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REVIEW

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Inhaled vaccine delivery in the combat against respiratory viruses: a 2021 overview of recent developments and implications for COVID-19

Rick Heida, Wouter LJ Hinrichs and Henderik W Frijlink

Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Groningen, The Netherlands

ABSTRACT

Introduction: As underlined by the late 2019 outbreak of severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), vaccination remains the cornerstone of global health-care. Although vaccines for SARS-CoV-2 are being developed at a record-breaking pace, the majority of those that are licensed or currently registered in clinical trials are formulated as an injectable product, requiring a tightly regulated cold-chain infrastructure, and primarily inducing systemic immune responses.

Areas covered: Here, we shed light on the status of inhaled vaccines against viral pathogens, providing background to the role of the mucosal immune system and elucidating what factors determine an inhalable vaccine's efficacy. We also discuss whether the development of an inhalable powder vaccine formulation against SARS-CoV-2 could be feasible. The review was conducted using relevant studies from PubMed, Web of Science and Google Scholar.

Expert opinion: We believe that the scope of vaccine research should be broadened toward inhalable dry powder formulations since dry vaccines bear several advantages. Firstly, their dry state can tremendously increase vaccine stability and shelf-life. Secondly, they can be inhaled using disposable inhalers, omitting the need for trained health-care personnel and, therefore, facilitating mass-vaccination campaigns. Thirdly, inhalable vaccines may provide improved protection since they can induce an IgA-mediated mucosal immune response.

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Dry powder vaccine; IgA; inhalation; MALT; mucosal immunity; pulmonary administration; respiratory viruses; SARS-COV-2; vaccines

1. Introduction

Since Edward Jenner's revolutionary approach to protect 8-year-old James Phipps from smallpox by injecting him with pus from a cowpox blister, vaccination is still of utmost importance in preventing society-disrupting viral infections around the globe [1]. With an increasing world population, globalization and concurrently increasing infrastructural needs as their primary catalysts, viral infections will continue to emerge, and most likely increase in incidence rate, as has been recently underlined by the sudden onset of the COVID-19 pandemic [2]. Additionally, global warming will likely contribute to an increased cross-over of endemic viral strains to remote regions around the world, posing an additional threat to human health [3,4].

Although needle-based vaccination has been the gold standard for vaccine administration, it poses several limitations. Firstly, injectable vaccines are either formulated as unstable liquids that require cold storage or as lyophilized powders for reconstitution. Secondly, it requires trained health-care personnel, which can be a problem specifically in non-industrialized countries and remote areas. Thirdly, there is a risk of needle-stick injuries and needle re-use, increasing the probability of cross contamination. Fourthly, compliance to needle-based vaccination may be low because of associated needle-phobia and pain at the injection site [5], and lastly, vaccination by injection predominantly induces systemic immune responses, that are not specifically directed at the pathogen's region of infection, such as mucosal sites [6]. Therefore, alternative ways of administration are highly desirable.

Over the years, various needle-free administration routes have been explored [5,7,8]. Of these, inhalation is an interesting way of administration, especially for vaccines against airborne transmissible micro-organisms that cause respiratory tract infections. Because of its large surface area of approximately 70– 100 m^2, its permeable epithelium, and its highly perfused nature, the respiratory tract mucosa is one of the most optimal targets for the uptake of biopharmaceuticals [9]. Due to a continuous exposure to foreign materials, the airways house a variety of antigen-presenting cells (APCs), such as alveolar macrophages and dendritic cells, which continuously scan their environment for every antigen that enters the body, in order to rapidly activate downstream immune responses [10]. Inhalation of vaccines in principle provides the opportunity to target all regions of the respiratory tract, including the pulmonary region. The advantage of this is that, in case of respiratory infections, the vaccine can be delivered directly at the pathogen's portal of entry, where it can induce a local immune response. The benefit of this is underlined by studies that show accumulation of preexisting virus-specific immune

CONTACT Wouter LJ Hinrichs W.L.J.Hinrichs@rug.nl Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands

Article Highlights

- Inhalation of vaccines is a promising strategy to prevent airborne infections.
- While COVID-19 has urged the development of novel vaccine concepts, a significant portion of the formulations used faces logistical challenges with regard to temperature management.
- Research on inhaled vaccine delivery should be directed more towards the development of stable dry vaccine formulations since they have the advantage of increased stability, and hence, can be stored for prolonged periods without decay under less strict temperature requirements.
- The implementation of aerosolized vaccines should be seriously considered as an option to condemn highly infectious viral diseases like measles, influenza and COVID-19.
- While extensive research has shown the benefits of inhaled vaccines, the vaccination dogma is still centered around needles.

cells in the lungs and, thus, might be worthwhile to target directly [11,12]. The feasibility of pulmonary vaccination was already proven in the sixties and seventies of the past century by pioneers like Waldman *et al*. [13–16], Haigh *et al*. [17,18], and McCrumb *et al*. [19,20] who have shown that the inhalation of influenza and measles vaccines resulted in adequate protection. However, these authors used classical nebulizers for the administration of liquid vaccine formulations that were unsuitable for larger vaccination campaigns because of stability issues. Around the onset of this century, the inhaled administration of aerosolized measles vaccine was shown to be successful in schoolchildren in several studies [21–23]. Furthermore, inhaled vaccines against tuberculosis were shown to be effective in humans [24,25].

Although the abovementioned studies support the benefits of respiratory tract administration of vaccines, the administration of stable dry-powder formulations remains largely unexplored. In this review, we will provide a 2021 overview of the status of inhaled vaccines, specifically against viral pathogens that infect the respiratory tract, including SARS-CoV-2. Also, we will elaborate on the hurdles to be overcome for pulmonary administration of stable dry-powder vaccines to become a successful alternative to the current needle-based vaccination strategy. The review was conducted using PubMed, Web of Science, and Google Scholar, with the oldest studies including seminal works on respiratory tract immunization in the 1960s of the 20th century. Our search strategy was based on (but not limited to) the following terms: respiratory viruses and viral respiratory tract infections, pulmonary vaccination, inhaled vaccines, respiratory tract immunization, immunoglobulin A, mucosal-associated lymphoid tissue, (induced) bronchial-associated lymphoid tissue, dry powder vaccines, and aerosolized vaccines.

2. The mucosal immune system of the respiratory tract

Mostly, inhaled materials that reach the airways, like dust particles and other inert substances, are subject to immunological tolerance, after which they are removed either by mucociliary clearance or via cough/sneeze reflexes. This tolerance toward innocuous materials, mediated by regulatory T-cell subsets, is highly important for maintaining immunological homeostasis [26,27]. However, when immune cells encounter pathogens, recognition of pathogen-associated molecular patterns (PAMPs, e.g. through toll-like receptors) or damage-associated molecular patterns (DAMPs, e.g. from infected cells) drives APCs into taking up the antigen. Hereafter, dendritic cells usually migrate via afferent lymphatic vessels to nearby draining lymph nodes, where the antigen is presented via MHC-II complexes to naïve T- and B-cells. Besides following the conventional route of antigen presentation, upon infection of the human respiratory system specialized tissues called inducible bronchus-associated lymphoid tissue (iBALT) can be formed, consisting of B-cell follicles and plasma cells, sometimes surrounded by densely packed T-cell zones and APCs. Here, antigens are efficiently presented to both naïve- and effector T- and B-cells without having to migrate through the lymphatic system [28–31]. These tertiary ectopic lymphoid tissue structures, which are induced upon stimulation with inflammatory stimuli following infection, and are maintained by follicular dendritic cells [32], are part of a larger network of interconnected mucosal-associated lymphoid tissues (MALT) that can be found alongside various mucosal sites of the human body [33]. An example of such a connected mucosal tissue within the respiratory tract is the constitutive nasal-associated lymphoid tissue (NALT). Together with secondary lymphoid organs such as the regional lymph nodes, the adenoids, and the tonsils (the latter making up Waldeyer's ring), these vascularized tissues take part in the common mucosal immune system of the respiratory tract, which is highly important for efficient processing of pathogenic antigens and for subsequent pathogen neutralization (Figure 1) [27,28,33]. In studies on influenza-infected mice, it has been shown that iBALT acts as an important niche for long-lived antigen-specific memory B-cells, plasma cells and virus-specific CD8 + T-cells, which can directly act upon secondary infection, independently of secondary lymphoid organs [31]. In a later study, it was shown that depletion of iBALT in mice even led to reduced numbers of serum antibodies with hemagglutinating capacity, indicating an important role for locally formed immune tissues in the long-lived B-cell-driven systemic humoral response [32]. Antigens can reach MALT either via transcytosis through antigen-delivering microfold (M)-cells, via environment-scanning dendritic cells, or, indirectly, via the lymphatic system. Also a role for mucus-producing goblet cells has been implicated [34].

Besides systemic IgG, an essential effector component of the mucosal immune system is mediated by dimeric IgA antibodies, which are formed by plasma cells in the sub-epithelial lamina propria that are differentiated under the influence of follicular helper-T-cell-(T_{FH})-derived and epithelial tissue-derived cytokines (e.g. TGF-β, IL-2, IL-5, IL-6, IL-10, and IL-21) [38,39]. Because we consider the formation of IgA antibodies next to IgG to be one of the most important measures of an active mucosal immune system, the cellular arm of immune

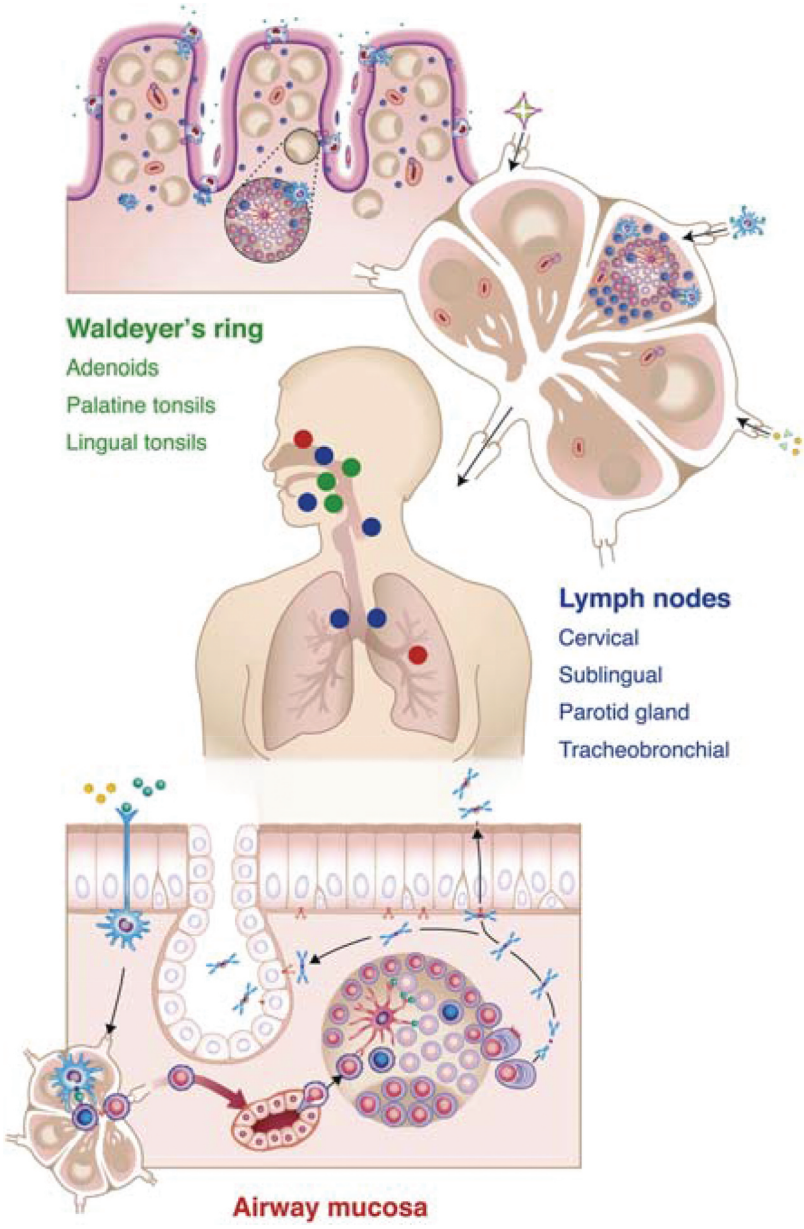

Figure 1. Overview of components involved in the mucosal immune system of the respiratory tract. In green, the localization of primary inductive lymphoid tissue sites, collectively known as Waldeyer's ring. In blue, the lymph nodes, which are important for generating T-cell-dependent systemic IgG-based immune responses. red, the localization of (inducible) ectopic tissue structures NALT and BALT, important for eliciting follicular helper-T-cell- and B-cell-mediated effector functions, related to secretory IgA antibodies. Figure reprinted with permission of [40].

defense will not be discussed in detail in this review. For this, we refer to some previous reviews [39,40]. IgA antibodies at mucosal tissues are usually dimeric of nature, in contrast to bone marrow-derived IgA in the blood [27]. An important aspect of dimeric IgA is that it can bind to specific polymeric immunoglobulin receptors (pIgR) on the basolateral side of mucosal epithelial cells. After receptor-mediated endocytosis, these IgA antibodies can cross the epithelial lining by means of vesicular transport. Upon dissociation from the pIgR at the apical surface of the epithelium, IgA molecules are functionalized with a part of the pIgR called the secretory component, which prevents proteolytic cleavage in the lumen [41]. The secreted dimeric IgA molecules in complex with the secretory component are known as secretory IgA (SIgA). In the lumen, part of the SIgA effector functions are initiated, together with IgM antibodies contributing to the principle of immune exclusion, which entails all mechanisms that prevent antigens from passing the respiratory tract epithelium (Figure 2) [42]. Firstly, SIgA antibodies can cross-link pathogens in the lumen, leading to sterical hindrance and thereby to a blockage in infectivity. Secondly, they can bind antigens that have already crossed the epithelial lining into the lamina propria and subsequently expel them via the abovementioned mechanism of receptor-mediated endocytosis. Thirdly, they can bind antigens inside infected cells and export them via vesicular transport [43]. SIgA antibodies can also activate innate leukocytes like monocytes, macrophages, and neutrophils, which carry the IgA receptor FcαR (CD89) [27].

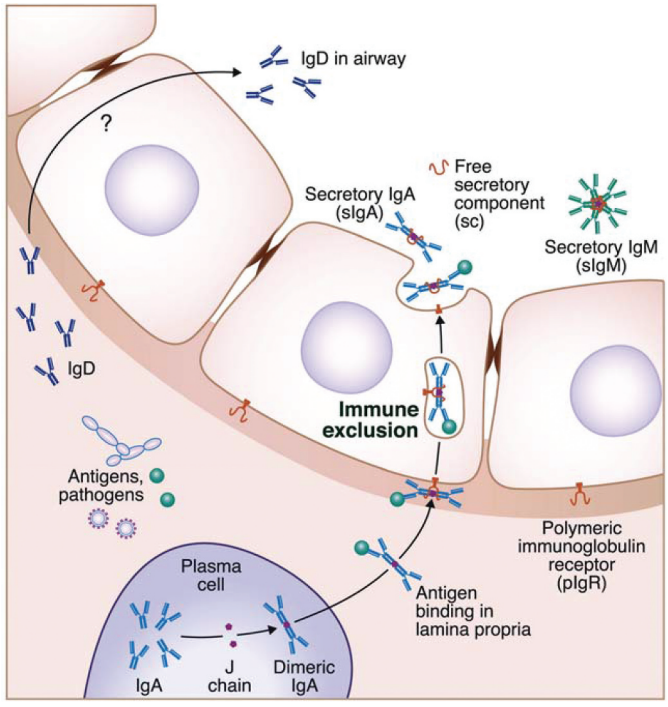

Figure 2. Production of dimeric IgA antibodies upon the encounter of antigens within mucosal sites such as BALT and NALT. The figure shows the principle of immune exclusion, where the antibodies function to expel antigens from the lamina propria back into the airway lumen. Besides the production of IgA, a minority of antibodies produced consists of secretory IgM, which is also dependent on the pIgR, as well as IgD antibodies. Figure reprinted with permission of [40].

An important property of the mucosal network is that antigenic priming at one particular mucosal region may induce a response at a distinct mucosal region where similar homing receptors are present as the initial site of infection. For instance, the microvasculature in the lamina propria near iBALT and NALT both contain the homing receptor VCAM-1. Antigenic priming in the nasal or oral mucosae may therefore also induce mucosal antibodies in distinct mucosal tissues of the lungs and vice versa. In other words: if an individual gets infected via the mucosa of the nose, this may also lead to increased levels of SIgA in the airways. This interplay of distinct mucosal sites is often named the common mucosal immune system [44–46].

In conclusion, administration of vaccines to the respiratory tract mucosa not only induces systemic IgG-mediated and cell-mediated responses but, more importantly, it opens up the possibility to induce a strong local immune response, supported in the foremost place by SIgA antibodies, which are of utmost importance to the respiratory tract's immune defense [40].

3. Prerequisites to inhalable vaccines

Inhalation is the most efficient way to target the respiratory tract. In order for an inhaled vaccine to be effective, it should preferably meet the following characteristics: 1) like any other vaccine, it would be advantageous if the inhalable vaccine is stable at ambient temperatures, not requiring a cold-chain and not requiring sterile water for reconstitution; 2) the vaccine should be delivered to the right site in the lungs; 3) the vaccine should be immunogenically active enough to ensure sufficient (mucosal) immune cell activation, i.e. it should overcome the barrier of immunotolerance; 4) the vaccine should not lead to adverse effects and should not lead to exacerbations of underlying illnesses such as asthma and COPD. Below, we will discuss several of these parameters which are of influence on the outcome of the vaccination strategy.

3.1. Advantages of dry vaccine formulations for inhalation

Inhalable vaccine formulations can roughly be divided into two categories: liquid formulations and powder-based formulations. In order to ensure effective dispersion of the formulation into an inhalable aerosol, both types of formulations have their own inhalation devices. Because the wide range of inhalation devices and their technological aspects have been reviewed elsewhere, it will not be discussed here in detail [47–50].

When considering vaccine stability, dry powder formulations have several advantages over liquid formulations. Firstly, if properly dried, dry vaccines can be more stable and may be stored at ambient condition, thereby circumventing the requirement for a cold - chain. This is highly beneficial for distribution to warmer climates and developing countries. Secondly, dry vaccines weigh less than liquid solutions which favours bulk transportation, and thirdly, dry powder formulations are suitable for use in disposable dry powder inhalers (DPIs), which prevents re-use, cross-contamination, and moisture-induced degradation, and, enables the delivery of reproducible doses in one or only a few inhalations compared to liquid formulations [49,50]. To obtain vaccines in the dry state,

there are several drying technologies, e.g.: spray drying, freeze- drying and spray-freeze drying. However, it is key to use a drying technique that can yield particles with a defined size range, suitable for inhalation [47]. In order to protect the vaccine from the harsh conditions during the drying process but also during storage, it is of importance to stabilize the vaccine properly with suitable excipients. This is often done by incorporating the vaccine in a protective matrix of glass-forming excipients like sugars: a technology that has been applied for numerous biopharmaceuticals [51–53]. The optimal choice for stabilizing excipients depends on the type of vaccine (e.g. WIV, subunit, vector-based) and on the drying conditions.

3.2. Influence of deposition site on efficacy of inhaled vaccines

Since air-transmissible pathogens infect specific regions along the respiratory tract, depending on the expression of their respective attachment receptor, it is important to know whether inhaled vaccines should reach these particular regions or not in order to confer immune protection. Key to their efficacy is that the vaccine formulations should be dispersed at a proper aerodynamic size range, suitable to penetrate the airways. Aerosols with an aerodynamic size range of 1–5 µm are considered to show penetration and deposition in the lung, whereas smaller particles are merely exhaled. Particles larger than 5 µm are mainly deposited in the throat or the upper respiratory tract because of inertial impaction [54,55]. Besides size, other factors, such as, shape, density, breathing pattern (inhalation flow rate), charge, and hygroscopicity may also affect the deposition behaviour of particulates in the lungs [9].

In recent years, a few studies have shown that vaccine deposition site is of minor relevance to its protective efficacy against influenza. One of the studies, performed in cotton rats, showed that both tracheal, as well as pulmonary administration of whole inactivated virus (WIV) vaccine, led to comparable protection upon challenge, as both systemic IgG as well as mucosal IgA antibodies were being formed [56]. Interestingly though, vaccine formulations that reached the lungs more effectively, did induce higher IgA titers. Arguably, this may be an indication for the formation of iBALT-like structures, which tend to form near bronchial regions. Supportive of this hypothesis is the fact that, upon pulmonary administration, vaccine-specific IgA antibodies were also detected in nasal washes, possibly reflecting trafficking of antigen-specific IgA-producing B-cells to distinct lymphoid tissues, and explaining why deposition site seems of minor relevance to influenza vaccines [56]. These findings are in line with the hypothesis of the common mucosal immune system.

In a subsequent study by Tomar *et al*. [57], the effect of deposition site on vaccine efficacy was assessed for both a vaccine against influenza A virus, which is an airborne pathogen, and for a vaccine against hepatitis B virus, a bloodborne pathogen [57]. Hereto, mice were vaccinated twice with either influenza A subunit vaccine, or with hepatitis B surface antigen (HBsAg). The vaccines were either administered intramuscularly (i.m.) or administered as a powder to both the upper- and to the lower respiratory tract, with a two-week interval. It was found that serum IgG titers were generally lower upon respiratory tract administration of the influenza vaccine than upon i.m. administration, regardless of whether the vaccine was administered to the upper respiratory tract or to the pulmonary region. While serum IgG titers were also higher upon i.m. administration of HBsAg than upon pulmonary administration, there was a significant difference in serum IgG titers between administration to the lower and the upper respiratory tract. Although administration to the lower respiratory tract led to increased IgG titers in the serum, thus comparable to the results obtained for influenza, no serum IgG titers were detected at all after administration to the upper respiratory tract [57]. When broncho-alveolar lavage (BAL) fluid was analyzed on day 28, IgG titers were again found to be higher upon i.m. vaccination than upon respiratory tract administration for both the influenza vaccine and HBsAg. Interestingly, though, again no IgG levels were detected after upper respiratory tract administration of HBsAg.

As a measure of the mucosal immune response, IgA titers were measured in the BAL fluid of animals vaccinated with the influenza subunit vaccine. As expected, no IgA antibodies were detected in the i.m. vaccinated group, while these were detected at comparable levels in the mice, when the vaccine was deposited in either the upper- or the lower respiratory tract. The authors therefore hypothesized that the site of deposition is of minor relevance for vaccines against airborne viruses that spread via the respiratory tract, such as influenza, while it is of relevance for non-airborne pathogens [57]. These results might relate to the absence of hepatitis B-specific receptors on the respiratory epithelium. As a consequence, IgG production probably relies on the uptake of the vaccine by alveolar dendritic cells and other scanning immune cells, capable to elicit a quick transit to the nearby lymph nodes.

While the above studies hypothesize that the site of influenza vaccine deposition in the respiratory tract is not relevant, a study by Minne *et al*. [58] argued that the deposition site is in fact relevant; suggesting that deposition to the deep lungs elicits a higher increase in local antibody titers. It should be noted, though, that this study did not compare the effect of deep lung deposition directly with the effect of deposition to the central airways and the trachea, but rather to the antibody levels induced by nasal administration [58].

The resemblance in the response between lower respiratory tract- and upper respiratory tract administration upon respiratory viral-infections may be related to a similar capacity of those regions to induce and to activate specialized lymphoid tissue structures like iBALT and NALT, and to stimulate isotype-switching of B-cells into IgA-producing plasma cells. For human influenza strains specifically, which mainly target α-2,6-linked sialic acid receptors expressed on the upper respiratory tract epithelium, it can also be hypothesized that the mucocilliary apparatus causes viral particles initially deposited to the lower respiratory tract, to eventually reach upper respiratory tract-associated attachment receptors.

Besides the above studies, which directly assessed the influence of deposition site, the fact that school children were successfully vaccinated with inhaled measles vaccine also supports the observation that for vaccines against airborne viruses, lung deposition is of limited relevance [21]. It

can be assumed that a group of 385 children in the age of 5 to 14 years old shows a highly variable inhalation behaviour. The observation that despite this fact still 84.6% of the children showed seroconversion after one month (compared to 78.8% after subcutaneous injection) shows that also for the measles vaccine the site of deposition in the lungs is hardly of relevance [21].

From the studies described here, it can be concluded that the deposition site seems mainly relevant for vaccines against systemic viral infections (e.g. hepatitis B) to work optimally. However, vaccines against airborne micro-organisms may be less dependent on the deposition site, probably owing to regions of interconnected MALT. Since these tissues are linked through a common mucosal immune system, B-cell priming in one particular region probably facilitates homing to distinct regions regardless of the mucosal site where the infection takes place. Because the influence of deposition site is an important parameter for inhaler design, the fact that vaccine deposition site may not be relevant for respiratory viruses might be of groundbreaking importance. Therefore, this observation should be further studied for other respiratory viruses as well, such as SARS-CoV-2, measles virus, and respiratory syncytial virus. If additional studies show a similar outcome, the design of a suitable inhaler for these vaccines may not necessarily need stringent requirements for achieving whole lung deposition, which would be highly beneficial.

3.3. Crossing the barrier of immunotolerance

Since the respiratory tract mucosa are continuously exposed to a plethora of innocuous substances from the outer environment, it is important to ensure a high degree of immunotolerance. However, this often comes at the expense of vaccine immunogenicity. For instance, a drawback of the use of viral subunits as vaccines, is that they are usually low in immunogenicity. In contrast to WIV and live-attenuated vaccines, subunit vaccines only consist of viral fragments, and thereafter, they lack the particulate nature of the intact virion. For this reason, subunit vaccines are considered not optimal stimulators of pattern recognition receptors, because they lack PAMPs [59]. While inhaled vaccines may provide an interesting alternative to parenteral vaccination, the immune response that is to be induced has to be strong enough to provide a significant benefit. Therefore, inhalable vaccine formulations may benefit from adjuvants. The need for adjuvants may depend on the intrinsic immunogenicity of the pathogen itself, but also on the vaccine type. Since the necessity for adjuvants in inhalable vaccine formulations is not yet fully elucidated, it would be worthwhile to study its influence on the mucosal immune response, both concerning local antibody production and the presence of immune cells, and, to see whether it improves the protective efficacy of the vaccine. However, not every adjuvant suitable for i.m. administration may be suitable for inhalation, like non-soluble alum and oil-based adjuvants. Therefore, potent adjuvants used along with parenterally administered vaccines should not be used if they are toxic to the mucosal environment of the respiratory tract.

As reviewed elsewhere, a range of adjuvants can be used together with vaccines to initiate or to strengthen the mucosal immune response upon administration to the respiratory tract [60–62]. For parenterally administered vaccines that have low intrinsic immunogenicity, such as subunit-based vaccines and peptides, it is considered essential to incorporate potent adjuvants to either stimulate innate immune cells (e.g. pattern recognition receptors such as toll-like receptors) or to directly stimulate B-cells and or T-helper-cells in a way to overcome immunotolerance and to increase IgA production [39]. It has been shown that influenza subunit vaccines formulated as a powder, with inulin as stabilizing excipient, were capable to induce a potent immune response without requiring adjuvants [63]. However, the study did not perform a challenge study and seroconversion was measured only systemically.

4. Studies on pulmonary vaccination

The majority of clinical studies on inhaled vaccines has focussed on liquid formulations. Most of the clinical studies involved vaccination against measles, with many studies reporting superior effectivity of (booster) vaccines when administered by aerosol, compared to administration via injection. Also studies on aerosolized influenza vaccines have shown clinical effectivity (Table 1). Another example of pulmonary vaccines that have been assessed in human trials is a nebulized virus-like particle vaccine against human papillomavirus type-16 [64]. An interesting outcome of this study was that in a small group of participants, immunization by aerosol led to SIgA antibodies in the genital secretions of the participants, potentially reflecting immune cell trafficking to distinct areas of MALT. However, the patient groups were small and the nebulizers used in this study probably did not lead to a high degree of peripheral lung deposition.

While the field of pulmonary vaccination using liquid aerosols has progressed steadily and has gathered accumulating evidence on the significance for especially aerosolized measles vaccines [65–68], those vaccines do not take away the stability and logistical issues that come along with liquid formulations. Regarding this aspect, dry powder vaccines have many advantages. Over the last two decades, several research groups have successfully managed to create dried vaccines, suitable for pulmonary delivery, with formulations retaining efficacy *in vivo* (Table 2). Yet, the amount of clinical studies is still limited. To date, the only clinical study performing pulmonary administration of dry-powder vaccines in humans is a study on dry powder measles vaccination in adult males [69]. The study assessed the efficacy of an inhaled dry powder measles vaccine, generated by carbon dioxide-assisted nebulization with a Bubble Dryer® (CAN-BD), which is a form of supercritical fluid drying [70]. The vaccine, which had proven its efficacy in macaques [71], was administered by two devices: the Puffhaler® (AktivDry LLC) and Solovent™ (Becton, Dickinson & Company). The formulation was administered to subjects seropositive for measles antibody, and its efficacy was compared with the currently licensed subcutaneous injection. Although the formulations did lead to increased antibody titers, comparable to the standard subcutaneous administration, the results were not considered convincing since

Table 1. Delivery of liquid-based viral vaccines by inhalation.

Virus	Vaccine strain and type	Study population	Humoral response measured	Outcome	References
Influenza	Bivalent WIV vaccine containing B/Massachusetts/66 and A/Aichi/68/H3N2	Males with age ranging from 15 to 64	Not specified	Significantly lower incidence of influenza for both immunization groups compared to control	[18]
	Bivalent WIV vaccine containing A/Japan/62/H2N2; A/Taiwan/64/H2N2; B/Massachusetts/66 vs. monovalent A/Hong Kong/68/H3N2	Young adults	Not specified	Significantly better protection after aerosol immunization compared to s.c. administration	[16]
	Bivalent vaccine not further specified	Adult males and children	IgG and IgA	Persisting levels of IgA antibodies after having received aerosolized immunization twice. Inhalation of small 1.5 µm particles led to systemic IgG production	[15]
	Bivalent WIV vaccine containing A/Japan/62/H2N2; A/Taiwan/64/H2N2; B/Massachusetts/66vs. monovalent A/Hong Kong/68/H3N2	Adults	Not specified	Aerosolized H3N2 virus was effective when given twice. Less effective for the bivalent vaccine	[14]
	Bivalent WIV vaccine containing A/Japan/62/H2N2; A/Taiwan/64/H2N2; B/Massachusetts/66	Adults	Not specified	79% less illness and shorter duration of illness after inhalation compared to 27% reduction upon s.c. administration	[13]
Measles/mumps/rubella*	MMR-SII vaccine (Edmonston-Zagreb live-attenuated measles vaccine, Leningrad-Zagreb live-attenuated mumps vaccine and RA 27/3 live-attenuated rubella vaccine); MMR-II vaccine (Attenuvax strain (comparable to Schwarz live-attenuated measles vaccine), Jeryl-Lynn live-attenuated mumps vaccine and RA 27/3 live-attenuated rubella vaccine)	Seropositive children between 6–7 years of age	Not specified	After administration of the booster dose, almost 100% of seropositivity was achieved. A small increase from baseline antibody levels was observed for measles and rubella. A significant increase was found in antibody levels against mumps. No significant differences were found between the groups receiving vaccine by injection and the groups receiving aerosolized vaccine. Antibody titers persisted one year after booster immunization.	[123,124]
	Edmonston-Zagreb live-attenuated measles vaccine	Seronegative infants	Not specified	After 91 days, seroconversion was 85.4% for aerosol group compared to 94.6% for s.c. group.	[67]
	MMR Triviraten vaccine (Edmonston-Zagreb live-attenuated measles vaccine, Rubini live-attenuated mumps vaccine and RA 27/3 live-attenuated rubella vaccine); MMR-II vaccine	Seropositive young adults between 18–25 years of age	Not specified	8 weeks post vaccination, the percentage of seropositivity had significantly increased for all vaccine groups (Triviraten aerosol, MMR-II s.c. and Triviraten s.c.) to almost 100%. The highest increase in antibody titers compared to baseline was found for the aerosol group (increase of 33% seropositivity compared to 24% for s.c. MMR-II and 13% for s.c. Triviraten). Post-vaccination seropositivity for mumps remained low after aerosol vaccination (increase of 6% from 27% to 33%) compared to an increase of respectively 51% and 32% for s.c. Triviraten and s.c. MMR-II. Seropositivity for rubella reached 100% for all vaccine groups, although this was already high at baseline. Seropositivity still persisted 1 year post-vaccination for measles and rubella while mumps antibody titers sharply decreased.	[125,126]
	Edmonston-Zagreb live-attenuated measles vaccine	9-month-old seronegative infants	Not specified	Measles-specific T cell responses were found in 42% of the aerosol-vaccinated group compared to 67% for the s.c. group, which was significantly lower. Seroconversion rates were 33% for the group receiving aerosol compared to 92% for the group receiving s.c. administerd vaccine. Lower seroconversion rates upon aerosol administration were contributed to vaccine dose.	[127]
	MMR vaccine (Edmonston-Zagreb live-attenuated measles vaccine, L-Zagreb live-attenuated mumps vaccine and RA 27/3 live-attenuated rubella vaccine)	Seropositive adults 18–50 years of age	Not specified	Aerosolized vaccine led to a superior response to all components (measles, mumps and rubella) compared to s.c. administration, regardless of preexisting antibodies	[128]
	Edmonston-Zagreb live-attenuated measles vaccine	12-month-old seronegative infants	Not specified	Measles-specific T cell responses were found in 72% of the aerosol-vaccinated group compared to 87% for the s.c. group. Seroconversion rates were 90% for the group receiving aerosol compared to 100% for the group receiving s.c. administered vaccine. Lower seroconversion rates upon aerosol administration were contributed to vaccine dose.	[129]
	Edmonston-Zagreb live-attenuated measles vaccine	Seropositive children 6–7 years of age	IgG and IgA	Significantly greater increase in serum IgG after 1 month upon aerosol administration than upon s.c. administration. No significant differences in serum IgA response. Significantly higher mean fold increase of IgG and sIgA in nasal secretions after administration by aerosol compared to the s.c. route, being illustrative of a robust mucosal immune response.	[130]
	Measles-Rubella (Edmonston-Zagreb live-attenuated vaccine and RA 27/3 strain)	Seropositive children 6–7 years of age	Not specified	For measles vaccine, significantly higher seroconversion rates and post-vaccination seropositivity was found for the group receiving aerosol compared to the group receiving s.c. vaccine. For rubella, similar seroconversion rates and post-vaccination seropositivity was detected between s.c. administration and administration by aerosol.	[131]

(*Continued*)

Table 1. (Continued).

Virus	Vaccine strain and type	Study population	Humoral response measured	Outcome	References
	Edmonston-Zagreb live-attenuated measles vaccine and Schwarz live-attenuated measles vaccine	Seropositive children between 5–14 years of age	IgG and IgM	After 1 month, 84.7% of the group immunized with aerosolized EZ vaccine showed seroconversion compared to 78.8% of group immunized s.c. with EZ vaccine. Superior titers in aerosol group compared to s.c. group 1 year post-vaccination. Antibody titers still present 6 years post-vaccination, with 86% seropositivity compared to 73% for the s.c. vaccinated group.	[21,72,133]
	Edmonston-Zagreb live-attenuated measles vaccine, Measles (EZ)-Rubella live-attenuated vaccine (RA 27/3 strain)	Seropositive children	Not specified	Superior seroconversion rates upon booster immunization by aerosol compared to injection. Aerosolized vaccine was more effective at low dose compared to the injected vaccine	[22]
	Edmonston-Zagreb live-attenuated measles vaccine	Children between 9 months and 15 years of age	Not specified	95.5% vaccine efficacy	[23]
	Edmonston-Zagreb live-attenuated measles vaccine and Schwarz live-attenuated measles vaccine	Seronegative infants	Not specified	S.c. administration led to seroconversion of 62% and 37% for EZ-strain and Schwarz strain respectively. Aerosol vaccination led to seroconversion of respectively 35% and 34%.	[134]
	Edmonston-Zagreb live-attenuated measles vaccine containing 1% human albumin and Edmonston-Schwarz live-attenuated measles vaccine containing hypertonic sugar solution	Children between 0–4 years of age	IgG	Successful immunization with presence of serum neutralizing antibodies, regardless of whether the vaccines had preexisting maternal antibodies. 100% seroconversion in the EZ group. Less effectiveness of the ES-vaccine in infants younger than 6 months	[132,135]
	RA 27/3 live attenuated rubella vaccine	Children between 3–5 years of age		Aerosolized rubella vaccine was equally effective in inducing serum antibody responses compared to the s.c. and intranasal route with 100% seroconversion rates.	[136]
	Edmonston strain	Seronegative young children between 1–15 years of age	Not specified	Successful immunization with presence of serum neutralizing antibodies upon nebulization.	[19,20]
Human papilloma virus	HPVL1 virus-like particle	Seronegative female adults between 18–45 years of age	IgG and IgA	Levels of seroconversion upon immunization by high-dose aerosol were comparable to those upon i.m. administration with equally high levels of serum IgG and IgA. Detectable sIgA in cervical secretions after immunization by aerosol. Inhaled vaccination was significantly more effective than nasal administration	[64]

* Results on measles, mumps and rubella were grouped since vaccines against those viruses are mostly administered in combination regimens. Several seminal works on successful immunization with aerosolized measles vaccines conducted in the 1960s and 1970s in Russia, Japan and the United States could not be accessed. Therefore, we decided to exclude these studies from the above table. For a recap of those early works, we would like to refer to comprehensive review papers [137,138]

Table 2. Pulmonary delivery of dry powder-based viral vaccines in pre-clinical studies.

Virus	Vaccine strain and type	Drying method	Administration method	Excipients/carrier	Model animal	Humoral response measured	Outcome	Reference
Influenza	A/CAL/H1N1 subunit	SD	Insufflator[a] vs. in-house device	Inulin	Mouse	IgG and IgA	Comparable serum IgG titers upon pulmonary administration, independent of deposition site. Low lung IgG titers compared to i.m. administration. Comparable lung IgA titers, independent of deposition site.	[57]
	A/CAL/H1N1 WIV	SFD	Insufflator	Inulin	Cotton rat	IgG and IgA	Serum IgG titers were comparable to i.m. vaccinated group. Mucosal IgG and IgA titers were lower upon powder administration compared to liquid	[56]
	A/PR/8/H1N1 WIV with adjuvants for comparison GPI-0100 and TLR-ligands Pam_3CSK_4; MPLA; CPG-ODN	SFD	Insufflator	Inulin	Mouse	IgG and IgA	All adjuvants except Pam_3CSK_4 increased serum and lung IgG titers. Only GPI-0100 WIV induced lung IgA titers	[62]
	A/HIR/H3N2; A/PR/8/H1N1 WIV with MPLA adjuvant	SFD	Insufflator	Inulin	Mouse	IgG and IgA	Serum IgG levels lower compared to i.m. vaccination. High levels of IgG and IgA in the lung compared to non-adjuvanted formulations	[73]
	A/HIR/H3N2; A/PR/8/H1N1 WIV	SFD	Insufflator	Inulin	Mouse	IgG and IgA	For A/PR/8/H1N1 comparable serum IgG titers between inhaled powder and i.m. formulations were found. Low amounts of IgG and IgA in lung and nose of powder-administered animals	[74]
	A/Panama/H3N2 subunit	SD and SFD	Insufflator	Inulin	Mouse	IgG	Superior IgG titers compared to liquid aerosols and i.m. administration	[75]
	A/Panama/H3N2 subunit	SFD	Insufflator	Inulin	Mouse	IgG, IgA and IgE	Superior serum, lung and nasal IgG and IgA responses compared to i.m. injection and liquid aerosolization. No IgE response	[63]
	A/WSN/H1N1 split	SD	Insufflator	Saturated lipids; HES; Lactose; $CaCl_2$	Rat	IgG and IgA	Local IgG production, but no IgA production	[76]
Measles	Edmonston-Zagreb live-attenuated vaccine	CAN-BD	PuffHaler and BD Soloventn.a. with silicone mask		Macaque	IgM, IgG, IgA	Induction of IgM, IgG and IgA. Complete protection upon challenge with wild-type virus one year later	[71]
	Edmonston-Zagreb live-attenuated vaccine	CAN-BD	Puffhaler	Myo-inositol	Cotton rat	Not specified	Immune response comparable to injection	[77]
	Edmonston-Zagreb live-attenuated vaccine	Study 1: SD; study 2: jet-milled	Study 1: intratracheal instillation; study 2: insufflator	Study 1: Trehalose; study 2: lactose	Macaque	Not specified	Levels of immunity were inferior compared to administration by nebulization and injection	[78]
Hepatitis B	Hepatitis B surface antigen	SD	Insufflator vs. in-house device	Inulin	Mouse	IgG	Deep lung administration led to high serum and lung IgG titers, as opposed to administration to the central airways.	[57]
	Recombinant Hepatitis B surface antigen	SD	Insufflator	PLGA-PEG nanoparticles with L-leucine	Guinea Pig	IgG and IgA	Serum IgG titers were slightly inferior to i.m. administration but a high IgA response was induced	[79]

Abbreviations: SD: spray-drying; SFD: spray-freeze-drying; Pam3CSK4: palmitoyl-3-cysteine-serine-lysine-4; MPLA: monophosphoryl lipid A; CPG-ODN: CPG-oligodeoxynucleotides; CAN-BD: CO_2-assisted nebulization with a bubble-dryer; PLGA: poly(lactic-co-glycolic acid); PEG: polyethylene glycol. [a]: Dry powder insufflator (Penn Century Inc., Philadelphia, PA, USA).

the subjects' baseline antibody levels were already high. Therefore, the authors concluded that the study should be reperformed in seronegative individuals [69]. Although in this study two devices, the Puffhaler® and Solovent™ were compared, no data were provided on the deposition behaviour of the powder, related lung bioavailability, and powder retention in the inhaler. Therefore, it would be interesting to know the vaccine particle size upon dispersion from the inhaler to assess the amount of vaccine that reaches the desired target region, and to assess whether the powder is effectively deagglomerated. In this light, it may be interesting to use disposable inhalers with known dispersion profiles.

5. Trends in inhaled vaccines: inhalation as administration route for COVID-19 vaccines

5.1. The current status of SARS-CoV-2 vaccine development

As 2020 and 2021 will be historically remembered for the COVID-19 pandemic, so will be the record-high pace at which novel vaccine candidates have been developed and evaluated [80,81]. While writing this review, two vaccines licensed by Pfizer/BioNtech and Moderna have received approval for use in the U.S [82,83]. as well as in Europe [84,85]. As a third vaccine the U.S. and Europe have granted market authorization for the Janssen vaccine and the AstraZeneca vaccine, respectively. The Janssen vaccine is currently awaiting marketing authorization in Europe. Both the Pfizer/BioNtech and the Moderna vaccine are based on single-stranded mRNA constructs, encoding for the SARS-CoV-2 spike protein, and have shown highly promising results with a protective effectivity of about 95% after two doses. However, a pitfall of both vaccines, which are liquid formulations, is that they have to be shipped at low temperatures of −70°C and −20°C, respectively, to prevent degradation of the constructs [86,87]. The AstraZeneca vaccine, which has been developed in collaboration with the University of Oxford, is an adenoviral-vector-based vaccine, that carries a DNA construct encoding for the SARS-CoV-2 spike protein [88]. Although its protective efficacy ranges between 60 and 90% depending on the dosing regimens, the vaccine is deemed stable for 6-months at refrigerated conditions, which is significantly more stable than the other vaccines [89–91]. Currently, AstraZeneca is looking into the option of teaming up with the Gamaleya Research Institute in Moscow, which produces the Russian Sputnik-V vaccine [92], as both vaccines use an adenoviral vector-based approach. Since the vaccines use different adenoviral vectors (of chimpanzee and human origin, respectively), combining both vaccines in a heterologous prime-boost approach may lead to synergistic outcomes [93]. The Janssen vaccine is also an adenoviral vector-based vaccine and has shown efficacy rates around 67%. The benefit of this vaccine is that it is a single-dose vaccine and that it can be stored under refrigerated conditions, which allows for a quicker vaccination and less strict logistical requirements [94].

5.2. Inhalation of SARS-CoV-2 vaccines

In addition to the mRNA- and vector-based vaccines described above, the vast majority of vaccine candidates that are currently being tested in clinical trials will be administered by injection [95]. Such liquid vaccines require a tightly regulated cold-chain infrastructure which poses a big challenge for the future distribution of vaccines across the globe, especially in remote- and tropical areas. Therefore, the question remains whether vaccine distribution will be effective enough to ensure mass-scale vaccination campaigns while minimizing costs. While pharmaceutical companies hint at producing a lyophilized version of their vaccine to increase vaccine stability, these still have to be reconstituted prior to administration. Although this partially resolves the stability issues of liquid formulations, it does not take away the downsides of injection. A potential solution to this may be found in inhalable dry powder vaccines.

At this stage, a clinical trial has been planned by Imperial College London and the University of Oxford to assess the effectiveness of an inhaled formulation of their viral vector-based vaccine candidates [96]. The vaccines will be delivered as anebulized aerosol at three increasing doses to a small group of healthy volunteers. To assess the potential benefits of the inhaled formulations compared to their injected counterparts, several samples will be taken, including bronchoscopy- and nasal samples, in order to screen for mucosal antibody responses including IgG and IgA. Blood will be drawn to assess systemic humoral responses and antigen-specific T-cell responses. Although this study may provide promising insights into the effectiveness of inhaled SARS-CoV-2 vaccines, to our best knowledge, no research has yet been planned towards the development of dried inhalable SARS-CoV-2 vaccines.

Interestingly, previous research may have opened up the possibility to make dry-powder COVID-19 vaccines, suitable for inhalation. In a seminal study by Lam and colleagues [97], mRNA was dried successfully by using the commonly used drying techniques, i.e. spray-drying and spray-freeze-drying. Reporting a relatively high yield, both techniques can also be used successfully to create powders that are suitable for pulmonary administration *in vivo*. The authors showed that the constructs, containing luciferase mRNA in complex with a novel delivery vector, retained *in vivo* activity upon administration to the lungs of mice. Notably, no short-term inflammatory effects or toxicity were found [97]. This may be an important step into the stabilization of future mRNA vaccines. Freeze-drying, a drying technique that is already applied for numerous pharmaceuticals, is deemed not suitable for inhalation since the powder that is being formed deviates from the physical prerequisites needed for inhalable powders. Usually, freeze-dried powders have to undergo a secondary milling step, which usually results in a heterogeneous mixture of particles [74]. Another application for pulmonary vaccine

delivery of SARS-CoV-2 vaccines may lie with the administration of more classical vaccine types like WIV or subunit vaccines as (sugar-glass) stabilized powder particles. This approach has already successfully been tested with influenza vaccines, as reviewed elsewhere [98]. A significant proportion of vaccine candidates that are currently evaluated in clinical trials entails protein subunit vaccines, one of which is licensed by Sanofi/GSK. Since their vaccine candidate, consisting of an adjuvanted recombinant spike protein, has been shown not to work properly in the elderly population, clinical studies have been suspended to optimize the antigen [99]. We believe that, in order to optimize immunogenicity, the vaccine may be a suitable candidate for pulmonary administration, as has been successfully done with influenza subunit vaccines [57,63,75]. Interestingly, the presence of regions of iBALT has been shown to prevent SARS-CoV-1 induced lethality *in vivo* [100]. To our knowledge, no research has been done regarding the protective role of iBALT in the clearance of SARS-CoV-2 infections. We therefore believe it would be of high interest to elucidate whether the presence of iBALT may have a beneficial effect on viral clearance and whether it is induced after vaccination. Future research should assess whether inhalable vaccines could be developed successfully to aid in the global battle against the COVID-19 pandemic.

6. Challenges for inhaled vaccines

Although pulmonary vaccination holds promise as an alternative to parenteral vaccination, there are also some critical factors that should be addressed. While the mucosal immune system likely plays a key role in defending the respiratory tract from pathogenic invasion, exuberant immune responses may deteriorate the lungs and counter-intuitively result in (antibody-dependent) enhancement of respiratory disease. The idea of an overreacting immune system in COVID-19 patients, for example, has been confirmed by studies showing that severe cases correlate with higher IgA titers [101–103] and are in some cases associated with excessive immune reactions leading to cytokine storms [36,37,104]. Therefore, the potential role of IgA in adverse events such as antibody-dependent cellular cytotoxicity or antibody-dependent enhancement should be carefully monitored during the evaluation of inhaled vaccines. Some studies have implicated that the presence of iBALT may lead to exacerbated immune responses in individuals infected with respiratory syncytial virus [30]. This might indicate that the presence of immunological memory can induce an over-reacting immune response upon vaccination. Notwithstanding this, a recent systematic review by Serazin et al., who investigated the incidence of acute respiratory distress syndrome (ARDS) after vaccination, revealed that no cases of ARDS after vaccination have been reported for any of the currently licensed vaccines [105]. Together with the lack of profound adverse events in studies about inhaled vaccination, we believe that the risks for such severe adverse events is minimal.

Another challenge toward the successful implementation of inhaled vaccines is that animal models for essential *in vivo* studies often require active administration of the vaccine since they simply cannot be instructed to perform the required inhalation manoeuvre. Moreover, commonly used devices for *in vivo* pulmonary administration often lead to low overall yields and poor dispersion and deposition profiles [106–108]. Therefore, the results gathered from *in vivo* studies using insufflation or intubation devices may not provide an accurate representation of the situation in humans. In relation to this, the efficacy of inhalers can only be assessed in human trials. It is therefore important that *in vivo* studies on inhaled vaccination are as reflective of the human situation as possible. During the last decades, some studies have tried to improve *in vivo* lung deposition of dry-powder vaccines using alternative administration devices [57,109]. An additional prerequisite towards *in vivo* studies is that vaccine candidates should be critically evaluated in animal models which most closely resemble humans, both concerning their respiratory tract anatomy, as well as their immunological response towards pathogens [9,110].

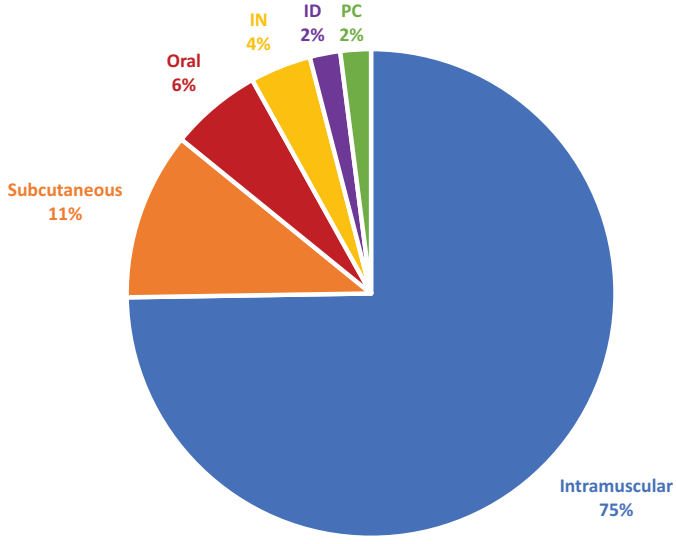

Figure 3. Pie diagram of the 81 currently FDA-licensed vaccines (excluding emergency use authorized vaccines such as SARS-CoV-2 vaccines) [139], divided by administration route. IN: intranasal; ID: intradermal; PC: percutaneous.

Another factor that influences the effectiveness of inhaled vaccines, is the inhalation manoeuvre of the patient [110]. While for airborne infections the deposition site of vaccines may not be of major relevance, this may be the case for vaccines against non-airborne infections. In order for a vaccine to reach the desired target region, the patient should be able to perform the inhalation manoeuvre correctly, considering a proper breathing flow rate, a correct hand-breath coordination, and potentially, being able to hold his or her breath. This requires a proper instruction of the inhalation manoeuvre. The inhalation technique may be optimized by the use of valved holding chambers [111]. This may also ensure that the individual receives the correct dose of vaccine. For DPIs, the inhaler resistance may be used as a tool to optimize the inhalation profile. On the other hand, DPIs may be less suitable for use in young children and infants, partly due to the fact that there have been no valved holding chambers developed for DPIs yet [47].

7. Conclusion

Of all viral respiratory tract infections, the only vaccines available for general use are directed against the influenza virus [39]. Since respiratory tract infections are still a major cause of morbidity and mortality around the globe, as underlined by the recent COVID-19 pandemic, we believe more focus should be put on alternative administration routes that more closely resemble the natural infection event, can elicit a broader immune response, and for which the vaccines can easily be distributed around the globe without large infrastructural difficulties and high demands for temperature management. With this in mind, we consider inhalation of stable dry powder vaccines a promising way of administration. A significant number of successful clinical studies on nebulized measles and influenza vaccines have already been undertaken, with convincing results for a majority of the vulnerable population and a high safety profile compared to the standard parenteral administration route. These studies have paved the way for research on other respiratory virus vaccines such as vaccines for SARS-CoV-2. Moreover, inhalation may also be a vaccination route worthwhile to consider for blood-borne viral pathogens (e.g. hepatitis B) since this might broaden the immune response toward IgA-mediated humoral responses and may provide an additional line of defense. To be able to safely distribute vaccine formulations around the world without losing efficacy, we are convinced stable dry-powder formulations could provide the next-generation of successful vaccines.

8. Expert opinion

Although vaccine delivery by inhalation may hold great potential for preventing highly contagious viral infections, the central dogma of vaccination is still centred around needle-based vaccination (Figure 3). While vaccine development has taken a giant leap forward and global efforts into finding a vaccine for SARS-CoV-2 have led to a record-high production speed and subsequent market authorization, the field of respiratory tract delivery, and mucosal immunization in general, is mostly ignored. Since the lungs are the primary portal of entry for numerous viruses, we argue that respiratory tract delivery of vaccines by means of inhalation deserves way more attention. Inhalation omits important causes for patient incompliance such as needle phobia and cannot lead to secondary infections from contaminated needles. Since inhalable vaccines provide the capacity to administer relatively high doses directly at the site of infection, they are very cost-effective. One of the main concerns for the development of vaccines suitable for inhalation is that they have to overcome the barrier of mucosal tolerance. Therefore, the use of adjuvants should be considered per vaccine to improve vaccine efficacy.

When considering the pulmonary route of vaccine administration, we believe that research regarding inhaled vaccines should primarily focus on dry powder formulations. Owing to their inherent stability, dry formulations provide the opportunity of stockpiling, and thus, they can be transported to remote tropical regions more efficiently without requiring cold-chain infrastructure. Also, dry powder formulations can be administered via patient-friendly disposable inhalers, thereby minimizing the need for trained health care personnel. While there are disposable nebulizers on the market, many still need an electricity-powered compressor, which hampers patient-friendliness compared to the pocket-size disposable DPIs (Figure 4). Once the vaccinee is instructed on how to use the inhaler, inhalable vaccines benefit from the ease of administration. In this way, self-vaccination is possible for large fractions of the population with minimal intervention. This would favour mass-vaccination campaigns.

In addition to the stability advantage of dried vaccine formulations, vaccines against respiratory tract infections, such as COVID-19, may highly benefit from local administration as locally administered vaccines induce immune responses that most closely resemble the natural infection event. In this regard, components of utmost importance are the mucosal immune system and virus-specific mucosal antibodies. Together with systemic IgG titers, the induced SIgA antibody response, accompanied by induction of specialized regions of iBALT, may lead to a synergistic response towards subsequent infections and, therefore, these parameters should be taken along in future research. Recent work by Sterlin *et al*. [112] suggests that IgA antibodies with mucosal homing properties may dominate the initial humoral response toward intruding SARS-CoV-2 particles, showing more effective virus neutralization compared to IgG antibodies. Although serum antibody levels waned over time, neutralizing IgA antibody titers were in some cases detectable for up to 73 days post- onset of symptoms [112]. Similar results were found by Nussenzweig and coworkers, who found that SIgA antibodies of the dimeric form, in contrast to monomeric IgA in the blood, more effectively neutralize SARS-CoV-2 particles, apparently by increased potency to cross-link viral spike proteins [113]. In addition, after natural infection, spike protein-specific IgG and IgA antibodies have been shown to persist for up to 8 months post-onset of symptoms, while memory B-cells likely persist even longer [114]. These results support the fact that the respiratory route should be more closely investigated for future vaccination strategies as this may lead to a broader immune response. Promising results for mucosal vaccination have already been reported by Hassan *et al*. who intranasally administered a

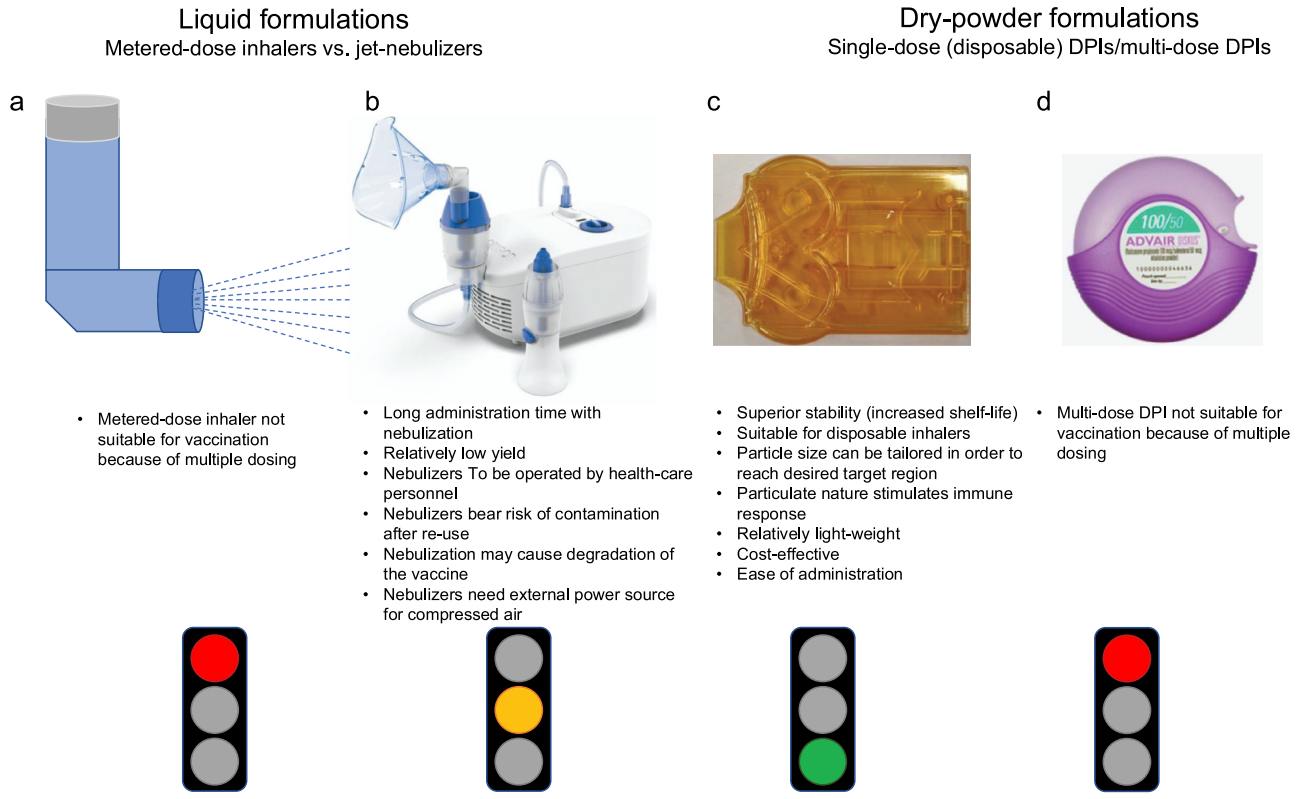

Figure 4. Justification of the most optimal inhaler device for pulmonary vaccination. (a) schematic representation of the metered-dose inhaler. (b) picture of an Omron C102 jet-nebulizer (Omron). (c) picture of a disposable DPI, the Twincer® (University of Groningen, The Netherlands [140]). (d) picture of a multi-dose DPI, the Diskus® (Advair).

chimpanzee adenovirus-vectored vaccine carrying a construct that encodes for the SARS-CoV-2 spike protein. The researchers found high levels of neutralizing IgG and IgA antibodies that led to almost complete protection against SARS-CoV-2 in both the upper and lower respiratory tract. In contrast, the intramuscular administered vaccine did not confer sterilizing immunity and did not produce SARS-CoV-2-specific IgA antibodies. Since comparable levels of serum-neutralizing antibodies were found for both immunization routes, the authors propose that the mucosal immune response accounted for the superiority of the intranasal route [115].

Although the effect of SARS-CoV-2 vaccines on long-term antibody levels remains to be investigated, eventual waning of antibody titers may be overcome with prime-boost vaccine regimens. Next to the classical form of homologous prime-boosting, by using two doses of the same vaccine, it may be worthwhile to consider heterologous prime-boost vaccination strategies. A recent example of an effective heterologous prime-boost vaccination strategy is the Russian Sputnik-V vaccine against SARS-CoV-2. The vaccine combines two different recombinant human adenoviral vectors, rAdV26 and rAdV5, which are administered separately with a 21-day interval, and has been reported in a phase 3 trial to be almost 92% effective [92]. While this approach is already highly effective, it may be worthwhile to aim for different administration routes, combining for example a parenterally induced systemic immune response with a pulmonary induced mucosal response. With such an approach, the developed immune response may be even broader. This approach has been investigated recently by Martini et al. who showed that by simultaneously administering i.m. and aerosolized influenza vaccine to pigs, a superior immune response is induced compared to the use of a single administration route, hinting at a synergistic effect [116]. Notwithstanding, we believe this strategy would not be the way to move forward when striving for needle-free immunization. Moreover, the use of separate administration routes would be challenging to implement in areas that already lack proper health - care infrastructure. Another potential disadvantage of administering booster doses via the respiratory tract might be that a primed immune system may cause an increase in local inflammation upon subsequent booster administration, potentially inflicting excessive lung damage.

A potential concern for the effective implementation of inhaled vaccines is that a representative *in vivo* model is often lacking [9,110]. While vaccines can be administered actively via intratracheal intubation devices, no animal can be instructed to perform the required inhalation maneuver, let alone, hold its breath. Therefore, preclinical studies should primarily focus on vaccine efficacy and safety, using animals that are susceptible to human infections, show resemblance with human respiratory tract anatomy, and respond in an immunologically comparable manner [9]. Regarding SARS-

CoV-2 vaccines, a suitable *in vivo* model may be found in minks, which have been repeatedly shown to be susceptible to SARS-CoV-2 infection and are even capable of transmitting the virus back to humans [117]. Alternatively, pigs may be a suitable model, as they closely match humans both with regard to their immune system as with regard to their respiratory tract anatomy. To our knowledge, two studies have assessed the effect of pulmonary administered viral influenza vaccines on pigs, using liquid aerosol [116,118]. However, ethical considerations and high costs make them a less commonly used model [119]. Therefore, clinical research should be regarded as an essential component.

Another factor that may hamper the successful implementation of inhalable vaccines is that health care officials might be unaware of the developments in the field. Since the demand for needle-based vaccines is still high, it may not be feasible to shift a company's production strategy toward inhalable vaccines. Furthermore, since needle-based vaccination is the worldwide gold standard for vaccination, the introduction of other administration routes may raise safety concerns for those suffering from allergies or other lung diseases. However, as discussed in this review, all clinical data on inhaled vaccines to date do not show profound adverse events following pulmonary administration of vaccines. Moreover, inhalable drugs are already widely used to treat and relieve a variety of lung diseases such as asthma and chronic obstructive pulmonary disease, without serious adverse events [110].

While vaccination strategies are ever-evolving, so are viruses. Recent reports on the emergence of several increasingly contagious strains of SARS-CoV-2 emerging from the UK [120], South - Africa [121] and Brazil [122] have taught us that the fight against the virus is not over yet. In the future, particular attention should be paid to pandemic preparedness, as current pandemic control has proven to be sub-optimal in many civilized countries. Although it is of utmost importance to create highly potent vaccines, it is of equal importance to inform people about precautionary safety measures, and to overcome vaccine hesitancy, because prevention will always be better than cure.

We expect that in five years from now, devices for pulmonary administration to animals have been improved, both with regard to vaccine deposition site and with regard to the emitted fine-particle fraction. These developments will be key in order to more accurately administer dry powder vaccines to small laboratory animals. We also believe that more research will be needed on the effective stabilization of complex vaccines like vector-based vaccines and mRNA vaccines in order to retain their vaccine efficacy upon drying. Moreover, as current dry powder-inhalers often lead to inadequate particle size distribution profiles, development of effective dry-powder inhalers suitable for vaccine inhalation will be an important challenge for the near future. As results so far have been overall convincing, we believe that aerosolized vaccines may provide an additional force in the eradication of the measles virus.

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ORCID

Rick Heida http://orcid.org/0000-0002-8425-394X
Wouter LJ Hinrichs http://orcid.org/0000-0002-1481-2769
Henderik W Frijlink http://orcid.org/0000-0001-7901-8198

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