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TNFR and LTβR agonists induce Follicle-Associated Epithelium and M cell specific genes in rat and human intestinal epithelial cells

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Abstract

M cells assist mucosal immune surveillance by transcytosis of particles to underlying lymphoid tissue, but the mechanisms of M cell differentiation are poorly understood. To develop a better defined cell culture model of M cell differentiation, we treated human (Caco-2BBE) and rat (IEC-6) intestinal epithelial cell lines with lymphotoxin beta receptor (LTβR) and TNF receptor (TNFR) agonists. Treated cells were studied for regulation of genes associated with M cell and Follicle-Associated Epithelium (FAE). We found that LTβR and TNFR agonists induce transcription of FAE specific genes (*Ccl20* and *Lamb3*) in Caco2-BBE cells and IEC-6 cells as well as rodent M cell specific genes such as *Sgnt-1/Scg5*, *Cldn4*, and *Gp2*. The cytokines have distinct but complementary effects; TNFR agonists mainly induced FAE specific genes, while the LTβR agonist induced M cell specific genes. The combination of cytokines showed additive induction of the FAE-associated *Ccl20*, *Lamb3* and a surprising induction of *CD137/Tnfrsf9*. On the other hand TNF agonists appeared to suppress expression of some LTβR-induced genes. Functionally, cytokine treatment led to the reorganization of microvilli and Claudin-4 redistribution. These studies suggest complex interactions between these cytokines in the context of either inflammation or tissue differentiation.

Keywords

lymphotoxin; tumor necrosis factor; mucosal immunity; epithelium

1. Introduction

M cells are specialized epithelial cells at mucosal surfaces that are important in immune surveillance [1–5]. Their developmental origins are very similar to that of the neighboring epithelial cells including absorptive enterocytes and FAE [6–8], 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

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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 [9–14]. 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, 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 [15–19]. These studies were based on the fact that M cells in vivo have a prominent basolateral pocket containing lymphocytes, mainly B cells, which are thought to provide important differentiation signals [20–22]. 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/TNF family, since genetic deficiencies in these genes cause a loss of secondary lymphoid tissue development [23–25]. The in vitro models remain relatively poorly defined, as they are based only on co-cultures of epithelial cells and lymphocytes, whether in direct contact [15,16] or across transwell filters [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 *Salmonella* and *Pneumococcus* can induce either increased numbers of M cells in FAE [26–28], or increased transcytosis activity with constant numbers of M cells [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 [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 in vitro models of M cell development and function. In the present report, we tested whether lymphotoxin agonists, thought to be responsible for secondary lymphoid tissue development in vivo [23–25], 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. Materials and Methods

2.1. Cell lines and tissue culture

Caco-2BBE 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-2BBE cells) and 6 well cluster plates (for IEC-6 cells). Cytokines, including recombinant TNF α (100 nanograms/ml, Peprotech) and LT β R agonist antibody (5 micrograms/ml, R&D Systems) were added to the medium immediately after the cells were plated. For consistent results with the LT β R agonist antibody, a crosslinking secondary donkey anti goat antibody (1 micrograms/ml, Southern Biotech) was added at the same time.

2.2. Quantitative PCR

Caco2-BBe and IEC-6 cells were divided into four groups according to the treatment: the control group, a TNF α treated group, an LT β R agonist treated group and a combined TNF α and LT β R 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 μ g 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 μ l of the reverse-transcribed RNA samples (from a 20- μ l 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 $\Delta\Delta$ CT method was used to determine the relative amount of gene expression.

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 CLDN4 (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.

2.4. 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.

3. Results

3.1 TNFR and LT β R agonist induces expression of FAE-associated genes in rat IEC-6 and human Caco2-BBe 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 LT β R 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 [32] and human Caco2-BBe cells [31]) 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* [33,34] and *Lamb3* [10].

We found that TNF α can induce *Ccl20* expression in both rat IEC-6 cells and Caco2-BBe cells (Figure 1a, b). LT β R alone also increased *Ccl20* expression in IEC-6 cells, but not in Caco2-BBe cells (Figs 1a, 1b). This result contrasts with previously reported results by Rumbo et al. [12,33] which suggested that an LT β R agonist could stimulate *Ccl20* expression in T84 cells; however, they did not compare with the effects of TNF α . This difference may only reflect cell line specific differences between the T84 cell line and the more uniform and differentiated Caco-2BBe subclone [31], as the rat IEC-6 cells did respond to both LT β R and TNF α ; 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 [10]. In this case, cytokine treatment of IEC-6 cells resulted in increasing expression in response to TNF α , with a lesser response to LT β R agonist (Fig 1c). The NF- κ B gene *relB* (*Relb*) has also been shown to be constitutively expressed by FAE in vivo [34] and, as might be expected for triggering of TNFR superfamily genes, both LT β R agonist and TNF α induced *Relb* expression, with additive effects (Figure 1d).

We also found, surprisingly, that TNFR and LT β R agonists can induce expression of a TNFR superfamily gene *Tnfrsf9* in Caco2-BBe cells. CD137, the protein encoded by *Tnfrsf9*, previously had been associated mainly with activated T cells as a costimulatory molecule [35–41], but not with FAE. Our data shows that TNF α can significantly induce *Tnfrsf9* expression up to 60 fold, while LT β R agonists show a similar but slightly less potent effect. Moreover, the combined use of TNF α and LT β R agonists shows an additive effect (Figure 1e). Similar results were found for IEC-6 cells (not shown).

3.2 TNFR and LT β R agonists affect expression of M cell-associated genes in rat IEC-6 and human Caco2-BBe 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 *Sgnc1*/*Scg5*, *Gp2*, and *Pgrrp1*/*Pgylrp1* [9–11,13,14]. Their expression in human M cells is not well documented, so while we have also studied the human Caco-2BBe 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 β R agonist induced both *Gp2* and *Scg5* expression in IEC-6 cells. In contrast, TNF α showed an inhibitory effect at 24 hours and 48 hours, though this effect was lost by 72 hours (Figure 2a,b). The expression of *Pgylrp1* 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 *Cldn4*) was also shown to be induced in Caco-2 cells in cocultures with Raji cells and also in M cells in vivo [10]. Similarly, in Caco2-BBe cells, we found that TNFR and LT β R agonists both induced *Cldn4* expression with a possible additive effect, though TNF α showed a stronger effect than LT β R agonist (Figure 2c).

3.3 TNFR and LT β R agonist 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. TNF α appeared to be more important in inducing FAE specific genes, including *Ccl20* and *Lamb3* (though this also included *Cldn4*), while having an inhibitory effect on M cell specific genes such as *Gp2* and *Scg5* (Figure 3a). By contrast, LT β R agonist by itself induced M cell specific genes, such as *Gp2*, *Scg5* and to a limited degree *Pglyrp1* (Figure 3b). The combination of these cytokines showed additive effects in the induction of the FAE-associated *Ccl20* and *Lamb3* (Figure 3c).

3.4 TNFR and LT β R agonists lead to functional changes in IEC-6 and Caco2-BBe cells

Because the cytokine-induced gene expression changes may also be associated with functional changes, we have begun to examine these effects in Caco2-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-2BBe cells, we found that combination cytokine treatment prevented the development of brush border microvilli when examined by transmission electron microscopy (Figure 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 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 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 c-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 5g-j).

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 [20], other studies [17,19] 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 [19]. However, it is unlikely that MIF is by itself sufficient for M cell differentiation 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 [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 lymphotoxin beta receptor triggering, but the possible ligands for this receptor (lymphotoxin alpha1/beta2 heterotrimers, LIGHT) are transmembrane proteins so are unlikely to be provided in soluble form 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 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 lymphotoxin beta receptor 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 TNF, while an M cell gene subset (*Gp2*, *Scg5*) was most effectively induced by the LT β R agonist.

Curiously, the LT β R-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 (e.g., ref. [45]) in which chronic transgene expression of TNF or lymphotoxin 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- κ B activation, and the LT β R and TNF receptors all signal through NF- κ B activation, this finding is not so surprising; indeed, it has been reported that the FAE is constitutively positive for *Relb* expression in vivo [34]. In the case of *Tnfrsf9* however, this is an unusual finding, since *Tnfrsf9* induction is usually associated with T cell activation [35,37,38]. However, since B cells and dendritic cells 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 in vivo [10]. We recently reported that the same cytokine treatment of airway and intestinal epithelial cells also increased their capacity to endocytose bacterial particles [42], and in some cases Claudin-4 was associated with the endocytosis vesicles.

The gene expression changes observed here in response to lymphotoxin/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. [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 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.

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Abbreviations

LTαR	lymphotoxin beta receptor
TNFR	TNF receptor
FAE	Follicle-Associated Epithelium

Gene nomenclature

<i>Pglyrp1</i>	PGRP-S
<i>Scg5</i>	sgne-1
<i>Cldn4</i>	Claudin-4
<i>Tnfrsf9</i>	CD137
<i>Anxa5</i>	Annexin 5
<i>Lamb3</i>	Laminin beta 3
<i>Relb</i>	relB
<i>Ccl20</i>	CCL20

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Figure 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 TNF α in both Caco-2BBE (A) and IEC-6 (B) cells, with an additive effect of TNF α plus LT β R agonist in IEC-6 cells. Similar effects were also seen for *Lamb3* (C), *Relb* (D), and *Tnfrsf9* (E).

Figure 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 LT β R agonist, and appeared to be transiently suppressed by TNF α alone. *Cldn4* (C) showed weaker induction by LT β R versus TNF α .

Figure 3. Summary of specific cytokine effects in IEC-6 cells

Data shown is a representative experiment from three independent experiments. TNF α (A) and LT β R (B) show distinct patterns of gene induction, with additive effects on *Ccl20* and *Lamb3* (C).

Figure 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 TNF α and LT β R 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 as described in reference [42]. Scale bar: 2 microns.

Figure 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-2BBE cells. Microscopy images were taken with 40x objective at original magnification.

Table 1

qPCR primer pairs

Human Ccl20	For	5'-GCCAATGAAGGCTGTGACATCAA-3'
	Rev	5'-CACTAAACCCTCCATGATGTGCAA-3'
Human Tnfrsf9	For	5'-AGCAGAGTGCCTGAGTTTAGGGT-3'
	Rev	5'-CAGGACAAAGGCAGAAGGTGTGA-3'
Human Cldn4	For	5'-CCTGCTAGCAAGAACAGAGTCC-3'
	Rev	5'-TGCAGGCAGATCCCAAAGTCA-3'
Human Relb	For	5'-TGAACCACTGACACTGGACTCGT-3'
	Rev	5'-CTCGCGGTAATGATTGGGGAACA-3'
Human Gapdh	For	5'-CATGAGAAGTATGACAACAGCCT-3'
	Rev	5'-AGTCCTTCCACGATACCAAAGT-3'
Rat Hprt1	For	5'-CAGCGAAAGTGAAAAGCCAAGT-3'
	Rev	5'-TGCCTACAGGCTCATAGTGCAA-3'
Rat Gp2	For	5'-CCACGTTCTTCCTGCCTGTCTT-3'
	Rev	5'-CATGACATCGGCTGTCAGGACAA-3'
Rat Scg5	For	5'-CAGCTGTCCTTGTCTCCATAGCA-3'
	Rev	5'-CAGCACAACCCAAAACGGCA-3'
Rat Pgylrp1	For	5'-CGAGATGTGGAACTCTGT-3'
	Rev	5'-ATTTTCAGGGTGGGAACTCCACA-3'
Rat Ccl20	For	5'-ACAGCTGGGGTTGGAGGTTTCA-3'
	Rev	5'-AGCGCCCTTCATAGATTGTGGGA-3'
Rat Lamb3	For	5'-CTGTGCTTGGCTGACCACACTA-3'
	Rev	5'-AGCTTCCAGGCTCCTGTCCAA-3'