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Review article

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The role of engineered materials in mucosal vaccination strategies

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Abstract

Mucosal pathogens, as exemplified by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis*, lead to substantial morbidity and mortality worldwide and pose serious threats to global health. Mucosal vaccination is crucial to combating mucosal pathogens because it enables the immune system to directly target and neutralize pathogens at their point of entry. Mucosal vaccines need to penetrate the mucus layer, reach the target tissue and activate robust immune responses in the mucosal tissues. Material-based strategies are necessary to meet these requirements. In this Review, we provide an overview of current mucosal vaccines, categorized by administration route, to highlight the importance of material design in overcoming the existing delivery challenges. We discuss the different classes of materials currently being used as vaccine carriers to induce antigen-specific mucosal immunity, including lipids, natural and synthetic polymers, inorganic materials and pathogen-inspired materials.

Sections

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Introduction

More than 90% of pathogens enter the body through mucosal sites[1]. Mucosal pathogens have the potential to cause epidemics, and their threat to public health is exemplified by the COVID-19 pandemic, which has also highlighted the critical role of vaccines in controlling infectious disease outbreaks[2]. Because mucosal pathogens replicate in mucosal tissues and transmit through contact with mucosal secretions[3,4], vaccination strategies that generate protection at mucosal sites can help to prevent infection, limit disease severity and reduce transmission[5,6].

The gastrointestinal tract (oral cavity, oesophagus, stomach, small intestine, colon and rectum) has a large surface area and is the most widely studied of all the mucosal tissues. The respiratory tract (nasal cavity, trachea, lung) is the target tissue for respiratory infections such as seasonal influenzas or the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2 virus). Although the female reproductive tract (uterus, vagina) has a small surface area compared with the gastrointestinal and respiratory tracts, it is a common site of entry of sexually transmitted infections. Table 1 summarizes mucosal pathogens and their route of infection (respiratory, gastrointestinal or urogenital tract), number of deaths and infections, and current vaccine coverage.

Many features of mucosal tissue pose challenges to vaccine delivery. Aside from being a physical barrier, the mucosa can degrade enzymes, rapidly clear substances and have an immunosuppressive microenvironment, variable pH level and diverse microbiota[7]. Therefore, material-based delivery platforms should be rationally designed to overcome the associated physiological constraints. A vaccine needs immunogenic components from the target pathogen to induce immunity. These components — including surface proteins, capsid proteins, toxins, surface polysaccharides, nucleic acids (DNA or mRNA), inactivated pathogens, live attenuated pathogens and recombinant proteins — are recognized by the immune system and stimulate the production of antibodies and activation of the immune cells. The vaccine formulations containing the immunogenic components need to prevent uncontrolled degradation until the vaccine reaches the target site, resist mechanical clearance and penetrate the mucus layer, be taken up by appropriate cell populations and be presented by immunogenic antigen-presenting cells (APCs), and have an effective adjuvant to overcome immune tolerance on mucosal surfaces[8–11]. The ability of biomaterials to be engineered to prevent antigen degradation, achieve specific localization, target cell populations, fine-tune release pH and kinetics, and achieve adjuvant effects make them desirable platforms for improving mucosal vaccines.

In this Review article, we highlight some of the key immunological characteristics governing mucosal immunity and their relevance in the development of vaccine delivery formulations. We focus on vaccine delivery vehicles used in oral (sublingual and buccal, or gastrointestinal); intranasal; pulmonary; and intrarectal, intravaginal and intrauterine mucosal administration routes. Additionally, we discuss a few vaccine delivery vehicles used in non-mucosal administration routes — including transcutaneous, subcutaneous and intramuscular — that are able to induce mucosal immunity.

Mucosal tissue structure and anatomy in mucosal vaccine design

To formulate efficacious mucosal vaccines, it is critical to understand the structure and properties of mucosal tissues. Mucosal tissues serve as barriers between the human body and the surrounding environment, and can be divided into type I and II[6] (Fig. 1a,b). Type I mucosa include tissues that line the gastrointestinal, respiratory and upper female reproductive tracts. Type II include tissues that line the corneal, oral, oesophageal and lower female reproductive systems[6].

Although the landscape of each mucosal surface is unique, the mucosa share a common architecture which can be subdivided into three distinct structural layers: the mucus, the epithelial layer and the lamina propria, which is the layer of tissue directly underlying the epithelium separated by a basement membrane.

The mucus layer, consisting of a network of hydrated mucins secreted by goblet cells in type I and by nearby glands in type II mucosal tissues[6], limits access of pathogens to the epithelium layer and acts as the first mucosal barrier[12]. The depth of the mucus layer varies widely at different mucosal tissues, such as ocular surfaces (cornea, conjunctiva), oral cavity (cheeks, lips, tongue), nasal cavity, respiratory tract, gastrointestinal tract, urogenital tract and female reproductive tract. Often, the depth of the mucus layer within the same tissue varies; for instance, its depth in the gastrointestinal tract is reported to range between about 50 and 450 μm in humans[13,14]. Therefore, mucus thickness at a given administration site should be considered when designing a mucosal vaccine, as it will affect how well the material can access the epithelial layer.

The epithelial layer, which ranges from 100 to 800 μm thick[12], acts as the second mucosal surface barrier. The epithelial layer of type I mucosal tissues consists of a simple columnar epithelium and mucin-secreting goblet cells. However, epithelial layers in type II mucosal tissues are composed of stratified squamous epithelia with multilayered keratinocytes. This multilayered cellular structure in type II tissues, compared with the single-cell layer in type I, provides greater protection against mechanical stresses. This structural difference suggests that vaccine formulations need to penetrate deeper in type II mucosal tissues to pass through the epithelial layer to reach the lamina propria to activate the immune cells.

The lamina propria contains a substantial number of dendritic cells, macrophages and complex networks of blood and lymphatic vasculature. Antigen that has breached the epithelium can be taken up by dendritic cells and brought to draining lymph nodes via the collecting lymph. Mucosa-associated lymphoid tissue (MALT) contains a sizable fraction of $CD8^+$ T cells, γδ T cells and antigen-sampling microfold (M) cells, which span the epithelium overlying B- and T-cell follicles to the lamina propria. Lymphocyte activation occurs in the MALT via M-cell sampling and transcytosis of luminal antigens, which are then presented by dendritic cells to naïve lymphocytes in the MALT[15]. MALT can be further subdivided into the gut-associated lymphoid tissue (which includes intestinal Peyer's patches and isolated lymphoid follicles), nasal-associated lymphoid tissue (including tonsils in humans) and inducible bronchus-associated lymphoid tissue. The relative abundance of tissue-resident antigen-experienced lymphocytes at mucosal surfaces provides robust local protection against mucosal pathogens.

Although the multilayered mucosal layers protect against pathogenic invasion, they are also barriers that vaccine formulations must overcome[16,17]. Additionally, the movement and shedding of mucus, aided by the beating of ciliated epithelium in the respiratory tract[18] or the peristalsis of the gastrointestinal tract, affects the diffusion of vaccine formulations. Therefore, to develop mucosal vaccines that effectively stimulate protective immune responses at the mucosal tissues, it is imperative to understand the characteristics of the mucosal tissue. Table 2 summarizes structural information of mucosal tissues, including organ system, tissue, administration route, mucosal tissue type, epithelium cell layer thickness, mucosal lymphoid tissue type, specialized

Table 1 | Mucosal disease burden (adapted from ref. 5)

Mucosal pathogen	Route of infection	Deaths	Infections	Vaccine coverage	Refs.
Respiratory syncytial virus	Respiratory tract	100,000–150,000 p.a.	33 million p.a.	None approved	186,187
Mycobacterium tuberculosis		1.5 million p.a.	10 million p.a.	Suboptimal coverage	188
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)		>2.6 million p.a.	>115 million p.a.	Suboptimal coverage	189
Streptococcus pneumoniae		1.2 million p.a.	>190 million p.a.	Suboptimal coverage	190
Bordetella pertussis		160,000 p.a.	>24 million p.a.	Suboptimal coverage	191
Haemophilus influenzae		48,000 p.a.	>40 million p.a.	Suboptimal coverage	192
Shigella	Gastrointestinal tract	200,000 p.a.	80–165 million p.a.	None approved	193
Enterotoxigenic *Escherichia coli*		>50,000 p.a.	~400 million p.a.	None approved	193
Helicobacter pylori		>15,000 p.a.	~3 billion p.a.	None approved	194
Rotavirus		~125,000 p.a.	>250 million p.a.	Suboptimal coverage	195
Salmonella		~60,000 p.a.	~500,000 p.a.	None approved	196
Clostridium		14,000 p.a.	~500,000 p.a.	None approved	197
Human immunodeficiency virus (HIV)	Urogenital tract	700,000 p.a.	1.7 million p.a.	None approved	198
Neisseria gonorrhoeae		Rare	>80 million p.a.	None approved	199
Human papillomavirus		>4,000 p.a.	>600,000 p.a.	Approved	200
Herpes simplex virus 2		Rare	>180 million p.a.	None approved	201
Treponema pallidum		>300,000 p.a.	>6 million p.a.	None approved	202
Hepatitis C virus		400,000 related p.a.	1.7 million p.a.	None approved	203,204

p.a., per annum

cell types and draining lymph nodes, as well as considerations for mucosal vaccine design.

Engaging mucosal defences in vaccine design

Strategies that establish protective immunity in mucosal tissues, such as mucosal vaccines, can combat mucosal pathogens[6,19]. Mucosal vaccines aim to eliminate pathogens at their entry sites and offer additional benefits over injectable vaccines, such as early local detection of infection, limited local and systemic spread of pathogen, reduced transmission and lower systemic exposure to vaccine doses[20]. Notably, for pathogens that predominantly reside locally in mucosal tissues, engaging mucosal immune defences can also effectively clear the infection[21,22].

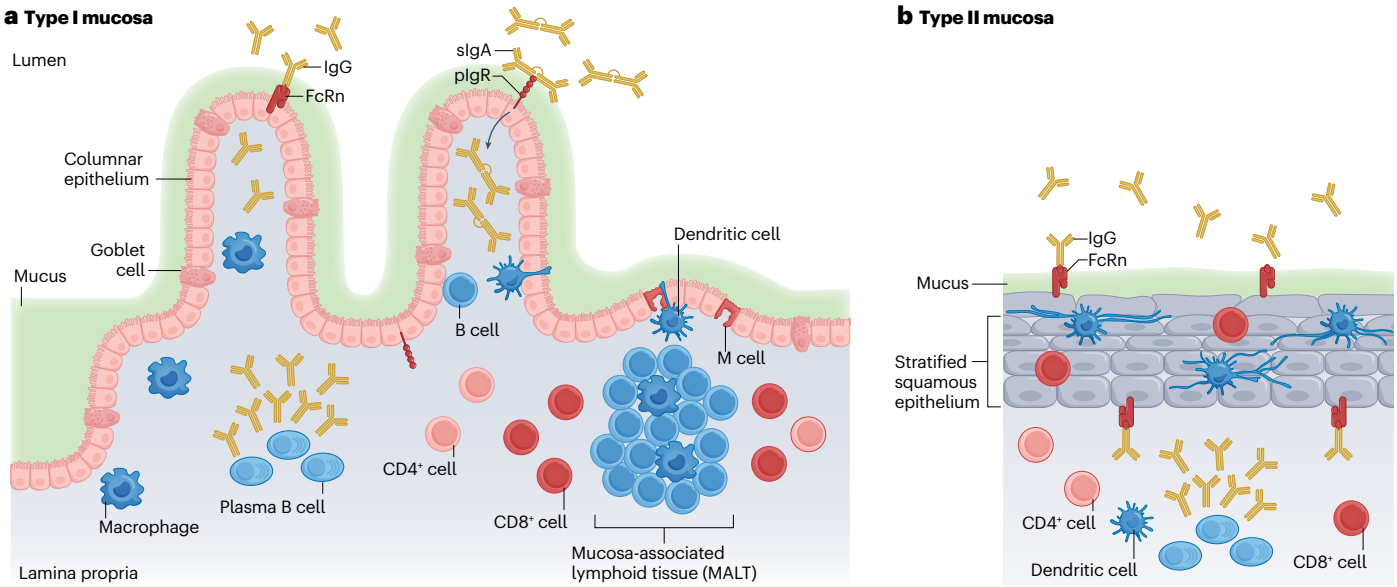
One of the most prominent metrics of mucosal immunity is the production of immunoglobulin A (IgA) in the mucosa[23]. Briefly, plasma cells produce secretory IgA, which is then transcytosed by secretory epithelial cells. However, IgA produced systemically is not transported into mucosal secretions[24]. This distinction is important, as some vaccines may elicit measurable serum IgA but may not concurrently elicit mucosal IgA production. Material-based approaches that effectively generate both are important, because pathogens enter the host via the mucosal layer and can spread systemically. Although the mechanisms for preferential IgA production require further elucidation, mucosal APC characteristics and local cytokine profiles in mucosal environments can be used to generate mucosal immunity[25]. Equally important, effective protection against pathogens necessitates the coordinated engagement of both the systemic and mucosal immune responses, employing production of both immunoglobulin G (IgG) and IgA antibodies. Therefore, throughout this Review, production of both IgA and IgG, as measures of vaccine effectiveness, is discussed.

Generation of highly site-specific tissue-resident memory T (T_{RM}) cells — which results from local antigen encounter — is another key metric to evaluate mucosal vaccine response[26]. Therefore, a protective immune response is generated at a specific tissue primarily when the antigen is encountered locally[27,28]. However, it is worth noting that multiple studies have reported a 'cross-protective' effect of mucosal vaccination, in which vaccination on one mucosal surface leads to protection (antigen-specific IgA and T_{RM} cells) of distant mucosal surfaces[29–32]. The route of administration substantially affects the mucosal IgA distribution[33–37] (Fig. 1c). This effect might be owing to the diffusion of administered material throughout the mucosa; however, a robust mechanism of pan-mucosal homing is not fully understood[29,30]. Using materials that can recapitulate mucosal IgA production at distant mucosal tissue can be beneficial in mucosal vaccine design.

Vaccine carriers to induce mucosal immunity

Vaccine formulations can be delivered directly to mucosal tissues via oral, intranasal, pulmonary, and intrarectal, intravaginal and intrauterine routes. They can also be delivered to the skin via transcutaneous, intradermal and subcutaneous routes, or injected to the muscles (intramuscular) or to the veins (intravenous). Mucosal vaccine delivery requires specialized formulations to overcome problems associated with anatomy of the mucosal tissues, for which different material formulations and devices, such as capsules, powders, sprays, creams, liquids (emulsions, solutions and suspensions) and microneedle patches have been used (Fig. 2a). Capsules are useful for oral (gastrointestinal) administration, as they can protect the vaccine against the

acidic and enzymatic environment of the gastrointestinal tract. Nasal sprays have been successful in intranasal delivery[38,39], as have powder formulations for both intranasal and pulmonary routes[40–43]. Topical cream vaccines applied to vaginal and rectal tissue can immunize against sexually transmitted diseases, and microneedle patches are effective with sublingual, buccal, transcutaneous and intradermal immunization[44]. Liquid formulations are commonly used in all of these administration routes.



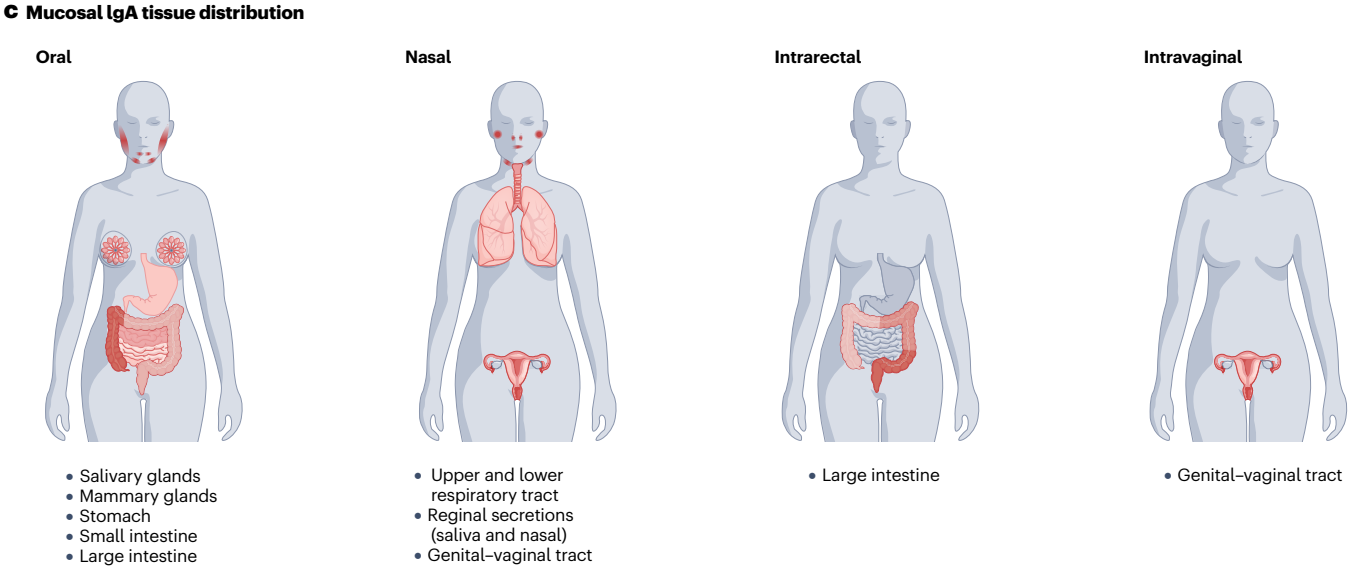


Fig. 1 | Schematic of type I and II mucosal tissues, and mucosal tissue IgA distribution. a, Type I includes tissues that line the gastrointestinal, respiratory and upper female reproductive tracts. Mucosal tissue type I is lined with a single layer of columnar epithelial cells, which express polymeric immunoglobulin receptor (pIgR) to transport dimeric immunoglobulin A (IgA) across the epithelium into the lamina propria, in addition to immunoglobulin G (IgG) transport by neonatal Fc receptor (FcRn). Secretory IgA (sIgA) is released into the lumen of the type I mucosa by the cleavage of the pIgR–IgA complex. Mucosa-associated lymphoid tissues (MALTs) are secondary lymphoid tissues that are predominantly found in type I mucosal tissues, whereby antigen taken up from the mucosal surface by microfold (M) cells activates naïve T and B cells. **b**, Type II includes tissues that line the corneal, oral, oesophageal and lower female reproductive systems. Mucosal tissue type II is lined with stratified squamous epithelium, which express only FcRn to transport IgG. **c**, Mucosal tissue IgA distribution following vaccination by different administration routes. IgA protection occurs in a characteristic pattern with the strongest response at the vaccine-exposed mucosa, followed by the second-strongest response at adjacent mucosae. Recent studies have found induction of immune responses in distant mucosal tissues, such as IgA production in vaginal and rectal tissue following intranasal administration. The strength of response is depicted through different levels of shading (darker shade indicates stronger response). Panels **a** and **b** adapted with permission from ref. 6, Annual Reviews. Panel **c** adapted from ref. 25, Springer Nature Limited.

Table 2 | Mucosal structure, cell types, draining lymph nodes and administration route access

Organ system	Tissue	Administration route	Mucosal tissue type	Epithelium cell layer thickness	Mucosal lymphoid structures	Specialized cell types	Draining lymph nodes	Considerations
Digestive	Oral mucosa	Sublingual	Type II mucosa Stratified squamous epithelium, non-keratinized[12,205]	100–200 µm (ref. 12)	Oral lymphoid foci[206]	Langerhans cells, myeloid dendritic cells, plasmacytoid dendritic cells, neutrophils and other immune cell types[207–209]	Cervical lymph nodes[210]	Thickness and permeability of mucosa
								Enzymatic degradation in the oral cavity
								Lack of M-cell sampling
								Saliva washout effects[211]
		Buccal		500–800 µm (ref. 12)				–
	Small intestine	Oral	Type I mucosa Columnar epithelium[12,212]	Single epithelial cell layer[12]	Peyer's patches	M cells, tissue-resident lymphocytes, IgA+ plasma cells, innate lymphoid cells etc.	Mesenteric lymph nodes, ileocolonic lymph nodes	Acidic environment in the stomach
		(Intragastric)						Oral tolerance and regional specification of lymph node properties[213,214]
	Colon	–			Lymphoid aggregates	T cells, B cells, natural killer/ natural killer T cells, myeloid cells etc.[215]	Mesenteric lymph nodes, ileocolonic lymph nodes	Elimination by peristalsis
	Rectum	Intrarectal					Rectal lymph nodes	Permeability of the epithelium and efficiency of delivery
							Mesenteric lymph nodes	Elimination by peristalsis
							Iliac lymph nodes	Patient comfort and clinical translatability
Respiratory	Nasal mucosa	Intranasal	Type I mucosa Columnar epithelium[12,212]	Single epithelial cell layer[12]	Waldeyer's tonsillar ring (nasal-associated lymphoid tissue)	M cells, dendritic cells, IgA+ plasma cells, other immune cell types[216]	Cervical lymph nodes	–
							Submaxillary nodes[217]	
	Lung	Aerosol/nasal nebulization			Bronchus-associated lymphoid tissue	M cells, alveolar macrophages and other immune cells	Tracheobronchial lymph nodes	Accessibility of material to the lower airway
Female reproductive	Uterus	Intrauterine	Type I mucosa Columnar epithelium[12,212]	Single epithelial cell layer[12]	lymphoid aggregates[218]	Tissue-resident T cells and other immune cell populations[219]	Iliac lymph nodes[220]	Effect of menstrual cycle on tissue characteristics and delivery efficacy
								Patient comfort and clinical translatability
	Vagina	Intravaginal	Type II mucosa[212] Stratified squamous epithelium	~28 cell layers varying by menstrual cycle[221]	–		Iliac lymph nodes	–
							Inguinal lymph nodes	–

This is a non-comprehensive list and, in many cases, the draining lymph nodes in humans can vary and remain to be fully understood. M cell, microfold cell.

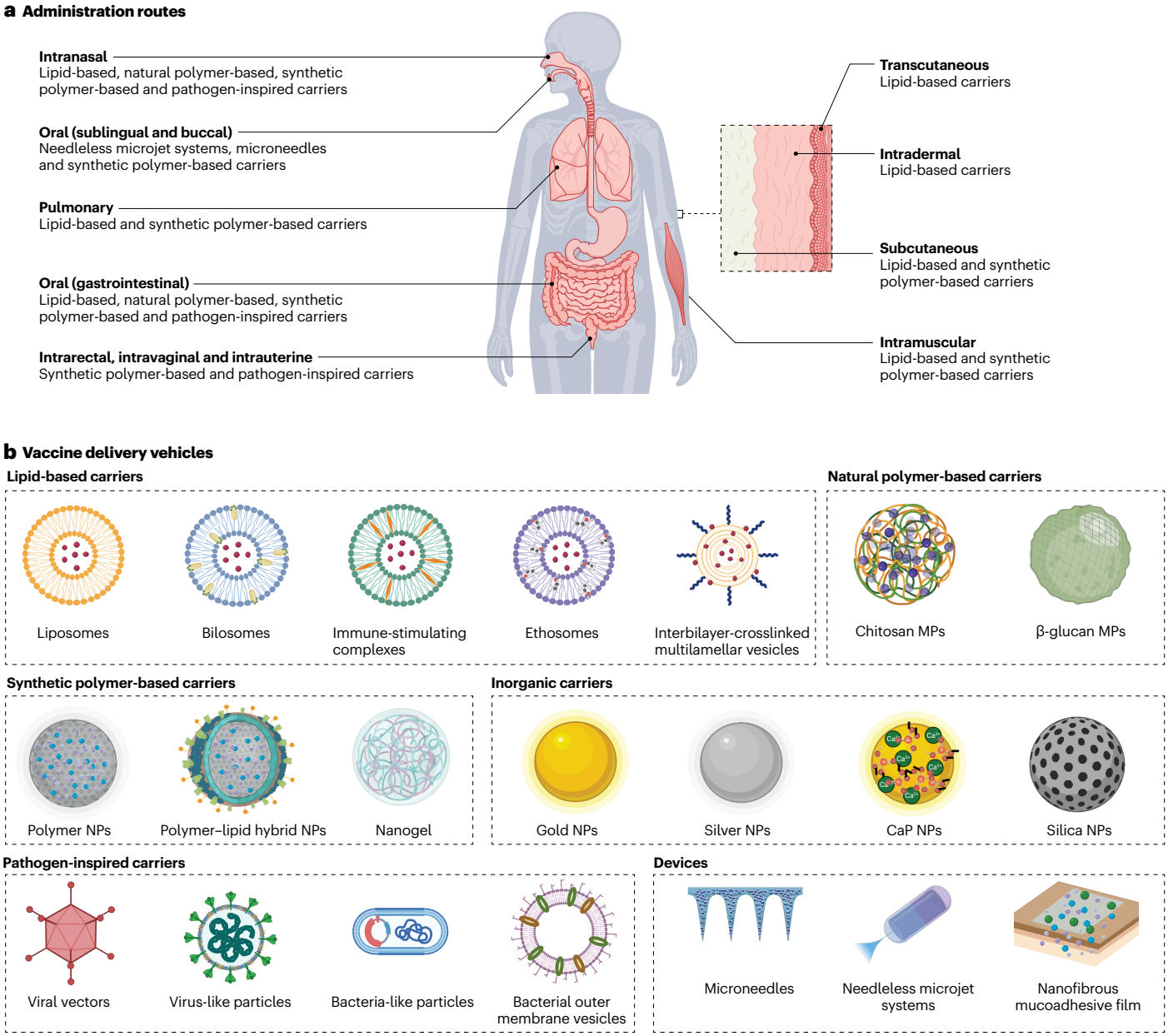


Fig. 2 | Vaccine administration routes and delivery vehicles. a, Types of vaccine carriers and devices used for each mucosal and non-mucosal administration route. **b**, Classes of materials used in vaccine delivery vehicles and devices. MPs, microparticles; NPs, nanoparticles.

Soluble antigens that are not encapsulated in a particle-based carrier face several challenges on administration, including rapid clearance, reduced immunogenicity, potential enzymatic degradation, need for higher doses, limited targeting to specific cells and possibility of allergenic reactions. Therefore, compared with naked and soluble antigen, antigens encapsulated in a particle-based carrier can be protected from degradation, can be better taken up by dendritic cells and APCs, can form an antigen depot and can be delivered along with adjuvant to the same APC. Certain self-adjuvant carriers such as virus-like particles can even deliver the antigens and act as adjuvant simultaneously. The materials used as vaccine carriers are critical to inducing the desired immune response. The size, surface charge and hydrophobicity of the vaccine delivery vehicles can all affect this immune response and thus must be optimized for each administration route[45]. Smaller carriers tend to enhance cellular uptake and antigen presentation, potentially leading to a stronger immune response. Positively charged surfaces can improve interactions with negatively charged cell membranes, aiding in cellular entry, whereas an optimal level of hydrophobicity can aid stability and controlled release of antigens, ensuring sustained immunogenicity. Nevertheless, depending on

the chemistry of the desired immunogenic vaccine components (that is, DNA, mRNA, protein, inactivated virus and so on), the physicochemical properties of the vaccine delivery vehicles need to be tailored to aid the components' encapsulation. Currently, a wide range of materials has been used to formulate mucosal vaccines (Fig. 2b). The discussion that follows categorizes vaccine delivery vehicles into two categories: mucosal and non-mucosal administration routes.

Mucosal administration routes

Oral (sublingual and buccal)

One way to elicit a mucosal immune response orally, while bypassing the complications associated with gastrointestinal vaccination, is to take advantage of the oral cavity mucosa, specifically the buccal (on the inside of the cheek) and sublingual (under the tongue) mucosal tissues. Vaccines administered sublingually or buccally can effectively induce mucosal immunity, owing to the abundant presence of immune cells in the oral mucosa[46]. However, the challenge in these routes is to overcome the thick mucus barrier and tight epithelial junctions designed to protect against native oral flora. Therefore, specialized mechanical platforms are needed to design sublingual and buccal vaccines capable of penetrating through this mucosal tissue.

For example, a buccal needleless microjet system was engineered for subepithelial delivery of ovalbumin antigen. This route demonstrated increased levels of oral mucosal tissue IgA and serum IgG compared with antigen delivered topically by a dropper in the buccal region[47]. The enhancement in the immune response is attributed to mucosal tissue penetration and direct delivery of vaccine to the underlying APCs. The device design includes two compartments. To administer the vaccine to the buccal cavity, the interior compartment (containing propellant and vaccine reservoirs sealed and separated by multiple membranes and a piston) is inserted into an exterior compartment (containing water). Water dissolves the polymeric membrane sealing the propellant reservoir and contacts the propellant, triggering a CO_2-generating chemical reaction. The increased pressure forces the piston towards the vaccine reservoir, breaking a nozzle membrane and ejecting a liquid jet of vaccine. This self-administered system also uses lyophilized antigen, allowing for a longer shelf life. A similar platform using the needleless Syrijet injector has been used for both sublingual and buccal HIV-1 vaccination in rhesus macaques[48]. Mucosal vaccination in the macaques elicited comparable IgG titres (systemic immunity) to subcutaneous vaccination but stronger rectal, vaginal and salivary IgA titres.

Microneedles have also been explored to access the buccal subepithelial space, including a strategy that used a 1D array of five microneedles made of stainless steel and coated with ovalbumin (as an antigen model) and two HIV antigens. The microneedle array was inserted into the rabbit dorsal tongue or rabbit inner lower lip for 2 minutes[49]. Other approaches have examined platforms with sustained release at the buccal mucosal surface, as opposed to one-time mechanical delivery through the mucus barrier. For example, a multilayered nanofibrous mucoadhesive film placed at the buccal and sublingual surfaces was able to slowly release poly(lactic-*co*-glycolic acid)–polyethylene glycol (PLGA–PEG) nanoparticles (NPs) into the oral mucosa, which penetrated locally and were transported to regional lymph nodes in a porcine model[50].

Oral (gastrointestinal)

Gastrointestinally administered vaccines represent the biggest challenge for mucosal vaccine development[51,52], owing to the harsh acidic and enzymatic gut environment that can degrade antigenic epitopes delivered in soluble form[53]. Overall, the gut environment is less conducive to robust immune activation than other mucosal surfaces such as the respiratory tract. The immune tolerance of the gastrointestinal tract can hinder vaccine immune response, and interference from gut microbiota can affect stability and efficacy of vaccines. Vaccine dilution by gastrointestinal contents and elimination by peristaltic movement can further contribute to the problem. Therefore, a substantially higher dose is required to generate immune response through gastrointestinal routes compared with peripheral administration. Adjuvant may also be necessary to enhance immune response. Overcoming these challenges is a complex task that requires careful vaccine formulation, design and delivery strategies. Different classes of materials have been used as mucosal vaccine carriers through gastrointestinal routes.

Lipid-based carriers. Liposomes mimic the natural structure of cell membranes, and their versatility, plasticity and biocompatibility make them a carrier of choice. They can encapsulate cargos with distinct properties by compartmentalizing them in different segments of the carrier, through entrapment in the hydrophilic core or intercalation into the hydrophobic lipid bilayer. This allows co-delivery of antigens (such as nucleic acids, proteins, peptides) and adjuvants. The tailorability of lipid compositions allows the liposome's features to be optimized for vaccine loading[54].

Cationic liposomes have been studied as effective vaccine carriers owing to their adjuvant effect[55]. For example, following three oral immunizations, cationic liposomes encapsulating DNA vaccine encoding for *Mycobacterium tuberculosis* protein induced expression of antigen in the epithelium, M cells, dendritic cells and Peyer's patches of mice intestine. Protection against an intravenous bacillus Calmette-Guérin (BCG) challenge was increased along with distinct reduction of tuberculosis burden in the lung[56]. Liposome-associated carrier, loaded with a recombinant protein, induced systemic and intestinal IgA and IgG response against *Salmonella* in chicken following two doses of oral administration. Additionally, it reduced the bacterial colonization in the intestinal tract and excretion[57]. Liposomes can be further modified with targeting moieties to enhance delivery efficiency. For instance, lectinized liposomes can be used to target M cells[58], and liposomes decorated with mannose derivatives can engage with mannose-binding receptors on APCs[59].

However, lipid-based platforms have several limitations for oral delivery, particularly the fragility of their structure against gastric acid, bile salts and pancreatic lipases in the gastrointestinal tract. Additionally, conventional liposomes exhibit poor permeability across intestinal epithelia. Modifications such as coupling with polymers[60], enteric coating with polymers, and alteration of lipid compositions have been used to address the stability and permeability problem. Bilosomes, for example, incorporate bile salts in the lipid bilayer to improve stability in the presence of bile salts found in the gastrointestinal tract[61]. Bilosomes have shown excellent capabilities to induce local and systemic immune response for oral delivery of antigens including influenza, hepatitis B, tetanus and diphtheria[62].

Immune-stimulating complexes (ISCOMs) are another class of lipid-based NPs with cage-like structures. ISCOMs are formed by self-assembly of phospholipids, saponin and cholesterol, and are considered second-generation liposomes because they have a built-in adjuvant effect owing to the presence of saponin. Their most defining characteristic is their ability to induce cellular immune response. Several studies have shown the potential of ISCOM-based formulations

for effective oral immunization against diseases including influenza[63], herpes simplex virus type 2 (HSV-2)[64] and diphtheria toxoid[65].

Natural polymer-based carriers. Particles made of natural polymers — including starch, hyaluronic acid, carbopol, alginate, β-glucan, chitosan and lectins — that encapsulate antigen can enhance the immune response compared with soluble antigen by increasing the retention time at mucosal sites[66]. Among biopolymers, alginate and chitosan have been most broadly evaluated owing to their mucoadhesive properties[67,68]. Chitosan is a biodegradable cationic polysaccharide that binds readily to mucosal surfaces. It is soluble in acidic environments (pH < 6); therefore, it is usually used with an enteric coating to avoid early dissolution under gastric conditions[69]. As an example, ovalbumin-loaded chitosan microparticles (MPs) coated with Eudragit L100, administered twice orally to mice, induced higher faecal ovalbumin-specific IgA than soluble ovalbumin or ovalbumin-loaded chitosan MPs without the coating[70]. Alginate-coated chitosan NPs containing hepatitis B recombinant protein and cytosine–phosphate–guanine (CpG) adjuvant, orally delivered in rats, were efficiently taken up into Peyer's patches[71], and in mice elicited high antigen-specific IgG titres in serum and secretory IgA in intestinal washing[72].

Yeast-derived β-glucan MPs are another class of materials used as oral delivery vehicles. These MPs with porous core–shell structures efficiently load antigen and feature receptor-targeted uptake by M cells and APCs, combined with the inherent adjuvant function of 1,3-β-D-glucans[73]. Ovalbumin-loaded β-glucan MPs orally administered three times in mice increased production of ovalbumin-specific serum IgG and IgA in intestinal fluid, and IL-17 and IFN-γ production in the splenocytes (which indicates the activation of specific immune pathways). Natural polymers, however, have limited tunability, and thus their properties might not be easily modified for controlled cargo release. Broad molecular weight distribution[74] and batch-to-batch variation[75] are additional concerns in clinical translation of natural materials.

A few non-polymeric natural materials have also been explored. Because secretory IgA can be transported to Peyer's patches by M cells when administered orally, secretory IgA itself was shown to serve as an antigen delivery vector and successfully induce systematic and mucosal immunity[76]. Plant-based formulations (such as MucoRice[77,78], pollen grains)[79] have the unique advantage of room-temperature stability. However, the low production, complex downstream processing for plant extract, unpredictable protein yield and limited regulatory framework limit their applications[80,81].

Synthetic polymer-based carriers. Synthetic polymers have seen widespread applications in drug delivery. PLGA is the most studied polymer for antigen delivery systems, owing to its biodegradability, biocompatibility and controlled release capabilities. PLGA has been studied as a potential carrier for oral delivery of antigens, such as ovalbumin or pertussis toxoid[82,83]. Although oral administration of antigen-encapsulated PLGA MPs reduced bacterial counts in the lungs after a challenge with *Bordetella pertussis*, multiple immunizations and high antigen dose were required. PLGA MPs have also been used for DNA vaccine delivery, such as rotavirus and HIV, and have shown induction of both antigen-specific humoral and mucosal response in mice. They further elicited some level of protection against a post-immunization mucosal challenge[84,85]. Additionally, a large-intestine-targeted delivery platform to induce rectal and vaginal immunity has been designed with pH-sensitive MPs (10–50 μm) made of Eudragit FS30D containing antigen-encapsulating PLGA NPs (300–500 nm)[86]. Upon two oral immunizations, increase of colonic antigen-specific $CD8^+$ T cell against HIV was observed, indicating colorectal immunity. Immunization by the NP-releasing platform also protected mice against a rectal and intravaginal challenge and reduced viral load equivalently to mice vaccinated via direct intrarectal route. One of the main challenges in antigen delivery using PLGA particles is that acidic byproducts form upon polymer hydrolysis. Developing efficient stabilizing strategies is therefore an active area of research[87,88].

An advantage of PLGA-based formulations lies in the ability to tune their encapsulation profile and release kinetics by modifying the molecular weight, ratio of polylactic acid (PLA) to polyglycolic acid (PGA) monomers, and functional end groups to suit the desired applications[89]. PLGA implants (such as Scenesse, Durysta and Ozurdex), MPs (such as Lupron Depot, Trelstar and Bydureon[90]) and NPs within in-situ-forming gels (such as Sublocade[91]) are long-acting drug delivery formulations that show promising direction for clinical development of PLGA-based vaccines[92].

Inorganic carriers. Nano- and microparticles of inorganic materials, including calcium phosphate (CaP), layered double hydroxides, gold, silver, carbon and silica, have been investigated as oral formulations owing to their inertness, rigidity and low toxicity[93]. Compared with soft organic materials, inorganic NPs offer a stable and rigid framework for antigen delivery in the acidic gastric environment. This prevents the early release of antigen that is commonly seen with polymeric NPs. Given the ability of gold NPs to be straightforwardly functionalized through thiol–gold interactions, chitosan-functionalized gold NPs have been studied for oral delivery of tetanus toxoid antigen along with adjuvant[94]. Following three doses of oral immunization, mice showed enhanced tetanus-toxoid-specific IgA in faeces and intestinal lavage.

CaP NPs are also promising delivery vehicles owing to their self-adjuvant property, biodegradability and safety profile[95]. For example, in mice, administering ovalbumin-encapsulated CaP NPs coated with chitosan and alginate enhanced systemic and mucosal immune response in faeces[96]. However, several challenges, such as low antigen-loading capacity and rapid aggregation of particles, limit the clinical translation of CaP NPs[97].

A biomimetic self-propelling micromotor has been recently developed as an oral delivery system to improve antigen delivery, tissue penetration, retention and uptake in the intestine[98]. The MP has a magnesium-based core and a TiO_2 shell fabricated by atomic layer deposition. The micromotor is then coated sequentially with a biomimetic cell membrane layer to load antigen, a layer of mucoadhesive chitosan and a layer of pH-responsive enteric polymer. Once exposed to pH around 5.5 in the intestine, the outer coating dissolves and motor propulsion is triggered, enabling spatial positioning of the micromotor. Mice orally administered with one dose of staphylococcal α-toxin-loaded micromotors showed enhanced faeces antitoxin IgA titres compared with static MPs.

Mesoporous silica NPs have the advantages of high porosity, large internal surface area, tunable pore size, straightforward surface functionalization, chemical stability, biocompatibility and low toxicity. As a case in point, the effect of particle physical properties on antigen release kinetics was systematically studied in bovine serum albumin (BSA)-loaded silica NPs with different particle sizes, morphologies and pore geometries[99]. High intestinal and salivary mucosal IgA titres were observed for all NP formulations in mice, a response absent in oral administration of free BSA and in parenteral administration of BSA emulsified in Freund's complete adjuvant. The specific responses generated

with different NPs were dependent on each NP's release profile: 430-nm NPs with honeycombed pores possessed the minimal initial burst and slowest release kinetics, and elicited the highest response.

Pathogen-inspired carriers. Viral vectors — modified viruses engineered to deliver genetic materials (DNA or mRNA) that encode a target antigen — are among the most effective platforms to breach the mucosal barriers and induce a strong immune response at the site of mucosal entry. Viral vectors present the antigens in a manner that mimics the natural infection. One promising state-of-the-art technology, developed by Vaxart, is a room-temperature-stable oral enteric-coated tablet containing a replication-incompetent recombinant adenovirus type 5 (Ad5) vectored vaccine. This formulation delivers two payloads of gene encoding the selected pathogen-specific protein antigen and a double-stranded RNA (dsRNA) as an adjuvant on the same viral vector. This two-payload strategy enhances immune response through the use of adjuvants, as dsRNA binds to Toll-like receptor 3 (TLR3) located on intestinal epithelial cells. This carrier has been shown to induce potent mucosal and systemic immunity against several enteric and respiratory pathogens in clinical trials, including norovirus (phase 1) [NCT02868073][100], H5N1 (phase 1) [NCT01335347][101] and SARS-CoV-2 (phase 1) [NCT04563702][102]. In a phase 2 influenza challenge study [NCT02918006][103,104], cellular, mucosal and humoral responses were induced after administration of an oral tablet based on an Ad5 vaccine that expresses influenza haemagglutinin along with dsRNA adjuvant; the protection against an intranasal wild-type H1N1 challenge 90 days post-vaccination was comparable to that of a licensed intramuscular vaccine (Fluzone Quadrivalent, Sanofi)[105]. This platform demonstrated that oral vaccines can imprint mucosal homing receptors on plasmablasts and generate antibody responses in the respiratory mucosal tissue.

A similar platform with an Ad5-based vaccine expressing spike protein and dsRNA adjuvant was successful in a golden hamster model[106]. After booster vaccination, orally and intranasally vaccinated hamsters showed higher serum neutralizing antibodies compared with intramuscular groups. Serum and bronchoalveolar lavage anti-S IgA were also enhanced in mucosally vaccinated groups, but not in intramuscular groups. These results translated into mucosally vaccinated animals having a decreased viral RNA in the nose and lungs, and decreased lung pathology score, accelerated viral clearance and reduced transmissibility compared with the intramuscularly vaccinated cohort upon a challenge study. The caveat with viral vectors, however, is the pre-existing vector-specific systemic immunity, which results in reduced immunogenicity of vector-based vaccines.

Bacteria-like particles (BLPs) are non-living particles derived from treated bacteria that maintain their peptidoglycan matrix, initial shape and size. They have been reported as gene delivery vectors for oral vaccination[107,108]. An example of this class is the platform developed by Symvivo (phase 1 clinical trial) [NCT04334980][109] that uses commensal bacteria, bacTRL, engineered to deliver plasmids containing synthetic DNA encoding spike protein of SARS-CoV-2. In this platform, the bacTRL product selectively colonizes colonic tissues upon oral administration and secretes plasmid DNA encoding transgene into the extracellular environment that will then be coordinated by a proprietary protein. The protein–plasmid DNA complex transfects colonic epithelium, localizes to the nucleus and expresses the antigen. Bacterial outer membrane vesicles are alternative pathogen-derived vehicles that have been effective against enteric pathogens including *Escherichia coli*, *Vibrio cholerae* and *Shigella*[110].

Virus-like particles are another promising candidate for oral delivery of antigen. Oravax is a virus-like particle-based triple antigen oral vaccine enclosed in a protective capsule that targets three SARS-CoV-2 virus surface proteins, including proteins less susceptible to mutation, to provide protection against emerging variants. Demonstrated to be safe and efficacious at triggering IgG and IgA responses in preclinical studies, this platform is being prepared for commencement of phase 1 and 2 clinical studies[111].

Intranasal

The nasal mucosa is the first barrier that airborne pathogens must pass. Therefore, induction of a robust and localized immune response is the key to combat airborne pathogens at their primary site of entry. This requires vaccine to reach the nasopharyngeal lymphoid tissue to initiate the immune response[112–116]. Intranasal delivery involves the introduction of the vaccine directly into the nasal cavity, targeting the upper respiratory tract. This can be achieved using various formulations, including nasal sprays and drops, and powders that are inhaled. However, rapid nasal mucociliary clearance leads to inconsistent vaccine doses and substantially reduces vaccine potency. It is therefore critical to develop new intranasal vaccine formulations that circumvent nasal clearance and promote strong mucosal immunity. For example, delivery carriers with mucoadhesive properties (such as being positively charged) prolong the retention time and penetration through mucosal tissue[117–119]. Additionally, nasal vaccines are a valuable tool in public health, offering advantages such as ease of administration, needle-free vaccination and the ability to induce both local and systemic immune responses.

Lipid-based carriers. Lipid-based vehicles can encapsulate antigen cargo to provide protection within the nasal environment and can be taken up by macrophages[120]. A lipid formulation of didodecyldimethylammonium bromide and dioleoyl phosphatidylethanolamine at a 1:1 molar ratio demonstrated enhanced mucin binding compared with liposomes with other molar ratios and compositions, and it improved delivery of an engineered three-component adjuvant (TriAdj) that has proven highly effective in a wide range of animal and human vaccines[121]. Intranasal administration of the lipid–TriAdj complex with ovalbumin in mice showed increased IgG and IgA titres in sera compared with TriAdj alone. Furthermore, this system exhibited an antigen dose-sparing effect, whereby at lower doses of antigen, the lipid formulation enabled a stronger response than did the non-lipid preparations of TriAdj. A different combinatory strategy used hybrid lipid–polymer NPs, coated with a mucoadhesive glycol chitosan adjuvant, for the delivery of *Chlamydia trachomatis* fusion antigen CTH522. When mice were vaccinated intranasally, the hybrid carriers increased IgG response in serum and IgA antibody secretion in the lungs compared with CTH522 adjuvanted with didodecyldimethylammonium/trehalose-6,6′-dibehenate liposomes[122].

Natural polymer-based carriers. In one approach, chitosan served as a carrier and adjuvant with immunostimulatory activity to encapsulate killed swine influenza A virus H1N2 antigens for intranasal administration in pigs[123]. Antigens encapsulated within chitosan NPs were more readily taken up by pig APCs than were soluble antigens (in vitro). Importantly, pigs vaccinated intranasally with the antigen-carrying NPs — compared with those vaccinated with only antigen — exhibited increased IgG titres in serum and enhanced mucosal response, resulting in IgA antibodies in nasal swabs, bronchoalveolar lavage fluid and lung lysates that were reactive against homologous (H1N2), heterologous (H1N1) and

heterosubtypic (H3N2) influenza A virus strains. Furthermore, intranasal vaccination with the chitosan system generated enhanced cellular immune responses in the respiratory tract, indicated by increased frequency of T helper memory cells and elevated secretion of the interferon gamma cytokine by tracheobronchial lymph nodes. In another example, a vaccine composed of receptor-binding-domain polypeptides formulated in chitosan solution induced receptor-binding-domain-specific mucosal IgA after intranasal delivery[124].

Synthetic polymer-based carriers. PLGA and PLA NPs are able to encapsulate hydrophobic molecules such as pattern recognition receptor ligands, which can specifically stimulate dendritic cells. One type of pattern recognition receptor, nucleotide-binding oligomerization domain (NOD)-like receptors, sense microbial products in the cytosol and thus can be used to design mucosal vaccines. In one example, PLA NPs, carrying NOD ligand adjuvants and coated with HIV-1 gag p24 antigen, were studied for intranasal administration in mice[125]. NOD-adjuvanted formulations induced higher IgG titres in sera than did p24-coated PLA NPs alone. In addition, although subcutaneous administration resulted in higher IgG titres compared with the intranasal route, only intranasal administration was able to induce high IgA titres in sera and vaginal lavages.

In another example, a cationic cholesteryl-group-bearing pullulan nanogel that self-assembles in water was used as intranasal vaccine[126]. Through complexation between the polymer amphiphile and protein, the hydrated nanomatrix can trap proteins without them aggregating and gradually release them in the native form. This cationic system efficiently delivered a model antigen to the anionic epithelial layer in the nasal cavity, where it was taken up by mucosal dendritic cells and induced strong antigen-specific immune responses. Intranasal administration of the cationic system in mice increased IgA-producing B cells in the lamina propria and paranasal sinuses of the nasal passages, and elicited antigen-specific IgA titres in nasal washes and antigen-specific serum IgG titres. In contrast, naked antigen vaccination produced a weak humoral response. In a follow-up study, nasal vaccination with pneumococcal nanogels led to robust antigen-specific antibody production, both systemically (IgG) and in mucosal linings (secretory IgA) in cynomolgus macaques. Additionally, it triggered cell-mediated responses through cytokine protection, along with increased expression of microRNA in serum and respiratory tract tissues, indicating enhanced T- and B-cell differentiation[127].

Pathogen-inspired carriers. A pertussis vaccine generated mucosal immunity by deploying immunostimulatory BLPs as the adjuvant[128]. Because protein antigens are poorly immunostimulatory when ingested or inhaled, immunostimulatory delivery systems and adjuvants are needed to enhance immune responses during intranasal immunization. BLPs are able to induce maturation and activation of APCs, making them suitable for delivery of the associated antigen for presentation by major histocompatibility complex class I and/or II. Alum-adjuvanted pertussis vaccine induced the highest IgG titres in sera; however, the intranasal administration of BLP-based vaccine amplified IgA titres in nasal wash compared with aluminium-adjuvanted vaccines. A subsequent intranasal challenge in mice with *Bordetella pertussis* cleared and inhibited bacterial growth in the lung, which could be ascribed to the high level of IgA, suggesting that BLPs may be promising mucosal adjuvants against whooping cough[128].

With respect to viral vectors[129–133], replication-competent adenovirus (Ad4)-vectored vaccine encoding influenza H5 HA, administered in a single intranasal dose, proved to be a promising platform in stimulating systemic and mucosal immunity (H5-specific IgG and IgA) against viral surface glycoprotein targets in a phase 1 clinical trial [NCT01443936 and NCT01806909][134–136]. Replicating vectors offer several advantages over replication-deficient vectors, including prolonged viral expression and ability to present viral glycoproteins at high valency, associated with induction of a robust and durable antibody response. Additionally, their ability to replicate at the same mucosal sites as the target virus can potentially lead to a more robust local cellular and humoral immunity. In a recent trial, administration of Ad4 expressing the influenza virus H5 HA presented higher and more durable levels of influenza-virus-specific neutralizing antibodies compared with oral administration[137].

Several studies have corroborated the advantages of intranasal over conventional intramuscular delivery[138]. Preclinical studies in mice of single-dose intranasal delivery of replication-incompetent chimpanzee adenovirus-vectored vaccine expressing the SARS-CoV-2 spike or its receptor-binding domain induced high levels of neutralizing antibodies, promoted systemic and mucosal IgA as well as $CD8^+$ T-cell response, and prevented SARS-CoV-2 infection in both the upper and lower respiratory tract[4]. In studies investigating the correlation between infection, viral shedding and possibility of onward transmission, the intranasal route provided superior protection over intramuscular delivery in both direct challenge and contact transmission with infected animals in hamsters and rhesus macaques[139]. In both animal models and challenges, the intranasal route resulted in higher neutralizing antibodies, reduced amount of infectious virus in nasal swab and attenuation of viral load in bronchoalveolar lavage. Additionally, preclinical studies in mice demonstrated the long-lived and cross-protective activity of a single intranasal dose of chimpanzee adenovirus-vectored vaccine against SARS-CoV-2 variants in mice, conferring complete protection in the upper and lower respiratory tract after challenges with variant viruses[140].

Pulmonary

Like intranasal vaccination, pulmonary vaccination is non-invasive and administered through the nasal cavity[141]. However, whereas intranasal vaccination targets the upper respiratory tract, pulmonary immunization targets the lower respiratory tract, which includes the trachea, bronchi and lungs. To target this region and trigger a stronger immune response in lungs, pulmonary vaccines are commonly administered in nebulized or aerosolized form[142]. The site-specific efficacy of a pulmonary vaccine is directly dependent on the nebulized particles' characteristics, such as size and surface charge of carriers[143,144]. For example, larger particles tend to target mucosal tissue in the upper respiratory tract, and smaller particles are more likely to reach the lower tract. In addition, because the mucus lining of the respiratory tract is negatively charged, positively charged carriers are more mucoadhesive and more likely to stay in the upper tract. Negatively charged carriers can be repelled from the mucus of the upper respiratory tract, raising the chance of the vaccine reaching the lower respiratory tract. Pulmonary administration exposes antigen to a large population of APCs in lung mucosa[145–147], which allows the vaccine to interact with lung-associated lymphoid tissue and results in the delivery of antigen to draining lymph nodes.

In a controlled analysis of the multifactorial and interdependent effects of particle size and hydrophobicity on pulmonary delivery of antigen using PLA or PLGA NPs, larger (>500 nm) and more hydrophobic particles were more efficiently internalized by rat alveolar macrophages, and hence induced higher serum IgG and mucosal

secretory IgA and endogenous cytokine levels post immunization against hepatitis B surface antigen (HBsAg)[148]. Another study used particle replication in non-wetting templates (PRINT) – a NP fabrication method that enables exquisite control over particle characteristics – to directly evaluate the effect of particle charge on immune response. PRINT was used to synthesize 200-nm, ovalbumin-conjugated poly(ethylene glycol) diacrylate hydrogel NPs with varied surface charges, while other physicochemical properties were kept constant. Cationic, amine-functionalized NPs were shown to induce a higher germinal centre B-cell population in the local lung draining lymph nodes (leading to high antigen-specific IgG in serum and high IgA in bronchoalveolar lavage fluid) following primary and secondary lung instillation in mice, compared with carboxylate-terminated, anionic particles. The inherent adjuvant effect of cationic NPs suggests that surface charge is one of the most critical design parameters for pulmonary delivery[149].

Interbilayer-crosslinked multilamellar vesicles (ICMVs), nanocapsules composed of stacked lipid bilayers that are stapled together through chemical crosslinking, offer better stability in vivo than traditional liposomes[150]. Compared with parenteral subcutaneous delivery, intratracheal immunization of mice with ovalbumin-loaded ICMVs combined with Toll-like receptor agonist adjuvants improved antigen trafficking to draining lymph nodes that persisted for 7 days, which led to enhanced T-cell priming and imprinting of the mucosal homing integrin α4β7. Furthermore, the ICMV nanocapsules generated a greater population of memory T cells in both systemic and mucosal compartments compared with soluble vaccine, and resulted in homing to the local lung tissue, systemic lymphoid compartments and distant mucosal sites (intestine and vaginal tract). The efficacy of this formulation was also assessed for simian immunodeficiency virus (SIV) gag antigen. Two immunizations protected mice against a post-mucosal challenge with SIV gag-expressing vaccinia virus and completely prevented the dissemination of virus to ovaries via generation of circulating and tissue-resident memory T-cell populations.

Coupling peptide antigens and adjuvants to an albumin-binding amphiphilic phospholipid moiety is another way to generate robust lung-resident memory T cells[151]. Endogenous albumin in the interstitial fluid binds to the lipid tail of the conjugate and serves as an efficient chaperone, promoting trafficking of antigen and adjuvant to lung-draining lymph nodes. Pulmonary administration of a viral gag peptide with adjuvant in mice elicited a prominent population of long-lived T_{RM} cells in the lung and a complete protection against a lethal challenge with vaccinia virus (4 months after the booster dose). The efficient generation of functional T_{RM} cells in the lung is attributed to the efficient uptake across the lung epithelium, and prolonged antigen and adjuvant accumulation in the lung and lymph nodes.

Intrarectal, intravaginal and intrauterine

Mucosal vaccines can also be delivered to the rectal, vaginal or uterine mucosal membranes using intrarectal, intravaginal or intrauterine routes[152]. The rectal mucosa is rich in blood vessels and has a relatively large surface area, allowing for efficient absorption of vaccine antigens[153]. However, the diverse microbiota[154] in the rectal mucosa affects vaccine efficacy and stability. Maintaining stability in the presence of commensal bacteria is crucial in vaccine design. Similar to rectal mucosa, the vaginal mucosa is highly vascularized and contains specialized immune cells, providing opportunities for efficient antigen uptake and immune stimulation. The vaginal pH is acidic, which can influence the stability of vaccines. Additionally, hormonal changes during the menstrual cycle can affect the permeability and immune environment of the vaginal mucosa. Vaginal vaccines should overcome these challenges to effectively induce the immune response[155,156]. The uterine mucosa undergoes cyclic changes in response to hormonal fluctuations, and variation in uterus anatomy among individuals can affect vaccine absorption and immune response. It should be noted that intrauterine administration[157] requires careful attention to safety to avoid potential harm to reproductive tissues and to ensure that no vaccine components adversely affect fertility or pregnancy[158,159].

A particle-based strategy to breach the thick mucosal lining of the vaginal epithelium and the epithelial barrier used a replication-deficient recombinant adenovirus (rAd) with a coating designed to balance mucus penetration and cellular transduction of epithelial cells. rAd encoding for HIV gag antigens was electrostatically coated with an anionic PEG-containing copolymer to prevent entrapment in the negatively charged mucin, and a positively charged cell-penetrating peptide for enhanced transduction efficiency. A comparison of intramuscular administration of rAd and intravaginal administration of rAd-nanocomplex in mice found that the latter led to increased IgG and IgA titres from a vaginal wash[160].

The route of administration can determine not only the tissue tropism of effector and memory cells, but also the tolerogenicity of a given antigen. One example is *Chlamydia trachomatis* (*Ct*), a sexually transmitted intracellular bacterium. Although mice infected with *Ct* acquired protective immunity on a re-challenge with live *Ct*, uterine exposure to ultraviolet-light inactivated *Ct* (UV-*Ct*) generated a pronounced tolerogenic response, resulting in increased bacterial burden on re-challenge. However, subcutaneous immunization provoked neither a tolerogenic response nor protection, even when combined with adjuvant[161]. An elegant approach to convert UV-*Ct* into an immunogen was achieved by complexing the negatively charged UV-*Ct* to a positively charged NP adjuvant through electrostatic interactions[161]. Intrauterine immunization with the adjuvanted UV-C*t*–NP complexes elicited long-lived genital protection in both conventional and humanized mice through rapid seeding of effector T cells in the uterine mucosa, establishment of T_{RM} cells and a robust systemic memory T-cell and *Ct*-specific antibody response. Interestingly, vaccination through distant intranasal mucosa evoked similar protection to intrauterine vaccination against genital *Ct* infection, whereas subcutaneous administration failed to do so. This result shows cross-mucosal protective and T-cell imprinting capability to engage the uterine recruitment pathway, presumably through α4β1 and other mucosal trafficking molecules (such as chemokines) that remain to be identified.

Non-mucosal administration routes

Although induction of mucosal immunity usually requires vaccines to be administered through mucosal routes, a few platforms have induced mucosal immunity through transcutaneous, subcutaneous and intramuscular administration routes[162,163].

Elastic liposomes loaded with HBsAg, when topically applied in a mouse model, were found to induce comparable IgG and higher IgA titres in sera compared to intramuscularly administered alum-adsorbed HBsAg[164]. The deformability of the elastic liposomes appeared to increase skin penetration and improve uptake by local immune cells. HBsAg loaded instead into ethosomes – carriers that are similar to liposomes but with the distinction of a high ethanol content – could penetrate more deeply in the skin than liposomes and were more effective at inducing serum IgA and IgG[165]. Topical vaccination strategies have also been tested in humans in preliminary clinical

Table 3 | List of mucosal vaccine clinical trials

Vaccine	Route of administration	Company or organization	Antigen type	Clinical number
Influenza (H5N1)	Intranasal	NIAID	Replication-competent Ad4 (HA)	NCT01806909 (phase 1)[136]
COVID-19		University of Oxford	Chimp Ad vector (spike)	NCT04816019 (phase 1)[222]
		Altimmune	Ad5 vector (RBD)	NCT04679909 (phase 1)[223]
		Bharat Biotech	Simian Ad vector (spike)	NCT04751682 (phase 1)[224]
		University of Hong Kong	Live attenuated influenza virus (RBD)	NCT04809389 (phase 1)[225]
		Codagenix	Live attenuated SARS-CoV-2	NCT04619628 (phase 1)[226]
		Tetherex Pharmaceuticals	Ad6	NCT04839042[227]
		Meissa Vaccines	Live attenuated respiratory syncytial virus RSV (spike)	NCT04798001 (phase 1)[228]
		CyanVac	Parainfluenza virus type 5 (spike)	NCT04954287[229]
		Laboratorio Avi-Mex	Recombinant NDV vector (spike)	NCT04871737[230]
		Center for Genetic Engineering and Biotechnology, Cuba	Protein subunit (RBD)	RPCEC00000345 (phase 1/2)[231]
Influenza (H1N1)	Oral	Vaxart	Ad5 (haemagglutinin, HA and double-stranded RNA (dsRNA) adjuvant, TLR3 agonist)	NCT02918006 (phase 2)[104]
Influenza (H5N1) (oral and tonsillar)		NIAID	Replication-competent Ad4 (H5 HA)	NCT01443936 (phase 1)[135]
Norovirus		Vaxart	Ad5 (GI.1 VP1 and dsRNA adjuvant)	NCT02868073 (phase 1)[100]
COVID-19		Vaxart	Ad5 (spike and dsRNA adjuvant)	NCT05067933 (phase 2)[232]
		Symvivo	Bacteria (DNA encoding spike)	NCT04334980 (phase 1)[109]
COVID-19	Oral+intramuscular	ImmunityBio	Ad5 (spike and nucleocapsid proteins)	NCT04732468[233]

trials. An immunization patch, consisting of heat-labile enterotoxin from *E. coli* adsorbed on gauze pads, was applied to the upper arm for 6 hours, with boosters at 12 and 35 weeks. This strategy successfully induced both systemic IgG and IgA responses[166]. A follow-up clinical trial found this prototype transcutaneous vaccine patch to be both safe and immunogenic in adults[167]. Overall, these studies support the feasibility of transcutaneous vaccine delivery to produce mucosal immune responses, and new studies aimed at HIV vaccination are warranted to use transcutaneous routes[168].

Mucosal immunity was also achieved by multiple subcutaneous injections of all-*trans*-retinoic acid (*at*RA) as a signalling molecule, along with antigen. Co-delivery of *at*RA with ovalbumin was shown to stimulate gut-homing receptors on T and B cells[169]. However, *at*RA administration is of concern owing to its toxic effects, low bioavailability[170] and low stability[171]. Furthermore, the need for multiple *at*RA injections to induce mucosal immunity makes its clinical translation unrealizable. To overcome these concerns, a liposome-based adjuvant containing two *at*RA delivery vehicles was designed[172]. One vehicle — a 220-nm nanocarrier made with a polar zwitterionic phosphocholine-lipid derivative, stabilized with PEG — acts as the fast-draining component to precondition local draining nymph nodes before they are presented with antigen by migrating dendritic cells and APCs. The second component, a cholesterol-stabilized cationic liposomal MP (~5 μm) containing antigen, forms depots and is designed for prolonged delivery of antigen by migratory APCs to the preconditioned lymph nodes. Using this system, mice vaccinated subcutaneously three times with recombinant *Chlamydia* antigen showed enhanced IgA response in the faeces, intestine and serum. Furthermore, vaccinated animals showed increased number of antigen-specific B cells in Peyer's patches, draining lymph nodes and spleen. *at*RA injected intramuscularly was also studied for mucosal imprinting. A nanocapsule comprising a squalene oil core and a hybrid PLGA/pH-sensitive lipid shell was engineered to enable co-delivery of *at*RA and electrostatically adsorbed, negatively charged antigen. This formulation enhanced antigen cross-presentation through the proton-sponge effect (achieved by incorporation of pH-sensitive lipids) and increased uptake by dendritic cells and intracellular delivery of the cargo. The NPs provided an antigen depot effect at the injection site with enhanced secretion of pro-inflammatory signals. Intramuscular administration of ovalbumin and recombinant enterovirus 71 in mice resulted in increased antigen-specific IgG, IgA in intestinal washes and gut-resident antigen-specific T-cell response compared with the antigen-alum-*at*RA mixture[173,174].

Inducing mucosal immunity with vaccines designed for non-mucosal (such as intramuscular or subcutaneous) routes presents several challenges. Non-mucosal-route vaccination does not provide direct exposure to the mucosal tissue, is less effective at inducing the production of secretory IgA, does not generate mucosal memory cells and does not protect at the pathogen entry sites. Because mucosal immune activation tends to require specific stimulatory signals that may not be naturally present, therefore, adjuvants are required to enhance the immune response when using non-mucosal routes, as discussed in this section. A key design goal for vaccine carriers is the ability to deliver adjuvants that increase immunostimulatory responses for downstream mucosal immunity.

Outlook

Despite substantial development of mucosal vaccines, only a few — most of which are orally administered — are currently FDA approved. Development of mucosal vaccines faces unique challenges of delivery, uptake, immunogenicity and safety[175]. To achieve sufficient protection, a mucosal vaccine needs to be effectively delivered across physical, chemical and anatomical mucosal barriers; be sampled and presented by APCs; be adjuvanted or have self-adjuvating effects to stimulate protective adaptive immune responses; and have low local and systemic toxicity. To further develop mucosal vaccine delivery systems, challenges associated with each route of administration (for instance, tolerating the acidic and enzymatic environment in gastrointestinal administration, or targeting the upper versus lower respiratory tract in intranasal versus pulmonary) and the nature of each type of mucosal tissue (for instance, the thick mucus and epithelium layers in oral mucosa, or the hormonally driven fluctuations in permeability and immune environment of the vaginal mucosa throughout the menstrual cycle) need to be considered. The physicochemical design of vaccine delivery vehicles — including size, shape, surface charge and the choice of materials — is key to overcoming these challenges and defining the biocompatibility, degradation rate, and vaccine stability and release kinetics. Adjuvants are also a critical parameter in mucosal vaccine design, as they help to stimulate a more robust immune response, activate the innate immune pathways (by triggering release of cytokines and chemokines), promote antigen uptake by APCs, enhance the production of mucosal secretory IgA, stabilize the immunogenic components of the vaccine, reduce antigen required in the vaccine dose and provide long-lasting immunity by production of memory immune cells.

The recent clinical achievements of mRNA and DNA vaccines, such as the mRNA–lipid NP vaccines used against COVID-19, guarantee their future application in next-generation mucosal vaccines[176,177]. Vaccines that deliver nucleic acids can be engineered to encode specific antigens, making them adaptable for targeting mucosal pathogens. Additionally, they can be administered locally and can stimulate both systemic and mucosal immune responses. Still, although mRNA and DNA vaccines hold great potential as mucosal vaccines, research is ongoing to address the optimal compositions and delivery mechanisms for reaching the mucosal tissues. For instance, a library of 720 biodegradable, ionizable lipids was investigated to optimize efficient delivery of mRNA–lipid NPs to the lungs through pulmonary administration[178]. Other research[179–181] has found that mRNA encapsulated within chitosan NPs, administered intranasally, can successfully penetrate the mucosal barriers to induce immune response, and that increasing the percentage of PEGylated lipids in gastrointestinally administered siRNA–lipid NPs led to more potent carriers. These studies exemplify how material design of vaccine carriers plays a crucial role in engineering new platforms[180].

The number of mucosal vaccines currently in clinical trials (Table 3) suggests that these vaccines have a bright future. To aid translation of mucosal vaccines to clinical phases, the production of novel vaccine carriers must be robust, large scale and low cost. Furthermore, formulations need to be stable and easy to store, ship and administer. To address the cold-chain storage and transport requirements of mRNA–lipid NP vaccines, a promising strategy is to load the vaccines in thermostable microneedle patches[182]. These patches offer a viable solution for keeping the vaccine stable, particularly in regions where cold storage infrastructure may be limited or unavailable. Lyophilization of mRNA–lipid NP vaccines is another way to streamline their distribution without loss of vaccine immunogenicity[183]. Moreover, inclusion of certain stabilizers and excipients, such as trehalose or sucrose, can improve stability and shelf life of vaccines. All of the considerations discussed in this section — from overcoming the mucosal barriers to fabrication, storage and shipment — can only be addressed by taking an interdisciplinary approach that emphasizes biomaterial engineering[184,185], nanotechnology, vaccinology[5,25], human biology and translational medicine.

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B.E., A.S., U.v.A., R.L. and A.J. conceptualized the manuscript. B.E. A.S., I.S. and Z.C. contributed to literature review, manuscript writing and figure composition. A.H.L., M.K., F.T. and D.M.F. contributed to literature review and manuscript writing. B.I. and G.L. contributed to

literature review. J.H. contributed to figure visualization. B.E., A.S., U.v.A., R.L. and A.J. edited and finalized the manuscript.

Competing interests

R.L. receives licensing fees (to patents on which he was an inventor) from, invested in, consults (or was on Scientific Advisory Boards or Boards of Directors) for, lectured (and received a fee), or conducts sponsored research at MIT for which he was not paid for the following entities: 611 Therapeutics; Abpro International; Acorda (formerly Civitas Therapeutics); Alfred University; Aleph Farms; Alivio Therapeutics; Alkermes; Allevi; Allurion; Alnylam Pharmaceuticals, Inc; Amberstone Bioscience; Amgen; aMoon; Apotex; Arcadia Biosciences, Inc; Arsenal Medical; Artificial Cell Technology, Inc; Avalon-Globocare; Bai Biosciences; BASF Corporation; Bayer; Balzan Foundation; Bexson Biomedical; Bilayer Therapeutics; Biogen; BioInnovation Institute (Novo Nordisk Founden); BioTE Medical; Blackrock; Blackstone (formerly Clarus); Boston Children's Hospital; CBC Group Investment Mgmt Group; Celanese; Celero; Cellink/BICO; Cellomics Technology LLC; Cellular Biomedical; CE&N/ACS; Charles River Laboratories, Inc.; Clontech Laboratories; Combined Therapeutics (CTx); Conference Forum; Cornell University; Crispr Therapeutics Ag; Crown Bioscience, Inc.; Daré Biosciences (formerly Microchips Biotech, Juniper Pharmaceuticals and Columbia Laboratories); Daros, Inc.; DeepBiome; Dewpoint Therapeutics; Dispendix; Eagle Pharmaceuticals; Earli; Edigene Biotechnology, Inc.; Editas Medicine, Inc.; ELC (Estee Lauder Companies); Eli Lilly; Eisai, Inc.; Entrega; EpiBone; Establishment Labs, SA.; Everlywell; Evox Therapeutics, Ltd.; Fate; Flagship Pioneering; Frequency Therapeutics, Inc.; GeneLeap Biotech; Genemedicine Co Lmtd; GenScript USA, Inc; Geneo Medicine; GENUV; Glaxosmithkline LLC; Glycobia; Glympse Bio; Goldman Sachs; Greenlight Biosciences; HCR (HealthCare Royalty Partners); HKF DNA Technologies; Hopewell Therapeutics; Horizon Discovery Group Plc; Humacyte, Inc.; IBEX Pharmaceuticals, Inc.; Immunai; ImmuneXcite, Inc.; Institute of Immunology Co. Ltd; Integrated DNA Technologies, Inc.; InVivo Therapeutics; IxBio; J.R. Simplot Company; Jnana Therapeutics; Kala Pharmaceuticals; Kallyope, Inc.; Kendall Capital; Kensa; Kodikaz Therapeutics; KAST (Korean Academy of Science and Technology); Ksq Therapeutics, Inc.; Kunlun Capital; Landsdowne Labs; LikeMinds; Lonza; Luminopia, Inc.; Luye (Shandong luye); Lyndra Therapeutics; Lyra Therapeutics (formerly 480 Biomedical); Maurice Marie Janot Award 2020; McGovern Institute; Medikinetics Co., Ltd.; Merck; MGH Ragon Institute; Micelle; Moderna Therapeutics; Momenta; Muse Biotechnologies, Inc.; Mylan; N2Tech; Nanobiosym; Nanobiotix; Neochromosone; Neoteny 4 LLP; NextRNA; Newbridge Ventures LLC; Noveome Biotherapeutics, Inc.; Novo Nordisk; Ohio State University; Olivo (acquired by Shiseido); Ovid Therapeutics; Particles for Humanity; Pfizer, Inc.; Pioneer Hi-Bred International, Inc.; Placon Therapeutics; Polaris Partners; Pontifical Academy of Sciences; Portal Instruments; Preceres LLC (acquired by Monsanto); PrognomIQ, Inc.; Pulmatrix; PureTech; Quris; ReLive; Rensselaer Polytechnic Institute/Department of Chemical and Biological Engineering; Reprocell USA, Inc. (formerly Stemgent); Replay Bio; Rubius Therapeutics; Satellite Bio; SBEF (Seoul Bio Economy Forum); Secant Medical, Inc.; Seer, Inc.; Selecta Biosciences; Senses LLC; Setsuro Tech Inc.; Seventh Sense Biosystems, Inc.; Shenzhen Rice Life Technology, Ltd; Shire Ag; Sigilon; Sigma Aldrich Co. LLC; SiO2 Materials Science; Ske S.R.L.; Soil Culture Solutions LLC (Dba Soilcea); Souffle Therapeutics; SQZ Biotechnologies; StemBioSys, Inc.; SuonoBio; T2 Biosystems; Taconic Biosciences, Inc. (formerly Taconic Farms); Taiwania Capital (Bio-Asia Taiwan Symposium); TARA; Tarveda Therapeutics; Teal Bio; Terasaki Institute; Tesio Pharmaceuticals; Third Rock Ventures; Tiba Biotech LLC; TissiuM (formerly Gecko); Transgenic, Inc.; Translate Bio (formerly Rana Therapeutics, Inc.); Trilink Biotechnologies, Inc.; Unilever (Living Proof); University of Bergen, Norway (Falch Lecture Honorarium); VasoRX; Verseau Therapeutics, Inc.; Virex Health; Vitakey; Vivtex Corporation; Westlake University; Whitehead Institute; Wiki Foods; Xenter; YourBio (formerly 7th Sense Biosystems); Yz Biosciences (Guangzhou), Inc.; Zenomics; ZWI Therapeutics. A.J. receives licensing fees (to patents on which she was an inventor) from, invested in, consults (or was on Scientific Advisory Boards or Boards of Directors) for, lectured (and received a fee), or conducts sponsored research at MIT for which she was not paid for the following entities: Moderna Therapeutics; OmniPulse Biosciences; Particles for Humanity; SiO2 Materials Science; Takeda (formerly Shire Ag); VitaKey. All the other authors declare no competing interests.

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