CHAPTER THREE

Epithelial Cell Contributions to Intestinal Immunity

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Abstract

The epithelial surfaces of the mammalian intestine interface directly with the external environment and thus continuously encounter pathogenic bacteria, fungi, viruses, and parasites. The intestinal epithelium is also closely associated with complex communities of symbiotic microorganisms. Intestinal epithelial cells are thus faced with the unique challenge of directly interacting with enormous numbers of microbes that include both pathogens and symbionts. As a result, gut epithelia have evolved an array of strategies that contribute to host immunity. This chapter considers the various mechanisms used by epithelial cells to limit microbial invasion of host tissues, shape the composition of indigenous microbial communities, and coordinate the adaptive immune response to microorganisms. Study of intestinal epithelial cells has contributed fundamental insights into intestinal immune homeostasis and has revealed how impaired epithelial cell function can contribute to inflammatory disease.

1. INTRODUCTION

1.1. Overview of epithelial-microbial interactions in the mammalian intestine

The epithelial surfaces of the mammalian intestine interface directly with the external environment. As a result, these tissues continuously encounter bacteria, fungi, viruses, and parasites that are ingested in food and water and may be potential pathogens. The epithelium separating these microorganisms from internal tissues comprises a single-cell layer and encompasses an enormous surface area (~200 m² in humans) (Neish, 2002). Thus, the intestinal epithelium is faced with the unique challenge of defending a large surface area in order to prevent pathogen invasion.

The mammalian intestine is also home to a diverse community of indigenous microorganisms numbering in the trillions. This community is known as the microbiota. These organisms establish symbiotic relationships with their hosts, making critical contributions to mammalian metabolism while occupying a protected, nutrient-rich environment. Despite the symbiotic nature of this relationship, the enormous numbers of intestinal bacteria present a continuous threat of host barrier breach, with the single-cell epithelial layer and large surface area compounding this threat. Such opportunistic invasion of host tissues by resident bacteria can subvert the symbiotic host-microbial relationship and lead to pathologies such as bacteremia or
chronic inflammation. At the same time, the intestinal epithelium must avoid potentially harmful overreactions that could unnecessarily damage intestinal tissues or alter the essential metabolic functions of the microbiota.

In order to meet these challenges, epithelial cells have evolved a number of strategies for managing the dense bacterial loads. These include epithelial cell-intrinsic innate immune defenses such as secretion of mucus and antimicrobial proteins and activation of autophagy, as well as strategies for shaping downstream adaptive immune responses. As a consequence, epithelial cells play a central role in limiting bacterial invasion of host tissues, in shaping the composition and location of indigenous microbial communities, and in coordinating the responses of subepithelial immune cell populations. This chapter explores each of these functions in detail, as well as how these mechanisms can become dysregulated in disease.

1.2. The intestinal microbiota

During the past decade, the development of molecular profiling techniques has allowed the acquisition of a comprehensive view of the composition of gut microbial communities. Numerous molecular profiling studies using ribosomal DNA sequencing methods have revealed that the human intestinal microbiota includes hundreds to thousands of distinct bacterial species, with prominent representation of both Gram positive and Gram negative bacteria (Eckburg et al., 2005). The species composition of the intestinal microbiota can vary widely between individuals, and changes in response to age and diet (Eckburg et al., 2005; Faith et al., 2013; Ley et al., 2005; Turnbaugh et al., 2006; Yatsunenko et al., 2012). An additional layer of complexity comes from microbes that gain entry to the intestinal ecosystem through host intake of food and water, but which are not stable members of the gut microbiota.

Mammalian intestinal microbial communities are comprised predominantly of species from two phyla: the Firmicutes and the Bacteroidetes (Eckburg et al., 2005; Ley et al., 2008). The Firmicutes are Gram positive bacteria that include species belonging to the Clostridia class, as well as members of the Enterococcaceae and Lactobacillaceae families and Lactococcus species. The Bacteroidetes are Gram negative bacteria that include several Bacteroides species such as Bacteroides thetaiotaomicron, Bacteroides fragilis, and Bacteroides ovatus (Eckburg et al., 2005). The remaining intestinal bacteria account for less than 10% of the population and belong predominantly to the Proteobacteria and Actinobacteria phyla (Eckburg et al., 2005).
This microbial community makes several important contributions to human health and development. A key function of the microbiota is to increase the efficiency of host digestion (Ley et al., 2008; Wostmann, Larkin, Moriarty, & Bruckner-Kardoss, 1983). Gut bacterial societies are metabolically active, degrading dietary substances that would otherwise be indigestible by the host (Martens et al., 2011; Turnbaugh et al., 2006). Certain members of the microbiota, such as \textit{B. thetaiotaomicron}, produce a diverse repertoire of glycosyl hydrolases that metabolize complex plant polysaccharides, thereby liberating simple carbohydrates for uptake by the host (Gill et al., 2006; Martens et al., 2011; Xu et al., 2003). This effectively increases the caloric value of the diet and would thus be favorable in an environment where nutrients are in short supply.

Enhanced host digestive efficiency is thought to be the primary driving force behind the evolution of microbiota associations with mammalian hosts. However, millions of years of co-evolution have led to the interweaving of many aspects of mammalian and microbial physiology, and thus intestinal microbes influence numerous aspects of host physiology and development. For example, the mammalian microbiota has a profound influence on the immune system. Intestinal bacteria provide instructive signals for the development of several lymphocyte subsets, including T helper 17 (TH17) cells (Ivanov et al., 2008, 2009), T regulatory cells (Atarashi et al., 2011), and B cells (He et al., 2007). Additionally, intestinal bacteria impact systemic immune responses by influencing the ratio of TH1 and TH2 effector cells (Mazmanian, Liu, Tzianabos, & Kasper, 2005). The microbiota also has pronounced effects on the nervous system and brain, with studies in mice suggesting that intestinal bacteria modulate susceptibility to neurodevelopmental defects such as autism spectrum disorders (Mazmanian et al., 2005). Finally, the microbiota plays an essential role in regulating host metabolic processes such as fat storage (Bäckhed et al., 2004).

1.3. Germ-free mice as experimental tools

Animals raised in microbiologically sterile (germ–free) conditions are important experimental tools for the study of epithelial contributions to intestinal immunity. Such animals are reared in sterile isolators that control their exposure to microorganisms, including bacteria, viruses, and eukaryotic parasites. Germ–free animals can be studied in their microbiologically sterile state or can be used as living test tubes for the establishment of microbial ecosystems that range from simple to complex. The technology has come to be known
as “gnotobiotics,” a term derived from Greek roots and meaning “known life.” Important insights about how resident microbial communities impact epithelial cell function have come from experimental comparisons of germ-free and colonized mice (Cash, Whitham, Behrendt, & Hooper, 2006; Hooper et al., 2001). These include the discovery of microbe-dependent antimicrobial protein expression (Cash et al., 2006; Hooper, Stappenbeck, Hong, & Gordon, 2003) and bacterial activation of epithelial cell autophagy (Benjamin, Sumpter, Levine, & Hooper, 2013). Studies of gnotobiotic animals have also produced important insights into how the microbiota shapes the development of adaptive immune cell populations (Geuking et al., 2011; Ivanov et al., 2008; Mazmanian et al., 2005).

2. CELLULAR MAKEUP OF THE INTESTINAL EPITHELIAL BARRIER

The internal tissues of the intestine are separated from the microbe-filled lumen by a single-epithelial layer that is ~20 μm thick. Intestinal epithelial surfaces are composed of several distinct cell lineages, each of which makes unique contributions to barrier integrity and mucosal immunity.

2.1. Enterocytes

The enterocyte is the most abundant epithelial cell lineage in both the small and the large intestines. Enterocyte membranes, as well as the tight junctions that form between the cells, present a significant physical barrier to microbial invasion. However, enterocytes also assume an active role in defending epithelial surfaces. This occurs in several ways, each of which will be discussed in detail in subsequent sections. First, enterocytes secrete a variety of antimicrobial proteins that directly attack and kill bacteria (discussed in detail in Section 5). Second, they support cellular processes, such as autophagy (Benjamin et al., 2013; Conway et al., 2013; Wlodarska et al., 2014), which defend against invading bacteria (discussed in Section 6). Third, enterocytes produce cytokines that coordinate responses from subepithelial immune populations (discussed in Section 7.2). Fourth, they transport secretory immunoglobulin A from the basolateral epithelial surface to the apical surface of the epithelium for discharge into the lumen (discussed in Section 7.1). This IgA plays an essential role in maintaining homeostasis between host tissues and intestinal microbial communities (Macpherson et al., 2000).
2.2. Goblet cells

Goblet cells are a secretory epithelial cell lineage found in both the small and the large intestines. A major function of goblet cells is the production of mucus, which forms a protective gel-like layer over the surface epithelium and protects against bacterial invasion (Johansson et al., 2008) (discussed in detail in Section 4). Other secreted products of goblet cells include resistin-like molecule β (Artis et al., 2004), which modifies T cell-mediated immunity (Nair et al., 2008), and trefoil factor, which promotes epithelial restitution after mucosal injury (Farrell et al., 2002; Mashimo, Wu, Podolsky, & Fishman, 1996; Playford et al., 1995). Finally, recent studies have revealed that goblet cells can acquire soluble antigens from the intestinal lumen and deliver them to subepithelial dendritic cells (McDole et al., 2012). Thus, goblet cells participate in antigen uptake and presentation to underlying immune cells, which was previously thought to be an exclusive function of intestinal M cells (discussed later).

2.3. Paneth cells

Paneth cells are an epithelial lineage unique to the small intestine. These secretory cells are positioned at the base of small intestinal crypts of Lieberkühn and contain abundant secretory granules containing a number of microbicidal proteins including α-defensins, C-type lectins, lysozyme, and phospholipase A2. As a result, this cell lineage is responsible for a large proportion of the small intestinal antimicrobial output. Upon detection of microbial signals, Paneth cells release their microbicidal granule contents into the gut lumen (Ayabe et al., 2000).

Paneth cells also play a central role in regulating small intestinal epithelial renewal. Paneth cells are positioned in crypts alongside the multipotent stem cells that give rise to all of the lineages of the differentiated intestinal epithelium. By secreting factors such as EGF, Wnt3, and the Notch ligand Dll4, Paneth cells sustain proliferating epithelial stem cells and thus contribute to epithelial renewal (Clevers & Bevins, 2013; Sato et al., 2011).

2.4. Enteroendocrine cells

Enteroendocrine cells are scattered throughout the small intestine and comprise about 1% of the epithelial cell population (Sternini, Anselmi, & Rozengurt, 2008). Like goblet cells and Paneth cells, enteroendocrine cells are specialized for secretion. They sense luminal contents, particularly nutrients, and secrete multiple regulatory factors such as gastric inhibitory
peptide, glucagon-like peptide, and vasoactive intestinal peptide that regulate digestion, intestinal motility, and food intake (Moran, Leslie, Levison, Worthington, & McLaughlin, 2008). Although enteroendocrine cells are scattered throughout the intestine, taken together they constitute one of the largest endocrine systems in the body.

### 2.5. M cells

Microfold cells, or M cells, are intestinal epithelial cells that are specialized for antigen sampling. They are found predominantly in the follicle-associated epithelium overlying the surfaces of intestinal lymphoid tissues such as Peyer’s patches and isolated lymphoid follicles. M cells function in transepithelial transport of both luminal antigens and intact microorganisms, which are presented to the immune cells of the lymphoid follicle in order to generate an immune response (Kraehenbuhl & Neutra, 2000). Although M cells are important for the generation of a strong immune response, they also represent a weak point in the intestinal epithelium as many pathogens exploit them as a portal of entry (Tahoun et al., 2012).

### 3. EPITHELIAL CELL SENSING OF INTESTINAL MICROBES

#### 3.1. Epithelial detection of microbes by pattern recognition receptors

Several epithelial cell-intrinsic mechanisms of innate defense are activated by direct bacterial recognition by epithelial cells. Recognition of microorganisms is mediated by host-encoded receptors, known as pattern recognition receptors, which recognize conserved microbial molecular patterns unique to prokaryotes. These molecular patterns include bacterial cell wall components such as lipopolysaccharide (LPS) and peptidoglycan, as well as protein components of specialized bacterial structures such as flagella (Ronald & Beutler, 2010). Certain pattern recognition receptors recognize viruses, mainly through the detection of viral nucleic acids (Yan & Chen, 2012). Ligand binding to pattern recognition receptors activates signaling cascades that control transcription of defensive or proinflammatory genes (Ronald & Beutler, 2010).

Toll-like receptors (TLRs) are a family of membrane-bound pattern recognition receptors that play a central role in microbial pattern recognition in mammals. Twelve mouse and ten human TLRs have been identified to date (Ronald & Beutler, 2010). At least four TLRs recognize molecular
patterns of bacteria. TLR2 and TLR4 recognize the bacterial cell wall components—lipoteichoic acid and LPS, respectively (Ronald & Beutler, 2010; Takeuchi et al., 2002). TLR5 detects flagellin, the major protein component of Gram negative flagella (Gewirtz, Navas, Lyons, Godowski, & Madara, 2001; Hayashi et al., 2001). TLR9 binds to unmethylated CpG DNA, which is present in bacteria but not in eukaryotic cells (Hemmi et al., 2000). TLR11 recognizes both profilin, which is a molecular signature of protozoan parasites (Yarovinsky et al., 2005), and flagellin from Salmonella typhimurium (Mathur et al., 2012). Upon ligand binding, TLRs initiate signaling cascades that trigger the nuclear translocation of the transcription factor NFκB, which directs expression of proinflammatory factors such as tumor necrosis factor and interleukin (IL)-8.

Signaling through several TLRs occurs through an adaptor protein, MyD88. MyD88 is recruited to the TLR cytoplasmic domain and signals through IRAK (Akira, Uematsu, & Takeuchi, 2006). Studies of MyD88-deficient mice have produced insight into the broad role of TLRs in intestinal epithelial cells, and several studies have identified an epithelial cell-intrinsic role for MyD88 in epithelial cell-mediated immunity. For example, MyD88−/− mice are deficient in expression of several epithelial proteins, including the antimicrobial lectin RegIIIγ (Brandl, Plitas, Schnabl, DeMatteo, & Pamer, 2007; Rakoff-Nahoum & Medzhitov, 2007; Vaishnava, Behrendt, Ismail, Eckmann, & Hooper, 2008; Vaishnava et al., 2011). Forced expression of a MyD88 transgene in Paneth cells specifically restored Paneth cell expression of RegIIIγ, indicating that Paneth cells directly sense bacteria through MyD88-dependent pathways (Vaishnava et al., 2008). Similarly, mice with MyD88 deleted specifically in enterocytes had lowered RegIIIγ expression (Vaishnava et al., 2011), emphasizing the importance of epithelial cell-intrinsic MyD88 signaling for regulating antimicrobial protein expression. The same epithelial cell-specific MyD88−/− mice revealed a role for epithelial cell-intrinsic MyD88 in bacterial activation of epithelial cell autophagy (Benjamin et al., 2013). Finally, the activation of epithelial TLR4 increased expression of TLR-dependent cytokines, such as CCL20, CCL28, and APRIL, which promoted increased B cell recruitment and differentiation in the intestine (Fukata et al., 2011; Shang et al., 2008). Together, these studies demonstrate the importance of epithelial cell-intrinsic recognition of microbial signals through TLRs for key immune functions of epithelial cells.

Several studies have analyzed epithelial cell-specific deletions of signaling molecules that lie upstream of the transcription factor NFκB. These include
the inhibitor of NFκB (IκB) kinase (IKK) complex and the NFκB modulator NEMO. Epithelial cell-specific deletion of the genes encoding either of these proteins produced increased susceptibility to induced and spontaneous colitis in mice (Nenci et al., 2007; Zaph et al., 2007). These studies reveal an essential role for epithelial cell-intrinsic NFκB signaling pathways in maintaining immune homeostasis in the intestine.

The nucleotide-binding oligomerization domain-like receptors (NLRs) are a second major group of proteins involved in microbial pattern recognition. In contrast to the membrane-bound TLRs, NLRs are located in the host cell cytoplasm. NOD2 was originally identified as the first genetic susceptibility locus for Crohn’s disease, which is characterized by chronic inflammation of the distal small intestine or proximal colon, or both (Hugot et al., 2001; Ogura et al., 2001). NOD1 and NOD2 were subsequently found to activate inflammatory signaling pathways that depend on sensing microbial molecular patterns (Girardin et al., 2003; Inohara et al., 2003). Both receptors are expressed in intestinal epithelial cells (Kim, Lee, & Kagnoff, 2004; Kobayashi et al., 2005; Lala et al., 2003; Ogura et al., 2003), and signal in response to muramyl peptides that are components of bacterial peptidoglycan (Girardin et al., 2003; Inohara et al., 2003). While NOD1-dependent signaling requires muramyl tripeptides that are unique to Gram negative bacteria (Girardin et al., 2003), NOD2-dependent signaling requires activation by a specific muramyl dipeptide common to both Gram positive and Gram negative bacteria (Inohara et al., 2003). Interestingly, recent findings indicate that NOD1 functions as part of a multiprotein complex, or “nodosome,” that includes small Rho GTPases and HSP90. It is this complex that detects peptidoglycan fragments in the cell cytoplasm and initiates the signaling cascades that activate NFκB (Keestra & Baemler, 2014; Keestra et al., 2013).

Other members of the NLR family are involved in inflammasome activation. Inflammasomes are multiprotein complexes that incorporate a sensor protein such as an NLR family member, an adaptor protein (ASC—apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)), and a caspase. These complexes function as platforms for the activation of caspases, such as caspase-1, which drive proinflammatory responses by processing the proinflammatory cytokines IL-1β and IL-18 (Martinon, Burns, & Tschopp, 2002). NLRP6 inflammasomes are of particular importance in the regulation of mucus production by goblet cells (Wlodarska et al., 2014) and likely play other important roles in gut epithelial biology (Elinav et al., 2011).
Although epithelial cell-intrinsic recognition of microbes can activate protective immune responses, overstimulation of these pathways can be detrimental by provoking tumorigenesis. For example, the activation of epithelial cell TLR4 can promote the development of colorectal tumors that are associated with colitis (Fukata et al., 2007). Similarly, chronic epithelial cell-intrinsic activation of IKKβ, which lies directly upstream of NFKB, leads to tumorigenesis that is secondary to colitis (Greten et al., 2004).

3.2. Tissue-specific modulation of epithelial cell-specific innate immune responses

The abundance of intestinal bacteria and their proximity to intestinal tissues provokes the question of how the intestine avoids overactive inflammatory responses to bacterial signals. Several mechanisms appear to limit activation of intestinal immune responses. First, there is an additional physical barrier imposed by the mucus layer, which limits bacterial access to the intestinal epithelium (Johansson et al., 2008; Vaishnava et al., 2011). Thus, it is possible that epithelial cell TLRs are stimulated only when the numbers of surface-associated microbes become large enough to pose a significant threat. Second, epithelial cells are polarized, allowing compartmentalization of certain pattern recognition receptors. For example, TLR5 is restricted to the basolateral surface of epithelial cells and thus can only sense bacteria that have invaded host tissues (Gewirtz et al., 2001). The polarity of epithelial cells also allows differential responses depending on whether bacterial signals are detected on the apical or the basolateral epithelial surface. For example, basolateral detection of ligands by epithelial TLR9 leads to activation and nuclear translocation of NFKB, while apical detection of ligands inhibits NFKB activation by stabilizing IκB (Lee et al., 2006). Consequently, the apically activated cells become refractory to further microbial stimulation.

A third mechanism that limits overactivation of epithelial inflammatory pathways is expression of factors that modulate pattern recognition receptor signaling. Studies in zebrafish have shown that intestinal alkaline phosphatase (IAP) alters bacterial LPS and thus reduces its proinflammatory potential (Bates, Akerlund, Mittge, & Guillemin, 2007). In this way, IAP may modulate the concentrations of LPS required to activate epithelial cell inflammatory signaling. This threshold concentration would be governed both by the affinity of LPS binding to its receptor(s) and by the rate at which IAP dephosphorylates LPS (Vaishnava & Hooper, 2007).
Another protein that modulates inflammatory signaling pathways is A20, a zinc-finger protein whose expression is controlled by NFκB (Krikos, Laherty, & Dixit, 1992). A20 is an ubiquitin-modifying enzyme that inhibits NFκB activation by downregulating key polyubiquitination-dependent inflammatory mediators (Wertz et al., 2004). A20 targets include TNF-receptor-associated factor 6 (TRAF6) (Deng et al., 2000) and receptor-interacting protein kinase (Li, Kobayashi, Blonska, You, & Lin, 2006). A20-deficient (Tnfaip3−/−) mice are highly susceptible to intestinal inflammation, suggesting that A20 plays an essential role in regulating the immune activation threshold in the intestine (Lee et al., 2000; Turer et al., 2008).

By expressing factors such as IAP and A20, intestinal epithelia may modulate the threshold bacterial density that is required to elicit an innate immune response. Such strategies may contribute to the relative tolerance of intestinal surfaces to the presence of high bacterial loads.

4. MUCUS PRODUCTION BY THE INTESTINAL EPITHELIUM

4.1. Secretion and assembly of the mucus layer

Goblet cells, found in both the small and large intestines, secrete large quantities of mucin proteins (Fig. 1). Mucins are highly glycosylated proteins that assemble to form a protective layer of viscous mucus that acts as an additional physical barrier between the epithelial surface and the bacterial communities in the intestine. The mucus layer extends up to 150 μm from the epithelial surface and is composed of two distinct strata (Johansson et al., 2008). The outer layer is heavily colonized with bacteria, while the inner layer is resistant to bacterial penetration, resulting in a protected zone directly adjacent to the epithelial surface (Johansson et al., 2008). Bacterial penetration of the inner mucus layer is also controlled by antibacterial proteins that are secreted by epithelial cells and retained in the mucus layer (Meyer-Hoffert et al., 2008; Vaishnava et al., 2011). Mice lacking the mucin glycoprotein MUC2 are unable to limit bacterial contact with the epithelium and consequently have severe intestinal inflammation (Johansson et al., 2008). Thus, the mucus barrier is essential for maintaining a beneficial symbiotic relationship with the luminal microbiota.

4.2. Regulation of mucus production

Inflammasomes are multiprotein complexes that regulate the processing and secretion of proinflammatory cytokines. Their assembly is triggered when a
Epithelial cells perform several cell-intrinsic innate immune functions that regulate interactions between luminal microorganisms and host tissues. Paneth cells secrete numerous antimicrobial proteins, such as α-defensins. RegIIIα (human) and RegIIIγ (mouse) are antimi-
crobial lectins that are secreted by Paneth cells and enterocytes (Cash et al., 2006). The RegIII lectins bind to peptidoglycan on Gram positive bacteria and kill the bacteria by forming a hexameric pore in the bacterial membrane (Cash et al., 2006; Lehotzky et al.,
2010; Mukherjee et al., 2014). Goblet cells secrete mucin glycoproteins that assemble into a viscous mucus layer that limits bacterial interactions with the epithelial surface (Gum, Hicks, Toribara, Siddiki, & Kim, 1994; Johansson et al., 2008). Autophagy is activated in response to invasive bacteria and prevents bacterial dissemination to deeper tissues (Benjamin et al., 2013; Conway et al., 2013). Epithelial antibacterial autophagy depends on the TLR signaling adaptor MyD88 (Benjamin et al., 2013) as well as the essential autophagy factors ATG5 and ATG16L1 (Benjamin et al., 2013; Conway et al., 2013).
member of the NOD-like receptor (NLR) family senses stress or damage-associated molecular patterns (Schroder & Tschopp, 2010). This leads to recruitment of the adaptor protein ASC into a multiprotein complex that regulates the activity of caspase-1, a protease that cleaves and activates proinflammatory cytokines such as IL-1β and IL-18 (Agostini et al., 2004; Martinon et al., 2002).

Recent findings have revealed that inflammasome formation regulates mucus secretion by goblet cells. The NLR family member NLRP6 is an important regulator of inflammasome formation in the intestine. Genetic deletion of NLRP6 resulted in reduced intestinal IL-18 production and an altered microbiota. These phenotypes were associated with spontaneous intestinal hyperplasia, inflammatory cell recruitment, and enhanced susceptibility to chemically induced colitis (Elinav et al., 2011). Subsequently, NLRP6-dependent inflammasomes were found to regulate mucus secretion by goblet cells. Deletion of NLRP6 led to defective autophagy, which resulted in defective mucin granule exocytosis and made the mice susceptible to persistent infection by Citrobacter rodentium (Wlodarska et al., 2014). Although it is not yet clear whether epithelial cell-intrinsic inflammasome formation is responsible for this phenotype, these findings reveal an interesting connection among inflammasome activation, autophagy, and mucus production by the epithelium.

Goblet cell mucin secretion is also regulated by proteins that govern the autophagy pathway. In colonic goblet cells, proteins essential for autophagosome formation, such as ATG5, are required for efficient mucus secretion (Patel et al., 2013). This process is dependent on the endosomal pathway and the generation of reactive oxygen species (ROS). Perturbation of these pathways leads to an abnormal accumulation of mucin granules in goblet cells (Patel et al., 2013). Future studies will be required to assess the impact of mucin granule accumulation on the formation of the intestinal mucus layer and its ability to control interactions with the intestinal microbiota.

5. EPITHELIAL ANTIMICROBIAL PROTEINS

Epithelial antimicrobial proteins play a central role in allowing epithelial surfaces to cope with the challenge of being closely associated with a dense microbial community (Fig. 1). These natural antibiotics are an evolutionarily ancient defense system that is present in virtually all plants and animals. Mammalian antimicrobial proteins rapidly kill or inactivate
microorganisms and are members of a diverse group of protein families. The epithelial cells lining the intestine produce a rich array of antimicrobial proteins, likely reflecting the complexity of the microbial communities that challenge the intestinal surface.

Most antimicrobial peptides and proteins target the cell walls of microorganisms. As discussed later, several groups of antimicrobial proteins kill bacteria by disrupting their membranes. Other antimicrobial factors are enzymes that kill bacteria by digesting specific cell wall structures. Finally, some antimicrobial proteins work through other mechanisms such as nutrient sequestration. The presence of multiple antimicrobial protein families and the use of diverse killing strategies is likely important for limiting the evolution of resistance to multiple antimicrobial factors. In addition, the targeting of essential cell wall or cell membrane structures might promote the continued effectiveness of endogenous antimicrobial proteins over evolutionary timescales, as bacteria cannot readily alter these structures without compromising fitness.

5.1. Epithelial antimicrobial protein families

5.1.1 Defensins

A key mechanism by which antimicrobial proteins kill bacteria is through nonenzymatic disruption of microbial membranes. Defensins constitute the major family of membrane-disrupting peptides in mammals and are one of the most diverse and highly expressed protein families in the intestinal epithelium. The defensins are small peptides (2–3 kDa) with a conserved three-dimensional structure that has a characteristic amphipathic arrangement of cationic and hydrophobic amino acids (Zasloff, 2002). This arrangement produces a positively charged surface that is spatially separated from neighboring hydrophobic regions, thus facilitating insertion into negatively charged microbial membranes.

Defensins are classified into three major groups - α, -β and -θ - that vary in their disulfide bond arrangements and cysteine residue spacing (Selsted & Ouellette, 2005). The spectrum of antimicrobial activity varies for each particular protein, but in general, defensins exhibit a broad spectrum of activity against both Gram positive and Gram negative bacteria and in some cases are active against fungi, viruses, and protozoa (Selsted & Ouellette, 2005); however, individual defensins have marked differences in their activity spectrum and expression patterns (Ouellette, 2011).

The expression of α-defensins in the gastrointestinal tract is restricted to Paneth cells in small intestinal crypts and is lacking from other epithelial cell
lineages (Selsted & Ouellette, 2005). Because of their localization in the crypt, mouse α-defensins are termed “cryptdins.” In addition to cryptdins, mice encode a family of diverse cryptdin-related sequence (CRS) peptides. CRS peptides have four intramolecular disulfide bridges and further form covalent dimers by an additional intermolecular disulfide bridge (Hornef, Pütsep, Karlsson, Refai, & Andersson, 2004). These dimeric peptides exhibit potent antimicrobial activity against both Gram positive and Gram negative bacteria (Hornef et al., 2004). CRS peptides can form both heterodimers and homodimers, thus increasing their combinatorial diversity.

In contrast to α-defensins, β-defensins are expressed in enterocytes of the large and small intestines (O’Neil et al., 1999). The human genome contains at least 28 β-defensins, 8 of which are expressed (Schutte et al., 2002). A subset of these is expressed in intestinal epithelial cells (Fahlgren, Hammarström, Danielsson, & Hammarström, 2003; O’Neil et al., 2000; Wehkamp et al., 2002). There are reports that β-defensins also may help to recruit immune cells such as dendritic cells and T cells (Biragyn et al., 2002).

5.1.2 Lectins
Soluble lectins are a second group of antibacterial proteins that kill by non-enzymatic disruption of bacterial membranes. RegIIIγ and its human ortholog, RegIIIα (also known as hepatocarcinoma-intestine-pancreas/pancreatic-associated protein, or HIP/PAP), are expressed in multiple small intestinal epithelial lineages, including enterocytes and Paneth cells (Fig. 1) (Cash et al., 2006; Christa et al., 1996). Both proteins bind to peptidoglycan and have intrinsic bactericidal activity (Cash et al., 2006; Lehotzky et al., 2010). In contrast to defensins, the RegIII lectins are selective for Gram positive bacteria (Cash et al., 2006). This is consistent with the fact that peptidoglycan is accessible for binding on the outer surfaces of Gram positive bacteria, but it is buried in the periplasmic space of Gram negative bacteria.

The bactericidal action of the RegIII lectins is mediated by membrane disruption (Fig. 1) (Mukherjee et al., 2014). Similar to defensins, RegIIIα interacts with the charged bacterial membrane through electrostatic interactions. Upon contact with the lipid bilayer, RegIIIα oligomerizes to form a hexameric, membrane-penetrating pore (Mukherjee et al., 2014). The closely related lectin RegIIIβ is usually coexpressed with RegIIIγ in mice. RegIIIβ also binds to peptidoglycan (Lehotzky et al., 2010), although recent studies suggest that it can also bind to carbohydrate moieties on LPS and thus kill Gram negative bacteria (Miki, Holst, & Hardt, 2012; Stelter et al., 2011).
As several other members of the Reg family of C-type lectins are expressed in gastrointestinal tissues (Dieckgraefe et al., 2002), it seems likely that the Reg lectins represent a general mechanism of antibacterial defense at the mucosal surface.

Members of the galectin family of lectins also have antibacterial functions. Galectin-4 and galectin-8 are expressed in the gastrointestinal tract and specifically recognize and kill Escherichia coli that express carbohydrate structures that mimic human blood group antigens. Bacterial killing is accompanied by disruption of the bacterial membrane (Stowell et al., 2010), although the mechanism of membrane disruption remains to be defined. It is possible that these bactericidal lectins evolved as an outcome of a host-microbial arms race, as mimicry of host antigenic structures is a mechanism of pathogen evasion. By specifically recognizing such structures, these galectins may promote killing of microorganisms that are especially prone to evade the adaptive immune system (Stowell et al., 2010).

5.1.3 Cathelicidins
Cathelicidins are a third general class of epithelial antimicrobial peptides that are expressed in the intestinal epithelium and kill microorganisms by membrane disruption (Hase, Eckmann, Leopard, Varki, & Kagnoff, 2002). They are cationic, α-helical peptides with a conserved 14-kDa N-terminal “cathelin” (cathepsin L inhibitor)-like domain and a variable C-terminal region. The single cathelicidin gene (CAMP in humans) encodes a precursor protein (hCAP18) (Larrick et al., 1996). This protein can be cleaved at an alternate site to generate several active antimicrobial proteins, including the 37 amino acid peptide LL-37 (Gudmundsson et al., 1996) and the murine peptide CRAMP (cathelin-related antimicrobial peptide) (Gallo et al., 1997).

Cathelicidins exhibit biological activities similar to those of the defensin family. They kill bacteria by first binding to bacterial membranes via charge–charge interactions, followed by membrane insertion and disruption (Bals & Wilson, 2003). Both LL-37 and CRAMP exhibit antimicrobial activity against Gram positive and Gram negative bacteria as well as fungi (Bals & Wilson, 2003). Like β-defensins, LL-37 has biological functions that are independent of its bactericidal activity. For example, it has been shown to be chemotactic in vitro for immune cells, including monocytes, macrophages, and T cells, and induces cytokine secretion by dendritic cells (Davidson et al., 2004).
5.1.4 Lysozyme and phospholipase A2
Enzymes that kill bacteria through enzymatic attack on microbial cell walls constitute another key group of antimicrobial proteins. One such enzymatic protein, lysozyme, is abundantly expressed and secreted by Paneth cells. Lysozyme is a glycosidase that hydrolyzes the 1,4-β-glycosidic linkages of peptidoglycan. Lysozyme is more effective against Gram positive bacteria, whose peptidoglycan is on the outer cell wall surface and therefore more easily accessible than the peptidoglycan that is present in the periplasmic space of Gram negative bacteria (Ganz, 2004). Like lysozyme, secretory phospholipase A2 (sPLA2) is expressed in Paneth cells (Harwig et al., 1995). sPLA2 rapidly kills bacteria by hydrolyzing bacterial membrane phospholipids, thus compromising the integrity of the microbial membrane (Koprivnjak, Peschel, Gelb, Liang, & Weiss, 2002).

5.1.5 Lipocalin
A limited subset of antimicrobial factors function by depriving bacteria of essential nutrients, thus promoting what is known as “nutritional immunity” (Hood & Skaar, 2012). During infection, bacteria acquire much of their iron from the host through the production of siderophores that transport iron into the pathogen (Faraldo-Gómez & Sansom, 2003). Lipocalin binds and sequesters iron-bound siderophores, such as enterocalin, and thus inhibits bacterial growth (Flo et al., 2004).

5.1.6 RNases
The molecular mechanisms underlying the antibacterial activity of other intestinal microbicidal proteins remain unclear. Angiogenin-4 (Ang4) is a member of the ribonuclease family and is expressed exclusively in Paneth cells. Ang4 has broad-spectrum bactericidal activity against Gram positive and Gram negative bacteria (Hooper et al., 2003) and is thus similar to other bactericidal RNases, including RNase 7 (Harder & Schroder, 2002) and eosinophil cationic protein (Rosenberg, 1995). Although Ang4 has RNAse activity, it remains unclear whether this enzymatic activity is required for bactericidal function.

5.2. Regulation of epithelial antimicrobial proteins
Many antimicrobial proteins are toxic to mammalian as well as microbial cell membranes. Thus, the expression, secretion, and activity of most epithelial antimicrobial proteins are tightly controlled. This can occur through transcriptional and posttranslational regulation mechanisms (Fig. 2).
5.2.1 Transcriptional regulation of epithelial antimicrobial protein expression

Studies of germ-free mice have shown that some intestinal antimicrobial proteins are expressed independently of the microbiota whereas others require bacterial signals for their expression. For example, the majority of intestinal α-defensins require the Wnt pathway transcription factor TCF4 (van Es et al., 2005) but are expressed independently of the microbiota (Fig. 2) (Putsep et al., 2000). Similarly, expression of lysozyme, sPLA2, and certain members of the β-defensin family does not require microbial signals (Hooper et al., 2001, 2003; O’Neil et al., 1999). The cathelicidin LL-37 is expressed in human epithelial cells independently of the microbiota, although it is modestly upregulated by invasive microorganisms (Hase et al., 2002).

The expression of other epithelial antimicrobial proteins requires microbial stimulation. Members of the CRS family of peptides show increased levels of expression in conventionally raised mice compared with germ-free mice (Putsep et al., 2000). Similarly, members of the human β-defensin family, including hBD2, are expressed under the control of bacterial signals (O’Neil et al., 1999). Finally, the expression of both Ang4 and RegIIIγ is virtually absent in germ-free mice and is increased upon microbial colonization (Cash et al., 2006; Hooper et al., 2003).

As discussed earlier, host pattern recognition receptors direct the expression of some of these bacterially regulated epithelial antimicrobial proteins. For example, stimulation of TLRs is required for RegIIIγ mRNA expression by intestinal epithelial cells (Fig. 2). Studies of mice lacking MyD88, an adaptor molecule common to several TLRs, have revealed that RegIIIγ and RegIIIβ are expressed under the control of TLRs in vivo (Brandl et al., 2007; Rakoff-Nahoum & Medzhitov, 2007; Vaishnava et al., 2008, 2011). Further, the MyD88 dependence is intrinsic to epithelial cells (Brandl et al., 2007; Vaishnava et al., 2008, 2011). This suggests that epithelial cells, including enterocytes and Paneth cells, directly sense bacteria through TLRs and upregulate expression of RegIIIγ and RegIIIβ in response. Expression of these antimicrobial proteins is likely triggered by any of several TLRs, as mice deficient in individual TLRs do not have defects in RegIIIγ or RegIIIβ expression (Vaishnava et al., 2008). This is consistent with the fact that both LPS and flagellin (which bind TLR4 and TLR5, respectively) are sufficient to trigger RegIIIγ expression (Brandl et al., 2007; Kinnebrew et al., 2010; Vaishnava et al., 2008).
Figure 2 See legend on next page.
Intestinal epithelial cell expression of RegIIIγ also requires signals from at least one subepithelial immune cell lineage. Innate lymphoid cells (ILCs) reside in the lamina propria and phenotypically resemble natural killer cells (Sanos, Vonarbourg, Mortha, & Diefenbach, 2011). ILCs produce the cytokine IL-22, which binds to IL-22 receptors on epithelial cells to modulate epithelial cell function (Wolk et al., 2004). ILCs from germ-free mice produce low levels of IL-22 (Sanos et al., 2008), indicating that intestinal bacteria drive IL-22 expression in these cells. Interestingly, ILC-derived IL-22 is required for epithelial cell expression of RegIIIγ mRNA (Fig. 2)(Sanos et al., 2008). Thus, RegIIIγ expression is dependent on both epithelial cell-intrinsic TLR signaling through MyD88 and IL-22 produced by ILCs. It may be possible to reconcile these disparate observations by proposing that IL-22 functions as an environmental cue that licenses epithelial cells to express RegIIIγ. Epithelial cells must then receive an additional direct bacterial signal through TLRs in order to express RegIIIγ. Further studies will be required to unravel this regulatory network.

The expression of other intestinal antimicrobial proteins is regulated by NOD2, an intracellular pattern recognition receptor that is expressed in Paneth cells (Ogura et al., 2003). MDP has been shown to control the production of certain α-defensins (Kobayashi et al., 2005) and to enhance the bactericidal activity of Paneth cells (Fig. 2)(Petnicki-Ocwieja et al., 2009). Additionally, Nod2−/− mice have alterations in the composition of their small intestinal microbiota (Petnicki-Ocwieja et al., 2009), as well as increased susceptibility to oral challenge with the pathogen Listeria.
monocytogenes (Kobayashi et al., 2005). These results indicate that NOD2-stimulated antimicrobial defenses shape microbiota composition and protect the epithelial barrier from pathogen invasion.

Together, these findings reveal that different subsets of antimicrobial proteins are regulated via distinct mechanisms. A constitutive chemical barrier is established at the mucosal surface by the subset of antimicrobial proteins that are expressed independently of bacterial signals. The regulated expression of other proteins through TLR and NOD2 activation suggests that a subset of antimicrobial responses may be more precisely titrated in response to microbial numbers or the composition of the intestinal microbial community. Strict regulation of certain antimicrobial responses by bacterial signals could also protect against overproduction of antimicrobial proteins that could interfere with intestinal ecology and thus undermine the beneficial contributions of the microbiota.

5.2.2 Developmental regulation of antimicrobial protein expression

At least two epithelial antimicrobial proteins are developmentally regulated. Both Ang4 and RegIIIγ are strongly induced in the small intestine during early postnatal life. In conventionally raised mice, the level of Ang4 expression increases approximately 20-fold during weaning (day 17–21 in mice) and remains at adult levels thereafter (Hooper et al., 2003). The level of expression of RegIIIγ in mice increases by a remarkable 3000-fold during the same period (Cash et al., 2006). These findings suggest that Ang4 and RegIIIγ could function in part to maintain mucosal homeostasis in the face of the changing microbial ecology and withdrawal of maternal passive immunity that is associated with weaning.

5.2.3 Posttranslational regulation of antimicrobial protein function

Because membrane-toxic antimicrobial proteins can also target mammalian cell membranes (Lichtenstein, Ganz, Selsted, & Lehrer, 1986), the activities of many antimicrobial proteins are suppressed during storage in membrane-bound secretory granules. α-Defensins are stored in Paneth cell granules as inactive propeptides and are processed at their N-termini by matrix metalloproteinase-7 (MMP7) to produce mature bactericidally active peptides (Wilson et al., 1999). In humans, trypsin cleaves α-defensins to their mature forms (Ghosh et al., 2002). RegIIIγ also requires N-terminal proteolytic processing by trypsin to yield a bactericidally active protein (Mukherjee et al., 2014). Similarly, β-defensins are expressed as propeptides, but the processing mechanism remains to be established (Schutte & McCray, 2002).
The distinctive reducing environment of the intestinal lumen provides another mechanism of posttranslational regulation that likely protects host cells during storage of antimicrobial proteins. Under the oxidizing conditions present inside host cells, the antimicrobial protein human β-defensin 1 (hBD1) has weak antimicrobial activity. However, under the reducing conditions that are characteristic of the intestinal lumen, hBD1 undergoes marked structural changes that unmask a potent antimicrobial activity (Schroeder et al., 2011).

5.2.4 Regulation of antimicrobial protein secretion

The process of antimicrobial protein secretion is also controlled by bacterial signals. As discussed earlier, Paneth cells produce most of the antimicrobial proteins in the small intestine, including a diverse array of α-defensins, Ang4, and lysozyme. Paneth cells secrete their granule contents in response to exposure to live bacteria or to bacterial molecules such as LPS (Fig. 2) (Ayabe et al., 2000). Thus, antimicrobial protein release is precisely regulated in response to bacterial signals, though the mechanisms of bacterial sensing that control Paneth cell secretion are not yet clear.

More recently, proteins of the autophagy pathway have been found to play an important role in regulating granule exocytosis in Paneth cells. ATG16L1 and ATG5 are proteins that are each essential for autophagy (Cadwell et al., 2008). Mice-lacking epithelial cell expression of ATG16L1 or ATG5 exhibits defective Paneth cell granule packaging and exocytosis, indicating a role for these autophagy proteins in the Paneth cell secretory pathway (Cadwell et al., 2008). Interestingly, a single coding variation (T300A) in the ATG16L1 protein is strongly associated with the risk of Crohn’s disease, and patients homozygous for the risk allele also show abnormal Paneth cell granule formation and exocytosis (Fig. 2) (Cadwell et al., 2008). This suggests that defective Paneth cell granule secretion may be one factor that leads to Crohn’s disease pathogenesis.

5.3. In vivo functions of epithelial antimicrobial proteins

Studies of genetically engineered mouse models have offered insight into how epithelial antimicrobial proteins function in vivo. These proteins not only protect against pathogen colonization but also allow mammalian hosts to control the composition and location of their resident microbial communities.
5.3.1 Protection against pathogens
Experiments in mice have yielded key insight into the importance of intestinal antimicrobial proteins in pathogen protection in vivo. MMP7 is required to generate bactericidally active α-defensins in mice, and consequently, Mmp7<sup>-/-</sup> mice have elevated susceptibility to oral challenge with the intestinal pathogen <i>S. typhimurium</i> (Wilson et al., 1999). A second mouse model that has illuminated the in vivo function of α-defensins is a transgenic mouse overexpressing human α-defensin–5 (DEFA5) in Paneth cells. DEFA5-expressing mice show greater resistance to oral infection with <i>S. typhimurium</i> than wild-type mice, demonstrating an essential role for α-defensins in limiting pathogen colonization (Fig. 3) (Salzman, Ghosh, Hutner, Paterson, & Bevins, 2003). Finally, antibody-mediated inactivation of RegIIIγ shows that this antimicrobial lectin is important for limiting colonization by Gram positive intestinal pathogens such as <i>L. monocytogenes</i>.

![Figure 3 Functions of antimicrobial proteins in the intestine.](image)

Forced expression of a human α-defensin 5 (DEFA5) transgene in Paneth cells limits colonization by <i>S. typhimurium</i> (Salzman et al., 2003) and controls microbiota composition in the small intestine (Salzman et al., 2010). The antimicrobial lectin RegIIIγ helps to confine bacteria to the outer mucus layer, thus limiting bacterial interactions with the epithelial surface of the small intestine (Vaishnava et al., 2011).
(Brandl et al., 2007) and vancomycin-resistant Enterococcus faecalis (Brandl et al., 2008).

5.3.2 Shaping microbiota composition

α-Defensins also regulate the composition of the intestinal bacterial community. Mmp7⁻/⁻ and DEFA5-transgenic mice each show marked α-defensin-dependent changes in the composition of their microbial communities compared with wild-type mice (Fig. 3) (Salzman et al., 2010). Further, the defensin-deficient Mmp7⁻/⁻ mice and the defensin-complemented DEFA5-transgenic mice show reciprocal differences in community composition. These changes include altered proportions of Firmicutes, the major Gram positive phylum, and Bacteroidetes, the major Gram negative phylum, of the mouse intestine (Salzman et al., 2010). The DEFA5-transgenic mice also showed a loss of segmented filamentous bacteria (SFB) relative to wild-type mice. Consistent with the fact that SFB promotes the development of intestinal IL 17-producing T_h17 cells (Ivanov et al., 2009), the DEFA5-transgenic mice had lower frequencies of lamina propria T_h17 cells compared with wild-type mice (Salzman et al., 2010). Thus, α-defensins shape the intestinal microbiota composition and control the level of immune stimulation.

5.3.3 Limiting bacterial-epithelial cell contact

A key mechanism by which the mammalian intestine maintains homeostasis with its associated bacterial communities is to minimize contact between the bacteria and the host tissues. As discussed in Section 4, the mucus layer plays an essential role in limiting direct contact between microbiota and host. Further, most antimicrobial activity is confined to the mucus layer and is essentially absent from the luminal content (Meyer-Hoffert et al., 2008). Thus, in addition to acting as a physical barrier, the mucus layer may also limit bacterial access to the epithelium by forming a diffusion barrier that concentrates antimicrobial proteins near the epithelial cell surface.

Consistent with this idea, studies of RegIIIγ⁻/⁻ mice exhibit suggest that RegIIIγ interacts with the mucus layer to limit bacterial contact with the intestinal epithelial cell surface (Fig. 3). RegIIIγ⁻/⁻ mice are characterized by increased colonization of the small intestinal epithelial surface by Gram positive bacteria, consistent with the specificity of RegIIIγ for Gram positives (Cash et al., 2006). Interestingly, these differences do not extend to the luminal bacterial communities, which are similar when comparing RegIIIγ⁻/⁻ and wild-type littermates. This suggests that the antibacterial...
effects of RegIIIγ are confined to the inner mucus layer. The niche-specific activity of RegIIIγ could arise from restricted diffusion of RegIIIγ through the mucus barrier, from binding interactions between RegIIIγ and mucus glycoproteins or because RegIIIγ requires environmental conditions that are unique to the mucosal surface niche.

6. INTESTINAL EPITHELIAL CELL AUTOPHAGY

Autophagy is an evolutionarily ancient process in which cytoplasmic materials are targeted to the lysosome for degradation. Portions of the cytoplasm are sequestered into double-membrane structures, called autophagosomes, which fuse with lysosomes, delivering their contents for degradation by lysosomal enzymes (Deretic & Levine, 2009). The process involves the concerted action of several cytoplasmic proteins. A primary function of autophagy is to maintain cellular homeostasis by degrading cytoplasmic contents during cellular starvation and by recycling damaged organelles and proteins (Rabinowitz & White, 2010). However, autophagy has also been shown to be critical for the recognition and degradation of intracellular pathogens, thus functioning as an innate barrier to infection (Benjamin et al., 2013; Conway et al., 2013; Deretic & Levine, 2009; Levine, Mizushima, & Virgin, 2011).

In the mammalian intestine, the autophagy pathway mediates at least two distinct functions. First, it acts as an innate barrier to the dissemination of invasive bacteria, such as *S. typhimurium*. Second, the proteins that regulate classical autophagy activation are also essential for proper granule formation and protein secretion in intestinal secretory cell lineages such as goblet cells and Paneth cells (Cadwell et al., 2008; Patel et al., 2013). Both of these functions are discussed in this section.

6.1. Autophagy as a barrier to bacterial dissemination

Recent studies have shown that autophagy is an important epithelial cell-autonomous mechanism of antibacterial defense that protects against dissemination of intestinal bacteria. Epithelial autophagy is activated in the mouse intestinal epithelium by a pathogen, *S. typhimurium*, as well as by *E. faecalis*, an opportunistically invasive commensal (Benjamin et al., 2013; Conway et al., 2013). Autophagy is specifically triggered by bacterial invasion of epithelial cells and remains at baseline levels in response to the microbiota normally present in specified pathogen-free mice. Epithelial cell autophagy is also tightly regulated, requiring epithelial cell-intrinsic MyD88 signaling.
Finally, epithelial autophagy activation depends on the presence of the autophagy factors ATG5 and ATG16L1, as deletion of either of these factors leads to increased extraintestinal spread of *S. typhimurium* (Fig. 1) (Benjamin et al., 2013; Conway et al., 2013).

There may also be a role for autophagy in limiting colonization of non-invasive pathogens. *C. rodentium* is an attaching and effacing pathogen which forms lesions on the apical surface of the colonic epithelium but does not enter epithelial cells in large numbers (Mundy, MacDonald, Dougan, Frankel, & Wiles, 2005). Epithelial cell expression of the autophagy protein ATG7 confers protection against luminal colonization by *C. rodentium*, although it is not yet clear whether *bona fide* autophagy is involved in limiting *C. rodentium* intestinal colonization and pathogenesis (Inoue et al., 2012).

### 6.2. Autophagy-dependent regulation of protein secretion

The autophagy machinery is also involved in cellular functions that are distinct from classical autophagy involving the targeting of bacteria to autophagosomes (Zhao et al., 2008). As discussed earlier, mice lacking epithelial cell expression of ATG16L1 or ATG5 exhibit defective Paneth cell granule formation and exocytosis, indicating a role for these autophagy proteins in the Paneth cell secretory pathway (Cadwell et al., 2008). Mice with an epithelial cell-specific deletion of ATG5 also exhibit defective exocytosis of goblet cell granules, leading to altered mucus secretion (Patel et al., 2013).

A point mutation (T300A) in the critical autophagy gene ATG16L1 is associated with a predisposition to Crohn’s disease in humans (Hampe et al., 2007; Rioux et al., 2007; Wellcome Trust Case Control Consortium, 2007). The T300A mutation both reduces antibacterial autophagy (Kuballa, Huett, Rioux, Daly, & Xavier, 2008; Lassen et al., 2014) and disrupts granule packaging and protein secretion in Paneth cells (Cadwell et al., 2008, 2010). Such defects could lead to inflammation through reduced antimicrobial protection at the epithelial surface.

### 7. EPITHELIAL REGULATION OF ADAPTIVE IMMUNITY

In addition to epithelial cell autonomous functions that kill bacteria and control the microbiota, epithelial cells also communicate with underlying immune cells to regulate and coordinate adaptive immune responses. These functions include transcytosis of immunoglobulin A, secretion of cytokines that direct adaptive immune responses, and delivery of antigen to the adaptive immune system (Fig. 4).
7.1. Transcytosis of immunoglobulin A

One important way in which epithelial cells contribute to immune defense is through transcytosis of secretory immunoglobulin A (IgA) across the epithelial barrier. IgA is the most abundant immunoglobulin isotype in the intestine and is essential for maintaining luminal compartmentalization of intestinal bacteria and preventing their penetration into host tissue (Macpherson et al., 2000; Macpherson & Uhr, 2004; Suzuki et al., 2004). Much of this IgA is specific to intestinal bacteria and is produced by IgA-secreting plasma cells that develop in lymphoid tissues and then home to the lamina propria. The plasma cells secrete dimeric IgA that binds to the polymeric immunoglobulin receptor (pIgR) on the basolateral surface of epithelial cells. The pIgR–IgA complex is transcytosed across the epithelium, and the IgA is deposited on the apical epithelial surface. IgA plays an essential role in confining bacteria to the intestinal lumen (Macpherson et al., 2000), though the exact mechanisms remain unclear. Goblet cells deliver small soluble antigens from the intestinal lumen to lamina propria DCs (McDole et al., 2012).
the lamina propria. The plasma cells secrete dimeric IgA that is then bound to the polymeric immunoglobulin receptor (pIgR), which is positioned on the basolateral surface of epithelial cells. pIgR is itself expressed under the control of microbiota signals that are transmitted through MyD88 and NFκB (Bruno, Frantz, Rogier, Johansen, & Kaetzel, 2011; Johansen & Kaetzel, 2011). The pIgR–IgA complex is transcytosed across the epithelium, and the IgA is deposited on the apical epithelial surface of the epithelium (Fig. 4). The exact mechanisms by which IgA confines symbiotic bacteria to the intestinal lumen remain unclear but may involve retention of bacteria in the mucus layer or promoting phagocytic clearance of organisms that have breached the epithelial barrier.

7.2. Cytokine secretion

Several cytokines produced by intestinal epithelial cells under the control of microbiota signals promote adaptive immune responses (Fig. 4). For example, APRIL (a proliferation-inducing ligand) is secreted by epithelial cells in a MyD88-dependent manner and drives T cell-independent IgA2 class switching (He et al., 2007). BAFF (B cell-activating factor of the TNF family) is another cytokine-like factor that is closely related to APRIL, is secreted by epithelial cells in response to bacterial signals, and regulates B cell maturation, survival, and function (Cerutti, Puga, & Cols, 2011; Xu et al., 2007). Another epithelial-derived cytokine is TSLP (thymic stromal lymphopoietin), which has a critical immunoregulatory role in both inflammatory settings and in response to pathogenic worm infections (Rimoldi et al., 2005; Taylor et al., 2009; Zeuthen, Fink, & Frokiaer, 2008; Ziegler & Artis, 2010). TSLP causes dendritic cells and macrophages to adopt tolerogenic phenotypes and to secrete cytokines such as IL-10 (Rimoldi et al., 2005).

7.3. Antigen delivery to subepithelial immune cells

A key function of intestinal epithelial cells is participating in antigen sampling and presentation to the adaptive immune system. This results in directed adaptive immune responses to commensal or pathogenic bacteria. Classically, M cells that overlie Peyer’s patches and isolated lymphoid tissues have been associated with antigen sampling. As discussed in Section 2, these specialized cells mediate the sampling of antigens and microorganisms from the intestinal lumen, with presentation to immune cells that underlie the epithelium (Kraehenbuhl & Neutra, 2000). M cells have also been reported to be distributed in the villus epithelium and may thus
provide an alternate route of antigen entry in the intestinal epithelium (Jang et al., 2004).

Goblet cells have recently been shown to participate in luminal antigen sampling (Fig. 4). These cells deliver small soluble antigens from the intestinal lumen to CD103+ dendritic cells (DCs) in the underlying lamina propria (McDole et al., 2012). These CD103+ DCs are a specialized DC subset that promote IgA production, imprint gut homing on lymphocytes, and induce the development of T regulatory cells (Coombes et al., 2007; Jaensson et al., 2008; Johansson-Lindbom et al., 2005; Sun et al., 2007; Uematsu et al., 2008). Although more work is required to understand this process and its consequences, goblet cell antigen presentation is likely to have important effects on mucosal immunity.

Enterocytes participate in antigen presentation to the immune system in at least two ways. First, enterocytes facilitate the extension of the dendrites of subepithelial mononuclear phagocytes by expressing tight junction proteins that “pry” open the tight junctions between intestinal epithelial cells, allowing dendrite extension into the lumen for direct sampling of microorganisms at the epithelial apical surface (Rescigno et al., 2001). A second way that enterocytes participate in antigen presentation is through the expression of CD1d. CD1d presents lipid antigens, derived from either “self” or from bacteria, to natural killer T (NKT) cells (Colgan, Hershberg, Furuta, & Blumberg, 1999; Heller, Fuss, Nieuwenhuis, Blumberg, & Strober, 2002; Wingender & Kronenberg, 2008). NKT cells play important roles in the development of intestinal inflammatory responses and are involved in pathogenic inflammation in both animal models and human IBD (Heller et al., 2002). Epithelial CD1d expression suppresses proinflammatory NKT cell functions and thus reduces intestinal inflammation (Olszak et al., 2014). This is in contrast to CD1d-mediated antigen presentation through bone marrow-derived cells, which stimulates proinflammatory functions of NKT cells (Olszak et al., 2014).

8. BACTERIAL STIMULATION OF EPITHELIAL CELL REPAIR

The epithelium is a critical physical barrier against microbial penetration. This function can be perturbed when there is epithelial damage from environmental insults such as toxins or pathogenic bacteria. The presence of large indigenous bacterial populations leads to a significant risk for bacterial invasion, inflammation, and sepsis following intestinal epithelial damage. The intestinal epithelium must therefore be able to recognize and repair
8.1. MyD88-dependent epithelial repair

The mechanisms underlying intestinal epithelial repair have relied largely on the analysis of the dextran sulfate sodium (DSS)-induced model of epithelial injury. In this model, epithelial injury is initiated in the colons of mice through administration of DSS in drinking water. Epithelial damage is visible through the appearance of focal colonic lesions after a few days of DSS administration, is accompanied by increased mucosal permeability, and can be detected prior to the ensuing inflammatory response. After removal of DSS, a complex tissue repair process is initiated, resulting in vigorous epithelial cell proliferation and restoration of an intact epithelial barrier (Chen, Chou, Fuchs, Havran, & Boismenu, 2002).

Efficient colonic epithelial repair requires the presence of resident gut bacteria. Mice lacking most of their intestinal microbiota due to antibiotic treatment are more susceptible to DSS-induced epithelial injury than fully colonized mice (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004). The effect is reversible, as recolonization of antibiotic-treated mice with commensal bacteria restores normal epithelial repair processes. Mice lacking the TLR signaling adaptor MyD88 exhibit defective epithelial repair in response to DSS-induced mucosal damage, showing that TLR signaling is required for efficient epithelial repair (Rakoff-Nahoum et al., 2004). These findings indicate that bacterial activation of TLR signaling pathways is essential for colonic tissue repair processes.

The DSS injury model has also provided essential clues about the intestinal cell populations that promote microbe-regulated epithelial repair. Analysis of bone marrow chimeric mice revealed that the MyD88-dependent signals that drive epithelial repair derive from bone marrow-derived cells (Rakoff-Nahoum, Hao, & Medzhitov, 2006). This finding was further refined by studies of mice lacking specific immune cell populations, which showed that macrophages are required for the colonic epithelial proliferative response (Pull, Doherty, Mills, Gordon, & Stappenbeck, 2005). After DSS-induced injury, colonic macrophages are recruited to sites of active epithelial proliferation where they become localized next to epithelial progenitor cells and express factors that stimulate cellular proliferation (Pull et al., 2005). Together, these results suggest a model in which epithelial repair is driven by bacterial stimulation of mobile
subepithelial myeloid cells that migrate to damaged regions and produce factors that promote epithelial restitution.

### 8.2. Activation of epithelial repair by reactive oxygen species

Bacteria also promote epithelial repair by inducing ROS in intestinal epithelial cells. Bacteria stimulate the formyl peptide receptor on epithelial cells, activating NADPH-oxidase 1 (NOX1) and enhancing ROS generation (Alam et al., 2013). Through the inactivation of redox-sensitive tyrosine phosphatases, ROS promote the formation of focal matrix adhesions that are necessary for the repair of epithelial damage (Swanson et al., 2011). This in turn stimulates the migration and proliferation of enterocytes that are adjacent to sites of epithelial damage (Leoni et al., 2013). Similar pathways operate in Drosophila, suggesting that this is an evolutionarily ancient mechanism of epithelial restitution (Hochmuth, Biteau, Bohmann, & Jasper, 2011; Jones et al., 2013; Lee, 2009).

### 9. EPITHELIAL DYSFUNCTION IN INFLAMMATORY DISEASE

Inflammatory bowel disease (IBD) is characterized by severe inflammation of the colon or the distal small intestine, or both. Although the exact causes of IBD remain poorly understood, its pathologic characteristics indicate that disease arises in part from dysregulated bacterial interactions with the intestinal epithelial surface. As evidence of this, IBD patients frequently show increased numbers of bacteria in direct contact with the intestinal epithelium (Swidsinski, Weber, Loening-Baucke, Hale, & Lochs, 2005). This suggests that IBD is characterized in part by a failure of mechanisms that normally limit microbiota–epithelial contact.

Consistent with this idea, several IBD risk alleles alter epithelial cell function by impairing production of antimicrobial peptides or mucus (Fig. 5). NOD2 was identified as the first genetic susceptibility locus for Crohn’s disease (Hugot et al., 2001; Ogura et al., 2001). Patients with NOD2 defects exhibit reduced α-defensin antimicrobial peptide expression in Paneth cells, coincident with severe intestinal inflammation (Wehkamp et al., 2005). One possible model to explain the inflammatory phenotype is that reduced α-defensin production leads to increased numbers of epithelium–associated bacteria, which could contribute to uncontrolled inflammation in conjunction with other genetic defects. As discussed in Section 6, ATG16L1 is a Crohn’s disease risk allele that disrupts antibacterial autophagy and impairs
packaging and exocytosis of Paneth cell secretory granules, thus inhibiting antimicrobial protein release (Cadwell et al., 2008). Defective antibacterial autophagy as well as impaired Paneth cell secretion of antimicrobial proteins could diminish the capacity of the epithelium to manage interactions with bacteria, thereby increasing the likelihood of bacterial penetration and mucosal inflammation.

Finally, the transcription factor XBP1 participates in the response of the endoplasmic reticulum to stress and is required for normal development of Paneth cells and goblet cells (Kaser et al., 2008). Polymorphisms in the corresponding genes are associated with an increased incidence of inflammatory bowel disease (Cadwell et al., 2008; Hugot et al., 2001; Kaser et al., 2008; Koslowski et al., 2009; Ogura et al., 2001; Wehkamp et al., 2005). This could be due to reduced production of antimicrobial proteins that normally control the microbiota and limit bacterial contact with the intestinal epithelium, as well as impaired antibacterial autophagy.

Figure 5  *Dysregulation of epithelial cell function in inflammatory disease.* NOD2, TCF4, XBP1, and ATG16L1 each promote antimicrobial protein expression and secretion by Paneth cells (Cadwell et al., 2008; Kaser et al., 2008; Kobayashi et al., 2005; Petnicki-Ocwieja et al., 2009; van Es et al., 2005), and ATG16L1 is also critical for antibacterial autophagy (Conway et al., 2013; Lassen et al., 2014). Polymorphisms in the corresponding genes are associated with an increased incidence of inflammatory bowel disease (Cadwell et al., 2008; Hugot et al., 2001; Kaser et al., 2008; Koslowski et al., 2009; Ogura et al., 2001; Wehkamp et al., 2005). This could be due to reduced production of antimicrobial proteins that normally control the microbiota and limit bacterial contact with the intestinal epithelium, as well as impaired antibacterial autophagy.

Figure 5  *Dysregulation of epithelial cell function in inflammatory disease.* NOD2, TCF4, XBP1, and ATG16L1 each promote antimicrobial protein expression and secretion by Paneth cells (Cadwell et al., 2008; Kaser et al., 2008; Kobayashi et al., 2005; Petnicki-Ocwieja et al., 2009; van Es et al., 2005), and ATG16L1 is also critical for antibacterial autophagy (Conway et al., 2013; Lassen et al., 2014). Polymorphisms in the corresponding genes are associated with an increased incidence of inflammatory bowel disease (Cadwell et al., 2008; Hugot et al., 2001; Kaser et al., 2008; Koslowski et al., 2009; Ogura et al., 2001; Wehkamp et al., 2005). This could be due to reduced production of antimicrobial proteins that normally control the microbiota and limit bacterial contact with the intestinal epithelium, as well as impaired antibacterial autophagy.
Together, these studies suggest that defects leading to reduced antimicrobial protein or mucus production may increase the likelihood of bacterial invasion of the epithelial barrier with consequent inflammation. However, it is important to note that epithelial cell defects, such as genetic Paneth cell ablation, are insufficient to produce inflammation in mice (Garabedian, Roberts, McNevin, & Gordon, 1997). This suggests that the development of inflammatory disease in humans may require additional genetic defects that impact, for example, the ability of phagocytic cells to remove bacteria that breach the epithelial barrier. Thus, multiple genetic lesions that target different levels of immune control of the microbiota may be required before inflammatory disease is manifested.

10. FUTURE PERSPECTIVES

The mammalian intestinal epithelium is faced with a complex and dynamic microbial challenge that is unique among tissues. The studies discussed in this chapter highlight the diverse array of strategies used by epithelial cells to maintain homeostasis with the enteric microbiota and to prevent pathogen invasion. These strategies include epithelial cell-intrinsic functions such as antimicrobial protein production, mucus secretion, and autophagy activation. At the same time, the intestinal epithelium plays a central role in stimulating and coordinating adaptive immune responses to intestinal microorganisms. Finally, it is clear that disrupting epithelial cell functions can have profound consequences for host health.

Most of our current understanding of epithelial cell immune function is derived from studies of the gastrointestinal tract. However, other body surfaces, such as the skin (Grice & Segre, 2011), respiratory tract (Dickson, Erb-Downward, & Huffnagle, 2013), and urogenital tract (Ma, Forney, & Ravel, 2012), are also home to diverse communities of indigenous microorganisms that are in close contact with epithelial cells. These tissues are thus also likely to be sites where epithelial immune functions have profound effects on host health. Future studies of other body surfaces will therefore be critical for obtaining a comprehensive picture of epithelial cell contributions to immunity.

Finally, the majority of studies performed to date have focused on interactions between epithelial cells and intestinal bacteria. However, the intestinal microbiota also includes eukaryotic viruses (Virgin, 2014), bacteriophage (Reyes et al., 2010), and eukaryotic organisms such as fungi (Iliev et al., 2012). These other elements of the microbial community are
undoubtedly also important targets of epithelial cell-intrinsic immune processes and likely profoundly influence epithelial function. These other microbiota components will provide fascinating targets for further exploration of epithelial cell contributions to intestinal homeostasis. Ultimately, such efforts should produce deeper insight into how mammalian hosts manage interactions with diverse microbial communities and provide new opportunities to improve human health.

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