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Targeting Cellular Receptors as a Strategy for the Development of New Generation Mucosal Vaccines

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ABSTRACT

Mucosal pathogens are a primary source of infection that require the development of vaccines to induce a specific, protective and long lasting immune response. Despite an urgent need, development of effective mucosal immunization strategies has proven difficult. The literature was reviewed to elucidate cell specific targets that may be utilized to increase the immunogenicity of protective antigens through mucosal vaccination. Vaccine vehicles such as liposomes, outer membrane vesicles and Poly Lactic Acid (PLA) nanoparticles serve as antigen delivery systems as well as immuno-stimulatory agents. Even with the advances in mucosal vaccine delivery mechanisms many systems lack specificity and fail to generate a protective immune response. Hence, targeting extracellular receptors by harnessing antibody specificities, bacterial molecules, or toxins in conjunction with protective antigens provides an attractive alternative to conventional vaccine regiments that may circumvent the need for delivery systems and adjuvants. Fusion proteins targeting M cells, dendritic cells, or lymphocytes, are promising candidates for enhancing antigenic trafficking across the mucosal membrane and subsequent uptake by antigen presenting cells. Directing antigens to extracellular receptors of epithelial and immune cells via mucosal immunization provides an attractive model for generating a protective memory immune response that is essential to a successful vaccine regiment.

Keywords: Mucosal Vaccines, Targeting, Cell Receptors, Poly Lactic Acid (PLA)

1. INTRODUCTION

The vast majority of infectious diseases are caused by pathogens that invade the body via the mucosal surfaces. Thus, there is an urgent need for vaccines that will induce protective and long-lasting immunity against mucosal pathogens, especially in infants and children. However, despite its attractive, needle-free administration, effective mucosal immunization has proven difficult. Mucosal vaccines have been known to get diluted in mucosal fluids or get stuck within the mucus gel and subsequently get degraded by proteases (Neutra and Kozlowski, 2006). In addition, deciphering the dose and route of vaccination, the timing of boosters and the choice of adjuvant may make the all-important difference between establishing a protective, memory immune response and unwillingly achieving mucosal tolerance.

Mucosal surfaces are protected from pathogenic organisms by physicochemical and cellular barriers that comprise the innate immune system, as well as by the highly specific and inducible adaptive responses. Epithelial cells line most mucosal surfaces creating a complex cellular mesh that blocks pathogen invasion. In addition, antibodies produced by B lymphocytes, have

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neutralizing properties against bacterial and viral molecules, blocking pathogen attachment on mucosal surfaces and facilitating aggregation and opsonophagocytosis of the microbes. These effector functions are mediated primarily by IgA which dominates in the mucosal surfaces in its secretory form (sIgA) (Lamm, 1997).

Although the ability to elicit a strong antibody response is key for a successful vaccine, cellular immunity at mucosal surfaces has also been proven critical in both regulating immune responses and driving direct effector functions. For example, in the case of a live attenuated influenza vaccine, cytotoxic CD8+ T cell responses are thought to contribute significantly to cross-reactive protection against various influenza strains (Sun *et al*., 2011), while TH17 cells have been shown to block bacterial colonization during *Salmonella* infection in the intestines of immunized mice (Godinez *et al*., 2008).

The initial step of vaccine development entails the identification of a protective antigen, the choice of an adjuvant and the decision upon the route of administration. Ideally, a successful mucosal vaccine should: (1) preserve vaccine antigens from enzymatic degradation, (2) limit their elimination or dilution in the organisms, (3) facilitate its transport through the mucosal epithelial linings and (4) ensure an efficient uptake by professional Antigen Presenting Cells (pAPCs), such as Dendritic Cells (DCs) and macrophages. Professional APCs will process and present the vaccine antigens to T cells via in an MHC dependent fashion, thus, driving the initiation of a protective and long-lasting adaptive immune response.

The paucity of mucosal adjuvants and delivery systems has led scientists to study vaccine approaches that target protective antigens to specific cellular receptors. Doing so, we can increase the immunogenicity of an antigen and bias the immune system to develop adaptive immune responses specific to that particular antigen, hence, eliciting long-lasting protection against subsequent challenge with the infectious agent.

Targeting to cellular receptors is usually achieved by exploiting antibody specificities. Antigens have been physically, chemically, or genetically linked to antibody molecules, or Fab' fragments, that target specific cellular receptors. If these receptors are expressed on epithelial cells, such as the specialized M cells, then the vaccine has the advantage of easily crossing the epithelial barrier. If, on the other hand, the vaccine targets activating receptors on pAPCs, such as DCs, then enhanced antigen uptake and presentation will be accompanied by maturation and activation of the pAPCs that will in turn drive the generation of effective lymphocyte responses.

In this review we will focus on the various vaccine strategies that target antigens to different extra-cellular receptors in order to facilitate their trafficking through the epithelial cell lining and their subsequent uptake by pAPC for processing and presentation. Such approaches have yielded promising results in a wide range of infectious models that use both bacterial and viral mucosal pathogens.

1.1. Mucosal Adjuvants and Vaccine Delivery Modes

Vaccines comprised of attenuated microorganisms, although relatively immunogenic, run the risk of causing disease. This caveat has led research to identify microbial fractions, or even isolated specific proteins, as vaccine candidates to replace the use of whole microorganisms. The shortcoming of this approach is that these subunit vaccines are usually poorly immunogenic, hence requiring the use of adjuvants that will boost or enhance specific immune responses, ultimately establishing protective immunity (Kensil *et al*., 2004; McCluskie *et al*., 1998; Vogel, 2000). Although innate immune responses usually suffice to protect against mucosal pathogens, the ability of the latter to evade effector responses, including phagocytosis and complement mediated lysis, renders the adaptive branch of the immune system critical to resolving mucosal infections (Blank *et al*., 2011; Cooper *et al*., 2002; Klinman *et al*., 1999). Thus, it is important that adjuvants will enhance specific T and B lymphocyte responses while establishing immunologic memory.

Vaccine adjuvants can be divided into two broad categories: Immune modulators and delivery systems (Kensil *et al*., 2004). Currently, the only FDA approved adjuvant in the United States is the hydrated potassium aluminium sulfate (Alum) which is impractical for the delivery of mucosal vaccines and also fails to generate robust cellular immune responses as it initiates a TH2 biased immunologic profile (Brewer *et al*., 1999). Consequently, there is an unmet need for safe and effective mucosal immunization vaccine strategies and for the identification of alternative, yet safe mucosal vaccine adjuvants.

Current research has utilized various vaccine vehicles that not only deliver vaccines to and through mucosal surfaces but also have immunostimulatory abilities.

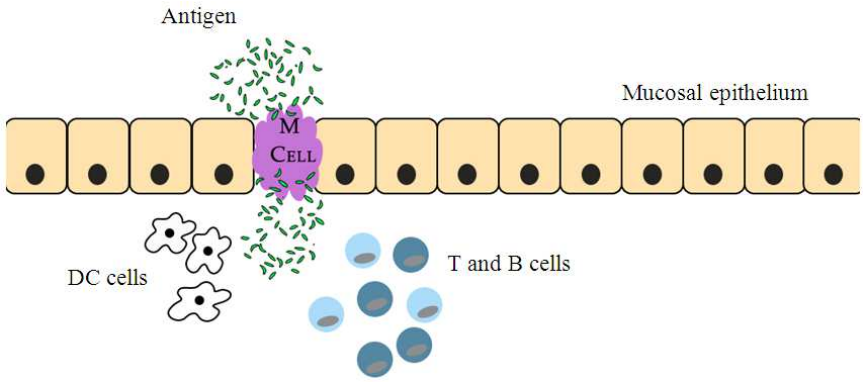

Fig. 1. M cells are important carriers of antigens through the epithelial layers to the mucosa-associated lymphoid tissues, making them available for uptake by pAPCs, such as DCs, processing and presentation to T and B lymphocytes

For example, liposomes, Immune Stimulating Complexes (ISCOMs), monoglycerides with fatty acids and Outer Membrane Vesicles (OMV) have been evaluated giving promising results (Hu *et al*., 2001; Mann *et al*., 2009; Mitragotri, 2005). In addition, antigens encapsulated in PLA or PLGA nanoparticles have generated strong mucosal and systemic immune responses, both in mice (Song *et al*., 2008) and humans (Katz *et al*., 2003). Nanocarriers, having comparable dimensions to bacterial pathogens, are efficiently taken up by phagocytosis, thus delivering the antigens to pAPCs. Alternatively, recombinant or attenuated strains of various bacteria, such as *Salmonella*, *E. coli*, or *Listeria*, have been also used as vectors to deliver antigens to the Mucosal Associated Lymphoid Tissue (MALT) (Hall *et al*., 2009; Boyer *et al*., 2005). These vectors not only target the DNA or peptide vaccine construct to pAPCs, but also provide a strong danger signal, acting as natural adjuvants, thereby promoting maturation and activation of DCs (Mayr *et al*., 2005).

However, despite the significant advances in the area of vaccine delivery, most approaches lack in specificity, thus creating potential hypersensitivity issues, or they are simply unable to specifically moderate the immune system towards a desired, protective immune response. Targeting antigens to receptors expressed on epithelial cells and pAPCs, not only circumvents the need for an adjuvant or a delivery system, but also provides us with the ability to specifically manipulate the immune system to our advantage, eliciting the best possible protection against mucosal pathogens.

1.2. M Cells-The Gateway to Mucosal-Associated Lymphoid Tissues

M cells are specialized epithelial cells responsible for sampling antigens at the interface of mucosal surfaces and the environment and presenting them to the mucosal immune system (**Fig. 1**). Once antigens are trancytosed by M cells they can quickly move to the basolateral membrane and interact with the underlying lymphoid aggregates (Owen, 1977). Thus, the high transcytotic ability of M cells and their strategic location at the mucosal interface, make them an attractive target for mucosally delivered vaccines. Below we describe a number of delivery systems, bacterial molecules, toxins and antibodies that have been used to target M cells.

1.3. Lectin-Mediated Targeting

Lectins, often expressed on M cells, are a structurally diverse group of proteins and glycoproteins that bind on carbohydrate moieties of cell-surface proteins and lipids. Targeting lectins on M cells has been shown to increase antigen uptake and transport through the epithelium (Clark *et al*., 2000; Jepson *et al*., 2004; Azizi *et al*., 2010; Chen *et al*., 1996). Indeed, these studies have shown that nanoparticles coated with the lectin-ligands such as Wheat Germagglutinin (WGA) and Concanavalin A (ConA) enhanced the endocytosis of nanoparticles by murine intestinal epithelial cells. One of the most investigated lectin is *Ulex Europeans* Agglutinin 1 (UEA-1), which binds to a-L-fucose residues expressed on the apical surface of M cells in mice (Gupta *et al*., 2007). Upon oral immunization of mice with UEA-1, chemically anchored to the surface of Hepatitis B surface Antigen (HBsA)-loaded nanoparticles, increased HBsA-specific sIgA responses and mediated a strong TH1-like immune response (Gupta *et al*., 2007). In a different study, mice immunized via the oro-gastric route with either UEA-1-agglutinated *H. pylori* or UEA-1-agglutinated *C. jejuni* showed significantly enhanced bacteria-specific IgG and

IgA antibody responses in their vaginal washings, compared to mice receiving non-agglutinated bacteria (Chionh and Sutton, 2010). However, because UEA-1 also reacts strongly with goblet cells and the mucous layer covering the intestinal epithelium due to the existence of abundant sialic acids (Kandori *et al*., 1996), scientists have developed monoclonal antibodies that specifically target lectins expressed solely on M cells. For example, Nochi and colleagues developed an oral vaccine comprising of the Botulism Toxoid (BT)-conjugated to the antibody NKM 16-2-4, which shows specificity for α(1,2)-fucosylated M cells. Oral immunization of Chinese hamsters with this vaccine construct resulted in robust anti-BT specific antibody responses which were significantly higher compared to the use of UEA-1 or the BT toxoid alone.

However utilization of lectins as targeting molecules for mucosal immunization presents challenges for oral vaccine delivery due to their potential toxicity and possible degradation of the lectin once anchored onto nanoparticles (Russell-Jones, 2001). Vaccine delivery could be further limited as intestinal glycosylation patterns of M cells vary at differing locations within the intestine (Lavelle and O'Hagen, 2006). In addition, some lectins have been found to be highly immunogenic which may inhibit uptake of the constructs by mucosal epithelial cells due to the generation of local innate immune responses against the lectin-targeting molecule (Clark *et al*., 2000). However, the immunostimulatory capacity of lectins may present an alternate role for lectin ligands as mucosal adjuvants.

1.4. Targeting Pattern Recognition Receptors (PRRs)

PRRs on various cell types, including pAPCs and epithelial cells, can recognize Pathogen Associated Molecular Patterns (PAMPs), such as LPS, enhancing attachment and phagocytosis of microorganisms (Chadwick *et al*., 2009). For instance, TLR-4, the natural ligand of LPS, has been shown to be expressed on M cells and be involved in bacterial translocation (Neal *et al*., 2006; Tyrer *et al*., 2006). Unsurprisingly, nanoparticles coated with LPS were uptaken more effectively by mouse M cells in the intestinal epithelial compared to their control counterparts, indicating a novel targeting and transport pathway through the mucosal layers (Gribar *et al*., 2008).

1.5. Use of Bacterial Toxins for M Cell Targeting

Cholera Toxin (CT) from Vibrio cholera and the heat-Labile Enterotoxin (LT) from Enterotoxigenic E. Coli (ETEC) are structurally related AB_5 enterotoxins, composed of an enzymatically active A subunit, which mediates their toxicity and a pentameric B subunit that binds with high affinity to ganglioside receptors expressed on the apical surface of M cells (Cheesman *et al*., 2004). Intranasal or oral immunization of mice with attenuated pathogens, or protective antigens, chemically or genetically conjugated to these toxins, or their respective B subunits, generated protection against subsequent lethal challenge with these pathogens (Jang *et al*., 2004; Bergquist *et al*., 1998; Rask *et al*., 2000). Moreover, coupling of Cholera Toxin B subunit (CTB) to antigen-loaded liposomes generated enhanced mucosal immune responses in mice as compared to untargeted antigen loaded particles (Harokopakis *et al*., 1998). Similarly, Clostridium Perfringens Enterotoxin (CPE), specifically binds with high affinity to Claudin-4, a protein associated with M cell endocytosis, thus presenting an alternative toxin utilized for M cell targeting.

1.6. Utilization of other Receptors as Targeting Candidates for Vaccine Transport Across the Mucosal Epithelia

Different studies have shown the expression of other receptors on M cells that could provide additional candidates for targeting vaccine approaches. For example, when latex nanoparticles coated with purified invasin (binds to b1 integrins) from Yersinia pestis were administered orally to rats, six times more invasin-coated nanoparticles were found in the circulation than uncoated ones (Hussain and Florence, 1998), confirming the expression of integrin receptors on epithelial cells. Similarly, the coating of nanoparticles with fibronectin, an endogenous ligand for a5b1 integrin, significantly increased the binding of nanoparticles to the Peyers patches and their active transport across the intestinal epithelium (Gullberg *et al*., 2006).

In addition, some complement receptors have been identified on epithelial cells and utilized for antigen targeting. For instance, Kim *et al*. (2011) showed the expression of C5a complement receptor on the surface of M cells. In this novel approach, oral administration of *Yersinia enterolytica* expressing Outer membrane Protein H (OmpH), a ligand of C5aR, led to enhanced antigen-specific local and systemic immune responses to the pathogen.

Finally, adult epithelial cells in both mice and humans express the neonatal Fc Receptor (FcRn) which binds with variable affinities to the Fc portion of the different IgG antibody sub-classes, thus mediating the trans-epithelial transport of IgG (Roopenian and Akilesh, 2007; Yoshida *et al*., 2004; Ward and Ober, 2009).

Hence, mucosal vaccination with Immune Complexes (IC) formed between IgG antibodies and whole, attenuated microorganisms, or merely protective antigens, can enhance the transport of the vaccine through the epithelial layers, therefore increasing its accessibility to the lymphoid tissues. Indeed, in a study carried out by Chopra (1998), intranasal immunization with inactivated *Francisella tularensis* (iFt) coupled with an anti-LPS IgG2a mAb, increased protection against subsequent lethal challenge with the live bacterium, compared to immunization with iFt alone. This protection was abrogated when FcRn-genetically deficient mice were used. In a similar study, mucosal immunization with the HIV protein, *gag*, genetically fused to the heavy chain of IgG (Gag-Fc), increased the transport of the protein through the epithelial layers, thus increasing its immunogenicity (Singh *et al*., 2001).

1.7. Targeting Dendritic Cells (DCs)-Building a Link between Innate and Adaptive Immunity

Presentation of antigen to T lymphocytes by DCs is highly efficient due to the constitutive, high expression of MHC class II and other costimulatory molecules on the surface of these pAPCs. Thus, targeting DCs as a vaccine strategy has been the focus of many research groups. Apart from the obvious advantages of antigen uptake and presentation to T cells, DCs have been recently shown to be involved in the transport of antigens through mucosal surfaces, mainly in the absence of differentiated M cells, via their dendrites that protrude across the intestinal epithelial layer and into the lumen (Rescigno and Sabatino, 2009). The "ferried" antigens can then be presented to conventional CD4+and CD8+T cells of the MALT, thus ensuring a specific, adaptive immune response. Subsequent activation of lymphocytes will drive robust effector functions and also lead to the expression of tissue-homing receptors that will allow lymphocytes to traffic to areas of antigenic exposure (Iwasaki, 2007; Johansson-Lindbom *et al*., 2005; Mora *et al*., 2006; Devriendt *et al*., 2001). The importance of DCs in mucosal immune responses was further demonstrated by a study from Fahlen-Yrlid *et al*. (2009) in which administration of OVA intranasally led to the production of sIgA-specific antibodies only in the presence of Conventional DCs ($CD11c^{high}$).

It is noteworthy that DCs of the Respiratory Track (RTDCs) have been shown to play different roles in controlling the immunological homeostasis of inhaled antigens (Stumbles *et al*., 2003). Recent findings have suggested that the DC subsets of the airway mucosa do not have identical abilities to sample and present antigen, thus, understanding the role played by each DC subpopulation will be key for the development of mucosal vaccines (Garnier and Nicod, 2009; Garnier *et al*., 2007; Wikstrom and Stumbles, 2007).

Hence, it is evident that targeting antigens to DCs in the mucosa is a way to understand and exploit the specific functions of the various DC subtypes residing or recruited to these tissues, leading to the generation of better vaccines. Unfortunately, there are very few, if any, DC-specific markers. Nevertheless, the majority of receptors expressed on the surface of DCs are involved in endocytosis and antigen presentation, therefore presenting attractive candidates for vaccine targeting.

1.8. C-Type Lectin (CLR) Targeting

CLRs are pattern recognition receptors that are widely expressed on various APC populations (Gordon, 2002; Pyz *et al*., 2006). Through their carbohydrate-recognition domains, CLRs selectively bind to glycans associated with microorganisms and lead to rapid internalization (Brown *et al*., 2003; Mansour *et al*., 2006). CLRs include the Mannose Receptors (MR), DEC-205 and DC-SIGN (DC-specific ICAM-grabbing non-integrin). MR expression is abundant on mouse bone-marrow derived DCs, human monocyte-derived DCs and interstitial DCs, such as Langerhans cells (Sallusto *et al*., 1995; Uccini *et al*., 1997). Ligands containing mannan or mannose have been successfully used in various forms to direct antigens to APCs, thus generating more efficient immune responses (Agnes *et al*., 1998; Keler *et al*., 2004).

Another CLR, DEC-205, is highly expressed on human and murine DCs, especially in the T-cell rich areas of the secondary lymphoid organs (Jiang *et al*., 1995). Initial studies demonstrated the ability of rabbit antibodies, specific for DEC-205, to be presented to reactive T-cell hybridomas 100-fold more efficiently than irrelevant antibodies (Jiang *et al*., 1995). Despite its "in vitro" promise, generation of protective immune responses in infectious models following targeting to DEC-205 requires co-administration of an adjuvant, such as anti-CD40, heat-shock protein 70 (HSP-70), poly-IC, or a TLR agonist (Stylianou *et al*., 2011; Bonifaz *et al*., 2002; Lazarevic *et al*., 2003; Wang *et al*., 2001; 2009; Uehori *et al*., 2005). It is important to note that in the absence of adjuvants, targeting antigens to DEC-205 alone may result in mucosal tolerance; a detrimental outcome in vaccine research.

Another receptor utilized in antibody-mediated C-lectin targeting on DCs is DC-SIGN. Antigens targeted to this CLR are uptaken rapidly by multiple DC populations contributing to T-cell priming and activation (Schjetne *et al*., 2002; Dakappagari *et al*., 2006). Indeed,

the rapidly internalized antigen and its subsequent routing to the lysosomal compartments would lead to effective processing and presentation to T cells. Nevertheless, there is a paucity of studies assessing this targeting approach in infectious models "*in vivo*".

1.9. Targeting Toll-like Receptors (TLRs)

TLRs are an essential family of pattern-recognition receptors, abundantly expressed in murine and human DCs, either intracellularly or extracellularly. A number of studies have highlighted the importance of TLRs and the critical role they play in integrating innate and adaptive immunity (Reise-Sousa, 2004; Mazzoni and Segal, 2004). These novel insights have provided impetus for developing new vaccines against various mucosal pathogens that target TLRs. For example, when the Cytomegalovirus (CMV) vaccine, gB, was administered in mice with CpG, the natural ligand for TLR-9, it resulted in the generation of long-lived Ag-specific T cells and antibodies with cross-neutralizing activity against heterologous CMV strains (Dasari *et al*., 2011). In a similar study, murine administration of HIV *gag* and *nef* proteins with CpG increased the maturation levels of DCs, as depicted by the expression levels of costimulatory molecules, while it resulted in the generation of HIV-specific CD8+ T cells, providing evidence for cross-presentation (Lore *et al*., 2003). However, the relatively scarce expression of TLR-9 on human DCs (Jarrossay *et al*., 2001; Krug *et al*., 2001) led Wille-Reece and colleagues to co-administer the TLR-7/8 agonist, R848, together with the HIV proteins and CpG. Indeed, the generation of HIV-specific CD8+ T cells and the production of TH1-like cytokines in the sera of immunized mice were improved significantly compared to the previous study in which TLR-9 was the targeted receptor. The potential superiority of TLR-7 over TLR-9 was further demonstrated in a murine influenza model study by Schmitz *et al*. (2012), in which mice were immunized with the viral extracellular domain, M2, targeted to TLR-7. The resulting anti-influenza IgG2c high titer was indicative of a protective, TH-1 like response.

Another example of TLR targeting via its natural ligand is TLR-5. Bacterial flagellin binds to a leucine-rich repeat of TLR-5 and induces downstream signaling in a MyD88-dependent manner (Mizel *et al*., 2003; Hayashi *et al*., 2001). The adjuvant effect of flagellin leads to increased cytokine production (Applequist *et al*., 2005; Bachmann *et al*., 2006; Didierlaurent *et al*., 2004; Cuadros *et al*., 2004), increased lymphocyte trafficking in the draining lymph nodes (Bates *et al*., 2008) and activation of $CD11c^{high}$ DCs (Bates *et al*., 2009). Consequently, vaccination of mice with fusion proteins comprising of flagellin and bacterial or viral antigens results in high antibody titres and occasionally protection against a wide range of mucosal pathogens, including but not limited to: *Yersinia pestis* (Mizel *et al*., 2009), West Nile virus (McDonald *et al*., 2007), *Pseudomonas aeruginosa* (Weimer *et al*., 2009), Vaccinia virus (Delaney *et al*., 2010) and *Streptococcus pneumoniae* (Nguyen *et al*., 2011).

Furthermore, vaccines containing TLR-2 agonists, or its natural ligands, such as *Neisseria meningitides* PorB, protected mice against herpes simplex type 2 challenge and experimental tularemia (Zhang *et al*., 2009; Chiavolini *et al*., 2008). The high-expression of TLR-2 on genital track DCs would make this targeted receptor an attractive candidate, especially for intravaginal immunization regimes.

Finally, TLR-4, although widely expressed on various DC sub-populations, has been met with skepticism as a vaccine targeted molecule. Its natural ligand, Lipopolysaccharide (LPS), causes activation of innate cells and secretion of pro-inflammatory cytokines, although it initially plays a significant protective role against intracellular pathogens, prolonged exposure may lead to microbial sepsis and tissue necrosis. Hence, the effect of a vaccine strategy targeting TLR-4 should be tightly regulated in order to avoid any unfavorable toxic side-effects.

1.10. Targeting Fc Receptors (FcR)

FcR are immunoglobulin receptors, widely expressed throughout the immune system. By binding to the Fc portion of antibodies, FcR provide a link between the powerful effector functions of the innate immune cells and the specificity of the adaptive immune system. Numerous studies have demonstrated that targeting antigens to FcR and in particular the activating FcγRI, FcγRIIA (in humans) and FcγRIII, expressed on monocytes, macrophages, DCs, basophils and mast cells, enhanced humoral and cellular immune responses both "*in vitro*" and "*in vivo*" (**Fig. 2**) (Adamova *et al*., 2005; Gosselin *et al*., 1992; Guyre *et al*., 1997; Keler *et al*., 2000; Liu *et al*., 1996; Rawool *et al*., 2008; Snider *et al*., 1990; Walsh *et al*., 2003). Cross-linking FcγR transduces signals to the interior of the cell, which can be stimulatory or inhibitory in nature depending on what signaling molecules are associated with the cytoplasmic tails of the FcR in question.

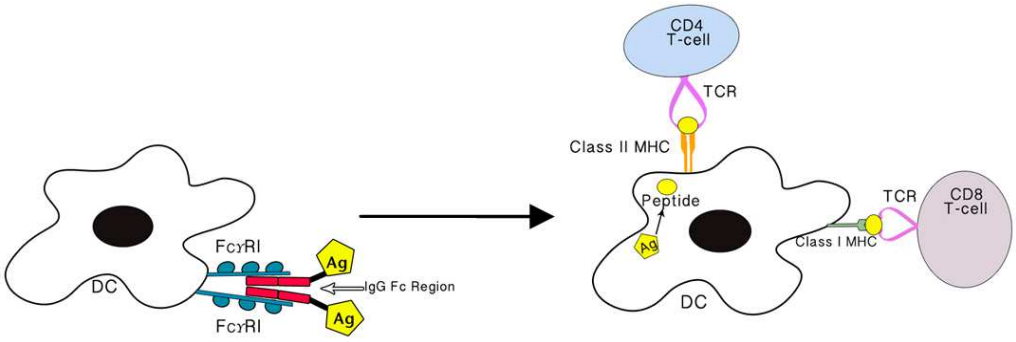

Fig. 2. Cross-linking the activating FcγRI receptors expressed on pAPCs, such as DCs, will enhance the uptake, processing and presentation of antigens to T lymphocytes

For example, the activating FcγRI generates stimulatory signals via the immunoreceptor Tyrosine-Based Activation Motif (ITAM), while inhibitory signals are generated via the immunoreceptor tyrosine-based Inhibition Motif (ITIM), following cross-linking of FcγRIIB, both in humans and mice (Daeron, 1997; Gessner *et al*., 1998). Thus, FcRs have a dual function on DCs: First, they will bind Immune Complexes (IC) facilitating uptake, processing and presentation of the antigen via MHC molecules, leading to T lymphocyte priming. Second, ICs will generate activating or inhibitory signals depending upon which FcRs they will cross-link on the DCs (Dhodapkar *et al*., 2002; Gosselin *et al*., 2009; Kalergis and Ravetch, 2002; Rafiq *et al*., 2002), hence affecting the outcome of the immune response. Consequently, FcRs are involved in regulating a multitude of innate and adaptive immune responses, which makes them attractive targets for vaccine strategies. Indeed, immunization with hepatitis B antigen-Ab complex enhanced antigen-specific IgG1 production, while in a different study, intranasal immunization of mice with *Streptococcus* mutants-Ab complex influenced the immunoglobulin isotype and humoral immune response against the pathogen (McCliskie and Davison, 1990; Brady *et al*., 2000; Rhodin *et al*., 2004). In our own studies, in, immunization of mice with fixed *F. tularensis*, LVS and anti-LPS IgG2a mAb ICs generated a protective, TH1-like response, against subsequent lethal LVS challenge (Rawool *et al*., 2008).

A significant disadvantage of utilizing an IC or Fc-Ag fusion protein techniques is the lack of specificity, allowing for targeting of the inhibitory FcγRIIB receptor, potentially suppressing the immune response. This problem can potentially be circumvented by using recombinant DNA technology: In one of our recent studies, we targeted the *S. pneumoniae* protective antigen, PspA, to human FcγRI in a transgenic mouse model, generating a pathogen-specific cellular and humoral immune response that protected mice against infection with *S. pneumoniae* serotype 3 (Bitsaktsis *et al*., 2012). By utilizing this specific targeting approach we avoided binding and activation of the inhibitory FcγRIIB, thus generated enhanced protective immune responses.

It is also important to note that the majority of IgG Ab subclasses bind the neonatal receptor, FcRn, expressed on epithelial cells. Hence, targeting to FcγRs is effective not only for its direct activating capabilities on pAPCs, such as DCs, but also because it facilitates trafficking through the epithelial layers and into the lymphocyte-rich mucosa-associated lymphoid tissues, leading to the generation of effective adaptive immune responses. Thus, construction of a fusion protein that specifically targets both the activating FcγRI, as well as the epithelial transport pathways (via the M cells) would provide a superior approach in the generation of FcR-targeted mucosal vaccines.

1.11. Targeting Ganglioside Receptors

Apart from on M cells, ganglioside receptors are also expressed on many subpopulations of DCs and are shown to be involved in antigen uptake (Grdic *et al*., 2005; Kawamura *et al*., 2003). In addition, ligation of ganglioside receptors on DCs causes the activation and maturation of the latter via an NF-kappaB-mediated pathway (Kawamura *et al*., 2003). Thus, targeting antigens to ganglioside receptors, mainly by fusing them genetically or chemically to AB_5 enterotoxins, enhances immune responses specific to the antigen. Due to the potential side effects of these toxins, various toxin subunits have been utilized that circumvent the problem

of toxicity while maintaining the adjuvanicity of the toxin. More specifically, intravaginal immunization of mice with a fusion protein comprising of the *Chlamydia* protective antigen, MOMP and the non-toxic subunit of cholera toxin, CTA2B, induced DC maturation and proliferation, while it established long-term, protective anti-chlamydial immunity (Ekong *et al*., 2009). Similarly, intranasal co-administration of fixed *F. tularensis* bacteria with CTB, a different non-toxic subunit of CT, has also proven effective against lethal infection with *F. tularensis*, LVS, via the generation of a strong TH1-like response (Bitsaktsis *et al*., 2009). In the latter study, CTB was not linked to the antigen, thus it is likely that the immunostimulatory effect was via binding of CTB to other receptors as well, such as TLRs, causing a more generalized activation of the innate immune system. Although these approaches have proved promising, the potential use of such toxins in the clinical setting raises regulatory concerns. Intranasal administration of CT and CTB has been shown to promote uptake of vaccine proteins in epithelial olfactory nerves and subsequent transportation into olfactory bulbs, increasing the possibility of neuronal damage. A way to circumvent the problem of toxin accumulation in the olfactory nerves, cholera toxin has been incubated with its ligand, GM1, prior to intranasal administration in order to minimize binding to the neuronal cells.

1.12. Targeting other Novel DC Ligands

In order to establish new vaccine platforms that target DCs, a phage-display peptide library has been used to identify various 12mer DC-specific binding peptides (Curiel *et al*., 2004). Fusion of these peptides with protective antigens from mucosal pathogens has given promising results in different infectious models. For example, genetic fusion of one of these peptides to hepatitis C virus non-structural protein, (NS)3, showed significantly improved immunogenicity as compared to a (NS)3 control fusion protein or (NS)3 protein alone (Mohamadzadeh *et al*., 2008). In a different study published from the same group, oral administration of a *Bacillus anthracis* protective antigen (PA) fused to a novel DC-binding peptide activated mucosal pAPCs, which in turn induced neutralizing anti-*Bacillus* Antibodies (sIgA) and pathogen-specific T cell immunity (Mohamadzadeh *et al*., 2009). In these studies, the novel, DC-targeting fusion proteins are delivered via probiotic bacteria, such as *Lactobacillus*. This innovative delivery approach has the following advantages: 1. *Lactobacilli* are part of the normal gut flora, 2. they are efficiently uptaken by M cells and transported through the epithelium layers to the mucosal DCs and 3. They have the ability to induce antigen-specific IgA in the mucosa and mesenteric lymph nodes (Robinson *et al*., 1997; Wells and Mercenier, 2008).

Studies are in progress to identify and characterize the molecular nature of these new DC-specific peptide ligands. It is of importance to note that the majority of these peptides are strongly involved in the endocytic pathway, allowing faster and more efficient transport of the immunogenic material into the cells without impairing DC functions. Further identification of these peptides will undoubtedly create new vaccine platforms for targeting DCs via these novel DC-binding peptides.

1.13. Lymphocyte Targeting-A Less Travelled Vaccine Approach

In order for lymphocytes to drive effective adaptive immune responses they require at least two activation signals: The first signal is generated following recognition of antigens via the T-cell or B-cell receptors. The second signal is provided by the ligation of co-stimulatory molecules, such CD28 and CD40 on T and B lymphocytes, respectively. Hence, targeting these extracellular receptors with agonistic antibodies would significantly enhance both cellular and humoral immune responses; a strategy often employed and tested in cancer treatments. The main caveat of this approach, apart from by-passing the opportunity to stimulate the innate immune system, is that lymphocyte activation is usually non-specific, creating potential autoimmune complications. Nevertheless, a small number of studies have utilized agonistic antibodies against lymphocyte receptors as vaccine adjuvants in order to increase the immunogenicity of an antigen, or to generate protective immune responses against various infectious agents.

1.14. CD21 (Complement Receptor 2) Targeting

CD21 exists as a signal-transducing complex together with the B-cell membrane protein CD19 (Bradbury *et al*., 1992; Matsumoto *et al*., 1991). In the CD21/CD19 complex, CD21 functions as the ligand-binding subunit, while CD19 is responsible for transmitting the signal intracellularly.

When antigens coated with the complement factor C3d interact with B cells, the antigen binds the BCR while C3d, the natural ligand of CD21, binds the latter receptor simultaneously. Thus, immunization with antigen-C3d fusion proteins may have a dual effect: It can facilitate the role of B cells as pAPCs by enhancing antigen uptake and presentation to T cells, while

simultaneously enabling B cells to carry out their antibody secretion effector functions. Indeed, using complement tagged proteins as antigens has shown great promise in infectious models. It has been established that complement activation plays an important role in the induction of humoral immune responses, which are key for protection especially against extracellular pathogens (Fearon, 1998; Nielsen *et al*., 2000). Dempsey *et al*. (1996) demonstrated that a recombinant protein containing three copies of C3d attached to the carboxy-terminal of Hen-Egg Lysozyme (HEL) elicited a primary response at a 10,000-fold lower concentration than that required by unmodified HEL. In a Bovine Viral diarrhea Model (BVD), immunization of cattle with a C3d-E2 viral envelope protein increased the immunogenicity of E2 by 1000-fold, as well as the levels of BVD neutralizing antibodies (Wang *et al*., 2004).

In studies of greater clinical significance, administration of DNA vaccines expressing secreted forms of Haemagglutinin (sHA) of influenza virus, sHA of measles virus and HIV gp120 fused to C3d all elicited higher titres of pathogen-specific neutralizing antibodies compared to immunization with the unmodified proteins (Mitchell *et al*., 2003; Ross *et al*., 2000; 2001; Green *et al*., 2003; 2001). Therefore, targeting protein antigens to CD21 not only increases their immunogenicity but also provides a cost-effective approach due to the significantly lower antigen concentrations required to elicit robust and protective immune responses.

1.15. 5.2 OX40 (CD134) Targeting

O×40 is a member of the Tumor Necrosis Factor (TNF) receptor super family and is transiently expressed by T cells after ligation of their TCR (Mallett *et al*., 1990; Calderhead *et al*., 1993; Godfery *et al*., 1994). Ligation of OX40 by OX40L, mainly expressed on pAPCs, contributes to optimal T-cell function including survival and activation of effector T cells as well as generation of memory cells (Gramaglia *et al*., 2000; Maxwell *et al*., 2000; Rogers *et al*., 2001). Indeed, injection of OX40L-immunoglobulin fusion protein into mice that were infected with *Cryptococcus neoformans* increased the number of interferon-γ (IFN-γ)-secreting CD4+T cells while it decreased the *C. neoformans* burden in the lungs (Humpherys *et al*., 2003). In a similar study, injection of mice with an agonistic anti-OX40 antibody prior to intranasal challenge with vaccinia virus increased the frequency of memory CD8+T cells in the lungs that were shown to mediate anti-viral properties protecting mice against lethal challenge with the virus (Salek-Ardakani *et al*., 2011). Hence, it is quite plausible that production of fusion proteins consisting of protective antigens and anti-OX40 antibodies may be a promising approach for targeted vaccines against various mucosal pathogens.

2. CONCLUSION

Prevention and cure of diseases caused by mucosal pathogens remain a challenge for science and research. Undoubtedly, the generation of protective, robust and cost-effective mucosal vaccines would revolutionize medicine.

In this review, we summarized the various mucosal vaccine approaches that target extra-cellular receptors with the intention to improve transport of antigens through the mucosal epithelia, mainly by targeting M cells, or to enhance long-term, protective immune responses against mucosal pathogens by specifically targeting DCs or lymphocytes (**Table 1**). Indeed, a wide range of cellular receptors have been exploited as candidates for targeted vaccines with variable success. The main drawbacks of most studies include the generalized over-stimulation of the immune system which could prove damaging to the host as well as the potential toxicity of some ligands. Alleviating these issues by using clinically safe molecules that can target and generate specific, robust and controlled immune responses is critical in creating successful mucosal vaccination regiments.

Understanding the mechanism of luminal antigen uptake that will deliver the antigen to the immune inductive sites is of high importance, as introduction of an antigen to the mucosal system does not guarantee the generation of an effective immune response. In addition, mucosal immunization, mainly in humans, results in compartmentalized immune responses that often lack in reproducibility. For instance, targeting DC populations in the lung parenchyma may limit the efficiency of a vaccine, owing to a relative excess of inhibitory macrophages. By contrast, delivering antigens to airway mucosal DCs, the most frequent and efficient pAPCs in the larger airways, may enhance immune responses against a pathogen. Thus, the need for a method that will analyze and assess human mucosal immune responses to vaccines remains unmet.

Furthermore, the majority of the vaccine studies utilize the specificity of antibodies to target cellular receptors. Despite this being a straight-forward approach, it is a very expensive strategy for the clinical setting. Results have indeed been promising, but vast production of these mAbs for immunization purposes would not be cost-effective, especially for Third World countries.

Table 1. Summary of extra-cellular receptors expressed on M cells, DCs and lymphocytes that can be targeted in order to enhance transport and immunogenicity of mucosal vaccines

Cell type	Receptor	Ligand	Associated Response
M Cells	Lectin	Germagglutinin (WGA)	Enhanced antigen endocytosis and transport through epithelia
	Lectin	Concanavalin A (ConA)	
	Pattern Recognition	Lipopolysaccaride (LPS)	
	Ganglioside	Cholera toxin (CT)	
	Ganglioside	Heat-labile enterotoxin (LT)	
	Claudin-4	Clostridium Perfringens enterotoxin (CPE)	
	Integrins	Fibronectin	
	Integrins	Invasin	
	Complement (C5a)	Outer membrane protein H (OmpH)	
	Neonatal Fc (FcRn)	Fc of IgG subclasses	
Dendritic cells	C-type lectin (CLR)	Mannan	Activation of innate and adaptive immune response characterized by antigen specific cellular and humoral response
	C-type lectin (CLR)	Mannose	
	Toll-like receptor (TLR)	Bacterial flagellin	
	Toll-like receptor (TLR)	CpG	
	Toll-like receptor (TLR)	Lipopolysaccharide (LPS)	
	Toll-like receptor (TLR)	Viral extracellular domain (M2)	
	Fc receptors (FcR)	Antibody Fc domain	
	Ganglioside	AB5 enterotoxins	
Lymphocytes	Complement receptor 2 (CD21)	Complement factor (C3d)	Enhanced B-cell antigen presentation and antibody production (CD21)
	OX40	OX40L	Enhanced T-cell activation and memory cell formation (OX40)

Hence, alternative strategies for production of vaccine fusion proteins should be employed, such as utilization of recombinant DNA technology.

Finally, we have to keep in mind that the majority of these vaccine studies are done on animal models and often cannot be extrapolated to human due to the very significant differences in anatomy, physiology and immunogenicity among the different species. However, multi-disciplinary efforts and collaborations between basic scientists and clinical vaccinologists may bridge these biological gaps and enhance our understanding of the human mucosal immune system.

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