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EXPERT REVIEWS

Innate sensors of influenza virus: clues to developing better intranasal vaccines

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Mucosal immunity acquired by natural infection with influenza viruses at the respiratory tract is more effective and cross-protective against subsequent variant virus infection than systemic immunity induced by parenteral immunization with inactivated vaccines. To develop an effective influenza vaccine, it is beneficial to mimic the process of natural infection that bridges innate and adaptive immune systems. The innate immune system that recognizes influenza virus infection consists of several classes of pattern-recognition receptors, including the Toll-like receptors, the retinoic acid-inducible gene-I-like receptors and the NOD-like receptors. Here, we review our current understanding of the mechanism of innate recognition of influenza and how the signals emanating from the innate sensors control adaptive immunity. Further, we discuss the potential roles of these receptors in developing intranasal influenza vaccines.

KEYWORDS: adaptive immune response • adjuvant • IL-1β • inflammasome • influenza • innate immune response • intranasal vaccine • NALP3 • NOD-like receptor • RIG-1-like receptor • Toll-like receptor

Influenza virus is responsible for annual epidemics that cause severe illnesses in approximately 5 million people worldwide. Lethal pneumonia and encephalopathy caused by influenza virus have now become a serious problem, especially among the elderly and children, respectively [1,2]. Furthermore, the H5N1 highly pathogenic avian influenza virus associated with a high fatality rate (greater than 60%) has been reported in Southeast Asia, Europe and Africa. From January 2003 to June 2008, 243 deaths among 385 cases of laboratory-confirmed infection with highly pathogenic H5N1 avian influenza were reported to the WHO [201]. In some instances, human-to-human transmission of the H5N1 virus appears to have occurred [3], suggesting that this virus has the potential to cause a influenza pandemic [4]. Furthermore, an H5N1 virus (A/Hanoi/30408/2005) resistant to oseltamivir was isolated from a Vietnamese girl [5], and H5N1 viruses isolated from individuals in Hong Kong in 1997 were found to be resistant to interferons and TNF-α [6]. Therefore, there is an urgent and important public-health need to develop effective vaccines against not only annual seasonal influenza viruses but also highly pathogenic H5N1 avian influenza viruses.

Toll-like receptor-mediated influenza viral recognition & innate defense

It is well established that Toll-like receptors (TLRs) on the cell surface and endosomal compartments play a crucial role in the recognition of microbial pathogens. TLR signaling leads to the induction of immediate innate responses to clear the virus. dsRNA, a molecular pattern associated with viral infection, and influenza genomic ssRNA are recognized by TLR3 and TLR7, respectively (FIGURE 1) [7–9]. In mice that are genetically deficient in TLR3 or its adapter molecule, Toll/IL-1 domain-containing adaptor-inducing IFN-β (TRIF) were not impaired in innate immune responses against ssRNA viruses [10]. It is possible that this is due to the fact that not all negative-strand RNA viruses generate dsRNA in their life cycle [11,12].

Plasmacytoid dendritic cells (pDCs) specialize in the robust secretion of type I IFNs, which act as a natural mucosal adjuvant of an intranasally administered influenza vaccine [13]. pDCs are specifically activated by recognition of viral genomic RNA and DNA by means of TLR7/8 and TLR9, respectively (FIGURE 1) [8,9,14,15]. Heat-inactivated [8], formaldehyde-fixed [16] or UV-irradiated [17] influenza virus can induce IFN-α responses comparable with their live

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counterparts in pDCs. These findings indicate that the TLR-mediated recognition of influenza virus in pDCs occurs without direct infection, and that the presence of influenza viral genomic RNA within the endosomal/lysosomal compartments is sufficient to activate antiviral pathways [18]. By contrast, some other ssRNA viruses, such as vesicular stomatitis virus and Sendai virus, are recognized in an autophagy-dependent manner by pDCs through TLR7 [17,19–21]. Although the importance of pDCs as a source of type I IFNs *in vivo* has been shown for other viruses, direct identification of IFN-producing cells during influenza infection in the lung has not been achieved. Recently, Kumagai *et al.* established a knock-in mouse in which green fluorescence protein (GFP) was expressed under the control of the *Ifna6* promoter [22]. Interestingly, intranasal infection with Newcastle disease virus (NDV) did not induce IFN-α from pDCs. Instead, alveolar macrophages (AMs) and conventional DCs were the major inducers of IFN-α [22,23].

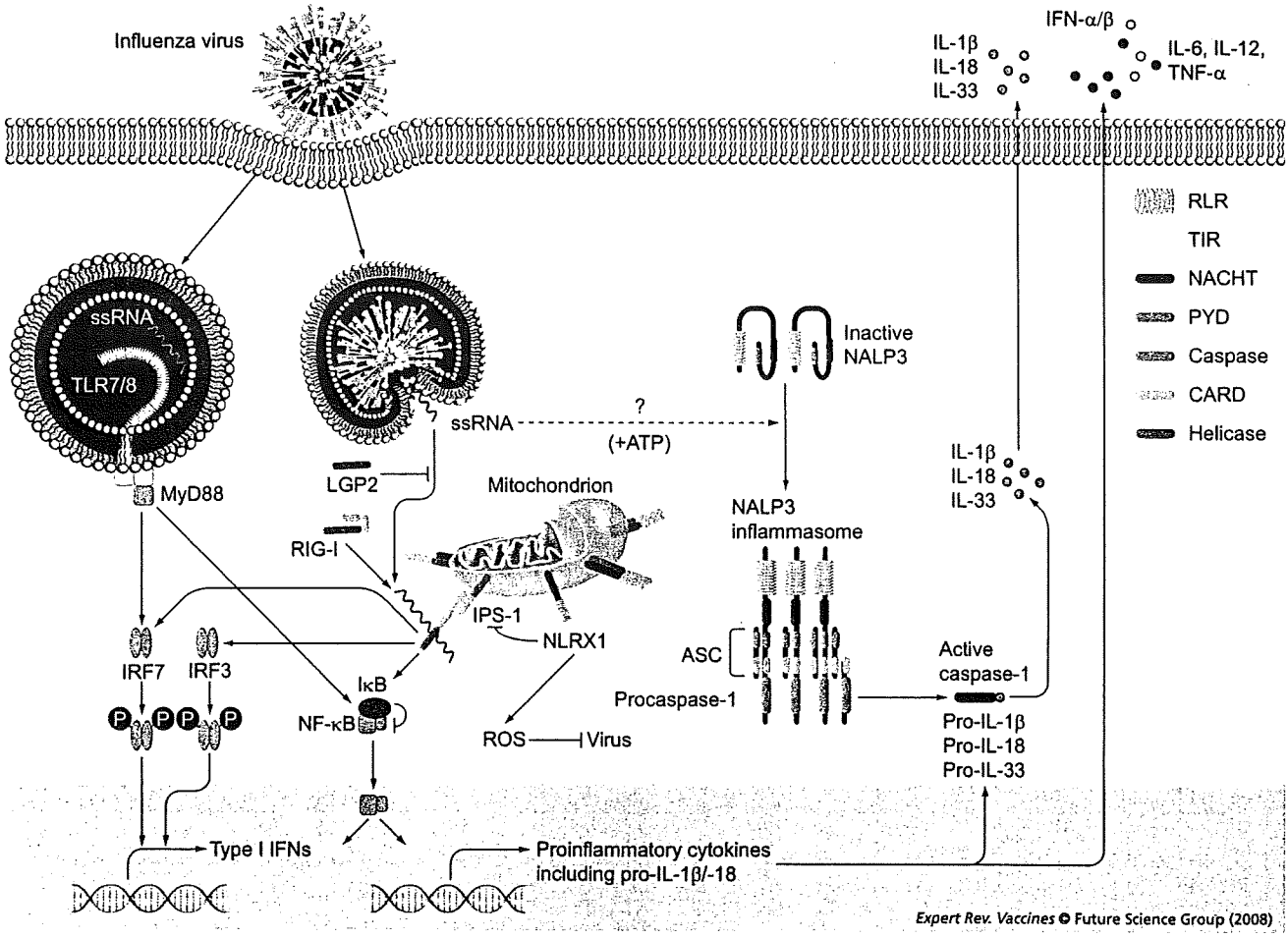

Figure 1. Innate immune recognition of influenza virus infection. TLRs: TLR7 and TLR8 detect ssRNA. After ssRNA recognition, TLR7/8 recruits an adaptor molecule, myeloid differentiation primary response gene 88 (MyD88) containing TIR domain through TIR–TIR interaction. Signaling through these adaptors leads to the phosphorylation and proteasomal degradation of IκB protein and the release of a transcription factor NF-κB to the nucleus to regulate the expression of its target genes, which include proinflammatory cytokines, such as IL-6 and TNF-α, or IRF7 phosphorylation. Phosphorylated IRF7 forms a dimer and translocates into the nucleus to express *IFN*-α and *IFN*-β genes. **RLRs:** After recognition of viral RNA, retinoic acid-inducible gene-I recruits IPS-1 via CARD–CARD interaction. IPS-1 is localized to mitochondria and acts as an adaptor that links RLRs and target genes, which include proinflammatory cytokines and type I IFNs. LGP2, a related family member, acts as a negative regulator of these receptors by sequestering RNA. NLRX1 has been recently shown to act as a negative regulator of IPS-1 signaling, possibly by blocking the interaction of RIG-I with IPS-1. However, NLRX1 also promotes ROS production at the mitochondria, which consequently helps to fight bacteria and viruses. **NLRs:** NALP3 forms multimolecular complexes termed 'inflammasomes' following their activation. NALP3 might recognize influenza virus infection and recruits ASCs that, in turn, recruits procaspase-1, which is activated by autocatalytic cleavage. Cleaved caspase-1 catalyzes proteolytic processing of pro-IL-1β, pro-IL-18 and pro-IL-33 into the active cytokines that are then released.
ASC: Antigen-secreting cell; CARD: Caspase recruitment domain; IFN: Interferon; IPS: IFN-β promoter stimulator; IRF: IFN regulatory factor; NF: Nuclear factor; NLR: NOD-like receptor; RLR: Retinoic acid-inducible gene-I-like receptor; ROS: Reactive oxygen species; TIR: Toll/IL-1 receptor; TLR: Toll-like receptor.

In IFN-β promoter stimulator (IPS)-1-deficient *Ifna6gfp* mice, AMs did not produce IFN-α, indicating that the AM relies on the Rig-1-like receptor (RLR)–IPS-1 system to detect viruses (RLR pathways will be discussed later). On the other hand, pDCs produced IFN-α when AMs were depleted, suggesting that pDCs function when the first defense line is broken [22,23]. Thus, AMs act as a type I IFN producer that is important for the initial responses to NDV infection in the lung. Since the nonstructural-1 (NS1) protein of influenza virus was found to suppress IFN-α/β production from conventional DCs or lung epithelial cells by interacting with retinoic acid-inducible gene-I (RIG-I) [12,24–26], it is possible that influenza virus suppresses AM-mediated antiviral responses, disseminates more rapidly than NDV and, finally, induces pDC-mediated antiviral response in the lung. The critical inducer of type I IFNs following influenza virus infection should be determined by future studies.

Recognition of influenza virus by RLRs & innate defense

It is becoming increasingly clear that the immune system has evolved redundant mechanisms in innate viral recognition. Unlike the TLRs that recognize viral nucleic acids in the endosomes, the RLRs recognize signatures of virus replication within the cytosol of infected cells. Most viruses produce dsRNA in infected cells. Initially, both RIG-I and melanoma differentiation-associated gene (MDA)5 were identified as sensors of a synthetic analog of viral dsRNA, poly(I:C). RIG-I was reported to be involved in the detection of poly(I:C), and the subsequent activation of the transcription factors NF-κB, IFN regulatory factor (IRF)-3 and -7, leading to inflammatory cytokine and type I IFN production (Figure 1) [27,28]. Another sensor of cytosolic viral recognition is MDA5, which can induce the production of IFN-β upon binding to poly(I:C) [29,30]. After recognition of viral RNA, RIG-I and MDA5 bind to IPS-1 (also known as MAVS, Cardif and VISA) via the caspase recruitment domain (CARD–CARD) interaction. IPS-1 is localized to the mitochondria and acts as an adaptor that links RLRs to type I IFN induction (Figure 1) [31–34]. Another mitochondria-targeted protein, NLRX1, was shown recently to act as a negative regulator of IPS-1 signaling by disrupting virus-induced IPS-1–RLRs interactions (Figure 1) [35]. Therefore, the dissociation of IPS-1 from NLRX1 is crucial to transmit RIG-I-mediated signaling. However, NLRX1 also promotes reactive oxygen species (ROS) production induced by TNF-α, *Shigella* infection and dsRNA at mitochondria, which consequently helps to fight bacteria and viruses (Figure 1) [36]. Thus, the precise mechanism by which NLRX1 controls antiviral functions remains to be determined.

A seminal work by Kato *et al.* analyzed RIG-I- and MDA5-deficient mice to dissect the differential roles of RIG-I and MDA5 in influenza virus detection, and found that MDA5 recognized positive-sense RNA viruses of the *Picornaviridae* family [37]. On the other hand, RIG-I, but not MDA-5, was essential for the production of IFNs in response to influenza virus (Figure 1) [38]. In addition, RIG-I was found to recognize 5′-triphosphate ssRNA present in the influenza ssRNA genome [12,39]. Original reports indicated that RIG-I could also recognize dsRNA in addition to 5′-triphosphagte ssRNA [27,40]. Recently, another study by Kato *et al.* provided evidence for size-based discrimination of dsRNA by RLRs and suggested that MDA5 and RIG-I selectively recognized long and short dsRNAs, respectively [41]. In addition, Gale and colleagues demonstrated that the polyuridine motif of the hepatitis C virus (HCV) genome 3′-nontranslated region and its replication intermediate serves as the substrate of RIG-I, and showed that similar homopolyuridine or homopolyriboadenine motifs present in the genomes of RNA viruses are the main feature of RIG-I recognition and immune triggering in human and murine cells [42]. Since influenza virus is reported not to produce dsRNA [11,12,41], triphosphate ssRNA of influenza viral genome is considered to be the substrate of RIG-I, leading to the induction of type I IFNs [43]. *In vivo*, the induction of type I IFNs in response to intranasal influenza A virus infection is abrogated in the absence of both myeloid differentiation primary response gene (MyD)88 and IPS-1, although mice lacking either of these molecules are capable of producing type I IFNs [44]. These results have significant implications on the design of effective adjuvants in the future.

Control of adaptive immunity by TLRs versus RLRs

The TLRs and RLRs not only recognize influenza virus infection and provide innate defense, but also play an important role in the generation of adaptive immunity. DCs play a crucial role in linking the innate and adaptive immune systems. Microbial antigens are recognized by pattern-recognition receptors (PRRs) expressed by DCs and this triggers a maturation program in the DC that ultimately determines how a naive T cell will respond to antigen. Signaling from TLRs leads to the upregulation of costimulatory molecules required for naive T-cell activation and the generation of proinflammatory cytokines, such as IL-6, IL-12 and TNF-α, which influence the type of T cells generated [45]. There are several subsets of DCs that can influence the type of T cells activated, as well as the type of response generated [46]. In addition to DCs, TLR signaling in B cells are important in the induction of adaptive immune responses [47]. Recently, two papers revealed the importance of lung DC subsets in adaptive immune responses [48,49]. McGill *et al.* showed that newly recruited lung DCs after influenza virus infection play an important role for pulmonary $CD8^+$ T-cell responses and the clearance of influenza virus from the lung. GeurtsvanKessel *et al.* showed that the development of virus-specific $CD8^+$ T cells was impaired when lung $CD11c^{hi}$ DCs and AMs or $langerin^+CD11b^-CD11c^{hi}$ DCs were depleted using either CD11c–diphtheria toxin receptor (DTR) or langerin–DTR mice, respectively. By contrast, there was no effect when pDC was depleted by 120G8 monoclonal antibody. Rather, in pDC-depleted mice, there was a reduction in virus-specific serum antibodies after viral clearance in the lung.

In mice that are genetically deficient in TLR3 or its adapter molecule, TRIF was not impaired in the development of $CD4^+$ or $CD8^+$ T-cell or B-cell responses upon influenza viral infection [44,50]. It is possible that this is because influenza A virus infection does not generate dsRNA [11,12]. Therefore, TLR3 might

be dispensable for the induction of adaptive immune response after live influenza virus infection, although TLR3 agonists serve as an important adjuvant for influenza vaccines [51–53].

Previous studies have examined the requirement for TLR7 and MyD88 in adaptive immunity to influenza virus infection with conflicting results. Lopez *et al.* found no requirement for MyD88 on T cells or immunoglobulin responses to aerosolized influenza virus challenge [10]. By contrast, Akira and colleagues showed that TLR7 and MyD88 are required for the induction of CD4 Th1 responses, and virus-specific total IgG and IgG_{2a} responses, but not cytotoxic T-lymphocyte (CTL) responses [44]. Similarly, Marsland's groups demonstrated that IgG_{2a} and IgG_{2c} responses to influenza were impaired in MyD88-deficient mice [50]. They suggested that TLR7 regulates appropriate antibody isotype switching directly through signaling in B cells, and indirectly through the induction of IFN-α by pDCs [50]. All studies found IgG_1 antibodies to be elevated in MyD88-deficient mice [10,44,50], as well as in TLR7-deficient mice [50]. Together, these studies indicated that activation of primary CD8 T cells during anti-influenza immune response relies on mechanisms other than the TLR7 and MyD88 [10,44,50], while $IgG_{2a/c}$ and CD4 T-cell responses depend on MyD88 [44,50].

The differences in the three reported studies may lie in the experimental differences. In the previous study, the authors collected serum to measure virus-specific antibodies titers at 14 days postinfection [10]. On the other hand, Koyama *et al.* collected serum from infected mice at 14 and 21 days postinfection [44]. In the latter study, although there was no significant difference in antibodies titer among wild-type, MyD88-, IPS-1- and TRIF-deficient mice at 14 days postinfection, a clear difference was observed in MyD88-deficient mice at 21 days postinfection [44]. With respect to $CD4^+$ T-cell responses, while Lopez *et al.* used virus-infected splenocytes as antigen-presenting cells (APCs), Koyama *et al.* stimulated splenocytes as APCs with $CD4^+$ T-cell peptide derived from nucleoprotein. Further studies are required to resolve these issues since these two PRR systems probably contribute differentially depending on the route or dose of virus infection [22,37]. In contrast to the role of TLR7/MyD88, IPS-1-deficient mice showed no obvious defects in CD4, CD8 or antibody responses [44].

NOD-like receptors as a novel sensor of influenza viral infection

NOD-like receptors (NLRs) comprise a large family of intracellular PRRs that regulates innate immunity in response to recognition of various self and nonself molecules [54–59]. The first NLR proteins identified, NOD1 and NOD2, have been shown to detect bacterial peptidoglycan and to activate NF-κB [60]. Structurally similar proteins of the NALP family were further identified and shown to form inflammasomes leading to caspase-1 activation. Caspase-1, also known as IL-1β-converting enzyme, is an essential regulator of inflammatory response through its capacity to process and activate pro-IL-1β, pro-IL-18 and pro-IL-33 (Figure 1) [55,61,62]. IL-1β and IL-18 are potent proinflammatory cytokines, and IL-33 promotes response mediated by Th2 cells [63,64]. Upon cleavage of their proforms by caspase-1, these cytokines become active and are secreted by poorly understood mechanisms [61]. Therefore, caspase-1 is considered as a critical factor for the inflammatory response.

Recent reports suggested that NALP3, which is also known as NLRP3/cryopyrin/CIAS1/PYPAF1 [65], forms a caspase-1 activating molecular complex termed the inflammasome (Figure 1). The NALP3 inflammasome can be activated by endogenous signals from dying cells (ATP or uric acid crystals) as well as compounds associated with pathogens such as bacterial RNA or peptidoglycan [66–71]. The mature IL-1β formation requires at least a two-step activation mechanism: first, transcriptional and translational upreguraton of pro-IL-1β that is regulated by TLR signaling; and a second signal that leads to the activation of caspase-1 (Figure 1). This second signal can be activated by the NALP3 inflammasome. Although there are several candidates that activate NLAP3 inflammasome, it is unclear which ligand directly activates the NALP3 inflammasome. However, it is likely that K^+ efflux is an upstream trigger of NALP3 inflammasome activation [72–77]. Pharmacological studies have revealed that IL-1β secretion induced by ATP is mediated by K^+ efflux, the release of intracellular Ca^{2+} stores and the activities of protein tyrosine kinases and calcium-independent phospholipase A2. The relationship between these events and the processing and release of IL-1β are still not clear, although NALP3 may recognize an endogeneous molecule generated by these processes.

Recent reports indicate that virus infection also results in the activation of inflammasomes [78–80]. Kanneganti *et al.* showed that Sendai and influenza viruses activated the NALP3 inflammasome in macrophages pulsed transiently with ATP for 30 min *in vitro* (Figure 1) [78]. Influenza virus infection is accompanied by IL-1β production in bronchoalveolar washing of mice from 2–4 days postinfection [81,82]. Furthermore, influenza virus infection activates IL-1β and IL-18 production in human macrophages [83]. IL-1β enhanced survival of the mice during influenza virus infection [84]. IL-1β appeared not to influence the killing of virus-infected cells but to enhance the priming of CD4 T-helper cells, production of antiviral IgM antibodies by B cells and recruitment of lymphocytes to the site of infection. Not surprisingly, influenza viruses have evolved numerous strategies to inhibit or dampen the host immune response to infection, including the activation of inflammasomes. NS1 protein of influenza virus suppressed caspase-1 activation, maturation of pro-IL-1β and pro-IL-18, and caspase-1-dependent apoptosis in infected primary human macrophages [85].

Uric acid crystals activate NALP3 inflammasome [69]. Interestingly, influenza virus also was reported to induce uric acid in bronchoalveolar lavage fluid and serum in mice [86]. Shi *et al.* demonstrated that uric acid, which is released from injured cell stimulated DC maturation and, when coinjected with particulate HIV-1 gp120 antigen *in vivo*, significantly enhanced CD8 T-cell responses [66]. Eliminating uric acid by uricase *in vivo* inhibited the immune response to antigens associated with injured cells, but not to antigens presented by activated DCs. Another report showed that uric acid increased IgG_1-based humoral immunity when added to dying tumor cells or with ovalbumin (OVA) antigen [87]. These results imply that uric acid from influenza

virus-infected cells could play a role in generating virus-specific CD8 T-cell responses. However, the role of NLR in the control of adaptive immune response to influenza virus is unknown.

Advantages of mucosal vaccines over parenteral vaccines

When developing a vaccine, both prophylactic effectiveness and safety should be considered. Mucosal immune system of the upper respiratory tract, which is a primary site of influenza viral infection, provides the first immunological barrier against infection. Inactivated split vaccines against the influenza virus have been administered parenterally to induce serum antihemagglutinin IgG antibodies, which are highly protective against homologous virus infection, but are less effective against heterologous virus infection [88]. By contrast, a number of studies have shown that the mucosal immunity acquired by natural infection, which is mainly mediated by the secreted form of IgA (sIgA) in the respiratory tract is more effective and cross-protective against heterologous virus infections than systemic immunity induced by parenteral vaccines in humans and mice [88-90]. It is believed that IgA is more cross-protective against heterologous influenza compared with IgG based on the divalency (higher avidity) and their location [88]. The sIgA antibodies consist of dimeric IgA (dIgA) joined by a J-chain, coupled to a secretory component. sIgA is produced by B cells within the lamina propria underneath the mucosal epithelium. The dIgA antibodies secreted by B cells first bind to the polymeric immunoglobulin receptor (pIgR) on the basolateral surface of the epithelial cells and are transcytosed to the apical surface. They are released into the lumen as sIgA antibodies upon cleavage by a specific protease that releases extracellular region of pIgR (forming the secretory component). The blockade of transepithelial transport of dIgA in pIgR-knockout mice results in a dramatic reduction in protection against challenge with heterologous influenza viruses [89]. Furthermore, the dIgA antibodies can bind to newly synthesized viral proteins within the epithelial cells to prevent viral assembly [91]. The influenza virus-specific sIgA antibodies are present at high levels in the upper respiratory tract within the mucus of nose, trachea, bronchi and bronchioles, whereas the specific IgG antibodies, which could access the mucosal surfaces by passive diffusion from serum, are found predominantly in the lower respiratory tract in the serous fluid of alveolar epithelia [89,92]. Thus, sIgA antibodies are involved primarily in the prevention of influenza in the upper respiratory tract. Collectively, induction of sIgA at the respiratory tract has a great advantage in protecting against unpredictable epidemics of influenza over conventional parenteral vaccines.

Adjuvant-combined intranasal influenza vaccines

In order to induce mucosal immunity, immunization at the mucosal surface is often required. Since antigen alone is often insufficient to elicit proper immune responses at the mucosal surface, adjuvant is required for a given vaccine to induce immune responses. In developing intranasal vaccines, cholera toxin (CT) and *Escherichia coli* heat-labile toxin (LT) have been used as adjuvant to enhance mucosal immune responses [93]. Although CT and LT are effective adjuvants to enhance mucosal immune responses including sIgA responses, they have some side effects in humans, including Bell's palsy and nasal discharge [94,95]. Therefore, other adjuvants that are as effective as CT or LT and are also safe for human use have been developed for clinical application with intranasal influenza vaccine [51-53,96-102].

Previous studies have demonstrated that various elements of the innate immune system play roles in protection against influenza virus infection. For example, the collectin family of mammalian C-type lectins including surfactant proteins A and D, mannose-binding protein and conglutinin, which are expressed in the airway and alveolar epithelium, can bind to influenza virus, act as opsonins and inhibit influenza virus HA activity [103-107]. The extracellular matrix protein mindin can interact with influenza virus particles directly and enhance the clearance of influenza virus in the lung tissue [108]. Defensins can directly inactivate influenza A virus [109] and the θ-defensin retrocyclin-2 inhibits influenza virus infection by blocking membrane fusion mediated by the viral HA [110]. However, it is unknown whether these anti-influenza virus effectors have the capacity to generate adaptive immune responses and thereby serving as adjuvants for intranasal vaccine.

In contrast to CT, LT or innate effector proteins outlined previously, the agonists of TLR, RLR and NLR are likely to be an ideal candidate for influenza vaccine adjuvants, owing to the fact that these sensors are used by the host to recognize and mount robust protective immunity during the natural course of influenza infection. As described previously, dsRNA acts as a molecular mimic associated with viral infection. In response to dsRNA, endosome-localized TLR3 and the cytoplasmic RLRs, RIG-I and MDA5 all activate type-I IFN and the NF-κB pathways [7,37,44,111]. Type I IFNs, consisting of IFN-α, IFN-β, IFN-ε, IFN-ξ and IFN-ω, are the most potent antiviral cytokines that mediate innate protection against a myriad of viruses. Type I IFNs also contribute to the generation of adaptive immunity, such as CTL and antibody responses against subcutaneous immunization of soluble materials [112,113].

It has been demonstrated that the synthetic dsRNA poly(I:C), an agonist of TLR3 and MDA5 [7,37], has mucosal adjuvant activity when coadministered intranasally with an inactivated influenza vaccine, and increases both the mucosal and systemic humoral responses, resulting in complete protection against homologous and heterologous variant influenza viruses, including the highly pathogenic H5N1 avian influenza virus [51-53,99]. Sloat *et al.* also reported that mice immunized intranasally with recombinant anthrax protective antigen combined with poly(I:C) developed strong systemic and mucosal anti-anthrax antigen responses with lethal toxin-neutralization activity [114]. The adjuvant effects of poly(I:C) require a co-operative activation of TLR3 and cytoplasmic RNA helicase MDA5 pathways when mice were administered intraperitoneally with OVA plus alum together with poly(I:C) [115]. However, while poly(I:C) is a potent mucosal adjuvant that induces type I IFNs and has the potential to bridge innate and adaptive immunity [46], it has been associated with serious adverse events during clinical trails. Poly(I:C) induced a number of side effects in humans, including renal failure and

hypersensitivity reactions in some patients in a previous clinical trial in patients with leukemia or solid tumors using as high as 75 mg of poly(I:C)/m^2 of tumor mass on day 0 and then daily from day 7 to a maximum of 35 days [116]. By contrast, polyI:polyC$_{12}$U (Ampligen®), which is similar to poly(I:C), has a good safety profile based on clinical trials, including a recently conducted double-blind, placebo-controlled Phase III clinical trial [117]. To date, more than 75,000 doses of Ampligen have been administered to humans, at an average dose of 400 mg, and it has been generally well tolerated. Recently, we showed that Ampligen has mucosal adjuvant activity when coadministered intranasally with formalin-inactivated H5N1 influenza whole-virion vaccine or trivalent inactivated influenza vaccine licensed in Japan for the 2005–2006 season – it increased both the mucosal and systemic humoral responses that protected mice against homologous and heterologous highly pathogenic H5N1 avian influenza viruses [52,53]. We also observed that coadministration of H5N1 vaccine with Ampligen as mucosal adjuvant elicited high levels of vaccine-specific IgA titer in saliva and IgG titer in the serum in cynomolgus macaques [Ichinohe T *et al.*, Unpublished Data]. Plans to test the efficacy of the adjuvant-combined intranasal influenza vaccine in human clinical trials will be underway as early as 2010 in Japan [90,202].

In 2003, the first live-attenuated, cold-adapted influenza virus vaccine (FluMist®, MedImmune Vaccines, Inc., MD, USA), which replicates in the upper respiratory tract of a vaccinated individual, was licensed in the USA for healthy individuals 2–49 years of age. It has been reported that cold-adapted, live-attenuated influenza virus (LAIV) has a higher relative efficacy compared with trivalent inactivated influenza vaccine in children under 5 years of age [118–120]. Since children infected with wild-type influenza virus can shed virus in nasal secretion within 14 days after symptom onset [121–124], the potential for transmission of vaccine viruses from vaccinated contacts to unvaccinated infants has been raised as a concern. Since the NS1 protein of the influenza A virus can inhibit the RIG-I-mediated induction of IFN-β [12,24–26], it also remains possible that NS1 reduces the effectiveness of the LAIV. However, Talon *et al.* clearly showed that immunization with influenza virus lacking the viral *NS1* gene induces comparable levels of virus-specific antibody and T-cell responses to wild-type influenza virus and protects mice from subsequent lethal viral challenge [125].

Expert commentary

Adjuvants are essential for the development of modern vaccines that promote various types of immune responses. Adjuvants probably operate through triggering one of the PRRs that evolved to recognize pathogens. For example, some live-attenuated viral vaccines are effective because they contain sufficient PRR agonists, whereas other vaccines are less effective, presumably due to the lack of their ability to mimic viral infection through PRR activation.

Avian H5N1 influenza A is an emerging pathogen with the potential for great harm to humans. Preparation of vaccines that provide protective activity against not only homologous but heterologous highly pathogenic influenza viruses is important for the control of new pandemic influenza. Although current parenteral influenza vaccines limit severity of disease, they do not prevent viral infection itself due to their inability to induce mucosal IgA responses at the site of infection. Since it is difficult to predict which strain of influenza will cause a pandemic in the future, it is advantageous to produce vaccines that confer cross-protective immunity. It is becoming increasingly clear that mucosal influenza vaccination induces cross-protective immunity against variant influenza viruses by inducing secretory IgA at the site of infection. Intranasal vaccination coadministered with an adjuvant appears to offer an effective strategy against an influenza pandemic, regardless of the strain of H5N1. The potential shortage of vaccines during a pandemic might also be addressed by preparing a stock of intranasal vaccines in advance using the US FDA 'animal rule' as a global model for regulatory approval by various governmental regulatory agencies.

In our opinion, polyI:polyC$_{12}$U-combined intranasal influenza vaccine provides the most promising preventive tool for influenza virus infection. Since TLR3, the sensor of polyI:polyC$_{12}$U [126], is localized to the endosomal compartment, concomitant administration of polyI:polyC$_{12}$U and liposome may be more effective than polyI:polyC$_{12}$U alone. In fact, chitin microparticles, as a carrier for poly(I:C), enhanced antibody responses and provides protection against lethal H5N1 influenza virus challenge in conjunction with poly(I:C) [99]. Furthermore, the role of inflammasome in both innate and adaptive immune protection against influenza is largely unknown. Since the Th2-inducing activity of the most commonly used adjuvant, alum [127], is mediated through inflammasome activation [128], inflammasome activation may prove to be important in vaccine designs in the future. We still do not know how well the efficacy of vaccines tested in mice translates to humans. However, it is clear that development of biocompatible materials that enhance the adhesion and uptake of vaccine by the nasal cavity and the respiratory tract would be a beneficial advancement, since the nasal cavities differ in size between mice and humans and most of the nasal spray-administered vaccine ends up being ingested when they are administered to humans.

Key issues

- Highly pathogenic H5N1 avian influenza A virus has the potential to cause the next influenza pandemic.
- There is an urgent and important public-health need to develop effective vaccines against highly pathogenic H5N1 avian influenza virus.
- Although currently licensed human vaccines are subtype specific, we cannot predict the strain that is most likely to cause the next pandemic.
- Intranasal vaccine can provide safe and effective mucosal immunity against variant influenza virus infection.
- Adjuvants are essential for the development of intranasal inactivated influenza vaccine.

Five-year view

The influenza virus changes its antigenicity every year and one cannot predict the strain of the new emerging influenza virus in humans. Cross-protective immunity can limit infection and spreading of variant viruses. The advantage of mucosal immunity in the prevention of influenza is already clear; however, until recently, there were no human-applicable adjuvants for mucosal vaccines.

Knowledge of the mechanisms by which influenza viruses are recognized by immune systems and how adaptive immune responses are generated after infection provides us with a better understanding to develop new effective adjuvant-combined intranasal influenza vaccines. We hope to make needle-free adjuvant-combined intranasal influenza vaccine a reality within 5 years.

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Development of mucosal adjuvants for intranasal vaccine for H5N1 influenza viruses

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Abstract: An increasing number of infections of highly pathogenic avian influenza virus (H5N1) in humans has been reported in South-East Asia and other areas of the world. High mortality (>60%) of this viral infection and its pathosis of systemic infection are features of this new human disease. Moreover, there is great concern that this avian H5N1 virus could cause a pandemic of new influenza in humans, once it acquires the ability for human to human transmission. To prevent such highly contagious infectious diseases as influenza, it is essential to prepare effective vaccines. Especially in the case of new influenza virus, we cannot predict the strain which will cause the pandemic. In such a situation, a vaccine that induces cross-protective immunity against variant viruses is extremely important. However currently used parenteral seasonal influenza vaccine is strain-specific, and is less effective against variant viruses. In order to overcome the weakness of current vaccines we need to learn from the immune responses induced by natural infection with influenza viruses. In the case of mucosally acquired acute respiratory infection such as influenza, mucosal immunity induced by natural infection plays important role in protection against the infection, as mucosal secretory IgA antibody plays an important role in cross-protection. In this review we describe the advantages and development of mucosal vaccine against highly pathogenic H5N1 influenza viruses.

Keywords: influenza virus, mucosal immunity, secretory IgA antibody, adjuvant

Influenza virus and its infection signal

Influenza is a contagious acute respiratory disease of birds and mammals caused by infection of the upper respiratory tract by viruses of the family Orthomyxoviridae. Types A and type B infect humans and cause respiratory symptoms and also encephalopathy in infants. Recently it has been reported that infection by highly pathogenic influenza viruses (HPIV) and the avian influenza virus (H5N1) in humans can be fatal. In cases where infection sites were not restricted to the respiratory system, it spread systemically including the gastrointestinal (GI) tract.[1] Although most human H5N1 infections have been caused by the direct transmission of virus from infected poultry, there is fear that a pandemic could result if subsequent transmission of H5N1 virus occurred between infected humans.[2] Therefore, there is an urgent and important public health need to develop effective vaccines against this highly pathogenic strain of avian influenza virus.

Annual epidemics of influenza are caused when the antigenic properties of the viral surface proteins hemagglutinin (HA) and neuraminidase (NA) are altered. HA is involved in binding of the virus to sialic acids on the surface of susceptible cells.[3] NA cleaves terminal sialic acid residues from carbohydrate moieties on the surfaces of infected cells, promoting the release of progeny viruses from infected cells. It has been shown that both HA and NA are among the most protective of the various viral proteins against influenza when immunized with plasmid DNAs encoding HA and NA.[4]

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Influenza virus has single-stranded RNA as its genome, and this single-stranded RNA is recognized as an infection signal by host cells through Toll-like receptor 7 (TLR-7).[5] In the course of viral replication, double-stranded RNA is produced, which is recognized by TLR-3 as an infection signal. Thus, influenza virus is recognized by host immune cells at the very early stage of infection by the host through pathogen signals, and these receptors and the host immune system initiate the mucosal and systemic immune system against present and future viral infection. By verifying a series of events occurring at the infection site, we use our increased understanding of the immune response to develop and apply strategies to combat influenza viral infection.

Innate immunity and adjuvant effect

Innate immunity is a set of nonspecific mechanisms that constitute the body's naturally occurring immune response to infection by microbes at any site. In influenza virus infection, the upper respiratory mucosal surface is the effector site of the innate immune system. The mechanical barrier of the mucosal epithelium, surface mucus, secretion of antimicrobial peptides such as defensins, secretion of type I and II interferons (IFNs), natural killer cells, and complement factor all play important roles in innate immunity at the respiratory mucosa (Figure 1). Among these, the IFN response is required to signal viral infection. During influenza virus infection, genomic single-stranded RNA, and double-stranded RNA produced during viral replication, have been implicated as the molecular signals of infection that trigger IFN production.

The innate immune system senses viral infection by recognizing a variety of viral components, including double-stranded (ds) RNA, and triggering antiviral responses. The cytoplasmic helicase protein retinoic-acid-inducible protein I (RIG-I, also known as Ddx58) and melanoma differentiation-associated gene 5 (MDA5, also known as Ifih1 or Helicard) have been implicated in recognition of viral dsRNA. Viral dsRNA binds to RIG-I and MDA5 in the cytoplasm, which leads to activation of IFN regulatory factors.[6] In vitro studies suggest that RIG-I and MDA5 recognize both RNA viruses, and polyinosine-polycytidylic acid (poly(I:C)), a synthetic dsRNA analog. RIG-I is essential for the production of IFNs in response to RNA viruses, including paramyxovirus, influenza virus, and Japanese encephalitis virus, whereas MDA5 is critical for the detection of picornavirus.[6] The recognition of viral infection by the innate immune system

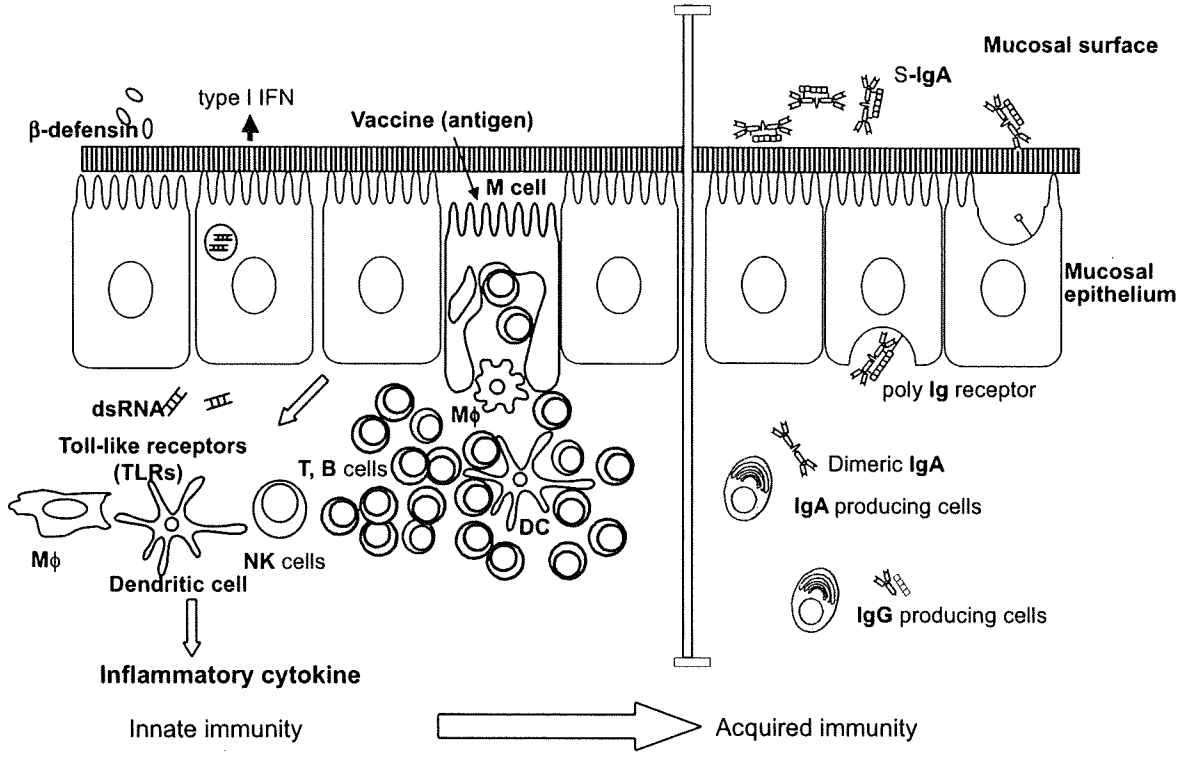

Figure 1 Defence mechanism at mucosal site, innate and adaptive immunity.

bridges the transition between innate and adaptive immune responses. This is a particularly important facet of innate immunity involved in mucosal immune responses. We can take advantage of the mucosal innate immune response to enhance vaccine efficacy, which we will discuss later in this review.

Among the several innate immune receptors, the Toll-like receptor family plays a central role in the recognition of viral nucleic acid. This recognition leads to the induction of type I IFN. We previously demonstrated that the synthetic double-stranded RNA (dsRNA) poly(I:C), a TLR-3 agonist, has mucosal adjuvant activity when co-administered intranasally with an influenza HA vaccine, and increases both the mucosal and systemic humoral response, resulting in complete protection against challenge by homologous avirulent (H1N1) and highly pathogenic (H5N1) influenza viruses in mice.[7,8] Sloat and Cui[9] also reported that mice immunized intranasally with recombinant anthrax protective antigen adjuvanted with poly(I:C) developed strong systemic and mucosal anti-anthrax antigen responses with lethal toxin neutralization activity. Thus, the signals conducted by innate immune receptors work as adjuvants which act as a bridge between innate immunity and acquired immunity.

Mucosal vaccine

Seasonal influenza vaccines are prepared based on the prediction of the expected strain of epidemic of the next season. These are parenterally injected vaccines which does not prevent the infection itself, which reduce the severity and complications after the infection. Parenteral vaccines can induce the neutralizing IgG antibody in the serum but they cannot induce the secretory IgA antibody which acts on the mucosal surface. Secretory IgA antibodies on the mucosal membrane surface are highly effective for preventing infection because they react on the surface of the mucosal membrane before the pathogens attach to the epithelial cell surface, which is the first target of influenza viral infection. Moreover, serum IgG antibodies are less effective against drifted viral strains because they act more specifically than secretory IgA antibodies. Secretory IgA antibodies have cross-protective effects against variant strains of the influenza virus. The exact mechanism of the cross-reactive effects of IgA is still unknown, but this phenomenon is a great advantage in preventing infection. In fact, natural influenza virus infection was shown to be superior to vaccination with inactivated virus in inducing cross-protection against infection by mutated viruses within a particular subtype of the A-type virus in humans.[10–12] Another reason why the mucosal immune system is adept at preventing infection is that the effector sites are not restricted to the originally sensitized mucosa. IgA-specific antibody forming cell (AFC) precursors migrate from mucosal sites throughout the entire body via site-specific homing pathways. This system is referred to as the common mucosal immune system.[13–17] Because of the advantages of induction of mucosal immunity for preventing influenza, several strategies have been used to attempt to development a mucosal vaccine. For effective induction of secretory IgA by inactivated vaccine, mucosal co-administration of vaccine with adjuvant is necessary. As a mucosal adjuvant, a bacterial toxin such as cholera toxin (CT) or *Escherichia coli* heat-labile toxin (LT) have been used experimentally.[18,19] Although LT is an effective adjuvant for the production of mucosal IgA, it has adverse clinical side effects, such as facial paralysis (Bell's palsy).[20] New, clinically safe and effective adjuvants are necessary for the administration of intranasal influenza vaccines to humans. The most promising candidate for mucosal adjuvant is PolyI:PolyC_{12}U (Ampligen®), which is synthetic dsRNA and has a good safety profile based on clinical trials, including a recent double-blind, placebo-controlled Phase III clinical trial for chronic fatigue syndrome (CFS).[21–23] To date, >75,000 doses of Ampligen® have been administered to humans, at an average dose of 400 mg, and it has been generally well tolerated. Recently, it was shown that PolyI:PolyC_{12}U was as effective as poly(I:C) in inducing maturation of human monocyte-derived dendritic cells in vitro.[24] So PolyI:PolyC_{12}U (Ampligen®) was examined as an adjuvant for mucosal influenza H5N1 vaccine administered intranasally in mice together with synthetic dsRNAs (poly(I:C) and Ampligen®) as powerful TLR-3 agonists.

Highly pathogenic avian influenza virus H5N1

The first outbreak of the highly pathogenic avian influenza virus H5N1 was reported in humans and birds in Hong Kong in 1997, during which six out of 18 infected people died.[25] Subsequently, re-emergence of the H5N1 virus associated with a high fatality rate (greater than 60%) has been reported in southern China, Vietnam, Thailand, Cambodia, Indonesia, Turkey, and Iraq. From January 2003 to September 2008, 387 laboratory-confirmed human cases of H5N1 were reported to the World Health Organization (WHO). Although most human H5N1

infections have been caused by the direct transmission of virus from infected poultry, there is fear that a pandemic could result if subsequent transmission of H5N1 virus occurred between infected humans.[2] Because the ability to be transmitted from human to human represents the final barrier to a new pandemic of H5N1, there is an urgent and important public health need to develop effective vaccines in preparation for such a pandemic. However developing a vaccine against the H5N1 virus poses a number of problems. A highly contained facility is required, and the virus grows very poorly in embryonated eggs because it kills chickens. Attenuation of the vaccine strain is necessary to eliminate these problems. Currently licensed human vaccines are strain-specific and do not protect against heterotypic influenza viruses. This is problematic, because influenza A (H5N1) continues to evolve into antigenically distinct clades. The question remains of how an effective vaccine can be prepared for an impending pandemic of a new influenza, which might be caused by a highly pathogenic strain of avian influenza virus. Influenza virus A (H5N1) is not the only strain that could cause a new pandemic in humans.

H5 vaccine candidates must be continually updated to match the antigenicity of circulating viruses because of the differences in HA antigenicity among 1997, 2003, and 2004 H5 viruses.[26] In addition, it is difficult to predict which strain of virus (H5 or other avian-associated HA) will be responsible for a pandemic. In such circumstances, the ideal approach is to prepare a vaccine that confers strong cross-protective immunity against variants of a particular virus strain. Mucosal immunity induced through natural infection by influenza virus has potent cross-protective activity, compared with subcutaneous vaccination-induced systemic immunity. Cross-protective activity is correlated with mucosal secretory IgA, which is not induced after subcutaneous vaccination.[27] In order to induce cross-protective mucosal immunity through influenza vaccination, we have examined the effect of intranasal administration of an inactivated viral vaccine with various adjuvants, and found that mucosal IgA plays an important role in cross-protection against variant influenza A and B virus infection.[7,28–30] Nicholson and colleagues reported that the H5N1 vaccine is poorly antigenic in humans, and requires adjuvant to elicit a detectable antibody response.[31] Several groups looking at avian influenza H5N1 vaccines have reported that intranasal administration of a formalin-inactivated whole virus vaccine with or without mutant *E. coli* LT adjuvant (R192G), or an adenoviral vector-based influenza vaccine, protected mice from lethal challenge by a heterologous H5N1 virus.[32–34]

Development of adjuvant-combined inactivated nasal vaccines

Subcutaneous injection of inactivated vaccines would be an effective strategy in an epidemic caused by a homologous virus, as it induces specific serum IgG, but would be less effective in an epidemic caused by a heterologous virus. On the other hand, live attenuated vaccines effectively protect against heterologous virus infection by inducing secretory IgA, IgG, and cytotoxic lymphocyte (CTL) responses. However, because their safety has been proven only in healthy people between the age of 5 and 49, their use is approved only for this group of people in the US. Intranasal administration of inactivated vaccines represents a potential solution to overcoming these problems.

In clinical trials, inactivated whole virus particles and split-product vaccines have been shown to be effective in preventing live virus infection when administered intranasally.[35–39] Moreover, intranasal administration of an inactivated whole virion vaccine induced a broad spectrum of heterosubtypic immunity in mice, which was not observed using an ether-split vaccine.[33] The stronger immunogenicity of the inactivated whole virion vaccine was likely due to the stimulation of innate immunity by genomic single-stranded RNA, via TL-R7.[5,40]

Intranasal administration of an inactivated ether-split vaccine and the synthetic dsRNA poly(I:C) conferred effective cross-protection in the upper respiratory tract (RT) against viral variants (drift viruses) of influenza A, or B-type viruses.[7] Because most viruses produce dsRNA during replication,[41] synthetic dsRNA likely acts as a molecular mimic of viral infection. The mammalian TLR-3 receptor recognizes dsRNA, and activates the NF-κB[42] pathway, resulting in activation of type I IFN, which in turn enhances the primary antibody response to subcutaneous immunization of soluble materials.[43] This adjuvant activity of type I FN appears to play an important role in bridging the gap between innate and adaptive immunity.[43]

In mice, intranasal administration of an ether-split vaccine from PR8 (influenza strain H1N1) and poly(I:C) adjuvant induced a strong anti-HA IgA and IgG response in nasal washes and serum, respectively, while vaccination without poly(I:C) induced very little response. In addition, administration of either an A/Beijing (H1N1) or A/Yamagata (H1N1) vaccine and poly(I:C) conferred complete protection against PR8 virus challenge in a mouse model of nasal infection,

suggesting that intranasal vaccination with poly(I:C) adjuvant confers cross-protection against variant viruses. Although the systemic antigen-specific T-cell responses were induced by intranasal vaccination with poly(I:C) adjuvant, T-cell responses against heterologous influenza viruses were weak. Moreover, TLR3, which is a receptor for dsRNA in nasal-associated lymphoid tissue (NALT), was upregulated at the level of mRNA expression upon intranasal administration of a split vaccine and poly(I:C).[7] Recently, a clinically safe dsRNA, PolyI:PolyC_{12}U (Ampligen®), was investigated as a dsRNA adjuvant for intranasal avian flu vaccines.

To evaluate the adjuvant effect of Ampligen®, the protective effect of intranasal administration of vaccine and Ampligen® adjuvant against homologous (A/Vietnam) and heterologous (A/Hong Kong and A/Indonesia) H5N1 influenza virus challenge was examined[44] (Figure 2). Two groups of mice were immunized either intranasally or subcutaneously with 1 μg of vaccine from Vietnam strain and 10 μg of Ampligen®, then challenged by intranasal administration of 1000 PFU of H5N1 influenza virus at 2 weeks after the final immunization. A third group of control mice was immunized intranasally with 10 μg of Ampligen® alone. In response to homologous viral challenge, all the mice immunized intranasally with vaccine and Ampligen® completely cleared viruses in their nasal cavity. By contrast, significantly higher levels of virus in nasal wash were detected in mice immunized subcutaneously with vaccine and Ampligen®. All mice of both groups survived after homologous A/Vietnam/1194/2004 viral challenge. In the heterologous virus challenge group, virus titers in nasal wash of intranasal vaccination group were significantly lower than in the subcutaneous vaccination group after A/Hong Kong or A/Indonesia viral challenge. Consequently, although intranasally immunized mice survived a potentially lethal infection with A/Hong Kong or A/Indonesia viruses, most influenza-challenged mice died (Figure 2). These results clearly indicated that intranasal administration of H5N1 vaccine and Ampligen® adjuvant is more effective than subcutaneous vaccination against homologous and heterologous H5N1 influenza virus challenge.

BALB/c mice were immunized three times intranasally or subcutaneously with trivalent inactivated influenza vaccine licensed in Japan for the 2005–2006 season.[45] The vaccine included A/New Caledonia/20/99 (H1N1), A/New York/55/2004 (H3N2), and B/Shanghai/361/2002 viral strains and was administered with PolyI:PolyC_{12}U (Ampligen®) as an adjuvant. The immunized mice were challenged with A/Hong Kong, A/Vietnam, or A/Indonesia H5N1 influenza viruses 2 weeks after the final immunization. Mice immunized intranasally manifested cross-reactivity of mucosal IgA and serum IgG with H5N1 virus as well as a reduced H5N1 viral titer in nasal wash, and their survival was higher after H5N1 virus challenge compared with nonimmunized animals. Subcutaneous immunization did not induce a cross-reactive IgA response and did not afford protection against H5N1 viral infection. These results suggest that intranasal immunization with annual influenza vaccine may overcome the problem of a limited supply of H5N1 virus vaccine by providing cross-protective mucosal immunity in humans against H5N1 viruses with pandemic potential.

Cross-protection by other vaccines

Parenteral inactivated vaccines, including split-product, subunit vaccines and whole virion vaccines, induce mainly serum IgG antibodies that are weakly cross-protective against drift viruses within a subtype. These IgG antibodies would be effective against an epidemic of homologous virus, but would rarely be effective against an epidemic caused by a heterologous virus. Thus, an inactivated parenteral vaccine can effectively protect against an epidemic caused by a homologous virus, but would be relatively ineffective against an epidemic caused by a heterologous virus.

On the other hand, a cold-adapted, live-attenuated virus vaccine licensed in Russia and in the USA[46–48] appeared to mimic the natural course of infection, and provided cross-protective immunity against different subtypes of viruses by inducing secretory (S)-IgA antibodies, serum IgG antibodies, and a CTL response.[49,50] The advantage of live viral vaccines is that they induce not only mucosal IgA and serum IgG antibody responses, but also CTL responses, and confer cross-protection against different subtypes of influenza virus. Current cold-adapted (ca) live-attenuated influenza virus vaccines are growth-restricted to the upper RT. Using reverse genetics, a live attenuated vaccine was generated that encodes a modified form of H5 HA and wild-type-type N1 neuraminidase from influenza A virus strain H5N1, with the remaining gene segments derived from the cold-adapted (CA) influenza A vaccine donor strain. This vaccine was immunogenic in mice.[51] Four weeks after receiving a single intranasally administered dose of CA vaccine, mice were fully protected from lethal challenge with homologous and antigenically distinct, heterologous wt strain H5N1 viruses from different genetic sublineages.[51] Because live attenuated vaccine can induce immune responses equivalent to those induced by natural infection, a live vaccine that has no side

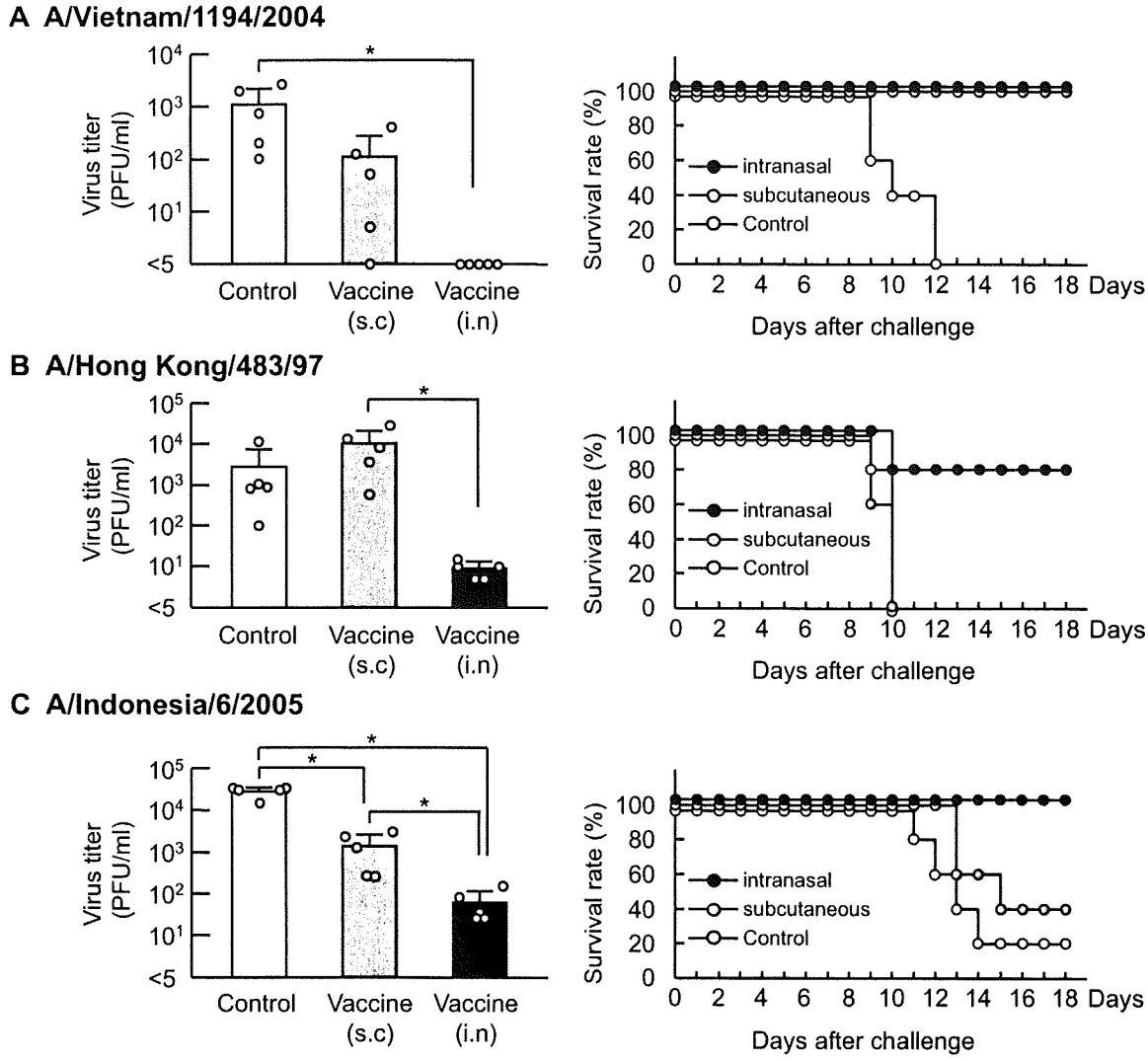

Figure 2 H5N1 virus titers in nasal washes and survival rates after lethal challenge with homologous A/Vietnam, heterologous A/Hong Kong, or heterologous A/Indonesia viruses. Mice were immunized intranasally (solid bar) or subcutaneously (gray bar) with vaccine and Ampligen®, then challenged by intranasal administration of 1000 PFU of A/Vietnam (**A**), A/Hong Kong (**B**), or A/Indonesia (**C**) virus 14 days after the final immunization. Nasal washes were collected three days post infection (d.p.i), and virus titers were measured by plaque assay. Each bar represents the mean ± SD of five mice and open circles indicate individual animals. For statistical analysis, virus titers were compared to those from control mice (open bar) that received intranasal administration of 10 μg of Ampligen® alone. Survival rates were monitored for 18 days. Copyright © 2007. Reproduced with permission from Ichinohe T, Kawaguchi A, Tamura S, et al. Intranasal immunization with H5N1 vaccine plus Poly I:Poly C12U, a Toll-like receptor agonist, protects mice against homologous and heterologous virus challenge. *Microbes Infect.* 2007; 9:1333–1340.
Note: *$p < 0.05$.

effects would be good candidate of pandemic vaccine, if it could be produced.

Conclusion

Now that a pandemic of new influenza virus seems possible, and because it will be difficult to know when a pandemic will occur or which strain of virus will be the cause, it is in our best interests to develop broadly effective and safe vaccines against the influenza virus. For the development of a broadly effective vaccine, induction of mucosal immunity is an inevitable requirement, as mucosal secretory IgA plays an important role in cross-protection. Vaccines designed to induce mucosal immunity are necessary to combat a new influenza pandemic. As stated above, one of the requirements for inducing mucosal immunity is administration of the vaccine at mucosal sites, such as the nasal mucosa. For this reason, intranasal administration of inactivated vaccine plus adjuvant, or live attenuated vaccines, are promising candidates for

inducing cross-protective immunity against variant influenza viruses. However, for safety reasons, the ideal vaccine for induction of cross-protective mucosal immunity may be an inactivated vaccine. Recently, several candidate adjuvants that are effective in mucosal vaccine administration have emerged, including dsRNA (Ampligen®),[7,8] CMPs, SMPs,[30] and mutant CT.[52] These mucosal adjuvants represent promising approaches to the development of safe and effective vaccines for a potential influenza pandemic.

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