Title: Functional differences between M cells and enterocytes in sampling luminal antigens

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Abstract:

Oral delivery of agents such as vaccines offers a number of significant advantages over parenteral routes, yet only a small number of oral vaccines are routinely available today. The small intestine contains lymphoid aggregates that are overlaid by M cells. These aggregates are part of the gut-associated lymphoid tissues and are important for determining host responses to particulate antigenic material within the small intestine. Differentiating the receptor requirements for M cell uptake and transcytosis of bacterial antigen from the intestine has progressed although the specific signalling mechanisms that initiate antigen uptake and specifically target antigen to these cells is still relatively unknown. Microbial pathogen-associated molecular patterns (PAMPs) are recognised by the innate immune system through pattern recognition receptors (PRRs) either through direct receptor-bacterial ligand or endogenous adaptor-bacterial molecule interactions. PRRs on the surface of M cells that have been identified as important in antigen transcytosis include toll-like receptor-4, platelet activating factor receptor and $\alpha 5\beta 1$ integrin. A number of these PRRs are also found on neighbouring enterocytes and therefore the pathways signalled by receptor-ligand binding may differentially trigger different transduction pathways. Elucidation of these signalling pathways may assist in the design of effective oral vaccines that target the gut-associated lymphoid tissue.

Introduction

Exposure to microbial agents and other environmental antigens occurs mostly through mucosal surfaces. Thus it is logical that targeting the first exposure to the mucosa would be a very effective vaccination strategy. Induction of mucosal immune responses occur best through priming of mucosal associated lymphoid aggregates, which selectively sample the milieu bathing the mucosal surface. The ability to oral deliver agents such as vaccines offers a number of significant advantages. Among the practical advantages is that needles are unnecessary, thus the cost and hazard associated with their use and the need for trained personnel for vaccine administration are reduced, improving the potential sustainability of global immunisation programs as required in the current WHO global immunisation strategy [1]. Excellent examples include the oral polio vaccine program which is one-tenth the cost of the injectable polio program [2] and the oral cholera vaccines which are more efficacious (although still suboptimal), cost effective and safer when compared to the previous injectable vaccine [3].

Despite such advantages, only a small number of oral vaccines are routinely available today, since oral vaccine trial results have often been suboptimal and highly variable at best. Most approaches rely on delivery of large amounts of antigen to the small intestine in the hope that there will be sufficient random uptake to induce an effective immune response. Along the small intestine the lymphoid aggregates are overlaid by M cells that determine how the host will subsequently respond to particulate antigenic material in the small intestine. Differentiating the receptor requirements for M cell uptake and transcytosis of bacterial antigen from the intestine has progressed in recent years, however knowledge about the specific signalling mechanisms that initiate antigen uptake and specifically target antigen to these cells is still relatively unknown [4-7].

The significance of understanding M cell receptors and the signalling mechanisms in order to improve antigen delivery systems has far reaching benefits. The ability to directly target M cells would assist the development of optimal delivery technologies of a number of potential oral viral, bacterial and synthetic vaccines. Such a development would enable optimization of oral

immunization schedules for more consistent and predictable immune responses. In addition to potential vaccine developments, such knowledge has significance for a number of human disease conditions. Knowledge of the various pathways induced by different microbial and environmental antigens could potentially lead to the design of immune modulation strategies for autoimmune diseases and inflammatory conditions of the bowel. Other potential applications include how microbes may modify enterocyte gene expression to favour the development of a carcinoma, such as caused by *Helicobacter pylori* [8], or interact with receptors on the apical surface of M cells to breach the mucosal barrier, such as in the case of the Human Immunodeficiency Virus [9].

Transcytosis by M cells

One of the most fundamental gaps in our knowledge of induction of immune responses at the mucosal surface relates to how M cells sample the luminal milieu, the intracellular biochemical processes involved in transcytosis of the sampled material and finally, how the material is presented to the underlying lymphoid apparatus. Until fairly recently, research into the way M cells in the intestine sample antigens had been hampered due to the lack of appropriate *in vivo* and *in vitro* experimental models. The relatively low numbers of M cells in the gastrointestinal tract (GIT) and the inter-species variability in surface receptors have made *in vivo* systems difficult to establish [10].

Our laboratory further developed an *in vitro* model that was originally described in 1997 [11] into an experimental system which was biochemically and physiologically verified; was reliable and reproducible; produced quantifiable data [5]; and provided a sufficient proportion of M cells in the cocultures (30%) to enable study of their function, surface receptors and intracellular biochemical pathways. This work verified both microscopically and by the presence of cell characteristics known to be specific to intestinal M cells the proportion of M cells within Caco-2 cell co-cultures *in vitro* [5].

M cells are structurally dissimilar to their neighbouring enterocytes with fewer lysosomes, more mitochondria, a lack of mucous glycocalyx covering their surface and a basolateral extracellular

space that surrounds interdigitated lymphoid cells. They have a different dominance and pattern of cell surface receptors compared with enterocytes [4, 12, 13]. Unlike enterocytes, M cells are adept at transcytosing material from their apical surface to the basolateral membrane. This is thought to occur by mechanisms such as endocytosis of clathrin-coated vesicle, an actin-dependent phagocytosis and engulfment by a fluid-phase pinocytosis or macropinocytosis [14]. Studies that focus on the specificity of antigen transcytosis have provided evidence that human M cells utilise receptor dependent transport mechanisms for both viral [9] and bacterial uptake [5]. It is now recognised that transcytosis is a receptor-mediated process performed by the M cells.

The study of the way different bacteria, such as the respiratory pathogen nontypeable *Haemophilus influenzae* (NTHi) and the gut commensal/pathogen *Escherichia coli*, identified differences in transcytosis between normal intestinal enterocytes and M cells. These bacteria were poorly transcytosed by Caco-2 monocultures (a measure of transfer due only to enterocytes) but were actively transcytosed in M cell co-cultures (increase was associated with the activity of the M cells) [4]. In contrast, the bacterium *Pseudomonas aeruginosa* showed no differentiation in transcytosis in the different cell cultures, being similarly endocytosed/transcytosed and to a more limited degree by both cell types (unpublished data). *P. aeruginosa* can bind to toll-like receptor (TLR)-5 on polarised Caco-2 cells and villus enterocytes [15] whereas NTHi cannot [16] and such a difference may explain the variations in transcytosis between the bacteria. Such results are not an artefact of concentration because the studies were able to show that the bacterial uptake was saturable [4] and using an *ex vivo* model of murine isolated intestinal segments with Peyer's patches (in comparison to villus), the same findings occurred for NTHi and *P. aeruginosa*.

Pattern recognition receptors

Prokaryotes contain molecular motifs that are unique to microorganisms. These motifs, known as common microbial pathogen-associated molecular patterns (PAMPs), are recognised by the innate immune system through pattern recognition receptors (PRRs) either through direct receptor-bacterial ligand or endogenous adaptor-bacterial molecule interactions [17]. For example, TLR-4, in

association with CD14 and MD-2, recognises lipopolysaccharide (LPS) expressed by Gram

negative bacteria [18]; TLR-2 recognises lipotechoic acids expressed by Gram positive bacteria

[19]; platelet-activating factor receptor (PAFR) binds to the phosphorylcholine moiety of bacterial

surface lipooligosaccharide (LOS) molecules [20]; and, $\alpha 5\beta 1$ integrin binds, through endogenous

fibronectin, to fibronectin-binding proteins expressed by many bacteria [21].

Adherence is the first step in the antigen-uptake process and it is believed to be mediated by

PRRs and lectin-like microbial adhesins that can bind specific glycoprotein or glycolipid molecules

on the apical surface of cells [22]. These binding sites include glycosphingolipid asialo-GM1, O-

linked sialylglycoproteins and other galactosyl- and sialic acid containing oligosaccharides, most of

which are distributed widely on intestinal epithelial cells [23].

While very little is known about receptors that are specific to human M cells, there are

differences in the specificity of antigen uptake by M cells versus enterocytes that appears to be due

to the location and concentration of most receptors, rather than merely their presence on a particular

cell type [4]. Such a difference can be demonstrated by the distribution of the receptor $\alpha 5\beta 1$

integrin, which is found on the lateral and basolateral surfaces of enterocytes but only on the apical

surfaces of M cells [5, 12]. $\alpha 5\beta 1$ integrin assists preferential uptake by M cells *in vivo* of

enteropathogenic organisms, such as *Yersinia spp*, via a receptor/bacterial invasion interaction [24-

26].

Research in our laboratories using the *in vitro* M cell model and confirmation in *ex vivo* isolated

murine intestinal segments demonstrate that M cells, but not Caco-2 cell monocultures, uptake and

transcytose NTHi by specific receptor driven mechanisms. TLR-4, PAFR and $\alpha 5\beta 1$ integrin are all

involved in mediating uptake [4]. Not only does the pattern of expression of these receptors differ

on M cells and enterocytes, experiments from our laboratories and recent experiments by Gullberg

[12] demonstrated significantly reduced bacterial translocation using specific receptor inhibition

studies [4].

This work has identified receptors that perform a role in transcytosis within M cells located on

the luminal surface of intestinal Peyer's patches (Fig 1). These receptors are associated with several different cell-signalling pathways, however, how these pathways function, whether synergistically or independently to facilitate transcytosis is currently unknown.

Role of α5β1 integrin on the apical surface of M cells

Redistribution of α5β1 integrin to the apical surface is an important factor in enterocyte to M cell transformation and inhibition of α5β1 binding significantly inhibited transcytosis [4, 12]. Thus, signalling pathways that are activated by this receptor may be very important for M cell function. α5β1 integrin is believed to activate several signalling pathways (Figure 1) through its two-subunit domains. Whether these pathways function in M cells or how they may contribute to the transcytotic capability of M cells is unknown. α5β1 integrin is normally associated with the basal-lateral surfaces in normal enterocytes and other cell types. The functional significance in triggering intracellular signalling pathways associated with the relocation to the apical surface on M cells compared with the functional roles associated with basal-lateral expression is unknown.

Most of the limited amount of information on signalling through the α5 and β1 domains is known as a result of studies to understand other cellular phenomena. Activation of the α5β1 signal involves the Rho family of small GTP binding proteins and cell signalling via phosphatidylinositol-3 kinase (PI$_3$K)/Akt pathway, the focal adhesion kinase (FAK) pathway activating the anti-apoptotic protein Bcl-2 and/or the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) IV pathway [27-31]. In Caco-2 cells, the β1-integrin subunit, but not the α5 domain, activated mitogen activated protein kinase (MAPK), p38 and FAK in response to cyclic strain [32]. The results of studies presented in the literature suggest that the role of expression of integrins, and in the case of M cells α5β1-integrin, is complex.

Expression differences between M cells and enterocytes

A recent study analysed changes in gene expression between Caco-2 and M cell-like cells generated using the Raji B cell cocultures [33]. This study found 180 differently transcribed genes, mainly associated with cell differentiation, endocytosis and intracellular transport, surface

molecules and inflammatory responses. The most significant finding reported was the identification of galectin-9 (gal-9) on the surface of M cells. Galectins are a highly diverse family of β-galactoside-binding lectins usually located in the cytoplasm, nucleus and sometimes the extracellular matrix. Gal-9 has been identified as a cellular maturation marker, as a result of signalling through p38 MAPK [34], with affinity for both branched N-glycans and repeated oligolactosamines [35] with lactose as a natural ligand for gal-9. The identification of gal-9 on the surface of M cells may be associated with some of the binding differences observed with the LOS mutants of NTHi [4]. Although the genes associated with the epitopes on NTHi LOS are phase variable, Lic2A adds the terminal β-galactose to the lactose of LOS [36]. This evidence, in combination with our observations that a phosphorylcholine (PC) mutant NTHi strain [4] was poorly transcytosed, indicates that a role for specific LOS structures in M cell targeting is also worthy of further study.

The different receptors and receptor-mediated signalling pathways may act synergistically during M cell transcytosis. Studies in our laboratories found that when TLR-4 and the PAF receptor were blocked there was a reduction in the ability of the M cell co-cultures to transcytosis the bacterium, NTHi [4]. NTHi expresses a bacterial LOS that has been shown to mediate uptake in other cells via the PAF receptor [20, 37]. PAF is released by various cells and may have a regulatory role in the GIT as it is known to increase the mucosal permeability of the intestine [38]. PAF utilises tyrosine phosphorylation of E-cadherin and cytoskeletal rearrangement of enterocytes to mediate the movement of macromolecules across the mucosal barrier [38]. Bacterial LOS binding to the PAF receptor may activate the calcium/inositol phosphate (Ca/IP$_3$) pathways through the G-protein G$_{qi}$. Activation of the PAF receptor by bacterial LOS or LPS may not be straightforward. One study has shown that LPS, and not PAF, activates transcription factor NFκB in the intestine [39]. Studies in our laboratory have shown that the GIT commensal *E. coli* (LPS+) was preferentially transported by M cell co-cultures both *in vitro* and in mouse *ex vivo* studies, and that antagonists to both the PAF receptor and α5β1 integrin were able to block transcytosis of both NTHi (LOS+) and *E. coli*. The

lack of transport difference between enterocyte and M cell cultures for *P. aeruginosa* (LPS+) was not necessarily specific for differences between LOS (NTHi) and LPS. Other studies have also reported similar differences with bacterial transcytosis. *Salmonella typhimurium* was preferentially transported whereas enteropathic *E. coli* was not [40], whereas uropathogenic *E. coli* IH11128 specifically entered via $\alpha5\beta1$-integrin [41] and *Listeria monocytogenes* transcytoses through M cells independent of the action of classical virulence factors [42].

Conclusion

There is now a strong body of evidence that bacterial ligands bind to cellular receptors on M cells to initiate transcytosis. Binding to cellular receptors initiates biochemical pathways specific to that receptor, combinations of receptors and/or co-stimulatory molecules act to initiate the subsequent cellular events. Existing evidence supports the hypothesis that it is the triggering of specific pathways that may distinguish the outcome of transcytotic antigen uptake and subsequent presentation to lymphoid tissue. A number of receptors on human M cells are also found on neighbouring enterocytes and therefore the pathways signalled by receptor-ligand binding may differentially trigger different transduction pathways. Two key differences between enterocytes and M cells is the redistribution of $\alpha5\beta1$ integrin to the apical surface [5] and the expression of gal-9 [33]. Further studies are needed that focus on the pathways that are activated or suppressed during M cell transcytosis. The select features of M cells identified by the presence/absence and relative importance of specific signal transduction pathways associated with an enhanced capacity to transcytose bacteria will give insight into the cellular mechanisms in the gastrointestinal tract that allow antigen sampling and priming of the immune system resulting in prevention of disease.

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Figure Legend

Figure 1. Schematic representation of the luminal surface of an intestinal M cells showing the apically expressed PRRs that recognise microbial PAMPs. Downstream signalling pathways known to be associated with activation of these PRRs are shown. Whether these pathways are activated in M cells during transcytosis of bacteria is currently unknown.

Figure 1.