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

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Abstract

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-1 β ; inflammasome; influenza; innate immune response; intranasal vaccine; NALP3; NOD-like receptor; RIG-I-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 intra nasally 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 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 [1719–21]. 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 macro phages (AMs) and conventional DCs were the major inducers of IFN- α [22,23].

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- κ B, 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'-triphosphate 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 IgG₁ 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 post infection, 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- κ B [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-1 β -converting enzyme, is an essential regulator of inflammatory response through its capacity to process and activate pro-IL-1 β , pro-IL-18 and pro-IL-33 (Figure 1) [55,61,62]. IL-1 β 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-1 β formation requires at least a two-step activation mechanism: first, transcriptional and translational upregulation of pro-IL-1 β 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 NALP3 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-1 β secretion induced by ATP is mediated by K⁺ efflux, the release of intracellular Ca²⁺ 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-1 β are still not clear, although NALP3 may recognize an endogenous 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-1 β production in bronchoalveolar washing of mice from 2–4 days postinfection [81,82]. Furthermore, influenza virus infection activates IL-1 β and IL-18 production in human macrophages [83]. IL-1 β enhanced survival of the mice during influenza virus infection [84]. IL-1 β 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-1 β 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 cells 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 IgG₁-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 under-neath 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- κ B 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 trials. 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)/m² of tumor mass on day 0 and then daily from day 7 to a maximum of 35 days [116]. By contrast, polyI:polyC₁₂U (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 tri valent 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:polyC₁₂U-combined intranasal influenza vaccine provides the most promising preventive tool for influenza virus infection. Since TLR3, the sensor of polyI:polyC₁₂U [126], is localized to the endosomal compartment, concomitant administration of polyI:polyC₁₂U and liposome may be more effective than polyI:polyC₁₂U 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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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.