Review

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The Mechanisms of M-cell Differentiation

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Intestinal M (microfold or membranous) cells are an enigmatic lineage of intestinal epithelial cells that initiate mucosal immune responses through the uptake and transcytosis of luminal antigens. Due to their rarity, the mechanisms of M-cell function and differentiation are poorly understood. To overcome this problem, experimental strategies to enrich for M-cells have been established. Transcriptome analyses have provided valuable insight, especially on the receptors for antigen uptake, and such studies have broadened our knowledge of M-cell function. In another line of investigation, we and others have begun to dissect the molecular pathways of M-cell differentiation. Among them, receptor activator of NF-κB ligand (RANKL) has been identified as an essential factor for M-cell differentiation. We have focused on the M-cell inducible activity of RANKL and have been able to observe temporal transitions during M-cell differentiation by using *in vivo* ectopic M-cell differentiation induced by exogenous RANKL treatment. We have found that the ets-family transcription factor Spi-B is essential for functional maturation of M cells. In the absence of Spi-B, the immune response to *Salmonella* Typhimurium is severely impaired, suggesting that M cells are important for maintaining intestinal homeostasis.

Key words: M cell, Peyer's patch, follicle-associated epithelium, Spi-B

INTRODUCTION

The mucosal surface of the mammalian gut is continuously exposed to a variety of foreign proteins and microorganisms, some of which are potentially harmful to the host. To protect itself from these dangers, the host has evolved specialized organized lymphoid tissue, gut-associated lymphoid tissue (GALT), which includes Peyer's patches (PPs) and isolated lymphoid follicles. GALT is the inductive site for intestinal immunity but is different from other peripheral lymphoid tissues; it lacks afferent lymphatics and instead directly samples mucosal antigens across the epithelial barrier to initiate immune responses. This task is thought to be mainly accomplished by specialized epithelial cells known as M cells (microfold or membranous), which are found in the follicle-associated epithelium (FAE) covering the lymphoid follicles of GALT [1]. M cells have a tremendous capacity for antigen uptake and transcytosis, functions that allow the rapid transport of antigens to underlying lymphoid tissue, especially to antigen-presenting cells. Processed antigens are then presented to T cells, which support cognate B-cell activation, ultimately leading to the generation of plasma cells that produce polymeric immunoglobulin A (IgA) [2]. Thus, M-cell-mediated transport of antigens is an important step in the initiation of mucosal immune responses.

To better understand the biological significance of M cells in host defense, the importance of an M-cell deficient model cannot be overestimated. One plausible approach to achieve such a model would be to inactivate genes critically involved in M-cell differentiation. Although the functional and morphological features of M cells were initially described nearly 40 years ago, many basic questions about M-cell differentiation and function remain unsolved [3–5]. In this review, we introduce the basics of M-cell biology and introduce recent findings concerning M-cell differentiation.

DETECTION OF M CELLS AND IDENTIFICATION OF M-CELL SPECIFIC MOLECULES

For a long time, the detection of M cells depended on morphological analysis using electron microscopy. M cells have characteristic morphological features that set them apart from other subsets of intestinal epithelial cells. They have shorter and irregular microvilli on their

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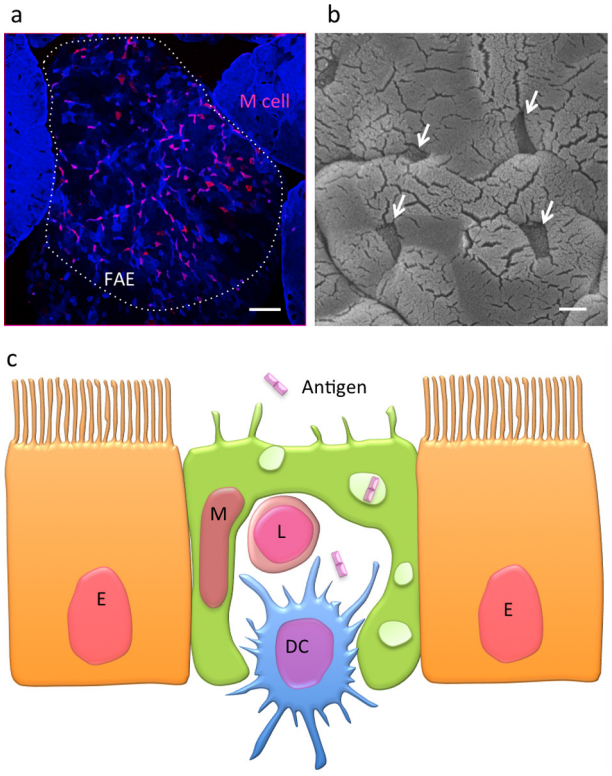

Fig. 1. The features of M cells.

(a) M cells are distributed in the FAE. M cells were visualized with anti-GP2 antibody. The dotted line shows the FAE. Scale bar = 80 µm. (b) Scanning electron microscopic image of M cells. Arrows indicate M cells harboring sparse and irregular microvilli. Scale bar = 3 µm. (c) Illustration of the morphological features of M cells. M cells (M) possess irregular microvilli and form a pocket-like invagination of the basolateral plasma membrane harboring lymphocytes (L) and dendritic cells (DC). The cytoplasm of M cells is thin compared with that of enterocytes (E), resulting in a "membranous" morphology.

apical surface and a pocket-like basolateral invagination of the plasma membrane that houses lymphocytes and antigen-presenting cells (Fig. 1). These morphological features enable the recognition of M cells by electron microscopy and are likely to be conserved in M cells throughout GALT in the small and large intestine, such as isolated lymphoid follicles and colonic patches [6, 7]. In some species, cytoskeletal proteins such as actin, villin, cytokeratin and vimentin are used for the detection of M cells [8–12]. In addition, *Ulex europaeus* agglutinin-I (UEA-I), which binds α-1,2 fucosylated residues, has become a classical marker for murine M cells [13, 14]. Although useful as M-cell markers, these molecules are not uniformly applicable among different species, thus hindering a global understanding of M-cell biology. Several approaches for identifying molecules highly and specifically expressed in M cells have been used to overcome this problem and find universal M-cell markers. We summarized the M-cell markers identified so far in Table 1 [2, 15–22]. Hase et al. established a method to isolate FAE from murine PPs and compared the gene expression profile of these cells with that of the much more abundant villous epithelial cells. Based on this analysis, they found that secretory granule, neuroendocrine protein 1 (sgne-1), encoded by the *Scg5* gene, is selectively expressed in M cells [18]. Verbrugghe et al. also performed a similar type of analysis and reported that annexin A5 is abundantly expressed by murine M cells [15]. Terahara et al. developed an M-cell recognizing monoclonal antibody (NKM 16-2-4) by immunizing a rat with UEA-I$^+$ cells from the murine small intestine and used it to sort out a putative M-cell fraction from murine PPs for gene expression profiling [17, 23]. The identification of such M-cell-specific molecules has enabled us to detect M cells relatively easily by immunohistochemical analysis.

Table 1. The list of published M-cell markers

Name	References
Annexin A5	[15]
CCL9	[16]
GP2	[2, 17]
Marcksl1	[17]
M-Sec	[19]
PGRP-S	[20]
PrP^c	[21]
Sgne-1	[18]
Umod	[22]

ANTIGEN-UPTAKE RECEPTORS EXPRESSED ON THE M-CELL SURFACE

M cells provide an efficient portal through which gut luminal antigens can be transported into the underlying lymphoid tissue. In keeping with this function, M cells have a high capacity for phagocytosis and transcytosis. To identify the molecules involved in these functions, gene expression profiling of M cells as described above has been utilized. Two research groups have independently identified glycoprotein-2 (GP2) as an M-cell-specific molecule because of its prominent expression on M cells [2, 17]. GP2 is a glycosylphosphatidylinositol (GPI)-anchored protein and selectively binds to the type I pili on the outer membrane of certain bacteria, such as *Escherichia coli* and *Salmonella* Typhimurium. In the absence of GP2, uptake and transcytosis of

these bacteria and the subsequent immune response are severely impaired, suggesting that GP2 is an important immunosurveillance receptor for luminal antigens [2].

Cellular prion protein (PrP^c), another GPI-anchored protein highly expressed on M cells, was also identified as an antigen-uptake receptor. PrP-deficient mice have a defect in uptake of the gram-negative bacterium *Brucella abortus* into PPs. Antigen uptake in this case seems to depend on the affinity of PrP for heat-shock protein 60 (Hsp60) expressed by *B. abortus* [24]. Many other proteins in addition to GP2 and PrP have been identified on the M-cell surface. To clarify the molecular mechanisms of antigen uptake, the interaction between these molecules and luminal antigens should be investigated.

M CELLS DERIVE FROM LGR5+ INTESTINAL EPITHELIAL STEM CELLS IN THE CRYPTS

All intestinal epithelial lineage cells, including absorptive enterocytes, goblet cells, Paneth cells (only in the small intestine) and enteroendocrine cells originate from intestinal epithelial stem cells located at the bottom of the intestinal crypts of Lieberkühn. These intestinal epithelial stem cells express the leucine-rich repeat-containing G protein-coupled receptor 5 (Lgr5) [25]. Lineage-tracing studies using transgenic mice expressing a reporter gene (*LacZ*) under control of the *Lgr5* promoter have confirmed that all epithelial cells within the FAE, including M cells, are also derived from Lgr5+ intestinal epithelial stem cells [26]. Different from other epithelial cell lineages, specification into the M-cell lineage from stem cells or transit-amplifying cells, which directly derive from stem cells and rapidly proliferate, has been mainly examined with respect to extrinsic factors from the adjacent microenvironment, as described below.

THE EXTRINSIC FACTORS FOR M-CELL DIFFERENTIATION

Hematopoietic cells (B cells)

In general, M cells are mostly confined to the FAE overlaying the GALT, indicating that immune cells in GALT have a substantial role in M-cell differentiation. In particular B cells have been thought to be important for M-cell differentiation because they are abundantly observed adjacent to M cells [27], and because B-cell-deficient mice have fewer M cells in the FAE than wild-type mice [28]. In addition, cells of the human adenocarcinoma cell line Caco-2 have been reported to convert into M-like cells in the presence of Raji cells, a human B-cell Burkitt's lymphoma cell line [29]. These observations suggest that B cells have the potential to induce M cell differentiation. It is also known that C-C chemokine receptor 6 (CCR6)-deficient mice show a significant decrease in M cells in the FAE [30]. This M-cell reduction has been shown to be due to a significant reduction in the accumulation of a unique $CD11c^{int}$ B cell subset into the subepithelial dome (SED) region of the PP [31]. Of note, however, M cells can be induced in intestinal crypt organoid culture, in the absence of lymphoid cells, upon RANKL treatment [26], suggesting that B cells (including $CD11^{int}$ B cells) are dispensable for M-cell development. Taken together, B cells may support M-cell differentiation indirectly and/or may be required for the maintenance of differentiated M cells once they mature.

Bacterial exposure

Intestinal microbes are also thought to trigger M-cell development. For example, simple relocation of mice kept under specific pathogen free (SPF) conditions to a "dirtier" i.e., more normal, conventional facility increased the M-cell number [32]. In addition, pathogenic bacteria can also affect M-cell development and/or function, e.g., *Streptococcus pneumoniae* in the case of rabbit M cells and *S.* Typhimurium in the case of germ-free mice [33, 34]. A very recent study has demonstrated that the type III secretion system effector protein SopB produced by *S.* Typhimurium activates the transition of enterocytes into M cells [35].

Receptor activator of nuclear factor-κB ligand (RANKL)

Taylor et al. showed that RANKL, which was originally reported to induce osteoclast development, is highly expressed by stromal cells distributed in the SED of the PP [36]. A subsequent study from the same group showed that RANKL has an essential role in M-cell differentiation. Indeed, RANKL-deficient mice have considerably fewer M cells, and exogenous administration of recombinant RANKL restores the number of M cells in these mice. Furthermore, treatment with RANKL can induce ectopic differentiation of the villous epithelium (VE) into M cells in wild-type mice [37].

TRACING M-CELL DIFFERENTIATION AFTER EXOGENOUS RANKL TREATMENT

As there are normally no M cells in the VE, we thought that RANKL-induced ectopic M-cell differentiation would enable us to observe the transition stages in the process of M-cell differentiation. As expected, the expression kinetics of M-cell markers after treatment with RANKL

are distinct; the expressions of myristoylated alanine-rich C kinase substrate like 1 (Marcksl1), CCL9 and GP2 peak after 1, 2 and 3 days, respectively. Furthermore, the localization of these M-cell markers moves from the crypt zones toward the tips of the villi with time after RANKL treatment, with Marcksl1 limited to the crypt-villus junction at day 1 and GP2 expression restricted to the upper part of the villi on day 3. Given that the position of the cells along the intestinal crypt-villus axis reflects their degree of maturation, these observations indicate that Marcksl1 expression is initiated at an early stage of M-cell differentiation, whereas expression of CCL9 and GP2 requires further maturation of the RANKL-induced M cells [16]. Our findings parallel the tempo of expression of these M-cell marker molecules during the physiological development of M cells in ontogeny and provide novel insight with respect to M-cell differentiation.

In terms of the distribution of RANKL-induced M cells in the VE, these cells seem to be similar to villous M-like cells. Actually, it has been shown that villous M-like cells harbor the typical M-cell morphologies and capacity for antigen uptake [38]. To define the features of villous M-like cells, the gene expression profile of villous M-like cells was compared with those of VE or PP M cells. Although villous M-like cells share traits with PP M cells in terms of chemokine expression, typical M-cell markers such as GP2 were not expressed in villous M-like cells [17]. Considering that RANKL-induced M cells mimic the gene expression profile of PP M cells, villous M-like cells are distinct from RANKL-induced M cells.

EXPRESSION OF THE ETS-FAMILY TRANSCRIPTION FACTOR SPI-B DURING M-CELL DIFFERENTIATION

Identification of lineage-specific transcription factors expressed during M-cell differentiation is a key to elucidating the molecular mechanisms of the process. Many transcription factors are known to be involved in the cell-fate decisions made as intestinal epithelial stem cells in the crypt differentiate into one of the recognized types of terminally differentiated intestinal epithelial cells. Atoh1, which is repressed by the Notch effector transcriptional repressor Hes1 [39] is essential for commitment of epithelial progenitor cells into the secretory lineages, including goblet cells, Paneth cells, enteroendocrine cells and tuft cells [40]. Downstream of Atoh1, the specification of the individual secretory cell lineages requires at least one additional transcription factor: KLF4 is required for the maturation of goblet cells [41]; Sox9 is required for the maturation of Paneth cells [42, 43]; and neurogenin 3 is required for the maturation of enteroendocrine cells [44]. We hypothesized that M-cell differentiation also requires regulation by one or more distinct transcription factors. To identify the transcription factor (s) involved in M-cell differentiation, we performed gene expression profiling during RANKL-induced M-cell differentiation. We focused on transcription factors upregulated early during M-cell differentiation and found that the ets-family transcription factor Spi-B is highly expressed in M cells [16]. The expression of Spi-B is conserved in multiple species, including human M cells and in an *in vitro* bovine M-cell model [35], indicating its crucial role in M-cell differentiation and function.

SPI-B IS ESSENTIAL FOR THE MATURATION OF M CELLS

To clarify the role of Spi-B in M-cell development, we examined Spi-B-deficient mice and found that in the PP, GP2+ M cells were completely absent from the FAE, whereas Marcksl1 and annexin A5 were not silenced. Based on the kinetics of appearance of these markers during M-cell differentiation, it seems likely that expression of GP2 requires terminal maturation of M cells. We have therefore hypothesized that the Marcksl1+ annexin A5-positive cells belong to a functionally immature stage in the M-cell lineage. Consistent with this hypothesis, these cells lack typical features of M cells, such as the disorganized microvilli on their apical surface and the pocket-like structure harboring immune cells on their basolateral side. In addition to these morphological abnormalities, M cells in Spi-B-deficient mice exhibited functional defects in the transport of antigens, such as a significant reduction in transport of orally administered *S.* Typhimurium and *Yersinia enterocolitica* to PPs [16].

We also found that Spi-B was expressed in M cells of other organized lymphoid tissues in the gut, such as isolated lymphoid follicles in the small intestine and colonic patches in the colon [16]. To investigate the contribution of Spi-B in non-gut M-cell differentiation, we assessed Spi-B expression in nasal-associated lymphoid tissues (NALT). As we expected, M cells within the epithelium covering (NALT) also expressed Spi-B (unpublished data). These observations indicate that the expression of Spi-B is also conserved throughout mucosal surfaces.

THE LACK OF FUNCTIONALLY MATURE M CELLS IMPAIRS MUCOSAL IMMUNE RESPONSES

The severe reduction in transport of pathogenic bacteria into PPs in Spi-B-deficient mice raises the possibility that bacteria-specific immune responses are impaired in the absence of functionally mature M cells. To examine this possibility, we evaluated the *S.* Typhimurium-specific immune response by using adoptive transfer of T cells from an *S.* Typhimurium-specific T-cell receptor transgenic mouse, SM1. In the absence of Spi-B, T-cell activation by orally administered *S.* Typhimurium and subsequent proliferation were significantly impaired [16]. These findings indicate that the transport of antigen via M cells is essential for inducing normal mucosal immune responses (Fig. 2), thus possibly resolving the issue that has been debated for many years.

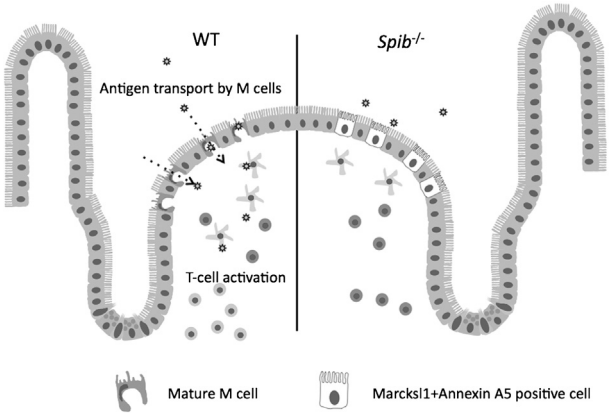

Fig. 2. Absence of Spi-B impairs bacterial translocation and subsequent immune responses.

Spi-B-deficient (*Spib*$^{-/-}$) mice lack GP2+ mature M cells overlaying the PPs. This significantly decreases the translocation of *S.* Typhimurium into the PPs, resulting in the impairment of *S.* Typhimurium-specific T-cell activation.

SPI-B-INDEPENDENT REGULATION OF M-CELL DIFFERENTIATION

Apart from our study, Sato et al. more recently reported that there is a population of PP M cells that arises independently from Spi-B, as they found that *Alcaligenes* spp., which are Gram-negative bacteria, were taken up into PPs in the absence of Spi-B [22]. Another research group demonstrated that Spi-B transduction is insufficient to give rise to M cells in the intestinal epithelial cells [26]. In addition, we have identified Marcksl1+ annexin A5-positive cells in Spi-B-deficient mice. Taken together, other factors are likely required for the development of M cells. In support of this hypothesis, it has been reported that the noncanonical NF-κB pathway evokes Spi-B in response to RANKL [35]. This pathway may induce other transcription factors as well as Spi-B for M-cell differentiation.

PERSPECTIVES OF FUTURE M-CELL STUDIES

The main functions of M cells are antigen uptake and transcytosis. As described above, a few receptors for specific antigen uptake have been identified, and the molecular basis for antigen uptake has been partially clarified. On the other hand, there is much less information with respect to the transcytosis of antigens. Recently, Asai et al. used the *in vitro* M-like cell culture derived from the Caco-2 cell system to demonstrate that the SRC family tyrosine kinase HCK is involved in transcytosis of antigens [45]. They also suggest that Spi-B regulates HCK-dependent transcytosis. Other than HCK, it seems likely that multiple molecules regulated by Spi-B are involved in transcytosis. These initial studies suggest that determining Spi-B targets in M cells may provide important new insight into the mechanisms of transcytosis. Recently, a novel *in vitro* M-cell differentiation model has been established by using organoid cultures from intestinal crypts [26]. These *in vitro*-derived M cells exhibited similar gene expression profiles as *in vivo* M cells and may allow for advanced analyses of M-cell biology.

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