3 PFU) than C57BL/6J mice ($logMLD_{50}$ = 1.8 PFU). Thus, increased virulence of H5N1 influenza virus for BALB/c mice becomes visible, in contrast to Figure 1A, where the infection dose used was six times higher in terms of MLD_{50} for BALB/c than C57BL/6J mice.

In summary, seasonal H1N1 influenza is not lethal for both BALB/c and C57BL/6J mice. H5N1 influenza is more pathogenic for BALB/c than C57BL/6J mice. In contrast, C57BL/6J animals are more susceptible to 2009 pH1N1 influenza viruses displaying differential pathogenicities, which is not observed in BALB/c mice. Our data further suggest that host determinants contributing to 2009 pH1N1 pathogenesis are different from those responsible for H5N1 pathogenesis.

Figure 1. Pathogenicity in BALB/c and C57BL/6J mice. BALB/c (**A** and **B**) or C57BL/6J (**C** and **D**) mice were infected with 10^5 PFU of seasonal H1N1 ($n = 14$), HH05 (pH1N1) ($n = 21$), and HH15 (pH1N1) ($n = 21$) and with 10^2 PFU of human H5N1 ($n = 20$) and observed for survival and weight loss for 14 days. Survival and weight loss are presented for infected BALB/c (**A** and **B**, respectively) and C57BL/6J (**C** and **D**, respectively) mice. Control groups received PBS.

Elevated Virus Lung Titers Correlate with Enhanced Virulence in BALB/c but Not in C57BL/6J Mice

We then analyzed organ titers of seasonal H1N1, HH05, and HH15 and human H5N1 influenza virus–infected BALB/c and C57BL/6J mice (Figure 2, A and B).

At 3 days p.i., no significant differences in lung titers among seasonal H1N1, HH05, HH15, or H5N1 influenza virus–infected BALB/c mice were observed (Figure 2A). At 6 days p.i., lung titers were lowest in BALB/c mice infected with seasonal H1N1, followed by HH05 influenza. The highest lung titers were detected in animals infected with HH15 and H5N1. While infection with seasonal H1N1, HH05, and HH15 was mainly restricted to the lung, H5N1 infection spread to extrapulmonary organs, such as the brain and gut of BALB/c mice (Figure 2A).

In C57BL/6J mice, on days 3 and 6 p.i., no significant differences in the overall lung titers were observed in seasonal H1N1-, HH05-, HH15-, and H5N1-infected mice (Figure 2B). However, significantly high virus titers were detected in the gut of HH15-infected mice on day 6 p.i., but not in seasonal H1N1- or HH05-infected animals (Figure 2B).

Taken together, levels of virus lung titers correlate with the grade of virulence in infected BALB/c mice, suggesting that virus lung titers are an important marker to predict lethality of a virus strain in this host. In contrast, virus lung titers do not correlate with enhanced lethal outcome in C57BL/6J mice. This suggests that, in C57BL/6J mice, additional host factors contribute to lethal outcome.

Table 1. MLD_{50} in BALB/c and C57BL/6J Mice

Mice	MLD_{50} (log PFU)			
	Seasonal H1N1	HH05 (pH1N1)	HH15 (pH1N1)	Human H5N1
BALB/c	>6	>6	>6	0.3
C57BL/6J	>6	5.2	3.5	1.8

BALB/c and C57BL/6J mice were infected with serial 10-fold virus dilutions (10^6 to 10^0 PFU) of seasonal H1N1, HH05 (pH1N1), and HH15 (pH1N1) and human H5N1 and observed for 14 days p.i. The MLD_{50} was calculated as described by Reed and Muench.[19]

Similar Lung Pathological Features and Virus Tropism in BALB/c and C57BL/6J Mice

BALB/c mice (Figure 3, A–E) infected with seasonal H1N1 influenza (Figure 3B) presented only few local mononuclear infiltrations and single virus-infected cells compared with uninfected controls (Figure 3A). HH05-infected BALB/c mice (Figure 3C) revealed comparably more infected alveolar epithelial cells in association with inflammatory cells than those infected with seasonal H1N1. More virus-positive alveolar epithelial cells, relatively more plasma cells and macrophages, and extended capillaries were found in HH15-infected mice (Figure 3D) compared with HH05-infected animals. The H5N1-infected animals (Figure 3E) presented the most virus-positive alveolar and bronchial epithelial cells, with

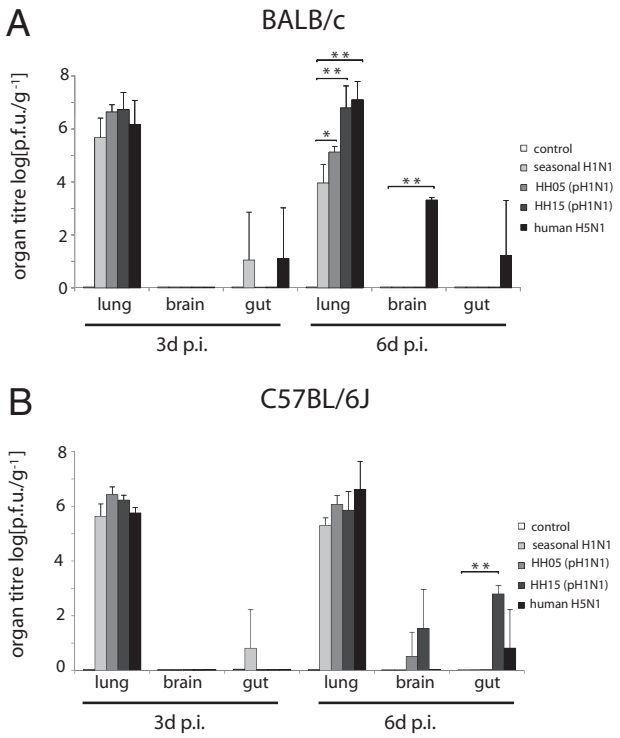

Figure 2. Organ tropism in BALB/c and C57BL/6J mice. BALB/c (**A**) or C57BL/6J (**B**) mice were infected with 10^5 PFU of seasonal H1N1, HH05, and HH15 (pH1N1) and with 10^2 PFU of human H5N1. Virus titers in lung, brain, and gut homogenates of three animals per time point were determined by plaque assays on day 3 or 6 p.i. No virus was detected in uninfected control groups. The statistical significance of differences in virus titers was calculated by the Student's *t*-test (*$P < 0.05$ and **$P < 0.01$).

pathological features comparable to HH15-infected mice revealing diffuse hemorrhages.

C57BL/6J mice (Figure 3, F–J) infected with seasonal H1N1 (Figure 3G) presented focal areas with mononuclear infiltrates compared with uninfected controls (Figure 3F) and single virus-positive alveolar and bronchial epithelial cells. HH05-infected animals presented more infected alveolar epithelial cells and mononuclear inflammatory areas with extended capillaries (Figure 3H) compared with animals infected with seasonal H1N1. Lung pathological features were more severe in HH15- than in HH05-infected mice, with numerous virus-positive bronchial and alveolar epithelial cells and inflamed and destroyed alveolar structures (Figure 3I). Severe diffuse alveolar destruction and hemorrhages with numerous infected alveolar epithelial cells were observed in H5N1-infected mice (Figure 3J).

In summary, no differences in lung pathological features and virus tropism are observed with the respective virus strain in both mouse models. Seasonal H1N1 leads to marginal inflammation, followed by HH05 infection. HH15 and H5N1 viruses cause severe pneumonia in both mouse models. These findings further suggest that, in addition to the virus ability to replicate in the lung, other host factors contribute to the differential pathogenicity in BALB/c and C57BL/6J mice.

Enhanced Viral Pathogenicity Correlates with Lymphopenia in the Respective Mouse Model

Blood cell parameters were compared for infection of BALB/c and C57BL/6J mice (Table 2 and Table 3, respectively).

In BALB/c mice (Table 2) infected with seasonal H1N1, HH05, and HH15, white blood cells slightly decreased at 3 days p.i. but increased again at 6 days p.i., comparable to uninfected controls. White blood cell counts were significantly reduced in H5N1-infected animals at 6 days p.i. ($P < 0.01$) but not in HH05- or HH15-infected animals compared with seasonal H1N1–infected groups. Hb levels were significantly reduced in H5N1-infected mice at 3 days p.i. ($P < 0.02$). Furthermore, in H5N1-infected animals, hematocrit levels were significantly elevated at 3 days p.i. ($P < 0.02$) and 6 days p.i. ($P < 0.01$). Severe lymphopenia, with a decrease of >80% in lymphocyte counts, was observed in BALB/c mice infected with human H5N1 influenza at 6 days p.i. ($P < 0.002$). No significant differences were detected in other parameters assessed in BALB/c mice.

In C57BL/6J mice (Table 3) infected with HH05 and HH15, white blood cell counts significantly decreased at 3 days p.i. ($P < 0.01$ and $P < 0.006$, respectively) compared with seasonal H1N1 influenza–infected animals. In HH05-infected animals, white blood cells increased again at 6 days p.i., similar to seasonal H1N1–infected mice and uninfected controls. In contrast, HH15-infected mice presented prolonged reduced white blood cells at 6 days p.i. ($P < 0.005$), similar to H5N1-infected animals ($P < 0.009$). Accordingly, HH05- and HH15-infected mice presented significantly reduced lymphocytes at 3 days p.i. ($P < 0.009$ and $P < 0.002$, respectively) compared with seasonal H1N1-infected animals. In HH05-infected animals, lymphocyte levels increased again at 6 days p.i., similar to seasonal H1N1-infected groups and uninfected controls. HH15-infected mice presented severe lymphopenia, with a decrease in lymphocyte counts by >50%, comparable to H5N1-infected animals at 6 days p.i. ($P < 0.002$ and $P < 0.002$, respectively).

Taken together, these observations show that HH15 infection leads to prolonged lymphopenia in C57BL/6J mice, in contrast to HH05. H5N1 leads to severe lymphopenia in BALB/c mice and, to a lesser extent, in C57BL/6J mice. Thus, grade of lymphopenia correlates with lethal outcome in the given host.

Enhanced pH1N1 Pathogenicity Correlates with a Depressed Cytokine Response, whereas Increased H5N1 Pathogenicity Correlates with an Elevated Proinflammatory Cytokine Response

It was described before that BALB/c mice were genetically prone to mount a predominantly type 2 helper T-cell (Th2) response, whereas C57BL/6J mice generate a predominantly Th1 response.[23] Therefore, we determined local (Figure 4, A and B) and systemic (Figure 4, C and D)

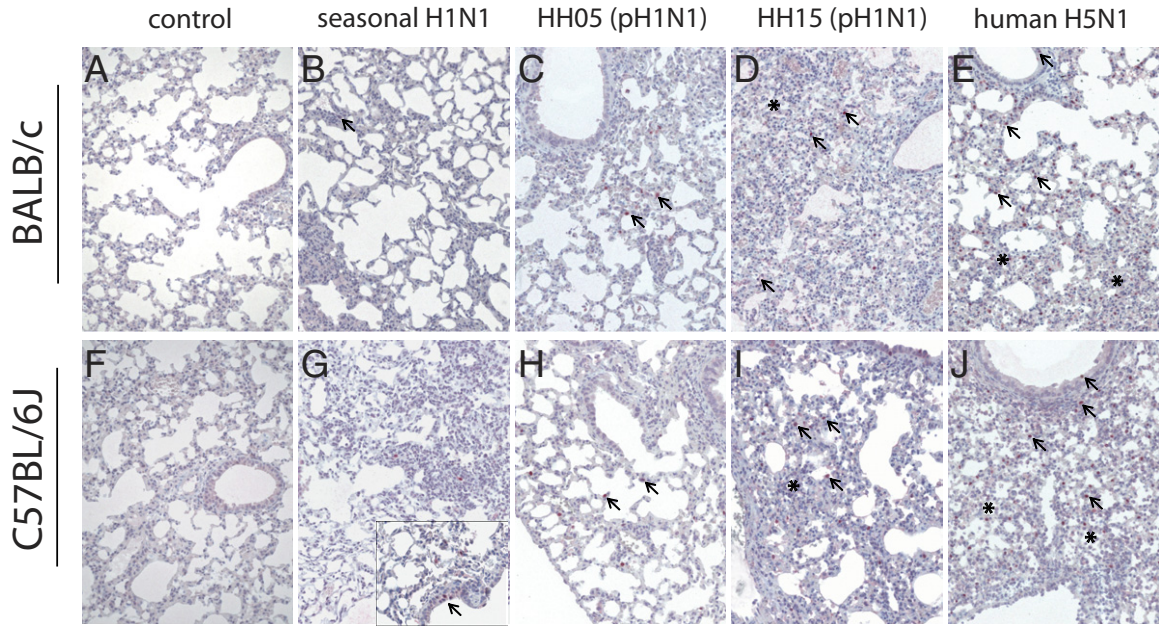

Figure 3. Lung pathological features in BALB/c and C57BL/6J mice. Mice were infected with 10^5 PFU of seasonal H1N1 (**B** and **G**), HH05 (**C** and **H**), and HH15 (**D** and **I**) (pH1N1) and with 10^2 PFU of human H5N1 (**E** and **J**). Control groups received PBS (**A** and **F**). IHC stainings were performed from the lungs of three infected BALB/c (**top**) and C57BL/6J (**bottom**) mice obtained on day 6 p.i. using a polyclonal antibody, as previously described.[18,21] Counterstaining with hematoxylin was performed as previously described.[18] Antigen-positive cells are red-brown. **Arrows** indicate some infected cells; and **asterisks**, largely destructed and infiltrated areas. Original magnification, ×400 (all images were obtained using a light microscope).

levels of Th1 cytokines (ie, TNF-α, IFN-γ, and MCP-1) and Th2 cytokines (ie, IL-4, IL-6, and IL-10)[23,24] in infected BALB/c and C57BL/6J mice (Figure 4).

Local cytokine levels in the lung revealed that BALB/c mice were generally able to mount cytokine responses of greater magnitude than C57BL/6J mice upon infection with HH05, HH15, or human H5N1 influenza viruses. No significant differences in the host innate immune response were observed in seasonal H1N1-infected BALB/c and C57BL/6J mice, with the exception of IFN-γ, which was significantly increased in BALB/c mice (Figure 4A). Levels of TNF-α, IFN-γ, and especially IL-10 were highly elevated in HH05- and HH15-infected BALB/c mice, in contrast to C57BL/6J mice. IL-4 levels were significantly increased in HH05-infected BALB/c compared with C57BL/6J mice. Interestingly, HH05 infection led to significantly higher induction of IL-10 compared with HH15 infection in BALB/c, but not in C57BL/6J, mice. In contrast to pH1N1 infections, no alterations in IL-4 and IL-10 levels were observed in H5N1-infected BALB/c or C57BL/6J mice (Figure 4B). Furthermore, in contrast to pH1N1 infections, H5N1-infected animals presented highly elevated proinflammatory cytokine levels of TNF-α, IFN-γ, MCP-1, and IL-6 in BALB/c mice and, to a lesser extent, in C57BL/6J mice. Systemic cytokine levels detected in the serum specimens of infected animals were generally lower compared with cytokine responses in the lungs (Figure 4, C and D). With the exception of IL-4, cytokine levels were either not detectable or slightly higher than detection limits in seasonal H1N1–infected BALB/c and C57BL/6J mice. A significantly elevated cytokine response was only detected for IFN-γ in HH05- and HH15-infected BALB/c mice, consistent with local lung response. Highest levels were detected for IFN-γ

Table 2. Blood Cell Count in BALB/c Mice Infected with 10^5 PFU of Seasonal H1N1, HH05 (pH1N1), and HH15 (pH1N1) and with 10^2 PFU of Human H5N1

Type of blood cell	Controls	Seasonal H1N1		HH05 (pH1N1)		HH15 (pH1N1)		Human H5N1	
		3 days p.i.	6 days p.i.	3 days p.i.	6 days p.i.	3 days p.i.	6 days p.i.	3 days p.i.	6 days p.i.
WBCs (10^3/mm^3)	7.3 ± 1.8	5.3 ± 1.1	7.3 ± 1.3	5.0 ± 1.4	7.4 ± 1.3	4.8 ± 0.4	7.8 ± 0.7	6.7 ± 1.0	2.5 ± 0.6*
RBCs (10^3/mm^3)	10.7 ± 0.7	9.4 ± 0.4	9.9 ± 0.1	10.4 ± 1.6	11.3 ± 0.8	10.8 ± 0.6	11.1 ± 0.3	11.4 ± 0.4	11.5 ± 0.7
Hb (g/dL)	19.0 ± 1.2	17.3 ± 1.0	17.4 ± 0.4	18.7 ± 2.4	19.9 ± 1.2	19.2 ± 1.0	19.8 ± 0.3	20.0 ± 0.7*	20.0 ± 1.6
HCT (%)	49.6 ± 1.7	46.4 ± 3.0	49.8 ± 0.3	48.0 ± 7.2	52.1 ± 3.9	49.7 ± 3.7	50.8 ± 1.6	52.9 ± 0.3*	54.8 ± 2.1*
PLTs (10^3/mm^3)	823 ± 227	1007 ± 218	967 ± 101	620 ± 410	778 ± 484	591 ± 180	598 ± 251	870 ± 308	1005 ± 25
LYMs (10^3/mm^3)	5.4 ± 1.1	3.6 ± 0.7	4.8 ± 1.0	3.5 ± 1.2	4.1 ± 1.2	3.2 ± 0.4	4.3 ± 0.8	4.0 ± 0.3	0.7 ± 0.0*
GRAs (10^3/mm^3)	1.9 ± 0.9	1.7 ± 0.5	2.5 ± 0.4	1.5 ± 0.3	3.3 ± 0.2	1.6 ± 0.0	3.5 ± 0.6	2.7 ± 0.7	1.8 ± 0.6

Data are given as the mean ± SD frequency using individual analysis. Blood samples were taken from three animals per time point, and automated blood cell counts were performed. Statistical analysis was performed with the Student's *t*-test.

*Asterisk indicates significance.

GRA, granulocyte; HCT, hematocrit; LYM, lymphocyte; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

Table 3. Blood Cell Count in C57BL/6J Mice Infected with 10^5 PFU of Seasonal H1N1, HH05 (pH1N1), and HH15 (pH1N1) and with 10^2 PFU of Human H5N1

Type of blood cell	Controls	Seasonal H1N1		HH05 (pH1N1)		HH15 (pH1N1)		Human H5N1	
		3 days p.i.	6 days p.i.	3 days p.i.	6 days p.i.	3 days p.i.	6 days p.i.	3 days p.i.	6 days p.i.
WBCs (10^3/mm^3)	9.4 ± 2.8	10.3 ± 1.2	9.8 ± 1.0	5.5 ± 1.4*	10.2 ± 0.5	5.9 ± 0.8*	5.8 ± 0.8*	10.3 ± 0.5	5.8 ± 1.1*
RBCs (10^3/mm^3)	9.8 ± 1.3	10.9 ± 0.3	9.3 ± 6.0	11.3 ± 0.8	9.9 ± 0.7	11.7 ± 1.0	11..3 ± 0.6	10.4 ± 0.1	10.4 ± 0.4
Hb (g/dL)	15.1 ± 2.0	16.8 ± 0.4	14.6 ± 0.7	17.7 ± 1.4	15.4 ± 0.8	18.5 ± 1.3	16.9 ± 0.6	16.2 ± 0.1	16.6 ± 0.6
HCT (%)	48.1 ± 6.9	47.6 ± 1.5	40.7 ± 2.0	48.6 ± 3.9	41.7 ± 2.9	50.9 ± 4.1	47.9 ± 2.0	45.3 ± 0.2	45.2 ± 1.3
PLTs (10^3/mm^3)	1051 ± 303	779 ± 260	1248 ± 76	1541 ± 172	1417 ± 289	1479 ± 85	1334 ± 223	1137 ± 105	1332 ± 262
LYMs (10^3/mm^3)	7.9 ± 2.3	8.2 ± 1.0	8.0 ± 0.8	3.8 ± 1.3*	6.1 ± 0.8	3.7 ± 0.7*	3.5 ± 0.7*	7.9 ± 1.1	3.6 ± 0.7*
GRAs (10^3/mm^3)	1.6 ± 0.6	2.1 ± 0.4	1.8 ± 0.2	1.6 ± 0.2	4.1 ± 0.8	2.2 ± 0.3	2.3 ± 0.6	2.3 ± 0.9	2.2 ± 0.4

Data are given as the mean ± SD frequency using individual analysis. Blood samples were obtained from three animals per time point, and automated blood cell counts were performed. Statistical analysis was performed with the Student's *t*-test.

*Asterisk indicates significance.

GRA, granulocyte; HCT, hematocrit; LYM, lymphocyte; PLT, platelet; RBC, red blood cell; WBC, white blood cell.

and IL-10 in H5N1-infected BALB/c mice and for MCP-1 in C57BL/6J mice (Figure 4, C and D).

Taken together, increased susceptibility observed in pH1N1-infected C57BL/6J mice correlates with significantly lowered production of Th1 and Th2 cytokines. In contrast, increased H5N1 pathogenicity in BALB/c mice correlates with elevated levels of proinflammatory cytokines, such as TNF-α, IFN-γ, MCP-1, and IL-6. These findings suggest that an inappropriate or absent cytokine response might significantly contribute to increased susceptibility of C57BL/6J mice to pH1N1 influenza.

Discussion

Herein, we analyzed the pathogenesis, virus replication, virus tropism, and innate immune responses in BALB/c and C57BL/6J mouse strains infected with two 2009 pH1N1 isolates (ie, HH05 and HH15), a seasonal H1N1, and a human H5N1 influenza virus isolated from a fatal case.[17] We show that C57BL/6J mice are more susceptible to pH1N1 influenza isolates, whereas BALB/c mice are mostly resistant. No differences in susceptibility were observed for seasonal H1N1 influenza in both mouse models. In contrast, H5N1 virus infection was more virulent in BALB/c than C57BL/6J mice.

Both of the clinical pH1N1 isolates (ie, HH05 and HH15) were obtained from patients who required hospitalization but recovered 5 to 6 days after admission to the hospital. However, it is difficult to assess the virulent potential of these isolates because both patients were treated with oseltamivir immediately after hospitalization. Both patients were healthy, without any underlying medical conditions. It is tempting to speculate about a potential more severe outcome in the absence of oseltamivir treatment or in patients with known risk factors for influenza. The viruses used in our experimental studies were isolated from patients before oseltamivir treatment.

The pathogenesis of 2009 pH1N1 isolates has been studied in mice by several groups. However, most of these studies[14,15,25] were performed in BALB/c mice; in these mice, the viruses replicated more efficiently compared with seasonal H1N1 influenza, but were generally not lethal. These data are consistent with our observations in BALB/c mice. Also, the pH1N1 isolate MX/4482, from a severe case, was not pathogenic in BALB/c mice ($\log MLD_{50}$ >6 PFU) but killed 50% of infected ferrets.[15] On the other hand, WSLH049 (pH1N1), isolated from a patient who required hospitalization, had a $\log MLD_{50}$ of 4.5 PFU in BALB/c mice but was not studied in other mammalian models.[14] The Neth/09 pH1N1 strain was previously shown to be lethal for C57BL/6 mice.[26] Recently, some 2009 pH1N1 strains presented heterogeneous virulence in a macaque model.[27] Clearly, more studies using several pH1N1 isolates from mild and severe cases are needed in BALB/c and C57BL/6J mice and other mammalian models to identify an adequate model for the studies of pH1N1 pathogenesis and their correlation with clinical outcomes in humans.

Previously described markers predictive of virus adaptation to humans were not observed in 2009 pH1N1 viruses, suggesting that unrecognized novel determinants are responsible for their increased pathogenicity in several animal models.[5] Growth kinetics in human lung cells revealed that HH15 grows to 10 times higher titers compared with HH05 (see Supplemental Figure S1 at *http://ajp.amjpathol.org*). However, the overall growth of pH1N1 isolates was similar to seasonal H1N1 influenza, albeit high titers were detected during later time points with HH15. In contrast, human H5N1 influenza growth exhibited pH1N1 and seasonal H1N1 virus titers by more than four magnitudes. The HH05 and HH15 viruses differ by 12 amino acid exchanges in the genes encoding for PB2, PA, NP, HA, NA, and NS1 (see Supplemental Table S1 at *http://ajp.amjpathol.org*). The contribution of these substitutions to increased virulence in C57BL/6J mice is being investigated. Recently, the importin-α7 gene has been shown to be a key regulator of 2009 pH1N1 but not H5N1 influenza pathogenicity in transgenic mouse models in mixed 129×1/SvJ-C57BL/6 backgrounds.[21] This further underlines that viral and host determinants responsible for H5N1 influenza pathogenicity are different from those determining 2009 pH1N1 influenza. Clearly, further studies are needed to identify viral and host determinants responsible for 2009 pH1N1 and human H5N1 influenza pathogenicity in experimental animal models.

We observed that virus infection was predominantly restricted to the lungs of HH05- and HH15-infected BALB/c mice, similar to seasonal H1N1 infection. In con-

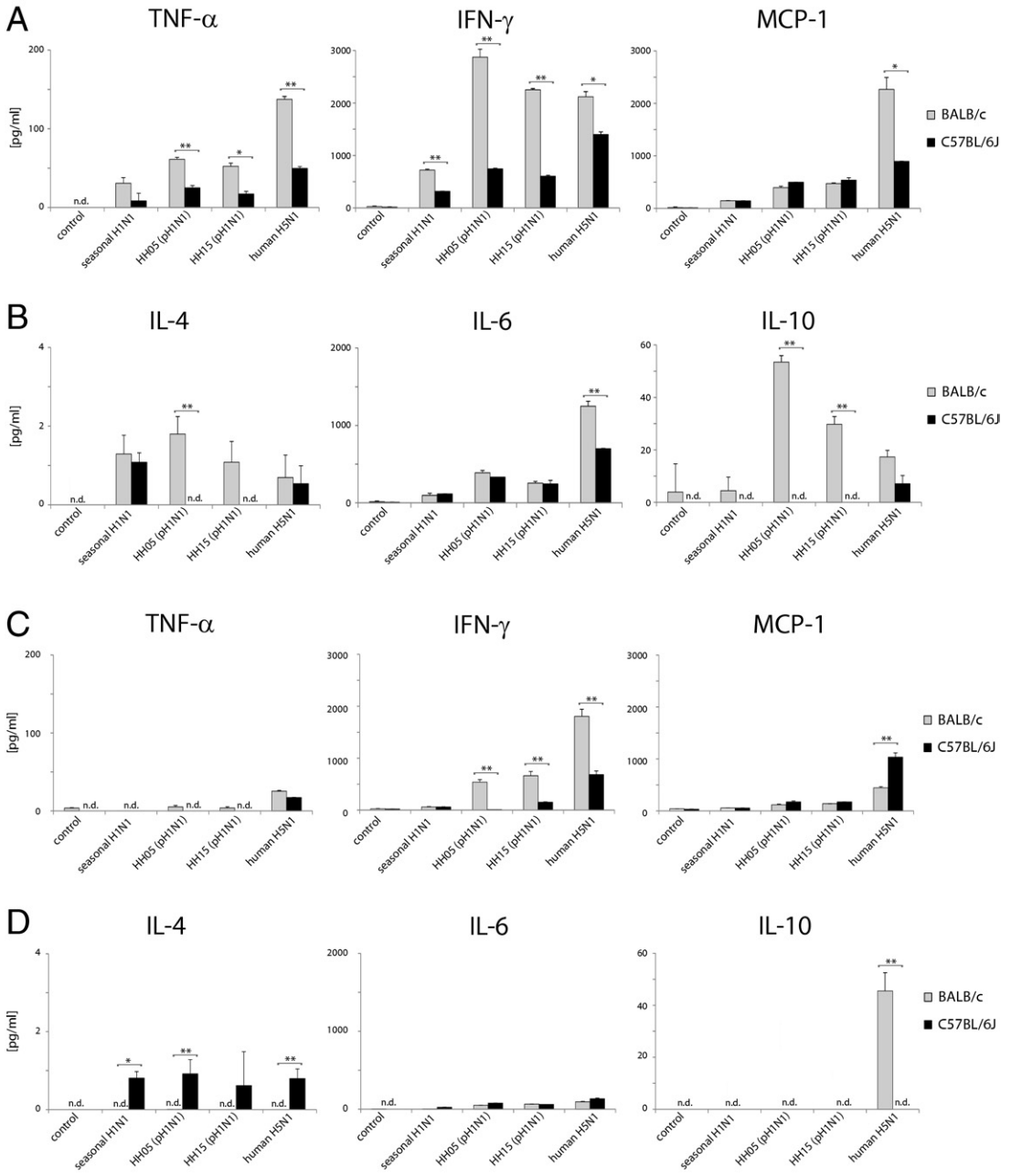

Figure 4. Cytokine response in BALB/c and C57BL/6J mice. BALB/c and C57BL/6J mice were infected with 10^5 PFU of seasonal H1N1, HH05, and HH15 (pH1N1) and with 10^2 PFU of human H5N1. Control groups received PBS. Levels were measured for Th1 cytokines (TNF-α, IFN-γ, and MCP-1) (**A** and **C**) and Th2 cytokines (IL-4, IL-6, and IL-10) (**B** and **D**) in BALB/c (gray bars) and C57BL/6J (black bars) mice by enzyme-linked immunosorbent assay. Bars represent mean ± SD cytokine concentrations of lung homogenates (**A** and **B**) from three animals or pooled serum samples (**C** and **D**) from four to seven animals on day 6 p.i. n.d. indicates not detected (ie, cytokine levels less than the detection limit). The statistical significance of differences in cytokine levels between BALB/c and C57BL/6J mice was calculated by the Student's *t*-test (*$P < 0.05$ and **$P < 0.01$).

trast, significantly high virus titers were detected in the gut of HH15-infected C57BL/6J mice. However, no replicating virus could be detected in the gut tissues of HH15-infected C57BL/6J mice by IHC staining (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). This suggests that extrapulmonary virus titers detected in pH1N1-infected C57BL/6J mice are likely representing different grades of viremia. Replicating virus in the gut was previously shown in cats fed with H5N1-infected chicks but not in animals infected via the respiratory tract.[28] This further suggests that, upon respiratory tract infection, highly pathogenic strains can spread through the blood stream, as was previously shown for H7N7 and H5N1 isolates by several groups.[18,28,29] The severity of lung damage observed correlated with lung titers detected in BALB/c mice. The lungs of HH15- and H5N1-infected animals were more severely infiltrated and even partially hemorrhagic compared with HH05 in BALB/c and C57BL/6J mice. Interestingly, the finding that HH15 causes more lung damage than HH05 was observed in both mouse

models. Furthermore, viral lung titers did not correlate with disease outcome in C57BL/6J mice, in contrast to BALB/c mice, because lung pathological features and virus tropism were similar in both mouse models. Thus, viral lung titers are not a reliable indicator of virus pathogenicity in C57BL/6J mice. Our findings strongly suggest that lethal outcome in C57BL/6J mice is not only affected by virus replication but also by other determinants (ie, host immune responses) significantly contribute to disease outcome.

Another important factor in pathogenesis is lymphopenia, which has been a hallmark for lethal influenza virus infection in our studies in the given host. HH15-infected animals underwent prolonged lymphocyte depletion in C57BL/6J mice, with a lethal outcome. pH1N1-caused lymphopenia was not previously reported because of the lack of an appropriate animal model to study pH1N1 pathogenesis. Accordingly, H5N1 infection also led to lymphopenia in C57BL/6J mice and more severely in BALB/c mice. These observations indicate that virulent pH1N1 isolates and H5N1 viruses use immunosuppression, resulting from lymphocyte depletion as a mechanism to fuel the infection process, albeit in different host genetic backgrounds.

BALB/c mice are genetically primed to mount a Th2 response, whereas C57BL/6J mice predominantly generate a Th1 response.[23] Therefore, we compared levels of Th1 with levels of Th2 cytokines[23,24] in lung homogenates from BALB/c and C57BL/6J mice. Th1-primed cells play a predominant role in cellular immune responses, whereas Th2-primed cells are important for the generation of a humoral immune response.[24] Th1 TNF-α, MCP-1, and Th2 IL-6 also have proinflammatory functions.[24] These cytokines play an important role in virus clearance but can be detrimental if they are excessively induced, as was previously described for H5N1.[30,31] After human H5N1 infection, Th1/Th2 hypercytokinemia and severe lymphopenia were observed in BALB/c mice and, to a lesser extent, in C57BL/6 mice. Similar observations were previously described for H5N1 and H7N7 influenza viruses in mice.[18,32] H5N1-induced hypercytokinemia and high virus load have been associated with lethal outcome in humans.[31] However, the absence of single cytokines [ie, chemokine ligand 2 (MCP-1), IL-6, and TNF-α] in single-knockout mice was not protective, but the double knockout of TNF receptor 1/2 reduced lethality in H5N1-infected mice,[33] suggesting that additional determinants contribute to H5N1 pathogenesis.

Remarkably, we observed that increased pH1N1 pathogenicity in C57BL/6J mice correlated with reduced Th1 and Th2 cytokines compared with BALB/c mice. In general, no significant differences in cytokine levels were observed between HH05- and HH15-infected C57BL/6J mice. In pH1N1-infected BALB/c mice, only systemic IFN-γ levels were increased, consistent with the local lung response. Interestingly, IL-10 and IFN-γ levels were increased in the lungs of HH05- and HH15-infected BALB/c mice and systemically in H5N1-infected BALB/c mice. However, controversial findings were reported on the role of IL-10 in influenza virus infection. Blocking the IL-10 response has increased morbidity and lung inflammation[34]; on the other hand, it may reduce mortality in influenza virus–infected mice.[35] The exact role of IL-10 in pH1N1 and H5N1 influenza virus infection needs further investigation to determine whether the host genetic background influences the beneficial or detrimental role of cytokine responses.

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