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ORIGINAL ARTICLE

The zoonotic potential of avian influenza viruses isolated from wild waterfowl in Zambia

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Abstract Whilst remarkable progress in elucidating the mechanisms governing interspecies transmission and pathogenicity of highly pathogenic avian influenza viruses (AIVs) has been made, similar studies focusing on low-pathogenic AIVs isolated from the wild waterfowl reservoir are limited. We previously reported that two AIV strains (subtypes H6N2 and H3N8) isolated from wild waterfowl in Zambia harbored some amino acid residues preferentially associated with human influenza virus proteins (so-called human signatures) and replicated better in the lungs of infected mice and caused more morbidity than a strain lacking such residues. To further substantiate these observations, we infected chickens and mice intranasally with AIV strains of various subtypes (H3N6, H3N8, H4N6, H6N2, H9N1 and H11N9) isolated from wild waterfowl in Zambia. Although some strains induced seroconversion, all of the tested strains replicated poorly and were nonpathogenic for chickens. In contrast, most of the strains having human signatures replicated well in the lungs of mice, and one of these strains caused severe illness in mice and induced lung injury that was characterized by a severe accumulation of polymorphonuclear leukocytes. These results suggest that some strains tested in this study may have the potential to infect mammalian hosts directly without adaptation, which might possibly be associated with the possession of human signature residues. Close monitoring and evaluation of host-associated signatures may help to elucidate the prevalence and emergence of AIVs with potential for causing zoonotic infections.

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Introduction

It is widely acknowledged that avian influenza viruses (AIVs) circulating in wild waterfowl reservoirs such as ducks, gulls and shorebirds are occasionally transmitted to land-based avian hosts and mammals [24, 33]. Most AIVs found in the reservoir generally cause asymptomatic or less-severe diseases in poultry and are thus referred to as low-pathogenic AIVs (LPAIVs). In contrast, highly pathogenic AIVs (HPAIVs) cause an acute systemic disease in poultry with a mortality rate that often approaches 100 % [1]. Currently, only some AIVs of the H5 and H7 subtypes are known to become HPAIVs under natural conditions. It is also known that AIVs of limited subtypes (H1-H3) have so far established stable lineages in some non-avian species such as humans, pigs and horses [25, 33].

In recent years, repeated zoonotic transmissions of AIVs (mostly H5, H7 and H9 subtypes) from terrestrial birds into humans have occurred [7, 25]. In fact, prior to the emergence of the 2009 H1N1 pandemic virus, the unprecedented impact on animal and public health of the Asian-origin H5N1 HPAIV led to the prediction that a virus of subtype H5N1 might cause the next pandemic [21]. These zoonotic transmissions have been influential in heightening investigations into the virulence and host-range determinants of AIVs, particularly those for HPAIVs [2, 21]. Whereas remarkable progress has been made in understanding the pathogenesis of HPAIVs, information on virulence and host-range determinants of LPAIVs isolated from the waterfowl reservoir is minimal. This is despite the fact that the past documented pandemics (i.e., 1918 H1N1, 1957 H2N2, 1968 H3N2 and 2009 H1N1) were caused by viruses of non-highly-pathogenic subtypes and possessed some genes of avian origin. Moreover, the recent pandemic threat due to a novel reassortant avian-origin influenza A (H7N9) virus found in China is of low pathogenic pathotype [12]. Furthermore, there is mounting evidence suggesting that LPAIVs of multiple subtypes can infect mammals, including humans, under natural and experimental conditions, with appreciable degrees of morbidity and mortality [7, 9, 14, 17, 20, 22, 25, 30]. In the context of influenza pandemic preparedness, these data indicate that prudent pandemic plans should involve research and surveillance efforts targeting most influenza virus subtypes worldwide.

To date, the key amino acid substitutions that may enable an AIV to cause interspecies transmissions into other hosts remain obscure. By analyzing a large data set of influenza virus sequences, several studies have identified host-specific, conserved amino acids at particular positions, so-called genetic signatures [5, 6, 10, 27]. It has been hypothesized that these specific substitutions might be associated with the ability of AIVs to efficiently infect mammalian hosts, including humans. However, this notion has not been comprehensively tested. We previously reported that some AIV strains isolated from wild waterfowl in Zambia harbored residues frequently observed in human influenza viruses [28]. In experimental infection of mice, two AIV strains possessing the human signature residues showed higher levels of virus replication in the lungs of infected mice and caused more morbidity as measured by weight loss than a strain lacking such residues. To further substantiate these observations, we assessed the replicative and pathogenic potential of several Zambian AIV strains (subtypes H3N6, H3N8, H4N6, H6N2, H9N1 and H11N9) in chicken and mouse models.

Materials and methods

Ethics statement

All animal experimental procedures were conducted in the biosafety level 2 and 3 facilities at Hokkaido University Research Center for Zoonosis Control, Japan, in strict accordance with the guidelines of the institutional animal care and use committee of Hokkaido University.

Viruses

AIVs used in this study (Table 1) were isolated from wild waterfowl in Zambia between 2006 and 2009 [28, 29]. Before being used in this study, these viruses were passaged once in 10-day-old specific-pathogen-free (SPF) embryonated eggs and then titrated to determine the EID_{50}, which was calculated by a method described previously [26]. The viruses were appropriately diluted with sterile phosphate-buffered saline (PBS) to adjust the virus titer to $10^{7.5}$ EID_{50}/ml.

Experimental infection of chickens

Viruses were inoculated intranasally (i.n.) into 6-week-old SPF chickens (Boris brown) at $10^{7.5}$ EID_{50}/ml (0.1 ml). There were nine groups (including a PBS-inoculated control group) in total, consisting of six chickens each. On day 3 post-inoculation (p.i.), three chickens were euthanized, and tracheas, lungs and colons were sampled for virus

Table 1 Seroconversion of chickens inoculated with LPAIVs isolated from wild waterfowl[1]

Virus	Abbreviation	HI titers of three individual chickens 14 days after inoculation		
A/pelican/Zambia/01/06 (H3N6)	Zb01 (H3N6)[2]	16	8	16
A/goose/Zambia/05/08 (H3N8)	Zb05 (H3N8)[3]	<2	8	4
A/goose/Zambia/07/08 (H4N6)	Zb07 (H4N6)[3]	16	64	<2
A/duck/Zambia/03/08 (H6N2)	Zb03 (H6N2)[2]	<2	<2	<2
A/duck/Zambia/10/09 (H6N2)	Zb10 (H6N2)[3]	<2	128	64
A/pelican/Zambia/13/09 (H9N1)	Zb13 (H9N1)[3]	8	<2	<2
A/duck/Zambia/11/09 (H11N9)	Zb11 (H11N9)[2]	<2	32	<2
A/duck/Zambia/12/09 (H11N9)	Zb12 (H11N9)[2]	32	<2	<2
	PBS	<2	<2	<2

[1] Sera with HI titers ≥16 were considered positive

[2] Virus with no apparent human/mammalian-associated residues in its proteins

[3] Virus having at least one human/mammalian-associated residue in its proteins

titration in eggs. The rest of the chickens were monitored for clinical signs for 14 days. At the end of this period, serum was obtained for antibody titration by a standard haemagglutination inhibition (HI) test.

Experimental infection of mice

The methods used to investigate the replicative and pathogenic capacity of these viruses in mice were essentially the same as those described previously [28], with slight modifications. Here, 6-week-old female BALB/c mice (15 mice per group) were inoculated i.n. with 0.05 ml of Zb01 (H3N6), Zb05 (H3N8), Zb07 (H4N6), Zb13 (H9N1), Zb11 (H11N9) or Zb12 (H11N9) ($10^{7.5}$ EID_{50}/ml). Mock-infected mice received the same volume of PBS. Five mice were euthanized on days 1 and 3 p.i. for virus titration in the lungs and spleens. Monitoring of mice for clinical signs and virus titration in organs were done as described previously [28].

Histopathology and immunohistochemistry

BALB/c mice (three mice per group) were infected i.n. with 0.05 ml of $10^{7.5}$ EID_{50}/ml of Zb01 (H3N6) and Zb07 (H4N6), or mock-infected with sterile PBS. On day 3 p.i., mice were euthanized, and whole lung tissues were collected and fixed with buffered neutral formalin and then embedded in paraffin. Formalin-fixed paraffin-embedded tissues were cut into sections of 3-μm thickness and mounted on glass slides for histopathological and immunohistochemical examination. For histopathological assessment, the sections were stained with hematoxylin and eosin or only hematoxylin and microscopically examined. For immunohistochemical staining, we used a rabbit polyclonal hyperimmune serum against influenza A virus nucleoprotein (NP) (1:5000; the rabbit was immunized with amino acids 428-441 of NP) for detection of influenza viruses. For immunohistochemical staining of viral NP antigens, retrieval was performed with a pressure cooker using 0.01 M citrate buffer (pH 6.0). After cooling and washing in PBS, endogenous peroxidase activity was blocked by treatment with 3 % hydrogen peroxide in methanol for 15 minutes at room temperature (RT). The sections were then washed in PBS and incubated for 10 minutes with normal goat serum (Nichirei, Tokyo, Japan) at RT and for 30 minutes with primary antibody (anti-NP rabbit antiserum) at RT. The sections were then incubated for 30 min at RT with a secondary antibody labeled with horseradish peroxidase (Histofine Simple Stain MAX-PO(R); Nichirei). Immunoreactivity was detected using a 3,3′-diaminobenzidine substrate.

Results

Waterfowl LPAIVs replicated poorly and caused no disease in chickens

To investigate the ability of the LPAIV strains isolated from waterfowl in Zambia to replicate in terrestrial birds, chickens were inoculated i.n. with each of the viruses listed in Table 1. Among the viruses tested in chickens, Zb01 (H3N6), Zb03 (H6N2) and Zb11 (H11N9) apparently possessed no human signature residues, whilst the rest of the viruses had at least one such residue (Table 2). During the observation period, none of the chickens showed any symptoms of illness. Virus was not detected in any of the trachea, lung and colon samples collected on day 3 p.i., suggesting their poor replicative capacity in this animal model. However, at least one of the three chickens inoculated with Zb01 (H3N6), Zb07 (H4N6), Zb10 (H6N2), Zb11 (H11N9) and Zb12 (H11N9) produced virus-specific antibodies, as indicated by increased HI titers that ranged from 16 to 128 (Table 1). These results suggest that the seroconverted chickens had been infected despite no recovery of inoculated viruses from their tissue samples. With regard to the replicative and/or pathogenic potential of the viruses tested, we did not discern any appreciable differences between viruses possessing and lacking human signatures.

Table 2 Human-associated amino acids identified in viral proteins of AIVs isolated in Zambia

Protein	Position[1]	Host		Isolate[2]
		Avian	Human	
PB2	475	L	M	Zb10 (H6N2)
PB1-F2	66	N	S[3]	Zb13 (H9N1)
	76	V	A	Zb13(H9N1)
	82	L	S	Zb05 (H3N8)
				Zb10 (H6N2)
				Zb13 (H9N1)
	87	E	G	Zb10 (H6N2)
M2	55	L	F	Zb07 (H4N6)
				Zb10 (H6N2)
				Zb12 (H11N9)

[1] Human-associated residues at these specific positions were described previously [5, 10, 27]

[2] Names of isolates possessing human-associated amino acid residues

[3] The amino acid serine at position 66 of the PB1-F2 protein is not a human-associated residue but was previously shown to increase virulence in mice [8]

Waterfowl LPAIVs possessing human signatures replicated well in lungs of infected mice

Next, we investigated the ability of the LPAIV strains to replicate in mouse lungs. Since the replicative and pathogenic profiles of Zb03 (H6N2) and Zb10 (H6N2) were determined previously (i.e., Zb10 (H6N2), possessing 4 human signature residues, replicated better and caused more severe disease in mice than Zb03 (H6N2), which lacks such residues) [28], these viruses were excluded in this mouse experiment. On day 1 p.i., all of the viruses tested were recovered from the lungs of all infected mice with virus titers ranging from $10^{3.5}$ to $10^{5.3}$ EID_{50}/g (Table 3). Marked differences in lung virus titers among the viruses were observed on day 3 p.i.; Zb01 (H3N6), Zb11 (H11N9) and Zb12 (H11N9) displayed poor replication (virus titers were $\leq 10^{1.8}$ EID_{50}/g), whereas Zb05 (H3N8), Zb07 (H4N6) and Zb13 (H9N1) exhibited higher levels of virus replication, with virus titers ranging from $10^{2.7}$ to $10^{3.9}$ EID_{50}/g (Table 3). It was also noted that viruses were detected in the lungs of all the mice inoculated with Zb05 (H3N8), Zb07 (H4N6) and Zb13 (H9N1), whereas in Zb01 (H3N6), Zb11 (H11N9) and Zb12 (H11N9)-inoculated mice, virus was detected only in one, two and three of the five mice, respectively (Table 3). None of the viruses was detected in the spleen, suggesting that the viruses could not efficiently spread systemically. An apparent correlation between possession of human signatures and capacity to replicate efficiently in the lungs of mice was observed in Zb05 (H3N8)-, Zb07 (H4N6)- and Zb13 (H9N1)-inoculated mice. However, whilst having a human signature residue, Zb12 (H11N9) replicated poorly in the lungs of infected mice.

Table 3 Replication of LPAIVs isolated from wild waterfowl in mice

Virus	Mean titers (log_{10} EID_{50}/g) of virus-positive samples		
	Lung		Spleen
	Day 1	Day 3	Day 3
Zb01 (H3N6)[1]	3.7 (5/5)	1.5 (1/5)	<1.5
Zb05 (H3N8)[2]	3.9 (5/5)	2.7 (5/5)	<1.5
Zb07 (H4N6)[2]	5.3 (5/5)	3.9 (5/5)	<1.5
Zb11 (H11N9)[1]	4.0 (5/5)	1.8 (2/5)	<1.5
Zb12 (H11N9)[2]	4.0 (5/5)	1.7 (3/5)	<1.5
Zb13 (H9N1)[2]	3.5 (5/5)	3.4 (5/5)	<1.5

[1] Virus with no apparent human/mammalian-associated residues in its proteins

[2] Virus having at least one human/mammalian-associated residue in its proteins

Waterfowl LPAIVs induced illness in mice

We then investigated the capacity of Zb01 (H3N6), Zb05 (H3N8), Zb07 (H4N6), Zb13 (H9N1), Zb11 (H11N9) and Zb12 (H11N9) to cause illness in mice. Mice were monitored for clinical signs such as inappetence, labored breathing, weight loss, ruffled fur and hunching for 14 days. Except for Zb07 (H4N6)-infected mice, which exhibited severe weight loss, a transient weight loss of up to about 11 % was observed between days 1 and 4 in infected mice, and their weight returned to baseline by day 8 p.i. (Fig. 1). It was noted that a considerable reduction in body weight (more than 10 %) occurred in Zb05 (H3N8)- and Zb07 (H4N6)-infected mice. Moderate ruffling of the fur was seen in mice infected with Zb01 (H3N6), Zb05 (H3N8) and Zb13 (H9N1). Zb07 (H4N6)-infected mice showed severe ruffling of the fur, labored breathing and hunching for the most of the observation period. All of the mice survived the infection for the 14-day observation period. It is noteworthy that in an independent experiment in which undiluted chorioallantoic fluid was used, mice infected with Zb07 (H4N6) (10^9 EID_{50}/ml/mouse) died within 5 days p.i., whilst all mice infected with other viruses survived the infection (data not shown).

Zb07 (H4N6)-infected mice exhibited severe pulmonary lesions

To examine the lung pathology induced by Zb07 (H4N6), mice were infected i.n. with Zb01 (H3N6) and Zb07 (H4N6). Zb01 (H3N6) was selected for histopathological analysis because it induced the most weight loss among the strains lacking human signatures. On day 3 p.i., a time

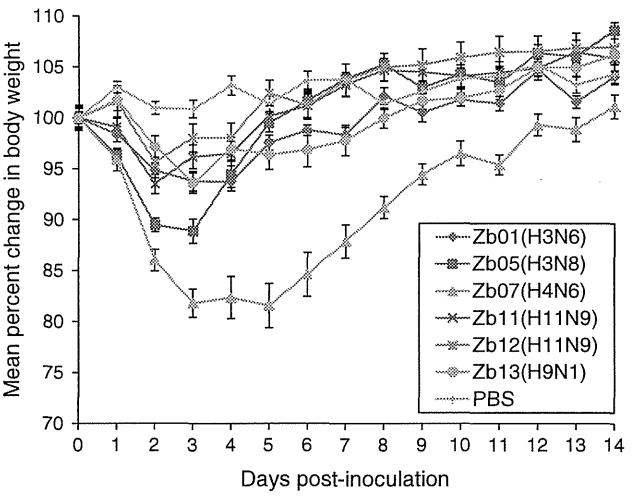

Fig. 1 Weight loss in mice infected with LPAIVs isolated from wild waterfowl in Zambia. Data are presented as mean body weight change per group ± standard deviation

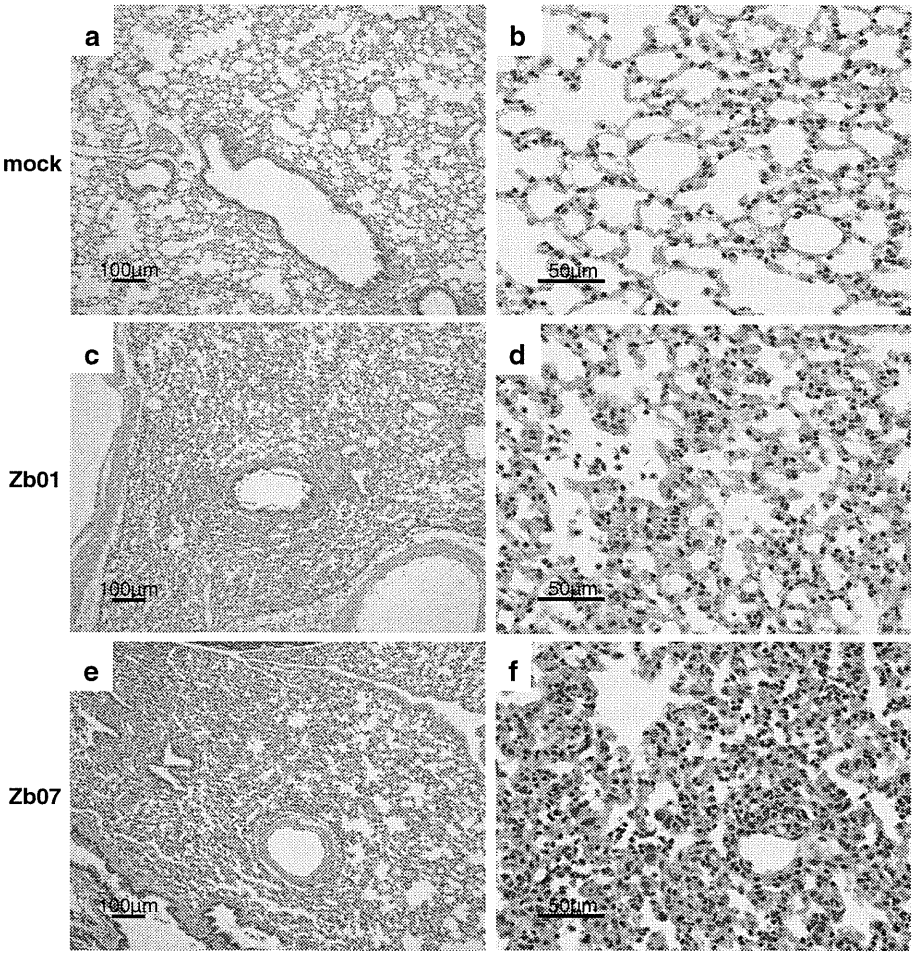

Fig. 2 Representative histopathological images of lung tissues of mice infected i.n. with Zb01 (H3N6) or Zb07 (H4N6). On day 3 p.i., lung samples were collected from mock-infected (**a** and **b**), Zb01 (H3N6)-infected (**c** and **d**) and Zb07 (H4N6)-infected (**e** and **f**) mice. The tissue sections were stained with hematoxylin and eosin

point at which most isolates from Zambia caused maximal weight loss, mice were euthanized and whole lung tissues were collected and processed for histopathological examination. Lungs of mice infected with Zb01 (H3N6) showed moderate interstitial and perivascular infiltration of inflammatory cells as well as desquamation of the bronchial epithelium and migration of macrophages to the alveolar space (Fig. 2c, d). On the other hand, lung tissues of mice infected with Zb07 (H4N6) exhibited severe infiltration of inflammatory cells into the perivascular and interstitial space (Fig. 2e, f). Interstitial edema and migration of inflammatory cells to the alveolar space were also observed in lung tissues of Zb07 (H4N6)-infected mice (Fig. 2f). Though perivascular infiltration of inflammatory cells was observed in lungs of mice infected with Zb01 (H3N6) and Zb07 (H4N6), interestingly, the inflammatory cellular compositions in these areas were different. We observed that infiltration of polymorphonuclear leucocytes (PMNs), mostly neutrophils, in the lung tissues of Zb07 (H4N6)-infected mice was more prominent than in those of Zb01 (H3N6)-infected mice. To confirm this observation, we prepared sections stained with only hematoxylin and analyzed the inflammatory cellular compositions of perivascular areas morphologically (Fig. 3). We found that inflammatory cells were mainly lymphocytes in most of the perivascular areas of the lungs of Zb01 (H3N6)-infected mice (Fig. 3a). On the other hand, PMNs were predominantly found in perivascular areas of the lung tissues of Zb07 (H4N6)-infected mice (Fig. 3b). By immunohistochemistry for the NP antigen, positive signals were observed in bronchial and alveolar epithelium cells of the lungs of both Zb01 (H3N6) and Zb07 (H4N6)-infected mice (Fig. 3c, d).

Discussion

In this study, we utilized animal models (chickens and mice) to biologically characterize LPAIV strains isolated from wild waterfowl in Zambia. In chickens, all of the tested viruses replicated poorly, whilst five isolates induced seroconversion in at least one of the three virus-inoculated chickens (Table 1). This finding may be consistent with AIVs that are not adapted to gallinaceous poultry.

However, in the context of avian influenza surveillance and control, the existence of wild waterfowl AIVs that can directly infect chickens but cause no obvious signs of disease poses an epidemiological challenge in intensive monitoring of commercial or backyard poultry. Frequent introduction of such viruses into poultry may lead to an increased risk that AIVs acquire mutations that may allow them to expand their host range [15]. In fact, a serological study has recently provided evidence of human infections with LPAIVs (subtype H4 and H11) among backyard poultry growers [18]. As clearly demonstrated by the recent human cases of H7N9 LPAIV infection in China, contact with unrecognized LPAIV-infected domestic birds may indeed lead to human infections with dire consequences.

In stark contrast to the findings in chicken experiments in which there were no appreciable differences between viruses possessing and lacking human signature residues, most LPAIV strains having human signatures exhibited better replication in mice than those lacking such residues (Table 3). Importantly, all of the tested viruses induced illness in mice. With the exception of Zb07 (H4N6), which caused the most severe disease in mice, there were no marked differences in the severity of disease between mice infected with viruses either possessing or lacking human signatures. Taken together, these data suggest that even wild waterfowl AIVs displaying poor replicative capacity in gallinaceous poultry may have the potential to directly switch hosts and infect mammals without prior adaptation. Although most reported human infections with AIVs occurred as a result of close contact with domestic poultry, serologic evidence of AIV infection (subtype H11N9) in three individuals with a history of substantial exposure to wild waterfowl and game birds does support the idea of direct transmission of AIVs from their natural reservoirs into humans [13].

Whilst it might be reasonable to assume that the presence of human signature residues in some of the waterfowl LPAIV strains tested may have influenced the observed improved replication in mice, further studies employing techniques such as reverse genetics and site-directed mutagenesis are required to reach such a conclusion. Moreover, despite having a human signature residue, Zb12 (H11N9) replicated poorly in the lungs of infected mice. Even as this finding may indicate that the enhanced replication of LPAIVs in mice observed in the current study may not solely be accounted for by possession of human signature residues, it may also imply that some of these residues may not be critical factors in host-switch mechanisms of AIVs. Other viral (e.g., subtype; H11 viruses are rarely detected in non-reservoir hosts) and host factors may be involved. Regardless, our studies provide impetus to investigate how AIVs may acquire human/mammalian-associated residues in nature and the potential role of such residues in virulence and/or host-switch mechanisms of AIVs.

Histopathological examination revealed that wild-bird LPAIVs, Zb01 (H3N6) and Zb07 (H4N6), induced moderate and severe pulmonary lesions in mice, respectively, a

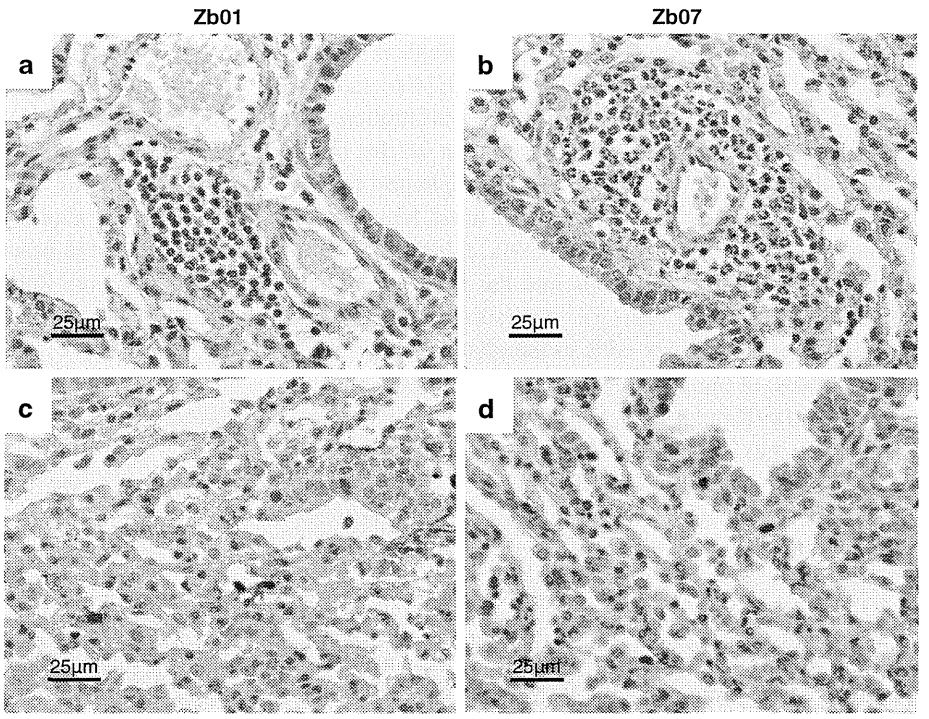

Fig. 3 Representative nuclear staining and immunohistochemical images of lung sections of mice infected i.n. with Zb01 (H3N6) or Zb07 (H4N6). On day 3 p.i., lung samples were collected from Zb01 (H3N6)-infected (**a** and **c**) and Zb07 (H4N6)-infected (**b** and **d**) mice. The tissue sections were stained with hematoxylin alone for nuclear stain (**a** and **b**), or rabbit polyclonal antibody against viral NP antigens for immunohistochemistry (**c** and **d**)

result that appeared to correlate with the magnitude of virus replication and morbidity caused by these viruses (Table 3; Fig. 1). The finding that lung lesions of Zb07 (H4N6)-infected mice were severer than those of mice infected with Zb01 (H3N6) may also be related to the greater early inflammatory response characterized by a severe accumulation of PMNs observed in the perivascular lesions of Zb07 (H4N6)-infected mice than those of Zb01 (H3N6) (Fig. 3). Whereas these cells have been shown to play a significant role in preventing influenza virus propagation in the lungs following primary pulmonary infection, excessive influx of PMNs, particularly neutrophils, may contribute to acute lung injury in influenza virus-induced pneumonia [11, 23, 31]. It is unclear why Zb07 (H4N6) induced a greater influx of PMNs into mouse lung than Zb01 (H3N6). Although the level of virus replication may be involved, the expression of inflammatory mediators such as complement factors and chemokines may also play a role in inducing PMNs migration into the mouse lung [32]. It is also worth noting that Zb07 (H4N6) possesses human-associated residues in the matrix 2 (M2) protein. The M2 protein has been shown to induce secretion of the pyrogenic cytokine IL-1β via stimulation of the NLRP3 inflammasome pathway [16]. This may contribute to uncontrolled deleterious inflammation (a so-called cytokine storm), which may exacerbate lung immunopathology and disease of influenza [3]. Furthermore, IL-1 plays an important role in hemostasis deregulation through tissue factor induction. Since hemostasis deregulation emerges as a key pathway in the cytokine storm induced by influenza viruses [3, 4, 19], it seems reasonable to speculate that human signature residues in the M2 protein of AIVs may be involved in cytokine storm via IL-1 production and hemostasis deregulation.

In this study, we have demonstrated that some of the LPAIVs isolated from wild waterfowl in Zambia may have the potential to infect chickens and mice without adaptation. We have also shown a possible correlation between viral replication capacity in mice and possession of human signature residues in viral proteins, which may be associated with the potential to cause interspecies transmission. Our study emphasizes the need for close monitoring and evaluation of host-associated signatures in AIVs to better understand the emergence of strains capable of causing zoonotic infections.

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Protective Efficacy of Passive Immunization with Monoclonal Antibodies in Animal Models of H5N1 Highly Pathogenic Avian Influenza Virus Infection

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Abstract

Highly pathogenic avian influenza (HPAI) viruses of the H5N1 subtype often cause severe pneumonia and multiple organ failure in humans, with reported case fatality rates of more than 60%. To develop a clinical antibody therapy, we generated a human-mouse chimeric monoclonal antibody (MAb) ch61 that showed strong neutralizing activity against H5N1 HPAI viruses isolated from humans and evaluated its protective potential in mouse and nonhuman primate models of H5N1 HPAI virus infections. Passive immunization with MAb ch61 one day before or after challenge with a lethal dose of the virus completely protected mice, and partial protection was achieved when mice were treated 3 days after the challenge. In a cynomolgus macaque model, reduced viral loads and partial protection against lethal infection were observed in macaques treated with MAb ch61 intravenously one and three days after challenge. Protective effects were also noted in macaques under immunosuppression. Though mutant viruses escaping from neutralization by MAb ch61 were recovered from macaques treated with this MAb alone, combined treatment with MAb ch61 and peramivir reduced the emergence of escape mutants. Our results indicate that antibody therapy might be beneficial in reducing viral loads and delaying disease progression during H5N1 HPAI virus infection in clinical cases and combined treatment with other antiviral compounds should improve the protective effects of antibody therapy against H5N1 HPAI virus infection.

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Competing Interests: MK is employed by Shionogi & Co., Ltd., a company developing the neuraminidase inhibitor used in this study. This does not alter our adherence to all PLOS Pathogens policies on sharing data and materials.

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Introduction

Influenza A viruses are divided into subtypes based on the antigenicity of two envelope glycoproteins, hemagglutinin (HA) and neuraminidase (NA). To date, H1-H16 HA and N1-N9 NA subtypes have been found in wild aquatic birds, the natural reservoir of influenza viruses [1–3]. Of these HA subtypes, only some avian influenza viruses of the H5 and H7 subtypes are known to become highly pathogenic avian influenza (HPAI) viruses under natural conditions. While HPAI viruses cause an acute systemic disease in poultry with a mortality rate that often approaches 100%, avian to human transmission of HPAI viruses is limited and HPAI viruses had never been reported to cause lethal infection in humans until the first emergence of an H5N1 HPAI virus in southern China in 1996.

The H5N1 HPAI virus has been circulating in poultry for more than a decade since its reemergence in southern China in 2003, and has caused unprecedented outbreaks in wild birds and poultry in Asia, the Middle East, and Africa [4–10]. The H5N1 HPAI virus

Author Summary

The H5N1 highly pathogenic avian influenza virus has been circulating in poultry in Asia, the Middle East, and Africa since its first appearance in southern China in 1996. This virus occasionally infects humans with a high case mortality rate and poses a significant pandemic threat. Since neutralizing antibodies generally play a major role in protective immunity against influenza viruses, antibody therapy is a potential option for preventing highly lethal infection with the H5N1 virus in humans. Here we evaluated the protective potential of a human-mouse chimeric monoclonal antibody with strong neutralizing activity against H5N1 viruses in mouse and nonhuman primate models of lethal H5N1 virus infection. The therapeutic use of the neutralizing antibody resulted in reduced viral loads and improved survival in animals infected with highly pathogenic H5N1 viruses. It was noted that the protective effects were more prominent in immunosuppressed macaques, which might provide a model of protection against severe clinical disease in immunocompromised patients. In addition, combination therapy together with an antiviral drug reduced the selection of escape mutants. Collectively, this study suggests that antibody therapy may have beneficial effects in clinical cases of H5N1 HPAI virus infection in humans.

occasionally infects humans with a high case mortality rate and poses a significant pandemic threat [11,12,13]. Since 2003, 641 laboratory-confirmed human cases of H5N1 HPAI virus infection have been reported from 15 countries, with 380 fatal cases (as of October 8, 2013) [12]. In fact, prior to the emergence of the swine-origin H1N1 pandemic virus in 2009, the impact on animal and public health of the Asian origin H5N1 HPAI virus led to the prediction that a virus of the H5 subtype might cause the next pandemic, since this HA subtype is distinct from those of viruses circulating in the human population (i.e., subtypes H1 and H3) [13].

In recent years, passive immunization with human or humanized monoclonal antibodies (MAbs) specific to viral proteins has been tested in animal models and clinical trials, providing evidence of the effectiveness of MAbs for prophylaxis or treatment of infectious diseases [14]. Indeed, a humanized MAb specific to Respiratory syncytial virus F protein is already approved by the US Food and Drug Administration and used in clinical cases. Importantly, particular attention has been paid to antibody therapy against highly lethal diseases such as rabies [15–17], severe acute respiratory syndrome [18,19], Hendra [20], Nipah [21], and Ebola viruses [22–25].

It is known that HA, which is responsible for both receptor binding and fusion of the virus envelope with the host cell membrane, is the primary target of neutralizing antibodies against influenza viruses. Since antibodies generally play a major role in protective immunity against influenza virus infection [26], antibody therapy might be a potential option for preventing lethal infection of humans by the H5N1 HPAI virus. In this study, we genetically modified a mouse MAb (m61) neutralizing the infectivity of H5N1 HPAI viruses to create human-mouse chimeric MAb (ch61), aiming at clinical application, and evaluated its protective potential in mouse and nonhuman primate models of H5N1 HPAI virus infection.

Materials and Methods

Viruses and cells

HPAI virus strains A/Hong Kong/483/1997 (H5N1) (HK483), A/Viet Nam/1194/2004 (H5N1) (VN1194), and A/Vietnam/UT3040/2004 (H5N1) (VN3040) from the repository of our laboratory, were propagated in Madin-Darby canine kidney (MDCK) cells from the repository of our laboratory and stored at −80°C until use. HK483, VN1194, and VN3040 belong to clades 0, 1, and 1 in a phylogenetic tree, respectively [27]. MDCK cells were grown in Eagle's minimal essential medium supplemented with 10% calf serum. All experiments using infectious viruses were performed in the biosafety level 3 facilities of the Hokkaido University Research Center for Zoonosis Control and Research Center for Animal Life Science, Shiga University of Medical Science.

Generation of mouse monoclonal antibodies

Mouse MAb 61-2-1 (m61), was generated according to standard procedures. Briefly, six-week-old female BALB/c mice (Japan SLC) were immunized intramuscularly two times with 100 μg of formalin-inactivated purified virions and boosted intraperitoneally [23]. Spleen cells harvested 3 days after boosting were fused to P3U1 myeloma cells according to standard procedures. Hybridomas were screened for secretion of HA-specific MAbs by enzyme-linked immunosorbent assay (ELISA), and cloned by limiting dilution. The resulting cell clones were inoculated into BALB/c mice intraperitoneally to produce ascites. Antibodies were purified from ascites using the Affi-Gel Protein A MAPS II Kit (Bio-Rad). Mouse MAbs ZGP133 and ZGP226 used as control antibodies were generated as described previously [23].

Generation of human-mouse chimeric monoclonal antibodies

Human-mouse chimeric MAb ch61 was generated and purified from culture supernatants as described previously [23]. Briefly, total RNA was extracted from mouse hybridoma cells producing MAb m61, and the variable heavy- and light-chain regions were amplified by RT-PCR with primers designed for the antibodies. The PCR products were cloned into an expression vector. Stable cell lines expressing recombinant MAb ch61 were obtained by transfection of CHO DG44 cells (Invitrogen, Carlsbad, CA). Chimeric MAbs (ch133 and ch226) specific for the Ebola virus glycoprotein were generated as control MAbs using the same methodology [23]. These human-mouse chimeric MAbs were purified from culture supernatants using rProtein A Sepharose Fast Flow (GE Healthcare) and EndoTrap red (Profos AG). MAb purity (>98%) and endotoxin levels (<1.0 EU/ml) were confirmed by performing SDS-PAGE and with an Endospecy ES-50M kit (Seikagaku Corporation), respectively.

Neutralization assay

Serially diluted antibodies (100 μl) were mixed with 200 plaque forming units (PFU) of H5N1 viruses for 1 h at room temperature, and inoculated onto MDCK cells. After 1 h, the inoculum was removed and the cells were overlaid with 1% Bacto-Agar (BD) in Eagle's minimal essential medium (MEM). Two days later, the number of plaques was counted and the percentage of plaque reduction was calculated.

Selection of escape mutants *in vitro*

Escape mutants were selected by culturing VN1194 in MDCK cells in the presence of MAb m61. Serial dilutions of VN1194 were mixed with purified MAb m61 (final concentration of 10 μg/ml), incubated for 1 h, and the mixtures were inoculated into confluent MDCK cells in 6-well tissue culture plates. After 1 h adsorption, the cells were overlaid with MEM containing 1% agar and MAb m61 ascites (final dilution of 1:1000), and then incubated for 2 days at 35°C. Eight escape mutants were purified from single

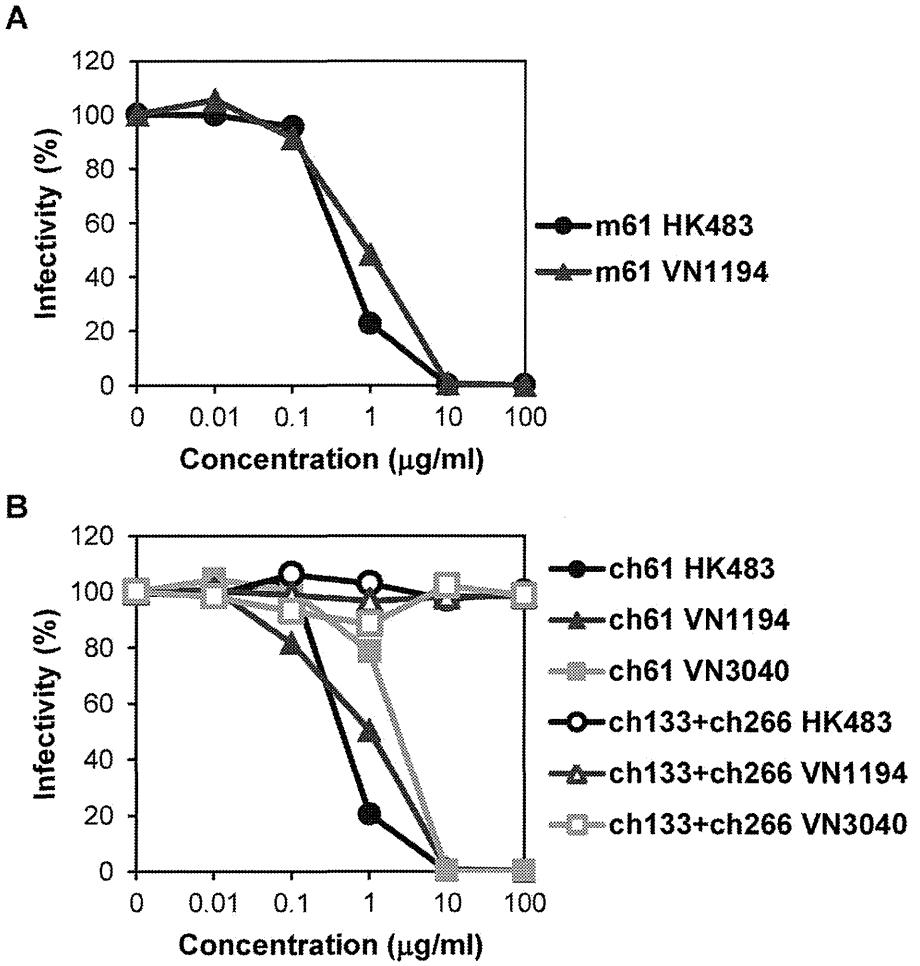

Figure 1. Neutralizing activities of MAbs m61 and ch61 against H5N1 HPAI viruses. Purified MAbs m61 (A) and ch61 (B) at the indicated concentrations were mixed with HK483, VN1194, or VN3040 and inoculated onto MDCK cells. A mixture of MAbs ch133 and ch226 was used as a control. The percentage of infectivity was calculated as follows: Infectivity (%) = the number of plaques with antibody/the number of plaques without antibody ×100. Averages of three independent experiments are shown.
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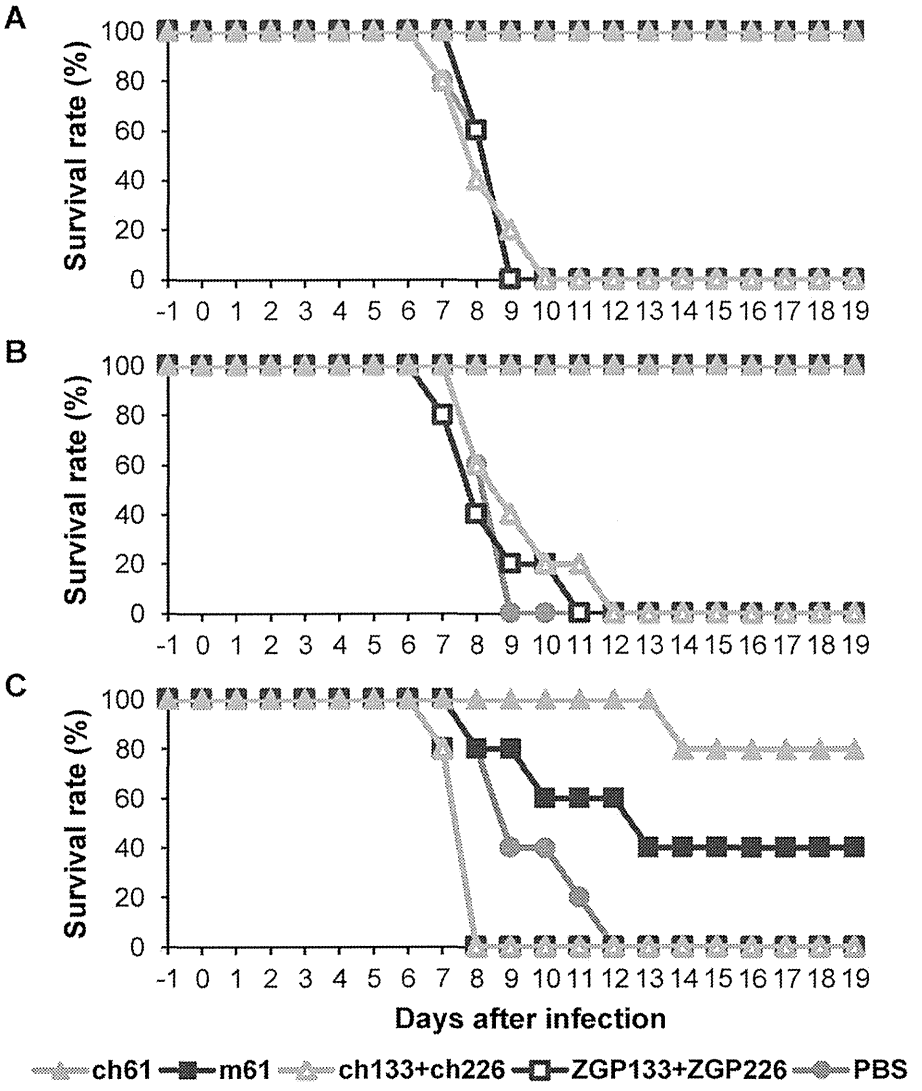
isolated plaques, and propagated in MDCK cells with serum-free MEM containing trypsin. The nucleotide sequences of the HA genes of the parent strains and the escape mutants were determined and the deduced amino acid sequences were compared among these viruses (H3 numbering).

Passive immunization and protection tests of mice

Six-week-old female BALB/c mice were passively immunized by intraperitoneal injection with 200 μg of purified MAbs m61 or ch61 24 hours before, or 24 hours or 72 hours after intranasal challenge with 50 μl of 12.5×50% mouse lethal dose of HK483 under anesthesia with isoflurane. Control groups were administered with control antibodies (mixture of MAbs ZGP133/ZGP226 or ch133/ch226) or phosphate-buffered saline (PBS). Animals were monitored daily for weight loss and clinical signs. Five days after the challenge, mice were euthanized to obtain lung tissue samples. Lung homogenates (10% w/v) prepared in MEM were centrifuged at 3,000× g for 10 min, and then the supernatants were examined for virus infectivity. Virus titers were measured by a plaque assay using MDCK cells.

Preparation of nonhuman primate study

The animal experiments were conducted in strict compliance with animal husbandry and welfare regulations. Food pellets of CMK-2 (CLEA Japan) provided once a day after recovery from anesthesia and drinking water were available *ad libitum*. Animals were singly housed in the cages equipping bars to climb up and puzzle feeders for environmental enrichment under controlled conditions of humidity (60±5%), temperature (24±1°C), and light (12 h light/12 h dark cycle, lights on at 8:00 A.M.). Five- to seven-year-old female cynomolgus macaques (*Macaca fascicularis*) from the Philippines (Ina Research) were used. The cynomolgus macaques used in the present study were healthy adults. The absence of influenza A virus NP-specific antibodies in their sera was confirmed before experiments using an antigen-specific ELISA, AniGen AIV Ab ELISA (Animal Genetics), for currently circulating influenza virus. Three weeks before virus inoculation, a telemetry probe (TA10CTA-D70, Data Sciences International) was implanted in the peritoneal cavity of each macaque under ketamine/xylazine anesthesia followed by isoflurane inhalation to monitor body temperature. The macaques used in this study were free from herpes B virus, hepatitis E virus, *Mycobacterium tuberculosis*, *Shigella* spp., *Salmonella* spp., and *Entamoeba histolytica*. Individual macaques were distinguished by treatments and numbers: C: macaques injected with control MAbs, T: macaques treated with MAb ch61, IC: immunosuppressed macaques injected with control MAbs, IT: immunosuppressed macaques treated with



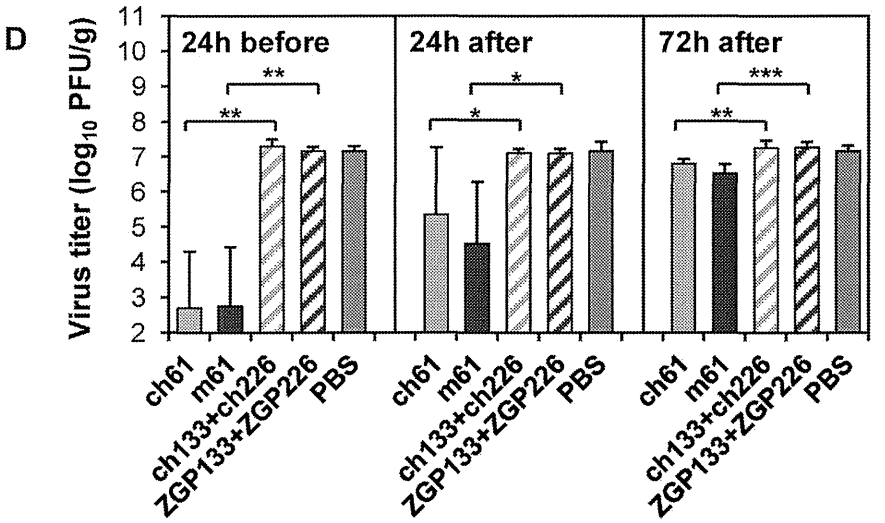


Figure 2. Protective efficacy of passive immunization with MAbs m61 and ch61 against HK483 in a mouse model. Ten mice in each group were treated intraperitoneally with 200 μg of purified MAb m61 or ch61 24 hours before (A), 24 hours after (B) or 72 hours after (C) virus challenge with a lethal dose of HK483 and 5 mice were monitored for their clinical signs/survival and another 5 mice were used for virus titration.

Control mice were given control antibodies (ZGP133+ZGP226 or ch133+ch226) or PBS. Five days after the challenge, lung tissue samples were collected to measure virus titers (D). The averages titers and standard deviations of 5 mice are shown. Titers below the limit of detection were assigned a value of 2. Significant differences (Student *t*-test) were indicated by asterisks (*** $p<0.001$, ** $p<0.01$, * $p<0.05$).
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MAb ch61, ICP: immunosuppressed macaques injected with MAbs and peramivir, ITP: immunosuppressed macaques treated with MAb ch61 and peramivir.

Antibody treatments and protection tests of macaques

Macaques (2.4–3.1 kg) were inoculated (day 0) with VN3040 (total 3×10^6 PFU/7 ml) in their nasal cavities (0.5 ml for each nostril) and on their tonsils (0.5 ml for each tonsil) with pipettes and into the trachea (5 ml) with catheters under ketamine/xylazine anesthesia. MAb ch61 or control MAbs (a mixture of MAbs ch133 and ch226) were administered intravenously twice (20 mg/head/dose; 6.5–8.3 mg/kg) on days 1 and 3 after infection. Animals were monitored daily (approximately every 12 hours) for clinical scoring (Table S1). Serum samples were obtained on days −1, 1, 3, 5, and 7. For virus titration, cotton sticks (TE8201, Eiken Chemical) were used to collect fluid samples from the nasal cavities and tracheas under ketamine/xylazine anesthesia, and the sticks were subsequently immersed in 1 ml of PBS containing 0.1% bovine serum albumin (BSA) and antibiotics. A bronchoscope (Machida Endoscope) and cytology brushes (Olympus) were used to obtain bronchial samples. The brushes were immersed in 1 ml of PBS with BSA. Viral titers were determined by the tissue culture infectious dose ($TCID_{50}$) in MDCK cells [28]. For immunosuppressive treatments of macaques, cyclophosphamide (CP) (Nacalai Tesque) and cyclosporine A (CA) (Novartis Pharma) were used [29]. CP (40 mg/kg) was administered intravenously by bolus injection on days −7, −5, −3, −1, and 0. CA (50 mg/kg) was administered orally into stomach using a catheter from day −7 to day 6. We confirmed that the treatment with CP and CA decreased the number of white blood cells in the macaques (Fig. S1). In some experiments, peramivir hydrate (30 mg/kg/dose, provided by Shionogi & Co., Ltd.) was administered intravenously by bolus injection once a day from day 1 to day 5 after infection [30]. Since patients with a severe respiratory illness might have a difficulty to intake or inhale drugs, we chose peramivir hydrate as an antiviral agent with intravenous injection. The concentrations of cytokines in sera and tissue homogenates were measured using the Milliplex MAP nonhuman primate cytokine panel and Luminex 200 (Millipore). Although the experiment was originally designed to collect samples from all animals for virology and immunology studies terminating on day 7, some animals were euthanized when their clinical scores reached 15 (a humane endpoint) and subjected to autopsy to collect tissue samples. Macaques that were unfortunately found dead during the intervals of the monitoring time points were also immediately subjected to autopsy. These animals (i.e., euthanized or dead) were counted as nonsurvivors.

Histological examination

After autopsy on indicated days after virus infection, lung tissue samples were fixed with 10% formalin, and embedded in paraffin. Sections were stained with hematoxylin and eosin (H & E). Influenza virus nucleoprotein (NP) antigens were stained with antisera of rabbits immunized with an NP synthetic peptide (AFTGNTEGRTSDMR at positions 428–441 of the NP sequence: GenBank accession number, ADC34563) after treatment in a pressure cooker in 0.01 M citrate-phosphate buffer. After incubation with anti-rabbit immunoglobulin antibody conjugated with horseradish peroxidase (Nichirei Bioscience Inc.), NP was detected with diaminobenzidine (Nichirei Biosciences Inc.).

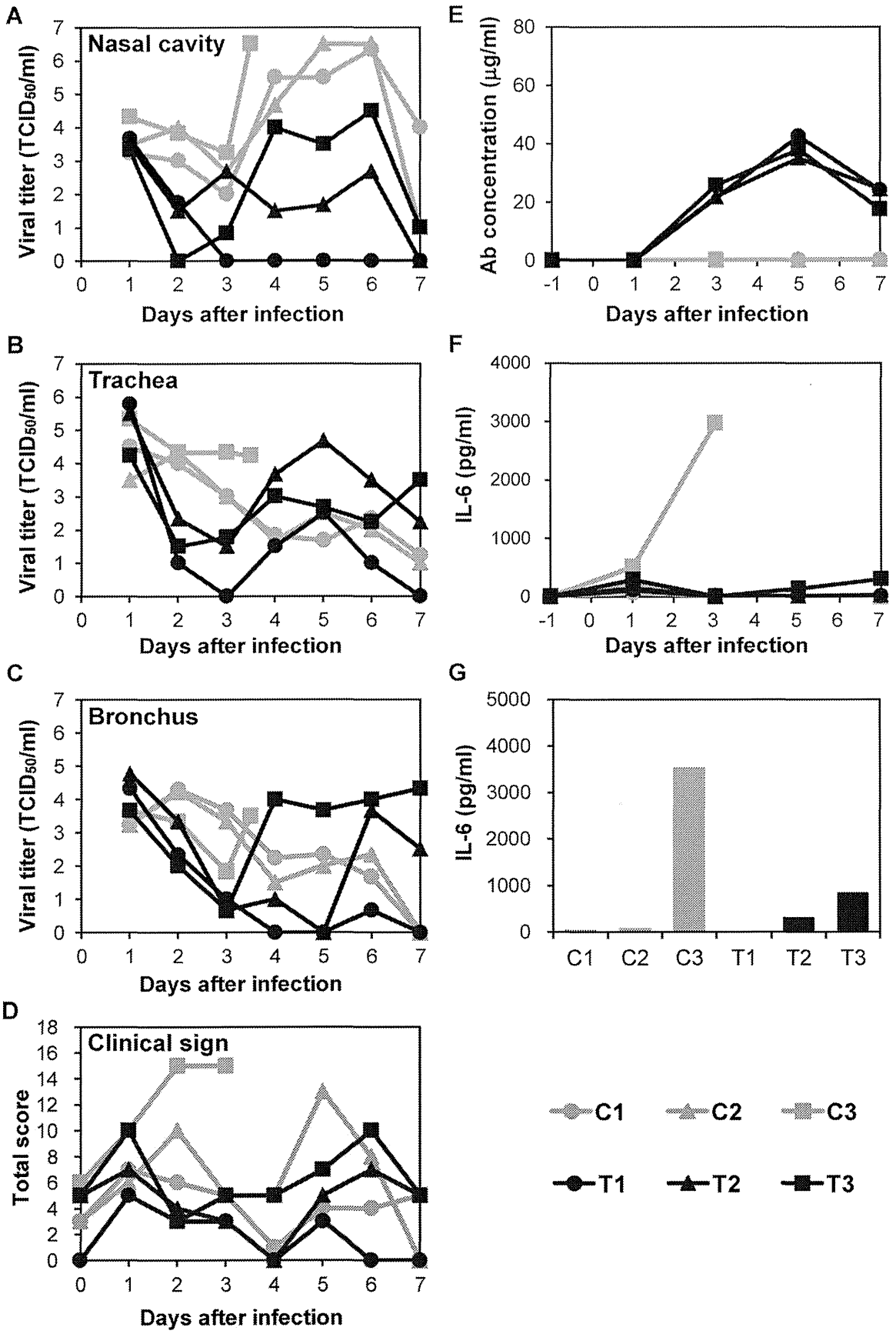
Ethics statement

Animal studies were carried out in strict accordance with the Guidelines for Proper Conduct of Animal Experiments of the Science Council of Japan. The animal experiments were conducted in strict compliance with animal husbandry and welfare regulations. The mouse study was approved by the Hokkaido University Animal Care and Use Committee (Permit number: 08-0234). The nonhuman primate study was also carried out in strict accordance with the Guidelines for the Husbandry and Management of Laboratory Animals of the Research Center for Animal Life Science at Shiga University of Medical Science and Standards Relating to the Care and Management, etc. of Experimental Animals (Notification No. 6, March 27, 1980 of the Prime Minister's Office, Japan). The protocol was approved by the Shiga University of Medical Science Animal Experiment Committee (Permit number: 2011-6-9HHH). All procedures were performed under ketamine and xylazine anesthesia, and all efforts were made to minimize suffering. Regular veterinary care and monitoring, balanced nutrition, and environmental enrichment were provided by the Research Center for Animal Life Science at the Shiga University of Medical Science. Macaques were euthanized at endpoint (7 days after virus inoculation for immunological and virological analysis) using ketamine and xylazine anesthesia followed by intravenous injection of pentobarbital (200 mg/kg). Animals were monitored twice a day during the study to be clinically scored as shown in Table S1. Animals would be euthanized if their clinical scores reached 15 (a humane endpoint).

Results

In vitro characterization of anti-H5 MAbs m61 and ch61

MAb m61 showed neutralizing activities against HK483 and VN1194 (Fig. 1A). The 50% inhibitory concentrations of MAb m61 against HK483 and VN1194 were 0.42 and 0.92 μg/ml, respectively. To determine the epitope for MAb m61, escape mutants of VN1194 were selected in the presence of this MAb and the deduced amino acid sequences of the parent virus and mutants were compared. Lysine to threonine, asparagine, and glutamic acid substitutions were found at position 193 in 12.5, 25.0, and 12.5% of the cloned mutants, respectively, and 50% of the mutants had substitution from lysine to glutamic acid at position 222 (data not shown). The amino acid residue at position 193 is located near the receptor-binding site on the antigenic sites of HA molecules [31–34]. Accordingly, MAb m61 showed hemagglutination-inhibition activity (data not shown). We then converted MAb m61 into the human-mouse chimeric MAb ch61, and its neutralizing activities against HK483, VN1194, and VN3040 were analyzed in vitro (Fig. 1B). MAb ch61 significantly reduced the infectivity of these H5N1 viruses in a dose-dependent manner, whereas the negative control MAbs did not. The 50% inhibitory concentrations of MAb ch61 against HK483, VN1194, and VN3040 were 0.43, 1.00, and 2.29 μg/ml, respectively. These values were similar to those of the original mouse MAb m61, indicating that genetic modification of this MAb did not significantly affect the neutralizing activity *in vitro*.


A
Nasal cavity
Viral titer (TCID50/ml)
Days after infection
B
Trachea
Viral titer (TCID50/ml)
Days after infection
C
Bronchus
Viral titer (TCID50/ml)
Days after infection
D
Clinical sign
Total score
Days after infection
E
Ab concentration (μg/ml)
Days after infection
F
IL-6 (pg/ml)
Days after infection
G
IL-6 (pg/ml)
C1
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T2
T3

Figure 3. Protection of immunocompetent macaques treated with MAb ch61 against VN3040 infection. Macaques infected with VN3040 (3×10^6 PFU) on day 0 were injected intravenously with control MAbs (C1–C3, orange) or MAb ch61 (T1–T3, blue) on days 1 and 3. Viral titers in nasal (A), tracheal (B), and bronchial (inside lungs) (C) swab samples were determined using MDCK cells. Viral titers under the detection limit are indicated as 0. Clinical signs were scored with the parameters shown in Table S1 (D). Serum samples were collected from macaques during the period of the experiments and antibodies specific to influenza virus HA were quantified by ELISA [23] (E). IL-6 concentrations in serum samples (F) and lung tissue samples (G) were measured as described in the Materials and Methods section. Lung tissue samples of macaque C3 and other macaques were collected at autopsy on day 3 and on day 7, respectively, and IL-6 concentrations in 10% (w/v) homogenates in saline were measured.
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Table 1. Summary of treatments and survival rates of macaques.

Exp.	Animal ID	CP and CA	MAb	Peramivir	Survival/Total
#1	C1–C3	−	ch113+ch226	−	2/3
	T1–T3	−	ch61	−	3/3
#2	IC1–IC3	+	ch113+ch226	−	0/3
	IT1–IT5	+	ch61	−	3/4[a]
#3	ICP1–ICP3	+	ch113+ch226	+	1/3
	ITP1–ITP3	+	ch61	+	2/3
All[b]	C, IC, ICP	− or +[c]	ch113+ch226	− or +[d]	3/9[e]
	T, IT, ITP	− or +	ch61	− or +	8/10[e]

[a]One animal (IT3) that died most likely of bacterial infection was excluded.
[b]Sums of macaques used in three experiments are shown.
[c]CP and CA for immunosuppression were used in Exp. #2 and #3 but not in #1.
[d]Peramivir treatments were combined in Exp. #3 but not in #1 and #2.
[e]Significantly different survival rates between the two groups (chi-square test, $p<0.05$).
doi:10.1371/journal.ppat.1004192.t001

Table 2. Virus titers in the lungs of macaques.

		Log_{10} $TCID_{50}/g$[a]					
Animal	Autopsy	RU[b]	RM[b]	RL[b]	LU[b]	LM[b]	LL[b]
C1	Day 7	1.67	2.23	4.00	ND[c]	ND	2.00
C2	Day 7	ND	1.67	ND	2.00	ND	1.67
C3	Day 3	3.33	3.33	4.50	3.50	3.67	4.67
T1	Day 7	ND	ND	ND	ND	ND	ND
T2	Day 7	ND	4.23	6.00	4.50	ND	4.00
T3	Day 7	4.67	5.50	5.50	4.67	3.00	4.83
IC1	Day 3	6.00	7.33	7.67	8.00	6.67	7.50
IC2	Day 5	6.00	5.50	5.67	3.23	4.50	5.67
IC3	Day 4	4.50	2.23	4.50	2.33	3.50	4.67
IT1	Day 7	5.00	4.00	4.67	3.33	2.67	3.33
IT2	Day 7	3.50	3.50	1.83	1.67	2.00	4.23
IT3	Day 6	ND	ND	ND	2.33	ND	ND
IT4	Day 4	ND	ND	ND	2.50	1.67	3.67
IT5	Day 7	ND	3.00	2.67	ND	5.33	ND
ICP1	Day 5	ND	ND	ND	3.00	ND	ND
ICP2	Day 7	ND	ND	ND	ND	ND	ND
ICP3	Day 4	ND	ND	ND	ND	ND	1.67
ITP1	Day 7	ND	ND	ND	ND	ND	ND
ITP2	Day 7	ND	ND	ND	ND	ND	ND
ITP3	Day 3	1.67	ND	ND	ND	ND	ND

[a]After autopsy on indicated days after virus infection, lung tissue samples were collected.
The lung tissue homogenates were prepared and used to infect MDCK cells to determine $TCID_{50}$ per gram of tissue.
[b]RU, right upper lobe; RM, right middle lobe; RL, right lower lobe; LU, left upper lobe; RM, right middle lobe; LL, left lower lobe.
[c]Not detected (detection limit was $10^{1.5}$ $TCID_{50}/g$).
doi:10.1371/journal.ppat.1004192.t002

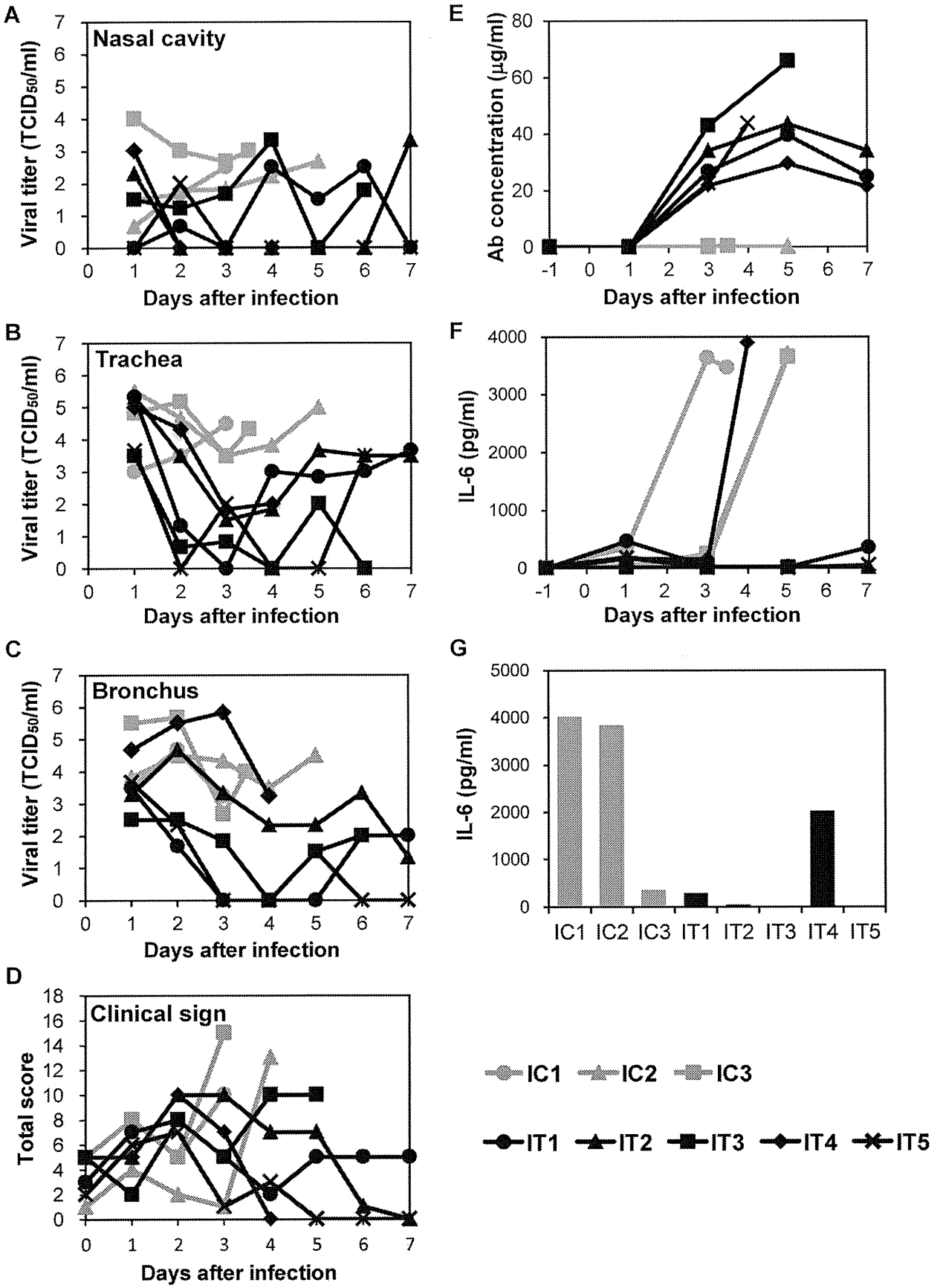
A
Nasal cavity
Viral titer (TCID50/ml)
Days after infection
B
Trachea
Viral titer (TCID50/ml)
Days after infection
C
Bronchus
Viral titer (TCID50/ml)
Days after infection
D
Clinical sign
Total score
Days after infection
E
Ab concentration (μg/ml)
Days after infection
F
IL-6 (pg/ml)
Days after infection
G
IL-6 (pg/ml)
IC1 IC2 IC3 IT1 IT2 IT3 IT4 IT5
IC1 IC2 IC3
IT1 IT2 IT3 IT4 IT5

Figure 4. Protection of immunocompromised macaques treated with MAb ch61 from VN3040 infection. Macaques pretreated with CP and CA were infected and injected intravenously with control MAbs (IC1–IC3, orange) and MAb ch61 (IT1–IT5, blue) as described in the legend of Figure 3. Viral titers in nasal (A), tracheal (B), and bronchial (C) swab samples were determined using MDCK cells. Viral titers under the detection limit are indicated as 0. Clinical signs were scored with the parameters shown in Table S1 (D). Concentrations of MAb ch61 (E) and IL-6 (F: serum and G: lung) were examined as described in the legend of Figure 3. Lung tissue samples were collected at autopsy from IC1 on day 3, from IC2 on day 5, from IC3 on day 4, from IT3 on day 6, and from IT4 on day 4.
doi:10.1371/journal.ppat.1004192.g004

Protective efficacy of passive immunization with MAbs m61 and ch61 in mice

We next investigated the potential of MAbs m61 and ch61 to protect mice from infection by HK483, known to be highly virulent for mice [35,36]. Mice treated with these antibodies 1 day before or 1 day after virus challenge with a lethal dose of HK483 survived without clinical symptoms, whereas all control mice died (or were euthanized) within 9 days after the challenge (Figs. 2A, B). Control mice uniformly showed severe weight loss (>25%) (data not shown). Treatment at 3 days after infection also partially protected the mice (Fig. 2C), although 2 surviving mice treated with m61 showed moderate weight loss (<15%) (data not shown). All control mice exhibited severe weight loss (>25%) and succumbed to HK483 infection. Consistent with the survival data, lung virus titers of mice treated with these anti-H5 HA MAbs 1 day before virus challenge were significantly lower than those of mice given the respective control antibodies (Fig. 2D). While of statistical significance, treatment after infection only modestly reduced the titers (Fig. 2D). These results indicated that MAbs m61 and ch61 were highly protective against H5N1 HPAI virus in mice.

Efficacy of chimeric anti-H5 MAb ch61 in immunocompetent macaques infected with H5N1 HPAI virus isolated from a human patient

To examine therapeutic efficacy of MAb ch61 in a nonhuman primate model of H5N1 HPAI virus infection, VN3040 was used, since this virus causes severe, often lethal, disease in cynomolgus macaques [37]. Macaques were infected with VN3040 on day 0 and treated with MAb ch61 or control MAbs twice on days 1 and 3 after infection. Body temperatures rose upon infection and decreased after the first injection of MAb ch61, but rose again on days 4–5 (Fig. S2). One of three macaques injected with control MAbs (C3) died on day 4, whereas all three macaques treated with MAb ch61 survived until day 7 after infection (Table 1, Exp. #1). The viral titers in nasal, tracheal, and bronchial samples of macaques treated with MAb ch61 were lower than those of macaques injected with control MAbs after the first injection of MAbs (i.e., on days 2 and 3) (Figs. 3A–C). In one of the MAb ch61-treated macaques (T1), the virus was only slightly detected in the nasal and bronchial samples on days 3–7 (Figs. 3A, C). Although the virus was recovered from the nasal samples of the other treated macaques (T2 and T3), the titers were lower than those of macaques injected with control MAbs (C1, C2, and C3) (Fig. 3A). Infectious viruses were recovered from lungs of most of the macaques even on day 7 (Table 2). Interestingly, the viral titers in nasal, tracheal, and bronchial samples drastically increased after day 4 in one macaque treated with MAb ch61 (T3) (Figs. 3A–C). Similar phenomenon was partially observed in the other treated macaques. Viral titers in tracheal and bronchial samples were often higher in T2 and T3 than in control macaques on days 4–7 (Figs. 3B, C). Accordingly, relatively high titers of the virus was detected in their lungs collected on day 7 (Table 2). Two of the treated macaques (T2 and T3) lost their appetite after virus infection and their clinical scores were increased, but they temporally recovered after injection of MAb ch61 (Fig. 3D). These results indicated that MAb ch61 reduced viral titers in the respiratory secretions of all the treated macaques, although inhibition of viral propagation was temporary in two of the treated macaques. We confirmed that the MAb concentrations on days 3–7 after challenge were maintained at above 20 μg/ml in all treated macaques up to day 7 (Fig. 3E). Thus, to examine the appearance of escape mutants, we sequenced viral RNAs extracted from the tracheal samples collected from MAb ch61-treated macaques on day 5. We found amino acid substitutions identical to those seen in the escape mutants selected in vitro (i.e., K193N or K193E) in 83% (5/6) and 25% (3/12) of the cloned viral genes obtained from T1 and T3, respectively, indicating that viral escape occurred during the treatment period.

Efficacy of anti-H5 antibody treatment in immunocompromised macaques infected with H5N1 HPAI virus isolated from a human patient

To further examine the protective potential of MAb ch61, we used an immunocompromised macaque model with influenza virus infection [29]. Macaques were pretreated with CP and CA and then infected with VN3040 on day 0. Increased body temperature was observed after infection in most of the macaques (IC1, IC2, IC3, IT1, IT2, IT3, and IT5) (Fig. S3). Body temperatures that rose upon infection decreased after the treatment with MAb ch61 in IT1 and IT2, but rose again on days 6–7. All three macaques injected with control MAbs succumbed to infection by day 5 (IC1, IC2, and IC3), whereas two (IT3 and IT4) of the five macaques injected with MAb ch61 also died on days 6 and 4, respectively (Table 1, Exp. #2).

Infectious viruses were consistently detected in the nasal, tracheal, and bronchial samples of macaques injected with control MAbs until death (Figs. 4A–C). On the other hand, the viral titers in the nasal samples of IT2, IT4, and IT5, and those in the tracheal samples of all five macaques treated with MAb ch61 decreased on days 2 and 3 (i.e., after injection of the antibody) (Figs. 4A, B). It was also noted that the viral titers in the bronchial samples of IT1, IT3, and IT5 were markedly reduced on days 2 and 3 (Fig. 4C). However, in the bronchial samples of IT2 and IT4, the titers on days 2 and 3 were similar to those of control macaques (Fig. 4C). Clinical scores in IT2 and IT5 were improved (clinical score = 0) on day 7 and IT1 slightly regained its appetite after MAb treatment (Fig. 4D). The viral titers increased on days 4–7 in the trachea and bronchial samples of some of the treated macaques (e.g., IT1 and IT2), as was the case with treatment of immunocompetent macaques. Furthermore, infectious viruses were detected in all lobes of their lungs, while the virus replication in the lungs of the other treated macaques was limited on day 7 (Table 2). Viruses with the K193R substitution in HA were recovered from the tracheal samples of IT1 and IT2 (11/11 and 17/18 of the cloned HA genes, respectively), whereas the concentrations of MAb ch61 circulating in the serum on days 3–7 after challenge were maintained at above 20 μg/ml in all treated macaques (Fig. 4E). These results indicated that treatment

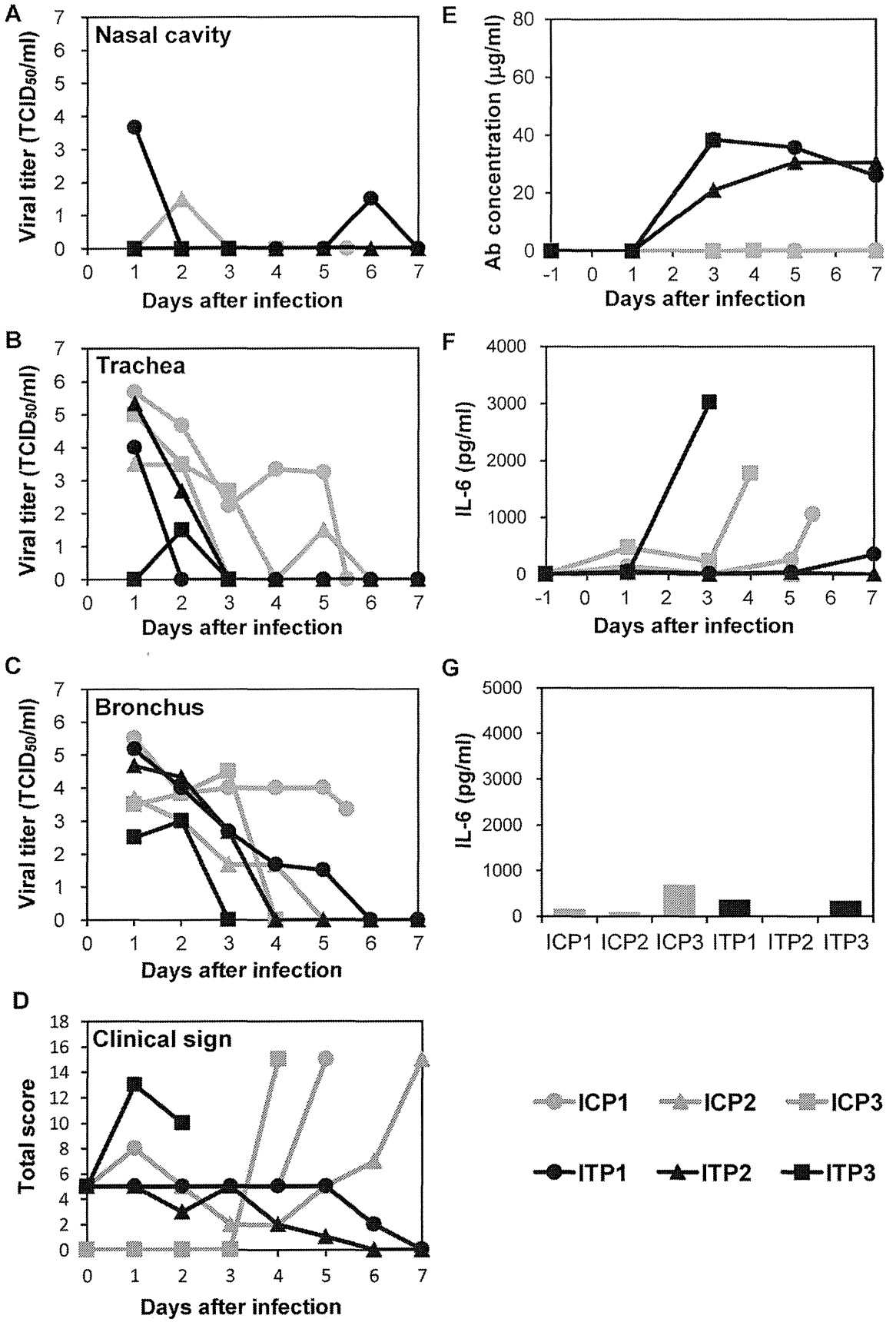
A
Nasal cavity
Viral titer (TCID50/ml)
Days after infection
B
Trachea
Viral titer (TCID50/ml)
Days after infection
C
Bronchus
Viral titer (TCID50/ml)
Days after infection
D
Clinical sign
Total score
Days after infection
E
Ab concentration (μg/ml)
Days after infection
F
IL-6 (pg/ml)
Days after infection
G
IL-6 (pg/ml)
ICP1 ICP2 ICP3 ITP1 ITP2 ITP3
ICP1
ICP2
ICP3
ITP1
ITP2
ITP3

Figure 5. Protection of immunocompromised macaques treated with MAb ch61 and peramivir from VN3040 infection. Macaques pretreated with CP and CA were infected and injected intravenously with control MAbs (ICP1–ICP3, orange) and MAb ch61 (ITP1–ITP3, blue) and with peramivir as described in the legend of Figure 3. Viral titers in nasal (A), tracheal (B), and bronchial (C) swab samples were determined using MDCK cells. Clinical signs were scored with the parameters shown in Table S1 (D). Viral titers under the detection limit are indicated as 0. Concentrations of MAb ch61 (E) and IL-6 (F: serum and G: lung) were examined as described in the legend of Figure 3. Lung tissue samples were collected at autopsy from ITP3 on day 3, from ICP3 on day 4, from ICP1 on day 5, and from other macaques on day 7.
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with MAb ch61 resulted in reduced viral loads and partial protection from lethal HPAI virus infection in immunosuppressed macaques, though this MAb treatment might select escape mutants.

Efficacy of combination therapy using anti-H5 antibody and a neuraminidase inhibitor in immunocompromised macaques infected with H5N1 HPAI virus isolated from a human patient

Since escape mutants were frequently selected in macaques treated with MAb ch61 alone, we examined combination therapy with MAb ch61 and the neuraminidase inhibitor peramivir to further reduce viral replication and the emergence of escape mutants. CP- and CA-pretreated macaques were infected with VN3040 and then MAbs were injected on days 1 and 3 in addition to continuous administration of peramivir on days 1–5. Two macaques treated with peramivir alone had to be humanely euthanized on days 5 and 4 (ICP1 and ICP3, respectively), whereas one macaque that received the combined treatment also died on day 3 (ITP3) (Table 1, Exp. #3). Increased body temperature was observed in two control and one ch61-treated macaques (ICP1, ICP3, and ITP2) (Fig. S4). The viral titers in the nasal and tracheal samples of macaques treated with both MAb ch61 and peramivir were almost undetectable after day 3 (Figs. 5A, B). Unlike MAb treatment alone (Figs. 3 and 4), no increase of the viral titer or body temperature was observed on days 4–7 in surviving macaques treated with MAb ch61 together with peramivir (Figs. 5A–C and Fig. S4) and the concentrations of MAb ch61 in the serum were maintained at above 20 μg/ml on days 3–7 after challenge in these macaques (Fig. 5E). Accordingly, infectious viruses were only slightly detected in the limited parts of lungs of the macaques (Table 2) and escape mutations (i.e., K193N or K193E) were not found in the cloned viral genes (0/11) obtained from the MAb ch61-treated macaques. Along with the reduced viral recovery from the samples, clinical scores in ITP1 and ITP2 were generally improved on day 7. These results indicated that combination therapy with MAb ch61 and peramivir inhibited viral propagation more efficiently than MAb or peramivir treatment alone, which might also result in reduced selection of escape mutants and improved survival after H5N1 HPAI virus infection in macaques.

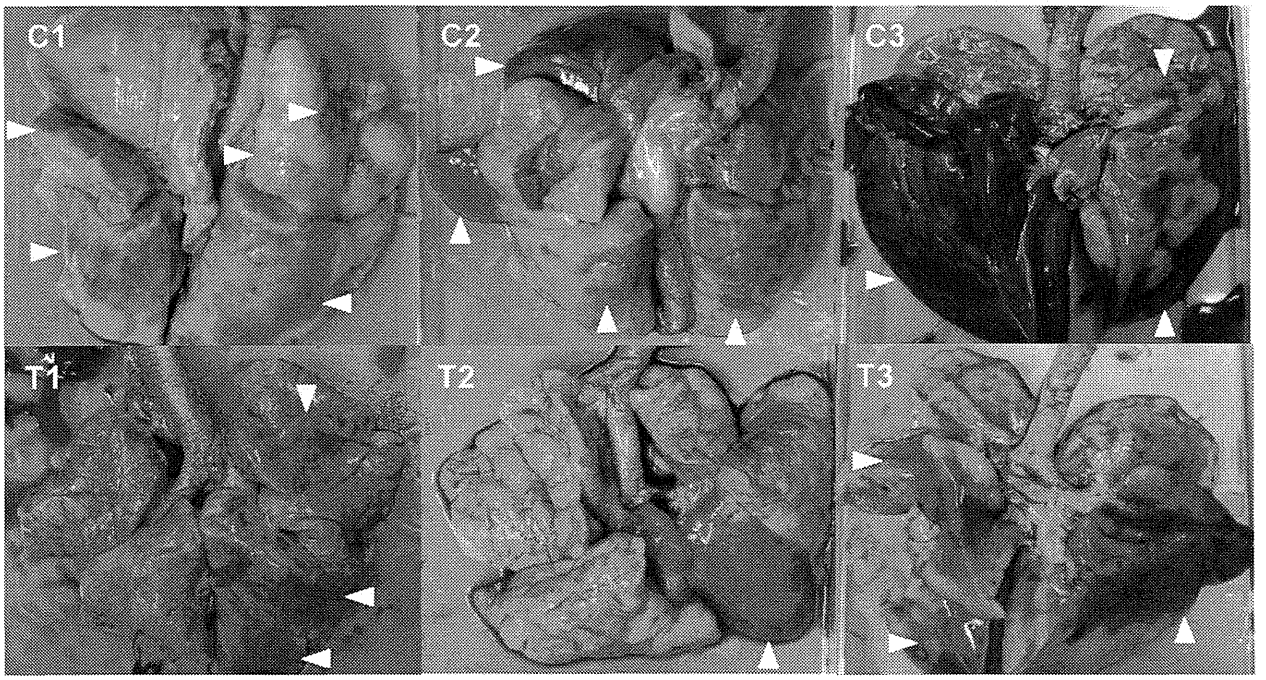

Figure 6. Gross pathological changes of the lungs of immunocompetent macaques infected with VN3040. Macaques were treated with control antibodies (C1–C3) or MAb ch61 (T1–T3). Macaque C3 was autopsied 3 days after virus infection. The other macaques were autopsied 7 days after virus infection. Dark red lesions indicated by white arrowheads show macroscopic inflammation, hemorrhage, and congestion.
doi:10.1371/journal.ppat.1004192.g006

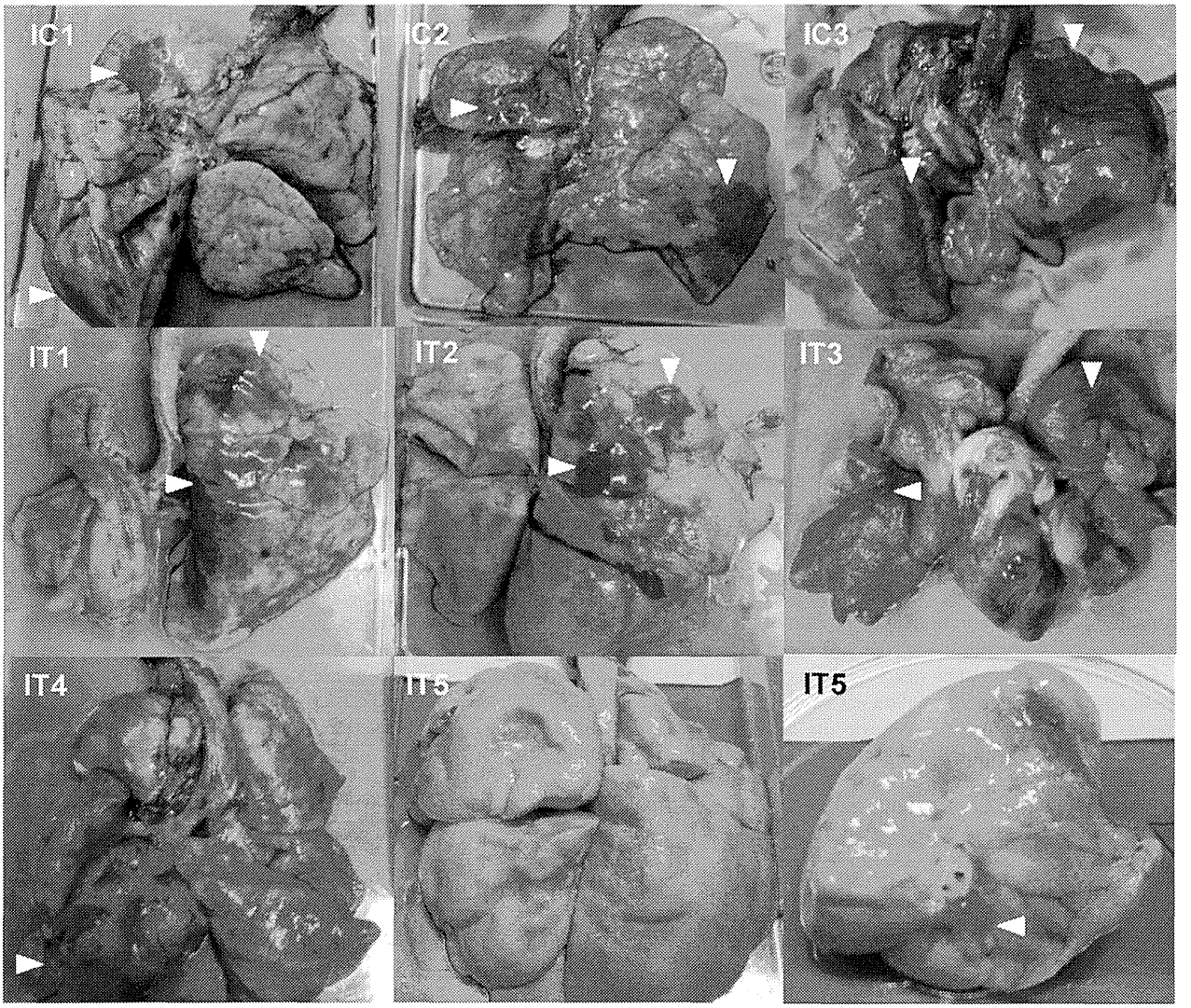

Figure 7. Gross pathological changes of the lungs of immunosuppressed macaques infected with VN3040. Immunosuppressed macaques were treated with control antibodies (IC1–IC3) or MAb ch61 (IT1–IT5). Macaques IC1, IC2, and IC3 were autopsied 3 days, 5 days, and 4 days after virus infection, respectively. Macaques IT3 and IT4 were autopsied 6 days and 4 days after virus infection, respectively. Macaques IT1, IT2, and IT5 survived during the observation period and were autopsied 7 days after virus infection. The upper lobe of the left lung of IT5 is shown in the lower right picture. Dark red lesions indicated by white arrowheads show macroscopic inflammation, hemorrhage, and congestion.
doi:10.1371/journal.ppat.1004192.g007

Correlation between disease severity and elevated levels of IL-6

To determine the cause of death of the macaques, we examined inflammation by measuring IL-6 production in sera and lung tissues. In the immunocompetent macaque model, an elevated IL-6 level was observed on day 3 in the serum of one macaque (C3) that died on day 4 but not in the other macaques (Fig. 3F). In addition, the lung IL-6 level of C3 on day 3 was markedly higher than those of the other macaques (Fig. 3G). In the immunocompromised macaque model, a marked increase of IL-6 was detected in the sera and/or lung tissues of all macaques injected with control MAbs (Figs. 4F, G). Similarly, increased IL-6 levels were detected in IT4. In a MAb ch61-treated macaque that died on day 6 (IT3), bacterial infection was detected in the cerebral ventricle (data not shown) and the rapid IL-6 response was not observed, suggesting that this macaque died of bacterial meningitis, not virus infection. In the combination therapy experiment, increased levels of IL-6 were detected in the sera of ICP1, ICP3, and ITP3, all of which were humanely euthanized or died after infection (Fig. 5F). IL-6 levels in lung tissues were relatively high in ICP3 and ITP3 (Fig. 5G). Consistent with some human cases previously described [38,39], these results suggested that increases of IL-6 in the serum and lungs might be associated with systemic inflammatory responses leading to death. While increased production of TNF-α and IL-1β were also seen in the macaques with severe disease, the other cytokines tested were unlikely correlated with disease severity of the macaques (Figs. S5, S6). Since elevated levels of IL-6, TNF-α and IL-1β are likely involved in a variety of systemic inflammatory states that are associated with endothelial barrier dysfunction, these cytokines could be important mediators of increased endothelial permeability, which might result in systemic organ failure caused by H5N1 HPAI virus infection.