Gene Expression Analysis of Host Innate Immune Responses during Lethal H5N1 Infection in Ferrets[▽][†]

Cheryl M. Cameron,[1,2,3] Mark J. Cameron,[1,2,3] Jesus F. Bermejo-Martin,[4] Longsi Ran,[2] Luoling Xu,[2]
Patricia V. Turner,[5] Ran Ran,[2] Ali Danesh,[1,2,3] Yuan Fang,[1,2,3] Pak-Kei M. Chan,[1,2,3] Nutan Mytle,[6]
Timothy J. Sullivan,[7] Tassie L. Collins,[7] Michael G. Johnson,[7] Julio C. Medina,[7]
Thomas Rowe,[1,2,3] and David J. Kelvin[1,2,3]*

*Division of Immunology, International Institute of Infection and Immunity, Shantou University Medical College, Shantou, Guangdong,
People's Republic of China[1]; University Health Network, Toronto, Ontario, Canada[2]; University of Toronto, Toronto, Ontario,
Canada[3]; Laboratorio de Immunologia de Mucosas, Universidad de Valladolid, Valladolid, Spain[4]; Department of Pathobiology,
Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada[5]; Southern Research Institute, Birmingham,
Alabama[6]; and Amgen, Inc., San Francisco, California[7]*

How viral and host factors contribute to the severe pathogenicity of the H5N1 subtype of avian influenza virus infection in humans is poorly understood. We identified three clusters of differentially expressed innate immune response genes in lungs from H5N1 (A/Vietnam/1203/04) influenza virus-infected ferrets by oligonucleotide microarray analysis. Interferon response genes were more strongly expressed in H5N1-infected ferret lungs than in lungs from ferrets infected with the less pathogenic H3N2 subtype. In particular, robust CXCL10 gene expression in H5N1-infected ferrets led us to test the pathogenic role of signaling via CXCL10's cognate receptor, CXCR3, during H5N1 influenza virus infection. Treatment of H5N1-infected ferrets with the drug AMG487, a CXCR3 antagonist, resulted in a reduction of symptom severity and delayed mortality compared to vehicle treatment. We contend that unregulated host interferon responses are at least partially responsible for the severity of H5N1 infection and provide evidence that attenuating the CXCR3 signaling pathway improves the clinical course of H5N1 infection in ferrets.

According to the World Health Organization, the H5N1 subtype of avian influenza virus has caused over 380 cases of human illness and over 240 deaths from 2003 to date. Person-to-person transmission of H5N1 virus appears to be a rare event (20, 37). Current therapeutic strategies for human H5N1 infection are restricted to vaccination, preventative or early treatment with antiviral agents, and use of corticosteroids. While antiviral treatments may benefit patients if administered early in the disease and the currently available H5N1 vaccines appear to be effective in animal models and safe in humans, neither approach guarantees protection in the face of an influenza pandemic. Newly emergent strains may not match stockpiled vaccine strains, and the virus is likely to develop resistance to antiviral agents.

Recent studies have suggested that aberrant host immune responses to H5N1 during acute infection may be partially responsible for the severity of disease in humans (11, 35). Very few mechanistic data, however, are available regarding host responses to H5N1 infection in humans or animal models. Treatments designed to correct a dysregulated host response and associated immunopathology during H5N1 infection therefore warrant further consideration in animal models.

In this study, a functional genomics approach was utilized to identify potential patterns of immune dysregulation during acute H5N1 influenza virus infection in ferrets by comparison to H3N2 infection. Ferrets were infected intranasally with one of two different influenza A viruses originally isolated from humans: A/Vietnam/1203/04 (H5N1) and A/Panama/2007/99 (H3N2). Three clusters of differentially expressed innate immune response genes that were highly enriched in interferon (IFN) response genes (IRGs) were found by microarray analysis of gene expression in lungs from H5N1- versus H3N2-infected animals. In particular, CXCL10 gene expression was significantly upregulated in H5N1-infected ferret lungs throughout the course of the study relative to mock-infected and H3N2-infected ferret lungs. This result prompted us to evaluate modulation of CXCL10 activity via blockade of CXCR3 signaling for efficacy in improving the morbidity associated with H5N1 infection in ferrets and as a potential avenue for future therapeutic intervention in humans.

MATERIALS AND METHODS

Ferret experiments. For the microarray study, 4- to 6-month old, neutered, descented, and influenza virus antibody-screened male fitch ferrets (Triple F Farms, Sayre, PA) were weighed and randomly assigned to one of three infection groups at the Southern Research Institute for intranasal instillation of either A/Vietnam/1203/04 H5N1 subtype (clade 1) or A/Panama/2007/1999 influenza virus at 1×10^6 50% egg infectious doses (EID_{50}) or for phosphate-buffered saline (PBS) mock infection, in a total volume of 1 ml. Infected ferrets ($n = 3$/group) were euthanized at 2 and 4 days postinfection (dpi) and at the end point (6 dpi or upon scoring as moribund due to 25% weight loss). Note that one H5N1-infected ferret was euthanized at 5 dpi under the definition of the end point (see Fig. S1 in the supplemental material). Mock-infected animals were euthanized on the day of infection. Clinical signs were monitored daily from 2

* Corresponding author. Mailing address: Division of Immunology, International Institute of Infection and Immunity, Shantou University Medical College, 22 Xinling Road, Shantou, Guangdong, P. R. China 515041. Phone: 86-754-8738699. Fax: 86-754-8544422. E-mail: dkelvin @uhnres.utoronto.ca.
† Supplemental material for this article may be found at http://jvi.asm.org/.
▽ Published ahead of print on 6 August 2008.

dpi. Viral loads in nasal turbinates and lung tissue from influenza virus-infected ferrets at necropsy were assayed by methods described previously (44) with a limit of detection of 1.5 EID_{50}/ml (log_{10}). For the AMG487 treatment study, the drug was kindly provided by Amgen (San Francisco, CA) and administered at a suggested one-third of the daily dose used safely in mouse studies (38). Ferrets were infected with H5N1 as described above and received either 1.65 mg/kg AMG487 in a 3-ml volume of PBS ($n = 8$) or PBS vehicle ($n = 9$) intraperitoneally every 12 h starting at 24 h postinfection and continuing until the end point (euthanasia upon scoring as moribund due to 25% weight loss). Nasal washes at 1, 3, and 5 dpi and lungs at necropsy were assayed for viral load in the AMG487 treatment study by methods described previously (44) with a limit of detection of 1.5 EID_{50}/ml (log_{10}). Weight loss, activity, temperature, and arterial O_2 saturation (SpO2) (pulse oximetry) were monitored daily from 2 dpi. Temperatures were monitored with subcutaneous transponders (IPTT-300; Biomedic Data Systems Inc, Seaford, DE).

Microarray analysis. Lung sections (~1 g) were obtained during necropsy and immediately homogenized in TriPure reagent, and total RNA was isolated according to the manufacturer's recommended protocol (Roche, Indianapolis, IN). RNA quality was assessed using a UV spectrophotometer and by formaldehyde gel electrophoresis. The quality of RNA isolated from a single H3N2-infected ferret's lung specimen collected at 4 dpi was insufficient for cDNA synthesis. Total RNA was amplified using MessageAMP kits (Ambion, Austin, TX). cRNA (15 μg) was labeled and hybridized to Affymetrix Canine 2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA). Canine arrays were chosen following analysis of 30 publically available ferret cDNA sequences and demonstration of high levels of homology (average of 89% identity) between canine and ferret nucleotide sequences (see Table S1 in the supplemental material). Probe-level analysis was performed using the Probe Logarithmic Intensity Error Estimate (PLIER) method in Array Assist V5.2 (Stratagene, La Jolla, CA). The raw intensity values for each target on the Affymetrix arrays were preprocessed with variance stabilization, log_2 transformation, and normalization against the data sets for mock infection excluding elements with median signal intensities of <6 log_2 units across samples. Sequential Student's t tests (time point versus mock) were used to identify genes significantly differentially expressed ($P \leq 0.05$ and $\geq$2-fold change) for at least one time point and group during H3N2 and H5N1 infection in ferrets. These genes were combined with significantly differentially expressed genes between H3N2- and H5N1-infected ferrets ($\geq$2-fold change for at least one time point and group and $P \leq 0.05$) identified using the Extraction and Analysis of Differential Gene Expression (EDGE) software tool (24). Hierarchical clustering was performed using GeneLinker Platinum 4.6 (Improved Outcomes, Kingston, Canada) and the Euclidean geometrical average of samples algorithm. Ingenuity Pathway Analysis 5.0 (IPA) (Ingenuity Systems, Redwood City, CA) was used to select, annotate, and visualize genes by function and pathway (gene ontology). Additional gene annotation was provided by the Interferon Stimulated Gene Database (12) and the Universal Protein Resource (UNIPROT) (URL:www.expasy.org/uniprot).

qRT-PCR. Quantitative real-time PCR (qRT-PCR) was performed on amplified RNA in triplicate using an ABI-Prism 7900HT and Sybr green master mix (Applied Biosystems, Foster City, CA). Primer sequences were as follows: for CXCL9, 5′-GGTGGTGTTCCTCTTTTGTTGAGT-3′ (forward) and 5′GGAA CAGCGTCTATTCCTCATTG-3′ (reverse); for CXCL10, 5′-CTTTGAACCA AAGTGCTGTTCTTATC-3′ (forward) and 5′-AGCGTGTAGTTCTAGAGA GAGGTACTC-3′ (reverse); for FCN1, 5′-CACCAAGGACCAGGACAATG A-3′ (forward) and 5′-CACCAGGCCCCCTGGTA-3′ (reverse); for SERPING1, 5′-GCCTCTCAGAGCCTGTATGG-3′ (forward) and 5′-CTTCCACTTGGCACT CAGGT-3′ (reverse); and for STAT1, 5′-AGCCTTGCATGCCAACTCA-3′ (forward) and 5′-GCAGTCTCAACTTCACGGTGAA-3′ (reverse). Gene expression levels for each sample were normalized to ferret beta-actin levels in the same sample. The quality of cDNA obtained from a single H5N1-infected ferret's lung specimen collected at the end point was insufficient for qRT-PCR validation.

Histochemistry. Lungs were routinely perfused, formalin fixed, and hematoxylin-and-eosin stained. Blinded serial sections were evaluated by a veterinary pathologist (P.V.T.).

Statistical methods. Survival curves were evaluated by Kaplan-Meier and log rank analysis. Other clinical indicators were compared by two-way analysis of variance (ANOVA) (Prism V3; GraphPad Software, San Diego, CA). Other tests used were Student's t test or the Mann-Whitney (nonparametric) rank sum test for two independent populations using SPSS for Windows V13.0 software (SPSS Inc., Chicago, IL) where noted. A P value of ≤ 0.05 was considered significant with all methods.

Microarray data accession number. Data sets are available publically at the Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/) under accession number GSE9606.

RESULTS AND DISCUSSION

To identify host responses specific to H5N1 virus infection and further examine the molecular basis of the high degree of lung pathology associated with H5N1 infection, we infected ferrets with either the H5N1 or H3N2 subtype of influenza A virus (see Fig. S1 and S2 in the supplemental material) and obtained tissue samples for gene expression analysis at various time points postinfection. Ferrets were inoculated intranasally with 10^6 EID_{50} of either A/Vietnam/1203/04 (H5N1) or A/Panama/2007/99 (H3N2) and examined daily for clinical signs of disease, including loss of activity, nasal discharge and respiratory distress, neurological signs, weight loss, and temperature. Overall, the clinical symptoms we observed in H5N1-infected ferrets were very consistent with previous studies using this particular virus strain (A/Vietnam/1203/04) or its reverse-genetics-derived recombinant form (15, 31). At 2 and 4 dpi and at the end point, ferrets were euthanized and lung tissue removed for RNA purification for gene expression analysis. RNA was quantified and assessed for integrity, and equal quantities of lung total RNA from each ferret were amplified and hybridized to oligonucleotide arrays. The resulting gene expression data were normalized directly to those for mock-infected ferrets.

Our analysis strategy, which identified genes either up- or downregulated during H5N1 and H3N2 infection and differentially expressed between H5N1 and H3N2 infection, resulted in a list of 2,295 genes with significantly changed expression in lungs from influenza virus-infected ferrets. Hierarchical clustering analysis revealed several groups of coordinately expressed genes in four prominent functional clusters as defined by IPA, including a cell growth and proliferation gene network cluster and three unique gene clusters related to IFN signaling and innate immunity (Fig. 1). Genes in the cell growth and proliferation gene cluster were generally upregulated in the lungs of H3N2-infected ferrets and downregulated in the lungs of H5N1-infected ferrets. Genes in two of the IFN signaling clusters were generally upregulated in the lungs of H5N1-infected ferrets beginning at 2 dpi relative to their expression in H3N2-infected ferrets. A third IFN/acute-phase response signaling cluster, enriched in IFN and complement genes, was expressed similarly in both groups. A few ferrets appeared to behave differently in terms of gene expression from others in their group. The outbred status of the ferrets used in this study may be at least partially responsible for variation in individual host responses.

Table S2 in the supplemental material lists all four functional clusters and the genes included in each one of them. No one signaling pathway dominated the cell growth and proliferation gene network cluster; however, it was interesting to note that several genes associated with T- and B-cell signaling were significantly downregulated in H5N1-infected ferret lungs relative to H3N2-infected ferrets throughout the study period, namely, CD45 (CfaAffx.17631.1.S1_s_at; EDGE $P = 0.046$), GRB2 (CfaAffx.8081.1.S1_s_at; EDGE $P = 0.010$), and a number of phosphoinositide-3-kinase genes and mitogen-activated
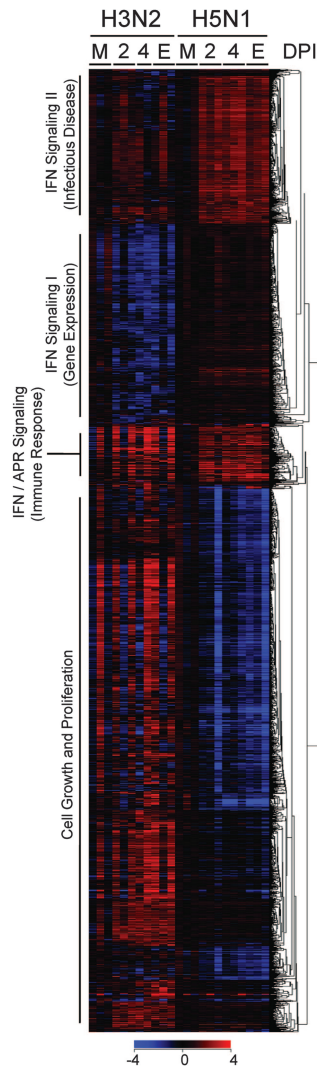
FIG. 1. Four prominent gene expression clusters identified in H3N2- and H5N1-infected ferrets. Sequential Student's *t* tests (time point versus mock) identified genes significantly differentially expressed ($P \le 0.05$ and ≥ 2-fold change) for at least one time point and group during H3N2 and H5N1 infection in ferrets. These genes were combined with significantly differentially expressed genes (≥ 2-fold change for at least one time point and group and $P \le 0.05$) identified by EDGE analysis of differential gene expression between H3N2- and H5N1-infected ferrets. As shown, 2,295 significantly different genes were analyzed by one-way (by gene) hierarchical clustering (red, upregulated; blue, downregulated). The most significant signaling pathway(s) (IFN and IFN/acute-phase response signaling) or network (cell growth and proliferation, gene expression, immune response, and infectious disease) according to IPA for the resulting clusters are noted. M, mock. E, end point as described in Materials and Methods.

protein kinase genes (see Table S2 in the supplemental material). The primary aim of this study was to perform a detailed analysis on the three clusters heavily enriched in innate-immunity-related genes.

Complement is a critical component of innate immunity and appears to play a role in the host response to influenza virus infection (4, 5, 43); however, the extent of the involvement of the complement cascade in highly pathogenic influenza virus infection is not known. In our study, SERPING1, C4, and C1QC (see Table 1 for full gene names) were significantly upregulated at time points throughout H5N1 and H3N2 infection relative to in the mock-infected ferrets but were not differentially expressed between H5N1- and H3N2-infected ferrets; however, C1QL3, C3, HF1, MASP1, and FCN1 were significantly differentially expressed between H3N2- and H5N1-infected ferrets by EDGE analysis (Table 1 and Fig. 2A). C1q is a charge pattern recognition receptor which recognizes innate immune system targets and forms a complex with C1r and C1s to constitute the first component of the serum complement system, C1 (14). C1 complex activation is regulated by the serine protease inhibitor SERPING1 (incidentally also considered an IRG), which forms a proteolytically inactive stoichiometric complex with C1r, C1s, and MASP proteases (9). The similar upregulation of SERPING1 in H5N1- and H3N2-infected ferrets relative to the mock-infected ferrets was confirmed by qRT-PCR (Fig. 2B). C4 is cleaved by activated C1 to produce C4b, an essential component of the C3 and C5 convertases of the classical pathway. Ficolins are key mediators of innate immunity that trigger the lectin pathway of the complement cascade via activation of MASP zymogens, which in turn cleave the C4 component (13, 26). Ficolin 1 (FCN1) was expressed in both H3N2 and H5N1 infection at 2 to 4 dpi to the end point, but to a significantly greater degree in H5N1-infected ferrets (Table 1). The significantly higher expression of FCN1 in H5N1-infected ferret lungs was confirmed by qRT-PCR (Fig. 2C). Accordingly, the expression of one of the targets of FCN1, MASP1, was significantly upregulated in the lungs of H5N1-infected ferrets relative to H3N2-infected ferrets (Table 1). In a visual summary of the these results, Fig. 2D shows that while genes from the classical arm of the complement pathway were similarly upregulated in both groups of influenza virus-infected ferret lungs, FCN1 and MASP1 (lectin pathway) and C3 (common to all three complement pathways) were differentially regulated between H5N1- and H3N2-infected animals. C3 gene expression was significantly downregulated in the lungs of H5N1-infected ferrets at the end point relative to H3N2-infected ferrets (Fig. 2D and Table 1). Interestingly, C3-deficient mice have been shown to be highly susceptible to primary infection with influenza A virus and exhibit delayed viral clearance and increased viral titers in the lung (23). Moreover, HF1, an inhibitor of the alternative pathway, was upregulated only in H3N2-infected ferrets at the end point, suggestive of a negative feedback loop in the lungs of H3N2-infected ferrets. Taken together, our results suggest that complement activation and regulation may be important to the success of the transition from innate to specific antiviral immune responses during influenza A virus infection.

We next looked at the expression of the large variety of IRGs identified in the three innate immunity-related clusters shown in Fig. 1. IRGs are a large family of IFN-signaling and IFN-stimulated immune mediators with pleiotropic downstream functions in innate antiviral responses and host defense (34). Figure 3A and Table 1 show that many IRGs, such as IFI44, ISG15 (G1P2), MX2, OAS1, OAS2, STAT1, TAP1, and UBE1L, were significantly upregulated throughout the study period in both H5N1- and H3N2-infected ferret lungs relative to the mock-infected ferret lungs. These genes, while coordinately expressed, have diverse functions in host defense. IFI44,

TABLE 1. Expression of selected genes in H3N2 versus H5N1-infected ferrets

Pathway and gene symbol	Name or description	Affymetrix identification	Entrez gene identification[a]	Mean gene expression (H3N2/H5N1) at[b]:			EDGE P value[c]
				2 dpi	4 dpi	End point	
IFN signaling							
CD274	CD274 molecule	CfaAffx.4085.1.S1_at	29126	**0.73**/0.19	**0.6**/0.58	**0.79**/**1.93**	0.036
COPB2	Coatomer protein complex, subunit beta 2	Cfa.3255.1.A1_s_at	9276	−0.05/−0.29	1.04/**0.69**	−0.17/0.21	0.890
CSF2RA	Colony-stimulating factor 2 receptor, alpha	CfaAffx.17240.1.S1_at	1438	−0.2/−0.04	**−1.36**/0.25	**−1.02**/0.31	0.002
CSNK1D	Casein kinase 1, delta	CfaAffx.10096.1.S1_s_at	1453	**0.63**/**1.35**	0.37/**1.62**	0.62/**1.48**	0.053
CXCL10	Chemokine (C-X-C motif) ligand 10	Cfa.16590.1.S2_at	3627	0.17/**0.98**	0.32/**0.93**	−0.34/**1.22**	0.022
FGG	Fibrinogen gamma chain	Cfa.5998.1.A1_x_at	2266	0.65/−0.36	0.25/0.38	0.27/1.68	0.044
FUBP1	FUSE binding protein 1	Cfa.19489.1.S1_at	8880	−0.58/0.18	−1.27/0.08	−0.95/**0.34**	0.030
GEM	GTP binding protein overexpressed in skeletal muscle	CfaAffx.14457.1.S1_at	2669	−0.01/**0.82**	**−1.25**/**0.81**	−0.3/**0.69**	0.032
GYS2	Glycogen synthase 2	Cfa.3601.1.S1_s_at	2998	−1.03/0.31	**−0.88**/0.46	0.07/0.35	0.007
HMMR	Hyaluronan-mediated motility receptor	CfaAffx.26316.1.S1_s_at	3161	−0.31/**0.44**	**−1.26**/**0.47**	**−0.89**/**0.66**	<0.001
IFI44	IFN-induced protein 44	CfaAffx.31148.1.S1_at	10561	**2.68**/1.01	2.97/**1.4**	1.5/**0.91**	0.494
IFI44L	IFN-induced protein 44-like	CfaAffx.31150.1.S1_at	10964	**1.57**/0.41	−0.21/**1.24**	0.08/0.92	0.201
IFI6	IFN-α-inducible protein 6	Cfa.20456.1.S1_at	2537	0.18/**1.48**	**−0.1**/**1.49**	−0.17/**1.09**	0.001
IFITM1	IFN-induced transmembrane protein 1	CfaAffx.10684.1.S1_at	10581	0.1/**1.15**	0.25/**1.61**	0.43/0.55	0.033
IRF4	IFN regulatory factor 4	CfaAffx.11513.1.S1_at	3662	0.25/**1.09**	−0.44/**1.55**	−0.42/**1.17**	0.009
ISG15	ISG15 ubiquitin-like modifier	Cfa.10757.1.S1_at	9636	**2.73**/**2.74**	**2.31**/**2.83**	0.51/**2.35**	0.266
JUN	Jun oncogene	CfaAffx.28854.1.S1_at	3725	1.7/**0.68**	3.07/**0.81**	1.69/**1.04**	0.691
MX2	Myxovirus (influenza virus) resistance 2	Cfa.3609.1.S1_s_at	4600	**0.55**/**0.75**	−0.04/**1.11**	0.16/**0.83**	0.193
OAS1	2′,5′-Oligoadenylate synthetase 1	Cfa.21191.1.S1_a_at	4938	0.01/0.92	**1.08**/**1.03**	0.3/0.55	0.708
OAS2	2′,5′-Oligoadenylate synthetase 2	CfaAffx.14097.1.S1_s_at	4939	**1.09**/0.81	1.36/**1.15**	0.51/**1.08**	0.433
PBEF1	Pre-B-cell colony-enhancing factor 1	Cfa.18345.1.S1_s_at	10135	0.42/−0.13	1.43/0.04	1.2/**1.55**	0.363
PIAS1	Protein inhibitor of activated STAT 1	Cfa.19469.1.S1_at	8554	−0.72/−0.05	−1.4/0.23	−1.15/0.2	0.041
PIM1	Pim-1 oncogene	CfaAffx.3073.1.S1_at	5292	0.37/**0.92**	−0.84/**1.09**	0.34/**0.83**	0.110
SERPING1	Serpin peptidase inhibitor, clade G, 1	Cfa.3117.2.A1_a_at	710	1.73/**1.34**	1.11/**1.63**	**2.15**/0.95	0.526
SERPING1	Serpin peptidase inhibitor, clade G, 1	CfaAffx.12560.1.S1_s_at	710	0.97/0.98	0.51/**1.62**	**1.48**/0.79	0.640
SOCS1	Suppressor of cytokine signaling 1	CfaAffx.28878.1.S1_at	8651	−0.32/**0.59**	**−1.65**/**0.48**	−0.34/**0.7**	0.048
STAT1	Signal transducer and activator of transcription 1	CfaAffx.15419.1.S1_at	6772	**0.92**/0.71	0.67/**1.21**	0.6/0.64	0.964
STAT2	Signal transducer and activator of transcription 2	CfaAffx.1159.1.S1_at	6773	**−0.8**/−0.15	**−1.01**/0.11	−0.24/0.07	0.002
TAP1	Transporter 1	Cfa.3155.1.S1_at	6890	2.29/0.84	**2.1**/**1.59**	1.37/**1.92**	0.401
UBE1L	Ubiquitin-activating enzyme E1-like	Cfa.3348.1.A1_s_at	7318	1.15/**2.05**	**1.11**/**2.47**	0.99/**1.87**	0.401
VAT1	Vesicle amine transport protein 1 homolog	CfaAffx.22532.1.S1_s_at	10493	**0.36**/**1.02**	0.09/**1.08**	−0.4/**0.87**	0.007
Complement							
C1QC	Complement component 1, q subcomponent, C chain	Cfa.10921.1.S1_at	714	0.81/**1.71**	**2.36**/**1.58**	1.47/**1.25**	0.848
C1QL3	Complement component 1, q subcomponent-like 3	Cfa.9757.1.A1_at	389941	−1.3/0.28	−1.3/0.28	−1.07/0.49	0.040
C3	Complement component 3	Cfa.12240.1.A1_at	718	−0.47/0.21	−1.15/0.48	**2.33**/**−1.6**	0.004
C3	Complement component 3	Cfa.13267.1.A1_s_at	718	−0.8/−0.17	**−1.37**/−0.44	0.41/−1.07	0.020
C4	Complement component 4	CfaAffx.1993.1.S1_s_at	720	2.34/**1.6**	0.94/**2.21**	**3.66**/1.17	0.342
CFI	Complement factor I (IF)	Cfa.14495.2.S1_at	3426	0.48/**1.15**	−0.46/**1.25**	0.06/**1.12**	0.084
FCN1	Ficolin 1	Cfa.13207.1.A1_s_at	2219	2.12/0.76	**2.9**/**3.22**	1.49/**4.24**	0.027
FCN1	Ficolin 1	CfaAffx.30397.1.S1_at	2219	0.8/0.21	**1.33**/**2.24**	**0.92**/**3.11**	0.001
HF1	Complement factor H	Cfa.20016.1.S1_at	3075	−0.46/−1.01	−0.36/−0.14	0.9/**−1.64**	0.019
MASP1	Mannan-binding lectin serine peptidase 1	CfaAffx.21390.1.S1_at	5648	**0.19**/**0.55**	0.18/**0.96**	**0.35**/**1.08**	0.016
SERPING1	Serpin peptidase inhibitor, clade G, 1	Cfa.3117.2.A1_a_at	710	1.73/**1.34**	1.11/**1.63**	**2.15**/0.95	0.526
SERPING1	Serpin peptidase inhibitor, clade G, 1	CfaAffx.12560.1.S1_s_at	710	0.97/0.98	0.51/**1.62**	1.48/**0.79**	0.640

[a] The Entrez gene identification corresponds to the human homolog. E, endpoint as described in Materials and Methods.
[b] Mean gene expression is normalized to the means for the corresponding mock infection ($\log_2$ ratio). Boldface indicates significantly differentially expressed genes ($P \leq 0.05$) versus the mock infection by the sequential Student's t tests.
[c] Statistical significance of gene expression differences between H3N2- and H5N1-infected ferrets determined by the EDGE analysis.

MX2, and OAS2 are antiviral IRGs induced mainly by type I IFNs (IFN-α and IFN-β), while OAS1 is induced by type I and II (IFN-γ) IFNs and mediates cellular resistance against viral infection (UNIPROT). UBE1L catalyzes the initial step of ISG15 activation, a target of influenza NS1B virus inhibition (41). ISG15 is an important ubiquitin-like modifier that conjugates with critical intracellular antiviral targets upon type I IFN stimulation, such as JAK1 and STAT1 (27). STAT1 is phosphorylated by Jak kinases upon type I and II IFN ligation of their cognate cell surface receptors, leading to IRG tran-

scription and initiation of an antiviral cellular state (25). The similar upregulation of STAT1 in H5N1- and H3N2-infected ferrets relative to the mock-infected ferrets was confirmed by qRT-PCR (Fig. 3B). TAP1, induced by IFN-γ, is critical for major histocompatibility complex class I antigen presentation, thereby facilitating the identification and eradication of virally infected cells (1).

Significant differences in the expression of other IRGs between H3N2- and H5N1-infected ferret lungs occurred throughout course of disease (Table 1). STAT2 gene expres-
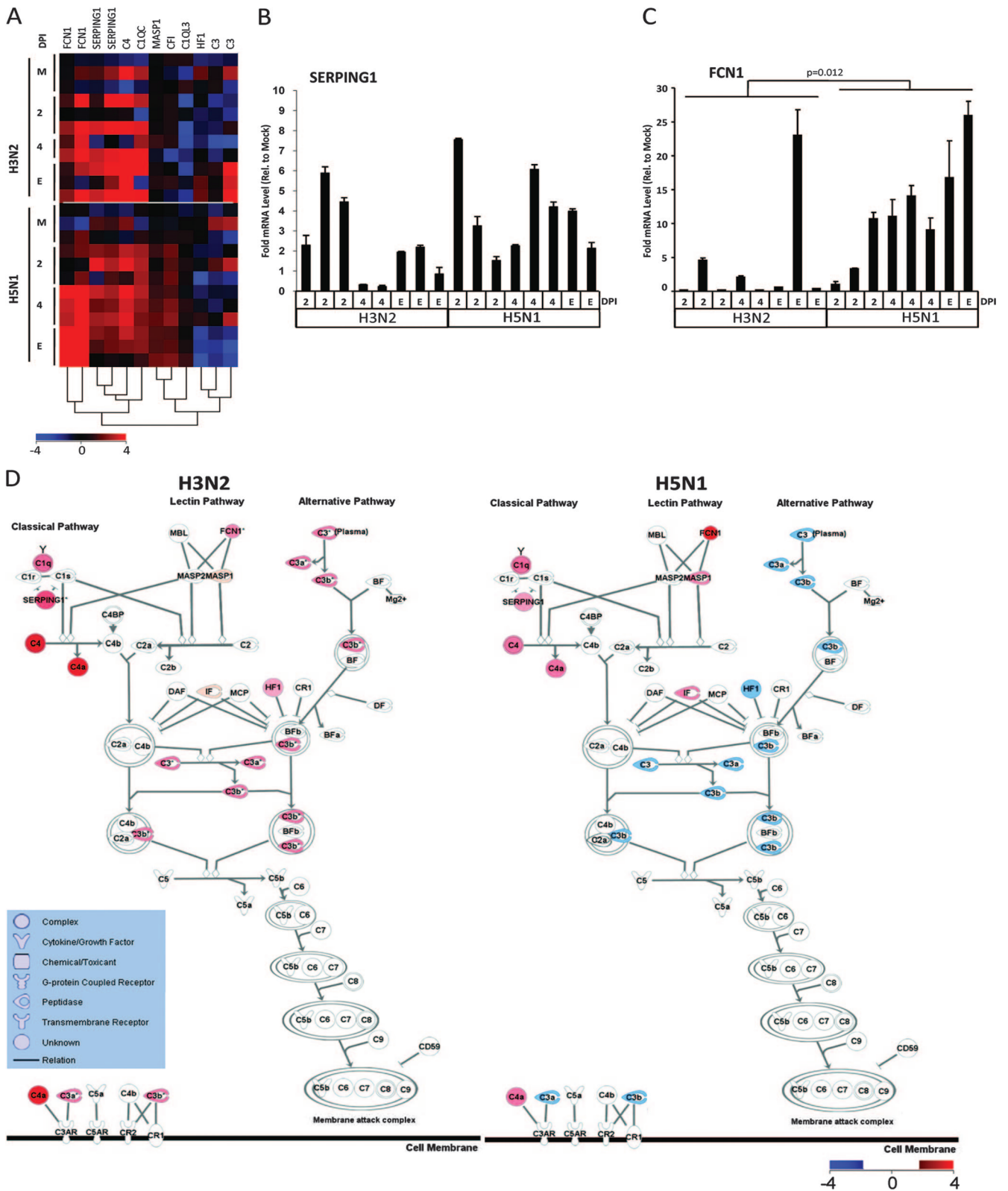
FIG. 2. Complement cascade activation in the lungs of influenza virus-infected ferrets. (A) One-way hierarchical clustering of complement-related genes selected by IPA from the three innate-immunity-related gene clusters in Fig. 1 (red, upregulated; blue, downregulated). (B) qRT-PCR analysis of SERPING1 gene expression performed in triplicate. Error bars indicate standard deviations. (C) qRT-PCR analysis of FCN1 performed in triplicate. Error bars indicate standard deviations, and the Mann-Whitney P value is shown. (D) IPA canonical pathway modeling of the complement system using the microarray analysis of gene expression data from the lungs of influenza virus-infected ferrets at the end point (red, upregulated; blue, downregulated). M, mock. E, end point as described in Materials and Methods.
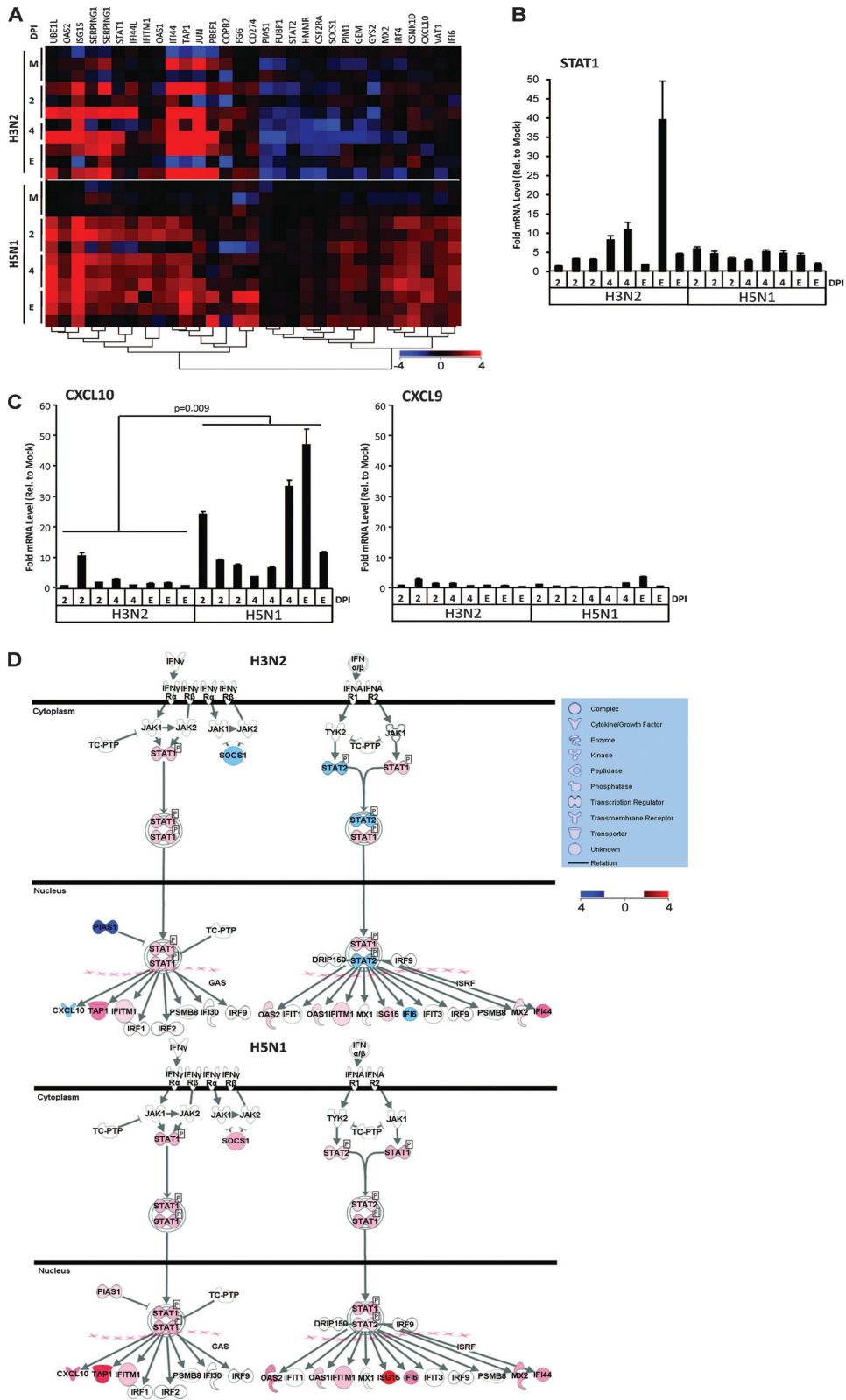
FIG. 3. IFN signaling pathway activation in the lungs of influenza virus-infected ferrets. (A) One-way hierarchical clustering of IRGs selected by IPA from the three innate immunity-related gene clusters in Fig. 1 (red, upregulated; blue, downregulated). (B) qRT-PCR analysis of STAT1 performed in triplicate. Error bars indicate standard deviations. (C) Left, qRT-PCR analysis of CXCL10. The Mann-Whitney P value is shown. Right, qRT-PCR analysis of CXCL9. Analyses were performed in triplicate, and error bars indicate standard deviations. (D) IPA modeling of the IFN signaling pathway using the microarray analysis of gene expression data from the lungs of influenza virus-infected ferrets at the end point (red, upregulated; blue, downregulated). M, mock. E, end point as described in Materials and Methods.
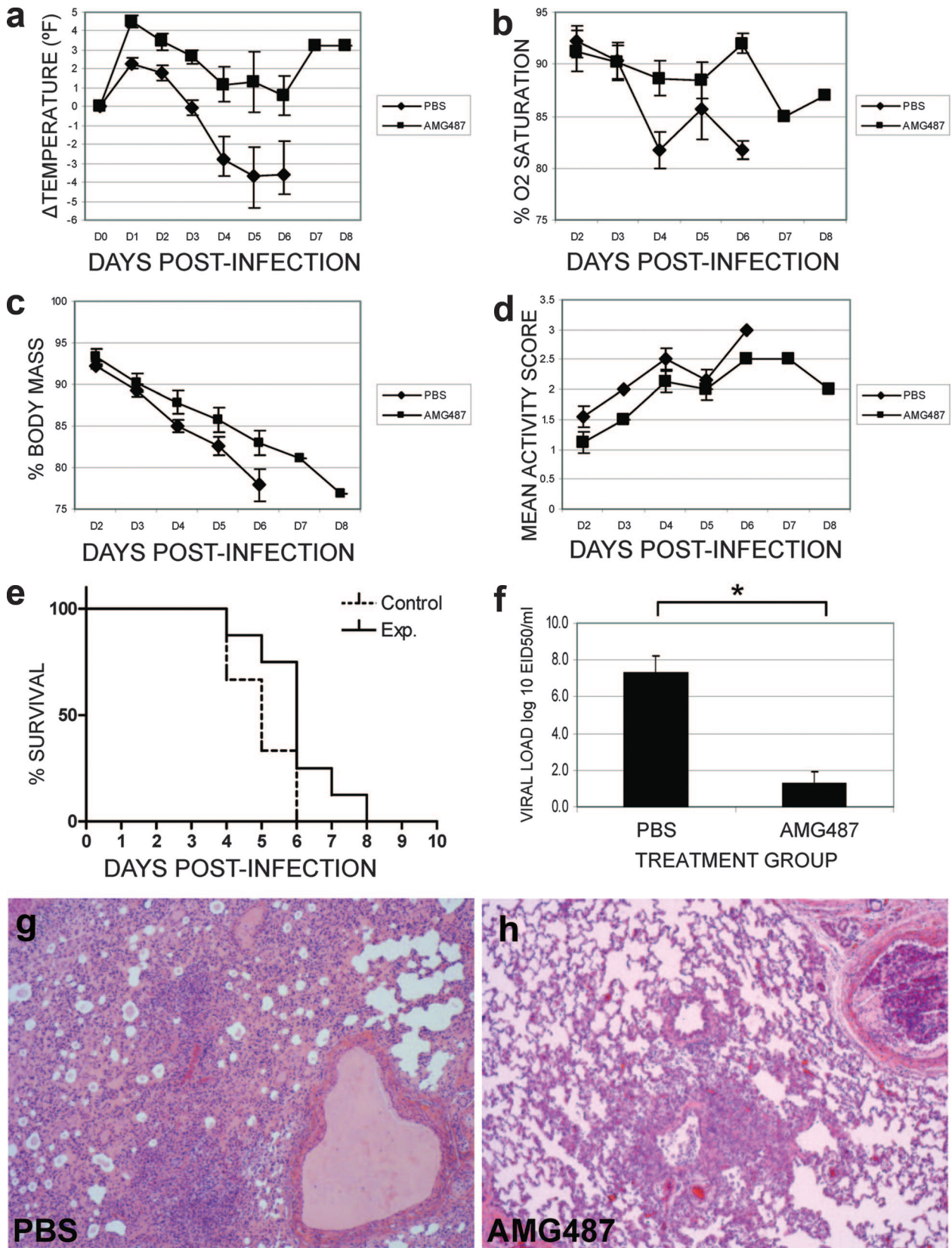
FIG. 4. AMG487 treatment improves respiratory function and delays mortality in H5N1-infected ferrets. Ferrets were infected with H5N1 as described in Materials and Methods and received either 1.65 mg/kg AMG487 in a 3-ml volume of PBS ($n = 8$) or PBS vehicle ($n = 9$) intraperitoneally every 12 h starting at 24 h postinfection and continuing until the end point. Weight loss, activity, temperature, and SpO2 (pulse oximetry) were monitored daily from 2 dpi. (a) Change in body temperature relative to baseline means ($P \leq 0.001$ by two-way ANOVA). (b) SpO2

sion was significantly downregulated in H3N2-infected ferrets versus H5N1-infected ferrets (Fig. 3A and Table 1). As modeled in IPA at the end point in Fig. 3D, increases in STAT2 transcription upon type I IFN stimulation may result in increased levels of STAT2 protein available for dimerization with STAT1 and serve to increase the transcription of many antiviral IRGs (34). Indeed, significant upregulation of CD274, IFI6 (G1P3), IFITM1, and IRF4 was noted in H5N1- versus H3N2-infected ferret lungs at time points throughout the course of disease (Table 1). CD274 (B7-H1) is upregulated on lymphocytes upon IFN-γ activation and plays a role in T-cell costimulation (UNIPROT) and apoptosis during viral infections (28). IFI6 is an antiviral response protein induced by type I IFNs with a particular potency in suppressing hepatitis C virus (17). IFITM1 is induced by type I and II IFNs and is involved in the control of cell growth and proliferation (10). Interestingly, IRF4 is not induced by IFNs but binds to the IFN-stimulated response element of the major histocompatibility complex class I promoter (UNIPROT) and has been implicated in the control of T-helper 2 (Th2) cell subset differentiation (36). SOCS1, a negative feedback regulator of cytokine signaling, was significantly downregulated in H3N2-infected ferrets and increased in the H5N1-infected ferrets (Table 1 and Fig. 3D). Likewise, PIAS1, a direct inhibitor of STAT1, is significantly attenuated in H3N2-infected ferrets and upregulated in H5N1-infected ferrets (Table 1 and Fig. 3D). Overall, our microarray analysis indicates that H5N1 and H3N2 influenza A virus infections result in notable IFN-mediated antiviral host responses in the lungs of ferrets. However, hyperinduction and persistent expression of certain IRGs in the lungs of H5N1-infected ferrets, despite functional negative feedback and perhaps in conjunction with suppressed T- and B-cell signaling (Fig. 1), may provoke the immune dysregulation characteristic of highly pathogenic H5N1 influenza virus.

CXCL10 gene expression was significantly upregulated in H5N1-infected ferret lungs throughout the course of the study relative to H3N2-infected ferret lungs as confirmed by qRT-PCR and EDGE analysis (Fig. 3C and Table 1). CXCL10 is a potent chemoattractant for activated Th1 lymphocytes and natural killer cells and is thought to play a role in the temporal development of innate and adaptive immunity in concert with type I and II IFNs (29). CXCL10's cognate receptor is CXCR3, which also binds CXCL9 and CXCL11. The expression of CXCL9 and CXCL11 could not be measured by microarray due to the absence of the probes on the Canine 2.0 array; however, by qRT-PCR we showed that CXCL9 expression was not induced to the level of CXCL10 expression during influenza virus infection and was not significantly different between H5N1- and H3N2-infected ferret lungs relative to the mock infection (Fig. 3C). The drug AMG487 is a potent antagonist of CXCR3 and can inhibit cell migration mediated by CXCL9,

CXCL10, and CXCL11, thereby blocking cellular recruitment (19) and tumor metastasis (38) in mice. We therefore tested whether attenuation of CXCR3 signaling by AMG487 could alter the disease course in H5N1-infected ferrets. We challenged 17 ferrets with H5N1 virus and randomly assigned 8 to treatment with AMG487 and 9 to treatment with PBS as per the schedule described in Materials and Methods. AMG487 treatment significantly abrogated hypothermia (Fig. 4a), increased SpO2 levels (Fig. 4b), decreased weight loss (Fig. 4c), and improved daily activity scores (Fig. 4d). AMG487 treatment also resulted in a significant shift in the length of survival (Fig. 4e) in that all vehicle-treated ferrets were deceased by the end of 6 dpi while 25% of the AMG487-treated ferrets survived until the end of 7 or 8 dpi. Nasal wash viral loads were not significantly different between AMG487- and vehicle-treated ferrets (data not shown); however, AMG487-treated ferrets exhibited significantly reduced viral loads in the lungs compared to controls at 6 dpi (Fig. 4f). Accordingly, AMG487-treated ferret lungs showed a marked reduction in interstitial and alveolar edema and infiltrate compared with controls at 6 dpi (Fig. 4g and h). Therefore, even though our model of infection of ferrets with H5N1 influenza virus at 1×10^6 EID$_{50}$ showed 100% lethality, blockade of the CXCL9, CXCL10, and CXCL11-CXCR3 signaling axis by AMG487 treatment caused a significant shift in the kinetics of viral replication in the lung and in the clinical course of disease.

Vaccines are the ideal means of protecting the population against an influenza outbreak, and a number of promising candidates have been recently tested (2, 16, 42). Unfortunately, influenza virus vaccine efficacy is variable in certain populations, such as the elderly (18), and the emergence of novel strains of influenza virus for which vaccines are not immediately available requires the development of other strategies. While antivirals may prove beneficial in the treatment of avian influenza virus disease in humans, circulating strains of H5N1 influenza virus exhibit variable susceptibility to antiviral agents, with full resistance to M2 inhibitors displayed by clade 1 and most clade 2 viruses and high levels of resistance to oseltamivir in clade 2 viruses. Ideally, a treatment which does not rely on virus strain specificity and one that does not allow for the development of virus resistance would complement vaccine and antiviral strategies. AMG487 has shown clinical promise in animal models and has been well tolerated in human phase II clinical trials (reviewed in reference 39), but its clinical potential in treating influenza is unknown. With appropriate future study, AMG487 treatment may demonstrate effectiveness in combination with other strategies, such as antiviral treatment, in reducing lung immunopathology, establishing effective antiviral IRG responses, and reducing viral burden during H5N1 infection.

One caveat of our study is that differences between H5N1

($P \leq 0.005$ by two-way ANOVA). (c) Weight loss (percentage of original body mass; $P \leq 0.001$ by two-way ANOVA). (d) Mean daily activity scores: 0, normal; 1, alert, playful when stimulated; 2, alert, not playful when stimulated; 3, neither alert nor playful when stimulated ($P \leq 0.05$ by two-way ANOVA). (e) Kaplan-Meier survival curves for AMG487 (Exp.)-versus vehicle (Control)-treated ferrets. Curves are significantly different ($P \leq 0.05$) by log rank analysis. (f) Lung viral titers in three ferrets from each treatment group euthanized at 6 dpi (∗, $P \leq 0.05$ by Student's t test). (g and h) Representative hematoxylin- and eosin-stained lung sections at 6 dpi (magnification, ×4). Error bars represent standard errors of the means in all panels.

and H3N2 pathogenicity in the lung during infection may not have been directly reflected by the microarray analysis, since H3N2 did not replicate to detectable levels in the ferret lungs. H3N2 infection served as an important background against which to identify potential host responses specific to H5N1 infection in ferrets for further analysis. Also, the lack of availability of ferret-specific reagents and sequences limited the extent to which we could validate our microarray results by qRT-PCR and/or protein assays. Nonetheless, our results are significant in that we have identified innate immune genes that are similarly expressed during H5N1 and H3N2 infection, as well as notable genes involved in complement and IFN signaling that are differentially expressed in the respiratory tracts of H5N1 influenza virus-infected ferrets and H3N2-infected ferrets.

Several recent microarray studies have highlighted the common involvement of IFN responses in the acute phase of uncomplicated influenza virus infections in humans, macaques, and rodents (3, 21, 30); however, insufficient knowledge exists regarding the mechanisms of host inflammatory cytokine and chemokine responses in severe H5N1 pathogenesis (11, 32, 33, 40). Mechanistically, we have attenuated the action of CXCL10 by blocking its activation of CXCR3 using AMG487 drug treatment, thereby reducing pulmonary viral load and pathology, improving respiratory function, and eliciting a modest yet statistically significant delay in mortality in H5N1-infected ferrets. Previous in vitro results show that H5N1-infected primary human alveolar and bronchial epithelial cells have a greater capacity for CXCL10 induction than those infected with common strains of human influenza virus (8). In agreement with our current results, it has also been shown that CXCL10 and not CXCL9 expression correlates with pharyngeal viral load in human H5N1 infections (11). Likewise, we have previously shown that high levels of CXCL10 and not CXCL9 are associated with persistent severe viral disease in patients with severe acute respiratory syndrome (6, 7). Finally, the severity and outcome of 1918 influenza virus infection in a macaque model may be determined by dysregulated IFN responses that arise during host innate immunity (22). Our results provide further evidence that IRGs, in particular CXCL10, may have pathological importance in H5N1 infection and are at least partially responsible for disease pathogenesis. The parallels among the immunopathologies of severe acute respiratory syndrome virus, 1918 influenza virus, and avian influenza virus (H5N1) suggest a common underlying mechanism in the natural disease course of these infections. In this regard, AMG487 treatment has unique possibilities in correcting dysregulated host responses during severe respiratory viral illnesses and warrants further exploration in complementing existing potential therapies.

REFERENCES

1. **Abele, R., and R. Tampe.** 2004. The ABCs of immunology: structure and function of TAP, the transporter associated with antigen processing. Physiology (Bethesda) **19:**216–224.
2. **Baras, B., K. J. Stittelaar, J. H. Simon, R. J. Thoolen, S. P. Mossman, F. H. Pistoor, G. van Amerongen, M. A. Wettendorff, E. Hanon, and A. D. Osterhaus.** 2008. Cross-protection against lethal H5N1 challenge in ferrets with an adjuvanted pandemic influenza vaccine. PLoS One **3:**e1401.
3. **Baskin, C. R., A. Garcia-Sastre, T. M. Tumpey, H. Bielefeldt-Ohmann, V. S. Carter, E. Nistal-Villan, and M. G. Katze.** 2004. Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (*Macaca nemestrina*). J. Virol. **78:**10420–10432.
4. **Beebe, D. P., R. D. Schreiber, and N. R. Cooper.** 1983. Neutralization of influenza virus by normal human sera: mechanisms involving antibody and complement. J. Immunol. **130:**1317–1322.
5. **Bjornson, A. B., M. A. Mellencamp, and G. M. Schiff.** 1991. Complement is activated in the upper respiratory tract during influenza virus infection. Am. Rev. Respir. Dis. **143:**1062–1066.
6. **Cameron, M. J., J. F. Bermejo-Martin, A. Danesh, M. P. Muller, and D. J. Kelvin.** 2008. Human immunopathogenesis of severe acute respiratory syndrome (SARS). Virus Res. **133:**13–19.
7. **Cameron, M. J., L. Ran, L. Xu, A. Danesh, J. F. Bermejo-Martin, C. M. Cameron, M. P. Muller, W. L. Gold, S. E. Richardson, S. M. Poutanen, B. M. Willey, M. E. Devries, Y. Fang, C. Seneviratne, S. E. Bosinger, D. Persad, P. Wilkinson, L. D. Greller, R. Somogyi, A. Humar, S. Keshavjee, M. Louie, M. B. Loeb, J. Brunton, A. J. McGeer, and D. J. Kelvin.** 2007. Interferon-mediated immunopathological events are associated with atypical innate and adaptive immune responses in patients with severe acute respiratory syndrome. J. Virol. **81:**8692–8706.
8. **Chan, M. C., C. Y. Cheung, W. H. Chui, S. W. Tsao, J. M. Nicholls, Y. O. Chan, R. W. Chan, H. T. Long, L. L. Poon, Y. Guan, and J. S. Peiris.** 2005. Proinflammatory cytokine responses induced by influenza A (H5N1) viruses in primary human alveolar and bronchial epithelial cells. Respir. Res. **6:**135.
9. **Cicardi, M., L. Zingale, A. Zanichelli, E. Pappalardo, and B. Cicardi.** 2005. C1 inhibitor: molecular and clinical aspects. Springer Semin. Immunopathol. **27:**286–298.
10. **Deblandre, G. A., O. P. Marinx, S. S. Evans, S. Majjaj, O. Leo, D. Caput, G. A. Huez, and M. G. Wathelet.** 1995. Expression cloning of an interferon-inducible 17-kDa membrane protein implicated in the control of cell growth. J. Biol. Chem. **270:**23860–23866.
11. **de Jong, M. D., C. P. Simmons, T. T. Thanh, V. M. Hien, G. J. Smith, T. N. Chau, D. M. Hoang, N. V. Chau, T. H. Khanh, V. C. Dong, P. T. Qui, B. V. Cam, D. Q. Ha, Y. Guan, J. S. Peiris, N. T. Chinh, T. T. Hien, and J. Farrar.** 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat. Med. **12:**1203–1207.
12. **de Veer, M. J., M. Holko, M. Frevel, E. Walker, S. Der, J. M. Paranjape, R. H. Silverman, and B. R. Williams.** 2001. Functional classification of interferon-stimulated genes identified using microarrays. J. Leukoc. Biol. **69:**912–920.
13. **Frederiksen, P. D., S. Thiel, C. B. Larsen, and J. C. Jensenius.** 2005. M-ficolin, an innate immune defence molecule, binds patterns of acetyl groups and activates complement. Scand. J. Immunol. **62:**462–473.
14. **Ghai, R., P. Waters, L. T. Roumenina, M. Gadjeva, M. S. Kojouharova, K. B. Reid, R. B. Sim, and U. Kishore.** 2007. C1q and its growing family. Immunobiology **212:**253–266.
15. **Govorkova, E. A., J. E. Rehg, S. Krauss, H. L. Yen, Y. Guan, M. Peiris, T. D. Nguyen, T. H. Hanh, P. Puthavathana, H. T. Long, C. Buranathai, W. Lim, R. G. Webster, and E. Hoffman.** 2006. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J. Virol. **80:**6195.
16. **Itoh, Y., H. Ozaki, H. Tsuchiya, K. Okamoto, R. Torii, Y. Sakoda, Y. Kawaoka, K. Ogasawara, and H. Kida.** 2008. A vaccine prepared from a non-pathogenic H5N1 avian influenza virus strain confers protective immunity against highly pathogenic avian influenza virus infection in cynomolgus macaques. Vaccine **26:**562–572.
17. **Itsui, Y., N. Sakamoto, M. Kurosaki, N. Kanazawa, Y. Tanabe, T. Koyama, Y. Takeda, M. Nakagawa, S. Kakinuma, Y. Sekine, S. Maekawa, N. Enomoto, and M. Watanabe.** 2006. Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. J. Viral Hepat. **13:**690–700.
18. **Jefferson, T., D. Rivetti, A. Rivetti, M. Rudin, C. Di Pietrantonj, and V. Demicheli.** 2005. Efficacy and effectiveness of influenza vaccines in elderly people: a systematic review. Lancet **366:**1165–1174.
19. **Johnson, M., A. R. Li, J. Liu, Z. Fu, L. Zhu, S. Miao, X. Wang, Q. Xu, A. Huang, A. Marcus, F. Xu, K. Ebsworth, E. Sablan, J. Danao, J. Kumer, D. Dairaghi, C. Lawrence, T. Sullivan, G. Tonn, T. Schall, T. Collins, and**

J. Medina. 2007. Discovery and optimization of a series of quinazolinone-derived antagonists of CXCR3. Bioorg. Med. Chem. Lett. **17:**3339–3343.

20. **Kandun, I. N., H. Wibisono, E. R. Sedyaningsih, Yusharmen, W. Hadisoedarsuno, W. Purba, H. Santoso, C. Septiawati, E. Tresnaningsih, B. Heriyanto, D. Yuwono, S. Harun, S. Soeroso, S. Giriputra, P. J. Blair, A. Jeremijenko, H. Kosasih, S. D. Putnam, G. Samaan, M. Silitonga, K. H. Chan, L. L. Poon, W. Lim, A. Klimov, S. Lindstrom, Y. Guan, R. Donis, J. Katz, N. Cox, M. Peiris, and T. M. Uyeki.** 2006. Three Indonesian clusters of H5N1 virus infection in 2005. N. Engl. J. Med. **355:**2186–2194.

21. **Kawada, J., H. Kimura, Y. Kamachi, K. Nishikawa, M. Taniguchi, K. Nagaoka, H. Kurahashi, S. Kojima, and T. Morishima.** 2006. Analysis of gene-expression profiles by oligonucleotide microarray in children with influenza. J. Gen. Virol. **87:**1677–1683.

22. **Kobasa, D., S. M. Jones, K. Shinya, J. C. Kash, J. Copps, H. Ebihara, Y. Hatta, J. H. Kim, P. Halfmann, M. Hatta, F. Feldmann, J. B. Alimonti, L. Fernando, Y. Li, M. G. Katze, H. Feldmann, and Y. Kawaoka.** 2007. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature **445:**319–323.

23. **Kopf, M., B. Abel, A. Gallimore, M. Carroll, and M. F. Bachmann.** 2002. Complement component C3 promotes T-cell priming and lung migration to control acute influenza virus infection. Nat. Med. **8:**373–378.

24. **Leek, J. T., E. Monsen, A. R. Dabney, and J. D. Storey.** 2006. EDGE: extraction and analysis of differential gene expression. Bioinformatics **22:**507–508.

25. **Leonard, W. J., and J. J. O'Shea.** 1998. Jaks and STATs: biological implications. Annu. Rev. Immunol. **16:**293–322.

26. **Liu, Y., Y. Endo, D. Iwaki, M. Nakata, M. Matsushita, I. Wada, K. Inoue, M. Munakata, and T. Fujita.** 2005. Human M-ficolin is a secretory protein that activates the lectin complement pathway. J. Immunol. **175:**3150–3156.

27. **Liu, Y. C., J. Penninger, and M. Karin.** 2005. Immunity by ubiquitylation: a reversible process of modification. Nat. Rev. Immunol. **5:**941–952.

28. **Muhlbauer, M., M. Fleck, C. Schutz, T. Weiss, M. Froh, C. Blank, J. Scholmerich, and C. Hellerbrand.** 2006. PD-L1 is induced in hepatocytes by viral infection and by interferon-alpha and -gamma and mediates T cell apoptosis. J. Hepatol. **45:**520–528.

29. **Neville, L. F., G. Mathiak, and O. Bagasra.** 1997. The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. Cytokine Growth Factor Rev. **8:**207–219.

30. **Pennings, J. L., T. G. Kimman, and R. Janssen.** 2008. Identification of a common gene expression response in different lung inflammatory diseases in rodents and macaques. PLoS One **3:**e2596.

31. **Salomon, R., J. Franks, E. A. Govorkova, N. A. Ilyushina, H. L. Yen, D. J. Hulse-Post, J. Humberd, M. Trichet, J. E. Rehg, R. J. Webby, R. G. Webster, and E. Hoffmann.** 2006. The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. J. Exp. Med. **203:**689–697.

32. **Salomon, R., E. Hoffmann, and R. G. Webster.** 2007. Inhibition of the cytokine response does not protect against lethal H5N1 influenza infection. Proc. Natl. Acad. Sci. **104:**12479–12481.

33. **Szretter, K. J., S. Gangappa, X. Lu, C. Smith, W. J. Shieh, S. R. Zaki, S. Sambhara, T. M. Tumpey, and J. M. Katz.** 2007. Role of host cytokine responses in the pathogenesis of avian H5N1 influenza viruses in mice. J. Virol. **81:**2736–2744.

34. **Takaoka, A., and H. Yanai.** 2006. Interferon signalling network in innate defence. Cell. Microbiol. **8:**907–922.

35. **To, K. F., P. K. Chan, K. F. Chan, W. K. Lee, W. Y. Lam, K. F. Wong, N. L. Tang, D. N. Tsang, R. Y. Sung, T. A. Buckley, J. S. Tam, and A. F. Cheng.** 2001. Pathology of fatal human infection associated with avian influenza A H5N1 virus. J. Med. Virol. **63:**242–246.

36. **Tominaga, N., K. Ohkusu-Tsukada, H. Udono, R. Abe, T. Matsuyama, and K. Yui.** 2003. Development of Th1 and not Th2 immune responses in mice lacking IFN-regulatory factor-4. Int. Immunol. **15:**1–10.

37. **Ungchusak, K., P. Auewarakul, S. F. Dowell, R. Kitphati, W. Auwanit, P. Puthavathana, M. Uiprasertkul, K. Boonnak, C. Pittayawonganon, N. J. Cox, S. R. Zaki, P. Thawatsupha, M. Chittaganpitch, R. Khontong, J. M. Simmerman, and S. Chunsutthiwat.** 2005. Probable person-to-person transmission of avian influenza A (H5N1). N. Engl. J. Med. **352:**333–340.

38. **Walser, T. C., S. Rifat, X. Ma, N. Kundu, C. Ward, O. Goloubeva, M. G. Johnson, J. C. Medina, T. L. Collins, and A. M. Fulton.** 2006. Antagonism of CXCR3 inhibits lung metastasis in a murine model of metastatic breast cancer. Cancer Res. **66:**7701–7707.

39. **Wijtmans, M., D. Verzijl, R. Leurs, I. J. de Esch, and M. J. Smit.** 2008. Towards small-molecule CXCR3 ligands with clinical potential. Chem. Med. Chem. **3:**861–872.

40. **Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza.** 2008. Update on avian influenza A (H5N1) virus infection in humans. N. Engl. J. Med. **358:**261–273.

41. **Yuan, W., and R. M. Krug.** 2001. Influenza B virus NS1 protein inhibits conjugation of the interferon (IFN)-induced ubiquitin-like ISG15 protein. EMBO J. **20:**362–371.

42. **Zangwill, K. M., J. J. Treanor, J. D. Campbell, D. L. Noah, and J. Ryea.** 2008. Evaluation of the safety and immunogenicity of a booster (third) dose of inactivated subvirion H5N1 influenza vaccine in humans. J. Infect. Dis.

43. **Zhang, H., Y. A. Su, P. Hu, J. Yang, B. Zheng, P. Wu, J. Peng, Y. Tang, and L. Zhang.** 2006. Signature patterns revealed by microarray analyses of mice infected with influenza virus A and Streptococcus pneumoniae. Microbes. Infect. **8:**2172–2185.

44. **Zitzow, L. A., T. Rowe, T. Morken, W. J. Shieh, S. Zaki, and J. M. Katz.** 2002. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J. Virol. **76:**4420–4429.