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Author
Wang, Jing

Publication Date
2011

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Gene Regulation in M Cell Lineages: *in Vitro* and *in Vivo*

A Dissertation submitted in partial satisfaction of the requirements for the degree of

Doctor of Philosophy

in

Biomedical Sciences

by

Jing Wang

June 2011

Dissertation Committee:

Dr. David Lo, Chairperson
Dr. Emma Wilson
Dr. Frances Sladek
The Dissertation of Jing Wang is approved:

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                      Committee Chairperson

University of California, Riverside
Acknowledgements

I'd like to thank Dr. David Lo, who taught me what I needed to know (including cooking recipes) and was endlessly enthusiastic about science and research as well as endlessly supportive.

My graduate advisor, Dr Monica Carson, thanks for the technical guidance, and for taking care of what needed to be done with the graduate program. Dr. Carson and Dr. Lo made me feel like part of a family.

I'd also like to thank my committee members, Dr Emma Wilson, Dr Frances Sladek and former committee memeber Dr Kathy Defea, who gave me crucial guidance along the way.

My colleagues in the lab, who made my five-year PhD course so colorful. Without their laughter and support, this thesis would not be possible. Veronica Gusti. Thanks for helping me with my experiment when I needed it. And thanks for your delicious food. With her help, I gained knowledge and weight. Gracie Hsieh. We grew up together in the lab, although again, some of that growing was food-related. Gracie also throws a great wedding. Kaila Benett. Thank you for correcting all my grammar mistakes. Without her help, I probably would make more English mistakes. Holly Eckelhoefer. You took care of all the lab stuff and made sure I got whatever I needed in the lab. Also, you taught me all the important U.S. holiday traditions, including the “singing” Halloween socks. Mary Stover-Hamer. You taught me how to take electron microscopy pictures, without which people, terrifyingly, would have had to rely on my
writing to understand my thesis. And you made sure I didn’t miss any pretty dog studio pictures. Nancy Appleby and Witney Carter in the lab, who helped me with the mice.

I’d also like to thank my friends from outside the lab for their support and company. Wei Wu and Xiaopei Cui. You guys made the U.S. feel like home, and you are my sisters. Robin Clark, you are my first American friend. You always offered help, and you always told me what you honestly thought, the way a friend should.

Jeff Beresford-Howe. You always think I am the best and were supportive no matter what I did. I’m so glad I missed my flight to San Francisco that day. I love you.

And most of all, I'd like to thank my parents, Huiping Fu and Jiacai Wang, who have loved and supported me from start to finish of this long, strange trip.
ABSTRACT OF THE DISSERTATION

Gene Regulation in M Cell Lineages: *in Vitro* and *in Vivo*

by

Jing Wang

Doctor of Philosophy, Graduate Program in Biomedical Sciences
University of California, Riverside, June 2011
Dr. David Lo, Chairperson

M cells are specialized epithelial cells that assist immune surveillance by transcytosis of particles and antigens to underlying lymphoid tissues. So far, four M cell phenotypes have been identified in airways and intestines (NALT, Peyer’s patch, respiratory and villous M cells). However, the mechanism of M cell differentiation is poorly understood, as well as the relationships between different M cell subtypes.

To address these issues, we treated human (Caco-2BBe) and rodent (IEC-6) intestinal epithelial cell lines with lymphotoxin beta receptor (LTβR) and TNF receptor (TNFR) agonists. Treated cells were then studied for regulation of genes previously shown to be associated with M cell and Follicle-Associated Epithelium (FAE). Moreover, we used a transgenic reporter mouse strain to follow the development of M cell subsets *in vivo*, and examined the de novo induction of M cells by cholera toxin.

We found that LTβR and TNFR agonists can induce transcription of FAE specific genes (*Ccl20* and *Lamb3*) as well as rodent M cell specific genes such as *Sgne-1/Scg5*, *Cldn4*, and *Gp2* in Caco-2 BBe cells and IEC-6 cells. These cytokines have distinct but
complementary effects; TNFR agonists induced FAE specific genes, while the LTβR agonist induces M cell specific genes. Combination usage of both cytokines showed additive induction of the FAE-associated Ccl20, Lamb3 and surprisingly the induction of CD137/Tnfrsf9. Functionally, cytokine treatment led to both the reorganization of microvilli as well as Claudin-4 redistribution.

With the help of the reporter transgenic mouse model, we found that M cells overlying intestinal Peyer’s patches and Nasal Associated Lymphoid Tissue (NALT) have distinct developmental origins, yet show convergent phenotypes, such as expression of a peptidoglycan recognition protein-S (PGRP-S) reporter in vivo. By contrast, though Peyer’s patch and Villous M cells are derived from intestinal crypt stem cells, their phenotypes are clearly distinct: Peyer’s patch M cells form an intimate M cell-dendritic cell functional unit while Villous M cells do not engage with underlying dendritic cells. Though B lymphocytes are thought to play a critical role in M cell function by forming a basolateral pocket and possibly signaling through CD137, initial commitment to M cell lineages is B lymphocyte- and CD137-independent. In addition, cholera toxin can cause rapid induction of new M cells in the airway and intestines without cell division, suggesting transdifferentiation from mature epithelial cells. cholera toxin-induced M cells exhibit the local M cell phenotype without evidence for mixed phenotypes. Finally, different M cell phenotypes display distinct properties; intestinal Peyer’s patch M cells are more efficient than Villous M cells in the uptake of Salmonella.

Our studies show that LTβR and TNFR agonists induce many features associated with M cell differentiation, and suggest that these agonists may be involved in secondary
lymphoid tissue development \textit{in vivo}. Additionally, we confirmed that the differentiation of M cells \textit{in vivo} toward strictly defined phenotypic subsets, and is consistent with functional specialization.
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<th>Description</th>
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<tbody>
<tr>
<td>BALT</td>
<td>Bronchus associated lymphoid tissue</td>
</tr>
<tr>
<td>CALT</td>
<td>Conjunctiva associated lymphoid tissue</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DsRedE2</td>
<td>DsRed Express2</td>
</tr>
<tr>
<td>EdU</td>
<td>5-ethynyl-2'-deoxyuridine</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAE</td>
<td>Follicle associated epithelium</td>
</tr>
<tr>
<td>Igh-6</td>
<td>Immunoglobulin heavy chain 6</td>
</tr>
<tr>
<td>IL-7R</td>
<td>Interleukin-7 receptor</td>
</tr>
<tr>
<td>LT</td>
<td>Lymphotoxin</td>
</tr>
<tr>
<td>LT-α</td>
<td>Lymphotoxin α</td>
</tr>
<tr>
<td>Lti</td>
<td>Lymphoid tissue inducer cells</td>
</tr>
<tr>
<td>LTβR</td>
<td>Lymphotoxin β receptor</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal associated lymphoid tissue</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>NALT</td>
<td>Nasal associated lymphoid tissue</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>PGRP</td>
<td>Peptidoglycan recognition protein</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor activator of NF-κB Ligand</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>Sgne-1</td>
<td>Secretory granule neuroendocrine protein 1</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscope</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll like receptors</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNFR associated factor</td>
</tr>
<tr>
<td>UEA-1</td>
<td>Ulex europaeus agglutinin-1</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction
1.1 Mucosal immunity

Our body constantly interacts with the outside world via our skin and mucosal surfaces, which line the digestive and urogenital tracts and the airways. The mucosal surfaces take in and expel substances and protect against damage to the body. However, they do this while remaining vulnerable to microorganisms, since there is only a single layer of epithelial cells. To combat this vulnerability, it is critical to engage in continuous and quick detection of potentially harmful agents and to rapidly deploy mechanisms for neutralizing or destroying the agents. Besides the natural barrier constituted by mucosal epithelial cells and other non-specific mechanisms such as the presence of mucus and digestive enzymes, specific protective effects usually come from highly organized mucosa-associated lymphoid tissue (MALT). MALT is further identified by location, e.g., gut-associated lymphoid tissue (GALT), nasal-associated lymphoid tissue (NALT), bronchus-associated lymphoid tissue (BALT) and conjunctiva associated lymphoid tissue (CALT).

Functional compartments of MALT include the overlying follicle-associated epithelium (FAE), lymphoid follicles, the interfollicular region and the subepithelial dome region. The FAE is separated from the follicles by a subepithelial dome region that contains lymphocytes, macrophages, and dendritic cells (DC). Germinal centers found inside the follicles contain mainly B-cells, but also follicular DCs and a few CD4+ T-cells. The interfollicular region has CD4+ T-cells, macrophages, DCs, a few B-cells and plasma cells, and high endothelial venules (Fig 1-1).
The initiation of antigen-specific immune responses occurs at various MALT sites that contain Membranous (or “Microfold”) cells, also called M cells. M cells constantly and selectively sample and transcytose antigens and microorganisms, presenting them from the lumen to the B and DCs in the follicle. This in turn leads to antigen-specific T-cell activation, B-cell IgA class switching and clonal expansion. After that, the activated T- and B-cells leave the lymphoid tissue and regional lymph nodes via the lymphatic system and enter the blood stream, then migrate to other distant mucosa (such as the lamina propria), where they leave the blood, differentiate into plasma cells and produce antigen-specific IgG and IgA. Secretory IgA is then secreted across the mucosal epithelium and leads to neutralization, agglutination and/or opsonization of the microorganisms.

1.2 M cells

1.2.1 What is an M cell?

In 1972, Robert Owen first described M cells by scanning electron microscopy (SEM) using human lymphoid follicles. Owen noted a subset of shorter cells that did not have closely packed microvilli like the enterocytes surrounding them; instead, irregular and short microfolds could be seen at the apical surface. According to the cell morphology revealed by SEM, these cells were named ‘microfold cells’, so called M cells\(^2\). However, at that time, it was not known what these cells were, and what their functions might be.
After their discovery in human Peyer’s patch, M cells were observed in many other species, including mice, rats, rabbits, guinea pigs, pigs, bovines and chickens. Although their microfold structure is not identical to that found in humans, they all have short and irregular microvilli on the apical surface. Moreover, all of them have a basolateral pocket, which is formed by invagination of the basolateral surface of the M cell. B cells, DCs, and sometimes T cells are accommodated inside the pocket. Since these specialized cells could not be seen by light microscopy, and because they separate underlying lymphoid tissue from the lumen as a membrane might, they subsequently came to be known as “membranous Cell”, further establishing the name of “M cell”.

M cells were mainly observed residing in the FAE covering the follicles at various MALT locations. After being discovered in Peyer’s patch in the gut, M cells were also found at all the other locations of GALT, including isolated lymphoid follicles, lymphoid tissue in the caecum, colon and rectum. Moreover, M cells were found on villi, at a very low incidence, as well as in NALT, BALT and CALT. In humans, who do not have NALT, M cells are also located in the tonsils. The relative abundance of M cells in vivo depends on the species and the locations of MALT. In mice, M cells comprise nearly 8% of Peyer’s patch epithelial cells but only 1% of intestinal epithelial cells; in rabbits, M cell account for as much as 50% of Peyer’s patch epithelium number. During pathological conditions such as infection and toxicity, M cell numbers can increase to approximately 20 to 30% in Peyer’s patch in mice. These M cells are involved in initiating the mucosal immune protection response but also important in tolerance of food antigens and commensal bacteria that live in the body.
M cells exhibit features distinct from neighboring epithelial cells which are due in part to their transcytosis function. The apical surface of M cells lack overlying mucus and do not have microvilli or cilia. This makes microorganisms and antigens more accessible to M cells. Moreover, the basolateral surface of M cells is invaginated to form a unique pocket where B and DCs reside. This can shorten the distance for M cell transcytosis. The low levels of digestive enzymes such as alkaline phosphatase found in M cells suggest that digestion and absorption of luminal contents are not a major function of M cells. Finally, the lack of lysosomes and reduced activity levels of acid phosphatase inside the cytoplasm of M cells avoid digestion of antigens and microorganisms and further allows particles to be transcytosed without significant degradation.

1.2.2 M cells from different sites

As previously discussed, M cells are mainly found in the FAE covering the follicles in lymphoid-associated tissue at various mucosal sites, including GALT and NALT. M cells can also be found on villi. Recently, Kim et al found a new subset of M cells in murine nasal passages that were capable of taking up respiratory pathogens. This new subset of M cells, respiratory M cells, was found in the single-layer epithelium that covers some regions of the nasal cavity. Regardless of location, M cells share common characteristics: they bind the Ulex europaeus agglutinin-1 (UEA-1); they have brush borders at the apical surface; and they have a readily apparent intraepithelial pocket containing mononuclear cells under TEM. However, although nearly all M cells share similar morphology and perform similar functions in immune surveillance, different M
cell populations have distinct gene expression profile and acquire unique functions as they encounter differing microorganisms.

While M cells from NALT and Peyer’s patch share the same function in mucosal immunity, subtle differences exist. Typically, NALT-targeted immunization induces antigen-specific immune responses in the respiratory\textsuperscript{27,28} and reproductive tracts\textsuperscript{29,30}, while Peyer’s patch-targeted immunization raise a protective immune response in the gastrointestinal tract\textsuperscript{31,32}. This may correlate with activation of B cells with different homing receptors. High-level expression of CCR10 and $\alpha_4\beta_1$-integrin IgA-committed B cells are found in nasally immunized mice\textsuperscript{33} and high level expression of CCR9, CCR10, as well as $\alpha_4\beta_7$ and $\alpha_4\beta_1$-integrins IgA-committed B cells, are found in Peyer’s patch-targeted immunization\textsuperscript{34}. Moreover, NALT and Peyer’s patch have distinct features of organogenisis. NALT tissue genesis begins after birth\textsuperscript{35}, whereas that of Peyer’s patch commences during the gestational period\textsuperscript{36}. Cytokines that contribute to NALT and Peyer’s patch development are also different. Although CD3$^+$CD4$^+$CD45$^+$ cells function as inducer cells in both Peyer’s patch\textsuperscript{37} and NALT\textsuperscript{35} organogenesis, the lymphotoxin $\beta$ receptor (LT$\beta$R) and interleukin-7 receptor (IL-7R)-mediated tissue-genesis program is only crucial for the formation and development of Peyer’s patch\textsuperscript{8,38,39}, but independent of NALT differentiation\textsuperscript{35}. All these differences in function and development between Peyer’s patch and NALT indicate that Peyer’s patch M cells and NALT M cells are also different from each other in origins, biological functions and factors involved in differentiation, which may be due to their anatomically and environmentally distinct locations. For example, instead of a rapid turnover rate and a crypt stem cell origin in
Peyer’s patch, airway epithelial cells are generated from a dispersed population of basal cells, and have half-lives up to weeks or months\(^4^0\).

Intestinal villous M cells are also different from Peyer’s patch M cells. Under normal circumstances, only a few villous M cells can be found on some villi (typically, one in 50 villi), either clustered or individually\(^1^6\). Their locations are usually closer to the upper half of the villus, suggesting the influence of microflora. During infection or while with stimuli, such as cholera toxin or pathogens, the numbers of villous M cells can greatly increase. As with other M cells, villous and Peyer’s patch M cells share the same features. It has been shown that villous M cells alone can induce an Ag-specific IgG response in Peyer’s patch-deficient mice, but their effect on IgA response is still unknown. Factors involved in villous M cell development are different from those in Peyer’s patch M cells. In LT-\(\alpha\) Knockout mice, where Peyer’s patch and FAE are missing, villous M cells can still be found, suggesting that development of villous M cells is independent of lymphotoxin \(\alpha\) (LT-\(\alpha\)) signal\(^4^1\), which contrasts with Peyer’s patch M cells. Comprehensive gene expression profiling of fluorescence-activated cell sorting (FACS)-sorted Peyer’s patch M cells, cholera toxin-induced villous M cells and intestinal epithelial cells\(^4^2\) shows that Peyer’s patch M cells share some but not all genes with both cholera toxin-induced villous M cells and intestinal epithelial cells\(^4^2\), indicating villous M cells and Peyer’s patch M cells are similar to each other to some extent, but still different. This may relate to different functions in vivo, such as the ability of transcytosing different types of bacteria. Since villous M cells usually appeared with
various stimuli, it may only respond to the pathogenic microorganisms, but not the commensal bacteria.

Respiratory M cells are less well understood. It has been shown that, as with villous M cells, respiratory M cells can be induced to increase in numbers. With the inhalation of respiratory pathogens such as Group A Streptococcus, cluster formation of respiratory M cells can be seen. Moreover, respiratory M cells alone can induce Ag-specific IgG and IgA production\(^3\). The gene expression of respiratory M cells has not yet been studied. Analogous to the difference between villous M cell and Peyer’s patch M cells, respiratory M cells and NALT M cells require different factors for development. In NALT deficient, binding/differentiation 2 KO mice, respiratory M cells can still be found. The frequency of their occurrence is similar to that of their littermate, \(\text{Id}2^{+/−}\) mice which have NALT \textit{in vivo}. This suggests that, respiratory M cells, like villous M cells, act as alternative entry site for various pathogens. And as a supplement, when necessary, they can be induced and trigger an alternative mucosal immune response.

\textbf{1.2.3 M cell associated endocytosis and transcytosis}

The most important function of M cells is to transcytose particles from the lumen to the DC and B cell inside the follicle. M cells can engulf various targets, including bacteria\(^4\), bacteria\(^5\), bacteria\(^6\), and virus\(^7\), as well as noninfectious particles such as latex beads\(^8\), microparticles\(^9\) and horseradish peroxidase\(^10\). For M cells to endocytose and transcytose various particles, binding seems to be the initial step (Fig.1-3). Experiments show that, tracer proteins that adhere to the apical membrane of M cells can be transcytosed to the baso-lateral pocket at least 50 times more efficiently than non-adherent tracers\(^11\).
are also experiments that show in rabbits, 30 nm particles were able to bind M cells but not neighboring enterocytes\textsuperscript{52}. This may be due to the lack of mucus or glycocalyx layer covering the cell surface or because the microfold apical surface on M cell is more accessible than wavy microvilli on enterocytes. However, this hypothesis alone cannot be used to explain the selectivity of M cell transcytosis. Some pathogenic bacteria, such as *Escherichia coli*\textsuperscript{53}, *Salmonella enterica*\textsuperscript{54} and *Shigella sonnei*\textsuperscript{55}, prefer to adhere to M cells than others. This also happens with viruses, including the mouse pathogen reovirus\textsuperscript{47}, the human pathogens poliovirus\textsuperscript{56} and HIV-1\textsuperscript{57,58}. All these are indicators that M cells may have a unique structure exposed to the luminal surface and the uptake may be receptor mediated. Though still not clear, many molecules have been proposed to be involved in the process, including $\beta$-1 integrin, TLRs, glycoprotein 2, etc.

$\beta$-1 integrin is thought to be involved in the M cell-mediated *Yersinia enterocolitica*-specific uptake. In enterocytes, $\beta$-1 integrin was found to be expressed at the basolateral surface. However, in M cells, the expression pattern was changed to the apical and lateral surface, which makes the integrin available for binding of *Yersinia*. It has been shown in mice that when given orally, large amounts of *Yersinia* were found with the FAE, but never in villous enterocytes. Of these *Yersinia* in FAE, the majority was seen associated with M cells. This M cell-specific invasion was due to the binding of invasin to the $\beta$-1 integrin, because an invasin-deficient mutant mouse lost its ability for M-cell invasion\textsuperscript{45}. This finding was also confirmed in an M cell like *in vitro* model, where blocking $\beta$-1 integrin on the epithelial cell also blocked the uptake of the bacteria\textsuperscript{59}.
Toll-like receptors (TLRs), members of the pattern-recognition receptors family, are also important for M cell-mediated bacteria uptake. So far, in mammals, the TLR family consists of at least 13 members. TLRs bind different ligands, for example, TLR2 recognizes lipoprotein, peptidoglycan and lipoteichoic acid from bacteria and zymosan from fungi; TLR3 binds to double-stranded RNA from virus and TLR4 mediates lipopolysaccharide signalling. It has been found that except for TLR3 and -6, other TLR members from TLR1 to TLR9 are all expressed in M cells. Some but not all TLRs have been investigated and shown to be important in M cell function. For example, although TLR2 is expressed both in villous epithelium and FAE, TLR2 activation can lead to receptor redistribution only in the FAE. Furthermore, the activation of TLR2 can enhance the transcytosis ability of M cells in a dose-dependent manner. Activation of TLR4 can also lead to the same result. Apical surface of M cells has high expression of TLR4. In the M cell in vitro model, uptake of a formalin-killed Nontypeable Haemophilus influenzae was three-fold higher than measured in the control group. Such increase could be blocked by blocking TLR4 but not TLR2 or α5β1 integrin, indicating the Nontypeable Haemophilus influenzae uptake is TLR4 dependent.

Glycoprotein 2, a major protein present in the zymogen granule membranes of the exocrine pancreas, was recently found to be also specifically expressed on the M cell apical surface both in human and mice. It was shown that, in mice, glycoprotein 2 could bind to FimH-expressing gram negative bacteria including Salmonella, E. coli. Such binding leads to the uptake of the bacteria, and in turn induce an antigen specific mucosal immune response. In glycoprotein 2−/− mice, the translocation of the FimH+ gram-negative
bacteria is significantly inhibited. Similar results are observed with the FimH+ bacteria. Together, these results suggest that glycoprotein 2 can serve as a transcytotic receptor on M cells for mucosal particles.

In addition to conventional receptors, tight junction proteins may also be related to M cell-mediated receptor-dependent endocytosis. Although M cells attach to adjacent enterocytes through tight junctions as conventional enterocytes do, the distribution of tight junction proteins in M cells are quite different. By using in situ hybridization as well as antibody staining, Lo et al found that, in M cells, claudin 4 also known as C. perfringens enterotoxin receptor, showed higher expression in UEA-1+ cells in FAE. More importantly, the distribution of claudin 4 is not restricted to tight junctions, but also observed in the cytoplasm. Although the function of claudin 4 in M cell mediated particle uptake is still unknown, as a bacterial enterotoxin receptor, it may be important in the process. Other tight junction proteins may also be important in M cell mediated endocytosis, such as junction adhesion molecule-A. It has long been known that reovirus can specifically adhere to M cell apical surface through σ protein 1, and junction adhesion molecule-A is found to bind to σ protein 1 directly, suggesting junction adhesion molecule-A may serve as receptor for the entry of reovirus into the M cells. However, the receptor function of junction adhesion molecule-A is still controversial, since some suggested that the interaction at the apical surface between type 1 sigma protein and M cell is through alpha2-3-linked sialic acid.

M cells can also endocytose other virus, including mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV) etc. For example, after infection,
MMTV was first observed in Peyer’s patch and then mesenteric lymph nodes. Moreover, B cell-deficient mice, which have fewer and non-functional M cells, were found to be resistant to the infection. However, the molecules involved in the infection process have not been identified. HIV has also been proved to be transported by M cells through lactosyl cerebroside and CXCR4 receptors in the Caco-2 co-culturing model.

After adhering to the apical surface, macromolecules or particles are then efficiently consumed by endocytosis and phagocytosis through M cells. However, the pathway of those particles into the M cells varies, which depends on the size and characteristics of the particle. Normally, large adherent particles and bacteria trigger phagocytosis, involving the extension of cellular processes and the reorganization of the submembrane actin network, while adherent viruses and macromolecules are taken up by clathrin dependent endocytosis. In contrast, nonadherent materials are taken up by fluid-phase endocytosis in either coated or uncoated vesicles.

To make things even more complicated, besides phagocytosis, some bacteria strains can also be taken up by M cells through different pathways, including macropinocytosis and clathrin-dependent endocytosis.

1.2.4 M cell gene expression profile

To understand the unique characteristics of M cells, and to identify the molecules that may be involved in M cell-specific development and function, several microarray studies have been conducted, that compare the gene expression profile in M cells with neighboring epithelial cells, and some M cell-specific genes were found. By using total gene expression analysis, Lo et al. compared the gene expression between the Peyer’s
patch dome, villi from normal BALB/c mice and Peyer’s patch dome from *Salmonella* treated mice. Among 19,000 different genes detected, 86 were either Peyer’s patch, or FAE or *Salmonella* treatment specific. Among the 86 genes, β3 laminin, peptidoglycan recognition protein (PGRP)–S and PGRP-L were found to be FAE specific. Moreover, in FAE, PGRP-S was expressed only in M cells. Verbrugghe *et al*\(^{78}\) also found 91 genes upregulated and 4 genes down-regulated in FAE with microarray analysis compare to villous enterocytes. Further in situ hybridization showed Annexin V, which has been reported to be important in endocytic transport and membrane scaffolding, is specifically expressed in M cell but not other enterocytes. Using the same technology, other FAE and M cell specific genes were found in different species, including secretory granule neuroendocrine protein 1 (*Scg5*) in mice\(^{79}\) M cells, and TNFAIP3 and CCL20, CCL23 in human FAE\(^{80}\) etc. However, the gene profiling in the Peyer’s patch M cell seems to be different to what happened in the villous M cell. For example, Myristoylated alanine-rich C kinase substrate like protein and glycoprotein 2 are only found in Peyer’s patch M cells but not in cholera toxin-induced villous M cells\(^{42}\), suggesting villous M cells may have different mechanisms for bacteria uptake.

**1.2.5 M cell lineage and differentiation**

Although M cells are an important entry site for various antigens and initiation of the mucosal immune response, their origin and differentiation is still controversial (Fig.1-4). Many scientists postulate that, like other enterocytes from the intestine, M cells are actually arising directly from the crypt stem cells. This hypothesis has been widely studied and confirmed by different groups. As early as 1984, Bye *et al* had already found
the transitional form of M cells at the neck of the crypts and upward. This transition form of M cells shares the morphology and functional properties of fully differentiated M cells. Other research groups support this finding with different cell markers and morphological parameters both in mouse and rabbit. Moreover, Gebert et al., using confocal laser scanning microscopy, compared the crypts at the base of Peyer’s patch dome with ordinary crypts. They found dome-associated crypts were morphologically distinct from ordinary crypts. M cell precursors (labeled by UEA-1) were only detected in dome-associated crypts. In addition, the two types of crypts were associated with radial strips of M cells in the dome, further indicating fully differentiated M cells are actually from the M cell precursors in dome associated crypts. Such relative distribution of M cells and dome enterocytes was also detected in rabbit intestine. However, this evidence still cannot rule out the possibility that with certain sets of stimuli, fully differentiated enterocytes cells can transition into functional M cells.

Others have argued that M cells are actually converted from fully differentiated enterocytes. Among those supporting that position, some think lymphoid cells from the underlying follicles trigger the enterocyte switching to an M cell phenotype. This hypothesis was confirmed by Kerneis et al., who injected the Peyer’s patch B lymphocytes into the duodenal mucosa of recipient mice. Nine days post injection, they found a Peyer’s patch-like structure and typical M cells with adjacent enterocytes at the site of injection. However, this is not sufficient to prove that the newly formed M cells are actually converted from the enterocytes since the turnover rate of intestine epithelial cells is 4-6 days. For instance, the typical M cells observed might have arisen from a
crypt cell that was influenced by the injected Peyer’s patch B lymphocytes. The acquisition of an M cell phenotype in the co-culture of Caco-2 and Peyer’s patch lymphocytes or Raji B cell model in vitro is used as more evidence for the conversion of M cells\textsuperscript{86}. But Caco-2 cells originate from cancer cells, which means they are poorly differentiated, crypt-like cells. The crypt cell-like feature of Caco-2 cells has already been confirmed by its expression of crypt cell markers as well as its potential for differentiation into different cell type in vitro\textsuperscript{88}. Therefore, even the co-culture model exhibits some of the M cell phenotypes; it does not necessarily mean the Peyer’s patch B lymphocytes can induce the trans-differentiation of fully differentiated enterocytes into M cells.

Besides the lymphocytes’ effect, it is also hypothesized that pathogens from the intestinal lumen can trigger M cell conversion. The effect of different pathogens has been examined, including the non-intestinal bacterium, Streptococcus\textsuperscript{89,90}, and the pathogenic intestinal bacteria, Salmonella\textsuperscript{91}. Borghesi et al showed by light, transmission electron, and scanning electron microscopy that after 2 hours of exposure to Streptococcus, the FAE showed dramatic changes in morphology and significantly increased M cell numbers and surface area\textsuperscript{89}. Meynell et al also showed that the increase in M cell numbers and surface area correlates with the upregulation of microsphere uptake\textsuperscript{90}. However, Streptococcus is a non-intestinal bacterium, which means such manipulation is not physiological. Also, the only characteristic feature of M cells examined in the experiment is lacking of microvilli, which may not be sufficient enough to prove these are indeed M cells rather than regular enterocytes infected by Streptococcus. In our
laboratory, a different strain of *Streptococcus* did not replicate the effect, indicating that such effect is limited to a certain strain of *Streptococcus*. In 1991, Savidge *et al*\(^91\) also showed that *Salmonella* could induce M-cell formation in germ-free mouse Peyer’s patch tissue. The increasing M cell numbers can be seen as early as 12 hours after exposure to *Salmonella in vivo* and last for 5 days. However, the increasing number of M cells coexists with the change of crypt depth and the doubling rate at which follicle crypt enterocytes migrate toward the apex, which suggests that the newly formed M cells may come from the newly differentiated crypt cells. Furthermore, the mice used in this experiment are germ free mice. Mice reared in a germ-free environment have a reduced number of Peyer’s patch\(^92\). Increasing the number of M cells may be an indirect effect of promoting the assembly of lymphoid follicles.

### 1.2.6 Factors involved in M cell differentiation

M cell differentiation is a fairly complicated process. In the intestine, both crypt cells and fully differentiated enterocytes may be involved in M cell development, while in NALT, basal cells may serve in the same capacity as crypt cells in the intestine, and engage in NALT M cell development. Other fully differentiated, ciliated cells might also convert into the M cell phenotype upon stimulation. Aside from the controversy about M cell origin, factors involved in M cell differentiation are also complicated. Many factors have been proven to be important, including lymphotoxin (LT), Tumor necrosis factor \(\alpha\) (TNF\(\alpha\)), B cells, receptor activator of NF-\(\kappa\)B Ligand (RANKL), CD137 and microbes etc.

LT-\(\alpha\), LT-\(\beta\) and TNF\(\alpha\) are all TNF family members. TNF\(\alpha\) and LT-\(\alpha\) homotrimer bind to TNFR1 and TNFR2, while LT\(\alpha1\beta2\) or LT\(\alpha2\beta1\) heterotrimer binds to
LTβR. After binding to its ligands, TNFR recruits TNFR associated factor (TRAF) 2 and 5, which in turn activates the canonical NFκB pathway (IKK/NFκB). LTβR recruits TRAF2,3,6 and activates the non-canonical NFκB pathway (P100/RelB). This leads to activation of gene transcription programs in the nucleus controlled in part by nuclear factor κB (NFκB). Although activates different NFκB pathway, LT-α, β and TNFα all have been shown to be indispensable in Peyer’s patch development. In LT-α and LT-β KO mice, Peyer’s patch formation is completely missing from the embryonic intestine38,93. This finding was further confirmed by Rennert et al 94,95, who injected LTβR-Ig blocking during gestation and showed that Peyer’s patch development was halted. However, blocking of TNFR by TNFR-Ig does not show significant effect on Peyer’s patch development, indicating LTαβ heterotrimer or LTβR may play a more important role than LTα3 homotrimer or TNFR.

The effect of TNFα is more complicated, since different strains of TNFα KO or deletion and TNFR KO showed different impacts on Peyer’s patch development. The appearance of Peyer’s patch in different TNFα KO and TNFR KO strains ranged from completely missing to reduced numbers with severely disorganized microarchitecture96,97,98. Although results vary, it is still clear that Peyer’s patch development needs a signal from both LT and TNF. Since M cells lie in the FAE covering the Peyer’s patch follicle, TNF and LT are likely to be important in M cell differentiation. However, whether these factors influencing M cell development direct or indirect is still unknown. Possible indirect effect include factors secondary secreted by B cells, DCs inside the follicle or cell contact between B cells, DCs with FAE.
RANKL, another TNF superfamily member, is also required in M cell development. Like other members in the family, RANKL, after binding to its receptor RANK, recruits TRAF6 and then activates NF-κB. There are two forms of RANKL: a transmembrane protein form and a soluble form. The latter is cleaved from the transmembrane protein by metalloprotease\(^9\). RANKL is crucial in bone development. RANKL-deficient mice showed severe osteopetrosis due to a lack of osteoclasts to support osteoclastogenesis\(^10\). In addition, RANKL plays an important role in the immune system. \textit{In vitro}, RANKL has been shown able to activate multiple immune cells, including myeloid-derived DCs and T cells\(^101,102\). \textit{In vivo}, in both RANKL\(^103\) and RANK KO\(^104\) mice, peripheral lymph nodes are missing, suggesting RANKL is also important in lymph nodes development. Peyer’s patch in RANKL KO mice were smaller than in the wild type. Most importantly, M cell numbers in RANKL KO were also significantly reduced (<2% of wild type mice)\(^105\). Such deficiencies of M cells can be restored by treatment with exogenous RANKL. Wild type mice treated with neutralizaing anti-RANKL antibodies reproduce the M cell developmental defects, further proving that RANKL is directly involved in M cell development rather than being part of an indirect effect caused by deficiencies in lymph nodes or Peyer’s patch development. Furthermore, injection of soluble RANKL can also increase functional villous M cell numbers \textit{in vivo}. All these are clear indicators that RANKL is necessary in M cell development and differentiation.

One unique feature of M cell morphology is the basolateral pocket where B cells are accommodated. The M cell-B cell interaction occurs at the edge of the dome, and has
been confirmed to be a life-long time relationship\textsuperscript{41}. Because of this, it is very likely that B cells are also important in M cell development. Co-culturing Peyer’s patch B cells that change the human colon adenocarcinoma cell line Caco-2 into an M cell like phenotype\textsuperscript{86} further supports this notion. However, both in B cell deficient immunoglobulin heavy chain 6 (Igh-6) mice\textsuperscript{73,106} and the recombination-activating gene KO mice (lacking B cell and T cell)\textsuperscript{107}, Peyer’s patch M cells can still be found, although the Peyer’s patch in these transgenic mice are fewer, smaller, and more disorganized. However, these M cells are dysfunctional in that the transfer of MMTV through the intestine wall is significantly impaired\textsuperscript{73}. It is therefore likely that B cells are not required for M cell lineage commitment, but are necessary to maintain M cell phenotypes.

Signals involved in B cell-M cell talk are not well studied. One possible molecule involved is CD137/4-1BB(TNFRSF9). CD137, which belongs to the TNFR family, is a receptor for CD137 ligand. It is mainly expressed on activated T cells and its most well understood function is T cell co-stimulation. Recent data in our lab shows that CD137 is also expressed by intestinal epithelial cells in response to TNF/LT signaling\textsuperscript{108}. Other groups confirm that CD137 ligand is expressed in activated B cells\textsuperscript{109}, suggesting possible cross talk between M cells and B cells. Moreover, in CD137-deficient mice, although the development of mucosal lymphoid tissue (both Peyer’s patch and NALT) appears normal, and UEA-1\textsuperscript{+} M cells could still be seen in FAE, M cells exhibit an abnormal morphology (lacking a basolateral pocket) and function, deficits in particle transcytosis by NALT epithelium. This indicates CD137 may be important for maintaining M cell function rather.
Another factor that may affect M cell development is microbes. Previous experiments showed increased numbers of M cells in transferring specific pathogen free (SPF) mice into conventional housing environment\textsuperscript{92}. In addition to commensal bacteria, the normally harmless bacteria inside our body, pathogenic bacteria can also promote M cell differentiation. Feeding germ-free mice with attenuated pathogen\textsuperscript{91} or injecting pathogen using an intestinal loop\textsuperscript{89,90} can both induce M cell number increase. In SPF mice, by introducing non-intestinal bacteria \textit{Streptococcus}\textsuperscript{89,90} and pathogenic intestinal bacteria, \textit{Salmonella}\textsuperscript{91} into the intestine lumen, the number of M cells is also rapidly and dramatically increased. All these results suggest that microbes may influence M cell generation and maintenance. However, even in germ-free mice, which totally lack microbes in the intestine, there are still M cells at Peyer’s patch\textsuperscript{91}, indicating microbes may not be necessary for M cell development.

Since various pathogens can increase M cell numbers, and the TLRs, which recognize pathogen-associated molecular patterns, have been shown to be expressed in M cells\textsuperscript{110} where they are important in M cell mediated transcytosis\textsuperscript{67}, it is reasonable to hypothesize that TLRs may be important in M cell development too. However, both the TLR2 and TLR4 KO mice were indistinguishable from the wild type in terms of M cell function and M cell numbers\textsuperscript{41}. One explanation is that the TLR signals may be redundant for M cell differentiation, To rule out this possibility, scientists checked M cell number in mice deficient in Myd88, the universal adaptor protein of TLRs. No difference in M cells was observed\textsuperscript{41}. Therefore, it is possible that TLRs are involved in M cell function but not M cell differentiation. Other pattern recognition receptos such as PGRP
have also been found to be specifically expressed in Peyer’s patch M cells\textsuperscript{77}, although, their function in M cell development has not yet been established.

1.2.7 \textit{In vitro model for M cell studies}

The small number of M cells in the gastrointestinal tract (less than 1\% of the intestinal surface in both human and mice) has significantly limited research on M cell differentiation and function. Also, the significant interspecies differences between animals as well as the lack of commonly used markers make interpretation of animal studies difficult. A new generation of \textit{in vitro} human models will be extremely helpful in understanding the differentiation of M cells and the mechanisms by which pathogens are captured and transcytosed.

To date, most \textit{in vitro} studies have been done using complex co-culture systems in which human Caco-2 monolayers are grown on permeable filter supports together with fresh murine Peyer’s patch-derived B lymphocytes or Raji B human lymphocytes. Kerneis \textit{et al}\textsuperscript{86} and Gullberg \textit{et al}\textsuperscript{111} showed in different experiments that in the co-culture system, lymphocytes could migrate and settle in the epithelial layer, inducing reorganization of the brush border, changing expression of potential human M cell markers (decreased alkaline phosphatase and increased Sialyl Lewis A antigen) and up-regulating temperature-dependent transport of particles and \textit{Vibrio cholerae}. To further simplify the \textit{in vitro} system, conditioned media was used in place of B lymphocytes, and showed the ability of inducing an M cell phenotype.

Although several \textit{in vitro} models have been established, none of them really mimic what happens \textit{in vivo}. M cell development is a complicated process beyond their
interaction with B-lymphocytes. Therefore, the co-culture of B-lymphocytes together with fully differentiated Caco-2 cells may not be adequate to represent all the features of M cells. Moreover, there are no universal markers specifically labeling M cells, which adds more difficulty to confirming the formation of M cells. Several markers have been used to label M cells \textit{in vivo}, but most of these are species specific. UEA-1 is the marker used for labeling M cells in mice\textsuperscript{22}, but does not bind to human M cells\textsuperscript{112}. Even in mice, UEA-1 does not have specificity for M cells, but also binds to goblet cells\textsuperscript{16}. Vimentin is used to label rabbit M cells\textsuperscript{19} and cytokeratin antibodies for bovine and rabbit M cells respectively (cytokeratin 18 for bovine\textsuperscript{8} and cytokeratin 20 for rabbit\textsuperscript{23}). Again, none bind to human M cells. Although some groups raise molecules such as glycoprotein 2 that are specifically expressed in human M cells\textsuperscript{113}, they have not yet been widely studied and accepted. So far, the most popular method to identify human M cells is still by morphology (loss of microvilli, formation of basolateral pocket) and function (increased ability of uptake particles including beads, \textit{vibrio cholerae} and \textit{salmonella}). But these characteristics are not M cell specific, as inflammation and other factors can lead to the same phenomenon in non-M cells. In addition, the complicated system of co-culturing two different cell types together adds to the complexity of analyzing M cell function in detail too.
1.3 Cholera toxin and immune adjuvant effect

1.3.1 Structure of Cholera toxin

Cholera toxin is secreted by the gram-negative bacterium *Vibrio cholera*. The toxin is composed of six subunits: one A subunit and five B subunits. The A subunit is the toxin unit, while the B subunits are the binding units that bind to the ganglioside GM1 receptor on epithelial cells. The two different types of subunits are connected by a disulfide bond. The A subunit is further composed of a globular A1 domain, which is responsible for the ribosyltransferase activity, and an A2 domain which interacts with the B subunit. After B subunits bind to the cells, the whole toxin is then transported through lipid raft/caveolae-dependent endocytosis, clathrin dependent endocytosis or an Arf6 associated endocytotic pathway\textsuperscript{114,115,116}. After endocytosis, cholera toxin travels to the endoplasmic reticulum via a Golgi dependent retrograde transport pathway\textsuperscript{117} associated with the sequence motif at the C-terminus of the A2 domain\textsuperscript{118}. Inside the endoplasmic reticulum, A subunit dissociates with B subunit; this is followed by a proteolytic cleavage of the trypsin sensitive loop between the A1 and A2 domains, which in turn facilitates the ADP ribosyltransferase activity of the A1 domain\textsuperscript{119}. The A1 domain then enters the cytosol and binds to ADP-ribosylation factor 6 (Arf6), which opens up the active site and enables its catalytic activity by driving a conformational change. After being activated, the A1 domain catalyses ADP ribosylation of the trimeric Gsα component of AC, which leads to a dramatic increase in cyclic AMP (cAMP) synthesis, causes secretory diarrhea (Fig.1-5).
1.3.2 Cholera toxin as immune adjuvant

Mucosal or parenteral co-administration of cholera toxin with immunization antigens can greatly enhance IgA and IgG production\(^{120}\). Therefore cholera toxin has long been known as a powerful immune adjuvant and has been widely used in experimental animals. Although the mechanisms are still unknown, it seems that cholera toxin can act on every step of the immune response. Lycke et al\(^ {121}\) showed that cholera toxin can increase gut permeability for molecules > 3000 Da, and that such an effect is associated with the adjuvant function by cholera toxin on the gut immune response. This was evident because the use of B subunit alone which could not enhance permeability failed to induce a local immune effect. The increase in gut leakage is caused by the A1 domain-induced adenylate cyclase/cAMP system, and may lead to increased access of antigens from the lumen to the gut mucosal immune system.

Besides increasing permeability, cholera toxin can also enhance antigen presentation. It has been shown that, after oral delivery of cholera toxin, there was a rapid, transient increase in immature subepithelial DCs in the intestinal villi epithelium, followed by a massive accumulation of mature DCs in mesenteric lymph node\(^ {123}\). Also, cholera toxin can induce DCs migrate into the follicle associated epithelium of Peyer’s patch, which will in turn promote capture of incoming antigens and pathogens by Peyer’s patch DCs\(^ {124}\). In addition, cholera toxin alone or with other stimuli such as LPS or a combination of IL-1 and TNF\(\alpha\) can promote DCs maturation\(^ {125,126}\). Moreover, exposure of DCs to cholera toxin can increase the costimulatory molecules CD80, CD86, and MHCII associated with T cell activation\(^ {127}\).
Additionally, co-administering cholera toxin can increase germinal center formation after immunization\textsuperscript{128} and promote B cell isotype switching\textsuperscript{128}, particularly IgA switching. This effect is mainly due to the B subunit, and was thought to be mediated through TGF-beta 1\textsuperscript{129}. Also, cholera toxin has been proven to enhance B7-1 and B7-2 expression on Peyer’s patch B cells, and such effect requires initial binding of B subunit to B cells through GM1\textsuperscript{130}.

Moreover, the immune adjuvant effect of cholera toxin may also due to its ability to activate a skewed cytokine production. However, whether this is a Th2 response or a Th1 response is still controversial. Some research groups report that cholera toxin can promote a strong production of IL-4, IL-5 and IL-10 but little IFN-\(\gamma\)\textsuperscript{131,132}, indicating a Th2 response. Others have proposed that cholera toxin could indeed suppress IL-12 and IFN-\(\gamma\) production \textit{in vitro} or \textit{in vivo} while delivered intraperitoneally\textsuperscript{133}. This inhibitory effect may due to the suppression of IL-12 p70 gene transcription in monocytes or monocyte-derived DCs and the inhibition of the \(\beta1\) and \(\beta2\) chains of the IL-12 receptor on T cells. However, some studies showed that cholera toxin could induce a mixed Th1 and Th2 response with the production of both IFN-\(\gamma\) and IL-4 while delivered orally and intranasally\textsuperscript{134,135}. Such difference may due to different methods of cholera toxin administration: Oral and intranasal administration may lead to activation of a mucosal immune response, while intraperitoneal administration may cause a systemic immune response. Moreover, cholera toxin can also induce a Th17-dominated T cell response\textsuperscript{136} or an IL-10 secreting regulatory T cell response\textsuperscript{137}. Th17-type T cells are important for inflammatory responses and they produce host defenses against bacterial and fungal
infections at mucosal surfaces\textsuperscript{138,139}. Meanwhile, neutralizing IL-10 will inhibit antigen-specific antibody production\textsuperscript{140}. All these may also help to explain cholera toxin’s immune adjuvant effect.

The immunomodulatory effect of the A or B subunit alone adds even more complexity. Studies show that the non-toxic B subunit can exhibit both immunostimulatory and immunosuppressive effects while conjugated to different antigens. Yuki \textit{et al} demonstrated that mice intranasally immunized with a recombinant B subunit fusion protein linked with an auto-antigen T cell epitope of an insulin B chain peptide can significantly inhibit the development of diabetes\textsuperscript{141}. Odumosu \textit{et al} further showed that incubation of recombinant B subunit-INS fusion protein with human immature DCs could completely suppress their activation\textsuperscript{142}. However, conjugating or co-administering B subunit with Streptococcal surface protein antigen, can actually induce an immunostimulatory effect, represented by the generation of IgA antibody responses expressed at several mucosal sites including gut, saliva and the tracheal\textsuperscript{143}. Mechanisms underlying the differential effect of B subunit with different antigens remained to be discovered. This may be related to the properties of the antigen itself, or to the route of delivery and or the immunization protocol. Although nasal delivery of A subunit with ovalbumin has been shown to elicit both mucosal and systemic antibody response\textsuperscript{144}, unmodified A subunit is seldom used as immune adjuvant due to its toxicity. Agren \textit{et al} developed a A1 domain-DD molecule by removing the B subunit of cholera toxin and genetically linking two Ig-binding domains DD of staphylococcal protein A at its C-terminal end\textsuperscript{145}. \textit{In vivo}, this molecule exhibits a strong ADP-ribosyltransferase
activity and has the adjuvant ability to enhance systemic IgG as well as mucosal IgA responses. Also, it has been proven to be involved in long term plasma and memory B cell development\textsuperscript{146}.

1.3.3 Cholera toxin and M cell development

Many hypotheses have been tested regarding cholera toxin’s immunomodulatory effect. In contrast, its action on M cells is not well studied. Because M cells are the initial sites for mucosal immune response and cholera toxin can serve as an immune adjuvant, it is likely that cholera toxin may act on M cells as well. So far, the only experiment that has been done to study cholera toxin’s effect on M cells was by Kiyona \textit{et al}. It was shown that, when cholera toxin was orally administered to BALB/c mice, UEA-1\textsuperscript{+} cells were dramatically increased in the villous epithelium and in the Peyer’s patch. Such effect was most significant in day 1 and gradually decreased to normal after day 3\textsuperscript{42,24}. However, in this experiment, the effect of cholera toxin on NALT M cells was not examined. The difference of the induction of villous versus Peyer’s patch M cell was also not established. Furthermore, the origin of these newly formed UEA-1\textsuperscript{+} M cell is not clear. Whether these cells were coming from direct cell division or trans-differentiated from other enterocytes is still a mystery. The mechanisms of how cholera toxin induces M cells are still unknown. Also, the M cells detection is currently dependent on UEA-1 staining and NKM 16-2-4\textsuperscript{+} staining. As previously discussed, these are markers for other cell types, indicating the apparent increase of M cells may not accurately represent the change of M cells \textit{in vivo}. Lastly, there is no proof from the experiment that these induced M cells actually exhibit the same characteristic of the other M cells.
Herein, I will discuss the effects of TNFa and LTβR agonist in M cell differentiation *in vitro*. Additionally, we will compare the phenotypes of M cells in different locations using our transgenic mouse model that has the ability to specifically label M cells *in vivo*. 
1.4 References


135. Yanagita, M. et al. Nasopharyngeal-associated lymphoreticular tissue (NALT) immunity: fimbriae-specific Th1 and Th2 cell-regulated IgA responses for the


Figure 1-1: Peyer’s patch structure and antigen presentation by M cell.

Peyer’s patch is composed of a follicle which contains a centrally located germinal center and interfollicular regions flanked the follicle. FAE is the epithelial layer that covers the follicle and is separated from the follicle by the subepithelial dome region. Germinal center contains mostly B-lymphocytes but also follicular DCs and few CD4+ T-cells. T cells, macrophage, DCs, a few B-lymphocytes, plasma cells and high endothelial venules can be seen in the interfollicular region (IFR). DCs and B cells can also be found in the subepithelial dome region. M cells reside in the FAE layer that covers the whole follicle. The main function of M cell is to capture and transcytose antigens such as microbes to the DCs in the basolateral pocket of M cells. The antigen is then processed in the DC and presented to CD4+ T. T cells will then prime the B cells in the follicle, inducing B cell class switching and clonal expansion. Activated B cells then leave the Peyer’s patch through efferent lymphatic tubes. (From Nature Reviews Immunology 8, 421-434. Reprinted permission from the publisher.)
Figure 1-2: M cell structure under electron microscope.

Compared with the neighboring enterocytes (E), the M cell (M) has short and irregular microvilli. The basolateral membrane is invaginated and forms a pocket in which lymphocyte (L) residing in it. (From the Anatomical Record 216:521-527 (1994). Reprinted permission from the publisher).
Figure 1-3
Figure 1-3: M cell mediated particle uptake

For M cell mediated particle uptake, binding seems to be the first step. Many molecules have been proposed to be involved in this process, including TLRs, glycoprotein 2, β-1 integrin and tight junction proteins etc. After adherence to the apical membrane of M cells, these bacteria and macroparticles are taken up by clathrin-dependent endocytosis, caveolin mediated endocytosis, macropinocytosis or phagocytosis.
Figure 1-4: M cell development

M cells are thought to stem from both crypt stem cells and mature enterocytes. Usually, crypt stem cells give rise to the enterocytes in the intestine. With the stimulation of various cytokine, crypt stem cells differentiate into immature M cells. B cells then help these immature M cells further differentiate into mature M cells. Meanwhile, microbes and other factors may also direct enterocytes transformation into M cells. Both enterocytes and M cells move along the dome, and undergo apoptosis at the apex of the follicle.
Figure 1-5

[Diagram showing the binding and clustering of GMI in lipid rafts, followed by cell-type dependent endocytosis, transport to the Golgi, fusion with Golgi, and transport to the ER.]

Nature Reviews | Microbiology
Figure 1-5: Structure of cholera toxin.

Cholera toxin is consists of six subunits: one enzymatically active A-subunit and five GM1 receptor binding B-subunits. After binding to the cell, cholera toxin entering the cell through a cell-type dependent endocytosis. Followed by that, cholera toxin travels to the endoplasmic reticulum via a Golgi dependent retrograde transport pathway. Inside the endoplasmic reticulum, A subunit dissociates with B subunit; the A1 domain from the A subunit is then freed by a proteolytic cleavage. After that, A1 doman enters the cytosol, activates ADP-ribosylate adenylate cyclase, and leads to synthesis of cAMP. (From Nature Reviews Microbiology 7, 110-119\textsuperscript{122}. Reprinted permission from the publisher.)
Chapter 2: TNFR and LTβR agonists induce Follicle-Associated Epithelium and M cell specific genes in rat and human intestinal epithelial cells

Permission from J. Wang, ML. Fraga, A. Rynko, DD. Lo, Cytokine (2009) 47 (1): 69-76
The author of this thesis contributed to Figure 2-1, 2-2, 2-3, and Table 2-1.
2.1 Introduction

M cells are specialized epithelial cells at mucosal surfaces that are important in immune surveillance\textsuperscript{1,2,3,4,5}. Their developmental origins are very similar to that of the neighboring epithelial cells including absorptive enterocytes and FAE\textsuperscript{6,7,8}, but specific triggers, presumably from underlying lymphoid cells in the mucosal lymphoid follicles (e.g., Peyer's patches), induce their alternative functional phenotype. This functional phenotype and its associated genetic program is not well understood, as the molecular genetics of M cell biology is only beginning to be studied in detail. Recent studies have now begun to identify genes associated with both the FAE and M cell phenotype\textsuperscript{9,10,11,12,13,14}. Such genes are important both for understanding of the triggers of M cell development but also for identifying the elements of the specialized M cell functions such as particle transcytosis.

Along with the progress in identification of M cell specific genes, \textit{in vitro} studies have begun to model their development and function by using intestinal epithelium cell lines, mainly established from human colon carcinoma cells co-cultured with B lymphocytes\textsuperscript{15,16,17,18,19}. These studies were based on the fact that M cells \textit{in vivo} have a prominent basolateral pocket containing lymphocytes, mainly B cells, which are thought to provide important differentiation signals\textsuperscript{20,21,22}. The nature of these differentiation signals is not known, but may be a combination of soluble cytokines and cell-bound signals. Candidate cytokines include members of the Lymphotoxin (LT)/TNF family, since genetic deficiencies in these genes cause a loss of secondary lymphoid tissue development\textsuperscript{23,24,25}. The \textit{in vitro} models remain relatively poorly defined, as they are based only on co-cultures
of epithelial cells and lymphocytes, whether in direct contact\textsuperscript{15, 16} or across transwell filters\textsuperscript{17,18}. Thus, the actual details of cytokine interactions between the epithelium and lymphocytes are not known.

A clear elucidation of these interactions is complicated by the fact that despite the progress in identification of M cell-specific genes, a clear consensus molecular definition of M cell differentiation has not yet been established. Indeed, it is conceivable that functions associated with M cells, such as particle transcytosis, may in fact be present in a variety of distinct cell phenotypes. Studies have suggested that microbes such as \textit{Salmonella} and \textit{Pneumococcus} can induce either increased numbers of M cells in FAE\textsuperscript{26,27,28}, or increased transcytosis activity with constant numbers of M cells\textsuperscript{29}, raising the possibility that microbes may induce different types of M cells. Other variations on the M cell phenotype include the identification of villous M cells\textsuperscript{4}, and the fact that M cells might not be equivalent in airway versus intestinal tissues.

To clarify the connections between FAE and M cell-associated genes and the inducing triggers for M cell development, we have begun studies to characterize \textit{in vitro} models of M cell development and function. In the present report, we tested whether LT agonists, thought to be responsible for secondary lymphoid tissue development \textit{in vivo}\textsuperscript{23,24,25}, could be direct inducers of the M cell genetic program and associated functions. We found that indeed, both FAE- and M cell-specific genes could be induced by these agonists, and that functional changes could also be identified that may be characteristic of the M cell role in immune surveillance. These studies will help lead to a
clarification of M cell phenotypes, and should also help identify the signals that determine the commitment to the M cell versus FAE or conventional enterocyte lineages.

2.2 Materials and Methods

2.2.1 Cell lines and tissue culture

Caco-2 BBe cells and IEC-6 cells were obtained from ATCC. T84 and HT-29 cells were the generous gift of Dr. Carl Ware. Cells were cultured using recommended media preparations. For immunostaining, freshly passaged cells were grown on transwell filters (0.4 micron pore polycarbonate filter, Corning). For qPCR analysis, one million cells were cultured in a 25cm² flask (for Caco-2 BBe cells) and 6 well cluster plates (for IEC-6 cells). Cytokines, including recombinant TNFα (100 nanograms/ml, Peprotech) and LTβR agonist antibody (5 micrograms/ml, R&D Systems) were added to the medium immediately after the cells were plated. For consistent results with the LTβR agonist antibody, a crosslinking secondary donkey anti goat antibody (1 micrograms/ml, Southern Biotech) was added at the same time.

2.2.2 Quantitative PCR

Caco-2 BBe and IEC-6 cells were divided into four groups according to the treatment: the control group, a TNFα treated group, an LTβR agonist treated group and a combined TNFα and LTβR agonist treated group. Before RNA was extracted using Trizol (Invitrogen), cells are subjected to the treatment for 24h, 48h and 72h. Using the Superscript III first-strand synthesis system (Invitrogen), two µg of total RNA from each
sample were used to generate cDNA. The SYBR Green assay (Biorad) and the Biorad CFX96 detection system (Biorad) were used to detect real-time PCR products from one μl of the reverse-transcribed RNA samples (from a 20-μl total volume). Primers used for the qPCR are summarized in Table 1. Human Gapdh was used as a reference gene for the Caco2-BBe cells and mouse Hprt1 for the IEC-6 cells. Each PCR reaction was optimized to ensure that a single band of the appropriate size was amplified. The PCR cycling conditions were performed for all samples as follows: 40 cycles for the melting (95C, 15 seconds) and annealing/extension (60C for 1 minute). PCR reactions for each template were done in duplicate in 96-well plates. The comparative ΔΔCT method was used to determine the relative amount of gene expression.

2.2.3 Immunohistochemistry and Confocal microscopy

Cell cultures were fixed using 1% paraformaldehyde/PBS, then permeabilized in PBS, 0.1% Tween and blocked in 0.1% Tween in Casein solution. Cells were stained with antibodies to claudin 4 (Abcam), E-cadherin and ZO-1 (Zymed), followed by secondary reagents Alexa Fluor 488-, Alexa Fluor 568- or Alexa Fluor 647- conjugated anti-mouse, anti-rabbit or anti-goat (Invitrogen) antibody, then fixed with a 4% paraformaldehyde/PBS and mounted with Prolong Gold antifade reagent (Invitrogen). DAPI was used as a nuclear counterstain.

Images were obtained using a spinning disk BD CARVII Confocal Imager (BD Biosystems) attached to a Zeiss Axio Observer inverted microscope. Images were acquired using the 40x objective. Hardware control, including microscope, confocal and digital camera (Diagnostic Instrument Xplorer – XS) was done using BD IPLab Imaging
Software. Image Z resolution was further optimized with Media Cybernetics Auto X deconvolution software, using the AutoDeblur 3D Blind algorithm.

### 2.2.4 Transmission Electron Microscopy (TEM)

Cell cultures were washed twice in PBS and fixed in 4% paraformaldehyde/1% glutaraldehyde. Ultra-thin sections of cell-covered filters were prepared for TEM analysis by standard methods. Observations were made using a Phillips Tecnai 12 microscope.

### 2.3 Results

#### 2.3.1 TNFR and LTβR agonist induces expression of FAE-associated genes in rat IEC-6 and human Caco-2 BBc cells

Both conventional intestinal epithelium absorptive enterocytes and specialized Peyer's patch FAE are derived from the same intestinal crypt stem cells, but it has been assumed that the commitment of cells to the FAE phenotype is influenced by factors provided either by lymphoid tissue inducer cells (LTi)\(^{23}\), or by cells such as lymphocytes that have accumulated within the Peyer's patch follicle\(^{20}\). As Peyer's patch formation is known to be dependent on TNFR and LTβR agonists, we studied the potential for these cytokines to induce expression of FAE-associated genes. In some studies, freshly passaged Caco-2 cells have been suggested to resemble crypt stem cells, contrasting with their differentiated gene expression phenotype once epithelial monolayers and tight junctions are well established\(^{30,31}\). Thus, to test whether specific cytokines can influence the differentiation of such putative crypt-like precursors, we treated intestinal epithelial...
cell lines (rat IEC-6 and human Caco-2 BBBe cells) for various periods after passage and then examined their RNA by qPCR analysis for expression of specific sets of genes. Among the genes studied, we focused especially on genes previously shown to be expressed by mouse Peyer's patch FAE in vivo, especially Ccl20 and Lamb3.

We found that TNFα can induce Ccl20 expression in both rat IEC-6 cells and Caco-2 BBBe cells (Figure 2-1A, B). LTβR alone also increased Ccl20 expression in IEC-6 cells, but not in Caco-2 BBBe cells (Figs 2-1A, B). This result contrasts with previously reported results by Rumbo et al. which suggested that an LTβR agonist could stimulate Ccl20 expression in T84 cells; however, they did not compare with the effects of TNFα. This difference may only reflect cell line specific differences between the T84 cell line and the more uniform and differentiated Caco-2BBBe subclone, as the rat IEC-6 cells did respond to both LTβR and TNFα; moreover, the effects of the two agonists on Ccl20 expression were additive in these cells.

Laminin beta 3 (Lamb3) is another gene specifically expressed in Peyer's patch FAE cells in mice, though originally identified as regulated in Caco-2 cells. In this case, cytokine treatment of IEC-6 cells resulted in increasing expression in response to TNFα, with a lesser response to LTβR agonist (Fig 2-1C). The NF-kB gene relB (Relb) has also been shown to be constitutively expressed by FAE in vivo and, as might be expected for triggering of TNFR superfamily genes, both LTβR agonist and TNFα induced Relb expression, with additive effects (Figure 2-1D).

We also found, surprisingly, that TNFR and LTβR agonists can induce expression of a TNFR superfamily gene Tnfrsf9 in Caco-2 BBBe cells. CD137, the protein encoded by
*Tnfrsf9*, previously had been associated mainly with activated T cells as a costimulatory molecule\textsuperscript{35,36,37,38,39,40,41}, but not with FAE. Our data shows that TNF$\alpha$ can significantly induce *Tnfrsf9* expression up to 60 fold, while LT$\beta$R agonists show a similar but slightly less potent effect. Moreover, the combined use of TNF$\alpha$ and LT$\beta$R agonists shows an additive effect (Figure 2-1E). Similar results were found for IEC-6 cells (not shown).

2.3.2 TNFR and LT$\beta$R agonists affect expression of M cell-associated genes in rat IEC-6 and human Caco-2 BBc cells

Although M cells in the Peyer's patch FAE are also produced from crypt stem cells, their point of divergence from FAE cells during development is not clear. However, while FAE cells are morphologically very similar to conventional absorptive enterocytes, M cells show unique morphological and functional characteristics *in vivo*, so M cell-specific gene expression patterns may show a dependence on different cytokines than FAE-specific genes. Several genes have already been identified as specifically expressed in mouse M cells, including *Sgne-1*/Scg5, *Gp2*, and PGRP-S/*Pglyrp1*\textsuperscript{9,10,11,13,14}. Their expression in human M cells is not well documented, so while we have also studied the human Caco-2BBc cell line, we focused mainly on the rat IEC-6 cells with the expectation that they would more faithfully reproduce expression patterns in mouse M cells. Using qPCR analysis, we found that the LT$\beta$R agonist induced both *Gp2* and *Scg5* expression in IEC-6 cells. In contrast, TNF$\alpha$ showed an inhibitory effect at 24 hours and 48 hours, though this effect was lost by 72 hours (Figure 2-2A, B). The expression of *Pglyrp1* was only slightly increased at 24 hours, and *Annexin V/Anxa5* was not found to change significantly with cytokine treatment (not shown).
The tight junction protein Claudin-4 (encoded by the gene \textit{Cldn4}) was also shown to be induced in Caco-2 cells in cocultures with Raji cells and also in M cells \textit{in vivo}^{10}. Similarly, in Caco-2 BBe cells, we found that TNFR and LTβR agonists both induced \textit{Cldn4} expression with a possible additive effect, though TNFα showed a stronger effect than LTβR agonist (Figure 2-2C).

### 2.3.3 TNFR and LTβR agonists have distinct but complementary effects

When the cytokine induced regulation of the various FAE- and M cell-associated genes examined are summarized together for IEC-6 cells, some patterns begin to emerge. TNFa appeared to be more important in inducing FAE specific genes, including \textit{Ccl20} and \textit{Lamb3} (though this also included \textit{Cldn4}), while having an inhibitory effect on M cell specific genes such as \textit{Gp2} and \textit{Scg5} (Figure 2-3A). By contrast, LTβR agonist by itself induced M cell specific genes, such as \textit{Gp2}, \textit{Scg5} and to a limited degree \textit{Pglyrp1} (Figure 2-3B). The combination of these cytokines showed additive effects in the induction of the FAE-associated \textit{Ccl20} and \textit{Lamb3} (Figure 2-3C).

### 2.3.4 TNFR and LTβR agonists lead to functional changes in IEC-6 and Caco-2 BBe cells

Because the cytokine-induced gene expression changes may also be associated with functional changes, we have begun to examine these effects in Caco-2 BBe cells. Studies on IEC-6 cells were unfortunately more limited, as these cells were less able to form effective tight junctions (not shown). In the case of Caco-2 BBBe cells, we found that combination cytokine treatment prevented the development of brush border microvilli
when examined by transmission electron microscopy (Figure 2-4A, B). These changes were also associated with a functional increase in the endocytosis of bacterial particles, as shown by the *Staphylococcus aureus* endocytosed by the cytokine-treated Caco-2BBe cells (Figure 2-4B). This effect has also been described in more quantitative studies.42

In a previous report, we found that Claudin-4 mRNA and protein expression was not only increased in M cells in vivo, but the protein also was found to be redistributed from its normal tight junction location in intestinal epithelium to the cytoplasm in M cells.10 Interestingly, treatment of Caco-2BBe cells with cytokines also caused redistribution of Claudin-4 to cytoplasmic vesicles (Figure 2-5A, B). In addition, similar redistribution of Claudin-4 was induced by cytokine treatment of two other intestinal epithelial cell lines HT-29 and T84 (Figures 2-5C-F). Despite this change in Claudin-4, distribution of other tight junction markers such as ZO-1 and E-cadherin in the Caco-2BBe cells was not affected (Figure 2-5G).

### 2.4 Discussion

Since the report by Kerneis et al.,15 showing the induction of M cell-like features in Caco-2 cells co-cultured with lymphocytes, several studies have begun to define the mechanisms responsible for M cell-like induction. For example, while M cells in vivo appear to require the presence of B cells in a basolateral pocket, other studies17,19 have suggested that soluble factors from B cells such as the Raji cell line are sufficient to induce M cells without direct cell contact. The identity of the soluble factors is beginning to be defined; for example, a recent report suggests that the soluble factor Macrophage
Inhibitory Factor (MIF) may be sufficient to induce particle transcytosis activity in Caco-2 intestinal epithelial cells\textsuperscript{19}. However, it is unlikely that MIF is by itself sufficient for M cell differentiation \textit{in vivo}, since MIF-deficient mice were found to have normal M cell development. In another case, mice lacking the chemokine receptor CCR6 also lack M cells\textsuperscript{43,44}, indicating a role for the chemokine ligand CCL20 in M cell development. In this case, CCL20 produced by FAE might be mainly involved in recruiting inducer cells (not yet identified) to the FAE rather than directly driving M cell development. The contribution of soluble versus cell-associated factors thus remains an open question. Interestingly, induction of the FAE-associated chemokine gene CCL20 depends on LTβR triggering, but the possible ligands for this receptor (LT alpha1/beta2 heterotrimers, LIGHT) are transmembrane proteins so are unlikely to be provided in soluble form \textit{in vivo}.

The studies presented here should help define the contribution of specific differentiation factors to the molecular profiles of specific M cell development pathways. We started with genes previously reported by ourselves and others as M cell- and FAE-associated molecular markers, all validated by confirmation of their expression in Peyer's patch epithelium \textit{in vivo}. Since many of these genes were identified in the mouse, and since they do not always have similar expression in human cells, we used both Caco-2BBe cells and the rodent IEC-6 cell line to examine their regulation. By treating the cells with agonists of the LTβR and the TNF receptors, we identified subsets of genes with distinct regulatory patterns. The most striking finding was that the subset of genes
associated with the FAE (Ccl20, Lamb3) were most effectively induced by TNFα, while an M cell gene subset (Gp2, Scg5) was most effectively induced by the LTβR agonist.

Curiously, the LTbR-induced expression of Gp2 and Scg5 was also suppressed in the presence of TNF, indicating a complex regulation of gene expression. These differences may be an indication that production of differentiation-inducing cytokines in vivo may be controlled in specific microenvironments, such as the M cell basolateral pocket versus the broader FAE dome. In this context, the production of variable combinations of related cytokines in inflammatory conditions such as Inflammatory Bowel Disease or Type 1 Diabetes might lead to both tissue destructive processes and abortive lymphoid tissue differentiation. For example, an interesting illustration of this principle was reported by Ruddle, Flavell, and colleagues in which chronic transgene expression of TNF or LT in the pancreas showed distinct inflammatory patterns; additional complexity in this setting is likely as the cytokines will induce secondary expression of other cytokines within the target tissue.

Since both sets of factors are likely to be present in the Peyer's patch environment, it was also striking to find that the combination of both cytokine agonists had additive effects on expression of Relb and Tnfrsf9 in Caco-2BBe cells. Since the expression of relB is regulated by NF-kB activation, and the LTβR and TNF receptors all signal through NF-kB activation, this finding is not so surprising; indeed, it has been reported that the FAE is constitutively positive for Relb expression in vivo. In the case of Tnfrsf9 however, this is an unusual finding, since Tnfrsf9 induction is usually associated with T
cell activation\textsuperscript{35,37,38}. However, since B cells and DCs both express the CD137Ligand, there may be a role for CD137/Tnfrsf9 expression in the FAE or M cells.

With respect to function of the epithelial cells in response to cytokine treatment, we did see some changes in morphology, such as loss of brush border microvilli, and redistribution of the tight junction protein Claudin-4, similar to the cytoplasmic distribution we reported for M cells \textit{in vivo}\textsuperscript{10}. We recently reported that the same cytokine treatment of airway and intestinal epithelial cells also increased their capacity to endocytose bacterial particles\textsuperscript{42}, and in some cases Claudin-4 was associated with the endocytosis vesicles.

The gene expression changes observed here in response to LT/TNF ligands provide a basis for defining the conditions for inducing differentiation of FAE and M cells. Considering the close proximity of these two cell types in the Peyer's patch, the actual factors necessary for specifying either phenotype is likely to be more complex, and may require additional soluble and cell-bound signals. For example, as shown by Tumanov et al.\textsuperscript{22}, the cellular sources of cytokines may have important local effects on lymphoid follicle development even if they are not locally required for M cell differentiation in the follicle epithelium. Thus, by comparing the effects of cytokines on the cell lines with expression patterns \textit{in vivo}, we will continue to get a more precise definition of the induction of FAE versus M cell differentiation, but spatial relationships between cells and the timing of cytokine production will also need to be taken into account.
2.5 References


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Peyer’s Patch Lymphocytes of Human Enterocytes into M Cells that Transport

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Figure 2-1

A. BBe CCL20 expression

B. IEC-6 CCL20 expression

C. IEC-6 LAMB3 expression

D. BBe RelB expression

E. BBe CD137 expression

Legend:
- Control
- LTβR agonist
- TNFα
- Both
Figure 2-1: Induction of FAE-specific genes in Caco-2BBe ("BBe") and IEC-6 cells.

Real-time PCR assays for human and rat Ccl20, rat Lamb3, and human Relb and Tnfrsf9 are shown as the average fold-induction from three independent experiments, with each experiment normalized to expression in untreated cells. Ccl20 was induced best by TNFa in both Caco-2BBe (A) and IEC-6 (B) cells, with an additive effect of TNFα plus LTβR agonist in IEC-6 cells. Similar effects were also seen for Lamb3 (C), Relb (D), and Tnfrsf9 (E).
Figure 2-2

A. IEC-6 GP2 expression

B. IEC-6 sgne-1 expression

C. BBc CLDN4 expression
**Figure 2-2: Induction of M cell-specific genes in Caco-2BBe ("BBe") and IEC-6 cells.**

Real-time PCR assays for rat *Gp2* and *Scg5*, and human *Cldn4* are shown as the average fold-induction from three independent experiments, with each experiment normalized to expression in untreated cells. Both *Gp2* (A) and *Scg5* (B) were best induced by LTβR agonist, and appeared to be transiently suppressed by TNFα alone. *Cldn4* (C) showed weaker induction by LTβR versus TNFα.
Figure 2-3

A. TNF induced gene expression

B. LTBR agonist induced gene expression

C. Both induced expression
Figure 2-3: Summary of specific cytokine effects in IEC-6 cells.

Data shown is a representative experiment from three independent experiments. TNFα (A) and LTβR (B) show distinct patterns of gene induction, with additive effects on Ccl20 and Lamb3 (C).
Figure 2-4
Figure 2-4: Cytokine effects on morphology and function in Caco-2BBe cells.

Caco-2BBe cells cultured for a few days already show evidence for apical microvillus development (A), while treatment with the combination of TNFα and LTβR agonist (B) prevents microvillus development, and enhanced uptake of bacterial particle (arrows). Caco-2BBe cell cultures were given *Staphylococcus aureus* particles for 12 hours prior to fixation. Scale bar: 2 microns.
Figure 2-5
Figure 2-5: Redistribution of Claudin-4 protein in treated intestinal epithelial cells.

Claudin-4 in Caco-2BBe (Claudin-4, red; A,B), HT-29 (Claudin-4, green; C,D), and T84 (Claudin-4, green; E,F) epithelial cells redistributes from the tight junctions (A,C,E) to vesicular structures in the cytoplasm (arrows; B,D,F) after treatment with cytokines. By contrast, distribution of tight junction markers ZO-1 (G,H) and E-cadherin (I,J) were not changed in Caco-2 BBe cells. Microscopy images were taken with 40x objective at original magnification.
Table 2-1: qPCR primer pairs

<table>
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<th>Species</th>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>Human CCL20</td>
<td>For</td>
<td>5’-GCCAATGAAGGCTGTGACATCAA-3’</td>
<td>Rev 5’-CACTAAACCCTCCATGATGTGCAA-3’</td>
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<tr>
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<td>For</td>
<td>5’-AGCAGAGTGCCCTGAGTTTAGGGT-3’</td>
<td>Rev 5’-CAGGACAAAGGCAGAAGGTGTGA-3’</td>
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<td>For</td>
<td>5’-CCTGCTAGCAAGAACAGAGTCCC-3’</td>
<td>Rev 5’-TGCAAGGAGATCCCCAAAGTCA-3’</td>
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<tr>
<td>Human RelB</td>
<td>For</td>
<td>5’-TGAACCTGACACTGGACTCGT-3’</td>
<td>Rev 5’-CTCGCGGTAATGATTGGAACA-3’</td>
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<td>Human GAPDH</td>
<td>For</td>
<td>5’-CATGAGAAGTATGACACAGCCT-3’</td>
<td>Rev 5’-AGTCCTTCCACGATACCAAGT-3’</td>
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<tr>
<td>Rat HPRT</td>
<td>For</td>
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<td>Rat Glycoprotein2</td>
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<td>Rev 5’-CATGACATCGGCTTGAGACAAA-3’</td>
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<td>Rat Sgne-1</td>
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<td>Rev 5’-AGCTTCCAGGCTTCCTGCA-3’</td>
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Chapter 3: PGRP-S DsRed Express2 Transgenic mice in M cell study
3.1 Introduction

M cells, as specialized epithelia cells, are mostly found in the follicle associated epithelial (FAE) layer covering mucosal associated lymphoid tissue (MALT) at various mucosal sites\textsuperscript{1,2,3,4,5}. The protective mucosal immune response initiated in the MALT, and it is the main site for IgA production. M cells are mostly responsible for immune surveillance by continuously and selectively transcytosing the microparticles from the lumen to the highly organized lymphoid cells inside the follicle.

To fulfill the task of immune surveillance, M cells exhibit distinctive morphological features when compared with the adjacent enterocytes under the electron microscope. While the apical surface of neighboring enterocytes or airway epithelial cells is decorated by microvilli or cilia, the apical surface of M cells have only shortened and irregular microfolds\textsuperscript{6}. Also, there is no mucus layer overlying the M cell apical surface\textsuperscript{7}. All these make M cells more accessible to microparticles. Moreover, TEM shows the basolateral surface of M cells to be invaginated to form a unique basolateral pocket\textsuperscript{1} where B cells and dendritic cells (DC) reside. This serves to shorten the distance for M cell mediated transcytosis. TEM also shows small volume fraction of dense bodies and absence of acid phosphatase\textsuperscript{8}, indicating reduced lysosomes, which allows particles to be transcytosed by M cells without extensive degradation.

Although the M cell has distinct features under electron microscopy, due to its attenuated apical surface, it can hardly be seen under the light microscope. Moreover, there is no universal M cell marker. So far UEA-1 is the most accepted tool for identifying M cells in mice. UEA-1 binds to many glycoproteins and glycolipids
containing an α-linked fucose residue. These were first found to be present in rabbit dome epithelial cells, and was analogous to the distribution of M-cells\textsuperscript{9}. Clark et al then showed that UEA-1 strongly stained 97.2\% of M-cells\textsuperscript{2}. However, UEA-1 also binds to goblet cells\textsuperscript{3}. This restricts its usefulness in identifying M cells \textit{in vivo}. Moreover, though UEA-1 has been shown to be able to label M cells in rodents, it does not label human M cells, which also limits its usage.

Other M cell markers have also been proposed including vimentin\textsuperscript{4} and cytokeratin 20\textsuperscript{10} for rabbit and cytokeratin 18 in bovines\textsuperscript{11}. Another antibody is NKM 16–2-4, which was developed by Nochi et al. Like UEA-1, it also binds to an α (1,2)-fucose-containing carbohydrate moiety. However, unlike UEA-1, this monoclonal antibody specifically binds to Peyer’s patch M cells but not goblet cells\textsuperscript{12}. Unfortunately, as NKM 16-2-4 still binds to the carbohydrate moiety at the cell surface, it may still be species restricted like UEA-1. Recently, glycoprotein 2 is also proposed to be specifically expressed in human and mouse M cells and is thought to be involved in the uptake of FimH\textsuperscript{+} gram-negative bacteria\textsuperscript{13}.

Moreover, although some of the antibodies discussed above can label M cells \textit{in vivo}, most of them only bind to the apical surface of the cell. No antibody so far can mark the whole cell body, thereby distinguishing M cells from the lymphocytes residing in the basolateral pocket. In addition, staining with antibody requires tissue fixation. This restricts the use of antibody for live imaging. Therefore, developing a transgenic mouse line with M cells labeled \textit{in vivo} would be helpful.
The mammalian Peptidoglycan Recognition Protein (PGRP) family is composed of four family members: PGRP-S, PGRP-L, PGRP-1\(\alpha\) and PGRP-1\(\beta\), all of which function in antibacterial defenses, innate immunity\(^\text{14}\), and to modulate inflammation and the immune response\(^\text{15}\). It has been confirmed that PGRP-S is highly expressed in the leukocytes and their precursors\(^\text{16,17}\), and PGRP-L is mainly found in the liver\(^\text{18}\), and low levels in oral epithelial cells\(^\text{19}\). Lo et al used total gene expression analysis to compare the gene expression in FAE and non-Peyer’s patch tissue, and found that PGRP-S expression is enriched in FAE. Further \textit{in situ} hybridization experiments confirmed its expression in FAE. Surprisingly, the expression of PGRP-S in FAE was exclusively co-localized with UEA-1\(^+\) M cells, suggesting that PGRP-S is an M cell specific protein\(^\text{20}\). When it is taken into consideration that M cells can selectively transcytose various bacteria into the follicle, PGRP-S may be involved in transcytosis vesicles. Alternatively, PGRP-S may serve as a bactericidal agent.

Since PGRP-S is specifically expressed in M cells in the intestine, the PGRP-S promoter should be able to drive M cell specific protein expression. Here we report a new transgenic mouse line, in which the fluorescent protein dsRed express2 (dsRedE2) as a reporter gene is connected downstream of the PGRP-S promoter, thereby labeling M cells \textit{in vivo} with red fluorescent protein.
3.2 Materials and Methods

3.2.1 Transgene Construction

To identify the PGRP-S promoter, 5kb of sequence upstream of the PGRP-S gene and PGRP-S intron sequence was obtained from the UCSC genome Bioinformatics database (http://genome.ucsc.edu/), and screened for putative promoter elements using the TRANSFAC Transcription Factor Binding Sites Database (http://gene-regulation.com/cgi-bin/pub/databases/transfac/search.cgi), with results in supplemental data. 4kb of candidate promoter sequence was then cloned by PCR from mouse genomic DNA using these primers: 5’- TTCCCAAG-CTTGCTGATAGCCTTTCTAAA ACTG-3’ and 5’- GTTCTCAGTGCTATCCATGCTGGACGTGC-CGCACAGG-3’. Based on conventional splice site sequences as well as the size of the intron, the second intron from PGRP-S was also cloned using the primers 5’-GTAAGTACATCCTGGACTG-3’ and 5’-CTGCAAGGGGAGGGACAAGATACAGA-3’, and inserted into the dsRedE2 coding sequence by overlap PCR. The insertion site of dsRedE2 was decided according to the conservative splice site AG at 5’ exon and G at 3’ exon. The dsRedE2 fragment was obtained from the pCMV-dsRed-Express2 vector (Clontech) and was inserted downstream of the PGRP promoter fragment by overlap PCR. The BGH polyA site fragment, obtained from pCDNA 3.1(+) (Invitrogen) was added to the end of the dsRedE2 coding sequence (Figure 3-1). Expression of dsRedE2 with the PGRP-S intron (in pCDNA) was checked by transfection into Caco-2BBBe cells before cloning downstream of the PGRP-S promoter.
3.2.2 Establish Transgenic Mice

To generate transgenic mice the 5.5 kb insert in pGEM was excised from the plasmid backbone by HindIII/KpnI digestion, gel purified and injected into fertilized oocytes of CB6 F2 mice. Founders were identified by PCR analysis of DNA isolated from tail clips using primers specific for dsRedE2 (5’-CGACATCCCCGACTACAAGAAGC-3’ and 5’-CTTCAGCTTCAGCGC-CTTGTGGAT-3’). Founders were bred to BALB/c wild type mice. All mice were bred in the UC Riverside vivarium under SPF conditions and were handled in accordance with institutional IACUC and NIH guidelines.

3.2.3 Neutrophil separation from peripheral blood

Peripheral blood samples were collected by retro-orbital puncture using heparinized capillary tubes and collected into 1.5 mL microcentrifuge tubes containing a final concentration of 3 mM EDTA-Na$_2$. PBNs were isolated from the anticoagulated blood by density centrifugation (modified from the protocol on ABI protocol. http://www.ambion.com/techlib/append/supp/wbc.html). Briefly, the blood sample was centrifuged at 2000g for 15 minutes at room temperature. After centrifugation, the upper layer of plasma was discarded, and the buffy coat was transferred to a slide by pipetting. A smear was then made with a spreader slide. The smear was fixed with 4% paraformaldehyde/PBS, and mounted with Prolong Gold antifade reagent (Invitrogen). DAPI was used as a nuclear counterstain. Images were obtained as described below.
3.2.4 Immunohistochemistry and Confocal microscopy

For cryostat sections, dissected NALT, Peyer’s patch or spleen tissue was fixed in 4% paraformaldehyde/PBS, flash-frozen for cryostat sections and stained in sections. For whole tissue mounts, dissected tissues were fixed in 4% paraformaldehyde/PBS and then stained in whole tissue fragments. For both cryostat sections and whole mounts, tissues were then permeabilized in PBS, 0.1% Tween and blocked in 0.1% Tween in Casein solution. Tissues were then stained with antibodies to Iba1 (eBiosciences) and fluorescein-conjugated or biotinylated Ulex Europaeus Agglutinin 1 Lectin (UEA-1) (Vector), followed by secondary reagents Alexa Fluor 488 conjugated anti-rabbit antibody, or streptavidin conjugated Alexa Fluor 647. Post-fixation was done with 4% paraformaldehyde/PBS. Tissue was then mounted with Prolong Gold antifade reagent (Invitrogen). DAPI was used as a nuclear counterstain. Images were obtained using a spinning disk BD CARVII Confocal Imager (BD Biosystems) on a Zeiss Axio Observer inverted microscope. Images were acquired using the 20x objective. Hardware control, including microscope, confocal and digital camera (Diagnostic Instrument Xplorer – XS) was done using BD IPLab Imaging Software. Image Z resolution was further optimized with Volocity software (PerkinElmer).

3.2.5 Isolation of peritoneal cells

Peritoneal cells were obtained as previously described. Briefly, mice were sacrificed after anesthetization with isoflurane, and 5 ml of ice cold 3%FBS/PBS were injected into the peritoneal cavity. The peritoneum was then gently massaged the to dislodge the attached cells into the solution. The fluid inside the peritoneal cavity was
then collected by a 5 ml syringe and deposited into pre-cooled tubes and kept on ice for further use. The peritoneal lavage was then repeated, after which the peritoneal cavity was cut open and the remaining fluid was collected. This combined fluid was centrifuged at 100g for 5 minutes at 4°C. The cell pellet was then resuspended for flowcytometry analysis.

3.2.6 Isolation of spleen cells

Splenocytes were prepared as previously described. Briefly, spleens were removed and cut into small pieces. Single-cell suspension was obtained from dissociation between frosted glass slides on ice. Cells were then treated with RBC lysing solution (0.15 M NH~Cl, 1.0 mM KHCO3, and 0.1mM Na2 EDTA) for 5 minutes to eliminate erythrocytes and resuspended at 1x 10^7 cells/ml in sorting buffer. (1% FBS, 1mM EDTA, 25mM HEPES in PBS)

3.2.7 Flow cytometry analysis

For flow cytometry analyses, cells were blocked (10^7/ML) with anti-FcR (5µg/mL) for 5 minutes. Cells were then stained with 1 µg/mL of FITC-, phycoerythrin (PE)-, or Alexa648-conjugated mAb, including anti-ly6G (eBioscience), CD11b (eBiosciences) and F4/80 (eBiosciences) for 30 min, and washed 3 times with staining medium. All processes were handled on ice. Flow cytometry data was acquired on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Data were analyzed with Flowjo.
3.3   Results

3.3.1   Transcription factor binding site for PGRP-S promoter

In order to define the correct PGRP-S promoter sequence, we first analyzed the putative promoter binding site using TRANSFAC Transcription Factor Binding Sites Database with 5kb genomic sequence preceding the start codon (Figure 3-2). According to the database query, there were a total of 11 transcription factor binding sites. Among them, there are three HNF-4, two NFkB, two c-Rel transcription factor binding sites, and one site each for AP-1 HNF-3, FOXD3 and NKx2-5. Since PGRP-S is mainly expressed in leukocytes and M cells, it is possible that factors involved in inflammation and infection may play an important role in driving PGRP-S expression. We therefore decided to use a 4kb sequence upstream of the starting codon of PGRP-S, containing all of the above mentioned transcription factor motifs, as our promoter sequence.

3.3.2   Neutrophils in transgenic mice express dsRedE2 in vivo

Since PGRP-S is highly expressed in the leukocytes and their precursors, we examined the expression of dsRedE2 in the neutrophil in the newly established transgenic mice. Among the 6 transgenic mice line that were being studied, two of them (named as E and F line) expressed strong red fluorescence in circulating neutrophils or "polymorphonuclear cell”. The red fluorescence was mainly seen in the cytoplasm. To confirm this finding, we examined splenic sections. In the spleen, polymorphonuclear cells specifically expressed dsRedE2 Moreover, the macrophage marker Iba1 did not bind to dsRedE2 expressing cells. (Figure 3-3).
The specificity of dsRedE2 expression in both the E and F lines was confirmed by flow cytometry. Peritoneal cells and splenocytes were collected for flow cytometry analysis. As compared with wild type BALB/c, most Ly6G\textsuperscript{+}CD11b\textsuperscript{+}F4/80\textsuperscript{-} cells from transgenic mice expressed red fluorescent protein, although some remained negative (Figure 3-4A), suggesting there maybe subset of the neutrophil population that does not express PGRP-S \textit{in vivo}. Greater red fluorescent protein expression was observed in the F as compared to the E line. However, when comparing the ratio of neutrophils and macrophages in the spleen, the F line showed a significantly lower ratio of neutrophils to macrophages compared with wild type mice (1.57 in F line vs 1.95 in WT) (Figure 3-4B). Moreover, in the F line, in the peritoneal cavity, the neutrophil population was completely missing (Figure 3-4C), suggesting a potential toxicity associated with high expression of dsRedE2. Meanwhile, identification of dsRedE2 expression cells, confirms that they are almost exclusively neutrophils, except a small number of macrophage/myeloid lineage cells (F4/80\textsuperscript{+}) (Figure 3-4D).

3.3.3 \textbf{DsRedE2 expression in M cells}

To check the dsRedE2 expression in the M cells in the transgenic mice, Peyer’s patch and NALT were collected. Red fluorescence was strong in nearly all UEA-1-staining epithelial cells in Peyer’s patch follicle epithelium. The red fluorescence signal was cytoplasmic, with UEA-1 binding at the apical surface (Figure 3-5). More importantly, the red fluorescent protein was only found in Peyer’s patch, but not in villi (data not shown), further evidence for Peyer’s patch M cell-specific expression of PGRP-S. Meanwhile, in NALT, the expression pattern is slightly different from Peyer’s patch.
In NALT, almost all the UEA-1-staining epithelial cells express red fluorescence. However, the red fluorescent was not restricted to the UEA-1\(^+\) M cells, but can be seen in other epithelial cells (Figure 3-5), though the identity and features of these UEA-1\(^-\) DsRedE2\(^+\) cells are still unclear.

3.4 Discussion

The lack of universal and specific M cell markers \textit{in vivo} have greatly hindered the advancement of studies on M cell function and development. Thus, the establishment of \textit{in vivo} models to study M cell functional mechanisms is of crucial importance and need. We have addressed this need by successfully establishing a transgenic mouse that contains both a PGRP-S promoter and dsRedE2 reporter gene that has the capability to specifically label the cytoplasm of M cells \textit{in vivo}.

PGRP-S is a member of a pattern recognition molecule family, and has mainly been expressed as soluble protein in neutrophils. Recently, PGRP-S has been shown to be specifically expressed in both M cells as well as other epithelial cells along the mucosal surface\(^23\). The main function of PGRP-S is bactericidal. Dziarski \textit{et al} showed that PGRP-S KO mice are more susceptible to infection by gram-positive bacteria. Moreover, Dziarski \textit{et al} concluded that this increase in susceptibility was not due to decrease of bacterial uptake, but rather a deficiency in the polymorphonuclear cells’ ability to kill and digest the ingested bacteria\(^24\). Given that polymorphonuclear cell are one of the main components in innate immune responses, together with the idea that mucosal epithelial
cells are highly susceptible to various bacterial infection, it is not all together surprising that PGRP-S at are expressed at these sites.

Since PGRP-S is expressed in the cells that uptake various pathogens, as well as playing an important role in innate immunity, it is reasonable to expect that the expression of PGRP-S is controlled by both inflammation and infection. In fact, most of the transcription factor-binding sites of PGRP-S promoter are directly related to inflammation, including NF-kB family members and AP-1. Two NF-kB and two c-Rel binding sites are contained in the PGRP-S promoter sequence, all belonging to the NF-kB family. A wide range of inflammation related stimuli, including pathogens, pro-inflammatory cytokines carry out activation of the NF-kB family. Similar to the NF-kB family, the AP-1 family is also comprised of several inflammation-related proteins, including Jun, Fos, ATF subfamilies. Besides their well-known function in cell proliferation, differentiation\textsuperscript{25} and transformation\textsuperscript{26}, the AP-1 family responds to bacterial and viral infection by triggering the production of cytokines such as IL-1\textsuperscript{27} and TNFα\textsuperscript{28} during immune response. Moreover, the activation of AP-1 can further regulate and enhance the expression of these inflammatory mediators\textsuperscript{29}, and form a positive feedback loop between AP-1 and inflammation.

Additionally, the PGRP-S promoter also contains binding sites for Hepatocyte nuclear factor (HNF). Specifically, the PGRP-S promoter contains three HNF-4 binding sites and one HNF-3 binding site. HNF-4 has been shown to be expressed mainly in liver, kidney and gut and plays a role in promoting intestinal epithelial differentiation\textsuperscript{30}. Furthermore, it has been suggested HNF-4 actually instructs the intestinal epithelial cell’s
to express a specific phenotype expression\textsuperscript{31}, and may drive the PGRP-S expression in M cells. Meanwhile, HNF-3, also named as FOXM1b or FoxA2, is a proliferation-associated transcription factor\textsuperscript{32,33}; it regulates genes that control proliferation at all stages, and may also regulate PGRP-S expression at different stages of proliferation.

Although all these transcription factors discussed above may be important in PGRP-S expression in M cells or neutrophils, they may also play distinct roles. NF-kB and AP-1 is universally expressed in various cell type, suggesting they may be required for PGRP-S expression both in M cells and neutrophils. However, c-Rel expression is restricted to haematopoietic cells and lymphocytes, indicating that c-Rel may be a neutrophil specific transcription factor for PGRP-S expression. Meanwhile, HNF-4 is known to be expressed in intestinal epithelial cells, liver and kidney however, it is not seen in neutrophils, indicating that HNF-4 maybe a M cell specific transcription factor. The role of HNF-3 still remains unknown: one study suggested that it may play a role in leukemia cell proliferation\textsuperscript{32}. It is possible that HNF-3 may serve as transcriptional factor in both neutrophils as well as M cells. However, the particular role of each transcription factor in PGRP-S expression is still undefined. To test the hypothesis, we need to examine the expression of these transcription factors in M cell and neutrophils. Moreover, deletion and mutation analysis can be used to identify the putative transcription element. Gel shift experiment can further confirm the binding of the transcription factor to the promoter.

Among all the transcription factors, it is likely some are more important than others, and different factors may play different roles at various situations. Unfortunately,
in our study, we did not perform promoter deletion experiment; instead, we took the whole 4kb sequence upstream of PGRP-S gene. Therefore, the specific role of single transcriptional sites remains unknown, and combination effects also remain unexplored. According to the known feature of each individual transcription factor, we speculate that HNF-3 or HNF4 may modulate PGRP-S expression during cell differentiation, NF-kB, c-Rel both function during inflammation and infection, while AP-1 be important in both situation. If so, it is possible that the expression of red fluorescent protein in neutrophils increases during bacterial challenge. This could be answered easily by flowcytometry in the future.

Although the specific sequence responsible for PGRP-S promoter activity is still unknown, the expression of red fluorescent protein in neutrophils and M cells indicates the 4kb sequence upstream of PGRP-S gene is sufficient to promote PGRP-S expression \textit{in vivo}. More importantly, with the red fluorescent protein labeling our transgenic mice offer a new opportunity to study neutrophil and M cell function under live imaging as well as \textit{in vivo} studies. The specificity of PGRP-S expression and the utilization of PGRP-S promoter may also provide a tool for studying individual gene function by specifically knocking down or knocking in genes of interest in neutrophils or M cells, which will be discussed in detail later on.
3.5 Reference:


Figure 3-1
Figure 3-1: PGRP-S-dsRedE2 construct

DsRedE2 coding sequence and inserted PGRP-S intron was added to downstream of the PGRP promoter fragment. The BGH polyA site fragment was attached to the end of the dsRedE2 coding sequence.
Figure 3-2
Figure 3-2: Transcription factor binding site for PGRP-S promoter.

There are a total of 11 transcription factor binding sites in what according to the MATCH program. These include three HNF-4 sites, two NFkB sites, two c-Rel sites and one site each for AP-1 HNF-3, FOXD3 and NKx2-5.
Figure 3-3: Neutrophil dsRedE2 expression in spleen and blood.

Confocal microscopy of splenic sections reveal polymorphonuclear cell expressing dsRedE2 (yellow arrows). And are stained for macrophages (Iba-1, green). Nuclei are shown in white to highlight polymorphic nuclei of neutrophils. Inset shows blood smear with lymphocyte (center) and two neutrophils (red, arrows), with nuclei shown in green.
Figure 3-4

A

CD11b

Ly6G

3.54

% of Max

0 10^1 10^2 10^3

DsRedE2

Wt, SP 2

F, SP 2

E, Sp 2

B

F4/80

ly6G

Wt

1.55

1.03

E

1.57

3.36

F

1.05

1.65

C

CD11b

Ly6G

Wt

0.97

E

1.04

F

0.672

D

SSC-H, SSC-Height

DsRedE2

CD11b

Ly6G

F4/80

4.62 91.2

4.97 91.6
Figure 3-4: Spleen and peritoneal neutrophil flow cytometry.

(A) Spleen cells gated on Ly6G⁺CD11b⁺ cells are positive for dsRedE2 fluorescence from PGRP-S-dsRedE2 mice but not control BALB/c mice. Cells from F line show higher expression of dsRedE2 than E line. Black, wild type Balb/c; red. E line; blue, F line. (B) F4/80⁺ and Ly6G⁺ F4/80⁻ cells in the spleen. Left panel, wild type Balb/c; middle panel, E line; right panel, F line. In F line, the frequency of Ly6G⁺F4/80⁻ cells is the lowest. (C) In the peritoneum, F line, Ly6G⁺CD11b⁺ cells are almost missing (right panel), while in wild type Balb/c (left panel) and E line (middle panel), there are around 1% of Ly6G⁺CD11b⁺ cells. (D) Cells from F line gated for dsRedE2 fluorescence show >90% staining for Ly6G, with a small population of Ly6G negative cells staining for F4/80 (probably macrophages) or CD11b. (E line shows the same pattern)
Figure 3-5
Figure 3-5: Expression of PGRP-S-dsRedE2 transgene in M cells of Peyer’s patch follicle and NALT epithelium.

(A) Peyer’s patch from PGRP-S-dsRedE2 mice shows that within the follicle epithelium, nearly all the UEA-1 (green) positive M cells express dsRedE2 fluorescence in the cytoplasm. (B) NALT from PGRP-S-dsRedE2 transgenic mice shows UEA-1⁺ NALT M cells (green) among epithelial cells expressing dsRedE2 (red).
Chapter 4: Convergent and divergent development among M cell lineages in mouse airway and intestine

Submitted, J. Wang, DD. Lo. The author of this thesis contributed to all the figures.
4.1 Introduction

M cells are specialized epithelial cells of the airway and intestinal mucosa that play a primary role in mucosal immune surveillance\textsuperscript{1,2,3}. Captured microparticles are transported (transcytosed) across the epithelium for uptake by underlying dendritic cells (DC) and stimulation of mucosal immunity. Most M cells are found in follicle-associated epithelium overlying organized mucosal lymphoid aggregates such as intestinal Peyer’s patches\textsuperscript{4} and Nasal Associated Lymphoid Tissues (NALT)\textsuperscript{5}. M cells are also present over inducible lymphoid aggregates such as Isolated Lymphoid Follicles\textsuperscript{6} in the intestine and Bronchus-Associated Lymphoid Tissue (BALT)\textsuperscript{7} in the lung. In addition, an inducible M cell population has been described at the tips of intestinal villi, called Villous M cells\textsuperscript{8}.

The regulation of M cell development and function are not well understood despite their assumed central role in mucosal immunity. In the constitutive mucosal lymphoid tissues, it would be reasonable to assume that lymphoid tissue inducer cells (LTi) responsible for the formation of Peyer’s patch\textsuperscript{9} and associated stromal cells are also involved in M cell development. Accordingly, LTi-type TNF and Lymphotoxin (LT) ligands have been reported to induce expression of genes in intestinal epithelium that were associated with follicle epithelium and M cells\textsuperscript{10}. Since the follicle epithelium turns over rapidly with a half-life of a few days\textsuperscript{11}, the regulation of M cell proportion among follicle epithelial cells requires dynamic regulation. Thus, the origin and induction of M cells has been subject to controversy. Various studies have supported the competing views that M cell induction is due to (a) a subset of crypt stem cells dedicated solely to M cell production\textsuperscript{12,13,14} or (b) transdifferentiation from existing mature enterocytes\textsuperscript{15,16}. The
situation becomes more complex in settings where immune stimulation by bacteria (e.g., *Strep. pneumoniae*)\(^\text{17}\) or their components (e.g., flagellin\(^\text{18}\) or cholera toxin\(^\text{19}\)) can induce rapid production of new M cells; here, the kinetics of induction challenge the idea that crypt stem cells directly account for all new M cell production.

Studies have suggested that M cell development in the intestine is dependent on B lymphocytes; indeed, a characteristic feature of Peyer’s patch follicle M cells is the presence of a basolateral pocket usually containing at least one B lymphocyte\(^\text{20,21}\). Co-cultures between Caco-2 intestinal epithelium and B lymphocytes have been shown to induce an M cell phenotype\(^\text{15,22}\), and mice lacking B lymphocytes appear to lack M cell transcytosis function\(^\text{21}\). However, in CD137-deficient mice that fail to develop M cell basolateral pockets and transcytosis function, the initial lineage commitment of progenitors to the M cell lineage appeared intact\(^\text{23}\). Thus, it is possible that M cell development is a two step process with the first lineage commitment step being B cell independent, followed by a step that is B cell-dependent.

The induction of the M cell phenotype is no less complex in the case of mouse NALT M cells. The apical membrane of intestinal M cells appears relatively membranous (hence the name “M” cell) compared to neighboring cells with an actin-based brush border. In contrast, airway M cells, also with a membranous apical surface, are surrounded by cells with long tubulin-based cilia\(^\text{5}\). Instead of a rapid turnover and crypt stem cell origin, airway epithelial cells are generated from a dispersed population of basal cells, and have half-lives on the order of weeks to months\(^\text{24,25,26,27}\). Despite their
distinct origins and turnover, airway and intestinal M cells show strikingly similar phenotype and functions, suggesting that overlapping genetic programs are induced.

In our studies here, we showed the convergent and divergent phenotype between NALT, Peyer’s patch and villous M cells in terms of PGRP-S expression and M cell-DC interaction. And we confirmed that the differentiation of M cells in vivo are toward strictly defined phenotypic subsets, and is consistent with functional specialization.

4.2 Materials and Methods

4.2.1 Animals

PGRP-S-dsRedE2 mice are established as previous described in chapter 3. B cell-deficient mutant mice on the BALB/c background (C.129S2-Igh-6tm1Cgn/J; referred to as “Igh-6” in this report) and BALB/c mice were obtained from The Jackson Laboratory. CD137-deficient mice on the BALB/c background were provided by Dr. B.S. Kwon. All mice were bred in the UC Riverside vivarium under SPF conditions and were handled in accordance with institutional IACUC and NIH guidelines.

4.2.2 Cholera toxin administration

Cholera toxin (Calbiochem) was reconstituted in PBS to a final concentration of 1µg/µl. Mice were starved for 12 hours before cholera toxin administration. 15 µg of cholera toxin19 was delivered either by gavage (in 200µl PBS) or intranasally (in 20µl PBS, 10µl each side). After 48 hours, mice were humanely killed and dissected for analysis.
4.2.3 Nucleoside analogue labeling and staining

Mice were injected i.p. with 200 µg of the nucleoside analogue 5-ethynyl-2’-deoxyuridine (EdU, Invitrogen) in 200 µl PBS at the same time cholera toxin was administered. Small intestine, Peyer’s patch, and NALT were harvested at 48h after injection. Dissected tissues were fixed in 4% formaldehyde/PBS for 15 min, followed by washing with 3% BSA/PBS twice. Tissue was then permeabilized with 0.5% Triton X-100/PBS for 20 mins and blocked with 6% BSA/PBS for another 30 mins before staining with Alexa fluor 488 azide click it reaction cocktail (Invitrogen) for 30 mins at room temperature. Tissue was washed with 3% BSA/PBS twice again, and used for immunohistochemistry staining as described below.

4.2.4 Immunohistochemistry and Confocal microscopy

For cryostat sections, dissected NALT or Peyer’s patch tissue was fixed in 4% paraformaldehyde/PBS, flash-frozen for cryostat sections and stained in sections. For whole tissue mounts, dissected tissues were fixed in 4% paraformaldehyde/PBS and then stained in whole tissue fragments. For both cryostat sections and whole mounts, tissues were then permeabilized in PBS, 0.1% Tween and blocked in 0.1% Tween in Casein solution. After that, tissues were stained with antibodies to glycoprotein 2 (Medical and Biological Laboratories) and Rhodamine, Fluorescein or biotinylated Ulex Europaeus Agglutinin 1 Lectin (UEA-1) (Vector), followed by secondary reagents Alexa Fluor 488 conjugated anti-rat antibody, or streptavidin conjugated Alexa Fluor 647. Post-fixation was done with 4% paraformaldehyde/PBS. Tissue was then mounted with Prolong Gold antifade reagent (Invitrogen). DAPI was used as a nuclear counterstain. Images were
obtained using a spinning disk BD CARVII Confocal Imager (BD Biosystems) on a Zeiss Axio Observer inverted microscope. Images were acquired using the 20x objective. Hardware control, including microscope, confocal and digital camera (Diagnostic Instrument Xplorer – XS) was done using BD IPLab Imaging Software. Image Z resolution was further optimized with Volocity software (PerkinElmer). M cell numbers were counted for every image and then normalized by surface area, which was measured by Volocity software.

4.2.5 Salmonella uptake in vivo

The attenuated *aroA S. typhimurium* strain transformed with the pnirGFP plasmid was kindly provided by C. Nagler, University of Chicago. Mice were gavaged with either PBS or cholera toxin 48 hours prior to the uptake studies. For analysis of uptake, mice were anesthetized by i.p. injection of 500 µl of Avertin (1 to 50 in warm PBS). Segments (10-15 cm long) of the small intestine of BALB/c or CD137KO mice were ligated at both ends with surgical thread and GFP-expressing bacteria (1 × 10⁹) suspended in 200 µl PBS were directly injected into the lumen. 30 minutes later, Peyer’s patch and the intestinal segments (without Peyer’s patch) were removed, extensively washed with PBS, then fixed in 4% paraformaldehyde and processed for confocal microscopy as described above.

4.2.6 Scanning Electron Microscopy (SEM)

For Scanning EM, tissues were dissected and fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences) for 3h and 1% OSO4 (Thermo) in PBS for anther 3h.
Tissue was then prepared by critical point drying and gold sputter coating. Samples were examined with an FEI XL30 field emission scanning electron microscopy (SEM) at 5 kV.

4.2.7 Statistics

Comparison of M cell numbers between cholera toxin treated and non-treated mice were performed using 2-tailed, non-parametric Mann-Whitney test. Comparison of M cell numbers among non-treated, cholera toxin gavaged, and cholera toxin intranasally treated mice were performed using one way ANOVA (non parametric) Kruskal-Wallis test followed by Dunn’s multiple comparison post test. All statistical analyses were performed using Prizm software (GraphPad).

4.3 Results

4.3.1 Expression of PGRP-S-dsRedE2 transgene in M cells of Peyer’s patch follicle epithelium and Nasal Associated Lymphoid Tissue

One challenge to the study of M cell development and function has been the lack of genetic markers of M cell differentiation. In the mouse, identification has depended on the lectin UEA-1 specific for fucose displayed largely on the apical surface of M cells. A monoclonal antibody against fucose has provided better specificity; though the association with a specific fucosyltransferase gene has been described, it is not yet clear that this gene’s expression is unique to M cells. We recently established a transgenic mouse line with PGRP-S promoter to drive expression of a reporter red fluorescent protein transgene, and showed that red fluorescence was strong in nearly all UEA-1-staining epithelial cells in Peyer’s patch follicle epithelium (Figure 4-1A,B) and Nasal
Associated Lymphoid Tissue epithelium (see below). This fluorescence correlated well with staining for gp2, another Peyer’s patch M cell-associated gene. At the margins of the follicle epithelium, newly generated M cells migrate from the adjacent intestinal crypts, so early M cell lineage commitment and maturation likely occurs in this zone. Here, the red fluorescence only slightly preceded the appearance of gp2 staining (Figure 4-1B), suggesting that these two genetic markers of M cell development are closely coordinated.

4.3.2 Peyer’s patch M cell lineage commitment and PGRP-S reporter expression is B lymphocyte- and CD137-independent

We recently reported that in CD137-deficient mice, M cell lineage commitment (i.e., appearance of UEA-1+ cells) appeared to be intact, but the characteristic B lymphocyte basolateral pockets failed to form, suggesting a requirement for a CD137-CD137L signal between M cells and B lymphocytes for pocket formation. This was associated with a failure to develop particle transcytosis function in both Peyer’s patch and NALT. This interaction between M cells and B lymphocytes is also a likely contributor to the observation that in B cell-deficient mice, M cell function is absent.

We backcrossed the PGRP-S-dsRedE2 transgene to CD137-deficient (Figure 4-2A) and B lymphocyte deficient Igh-6 (Figure 4-2B) mice and confirmed that UEA-1+ cells were still present in Peyer’s patch follicle epithelium. Due to the lack of B lymphocyte formation of basolateral pockets in both backcrosses, the UEA-1+ cells were unusually narrow; however, all of them were positive for cytoplasmic dsRedE2 expression. Thus, the M cell specific PGRP-S gene was still induced in UEA-1+ cells.
despite the lack of basolateral pocket formation and M cell transcytosis function in both knockout models. This result supports the interesting possibility that final acquisition of M cell transcytosis function could be dependent more on cytoskeletal changes associated with the formation of the basolateral pocket rather than activation of an M cell specific genetic program.

4.3.3 M cell - DC interactions suggest functional units in Peyer’s patch

While M cell functional development may be dependent on interaction with basolateral pocket B lymphocytes, their main role in immune surveillance is in providing luminal antigens to the Peyer’s patch antigen presenting cells. Therefore, to examine the relationship between M cells and underlying DCs, we crossed the PGRP-S-dsRedE2 transgene to the CX3CR1-EGFP reporter transgene that expresses a green fluorescent protein in DC\(^{30}\). Because the dsRedE2 and EGFP proteins fill the cytoplasm of the M cells and DC, their full morphology can be visualized by confocal microscopy. Interestingly, each red fluorescent Peyer’s patch M cell showed a specific association with an adjacent underlying green fluorescent DC, and dendritic processes can be identified extending up along the basolateral pocket of many M cells, where the local contact points between the cells appear as yellow fluorescence (Figure 4-3). This one-to-one association suggests the formation of an M cell-DC functional unit, though it is not yet clear if this association is stable over time, or changes with immune activation.
4.3.4  Cholera toxin induces de novo M cell transdifferentiation from enterocytes on Peyer’s patch

Several immune stimuli can activate the generation of new M cells within hours to days. This phenomenon has been controversial, with various authors arguing that the induction is due to (a) rapid activation of M cell production from crypt stem cells, (b) acute transdifferentiation from mature enterocytes, or alternatively, (c) increased activity of existing M cells. It is possible that under different experimental conditions, one or more of these mechanisms may be acting at the same time. In our studies we focused only on the actual numbers of UEA-1+ M cells across the Peyer’s patch follicle epithelium, and we found that cholera toxin was potent in inducing the development of 30-50% more M cells across the Peyer’s patch follicle epithelium within 48 hours (Figure 4-4A-C). Despite the greatly increased numbers of M cells in the follicle epithelium, they maintained a dispersed distribution across the epithelium, in contrast to the clustering of M cells seen with villous M cells (see below). Interestingly, the effect of cholera toxin on intestinal M cells was similar whether the cholera toxin was administered intranasally or orally (Figure 4-4C).

The induction of higher numbers of M cells by cholera toxin was not significant in the context of the CD137KO; however, here the non-treated M cell levels were already considerably higher than the background levels on wild type BALB/c (Figure 4-4D), suggesting that the knockout was associated with some chronic stimulation of M cells. In the case of B cell-deficient Igh-6 mice, cholera toxin induction of new M cells over follicle epithelium was clearly evident despite the absence of B cells or CD137 (Figure 4-
Thus, cholera toxin induction of new M cell lineage cells was largely along the lines of first step lineage commitment, independent of B cells.

The induction of new M cells was rapid, but sustained, consistent with the normal half-life of intestinal epithelium. The rapid increase in the number of M cells was consistent with the possibility that these new M cells were generated from follicle enterocytes already expressing a mature brush border. To examine this question, we administered cholera toxin along with the nucleoside analogue EdU. Within the 48 hours of the M cell induction, EdU uptake was clearly evident within the neighboring intestinal crypts, but not in any of the follicle epithelial cells further from the crypts, including the UEA-1+ dsRedE2+ cells (Figure 4-4F). Thus, the newly differentiated M cells appeared to arise by direct transdifferentiation from mature enterocytes.

4.3.5 Phenotypic and functional differences in cholera toxin-induced villous M cells

The apparent transdifferentiation of mature enterocytes to an M cell phenotype is especially prominent in the case of villous M cell development. In untreated mice, villous M cells are infrequently present at the tips of the intestinal villi (Figure 4-5A), but in response to cholera toxin, villi with clusters of UEA-1+ M cells are more easily identified (Figure 4-5B). Considering their location exclusively at the tips of villi, it was unlikely that they were newly generated from crypt stem cell precursors; accordingly, cholera toxin-induced villous M cells did not label with EdU during the induction period (Figure 4-5B), suggesting that these are also the product of transdifferentiation from mature enterocytes. Scanning electron microscopy confirmed the loss of brush border microvilli
(Figure 4-5A,B). In contrast to Peyer’s patch follicle M cells, they were consistently found in clusters rather than dispersed as single M cells, and there were few contacts with underlying DC (Figure 4-5C). Consistent with the differences in gene expression phenotype, the PGRP-S-dsRedE2 transgene was also not expressed in these M cells (Figure 4-5C). As with follicle M cells, cholera toxin induction was not dependent on the presence of B cells or CD137 (not shown).

The striking differences between follicle and villous M cells in their location, dispersion (i.e., singly versus clustered), and gene expression, are also evident in their function. Thus, when cholera toxin-induced M cells were tested for uptake of fluorescent Salmonella, the Peyer’s patch follicle M cells (Figure 4-6A) appeared to be far more efficient than villous M cells (Figure 4-6B). This functional difference may be due to several factors, such as the expression of the gp2 protein only by follicle M cells, which has been shown to bind fimbriae of some gram negative bacteria.

**4.3.6 NALT M cells express PGRP-S-dsRedE2 transgene and can also develop from Foxj1+ ciliated progenitors**

M cells are not unique to the intestine; in the mouse, they also are present in airway epithelium over NALT, and as with intestinal M cells, they present a membranous apical surface among a bed of ciliated epithelial cells. Thus, airway and intestinal M cells should show similarities in their development and genetic program. Similar to intestinal M cells, we found that NALT M cell initial lineage commitment is also independent of B cells and CD137. However, in view of the different developmental origins of M cell populations in the airway versus intestine, the M cell phenotype may be viewed as a
convergent development toward a common functional phenotype. For example, in contrast to intestinal epithelium, airway epithelial cells are generated from basal cell precursors\textsuperscript{24,25}, which give rise to both ciliated airway epithelium and Clara cells. Ciliated airway epithelium express the transcription factor Foxj1, associated with terminal differentiation, and they have a very long half life, on the order of weeks or months\textsuperscript{24,26,27,32}. In response to chemical injury, new Clara cells are quickly formed, and evidence suggests that they come from the basal cells and not from transdifferentiation of mature Foxj1+ ciliated epithelium. Although this has not been directly studied in the case of NALT epithelium, we expected similarities in Foxj1 expression and epithelial cell turnover.

As with the intestine, the PGRP-S-dsRedE2 reporter was expressed in a subset of airway epithelium, but expression was not limited to UEA-1\textsuperscript{+} cells (Figure 4-7A). cholera toxin can also induce rapid development of new M cells in the NALT epithelium (Figure 4-7B), and here too transdifferentiation from neighboring mature epithelial cells may be involved. In Foxj1-EGFP transgenic mice\textsuperscript{27}, NALT ciliated epithelial cells expressed the EGFP reporter, while UEA-1\textsuperscript{+} M cells were all negative for EGFP (Figure 4-7C). cholera toxin treatment significantly increased the number of NALT M cells, and they were all EGFP negative (Figure 4-7D). Expression of the PGRP-S-dsRedE2 transgene in the NALT was different from the intestine, as many dsRedE2\textsuperscript{+} cells were UEA-1 negative, suggesting that they were not mature M cells (Figure 4-7C). Interestingly, Foxj1-EGFP/PGRP-S-dsRedE2 double transgenic mice, numerous UEA-1 negative cells were found expressing both EGFP and dsRedE2, showing yellow fluorescence (Figure 4-7C).
When these mice were induced by cholera toxin, these double expressing cells seemed to be replaced by new NALT M cells (Figure 4-7D,E), as the cell counts showed that the double expressing cells were similar in number to the increased number of new M cells (Figure 4-7E). Moreover, none of the cells labeled with EdU (not shown), so proliferation of progenitors was unlikely. Thus, in contrast to the injury-induced production of Clara cells, at least some of the NALT M cell production may come from mature progenitors expressing both PGRP-S and Foxj1.

4.4 Discussion

The results of our studies here, along with other recent reports, support a hypothesis that mucosal M cells can be categorized into at least three distinct phenotypes: Peyer’s patch follicle epithelium M cells, inducible villous M cells, and Nasal Associated Lymphoid Tissue follicle epithelium M cells. A recent report identified a fourth type of M cell, a “respiratory” M cell, present in the upper airway, likely to be analogous to the villous M cell. Given the distinct morphological and functional differences among the phenotypes, we propose to define broad categories of M cells as “constitutive” (Peyer’s patch and NALT) versus “inducible” (villous and respiratory). The constitutive subsets are found on constitutive mucosal lymphoid tissues, and they appear to form functional units with underlying lymphoid tissue DCs. Despite the differences in their developmental origins and life span, one characteristic shared by these cells is their dispersed single-cell distribution across the face of the follicle epithelium. In addition,
they expressed a subset of specific genes that appear helpful to function in microbial uptake, including PGRP-S\textsuperscript{33} and gp2\textsuperscript{28}.

By contrast, the inducible subsets are scattered in various sites along the mucosal epithelium, not associated with organized lymphoid tissues. Because of their scattered distribution, it can be difficult to provide consistent quantification, but it appears that inducible M cells are dependent on immune stimuli to develop in significant numbers. This is probably driven in part by the inducible stromal cell expression of RANKL, as direct administration of RANKL was very effective in inducing significant numbers of villous M cells\textsuperscript{34}. Due to the appearance of inducible villous M cells away from lymphoid tissues, any associations with underlying DCs appeared random at best. Interestingly, in contrast to the dispersion of constitutive M cells, the inducible M cells were found in dense clusters, such as at the tips of intestinal villi. Finally, some function-associated genes (PGRP-S, gp2) were not expressed by these inducible cells, and this appears to correlate with some reduced transcytosis function.

The situation becomes more complex as we consider the acute induction of new M cells by cholera toxin treatment. The numbers of M cells belonging to both the constitutive and inducible subsets increased significantly. However, the striking result here was that the phenotype of the new Peyer’s patch and NALT M cells conformed strictly to the local phenotype; thus, new Peyer’s patch and NALT M cells all expressed the PGRP-S-dsRedE2 transgene, while new villous M cells were all negative for the transgene expression. Similarly, the dispersed single-cell distribution of M cells across
the face of the Peyer’s patch and NALT epithelium was retained despite the increased density.

The cholera toxin-inducible M cells in both airway and intestine all appeared to be rapidly produced without evidence for cell division (i.e., EdU uptake). The simplest explanation for the development of these cells is that they are the product of transdifferentiation from mature enterocytes or ciliated airway epithelium. Interestingly, the finding of double expressing Foxj1-EGFP/PGRP-S-dsRedE2 cells in the NALT epithelium raises the possibility that latent M cell precursors may already exist among mature mucosal epithelium. That is, a subset of differentiated ciliated airway epithelium may specifically retain the ability to convert to an M cell phenotype, losing their apical cilia. Unfortunately, there is no evidence for a similar follicle epithelium subset on Peyer’s patch, since all PGRP-S-dsRedE2 expressing epithelial cells were already also UEA-1⁺.

An important implication of the distinct phenotypes and functions of the constitutive versus inducible M cell subsets is that the differential uptake of specific types of microbes will change as the balance of M cell subsets changes. Since various inflammatory and infectious conditions (such as the microbial components described above) can trigger the production of the inducible phenotype, the nature of the mucosal immune response can change as local tissue responses change. There are two principal direct differences in the contributions of constitutive versus inducible M cells: First, since inducible M cells are not directly associated with underlying DCs and organized lymphoid tissues, uptake through these cells is far less likely to promote the production of
secretory IgA, as the isotype switching of B cells is mainly associated with the organized mucosal lymphoid tissues\textsuperscript{35,36}. Second, the inducible M cells do not express the sets of genes associated with selective microbe uptake (e.g., gp2 and PGRP-S), so despite their relative inefficiency, they will enable a different array of microbes to cross the mucosal barrier. Since any potential immune response is more likely to be initiated in draining lymphoid tissues such as the mesenteric lymph node, production of secretory IgA is likely to be less prominent, with more dependence on IgG and T cell responses.

While our studies were concerned with microbial (e.g., cholera toxin) triggers of M cell development, other settings involving mucosal inflammation (and TNF/LT cytokines) should also have an effect on the various M cell subsets present. However, since clear genetic markers for M cells have not been available until recently (at least for mouse), this question has not been closely studied in situations such as models of inflammatory bowel disease or ileitis. Here, the PGRP-S-dsRedE2 transgenic strain should be useful in identifying differential induction of M cell subsets \textit{in vivo}. The differential microbial uptake by M cell subsets may have an important effect on the progression or persistence of intestinal inflammation, in part through the differences in the induced mucosal immune response. As the relationship between M cell function and mucosal immunity becomes more clearly defined, these questions will begin to be addressed.
4.5 Reference


Figure 4-1:
Figure 4-1: Expression of PGRP-S-dsRedE2 transgene in M cells of Peyer’s patch follicle epithelium.

(A). Peyer’s patch from PGRP-S-dsRedE2 mice showing that within the follicle epithelium, nearly all the UEA-1 (green) positive M cells express dsRedE2 fluorescence in the cytoplasm. (B). Peyer’s patch section showing UEA-1 positive (green) M cells expressing dsRedE2 in the cytoplasm (red, arrow). Relative to the neighboring crypt, apical UEA-1 staining appears slightly earlier than cytoplasmic dsRedE2 fluorescence (asterisk). (C). Peyer’s patch section showing cytoplasmic dsRedE2 correlating well with apical staining for gp2 (green, arrow), though with nearby crypt at the far left, dsRedE2 fluorescence (asterisk) slightly preceded the appearance of gp2.
Figure 4-2: PGRP-S reporter expression in CD137KO and B lymphocyte deficient Igh-6 mice.

CD137KO/PGRP-S-dsRedE2 (A) or Igh-6/PGRP-S-dsRedE2 (B) Peyer’s patch sections show UEA-1⁺ (Green) M cells expressing dsRedE2 fluorescence (Red) in the cytoplasm (arrow).
Figure 4-3
**Figure 4-3: M cell - DC association.**

Section of CX3CR1-EGFP/PGRP-S-dsRedE2 double transgenic Peyer’s patch follicle epithelium shows UEA-1⁺ (cyan) dsRedE2⁺ (red) M cells with EGFP⁺ DCs (green) under each M cell, where close contact appears as yellow fluorescence (arrows).
Figure 4-4
Figure 4-4: Cholera toxin induces de novo M cell differentiation from enterocytes on Peyer’s patch follicle epithelium.

Peyer’s patch from BALB/c mice shows UEA-1$^+$ M cell numbers before (A) and after (B) cholera toxin treatment. SEM images below show M cells on Peyer’s patch follicle epithelium of BALB/c mice before (A bottom) and after cholera toxin (B bottom). M cells are apparent as cells lacking brush border microvilli. (C), Peyer’s patch follicle M cell density in control (n=42), cholera toxin gavaged (n=50) and cholera toxin intranasally delivered (n=24). Each measurement represents one follicle, three mice per treatment group. M cell density is significantly increased in the treated groups (P<0.001). (D), CD137KO Peyer’s patch follicle M cell density in control (n=41) versus cholera toxin gavaged (n=47). (E), Igh-6 mice Peyer’s patch follicle M cell density in control (n=12) versus cholera toxin gavaged (n=12). Here, cholera toxin induced a significant increase in M cell density (P=0.0194). (F) Peyer’s patch from cholera toxin-treated BALB/c mice given EdU at the same time. EdU (red) labeled nearby crypt cells (arrow) but not the UEA-1 positive (green) M cells.
Figure 4-5
Figure 4-5: Cholera toxin induces villous M cell differentiation from enterocytes. (A), Villous epithelium from untreated BALB/c mice stained with UEA-1 (green) showing villous M cells infrequently at the tips of the villi. Blue: nuclei. (B), Whole mount villous epithelium from BALB/c mice gavaged with cholera toxin, also injected with EdU at the same time. The picture shows increased numbers of UEA-1$^+$ villous M cells (green) clustered together at the villus tips. However, EdU (red) mainly labeled nearby crypt cells (arrow). SEM images of villous M cells in control villi (A bottom) and after cholera toxin (B bottom) showing villous M cells clustered at the tips of villi of mice. (C), Villous section from cholera toxin gavaged CX3CR1-GFP/PGRP-S-dsRedE2 double transgenic mice. UEA-1 positive villous M cells (green) did not express dsRedE2 fluorescence; only rarely contacting DCs (red, arrow). Grey: nuclei.
Figure 4-6
Figure 4-6: *Salmonella* uptake in Peyer’s patch and villous M cells.

*Salmonella* were administered into isolated intestinal loops of cholera toxin gavaged BALB/c mice. Peyer’s patch and villous sections show that UEA-1+ (green) Peyer’s patch follicle epithelium M cells (A) were more efficient than UEA-1+ (green) villous M cells (B) in terms of *Salmonella* uptake (red).
Figure 4-7

A. Image showing cellular structure with arrows indicating specific areas.

B. Graph comparing cell counts among different conditions: Control, CT Gavage, and CT IN. The graph shows a significant difference marked by ***.

C. Image with arrows highlighting specific cellular features.

D. Image showing a different cellular structure with 14µm scale.

E. Bar graph comparing cell counts: CT UEA-1, CT UEA-1 Foxp3+GRP, and CT UEA-1. CT UEA-1 shows a significantly higher cell count compared to the others.
Figure 4-7: NALT M cells can develop from Foxj1+ progenitors.

(A) NALT from PGRP-S-dsRedE2 transgenic mice shows UEA-1+ NALT M cells (cyan) among epithelial cells expressing dsRedE2 (red). (B) NALT M cell density in control (n=22), cholera toxin gavaged (n=20) and cholera toxin intranasally delivered (n=19). Each count represents one image, three mice per treatment group. M cell density was significantly increased in the treated groups (P<0.001). (C) NALT from Foxj1-GFP/PGRP-S-dsRed double transgenic mice. All the UEA-1+Foxj1negative NALT M cells (cyan) and some of the UEA-1negative Foxj1+ ciliated epithelial cells (green) expressed dsRedE2 (red, arrow). (D) NALT from Foxj1-GFP/PGRP-S-dsRed double transgenic mice gavaged with cholera toxin. After treatment, UEA-1+ NALT M cell numbers increased; moreover, there were no longer UEA-1Foxj1+ ciliated epithelial cells (green) expressing dsRedE2 fluorescence. (E) NALT M cell density count in Foxj1-GFP/PGRP-S-dsRedE2 double transgenic mice showing that UEA-1negative Foxj1-GFP/PGRP-S-dsRedE2 double expressing cells in control mice were similar in number to the increased number of new M cells in the cholera toxin gavaged mice. Counts were from individual confocal images (n=4 for UEA-1+ and UEA-1− untreated mice, n=6 for cholera toxin-treated mice), two mice per group.
Chapter 5: Conclusions and Perspectives
5.1 M cell differentiation and M cell model

Although M cells are critical in immune surveillance and the initiation of mucosal immune response, molecular studies on their development and differentiation pathway have been limited. Factors involved in M cell differentiation are complicated but not well understood. Many factors have been proven to be essential for the process, including TNFα⁴, LT²,³, RANKL⁴, microbes⁵, enterotoxin⁶ and B cells⁷, either in a direct or an indirect way. The progenitor cells that could differentiate into M cells are also controversial, with evidence for both crypt cell origin⁸,⁹,¹⁰,¹¹ and mature enterocytes origin¹²,¹³,¹⁴. In our study, we examined some of the factors implicated in M cell development in vitro and in vivo, and their role in M cell differentiation as well as function.

The ability of fully differentiated enterocytes to trans-differentiate into M cells is still under debate. In our studies (Chapter 2), we showed that enterocytes are able to transform into M cell-like cells both in vitro and in vivo. Caco-2 cells and the IEC-6 cell line were used for the in vitro models. These experiment showed that, with stimulation by TNFα and LTβR agonist, intestinal epithelial cells can exhibit at least some M cell features, including loss of microvilli; increased expression of M cell and FAE specific genes such as Ccl20, Gp2, Sgc5, Lamb3; redistribution of claudin4 and beta1 integrin; and enhanced uptake function. However, the Caco-2 cell line is a human carcinoma cell line that exhibits certain crypt cell characteristics¹⁴,¹⁵. Caco-2 cells have the potential to spontaneously differentiate into enterocytes, but this is dependent on the cell density as well as permeable supports¹⁶,¹⁷,¹⁸. Furthermore, the differentiation of Caco-2 cell exhibits
a mosaic pattern\textsuperscript{19,20,21}, indicating the lack of homogeneity of the cell population. Also, studies showed the differentiated Caco-2 cells actually resemble fetal but not adult enterocytes based on their enzymatic activity\textsuperscript{18}. All of these factors limit the utility of Caco-2 cells in representing the normal situation \textit{in vivo}. Moreover, the changing phenotype of enterocytes into M cells is not complete, with some of the M cell specific genes left unchanged, such as PGRP-S, though these may reflect species differences.

Therefore, we further tested the trans-differentiation ability of enterocytes into M cells \textit{in vivo} (Chapter 4). Not surprisingly, with the challenge of cholera toxin, the number of M cells significantly increased by about 30\%. Our \textit{in vivo} experiments further confirmed that fully differentiated enterocytes could trans-differentiate into M cells based on the following observations: First, the newly formed M cells are not labeled by nucleoside analogue EdU, indicating these M cells are not coming from cell division. Second, villous M cells are mainly found at the tip of the villi, suggesting these cells are not generating from crypt stem cells. Third, previous studies showed that following cholera toxin treatment the increase in M cell numbers peaks at 24h and gradually goes down until 72h\textsuperscript{6}. The fast increase of M cell numbers serves as another indirect proof for epithelial cell trans-differentiation.

We also examined factors that may be important in M cell differentiation. In addition to TNF\textalpha and LT\betaR agonist’s ability to transform Caco-2 and IEC-6 cells to M cell-like cells \textit{in vitro}, we also tested the effect of cholera toxin and B cells interaction on M cell differentiation \textit{in vivo} (Chapter 4). The result shows that cholera toxin can increase M cell numbers independent of B cells and CD137. This is consistent with
previous studies that B cells and CD137 are not required for M cell lineage commitment, but necessary for M cell function\textsuperscript{22,7}.

Combining our \textit{in vitro} and \textit{in vivo} studies outlining factors influencing M cell development. In the follicle (Figure 5.1), with cytokines from lymphoid tissue, crypt cells make their commitment to convert into M cell progenitors. The release of cytokines can either come from lymphoid tissue inducer cells during development or from other immune cells during inflammation with the help from various microbes. At the moment, in Peyer’s patch, the M cell progenitor, with Fut1-dependent fucose moiety production\textsuperscript{6}, binds to UEA-1 lectin, and may start to express CD137. Meanwhile, a subset of CCR6\textsuperscript{+} CD137L\textsuperscript{+} B lymphocytes, receiving signals from CCL20, migrate into the follicle epithelium, and meet the CD137 expressing M cell progenitor there, establishing a CD137/CD137L cross signaling. After that, the presence of the B cell, the cross signaling of CD137/CD137L, or other factors induce M cell maturation, including cytoskeletal changes (formation of the basolateral pocket), and the ability to transcytose various particles. At the same time, with external stimuli, such as \textit{Salmonella} and cholera toxin, fully differentiated enterocytes at FAE can trans-differentiate into M cells. A similar phenomenon can be seen in the villi. Under normal conditions, villous epithelium consists of enterocytes and goblet cells. Upon presentation of certain microbes or cholera toxin, these enterocytes can also turn into M cells. Interestingly, the newly formed M cells are usually found at the tip of the villi; the region where most of the microbes come
in contact. Both villous and Peyer’s patch M cell trans-differentiation may mediated by cholera toxin or microbe induced cytokine expression, possibly RANKL.

In NALT, there are subtle differences when compared with Peyer’s patch(Figure 5-2). NALT M cells arise from basal cells and have longer lifespan. Moreover, in normal NALT, there is an intermediate M cell type, which shares both the M cell and respiratory epithelial phenotype (when comparing the Foxj1 and PGRP-S expression). With stimulation, it is possible that this intermediate M cell loses its ciliated epithelial phenotype and differentiates into fully differentiated NALT M cells. The presence of this intermediate M cell type may lead to a faster response to various pathogens in the nasal passages. However, details of this intermediate M cells are still unknown. Whether they actually bear any M cell functions is still a mystery.

Knowing the essential factors involved in M cell development will be helpful in assessing and designing a valid in vitro M cell model. Currently, the most acceptable M cell in vitro model is co-culturing Caco-2 cells with Peyer’s patch B cells or Raji B cells. Unfortunately, this model shows only limited phenotypic similarities to M cells in vivo. In the intestine, besides categorizing M cells in to Peyer’s patch and villous M subtype, M cells can also be divided into inducible and constitutive M cell. Constitutive M cells reside in the FAE, and develop from dome associated crypt stem cells, while inducible M cells (usually villous M cell) are changing from fully differentiated enterocytes. However, in the Caco-2 co-culture system, the human adenocarcinoma cell line likely neither represent dome-associated crypt cells nor fully differentiated enterocytes. This restricts their ability to become either constitutive or inducible M cells. More importantly,
our results showed that in B cell deficient mice, both constitutive and inducible M cells were present before and after stimuli, indicating B cells are only required for maintaining M cell function, but not M cell lineage commitment. Other factors such as cytokines maybe more important in M cell development. Thus, B cells alone in the co-culture system are not sufficient to induce M cells in vitro.

Accordingly, a new in vitro model could be proposed. IEC-6 cells or Caco-2 cells will be cultured for rodent and for human M cell research respectively. TNFα and LTβR agonist mimicking the LTi factor will then be added into the media to induce development of M cells. B cells will be added 24 hours after cytokine treatment to maintain M cell function. The M cell phenotype can be examined by RT PCR to check for M cell specific gene expression, or by electron microscopy for morphology change. M cell function could also be examined according to transcytosis ability. Alternatively, since CD137 has been found to be important in M cell basolateral formation as well as transcytosis function, and is thought to be implicated in B cell/M cell crosstalk, it could be applied to the culture in the place of B cells. However, it is still unknown that whether the soluble or membrane bound form of CD137L is more important. Moreover, it is also possible that direct contact with B cells is required. These can all be tested in the in vitro model.

5.2 PGRP-S promoter in M cell function study

In addition to studying M cell development, we also designed a new transgenic mouse line with M cells labeled red in vivo. The mice bear a transgene with PGRP-S
promoter driven expression of red fluorescent protein dsRedE2. Peyer’s patch M cells but not villous M cells therefore specifically express dsRedE2 in these mice. In the NALT, red fluorescent protein can be seen both in M cells and some other epithelial cells. Furthermore, the expression of dsRedE2 in M cells is not restricted by M cell function. It was expressed immediately after crypt cells make the commitment to become M cells.

PGRP-S belongs to the pattern recognition molecule family and binds to the peptidoglycan of the bacterial cell wall. The main function of PGRP-S is bactericidal. It has been shown that PGRP-S can bind to the Gram-positive bacteria *S.aureus* as well as Gram-negative bacteria *E.coli*, and in turn inhibits *S.aureus* growth while having a synergistic antimicrobial effect against *E.coli*\(^{23}\). Since PGRP-S plays an important role in the innate immune response, it is not surprising to see PGRP-S mainly expressed in the neutrophil. PGRP-S’s expression in M cells may also correlate with its function. M cells are involved in sampling various pathogens. Expression of PGRP-S in the M cell may either serve as a receptor for M cell transcytosis, or to kill extracellular bacteria before they enter the follicle. Given that peptidoglycan is mainly expressed on the cell wall of Gram-positive bacteria, it is reasonable to suspect that nasal passages require more PGRP-S expression when compared with the intestine, since Gram-positive bacteria are usually involved in respiratory tract infection and Gram-negative bacteria for digestive tract infection. This correlates with our findings: in NALT, both M cells and some ciliated epithelial cells express PGRP-S.

PGRP-S promoter-driven gene expression specifically in Peyer’s patch M cells makes it a useful tool to examine the function of these cells and the differences between
Peyer’s patch M cells and villous M cells. To perform this experiment, we are going to use the Cre-\(loxP\) recombination system. We designed a construct as it is shown in figure 5-3. Briefly, the PGRP-S promoter is used to drive the expression of the gene. DsRedE2 with the PGRP-S intron is added right after the PGRP-S promoter. An inverted diphtheria toxin sequence is then connected to the 3’ of dsRedE2 construct. Before dsRedE2 and after diphtheria toxin sequence, two flanking loxp3 sites with opposite direction are inserted. The construct will then be injected into fertilized oocytes of CB6 F2 mice to establish the transgenic mouse line. Founders that be confirmed to express the transgene will then be crossbred to villin-Cre mice. Since villin is specifically expressed in the intestine, the expression of dsRedE2 in the neutrophil would not be affected. However, in the intestine, the Cre recombinase will mediate the inversion of the floxed transgene, and therefore leads to the expression of diphtheria toxin, and shuts off the expression of dsRedE2 (Figure 5-3). The expression of diphtheria toxin will then in turn specifically kill Peyer’s patch M cells. The mice then become Peyer’s patch M cell-deficient.

With the establishment of Peyer’s patch M cell deficient mice line, Peyer’s patch M cell function in mucosal immunity \textit{in vivo} can be checked in various aspects. For example, if we immunize the mice orally with an M cell targeted vaccine, and compare them with the wild type, the importance of M cells in IgA and IgG production can be evaluated. They can also be used to examine the role of Peyer’s patch M cells during infection. Moreover, M cells’ function in inflammatory bowel disease can be tested by inducing inflammatory bowel disease in Peyer’s patch M cell deficient mice. Furthermore, to understand villous M cell function, villous M cells can be induced in Peyer’s patch M
cell-deficient mice using cholera toxin. The function of villous M cells can then be tested independently. In addition, crossbreeding the transgenic mice with Foxj1-cre mice can generate NALT M cell deficient mice. However, since there is an intermediate stage of epithelial cell that also express PGRP-S in the NALT, they may be also killed by the expression of diphtheria toxin. This may affect the accuracy of the result.

Currently, there is no M cell KO model available. All M cell-deficient mice that have been generated are secondary lymphoid tissue deficient or Peyer’s patch deficient, and are usually lacking T cell or B cell function or deficient in certain cytokines, such as TNFα and LT. Since lymphocytes and cytokines play an essential role in generating a mucosal immune response, any immunology defect in these transgenic mice would not be caused only by M cells. However, with our model, we are measuring the pure M cell role in the immune response, since all lymphocytes and cytokines are kept intact in our mouse model. Furthermore, by crossingbreeding with different Cre mice, we are able to examine the function of M cells at different locations.

5.3 Possible application

Understanding M cell development will also be useful in developing effective mucosal vaccines. Since pathogens usually enter the body at epithelial barriers, manipulating immune responses at the inductive sites could therefore lead to better protection over parenteral immunization alone. For example, intranasal delivery of attenuated influenza in addition to trivalent inactivated influenza vaccine delivered parenterally to elderly patients in nursing homes significantly increases protection against
influenza. This could be explained by the production of secretory immunoglobulin A at the mucosal site. Mucosally delivered vaccines can generate an IgA response in addition to an IgG response. IgA will then be secreted into the mucosal surface, where it can then bind to invading pathogens. The protective role of IgA has already been shown in *Salmonella* and *Vibrio cholerae* infection. The rate of pathogen clearance is correlated with mucosal and serum IgA titers, which further confirms the importance of IgA in mucosal immunity. It is known that M cell transport plays a key role in induction of specific IgA lymphoblasts in Peyer’s patch. The efficacy of M cell-targeted vaccines has already been confirmed by UEA-1 (binds to α(1,2) fucose) and σ1 protein (binds to α(2,3) linked sialic acid) conjugated vaccines, which elicit strong antigen specific IgA and IgG production. With the understanding of M cell differentiation, mucosal immunization efficiency could potentially be further enhanced by increasing M cell numbers; this would also enhance the ability to transcytose antigen in vivo, which may leads to more IgA production, ultimately providing better protection.

It has been suggested that M cells may also play a role in inflammatory bowel disease. In Crohn’s disease, aphthoid lesions, one of the manifestations, are found to occur initially at the sites of lymphocyte accumulation. Moreover, erosions in the FAE can also be seen in Crohn’s disease using SEM. In addition, in spondylarthropathy-related ileitis inflammation patients, there is a significant increase in M cell numbers from 0-6% to 12-24%. Similar induction can also be seen in indomethacin-induced enteritis rats. Together, these indicate the possible importance of M cells in inflammatory bowel disease. Research has been hypothesized that the increased number
of M cells increases antigen and bacteria that penetrate the FAE layer, in turn amplifying the antigen-immune interaction. This initiates the inflammatory response, which will further increase M cell numbers and increase intestinal permeability that leads to inflammatory bowel disease\textsuperscript{38}. On the other hand, destruction of FAE and M cells may lead to inflammatory bowel disease, and induction of M cells may serve as a protective factor. M cells may also involved in regulating normal intestinal flora, which may be beneficial for preventing the development of inflammatory bowel disease. Therefore, understanding M cell development and factors involved in M cell induction as well as function may help to answer the question.
5.4 Reference


alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. J. Exp. Med 184, 1397-1411 (1996).


Figure 5-1
Figure 5-1: M cell development in the intestine.

In general, crypt cells give rise to both enterocytes and M cells. After the crypt cell makes the commitment of being an M cell with the influence of LTi, it becomes UEA-1+ CD137+. With the help of CCL20, a subset of B cell that is CCR6+CD137L+ moves to the M cell. The direct interaction of B cell/M cell, and the cross talk between B cell/M cell with CD137/CD137L promotes M cell maturation, including cytoskeleton change and gaining of transcytosis function. With the external stimuli such as cholera toxin, it will induce some unknown cytokine expression (possibly RANKL). The latter will then induce the trans-differentiation of enterocytes.
Figure 5-2

NALT

Foxj1+ PGRP-S cell

PGRP-S+UEA-1+ Mature M cell

Foxj1+ PGRP-S+ cell

Basal Cell

B cell

Cholera toxin

Basal Cell
**Figure 5-2: M cell development in NALT.**

Like the crypt cell in the intestine, basal cells give rise to the NALT epithelial cells, including ciliated epithelial cells and M cells. In addition, there is normally an intermediate ciliated epithelial cell in the NALT, which bear partial M cell features, exhibited as Foxj1+PGRP-S+. With cholera toxin stimulus, this intermediate cell can trans-differentiate into UEA-1⁺PGRP-S⁺ M cell, and lose its Foxj1+ phenotype. The factors involved in the pathway are yet to be determined, but are B cell and CD137L independent, and may include RANKL.
Figure 5-3.
Figure 5-3: PGRP-dsRedE2DT construct.

DsRed coding sequence and inserted PGRP-S intron was cloned following PGRP-S promoter. Flipped diphtheria toxin is then connected to the 3’ of dsRed construct. Opposite direction of flanking loxp3 sites was inserted before dsRed and after diphtheria toxin construct. After cross breed with Villin-Cre mice, DsRed-DT construct was inverted, diphtheria toxin will be expressed and leads to Peyer’s patch M cell’s death.