Minireview *Highlight Article*

Identifying human and murine M cells *in vitro*

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Impact statement

The study of M cells, a specialized epithelial cell type found in the follicle-associated epithelium, is hampered by the lack of a universal M cell marker. As such, many studies lack reliable and universally recognized methods to identify M cells in their proposed models. As a result of this it is difficult to ascertain whether the effects observed are due to the presence of M cells or an unaccounted variable. The outcome of this review is the thorough evaluation of the many M cell markers that have been used in the literature thus far and a proposed criterion for the identification of M cells for future publications. This will hopefully lead to an improvement in the quality of future publications in this field.

Abstract

M cells are an epithelial cell population found in the follicle-associated epithelium overlying gut-associated lymphoid tissues. They are specialized in the transcytosis of luminal antigens. Their transcytotic capacity and location in an immunocompetent environment has prompted the study of these cells as possible targets for oral drug delivery systems. Currently, the models most commonly used to study M cells are restricted to *in vivo* experiments conducted in mice, and *in vitro* studies conducted in models comprised either of primary epithelial cells or established cell lines of murine or human origin. *In vitro* models of the follicle-associated epithelium can be constructed in several ways. Small intestinal Lgr5+ stem cells can be cultured into a 3D organoid structure where M cells are induced with RANKL administration. Additionally, *in vitro* models containing an "M cell-like" population can be obtained through co-culturing intestinal epithelial cells with cells of lymphocytic origin to induce the M cell phenotype. The evaluation of the efficiency of the variations of these models and their relevance to the *in vivo* human system is hampered by the lack of a universal M cell marker. This issue has also hindered the advancement of M cell-specific targeting approaches aimed at improving the bioavailability of orally administered compounds. This critical review discusses the different approaches utilized in the literature to identify M cells, their efficiency, reliability and relevance, in the context of commonly used models of the follicle-associated epithelium. The outcome of this review is a clearly defined and universally recognized criteria for the assessment of the relevance of models of the follicle-associated models currently used.

Keywords: M cells, follicle-associated epithelium, organoid, enteroid, Gp-2, UEA-1

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Introduction

The gastrointestinal (GI) mucosal surface functions as the primary interface between the internal and external environment, absorbing luminal nutrients, serving a protective role against invading pathogens and mediating the gastrointestinal immune response.[1] The innermost layer of the GI mucosa is comprised of a monolayer of polarized epithelial cells conjoined by tight junctions (TJs), forming an effective barrier preventing the invasion of luminal pathogens into the body.[1,2] Due to the considerable bacterial burden imposed onto the GI mucosa, the mucosal immune system and its associated lymphoid structures, collectively termed the gut-associated lymphoid tissues (GALT), play an important role in maintaining homeostasis and mediating mucosal immune responses. GALT structures include isolated lymphoid follicles (ILFs) scattered throughout the epithelium of the small intestine and aggregated lymphoid nodules called the Peyer's Patches (PPs).[3]

PPs are aggregated nodules of lymphoid tissue composed of three distinct regions: the follicular area,

the inter-follicular area and the follicle-associated epithelium (FAE).[3] The follicular and interfollicular regions are comprised of organized lymphoid follicles with proliferating B lymphocytes, follicular dendritic cells and macrophages, with a germinal center forming the core of each follicle.[3] The FAE is the name given to the epithelium directly overlying GALT. The complex interplay between the cells of the FAE and associated lymphocytes are central to inducing the mucosal immune response.[3–5] The FAE differs from the regular villous epithelium, containing a large number of lymphocytes, fewer goblet cells and a subpopulation of specialized epithelial cells called the M (microfold or membranous) cells.[6–8]

M cells are specialized epithelial cells expressed in many mucosal tissues of the body; most prominently in the GI tract and the airways. Within the small intestine, M cells are located primarily in the epithelium covering the PPs and account for less than 5–10% of all the cells of the FAE, with the number of mature M cells decreasing with age.[9] These cells are highly specialized for the transcytosis of luminal contents into the underlying immuno-competent environment.[10] Due to their specialized function for the uptake of luminal antigens, M cells have long been considered an attractive target for delivery of mucosal vaccines and biologics.[11–13] Lineage tracing experiments using mice expressing a *LacZ* reporter gene under the control of the Lgr5 promoter have shown that M cells derive from Lgr5+ stem cells that reside at the bottom of the intestinal crypts.[6] It is postulated that M cell differentiation is regulated by several different factors including the binding of receptor activator of NF-κB ligand (RANKL), secreted by sub-epithelial stromal cells, to the RANK receptor expressed by epithelial cells (Figure 1). Studies have reported RANKL-deficient mice have a significantly diminished number of M cells in the FAE and consequently exhibit impaired transcytosis. Subsequent administration of recombinant RANKL can restore M cell numbers and functionality.[6,14] Though it is well established that the ligation of RANK by RANKL and the subsequent activation of the NF-κB pathway is essential for the differentiation of M cells, until recently the intracellular signaling mechanisms involved remained unclear. Recently, a study used TNF receptor associated factor (TRAF) 6-deficient mice to evaluate the role of TRAF6 in M cell differentiation. TRAF6 is known to play a crucial role in the RANK-mediated activation of the canonical NF-kB pathway.[15] Mice lacking TRAF6 specifically in the intestinal epithelium (TRAF6^{IEC-KO}) were generated by crossing mice with LoxP-flanked *Traf6* alleles with *Villin*-Cre transgenic mice. The expression of M cell specific markers was silenced in the PP FAE of TRAF6^{IEC-KO} mice throughout the length of the GIT, indicating that TRAF6-mediated activation of the canonical NFκB pathway was crucial to M cell development.[16] This observation was further confirmed using

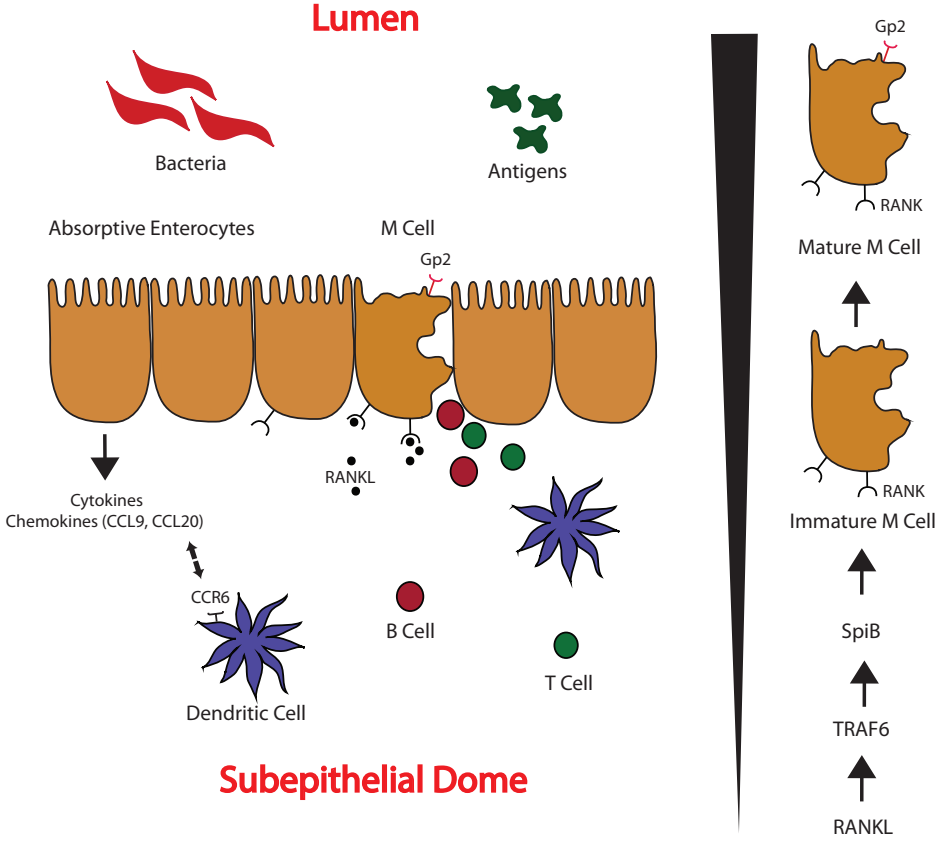

Figure 1. The differentiation of M cells is triggered by ligation of the RANK receptor expressed by cells of the intestinal epithelium to its ligand, RANKL. This causes the activation of TRAF6-mediated NF-kB signaling and the induction of Ets transcription *SpiB*. M cells express different markers at during early and late stage development, with Gp2 being indicative of fully differentiated, mature M cells. Cytokines secreted by the follicle-associated epithelium, such as CCL20 and CCL9, have a chemotaxic effect toward CD11b+ dendritic cells that are present in the subepithelial dome. (A color version of this figure is available in the online journal.)

human TRAF6$^{-/-}$ organoids generated by the CRISPR/Cas9 system; M cell specific marker expression was silenced. Downstream of RANK/RANKL signaling, research suggests SpiB, a highly conserved transcription factor belonging to the Ets (E26 transformation specific) family, is responsible for M cell differentiation, with SpiB$^{-/-}$ mice presenting with impaired PP formation and few to no detectable M cells.[6,17] SpiB$^{-/-}$ mice also display a significantly diminished expression of several previously reported M cell specific genes.[18] SpiB also appears to be implicated in human M cell development, with *in vitro* organoid cultures grown from human tissue showing an increased transcriptional expression of M cell specific genes in response to RANKL stimulation.[7,16] This research highlights the central role of SpiB in regulating the development of functional, mature M cells, though more recently it has been postulated that M cells can develop via SpiB-independent pathways.[18,19]

In the past several years, it has become evident that M cell differentiation remains a complex and incompletely understood process. Studies suggest that independently of SpiB, villous epithelial cells can develop an M cell-like morphology and function upon exposure to *Salmonella* Typhimirium and cholera toxin (CT).[20,21] These "villous" M cells express some, but not all, M cell marker genes, though it is unclear how similar in function they are to the PP M cells. In addition to this, SpiB$^{-/-}$ mice display a significantly diminished expression of the human and murine M cell-specific gene *Gp2* and the reported M cell specific gene *Umod*. However, other reported M cell specific genes *Fut1, Marcskl1* and *Pglyrp1*, remained comparably expressed both in WT mice and SpiB$^{-/-}$ mice.[18] These data suggest that a subset of cells phenotypically similar to M cells may differentiate independently of SpiB.[18,19] Despite this, SpiB is essential for the functional and structural maturation of M cells.[18] The villous "M cells" that develop independently of SpiB, often in response to a pathogen, do not display all of the hallmarks of mature M cells, including expression of Gp2 which is widely considered a terminal differentiation marker of human and murine M cells. As such these cells may in fact be deemed a separate cell population altogether, or a less functionally mature version of PP M cells.[18,22,23] Additionally, M cells generated independently of SpiB have an impaired ability for the uptake of particles compared to PP M cells, indicating a less functional phenotype than SpiB-regulated PP M cells.[6,18] This is equally true in other mucosal areas of the body; in the nasal-associated lymphoid tissues (NALT), CT can induce epithelial precursors to differentiate into M cells within a timeframe of several hours.[24] In the colon, inflammation triggered by exposure to *Citrobacter rodentium* can also induce the development of M cells.[25] These induced villous "M cells" do not faithfully recapitulate the genetic and proteomic signature of true PP M cells.[18] Though several studies have demonstrated the integral role M cells play in initiating the mucosal immune response, it is also clear that the diversity of factors that can lead to the differentiation of M cells encompass a complex and not yet fully understood process that likely has a significant impact on the phenotypic and genotypic profile observed.[19,22,26] A more comprehensive examination of the various developmental origins of M cells is outside the scope of this critical review. However, it is crucial to convey here that there appear to be different developmental origins of M cells, and this likely explains why there is no "blanket" M cell marker which applies across all of the models published in the literature.

Most biologics, including peptide and protein-based drugs, are delivered parenterally due to their poor GI permeability and oral bioavailability.[11] Oral delivery is preferred due to cost effectiveness and better patient compliance; hence, there is considerable current interest in formulating oral delivery systems which improve bioavailability of biological agents. Specific interest in developing oral delivery systems that target M cells has led to an increased demand for understanding of the molecular mechanisms regulating M cell function. As a result, M cells have been studied extensively in many species in both *in vivo* and *in vitro* systems, with varying degrees of success.[7,10,13,27–35] The most commonly used and best validated models for the *in vivo* study of M cells are murine models. In mice, several reported studies have successfully targeted murine M cell markers to enhance the bioavailability of an orally delivered compound *in vivo*.[11,13] However, variance between M cell markers and M cell frequency in different species (refer to Table 2) suggests that the use of data obtained from non-human models may not be relevant to human clinical applications. As such, numerous *in vitro* models have been developed to allow for the evaluation of agents designed to exploit the characteristics of M cells. A commonly used and easily reproducible human *in vitro* model of the FAE uses the intestinal colon-derived cell line Caco-2 co-cultured with B cells, most often Raji cells, to induce the M cell phenotype.[29,36] The two cell populations are typically cultured in a Transwell system separated by a porous membrane, as described previously.[36–38] The co-culturing of these two cell types induces the differentiation of a highly transcytotic sub-population of cells with a similar functional profile to the small intestinal PP M cells. It remains unclear how similar these cells are to PP M cells.[39] Commonly used and/or recently reported variations of the *in vitro* culture-based model initially pioneered by Gullberg et al.[37] are summarized in Table 1. Multiple approaches have been used in an attempt to identify M cells in such co-culture models. These include electron microscopy,[6,29] binding of lectins (UEA-1, WGA and PNA),[40] expression of glycoproteins (Gp-2),[10] alkaline phosphatase activity[36] and expression of cellular adhesion markers such as NCAM and VCAM.[21,37] Although it is well established that Gp-2 is the only marker of mature M cells cross-reactive between human and mouse known-to-date,[10,30] Caco-2/Raji co-culture-based systems have not successfully applied this marker systematically in the literature. This poses additional questions in regards to the possible genetic and proteomic disparities between *in vivo* human PP M cells and the "M-like cells" generated through the Caco/Raji co-culture.[19] As a result of these discrepancies in published data, it is imperative to establish a method of benchmarking *in vitro* models against one another to

Table 1. Commonly used and recently published variations of *in vitro* models of the follicle-associated epithelium and examples of M cell identification techniques used.

Model	M cell identification methods	Reference
Caco-2 cells are seeded on the apical membrane of Transwell inserts. The cells are cultured for 4–6 days and the model is inverted. Raji cells are added to the basolateral compartment of the inverted model on days 14–16 of culture. The co-culture is maintained for a further 4–6 days.	• Functional transport studies between Caco-2 monoculture controls and co-cultures • TEM • SEM	36,44
Caco-2 cells are seeded on the apical membrane of Transwell inserts. The cells are cultured for 14 days. Raji cells are added to the basolateral compartment on day 14 of culture. The co-culture is maintained for a further 4–6 days.	• Functional transport studies between Caco-2 monoculture controls and co-cultures • Immunofluorescence staining of β-1 integrins	38
Caco-2 and HT29-MTX cells are seeded on the apical membrane and allowed to reach confluence. Raji cells added to the basolateral compartment on day 14 of culture. The triple-culture is maintained for a further 4–6 days.	• Distribution of alkaline phosphatase on apical cell surface • Immunofluorescence evaluation of degree of binding of WGA • SEM	43,73,74
Caco-2 cells are pretreated with 25 ng/ml of epidermal growth (EGF) factor for 24 h. Then, Raji cells and EGF pretreated Caco-2 cells are allowed to interact in detached condition for 2 h at a 1:1 ratio and then plated in culture plates. Cells are cultured for 14 days.	• Functional transport studies between Caco-2 monoculture controls and co-cultures • Phase contrast imaging • SEM • Immunofluorescence of clusterin	29
Organoids are cultured from primary murine epithelial crypt stem cells. RANKL is added at a concentration of 200 ng/nl for 72 h to stimulate M cell differentiation.	• Immunofluorescence staining of UEA-1 and annexin V • TEM • SEM • Functional uptake studies of beads • Expression of M cell genes	6
Epithelial organoids are cultured from primary human small intestinal crypts. RANKL is added at a concentration of 200 ng/nl for 72 h to stimulate M cell differentiation.	• Immunofluorescence staining of Gp2, E-Cadherin and EPCAM • Functional uptake studies of microparticles • Expression of M cell genes	7

determine the overall relevance of different methods to the *in vivo* system.

Other models of the FAE include organoid and enteroid cultures; in an attempt to advance the standard Caco-2/Raji co-culture, a murine 3D organoid model of the FAE was introduced in which M cell differentiation was induced with soluble RANKL treatment.[6] This model used murine primary small intestinal epithelial stem cells to culture an epithelial organoid structure representative of the intestinal epithelial barrier. Subsequently, M cells were induced using soluble RANKL, which resulted in an increase of the genetic expression of both *SpiB* and *Gp-2*. To improve the translatability of this model, in 2016 Rouch et al. introduced a similar enteroid model with RANKL-induced M cells grown from human intestinal crypt cells, observing similar upregulation of M cell markers in response to RANKL.[7] Enteroid and organoid cultures are potentially more biologically relevant than the colon-derived Caco-2 cell line-based model as they are grown from primary, healthy tissue and findings using these models may therefore be more translatable to the human *in vivo* environment than cell line-based models and murine cultures. This is particularly due to the expression of M cell markers in the organoid and enteroid cultures that have not been validated in the cell line-based systems.[6,7]

As stated previously, a major impediment to studies involving M cells is the lack of a reliable and specific M cell marker that applies across all established models. Different pathways of M cell differentiation may indicate an inherent difference in the genetic and proteomic signatures, thus perhaps making a universal marker unlikely. Thus far, the identification of M cells in the literature has relied on a number of different strategies, making it difficult to compare data across different models and studies. As such it is imperative to describe a reliable and specific M cell identification approach that will apply across both human and murine *in vitro* models, as they are most

Table 2. M cell markers identified specifically in the gut-associated lymphoid tissue epithelium of different species.

Species	M cell markers	Reference
Human	Gp2 Clusterin (?) Morphological indicators Functional capacity	7,27,29
Mouse	Gp2 UEA-1 Clusterin Annexin V (immature) Morphological indicators Functional capacity	10,13,28,30, 31
Rabbit	Vimentin	32,33
Pig	Cytokeratin 18	34
Rat	Cytokeratin 8	35

commonly used to study M cells. This critical review aims to discuss the various methods employed in the literature for confirming the presence of human and murine M cells in *in vitro* models of the FAE.

Morphological indicators of M cell presence

Application of electron microscopy

The unique morphology of the M cell was first described in 1973 and compared between three different systems; the rabbit appendix, the chicken bursa of Fabricius and the mouse Peyer's Patches.[41] Transmission electron microscopy (TEM) revealed a morphologically distinct cell type associated with follicular lymphoid structures that exhibited increased capability of transporting ferritin and India ink tracers from the lumen of the small intestine.[41] Among the many morphological and structural differences displayed by M cells, the most obvious are a denser cytoplasm, irregular microvilli formation, vesicles in the apical cytoplasm and an overall reduction in size of the cell. These parameters are still used frequently to identify M cells in *in vitro* human M cell models.[29,38,43] Indeed, due to the lack of a universally applicable gene or protein markers, the most definitive way of confirming M cell presence is to visualize M cells with electron microscopy. For example, TEM is often used to visualize the characteristic disrupted brush border phenotype associated with the apical membrane of M cells.[44,45] Scanning electron microscopy (SEM) is also frequently used to visualize the apical cellular surface in an attempt to distinguish well-formed microvilli of regular epithelial cells from the mal-formed, disrupted brush border of M cells.[23,29,44] Loss of features associated with the epithelial cell phenotype, including increase in the expression of annexin V,[6] may imply these cells undergo a differentiation process that resembles a partial epithelial to mesenchymal (EMT) transition.[46] Morphological assessment may be the most reliable way of confirming the presence of a highly transcytotic cell population because, despite the variance in markers, the morphological characteristics of both fully mature PP M cells and villous "M cells" are relatively consistent.[22] However, though useful as a visual guide providing information about the morphologic appearance of the cell, TEM and SEM are laborious and cost inefficient techniques which provide mostly qualitative information.[47] The data acquired are hard to quantify objectively and as such it is generally accepted that the sole use of TEM or SEM is not sufficient and needs to be accompanied by other methods.[48]

Cytoskeletal structure

The cell cytoskeleton is comprised of organized networks of interacting filamentous proteins that serve three main functions; they maintain spatial organization of the internal cellular space, they generate forces enabling cell movement and change of shape and they connect the cell to the external environment.[49] There are three main filamentous proteins collectively comprising the building blocks of the cell cytoskeleton; actin filaments, microtubules and intermediate filaments.[49] All three cytoskeletal proteins are able to reorganize in response to external cues and thus rearrange the intracellular compartments. Actin filaments in particular are less rigid than other interacting proteins, yet able to assemble highly organized, stiff structures in the presence of cross-linkers. Actin filaments support protrusions, such as intestinal epithelial microvilli, and can generate forces involved in changing cellular shape, as is observed during phagocytotic events.[49] Furthermore, the actin cytoskeleton is subject to continuous assembly and disassembly in response to local signaling cues. During endocytosis, signals released by the invaginating plasma membrane trigger the disassembly and reorganization of local actin filaments allowing the membrane region to form an endocytotic vesicle.[49] The unique morphology of the M cells' disrupted apical brush border has been reported on frequently; electron microscopy techniques have revealed the loss of organization in the apical microvilli of M cells and the invaginations in the cells' basolateral surface.[50] The partial loss of apical microvilli suggests rearrangement of the internal actin filaments supporting microvilli protrusions, and as such actin filament stains can give indication of the presence of M cells. Phalloidin, a high-affinity filamentous actin probe, or villin, an actin binding protein, are among common immunohistological stains that can be used to stain epithelial cell monolayers in which discontinuous staining indirectly suggests the presence of M cells.[51] Recently, a study investigated the role of allograft inflammatory factor 1 (Aif1) in M cell mediated transcytosis after observing the depletion of Aif1 in $SpiB^{-/-}$ mice. Using $Aif1^{-/-}$ mice, the study showed that although *Aif1* deficiency did not impact the amount of Gp2+ M cells, it significantly impeded their uptake capacity of both microparticles and *Yersinia enterocolitica* and *S. Typhimurium*.[52] This was initially hypothesized to be a result of actin remodeling, as previous studies had shown that Aif1 is implicated in the cytosketelal development of cells,[53,54] however Aif1 deficiency did not alter the ultrastructural appearance of M cells nor their frequency, yet still remained crucial for antigen uptake.

Cell surface markers

Lectins

UEA-1 and WGA. Ulex europaeus agglutinin (UEA) 1 is a lectin which binds to glycoproteins and glycolipids containing α-linked fucose residues.[11] Several research studies have shown that UEA-1 is able to bind to sites of α-1,2 fucosylation on murine M cells with high affinity.[13,21,40] UEA-1 was first established as a positive marker for murine M cells in 1993 by Clark et al. who used fixed mouse small intestinal PPs to compare four lectins for their binding affinity to M cells within the FAE: UEA-1, psophocarpus tetragonolobus (WBA), arachis hypogaea (PNA) and triticum vulgaris (WGA). They reported UEA-1 bound strongly to M cells, without cross-binding with goblet cells and enterocytes. WBA exhibited similar staining patterns as UEA-1, but with a much weaker staining intensity. PNA was found to bind with strong affinity to goblet cells but not to any other cell type, whilst WGA

was found to stain goblet cells strongly, enterocytes moderately and M cells not at all.[31] As such, UEA-1 was reported to be a strong, positive M cell marker and WGA a reliable negative M cell marker for murine M cells in PPs.[31] However, a number of subsequent studies have reported that UEA-1 also binds strongly to goblet cells, which is inconsistent with the initial observation of Clark.[22,55,56] Currently, UEA-1 is used as a marker of goblet cells. Though goblet cells bind UEA-1, they also binds WGA, therefore M cells can be differentiated from goblet cells in murine models by co-staining with an additional marker. UEA-1 and WGA have been used to identify M cells in murine models frequently, though utilization of these known murine markers in human systems is infrequent in the literature and has yielded variable results. One study showed decreased staining in the co-cultures, which directly contradicts the observations made in murine systems,[57] whilst another showed a relatively large amount of non-specific binding across both monocultures and co-cultures.[30] This indicates UEA-1 may not be a reliable marker for human M cells generated through the standard co-culture.

Building on the work conducted in murine systems, fluorescent latex microspheres were coated with either UEA-1 or several different agents and their interaction with murine M cells measured using a murine intestinal gut loop model.[13] The microspheres were injected into gut loops excised from female BALB/c mice. Out of all the agents trialed, only UEA-1 coated microspheres demonstrated selective binding to M cells, being internalized at a 100-fold greater rate than the control BSA-coated microspheres. Furthermore, it was found that binding of UEA-1 with a-L-fucose prior to exposing the microspheres to M cells resulted in a similar level of binding as in the controls, suggesting UEA-1 interacted specifically with the carbohydrate receptor on M cells.[13] This work prompted abundant interest in M-cell specific targeting of bioactive agents. For example, microparticles containing an antigen and with and without UEA-1, were introduced into four mice intramuscularly or mucosally through oral, nasal or rectal administration and immunoglobulin[22] isotypes generated were measured by an ELISA test. Though, as expected, intramuscular administration elicited the highest Ig response, microparticles targeted using UEA-1 significantly enhanced the immune response in all mucosal routes trialed.[11]

In recent years several studies have reported successful lectin-mediated M cell targeting approaches in murine models.[11,13,21] Notably, human studies in this area are relatively few. Russell-Jones et al. reported enhanced transport of microspheres functionalized with different lectins in Caco-2 monolayers,[58] but otherwise there is a lack of data regarding M cell targeting in human models. Despite the reported successful applications of UEA-1-mediated targeting of M cells in non-human systems, little is known about this transcytotic pathway.[30] As such, there is a need for well-characterized, reproducible human models of the FAE to further study the molecular mechanisms of M-cell mediated transcytosis.[59]

NKM 16–2-4. NKM 16–2-4 is a monoclonal antibody (mAb) first developed in 2007 in an effort to develop a marker specific for the α(1,2) fucose containing carbohydrate moiety than UEA-1. NKM 16–2-4 has been shown to bind to murine M cells with a greater affinity than UEA-1.[60] The mAb NKM 16–2-4 conjugated to FITC was introduced into rat intestinal loop models containing PPs. Within 10 min of administration, NKM 16–2-4 specifically attached to the apical surface of M cells in the dome region of the PPs, whereas the rat IgG control did not, as assessed through confocal imaging of stained sections. As a result of the intended increased specificity, the mAb did not cross-bind to goblet cells.[60] The intended use of the NKM 16–2-4 mAb was to serve as an M cell targeting agent specifically for murine systems. It has since been used to identify M cells in both human and murine *in vitro* FAE models, though use in human models is infrequent and generates variable results.[10,30]

Proteins

Clusterin

Clusterin, also known as apolipoprotein J, is a highly conserved, disulfide-linked heterodimeric glycoprotein previously shown to be strongly expressed in murine PPs.[27] Normal villous epithelium samples and FAE samples were extracted from BALB/c mice and gene expression profiles were compared using microarray analysis. Clusterin was found to have a 7.76-fold increased expression in the FAE compared to the normal villous epithelium. This finding prompted a more comprehensive investigation into the expression of clusterin in inductive sites of human mucosal associated lymphoid tissue.[27] Clusterin expression was investigated in fixed surgical resection specimens of human non-pathologic terminal ileum containing PPs, colon, appendix, palatine and nasopharyngeal tonsils using immunohistochemistry and cryo-immunogold electron microscopy.[27] In human PPs, clusterin marked subcellular cytosolic space of cells thought to be M cells. To ensure the stained cells were M cells, a combination of high resolution immunofluorescence imaging and cryo-immunogold electron microscopy was conducted with results confirming clusterin was immunoreactive in the cytosol of M-like cells with the characteristic M cell phenotype. Although this suggests clusterin may be expressed in the cytosol of human M cells, Verbrugghe et al. used Caco-2 cells as a positive control for the clusterin antibody.[27] Caco-2 cells do express the *CLU* gene encoding for clusterin.[61] As such, although clusterin may mark human PP M cells, it may lack specificity as a marker for M cells generated in an *in vitro* Caco-2/Raji cell-based model. Despite this, clusterin was recently used to mark M cells in a proposed, novel Caco-2/Raji co-culture protocol published by Chaikhumwang et al.[29] (refer to Table 1). They reported no clusterin expression in Caco-2 cells alone, as demonstrated by Western blotting.[29] This disparity in published data indicates more studies are required to determine whether clusterin can be used as a reliable human M cell marker across various human models.

Annexin V

Annexin V (or annexin A5) is a cellular protein belonging to the annexin family of calcium-dependent phospholipid binding proteins.[62] Annexin V is most frequently known for its function as an apoptotic marker; it is used commonly in flow cytometry to detect apoptotic cells due to its ability to bind phosphatidylserine (PtdSer) on the outer leaflet of the plasma membrane.[28] Expression of PtdSet on the cellular membrane is an integral step in apoptosis as it signals nearby phagocytes to action. In 2004, a novel study proposed PtdSer may form a part of a yet undescribed pinocytotic pathway.[63] The study observed the internalization of annexin V into anti-Fas (an apoptosis signal receptor) stimulated Jurkat cells in a PtdSer dependent manner, whereby annexin V was transported into the cell and redistributed throughout the cytoplasm in endocytotic vesicles.[63]

The FAE-specific genetic expression of annexin V was reported by two different groups.[28,64] Gene expression profiles of the FAE and normal villous epithelium of BALB/c mice were compared through microarray analysis. The analysis revealed that within the FAE, 91 genes were upregulated and 4 downregulated by at least two-fold. This subset of genes was further confirmed through quantitative RT-PCR.[28] The *Anxa5* gene encoding Annexin V was upregulated more than 3 times in the FAE compared to the VE, as determined by microarray analysis. *Anxa5* was the second most expressed gene in the FAE, behind the gene encoding for clusterin. Immunohistochemical analyses revealed that whilst the VE was completely negative for annexin V immunoreactivity, strong immunoreactivity of annexin V co-localizing with UEA-1 was observed within the FAE. Other studies have shown that annexin V binds to the lipopolysaccharide of gram negative bacteria reducing endotoxin activity, suggesting that annexin V may play a role in M cell mediated bacteria uptake.[62] It appears that the role of annexin V as an M cell marker functions independently of SpiB, and thus may be more indicative of an immature/proliferating M cell phenotype. A 2012 study used RT-PCR to evaluate mRNA expression of M cell specific genes in WT and SpiB$^{-/-}$ mice. The transcriptional expression of M cell specific genes Gp2, CCL9, M-Sec and Sgne-1 was completely ablated in SpiB$^{-/-}$ mice and this expression was not restored by subsequent RANKL administration.[17] In contrast, mRNA expression for Marcksl1 and annexin V was maintained the SpiB$^{-/-}$ mice.[17] This observation suggests Gp2, CCL9, M-Sec and Sgne-1 are regulated by SpiB, whereas Marcksl1 and annexin V appear to be SpiB-independent and thus may be markers of a less mature M cell phenotype (Figure 2).[17]

In 2012, de Lau et al. developed an *in vitro* "M cell" organoid model using cells derived from either wildtype (WT) or SpiB-deficient mice that were stimulated with recombinant RANKL to induce M cell differentiation.[6] RANKL stimulation resulted in an increase of the genetic expression of *SpiB* within 24 h, as well as other M cell markers including *Gp-2*. This advanced model was validated using immunohistochemical analyses of UEA-1, annexin V and Gp-2, alongside transcriptional analyses of RANK, SpiB, Gp-2 and annexin V.[6] Though likely to be effective in murine models, annexin V remains unexplored in human systems. However, as previously highlighted, it is possible that annexin V, a known EMT marker, may be a marker of the phenotypic transition that epithelial cells undergo in order to adopt the "M cell" phenotype.

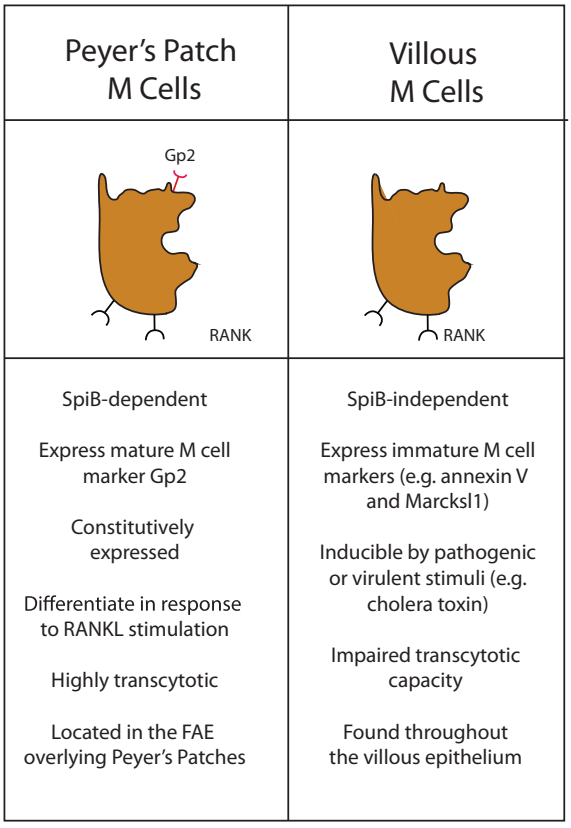

Figure 2. M cells constitutively expressed by the follicle-associated epithelium express the mature M cell marker Gp2. They differentiate in response to RANKL stimulation and induction of *SpiB*. Alternatively, regular villous enterocytes can be prompted to differentiate into "villous" M cells that are only partially reminiscent of the M cell phenotype. Villous M cells are not restricted to the follicle-associated epithelium, express different markers and develop independently of SpiB. They are void of Gp2 expression and thus have an impaired transcytotic ability. (A color version of this figure is available in the online journal.)

Glycoprotein-2

Glycoprotein-2 (Gp-2) is a protein secreted from intracellular zymogen granules and anchored to the cell membrane through glycosylphosphatidylinositol (GPI) linkages.[65] Gp-2 binds pathogens, including enterobacteria, and is thus central in mediating innate immunity.[10] Though initially thought to be expressed solely on mammalian pancreatic acinar cells,[65] microarray analysis conducted on the FAE and normal intestinal epithelium of mouse PP's has demonstrated an increased expression of *Gp-2* mRNA on the FAE.[64] Additionally Gp-2 has been shown to be localized on M cells of the FAE through *in situ* hybridization and immunostaining experiments showing co-localization of Gp-2 with UEA-1.[10] Comparison of immunostaining of mouse and human PP showed similar staining patterns in the FAE overlying the PP, with co-localization with another

proposed human M cell marker clusterin.[10] Thus Gp-2 may be the only M cell marker cross reactive between human and mouse that is currently available.[10] Gp-2 selectively binds to the FimH region of the type I pili on the outer membrane of bacteria including *E. coli.* In the absence of Gp-2, the transport of type I piliated bacteria across the FAE is impeded.[10] The expression of Gp2 was implicated as a pivotal factor in the transcytotic function of M cells in a 2015 study by Kimura and Iwanaga (2015).[66] Briefly, they assessed the uptake capacity of two subsets of M cells; Gp2-high "mature" M cells and M cells expressing a purported early M cell marker Tfnaip2, with low Gp2 expression. Ligated intestinal loop assays using 20 nm fluorescent latex beads revealed that the beads were internalized mostly by Gp2-high cells and only occasionally by Gp2-low cells.[66] This suggests that Gp2 expression by M cells is crucial for high transcytotic capacity.[66]

Though it has been previously reported *SpiB*$^{-/-}$ mice lack Gp-2 expressing M cells in the FAE, it has also been suggested the M cell phenotype can be induced in regular absorptive epithelial cells via a *SpiB* independent pathway.[18,19] One study showed that villous intestinal epithelial cells (IECs) could adopt an M cell phenotype when presented with an immunological challenge in the form of CT and *Salmonella* Thyphirium.[20,22] These CT-induced "M cells," designated as such due to being UEA-1^{+}, NKM 16–2-4^{+} and CD45^{-}, showed a different expression pattern of protein markers and cytokines as compared to PP M cells and normal IECs. Despite this, they were capable of increased transcytosis and developed an M cell-like morphology. In SpiB-dependent PP M cells, Gp-2 was highly expressed and co-localized with UEA-1, whereas in CT-induced "M cells" it was entirely absent. This is also true of another reported M cell marker Marcksl1. Additionally, cytokines that were abundantly expressed in PP M cells were only moderately expressed in CT-induced "M cells"; as an example, CCL9 was upregulated over 124 times in PP M cells compared to IECs, and only 7 times in CT-induced "M cells." This indicated that SpiB-dependent M cells of the FAE and SpiB-independent "M cells" may have different proteomic and genetic expression profiles, and a different functional capacity.[18,22] Taken together, it is likely that SpiB regulates Gp-2 expression, and additionally that, due to their lower numbers, absence of crucial markers and diminished functional capacity, these *S*piB-independent "M cells" may not have a mature, fully differentiated M cell phenotype.

In the context of the *in vitro* models used to study the FAE, both mouse and human organoid cultures have successfully used Gp-2 to identify M cells.[6,7] However, it has been reported that neither the *SpiB* gene nor the *Gp-2* gene are expressed in Caco-2 cells.[5] This indicates that "M cells" generated through the Caco-2/Raji co-culture method are likely differentiated by a SpiB-independent pathway and as such may not be fully mature nor functionally similar to Gp2^{+} PP M cells. Interestingly, one study does show the presence of Gp-2 on the apical surface of "M cells" generated through a Caco-2/Raji co-culture, but the study lacks gene expression data.[30] Except in this instance, Gp-2 staining of Caco-2/Raji co-cultures has not been successfully demonstrated in the literature. As such, Gp-2 can be used as a reliable, human and murine M cell marker, but the absence of Gp-2 in a particular model may indicate the presence of "M-like" cells generated independently of SpiB. It is important to note that though mechanisms of action of these cells may be similar, not enough is known about this subpopulation of cells to assume that they are a molecularly relevant model of the *in vivo* FAE microenvironment. Immortalized cell-line-based models are limited in their ability to recapitulate healthy human and murine tissues. Due to this, the *in vitro* organoid system cultured from isolated Lgr5+ intestinal stem cells and stimulated by RANKL appears to be a more appropriate model of the FAE, with reference to the genetic and proteomic signature reminiscent of *in vivo* PP M cells. The organoid system, pioneered by the Clevers group, is a controlled, *in vitro* organ model that, as a consequence of being cultured from primary stem cells, recapitulates much of the complexity of the original organ. Small intestinal organoids have, under certain growth conditions, been shown to spontaneously develop a crypt-villous structure and give rise to all of the main cell types found in the intestinal epithelium.[67] In the *in vitro* M cell organoid model presented by de Lau et al. (2012), Gp2 expression is demonstrated at both the genetic and proteomic level indicating the presence of mature, terminally differentiated M cell population.[6] This, coupled with the observation that Gp2 expression has not been definitively confirmed in the Caco/Raji co-culture model, suggests that the organoid system is a far more accurate representation of the FAE.

Functional indicators of M cell presence

Transcytotic capacity

The interest in studying M cells is prompted by their increased ability to transcytose luminal antigens and the potential applications of this to targeted oral vaccine delivery strategies. In the absence of a confirmed M cell protein marker, the most reliable indicator of M cell presence is the transcytotic capacity of the cells.[36,44] Functional studies evaluating the degree of uptake and transcytosis of particles are regularly used in *in vitro* models to confirm the presence of an M-cell like population. It is reported than in human cell-line-based assays in which M-like cells are induced through co-culturing epithelial cells with lymphoblastic cells, the presence of induced M-like cells can increase transcytotic capacity by approximately 800 times.[36] Furthermore, it is believed the transcytotic capacity of M cells is further exacerbated through contact with pathogenic materials. Through TEM, it has been shown that radiolabeled *Vibrio chloerae* were up taken by M cells in the PPs' of white rabbits, and that neutralization of *Vibrio chloerae* through acidification, heat, formalin or UV radiation reversed this effect.[68] It has also been shown that various strains of enterohemorrhagic *Escherichia coli* interact with *ex vivo* murine PPs, increasing their absorptive capacity, using the intestinal gut loop model.[10,26] *In vitro* functional transport studies are a relatively simple method of confirming M cell presence by evaluating the absorptive

capacity of the cell, providing also that the monolayer integrity remains intact.

Alkaline phosphatase

Alkaline phosphatase is a homodimeric protein enzyme secreted bi-directionally by villous enterocytes and is integral to the digestive function of enterocyte cells.[69] Many *in vitro* models of the FAE utilize the Caco-2 cell line to represent the absorptive enterocytes of the *in vivo* small intestinal epithelium.[36] Caco-2 cells spontaneously differentiate into polarized enterocyte-like cells expressing high levels of brush border enzymes typical of small intestinal absorptive enterocytes.[70] The activity of brush border digestive enzymes, such as alkaline phosphatase, peptidases and disaccharides, gradually increase as cells mature and reach confluence. Thus, the conversion of enterocyte-like cells to "M cells" generally involves a loss of digestive function and a decrease in the expression of digestive enzymes including alkaline phosphatase.[69] This suggests a decrease in alkaline phosphatase activity could serve as a negative marker of M cells. Smith et al. were among the first to attempt to quantify M cells in mice by investigating the decrease in alkaline phosphatase activity.[71] They examined the difference in number of M cells in BALB/c mice when the mice were transferred from a specific pathogen-free environment to a normal animal house environment. To quantify M cell numbers, they hypothesized that cells with the least amount of alkaline phosphatase activity were most likely to be M cells. This was supported by the observation that cells with the least amount of alkaline phosphatase activity change in numbers in a predictable fashion after the transfer of mice from a specific pathogen free environment into a normal animal house environment. This observation led them to suggest alkaline phosphatase is indicative of conversion of enterocyte-like cells into M cells.

Currently, many human *in vitro* models quantify alkaline phosphatase activity in order to measure the activity of intestinal cell lines.[43,70,72] In the context of the Caco-2/Raji co-culture model, a decrease in alkaline phosphatase activity is generally attributed to the presence of M cells.[43] Negative markers can be useful as complementary data to more established M cell identification methods, but cannot definitively be attributed to M cell conversion as a loss of brush border digestive enzymes indicates a decrease in digestive function of the original cell line and not necessarily the presence of M cells.

Conclusion

In vitro studies of the molecular mechanisms of function of the follicle-associated epithelium are made difficult by the lack of a universal M-cell specific marker that evaluates the degree of conversion of M cells and their transcytotic capacity. M cells are vital to mediating the intestinal immune response by regulating access of microorganisms and antigens circulating in the gut lumen to the underlying immune tissue. Despite their central role in intestinal immunity, the molecular mechanisms regulating the actions of these cells are still poorly understood. The importance of further study into the molecular actions of M cells is highlighted by the fact over 95% of human pathogenic microroganisms gain entry into host cells by crossing epithelial barriers.[2] Thus, inducing mucosal immunity by targeting M cells directly would be highly beneficial, despite the low number of M cells found *in vivo*. This critical review has discussed commonly used M cell markers, and outlined the necessity for developing a standardized approach to make the study of the M like cells generated *in vitro* more reliable. The current trend of using only imaging techniques or enzyme activity assays is not sufficient to demonstrate conclusively the conversion of enterocytes to mature M cells. Alternatively, the use of functional experiments showing elevated transport in M cell models compared to control models does not definitively confirm that the results observed are due to M cell activity without confirmation of M cell presence. Due to the lack of a single, universal M cell marker, an approach utilizing several techniques is recommended to confirm the presence of M cells, and expression of Gp2 in human and murine models can be considered indicative of a mature M cell population. Understanding the molecular mechanisms regulating M cells will be a crucial step towards advancing our knowledge of intestinal immunity and developing novel strategies to improve oral delivery of biologically active compounds.

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