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Oral mucosa immunity: ultimate strategy to stop spreading of pandemic viruses

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Global pandemics are most likely initiated via zoonotic transmission to humans in which respiratory viruses infect airways with relevance to mucosal systems. Out of the known pandemics, five were initiated by respiratory viruses including current ongoing coronavirus disease 2019 (COVID-19). Striking progress in vaccine development and therapeutics has helped ameliorate the mortality and morbidity by infectious agents. Yet, organism replication and virus spread through mucosal tissues cannot be directly controlled by parenteral vaccines. A novel mitigation strategy is needed to elicit robust mucosal protection and broadly neutralizing activities to hamper virus entry mechanisms and inhibit transmission. This review focuses on the oral mucosa, which is a critical site of viral transmission and promising target to elicit sterile immunity. In addition to reviewing historic pandemics initiated by the zoonotic respiratory RNA viruses and the oral mucosal tissues, we discuss unique features of the oral immune responses. We address barriers and new prospects related to developing novel therapeutics to elicit protective immunity at the mucosal level to ultimately control transmission.

KEYWORDS

oral mucosa, RNA viruses, pandemics, saliva immunity, mucosal vaccine

1 Zoonotic respiratory RNA viruses are linked to global pandemics

Pandemic refers to the explosive outbreaks of communicable diseases on a global scale (1–3). The scale, geographic location, and duration of pandemics are unpredictable (4, 5). Historically, the most devastating pandemics were initiated by cross-species transmission of pathogens, such as Justinian plague (541-542 AD), the Black Death (1347-1351), flu pandemics (Spanish flu in 1918, Asian flu in 1957, Hong Kong flu in 1968, Russian flu 1977, Swine flu in 2009), and the ongoing SARS-CoV-2 COVID-19 pandemics (2019-current) (6–9). Since most human populations are immunologically naive, wildlife pathogens that acquired a susceptibility to humans can spread rapidly (10). Still, cross-species transmission from animal to human is not as common and requires successful adaptation to maintain long-term human to human transmission (11–14). Wolfe et al. summarized five progressive stages of animal microbe's

human adaptation: 1) exclusivity to animals; 2) obtaining non-sustainable animal-to-human transmission; 3) limited human-to-human transmission; 4) sustained human-to-human transmission without the need for an intermediate host (influenza A, SARS, MERS, SARS-CoV-2,Vibrio cholerae, and dengue virus); and 5) exclusive circulation in humans (15). As humans encroach into the natural habitats of wildlife and as human population, travel, and trade increases, so does the risk of spillover events (16). Domestic animals serve as intermediate hosts to create novel zoonotic pathogens, increasing the chance of transmission from wildlife (17, 18). Emergence of the pandemic 2009 H1N1 virus (pdm09 H1N1) serves as a prime example where the novel virus was created by a triple genetic reassortment event (influenza genes derived from North American swine, humans, and birds) which most likely occurred in domesticated pigs (19–21). Exceptional mutation rates and short generation times are highly advantageous to RNA viruses, allowing them to adapt to new host systems and break the species barrier by compatibility to host cell receptors, cellular enzyme systems, or tissue tropism (22, 23). Mutation rates of RNA viruses can roughly occur at rates of six orders of magnitude greater than those of their cellular hosts (23). Across multiple studies, a critical part of emerging pathogens (25-44%) in humans is reported to be related to respiratory RNA viruses (24–27).

The global pandemics affecting all five continents almost simultaneously were initiated by zoonotic respiratory RNA viruses including influenza and the coronaviruses. Currently, vaccines are the most efficacious measure to reduce the disease severity and mortality of respiratory viral diseases (28, 29). However, due to the biased immunogenicity to elicit systemic neutralizing antibody response, vaccinations cannot stop the spread of the virus at mucosal surfaces (30–33). Silent spread of viruses among asymptomatic patients can further generate novel escaping mutants (34–37) and impact public health.

2 Salivary droplets as transmission source of zoonotic respiratory RNA viruses

Respiratory RNA viruses primarily infect and replicate at respiratory tracts, and the amplified viruses shed their progeny into mucosal droplets, often spread by coughing or sneezing (38, 39). Considering the poor stability of RNA and viral envelope structure, transmission of aerosolized particles had been, historically, less supported (40). Due to this belief, the efficacy of facial masks was questioned in preventing transmission of the respiratory viruses during the initial phase of the COVID-19 pandemic (41). The role of aerosolized particles in transmission of respiratory particles has been more supported as experiencing explosive incidence of the COVID-19 cases in indoor environments that are poorly ventilated, such as meatpacking factories, cruise ships, and churches (40).

For the transmission of highly attenuated SARS-CoV-2 variant strains, salivary droplets generated during speech have been increasingly considered as a major transmission vehicle for the asymptomatic carriers lacking respiratory symptoms (coughing and sneezing) (42, 43). While the SARS-CoV-2 virus is considered a respiratory pathogen, the virus is known to replicate in a variety of tissues and organs expressing the ACE2/TMPRSS receptors, including gingival tissues and salivary glands (44).

This is also consistent with human adapted influenza viruses and oral epithelium. It requires galactose linked to α-2,6-sialic acid, abundantly expressed on epithelial cells of the upper respiratory tract, including oropharynx (45). While avian influenza viruses preferentially bind to the α-2,3-SA expressed in the human lower respiratory tract, human adapted zoonotic influenza viruses replicate in the oropharyngeal airway and shed into the salivary droplets. Responsible for the 2009 pandemic, the A/(H1N1)pdm09 virus has been reported to bind to α-2,6-SA and, to a limited extent, to α-2,3-SA (45, 46). In the case of the highly pathogenic avian influenza virus H5N1 viruses, one of the most devastating candidate pandemic virus strains, can also infect and replicate in cells of the nasopharyngeal and oropharyngeal epithelia (47). Influenza is also known to be detected in saliva (48, 49). A recent study showed no significant difference in detection rate of influenza virus detection rate between saliva and nasopharyngeal swabs (48).

In the case of small virus-laden droplets (<30μm), highly sensitive laser light scattering observations have revealed that loud speech can emit thousands of oral fluid droplets per second (43). In a closed, stagnant air environment, they disappear from the window of view with time constants in the range of 8 to 14 min, corresponding to droplet nuclei of 4μm diameter, or 12 to 21μm droplets prior to dehydration (43). Virus-laden droplets less than 30μm could even spill over conventional facial masks. Spilled RNA virus particles maintain infectivity for hours in the air or on surfaces and infection virus was still detected up to 28 days later (50).The stability of coronaviruses varied between 1 hour to 24 hours depending on the humidity and temperature (51–55). In the case of animal coronavirus porcine enteric diarrhea virus (PEDV), the viral RNA in air was detectable at 16.1 km (56). Actual evidence of airborne transmission has also been demonstrated in *in vitro* and *in vivo* models. Kormuth et al. used humidity-controlled chambers and identified that the 2009 pandemic influenza A (H1N1) virus in suspended aerosols stationary droplets remain infectious for an hour across a wide range of humidities (23-98%) (57). Through a guinea pig model, transmission of influenza A/Panama/2007/1999 (H3N2) (58) virus through the air was measured as efficient as the fomite transmission (58). Collectively, active shedding of respiratory RNA viruses in saliva can be a major source of transmission from asymptomatic carriers lacking respiratory symptoms. Stability of RNA viruses in the air and potential of airborne transmission shows the ease of transmission of the zoonotic respiratory RNA viruses, emphasizing the need for induction of oral immunity (Figure 1).

3 Induction of oral immunity reduces respiratory viruses spread

The lack of effective measures to prevent entry of viral particles at the mucosal surfaces poses a major challenge in controlling

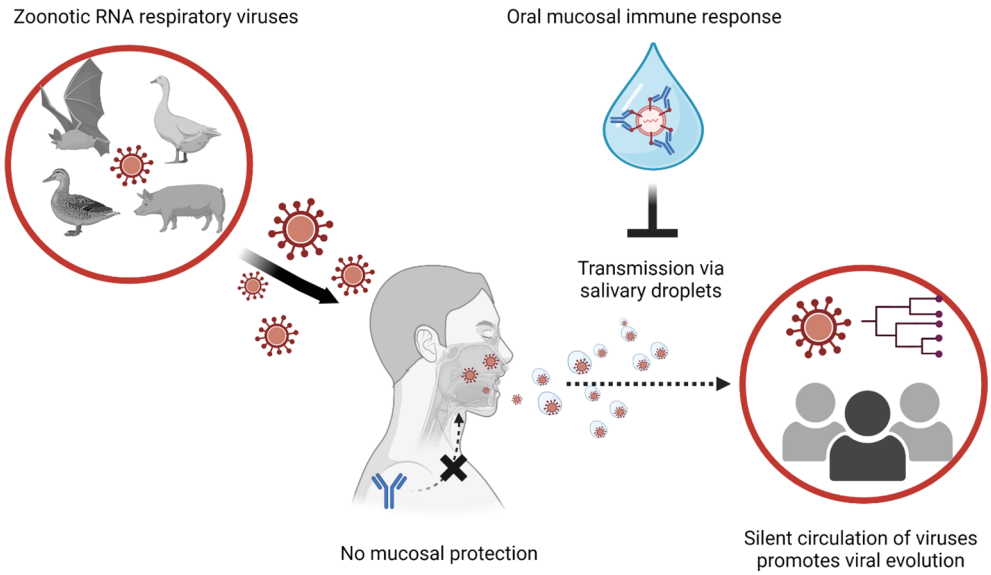

FIGURE 1
Global transmission viral transmission patterns could be interrupted by mucosal immune responses to manage zoonotic RNA respiratory viruses. Novel viral infections to humans are originated from animals. Especially when zoonotic respiratory RNA viruses gain human-to-human transmission capabilities, the novel infectious agent can explosively spread through the immunologically naive human population. In the case of human populations gaining partial immunity to the virus, systemic antibody response reduces the severity of clinical illness and mortality. However, systemic immune response cannot block the infections/transmission of the viruses at the mucosal surfaces (e.g., upper respiratory tract and oral cavity). In transmission of asymptomatic infection without respiratory symptoms (sneezing and coughing), virus-laden salivary droplets can act as major source of viral transmission. Thus, induction of protective immune response at the oral mucosal surface is instrumental to control transmission of the zoonotic respiratory RNA viruses.

zoonotic respiratory viruses. Vaccination is the most effective strategy to control zoonotic respiratory RNA viral disease, significantly lowering the disease severity and case-fatality rate (59–61). However, current vaccines administered via parenteral route cannot directly stimulate the mucosal immune system (29, 62). Systemic antibodies induced by vaccination provide partial protection to subjects but transportation from blood to mucosal epithelia surface is highly restricted to confer protection at mucosal surfaces (63–65). Instead, vaccinated individuals can carry the viruses without apparent symptoms and serve as asymptomatic carriers (66). As viruses are more attenuated and sheds easier without apparent symptoms, vaccination and symptom-based intervention strategies lose their efficacy and the viruses evolve to more divergent escaping mutants (67). The ultimate strategy to end the current pandemic and prevent future pandemics is to control transmission. Current efforts to control variant viruses are to induce sterilizing immunity, which in turn provides protective immune responses at both mucosal and systemic levels (68, 69). In theory, sterilizing immunity aims to induce neutralizing antibodies at the viral entry site, differentiated from the protective immunity which refers to prevention from symptomatic infections. Sterile immunity prevents the viral transmission, including the asymptomatic and presymptomatic carriers (68, 70). At the phase when the viruses are highly attenuated and asymptomatic transmission lacking respiratory symptoms (e.g., coughing or sneezing) is more frequent, induction of neutralizing IgA response at oral mucosa should be considered (43). While the oral immune system is known to be on the frontline of the gastrointestinal tract (GIT) and respiratory tract, it has been relatively less investigated (71–74). Novel strategies needed to induce oral mucosal immune responses are particularly scarce due to its unique role in preventing entry of external pathogens and hyperactivity to diet or to air exposure.

4 The oral mucosal immune system is driven by a unique features

Oral mucosa is the beginning of the GIT and shares anatomic and histologic characteristics with GIT (75–77). In addition to mucus produced by overall GIT, the oral cavity produces saliva (32, 78). The whole saliva is originally generated from serum exudates and supplemented with highly diverse molecules from mucosal cells, immune cells, and microbes (78). Continuous production and swallowing of saliva provide a mechanical clearance of pathogens (78). Also, saliva contains host defense proteins, primarily responsible for both adaptive and innate humoral immune response at oral mucosa (78).

Oral mucosa, like other mucosal tissues, can be divided into three major layers, epithelia, lamina propria, and specialized lymphoid tissues (visual summary in Figure 2) (73, 75). The epithelial layer of oral mucosa is stratified squamous epithelium, forming a thicker and denser mechanical barrier than the single layer of GIT epithelia (73, 75). The top portion of the oral epithelial layer forms a level of various levels of keratinization according to the anatomical location (73, 75). Some areas, such as pharynx and junctional epithelium at periodontal space, are non-keratinized and serve as a major point for the innate defense and homeostasis in oral microenvironments (79–81). Lamina propria (LP), a loose connective tissue containing blood and

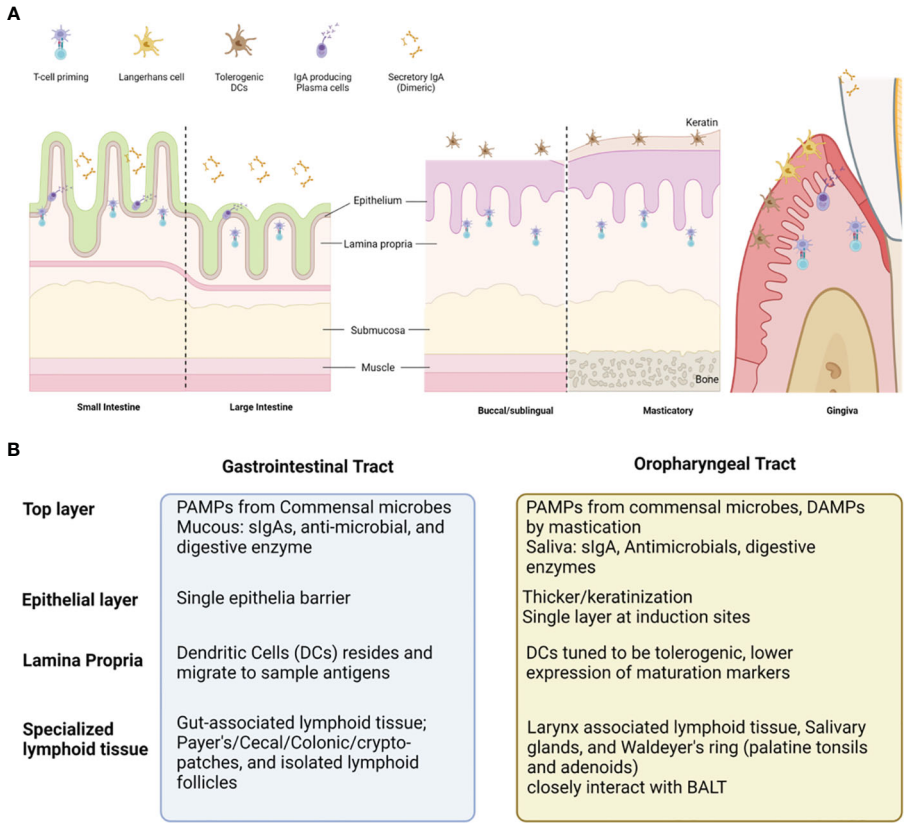

FIGURE 2
Comparison between oral vs gastrointestinal mucosal tissues and the cell populations contributing to overall immunity. Oral mucosa is the initial compartment for gastrointestinal tract (GIT). Overall structure of oral mucosa is like GIT, consisted with the shares common histologic structure; covered with commensal microbes and saliva filled with diverse antimicrobial, enzymes, and secretory IgAs (sIgAs) **(A)**. The top layer, epithelium, lamina propria, and specialized lymphoid tissues present distinct cells and functions according to the GI versus Oral tract **(B)**. Oral cavity presents unique traits (much thicker epithelial layer, presence of keratin layer, and tolerogenic dendritic cells), which can prevent vaccine antigen delivery and induction of virus-specific immune response at oral mucosal surfaces.

lymphatic vessels under epithelial layers, is a major inductive and effector site for immune cells (79). Steady-state dendritic cells (DCs) reside throughout the lamina propria and often migrate to sample auto-, and foreign antigens derived from commensal microbes, dietary components, mastication damage and pathogens (82). The steady-state DCs in oral tissues are tuned to be tolerogenic to most stimuli from the oral microenvironment, expressing low levels of maturation markers (CD80, CD83, and CD86) (83, 84). In certain conditions, such as invasion of pathogenic microbes, dysbiosis, or damage associated with molecular patterns (DAMPs), the DCs are activated and migrate to lymphoid tissues to induce T activation, such as buccal mucosa, salivary glands, and waldeyer's ring, is located and serves as major site for activation and expansion of lymphocytes (79). Activated antigen-specific IgA secreting B cells or CD8+ T cells relocate to the effector site, such as the epithelium, LP, and salivary glands, to mediate immune response. But mature DCs also limit T cell activation and promote immune tolerance in specific triggers, such as IL-27, IL-10, vitamin A, or ligands of the aryl hydrocarbon receptor (AhR) (85–88).

As the sIgA can block the viral replication cycle at the initial stage, virus specific sIgAs has been thought to be the most potent target to induce sterilizing immunity at mucosal surfaces (70). In oral mucosa, the sIgA is produced from plasma cells primarily residing in salivary glands and secreted as two monomers linked by a junctional chain via polymeric immunoglobulin receptors (pIgR) at the basolateral membrane of epithelial cells (89–91). In mucosa, the process of class switching to the IgA producing B cell occurs at the lymphoid tissues, such as nasopharyngeal-associated lymphoid tissues (NALT), tear duct associated lymphoid tissue (TALT), and peripheral lymphoid tissues. To elicit antibodies specific to the viral antigen with high affinity, the naive B cells go through the class switch recombination (CSR) by CD40-CD40L ligation in presence of the TGF-β and other co-stimulatory cytokines (IL-4,IL-5, IL-6, IL-10 and IL-21) mediated by CD4+ helper T cells (Th) (91, 92). Meanwhile, naive B cells can activate in response to the continuous stimuli from commensal microbes, metabolites and dietary antigens without involvement of T cells or hypersomatic mutations (93, 94). Two types of antigens have been known to induce the T cell-independent activation (95–97). Type I antigens are typically microbial products (e.g. bacterial LPS or DNA), directly activating B cells through the toll-like receptors on the B cell surface. Type 2 antigens are usually repetitive or highly cross-linked structures found on the surface of encapsulated bacteria, such as polysaccharides or glycolipids. Type II antigens do not have intrinsic activity to stimulate, but accumulation of BCRs and cross-activation of

the receptors can activate B lymphocytes, leading to the production of various cytokines, including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). Type II antigens can only activate mature B cells. Due to the lack of CSR, sIgAs produced by T cell independent processes present low affinity and low specificity to antigens (98). The source of cytokines involved in T cell independent class-switching is thought to come from subsets of innate immune cells, such as innate lymphoid cells (ILCs) (99). In addition to the sIgA response, commensal microbes are instrumental for the induction and/or tolerance of local immune responses (100–102). Oral mucosa possesses the second largest microbial community after the gut (103, 104). The symbiotic interaction between mucosal epithelial linings and microbes is crucial to maintain steady state of the oral mucosa (100–102).

Microbial colonies serve as primary barriers to inhibit the invasion of external microbes (105). Microbes and their metabolites can also modulate the tone of immune response to constant stimulation by the dietary and inhaled antigens (100–102). Metabolites produced from gut microbiota have been shown to directly influence both inflammatory cells (inflammatory Macs (iMacs), DCs, CD4 T helper (Th)1, CD4Th2, Th17, natural killer (NK) T cells, NK cells and neutrophils) and immuno-suppressive cells (e.g., tolerogenic T cells (T_{reg}), regulatory B cells (B_{reg}) and innate lymphocytes (ILCs)) (103). Accumulating evidence reveals that the dysbiosis in oral mucosa also contributes to the disease pathogenesis, especially for the respiratory viral infection (103, 106, 107). It is important to note that the oropharynx is the primary site of viral replication and immune induction and major source of the lung microbiome (103, 108). Also, infection with respiratory viruses, such as the SARS-CoV-2, impacts on enrichment of opportunistic pathobionts in the oral cavity (106, 109, 110). A recent cross-sectional study showed that the COVID-19 patients presented a distinctive microbiome profile, a decrease in the alpha-diversity and bacterial species richness in association with symptom severity (103).

The oral cavity maintains homeostatic inflammatory state, created by microflora. The local microflora habituated on the oral cavity is known to be more than 700 species of bacteria, viruses, fungi, and protozoa (111). The main inhabitants of a healthy oral cavity are gram positive and negative cocci and rods, such as Firmicutes, Bacillus, Proteobacteria and Actinomycets (111–113). In a homeostatic state, the microbial community acts as a barrier against colonization of foreign agents and aids differentiation/maturation of the oral immune system (114). For example, constant production of bacterial products and damage associated molecular patterns (DAMPs) constantly recruit and stimulate innate immune cells (eg. neutrophil). Also, bacterial products (LPS, DNA, or polysaccharides) serve as antigen to induce T cell independent low affinity sIgA response during normal state. The sIgAs produced from the healthy state play a pivotal role to prevent overt growth of microbiome. Another important regulator of the microbiome is the fibrin (115). Inflammation triggered by the microbiome results in constant fibrin deposition in oral mucosa. The fibrin activates neutrophil effector functions, harnessing overgrowth of bacteria and activating the plasmin-mediated fibrinolysis. Since the homeostatic inflammation is highly orchestrated by complex interaction among oral mucosa, microbiota, immune cells and clotting factors, dysbiosis and/or tissue damage created by viral infection can significantly impair the oral immune system and promote disease progress from local infection to the systemic illness (103, 115).

5 Induction of protective mucosal immune response is challenging: insights on the oral immunology

The oral mucosa is exposed to a variety of environmental insults, including pathogens, allergens, and toxins (77, 116). The oral mucosa is also the first line of defense against these insults, and it is essential that the oral mucosa is able to mount an effective immune response (77, 116). The immune response at mucosal surfaces is mediated by a variety of cells, including dendritic cells, macrophages, neutrophils, and B cells (117). These cells work together to generate an immune response that is specific to the pathogen or allergen that is being encountered (117). While the oral mucosa is constantly stimulated by foreign intakes, the symbiotic interactions among microbes epithelial cells and immune cells can also send signals to the system including clotting factors and microbiome intrusion (83, 84, 115). Due to the complexity, induction of antigen-specific immune response at the oral mucosal surface requires alternative approaches differentiated from conventional parenteral prophylactic or therapeutic strategies. The induction of mucosal immune responses is a complex process that is not fully understood, however, it is known that a number of factors can influence the ability of the oral mucosa to mount an effective immune response (83, 84, 115). These factors include: (i) the presence of pathogens or allergens; (ii) the integrity of the oral mucosa; (ii) the presence of IgA antibodies; (iii) the presence of cytokines; (iv) the presence of regulatory T cells (83, 84, 115, 116). Also, the oral mucosa is home to a variety of commensal bacteria that can interfere with the immune response (83, 84, 115).

The first challenge for inducing mucosal immune response is the multiple mechanical and chemical barriers. Specially, the oral cavity is composed of multiple layers of epithelial cells, most areas covered with keratinized cells, except the inductive sites (pharynx, tonsil, hard/soft palate, buccal-, and sublingual mucosa) (81, 83). Also, the continuous production and swallowing of saliva containing diverse enzymes interferes with stable delivery of vaccine antigens and adjuvants (118). To induce protective immune response, the immunogen needs to overcome such barriers and persist at the site to initiate cascades of immune responses that lead to protection, such as homeostasis and maintenance of health.

The second barrier is to elicit protective immune responses by overcoming oral tolerance without the risk of experiencing hypersensitivity (83, 119, 120). Oral tolerance refers to the process in which the immune system does not respond to orally administered antigens (83, 119). At least two different mechanisms have been identified to mediate development of oral tolerance (83, 119). One mechanism is the induction of regulatory T cells via production of TGF-β but that concomitant retinoic acid signaling

boosted this process by mucosal DCs (119). T cell anergy is another possible mechanism induced in high-dose oral tolerance. Anergic T cells are also known to contribute to oral tolerance (83, 119). One method to circumvent oral tolerance could be to apply antigen in another mucosal route, such as intranasal or sublingual route.

Additional considerations relate to inducing protective oral immune response from by influence of commensal microbes and microbiome derived signals (77, 82). Oral cavity maintains homeostatic inflammatory status against commensal microbiota (77, 121, 122). In oral vaccination, depletion of microbiota significantly reduced Th1 and Th17 response to the heat-labile enterotoxin of enterotoxigenic Escherichia coli as adjuvant (LT R192G/L211A) (123). Also, individuals who displayed more diverse gut microbiota tended to exhibit better response to vaccinations (124). In contrast, dysbiosis can result in reduction in vaccine efficacy (125–127). Probiotics have been suggested to enhance IgA and memory T cell response in COVID-19 management (128).

Above issues are the major barrier in developing prophylactic/vaccine strategies to induce oral mucosal immune system and stop the silent spread of the zoonotic respiratory viruses via salivary droplets. Next, we discuss novel approaches targeting influenza, and the SARS-CoV-2 viruses, under clinical trials to prove their efficacy in induction of oral mucosal immunity.

6 Novel approaches inducing mucosal immune response specific to the zoonotic respiratory RNA viruses

6.1 Delivery system

6.1.1 Direct sensitization of oral mucosa

Is the most efficient route to activate resident immune cells and induce antigen-specific IgA response (129). Novel delivery strategies have been designed to overcome multiple mechanical barriers (e.g., keratinized epithelium, clearance system), proteolytic activity of saliva, and tolerogenic mechanism of oral mucosa. A lipid based delivery system (i.e. liposome, lipid nanoparticles, emulsion and immunostimulatory complexes (ISCOMs)) is a promising vehicle, formulating immunogens in water-immiscible lipid, protecting enzymatic digestion, and enhancing absorption into the mucosal surfaces (130, 131). For COVID-19, the lipid nanoparticle-mRNA format was successfully introduced in an intramuscular injection format. To induce oral mucosal immunity to influenza and COVID-19, the lipid-based delivery system has been tested in *in vivo* studies (132–137).

6.1.2 Polymer-based delivery systems

Can increase the contact time of delivered adjuvant/immunogen, provide stability, and adjunctive effects (138, 139). Polymers can be divided into natural (chitosan, gamma polyglutamic acid, hyaluronic acid, and pullulan) and synthetic (PLGA, Polyethyleneimine, poly-ϵ-caprolactone, PCL, and Polypropylene sulfide). For influenza, the polymer-based vaccines have already been developed and proven their efficacy in animal models for the mucosal influenza vaccine development (140–144). Also, the polymeric-based nanoparticles system is under development for COVID-19 therapeutics and vaccines (145).

6.1.3 Sublingual vaccination

Is also a method of delivering vaccines directly under the tongue, absorbed by the mucous membranes (118, 146). Similar to sublingual vaccination, buccal vaccination is another method of delivering vaccines directly to the mucous membranes in the mouth (118, 146). However, instead of placing the vaccine under the tongue, the vaccine is placed on the inner cheek or buccal mucosa (118, 146). Both sublingual-, and buccal mucosa contains high level of antigen presenting cells, T-, and B-cells and attractive target as vaccine delivery (118, 146, 147). One potential advantage of buccal vaccination over sublingual vaccination is that it may offer more flexibility in terms of vaccine design and formulation. The buccal mucosa has a larger surface area than the sublingual mucosa, which may allow for the delivery of larger doses of the vaccine or the use of more complex formulations (148). There are ongoing research and development efforts to create sublingual vaccines for influenza and coronavirus (including SARS-CoV-2) (118, 149–151). Previous preclinical studies in animals have shown promising results for sublingual vaccines against influenza and coronaviruses, demonstrating the induction of robust immune responses and protection against infection. However, to date, no sublingual vaccine for influenza or coronavirus has been approved for use in humans. Development of the sublingual vaccines for influenza and coronaviruses remains an active area of research, there have been multiple clinical trials (Table 1).

6.1.4 Microbial display system

Microorganisms, such as virus, bacteria or yeast, can be used as a vaccine delivery system. The microbial display system can leverage their surface proteins as immunostimulants, enhancing immunogenicity of weakly immunogenic vaccine antigens. Also, the *in vitro* cultivation of vehicle microbes enables mass production in a cost-effective way. Bacteria, varial, and fungi have been widely investigated as delivery vehicles. The spore-based system is under clinical trial for the COVID-19 vaccine. (NCT05239923).

6.2 Mucosal vaccines

6.2.1 Live attenuated vaccines (LAV)

AV promote direct sensitization of the mucosal surface and have been the most efficacious way to elicit a protective immune response in the oral mucosa (152, 153). Also, live replicating viruses in epithelia stimulates innate and cell-mediated immunity, serving as a self-adjuvant and preserved from the mucosal clearance system. The LAV can be delivered via a variety of routes, including oral, nasal, and rectal (154). Oral delivery of live attenuated vaccines is particularly effective in inducing mucosal immunity (155–158). This attribute has distinct benefits from the parenterally delivered injectable vaccines, including, high efficacy at oral mucosa, ease of administration, and cost effectiveness (28, 159, 160). Also, the LAV

TABLE 1 Clinical Trials, Study Phase, and Types of Investigations Related to Oral Mucosa.

1	Phase	Study
Novel delivery system		
NCT04334980	Phase I/II	phase 1/2 trial evaluating the safety and immunogenicity of a sublingual influenza vaccine
NCT04625972	Phase I	phase 1 trial evaluating the safety and immunogenicity of a sublingual COVID-19 vaccine
NCT04563702	Phase I	phase 1 trial evaluating the safety and immunogenicity of a buccal COVID-19 vaccine
NCT04644782	Phase II	phase 2 trial evaluating the safety and efficacy of a sublingual COVID-19 vaccine
Live attenuated/vector vaccine canddiates		
NCT01982331	Phase II	phase 2 trial evaluating theReactogenicity, Safety and Immunogenicity of a Live Monovalent A/17/California/66/395 (H2N2)Influenza Vaccine
NCT02480101	Phase II	phase 2 trial evaluating theReactogenicity, Safety and Immunogenicity of a Live Monovalent A/17/Anhui/2013/61 (H7N9) Influenza Vaccine
NCT01841918	Phase II	phase 2 trial evaluating the Safety and Immunogenicity of Live Attenuated Influenza H5 Candidate Vaccine Strain A/17/Turkey/Turkey/05/133 (H5N2) in Healthy Thai Volunteers
NCT02229357	non-randominzed open label	non-randomized open label study evaluating thepriming Effects by Pandemic Live Attenuated Influenza Vaccine (LAIV Candidate Vaccine Strain A/17/Turkey/Turkey/05/133 (H5N2)) on the Subsequent Response to Inactivated H5N1 Vaccine in Healthy Thai Volunteers: A Non-Randomized, Open Label Study
NCT03300050	Phase I	phase 1 trial evaluating the Reactogenicity, Safety, and Immunogenicity of a Live Attenuated Universal Influenza Vaccine (cH8/1N1 LAIV) Administered as a Single Priming Dose Followed Three Months Later by a Single Booster Dose of an Inactivated Universal Influenza Vaccine (cH5/1N1 IIV) (Adjuvanted With AS03A or Unadjuvanted) in 18 Through 39 Year-old Healthy Subjects, Contrasted With a Two Dose Schedule of an Inactivated Universal Influenza Vaccine (cH8/1N1 IIV + AS03A Followed Three Months Later by cH5/1N1 IIV + AS03A)
NCT04619628	Phase I	phase 1 trial evaluating the safety and efficacy of a COVI-VAC COVID-19 vaccine
NCT04871737	Phase I	phase 1 trial evaluating the safety and efficacy of a Newcastle disease virus (NDV) vector vaccines expressing the spike protein of SARS-CoV-2
NCT04816019	Phase I	phase 1 trial evaluating the safety and efficacy of a intranasal ChAdOx1 nCoV-19 (AZD1222) COVID-19 vaccine
NCT05007275	Phase I	phase 1 trial evaluating the safety and efficacy of a aerosole ChAdOx1 nCoV-19 (AZD1222) COVID-19 vaccine
NCT04839042	Phase I	phase 1 trial evaluating the safety and efficacy of SC-Ad6-1 COVID-19 vaccine
Second generation vaccine: Adjuvnat-vaccine complex		
NCT05385991	Phase I	phase 1 trial evaluating the Safety and Immunogenicity of the ACM-SARS-CoV-2-beta With ACM-CpG Vaccine Candidate (ACM-001), Administered Intramuscularly or Intranasally as a Booster Dose in Healthy Adults Aged 18 to 55 Years, Who Were Previously Vaccinated Against SARS-CoV-2.
Oral antivirals/antiseptics		
NCT04405570, NCT04405739	Phase II/III	phase 2/3 trial evaluating the ribonucleoside analogue inhibitor of influenza viruses, MK-4482/EIDD-280 for influenza and SARS-CoV-2 viruses
NCT04405570	Phase Iia	phase 2a trial evaluating the Safety, Tolerability and Efficacy of EIDD-2801 to Eliminate SARS-CoV-2RNA Detection in Persons With COVID-19
NCT04497987	Phase III	phase 3 trial evaluating the Efficacy and Safety of LY3819253 Alone and in Combination With LY3832479 in Preventing SARS-CoV-2 Infection and COVID-19

is free from the issue of delivery since the vaccine virus attaches to the cellular receptor and is internalized into the mucosal epithelial surface. Activation of innate intracellular signaling pathways during internalization can add self-adjuvanting effects, mainly through the pathogen recognition receptor (PRRs) (161). For seasonal influenza, for example, FluMist has been used over decades as an intranasal spray vaccine (162). Also, there have been multiple live vaccine candidates, such as live attenuated vaccine format and or vector vaccines (163). However, the FluMist could not induce salivary IgA response (164), showing that nasal activation is not always effective.

Multiple live attenuated influenza vaccines have been developed against pandemic influenza strains H5Nx and H7N9 viruses, which is under clinical trials (H2N2: NCT01982331; H7N9: NCT02480101; H5Nx: NCT01841918 and NCT02229357) (165–168). As a next-generation influenza vaccine, a chimeric hemagglutinine-based universal influenza vaccine is also under clinical trial

(NCT03300050) (169). Since this novel antigen does not naturally occur, it can avoid the risk of back-mutation. For COVID-19, the COVI-VAC is under phase I clinical trial (NCT04619628).

6.2.2 Vector vaccines

Live vaccines can be designed by using viral vectors, such as Newcastle disease virus (NV), Vestibulo stomatitis virus (VSV), and adenoviruses (170–172). Viral vectors are genetically engineered to express novel antigens, such as the spike protein of the SARS-CoV-2 virus (170–173). As the LAVs, vector viruses attach and replicate directly on the target mucosal tissue, solving the issues of delivery, dosage, and deposition (174). Also, the replication of vector virus triggers the innate and cell-mediated immune system, providing an adjuvant effect for the vaccine antigen (175). A Newcastle disease virus (NDV) vector vaccine expressing the spike protein of SARS-CoV-2 is currently under phase I clinical study (NCT04871737). A replication-competent chimeric VSV-SARS-CoV-2 vaccine candidate by replacing the VSV glycoprotein (G) gene with a coding sequence for the SARS-CoV-2 Spike glycoprotein (S) (VSVΔG-SARS-CoV-2) also has proven efficacy in a hamster model (176). The ChAdOx1 nCoV-19 (AZD1222), developed by AstraZeneca and first approved as an intramuscular vaccine, is now under phase I clinical trial to be applied as intranasal vaccine (NCT04816019) and aerosols (NCT05007275). The SC-Ad6-1 is another adenovirus vector vaccine from Tetherex Pharmaceuticals Corporation also under phase I clinical trial (NCT04839042).

Novel live vaccine candidates under clinical trials are highly expected to be used to complement limitations of current parenteral vaccines. Their efficacy on stopping transmission of viruses is still an emerging topic in the vaccine industry and more accumulated data will be needed.

6.3 Mucosal adjuvants

Adjuvants are substances an agent that increases specific immune responses to an antigen (177). Mucosal adjuvants can enhance the immunogenicity of vaccines at the mucosal surface, as evidenced in AS03, MF59, and CpG-ODN (178–180). To enhance the immunogenicity of the vaccines at the mucosal surface, novel adjuvant strategies have been suggested, especially for the influenza vaccine (181–186). Novel approaches apply the microbiome and its byproduct as a source of innate signaling to enhance the antiviral immune response in mucosal surfaces. For example, PMAPs from antibiotic-killed bacteria could enhance antiviral-immune response in intranasal mucosa (187, 188). In a hamster model, Mao et al. applied antibiotic-killed intranasal and oral microbes to induce vaccine-specific nasal IgA and serum IgG responses to influenza and SARS-CoV-2 viruses in a dose-dependent manner (189).

Novel vaccines incorporate adjuvant molecules into vaccine candidates to enhance immunogenicity and delivery system. As a second-generation vaccine, the ACM-SARS-CoV-2-beta ACM-CpG vaccine candidate (ACM-001) is under clinical trial (ClinicalTrials.gov identifier: NCT05385991). The vaccine consists of recombinant Beta spike protein co-administered with synthetic CpG adjuvant. Both components are encapsulated within artificial cell membrane (ACM) polymersomes, synthetic nanovesicles efficiently internalized by antigen-presenting cells, including dendritic cells, enabling targeted delivery of cargo for enhanced immune responses. The ACM vaccine has proven enhanced serum IgG and neutralized response immunogenicity in C57BL/6 mice and Golden Syrian hamsters. In the oral cavity, the ACM-001 vaccination could not reduce the viral peak titer but shortened the viral shedding period (190).

6.4 Direct reduction of viral load by using oral antivirals/antiseptics

While the threat of current and future pandemic respiratory viruses is still ongoing, there has not been an effective strategy to induce oral mucosal immunity, especially to novel viruses. Focusing on reducing transmission of the viral spread through saliva droplets, direct administration of antivirals on the oral mucosa can be a temporary alternative strategy to reduce or block viral shedding at oral mucosa. For example, the ribonucleoside analog inhibitor of influenza viruses, MK-4482/EIDD-2801, reported the efficacy for both influenza and SARS-CoV-2 viral infection (currently in phase II/III clinical trials, NCT04405570 and NCT04405739, respectively). In a ferret model, the MK-4482/EIDD-2801 significantly reduced the replication level of the virus at the upper respiratory tract and completely prevented transmission to the contact controls (191). Molnupiravir is also antiviral under clinical trial (NCT04405570), which completely stopped virus shedding from the COVID-19 outpatients by day five after administration via oral route (192) and also active against other RNA viruses, such as influenza, SARS, and MERS. Paxlovid is also an oral antiviral test for COVID-19, reported to shorten the viral shedding period, but it cannot prevent viral infection (193). Antivirals can also be used as prophylaxis to prevent viral infection in the population with high exposure risk. In the case of the influenza virus, antiviral medications (amantadine and neuraminidase inhibitors) are allowed to be used as chemoprophylaxis in people at high risk of influenza complications and people with severe immune deficiencies or receiving immunosuppressive medications (194).

For SARS-CoV-2, repurposing of antivirals as prophylaxis is currently under clinical trial (study NCT04497987). In a stochastic model of early-phase viral infection, the combination of antivirals that block the viral entry and increase viral clearance was estimated to block the small load of viral inoculum (195). Still, the use of antivirals is highly restricted due to their potential side effects and genotoxicity (196). Also, in a primate model, incomplete use of Remdesivir induced a longer duration of viral shedding (197). The combination of Bromelain and Acetylcysteine (BromAc) is under clinical trial to be used as a nebulized form in Healthy volunteers (198). Bromelain, extracted from the pineapple plant (Ananas comosus), contains enzymes that hydrolyze glycosidic bonds in complex carbohydrates and has been shown to remove the spike and hemagglutinin proteins of Semliki Forest virus, Sindbis virus, mouse gastrointestinal coronavirus, hemagglutinating encephalomyelitis virus, and H1N1 influenza viruses (199–201). Acetylcysteine is known to destabilize virion structures by disulfide bridge disruption. The combination use of two molecules unfolds the

molecular structures of complex glycoproteins, thus allowing binding to occur because of the high affinity between RBD and ACE2 (198).

To directly reduce/remove viral particles from the oral cavity, antiseptics are also tested under clinical trials and considered to be used. Povidone Iodine has especially shown its efficacy for the oropharyngeal infection (202–204). Since Povidone Iodine has not shown side effects, While hydrogen peroxide can provide an antiseptic effect plus boost the innate immune response by stimulating toll-like receptor 3; the results have been conflicting on the reduction of viral load at the oral mucosal surface (205).

7 Future directions and synergistic effects from current vaccines and next-generation vaccines

As COVID-19 pandemic is not considered a "public health emergency", the risk of the virus spreading and evolving into new variant strains persists. Partial immunity provided by parenteral immunization greatly contributed to reducing the disease severity, but cannot fully stop the spread of the virus, constantly producing novel variant viruses. This review summarized unique characteristics of oral mucosal immunity and discussed strategies currently under clinical trials. Induction of the "sterilizing immunity" is not yet achieved, but there have been remarkable advances in understanding of oral mucosal immune system and vaccine/adjuvants. As a temporary measure to reduce active viral replication at oral mucosa, direct application of antiseptics/antivirals are also considered and under clinical trials. Albeit the limitations, current parenteral vaccines are still the most effective strategy to control pandemic viruses at this present, and emerging mucosal strategies are needed. Even though vaccination provides only partial immunity to mask apparent symptoms and contributes to the silent evolution of the zoonotic respiratory RNA viruses, vaccine-induced immunity reduces the viral load and limits the evolution pool of the viruses, which in turn can hamper transmission. In country-scale analyses on the SARS-CoV-2 genome, diversity of the SARS-CoV-2 virus showed an inverse correlation with the mass vaccine rate ($n = 25$ countries, mean correlation coefficient = −0.72, S.D. = 0.20) and viruses isolated from vaccinated COVID-19 patients presented significantly lower diversity in known B cell epitopes compared to those from unvaccinated COVID-19 patients (2.3-fold, 95% C.I. 1.4-3.7) (206). Also, pre-existing immunity built by parenteral immunization still provides a booster effect to the mucosal immunization. There have been multiple studies proving the combination of current parenteral vaccinations with mucosal vaccines, providinga synergistic effect on both systemic and mucosal responses (207–209).

Current open questions remaining in the mucosal immune response are 1) What is the sensitive, specific, and reproducible analyte to quantify protective mucosal immune response? 2) what is the complete mechanism involved in oral tolerance and hyperactivity? 3) the most efficient and safe delivery/adjuvant system for the oral mucosa, and 4) the oral microbiome which can contribute elicit protective immune responses. The COVID-19 pandemic has been a unique opportunity to explore diverse strategies against respiratory pathogens. Our current real challenge will be a continuous effort and investment in developing novel strategies to provoke mucosal immunity, especially at oral mucosal sites at a populational scale.

Author contributions

HJ conceptualized the topic of the article. HJ and MF contributed to build the outline of the paper. HJ and MF wrote the first draft of the paper and MM contributed manuscript revision. HJ and MM created figures, revised by MF. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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