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Innovative Mucosal Vaccine Formulations Against Influenza A Virus Infections

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Despite efforts made to develop efficient preventive strategies, infections with influenza A viruses (IAV) continue to cause serious clinical and economic problems. Current licensed human vaccines are mainly inactivated whole virus particles or split-virion administered via the parenteral route. These vaccines provide incomplete protection against IAV in high-risk groups and are poorly/not effective against the constant antigenic drift/shift occurring in circulating strains. Advances in mucosal vaccinology and in the understanding of the protective anti-influenza immune mechanisms suggest that intranasal immunization is a promising strategy to fight against IAV. To date, human mucosal anti-influenza vaccines consist of live attenuated strains administered intranasally, which elicit higher local humoral and cellular immune responses than conventional parenteral vaccines. However, because of inconsistent protective efficacy and safety concerns regarding the use of live viral strains, new vaccine candidates are urgently needed. To prime and induce potent and long-lived protective immune responses, mucosal vaccine formulations need to ensure the immunoavailability and the immunostimulating capacity of the vaccine antigen(s) at the mucosal surfaces, while being minimally reactogenic/toxic. The purpose of this review is to compile innovative delivery/adjuvant systems tested for intranasal administration of inactivated influenza vaccines, including micro/nanosized particulate carriers such as lipid-based particles, virus-like particles and polymers associated or not with immunopotentiatory molecules including microorganism-derived toxins, Toll-like receptor ligands and cytokines. The capacity of these vaccines to trigger specific mucosal and systemic humoral and cellular responses against IAV and their (cross)-protective potential are considered.

Keywords: influenza A virus, mucosal vaccines, adjuvant, delivery systems, intranasal immunization

INTRODUCTION

Despite progress in antiviral therapies, influenza viruses remain an important cause of respiratory tract (RT) infections in humans and animals worldwide (1). Influenza viruses are members of the *Orthomyxoviridae* family and are classified into four genera (A, B, C, D). Influenza A viruses (IAV), whose natural reservoirs are aquatic birds, can infect a broad spectrum of animal species including humans and poultry. Based on the molecular structure and genetic characteristics of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), IAV can be categorized into 18 HA subtypes and 11 NA subtypes.

IAV have a negative-sense, single-stranded RNA genome consisting of 8 segments encoding for at least 17 viral proteins (1). Each segment is associated with the viral nucleoprotein (NP) and the three polymerase components, namely the polymerase basic protein 1 (PB1) and 2 (PB2) and the polymerase acidic protein (PA). These ribonucleoprotein complexes are encapsidated by the matrix protein 1 (M1) beneath an envelope composed of a lipid bilayer derived from the host plasma membrane where are embedded the surface glycoproteins HA, NA and the matrix protein 2 (M2). HA is responsible for the binding of the virus to sialic acid moieties at the host cell surface. HA is a trimeric glycoprotein and each monomer is composed of two domains, a globular head (HA1) and a stalk domain (HA2). HA1, exposed at the surface of the virion is subject to a high degree of antigenic variations. HA2, more conserved across IAV, is involved in various steps of the virus life cycle, including the fusion between the viral envelope and the endosomal host membrane. NA is a tetrameric glycoprotein which enzymatically removes sialic acid residues from the surface of infected cells, allowing the release of budding virions. M2 is a tetrameric protein acting as a proton-selective ion channel which triggers the uncoating of the viral

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Abbreviations: Ab, antibody; Ag, antigen; APC, antigen-presenting cell; ASC, antibody-secreting cell; BALT, bronchus-associated lymphoid tissues; BCR, B cell receptor; CCL, (C-C motif) ligand; CCR, (C-C motif) receptor; CCS, ceramide carbamoyl-spermine; cDC, conventional dendritic cell; c-di-AMP, cyclic-di-adenosine monophosphate; cGAMP, 2',3'-cyclic-di-guanosine monophosphate-adenosine monophosphate; CDN, cyclic di-nucleotides; CpG-ODN, synthetic oligodeoxynucleotides composed of unmethylated CpG motifs; CT, cholera toxin; CTA1, A1 subunit of the cholera toxin; CTA1-DD, CTA1 fused to a synthetic dimer of the Ig binding D domain (DD) from *Staphylococcus aureus* protein A; CTL, cytotoxic T lymphocyte; DC, dendritic cell; DDAB, dimethyldioctadecylammonium bromide; DLN, draining lymph nodes; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; DOTAP, dioleoyl-3-trimethylammoniumpropane; DPPC, dipalmitoyl phosphatidylcholine; FAE, follicle-associated epithelium; GC, germinal center; HA, hemagglutinin; HA1, globular head domain of hemagglutinin; HA2, stalk domain of hemagglutinin; HAI, hemagglutination-inhibition; HIV, human immunodeficiency virus; HPAIV, highly pathogenic avian influenza viruses; HTCC, *N*-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride; IAV, influenza A viruses; IBV, influenza B virus; IFN, interferon; Ig, immunoglobulins; IIV, inactivated influenza viruses; IL, interleukin; i.n., intranasal; ISCOM, immune stimulating complex; ISCOMATRIX, immune stimulating complex matrix; LAIV, live-attenuated influenza viruses; LPAIV, low pathogenic avian influenza viruses; LRT, lower respiratory tract; LT, *Escherichia coli* heat-labile toxin; M cells, microfold cells; M1, matrix protein 1; M2(e), (ectodomain of the) matrix protein 2; MALT, mucosa-associated lymphoid tissues; mDC, myeloid dendritic cell; MHC, major histocompatibility complex; moDC, monocyte-derived dendritic cell; MPLA, monophosphoryl lipid A; NA, neuraminidase; NAIP, nucleotide oligomerization domain-like receptor family, apoptosis inhibitory protein; NALT, nasopharynx-associated lymphoid tissues; NK, natural killer; NLR, nucleotide oligomerization domain-like receptor; NLRP3, nucleotide oligomerization domain-like receptor family, pyrin domain containing 3; NP, nucleoprotein; N$_{RSV}$, nucleoprotein of the respiratory syncytial virus; PA, polymerase acidic protein; PAMP, pathogen associated molecular pattern; PB1, polymerase basic protein 1; PB2, polymerase basic protein 2; pDC, plasmacytoid dendritic cell; PEI, polyethylenimine; PG, phosphatidylglycerol; γ-PGA, poly (γ-glutamic acid); PLGA, poly (D, L-lactide-co-glycolide); poly (I:C), polyinosine-polycytidylic acid; PRR, pattern recognition receptor; RT, respiratory tract; SP-C, surfactant protein C; STING, stimulator of interferon genes; Th, T helper; TLR, Toll-like receptor; TMC, *N*-trimethyl-derivatives of chitosan; TRIF, Toll or interleukin-1 receptor domain-containing adaptor-inducing interferon-β; T$_{RM}$, resident memory T cell; URT, upper respiratory tract; VLP, virus-like particle; WIV, whole inactivated viruses.

ribonucleoprotein complexes necessary for the release of the viral genetic material into the host cytosol. Unlike HA and NA, the ectodomain of M2 (M2e) is sparsely expressed at the surface of the virion, less subjected to the host immune pressure and consequently more conserved across IAV (1).

Circulating IAV are continuously evolving, leading to the emergence of new strains expressing surface glycoproteins that have distinct antigenic properties (1). In particular, point mutations in the viral genome RNA result in the emergence of new strains responsible for seasonal epidemics ("antigenic drift"), and the co-infection of a host with multiple IAV strains can result in genetic reassortments responsible for the emergence of novel subtypes ("antigenic shift") that can give rise to strains with pandemic potential. The disease severity caused by IAV infections depends on several parameters such as viral and host factors. In humans, the virus initially targets the mucosa of the upper RT (URT) (nose, pharynx), leading to dry cough, nasal discharge, rhinitis, pharyngitis and fever, and can eventually reach the lower RT (LRT) (trachea, bronchi, bronchioles, alveoli) resulting in fatal pneumonia in severe cases. Seasonal influenza infections, which are mainly caused by H1N1 or H3N2 IAV strains, are responsible for 3–5 million human cases of severe infections and 290,000–650,000 fatal cases annually, most often in young children, the elderly and immunocompromised individuals (2). Pandemic IAV infections affect a broader category of populations and cause atypical and more severe clinical symptoms (1). In aquatic birds, low pathogenic avian influenza viruses (LPAIV) typically cause asymptomatic infections. In poultry, infections with LPAIV can be asymptomatic or provoke low to mild pathophysiological damages to the respiratory, digestive and reproductive systems (resulting in a drop in egg production), while infections with highly pathogenic avian influenza viruses (HPAIV) are characterized by high morbidity and mortality rates. Outbreaks in domestic poultry result in massive culling to control the viral spread and are thus responsible for important economic losses (3).

THE MUCOSAL IMMUNE RESPONSES AGAINST IAV INFECTIONS

The host RT is not only the initial point of entry and replication of IAV, but also the site of the host immune defenses. Components of the antiviral immune responses are located in the RT lining fluids and in the mucosa-associated lymphoid tissues (MALT) (4). Broadly, in mammals, the MALT of the RT include the nasopharynx-associated lymphoid tissues (NALT) located at the entrance to the nasopharyngeal duct in the URT (Waldeyer's ring in humans), the bronchus-associated lymphoid tissues (BALT) randomly distributed along the LRT but most consistently located at sites of bronchial tree bifurcation, and the draining lymph nodes (DLN). The NALT and BALT are globally composed of organized inductive sites, comprising B cell follicles and interfollicular T cell areas, as well as effector sites, consisting of the diffuse tissue of the lamina propria. The

MALT of the RT are overlaid by a surface epithelium made of ciliated cells and various transporter cells located in the follicle-associated epithelium (FAE), such as microfold (M) cells, which transfer the luminal antigens (Ags) to the subepithelial cells (5). MALT are also detected in the avian RT (6). Over the last decade, considerable progress have been done in the understanding of the mechanisms of host mucosal immune responses against IAV (mostly in experimental mouse models of IAV infections).

At the first steps of infection, IAV must face host innate immune molecules with antiviral and/or immunomodulatory activities such as mucins, lectins, complement molecules, natural immunoglobulins (Ig) and antimicrobial peptides (7). Once the virus reaches and infects the epithelium, the local immune cells detect the viral components [also called pathogen-associated molecular patterns (PAMPs)] via pattern recognition receptors (PRRs) including Toll-like receptors (TLRs) (7). These cells produce various cytokines and chemokines involved in the antiviral defense and in the recruitment and activation of innate effector cells, which establish an antiviral program and prime adaptive immune responses.

Neutrophils, natural killer (NK) cells, and monocytes/macrophages interfere with viral replication and spread within the RT, and Ag-presenting cells (APCs) including dendritic cells (DCs) initiate specific adaptive immune responses (7–9). Murine DC subsets can be classified into $CD11c^{hi}$ conventional DCs (cDCs), $CD11c^{lo}B220^+$ plasmacytoid DCs (pDCs) and inflammation-induced $CD11b^+Ly6C^+$ monocyte-derived DCs (moDCs). In mice, after capturing IAV Ags in the MALT of the RT, respiratory DCs (including $CD103^+CD11b^{lo}$ and $CD103^-CD11b^{hi}$ cDC subsets) acquire a mature phenotype, transport viral Ags to the DLN and simultaneously process the Ags and present Ag-derived peptides on class I or II major histocompatibility complex (MHC) molecules to naive $CD8^+$ and $CD4^+$ T cells, respectively (10–14). Beside migratory cDCs, $CD8\alpha^+CD11b^-$ resident cDCs are also involved in the cross-presentation of IAV Ags to naive $CD8^+$ T cells in the DLN (10, 15). Following IAV infection in mice, moDCs accumulate in the RT and they promote immune-induced pathology. However, their complete elimination is detrimental because they facilitate the optimal expansion of effector $CD8^+$ T cells in the infected lung (16). Finally, pDCs are a potent source of type I interferons (IFNs), they are involved in the generation of virus-specific antibody (Ab)-secreting cells (ASCs) and they ensure a full-magnitude $CD8^+$ T cell response (17–19). Subsets of $CD11c^+CD103^{lo}$ myeloid DCs (mDCs), including $CD1c^+$ mDC1 and $CD141^+$ mDC2 (equivalent to $CD103^-CD11b^{hi}$ and $CD103^+CD11b^{lo}$ mouse cDC subsets, respectively), and pDCs have been identified in the human RT. Studies have suggested that mDCs and pDCs could traffic to the site of IAV infection, but the functionality of the human DC subsets during IAV infection remains largely unknown (20).

Following primary IAV infection in mice, activated $CD4^+$ and $CD8^+$ T cells are detected in the DLN and spleen and the most highly activated and divided cell populations migrate to the airways and notably to the lamina propria of the MALT (21, 22). Activated IAV-specific $CD8^+$ T cells differentiate into effector cytotoxic T lymphocytes (CTLs) which kill virus-infected cells (23, 24). Activated IAV-specific $CD4^+$ T cells differentiate into various subsets which present heterogeneous antiviral functionalities and organ location, including T helper 1 (Th1), Th17 cells, follicular helper $CD4^+$ T cells and $CD4^+$ CTLs (25–27). Studies in humans have mainly examined IAV-specific T cell responses in the peripheral blood. Secondary responses are generally observed because most adults have encountered IAV Ags multiple times during infections and/or vaccinations. In the absence of specific Ab responses to newly-emerged IAV, the presence of pre-existing circulating cross-reactive $CD4^+$ or $CD8^+$ T cells exhibiting cytotoxic activities correlates with protection against experimentally or naturally mild H1N1 or H3N2 infections in humans (28–30). A recent study showed that rapid and robust IAV-specific $CD8^+$ T cell recall responses correlated with early recovery of patients from severe H7N9 disease (30). Most T cell epitopes are highly conserved across IAV and are located on internal proteins such as NP, M1 or polymerase subunits, but they are also present in surface glycoproteins HA and NA, and in M2 (9, 28, 31–33).

Follicular helper $CD4^+$ T cells support the generation and maintenance of the germinal centers (GCs) in the B cell follicles in cooperation with follicular DCs (34). In GCs, activated B cells, which have received helper signals from cognate $CD4^+$ T cells, experience intense proliferation and undergo processes of class switch recombination, somatic hypermutation and affinity selection. GC reactions usually result in the generation of specific long-lived ASCs producing high affinity switched protective Abs and memory B cells. Activated B cells can also participate in early GC-independent reactions, mainly characterized by the rapid generation of low-affinity/specificity ASCs/memory B cells (34). Primary IAV infection in mice results in the development of specific ASCs which present site-specific kinetics and isotype distribution (35, 36). Anti-IAV Abs display a variety of functions depending on their isotype, specificity, affinity, concentration and post-translational modifications. Secretory IgA, which are polymeric IgA produced by IgA ASCs in the lamina propria and secreted to the mucosal surface, prevent infection of the epithelial cells via extracellular or intracellular immune exclusion. In addition, polymeric IgA can cross-react with IAV heterovariants (different IAV viruses belonging to the same subtype) or heterosubtypic strains (different IAV viruses belonging to different subtypes) (37). This breadth of reactivity could be linked to the avidity conferred by the polymeric form and/or the effect of the constant heavy chain in modulating the specificity/affinity of the variable regions (37, 38). IgA are thus an essential protective front line of defense against highly variable IAV. Whereas, IgG likely play a minor role in supporting secretory IgA in the prevention of IAV infection in the URT, they are crucially involved in the protection of the LRT in mice (39). In humans with low serum hemagglutination-inhibition (HAI) Ab titers, nasal and serum IgA provide protection against an i.n. experimental H1N1 challenge (40). However, the relative role of mucosal IgA vs. IgG in the (cross)-protection against IAV infection in humans remains poorly characterized.

IAV-specific Abs generated after an infection are mostly directed against the variable HA1 and, in a lesser extent, against NA (41). Anti-HA Abs mainly interfere with the virus infectivity by preventing virus binding to the sialic acid molecules and entry in epithelial cells. High HAI Ab titers are traditionally considered as the primary correlate of protection against IAV in humans and in animal models (1). Anti-NA Abs prevent the release of newly formed virions. Broadly neutralizing Abs directed against the conserved HA2 can occasionally be elicited in IAV-infected individuals and they interfere with the virus infectivity through several mechanisms (42). Besides neutralizing functions, IAV-specific Abs also exhibit Fc-related effector functions including Ab-dependent cellular cytotoxicity, Ab-dependent phagocytosis and Ab-dependent complement mediated-lysis (43–45). Abs directed against HA2, NA, M2e, NP, and M1 isolated from humans or animals have the potential to mediate protection via Fc-dependent mechanisms (43–45).

The clearance of a primary viral infection results in the establishment of long-lasting memory immune cells detectable in the MALT and in other lymphoid tissues, in the circulation and in the bone marrow. These cells play a critical role in the fight against reinfections. Among influenza-specific memory T cell populations, lung-resident memory $CD8^+$ T ($CD8^+$ T_{RM}) cells are crucially involved in the heterosubtypic cross-protection against pulmonary infection in mice (46, 47). Virus-specific $CD8^+$ T_{RM} cells can also be generated in the URT after IAV infection in mice. This cell population can efficiently clear a secondary heterosubtypic IAV infection from the URT and consequently blocks the spread of the virus from the URT to the LRT and the subsequent development of severe pulmonary diseases (48). IAV infection also stimulates the generation of specific lung $CD4^+$ T_{RM} cells in mice (49) and adoptive transfer experiments demonstrate that these cells have a better protective potential against homologous reinfection than memory $CD4^+$ T cells isolated from non-MALT locations (50). IAV-specific lung $CD8^+$ T_{RM} cells are also detected in humans but due to practical and ethical limitations, the implication of mucosal T_{RM} cells in the resistance against IAV reinfection in humans is largely unknown (49, 51).

Whereas, long-lived ASCs serve as an immediate first line of defense against IAV homologous reinfections by secreting Abs with high specificity/affinity, memory B cells are more specialized to respond to antigenically divergent viruses. In particular, recent studies in mice have shown that GCs in the BALT select cross-reactive B cell repertoire, resulting in the generation of tissue-resident broad reactive memory B cells (52). The presence of these memory B cell populations at the site of infection facilitates their direct contact with intact influenza virions and could promote a faster production of high-affinity (cross-reactive) Abs through T-independent and PRR-dependent pathways (34). Ab responses induced by natural IAV infections in humans are relatively broad and long-lived (41). However, further investigations are needed to localize and identify cell subsets involved in these memory cross-reactive humoral responses (41).

PROBLEMATIC ISSUES RELATED TO CURRENT IAV VACCINES

Vaccination remains the most efficient and cost-effective means to protect human and animal populations against IAV (1). Most influenza vaccines available on the market are inactivated influenza A virus (IIV) vaccines administered via the parenteral route. Three types of IIV vaccines exist, namely whole inactivated virus (WIV) vaccines consisting of formaldehyde- or β-propiolactone-inactivated whole virion, split virus vaccines, and subunit vaccines. In split virus vaccines, the virus envelope is broken by diethyl ether or detergent treatment that disrupts the particulate organization and exposes all viral proteins, while subunit vaccines consist of surface proteins HA and NA separated from the nucleocapsid and lipids. While WIV preparations are commonly used in human pre-pandemic vaccines and in poultry, current human seasonal vaccines are mainly split virus or subunit vaccines. Current IIV vaccines predominantly induce virus-specific Ab responses directed against HA1 and do not stimulate efficient cellular immune responses. Thus, the efficiency of these vaccines is restricted to the protection against homologous/antigenically similar strains (1).

Beside IIV vaccines, live-attenuated influenza virus (LAIV) vaccines administered via the intranasal (i.n.) route are also available in humans. LAIV vaccines are composed of cold-adapted virus strain(s) restricted to the URT and causing only mild symptoms (1). LAIV and IIV vaccines are equally effective in adults, while studies have concluded that LAIV vaccines are more efficient in children, generating broader and longer-lived immune responses. Indeed, LAIV vaccines mimic the natural route of infection and consequently induce stronger mucosal IgA and broader T cell-mediated immune responses than IIV vaccines (1). A recent study in mice comparing two different licensed influenza vaccines showed that, in contrast to IIV vaccines injected parenterally, i.n. LAIV vaccines elicited the generation of lung T_{RM} cells, conferring long-term protection against various non-vaccine strains (53). Despite very promising results, some drawbacks have been associated with the use of LAIV vaccines (1). A suboptimal protection of LAIV vaccines in children against the 2009 H1N1 pandemic virus has been reported in the USA, which might be due to an impaired viral replication in the URT, resulting in a decreased stimulation of the host (mucosal) immune system. Also, for safety concerns, LAIV vaccines are contraindicated in children <2 years old and in immunocompromised individuals. Finally, the virus strain(s) composing LAIV vaccines could theoretically undergo genetic reassortments with circulating wild type viruses (1).

Thus, due to its ability to generate broadly reactive and long-term protective immune responses at the front line of virus entry, vaccination via the mucosal route is an appropriate strategy for the prevention and control of IAV infections. However, current mucosal vaccines need to be reformulated into safer, more refined and immunogenic preparations. In order to counteract the high variability of IAV, an active area of research area focusing on developing subunit vaccine candidates containing conserved surface or internal protein(s)/epitope(s) of the virus has garnered

much interest. These "universal" vaccines aim at generating cross-reactive Abs and/or cross-reactive T cell responses (1). Alongside WIV, split or protein subunit vaccines, nucleic acid-based subunit vaccines induce vaccine Ag production in the host itself and can engage both humoral and cell-mediated immune responses (1, 31). Pre-clinical and clinical studies have shown that nucleic acid-based IAV vaccines can be efficiency delivered by live viral vectors through parenteral or mucosal routes and viral-vectored vaccines are commercially available for the control of avian IAV in poultry (54, 55). Nucleic-acid based vaccines delivered via non-lived vectors mimic infection or immunization with live microorganisms while being non-infectious, egg/cell-free manufacturable (ensuring a rapid, cost-effective and scalable production) and they do not have to address the challenge of preexisting or induced anti-vector immunity which could prevent repeated immunizations (56–58).

ADJUVANTS/DELIVERY SYSTEMS FOR INTRANASAL VACCINATION AGAINST IAV INFECTIONS

The goal of vaccination is to generate potent and long-term protective immune responses against infections. Unlike attenuated live vaccines, inactivated vaccines (especially purified or recombinant subunit vaccines) usually require additional compounds to be effective. The role of an adjuvant is to increase vaccine efficacy by modeling the quality and the quantity of the host immune responses. In particular, the adjuvant can affect the magnitude, breadth, specificity, affinity, kinetics, longevity, and composition of the immune responses (59–61). The incorporation of adjuvant(s) into a vaccine formulation can decrease the amount of the vaccine Ag(s) and the number of doses required to induce protective immunity, and enhance vaccine efficacy in special populations (the elderly, neonates/infants, immunocompromised individuals) (59–61). Adjuvants were formerly classified as delivery systems, which function as vehicles to which the vaccine Ag(s) can be associated, and immune potentiators. However, there is now evidence that some delivery systems also exhibit immunostimulatory properties. Adjuvants exert their effect through various mechanisms including the formation of a depot at the site of injection, which guarantees a slow release of the vaccine Ag(s) and a constant stimulation of the host immunity (depot effect), the recruitment of immune cells, the activation of innate immune receptors, the enhancement of Ag uptake by APCs and the activation and the maturation of APCs (59–61). By targeting innate immune cell subsets and by generating a particular cytokine milieu (Th1, Th2, and/or Th17 for example), adjuvants can shape the nature of the subsequent adaptive immune responses to produce the most effective and protective reaction against a given pathogen.

Despite the relevance of the mucosal route of vaccination in the fight against respiratory pathogens, this method of administration faces several hurdles (62). In addition to encountering a tolerogenic mucosal environment, vaccine Ag(s) must resist degradation caused by the harsh mucosal environment characterized by the presence of proteases, nucleases and low pH. In addition, the Ag(s) is (are) likely to be diluted in the mucosal fluids and swept away by the mucociliary clearance. Consequently, mucosal vaccines have to be carefully formulated with adjuvants in order to breach the host mucosal barriers and allow the vaccine Ag(s) to reach and stimulate the cells of the underlying MALT (62). Mucosal adjuvants should ensure the integrity and the stability of vaccine Ag(s), exhibit mucoadhesive properties and allow Ag uptake by epithelial cells and/or M cells. The rationale and challenges for the development of IAV mucosal vaccines are presented in **Figure 1**.

In this section, we have compiled the delivery/adjuvant systems tested for i.n. administration of IIV vaccines with a special emphasis on experimental animal studies (see **Supplemental Table 1**). We have focused our work on non-replicative delivery/adjuvant systems (excluding viral and bacterial vectors).

Bacterial Enterotoxins and Derivatives

The most potent and studied mucosal adjuvants in pre-clinical studies are cholera toxin (CT), the closely related *Escherichia coli* heat-labile toxin (LT) and their derivatives (detoxified/non-toxic holotoxins, isolated subunits). CT and LT holotoxins are composed of a pentameric B subunit, which binds to GM1-ganglioside receptors ubiquitously present on the surface of most nucleated cells, non-covalently associated with an A subunit. The A1 portion of the A subunit enzymatically ADP-ribosylates the α subunit of the GTP-binding protein Gs (Gsα), causing a dramatic elevation of intracellular cyclic AMP responsible for the efflux of ions and water from the targeted cells associated with watery diarrhea. The mucosal adjuvant properties of enterotoxins rely on a better accessibility of the co-administered Ag(s) to the cells of the MALT, which is related to an increased permeability of the mucosal barriers and/or to an enhancement of the recruitment and activation of local APCs (63).

Experimental studies in mice have demonstrated that these holotoxins are potent immunostimulators for mucosal IIV vaccines (64, 65). For example, the addition of CT to H1N1 WIV vaccine administered to mice via the i.n. route enhanced the magnitude of the serum and mucosal Ab responses directed against homologous, H1N1 heterovariant and H3N2 heterosubtypic viruses (65). CT increased the breadth of protection of the vaccine and all mice receiving the adjuvanted formulation survived the i.n. H3N2 heterosubtypic challenge (65). The adjuvant also stimulated the generation of long-lived ASCs lodged in the bone marrow that secreted IgA and IgG reactive against the homologous and the H3N2 heterosubtypic viruses (65). Holotoxins engineered into less toxic molecules, through site-directed mutations of the enzymatically active A subunit, and administered with influenza WIV or subunit vaccines also retained adjuvant functions (66, 67). However, the i.n. delivery of vaccines formulated with native or even detoxified LT in humans resulted in the development of facial paralysis (Bell's palsy) in some individuals, stopping further clinical use of enterotoxin-based adjuvants (63).

Enterotoxins lacking B subunit are good alternatives to i.n. holotoxin-based adjuvants because they keep strong adjuvant

FIGURE 1 | Rationales and challenges for the development of IAV mucosal vaccines.
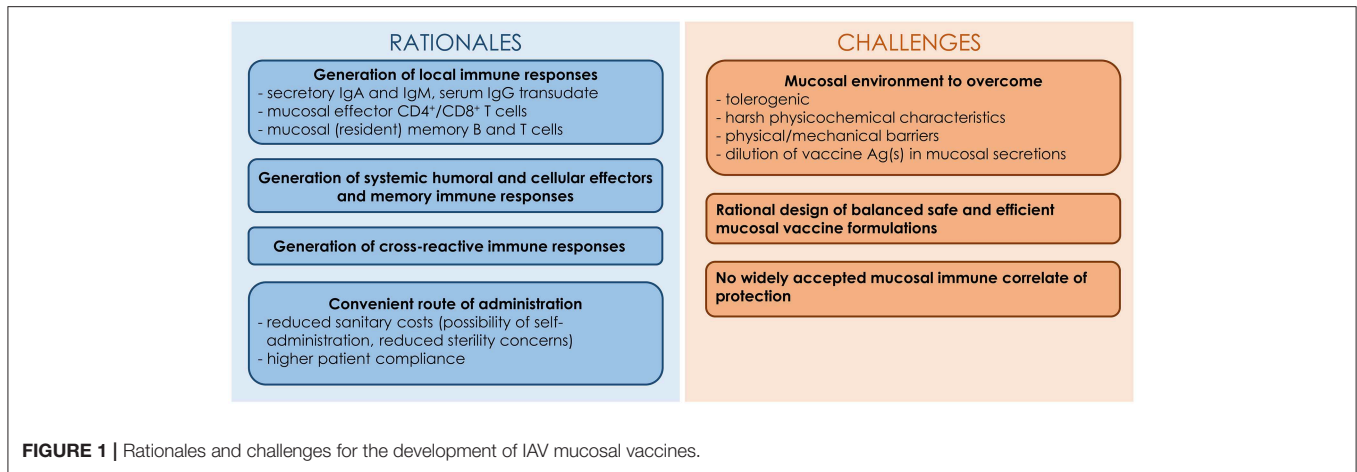
functions without toxic side effects (68, 69). For example, a study in mice evaluated the ability of the A1 subunit of the CT (CTA1) to potentiate the immunogenicity of a subunit vaccine composed of a portion of HA2 of a H5N1 virus [$HA2_{H5N1(15-137)}$] fused with an M2 consensus sequence spanning the residues of the extracellular and cytoplasmic domains of M2 from H1N1, H5N1 and H9N2 viruses ($consM2_{H1N1/H5N1/H9N2}$) (69). The fusion of CTA1 with the $consM2_{H1N1/H5N1/H9N2}$-$HA2_{H5N1(15-137)}$ chimeric protein increased the magnitude and persistence of M2/HA2-specific serum and mucosal humoral responses and M2/HA2-specific cellular immune responses in the spleen. Mice immunized with the CTA1-adjuvanted vaccine showed a better resistance to an i.n. challenge with divergent subtypes of IAV (H1N1, H5N1, H5N2, H7N3, H9N2) than mice immunized with the vaccine without CTA1 (69).

The chimeric protein composed of CTA1 fused with a synthetic dimer of the Ig binding D domain (DD) from *Staphylococcus aureus* protein A (CTA1-DD) is also a promising adjuvant for next generation mucosal vaccines against IAV. The DD portion of the molecule targets the B cell receptor (BCR) as well as the complement receptor CD21 on follicular DCs, resulting in an enhancement of GC formation (63, 70). In addition, experiments in mice and non-human primates have proven the safety of CTA1-DD (63). In pre-clinical tests, CTA1-DD increased the immunogenicity and/or the protective potential of various influenza subunit vaccines administered intranasally, either in fusion with M2e epitopes (71, 72), admixed with virus-like particles (VLPs) exposing M2e epitopes (73), or incorporated into lipid-based particles with IAV Ags (70). For example, the incorporation of CTA1-DD into immune stimulating complexes (ISCOMs) containing H1N1 HA and NA boosted the specific serum and mucosal humoral responses as well as type 1/type 2-cell-mediated immune responses in the spleen in mice. The immunostimulatory effects of CTA1-DD depended both on the enzymatic activity of the toxin and on B cell targeting (70). These encouraging results make CTA1-DD a very attractive adjuvant for mucosal anti-IAV vaccines for clinical or veterinary use (63).

Flagellin

Flagellin, the primary structural component of the bacterial flagellum, is another promising mucosal adjuvant for anti-IAV vaccines. Flagellin targets TLR5 expressed at the surface of various cells including airway epithelial cells, DCs and lymphocytes, as well as cytosolic detectors of the NAIP (nucleotide oligomerization domain-like receptor (NLR) family, apoptosis inhibitory protein) family proteins (74). The mucosal adjuvant properties of flagellin rely on the acceleration of the transepithelial transport of the co-administered Ag(s) by the FAE and on the stimulation of the migration of subepithelial DCs into the FAE (75).

The i.n. co-administration of recombinant flagellin derived from *Vibrio vulnificus* or *Salmonella typhimurium* with H1N1 WIV vaccine or a trivalent split vaccine composed of two IAVs (H1N1 and H3N2) and an influenza B strain (IBV) boosted the immunogenicity of the vaccine in mice (76, 77). In particular, the adjuvant increased the serum and/or mucosal Ab responses and elicited systemic type 2-biased-cell-mediated immune responses (76, 77). The incorporation of flagellin into the vaccine preparation enhanced protection of mice against a subsequent i.n. homologous challenge (76, 77). The adjuvant exhibited a safer profile than enterotoxins because it did not accumulate in the central nervous system (77).

Flagellin is also a potent mucosal adjuvant for influenza subunit vaccines (78–80). The association of flagellin with IAV epitopes, as free fusion proteins or as membrane-anchored form into influenza VLPs, significantly increased the immunogenicity and the (cross)-protective potential of these vaccines against IAV in mice (78–80). For example, the membrane incorporation of flagellin into H1N1 (HA/M1) VLPs enhanced serum and mucosal Ab titers and elicited systemic type 1/type 2-cell-mediated immune responses specifically directed against the homologous H1N1 strain (78). Moreover, the addition of flagellin to the vaccine formulation induced higher cross-neutralizing Ab titers to a H3N2 virus detectable in the serum and in the lung lavage (78). Accordingly, mice immunized with the adjuvanted vaccine were all resistant to the i.n. H3N2 heterosubtypic challenge in contrast to the unadjuvanted group (78). Another *in vitro* study

showed that the conjugation of flagellin on the surface of gold nanoparticles bearing a recombinant trimeric HA derived from a H3N2 virus stimulated the uptake of the vaccine by murine bone marrow-derived DCs and promoted the activation and the maturation of the cells (81). Mice intranasally immunized with such flagellin-adjuvanted vaccine developed higher influenza-specific immune responses and showed a better resistance against an i.n. homologous challenge (81, 82).

Interestingly, flagellin also significantly boosted the immunogenicity and the protective capacity of IIV vaccines in chickens, and notably when it was admixed with H5N2 WIV vaccine or fused with an epitope located in HA1 of a H7N9 virus (83, 84).

Clinical trials have proven the safety and the immunogenicity of flagellin-adjuvanted influenza subunit vaccines when administered via the parenteral route (85, 86). Further clinical investigations on the efficacy of this adjuvant in mucosal anti-IAV vaccination are needed.

Proteosome-Based Adjuvants

Proteosomes and Protollin are two other potent mucosal immunoactivators. Proteosomes are nanoparticles composed of a mixture of outer membrane proteins and traces of lipooligosaccharides derived from *Neisseria meningitidis* and Protollin consist of proteosomes complexed with *Shigella flexneri* lipopolysaccharides. These adjuvants stimulate Ag uptake by the FAE and cells of the MALT, and promote the maturation and activation of APCs via the engagement of various PRRs (87). Pre-clinical tests in mice concluded that proteosomes and Protollin were safe and strong mucosal adjuvants for split (88, 89) or subunit (89, 90) IAV vaccines. In addition, recent clinical trials showed that an i.n. proteosome-adjuvanted trivalent split vaccine was safe, immunogenic, and effective in healthy adults (91). The vaccination protected subjects against illness following an experimental H3N2 homologous challenge, and the protection was correlated with pre-challenge specific serum HAI Ab and nasal IgA titers (91). Thus, this class of adjuvants deserves to be harnessed for the development of mucosal anti-IAV vaccination.

Bacterial-Derived Vesicles

Bacterium-Like Particles

Bacterium-like particles, also known as Gram-positive enhancer matrix, are non-living microparticles derived from non-pathogenic food-grade bacteria *Lactococcus lactis*, and consist of bacterial-shaped peptidoglycan spheres deprived of intact surface proteins and intracellular content. The immunostimulating properties of bacterium-like particles are related to their ability to activate and stimulate the maturation of APCs (92). In mice, bacterium-like particles admixed with H1N1 or H3N2 split vaccine enhanced serum and mucosal humoral responses as well as systemic type 1-biased cellular responses directed against the vaccine strain (93, 94). Moreover, the presence of the particles in the vaccine formulation significantly increased the level of protection of mice against an i.n. homologous challenge (94). The adjuvanticity of bacterium-like particles was suggested to be TLR2-dependent (95). Successful pre-clinical tests were also

reported using bacterium-like particles non-covalently coupled with purified influenza epitopes (HA, M2e or NP) (92).

Finally, a phase I clinical trial has proven the safety and the immunogenicity of i.n. vaccine formulations composed of seasonal influenza trivalent split vaccine mixed with bacterium-like particles (92). In particular, the adjuvant boosted the specific serum HAI Ab and nasal IgA titers and increased the frequency of IFN-γ-producing peripheral blood mononuclear cells (92).

Outer Membrane Vesicles

Outer membrane vesicles are nanosized vesicles naturally produced by Gram-negative bacteria (96). They are composed of various PAMPs such as lipopolysaccharides, lipoproteins, peptidoglycan, flagellin monomers and nucleic acids, and they can stimulate the activation of respiratory CD103$^+$ DCs (96). The incorporation of outer membrane vesicles into a trivalent split vaccine boosted influenza-specific serum IgG and HAI Ab titers as well as IgG and IgA titers in the LRT in mice receiving the vaccine formulation via the i.n. route (96). In addition, the adjuvant stimulated type 1-cell-mediated immune responses in the lungs and spleen (96). All mice survived an i.n. homologous challenge and were significantly more resistant to H1N1 heterovariant or H5N2 heterosubtypic challenges than mice immunized with the unadjuvanted vaccine (96).

Lipid-Based Adjuvants

Liposomes

Liposomes are self-assembling bi- or multi-layered lipid vesicles with an aqueous core ranging from 10 nm to several μm in diameter (97). Various categories of lipids can be incorporated into liposomes such as phospholipids, sterols (cholesterol) or sphingolipids. Liposomes are common vaccine delivery vehicles because they protect the associated Ag(s) from the degradation or neutralization, they exhibit Ag depot effect and they are uptaken by APCs (97). Several physicochemical features of the liposomal formulations determine their immunomodulatory potentials including the lipid characteristics and the lipid composition of the liposomes (97). For example, the length and the degree of saturation of the hydrophobic tail of the lipids govern the fluidity/permeability and thus the stability of the particles. Cholesterol commonly modulates the stability of liposomes. In addition, the hydrophilic headgroups of the lipids determine the surface charge and, by extension, the ability of the liposomes to adhere/penetrate epithelial surfaces (in the context of a mucosal vaccination). The localization of the vaccine Ag(s) in the preparation, e.g., admixed with the liposomes, attached to the surface of the liposomes or encapsulated in the aqueous core of the liposomes, also influences the Ag-specific immune responses. Finally, the adjuvanticity of the liposomes can be improved by the incorporation of muco-adhesive/muco-penetrating polymers or PRR ligands into the formulation (97).

Both pre-clinical and clinical trials have demonstrated the remarkable adjuvant potential of liposomes in parenteral vaccination against IAV. Encouraging results have also been obtained in the context of mucosal vaccination (97, 98). Early studies showed that anionic dimyristoyl phosphatidylcholine (DMPC)/dimyristoyl phosphatidylglycerol (DMPG) liposomes

facilitated the association and uptake of co-administered molecules by macrophages *in vitro* (99). The co-encapsulation of H1N1 split vaccine in DMPC/DMPG liposomes with synthetic oligodeoxynucleotides composed of unmethylated CpG motifs (CpG-ODN, TLR9 ligands) boosted the magnitude of virus-specific IgG and IgA titers in the URT and LRT and induced systemic type 1-biased cell-mediated immune responses in intranasally immunized mice (99). In addition, mice receiving the liposome-formulated vaccine showed reduced lung viral loads after an i.n. heterovariant challenge (99). CAF01, another liposomal preparation containing the monocationic lipid dimethyldioctadecylammonium bromide (DDAB) and the immunostimulator α,α'-trehalose 6,6'-dibehenate was effectively uptaken by murine bone marrow-derived DCs *in vitro* and stimulated the maturation of the cells (100). By using the human bronchial epithelial Calu-3 cell culture model, CAF01 was shown to enhance the transport of co-administered Ag through the mucus layer and across the epithelial cells (101). Accordingly, in mice, the i.n. administration of CAF01 with H3N2 (100) or seasonal trivalent (101) split vaccines remarkably strengthened virus-specific mucosal and systemic immune responses. Finally, polycationic liposomes composed of ceramide carbamoyl-spermine (CCS) sphingolipids complexed with cholesterol enhanced the efficacy of H1N1 monovalent or seasonal trivalent split vaccines (102, 103). Mice intranasally immunized with the vaccine adjuvanted with CCS/cholesterol liposomes showed an increase in serum and mucosal HAI Ab titers directed against homologous or heterovariant viruses (102, 103). In addition the adjuvant increased type 1-cell-mediated immunity in the spleen (102, 103). Finally, the animals exhibited a better resistance to i.n. homologous or heterovariant challenges than mice immunized with the unadjuvanted vaccine (103). CCS/cholesterol liposomes presented similar adjuvant effects to CT, while having a better safety profile (103). Interestingly, CCS/cholesterol liposomes had stronger adjuvant properties than anionic DMPC/DMPG or monocationic dioleoyl-3- trimethylammoniumpropane (DOTAP)/cholesterol liposomes, which could be linked to an extended retention time of influenza Ags in the nasal cavity and in the lungs (102, 103). Finally, CCS/cholesterol liposomes also enhanced the immunogenicity of H1N1 split vaccine in aged mice, albeit with lower specific serum and mucosal Ab titers than those observed in adult mice (103). Clinical trials with i.n. liposome-based influenza split vaccine have been reported (97, 104).

Some studies have demonstrated that liposomes are also potent mucosal carriers for influenza subunit vaccines in mice (105–107). For example, an M2e consensus sequence incorporated into the lipid bilayer of liposomes composed of a mixture of phospholipids, cholesterol and monophosphoryl lipid A (MPLA) generated M2e-specific serum IgG and systemic type 1/type 2-cell mediated immune responses in mice vaccinated subcutaneously and intranasally (107). The animals were also significantly protected against an i.n. homologous challenge (107). Interestingly, the M2e-specific serum Ab response inhibited *in vitro* viral cell lysis by various IAV subtypes including H2N2, H3N2, H6N2, H5N9, and H1N1 IAV, highlighting the potential of liposomes as

mucosal delivery platforms for broadly-protective vaccines against IAV (107).

Preclinical and clinical studies have also concluded that cationic liposomes are attractive delivery platforms for nucleic-acid based IAV vaccines. Cationic lipids are known to efficiently complex the nucleic acids, facilitate cellular uptake and allow endosomal escape of nucleic acids into the host cytoplasm (57). The encapsulation of plasmid DNA encoding a HA protein derived from a H1N1 strain into cationic liposomes administered to mice via the i.n. route boosted the immunogenicity of the vaccine and all animals survived a subsequent i.n. homologous challenge unlike mice vaccinated with the naked plasmid DNA (105). Over the past decade, major technological advances have enabled mRNA vaccines to become promising candidates against IAV infections (57, 58, 108). In contrast to plasmid DNA, mRNA-based vaccines are delivered directly to the cytoplasmic site of function, eliminating the potential risk of integration into the host chromosome. The engineering of mRNA sequence and the development of separation and/or purification techniques can increase mRNA translation and stability and modulate its inherent immunogenicity (57, 58, 108). In addition, several delivery systems have been explored to improve the efficiency of mRNA vaccines, including liposomes (109). These carriers are usually composed of an ionizable cationic lipid, cholesterol, phospholipids, and lipid-linked polyethylene glycol which increases the half-life of the formulation (109). Liposome-encapsulated mRNA vaccines encoding IAV Ags such as HA, NP and/or M1 administered via intramuscular or intradermal routes generated significant B and/or T cell immune responses and conferred protection against i.n. homologous or heterosubtypic IAV challenge in various experimental animal models including mice, ferrets and/or non-human primates (110–112). A recent clinical study showed that mRNA vaccine encoding a HA protein derived from H10N8 or H7N9 viruses formulated with liposomes and administered via the intramuscular route was safe and immunogenic (111). While lipid nanoparticles are potent delivery systems for nasal mRNA vaccines in the context of anti-tumor vaccination (113), additional studies are needed to evaluate the efficacy of such carriers in mucosal vaccination against IAV.

Virosomes

Virosomes are a special category of liposomes which are composed of purified or synthetic lipids and viral envelope proteins such as HA and NA ("influenza virosomes") (98). Influenza virosomes are commercially available, safe and efficient vaccine platforms for parenteral vaccination in humans. However, there have been no new i.n. formulations on the market since the withdrawal of LT-adjuvanted virosomal vaccines (98). Among various strategies to potentiate the immunogenicity of i.n. virosomal vaccines against IAV, the incorporation of cyclic di-nucleotides (CDN) (see section Nucleotide-Based Adjuvants) has proven to be valuable in pre-clinical tests (114). Mice immunized with H5N1 virosomes admixed with cyclic-di-adenosine monophosphate (c-di-AMP) developed stronger, broader and more persistent anti-IAV immune responses than mice immunized with the unadjuvanted virosomes (114). In

particular, the animals showed higher mucosal IgA titers and higher serum HAI Ab titers reactive against homologous and heterovariant viruses. Moreover, the adjuvant increased the frequency of long-lived IgG ASCs lodged in the bone marrow. Mice also exhibited stronger systemic type 1/type 2/type 17 cellular immune responses, including higher frequency of multifunctional influenza-specific CD4+ T cells in the spleen (114). Finally c-di-AMP promoted protection of mice against i.n. homologous challenge (114). Clinical trials with virosome-based influenza vaccines administered via the nasal route are ongoing (98, 115).

Immune Stimulating Complexes (ISCOMs)/ Immune Stimulating Complex Matrix ISCOMATRIX

ISCOMs are negatively-charged pentagonal dodecahedrons with a ring-like stable structure about 40 nm in diameter which are spontaneously formed after mixing the vaccine Ag(s) with cholesterol, phospholipids and saponins from the *Quillaja saponaria* Molina tree (QuilA) which are potent immunostimulators (98). The corresponding structure without incorporated vaccine Ag is called ISCOMATRIX. ISCOM/ISCOMATRIX adjuvants are efficiently processed by various APCs and are thus widely-exploited vaccine delivery systems. The high affinity between saponins and cholesterol ensures the stability of the adjuvant (116). Vaccine formulations with more refined fractions of QuilA saponins have been developed in order to increase the safety and the tolerogenicity of the adjuvant (117). Pre-clinical studies have shown that this category of adjuvants markedly increases the immunogenicity and the protective potential of i.n. split or subunit IIV vaccines, with the viral Ags incorporated into ISCOMs or simply admixed with the ISCOMATRIX (70, 117, 118). Notably, mice intranasally immunized with an ISCOM/ISCOMATRIX-formulated H1N1 split vaccine presented higher nasal/lung IgA and serum IgG titers than mice vaccinated with the non-adjuvanted vaccine (118). Two other studies indicated that mice receiving envelope Ags derived from H1N1 virus in ISCOM/ISCOMATRIX formulations via the i.n. route exhibited stronger serum and mucosal Ab responses as well as higher systemic type 1/type 2 cellular immunity than mice receiving the free Ags (70, 117). The immunogenicity of ISCOM/ISCOMATRIX preparations can be boosted by the inclusion of immunostimulators (117). The addition of CTA1-DD into ISCOM/ISCOMATRIX formulations resulted in generating attractive and versatile mucosal delivery platforms for IAV Ags by targeting both DCs and B cells (117). Of note, a CTA1-3M2e-DD/ISCOM preparation could be kept 1 year at 4°C or as freeze-dried powder without altering the immunogenicity of the vaccine (117). ISCOMs are thus promising adjuvant for the development of cold chain-independent vaccines. Finally we can mention that ISCOMATRIX is also a suitable mucosal adjuvant for H5N1 WIV vaccines in chickens (119). Clinical trials with i.n. ISCOM-based influenza vaccines are currently under way (98, 116).

Other Lipid-Based Adjuvants

Surfacten is a modified bovine pulmonary surfactant forming unilamellar vesicles about 300–1,000 nm, which is widely used in premature babies with respiratory distress syndrome without significant adverse effects. It also displays potent mucosal adjuvant properties toward influenza split vaccines in mice (120, 121). More precisely, Surfacten enhanced the uptake of influenza Ags by bone marrow-derived DCs *in vitro*, and increased the distribution and the retention time of influenza Ags as well as the maturation of CD11c+ cells in the nasal cavity *in vivo* (120, 121). The adjuvant boosted both virus-specific local and systemic humoral and cell-mediated immunity and enhanced the protection of mice against i.n. homologous or heterovariant challenge (120, 121). Three major lipids, namely dipalmitoyl phosphatidylcholine (DPPC), phosphatidylglycerol (PG) and palmitate, and the surfactant protein C (SP-C) play an essential role in the adjuvant properties of Surfacten (121).

SF-10, a synthetic surfactant composed of DPPC/PG/palmitate lipids, a cationic SP-C-related peptide and a mucoadhesive carboxyvinyl polymer (CVP) is also a safe and effective delivery vehicle for IAV split vaccines in mice and non-human primates (122–124). *In vivo* experiments in mice showed that SF-10 stimulated the delivery of co-administered Ags to epithelial cells and APCs localized in the NALT, including CD8+CD11c+ DCs known to be involved in the cross-priming of CD8+ T cells (123). Accordingly, the i.n. administration of SF-10 with H1N1 split vaccine strengthened virus-specific serum and mucosal Ab responses and T cell-mediated responses (122, 123). In particular, mice immunized with the SF-10-supplemented vaccine showed higher frequency of IFN-γ- and IL-4-secreting lymphocytes in the NALT and CD8+ CTLs in the spleen (122, 123). These mice were also more resistant to an i.n. heterovariant challenge than those immunized with the unadjuvanted vaccine, and the protection was significantly reduced after depletion of CD8+ or CD4+ T cells (123). The resistance was associated with higher cytolysis activities in the lungs and DLN in the early phase of infection (123). Finally, young cynomolgus monkeys immunized with H1N1 split vaccine admixed with SF-10 via the i.n. route developed humoral immune responses composed of nasal Abs which cross-reacted against H1N1 heterovariant and H3N2 heterosubtypic viruses, and serum HAI Abs (124). Moreover, the animals exhibited influenza-specific immunological memory (124). SF-10 is thus a very promising adjuvant for i.n. influenza vaccines intended for humans, and in particular for young children.

Endocine is an anionic adjuvant based on mono-olein and oleic acid lipids found ubiquitously in the human body. Clinical vaccine studies against diphtheria and the human immunodeficiency virus (HIV) have proven that Endocine is a safe and well-tolerated i.n. adjuvant (125). Several studies have demonstrated that it is also a promising mucosal adjuvant for IAV vaccines (125–127). The addition of Endocine to an i.n. split trivalent vaccine enhanced both local and systemic cross-reactive humoral and cell-mediated immune responses in adult mice (126). The adjuvant is also a potent immunoenhancer in immunocompromised mice (127). Aged mice immunized with an Endocine-adjuvanted H1N1 split vaccine exhibited increases in influenza-specific serum IgG and HAI Ab titers and lung IgG and IgA titers (127). Finally, ferrets intranasally immunized with H1N1 split or WIV vaccine formulated with Endocine

developed serum HAI and neutralizing Ab responses against homologous and H1N1 heterovariant viruses (125). Animals challenged intratracheally with the homologous strain were significantly protected from virus replication in the URT and LRT (125). Further (pre-)clinical investigations on the use of Endocine in i.n. IIV vaccine formulations are warranted.

Among other lipid-based adjuvants which have proven to be successful in mucosal vaccination against IAV in (pre)-clinical tests, we can cite the oil-in-water nanoemulsion $W_{80}5EC$, consisting of a highly refined soybean oil combined with non-ionic and cationic surfactants and ethanol. *In vitro* and *in vivo* studies in mice suggested that the adjuvanticity of $W_{80}5EC$ was related to its ability to increase the uptake of the antigenic payload by nasal epithelial cells, facilitating the subsequent engulfment of Ag-primed apoptotic epithelial cells by DCs, followed by activation and migration of DCs to the DLN (128, 129). The adjuvant activity of $W_{80}5EC$ is related, at least in part, to TLR2/TLR4 activation (130, 131). Accordingly, $W_{80}5EC$ enhanced the immunogenicity and the protective potential of i.n. H1N1 WIV vaccine in mice (132). The i.n. administration of $W_{80}5EC$ with a seasonal IAV split vaccine also boosted serum humoral responses directed against homologous and heterovariant viruses in ferrets (133). Finally, a clinical study conducted with healthy human adults revealed that $W_{80}5EC$ was safe, well-tolerated and boosted the influenza-specific serum and mucosal Ab responses when combined intranasally with a seasonal trivalent split vaccine (134). A recent study mentioned that an mRNA vaccine expressing a HA protein derived from a H1N1 virus formulated with an oil-in-water cationic nanoemulsion elicited broad and protective immune responses against homologous and heterologous challenge when delivered intramuscularly to mice or ferrets (135). However, the efficiency of such formulations in the context of a mucosal immunization remains to be evaluated.

Virus-Like Particles (VLPs)

VLPs are multimeric cage-like structures consisting of self-assembled structural viral proteins around a hollow interior space devoid of viral genetic material. VLPs mimic virions in shape, size, and molecular organization while being non-replicating and non-infectious (136, 137). VLPs can interact with APCs of the innate immunity. In addition, VLPs display highly repetitive epitopes and can stimulate B cell responses by BCR cross-linking (136).

Several pre-clinical studies have demonstrated that VLPs are potent i.n. delivery vehicles for IAV epitopes (79, 138). Influenza VLP vaccines are mostly produced by the recombinant baculovirus/insect cell expression system and consist of self-assembled M1 molecules surrounded by the lipid membrane derived from the insect cells where various combinations of influenza epitopes are anchored, including HA, NA and/or M2(e) (136, 137). Most of influenza VLPs fully protect animals against an i.n. homologous challenge, and several studies have reported their efficacy against heterologous viruses as well (136, 137). For example, the i.n. immunization of mice with VLPs incorporating H1N1 HA, NA and M1 [(HA/NA/M1) VLPs] induced the generation of IgG and IgA in the serum, the URT and LRT, which

cross-reacted with a H5N1 virus (139). Mice were fully protected against a subsequent i.n. homologous challenge and exhibited a partial but significant resistance to a challenge with the H5N1 virus (139). In contrast, all mice receiving the vaccine via the intramuscular route succumbed to the heterosubtypic challenge. Ferrets intranasally immunized with the same vaccine survived the H5N1 challenge and displayed reduced nasal viral loads, unlike animals vaccinated via the parenteral route (139).

Numerous pre-clinical studies have proven that i.n. influenza VLP vaccines elicit long-lasting (cross-) protective immune responses (140–142). For example, long-lived IgA/IgG ASCs and memory $CD4^+/CD8^+$ T cells were detected in the bone marrow and in the spleen of mice immunized with H1N1 (HA/M1) VLPs, respectively (140). All mice survived an i.n. homologous or heterovariant challenge up to 5 months post-immunization (140). Also, a recent study showed that the i.n. administration of a mixture of (HA/M1) VLPs individually displaying H1, H3, H5, and H7 HA epitopes significantly protected adult mice against an i.n. homologous (H1N1 or H7N1 virus), heterovariant (H5N1 or H7N9 virus), or heterosubtypic (H2N1, H7N1, H10N1, or H11N1 virus) challenge (142). The VLP cocktail protected mice against a challenge with the H7N9 or H10N1 virus until 6 months post-vaccination. Passive serum transfer experiments suggested an involvement of anti-HA Abs in the resistance against homologous or heterovariant challenge; however the correlates of protection against the heterosubtypic challenge remain unknown (142). Interestingly, a partial protection was also observed in aged mice against the H10N1 challenge (142).

Other VLP platforms presenting conserved IAV epitopes (M2e or epitopes located in HA2) have been successfully tested in pre-clinical tests with the hepatitis B virus core protein (73, 143, 144), the coat protein of bacteriophage Qβ (Qβ-VLPs) (145), the protrusion domain of the capsid protein of norovirus (P-VLPs) (138, 146) or the NP of respiratory syncytial virus (N_{RSV}-VLPs) (147) as VLP scaffold.

Experimental studies in mice have allowed a better understanding of the adjuvant mechanisms of Qβ-VLPs at the mucosal surfaces (148, 149). During the self-assembly process, Qβ-VLPs are packaged with RNA derived from *E. coli* which typically mediate TLR3/7 signals. A first study concluded that Qβ-VLPs applied via the i.n. route were captured by alveolar macrophages and lung DCs, transported to the DLN and subsequent mucosal IgA responses required macrophage/DC activation via TLR7 stimulation (148). A second study established that Qβ-VLPs administered intranasally could also be taken up by the lung B cells, transported to the spleen and delivered into B cell follicles (149). In accordance with these results, mice intranasally immunized with Qβ-VLPs incorporating M2e epitopes developed significant mucosal and serum M2e-specific humoral responses, and all animals survived a subsequent i.n. lethal H1N1 challenge (145). Besides Qβ-VLPs, P-VLPs are also efficient mucosal delivery platforms for M2e epitopes in mice (138) and in chickens (146). Chickens intranasally immunized with P-VLPs carrying multiple repetitions of an avian M2e consensus sequence showed a significant reduction in virus shedding from the trachea and the cloaca after an i.n. challenge with avian H5N2 or H7N2 viruses (146). However, no anti-M2e

Ab were detected in the serum or in the nasal washes and the protective immune mechanisms remain unknown. Finally, in our research group, we have demonstrated that mice intranasally immunized with three repetitions of M2e sequence from a H1N1 virus exposed at the surface of N_{RSV}-VLPs developed strong local and systemic M2e-specific Ab responses and were successfully protected against an i.n. homologous challenge (147). Only the i.n. route generated mucosal IgA titers and led to the protection of animals (147).

Although most of the aforementioned influenza VLPs trigger potent host immune reactions by themselves, several studies have indicated that the incorporation of immunostimulating molecules such as enterotoxins (64, 73), PRR ligands (78, 79, 81, 82, 150) or cytokines/chemokines (151), either admixed or anchored at the surface of VLPs, enhances the magnitude, the duration and/or the breadth of the protective immune responses.

Clinical trials have attested the safety and the efficacy of influenza VLPs in parenteral vaccination (136). The promising results obtained in mucosal vaccination during experimental animal studies support the rationale for developing influenza VLPs for human mucosal vaccination as well.

Organic Polymers
Chitosan
Chitosan are natural cationic polysaccharides composed of glucosamine and N-acetylglucosamine resulting from the partial deacetylation of chitin (152). Diverse forms of chitosan are available with various degrees of acetylation, molecular weight and chemical modifications affecting their solubility, biocompatibility, biodegradation and immunogenicity (152). Chitosan polymers and their derivatives have been widely used as mucosal adjuvants under different formulations such as solutions, powders, micro/nanoparticles and gels (152). Because of electrostatic interactions of positively charged chitosan with the negatively charged components of the mucus, chitosan polymers exhibit strong mucoadhesive properties. In addition, chitosan-based adjuvants enhance the permeability of the epithelial barrier by inducing a reversible opening of tight junctions, and consequently promote the interactions of the co-administered Ag(s) with the MALT. Finally, chitosan polymers directly stimulate the activation and the maturation of APCs including DCs via TLR4-, NLR family pyrin domain containing 3 (NLRP3)-, and/or stimulator of interferon genes (STING)-dependent pathway (152, 153).

Chitosan-based adjuvants have successfully improved the immunogenicity and the protective potential of i.n. influenza split vaccines in pre-clinical tests (154–157). For example, N-trimethyl-derivatives of chitosan (TMC) boosted the immunogenicity of H1N1 split vaccine reflected by increased serum IgG and HAI Ab titers and mucosal IgA titers (154). An optimal immune response was obtained when influenza Ags were conjugated on TMC nanoparticles instead of being encapsulated into the TMC nanoparticles (154). In another study, a thermo-sensitive chitosan-based hydrogel (a solution of N-[(2-hydroxy-3-trimethylammonium) propyl] chitosan chloride (HTCC) plus α,β-glycerophosphate) formulated with H5N1 split vaccine extended the retention time of influenza

Ags in the nasal cavity of mice (156). This could be due to the mucoadhesive properties of HTCC and/or the reduction of the ciliary beating frequency caused by the overlaying of epithelial surface with the semi-solid hydrogel solution (156). In addition, the adjuvant efficiently increased the intercellular penetration of influenza Ags through the mucosa (156). Consequently, mice receiving the hydrogel-adjuvanted vaccine presented a boost in influenza-specific serum and mucosal Ab titers as well as in cell-mediated immune responses, including an increase in type 1/type 2 cellular responses in the spleen and in the frequency of memory $CD8^+$ T cells in the NALT (156). TMC admixed with H5N1 split vaccine also boosted serum Ab titers reactive against the vaccine strain and H5N1 heterovariant viruses in ferrets and the adjuvanted vaccine protected animals against a subsequent intratracheal homologous challenge (157).

Pre-clinical experiments in mice and chickens have also revealed that chitosan polymers are efficient mucosal adjuvants for DNA (158) or protein influenza subunit vaccines either admixed with influenza Ags (144, 159) or as coating agents on the surface of polyamines (69, 160) or polyesters (161, 162) nanoparticles containing influenza Ags (see sections Chitosan and Polyamine polymers). Chitosan-containing lipid nanoparticles complexed with mRNA encoding H5 HA or NP proteins also boosted the immunogenicity of IAV Ags after subcutaneous immunization in mice and rabbits (163). These experiments have to be performed in the context of a mucosal vaccination.

Clinical studies have proven the safety and the adjuvant potential of chitosan for various i.n. vaccines including IIV vaccines, but further investigations are needed (164, 165).

Polyamine Polymers
Poly (γ-glutamic acid) (γ-PGA) polymers are anionic biodegradable bacterial polymers with mucoadhesive properties due to the hydrogen bonding between the carboxylate group of γ-PGA and the hydroxyl group of the mucus glycoproteins (166). In addition, γ-PGA nanoparticles are efficiently taken up by APCs and they enhance the activation, the maturation and the functions of APCs (167).

Experiments in mice have demonstrated that γ-PGA polymers are potent mucosal adjuvants/carriers for split or subunit influenza vaccines (69, 160, 167, 168). For example, a first study established that the supplementation of an i.n. H1N1 split vaccine with γ-PGA nanoparticles enhanced serum and mucosal neutralizing Ab responses and type 1/type 2-cell mediated immune responses in the spleen (168). These responses were specifically directed against the vaccine strain or a heterovariant virus (168). Mice immunized with the adjuvanted vaccine were fully resistant to i.n. homologous or heterovariant challenge (168). In a second study, the incorporation of consM2$_{H1N1/H5N1/H9N2}$-HA2$_{H5N1(15-137)}$ into a formulation consisting of γ-PGA polymers associated with MPLA and QS21 (a specific fraction of QuilA saponins) generated higher and more persistent HA2/M2-specific immune responses than a free consM2$_{H1N1/H5N1/H9N2}$-HA2$_{H5N1(15-137)}$ fusion protein vaccine (167). The adjuvanted vaccine conferred a full and/or long-lasting protection to mice against an i.n. challenge with different

subtypes of IAV including H5N1, H1N1, H5N2, or H9N2 viruses (167). Notably, the adjuvant effect of the γ-PGA/MPLA/QS21 formulation was similar as CT (167).

Polyethyleneimine (PEI) polymers are a family of synthetic cationic molecules widely used as gene transfer agents because of their ability to complex, condense and protect nucleic acids against enzymatic degradation, to promote transfection of cells and to facilitate endosomal escape of nucleic acids (166). *In vivo* bioluminescence imaging of luciferase performed in mice after the i.n. administration of luciferase-encoding DNA formulated with PEI or unformulated DNA revealed that PEI significantly improved the efficiency of gene transfer in the RT (169). Accordingly, PEI efficiently boosted the magnitude, the breadth and the protective potential of influenza DNA vaccines administered via the i.n. route in mice (169). In particular, the combination of PEI with a DNA vaccine encoding a HA derived from a H5N1 virus increased the serum and mucosal Ab responses against the vaccine strain (169). Furthermore, the animals presented higher frequency of HA-specific memory IFN-γ-secreting CD4$^+$ and CD8$^+$ T cells in the lungs and spleen than mice vaccinated with the naked DNA (169). In accordance with these observations, mice immunized with the adjuvanted vaccine were more resistant to an i.n. homologous challenge (169). Finally, the PEI-complexed vaccine generated cross-reactive immune responses and conferred partial cross-protection to mice against an i.n. H5N1 heterovariant challenge (169). A recent study has demonstrated that PEI conjugated with β-cyclodextrin (a cyclic polysaccharide) is an excellent platform material for i.n. mRNA vaccination in mice (170). *In vivo* imaging showed that the complexation of anionic mRNA encoding the envelope glycoprotein gp120 of HIV with the PEI-cyclodextrin polymer prolonged the nasal residence time of the mRNA and increased uptake of the mRNA by nasal epithelial cells (170). In addition, *in vitro* observations suggested that the polymer facilitated the i.n. delivery of the mRNA cargo through a paracellular route by reversibly opening tight junctions while preserving the integrity of the epithelial barrier (170). Consequently, this polymeric delivery system boosted the gp120-specific serum and mucosal humoral and cellular responses (170). In mice, PEI is a potent adjuvant for subcutaneous or intramuscular mRNA vaccines encoding IAV HA or NP Ags (171, 172). However, the ability of PEI to transport IAV mRNA vaccines through the mucosal route awaits further investigation.

Likewise, PEI polymers are potent mucosal adjuvants for influenza WIV and protein subunit vaccines in mice and chickens, with similar potency as bacterial-derived adjuvants (84, 173). *In vitro* analysis showed that the combination of PEI with H9N2 WIV vaccine increased the adhesion of the viral Ags to Calu-3 epithelial cells (173). In addition PEI enhanced the cellular uptake and endosomal escape of the viral Ags in murine bone marrow-derived DCs, and stimulated the maturation of the cells (173). Accordingly, mice intranasally immunized with the PEI-complexed WIV vaccine developed higher local and systemic influenza-specific immune responses than mice immunized with the uncomplexed WIV vaccine (173). Finally, a recent study demonstrated (172, 173) that chickens immunized with a portion of the HA1 domain of a H7N9 virus formulated with PEI

developed higher serum, nasal and lung anti-HA Ab titers than animals immunized with the unadjuvanted formulation (84). The birds also showed reduced viral loads in the cloaca and throat after an i.n. homologous challenge in comparison with the birds of the unadjuvanted group (84).

Because of safety issues related to the non-biodegradable nature of PEI and to the positive charge of these polymers (which can interact electrostatically with cellular anionic macromolecules and interfere with normal cellular functions), less toxic PEI derivatives have been developed such as deacylated PEI. These modified PEI polymers retain adjuvant properties toward HA DNA vaccines in mice (174).

Polyesters
PLGA, a copolymer of poly (D, L-lactide-co-glycolide) approved for clinical use, has been widely exploited as a nanoparticle delivery system because of its biocompatibility, biodegradability, safety, and controlled release properties (166). Hydrophilic polymeric materials can be added on the surface of PLGA nanoparticles to increase the stability and the transfer of the antigenic payload across mucosal surfaces. These stabilizers are usually polyethylene glycol, polyvinyl alcohol or chitosan (175). Experimental studies in animals have demonstrated that PLGA nanoparticles are good mucosal adjuvants for i.n. IAV WIV vaccines (162, 176). A first study showed that chickens immunized with an aerosol vaccine composed of H9N2 WIV admixed with polyvinyl alcohol-modified PLGA nanoparticles incorporating PEI-CpG-ODN complexes generated higher virus-specific serum IgY and HAI Ab titers and mucosal IgA titers than birds immunized with the non-adjuvanted WIV vaccine (176). A second study concluded that chickens immunized via i.n. and intraocular routes with polyvinyl alcohol/chitosan-modified PLGA nanoparticles encapsulating a H4N6 WIV with PEI-CpG-ODN complexes developed higher influenza-specific serum and mucosal Ab responses than chickens of the unadjuvanted group (162).

Poly-(ε-caprolactone), another biodegradable and biocompatible polyester polymer, was also described as a potent carrier system for mucosal vaccines against IAV in pre-clinical tests (161). Notably, mice intranasally vaccinated with chitosan-coated poly-(ε-caprolactone) nanoparticles incorporating a recombinant HA derived from a H1N1 virus exhibited higher HA-specific serum and mucosal Ab titers and systemic type 1/type 2-cell-mediated immune responses than mice vaccinated with uncoated nanoparticles or with the unadjuvanted recombinant HA (161).

Other Organic Polymers
Poly (*N*-vinylacetamide-*co*-acrylic acid) (PNVA-co-AA) bearing D-octaarginine are biocompatible cationic oligopeptides which enhance permeation of co-mixed Ag(s) through mucosal barriers (177). The co-administered Ag(s) are suggested to be taken up into cells via macropinocytosis through the biorecognition of the peptidyl branches in the polymer backbone, while the polymer remains on the cell membrane (177). The i.n. co-administration of H1N1 WIV vaccine with D-octaarginine-linked PNVA-co-AA

in mice boosted the HA-specific nasal IgA titers which cross-reacted *in vitro* with recombinant HAs from H1N1 heterovariant and H3N2 or H5N1 heterosubtypic viruses (178, 179). Mice vaccinated with the adjuvanted preparation showed a better resistance to an i.n. homologous challenge than mice immunized with H1N1 WIV alone (178, 179). The cross-protective potential of such polymers remains to be evaluated *in vivo*.

Inorganic Nanoparticles

During the last years, gold nanoparticles are appearing increasingly attractive (mucosal) vaccine delivery vehicles (180). As immunologically inert molecules, they do not induce competing carrier-specific immune responses. Also, they can be chemically synthesized with tight control over the nanoparticle size and are easily functionalized with vaccine epitopes/immunopotentiators (180). A series of recent publications has demonstrated that gold nanoparticles are potent mucosal delivery platforms for influenza Ags in mice (81, 82, 181, 182). By designing dual-linker nanoparticles, a research group grafted a recombinant trimeric HA derived from a H3N2 virus and a recombinant flagellin monomer on gold nanoparticles by click chemistry and metal-chelating reactions with high conjugation efficiency (81, 82). The conjugation of the vaccine epitopes on the nanoparticles increased the uptake of influenza Ags by DCs and stimulated the activation, maturation and function of DCs *in vitro* (82). With respect to these observations, the vaccine formulation intranasally administered to mice generated stronger influenza-specific immune responses than a vaccine composed of free trimeric HA mixed with flagellin (81). Notably, mice immunized with the nanoparticles showed elevated serum IgG and HAI Ab titers, higher IgG and IgA titers in the URT and LRT and higher frequency of long-lived IgG/IgA ASCs in the spleen (81). In addition, animals exhibited higher influenza-specific systemic and local cell-mediated immune responses. In particular, mice showed a boost in type 1/type 2/type 17-cell-mediated immune responses and in the frequency of CD8$^+$ CTLs in the spleen, an increase in type 1-biased cellular responses in the NALT and higher frequency of IFN-γ-expressing CD4$^+$/CD8$^+$ T cells in the DLN (81). All mice survived a lethal i.n. homologous challenge and showed reduced lung viral loads in contrast to animals receiving the vaccine formulation uncoupled to the nanoparticles (81). The attachment of a human M2e consensus sequence to gold nanoparticles also significantly strengthened the immunogenicity and the protective potential of the M2e epitopes in mice (181, 182). Hence, these nanocarriers are interesting mucosal delivery platforms for broadly-protective vaccines against IAV.

Nucleotide-Based Adjuvants

Polyinosine-polycytidylic acid [poly (I:C)] is a synthetic analog for double-stranded RNA that mimics viral RNA and activates various APCs in a TLR3/Toll or interleukin (IL)-1 receptor domain-containing adaptor-inducing interferon-β (TRIF)-dependent pathway (61). Numerous studies have demonstrated that the incorporation of poly (I:C) into IAV WIV, split or subunit vaccines enhanced the magnitude and/or the breadth of the specific immune in mice and chickens (183–186).

A recent study showed that the i.n. administration of poly (I:C) with H1N1 split vaccine in mice boosted serum IgG and mucosal IgA Ab responses in a TLR3/TRIF-dependent pathway (183). The adjuvant also increased the frequency of virus-specific IFN-γ-secreting CD4$^+$ and CD8$^+$ T cells in the spleen (183). The mucosal adjuvanticity of poly (I:C) relied on its ability to stimulate the activation and the maturation of CD103$^+$ DCs located in the NALT, which were crucially involved in the generation of local humoral and T cell-mediated immune responses (183). In particular, the addition of poly (I:C) to the vaccine formulation enhanced the frequency of GC B cells and follicular helper T cells in the NALT and the local expression of various cytokines involved in IgA class switching (183). Mice immunized with the adjuvanted vaccine were fully protected against an i.n. H1N1 heterovariant challenge in contrast to TRIF$^{-/-}$ mice or mice immunized with the unadjuvanted vaccine (183).

Poly (I:C) was also tested in a HA2-directed vaccine strategy in mice (185). The methodology consisted of sequential immunizations with different chimeric HA molecules which have the same H1 HA2 but different non-H1 HA1s. The adjuvant boosted the induction of HA2-specific serum IgG and nasal IgA directed against the HA vaccine strain (185). In addition the adjuvant enhanced the generation of serum Abs which cross-reacted with HA molecules from H1N1 heterovariant and H5N1 and H2N2 heterosubtypic viruses (185). The adjuvanted vaccine fully protected mice against i.n. homologous and H1N1 heterovariant challenges (185).

We can mention that poly (I:C) is also an interesting mucosal adjuvant for influenza vaccines in chickens (186). The i.n. co-administration of poly (I:C) with H5N1 IIV vaccine enhanced influenza-specific serum IgG and HAI Ab titers and IgA titers in the nose and in the trachea of birds (186).

Finally, poly (I:C$_{12}$U), an analog of poly (I:C) exhibiting a safer profile, gave promising results in pre-clinical (187) and clinical trials (188). In particular, the i.n. immunization of healthy humans with a seasonal LAIV vaccine in association with poly (I:C$_{12}$U) induced the generation of nasal IgA which reacted with homologous viruses as well as H5N1, H7N9, and H7N3 HPAIV with pandemic potential for humans (188).

Another class of nucleotide-based adjuvant, CDN, exhibited comparable efficacy to poly (I:C) in enhancing the immunogenicity of mucosal IAV split or subunit vaccines in mice (114, 189–192). CDN are bacterial second-messenger molecules detected by the innate immune system via various sensors including STING (189). The inclusion of 2′,3′-cyclic-guanosine monophosphate-adenosine monophosphate (cGAMP) in i.n. H1N1 split vaccine stimulated the activation of innate and adaptive immunity in the NALT, the spleen and the DLN, and promoted GC formation in the NALT in a STING-dependent pathway (189). It resulted in an increase in specific serum and mucosal humoral responses and in systemic type 1/type 2/type 17 cellular immune responses, including higher frequency of IFN-γ-producing CD4$^+$ and CD8$^+$ T cells in the spleen (189). Other studies demonstrated that the addition of CDN to recombinant H5N1 HA or H1N1 NP subunit vaccines enhanced the magnitude and/or the breadth of the

humoral and cellular anti-IAV immune responses in mice (190, 192).

CpG-ODN are TLR9/TLR21 agonists which activate various APCs and commonly generate pro-inflammatory and Th1-biaised immune milieu (193). These compounds have thus been widely exploited as vaccine enhancers in numerous pre-clinical and human clinical trials (193). Several studies in mice and chickens have demonstrated that CpG-ODN alone or in combination with other immunopotentiators/delivery systems are suitable mucosal adjuvants for IAV WIV, split or subunit vaccines (64, 83, 99, 176, 181, 182, 186, 194–197). By using an *in vitro* DCs:Calu-3 cells co-culture model and by doing i.n. instillation in mice *in vivo*, a recent study proposed a mechanism for the mucosal adjuvanticity of CpG-ODN toward a H9N2 WIV vaccine. The authors suggested that CpG-ODN stimulated the secretion of the chemokine (C-C motif) ligand 20 (CCL20) by epithelial cells in a TLR9-dependent pathway, which enhanced DC recruitment to the nasal epithelium and promoted the formation of transepithelial dendrites involved in the capture of luminal influenza Ags (197). Clinical trials have proven the safety and immunogenicity of CpG-ODN-adjuvanted IIV vaccines when administered via the parenteral route (85). In view of the very promising results in animal models, the efficacy of CpG-ODN in mucosal anti-IAV vaccination should be evaluated in humans.

Cytokines/Chemokines

Cytokines and chemokines are signaling molecules released by a wide range of immune and non-immune cells (198). They are critically involved in host defense against infections by modulating the activation, maturation, differentiation, migration, survival and functions of innate and adaptive immune cells (198). These molecules have been considered as interesting alternatives to pathogen-derived adjuvants for anti-IAV vaccines (198).

Various cytokines such as IL-2, IL-12, IL-1 family cytokines (IL-1α/β, IL-18, and IL-33), IL-23 and type I IFNs are efficient and safe mucosal adjuvants when admixed with IAV WIV, split or subunit vaccines in mice (198, 199). For example, the incorporation of type I IFNs to an i.n. H1N1 split vaccine enhanced the magnitude and the duration of virus-specific serum and mucosal humoral responses and increased the protection of mice against an i.n. homologous challenge (200). The mucosal adjuvanticity of type I IFNs was suggested to be related to the ability of the cytokines to stimulate the uptake of co-administered Ags by phagocytes of the nasal mucosa (200). In another study in mice, the i.n. co-administration of cytokines belonging to the IL-1 family with recombinant HA derived from a H1N1 virus increased the magnitude of serum and mucosal anti-HA Abs titers in serum and in mucosal secretions as well as the frequency of type 1/type 2 cytokine-producing cells and CD8^{+} CTLs in the spleen (201). The adjuvant remarkably enhanced the level of protection of mice against an i.n. heterovariant challenge (201). Mucosal vaccine formulations with cytokines/chemokines anchored into influenza WIV or VLPs have also been successfully tested in mice (151, 202–204). The incorporation of cytokines/chemokines in nanoparticle vaccine formulations could overcome the relatively short half-life of these molecules (198). Recent studies have been focused on CCL28, which is highly expressed by epithelial cells and selectively attracts T and B cell subsets (including IgA ASCs) at mucosal surfaces (151, 203). CCL28 anchored in H3N2 (HA/M1) VLPs showed *in vitro* chemotactic activity toward chemokine (C-C motif) receptor 3 (CCR3)/CCR10-expressing mouse splenocytes and lung cells (203). In mice, the chemokine enhanced the magnitude, the avidity, the functionality and the duration of serum and mucosal humoral responses specifically directed against homologous and heterovariant viruses (151, 203). Accordingly, the adjuvanted vaccine conferred a higher and more persistent protection in animals against an i.n. homologous or heterovariant challenge (151, 203).

Although cytokines/chemokines are promising adjuvants for mucosal anti-IAV vaccines, these molecules may present some drawbacks (198). The pleiotropic effects of these proteins on immune cells may result in unwanted adverse effects. In addition, the adjuvant effects observed in animal models may not be directly translated to humans as it was reported for an i.n. influenza split vaccine formulated with type I IFNs (205).

Adjuvants and Trained Immunity

Studies listed so far in this review have evaluated the efficacy of mucosal adjuvants on their ability to enhance adaptive immune responses (T and/or B cell responses) specifically directed against influenza Ags (**Supplemental Table 1**). However, a growing body of evidence suggests that some categories of adjuvants may also induce long-lasting functional state within innate immune cells, resulting in an increase in non-specific host defense against a range of pathogens. This concept has been named "trained immunity" (206). Some studies have indicated that prophylactic mucosal administration of bacterial-derived molecules, in particular enterotoxins, TLR3 and TLR9 ligands, or type I IFNs protected mice against a subsequent IAV challenge (73, 207–209). For example, mice intranasally treated with CT, LT (R192G) or CpG-ODN showed reduced mortality and/or lung viral loads subsequently to a H1N1 challenge 24 h after the last treatment. The improved resistance was associated with higher frequencies of CD4^{+} T cells, B cells and DCs in the airways and the generation of BALT-like structures in the lungs (207). Furthermore, the i.n. administration of chitosan fully protected mice against lethal challenge with H7N9 or diverse H1N1 viruses (210). The protection was associated with infiltration of leukocytes in the bronchoalveolar lavage and an enhanced expression of inflammatory cytokines in the LRT (210). Mice were significantly protected against H7N9 challenge even 10 days after the chitosan administration (210). Whether the protective efficacy of the mucosal IAV vaccine formulations compiled in this review was influenced by trained immunity remains to be determined.

CONCLUSIONS

Vaccination via the i.n. route has the potential to elicit long-lasting and cross-protective humoral and cellular immune responses at the portal of entry of respiratory pathogens. It is thus a suitable strategy to prevent infections caused by highly variable IAV. However, the i.n. vaccine formulations have to be rationally elaborated because the respiratory mucosal surfaces restrain the immunoavailability and the immunogenicity of vaccine Ag(s). Numerous experimental animal studies have demonstrated the promising potential of various adjuvant/delivery systems for the development of i.n. human and veterinary (chickens) vaccines against IAV. They consist of immunopotentiatory molecules, such as bacterial-derived components, synthetic nucleotides and cytokines/chemokines, associated or not with particulate carriers, such as lipid-based particles, VLPs, organic polymers and inorganic nanoparticles. These adjuvant systems boost the efficacy of current IIV vaccines or new subunit vaccine candidates by increasing the magnitude, the persistence and/or the breadth of the host (protective) anti-IAV immunity. Some of these novel mucosal vaccine formulations are safe and immunogenic in early phase clinical trials but they have to overcome several hurdles before reaching the market, including regulatory and economic restrictions and the lack of appropriate correlates of protection. Standardized assays taking into account the protective role of non-HAI Abs and cell-mediated immunity need to be developed to adequately evaluate the efficacy of these new mucosal formulations. Needle-free mucosal vaccines that provide a broad-coverage against IAV and do not require annual re-vaccination are promising alternatives to current IAV vaccines and should increase awareness about the benefits of influenza vaccination among the general public.

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SUPPLEMENTARY MATERIAL

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Supplemental Table 1 | Adjuvants/delivery systems tested in intranasal vaccination against IAV infections in pre-clinical and clinical studies.

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