博士論文（要約）

Analysis of the host responses to influenza virus infection and their application to the development of vaccines

（インフルエンザウイルスに対する宿主応答の解析とワクチン開発への応用）

桂　廣亮

CONTENTS

PREFACE

On account of the affluence and medical advances of developed countries, we now have an average life expectancy of 80 years. Then, the risk of various diseases such as cancers, cardiac disorders, apoplexy, and diabetes increases. Meanwhile, infectious diseases have been a threat to all mankind regardless of age and sex since time began. Influenza is one of the most familiar infectious diseases. It is a respiratory disorder caused by influenza viruses. Influenza can cause severe clinical symptoms compared with other respiratory infectious diseases, and it is highly contagious. Accordingly, the influenza virus is a considerable burden on medical and public health worldwide.

Influenza viruses belong to the *Orthomyxoviridae* family and possess eight segments of negative-stranded viral RNA (vRNA). They are classified into three major types, influenza A, B, and C viruses, based on the antigenicities of their internal proteins (Wright et al., 2013). Influenza A and B viruses have been circulating in humans. Because influenza A viruses have the potential to cause pandemics, these viruses have been extensively studied from global surveillance to their interactions with host cell systems.

Influenza A viruses are further classified into subtypes based on the antigenicities of their two surface proteins, hemagglutinin (HA) and neuraminidase (NA). Currently, 18 HA and 9 NA subtypes have been identified (Tong et al., 2012; Tong et al., 2013; Wright et al., 2013). During the 20th century, we experienced three pandemics caused by influenza A virus: Spanish influenza in 1918 (H1N1 subtype), Asian influenza in 1957 (H2N2 subtype), and Hong Kong influenza in 1968 (H3N2 subtype). Although the origin of the Spanish influenza remains controversial, the H2 HA, N2 NA, and PB1 gene segments of the Asian influenza and the H3 HA and PB1 gene segments of the Hong

Kong influenza came from avian influenza viruses (Scholtissek et al., 1978; Kawaoka et al., 1989). In 2009, the newly emerging swine origin influenza A virus (H1N1) rapidly spread all over the world and resulted in the first pandemic of this century. The World Health Organization (WHO) defined this virus as A(H1N1) pdm09. At that time, a triple reassortant virus between human H3N2, North American avian, and classical swine viruses was circulating in the North American pig population. A(H1N1) pdm09 emerged by further reassortment of the triple reassortant virus with a Eurasian avian-like swine virus (Dawood et al., 2009; Smith et al., 2009). Thus, newly emerging viruses that result from the reassortment of viral gene segments derived from distinct viruses circulating in various animals can occasionally cause pandemics. This is a key characteristic of influenza viruses.

Since the first case of human infection with an H5N1 highly pathogenic avian influenza virus was identified in Hong Kong in 1997 (Claas et al., 1998), H5N1 viruses have undergone rigorous surveillance. Avian influenza viruses are thought to have a low probability of airborne human-to-human transmission due, in part, to the difference in receptor binding preference between human and avian viruses (Rogers et al., 1983); indeed, only sporadic cases of infection have been reported. Nevertheless, the case fatality rate of infection with H5N1 highly pathogenic avian influenza viruses is estimated to be ~60%. Therefore, a pandemic caused by one of these viruses would inflict enormous damage on human society. Recently, several mutations in H5 HA that allow transmission between mammals via the airborne route were identified in a ferret model (Herfst et al., 2012; Imai et al., 2012). Importantly, H5N1 avian influenza viruses that possess some of these mutations have been isolated in nature (Neumann et al., 2012).

In addition to H5N1 subtypes, H7N9 avian influenza virus infection of humans

was reported in China in 2013 (Chen et al., 2013; Gao et al., 2013; Li et al., 2013). Because viruses of the H7N9 subtype have not previously circulated in humans, no one has specific immunity against these viruses (Watanabe et al., 2013). To make matters worse, limited transmissibility of H7N9 viruses isolated from human patients was observed in a ferret model (Belser et al., 2013; Richard et al., 2013; Watanabe et al., 2013). If influenza viruses we have never encountered acquire the ability to transmit more readily via the airborne route from person to person, it is highly likely that such viruses will cause a pandemic. It is difficult to predict which subtype of virus will cause a pandemic; therefore, we have to continue the global surveillance and basic research of influenza viruses.

Influenza viruses infect the respiratory tract. Avian influenza viruses preferentially recognize α2,3-linked sialic acid (SA) and human isolates recognize α2,6-linked SA as receptors (Rogers et al., 1983). After binding and internalization, viruses are transported via the endocytosis pathway (Matlin et al., 1981; Sieczkarski et al., 2002), and then viral RNAs (vRNAs) are released from virions after virus-host membrane fusion. During the influenza virus replication cycle, vRNAs are sensed by pattern recognition receptors (PRRs), such as retinoic acid inducible gene-I (RIG-I) (Hornung et al., 2004; Pichlmair et al., 2004) and toll-like receptor (TLR) 7 (Diebold et al., 2004), and subsequent antiviral responses, including the production of type I interferon, pro-inflammatory cytokines, and chemokines, are elicited (Kawai et al., 2006). Then, various immune cells such as monocytes and neutrophils infiltrate the sites of infection depending on the secreted chemokines to contain the spread of virus and remove the virus-infected cells. The inflammatory responses then resolve due to the action of anti-inflammatory cytokines such as interleukin (IL)-10, and adaptive immunity, which is dominated by T cells and B cells, is activated. In this doctoral thesis, I focused on three points: (1)

influenza virus infection within host cells and the induction of antiviral responses *in vivo*, (2) the resolution of inflammation, and (3) the induction of virus-specific adaptive immunity.

Little is known about how an influenza virus infection spreads throughout the body over time due to the lack of a suitable reporter virus that would allow us to detect virus-infected cells easily in the body. It is difficult to stably express a foreign gene because the size of the viral genome is limited and the mechanisms for incorporation of segmented viral genomes into virions are not fully understood. In 2010, a replication-competent recombinant virus that expressed green fluorescent protein (GFP) in infected cells was reported (Manicassamy et al., 2010). This reporter virus was very useful, but had several problems in terms of stability of GFP expression. For these reasons, our group improved the expression of the fluorescent protein in this reporter virus. In chapter I, I will describe the characterization of the improved reporter virus and my analysis of the host responses obtained when using this reporter virus.

To prevent infection with influenza viruses, vaccination is one of the most effective measures. Two types of influenza vaccines are available, inactivated vaccines and live attenuated vaccines; however, there is still room for improvement in terms of their immunogenicity and safety, respectively (Cox et al., 2004). In chapter II, I show the results of my assessment of the efficacy of a new vaccine based on a replication-incompetent virus. Specifically, I generated a replication-incompetent virus that was deficient in HA membrane fusion activity and assessed its vaccine efficacy.

In chapter III, I attempted to develop a bivalent vaccine based on a replication-incompetent virus that carried an antigen for another respiratory infectious agent, *Streptococcus pneumoniae*. I selected *S. pneumoniae* because it is a causative agent of

not only community-acquired pneumonia but also a bacterial pneumonia that follows influenza virus infection (Morens et al., 2008; Jambo et al., 2010; Gill et al., 2010). I generated a virus that expresses pneumococcal surface protein A (PspA) in infected cells, and then assessed its vaccine efficacy in mice. I conclude my thesis by suggesting new possibilities for replication-incompetent viruses.

CHAPTER I

Characterization of a recombinant influenza A virus carrying the Venus gene and its application for the analysis of the infection dynamics in the mouse lung.

Chapter I は雑誌掲載のため公表できません。

CHAPTER II

A replication-incompetent virus possessing an uncleavable hemagglutinin as an influenza vaccine.

Abstract

Vaccination is one of the most effective measures to protect against influenza virus infection. Inactivated and live-attenuated influenza vaccines are available; however, their efficacy is suboptimal. To develop a safe and more immunogenic vaccine, I produced a novel replication-incompetent influenza virus that possesses uncleavable hemagglutinin (HA) and tested its vaccine potential. The uncleavable HA was engineered by substituting the arginine at the C-terminus of HA1 with threonine, which prevents cleavage of HA into its HA1 and HA2 subunits, preventing fusion between the host and viral membranes. Although this fusion-deficient HA influenza virus that possesses uncleavable HA (uncleavable HA virus) could undergo multiple cycles of replication in only wild-type HA-expressing cells, it could infect normal cells and express viral proteins in infected cells, but could not generate infectious virus from infected cells due to the uncleavable HA. When C57BL/6 mice were intranasally immunized with the uncleavable HA virus, influenza-specific IgG and IgA antibodies were detected in nasal wash and bronchoalveolar lavage samples and in serum. In addition, influenza-specific $CD8^+$ T cells accumulated in the lungs of these mice. Moreover, mice immunized with the uncleavable HA virus were protected against a challenge of lethal doses of influenza virus, unlike mice immunized with a formalin-inactivated virus. These findings demonstrate that this fusion-deficient virus, which possesses uncleavable HA, is a suitable influenza vaccine candidate.

Introduction

Influenza viruses cause acute respiratory disease and are responsible for epidemics and occasional pandemics as exemplified in 2009 (Dawood et al., 2009; Neumann et al., 2009). Although medical treatment options are well developed, influenza continues to be a great burden on public health and economies worldwide.

Currently, inactivated and live-attenuated vaccines are available. While inactivated vaccines are safe and induce virus-specific immunoglobulin G (IgG) in sera because of intramuscular administration, live-attenuated vaccines, which bear introduced mutations that are responsible for temperature sensitivity and viral attenuation (Maassab et al., 1968; Chen et al., 2006; Hoffmann et al., 2005; Jin et al., 2003; Jin et al., 2004), are more effective because they induce not only virus-specific IgG but also secretory Immunoglobulin A (IgA) at the site of infection following intranasal administration (Cox et al., 2004). In addition to inducing antibodies, live-attenuated vaccines elicit the virus-specific cytotoxic T lymphocytes (CTLs). CTLs specifically lyse infected cells and prevent viral spread. CTLs are also known to have cross-reactivity between different subtypes of influenza A virus. Thus, the induction of CTLs has attracted attention in vaccine development (Rimmelzwaan et al., 2007). Nevertheless adverse effects, such as runny nose, due to replication of the live-attenuated vaccine virus are an issue (Ambrose et al., 2008), and importantly, this live vaccine is not recommended for children under the age of 2, adults aged 50 or over, pregnant women, or immunocompromised patients (Fiore et al., 2010). To overcome current limitations, an ideal influenza vaccine continues to be sought and various strategies have been employed such as virus-like particles (VLPs) (Lambert et al., 2010).

To develop a safe and more effective vaccine that has the merits of inactivated and live-attenuated vaccines, we previously proposed a replication-incompetent virus as a novel influenza vaccine. We reported that NS2-knockout VLPs could efficiently protect mice against lethal doses of influenza virus (Watanabe et al., 2002). However, because of the lack of a cell line that stably expresses NS2 protein, we have to generate VLPs by transfecting plasmids each time.

To overcome this drawback, here I developed a novel replication-incompetent virus that possesses an uncleavable HA (uncleavable HA virus) and assessed its immunogenicity and vaccine efficacy in mice.

Materials and methods

Plasmids. To generate the construct encoding the uncleavable HA gene, we used a plasmid containing the HA gene of A/California/04/09 (CA04) as a template for PCR and primers containing nucleotide replacements at positions 1031 and 1032 of the HA gene, which introduce amino acid substitutions at the cleavage site of HA. This mutated HA gene was cloned into a plasmid under the control of the human polymerase I promoter and the mouse RNA polymerase I terminator (referred as PolI plasmid) as described previously (Neumann et al., 1999). In addition, a plasmid for the expression of wild-type CA04 HA was also constructed by cloning the open reading frame of the CA04 HA gene into an expression plasmid pCAGGS (Niwa et al., 1991).

Cells and viruses. Madin-Darby canine kidney (MDCK) and human embryonic kidney 293T (HEK293T) cells were maintained in minimum essential medium (MEM) containing 5% of newborn calf serum (NCS) and in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, respectively. MDCK cells expressing HA from CA04 [HA-MDCK (CA04)] and from A/WSN/33 (WSN) [HA-MDCK (WSN)] were established as follows: for HA-MDCK (CA04) cells, MDCK cells were co-transfected with plasmids for the expression of CA04-HA and puromycin *N*-acetyltransferase, and were cultured in MEM containing 5% NCS and 5 μg/ml puromycin dihydrochloride (Nacalai Tesque). Expression of HA in selected cell clones was confirmed by means of immunostaining with an anti-CA04 HA antibody. For HA-MDCK (WSN), MDCK cells were transfected with plasmids expressing WSN-HA and WSN-neuraminidase (NA) along with pcDNA3.1(+) (Invitrogen), and were cultured in MEM containing 5% NCS

and 0.8 mg/ml Geneticin. Surviving cell colonies were picked and were tested for HA and NA expression by means of immunostaining with anti-WSN HA and NA antibodies. Antigen-positive cells were then cloned by using a limited dilution assay. Expression of HA but not NA protein was confirmed in the resulting cell clones. All cells were maintained at 37°C in 5% CO_2. Mouse-adapted CA04 virus (Sakabe et al., 2011) was propagated in MDCK cells as previously described.

Generation of uncleavable HA virus. The uncleavable HA virus was artificially generated by using plasmid-based reverse genetics as described previously (Neumann et al., 1999). This virus was a reassortant between CA04 and A/Puerto Rico/8/34 (PR8) viruses. Briefly, eight PolI plasmids (mutant HA and NA (Sakabe et al., 2011) from CA04 and all others from PR8 (Horimoto et al., 2007) were transfected with five protein-expressing plasmids for the expression of the three polymerases, nucleoprotein (NP) (Horimoto et al., 2007), and wild-type CA04 HA into HEK293T cells. Forty-eight hours after transfection, the supernatants containing the uncleavable HA virus were harvested and propagated once in HA-MDCK (CA04) at a multiplicity of infection (MOI) of 0.001 at 35°C for 48 h in MEM containing L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (0.8 μg/ml) and 0.3% bovine serum albumin (BSA) (Sigma Aldrich). Cell debris was removed by centrifugation at 3,500 rpm for 20 min at 4°C and the supernatants were stored at -80°C until use.

Preparation of formalin-inactivated virus. For the formalin-inactivated virus, a reassortant virus between CA04 (wild-type HA and NA genes) and PR8 (the rest of the genes) (CA04 2:6 virus) was generated by using plasmid-based reverse genetics as

described above. The virus was propagated in MDCK cells and the culture supernatants containing the virus were treated with 0.1% formalin (final concentration) (Sigma Aldrich) at 4°C for a week to inactivate infectivity. Inactivation of the virus was confirmed by the absence of detectable infectious virus following inoculation of formalin-treated virus into MDCK cells. After confirmation of inactivation of infectivity, formalin-treated virus was purified by sucrose-gradient ultracentrifugation at 100,000 × *g* for 2 h at 4°C and stored at -80°C until use.

Immunostaining assay. Twenty-four hours after infection with viruses, cells were washed twice with phosphate-buffer saline (PBS) and fixed with 100% methanol for 30 min at room temperature. To detect CA04 HA-expressing cells, these cells were reacted with anti-CA04-HA antibody. To determine the viral titer of the uncleavable HA virus, we conducted the same procedure after performing a plaque assay with HA-MDCK (WSN) cells.

Immunization and protection test. Four-, six-, or eight-week-old female C57BL/6 mice (Japan SLC) were intranasally immunized with 50 μl of 1.7 x 10^5 PFU (equivalent to 16 hemagglutination units (HAU)) of the uncleavable HA virus three times, twice, or once, respectively, at 2-week intervals. As control groups, female C57BL/6 mice (4-week-old) were intranasally immunized with 50 μl of 16 HAU of the formalin-inactivated virus (FI) or with medium three times at 2-week intervals. Three weeks after the final vaccination, six mice per group were euthanized to obtain sera, bronchoalveolar lavage (BAL), and nasal washes. In addition, three mice per group were euthanized to obtain lungs and spleen for detection of viral-specific $CD8^+$ T-lymphocytes. Also three

weeks after the final vaccination, mice were challenged with 10 or 100 times the 50% mouse lethal dose (MLD_{50}) of mouse-adapted CA04 virus. Eight mice per group were monitored for survival and body weight changes for 14 days after challenge. Lungs and nasal turbinates from three mice per each group were collected on days 3 and 6 after challenge to determine virus titers. Virus titers were determined on MDCK cells.

Detection of virus-specific antibodies. Virus-specific antibodies in nasal wash, BAL, and serum were detected by using an enzyme-linked immunosorbent assay (ELISA) (Kida et al., 1982). I used undiluted samples (nasal washes and BAL) and 1:10 diluted samples (serum). In this assay, 96-well ELISA plate wells were coated with approximately 200 HAU (in 50 μl) of purified CA04 virus treated with disruption buffer (0.5M Tris-HCl [pH 8.0], 0.6M KCl, and 0.5% Triton X-100). After incubation of the samples on virus-coated plates, goat anti-mouse IgA or IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratory Inc) was added to detect bound antibody.

Detection of virus-specific $CD8^+$ T lymphocytes. A tetramer assay was used to detect virus-specific $CD8^+$ T lymphocytes. Single cell suspensions of lung and spleen were prepared from inoculated mice three weeks after their final vaccination. After being incubated with anti-CD16/32 (BD Bioscience), the cells were mixed with a Phycoerythrin (PE)-conjugated H-$2D^b$ tetramer specific to the NP epitope (amino acid positions 366–374, ASNENMETM) (MBL) at room temperature for 20 min. Cells were then incubated with Allophycocyanin-cyanine 7 (cy7)-conjugated anti-CD3ε antibody (BD-Bioscience), PE-cy7-conjugated anti-CD8α antibody (BD Bioscience), and via-probe (BD Bioscience)

for 30 min at 4°C and then washed with PBS containing 0.5% BSA and 2 mM EDTA (pH 7.2). Cells were analyzed with FACSAria II (Becton, Dickinson and Company) and FlowJo software (Tree Star, Inc.).

Results

Replicative ability and viral protein expression of the uncleavable HA virus in vitro. To generate the uncleavable HA, the arginine at the cleavage site of HA1, which is important for HA cleavage by trypsin-like protease, was changed to threonine. Since the cleavage of HA protein is essential for membrane fusion (Skehel et al., 2000), a virus possessing this uncleavable HA should be unable to complete its replication cycle. To assess this, I examined if the uncleavable HA virus replicated in MDCK and HA-MDCK (CA04) cells: the latter cells constitutively express CA04 virus HA. As expected, CA04 2:6 reassortant virus, serving as a control, replicated to a similar extent in both MDCK and HA-MDCK (CA04) cells (Fig. 1). By contrast, although no infectious particles of the uncleavable HA virus were detected in the supernatant of MDCK cells, the uncleavable HA virus could replicate efficiently in HA-MDCK (CA04) cells, to a level comparable to that of CA04 2:6 reassortant virus (Fig. 1). I also tested whether the virus could revert to the wild-type by passaging it in MDCK cells. After three passages, I did not observe any cytopathic effect (data not shown), suggesting that the mutation was stable.

To further ensure that the uncleavable HA virus replicated in HA-MDCK cells, but not in MDCK cells, I visualized the infected cells by immunostaining (Fig. 2). Whereas CA04 2:6 virus efficiently spread in both cell types (Fig. 2, left panels), the uncleavable HA virus only infected and expressed viral protein (HA in this case) in individual MDCK cells (Fig. 2, upper middle panel) although it efficiently spread in HA-MDCK (WSN) cells (Fig. 2, lower middle panel). These results indicate that the uncleavable HA virus is replication-incompetent, capable of only a single cycle of

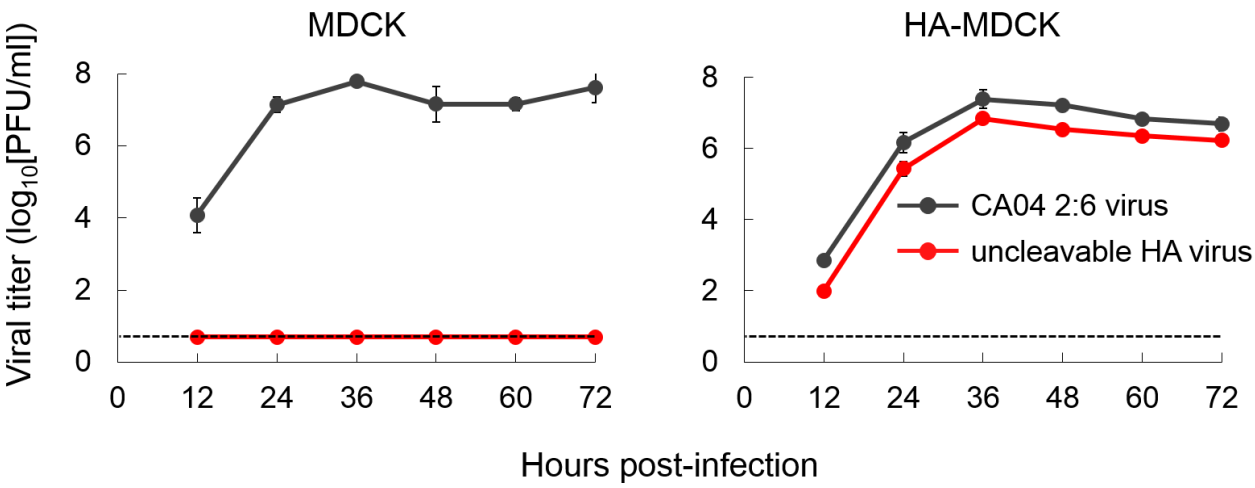

Fig. 1. Growth kinetics of CA04 2:6 and uncleavable HA viruses in MDCK and HA-MDCK cells.

Both cell types were infected with viruses at an MOI of 0.001. Supernatant was collected every 12 h, and viral titers in the supernatants of both cell types were determined by means of the conventional plaque assay or a plaque assay with immunostaining with an anti-HA antibody using HA-MDCK cells. The broken line indicates the detection limit for virus titers (5 PFU/ml).

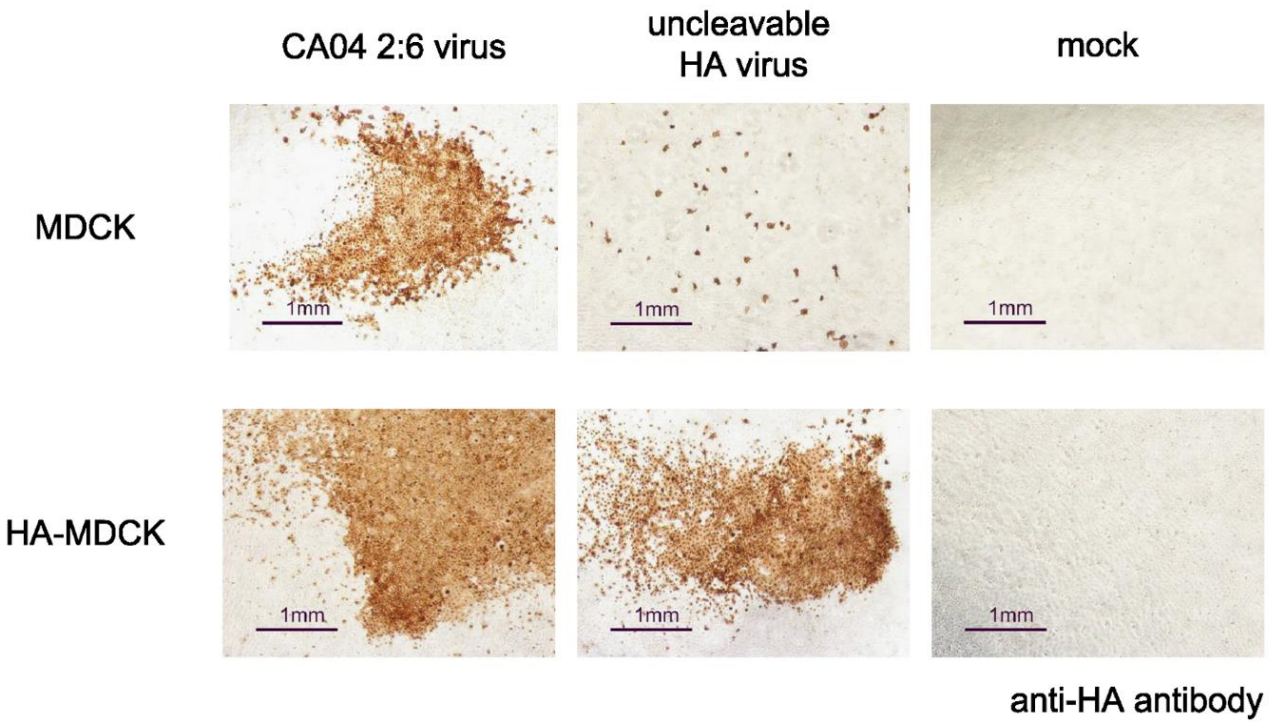

Fig. 2. HA protein expression in virus-infected cells.

MDCK and HA-MDCK (WSN) cells were infected with CA04 2:6 and uncleavable HA viruses; 24 h after infection, the cells were fixed and stained by using an anti-CA04 HA antibody. Cells colored brown are CA04 HA-expressing cells.

infection and unable to produce infectious progeny in normal MDCK cells.

Induction of virus-specific immunity by the uncleavable HA virus in mice. To assess the ability of the uncleavable HA virus to induce virus-specific immunity against influenza virus, I immunized mice with the uncleavable HA virus or FI. To examine virus-specific antibody production, IgG and IgA against CA04 in the nasal wash, BAL, and serum of immunized mice were measured by using an ELISA (Fig. 3). Although only low levels of antibodies were detected in mice immunized three times with FI or inoculated with medium, antibody levels increased as the number of vaccinations increased in mice immunized with the uncleavable HA virus. Notably, both the virus-specific IgG and IgA titers in BAL, and the IgG in serum from mice immunized only once with the uncleavable HA virus were higher than the respective titers from mice immunized with FI three times.

I also examined the virus-specific (NP in this case) $CD8^+$ T cell response by using a tetramer assay. NP-specific $CD8^+$ T cells were identified in the lungs and spleens only of mice immunized with the uncleavable HA virus (Fig 4). Of note, NP-specific $CD8^+$ T cells predominantly accumulated in the lungs rather than the spleens of the immunized mice (Fig. 4), and the number of NP-specific $CD8^+$ T cells increased as the number of vaccinations increased (Fig. 4B).

These results demonstrate that the uncleavable HA virus could elicit both humoral and cellular immunity more efficiently than FI against influenza virus at the local sites where influenza virus replicates.

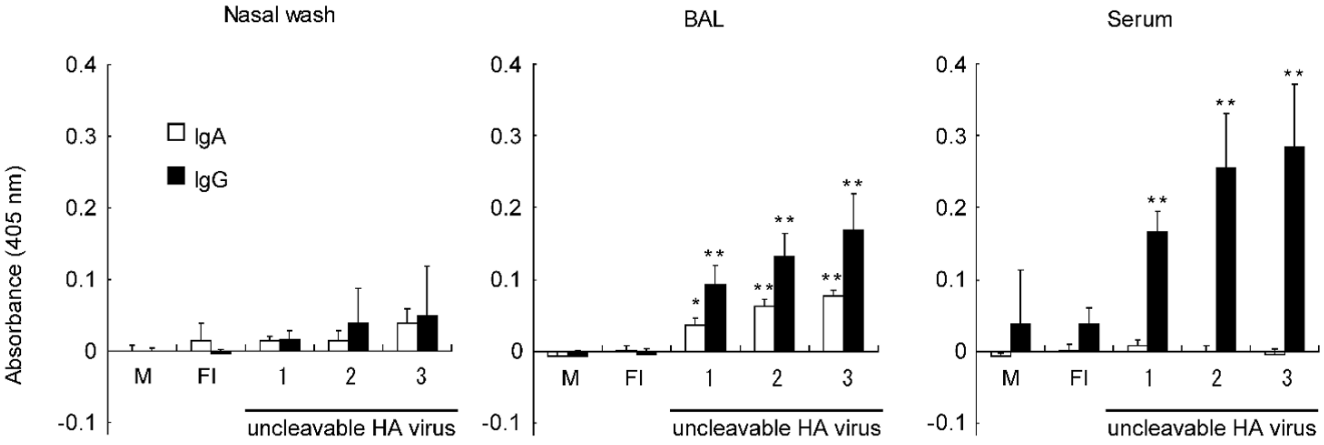

Fig. 3. Induction of virus-specific IgG and IgA in nasal wash, BAL, and serum of mice immunized with the uncleavable HA virus.

Virus-specific antibodies were detected by means of an ELISA. Samples from six mice from each group were obtained 3 weeks after the final vaccination. Results are expressed as the mean absorbance (± standard deviations) of undiluted samples (nasal wash and BAL) or of 1:10 diluted samples (serum). Statistically significant differences among the groups were assessed by using the Tukey-Kramer method. Asterisks (* or **) indicate a significant difference from samples from mice inoculated with FI (*, $P<0.05$; **, $P<0.01$). The numbers on the x-axis indicate the times of vaccination of uncleavable HA virus. M: medium. FI: Mice were immunized with formalin-inactivated vaccine three times with 2 week intervals.

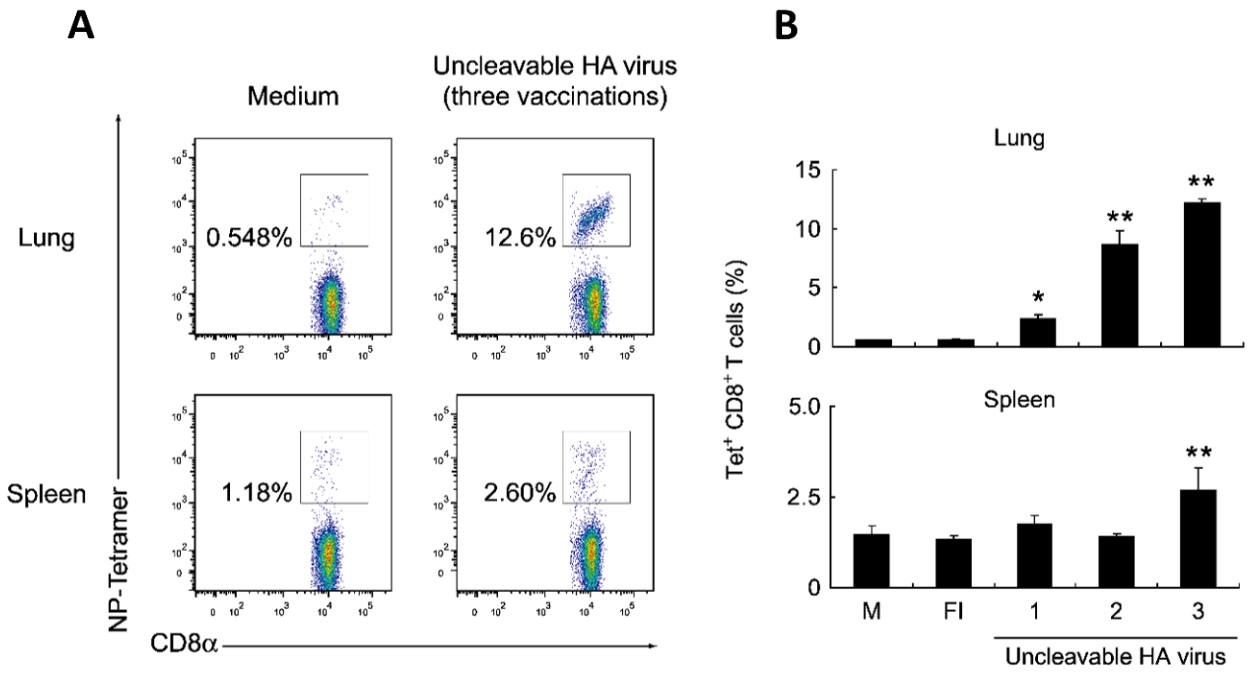

Fig. 4. Induction of virus-specific CD8$^+$ T cells in mice immunized with the uncleavable HA virus.

Virus-specific CD8$^+$ T cells were detected in the lungs and spleen of inoculated mice by using a tetramer assay. (A) Representative dot plot for CD3ε$^+$ CD8α$^+$ cells from the spleen or lungs of a mouse inoculated with medium or with the uncleavable HA virus. (B) The percentage of virus-specific CD8$^+$ T cells (NP-tetramer$^+$ CD8α$^+$ population) in the CD3ε$^+$ CD8α$^+$ cell populations in the lungs and spleen of inoculated mice. Results are expressed as the mean values (± standard deviations). Statistically significant differences among the groups were assessed by using the Tukey-Kramer method. Asterisks (* or **) indicate significant differences from samples from mice inoculated with FI (*, P<0.05; **, P<0.01).

Protection against influenza virus in mice immunized with the uncleavable HA virus. Finally, I evaluated the protective ability of the uncleavable HA virus. To this end, I monitored body weight changes and survival of mice immunized with the uncleavable HA virus after challenge. The body weights of mice inoculated with medium decreased rapidly to approximately 70%–80% (Fig. 5A) and most mice (7 out of 8) succumbed to their infection (Fig. 5B). The body weights of mice immunized with FI also clearly decreased to 80% (Fig. 5A) and several mice (3 and 5 out of 8 with 10 and 100 MLD_{50}, respectively) died (Fig 5B). By contrast, with 10 MLD_{50}, mice immunized once with the uncleavable HA virus showed slightly reduced body weights, and mice immunized twice and three times with this virus showed no body weight changes (Fig. 5A left panel). Moreover, all mice immunized with this virus survived (Fig. 5B left panel). With 100 MLD_{50}, although mice immunized once with this virus showed similar body weight reductions as mice immunized with medium and FI, the mice immunized twice and three times with the uncleavable HA virus showed only slight or no body weight loss, respectively (Fig. 5A, right panel). All mice immunized with the uncleavable HA virus survived, except for 3 out of 8 mice immunized only once with the uncleavable HA virus (Fig. 5B, right panel).

I also determined virus titers in the lungs and nasal turbinates of mice immunized with the uncleavable HA virus after challenge (Table 1). Virus titers in both organs of mice immunized with the uncleavable HA virus were appreciably lower as the number of vaccinations increased, especially on day 6 post-infection, than those in organs of mice immunized with medium or with FI. Taken together, these results indicate that the uncleavable HA virus has potency as an influenza vaccine.

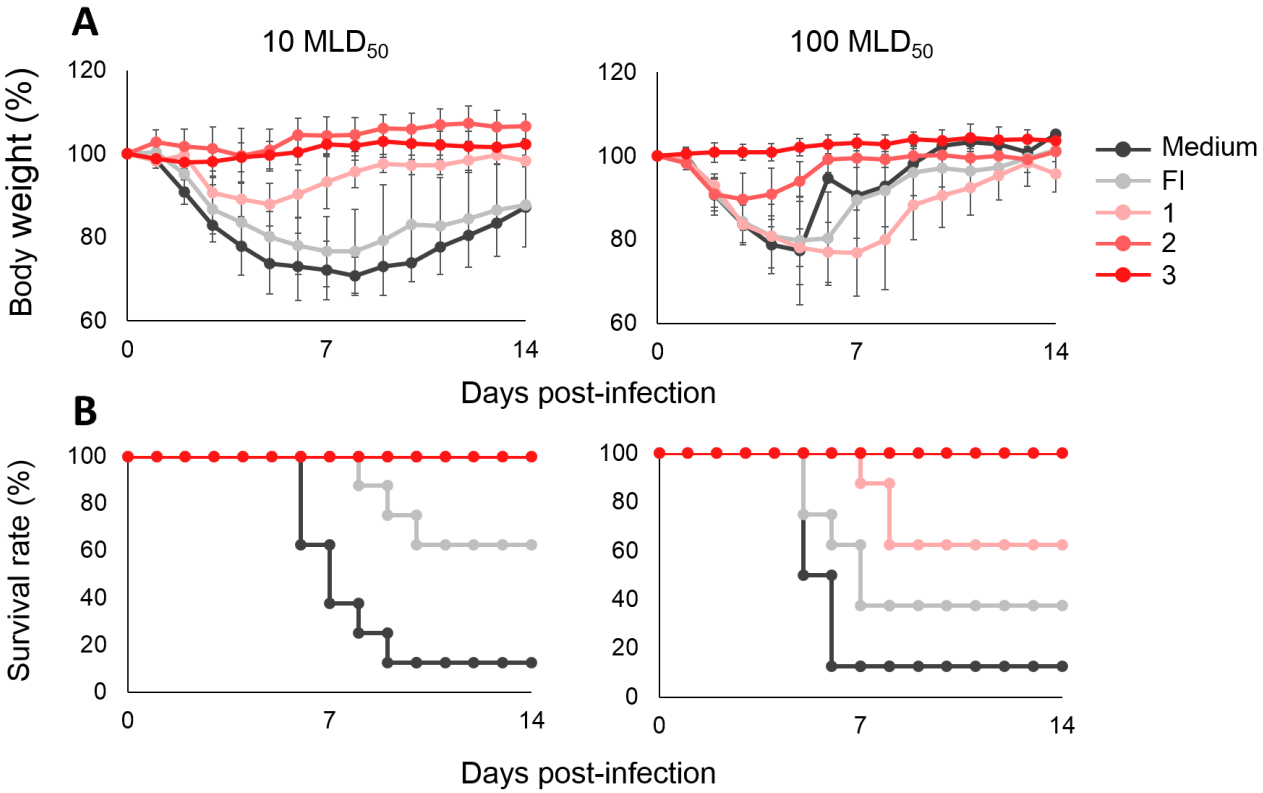

Fig. 5. Body weight changes and survival curves for mice challenged with wild-type virus.

Eight mice per group were intranasally challenged with 10 or 100 MLD_{50} of mouse-adapted CA04 3 weeks after the final vaccination. Body weights were measured (A) and survival rate was monitored (B) for 14 days after challenge.

Table. 1. Protection against challenge with lethal doses of influenza virus in mice immunized with an uncleavable HA virus[a]

challenge dose	inoculum	days post-infection	organ	Virus titer (mean ±SD log_{10}[PFU/g])
$10MLD_{50}$	Medium	3 dpi	NT[b]	5.1 ± 0.6
			Lung	7.0 ± 0.4
		6 dpi	NT	4.2 ± 0.3
			Lung	6.4 ±0.4
	FI[d]	3 dpi	NT	4.6 ± 0.5
			Lung	7.3 ± 0.0
		6 dpi	NT	4.0 ± 0.2
			Lung	6.0 ± 0.3
	Once	3 dpi	NT	5.2, 5.8
			Lung	7.8 ± 0.2
		6 dpi	NT	ND[e]
			Lung	3.4, 3.2
	Twice	3 dpi	NT	4.3, 3.9
			Lung	6.1 ± 0.8
		6 dpi	NT	ND
			Lung	2.5, 2.5
	Three times	3 dpi	NT	ND
			Lung	2.2
		6 dpi	NT	ND
			Lung	2.0

challenge dose	inoculum	days post-infection	organ	Virus titer (mean ±SD log_{10}[PFU/g])
$100MLD_{50}$	Medium	3 dpi	NT	5.7 ± 0.4
			Lung	7.5 ± 0.2
		6 dpi	NT	NA[c]
			Lung	NA
	FI	3 dpi	NT	5.7 ± 0.7
			Lung	7.1 ± 0.7
		6 dpi	NT	3.9 ± 0.5
			Lung	5.3 ± 0.4
	Once	3 dpi	NT	4.9 ± 0.4
			Lung	7.5 ± 0.5
		6 dpi	NT	3.3, 3.5
			Lung	3.7 ± 2.3
	Twice	3 dpi	NT	6.0
			Lung	1.6, 6.5
		6 dpi	NT	1.6
			Lung	ND
	Three times	3 dpi	NT	ND
			Lung	5.8, 2.4
		6 dpi	NT	ND
			Lung	ND

[a] Twelve mice from each group were intranasally inoculated with 10 or 100 MLD_{50} of mouse-adapted CA04 (50 μl per mouse) 3 weeks after the final vaccination. Three mice per group were sacrificed on days 3 and 6 post-infection, and lungs and nasal turbinate were collected to determine virus titers. Results are expressed as the mean titer (log_{10} PFU/g) ± standard deviations. When virus was not recovered from all three mice,

individual titers are given.

[b] NT: Nasal turbinate

[c] NA: Not applicable because the mice died

[d] FI: formalin-inactivated virus. Mice were immunized three times with two week intervals.

[e] ND: Not detected (detection limit, 10 PFU/lung or 5 PFU/NT)

Discussion

Currently, embryonated hen's eggs are typically used to produce both inactivated and live-attenuated vaccines, although cell-based vaccines are approved in Europe. However, vaccine production in eggs has several issues. One problem is the alteration of antigenicity from that of the circulating virus during cultivation of the vaccine strain in embryonated hen's eggs (Katz et al., 1987; Newman et al., 1993; Williams et al., 1993). Another is allergic reactions to components derived from eggs present in vaccines grown in eggs (Gruenberg et al., 2011). In contrast, the antigenicity of viruses grown in cells closely matches that of viruses isolated from influenza patients (Williams et al., 1993; Katz et al., 1989). Since the uncleavable HA virus in this study only grew in HA-expressing cells and to a level comparable to a virus possessing wild-type HA (Fig. 1), production of this vaccine should not only be feasible, but this type of vaccine should maintain its antigenicity; however, the cell line needs to be validated to ensure that it is free of anything harmful, such as tumorigenicity.

I demonstrated that antibody responses (IgA and IgG) were efficiently induced by the uncleavable HA virus (Fig. 3), like NS2-knockout VLPs (Watanabe et al., 2002). In addition to antibody responses, I tested the virus-specific CTL response, which we did not examined with our NS2-knockout VLPs, and found that a CTL response to an internal viral protein (i.e., NP) was also induced by this virus (Fig. 4). The reason for this induction is that viral proteins (NP in this case) are expressed in cells infected with this replication-incompetent virus, suggesting that this virus could act similarly to a live-attenuated virus in terms of induction of immunity despite not generating infectious progeny.

My findings suggest that a combination of this type of virus and cells expressing

a seasonal virus HA can serve as a platform for a bivalent vaccine as follows: If an uncleavable H5 HA gene is partnered with the rest of the genes from a seasonal influenza virus, the resulting virus can serve as a vaccine against H5 and seasonal influenza viruses. That is, antibody responses against H5 HA (expressed from the viral HA gene) and HA (derived from the cell line) would likely be induced as would CTL responses against H5 HA and the internal viral proteins (expressed from the genes of the seasonal virus). Likewise, if a gene, encoding a protein responsible for inducing immunity, from another respiratory pathogens, such as *Streptococcus pneumoniae*, which causes secondary bacterial infections following influenza virus infection, or respiratory syncytial virus, which causes severe manifestations in infants, is inserted into the HA gene, the resultant virus may be used as a vaccine against both influenza virus and the other respiratory pathogen.

In conclusion, a replication-incompetent virus that possesses a modified HA gene, when partnered with an HA-expressing cell line, has potential as a novel vaccine candidate.

CHAPTER III

A bivalent vaccine based on a replication-incompetent influenza virus protects against *Streptococcus pneumoniae* and influenza virus infection.

Chapter III は雑誌掲載のため公表できません。

CONCLUDING REMARKS

Influenza viruses cause epidemics and occasional pandemics. Since the beginning of the 20th century, we have experienced four pandemics (Wright et al., 2013). In addition, sporadic infections caused by H5N1 and H7N9 viruses have been reported in recent years. Despite advances in medicine and the availability of new antivirals, influenza viruses continue to be a great threat to our lives. To develop novel antivirals and vaccines to control influenza, we must continue to conduct basic research on influenza virus infection. In this thesis, I focused on host responses to influenza virus infection and novel vaccine production based on genetic recombination technology of influenza virus.

In chapter I, I characterized an improved recombinant influenza A virus carrying a reporter gene (NS1-Venus PR8 MA virus). I demonstrated that NS1-Venus PR8 MA virus stably expressed the Venus protein at high levels. By using NS1-Venus PR8 MA virus, I could readily detect virus-infected cells. I believe that NS1-Venus PR8 MA virus could be useful not only in intravital live imaging experiments, which has not been successful to date, but also in screens for new antivirals.

Vaccination is effective in the prophylaxis of influenza virus infection. However, there is still room for improvement in the efficacy and safety of our current vaccines. In chapter II, I generated a replication-incompetent influenza virus that lacks membrane-fusion ability to evaluate as an influenza vaccine. I demonstrated that mice inoculated with this uncleavable HA virus could successfully elicit not only virus-specific antibodies at the surface of respiratory mucosa but also cytotoxic T lymphocytes in the lung. I also showed that the uncleavable HA virus completely protected mice from lethal infection with influenza virus.

On the basis of the results described in chapter II, in chapter III, I generated a bivalent vaccine designed to protect against influenza virus and *S. pneumoniae* infection. By using the packaging signal of the HA segment, I produced HA-KO/PspA virus, which expresses PspA protein as an antigen of *S. pneumoniae* in infected cells. I demonstrated that HA-KO/PspA virus could prevent nasal colonization and lethal infection due to *S. pneumoniae* as well as lethal infection by influenza virus. Therefore I believe that a replication-incompetent virus can serve as a bivalent vaccine.

Despite the intense efforts of influenza virus researchers, several important problems remain, including the emergence of antiviral-resistant viruses and inefficient influenza vaccines. Moreover, we cannot predict which subtype of virus will cause the next pandemic. To better understand influenza virus, we must continuously conduct intensive basic research. I hope that the results presented here will contribute to the elucidation of the pathogenesis of influenza virus and help establish new prophylactic and therapeutic measures to control influenza virus infection.

ACKNOWLEDGEMENTS

I would like to express my deepest appreciation to Prof. Yoshihiro Kawaoka, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, for his detailed suggestions, tremendous support, and considerable encouragement. I would also like to express my sincere gratitude to Dr. Satoshi Fukuyama, Group Leader of the ERATO Infection-induced Host Responses Project, Japan Science and Technology Agency, for his continual help and extensive discussions. I am deeply grateful to Dr. Shinji Watanabe, Graduate School of Medicine and Veterinary Medicine, University of Miyazaki, for his valuable advice, and Dr. Iwatsuki-Horimoto Kiyoko, Division of Virology, Institute of Medical Science, The University of Tokyo, for her technical help. I would also like to thank to Dr. Taisuke Horimoto, Graduate school of Agriculture and Life Science, The University of Tokyo, for his solid suggestions.

I am grateful for my collaborations with Professor Kazunori Oishi, Dr. Yukihiro Akeda, and Dr. Zhenyu Piao, Laboratory of Clinical Research on Infectious Disease, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, for their kind support during the experiments on *S. pneumoniae* infection.

I also thank Susan Watson for scientific editing, and Dr. Seiya Yamayoshi, Dr. Shin Murakami, Dr. Saori Sakabe, Dr. Ryo Takano, Dr. Takeo Gorai, and Ryuta Uraki for helpful discussions. I express my gratitude to the members of the Division of Virology, the Institute of Medical Science, The University of Tokyo, and the members of the ERATO Infection-induced Host Responses Project, JST.

Finally, I would like to offer my special thanks to my friends and my family, especially my mother and father for their continuing support.

REFERENCES

Ambrose CS, Luke C, Coelingh K. 2008. Current status of live attenuated influenza vaccine in the United States for seasonal and pandemic influenza. Influenza Other Respi Viruses **2:**193-202.

Baskin CR, Bielefeldt-Ohmann H, Tumpey TM, Sabourin PJ, Long JP, García-Sastre A, Tolnay AE, Albrecht R, Pyles JA, Olson PH, Aicher LD, Rosenzweig ER, Murali-Krishna K, Clark EA, Kotur MS, Fornek JL, Proll S, Palermo RE, Sabourin CL, Katze MG. 2009. Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. Proc Natl Acad Sci U S A **106:**3455-3460.

Belser JA, Gustin KM, Pearce MB, Maines TR, Zeng H, Pappas C, Sun X, Carney PJ, Villanueva JM, Stevens J, Katz JM, Tumpey TM. 2013. Pathogenesis and transmission of avian influenza A (H7N9) virus in ferrets and mice. Nature **501:**556-559.

Bettelli E, Das MP, Howard ED, Weiner HL, Sobel RA, Kuchroo VK. 1998. IL-10 is critical in the regulation of autoimmune encephalomyelitis as demonstrated by studies of IL-10- and IL-4-deficient and transgenic mice. J Immunol **161:**3299-3306.

Blander JM, Medzhitov R. 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature **440:**808-812.

Bogaert D, De Groot R, Hermans PW. 2004. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. Lancet Infect Dis **4:**144-154.

Briles DE, Hollingshead SK, Paton JC, Ades EW, Novak L, van Ginkel FW, Benjamin WH. 2003. Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with Streptococcus pneumoniae. J Infect Dis **188:**339-348.

Briles DE, Novak L, Hotomi M, van Ginkel FW, King J. 2005. Nasal colonization with Streptococcus pneumoniae includes subpopulations of surface and invasive pneumococci. Infect Immun **73:**6945-6951.

Chaussee MS, Sandbulte HR, Schuneman MJ, Depaula FP, Addengast LA, Schlenker EH, Huber VC. 2011. Inactivated and live, attenuated influenza vaccines protect mice against influenza: Streptococcus pyogenes super-infections. Vaccine **29:**3773-3781.

Chen Y, Liang W, Yang S, Wu N, Gao H, Sheng J, Yao H, Wo J, Fang Q, Cui D, Li Y, Yao X, Zhang Y, Wu H, Zheng S, Diao H, Xia S, Chan KH, Tsoi HW, Teng JL, Song W, Wang P, Lau SY, Zheng M, Chan JF, To KK, Chen H, Li L, Yuen KY. 2013. Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. Lancet **381:**1916-1925.

Chen Z, Aspelund A, Kemble G, Jin H. 2006. Genetic mapping of the cold-adapted phenotype of B/Ann Arbor/1/66, the master donor virus for live attenuated influenza vaccines (FluMist). Virology **345:**416-423.

Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, Krauss S, Shortridge KF, Webster RG. 1998. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet **351:**472-477.

Cox RJ, Brokstad KA, Ogra P. 2004. Influenza virus: immunity and vaccination strategies. Comparison of the immune response to inactivated and live, attenuated influenza vaccines. Scand J Immunol **59:**1-15.

Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM, Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team. 2009. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med **360:**2605-2615.

de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha dQ, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J. 2006. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med **12:**1203-1207.

Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. 2004. Innate antiviral

responses by means of TLR7-mediated recognition of single-stranded RNA. Science **303:**1529-1531.

Do-Umehara HC, Chen C, Urich D, Zhou L, Qiu J, Jang S, Zander A, Baker MA, Eilers M, Sporn PH, Ridge KM, Sznajder JI, Budinger GR, Mutlu GM, Lin A, Liu J. 2013. Suppression of inflammation and acute lung injury by Miz1 via repression of C/EBP-δ. Nat Immunol **14:**461-469.

Ezoe H, Akeda Y, Piao Z, Aoshi T, Koyama S, Tanimoto T, Ishii KJ, Oishi K. 2011. Intranasal vaccination with pneumococcal surface protein A plus poly(I:C) protects against secondary pneumococcal pneumonia in mice. Vaccine **29:**1754-1761.

Ferreira DM, Darrieux M, Oliveira ML, Leite LC, Miyaji EN. 2008. Optimized immune response elicited by a DNA vaccine expressing pneumococcal surface protein a is characterized by a balanced immunoglobulin G1 (IgG1)/IgG2a ratio and proinflammatory cytokine production. Clin Vaccine Immunol **15:**499-505.

Fiore AE, Uyeki TM, Broder K, Finelli L, Euler GL, Singleton JA, Iskander JK, Wortley PM, Shay DK, Bresee JS, Cox NJ. 2010. Prevention and control of influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2010. MMWR Recomm Rep **59:**1-62.

Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J. 2005. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc

Natl Acad Sci U S A **102:**18590-18595.

Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, Chen J, Jie Z, Qiu H, Xu K, Xu X, Lu H, Zhu W, Gao Z, Xiang N, Shen Y, He Z, Gu Y, Zhang Z, Yang Y, Zhao X, Zhou L, Li X, Zou S, Zhang Y, Yang L, Guo J, Dong J, Li Q, Dong L, Zhu Y, Bai T, Wang S, Hao P, Yang W, Han J, Yu H, Li D, Gao GF, Wu G, Wang Y, Yuan Z, Shu Y. 2013. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med **368:**1888-1897.

Gill JR, Sheng ZM, Ely SF, Guinee DG, Beasley MB, Suh J, Deshpande C, Mollura DJ, Morens DM, Bray M, Travis WD, Taubenberger JK. 2010. Pulmonary pathologic findings of fatal 2009 pandemic influenza A/H1N1 viral infections. Arch Pathol Lab Med **134:**235-243.

Gorai T, Goto H, Noda T, Watanabe T, Kozuka-Hata H, Oyama M, Takano R, Neumann G, Watanabe S, Kawaoka Y. 2012. F1Fo-ATPase, F-type proton-translocating ATPase, at the plasma membrane is critical for efficient influenza virus budding. Proc Natl Acad Sci U S A **109:**4615-4620.

Gruenberg DA, Shaker MS. 2011. An update on influenza vaccination in patients with egg allergy. Curr Opin Pediatr **23:**566-572.

Hama H, Kurokawa H, Kawano H, Ando R, Shimogori T, Noda H, Fukami K, Sakaue-Sawano A, Miyawaki A. 2011. Scale: a chemical approach for fluorescence

imaging and reconstruction of transparent mouse brain. Nat Neurosci **14:**1481-1488.

Hatta M, Gao P, Halfmann P, Kawaoka Y. 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. Science **293:**1840-1842.

Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y. 2007. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog **3:**1374-1379.

Herfst S, Schrauwen EJ, Linster M, Chutinimitkul S, de Wit E, Munster VJ, Sorrell EM, Bestebroer TM, Burke DF, Smith DJ, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2012. Airborne transmission of influenza A/H5N1 virus between ferrets. Science **336:**1534-1541.

Hoffmann E, Mahmood K, Chen Z, Yang CF, Spaete J, Greenberg HB, Herlocher ML, Jin H, Kemble G. 2005. Multiple gene segments control the temperature sensitivity and attenuation phenotypes of ca B/Ann Arbor/1/66. J Virol **79:**11014-11021.

Horimoto T, Murakami S, Muramoto Y, Yamada S, Fujii K, Kiso M, Iwatsuki-Horimoto K, Kino Y, Kawaoka Y. 2007. Enhanced growth of seed viruses for H5N1 influenza vaccines. Virology **366:**23-27.

Hornung V, Ellegast J, Kim S, Brzózka K, Jung A, Kato H, Poeck H, Akira S,

Conzelmann KK, Schlee M, Endres S, Hartmann G. 2006. 5'-Triphosphate RNA is the ligand for RIG-I. Science **314:**994-997.

Huber VC, Peltola V, Iverson AR, McCullers JA. 2010. Contribution of vaccine-induced immunity toward either the HA or the NA component of influenza viruses limits secondary bacterial complications. J Virol **84:**4105-4108.

Imai M, Watanabe T, Hatta M, Das SC, Ozawa M, Shinya K, Zhong G, Hanson A, Katsura H, Watanabe S, Li C, Kawakami E, Yamada S, Kiso M, Suzuki Y, Maher EA, Neumann G, Kawaoka Y. 2012. Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5 HA/H1N1 virus in ferrets. Nature **486:**420-428.

Iwasaki A. 2012. A virological view of innate immune recognition. Annu Rev Microbiol **66:**177-196.

Jambo KC, Sepako E, Heyderman RS, Gordon SB. 2010. Potential role for mucosally active vaccines against pneumococcal pneumonia. Trends Microbiol **18:**81-89.

Jedrzejas MJ. 2007. Unveiling molecular mechanisms of bacterial surface proteins: Streptococcus pneumoniae as a model organism for structural studies. Cell Mol Life Sci **64:**2799-2822.

Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, Kemble G, Greenberg H. 2003. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains (FluMist) derived from cold-adapted A/Ann Arbor/6/60. Virology **306:**18-24.

Jin H, Zhou H, Lu B, Kemble G. 2004. Imparting temperature sensitivity and attenuation in ferrets to A/Puerto Rico/8/34 influenza virus by transferring the genetic signature for temperature sensitivity from cold-adapted A/Ann Arbor/6/60. J Virol **78:**995-998.

Joller N, Hafler JP, Brynedal B, Kassam N, Spoerl S, Levin SD, Sharpe AH, Kuchroo VK. 2011. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. J Immunol **186:**1338-1342.

Kadioglu A, Weiser JN, Paton JC, Andrew PW. 2008. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. Nat Rev Microbiol **6:**288-301.

Katsura H, Iwatsuki-Horimoto K, Fukuyama S, Watanabe S, Sakabe S, Hatta Y, Murakami S, Shimojima M, Horimoto T, Kawaoka Y. 2012. A replication-incompetent virus possessing an uncleavable hemagglutinin as an influenza vaccine. Vaccine **30:**6027-6033.

Katz JM, Naeve CW, Webster RG. 1987. Host cell-mediated variation in H3N2

influenza viruses. Virology **156:**386-395.

Katz JM, Wang M, Webster RG. 1990. Direct sequencing of the HA gene of influenza (H3N2) virus in original clinical samples reveals sequence identity with mammalian cell-grown virus. J Virol **64:**1808-1811.

Katz JM, Webster RG. 1989. Efficacy of inactivated influenza A virus (H3N2) vaccines grown in mammalian cells or embryonated eggs. J Infect Dis **160:**191-198.

Kawai T, Akira S. 2006. Innate immune recognition of viral infection. Nat Immunol **7:**131-137.

Kawamoto S, Tran TH, Maruya M, Suzuki K, Doi Y, Tsutsui Y, Kato LM, Fagarasan S. 2012. The inhibitory receptor PD-1 regulates IgA selection and bacterial composition in the gut. Science **336:**485-489.

Kawaoka Y, Krauss S, Webster RG. 1989. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol **63:**4603-4608.

Keir ME, Butte MJ, Freeman GJ, Sharpe AH. 2008. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol **26:**677-704.

Kida H, Brown LE, Webster RG. 1982. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of

A/Seal/Massachusetts/1/80 (H7N7) influenza virus. Virology **122:**38-47.

King C, Tangye SG, Mackay CR. 2008. T follicular helper (TFH) cells in normal and dysregulated immune responses. Annu Rev Immunol **26:**741-766.

Kittel C, Sereinig S, Ferko B, Stasakova J, Romanova J, Wolkerstorfer A, Katinger H, Egorov A. 2004. Rescue of influenza virus expressing GFP from the NS1 reading frame. Virology **324:**67-73.

Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M, Feldmann F, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H, Kawaoka Y. 2007. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature **445:**319-323.

Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. Cell **75:**263-274.

La Gruta NL, Kedzierska K, Stambas J, Doherty PC. 2007. A question of self-preservation: immunopathology in influenza virus infection. Immunol Cell Biol **85:**85-92.

Lambert LC, Fauci AS. 2010. Influenza vaccines for the future. N Engl J Med **363:**2036-2044.

Li Q, Zhou L, Zhou M, Chen Z, Li F, Wu H, Xiang N, Chen E, Tang F, Wang D, Meng L, Hong Z, Tu W, Cao Y, Li L, Ding F, Liu B, Wang M, Xie R, Gao R, Li X, Bai T, Zou S, He J, Hu J, Xu Y, Chai C, Wang S, Gao Y, Jin L, Zhang Y, Luo H, Yu H, Gao L, Pang X, Liu G, Shu Y, Yang W, Uyeki TM, Wang Y, Wu F, Feng Z. 2013. Preliminary Report: Epidemiology of the Avian Influenza A (H7N9) Outbreak in China. N Engl J Med.

Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K. 2005. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J Virol **79:**12058-12064.

Lozach PY, Huotari J, Helenius A. 2011. Late-penetrating viruses. Curr Opin Virol **1:**35-43.

Maassab HF. 1968. Plaque formation of influenza virus at 25 degrees C. Nature **219:**645-646.

Manicassamy B, Manicassamy S, Belicha-Villanueva A, Pisanelli G, Pulendran B, García-Sastre A. 2010. Analysis of in vivo dynamics of influenza virus infection in mice using a GFP reporter virus. Proc Natl Acad Sci U S A **107:**11531-11536.

Mantovani A, Biswas SK, Galdiero MR, Sica A, Locati M. 2013. Macrophage plasticity and polarization in tissue repair and remodelling. J Pathol **229:**176-185.

Martin K, Helenius A. 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M1) promotes export and inhibits import. Cell **67:**117-130.

Matlin KS, Reggio H, Helenius A, Simons K. 1981. Infectious entry pathway of influenza virus in a canine kidney cell line. J Cell Biol **91:**601-613.

McCullers JA. 2006. Insights into the interaction between influenza virus and pneumococcus. Clin Microbiol Rev **19:**571-582.

Moffitt KL, Malley R. 2011. Next generation pneumococcal vaccines. Curr Opin Immunol **23:**407-413.

Morens DM, Taubenberger JK, Fauci AS. 2008. Predominant role of bacterial pneumonia as a cause of death in pandemic influenza: implications for pandemic influenza preparedness. J Infect Dis **198:**962-970.

Nabors GS, Braun PA, Herrmann DJ, Heise ML, Pyle DJ, Gravenstein S, Schilling M, Ferguson LM, Hollingshead SK, Briles DE, Becker RS. 2000. Immunization of healthy adults with a single recombinant pneumococcal surface protein A (PspA) variant stimulates broadly cross-reactive antibodies to heterologous PspA molecules. Vaccine **18:**1743-1754.

Nagai T, Ibata K, Park ES, Kubota M, Mikoshiba K, Miyawaki A. 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological

applications. Nat Biotechnol **20:**87-90.

Narasaraju T, Yang E, Samy RP, Ng HH, Poh WP, Liew AA, Phoon MC, van Rooijen N, Chow VT. 2011. Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. Am J Pathol **179:**199-210.

Neumann G, Noda T, Kawaoka Y. 2009. Emergence and pandemic potential of swine-origin H1N1 influenza virus. Nature **459:**931-939.

Neumann G, Watanabe T, Ito H, Watanabe S, Goto H, Gao P, Hughes M, Perez DR, Donis R, Hoffmann E, Hobom G, Kawaoka Y. 1999. Generation of influenza A viruses entirely from cloned cDNAs. Proc Natl Acad Sci U S A **96:**9345-9350.

Newman RW, Jennings R, Major DL, Robertson JS, Jenkins R, Potter CW, Burnett I, Jewes L, Anders M, Jackson D. 1993. Immune response of human volunteers and animals to vaccination with egg-grown influenza A (H1N1) virus is influenced by three amino acid substitutions in the haemagglutinin molecule. Vaccine **11:**400-406.

Niwa H, Yamamura K, Miyazaki J. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193-199.

Ochs MM, Bartlett W, Briles DE, Hicks B, Jurkuvenas A, Lau P, Ren B, Millar A. 2008. Vaccine-induced human antibodies to PspA augment complement C3 deposition

on Streptococcus pneumoniae. Microb Pathog **44:**204-214.

Oma K, Zhao J, Ezoe H, Akeda Y, Koyama S, Ishii KJ, Kataoka K, Oishi K. 2009. Intranasal immunization with a mixture of PspA and a Toll-like receptor agonist induces specific antibodies and enhances bacterial clearance in the airways of mice. Vaccine **27:**3181-3188.

Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. 2011. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol **29:**71-109.

Palacios G, Hornig M, Cisterna D, Savji N, Bussetti AV, Kapoor V, Hui J, Tokarz R, Briese T, Baumeister E, Lipkin WI. 2009. Streptococcus pneumoniae coinfection is correlated with the severity of H1N1 pandemic influenza. PLoS One **4:**e8540.

Pichlmair A, Schulz O, Tan CP, Näslund TI, Liljeström P, Weber F, Reis e Sousa C. 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. Science **314:**997-1001.

Rawlins EL, Hogan BL. 2006. Epithelial stem cells of the lung: privileged few or opportunities for many? Development **133:**2455-2465.

Ren B, Szalai AJ, Hollingshead SK, Briles DE. 2004. Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface.

Infect Immun **72:**114-122.

Richard M, Schrauwen EJ, de Graaf M, Bestebroer TM, Spronken MI, van Boheemen S, de Meulder D, Lexmond P, Linster M, Herfst S, Smith DJ, van den Brand JM, Burke DF, Kuiken T, Rimmelzwaan GF, Osterhaus AD, Fouchier RA. 2013. Limited airborne transmission of H7N9 influenza A virus between ferrets. Nature **501:**560-563.

Rimmelzwaan GF, Fouchier RA, Osterhaus AD. 2007. Influenza virus-specific cytotoxic T lymphocytes: a correlate of protection and a basis for vaccine development. Curr Opin Biotechnol **18:**529-536.

Rodgers GL, Klugman KP. 2011. The future of pneumococcal disease prevention. Vaccine **29 Suppl 3:**C43-48.

Rogers GN, Paulson JC. 1983. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology **127:**361-373.

Sakabe S, Ozawa M, Takano R, Iwastuki-Horimoto K, Kawaoka Y. 2011. Mutations in PA, NP, and HA of a pandemic (H1N1) 2009 influenza virus contribute to its adaptation to mice. Virus Res **158:**124-129.

Saraiva M, O'Garra A. 2010. The regulation of IL-10 production by immune cells.

Nat Rev Immunol **10:**170-181.

Scholtissek C, Rohde W, Von Hoyningen V, Rott R. 1978. On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology **87:**13-20.

Schulz O, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, Azuma YT, Flavell RA, Liljeström P, Reis e Sousa C. 2005. Toll-like receptor 3 promotes cross-priming to virus-infected cells. Nature **433:**887-892.

Shinya K, Fujii Y, Ito H, Ito T, Kawaoka Y. 2004. Characterization of a neuraminidase-deficient influenza a virus as a potential gene delivery vector and a live vaccine. J Virol **78:**3083-3088.

Short KR, Kroeze EJ, Fouchier RA, Kuiken T. 2013. Pathogenesis of influenza-induced acute respiratory distress syndrome. Lancet Infect Dis. (in press)

Sica A, Mantovani A. 2012. Macrophage plasticity and polarization: in vivo veritas. J Clin Invest **122:**787-795.

Sieczkarski SB, Whittaker GR. 2002. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. J Virol **76:**10455-10464.

Skehel JJ, Wiley DC. 2000. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem **69:**531-569.

Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, Ma SK, Cheung CL, Raghwani J, Bhatt S, Peiris JS, Guan Y, Rambaut A. 2009. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 459:1122-1125.

Snelgrove RJ, Godlee A, Hussell T. 2011. Airway immune homeostasis and implications for influenza-induced inflammation. Trends Immunol **32:**328-334.

Snelgrove RJ, Goulding J, Didierlaurent AM, Lyonga D, Vekaria S, Edwards L, Gwyer E, Sedgwick JD, Barclay AN, Hussell T. 2008. A critical function for CD200 in lung immune homeostasis and the severity of influenza infection. Nat Immunol **9:**1074-1083.

Teijaro JR, Walsh KB, Cahalan S, Fremgen DM, Roberts E, Scott F, Martinborough E, Peach R, Oldstone MB, Rosen H. 2011. Endothelial cells are central orchestrators of cytokine amplification during influenza virus infection. Cell **146:**980-991.

Tong S, Li Y, Rivailler P, Conrardy C, Castillo DA, Chen LM, Recuenco S, Ellison JA, Davis CT, York IA, Turmelle AS, Moran D, Rogers S, Shi M, Tao Y, Weil MR, Tang K, Rowe LA, Sammons S, Xu X, Frace M, Lindblade KA, Cox NJ, Anderson LJ, Rupprecht CE, Donis RO. 2012. A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A **109:**4269-4274.

Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, Yang H, Chen X, Recuenco S, Gomez J, Chen LM, Johnson A, Tao Y, Dreyfus C, Yu W, McBride R, Carney PJ, Gilbert AT, Chang J, Guo Z, Davis CT, Paulson JC, Stevens J, Rupprecht CE, Holmes EC, Wilson IA, Donis RO. 2013. New world bats harbor diverse influenza A viruses. PLoS Pathog **9:**e1003657.

Wang J, Li F, Sun R, Gao X, Wei H, Li LJ, Tian Z. 2013. Bacterial colonization dampens influenza-mediated acute lung injury via induction of M2 alveolar macrophages. Nat Commun **4:**2106.

Watanabe T, Kiso M, Fukuyama S, Nakajima N, Imai M, Yamada S, Murakami S, Yamayoshi S, Iwatsuki-Horimoto K, Sakoda Y, Takashita E, McBride R, Noda T, Hatta M, Imai H, Zhao D, Kishida N, Shirakura M, de Vries RP, Shichinohe S, Okamatsu M, Tamura T, Tomita Y, Fujimoto N, Goto K, Katsura H, Kawakami E, Ishikawa I, Watanabe S, Ito M, Sakai-Tagawa Y, Sugita Y, Uraki R, Yamaji R, Eisfeld AJ, Zhong G, Fan S, Ping J, Maher EA, Hanson A, Uchida Y, Saito T, Ozawa M, Neumann G, Kida H, Odagiri T, Paulson JC, Hasegawa H, Tashiro M, Kawaoka Y. 2013. Characterization of H7N9 influenza A viruses isolated from humans. Nature **501:**551-555.

Watanabe T, Watanabe S, Neumann G, Kida H, Kawaoka Y. 2002. Immunogenicity and protective efficacy of replication-incompetent influenza virus-like particles. J Virol **76:**767-773.

Watanabe T, Watanabe S, Noda T, Fujii Y, Kawaoka Y. 2003. Exploitation of nucleic acid packaging signals to generate a novel influenza virus-based vector stably expressing two foreign genes. J Virol **77:**10575-10583.

Williams SP, Robertson JS. 1993. Analysis of the restriction to the growth of nonegg-adapted human influenza virus in eggs. Virology **196:**660-665.

Wright P, Neumann G, Kawaoka Y. 2013. Orthomyxoviruses, Fields Virology 6th edition
(Lippincott Williams & Wilkins).

Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D, Grogan JL. 2009. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol **10:**48-57.