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Global Host Immune Response: Pathogenesis and Transcriptional Profiling of Type A Influenza Viruses Expressing the Hemagglutinin and Neuraminidase Genes from the 1918 Pandemic Virus

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To understand more fully the molecular events associated with highly virulent or attenuated influenza virus infections, we have studied the effects of expression of the 1918 hemagglutinin (HA) and neuraminidase (NA) genes during viral infection in mice under biosafety level 3 (agricultural) conditions. Using histopathology and cDNA microarrays, we examined the consequences of expression of the HA and NA genes of the 1918 pandemic virus in a recombinant influenza A/WSN/33 virus compared to parental A/WSN/33 virus and to an attenuated virus expressing the HA and NA genes from A/New Caledonia/20/99. The 1918 HA/NA:WSN and WSN recombinant viruses were highly lethal for mice and displayed severe lung pathology in comparison to the nonlethal New Caledonia HA/NA:WSN recombinant virus. Expression microarray analysis performed on lung tissues isolated from the infected animals showed activation of many genes involved in the inflammatory response, including cytokine, apoptosis, and lymphocyte genes that were common to all three infection groups. However, consistent with the histopathology studies, the WSN and 1918 HA/NA:WSN recombinant viruses showed increased up-regulation of genes associated with activated T cells and macrophages, as well as genes involved in apoptosis, tissue injury, and oxidative damage that were not observed in the New Caledonia HA/NA:WSN recombinant virus-infected mice. These studies document clear differences in gene expression profiles that were correlated with pulmonary disease pathology induced by virulent and attenuated influenza virus infections.

The influenza pandemic of 1918 to 1919 is one of the single deadliest infectious disease outbreaks in human history and was responsible for up to 40 million deaths worldwide, with over 600,000 deaths in the United States alone (59, 67). Amazingly, approximately 4 out of every 10 deaths of the U.S. troops engaged in World War I were the result of influenza virus infection. Another unusual feature of the 1918 pandemic was a disproportionate mortality rate in young adults (24, 64, 77). Modern histopathological analysis of fixed human lung tissues from 1918 influenza virus fatalities revealed significant damage to the lungs, with acute focal bronchitis and alveolitis that were often associated with massive pulmonary edema and hemorrhage and rapid destruction of the respiratory epithelium. The causes of the high lethality and profound lung pathology that were consequences of 1918 influenza cases remain largely unknown. The emergence of novel influenza viruses and associated epidemics in human populations is thought to occur largely from zoonotic transfer from animal strains (4, 43, 46, 63, 74–76), and additional influenza pandemics have occurred in 1957 with the "Asian" influenza virus (H2N2) and in 1968 with the "Hong Kong" influenza virus (H3N2), which were estimated to be responsible for approximately 70,000 and 34,000 additional influenza-related deaths, respectively, in the United States (49, 56, 60, 62, 78). This is further evidenced by the recent emergence of highly pathogenic avian H5N1 influenza viruses in Hong Kong in 1997, which showed unusually high lethality in humans. Fortunately, only a few cases of human infections occurred with these H5N1 viruses in 1997, 2003, and 2004. Even with the development of influenza vaccines, influenza viruses still account for approximately 36,000 deaths in the United States annually, and the with the constant threat of emerging highly pathogenic strains such as the H5N1 "Hong Kong" viruses, pandemic outbreaks remain a serious public health concern (15–17, 70).

Influenza A virus is a member of the *Orthomyxoviridae* family of segmented, negative-stranded RNA viruses, with a genome composed of eight segments encoding up to 11 proteins and spanning approximately 15,000 nucleotides (18, 41). The major antigenic sites of influenza A virus are the hemaggluti-

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nin (HA) and neuraminidase (NA) proteins, which are expressed as envelope glycoproteins (41). The HA gene encodes an approximately 62,000-Da protein that binds *N*-acetylneuraminic acid (sialic acid) moieties on target cell membrane proteins, resulting in viral attachment (41). The NA gene encodes an approximately 50,000-Da protein that is responsible for promoting release of virus from infected cells by catalyzing the hydrolysis of sialic acid residues that would otherwise cause virus aggregation in host cells (41). As HA is the major antigenic site for the production of neutralizing antibodies, influenza pandemics are correlated with dramatic changes in the HA gene segment through the process of antigenic shift (34, 48). Determination of the sequences of the 1918 influenza virus genes has allowed the reconstruction of recombinant influenza viruses expressing genes from the 1918 virus by using reverse genetics (8, 57, 58, 68). Recent work from our laboratories using such a surrogate system demonstrated that the NS1 protein of influenza A/Brevig Mission/1/18 virus expressed in the context of a recombinant influenza A/WSN/33 virus (WSN) was a more potent alpha/beta interferon (IFN-α/β) antagonist than the parental WSN NS1 protein in infected human respiratory epithelial (A549) cells (29). In addition to the IFN-antagonistic properties of the NS1 protein, the surface glycoproteins of influenza virus (HA and NA) have been shown to be important virulence factors in mice and birds (30, 31, 33, 41, 52, 61, 65, 71, 73). As the HA and NA sequences of the 1918 virus have been elucidated (57, 58), we next determined the contribution of the 1918 HA and NA genes to viral pathology in the mouse model by examining infection outcome, lung histology, and gene expression changes by expression microarray analysis.

For these studies, we have used recombinant WSN influenza viruses that express the HA and NA genes of the 1918 pandemic influenza virus described previously (69). Our previous studies showed that 1918 HA/NA:WSN and parental WSN recombinant viruses were both highly pathogenic in mice (50% lethal dose [LD_{50}], $<10^3$ PFU), while a control WSN virus expressing the HA and NA genes of the human influenza A/New Caledonia/99 H1N1 virus was significantly attenuated (LD_{50}, $>10^6$ PFU). Histology performed on the lungs of infected animals demonstrated that the 1918 and WSN recombinant viruses caused significantly more severe pathology in mice than the New Caledonia HA/NA:WSN recombinant virus. Expression microarray analysis performed on RNA isolated from whole, infected lungs showed many common markers of viral infection, including the marked up-regulation of Stat1, interleukin-2 (IL-2) receptor, and IFN regulatory factor 1 (Irf1). Moreover, infection with highly virulent 1918 HA/NA:WSN and WSN recombinant viruses resulted in the significant up-regulation of many proinflammatory genes, activated lymphocyte genes, and stress-induced genes.

MATERIALS AND METHODS

Recombinant influenza viruses. Recombinant wild-type WSN, 1918 HA:NA/WSN, and New Caledonia HA:NA/WSN viruses were previously described (69). The 1918 HA and NA genes correspond to the published sequences of the influenza A/South Carolina/1/18 virus (H1N1) HA open reading frame (57) and the influenza A/Brevig Mission/1/18 virus (H1N1) NA open reading frame, respectively (58). The noncoding regions of each segment are identical to those of the corresponding segments of influenza A/WSN/33 virus (H1N1). All experiments with live virus were performed under biosafety level 3 (agricultural) (BSL-3Ag) containment (7).

Mouse experiments. Male BALB/c mice, 6 to 7 weeks old (Simonsen Laboratories, Gilroy, Calif.), were anesthetized with ketamine-xylazine (1.98 and 0.198 mg per mouse, respectively) and inoculated intranasally with the indicated virus dose. Mice were housed in cages inside stainless steel isolation cabinets that were ventilated under negative pressure with HEPA-filtered air. All animal work was performed in specially separated negative-pressured, HEPA-filtered rooms within the larger BSL-3Ag building. All personnel wore half-body Rocal hoods with backpack HEPA-filtered air supplies.

Histopathology. Five mice from each group were euthanatized at 6, 24, and 72 h postinfection (p.i.). A 5-mm lung piece from the ventral end of each left lobe was collected for histopathology. By using standard procedures, tissues were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. The results were scored as follows: 1, no lesion; 2, mild lesion; 3, moderate lesion; 4, severe lesion. Scores obtained by blind screening of the samples by three pathologists (D.E.S., R.M.P., and J.K.T.) were averaged (see Table 1). Duplicate sections were stained by immunohistochemistry methods to determine influenza virus antigen distribution in lung tissues, as described previously (53).

Total RNA isolation and RNA amplification. Lungs were received frozen in individual tubes, stored in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M β-mercaptoethanol) (21). They were stored in house at −70°C and thawed on ice prior to RNA extraction. Each tissue sample was homogenized for at least 30 s (or until all solid material was homogenized) with a Kinematica Polytron PT1200 instrument. The homogenized material was transferred to a fresh 15-ml tube and brought to 6.5 ml with ice-cold solution D. Added to this were 650 μl of 2 M sodium acetate, 6.5 ml of water-saturated phenol, and 1.3 ml chloroform-isoamyl alcohol (49:1). Tubes were vortexed and gently rocked for 10 min at room temperature. The mixture was then centrifuged for 30 min at 8,500 rpm and 4°C by using a Beckman L8-80 M ultracentrifuge with a JA18 rotor. The resulting aqueous layer was transferred to a new tube and precipitated with an equal volume of cold isopropanol at −20°C overnight. Centrifugation was performed again as described above. The resulting pellet was rinsed in 75% ethanol and resuspended in 150 μl of RNase-free water. A Beckman Coulter DU 640B spectrophotometer was used to quantify total RNA. One hundred micrograms of total RNA was purified with an RNA-Easy column according to the manufacturer's specifications. A Hewlett-Packard Kayak XM600 bioanalyzer was used to check the purity of the RNA prior to amplification. One round of RNA amplification was performed for each infection sample with a RiboAmp kit (Arcturus KIT0201) to generate amplified RNA (aRNA), according to the manufacturer's specifications. Capillary gel electrophoresis (Hewlett-Packard Kayak XM600 bioanalyzer) was used to check the purity of the aRNA prior to probe labeling.

Probe labeling and cDNA array slide hybridization. Fluorescent cDNA probes were prepared as previously described (27–29). Briefly, approximately 3 μg of aRNA was used to generate Cy3- and Cy5-labeled cDNA probes. The aRNA was incubated at 70°C for 10 min in the presence of 250 ng of random 9-mers (catalog no. 10336022; Invitrogen) per μl and 2 μM dT and then placed on ice for 30 s. The tubes were incubated for 10 min at room temperature in the presence of 1× enzyme buffer (catalog no. 18064-014; Invitrogen); 10.5 mM DTT; 263 μM dATP, dGTP, and dTTP; 52.6 μM dCTP; RNasin (1:38) (catalog no. N2511; Promega); and 52.6 μM Cy3- or Cy5-labeled dCTP (catalog no. PA53021 and PA55021; Amersham). Superscript III (Invitrogen) was then added (1:20), and the reaction mixture was incubated in the dark for 4 h at 42°C. The aRNA portion of the resulting cDNA-aRNA hybrid was degraded by adding 5 N NaOH and incubating for 10 min at 37°C, followed by treatment with 2 M MOPS (morpholinepropanesulfonic acid) to neutralize the reaction. The cDNA probe was purified by using a 96-well plate (Millipore MAFBNOB50) pretreated with 100 μl of binding buffer (150 mM potassium acetate, 5.3 M guanidine-HCl [pH 4.8]). Two elution steps with 10 mM Tris (pH 8.0) were performed. cDNA yield and Cy3 and Cy5 dye incorporation were measured with a Shimadzu UV-1601 spectrophotometer and corresponding probe samples normalized based on cDNA concentrations. The samples were then passed through a G50 column to remove unincorporated dye and other impurities. Probe samples were dried for 90 min at 50°C with a Savant SPD111V Speed-Vac. Mouse cDNA arrays were obtained from the University of Washington Center for Expression Arrays (see below). The arrays were pretreated by dipping in water and drying quickly with wall air. Probe samples were resuspended in 25 μl of warmed hybridization buffer [50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 100 μg of CotI DNA per ml, and 20 μg of poly$(A)_{72}$]. The probe was boiled for 3 min and placed on ice. Appropriate probe samples were combined, and 50 μl was used to

hybridize each slide. Slides were incubated in a dark, humid chamber for 16 h at 42°C. The slides were washed once in prewarmed 1× SSC plus 0.2% SDS (10 min with rocking), twice in prewarmed 0.1× SSC plus 0.2% SDS (10 min each with rocking), and twice in 0.1× SSC at room temperature (1 min each with rocking) and then dipped twice in distilled water. Slides were dried by using wall air and submitted to the University of Washington Center for Expression Arrays to be scanned with a scanner (Molecular Dynamics). Raw data were combined and processed by using the in-house programs Spot-on Image and Expression Array Manager.

Expression microarray and statistical analysis. Microarrays were constructed at the University of Washington Center for Expression Array Technology with 6,913 unique PCR products generated from sequence-verified IMAGE clones of the National Institute of Aging (NIA) murine 15k gene set, which can be found at http://ra.microslu.washington.edu/genelist/documents/mouse/Mouse_1-18_control_MU-OD-1_020502.htm. Briefly, a single experiment comparing two samples was performed by using the dye label reverse technique with hybridization to the cDNA microarrays, allowing for the calculation of mean ratios between expression levels of each gene in the analyzed sample pair, standard deviations, and *P* values for each experiment. All data were entered into a custom-designed Oracle 9i-backed relational database, Expression Array Manager, and were then uploaded into Resolver System 3.0 (Rosetta Biosoftware, Kirkland, Wash.) and Decision Site (Spotfire, Somerville, Mass.). Primary analysis of expression microarray data was performed by using Resolver, with supplemental analysis and figure preparation with the SpotFire Decision Site 7.1.1. Data normalization and the Resolver System error model specifically developed for our slide format are described on the website http://expression.microslu.washington.edu. This website is also used to publish all primary data in accordance with the proposed standards (12).

RESULTS

Virus construction and characterization. Recombinant WSN viruses were created by using reverse genetics, and viruses expressing the HA and NA genes from the 1918, New Caledonia, or parental WSN influenza virus were characterized as previously described (69). Sequence identity of all of the viruses was confirmed by reverse transcription-PCR (data not shown). The New Caledonia HA and NA sequences were chosen as controls because they represent genes from a recent human H1N1 virus. Previous studies demonstrated that the 1918 HA/NA:WSN and parental WSN viruses were highly pathogenic in mice, with an LD_{50} of 2.75 (expressed as the log of the PFU required to give 1 LD_{50}), compared to an LD_{50} of >6 for the New Caledonia HA/NA:WSN virus (69). In this study, mice were infected intranasally with 10^6 PFU of virus and body weight was measured daily for days 1 to 5 (data not shown). Mice infected with both the 1918 HA/NA:WSN and parental WSN viruses showed dramatic weight loss between days 2 and 5 p.i. (~25% by day 5), while mice infected with the New Caledonia recombinant HA/NA virus showed a markedly smaller loss of body weight (10% by day 5). Additional infected mice (six per group) were observed for 14 days, and mice infected with the 1918 HA/NA:WSN and parental WSN viruses showed 100% mortality by day 9 p.i. In contrast, mice infected with the New Caledonia HA/NA:WSN recombinant virus showed 0% mortality. To investigate further the differences in pathogenesis of the recombinant H1N1 viruses, we examined lung pathology, distribution of virus antigens, and overall gene expression in lung tissue isolated at 24 and 72 h p.i.

Mouse lung histopathology. The histopathology data as determined in coded fashion by three independent pathologists are summarized in Table 1. Mock-inoculated mice lacked lesions in the lungs, except for a few foci of purulent bronchitis consistent with inhaled keratin (hair) foreign body (Fig. 1A). Mice infected with New Caledonia HA/NA:WSN recombinant virus had minimal to moderate necrotizing bronchitis with lymphocytic-to-histiocytic peribronchitis (Fig. 1B), and the alveoli adjacent to affected bronchi showed mild histiocytic inflammation. Mice infected with either the WSN or 1918 HA/NA:WSN recombinant virus had similar pulmonary lesions consisting of moderate to severe necrotizing bronchitis that was peribronchial to diffuse, moderate-to-severe histiocytic alveolitis with associated moderate-to-severe pulmonary edema, and some alveolar neutrophils (Fig. 1C and D). With individual WSN- and 1918-infected mice, alveolitis varied from mild to severe and the distribution varied from a single focus to affecting the entire lung lobe. However, at 72 h p.i. differences between the 1918 HA/NA:WSN and WSN infections could be observed, including a more prominent neutrophilic component of the alveolitis in the 1918 HA/NA:WSN-infected mice (reflecting a slightly higher histopathology score, as seen in Table 1). Additionally, the lungs of several of the parental WSN-infected mice showed some evidence of resolving bronchopneumonia at 72 h p.i., although the tissue damage would have ultimately been fatal in these animals. In contrast, the 1918 HA/NA:WSN-infected mice showed active diffuse pneumonitis in aggregate, suggesting that the 1918 HA/NA:WSN virus maintains an active inflammatory process longer than the recombinant wild-type WSN virus. For mice infected with New Caledonia HA/NA:WSN recombinant virus, influenza virus antigen was frequently demonstrated by immunohistochemical staining in bronchial epithelial cells and associated luminal inflammatory cells but was rarely seen in alveolar macrophages (data not shown). By contrast, lungs from WSN- and 1918 HA/NA:WSN-infected mice had influenza virus antigen commonly in necrotic cellular debris within bronchial lumina and alveoli and frequently within alveolar macrophages. Such influenza virus antigen was most intensely seen in the 1918 HA/NA:WSN-infected mice (data not shown). To better understand the mechanisms responsible for these dramatic differences in lung pathology between the attenuated New Caledonia HA/NA:WSN and lethal 1918 HA/NA:WSN and parental WSN recombinant viruses, the genetic basis of these responses was next determined by transcriptional profiling of whole lungs from infected mice.

TABLE 1. Average histopathology scores for lungs from mice inoculated with H1N1 recombinant influenza viruses

Time (h)	Avg score (n = 5)[a] for the following infection group:			
	Mock	New Caledonia HA/NA	WSN HA/NA	1918 HA/NA
24	1.0 (0)	2.8[b] (4)	2.7 (5)	2.3 (4)
72	1.4 (2)	2.2 (4)	2.4 (4)	3.1 (5)

[a] 1, no lesion; 2, mild lesion; 3, moderate lesion; 4, severe lesion. Numbers in parentheses indicate the number of animals in that group with pathological signs of infection.

[b] One mouse had foreign keratin body identified with purulent bronchitis and was excluded.

Overview of expression microarray analysis. To identify changes in gene expression associated with the increased severity of necrotizing bronchitis and alveolitis observed in mouse infections with the 1918 and WSN recombinant viruses relative to the New Caledonia virus, total RNAs were isolated from the lungs of five individual mice at 24 and 72 h postinoculation. The RNAs were quantified and analyzed for integrity, and equal masses of high-quality RNA from each infection

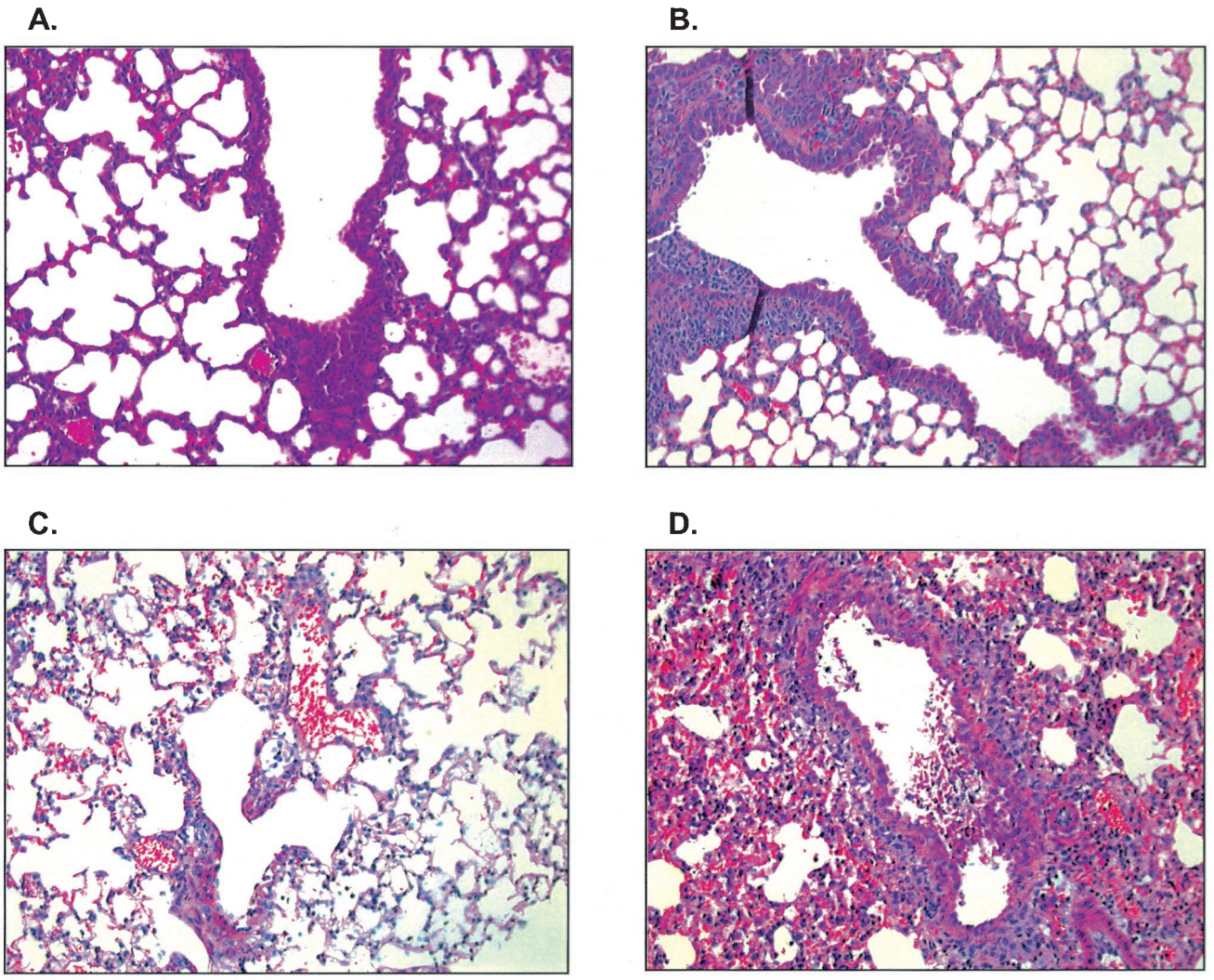


FIG. 1. Photomicrographs of hematoxylin-and-eosin-stained lung sections of mice inoculated with New Caledonia, WSN, and 1918 recombinant influenza viruses at 72 h postinoculation. (A) Normal morphology observed in mock-infected murine lung. (B) Moderately severe purulent bronchitis with epithelial necrosis and moderate lymphocytic peribronchitis after infection with New Caledonia recombinant virus. (C) Moderate necrotizing bronchitis with moderate histiocytic alveolitis and edema after infection with WSN virus. (D) Moderate necrotizing bronchitis with severe histiocytic alveolitis after infection with 1918 HA/NA:WSN recombinant virus.

group were pooled and amplified by using the method developed by Phillips and Eberwine (55). Following amplification, RNA integrity was verified by capillary gel electrophoresis, and fluorescent first-strand cDNA probes were prepared and hybridized to glass-immobilized cDNA comprised of the NIA murine 15k set, as described in Materials and Methods and previously (26–28). As shown in Fig. 2, 3,063 genes on the array showed a greater-than-1.5-fold up- or down-regulation from 24 to 72 h p.i. relative to mock-infected mouse lung at any single point. Marked alterations in gene expression could be observed in the 1918 HA/NA:WSN and WSN infections at 24 h postinoculation. This is in contrast with the New Caledonia virus-infected animals, which showed few significant changes in gene expression at 24 h after infection. These data closely parallel the severity of lung pathology observed at 24 h p.i. By 72 h p.i., all of the infection groups showed marked activation and repression of gene expression.

Gene expression changes at 24 h p.i. As shown in Fig. 3A, by 24 h p.i. the vast majority (83%) of genes showing a greater than 1.5-fold regulation in 1918 HA/NA recombinant virus-infected lung were common to the WSN infections, compared to 1% in common with New Caledonia virus infections and 6% in common in all infection groups. This analysis showed a high degree of correlation between altered host gene expression and infection pathogenicity. For all microarray studies described in this report, we focused our analysis on the genes with available known or inferred functional annotations. As shown in Fig. 3B, several genes were shown to be preferentially regulated in the 1918 HA/NA:WSN infections, including that for the inflammatory mediator Muc1. Most significant was the dramatic increase in the number of genes that were preferentially regulated in both the 1918 HA/NA:WSN and WSN infections, as shown in Fig. 3C. Given the large number of genes in this set (745 out of a total of 6,913), we focused on genes that

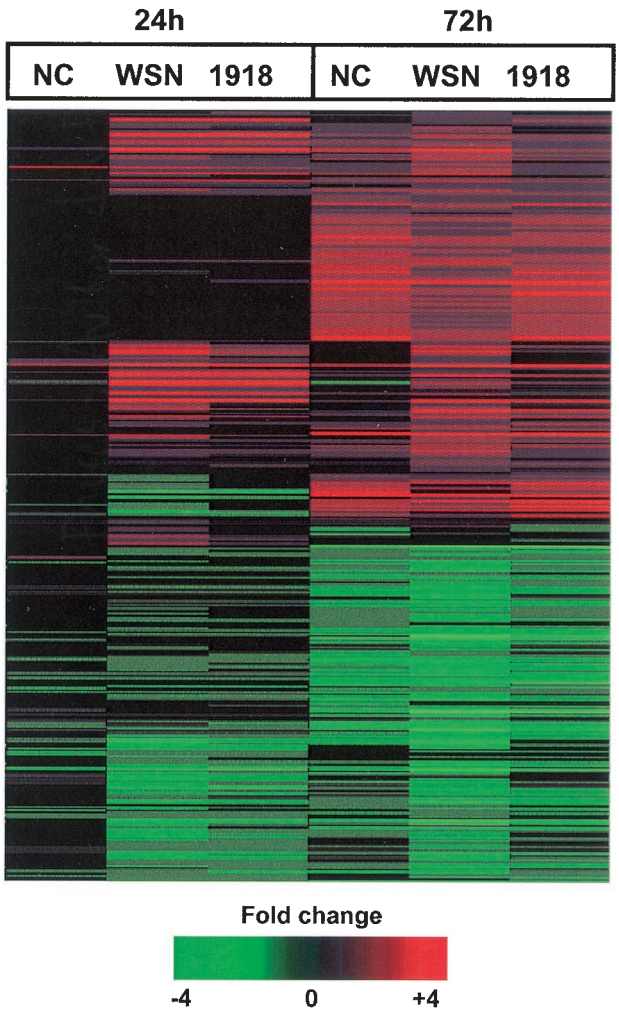


FIG. 2. Pattern of gene expression in whole lungs of mice inoculated with New Caledonia (NC), WSN, and 1918 HA/NA:WSN recombinant influenza viruses. A matrix of 3,063 genes regulated ≥1.5-fold ($P \leq 0.05$; $n = 4$) in at least one experiment at 24 and 72 h p.i., as analyzed using the Resolver analysis system (Rosetta Biosoftware), is shown. Fluorescent cDNA probes were prepared from amplified RNA constructed from pooled equal masses of total RNAs from five individual animals and were used to interrogate murine cDNA arrays containing the NIA 15K gene set compared to total lung from uninfected mice (see Materials and Methods). Genes shown in red were up-regulated, while genes shown in green were down-regulated, in infected compared to uninfected lung.

were marked discordant or anticorrelated with the New Caledonia HA/NA:WSN infections. This analysis demonstrated that many genes involved in the inflammatory response and necrosis were identified to be substantially up-regulated following infection with highly virulent 1918 HA/NA and WSN recombinant viruses. Several tumor necrosis factor (TNF)-related genes were found to be markedly up-regulated at 24 h, including those for TNF alpha (TNF-α), TNF-α-converting enzyme (EST_H3010D10), and TNF-α-induced protein 2 (Tnfaip2), an inhibitor of the transcription factor NF-κB (Nfkbia), in addition to the cytokine-induced RNA binding motif protein 3 (Rbm3). Moreover, many genes involved in macrophage and neutrophil activation and recruitment were identified, including those for platelet selectin (Selp), colony-stimulating factor 1 (Csf1), mitogen-responsive 96-kDa phosphoprotein (Dab2), and the IL-2 receptor (Il2rg). Other genes of interest included those for heme oxygenase (Hmox1), which is involved in the response to reactive oxygen species formation; Ctla2b, a marker of activated mouse cytotoxic T cells; Sdc2, which is involved in cholesterol metabolism in macrophages; and beta-2 microglobulin, which is involved in expression of major histocompatibility complex (MHC) class I molecules. Taken together, these results demonstrated that infection with highly virulent 1918 HA/NA:WSN and WSN viruses resulted in the activations of many proinflammatory and pronecrotic genes that were both higher in magnitude and more sudden in onset than in the lungs of mice infected with New Caledonia HA/NA:WSN recombinant virus. As shown in Fig. 3D, New Caledonia HA/NA:WSN infection resulted in a subtle preferential regulation of several stress- and immune-related genes, including repression of thymus chemokine 1 (Ppbp) and activation of heat shock 70-kDa protein 5 (Hspa5) and the Ca^{2+}-dependent adhesion protein protocadherin 7 (Pcdh7). As shown in Fig. 3E, examination of genes that were commonly regulated in all three infections showed many genes involved in inflammation, including the up-regulation of IFN regulatory factor 1 (Irf1), the stress-induced transcriptional repressor Atf3, the T lymphocyte- and IFN-inducible protein Tgtp, GRO1 oncogene (EST_H3051F10), the C-type lectin Clecsf9, and the IFN-responsive transcriptional activator Stat1. Other findings of interest include down-regulation of NADP-dependent leukotriene β4 12-hydroxydehydrogenase (EST_H3040C04), which is involved in monocyte trafficking to sites of inflammation; the oxidative stress response gene glutathione *S*-transferase (Gstp2), the apoptosis inhibitor Birc5, and two cell adhesion molecules integrin beta 1 and 4 (Itgb1 and Itgb4). Taken together, these data revealed that at the times sampled in this study, the most dramatic differences between high- and low-pathogenicity influenza viruses were observed by 24 h p.i., and they suggested that many important events regarding influenza virus pathogenicity occur early in infection.

Gene expression changes at 72 h p.i. As shown in Fig. 4A, at 72 h postinoculation, the majority of genes regulated more than 1.5-fold during 1918 HA/NA:WSN virus infection (70%) were common to all infections, with 20% in common with WSN and 4% in common with New Caledonia HA/NA:WSN infections. A closer inspection of the genes preferentially regulated greater than 1.5-fold ($P < 0.05$; $n = 4$) in the 1918 HA/NA:WSN infections showed elevated expression of the protease cathepsin H (Ctsh); several transcription factors, such as Sp1 and Satb1; and the transforming growth factor β family member Gdf3 (data not shown; see full data at http://expression.microslu.washington.edu). However, this analysis indicated that the genes preferentially regulated in the 1918 HA/NA:WSN infections were only modestly changed, with respect to magnitude but not direction, compared to changes observed for the New Caledonia HA/NA:WSN and parental WSN recombinant viruses. These data indicate that few alterations in the expression of genes present on the microarrays employed in these studies were uniquely attributable to infections with the 1918 HA/NA:

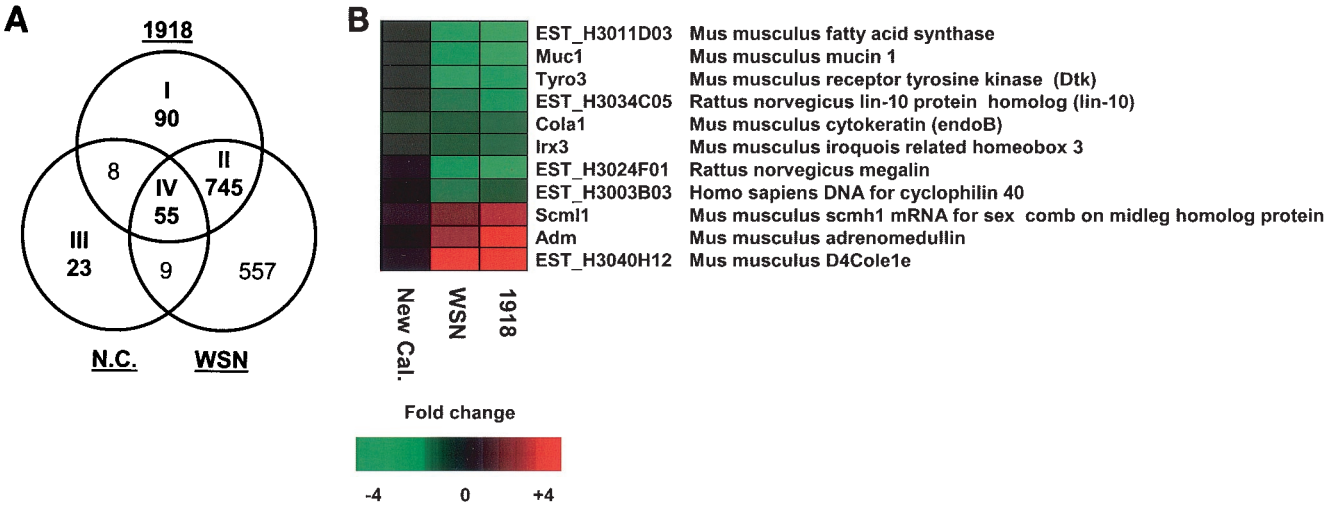
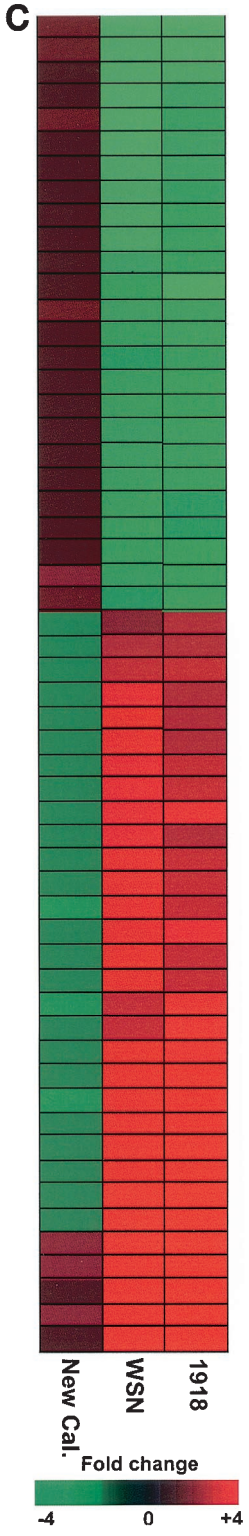


FIG. 3. Analysis of host response to influenza virus infection at 24 h p.i. (A) Venn diagram showing the overlap of genes with ≥1.5-fold ($P \leq 0.05$; $n = 4$) regulation in any single experiment in mouse lung tissue isolated 24 h following infection with 1918 HA/NA:WSN, WSN, or New Caledonia (N.C.) HA/NA:WSN. (B) Hierarchical clustering of genes preferentially regulated during 1918 HA/NA:WSN infection. (C) Hierarchical clustering of selected genes preferentially regulated during 1918 HA/NA:WSN and WSN infections (D) Hierarchical clustering of genes preferentially regulated during New Caledonia HA/NA:WSN infection. (E) Hierarchical clustering of genes preferentially regulated in all three influenza virus infections. Genes shown in red were up-regulated, while genes shown in green were down-regulated, in infected compared to uninfected lung.

WSN virus (see Materials and Methods). However, when we examined the population of genes that showed preferential regulation of greater than 1.5-fold ($P < 0.05$; $n = 4$) in the 1918 HA/NA:WSN and WSN infections, clear differences could be observed in comparison with the New Caledonia HA/NA:WSN infections. By using set analysis, 384 genes that were regulated more than 1.5-fold only in the lungs of mice infected with 1918 HA/NA and WSN viruses were identified, with approximately 190 genes that had available functional annotation. A closer inspection of these data showed 19 annotated genes whose expression was anticorrelated between the two high-pathogenicity infections and New Caledonia HA/NA:WSN (Fig. 4B). The genes that showed increased expression in the 1918 HA/NA:WSN and WSN infections but were down-regulated in the New Caledonia infection included several cytokine-regulated genes, including those for TNF-α-induced protein 2 (Tnfaip2), the cold shock RNA binding protein Rbm3, and the IL-10-induced metalloprotease ADAMTS4 (EST_H3022E09). Also included in this group of genes that were up-regulated during highly pathogenic influenza infections were the oxidative stress response glutathione peroxidase 3 (Gpx3) gene and the DNA damage-induced growth arrest Gadd45g gene. These data suggested that infections with the 1918 HA/NA:WSN and WSN viruses resulted in a more significant inflammatory response and reactive oxygen species-induced stress, as was similarly observed at 24 h p.i. When we examined the genes that showed preferential regulation in the New Caledonia HA/NA:WSN infections, we identified 200 genes, of which 97 had at least inferred functional annotation. This analysis further showed that the expression of 11 genes was anticorrelated with either the 1918 HA/NA:WSN or parental WSN infections. However, as shown in Fig. 4C only a macropain protease subunit (Psma6) showed anticorrelation with both of the highly virulent recombinant influenza viruses. Given the minimal correlation of these genes with the previously reported virulence of these viruses, a direct relationship between virus-induced regulation of these genes could not be inferred (69). We next examined the genes whose expression was regulated more than 1.5-fold in all of the recombinant influenza virus infections. To identify the dominant viral response genes, we focused on the expression of genes with significant regulation (>4-fold with $P < 0.05$ [$n = 4$]) at 72 h that were common to all infection groups (Fig. 4D). Many of the genes commonly regulated by all three infection groups were those involved in immune responses. These include the genes for the IL-2 receptor (EST_H3024G06), which is involved in T-cell activation and proliferation; cytochrome b_{245} (EST_H3060F11), a critical component of membrane-bound oxidase activity of phagocytes (macrophages and neutrophils); the transcription factor Stat1, which is a critical component of the IFN-α/β and IFN-γ response; the Gro1 oncogene (EST_H3051F10), which is the mouse orthologue of human CXCL1, a potent neutrophil chemokine; the C-type lectin Clecsf9, a downstream target of NF–IL-6 that plays roles in macrophage function; and the IFN-regulated transcription factor Irf1, which activates expression of IFN-α/β and IFN-inducible MHC class I genes. Other commonly up-regulated genes include those for cyclin D2 (Ccnd2), which is essential for control of the cell cycle at the G_1-to-S transition and is an indicator of cell proliferation. Taken together, the pattern of these gene expression data showed that clear differences were observed between the highly virulent 1918 HA/NA:WSN and parental WSN recombinant viruses and the attenuated New Caledonia HA/NA:WSN recombinant virus, which mirrored the histopathology data, and that many critical events determining the severity of viral pathogenicity occurred relatively early in infection (by 24 h p.i. in our studies).



Pmm1	Mus musculus phosphomannomutase Sec53p homolog
EST_H3024F01	Rattus norvegicus megalin
Scd2	Mus musculus stearoyl-Coenzyme A desaturase 2
Rgs11	Mus musculus regulator of G-protein signaling 11
Gpam	Mouse glycerol-3-phosphate acyltransferase
LOC56435	Mus musculus mRNA for mPACPL1
Ero1l-pending	Mus musculus ERO1-like (S. cerevisiae) (Ero1l-pending)
EST_H3030B09	Human monoamine oxidase (MAOA)
EST_H3010C05	Homo sapiens myelin protein zero-like 1
Fus1	Mus musculus Fus1 gene
EST_H3071D09	Mus musculus tetratricopeptide repeat domain (Ttc3)
EST_H3058B10	Homo sapiens centrin, EF-hand protein, 2 (CETN2)
Acadm	Mus musculus medium-chain acyl-CoA dehydrogenase
Slc25a17	Mus musculus mRNA for peroxisomal integral membrane protein PMP34
EST_H3003B03	Homo sapiens DNA for cyclophilin 40
Emb	Mus musculus embigin
EST_H3028C09	Mus musculus adenosine kinase (Adk)
Hmg4	Mus musculus high-mobility group protein 4
Ppib	Mus musculus peptidylprolyl isomerase B
EST_H3059E05	Mus musculus malate dehydrogenase, soluble (Mor2)
EST_H3025C10	Homo sapiens HSPC194
Soat1	Mus Musculus acyl-coenzyme A:cholesterol acyltransferase
Nucb2	Mus musculus nucleobindin 2
EST_H3042C06	Mus musculus progressive ankylosis (ank)
EST_H3051B05	Homo sapiens F-box only protein 5 (FBXO5)
Hmox1	Mus musculus Heme oxygenase 1
EST_H3010D10	Mus musculus tumor necrosis factor alpha converting enzyme (TACE)
EST_H3038D05	Homo sapiens similar to kinesin-like 4
EST_H3049A11	M.musculus rab20
Ctla2b	Mouse cytotoxic T lymphocyte associated-2 beta
LOC55989	Mus musculus SIK similar protein
EST_H3022D10	Homo sapiens brain acid-soluble protein 1 (BASP1)
Plaur	M.musculus muPAR1
Gpcr26	Mus musculus lysophosphatidic acid receptor (vzg-1)
G3bp2-pending	Mus musculus RNA-binding protein isoform G3BP-2a
EST_H3010H07	Mus musculus pellino 1 (Peli1)
EST_H3029A04	Mouse scip POU-domain protein
Ninj1	Mus musculus ninjurin 1
ESET	M.musculus mRNA for testis-specific thymosin beta-10
Rbm3	Mus musculus RNA binding motif protein 3
Cpsf2	Mus musculus cleavage and polyadenylation specific factor 2 (100kD
Tnfaip2	Mus musculus tumor necrosis factor, alpha-induced protein 2
EST_H3002E01	Mus musculus selenoprotein R (Sepr)
Cyp1b1	Mus musculus cytochrome P450, 1b1, benz[a]anthracene inducible
Lmnb1	Mus musculus lamin B1
Il2rg	Mus musculus mRNA for interleukin 2 receptor gamma chain
Csf1	Mus musculus colony stimulating factor 1 (macrophage)
EST_H3008E07	Homo sapiens 7-60 protein
Selp	Mus musculus selectin, platelet
Nfkbia	Mus musculus I kappa B alpha
Upp	Mus musculus uridine phosphorylase
Wars	M.musculus (clone S5) WRS mRNA for tryptophan--tRNA ligase
B2m	Mus musculus beta-2 microglobulin
Tnf	TNF alpha
EST_H3020F06	Homo sapiens sorting nexin 10 (SNX10)
Mlp	Mus musculus MARCKS-like protein

FIG. 3—*Continued*.

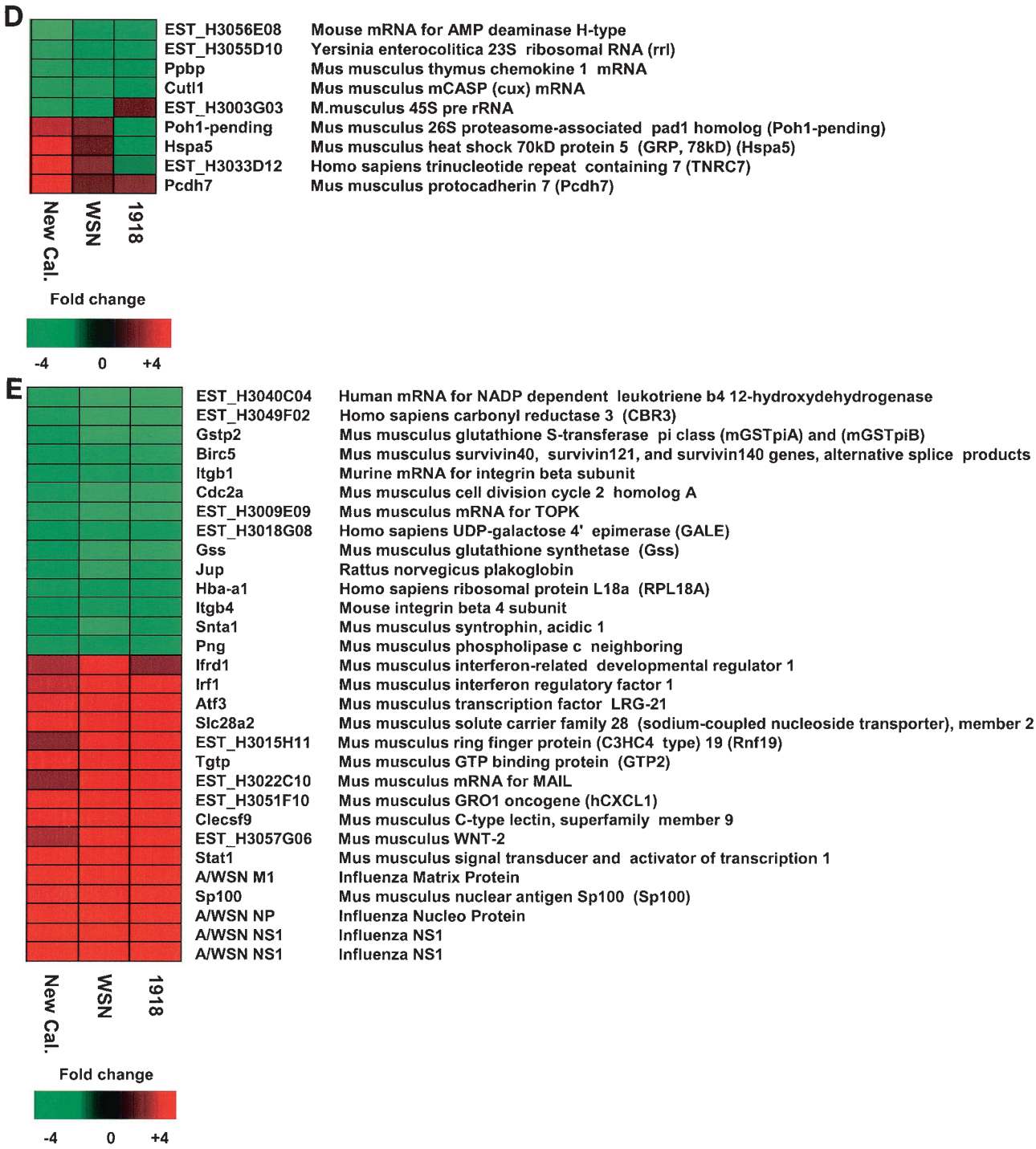


FIG. 3—*Continued*.

DISCUSSION

In an uncomplicated influenza virus infection, the cytopathic effect of viral infection is most often observed as degenerative and necrotic changes in epithelial cells of the bronchial and bronchiolar mucosa (22). Severe influenza infections are associated with significant changes in alveolar cells, typified by acute, focal alveolitis (22). Inflammation and necrosis of the alveoli are often characterized by a rapid course of infection, with death resulting within days of the onset of clinical symptoms (22). Modern pathological analysis of lung tissues collected from patients who died from primary influenza pneumonia in 1918 showed acute focal bronchiolitis and alveolitis associated with acute massive pulmonary edema. Additional variations of this 1918 pathology were acute massive pulmo-

nary hemorrhage and secondary bacterial pneumonia with total destruction of alveolar architecture. In the present study the lungs of mice infected with highly virulent 1918 HA/NA:WSN and parental WSN recombinant influenza viruses showed profound changes in morphology, including moderate to severe necrotizing bronchitis and acute histiocytic alveolitis. In contrast, the lungs of mice infected with attenuated New Caledonia HA/NA:WSN recombinant virus showed minimal to modest necrotizing bronchitis and mild alveolitis that was typically associated with the areas of bronchial necrosis. Pronounced chemotaxis and recruitment of neutrophils were observed in the 1918 HA/NA:WSN and WSN infections and appeared to be slightly more common in the lungs of mice infected with 1918 HA/NA:WSN virus. We have used microarray technology to better understand the genetic contribution of the host response during severe influenza pneumonia. We have shown clear differences in the transcriptional profiles of the lungs of mice infected with highly virulent and attenuated influenza viruses, including many inflammation, necrosis, and stress response genes.

Influenza virus HA and NA genes as virulence factors and viral fitness. Both the HA and NA genes of influenza virus have been previously shown to be important virulence factors (30, 31, 33, 41, 52, 61, 65, 71, 73). Sequence analysis of the 1918 HA (H1) and NA (N1) genes has demonstrated that neither the HA gene nor the NA gene possessed any obvious features that could be related directly to virulence in avian or mouse-adapted influenza viruses. However, pathology analysis showed a slightly more severe pathology in mice infected with the 1918 HA/NA:WSN virus at 72 h p.i. compared to the parental WSN, suggesting that additional, unidentified virulence markers may be present in these glycoproteins. However, virulence is a complex interaction between the host and pathogen, encompassing issues of viral fitness, relating to how well a series of gene segments are adapted to work together, and issues of host adaptation, involving a particular combination of segments working in a particular host. As WSN NS1 is a critical factor in virulence, it is possible that the 1918 HA and NA sequences, being more similar to those in WSN, act in a permissive manner to produce a fit and adapted virus for mouse virulence and that New Caledonia glycoproteins result in a virus that is either not as fit (i.e., has a slower replication rate), not sufficiently mouse adapted, or both. However, it is clear that without any previous mouse adaptation, expression of the 1918 HA and NA genes in the WSN virus background retained high WSN murine virulence. This is consistent with the hypothesis that the HA and NA proteins contributed to the virulence and unusual pathology of the 1918 influenza virus by facilitating viral replication, broadening cellular tropism, and/or triggering a more severe and sustained inflammatory response. Studies are planned to determine the contributions of the 1918 HA and NA genes to influenza virus virulence and pathology in a primate model.

Inflammatory responses and recruitment of immune cells. Epithelial cells provide a first line defense against microbe infections and function to help recruit inflammatory cells to sites of infection by the expression and secretion of cytokines, chemokines, and adhesion molecules. Histopathological analysis of the lungs from 1918 HA/NA:WSN and WSN infections showed increased recruitment and accumulation of neutrophils. Accordingly, we observed many significant increases in the expression of genes associated with phagocytic cell activation and chemotaxis, including those for Gro1 (the mouse orthologue of human CXCL1), the heparin sulfate proteoglycan syndecan-4, the C-type lectin Clecsf9, and both P- and E-selectins. Moreover, we identified a marked increase in the expression of the monokine Scya4 (also known as MIP-1b), which through the binding of CCR5 and CCR8 plays important regulatory roles in lymphocyte activation. Initiation of inflammation triggered by the production of IL-1 or TNF-α results in the expression of lymphocyte growth factors, and our analysis has demonstrated that infection with highly virulent influenza viruses was associated with up-regulation of Csf1 mRNA expression as well as that of the Csf1 receptor signaling protein Dab2. We further observed the elevated expression of activated T-cell markers, including cytotoxic T lymphocyte-associated protein 2 alpha and beta (Ctla2a and Ctla2b). Taken together, these results point to the profound activation of the inflammatory response in the lungs of mice infected with the highly pathogenic 1918 HA/NA:WSN and parental WSN recombinant viruses.

Genetic markers of highly virulent influenza virus infection. Transcriptional profiling performed on mouse lung tissues demonstrated significant up-regulation of many genes involved in necrosis, lymphoid cell activation and recruitment, and oxidative damage in the lungs of mice infected with 1918 HA/NA:WSN or parental WSN recombinant virus compared to infection with attenuated New Caledonia HA/NA:WSN virus. Perhaps the most significant of these changes was the observed up-regulation of genes involved in TNF signaling, including those for TNF-α, TNF-α-converting enzyme (EST_H3032H05), and TNF receptor (Tnfrsf12). The expression of TNF during influenza virus infection has been shown to be important for pathogenicity (10, 35–37, 39, 42, 47, 51). A recent study of the effect of highly pathogenic avian 1997 Hong Kong H5N1 viruses in monocyte-derived human macrophages demonstrated that the 1997 H5N1 viruses were potent inducers of proinflammatory cytokines, the most significant of which was TNF-α, with the peak in TNF-α secretion reported to be at between 12 and 24 h p.i. and with a nearly 50% drop by 36 h (19). In the present study, these kinetics parallel the steady-state levels of TNF mRNA that we observed at 24 h and 72 h p.i. In addition to its role as a key necrosis mediator, TNF-α also acts as an important proinflammatory signal, and activation of this pathway has been implicated in the pathogenesis of acute respiratory infections. Interestingly, TNF antagonists have been employed as effective treatments for the inflammatory diseases rheumatoid arthritis and Crohn's disease, and they have also been shown to reduce the weight loss and illness in primary influenza A infection in mice (36). In addition, infection with 1918 HA/NA:WSN and WSN recombinant viruses also resulted in up-regulated expression of Nfkbia, an inhibitor of the TNF receptor-activated transcription factor NF-κB. This observation was important because loss of NF-κB activity has been shown to increase the cytotoxic effects of TNF, resulting in increased cell death (9). It is conceivable that the combination of high levels of TNF expression and the inhibition of NF-κB activation (inferred from up-regulated Nfkbia expression) act synergistically to cause signifi-

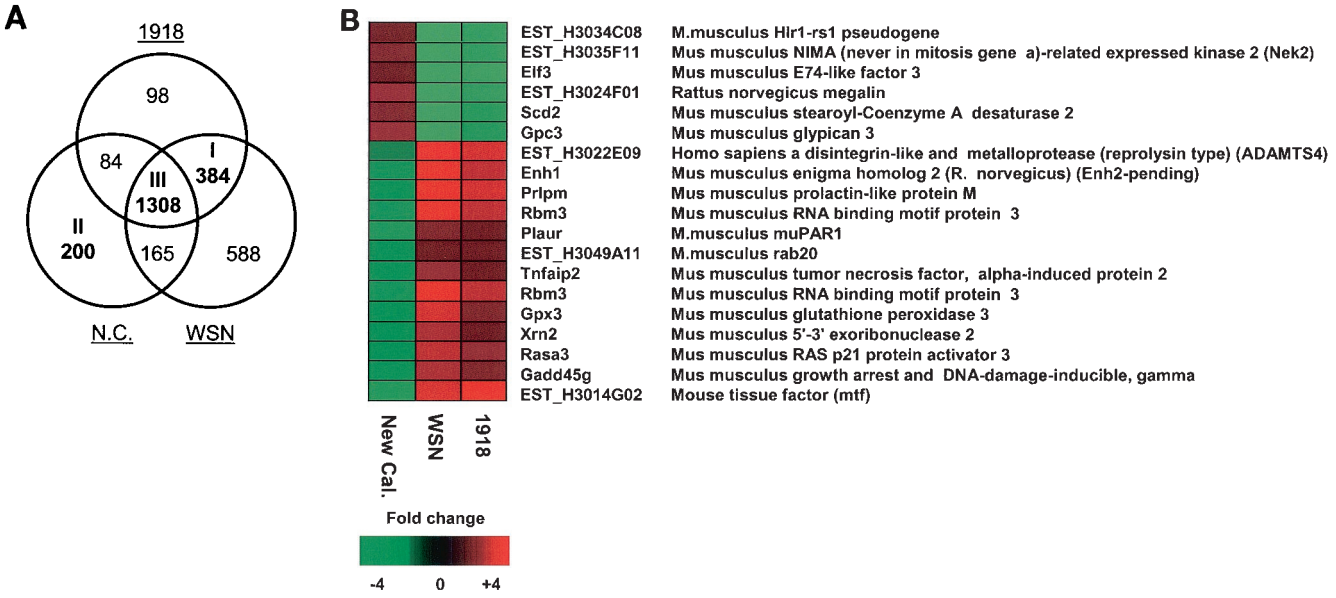


FIG. 4. Analysis of host response to influenza virus infection at 72 h p.i. (A) Venn diagram showing the overlap of genes with ≥1.5-fold ($P \leq 0.05$; $n = 4$) regulation in any single experiment in mouse lung tissue isolated 72 h following infection with 1918 HA/NA:WSN, WSN, or New Caledonia (N.C.) HA/NA:WSN. (B) Hierarchical clustering of selected genes preferentially regulated during 1918 HA/NA:WSN and WSN infections. (C) Hierarchical clustering of selected genes preferentially regulated during New Caledonia HA/NA:WSN infection. (D) Hierarchical clustering of selected genes that showed ≥4-fold regulation in all three infections. Genes shown in red were up-regulated, while genes shown in green were down-regulated, in infected compared to uninfected lung.

cant bronchial and alveolar epithelial cell necrosis during highly virulent influenza virus infections. The production of reactive oxygen species, especially superoxide radicals, and the subsequent oxidative damage of cells and tissues are recognized as key contributors to the viral pathogenesis (1, 2, 5, 13, 25, 38, 40, 44, 45, 54). Specifically, the generation of oxygen radicals has been shown to be an important aspect of influenza virus virulence and pathology (3, 6, 13, 14, 20, 50, 79). Accordingly, we have identified the up-regulation of several important genes involved in the production of and response to superoxide radicals in the lungs of mice infected with influenza viruses. Principal among these is the increased expression of a critical component of the membrane-bound oxidase of neutrophils and macrophages, cytochrome b_{245} (EST_H3060F11 or Cybb), that generates superoxide radicals. The increased expression of cytochrome b_{245} in the lungs of mice infected by all of the influenza viruses used in this study points to the increased production of oxygen radicals and the activation of phagocytic cells. Moreover, the lungs from mice infected with highly virulent influenza viruses expressed elevated levels of indicator genes for significant oxidative damage, including heme oxygenase-1 (Hmox1), and the down-regulation of the antioxidants glutathione peroxidase (Gpx3) and peroxiredoxin V (Prdx5). Taken together, these data suggest that an important component of the severe pulmonary pathology that occurs during highly pathogenic influenza virus infection is increased damage caused by significant production of TNF and oxygen radicals.

At present we do not understand the contributions that individual cell types make to the aggregate gene expression changes identified in whole lungs. Analysis of the lungs from human patients who died from causes unrelated to pulmonary complications showed that five major cell types were present in the parenchyma, consisting of 15% type I and II alveolar epithelial cells, 30% capillary epithelial cells, 37% interstitial cells, and between 5 and 20% alveolar macrophages (23). Assuming a similar cellular composition in the mouse lung, the percentage of cells susceptible to influenza virus, primarily the alveolar epithelial cells, is approximately 15%. Therefore, the contribution of viral replication in bronchial and alveolar epithelial cells to the overall transcriptional profile of an intact lung is difficult to determine from the data presented in this report. However, previous work from our laboratories has shown that infection of a type II alveolar epithelial cell line (A549) with influenza virus resulted in suppression of the cellular innate antiviral response (11, 26, 27, 29, 32, 66, 72). It is reasonable to conclude that many of the gene expression changes correlated with the severity of infection, particularly those of the TNF-α gene, arise from the influx of inflammatory cells, such as T cells, macrophages, and neutrophils. Additional studies using bronchoalveolar lavages could be used in the future to better determine the contribution that macrophages and neutrophils make to the gene expression profiles observed in these infection groups. However, our studies demonstrated clear and dramatic differences in gene expression in lungs of mice infected with highly virulent and attenuated influenza viruses, regardless of cellular mRNA origin.

Gene expression profiling and predictors of infection outcome. From the data presented in this report, a model for the relationship between influenza virus virulence, pulmonary gene expression profiles, and infection pathology can be surmised. As shown in Fig. 5, both virulent and attenuated influenza viruses result in mild necrotizing bronchitis and minimal alveolitis. The highly virulent influenza viruses also trigger a markedly more robust inflammatory response, as indicated by high levels of expression of TNF, Csf1, IL-2 receptor, selectins,

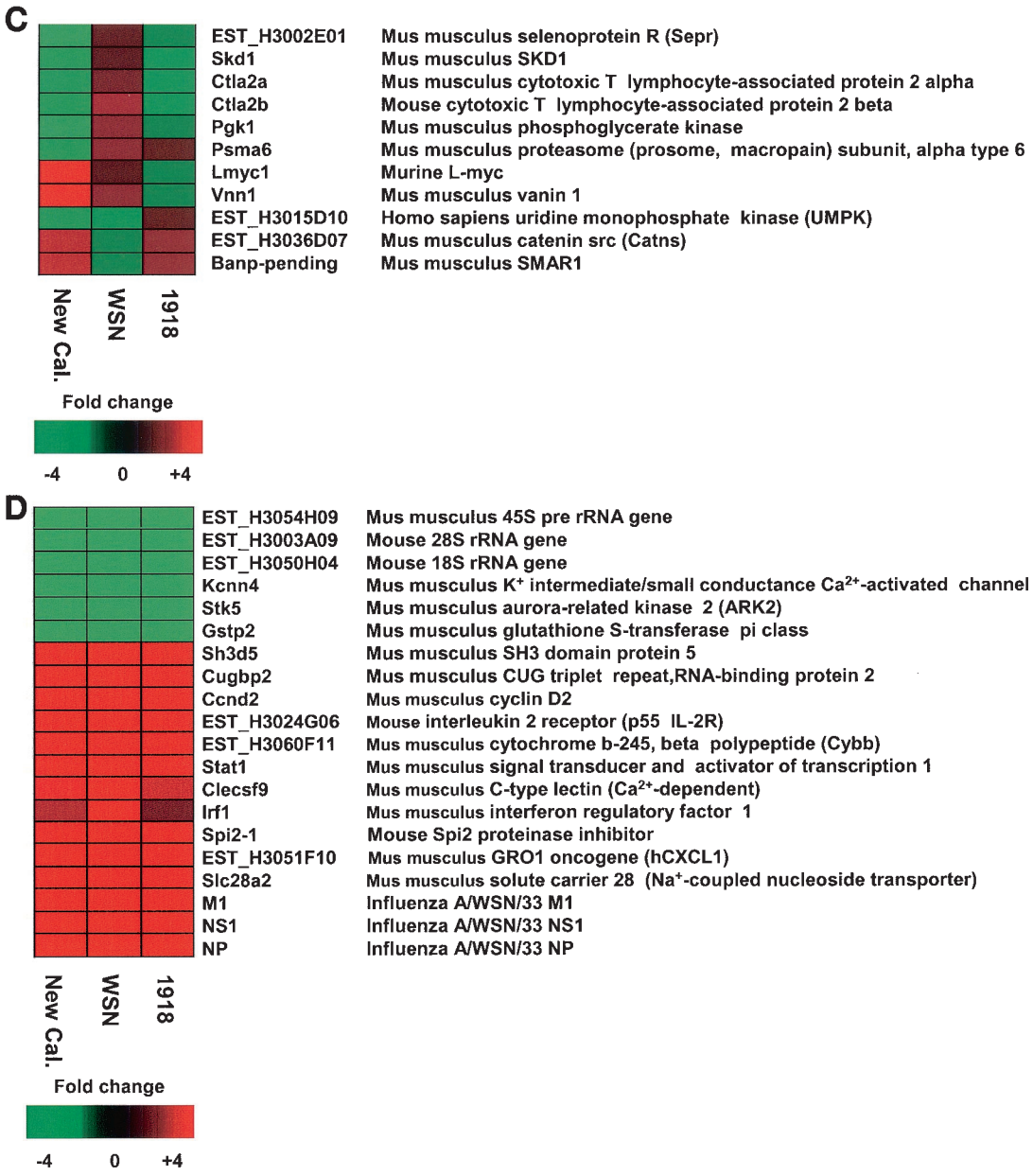


FIG. 4—*Continued.*

and class I MHC (via beta-2 microglobulin [B2m]). This is compounded by significant necrosis and elevated expression of TNF, Nfbia, and Gadd45g, coupled with increased oxidative stress, as indicated by up-regulated expression of Hmox1 and down-regulation of the antioxidants Gpx3 and Prdx5. The potent activation of these pathology-associated pathways in a highly virulent influenza virus infection results in the development of severe necrotizing bronchitis and acute alveolitis, which are more likely to result in a poor infection prognosis. In order to determine whether these gene expression changes are causative features of a highly pathogenic influenza virus infection, infections with the viruses described in this paper could be performed in mutant mice with knockouts in pathogenesis-related genes, some of which are shown in Fig. 5. If the model presented in Fig. 5 is accurate, infection with the 1918 HA/NA:WSN and WSN viruses would result in less severe pathology and viral attenuation. Combined with our observations of marked up-regulation of TNF expression during infection with highly virulent influenza viruses, these studies show the importance of TNF in the induction of serious complications resulting from an exaggerated inflammatory response. Further studies are needed to explore the potential role of the other pathogenesis genes documented in the present study.

Taken together, the data presented in this study show that differences in the severity of influenza virus infection were associated with marked differences in lung morphology and host gene expression. These analyses further suggested that the lungs of mice infected with the 1918 HA/NA:WSN virus dis-

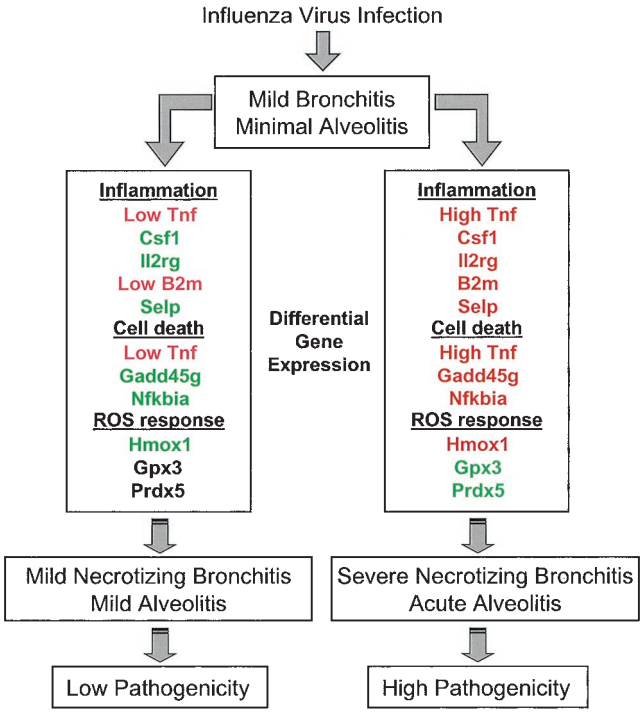


FIG. 5. Model of pulmonary gene expression and influenza virus virulence. The relationship between differential gene expression, inflammatory responses, cell death, oxidative damage, and pathogenicity of influenza virus infection in mice is shown.

played in aggregate a slightly more severe pathology, suggesting that an intrinsic property of the 1918 HA and NA proteins may be the production of a longer and more severe immune response culminating in a far more destructive viral infection. Moreover, transcriptional profiling showed that a marked upregulation of genes involved in inflammation, cell death, and oxidative stress could be correlated with influenza virus virulence and was manifested early in infection.

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