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Role of Host Cytokine Responses in the Pathogenesis of Avian H5N1 Influenza Viruses in Mice[▽]

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Highly pathogenic avian H5N1 influenza viruses are now widespread in poultry in Asia and have recently spread to some African and European countries. Interspecies transmission of these viruses to humans poses a major threat to public health. To better understand the basis of pathogenesis of H5N1 viruses, we have investigated the role of proinflammatory cytokines in transgenic mice deficient in interleukin-6 (IL-6), macrophage inflammatory protein 1 alpha (MIP-1α), IL-1 receptor (IL-1R), or tumor necrosis factor receptor 1 (TNFR1) by the use of two avian influenza A viruses isolated from humans, A/Hong Kong/483/97 (HK/483) and A/Hong Kong/486/97 (HK/486), which exhibit high and low lethality in mice, respectively. The course of disease and the extent of virus replication and spread in IL-6- and MIP-1α-deficient mice were not different from those observed in wild-type mice during acute infection with 1,000 50% mouse infective doses of either H5N1 virus. However, with HK/486 virus, IL-1R-deficient mice exhibited heightened morbidity and mortality due to infection, whereas no such differences were observed with the more virulent HK/483 virus. Furthermore, TNFR1-deficient mice exhibited significantly reduced morbidity following challenge with either H5N1 virus but no difference in viral replication and spread or ultimate disease outcome compared with wild-type mice. These results suggest that TNF-α may contribute to morbidity during H5N1 influenza virus infection, while IL-1 may be important for effective virus clearance in nonlethal H5N1 disease.

Highly pathogenic avian (HPAI) influenza A H5N1 viruses were first recognized to be capable of infecting humans in 1997, when wholly avian influenza viruses were transmitted directly from birds to humans in Hong Kong, causing 18 cases of respiratory disease, including six deaths (1, 8, 24, 48, 51, 52). Two additional human cases of H5N1 virus infection were identified in Hong Kong in early 2003 (37). Since late 2003, HPAI H5N1 viruses have spread across Asia to the Middle East and into Europe and Africa, causing outbreaks of disease and death in domestic poultry and wild birds. As of September 2006, approximately 240 laboratory-confirmed human cases of H5N1 virus infection have been reported by the World Health Organization. Over 50% of the human infections have been fatal (3, 58; Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO, World Health Organization 2006, http://www.who.int/csr/disease/avian_influenza/country/cases_table_2006_09_19/en/index.html).

As in the 1997 outbreak, the recent human H5N1 infections have occurred primarily among previously healthy children and younger adult patients, who typically developed fever, respiratory symptoms, leukopenia, and lymphopenia before progressing to primary viral pneumonia complicated by acute respiratory distress syndrome (3, 6, 58, 63). Limited reports of viremia and detection of viral RNA in extrapulmonary tissues and fluids suggest that H5N1 viruses can disseminate systemically in humans, at least in some individuals (7, 11, 60). Elevated levels of serum cytokines and chemokines accompanied these clinical manifestations. The occurrence of this "cytokine storm" has been proposed to contribute to the increased severity of the disease caused by these viruses (10, 37, 56, 63). In support of this hypothesis, H5N1 viruses were found to elicit substantially higher expression of proinflammatory chemokines and cytokines, in particular tumor necrosis factor alpha (TNF-α), in human primary macrophages compared with some H1N1 or H3N2 human influenza viruses (5). To assess the contribution of the proinflammatory cytokine responses to severe disease in vivo, we used a mouse model which generally reflects the severity of human H5N1 virus disease (30, 32, 59), although the central nervous system involvement typical of highly virulent H5N1 strains in mice may only be a rare complication in H5N1 virus-infected humans (11). Although mouse and human respiratory tissues differ in their overall distribution and type of sialic acid receptor expression, α-2,3-linked sialic acid, preferentially recognized by avian influenza viruses, is present in the lower respiratory tract of both species (21).

In BALB/c mice, H5N1 viruses isolated from humans in 1997 were found to replicate efficiently without the need for adaptation and could be characterized as being of high or low pathogenicity (14, 18, 24, 25, 32, 59). Systemic spread of virus, cytokine dysregulation, lymphopenia, severe tissue pathology, and death were characteristic of the infection of mice with the high-pathogenicity phenotype, represented by A/Hong Kong/483/97 (HK/483) virus, whereas viruses of the low-pathogenic-

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ity phenotype, represented by A/Hong Kong/486/97 (HK/486) virus, did not to replicate systemically and were generally not lethal for mice. Expression of proinflammatory cytokines and chemokines, including interleukin-1β (IL-1β), TNF-α, IL-6, and macrophage inflammatory protein 1 alpha (MIP-1α), in the brain of HK483 virus-infected mice was associated with the lethal phenotype of this virus (2, 36, 53, 59). These findings suggest that the mouse is suitable as a model to investigate the role of the host's proinflammatory response in the exceptional virulence of the avian H5N1 influenza viruses in mammals.

Here we use transgenic C57BL/6 (B6) or B6/129 mice deficient in TNF receptor 1 (TNFR1), IL-1 receptor (IL-1R), IL-6, and MIP-1α to better understand the role that these cytokines and chemokines play during H5N1 influenza virus infection. We found that a lack of IL-6 or MIP-1α had no effect on the kinetics of the disease process or virus replication in H5N1 virus-infected mice, whereas a lack of IL-1R signaling delayed viral clearance and enhanced the lethality of the low-pathogenicity virus. In contrast, absence of TNFR1 signaling significantly delayed morbidity but had no effect on virus replication or on the ultimate outcome of H5N1 disease in mice. These results provide insight into the mammalian host immune response during avian H5N1 influenza A virus infection which is important for the design of immunomodulatory therapies for H5N1 infection.

MATERIALS AND METHODS

Viruses. The influenza viruses used in this study were the H5N1 viruses A/Hong Kong/483/97 (HK/483) and A/Hong Kong/486/97 (HK/486). Virus stocks were propagated in the allantoic cavity of 10-day-old embryonated chicken eggs at 37°C for 24 h. The allantoic fluids were harvested, aliquoted, and stored at −70°C until use.

Laboratory facility. Because of the potential risk to humans and poultry, all experiments using infectious pathogenic avian H5N1 viruses, including work with animals, were conducted under conditions of biosafety level 3 containment that included enhancements required by the U.S. Department of Agriculture and the Select Agent Program (42). Laboratory workers were required to wear appropriate respirator equipment (RACAL Health and Safety Inc., Frederick, MD).

Mice. IL-1R (B6; 129S1-*Il1r1*^{tm1Roml})-, IL-6 (B6; 129S2-*Il6*^{tm1Kopf})-, MIP-1α (B6; 129P2-*Ccl3*^{tm1Unc}/J)-, and TNFR1 (B6; 129S-*Tnfrsf1a*^{tm1Imx}*Tnfrsf1b*^{tm1Imx})-deficient mice aged 6 to 8 weeks and age-matched C57BL/6J (B6) or B6129SF2/J (B6/129) control mice (Jackson Laboratories, Bar Harbor, ME) were used for these studies. Animal research was conducted under the guidance of the CDC's Institutional Animal Care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited animal facility.

Viral challenge. Mice were lightly anesthetized with CO_2 and infected intranasally with 1,000 50% mouse infectious doses (MID_{50}) ($10^{3.75}$ egg infectious doses [EID_{50}] of HK/483 and $10^{5.25}$ EID_{50} of HK/486) of virus stocks diluted in phosphate-buffered saline (PBS) in a 50-μl volume. The MID_{50} was determined for each mouse strain as previously described (30). Mice were monitored daily for morbidity and measured for weight loss and mortality for up to 14 days postinfection (p.i.). Any mouse that lost more than 25% of its body weight was euthanized. Due to the limited availability of the transgenic mouse strains a separate experiment was conducted to evaluate virus replication. Mice were infected with 1,000 MID_{50}, and lung, brain, spleen, and thymus samples were collected from four mice each on days 3 and 6 p.i. The samples were immediately frozen and stored at −70°C for subsequent determination of infectious virus and cytokine protein content.

Viral titration. Tissues were homogenized in 1 ml of cold PBS. For analysis of infectivity, the titers of the virus were determined using clarified tissue homogenates in eggs from initial dilutions of 1:10. The limit of virus detection was $10^{1.5}$ EID_{50}/ml. Virus titers were calculated by the method of Reed and Muench and are expressed as the mean $\log_{10}$ EID_{50} per milliliter ± standard errors (SE) of the means (41). Tissues in which no virus was detected were given a value of $10^{1.5}$ EID_{50}/ml for calculation of the mean titer.

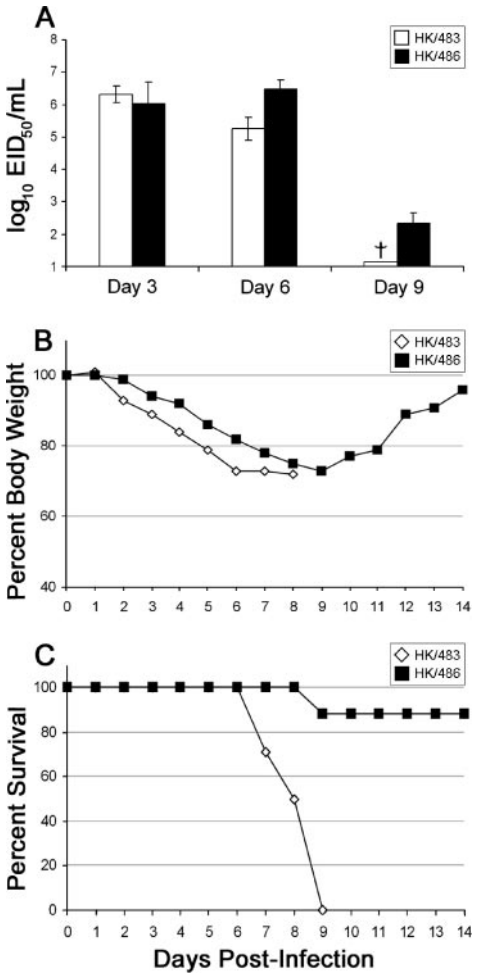

FIG. 1. Characterization of H5N1 pathogenicity in B6/129 mice. (A) B6/129 mice were infected with 1,000 MID_{50} of HK/483 or HK/486 virus. Four mice from each virus-infected group were euthanized on day 3, 6, or 9 p.i., and viral titers of individual tissues were determined. Data are expressed as $\log_{10}$ of mean titers ± SE. The limit of virus detection was $10^{1.5}$ EID_{50}/ml. †, HK/483 virus-infected mice did not survive to day 9 postinfection. (B and C) Ten mice were observed for weight loss (B) and survival (C) for 14 days p.i. Data are expressed as a percentage of mean starting weight or total survival.

Quantification of cytokines and chemokines. Tissue homogenates from days 3 and 6 p.i. were analyzed by use of an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. Tissue homogenates were analyzed for levels of IL-1β, IL-6, MIP-1α, and TNF-α.

TNF-α neutralization in mice. C57Bl/6 mice were given three doses of either 0.2 ml of rabbit anti-mouse TNF-α polyclonal sera (representing 250,000 neutralizing units) (kindly provided by Michael Luster, National Institute for Occupational Safety and Health, CDC) or 200 μg of rat neutralizing antisera raised against recombinant mouse TNF-α (Upstate Biotechnology, Chicago, IL) by the intraperitoneal route (31, 33). Control mice were injected with normal rabbit or rat sera or PBS. Mice received treatment 1 day before infection and on the day of infection and 1 and 4 days p.i. Mice were bled from the orbital plexus at different intervals to ensure that saturating levels of antibody were achieved.

Histopathologic and immunohistochemical analysis. Four mice were euthanized on days 3 and 6 p.i. Brain, lung, spleen, and thymus tissues from each time point and each experimental group were removed and fixed in formalin, routinely processed, and embedded in paraffin. Histopathologic examination was performed by using hematoxylin- and eosin (H&E)-stained sections. For antigen staining, a colorimetric indirect immunoalkaline phosphatase immunohisto-

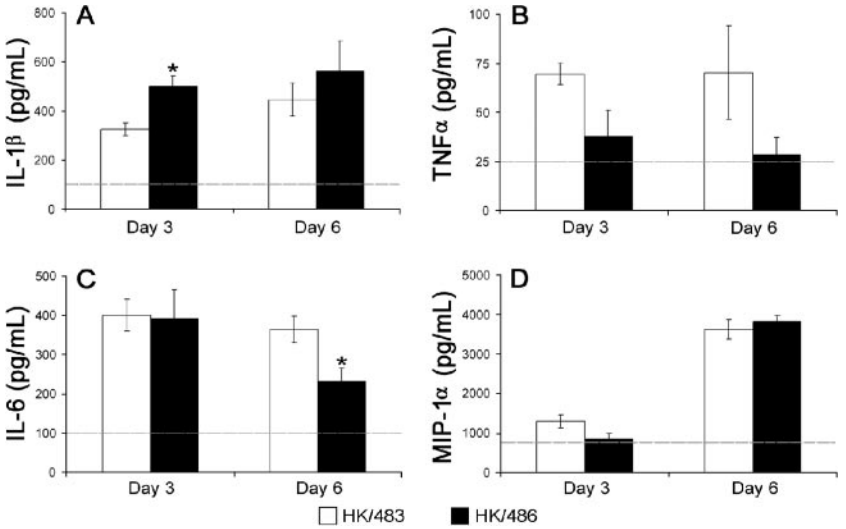

FIG. 2. Determination of proinflammatory cytokine levels in lungs of B6/129 mice. B6/129 mice were infected with 1,000 MID_{50} of HK/483 or HK/486 virus. Four mice from each virus-infected group were euthanized on either day 3 or day 6 p.i., and tissue homogenates were generated for analysis. IL-1β (A), TNF-α (B), IL-6 (C), and MIP-1α (D) levels were assayed by ELISA as described in Materials and Methods. Bars represent mean cytokine concentrations (in picograms per milliliter) ± SE. The horizontal lines denote constitutive levels present in uninfected B6/129 mice. *, $P \leq 0.02$.

chemistry method was developed as previously described (64, 65). The primary antibody used in the immunohistochemistry assay was a goat antiserum to A/Tern/South Africa/61 (H5N3) (National Institute of Allergy and Infectious Diseases, Bethesda, MD) that recognizes both surface glycoproteins and internal proteins of the virus.

Peripheral blood leukocyte counts. On days 0, 3, and 6, blood samples were collected from the orbital plexus of four mice per group. Absolute leukocyte counts were performed with heparinized blood diluted 1:10 with Turk's solution (2% acetic acid, 0.01% crystal violet [vol/vol], double-distilled water). For differential counts, peripheral blood smears were stained with Hema-3 stain (Fisher Diagnostics, Orangeburg, NY), and the numbers of monocytes, neutrophils, and lymphocytes were determined.

Statistical analysis. The statistical significance of viral titer and morbidity data was determined by using a two-tailed, paired Student's *t* test. The statistical significance of mortality data was determined by using the null model likelihood ratio test and the Mann-Whitney test.

RESULTS

Characterization of H5N1 virus in B6/129 wild-type mice. Although H5N1 viruses are known to replicate efficiently in BALB/c mice (12, 30, 32), replication and lethality of H5N1 viruses in B6 or B6/129 mouse strains, typically used to derive cytokine- or chemokine-deficient mice, have not been reported previously. In preliminary experiments, we determined that B6 mice exhibited weight loss and lethal disease following infection with H5N1 viruses comparable to that observed with the established BALB/c mouse model. Titers (50% lethal doses) for the highly lethal HK/483 virus were identical in the two mouse strains ($10^{0.5}$ EID_{50} per 50 μl). We next characterized the relative virulence of HK/483 and HK/486 viruses for B6/129 mice. Mice were inoculated intranasally with 1,000 MID_{50} of either of the H5N1 influenza A viruses, a dose that was determined previously to consistently produce the two distinct pathogenicity phenotypes in mice, and the kinetics of virus replication, morbidity, and mortality were determined. Lung virus titers measured on days 3 and 6 p.i. were high ($10^{5.25}$ to $10^{6.5}$ EID_{50}/ml) for both viruses and were not significantly different from each other (Fig. 1A). However, by day 9 p.i. all HK/483 virus-infected mice had succumbed, while 90% of HK/486 virus-infected mice survived (Fig. 1C) and exhibited a mean 4 $\log_{10}$ reduction in lung virus titers compared with day 6 titer results (Fig. 1A), indicating that these mice were effectively clearing the viral infection. Furthermore, HK/486 virus-infected mice began to regain weight by day 9 p.i. and had almost returned to preinfection weights by the end of the experiment (Fig. 1B). Therefore, both B6 and B6/129 mice reproduce the pathogenicity phenotypes for H5N1 viruses previously established for the BALB/c mouse.

Expression of cytokines in lungs of H5N1-infected B6/129 mice. To determine the proinflammatory cytokine and chemokine levels following H5N1 virus infection in B6/129 wild-type mice, lungs from mice infected with 1,000 MID_{50} of HK/483 or HK/486 H5N1 influenza A viruses were collected on days 3 and 6 p.i., and homogenates were subsequently assayed for IL-1β, IL-6, TNF-α, and MIP-1α levels by ELISA. The levels of all cytokines were substantially greater than constitutive levels in the lungs of B6/129 mice by day 3 p.i. (Fig. 2). However, in HK/483 virus-infected mice, IL-1β levels were significantly reduced ($P \leq 0.02$) (Fig. 2A), whereas IL-6 ($P \leq 0.02$) and TNF-α levels were elevated, although the latter result was not significant compared with levels found in HK/486 virus-infected mice (Fig. 2B and 2C). The levels of MIP-1α induced by either H5N1 virus in the lungs on day 6 p.i. were also significantly elevated compared with levels in normal mice (Fig. 2D). The cytokine and chemokine patterns detected in the brain were similar to those previously observed in BALB/c mice in that they were detected in HK/483-infected mice but not in HK/486-infected mice (data not shown) (2, 56). Therefore, the data from the B6/129 mouse model of H5N1 virus infection are consistent with our previous findings for the BALB/c mouse and suggest that differential induction of proinflammatory cytokines by HK/483 and HK/486 viruses may contribute to the

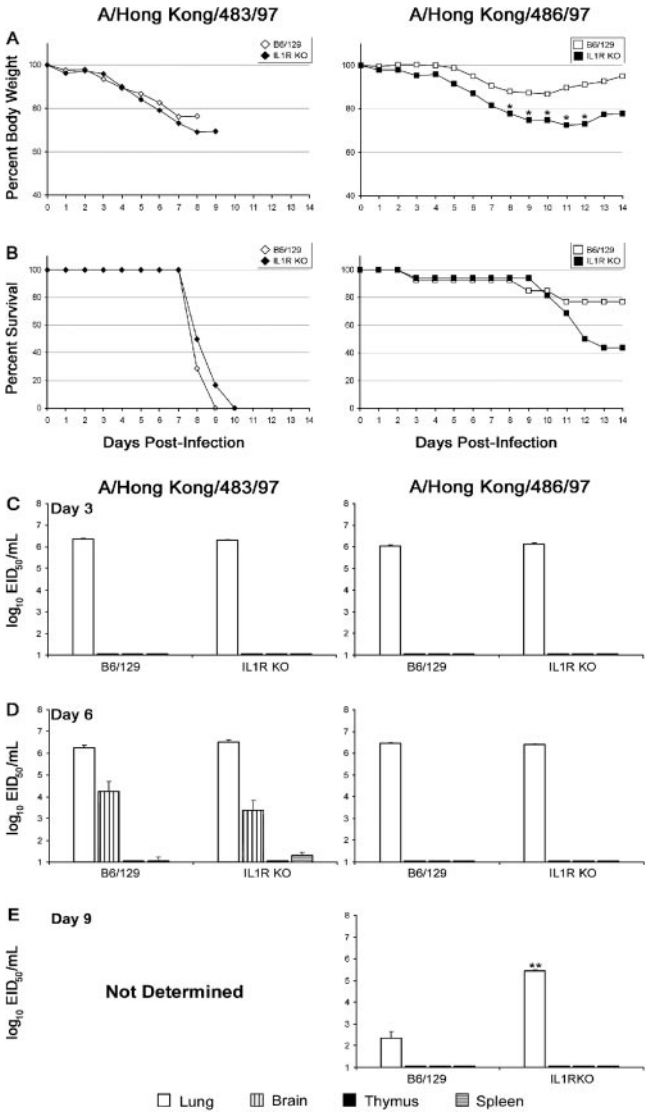

FIG. 3. Effect of IL-1R deficiency on H5N1 disease outcome. B6/129 and IL-1R-deficient mice were infected with 1,000 MID_{50} of HK/483 or HK/486 virus. (A and B) Ten to 15 mice were observed for weight loss (A) and survival (B) for 14 days p.i. Data are expressed as a percentage of mean starting weight or total survival. (C to E) Four mice from each virus-infected group were euthanized on day 3 (C), day 6 (D), or day 9 (E) p.i., and viral titers of individual tissues were determined. Data are expressed as $\log_{10}$ values of mean viral titers ± SE. *, $P \leq 0.02$; **, $P \leq 0.0001$.

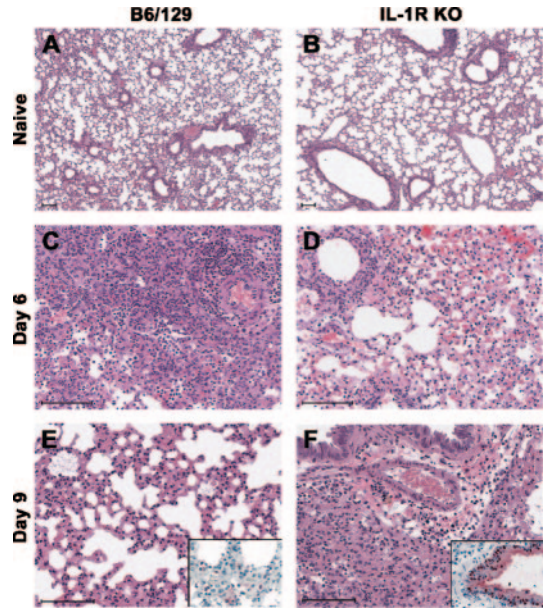

FIG. 4. Representative images of the histopathologic effect of IL-1R deficiency in mice infected with HK/486 virus. B6/129 and IL-1R-deficient mice were infected with 1,000 MID_{50} of HK/483 or HK/486 virus. Tissues were removed on the indicated day p.i. and routinely processed for histopathologic and immunohistochemical analyses. (A and B) Representative H&E images showing similar histologic features in lungs of naïve B6/129 (A) and IL-1R-deficient (B) mice. (C to F) Representative H&E images showing inflammatory infiltrates in the lungs of B6/129 (C and E) and IL-1R-deficient (D and F) mice on day 6 and 9 p.i. with HK/486 virus. Immunostaining of influenza virus antigen was observed in bronchiolar epithelial cells of IL-1R-deficient mice on day 9 p.i. (F, inset), whereas B6/129 mice showed only scattered antigen staining in alveolar cells (E, inset). Bars, 100 μm.

observed differences in severity of disease caused by these H5N1 viruses (59). Thus, B6 or B6/129 cytokine- or chemokine-deficient mice can be used to investigate the role of individual cytokines or chemokines in the H5N1 virus disease process.

IL-6 and MIP-1α have little effect on the outcome of H5N1 influenza virus disease. Because of the elevated levels of IL-6 and MIP-1α found during H5N1 virus infection, we first examined the role of these immune products in mice deficient in either IL-6 cytokine or MIP-1α chemokine. IL-6- or MIP-1α-deficient mice or B6 wild-type control mice were infected with 1,000 MID_{50} of HK/483 or HK/486 H5N1 viruses and were monitored for 14 days. IL-6- and MIP-1α-deficient mice and wild-type mice infected with HK/483 or HK/486 virus exhibited similar kinetics of weight loss and mortality (data not shown). Furthermore, viral titers in lung, brain, spleen, or thymus tissue on days 3 and 6 p.i. for either H5N1 virus in IL-6- or MIP-1α-deficient mice were comparable to wild-type control titers (data not shown). These results suggest that a lack of IL-6 or MIP-1α does not affect the outcome of H5N1 virus disease in mice.

IL-1 promotes clearance and protects against virus-induced mortality. Because of the reduced levels of IL-1β in B6/129 mice during HK/483 virus infection, we next determined what role IL-1 may have during H5N1 disease in IL-1R-deficient mice infected with 1,000 MID_{50} of HK/483 or HK/486 viruses. There was no difference in weight loss, survival, or viral titers on days 3 or 6 p.i. observed between HK/483 virus-infected IL-1R-deficient and control mice. In contrast, IL-1R-deficient mice infected with HK/486 virus lost significantly more weight ($P \leq 0.02$) and a greater percentage of HK/486 virus-infected IL-1R-deficient mice succumbed to infection after day 8 p.i. compared with B6/129 wild-type mouse results, although this difference did not reach statistical significance (Fig. 3B). There was no difference in viral titers between the IL-1R-deficient and control mice on day 3 or 6 p.i. (Fig. 3C and D). However, because the differences observed in weight loss and lethal outcome between IL-1R-deficient mice and wild-type controls in-

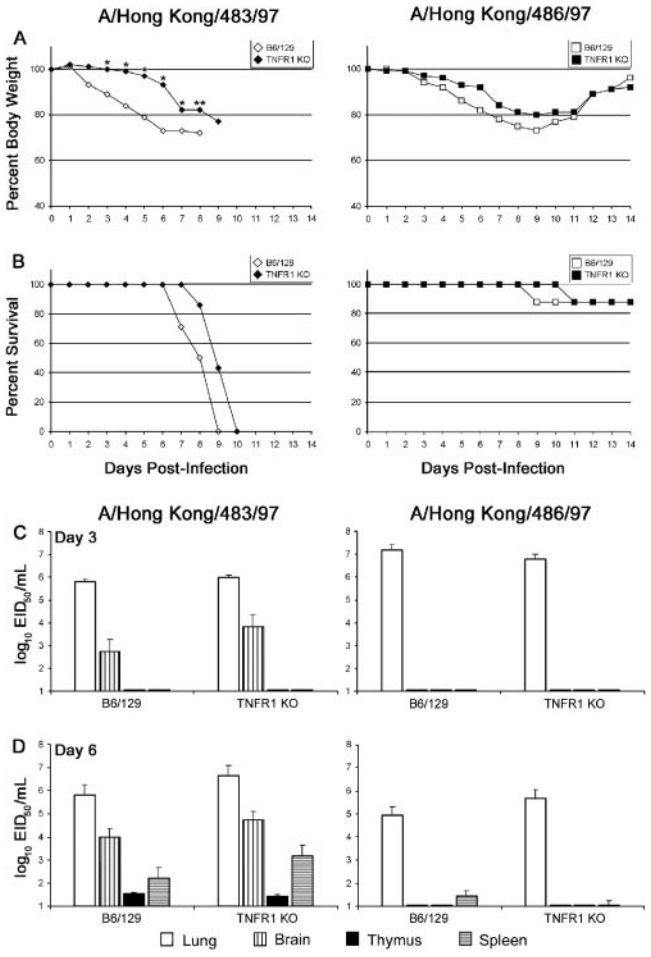

FIG. 5. Effect of TNFR1 deficiency on H5N1 virus disease outcome. B6/129 and TNFR1-deficient mice were infected with 1,000 MID_{50} of HK/483 or HK/486 virus. (A and B) Ten mice were observed for weight loss (A) and survival (B) for 14 days p.i. Data are expressed as a percentage of mean starting weight or total survival. (C and D) Four mice from each virus-infected group were euthanized on either day 3 (C) or day 6 (D) p.i., and viral titers of individual tissues were determined. Data are expressed as $\log_{10}$ of mean viral titers ± SE. *, $P \leq 0.001$; **, $P \leq 0.02$.

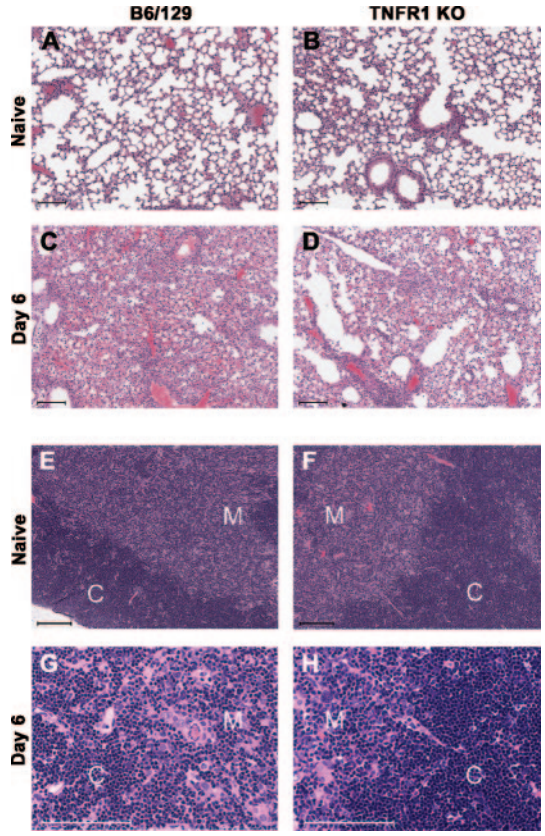

FIG. 6. Effect of TNFR1 deficiency on HK/483 virus pathology in lung and thymus. B6/129 and TNFR1-deficient mice were infected with 1,000 MID_{50} of HK/483 or HK/486 virus. Tissues were removed on the indicated day p.i. and routinely processed for histochemical analyses. Representative H&E images show similar histologic features in naïve lungs and thymuses of B6/129 (A and E) and TNFR1-deficient (B and F) mice. Representative H&E images show similar amounts of inflammatory infiltrates in the lungs of B6/129 (C) and TNFR1-deficient (D) mice on day 6 p.i. with HK/483 virus. Representative H&E images show a higher density of thymocytes in the thymic cortex of TNFR1-deficient mice (H) compared with B6/129 mouse results (G) on day 6 p.i. with HK/483 virus. Bars, 100 μm. C, cortex; M, medulla.

fected with HK/486 virus occurred late in the time course of infection, we also assessed viral titers on day 9 p.i. At this time point, HK/486 lung viral titers in IL-1R-deficient mice were 1,000-fold higher than those in wild-type mice (Fig. 3E; $P \leq 0.0001$). The lungs from HK/486 virus-infected mice of either strain, collected on days 6 and 9 p.i., were also examined histologically. On day 6 p.i., the lungs from wild-type control mice exhibited more inflammatory infiltrate than those from the IL-1R-deficient mice, although differences were modest (Fig. 4C and D), and immunohistochemical analyses revealed no visible differences in antigen amounts or distribution between the two groups of mice (data not shown). Conversely, by day 9 p.i. the lungs from IL-1R-deficient mice displayed more-extensive inflammation than those from control mice (Fig. 4E and F). Furthermore, immunohistochemical analyses revealed more-abundant viral antigen in the bronchiolar epithelial cells in IL-1R-deficient mice, which is consistent with the increased viral titers detected in these mice versus control mice at day 9 p.i. (Fig. 4E, inset; Fig. 4F, inset; and Fig. 3E). These data suggest that the IL-1 response may be important for effective clearance of the less-virulent H5N1 virus in mice.

TNF-α contributes to H5N1 virus disease. TNF-α, a key regulator of inflammation (58), is produced in mouse organs in response to H5N1 virus replication. To assess the contribution of TNF-α production in H5N1 virus pathogenesis, TNFR1 knockout mice were infected with 1,000 MID_{50} of HK/483 or HK/486 virus and the levels of virus replication, morbidity, and mortality were evaluated as described above. Infection of TNFR1-deficient mice with the highly virulent HK/483 virus resulted in a significant delay in weight loss compared with wild-type mouse infection results from day 3 to 7 p.i. ($P \leq 0.001$ to 0.02) (Fig. 5A), although the data with respect to mean maximum weight lost by the two group of mice were not significantly different and HK/483 virus infection was ultimately lethal for either group (Fig. 5B). A similar delay in weight loss was observed for TNFR1-deficient mice infected

TABLE 1. Analysis of lymphocyte populations in thymus and blood of TNFR1-deficient and wild-type control mice infected with HK/483 virus[a]

Mouse	Virus	Total no. of cells per thymus sample[a]	Mean % of total thymocytes ± SE			No. of leukocytes/μl	% Lymphocytes in peripheral blood[c]
			$CD4^+$ $CD8^+$	$CD4^+$ $CD8^-$	$CD4^-$ $CD8^+$		
B6/129	None	1.02×10^8	89.0 ± 0.6	6.5 ± 0.9	2.0 ± 0.1	7.0×10^3	66.2
B6/129	HK/483	1.26×10^{7b}	42.5 ± 13.7[b]	32.0 ± 9.8	14.8 ± 2.5[b]	1.1×10^{3d}	24.2[b]
TNFR1KO	None	2.21×10^8	91.0 ± 0.6	5.5 ± 0.3	1.5 ± 0.3	6.2×10^3	63.8
TNFR1KO	HK/483	1.18×10^8	63.0 ± 7.9[b]	18.8 ± 4.8	7.8 ± 1.9[b]	1.6×10^{3d}	44.7

[a] Four mice were infected with 1,000 MID_{50} of HK/483 virus; 6 days later, animals were euthanized for collection of blood and thymus. A single-cell suspension of thymocytes was prepared from each mouse; cells were then counted and subjected to flow cytometric analysis as described in Materials and Methods by gating of the lymphocyte population.

[b] $P \leq 0.05$ compared with uninfected group.

[c] Four mice from each group were bled via the orbital plexus on days 0 and 6 p.i. Total peripheral white blood cell count analyses were performed using Turk's solution as previously described (59). Percentages of lymphocytes were determined by counting 100 white blood cells on fixed blood smear slides.

[d] $P \leq 0.06$ compared with uninfected group.

with HK/486 virus (Fig. 5A), but the majority of animals survived infection with this strain (Fig. 5B). Despite these differences in clinical illness, there was no significant difference in viral titers in lung, brain, spleen, and thymus tissue for either H5N1 virus between TNFR1-deficient mice and wild-type controls on day 3 or 6 p.i. (Fig. 5C and D). Results similar to those obtained with the TNFR1-deficient mice were obtained with wild-type B6 mice infected with HK/483 virus and administered neutralizing anti-mouse TNF-α polyclonal or monoclonal sera (31, 33). In two separate experiments, mice receiving neutralizing anti-TNF-α sera lost significantly less weight by day 5 p.i. ($P \leq 0.01$) but showed no significant differences in mortality or viral titers in lung, brain, spleen, or thymus tissue compared with mice that received normal rabbit sera (data not shown). Similarly, there was no substantial difference in the inflammation observed in lungs from either strain of mice on day 6 p.i. (Fig. 6C and D). These results suggest that TNF-α may contribute to symptoms of severe disease in H5N1 virus-infected mice but does not directly influence viral replication or the ultimate outcome of disease in this model.

It was of interest to find in the present study that TNFR1-deficient mice did not exhibit the same extent of thymic involution observed in wild-type B6/129 mice 6 days p.i. with HK/483 virus. Previous work from our group determined that infection of BALB/c mice with the more virulent H5N1 virus, HK/483, led to peripheral lymphopenia as well as a decrease in overall cellularity of the thymus and, in particular, a significant decrease in the percentage of double-positive (DP) ($CD4^+$ $CD8^+$) lymphocytes relative to the results obtained for uninfected or HK/486-infected mice (59). This observation led us to hypothesize that increased production of TNF-α during H5N1 virus infection contributed to the peripheral lymphopenia, of which thymic involution was an indicator. To test this hypothesis, we first performed histological analysis of thymic tissues from HK/483 virus-infected mice. The thymuses of wild-type B6/129 mice exhibited decreased cellularity in the cortex (Fig. 6G) compared with thymuses from TNFR1-deficient mice (Fig. 6H). Consistent with the histological analyses, total thymocyte counts in HK/483 virus-infected B6/129 mice were eightfold lower ($P \leq 0.05$) than those seen with uninfected mice in comparison with a less than twofold reduction in thymocyte numbers in HK483 virus-infected TNFR1-deficient mice (Table 1). Flow cytometric analysis of thymocytes indicated that HK/483 virus infection resulted in a decrease in frequency of the DP thymocyte population relative to the results seen for uninfected animals in either B6/129 wild-type or TNFR1-deficient mice, although the overall effect with respect to a decrease in total DP cell numbers was more pronounced in the wild-type mice (Table 1). However, no differences were detected in total blood leukocyte counts between the B6/129 control animals and their TNFR1-deficient counterparts, as both groups exhibited substantial leukopenia and lymphopenia 6 days after infection with HK/483 virus (Table 1). Taken together, these results suggest that the TNF-α response induced in H5N1 virus-infected animals contributes to thymic involution but does not affect leukocyte depletion in peripheral blood.

DISCUSSION

The widespread distribution of highly pathogenic avian H5N1 influenza A viruses in wild birds and, in particular, in domestic poultry populations continues to pose a threat to public health. Severe respiratory disease and a high case-fatality rate have become a hallmark of H5N1 infection in humans as well as in other mammalian species (19, 40, 54, 55). Detection of increased levels of cytokine expression, also referred to as a "cytokine storm," in blood or tissues of H5N1 virus-infected patients has prompted the hypothesis that an exacerbated proinflammatory response may contribute to the severity of H5N1 disease in humans (5, 37, 56, 60, 63). Clinical features that are prominent in human H5N1 infections such as acute respiratory distress syndrome and multiple organ dysfunction have previously been associated with cytokine dysregulation (4, 6, 37, 56, 58). In this study, we investigated the contribution of individual cytokines or chemokines to the H5N1 viral disease process in mice. We first established morbidity, lethality, viral replication and spread, and cytokine and chemokine expression in the B6/129 mouse, a strain for which numerous cytokine-deficient mice have been bred. We also determined that there were no substantial differences in cytokine and chemokine expression between wild-type B6/129 and the knockout mice used in this study during H5N1 virus infection except for the expected lack of IL-6 or MIP-1α in the respective cytokine-deficient mouse strains (data not shown). The kinetics and outcome of infection with HK/483 and HK/486, two H5N1

viruses that show different virulence phenotypes in wild-type mice, were found to be essentially unaffected in mice deficient in IL-6 or MIP-1α expression. In contrast, mice deficient in IL-1R displayed delayed clearance of the less virulent HK/486 virus, while mice deficient in TNFR1 exhibited delayed weight loss and death following infection with the more virulent HK/483 virus. These results suggest that TNF-α may contribute to early disease severity, whereas IL-1 may play a role in viral clearance late in H5N1 virus infection.

In contrast to the noted effects of IL-1β and TNF-α response and the fact that both H5N1 viruses elicited strong IL-6 production, lack of IL-6 expression did not appreciably affect the outcome of H5N1 infection. This may be due, in part, to the redundancy of the biological effects of IL-6 in mammalian systems. Our findings were consistent with those of Kozak et al., who observed no significant difference in weight loss results between IL-6-deficient and wild-type mice infected with mouse-adapted A/Puerto Rico/8/34 (PR/8; H1N1) influenza A virus (27). We also failed to detect any significant differences between MIP-1α-deficient mice and wild-type mice following infection with either of the H5N1 viruses despite the strong induction of this chemokine in lungs of wild-type mice. Cook et al. had previously demonstrated that MIP-1α-deficient mice infected with a sublethal dose of PR/8 virus exhibited less pulmonary inflammation and a modest delay in clearance of virus from the lungs compared with wild-type controls, suggesting that MIP-1α-mediated recruitment of inflammatory mediators contributed to viral clearance (9). Although MIP-1α does not appear to be critical for the induction and function of influenza virus nucleoprotein-specific $CD8^+$ T cells during primary influenza virus infection, it may influence $CD8^+$ T-cell recruitment to the site of infection in the lungs (23). The fact that we observed no difference in the kinetics of weight loss, recovery rate, or viral replication in MIP-1α-deficient mice infected with HK/483 or HK/486 virus suggests either that MIP-1α does not contribute directly to the control of viral clearance during the primary immune response to H5N1 virus infection or that other chemokines with overlapping functions can compensate for its loss.

IL-1 acts locally and systemically during acute phase responses to bacterial and viral infection or other inflammatory stimuli, causing fever, anorexia, and lethargy in the host (reviewed in reference 12). We observed that IL-1R-deficient mice did not differ from wild-type mice in the outcome of infection with the highly lethal H5N1 virus HK/483. In contrast, following infection with the less virulent HK/486 virus, IL-1R-deficient mice lost significantly more weight, exhibited delayed clearance of virus, and had a greater proportion of animals succumb to infection than their immunocompetent counterparts. In other studies, mice deficient in IL-1R or IL-1β and infected with PR/8 virus were reported to exhibit enhanced mortality which was not associated with enhanced weight loss, and in the case of IL-1R-deficient mice, a modest delay in viral clearance (28, 44). The significant weight loss observed for HK/486 virus-infected IL-1R-deficient mice in the present study occurred late in infection, when relatively high virus titers were still present in the lungs of IL-1R-deficient mice but not in those of wild-type mice. Others have reported that IL-1R-deficient mice mount diminished or altered antigen-specific T-cell responses but similar humoral responses compared with wild-type mice (29, 43, 44). Similarly, we observed no differences in H5 hemagglutinin-specific serum immunoglobulin M (IgM), IgG, IgA, or hemagglutination inhibition antibody titers between IL-1R-deficient mice and B6/129 control mice on either day 10 or 21 p.i. (data not shown). In contrast, Schmitz et al. detected reduced serum and bronchoalveolar lavage virus-specific IgM responses, but not bronchoalveolar lavage cytotoxic T-cell effector function, on day 10 p.i. in IL-1R-deficient mice infected with PR/8 virus (44). The differences in IgM responses between the results of the two influenza studies may be due to differences in viral growth kinetics of H5N1 versus H1N1 viruses or in the specificity of IgM antibody response measured. Nevertheless, our results suggest that IL-1 is important for the induction of effective adaptive immune responses against H5N1 viruses that aid in viral clearance and that the reduced IL-1β production observed for HK/483 virus-infected wild-type (Fig. 2A) mice may contribute to the rapid and lethal disease outcome.

Both H5N1 viruses used in this study, like other influenza A viruses, induce elevated amounts of TNF-α following productive replication of the virus in the lungs of mice. The early delay in weight loss observed in H5N1 virus-infected TNFR1-deficient mice is consistent with a role for TNF-α in induction of cachexia and may explain the modest delay in time to death for HK/483 virus-infected mice, since no effect on viral replication or spread was observed (57). These results are consistent with those of other studies using mouse-adapted influenza A viruses in which treatment with TNF-α neutralizing antibodies delayed mouse weight loss and/or the time to death by approximately 1 day but did not alter viral replication in infected lungs (20, 38). In contrast, TNF-α was reported to have potent antiviral properties for human influenza A viruses in porcine lung epithelial cells, whereas H5N1 viruses were resistant to the antiviral effects of TNF-α as well as of alpha interferon (IFN-α), IFN-β, and IFN-γ (45, 46). The resistance of H5N1 viruses to the antiviral effects of these cytokines was proposed to contribute to the virulence of H5N1 viruses in vivo. However, in our mouse H5N1 model, TNF-α does not appear to have affected virus replication or spread directly but rather promoted the clinical severity of the disease. The antiviral activity of TNF-α in epithelial cell cultures may be due to its ability to activate the IFN response pathway by the direct induction of IFN β expression and/or indirectly through the upregulation of Toll-like receptor 3 and RIG-I expression (22, 34). Thus, the lack of an antiviral effect of TNF-α in influenza virus-infected inbred mice may in part be due to the lack of a functional Mx gene, an interferon-inducible gene that is known to play a role in the host's antiviral response to influenza virus infection (13, 50). The role of type I interferon in the pathogenesis of H5N1 viruses in this murine model is currently under investigation.

TNFR1-deficient mice infected with HK/483 virus did not exhibit the same extent of thymic involution or depletion of DP thymocytes as wild-type B6/129 or BALB/c mice (59), suggesting that TNF-α signaling may contribute to the loss of thymocytes in H5N1 virus-infected mice. Thymic involution and alteration in the frequency of thymocyte populations have been observed previously in mice during viral infection (17, 26, 35). Uninfected transgenic mice that overexpress TNF-α in thymocytes and T cells also exhibit reduced thymic mass and deceased populations of DP cells (15, 16, 39). TNF-α and related

TNF-superfamily members, including TNF-related apoptosis-inducing ligand, are known to mediate apoptosis of T cells and, in particular, thymocytes (49, 61). This fact, together with our observations in this study, suggests that the thymic depletion observed in immunocompetent H5N1 virus-infected mice is primarily due to the production of increased levels of TNF-α produced in response to H5N1 infection rather than to a direct cytolytic effect of the virus itself.

The demonstration that H5N1 viruses are potent inducers of certain cytokines and chemokines in primary human alveolar and bronchial epithelial cells and monocyte-derived macrophages has provided support for the hypothesis that host innate immune responses may contribute, at least in part, to the severity of disease induced by H5N1 virus infection in humans (4, 5). The results of the present study confirm that TNF-α and a lack of IL-1β signaling can influence the course of H5N1 disease in vivo, although intrinsic properties of H5N1 viral replication, including a greater efficiency of replication in mouse cells associated with the E627K substitution in the PB2 gene found in the highly pathogenic HK/483 virus, may influence the overall outcome of infection (47). Because of the functional redundancy of some cytokine and chemokine products, it is possible that depletion of more than one cytokine and/or chemokine may have even more substantial effects on H5N1 disease than those described here. In this respect the mouse offers a suitable preclinical model for the evaluation of therapies using antibodies or receptor analogs that may modulate the inflammatory response induced by H5N1 viruses, either alone or in combination with antiviral therapy that would promote viral clearance (5, 37, 62).

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