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Mechanisms of M Cell Differentiation: New Insights Acquired with Enteroid Cultures

By
Lara Megan Beers Wood

Doctor of Philosophy
Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

_____
Ifor Williams, M.D. Ph.D.
Advisor

_____
Lawrence Boise, Ph.D.
Committee Member

_____
Timothy Denning, Ph.D.
Committee Member

_____
Andrew Neish, M.D.
Committee Member

_____
David Weiss, Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

Mechanisms of M Cell Differentiation: New Insights Acquired with Enteroid Cultures

By

Lara Megan Beers Wood

B.S., University of Georgia, 2010

Advisor: Ifor Williams, M.D., Ph.D.

An abstract of a dissertation submitted to the Faculty of the James T. Laney Graduate School of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences Program
Immunology and Molecular Pathogenesis
2016

Abstract

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The primary function of the microfold (M) cell is to take up particulate antigen from the intestinal lumen and present it to the immune cell residing in its basolateral pocket. The M cell is unique to the epithelium overlying the Peyer's patch (PP), isolated lymphoid follicles, and cecal patch of the intestine where the epithelium is exposed to RANKL. M cell differentiation is dependent on RANKL induction of Spi-B and Spi-B dependent genes. RANKL also induces a distinctive gene expression pattern in the follicle-associated epithelium (FAE). M cell specific gene expression and differentiation have been difficult to study *in vivo* due to low numbers on restricted epithelial sites. RANKL stimulation of intestinal enteroids overcomes this problem by providing a larger number of M cells to study *in vitro* in a model that replicates M cell specific gene expression and increased transcytosis. The following studies increase the understanding of M cell differentiation in this model by introducing TNF-α to the RANKL stimulation of enteroids. TNF-α activates the canonical pathway of NF-κB leading to increased translation of the precursors for noncanonical NF-κB, *Relb* and *Nfkb2*. RANKL can then activate more noncanonical NF-κB heterodimers which results in increased and more rapid M cell specific gene expression. This model is an important tool to continue to study the early steps of M cell differentiation.

One of the advantages of the enteroid culture system is that it allows the culture of intestinal epithelium from mice that are not viable past weaning. This advantage was exploited with the study of a conditional knockout of Atoh1 in the small intestinal epithelium. Mice with this mutation lack all secretory cells and rarely live to adulthood. Atoh1 deficient enteroids do not express M cell specific genes upon stimulation with RANKL. The role of Atoh1 in M cell differentiation suggests it belongs to the secretory lineage of intestinal epithelial differentiation. The M cell belonging to the secretory cell type will change the approach to further studies of the M cell as it shares early differentiation steps with a diverse group of specialized epithelial cells

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Introduction

The M (Microfold) cell is responsible for sampling antigens from the lumen of the small intestine (SI) and presenting them to the immune system. The morphology and functional capabilities of the M cell are particularly suited to this role. Electron microscopy shows that M cells lack the normal microvilli present on the luminal side of most absorptive enterocytes.[1] Also, they have an invagination on the basolateral surface that can house immune cells such as a dendritic cell or lymphocyte. A vast amount of vesicles can be seen in the cytoplasm.[2] Each of these morphologic features is partly responsible for the M cell's ability to take in antigens from the lumen and present them to the immune system. The stubby microvilli on the luminal surface are accompanied by an altered glycocalyx and thinner mucus layer which allow for access of the cell membrane to the luminal contents.[3] The presence of a vesicular network was one of the first clues that M cells are extremely skilled at macropinocytosis. M cells have since been shown to take up bacteria and fluorescent beads and pass them into the Peyer's patch (PP). [4-6] This uptake, coupled with the presence of an immune cell in the basolateral pocket of the M cell, provides evidence of interactions between the epithelium and the underlying immune system. These cells are found in the follicle associated epithelium (FAE), the epithelium over isolated lymphoid follicles, and on immune follicles at other mucosal sites such as the conjunctiva, tonsil, and NALT. [7-9]

M cells are known to be an important bridge to the immune system affecting oral tolerance and IgA responses. The PP is the major inductive site for IgA production in the gut.[10] M cells take up antigen and pass it to immune cells such as dendritic cells, which

can then present the antigen to T cells and influence class switching in the germinal centers within the PP[11, 12]. In mice conditionally lacking M cells in the small intestinal epithelium, germinal center formation in PP is reduced and the acquisition of IgA to the commensal microbiota is delayed after weaning. [6] Thus, the M cell serves an important function as an intermediary between the microbiota and the immune system. Many investigators have sought to exploit this transcytotic role by targeting oral vaccines or allergens to M cells as a means to incite or repress an immune response.[11, 13, 14] Learning more about the function and development of the M cell could contribute to these areas of inquiry.

M cells have been notoriously hard to study *in vivo* due to the low numbers of naturally occurring cells and the lack of detectable surface markers. Historically, one popular marker used to identify M cells in mice was a surface alpha 1,2 fucosylation recognized by the *Ulex europeaus* 1 (UEA-I) lectin.[15] Since, goblet cells can also be stained with this lectin, researchers then relied on cell morphology to determine the difference in cell type. Recently, glycoprotein 2 (GP2), a receptor for FimH+ bacteria, was determined to be M cell specific.[4, 16] It is now accepted that UEA-I+, GP2+ cells on the PP dome are M cells. A previous attempt at an *in vitro* system to study M cell differentiation, the co-culture of Caco-2 epithelial cells and Raji B cells, replicated the transcytotic function of M cells but did not result in M cell specific gene expression. [17]

Using Enteroid Culture to Study the Intestinal Epithelium. The single layer of epithelial cells which makes up the complex barrier of the small intestinal epithelium develops through an intricate, concerted effort on the part of stem cells and fully differentiated cells. For many years, the differentiated cell types of the SI epithelium have

been studied *in vivo,* where they are bombarded by signals from the immune system and microbiota, or *in vitro* using transformed cell lines. Both strategies of study have associated challenges. The advent of the enteroid culture system has allowed for *in vitro* study of this important epithelial barrier.[18] Enteroids retain the morphology of the tissue with the ability of the cells to differentiate while minimizing confounding environmental signals. [19]

The small intestinal epithelium is composed of crypts and villi with occasional organized lymphoid structures called PP inducing specialized adaptations in the overlying epithelium. Each crypt is home to a heterogeneous pool of intestinal stem cells (ISCs) responsible for renewal of all cell types in the intestinal epithelium. New cells mature and differentiate as they move up and out of the crypts toward the tips of the villi. As the cells die, they are shed off the tip of the villus in a process called anoikis. In this way, the whole intestinal epithelium turns over approximately every four days. At the base of the crypt, the fully differentiated Paneth cell releases antimicrobial peptides as well as Wnt ligands that help to organize the crypt.[20, 21] Between the Paneth cells lay the LGR5+ crypt base columnar cells that have been established as stem cells with the ability to give rise to all cell types in the intestinal epithelium *in vitro* in enteroid culture.[19] Four cells above the base of the crypt, in the +4 position, are located the quiescent stem cells.[22] Then, above these cells on the crypt to villus axis, are short-term label retaining cells comprised of secretory and absorptive enterocyte precursors.[23] These cells can dedifferentiate in to LGR5+ stem cells after injury. [24, 25] Above the precursors are the rapidly dividing transit amplifying cells from which all differentiated cell types in the intestinal epithelium develop by asymmetrical division. [22, 26]

The enteroid culture system is changing the way scientists are able to study the development of the intestinal epithelium. Lineage tracing in the LGR5-GFP reporter mouse allowed researchers to identify LGR5+ stem cells capable of generating all of the cell types in the intestinal epithelium.[19] Sorted and fluorescently labeled, LGR5+ stem cells were used to seed a 3-D cell culture system using a collagen, laminin and entactin matrix called Matrigel. [19, 27] The matrix acts as a basement membrane surrogate that the cells can use to make a 3-D complex with an enclosed lumen mimicking the apical-basolateral polarization of IECs *in vivo*. The stem cells were cultured in this matrix in a media supplemented with EGF, R-spondin1 and Noggin. These factors bathe the stem cells in signals that replicate all of the signaling cascades needed for renewal and differentiation of the crypt cells *in vivo*. EGF binds to EGFR leading to proliferation and survival signaling for the cell culture.[28] R-spondin1binds to the LGR5 receptor which sequesters ZRNF3 from degrading components of the Wnt receptor resulting in increased Wnt/β-catenin signaling. [29] Noggin inhibits the natural BMP signal which antagonizes Wnt signaling.[28] As the LGR5+ cells divide, they form a sphere that then begins to form crypt-like structures that bud off of a central space analogous to the intestinal lumen.[28] These organoids are made up of absorptive enterocytes, secretory cells, and crypt stem cells as confirmed by immunohistochemistry. [28, 30]The organoids can be kept in culture, disrupted, split, and frozen much like normal cell culture. [31, 32]

While the organoid culture technique can be implemented from a single stem cell type, whole crypts can also be isolated from experimental animals and used to seed the cultures. [28] This allows the study of genotypes of mice which may not be viable to adulthood or may require special housing. The organoid culture system also provides

researchers with cells that are receptive to drug and cytokine treatment. For instance, enteroids treated with IL-22 express IL-22 responsive genes and undergo proliferation.[33, 34] When enteroids are made from STAT3-/- mice, the effect of IL-22 is blocked.[33] Thus, the organoid culture system is an easily manipulated cell culture system and is changing the way the development of the intestinal epithelium is studied.

Secretory Cell Development. The vast majority of intestinal epithelial cells are absorptive enterocytes. However, secretory cells are a heterogeneous class of intestinal epithelial cells that provide many necessary functions. The class of secretory cells encompasses goblet, Paneth, tuft, and enteroendocrine cells.[21, 35, 36] Each type of secretory cell develops from the same stem cell origin as the absorptive enterocyte. However, different developmental signals that induce unique transcription factor profiles lead to the maturation of these distinctive cell types. The absorptive enterocyte is thought to be the default developmental program for a cell differentiating from the crypt. The engagement of NOTCH1 or NOTCH2 upregulates the expression of HES1 leading to the absorptive enterocyte developmental lineage.[24, 37] HES1 then suppresses Atonal Homolog1 (ATOH1, *Atoh1*) preventing all other developmental programs.[38] In an adjacent cell expressing a Notch ligand such as DLL1, ATOH1 is upregulated and suppresses HES1. ATOH1 is required for a developing enterocyte to differentiate into one of the secretory cell types.[36, 39] Following ATOH1 expression, each cell type expresses a unique set of transcription factors that is required for its endstage differentiation. For instance, a cell that will become a goblet cell expresses ATOH1 followed by GFI1 then SPDEF along its differentiation.[40] It is believed that the cells undergo lateral inhibition patterned by Notch/ Delta signaling which prevents multiple

secretory cells from developing next to each other.[37] The only intestinal epithelial cell whose differentiation program has not been fully elucidated is the M cell. The role of Atoh1 or Hes1 during its development on the FAE has not yet been studied.

The Development of the Peyer's Patch and the Follicle Associated Epithelium. The main immune structures in the small intestine are the PP. Each PP houses several B cell follicles as well as many T cells and dendritic cells. The PP develop through a coordinated effort of lymphoid tissue inducer cells expressing the lymphotoxin αβ, TNF-α and RANKL.[41, 42] Each of these cytokines is important for aggregation, B cell follicle formation, and epithelial patterning, respectively.[43] Efferent lymphatics leading to the mesenteric lymph nodes are the only route of egress from the PP to the rest of the immune system. The source of antigen for priming dendritic cells and B cell germinal centers comes across the intestinal epithelium from the lumen of the intestine. Several pathways exist for the antigens to cross the epithelial barrier. The most widely accepted pathway for particulate antigen is uptake through M cells. An alternative pathway that has also been suggested is uptake by dendritic cells that are able to extend dendrites through the epithelium to directly sample luminal antigens.[44] Goblet cells may also sample soluble antigen during their change in structure following the release of mucus. [45] While each of these avenues contributes to antigen uptake, the M cell is the primary route for uptake of particulate antigens.

The specialized FAE overlaying the follicles of PP is the only type of epithelium that represents a major departure from the typical crypt-villus epithelial organization. Several signals from the surrounding tissue are responsible for this change. While RANK is expressed on all IECs, RANKL is only expressed in the subepithelial dome of the PP

restricting RANKL-RANK signaling to the FAE in the normal SI.[46] The immune cells encased in the PP release cytokines constantly in an effort to create and sustain germinal centers. These signals result in an FAE enriched for M cells and deficient in most other secretory cells relative to the villous epithelium.[47] Goblet cells are still present but at a much lower concentration than the villous epithelium.[48] The lack of the goblet cells results in a thinning of the mucus layer allowing for more interaction between the lumen and the epithelium.

RANKL–RANK signaling leads to many of the differences between the intestinal epithelium and the FAE. The restriction of RANKL to the subepithelial dome is thought to be the main reason that M cell expression is unique to the FAE.[46, 47] When mice are given exogenous RANKL, they not only develop more M cells on each PP, but they also develop UEA-I+ M cells through out the villous epithelium. [46] This discovery helped to circumvent some of the previous problems associated with studying M cells, especially their paucity *in vivo*. Following RANKL treatment, the intestinal epithelium could now be sorted based on UEA-I staining.[49] Microarrays on the RANKL-treated tissue as well as sorted UEA-I+ cells have led to the discovery of more M cell specific genes. Most notably, the transcription factor Spi-B was discovered through these studies and is now thought to be the master regulator of M cell development.[49-51]

The Ets transcription factor Spi-B leads to the endstage differentiation of an M cell. Spi-B-/- mice lack M cells on the PP of the small intestine.[50] This phenomenon appears even following exogenous RANKL treatment.[50] Thus, Spi-B is thought to be a transcription factor downstream of RANK-RANKL signaling that is responsible for the development of M cells. Several M cell specific genes have been shown to be Spi-B

dependent. One of these is the gene encoding CCL9, a ligand for CCR1 that can recruit certain subsets of immune cells.[52] The aforementioned protein marker, GP2, is also Spi-B dependent. Not all RANKL induced, M cell specific genes are Spi-B dependent. MARCKSL1 is absent in the PP of the small intestine of Spi-B-/- mice; however, it is still induced by treatment with exogenous RANKL.[51] Thus, the expression of this gene is independent of Spi-B. The strongest evidence that Spi-B is the master regulator of M cell differentiation is the loss of phagocytic function observed in the PP of Spi-B-/- mice.[30, 50] This finding is illustrated by both a fluorescent particle uptake experiment as well as an assay using SM1 T cell activation as a readout of immune response to *S. typhimurium*.[50] Recently, Spi-b -/- and wild type whole crypts were used to make enteroids. Once the enteroids formed, they were subjected to treatment with RANKL. Several of the cells in the wild type organoids differentiate into M cells based on gene expression and function. The wild-type organoids upregulated many RANKL responsive genes including Spi-B and GP2 while the Spi-B -/- organoids could not respond in the same manner.[30]

Spi-B was originally discovered in a Burkitt's lymphoma B cell line where it was thought to be redundant for the closely related Ets transcription factor PU.1.[53] In fact, PU.1 and Spi-B have identical DNA binding sites that differ from the other Ets transcription factors.[54] In Spi-B-/- mice, PU.1 is present at wild type levels yet is unable to prevent defects in B cell proliferation and M cell differentiation.[54] Therefore, the roles of these closely related proteins may be similar but are not entirely redundant. PU.1 and Spi-B possess differences in their non-DNA binding domains allowing for interaction with divergent proteins.[55] These interactions may account for some of their

disparate tissue expression as well. Both transcription factors are present in B cells. PU.1 is also expressed in neutrophils, monocytes, megakaryocytes, and mast cells.[53] Spi-B is not expressed in these immune cells. Instead its tissue distribution is quite diverse. Spi-B is known to be expressed alone in plasmacytoid dendritic cells, medullary thymic epithelial cells (mTECs) and M cells[56-58]. Spi-B has two isoforms that can be transcribed from two different promoters.[59] Promoter 1 is upstream of the first exon while promoter 2 is TATA-less and contained within the first intron. Promoter 2 contains an octamer binding site. So, it is thought to be specifically targeted by OBF1.[60] Most of the information provided by the original literature on Spi-B comes from the comparison of Spi-B to PU.1. The importance of Spi-B in mTEC, plasmacytoid dendritic cell and M cell differentiation will likely shed more light on this transcription factor.

RANKL-RANK Signaling. While the M cell is a good example of a specific way in which the FAE is altered from the surrounding epithelium, the FAE also shows differential gene expression. Microarrays of FAE excluding M cells led to the discovery of these genes as FAE specific. For instance, CCL20 and CXCL16 are expressed by the FAE to recruit immune cells.[47, 61] These genes were previously assumed to be M cell specific. The discovery that the FAE differs in more ways than just the presence of M cells opens a new field of inquiry into the role of the FAE as the only section of epithelium exposed to RANKL. Thus, RANKL-RANK signaling likely contributes to some of the unique gene expression observed in the FAE.

Receptor activator of NF-κB (RANK) was first described as a homolog for the TNF receptor in dendritic cells. Its ligand was discovered first and originally called TRANCE for TNF-related activation-induced cytokine.[62] The name was changed to

RANKL following the discovery of its receptor. RANK and its ligand are not the only two players in this system. Osteoprotegerin (OPG) is a decoy receptor for RANKL.[63] These three proteins allow for activation and regulation of NF-κB in several cell types. RANKL-RANK signaling has been thoroughly studied in osteoclastogenesis where it was found to be important for differentiation and survival of monocytes as they become osteoclasts.[64] It has also been studied in the mammary epithelium, medullary thymic epithelium and the intestine.[56, 65, 66] In each of these disparate cell types, RANKL binds to RANK but is kept in check by the feedback expression of OPG downstream of RANKL-RANK signaling. [67]

RANK specifically signals through the adapter protein TRAF6 to induce activation of NF-κB.[68] RANK has been shown to signal through other TRAF family members, TRAF2 and TRAF5 as well. [69] Downstream of TRAF6, JNK, p38, PI3K - Akt, and both the canonical and noncanonical pathways of NF-κB activation are all induced by RANKL ligation of RANK.[70] These signaling pathways result in the activity of transcription factors including NF-κB, NFATc1, and the c-fos subunit of AP-1.[71] PU.1, the transcription factor closely related to Spi-B and the transcription factor necessary for maturation of M cells, can lead to induction of RANK during osteoclast differentiation. [72]While RANKL-RANK signaling initiates a cascade of events causing many changes in the cells as diverse as monocytes and intestinal epithelial cells, the most important pathway for M cell differentiation is the non-canonical pathway of NF-κB activation.[4]

Nuclear factor κB is a set of heterodimeric transcription factors that can be predominantly made from four subunits. The canonical heterodimer is made up of p65

and p50 while the noncanonical heterodimer is made up of Relb and p52.[73] Both heterodimers can bind to similar κB binding sites to induce transcription of target genes.[74] Relb and the precursor to p52, p100, are sequestered in the cytoplasm by IKKα.[75] NF-κB-inducing Kinase (NIK) is required for the degradation of IKKα and the processing of p100 to p52.[75] NIK is normally ubiquitinated but can be activated by signaling through a subset of receptors in the TNF receptor superfamily such as BAFFR, CD40, RANK, and LTβR.[76] The time course of genes induced by RANKL-RANK signaling through the non-canonical and canonical pathways is different as non-canonical NF-κB is activated slower than canonical NF-κB by several hours.[77] The noncanonical pathway of NF-κB is an important pathway downstream of RANKL-RANK ligation that must be studied when discussing the effects of RANK signaling on M cell differentiation.

The goal of the following studies is to further illuminate the mechanisms of M cell differentiation. In Chapter 1, a model is established to study M cell differentiation *in vitro* using RANKL treated enteroids. Previously, RANKL treated enteroids were shown to express a few M cell specific genes and increased transcytosis.[30] In this study, the RANKL treated enteroid model is expanded by showing a wider range of M cell and FAE specific gene expression and by adding TNF-α to the culture conditions which allows for greater expression of both FAE and M cell specific genes. In Chapter 2, the enteroid system is used to study the effect of RANKL induced M cell specific gene expression in intestinal epithelium lacking the transcription factor Atoh1 to investigate whether M cells are best considered to be a part of the absorptive or secretory lineage of intestinal epithelial cell differentiation. These inquiries provide valuable insights into the

development of the FAE and M cell which can be used to further the understanding of the differentiation and function of the M cell.

Chapter 1

TNF-α Augments RANKL-Dependent Intestinal M Cell Differentiation in Enteroid Cultures

Megan B. Wood, Daniel Rios, Ifor R. Williams

Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA.

E-Published ahead of print in AJP Cell Physiology. July13, 2016. doi: 10.1152/ajpcell.00108.2016

All experiments in this study were performed by Megan B. Wood.

This manuscript was written by Megan B. Wood and Ifor R. Williams.

ABSTRACT

M cells are phagocytic intestinal epithelial cells in the follicle-associated epithelium (FAE) of Peyer's patches (PPs) that transport particulate antigens from the gut lumen into the subepithelial dome. Differentiation of M cells from epithelial stem cells in intestinal crypts requires the cytokine RANKL and the transcription factor Spi-B. We used three-dimensional enteroid cultures established with small intestinal crypts from mice as a model system to investigate signaling pathways involved in M cell differentiation and the influence of other cytokines on RANKL-induced M cell differentiation. RANKL addition to enteroids induced expression of multiple M-cell-associated genes by 1 day, including *Spib*, *Ccl9*, *Tnfaip2*, *Anxa5* and *Marcksl1*. The mature M-cell marker *Gp2* was strongly induced by 3 days and expressed by 11% of cells in enteroids. The noncanonical NF-κB pathway was required for RANKL-induced M cell differentiation in enteroids, as addition of RANKL to enteroids from mice with a null mutation in the *Map3k14* gene encoding NF-κB-inducing kinase failed to induce M-cell-associated genes. While the cytokine TNF-α alone had little if any effect on expression of M-cell-associated genes, adding TNF-α to RANKL consistently resulted in 3- to 6-fold higher levels of multiple M-cell-associated genes compared to RANKL alone. One contributing mechanism is the rapid induction by TNF-α of *Relb* and *Nfkb2*, genes encoding the two subunits of the noncanonical NF-κB heterodimer. We conclude that endogenous activators of canonical NF-κB signaling present in the GALT microenvironment including TNF-α can play a supportive role in the RANKL-dependent differentiation of M cells in the FAE.

INTRODUCTION

Microfold (M) cells are specialized epithelial cells found in the follicle-associated epithelium (FAE) of Peyer's patches (PPs). M cells are responsible for the highly efficient uptake of particulate antigens into gut-associated lymphoid tissue (GALT) structures such as PPs and isolated lymphoid follicles (ILFs) that serve as inductive sites for mucosal immunity in the intestine [1]. Mature M cells in the FAE are defined by unique morphological features including blunted microvilli and an intraepithelial pocket, their capacity for efficient uptake of particulate antigens, and expression of a set of genes that distinguish M cells from both neighboring FAE enterocytes and the other types of specialized enterocytes found in villous intestinal epithelium. M cells develop from $Lgr5^{+}$ stem cells present in crypts surrounding the FAE [2]. Differentiation of precursor cells into the M-cell lineage requires receptor activator of NF-κB ligand (RANKL) signaling through the RANK receptor [3], followed by induction of the Ets transcription factor Spi-B, which is restricted to the M cell lineage among enterocytes and required for full differentiation of M cells and acquisition of markers found on mature M cells such as GP2 [4]. Not all M-cell associated markers require Spi-B expression; the selective expression of *Marcksl1* and *Anxa5* by M cells is independent of Spi-B [4, 5]. Mice with conditional deletion of the *Tnfrsf11a* gene encoding RANK in the intestinal epithelium have a phenotype characterized by absence of intestinal M cells, reduced formation of germinal centers in PP, and substantial impairment in development of a secretory IgA response after weaning [6]. The scarcity of M cells within the entire intestinal epithelium has consistently presented a formidable obstacle to the development of *in vitro* approaches to study M-cell differentiation and function.

An *in vitro* model system that has been used widely to study M-cell biology is co-culture of the human Caco-2 colonic adenocarcinoma cell line with a source of B lymphocytes in polarized Transwell cultures [7]. In the presence of B cells, a subset of the Caco-2 cells exhibit enhanced transcytosis of particulate antigens that resembles one of the main phenotypic features of natural M cells in the FAE. While the original version of this co-culture model of M cell-like cells added freshly isolated mouse Peyer's patch cells to the Caco-2 cells, addition of human Raji B lymphoblastoid cells to Caco-2 cells was an alternate technique that also yielded epithelial monolayers with enhanced transcytotic function [7]. Variations of the original Caco-2/Raji co-culture model have been used widely to study transcytosis of nanoparticles and bacteria [8-10]. A weakness of the Caco-2/Raji co-culture model is that most of the genes selectively expressed by natural intestinal M cells are not induced in this *in vitro* model compared to monocultures of Caco-2 cells [11]. There continues to be a need for additional *in vitro* models for studying intestinal M cells that use nontransformed cells and more faithfully replicate the transcriptional signature of the M cell lineage.

The enteroid culture system is a three-dimensional culture technique using a Matrigel scaffold with defined growth factors to enable the survival and expansion of stem cells present in freshly harvested intestinal crypts [12]. Enteroids can be used to study the differentiation of specialized enterocytes found in the small intestinal epithelium by allowing maintenance of some intestinal stem cells in a reconstituted stem cell niche while permitting differentiation of some progeny of the precursor cells into specialized absorptive and secretory cell types naturally found in the intestinal epithelium

[13]. The enteroid system allows for the study of the intestinal epithelium without confounding signals from the microbiota and immune system, thus providing a physiologically relevant model for renewal and differentiation of the isolated intestinal epithelium. Addition of RANKL to mouse and human enteroid cultures was previously shown to induce M-cell-associated gene expression and enhanced transcytosis of microspheres and bacteria [2, 6, 14]. In the current study, we have used the RANKL-supplemented enteroid culture system to further investigate signaling pathways involved in the differentiation of M cells. We find that RANKL acts through the noncanonical NF-κB pathway to induce *Spib* expression, followed by expression of both Spi-B-dependent and Spi-B-independent M-cell-associated genes. We also show that while TNF-α alone does not induce M cell differentiation in enteroid cultures, combining TNF-α and RANKL boosts the expression of multiple M-cell-associated genes compared to RANKL alone.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used for wild-type enteroid cultures. Alymphoplasia mice *(aly/aly)* and *aly/+* controls were bred

in our mouse colony at Emory University starting with *aly/+* mice backcrossed onto the C57BL/6 background provided by Drs. Mandy Ford and Kenneth Newell (Emory University). Mice were genotyped for the wild type and *aly* mutant alleles of the *Map3k14* gene by running two separate PCR reactions with allele-specific forward primers (*aly* Forward 5'-CACATCCCGAGCTACTTCAACA-3' or WT NIK Forward 5'-CACATCCCGAGCTACTTCAACG-3') and a common reverse primer (NIK Reverse 5'-CCTTCGGGGACTCTACAGGC-3') [15]. The mutant and wild type NIK alleles both yielded 266 bp PCR products. Mice with conditional deletion of the *Tnfrsf11a* gene encoding RANK in intestinal epithelial cells ($RANK^{\Delta IEC}$) mice and $RANK^{F/F}$ littermate controls were bred at Emory University and genotyped as previously described [6]. The animal studies were reviewed and approved by the Emory University Institutional Animal Care and Use Committee.

Crypt isolation and enteroid culture. The distal 10 cm of the small intestine excluding any PPs was excised, opened and washed. The intestine was then incubated with 5 mM EDTA for 20 min with shaking at 4°C. The epithelium was removed by manual disruption for 2 minutes in a solution of 43.4 mM sucrose (Thermo Fisher Scientific, Waltham, MA) and 54.9 mM D-sorbitol (Thermo Fisher Scientific) in Dulbecco's PBS (Corning Life Sciences, Tewksbury, MA). Following filtration through 70 μm mesh and a 4 min 200 g spin, the sedimented crypts were resuspended in 50 μl Matrigel (Corning Life Sciences) and placed in the center of wells in a 24-well plate. The plates were incubated at 37°C for 30 min to allow for polymerization of Matrigel before adding 500 μl per well of culture media as previously described [13]. The culture media (abbreviated

as ENR media because it includes EGF, Noggin and R-spondin) consisted of 50:50 DMEM-Ham's F-12 (Corning Life Sciences), 1% N-2 Plus Media Supplement (R&D Systems, Minneapolis, MN), 2% B-27 serum-free supplement (Thermo Fisher Scientific), 1% penicillin/streptomycin (Corning Life Sciences) and 10 mM HEPES (Thermo Fisher Scientific), 50 ng/ml EGF (Peprotech, Rocky Hill, NJ), 100 ng/ml Noggin (Peprotech) and 10% R-spondin2 conditioned media obtained from the HEK-Rspo2AP cell line provided by Dr. Jeffrey Whitsett (Cincinnati Children's Hospital Medical Center, Cincinnati, OH) [16]. All of the ENR media used also contained 3 ng/ml ROCK inhibitor Y-27632 (BD Biosciences, Franklin Lakes, NJ) because inclusion of this compound improved the viability of cultured enteroids. Newly established enteroids were cultured for 3 days before media above the Matrigel was changed and the cultures were stimulated with 100 ng/ml murine RANKL (Peprotech) for 1 or 3 days. In some experiments, enteroids were stimulated with 50 ng/mL murine IL-22 (Peprotech) or 50 ng/ml murine TNF-α (Peprotech) alone or in conjunction with RANKL. TNF-α was not used at concentrations above 50 ng/ml because higher concentrations led to increased enterocyte death due to apoptosis and compromised recovery of RNA [17].

Antibodies. Ultra-LEAF grade purified anti-mouse TNF-α antibody (MP6-XT22; BioLegend, San Diego, CA) was added to some enteroid cultures at a final concentration of 5 μg/ml to neutralize TNF-α. Unconjugated monoclonal rat anti-mouse GP2 (clone 2F11-C3, MBL International, Woburn, MA) was used to stain frozen sections of enteroids. Alexa Fluor 546-conjugated goat anti-rat secondary antibody (Invitrogen, Carlsbad, CA) was used to detect the anti-GP2 primary. FITC-conjugated monoclonal

anti-E-cadherin antibody (clone 36, BD Biosciences) was used to stain cell junctions of enteroids on frozen sections.

Quantitative real-time PCR. Enteroids in Matrigel were incubated with Cell Recovery Solution (Corning Life Sciences) with shaking for 1 h at 4°C to dissolve Matrigel prior to RNA extraction. The contents of 3 separate replicate wells were pooled for each experimental condition. After 2 PBS washes, RNA extraction was carried out using the RNeasy Mini Kit (Qiagen, Valencia, CA). Between 0.5 and 1.0 μg of RNA was used to make cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA), with the same amount of RNA used from all samples in each experiment. The iTaq Universal SYBR Green Supermix (Bio-Rad) was used for PCR reactions run on a CFX Connect Thermocycler (Bio-Rad). All PCR primers used are listed in Table 1. The amplicons from all primer pairs span at least one intron in the target gene to avoid amplification of genomic DNA. Each time a new primer pair was used for the first time, the size of the amplicon was confirmed on an agarose gel. Thereafter, the melting curves of the amplicons were used to confirm primer specificity. All amplifications were run in triplicate. Cycle threshold (Ct) results were normalized by comparison to the housekeeping genes *Gapdh* and *Rpl13a*. The ΔΔCt method was used to determine fold induction of a gene of interest in a comparison of two samples, with normalization of each experimental Ct result to the geometric mean of the Ct values of *Gapdh* and *Rpl13a* [18].The relative expression of genes was reported in comparison to *Gapdh* because this is a widely used standard. Relative expression was determined by normalizing the average cycle threshold of each sample to the *Gapdh* result and setting the expression

level of *Gapdh* at 1. The baseline relative expression of M-cell- associated genes such as *Spib* and *Gp2* in enteroids harvested after 4 days of culture was similar to the level of expression detected in freshly isolated villous epithelium (data not shown).

Cloning of PCR-amplified Spib transcripts. cDNA amplified from RANKL-treated enteroids using Spib-1 or Spib-2 specific primers was ligated into pJET1.2 using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific). Plasmids with inserts of the correct size were sequenced by automated dideoxy sequencing. The sequences of the Spib-1 and Spib-2 amplicons confirmed usage of the expected promoters and splice sites.

Immunofluorescent microscopy. Enteroids were removed from Matrigel by treating the Matrigel with 500 μl of Cell Recovery Solution (Corning Life Sciences) with shaking for 1 h at 4°C followed by washes. The recovered enteroids were embedded in OCT compound (Thermo Fisher Scientific) and snap frozen in isopentane on dry ice. Blocks containing the enteroids were sectioned on a cryostat to yield 5 μm thickness frozen sections. Slides were fixed with 4% paraformaldehyde for 20 min at room temperature. Sections were stained overnight with rat anti-mouse GP2 monoclonal antibody followed by a 2 h incubation with goat-anti-rat IgG Alexa Fluor 546 secondary and monoclonal FITC-anti-E-cadherin. DAPI dye (EMD Millipore, Billerica, MA) was used to stain nuclei. Fluorescence staining images were acquired with a Nikon 50i microscope using an oil immersion 40X objective.

Statistical analysis. Mean values of relative expression in qPCR experiments were compared by a two-tailed Student's *t*-test with Prism (GraphPad Software, La Jolla, CA). A P value of < 0.05 was considered significant.

RESULTS

Enteroids stimulated with RANKL express M-cell-associated genes. Three-dimensional enteroid cultures were established using C57BL/6 small intestinal crypts and cultured for 3 days in ENR media. The media was replaced at 3 days with ENR or ENR supplemented with RANKL to induce expression of M-cell-associated genes. At 1 day after RANKL addition, mRNA for several genes known to be selectively expressed by M cells and/or the FAE was strongly up-regulated including *Spib*, *Ccl9*, *Tnfaip2*, *Marcksl1* and *Ccl20* (Fig. 1A). Induction of *Ccl9* and *Tnfaip2* in M cells was previously shown to be Spi-B-dependent, while induction of *Marcksl1* is independent of Spi-B [4]. Another gene strongly induced by RANKL at 1 day was *Tnfrsf11b*, which encodes osteoprotegerin (OPG), a soluble decoy receptor for RANKL that functions as an antagonist of RANKL-mediated signaling. Induction of *Tnfrsf11b* by RANKL was previously demonstrated in thymic medullary epithelial cells [19]. After 3 days of stimulation with RANKL, additional M-cell-associated genes expressed by mature M cells were induced including *Gp2* and *Anxa5* (Fig. 1B). To ascertain the frequency of M cell differentiation within enteroids after addition of RANKL, sections of enteroids stimulated with RANKL for 3 days were stained for GP2 (Fig. 1C). GP2 was detected predominantly on the apical surface of an average of 3-4 cells per RANKL-stimulated enteroid, and 11% of the total cells examined. No GP2 expression was detected in control cultures that did not receive RANKL. The $GP2^{+}$ M cells within each enteroid were usually not found adjacent to other M cells, which resembles the pattern of distribution of M cells within the FAE of PP. The M-cell-associated genes induced by RANKL *in vitro* in enteroid cultures were expressed

in the same sequence previously described *in vivo* for the small intestinal villous epithelium following systemic RANKL injection [4].

RANKL, IL-22 and TNF-α induce distinct patterns of gene expression in enteroids. IL-22 has potent effects on intestinal epithelial cells *in vivo* and in enteroid cultures, signaling through a heterodimeric receptor consisting of IL-22R1 and IL-10R2 to activate STAT3 and induce epithelial proliferation and expression of antimicrobial proteins [20, 21]. To demonstrate the specificity of RANKL-induced gene expression in the enteroid system, cultures were stimulated for 1 day with IL-22 or RANKL, or maintained in the base ENR media. Addition of IL-22 strongly induced known IL-22 responsive genes including *Reg3g* and *Saa1*, but did not induce *Spib* (Fig. 2, A-C). No induction of *Reg3g* and *Saa1* was observed after RANKL stimulation, under conditions that resulted in strong induction of *Spib*. TNF-α is a cytokine in the TNF superfamily that rapidly activates the canonical NF-κB pathway but not the slower noncanonical NF-κB pathway [22-24]. Stimulation of enteroids with TNF-α induced *Ccl20* expression at 4 h and 1 day, but failed to induce *Spib* expression at 1 day (Fig. 2, D-F). RANKL also induced *Ccl20* expression, but not until the 1 day time point (Fig. 2, E-F). While the enteroid cultures responded to all 3 cytokines tested, only RANKL induced an increase in *Spib* expression.

RANKL-induced M-cell differentiation depends on the noncanonical NF-κB signaling pathway. RANK is one of several receptors in the TNF receptor superfamily that signals primarily through the noncanonical NF-κB signaling pathway, which depends on activation of NF-κB-inducing kinase (NIK) and nuclear translocation of p52-RelB

heterodimers [23, 25-27]. To determine if RANKL-induced M cell differentiation in enteroids also requires the noncanonical NF-κB pathway, enteroids from *aly/aly* mice with a nonfunctional NIK allele and *aly/+* control mice were stimulated with RANKL, IL-22, or TNF-α (Fig. 3). RANKL failed to induce *Spib* or *Gp2* expression in *aly/aly* enteroids. However, *aly/aly* and *aly/+* enteroids showed nearly equivalent induction of *Reg3g* by IL-22. In addition, TNF-α induction of *Ccl20* via the canonical NF-κB pathway was maintained in *aly/aly* enteroids (Fig. 3D). These results show that RANKL-induced M cell differentiation in enteroids is abrogated when noncanonical NF-κB signaling is blocked.

RANKL induces the NF-κB-dependent Spib-1 transcript variant. In B lymphocyte cell lines, transcription of the mouse *Spib* gene can be initiated from two distinct promoters yielding transcript variants designated Spib-1 and Spib-2 [19, 28, 29]. The promoter regulating expression of the Spib-1 transcript is located upstream of the first exon, while the promoter of the Spib-2 transcript is found within the first intron (Fig. 4A). The Spib-1 promoter contains a consensus κB site (GGGGATCCCC) 149 bp upstream of a consensus TATA box sequence (TATATATA) located just 5' to the transcriptional start site. The Spib-2 promoter includes a recognition site for octamer transcription factors (ATTTGCAT), but does not include a TATA box [28, 29]. Because the primer pair used in our previous qPCR experiments amplifies both the Spib-1 and Spib-2 transcripts, we used isoform-specific forward primers in combination with a common reverse primer to determine which *Spib* transcript was induced by RANKL in enterocytes (Fig. 4B,C). The isoform-specific qPCR amplifications revealed that RANKL exclusively induced the

Spib-1 transcript. A low constitutive level of Spib-2 mRNA was detected in enteroids, but no additional Spib-2 mRNA was induced by RANKL addition (Fig. 4C). The previously reported splice junctions between the first and second exons of the Spib-1 and Spib-2 transcripts were confirmed by cloning and sequencing the isoform-specific Spib-1 and Spib-2 amplicons (M. B. Wood, data not shown). Thus, RANKL-induced *Spib* expression in enterocytes depends on noncanonical NF-κB induction of the Spib-1 transcript.

TNF-α enhances RANKL-induced M-cell-associated gene expression. TNF-α signaling through the canonical NF-κB pathway can synergize with RANKL in the *in vitro* induction of genes associated with osteoclast differentiation [30-33]. TNF-α also plays an essential, supportive role in the normal maturation of B cell follicles in lymphoid tissues including PPs [34-36]. To determine whether TNF-α can also support RANKL-induced M cell differentiation, enteroid cultures were stimulated with RANKL, TNF-α, or both TNF-α and RANKL. While stimulation with TNF-α alone resulted in little if any increase in the expression of the M cell-associated genes studied, the combination of TNF-α and RANKL consistently resulted in a 3- to 6-fold further boost in the expression of M cell-associated genes over the considerable induction achieved with RANKL alone (Fig. 5). This robust increase in the expression of M cell-associated genes after combined stimulation with TNF-α and RANKL was associated with only a small increase in the frequency of $GP2^+$ cells in enteroids (to 14% compared to 11% with RANKL only) that was not statistically significant (Fig. 6). These results indicate that the observed enhancement of M-cell associated gene expression by combined stimulation with TNF-α

and RANKL is primarily achieved by more rapid induction of M cell-associated gene expression rather than recruitment of additional precursor cells into the M cell lineage.

TNF-α fails to induce M cell specific gene expression in the absence of RANKL-RANK signaling. A slight induction of *Spib* and several Spi-B-dependent genes was seen in some TNF-α stimulation experiments using C57BL/6 enteroids, perhaps as a result of TNF-α enhancing the response to a small amount of residual endogenous RANKL in the enteroids. This low and variable level of induction of M-cell-associated genes by TNF-α alone was not detected in enteroids cultured from $RANK^{\Delta IEC}$ mice in which any endogenous RANKL present would be unable to signal (Fig. 7). TNF-α was able to normally induce *Ccl20* expression in enteroids from $RANK^{\Delta IEC}$ mice, confirming that the canonical NF-κB pathway remained intact.

TNF-α stimulates transcription of Relb and Nfkb2 in enterocytes. TNF-α signaling through the canonical NF-κB pathway is known to induce transcription of *Relb* in several cell types [37, 38]. We determined whether TNF-α stimulation of enterocytes induced the genes encoding the RelB and p52 components of the noncanonical NF-κB heterodimer. After 4 h of stimulation with TNF-α, expression of both *Relb* and *Nfkb2* were significantly increased (Fig. 8). While stimulation with RANKL did not induce increases in *Relb* or *Nfkb2* at 4h, enteroids treated for 1 day with RANKL showed significant induction of *Relb* and *Nfkb2* expression. The combination of TNF-α and RANKL resulted in the more induction of *Relb* and *Nfkb2* at 1 day than RANKL alone. The early induction of *Relb* and *Nfkb2* by TNF-α results in greater availability of the two proteins

that comprise the noncanonical NF-κB heterodimer that translocates to the nucleus after RANKL-induced activation of NIK.

RANKL-induced M-cell-associated gene expression does not depend on endogenous TNF-α.

The enhancing effect of TNF-α on RANKL-induced M-cell differentiation raised the possibility of small amounts of endogenous TNF-α present in enteroids partially supporting the observed effects of RANKL. To test this possibility, we stimulated enteroid cultures with RANKL in the presence of a TNF-α neutralizing antibody (Fig. 9). Anti-TNF-α did not reduce the RANKL-induced expression of either *Spib* or *Ccl20*. The neutralizing activity of the anti-TNF-α in the enteroid system was confirmed by its ability to reduce expression of *Ccl20* in TNF-α treated enteroids down to the level of the untreated control. Thus, RANKL-induced M cell differentiation in the enteroid culture system is independent of endogenous TNF-α.

DISCUSSION

The epithelial lining of the mammalian intestine consists of multiple specialized types of enterocytes that arise following differentiation of intestinal stem cells (ISCs) residing near the base of intestinal crypts. The identification of a defined, serum-free media capable of supporting the *in vitro* growth of isolated ISCs in a three-dimensional matrix ("organoid" cultures) was an important technical advance in stem cell and epithelial cell biology [13, 39, 40]. *In vitro* studies with enteroids (i.e. organoids established with small intestinal ISCs) are providing new insights into how ISCs differentiate into various specialized absorptive and secretory lineages [12]. Antigen-sampling M cells belong to a highly specialized enterocyte lineage that is normally restricted to the FAE overlying GALT structures. While M cells are not detected in standard enteroid cultures, previous work has shown that RANKL supplementation of mouse and human enteroids is sufficient to elicit the appearance of a subset of cells expressing signature M cell genes (e.g. *Spib* and *Gp2*) and capable of enhanced phagocytic activity [2, 6, 14].

Because expression of *Spib* and *Gp2* is strongly induced by RANKL in the mouse enteroids, we investigated which other known M-cell associated genes were activated during the course of *in vitro* M cell differentiation. We found that both Spi-B-dependent (*Ccl9* and *Tnfaip2*) and Spi-B-independent M-cell-specific genes (*Marcksl1* and *Anxa5*) were efficiently induced by RANKL. Because M cells found in RANKL-supplemented enteroids faithfully replicate the pattern of gene expression of natural M cells, the

enteroid system is a significant improvement over the Caco-2/Raji co-culture system for both gene discovery applications and functional studies of bona fide M cells [11]. The RANKL-supplemented enteroid system provides a new discovery tool for identification of novel M-cell lineage-restricted genes in multiple species, and can also be used to determine whether putative M-cell-associated genes identified by other approaches are part of the RANKL-activated differentiation program.

Absorptive enterocytes within the FAE exhibit a different pattern of gene expression from absorptive enterocytes found on villi [41], and CCL20 is one of the best-characterized markers selectively expressed by FAE enterocytes [42, 43]. RANKL induced *Ccl20* expression in enteroid cultures, indicating that RANKL is one of the endogenous signals contributing to the specific gene expression pattern characteristic of FAE enterocytes. Since other cytokines including IL-1, TNF-α, and LT$\alpha_1\beta_2$ also strongly induce *Ccl20* expression by enterocytes [22, 44], the combined effects of RANKL and additional cytokines present in the local PP microenvironment are likely to be responsible for the pattern of gene expression characteristic of FAE enterocytes. RANKL stimulation of thymic epithelial cells activates expression of the *Tnfrsf11b* gene encoding the soluble RANKL decoy receptor osteoprotegerin [19]; therefore, we tested whether RANKL also induced *Tnfrsf11b* in enterocytes. RANKL strongly induced *Tnfrsf11b* in enterocytes, which may be part of an inhibitory feedback loop that regulates the enterocyte response to RANKL. We have not determined whether the RANKL-induced expression of *Tnfrsf11b* occurs throughout the FAE or just in M cells.

We also used the mouse enteroid system to investigate the relative roles of the canonical and noncanonical NF-κB signaling pathways in RANKL-induced M cell differentiation. RANKL failed to induce the M-cell-specific genes *Spib* and *Gp2* in enteroid cultures established from *aly/aly* mice homozygous for a null mutation in the *Map3k14* gene encoding NIK, extending previous *in vivo* results obtained after injection of RANKL into *aly/aly* mice [45]. The block in M cell differentiation in *aly/aly* mice shows that M cell differentiation has the same dependence on the noncanonical NF-κB pathway as most other RANKL-dependent responses including the induction of *Spib* in mouse thymic epithelial cells [19]. One of the important early targets for the noncanonical NF-κB heterodimer induced following RANKL stimulation is likely to be the κB site in the Spib-P1 promoter upstream of the first exon of the mouse *Spib* gene. Addition of RANKL to enteroids induced the Spib-1 mRNA isoform transcribed from this promoter rather than the Spib-2 mRNA transcribed from the Spib-P2 promoter located upstream of the second exon. Spib-P1 was previously shown to be the Spib promoter activated by RANKL in thymic epithelial cells [19].

Experiments comparing the responses of enteroid cultures to RANKL alone or RANKL plus TNF-α demonstrated that the combination of TNF-α and RANKL consistently resulted in a 3- to 6-fold boost in the expression of M-cell-associated genes above the level achieved with RANKL alone. Since TNF-α signals through the canonical NF-κB pathway and not the noncanonical pathway [46], this result indicates that strong activation of the canonical NF-κB pathway can play a supporting role in RANKL-stimulated M cell differentiation. One potential mechanism for this effect is the ability of

canonical NF-κB activation to rapidly induce enhanced expression of the *Relb* and *Nfkb2* genes encoding the RelB and p52 subunits of the noncanonical NF-κB heterodimer [38, 47]. After RANKL-RANK signaling activates NIK allowing for the p100 precursor protein to be processed into the active p52 subunit and associate with RelB, the presence of more RelB and p100 protein increases the number of potential noncanonical NF-κB heterodimers that can form to mediate the downstream effects of RANKL-dependent NIK activation. Alternatively, some of the κB sites that regulate transcription of genes involved in M cell and FAE differentiation may be responsive to binding of either canonical p65-p50 or noncanonical RelB-p52 heterodimers, potentially enabling synergistic gene induction when nuclear translocation of both canonical and noncanonical NF-κB complexes occurs at the same time.

Our finding that TNF-α enhances RANKL-induced expression of the full spectrum of M-cell-associated genes in enteroids has several implications for future studies of M cell differentiation. Combined stimulation of enteroids with RANKL and TNF-α may assist in the discovery of additional M-cell associated genes by enhancing the sensitivity of transcriptomics to detect genes that are less strongly induced. The supportive role of TNF-α demonstrated *in vitro* also raises the possibility that TNF-α or even other inducers of the canonical NF-κB pathway present in the PP microenvironment could serve a similar function during *in vivo* M cell differentiation. However, our studies clearly show that a cytokine such as TNF-α, which can play an accessory role in M cell differentiation through activation of the canonical NF-κB pathway, is not capable of

inducing *Spib* and the rest of the Spi-B-dependent M cell differentiation program on its own.

ACKNOWLEDGEMENTS

We thank Drs. Benyue Zhang and Andrew Gewirtz at Georgia State University for their assistance with techniques and reagents that enabled us to successfully establish the enteroid culture technique in our laboratory. We thank Drs. Mandy Ford and Kenneth Newell at Emory University for providing us with the *aly*/+ mice used to establish a breeding colony in our mouse facility.

GRANTS

This work was supported by National Institutes of Health grants R01-DK64730 and R21-AI111388 (to I.R. Williams) and a Senior Research Award from the Crohn's & Colitis Foundation of America (to I. R. Williams).

REFERENCES

1. Hase, K., et al., *Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response.* Nature, 2009. **462**(7270): p. 226-30.
2. de Lau, W., et al., *Peyer's patch M cells derived from Lgr5(+) stem cells require SpiB and are induced by RankL in cultured "miniguts".* Mol Cell Biol, 2012. **32**(18): p. 3639-47.
3. Knoop, K.A., et al., *RANKL is necessary and sufficient to initiate development of antigen-sampling M cells in the intestinal epithelium.* J Immunol, 2009. **183**(9): p. 5738-47.
4. Kanaya, T., et al., *The Ets transcription factor Spi-B is essential for the differentiation of intestinal microfold cells.* Nat Immunol, 2012. **13**(8): p. 729-36.
5. Sato, S., et al., *Transcription factor Spi-B-dependent and -independent pathways for the development of Peyer's patch M cells.* Mucosal Immunol, 2013. **6**(4): p. 838-46.
6. Rios, D., et al., *Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria.* Mucosal Immunol, 2015.
7. Kerneis, S., et al., *Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria.* Science, 1997. **277**(5328): p. 949-52.
8. Gullberg, E., et al., *Expression of specific markers and particle transport in a new human intestinal M-cell model.* Biochem Biophys Res Commun, 2000. **279**(3): p. 808-13.

9. des Rieux, A., et al., *Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium.* Eur J Pharm Sci, 2005. **25**(4-5): p. 455-65.

10. Martinez-Argudo, I. and M.A. Jepson, *Salmonella translocates across an in vitro M cell model independently of SPI-1 and SPI-2.* Microbiology, 2008. **154**(Pt 12): p. 3887-94.

11. Mabbott, N.A., et al., *Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium.* Mucosal Immunol, 2013. **6**(4): p. 666-77.

12. Stelzner, M., et al., *A nomenclature for intestinal in vitro cultures.* Am J Physiol Gastrointest Liver Physiol, 2012. **302**(12): p. G1359-63.

13. Mahe, M.M., et al., *Establishment of gastrointestinal epithelial organoids.* Curr Protoc Mouse Biol, 2013. **3**: p. 217-240.

14. Rouch, J.D., et al., *Development of Functional Microfold (M) Cells from Intestinal Stem Cells in Primary Human Enteroids.* PLoS One, 2016. **11**(1): p. e0148216.

15. Wang, J., et al., *Donor lymphoid organs are a major site of alloreactive T-cell priming following intestinal transplantation.* Am J Transplant, 2006. **6**(11): p. 2563-71.

16. Bell, S.M., et al., *R-spondin 2 is required for normal laryngeal-tracheal, lung and limb morphogenesis.* Development, 2008. **135**(6): p. 1049-58.

17. Garside, P., et al., *Analysis of enteropathy induced by tumour necrosis factor alpha.* Cytokine, 1993. **5**(1): p. 24-30.

18. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome Biol, 2002. **3**(7): p. RESEARCH0034.

19. Akiyama, N., et al., *Limitation of immune tolerance-inducing thymic epithelial cell development by Spi-B-mediated negative feedback regulation.* J Exp Med, 2014. **211**(12): p. 2425-38.

20. Nagalakshmi, M.L., et al., *Interleukin-22 activates STAT3 and induces IL-10 by colon epithelial cells.* Int Immunopharmacol, 2004. **4**(5): p. 679-91.

21. Lindemans, C.A., et al., *Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration.* Nature, 2015. **528**(7583): p. 560-4.

22. Fujiie, S., et al., *Proinflammatory cytokines induce liver and activation-regulated chemokine/ macrophage inflammatory protein-3alpha/ CCL20 in mucosal epithelial cells through NF-kappaB.* International Immunology, 2001. **13**(10): p. 1255-1263.

23. Novack, D.V., et al., *The IkappaB function of NF-kappaB2 p100 controls stimulated osteoclastogenesis.* J Exp Med, 2003. **198**(5): p. 771-81.

24. Grabinger, T., et al., *Ex vivo culture of intestinal crypt organoids as a model system for assessing cell death induction in intestinal epithelial cells and enteropathy.* Cell Death Dis, 2014. **5**: p. e1228.

25. Claudio, E., et al., *BAFF-induced NEMO-independent processing of NF-kappa B2 in maturing B cells.* Nat Immunol, 2002. **3**(10): p. 958-65.

26. Coope, H.J., et al., *CD40 regulates the processing of NF-kappaB2 p100 to p52.* EMBO J, 2002. **21**(20): p. 5375-85.

27. Dejardin, E., et al., *The lymphotoxin-beta receptor induces different patterns of gene expression via two NF-kappaB pathways.* Immunity, 2002. **17**(4): p. 525-35.

28. Chen, H., et al., *Two promoters direct expression of the murine Spi-B gene, an Ets family transcription factor.* Gene, 1998. **207**(2): p. 209-218.

29. Bartholdy, B., et al., *The Ets factor Spi-B is a direct critical target of the coactivator OBF-1.* Proc Natl Acad Sci U S A, 2006. **103**(31): p. 11665-70.

30. Spahn, T.W., et al., *Induction of oral tolerance to cellular immune responses in the absence of Peyer's patches.* Eur J Immunol, 2001. **31**(4): p. 1278-87.

31. Komine, M., et al., *Tumor necrosis factor-alpha cooperates with receptor activator of nuclear factor kappaB ligand in generation of osteoclasts in stromal cell-depleted rat bone marrow cell culture.* Bone, 2001. **28**(5): p. 474-83.

32. Fuller, K., et al., *TNFalpha potently activates osteoclasts, through a direct action independent of and strongly synergistic with RANKL.* Endocrinology, 2002. **143**(3): p. 1108-18.

33. Zhang, Y.H., et al., *Tumor necrosis factor-alpha (TNF) stimulates RANKL-induced osteoclastogenesis via coupling of TNF type 1 receptor and RANK signaling pathways.* J Biol Chem, 2001. **276**(1): p. 563-8.

34. Pasparakis, M., et al., *Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF 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, 1996. **184**(4): p. 1397-411.

35. Pasparakis, M., et al., *Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient for tumor necrosis factor and its 55-kDa receptor.* Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6319-23.

36. Kuprash, D.V., et al., *Novel tumor necrosis factor-knockout mice that lack Peyer's patches.* Eur J Immunol, 2005. **35**(5): p. 1592-600.

37. Bren, G.D., et al., *Transcription of the RelB gene is regulated by NF-kappaB.* Oncogene, 2001. **20**(53): p. 7722-33.

38. Zhao, Z., et al., *TNF Induction of NF-kappaB RelB Enhances RANKL-Induced Osteoclastogenesis by Promoting Inflammatory Macrophage Differentiation but also Limits It through Suppression of NFATc1 Expression.* PLoS One, 2015. **10**(8): p. e0135728.

39. Sato, T., et al., *Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche.* Nature, 2009. **459**(7244): p. 262-265.

40. Sato, T. and H. Clevers, *Primary mouse small intestinal epithelial cell cultures.* Methods Mol Biol, 2013. **945**: p. 319-28.

41. Hase, K., et al., *Distinct gene expression profiles characterize cellular phenotypes of follicle-associated epithelium and M cells.* DNA Res, 2005. **12**(2): p. 127-37.

42. Zhao, X., et al., *CCL9 is secreted by the follicle-associated epithelium and recruits dome region Peyer's patch CD11b+ dendritic cells.* Journal of immunology (Baltimore, Md. : 1950), 2003. **171**(6): p. 2797-2803.

43. Anderle, P., et al., *Novel markers of the human follicle-associated epithelium identified by genomic profiling and microdissection.* Gastroenterology, 2005. **129**(1): p. 321-7.

44. Rumbo, M., et al., *Lymphotoxin beta receptor signaling induces the chemokine CCL20 in intestinal epithelium.* Gastroenterology, 2004. **127**(1): p. 213-23.

45. Kimura, S., et al., *Visualization of the entire differentiation process of murine M cells: suppression of their maturation in cecal patches.* Mucosal Immunol, 2015. **8**(3): p. 650-60.

46. Oeckinghaus, A., M.S. Hayden, and S. Ghosh, *Crosstalk in NF-kappaB signaling pathways.* Nat Immunol, 2011. **12**(8): p. 695-708.

47. Schjerven, H., et al., *De novo synthesized RelB mediates TNF-induced up-regulation of the human polymeric Ig receptor.* J Immunol, 2004. **173**(3): p. 1849-57.

48. Cui, W., D.D. Taub, and K. Gardner, *qPrimerDepot: a primer database for quantitative real time PCR.* Nucleic Acids Res, 2007. **35**(Database issue): p. D805-9.

49. Spandidos, A., et al., *PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification.* Nucleic Acids Res, 2010. **38**(Database issue): p. D792-9.

50. Natividad, J.M., et al., *Differential induction of antimicrobial REGIII by the intestinal microbiota and Bifidobacterium breve NCC2950.* Appl Environ Microbiol, 2013. **79**(24): p. 7745-54.

51. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-98.

52. Cervia, D., et al., *Modulation of the neuronal response to ischaemia by somatostatin analogues in wild-type and knock-out mouse retinas.* J Neurochem, 2008. **106**(5): p. 2224-35.

53. Larderet, G., et al., *Human side population keratinocytes exhibit long-term proliferative potential and a specific gene expression profile and can form a pluristratified epidermis.* Stem Cells, 2006. **24**(4): p. 965-74.

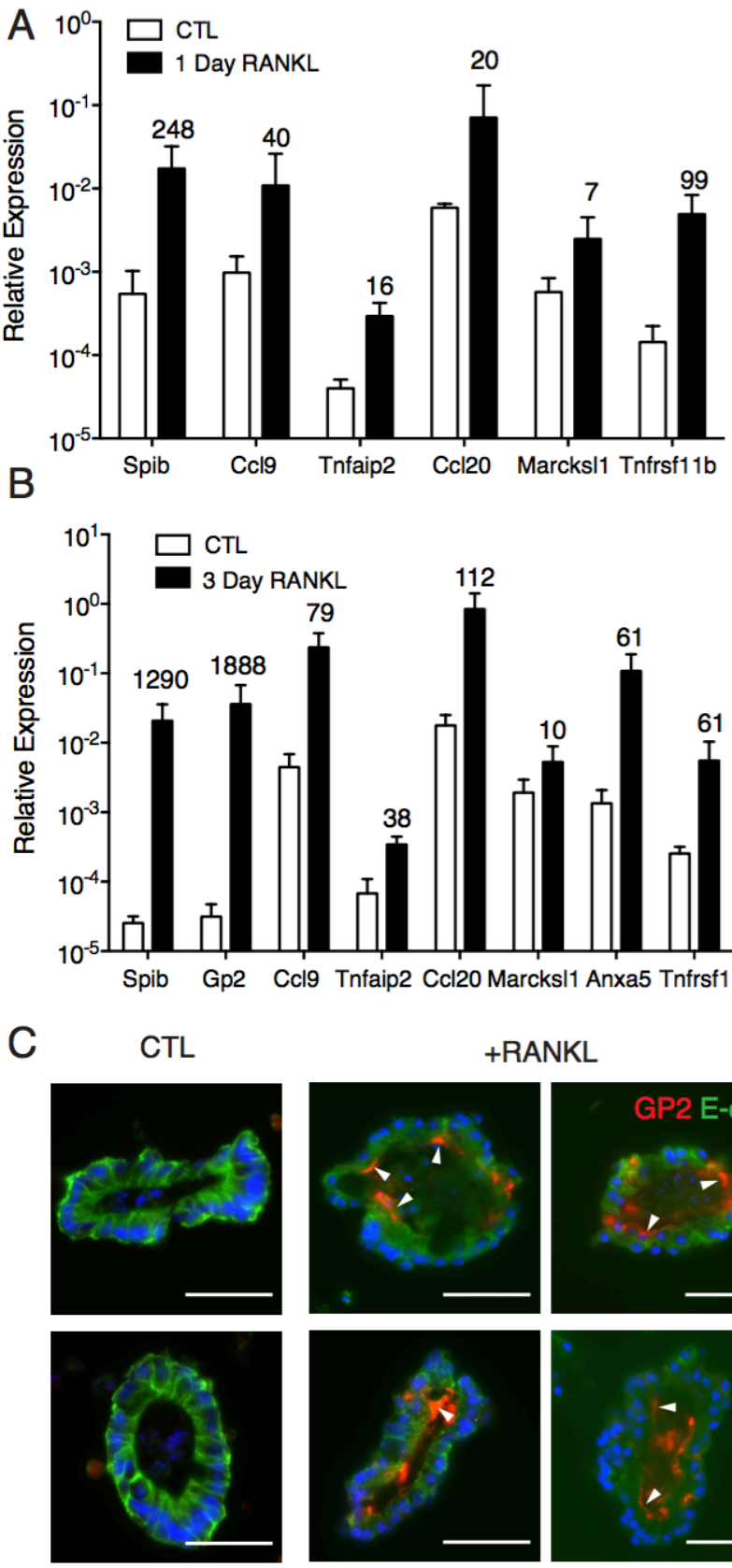

A
CTL
1 Day RANKL
Relative Expression
248
40
16
20
7
99
Spib
Ccl9
Tnfaip2
Ccl20
Marcksl1
Tnfrsf11b
B
CTL
3 Day RANKL
Relative Expression
1290
1888
79
38
112
10
61
61
Spib
Gp2
Ccl9
Tnfaip2
Ccl20
Marcksl1
Anxa5
Tnfrsf11b
C
CTL
+RANKL
GP2 E-cad

Fig. 1. Enteroids stimulated with RANKL express M-cell-associated genes. A and B: Enteroids were stimulated with 100 ng/ml RANKL for 1 day (A) or 3 days (B). Expression of genes was determined by quantitative PCR and reported as relative expression normalized to *Gapdh*. Numbers above bars indicate fold induction of M-cell-associated genes compared to untreated controls. Values reported are mean ± SE of 4 experiments. Relative expression of all genes examined was increased after RANKL treatment compared to controls with $P < 0.05$ for each gene. C: Immunofluorescence of control enteroids and enteroids treated with 100 ng/ml RANKL for 3 days. DAPI (blue), E-cadherin (green), and GP2 (red). Arrowheads indicate apical GP2 staining on single cells. All scale bars 50 μm.

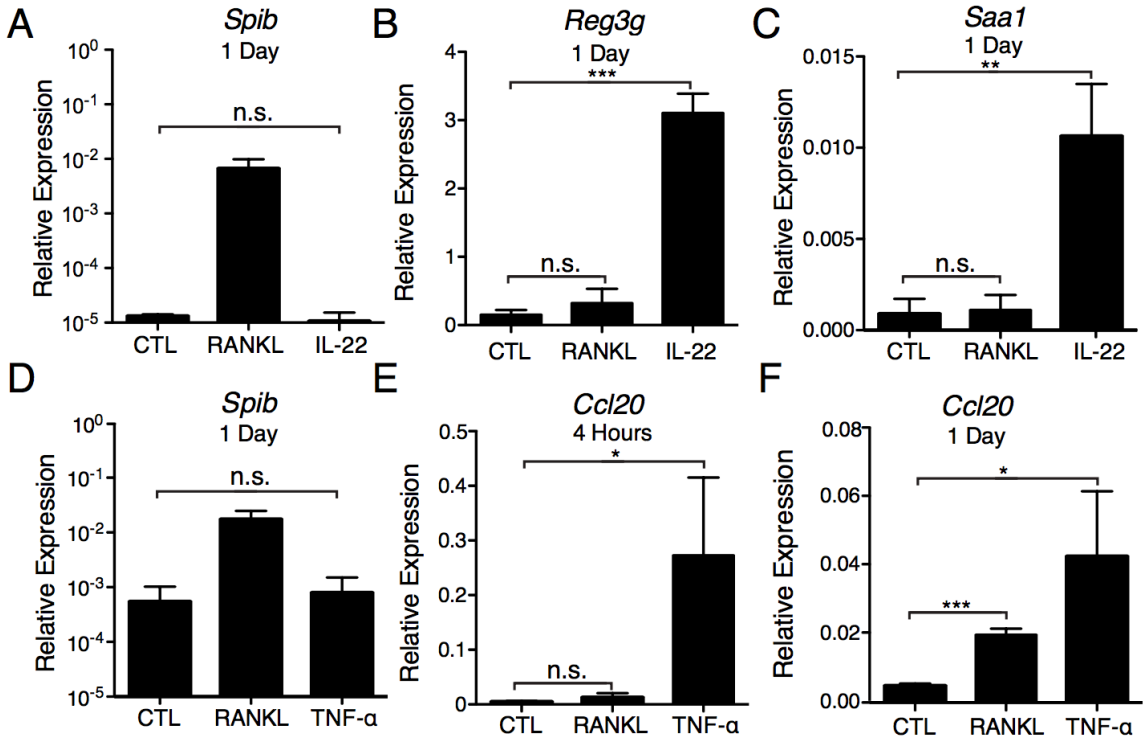


Fig. 2: RANKL specifically induces *Spib* in enteroids. A-C: Enteroids were treated with 50 ng/ml IL-22 or 100 ng/ml RANKL for 1 day. Relative expression of *Spib*, *Reg3g*, and *Saa1* was determined in untreated, RANKL-treated and IL-22 treated enteroids. Data reported as mean ± SE of 3 experiments. D- F: Enteroids were treated with 50 ng/ml TNF-α or 100 ng/ml RANKL for 1 day or 4 hours. Relative expression of *Spib* and *Ccl20* was determined in untreated, RANKL-treated and TNF-α-treated enteroids at 1 day (D, F) or 4 hours (E). Data represented as mean ± SE of 4 experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

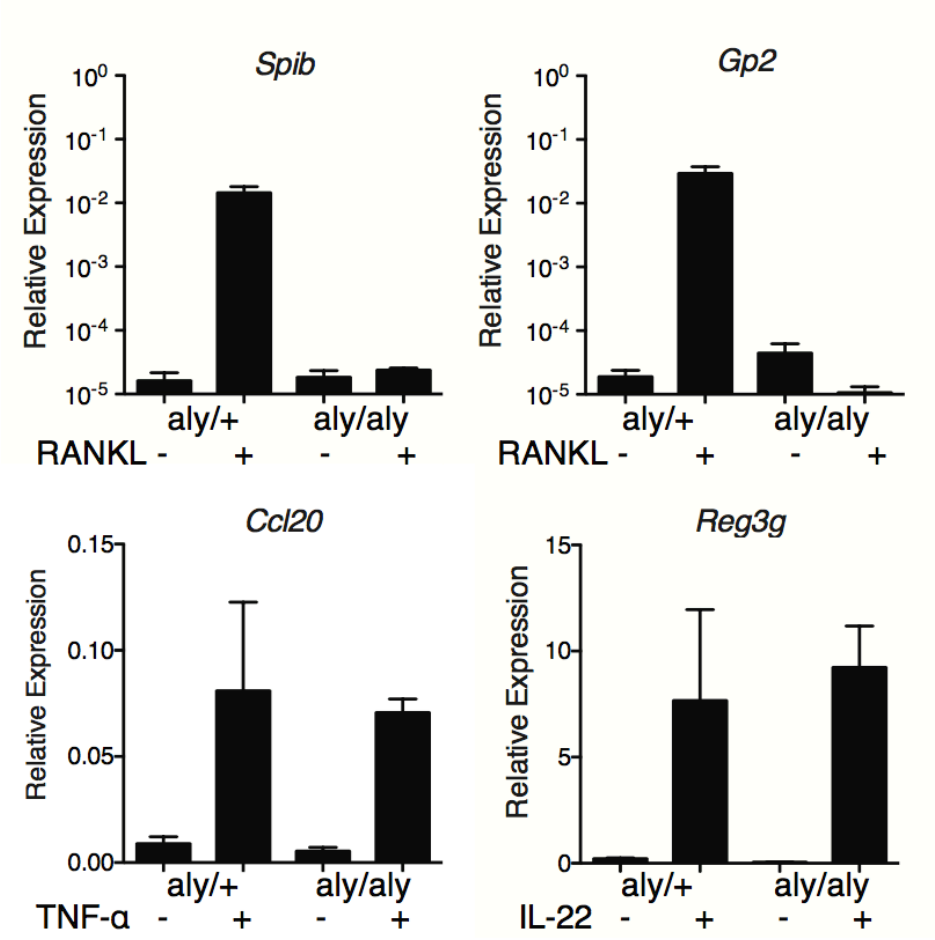


Fig. 3: *Aly/aly* enteroids do not express M-cell-specific genes when stimulated with RANKL. A and B: Relative expression of *Spib* and *Gp2* following 100 ng/ml RANKL stimulation for 3 days in *aly/+* control and *aly/aly* enteroids. Data represented as mean ± SE of 3 experiments. C: Relative expression of *Ccl20* following treatment with 50 ng/ml TNF-α for 4 h in *aly/+* control and *aly/aly* enteroids. Data represented as mean ± SE of 3 experiments. D: Relative expression of *Reg3g* following treatment of *aly/+* control and *aly/aly* enteroids with 50 ng/ml IL-22 for 1 day. Data reported as mean ± SE of 2 experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

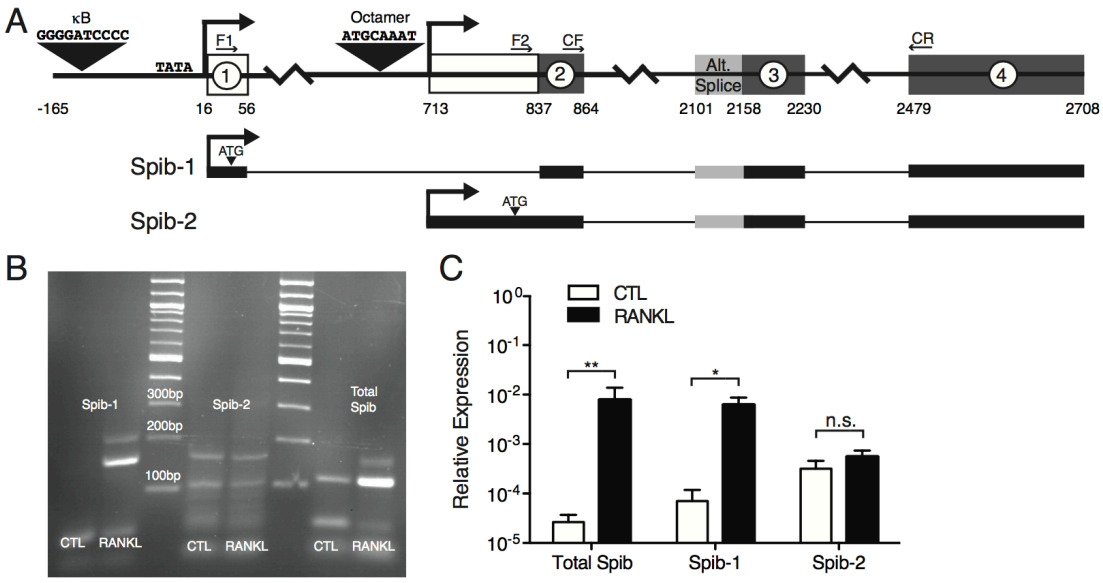
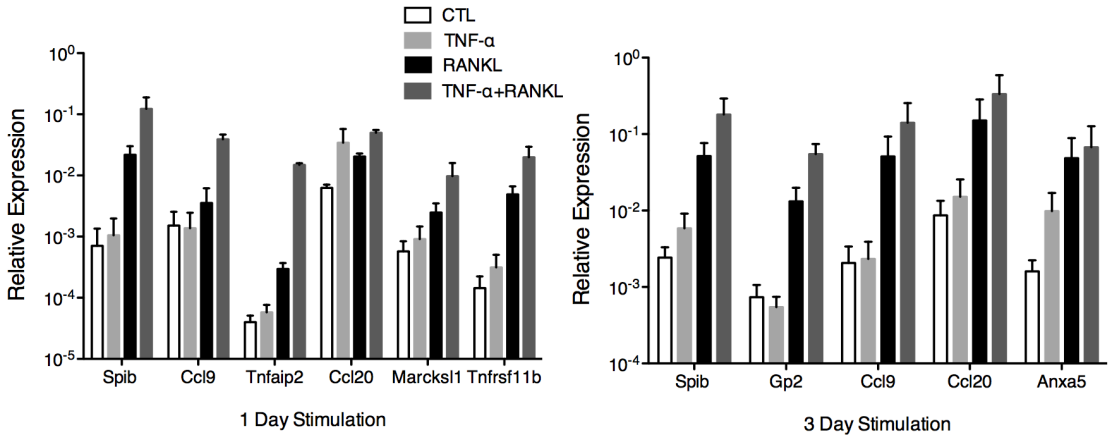


Fig. 4. RANKL stimulation of enteroids induces the Spib-1 transcript of *Spib*. A: Schematic of mouse chromosome 7 genomic DNA containing the promoters and first 4 exons of *Spib*. The exon-intron boundaries for the first 4 exons are numbered based on aligning the NCBI Reference Sequence mRNA for mouse *Spib* (NM_019866.1) with 6092 bp of C57BL/6 genomic chromosome 7 sequence (NC_000073.6, 44525995..44532086, complement), with nucleotide position 1 assigned based on the predicted 5' end of a *Spib* mRNA with the maximum amount of 5' untranslated sequence. Large arrowheads indicate the κB and octamer binding sites located in the first and second promoters, respectively. Individual exons are indicated by numbers in white circles. Exons or regions of exons in white boxes are unique to the Spib-1 and Spib-2 transcripts transcribed from promoters 1 and 2, respectively; exons in dark grey boxes are present in both Spib-1 and Spib-2. The light grey portion at the 5' end of exon 3 is an alternatively spliced region included in some *Spib* splice variants. F1= Spib-1 specific forward primer; F2 = Spib-2 specific forward primer; CF = Spib common forward primer; CR = common reverse primer. B: Agarose gel of PCR products obtained with

Spib-1-specific, Spib-2-specific, and common Spib forward primers using cDNA from control enteroids and enteroids stimulated with 100 ng/ml RANKL for 1 day. C: Relative expression of total *Spib*, Spib-1 and Spib-2 after 1 day of stimulation with 100 ng/ml RANKL. Data reported as mean ± SE of 3 experiments. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.



Fold Induction Over Unstimulated Control

Condition	Spib		Gp2		Ccl9		Tnfaip2		Marcksl1		Ccl20		Anxa5		Tnfrsf11b	
	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3	Day 1	Day 3
TNF-α	2.4	2.3	N/A	1.4	5.0	1.6	2.9	N/A	2.0	N/A	4.8	2.3	N/A	6.4	5.8	N/A
RANKL	237.9	17.4	N/A	25.1	37.0	21.6	15.7	N/A	7.4	N/A	11.2	12.2	N/A	23.3	99.4	N/A
TNF-α+RANKL	578.9	70.0	N/A	192.7	227.8	85.2	93.1	N/A	39.6	N/A	41.5	53.7	N/A	36.2	585.3	N/A

Fig. 5. TNF-α enhances RANKL-induced M-cell-associated gene expression. A: Relative expression of M-cell-specific and FAE-specific genes after no stimulation or 1 day of stimulation with 50 ng/ml TNF-α, 100 ng/ml RANKL, or 50 ng/ml TNF-α plus 100 ng/ml RANKL. Data reported as mean ± SE of 3 experiments. Expression of all genes was increased in TNF-α plus RANKL-treated enteroids compared to RANKL-treated enteroids, $P < 0.05$. B: Relative expression of M-cell-specific genes after 3 days of stimulation with 50 ng/ml TNF-α, 100 ng/ml RANKL, or 50 ng/ml TNF-α plus 100 ng/ml RANKL. Data reported as mean ± SE of 3 experiments. Expression of all genes except *Anxa5* was significantly increased in TNF-α plus RANKL-treated enteroids compared to RANKL-treated enteroids, $P < 0.05$. C: Table comparing average fold induction of M-cell-specific and FAE-specific genes by RANKL vs. TNF-α plus RANKL.

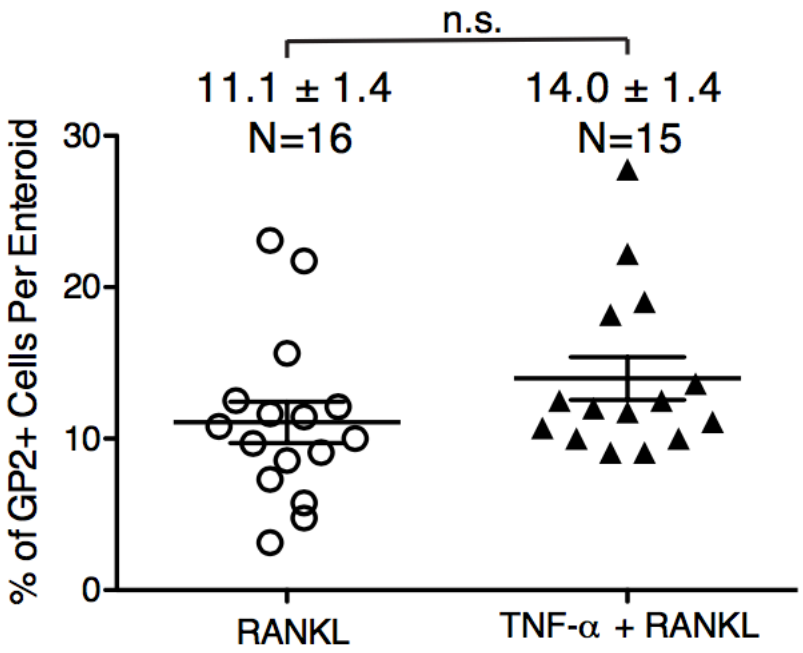


Fig. 6. Addition of TNF-α does not increase the frequency of $GP2^+$ M cells in RANKL-treated enteroids. The percentage of $GP2^+$ cells out of the total number of $DAPI^+$ nuclei was compared in sections of enteroids treated for 3 days with 100 ng/ml RANKL or 50 ng/ml TNF-α combined with 100 ng/ml RANKL. The results are presented as a scatter plot of data collected from individual enteroids, with the mean ± SE of each group and the number of enteroids analyzed listed at the top. n.s., not significant.

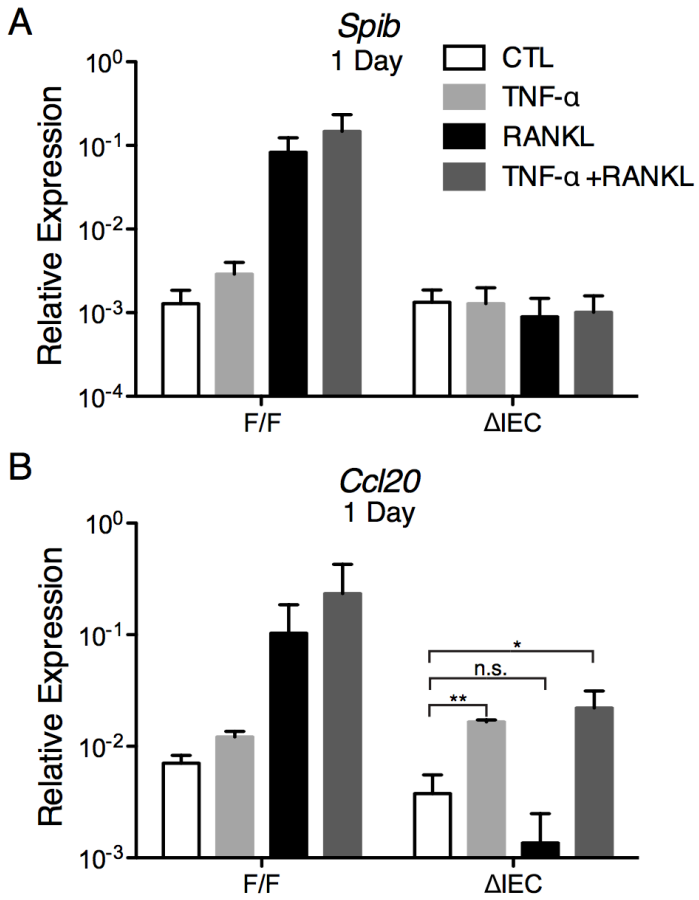


Fig. 7. TNF-α does not induce *Spib* expression in RANK$^{\Delta IEC}$ enteroids. A and B: Relative expression of *Spib* (A) and *Ccl20* (B) by RANK$^{F/F}$ and RANK$^{\Delta IEC}$ enteroids that were untreated or stimulated with 50 ng/ml TNF-α, 100 ng/ml RANKL, or 50 ng/ml TNF-α plus 100 ng/ml RANKL. Data reported as mean ± SE of 3 experiments. *, $P < 0.05$; **, $P < 0.01$; n.s., not significant.

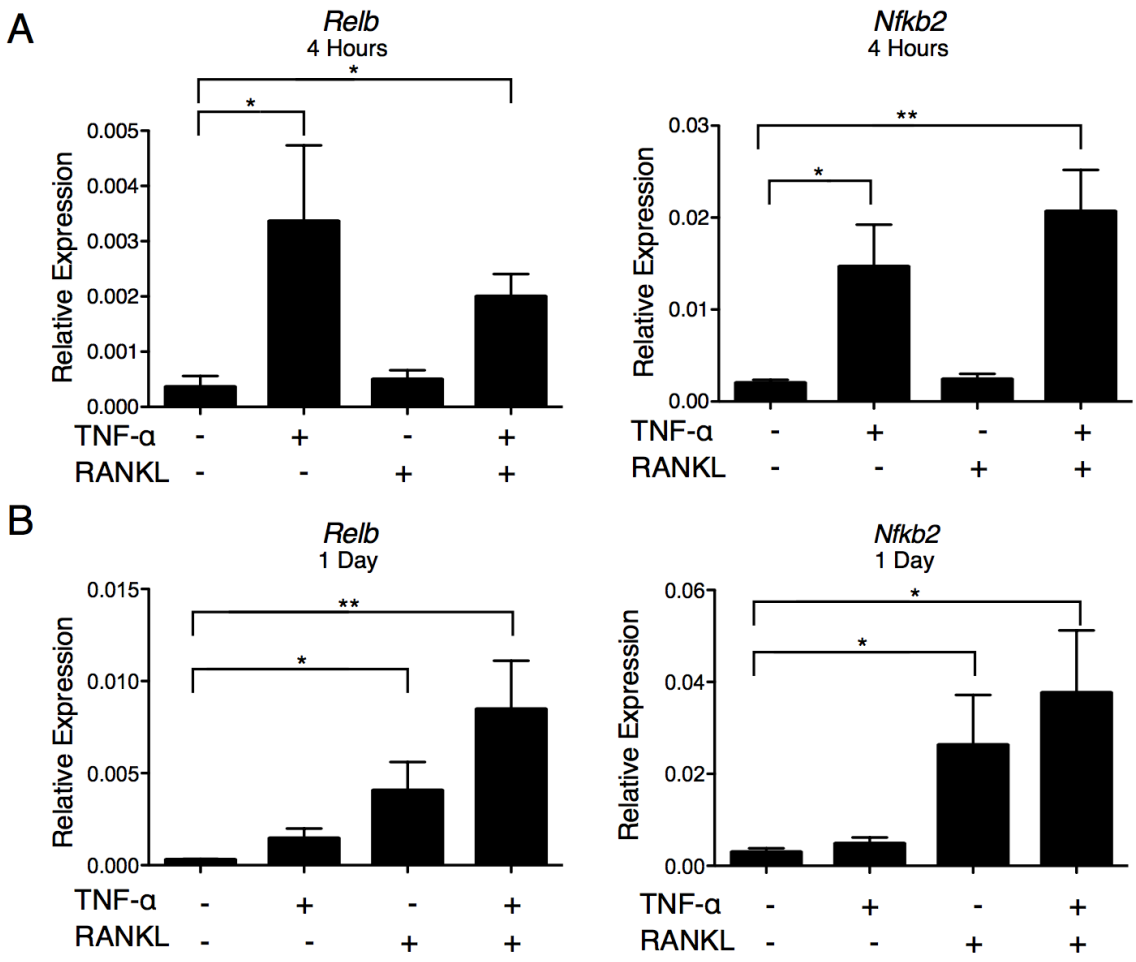


Fig. 8. TNF-α induces *Relb* and *Nfkb2* expression and enhances RANKL-induced *Relb* and *Nfkb2*. A: Relative expression of *Relb* and *Nfkb2* after 4 h of no stimulation or stimulation with 50 ng/ml TNF-α, 100 ng/ml RANKL, or 50 ng/ml TNF-α plus 100 ng/ml RANKL. Data represented as mean ± SE of 3 experiments. B: Relative expression of *Relb* and *Nfkb2* after 1 day of no stimulation or stimulation with 50 ng/ml TNF-α, 100 ng/ml RANKL, or 50 ng/ml TNF-α plus 100 ng/ml RANKL. Data represented as mean ± SE of 4 experiments. *, $P < 0.05$; **, $P < 0.01$.

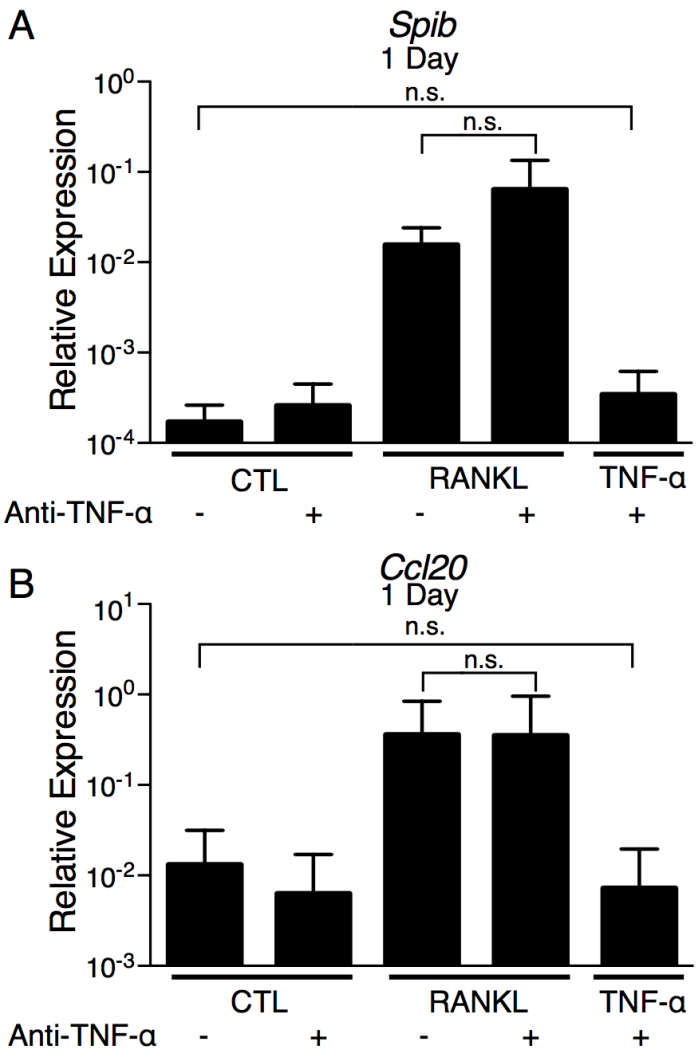


Fig. 9. RANKL induces M-cell-specific gene expression in the absence of endogenous TNF-α. A and B: Relative expression of *Spib* and *Ccl20* by enteroids after 1 day of no stimulation or stimulation with 50 ng/ml TNF-α or 100 ng/ml RANKL with or without 5 μg/ml of a neutralizing anti-TNF-α antibody. Data reported as mean ± SE of 3 experiments. n.s., not significant.

TABLE 1. Primers Used for Quantitative PCR

Primer Name	**5'-3' Sequence**	**Source of Primers**
Common *Spib* Forward	GCCCACACTTAAGCTGTTTGTA	Sato et al. [40]
Spib-1 Forward	CTCTGAACCACCATGCTTGCT	Bartholdy et al. [29]
Spib-2 Forward	AGGGCGGCCCTGACAT	
Common *Spib* Reverse	CTGTCCAGCCCCATGTAGAG	Sato et al. [40]
Gp2 Forward	CTGCTACCTCGAAGGGGACT	qPrimer Depot [48]
Gp2 Reverse	CATTGCCAGAGGGAAGAACT	
Ccl9 Forward	TACTGCCCTCTCCTTCCTCA	Kanaya et al. [4]
Ccl9 Reverse	TTGAAAGCCCATGTGAAACA	
Anxa5 Forward	ATCCTGAACCTGTTGACATCCC	PrimerBank [49]
Anxa5 Reverse	AGTCGTGAGGGCTTCATCATA	
Ccl20 Forward	TCCAGAGCTATTGTGGGTTTCA	PrimerBank [49]
Ccl20 Reverse	GAGGAGGTTCACAGCCCTTTT	
Tnfrsf11b Forward	GGGCGTTACCTGGAGATCG	Akiyama et al. [19]
Tnfrsf11b Reverse	GAGAAGAACCCATCTGGACATTT	
Tnfaip2 Forward	TACTGCCCTCTCCTTCCTCA	qPrimerDepot [48]
Tnfaip2 Reverse	TTGAAAGCCCATGTGAAACA	
Reg3g Forward	CGTGCCTATGGCTCCTATTGCT	Natividad et al. [50]
Reg3g Reverse	TTCAGCGCCACTGAGCACAGAC	
Marcksl1 Forward	GGCAGCCAGAGCTCTAAGG	Sato et al. [40]
Marcksl1 Reverse	TCACGTGGCCATTCTCCT	
Saa1 Forward	CATTTGTTCACGAGGCTTTCC	Ivanov et al. [51]
Saa1 Reverse	GTTTTTCCAGTTAGCTTCCTTCATGT	
Relb Forward	ACTGGATGCCCAGGTTGTTA	qPrimerDepot [48]
Relb Reverse	CCTGGTGTGGAAGGACTGG	
Nfkb2 Forward	GGCCGGAAGACCTATCCTACT	PrimerBank [49]
Nfkb2 Reverse	CTACAGACACAGCGCACACT	
Rpl13a Forward	CACTCTGGAGGAGAAACGGAAGG	Cervia et al. [52]
Rpl13a Reverse	GCAGGCATGAGGCAAACAGTC	
Gapdh Forward	TTCACCACCATGGAGAAGGC	Larderet et al. [53]
Gapdh Reverse	GGCATGGACTGTGGTCATGA	

Chapter 2

Intestinal M Cell Differentiation is Compromised in the Absence of Atoh1: Evidence that M Cells Belong to the Secretory Enterocyte Lineage

Megan B. Wood and Ifor R. Williams MD, PhD

Manuscript in preparation for submission to Mucosal Immunology, 2016.

All experiments in this study were performed by Megan B. Wood.

This manuscript was written by Megan B. Wood and Ifor R. Williams.

ABSTRACT

The Microfold (M) cell performs an important function as the intermediary between the immune system and the intestinal lumen. While the function and late-stage differentiation of the M cell have been studied, the early requirements for the differentiation of this unique cell are unknown. Here, we use enteroids derived from the intestinal epithelium of a mouse with a conditional knockout of Atoh1 in the small intestinal epithelium to examine the role of Atoh1 in M cell differentiation. M cell differentiation in enteroids treated with RANKL is blocked in the absence of Atoh1. Atoh1 deficient enteroids lack Paneth cells. So, Wnt3a and a GSK3 inhibitor were included in some experiments to replace the natural Wnt signal provided by Paneth cells. We find that the stabilization of β-catenin with these additives inhibits differentiation of M cells in wildtype enteroids and causes mild responsiveness to RANKL in the Atoh1 deficient enteroids. However, RANKL treatment of mice with Atoh1 deficient intestinal epithelium does not result in M cell specific gene expression. The dependence of the M cell on Atoh1 expression places the M cell in the secretory lineage of intestinal epithelial cell development along with Paneth, goblet, enteroendocrine, and tuft cells.

INTRODUCTION

The Microfold (M) cell is a specialized epithelial cell in the small intestine specifically found in the Follicle associated epithelium (FAE) or isolated lymphoid follicle epithelium. The M cell is markedly different from the absorptive enterocyte in both morphology and function. The apical surface exhibits stubby microvilli while the cytoplasm is filled with an advanced vesicular network; [1] M cells use this network if vesicles to mediate the uptake of particulate antigen from the apical surface to an immune cell residing in the cell's basolateral pocket. [2] A loss of M cells prevents uptake of beads or fluorescent bacteria in to the Peyer's patch. [3]The M cell pathway of antigen uptake is necessary for the timely acquisition of fecal IgA in mice.[4] Thus, the M cell is an important mediator of the immune response to luminal contents.

The intestinal epithelium of the small intestine is overwhelmingly composed of absorptive enterocytes that facilitate nutrient uptake and have a regimented brush border composed of microvilli. 10% of the small intestinal epithelium is made up of specialized epithelial cells collectively termed secretory cells.[5] Cells in this category include goblet cells, Paneth cells, enteroendocrine cells and the recently discovered tuft cell. [6] Each of these cells has a known function that is necessary for homeostasis of the intestinal epithelial barrier.

Both the absorptive and secretory cells types develop from small intestinal crypts with each lineage having a defined developmental program. Wnt signaling supported by Paneth cells leads to the renewal of the stem cell niche. [7] The first stage of differentiation for an absorptive enterocyte is Notch signaling which upregulates the

transcription factor Hes1, actively repressing the secretory cell lineage by inhibiting expression of the transcription factor Atoh1. Notch and Wnt signaling are abrogated in secretory precursors with Atoh1 expression.[8] A cell that will become part of the secretory lineage expresses a Delta-like ligand, preventing Notch signaling and allowing for expression of Atoh1. Atoh1 expression is required for precursor cells to differentiate into a cell of the secretory lineage. [9] Following Atoh1 expression, each secretory cell will express its own master regulator transcription factor.

Absorptive enterocytes, secretory cells, and M cells in the small intestine develop from LGR5+ stem cells as confirmed by fate mapping. [10] RANKL-RANK signaling has been shown to be required for differentiation of the M cell after the transit amplifying cell stage. [3, 4, 10] RANKL signaling induces the transcription factor Spi-B leading to end stage M cell specific gene expression such as *Gp2*.[1]. However, it is not known whether the M cell shares its precursor stage with that of the secretory or absorptive lineage. Atoh1 and Hes1 are both important genes in multiple tissues making a full knockout of either gene embryonic lethal.[11] Therefore, a tissue specific deletion of Atoh1 in the small intestine was used to study the contribution of Atoh1 to M cell development. The enteroid culture system allows for study of all stages of intestinal epithelial differentiation and M cell differentiation with the added ability to manipulate the stem cell niche and study multiple tissues from wildtype and mutant mice. We are able to show that Atoh1 is important for M cell differentiation in intestinal enteroids as well as intestinal epithelium *in vivo*. Understanding more about the developmental program of the M cell will bring more insights into its unique morphology and function.

RESULTS

Intestinal epithelium from Atoh1$^{\Delta IEC}$ mice lack secretory cells yet form functional enteroids. In order to study the effect of Atoh1 on M cell differentiation, we crossed the Atoh1$^{F/F}$ to a mouse that uses the Villin promoter to drive Cre recombinase. The VilCre+Atoh1 fl/fl (Atoh1$^{\Delta IEC}$) mouse appears runted as compared to littermate controls (Fig. 1A). Histology shows a lack of goblet and Paneth cells in the small intestine while the architecture of the epithelium is much the same (Fig. 1 B,C). Villin driven Cre is known to have mosaic expression in the colon and cecum. [12] Atoh1$^{\Delta IEC}$ mouse has mosaic expression of Atoh1 in the colon, as areas of goblet cells are clearly present in the colon. However, there is little to no mosaicism in the small intestine (Fig. 1C). Thus, by using small intestinal crypts to seed enteroid cultures, we are able to study the dynamics of a pure population of Atoh1$^{\Delta IEC}$ epithelial cells. Enteroids were cultured from 14 -21 day old Atoh1$^{F/F}$ or Atoh1$^{\Delta IEC}$ mice. The Atoh1$^{\Delta IEC}$ enteroids grew similarly to Atoh1$^{F/F}$ controls (Fig. 1C); however, the overall output of enteroids derived from such young mice was lower than from adult wildtype mice due to the size and fragility of the small intestinal tissue.

Atoh1$^{\Delta IEC}$ enteroids do not express M cell specific genes when stimulated with RANKL. After 1 day of stimulation with RANKL, the Atoh1$^{F/F}$ enteroids showed increased expression of *Spib*, *Ccl9*, and *Ccl20*. However, the Atoh1$^{\Delta IEC}$ enteroids did not respond to RANKL stimulation by expression of *Spib* or *Ccl9* (Fig. 2A). Following 3 days of stimulation with RANKL, Atoh1$^{\Delta IEC}$ did not express *Spib*, or its dependent genes *Gp2* and *Ccl9* (Fig. 2B). Atoh1$^{\Delta IEC}$ enteroids did respond to RANKL by expressing *Ccl20*

at a 1 day timepoint suggesting that the Atoh1$^{\Delta IEC}$ enteroids could still express FAE specific but not M cell specific genes. Atoh1$^{F/F}$ enteroids expressed GP2 on the surface of a several cells per enteroid after 3 days of stimulation with RANKL much like wildtype enteroids treated with RANKL. However, Atoh1$^{\Delta IEC}$ enteroids did not express any detectable GP2 (Fig. 2C).

The addition of TNF-α did not enhance expression of M cell specific gene expression in Atoh1$^{\Delta IEC}$ enteroids. Previously, we showed that TNF-α +RANKL stimulation enhanced M cell specific gene expression such as *Spib* and *Ccl9* as well as FAE specific gene expression such as *Ccl20*.[13] Atoh1$^{F/F}$ enteroids responded similar to wildtype enteroids by showing enhancement of *Spib*, *Ccl9*, and *Ccl20* (Fig. 3A). However, stimulation with TNF-α+RANKL did not induce gene expression of *Spib* or *Ccl9*, but still induced similar expression of *Ccl20* to the Atoh1$^{F/F}$ enteroids (Fig. 3A). TNF-α alone was able to induce *Ccl20* expression in both Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ as had previously been seen in wildtype mice. Following 1 day of stimulation with IL-22, both Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids showed increased expression of *Saa1* and *Reg3g* indicating that the enteroids from these mice responded normally to IL-22 stimulation (Fig. 3b).[14]

Atoh1$^{\Delta IEC}$ mice do not respond to in vivo treatment with RANKL. Previously, it has been shown that treatment with exogenous RANKL for 3 days results in expression of M cell specific genes on the villous epithelium. [3] Since the Atoh1$^{\Delta IEC}$ mice were runted, the protocol was amended to 24 hours so that early M cell specific gene expression could be evaluated based on qPCR of RNA extracted from whole tissue.[1] Atoh1$^{F/F}$ mice responded to RANKL treatment with induction of *Spib*, *Ccl9*, *Relb* and

Nfkb2 (Fig. 4). Atoh1ΔIEC mice did not express *Spib* or *Ccl9* but did express *Relb* and *Nfkb2* following treatment with RANKL (Fig. 4). *Relb* and *Nfkb2* are expressed following signaling of canonical and non-canonical NF-κB.[15, 16] The Atoh1$^{\Delta IEC}$ pattern of gene expression following treatment with RANKL suggests that the intestinal epithelium can undergo NF-κB signaling but this does not result in M cell specific gene expression.

β-catenin stabilizing additives affect differentiation of enteroids. Enteroids derived from the small intestine require Noggin, EGF, and R-spondin (ENR-media) for prolonged culture; however, recombinant Wnt3a (W-ENR media) and GSK3 inhibitors (GSK3i), such as Chiron99021, (G-ENR media) may be added to the media to boost β-catenin levels increasing viability and number of the enteroid cultures.[17, 18] Furthermore, it has been suggested that the addition of recombinant Wnt3a and/or a GSK3 inhibitor can skew the entire enteroid toward crypt stem cells.[17] Figure 5A shows that SI enteroids cultured in W-ENR have a dampened induction of *Spib* and *Gp2* after stimulation with RANKL for 3 days. Similarly, enteroids cultured with G-ENR do not respond to RANKL stimulation (Fig. 5B). If Wnt3a or the GSK3i are not present during the stimulation with RANKL, the wildtype response is mostly rescued. (Fig. 5A, B) Since, both recombinant Wnt3a and GSK3 inhibitors act to reduce budding of the enteroids and increase stemness of crypt cells, enteroids may be prevented from starting the differentiation process into M cells by expressing *Spib* following stimulation with RANKL.

β-catenin stabilization allows Atoh1$^{\Delta IEC}$ enteroids to respond to RANKL by expressing M cell specific genes. The Atoh1$^{\Delta IEC}$ epithelium has no Paneth cells as an endogenous source of Wnt ligands. We added Wnt3a (Fig. 6A) or a GSK3i (Fig. 6B) to

the media for the first 3 days of culture. Before beginning stimulation with or without RANKL, media was changed to basic ENR containing media. Atoh1$^{\Delta IEC}$ enteroids not treated with Wnt3a or GSK3i responded as before by not expressing *Spib* or *Ccl9* upon stimulation with RANKL for one day (Fig. 6). However, pretreatment with Wnt3a or a GSK3i allows Atoh1$^{\Delta IEC}$ enteroids to respond to RANKL stimulation by expressing *Spib* and *Ccl9*. Following treatment with Wnt3a for 3 days and a 3 day stimulation with RANKL, both Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids express GP2 on the apical surface of isolated cells.(data not shown)

β-catenin stabilization increases intestinal stem cell marker expression in Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids. Adding Wnt3a or a GSK3i to Atoh1$^{\Delta IEC}$ enteroids should replace Wnt ligands not available from endogenous Paneth cells, instead these manipulations seem to bypass the requirement of Atoh1 for M cell specific gene expression. In an effort to understand the effect of β-catenin stabilization on the Atoh1$^{\Delta IEC}$ enteroids and M cell differentiation, Wnt3a or a GSK3i to the media then looked at the effect of these additives to the stem cell compartment. *Lgr5* and *Snai1* are expressed by intestinal stem cells, and both genes are targets of Wnt signaling. [19, 20] The ratio of differentiated cells to stem cells in the intestine and enteroids is quite high; therefore, *Lgr5* and *Snai1* expression reflects the small number of stem cells in their low expression level. When Wnt3a or a GSK3i was added for the first 3 days of culture, followed by a one day rest, *Lgr5* and *Snai1* expression significantly increased in both Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids (Fig. 7). When the enteroids were stimulated with RANKL instead of resting, *Lgr5* and *Snai1* expression was still increased after treatment with Wnt3a or GSK3i in the Atoh1$^{\Delta IEC}$ enteroids (Fig. 7). Treatment with Wnt3a or a

GSK3i stabilizes β-catenin resulting in increased stem cell specific gene expression. Taken with the data that the presence of Wnt3a or a GSK3i blunts the response of wildtype enteroids to RANKL, increased *Lgr5* expression suggests the enteroids are made up of mostly multipotent stem cells.

IL-22 signaling does not rescue the ability of Atoh1$^{\Delta IEC}$ enteroids to respond to RANKL. IL-22 is known to have profound effects on the intestinal epithelium that lead to the release of antimicrobial peptides and wound healing. Enteroids treated with IL-22 show increased proliferation and an expansion of intestinal stem cells.[21] Since IL-22 aids in enteroid formation from Atoh1$^{\Delta IEC}$ epithelium via STAT3 signaling, IL-22 was used to pre-treat Atoh1$^{\Delta IEC}$ enteroids before RANKL stimulation (Fig. 8).[21] IL-22 pretreatment did not rescue the ability of Atoh1$^{\Delta IEC}$ enteroids to respond to RANKL nor did it increase the response to RANKL in Atoh1$^{F/F}$ enteroids (Fig. 8A, B). Therefore, the ability of Atoh1$^{\Delta IEC}$ enteroids to respond to RANKL following treatment with Wnt3a or a GSK3i is independent of the STAT3 pathway.

DISCUSSION

Enteroids derived from pre-weaned Atoh1$^{\Delta IEC}$ mice allow the study of M-cell differentiation in the absence of Atoh1 signaling to address whether Atoh1 is required for M-cell differentiation. We found that both *in vitro* and *in vivo,* Atoh1 was necessary for *Spib* and Spi-B-dependent gene expression. The abrogation of *Spib* expression was not due to a lack of NF-κB signaling as *Ccl20* was expressed *in vitro* while *Relb* and *Nfkb2* were induced following RANKL treatment *in vivo.* These findings suggest that the M cell develops from the secretory lineage, which is dependent on Atoh1.

The developmental origin of the M cell has been speculated in the past leading to a number of theories. One of the earliest theories on how the M cell differentiates was based on the observation that M cell uptake of fluorescent beads is increased following exposure to pathogenic bacteria. This led to the belief that upon insult by a pathogen, FAE cells must quickly transdifferentiate into M cells to increase antigenic traffic to the immune cells of the PP.[22, 23] Exogenous treatment with RANKL shows that M cell specific genes are expressed in a distinct order over time as cells move up from the crypt to become villous M cells.[1] Our data also shows that treatment with RANKL results in the sequential expression of Spi-B and its dependent genes over a 3 day period. Our enteroid model replicates the constant RANKL-RANK signaling present during the development of the FAE from crypts surrounding the Peyer's patch. Therefore, we conclude that M cells develop from the crypt through the secretory lineage to become a mature specialized epithelial cell.

Our work sheds further light on the complex regulation of epithelial development and the interplay between this complicated barrier tissue and the immune system. Additionally, Atoh1 and RANKL signaling are important players in other epithelial systems such as the inner ear and the medullary thymic epithelium.[11, 24] Thus, our findings could hold important clues to the differentiation of specialized epithelial cell types in other tissues in close proximity to immune structures. Our model also provides a mechanism to further study the early differentiation of the M cell as opposed to other secretory cells. This could allow for further study of how RANKL-RANK affects the function of the FAE.

Modifying the culture conditions to increase stemness of the enteroid abrogates the response to RANKL. The addition of Wnt3a or a GSK3 inhibitor to the enteroids preferentially enhances stem cell survival by stabilizing β-catenin. Removing the stem-inducing factors during RANKL stimulation allows the cells in the enteroid to undergo M cell differentiation. Adding Wnt3a to Atoh1$^{\Delta IEC}$ enteroids expands the stem cell compartment, as evidenced by the increased expression of stem cell specific genes *Lgr5* and *Snai1*, allowing for the expression of M-cell specific genes following stimulation with RANKL. Wnt3a or a GSK3i boosts the number of Atoh1$^{\Delta IEC}$ enteroids in culture by replicating the Wnt signal normally provided by the Paneth cell in wildtype enteroids.[7] Treatment with IL-22, which induces stem cell proliferation, does not rescue Atoh1$^{\Delta IEC}$ responsiveness to RANKL indicating that this phenomenon is mediated specifically through Wnt signaling. Thus, we believe that the return of sensitivity to RANKL in Atoh1$^{\Delta IEC}$ enteroids is due to the stabilization of β-catenin, increased plasticity and greater proportion of stem cells per enteroid. Atoh1 is known to block Wnt signaling as a

step towards reducing proliferation and ultimately differentiation.[25] So, it is also possible that the lack of Atoh1 signaling in Atoh1$^{\Delta IEC}$ leads to increased responsiveness to Wnt agonists. The use of Wnt3a and GSK3 inhibitors is often suggested when enteroid cultures are difficult to maintain; however, if the goal of the study is to examine differentiation, these factors must be used with caution.

The secretory cells of the intestinal epithelium, while varying in function, arise from a common precursor and exhibit complex cytoskeletal rearrangement and complex vesicular networks. The M cell has the unique function of taking up particulate antigen in the restricted FAE exposed to RANKL. However, it is also a cell marked by distinctive morphology and extensive system of vesicles used to move antigen across the epithelium. By showing that Atoh1 is an important part of M cell differentiation, this work should cement the idea of the M cell as part of the secretory lineage of intestinal epithelial differentiation whose rare gene expression profile is dependent on RANKL restriction to the Peyer's patch.

MATERIALS AND METHODS

Mice

Villin-Cre mice from our colony were bred with B6.129S7- Atoh1^{tm3Hzo}/J mouse (Jackson Laboratories, Bar Harbor, ME) resulting in a mouse which has no functional Atoh1 in the intestinal epithelium.[5] These mice are referred to as Atoh1$^{\Delta IEC}$ in the text. Atoh1$^{f/f}$ mice lacking the Villin-Cre transgene were used as controls. In experiments where no genotype is indicated adult female C57Bl/6 mice (Jackson Laboratories) were used. Animal studies were reviewed and approved by the Emory University Institutional Animal Care and Use Committee.

Histology

Following excision and fixation in 10% formalin, small intestine and colon were embedded in paraffin, sectioned and stained by the Cancer Tissue and Pathology Shared Resource of Winship Cancer Institute of Emory University. Small intestine was co-stained with PAS and Alcian blue for visualization of Paneth and goblet cells. Colon samples were stained with Alcian blue for visualization of goblet cells.

Enteroid Culture

Enteroid culture was performed on small intestine as previously described with a few revisions. [17] Briefly, whole crypts taken from the small intestine were cultured in Matrigel and ENR media. Enteroids were cultured for 3 days before stimulation with cytokines was initiated. RANKL (Peprotech, Rocky Hill, NJ) stimulation was performed with 100ng/mL for 1 or 3 days. TNF-α (Peprotech) stimulation was performed with 50 ng/mL. 50ng/mL of IL-22 (Peprotech) was used for stimulations where indicated. W-

ENR media consisted of the ENR media containing accepted proportions of EGF, Noggin and R-spondin with 100ng/mL rWnt3a (Peprotech). G-ENR media contained ENR media supplemented with 10 μM Chiron 99021(Stemgent, Lexington, MA).

Fluorescent Imaging

Enteroids stimulated with RANKL for 3 days were liberated from Matrigel (Corning Life Sciences, Tewksbury, MA) with Cell Recovery Solution (Corning) and frozen in OCT. 5μm sections were stained with unconjugated rat anti-mouse GP2 (clone 2F11-C3, MBL International, Woburn, MA) overnight. The next day, sections were stained with DAPI, Goat anti-Rat Alexa 546 conjugated secondary antibody (Invitrogen, Carlsbad, CA), and Fitc-conjugated anti-Ecadherin(clone 36, BD Biosciences) for 2 hours.

qPCR and primers

Following extraction from Matrigel with Cell Recovery Solution(Corning), all RNA was extracted using RNEasy kit (Qiagen, Valencia, CA) and cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). qPCR was performed on a CFX connect thermocycler using the iTaq SYBR Green Universal mastermix(Bio-Rad) and the primers listed in Table 2. Fold induction was calculated by normalizing to two housekeeping genes, *Rpl13a* and *Gapdh* using the ΔΔCt method.[26] Relative expression was calculated by normalizing triplicate cycle threshold (Ct) values to *Gapdh* then setting the expression value of Gapdh as 1.

In vivo RANKL treatment

Exogenous recombinant RANKL treatment has been previously reported. [3] As the mice were used in these experiments were younger and smaller than an adult mouse,

the dosage of RANKL-GST was amended to 2.5ug/g of body weight. The first dose of RANKL was given i.p. at hour 0 with another dose at hour 16. The intestine was excised and whole tissue RNA was extracted using the RNEasy Kit (Qiagen) at 24hrs.

Statistical Analysis

A student's t test generated with Prism (GraphPad Software, La Jolla, CA) was used to determine statistical significance between means of relative expression. Significance was set at $P<0.05$.

TABLE 2. Primer sequences used in qPCR studies.

Primer Name	**Primer Sequence**	**Primer Source**
Spib Forward	GCCCACACTTAAGCTGTTTGTA	Sato, et al. [27]
Spib Reverse	CTGTCCAGCCCCATGTAGAG	
Ccl9 Forward	TACTGCCCTCTCCTTCCTCA	Kanaya, et al. [1]
Ccl9 Reverse	TTGAAAGCCCATGTGAAACA	
Ccl20 Forward	TCCAGAGCTATTGTGGGTTTCA	PrimerBank[28]
Ccl20 Reverse	GAGGAGGTTCACAGCCCTTTT	
Gp2 Forward	CTGCTACCTCGAAGGGGACT	qPrimer Depot[29]
Gp2 Reverse	CATTGCCAGAGGGAAGAACT	
Saa1 Forward	CATTTGTTCACGAGGCTTTCC	Ivanov, et al. [30]
Saa1 Reverse	GTTTTTCCAGTTAGCTTCCTTCATGT	
Reg3g Forward	CGTGCCTATGGCTCCTATTGCT	Natividad, et al.[31]
Reg3g Reverse	TTCAGCGCCACTGAGCACAGAC	
Relb Forward	ACTGGATGCCCAGGTTGTTA	qPrimerDepot[29]
Relb Reverse	CCTGGTGTGGAAGGACTGG	
Nfkb2 Forward	GGCCGGAAGACCTATCCTACT	PrimerBank[28]
Nfkb2 Reverse	CTACAGACACAGCGCACACT	
Lgr5 Forward	CCTACTCGAAGACTTACCCAGT	Schumacher, et al.[32]
Lgr5 Reverse	GCATTGGGGTGAATGATAGCA	
Snai1 Forward	CACACGCTGCCTTGTGTCT	Primerbank[28]
Snai1 Reverse	GGTCAGCAAAAGCACGGTT	
Rpl13a Forward	CACTCTGGAGGAGAAACGGAAGG	Cervia, et al. [33]
Rpl13a Reverse	GCAGGCATGAGGCAAACAGTC	
Gapdh Forward	TTCACCACCATGGAGAAGGC	Larderet, et al. [34]
Gapdh Reverse	GGCATGGACTGTGGTCATGA	

REFERENCES

1. Kanaya, T., et al., *The Ets transcription factor Spi-B is essential for the differentiation of intestinal microfold cells.* Nat Immunol, 2012. **13**(8): p. 729-36.
2. Owen, R.L., *Uptake and transport of intestinal macromolecules and microoganisms by M cells in Peyer's Patches- a personal and historical perspective.* Seminars in Immunology, 1999. **11**: p. 157-163.
3. Knoop, K.A., et al., *RANKL is necessary and sufficient to initiate development of antigen-sampling M cells in the intestinal epithelium.* J Immunol, 2009. **183**(9): p. 5738-47.
4. Rios, D., et al., *Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria.* Mucosal Immunol, 2015.
5. Shroyer, N.F., et al., *Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis.* Gastroenterology, 2007. **132**(7): p. 2478-88.
6. Gerbe, F., et al., *Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium.* The Journal of cell biology, 2011. **192**(5): p. 767-780.
7. Farin, H.F., J.H. Van Es, and H. Clevers, *Redundant sources of Wnt regulate intestinal stem cells and promote formation of Paneth cells.* Gastroenterology, 2012. **143**(6): p. 1518-1529 e7.

8. Tian, H., et al., *Opposing activities of Notch and Wnt signaling regulate intestinal stem cells and gut homeostasis.* Cell Rep, 2015. **11**(1): p. 33-42.

9. Yang, Q., et al., *Requirement of Math1 for secretory cell lineage commitment in the mouse intestine.* Science, 2001. **294**(5549): p. 2155-8.

10. de Lau, W., et al., *Peyer's patch M cells derived from Lgr5(+) stem cells require SpiB and are induced by RankL in cultured "miniguts".* Mol Cell Biol, 2012. **32**(18): p. 3639-47.

11. Huang, W.H., et al., *Atoh1 governs the migration of postmitotic neurons that shape respiratory effectiveness at birth and chemoresponsiveness in adulthood.* Neuron, 2012. **75**(5): p. 799-809.

12. Madison, B.B., et al., *Cis elements of the villin gene control expression in restricted domains of the vertical (crypt) and horizontal (duodenum, cecum) axes of the intestine.* J Biol Chem, 2002. **277**(36): p. 33275-83.

13. Wood, M.B., D. Rios, and I.R. Williams, *TNF-alpha Augments RANKL-Dependent Intestinal M Cell Differentiation in Enteroid Cultures.* Am J Physiol Cell Physiol, 2016: p. ajpcell 00108 2016.

14. Farin, H.F., et al., *Paneth cell extrusion and release of antimicrobial products is directly controlled by immune cell-derived IFN-gamma.* J Exp Med, 2014. **211**(7): p. 1393-405.

15. Schjerven, H., et al., *De novo synthesized RelB mediates TNF-induced up-regulation of the human polymeric Ig receptor.* J Immunol, 2004. **173**(3): p. 1849-57.

16. Zhao, Z., et al., *TNF Induction of NF-kappaB RelB Enhances RANKL-Induced Osteoclastogenesis by Promoting Inflammatory Macrophage Differentiation but also Limits It through Suppression of NFATc1 Expression.* PLoS One, 2015. **10**(8): p. e0135728.

17. Mahe, M.M., et al., *Establishment of gastrointestinal epithelial organoids.* Curr Protoc Mouse Biol, 2013. **3**: p. 217-240.

18. Sato, T. and H. Clevers, *Primary mouse small intestinal epithelial cell cultures.* Methods Mol Biol, 2013. **945**: p. 319-28.

19. Yin, X., et al., *Niche-independent high-purity cultures of Lgr5+ intestinal stem cells and their progeny.* Nat Methods, 2014. **11**(1): p. 106-12.

20. Horvay, K., et al., *Wnt signaling regulates Snai1 expression and cellular localization in the mouse intestinal epithelial stem cell niche.* Stem Cells Dev, 2011. **20**(4): p. 737-45.

21. Lindemans, C.A., et al., *Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration.* Nature, 2015. **528**(7583): p. 560-4.

22. Meynell, H.M., et al., *Up-regulation of microsphere transport across the follicle-associated epithelium of Peyer's patch by exposure to Streptococcus pneumoniae R36a.* FASEB J, 1999. **13**(6): p. 611-9.

23. Borghesi, C., et al., *Modifications of the follicle-associated epithelium by short-term exposure to a non-intestinal bacterium.* J Pathol, 1996. **180**(3): p. 326-32.

24. Akiyama, N., et al., *Limitation of immune tolerance-inducing thymic epithelial cell development by Spi-B-mediated negative feedback regulation.* J Exp Med, 2014. **211**(12): p. 2425-38.

25. Leow, C.C., et al., *Hath1, down-regulated in colon adenocarcinomas, inhibits proliferation and tumorigenesis of colon cancer cells.* Cancer Res, 2004. **64**(17): p. 6050-7.

26. Vandesompele, J., et al., *Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes.* Genome Biol, 2002. **3**(7): p. RESEARCH0034.

27. Sato, S., et al., *Transcription factor Spi-B-dependent and -independent pathways for the development of Peyer's patch M cells.* Mucosal Immunol, 2013. **6**(4): p. 838-46.

28. Spandidos, A., et al., *PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification.* Nucleic Acids Res, 2010. **38**(Database issue): p. D792-9.

29. Cui, W., D.D. Taub, and K. Gardner, *qPrimerDepot: a primer database for quantitative real time PCR.* Nucleic Acids Res, 2007. **35**(Database issue): p. D805-9.

30. Ivanov, II, et al., *Induction of intestinal Th17 cells by segmented filamentous bacteria.* Cell, 2009. **139**(3): p. 485-98.

31. Natividad, J.M., et al., *Differential induction of antimicrobial REGIII by the intestinal microbiota and Bifidobacterium breve NCC2950.* Appl Environ Microbiol, 2013. **79**(24): p. 7745-54.

32. Schumacher, M.A., et al., *The use of murine-derived fundic organoids in studies of gastric physiology.* J Physiol, 2015.

33. Cervia, D., et al., *Modulation of the neuronal response to ischaemia by somatostatin analogues in wild-type and knock-out mouse retinas.* J Neurochem, 2008. **106**(5): p. 2224-35.

34. Larderet, G., et al., *Human side population keratinocytes exhibit long-term proliferative potential and a specific gene expression profile and can form a pluristratified epidermis.* Stem Cells, 2006. **24**(4): p. 965-74.

FIGURES

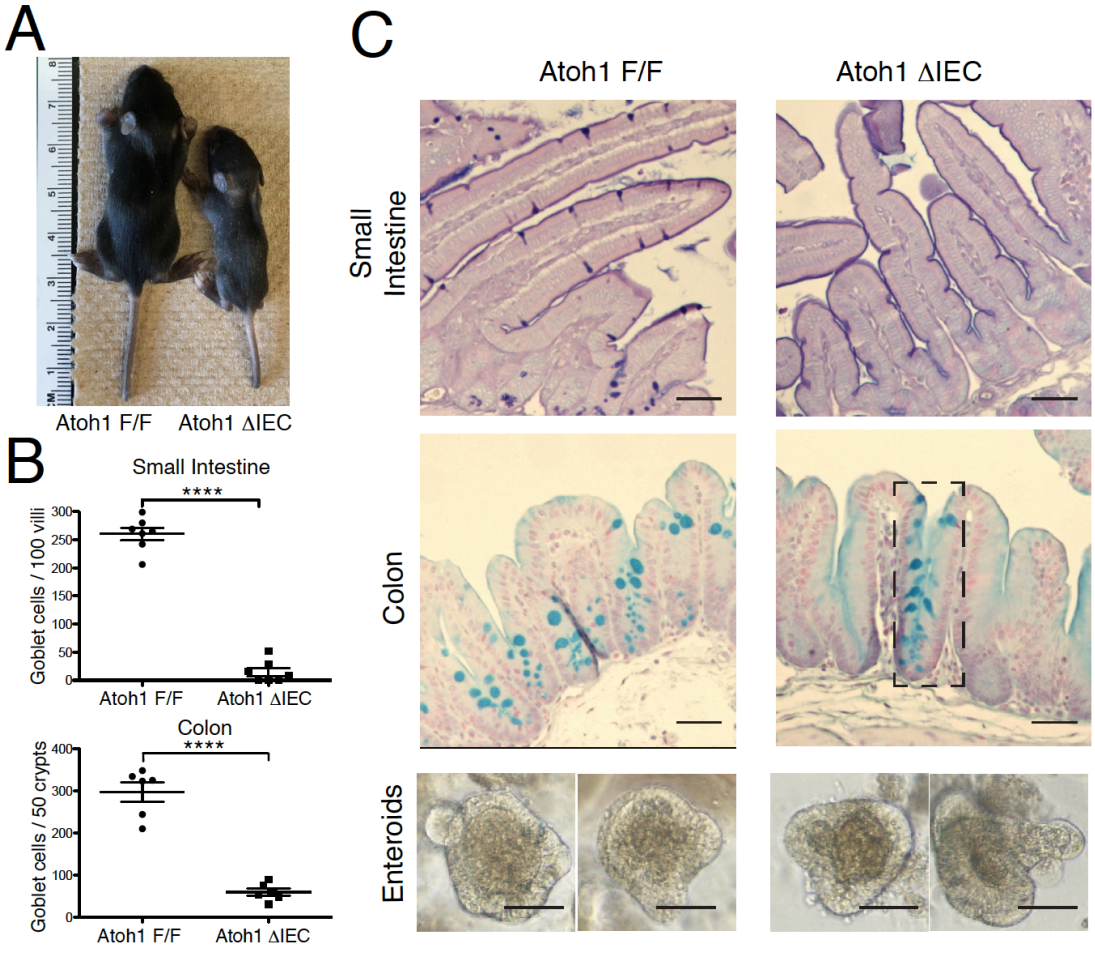


Figure 1: Atoh1$^{\Delta IEC}$ lack secretory cells in the small intestine, but the epithelium responds normally

A) Histology of small intestine and colon of Atoh1$^{F/F}$ control and Atoh1$^{\Delta IEC}$ mice visualization of goblet cells. Small intestine co-stained with PAS and Alcian blue. Colon stained with Alcian blue. Bright field images of enteroids derived from Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids after 6 days of culture. B) Goblet cell counts in the small intestine and colon. Goblet cells were counted in a representative 100 villi of the small intestine or 50 crypts in the colon. D) 14 day old Atoh1$^{F/F}$ control and Atoh1$^{\Delta IEC}$ mice from the same litter.

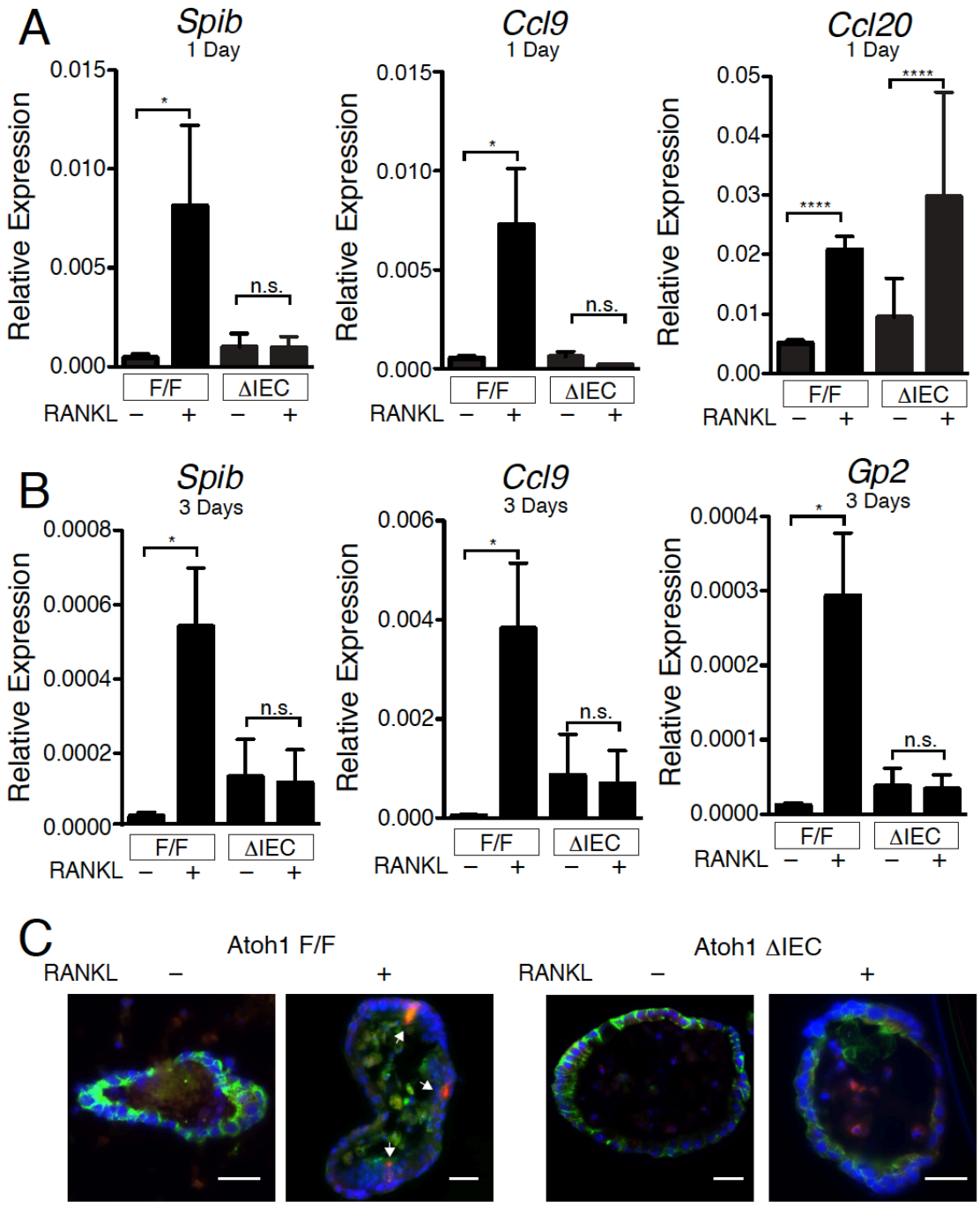


Figure 2: Atoh1$^{\Delta IEC}$ enteroids do not respond to RANKL treatment

A) Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids were treated with 100 ng/mL RANKL for 1 day and relative expression of *Spib, Ccl9,* and *Ccl20* were compared. Mean ± s.e.m. of 3 experiments. B) Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids were stimulated with 100 ng/mL

RANKL for 3 days and relative expression of *Spib*, *Gp2*, and *Ccl9* were compared. Mean+s.e.m. of 3 experiments. All relative expression normalized to *Gapdh*. *, $P < 0.05$; ****, $P < 0.0001$; n.s., not significant. C) Immunoflouresent imaging of $Atoh1^{F/F}$ and $Atoh1^{\Delta IEC}$ 3-day RANKL-treated enteroids. DAPI (blue), E-cadherin (green), and GP2 (red). Scale bars 50 μm. Arrows indicate GP2+ cells.

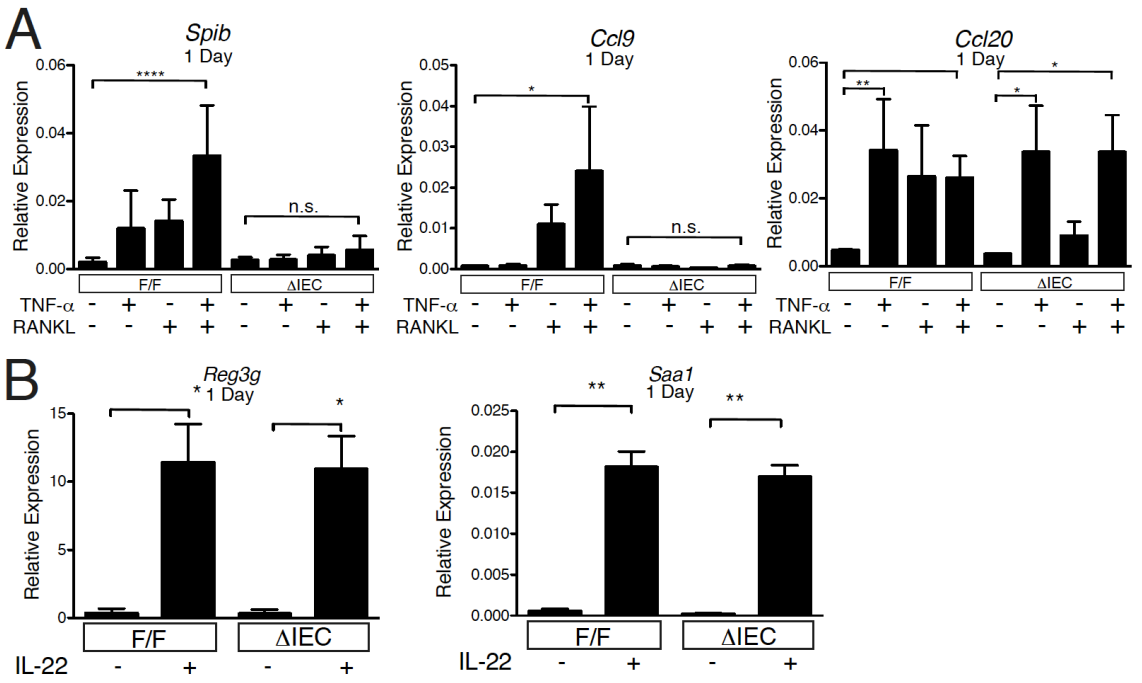


Figure 3: Atoh1$^{\Delta IEC}$ enteroids respond normally to IL-22 and TNF-α

A) *Spib*, *Ccl9*, and *Ccl20* expression were compared in Atoh1$^{F/F}$ or Atoh1$^{\Delta IEC}$ enteroids stimulated with 50ng/mL TNF-α alone, 100 ng/mL RANKL alone or 100ng/mL RANKL plus 50ng/mL TNF-α for 1 day. All relative expression normalized to *Gapdh* and shown as a mean ± s.e.m of 3 experiments. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; n.s., not significant. B) *Reg3g* and *Saa1* expression were compared in Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids treated with 50ng/mL IL-22 for 1 day. Mean ± s.e.m. of 2 experiments. Relative expression compared to *Gapdh*. *, $P < 0.05$; **, $P < 0.01$.

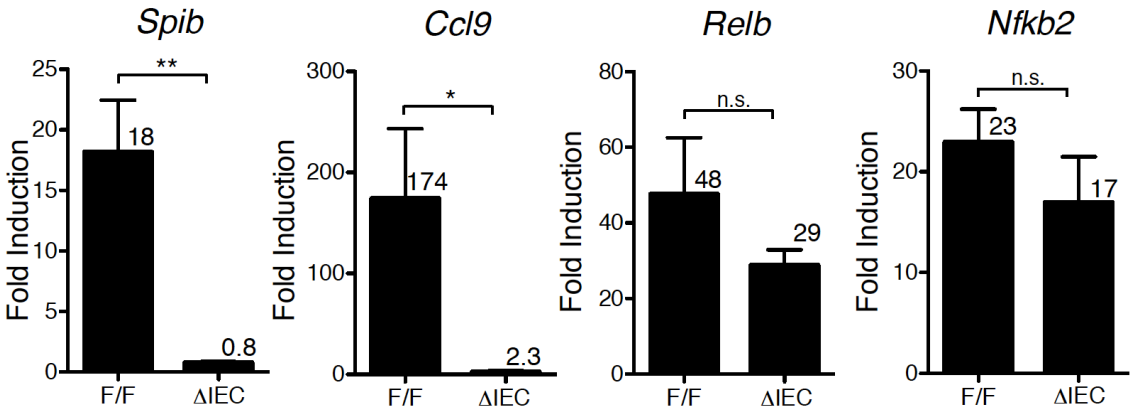


Figure 4: Atoh1$^{\Delta IEC}$ mice do not respond to *in vivo* treatment with RANKL

Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ mice were treated with exogenous RANKL at 2.5 μg/g of body weight for 24 hours. qPCR was run on whole small intestinal tissue for *Spib*(A), *Ccl9*(B), *Ccl20*(C), *Relb* (D), and *Nfkb2*(E). All relative expression expressed as mean ± s.e.m of 2 experiments and normalized to *Gapdh*. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$; n.s., not significant.

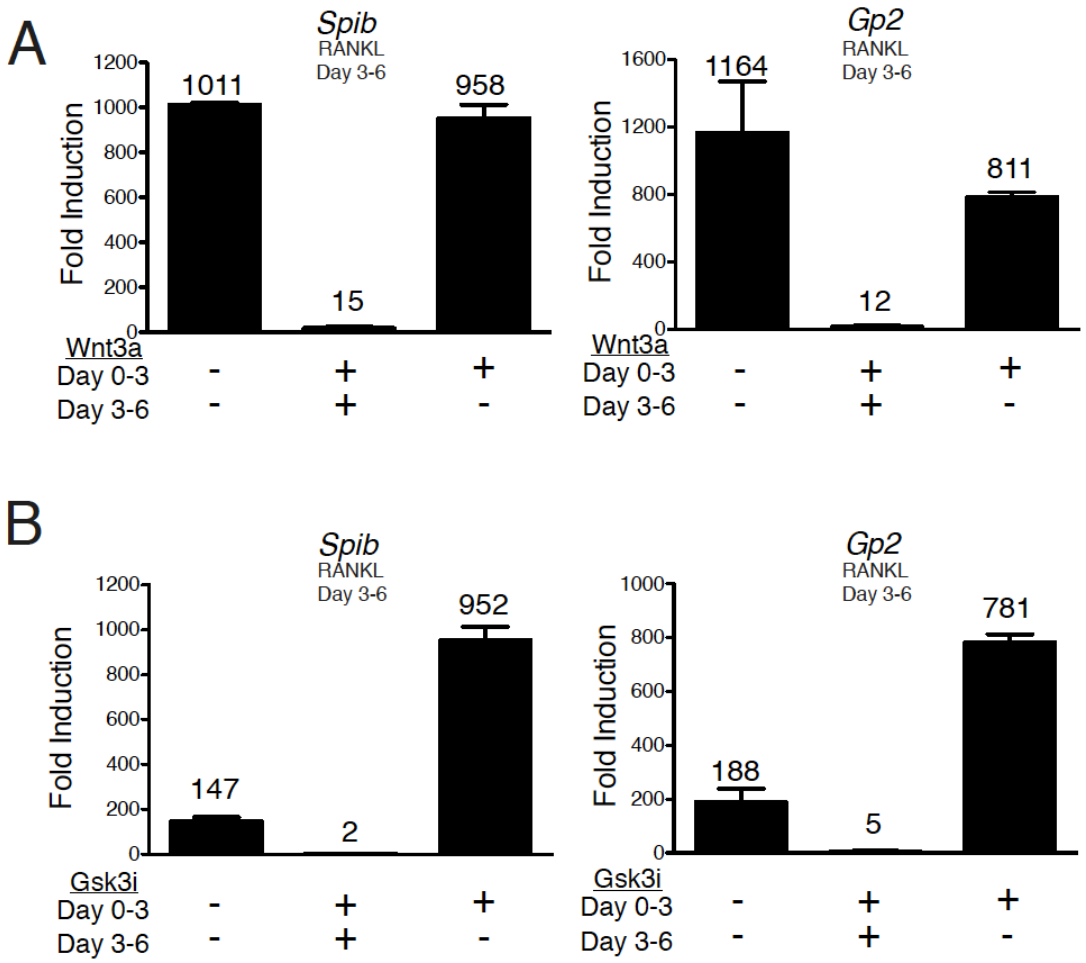


Figure 5: Wnt3a or inhibition of GSK3 prevents small intestinal enteroids from responding to RANKL

A) ENR enteroids were cultured in ENR media and stimulated with 100ng/mL RANKL for 3 days. W-ENR enteroids were cultures in ENR media supplemented with 100ng/mL Wnt3a through out the 3 day stimulation with 100ng/mL RANKL. W-ENR -> ENR enteroids were cultured in ENR media with 100ng/mL Wnt3a for 3 days then were switched to ENR media for the 3 day stimulation with 100ng/mL RANKL. *Spib* and *Gp2* expression was compared to unstimulated controls and normalized to *Rpl13a* and *Gapdh*. Average fold induction indicated by numbers above columns. Fold induction expressed

as a mean ± of 3 experiments. B) ENR enteroids were cultured in ENR media and stimulated with 100ng/mL RANKL for 3 days. G-ENR enteroids were cultured in ENR media supplemented with 10μM GSK3i (Chiron99021) through out the 3 day stimulation with 100ng/mL RANKL. G-ENR -> ENR enteroids were cultured in ENR media with 10μM GSK3i for 3 days then were switched to ENR media for the 3 day stimulation with 100ng/mL RANKL. *Spib* and *Gp2* expression was compared to unstimulated controls and normalized to *Rpl13a* and *Gapdh*. Average fold induction indicated by numbers above columns. Fold induction expressed as a mean ± of 3 experiments.

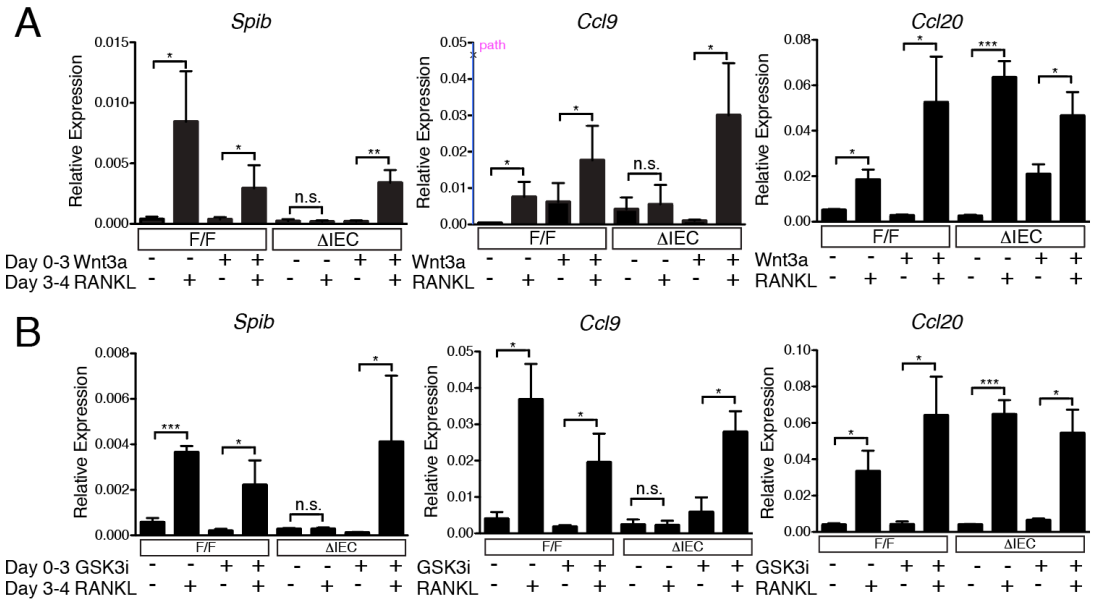


Figure 6: Stabilization of β-catenin allows Atoh1$^{\Delta IEC}$ mice to respond to RANKL stimulation

A) 100 ng/mL Wnt3a was added to cultures for 3 days, and then media was changed to basic ENR media during stimulation with 100 ng/mL RANKL for 1 day. Gene expression of *Spib*, *Ccl9*, and *Ccl20* were analyzed by qPCR as a mean ± s.e.m. of 3 experiments.

B) A GSK3i was added to cultures at a concentration of 10 μM for 3 days. After a change to ENR media, enteroids were stimulated with 100 ng/mL RANKL for 1 day. Gene expression of *Spib*, *Ccl9*, and *Ccl20* were analyzed by qPCR as a mean ± s.e.m. of 3 experiments. All relative expression normalized to *Gapdh*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

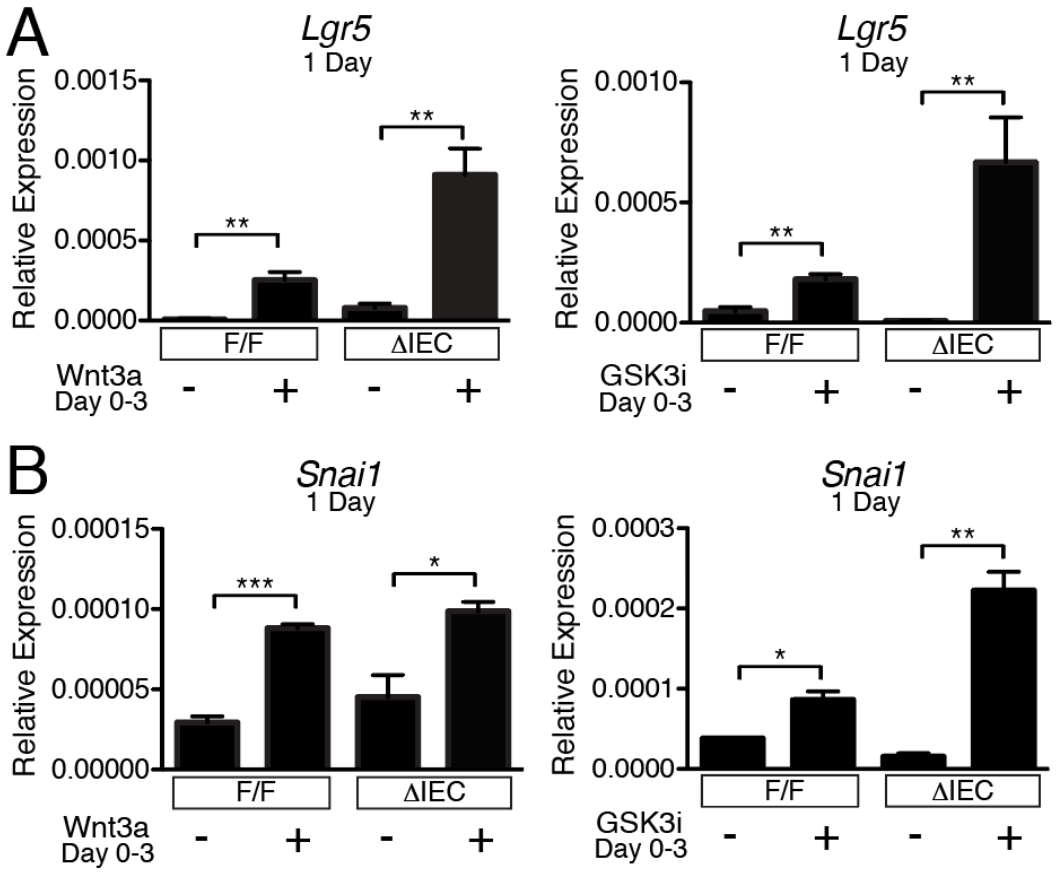


Figure 7: β-catenin stabilization results in increased expression of stem cell marker *Lgr5* A) 100 ng/mL Wnt3a was present in Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ cultures for 3 days, but was removed during a 1 day stimulation with 100 ng/mL RANKL. Then, *Lgr5* and *Snai1* expression was assessed from a mean ± s.e.m. of 3 experiments.. B) 10 μM GSK3i was present in Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ cultures for 3 days, but was removed during a 1 day stimulation with 100 ng/mL RANKL. Then, *Lgr5* and *Snai1* expression was assessed from a mean ± s.e.m. of 3 experiments. All relative expression normalized to *Gapdh*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

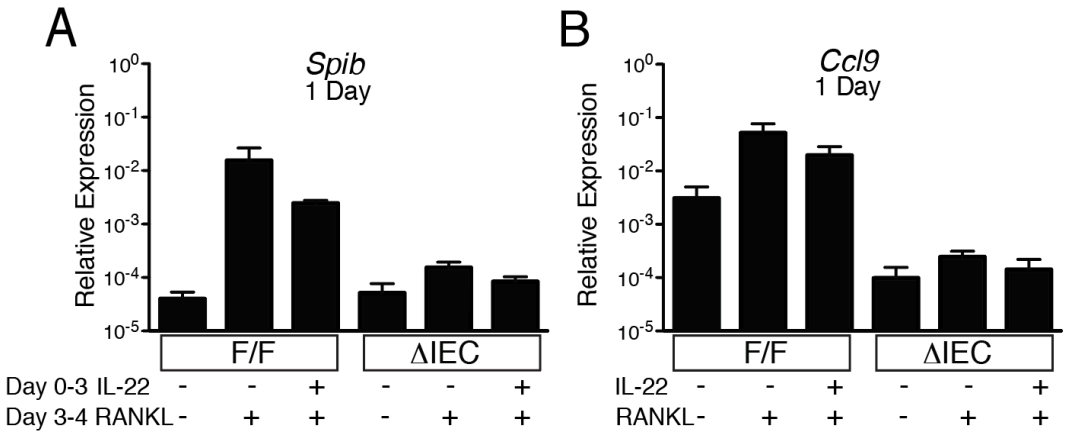


Figure 8: IL-22 does not restore responsiveness to RANKL in Atoh1$^{\Delta IEC}$ enteroids. Atoh1$^{F/F}$ and Atoh1$^{\Delta IEC}$ enteroids were unstimulated, treated with 100ng/mL RANKL for 1 day, or treated with 50ng/mL IL-22 for the initial 3 days of culture then changed to ENR media and stimulated with 100ng/mL RANKL for 1 day. Relative expression of RANKL-responsive genes *Spib*(A) and *Ccl9*(B) were analyzed. Mean ± s.e.m. of 2 experiments on a log scale. Relative expression compared to *Gapdh*.

Discussion

M cells are an important participant in immune responses to the commensal bacteria in the intestine. The presence of M cells at multiple mucosal sites indicates that the function of the M cell is important at mucosal epithelial barriers. Mice with a specific deficiency in intestinal M cell differentiation exhibit defects in secretory IgA responses that help to pattern the commensal microbiota and respond to pathogens.[6] However, the genes responsible for an M cells form and function as well as the early developmental program of the M cell remain largely unknown. The work presented here establishes an *in vitro* enteroid culture model that can be used to elucidate the full suite of M cell specific gene expression. This model was also used to explore one of the key early stages of M cell differentiation and identifies the M cell belonging to the secretory lineage in the small intestine.

Chapter 1 describes a new way in which the unique positioning of the FAE over Peyer's patch B cell follicles affects cell differentiation. Previous studies in this lab had identified RANKL as necessary for M cell differentiation.[46] Here we show that other cytokines, such as TNF-α which stimulates the canonical pathway of NF-κB activation, can increase the available non-canonical NF-κB heterodimers able to respond to RANKL.(Figure 1B) The combination of RANKL and TNF-α in the enteroid system creates a more complete *in vitro* model of M cell differentiation in which it may be possible to discover a wider range of M cell and FAE specific genes than detected with RANKL alone as more M cell specific genes known to be expressed highly *in vivo* are expressed in this system. A 1 day RANKL stimulation induces the M-cell-specific

expression of *Spib* and *Ccl9* and the FAE specific expression of *Ccl20*. After 3 days of stimulation, *Anxa5* and *Gp2* are also induced by RANKL alone. RANKL alone did not induce *Tnfaip2* or *Marcksl1* as highly as had been previously reported. [47, 50] The addition of TNF-α boosted the expression of all M cell and FAE specific genes with the most impact on these previously lowly induced genes, *Tnfaip2* and *Marcksl1*. TNF-α signals through the canonical pathway to increase transcription of the genes responsible for the noncanonical pathway, *Relb* and *Nfkb2*. (Figure 1B) Therefore, the canonical and noncanonical pathways of NF-κB activation can work together to induce M-cell-specific gene expression. This suggests that the FAE *in vivo* can respond to multiple cytokines produced by the stromal and immune cells within the Peyer's patch that affect its unique development.

In the past, M cell differentiation has been explained both as transdifferentiation of FAE enterocytes to M cells and as differentiation from a crypt precursor. Chapter 2 suggests that M cells differentiate from the crypts surrounding the PP through the secretory lineage and not as a result of transdifferentiation. The first reports of M cells developing through transdifferentiation came from observations that the M cell population in the FAE expanded rapidly upon exposure to a pathogenic bacterium.[78, 79] This idea was furthered by the observation that *Salmonella typhimurium* could increase the number of M cells on the PP by inducing epithelial-mesenchymal transformation (EMT) in FAE enterocytes leading to M cell specific gene expression.[80] However, it has also been shown that treatment of mice with exogenous RANKL does not result in villous M cell differentiation in the same manner. Instead, as cells exposed to and capable of responding to RANKL arise from the crypt, they express a sequential

array of M cell specific genes from early expression of Spi-B to endstage expression of *Gp2*.[50] The studies in Chapter 1 of RANKL and TNF-α treated enteroids, the same pattern of gene expression from *Spib* and *Ccl9* at 1 day to GP2 expression at day 3 is seen. Thus, the natural pathway for M cell differentiation is from the crypts surrounding the PP that are exposed to RANKL.

In the intestine, Wnt signaling that is superseded by Notch-Delta signaling as the cell begins to differentiate controls the LGR5+ stem cell compartment. The next checkpoint is the expression of Hes1 or Atoh1.[36] Atoh1 and Hes1 belong to the basic helix loop helix (bHLH) family of transcription factors. Atoh1 binds as a heterodimer with the helix loop helix protein E47 to enhancer regions of target genes. [81] (Figure 1A) Hes1 expression marks the beginning of the differentiation of an absorptive enterocyte while Atoh1 is expressed in secretory cell precursors. If the tissue had been unspecified, this sequence of events could belong to any one of several epithelial tissues. Atoh1 expression is also seen in specialized epithelial cells in the inner ear and small intestine. In both these tissues, cells that have expressed Atoh1 undergo morphological changes resulting in such cells as the ciliated inner ear hair cell, the Merkel cell in the skin, the tuft cell, mucus secreting goblet cell, Paneth cell, and enteroendocrine cell.[36, 82, 83] To date, the only specialized small intestinal epithelial cell that was not considered to be a part of the Atoh1-dependent secretory cell lineage is the M cell. The enteroid system provided an opportunity to study a tissue specific knockout of Atoh1 as a global knockout of Atoh1 is embryonic lethal and mice deficient for Atoh1 in the intestine have a low survival rate past 14 days of age.[21] Functional enteroids can be made from intestinal epithelium lacking Atoh1 when supplemented with Wnt3a.[84]

RANKL treatment of Atoh1$^{\Delta IEC}$ derived enteroids not supplemented with Wnt3a did not yield M cell specific gene expression. Furthermore, mice treated with exogenous RANKL for 24 hours did not have increased *Spib* expression in the small intestine. Thus, Atoh1 expression is an important step in the differentiation of the M cell from a secretory precursor. (Figure 1) Specific Atoh1 target genes have been studied in both inner ear hair cells and cerebellar neurons.[85, 86] Similar studies could now be done on Atoh1 expressing M cell precursors from enteroids to better understand how the network of Atoh1 target genes overlaps in these disparate cell types.

The 3-D enteroid system allows the study of multiple epithelial cell type in the small intestine. Variations in the supplemented media used in this type of organoid culture have also allowed for the study of mammary and inner ear epithelium.[87] [88] The hallmark of the enteroid/organoid culture system's ability to model *in vivo* tissue is that the supplemented media supports tissue-specific stem cells by replicating the *in vivo* signals needed for full differentiation of every cell type of that associated epithelium. For intestinal enteroids, these additives are epidermal growth factor, Noggin and R-spondin. Each of these signals is balanced to provide support for cell type differentiation and plasticity of the stem cell compartment. The addition of Wnt3a or a GSK3i can aid in the vitality of enteroid cultures by increasing proliferation and preventing differentiation by encouraging stem cell gene expression. Use of this manipulation is necessary when an investigator wishes to transfect enteroids or when the epithelium used to derive enteroids lacks Paneth cells.[31, 84] However, as shown in Chapter 2, it should be used with caution as increased Wnt signaling also can inhibit differentiation when both a Wnt signaling and a differentiation signal, such as RANKL, are present at the same time.

The enteroid culture model of M cell differentiation will allow for future investigators to study the gene expression changes within the first day of RANKL stimulation. This could reveal important new information as to the exact contribution of Atoh1 to the differentiation of the M cell. The role of Atoh1 in specialized epithelial cell development could then be expanded to other Atoh1 dependent cell types in the multiple tissues. Complex epithelium that transmits signals across a barrier provides an important function in a variety of areas of the human body. The skin, inner ear, mammary epithelium, tongue, olfactory epithelium, thymus, and small intestine are all examples of complex epithelial tissues that transmit particles or signals to affect the nervous and immune system. Each of these tissues has its own stem cell compartment that undergoes differentiation into multiple cell types.

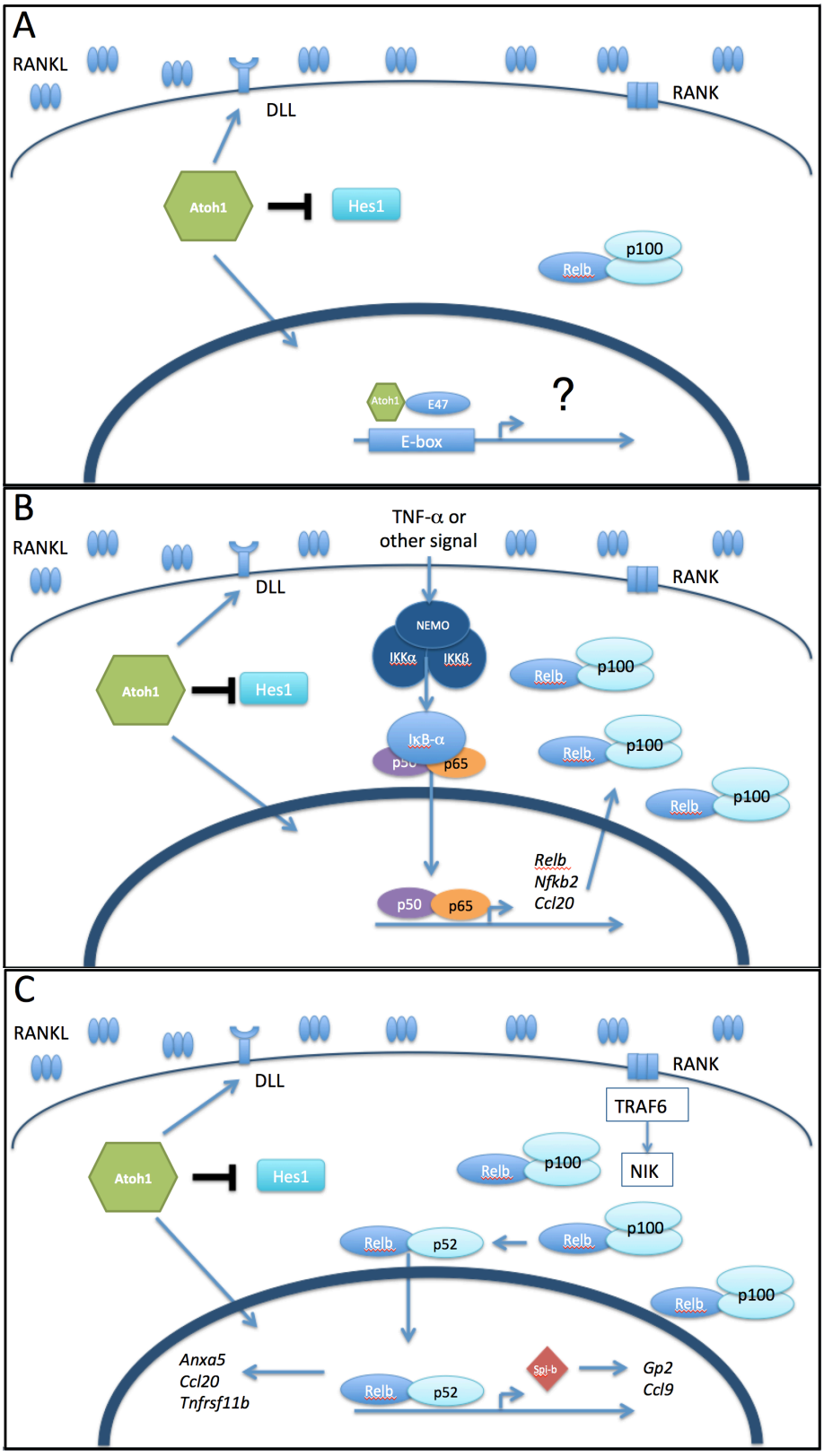
Exciting new advances have been made in the enteroid field that could further the understanding of the function of the M cell. The intestine is a complicated tissue that is responsible for both absorption of nutrients and barrier defense. Multiple cell types are in contact to allow for proper functioning of the small intestine and its communication with the nervous, circulatory, and immune system. The small intestinal epithelium is known to interact with these other systems. For instance, the enteroendocrine and tuft cells release products that can affect the nervous system.[35, 89] The M cell has been shown to have close interaction with the immune system in the Peyer's patch which results in effects on the systemic immune system. Enteroid culture can now support co-culturing of multiple cell types such as dendritic cells or intraepithelial lymphocytes with intestinal epithelial cells.[90, 91] Since enteroid M cells have been shown to be functional in assays with beads micro-injected into the lumen of the enteroid, a co-culture of a TNF-α and RANKL

stimulated enteroid with immune phagocytic cells such as dendritic cells could help to understand the interaction of the M cell with the cell housed in its basolateral pocket.[30, 92] A co-culture of M cells and immune cells could also show the state of the immune cell after interaction with the M cell which would increase understanding of the acquisition of peripheral tolerance.

Enteroid culture also represents an exciting opportunity to scale up the amount of M cells that could be studied at one time. Since enteroids can be split and re-cultured for several cycles, basic enteroids could be passaged until a large number are viable then they could be treated with TNF-α and RANKL for 3 days and screened. GP2+ cells could be sorted and RNA sequencing could be performed which would definitively reveal the genes expressed by a fully differentiated M cell. Additionally, 3 day treated enteroids could be microinjected with a multitude of antigens. This type of assay could be especially helpful in attempting to discover ligands for M cell uptake that could be used as M cell targeted adjuvants in oral vaccination. Since M cells are thought to contribute to oral tolerance of food antigens[11], a scalable *in vitro* model of functional M cells will also allow for the study of the interaction of well-known food allergens with the FAE.

While it is currently known that M cells are important for crosstalk between the intestinal lumen and the mucosal immune system, many of the genes needed for M cells perform this function are unknown. Following the advancements of understanding the need for RANKL and Spi-B for M cell differentiation, the study of M cell differentiation has slowed mostly due to low numbers of observable M cells *in vivo*. The work shown here puts forth an *in vitro* model with the capability of replicating M cell specific gene expression and function. This model is a powerful tool which should lead to the

discovery of genes related to the M cell's ability to take up antigen and pass it to the immune system while mitigating the response to commensal and pathogenic antigens. The RANKL-treated enteroid model was used to determine that the M cell is a part of the secretory lineage of the small intestine. This discovery changes the way M cells can be viewed as a specialized epithelial cell with commonalities, such as an extensive vesicular network, with the other secretory cells of the intestinal epithelium. With these new building blocks for future M cell research in place, the table has been set for further advances in the understanding and *in vivo* manipulation of M cell function.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Ccl20
Tnfrsf11b
Relb
p52
Spi-b
Gp2
Ccl9

Figure 1. Model for M cell differentiation in enteroid cultures.

A) As the future M cell moves out of the crypt, Atoh1 is expressed. Atoh1 suppresses Hes1, the transcription factor necessary for an absorptive enterocyte fate. Atoh1 induces expression of Delta-like ligand on the surface of the cell which prevents Notch signaling. Atoh1 acts with its co-factor E47 on E-box containing promoters of as yet unknown target genes. B) TNF-α or another signal that stimulates the canonical pathway of NF-κB activation leads to the translocation of the canonical heterodimer, p65-p50 to the nucleus. p65-p50 acts on the promoters of *Relb* and *Nfkb2*, genes that encode the components of the noncanonical NF-κB heterodimer as well as the FAE specific gene *Ccl20*. This leads to a larger amount of inactive noncanonical NF-κB in the cytoplasm. C) RANKL-RANK signals through TRAF6 to activate NIK. NIK is then able to process Relb-p100 to the active heterodimer, Relb-p52. Relb-p52 acts on the promoter one to induce *Spib* expression as well as directly on the promoters of other non-Spi-b dependent genes(*Anxa5, Ccl20, Tnfrsfllb*). Spi-b is necessary for transcription of M cell specific genes such as *Gp2* and *Ccl9*.

Literature Cited in Introduction and Discussion

1. Owen, R.L. and A.L. Jones, *Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles.* Gastroenterology, 1974. **66**(2): p. 189-203.
2. Owen, R., *Uptake and transport of intestinal macromolecules and microorganisms by M cells in Peyer's patches--a personal and historical perspective.* Seminars in immunology, 1999. **11**(3): p. 157-163.
3. Owen, R.L., *Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study.* Gastroenterology, 1977. **72**(3): p. 440-51.
4. Kimura, S., et al., *Visualization of the entire differentiation process of murine M cells: suppression of their maturation in cecal patches.* Mucosal Immunol, 2015. **8**(3): p. 650-60.
5. Inman, L.R. and J.R. Cantey, *Specific adherence of Escherichia coli (strain RDEC-1) to membranous (M) cells of the Peyer's patch in Escherichia coli diarrhea in the rabbit.* J Clin Invest, 1983. **71**(1): p. 1-8.
6. Rios, D., et al., *Antigen sampling by intestinal M cells is the principal pathway initiating mucosal IgA production to commensal enteric bacteria.* Mucosal Immunol, 2015.
7. Liu, H., et al., *M cells in the follicle-associated epithelium of the rabbit conjunctiva preferentially bind and translocate latex beads.* Invest Ophthalmol Vis Sci, 2005. **46**(11): p. 4217-23.

8. Mutoh, M., et al., *RANKL regulates differentiation of microfold cells in mouse nasopharynx-associated lymphoid tissue (NALT).* Cell Tissue Res, 2016. **364**(1): p. 175-84.

9. Knoop, K., et al., *Distinct developmental requirements for isolated lymphoid follicle formation in the small and large intestine: RANKL is essential only in the small intestine.* The American journal of pathology, 2011. **179**(4): p. 1861-1871.

10. Macpherson, A.J., et al., *The immune geography of IgA induction and function.* Mucosal Immunol, 2008. **1**(1): p. 11-22.

11. Worbs, T., et al., *Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells.* J Exp Med, 2006. **203**(3): p. 519-27.

12. Macpherson, A.J. and T. Uhr, *Induction of protective IgA by intestinal dendritic cells carrying commensal bacteria.* Science, 2004. **303**(5664): p. 1662-5.

13. Nochi, T., et al., *A novel M cell-specific carbohydrate-targeted mucosal vaccine effectively induces antigen-specific immune responses.* J Exp Med, 2007. **204**(12): p. 2789-96.

14. Tyrer, P.C., et al., *Receptor mediated targeting of M-cells.* Vaccine, 2007. **25**(16): p. 3204-9.

15. Clark, M., et al., *Differential expression of lectin-binding sites defines mouse intestinal M-cells.* The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society, 1993. **41**(11): p. 1679-1687.

16. Hase, K., et al., *Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response.* Nature, 2009. **462**(7270): p. 226-30.

17. Lo, D., et al., *Cell culture modeling of specialized tissue: identification of genes expressed specifically by follicle-associated epithelium of Peyer's patch by expression profiling of Caco-2/Raji co-cultures.* Int Immunol, 2004. **16**(1): p. 91-9.

18. Sato, T. and H. Clevers, *Primary mouse small intestinal epithelial cell cultures.* Methods Mol Biol, 2013. **945**: p. 319-28.

19. Sato, T., et al., *Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche.* Nature, 2009. **459**(7244): p. 262-265.

20. Farin, H.F., et al., *Paneth cell extrusion and release of antimicrobial products is directly controlled by immune cell-derived IFN-gamma.* J Exp Med, 2014. **211**(7): p. 1393-405.

21. Shroyer, N.F., et al., *Intestine-specific ablation of mouse atonal homolog 1 (Math1) reveals a role in cellular homeostasis.* Gastroenterology, 2007. **132**(7): p. 2478-88.

22. Srinivasan, T., et al., *Notch signalling regulates asymmetric division and inter-conversion between lgr5 and bmi1 expressing intestinal stem cells.* Sci Rep, 2016. **6**: p. 26069.

23. Li, N., et al., *Mouse Label-Retaining Cells Are Molecularly And Functionally Distinct From Reserve Intestinal Stem Cells.* Gastroenterology, 2016.

24. Tian, H., et al., *Opposing activities of Notch and Wnt signaling regulate intestinal stem cells and gut homeostasis.* Cell Rep, 2015. **11**(1): p. 33-42.

25. Tetteh, P.W., et al., *Replacement of Lost Lgr5-Positive Stem Cells through Plasticity of Their Enterocyte-Lineage Daughters.* Cell Stem Cell, 2016. **18**(2): p. 203-13.

26. Slorach, E.M., F.C. Campbell, and J.R. Dorin, *A mouse model of intestinal stem cell function and regeneration.* J Cell Sci, 1999. **112 Pt 18**: p. 3029-38.

27. Hughes, C., L. Postovit, and G. Lajoie, *Matrigel: a complex protein mixture required for optimal growth of cell culture.* Proteomics, 2010. **10**(9): p. 1886-1890.

28. Mahe, M.M., et al., *Establishment of gastrointestinal epithelial organoids.* Curr Protoc Mouse Biol, 2013. **3**: p. 217-240.

29. Chen, P.-H., et al., *The structural basis of R-spondin recognition by LGR5 and RNF43.* Genes & development, 2013. **27**(12): p. 1345-1350.

30. de Lau, W., et al., *Peyer's patch M cells derived from Lgr5(+) stem cells require SpiB and are induced by RankL in cultured "miniguts".* Mol Cell Biol, 2012. **32**(18): p. 3639-47.

31. Andersson-Rolf, A., et al., *A video protocol of retroviral infection in primary intestinal organoid culture.* J Vis Exp, 2014(90): p. e51765.

32. Ootani, A., et al., *Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche.* Nature medicine, 2009. **15**(6): p. 701-706.

33. Lindemans, C.A., et al., *Interleukin-22 promotes intestinal-stem-cell-mediated epithelial regeneration.* Nature, 2015. **528**(7583): p. 560-4.

34. Nagalakshmi, M.L., et al., *Interleukin-22 activates STAT3 and induces IL-10 by colon epithelial cells.* Int Immunopharmacol, 2004. **4**(5): p. 679-91.

35. Gerbe, F., et al., *Distinct ATOH1 and Neurog3 requirements define tuft cells as a new secretory cell type in the intestinal epithelium.* The Journal of cell biology, 2011. **192**(5): p. 767-780.

36. Yang, Q., et al., *Requirement of Math1 for secretory cell lineage commitment in the mouse intestine.* Science, 2001. **294**(5549): p. 2155-8.

37. Hsieh, E.H. and D.D. Lo, *Jagged1 and Notch1 help edit M cell patterning in Peyer's patch follicle epithelium.* Dev Comp Immunol, 2012. **37**(2): p. 306-12.

38. Zheng, J.L., et al., *Hes1 is a negative regulator of inner ear hair cell differentiation.* Development, 2000. **127**(21): p. 4551-60.

39. Stamataki, D., et al., *Delta1 expression, cell cycle exit, and commitment to a specific secretory fate coincide within a few hours in the mouse intestinal stem cell system.* PLoS One, 2011. **6**(9): p. e24484.

40. Noah, T.K., et al., *SAM pointed domain ETS factor (SPDEF) regulates terminal differentiation and maturation of intestinal goblet cells.* Exp Cell Res, 2010. **316**(3): p. 452-65.

41. Lorenz, R.G., et al., *Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin beta receptor, and TNF receptor I function.* J Immunol, 2003. **170**(11): p. 5475-82.

42. Taylor, R.T., et al., *Intestinal cryptopatch formation in mice requires lymphotoxin alpha and the lymphotoxin beta receptor.* J Immunol, 2004. **173**(12): p. 7183-9.

43. Pasparakis, M., et al., *Peyer's patch organogenesis is intact yet formation of B lymphocyte follicles is defective in peripheral lymphoid organs of mice deficient*

for tumor necrosis factor and its 55-kDa receptor. Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6319-23.

44. Chieppa, M., et al., *Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement.* J Exp Med, 2006. **203**(13): p. 2841-52.

45. Knoop, K.A., et al., *Microbial sensing by goblet cells controls immune surveillance of luminal antigens in the colon.* Mucosal Immunol, 2015. **8**(1): p. 198-210.

46. Knoop, K., et al., *RANKL is necessary and sufficient to initiate development of antigen-sampling M cells in the intestinal epithelium.* Journal of immunology (Baltimore, Md. : 1950), 2009. **183**(9): p. 5738-5747.

47. Kobayashi, A., et al., *Identification of novel genes selectively expressed in the follicle-associated epithelium from the meta-analysis of transcriptomics data from multiple mouse cell and tissue populations.* DNA Res, 2012. **19**(5): p. 407-22.

48. Ermund, A., et al., *Mucus properties and goblet cell quantification in mouse, rat and human ileal Peyer's patches.* PLoS One, 2013. **8**(12): p. e83688.

49. Terahara, K., M. Yoshida, and O. Igarashi…, *Comprehensive gene expression profiling of Peyer's patch M cells, villous M-like cells, and intestinal epithelial cells.* The Journal of …, 2008.

50. Kanaya, T., et al., *The Ets transcription factor Spi-B is essential for the differentiation of intestinal microfold cells.* Nat Immunol, 2012. **13**(8): p. 729-36.

51. Sato, S., et al., *Transcription factor Spi-B-dependent and -independent pathways for the development of Peyer's patch M cells.* Mucosal immunology, 2012.

52. Zhao, X., et al., *CCL9 is secreted by the follicle-associated epithelium and recruits dome region Peyer's patch CD11b+ dendritic cells.* Journal of immunology (Baltimore, Md. : 1950), 2003. **171**(6): p. 2797-2803.

53. Ray, D., et al., *Characterization of Spi-B, a transcription factor related to the putative oncoprotein Spi-1/PU.1.* Molecular and cellular biology, 1992. **12**(10): p. 4297-4304.

54. Garrett-Sinha, L., et al., *PU.1 and Spi-B are required for normal B cell receptor-mediated signal transduction.* Immunity, 1999. **10**(4): p. 399-408.

55. Rao, S., et al., *SPI-B activates transcription via a unique proline, serine, and threonine domain and exhibits DNA binding affinity differences from PU. 1.* Journal of Biological Chemistry, 1999.

56. Akiyama, N., et al., *Limitation of immune tolerance-inducing thymic epithelial cell development by Spi-B-mediated negative feedback regulation.* J Exp Med, 2014. **211**(12): p. 2425-38.

57. Karrich, J., et al., *The transcription factor Spi-B regulates human plasmacytoid dendritic cell survival through direct induction of the antiapoptotic gene BCL2-A1.* Blood, 2012. **119**(22): p. 5191-5200.

58. Sasaki, I., et al., *Spi-B is critical for plasmacytoid dendritic cell function and development.* Blood, 2012. **120**(24): p. 4733-4743.

59. Chen, H., et al., *Two promoters direct expression of the murine Spi-B gene, an Ets family transcription factor.* Gene, 1998. **207**(2): p. 209-218.

60. Bartholdy, B., et al., *The Ets factor Spi-B is a direct critical target of the coactivator OBF-1.* Proc Natl Acad Sci U S A, 2006. **103**(31): p. 11665-70.

61. Wang, J., et al., *TNFR and LTbetaR agonists induce follicle-associated epithelium and M cell specific genes in rat and human intestinal epithelial cells.* Cytokine, 2009. **47**(1): p. 69-76.

62. Bachmann, M.F., et al., *TRANCE, a tumor necrosis factor family member critical for CD40 ligand-independent T helper cell activation.* J Exp Med, 1999. **189**(7): p. 1025-31.

63. Simonet, W.S., et al., *Osteoprotegerin: a novel secreted protein involved in the regulation of bone density.* Cell, 1997. **89**(2): p. 309-19.

64. Shinohara, M., et al., *Tyrosine kinases Btk and Tec regulate osteoclast differentiation by linking RANK and ITAM signals.* Cell, 2008. **132**(5): p. 794-806.

65. Hikosaka, Y., et al., *The cytokine RANKL produced by positively selected thymocytes fosters medullary thymic epithelial cells that express autoimmune regulator.* Immunity, 2008. **29**(3): p. 438-50.

66. Thomas, E., et al., *Receptor activator of NF-kappaB ligand promotes proliferation of a putative mammary stem cell unique to the lactating epithelium.* Stem Cells, 2012. **30**(6): p. 1255-64.

67. Schneeweis, L., D. Willard, and M. Milla, *Functional dissection of osteoprotegerin and its interaction with receptor activator of NF-kappaB ligand.* The Journal of biological chemistry, 2005. **280**(50): p. 41155-41164.

68. Wong, B., et al., *TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src.* Molecular cell, 1999. **4**(6): p. 1041-1049.

69. Wong, B.R., et al., *The TRAF family of signal transducers mediates NF-kappaB activation by the TRANCE receptor.* J Biol Chem, 1998. **273**(43): p. 28355-9.

70. Yi, T., et al., *Epidermal growth factor receptor regulates osteoclast differentiation and survival through cross-talking with RANK signaling.* J Cell Physiol, 2008. **217**(2): p. 409-22.

71. Kwak, H.B., et al., *Pyridone 6, a pan-Janus-activated kinase inhibitor, suppresses osteoclast formation and bone resorption through down-regulation of receptor activator of nuclear factor-kappaB (NF-kappaB) ligand (RANKL)-induced c-Fos and nuclear factor of activated T cells (NFAT) c1 expression.* Biol Pharm Bull, 2009. **32**(1): p. 45-50.

72. Kwon, O.H., et al., *The hematopoietic transcription factor PU.1 regulates RANK gene expression in myeloid progenitors.* Biochem Biophys Res Commun, 2005. **335**(2): p. 437-46.

73. Karrer, U., et al., *Immunodeficiency of alymphoplasia mice (aly/aly) in vivo: structural defect of secondary lymphoid organs and functional B cell defect.* European journal of immunology, 2000. **30**(10): p. 2799-2807.

74. Nischan, J., et al., *Binding sites for ETS family of transcription factors dominate the promoter regions of differentially expressed genes in abdominal aortic aneurysms.* Circ Cardiovasc Genet, 2009. **2**(6): p. 565-72.

75. Xiao, G., A. Fong, and S.C. Sun, *Induction of p100 processing by NF-kappaB-inducing kinase involves docking IkappaB kinase alpha (IKKalpha) to p100 and IKKalpha-mediated phosphorylation.* J Biol Chem, 2004. **279**(29): p. 30099-105.

76. Dejardin, E., *The alternative NF-kappaB pathway from biochemistry to biology: pitfalls and promises for future drug development.* Biochem Pharmacol, 2006. **72**(9): p. 1161-79.

77. Sun, S.C., *Non-canonical NF-kappaB signaling pathway.* Cell Res, 2011. **21**(1): p. 71-85.

78. Borghesi, C., et al., *Modifications of the follicle-associated epithelium by short-term exposure to a non-intestinal bacterium.* J Pathol, 1996. **180**(3): p. 326-32.

79. Meynell, H.M., et al., *Up-regulation of microsphere transport across the follicle-associated epithelium of Peyer's patch by exposure to Streptococcus pneumoniae R36a.* FASEB J, 1999. **13**(6): p. 611-9.

80. Tahoun, A., et al., *Salmonella transforms follicle-associated epithelial cells into M cells to promote intestinal invasion.* Cell Host Microbe, 2012. **12**(5): p. 645-56.

81. Scheffer, D., et al., *The alpha1 subunit of nicotinic acetylcholine receptors in the inner ear: transcriptional regulation by ATOH1 and co-expression with the gamma subunit in hair cells.* J Neurochem, 2007. **103**(6): p. 2651-64.

82. Wright, M.C., et al., *Unipotent, Atoh1+ progenitors maintain the Merkel cell population in embryonic and adult mice.* J Cell Biol, 2015. **208**(3): p. 367-79.

83. Driver, E.C., et al., *The Atoh1-lineage gives rise to hair cells and supporting cells within the mammalian cochlea.* Dev Biol, 2013. **376**(1): p. 86-98.

84. Durand, A., et al., *Functional intestinal stem cells after Paneth cell ablation induced by the loss of transcription factor Math1 (Atoh1).* Proc Natl Acad Sci U S A, 2012. **109**(23): p. 8965-70.

85. Cai, T., et al., *Characterization of the transcriptome of nascent hair cells and identification of direct targets of the Atoh1 transcription factor.* J Neurosci, 2015. **35**(14): p. 5870-83.

86. Klisch, T.J., et al., *In vivo Atoh1 targetome reveals how a proneural transcription factor regulates cerebellar development.* Proc Natl Acad Sci U S A, 2011. **108**(8): p. 3288-93.

87. Liu, X.P., et al., *Functional development of mechanosensitive hair cells in stem cell-derived organoids parallels native vestibular hair cells.* Nat Commun, 2016. **7**: p. 11508.

88. Kim, N.D., K.J. Paik, and K.H. Clifton, *Inhibitory effects of retinoids on development of squamous metaplasia in rat mammary epithelial organoids cultured in Matrigel.* Cancer Lett, 1996. **110**(1-2): p. 217-23.

89. Grunddal, K.V., et al., *Neurotensin Is Coexpressed, Coreleased, and Acts Together With GLP-1 and PYY in Enteroendocrine Control of Metabolism.* Endocrinology, 2016. **157**(1): p. 176-94.

90. Nozaki, K., et al., *Co-culture with intestinal epithelial organoids allows efficient expansion and motility analysis of intraepithelial lymphocytes.* J Gastroenterol, 2016. **51**(3): p. 206-13.

91. Chng, S.H., et al., *Ablating the aryl hydrocarbon receptor (AhR) in CD11c+ cells perturbs intestinal epithelium development and intestinal immunity.* Sci Rep, 2016. **6**: p. 23820.

92. Rouch, J.D., et al., *Development of Functional Microfold (M) Cells from Intestinal Stem Cells in Primary Human Enteroids.* PLoS One, 2016. **11**(1): p. e0148216.