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REVIEW

Mucosal delivery of nanovaccine strategy against COVID-19 and its variants



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Liposome

Abstract Despite the global administration of approved COVID-19 vaccines (*e.g.*, ChAdOx1 nCoV-19®, mRNA-1273®, BNT162b2®), the number of infections and fatalities continue to rise at an alarming rate because of the new variants such as *Omicron* and its subvariants. Including COVID-19 vaccines that are licensed for human use, most of the vaccines that are currently in clinical trials are administered *via* parenteral route. However, it has been proven that the parenteral vaccines do not induce localized immunity in the upper respiratory mucosal surface, and administration of the currently approved vaccines does not necessarily lead to sterilizing immunity. This further supports the necessity of a mucosal vaccine that blocks the main entrance route of COVID-19: nasal and oral mucosal surfaces. Understanding the mechanism of immune regulation of M cells and dendritic cells and targeting them can be another promising approach for the successful stimulation of the mucosal immune system. This paper reviews the basic mechanisms of the mucosal immunity elicited by mucosal vaccines and summarizes the practical aspects and challenges of nanotechnology-based vaccine platform development, as well as ligand hybrid nanoparticles as potentially effective target delivery agents for mucosal vaccines.

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1. Introduction

The number of COVID-19 infections and fatalities has rapidly increased worldwide, and the World Health Organization (WHO) declared it as a global pandemic on March 11, 2020. More than 386,548,962 cases of infection, including 5,705,754 deaths, have been reported till February 1, 2022. These numbers continue to increase at an alarming rate¹. Considering its long-lasting, expanding socioeconomic impact on global society, a novel strategy and convenient global distribution of vaccines are imperative².

COVID-19 belongs to the genus *Betacoronavirus* of the *Coronaviridae* family, which comprises enveloped, single positive-strand RNA viruses infecting the respiratory system, with a genome size of 26–32 kb³. Under an electron microscope, the virus envelope expresses a club-shaped glycoprotein projection, exhibiting the shape of the solar corona. The genome of COVID-19 encodes four structural proteins, spike (S), envelope (E), membrane (M), and nucleocapsid (N) proteins, in addition to 16 nonstructural proteins (NSPs)⁴. In the early stages of infection, the receptor-binding domain (RBD) of the S1 subunit of the S protein binds to angiotensin-converting enzyme 2 (ACE2) in the host cell membrane, which subsequently leads to the fusion of the virus with the membrane through the S2 subunit, followed by clathrin-mediated endocytosis and dynamin-independent endocytosis⁵. As ACE2 is expressed on the membrane of the vascular endothelium, respiratory epithelium, alveolar monocytes, and macrophages, COVID-19 tends to exhibit a tropism toward such cells⁶. Typically, during viral infection, entry of the viral particles into the epithelial cells of the airway leads to the activation of cytoplasmic RNA sensors and subsequent activation of transcription factors, such as TNF-receptor associated factor (TRAF) 3 and TRAF6⁷. This results in mediating the activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B) and interferon regulatory factor (IRF) 3 and IRF7 and subsequently activates the antiviral response against the virus⁷. However, COVID-19 suppresses the activation of TRAF3/6, thereby suppressing the antiviral response⁷. Consequently, the viral particles can readily replicate in the respiratory epithelial cells without hindrance by the immune system. Excessive replication of the virus triggers cell death that results in the exposure of the viral particles and cell debris with the subsequent activation of inflammatory cytokines⁸ (type 1 IFN, IL-1 β , IL-6, TNF- α) and the recruitment of immune cells like neutrophils, macrophages, and monocytes. Furthermore, induction of the IgG antibodies against COVID-19 can cause additional cell damage, which is known as the “antibody-dependent enhancement (ADE) effect”⁹. The immune complex formed by viral particles and IgG antibody bind to Fc γ R of macrophages and induces the production of inflammatory cytokines that can lead to further destruction of the affected organ¹⁰. Thus, viral replication in the immune-suppressed alveolar epithelial cells coupled with the recruitment of activated inflammatory cytokines and immune cells is the key immunological pathophysiology of COVID-19.

The clinical features of COVID-19 are known to be the consequence of the massive immune response caused by the damage to the alveolar epithelial cells and vascular endothelial cells. The most common symptoms at the onset of the disease include fever, cough, fatigue, myalgia, and dyspnea¹¹. However, the clinical manifestations range from mild pneumonia to critical conditions such as acute respiratory failure, systemic shock, or multi-organ failure¹². Although ~80% of the patients exhibit mild

symptoms (with mild or no pneumonia)¹³, 20%–30% of patients who undergo hospitalization with pneumonia require intensive care for respiratory support, which can rapidly exacerbate into severe hypoxia, acute respiratory distress syndrome (ARDS), and conditions requiring mechanical ventilation^{14,15}. Patients can also exhibit extra-pulmonary symptoms such as thrombotic complications, myocardial dysfunction, coronary syndrome, gastrointestinal symptoms, neurologic illness, and dermatologic complications¹⁶.

The major route of transmission of COVID-19 is respiratory droplets (>5–10 μ m in diameter) that enter through the mucosal membranes of the nose, eyes, and mouth. Abundant expression of ACE2 in the nasal and oral mucosa further supports its mode of transmission and the resulting pulmonary and gastrointestinal symptoms^{17,18}. As asymptomatic carriers of COVID-19 are able to transmit the virus, policy measures such as social distancing and travel restrictions have been implemented to curb the spread of the virus. However, these methods are temporary, and it is important to enhance and boost the immune system to combat the virus regardless of exposure, which necessitates the development of a vaccine against COVID-19¹⁹.

In-depth research and understanding of the pathology and molecular biology of COVID-19 have stimulated the rapid development of therapeutics and vaccines against COVID-19. Currently, ten vaccines, including CoronaVac®, ChAdOx1 nCoV-19®, mRNA-1273®, and BNT162b2®, have been approved as COVID-19 vaccines for human use and have entered phase 4 clinical trials for their verification and monitoring of efficacy and safety²⁰. Despite the global vaccine administration, there are still several challenges in achieving the global control of COVID-19. To enable successful usage of vaccines and increase their accessibility worldwide, four aspects should be fulfilled: development and production, affordability, allocation, and deployment¹⁹. After proving their efficacies, the vaccines are affordably produced on a large scale; then, they are broadly distributed and strictly stored for easy administration. However, the lack of COVID-19 vaccine supply due to the limited manufacturing capacity, difficulty of universal allocation, scarcity of storage facilities, lack of medical infrastructure for the vaccination, in addition to hesitancy and doubts toward vaccination, are hampering the administration in the global population¹⁹. In this aspect, a needle-free formulation that enables the mucosal administration of the vaccine can provide safe, convenient, low-cost, and accessible vaccines. Needle-free non-invasive formulations enable low-cost mass production. Moreover, the ease of administration enhances patients' compliance by enabling the patient to self-inoculate the vaccine without visiting the hospital²¹. In addition, needle-free mucosal vaccines eliminate the risk of infections caused by the needle. However, among the 170 vaccine candidates that entered clinical trials until August 2022, only 20 vaccines can be administered *via* the needle-free mucosal route, five vaccines *via* the oral route, 12 vaccines *via* the intranasal route, one vaccine *via* aerosol, and two vaccines *via* inhalation. Approximately 154 vaccines are administered systematically; five *via* the subcutaneous route, nine *via* the intradermal route, and 140 *via* the intramuscular route. The fact that most vaccines depend on injection implies the urgent need for the development of needle-free vaccines to enhance the accessibility of vaccines and thus achieve herd immunity.

The effectiveness of needle-free mucosal vaccines is not limited to practical and economic aspects but has advantages in

inducing both local mucosal and systemic immunity. As the primary entrance route of COVID-19 infection is through the mucosal membranes of the nose or mouth, and owing to the abundant expression of ACE2 in the nasal and oral mucosa, blockage of the viral entry through the immune induction of the local mucosa is crucial¹⁷. Moreover, immunization through the mucosal route (intranasal or oral vaccine) is able to elicit a strong mucosal antibody response along with the systemic immune response^{22,23}; thus, mucosal vaccines can be an effective strategy for sterilizing immunity and prophylaxis against COVID-19.

Despite its practical and clinical importance, there are several challenges facing the development of mucosal vaccines. To deal with those challenges, great effort has been made to develop platforms that induce potent mucosal immunity without being inactivated by the harsh mucosal environment. In this regard, nanotechnology is now being employed as an effective delivery platform to protect vaccine antigens from degradation and selectively deliver antigens to the mucosal immune system. The stability of nanotechnology-based formulations, encapsulation of antigens with adjuvants, and suitability of attaching various ligands or receptors enable the induction of mucosal immunity. Thus, the main aim of this review was to describe the basic mechanisms of mucosal immunity elicited by mucosal vaccines and summarize the practical aspects and challenges of developing nanotechnology-based vaccine platforms, as well as ligand hybrid nanoparticles as potentially effective target delivery agents for mucosal vaccines.

2. Currently available COVID-19 vaccine platforms

2.1. Overview of COVID-19 vaccine platforms in clinical trials

For the successful development of vaccines against COVID-19, the selection of a suitable antigen, delivery agent, adjuvant, and route of administration is crucial²⁴. In the aspect of vaccine antigens, S protein, a key molecule for viral entry, is the only surface protein of coronaviruses, making it an attractive target for vaccine development against COVID-19. Antibodies targeting the S protein, especially the receptor-binding domain (RBD), can not only neutralize the virus but also prevent the virus from binding to the host cell²⁵. Currently, a majority of the vaccines under development utilize the S protein as an antigen and can be categorized into three classes depending on the mode of antigen delivery (Fig. 1)^{25–27}. First, the mode of delivery in inactivated, live, and recombinant protein vaccines is the direct introduction of the antigen itself. Second, viral vector vaccines utilize a viral vector (mainly adenovirus), with or without a replication potential, that is genetically modified to express the S protein on its surface. Lastly, DNA and RNA vaccines have a relatively new mode of delivery, where the S protein-encoding nucleic acid is translated into S protein upon delivery to the target cell. Currently, 170 candidate vaccines have entered clinical trials (Table 1). Among the various types of vaccine platforms, ten vaccine candidates are currently approved for human use by WHO-recognized regulatory authorities; of them, two are mRNA vaccines, three are inactivated virus vaccines, four are non-replicating viral vector vaccines, and one protein is subunit vaccine²⁰. They are currently undergoing phase 4 clinical trials for their verification and monitoring of efficacy. Interim reports of efficacy are summarized in Table 2.

2.2. Challenges and limitations of the current major vaccine platforms

The main mechanism that endows the human body a function of protection from the virus is the induction of antibody response through natural infection or vaccination. Natural infection with COVID-19 induces both mucosal antibody responses (secretory IgA) and systemic antibody responses (IgG)²⁵. While secretory IgA is known to protect the upper respiratory tract, IgG is known to protect the lower respiratory tract. By contrast, intramuscular or intradermal administration of vaccines mainly induces IgG without inducing the secretory IgA²². Considering that the SARS-CoV-2 *Omicron* variant (B.1.1.529) preferentially infects and replicates in the upper respiratory airways, inducing high levels of neutralizing antibodies in the nasal mucosa is vital for the prevention of viral transmission^{41,69}. The neutralizing potency of secretory IgA in the nasopharynx is 7.5-fold higher than that of IgG in serum⁷⁰. In this aspect, while intramuscular or intradermal administration of vaccines leads to the prevention of severe lower respiratory infections, it is less likely that these vaccines would induce local mucosal immunity. For instance, a study on adenoviral vector vaccine in chimpanzees (ChAd) revealed that the intramuscular administration of vaccines to the BALB/c mouse model did not induce secretory IgA response²². Even though these vaccines may prevent the onset of the symptomatic severe lower respiratory tract infections, a possibility of mild upper respiratory tract infections remains. Likewise, evaluation of the currently used intramuscular COVID-19 vaccines revealed that all vaccines exhibited high efficacy in the prevention of severe infections in all age groups, while efficacy in prevention of symptomatic infection and transmission remains low and shows variable results (Table 2)⁵⁹. Thus, intramuscular or intradermal administration of vaccines does not necessarily lead to sterilizing immunity of the whole population. In comparison, intranasal administration of vaccines can induce both secretory IgA in the upper respiratory tract and IgG in the lower respiratory tract²⁵. Such a dual effect of intranasal vaccines can provide mucosal immunity in both the upper and lower respiratory tracts, thereby leading to the sterilizing immunity of the whole population²⁵. Different immunological effects of the parental and intranasal routes are summarized in Fig. 2.

2.3. Urgent necessity of mucosal COVID-19 vaccine and limitations of the current major vaccine platforms as potential mucosal vaccines

Mucosal vaccines licensed for human use still heavily rely on whole-cell inactivated or live attenuated vaccine platforms⁷¹. In the case of COVID-19, among the 170 vaccine candidates that have undergone clinical trials, only five vaccine candidates are administered orally, and 11 are administered intranasally (Table 3). As seen in Table 1, despite the current broad shift of intramuscular vaccine platforms from whole virus vaccines (live attenuated or inactivated) toward viral vector, nucleic acid, and subunit vaccines, only a small percentage of these three platforms are administered *via* the mucosal route. Such discrepancy in vaccine platform landscapes between injectable and mucosa-administered vaccines is due to the protective barriers and immunotolerant characteristics of the mucosa. Viral-vectored and live attenuated vaccines use the natural pathogen as a vector. Since the natural pathogen includes various types of pathogen-associated molecular patterns (PAMPs) and is able to evade or breach the mucosal barrier, the natural pathogen as a vector acts as a strong adjuvant for

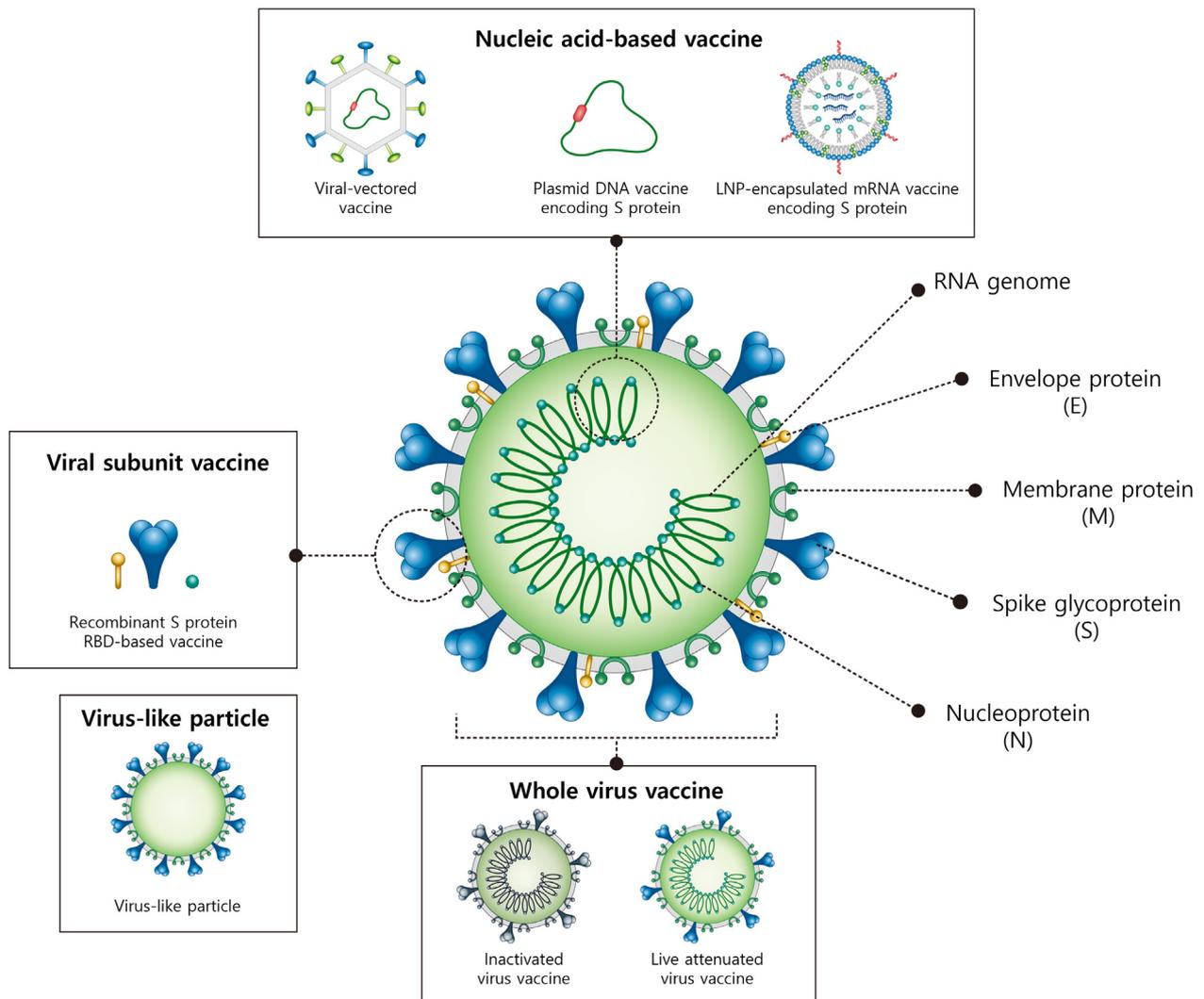


Figure 1 COVID-19 virus is composed of genomic RNA covered by nucleoprotein (N protein), membrane protein (M protein), an envelope protein (E protein) and spike glycoprotein (S protein). The development of various vaccine platforms is based on the structure of the COVID-19 virus particle. This includes nucleic acid-based vaccine (replicating, non-replicating vaccine, DNA plasmid, vaccine, mRNA vaccine), whole virus vaccine (inactivated virus vaccine, live attenuated virus vaccine), viral subunit vaccine (recombinant S protein, RBD-based vaccine) and virus-like particle. RBD, receptor-binding domain.

inducing a potent immune response and enables the effective antigen delivery to the mucosal immune inductive sites^{19,71}. However, despite their prevalent usage as mucosal vaccines, the safety of the viral-vectored, live attenuated, and whole-cell killed vaccines is their major limitation^{72,73}. Due to the potential reversion or recovery of virulence, these vaccine platforms pose risks to immunocompromised patients and require strict quality control and high manufacturing costs^{74,75}. By contrast, nucleic acid-based vaccines and protein subunit vaccines are purified molecules. Without protective delivery agents, these molecules are prone to degradation in harsh mucosal environments and lack the capability to induce an immune response. However, protein subunit and nucleic acid-based vaccines can be easily manufactured on a large scale and reduce the potential safety issue caused by virulence reversion⁷⁴. Thus, there are ongoing attempts to develop effective delivery agents for the successful mucosal administration of protein subunit vaccines and nucleic acid-based vaccines⁷⁶.

Viral-vectored vaccines are the commonly used vaccine platform developed for mucosal vaccines, owing to their ease of

manufacturing, well-described biochemical traits, potent ability to induce T cell and B cell responses, and the broad availability of administration routes (aerosol, oral, intradermal, and intramuscular)^{26,71,77}. As the natural adenovirus can breach through the mucosal layer of the respiratory tract, the intranasal administration of aerosol adenoviral vector enables the safe and effective delivery of antigens to the immune inductive site of the respiratory tract⁷⁷. In this manner, the capability of breaching and safe delivery of antigens across the mucosa of the host has made the viral vector vaccine the most utilized platform for potential mucosal vaccines against COVID-19. Additionally, the adenoviral vector itself can elicit B cell and T cell responses and act as a self-adjuvant for the induction of immune response. The critical disadvantages of the adenoviral vector vaccine are the lack of long-term efficacy and the issue of safety triggered by pre-existing adenoviral immunity⁴². Pre-existing immunity against the adenovirus can act as a double-edged sword for the efficacy and safety of the vaccine. Induction of the anti-adenoviral effector memory T cell triggered by pre-existing adenovirus immunity can degrade the vaccine-

Table 1 Overview of efficacy, safety, manufacture, storage, and proportion of each vaccine platform in clinical trials.

Platform	Efficacy	Safety	Manufacture & storage	Candidate vaccine (no. and %)	
PS	<ul style="list-style-type: none"> - Weaker immune response compared to whole virus vector and viral vector vaccine due to lack of PAMPs. Requires the addition of adjuvants or administration of booster dose to enhance immunogenicity. The utilization of aluminum hydroxide as an adjuvant is common. CpG1018 and Matrix-M1 were also used as adjuvant²⁸⁻³⁰ 	<ul style="list-style-type: none"> - Well tolerated compared to whole virus and viral vector vaccines and safely administered to immunocompromised people^{29,30} 	<ul style="list-style-type: none"> - Expression system of protein influences quality and quality of protein subunit vaccines due to post-translational modification in mammalian cell³¹⁻³³ - Safely produced without having to handle viral particles²⁹ - Stable and do not require storage in low-temperature storage facilities^{30,33} 	54	32%
RNA	<ul style="list-style-type: none"> - Lower immunogenicity and requirement of booster doses due to waning vaccine effectiveness^{31,34} - Exhibits the highest efficacy to prevent symptomatic COVID-19 compared to other vaccines³⁵ - Requires carrier molecules (lipid- or polymer-based nanoparticles) to protect RNA from RNase degradation and increase efficacy^{4,36,37} - Activation of innate immunity induces the type 1 interferon pathway, which degrades mRNA and inhibits translation of mRNA, leading to a decrease in the efficacy of the vaccine. Sequence optimization is required to improve efficacy^{4,38} 	<ul style="list-style-type: none"> - No interaction with host-cell DNA, which does not lead to genomic integration and subsequent insertional mutagenesis (non-integrating platform)^{4,31} - Naturally degraded without metabolic toxicity³⁷ - Activation of interferon signaling due to exogenous RNA induces inflammation^{4,31} 	<ul style="list-style-type: none"> - High adaptability to new pathogens and variants due to scalability and ease of mass production through high yield of <i>in vitro</i> transcription.⁴ - Safely produced without having to handle viral particles or cell cultures and animal matrix³⁷ - Unstable and requires ultra-cold storage facility^{29,39} 	41	24%
VV	<ul style="list-style-type: none"> - Stronger immune response. Elicit robust humoral and cellular responses with a single dose by mimicking natural infection, providing long-term immune response^{40,41} - Pre-existing immunity against human viral vectors can attenuate immune responses^{42,43} 	<ul style="list-style-type: none"> - Potential risk for infection and inflammation that can cause adverse reactions^{44,45} - Risk of genomic integration³¹ 	<ul style="list-style-type: none"> - Optimization of the cellular system is required for efficient viral vector³¹ - Safely produced without having to handle viral particle²⁹ - Can be easily produced on large scale⁴⁶ - Stable and do not require stringent storage conditions⁴¹ 	21 (VVnr) 4 (VVr) 2 (VVr + APC) 1 (VVnr + APC)	16%
IV	<ul style="list-style-type: none"> - Weaker immune response compared to live attenuated vaccines, requiring a higher dose, booster dosages, or adjuvants^{29,47,48} - Induction of long-lived humoral and cellular immune responses with several booster doses (results of nonrandomized trial among health care workers administered with BBIBP-CorV)⁴⁹ 	<ul style="list-style-type: none"> - Less risk of virulence reversion compared to live attenuated virus vaccine if properly inactivated, which is also suitable for immunocompromised patients²⁹ - Potential induction of vaccine-induced eosinophilic pulmonary response⁵⁰ 	<ul style="list-style-type: none"> - Require high levels of live pathogens for production⁵¹ - Inactivation process can affect the immunogenicity of the antigen due to structural deformation^{29,31} - Ease of transport and storage. Inactivated virus vaccine can be stored at 2–8 °C, enabling storage in countries with limited cold storage facilities⁴⁸ 	22	13%
DNA	<ul style="list-style-type: none"> - Induce humoral and cellular responses, but weaker immune response compared to live attenuated vaccines and viral vector vaccines^{31,52} - Difficulty in nuclear membrane barrier penetration causes low immunogenicity⁵² 	<ul style="list-style-type: none"> - Non-living, non-replicating, non-transmitting DNA plasmids are used⁵² - Well tolerated compared to whole virus vaccine and viral vector vaccines⁵² - Potential risk of genetic integration leading to insertional mutagenesis^{52,53} 	<ul style="list-style-type: none"> - High adaptability to new pathogens and variants due to scalability and easiness of mass production²⁹ - Safely produced without having to handle viral particles - Stable under room temperature and easy to store⁵² 	16	9%

(continued on next page)

Table 1 (continued)

Platform	Efficacy	Safety	Manufacture & storage	Candidate vaccine (no. and %)	
VLP	- Stronger immune response compared to protein subunit vaccines due to self-adjuvant properties owing to epitope similar to native virus ^{31,54,55} - Particulate size resembling respiratory viruses (20–200 nm), which is optimal for uptake by antigen-presenting cells ^{54,56}	- Overcome safety issues from the reversion of virulence caused by inactivated virus vaccine and live attenuated virus vaccine ^{54,55}	- Scalability and ease of mass production ⁵⁴ - Safely produced without involving live virus or inactivation steps ³¹ - Difficulty in particle assembly ⁵⁵ - Low stability in environmental change (temperature, shear stress, and fluid dynamics) ⁵⁵	6	4%
LAV	- Stronger immune response. Robust cellular and humoral immune responses without the usage of adjuvants, resulting in long-lasting immune responses ^{29,57}	- Possibility of virulence reversion and safety problems in immunocompromised patient ³¹	- Difficulty in achieving attenuated strains ²⁹ - Needs to be stored at low temperatures to remain stable ⁵⁸ - Low stability under environmental changes (temperature, shear stress, and fluid dynamics) ⁵⁶	2	1%

PS, protein subunit; VV, viral vector; VVnr, non-replicating viral vector; VVr, replicating viral vector; VVr + APC, replicating viral vector + antigen presenting cell; VVnr + APC, non-replicating viral vector + antigen presenting cell; IV, inactivated virus; VLP, virus-like particle; LAV, live attenuated vaccine.

infected cell, hindering the long-term actions of the vaccine⁴². In the phase 1 study of recombinant adenovirus type 5 (Ad5)-vectored vaccine, individuals with high baseline Ad5 neutralizing antibody titer (>1:200) exhibited weaker antibody and T cell responses compared to those with low baseline neutralizing antibody titer (\leq 1:200)⁴³. Moreover, severe side effects of the adenoviral vaccine, such as Bell's palsy, Guillain-Barre Syndrome, gait instability, and pneumonia, can be linked with the pre-existing immunity of the adenovirus²⁶. For instance, a recent study has proposed the potential causality between thrombocytopenic thrombosis and injection of ChAdOx1 nCoV-19 vaccine in a similar manner to heparin-induced thrombocytopenia^{44,45}.

Live attenuated vaccine is developed by attenuating the virulence of the whole virus while keeping it viable by mutating the pathogenic component of the viral genome. As live attenuated vaccine preserves immunogenic components of the natural pathogen, it induces robust and long-lasting immune responses without requiring the usage of adjuvants or administration of multiple boosters^{48,57}. However, the safety issue dealing with virulence reversion remains the main limitation of the live attenuated vaccine platform.

Protein subunit vaccines can be easily manufactured on a large-scale and are relatively more tolerable compared to viral-vectored and live attenuated vaccines^{74,78}. However, subunit vaccines are prone to enzymatic degradation in the mucosal tract and are relatively less immunogenic due to the lack of immune-stimulating antigens. In this aspect, equipping adjuvants and delivery agents is necessary to enhance the mucosal immunogenicity of subunit vaccines²⁸.

Nucleic acid vaccines, another major vaccine platform utilized for COVID-19, deliver a nucleic acid that encodes a specific protein to the cell. The mRNA in the cytosol is constantly translated into specific proteins that induce the host immune response⁷⁹. Such proteins include antigens of a certain pathogen, antibodies of a specific antigen, and immunostimulatory

molecules (CD70, GM-CSF). The mRNA vaccine can be readily manufactured through *in vitro* transcription. Such easiness of vaccine production through *in vitro* transcription enables the prompt development of vaccines against various viral antigens⁷⁹. Additionally, its safe manufacturing process compared to the live attenuated viral vaccine or the inactivated viral vaccine makes it an attractive new-generation vaccine platform for future pandemics. However, there are no approved nucleic acid vaccines for mucosal administration so far. The major limitations of the mRNA vaccine lie in the innate instability of the RNA, difficulty of intracellular delivery, and induction of innate immunity⁴. The naked mRNA is an unstable molecule and can be degraded by RNase that is widely distributed in our body. Moreover, the negative charge of the RNA vaccine hinders its intracellular delivery⁵². Furthermore, ssRNA or dsRNA in the cytosol can be recognized by toll-like receptors (TLRs), inducing innate immunity of the cell⁸⁰. The activation of innate immunity induces the type 1 interferon pathway, which degrades mRNA and inhibits mRNA translation, decreasing the efficacy of the vaccine^{4,38}. To overcome these difficulties, it is crucial to select delivery agents that protect RNA molecules from degradation by RNases and facilitate intracellular delivery^{81,82}. One of the major delivery agents for mRNA vaccine is the lipid-based delivery platform, which includes cationic lipids, cholesterol, and PEG⁴. The lipid nanoparticle platform-based mRNA-1273 and BNT162b2 also utilize ionizable cationic lipid, helper lipid, and PEG-lipid (Fig. 3)⁸³. This nanocarrier not only stably encapsulates the RNA but also enables its endocytosis into the cytosol. Other delivery platforms include polymer-based delivery, peptide-based delivery, and cationic nanoemulsion^{81,84}. However, there are no licensed mRNA vaccines or candidates undergoing clinical trials for the mucosal route of administration. The absence of target delivery platforms toward the immune inductive site can hinder the full efficacy of the mRNA vaccine due to the nonselective delivery of the mRNA toward a non-targeted cell^{85,86}.

Table 2 Overview of licensed COVID-19 vaccines.

Approved vaccine	Platform	Manufacturer	Route of administration	Variants of concern	Prevention of symptomatic infection (%)	Prevention of severe infection (%)	Ref.
BNT162b2	mRNA	Pfizer-BioNTech	Intramuscular	Alpha	78–95	95	59,60
				Beta	75	95	
				Delta	42–79	94	
				Omicron	44 (primo) 75 (booster)	72 (primo) 90 (booster)	
mRNA-1273	mRNA	Moderna	Intramuscular	Alpha	84–99	97	59,61
				Beta	96	97	
				Delta	76–84	97	
				Omicron	48 (primo)	73 (primo) 90 (booster)	
AZD1222	Viral vector	AstraZeneca-Oxford	Intramuscular	Alpha	75	94	59,62
				Beta	10	NA	
				Delta	67	92	
				Omicron	36 (primo) 75 (mRNA booster)	71 (primo)	
Ad26.COV-2-S	Viral vector	Janssen	Intramuscular	Alpha	72	86	59,63
				Beta	65	76	
				Delta	64	76	
				Omicron	33	57	
Ad5-nCoV (convidecia)	Viral vector	CanSino Biologics	Intramuscular	Omicron	64	96	59,64
BBV152 (covaxin)	Inactivated virus	Bharat Biotech	Intramuscular	Delta	78	100	59,65
WIBP-CorV	Inactivated virus	Sinopharm	Intramuscular		73	100	59,66
CoronaVac	Inactivated virus	Sinovac Biotech	Intramuscular	Alpha	47	50	59,67
				Beta	NA	NA	
				Delta	59	NA	
				Omicron	NA	NA	
BBIBP-CorV	Inactivated virus	Sinopharm	Intramuscular	Alpha	68	73	59,66
				Beta	NA	NA	
				Delta	67	NA	
				Omicron	NA	NA	
NVX-CoV2373	Protein subunit	Novavax	Intramuscular	Alpha	86	89	59,68
				Beta	86–93	86	
				Delta	NA	NA	
				Omicron	43	65	

Consequently, the lack of targeted mRNA delivery agents hinders the development of mucosal mRNA vaccines, which further necessitates the development of a nanoparticle-based targeted delivery system.

Considering the safety issue of commonly used mucosal vaccine platforms (viral vector, live attenuated virus, and inactivated virus), protein subunit and nucleic acid vaccines can be safe alternatives. Additionally, the ease of scale-up and manufacturing of protein subunit vaccine and mRNA vaccine would offer high adaptability to new variants or pathogens. However, weak inherent immunogenicity and prompt degradation of mRNA vaccine and protein subunit vaccine in the mucosal environment remain the main obstacles. Considering the clinical importance and necessity of mucosal vaccines, it is crucial to study the target cell for antigen delivery, prepare a suitable formulation for effective antigen protection, and choose an adjuvant for the successful induction of mucosal immunity. This necessitates the research and development of the components and mechanisms of mucosal immune systems and various mucosal vaccine platforms for the effective induction of mucosal immunity.

2.4. COVID-19 mucosal vaccines in the preclinical study and clinical trials

Despite the absence of approved COVID-19 vaccines for intranasal administration, 11 intranasal vaccines are currently undergoing clinical trials¹⁹. Among the 11 intranasal COVID-19 vaccines, eight vector vaccines, two protein subunit vaccines, and one live attenuated vaccine are now being investigated (Table 4)^{19,28}.

Viral vector vaccines are the most prevalent vaccine platform developed for mucosal COVID-19 vaccines. For instance, intranasal vaccination of nonreplicating human type 5 adenovirus encoding the full-length SARS-CoV-2 S protein developed by CanSino Biologics (Tianjin, China) (Ad5-nCoV) has induced S-specific IgG and IgA as well as IFN γ , TNF α , IL-2 responses in splenic CD8⁺ and CD4⁺ T cells in BALB/c mice⁸⁷. Activation of the local and systemic immune system has prevented infection in both the upper and lower respiratory tract²². Moreover, intranasal administration of chimpanzee adenovirus (simian Ad-36) encoding a stabilized prefusion form of the S protein (ChAd-SARS-

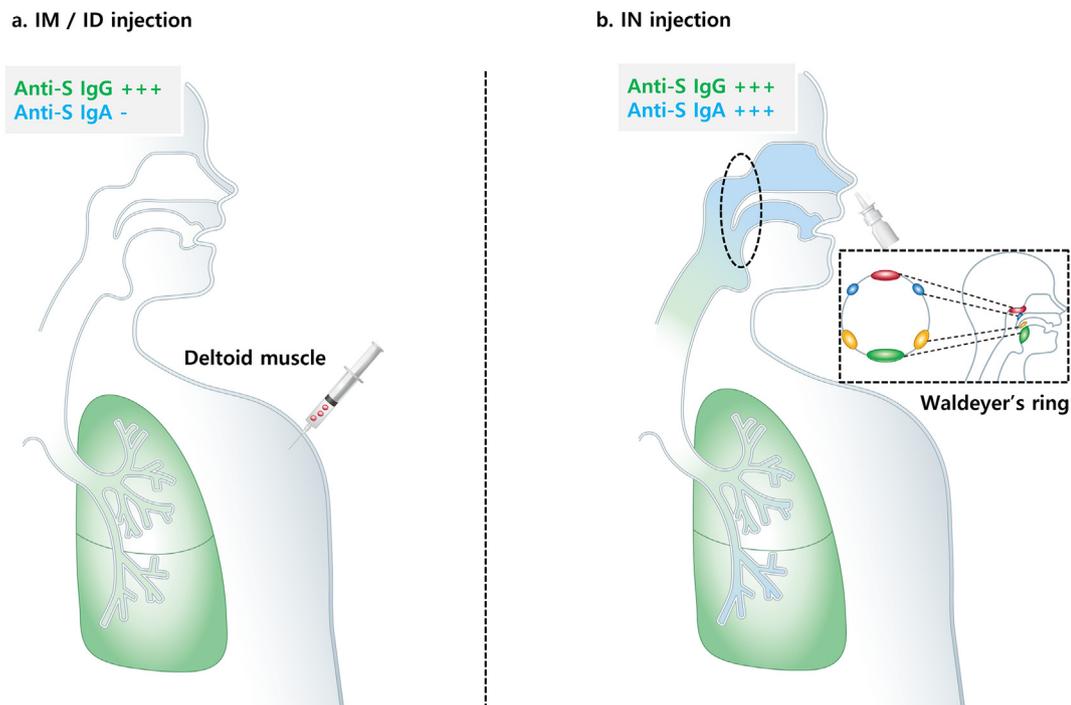


Figure 2 The lower respiratory tract (LRT) is mostly protected by systemic immune response mediated by serum IgG, while the upper respiratory tract (URT) is mostly protected by local immune response mediated by secretory IgA1 (sIgA1) in mucosal surface. (a) Parenteral (intramuscular, intradermal, subcutaneous) administration of vaccine leads to potent induction of serum IgG rather than sIgA. Such deficiency of sIgA in URT leaves the individual more susceptible to symptomatic infection and transmission, especially the *Omicron* variant (B.1.1529) which preferentially infects and replicates in URT. (b) Intranasal administration of vaccine leads to dual induction of both sIgA in mucosal surface and IgG in serum, which subsequently leads to sterilizing immunity in both URT and LRT. However, the potency of systemic immune response is often lower than the parentally administered vaccine.

CoV-2) has also induced the production of anti-S and anti-RBD neutralizing antibodies and T cell responses in nasal swabs, bronchoalveolar lavage fluid (BAL), and lung tissues of rhesus macaques⁸⁸. Other viral vectors include attenuated influenza virus, parainfluenza virus 5 (PIV 5), vesicular stomatitis virus (VSV), live Newcastle disease virus (NDV), and lentivirus. Live recombinant NDV expressing a stable version of the S protein in pre-fusion conformation (AVX/COVID-12-HEXAPRO) was administered to pigs intranasally, intramuscularly, and by a combination of the two routes⁸⁹. Administration of vaccine-induced antibodies against the S protein and variants of concern (B.1.1.7,

B.1.351, P.1) blocked ACE2-RBD interactions⁸⁹. Viral-vectored intranasal vaccines in the clinical phase are summarized in Table 4. While most clinical data are yet to be released, AVX/COVID-12 HEXAPRO was administered twice intramuscularly (IM-IM), twice intranasally (IN-IN), or intranasally (IN), followed by intramuscularly (IN-IM) to 91 healthy volunteers in an open-label non-randomized non-placebo-controlled phase 1 clinical trial (NCT04871737). Potent antibody and cellular immune responses were detected in the IM-IM/IN-IM high-dose group⁹⁰. All administration routes at all dose levels were well tolerated and were not associated with severe adverse events⁹⁰. Based on this result, a phase II study on AVX/COVID-12 HEXAPRO is ongoing for the evaluation of safety and immunogenicity (NCT05205746). Two intranasal doses of live attenuated influenza virus-vectored vaccine encoding RBD (DelNS1-2019-nCoV-RBD-OPT1) in double-blind, randomized, placebo-controlled phase 1 and 2 trials have also demonstrated immunogenicity and safety, which was well tolerated in healthy adults and induced RBD-specific IgG, secretory IgA and T cell immunity (ChiCTR2000037782, ChiCTR2000039715, ChiCTR2100048316)⁹¹.

In the case of viral-vectored vaccines developed for oral administration, VXA-CoV2-1 developed by Vaxart, an adenovirus type 5 (rAd5) vector containing full-length SARS-Cov-2 S gene with cytomegalovirus promoter, and full-length SARS-Cov-2 N gene with a human beta-actin promoter (Ad5 S + N vaccine) has

Table 3 Route of administration of vaccine candidates in clinical trials.

Route of administration	Candidate vaccine (no. and %)	
Oral	5	3%
Intranasal	12	7%
Aerosol	1	1%
Inhaled	2	1%
Subcutaneous	5	3%
Intradermal	9	5%
Intramuscular	140	82%
No data (ND)	11	6%

Table 4 Overview of COVID-19 mucosal vaccines in clinical trials.

Vaccine platform	Candidate vaccine	Route of administration	Manufacturer	Phase	Clinical trial No.
Ad-vectored vaccine	ChAdOx1	IN	University of Oxford/Vaxart	Phase 1	NCT04816019
	Ad5-nCoV	IN or IM	Cansino/Beijing institute	Phase 1/2	NCT04840992
	BBV154	IN	Bharat Biotech International	Phase 3	CTRI/2002/02/040065
	VXA-CoV2-1	Oral	Vaxart	Phase 2	NCT05067933
LAIV-vectored vaccine	hAd5 S-Fusion + N-ETSD	SC, Oral, or SL	ImmunityBio	Phase 1/2	NCT04843722
	DelNS1-2019-nCoV-RBD-OPT1 (intranasal flu-based-RBD)	IN	University of Hongkong/Xiamen University/Beijing Wantai Biological Pharmacy	Phase 3	ChiCTR2100051391
	CVXGA1	IN	CyanVac LLC	Phase 1	NCT04954287
PIV5- vectored vaccine	MV-014-212	IN	Meissa Vaccines, Inc.	Phase 1	NCT04798001
RSV-vectored vaccine	AVX/COVID-12-H EXAPRO	IN, IM or IN + IM	Laboratorio Avi-Mex	Phase 1	NCT04871737
NDV-vectored vaccine	NDV-HXP-S	IN	Sean Liu/Icahn School of Medicine at Mount Sinai	Phase 2/3	NCT05354024
PS	CIGB-669 (RGB + AgnHb)	IN or IN + IM	Center for Genetic Engineered Biology (CIGB)	Phase 1/2	RPCE00000345
	Razi Cov pars, (recombinant S protein)	IM + IN	Razi Vaccine/Serum Research Institute	Phase 3	IRCT20210206050259N3
LAV	CoV2-OGEN1	Oral	USSF/Vaxform	Phase 1	NCT04893512
	COVI-VAC	IN	Codagenix/Serum Institute of India	Phase 3	ISRCTN15779782
DNA	BacTRL-spike DNA vaccine	Oral	Symvivo Corporation	Phase 1	NCT04334980

Ad, adenovirus; LAIV, live attenuated influenza virus; PIV, parainfluenza virus 5; RSV, respiratory syncytial virus; NDV, Newcastle disease virus; PS, protein subunit; LAV, live attenuated vaccine; IN, intranasal; IM, intramuscular; SC, subcutaneous; SL, sublingual.

shown promising preclinical data⁹². Two doses of VXA-Cov-2-1 were orally administered (oral gavage) to Syrian hamsters before intranasal challenge with SARS-CoV-2. Histopathological analysis of the lung samples showed that two oral doses of VXA-CoV-2-1 elicited a reduction in the inflammatory response and viral load by more than 4 logs in the lungs after intranasal challenge with SARS-CoV-2. Vaccination also induced robust S1-specific serum IgG response and mucosal IgA with the reduced transmission of SARS-CoV-2 in hamsters^{92,93}. In this regard, a single-site, dose-ranging, open-label phase 1 trial of the VXA-CoV-2-1 oral tablet was administered to healthy volunteers aged 18–54 years old (NCT04563702). The results have shown that two oral vaccinations of VXA-CoV-2-1 elicited mucosal secretory IgA in the majority of vaccine recipients, which was highly cross-reactive to variants and persisted for up to 360 days⁹⁴. A phase II clinical trial for dose and age escalation is currently ongoing to evaluate the safety and immunogenicity of VXA-Cov-2-1 (NCT 05067933). Another type of Ad5 S + N vaccine developed for oral delivery is the hAd5-S-Fusion + N-ETSD vaccine developed by ImmunityBio. hAd5-S-Fusion + N-ETSD is a bivalent human adenovirus type 5 vector containing an S sequence modified for optimal cell surface expression (S-Fusion) and N antigen modified with an enhanced T-cell stimulation domain (ETSD) for the optimal transportation *via* endosomal/lysosomal route for MHCI/II presentation^{95,96}. Subcutaneous prime vaccination followed by two oral booster dosages of hAd5-S-Fusion + N-ETSD in rhesus macaques induced neutralizing anti-S IgG and Th1-based T cell responses. This led to the

inhibition of viral replication of SARS-Cov-2 in the upper and lower respiratory tract⁹⁶. A phase 1 clinical trial was conducted on hAd5-S-Fusion + N-ETSD, and the results showed ten-fold T cell-specific IFN- γ secretion in recipients compared to naïve participants with cross-reactivity found in B.1.1.7, B.1.429, P.1, and B.1351 strains (NCT04591717)⁹⁷. hAd5-S-Fusion + N-ETSD is currently ongoing clinical trials for diverse administration routes, such as sublingual, oral, and subcutaneous.

In the case of intranasal protein subunit vaccines, trimeric or monomeric S protein adjuvanted with cyclic GMP-AMP synthase and the stimulator of interferon gene agonist (STINGa) was encapsulated with liposome⁹⁸. Intranasal administration of adjuvant encapsulated liposome to BALB/c mice elicited neutralizing antibody in the serum, secretory IgA in the upper and lower airway, and S-specific T cells in the spleen and lungs⁹⁸. Protein subunit vaccines in the clinical phase are CIBG-669, which constitutes an RBD protein with an HBV nucleocapsid antigen (AgnHB), and Razi Cov Pars, which constitutes a recombinant S protein. Intranasal administration of CIBG-669 in phase 1/2 trials induced an anti-RBD response by 4-fold with no severe adverse effects (RPCEC00000382). Razi Cov Pars is currently undergoing a phase 3 study (IRCT20210206050259N3). CoV2-OGEN1, a recombinant protein subunit vaccine developed for oral administration, is currently ongoing a phase 1 trial to evaluate the safety of a 2-dose vaccination schedule of oral suspension (NCT04893512).

COVI-VAC, a live-attenuated vaccine with 283 silent mutations in the gene encoding the S protein is now being tested as an

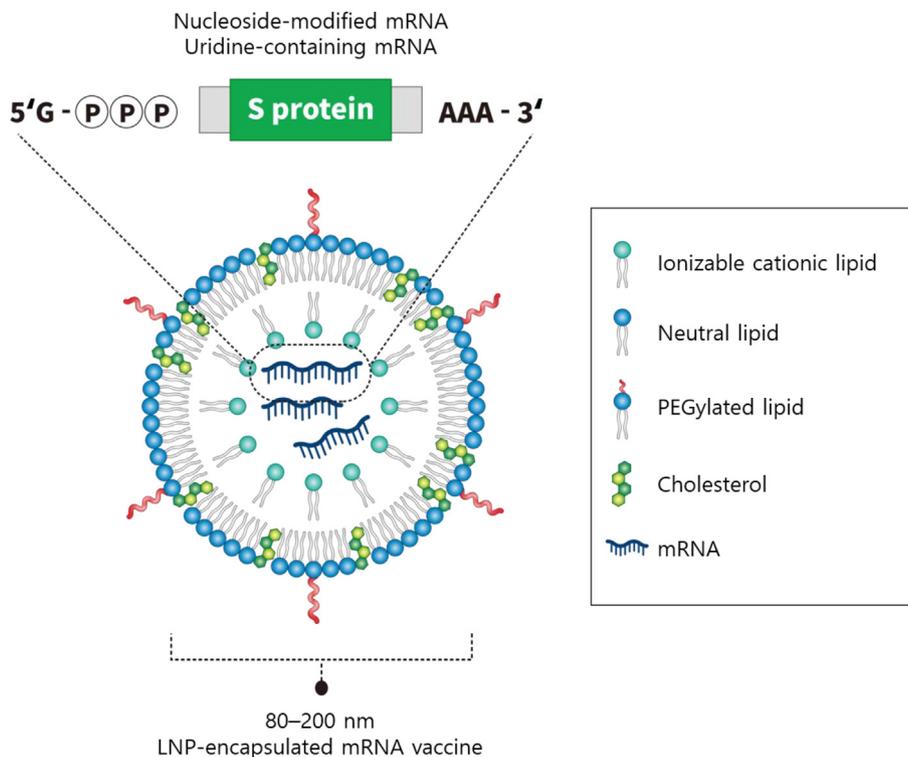


Figure 3 Elements used in a lipid nanoparticle (LNP)-encapsulated mRNA vaccine. LNP protects mRNA from external degrading factors (H_2O , pH, nucleases) and consists of ionizable cationic lipid, neutral lipid, PEGylated lipid, and cholesterol, ionizable cationic lipid stabilizes negatively charged mRNA, cholesterol provides structural stability of LNP, and PEGylated lipid enables long-term circulation of LNP.

intranasal formulation⁴⁸. Phase 1 randomized, double-blind, placebo-controlled, dose-escalation trial of a single intranasal dose of COVI-VAC has demonstrated that administration of COVI-VAC was not associated with substantial adverse events and stimulated serum and mucosal immune responses (NCT04619628)⁴⁸. Currently, COVI-VAC is undergoing a phase 3 clinical trial (ISCRTN15779782).

Finally, the bacTRL-spike oral DNA vaccine developed by Symvivo corporation (Burnaby, BC V5J 5J8, Canada) utilizes the unique bacTRL™ platform, which is commensal bacteria (*Bifidobacterium longum*) engineered to deliver synthetic plasmid DNA encoding spike protein. A commensal *Bifidobacterium* selectively colonizes in the lower gastrointestinal tract and expresses transport protein which binds to plasmid DNA. Transport protein-bound plasmid DNA is secreted to the extracellular environment and subsequently transfects the colon epithelium. Transfected colon epithelium expresses antigen, inducing antigen-presenting, followed by activation of the immune response. Oral administration of bacTRL-spike-1 is currently undergoing the phase 1 trial to evaluate safety and tolerability in healthy adults (NCT04334980).

3. Basic mechanism of mucosal immunity and mucosal vaccines

3.1. Antigen sampling in mucosal inductive sites

The organized lymphoid tissue in respiratory and gastrointestinal mucosa plays a critical role in the sampling of antigens and induction of mucosal immunity⁹⁹. The organized mucosal

inductive site, mainly located at the site of pathogen exposure (palatine tonsil, lingual tonsil, adenoid, Peyer's patch)¹⁰⁰ has unique epithelial cells called 'follicle-associated epithelium (FAE)' along with the lymphoid follicle. FAE, a specialized epithelial cell differentiated from the mucosal lymphoid follicle¹⁰¹, possesses a distinct ability of antigen capture, delivery, or presentation, which is mediated by the M cell (microfold cell)^{102,103}. The M cell, named after its scarce microvilli present on the apical side compared to other absorptive epithelial cells, possesses high endocytic activity and effectively captures foreign materials from the lumen of the mucosal surface and delivers them to the basolateral side (Fig. 4)²¹. The basolateral side of the M cell has a pocket shape structure, which enables the localized recruitment of DC, B cell, and T cell inside the pocket¹⁰⁴.

The primary inductive sites for gastrointestinal adaptive immune response are Peyer's patch, cecal patch, and colonic patch, which are the largest MALT in the digestive tract, and are found in the submucosa of the small intestine, cecum, and colon¹⁰⁵. The primary inductive site for the upper respiratory adaptive immune response is NALT in mice, which is found on the dorsal side of the soft palate. Humans also have organized mucosal lymphoid tissues called Waldeyer's ring. Peyer's patch and NALT exhibit highly similar structures composed of follicle-associated epithelium, subepithelial dome, B cell follicles with germinal centers, and interfollicular zone. The chemokines CCL20 and CCL9 produced by FAE recruit lymphocytes and dendritic cells expressing the corresponding counter receptor (CCR6 and CCR1)¹⁰⁶. Below the epithelium is the subepithelial dome (SED), where DCs encounter antigens transported by enterocytes and M cells. Beneath the SED

are interfollicular T cell zones and B cell follicles with a germinal center. In the interfollicular T cell zones, DCs present antigens and activate effector CD4⁺ and CD8⁺ T cells. Activated CD4⁺ T cells induce the formation of an IgA⁺ plasma cell through isotype class switching and affinity maturation in the germinal center of the B cell follicle (Fig. 5).

3.2. Immune responses in mucosal effective sites

Effector B and T cells activated by DC express tissue-specific adhesion molecules and chemokine receptors. Such a receptor-mediated homing property of lymphocytes enables the localized concentration of mucosal immune response at the site of antigen exposure as well as distant organs, which express the corresponding receptor or ligand of the molecule¹⁰⁷. Effector B and T cells stimulated in inductive sites are preferentially transferred to related mucosal-effector sites. For instance, IgA⁺ plasma cells induced by NALT express $\alpha 4\beta 1$ and CCR10, which migrate to the respiratory and genitourinary tracts expressing VCAM-1 and CCL28^{108,109}. This elicits local recruitment and focusing of IgA⁺ B cells in the respiratory tract. IgA⁺ plasma cells localized in the respiratory tract secrete antigen-specific IgA⁺ to the lumen, which prevents viral entry into the respiratory epithelium (Fig. 5). IgA⁺ plasma cells activated from Peyer's of the intestine express $\alpha 4\beta 7$ integrin, which strongly binds to MADCAM1 of the small intestinal venule^{107,109}. This elicits local recruitment and focusing of IgA⁺ B cells in the intestine, preventing viral entry into the digestive tract. In contrast, systemic immunization of the antigen did not induce the expression of $\alpha 4\beta 7$ integrin and CCR10 in B cells, which further corroborates the ineffectiveness of systemic immunization for the induction of mucosal immunity.

As such "counter receptors" are widely expressed in various types of mucosal organs, induction of the mucosal immunity in a primary organ can elicit immunity in distant organs (Fig. 6). For instance, IgA-secreting B cells activated from MALT expresses CCR10, which enables its recruitment towards various organs expressing the corresponding CCL28 (the small intestine, large intestine, salivary glands, tonsils, and bronchi)¹⁰⁷. This is mediated through a mechanism termed as "common mucosal immune system (CMIS)"^{110,111}. This enables oral or intranasal administration of the vaccine to induce mucosal immunity in distant organs such as vaginal mucosa, which can be applied in the development of the orally administered human immunodeficiency virus (HIV) vaccine¹¹². Moreover, among the various kinds of mucosal administration of hybrid HIV peptide and cholera toxin as an adjuvant, the intranasal administration induced a relatively potent anti-HIV antibody response compared to the other routes^{100,113}. In the case of the COVID-19 vaccine in a preclinical study, a recombinant SARS-CoV-2 RBD-based subunit vaccine adjuvanted with Alhydrogel® (aluminum oxyhydroxide gel) was administered intranasally. The results have shown induction of RBD-specific secretory IgA at nasal washes, bronchoalveolar lavage (BAL), as well as the intestine and vaginal wash¹¹⁴. Although Peyer's patch and NALT show similarities in structure and mechanism of antigen sampling, the major difference between the two is associated with lymphocyte homing¹¹⁵. Owing to the differences in receptor and chemokine profiles of activated B cells and T cells depending on the route of administration, different routes of mucosal administration lead to different localization of lymphocytes toward each mucosal organ. Intranasal vaccination induces a robust immune response in respiratory and genitourinary tracts while inducing a weak immune response

in the intestinal tract^{116,117}. Mucosal B cells induced by NALT express $\alpha 4\beta 1$ and CCR10, which migrate to the respiratory and genitourinary tracts expressing VCAM-1 and CCL28^{108,109}. B cells induced by Peyer's patch and isolated lymphoid follicle express $\alpha 4\beta 7$ and CCR9, which migrate to intestinal tracts expressing CCL25 and MADCAM-1^{107,116}. The difference in the imprinting of homing specificity depends on the presence of retinoic acid. Retinoic acid is uniquely produced by intestinal DC and acts as a key signaling molecule for the expression of gut-homing receptor $\alpha 4\beta 7$ in immune effector cells¹¹⁸. On the contrary, the absence of retinoic acid leads to the expression of $\alpha 4\beta 1$ and CCR10, which guides B cells to the airway and reproductive mucosa¹¹⁸. In this regard, oral vaccination would induce a potent mucosal immune response in the intestinal tract and salivary glands, while mucosal immune activity in the upper respiratory tract would remain relatively low compared to intranasal vaccine^{116,119}. Thus, it is important to consider the potency of localized mucosal responses of each organ while determining the site of administration. In this aspect, intranasal vaccination would be a rational approach for the prevention of SARS-CoV-2 infection compared to oral vaccination.

Although it is obvious that secretory IgA and virus-neutralizing antibodies block the viral entry site and provide sterilizing immunity, the recent advent of highly contagious *Omicron* variants (B.1.1.529) evading neutralizing antibodies is raising a new concern¹²⁰. In this aspect, CD8⁺ T cell, despite its non-sterilizing immunity and redundant protective effect with neutralizing antibodies, recognizes highly conserved epitopes among various types of SARS-Cov-2 variants^{121,122}. Specifically, lung resident and systemic memory T cells showed effective protective immunity against antibody-resistant *Beta* (B1.351) variants in the absence of neutralizing antibodies¹²¹. Thus, inducing both neutralizing antibodies and memory T cells can be a new paramount of the COVID-19 vaccine by endowing both sterilizing immunity and high cross-reactivity¹²¹. Tissue-resident memory T cells (T_{RM}), long-lived memory T cells residing in various types of tissues (the gut, skin, lungs, reproductive tract, liver, and brain), are crucial for the prevention of respiratory infection by their rapid response to re-infection stimuli. T_{RM} develops from effector memory T cell (T_{EM}) precursors in response to local antigen recognition and exposure to TGF- β and IL-15¹²³. Despite its functional similarity with T_{EM}, T_{RM} exhibits distinct transcription profiles for its long-term survival in the tissue microenvironment and induces stronger protective mucosal immunity than T_{EM}^{124,125}. Specifically, lung CD8⁺ T_{RM} cell produces a significant amount of IFN γ compared to T_{RM} cells in other types of tissue, which has a pivotal role in protection from respiratory virus infection¹²⁶. Lung CD8⁺ T_{RM} cells can be divided into two subpopulations known as airway T_{RM} and interstitial T_{RM}. Within these two subgroups, airway T_{RM} is known to be the less cytolytic and determines the efficacy of lung cellular immunity^{126,127}.

Despite its clinical importance, lung CD8⁺ T_{RM} tends to exhibit a short lifespan and is lost a few months after acute infection, thereby leaving the host susceptible to secondary infection¹²⁸. A study demonstrated that the short lifespan of lung CD8⁺ T_{RM} is associated with epigenetics and transcriptional changes of the airway T_{RM} cells induced by the airway environment, which promotes apoptosis due to amino acid starvation and activation of an integrated stress response¹²⁴. Thus, sustaining the lifespan of lung T_{RM} cells can be a key strategy of mucosal vaccines for the prevention of SARS-CoV-2 infection. With

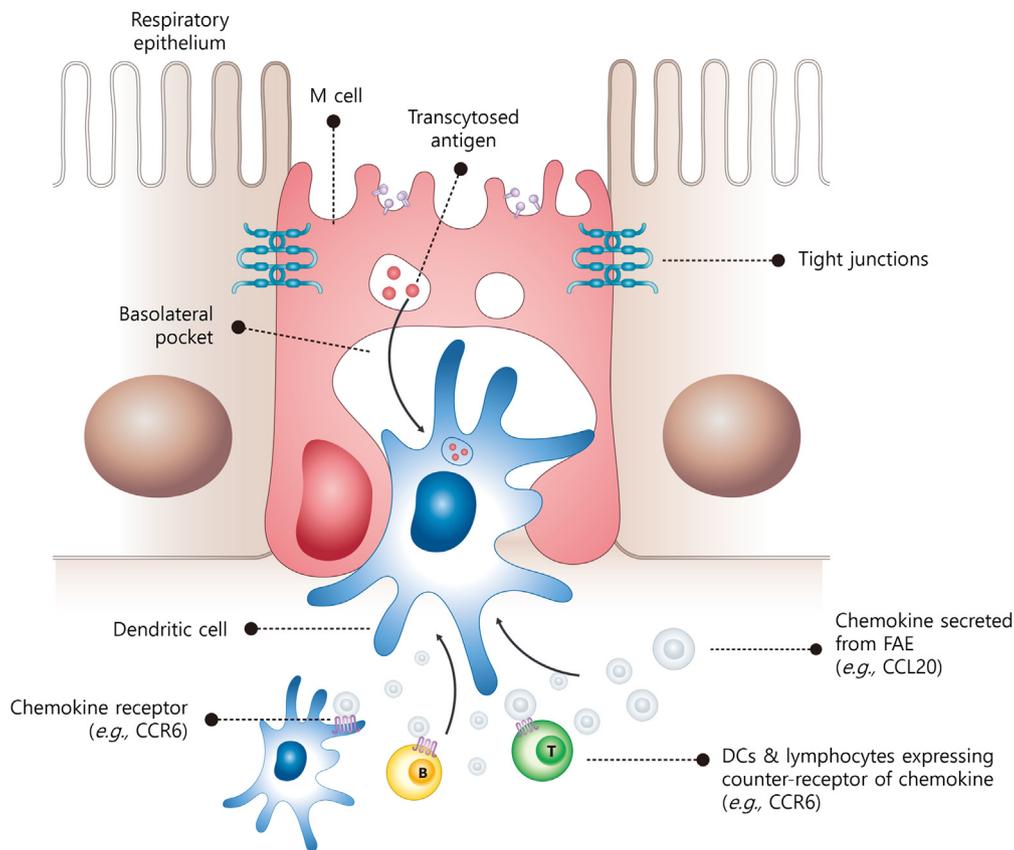


Figure 4 Morphological features of M cells. M cell is characterized by a sparse amount of microvilli and short microvilli showing microfold pattern on apical side and deep invagination of the basolateral side, which acts as localization space of dendritic cells and lymphocytes. Recruitment of dendritic cells and lymphocytes is mediated by chemokine (e.g., CCL20) secreted from the follicle-associated epithelium (FAE) and its counter-receptor (e.g., CCR6).

respect to this strategy, an Adenovirus vector expressing influenza nucleoprotein (AdNP) was administered intranasally to increase the longevity of lung $CD8^+ T_{RM}$ ¹²⁹. The results have shown larger populations of lung T_{RM} and a longer population maintenance period (1-year post-vaccination) compared to influenza infection. Such expansion of longevity is induced by persistent antigen expression after AdNP vaccination¹³⁰. These studies further support the necessity of intranasally delivered vaccines and suitable delivery agents for the sufficient activation and long-term maintenance of lung T_{RM} cells.

3.3. M cell and dendritic cells – key target cells for mucosal vaccine delivery

In the mucosal membrane, potentially harmful antigens are captured by various modalities of the mucosal surface and specific antigens induce immune surveillance of the luminal components¹³¹. Among the various modalities of capturing luminal antigens, one of the most intriguing mechanisms is capturing and transcytosis of antigens mediated by M cell, a highly specialized epithelium that plays an important role in mediating the induction of mucosal immunity¹³². Transported antigens are effectively transferred to antigen-presenting cells or dendritic cells localized in the basolateral pocket and initiate the activation of mucosal immune responses. The M cells are present in the FAE of gut-associated lymphoid tissue (GALT) and nasal-associated lymphoid tissue (NALT). Importantly, various studies have

proven the importance of NALT M cells in the immune induction of the common mucosal immune system^{133,134}. In a CCR6-deficient murine model that exhibits a reduced number of NALT M cells, antigen-specific nasal IgA was not detected after nasal immunization of attenuated vaccine strain of *Salmonella typhimurium*, whereas the nasal immunization in the wild-type CCR6 model successfully induced the secretion of nasal IgA¹³³. For the development of mucosal vaccines, understanding the unique structural features of M cells and the cellular mechanisms underlying antigen capturing, transportation, and presentation are crucial.

The M cell exhibits close contact with columnar epithelial cells through tight junctions and desmosomes¹³⁵. Compared to the adjacent columnar epithelial cells, the morphology of the M cell is characterized by a sparse amount of microvilli and short microvilli showing a microfold pattern on the apical side and deep invagination of the basolateral side that acts as a localization space of antigen-presenting cells^{103,136}. Such unique structures of M cells enable the efficient sampling of antigens and transportation of antigens to APC located in the basolateral pocket. Since microvilli have negative charges owing to their glycoprotein structure, the columnar epithelial cells abundant in proximity to the microvilli tend to show electric repulsion with the bacterial peptide. As a scarce amount of M cells exists on the apical side, electrical repulsion between the bacterial peptides and the M cell is less likely to occur. Thus, the bacterial peptides or antigens are constantly repelled from the columnar epithelial cells and finally

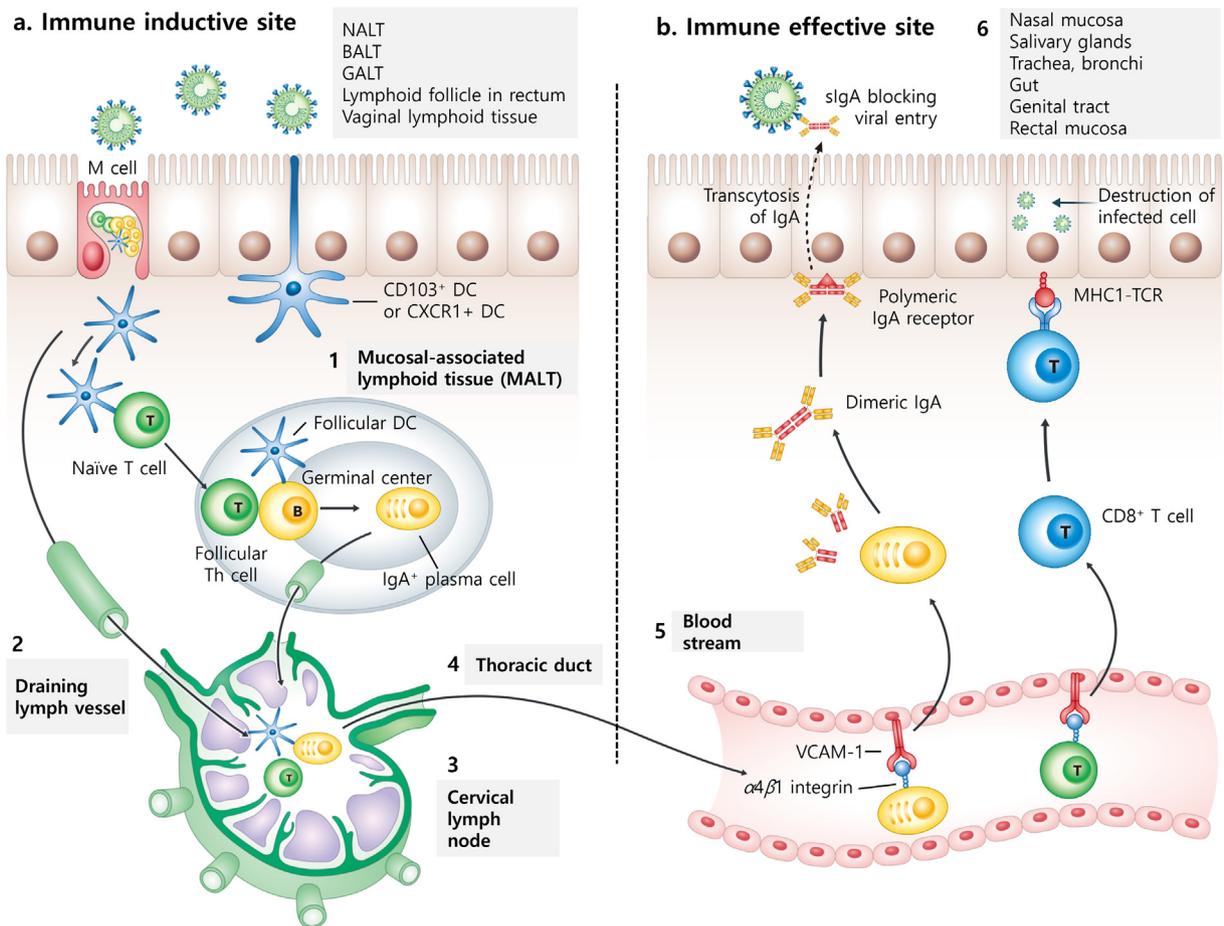


Figure 5 The antigen is captured by M cell and transcytosed to dendritic cells in the basolateral pocket. Subsequently, dendritic cell interacts with lymphocytes, which subsequently lead to the activation of plasma cell in B cell follicle. IgA + B cell and T cell activated by DCs express tissue-specific adhesion molecule and chemokine receptor and localize to the distant organ, which expresses the corresponding receptor or ligand of the molecule. NALT, nasal-associated lymphoid tissue; BALT, bronchus-associated lymphoid tissue; GALT, gut-associated lymphoid tissue; CXCR1, C-X-C motif chemokine receptor 1; FDC, follicular dendritic cell; VCAM-1, vascular cell adhesion molecule-1; MHC1, major histocompatibility complex class 1; TCR, T cell receptor.

reach the M cells, where less electrical repulsion is present (M cell trap)¹³¹. Additionally, the basolateral pocket of the M cell provides a space for the interaction of various immune cells. Antigen transported by M cell is effectively captured by APCs or DCs in the basolateral pocket and migrates to the interfollicular T cell zone or draining lymph node. This induces the activation of Naive T cells, class switching, affinity maturation of B cell response, and memory response of lymphocytes. Activated T cells and IgA-producing B cells localize to effector sites producing antigen-specific IgA in the nasal cavity¹¹⁶. In this aspect, the distinct role of the M cell in capturing the luminal foreign antigen and providing direct entry to the mucosal immune inductive site makes the M cell an attractive target for mucosal vaccine delivery¹³⁷.

The population of DCs, which comprises heterogeneous subgroups, performs a pivotal role in guiding the immunity towards activation or tolerance¹³⁸. Since the mucosal environment is constantly exposed to diverse commensal and foreign antigens, it is important for the immune system to distinguish between harmless and harmful antigens. In this regard, DCs in the mucosal immune system subtly regulate the balance between immune activation and tolerance¹³⁹. Therefore, for the successful induction of immune response by the mucosal vaccine, it is important to understand and utilize the mechanism of immune regulation

mediated by DCs. Moreover, delivering antigens to the proper subtype of mucosal dendritic cells and skewing the balance of the immune system to trigger its activation is crucial.

Within the mouse NALT, a large population of DCs was found to be adjacent to the sub-epithelium and peri-vascular and perilymphatic spaces, with some DCs extending their dendrites to the epithelium¹¹⁵. Three major subsets of dendritic cells were identified: CD103⁺ CD11b⁻ cells, CD103⁺ CD11b⁺ cells, and CD103⁻ CD11b⁺ cells^{115,116}. Among the three subsets, CD103⁻ CD11b⁺ cells, located mostly in the FAE or NALT, predominate over other types of DCs¹¹⁵. After nasal immunization or infection, CD103⁻ CD11b⁺ cells migrate to cervical lymph nodes and present antigens to CD4⁺ T cells^{116,140–142}. The CD103⁺ CD11b⁻ DCs, which are mostly distributed along the peripheral site of the NALT, skin, lungs, and intestine, efficiently uptake viral antigens and subsequently activate CD8⁺ T cells *via* cross presentation^{115,143,144}. Furthermore, studies on nasal CD103⁺ DCs have proved that activating CD103⁺ DCs in NALT stimulates the vaccine-specific IgA induction^{145,146}. Specifically, the intranasal administration of influenza hemagglutinin subunit with polyinosine-polycytidylic (polyI:C) enhanced the production of vaccine-specific IgA¹⁴⁶. A mechanistic study revealed that polyI:C was endocytosed into CD103⁺ DCs and activated the TLR3

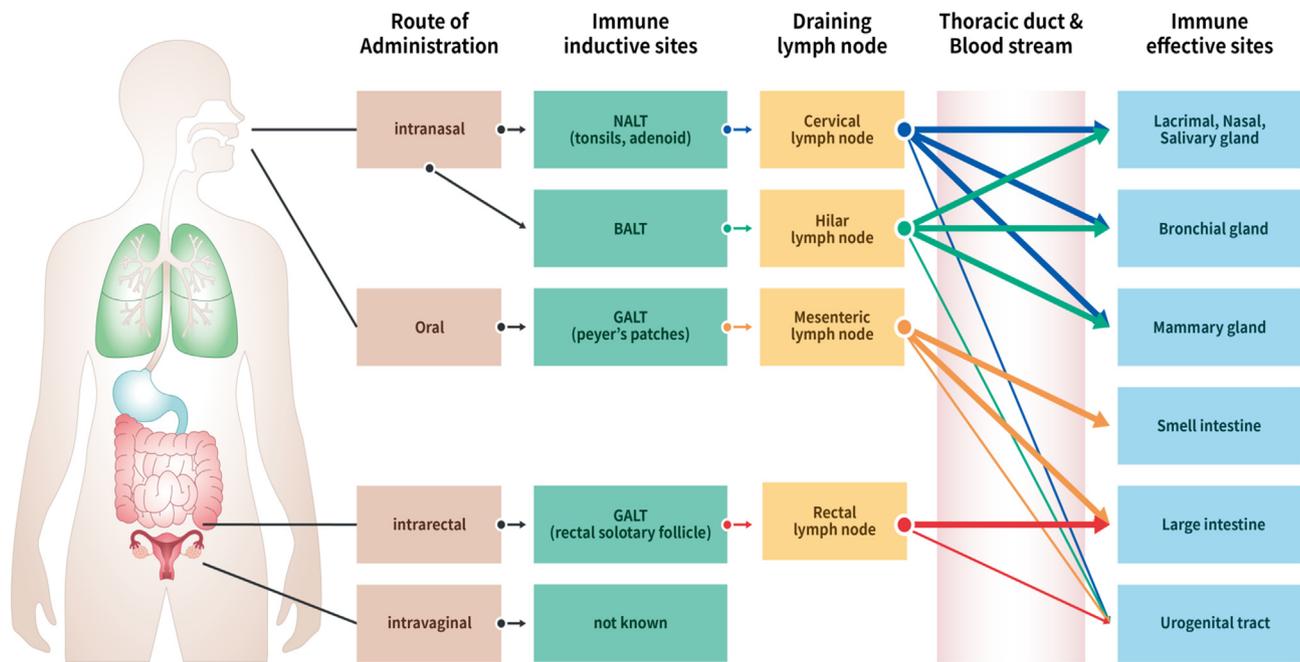


Figure 6 Different routes of administration localize antigens to specific immune inductive sites. Mucosal immune inductive sites consist of NALT (tonsils, adenoid), BALT (no known equivalent tissue in human bronchi), GALT (Peyer's patch), and rectal solitary follicle of the rectum. Each immune inductive site activates antigen-specific lymphocytes, which are subsequently drained to the corresponding lymph nodes (cervical lymph node, hilar lymph node, mesenteric lymph node, rectal lymph node) and circulate through the bloodstream. Circulating effector T cells and B cells are preferentially recruited to the site of antigen exposure than other immune effective sites. However, tissue-specific adhesion molecules and chemokine receptors of lymphocytes enable localization to distant organs that express the corresponding receptor or ligand. Such receptors or ligands are widely expressed in various types of mucosal organs, thereby eliciting immunity in distant organs.

signal, which subsequently induced T cell-dependent IgA production¹⁴⁶. Accordingly, targeting nasal CD103⁺ DCs can be an attractive approach for inducing immunity in the nasal mucosa.

Peyer's patch follows a similar pattern to NALT. Vaccine or viral antigens transported by M cells can be captured by CD11b⁺ DCs in the basolateral side of the M cells. Capturing of the antigen induces expression of CCR7 in CD11b⁺ DCs, which triggers migration to the interfollicular region and presentation of antigen to the interfollicular T cell area. Activation of effector T cells induces isotype class switching and affinity maturation in B cell follicles¹⁴⁷. CD103⁺ DCs in the Peyer patch migrates into the afferent lymphatic vessels and cross presents antigen to CD8⁺ T cells in the mesenteric lymph node. Interaction of CD103⁺ DC and T cells also stimulates expression of CCR9 and $\alpha 4\beta 7$ integrin of T cells, which act as gut-homing receptors¹⁴⁸.

Despite their efficient antigen cross-presentation and CD8⁺ T cell activation, CD103⁺ DCs are also known as efficient inducers of FOXP3⁺ T_{reg} cells in the intestine^{141,149}. Specifically, mesenteric lymph node-derived CD103⁺ DCs exhibit high expression of tissue plasminogen activator, TGF β 2, and latent TGF- β -binding protein 3, which are crucial for the activation, secretion, and localization of latent TGF- β ¹⁴⁴. Activation of TGF- β stimulates differentiation and maintenance of FOXP3⁺ T_{reg} cells (induced T_{reg}, iT_{reg})¹⁵⁰. Additionally, CD103⁺ targeting antibody M290 coupled with ovalbumin (M290. OVA) was administered intratracheally¹⁵¹. Administration of M290. OVA significantly reduced airway eosinophilia, Th2 cytokines, and anti-OVA IgE antibody responses, which implicates induction of immunotolerance *via* CD103⁺ targeting¹⁵¹.

These observations implicate that CD103⁺ DC exerts two opposing effects on the mucosal immune system: (1) cross-

presentation of foreign antigens with subsequent activation of CD8⁺ T cell, leading to protection from viral infections and (2) induction of immunotolerance by triggering differentiation of FOXP3⁺ T_{reg} cells (induced Treg, iTreg). Such counter-effects of CD103⁺ DCs can be explained by two hypotheses: 1) cross-presentation of foreign antigens or self-antigens and 2) exposure to differing molecular cues and environmental conditioning in each mucosal organ¹⁴⁴. Firstly, the cross-presentation of antigens to CD8⁺ T cells leads to one of two different pathways: cross-priming or cross-tolerance¹⁴⁴. While cross-priming would lead to the cellular immune responses against the foreign antigen, cross-tolerance would lead to T cell deletion or T cell anergy, which subsequently induces tolerance. Cross-tolerance is induced through the phagocytosis of apoptotic cells by CD103⁺ DCs¹⁴⁴. Secondly, in the gut microenvironment, a significant amount of retinoic acid is formed by epithelial enzymes¹⁵². Retinoic acid acts as a crucial cofactor for the induction of intestinal iT_{reg} mediated by TGF- β . While *in vitro* cultures of MLN CD103⁺ with retinoic acid and TGF- β have sufficiently induced T_{reg}, the addition of retinoic acid inhibitors significantly reduced the induction of T_{reg}¹⁵³. Moreover, a study demonstrated that CD103⁺ DCs adjacent to intestinal epithelial cells obtain a tolerogenic phenotype, which leads to the activation of T_{reg}¹⁵⁴. This further supports the hypothesis that environmental conditioning and molecular cues adjacent to CD103⁺ DCs determine the immunological pathway.

In this aspect, further studies on CD103⁺ DCs and characterization of the factors that determine the state of immune activation or immune tolerance would enable control of the activity of the immune system *via* DC targeting. In the case of mucosal COVID-19 vaccines, skewing the balance to immune activation would be

an important strategy for protection against respiratory infections. Thus, it is important to consider such bidirectional capacity and the heterogeneous subgroups of DCs.

4. Nanotechnology-assisted COVID-19 mucosal vaccine

4.1. Challenges and practical strategies for mucosal vaccination

Challenges and the rational design of a mucosal vaccine are closely related to the distinct immunological traits of the mucosal system. Since the mucosal system is constantly exposed to various types of environmental irritants, pathogens, and allergens, it has developed its own measures to guard the human body against various types of environmental pathogens¹⁵⁵. Exposure to various types of antigens renders the mucosal system with three distinct protective mechanisms to keep the antigens from entering and harming the human body. Firstly, the mucosal surface exhibits a harsh environment through its innate defense mechanisms^{156,157}. Antigens can be diluted, captured, or removed from the mucosa through mucus, enzymatic degradation, and ciliary clearance. Secondly, the mucosal system is able to distinguish harmless antigens from harmful counterparts and induce immune tolerance against them. This prevents unnecessary inflammation against harmless antigens. Thirdly, the mucosal surface has its own specialized epithelial cells (FAE) and organized lymphoid tissues. Through this specialized compartment known as MALT, the mucosal immune system is able to sample antigens from the mucosal surface (immune inductive site) and recruit the activated effector lymphocytes to the lamina propria of various mucosa and form cytokine and secretory IgA at the immune effector site¹⁰⁰. The secretory IgA neutralizes harmful toxins and pathogens in the mucosal environment, while the effector T and B cells retain long-term immune activity against specific pathogens¹⁵⁸. The distinct immunological features of the mucosa guide the effective design of the mucosal vaccine in three aspects: the vaccine should remain stable under physical and chemical barriers of a harsh mucosal environment, breach the epithelium through precise targeting of the sampling site of the mucosal immune system, and subsequently induce the immune activation rather than immune tolerance. Accordingly, the formulation of a mucosal vaccine should possess stability, capability of targeted delivery, and immunogenicity to achieve effective mucosal immunity against pathogens. Thus, nanotechnology-based delivery agents can be utilized to fulfill the following three aspects: (1) evasion and protection from mucosal barriers, (2) targeted delivery to the specific types of cells, and (3) activation of the immune system. The nanotechnology-based formulation is expected to impart favorable traits (protection of antigen, targeted delivery, and immunogenicity) for mucosal vaccine *via* several strategies: (1) modification of the physicochemical properties (such as size, shape, charge) of the nanoparticles, with a size of 20–200 nm in diameter and positively charged nanoparticle showing stronger intranasal immune response^{75,159}, (2) encapsulating or attaching antigens to the nanoparticle, (3) targeting specific cells through targeting moieties or ligands, and (4) using nanoparticle with innate adjuvant activity and equipping nanoparticles with immune activating signals or molecules (Fig. 7).

Pathogens, foreign materials, as well as mucosal vaccines are generally exposed to the harsh environment of the mucosal surface, which includes dilution of materials through mucus, degradation mediated by protease and nuclease, and mucociliary clearance. As a

result, soluble non-adherent antigens are removed and diluted by the constant renewal of the mucus and are not recognized as non-self, leading to immune tolerance toward the antigens¹⁰⁰. Thus, designing effective delivery agents or formulations that have the ability to protect the antigen and induce mucosal immunity is crucial to achieving a successful induction of mucosal immunity. Formulation of mucosal vaccines requires that the formulation should reach FAE without being degraded or removed by the harsh mucosal environment and host defense mechanism (stability). After being delivered to FAE, the formulation should enable the effective uptake of antigens and mucosal adjuvants (antigen uptake). Subsequently, antigen and adjuvant should induce potent mucosal immunity through antigen presentation and lymphocyte activation (immunogenicity)²¹. Therefore, antigen encapsulated with a well-designed particle can be easily recognized by the innate immune system and act as a stronger inducer of mucosal immune response compared to its soluble counterparts^{24,160}.

Considering that ACE2 receptors recognized by SARS-CoV-2 are highly expressed on the oral and mucosal epithelium, inducing protective immunity *via* oral and intranasal delivery routes can be possible candidates for administration¹⁶¹. Within oral and intranasal routes, intranasal vaccination induces robust immune activation in the upper respiratory tract and extended lifespan of lung T_{RM} cells as discussed above. By contrast, as mentioned above, oral immunization induces strong immune responses in the digestive tract and salivary glands rather than in the upper respiratory tracts. In addition, low bioavailability owing to gastric degradation and first-pass metabolism and dilution of the vaccine due to intestinal content further hinders the efficacy of oral vaccines^{29,162}. In this aspect, nasal vaccine delivery can be a suitable route of administration for the prevention of SARS-CoV-2 infection. For the development of intranasal nanovaccines, the target cell and formulation of the vaccine are crucial factors to be considered.

4.1.1. Polymeric nanoparticles

By modifying the physicochemical properties and surface molecules of biodegradable and biocompatible polymeric nanoparticles, target delivery toward MALT can be achieved. Specifically, equipping nanoparticles with mucoadhesive materials (passive targeting) and anchoring certain lectins, ligands, or specified antibodies that can selectively bind to M cells are commonly utilized methods.

The first strategy is modifying the surface property of the nanoparticles by incorporating and anchoring mucoadhesive material to the nanoparticle. The mucoadhesive property of such formulations increases the chance of antigen uptake by prolonging the contact time of the vaccine and mucosal surface without being degraded²⁴. The mucus is composed of glycoprotein, lipid, inorganic salt, and water. It is formed by crosslinking the layers of mucin fiber, which is a polymer formed by disulfide bonds between the mucin monomers. The negative charge of mucin is formed by a glycoprotein consisting of numerous sialic acids and sulfates. In addition, its cysteine-rich domain of the naked protein region forms a hydrophobic domain. Thus, a particle having a positive charge and a hydrophobic property adheres to the negatively charged mucus through electrostatic and hydrophobic interactions.

Secondly, active targeting of cells and organs through receptor-ligand interaction can be an attractive strategy for antigen delivery. Through surface modification, nanoparticles can be selectively delivered to FAE or mucosal surfaces. The typical target cell of such a delivery platform is the M cell, a specified epithelial cell located in FAE, acting as a portal of entry for antigens into APCs

and lymphocytes. Its specialized ability to capture antigens and transport them to APCs in the basolateral pocket makes it an interesting potential target site for mucosal vaccines. However, the inadequate amount of M cells in the human body implies that specific targeting and binding of M cells is crucial for the successful induction of mucosal immunity¹⁶³. Thus, by modifying the surface of nanoparticles with certain lectins, ligands, or specified antibodies that can selectively bind to M cells, the antigen can be effectively delivered to the site of induction (Fig. 8)¹⁰². In addition, coating the outer membrane of the mucosa-breaching bacteria and viruses with a nanoparticle surface enables the evasion of the host defense mechanism, strong adhesion to M cells, and strong induction of mucosal immunity. Such formulations include a virus-like particle (VLP) and membrane-coated nanoparticle.

Polymeric nanoparticle, a particulate delivery agent encapsulating antigens, is able to fulfill the aforementioned features by endowing a wide variety of physicochemical properties and binding capacity to certain target tissues¹⁶⁴. Due to their structural stability, strong immunogenicity, and capability of controlled release, various types of polymeric nanoparticles and microparticles are now being studied as delivery agents of mucosal vaccines¹⁶⁵. Among the various types of polymer materials, synthetic polymeric nanoparticles, poly(D,L-lactide-co-glycolide) (PLGA), poly(ϵ -caprolactone) (PCL), and poly(D,L-lactide) (PLA) are widely investigated due to their high biocompatibility, protective function for antigen and adjuvant, capability of cell and organ targeting, capability of controlled release, and low toxicity^{165,166}. PLGA is the most extensively studied polymeric nanoparticle for the delivery of vaccine antigens. Specifically, PLGA-encapsulating TLR agonists induced strong antigen-specific mucosal immune response against various types of pathogens¹⁶⁶. Also, surface modification of PLGA with mucoadhesive materials and M cell targeting moieties enables efficient induction of mucosal immunity^{167,168}.

Another type of synthetic polymeric nanoparticle is dendrimers, which contain a central core surrounded by hyperbranched nano-sized symmetric macromolecules with a monodisperse structure¹⁶⁹. As branched elements are built around the central core, dendrimers form internal cavities for the encapsulation of antigens and distinctive size, shape, and surface charges. Due to such features, physicochemical and biological properties can be customized through the introduction of different functionalized surface moieties¹⁶⁹. For instance, intranasal immunization of the HIV-1 gp120 peptide complexed with fourth-generation polyamidoamine dendrimer (G4-PAMAM) induced peptide-specific IgG and IgA responses in the serum, nasal wash, and vaginal fluid of mice¹⁷⁰.

Natural polymeric nanoparticles exhibiting bioadhesive properties include chitosan, maltodextrin, alginate, hyaluronic acid, carboxymethyl cellulose, hydroxyethyl cellulose, and pectin^{78,171}. For instance, chitosan, a cationic polymer synthesized by deacetylation of chitin, has widely been used for mucosal vaccines¹⁷². Chitosan is able to adhere to the mucosal membrane for a long period and enhance paracellular transportation without being affected by mucociliary clearance. Chitosan also possesses adjuvant properties and induces mucosal immunity and further enhances the immunogenicity of the mucosal vaccine^{156,173}. However, since chitosan exhibits hydrophobicity at physiological pH, a trimethylated form of chitosan (TMC) has been used for enhancement of hydrophilicity and was employed in the nasal vaccine platform^{174,175}. Moreover, PLGA particles exhibit low bioadhesive capability. Thus, chitosan can also be employed to enhance the mucosal adhesive property of PLGA particles, further enhancing the mucosal immunogenicity of the vaccine¹⁶⁸.

4.1.2. Liposomes

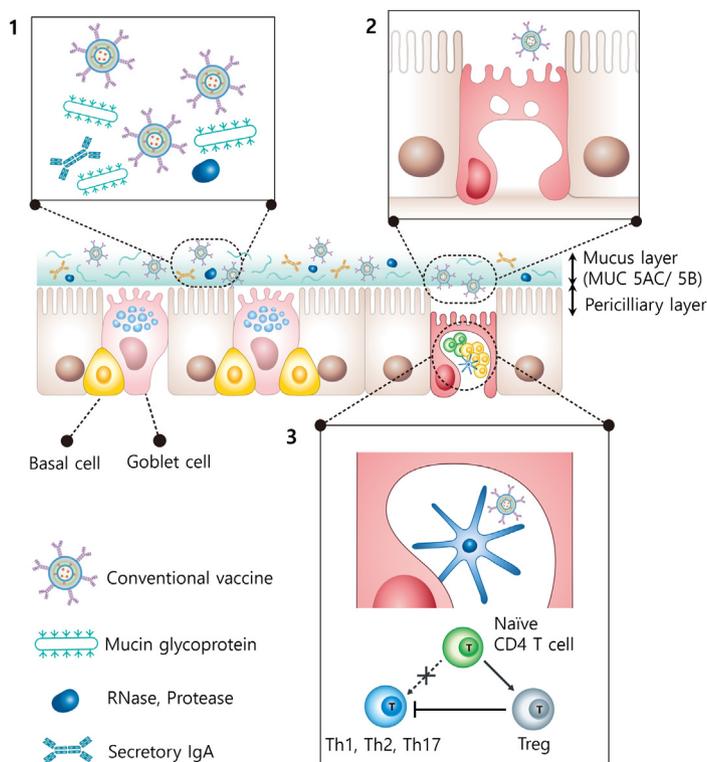
Liposomes consist of biodegradable, non-toxic, and non-immunogenic phospholipids. Their wide variety of physicochemical properties, including various sizes, lipid composition, and electrical charges, enables a rational vaccine design¹⁷⁶. Additionally, liposomes can protect antigens from being degraded by the harsh mucosal environment by encapsulating the antigens in the hydrophilic core or complexing antigens with acyl chains or electrically charged surfaces¹⁷⁶. Importantly, coating or modifying the surface of the liposomes with certain ligands and adjuvants enable the selective delivery of liposome toward certain organs with the subsequent induction of a stronger immune response^{177,178}. This leads to a more potent and selective immune response against the encapsulated antigen. In this context, liposomes can be an attractive delivery platform for mucosal vaccines since M cell targeting can also be achieved through the modification of liposomes with specific ligands. As mentioned in the polymerized nanoparticle, lectin, M cell-specific ligand, and immunoglobulin can be utilized in the polymerized liposome for active targeting of M cells (Table 5, Fig. 8). A typical example of such a platform is BSA-encapsulated liposomes coated with UEA-1, which has shown immune-stimulating results in a mouse model compared to the uncoated liposomes¹⁷⁹.

4.1.3. Inorganic nanoparticles

Among inorganic nanoparticles, gold nanoparticles (AuNPs) are commonly used for vaccination, due to their efficient internalization by antigen-presenting cells and inherent immunostimulatory effects, inducing a subsequent immune activation^{180–182}. Further, the ease of size and morphology customization and functionalization makes gold nanoparticles potential antigen delivery agents¹⁸¹. Using the stable bond between the thiol groups and gold, AuNPs can be easily functionalized or conjugated with antigens or adjuvants⁷⁵. Specifically, peptide consensus M2e of influenza virus A, which has a cysteine residue at the C-terminal, was conjugated to AuNPs through a bond between the thiol group and gold (M2e-AuNP)¹⁸³. Intranasal administration of M2e-AuNP adjuvanted with CpG (cytosine-guanine rich oligonucleotide) induced M2e-specific IgG serum antibodies with cross-reactivity to various types of influenza viral strains¹⁸³. Another method of AuNP functionalization is polyelectrolyte multilayer (PEM) coating of AuNPs. The gold nanoparticle template was coated layer by layer, with alternating deposition of anionic adjuvant polyinosinic-polycytidylic acid (polyIC) and SIINFEKL peptide antigen modified with nona-arginine. The former acts as a poly-anionic TLR agonist, and the latter as an antigen with a cationic anchor. The resulting (immune-PEM coated) AuNPs were efficiently internalized by DCs and induced a high level of circulating antigen-specific CD8⁺ T cells¹⁸⁴. Despite its wide range of functionalization capacity, the safety of gold nanoparticles remains an obstacle. As gold nanoparticles can accumulate in specific organs such as the liver and spleen in the long term, the distribution of gold nanoparticles and toxicity of accumulated gold nanoparticles in various cell types should be addressed^{185,186}.

Another type of inorganic nanoparticle, carbon nanotube (CNT), is a cylindrical structure composed of carbon atoms arranged in a planar honeycomb lattice. Carbon nanotubes can be classified into single-wall carbon nanotubes (swCNTs) with an approximate diameter of 1–2 nm and multi-wall carbon nanotubes (mwCNTs) with an approximate diameter of 2–100 nm¹⁸⁸. Recently, CNTs have been extensively studied as vaccine carriers due to their high stability, high loading capacity, diverse ways of functionalization, and ease of mass production^{187,188}. However,

a. Challenges of mucosal vaccines



b. Designing of mucosal nanovaccines

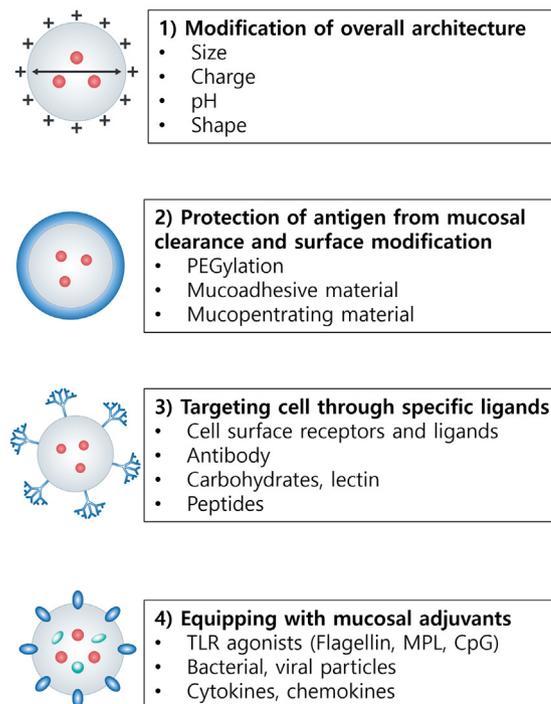


Figure 7 Challenges of mucosal vaccines and strategies of mucosal nanovaccines. (a) Presence of physical, chemical epithelial barrier in the mucosa (mucin, RNase, protease, antibody, and ciliary clearance), (b) Off-targets due to lack of antigen sampling cells in the mucosal epithelium, (c) Immunotolerant property of the mucosal immune system. To overcome such obstacles, several strategies can be employed: designing mucosal nanovaccines including modification of overall nanoparticle architecture, protection of antigen from the external environment through surface modification, targeting specific cells through ligands, and equipping nanoparticles with mucosal adjuvants. MUC, mucin.

due to their hydrophobicity and toxic effects on macrophages, CNTs are generally functionalized to reduce toxicity and increase biocompatibility. Common functionalization methods of CNTs include the introduction of a carboxylic acid or amino group to their surface. The carboxylic acid group or protonated amine group of CNTs can increase biocompatibility and act as a moiety to conjugate or electrostatically adsorb protein antigens or DNA¹⁸⁷. Functionalized CNTs possess inherent immunogenic activity, which is efficiently captured by antigen-presenting cells and induce the production of pro-inflammatory cytokines, leading to robust immune responses¹⁸⁸. Moreover, targeting M cells or DCs through CNT surface modification can be a potential strategy to induce a potent mucosal immune response¹⁸⁹. However, the safety of CNTs remains a concern. Due to its pro-inflammatory effect, airway exposure to CNTs has led to the exacerbation of lung injury in a mouse model¹⁹⁰. Despite a successful reduction of toxicity *via* functionalization, further studies on the toxicology of CNTs and assessment of regimen and functionalization strategies to control an appropriate level of immune activity are required¹⁹¹.

4.2. Nanotechnology-assisted COVID-19 mucosal vaccine for M cell targeting

4.2.1. Target tissue and cell of mucosal vaccine

Since the major route of transmission of COVID-19 is the respiratory droplets entering the mucosal membranes of the nose, eyes,

and mouth, stimulating the mucosal immunity in the luminal surface of such organs can be a key strategy to achieve sterilized immunization. NALT is one of the major constituents of MALT, which acts as an induction site of mucosal antigen-specific immune responses¹⁹². The Waldeyer's ring, composed of paired palatine, nasopharyngeal tonsils, and lingual tonsils, is considered the human anatomical equivalent of the NALT in mice (Fig. 9a)¹⁹³. NALT is known to contain all immunocompetent cells required for the induction of antigen-specific T and B cell responses¹⁹⁴. Components of nasal mucosal immunity are summarized in Table 6. Specifically, human tonsils consist of lymphoid follicles and interfollicular tissues supported by reticular cells¹⁹⁵. Lymphoid follicles contain germinal centers filled with follicular DCs and B cells. The interfollicular region contains substantial amounts of APCs and $\alpha\beta$ T cells (Fig. 9b). Due to its specialized structure, the intranasal administration of the vaccine-induced mucosal immune responses in NALT and was verified to induce strong mucosal immunity in the respiratory immune system in addition to the systemic immunity^{117,194}. For example, the intranasal administration of live attenuated influenza virus vaccine has proven to be effective in preventing seasonal influenza infection¹⁹⁶. Additionally, it has been demonstrated that nasal immunization induces the activation of CCR10 and $\alpha4\beta1$ integrin expressing IgA-secreting B cells, which can migrate and localize to CCL28 and VCAM1 expressing organs (respiratory tract and genital tract)^{108,197}. In addition, nasal administration requires a

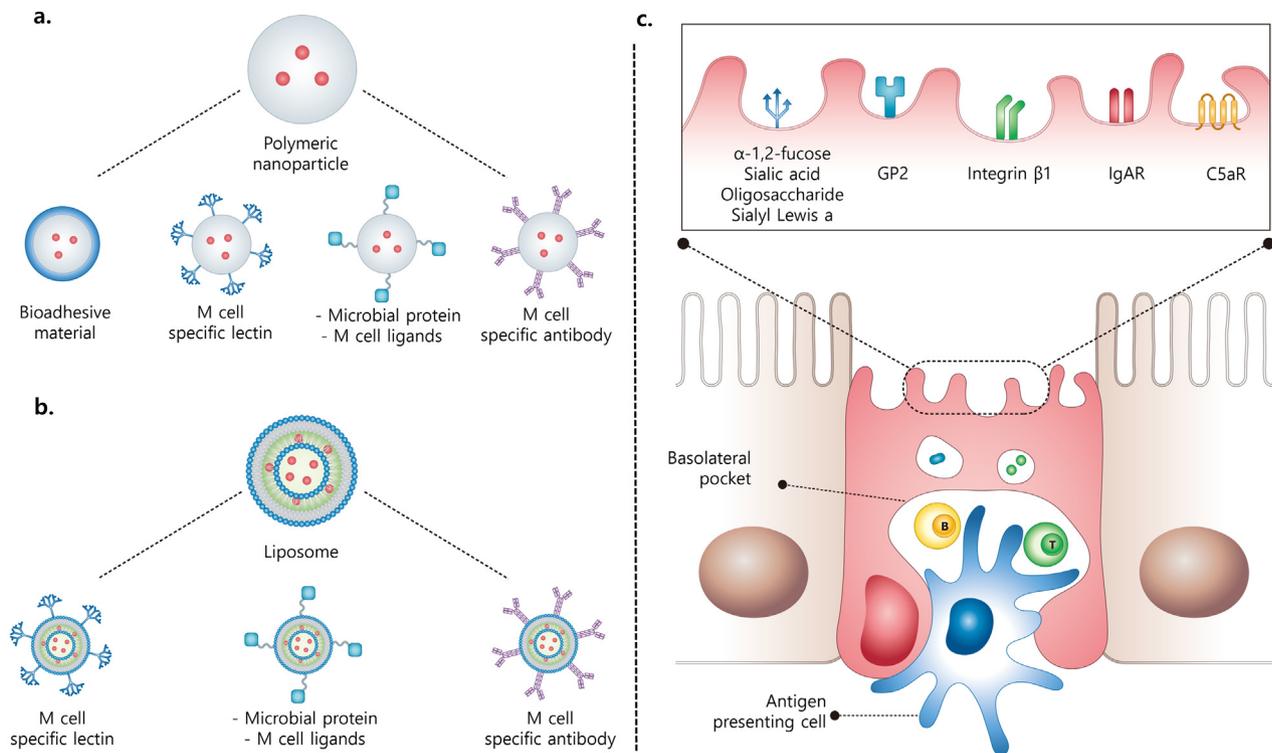


Figure 8 Schematic diagram of polymeric nanoparticle or polymerized liposomes modified by various M cell ligands (lectins, microbial proteins, antibodies). Modified particles and liposomes selectively bind to apical receptors of the M cell and are readily transcytosed through the M cell. This enables effective antigen delivery to mucosal immune inductive sites. GP2, glycoprotein 2; IgAR, IgA receptor; C5aR, C5a receptor.

smaller dose of the antigen compared to oral administration since antigens are not exposed to the digestive enzymes of the gastrointestinal tract, which exhibits a less hostile environment¹⁹⁴.

4.2.2. Nanotechnology-assisted mucosal vaccine for M cell targeting

For the successful antigen capture and transfer mediated by the M cell, three requirements need to be fulfilled. Firstly, the vaccine should reach and strongly bind to the apical membrane of the M cell. Secondly, the antigen and adjuvants should be effectively delivered into the M cell and transferred to the APCs. Thirdly, the antigen and adjuvants should induce a potent immune response.

Accordingly, either non-specific binding or receptor-mediated specific binding to the M cell can be considered the main delivery strategy. In the case of non-specific binding, the surface potential of the particle and hydrophobicity would act as important factors for interaction. Specifically, a particle with high hydrophobicity or positive charge is known to strongly interact with the M cell¹⁹⁸. Nevertheless, since the chemical and physical environment of the mucus and the adjacent luminal protein constantly fluctuates, it is likely that the physiochemical property of the delivery agent would also change, leading to inconsistent efficacy. Despite its specialized role in capturing the macromolecules and particles, the proportion of the M cells in the respiratory epithelium is very low¹⁶³. Thus, active targeting of the M cell through a specific receptor-ligand interaction would lead to consistent and accurate delivery of the antigen to the M cell¹⁰². Such modalities can be achieved through nanoparticle coating of M cells binding ligands or mimicking the outer surface of the M cell breaching virus or bacteria through membrane-coated nanoparticles.

For effective sampling and uptake of antigen, M cell expresses various types of receptors. A typical example of such a receptor is

glycoprotein 2 (GP2), which not only acts as a specific marker of the M cell but also as a receptor of the Fim H component of Type I pili, which enables the effective uptake of microbes. Type I piliated bacteria *E. coli* and *S. typhimurium* do not undergo transcytosis in GP2-deficient M cells¹⁹⁹, which implicates the crucial role of the glycoprotein and receptor-ligand interaction in the M cell-mediated antigen capture. Furthermore, prion protein (PrPc), a protein that is highly expressed on the apical membrane of the M cell, is known to interact with Hsp60-containing bacteria such as *Brucella abortus*, which subsequently leads to the internalization of the bacteria²⁰⁰. In addition, ANXA 5 of the M cell specifically recognizes lipid A of LPS, which enables the uptake of gram-negative bacteria and peptidoglycan recognition protein 1 (PGLRP-1), which is highly expressed on the M cell, recognizing the peptidoglycan of bacteria²⁰¹.

Thus, understanding the receptor-ligand interaction between the M cell and antigen is crucial in designing a vaccine that is easily recognized by M cells and efficiently transferred to the APCs on the basolateral side. Such target receptors or glycoproteins include α -L-fucose, claudin 4 protein, integrin- β 1, GP2, TNF α -induced protein 2 (Tnfaip2), and C-C motif chemokine ligand 9^{192,202}. The investigated M cell-specific molecules and their ligands are summarized in Table 7²⁰⁴, while the nanotechnology-based M cell targeting strategies are illustrated in Fig. 8.

4.2.2.1. Lectin-coupled polymeric particle for M cell targeting. The M cell has a specific glycocalyx structure on its cell surface, unlike other epithelial cells. Thus, M cell-specific carbohydrate residues can be potential targets for lectin-mediated delivery²⁰⁴. An example of M cell-specific carbohydrate residues is α -L-fucose. By incorporating lectin that can bind with α -L-fucose of the M cell through surface modification of

Table 5 Functionalizing liposomes with lectins or ligands for M cell-targeted delivery (cited from Ref. 21).

Strategy to enhance M cell delivery	Lectin or ligands	Receptor	Conjugation
Lectin-coupled liposomes	UEA-1	α -Fucose residues	Liposomes Liposomes encapsulated with HbsAg
Ligands-modified liposomes	WGA	(D-GlcNAc) ₂ , sialic acid	Liposomes
Antibody-coated liposomes	CTB subunit	Ganglioside GM1	Liposomes
	Secretory IgA		Liposomes

UEA-1, *Ulex europaeus* agglutinin 1; WGA, *Triticum vulgare* wheat germ agglutinin; CTB, cholera toxin B subunit; D-GlcNAc, *N*-acetylglucosamine; GM1, monosialotetrahexosylganglioside.

nanoparticle, the antigen can be successfully delivered to the M cell^{171,205}. The most investigated candidates are lectin *Ulex europaeus* 1 (UEA-1) and LTA. In particular, the M cell targeting vaccine using UEA-1 coupled antigen-encapsulated liposome has successfully induced antigen-specific secretory IgA response²⁰⁶. Unfortunately, UEA-1 cannot be used for human M cell-targeting vaccines due to the lack of specific receptors in human cells²¹. In addition, coupling the antigen with NKM 16-2-4, a monoclonal antibody exhibiting higher affinity towards α -L-fucose compared to UEA-1, resulted in the successful induction of the antigen-specific IgA response²⁰⁷.

4.2.2.2. Microbial protein-mediated M cell targeting. Another strategy for the targeted delivery of antigen is modifying the polymeric nanoparticle with an M cell-specific ligand or microbial adhesin. Diverse types of microorganisms utilize M cells as a route for host invasion¹⁰². Since these microorganisms use

microbial adhesin to bind with M cells, microbial adhesin has been exploited as a delivery guide to target M cells. Such microorganisms tend to survive in hostile mucosal environments, implying that microbial adhesins are generally resistant to mucosal degradation. Additionally, some microbial adhesins tend to be internalized into epithelial cells in addition to adhesion, thus further enhancing the induction of mucosal immunity¹⁰².

An example of such microbial adhesion is the invasion of *Yersinia* species. *Yersinia* is one of the typical microbes that exploit the intestinal M cell as an entry route for invasion. The mechanism of M cell targeting is mainly mediated by protein invasion, which can selectively bind with the β 1 integrin of the M cell²⁰⁸. By applying this mechanism to a mucosal vaccine, a study has proved the effective binding of the M cell and the increased uptake of antigen by coating an invasion-coated membrane to a polystyrene particle²⁰⁹. Moreover, the CPE30 peptide derived from the C-terminal domain of *Clostridium perfringens*

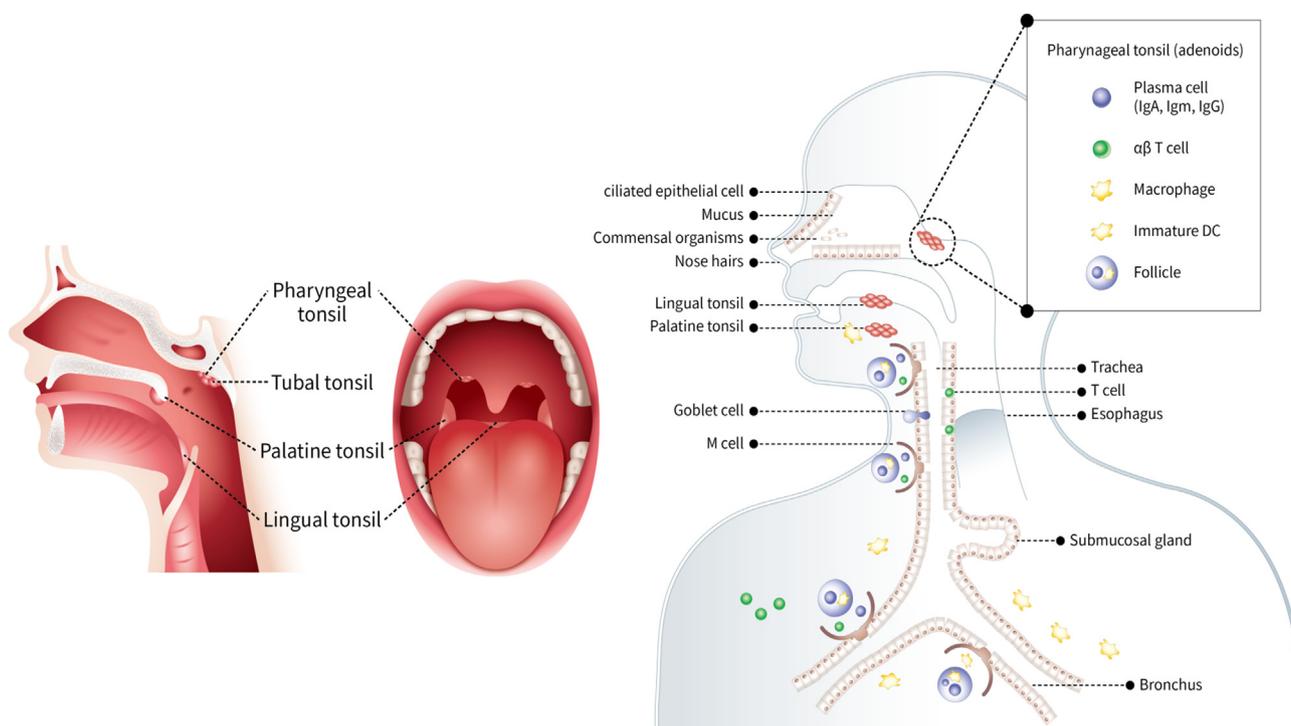


Figure 9 (a) Four tonsils comprise Waldeyer's ring (pharyngeal tonsil, tubal tonsil, palatine tonsil, and lingual tonsil). (b) Human tonsils are composed of the follicle and interfollicular tissue. The follicle contains follicular dendritic cells and B cells, which induce activation and affinity maturation of B cells. Interfollicular tissue includes substantial amounts of antigen-presenting cells.

Table 6 Components of the nasal and gastrointestinal mucosal immune system (cited from Ref. 116).

Immune system	Nasal mucosal immune system	Intestinal mucosal immune system
Inductive sites	Nasal-associated lymphoid tissue (NALT)	Peyer's patch and isolated lymphoid follicles (ILF)
Effective sites	Upper airways, lacrimal, nasal and salivary glands	Small bowel, large bowel
T cells	Naïve T cell	Memory T cell
B cells	CD62L, $\alpha 4\beta 1$, CCR7, CCR10	$\alpha 4\beta 7$, $\alpha 4\beta 1$, CCR9, CCR10
DCs	CD103 ⁺ CD11b ⁻ CD103 ⁺ CD11b ⁺ PDCA-1 ⁺ CD11c ⁺ pDC CD11b ⁺ CD64 ⁻ F4/80 ⁻	CD103 ⁻ CD11b ⁻ CD103 ⁺ CD11b ⁻ CD8 α ⁺ CX3CR1 ⁻ CD103 ⁺ CD11b ⁺ CX3CR1 ^{low} CD103 ⁻ CD11b ⁺ CX3CR1 ^{int}
Macrophages	CD11b ⁺ CD64 ⁺ F4/80 ⁺	CD64 ⁺ CX3CR1 ^{hi}
Immune response	Th1/Th2	Th2 > Th1

CCR, C-C chemokine receptor; PDCA-1, plasmacytoid dendritic cell antigen-1; pDC, plasmacytoid dendritic cell.

enterotoxin selectively binds to claudin-4 in the M cells, a tight junction transmembrane protein that is highly expressed in Peyer's patch M cells²¹⁰. PLGA nanoparticle incorporating a fusion protein with influenza hemagglutinin fused with CPE30 has shown enhanced delivery through the M cell *in vivo*²¹¹.

Another example of microbial adhesion is the reovirus protein $\sigma 1$. Reovirus is one of the viruses that are typically known to adhere to the M cell and is endocytosed by the intestinal M cells *in vivo*²¹², where its mechanism is mediated by the viral haemagglutinin $\sigma 1$ protein²¹³. By incorporating $\sigma 1$ protein into a liposome, enhanced adhesion to rat Peyer's patch tissues has been observed²¹⁴.

4.2.2.3. Immunoglobulin-mediated M cell targeting. Secretory IgA can also adhere to the apical membrane of the M cell, which promotes the uptake of antigen²¹⁵. The mechanism of adherence and cellular uptake is mediated by Dectin-1, a small type II transmembrane protein of the C-type lectin family²¹⁶. By taking this mechanism into account, chitosan-dextran sulfate nanoparticles loaded with pertussis toxin and sIgA were developed for the intranasal delivery of proteins, which showed an enhanced uptake of nanoparticles by the M cells²¹⁶.

A typical example of an M cell-targeting vaccine platform is a study on the UEA-1-conjugated PLGA nanoparticle that uses the TLR agonist monophosphoryl lipid A (MPL) as a mucosal adjuvant (Fig. 10.)²¹⁷. Since UEA-1 of PLGA nanoparticles can interact with α -fucose of the M cell, the vaccine is effectively bound to the M cell, delivered to DCs, and successfully induced mucosal IgA and serum IgG responses¹⁶⁷. In addition, the lectin-anchored HbsAg-loaded PLGA nanoparticle effectively binds to α -fucose of the M cell and can be utilized as an oral immunization platform²¹⁸. A PEG-PLGA nanoparticle with the RGD motif (RGD-decorated PEG-PLGA NP) bound to $\beta 1$ integrin of M cells and enhanced the cellular uptake of the antigen with adjuvant activity²¹⁹. Another application of nanoparticle used glycochitosan surface-coated mucoadhesive PLA nanosphere, which successfully induced mucosal immunity²²⁰.

4.2.3. Nanotechnology-assisted COVID-19 mucosal vaccine for DC targeting

Considering the different roles of each DC subset and the bidirectional capacity of DC, two main aspects should be fulfilled for successful immune activation: (1) selective delivery of antigen toward a proper type of DC *via* modulating the surface molecules of the nanoparticles that bind to the receptors of DCs and (2) shifting the balance of DCs toward immune activation *via*

equipping nanoparticles with proper signals and stimuli, such as activating adjuvants and transcription factors.

4.2.3.1. Modifying the physical properties of nanoparticle for DC-mediated immune activation. One of the major strategies for immune activation is through optimizing the charge, size, shape, and rigidity of nanoparticles. Firstly, positively charged nanoparticles are known to exhibit immune-activating traits through interaction with negatively charged membranes of the APCs (Fig. 11)^{221–223}. In liposomes, cationic lipids such as 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) and 3β -[N-(N',N'-dimethylaminoethane)-carbonyl]cholesterol (DC-Chol) exhibited DC-activating effects by inducing the expression of DC maturation markers and production of proinflammatory cytokines¹³⁸. By contrast, negatively charged nanoparticles containing

Table 7 M cell-specific receptors and their corresponding ligands (cited from Ref. 203).

Ligand	Target receptor of M cells
<i>Ulex europaeus</i> 1 (UEA-1)	α -1,2-Fucose
<i>Aleuria aurantia</i> (AAL)	α -L-Fucose
Galectin-9	N-Glycans/repeated oligosaccharide
Petpide Co1 (SFHQLPARSPLP)	C5aR
Cathelicidin LL-37	P2X7 receptor, formyl peptide receptor 2
Antibody NKC 16-2-4	α -1,2-Fucose-containing carbohydrate
Antibody LM112	Sialyl-Lewis A
Antibody 3G7-H9	Glycoprotein 2
$\sigma 1$ Protein (reovirus)	σ -2,3-Sialic acid
Invasin (<i>Yersinia</i>)	$\beta 1$ Integrin
Long polar fimbriae (<i>E. coli</i> , <i>Salmonella</i>)	Unknown
FimH (<i>E. coli</i> , <i>Salmonella</i>)	Glycoprotein 2/uromodulin
OmpH (<i>Yersinia</i>)	C5aR
Lipoteichoic acid	TLR-2
Phosphorylcholine moiety of LPS	PFAR
Hsp60 of <i>Brucella abortus</i>	Cellular prion protein
Lipid A domain of LPS	ANXA5
Bacterial peptidoglycan	PGLRP-1
SIgA	Unknown
C-Terminal domain of enterotoxin (<i>Clostridium perfringens</i>)	Claudin 4

phosphatidyl serine (PS) showed an immunotolerant effect by mimicking the apoptotic cells^{224,225}.

Secondly, antigen uptake, processing, and presentation mechanisms mediated by APCs are determined by the particle size. Particles smaller than 100 nm tend to go through clathrin-mediated endocytosis, while particles larger than 200 nm tend to go through phagocytosis. The different entry mechanism of particles leads to different endocytic routes of the nanoparticles. Specifically, the cargo of the nanoparticles taken up by phagocytosis is prone to be processed through the endo-lysosomal route and presented *via* the MHC II pathway, which leads to the activation of CD4⁺ T cells. By contrast, nanoparticles smaller than 100 nm resemble the entry mechanism and processing pathway of the virus, and the Th1 response predominates over the Th2 response.

Thirdly, the uptake mediated by APCs is known to be influenced by shape more than size²²⁶. Specifically, in the case of solid nanoparticles, rod-shaped nanoparticles are reported to induce a proinflammatory response compared to their spherical counterparts.

4.2.3.2. Use of DC-specific ligands for DC targeting and DC-mediated immune activation. Targeting DC receptors can be another promising approach for stimulating the mucosal immune system. The ability of DCs to recognize and internalize antigens is attributed to their receptors. DC receptors sense foreign materials, pathogen-associated molecular patterns (PAMPs), and danger-associated molecular patterns (DAMPs) *via* various types of pattern recognition receptors (PRRs). Typical examples of PRRs are TLRs, C-type lectin receptors (CLRs), nucleotide-binding and oligomerization domain-like receptors (NLRs), and retinoic acid-inducible gene 1-like helicases receptors (RLRs)²²⁷. In addition, other types of receptors, such as Fc γ receptor (Fc γ R) and Siglecs, act as important regulators of the immune response. After encountering antigen through the receptor, immature DCs undergo maturation and activation process. Mature DCs migrate to

secondary lymphoid organs *via* chemokine receptors and express co-stimulatory molecules (CD80, CD86, and CD40) to provide a second signal for T cells in the secondary lymphoid organs²²⁷. In this regard, nanoparticles equipped with targeting ligands for specific types of DC receptors can enhance antigen internalization, processing, and presentation. Since different types of DC receptors activate different subtypes of T cells and consequently lead to either immune activation or tolerance, it is important to categorize which receptor to target to obtain the intended outcome.

Firstly, among the various types of receptors, CLRs are an important group of DC receptors that recognize glycosylated antigens. Typical examples of CLRs include DC-specific ICAM-3 grabbing non-integrin (DC-SIGN), dendritic cell natural killer lectin group receptor-1 (DNGR-1), mannose receptor (MR), and a 205-kDa membrane protein (DEC-205) (Fig. 11). Interaction of CLRs with glycosylated antigens are mainly mediated by carbohydrate recognition domains (CRDs), which binds to carbohydrates such as mannose, fucose, galactose, Lewis X, and glucan^{228,229}. Important features of CLRs include phagocytosis and cross-presentation of antigens. Particularly, CLRs activate tyrosine kinase and downstream signaling, which stimulates the NF- κ B pathway and activates immune responses, whereas some types of CLRs, such as DC immunoreceptor (DCIR), contain immune-receptor tyrosine-based inhibitory motifs (ITIMs), which inhibit activation of the immune responses²³⁰. Considering this aspect, targeting specific types of CLRs has been used as a major immune-activating modality for DC-targeting vaccines. Specifically, the intranasal administration of *Yersinia pestis* Lcr V protein fused to anti-DEC-205-antibody with poly I:C as an adjuvant induced IFN γ secreting CD4⁺ T cells in the airway as well as pulmonary IgG and IgA antibodies²³¹.

Secondly, other important target DC receptors are TLRs, which also constitute an important group of receptors that recognize PAMPs. Specifically, the intranasal administration of influenza hemagglutinin subunit with polyinosine-polycytidylic (polyI:C)

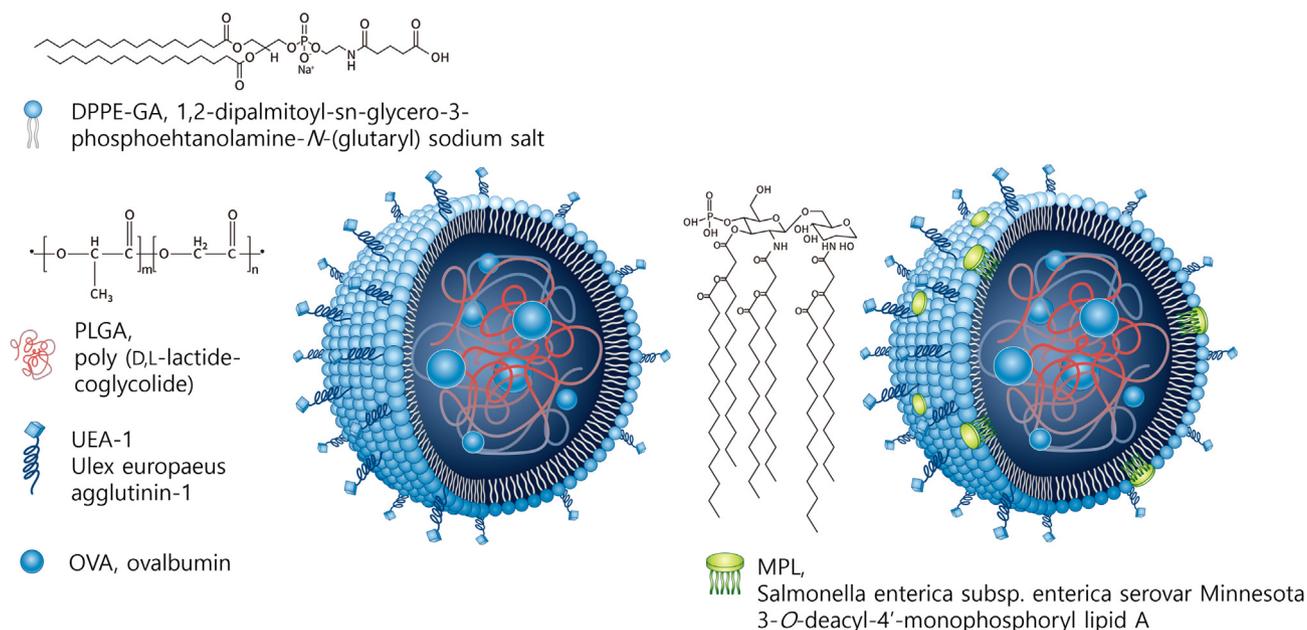


Figure 10 The uniform-sized polymeric lipid NPs were prepared using PLGA and head-group carboxylic lipids. UEA-1 was conjugated on the PLGA nanoparticle surface by carbodiimide chemistry adjuvanted with TLR-agonist MPL. UEA-1, *Ulex europaeus* agglutinin-1; PLGA, polylactide-co-glycolide, TLR, toll-like receptor; MPL, monophosphoryl lipid A.

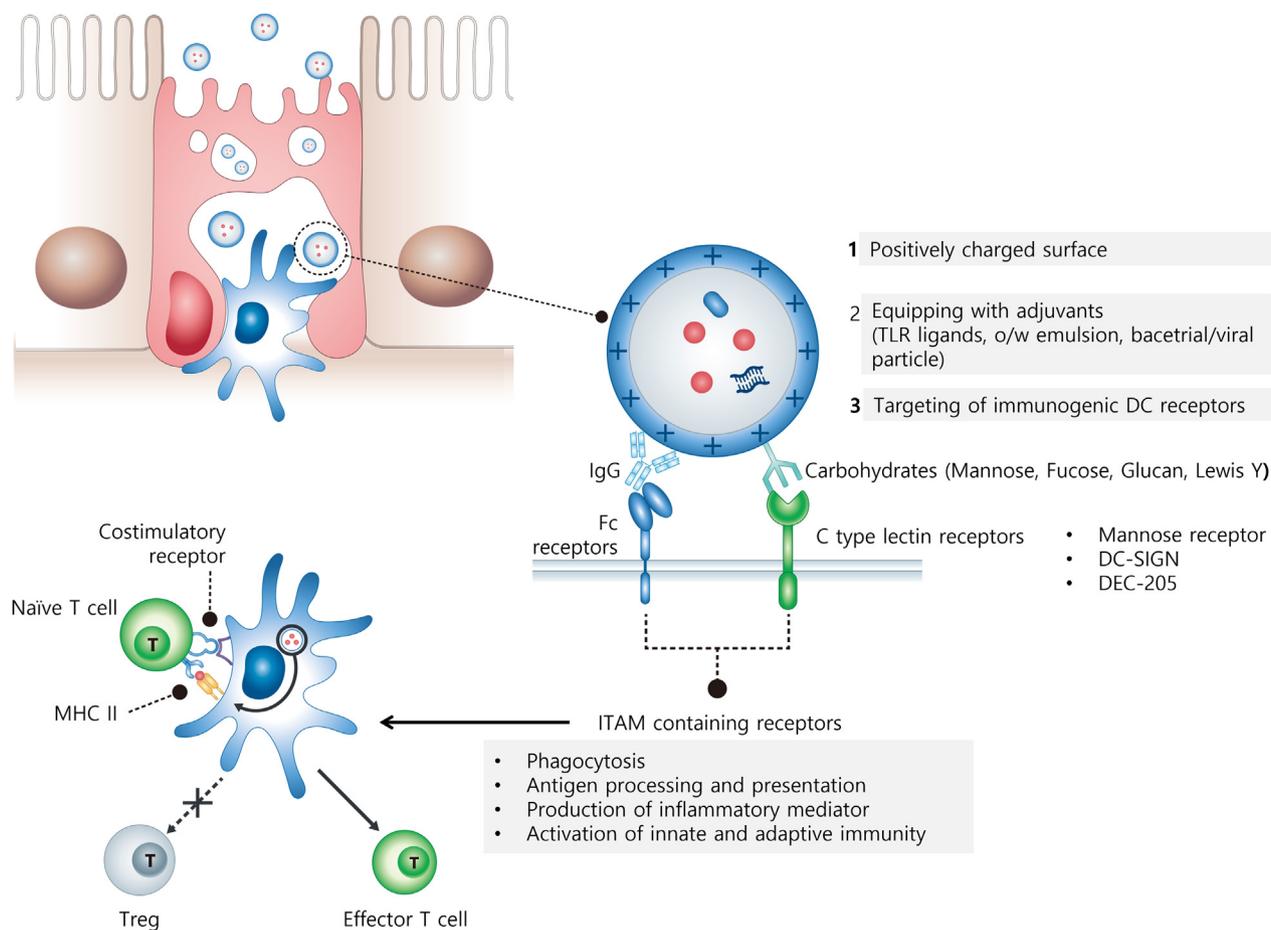


Figure 11 Schematic illustration of the mechanism of DC targeting nanoparticle. Three major strategies can be employed to enhance the immune-activating capacity of DC. Firstly, the positively charged nanoparticles exhibit DC-activating properties through interaction with the negatively charged membrane of DC. Secondly, equipping nanoparticle with immunostimulatory adjuvants leads to the activation of pattern recognition receptors (PRRs) of DC, which subsequently induce DC maturation, DC-mediated antigen processing, antigen presenting, and immune activation. Thirdly, targeting immunogenic DC receptors (FcR, CLR, TLR) with specific ligands induces efficient phagocytosis, antigen processing, and promotes the binding with the immunogenic DC receptors leading to phosphorylation of ITAM, which subsequently induces the signaling pathways that mediate phagocytosis, inflammatory mediator production, and activation of immunity. FcR, Fc receptor; CLR, C-type lectin receptor; TLR, toll-like receptor; DC-SIGN, DC-specific ICAM-3 grabbing non-integrin; DEC-205, a 205-kDa membrane protein; ITAM; immunoreceptor tyrosine-based activation motif; Treg, regulatory T cell.

enhanced the production of vaccine-specific IgA¹⁴⁶. A mechanistic study has revealed that polyI:C was endocytosed into CD103⁺ DCs and activated the TLR3, which subsequently induced T cell-dependent IgA production¹⁴⁶.

Thirdly, Fc γ R is also a crucial receptor that regulates the immune response and induces the maturation of DCs (Fig. 11). As IgG binds to specific antigen, the Fc domain of the antigen-specific IgG binds to Fc γ R. This subsequently induces the activation of phagocytosis and the production of cytokines and chemokines and consequently leads to the activation or inhibition of T and B cells. Within the various types of Fc γ R, Fc γ RI, Fc γ RIIa, Fc γ RIIc, and Fc γ RIIIa are immune activating receptors, whereas Fc γ RIIb is the immune inhibitory receptor due to its ITIM motif²³². Specifically, a study has demonstrated that intranasal administration of Fc γ R receptor-targeted mucosal vaccine (inactivated *Francisella tularensis*-antibody complex) led to enhanced DC maturation, internalization, processing, and presentation of antigen and activation of IFN γ secreting memory CD4⁺ T cell in mice²³³.

4.3. Mucosal adjuvants for fostering immunogenicity of mucosal vaccine

Intranasal administration of the vaccine alone is not sufficient to stimulate NALT-induced mucosal immunity¹⁰⁸, necessitating the use of an adjuvant. An adjuvant is defined as a component co-administered with a certain antigen to increase or modulate a specific immune response. Since an adjuvant can be a major determinant for the development of long-lived memory B cells and effector plasma B cells²⁴, selecting the appropriate adjuvant should be taken into account. Similar to the formulation of a vaccine, an effective mucosal adjuvant should protect the antigen from mucosal defense mechanisms, possess a mucoadhesive property, activate the cellular uptake of the antigen, and possess an immunostimulatory property to induce mucosal immunity. The majority of adjuvants are TLR agonists possessing a pattern-associated molecular pattern (PAMP) that activates the innate immune system. The activated innate immune system further mediates the stimulation of adjuvants such as flagellin—a ligand

of TLR5, monophosphoryl lipid A—a ligand of TLR4, and CpG-oligodeoxynucleotide (ODN)—a ligand of TLR9.

Another well-known adjuvant of the mucosal vaccine is bacterial enterotoxin, which includes cholera toxin, and *E. coli* heat-labile toxin. Such enterotoxins possess an AB complex, with the A portion exhibiting major toxic activity while the B portion binding to ganglioside (GM1) of the mucosal epithelium. Following the interaction between ganglioside and enterotoxin, FAE can readily uptake the enterotoxin. However, since ganglioside is expressed in various types of cells, binding of enterotoxin to off-target cells can possibly trigger various types of side effects. One of these side effects is Bell's palsy, a paralysis of the facial nerve (Cranial Nerve VII) caused by the interaction between the ganglioside of the nerve cell and enterotoxin. To decrease the toxicity of the enterotoxin, a site-directed mutated form of A1, the A2 subunit of cholera toxin, was developed. Moreover, the B subunit of cholera toxin was removed and substituted by D-fragment of protein A of *Staphylococcus aureus* (CTA1-DD). This adjuvant can effectively bind to CD21 of the follicular DCs and activate the maturation of B cells in germinal center formation¹²⁹.

5. Discussion and perspectives

The aim of this review was to summarize various vaccine platforms available against COVID-19 and to emphasize the necessity of the needle-free mucosal vaccine. Most of the vaccines that are currently undergoing clinical trials, including COVID-19 vaccines licensed for human use, are administered parentally. The lack of conveniently administered formulations and advance of new variants has led to the limited accessibility and production of the vaccines, which is hampering the formation of herd immunity. Factors resulting in low accessibility of vaccines include scarcity of manufacturing or storage facilities, limited accessibility to medical professionals for the administration of injectable vaccines, and high cost of vaccines. Furthermore, since it is demonstrated that the parenteral vaccines do not induce localized immunity in the upper respiratory mucosal surface, administration of the currently approved vaccines does not lead to sterilizing immunity. This further supports the need for a mucosal vaccine that blocks the main entrance routes of COVID-19, that is, the nasal and oral mucosal surfaces.

The later part of this review describes the basic mechanisms and traits of mucosal immunity and further extends to the discussion of the challenges and strategies of the mucosal vaccine. Since the mucosal surface exhibits a harsh environment for foreign materials and shows relatively weak immunogenicity, the mucosal vaccine should fulfill high stability and selective delivery of antigens to the mucosal immune inductive site. In this aspect, nanoparticle-based vaccines can offer stability, targeted delivery, and immunogenicity. For the successful delivery of the antigen and activation of mucosal immunity, the antigen should be transported from the luminal surface to the specialized sub-epithelial lymphoid organs. For this purpose, the target cell, administration route, formulation, and mucosal adjuvants should be carefully considered.

The M cell, a highly specialized epithelium presenting in the FAE of GALT and NALT, can act as a key player in the induction of mucosal immunity. The distinctive role of the M cell in capturing the antigen and its transcytosis to APCs localized in the basolateral pocket renders it an attractive target for mucosal vaccines. Since the proportion of M cells in the mucosal epithelium is significantly low, designing a formulation that enables the

selective binding and delivery of antigens is crucial. A typical strategy for the M cell-targeted delivery is through targeting specific receptors of the M cell by modifying the surface of nanoparticles with a certain lectin, ligand, or antibody that can selectively bind to the M cell. In this regard, various surface modifications of polymeric nanoparticles and liposomes have enhanced the antigen delivery to APCs with the subsequent induction of mucosal immunity²⁰³.

However, there are still some challenges facing the development of an M cell-targeted delivery platform. Firstly, a significantly low number of M cells present in the mucosal epithelium hamper the M cell-targeted delivery. To address this issue, receptor activator of nuclear factor kappa-B ligand (RANKL), a factor that induces the differentiation of the M cell from a precursor epithelial cell, can be exploited to increase the number of M cells in the mucosal epithelium²³⁴. Secondly, the lack of an animal model for M cells owing to the difference in the receptor profile of M cells between humans and other mammals should be overcome for strict preclinical study of the mucosal vaccine. Thirdly, the bidirectional capacity of M cell delivery is a challenge that needs to be addressed. Strictly, since the role of the M cell is limited to antigen capturing and transportation, the M cell-targeted delivery can induce both immune activation and tolerance. However, factors that determine whether M cell-targeted delivery results in the stimulation of immune response or immune tolerance remain elusive. Specifically, an animal study has demonstrated that oral and intranasal administration of ovalbumin fused to reovirus protein $\sigma 1$ (OVA- $\sigma 1$) induces immune tolerance by reducing anti-OVA Ab and CD4⁺ T cell response in mucosal and systemic lymphoid tissues^{235,236}. In this aspect, further studies on M cell delivery should focus on investigating and standardizing various factors that determine immune activation or tolerance. Additionally, encapsulating or crosslinking mucosal adjuvants with nanoparticles can impart a mucosal immunostimulatory property of vaccines, which includes TLR agonists and microbial toxins.

DCs, initiators and key regulators of the immune response, are other attractive target cells to enhance the induction of immune response. Since DCs are a heterogeneous group of cells that induce both immune activation and immune tolerance in addition to activating different types of lymphocytes depending on the processing pathways, it is important to deliver antigens toward the proper type of DCs and promote immune activation. The typical strategy of DC-targeted delivery includes modulating the physicochemical properties (charge, size, and shape) of nanoparticles and equipping the nanoparticles with various types of specific ligands of the DCs.

For sophisticated modulation of the immune response, a profound understanding of the role of each subset of DCs and factors that influence the bidirectional capacity of DCs are necessary. For instance, the distinctive role of different subsets of DCs in NALT and the receptor and marker profiles of each subset are crucial. In addition, similar to the M cell-targeted delivery, the physical and chemical properties that determine the immune activation or tolerance are also important but remain unclear. Furthermore, since the role of DCs is affected by the interaction with various types of immune cells and cytokines within the immune system, research on how external signals and cell-to-cell interactions guide DCs to immune activation or tolerance should also be investigated.

Despite the various limitations and obstacles, this study recommends carrying out further research on mucosal vaccines and nanoparticle-based M cell vaccines. Moreover, DC-targeting

platforms might act as a crucial strategy to combat COVID-19 and upcoming new variants.

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Author contributions

Junwoo Lee collected relevant research articles and summarized the literatures and drafted the manuscript. Dongwoo Khang conceived, supervised and edited the review.

Conflicts of interest

The authors have no conflicts of interest to declare.

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