Vaccination is the most efficient way to combat and prevent infectious diseases. However, most vaccines are administered systemically and are ineffective in eliciting protective immunity at mucosal sites. By contrast, oral delivery of therapeutic or prophylactic vaccines induces both systemic and mucosal immune responses. Additionally, oral delivery offers several advantages over systemic vaccination, such as ease of administration and increased safety. Despite these advantages, progress in oral vaccination has been rather slow due to the many hurdles posed by the gastrointestinal tract. To be effective as oral vaccine, antigens need to be resistant against or protected from acidic and enzymatic denaturation before reaching their target site, where their uptake should be enhanced, resulting in an increased immunogenicity. Despite the development of numerous delivery systems, their uptake by the intestinal epithelium remains poor. Most efforts are focussed on strategies to augment M cell mediated uptake. In the current review we discuss the possible strategies to target transcytotic receptors expressed on the apical surface of not only M cells, but also enterocytes to facilitate the uptake of antigen-loaded biodegradable microparticles, which could result in the induction of robust protective immune responses in multiple species.
challenging and time-consuming effort of identifying protective antigens. Additionally, the proper route of vaccine administration must be carefully selected to induce both local and systemic robust pathogen-specific immunity. Indeed, most infectious diseases are caused by pathogens that colonise and invade the host at mucosal surfaces and require the induction of pathogen-specific secretory IgA (sIgA) at the site of infection for an effective protection of the host through sIgA-mediated immune exclusion of this pathogen or its toxins. Nevertheless, in spite of this, the vast majority of commercial vaccines are delivered systemically by injection and in general fail to induce a pathogen-specific mucosal immunity due to the systemic homing specificity of effector lymphocytes activated in the peripheral lymph nodes. Therefore, in order to induce a protective immunity against intestinal pathogens, vaccines should be delivered to the intestinal mucosa via the oral route. This is especially true for non-invasive pathogens, such as ETEC, to which vaccine-induced protection is almost exclusively mediated by locally produced sIgA antibodies [3]. Although other mucosal administration routes (sublingual, nasal, pulmonary, rectal, genital) are available with the potential to induce immune responses at distant mucosal surfaces, they are less robust and less efficient to combat intestinal infections. Indeed, a remarkable compartmentalisation of the mucosal immune system exists due to the homing specificity of immune cells. In general, it appears that primed immune cells preferentially home to effector sites corresponding to the inductive sites where APCs initially were triggered by antigens [4]. On top of the induction of a local mucosal immunity at the site of infection, the oral route for vaccine delivery has several advantages over more traditional routes of administration, including systemic delivery. Oral delivery avoids the use of needles, thereby increasing patient compliance, reducing the need for trained personnel and averting vaccine-related infections correlated to the disposal and reuse of needles in systemic delivery. In addition, oral vaccine formulations could potentially avoid the cold chain for storage, which would benefit the developing world.

Notwithstanding these advantages, the oral route for vaccine delivery is the most challenging and the most difficult to achieve and progress in oral vaccine development has been rather slow. Indeed, vaccines based on inactivated pathogens are sometimes ineffective and, although vaccines containing live microorganisms (attenuated or vectors) are more effective, they carry the risk of reversion to virulence and the induction of disease in immunocompromised individuals. Due to these reactogenicity problems, novel vaccines consisting of protein antigens are currently being developed. However, the effectiveness of these subunit vaccines is hampered due to several physiological and immunological barriers posed by the gastrointestinal tract. For instance, proteins not only have to survive the low gastric pH and degradation by proteolytic enzymes present in the gastrointestinal tract, they often have to circumvent the interference by the lactogenic immunity, such as neutralising antibodies and milk factors. Other problems associated with oral vaccine delivery are the poor transport of antigens across the intestinal epithelium to reach the underlying gut-associated lymphoid tissue (GALT) and the induction of oral tolerance instead of protective immunity by the GALT. Indeed, to be effective as vaccine, antigens have to activate the innate immune system and subsequently evoke intestinal adaptive immune responses. However, oral administration of antigens generally results in a state of immunological hyporesponsiveness or oral tolerance [3,5]. A promising strategy to surmount these hurdles is the encapsulation of vaccine antigens in biodegradable particulate delivery systems, which can protect antigens from digestive enzymes and the maternal immunity. Uptake of these particulate delivery systems by the intestinal epithelium is rather poor and although modification of their size, surface charge or hydrophobicity can increase the efficiency of epithelial uptake, surface decoration of the antigen-loaded particulates with targeting ligands, specific for epithelial receptors, could further enhance the uptake and transcellular transport of antigens [6,7]. Moreover, this could potentially subdue the induction of oral tolerance since receptor-mediated endocytosis mostly induces antigen-specific mucosal immune responses. In addition, the incorporation of mucosal adjuvants in particulate delivery systems could lead to a more potent activation of the intestinal innate and adaptive immune system.

### 3. Oral antigen delivery systems

The efficacy of oral vaccines is currently limited by the poor immunogenic properties of the vaccine antigens and a very inefficient delivery of these antigens to the intestinal surfaces, which can be mainly attributed to gastrointestinal degradation and poor uptake by intestinal epithelial cells and antigen presenting cells (APC). A wide variety of particulate delivery systems, including polymer-based nano- or microparticles, immune-stimulating complexes (ISCOMs) and liposomes, have been developed to enhance the bioavailability and immunogenicity of vaccine antigens in human and livestock species [6,7,24–26]. Besides these delivery systems, the recent development of yeast ghosts, consisting mainly of...
Antigen sampling routes in the intestine and potential targeting of the molecular machinery involved in this sampling to augment particle uptake by the epithelium. Vaccine delivery particles can be internalised irrespective of the presence of targeting ligands on their surface either by M cells, present in the FAE of the Peyer’s patches, or by LP phagocytes, which extend dendrites into the lumen to sample this environment. This could enable these cells to endocytose delivery vehicles directly from the lumen. Although M cells at PPs efficiently take up particles, selective targeting to M cell-specific apical receptors could enhance this uptake and the subsequent delivery to APCs in the SED. Likewise, the internalisation of particles, targeted to LP APC-specific receptors present on the balloon bodies of protruding dendrites, could be facilitated. Enterocytes display several receptors involved in the transcytosis of macromolecules, e.g., FcR, and selective targeting to these receptors could enhance the internalisation and transcytosis of vaccine delivery vehicles [130]. APC: antigen-presenting cell, FAE: follicle-associated epithelium, LP: lamina propria, PP: Peyer’s patch, SED: subepithelial dome.

Lectins are a structurally diverse group of proteins and glycoproteins, which bind reversibly and with relatively high affinity to specific carbohydrate residues present on cell surface proteins or lipids. Since cell surface carbohydrate expression exhibits considerable regional and even cell-type specific differences, the use of lectins may permit targeting to specific locations within the intestinal tract. These lectins are mainly of plant or bacterial origin (Table 1) and a number of these lectins are relatively resistant to intestinal degradation [31].

Most research regarding lectin-mediated targeting of delivery systems has focussed on M cells, although some lectins have been

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**Table 1**

Overview of plant lectins and microbial adhesins used for targeted delivery.

<table>
<thead>
<tr>
<th>Plant lectin (source)</th>
<th>Abbreviation</th>
<th>Sugar specificity</th>
<th>Cell line or tissue targeted</th>
<th>Conjugation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ulex europaeus 1 (gorse)</td>
<td>UEA-1</td>
<td>α-L-fucose</td>
<td>Murine PP M cells</td>
<td>Liposomes</td>
<td>[43,44]</td>
</tr>
<tr>
<td>Triticum vulgaris (wheat germ)</td>
<td>WGA</td>
<td>(D-glCNAc)₂-sialic acid</td>
<td>Murine M cells and enterocytes</td>
<td>Liposomes</td>
<td>[36]</td>
</tr>
<tr>
<td>Lycopersicon esculentum (tomato)</td>
<td>TL</td>
<td>(glCNAc)₂</td>
<td>Caco-2 cells</td>
<td>Nanoparticles</td>
<td>[35]</td>
</tr>
<tr>
<td>Canavalia ensiformis (jack bean)</td>
<td>ConA</td>
<td>α-D-mannose, α-D-glucose</td>
<td>Porcine enterocytes, rat intestine, Caco-2 cells</td>
<td>Polystyrene microparticles</td>
<td>[39,40]</td>
</tr>
<tr>
<td>Aleuria aurantia (orange peel mushroom)</td>
<td>AAL</td>
<td>α-L-fucose</td>
<td>Murine PP M cells</td>
<td>Polystyrene microparticles</td>
<td>[35]</td>
</tr>
<tr>
<td><em>Wisteria floribunda</em> (Japanese wisteria)</td>
<td>WFA</td>
<td>sialic acid</td>
<td>Mouse intestine, M cells</td>
<td>HSA FedF</td>
<td>[57,58]</td>
</tr>
</tbody>
</table>

**Microbial adhesins (pathogen/host)**

<table>
<thead>
<tr>
<th>Fimbriae</th>
<th>Abbreviation</th>
<th>Sugar specificity</th>
<th>Cell line or tissue targeted</th>
<th>Conjugation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>F4 (K88)</td>
<td>(ETEC/pig)</td>
<td>Sialic acid containing glycoproteins</td>
<td>Porcine enterocytes and M cells</td>
<td>HSA FedF</td>
<td>[57,58]</td>
</tr>
<tr>
<td>F5 (K99)</td>
<td>(ETEC/pig, ruminants)</td>
<td>NeuGc-sialylneolactotetraosyl ceramide</td>
<td>Mouse intestine</td>
<td>BSA</td>
<td>[47]</td>
</tr>
<tr>
<td>F6 (987P)</td>
<td>(ETEC/Pig)</td>
<td>glycopolypeptide, sulphatide</td>
<td>Mouse intestine</td>
<td>BSA</td>
<td>[47]</td>
</tr>
</tbody>
</table>

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* Many lectins with diverse sugar specificities have been investigated as potential adhesins for intestinal epithelial cells. This table gives examples of those lectins which have been shown to enhance uptake of particles *in vitro* or *in vivo*.

* Both the F5 and F6 receptors are expressed in the porcine small intestine, but have not been identified in the mouse intestine. BSA, bovine serum albumin; ETEC, enterotoxigenic E. coli; FedF, adhesin subunit of F18 fimbriae; glCNAc, N-acetylglucosamine; HSA, human serum albumin; PLGA, poly(lactide-co-glycolic acids); PP, Peyer’s patch.
identified which bind to enterocytes [30,32–34]. For instance, both wheat germ agglutinin (WGA) and concanavalin A (ConA) enhanced the transcytosis of nanoparticles (NP) through Caco-2 monolayers and WGA also enhanced in vivo the endocytosis of polymerised liposomes by murine intestinal epithelial cells [35,36]. Porcine enterocytes also internalise WGA in vitro, although oral immunisation with WGA-coated antigen–loaded Gantrez NP failed to enhance the protective immunity against porcine ETEC [37]. The possibility of using a tomato-derived lectin (TL) as an intestinal bioadhesin has also been investigated. TL specifically binds to rat, porcine and human enterocytes in vitro and TL-modified nanoparticles were in vivo even more efficiently absorbed from the rat gut than unmodified particles. This uptake was mainly associated with non-lymphoid tissues, such as enterocytes [38–40].

The most investigated lectin is *Ulex europaeus* agglutinin 1 (UEA-1), which binds to α-L-fucose residues expressed on the apical surface of M cells, but also goblet and Paneth cells, in mice [41]. Both liposomes and polystyrene microspheres covalently linked with UEA-1 showed selective binding to and rapid uptake by murine M cells upon oral delivery [42,43]. Gupta et al. [41,44] chemically anchored UEA-1 to the surface of hepatitis B surface antigen–loaded poly(lactide–glycolic acid) (PLGA) nanoparticles or liposomes, which predomi-

nantly associated with PP M cells.

Upon oral immunisation, these particles elicited a significantly higher IgA response compared to untargeted particles or intramuscular immunisation, illustrating the potential of orally delivered M cell-targeted lectinised nanoparticles to induce appropriate mucosal immune responses. In addition, these particles also enhanced the cell-mediated immunity as evidenced by significant increases in the expression of the Th1 polarising cytokines IL-2 and IFN-γ. A similar increase in the expression of IFN-γ was observed upon oral administration of PLGA microparticles conjugated with a lectin derived from the edible orange peel mushroom *Aleuria aurantia* (AAL) in an oral birch pollen immunotherapy. Just as UEA-1, AAL also has specificity for α-L-fucose residues and targets murine M cells [45,46].

The major drawbacks of these plant lectins as targeting ligand for oral vaccine delivery are their toxicity, their anti-nutritional properties and their potential susceptibility to degradation once coated on the surface of microparticles. Indeed, feeding large quantities of the kidney bean lectin (*Phaseolus vulgaris* agglutinin; PHA) to rats had a deleterious effect on the nutritional status and in pigs often caused significant weight losses. Similar toxicities have been found for orally administered ConA, WGA and other lectins [47,48]. Besides this toxicity, some lectins, such as mistletoe lectin 1, are also highly immu-
genic and may therefore have potential as mucosal adjuvants. Nevertheless, this immunostimulatory capacity has to be taken into account, since the possibility exists that a strong local antibody re-
sponse directed against the targeting molecules could prevent the up-
take of the delivery system or could elicit an allergic response to the targeting ligand [33]. To overcome these problems, a high throughput screen of mixture-based synthetic combinatorial compound libraries in a UEA-1 competitive binding assay to human IEC has been devel-
oped, which identified stable non-toxic small lectin mimetics with a high specificity for α-L-fucose residues [49]. Although these lectin mimetics could have potential in oral vaccine targeting, the fact still re-

mains that the α-L-fucose receptor is not expressed on human PP M cells, whilst in pigs its expression is not restricted to PP M cells [9,50,51]. Furthermore, most lectins will interact with the highly glyco-
syalted mucus, which itself has high turn-over rate. Some studies suggest however that the prolonged residence time caused by mucus adhesion could enhance absorption by IEC. Indeed, mucus en-
trapment of lectin-conjugated microparticles resulted in improved absorption by rat enterocytes [49]. Although promising results have been obtained with lectins as targeting ligands, their use in vaccine delivery could be limited as the glycosylation pattern in the intestine differs between enterocytes and M cells at different intestinal locations (presumably influenced by the local microflora), with age and between species [30,50].

4.2. Bacterial adhesins

As an alternative for these plant lectins, it seems possible to ex-
ploit microbial adhesins, which mediate the attachment of microor-
ganisms to intestinal epithelial cells, for intestinal vaccine delivery, especially since these microbial adhesins are relatively resistant to in-
testinal degradation. For instance, several ETEC express fimbriae on their surface allowing adhesion to the intestinal epithelium [52]. In the case of F4* ETEC, F4 fimbriae bind to specific F4 receptors (F4R), present on the surface of porcine enterocytes. These receptors are necessary to induce a protective mucosal immunity following oral administration of purified F4 fimbriae to piglets, as in F4R animals, F4 fimbriae tend to induce oral tolerance [53,54]. The F4R mediates transcytosis of F4 fimbriae across the intestinal epithelium, suggest-
ing that fimbriae could be used as a carrier system [55,56]. In fact, conjugation of heterologous antigens to F4 fimbriae has been shown to induce enhanced mucosal antigen-specific antibody responses upon oral administration [57,58]. Unfortunately, F4 fimbriae cannot be used as a universal carrier system for the oral immunisation of all piglets, since some animals lack the F4 receptor [54].

Recently, glycoprotein 2 (GP2) has been identified as an M cell-
specific apical receptor in human and mice. This GP2 receptor is con-
served among mammals and its interaction with the FimH adhesin of type I-fimbriated bacteria (*E. coli, Yersinia, Salmonella*) is required for the M cell-mediated bacterial transcytosis as well as the subsequent induction of mucosal immune protection. This indicates that GP2 could provide a new approach for selective M-cell targeting in vacci-
nation protocols to prevent infectious diseases and it might be con-

sidered to determine if FimH or other GP2 ligands could direct antigens to M cells to elicit antigen-specific mucosal immunity [34,59,60].

However, bacterial adhesins are potent immunogens, raising the possibility that an immune response could be generated which is di-
rected against the targeting molecules on the surface of the delivery system, thereby decreasing the endocytosis of the vaccine delivery systems and the efficient induction of immune responses. Additional-
ly, since these adhesins originate from pathogens, the efficacy of the targeting system could be doubtful in the presence of a preexist-
ing immunity to the pathogen.

4.3. Adopting invasive strategies from enteric pathogens

M cells are considered as the gateways for antigen entry to the un-
derlying mucosal tissues and are therefore exploited by various en-
teric pathogens as a route of entry to the underlying host tissue, predominately by hijacking the endocytosis machinery of M cells [8]. The invasiveness of these viral or bacterial pathogens is mediated by specific pathogen–host interactions, which could be adopted to deliver antigens into the GALT [9,61]. Indeed, the adhesins required for pathogen attachment and invasion to specific epithelial receptors are currently being investigated to enhance oral vaccine delivery via antigen-encapsulated microspheres.

Reovirus can invade intestinal M cells in rodents and rabbits through interactions of the outer capsid protein σ1 with α(2,3)-linked sialic acid containing glycoconjugates on the apical membrane [62]. Incorporation of recombinant σ1 into liposomes enhanced binding to rat PP, whilst a recent study demonstrated the enhanced binding of an OVA-σ1 fusion protein to murine M cells, indicating that reovirus σ1 protein could be used to coat antigen-loaded microparticles to induce mucosal immune responses [63,64].

Yersinia on the other hand binds with its outer-membrane protein invasin to β1 integrin, which is expressed on the apical surface of enterocytes and M cells [65]. When this invasion protein is conjugated to the surface of latex nanoparticles, it improves the uptake of these
particles by M cells [66]. Furthermore, recombinant bacterial strains expressing *Versinia* invasin elicited both mucosal and systemic protection upon i.n. and oral immunisation, respectively, indicating the feasibility of β1 integrin targeting to induce protective immunity [67,68].

Tyer et al. [69] demonstrated the importance of M cell-specific apical α5β1 integrin in bacterial uptake and translocation by human and murine M cells. The endogenous ligand for α5β1 integrin is fibronectin through interactions with RGD (Arg-Gly-Asp) peptide motifs, which could provide the opportunity to develop M cell-specific targeting strategy [61]. Indeed, microspheres coated with RGD peptide motifs were more efficiently transported through the human FAE than peptide-coated microspheres which were unable to bind α5β1 integrin [70]. Recently, two studies confirmed the efficient endocytosis of PEGylated PLGA-based nanoparticles that display surface RGD peptide motifs or peptidomimetics targeting α5β1 integrins on human M cells in *vitro* and murine M cells in *vivo*, resulting in higher antigen-specific serum Ab responses following intraduodenal injection [71,72].

Attempts to mimic pathogen entry routes could lead to the development of ligand-mediated targeting of particulate vaccine antigens to M cells or enterocytes thereby increasing their endocytosis and the efficient initiation of mucosal immune responses. The efficacy of such an approach depends however on the species-specific expression of pathogen receptors, which hampers the development of a species-independent carrier or targeting ligand. Interestingly, expression in the FAE of the *Clostridium perfringens* enteroxin receptor, claudin 4, is conserved among mouse and human PP. Targeting of PLGA nanoparticles to claudin 4 enhances their in vivo uptake, indicating that this tight junction protein can be used to deliver bioactive compounds to the FAE [50,73].

4.4. Bacterial toxins

Cholera toxin (CT) from *Vibrio cholerae* and the heat-labile enterotoxin (LT) from ETEC are structurally related AB, enterotoxins, composed of an enzymatically active A subunit with ADP-riboseyltransferase activity, which mediates their toxicity, and a pentameric B subunit that binds with high affinity to gangliosides present on the apical surface of IEC [74]. These enterotoxins can be further classified into two major types on the basis of genetic, biochemical and immunological properties. The type I subfamily contains CT and LT-1, whilst the type II subfamily comprises LT-lla and LT-llb [75].

The main difference between these two subfamilies arises from a significant divergence in the amino acid sequence of their B subunits, resulting in differential ganglioside-receptor binding profiles. Whilst type I enterotoxins bind with high affinity to GM1-gangliosides, LT-lla binds with decreasing affinity to GD1b, GD1a- and GM1-gangliosides. On the other hand, LT-llb only binds avidly to GD1a [75,76]. In contrast to their respective holotoxins, the B subunits of LT-lla and LT-llb bind to TLR2 and activate TLR2-mediated cellular activation [77]. LT and CT both act as potent mucosal adjuvants and display strong immunomodulatory properties, which potentely enhance antigen-specific mucosal and systemic immune responses to co-administered antigens upon oral administration in rodents and in pigs, resulting in a long-term immunological memory to themselves and to bystander proteins [57,58,78,79]. However, significant toxicity issues could arise with the use of native toxins. Therefore, toxin mutants have been generated by eliminating or reducing the enzymatic activity of the A subunit [80]. The latter are less toxic, but still retain their mucosal adjuvant capacity, enhancing immune responses to antigens co-administered with or conjugated to these B subunits [81,82]. Moreover, these B subunits can have significant potential as carrier molecules. Indeed, the cholera toxin B subunit (CTB) undergoes transcytosis across human intestinal epithelial cell and enhances mucosal immune responses to conjugated antigens in humans following intranasal or oral administration [11,83,84]. Moreover, coupling of CTB to antigen-loaded liposomes generated enhanced mucosal immune responses in mice as compared to untargeted antigen-loaded particles [85]. As for CTB, conjugation of LTB to nanoparticles resulted in an enhanced uptake following oral delivery to rats [47]. Notwithstanding the great potential of these toxins or their B subunits as mucosal adjuvants or carrier molecules, the reported interactions of GM1-binding toxins with neuronal tissues and the subsequent B subunit-dependent retrograde transport to the brain after intranasal immunisation, poses a serious risk to their clinical use [86,87].

4.5. Pattern-recognition receptors

Pattern-recognition receptors (PRRs) on various cell types, including antigen presenting cells and epithelial cells, can recognise microorganism-associated molecular patterns (MAMPs), such as lipopolysaccharide (LPS), to enhance attachment and phagocytosis of microorganisms [12]. For instance, Toll-like receptor (TLR) 4, the receptor for LPS, mediates bacterial translocation through enterocytes and M cells [16,69]. In addition, in *vitro* experiments demonstrated that the rat enterocyte cell line IEC-6 internalised LPS-coated, but not uncoated latex beads in a TLR4-dependent manner, indicating that LPS-coated particles may provide yet another alternative for targeted delivery of antigen-loaded particulates to the intestinal epithelium, albeit that reduced immune responses were observed in comparison to uncoated NP [16,88]. In contrast to TLR4 targeted particles, the coating of NP with *Salmonella*-derived flagellin enhanced their uptake by Peyer’s patches and elicited higher systemic and mucosal antibody responses upon oral administration [89]. Besides TLRs, epithelial cells express a variety of mammalian lectins, which bind particular carbohydrates, and conjugating carbohydrates on the surface of particles could target these to the enterocytes. Indeed, mannosylation of nanoparticles enhanced both the transepithelial transport of these particles across Caco-2 monolayers and the uptake by rat Peyer’s patches [72,90].

4.6. Antibody-mediated targeting

An alternative technique for achieving intestinal epithelial targeting would be to exploit antibodies directed against apical cell surface antigens. The feasibility of such an approach was shown almost two decades ago by Pappo et al. [91], who demonstrated that the internalisation of polysytrene microparticles was increased by coating with a mAb against an apical surface antigen expressed on both enterocytes and M cells. Recently, Nochi et al. [92] developed an M cell-specific mAb (NKM 16-2-4) as a carrier to target vaccine antigens to M cells. Upon oral administration to mice, the NKM 16-2-4 conjugated with botulinum toxin induced strong Ag-specific mucosal IgA responses, which protected the animals against a lethal challenge with the toxin. However, as with UEA-1 and AAL, its application as a universal M cell-specific marker will be limited, since this mAb specifically detects fucose-containing carbohydrate structures on murine M cells. These findings nonetheless illustrate the potential of antibody-targeted delivery of particles to epithelial cell surface antigens to enhance internalisation and the induction of protective immunity. Similarly, antibody-mediated targeting of particulate delivery systems to galectin-9, which is a human M cell-specific marker [70,93], could potentially enhance vaccine delivery to the human intestine, although the induction of a protective immunity by this approach seems unlikely as galectin-9-mediated signals promote regulatory T cell differentiation and suppress Th17 cell differentiation [94].

As mentioned earlier, F4 fimbriae bind to specific F4R, which mediate transcytosis of the fimbriae across the intestinal epithelium. This F4R has been identified as a glycoprotein and certain carbohydrate structures present on this protein are important for F4 fimbrial binding [95]. The inability of F4 fimbriae to attach to the epithelium of F4R-piglets could be attributed to the absence of these F4-specific
5. FcRn as a candidate receptor for targeted antigen delivery

The neonatal Fc receptor, FcRn, was first described in the intestine of neonatal rats [98]. Since then FcRn expression has been described in many species, including human, primates, mice, rabbits, ruminants and pigs and has been detected in several tissues, such as kidney, mammary gland, lung, placenta, vascular endothelium and intestinal epithelium of these species [99–101]. FcRn structurally resembles MHC class I molecules, although the peptide-binding groove in FcRn is occluded, indicating that FcRn-mediated Ag presentation to T cells is highly unlikely to occur [102]. In contrast, FcRn binds the Fc portion of IgG with high affinity at slightly acidic pH (pH 6.5), but not at physiological pH (7.4) [103]. Several functions have been ascribed to FcRn, which are a direct consequence of this unique pH-dependent IgG binding. First, FcRn is involved in the transfer of maternal IgG from the mother to her offspring to confer on them a short-term passive lactogenic immunity. This FcRn-mediated IgG transfer occurs through placental and/or intestinal pathways depending on the species [100,104,105]. Second, this receptor plays an important function as a homeostatic regulator of two important serum proteins, IgG and albumin, in adult mammals [100,106]. In contrast to the transfer of maternal IgG, this regulation occurs via a highly similar mechanism in all species thus far analysed. Endothelial cells express FcRn and upon internalisation of serum proteins by these cells, endosomal FcRn can bind internalised IgG after acidification, thereby protecting IgG from lysosomal degradation and thus prolonging its serum half-life to 22 days in humans and 45 days in mice [107,108].

More recently, in vitro and in vivo studies have demonstrated that FcRn could play a prominent role in mucosal immunity as an immunological sensor [101]. In rodents, the intestinal expression of FcRn rapidly declines after weaning, whereas in humans, pigs and other species, FcRn expression occurs both in neonates and adults [17,18]. Furthermore, FcRn is expressed in human and porcine intestinal epithelial cell lines and transcytoses IgG molecules bidirectionally across these polarised cell monolayers [109–112]. The potential role of FcRn in the adult intestine has been studied with transgenic mice models, in which endogenous FcRn has been knocked out, whilst human FcRn is expressed in intestinal epithelial cells. The slightly acidic environment of the small intestine allows FcRn, which is expressed at the apical surface of the enterocytes, to bind both IgG and antigen-IgG immune complexes (IC). FcRn then transcytoses these IC through the epithelial barrier and releases them in the underlying extracellular space, which is at physiological pH. Following transcytosis from the intestinal lumen to the underlying GALT, these IC are endocytosed by CD11c⁺ lamina propria dendritic cells, which then migrate to the mesenteric lymph nodes to activate CD4⁺ T cells. Alternatively, FcRn can also deliver pathogen-specific IgG to the intestinal lumen, which subsequently enhances the resistance of mice to an intestinal infection [17,113]. These findings clearly illustrate the role of FcRn-mediated transepithelial transport as an important mechanism by which DC can sample luminal antigen for the control of mucosal pathogens. Kobayashi et al. [114] expanded this view by demonstrating that DC, which acquire luminal antigen, in this case flagellin, by this mechanism can initiate Ab responses that aggravate an ongoing inflammation. Intriguingly, it has recently been demonstrated that i.p. administration of Cry1Ac, a toxin derived from Bacillus thuringiensis, exerts potent mucosal immunogenic properties and induces FcRn expression in intestinal epithelial cells from adult mice. This finding implies that FcRn expression can be upregulated in response to intestinal infection to enhance transcytosis of pathogen-specific IgG to the intestinal lumen to combat these intestinal infections [115]. Whilst these studies provide evidence that FcRn can transport immune complexes to small intestinal Lp DC, which can subsequently induce protective immunity, the usefulness of this FcRn-mediated transport for targeted oral delivery of particulate antigens to intestinal DC and the induction of a protective SlgA immune response in humans and domestic animals still remains to be determined.

5. PRR ligands as oral adjuvant

The intestinal environment is extremely rich in food antigens and antigens derived from the commensal microflora. An increasing body of evidence indicates that intestinal epithelial cells (IEC), which were once considered as merely providing a physical barrier to the external environment, play a more cardinal role in the induction of innate and adaptive immunity and the regulation of immune homeostasis [116,117]. Because of their barrier function, IEC are the first cells encountering intestinal pathogens and they can act as immunological sensors detecting pathogens through different classes of pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) [118,119]. In general, PRR engagement by MAMPs elicits the secretion of proinflammatory cytokines and chemokines, the subsequent recruitment of APCs and the priming of effector T and B cell responses. Despite the importance of these PRRs in pathogen detection, most MAMPs are however shared by both pathogens and the commensal flora. Consequently, even under homeostatic conditions, intestinal epithelial cells (IEC) are continuously exposed to many PRR ligands and several mechanisms have evolved to dampen and modulate the response of IEC toward gut flora-derived MAMPs. For instance, the prolonged exposure of IEC to MAMPs results in tolerance and cross-tolerance to other PRR ligands. However, the recognition of these commensal-derived MAMPs by epithelial TLRs is nevertheless crucial for maintaining the intestinal immune homeostasis [120,121]. Recent evidence indicates that an intensive crosstalk between IEC, commensals and intestinal DC creates a tolerogenic environment, which dampens and modulates the cytokine expression by IEC and the activity of DC, resulting in a state of immunological unresponsiveness to harmless commensals [122]. This is a major obstacle for the development of oral vaccines, which should overcome these tolerance mechanisms by the incorporation of immunopotentiators or adjuvants [117].

The best known mucosal adjuvants are CT and LT, however, as mentioned earlier, some safety issues arise with the use of these toxins in mucosal vaccines for human application. Alternatively, TLR ligands have a strong adjuvant activity when administered systemically and some even display strong adjuvant properties at mucosal sites in both experimental and domestic animals. Among these, unmethylated CpG oligodeoxy-nucleotides (ODN), which are recognised by TLR9, display strong immunomodulatory and adjuvant properties. Indeed, upon mucosal administration these CpG ODN enhanced innate and adaptive immune responses in animal models [78,123]. Another MAMP with promising adjuvant properties is the TLR5 ligand, flagellin [124]. TLR5 is expressed by intestinal epithelial cells and triggers the latter to secrete pro-inflammatory cytokines, such as CCL20, a chemotractant for DC [125,126]. Moreover, flagellin induces the maturation of intestinal DC, activates CD4⁺ T cells in vivo and promotes the development of mixed effector T cell responses [124,127,128]. With respect to flagellin, this offers the opportunity to combine enhanced uptake by the epithelium (see Section 4.5) and...
activation of the intestinal immune system by targeting PRRs via PRR ligand surface modification of microparticles. It should be noted however that PRR activation does not necessarily coincide with any potential ad- hesive properties as some PRRs reside in intracellular compartments, such as endosomes, whilst others are only accessible upon transcytosis through the intestinal epithelium.

Thus, it seems that these TLR ligands have an intrinsic adjuviant capacity, presumably because they activate key innate signalling pathways and stimulate IEC or the appropriate mucosal DC, which in turn orchestrate adaptive immune responses to combat infections. However, live attenuated vaccine vectors are still superior as mucosal vaccines, in part due to their ability to activate multiple innate re- sponses through the presence of several PRR ligands [129]. This im- plies that oral vaccines should incorporate several mucosal adjuvants, which can activate multiple innate signalling pathways, to induce adequate adaptive immunity.

6. Conclusions and perspectives

The tremendous impact of vaccination programs on global health is without question. Oral vaccination has great potential and many benefits in comparison to systemic delivery, including ease of admin- istration and the induction of a local protective immunity at mucosal surfaces. However, despite many efforts, vaccinologists still struggle to develop highly efficient oral subunit vaccines owing to the highly degradative environment in the gastrointestinal tract and the pres- ence of a general tolerogenic microenvironment in the GALT. The en- capsulation of antigens in biodegradable microparticulate delivery systems has the potential to accelerate the development of oral vac- cines as this will render antigens more immunogenic, mimicking polymeric antigens and pathogen dimensions and protecting them against proteolytic degradation and neutralisation by maternal anti- bodies. However, uptake of these particles by M cells and enterocytes remains poor. Further research to define the optimal properties of mi- croparticles, such as hydrophobicity, size and surface charge, to en- hance not only their interaction with the intestinal epithelium, but also their internalisation by epithelial cells, seems necessary to ac- celerate oral vaccine design. The latter could be improved by targeting strategies, which are not solely focused on M cells, but are also ex- panded to enterocytes. Genome-wide surveys could identify novel epithelial transcytotic receptors, which are conserved across mam- mals, for the selective targeting of oral vaccines, as this would allow the generation of a multispecies vaccine platform. However, the age-related variation in expression levels of these receptor candidates should be assessed to ensure that the targeting strategy is applicable in both children and elderly. In this respect, FcγR seems a plausible receptor candidate for the selective targeting of vaccine antigens. This receptor is involved in antigen transcytosis across the epithelial barrier, is conserved among different species and is expressed throughout life by intestinal epithelial cells. In addition, FC-fragment mediated FcγR targeting could enhance uptake of FC-decorated mi- croparticles by intestinal APCs [130]. A potential pitfall associated with oral vaccination is the presence of a tolerogenic environment in the intestine maintained by a triadoule between commensals, in- testinal epithelial cells and antigen-presenting cells. Proper activation of IECs seems essential to allow a switch from a tolerogenic to an immune-inductive environment. This necessitates the development and co-encapsulation of efficient mucosal adjuvants, such as CpG or flagellin, although the proteinaceous nature of the latter could be a drawback. Nevertheless, selective targeting to conserved epithelial receptors involved in transcytosis would enhance the efficiency by which antigen/adjuvant-loaded microparticles induce robust protec- tive immune responses.

To our knowledge so far none of the described approaches have currently been translated to a human or large animal setting. This can be explained because mice have a limited predictive value in the context of mucosal vaccine studies and as a model seem inapt to grasp the fine-tune regulation of the innate and adaptive immune system. For example, human and mice differ more form each other in physiology, intestinal (micro-)architecture, molecular immune system than pig and human [131,132]. This makes the translation to human or large animals more difficult and calls for the use of more appropriate animal models to study the efficacy of novel oral vaccina- tion strategies.

7. Uncited references

[20]

Acknowledgements

FWO-, IWT-Vlaanderen and UGent are acknowledged for their financial support.

References


