In vitro studies are elucidating the receptor basis of human Peyer's patch M cell pathogen entry with a view to discovering new targets to enable oral vaccination with particle-entrapped antigens

Keynote review:

Intestinal Peyer's patch M cells and oral vaccine targeting

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Specialized M cells in the follicle-associated epithelium of intestinal Peyer's patches serve as portals for diverse particulates. Following antigen handover to dome lymphocytes, a protective mucosal antibody secretion ensues. One approach to oral vaccine delivery is to mimic the entry pathways of pathogens via M cells. The paucity of human tissue for *in vitro* investigation has hampered the discovery of M-cell pathogen receptors; however an *in vitro* human M like-cell culture model displays many expected phenotypic features. Comparative studies using microarrays reveal several novel M-cell surface receptors that could be used to potentially target orally delivered antigens.

Although intestinal Peyer's patches (PP) were first described by Johan Peyer more than 300 years ago, the detailed cellular structure of these groups of lymphoid follicles only began to be deciphered in the 1970s with the advent of microscopic techniques that permitted elucidation of component surface epithelial cell types [1]. PP populations vary with respect to species, anatomical location, with age/developmental stage and as a consequence of exogenous factors. In humans, the number of PP along the length of the gastrointestinal tract increases to ~300 at puberty and then declines thereafter [2]. PP are at their highest density in human ileum where they comprize 10–1000 individual follicles organized into discrete lymphoid structures overlaid by a follicle-associated epithelium (FAE). Individual PPs are an example of organized gut-associated lymphoid tissue (GALT). They are visible to the naked eye as rounded or elongated structures, apparent on the luminal surface of the intestine. Their average diameters range from an eighth of a centimetre in mice, to one centimetre in dogs and man, and up to tens of centimetres in ruminants. However, their borders are not distinct and can be difficult to identify macroscopically. PP and

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lymphoid follicles are also found in the human colon but their function remains unknown. Within the FAE, M cells (or 'microfold' or 'membranous' cells), enterocytes and goblet cells comprize subsets. In 1922, rabbit PP were shown to be sites of uptake of *Mycobacterium tuberculosis* [3] but this was dismissed as a non-specific process of little importance.

Up to 95% of pathogens cross epithelial barriers, therefore, attempts to manipulate specific immune responses at inductive sites, such as PP, could lead to new mucosal vaccines against established and emerging diseases [4,5]. Methods to generate specific immunity can be informed by understanding antigen-presenting mechanisms at these invasion site(s). Existing examples of mucosal inductive sites employed in this way include intradermal delivery of antigens to dendritic cells in human skin [6], as well as delivery of mucosal vaccines via nasal-associated lymphoid tissue [7]. It is anticipated, therefore, that a proper molecular and functional analysis of these portals of antigen exposure will lead to rational design of novel mucosal vaccine formulations comprizing appropriate targeting strategies and adjuvants.

Here, we review the role of the M cell in initiating mucosal immunity and update current knowledge of M-cell structure and function across a range of species as it impacts on the feasibility of targeting of oral vaccines. Recognizing that other sampling cells and mechanisms also contribute to mucosal immunity, we review the evidence for M-cell-specific uptake of a range of particulates including microbes. We discuss how these events relate to immunological outcome. Finally, we describe the potential of a human M-like cell model for production of new vaccine receptors for oral targeting.

M cells and immunity

In common with other M-cell-containing GALT, including tonsils and appendix, at one time PP were erroneously regarded by some as vestigial organs. However, this view has changed. The immunology of mammalian PP has been recently reviewed [8], specifically with respect to tolerance and food allergy [9] and also to parasite infection [10]. FAE-located M cells sample particulates from the luminal side of the gastrointestinal tract, presenting them to the lamina propria, which contains dense populations of lymphocytes, macrophages and dendritic cells. Using *in vitro* and *in vivo* methods, it has been shown that PP can absorb a wide variety of particulates, including proteins [11] and antigens [12]. This is important because the epithelium is considered a barrier to the entry of non-nutritional macromolecules from the lumen. General agreement on M-cell function fails to go much further than this. At the same time, the structure and function of PP express species-specific features, which contribute to host–pathogen biology. Although M-cell numbers are thought to be regulated by bacterial challenge, there are abundant PP-like follicles in the sterile small intestine of neonatal ruminants and pigs. These B-cell-rich follicles, which disappear by young adulthood, can achieve a mass equivalent to that of the thymus representing up to 1% of total body weight [13]. The principal contribution of such transient structures might be in the basic education of the immune system, including a contribution to the development of immunological tolerance [14]. A second mammalian population of PP-like lymphoid follicles tend to reduce with age, albeit much more slowly. Species-specific patterns of PP development have been described [13] but strict functional interpretation awaits elucidation.

BOX 1

Requirements for a successful targeted oral vaccine

- High entrapment efficiency in particulate formulation
- Antigen stability in particle retained
- Protection of antigen from intestinal metabolism
- Uptake of vaccine by M cells through targeting and/or adjuvants
- Antigen trafficking to dome lymphoid and dendritic cells
- Stimulate durable systemic and mucosal immunity
- Protect animal model against challenge
- Scale-up formulation process
- Phase I trial

M cells and other routes of antigen trafficking across the intestine

It is generally accepted that the epithelium overlaying PP and, more specifically, the M cell contributes in an active manner to antigen trafficking. Thus, in the case of neonatal large farm animals the rate of uptake by FAE could be of a magnitude that is enough to permit effective passive transfer of maternal immunoglobulins [15]. However, the proportional contribution to overall function of the immune system (as well as opportunities for vaccine strategy) is not well understood. As a portal of entry, M cells therefore represent generic sampling sites. Their principal role is to deliver exogenous (luminal) material to subepithelial compartment(s). Questions remain as to whether M-cell translocation of immunogens has a specific role in initial immune induction and also in the response to challenge of a primed system.

M-cell translocation of luminal material appears to represent a breach in the innate immune system. However, this might be of advantage to the host if such delivery is coupled to immune protection of a previously primed mucosal immune system. For example, M cells in PP are regarded as being instrumental in initiating mucosal immunity against pathogens invading across epithelial barriers [16]. From studies with IgA knockout mice, it is established that the production of secretory IgA in response to specific viral pathogens is an effective way to prevent subsequent attachment of the agent to the PP mucosa and that this protects against oral challenge [17]. Therefore, M-cell uptake of antigen and subsequent hand-over to professional antigen presenting cells appears to be a key

trigger of the process of inducing a range of outcomes, including tolerance as well as systemic and mucosal immunity.

M cells represent potential 'targets' with which to increase the effectiveness of oral vaccines. Protein, peptide subunits or DNA vaccines can be delivered in similar particulate formats as the many pathogens to which mammals have evolved successful immunological strategies. These advantages can fulfil criteria for oral vaccines (Box 1). In addition, it might be possible to encapsulate M-cell targeted antigens in particles containing heat stable sugars that will address the issue of the refrigeration requirement for distribution of vaccines in the hot climates of the developing world. Additional advantages include elimination of the syringe delivery system, reduced requirements for training of personnel and reduced stringency of formulation (for non-parenteral administration).

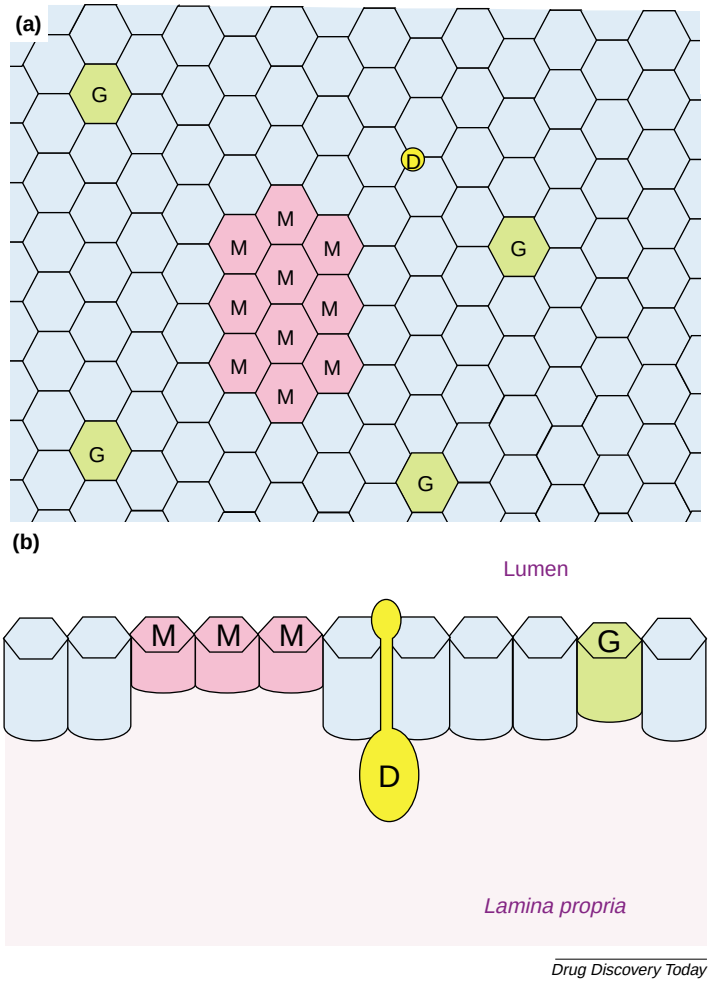

FIGURE 1

M-cell uptake of macromolecules. These cartoons offer **(a)** an 'antigen-eye' view of the luminal surface of intestinal epithelium and **(b)** a cross-sectional representation through the epithelium. Most nutrient-absorbing enterocytes (blue) have apical membranes covered with microvilli, glycocalyx and mucopolysaccharide gels. Goblet cells (G) secrete mucous in tandem with protective ion and fluid secretion by enterocytes. M cells (M) are interspersed between enterocytes in the follicle-associated epithelium (FAE). Projections from dendritic cells (D, yellow) cross intercellular junctions to span the luminal and sub-epithelial spaces. For simplicity, this representation ignores the complicated architecture of the gut wall.

M cells are not the only cells involved in sampling bacteria from the intestinal lumen. A newly discovered route suggests that dome-derived dendritic cells (DCs) can extend projections into the lumen to capture antigens for presentations to intraepithelial lymphocytes [18]. DCs also express tight junction proteins, therefore, it is tempting to speculate that some pathogen-derived secretions including *Clostridium perfingens* enterotoxin might selectively adhere to these targets in the intestinal lumen in advance of reaching the epithelium. This M-cell-independent mechanism was demonstrated by using a co-culture of mouse DCs with human Caco-2 monolayers, together with bacterial challenge to the apical side. Invasive, but not commensal, bacteria were able to induce the epithelium to release type-1 cytokines, which in turn act to induce DC maturation. A widely held view is that the DC 'extension' bacterial monitoring model might have a complementary role in tolerance induction, as well as in the adaptive immune response.

M-cell structure and function across different species

The significance of comparative biology to understanding M-cell function is twofold. First, traditional reliance on non-human species for fundamental and applied research has generated a much greater archive of literature on rodent and rabbit PP than those of humans. Second, and more optimistically, it is likely that survival-linked evolution has resulted in a reasonable degree of conservation of the fundamental mechanisms of M-cell function across species. It might be useful to consider 'conserved' and 'non-conserved' characteristics of M cells that exhibit species-specific, age- and site-dependent variations. First, M cells exhibit some structural similarities across a range of species; they are typically characterized by short truncated microvilli, a thin glycocalyx and an invaginated basolateral pocket, which houses lymphocytes and a reduced level of intracellular lysosomes [19]. However, a reduced brush border is not always a feature of FAE [20]. It is likely that alterations in the glycocalyx and brush border at different GALT regional sites arise due to the influence of luminal microbes. Truncated microvilli should promote sampling function because particle access to the apical membrane should enable transcellular passage across the basolateral membrane to strategically located sub-epithelial lymphocytes or dendritic cells. By contrast, the large surface area of intestinal microvilli is better designed to absorb greater amounts of soluble nutrients, electrolytes and water. Some of these aspects can be considered from a luminal viewpoint (Figure 1).

A second conserved characteristic of M cells is their contribution to acquired immunity. There is some evidence that PP can both process and present antigens because MHC Class II determinants are expressed on the basolateral sides of rat M-cell membranes [21] and also in human FAE [22].

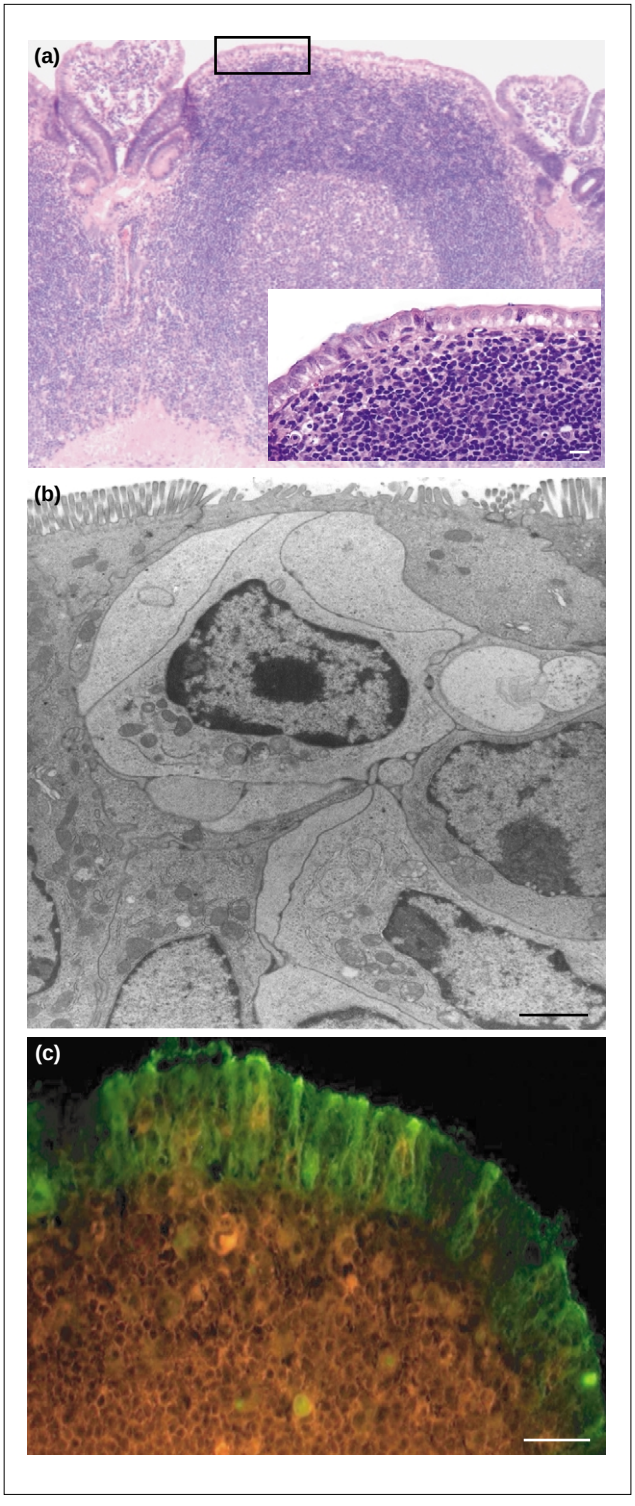

FIGURE 2

Locating M cells in the follicle-associated epithelium of rodents. **(a)** Haemotoxylin-eosin stain of a rat Peyer's patch and overlying follicle-associated epithelium (FAE). Lymphoid follicles in the submucosa underlie the dome areas nestling between villi. FAE is present overlying the dome lymphocytes. Scale bar = 10 μm. **(b)** Transmission electron micrograph of rat M cell (centre) flanked by two enterocytes. Scale bar = 1 μm. **(c)** Murine M cells closely apposed by B cells as shown by dual immunohistochemical staining. M cells are labelled with FITC-UEA. B cells are labelled with monoclonal antibody, M5/114.15.2, reacting with I-A and I-E sub-region encoded glycoproteins of MHC Class II. Scale bar = 10 μm. (Fluorescent photograph for Figure 2c provided by David Lo of the La Jolla Institute for Allergy and Immunology, USA).

Initiation or completion of antigen processing will be regulated by other factors in the case of a primary response, or by immunological history in the event of occasional or chronic exposure to luminal bacteria. Overall, M cells fulfil many of the criteria for antigen presenting cells according to current definitions.

The proportion of M cells in FAE in PP from different species is variable. Expressed as a percentage of M cells out of the total cells of the FAE, rabbits and mini-pigs are well-endowed (>20%). By contrast, there are proportionally fewer M cells in the PP FAE of rodents (~10%) and humans (<5%) [23]. Along with the potential for dilution in the intestinal tract, the low number of M cells available in man might impact on achieving significant antigen targeting. Some functional differences have also arisen from inter-species comparisons. For example, rabbit M cells absorb polystyrene microparticles especially well, whereas human and mouse M cells do so less avidly [24]. Furthermore, confocal microscopy studies of M cells in different PP from the same individual mouse reveals considerable variability in the degree of particle adherence [25]. Together with intra-species variations in transit time and PP:intestine surface area ratio, predictions of immune responses to oral vaccines in man are clearly difficult to make on the basis of particle uptake by M cells in laboratory animals.

Differences in PP structure and function across species appear to outnumber the similarities. The current PP M-cell structural model is based primarily on microscopic data from rodents and rabbits. In contrast to human and rodents, sheep and other ruminants display 4–8 large discrete PP in the jejunum and, in addition, have a long continuous temporary PP stretching from the jejunum to the ileo-caecal opening [26]. Within the FAE, M cells are flanked and outnumbered by columnar enterocytes (Figure 2). Mucus-secreting goblet cells tend to be present in similar low quantities as M cells in FAE in most species but there is no strict correlation between them. For example, rabbit FAE contains no goblet cells despite a large amount of M cells. In addition, Jang *et al.* [27] have described the existence of intestinal villous M cells in mice and provided evidence that these cells perform a role in PP-independent induction of antigen-specific immunity. These non-PP M cells appear to differ from rabbit vimentin- and lectin-positive villous (cup) cells because the latter do not perform any endocytotic function [28].

M-cell ontogeny

M-cell numbers appear to be regulated by endogenous (host) factors and exogenous (environment) factors. As with the underlying lymphoid tissues, PP FAE exhibit an organized distribution. In rabbit ileal FAE, the majority of M cells are located along the flanks where pores in the basement membrane enable the migration of leucocytes [29]. Using deoxyuridine as a tracer, M cells were then shown to migrate up to the top of the dome before being sloughed off into the lumen. There is some suggestion

TABLE 1

Receptors and markers on FAE or M cells from various species[a]

Receptor	FAE / M-cell models	Sample reference
ICAM-1	Human colon lymphoid tissue	[94]
Reduced alkaline phosphatase	Mouse, rabbit, human FAE	[44]
SLAA	Human?	[45]
α L-fucose	Mouse M cells	[48]
GM_1 for cholera toxin B	Ubiquitous	[47]
β1 integrin	Mouse M cells	[95]
Muc-2	Rabbit M cells	[61]
β catenin, α actinin, E-cadherin	Mouse FAE	[54]
α2–3-sialic acid	Rabbit FAE	[83]
CCR5 receptor	M-like cells	[96]
CD155 receptor	Human, primate FAE	[77]
CPE-R, MMP-15	Human, primate FAE	[46]
Laminin β3	Human, mouse FAE	[68]
PGRP-S	Mouse M cells	[39]
IgA-specific receptor	Mouse M cells, possibly human	[42]

[a]Exclusive M-cell expression by any of these markers is unlikely; some are expressed on FAE and others are also expressed in villous epithelia.

Abbreviations: FAE, follicle-associated epithelium; ICAM-1, Intracellular Adhesion Molecule-1; SLAA, Sialyl Lewis Antigen A; GM_1, ganglioside receptor for CTB; *Muc-2*, rabbit equivalent of human mucin *Muc2*; α2–3-sialic acid, receptor for reovirus; CCR5, receptor for HIV-1; CD155, receptor for poliovirus; CPE-R, *C. perfringens* enterotoxin receptor; MMP-15, Matrix Metalloproteinase 15; Laminin β3, putative receptor for prions; PGRP-S, Peptidoglycan Recognition Protein-S.

that M cells differentiate during this journey and are fully phagocytic at the apex of the dome for several days. Such evidence is consistent with the hypothesis that M cells can differentiate from stem-like cells in the intestinal crypts.

Lymphocytes influence many aspects of epithelial function and this appears to include FAE growth and differentiation [30]. It seems that B cells are not an absolute requirement for initial FAE formation, although they promote its full development. For example, in B-cell-deficient mice, PP are reduced in number and size [31]. This is further supported by the observation that B-cell-derived lymphotoxin and tumour-necrosis factor are required for the development of lymphoid follicles in PP but these are not obligatory for FAE maintenance [32]. Lambs whose ileal PP have been surgically removed become deficient in antibody-bearing lymphocytes, suggesting that communication between lymphocytes and FAE might be bi-directional [33]. Debard *et al.* [34] have suggested the existence of antigen-independent (constitutive) formation of FAE and M cells before birth followed by environment-dependent recruitment of lymphocytes into the dome region in the neonate. Therefore, B cells are thought to perform a dual role in GALT, namely an immune role in the secretion of antibodies and in antigen presentation, as well as a developmental role in assisting M-cell differentiation towards the optimum function in antigen translocation.

Exogenous factors might also regulate M-cell development. Following bacterial exposure, several *in vivo* studies suggest that an increased proportion of M cells in the FAE can be induced rapidly. For example, exposure of germ-free mice to S*almonella typhimurium* mutants caused a twofold increase in M-cell numbers (suggested by increased numbers of cells with little alkaline phosphatase activity) and an increase in CD4 and CD8 counts [35]. In rabbits exposed to *Streptococcus pneumonia R36A* for one hour, M-cell numbers along the flanking regions of PP appear to be increased along with a doubling in particle uptake capacity [36]. In addition, using quantitative immunohistochemistry, Gebert *et al.* [37] gave an alternative analysis by demonstrating that short-term exposure of rabbit PP to *S. pneumonia* R36A leads to increased transport capacity of individual M cells without increasing their number by *de novo* induction. Bacterial exposure therefore increases either the number of sampling M cells or the further differentiation of predetermined M cells in the FAE. Whether M-cell genesis derives from conversion of pre-existing dome enterocytes or from independently migrating undifferentiated crypt cells of specific lineage is clearly a subject of continuing debate. The evidence for both sides has been reviewed recently leading to a conclusion that the arguments are not mutually exclusive [38]. From an M-cell targeting perspective, it is important to gain a greater understanding of the M-cell differentiation process. Either way, bacterial exposure might provide a convenient method to increase the amount of M-cell RNA for microarray analysis to aid identification of putative vaccine receptors [39]. Furthermore, by boosting M-cell growth and differentiation, non-pathogenic microorganisms or probiotics might serve to promote antigen uptake.

M-cell apical membrane receptors

Phagocytosis is a process that is enhanced by receptors that recognize evolutionary conserved microbial patterns. These sites have been termed 'microbial-associated molecular patterns'. Examples of PP receptors with the capacity to bind a wide variety of microbial cell wall components include *Toll*-like receptor (TLR) 2 on swine M cells [40] and also TLR-9 on swine FAE [41]. Furthermore, because M cells bind and endocytose antibodies located in the lumen, there is interest in ascertaining whether there are specific Ig receptors on M cells and the FAE. A novel IgA receptor was recently discovered in mouse M cells and it is thought that it might facilitate transport of secretory IgA from luminal secretions into GALT [42]. Similarly, it is likely that human neonatal Fc receptors might line the FAE (as well as villous epithelium) with a function of transporting IgG-antigen complexes across the epithelium for processing [43].

There is growing interest to discover if FAE and M cells in different species might have a common set of conserved apical membrane target proteins (Table 1). Originally, this effort was made for the purpose of generating ligands with

which to label M cells, which could then be used in M-cell targeting and differentiation studies. Some distinct epitopes have been described for individual species but there is still no broadly applicable conserved species-independent label. To date, the most reliable – although not universal – FAE marker remains a negative stain for alkaline phosphatase, denoting the reduced brush border and poorly differentiated epithelium [44]. In 1999, there was some excitement with the discovery that sialylated Lewis A antigen (SLAA) appeared to be expressed selectively on M cells from a small number of biopsies of human Peyer's and caecal patches [45]. Others have not yet been able to reproduce those findings in human intestinal biopsy sections [46]. Notwithstanding the lack of a 'universal' M-cell marker, some exciting results from targeting experiments have been achieved. For example, the coupling of recombinant cholera toxin B subunit to liposomes containing *Streptococcus mutans* antigens generated enhanced mucosal immunization in mice compared to untargeted antigen-loaded particles [47].

Ulex europaeus agglutinin 1 (UEA-1) has high specificity for the carbohydrate moiety, α-L-fucose, located on the apical membranes of mouse M cells [48,49]. There have been successful efforts made into *in vivo* targeting to mouse M cells by conjugating the lectin to polymerized liposomes [25] and also to latex particles [50]. Interestingly, Foster and Hirst recently reported results of the oral vaccination of mice with latex particles coated with albumin and UEA-1, where these induced an enhanced level of serum IgG and IgM compared to untargeted BSA-coated particles [4]. UEA-1 is of limited value in vaccine delivery because the lectin is toxic, is subject to intestinal degradation, and its receptor is not expressed in human PP [45] and not even by all murine M cells [48]. From a high throughput screen of mixture-based compound libraries in a competitive UEA-1 binding assay, a stable low molecular weight 4-copy gallic acid residue was identified that appears to have high affinity for the fucose receptor on murine M cells [51]. While stable non-toxic small molecule mimetics of lectins could have potential in oral vaccine targeting, demonstration of reproducible receptor expression in human PP is a prerequisite.

As another alternative to the lectin UEA-1, the edible orange peel mushroom *Aleuria aurantia* was used to target the α-L-fucose receptor. Coated poly(D,L-lactide-co-glycolide) (PLG) particles were entrapped with birch pollen antigens and administered to mice as a potential oral allergen immunotherapy [52]. In pollen-sensitized mice, oral administration of this formulation led to increases in interferon-γ and IgG2a antibody. Convincing demonstrations that antigen-loaded M-cell fucose receptor-targeted biocompatible particles can genuinely enhance systemic and mucosal immunity in mice would provide a stronger case to support the linkage between targeting and vaccine efficacy. However, human M-cells are unlikely to show specific UEA-1 lectin binding and it will be interesting to see if regulatory authorities will permit human Phase I testing of a UEA-mimetic with an antigen loaded particle, even if murine immune data has turned out to be positive [53].

M cells can also be discriminated from enterocytes in the FAE on the basis of altered *adherens* junction protein expression [54]. This included increased expression of polymerized actin, β-catenin, E-cadherin and α-actinin, all proteins that are involved in the maintenance of tight junctions, as well as in endocytic function of cells. Importantly, intercellular tight junctions (which are shared between M-cells and adjacent enterocytes) have been described as pathogen binding sites [55]. Evidence from rabbit FAE supports the hypothesis that FAE tight junctions are different from those of non-FAE intestinal epithelia, as they appear to display a greater number of junctional strands associated with their *zonula occludens* [56]. Related studies using isolated rabbit mucosae suggest that their FAE tight junctions display higher transepithelial electrical resistance than control intestinal mucosae, with an associated outward short-circuit current response to cholinomimetics [57]. As regards paracellular transport of microbes across M cells, little is known relating to potential *adherens* junction receptor targets or the capacity for these pathways to be pharmacologically regulated in PP. The evidence of extremely well-differentiated tight junctions on M cells under normal physiological conditions indirectly corroborates the presence of transcellular endocytotic uptake mechanisms for many pathogens and particles. Intuitively, a more robust set of intercellular connections should be required to facilitate transcellular particulate transport into the invaginating pocket [58].

Other 'non-receptor' dependent apical surface specialization of M cells might contribute to translocation. These include topographical variation, surface charge, unstirred layer(s) and post-translational modification of surface proteins. Few of these have been studied systematically. Both the filamentous brush border glycocalyx on enterocytes and the thin glycoprotein coat on M cells might act as size-selective barriers restricting penetration by microparticles [59]. A reduced mucous gel on PP epithelia has also been reported [60] but the significance of this is unclear and the regulation of mucus synthesis and degradation at these sites has not been examined. An intriguing recent discovery was that the mucin, *Muc2*, was differentially expressed on rabbit M cell membranes [61]. Many host–pathogen interactions involve carbohydrate moieties, so the M cell apical membrane might compete with mucus glycoproteins for pathogen binding.

A human M-like cell model

M cells have been modelled *in vitro* using cell cultures [62]. The phenotype conversion is based on the premise that lymphocytes are important inducers of PP differentiation. Two configurations have been described. In the most detailed description of the model [63], human Caco-2

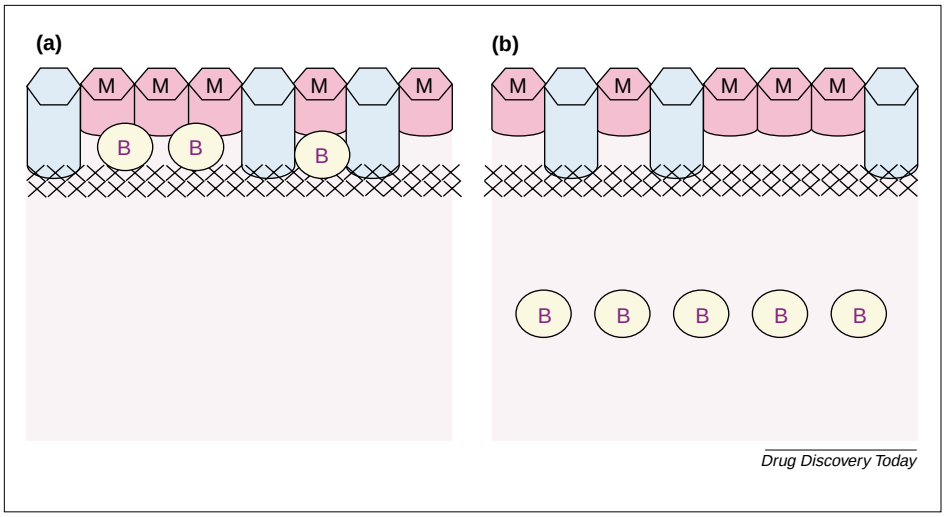

FIGURE 3

Configurations of an M-like cell model. (a) Co-culture model [62], in which B cells are intercalated within Caco-2 monolayer. **(b)** Separate compartment model [64], in which B cells do not have physical contact with monolayer.

intestinal epithelial monolayers or a Caco-2 sub-clone (TC-7) were grown on the downside of a Transwell® (Costar, UK) permeable filter for 14 days and were then exposed to fresh murine PP-derived B lymphocytes on the top (well) side (Figure 3a). The filter cup was then inverted for up to 7 days so that the B cells could intercalate within the monolayer. The brush border appeared to lose microvilli and displayed reduced sucrose isomaltase and villin expression, consistent with generation of M-like cells. In addition, at a functional level, the co-culture transported 200 nm FITC-conjugated latex beads and the bacterium, *Vibrio cholerae*, to a greater extent than Caco-2 mono-cultures at an optimum time of between days 2 and 4 of co-culturing. Neither was the construct specific to mouse-derived PP lymphocytes because replacement with Raji B human lymphocyte cultures also demonstrated the M-like cell phenotype, whereas replacement with Jurkat T cells did not [63]. These variations in lymphocyte source meant that researchers could now use an entirely human model to examine human M-like cell function. Furthermore, intimate contact by B cells with the epithelium was not obligatory because the phenotype was also expressed in the presence of B cells as a suspension below the filter upon which Caco-2 are grown [64] (Figure 3b). Raji-B -conditioned medium also partly induced the M-cell phenotype (D. J. Brayden and A.W. Baird, unpublished data), therefore, this raises the possibility of accurately identifying soluble Type 1 cytokines that can rapidly upregulate antigen uptake in the co-culture. This could be a useful pre-screening assay for oral adjuvant development. Intriguingly, Man *et al.* [38] have suggested that some of the bacterial vectors currently being used to deliver oral vaccines in preclinical studies might also be selected according to their potential to induce the differentiated M-cell phenotype indirectly via inflammatory local cytokine production. A downside to this argument is that increasing the numbers of M cells might lead to an inappropriate immune response against strains of commensal bacteria.

Significant data in support of conversion of Caco-2 monolayers to an M-like cell phenotype has been obtained, (Box 2). In respect to particle translocation, size and temperature dependence has been clearly demonstrated for latex microspheres [62–65]. This supports the view that particle transport is predominantly transcellular, endocytotic and energy dependent. The optimum size for latex particle uptake by the co-culture appears to be <1 μm in diameter [64], similar to that seen in mouse gut loops [25]. Similarly, uptake of fluorescent chitosan microparticles by cultured human M-like cells is comparable with that demonstrated in murine PP and greater than that seen in monocultures [66].

In addition to functional similarities, the M-like cell co-culture is comparable with PP FAE in several ways. For example, there is reduced expression of brush border alkaline phosphatase, and increased expression of SLAA and $\beta_1$integrin. Importantly, antibody-detectable expression of the α-L-fucose receptor for the lectin UEA-1 was not increased overall in the co-culture [64], confirming the negative data in a co-culture gene profiling study [39] and from human intestinal biopsies [45]. Upregulated expression of β_1 integrins on the co-culture apical membranes (including apparent distribution from basolateral membranes) further supports generation of an M-cell phenotype [65,67]. A feature of integrin-mediated *Yersinia* uptake *in vitro* was redistribution of β_1 expression from both membranes of Caco-2 monolayers to just the apical membrane of the epithelium in the presence of B lymphocytes. However, significant β_1 integrin-independent *Yersinia* has been described [67] and apical β_1 integrin expression has not yet been demonstrated on human PP biopsies (Brayden D.J. unpublished data).

BOX 2

Characteristics of cultured human M-like cells

- Truncated microvilli
- Increased adherence and uptake of latex nanoparticles and liposomes
- Reduced expression of alkaline phosphatase and UEA-1
- Increased expression of sialylated Lewis Antigen A and β1 integrin
- Increased binding of viruses: polio and HIV
- Increased binding of bacteria: *V. cholera* and *S. typhimurium*
- Increased transport of scrapie
- Discovery of targets by gene expression: correlation with primate tissue
- B cell-derived factors influence epithelial gene expression

M-cell targets from gene expression studies

Further evidence that the co-culture model has many characteristics of PP has emerged recently from gene expression studies. The co-culture construction using suspensions of B cells in the compartment below the Caco-2 monolayer is uniquely suited for such an approach because the cells to be genotyped are physically separated. Recently, Lo and colleagues [46] demonstrated that a range of epithelial genes that were upregulated in co-culture correspond to genes expressed selectively in mouse FAE, data that were confirmed by PCR and *in situ* hybridization. These included the *C. perfingens* enterotoxin receptor (claudin 4), laminin β3, tetraspan TM4SF3 and a matrix metalloproteinase, MMP15. Of these, comparisons between co-cultures and mouse PP with human PP revealed cross-species conservation of claudin 4 and TM4SF3 expression in FAE. That claudin-4 might be expressed across the entire FAE is intriguing. It appears to have a dual location at tight junctions (M cell–enterocyte) and as an M-cell and enterocyte cytoplasmic receptor. It could have a role in trafficking pathogens across M cells to lymphocytes or dendritic cells. Of other potential targets, peptidoglycan recognition protein (PGRP)-S was shown to be co-localized with UEA-positive cells in microdissected mouse PP, whereas PGRP-L and the laminin β3 receptor were located in the FAE [39]. The 67 kDa laminin β3 receptor is a receptor that binds prions [68] and its expression on FAE is entirely consistent with M cells as a portal of scrapie and/or BSE entry in sheep. It also seems that PP lymphocytes can promote M-cell differentiation in the direction of antigen sampling by specifically modulating FAE gene expression *in vitro* and *in vivo* [69]. An as yet unexamined interplay is the likely influence of the epithelial cells on the co-cultured B cells. It remains to be seen, however, if this exciting new *in vitro* model truly mimics *in vivo* mechanisms for M-cell uptake of those pathogens partial to this route. In overall context, it seems that many pathogens can be absorbed directly by enterocytes, M cells or DCs, or through compromized barriers, or via combinations thereof.

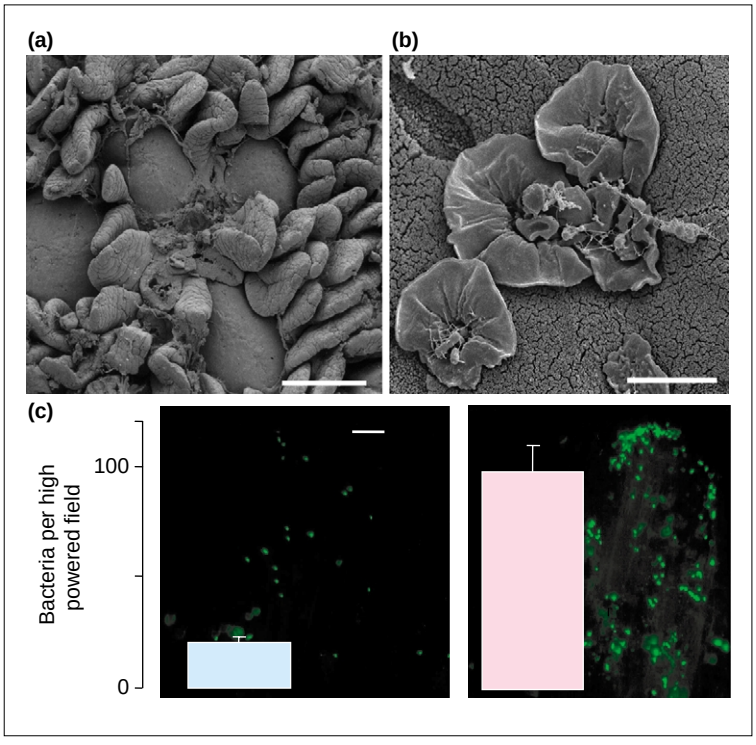

FIGURE 4

Interactions between *Salmonella typhimurium* and mouse PP and human M-like cells. (a) At low magnification four 'domes' can be seen nestling between villi of a mouse Peyer's patch. The follicle-associated epithelium (FAE) contains M cells. Scale bar = 200 μm. **(b)** *S. typhimurium*-induced changes in M-cell surface morphology seen at higher magnification. M cells in the centre of the image exhibit prominent membrane protrusions ('membrane ruffles'). Bacteria can be seen associated with membrane ruffles (e.g. lower left). Two M cells with normal morphology appear recessed between normal enterocytes due to their shorter microvilli (bottom left and top left). Scale bar = 5 μm. Figure reproduced from Ref. [105] with permission from Elsevier. **(c)** Adherence of fluorescently-labelled *S. typhimurium* to cultured epithelial sheets can be quantified. Significantly greater levels of interaction occur when human M-like cells are exposed to the bacteria (right) than for control Caco-2 monolayers (left). Scale bar = 10 μm.

M-cell adherence of other pathogens

Salmonella typhimurium has been shown to adhere preferentially to the co-culture (Figure 4). *In vivo*, the bacterium targets murine M cells at least in part via long polar fimbriae [70]. M-cell infection by *Salmonella* leads to their destruction, which is accompanied by widespread FAE loss, the cause of which remains unknown [71]. *Salmonella* also appears to cause a 'ruffling' type rearrangement of murine M-cell apical membranes similar to those seen in infection of cultured epithelia [72]. A type III secretion system, which is required for *Salmonella* invasion of cultured cells, also contributes to, but is not essential for, M-cell uptake [71]. Considerable research is being conducted to determine what host cell and bacterial molecules contribute to *Salmonella* infection of the co-culture M-cell model. Co-culture infection of S. *typhimurium* mutants could thus be used to investigate *Salmonella* virulence determinants, as well as assessing potential vaccine strains. Various *Salmonella* species are continually under investigation as live attenuated vaccines and also as vectors for oral delivery of other antigens to M cells, therefore, it is not surprising that there is considerable study into promoting receptor-based interactions between the species and M cells and enterocytes. The findings with *S. typhimurium* are not, however, universal for all bacterial pathogens. In marked contrast to the increased adherence to the co-culture and to mouse PP by *Salmonella*, the *in vitro* model was used to support the fact that *Listeria monocytogenes* does not require or use M cells to invade. It prefers ultimately to attach and spread from luminally-exposed basolateral membrane of the periodically damaged small intestine [73].

Recently, the co-culture was shown to transport scrapie extremely efficiently and to a greater extent than Caco-2 monolayers [74], data which suggested that M cells might have a key role in oral prion transmission. Moreover, the

TABLE 2

Association between pathogens and FAE/M cells in different species

Agents	Models	Mechanism	Sample reference
Bacteria			
S. typhimurium	Mouse and calf gut loops	LPF	[97]
S. typhi	Mouse and rat gut loops	Unknown/lack LPF	[98]
Y.pseudotuberculosis *Y. enterocolitica*	Mouse and rabbit gut loops; human M-like cells	*Inv*-binding β_1-integrins	[95]
Shigella flexneri	Rabbit and mouse gut loops		[97]
M. paratuberculosis	Calf, goat and mouse gut loops	FAP-P binds integrins via fibronectin bridge	[99]
RDEC-1; EPEC	Rabbit (orogastric inoculation)	AF/R1 pili	[100]
V. cholerae	Rabbit gut loops; M-like cells		[101]
C. jejuni	Rabbit gut loops		[102]
Viruses			
Type I reovirus	Rabbit gut explants	σ1 protein binding to α2–3-linked sialic acid	[83]
HIV-1	Rabbit, mouse gut explants; M-like cells	lactosyl cerebroside and CXCR4	[96]
Polio virus	Human gut explants; M-like cells		[76]
Protozoa			
Cryptosporidium	Infected guinea pigs		[103]
Prions			
Scrapie PrP^{sc}	Sheep; M-like cells		[104]

Abbreviations: FAE, follicle-associated epithelium; LPF, long polar fimbrae; FAP-P, Fibronectin Attachment Protein-P; CXCR4, chemokine receptor.

transported prions were capable of infection according to a bioassay comprising intracerebral injection to prion-sensitive mice. It was unlikely that the Raji B cells were the target of the translocated prions because those cells are largely restricted to the basolateral side of the filter of the intercalated co-culture model, whereas the epithelium was exposed to prions on the apical side. Consistent with the co-culture data, PP appear to be key sites of initial prion infection and propagation in scrapie-sensitive sheep but not in their genetically-resistant counterparts [75]. Perhaps natural prion infection occurs by the oral route via PP infection followed by replication in GALT. Uptake of prions by PP could then lead to three potential routes to the brain: (i) via local enteric cholinergic innervation in apposition to the FAE, (ii) via access to the lymphatic system and (iii) via the blood-borne route. It will be interesting to see whether PP M-cell trafficking is a common and significant pathway in prion disease transmission between and within species.

A summary of pathogens thought to interact with FAE or M cells is shown in Table 2. Several of these agents adhere selectively to the apical membranes of the M-like cell co-culture, as well as to murine and/or macaque PP *in vivo*. For example, Type 1 polio virus transcytosed across the co-culture to a greater extent than across control Caco-2 monolayers and the entry step appeared to depend upon apical membrane expression of the CD155 receptor [76]. Thus, the co-culture expresses an upregulated receptor on M-like cells for poliovirus, which appears to correlate with its level of FAE expression in primates [77]. The corollary was that weak enterocyte expression of CD155 protects mice from oral (but not parenteral) challenge with polio virus [78].

M cells and diarrhea-inducing viruses

One area of applied research of distinct importance is in generating immunity to viruses. In this regard, polio virus offers a convincing proof-of-principle, which can be extended to common viruses that cause diarrhoea. Rotavirus gastroenteritis is estimated to cause >600 000 deaths a year, primarily in the developing world. Conservatively, this represents over one-third of all diarrhea-related deaths and is responsible for 20–50% of worldwide diarrhea-related hospitalizations [79]. Rotavirus virions comprize a three-layered protein capsid in which the conserved antigen VP6 coats the RNA core, while VP4 and VP7 are outer capsid surface proteins capable of stimulating protective neutralizing antibody responses. In 1999, a live attenuated tetravalent oral vaccine (RotaShield®, Wyeth Lederle) was withdrawn due to an association with a potentially fatal bowel obstruction, intussusception. However, there is now considerable evidence that two new oral vaccines might be able to overcome the safety issues of RotaShield® while retaining the efficacy. One of these, the oral vaccine Rotarix® (GlaxoSmithKline) is a live attenuated human version of the predominant G1 serotype and was approved in Mexico in 2004. It is expected to treat up to 100 million infants per year in the developing world to yield expected revenues of US$250 million [80]. Merck's RotaTeq® is an oral re-assortment

vaccine expressing VP7 and VP4 from five common human serotypes surrounded by a bovine capsid. It has completed Phase III trials and will be targeted at up to 8 million infants per year in the developed world to give expected annual revenues of US$1 billion [80].

Studies of the interactions between native virus and PP suggest that M cells are important in the induction of mucosal immunity by live attenuated rotavirus vaccines. When mice were orally inoculated with murine rotavirus, antigen was detected predominantly in the FAE for 3–7 days post-infection before migrating to sub-epithelial and interfollicular areas [81]. There was indirect evidence of location of antigen in lymphoid follicles and the subsequent development of local antibody responses in different segments of small intestine. Oral vaccination with porcine rotavirus antigens in microspheres also suggests a key role for M cells. Kim *et al.* [12] demonstrated recently that alginate polysaccharide microspheres encapsulating the rotavirus antigen VP6 were taken up selectively by PP in a sheep intestinal loop model. Similar to natural porcine rotavirus infection, this delivery system also induced secretion of faecal VP-6 specific IgA in mice following oral immunization. Others showed that a single oral vaccination of mice with a DNA vaccine encoding VP6 formulated in PLG microspheres achieved protection against challenge 12 weeks later [82]. Thus, it appears that PP FAE is an important uptake and immune inductive site for both native and attenuated rotavirus, as well as for particulate formulations of subunit and DNA antigens.

While the dissection of the route of M cell entry by specific rotavirus epitopes remains to be elucidated, there is more detailed knowledge on the mechanism of PP uptake of another diarrhea-inducing group of organisms, icosohedral Type 1 reoviruses. Selective binding to rabbit M cells appears to involve attachment of the viral hemagglutinin adhesin, σ1 protein, to M cell apical membrane glycoconjugates containing α2–3-linked sialic acid [83]. These lectin receptors are present on apical membranes of M cells, as well as enterocytes in the intestine. The attachment appears to be M-cell specific only when presented on the surface of a reovirus particle, perhaps due to reduced access to villous epithelia arising from the overlying thick glycoprotein layer. Passive oral immunization of mice with IgA and IgG antibodies against reovirus Type 1 Lang σ1 (T1L) antigen prevented PP viral uptake and infection [84]. In addition, there is evidence that T1L is present in mouse PP sub-epithelial dome dendritic cells following reovirus infection, suggesting that these cells can capture the antigen from FAE handover [85]. These examples illustrate that understanding pathogen entry pathways across M cells has the potential to lead to vaccination strategies. While imitating pathogen interactions with M cells might be a smart way to develop oral vaccines, it will still be a tall order to mimic the elegance of reovirus, which undergoes intra-luminal peptidase-mediated alteration of its outer capsid to release its infectious sub-viral particles for binding M cells [86]. Thus, the virus has evolved as an enteric-coated particle polymer formulation, whose cargoes (DNA or RNA) are ultimately and effectively released in selected intestinal regions.

M-cell uptake of particulate oral vaccines: the link to mucosal immunity

Current internationally licensed vaccines against mucosal infections, including polio, cholera, rotavirus and influenza, are predominantly live-attentuated and are mostly administered by mucosal routes [87]. However, there have been few successful oral vaccine trials in man using non-live antigens in particles. Mixed results were obtained in a limited number of human subjects dosed with untargeted PLG microspheres containing the highly potent *Escherichia coli* colonization factor antigen II as potential vaccine for enterotoxigenic *E. coli* [88]. Although antibody responses were achieved in 5 out of 10 subjects, the levels generated appeared to be insufficient for protection. A follow-up oral Phase I trial using the CS6 antigen in PLG yielded antibody responses in 4 out of 5 subjects but demonstration of a true particle effect was unclear [89]. However, these studies suggest indirectly that there is likely to be some particle uptake in man, although it is not possible to discriminate the efficiency of the process from the likely variability in immune response generation. A quantifiable relationship between enhanced M-cell targeting of vaccine loaded particles and an enhanced immune outcome therefore remains illusive. Aside from the immunology issues, pharmaceutical design hurdles are also present for antigen-loaded particles. These include antigen stability issues, premature antigen release from particles in the intestine and incomplete antigen release within Peyer's patch at the right time.

A Phase I trial of a single shot tetanus toxoid (TT) and diphtheria toxoid in Poly-(lactide)/PLG microspheres is still some way off, even though outcomes in mice and guinea-pigs showed positive and durable immune responses using antigen-loaded microparticles [90]. It is possible that particulate antigens, when targeted to inductive immune sites, might perform even better in man than in laboratory animals because the pathways of antigen presentation by human dendritic cells are relatively well established and might even be superior [91]. Other concerns relate to long-term antigen stability in PLG microparticles and to scale-up of the formulation process [91]. Advancing non-live oral vaccine delivery systems into human trials is, therefore, a fraught business reliant on cost of goods, formulation scale-up and antigen stability. Commercially viable production of vaccine particle formulations is possible, however, as indicated by approvals of nasally administered liposomal-based influenza and hepatitis A-entrapping virosomes [92].

Concluding remarks

In the past 20 years, advances in understanding the remarkable M cell have taken place. A testament to this

fact is that the once common surgical intervention of tonsillectomy and appendectomy are performed less frequently as the role of GALT in mucosal immunity is becoming better understood. However, since the original description of the PP as a route of mycobacterial uptake nearly a century ago, there is relatively little clarity regarding PP or M-cell contribution to pathogen invasiveness and infection or to the mucosal immune responses to intestinal challenge. Research in the 1980s suggested that PP could be a potential route of entry for oral peptides into the lymphatic system but commercial interest was stymied due to the low uptake capacity. In the 1990s, the role of the M cell in antigen uptake made it a prime target for researchers in oral vaccines because it was shown that particles had an inherent affinity for M cells and, in addition, it seemed that only a small amount of antigen uptake might be required to induce memory-driven responses. Interest waned when it was shown, through the use of confocal microscopy techniques, that particle uptake by M cells *in vivo* was at a lower level than previously thought and that it was limited to sub-micron particles, which were difficult to load and to generate reproducible antigen release patterns from. Although there is now evidence that targeting ligand-coated particles to mouse M cells can lead to increased uptake *in vivo*, translating this to a reproducibly enhanced immune response requires more data to convince. Attempts to mimic pathogen entry routes into M cells and other routes of antigen uptake could assist in the development of ligand-mediated targeting of particulate vaccine cargoes. Thus, the oral administration of non-live vaccines to successfully stimulate mucosal immune responses might eventually contribute to the achievement of one of the priority research goals of vaccinology, namely the incorporation of additional routes of delivery [93].

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