Mucosal vaccines — fortifying the frontiers

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Abstract | Mucosal vaccines offer the potential to trigger robust protective immune responses at the predominant sites of pathogen infection. In principle, the induction of adaptive immunity at mucosal sites, involving secretory antibody responses and tissue-resident T cells, has the capacity to prevent an infection from becoming established in the first place, rather than only curtailing infection and protecting against the development of disease symptoms. Although numerous effective mucosal vaccines are in use, the major advances seen with injectable vaccines (including adjuvanted subunit antigens, RNA and DNA vaccines) have not yet been translated into licensed mucosal vaccines, which currently comprise solely live attenuated and inactivated whole-cell preparations. The identification of safe and effective mucosal adjuvants allied to innovative antigen discovery and delivery strategies is key to advancing mucosal vaccines. Significant progress has been made in resolving the mechanisms that regulate innate and adaptive mucosal immunity and in understanding the crosstalk between mucosal sites, and this provides valuable pointers to inform mucosal adjuvant design. In particular, increased knowledge on mucosal antigen-presenting cells, innate lymphoid cell populations and resident memory cells at mucosal sites highlights attractive targets for vaccine design. Exploiting these insights will allow new vaccine technologies to be leveraged to facilitate rational mucosal vaccine design for pathogens including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and for cancer.

The global burden of mortality and morbidity associated with infectious diseases caused by mucosal pathogens remains unacceptably high. Indeed, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic provides a brutal reminder of the continual threat of novel mucosal infectious challenges, in addition to the threat posed by many widespread mucosal infections for which no or only suboptimal vaccines exist. Now more than ever, there is a clear focus on vaccine requirements for respiratory pathogens but, importantly, new and improved vaccines are also urgently needed for numerous enteric pathogens and other agents such as those causing sexually transmitted diseases (STDs) and oncogenic viruses that gain access through the mucosae.

Respiratory pathogens remain a prominent cause of global mortality, with lower respiratory tract infections constituting the fourth leading cause of death worldwide[1]. Lower respiratory tract infections are responsible for approximately 2.4 million deaths per annum, with *Streptococcus pneumoniae*, respiratory syncytial virus (RSV), *Haemophilus influenzae* B and influenza virus taking a particularly high toll on the young (<5 years old) and older people[2]. There is currently no approved vaccine for RSV infection, which is particularly prevalent in children and infants[2–4], and although there are licensed vaccines targeting respiratory pathogens such as *Mycobacterium tuberculosis*, *S. pneumoniae*, *Bordetella pertussis* and influenza virus, improved vaccines for these pathogens are required to enhance suboptimal protection, particularly at the site of infection, and to increase coverage (FIG. 1). There are indications that innovative mucosal vaccine approaches offer promise for these infections. For example, live attenuated influenza vaccines given intranasally are now an integral part of influenza vaccination strategies with particular application to children[5,6], intranasally administered *B. pertussis* vaccines have entered phase II trials[7,8] (Supplementary Table 1) following successful phase I completion, and preclinical data investigating the intranasal delivery of Bacillus Calmette–Guérin for *M. tuberculosis* have yielded promising results[9]. The emergence of SARS-CoV-2 has firmly demonstrated how deadly and disruptive respiratory pathogens can be, with approximately 2.6 million deaths attributed to this pathogen at the time of writing[10] and estimates that the SARS-CoV-2 pandemic will continue to stunt global economic growth in 2021, particularly in low-income countries[11]. Although an array of effective SARS-CoV-2 vaccines have been designed and implemented, challenges in mass production and deployment

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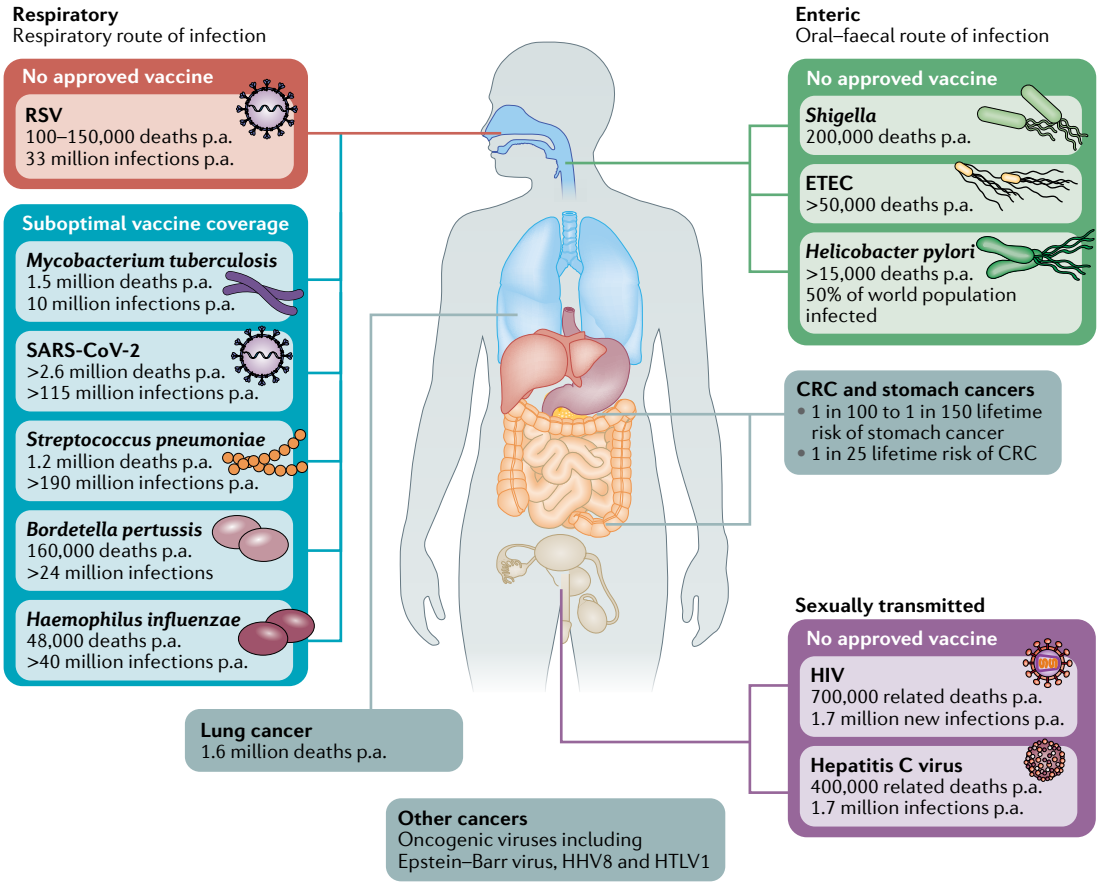

Fig. 1 | **Burden of mucosal diseases with unmet vaccine needs.** Respiratory, enteric and sexually transmitted infections constitute prominent causes of death worldwide, and this is exacerbated in low-income regions. Aetiological agents shown are vaccine targetable, but there remains an unmet need for new or improved vaccination approaches to address global vaccine coverage. Mucosal vaccination strategies hold promise to address this unmet need, providing more robust mucosal immunity and an alternative to parenteral vaccination. In addition to their centrality in the pathogenesis of infectious disease, mucosal tissues are frequent sites of tumour development and mucosal vaccination strategies may play a role in the prophylactic and therapeutic targeting of these malignancies. CRC, colorectal cancer; ETEC, enterotoxigenic *Escherichia coli*; p.a., per annum; RSV, respiratory syncytial disease; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

still provide an unmet need for global coverage (FIG. 1). New vaccines could help to circumvent these issues. In particular, orally delivered SARS-CoV-2 vaccines would be suited to global vaccination attempts, especially in lower-income countries, as these vaccines will not only allow for enhanced convenience and compliance but also the intestine may represent a viral target organ[12]. Indeed, the development of 'universal' mucosal vaccines targeting conserved antigens on coronaviruses[13] and influenza viruses, although challenging, may be a viable option for prevention of future pandemics.

Enteric pathogens causing diarrhoeal disease are the eighth leading cause of death worldwide, with children, in particular, at risk[14]. Among these, *Shigella* and enterotoxigenic *Escherichia coli* (ETEC) have an urgent vaccine requirement (FIG. 1). Enteric pathogens and associated acute and chronic infections have a stark impact on the livelihoods of at-risk individuals in lower-income countries. Aside from diarrhoeal disease, the impact of such infections on physical and cognitive development is becoming more apparent[14], not only highlighting the need for vaccine development but also impacting how we determine vaccine efficacy. Lack of moderate-to-severe symptoms may not be an adequate correlate of protection — prevention of colonization and/or low-grade infection may be the crucial determinant. The World Health Organization (WHO) has endeavoured to end cholera by 2030 through implementation of widespread preventive measures, including vaccination[15], providing a challenge to oral cholera vaccine manufacturers globally. This may be addressed through successful development of lower-cost alternative oral cholera vaccines such as Hillchol, which is currently under clinical evaluation[16].

Mucosal vaccines targeting the genital tract have the potential to combat STDs and local tumours, which is important given that cervical cancer represents the fourth most common cancer in women[17]. Clearly, there is an enormous need for an effective HIV vaccine and given the intestinal tropism of the virus[18], mucosal vaccine strategies are warranted[19]. Additionally, the emergence of multidrug-resistant STDs is of concern and could be combatted through preventive mucosal vaccine strategies.

Whereas most licensed vaccines are currently administered by injection, mucosal vaccines can outperform parenteral vaccination strategies in eliciting protective mucosal immune responses that block infection or transmission[20,21]. The nature of the infection must be considered from the outset in designing mucosal vaccines or, indeed, in assessing whether targeting the mucosal route is necessary. For example, in the case of enteric pathogens, the infections may be invasive (as is seen in typhoid and polio), partially/locally invasive (as seen in shigellosis) or strictly mucosal (as seen for cholera and ETEC infections)[22,23]. This will impact on whether a systemic immune response is an appropriate objective, or whether predominantly mucosal or both mucosal and systemic immunity would be more effective. In this context, the nature of the mucosal surface (for example, the uninflamed small intestine versus the lower respiratory tract) will influence the accessibility of circulating antibodies, the nature of the dominant antibody isotype and the transport mechanism governing access of antibodies to mucosal infectious pathogens[24,25].

Strong mucosal cellular and humoral immune responses have the potential to induce sterilizing immunity by impeding pathogen binding to and uptake across epithelial surfaces. However, there are significant hurdles to mucosal vaccine development, including incomplete knowledge of the nature of protective mucosal immune responses. Advancing new mucosal vaccines and improving existing vaccines requires innovative adjuvant approaches and delivery strategies, which is the focus of this Review. Given the specific architecture of the mucosal surfaces and their unique cellular composition, vaccine strategies should be specifically tailored for the target site rather than redeploying effective injectable vaccines. In any case, many adjuvants that are effective by injection are not optimal for mucosal delivery.

Lessons from licensed mucosal vaccines

Over recent decades, there has been a broad shift from injectable whole-cell killed and attenuated vaccines towards adjuvanted subunit and, more recently, viral vectored, RNA and DNA vaccines[26,27]. This can reduce the potential for excessive reactogenicity and is facilitated by advances in antigen discovery, adjuvants and delivery systems. However, the landscape for mucosal vaccines is very different. Of the nine mucosal vaccines approved for use in humans — eight oral and one intranasal — all are either live attenuated or whole-cell inactivated vaccine formulations (FIG. 2). This current dichotomy in approaches is, in part, due to greater tolerability of orally administered whole-cell killed antigens, the susceptibility of unprotected subunit antigens to degradation and clearance, and, crucially, a lack of proven mucosal adjuvants.

Currently, the only subunit antigen included in a licensed mucosal vaccine is cholera toxin B subunit (CTB), included as an additional component of the killed whole-cell *Vibrio cholerae* vaccine Dukoral. CTB cannot be regarded as a 'model' subunit antigen as this is the binding component of cholera toxin — it binds with high affinity to GM1 on epithelial cells and is highly immunogenic[28–30] (BOX 1). Nevertheless, this does indicate that potent immune responses can be induced against an orally administered purified protein even if this is in the presence of whole bacteria. The tolerability of oral whole-cell antigens is instructive as, although adjuvanted subunits are preferable for parenteral formulations, leveraging potent mucosal adjuvants with whole-cell antigens may be a more productive approach for mucosal vaccination, especially via oral routes.

Developing whole-cell antigens as a platform offers potential for combination with purified subunits but also, perhaps more encouragingly, for overexpression of antigens on whole cells — ETVAX provides a stellar example of this[31]. Developed at the University of Gothenburg in collaboration with Scandinavian Biopharma, ETVAX comprises four *E. coli* strains engineered to overexpress colonization factor antigens on the bacterial surface, namely CFA/I, CS3, CS5 and CS6, in combination with LCTBA[31,32] (a CTB and *E. coli* heat-labile enterotoxin B subunit (LTB) hybrid; see BOX 1). Overexpression of antigens on inactivated whole bacteria is thus a promising approach to increase immunogenicity, leveraging the adjuvanticity of inactivated bacteria while helping to minimize the vaccine dose required. This approach may also be applied to inactivated viruses or virus-like particles, taking advantage of their inherent immunogenicity, particulate antigen presentation and well-established expression systems[33,34].

Aside from Dukoral, Euvichol and Shanchol, the other licensed mucosal vaccines are all live attenuated bacteria (*Salmonella enterica* subsp. *enterica* serovar Typhimurium) or attenuated and/or reassortant viruses delivered orally (polio vaccine, rotavirus) or nasally (influenza A and influenza B viruses). Overall, this highlights the tolerability and effectiveness of mucosal attenuated and whole-cell vaccines but also points to the key question of why the successful shift to more modern vaccine strategies has not yet occurred for mucosal vaccines.

Vaccine lessons from mucosal tissues

Unique aspects of mucosal immune responses. There are many distinctive features of mucosal immune responses that impact on mucosal vaccine design, ranging from the structure and location of immune inductive sites to the type of effector and memory cells induced and their longevity and location. The mucosal immune system can be broadly categorized into inductive sites where antigen-specific B cell and T cell responses are initiated and effector sites (such as the lamina propria and epithelium) where the adaptive immune responses are mediated. The nature of the inductive sites varies between species and also between different mucosae. In the case of the intestine, the inductive sites are the gut-associated lymphoid tissue and the intestine-draining mesenteric lymph nodes. Gut-associated lymphoid tissue in humans and mice comprises Peyer's patches and isolated lymphoid follicles[35]. The connection between inductive and effector sites is facilitated by selective expression of integrins and chemokine receptors on B cells[36] and T cells[37]. For example, in the case of the small intestine, imprinting of α4β7 integrin and CC-chemokine receptor 9 (CCR9) expression on lymphocytes is key for tissue-specific homing of

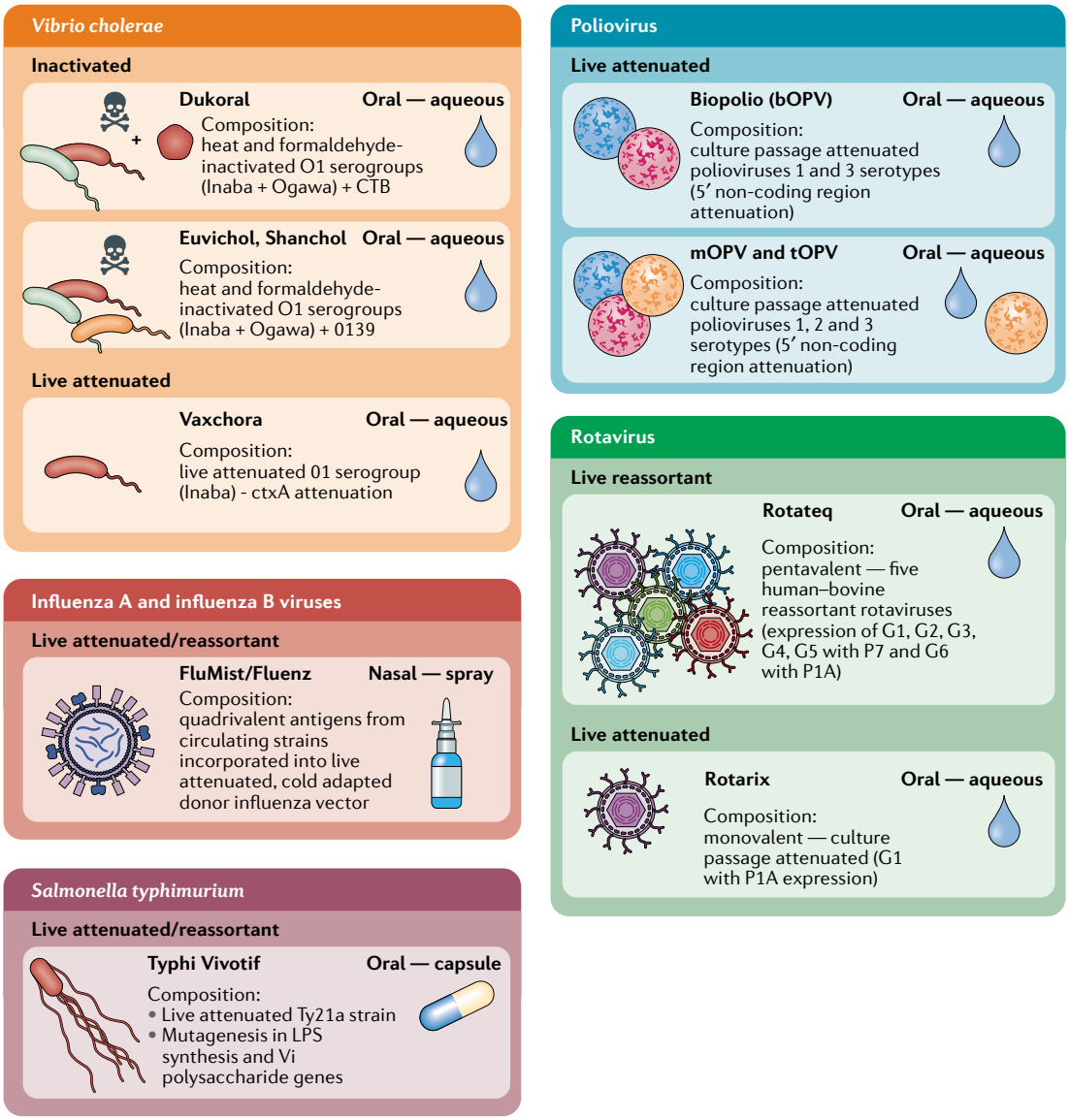

Fig. 2 | **Licensed mucosal vaccines.** Eight oral vaccines are currently licensed for use against cholera, salmonella, poliovirus and rotavirus. Live attenuated influenza vaccines remain the sole licenced intranasal vaccines. To date, live attenuated and inactivated vaccines have proved the most successful platforms for mucosal vaccine design. CTB, cholera toxin B subunit; LPS, lipopolysaccharide.

cells. Although mucosal immune responses are compartmentalized, there is crosstalk between different mucosae, and it is thus possible to vaccinate at a single mucosal site but also promote immune responses at distant mucosal sites[38]. Understanding the nature of the signals regulating such homing in a human context is critical to allow design of novel mucosal vaccines that can potentially target mucosae distant from the site of vaccination.

Mucosal sites cover a surface area of 30–40 m^2 in humans[39] predominantly in the gastrointestinal tract, respiratory tract, urogenital tract and ocular cavities, playing a crucial role in homeostasis and interactions with the microbiome, dietary antigens and other environmental material. As a result, they constitute prominent points of pathogen entry and are often sites of tumour development. This high antigenic load and constant exposure to microbes necessitates mucosal immunoregulatory mechanisms that are vital to maintain homeostasis and prevent damaging chronic inflammatory responses[40]. This has significant implications with regard to vaccine development, for example, many Toll-like receptor (TLR) agonists that are effective adjuvants when injected parenterally have poor efficacy when administered orally. This results, at least in part, from tolerization of intestinal antigen-presenting cells (APCs) to pathogen-associated molecular patterns, particularly TLR ligands to which they are continuously exposed via the microbiome[41], and also as a result of the basolateral rather than luminal expression of TLRs at epithelial surfaces. Detailed knowledge on mucosal responsiveness to pathogen-associated molecular patterns, responsive target cells and their location is critical so that productive target pathways can be identified for adjuvant discovery and optimization.

A recent report demonstrated that proximal intestinal gut-draining lymph nodes preferentially supported regulatory T cell responses whereas distal gut-draining lymph nodes supported the induction of effector T helper cells[42]. These insights into the balance of regulatory and effector responses can inform vaccine design — if antigen uptake in proximal regions of the small intestine preferentially enhances tolerogenic responses, delivery of oral vaccines in solution may not be optimal and targeting of antigens to the distal small intestine or large intestine may be more effective. This preferential induction of regulatory T cells in the proximal intestine could also be affected by the presence of adjuvants or the nature of the orally administered antigen. Vaccines could thus potentially over-ride the tolerogenic environment in the proximal intestine by inducing an inflammatory signature to allow the induction of effector T cell responses, although overall there may be an advantage in targeting the distal intestine.

One such large intestine-targeted oral delivery system was described where vaccine nanoparticles were delivered within pH-dependent microparticles. This oral construct induced protective antiviral immunity in the rectum and vagina comparable with levels seen with colorectal vaccination and protected against rectal and vaginal viral challenge[43], providing a potentially productive route for mucosal vaccination for STDs. Although compartmentalization of mucosal immune responses is well established, confirming that the same subdivisions and connections apply from rodents to humans can be a challenge. However, it was shown that oral Dukoral vaccination increased the numbers of circulating IgA^+ memory B cells with a surface marker expression profile indicative of homing to the large intestine and respiratory tract[38]. Furthermore, translation of vaccine delivery strategies from small animal models to humans can be challenging owing to differences in parameters including gastrointestinal pH, gastrointestinal tract residence times and intestinal surface area. Some of these factors have been addressed in the case of oral drug delivery, but it is quite clear that in the absence of immunogenic antigens and effective adjuvants, addressing delivery challenges in isolation offers modest benefits and the vaccine components must be optimized for the targeted mucosal pathogen and its site of infection. The nature of the antigen is also a major determinant of responses, whether living or non-living, soluble or particulate. This can dictate the nature of antigen uptake pathways at mucosal sites and should be a principal design feature in the development of mucosal vaccines (FIG. 3). Particulate antigens — whether as whole bacterial cells, attenuated or inactivated viruses, virus-like particles or synthetic particulate formulations — are more immunogenic than purified proteins[33] and, in addition to their greater inherent immunogenicity, when delivered mucosally their particulate nature can impact on the site of uptake and APC targeting.

Antigen-presenting cells in mucosal tissues. APC populations at mucosal sites are highly dynamic. In addition to tissue-resident dendritic cells and macrophages, during inflammatory responses or infection, further APCs are recruited that can engage with absorbed antigen and contribute to effector responses[44]. Indeed, there is evidence that during inflammation, monocytes in the gut and lungs can upregulate CCR7, migrate to lymph nodes and prime T cell responses[45], and recruited monocytes or immature macrophages can produce inflammatory cytokines that contribute to T helper 1 (T_H1) cell and T_H17 cell responses[44]. Local inflammation triggered by mucosal vaccines and/or adjuvants could thus contribute to enhanced adaptive immunity by recruiting APCs. Although tissue-resident macrophages do not migrate to lymph nodes and are thus unlikely to directly prime T cells, antigen sampling CX_3C-chemokine receptor 1 (CX_3CR1^+) mononuclear phagocytes can transfer antigen to dendritic cells[46] (FIG. 3) and colonic CX_3CR1^+ mononuclear phagocytes were shown to be required for induction of T_H17 cell and antibody responses to intestinal fungi[47].

Two key dendritic cell populations in gut-draining lymph nodes — $CD103^+CD11b^-$ dendritic cells and $CD103^+CD11b^+$ dendritic cells — have been associated with tolerogenic or pro-inflammatory immune responses, respectively[48]. Assigning specific roles to dendritic cell and macrophage populations in the intestine can be challenging as this is context-dependent. A recent study in a model of *S.* Typhimurium infection found that intestinal CX_3CR1^+ macrophages were superior to conventional type 1 dendritic cell (cDC1) and cDC2 populations in promoting the production of *S.* Typhimurium-specific IgA[49]. Furthermore, these broad categories of dendritic cell populations may not capture the true complexity of responses in the intestine, and subsets of these populations can contribute to the recruitment and activation of T cells and B cells at the site of infection. Differential gene expression profiles in cDC1 and cDC2 populations from different gut regions were reported[42], indicating that

Box 1 | CTB — mucosal subunit antigen or adjuvant?

The pentameric cholera toxin B subunit (CTB) has been successfully and safely incorporated in recombinant form into the oral cholera vaccine Dukoral since 1991. It represents the only subunit antigen incorporated in a licensed mucosal vaccine to date. CTB binds to the membrane ganglioside GM1 (REF.[143]) and fucosylated glycans[144,145] on cells including enterocytes, microfold (M) cells, macrophages and dendritic cells. As a result, CTB has the potential to promote effective delivery of bound antigens to mucosal antigen-presenting cells (APCs)[146]. Although CTB has in the past been classed as an adjuvant, this definition was complicated by the presence of residual cholera toxin or lipopolysaccharide (LPS) in CTB preparations. Indeed, CTB is now well established as an effective mediator of tolerance to attached or admixed antigens by oral and intranasal routes[147–149]. It was also proposed that orally delivered CTB can promote intestinal repair and healing responses[150]. In human studies, CTB has been shown to also promote induction of antigen-specific local IgA and systemic IgG responses when administered via rectal and intranasal routes[151,152]. Its inclusion in Dukoral is primarily to induce intestinal and circulating cholera toxin-specific antibodies, which can contribute to short-term protection against cholera[153] and cross-protection against enterotoxigenic *Escherichia coli* (ETEC) via shared epitopes in *E. coli* heat-labile enterotoxin B subunit (LTB)[154,155]. Similarly, LCTBA (a CTB and LTB hybrid) is included in the candidate ETEC vaccine ETVAX, expanding the number of antigenic targets and cross-protection[31,32]. Recently, CTB has been shown to promote activation and expansion of polyfunctional T helper 1 (T_H1) cells and T_H17 cells when given intradermally alongside a $DEC205^+$ dendritic cell-targeted antigen; notably, this included induction of local and intestinal protective tissue-resident memory T (T_{RM}) cells[29]. This highlights the potential for incorporation of CTB in parenteral–mucosal push–pull vaccination strategies.

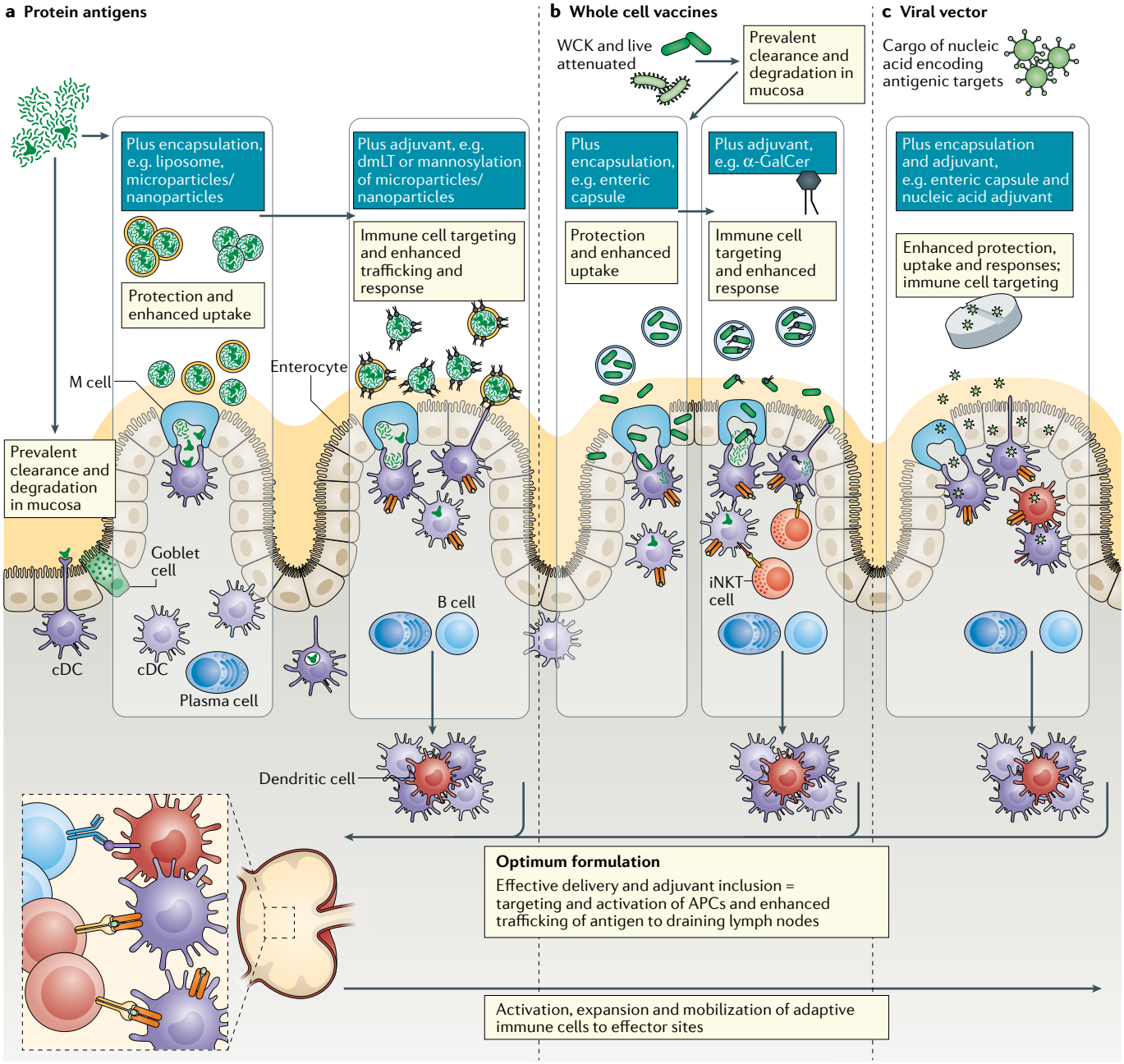

Fig. 3 | **Vaccine uptake at mucosal sites.** Nature of antigen uptake in the intestine is dependent on the type of vaccine components used, whether soluble or particulate, inert or live. Innovative encapsulation and targeting strategies have the potential to protect antigens while enhancing uptake and delivery to optimal intestinal regions. Inclusion of an effective mucosal vaccine adjuvant can confer multiple benefits from preventing tolerogenic responses to antigens, recruiting and activating antigen-presenting cells (APCs) and engaging other innate immune cells that contribute to protective immunity. Although there are currently few safe and effective adjuvants, cell-targeting adjuvants such as *Escherichia coli* double-mutant heat-labile toxin (dmLT), which binds to gangliosides on microfold (M) cells and dendritic cells, or α-galactosylceramide (α-GalCer), which can promote activation of invariant natural killer T (iNKT) cells locally and in draining lymph nodes via dendritic cell-mediated presentation, offer promise. Optimal formulations will address antigen design, adjuvanticity and antigen protection and targeting to address the unique challenges of intestinal delivery in the case of protein antigens (part **a**), whole cell vaccines (part **b**) and viral vector vaccines (part **c**). cDC, conventional dendritic cell; WCK, whole-cell killed.

not only the type of gut dendritic cell but also its precise tissue location may be key for the outcome of oral vaccination. The latter study also found that, compared with proximal gut-draining lymph nodes, distal gut-draining lymph nodes are more efficient in promoting the differentiation of T_H17 cells. Given the importance of T_H17 cells for defence against extracellular pathogens and for support of intestinal IgA secretion, this finding may be instructive for delivery of oral vaccines. It is of note that cDC1 and cDC2 frequencies remain relatively stable

Chitosan
A cationic polymer derived from chitin with mucosal adjuvant properties. In addition to its mucoadhesive attributes, chitosan promotes adaptive immunity through activation of the cGAS–STING and NLRP3 inflammasome pathways.

Enchained growth
A process where high-affinity IgA specific for surface antigens cross-links bacteria, preventing daughter cell separation after division and contributing to clearance of mucosal pathogens.

Microfold (M) cells
Specialized antigen sampling epithelial cells generally found in the follicle-associated epithelium overlying organized mucosal lymphoid tissue.

throughout life[50] (TABLE 1), an important consideration for cDC-targeted vaccine strategies. In the nasal mucosa, resident cDCs are vital for maintaining non-responsiveness to harmless inhaled antigens but viral infection promotes recruitment of monocyte-derived dendritic cells (moDCs) that can mediate T cell priming[51]. This study utilized a nasally administered chitosan hydrogel vaccine platform to trigger the inflammatory recruitment of moDCs coupled to the sustained release of antigen, which successfully promoted antigen-specific $CD8^+$ T cell activation and expansion. This suggests that in addition to potentially targeting specific populations of mucosal dendritic cells, vaccines can also aim to promote recruitment of monocytes and moDCs to mediate protective mucosal immunity. Indeed, with subunit antigens, adjuvants may be critical to overcome tolerance induction and may also contribute to recruitment of 'unconditioned' APCs that prime antigen-specific T cells and B cells. Assessing the capacity of mucosal adjuvants to alter the activation states of tissue-associated or lymph node dendritic cells and to recruit monocytes and other APCs may be a useful indicator of efficacy, certainly more so than in vitro screening of cultured dendritic cells or macrophages that may poorly reflect responses of mucosal APCs following vaccination.

Induction of IgA and other mucosal antibodies. In terms of correlates of immunity following mucosal vaccination, induction of antigen-specific IgA is a crucial consideration. IgA is the dominant antibody at many mucosal sites and mediates protection against a range of enteropathogens. Recently, the importance of dimeric IgA in neutralization of SARS-CoV-2 has been highlighted in patients infected with the virus[52] and high-avidity IgA can protect against enteropathogens by processes including agglutination and the recently described process of 'enchained growth'[53]. For oral vaccination with adjuvanted subunit antigens, multiple doses (at least three) are needed to induce effective secretory IgA responses[21]. Induction of antigen-specific IgA was observed following two doses of ETVAX, with the addition of the *E. coli* double-mutant heat-labile toxin (dmLT) adjuvant enhancing the kinetics of induction[31]. This suggests that suitable adjuvants may exert a dose-sparing effect in mucosal IgA induction. Vaccines utilizing viral vectors and attenuated viruses may induce IgA responses more efficiently — for example, one dose of intranasal live attenuated influenza vaccine can elicit mucosal IgA[54] in recipients, and faecal/salivary IgA was observed in recipients of the Vaxart norovirus vaccine candidate, comprising an adjuvanted enterically stable adenovirus type 5 (Ad5) vector[55]. Although detailed assessment of antigen-specific mucosal immunity is more challenging in humans than in preclinical models, recent advances are facilitating novel means of determining vaccine-induced mucosal IgA responses in clinical trials (BOX 2).

Although the importance of mucosal dendritic cell-mediated antigen sampling and trafficking to draining lymph nodes for induction of IgA responses has long been appreciated, Komban et al. recently uncovered a new layer of antigenic crosstalk between microfold (M) cells and B cells in the subepithelial dome region of Peyer's patches. $CC26^+CCR1^+GL7^-$ B cells were shown to be capable of sampling antigen directly from M cells and trafficking to germinal centres where their activation and population expansion occurs, challenging the idea of dependence on cDC-mediated antigen transfer for optimal antigen-specific IgA induction[56]. However, whereas transport of secretory antibody (IgA, IgM) via the polymeric immunoglobulin receptor is an essential and highly efficient process in the intestine, this is not the case at all mucosal sites. In the female reproductive tract, IgG rather than IgA can be critical for protective immunity to viral infection[57]. Likewise, IgG plays an

Table 1 | **Frequency of human innate and innate-like immune cell populations at different mucosal sites**

Immune cell population	Frequency at mucosal tissue site					Refs
	Upper respiratory tract/oral gingivae	Lungs/lower respiratory tract	Duodenum/jejunum	Ileum	Colon	
ILC[a]	3.5% of total $CD45^+$ cells (80% ILC3, 20% ILC1, <1% ILC2)	0.19% of total $CD45^+$ cells (60% ILC3, 30% ILC2, 10% ILC1)	1% of total $CD45^+$ cells (ILC1 dominant)	0.8% of total $CD45^+$ cells (ILC3 dominant)	1.1% of total $CD45^+$ cells (ILC3 dominant)	128–131
cDC1	NA	0.05% of total $CD45^+$ cells	0.025% of total $CD45^+$ cells	0.021% of total $CD45^+$ cells	0.0075% of total $CD45^+$ cells	50
cDC2	NA	0.25% of total $CD45^+$ cells	0.1% of total $CD45^+$ cells	0.07% of total $CD45^+$ cells	0.06% of total $CD45^+$ cells	50
pDC	NA	0.03% of total $CD45^+$ cells	0.0015% of total $CD45^+$ cells	0.004% of total $CD45^+$ cells	0.015% of total $CD45^+$ cells	50
Natural killer cell	NA	10–15% of total $CD45^+$ cells	1–2% of total $CD45^+$ cells	0.9% of total $CD45^+$ cells	1% of total $CD45^+$ cells	128–131
γδ T cell	NA	ND in humans[b]	13% of total IELs	12% of $CD3^+$ IELs and 35% of $CD3^+$ LPLs	30–40% of $CD3^+$ IELs and 5% of $CD3^+$ LPLs	132–137
NKT cell	NA	ND in humans[b]	0–2% of IELs and LPLs	0–2% of IELs and LPLs	0–2% of IELs and LPLs	138
MAIT cell	NA	2–4% of $CD3^+$ cells	2–11% of $CD3^+$ cells	2–11% of $CD3^+$ cells	0.3–5% of IELs and 1–3% of LPLs	139–142

cDC1, conventional type 1 dendritic cell; IEL, intraepithelial lymphocytes; ILC, innate lymphoid cell; LPL, lamina propria lymphocyte; MAIT, mucosal-associated invariant T cell; NKT cell, natural killer T cell; NA, not available; ND, not defined; pDC, plasmacytoid dendritic cell. [a]Excluding classical natural killer cells. [b]γδ T cells have not been defined in human lung tissue but have been measured in bronchoalveolar lavage fluid.

Box 2 | **Methodologies and challenges in studying human mucosal immune responses following vaccination**

Although mucosal immune responses can be characterized preclinically in great detail in tissues and secretions, this is challenging in a clinical context. Therefore, establishing robust correlates of vaccine-induced adaptive immunity is a priority[156–158]. Assessing antibody responses in mucosal secretions has been a predominant approach. Indeed, vaccine-induced IgA responses in saliva[159], nasal wash[160] and faecal samples[161] are frequently determined. Salivary IgA sampled from the submandible/sublingual region has also been shown to correlate well with intestinal IgA responses in an enterotoxigenic *Escherichia coli* (ETEC) challenge study[162]. The potential to determine mucosal cellular immune responses is restricted by access to tissues, and as sampling from mucosal sites such as the lungs and intestines is invasive and unpleasant for trial volunteers, blood collection is predominantly relied upon to identify vaccine-induced migrating effector cells in peripheral blood mononuclear cells. Circulating mucosal effector cell populations can be characterized using lineage and effector markers, alongside mucosal homing marker expression by flow cytometry. ELISPOT assays can be used to measure antibody-secreting cells[163] in isolated peripheral blood mononuclear cells; alternatively, supernatants from cultured peripheral blood mononuclear cells can be harvested for evaluation of antibodies in lymphocyte secretion (antibody in lymphocyte supernatant (ALS))[164] by ELISA or multiplex assays. As the circulation of mucosa-derived lymphocytes in the blood is a dynamic and transient process, optimization of kinetics is critical. Assessing responses 5 days following oral booster vaccination has been suggested as optimal for detecting antibody-secreting cells/ALS responses[161], which have been shown to correlate with mucosal immune responses in the case of challenge or vaccination trials with ETEC and cholera vaccines[165–168]. Logistically, ALS methodologies are advantageous as supernatants can be frozen for later analysis. Mottram et al.[169] recently identified B cell maturation antigen (BCMA) as a biomarker for the induction of vaccine-specific IgA and memory B cell responses to multiple antigens when measured via ELISA in ALS samples following oral vaccination; this simplified assay may prove especially useful when only low blood volumes are available, for example, in paediatric samples.

important role in protective immunity in the lower respiratory tract whereas IgA is relatively more important in the nasal compartment[58].

Tissue-resident memory T cells in mucosal tissues. Tissue-resident memory T (T_{RM}) cells have been identified at multiple mucosal sites[59] and are thought to play decisive roles in rapid responses to infection[60] and cancers (BOX 3), thus providing another important correlate for mucosal vaccine efficacy. The human duodenal $CD4^+$ T cell compartment was recently shown to be enriched with a population of polyfunctional T_H1 cells, which survive for at least 1 year[61]. This offers significant hope for inducing sustained protective cellular immunity if optimal oral vaccine strategies are designed. There are clearly significant tissue-specific differences in the nature of T_{RM} cell populations so this must be considered in the design of new vaccine approaches. $CD8^+$ T_{RM} cells in the lungs are pivotal for protection against respiratory viral infections but these cells are generally relatively short-lived, and this can compromise responses to subsequent infection[62]. The latter study found that systemic vaccination (intravenous administration of *Listeria monocytogenes* expressing influenza virus-associated antigen) could enhance lung T_{RM} cells in mice previously infected with influenza virus by increasing numbers of circulating effector memory T cells. This clearly has implications regarding the potential for systemic booster vaccinations in previously infected or mucosally primed populations to sustain resident memory $CD8^+$ T cells in the lungs. A population of lung-resident helper T cells was recently characterized that was required to support tissue-resident memory B cells and $CD8^+$ cells following influenza virus infection[63,64]. These cells, which are induced locally in the lung, may be key for promoting long-lived cellular and humoral immunity following vaccination in the respiratory tract, so the optimal strategy for their induction should be addressed. Recent evidence indicates that long-term maintenance of lung T_{RM} cells requires airway vaccination and sustained antigen presence in the lungs, which was facilitated by an adenovirus vector vaccine[65]. It was recently shown in mouse models that T_{RM} cells migrate to the mediastinal lymph nodes from the lungs during infection in a process termed 'retrograde migration'. These cells retained a T_{RM} cell phenotype and provided long-term protection[66]. This may be an important consideration following intranasal vaccination strategies. Further studies from the same group demonstrate that, upon restimulation, T_{RM} cells can undergo retrograde migration and give rise to effector memory T cells and central memory T cells that have a predisposition for homing to their tissue of origin[67].

Targeting the genital tract. Although oral, sublingual and nasal routes are more convenient and there are currently no vaccines that specifically target the genital tract in clinical use, vaccination in the genital tract could have significant advantages in targeting STDs, even as a vaccine-boosting approach. In mouse models, vaginal immunization with herpes simplex virus 2 (HSV-2) glycoprotein D antigen and the adjuvant α-galactosylceramide (α-GalCer) induced protective immunity against HSV-2 challenge[68]. A combined vaccination approach using recombinant influenza virus–HIV vectors administered via intranasal and intravaginal routes (in mice) resulted in HIV-specific $CD8^+$ T_{RM} cells in the vaginal mucosa[69]. Vaginal immunization of mice with an attenuated HSV-2 strain resulted in the induction of a population of $IFN\gamma^+CD4^+$ T_{RM} cells, which promoted CXCL9-mediated and CXCL10-mediated recruitment of memory B cells upon secondary challenge[70]. By contrast, primary vaccination did not result in the induction of a tissue-resident population of plasma cells in the female reproductive tract. Thus, vaginal booster vaccination or, possibly, booster vaccination in the large intestine may be an effective strategy following systemic priming to trigger genital tract responses, although these findings must first be confirmed in a human setting. Promising recent data showed that vaginal delivery (by intramucosal vaginal injection or spray) of recombinant glycosylated IL-7 to rhesus macaques acted as an effective mucosal adjuvant, enhancing the induction of antigen-specific IgA/IgG in the vaginal mucosa following subsequent vaginal delivery of diphtheria toxoid[71]. This could be a broadly applicable strategy that may overcome hypo-responsiveness to vaginal vaccine delivery.

Immune cell populations targeted by mucosal vaccines. Mucosal adjuvants should aim to activate and target local or recruited APCs (FIG. 3) or populations of immune cells enriched in the mucosa (TABLE 1) in order to mount effective mucosal responses. Innate lymphoid cells (ILCs),

mucosal-associated invariant T cells, natural killer T (NKT) cells and γδ T cells are abundant in mucosal tissues and can play crucial roles in mediating and shaping mucosal immunity[72–79]. Adjuvants can also be exploited in parenteral–mucosal push–pull strategies; for example, dmLT has been shown to imprint mucosal homing markers on T cells when injected[80]. Similarly, retinoic acid has been identified as a suitable adjuvant in such strategies, imprinting gut-homing markers on T cells and leading to protective intestinal responses following subcutaneous vaccination[81]. There are currently two ongoing trials investigating parenteral–mucosal push–pull strategies for SARS-CoV-2 vaccination: NCT04732468 and IG/VPIN/CVD19/2001. The former trial involves investigating combinations of oral and subcutaneous immunization with a human adenoviral vector expressing modified SARS-CoV-2 spike and nucleocapsid proteins. By contrast, the latter trial involves combinations of intranasal and intramuscular immunization, with the vaccine composed of the receptor-binding domain of SARS-CoV-2 spike protein adjuvanted with hepatitis B virus nucleocapsid protein when given intranasally and with alum when given intramuscularly.

Mucosal adjuvant approaches

Enhancing the efficacy of subunit and inactivated antigens. Toxoid adjuvants are the best-characterized class of mucosal adjuvants and the development of safe yet potent derivatives of *E. coli* heat-labile toxin and cholera toxin (BOX 4) has paved the way for their safe incorporation in vaccine formulations. Incorporation of dmLT has been shown to improve clinical responses to several whole-cell antigens, as seen with ETVAX and ACE527 (REFS[31,82]). Excellent overviews of the development and clinical application of dmLT are provided by Clements and Norton[80] and Qadri et al.[31]. Based on a similar approach, the adjuvant multiple mutated cholera toxin (mmCT) has been proposed as an alternative to dmLT[83], and in preclinical studies mmCT has been shown to enhance T_H1 cell and T_H17 cell responses in addition to mucosal and serum antibodies to a whole-cell *Helicobacter pylori* antigen[84]. CTA1DD is a cholera toxin-derived adjuvant that was designed to combine the beneficial immunostimulatory effects of the CTA subunit enzyme with the B cell-targeting properties of a D-domain dimer from *Staphylococcus aureus*, to reduce off-target effects and toxicities[85,86]. It was recently shown that CTA1DD enhanced the maturation of follicular dendritic cells in lymph nodes following mucosal vaccination in neonatal mice and that oral priming with a construct incorporating the influenza virus M2e antigen (CTA1-3M2e-DD) induced protective immunity in neonates against influenza challenge[87]. Combination of lipid nanoparticles and CTA1-3M2e-DD generated a highly effective nasal vaccination system that conferred protective immunity against influenza virus infection in mice[88]. This combination adjuvant was particularly effective in promoting respiratory tract IgA, T_H1 cell and T_H17 cell responses, holding promise for universal influenza vaccination applications[88]. However, the efficacy and safety of CTA1DD remains to be determined clinically. The use of toxoid adjuvants intranasally has been somewhat marred by the clinical emergence of Bell's palsy in some recipients of influenza vaccines adjuvanted with wild-type *E. coli* heat-labile toxin[89] or LTK63, a genetically detoxified *E. coli* heat-labile toxin derivative[90]. An alternative is sublingual vaccination, which has shown significant promise as a means of promoting protective immunity in animal models[91,92] although immune responses to sublingual dmLT were modest in a clinical trial[93]. There may be scope to enhance such responses by formulation with agents such as chitosan to enhance antigen and adjuvant residence times (BOX 2).

These studies would suggest that nasal delivery of ganglioside-targeting toxoid adjuvants is inadvisable. However, results from a phase II clinical trial on a trivalent influenza vaccine, composed of haemagglutinin and adjuvanted with LThαK, a detoxified *E. coli* heat-labile toxin derivative, were recently reported[94,95] (NCT03784885). An acceptable safety profile was reported following two nasal vaccinations, which induced higher antigen-specific nasal IgA responses than the non-adjuvanted antigen[94]. LThαK is reported to have no ribosylating activity, correlating with enhanced retention in the nasal passages and the enhanced safety profile. Phase III trials will investigate efficacy in a challenge setting and will further elucidate the safety of LThαK for intranasal incorporation in a larger patient cohort. Importantly, nasal delivery of CTA1DD did not result in trafficking to the olfactory bulb, indicating its safety as a nasal vaccine adjuvant[88]. In summary, toxoid adjuvants are the most advanced mucosal adjuvants,

Box 3 | Vaccine approaches for mucosal cancers

Malignancies commonly emerge at the mucosae, providing a rationale for mucosal vaccine targeting. Incidences of mucosal cancers are increasing, with cancers of the head and neck and the reproductive, respiratory and digestive tracts estimated to cause 8.52 million deaths per annum by 2040 compared with a current estimate of 5.15 million deaths per annum[170]. Effective tumour immunosurveillance and elimination relies on tumour-specific $CD8^+$ T cells. Therefore, mucosal vaccine strategies that effectively mobilize cell-mediated immunity with generation of sentinel tissue-resident memory T cells (T_{RM} cells) are required[171,172]. Mucosal vaccination strategies have proved more effective than parenteral vaccination routes[171,173,174]. Nizard et al. demonstrated that an intranasal dendritic cell-targeted vaccine was more protective than parenteral vaccination in an orthotopic head and neck tumour model (HPV16 $E6^+$ and $E7^+$ expressing TC-1), and this effect was dependent on the presence of mucosal antigen-specific $CD8^+$ T_{RM} cells[171]. Optimal targeting of dendritic cell subsets is vital for such approaches: conventional type 1 dendritic cell (cDC1) populations including $CD103^+$ non-lymphoid dendritic cells (analogous to $CD141^+$ dendritic cells in humans) and $CD8\alpha^+$ lymphoid dendritic cells are efficient at cross-priming cytotoxic T lymphocytes[175] and in imprinting mucosal homing receptors, displaying antitumour functionality[176,177]. Furthermore, the proficiency of $CD103^+$ dendritic cells in trafficking intact antigens from tumours to tumour-draining lymph nodes and their importance in the context of checkpoint blockade responses has been highlighted[176,178,179]. Vaccines/adjuvants that effectively target these subsets and/or strategies to expand their number prior to vaccination should prove most successful. With the exception of virally induced cancers where viral antigens are vaccine targetable, antigen selection is problematic in prophylactic vaccination. Targeting tumour neoantigens is an attractive concept yet it is unlikely to ever be a 'one size fits all approach' as the degree and composition of mutational burden is highly patient-specific and tumour-specific[180,181]. Therapeutic mucosal cancer vaccines can circumvent these issues via personalized medicine-based vaccine design[182,183] or, possibly, through local antigen release for a more general approach[184]. Mucosal cancer vaccines not only have potential to prevent and treat mucosal tumours, but also to prevent infection with viruses linked to non-mucosal malignancies, such as Epstein–Barr virus and hepatitis viruses.

Box 4 | **Enterotoxin-derived mucosal adjuvants: dmLT and mmCT**

Cholera toxin and *Escherichia coli* heat-labile toxin are the gold-standard mucosal adjuvants. However, their toxicity necessitated strategies to enhance safety whilst retaining adjuvanticity, culminating in generation of *E. coli* double-mutant heat-labile toxin (dmLT)[185] and multiple-mutated cholera toxin (mmCT)[83]. The introduced mutations target the ADP ribosyltransferase activity of the toxin A subunit[83,185] and both molecules are powerful mucosal adjuvants that enhance mucosal IgA and serum IgG responses in addition to CD4$^+$ T cell responses, particularly T helper 17 (T_H17) cells[80,84]. Interaction of cholera toxin with GM1 on gut dendritic cells is required for its oral adjuvanticity[146], although the precise mechanisms of action are not fully resolved. NF-κB activation was required for the adjuvanticity of mmCT, with cyclic AMP–protein kinase A (cAMP–PKA) signalling proposed to be required for NF-κB activation in mmCT-stimulated dendritic cells in vitro[186], although PKA may be dispensable for dendritic cell activation by dmLT[187]. Both dmLT and mmCT required cAMP–PKA-dependent inflammasome activation to promote human T_H17-type responses[188]. There has been extensive evaluation of dmLT in clinical trials as an oral vaccine adjuvant, with results indicating an acceptable safety profile and strong adjuvanticity[31,32,80,82]. Furthermore, a National Institute of Allergy and Infectious Diseases (NIAID)-sponsored trial investigating the safety and adjuvanticity of three doses of dmLT by oral, sublingual and intradermal routes has begun recruitment in an enterotoxigenic *E. coli* (ETEC) endemic area of Bangladesh (NCT03548064). The inclusion of toxoid-derived adjuvants in mucosal vaccines may improve responses in low-responding demographics, such as older people and young children[31]. Toxoid-derived adjuvants may also potentially help in addressing the lower responses to oral vaccines that are often seen in endemic regions (known as the 'tropical barrier') compared with the higher income countries where early-stage clinical trials are frequently conducted. Although existing antibodies to dmLT do not appear to impair its adjuvanticity upon booster vaccination[80], different outcomes have been observed in trials between Swedish and Bangladeshi recipients[31,32] that require further evaluation on the potential impact of previous exposure.

having demonstrated impressive efficacy in clinical trials for oral whole-cell killed vaccines.

Aside from toxoid adjuvants, there are a small number of mucosal adjuvants with demonstrated safety and efficacy. The invariant NKT cell activator α-GalCer is a promising mucosal adjuvant and potentially an indicator of the potential for targeting innate-like lymphocytes to produce a new generation of mucosal adjuvants. We have demonstrated that oral delivery of a whole-cell killed *H. pylori* antigen adjuvanted with α-GalCer induced protective immunity from gastric bacterial challenge, characterized by induction of local IgA and T_H1 cell immunity, comparable with a cholera toxin adjuvanted vaccine[96]. The induction of antigen-specific T_H1 cell responses was dependent on CD1d, IL-1R1 and IL-17R signalling; therefore, α-GalCer provides a proof of principle for targeting the relatively abundant mucosal invariant NKT cell populations for effective adjuvanticity. We have further characterized α-GalCer as an effective adjuvant with oral whole-cell killed ETEC and cholera antigens including the CFA/I overexpressing JT-49 ETEC vaccine combined into enterically stable smPill mini-spheres. Potent induction of intestinal CFA/I-specific IgA was observed in addition to serum IgG responses[97]. Oral vaccination with Dukoral and α-GalCer induced stronger intestinal IgA and serum IgG responses than Dukoral alone and was comparable with cholera toxin adjuvanted Dukoral[98]. Finally, incorporation of α-GalCer in a novel multi-antigen cholera vaccine composed of whole-cell killed double-lipopolysaccharide (LPS) antigen expressing cholera vaccine with CTB promoted robust mucosal immunity with concomitant systemic antibody production, outperforming Dukoral and the whole-cell killed cholera alone[98]. Our preclinical data provide a rationale for the inclusion of α-GalCer in future whole-cell oral vaccines, which may lead to more durable protection, addressing shortcomings in immunity and response rates.

Chitosan is well established as a mucosal adjuvant/delivery system given its mucoadhesive properties and immunostimulatory effects[99]. We have demonstrated the effectiveness of chitosan as an intranasal adjuvant in mouse models. Intranasal vaccination with chitosan and pneumococcal surface protein A (PspA) led to the induction of lung PspA-specific IFNγ and IgG1, IgG2c and IgA responses that were dependent on STING signalling[100]. STING-activating cyclic dinucleotides have been trialled as mucosal adjuvants. An intranasal synthetic cyclic dinucleotide (cyclic diguanylate) adjuvanted subunit vaccine induced protective immunity against *M. tuberculosis* in mice, correlating with potent induction of T_H17 cells[101]. Other cyclic dinucleotides — including cyclic di-AMP and cyclic di-GMP — have also shown promise as mucosal adjuvants[102,103]. Mansouri et al. recently highlighted roles for two lung cDC2 populations in intranasal cyclic di-GMP adjuvanticity. Antibody responses were dependent on activation of moDCs by TNFR2$^-$ cDC2 populations, with subsequent T follicular helper cell and germinal centre B cell activation, whereas induction of T_H1 cell and/or T_H17 cell responses was dependent on their TNFR$^+$ cDC2 counterparts[104]. These studies provide a strong rationale for further development of mucosal adjuvants targeting the STING pathway. In this context, chitosan has specific advantages in its record of clinical use and mucoadhesive properties in addition to STING-dependent adjuvanticity.

Whereas emulsion-based adjuvants have been highly successful in injectable vaccines, such approaches have not reached clinical application mucosally. Bluewillow Biologics currently have a phase I trial underway (NCT04148118) utilizing an intranasally administered nanoemulsion (oil in water emulsion) adjuvanted recombinant protein vaccine against anthrax (BW-1010). Preclinically, this vaccine has previously been shown to be protective in guinea pig models of infection, correlating with induction of systemic and local antibody induction[105]. The nanoemulsion adjuvant has also been shown to promote T_H1 cell immunity and T_H17 cell immunity in anthrax and *M. tuberculosis* vaccine formulations, respectively[105,106]. Kimoto et al. recently reported a promising mucosal adjuvant with possible applications in oral and intranasal vaccination routes. Two oral doses of HAv (haemagglutinin-based vaccine) adjuvanted with SF10 (a synthetic surfactant adjuvant) led to induction of antigen-specific mucosal IgA and protection from influenza in a preclinical model, outperforming cholera toxin[107]. These recent studies highlight the promise of adjuvanted mucosal vaccines, with many taking inspiration from bacteria-derived virulence factors and showing promise for inclusion not only in subunit but also whole-cell formulations.

Mucosal nucleic acid and viral vectored vaccines. Until very recently, there were no licensed nucleic acid vaccines for clinical use. However, mRNA vaccines against

Polymersomes
Hollow vesicles generally comprising block copolymers that can incorporate vaccine antigens and adjuvants in the aqueous phase.

SARS-CoV-2 have now been successfully trialled and rolled out for parenteral vaccination, displaying impressive efficacy and paving the way for others to follow[108]. Mucosal vaccination utilizing nucleic acids poses a greater challenge, as successful candidates must penetrate the mucus layer, translocate into target cells and evade extracellular and intracellular degradation. Vaccination via the oral route poses an added challenge with the low gastric pH and difficulty in ensuring release of the nucleic acid payload at the appropriate location. Innovative protective delivery strategies for nucleic acids have been developed using nanocarriers and biomaterials[109–111], and in particular the complexing of nucleic acids with polycationic materials including chitosan and polyethylenimine (PEI) and encapsulation of the nucleic acid cargo utilizing liposomes and polymersomes are showing potential. Lipidoid nanoparticles have been shown to effectively deliver small interfering RNA molecules to intestinal epithelial cells in the lower small intestine and colon following oral administration[112]. Additionally, intranasal delivery of chitosan nanoparticles encapsulating mRNA with a viral protein coating elicited protection from avian influenza in chickens[113]. The coming years are likely to see great activity in this space, particularly around mobilizing solid lipid nanoparticles for mucosal RNA vaccine development.

Viral vectors are among the most promising strategies for mucosal vaccination, owing to their capacity for intracellular delivery, versatility and intrinsic immunogenicity. Viral vector strategies are applicable to oral vaccination when protection from conditions of the gastrointestinal tract and effective release are addressed. This is exemplified by the technology from Vaxart, whose oral influenza vaccine candidate VXA-A1.1 utilizes an enterically stable tableted delivery system, carrying a cargo of haemagglutinin encoding adenoviral vectors and a double-stranded RNA adjuvant. Data from phase I (NCT01688297) and phase II (NCT02918006) clinical trials demonstrated that VXA-A1.1 is well tolerated[114] and, crucially for future adenoviral vector strategies, is not hindered by pre-existing adenoviral immunity when given orally[114]. Oral vaccination with VXA-A1.1 induced superior protection from influenza A virus challenge compared with the conventional intramuscularly delivered FluZone vaccine[115]. Whether this platform can be used to develop an effective oral quadrivalent influenza vaccine remains to be demonstrated. Additionally, orally administered RSV vaccines are in preclinical development (VXA-RSV-f)[116], and an oral Norovirus vaccine (VXA-G1.1-NN) showed favourable safety and immunogenicity in a phase 1 trial (NCT02868073)[55]. More recently, an orally administered SARS-CoV-2 vaccine (VXA-CoV2-1) was described that uses the same formulation and is currently in phase I trials (NCT04563702). The efficacy of the double-stranded RNA adjuvant included in this platform is also very promising as an alternative to the canonical toxoid-based adjuvants in various stages of development and broadens the range of PRR targets that can be exploited for oral vaccination. This double-stranded RNA adjuvant will likely effectively target dendritic cell populations for activation owing to their high TLR3 expression. This capacity to successfully adjuvant viral vectors may be critical as they will likely be less effective when given mucosally compared with parenteral routes. Further to this point, it has been recently shown that responses to viral vector (MVA) antigens can be enhanced by the saponin-containing adjuvant matrix M following subcutaneous vaccination[117].

Viral vector approaches also hold potential for vaccination in the respiratory tract. Intranasal vaccination of mice with an adenoviral vector encoding influenza virus nucleoprotein induced a population of $CD8^+$ T_{RM} cells in the lungs that was sustained for longer than 1 year[65]. This was dependent on respiratory vaccination and sustained antigen expression, and contrasted with the situation following parenteral influenza virus infection, where the local $CD8^+$ T_{RM} cell population was rapidly lost. The authors suggested that induction of robust local cellular immunity may address issues surrounding the reliance on systemic antibody responses to haemagglutinin associated with parenteral influenza vaccination[65]. Nasal delivery of chimpanzee adenoviral (ChAd) vectors may also have potential in SARS-CoV-2 vaccines. Nasal delivery of ChAd-SARS-CoV-2 expressing homotrimeric spike antigen induced promising results in a murine infection model (K18-hACE2 mice). A single dose provided protection from upper and lower respiratory tract infection, correlating with induction of neutralizing antibody titres in the serum and bronchoalveolar lavage alongside the induction of $IFN\gamma^+$ and granzyme B^+ $CD8^+$ T cells[118]. Whether efficacy would be sufficient clinically with viral vectors alone is unclear but, as with oral delivery, there may be scope to enhance responses with appropriately targeted mucosal adjuvants. With viral (or bacterial) vectored vaccines, the capacity of vaccine-induced secretory antibody responses to compromise responses to booster vaccination must be considered[119]. However, data from preclinical models have shown that pre-existing intestinal immunity did not compromise efficacy of an oral experimental viral vectored rabies vaccine[120]. Furthermore, compelling recent clinical data found no detrimental effect of pre-existing influenza-specific nasal IgA responses on the efficacy of nasal live attenuated influenza virus vaccination in children[121], and recently Janssen reported no clear impact of pre-existing immunity to their Ad26 vectored vaccine platform on efficacy following priming or boosting vaccinations[122]. This must be ascertained for each specific vaccine but, moving forwards, the availability of numerous mucosal viral vectors and adjuvant strategies would allow a heterologous prime–boost approach to overcome pre-existing immunity if required.

Concluding remarks

We are currently in the midst of a revolution in vaccine research and development. Cutting-edge research and advances into nucleic acid and viral vector vaccine technologies allowed SARS-CoV-2 vaccines to be developed and produced in an unprecedented short period of time. These advances are yet to impact on clinically used mucosal vaccines, but this will likely change in the near future. Mucosal vaccines offer the significant

benefit of triggering immune responses at the principal sites of infection, offering scope for sterilizing immunity achieved by local secretory antibody responses and resident populations of $CD4^+$ and $CD8^+$ T cells. Thus, the outstanding obstacles to mucosal vaccine development are worth the effort as they are far outweighed by the potential immunological and logistical benefits in terms of ease of delivery. One of the major challenges that requires innovative solutions is the 'tropical barrier', where responses to oral vaccines in low- and middle-income countries can be lower than those seen throughout clinical trials in high-income countries. Interventions to address this problem are urgently required[123] and may include implementation of probiotic supplements prior to or during vaccination[124]. The potential for adjuvants to overcome suboptimal responses must be addressed as this and increased antigen doses may have a greater impact than other proposed strategies. Indeed, the most advanced mucosal adjuvant, dmLT, has demonstrated efficacy in both high-income and low-income countries[31,32]. Identifying whether other candidate adjuvants can also increase efficacy of existing oral vaccines as well as facilitating the development of novel vaccines is a priority. Targeting mucosally abundant cellular populations such as ILCs, mucosal-associated invariant T cells and NKT cells has significant promise but clinical validation of these approaches is required. A recent study in mice found that intestinal ILCs can migrate via the lymph to the mesenteric lymph nodes, and in response to infection with *S.* Typhimurium these migrating ILCs exhibited greater levels of activation and cytokine production. Mobilizing this population using ILC-targeting adjuvants may have significant potential to bolster mucosal immune responses[75]. In addition to stand-alone mucosal vaccine approaches, parenteral mucosal prime–boost strategies offer promise. These may be enhanced with injectable vaccines that imprint a degree of mucosal homing, for example, with dmLT[125] or retinoic acid[81], and their relative ability to enhance tissue-resident T cell responses may be key to success. In some cases, antigen alone may be sufficient for mucosal boosting[126] although this will depend on the nature and immunogenicity of the antigen and it is likely, in most cases, that an effective adjuvant will be required. In summary, although the leaps forward in injectable vaccine strategies have not yet been seen with mucosal vaccines, this is likely to change in the near future. Advances in our understanding of mucosal protective immunity, developments in measuring human mucosal immunity[127] and antigen and adjuvant discovery offer hope that novel mucosal vaccines for infectious diseases and cancer are on the horizon.

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