JUNE 2023 Volume 2, Issue 6

VACCINE INSIGHTS

SPOTLIGHT ON
Advances in formulation and administration

Guest Editor
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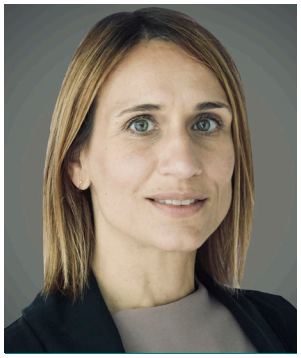
VACCINEINSIGHTS

ADVANCES IN FORMULATION & ADMINISTRATION

SPOTLIGHT

Spotlight on advances in formulation & administration

Ana Jaklenec



"The recent SARS-CoV-2 pandemic highlighted our ability to develop and administer novel mRNA-based vaccines in unprecedented time and magnitude."

FOREWORD

Vaccine Insights 2023; 2(6), 255–257

DOI: 10.18609/vac.2023.038

The recent SARS-CoV-2 pandemic highlighted our ability to develop and administer novel mRNA-based vaccines in unprecedented time and magnitude. However, it also amplified the disparities in vaccine access across the globe, especially between low- and high-income countries, and emphasized the poor thermostability and durability of the mRNA vaccines. These challenges have energized vaccine research as scientists rigorously work on identifying and addressing the complex problems related to vaccine development, especially in the area of mucosal vaccines and adjuvants. Their work has the potential to uncover the unique mechanisms at the forefront of transforming vaccine development, delivery, and efficacy.

In this month's Spotlight on Advances in Formulation and Administration, we discuss approaches to address vaccine inequity with Christopher Fox (Access to Advanced Health Institute). This comprehensive interview covers sustainable sourcing of excipients for vaccine formulations, nasally delivered vaccines, dry thermostable vaccine formulations, and mucosal TB vaccines. Advances in alternative administration routes, including transdermal, intranasal, and inhaled, offer improved convenience, increased patient compliance, and mucosal protection. Eliminating cold chain logistics with lyophilization has the potential to transform vaccine storage and distribution. These advancements allow for extended storage, easier transportation, and increased accessibility to vaccines, particularly in remote or resource-limited areas.

Elsewhere in the Spotlight, Aneesh Thakur (University of Saskatchewan) identifies key challenges with current vaccines and explores the advantages of inhalable vaccines which have the potential to induce protective immunity where the infection starts.

Lisa A Morici and James B McLachlan (Tulane University School of Medicine) highlight the need for improved vaccines that can elicit long-lasting mucosal immunity. These experts comprehensively analyze emerging evidence from pre-clinical studies that warrant further mechanistic investigation to improve next-generation vaccines against mucosal pathogens, especially those with pandemic potential. They highlight the need to uncover the underlying mechanisms by which adjuvants stimulate immune cells and produce more effective vaccines. By identifying key adjuvant properties, they suggest, researchers can develop novel adjuvants that optimize immune responses against specific diseases. This research is crucial for designing vaccines with improved efficacy, longer-lasting immunity, and broader protection against evolving pathogens.

To this end, Erica L Stewart, Anneliese S Ashhurst, and Warwick J Britton (University of Sydney) discuss the need for mucosal vaccines capable of reducing viral transmission as well as disease severity. They propose the use of subunit vaccines due to their targeted approach and customizable nature. Tailoring adjuvants to specific populations has emerged as a promising approach, resulting in vaccines that are both safer and more effective for vulnerable populations. Adjuvants can be customized to elicit optimal immune responses in high-risk groups, addressing their unique immunological challenges.

Additionally, Ed Lavelle (Trinity College, Dublin), describes how to induce innate immune responses with particulate adjuvants. In his interview, he illuminates the growing understanding of adjuvant mechanisms, mucosal vaccines, and why size matters for nanoparticle adjuvanticity. Lavelle discusses the advantages of particulates and their availability to change certain parameters that will preferentially induce CD4 or CD8 T cells, or antibody responses.

This issue highlights a growing field within vaccine research and development, focusing on addressing challenges related to vaccine efficacy that will not only make vaccines more stable and protective but will also bring us closer to vaccine equity and in time make preventive health care a privilege available to all across the globe.

BIOGRAPHY

DR ANA JAKLENEC is a Principal Research Scientist and PI at Massachusetts Institute of Technology at the David H Koch Institute for Integrative Cancer Research. Her group is focused on engineering delivery systems for global health. Dr Jaklenec has over 20 years of experience in the area of bioengineering, materials science, micronutrient and vaccine stabilization and delivery. Dr Jaklenec holds a BSc in Biomedical Engineering from Boston University and a PhD in Biomedical Engineering from Brown University. She is the recipient of the Ruth L Kirschstein National Research Service Award (NRSA) from the National Institutes of Health (NIH). Dr Jaklenec was elected to the American Institute for Medical

DOI: 10.18609/vac.2023.038

and Biological Engineering (AIMBE) College of Fellows in 2022 for her work in controlled delivery of vaccines and heat-stable micronutrients for global health that can change the world. She was also elected to the Controlled Release Society (CRS) College of Fellows in 2022 for her research at the interface of engineering and immunology that utilizes precise fabrication and design of materials at the nano- and micro-scale for use in controlled drug delivery for global health. She has supervised over 50 pre- and postdoctoral students and has written over 40 articles in high-impact journals and has over 50 issued and pending patents worldwide. She is an active member of the Biomedical Engineering Society, the Controlled Release Society, and the Society for Biomaterials.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.
Acknowledgements: None.
Disclosure and potential conflicts of interest: Jaklenec A receives funding from the Gates foundation. Jaklenec A is a consultant for Moderna.
Funding declaration: The authro received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.
Revised manuscript received: JuL 5, 2023; **Publication date:** Jul 20, 2023.

INTERVIEW

Formulation innovation: thermostable vaccines, sustainable materials & mucosal delivery

Charlotte Barker, Editor, *Vaccine Insights*, speaks to **Christopher Fox,** Senior Vice President of Formulations, Access to Advanced Health Institute (AAHI), about his work developing innovative vaccine formulations that combine stability and accessibility.

Vaccine Insights 2023; 2(6), 243–248

DOI: 10.18609/vac.2023.036

Q How did you get involved in the area of vaccine formulation?

CF: My background is in bioengineering. During graduate school, it became clear to me that I wanted to focus on applied work—making products that would benefit people in their daily lives. Vaccine formulation offers an opportunity to apply science in a practical field where you have the potential for real impact. I found that very rewarding.

Q How have your interests evolved?

CF: When I started, I was doing very fundamental benchwork on formulation science. As time went on, my research became more and more translational and product-driven, focusing on how to get formulations to clinical testing.

Now we are looking into what I think are even more important and impactful topics, like the sustainability of the raw materials that go into these formulations and how to make formulations thermostable so they are accessible across the world. Can we deliver vaccines through needle-free routes of administration? Can we tech transfer vaccine formulations so that they can be manufactured in local settings with widely available equipment? We want formulations that are cost-effective and can be made across the world. These practical aspects drive my work now.

Q What projects are you working on at the moment?

CF: We have a number of exciting projects. One involves finding a sustainable non-animal source for squalene—an ingredient that is commonly found in vaccine adjuvant formulations and is currently derived from shark liver. Working with key partners such as Amyris, Inc., we have made great progress in identifying not only sustainable sources of squalene itself but also analog molecules that may work even better [1].

Another project we are working on is a nasally delivered vaccine candidate against *Entamoeba histolytica*, the causative agent of the enteric disease amebiasis [2]. Amebiasis is a neglected disease that mostly affects young children in very poor countries. To date, there has never been a vaccine tested in humans for amebiasis. However, we are making good progress on a vaccine formulation containing a protein antigen developed by the University of Virginia and a lipid-based adjuvant formulation developed by AAHI and 3M, which can be delivered via a nasal spray. We chose intranasal administration to generate mucosal immunity to this enteric pathogen. We have plans to progress to Phase 1 clinical testing in the coming years.

Finally, we are working on next-generation tuberculosis (TB) vaccines. In partnership with the University of Alberta, we are preclinically testing a spray-dried powder formulation that can be delivered to the nose or lungs [3]. Since it is a dry powder, it also offers the benefit of being thermostable and thus able to be stored for some time outside of the cold chain.

Most recently, we published results from a Phase 1 clinical trial on another dried formulation of the same vaccine [4]. In this case, instead of being a spray-dried powder, it is a freeze-dried (lyophilized) cake. It is designed for reconstitution with water and then injection.

Q What were the key challenges in developing a dried formulation?

CF: It is not trivial to make dried formulations that are stable and maintain their biological activity. Our lyophilized TB vaccine was a 10-year effort. It started with a systematic and rigorous formulation design. We evaluated 37 different excipients for compatibility with the protein antigen and adjuvant formulation, which is an oil-in-water emulsion with a toll-like receptor (TLR) agonist.

DOI: 10.18609/vac.2023.036

Once we had identified several lead excipients, we combined them in different ways to generate the most stable formulation that was most easily lyophilized. Then we took our lead candidates and evaluated them for biological activity in preclinical models. We identified a lead candidate formulation and scaled up and refined the lyophilization process. Then we manufactured the material for clinical testing.

The most challenging aspect was the analytical characterization. The active ingredients are at extremely low concentrations and quantifying them in a complex oil-in-water emulsion that had been dried was very difficult.

Ultimately, we were able to show that the dried formulation was stable for 3 months at 37 °C, which was our objective. No currently licensed vaccine comes close to that stability profile.

In the Phase 1 clinical trial, we were aiming to show that the new dried formulation maintained the same safety and immunogenicity profile as the previous liquid non-thermostable formulation. To our surprise, the dried formulation resulted in significantly higher antibody titers and B cells, and we maintained a robust T cell response. We were very happy with those results.

Q You have previously worked on RNA vaccines. How does the composition of the nanostructured lipid carrier (NLC) you developed overcome stability challenges?

CF: This is certainly a hot topic right now. Lipid nanoparticles (LNPs) are used with the currently licensed RNA vaccines, but we took a different approach. Instead of encapsulating the RNA inside LNPs, we developed an NLC formulation that can complex the RNA on the surface of the particle and still protect the RNA from degradation.

We found some advantages in this approach in terms of versatility. For instance, you can make the NLC formulation on its own without the RNA and complex the RNA later, even immediately before immunization. That might allow stockpiling of the NLC in preparation for a pandemic so that only the RNA itself needs to be manufactured at speed.

The NLC formulation also has a different excipient content than you would find in LNPs. It is more similar to the excipient content that you might find in an oil-in-water emulsion, which is commonly used in the vaccine adjuvant field. In fact, the NLC is made on the same type of equipment that is used to make oil-in-water emulsions, so the manufacturing aspects are very familiar to adjuvant makers.

Finally, we took the lessons we learned from working on our lyophilized TB vaccine and applied them to our RNA vaccine. We were able to lyophilize our RNA formulation with the RNA and NLC, allowing enhanced stability outside of a cold chain for months.

Q What is the most promising approach to improve the stability of current RNA vaccines?

CF: I think the most promising avenue for LNP-based RNA vaccines would be a lyophilized formulation. It might not have ambient-temperature stability, but even refrigerated stability would be a huge step forward, especially for resource-poor areas. That is probably the first advance we will see in the licensed RNA vaccines.

"Maintaining the physical stability of the LNPs, ensuring no chemical degradation of the RNA, and developing a lyophilization process that is fast and affordable are all key factors."

However, it is going to take a lot of formulation and process optimization to make it work. Maintaining the physical stability of the LNPs, ensuring no chemical degradation of the RNA, and developing a lyophilization process that is fast and affordable are all key factors.

Q What vaccine formulation advances do you hope to see in the future?

CF: There is an urgent need to advance some of these promising technologies to licensed products. I hope we will see more of the thermostable dried formulations that have shown enhanced stability in RNA vaccines and adjuvant-containing protein vaccines. I would also like to see more vaccines administered via alternative routes—whether mucosal delivery or microneedle patches, it is a very active and exciting area.

As a result of the pandemic, we have learned to think more broadly and creatively about combining vaccine platforms to achieve a more comprehensive immune response. RNA vaccines, for example, could be used as the first line of defense in an emerging pandemic since they can be manufactured and deployed rapidly. These could be followed by vaccines that take longer to develop but offer advantages in terms of the durability of immune response (e.g., adjuvanted protein vaccines) or mucosal routes of immunization that generate responses that cannot typically be obtained from injected vaccines (e.g., mucosal responses).

Combining approaches and designing vaccines that complement each other instead of competing would be very powerful. That is easier said than done, but it could really benefit the end user.

As new technologies are advanced, it is critical to transfer manufacturing capacity around the world to build local capacity and allow vaccines to be produced where they are needed.

Q What is next for your group?

CF: We plan to progress some of our technologies into clinical testing. For instance, the sustainable squalene alternatives, the nasally delivered amebiasis vaccine candidate, and the spray-dried TB vaccine candidate.

As for the product candidates that are already in clinical testing, we are focusing on advancing those further into Phase 2 and 3 clinical trials and getting them into licensed products with appropriate partners. That is where the rubber meets the road. We will be working very hard on getting our technology through that last, most difficult mile to product licensure.

DOI: 10.18609/vac.2023.036

BIOGRAPHY

CHRISTOPHER FOX leads the development of vaccine adjuvant formulations for clinical testing against a variety of infectious diseases. Since joining AAHI in 2007, he has served as Principal Investigator on multiple vaccine development grants and contracts involving pragmatic solutions to ensure equitably accessible products, such as temperature-stable formulations, intranasally administered vaccines, manufacturing process scale-up, and sustainable raw material sourcing. Dr Fox also directs AAHI's efforts to transfer adjuvant formulation manufacturing know-how and processes around the world to build local capacity. With 20 years of experience and over 120 publications, he is a recognized expert in the field of vaccine adjuvant technology.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given his approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: Fox C discloses he has a grant from the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) (R01AI135673). He also has a contract from the Department of Health and Human Services (HHSN272201400041c and HHSN272201800025c). Fox C received travel support from Vaccine Formulation Institute for a 10-year anniversary meeting (Apr 2023) and received travel support from the Industrial Advisory Board of Department of Biological Engineering at Utah State University (Apr 2023). Fox C is also a paid member of the scientific advisory board of MaxVax LLC and a unpaid member of the Industrial Advisory Board of Department of Biological Engineering at Utah State University.

Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is based on an interview with Christopher Fox carried out on May 24, 2023.

Interview held: May 24, 2023; **Revised manuscript received:** Jul 6, 2023; **Publication date:** Jul 19, 2023.

DOI: 10.18609/vac.2023.036

VACCINEINSIGHTS

ADVANCES IN FORMULATION & ADMINISTRATION

SPOTLIGHT

Inhalable vaccines: inducing protective immunity where the infection starts

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"The translation of inhalable vaccines from research and development to clinics requires a concerted approach from academia, industry, and government agencies as seen for COVID-19 vaccines."

VIEWPOINT

Vaccine Insights 2023; 2(6), 197–200

DOI: 10.18609/vac.2023.031

The global pandemic of COVID-19, caused by SARS-CoV-2, has for the first time led to the testing and development of almost all possible available vaccine platform technologies. However, it is also clear that the current-generation vaccines are not fully protective [1], do not induce mucosal immunity [2], have low-temperature storage requirements [3],

and need frequent booster immunizations [4]. Therefore, an important grand challenge for future vaccination programs is the ability of the vaccines to induce protective immunity at the sites where the infection first starts, e.g., in the lungs for several respiratory infections that are transmitted by infected aerosols.

The majority of the current vaccines in the global childhood vaccination program of the WHO are based on liquid formulations and are given through parenteral administration. Many of these vaccines are successful and induce protective immunity. However, for respiratory infections such as influenza, tuberculosis, and SARS-CoV-2, it is known that systemic immunity induced by parenteral immunization is not able to induce complete protection against infection [4]. On the other hand, mucosal vaccines can induce mucosal antibodies (IgA and IgG) [5], tissue-resident memory lymphocytes (T and B cells) [6], and trained innate immune cells (trained immunity) [7]. This multifaceted immunity can induce robust localized protective immunity at mucosal surfaces. Moreover, owing to the common mucosal immune system, antigen-specific lymphocytes induced in a mucosal site can migrate to other mucosal sites as effector cells to induce antigen-specific protection in all mucosal tissues [8]. However, different respiratory pathogens have distinct immunological requirements for protection and thus identifying mucosal immune correlates that prevent the acquisition and onward transmission of infection remains critical [9].

Another considerable challenge for global immunization programs, as observed during the COVID-19 pandemic, is the distribution, storage, and usage of vaccines. The design of thermostable vaccine dosage forms that can be stored, shipped, and distributed independently of an expensive cold chain remains a priority and has been recognized as a quality target product profile for vaccines against respiratory pathogens by the WHO [10]. The majority of vaccines are formulated as liquid dosage forms and should be stored at cold or ultra-cold temperatures during manufacture, transport, and storage until their use. Solid dosage forms of the vaccines manufactured by drying techniques such as freeze drying or spray drying in the presence of sugar-based excipients preserve antigen stability and potency [11]. Such solid dosage forms of vaccines can be stored at ambient temperatures for several months and can dramatically reduce the overall cost of the vaccine product.

Inhalable vaccines show enormous potential as the ideal next generation of vaccines for respiratory pathogens [12]. Inhalable vaccines can be formulated as liquid or solid dosage forms and administered through disposable devices such as nebulizers or dry powder inhalers. Inhalable vaccines can also induce a localized immune response targeting the mucosal surfaces where pathogens enter. The localized delivery of inhalable vaccines allows a dose-sparing effect as compared to parenteral vaccination. Moreover, inhalable vaccines have minimum storage requirements for mass vaccination programs and are apt for prophylactic strategies in low- and middle-income countries and for swift pandemic response. However, inhalable vaccines must surmount impending challenges associated with their clinical translation, including crossing pulmonary biological barriers such as mucus and pulmonary surfactant, design of stable formulations that can withstand drying and aerosolization, and identifying mucosal immune correlates that are concordant with systemic immune responses [13].

The emergence of SARS-CoV-2 variants of concern, the incidence of breakthrough infections, and the need for frequent booster immunizations have underlined the importance of developing next-generation vaccines that can induce durable protective immunity. Inhalable vaccines represent one such attractive platform that can induce robust protective immunity at the first site of pathogen invasion. A sustained immunological memory induced by inhalable vaccines in lung tissues can thwart the establishment and dissemination of infection by respiratory pathogens. The inhalable vaccines can not only be used as a stand-alone vaccination approach but also as

DOI: 10.18609/vac.2023.031

supplemental mucosal vaccines to stimulate specific upper airway immunity or as prime–boost vaccination approaches. The successful realization of inhalable vaccines will require an interdisciplinary approach focused on lung physiology, immunology, vaccinology, and drug delivery. The translation of inhalable vaccines from research and development to clinics requires a concerted approach from academia, industry, and government agencies as seen for COVID-19 vaccines.

BIOGRAPHY

ANEESH THAKUR is a principal scientist and group leader at the Vaccine and Infectious Disease Organization. His background includes training in veterinary medicine, microbiology and immunology, and drug delivery. His research focuses on fundamental understanding of the design requirements for vaccines and adjuvants for mucosal immunization and nanoparticle-based delivery systems for mRNA vaccines.

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given his approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: The author has no conflicts of interest.

Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited.

Revised manuscript received: May 26 2023; **Publication date:** Jun 13 2023.

DOI: 10.18609/vac.2023.031

VACCINEINSIGHTS

ADVANCES IN FORMULATION & ADMINISTRATION

SPOTLIGHT

EXPERT INSIGHT

Non-mucosal vaccination strategies to enhance mucosal immunity

Lisa A Morici & James B McLachlan

The SARS-CoV-2 pandemic has highlighted the need for improved vaccines that can elicit long-lasting mucosal immunity. Although mucosal delivery of vaccines represents a plausible method to enhance mucosal immunity, recent studies utilizing intradermal vaccine delivery or incorporation of unique adjuvants suggest that mucosal immunity may be achieved by vaccination via non-mucosal routes. In this expert insight, we highlight emerging evidence from pre-clinical studies that warrant further mechanistic investigation to improve next-generation vaccines against mucosal pathogens, especially those with pandemic potential.

Vaccine Insights 2023; 2(6), 229–236

DOI: 10.18609/vac.2023.034

INTRODUCTION

Despite the availability of vaccines and other therapeutics, infectious diseases continue to plague people in all regions of the world. Most infections occur in mucosal tissues, and these infections remain one of the leading causes of mortality in children under the age of five [1]. Notably, the majority of lethal infections in children manifest as pneumonia, followed closely by diarrhea. The Global Enteric Multicenter Study (GEMS) found that a majority of moderate-to-severe diarrhea was caused by just four pathogens: rotavirus, Cryptosporidium, *Shigella*, and Enterotoxigenic *E. coli* [2]. Of these, a licensed vaccine exists only for rotavirus. Respiratory infections are even more prevalent in young children and, while there are a variety of vaccines against respiratory pathogens, there remains a significant lack of effective vaccines for some of the most severe pulmonary pathogens. Tuberculosis is predicted to infect one-third of the world's

population and yet the current vaccine, Bacille Calmette-Guerin (BCG), exhibits low to moderate protective efficacy against pulmonary disease, which varies geographically [3]. The current vaccine against whooping cough includes an acellular component for Pertussis that prevents disease but does not limit bacterial mucosal colonization or infection spread [4]. Most obviously, the SARS-CoV-2 pandemic highlights the need to discover and understand how vaccine design can better target protective immunity to the respiratory mucosa [5–10]. While less prevalent in children, sexually transmitted diseases (STDs) are no less important and can have lasting effects in adults. That vaccines can be effective against STDs is evidenced by the highly successful human papillomavirus (HPV) vaccine, which has significantly decreased HPV infection and reduced the incidence of HPV-caused cervical cancer in women [11,12]; however, there exists a need for new STD vaccines that can target the female reproductive tract (FRT). For example, there is no vaccine for genital herpes or HIV, demonstrating a need for new vaccines that target these pathogens. Further, while bacterial STDs (e.g., gonorrhea, Chlamydia, and syphilis) are currently treated with antibiotics, the increase in antibiotic resistance and the fact that asymptomatic people can unknowingly spread the disease to partners makes vaccine development crucial [13]. It is highly likely that targeting the immune response directly to the FRT mucosa would increase the effectiveness of such vaccines [14].

THE CURRENT PARADIGM FOR INDUCING MUCOSAL IMMUNITY

The mucosal immune response is traditionally initiated when antigen-presenting cells encounter foreign antigen in the mucosal compartment (e.g., intestinal lumen or airway), where antigen-presenting cells, particularly dendritic cells (DCs), directly sample antigen through surveillance of the mucosal luminal space [15–19]. These DCs then have the potential to induce a mucosal homing phenotype on T cells. For example, in the intestine, CD103+ DCs sample antigen in the gut, migrate into the mesenteric lymph nodes, and impart upregulation of gut-specific homing receptors, $\alpha4\beta7$, and CCR9, on T cells [20,21]. These cells are then primed to migrate back into the intestine where they can elicit antimicrobial function. The CXCL16–CXCR6 axis has been reported to be required for migration and differentiation of resident memory T cells in the lung [22–24]. A role for CXCR6 has also been shown for resident cells of the skin [25]. Further, recent work has demonstrated that CXCR6 not only effects T cell migration but is also involved in differentiation of resident T cells once they take up residence in the lung [26]. While there are clearly mucosal T cells in the FRT, the homing markers that drive those cells into the FRT are less obvious, with CD11c or CXCR3 and CCR5 being potential markers of interest [27,28]. What is increasingly acknowledged is that, like within the gut, specialized tissue resident DCs take up infectious luminal antigen, migrate to the mucosal tissue draining lymph nodes (mediastinal lymph nodes for the lung; iliac lymph nodes for the FRT), and activate CD4 or CD8 T cells to migrate back into the mucosal tissues. This 'mucosal to mucosal' cycle is the hallmark of inducing immunity in these tissues and has long served as the standard for how mucosal immunity is achieved.

MUCOSAL ADMINISTRATION OF VACCINES

Based on what is known, it would appear to be sensible to administer vaccines mucosally to drive the desired immune response at a particular mucosal site; however, this approach has some caveats that can preclude mucosal vaccination. For example, while some vaccines are delivered mucosally (predominantly orally) and are efficacious in

DOI: 10.18609/vac.2023.034

developed countries, they often fail to protect children in developing countries, making oral vaccination impossible. A prime example of this is the oral polio vaccine, which requires many more immunizations to achieve equivalent protective levels of immunity for children in developing countries compared to children in developed countries [29,30]. Multiple factors appear to be responsible for this; however, inadequate colonization of the intestinal mucosa due to ongoing diarrheal disease appears to play a significant role, as does oral tolerance [31,32]. It is known that the most effective classical 'mucosal adjuvants', such as cholera toxin (CT—derived from the enteric pathogen *Vibrio cholerae*), delivered either orally or intranasally in mice, can induce potent cell-mediated and antibody responses in the mucosal compartment [33–36]; however, while this approach is attractive in terms of initiating an immune response at pathogen sites of entry, it carries limitations: mucosally delivered CT and other GM1 receptor-binding bacterial-derived toxins have known side effects, such as inducing facial paralysis, when administered intranasally [37,38]. Whole toxin adjuvants, while immunologically effective, are dangerous when delivered orally, which is unsurprising given their enteric pathogen origin [39]. In some cases, mucosal vaccination is limited by the harsh environment of mucosal tissues (e.g., acidity in the stomach) or the impractically of immunization (i.e., intravaginally), and concerns exist about ensuring that the vaccine correctly targets the inductive mucosal immune tissues [40,41]. With these caveats in mind, a parenteral (non-mucosal) approach may be more attractive. Importantly, while inducing mucosal immunity in mucosal tissues may be achievable by immunizing individually directly into each of these sites, the ability to induce mucosal immunity in all mucosal tissues via a standardized parenteral formulation would circumvent this need for site-specific immunization. This would potentially avert the need to design a unique, separate vaccine for each mucosal tissue.

PARENTERAL VACCINE ADMINISTRATION & MUCOSAL IMMUNITY

It is becoming clear that novel vaccines must be designed such that non-mucosal immunization might lead to mucosal immunity. Recent work from our group and others has established that this non-mucosal to mucosal link can be established predominantly via intradermal immunization [42–44]. This is vital because most currently licensed vaccines are delivered parenterally (predominantly intramuscularly). While these vaccines induce systemic immunity, the mucosal immune response to these vaccines is often limited at best. It flies in the face of conventional immunology that intradermal (ID) immunization elicits a mucosal response; with rare exceptions, it was previously believed mucosal immune responses must be elicited mucosally. These findings suggest there may be some crosstalk between the skin and the mucosal immune system. In fact, it is becoming clearer that both skin and mucosal immune tissues share many of the same inductive cell types. For example, $CD103^+$ DCs from both skin and gut can activate naïve T cells and elicit effector function [45–48]. CD103 DCs display different frequencies and properties in humans and mice so this must be taken into consideration when extrapolating murine results to humans [49]. Most of the studies assessing ID immunization affecting mucosal responses have been limited to the quantification of IgA and/or IgG responses in the mucosal compartment, with little attention given to the Ag-specific cellular response at these sites. More recent publications showing CD4 T cell migration to the gut and lung in response to ID immunization are compelling and warrant further investigation on how parenteral immunization effects cell migration and mucosal immunity [50,51]. Other recent work from our group has also found that ID vaccination can induce antigen-specific

T cell migration into the FRT (personal communication).

What is also becoming understood is that the correct parenteral route must be combined with an appropriate adjuvant to achieve mucosal immunity. It is now appreciated that bacterial ADP-ribosylating toxin adjuvants such as CT can achieve this type of mucosal immune response. Indeed, we recently showed that the non-toxic ADP-ribosylating adjuvant double mutant heat-labile toxin (dmLT) can engage skin CD103+ DCs to drive vaccine-specific CD4 T cells in the gut mucosa (50), as well as elicit vaccine-specific lung CD4 T cells [51]. Mice that lack the transcription factor Batf3 also are deficient in the CD103+ DC subset in both the skin and the gut [52]. It is now known that this DC subset, while comprising only 3% of the total DCs in the skin, is essential to drive CD4 T cells initiated with ID immunization into the lamina propria of both the small and large intestines. Interestingly, recent findings from our group have also shown that dmLT-adjuvanted ID immunization can elicit vaccine-specific B cells to migrate into both the large intestines and the lungs [53]. Notably, these cells were non-circulating and, as such, are most likely resident in both tissues. As was found with T cell migration and activation, Batf3 appears to be required for both the full migration and class switch recombination of B cells, a process controlled by cytokines and ligation of CD40 on B cells with its ligand CD40L on T cells [54]. Unlike with T cells, this effect did not appear to depend on the presence of CD103+ DCs and instead was likely B cell-intrinsic, showing that, while Batf3 plays a role in mucosal migration of both immune cell types, this role appears distinct between T and B cells. While getting cells to the mucosa is the first step, induction of the appropriate cellular or humoral response against different pathogens is essential for clearance. For protective mucosal humoral responses, the most critical antibody isotype is secretory IgA. Importantly, dmLT and similar adjuvants are known to induce both systemic IgG and mucosal IgA, as well as CD4 T cells that can aid in class switch recombination, even when delivered parenterally [55–57]. Additionally, targeting the lymph node germinal center, where memory B cells and long-lived plasma cells develop, is important for effective vaccine development [58]. We have found that, at the same dose, ID immunization is superior to oral immunization at eliciting vaccine-specific systemic IgG and fecal IgA responses when using dmLT as the adjuvant [53]. Interestingly, this same antibody response could not be achieved by either route using the TLR9 agonist adjuvant CpG oligodeoxynucleotide at the same dose. Thus, using novel adjuvants or adjuvant combinations that can access the connection between the skin and the mucosal tissues has the potential to change how we think about vaccine design and to exploit this link to create more efficacious mucosal vaccines that can be delivered parenterally. Additionally, these adjuvants can lead to dose sparing, especially when used intradermally, allowing for more equitable, and cost-effective, distribution of vaccines around the world [59,60].

TRANSLATION INSIGHT

The concept of specifically targeting mucosal surfaces during the effector phase by using adjuvants to manipulate the inductive phase of systemic vaccination is uncharted territory, and consequently, unresolved questions remain: What additional mechanisms determine migration to mucosal tissues? What are the quality and protective efficacy of the T and B memory cells generated in the mucosa after parenteral immunization? Are T and B cells in the mucosa transient or resident? Can combinations of different adjuvants manipulate the T and B cell response to be more protective? Future work to address these questions using adjuvants or adjuvant combinations to drive mucosal immunity and elicit robust and broad immune responses encompassing all arms of the adaptive immune system will be essential

DOI: 10.18609/vac.2023.034

for the next generation of vaccines. Further, exploration of how vaccination at a single parenteral site can elicit immunity at all mucosal surfaces has the potential to change our current understanding and approach to novel vaccine formulations.

BIOGRAPHIES

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: All named authors take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: Morici LA discloses she has the following grants/contracts (HDTRA1-19-C0013; NIH 272201800045C; NIH/NIAID R01 AI166756-01). She receives consulting fees and payment for expert testimony from AP Experts LLC. McLachlan JB discloses he has the following grant/contract (NIH 01AI166756). Lastly, Tulane has an issued patent on vaccine adjuvant technology for which Morici LA and McLachlan JB are inventors.

Funding declaration: The authors received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.

Submitted for peer review: Apr 28, 2023; **Revised manuscript received:** Jun 8, 2023; **Publication date:** Jul 12, 2023.

DOI: 10.18609/vac.2023.034

VACCINEINSIGHTS

ADVANCES IN FORMULATION & ADMINISTRATION

SPOTLIGHT

EXPERT INSIGHT

Arming the airways: an update on the clinical status of intranasal vaccines & the role of mucosal adjuvants

Erica L Stewart, Anneliese S Ashhurst & Warwick J Britton

The COVID-19 pandemic highlighted the need for mucosal vaccines capable of reducing viral transmission as well as disease severity. As such, there are an unprecedented number of intranasal vaccines undergoing clinical testing. Due to their scalability and cost-effectiveness, viral vectors dominate the intranasal vaccine clinical trial landscape. However, concerns surrounding safety and pre-existing anti-vector immune responses support the development of other vaccine technologies. Subunit vaccines are one such strategy, given their targeted approach and capacity for tailoring via selection of appropriate adjuvants. One limitation, however, is the lack of safe and effective mucosal adjuvants. This review outlines the current progress in clinical research of intranasal vaccines with a focus on mucosal adjuvants. Given the ongoing impact of respiratory pathogens, it is imperative that the current momentum for the development of mucosal vaccines continues and is broadened to include diseases beyond COVID-19.

Vaccine Insights 2023; 2(6), 213–228

DOI: 10.18609/vac.2023.033

MUCOSAL VACCINATION

Vaccine delivery to mucosal sites is a strategy that has been employed since 10th century China [1], whereby smallpox lesions were administered intranasally (IN) to generate a protective immune response. More recently, vaccine delivery to a variety of mucosal sites is being researched for a number of infections: oral administration of the cholera vaccine, urogenital/rectal administration for human immunodeficiency virus (HIV), and

IN/intrapulmonary delivery for respiratory pathogens such as measles, SARS-CoV-2, influenza and *Mycobacterium tuberculosis* [2]. *M. tuberculosis* has been researched particularly in-depth for mucosal vaccination since it is an especially difficult pathogen to vaccinate against [3–7]. All these vaccines target the site of pathogen entry, thought to result in immune memory that is more rapid and effective than a systemic immune response [8].

The major advantages of mucosal delivery over parenteral immunization are the induction of tissue-resident memory T cells (TRMs) and secretory IgA that are strategically located to rapidly identify and engage invading pathogens [9–11]. In addition, mucosal vaccine delivery has been observed to generate systemic and lung-local IgG antibodies that are particularly important for the generation of neutralizing antibody responses that protect against severe disease in respiratory viral infections, such as influenza and SARS-CoV-2 [12,13]. However, an important benefit of mucosal vaccination is the generation of pathogen-specific IgA antibodies that are associated with protection against upper respiratory tract (URT) infections and reduction of viral transmission [11,14,15]. Mucosal vaccination is also attractive for its needle-free delivery, which may allow the targeting of vaccine-hesitant populations [16–18]. In addition, there is potential for self-administration which could negate the requirement for trained healthcare workers and further enhance vaccine accessibility [19]. Despite these advantages, however, there are significant barriers to the development of mucosal vaccines. Since mucosal sites exist at the environmental interfaces of the body, they contain many mechanisms to destroy and eject invading pathogens. For example, the respiratory tract employs mucociliary clearance to eject inhaled threats and stomach acid destroys or damages all but the toughest microbes [2,20]. In addition, mucosal immune sites contain safeguards to dampen immune responses and avoid constant inflammation [21]. A summary of the immune responses to nasal vaccine administration is outlined in Figure 1. Thus, mucosal vaccines must survive the environment they are delivered to and be sufficiently immunogenic without causing damaging inflammation.

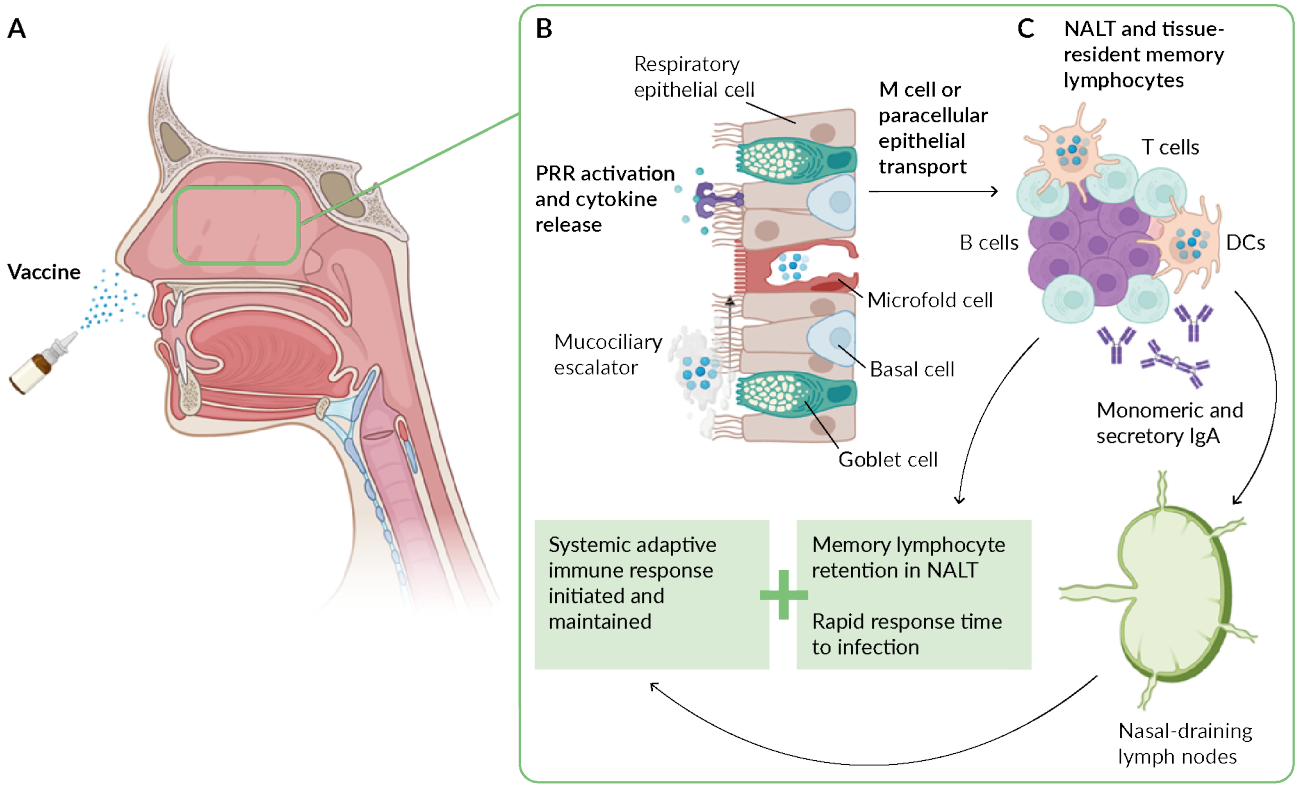
Few mucosal vaccines have been approved for clinical use thus far, and these have been for either oral or IN delivery. Oral-delivered vaccines must survive the inhospitable conditions of the digestive tract to reach the immune cells of the intestine, where they face the additional challenge of overcoming tolerogenic responses. The approved mucosal vaccines for oral administration include those against *Vibrio cholerae*, *Salmonella enterica*, poliovirus, and rotavirus [2]. IN vaccines must overcome barriers such as the mucociliary escalator and have the additional safety consideration of potentially inducing inflammatory responses close to the olfactory nerve [2]. Nevertheless, prior to the COVID-19 pandemic, three IN vaccines have been approved, and all were for influenza. The quadrivalent live-attenuated influenza vaccine (LAIV) FluMist® Quadrivalent (aka Fluenz® Tetra) (AstraZeneca, UK) is approved for IN use in the US, UK, and Europe [11,22,23]. Also approved in China in 2020 was the GanWu® freeze-dried LAIV (Changchun BCHT Biotechnology, China), while Nasovac is an H1N1 pandemic influenza vaccine developed by the Serum Institute of India that was approved in 2010 [24,25].

Since the beginning of the coronavirus disease (COVID-19) pandemic in 2020, three more respiratory mucosal vaccines have met regulatory approval and are in clinical use. The first was the adenovirus type 5 vector vaccine developed by CanSino Biologics, Convidecia™ Air, which is inhaled using a nebulizer and was approved by the World Health Organization (WHO) for emergency use listing. As such this is now being used in multiple countries including China (NCT05517642). The IN spray vaccine iNCOVACC® (NCT05522335; developed by Bharat Biotech) was also approved for use in India, and the IN spray Razi

DOI: 10.18609/vac.2023.033

FIGURE 1

Immune responses to intranasal vaccine delivery.



A) Intranasal vaccines are delivered as a liquid spray, drops, or dry powder spray where they are inhaled into the nasal cavities. B) The respiratory epithelium consists of ciliated epithelial cells and goblet cells secreting mucus held together by tight junctions that facilitate expulsion of inhaled particles via mucociliary clearance. Thus, vaccine antigen must either traverse microfold cells or may undergo paracellular epithelial transport that is promoted by some adjuvants, such as heat-labile enterotoxins, to reach the underlying nose-associated lymphoid tissue (NALT). Vaccine adjuvants may also activate pattern recognition receptors (PRR) on respiratory epithelial cells that leads to recruitment of innate immune cells. C) Vaccine antigen is internalised by tissue-resident dendritic cells, where it is then presented to naïve lymphocytes in the NALT. In addition, dendritic cells will transport vaccine antigen to the nasal-draining lymph nodes for antigen presentation. Nasal delivery promotes the retention of tissue-resident memory $CD4^+$ and $CD8^+$ T cells in the NALT, as well as IgA^+ memory B cells that generate a rapid response to infection.
Figure created using Biorender.com.

Cov Pars (IRCT20201214049709N1; Razi Vaccine and Serum Research Institute) was approved for emergency use in Iran [26] (Table 1). Both Convidecia Air and iNCOVACC utilize adenoviral vectors, favored for their capacity for rapid scale-up, relative ease of modification, and cost-effectiveness [27]. Razi-Cov Pars consists of recombinant SARS-CoV-2 spike protein with the oil-in-water adjuvant system RAS-01 [28].

INTRANASAL VACCINE DELIVERY

The finding in preclinical studies that vaccine delivery to the respiratory tract may reduce viral shedding and spread in SARS-CoV-2 vaccine models has piqued interest in IN vaccine delivery [29,30]. Traditional vaccine delivery routes, such as intramuscular and subcutaneous injection, generate systemic immunity whereby immune memory cells home to the secondary lymphoid organs such as the spleen and lymph nodes. In contrast, it has been found that tissue-resident memory in the respiratory mucosa can only be stimulated if antigen recognition occurs in that tissue [11,31,32]. After IN administration, inhaled particles arrive in the nasal cavity, a highly vascularized region with large surface area containing the respiratory

TABLE 1

Recent intranasal vaccine clinical trials.

Vaccine type (Key: Live attenuated / Subunit / Viral vector)	Adjuvant or vector type	Disease	NCT number	Vaccine name	Study phase	Sponsor	Completion date
Live attenuated		COVID-19	NCT04619628/ NCT05233826	COVI-VAC™	1	Codagenix, Inc	2022
Live attenuated		Influenza	NCT05163847	Cam2020 MSR2	1	FluGen Inc	2022
Live attenuated		Influenza	NCT04146623	CodaVax™	1	Codagenix, Inc	2020
Live attenuated		Influenza	NCT02251288	Live attenuated A/H7N9 Influenza Virus vaccine	1	National Institute of Allergy and Infectious Diseases (NIAID)	2018
Live attenuated		Influenza	NCT04650971	UniFluVec	1	Pharmenterprises Biotech LLC	2020
Live attenuated		Influenza	NCT04960397	Sing2016 M2SR	1	National Institute of Allergy and Infectious Diseases (NIAID)	Ongoing
Live attenuated		RSV	NCT04909021/ NCT04690335	MV-012-968	1/2	Meissa Vaccines, Inc.	Ongoing
Live attenuated		RSV	NCT04491877		2	Sanofi Pasteur, a Sanofi Company	Ongoing
Live attenuated		RSV	NCT05687279	RSVt	1/2	Sanofi Pasteur, a Sanofi Company	Ongoing
Live attenuated		Whooping cough	NCT05116241/ NCT05461131	BPZE1	2	ILiAD Biotechnologies	Ongoing
Subunit		Influenza	NCT03594890	OVX836	1	Osivax	2019
Subunit	Nanovax/NE01	Anthrax	NCT04148118	BW-1010	1	BlueWillow Biologics	2021
Subunit	Hepatitis B nucleocapsid protein	COVID-19	RPCEC00000345	CIGB-669/Mambisa	2	Center for Genetic Engineering and Biotechnology (CIGB), Havana	2021
Subunit	Outer membrane vesicle	COVID-19	NCT05604690	Avacc 10®	1	Intravacc B.V.	Ongoing
Subunit	Artificial cell membranes (ACM) and CpG7909	COVID-19	NCT05385991	ACM-001	1	ACM Biolabs	Ongoing
Subunit	RAS-01 (oil-in-water emulsion)	COVID-19	IRCT20201214049709N3	Razi-Cov Pars	3	Razi Vaccine and Serum Research Institute	Ongoing
Subunit	Heat-labile enterotoxin (LT)-derived from *E. coli* (LTh(αK))	Influenza	NCT03784885	AD07030	2	Advagene Biopharma Co. Ltd./The Development Center for Biotechnology (DCB)	2018
Subunit	Nanoemulsion	Influenza	NCT05397119	BW-1014	1	BlueWillow Biologics	Ongoing
Subunit	Endocine™	Influenza	NCT03437304	Immunose™ FLU	1/2	Eurocine Vaccines AB	2018
Viral vector	Adenovirus	COVID-19	NCT04679909	AdCOVID™	1	Altimmune, Inc.	2022
Viral vector	Adenovirus	COVID-19	NCT05522335	BBV154/iNCOVACC®	3	Bharat Biotech International Limited	Ongoing
Viral vector	Adenovirus	COVID-19	NCT04816019	ChAdOx1 nCOV-19	1	University of Oxford	2022
Viral vector	Adenovirus	COVID-19	NCT05248373	Gam-COVID-Vac	1/2	Gamaleya Research Institute of Epidemiology and Microbiology, Health Ministry of the Russian Federation	Ongoing
Viral vector	Adenovirus	COVID-19	NCT04839042	SC-Ad6-1	1	Tetherex Pharmaceuticals Corporation	Ongoing
Viral vector	Influenza	COVID-19	NCT05696067	Corfluvec	1/2	Tatyana Zubkova	Ongoing
Viral vector	Parainfluenza virus 5	COVID-19	NCT04954287/ NCT05736835	CVXGA1	1/2	CyanVac LLC	Ongoing
Viral vector	Influenza	COVID-19	NCT05200741	DelNS1-2019-nCoV-RBD-OPT1	2	The University of Hong Kong	Ongoing
Viral vector	Newcastle disease virus	COVID-19	NCT05181709	NDV-HXP-S	1	Sean Liu/Laboratorio Avi-Mex, S.A. de C.V.	Ongoing
Viral vector	Newcastle disease virus	COVID-19	NCT05205746	AVX/COVID-12	2	Laboratorio Avi-Mex, S.A. de C.V.	Ongoing
Viral vector	Respiratory syncytial virus	COVID-19	NCT04798001	MV-014-212	1	Meissa Vaccines, Inc.	2022
Viral vector	Influenza virus	COVID-19	NCT04809389	DelNS1-nCoV-RBD LAIV	1	The University of Hong King	Ongoing
Viral vector	Adenovirus	HIV	NCT03878121	Ad4-HIV	1	National Institute of Allergy and Infectious Diseases (NIAID)	Ongoing

TABLE 1 (CONT

Recent intranasal vaccine clinical trials.

Vaccine type		Adjuvant or vector type	Disease	NCT number	Vaccine name	Study phase	Sponsor	Completion date
Key:	Live attenuated Subunit Viral vector							
Viral vector		Adenovirus	Influenza	NCT01806909	AD4-H5-VTN	1	National Institute of Allergy and Infectious Diseases (NIAID)	2019
Viral vector		Pseudo-adenovirus	Influenza	NCT04034290	GamFluVac	2	Gamaleya Research Institute of Epidemiology and Microbiology, Health Ministry of the Russian Federation	2020
Viral vector		Modified vaccinia Ankara	RSV	NCT04752644	MVA-BN-RSV	2	Bavarian Nordic	2021
Viral vector		Parainfluenza virus 5	RSV	NCT05281263/ NCT05655182	BLB-201	1/2	Blue Lake Biotechnology Inc.	Ongoing
Viral vector		Sendai virus	RSV	NCT03473002	SeVRSV	1	National Institute of Allergy and Infectious Diseases (NIAID)	2019

epithelium and specialized immune structures [33].

One such structure is the nose-associated lymphoid tissue (NALT). The NALT is a tertiary lymphoid tissue consisting of lymphoid follicles that are a site for antigen presentation, lymphoid proliferation, and retention of memory lymphocytes [34–36]. Its presence bypasses the need for immune cells to travel to the draining lymph node and thereby allowing faster initiation of adaptive immune responses. It is also a site of IgA class switching, an important benefit of mucosal vaccine delivery [37,38]. In addition, the NALT is a site for naïve $CD4^+$ T cell priming, and the homing site for both $CD4^+$ and $CD8^+$ TRM cells where they are in an ideal position to re-encounter antigen [34–36,39]. In humans, the NALT most closely corresponds to Waldeyer's Ring, comprised of the nasopharyngeal tonsils, palatine tonsils, and bilateral lingual tonsils in the nasal passages [33]. Thus, the direct delivery of vaccine antigen to the specialized immune structures of the nasal passages promotes tissue-resident immune memory.

The URT also contains epithelial cells expressing various pattern-recognition receptors (PRRs) that initiate local inflammatory responses [40]. Thus, airway epithelial cells (AECs) are often major orchestrators of the chemokine and cytokine responses to IN vaccines [41–43]. In addition, specialized mucosal epithelial cells known as microfold (or 'M') cells exist on the periphery of NALT structures where they can transfer internalized antigens to underlying antigen-presenting cells (APCs) [44,45]. Many IN vaccines utilize viral vectors including adenovirus, influenza virus, and respiratory syncytial virus that have endogenous adjuvant activity. They can stimulate PRRs on epithelial cells including toll-like receptors (TLRs) and cytosolic DNA sensors, leading to expression of proinflammatory cytokines and interferons [46].

In contrast, since the delivery of protein alone is not sufficient to stimulate an immune response, subunit vaccines require vaccine adjuvants that will be able to withstand and stimulate the mucosal immune system. In some cases, these adjuvants will directly stimulate AECs and pulmonary immune cells via their PRRs, such as TLR agonists including flagellin that stimulates TLR5 and dipalmitoyl-S-glycerylcysteine (Pam_2Cys) that activates TLR2/TLR6 [41,47]. Other mucosal adjuvants aim to utilize existing features of the respiratory mucosa. For example, chitosan particles have been shown to be an effective IN adjuvant due to their mucoadhesive properties that promote vaccine penetration of the respiratory epithelium [48]. Similarly, pulmonary surfactant-mimetic liposomes containing cGAMP were used to direct H5N1 vaccine through the pulmonary surfactant layer to the underlying AECs [49], and a recent study fused the receptor binding domain of SARS-CoV-2 to the C-terminus of *C. perfringens* enterotoxin, which targets M cells [44]. Thus, IN vaccines take advantage of the native immune features of the respiratory mucosa to generate local immune memory responses.

INTRANASAL VACCINES IN THE CLINIC

Prior to the COVID-19 pandemic, the only IN vaccines to be approved for clinical use were live attenuated influenza vaccines (LAIVs). LAIVs are replication-competent in the URT, but do not replicate in the lower respiratory tract, enhancing their safety profile, and are usually administered as a single-dose IN spray [22]. Thus far they have been approved for use in Russia, USA, Canada, EU, UK, India, and China [33,50]. The efficacy of LAIVs has been found to range from approximately 40–80% or higher depending on the age of the immunized population and are most effective in children [22,51]. In the US 2014/2015 influenza season, LAIV had vastly reduced effectiveness caused by issues with the heat stability of the strain used [23]. The variable efficacy of LAIVs is also related to pre-existing immunity against influenza in the URT of adults, and this is one reason that some countries have recommended LAIVs for use in children. In addition, the less invasive IN delivery method is an attractive option for childhood immunization [22,23,51]. LAIVs are an important supporting precedent for IN vaccination, as they have a documented safety record and demonstrated efficacy [52,53]. LAIVs elicit mucosal IgA antibodies and T cell IFN-γ responses. In particular, the induction of mucosal IgA by IN LAIV was found to be a correlate of protection against influenza [54].

Since the COVID-19 pandemic, there have been three more mucosal vaccines approved for clinical use, two of which are virus-vectored, and one subunit vaccine. The significant interest in the development of IN vaccines for SARS-CoV-2 lies in the potential for the generation of a more broadly protective immune response against emerging variants of concern (VOC) and enhanced prevention of viral transmission [11,55]. Thus, COVID-19 has progressed the field of IN vaccination significantly, with a large number of IN COVID-19 vaccines currently progressing through clinical trials (Table 1).

Despite the enthusiasm surrounding the approval of inhaled SARS-CoV-2 vaccines, the immunogenicity profiles after immunization remain understudied, particularly in the mucosa. Clinical studies with Convidecia Air reported reduced adverse events paired with increased serum neutralizing antibodies after aerosol boosting of two intramuscular inactivated SARS-CoV-2 immunizations, when compared with the effects of a homologous intramuscular booster [56]. In these clinical studies, it was stated that mucosal IgA responses were not measured because of the

lack of established and validated methods in humans. In rhesus macaques, however, it was found that aerosol Convidecia promoted significant levels of IgA in the bronchoalveolar lavage (BAL) and serum [56–58]. Similarly, in a phase 3 trial of iNCOVACC, fewer side effects and increased salivary IgA were observed in subjects receiving the mucosal vaccine [59]. In the major preclinical immunogenicity assessment of iNCOVACC in multiple animal models, it was found that IN delivery promoted IgG and IgA in the BAL, along with a Th1-skewed phenotype in the spleen [60]. Hamster studies of the RaziCov Pars vaccine reported that an IN booster after two IM vaccinations significantly boosted serum and salivary anti-receptor binding domain (RBD) IgA coupled with a systemic Th1/Th17 cellular response [28]. Thus, the major differentiator of the recently approved mucosal SARS-CoV-2 vaccines is their induction of respiratory tract immunoglobulins, but this has not always been evaluated in clinical studies because of the lack of standardized methods for testing responses at mucosal site.

IN vaccines are also being developed for a variety of different mucosal pathogens including *Bordetella pertussis*, HIV, respiratory syncytial virus (RSV), and influenza. The clinical trials of 'intranasal vaccines' registered at clinicaltrials.gov over the past 5 years are summarized in Table 1. Comprehensive summaries of all IN vaccines that have progressed through clinical trials have been reported elsewhere [1,33]. Most of the vaccines shown in Table 1 are live attenuated or viral vector, likely due to the respiratory tropism of many viral vectors, the precedent set by the LAIVs already in use, as well as their scalability and cost-effectiveness. However, the development of viral vectors requires strategies, such as the use of rare serotypes or viruses from other species including chimpanzees, to reduce the risk of the vaccine being eliminated by pre-existing immune responses before a sufficient response is achieved [27, 60–62]. In contrast, subunit vaccines could be considered more targeted in that they deliver only the protein specific to the pathogen of interest along with an immune-stimulating adjuvant. Therefore, recipients can readily receive multiple homologous doses as prime and/or booster vaccines. There are only a handful of IN subunit vaccines that have recently been explored in clinical trials (Table 1), probably related to a lack of suitable mucosal adjuvants. Some of the mucosal adjuvants currently moving through clinical trials and their proposed mechanisms of action are detailed in Table 2.

MUCOSAL ADJUVANTS

The creation of effective mucosal adjuvants has long been challenging for vaccine developers since they must be able to withstand and activate/penetrate the respiratory mucosae without causing damaging inflammation. The development of adjuvants for IN application proves especially challenging, due to the proximity of the nasal passages to the olfactory nerves. Thus, there is a justified focus on the development of safe mucosal adjuvants.

There are a variety of mucosal adjuvants currently moving through the clinical pipeline (Table 2). Nanovax®/NE01 (BlueWillow Biologics, Inc., USA) is a soybean oil-in-water nanoemulsion being tested in IN anthrax and influenza vaccines. In preclinical mechanistic studies, it was shown to be internalized by ciliated epithelial cells after IN administration, leading to local DC uptake and proinflammatory cytokine production, promoting robust serum antibody production and mixed Th1/Th2/Th17-polarised responses in splenocytes [42,63]. In preclinical influenza vaccine trials, NE01 in combination with recombinant H5 hemagglutinin antigen administered IN in ferrets generated protection against heterologous H5N1 challenge, with the development of significant HAI and H5-specific IgG titers in serum and IgA in the BAL [69]. It is currently undergoing a phase I clinical trial to assess its safety and immunogenicity in BW-1014, a recombinant H5 vaccine (NCT05397119). Another oil-in-water

DOI: 10.18609/vac.2023.033

TABLE 2

Intranasal adjuvants in the clinical trial pipeline.

Adjuvant	Type	Proposed mechanism of action	Developer	Reference
Nanovax®/NE01	Nanoemulsion	Uptake by ciliated epithelial cells leading to apoptosis and upregulation of chemotactic factors	Blue Willow Biologics	[42,63]
OMV	Outer membrane vesicle	Activation of pattern recognition receptors via detoxified LPS and other lipoproteins	Intravacc B.V.	[64]
ACM and CpG7909	Artificial cell membranes (ACM) and TLR agonist	ACM uptake and TLR9 activation leading to DC activation	ACM Biolabs	[65]
RAS-01	Oil-in-water emulsion	Not determined	Razi Vaccine and Serum Research Institute	[28]
LTh(αK)	Heat-labile enterotoxin (LT)-derived from *E. coli*	LT adjuvants disrupt cellular tight junctions leading to enhanced mucosal antigen uptake (exact MOA of LTh(αK) not determined)	Advagene Biopharma Co. Ltd./The Development Center for Biotechnology (DCB)	[33, 66]
EndocineTM	Endogenous lipid	Ribonucleic acid release leading to DC activation in draining lymph nodes	Eurocine Vaccines AB	[67]
INNA-051	TLR2/6 agonist	Epithelial cell TLR2 activation leading to early immune cell recruitment	ENA Respiratory	[43]
AgnHb	Hepatitis B nucleocapsid	Encapsulated RNA thought to stimulate PRR	Center for Genetic Engineering and Biotechnology (CIGB)	[68]

adjuvant system is RAS-01, used in the IN SARS-CoV-2 vaccine Razi-Cov Pars approved for emergency use in Iran. In preclinical evaluation, IN-administered RAS-01 was found to induce salivary IgA and a Th1/Th17 immune signature in splenocytes [28]. A different strategy being explored by Intravacc (Netherlands) is that of outer membrane vesicles (OMVs). OMVs are non-replicative, lipid nanoparticles containing immunogenic components, such as LPS and lipoproteins, produced by numerous gram-negative bacteria. In their native form, they contain endotoxin that is highly inflammatory [70]. As such, the OMVs produced by Intravacc in the SARS-CoV-2 vaccine Avacc-10, derived from *Neisseria meningitidis*, are modified such that the LPS is genetically detoxified via deletion of the lpxL1 gene [71,72]. In preclinical studies, IN immunization led to higher serum IgG than intramuscular delivery, in addition to lung and nasal IgA [72]. Heat-labile enterotoxin (LT)-derived from *E. coli* (LThαK)) is another adjuvant strategy using bacterial-derived factors to stimulate the immune system. LT adjuvants are favored for their ability to enhance epithelial cell permeability for antigen to penetrate the mucosal barrier, but have had significant safety issues in the past [33]. Lth(αK) is a form of LT that has been detoxified so that the ADP-ribosylating enzyme, thought to be responsible for much of its toxicity, is completely inactivated [73,74]. Animal studies showed that after IN administration to mice, the majority of the adjuvant remained in the nasal passages without evidence of it entering the olfactory bulb or brain [74]. LTh(αK) is currently being explored in the Advagene Biopharma influenza vaccine AD07030 (NCT03784885) and as an immunomodulatory agent for COVID-19 (NCT05069610).

Endocine™ is an anionic lipid-based adjuvant consisting of the endogenous lipids mono-olein and oleic acid produced by Eurocrine Vaccines (Sweden). It is currently being tested in an influenza vaccine (Immunose™ FLU) in clinical trials and was also previously tested in an HIV vaccine candidate, both delivered IN [75–77]. In preclinical studies, it was found to enhance humoral responses in the serum and respiratory mucosa when administered IN to both young and old mice. In addition, Endocine was found to promote the generation of IL-2 and IFN-γ producing cells in the spleen [76]. The mechanism of action of Endocine was investigated by Hayashi *et al.* who examined its adjuvanticity in a selection of knockout mice and found that adjuvant activity was abrogated after RNAse treatment and in TANK-binding kinase 1 (involved in nucleic acid sensing) deficient mice [67]. Thus, the cellular release of nucleic acids leading to PRR activation is thought to be its mechanism of adjuvanticity.

Other studies are using TLR agonists as more targeted adjuvants to modulate the mucosal immune system. The SARS-CoV-2 vaccine candidate ACM-001 utilizes artificial cell membranes (ACM) to deliver recombinant beta variant spike protein and synthetic cytosine-phosphate-guanosine oligodeoxynucleotide (CpG), developed by ACM Biolabs (Singapore). The ACMs are thought to potentiate uptake of the particles by dendritic cells (DCs), with CpG, a potent TLR9 agonist, activating the DCs and promoting Th1 polarization [65,78]. IN administration to hamsters was found to induce systemic IgG and neutralizing antibodies, and subcutaneous delivery led to IFN-γ, TNF, and IL-2-expressing splenocytes [65,78]. The TLR2 agonists, Pam_2Cys and Pam_3Cys, when fused with an *M. tuberculosis* secreted protein and delivered to the lungs of mice induced protective immunity against *M. tuberculosis* [79]. As such, Pam_2Cys is now being assessed as an adjuvant for IN COVID-19 vaccination in preclinical trials. When Pam_2Cys was delivered with SARS-CoV-2 spike protein as an IN vaccine, the adjuvant stimulated induction of robust SARS-CoV2-specific IgA and IgG, neutralizing antibodies and Th17-polarised T cell responses in the respiratory mucosa that provided sterilizing protection against lethal viral challenge [41]. Pam_2Cys is also being explored in clinical trials to activate innate immune responses against influenza in the modified form of INNA-051 (ENA Respiratory (Australia)) (NCT05255822) [43]. The effect of INNA-051 relies primarily on respiratory epithelial cell TLR2 recognition, leading to proinflammatory cytokine expression and early recruitment of macrophages and neutrophils [43]. In a ferret model of SARS-CoV-2 infection, pre-treatment with IN INNA-051 significantly reduced viral burden [80]. Furthermore, in a recent phase 1 clinical trial, INNA-051 was found to be safe and tolerated after IN administration in healthy adults and is being evaluated for the prevention of influenza infection in a phase 2a trial (NCT05255822). It was also to be tested for the prevention of COVID-19, but this trial has since been withdrawn (NCT05118763) [81].

In addition to the mucosal adjuvants currently progressing through clinical trials, there are a variety of strategies being explored in preclinical animal models. Particle-based adjuvants and delivery systems have been utilized for their capacity to adhere to and withstand mucous membranes [82]. Chitosan-based adjuvants have long been studied for their mucoadhesive properties and have been found to promote IgA and Th1 responses in the lungs and blood [83–85]. Delta inulin, or Advax, is another particulate adjuvant shown to stimulate broad immune cell recruitment to the lungs and pulmonary vaccine-specific IgG, IgA, and Th17 cells [6,7,10]. Lung-localized immunoglobulin and Th17 responses are also induced by poly(lactic-co-glycolic acid) (PLGA) particle-based vaccines that can be formulated with additional immune-stimulatory components, as developed for *M. tuberculosis* and SARS-CoV-2 vaccines [86,87]. In addition, other PRR-stimulating adjuvants have also

DOI: 10.18609/vac.2023.033

been explored for mucosal delivery. A recent study of an IN SARS-CoV-2 vaccine utilized a synthetic dsRNA TLR3 agonist to generate neutralizing antibodies in the nasal passages and lungs [44]. Stimulator of interferon genes (STING)-activating adjuvants also generate vaccine-specific Th1/Th17 and IgA responses in the mucosa after IN delivery [88–90].

TRANSLATION INSIGHT: BARRIERS TO INTRANASAL VACCINE DEVELOPMENT

There are now a variety of promising IN vaccine strategies progressing through the pre-clinical and clinical pipeline that are being accelerated by the push for more effective COVID-19 vaccines. Some barriers remain, however, and overcoming these will allow the adoption of mucosal vaccines for widespread application to prevent a variety of respiratory infections.

Safety continues to be of primary concern, especially for the development of mucosal adjuvants. Individuals receiving the LT-adjuvanted influenza vaccine Nasalflu (Berna Biotech, Switzerland) in the 2000–2001 Switzerland influenza season were found to be at significant risk of transient facial nerve paralysis, leading to its withdrawal [91]. LTK63, a genetically detoxified version of the LT adjuvant used in Nasalflu, was also found to cause transient Bell's palsy, leading to the termination of HIV and tuberculosis vaccine trials (NCT00440544, NCT00369031) [92]. It has been previously suggested that more comprehensive investigation and standardization of safety testing in preclinical studies could allow more confident progression of IN vaccine candidates to clinical trials [93]. Most preclinical safety testing of vaccines historically focused on acute damage and systemic markers of tissue damage and inflammation; however, a shift towards studying respiratory-specific measurements could better select mucosal vaccines for clinical trials [78,93,94].

Secondly, there is a significant need for the development of more effective subunit IN vaccines, using adjuvants or excipients designed specifically for mucosal use. While viral vectors have shown promising results for IN vaccines, pre-existing immune responses may prevent peak performance [1]. In addition, the anti-viral immune responses induced by viral vectors are not optimal for protection against all respiratory pathogens [95,96]. Thus, there is demand to broaden the repertoire of available mucosal adjuvants. A difficulty in assessing mucosal vaccines and adjuvants, however, is a lack of established immunogenicity correlates. Unlike traditional vaccine delivery, mucosal delivery promotes humoral and cellular immune responses in respiratory tissues that are less amenable to sampling in people.

An advantage of IN delivery is that sampling of the nasal passages is significantly less invasive than that of the lower airways. In previous studies of mucosal vaccines, nasal and saliva swabs were used to measure mucosal IgA responses [59,97]. Despite this, some recent clinical studies of mucosal SARS-CoV-2 vaccines did not include this measurement of local immune responses [56,57]. Cellular immune responses in respiratory tissues, while an important measurement in preclinical vaccine studies, have historically been difficult to measure in the nasal passages owing to the low recovery of cells [98,99]. Furthermore, lower airway sampling, while possible and yielding exciting results in a recent aerosol TB vaccine clinical study, requires invasive bronchoscopy [100]. Establishment of standardized methods of measuring soluble factors in the respiratory tract such as antibodies and cytokines would likely increase accessibility of these assays to be readily available for clinical studies. In addition, an expectation that samples should be collected from mucosal tissues during clinical trials involving mucosal vaccine delivery would greatly improve the field. Recent studies have shown reliable methods of collecting immune cell samples from the nasal passages for flow cytometric analysis, alongside the collection of nasal washes for analysis of soluble factors, such as immunoglobulins and cytokines [98,101]. Roukens *et al.*

used nasal curettage and CyTOF analysis to identify 28 immune populations in the nasal passages of COVID-19 patients and were able to show the retention of antigen-specific nasal TRMs in convalescent individuals using this technique [101]. Another technique that could be more utilized and used previously in SARS-CoV-2 studies is the measurement of viral load using real-time polymerase chain reaction assays [102,103]. As such, the analysis of nasal immune responses combined with the measurement of viral load would provide more reliable correlates of vaccine efficacy than serum responses alone and these should be included in future clinical studies of mucosal vaccines.

The potential for inhaled SARS-CoV-2 vaccines to reduce viral transmission and generate broader immunity against VOCs has resulted in an unprecedented number of IN vaccines entering the clinical pipeline [55]. Mucosal vaccines also offer the prospect of enhanced vaccine accessibility because of their potential for self-administration and catering to needle-hesitant populations. This accessibility could be further broadened by the development of delivery devices validated for self-administration and vaccine formulations that do not require cold-chain transport. To make these possibilities a reality, continued government funding for mucosal vaccines for a range of respiratory pathogens, not only SARS-CoV-2, is required. In addition, increased focus on performing preclinical studies to understand the mechanism of action of adjuvants in the respiratory mucosa will provide the foundations for the rational design of more effective mucosal adjuvants.

CONCLUDING REMARKS

Respiratory pathogens have long been responsible for millions of deaths annually and will continue to be a serious global health burden until better vaccines can be developed. At this time in history, there is an unprecedented focus on mucosal immunization, and especially respiratory vaccine delivery, with three inhaled vaccines recently undergoing approval. IN vaccine delivery is deservedly a highly topical and exciting concept for its potential to enhance protection and reduce disease transmission, as well as the prospect of accessing vaccine-hesitant populations with needle-free delivery. As such, it is of primary importance for government agencies and vaccine developers to maintain the current focus and momentum on the research and translation of mucosal vaccines for SARS-CoV-2 and other respiratory pathogens.

BIOGRAPHIES

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DOI: 10.18609/vac.2023.033

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AUTHORSHIP & CONFLICT OF INTEREST

Contributions: All authors contributed to the planning and conceptualization of this review. Stewart EL wrote the first draft, Ashhurst AS and Britton WJ edited and provided critical feedback of the manuscript.

Acknowledgements: The figure was produced in BioRender.com licensed to Professor JA Triccas.

Disclosure and potential conflicts of interest: This work was supported by grants from the New South Wales Office for Health and Medical Research (WJB, ASA), and the National Health and Medical Research Council, APP1153493 (WJB) and APP2011467 (WJB) and University of Sydney. Ashhurst AS discloses she has grants/contracts from NSW Health, the Office for Health and Medical Research (2022–2024), University of Sydney Drug Discovery Initiative and Sydney Institute of Infectious Diseases (2020–2023). National Health and Medical Research Council of Australia (APP2002727, 2021–2023). Group for Research and Assessment of Psoriasis and Psoriatic Arthritis (2020). She also received funding for travel/meeting support from Australasian Society for Dermatology Research (2022), Australian peptide Association (2022), Australian and New Zealand Society for Immunology (2021), Molecular and Experimental Pathology Society (2020). Lastly, she is councillor at Molecular and Experimental Pathology Society of Australasia since 2018. Britton WJ discloses he has grants/contracts from NIH Advancing Vaccine Adjuvant Research for Tuberculosis Contract (BAA75N93022C00041, (2022–2027), NSW Dust Diseases Board. Defining new and effective therapies against thepathogenesis of accelerated silicosis (2023–2024) and NHMRC Ideas Grant (APP2011467) Defining the impact of chronic lung disease on COVID-19 (2022–2024).

Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: Invited; externally peer reviewed.

Submitted for peer review: Apr 20, 2023; **Revised manuscript received:** Jun 7, 2023; **Publication date:** Jul 12, 2023.

DOI: 10.18609/vac.2023.033

VACCINEINSIGHTS

ADVANCES IN FORMULATION & ADMINISTRATION

SPOTLIGHT

INTERVIEW

Inducing innate immune responses with particulate adjuvants

In this episode, **Charlotte Baker,** Editor, *Vaccine Insights,* speaks to **Ed Lavelle,** Professor, Trinity College Dublin, about our growing understanding of adjuvant mechanisms, mucosal vaccines, and why size matters for nanoparticle adjuvanticity.

Vaccine Insights 2023; 2(6), 191–196

DOI: 10.18609/vac.2023.030

Q How did you get involved in working with vaccine adjuvants?

EL: During my PhD, I was working on a project trying to make oral vaccines for fish. We collaborated with a lab in Nottingham that specialized in encapsulation of antigens in microparticles and hoped to facilitate controlled release with an antigen depot, and subsequently achieve sustained antibody responses.

This sparked my interest, and after my PhD, I joined the Nottingham lab to work on biodegradable particles as vaccine delivery systems. Over the years, I kept coming back to the question of how these particles enhance immune responses. Trying to understand exactly how the properties of the particle regulate immune responses has taken me deeper and deeper

into immunology, and the question has become more and more interesting as we've learned that we can modulate specific arms of the innate immune response using particulates.

Q What is the overarching goal of your research?

EL: The goal is to make better vaccines for infectious diseases and cancer, with a focus on cellular immune responses. We have developed a number of systems that are very effective in driving humoral immune responses but, historically, we have been less successful (at least with non-living vaccines) in enhancing antigen-specific cellular immunity, such as cytotoxic T cells and Th1-type responses.

We aim to engineer particulate systems in a way that is optimal for inducing specific types of immune responses. That clearly depends on exactly what the objective of the vaccine is—there is an appreciation now that different types of immune responses do different things in terms of effector responses, and different types of responses are required for different conditions.

Particulates are attractive because the technology is available to change certain parameters that will preferentially induce CD4 or CD8 T cells, or antibody responses. We want to resolve the relationship between the characteristics of the particulate and its ability to induce specific innate and adaptive immune responses.

Q What are the biggest gaps in our knowledge regarding adjuvants?

EL: Over the last couple of decades, we have obtained a very clear view of how certain adjuvants work, such as toll-like receptor ligands monophosphoryl lipid (MPL) or cytosine phosphoguanine (CpG) adjuvants. We know that they bind to specific receptors on or within antigen-presenting cells or other cells, which trigger specific signaling pathways.

In contrast, most vaccine adjuvants consist of particles, polymers, emulsions, or aluminum salts, which are not as well understood—we know that they are effective, but how do they drive specific immune responses?

That question has been partly addressed by the emerging field of systems vaccinology. We want to understand precisely how any adjuvant works and find the key factors that lead to, for example, a protective antibody response, a protective circulating T cell response, or a resident memory T cell response.

It is a difficult question to answer because adjuvants do many different things. When you inject an adjuvant-containing vaccine, there are huge changes in gene expression, metabolism, serum proteome, and so on. We are trying to pin down exactly which of those changes are responsible for what happens 6 months or a year later. Is it triggering the right type of response at the injection side? Is it triggering a specific type of response in the draining lymph node? Is it targeting a specific population of dendritic cells? Are the key sensors really the antigen-presenting cell or could it be other cells, like the injection-site muscle cells, endothelial cells, or target cells in the lymph node?

DOI: 10.18609/vac.2023.030

"We want to understand precisely how any adjuvant works and find the key factors that lead to, for example, a protective antibody response, a protective circulating T cell response, or a resident memory T cell response."

Modeling all of that complexity can be challenging, but systems vaccinology has taught us a huge amount about what happens in the first couple of days after vaccination. Some of that information has been surprising—we know now that even factors like the microbiome can impact vaccine efficacy.

Overall, we need to get a more rational picture of exactly how adjuvants imprint these early effects and how they associate with the desired effects, like neutralizing antibodies or sustaining resident T cells and mucosal cells.

Q How is your research helping to close some of those gaps in knowledge?

EL: We have focused a lot on mechanisms over the last 10–15 years. We are working on a couple of systems (particularly biodegradable particles and chitin-derived polymers) and trying to determine the factors associated with enhanced adaptive immune responses. From there, we are identifying the right particle size, particle charge, or polymer charge that provides the best B or T cell response, and why.

We have made some progress in understanding what type of dendritic cells are involved and what type of innate signaling pathways are being activated. That knowledge is beneficial because if you know what the mechanism is, you can set up *in vitro* systems that would associate with that potency, allowing the vaccine industry to rapidly screen a wide range of different particles or polymers to find those that drive optimal immune responses.

Q What applications are you working on right now?

EL: We have a few projects on mucosal vaccines, which have garnered a lot of interest since COVID. We have come to the realization that long-term protection against SARS-CoV-2 infection is probably best achieved through mucosal vaccination or a combination of vaccine injection and mucosal boosting but there are no adjuvants in any approved mucosal vaccine at the moment, so there is a long way to go.

Secondly, we are interested in adjuvants for cancer vaccines. We have been developing systems that are good at driving Th1 responses and cytotoxic T cells, and we believe these might be applicable to cancer. We are interested in pursuing that research further, especially on the cytotoxic T cell side.

In the next couple of years, we hope to move toward clinical application in cancer vaccines, especially with chitin-derived polymers.

Q What was the goal of your recent study exploring the adjuvanticity of nanoparticles [1]?

EL: This research took us the best part of 10 years from start to finish. The big question we asked was: does particle size matter in terms of adaptive immune responses? That has been a challenging question to answer over the years. If you look at the literature, you will find papers answering yes and no.

We set out to find a definitive answer to that question, exploring whether or not particle size matters for specific aspects of the immune response. We found that the importance of particle size depends on which aspect of the immune response you're measuring. For antibody responses, although there are slight degrees of variation, a broad range of particle sizes can generate responses. This explains why previous studies focusing on antibody response have given mixed results.

In contrast, for cytotoxic T cell responses, the answer is black and white. There is a very specific particle size that generates T cell responses: 50–60 nm, around the size of a virus. Why is size so important for cytotoxic T cells? By looking at multiple pathways over a long time, we eventually found that particles of that size drive immunogenic cell death via the pyroptotic pathway, demonstrated by the fact that Caspase-11 knockouts lost the cytotoxic T cell response.

Ultimately, whether particle size is important depends on what you are trying to achieve. If the objective is sustained antibody responses, you don't necessarily need a very small particle size. However, if you are targeting cytotoxic T cells, for example in cancer vaccines, we argue that particle size is pivotal.

Q How are you following up on the paper?

EL: A lot of the paper was based on polystyrene nanoparticles, and several people have asked if we duplicated the effects with biodegradable particles. While we duplicated some parts of the paper with biodegradable poly(lactic-co-glycolic acid) particles showing that the parameters were comparable, we are now working on a more comprehensive comparison.

The other question we are often asked is how you translate this into a formulation that could be used in humans. We are grappling with this now because clearly, there are issues in terms of formulation, scale-up, and stability long-term.

Q What's next for your work?

EL: My main focus will be translating our work with particles and chitin-derived polymers towards the clinic. Also, there is still a lot more to learn about these mechanisms; for example, which dendritic cell subsets are pivotal and whether we can further enhance

DOI: 10.18609/vac.2023.030

targeting of the draining lymph nodes. Our recent work highlights the value of a mechanistic approach that can inform vaccine design.

Plus, most of the work to date has been done by injection so we want to find out if we can achieve a successful mucosal vaccination with some of these systems, either as a standalone mucosal vaccine or in prime-boost strategies.

BIOGRAPHY

ED LAVELLE is the current Professor of Vaccine Immunology in Trinity College Dublin. He was elected a member of the Royal Irish Academy (MRIA) in 2021 is currently President of ECI2024 and former President of the Irish Society for Immunology and head of the School of Biochemistry and Immunology at Trinity College Dublin, Ireland. He graduated with a BSc in Microbiology from University College Galway and a PhD in Immunology from the University of Plymouth and carried out postdoctoral research at the University of Nottingham, Rowett Research Institute, Maynooth University and Trinity College Dublin on vaccine adjuvants and immunomodulation. He was appointed at Trinity College Dublin as a lecturer in 2004, associate Professor in 2012, Professor in Immunology in 2015 and Professor of Vaccine Immunology in 2022. His research has led to the development of adjuvants suitable for inclusion in injectable and mucosal vaccines for infectious diseases and resolving their mode of action. The lab is also focused on developing therapeutic vaccines for cancer and investigating vaccine strategies that promote immunogenic cell death, leading to enhanced protective immunity.

AFFILIATION

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REFERENCE

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Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given his approval for this version to be published.

Acknowledgements: None.

Disclosure and potential conflicts of interest: Lavelle E has the following patents: WO 2022/123430 A1 (Polymeric nanoparticles as vaccine adjuvants) and WO 2022/113022 A1 (Immunotherapy for cancer). He also discloses he is founder of AilseVax (start up company on immune-oncology vaccines).

Funding declaration: The author received no financial support for the research, authorship and/or publication of this article.

ARTICLE & COPYRIGHT INFORMATION

Article source: This article is based on a podcast with Ed Lavelle which can be found here.

Interview held: May 3, 2023**; Revised manuscript received:** May 26, 2023; **Publication date:** Jul 26, 2023.

DOI: 10.18609/vac.2023.030