Bioconjugate Chemistry

Review

Cite This: *Bioconjugate Chem.* 2018, 29, 613–623

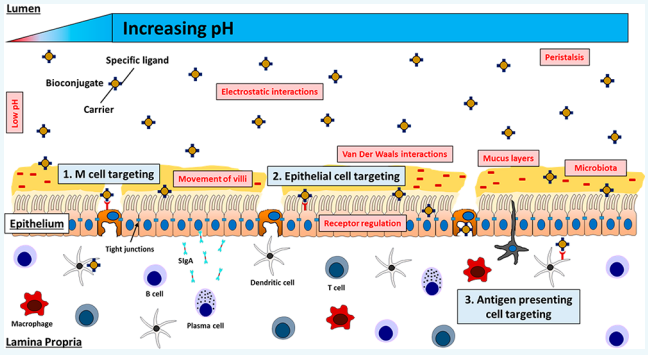
pubs.acs.org/bc

Targeted Strategies for Mucosal Vaccination

Stephanie Longet,[†] Mimmi L. E. Lundahl,[†] and Ed C. Lavelle*[,†,‡]

[†]Adjuvant Research Group, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute and [‡]Centre for Research on Adaptive Nanostructures and Nanodevices (CRANN) & Advanced Materials Bio-Engineering Research Centre (AMBER)Trinity College Dublin, D02 R590, Dublin 2, Ireland

ABSTRACT: Mucosal immune responses are in the first line of defense against most infections and protective mucosal immunity can be achieved by mucosal vaccination. However, mucosal tolerance and physicochemical features of the mucosal environment pose challenging obstacles to the development of mucosal vaccines. Vaccine formulations must be designed to enhance stability at the mucosae and incorporate features that induce innate immunity at mucosal inductive sites. To face these challenges, a number of novel delivery systems for targeting of mucosal vaccines to specific mucosal locations have been developed. In addition, specific mucosal immune cell targeting can potentially be achieved with ligand−antigen bioconjugates, in particular, those directed to specific receptors expressed on Microfold (M) cells, mucosal epithelial cells, or mucosal antigen presenting cells (APCs). In this topical review, targeted strategies to enhance the effectiveness of mucosal vaccines are addressed, and obstacles to the design and progression of effective ligand-mediated mucosal vaccines are highlighted.



1. INTRODUCTION

Vaccination against a range of infectious diseases has significantly reduced mortality and morbidity across the globe.[1] Even though most human pathogens initiate infection at mucosal surfaces, most licensed vaccines are administered by injection, which preferentially induces systemic immune responses.[2] Indeed, protective mucosal immune responses are most effectively elicited by mucosal vaccination.[3] Furthermore, mucosal vaccines are attractive by being needle-free, non-invasive, and able to improve patient compliance.[2] Despite these clear benefits, only a few live attenuated and inactivated oral and intranasal vaccines have been successfully developed for use in humans.[4,5] This is in part due to specific features of mucosal tissues that make mucosal vaccine development challenging.

Mucosal tissues maintain a fine equilibrium with the microbiota and facilitate the induction of tolerance against environmental and dietary antigens while mediating effector responses against pathogens.[6,7] Protection of these surfaces is facilitated by a combination of mechanical, physicochemical, and immunological barriers.

Mechanical and physicochemical barriers include the presence of mucus produced by goblet cells in the gastrointestinal, reproductive, and respiratory tracts as well as antimicrobial peptides produced by Paneth cells,[8] proteolytic enzymes, and low gastric pH in the gastrointestinal tract. Antigen-specific immune responses are induced in the mucosa-associated lymphoid tissues (MALTs) (e.g., Peyer's patches in the intestinal mucosa) that are covered by an epithelium which in the gut, lungs, nasopharynx, and tonsils contains Microfold (M) cells. These cells are specialized in the transport of antigens/microorganisms from the lumen to underlying dendritic cells (DCs) located in the subepithelial dome region (SED).[9,10] DCs present antigens to $CD4^+$ T cells in the interfollicular regions (IFRs). Activated $CD4^+$ T cells can subsequently support class-switch recombination and somatic hypermutation in naïve B cells in the germinal center (GC), resulting in the generation of IgA-expressing B cells. Retinoic acid plays a key role in imprinting the expression of mucosal homing molecules on IgA^+ B cells, allowing them to traffic to mucosal effector sites. At these sites, IgA^+ B cells mature into IgA producing plasma cells.[11] In addition to T-cell dependent IgA responses, T-cell independent IgA class switching can also take place by a direct action of DCs on B cells.[12] Finally, upon binding to the polymeric immunoglobulin receptor (pIgR) expressed on epithelial cells, IgA is translocated from the lamina propria into the lumen as secretory IgA (SIgA).[13,14] Under specific conditions, antigen-specific local $CD8^+$ T cell responses can also be induced (Figure 1). $CD8^+$ T cells have a cytotoxic role and are able to kill cells infected by viruses or intracellular bacteria as well as cancer cells. For instance, following oral infection by rotavirus, antigen-specific $CD8^+$ T cells can be detected within Peyer's patches.[15] Intrarectal immunization with a HIV-1 gp160 synthetic peptide vaccine and cholera toxin as adjuvant was shown to induce antigen-specific $CD8^+$ T cells

Special Issue: Bioconjugate Materials in Vaccines and Immunotherapies

Received: November 24, 2017
Published: January 4, 2018

DOI: 10.1021/acs.bioconjchem.7b00738
Bioconjugate Chem. 2018, 29, 613–623

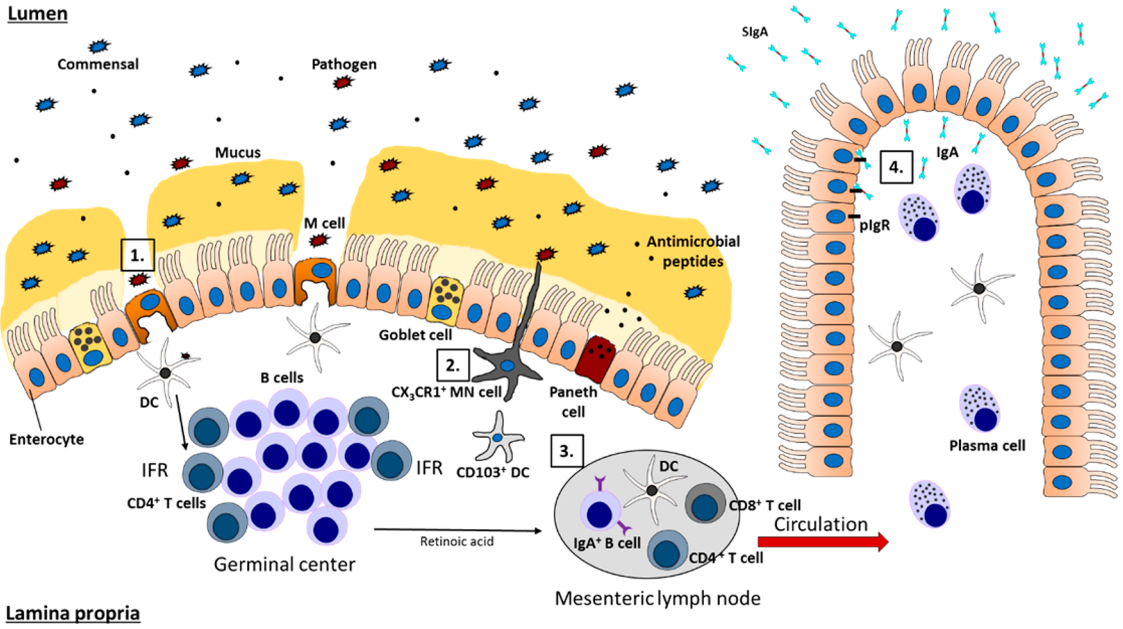


Figure 1. Induction of mucosal immune responses in gut-associated lymphoid tissues. (1) M cells sample luminal antigens and transfer them to dendritic cells (DCs). After their maturation, DCs migrate to the T cell zones (interfollicular regions, IFR). (2) CX_3CR1^+ mononuclear (MN) cells are also able to directly internalize antigens by extending their dendrites through tight junctions between epithelial cells. (3) Depending on the context (inflammation, tolerance), various cytokines are produced by DCs. (4) Activated T cells stimulate B cells and, in the lamina propria, plasma cells produce polymeric IgA molecules that are translocated as SIgA following binding to the polymeric Ig receptor (pIgR).

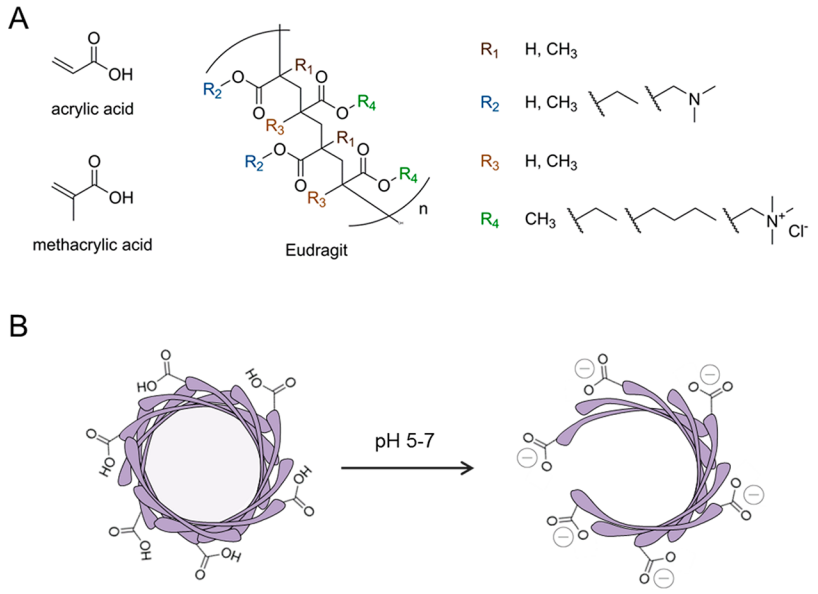


Figure 2. pH dependent dissolution of enteric coatings with Eudragit as an example. (A) Structures of various Eudragit polymers and primary monomers composed of acrylic acid and methacrylic acid. (B) Deprotonation of the Eudragit coating at a certain pH causes the carboxylic acid groups to become charged and enables polymer dissolution.

in intestinal inductive sites (Peyer's patches) and effector sites (lamina propria).[16] Recently, Hu and colleagues demonstrated the induction of protective lung-resident antigen-specific $CD8^+$ T cells after intranasal immunization of mice with a recombinant Sendai virus encoding for the *Mycobacterium tuberculosis* antigen Ag85A/B.[17]

Vaccine development is currently focused on subunit vaccines (e.g., purified proteins including toxoids, synthetic peptides, DNA, or conjugate vaccines) which are generally regarded as less reactogenic than killed or live attenuated vaccines[18] although this is challenging for the development of effective mucosal vaccines. To be successful, the design of mucosal vaccines should take into account the biology of specific mucosal tissues. Ideally, mucosal vaccines should be (1) stable in a highly enzymatic environment and resistant to site-specific pH; (2) delivered to specific immune inductive sites;[5] (3) adapted to interactions with mucus; (4) able to be transported through the epithelial barrier;[19] (5) captured by appropriate antigen presenting cells (APCs) and able to overcome mucosal tolerance. In order to face these challenges, several research groups are currently working on the identification of effective mucosal adjuvants able to enhance

DOI: 10.1021/acs.bioconjchem.7b00738
Bioconjugate Chem. 2018, 29, 613−623

the development of long-term mucosal immune responses alongside systemic immunity.[7,9] In addition, passive and active targeting strategies have been evaluated to enhance adaptive immune responses. Passive strategies are mainly based on the development of site-specific release delivery systems to direct mucosal vaccines to specific mucosal locations. These strategies are particularly useful for oral vaccines. Active strategies are based on the design of ligand-mediated vaccine delivery systems to target specific immune cells. This topical review addresses the state of the art and highlights considerations in the design and optimization of targeted vaccine strategies.

2. SITE-SPECIFIC DELIVERY SYSTEMS

Site-specific delivery systems are particularly appropriate for the delivery of oral vaccines to the gastrointestinal tract as the key sampling sites are grouped in specific anatomical locations named Peyer's patches (PPs).[20] Established technologies explored in the context of oral drug delivery are currently being tested for oral vaccine delivery.[21] Such systems may facilitate antigen protection under acidic gastric conditions and facilitate release in specific intestinal regions (e.g., jejenum, ileum). Site-specific release can be achieved through the application of pH-dependent coatings such as shellac (polyesters composed of various sesquiterpenoid acids esterified with hydroxy fatty acids, prominently aleurtic acid[22]), cellulose acetate phthalate, cellulose acetate trimellitate, poly(vinyl acetate phthalate), or hydroxypropyl methylcellulose phthalate.[23] The enteric properties of these polymers are attributed to the presence of carboxylic acid groups that are mostly protonated in water and acidic solutions, causing the formation of a water-insoluble film that is resistant to gastric juices.[24] Deprotonation of the carboxylic acids from pH 5 to 7, however, yields the water-soluble, charged carboxylate form; as such, the pK_a and notably the density of the carboxyl groups largely dictate the pH at which polymer dissolution occurs. Eudragit is one of the most common coatings used and it is derived from esters of acrylic acid and various methacrylic acids, whose physicochemical features are determined by which monomers are chosen and the subsequent nature and density of the carboxylic acid functional groups.[24] A wide range of Eudragit polymers with different dissolution properties have been developed[25] (Figure 2).

Eudragit polymers soluble in intestinal fluid (pH 6−7.4) such as Eudragit L30-D55 have been used in drug formulations including enteric coated tablets, capsules, films, nano/microparticles, and microspheres.[26] In the context of oral vaccination, delivery of Eudragit L30-D55 coated microspheres containing a *Mycoplasma hyopneumoniae* antigen to the piglet small intestine[27] induced systemic antigen-specific IgG responses. Similarly Pastor and colleagues demonstrated that oral delivery of Eudragit L30-D55 coated microparticles containing heat killed *Vibrio cholerae* O1 to rats induced high titers of serum *Vibrio cholerae* lipopolysaccharide-specific antibodies.[28] We recently demonstrated the efficacy of novel Eudragit L30-D55 coated minispheres (SmPill) containing a candidate enterotoxigenic *Escherichia coli* antigen and the adjuvant α-Galactosylceramide. Oral vaccination with this system generated higher antigen-specific serum IgG and mucosal IgA immune responses compared to the vaccine delivered to mice in solution.[29]

Colon-specific delivery systems have been also developed for local treatment of intestinal disorders such as intestinal inflammatory diseases and intestinal cancers.[30] In the context of mucosal vaccination, it is known that some pathogenic microorganisms used the vaginal and rectal routes of transmission (e.g., HIV, Herpes Simplex Virus). Intracolorectal vaccination strategy has been proposed as a strategy to drive protective immunity against pathogens of the genital tract and rectum. One established approach for colon targeting is the exploitation of colon-resident bacteria which produce enzymes that can digest polymers. Specifically, biodegradable delivery systems comprising azo-aromatic or acetyl derivatives of guar gum polymers are used.[31,32] Colon-specific bioadhesive polymers such as polycarbophils, polyurethanes, and poly(ethylene oxide) or pH-sensitive polymers such as Eudragit designed for colon-targeting (e.g., Eudragit FS 30 D) have also been explored for colon-targeted drug delivery.[30] In the context of oral vaccination, encapsulation of the antigen PCLUS3−18IIIB, which is a $CD4^+$ T cell helper epitopes fused with HIV Env $CD8^+$ T cell epitope, and Toll-like Receptor (TLR) agonist ligands (MALP2+poly(I:C)+CpG) in poly(lactic-*co*-glycolic acid) (PLG(A)) particles coated with Eudragit FS 30 D has been shown to induce antigen-specific $CD8^+$ T cell responses in the large intestine and antibody-mediated resistance to virus infection in the vaginal and rectal tracts.[33]

3. LIGAND-MEDIATED VACCINE DELIVERY SYSTEMS

While site-specific delivery technologies can help to direct vaccines to discrete mucosal regions, directing vaccine components to specific target cells can potentially be achieved with ligand−antigen bioconjugates, in particular, those directed to specific receptors expressed on M cells, mucosal epithelial cells, or mucosal APCs.

M Cell Targeting. Most surface proteins and lipids on cell membranes are glycosylated. The ability of plant lectins to specifically bind to glycans and thus facilitate bioadhesion has made them an attractive candidate for mucosal drug delivery.[34] In the context of mucosal vaccine delivery, one of most studied plant lectins is *Ulex europaeus* agglutinin-1 (UEA-1) which can bind to α-L-fucose residues expressed on murine M cells. This lectin has been conjugated to several carriers and the efficacy of these systems has been tested in preclinical models. Oral administration of UEA-1 conjugated to PLG(A) particles loaded with Hepatitis B surface antigen led to enhanced antigen-specific SIgA responses in mucosal secretions compared to responses elicited by nonlectinized particles in mice.[35] Enhanced mucosal and systemic immune responses against HIV peptides entrapped with UEA-1 in PLG particles was observed after intranasal vaccination of mice.[36] UEA-1 was covalently linked to polymerized liposomes[37,38] and was found to result in a significant increase of liposomal M-cell targeting in mice. We recently reported that polystyrene nanoparticles attached to UEA-1 or a UEA-1 mimetic through an avidin−biotin linker enhanced cellular immunity and protection against *Staphylococcus aureus* challenge following nasal coadministration with the antigen clumping factor A (ClfA). It was also shown that lectin targeting of the particles enhanced the effectiveness of nasal and oral vaccination with the antigen ovalbumin (OVA) and the system selectively enhanced cellular as opposed to humoral antigen-specific immunity.[39] As many mucosal pathogens gain entry to the host through M cells, M cell receptors used by bacteria have also been studied. For instance, *Escherichia coli* and *Salmonella enterica* serovar *typhimurium* are able to bind glycoprotein 2 (GP2) which is expressed on the apical plasma membrane of murine and human intestinal M cells, through FimH, a component of type I

DOI: 10.1021/acs.bioconjchem.7b00738
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Table 1. Overview of Ligands Used for M Cell-Targeting of antigens

ligands	receptors on M cells	carriers/fusions	administration routes/ models	delivery/immune responses	refs
Ulex europaeus agglutinin-1 (UEA-1)	α-L-fucose	PLGA particles	Oral (mouse)	- Mucosal SIgA, - Splenic Th1 responses	35
UEA-1	α-L-fucose	PLG particles	Intranasal (mouse)	- Systemic IgA/IgG - Mucosal IgA responses	36
UEA-1	α-L-fucose	Liposomes	Gut loop model (mouse)/*in vitro* assays	- Delivery - Binding	37,38
UEA-1	α-L-fucose	Polystyrene nanoparticles	Intranasal (mouse)	Protection against bacterial challenge	39
FimH (*Escherichia coli, Salmonella enterica*)	GP2	----	*In vitro* binding assay (mouse)	Delivery	40
OmpH (*Yersinia enterolitica*)	C5aR	Fusion with antigen enhanced green fluorescent protein (EGFP)	*In vitro* model/Oral (mouse)	- Binding - Systemic IgG and faecal IgA responses	55
Anti-glycoprotein 2 (GP2)	GP2	Fusion with ovalbumin (OVA) antigen	Ileal loop model/oral (mouse)	- Delivery - Faecal SIgA responses - Protection	41
C-terminal domain of *Clostridium perfringens* enterotoxin	Claudin-4	PLGA particles/Nanoparticles or fusion with hemagglutinin (HA)	Intranasal (mouse)	- Serum IgG - Mucosal IgA responses	53,54
Reovirus protein σ1	Junctional adhesion molecule-A (JAM-A)	Conjugation with polylysine and DNA vaccine	Intranasal (mouse)	- Serum IgG - Mucosal IgA responses - Cellular responses	56
RGD motif	β_1-integrin	PLGA-based nanoparticles	*In vitro* model/oral (mouse)	Serum IgG titers	42
Tetragalloyl-D-lysine dendrimer	Fucose receptor ?	No carrier	Inoculation at a site of the ileum (macaque)	Faecal IgA responses	43
CKSTHPLSC (CKS9)	Unknown	Nanoparticles	*In vitro* assays/Ileal loop (rat)	Binding	44
Peptide Co1	C5aR	Fusion with EGFP	*In vitro* assays/Ileal loop/oral (mouse)	- Binding - Serum IgG - Faecal IgA responses - Splenic and PP responses	57
Peptide motif (GWKERLSSWNRF)	GM1 ganglioside	Fusion with EGFP	*Ex vivo* intestine/oral (mouse)	- Binding - Mucosal and systemic immune responses	58
Antibody NKM 16-2-4	α (1,2)-fucose-containing carbo-hydrate moiety	Fusion with botulinum toxoid	Intestinal loop/oral (mouse)	- Serum IgG - Mucosal IgA responses	48
Antibody 5B11	Unknown	Polystyrene microparticles	Ileal loop model (rabbit)	Binding	47
Secretory Immuno-globulin A (SIgA)	Dectin-1	Fusion with p24 HIV antigen	Ileal loop model/oral (mouse)	- Binding - Serum and mucosal IgG and IgA responses - T cell responses - Protection	52

pili on the bacterial outer membrane.[40] Conjugation of an anti-GP2 monoclonal Ab (mAb) to OVA resulted in effective M cell targeting and oral vaccination with this system triggered enhanced faecal OVA-specific SIgA responses compared to the antigen alone.[41] In addition to lectins or bacterial ligands, the efficacy of other types of M cell targeting molecules has also been investigated. After oral administration, the tripeptide RGD motif exhibited binding to β-integrins on M cells[42] and a tetragalloyl-D-lysine dendrimer (TGDK) targeting murine, human, and nonhuman primate M cells[43] led to enhanced antigen-specific serum IgG responses in mice[42] and faecal antigen-specific IgA responses in macaques,[43] respectively. The M cell targeting capacity of the peptide CKS9 identified by phage display technique[44] was demonstrated in a rat ileal loop assay. Nakato and colleagues demonstrated that a comparative transcriptomic analysis of the follicle-associated epithelium (FAE) could help to identify novel M cell-specific molecules that could be targeted. A comparative gene expression profiling of chicken and mouse FAE revealed that cellular prion protein (PrP^c) was expressed on the luminal side of the apical plasma membrane of murine M cells.[45] PrP^c was shown to be used by *Brucella abortus* to invade the host through M cells in a mouse model.[46] Consequently this protein could also be a potential vaccine target. The efficacy of M cell-specific antibodies has also been analyzed. Conjugation of the anti-M cell antibody 5B11 to polystyrene microspheres enhanced particle uptake by rabbit M cells,[47] while oral vaccination with a conjugate of the NKM 16-2-4 antibody to botulinum toxoid (BT) enhanced BT-specific serum IgG and mucosal IgA responses as well as protective immunity against lethal challenge with BT in mice.[48] Furthermore, it has been demonstrated that SIgA-based HIV-1 p24 antigen complexes were taken up by M cells through DC-associated C-type lectin (Dectin)-1 receptor in mice.[49] Dectin-1 has been described as a small type II transmembrane protein

DOI: 10.1021/acs.bioconjchem.7b00738
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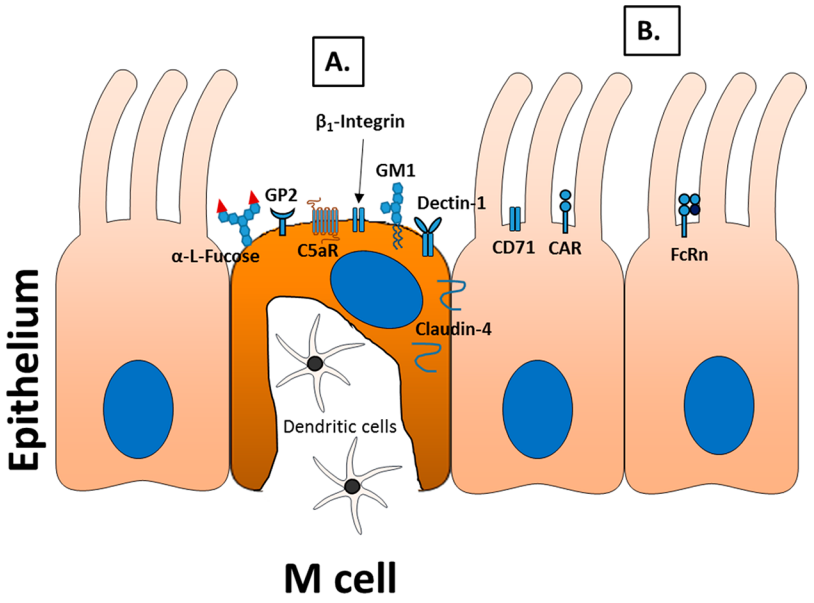

Figure 3. M cell and epithelial cell receptors used for targeting strategies in mucosal vaccination: (A) M cell receptors and adhesion molecules: α-L-fucose moieties are recognized by *Ulex europaeus* agglutinin-1 and the mAb NKM 16-2-4. Glycoprotein 2 (GP2) can be targeted by adhesins such as FimH expressed on some bacteria (*Salmonella enterica* serovar *typhimurium*, *Escherichia coli*) or the anti-GP2 mAb. OmpH expressed on bacteria such as *Yersinia enterocolitica* and the ligand peptide "Co1" are able to interact with the C5a receptor (C5aR). β_1-Integrin can be targeted by a tripeptide based on the RGD motif. The GM1 ganglioside receptor, which can bind cholera toxin, can be targeted by small peptides designed as ligands. The C-terminal domain of *Clostridium perfringens* enterotoxin has been used to target claudin-4 expressed in the cytoplasm of mouse and human M cells. Finally, Dectin-1 has been recently described as a SIgA receptor which mediates the transport of SIgA-immune complexes suggesting the possibility of using SIgA as a carrier. (B) Epithelial cell receptors: Transferrin can be used as a ligand to target CD71. IgG Fc has been tested as a carrier to transport antigens through the neonatal receptor (FcRn) and Coxsackie-adenovirus receptor (CAR) is able to bind the Adenovirus 2 fiber protein (Ad2F) ligand.

of the C-type lectin family, expressed by DCs, macrophages, and neutrophils.[50] This receptor recognizes β-glucans in fungal cell walls and transduces signals triggering antimicrobial activity such as phagocytosis and production of reactive oxygen species.[51] Its presence on M cells was shown to play a role in SIgA capture and internalization.[49] As SIgA is naturally adapted to the mucosal environment, the use of this antibody as a carrier for mucosal vaccine delivery is of significant interest. It was demonstrated that oral vaccination of mice with the p24 HIV antigen chemically bound to SIgA resulted in rapid delivery into intestinal tissues and the induction of enhanced systemic and mucosal antibody and cellular responses.[52]

Finally, the potential of directing antigens to an intracellular M cell target has also been tested. Nasal vaccination of mice with a conjugate between the C-terminal domain of *Clostridium perfringens* enterotoxin that binds claudin-4 expressed in the cytoplasm of mouse and human M cells, and influenza hemagglutinin (HA) induced higher HA-specific serum and mucosal antibody responses compared to HA alone.[53,54] An overview of ligands used for M cell-targeting of antigens is reported in Table 1 and Figure 3.

In order to design successful M cell targeting strategies, specific M cell characteristics should be taken into account: (1) glycosylation patterns on M cells differ between intestinal locations, ages and, species.[59] So far, most conjugates have been tested in animal models but the use of human biopsies can help to determine receptors expressed on human M cells; (2) the low number of M cells (e.g., 5% of PP cells in follicle-associated epithelium in humans and 10% in mice) may reduce the probability of interactions between the bioconjugates and M cells; (3) as excellently highlighted by Lo in 2013,[19] interactions between the vaccine particles in suspension and the intestinal surfaces especially short-range van der Waals interactions profoundly differ between the M cell surface and those of neighboring epithelial cells due to the lack of microvilli[59,60] and reduced mucus thickness;[61] (4) the size of bioconjugates, which may be related to the length of linkers, should be carefully studied in order to optimize their uptake by M cells which can transport microparticles of up to 1 μm in size.[62]

Mucosal Epithelial Cells. Receptors differentially expressed on mucosal epithelial cells have also been explored for bioconjugate targeting in the context of mucosal vaccination. The natural transcytotic and recycling transferrin receptor (CD71) located in the nasal epithelium was used to target a trimeric HIV gp140 antigen conjugated to transferrin. Nasal vaccination with this conjugate induced high titers of antigen-specific serum IgG as well as female genital tract IgA and IgG.[63] Staats and colleagues tested the ability of Adenovirus 2 fiber protein (Ad2F) to target enterocytes through the Coxsackie-adenovirus receptor (CAR). They reported enhanced immunogenicity of a botulinum neurotoxin A antigen fused to Ad2F when nasally administered to rabbits.[64] F4 fimbriae expressed on enterotoxigenic *Escherichia coli* can efficiently target receptors expressed on the surface of porcine enterocytes and have been suggested as a potential porcine oral vaccine candidate.[65−68] However, not all species express F4 receptors. In contrast, the neonatal Fc receptor (FcRn) has been described in several species including humans, primates, pigs, ruminants, rabbits, and mice and is expressed in several tissues including the lung, placenta, vascular epithelium, kidney, mammary glands, and the intestinal epithelium. This receptor binds the Fc portion of IgG with high affinity at pH < 6.5. FcRn facilitates the transport of IgG from mother to fetus

DOI: 10.1021/acs.bioconjchem.7b00738
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Table 2. Bioconjugates for Targeting Mucosal Epithelial Cells

ligands	receptors	conjugates	administration routes/models	immune responses	refs
Transferrin	CD71	Fusion with HIV gp140	Intranasal (mouse)	- Serum IgG - Vaginal IgG and IgA responses	63
Adenovirus 2 fiber protein (Ad2F)	Coxsackie-adenovirus receptor (CAR)	Fusion with botulinum neurotoxin A	Intranasal (rabbit)	Serum IgG responses	64
Adenovirus 2 fiber protein (Ad2F)	Coxsackie-adenovirus receptor (CAR)	Fusions with OVA and botulinum neurotoxin A	Sublingual (mice)	- Serum IgG/IgA responses - Mucosal IgA responses - Increase of cytokine-forming cells	73
Mouse IgG Fc fragment	FcRn	Fusion with herpes simplex virus type-2 glycoprotein gD	Intranasal (mouse)	- Serum IgG - Local B/T cell responses - Protection	70
Mouse IgG Fc fragment	FcRn	Fusion with HIV Gag (p24) protein	Intranasal (mouse)	- Serum and mucosal IgG responses - T and B cell responses - Protection	71
Human IgG Fc fragment	FcRn	Fusion with a protein containing a receptor binding domain of Middle East Respiratory Syndrome Coronavirus	Intranasal (mouse)	- Serum and local IgG/IgA - T cell responses	72

through placental or intestinal routes and transports IgG across mucosal surfaces in adult life suggesting the possibility to use IgG Fc as a carrier of antigens across the mucosal epithelial barrier.[69] Ye and colleagues fused the antigen, herpes simplex virus (HSV) type-2 glycoprotein gD, to an IgG Fc fragment and demonstrated that its nasal administration to mice could elicit mucosal B cell responses, $CD4^+$ and $CD8^+$ T cell responses as well as protect from intravaginal HSV-2 challenge[70]). A fusion between HIV Gag (p24) protein and the Fc region of IgG was also shown to induce mucosal and systemic Gag-specific IgG and $CD4^+$ and $CD8^+$ T cell responses as well as protection against an intravaginal challenge with vaccinia virus expressing the HIV Gag protein, after intranasal vaccination.[71] Ma and colleagues demonstrated that nasal delivery of a fusion between a protein containing a receptor binding domain of Middle East Respiratory Syndrome Coronavirus and the Fc region of human IgG to mice enhanced antigen-specific systemic and mucosal antibody responses as well as cellular responses.[72] An overview of bioconjugates for targeting mucosal epithelial cells is reported in Table 2 and Figure 3.

Epithelial cell targeting may be an advantage compared to M cell targeting due to the higher number of such cells, providing a greater number of targets for bioconjugates to interact with. However, the movement of villi and the presence of thick mucus layers on mucosal epithelial cells can modify the kinetics of bioconjugate diffusion. In addition, rapid epithelial cell turnover as well as the regulation of site- and species-specific expression of conjugate targeted receptors should be considered. In the context of oral vaccination, given that intestinal enterocytes are able to absorb particles smaller than <500 nm by endocytosis,[74] the size of bioconjugates should be adjusted for the uptake by intestinal enterocytes.

Bioconjugates for Targeting Antigen Presenting Cells. Given the essential role of APCs, and especially DCs, in the initiation of adaptive immune responses, the potential to directly target DCs by designing *in vivo* DC targeted vaccines has been considered. However, most bioconjugate vaccines have been tested through parenteral routes of administration. Some conjugates between antigens and Pattern Recognition Receptor (PRR)-targeting TLRs have been explored including TLR9-[75−77] and TLR5-ligands.[78] Ligands targeting C-type lectin receptors (CLRs) or Fc receptors expressed on DCs have also been evaluated. DC-SIGN expressed on DCs was targeted with fusions of Lewis X oligosaccharides and antigens (e.g., OVA, Heparanase, HIV gp120) and this approach resulted in induction or enhancement of systemic $CD4^+$ and $CD8^+$ T cell responses in mice.[79,80] Conjugates between antigens and mAbs targeting DEC205/CD205,[81] DNGR-1,[82] or CD11c[83] have been shown to activate DCs and elicit T cell responses and/or antigen-specific antibodies. Ruane and colleagues demonstrated that this approach could also be used in a mucosal context. Indeed nasal vaccination of mice with a fusion of an anti-CD205 antibody and the HIV p24 antigen, elicited HIV-specific effector and memory T cell responses locally and at distant mucosal sites including the gut.[84] Recently, the receptors Dectin-1 and SIGNR3 expressed on intestinal $CD11c^+CD11b^+MHCII^+F4/80^-CD8^-$ DCs were reported to bind SIgA and led to uptake of bacteria coated with SIgA into these cells.[85] Consequently, SIgA used as a carrier for mucosal vaccine delivery could also help to target DCs in mucosal tissues.

Macrophages also play a critical role in innate immunity. For example, mannose motifs found on the membrane of bacterial, viral, fungal, and parasitic pathogens could be potential ligands to specifically target mucosal macrophages. Indeed macrophages express a number of PRRs such as CLRs (e.g., Mannose Receptor (CD206), Dectin-2 and macrophage-inducible C-type lectin (Mincle) as well as TLRs including TLR-2 and -4 which can recognize these motifs.[86] In the context of mucosal vaccination, mannose-modified microspheres loaded with *Pseudomonas aeruginosa* outer membrane protein were shown

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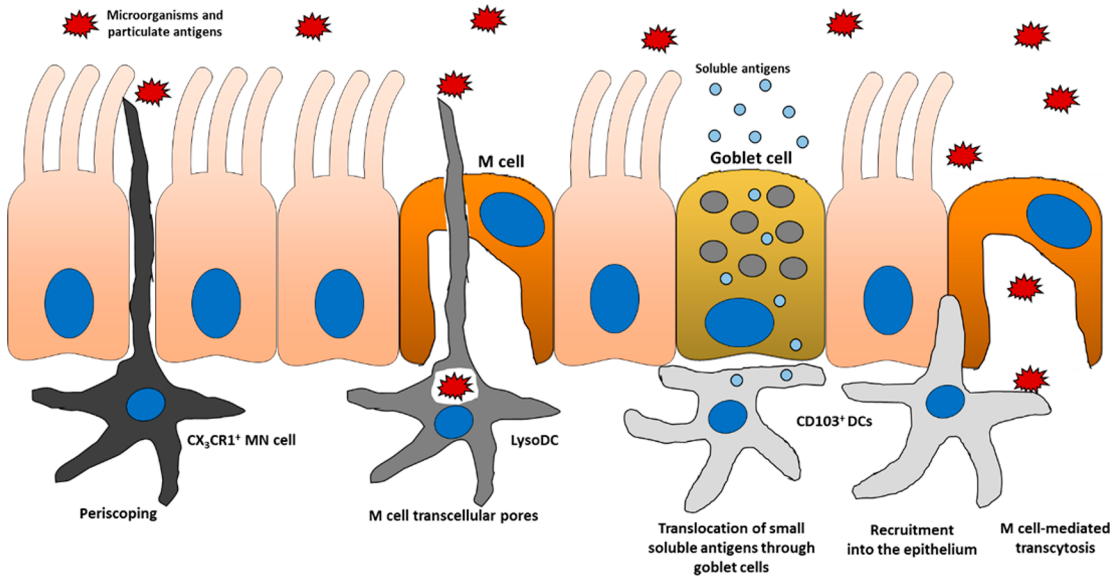

Figure 4. Luminal antigen sampling in the intestine by CX_3CR1^+ MN, $CD103^+$ DCs, and LysoDCs CX_3CR1^+ MN cells are able to insert their dendrites between enterocytes to directly sample the luminal content. LysoDCs have the ability to extend their dendrites through transcellular pores in M cells. $CD103^+$ DCs may be recruited into the gut epithelium to sample bacterial antigens via M cell-mediated transcytosis or by using their dendrites. Soluble antigens can be delivered to $CD103^+$ DCs through goblet cells. These DC subsets might be targeted by oral vaccines.

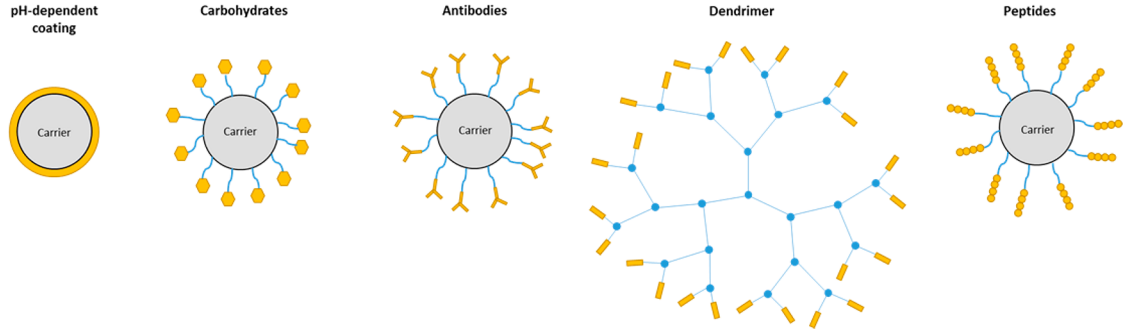

Figure 5. Examples of strategies used for targeting mucosal vaccines Some pH-dependent coatings can be used to deliver vaccines into specific mucosal locations. In addition, specific receptors or moieties can be targeted by ligands. For this purpose, different types of molecules can be used such as carbohydrates, antibodies, or peptides. These molecules can be linked to a carrier such as particles or liposomes but can also be directly conjugated to an antigen. Constructs such as dendrimers have also been tested to deliver mucosal vaccines.

to target macrophages through the Mannose Receptor. Intranasal immunization with this system triggered antigen-specific SIgA responses in nasal washes, bronchoalveolar and intestinal lavages, as well as serum IgG.[87]

The main challenge for mucosal APC targeting strategies is the accessibility of these cells. In the gastrointestinal tract, CX_3CR1^+ mononuclear cells are able to extend their dendrites between epithelial cells into the lumen to directly sample microorganisms and soluble bacterial antigens.[88] In addition, intraepithelial $CD103^+$ DCs can be recruited into the intestinal epithelium by luminal bacteria and can sample microorganisms via M cell-mediated transcytosis.[89] This DC subset is also able to phagocytose bacteria using intraepithelial dendrites and sample bacterial antigens for presentation. However, $CD103^+$ DCs do not sample soluble antigens efficiently.[90] Soluble antigens can be delivered to $CD103^+$ DCs in the lamina propria through goblet cells.[91] Interestingly, CX_3CR1^+ mononuclear cells can also directly transfer soluble antigens to $CD103^+$ DCs.[92] LysoDCs, a subset of DCs expressing high levels of lysozymes and having a strong phagocytic activity, can extend their dendrites through M cell-specific transcellular pores to the intestinal lumen[93] (Figure 4). Consequently, a suggested strategy for oral vaccination might be to target these particular cell subsets by using specific ligands.

4. CONSIDERATIONS IN THE DESIGN OF BIOCONJUGATES FOR MUCOSAL VACCINATION

Targeted mucosal vaccines allow the potential for enhanced and directed control of antigen/adjuvant delivery. This could lead to a reduction of vaccine doses and subsequently a diminution of adverse effects.[94] However, this point is dependent on intrinsic effects of the targeting ligands; for example, some plant lectins can exert toxic effects at high doses.[95] However, many lectins are components of the diet and the use of lectin mimetics is an approach to avoid use of the native molecules.

The complexity of mucosal tissues and their specific features can provide challenging obstacles to the design of effective ligand-mediated mucosal vaccines. The choice of passive or active targeting strategies is a primary issue (Figure 5). Indeed for some mucosal vaccines a site-specific delivery system may be sufficient and could reduce costs in comparison to the

DOI: 10.1021/acs.bioconjchem.7b00738
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design of specific bioconjugates. If an active strategy is selected, the design of conjugate will be based on the nature of ligand, carrier, and method of conjugation.[96] The density and spatial orientation of ligands on the carrier may also impact on the binding of conjugates to the target and subsequent immune responses generated.[97] To our knowledge, no studies have yet evaluated these potential differences in mucosal vaccine models. For instance, an increased number of lectin-based ligands on a carrier increases overall binding avidity and thus raises the probability of binding to a specific receptor but could also dramatically augment the risk of nonspecific binding to glycans found in mucus and consequently reduce the number of bioconjugates reaching the targeted receptors. Excessive ligand density can moreover negatively impact interactions with the target receptor[98] and furthermore augment aggregation. In addition to these variables, different outcomes may be expected if the targeted carrier is, for example, a liposome, a protein, a particle, or a dendrimer. The shape of the carrier, its size, and its charge play a role in interactions with cells.[99] The charge and size of carriers had a significant impact on their diffusion coefficient in mucus.[100] In addition, the size of bioconjugates should be considered depending on the cells which are targeted and their capacity for carrier uptake.[101] For instance, the length of linkers between the carrier and the ligand may be adjusted according to the characteristics of the targeted cells.

When the carrier is a protein, the stability of fusions should be evaluated. For example, the presence of enzymes may cause the degradation of bioconjugates, particularly in the gastrointestinal tract. In addition, pH variations impact on the charge of peptides and may lead to peptide denaturation and/or aggregation,[102] which may modify interactions between the peptide bioconjugates and the receptors, as well as other mucosal components.

Consequently, bioconjugate design must be optimized to reach and facilitate uptake by the target cells. For example, in the intestine, if the targeted cells are M cells or intestinal epithelial cells, facilitating subsequent uptake by DCs to mount efficient immune responses should also be considered. When the targeted cells are APCs, the transcytosis of the bioconjugate into the lamina propria should be taken into account.

5. CONCLUSIONS AND PERSPECTIVES

Ligand−antigen bioconjugate mucosal vaccines have clear benefits in directing antigen/adjuvant delivery into specific mucosal locations and can induce protective immune responses. To design the most effective ligand-mediated mucosal vaccines the nature of the ligand, carrier, and method of conjugation, as well as the size of the bioconjugate, its charge and the shape of the carrier should all be considered. Furthermore, the use of relevant animal models to test the bioconjugates is also essential. Currently, most constructs are tested in rodents. However, it is known that glycosylation patterns and receptors may be species-specific. For the development of veterinary ligand−antigen bioconjugate mucosal vaccines, the target species should be used: such as pigs, sheep, or cattle. However, for human targeted mucosal vaccines, it is less straightforward. A screening of receptors by using human intestinal biopsies could help to determine optimal human mucosal receptors that could be targeted, and the use of "humanized" mice might be considered. Even though there is evidence for the efficacy of ligand−antigen bioconjugate mucosal vaccines *in vitro* and in *in vivo* models, their design for clinical application is challenging. A close collaboration between chemists, immunologists, and clinicians is crucial to design and formulate human targeted mucosal vaccines.

AUTHOR INFORMATION

Corresponding Author

*E-mail: lavellee@tcd.ie. Phone: +353 1 8962488.

ORCID

Stephanie Longet: 0000-0001-5026-431X

Ed C. Lavelle: 0000-0002-3167-1080

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors wish to thank Science Foundation Ireland (SFI) [grant number 12/IA/1421] to support mucosal vaccine work in Prof. Ed Lavelle's lab. In addition, S.L is supported by the Swiss National Science Foundation [grant number P300PB-164740] and M.L.E.L by a Trinity College Dublin awarded postgraduate studentship.

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DOI: 10.1021/acs.bioconjchem.7b00738
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DOI: 10.1021/acs.bioconjchem.7b00738
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