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Mucosal vaccines

Novel strategies and applications for the control of pathogens and tumors at mucosal sites

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The mucosal immune system displays several adaptations reflecting the exposure to the external environment. The efficient induction of mucosal immune responses also requires specific approaches, such as the use of appropriate administration routes and specific adjuvants and/or delivery systems. In contrast to vaccines delivered via parenteral routes, experimental, and clinical evidences demonstrated that mucosal vaccines can efficiently induce local immune responses to pathogens or tumors located at mucosal sites as well as systemic response. At least in part, such features can be explained by the compartmentalization of mucosal B and T cell populations that play important roles in the modulation of local immune responses. In the present review, we discuss molecular and cellular features of the mucosal immune system as well as novel immunization approaches that may lead to the development of innovative and efficient vaccines targeting pathogens and tumors at different mucosal sites.

Introduction

Mucous membranes comprise an extensive surface area that lines cavities and internal organs directly facing the external environment. Such high exposure makes the mucosa the port of entry of a large number of infectious agents, requiring prompt and effective defense mechanisms. However, mucosal tissues also need to acquire tolerance against non-dangerous inhaled or orally absorbed antigens and maintain a dynamic equilibrium with the microbiota.[1] Indeed, the mucosal immune system has evolved tightly controlled surveillance and immunity induction mechanisms. The large number of deaths attributed to mucosal infections in children, approximately 10 million annually,[2] reinforces the challenge for the induction of an efficient immune response at mucosal sites. Although several mucosal infections have great epidemiological impact, only few mucosal vaccines against mucosal pathogens (poliovirus, *Vibrio cholerae*, *Salmonella typhi*, rotavirus, and influenza) have been commercially approved (**Table 1**).[3] This situation may change as some clinical trials are ongoing for mucosally administered vaccines to prevent infections against influenza virus, *Bordetella pertussis*, Enterotoxigenic *Escherichia coli* (ETEC), *Vibrio cholerae*, *Shigella sonnei*, and norovirus[4,5]. In addition, cancer vaccines against tumors located at mucosal sites (colon, head and neck, lung, genital tract) have failed to provide clinical benefits when administered by systemic routes.[6,7] The increasing understanding of the mucosal immune system and the current mucosal vaccine strategies should lead to the development of innovative and potent vaccines against mucosal pathogens and tumors located at mucosal sites.[8,9]

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The Mucosa-Associated Lymphoid Tissue

The mucosal immunological components can be divided into two main parts: organized mucosa-associated lymphoid tissues (MALTs), where antigen-specific immune responses are initiated, and diffuse lamina propria regions, which are the effector sites for antibody production (IgA) and T cell responses.[10] MALTs represent a complex immunological network structure, situated along the surfaces of various kinds of mucosal tissues, including the gut-associated lymphoid tissues (GALT) called the Peyer's Patches (PPs), the nasopharynx- associated lymphoid tissue (NALT), the bronchus associated lymphoid tissue (BALT), the conjunctiva-associated lymphoid tissue (CALT), and the vaginal-associated lymphoid tissue (VALT). The organization of each MALT is similar to that of a lymph node (LN) with B-cell-rich follicles and T-cell-rich interfollicular areas in close contact with dendritic cells (**Fig. 1**). Some interspecies differences exist in the nature and the regulation of MALT. For example, as opposed to rodents, humans generally do not have NALT anatomically, but they possess oropharyngeal lymphoid tissues, which seem to correspond functionally to NALT.[8] In addition, BALT is not constitutively present in all mammalian species, but is induced in response to microbial exposure or other types of pulmonary

Table 1. List of commercially available vaccines administered by the mucosal routes*,**

Name of the Vaccine (Company)	Composition	Pathogens targeted	Route of vaccination
Dukoral (SBL Vaccin AB)	Recombinant B subunit of cholera toxin and inactivated vibrio cholerae O1(Inaba and Ogawa serotype)	Vibrio Cholerae	Oral
Shanchol (Shantha Biotechnics)	bivalent inactivated vaccine containing killed whole cells of *V. cholerae* O1 and *V. cholerae* O139	Vibrio Cholerae	Oral
Rotateq (Sanofi-Pasteur-MSD)	Live attenuated rotavirus type p1a (8), g1-g4	Rotavirus	Oral
Rotarix (GSK)	Live attenuated rotavirus type rix 4414	Rotavirus	Oral
Vivotif (Crucell)	Attenuated live strain of Salmonella typhi Ty21a	Salmonella thyphi	Oral
Flumist (Medimmune)	Live attenuated influenza virus	Influenza	Nasal

*The oral Polio Vaccine Sabin is not commercially available except in epidemic context; ** Other mucosal vaccine candidates for pathogens (*Bordetella pertussis*, Enterotoxigenic *Escherichia coli* (ETEC), *Vibrio cholerae*, *Shigella sonnei*, *Helicobacter pylori*, *campylobacter*, *Salmonella Typhi* and *Paratyphi*, *hemophilus influenzae type* B, and norovirus) have been recently tested in human trials.

inflammation.[11] These inducible tissues may be more properly referred to as a tertiary or ectopic lymphoid tissue and are composed by organized structures with T- and B-cell areas, high content of endothelial venules (HEVs) in the T-cell zone and an overlying lymphoepithelium.[12]

Antigen Sampling and Presentation at Mucosal Surfaces

The epithelial cell layers associated with mucous membranes form physical and immunological barriers that are not impenetrable, but control the cross-talk between the lumen and the lamina propria using multiple antigen sampling strategies.[13] The epithelial cells lining the mucous membranes express pattern recognition receptors and antimicrobial effector molecules, which enables them to respond to microorganisms. These mucosal epithelial cells initiate the first steps in the host-pathogen interaction and largely influence the type of immune response elicited by the host.[14,15] In intestinal and airway epithelia, whose intercellular spaces are sealed by tight junctions, specialized epithelial microfold cells (M cells) deliver samples of foreign material by transepithelial transport from the lumen to the MALT.[16] M cells have reduced microvilli, a thin mucus layer and a pocket-like cell structure that holds dendritic cells (DCs) and/or lymphocytes, allowing an easier contact with pathogens and enhancing the contact with antigens.[17]

DCs work as the MALT sentinels, moving into the epithelium in close contact with M cells, sampling luminal antigens, and, then, migrating back to local (MALTs) or distant organized lymphoid tissues of draining lymph nodes (DLN). DCs are involved in the induction of immune responses against pathogens, as well as tolerance to commensal microbiota and food.[18,19] The tolerogenic functions of intestinal DCs are associated with higher IL-10 secretion, compared with splenic DCs[20] and induction of increased IL-4 and IL-10 production by naïve CD4 T cells,[21] properties likely associated with their tolerogenic functions. In mice PPs tolerogenic DCs are mainly represented by CD11b+CD8- cells, whereas CD11b-CD8- and CD11b-CD8+ DCs produce IL-12 and prime T cells for IFNγ production.[22] Another DC subpopulation described to play a specialized role in mucosal tissues, is represented by CD11b+CD103+ cells, which are highly capable of migrating from the lamina propria to DLN in a CCR7-dependent manner.[23] Through this process, CD103+ DCs transport antigens from mucosal sites and present them to CD8+ and CD4+T cells in DLNs, resulting also in the expression of homing molecules on these cells (imprinting).[21] Mucosal DCs also express FcRn, a receptor which binds the Fc portion of IgG at acidic pH, and play a major role in antigen cross-presentation leading of potent mucosal CD8+T cell responses.[24]

Effector immune responses at mucosal tissues

Mucosal antibody response

IgA is found in the serum and in secretions (sIgA). In human serum, the predominant form of IgA is IgA1 of which around 90% is monomeric and 10% is dimeric or polymeric. The IgA2 subclass predominates over the IgA1 subclass in the mucosae. IgA monomer requires the joining chain (J-chain produced by IgA-plasma cells) to form a dimer or a polymer. A particular hallmark of mucosal immunity is the local induction and production of secretory IgA (S-IgA) by activated B cells in MALT germinal centers or lymph nodes. B cell isotype switching for IgA production is mainly stimulated by TGF-β, while retinoic acid, IL-4, IL-10, and IL-6 are important co-factors for differentiation into plasma cells and the enhancement of IgA secretion.[25,26] These mediators are abundantly present at most mucosal surfaces, and are produced by DCs, epithelial cells, stromal cells, and mucosal lymphocytes.[27-29] MALT post-switched IgA$^+$ B cells disseminate in the blood via the efferent lymphatic vessel and some reach the mucosal effector tissues,[30,31] where they differentiate into IgA-producing plasma cells. In mucosal tissue dimeric or polymeric IgA, but not monomeric IgA show a high affinity for the polymeric Ig receptor (pIgR) localized at the basolateral surface of epithelial cells, which actively transport IgA across the mucosal epithelium to the lumen. In the mucosae and not in the blood, dimeric or polymeric IgA also include a secretory component (SC) corresponding to a part of the pIgR.[32] The final

S-IgA has unique properties, including acid and protease resistance and interactions with mucus and effector leukocytes.[26,33] Interestingly, IgA antibodies are unable to fix complement, functioning mainly as a neutralizing antibody, which results in a non-inflammatory immune response that limits the damage at mucosal tissues. IgA antibodies secreted onto the mucosal surfaces are particularly important in preventing infection by inhibiting the adhesion of bacteria, viruses, or other pathogens to epithelial cells.[34,35] The IgA complex, including the SC, bind to M cells and may have a role of 'selection', excluding pathogenic bacteria or fungi from the epithelial surface through its anchoring within the mucus and favoring biofilm formation of non-pathogenic bacteria in the space in close contact with epithelial cells.[36] Other immunoglobulin isotypes may also play a role in mucosal tissues and MALT B cells may switch to different immunoglobulin isotypes according to the anatomical site and the specific inducing conditions. For example in some species, IgG-secreting cells predominate over IgA-secreting cells in BALT.[37]

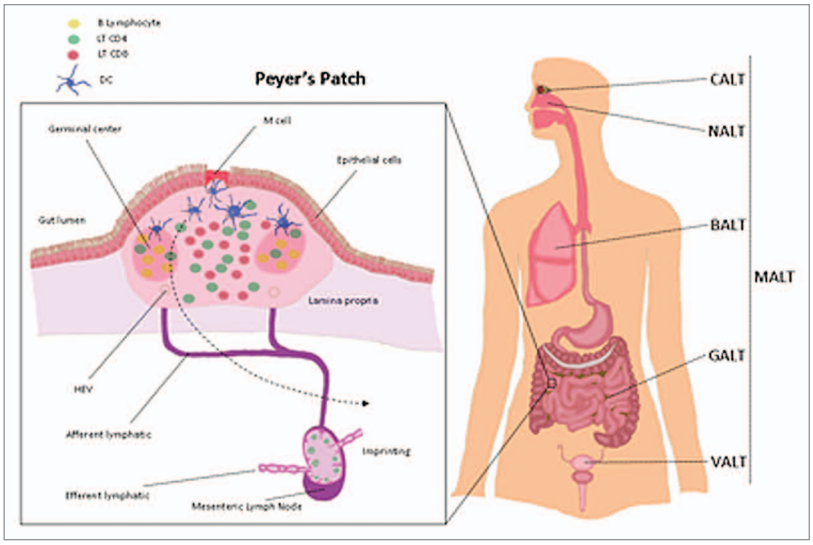

Figure 1. Mucosal associated lymphoid tissue (MALT) are organized lymphoid structures present in surface area in contact with environment such as the lung (bronchus-associated lymphoid tissue (BALT), the nose (Nasal-associated lymphoid tissue (NALT) and the gut (Gut-associated lymphoid tissue (GALT). Peyer's patch present in the GALT are often presented as a model of the MALT organization. It is located in the lamina propria layer of the small intestine and in the ileum in humans. This lymphoid structure between the lumen of the intestine and the mesenteric lymph node is the place of the priming of a mucosal immune response.

Cellular immune responses at mucosal sites

T cells receive antigenic activation and co-stimulation by antigen-presenting cells (APCs) that support their clonal expansion, as well as the cytokine cues that dictate their differentiation and homing to peripheral tissues.[38,39,38,40] Activated and memory T cells downregulate molecules used for LN entry and upregulate molecules involved in migration to non-lymphoid tissues, such as the skin and the intestinal mucosa.[40] This memory T cell population, called tissue resident memory cells (T_{RM}), has been shown to stably reside in the peripheral tissues and not to enter the circulation for a prolonged period of time.[40] The presence of T_{RM} cells has been described in many mucosal tissues including skin,[41-43] lungs,[44,45] salivary glands,[46] and intestinal epithelium.[47] T_{RM} rapidly acquire effector functions upon secondary antigenic stimulation and are highly protective against subsequent local infection.[48,49] The presence of T_{RM} in the mouse lungs has been described as a better surrogate marker, than memory specific T cells in the blood, for reinfection protection in different preclinical studies in mice.[50] In non-human primates, the presence of T_{RM} against SIV was essential to control the viral load.[51] The DC-mediated imprinting of specific homing molecules on T cell surface is influenced by the LN microenvironment and, particularly, by the local production of TGF-β (**Table 2**).[38] Gut and skin tissue-specific homing molecules expression on T cells also requires vitamin A/Retinoic Acid and vitamin D-derived metabolites, respectively.[52,53] Homing markers expression and their regulation is still not well defined for all mucosal tissues, but it is well established that T cell expression of α4β7, which binds to intestinal MADCAM-1 on endothelial cells, and CCR9, attracted by intestinally produced CCL25, enable T cells entry into the small intestine.[54,55] Furthermore, expression of CCR10 and P- and E-selectin ligands, induces T-cell skin homing mediated by cutaneous CCL27 and P- and E-selectins (**Table 2**).[56-58]

Compartmentalization and immunity at distant mucosal sites

Mucosal invasion by pathogens prime immune responses at both mucosal and systemic compartments. Similarly, several vaccine studies showed that mucosal immunization has also been efficient in generating immune responses detectable at distant mucosal tissues and in the blood.[58] In contrast, immunization via parenteral administration routes usually fails to induce mucosal immunity or induce weaker responses than those detected after mucosal immunization.[58-60] Mucosal immune stimulation after pathogen invasion or vaccination, besides being capable of inducing systemic immunity, activates B and T cells that may migrate to peripheral environments different from the one where stimulation was initiated. The possible interconnection between immunity at different mucosal surfaces, raised the concept of a "common mucosal immune system." In fact, mucosally primed T and B cells display preferential migration patterns to specific mucosal surfaces or tissues, that are shared among mucosal sites.[35,61] For example, IgA+B cells in the MALT usually express CCR10 on their surfaces, which favors migration to CCL28 expressed by epithelial cells in the gut, lung, breast, vaginal, and salivary glands.[62,63] Interestingly, the female sex hormone estradiol regulates the CCL28 expression.[64] Intranasal (i.n) or sublingual immunization induced specific IgA secretion and activation of CD8+T cells in the genital tract and in some cases in the intestine.[65,66] Indeed, DCs activated

Table 2. Tissue-resident CD8+ T cells key homing markers involved in migration to different sites

	Homing receptor	Tissue ligand
Gut	α4β7	MADCAM-1
(ref. 47, 46, 55)	CCR9	CCL25
	CD103	E-cadherin
Skin	CCR10	CCL27
(refs. 41–43, 56, 57)	P- and E- selectin ligands	P- and E- selectin
	CD103	E-cadherin
Lungs	CD103	E-cadherin
(refs. 45, 178, 141)	CD49a	Collagen IV
	α4β1	VLA4
	CXCR6	CXCL16
	LFA-1	ICAM-1
Salivary glands	CD103	E-cadherin
(ref. 46)	E-cadherin	
Genital tract	CD103	E-cadherin
(refs. 179, 180)	CD49a	Collagen IV
	E selectin ligand	E Selectin

at a particular immunization site, recirculate to distant lymphoid organs and induce distant T cell priming, explaining the genital immune response observed after i.n immunization with the B subunit of cholera toxin (CTB) coupled to OVA.[67] In clinical settings, however, an intravaginal boost was required to attract T cells to the genital tract after i.n immunization with the cholera toxin coupled to OVA.[68] Additionally, oral, nasal or intravaginal immunization with CTB were able to induce comparable secreted IgA levels in the vaginal tract but no cellular immune responses.[69] In fact, inducing mucosal immunity at different anatomical sites after local priming may vary according to the specific mucosal tissue, type of immune response or vaccination approach.

Potential Role of Microbiota in the Activity of Mucosal Vaccines

Mucosal tissues are colonized by a vast number of non-pathogenic microbes that exert a significant influence on mucosal immune regulation and, therefore, can be a factor in determining the effectiveness of mucosal vaccines. For example, the manipulation of the intestinal flora was shown to play a large role in T cell regulation.[70,71] Oral vaccines against rotavirus, *V. cholerae* and *Escherichia coli* (ETEC) have been shown to be less effective in developing countries. Difference in the nutritional status, but also in gut microflora, as well as ongoing persistent infections with helminths and parasites have been raised as determinant hypothesis to explain this differential efficiency.[72] For the future, we could expect the development of programs to deeply analyze and correlate the individual and geographical characteristics of the microbiota and the efficacy of mucosal vaccines.[27]

Strategies to Induce Mucosal Immune Responses

Immunization routes

DC priming at one specific mucosal site determines the subsequent homing of T cell to specific mucosal sites.[73] Subsequent studies confirmed that this phenomenon applies to different mucosal administration routes such as the intranasal, intratracheal, oral, intrarectal, sublingual, intravaginal, and intraocular routes. Various groups showed that the mucosal delivery, especially by the oral route, of mucosal non-pathogenic live recombinant bacteria (*Listeria monocytogenes*, *Salmonella typhimurium*, *Bacillus subtilis*, lactic acid bacteria…) expressing antigen is efficient to protect against mucosal pathogens.[74-78] The intranasal immunization route has been intensively studied and shown to induce robust humoral and cellular immune responses at the lung mucosa against virus and bacterial infection.[79-82] In addition, the i.n. immunization route favors Th17 polarization,[83] known to be involved in the generation of protective immunity against funghi and bacteria located at mucosal sites.[84] A comparative clinical trial performed with children immunized with a live attenuated influenza vaccine (Flumist) showed that administration by the i.n route was more efficient, than the same formulation delivered intramuscularly in the mounting of protective immunity.[85] These results led to the approval of the vaccine administered by the i.n. route by the US Food and Drug Administration (FDA), a breakthrough for mucosal vaccines in which the oral administration route had long been the only alternative for vaccination in humans.

Other mucosal immunization routes have also raised interest and generated encouraging results. In mice, the sublingual vaccination proved to induce strong mucosal pulmonary and genital immune responses associated with protection against influenza, RSV or SARS lethal virus challenges and genital infections.[86-88] However, in preclinical models, the sublingual route of immunization appeared to be less powerful than the i.n route in eliciting protective mucosal immune responses.[89] In humans, however, an HPV vaccine administered by the sublingual route induced less anti-HPV antibodies in the cervix, than the i.m. route.[90,91]

The intravaginal route may be less effective in the induction of mucosal immune response. Intravaginal immunization with subunit vaccines elicit weak or no mucosal immune response both in rodents and in humans.[92,93] In most cases, only live vectors have been shown to elicit mucosal immune response by this immunization route.[88,94] Intravaginal immunization with live HPV pseudovirions particles or systemic immunization combined with intravaginal administration of TLR agonist are promising

approaches against sexually transmitted pathogens, because of its ability to induce local CD8+ T[88,95] and CD4+T cell responses, as well as antibody responses. However, the efficacy of this route may depend on the hormonal cycle of the women, as it has been shown that the estradiol inhibits CD8+T cell priming.[96] Proteins or subunit vaccines could be administered by the intrarectal route but it would require the association of an adjuvant to elicit mucosal immune response and protection against infectious challenge.[97,98] Nevertheless, intrarectal immunization with subunit vaccines also seem to be less efficient than those comprising recombinant virus-based vaccines.[99,100] Intrarectal immunization with a peptide prime and recombinant vaccinia boost regimen activated high avidity CD8+ CTLs in the gut mucosa, that correlated with lower SIV virus dissemination in macaques.[51,101] The administration of an anti-HSV vaccine at the ocular mucosa induced both antibody (systemic and mucosal) and cellular immune responses leading to protective immunity to ocular virus challenge in mice.[101] In general, mucosal routes of immunizations are capable to induce mucosal and systemic immune responses, while systemic immunization induce systemic and, in a clearly lesser extent, mucosal immune responses.

Mucosal adjuvants

As with vaccines administered via parenteral routes, immune responses induced by vaccines delivered via mucosal routes can be drastically enhanced with the use of adjuvants. In preclinical models, the most largely employed adjuvants to induce mucosal immune responses have been non-toxic derivatives of cholera toxin (CT) and *Escherichia coli* heat labile enterotoxin (LT).[102,103] Although devoid of their enterotoxicity and capable to efficiently increase both antibody and cellular immune responses, LT-derivatives proved to be toxic to humans after i.n. delivery due to the nerve tissue tropism leading to accumulation in the olfactory nerve and the bulb and inducing transient facial paralysis.[104] Some groups attempt to preserve the adjuvant activity of LT while decreasing its toxicity.[105,106] Toll-like receptor (TLR) ligands have been described to enhance mucosal immunity when co-administered with antigens by mucosal routes. Recently, US FDA has approved the use of a TLR4 agonist, the Monophosphoryl lipid A (MPL), combined with alum (AS04) for intramuscular immunization of the Cervarix prophylactic vaccine against HPV infections, which promotes Th1-biased response.[107,108] AS01, an adjuvant system containing liposomes, MPL and saponin, increased mucosal immunity in non-human primates.[109] Accordingly, i.n. vaccination with TLR4 agonists resulted in robust immune responses to both the carrier protein and bacterial polysaccharide components of the *Hemophilus influenza* type B virus in murine models.[110] Micellar and emulsion formulations of a synthetic TLR4 agonist, Glucopyranosyl Lipid Adjuvant (GLA) administered by the i.n route induced Th17-biased systemic and mucosal antibody responses.[111] The TLR9 ligand CpG, induce Th1-biased immune responses after sublingual or i.n administration.[112,113] In a preclinical model, CpG administration was possibly linked to enhanced hepatitis pathogenesis induced by concanavalin-A production, but the CpG was not administered by a mucosal route in this case.[114]

Another TLR ligand, the TLR3-specific double-stranded RNA oligonucleotide has been shown to modulate local immune responses after i.n administration.[115] In addition, poly(I:C) proved to be an effective adjuvant for an i.n. delivered influenza vaccine.[116] The TLR5 ligand, flagellin, combined with various antigens has been shown to enhance IgA response after i.n administration and protect against various toxins and pathogens challenge in preclinical models.[117-119] Indeed, *Salmonella* flagellins has been shown to be flexible adjuvants leading to activation of antibody and T cell responses based on the administration via mucosal and parenteral routes either as recombinant protein genetically fused with the target antigen or admixed with the soluble or particulate antigens.[120] Recently, it has been shown that the i.n delivery of virus like particles (VLPs) in combination with TLR7 or TLR9 agonists led to a significantly better dose-sparing effects than TLR3, TLR5 or TLR8 agonists for the induction of specific and functional antibody responses in the respiratory, gastrointestinal, and reproductive tracts.[121]

Chitosan microparticles and cationic chitosan derivatives are obtained from natural crab shells, composed of chitin derivatives, and are potent activators of macrophages and NK cells. Their administration by the i.n route combined with antigen induced high levels of IgA in the serum of mice and non-human primates.[122] Chitosan seems to favor a Th2 biased immune response and its safety has already been validated in mice and humans.[122,123] Recent findings have evidenced α-Galactosylceramid (α-GalCer) as a promising mucosal adjuvant. This CD1d ligand activates natural killer T (NKT) invariant cells, promoting DC maturation and cross-presentation.[124,125] When administered via the i.n route in combination with various antigens, α-GalCer induced mucosal antibody responses, as well as CD8+T cells responses,[126,127] devoid of measurable toxicity and without redirecting antigen to the nervous system, as shown for cholera toxin.[128] Administration of α-GalCer by the i.n route, but not by the systemic route, allowed repeated stimulation of NKT in the lung.[129] This adjuvant has also been used to sensitize DC to activates NKT cells in patients presenting recurrent head and neck carcinoma, without described associated toxicity.[130] Another class of promising mucosal adjuvants is represented by some already clinically used antibiotics such as polymyxin B (PMB) and colistin (CL). Intranasal immunization with PMB or CL with ovalbumin (OVA), increased OVA-specific antibody responses in a dose-dependent manner both at mucosal and the systemic compartments without detectable inflammatory damages.[131] Overall, novel mucosal adjuvants are particularly promising in preclinical models and, at least some of them (such as TLR3, TLR4, TLR5, TLR9 agonists and αGalCer) are also strong inducers of mucosal cellular immune responses.

Mucosal Homing Signals Delivered by Adjuvants or Immunomodulators

Several studies suggest that the local delivery of a mucosal stimulus or a peripheral mucosal imprinting of T cells favor their

homing to mucosal sites. Shin and colleagues were the first to show that parenteral vaccination, which elicited systemic T-cell responses (prime), followed by recruitment of activated T cells through topical chemokine application in the genital tract (pull), reduced the spread of HSV-2 infection to the sensory neurons and prevented the development of clinical disease.[132] Mucosal signaling also appear necessary to recruit anti-tumor T cells after systemic vaccination for the generation of anti-tumor protective immunity at mucosal sites. Indeed, subcutaneous (s.c) immunization against HPV-associated tumors had no significant effect in the regression of genital tumors expressing the E6 and E7 proteins, while the same vaccination associated with intravaginal administration of CpG led to the regression of more than 75% of the genital tumors in mice.[95] Other studies showed that s.c immunization with protein-based vaccine combined with all-trans retinoic acid, induced robust upregulation of gut homing receptors, resulting in T and B cells migration to the gut and protecting mice from cholera toxin–induced diarrhea.[133]

Targeting Mucosal M Cells and Dendritic Cells

To improve the efficacy of mucosal vaccines, other strategies aim to deliver antigens to M cells or mucosal DCs. M cells are one of the intestinal barriers for the efficient delivery of vaccines to mucosal tissues, thus, targeting M cells improves the transcytosis of antigen to the MALT, such as intestinal PPs. A peptide sequence, identified as having M cells directing properties, when coupled to various vaccine compounds, increased humoral immune response.[134,135] Some bacterium species, such as *Lactobacillus acidophilus*, have been engineered to express antigen fused to a DC targeting peptide.[136] After oral delivery, this vaccine strategy improves mucosal immunity and protects against a lethal anthrax challenge.[136] Similar genetically modified spores of *Bacillus subtilis* engineered to express adhesins capable to recognize receptors expressed on gut epithelial cells and M cells enhance the induction of mucosal and systemic immune responses to an encoded antigen target.[137] Our group has developed a vector, composed of the non-toxic B subunit of Shiga toxin which target DCs and favor antigen cross-presentation.[138-140] We showed that i.n delivery of antigen coupled to STxB target mediastinal LNs DC cells and induced potent mucosal $CD8^+$T cells responses and antigen specific IgA at mucosal sites.[141] In contrast to other toxin-derived delivery vehicules, we did not observe toxicity after StxB-based vaccines intranasal administration in mice during a 6 mo follow up (unpublished results). This extends the good safety profile reported by our group and others when STxB-based vaccine were administered by systemic routes.[142-145]

Particulate Delivery Systems

Vaccines administered by mucosal routes face physical, chemical, and microbiota-imposed constraints, augmenting the risk of antigen degradation. Therefore, vaccine entrapment in non-viral particules can protect the antigens from degradation or denaturation, enhance their sustained release and allow the co-delivery of antigens and adjuvants. In addition, non-pathogen based vehicules elicit a less pronounced anti-vector immunity which allows repetitive immunization. PLA (poly(lactic acid) or PLGA (poly(lactic-co-glycolic acid) nanoparticles are interesting protein carriers that offer antigen protection, increased penetration across mucosal surfaces and controlled release of encapsulated antigen.[13,146] In humans, the oral delivery of PLG-encapsulated CS6 antigen from *E. coli* induced mucosal IgA responses, but complementary studies are required to assess its potency compared with free.[147] In preclinical models, other modified lipid-based delivery systems such as liposomes, ISCOMS, virosomes, proteosomes have shown encouraging results in mucosal vaccination settings.[13,148] In healthy volunteers, the intranasal administration of a proteosome-based influenza vaccine seemed to be efficacious and well tolerated.[149]

Lastly, β-glucans composed of carbohydrate polymers found in the cell walls of fungi, yeast, plants, and bacteria, which bind to dectin-1 and CR2, have demonstrated intrinsic adjuvant activity for the enhancement of humoral and cellular.[150] β-glucan particles also act as a delivery platform for mucosal antigens. Oral administration of ovalbumin-loaded glucan microparticulates increased the levels of OVA-specific IgA, and secretory IgA in the intestinal fluids.[151]

Relevance of Inducing Mucosal Immunity against Pathogens

The mucosal immunity acquired by natural influenza infection, primarily due to the production of S-IgA in the respiratory tract, is more effective and cross-protective against subsequent viral infection than the systemic immunity induced by parenteral vaccines in humans and mice.[152] In agreement with this observation, many comparative studies of mucosal (i.n, rectal, oral) and parenteral (i.m. or s.c) administration routes with different vaccines have demonstrated the superior efficacy of mucosal routes in obtaining mucosal immune responses at local or even remote sites.[153] In these studies, the mucosal route of immunization led to higher protection levels against mucosal pathogens, such as herpes virus, influenza virus, and *Mycobacterium tuberculosis*.[154-156] In humans, the oral vaccine against poliomyelitis, based in attenuated virus strains, induces the production of secretory IgA in intestinal mucosa, preventing the infection of enterocytes and virus spreading. Instead, the inactivated version of the vaccine administered via a parenteral route protects against neurological forms of the disease, but does not affect intestinal virus replication.[30] Nevertheless, other studies have shown that parenteral vaccination may induce mucosal immunity, being effective against some mucosal infections.[157] In most cases, these studies have been performed with live viral vectors, which when administered by the systemic routes, can diffuse to mucosal sites. Nonetheless, a direct comparison of the relative effectiveness of mucosal and parenteral administration routes has not been conducted. Moreover, it is necessary to differentiate vaccines designed to induce the production of antibodies from those developed for

higher activation of CD8+T-cell immunity.[158] Indeed, antibodies induced by parenteral vaccines may be found at mucosal sites by passive transudation of circulating antibodies. This observation explains the effectiveness of certain anti-influenza or anti-HPV vaccines that are administered by parenteral routes and protect against respiratory and genital infections. Moreover, it suggests a possible greater constraint in the phenomena of recirculation of T cells than of antibodies.

Mucosal Cancer Vaccines

Various cancer vaccines have been developed against mucosal tumors, including lung cancer.[159] The vaccines Mage-A3 and Stimuvax have recently been tested in phase II and III clinical trials, respectively, but failed to demonstrate a clinical benefit in the treatment of non-small cell lung cancer.[160,161] Our group has monitored the effects of a lung cancer vaccine based on the use of a recombinant vaccinia virus encoding the Muc 1 antigen expressed by adenocarcinomas of the lung. Although activation of anti-Muc-1 CD8+ T cells were detected in the blood after vaccination, the clinical response was not significant.[159,162] One hypothesis relies on the necessity to elicit anti-tumor T cells at the tumor mucosal sites, rather than only in the peripheral blood. Since all these lung cancer vaccines were administered by the i.m. route, we tested in murine models if mucosal immunization would impact the efficacy of cancer vaccines against tumors located in the lung or in the tongue. In the first experiments, using an STxB-based vaccine, we demonstrated that the i.n route of vaccination was more efficient to induce mucosal immune responses in the mediastinal and cervical lymph node, as well as in the broncho-alveolar lavage, than i.m. immunization.[141] In line with these results, we observed that the i.n route of immunization was more efficient to inhibit the growth of head and neck or lung cancer, than the i.m. or s.c route. The i.n (mucosal) immunization also led to a more robust tumor infiltration by anti-tumor CD8+ T, cells than the i.m. route. Finally, the i.n administration of the cancer vaccine specifically stimulated the expression of the mucosal integrins CD49a and CD103 on CD8+ T cells, which was not observed following systemic immunization. Blockade of CD49a decreased both CD8+ T-cell tumor infiltration and the therapeutic efficacy of the vaccine. A link was thus established between the route of vaccination and the imprinting of a mucosal homing program on vaccine induced CD8+ T cells. Interestingly, we showed that lung DCs, but not the spleen DCs were able to induce the CD49a expression on the surface of CD8+ T cells.[141] A similar pattern of T cells homing molecules was also reported after intrapulmonary vaccination.[163] This imprinting ability of lung DCs was confirmed in humans, as it was shown that CD1c lung DCs co-cultured with CD8+T cells promote upregulation of CD103 and CD49a on these cells.[164]

Other studies support the role of mucosal immunity in protecting against mucosal tumors. The oral administration of antigen combined with cholera toxin generated CD8+ T cell responses capable of controlling the growth of gastric tumors expressing ovalbumin, whereas the same vaccine formulation did not reproduce the anti-tumor effect when administered subcutaneously.[165] The adoptive transfer of anti-tumor CD8+ T cells induced by s.c. immunization could protect against the development of subcutaneous tumors but not gastric tumors.[166] Similarly, the immunization of mice with DCs by the s.c. route allowed the control of subcutaneous tumors, but not lung metastases.[167] Also, intrarectal vaccination with the CEA antigen vectorized in a recombinant vaccinia virus enhanced mucosal CEA-specific IgA antibody titers and cytotoxic CD8+T cells activation, and more importantly, the prevention of the progression of spontaneous colorectal cancer.[168] Other studies showed that the immunization route should be tailored to each mucosal tumor site. As an example, both s.c. and intravaginal, but not i.n vaccination, induced high numbers of anti-tumor CD8+T-cells in the bladder, as well as bladder tumor regression.[169] In contrast, studies conducted by the Nardelli-Haefliger's group showed that parenteral immunization was more effective than mucosal immunization to control the growth of intrauterine tumor.[170] In this model, it is possible that other effectors, such as antibodies, play a role in the tumor rejection, as the phenomenon of compartmentalization is less evident in humoral responses. Furthermore, the intravaginal immunization route is generally less effective in inducing effector T cell responses, which can be related to the low presence of lymphoid structures in the vagina, since the majority of structures are diffuse clusters of immune cells, and also to the hormonal dependence of this route. Nevertheless, the induction of mucosal CD8+T cell response after intravaginal vaccination has been previously reported.[92] In humans, the i.m. administration of long peptides derived from the E6 and E7 HPV proteins led to the regression of high grade vulvar dysplasia. However, as no control groups were included, it is difficult to conclude the real efficacy of this vaccine.[171] Except for this last study, no cancer vaccine administered by the systemic route showed any clinical benefit in controlling genital tumors. In general, these examples show that immunizations via mucosal routes are usually more effective than immunizations performed by parenteral routes in the development of protective immunity to mucosal tumors. In humans, some positive correlations have been reported between the CD8+T cell infiltration and the prognosis of some mucosal tumors (colorectal cancer, lung tumors),[159,172] supporting the role of CD8+T cells in tumor growth control. However, human cancer vaccines targeting tumors at mucosal sites have not sought to evaluate the usefulness of the mucosal immunization routes up to now. Of course other parameters, such as the difference in the microenvironment of mucosal and non-mucosal tumors and the possibility of tolerance against self-mucosal tumor antigens, have also to be considered for the success of cancer vaccines.[144,171,173,174]

Conclusion

Prophylactic vaccines against mucosal pathogens (influenza, rotavirus, HPV..) require the presence of antibodies at the mucosal pathogen entry site, either directly induced by the mucosal

route of immunization, or after transudation of serum antibodies elicited by the conventional routes of immunization.[175] In most prophylactic vaccines, the induction of antibody leads to a good and durable protection against pathogens. Of note, in rare disease models, the induction of humoral immunity or a Th2 polarization may not be beneficial.[176,177] For chronic mucosal infection and cancer, cell mediated immunity, especially based in CD8+T cell activation, seems to be mandatory for the success of a vaccine. In contrast to the antibody induction, the level of T cell responses compartmentalization may explain the importance to elicit them directly at the tumor or chronic infection mucosal site. Mucosal vaccine strategies aiming to direct the immune response to the site of pathogen invasion or to the anatomic site of tumor location should thus maximize the efficiency of the immune responses against pathogens and tumors. Various approaches and tools, such as immunization routes and incorporation of adjuvant and/or delivery systems, are already available for the design of optimal mucosal anti-infectious or anti-cancer vaccines. Considering the infectious diseases, these systems have been broadly exploited, but will be extended in the future. Since all therapeutic cancer vaccine trials failed to provide clinical benefit, the development of mucosal cancer vaccine has to be evaluated to strengthen their efficacy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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