Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium

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Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium

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SUMMARY

The transcytosis of antigens across the gut epithelium by microfold cells (M cells) is important for the induction of efficient immune responses to some mucosal antigens in Peyer's patches. Recently, substantial progress has been made in our understanding of the factors that influence the development and function of M cells. This review highlights these important advances, with particular emphasis on: the host genes which control the functional maturation of M cells; how this knowledge has led to the rapid advance in our understanding of M-cell biology in the steady-state and during aging; molecules expressed on M cells which appear to be used as "immunosurveillance" receptors to sample pathogenic microorganisms in the gut; how certain pathogens appear to exploit M cells to infect the host; and finally how this knowledge has been used to specifically target antigens to M cells to attempt to improve the efficacy of mucosal vaccines.

INTRODUCTION

A single layer of epithelial cells provides a protective barrier against the substantial bacterial burden within the intestinal lumen. However, the epithelia overlying the organized lymphoid follicles of the gut-associated lymphoid tissues (GALT) including the Peyer's patches, their equivalents in the caecum and colon, and isolated lymphoid follicles (ILFs), are specialized for sampling the lumenal contents. Under steady-state conditions approximately 10 % of the epithelial cells within these follicle-associated epithelia (FAE) are microfold (M) cells[1-3]. These cells have unique morphological features including the presence of a reduced glycocalyx, irregular brush border and reduced microvilli. Furthermore, in contrast to the neighbouring enterocytes within the FAE, M cells are highly specialized for the phagocytosis and transcytosis of gut lumen macromolecules, particulate antigens and pathogenic or commensal microorganisms across epithelium (Figure 1). Following their transcytosis across the FAE, antigens exit into the intraepithelial pocket beneath the M-cell basolateral membrane which contains various populations of lymphocytes and mononuclear

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phagocytes (MNP, a heterogenous population of macrophages and classical dendritic cells[4-6]). This specialized microenvironment beneath the M-cell enables the efficient transfer of lumenal antigens to MNP[7, 8]. Studies show that in the absence of M cells, or antigen sampling by M cells, antigen-specific T-cell responses in the Peyer's patches of mice orally-infected with *Salmonella enterica* serovar Typhimurium are reduced[2, 9]. Thus, the efficient M-cell-mediated sampling of gut lumenal antigens is considered an important initial step in the induction of some mucosal immune responses[2, 7-9].

In this review we mostly describe the recent progress that has been made in our understanding of the immunobiology of M cells in the intestine, but it is important to note that M cells are not only restricted the FAE overlying the GALT. For example, cells with typical features of M cells have been reported in the murine nasal passage epithelium, identifying a novel, NALT-independent mode of antigen sampling in the respiratory tract[10]. In the intestine M cells have also been described within the villous epithelium[8, 11,12], but whether these cells are functionally equivalent to those within the FAE overlying the organised lymphoid follicles remains to be determined[13].

M cells differentiate from Lgr5$^+$ stem cells in the crypts in a RANKL- and Spi-B dependent manner

Since M cells are considered to play an important role in the induction of specific mucosal immune responses in the Peyer's patches[2, 9], their manipulation may improve the efficacy of mucosal vaccines or help develop strategies to block transmission of some orally-acquired infections (discussed below). Many studies have therefore attempted to elucidate the molecular mechanisms which regulate M-cell differentiation in the gut epithelium. In the intestine almost all epithelial cell lineages develop from intestinal epithelial stem cells within the crypts. The dome-associated crypts surrounding the FAE, and the villous crypts at the base of the villi, each contain cycling leucine-rich repeat-containing G protein-coupled receptor-(Lgr5-) positive$^+$ stem cells intermingled amongst Paneth cells[14]. These stem cells produce highly proliferative daughter cells which upon appropriate stimulation can differentiate into all the epithelial cell populations within the small intestine including the enterocytes, goblet cells, enteroendocrine cells, tuft cells and Paneth cells. Lineage-tracing studies using transgenic mice expressing a reporter gene (*LacZ*) under control of the *Lgr5* promoter have confirmed that all the epithelial cells within the FAE, including M cells, are derived from the cycling Lgr5$^+$ stem cells within the dome-associated crypts[15-17] (Figure 2).

In the mouse intestine under steady-state conditions M cells are mostly confined to the FAE overlying the organized lymphoid follicles of the GALT. Since both the dome-associated crypts and the villous crypts throughout the intestine contain cycling Lgr5$^+$ stem cells, additional signals from the cells beneath the FAE in the subepithelial dome (SED) are required to induce M-cell differentiation, and in doing so restrict these cells to the epithelia overlying organized lymphoid follicles. The TNF superfamily member receptor activator of NF-κB ligand (RANKL), signals via its receptor RANK (receptor activator of NF-κB), which is expressed by epithelial cells throughout the intestine. However, in the intestine RANKL is selectively expressed by the subepithelial stromal cells beneath the FAE[18, 19]. Analysis of RANKL-deficient mice, and administration of exogenous RANKL *in vivo*, show this cytokine is a critical factor that controls the differentiation of RANK-expressing enterocytes into M cells[20]. Under steady-state conditions, the only RANK-expressing intestinal epithelial cells that are exposed to sufficient RANKL from any source to respond in a detectable way are those in the FAE that are immediately above the RANKL-expressing subepithelial stromal cells. These data imply that in the context of M-cell differentiation, much of the RANKL-RANK signalling is delivered by the intact transmembrane cytokine following direct cell contact, rather than systemic dissemination of soluble RANKL. However, the possibility cannot be entirely excluded that both the membrane and soluble

forms of RANKL are expressed by SED stromal cells. Accordingly, in the steady state, villous enterocytes situated distant from organized lymphoid structures are not exposed to enough RANKL to show any induction of the genes that regulate M cell-differentiation[2, 20]. Thus, the restriction of RANKL-expression to the subepithelial stromal cells ensures that the differentiation of $Lgr5^+$ stem cells into M cells is spatially restricted to the FAE overlying GALT (Figure 2). Consistent with this theory, the systemic treatment of mice with RANKL induces the ectopic differentiation of enterocytes into M cells in the villous epithelium[2, 20]. To help to focus the effects of soluble RANKL to the overlying FAE, it is plausible that the range of its activity is further restricted by binding to osteoprotegerin, a secreted decoy receptor of RANKL[21].

Distinct transcription factors control the expression of the sets of genes which ultimately determine the differentiation of crypt stem cell-derived progenitor cells into terminally differentiated intestinal epithelial cell populations. The same is true for M cells. The RANKL-induced, M cell-intrinsic, expression of the ETS transcription factor Spi-B specifically regulates the maturation of immature M cells into functionally mature M cells. While immature M-cell differentiation appeared to be unaffected in Spi-B-deficient (Spi-$B^{-/-}$) mice, their FAE were are almost completely deficient in functionally mature M cells[2, 15, 22]. Thus, the terminal differentiation of M cells, as for other intestinal epithelial cell lineages, is regulated by a specific transcription factor (Figure 2).

Transcriptomic profiling studies have helped to identify those genes which are expressed by functionally mature M cells and regulated by Spi-B, including: *Gp2*, *Tnfaip2* and *Ccl9* (discussed below). Others such as *Anxa5* and *Marcksl1* (which encode for annexin A5 and MARCKS-like protein 1, respectively) are expressed by immature (differentiating) M cells in response to RANKL-stimulation but are regulated independently of Spi-B[2, 13, 15, 23-26]. Although their precise roles are uncertain, the immature M-cell markers such as ANXA5 and MARCKS-like protein 1 may be involved in cytoskeletal regulation to help adapt the differentiating epithelial cell towards one specialized for transcytosis[27, 28].

An independent study has also investigated the requirement for Spi-B in M-cell differentiation[22]. In this study two distinct monoclonal Abs were used to detect M cells: one mAb specific for glycoprotein 2 (GP2, discussed below); and mAb NKM 16-2-4 which specifically recognises sites of α-1,2 fucosylation[25, 29] and has a greater degree of specificity for M cells than the lectin *Ulex europaeus* agglutinin I (UEA-1, which also binds α-1,2 fucose with high-affinity). While the expression of the mature $GP2^+$ M cells was completely absent in the FAE of Spi-$B^{-/-}$ mice, a small number of mAb NKM 16-2-4^+ M cells were evident (~10 mAb NKM 16-2-4^+ M cells/FAE)[22]. One interpretation of these apparently contradictory data is that a small population of M cells in the FAE may mature independently of Spi-B and as a consequence do not express proteins such as GP2 on their surfaces[22]. Alternatively, it is plausible that the remaining mAb NKM 16-2-4^+ cells in the FAE of Spi-$B^{-/-}$ mice were not fully differentiated M cells, or represented other populations of fucosylated enterocytes.

M cells appear to be regularly spaced in the FAE implying that their distribution is highly regulated (Figure 1b). Indeed, while all the epithelial cells in the FAE derive from $Lgr5^+$ stem cells[15] and are exposed to high levels of RANKL-stimulation from the stromal cells in the SED[20], only a proportion of the cells are destined to become M cells. Hsieh and colleagues have questioned how this distribution may be regulated[30]. Notch receptors and their ligands play important roles in determining cell fate and tissue patterning. For example, direct cell-cell signalling, where the expression of a Notch ligand by one cell, may influence the fate of an adjacent cell expressing a Notch receptor. Consistent with this role, Notch and the ligand Jagged1 also appear to influence the patterning of M cells across the FAE[30]. The

optimal distribution of M cells across the FAE may be important to ensure efficient immune surveillance, while at the same time maintaining the integrity of the FAE.

Analysis of cleaved caspase-3 expression in the intestinal epithelia of rats suggested that almost all the epithelial cells within the villi appear to be undergoing apoptosis, whereas the onset of apoptosis within the FAE has been reported to be delayed[31]. The differentiation and maturation of M cells from $Lgr5^+$ stem cells within the crypts takes ~3-4 days[2, 20]. Whether this apparent delay to the progression of apoptosis in the FAE enables M-cell differentiation to be sustained for longer periods is uncertain.

Lympho-epithelial factors influence M cell differentiation in the FAE

In addition to the important stimuli provided to the differentiating M cells in the FAE by SED stromal cells, lympho-epithelial cell interactions also influence their development. Indeed, a role for haematopoietic cells such as B cells in inducing M-cell differentiation is well established[32, 33]. In the small intestine the chemokine CCL20 is specifically expressed by the FAE[34], and in Peyer's patches it mediates the chemoattraction of CCR6-expressing lymphocytes and leukocytes towards the FAE[35]. Mice deficient in CCR6 ($CCR6^{-/-}$ mice), the sole receptor for CCL20, have a significantly reduced frequency of mature $GP2^+$ M cells in the FAE[36-38], implying that the impaired migration of CCR6-expressing lymphocytes or leukocytes towards the FAE impedes M cell differentiation due to reduced lympho-epithelial cell interactions. Alternatively, this may also be due to an altered cytokine milieu in the SED. In the Peyer's patches a unique subset of $CCR6^{hi}CD11c^{int}$ B cells has been reported to specifically migrate towards FAE-derived CCL20 and promote M-cell differentiation[37] (Figure 2). In the absence of CCR6 these cells could not migrate towards the FAE and M-cell differentiation was impeded, whereas their transfer into $CCR6^{-/-}$ mice coincided with restored M-cell maturation. Further analysis shows that although mature M cells are dramatically reduced in the FAE of $CCR6^{-/-}$ mice, immature ANXA5-expressing M cells are retained[36-38]. These data imply that CCL20-CCR6-signaling blockade in $CCR6^{-/-}$ mice does not impact on the initial induction of M-cell differentiation, but specifically impedes their functional maturation. Enteroinvasive bacteria including *S.* Typhimurium and the pathogenic bacteria *S.* Enteritidis and *Listeria monocytogenes*[39] have each been shown to induce CCL20 expression by intestinal epithelial cells. This pathogen-mediated stimulation of CCL20 expression may be part of the host's response to infection to attempt to enhance the "immunosurveillance" of the gut lumen. Alternatively, these pathogens may exploit this potentially aid their uptake from the gut lumen by stimulating the differentiation of enterocytes into M cells.

The factors provided by B cells (or other haematopoietic cell populations) that stimulate M-cell differentiation are uncertain, but may involve signalling between B cells and the M cell progenitor cells via CD137L/CD137[8, 40] or macrophage migration inhibitory factor[41]. The expression of CCL20 in the intestinal epithelium is rapidly induced by RANKL-stimulation (Figure 3) and enhanced by TNFR and LTβR stimulation[2, 42-44]. However, haematopoietic cell populations within Peyer's patches, including the $CD11c^+$ B cells, do not express RANKL[37], and B cell-derived TNF and lymphotoxin are each dispensable for the development of the FAE and M cells[45]. While the above studies together provide a plausible model by which lympho-epithelial interactions may contribute to the functional maturation of M cells, it is important to note that M cell maturation can be induced in an *in vitro* "gut organoid" model system which contains only epithelial cell elements[15]. Although the *in vivo* environment may differ significantly from that of an *in vitro* gut organoid, these data suggest that these lympho-epithelial interactions most likely provide accessory factors that enhance M-cell differentiation (Figure 2).

M cells are equipped with an array of molecules to sample lumenal antigens

M cells provide an efficient portal through which gut lumenal antigens can be delivered to the underlying mucosal lymphoid tissues. In order to achieve this, M cells in contrast to enterocytes, have a high endocytic and transcytotic capacity. Whether the sampling and transcytosis of microoganisms/antigens by M cells occurs predominantly in a non-selective manner as a consequence of constitutive "bulk sampling" of the lumenal contents, specifically via receptor mediated endocytosis, or both is uncertain. However, data from many studies suggest that M cells express a variety of "immunosurveillance" receptors on their apical surfaces which enable them to sample a diverse range of microbial pathogens and antigens.

Glycoprotein 2—Independent sets of gene profiling experiments aimed at identifying molecules that were expressed highly by M cells, but not by other enterocyte populations, identified GP2 as a specific M-cell marker[24, 25]. This GPI-anchored protein is highly expressed on the apical surface of M cells and selectively binds to the FimH component of the type I pili on the outer membrane of certain pathogenic and commensal bacteria such as *Eschericia coli* and *S.* Typhmurium. In the absence of GP2 expression by M cells, the transcytosis of type-I-piliated bacteria was impeded[9]. Furthermore, GP2-dependent bacterial transcytosis by M cells was shown to be important for the immunosurveillance of $FimH^+$ bacteria in the gut lumen[9].

Uromodulin—A homolog of GP2, uromodulin (UMOD or Tamm-Horsfall protein, THP), has also been reported to be specifically expressed by M cells[15, 22]. Secreted UMOD is a urinary protein which can also bind specifically to type-I-piliated *E. coli* and may function to prevent these bacteria from binding to uroepithelial cells[46]. Although the expression of *Umod* by Peyer's patch M cells is much lower than *Gp2* their similar characteristics imply that each protein may aid the sampling of $FimH^+$ bacteria in the gut lumen[9, 22].

Cellular prion protein—Continuing with the immunosurveillance theme, the cellular prion protein (PrP^C) is also expressed highly on the apical membrane of M cells[26] and has been implicated in the sampling of microorganisms from the gut lumen[47]. The GPI-anchored PrP^C protein is expressed highly by many cell populations in mammals, but its precise function remains uncertain as PrP-deficient mice do not show an obvious phenotype. The Gram-negative bacterium *Brucella abortus* is a facultative intracellular pathogen and can express the heat shock protein 60 (Hsp60) on its cell surface. Data show that PrP^C can interact with Hsp60, and may play an important role in the binding and internalization of *B. abortus* into M cells[47]. Thus, PrP^C may also act as an uptake receptor for pathogenic bacteria which express Hsp60.

Other M-cell-specific proteins—A number of other M-cell-specific proteins have been reported which may similarly selectively bind to components of potentially pathogenic microorganisms. However, experiments are required to determine their *in vivo* functions in M cells. ANXA5 is expressed by immature and mature M cells[48], and can bind to Gram-negative bacteria with high affinity via the lipid A domain of LPS[49]. The endotoxin activity of LPS is blocked when bound to ANXA5 implying an important anti-inflammatory role to protect the host against LPS-mediated endotoxemia. Alternatively, it is plausible that ANXA5 on M cells by binding LPS acts as an uptake receptor for Gram-negative bacteria. However, further studies are necessary to determine whether the lipid A domain of LPS would be directly accessible to M-cell-associated membrane receptors, or whether lipid A only becomes accessible after bacterial degradation. Peptidoglycan recognition protein (PGLRP)-1 (encoded by *Pglyrp1*, also known as PGRP-S) is an innate pattern recognition protein that binds to bacterial peptidoglycan, and is expressed highly by M cells[8]. Little is

known of the *in vivo* functions of the four mammalian PGLRPs, but PGLRP1 has bactericidal activity against Gram-positive bacteria and is not known to be involved in cell-signalling[50]. Whether the PGLRP1 secreted by M cells exhibits a similar bactericidal activity to help protect the host against internalized bacteria remains to be determined? The potential roles of other M-cell expressed proteins are less discernible such as fatty acid binding protein 5[51], clusterin[52] and secretogranin V (encoded by *Scg5*, also known as Sgne-1)[24].

A phage-display screen of potential M-cell ligands identified one peptide, termed “Co1”, which shared high sequence homology with the α1 helix of the outer membrane protein H (OmpH) of *Yersinia enterocolitica*[53]. The same region of OmpH is also homologous to that of an *E. coli* chaperone protein, Skp, which can act as a complement C5a receptor (C5aR) chemotactic ligand. Data from the combined analysis of an *in vitro* M-cell-like model and mouse Peyer’s patch M cells *in vivo*, have suggested that OmpH β1α1 binds to C5aR on the apical M-cell surface and enhances the transcytosis of antigens into M cells[54]. However, whether direct molecular interactions between OmpH β1α1 and C5aR (and homologous ligands) occur on M cells is uncertain.

Secretory IgA—Secretory IgA provides a first line of defence in the mucosal immune system by preventing the access of specific pathogens and their toxins across the intestinal epithelium, and is an important regulator of the composition of the commensal gut microflora[55]. For example, secretory IgA is essential for protection from the effects of lumenal cholera toxin exposure[56]. Secretory IgA can block lumenal pathogens from binding to gut epithelial cells and in doing so can provide protection against intestinal reovirus infection[57]. Similarly, secretory IgA may also mediate the lumenal trapping of certain bacteria such as *Shigella flexneri* retaining them within the mucus layer overlying the intestinal epithelium[58]. Prevention of infection against oral infection with *S. typhimurium* has also been described[59]. The targeting of secretory IgA-containing immune-complexes to M cells appears to lead to their subsequent transcytosis through to the MNP and lymphocytes in the SED[60, 61]. Thus, in addition to the role of secretory IgA in facilitating the immune-exclusion of lumenal pathogens and their antigens, the transcytosis of IgA by M cells may also aid antigen sampling and modulate mucosal immune responses. Whether the binding and uptake of secretory IgA and/or IgA-containing immune complexes by M cells occurs specifically is uncertain. No putative IgA receptor has been reported to be expressed by M cells. The possibility therefore cannot be excluded that the enhanced pinocytic and transcytotic activity of M cells alone is sufficient to efficiently internalize secretory IgA and/or IgA-containing immune complexes. Following their uptake from the gut lumen IgA-containing immune-complexes do not appear to disseminate beyond the mesenteric lymph nodes[61]. It is plausible that the secretory IgA-mediated, selective delivery of potential gut pathogens to Peyer’s patches, may act to enable antigen recognition under neutralizing and non-inflammatory conditions without causing widespread tissue dissemination.

Attraction to, and communication with, immune cells in the basolateral pocket

The M-cell basolateral pocket is a specialized microenvironment beneath the FAE which enables close interactions between M cells, MNP, B cells and T cells[7, 8]. These cellular associations ensure that transcytosed microorganisms or macromolecules are readily acquired by specific immune cell populations to induce an appropriate immune response. M cells may even establish intercellular communication channels in order to transport cellular components between themselves and the cells within the basolateral pocket[62]. Tunneling nanotubes (TNT) are thin membranous intercellular conduits which enable the transport of cellular components between cells. Pathogens may also exploit these TNT conduits to aid their dissemination between host cells[63, 64] or to manipulate the host’s response to infection

by shuttling pathogen-derived immunosuppressive factors from infected cells to immune cell populations[65]. Tumour necrosis factor alpha-induced protein 2 (TNFAIP2, also known as M-Sec) is expressed specifically by mature M cells in a Spi-B-dependent manner[2, 24]. In Peyer's patches TNFAIP2 is associated with TNT-like structures which appear to interconnect the M cells in the FAE[62]. Data show that TNFAIP2 acts as a key regulator of TNT formation between immune cells. Thus, the close association of M cells with MNP raises the possibility that some microorganisms or macromolecules may be directly transferred from the gut lumen to MNP via M-cell-derived TNT.

Chemokines and chemokine receptors play important roles in attracting lymphocytes and leukocytes to lymphoid tissues and controlling their positioning within them. In addition to CCL20[34], several other chemokines are also expressed by the FAE, some of them specifically by M cells including CCL9[2, 25, 66] and CXCL16[25, 67] (Figure 3). Retrospective analysis of the magnitude of all the chemokine encoding genes expressed by microarray data sets derived from Peyer's patch M cells[25], the FAE[26] and RANKL-stimulated villous epithelia[2] also indicates significant expression of CXCL11 when compared to the small intestine villous epithelium. The detection of a specific set of chemokines implies their expression is regulated by an underlying transcriptional program.

The wide range of chemokines and receptors expressed in mammals can lead to redundancy whereby several chemokines may bind and activate certain chemokine receptors. However, despite this apparent redundancy, mice deficient in the sole receptor for CCL20 ($CCR6^{-/-}$ mice) show a significant deficiency in M-cell maturation in the FAE[36, 37]. These data suggest that the other FAE- and M-cell-expressed chemokines most likely have accessory roles, such as the attraction of specific cell populations to the basolateral pocket to sample antigens and mount an immune response. The chemokine CCL9 appears to be preferentially expressed by mature M cells and may aid the recruitment of $CD11b^{+}$ MNP to the SED of Peyer's patches[2, 66]. CXCL16 expression may promote interactions between the FAE and CXCR6-expressing T cells[67], and may plausibly also act as an antimicrobial peptide in the mucosal epithelium[68]. CCL25 expression may help mediate the homing of IgA antibody-secreting cells[69], whereas CXCL11 may stimulate the attraction of CXCR3-expressing Th1 cells[70].

Pathogens can exploit M cells to invade the host

Pathogenic microorganisms have evolved a wide range of mechanisms to evade or even exploit immune cells to infect the host. M cells are no exception to this as their efficient transcytotic activity is exploited by numerous pathogens to breach the gut epithelium. In the absence of M cells, oral infections with *Y. enterocolitica*[36], prions[71] or retrovirus[33] are blocked or impeded. A role for M cells as portals for the entry of *Listeria monocytogenes*[72], *Mycobacterium avium* subsp. *paratuberculosis*[73, 74], *S.* Typhimurium[75], human immunodeficiency virus type 1[76], influenza virus[77], polio virus[78] and reovirus[79] have also been described. *Staphylococcus aureus* secretes a number of virulence factors such as the enterotoxins which can help subvert the host immune system. Data from *in vitro* studies imply that M cells may also aid the passage of staphylococcal enterotoxin A across the gut epithelium[80]. Together, these examples show that whereas the sampling of intestinal bacteria by M cells may be important for the induction of specific immunity in Peyer's patches, this function has the potential to be a critical Achilles heel by enabling the safe passage of some pathogenic microorganism or their toxins across mucosal epithelia.

Pathogens can manipulate M cells to invade the host

While some pathogenic microorganisms may use molecules expressed on the apical surfaces M cells as invasion receptors to infect the host, others appear to have evolved mechanisms

that exploit the plasticity of enterocytes to specifically induce M-cell differentiation in the gut epithelium. For example, apparent increases in M-cell-density or antigen transcytosis in the FAE have been described following exposure to *S.* Typhimurium[81] and *Streptococcus pneumoniae* R36a[82, 83].

The RANKL- and Spi-B-dependent differentiation of M cells from immature precursors in the crypts typically takes approximately 4 days[2, 84], yet the studies above suggest that some pathogenic bacteria can achieve this within hours of exposure[81-83]. These characteristic suggest that it is highly unlikely that terminally-differentiated enterocytes or crypt stem cells could be converted by bacteria into mature M cells in such a short time-frame. A more plausible explanation is that the bacteria produce factors which act on cells in the FAE that are already destined to become functionally mature M cells (eg: immature, differentiating M cells). Recent data support this hypothesis, and show how a specific factor secreted by *S.* Typhimurium is used by the bacterium to enable the rapid trans-differentiation of FAE enterocytes into M cells to permit host invasion[3]. Specifically, the SPI-1 encoded type III effector protein SopB secreted by *S.* Typhimurium induced the epithelial-mesenchymal transition of FAE enterocytes into M cells. This cellular trans-differentiation occurred via the SopB-dependent activation of Wnt/β-catenin signalling and led to the induction of both RANKL and its receptor RANK. The autocrine RANKL-RANK activation of the cells induced the expression of Spi-B and also the epithelial-mesenchymal transition-regulating transcription factor Slug (encoded by *Snai2*) that triggered the FAE enterocytes to trans-differentiate into M cells[3].

Aging impedes the functional maturation of M cells

The mucosal immune response in the intestine is significantly compromised by aging[85-88]. This age-related decline in immune competence is associated with diminished antigen-specific IgA antibody titres in the intestinal lumen[86, 89] and a decreased ability to generate tolerance to harmless antigens[87]. Despite the important role of M cells in mucosal immunity in Peyer's patches, nothing was known about how aging influenced their development and function. Using an immunosenescent mouse model we have recently shown that there is a dramatic decline in the functional maturation of $GP2^+$ M cells in the Peyer's patches of aged mice (≥ 600 days old). Furthermore, the transcytosis of particulate antigen across the FAE was likewise impeded[38]. In order to understand the mechanism underlying this aging-related decline in M-cell status, we also examined effects on the stimuli responsible for their induction and maturation. Aging did not affect RANKL expression by the SED stromal cells or the initial induction of M cell differentiation, but specifically impaired the expression of Spi-B and down-stream functional maturation of $GP2^+$ M cells. Expression of the chemokine CCL20 was also reduced in the FAE of aged Peyer's patches, consistent with data from an independent study of the effects of aging on ILFs[90]. As a consequence of the impaired CCL20 expression, the attraction cells (including the "M-cell-inducing" $CD11c^+$ B cells[37]) towards the FAE was impaired. Since both the M-cell intrinsic expression of Spi-B[2, 15, 22] and CCL20-CCR6 stimulation[36, 37] each play important roles in M-cell differentiation, the effects of aging on Spi-B and CCL20 expression appear to dramatically impede the functional maturation of M cells.

Further studies are required to determine whether this aging-related deficiency in the mucosal immune system's ability to sample lumenal antigens contributes to the aging-related decline in impaired antigen-specific mucosal immune responses[86]. However, since several pathogens such as *S.* Typhimurium[75] and prions[71] (as described above) exploit M cells to invade the host, the effects of ageing on M-cell status may paradoxically also have unexpected protective benefits. For example, the effects of aging on M-cell status may impede the early M-cell-dependent uptake of these pathogens into the Peyer's patches, and in doing so significantly reduce disease susceptibility[91, 92].

M-cell-binding ligands as novel mucosal vaccine targets

The significant advances that have been made in the identification of M-cell-specific surface markers or "immunosurveillance" receptors have focussed attention on the development systems to specifically deliver antigens to M cells in order to improve the efficacy of mucosal vaccines. A number of examples are discussed below. **Lectins**: The lectin UEA-1 binds to sites of α-1,2 fucosylation on the surfaces of M cells with high-affinity. Initial attempts using UEA-1 to target antigen to M cells showed that such approaches were effective in inducing antigen-specific mucosal IgA responses[93-96]. Along a similar theme, oral vaccination with antigen-encapsulated liposomes coupled to UEA-1 (termed *lectinized* liposomes) also led to increased uptake by Peyer's patch M cells and the induction of higher antigen specific secretory IgA responses[97].

Alternative approaches to target antigens to the α(1,2)-fucose-containing carbohydrate moiety on M cells have also been explored, for example, by using the non-toxic, small molecular UEA-1 mimetic, tetragalloyl-D-lysine dendrimer[98]. The mAb NKM 16-2-4 specifically recognises the α(1,2)-fucose-containing carbohydrate moiety on M cells with a greater affinity than that of UEA-1. Oral vaccine formulations comprising antigen coupled to mAb NKM 16-2-4 (with cholera toxin as a mucosal adjuvant) have been shown to be effective in inducing high antigen-specific mucosal IgA responses. Furthermore, oral vaccination with botulinum toxin-conjugated mAb NKM 16-2-4 induced protective immunity against oral exposure to a lethal toxin dose[29].

Claudin 4—Although claudin 4 is a component of tight junctions, this protein may also be redistributed to the cytoplasm of M cells and play a role in endocytosis. A peptide derived from the c-terminal domain of the *Clostridium perfringens* enterotoxin binds to the external domain of claudin 4 and can mediate uptake by M cells *in vivo*[99, 100]. Furthermore, this claudin 4-binding peptide when coupled to antigen is effective in enhancing mucosal antigen-specific secretory IgA responses after intranasal immunization[100].

C5aR ligands—As described above, the M-cell ligand "Co1" shared high sequence homology with OmpH of *Y. enterocolitica*, and each of these peptides appeared to bind to a C5aR on the apical surface of M cells[54]. The conjugation of antigens to either Co1 or OmpH could mediate their delivery to M cells in Peyer's patches and induce enhanced mucosal immune responses after oral administration[53, 54].

Reovirus hemagglutinin protein σ1—DNA vaccines have also been shown to confer protective immunity against a range of pathogenic microrganisms, but this approach has proved to be poor at inducing mucosal immunity. M-cell targeting has also been explored to attempt to improve the efficiency of the delivery of a DNA vaccine to a mucosal inductive tissue. Since reovirus is considered to bind M cells *in vivo*[79], a recombinant reovirus hemagglutinin protein σ1 conjugated to polylysine was used to target a DNA vaccine to NALT M cells[101]. Intranasal immunization with protein σ1-polylysine-DNA was effective in inducing prolonged antigen-specific mucosal IgA responses, illustrating that M-cell targeted peptide or DNA vaccinations may each represent effective means of inducing antigen-specific mucosal immunity.

However, in addition to their role in the induction of specific mucosal immunity, M cells may also be involved in the induction of oral tolerance. Independent studies show that mucosal administration of low doses of reovirus protein σ1 conjugated to ovalbumin (OVA) induced systemic unresponsiveness and failed to elicit an OVA-specific mucosal immune response. Under these conditions the mice were shown to be tolerogenic to OVA and were

resistant to peripheral challenge due to the generation of antigen-specific regulatory T cells[102, 103].

Concluding remarks

Significant recent advances have been made in the identification of M-cell-specific surface markers, and the characterization of the range of pathogen-derived ligands these "immunosurveillance" receptors can bind. By using similar approaches to those described above it may be possible to significantly improve the efficacy and specificity of the M-cell targeting and elicit long-lived and protective mucosal immunity, or tolerance. The identification of the molecular mechanisms that some pathogens use to exploit M cells to invade the host may also lead to the development of specific strategies to block the initial host colonisation. While the targeted delivery of vaccine antigens to M cells offers potential as an effective means of inducing antigen-specific mucosal immune responses, the observation that the functional maturation of M cells is dramatically impaired in aged mice implies that these strategies may be much less effective when used to immunize the elderly[38]. An understanding of the molecular mechanism that underpins this aging-related decline in M cell status will help identify novel approaches to stimulate M cell differentiation and potentially improve mucosal immunity in the elderly.

The use of various M-cell-deficient mouse models has greatly improved our understanding of the immunobiology of M cells in immunity and disease pathogenesis *in vivo*. However, in many of these instances the possibility that M-cell-independent factors significantly contribute to the effects described cannot be entirely excluded. For example, RANKL-neutralization dramatically depletes M cells in the FAE *in vivo*[20]. However, RANKL may influence gene expression by other cells in the FAE[13] which may also play a role in the uptake of lumenal antigens[104]. Similarly, the maturation of M cells is impaired in the FAE of CCR6$^{-/-}$ mice[36-38], but effects on mucosal immunity or disease susceptibility due to the reduced size of their Peyer's patches or defective T-cell responses cannot be entirely excluded. The independent demonstrations that expression of the transcription factor Spi-B is critical for the functional maturation of M cells[2, 15, 22] has identified the Spi-B$^{-/-}$ mouse as an alternative approach to study the immunobiology of M cells. However, Spi-B also plays an important roles in the differentiation of plasmacytoid DC and B cells[105, 106]. In order to completely avoid these off-target effects conditional knockout mice with genes encoding RANK or Spi-B specifically-ablated in the intestinal epithelium are crucial to study mucosal immunity in the specific absence of M cells.

An *in vitro* M-cell-like model system in which a monolayer of the differentiated human enterocyte cell line Caco-2 is cultivated with B cells has been widely used to study M cells[32]. However, although these cells display efficient transcytotic activity in contrast to the undifferentiated enterocytes, it is uncertain whether these co-cultivated epithelial cells accurately represent the many characteristics of M cells *in vivo*. Furthermore, analysis of the genes expressed by "M-cell induced" Caco-2 cells shows that while they express *CCL20* highly, they lack expression of many mature M cell marker genes such as *GP2* and *SPIB* (Table 1). Recently, exciting progress was reported on the development of a novel, potentially physiologically relevant *in vitro* M-cell model system. In this system, RANKL-stimulation induces M-cell differentiation in gut organoid cultures established from intestinal crypts or single Lgr5^{+} crypt stem cells[15]. As well as exhibiting high transcytotic activity, the range of genes expressed by these organoid cultures closely resembles those of M cells *in vivo* (Table 1). These M-cell containing organoid cultures offer a simple and physiologically relevant *in vitro* model system in which to study the immunobiology of M cells from a range of mammalian species.

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Finally, many studies have primarily focussed on M cells as the main sites of antigen sampling in mucosal epithelia, but several studies are challenging the assertion that this activity is so restricted (Figure 4). Data from the ultrastructural analysis of Peyer's patches identified a novel, previously unrecognised, potentially important component of the normal machinery for antigen sampling in the GALT. This study suggested that FAE enterocytes can also acquire certain gut lumenal antigens, and exocytose the intravascular contents of their large late endosomes into the extracellular space of the SED where they are acquired by MNP[104]. Goblet cells are situated throughout mucosal epithelia and until recently were mainly considered to function as mucin secreting cells. However, an elegant study shows that intestinal goblet cells can also function as passages for the delivery of low molecular weight soluble antigens to $CD103^+$ MNP in the lamina propria[107]. The sampling of lumenal antigens may also not be entirely restricted to epithelial cell populations. Although the incidence is rare in the steady-state, certain inflammatory stimuli appear to recruit MNP from the lamina propria to the gut epithelium where they insert their dendrites through the tight junctions between enterocytes to directly sample the luminal contents[108, 109]. In addition to this "periscoping" activity, another study has even proposed that MNP sample lumenal antigens by extending their dendrites directly through "transcellular pores" in the M cells themselves[7].

The precise immunological consequences of these alternative antigen sampling mechanisms are uncertain and require further analysis. Unlike macrophages which rapidly phagocytose microorganisms and destroy them in their phagosomes, classical DC appear to be equipped with both degradative and nondegradative antigen uptake pathways to facilitate antigen presentation to both T and B cells[110, 111]. Indeed, classical DC can capture and retain unprocessed antigen *in vitro* and *in vivo* and can transfer this antigen to naïve B cells to initiate a specific antibody response[112]. Viable commensal bacteria and unprocessed antigen can also be recovered from classical DC migrating within afferent mesenteric lymph[113, 114]. These contrasting characteristics raise the question of whether the cells through which antigens are initially sampled across the intestinal epithelium (M cells, enterocytes, goblet cells), determine whether lumen antigens are initially transferred directly into Peyer's patches (such as after M-cell-mediated transcytosis) or into villi and onwards to the mesenteric lymph nodes. Similarly, it is also plausible that in the steady-state these routes also direct lumenal antigens to specific MNP populations, and as a consequence, fundamentally influence the nature of the immune response directed to those antigens.

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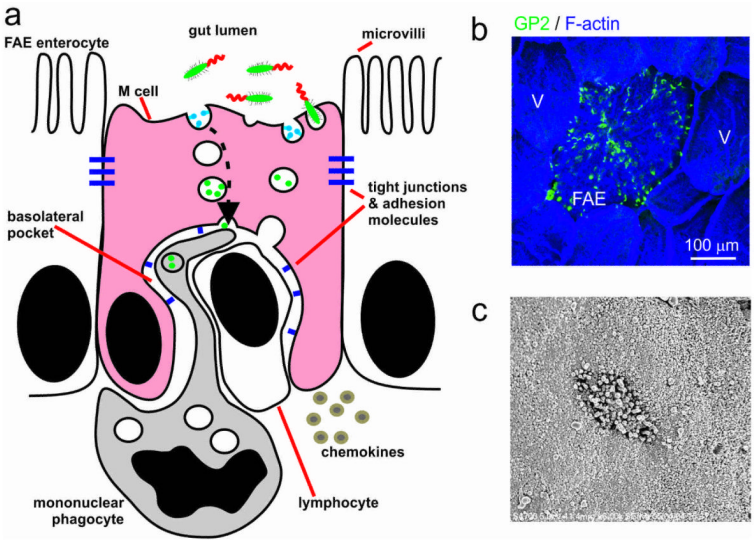

Figure 1.

The morphological features of M cells. (**a**) Cartoon illustrating the morphological features of M cells. Note the lack of microvilli and basolateral pocket containing a mononuclear phagocyte and a lymphocyte. (**b**) Whole-mount immunohistochemical analysis of $GP2^{+}$ mature M cells (green) in the FAE of a mouse Peyer's patch. Note the regular distribution of the M cells across the FAE. Note also that in the steady-state M cells are restricted to the FAE and are mostly absent from the surrounding villi. Tissue counterstained to detect f-actin (blue). V, villi. (**c**) Scanning electron micrograph of the apical surface of a M-cell. Note characteristic lack of/blunted microvilli.

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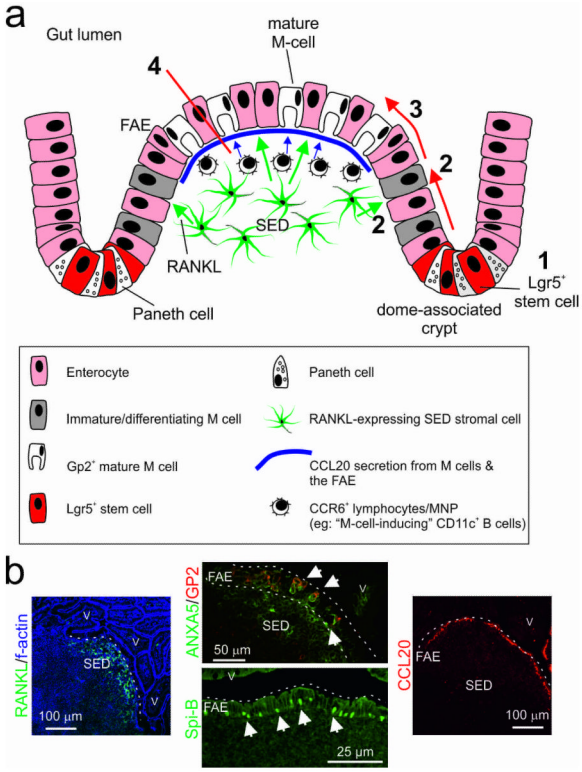

Figure 2.

M cells differentiate from Lgr5^{+} stem cells in the crypts in a RANKL- and Spi-B dependent manner. (**a, 1**) All epithelial cell lineages, including M cells, develop from Lgr5^{+} intestinal epithelial stem cells within the crypts[15]. (a, **2**) In the intestine RANKL is selectively expressed by the subepithelial stromal cells beneath the FAE[18, 19]. RANKL controls the differentiation of Lgr5^{+} stem cell-derived RANK-expressing enterocytes, into M cells[15, 20]. (**a, 3**) RANKL also induces the expression of the ETS family transcription factor Spi-B[2]. The subsequent differentiation of the ANXA5^{+}, MARCKS-like protein 1^{+} immature M cells into functionally mature GP2^{+}, TNFAIP2^{+} and CCL9^{+} M cells is regulated by intrinsic expression of Spi-B[2, 15, 22]. (**a, 4**) The chemokine CCL20 is specifically expressed by the FAE[34], and in Peyer's patches it mediates the chemoattraction of CCR6-expressing lymphocytes and leukocytes towards the FAE[35]. When CCL20-CCR6 signalling is impeded, M-cell maturation is likewise impeded[36-38]. Data suggest that a unique subset of CCR6^{hi}CD11c^{int} B cells specifically migrates towards FAE-derived CCL20 and promotes M-cell differentiation[37]. However, since M-cell maturation can be induced in an *in vitro* "gut organoid" model system containing only epithelial cell elements[15], it plausible that these CCL20-CCR6-mediated lympho-epithelial interactions most likely provide accessory factors that enhance M-cell differentiation. (**b**) Immunohistochemical analysis of the distribution of RANKL (green, left-hand panel), ANXA5, GP2 (green and red, respectively, upper-middle panel), Spi-B (green, lower-middle panel) and CCL20 (red, right-hand panel) in mouse Peyer's patches. Dotted lines indicate the boundary of the FAE. Arrows indicate positively immunostained M cells. V, villi.

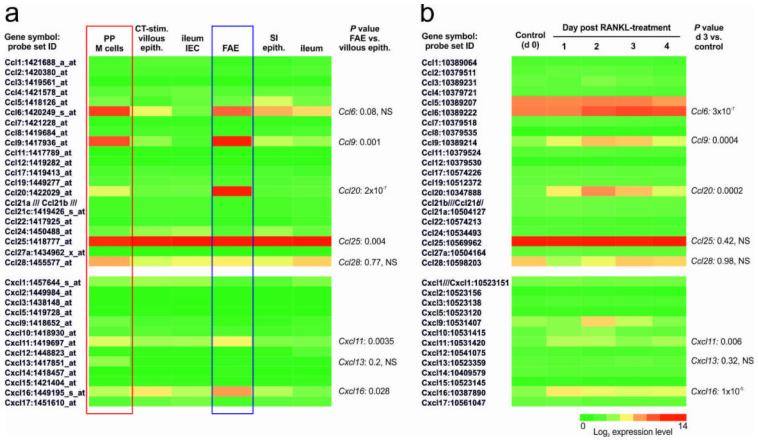

Figure 3.

Retrospective comparison of chemokine gene expression by M cells and the FAE. (a) Heat map showing the mean expression of profile of multiple chemokine-encoding probe sets in samples of Peyer's patch (PP) M cells, cholera-toxin-induced (CT-stim.) villous M cells and ileum intestinal epithelial cells (GSE7838)[25], FAE and M cells[26] and ileum (GSE13908)[68]. These data were performed on Affymetrix MOE430_2 mouse genome expression arrays. Each column represents the mean probe set intensity ($\log_2$) for all samples from each source (n = 2-4). Significant differences between groups were sought by ANOVA. P values for those genes which were expressed significantly ($P < 0.05$) within the FAE and by M cells at levels > 2.0 fold when compared to the villous epithelium are indicated. (b) Effect of RANKL-stimulation on the expression of chemokine-encoding genes in the villous epithelium. These data (GSE37861)[2] were performed on Affymetrix mouse gene 1.0 ST expression arrays (equivalent chemokine-related probe sets are shown). Each column represents the mean probe set intensity ($\log_2$) for all samples from each source (n = 3). P values for those genes which were significantly upregulated > 2.0 fold at d 3 after RANKL-treatments when compared to controls (d 0) are indicated. Representative probe set are shown when multiple probe sets for a gene were present on the arrays. NS, not significant.

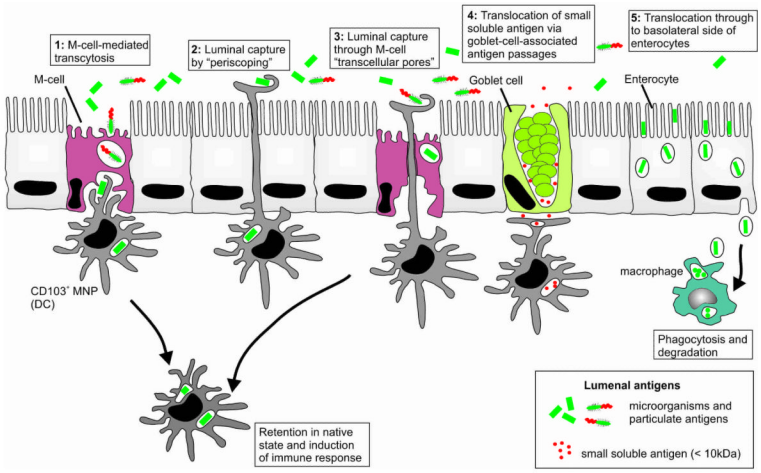

Figure 4.

Potential routes of lumenal antigen sampling in the intestine by epithelial cell subsets and MNP. (**1**) Sampling of gut lumenal microorganisms and macromolecules via M-cell-mediated transcytosis. (**2**) Although the incidence is rare in the steady-state, certain inflammatory stimuli appear to recruit MNP from the lamina propria where they insert their dendrites through the tight junctions between enterocytes to directly sample the luminal contents[108, 109]. (**3**) Another study has reported that MNP may even sample lumenal antigens by extending their dendrites directly through “transcellular pores” in the M cells themselves[7]. (**4**) Mucin secreting goblet cells can also function as passages for the delivery of low molecular weight soluble antigens to $CD103^+$ MNP in the lamina propria[107]. (**5**) Ultrastructural analysis suggests that FAE enterocytes can also acquire certain gut lumenal antigens, and exocytose the intravascular contents of their large late endosomes into the extracellular space of the SED where they are acquired by MNP[104].

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Table 1

Comparison of M-cell-related genes expressed in the villous epithelium in response to RANKL-stimulation, RANKL-stimulated *in vitro* intestinal organoids and *in vitro* M-cell-like differentiated Caco-2 cells.

Gene symbol	Gene title	Up-regulated in villous epithelium by RANKL (Fold Change)[a]	Day of peak expression level	Up-regulated in small intestinal organoids by RANKL *in vitro* (Fold Change)[b]	Up-regulated in M-cell-like cells *in vitro* (Fold Change)[c]
Gp2	glycoprotein 2 (zymogen granule membrane)	46.9	3	56.5	Not expressed
Spib	Spi-B transcription factor (Spi-1/PU.1 related)	31.9	2	18.5	Not expressed
Anxa5	annexin A5	12.7	2	35.5	Unchanged
Ccl20	chemokine (C-C motif) ligand 20	11.3	2	158.8	8
Tnfaip2	tumor necrosis factor, alpha-induced protein 2 (M-Sec)	7.7	2	13.9	Unchanged
Ccl9	chemokine (C-C motif) ligand 9	4.9	2	32.0	n/a
Prnp	prion protein	3.7	2	60.2	Unchanged
Marcksl1	MARCKS-like 1	3.6	1	7.0	Unchanged

[a] These data (GSE37861)[2] were performed on Affymetrix mouse gene 1.0 ST expression arrays. Fold-change in gene expression level at peak day when compared to controls (d 0).

[b] These data were performed on Agilent 4x44K whole mouse genome expression arrays and equivalent gene probe sets were compared (GSE38785)[15]. Fold-change in gene expression when compared to unstimulated control organoids.

[c] Performed on Affymetrix human genome U133 plus 2 expression arrays and equivalent gene probe sets were compared. Fold-change in gene expression by M-cell-like differentiated Caco-2 cells when compared to undifferentiated controls (GSE25330, GSE7745)[115]. n/a; an equivalent annotated probe set was not present on the array.