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REVIEW

Engineering drug delivery systems to overcome mucosal barriers for immunotherapy and vaccination

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ABSTRACT

Mucosal surfaces protect our bodies from pathogens and external irritants using a system of biological barriers. Overcoming these barriers is a significant drug delivery challenge, particularly for immunotherapies that aim to modulate the local immune response. Reaching local lymphoid tissues and draining lymph nodes (LNs) requires crossing the mucus mesh, mucosal epithelium, and either targeting M cells covering lymphoid tissues or utilizing lymphatic transport that shuttles molecules and particulates from the periphery to the LN. We first highlight the barrier properties of mucus and mucosal epithelium, and the function of the mucosal immune system. We then dive into existing drug delivery technologies that have been engineered to overcome each of these barriers. We particularly focus on novel strategies for targeting lymphoid tissues, which has been shown to enhance immunotherapies and vaccinations, via directly targeting LNs, lymphatic vessels, and M cells that transport samples of mucosal content to the lymphoid tissues.

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Introduction

Mucosal surfaces are the largest organs protecting our internal body surfaces from exposure to the external environment, preventing pathogens and macromolecules from reaching the internal surfaces of the body when the entry is undesired.[1–4] The mucosal surfaces are made up of a mucus layer covering the mucosa, or mucosal epithelium, mucosal immune cells and lymphoid organs, and underlying blood and lymphatic vasculature. All of these are tightly regulated and form a formidable barrier against pathogens and particulates.[3,5–7] Firstly, mucus has a tight mesh structure, and the charged mucins effectively trap positively charged particulates and pathogens.[2] Secondly, the mucosal epithelium tightly regulates the transport of molecules, such as, e.g. digested food products in the GI tract, ensuring no pathogen can cross into the body's interior. And thirdly, the local immune system, including immune cells located in the mucosal-associated lymphoid tissues and lymph nodes, destroys pathogens upon encounter.[1] While the mucosal surfaces provide a *desired* barrier for pathogens, they also form a barrier to nanoparticle-based drug delivery: any particulate needs to first successfully pass through by mucus and epithelium, to reach its therapeutic target. For immunotherapies and vaccination at mucosal surfaces, getting to the local immune cells and lymph nodes is key to induce the desired immune response.[1,8–11] Recognizing this, researchers in the past 10+ years have set out to design carriers to overcome the mucus barrier, pass through the epithelium, and target lymph nodes and lymphoid structures through a variety of mechanisms. This review first summarizes the relevant barriers and targets for immunotherapy and vaccination at the mucosal surfaces, and then delves into existing drug delivery technologies that have been engineered to overcome these barriers.

Overview of mucosal immunity

Mucosal surfaces are constantly exposed to microbes – both commensal and pathogenic and a fine balance has to be struck between protecting the body from the pathogenic microbes to not eliminating the commensal ones at the same time. Generally, when no pathogenic microbes are around, the immune response is downregulated at the mucosal surfaces through a variety of immune mechanisms.[12] In this section,

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we will discuss the role of the physical barriers, the immune cell composition, and their various functions at mucosal surfaces (Figure 1).

Mucus is our first line of defense at the mucosal surfaces, effectively trapping pathogens and particulates that are rapidly cleared and expelled through the mucus clearance mechanisms that evolved to further prevent pathogens and harmful particulates from reaching the surfaces of our mucosal epithelia. Mucus is a porous hydrogel that takes advantage of size and electrostatic and hydrophobic interactions to trap microbes and particulates, and also some secreted antibodies.[5,13,14] Mucus is made up of mucin fibers, peptidoglycans 0.3–2 MDa in size with an overall negative charge.[2,5] This effectively traps many pathogens or particulates that have a positive charge. In addition to the glycosylated regions, the mucin peptides also contain hydrophobic regions that bundle mucin fibers into cables, and effectively trap hydrophobic particulates.[2,5] Mucin fibers are linked together to form fibers several microns long, leading to a gel-mesh that excludes particulates of larger sizes. The types of components, their ratios, the mucin type, the mucus turnover rate, and the mucus thickness vary at the different mucosal surfaces, and some of the specific composition particularly mucin fibers are summarized in Table 1 and have been discussed in several other review articles.[2,5,6,34,49] A second protective property of mucus is its lubrication. Mucus gels are shear-thinning, which occurs when a slippage plane forms between two moving surfaces, such as the epithelium and food bolus moving through the intestine,[5] and provides lubrication that serves to prevent any mechanical damage that could be induced, e.g. during digestion, the closing of eyes, or sexual intercourse. Thus, mucus is the first physical barrier at mucosal surfaces, protecting both from mechanical damage, and trapping particulates and pathogens, thus preventing them from reaching and entering the mucosal epithelium.

The **mucosal epithelium** is the next barrier that needs to be overcome. The mucosal epithelium is a mostly non-keratinized epithelium (except in some areas, such as the oral cavity) that is highly regulated to prevent pathogens from entering the body.[3,12] It

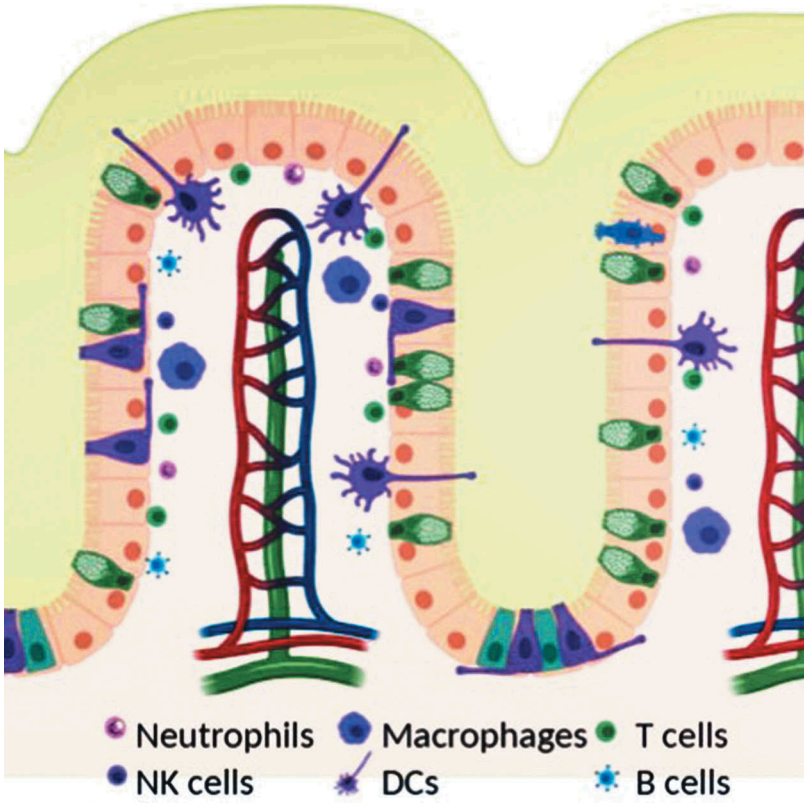

Figure 1. Schematic of mucosal surface including epithelium (orange), blood vessels (red and blue), lymphatic vessels (green), and immune cells (see legend). Created with BioRender.com.

Table 1. Mucus composition at different mucosal surfaces.

Mucosal Surface	Secreted Mucins	Adherent mucins	Other significant components	Reference
Respiratory tract	MUC2, MUC5AC, MUC5B, MUC19, MUC7	MUC1, MUC4, MUC12, MUC15, MUC20	Microbes, surfactants, phospholipids, immunoglobulins	15–25
Stomach	MUC5AC, MUC6	MUC1, MUC4, MUC12, MUC13, MUC15, MUC17	HCl, phospholipids	16,20,21,26–28
Small intestine	MUC2, MUC6	MUC1, MUC3A/B, MUC12, MUC13, MUC15, MUC17	Bile salts, phospholipids, microbes, immunoglobulins	21,25,27-33
Large intestine	MUC2	MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC17, MUC20	Microbes, phospholipids, immunoglobulins	16,20,21,25,28–35
Cervicovaginal tract	MUC5AC, MUC5B, MUC6	MUC1, MUC4, MUC12, MUC16	Lactobacilli and other microbes, immunoglobulins	16,17,20,25,27,36–42
Eye	MUC2, MUC5AC, MUC19	MUC1, MUC4	Antimicrobial peptides, immunoglobulins	16,20,25,43–48

serves varying functions at the different mucosal surfaces. For instance, in the lungs, the mucosa protects against pathogens in the upper airways, while it allows gas exchange in the lower airways. In the gastrointestinal tract, the mucosa not only protects us from our own microbiome infecting the tissue, but also is responsible for nutrient absorption.[4] To perform these functions and regulate the unwanted entry of pathogens or molecules, the epithelium contains numerous tight and cell-cell junctions that form a sort of fence between the material or pathogen attempting to cross to the underlying tissue. Cell-cell and tight junctions serve as the primary barrier for paracellular transport of material or pathogen attempting to cross the mucosal epithelium. Claudins are a key component of epithelial tight junctions and primarily reside at the most apical side of the junction.[50] There are over 20 different types of claudins expressed in humans, with many types present on epithelia at mucosal surfaces.[51] Claudin-1 can be found in the intestinal epithelium and is widely implicated in strengthening tight-junction barrier properties. In the colon, Claudin-3 serves to limit the transport of solute through this epithelial layer, however, when expressed in the lung alveolar epithelia an opposite effect on the regulation of paracellular transport is shown.[52,53] It is naturally unsurprising then that many drug delivery strategies have been designed to interface with these claudin proteins to improve penetration through the epithelial barrier. Two clinically available claudin-disrupting molecules are sodium caprate and mannitol, that when administered enhance paracellular absorption of a drug.[54] In addition to being a physical barrier, epithelial cells can sense their microenvironment, e.g. through receptors for pathogen-associated molecular patterns and can secrete 'danger signals' to activate an immune cascade such as pro-inflammatory cytokines and also antimicrobial peptides to initially fend off infection. The epithelium thus is the first line of *cellular* defense against pathogens.

In aid to the epithelial cells come the various **immune cells** present within the epithelium, also known as intraepithelial lymphocytes (IEL), and the lamina propria (Figure 1). The dominant cell type within the epithelial layer are CD8 + T cells that have an effector or memory phenotype.[55,56] In the gut as many as 5–15 lymphocytes can be found for every 100 epithelial cells in this layer. The lamina propria contains a variety of different immune cells including plasma cells, mainly responsible for producing the large quantities of antibodies present in the mucus and throughout the mucosal surfaces, conventional CD4+ and CD8 + T cells, dendritic cells, macrophages, innate lymphoid cells, and mast cells.[1,57,58] The dominant cell type here is CD4 + T cells and plasma cells.[12] Antibodies produced by plasma cells are secreted into the mucus gel via transcytosis from the basolateral side of the epithelium.[58] The predominant type is IgA, which has been shown to interact with the mucus gel.[42,59] In fact, antibodies secreted into mucus greatly enhance the ability to trap pathogens and toxins in mucus gel: They diffuse rapidly through the gel, retarded only slightly by transient, low-affinity bonds with the mucus gel.[60] However, when they accumulate on the surface of a pathogen they form enough multivalent adhesive interactions with the gel to trap the pathogen, thus serving their purpose of preventing infections.[61,62]

When foreign bodies are encountered at the mucosal surfaces and they, or their debris make it

past the mucus gel, their antigens will be taken up by **antigen-presenting cells**, including both traditional cells such as **dendritic cells** and **macrophages**, as well as non-traditional cells such as M cells on Peyer's patches in the GI tract. Macrophages at the mucosal surfaces primarily phagocytose and scavenge antigens, and help maintain antigen-specific tolerance locally by the production of tolerogenic factors such as IL-10.[1,12,25,58] Unlike in other peripheral tissues, macrophages at mucosal surfaces often do not migrate to the lymph nodes, but instead stay in the tissue to perform their tissue-specific responses.[56] These are particularly important for maintaining the 'down-regulated' state of the immune response so that there is no response formed to commensal microbes.[56] Dendritic cells, in contrast, scavenge antigen and migrate to the local draining lymph nodes for T cell education.[63,64] DCs not only phagocytose antigens, but also can acquire antigens from the non-traditional antigen-presenting cells. When an infectious agent is detected, epithelial cells secrete factors that will recruit DCs into the epithelial layer, sample antigens, and proceed to migrate to the LNs.[58] In the absence of an infectious agent, the same migrating DCs present antigens and educate **T cells** to form regulatory T cells that help maintain the tolerogenic environment toward commensal microbes.[12,56,58,65] In the lymph nodes, antigen-specific T cells are educated and these begin to expand and circulate back to the mucosal surface. Once pathogens are encountered some of these T cells stay behind as effector/memory lymphocytes.[56] In fact, at mucosal surfaces effector and memory lymphocytes as well as plasma **B cells** are the dominant types found even when no infectious agent is present.[55,56] This likely accounts for the ability to mount a quick immune response upon re-encounter of pathogens.

In addition to the classic immune response, mucosal surfaces have secondary lymphoid tissues directly associated with them, usually called **mucosal-associated lymphoid tissues**, or MALT. These include Peyers patches in the GI tract, bronchus-associated lymphoid tissues in the lungs, tonsils, adenoids, and other gut-associated lymphoid tissues.[12,58] These organs serve to rapidly provide an adaptive immune response to pathogens, serving to educate T and B cells into antigen-specific cells that will later on reside in the tissue.[66–71] Additionally, the secondary lymphoid tissues are also thought to help maintain the memory T cells that dominate the mucosal lymphocytes. These lymphoid tissues are usually connected to the lymph nodes as well via lymphatic vessels, leading to further distribution of antigens and allowing DC trafficking from the more local lymphoid tissue to the further downstream lymph nodes.[63,64] Combined, the local barriers such as mucus, epithelium, and the cellular immune response that is mounted by innate and adaptive cells stemming from the systemic circulation, lymph nodes, and local lymphoid organs, can mount a rapid response to rid the body of the pathogens we are constantly encountering at mucosal surfaces.[1,4,57,58] Targeting this response for immunomodulation is of vital interest to prevent and treat a variety of mucosal diseases.

Engineering systems to enhance immunotherapy and vaccination at mucosal surfaces

Therapeutic treatments targeting the immune system are becoming more and more prevalent. They range from classic vaccines and allergen immunotherapy to the cancer immunotherapies that have raised hopes of defeating this devastating disease. New immunotherapy treatments are constantly being developed, and many are applied to treat diseases of mucosal surfaces. These include the above-mentioned allergies and cancer, along with other diseases like inflammatory bowel disease and pulmonary fibrosis. In diseases where the immune response must be controlled and reduced, such as allergies, transplantation, and inflammatory bowel disease, immunosuppressive therapies are generally employed. Immunosuppressive drugs include antibodies that, e.g. block pro-inflammatory cytokines or prevent lymphocyte interaction with antigen-presenting cells, molecules that block cell division of B and T cells (cytostatics), and corticosteroids that prevent transcription of genes of pro-inflammatory cytokines.[72] In contrast, pro-inflammatory immunotherapies are used to turn on immune responses that have either been suppressed, like in cancer, or to induce responses to specific antigens such as during vaccination. These immunotherapies include antibodies that activate lymphocytes by, e.g. targeting checkpoint inhibitors that serve to turn off the

immune response, pro-inflammatory cytokines that activate immunity, and molecules that activate antigen-presenting cells, e.g. toll-like receptor agonists, and thus cause a down-stream immune cascade.[72,73]

In addition to these more classic immunotherapies, another application that seeks to take advantage and modulate local immunity at mucosal surfaces are vaccinations. Indeed, effective mucosal vaccination could significantly contribute to global health by protecting against both mucosal infections as well as those entering through the mucosal route but targeting other organs, such as HIV. Mucosal vaccination has several advantages over intramuscular vaccination strategies: it can induce a local immune response through mucosal-associated lymphoid tissues structures; mucosal vaccination at one mucosal surface can induce immune responses at multiple mucosal surfaces; and mucosal vaccination does not require needles and thus both prevents potential spread of blood borne infection by contaminated needles and allows easy dissemination even potentially allowing administration in the comfort of the home.[74] Despite these many advantages, not many mucosal vaccines are commercially available. Examples include those using live attenuated virus such as the vaccine for cholera, influenza, polio, rotavirus, and salmonella,[10] which are either administered intranasally or orally. For the case of oral administration, one major barrier is the stomach acid that will destroy most infectious and therapeutic agents and thus significantly limits applicability. Additionally, while live attenuated virus appears to be most effective for mucosal vaccines, potential reactivation of the attenuated virus poses a significant problem and could lead to the presentation of the actual disease after vaccination.[10] Subunit vaccines, the most common intramuscular vaccines, tend to be less effective because fragmented proteins are less able to withstand stomach acids and digestive enzymes, and do not transport well across epithelium or mucosal-associated lymphoid tissues. Both the potential for 'digestion', particularly for oral vaccination, and inability to cross the epithelial barrier are major issues to designing effective mucosal vaccines. These are barriers not only for vaccines but also for traditional immunotherapies. Technologies that have emerged to traverse the sticky mucus mesh and mucosal epithelium, protect vaccination cargo (e.g. via encapsulation in nanoparticles), and more recently to further potentiate vaccination via targeted delivery to lymph nodes or M cells. Figure 2 summarizes some of the known design criteria for vaccine and immunotherapy carriers to cross the mucosal barriers, and also to enter lymphatic vessels, and these technologies are further discussed in the following sections. We further direct the readers to several excellent reviews that include lists of commercially available mucosal-targeting drugs.[75–77]

Traversing the mucus mesh barrier: penetration technologies

The first barrier our technology must overcome is the mucus mesh barrier. The mucus mesh is the first layer of defense at the mucosal surfaces. It is a sticky, mesh-like filter system that effectively traps pollutants, pathogens, and irritants that are subsequently cleared with the mucus. This system also effectively traps nanoparticle systems used to enhance local drug delivery to the mucosal surfaces. Efforts from the last ~10+ years, spear-headed by the Hanes Lab, have led to the development of nanoparticle systems that slip through, rather than adhere to, the mucus mesh. Nanoparticles are able to slip through the mucus mesh by fulfilling two main characteristics: 1) they are smaller than the

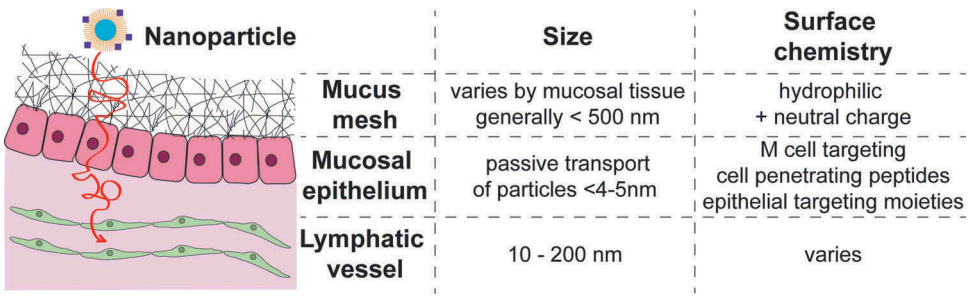

Figure 2. Schematic of mucosal barriers and known design criteria for crossing each barrier.

mucus mesh spacing, and 2) they have an overall hydrophilic and neutrally charged surface.[7,78] The size can be controlled by nanoparticle synthesis conditions and a hydrophilic and neutral surface can be achieved by choice of surface coating, e.g. by densely coating nanoparticles with hydrophilic PEG.[7,78–85] Nanoparticle systems that do not adhere to the mucus mesh have been shown to improve distribution on mucosal surfaces, including the cervicovaginal tract,[78,79,81–83] lungs,[84,85] and gastrointestinal (GI) tract,[82,83] and have improved drug levels in tissues as well as systemic drug delivery. We refer the reader to more extensive reviews of these systems.[86–88]

Traversing the mucosal epithelial barrier

The mucosal epithelial layer is a key barrier to the outside environment that putative drug delivery systems must cross. The structure of mucosal epithelial layers in the body vary by location; the GI tract and endocervix contain a simple layer of stratified columnar epithelial cells, the respiratory tract is composed of a pseudostratified columnar layer with the vagina, exocervix, and buccal mucosa comprised of a layer of stratified squamous epithelial cells.[2,5] The integrity of these cell layers is maintained through the expression of tight junction proteins including: claudins, E-cadherins, and occludins.[4] A large body of work exists on designing delivery systems that attempt to overcome the epithelial barrier for protein delivery,[89] and many efforts have focused on two technologies: permeation enhancers, which temporarily affect cell junctions or membranes, and cell-penetrating peptides, which interact with the cell membrane for internalization through various mechanisms. We briefly summarize some of the most promising systems here, including those surface markers that have been targeted for immunomodulation (Table 2), and refer readers to several excellent reviews for more in-depth discussion of permeation enhancers (PE) and cell-penetrating peptides (CPP).[89,97–99]

Permeation enhancers

PEs promote the delivery of therapeutics across the epithelium by either temporarily modulating tight junction properties or perturbing cell membranes or, in some cases, by both of these methods. PEs have been met with skepticism in terms of their safety, though most have shown little toxicity at concentrations needed to perform their functions in vivo, likely due to the transient nature of their effects.[89,100] PEs require proximity of the therapeutic to effectively traffic it across the epithelium, so co-delivery in proximity to the epithelium is key. Paracellular PEs have a variety of mechanisms including targeting tight junction structures such as claudins, E-cadherins, and occludins, while others modulate cytoskeletal reorganization that affects tight junction permeability.[89] Transcellular PEs that have been studied are usually varying classes of surfactants that directly disrupt the cellular membrane. Increasing interest lies in administering particulate drug delivery systems orally, though the combinations of particulates with PEs in combination has been limited. The most notable formulation combined insulin-loaded micelles with the surfactant sucrose erurate suspended in soybean oil with sodium cholate and sucrose laurate. The micelles are quite stable in water and effectively reduce blood sugar in rats.[101,102] Several other PEs have effectively enhanced the delivery of larger cargo, such as protein-complexes, liposomes, or other nanoparticles

Table 2. Epithelial surface markers targeted for immunomodulation.

Targeting Agent/Ligand	Target	Application	Reference
P6 *Haemophilus influenzae* membrane liporprotein	TLR2	1) NF-κB mediated transcription of MUC5AC, 2) Production of β-defensin 2 antimicrobial protein.	90,91
F-protein from Respiratory Syncytial Virus	TLR4	Vulnerability to lipopolysacchardide-mediated inflammation	92
FPS-ZM1	Inhibitor for receptor for advanced glycan end products (RAGE)	Anti-inflammatory and anti-oxidative stress mediator.	93
Transferrin	Transferrin receptor	Improves nanoparticle delivery to and across Caco-2 cells	94
Fc antibody region	Bind to Neonatal Fc Receptor (FcRn)	Mediates transport across epithelial barrier.	95
alpha-Galactosylceramide	Galactosyl ceramide receptor	Mediates transport across epithelial barrier and modulates immunity	96

including $C_{12}E_9$ and sodium deoxycholate.[103–106] Citric acid and other acidifying organic acids have also been shown to improve the delivery of peptides through the GI mucosal epithelium by lowering the optimal pH for proteolysis and while improving solubility.[107]

Cell-penetrating peptide technologies

CPPs are a class of molecules that improve transcellular or paracellular transport through epithelial cells. Natural and synthetic peptides have been employed for the delivery of genes and protein into and across the epithelial cell barrier.[98] CPPs share some unifying characteristics despite having a variety of primary sequences and secondary structures. Generally, CPPs are less than 30 amino acids in length, rich in positively charged amino acid residues, and contain hydrophobic tryptophan residues to help membrane translocation of the CPP. There are several different classes, including protein derived CPPs, such as the HIV transactivator of transcription peptide (TATp), chimeric peptides such as transportan, and synthetic peptides such as octa-arginine.[89,100] Most of these operate by initiating endocytosis, direct translocation, tight junction loosening, or formation of channels in the cell membrane. CPP technology has been successfully combined with nanotechnology to enhance systemic absorption of a cargo, often the 'test cargo' insulin.[89,100] For instance, a cargo of penetrating-modified insulin complexed with a polymer (HMPA) that did not interact with the mucus mesh led to 20-fold higher insulin absorption in vivo, which was followed by a decrease in blood glucose.[108] Similarly, a nanoparticle system for insulin delivery that contained a peptide targeting goblet cells (CSKSSDYQC) was shown to co-localize with goblet cells in an in vivo intestinal loop model.[109] The hypoglycemic effects were more limited than the penetrating-based system, but still showed a 1.5-fold increase compared to non-targeting nanoparticle controls.[109] More recently, systems combining cell and mucus penetration have emerged. For instance, Porsio et al. demonstrated that nanoparticles coated with PEG (to make them mucoinert) and TAT peptide (to enhance cell permeation) enhanced both penetrations through artificial cystic fibrosis mucus, and across lung epithelial cells.[110] A system developed by Tan et al. uses PEG-coated 170 nm silica nanoparticles that were loaded with cell-penetrating peptide, penetratin, along with therapeutic peptide.[111] These particles not only penetrated mucus, but also showed improved cellular uptake, exocytosis and transcellular permeation across mucosal epithelium compared to particles that were able to either penetrating the mucus barrier or contain CPPs.[111]

Targeting lymphoid tissue for potentiation of immunomodulatory treatments

Lymphoid tissues, including the lymph nodes (LNs) and mucosal-associated lymphoid tissues have recently become a target for immunotherapeutic treatments and vaccinations, since efforts have demonstrated significant potentiation of treatments when targeting tissues where immune cell education is occurring. In this section, we summarize technologies developed to target both lymph nodes and M cells that coat the mucosal-associated lymphoid tissues for enhanced immunotherapy and vaccination.

Lymph node targeting systems

Usually, LNs are targeted by direct injection into the lymph node or nearby lymphatic vessels. One of the early approaches targeted LNs for allergen immunotherapy, work that has led to a number of clinical trials that demonstrated enhanced efficacy of the treatment while simultaneously requiring much lower dosages, reducing the likelihood of adverse reactions such as anaphylaxis.[112,113] Another area that has had considerable developments in lymph node targeting approaches is cancer immunotherapy. Smith et al. demonstrated that intra-lymph node immunization of tumor peptide with adjuvant induces tumor regression and induces antigen-specific T cell response, with ~15% of recirculating CD8 + T cells being tumor antigen-specific.[114] Strikingly, 90% of mice immunized with intra-LN vaccines remained protected against tumor induction. Similarly, Liu et all designed cancer vaccines that have albumin 'hitchhiking' components, that is adjuvants and peptides conjugated to albumin that natural accumulates in

the LN.[115] These albumin hitchhiking vaccines were shown to reduce tumor burden in mice and enhance cytolytic activity and cytokines production in antigen-specific CD8 + T cells.

Several studies from the Kündig group have shown that LN targeting injections of CpG enhance its therapeutic potential, yielding a higher CD8 + T cell response to OVA compared to a subcutaneous injection with a 100-fold lower dose, and induced a higher anti-tumor response.[116] DNA vaccines also further enhanced the CD8+ cytotoxic T cell antitumor response by 100–1000 fold.[117] Furthermore, peptide vaccines injected into LN with MHC class I binding peptides from lymphocytic choriomeningitis virus enhanced the immunogenicity of the peptide by 10^6 fold compared to subcutaneous or intradermal injection.[118] This was evidenced by the significantly stronger CD8 + T cell response with greater cytotoxic activity and IFNy production, as well as long-term protection against viral infections and tumor growth.

Finally, studies in transplantation by Komori et al. demonstrated that the LN can serve as a transplantation site of different tissues and this will reduce the chances of rejection.[119] For instance, hepatocytes and thymocytes transplanted into mouse jejunal lymph nodes induced survival in mice with lethal metabolic disease and restored a functional immune system in athymic mice. While these data suggest that tolerance was induced to these cells, there is no mention of how the transplantation into the LN actually altered the immune response.[119] Their work suggested that LN transplantation favors vascularization, one of the keys to graft survival, and that transplantation of cell/tissue types into only one lymph node may be sufficient for successful treatment. Taken together, this suggests that targeting lymph nodes directly can significantly enhance the desired immune response, and thus can potentiate immunotherapeutic, vaccination, and even transplantation treatments.

Lymphatic targeting technologies for indirect drug delivery to the lymph nodes

More recently, technologies have emerged that indirectly target lymph nodes by targeting lymphatic transport.[120] The rationale behind targeting the lymphatic vessels is due to the transport functions of these vessels. Cells, fluids, protein, and small molecules are transported from peripheral tissues to the draining lymph nodes where adaptive immune responses are formed. Convective fluid flow in the interstitium drives fluid and molecules toward lymphatic vessels.[120] This fluid flow becomes increasingly important with increased molecular size, as the opposing movement toward blood capillaries by diffusion decreases for larger molecules.[120] At the same time, the extracellular matrix holding together the interstitium needs to be traversed, so particles need to be small enough to get across this extracellular matrix and reach lymphatic vessels and their downstream dLNs.

The size is necessary for particles to enter the lymphatics have been well established, with combined studies suggesting that particles ranging from 10 to 250 nm are ideal for transport into the lymphatics. Work by Reddy et al. demonstrated the optimal nanoparticle size for targeting lymphatics via intradermal injection in mice is between 5 and 50 nm. Intradermally delivered PEG-stabilized poly(propylene sulfide) nanoparticle 20 nm and 45 nm in size efficiently drained to local lymphatics and were recovered in high amounts in the dLNs.[121] Manolova et al. showed that larger particles, up to 200 nm, could also freely travel to the dLNs.[122] Similarly, Varypataki et al. showed that vaccine response could be enhanced in vivo by using smaller size cationic liposomes (150–200 nm) compared to larger poly-(lactic-co-glycolic-acid) (PLGA) nanoparticle (250–350 nm).[123] Interestingly, work by Kobayashi et al. demonstrated that nanoparticle systems smaller than 10 nm in size are *inefficiently* retained in lymphatic vessels, suggesting that these were too small to take advantage of convective fluid flow in the interstitial space.[124]

The effects of surface charge on targeting lymphatic transport remains poorly understood. As mentioned, Varypataki et al. showed that cationic liposomes effectively drained into lymphatic vessels.[123] Rao et al. demonstrated that hydrophobic, negatively charged PLGA and PLGA-poly(lactic acid)-PEG nanoparticles both accumulated in the dLN after 3h, with the PEGylated nanoparticles having higher accumulation.[125] However, this study on PLGA-PEG nanoparticles was far from

conclusive. The fairly large injection volume in this work, though, likely led to enhanced convective flow toward lymphatics, and the highly negative ζ-potential of the PEGylated nanoparticles, −30 mV, indicating the particles were not densely PEGylated, may have obscured some of the effects of surface charge and PEGylation on lymphatic transport. Finally, another study by Zeng et al. showed that cationic nanoparticles can also promote LN retention of nanoparticles, though the question remains if this was due to lymphatic transport or enhanced uptake of these nanoparticles by antigen-presenting cells.[126] More recently, DeKoker demonstrated that PEGylating 200 nm polymethacrylic acid nanoparticles drastically enhanced nanoparticle accumulation in the lymph nodes after 12 and 48 h, though whether this is by the mechanism of cellular migration or transport via lymphatic vessel remains to be explained.[127] Additionally, Mao et al. formulated chylomicron mimicking mesoporous silica nanoparticles containing the antiretroviral drug lopinavir, and demonstrated that these were able to effectively translocate across intestinal epithelium in vitro and in vivo in mice.[128] Furthermore, their formulation accumulated in the mesenteric lymph nodes, which could be inhibited using a lymph transport inhibitor, cycloheximide, which interferes with the chylomicron formation pathways, suggesting that chylomicron imitation could indeed be used to target the local gut draining lymph nodes.[128] Interestingly, a recent publication from Triacca et al. reveals transcytosis to be a key mode of transport into lymphatic vessels. Inhibiting clathrin and caveolin-mediated uptake was demonstrated to prevent the transport of albumin across lymphatic endothelial cells to a similar extent as tightening of cell-cell junctions using adrenomedullin.[129] To date, little is known about the mechanisms of nanoparticle transport across lymphatic vessels, and many more thorough studies are needed to better understand requirements, other than size, for effectively targeting nanoparticles to lymphatic vessels.

M cell-targeted drug delivery

Mucosal associated lymphoid tissues (MALT) are considered an alternate target to lymph nodes at the mucosal surfaces, as these perform similar functions in activating and educating local adaptive immunity. In particular, targeting M cells that cover the GALT, BALT, and NALT have been explored. M cells phagocytose antigens from the apical side of the epithelium and transfer them to the basal side, where the underlying antigen presenting cells take them up for lymphocyte education and activation.[12] This specific function makes them a particularly appealing target for immune modulatory treatments. Approaches usually target molecules specifically expressed on M cell surface. Perhaps the most commonly used example is targeting α-L-fucose expressed on M cells specifically using a variety of lectins including Ulex europaeus agglutinin 1 and Aleuria aurantia. Lectins are carbohydrate-binding proteins that specifically recognize certain sugar molecules and have a variety of functions including cell adhesion and receptors for recognition of sugars in the systemic circulation, e.g. by liver cells.[8,9,130] Lectins have been used for nanoparticle-based delivery including biodegradable systems, such as PLGA and liposomes, as well as latex particles. These systems induced robust immune responses that included the production of IgA and type 1 cytokines such as IFNy to the antigen ovalbumin.[131–135] Despite these promising studies, clinical translation proves difficult due to the immunogenicity inherently associated with these lectins.[8] This brought about the development of other molecules to target M cells including RGD peptides that adhere to the integrins expressed by M cells, and antibodies and/or their fragments that have been selected to target M cells specifically. Targeting mechanisms have been exploited for a variety of applications including vaccination against viral or bacterial targets (Table 3), and cancer, targeting responses against biological toxins like botulinum toxin, and induction of antigen-specific antibody responses to induce robust immunity against foreign molecules such as ovalbumin.[131–135] Table 3 summarizes targeting molecules and their application.

Conclusion

In summary, the mucosal surfaces form a formidable barrier to pathogens that also need to be overcome for mucosal drug delivery for immunomodulation. A variety of technologies have been developed to

Table 3. Summary of materials used to target M cells, their target on the M cell surface, and applications.

Targeting agent	Type of targeting	Application	Reference
BSK02^{TM} *Aleuria aurantia* lectin	Ligand with high affinity to α-L-fucose on M cell surface	Vaccination against ovarian cancer, vaccination reduced/retarded tumor volume and enhanced CD8+ lymphocyte response	136
M cell homing peptide (CKSTHPLSC)	High M cell affinity	Identified via phage display using in vitro M cells; displayed enhance in vivo transcytosis of cargo, vaccination against pathogenic intestinal spirochete *Brachyspira hyodysenteriae*	137,138
Ulex europaeus agglutinin 1 (UEA-1)	Ligand with high affinity to α-L-fucose on M cell surface	Immunization against GP5 antigen; nasal vaccination against Staph aureus protected against acute systemic infection without antibodies	139,140
OmpH β1α1 domain of *Yersinia enterocolitica*	Binds to a complement C5a receptor on the M cell surface	Vaccination against Infectious bursal disease virus (IBDV) using viral capsid protein; enhanced delivery of model antigen staph aureas nuclease using lactobacilli lactis expressing omph,	68,141
Secretory IgA (SIgA)	Binds to M cells (specificity not indicated)	Immunization using HIV p24gag protein induced humoral and cellular response against p24 (both cellular and antibody response)	142,143
RGD or GRGDS	Targets beta1 integrins on the apical side of M cells	Robust IgG response to OVA after oral delivery; Delivery of PR8 antigen induced antibody production; IgG and cellular response to OVA	131-133
Monoclonal antibodies NKM 16-2-4	antibody with high affinity to α-1,2-fucose on M cell surface	Oral delivery of tetanus toxin, botulinum toxoid and cholera toxin induce high levels of IgG and IgA responses and protected against botulinu toxin challenge	134
Antigen binding fragment from mouse antibody against glycoprotein 2 (GP2)	GP2, transcytotic antigen receptor specifically expressed on M cells	Immunization against OVA and Salmonella enterica lysate induced enhanced protection against bacteria	135,144
Full transmembrane mRANKL	Induces expression of RANK in M cells, then can target with mRANKL	Intraperitoneal sensitization then targeting induced oral immunization induced strong protective IgA and IgG	145

cross the mucus mesh and mucosal epithelium for drug delivery, and some have targeted mucosal immunity by, e.g., binding to M cells and targeting lymph nodes via lymphatic vessels. Many of these are quite promising and have been translated into clinical trials or products (Table), but often we are still faced with sub-optimal treatments or vaccines for many mucosal diseases such as cholera, allergies, and inflammatory bowel disease. We expect many more years of research and development of novel technologies are required until we have exhaustively targeted mucosal immunity for local treatment and prevention of diseases.

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