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Commensal-Epithelial Signaling Mediated via Formyl Peptide Receptor

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B.S., University of Georgia, 2005

Advisor: Andrew S. Neish, M.D.

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Abstract

Commensal-Epithelial Signaling Mediated via Formyl Peptide Receptors

By Christy Colleen Wentworth

Virtually all metazoan life exists in intimate contact with the prokaryotic kingdom, often in a symbiotic manner. In mammals, commensal bacteria are known to facilitate many homeostatic processes in the intestine, yet the molecular signaling mechanisms that mediate these events are largely unknown. The studies described in this thesis directly address this gap in our knowledge. We show that the bacterial specific peptide *N*-formyl-Met-Leu-Phe (fMLF), a component of gut luminal bacteria, is recognized by formyl peptides receptors (FPRs) located on the apical side of the intestinal epithelial cells. Recognition of fMLF serves to rapidly and potently activate the extracellular related kinase (ERK) MAPK signaling pathway in both cultured epithelial cells and the murine colon, inducing cellular proliferation in these cells. Importantly, no fMLF-induced activation of the pro-inflammatory NF-κB pathway, or the pro-apoptotic JNK pathway was detected, thus indicating specificity for FPR mediated signaling events towards ERK pathway mediated cellular responses. Furthermore, binding of fMLF by FPR induces rapid generation of reactive oxygen species (ROS) in epithelial cells. Physiological levels of ROS are known to function as signaling molecules that regulate protein activity by oxidizing hyper-reactive sensor cysteine residues within a subset of proteins. We show cellular ROS is rapidly induced by fMLF and is sufficient to oxidize a critical cysteine residue within the ERK specific MAP kinase phosphatase, DUSP3, rendering the enzyme catalytically inactive and thus unable to dephosphorylate and inhibit ERK pathway activity. Collectively, these data show that bacterial fMLF induces homeostatic signaling and responses in the intestinal epithelium by FPR dependant redox signaling.

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"Knowledge speaks, but wisdom listens.” Jimi Hendrix

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CHAPTER I: BACKGROUND

CHAPTER ONE: BACKGROUND

The mammalian intestinal lumen maintains a community of microbes numbering 10 to 100 trillion organisms representing hundreds of bacterial genera, broadly grouped into two taxa, the *Bacteroidetes* and *Firmicutes*. [1-4] The human microbiota thrives in the nutrient rich and thermostable environment provided by the host. In return, the microbiota has been shown to provide beneficial functions to the host including (but not limited to), vitamin syntheses, energy extraction from indigestible carbohydrates, stimulation of innate and adaptive immune system development, and competitive exclusion of pathogens. [3, 5-7] Additionally, studies have shown that the small intestinal crypts of axenic (germ-free) mice have a slower turnover of the epithelial cells causing the transit time from crypt to villus tip to double. [8-10] Villi of the small intestine of axenic mice are slender and uniform and the cecal mucosa is thinner and raised with short irregular villi. [11] Furthermore, epithelial regenerative responses to colonic injury are decreased in axenic animals, demonstrating a role for commensal microbes in the stimulation of wound restitution and response to injury. [12] The above examples demonstrate that the gut shares a mutually beneficial relationship with the microbiota, co-evolving over millennia. These findings have thus prompted interest in the development of therapeutic approaches where supplements of exogenous commensal bacteria are introduced to the background microbiota. This so-called probiotic therapy has been reported to ameliorate inflammation, strengthen epithelial barrier function and stimulate restitutional responses. [13-15] Additionally, abnormalities in the microbiota are generally accepted to be central to the pathogenesis of intestinal inflammation seen in inflammatory

bowel disease (IBD) [including Ulcerative colitis (UC) and Crohn's disease (CD)] and may be involved in other allergic, metabolic and infectious disorders. [16] Together, these data demonstrate compelling evidence for the essential role of commensal gut flora in maintaining intestinal homeostasis and health. However, the scientific evidence showing the positive influences of the gut microbiota is largely phenomenological, while the underlying molecular mechanism of how the host perceives commensal bacteria and how commensal bacteria mechanistically influence gut biology is unknown.

The gut possesses microbial monitoring receptors to respond to and manage threats from bacterial pathogens –and therefore, respond to and manage commensals. [17, 18] These molecules are known as "pattern recognition receptors" or PRRs and exhibit structural and functional homology across animal and plant kingdoms, indicating ancient evolutionary origin of these receptors. Transmembrane or intracytoplasmic PRRs recognize and bind to conserved "microbe associated molecular patterns" or MAMPs, which are invariant microbial macromolecular structures. Until now, investigations into bacterial (or bacterial product) recognition by PRRs have almost been entirely focused on the sensing of pathogenic bacteria that traverse the epithelial barrier and potentially infect mucosal compartments. PRRs that sense and respond to invading pathogens are either located on the basolateral surface of epithelial cells, or on the surface of macrophages that permanently reside within the intestinal mucosa. Recognition of MAMPs in the mucosa induces the activation of "alarm" pathways that stimulate the transcriptional upregulation of proinflammatory and anti-apoptotic effectors to manage bacterial numbers in intestinal mucosal tissues. Until recently, it was thought that intestinal

epithelial membrane spanning PRRs were only localized to the basolateral aspect of the epithelial cells, and not to the apical membrane of epithelial cells, thus avoiding constant and unchecked inflammatory response due to the constant sensing of the microbiota (Figure 1). However, recent investigations showed that a certain family of PRRs, namely the formyl peptide receptors (FPRs) spans the apical membrane of the intestinal epithelia and thus are continuously exposed to the microbiota. [19] Despite the constant sensing of microbial products in the lumen by FPRs, no overt intestinal inflammation is seen in the healthy gut. These observations lead us to formulate our hypothesis that sensing of the luminal microbiota by FPRs initiates signaling events that positively influence homeostasis in the intestinal epithelium.

1.1 Mammalian commensal bacteria

The first scientific notice of commensal bacteria is thought to have been produced by Antony van Leeuwenhoek when he observed dental scrapings under his microscope and detailed cells of differing shapes and sizes. However, most research has come in the 20th century due to the technical advances and thus a renewed interest in the field. One necessary technical advance that allowed the further study of commensal bacteria came from Robert E. Hungate. Through his research in the ecosystem of bovine rumen, he developed the roll tube technique to cultivate anaerobic bacteria, and by extension commensal bacteria. [20] Cultivation of commensal bacteria allowed the discovery of commensal ecological complexity by scientists such as Ed Moore, Lillian Holdeman, Sydney Finegold, and colleagues. Cultivation of anaerobic bacteria also led to the ability to associate commensal bacteria with their respective body site, pioneered by Ron

Gibbons in oral microbiota and Dwayne Savage in gut microbiota. [21] However, there is still only a small percentage of the microbiota that is cultivatable by standard microbiological techniques. Molecular phylogenic approaches based on sequencing of bacterial 16S ribosomal RNA and bulk genomic sequencing of entire communities or environments, allow for census-based, non–culture-dependent inventories of the entire microbiota population composition and component genes. [1, 22]

With the ability to associate microbiota both quantitatively and qualitatively with body site, the multitude and complexity of bacteria has now become apparent. The human body is comprised of approximately 10^{13} cells per individual compared to the approximate 10^{14} bacteria that an individual harbors at any one time. On the human body, commensal bacteria can be found on the skin, in the oral cavity, in the upper respiratory tract, in the urogenital tract, and the lower gastrointestinal tract. The skin has various microenvironments, and therefore the dermal commensal bacteria vary according to the character of the microenvironment, though usually consists largely of *S. epidermis*, *S. aureus*, *Micrococci*, *Diptheroids, Streptococci*, and to a lesser extent gram negative bacilli. The oral cavity and upper respiratory tract are colonized by many of the same bacteria such as *Streptococci, Lactobacilli, Spirochetes,* and *Bacteriodes*. In infants up to one month, Lactobacilli species predominate in the urogenital tract due to the low pH, and then pH levels increase and diptheroids, *S. epidermis, Streptococci*, and *E. coli* appear. Vaginal pH lowers during puberty and *Lactobacilli, Corynebacteria, Staphylococci, Streptococci,* and *Bacteriodes* predominate. After menopause, the pH level lowers again and flora returns to that of prepubescent species. [21, 23, 24] Finally, the

lower gastrointestinal tract has the largest population of bacteria consisting of up to 100 trillion organisms representative of over 500 genera from two taxa, *Bacteriodetes* and *Firmicutes*. Additionally, eukaryotic fungal species have also recently been identified as components of the microbiota. [25]

1.2 The gastrointestinal tract

The human gastrointestinal tract contains the highest concentration of bacteria and thus the dominant location for commensal-epithelial interactions to occur. In humans, microbial colonization of the intestine occurs just after birth. Development of the normal flora is a gradual process, that is initially determined by composition of the maternal gut microbiota, environment and also possibly by genetics. [26] When the diet changes from milk to solid foods, the bacterial flora change to due to the nutrients consumed and the bacteria it contains. The population composition is considerably stable at different locations along the gastrointestinal tract, but numbers vary greatly, ranging from $10^{2–3}$ in the jejunum and proximal ileum, $10^{7–8}$ in the distal ileum, and 10^{11} cells/g content in the ascending colon (Figure 2).

Stomach

The human gastrointestinal tract consists of the upper and lower tract. The upper gastrointestinal tract is comprised of the esophagus and stomach. Masticated food enters the stomach via the esophagus. The esophagus functions to move food and fluids from the pharynx to the stomach where gastric parietal cells secrete hydrochloric acid (HCl) and chief cells secrete pepsinogen, a weak protease that is activated in the low pH level

created by the presence of HCl. Pepsinogen then undergoes autocatalysis to form the highly active protease pepsin. The low pH helps denature food, making it easier for pepsin to hydrolyze food proteins into shorter chains of polypeptides, while traversing the stomach until it reaches the duodenum as a mixture of partially digested food, called chyme. Chyme leaves the stomach through the pyloric sphincter to enter the small intestine. [27, 28] Due to the acidity of its contents and the presence of proteolytic enzymes, the stomach is a preventative barrier for access of bacteria to the distal parts of the gastrointestinal tract. However, small numbers of bacteria can persist in these conditions, attached to the gastric epithelia or in the mucus, such as *H. pylori.* Though *H.pylori* is implicated as the causal agent of gastritis, gastric and duodenal ulcers, and gastric cancer, it is also present in 30-80% of healthy individuals. [24]

Small intestine/Small bowel

The lower gastrointestinal tract is comprised of the small intestine and the large intestine. The small intestine is where terminal digestion of carbohydrates, lipids, and proteins occurs, and where the products of digestion are absorbed into the blood. The small intestine has three contiguous parts, the duodenum, jejunum, and ileum. In the small intestine, the chyme mixes with mucosal digestive enzymes, pancreatic bicarbonate and hepatic bile to digest ingested macromolecules into smaller fragments.

Hallmark features of the small intestine are tiny finger-like mucosal projections called villi, on which the apical surfaces of each enterocyte are covered by microvilli. Microvilli are folding of the plasma membrane that forms cytoplasmic extensions on a cell. Villi

and microvilli function to increase the surface area of the small intestine so products of digestion can be more efficiently absorbed. Microvilli also aid in digestion by exposing active sites of enzymes embedded in the plasma membrane to the chyme. The small intestine digests chyme into absorbable molecular components, such as glucose, amino acids, and lipids. Glucose and amino acids are absorbed and enter the blood to go into the liver, and lipids are trafficked to the lymphatic system. Indigestible residual food material then enters the large intestine as waste products and/or fermentation substrates. [27, 28] For a bacterium to survive in the small intestine it needs to be bile resistant, then it can move on to a less volatile and thus colonizable environment of the large intestine.

Mucosal/Gut Immunity

The anatomical and functional components of the mucosal immune system are found mainly in the small intestine and are strongly influenced by the microbiota. [29-31] Mucosal defense can be divided into non-immunologic (intrinsic) and immunologic defense. Intrinsic defenses include gastric acidity, proteolytic enzyme activity, intestinal tight junction proteins (epithelial cell barrier), and mucin production. Mucus produced by goblet cells forms a viscoelastic layer on the epithelial layer, and increases in response to bacterial infection. [32] Immunologic defenses include both innate and adaptive immune systems. Intestinal cells are utilized for innate immune defenses and include Paneth cells located at the base of the crypts of the small intestine. Paneth cells secrete large quantities of antimicrobial proteins to protect the stem cells from bacterial contact. Another immune cell type involved in bacterial defense is the polymorphonuclear leukocyte (PMN). PMNs are short lived cells that are not normally found in the gut mucosa, though are recruited

by inflammatory signals to damaged tissues. PMNs phagocytose microbes that translocate into the mucosa and subsequently kill internalized pathogens. Other immune cells, which represent the adaptive immune system, reside in the intestinal subepithelia mucosa include macrophages and dendritic cells that, together with stroma cells, form the gut-associated lymphoid tissue (GALT) (Figure 2). [33] The GALT is the largest immune organ in the body, and is organized into distinct structures know as Peyer's patches. Peyer's patches are in effect mucosal lymph nodes overlaid with a specific epithelial cell type, the M cell, which takes up particulate antigens from the intestinal lumen. [34]

Large intestine/Colon

The small intestine empties into the large intestine or colon at the junction where the vestigial structures of the cecum and appendix remain. In herbivorous mammals, the cecum contains a large number of resident bacteria to aid in the enzymatic breakdown of plant cellulose into digestible components. The appendix is thought to be the degenerate terminal portion of the cecum that no longer maintains a primary function of cellulose breakdown due to a shift in diet from mainly plant sustenance to a more protein diet. The colonic surface or mucosa has no villi and is flat punctuated by tubular crypts that extend down to the underlying muscularis mucosa. [27, 28] The function of the colon is to absorb luminal vitamins, water, and electrolytes contained in the digested food and produced by the body to aid in digestion, compacting indigestible food stuffs into feces that pass into the rectum and then exit through the anus. Additionally, fermentation of complex carbohydrates occurs in the large intestine. However, complex carbohydrates are poorly digested by the human digestive system and require the microbiota for breakdown. The

end products of fermentation are a range of organic acids, including short chain fatty acids (SCFAs), such as butyrate, succinate, and propionate, and other terminal products. [35, 36] SCFAs are an important energy source for the colonic epithelium and provide the host with approximately 5%–15% of human energy requirements. [37]

The crypts of the colon and small bowel contain stem cells, enterocytes, goblet cells, enteroendocrine cells, and Paneth cells (limited to the small bowel). Stem cells reside at the base of the crypt and produce daughter cells mature during their migration along the crypt-villus axis in the transit amplifying compartment as the cells move up the sides of the crypt. Daughter cells differentiate into paneth cells (antimicrobial peptide producing cells), enterocytes (intestinal epithelial cells), goblet cells (mucus producing cells), and enteroendocrine cells (hormone secreting cells), and are eventually shed off into the lumen in an apoptotic process called anoïkis. The differentiation process takes appoximately 3-5 days to complete (Figure 3). [38, 39, 40] The surface of the epithelium of the colon is composed of columnar absorptive enterocytes, with smaller and less abundant microvilli to aid in absorption, and secretory goblet cells, which maintain a protective mucosal surface between intestinal cells and food and bacterial particles. [27, 28] Bacteria do not colonize the sterile environment of the crypts due to lack of access and the abundance of antimicrobial peptides. However, if bacteria have evolved to survive the transit from entering the mouth and surviving the acidic environment of the stomach and the bilic environment of the small intestine, they will colonize and produce biofilms in and on the mucus separating epithelial cells from digested food particles.

1.3. Gut Homeostasis

The microbiota that occupy the intestine are separated from the host systemic compartment by a single layer of epithelial cells, which respond to and manage commensals. [18] The microbiota thrives in a thermostable, nutrient rich environment and provides beneficial functions to the host including energy extraction of indigestible complex carbohydrates, vitamin and micronutrient syntheses, stimulation of immune development/function, and competitive exclusion of pathogens. [5, 6] Increasing evidence suggests that some commensal bacteria enhance intestinal epithelial homeostasis and barrier integrity. Studies with germ-free mice have revealed that the small intestinal crypts exhibit a slower turnover of the epithelial cells, with crypt to villus transit time double in germ-free animals as compared to conventionalized mice. [8] Additionally, regenerative responses to colonic injury are markedly attenuated in germ-free animals, [12] indicating a role of the microbiota in induction of epithelial proliferation and response to injury.

Recognition of this mutually beneficial relationship has prompted increasing interest in potential therapeutic benefits of supplementing the normal flora with exogenous viable bacteria. This approach, termed probiotics, has been reported to modulate inflammation, augment barrier function and stimulate reparative responses *in vitro*, and has shown promise as therapy in inflammatory and developmental disorders of the intestinal tract. [13] Probiotic bacteria can also stimulate several intestinal epithelial cell protective responses, including enhancement of epithelial barrier function, mucin synthesis and secretion, inhibition of enteropathic *E. coli* binding, and attenuation of apoptosis. Probiotics,

combined with formula are often administered in preterm babies in attempts to properly colonize the gastrointestinal tract. Probiotic supplements are also necessary in the absence of normal microbiota exposure from the mother due to c-section delivery and/or the inability to nurse properly since many times preterm babies do not have the strength to latch on to the nipple for feeding. Due to the beneficial effects described above, in adults, probiotics are thought to aid in digestion, improve immune function, prevent pathogen growth, and improve symptoms of IBDs. [13]

A commonly used probiotic in researching gut-microbial interactions is *Lactobacillus rhamnosus* GG (LGG). LGG (ATCC 53103) was isolated from the human intestinal tract in 1983 of a healthy patient by Sherwood Gorbach and Barry Goldin. LGG is a Gram-positive, facultative anaerobic bacillus that is both acid and bile resistant, allowing it to survive the stomach and the small intestine to traverse through or colonize the colon. LGG has been shown to affect many aspects of gut homeostatis, such as aiding in lactose metabolism, influencing intestinal microbiota due to its ability to adhere to the intestinal epithelia and competitively exclude other bacteria, secretion antimicrobial substances, and increasing intestinal barrier function. [41] Additionally, LGG is one of the best-studied strains used clinical trials for IBD and is clinically indicated for the treatment of UC and pouchitis. Consumed by thousands of people across the world, LGG is one of the most widely used and proficient probiotics, thus an excellent candidate to be used in studying commensal-epithelial interactions.

1.4 Bacterial signaling to the gut

Bacterial recognition by the gastrointestinal tract occurs mainly through pattern recognition receptors (PRRs) sensing microbial associated molecular patterns (MAMPs) secreted or attached to bacteria. Some of these PRRs include but are not limited to toll-like receptors (TLRs), NOD-like receptors (NLRs), and formyl peptide receptors (FPRs). Each receptor senses a specific class of molecule associated with a bacterium (yeast, fungi, etc), be it on the membrane surface as with TLRs and FPRs, or in the cytoplasm as with NLRs.

Toll-like Receptors

TLRs are a family of innate immune receptors that act as the first line of defense against microbial infection. The TLRs family in mammals is comprised of 12 members. TLRs are type I integral membrane glycoproteins characterized by an extracellular domain containing varying numbers of leucine-rich-repeats (LRRs) and a cytoplasmic signaling domain called Toll/IL-1R homology (TIR) domain. [42] TLRs can be further divided by localization, either extra- (TLR1, 2, 4, 5, and 6) or intracellulary (TLR3, 7, 8, and 9) localized. Many can be grouped together by which class of MAMPs is recognized. TLRs 1, 2, and 6 all recognize lipids, whereas TLRs 7, 8, and 9 recognize nucleic acids. TLR4 recognizes several structurally unrelated ligands, such as LPS, fibronectin, and heat-shock proteins. Other ligands, such as flagellin, are only recognized by one TLR, TLR5. Together, these receptors stimulate an innate immune response to bacterial, viral, or fungal infections either by invasive pathogens or exposure to normal flora due to injury.

NOD-like Receptors

Another family of mammalian PRRs is the NOD-like receptors (NLRs). Where TLRs recognize extracellular pathogens or phagocytosed organisms on the cell surface or in endosomes, NLRs recognize intracellular pathogens that have made their way into the cytoplasm. NLRs have three characteristic domains, an LRR domain involved in ligand recognition, a central NOD domain responsible for ATP independent dimerization, and the N-terminal domain comprised of protein-protein interaction cassettes responsible for the regulation of pro-apoptotic and pro-inflammatory signals. [43] The NLRs can be divided into two separate groups, NOD proteins (NOD 1 and 2) and inflammasome proteins, such as NALPs. NOD1 and NOD2 are broadly recognized by peptidoglycan-derived peptides, iE-DAP and MDP, respectively. [44] Inflammasome proteins recognize toxins and other proteins that have been gained cytoplasmic access to the cell via bacterial secretion or invasion mechanisms. [45] Internal signaling via NLRs ultimately leads to apoptosis or inflammation, indirectly through MAPK and NF-κB activation, as with NOD proteins, or through direct interaction with procaspases, as with inflammasome proteins.

There are other PRRs that detect a variety of other ligands, e.g. mannose receptors, RIG-I receptors, etc. but most of these receptors are either found on inflammatory cells or are responsible for mounting an antiviral and antifungal response. [46] And as discussed above, the TLRs and NLRs are mainly thought to respond to invading or pathogenic bacteria, mounting a pro-apoptotic or pro-inflammatory response in intestinal epithelia. Thus,

another receptor must be responsible for commensal-intestinal epithelial interactions, which formyl peptide receptors (FPRs) are a plausible candidate as will be discussed.

Formyl Peptide Receptors

Formyl peptide receptors (FPRs) are seven transmembrane receptors first described in neutrophils and macrophages. [47-49] The best characterized FPR ligands are prokaryotic translation products modified with N-formyl-methionine, known as formyl peptides, though FPRs are known to be promiscuous in ligand recognition. FPRs are represented in humans by the originally characterized FPR1, and the closely related FPR2/ALX and FPR3. FPR1 is the high affinity receptor for its best characterized ligand, N-formyl-Met-Leu-Phe (fMLF) (Figure 2), FPR2/ALX is the low affinity receptor, and FPR3, though similar in sequence to the other FPRs, has no affinity for the fMLF ligand. [49] Upon ligand binding in phagocytes, FPRs undergo a conformational change that allows recruitment of (pertussis toxin (PTx) sensitive) G proteins of the G_i family. This signaling causes 1) changes in actin dynamics and initiation of chemotaxis, 2) transcriptional upregulation of inflammatory effectors and cytokines, and 3) the activation of NADP(H) oxidase enzymes and ROS generation (respiratory burst). Thus, the FPRs are key PRRs that control the biological response of professional phagocytes to bacterial ligands.

FPRs can been found in other cell types, such as in neurons, follicular cells, hepatocytes, and epithelial cells. [50-54] Of these cell types, epithelial cells are most likely to encounter and respond to commensal bacteria. Recently, FPR was found to be localized on intestinal epithelial cells along the crypt-lumen axis in human mucosal biopsies, [19]

prompting interest that FPRs and related epithelial receptors may mediate physiological responses in the gut. It has been reported, in the presence of fMLF, FPR signals for activation of the phosphtidylinositol 3-kinase through Rac1 and Cdc42 and subsequent Ca+ influx, resulting in cell restitution and wound healing. [55]

MAPK signaling

Epithelial cellular signaling induced by most PRRs involves the highly conserved MAPK and related NF-κB systems. MAP kinase pathways (p38, JNK, ERK) are signaling relays that utilize three kinase phosphorylation cascades (MAPKKK to MAPKK to MAPK) to transduce a myriad of extracellular stimuli into intracellular signaling events, including initiating gene transcription, stimulating cytoskeletal alterations and other functions (Figure 4). [56] Enteric pathogenic bacteria, such as *Salmonella,* activate MAPK and NF-κB pathway signaling in polarized cultured epithelial cell monolayers. [57] However, in a normal healthy mammalian gut, the enteric commensal bacteria are in intimate contact with the epithelia, yet they do not activate epithelial cell pro-inflammatory or pro-apoptotic signaling pathways to levels that cause inflammation or tissue damage. Additionally, expression profiling studies of cultured cells and mice treated with commensal bacteria showed significant induction of a range of epithelial related genes, [58] thus indicating that whole commensal bacteria do activate some form of signaling transduction, thus MAPK ERK becomes a likely candidate for commensal induced signaling.

The extracellular related kinase (ERK) signaling relay is activated by a plethora of extracellular stimuli through binding to receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). Binding of these receptors results in the stimulation of small GTPases (Ras, Rac, and Cdc42), which in turn upregulates the ERK phosphorylation cascade of Raf (MAPKKK) to MEK1/2 (MAPKK) to ERK1/2 (MAPK). ERK 1 (44kDa) and ERK 2 (42kDa) are homologous isoforms that share the same substrate specificities *in vitro* which share 85% amino acid identity. Once activated, ERK proteins phosphorylate serine and threonine residues of both cytosolic proteins, such as paxillin, and nuclear target proteins (usually transcription factors), such as Elk-1 [59, 60]. ERK can either be sequestered in the cytosol where it can influence cytoskeletal dynamics and cell migration, or ERK can translocate across the nuclear membrane where it influences cell proliferation and survival dependent upon the type of ligand bound and the cell type.

ROS Signaling

Although the rapid generation of reactive oxygen species (ROS) is a cardinal feature of the cellular response of phagocytes to pathogenic and commensal bacteria, evidence is accumulating that ROS are also physiologically elicited in other cell types, including intestinal epithelia, in response to microbial signals. *Drosophila* requires commensal microbe-induced hydrogen peroxide (H_2O_2) to maintain gut epithelial homeostasis [12, 61, 62] and plants also utilize induced ROS in modulating signal transduction pathways in response to bacterial pathogens and symbionts. [63-65] In mammalian cells, physiologic ROS signaling regulates many necessary, homeostatic processes. ROS signaling has been implicated in regulating developmental processes in the fetus and

premature newborn, and requires tightly regulated changes in cellular localization and concentration. [66, 67] ROS have been shown to serve as critical second messengers in multiple signal transduction pathways in response to proinflammatory cytokines and growth factors. Proteins modulated by ROS include tyrosine phosphatases, antioxidants, members of the Ubc family, and DUSPs/MKPs. We have previously demonstrated that viable commensal bacteria cause transient oxidative inactivation of Ubc12 with resultant effects on neddylation of Cul-1.[14, 68] Specifically, in model intestinal epithelia, ROS has been shown to reduce inflammatory signaling through oxidative inactivation of Ubc12, a key enzyme regulating NF-κB activation. Ubc12 is responsible for activation of the specific ubiquitin ligase complex SCF-beta^{TRCP} through neddylation of its cullin-1 (Cul1) subunit. When Cul1 remains deneddylated, SCF-beta^{TRCP} fails to ubiquitinate the inhibitor of NF-κB (IκB-α), a modification that normally targets IκB-α for proteasomal degradation. NF-κB thus remains trapped in the cytosol by IκB-α, unable to translocate to the nucleus to activate transcription of inflammatory mediators. Additionally, ROS has been shown to affect cell migration and wound healing through oxidative inactivation of the tyrosine phosphatases LWM-PTP and SHP-2, enzymes that regulate focal adhesion activity. LWM-PTP and SHP-2 regularly act to dephosphorylate focal adhesion kinase (FAK). However, when LWM-PTP and SHP-2 are inactivated, there is sustained activation of FAK, and subsequent formation of focal adhesions and increase cell movement (Amrita Kumar, Ph.D., personal communication).

Thus, there is an evolutionarily conserved role of mild non-radical oxidants as signaling molecules which regulate cellular events, including stem cell maturation, cell division

and migration. [69-71] These functions require that oxidative events are physiologically compartmentalized and precisely regulated. Rarely, low pK_a of the active pocket surrounding a catalytic cysteine occurs due to the relative pK_a of surrounding amino acids and protein microenvironment, thus allowing the formation of hyper-reactive sensor cysteine residues. Cysteine is the most intrinsically nucleophilic amino acid in proteins. Its low oxidation–reduction (redox) potential in proteins means that the thiol side chain is highly sensitive to redox transformations and can exist in a number of reversible oxidation states. Thiols and disulfides are most well-studied, although sulfenic (RSOH), sulfinic (RSO_2H), and sulfonic (RSO_3H) acids have also been identified. [72] Importantly, redox sensitive hyper-reactive cysteine residues in proteins have diverse biochemical actions, and have significant roles in biological function. [73] Until recently, the unidentified consensus sequence defining functional cysteine residues in proteins hindered discovery of these enzymes. However, several current studies have used proteomics to profile the intrinsic reactivity of cysteine residues in native biological systems. [74, 75] It was found that hyper-reactive cysteine residues are rare, and undergo a variety of chemical modifications, such as oxidation. Importantly, hyper-reactive cysteine residues were identified in a number of proteins of unknown function, illustrating that cysteine oxoforms are utilized in cells to widen protein functional diversity.

MAPK Phosphatases

As mentioned, ROS can regulate cellular processes through transient oxidative inactivation of catalytic cysteine residues on key regulatory enzymes, influencing apoptosis, proliferation, and inflammatory signaling. A key group of such proteins are

dual specific phosphatase enzymes (DUSPs) which regulate MAPKs, known to influence these signaling aspects. DUSPs are a subset of protein tyrosine phosphatases that dephosphorylate threonine and tyrosine residues in the consensus motif Thr-X-Tyr (where X is Glu, Gly, or Pro) on MAPKs ERK, JNK, and p38, thus also referred to as MAPK phosphatases (MKPs). Once MAPKs are dephosphorylated, the signaling cascade becomes inactive. When the DUSP catalytic cysteine residues is oxidatively inactivated through the ROS product H_2O_2, the phosphatase activity of DUSPs is supressed leading to sustained activation of MAPKs. There are sixteen mammalian DUSPs that show dephosphorylation activity toward MAPK, of which ten target the ERK phosphorylation, indicative of a great deal of redundancy within the group. [76] The ERK specific targeting DUSP3 is a 185-amino acid (21kDa) long protein, cloned based on its homology with the *Vaccinia* virus H1 open reading frame, a key gene in vaccinia virus and poxvirus. [77, 78] DUSP3 is regulated during the cell cycle. [76, 79, 80] In particular, cells lacking DUSP3 arrest at G1/S and G2/M, and demonstrate morphological signs of senescence. The cell cycle arrest seen in cells lacking DUSP3 is dependent upon the activation of the DUSP3 substrates ERK and JNK. [76, 80]

Figure Legends

Figure 1: Intestinal cells: apical and basolateral aspects.

Figure 2: Diagram of bacterial load in the gut. Adapted from Neish, 2009

Figure 3: Structure of colonic crypts. Adapted from Barker, van der Wetering, and Clevers, 2008

Figure 4: Structure of N-formyl-Met-Leu-Phe. Adapted from Ye et al. 2009

Figure 5: FPR signaling.

Figure 6: MAPK signaling.

FIGURES

Figure 1

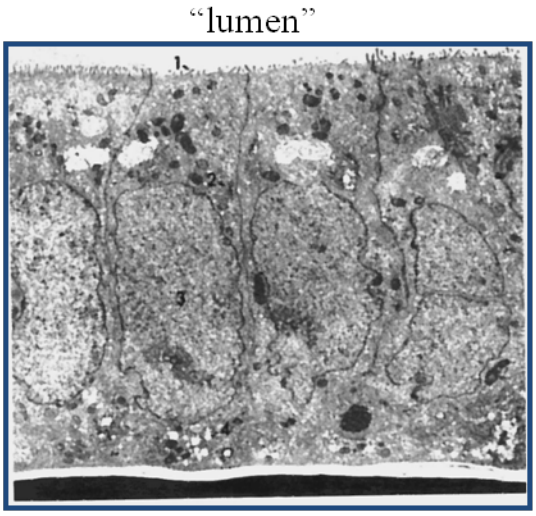


Figure 2

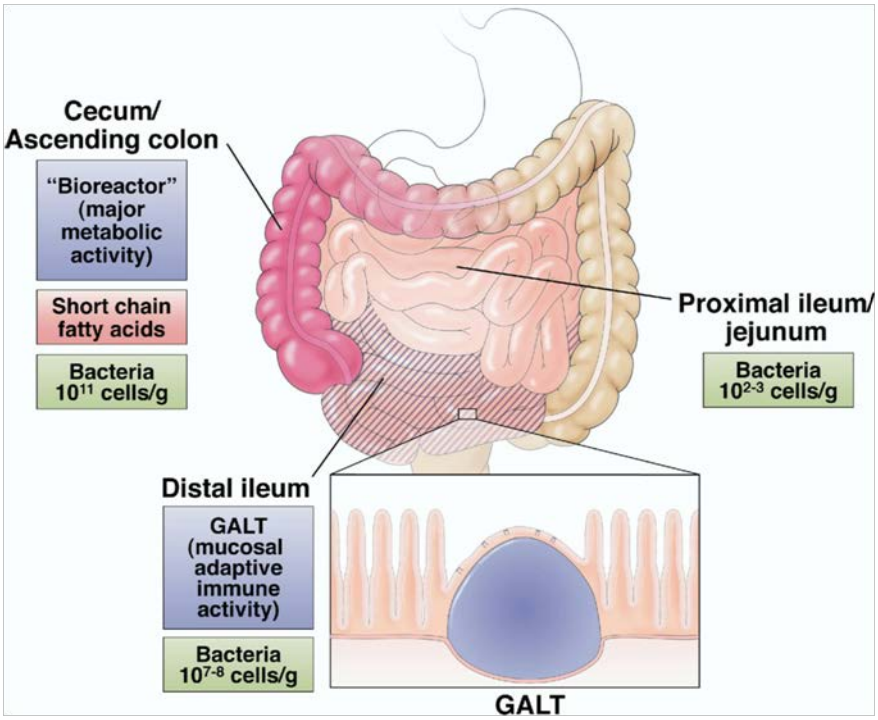

Cecum/
Ascending colon
"Bioreactor" (major metabolic activity)
Short chain fatty acids
Bacteria 10^{11} cells/g
Proximal ileum/ jejunum
Bacteria 10^{2-3} cells/g
Distal ileum
GALT (mucosal adaptive immune activity)
Bacteria 10^{7-8} cells/g
GALT

Figure 3

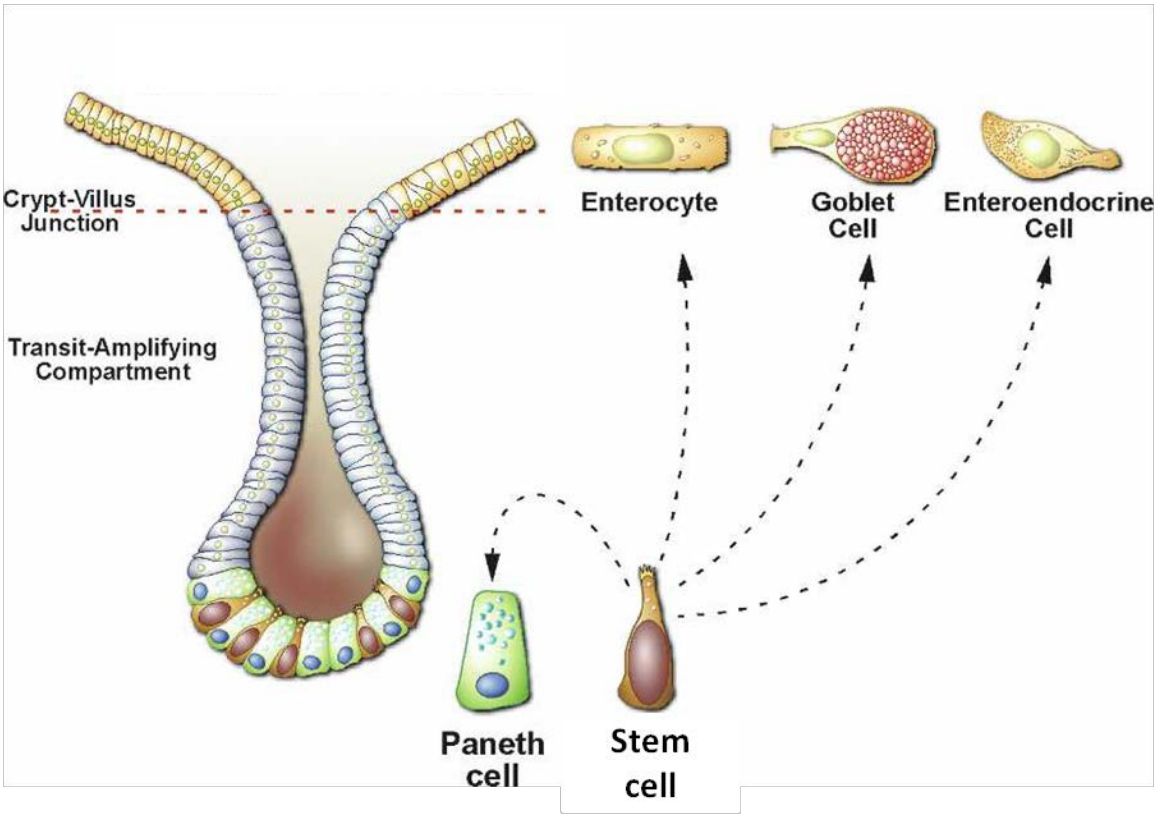

Crypt-Villus Junction
Transit-Amplifying Compartment
Enterocyte
Goblet Cell
Enteroendocrine Cell
Paneth cell
Stem cell

Figure 4

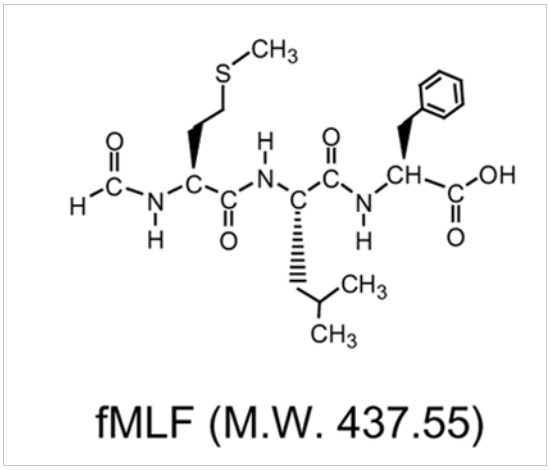


Figure 5

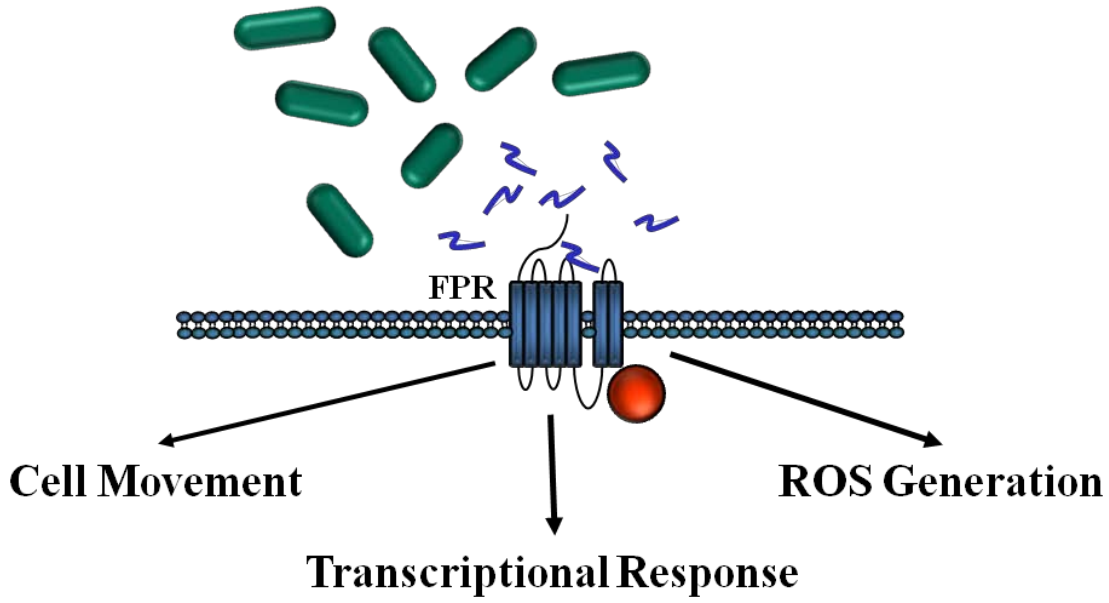
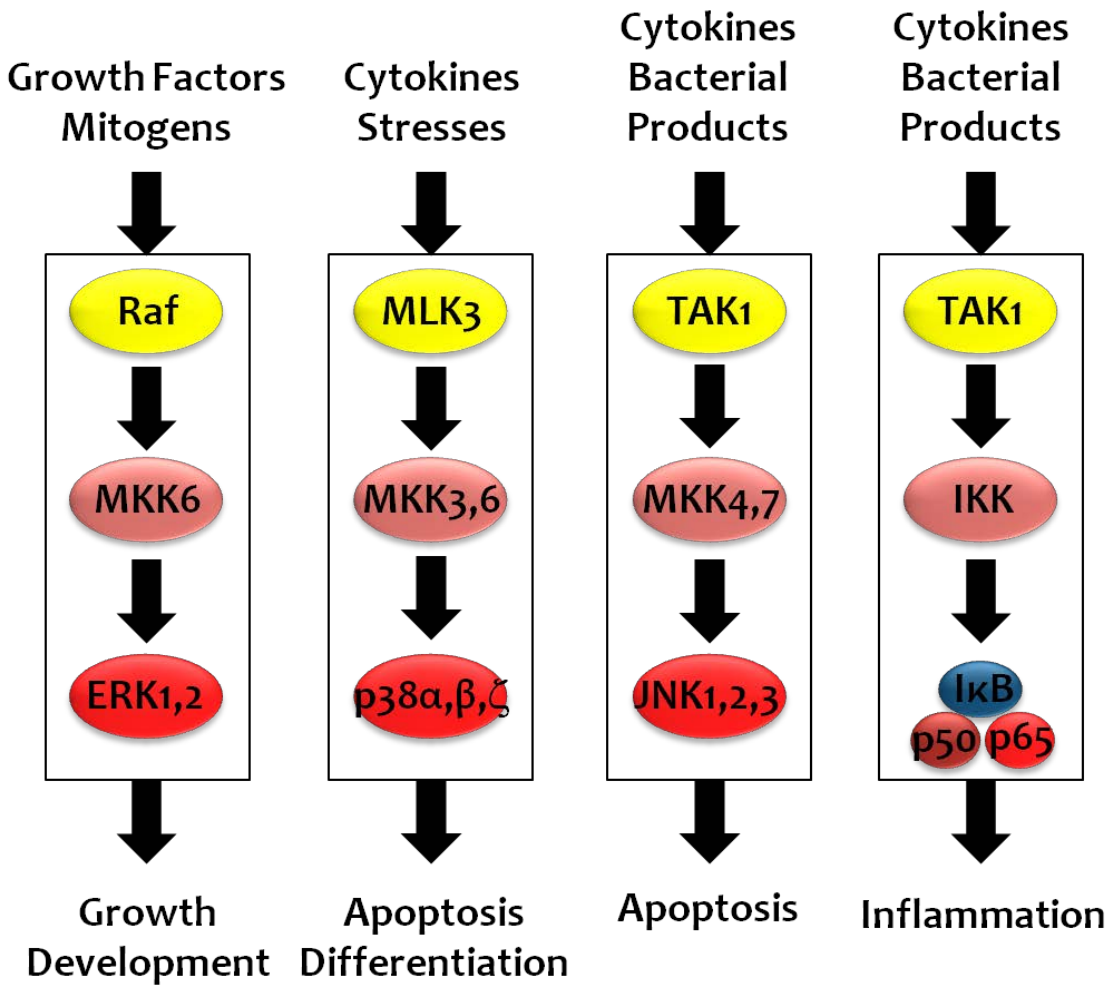

FPR
Cell Movement
ROS Generation
Transcriptional Response

Figure 6


Growth Factors
Mitogens
Cytokines
Stresses
Cytokines
Bacterial
Products
Cytokines
Bacterial
Products
Raf
MKK6
ERK1,2
MLK3
MKK3,6
p38α,β,ζ
TAK1
MKK4,7
JNK1,2,3
TAK1
IKK
IκB
p50
p65
Growth
Development
Apoptosis
Differentiation
Apoptosis
Inflammation

References

1. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE: Metagenomic analysis of the human distal gut microbiome. Science. 2006, 312:1355-1359

2. Xu J, Mahowald MA, Ley RE, Lozupone CA, Hamady M, Martens EC, Henrissat B, Coutinho PM, Minx P, Latreille P, Cordum H, Van Brunt A, Kim K, Fulton RS, Fulton LA, Clifton SW, Wilson RK, Knight RD, Gordon JI: Evolution of symbiotic bacteria in the distal human intestine. PLoS Biol. 2007, 5:e156

3. Tannock GW: New perceptions of the gut microbiota: implications for future research. Gastroenterol Clin North Am. 2005, 34:361-382, vii

4. Lee YK, Salminen S: Handbook of Probiotics and Prebiotics. Wiley & Sons, Inc.,. 2009,

5. Hooper LV, Bry L, Falk PG, Gordon JI: Host-microbial symbiosis in the mammalian intestine: exploring an internal ecosystem. Bioessays. 1998, 20:336-343

6. Neish AS: Microbes in gastrointestinal health and disease. Gastroenterology. 2009, 136:65-80

7. Gibson GR, Roberfroid MB: Handbook of Prebiotics. CrC Press, Boca Raton, Fla. 2008,

8. Hooper LV, Gordon JI: Commensal host-bacterial relationships in the gut. Science. 2001, 292:1115-1118

9. Khoury KA, Floch MH, Hersh T: Small intestinal mucosal cell proliferation and bacterial flora in the conventionalization of the germfree mouse. The Journal of experimental medicine. 1969, 130:659-670

10. Smith K, McCoy KD, Macpherson AJ: Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Seminars in immunology. 2007, 19:59-69

11. Jervis HR, Biggers DC: Mucosal Enzymes in the Cecum of Conventional and Germfree Mice. The Anatomical record. 1964, 148:591-597

12. Pull SL, Doherty JM, Mills JC, Gordon JI, Stappenbeck TS: Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. Proc Natl Acad Sci U S A. 2005, 102:99-104

13. Park J, Floch MH: Prebiotics, probiotics, and dietary fiber in gastrointestinal disease. Gastroenterol Clin North Am. 2007, 36:47-63, v

14. Kumar A, Wu H, Collier-Hyams LS, Hansen JM, Li T, Yamoah K, Pan ZQ, Jones DP, Neish AS: Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. EMBO J. 2007, 26:4457-4466

15. Lin PW, Myers LE, Ray L, Song SC, Nasr TR, Berardinelli AJ, Kundu K, Murthy N, Hansen JM, Neish AS: Lactobacillus rhamnosus blocks inflammatory signaling in vivo via reactive oxygen species generation. Free Radic Biol Med. 2009, 47:1205-1211

16. Sartor RB: Microbial influences in inflammatory bowel diseases. Gastroenterology. 2008, 134:577-594

17. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S: Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe. 2008, 3:352-363

18. Medzhitov R: Recognition of microorganisms and activation of the immune response. Nature. 2007, 449:819-826

19. Babbin BA, Jesaitis AJ, Ivanov AI, Kelly D, Laukoetter M, Nava P, Parkos CA, Nusrat A: Formyl peptide receptor-1 activation enhances intestinal epithelial cell restitution through phosphatidylinositol 3-kinase-dependent activation of Rac1 and Cdc42. J Immunol. 2007, 179:8112-8121

20. Chung KT, Bryant MP: Robert E. Hungate: pioneer of anaerobic microbial ecology. Anaerobe. 1997, 3:213-217

21. Tannock GW. *Normal Microflora: An introduction to microbes inhabiting the body.* First ed: Chapman and Hall; 2005.

22. Backhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI: Host-bacterial mutualism in the human intestine. Science. 2005, 307:1915-1920

23. (Editor) Baron S. *Medical Microbiology*. Fourth ed1996.

24. Tlaskalova-Hogenova H, Stepankova R, Hudcovic T, Tuckova L, Cukrowska B, Lodinova-Zadnikova R, Kozakova H, Rossmann P, Bartova J, Sokol D, Funda DP, Borovska D, Rehakova Z, Sinkora J, Hofman J, Drastich P, Kokesova A: Commensal bacteria (normal microflora), mucosal immunity and chronic inflammatory and autoimmune diseases. Immunol Lett. 2004, 93:97-108

25. Scupham AJ, Presley LL, Wei B, Bent E, Griffith N, McPherson M, Zhu F, Oluwadara O, Rao N, Braun J, Borneman J: Abundant and diverse fungal microbiota in the murine intestine. Appl Environ Microbiol. 2006, 72:793-801

26. Kirjavainen PV, Gibson GR: Healthy gut microflora and allergy: factors influencing development of the microbiota. Ann Med. 1999, 31:288-292

27. Raven PH, Johnson GB. *Biology*. Fifth ed: The McGraw-Hill Companies, Inc.; 1999.

28. Kumar V, Abbas AK, Fausto N. *Pathological Basis of Disease*. Seventh ed: Elserview Inc.; 2005.

29. Macdonald TT, Monteleone G: Immunity, inflammation, and allergy in the gut. Science. 2005, 307:1920-1925

30. Neutra MR, Mantis NJ, Kraehenbuhl JP: Collaboration of epithelial cells with organized mucosal lymphoid tissues. Nat Immunol. 2001, 2:1004-1009

31. Kelly D, King T, Aminov R: Importance of microbial colonization of the gut in early life to the development of immunity. Mutat Res. 2007, 622:58-69

32. Epple HJ, Kreusel KM, Hanski C, Schulzke JD, Riecken EO, Fromm M: Differential stimulation of intestinal mucin secretion by cholera toxin and carbachol. Pflugers Arch. 1997, 433:638-647

33. Niess JH, Brand S, Gu X, Landsman L, Jung S, McCormick BA, Vyas JM, Boes M, Ploegh HL, Fox JG, Littman DR, Reinecker HC: CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. Science (New York, N.Y. 2005, 307:254-258

34. Jang MH, Kweon MN, Iwatani K, Yamamoto M, Terahara K, Sasakawa C, Suzuki T, Nochi T, Yokota Y, Rennert PD, Hiroi T, Tamagawa H, Iijima H, Kunisawa J, Yuki Y, Kiyono H: Intestinal villous M cells: an antigen entry site in the mucosal epithelium. Proc Natl Acad Sci U S A. 2004, 101:6110-6115

35. Hooper LV, Midtvedt T, Gordon JI: How host-microbial interactions shape the nutrient environment of the mammalian intestine. Annu Rev Nutr. 2002, 22:283-307

36. Topping DL, Clifton PM: Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol Rev. 2001, 81:1031-1064

37. Bergman EN: Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiol Rev. 1990, 70:567-590

38. Corfield AP, Myerscough N, Longman R, Sylvester P, Arul S, Pignatelli M: Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease. Gut. 2000, 47:589-594

39. Marshman E, Booth C, Potten CS: The intestinal epithelial stem cell. Bioessays. 2002, 24:91-98

40. Barker N, van de Wetering M, Clevers H: The intestinal stem cell. Genes Dev. 2008, 22:1856-1864

41. Silva M, Jacobus NV, Deneke C, Gorbach SL: Antimicrobial substance from a human Lactobacillus strain. Antimicrob Agents Chemother. 1987, 31:1231-1233

42. Bowie A, O'Neill LA: The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. J Leukoc Biol. 2000, 67:508-514

43. Inohara, Chamaillard, McDonald C, Nunez G: NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu Rev Biochem. 2005, 74:355-383

44. Chamaillard M, Hashimoto M, Horie Y, Masumoto J, Qiu S, Saab L, Ogura Y, Kawasaki A, Fukase K, Kusumoto S, Valvano MA, Foster SJ, Mak TW, Nunez G, Inohara N: An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. Nat Immunol. 2003, 4:702-707

45. Martinon F, Burns K, Tschopp J: The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002, 10:417-426

46. Akira S, Uematsu S, Takeuchi O: Pathogen recognition and innate immunity. Cell. 2006, 124:783-801

47. Migeotte I, Communi D, Parmentier M: Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. Cytokine Growth Factor Rev. 2006, 17:501-519

48. Rabiet MJ, Huet E, Boulay F: The N-formyl peptide receptors and the anaphylatoxin C5a receptors: an overview. Biochimie. 2007, 89:1089-1106

49. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN, Murphy PM: International Union of Basic and Clinical Pharmacology. LXXIII.

Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol Rev. 2009, 61:119-161

50. Becker EL, Forouhar FA, Grunnet ML, Boulay F, Tardif M, Bormann BJ, Sodja D, Ye RD, Woska JR, Jr., Murphy PM: Broad immunocytochemical localization of the formylpeptide receptor in human organs, tissues, and cells. Cell Tissue Res. 1998, 292:129-135

51. McCoy R, Haviland DL, Molmenti EP, Ziambaras T, Wetsel RA, Perlmutter DH: N-formylpeptide and complement C5a receptors are expressed in liver cells and mediate hepatic acute phase gene regulation. The Journal of experimental medicine. 1995, 182:207-217

52. Rescher U, Danielczyk A, Markoff A, Gerke V: Functional activation of the formyl peptide receptor by a new endogenous ligand in human lung A549 cells. J Immunol. 2002, 169:1500-1504

53. Le Y, Hu J, Gong W, Shen W, Li B, Dunlop NM, Halverson DO, Blair DG, Wang JM: Expression of functional formyl peptide receptors by human astrocytoma cell lines. J Neuroimmunol. 2000, 111:102-108

54. Riviere S, Challet L, Fluegge D, Spehr M, Rodriguez I: Formyl peptide receptor-like proteins are a novel family of vomeronasal chemosensors. Nature. 2009, 459:574-577

55. Babbin BA, Parkos CA, Mandell KJ, Winfree LM, Laur O, Ivanov AI, Nusrat A: Annexin 2 regulates intestinal epithelial cell spreading and wound closure through Rho-related signaling. Am J Pathol. 2007, 170:951-966

56. Johnson GL, Lapadat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science. 2002, 298:1911-1912

57. Chang L, Karin M: Mammalian MAP kinase signalling cascades. Nature. 2001, 410:37-40

58. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI: Molecular analysis of commensal host-microbial relationships in the intestine. Science. 2001, 291:881-884

59. Ishibe S, Joly D, Zhu X, Cantley LG: Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. Mol Cell. 2003, 12:1275-1285

60. Caunt CJ, Finch AR, Sedgley KR, McArdle CA: Seven-transmembrane receptor signalling and ERK compartmentalization. Trends Endocrinol Metab. 2006, 17:276-283

61. Ha EM, Oh CT, Ryu JH, Bae YS, Kang SW, Jang IH, Brey PT, Lee WJ: An antioxidant system required for host protection against gut infection in Drosophila. Dev Cell. 2005, 8:125-132

62. Ha EM, Oh CT, Bae YS, Lee WJ: A direct role for dual oxidase in Drosophila gut immunity. Science. 2005, 310:847-850

63. Kotchoni SO, Gachomo EW: The reactive oxygen species network pathways:an essential prerequisite for perception of pathogen attack and the acquired disease resistance in plants. J Biosci. 2006, 31:389-404

64. Pauly N, Pucciariello C, Mandon K, Innocenti G, Jamet A, Baudouin E, Herouart D, Frendo P, Puppo A: Reactive oxygen and nitrogen species and glutathione: key players in the legume-Rhizobium symbiosis. J Exp Bot. 2006, 57:1769-1776

65. Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B: Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell. 2006, 18:1052-1066

66. Covarrubias L, Hernandez-Garcia D, Schnabel D, Salas-Vidal E, Castro-Obregon S: Function of reactive oxygen species during animal development: passive or active? Dev Biol. 2008, 320:1-11

67. Das KC: Redox control of premature birth and newborn biology. Antioxid Redox Signal. 2004, 6:105-107

68. Collier-Hyams LS, Sloane V, Batten BC, Neish AS: Cutting edge: bacterial modulation of epithelial signaling via changes in neddylation of cullin-1. J Immunol. 2005, 175:4194-4198

69. Forman HJ, Maiorino M, Ursini F: Signaling functions of reactive oxygen species. Biochemistry. 2010, 49:835-842

70. Janssen-Heininger YM, Mossman BT, Heintz NH, Forman HJ, Kalyanaraman B, Finkel T, Stamler JS, Rhee SG, van der Vliet A: Redox-based regulation of signal transduction: principles, pitfalls, and promises. Free radical biology & medicine. 2008, 45:1-17

71. Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L: Reactive

oxygen species produced by NADPH oxidase regulate plant cell growth. Nature. 2003, 422:442-446

72. Schafer FQ, Buettner GR: Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free radical biology & medicine. 2001, 30:1191-1212

73. Reddie KG, Carroll KS: Expanding the functional diversity of proteins through cysteine oxidation. Curr Opin Chem Biol. 2008, 12:746-754

74. Fomenko DE, Xing W, Adair BM, Thomas DJ, Gladyshev VN: High-throughput identification of catalytic redox-active cysteine residues. Science. 2007, 315:387-389

75. Weerapana E, Wang C, Simon GM, Richter F, Khare S, Dillon MB, Bachovchin DA, Mowen K, Baker D, Cravatt BF: Quantitative reactivity profiling predicts functional cysteines in proteomes. Nature. 2010, 468:790-795

76. Cerignoli F, Rahmouni S, Ronai Z, Mustelin T: Regulation of MAP kinases by the VHR dual-specific phosphatase: implications for cell growth and differentiation. Cell Cycle. 2006, 5:2210-2215

77. Guan KL, Broyles SS, Dixon JE: A Tyr/Ser protein phosphatase encoded by vaccinia virus. Nature. 1991, 350:359-362

78. Liu K, Lemon B, Traktman P: The dual-specificity phosphatase encoded by vaccinia virus, VH1, is essential for viral transcription in vivo and in vitro. J Virol. 1995, 69:7823-7834

79. Hao L, ElShamy WM: BRCA1-IRIS activates cyclin D1 expression in breast cancer cells by downregulating the JNK phosphatase DUSP3/VHR. Int J Cancer. 2007, 121:39-46

80. Rahmouni S, Cerignoli F, Alonso A, Tsutji T, Henkens R, Zhu C, Louis-dit-Sully C, Moutschen M, Jiang W, Mustelin T: Loss of the VHR dual-specific phosphatase causes cell-cycle arrest and senescence. Nat Cell Biol. 2006, 8:524-531

CHAPTER II: Commensal-Epithelial Signaling Mediated via Formyl Peptide Receptors

Commensal-Epithelial Signaling Mediated via Formyl Peptide Receptors

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Abstract

In health, commensal bacteria and/or their products engender beneficial effects to the mammalian gut, including stimulating physiological cellular turnover and enhancing wound healing, without activating overt inflammation. In the present study, we observed commensal bacterial mediated activation of the non-inflammatory ERK MAPK and Akt signaling pathways in gut epithelial cells and have delineated a mechanism for this bacterial activated signaling. All strains of commensal bacteria tested induced ERK phosphorylation without stimulating pro-inflammatory phospho-IκB or phospho-JNK, with Lactobacilli species being most potent. This pattern of signaling activation was recapitulated using the peptide, N-formyl-Met-Leu-Phe (fMLF), a bacterial product well known to stimulate signaling events in mammalian phagocytes. Sensing of fMLF by gut epithelial cells occurs via recently characterized formyl peptide receptors (FPRs) located in the plasma membrane. Both commensal bacteria and fMLF application to the apical surface of polarized gut epithelial cells resulted in specific FPR activation. In addition, pretreatment of model epithelia and murine colon with Boc2 (a specific peptide antagonist) or pertussis toxin (a G_i-protein inhibitor) abolished commensal-mediated ERK phosphorylation. Together, these data show that commensal bacteria specifically activate the ERK MAPK pathway in an FPR-dependent manner, delineating a mechanism by which commensal bacteria contribute to cellular signaling in gut epithelia.

Introduction

Commensal microbe-host interactions in the human gut have evolved over millennia. The diverse community of microbes that inhabit the human gastrointestinal tract comprise 10 to 100 trillion organisms representing about 500 genera of bacteria, grouped into two broad taxa, the Bacteroidetes and Firmicutes [1, 2]. The microbiota thrives in a thermostable, nutrient rich environment and provides beneficial functions to the host including energy extraction of otherwise indigestible complex carbohydrates, vitamin and micronutrient syntheses, stimulation of immune development/function, and competitive exclusion of pathogens [3, 4]. Additionally, studies with germ-free mice have revealed that the small intestinal crypts exhibit a slower turnover of the epithelial cells, with crypt to villus transit time double in germ-free animals [5]. Regenerative responses to colonic injury are markedly attenuated in germ-free animals [6], indicating a role of the microbiota in induction of epithelial proliferation and response to injury.

Recognition of this mutually beneficial relationship has prompted increasing interest in potential therapeutic benefits of supplementing the normal flora with exogenous viable bacteria. This approach, termed probiotics, has been reported to modulate inflammation, augment barrier function and stimulate reparative responses *in vitro*, and has shown promise as therapy in inflammatory and developmental disorders of the intestinal tract [7]. However, in other cases, the quantitative and/or qualitative abnormalities of the microbiota may be sufficient to provoke intestinal inflammation as seen in Inflammatory bowel disease (IBD) [which includes Ulcerative colitis (UC) and Crohn's disease (CD)] and contribute to other, systemic immune, allergic, metabolic and infectious disorders [8].

Thus, there is increasing evidence that the microbiota profoundly influences intestinal - and systemic- homeostasis and health. However, little is known of how the host perceives non-pathogenic bacteria, or how microbiota mechanistically influences gut biology.

The microbiota is separated from the host systemic compartment by only a single layer of epithelial cells, which are able to respond to and manage threats from bacterial pathogens -and by extrapolation, respond to and manage commensals [9, 10]. Transmembrane and intracytoplasmic receptors, such as the now well characterized Toll-like receptors and related Nod proteins, are designated "pattern recognition receptors" or PRRs, that recognize and bind to conserved structural motifs characteristic of a wide range of microbes, which are termed MAMPs, or "microbe associated molecular patterns". A different class of candidate pattern recognition receptors are the formyl peptide receptors (FPRs), which are seven transmembrane receptors originally described on neutrophils and macrophages [11-13]. The best characterized FPR ligands are formyl peptides, prokaryotic translation products modified with N-formyl-methionine, a bacterial specific amino acid. Upon ligand binding in phagocytes, FPRs undergo a conformational change that allows recruitment of pertussis toxin (PTx) sensitive G proteins of the G_i family. This signaling eventuates in 1) changes in actin dynamics and initiation of chemotaxis, 2) transcriptional upregulation of inflammatory effectors and cytokines, and 3) the activation of NADPH enzymes and ROS generation (respiratory burst). Thus, the FPRs are key PRRs that control the biological response of professional phagocytes to bacterial ligands. FPRs are represented in humans by the originally characterized FPR1, and the

closely related FPR2/ALX. Recently, we demonstrated FPR localization on epithelial cells along the crypt-lumen axis in human mucosal biopsies [14], prompting interest that FPRs and related epithelial receptors may mediate physiological responses in the gut. Interestingly, FPR2/ALX signals in a non-phlogistic fashion [15] and may thus be a candidate for commensal mediated homeostatic signaling. Indeed, Annexin 1, a ligand of FPR2/ALX, have been shown to have restitutive effects in wounded gut [16], and the anti-microbial protein BPI is stimulated in the gut by FPR signaling [17].

Epithelial cellular signaling induced by most PRRs involves the highly conserved MAPK and related NF-κB systems [18]. MAP kinase pathways (p38, JNK, ERK) are signaling relays that utilize three kinase phosphorylation cascades (MAPKKK to MAPKK to MAPK) to transduce a myriad of extracellular stimuli into intracellular signaling events, including initiating gene transcription, stimulating cytoskeletal alterations and other functions. We and others have previously reported that enteric pathogenic bacteria, such as *Salmonella,* activate MAPK and NF-κB pathway signaling in polarized cultured epithelial cell monolayers, and also identified TLRs as the likely signaling mediators [19]. However, in a normal healthy mammalian gut, the enteric commensal bacteria are in intimate contact with the epithelia, yet they do not activate epithelial cell pro-inflammatory or pro-apoptotic signaling pathways above tonic levels. Additionally, expression profiling studies of cultured cells treated with commensal bacteria showed significant induction of a range of epithelial related genes [20]. In this study, we sought to characterize how commensal bacteria could influence epithelial signaling events. Herein, we show that a spectrum of viable commensal gut bacteria is sensed by FPRs located on

enterocyte apical membranes of the intestine in vitro and in vivo. We show that bacterial/epithelial cell contact results in potent FPR and G protein dependent activation of the ERK signaling pathway. These findings describe a novel mechanism of non-pro-inflammatory bacteria-host communication, and define a previously unknown pattern recognition receptor in the mammalian gut.

Results

Enteric bacteria induce ERK phosphorylation in polarized T84 epithelial cells

To investigate enteric bacterial stimulation of epithelial signaling pathways, we tested pure cultures ($5x10^7$ cfu/ml) of individual candidate bacteria with known commensal or pathogenic behavior. Bacteria were apically applied to polarized T84 cultured cells, incubated for up to 30 min, and lysates analyzed by immunoblot for MAPK or NF-κB signaling pathway activation. For initial analysis, we assayed ERK activation, which we and others have shown is activated by both commensal and pathogenic bacteria [18, 19]. Each strain tested, both commensal and pathogenic, equally induced ERK phosphorylation (Figure 1A). Further studies were focused on *Lactobacillus rhamnosus* GG (LGG), which is used extensively as a probiotic, and has been demonstrated to affect cellular signaling processes [27, 28]. Apical LGG treatment robustly activated phosphorylation of ERK in a dose dependent manner with signal becoming undetectable at less than $5x10^4$ cfu/ml (approximate MOI=0.001) (Figure 1B). ERK pathway activation was also mediated by apical contact with non-viable sonicated bacterial cell wall preparations (Figure 1C). In comparison to ERK phosphorylation mediated by live cultures of LGG, which show transient upregulation over 1 hr, LGG cell wall preparation induced robust and persistent ERK activation (Figure 1C). The transient ERK activation seen with viable bacteria may be the result of active repression of signaling pathways, similar to our past observations showing commensal bacterial repressing NF-κB signaling [29]. These data show that LGG stimulated ERK phosphorylation is not dependant on live bacteria, and that a soluble epithelial cell stimulating factor is present in the LGG membrane.

L. rhamnosus GG or fMLF specifically activate ERK pathway signaling

Bacterially stimulated ERK activation is mediated by products released by bacteria, or by determinants on the bacterial surface (Figure 1). A potential candidate bacterial product is formyl-Met-Leu-Phe tripeptide (fMLF), which is a classical activator of ERK signaling in neutrophils [30]. Apically administered fMLF potently and specifically activated ERK pathway signaling, but had no stimulatory effects on the pro-apoptotic JNK, or the pro-inflammatory NF-κB pathways in T84 cells (Figure 2A and 2C). Interestingly, fMLF only weakly activated ERK pathway signaling when basolaterally applied to polarized T84 cells (Supplemental Figure 1). Significantly, T84 cells apically stimulated with $5x10^7$ cfu/ml LGG, or with LGG cell wall preparation did not activate the JNK or NF-κB signaling pathways, whereas robust activation of these pathways occurred in control cells stimulated with TNF-α (Figure 2A). In addition, activation of the proliferative/survival pathway Akt was mediated by live LGG, cell wall preparations and fMLF. This data with live LGG is in striking contrast to the effects of pathogenic *S. typhimurium*, which activates NF-κB and JNK signaling, in addition to ERK, consistent with past work from our laboratory (Figure 2A) [19, 29]. Interestingly, S. *typhimurium* did not activate Akt in this system. In order to demonstrate differential signaling between myeloid and intestinal epithelial cells, murine bone marrow derived macrophages stimulated with $5x10^7$ cfu/ml LGG and 500 nM fMLF over 30 min activated only ERK and JNK signaling pathways (Figure 2B). This is consistent with the role of macrophages as systemic immunocompetent cells, which are not in constitutive contact with bacteria as is the case with intestinal epithelial cells.

In the context of eukaryotic-prokaryotic interactions, the p38 and JNK pathways are generally considered pro-inflammatory and serve to activate innate immune responses. These MAPK classes terminate in activation of the AP-1 family of leucine zipper transcription factors (often functioning in concert with NF-κB) to stimulate transcription of pro-inflammatory effector genes, while the ERK MAPK pathway is preferentially activated by growth factors and other mitogenic stimuli and largely mediates proliferative and differentiation events [31]. The ERK MAPK is comprised of two serine-threonine MAP kinases, ERK 1/2 (or p44MAPK and p42 MAPK) that share 83% amino acid homology and are expressed constitutively and ubiquitously. The ERKs are largely functionally redundant, activated by a MAPKK, MEK1/2, and their substrates are vast, with over 160 potential targets described including nuclear transcription factors, cytoplasmic cytoskeletal regulators, receptors, signaling intermediates and other kinases [32]. Similarly, the Akt is a key component of a signaling pathway with well demonstrated survival and proliferative effects [33].

To further examine the specificity and establish a functional measure of LGG or fMLF induced ERK pathway activation, we employed transfection based ERK, JNK, and NF-κB dependent reporter assays. Elevated ERK pathway dependent luciferase activity was measured in transfected SK-CO15 cells stimulated with LGG and fMLF, whereas no JNK or NF-κB activity was detected in identically stimulated cells transfected with JNK or NF-κB dependent reporter (Figure 2D). EGF or TNF-α serves as an activating control for ERK and JNK/NF-κB, respectively. Thus, these data show that fMLF directly phenocopied LGG induced ERK pathway specific activation. Finally, to determine the

extent to which LGG and fMLF induce signaling to increase cellular proliferation, we assayed for EdU incorporation. Contact of cultured epithelial cells with viable LGG and fMLF for 12 hrs resulted in significant upregulation of EdU incorporation (Figures 2E and 2F).

Inhibition of G-protein coupling or formyl peptide receptor binding attenuates L. rhamnosus GG or fMLF induced ERK pathway activation

FPRs are seven transmembrane G-protein coupled receptors (GPCRs) present on the apical membrane of enterocytes [14]. Because both fMLF and LGG simulates ERK pathway activation, we sought to determine whether LGG induced ERK pathway activation is mediated via FPRs. We inhibited FPR activity using Pertussis toxin (PTx), a 105 kDa exotoxin from *Bordetella pertussis*, which catalyzes the ADP-ribosylation of $G\alpha_i$ subunits of GPCRs and blocks downstream cellular signaling stimulated by this class of receptor [34]. Polarized T84 cells were pretreated with 1 μg/ml PTx for 30 min, prior to apical stimulation with LGG or fMLF. PTx potently inhibited LGG or fMLF dependent ERK phosphorylation (Figure 3A), indicating that GPCR are required for LGG or fMLF dependent ERK phosphorylation.

To further functionally implicate FPR as a mediator of LGG induced ERK signaling, we used the Boc2 peptide, a fMLF peptidomimetic, which binds both FPR1 and FPR2/ALX with high affinity and has been shown to specifically block fMLF/FPR binding in neutrophils [16, 35]. Polarized T84 cells were incubated in the presence of Boc2 (100 ng/ml) for 30 min prior to apical treatment with LGG, fMLF (Figure 3A). Boc2 pretreatment

mitigated LGG or fMLF induced ERK phosphorylation (Figure 3A), but importantly did not inhibit EGF-induced ERK phosphorylation (Figure 3D), a control for non-GPCR activation of ERK phosphorylation. As Boc2 is a peptidomimetic, we observed a small degree of agonist activity under media control conditions. The same analysis was performed with the invasive enteric pathogen *S. typhimurium*. This bacterium was able to induced ERK as shown in (Figures 1A and 2A), but interestingly, this activation was not repressed by with Ptx and Boc2 (Figure 3A), consistent with the accepted view that invasive pathogens stimulate via TLR rather than apical FPRs.

We then investigated the inhibitory effects of PTx and Boc2 on LGG or fMLF induced ERK pathway activation using the previously utilized ERK dependent reporter gene assay. Transfected SK-CO15 cultured cells pretreated with either PTx or Boc2 before treatment with LGG of fMLF exhibited strong attenuation of ERK pathway dependent luciferase activity (Figure 3B). Neither PTx nor Boc2 significantly decreased EGF induced ERK pathway dependent luciferase activity, confirming PTx and Boc2 specific inhibition of GPCR and FPR respectively.

To further confirm the specificity of PTx and Boc2 toward GPCR and FPR respectively, we basolaterally stimulated polarized T84 cells with flagellin, a bacterial protein known to induce NF-κB activation via TLR5. Neither PTx nor Boc2 treated cells inhibited flagellin induced IκB phosphorylation (Figure 3C) demonstrating absence of PTx or Boc2 inhibitory activity toward TLR signaling.

L. rhamnosus GG or fMLF induce phosphorylation and activation of formyl peptide receptor in epithelial cells

The signaling cascade initiated by fMLF binding to FPR is mediated by phosphorylation of the receptor which, in turn, recruits the binding of accessory proteins, such as all three $G\alpha_i$ isoforms, to the second intracellular loop in the C-terminal region of FPRs [13]. To experimentally demonstrate induced FPR phosphorylation (and thus activation), SK-CO15 cells were stimulated for up to 30 min with LGG, or fMLF. Cell lysates were prepared and total FPR immunoprecipitated using an anti-FPR antibody NFPR1, which recognizes both FPR1 and FPR2/ALX [24]. Precipitated proteins were analyzed by immunoblot using an antibody against phosphorylated serine/threonine. Both LGG and fMLF rapidly induced the phosphorylation of FPR within 5-15 min of stimulation (Figure 4). Total amounts of FPR in the precipitated protein were analyzed using anti-FPR antibody, which detected a protein of about 60kDa that is invariant after agonist exposure, and is consistent with the predicted molecular mass of epithelial FPR (Figure 4). Next, we examined the effect of PTx or Boc2 pretreatment before stimulation of SK-CO15 cells with LGG or fMLF. Boc2, the competitive inhibitor of extracellular fMLF binding, potently inhibited FPR phosphorylation, whereas PTx, which inhibits G protein signaling downstream of FPR activation, did not (Figure 4). Together, these data demonstrate that LGG or fMLF activate FPR in cultured epithelial cells.

L. rhamnosus GG or fMLF treatment of murine colon stimulates ERK activation

We examined whether natural murine luminal contents could activate cell signaling events in vitro. Bacterial suspensions were prepared from mouse cecal contents.

Supernatants from both pelleted bacteria and bacterial suspensions themselves (diluted up to 8 fold) potently stimulated ERK activation in polarized T84 cells. Importantly, no upregulation of the pro-apoptotic JNK signaling pathways were detected (Figure 5A), consistent with previous data with isolated cultures of bacteria.

To examine LGG or fMLF induced activation of ERK signaling pathway in vivo, surgically constructed murine colonic loops were instilled with 100 µl LGG ($5x10^8$ cfu/ml) or fMLF (500 nM) for 7 min, consistent with the baseline *N*-formyl peptide content of the mammalian colon measured at 100 nM [36, 37]. The colon was then removed, cut longitudinally, epithelial cells scraped and lysed before immunoblot analysis. Murine intestinal epithelia incubated with LGG or fMLF show strong activation of ERK pathway signaling compared to mice treated with buffer control (Figure 5B). Again, no activation of JNK or IκB was observed. We then administered LGG or fMLF to murine colons via direct intrarectal administration. At 7 min post administration, colonic sections were resected, cut longitudinally, fixed and immunostained using antibodies against phosphorylated ERK. Both LGG and fMLF potently induced ERK pathway activation at the colonic villus tips, whereas control mice administered identical quantities of buffer did not (Figure 5C). We then systemically pretreated mice with 0.5 µg/0.1µl PTx via intraperitoneal injection for 18 hrs [38], or intrarectally pretreated 50 µg Boc2 to mice for 30 min before intrarectal administration of LGG or fMLF for 7 min. Resected colonic preparations were fixed and examined for ERK pathway activation by immunofluorescence. PTx and Boc2 pre-treatment resulted in markedly reduces levels of LGG or fMLF induced ERK phosphorylation in colonic

villus tips compared to mice administered LGG or fMLF alone (Figure 5C). These data demonstrate the recapitulation of effects observed in polarized cultured intestinal epithelial cells in a murine model. Finally, this experiment was repeated in mice with a germ line null mutation in MyD88, an adaptor necessary for TLR2, 4, and 5 signaling. Essentially similar results were obtained (Figure 5D), indicating fMLF and LGG induced ERK signaling was not via surface TLR perception of bacteria.

Discussion

In the colon, FPRs are localized to apical villus tips, exposed to luminal contents and thus the microbiota and its products, i.e. formyl peptides [14]. It is important to note that most, if not all, prokaryotic translation products are formylated, thus a very wide range of bacterial protein and peptide would be expect to activate FPRs. In addition, both FPR1 and the closely related FPR2/ALX are stimulated by these ligands, albeit with different affinities. In neutrophils, sensing of formyl peptides leads to increased motility (chemotaxis), ROS generation (oxidative burst), and transcriptional response. We propose that colonic sensing of gut luminal formyl proteins and peptides likely stimulates functionally homologous outcomes. We previously showed fMLF activates PI3K through Rac1 and Cdc42 and subsequent Ca+ influx in a wound healing model, which results in activation of epithelial cytoskeletal rearrangements and cellular movement [14]. Furthermore, our past work has shown that commensal bacteria [26, 39, 40] and fMLF (unpublished data) have the ability to activate ROS in intestinal epithelium, though direct FPR involvement has not been confirmed.

Our data is consistent with FPR functioning as a novel PRR in the gut. Bacterial sensing by the host is generally thought to occur via leucine rich repeats (LRR) bearing pattern recognition receptors such as the transmembrane Toll-like receptors and cytoplasmic Nod proteins. Our studies indicate FPRs are bona fide PRRs, though there are several unique features. One, among MAPKs, epithelial FPR signaling is limited to ERK and Akt activation and does not activate the typical pro-inflammatory MAPKs or NF-κB, as we showed in macrophages (Figure 2). Additionally, FPR signaling is largely (though not

solely) restricted to the apical aspect of polarized epithelia, unlike TLR signaling which occurs mainly on the basolateral surface [14]. While PRR mediated signaling clearly has a central and dominant role in initiating cellular inflammation during infection [9, 10], it is now also apparent that basal tonic TLR (and likely other PRR) mediated signaling in response to the microbiota and its products are necessary for mucosal health. Murine models with defective PRR signaling are hypersensitive to a variety of intestinal insults and stressors, and supplementation of TLR ligands such as CpG DNA and flagellin can have cytoprotective effects [41, 42]. This has led to the hypothesis that a constitutive degree of PRR signaling is necessary for normal gut homeostasis and underscores the importance of gut-prokaryotic interaction as a beneficial and necessary relationship. ERK activation is known to stimulate pro-proliferative gene regulatory events and initiate cytoskeletal rearrangements necessary for epithelial movement [31, 32], though it is not typically considered pro-inflammatory and would not be expected to mediate overt innate immune responses in IECs. As the normal microbiota (and therapeutic probiotic administration) is associated with epithelial growth and wound healing, FRP mediated activation of ERK signaling may represent a novel pathway, parallel to the TLR/Nod mediated pathways, by which microbial signals are transduced to affect epithelial physiology. This notion is consistent with observations where small peptides derived from *H. pylori* signal via FPRs in vitro and can accelerate mucosal gastric injury in vivo [43]. Furthermore, the FPR related receptor GPR43 has recently been identified as a receptor for short chain fatty acids –a product of the intestinal microbiota- and has a clear role in the resolution of intestinal inflammation [44]. Indeed, FPR2ALX was originally characterized by its anti-inflammatory and pro-resolving actions, where germline

mutations resulted in enhanced inflammation in a variety of models [45]. Thus, the presence of these receptors on gut epithelia, and exposed to an environment highly enriched in bacteria and their products suggest therapeutic stimulation of the FPRs may be relevant to treating intestinal inflammation/injury.

Acknowledgements

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Materials and Methods

Reagents

Flagellin was a generous gift from Dr. Andrew Gewirtz. Recombinant human TNF-α and EGF were purchased from R&D Systems (Minneapolis, MN), pertussis toxin (PTx) from Calbiochem (Darmstadt, Germany), and FPR antagonist *N-tert*-butoxycarbonyl-Met-Leu-Phe (Boc2) through MP Biomedicals (Aurora, OH).

Cell Culture

T84 epithelial cells were prepared on 5-cm^2 permeable filters as described previously [21] and were used 9-14 days after plating and achieving a stable transepithelial resistance of >1000 $\Omega \cdot cm^2$. The intestinal epithelial cell line SK-CO15 were grown and maintained as previously described [16]. Primary BMMs were prepared as previously described [22]. Marrow was extracted from femora and tibia of 8-week-old mice in a BL6 background and cultured in DMEM containing 10% FBS and 100 ng/ml M-CSF from Sigma (St. Louis, MI). Cells were incubated at 37°C for 5 days, then medium changed every 2 days until cell were confluent.

Cecal flora preparation

Murine cecal contents were removed and suspended in PBS. Fecal debris was pelleted following centrifugation at 100xg. Supernatant was transferred to a new tube and centrifuged at 10000xg to pellet bacterial flora. Supernatant was removed, and pelleted bacteria diluted in 1 ml PBS.

Bacterial strains

Lactobacillus rhamnosus GG (ATCC #11975), *Lactobacillus acidophilus* (ATCC #11975), *Lactobacillus casei* (ATCC #11578), *Bacteriodes thetaiotaomicron* (ATCC #12290), *Streptococcus thermophilus* (ATCC #14485), *Salmonella typhimurium* (ATCC #13311), and *Escherichia coli* (derived from DH5α strain from Invitrogen) were grown and prepared as previously described [23]. Unless otherwise noted, all bacteria were used at a cfu/ml of $5x10^7$.

Cell wall preparation

L. rhamnosus GG was grown to $5x10^7$ cfu/ml. Bacterial cells were disrupted by sonication, centrifuged at 1000xg to pellet bacterial debris. The resulting supernatant was collected and centrifuged at 30000xg to obtain the membrane fraction. The pelleted fraction was then resuspended in initial volume equivalent of DMEM.

Immunoblotting and Immunofluorescence

Antibodies were obtained as follows: anti IκB-α (Santa Cruz Biotechnology Inc.), phospho-JNK, phospho-ERK, and phospho-Akt (Cell Signaling), β-actin (Sigma-Aldrich), phosphoserine/threonine (AbCam 17464-50), fluorescein (FITC)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch), and HRP-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibody (GE Healthcare). Mouse anti-FPR mAb was a generous gift from Dr. Algeris Jesaitis. This antibody (NFPR-1) is a monoclonal antibody prepared against 305-GQDFRERLI-313 peptides present on both human FPR1 and FPR2/ANX and recognizes a ~60 kDa band in transfected CHO epithelial cells [24].

Immunoblot and immunofluorescent labeling slips was performed as previously described [25]. Nuclei were stained with To-Pro-3 iodide (Molecular Probes). Fluorescent images acquired by laser confocal microscopy through an x 63 objective.

Reporter gene assays

SK-CO15 cells were transiently transfected using Lipofectamine 2000 (Invitrogen Life Technologies) according to manufacturer's instructions. For luciferase reporter assays, cells were transfected with NF-κB dependent pNF-κB-Luc, ERK dependent Elk1, or JNK dependent c-Jun reporter plasmids (Luciferase Trans-Reporting Systems, Stratagene) according to manufacturer's instructions. Following cell treatment, cells were lysed in reporter lysis buffer (Promega) and activity determined using the Dual Luciferase Reporter Assay System (Promega).

EdU incorporation assay

SK-CO15 cells were grown to 90% confluency on glass coverslips. Following experimental treatment for 12 hrs, cells were treated with EdU according to manufacturer's instructions (Invitrogen Click-iT EdU Imaging 488). Nuclei were stained with To-Pro-3 iodide (Molecular Probes). Fluorescent images acquired by Confocal Microscopy through an x 63 objective. For quantitative analysis, 10 fields of view were randomly selected for each treatment.

Denaturing Immunoprecipitation

Following experimental treatment, epithelial cells were washed in cold HBSS, lysed in denaturing 1% SDS lysis buffer and heated to 95°C. SDS was then quenched to 0.1% by adding Triton X-100 lysis buffer. DNA was fragmented by passing lysate through a 25G needle and protein stabilized by incubation on ice. Samples were precleared 1 hr on ice before incubation with ~1 µg/ml FPR1 mAb (NFPR1) [24]. FPR1/antibody conjugates were then precipitated using 50% slurry of IgG coated agarose beads (ThermoScientific). Protein was released from the beads by incubation with 2x SDS lysis buffer at 95°C for 5 min. Samples were immunoblotted using an antibody against FPR1 or Rb pAb phosphoserine/threonine (AbCam 17464-50). Densitometric analysis was performed using Scion Image beta.

Mice

All murine experimental procedures were undertaken according to Emory University guidelines for ethical treatment of animals. Ileal loop analysis of 6- to 8-week-old BL6 (Jackson labs) or MyD88 -/- mice was conducted as previously described [26]. Briefly, the colon was opened along the mesenteric border, epithelial tissue scraped and collected prior to administration of PBS, *L. rhamnosus* GG, or fMLF for up to 7 min, lysed in RIPA buffer (100 mg tissue/ml of buffer) and centrifuged at 16,000 r.p.m. for 20 min at 4°C. Protein concentrations of supernatants were determined by protein assay (Bio-Rad). For analysis of colonic tissue by intrarectal (i.r.) treatment, 6- to 8-week-old B6 mice were anesthetized prior to administration of PBS, *L. rhamnosus* GG, or fMLF for up to 7 min. Subjects were euthanized, and tissues removed for analysis. The colon was opened

along the mesenteric border, placed in 4% paraformaldehyde 20 min, and subsequent colon whole mount preparation performed as described below. For control experiments, mice were systemically administered 1 µg/ml PTx via i.p. injection for 18 hr prior to *L. rhamnosus* GG treatment. For fMLF peptidomimetic control, mice were intrarectally administered 100 µg/ml Boc2 through soft catheter 30 min prior to *L. rhamnosus* GG treatment.

Colon whole mount preparation

Dissected murine tissues were fixed for 20 min in 4% paraformaldehyde, washed in PBS, permeabilized with 0.1% Triton X-100 for 5 min, and washed again. Samples were blocked in 5% normal goat serum for 1 hr before incubation with Rabbit anti-phospho-ERK (Cell Signaling) for 1hr at 37°C (or o/n at 4°C), and then with FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Cellular actin was stained with Alexa Flour® Phallodin-633 (Molecular Probes). Tissue was cut into 2 mm to 5 mm small pieces, mounted on slides and visualized by Laser Confocal Microscopy at 63X.

Reproducibility and data presentation

Differences of $p \leq 0.05$ using the student's t-test were considered significant. The results of statistical analyses are given in the figure legends.

Figure Legends

Figure 1. Apically applied enteric bacteria induce ERK phosphorylation in polarized T84 epithelial cells. All experiments are immunoblots of T84 lysates probed with a phospho-ERK specific antibody. **A:** Apical cell stimulation with 5 x10^7cfu/ml of *Lactobacillus rhamnosus* GG, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bateriodes thetaiotaomicron*, *Streptococcus thermophilus*, *Escherichia coli*, and *Salmonella typhmurium* strains over 30 min. **B:** Apical cell stimulation with *L. rhamnosus* GG titered as indicated over 30 min. **C:** Apical cell stimulation with *L. rhamnosus* GG [5x10^7 cfu/ml], or *L. rhamnosus* GG cell wall preparation (described in methods) over 1 hr. All immunoblot experiments were repeated at least 5 times.

Figure 2. Apically applied *L. rhamnosus* GG or fMLF specifically activate ERK/Akt pathway signaling. **A:** Immunoblot analysis of cultured T84 cells apically stimulated with *L. rhamnosus* GG [5x10^7 cfu/ml], L. *rhamnosus* GG cell wall preparation, fMLF [500 nM] or *S. typhimurium* [1x10^8 cfu/ml] or basolaterally with TNF-α [10 ng/ml] over 1 hr, lysed, and probed with the indicated antibodies. **B:** Murine bone marrow derived macrophages stimulated with *L. rhamnosus* GG [5x10^7cfu/ml] or fMLF [500 nM] over 30 min, lysed, and probed with antibodies indicated. **C:** Immunofluorescent analysis on fixed T84 cultured cells apically stimulated with *L. rhamnosus* GG [5x10^7 cfu/ml], or fMLF [500 nM] over 30 min. DNA (blue), Phospho-ERK (green). Confocal microscope magnification x 63. **D:** ERK, JNK, or NF-κB pathway specific luciferase reporter gene assay of transfected SK-CO15 cells treated with *L. rhamnosus* GG [5x10^7 cfu/ml], fMLF [500 nM]. EGF [200 ng/ml] or TNF-^{16}α [10 ng/ml] serve as activating controls for ERK

and JNK/NF-κB respectively. Data is shown as fold induction over unstimulated media control. * = P< 0.05 ** = P< 0.001. All immunoblot experiments repeated at least 5 times, immunofluorescence experiment repeated at least 3 times, luciferase assay n=6 for each experimental treatment. **E:** EdU incorporation into cultured SK-CO15 cells incubated for 12 h with *L. rhamnosus* GG [$5x10^7$ cfu/ml] or fMLF [500 nM]. DNA (blue), EdU (red). Confocal microscope magnification x 63 **F:** Quantitative representation of EdU positive cells in E. Number of EdU positive cells per 10 fields of view for 3 replicates per treatment. * = P<0.05.

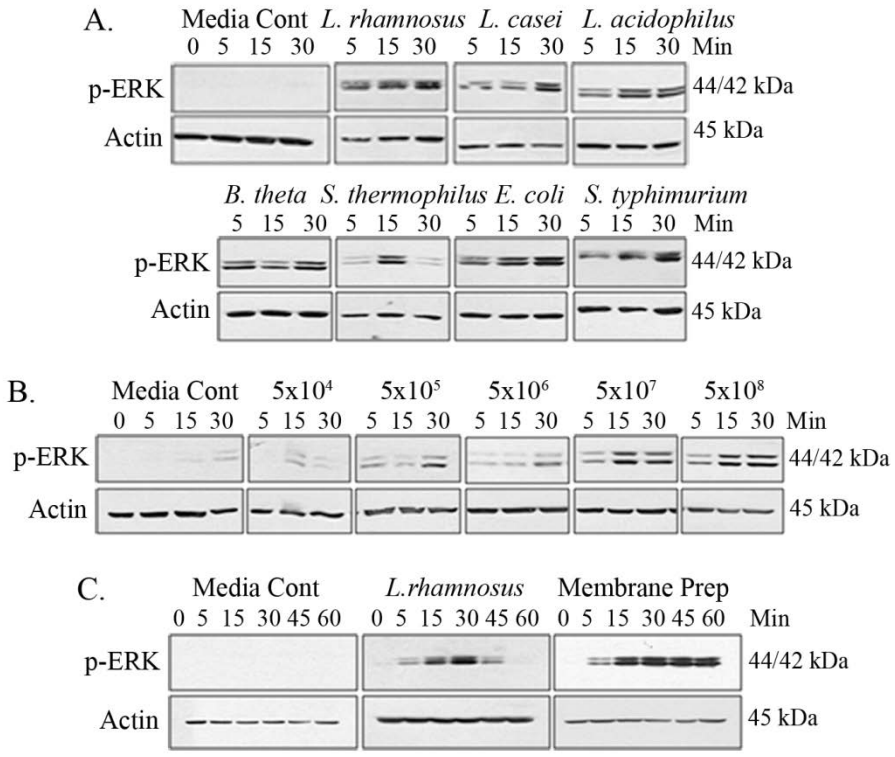
Figure 3. Inhibition of G-protein coupled receptors or formyl peptide receptors attenuates ERK phosphorylation in response to apical *L. rhamnosus* GG or fMLF stimulation. **A:** Immunoblot analysis of cultured T84 cells treated apically with PTx [1 μg/ml] or Boc2 [100 μg/ml] 30 min prior to apical stimulation with *L. rhamnosus* GG [$5x10^7$ cfu/ml], *S.typhimurium* [$1x10^8$ cfu/ml], or fMLF [500 nM] over 1 hr. **B:** ERK pathway specific luciferase reporter gene assay from transfected SK-CO15 cells treated with either PTx [1 μg/ml] or Boc2 [100 μg/ml] 30 min prior to *L. rhamnosus* GG [$5x10^7$ cfu/ml], fMLF [500 nM] or EGF [200 ng/ml] stimulation. Data is shown as fold induction over unstimulated media control.* = P< 0.05 ** = P< 0.001 **C:** Immunoblot analysis of T84 cells treated basolaterally with either PTx [1 μg/ml] or Boc2 [100 μg/ml] 30 min prior to basolateral stimulation with flagellin [100 ng/ml]. **D:** Immunoblot analysis of T84 cells treated apically with Boc2 [100 μg/ml] 30 min prior to basolateral EGF [200 ng/ml] stimulation. All experiments repeated at least 3 times.

Figure 4. Apical *L. rhamnosus* GG and fMLF induce phosphorylation of FPR. **A:** Immunoblot analysis of immunoprecipitated FPR from cultured SK-CO15 cells apically pretreated with either PTx [1 μg/ml], Boc2 [100 μg/ml] or DMEM 30 min prior to stimulation with *L. rhamnosus* GG [$5x10^7$ cfu/ml] or fMLF [500 nM]. Immunoprecipitated proteins were immunoblotted against antibodies to pSer/Thr and FPR. Immunoblot experiments repeated at least 3 times. Densitometric analysis normalized to background using Scion Image beta.

Figure 5. *L. rhamnosus* GG or fMLF treatment of murine colon stimulates ERK activation. **A:** Immunoblot analysis for phospho-ERK in T84 cells apically treated with cecal luminal preparations (as described in materials and methods) over 30 min. **B:** Immunoblot analysis for phospho-ERK in mouse colonic epithelial cell scrapings treated in vivo with 100 μl *L. rhamnosus* GG [10^7 cfu/ml] or fMLF [500 nM] for 7 min. **C:** Immunofluorescence of phospho-ERK of intestinal whole mount preparations (as described in material and methods) in either baseline intestinal mucosa or pretreated in vivo with either PTx [1 μg/ml] or Boc2 [100 μg/ml] 30 min prior to 100 μl *L. rhamnosus* GG [10^7 cfu/ml] or fMLF [500 nM] for 7 min. **D:** Experiment performed identically as in C, on a MyD88 null background. An $n \leq 3$ for each experimental murine treatment.

FIGURES

Figure 1



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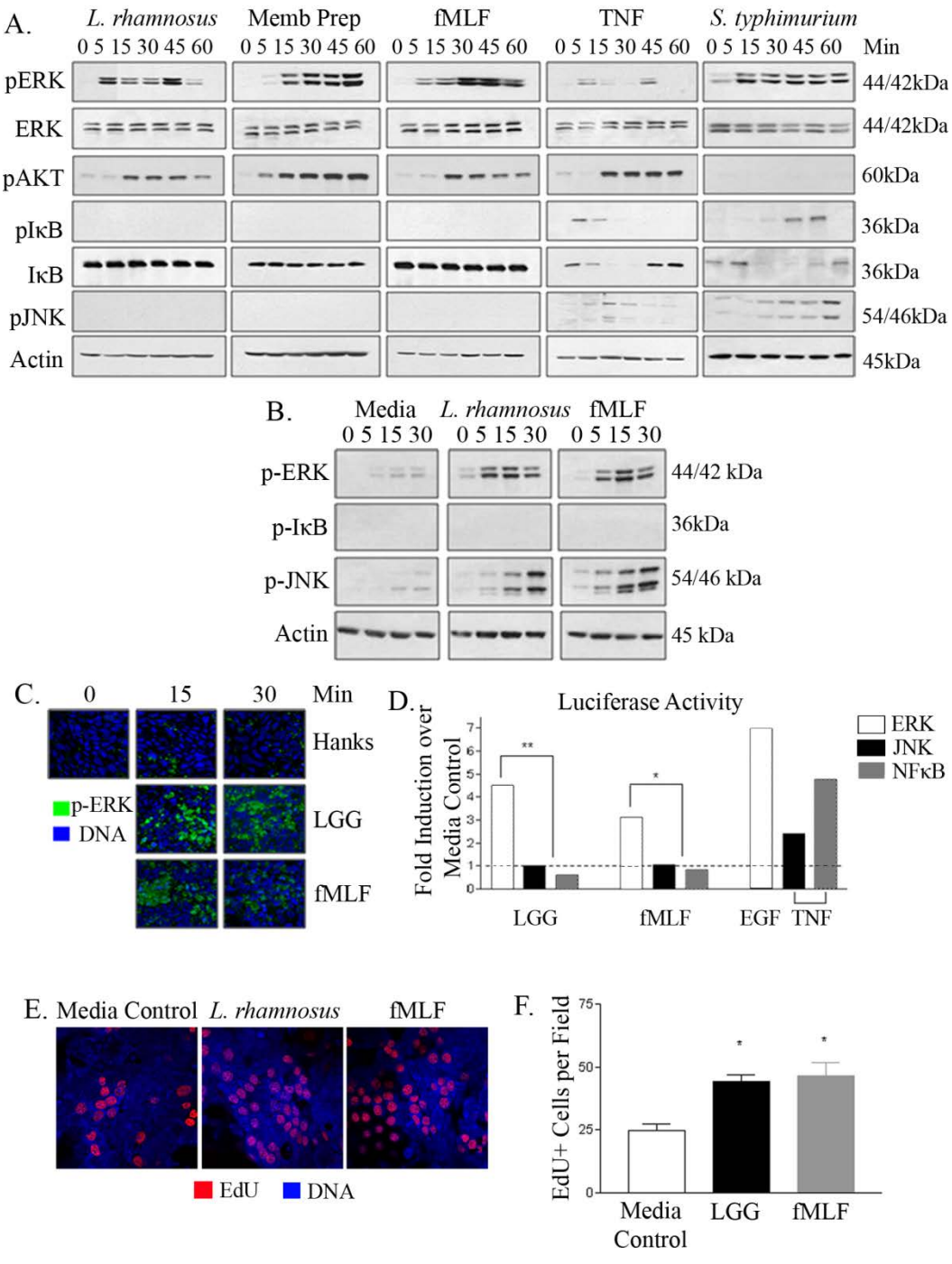
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Figure 3

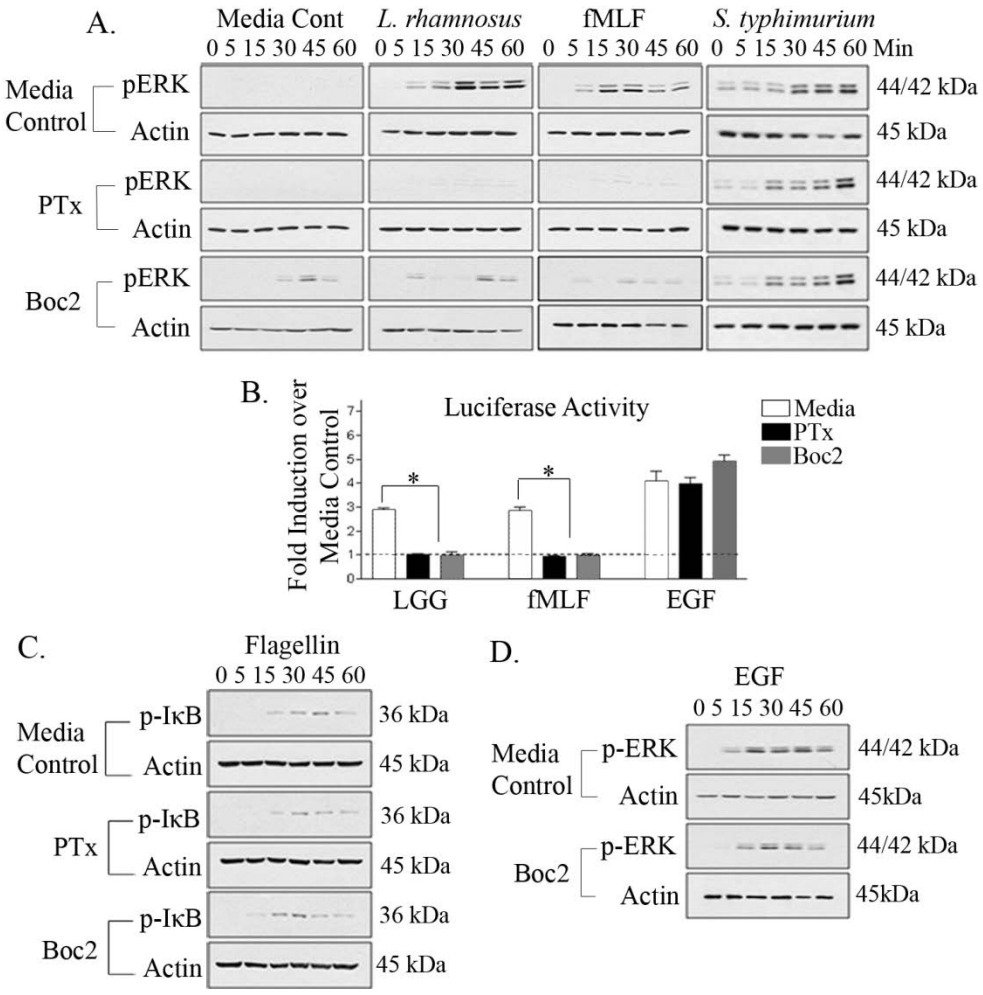
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Figure 4

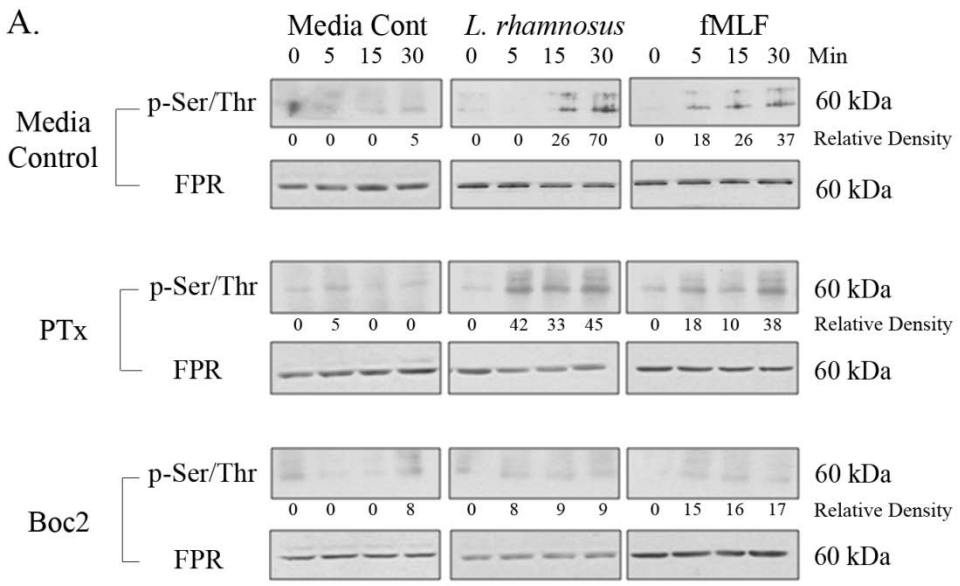
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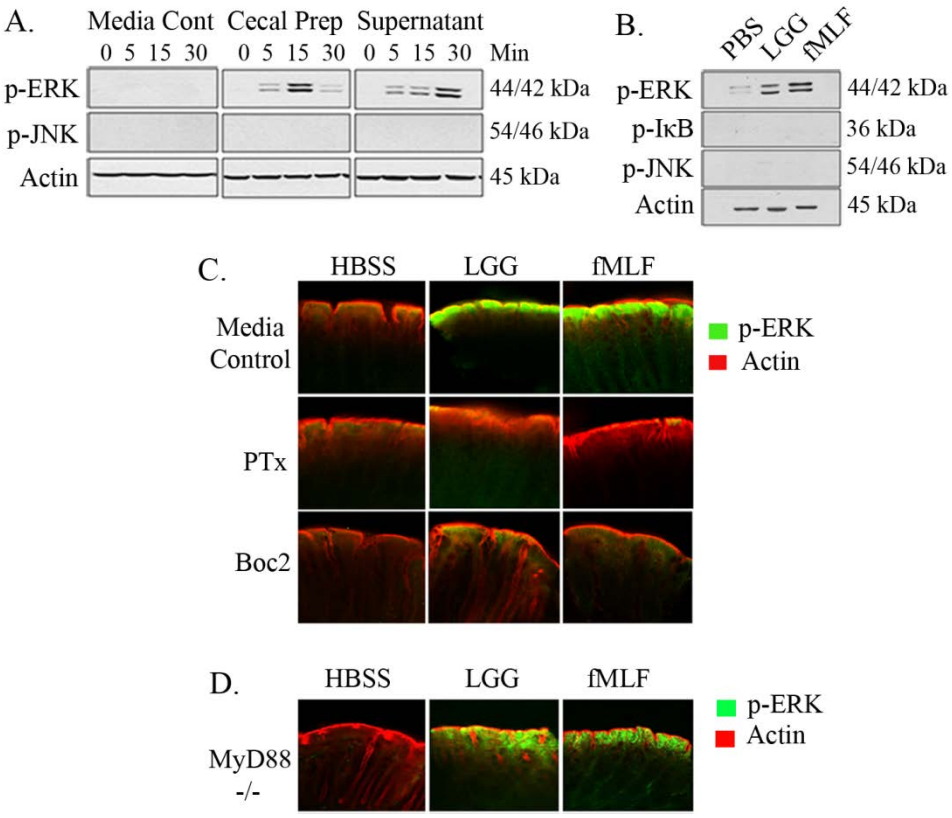
Figure 5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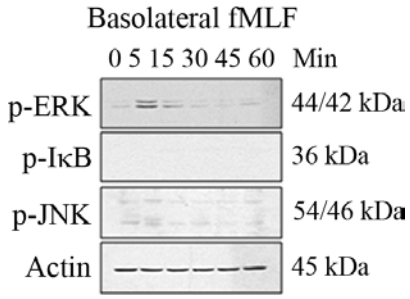
LGG
fMLF
p-ERK
p-IκB
p-JNK
Actin
44/42 kDa
36 kDa
54/46 kDa
45 kDa
C.
HBSS
LGG
fMLF
Media Control
PTx
Boc2
p-ERK
Actin
D.
HBSS
LGG
fMLF
MyD88 -/-
p-ERK
Actin

Supplemental Figure 1



Supplemental Figure 1. Basolaterally applied fMLF weakly activates ERK pathway signaling. Immunoblot analysis of cultured T84 cells apically stimulated with fMLF [500 nM] basolaterally over 1hr, lysed, and probed with the indicated antibodies. Immunoblot experiment repeated at least 5 times.

References

1. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE: Metagenomic analysis of the human distal gut microbiome. Science. 2006, 312:1355-1359

2. Xu J, Mahowald MA, Ley RE, Lozupone CA, Hamady M, Martens EC, Henrissat B, Coutinho PM, Minx P, Latreille P, Cordum H, Van Brunt A, Kim K, Fulton RS, Fulton LA, Clifton SW, Wilson RK, Knight RD, Gordon JI: Evolution of Symbiotic Bacteria in the Distal Human Intestine. PLoS Biol. 2007, 5:e156

3. Hooper LV, Bry L, Falk PG, Gordon JI: Host-microbial symbiosis in the mammalian intestine: exploring an internal ecosystem. Bioessays. 1998, 20:336-343

4. Neish AS: Microbes in gastrointestinal health and disease. Gastroenterology. 2009, 136:65-80

5. Hooper LV, Gordon JI: Commensal Host-Bacterial Relationships in the Gut. Science. 2001, 292:1115-1118

6. Pull SL, Doherty JM, Mills JC, Gordon JI, Stappenbeck TS: Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. Proceedings of the National Academy of Sciences. 2005, 102:99-104

7. Park J, Floch MH: Prebiotics, Probiotics, and Dietary Fiber in Gastrointestinal Disease. Gastroenterology Clinics of North America, Nutrition in Gastrointestinal Illness. 2007, 36:47-63

8. Sartor RB: Microbial Influences in Inflammatory Bowel Diseases. Gastroenterology. 2008, 134:577-594

9. Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S: Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe. 2008, 3:352-363

10. Medzhitov R: Recognition of microorganisms and activation of the immune response. Nature. 2007, 449:819-826

11. Migeotte I, Communi D, Parmentier M: Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. Cytokine & growth factor reviews. 2006, 17:501-519

12. Rabiet MJ, Huet E, Boulay F: The N-formyl peptide receptors and the anaphylatoxin C5a receptors: an overview. Biochimie. 2007, 89:1089-1106

13. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN, Murphy PM: International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacological reviews. 2009, 61:119-161

14. Babbin BA, Jesaitis AJ, Ivanov AI, Kelly D, Laukoetter M, Nava P, Parkos CA, Nusrat A: Formyl peptide receptor-1 activation enhances intestinal epithelial cell restitution through phosphatidylinositol 3-kinase-dependent activation of Rac1 and Cdc42. J Immunol. 2007, 179:8112-8121

15. Serhan CN, Chiang N, Van Dyke TE: Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol. 2008, 8:349-361

16. Babbin BA, Lee WY, Parkos CA, Winfree LM, Akyildiz A, Perretti M, Nusrat A: Annexin I regulates SKCO-15 cell invasion by signaling through formyl peptide receptors. The Journal of biological chemistry. 2006, 281:19588-19599

17. Canny G, Levy O, Furuta GT, Narravula-Alipati S, Sisson RB, Serhan CN, Colgan SP: Lipid mediator-induced expression of bactericidal/ permeability-increasing protein (BPI) in human mucosal epithelia. Proc Natl Acad Sci U S A. 2002, 99:3902-3907

18. Chang L, Karin M: Mammalian MAP kinase signalling cascades. Nature. 2001, 410:37-40

19. Zeng H, Carlson AQ, Guo Y, Yu Y, Collier-Hyams LS, Madara JL, Gewirtz AT, Neish AS: Flagellin is the major proinflammatory determinant of enteropathogenic Salmonella. J Immunol. 2003, 171:3668-3674

20. Hooper LV, Wong MH, Thelin A, Hansson L, Falk PG, Gordon JI: Molecular analysis of commensal host-microbial relationships in the intestine. Science. 2001, 291:881-884

21. Madara JL, Stafford J, Dharmsathaphorn K, Carlson S: Structural analysis of a human intestinal epithelial cell line. Gastroenterology. 1987, 92:1133-1145

22. Kim HJ, Zhao H, Kitaura H, Bhattacharyya S, Brewer JA, Muglia LJ, Ross FP, Teitelbaum SL: Glucocorticoids suppress bone formation via the osteoclast. J Clin Invest. 2006, 116:2152-2160

23. Collier-Hyams LS, Sloane V, Batten BC, Neish AS: Cutting edge: bacterial modulation of epithelial signaling via changes in neddylation of cullin-1. J Immunol. 2005, 175:4194-4198

24. Riesselman M, Miettinen HM, Gripentrog JM, Lord CI, Mumey B, Dratz EA, Stie J, Taylor RM, Jesaitis AJ: C-terminal tail phosphorylation of N-formyl peptide receptor: differential recognition of two neutrophil chemoattractant receptors by monoclonal antibodies NFPR1 and NFPR2. J Immunol. 2007, 179:2520-2531

25. Collier-Hyams LS, Zeng H, Sun J, Tomlinson AD, Bao ZQ, Chen H, Madara JL, Orth K, Neish AS: Cutting edge: Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. J Immunol. 2002, 169:2846-2850

26. Kumar A, Wu H, Collier-Hyams LS, Hansen JM, Li T, Yamoah K, Pan ZQ, Jones DP, Neish AS: Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. Embo J. 2007, 26:4457-4466

27. Resta-Lenert S, Barrett KE: Probiotics and Commensals Reverse TNF-[alpha]- and IFN-[gamma]-Induced Dysfunction in Human Intestinal Epithelial Cells. Gastroenterology. 2006, 130:731-746

28. Yan F, Cao H, Cover TL, Whitehead R, Washington MK, Polk DB: Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. Gastroenterology. 2007, 132:562-575

29. Neish AS, Gewirtz AT, Zeng H, Young AN, Hobert ME, Karmali V, Rao AS, Madara JL: Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. Science. 2000, 289:1560-1563

30. Torres M, Hall FL, O'Neill K: Stimulation of human neutrophils with formyl-methionyl-leucyl-phenylalanine induces tyrosine phosphorylation and activation

of two distinct mitogen-activated protein-kinases. J Immunol. 1993, 150:1563-1577

31. Johnson GL, Lapadat R: Mitogen-Activated Protein Kinase Pathways Mediated by ERK, JNK, and p38 Protein Kinases. Science. 2002, 298:1911-1912

32. Roux PP, Blenis J: ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. 2004, 68:320-344

33. He XC, Yin T, Grindley JC, Tian Q, Sato T, Tao WA, Dirisina R, Porter-Westpfahl KS, Hembree M, Johnson T, Wiedemann LM, Barrett TA, Hood L, Wu H, Li L: PTEN-deficient intestinal stem cells initiate intestinal polyposis. Nat Genet. 2007, 39:189-198

34. Bokoch GM, Gilman AG: Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. Cell. 1984, 39:301-308

35. Gavins FN, Yona S, Kamal AM, Flower RJ, Perretti M: Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. Blood. 2003, 101:4140-4147

36. Chadwick VS, Mellor DM, Myers DB, Selden AC, Keshavarzian A, Broom MF, Hobson CH: Production of peptides inducing chemotaxis and lysosomal enzyme release in human neutrophils by intestinal bacteria in vitro and in vivo. Scand J Gastroenterol. 1988, 23:121-128

37. Marasco WA, Phan SH, Krutzsch H, Showell HJ, Feltner DE, Nairn R, Becker EL, Ward PA: Purification and identification of formyl-methionyl-leucyl-

phenylalanine as the major peptide neutrophil chemotactic factor produced by Escherichia coli. The Journal of biological chemistry. 1984, 259:5430-5439

38. Hou W, Wu Y, Sun S, Shi M, Sun Y, Yang C, Pei G, Gu Y, Zhong C, Sun B: Pertussis toxin enhances Th1 responses by stimulation of dendritic cells. J Immunol. 2003, 170:1728-1736

39. Kumar A, Wu H, Collier-Hyams LS, Kwon YM, Hanson JM, Neish AS: The bacterial fermentation product butyrate influences epithelial signaling via reactive oxygen species-mediated changes in cullin-1 neddylation. J Immunol. 2009, 182:538-546

40. Lin PW, Myers LE, Ray L, Song SC, Nasr TR, Berardinelli AJ, Kundu K, Murthy N, Hansen JM, Neish AS: Lactobacillus rhamnosus blocks inflammatory signaling in vivo via reactive oxygen species generation. Free Radic Biol Med. 2009,

41. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E: Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol. 2006, 8:1327-1336

42. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R: Recognition of Commensal Microflora by Toll-Like Receptors Is Required for Intestinal Homeostasis. Cell. 2004, 118:229-241

43. de Paulis A, Prevete N, Rossi FW, Rivellese F, Salerno F, Delfino G, Liccardo B, Avilla E, Montuori N, Mascolo M, Staibano S, Melillo RM, D'Argenio G, Ricci V, Romano M, Marone G: Helicobacter pylori Hp(2-20) promotes migration and

proliferation of gastric epithelial cells by interacting with formyl peptide receptors in vitro and accelerates gastric mucosal healing in vivo. J Immunol. 2009, 183:3761-3769

44. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR: Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature. 2009, 461:1282-1286

45. Dufton N, Hannon R, Brancaleone V, Dalli J, Patel HB, Gray M, D'Acquisto F, Buckingham JC, Perretti M, Flower RJ: Anti-inflammatory role of the murine formyl-peptide receptor 2: ligand-specific effects on leukocyte responses and experimental inflammation. J Immunol. 2010, 184:2611-2619

Chapter III: Enteric Commensal Bacteria Induce ERK via FPR Dependent Redox Modulation of DUSP3

Enteric Commensal Bacteria Induce ERK via FPR Dependant Redox Modulation of DUSP3

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Abstract

The normal microbial occupants of the mammalian intestine contributes to diverse aspects of gut homeostasis, yet the mechanisms by which intestinal cells perceive and respond to the microbiota are largely unknown. Intestinal epithelial contact with commensal bacteria and/or their products has been shown to induce the activation of non-inflammatory signaling pathways, such as extracellular related kinase (ERK), thus potentiating events that influence the intestinal homeostatic processes. We previously showed that microbes stimulate ERK pathway activity via interaction with formyl peptide receptors (FPRs). In this study, we show that commensal bacteria initiate ERK signaling through rapid FPR dependant ROS generation that subsequently modulates the redox status of the MAP kinase phosphatases. We show ROS generation induced by commensal bacteria (e.g. *Lactobacillus rhamnosus* GG) and the FPR peptide ligand, N-formyl-Met-Leu-Phe (fMLF) was abolished in the presence of selective inhibitors for G-protein coupled signaling and FPR ligand interaction. In addition, pretreatment of cells with ROS inhibitors attenuated commensal bacteria- induced ERK signaling, indicating the requirement of generated ROS as intermediates in ERK activation. Analysis of the redox sensitive ERK specific protein phosphatase, VHR/DUSP3, showed that bacterial colonization elicited oxidative inactivation of DUSP3 and consequent stimulation of ERK signaling. Together these data show that commensal bacteria (and fMLF) activate ROS signaling in an FPR dependent manner and define a mechanism by which ROS influences the ERK pathway, and potentially other redox sensitive regulatory circuits.

Introduction

The mammalian intestinal epithelium coexists in intimate contact with up to 10^{14} prokaryotic organisms that comprise the gut microbiota. [1, 2] This prokaryotic community thrives in a generally symbiotic fashion, contributing to the host by providing vitamin and micronutrient synthesis, simulation of immune development and function, extraction of calories from otherwise indigestible complex carbohydrates, and competitive exclusion of pathogens. [3, 4] Recently, more direct effects on intrinsic epithelial processes have been described. For example, commensal bacteria have been shown to augment barrier function and stimulate reparative responses. [5-7] Germ-free mice show a slower turnover of intestinal epithelial cells, exhibiting a marked decrease in crypt to villus transit time. [8] In humans, commensal bacteria have also recently been used as a therapy (probiotics) for inflammatory and developmental disorders in the intestinal tract. [9] However, how the microbiota engages in cross talk with the intestinal epithelia to mediate gut homeostatic events and how this signaling is controlled is just now being elucidated.

Bacteria signal to the intestinal epithelia through transmembrane and intracellular receptors generally designated as pattern recognition receptors (PRRs), which bind conserved bacterial structural motifs, known as microbial associated molecular patterns (MAMPs). A class of newly characterized epithelial PRRs is the formyl peptide receptors (FPRs). First described on neutrophils and macrophages, FPRs are seven transmembrane receptors that bind N-formyl peptides, prokaryotic translation products modified with the bacterial specific amino acid N-formyl methionine. [10-12] Ligand bound

FPRs become phosphorylated causing them to undergo conformational changes that allows the recruitment of the G_i family of G proteins and initiates signaling resulting in 1) changes in actin dynamics initiating chemotaxis, 2) transcription of inflammatory effectors and cytokines, and 3) activation of NADPH oxidase enzymes resulting in the ROS production (respiratory burst). Recently, FPRs have been characterized on non-phagocyte cell types, including the intestinal epithelia, and our group has shown commensal bacteria directly signal through FPRs to activate the MAPK ERK signaling pathway without activating the proinflammatory IκB or proapoptotic JNK pathways. [13, 14] Additionally, we have shown commensal bacteria can modulate intestinal epithelial proinflammatory signaling through activation of reactive oxygen species (ROS). [6] It is unknown if ROS is stimulated in epithelial cells through FPRs in response to commensal bacteria, analogous to events well known in phagocytes, nor if ROS plays a role in homeostatic signaling.

The physiological generation of ROS by non-phagocyte NADPH oxidase enzymes (NOX) regulates diverse cellular processes through transient oxidative inactivation of catalytic cysteine residues on a spectrum of regulatory enzymes. [15-17] A well studied class of redox sensitive regulatory enzymes is the dual-specific phosphatases (DUSPs). [18] DUSPs are a subset of protein tyrosine phosphatases which dephosphorylate threonine and tyrosine residues in the consensus motif Thr-X-Tyr (where X is Glu, Gly, or Pro) on MAPKs ERK, JNK, and p38, thus are also referred to as MAPK phosphatases (MKPs). Once MAPKs are dephosphorylated, the signaling cascade becomes inactive. Conversely, when the DUSP catalytic cysteine residues are oxidatively inactivated

through localized ROS production, DUSPs phosphatase activity is reversibly inactivated, leading to sustained activation of MAPKs.

Here we show that commensal members of the microbiota, and their products, activate ROS signaling in the intestinal epithelia through FPRs and this induced ROS modulates ERK signaling through DUSP3 oxidative inactivation. These findings indicate that commensal bacteria activate ROS through intestinal FPRs and elucidate a mechanism by which the microbiota influences homeostatic signaling to the intestinal epithelia.

Results

fMLF and commensal bacteria induce the generation of ROS in cultured epithelial cells in an FPR dependent manner.

fMLF perception by FPRs is a classical inducer of ROS in neutrophils. We investigated if recognition of commensal bacteria or fMLF by FPRs situated on the apical side of epithelial cells mediated an analogous response. As a commensal bacterium, we utilized Lactobacillus rhamnosus GG (LGG), a member of the human microbiota and commonly used probiotic agent. Polarized cultured SK-CO15 colonic epithelial cells were treated with either 500nM fMLF or 5 x 10^7 cfu/ml LGG for up to 45 min, and ROS generation detected by oxidation of the redox sensitive dye CM-H2DCF-DA (DCF) as previously reported. [6] ROS was rapidly generated in fMLF or LGG treated cells following within 15 minutes of contact and peaking at 30 min (Figure 1). Note that ROS generation was detected in the cytoplasm of contacted cells, whereas no ROS generation was evident in the nucleus. The levels of ROS generated by fMLF or LGG were similar to those generated in response to contact with 250 μM levels of H_2O_2 (Fig. 1 Supplementary). Importantly, both fMLF and LGG- induced ROS generation was abrogated in cells pretreated with Boc2, a competitive inhibitor of fMLF binding to FPRs, and by pertussis toxin (PTx), an inhibitor the $G_{\alpha i}$ subunit of G-protein coupled receptors (Figure 1A and 1B). Pre-treatment of the stimulated cells with *N*-acetylcysteine (NAC), a glutathione (GSH) precursor and ROS sink, also abrogated fMLF and LGG- induced ROS generation. Together, these data show that fMLF or LGG induces the generation of physiological levels of ROS in contacted cells in an FPR dependent manner.

Dampening of cellular ROS levels attenuates L. rhamnosus *GG or fMLF induced ERK pathway activation and cellular proliferation.*

In a previous report by our research group, we showed that contact of the apical side of enterocytes by commensal bacteria or fMLF specifically activated the ERK signaling pathway, and induced cellular proliferation. [14] Having shown enterocytes also generate ROS in response to contact by fMLF and LGG, we next investigated the extent to which physiological levels of ROS influenced ERK signaling. Cultured SK-CO15 cells were treated with NAC (20mM), prior to treatment with 5 x 10^7 cfu/ml LGG or 500nM fMLF. Strikingly, whereas ERK is rapidly phosphorylated in response to LGG of fMLF, no ERK phosphorylation was detected in NAC treated cells, indicating a function for ROS in LGG or fMLF potentiated ERK signaling (Figure 2A). We also investigated ERK pathway activation in the presence of NAC using a transfection based ERK pathway dependent reporter assay. Consistent with the data from immunoblot analysis, pre-treatment with NAC inhibited LGG or fMLF- induced ERK pathway activation (Figure 2B).

We also previously reported that apical contact of polarized SK-CO15 cultured cells by LGG or fMLF induced cellular proliferation. [14] To determine whether ROS generated in response to contact by LGG and fMLF mediates these proliferative events, we assayed for EdU incorporation in cultured SK-CO15 cells pretreated with the antioxidant NAC before stimulation with either LGG or fMLF. We observed that antioxidant pretreatment significantly reduced the number of EdU positive cells induced by LGG and fMLF

cellular contact (Figure 2C and 2D) thus implicating a role for LGG and fMLF induced ROS generation in mediating the signaling events that induce cellular proliferation.

L. rhamnosus *GG or fMLF upregulate DUSP3 mRNA and protein.*

ROS modulate cellular signaling events via the oxidation of hyper-reactive cysteine residues within a subset of regulatory enzymes. [15] A well studied example are the family of MAPK phosphatases or DUSPs (dual specificity phosphatases), which dephosphorylate MAPK Tyrosine and Threonine residues, thus rendering MAPKs inactive. [18] Using microarray analysis, our research group showed that LGG treatment in 2 week old murine intestine induced the transcriptional upregulation of DUSP3, a family member that is known to specifically dephosphorylate ERK and thus inhibit ERK pathway signaling. [19] To confirm LGG and fMLF upregulation of DUSP3, we performed qPCR analysis on RNA isolated from LGG and fMLF treated SK-CO15 cells over 4 h, or on murine intestinal scrapings treated intrarectally for 30 min. In both *in vitro* and *in vivo* conditions, we saw a marked increase in DUSP3 mRNA synthesis (Figure 3). To determine if mRNA synthesis eventuates in protein synthesis and action, we probed SK-CO15 cells lysates treated with LGG and fMLF up to 1 h and intestinal scrapings at 30 min for DUSP3 protein. As DUSP3 is rapidly degraded by the proteosome, we incubated cells with MG-262, a proteosome inhibitor. Both LGG and fMLF treated cells exhibited an increase in DUSP3 total protein (Figure 3B). Together these data confirm intestinal epithelial DUSP3 upregulation in response to LGG and fMLF treatment suggesting DUSP3 as a potential target for redox regulation.

L. rhamnosus *GG or fMLF- induced generation of ROS oxidizes DUSP3.*

We then sought to establish the mechanism by which LGG or fMLF- induced generation of ROS influences ERK signaling circuitry. Hitherto, we have established that ROS is generated within minutes of LGG or fMLF contact with epithelial cells, and that ensuing events include the phosphorylation of ERK and the upregulation of ERK responsive genes, namely DUSP3. The known activity of DUSP3 is to catalyze the dephosphorylation of phosphorylated ERK, serving as a negative-feedback loop following pathway activation. [18] Under uninduced conditions, DUSP3 is expressed at tonic levels that are sufficient to suppress overt ERK pathway activation. The phosphatase activity of DUSP3 is dependent on a critical cysteine at position 124. Furthermore, it has been reported that this critical residue, conserved within the DUSP family of proteins, is also readily oxidized in response to elevated redox conditions, rendering DUSP family members catalytically inactive and unable to suppress MAPK pathway activity. [20] We thus hypothesized that the rapid generation of ROS in response to LGG and fMLF contact leads to the oxidation and inactivation of DUSP3, thus facilitating ERK phosphorylation and pathway activation. To test this hypothesis, we transfected cultured SK-CO15 cells with plasmids harboring wild type DUSP3, or a mutant and catalytically inactive form of DUSP3 (mDUSP3) where the critical catalytic cysteine residue at position 124 is mutated to an alanine residue, and assessed the ability to dephosphorylate active ERK. Whereas wild type DUSP3 potently repressed ERK phosphorylation to levels markedly lower than basal levels, transfection of a plasmid harboring mDUSP3, presumably acting as a dominant negative, resulted in clearly elevated levels of phosphorylated ERK (Figure 4A). Functionally, elevated ERK

pathway activity was measured by an ERK pathway specific luciferase reporter gene assay. Consistent with immunoblot analysis, wild type DUSP3 was effective in suppressing basal ERK transcriptional activity, whereas the mutant form, served to augment basal ERK transcriptional actively (Figure 4B).

Next, we tested if DUSP3 or mDUSP3 are oxidized in response to elevated cellular ROS levels. ROS generation was induced in SK-CO15 cultured cells transfected with plasmid constructs described in figure 4A, through treatment with LGG, fMLF, or control H_2O_2, and the oxidation status of DUSP3 examined by immunoblot analysis using non-reducing SDS-PAGE electrophoresis as described previously. [20] These conditions allow detection of higher molecular weight disulfide dimer and aggregate forms as previously described. Without stimulation, both DUSP3 and mDUSP3 migrate at 20 kDa. Both LGG and fMLF treatment resulted in a characteristic band shift indicative of DUSP3 oxidation and formation of dimeric and higher order aggregates at 15 and 30 min following initial contact by LGG or fMLF or with 1 mM H_2O_2 serving as positive control for oxidation (Figure 4C). Importantly, no band shift pattern was detected in lysates prepared from cells transfected with a plasmids harboring mDUSP3, where the redox sensor cysteine residue is mutated to an alanine residue, thus confirming that the critical cysteine residue on DUSP3 required for its ERK dephosphorylation activity is sensitive to cellular ROS levels (Figure 4C). Furthermore, pretreatment of transfected SK-CO15 cultured cells with NAC, and antioxidant, before ROS stimulation by LGG or fMLF, visibly reduced the amount of oxidized DUSP3 species, thus indicating that DUSP3 oxidation is a direct result of induced-ROS generation by LGG or fMLF (Figure 4C). In all cases, appearance

of oxidized forms of DUSP3 correlated with suppressed ERK activation, while mDUSP superinduced ERK phosphorylation. When DUSP3 oxidation was induced by soluble and transient stimulus (H_2O_2), removal of the stimulus resulted in the gradual reduction of oxidized forms up to four hours (Figure 4D). Together, these data show that LGG or fMLF induced ROS generation modulates the dynamic interaction between DUSP3 and its target substrate, phosphorylated ERK.

Antioxidant pretreatment inhibits L. rhamnosus *GG or fMLF induced phosphorylation of ERK in murine enterocytes.*

We previously reported that LGG or fMLF induced the phosphorylation of ERK in murine enterocytes. [14] In order to show that this activity is dependent on LGG or fMLF induced ROS-generation in the murine colon, we intrarectally pretreated mice with NAC before stimulation with 5 x 10^8 cfu/ml LGG or fMLF for 7 min. Strikingly, LGG or fMLF- induced ERK phosphorylation was completely abrogated in the colonic epithelium of NAC pretreated mice, compared to untreated control mice where ERK is phosphorylated within minutes of contact by LGG or fMLF (Figure 5A). To corroborate these observations, colonic epithelial scrapings from the mice treated as described above were examined by immunoblot analysis using an antibody against phosphorylated ERK. Consistent with immunofluorescent analysis, NAC pretreatment markedly reduced LGG or fMLF phosphorylation of ERK compared to non-pretreated samples (Figure 5B). Collectively, these data show that fMLF or LGG induce ERK via FPR dependant redox modulation of DUSP3.

Discussion

In this work, we describe a mechanism by which commensal bacteria can influence ERK MAPK, an important, non proinflammatory eukaryotic signaling pathway. In health, the intestinal epithelium is in constant contact with trillions of bacteria, and thus bacterial products, at any given moment. As described, the vast majority of host microbial contact is symbiotic or innocuous; however, the threat from overt enteric pathogens (e.g. Salmonella) is also constant. During co-evolution with constant bacterial contact, the intestine developed mechanism to perceive and respond to bacteria, including resident microbiota, and the occasional pathogen. While epithelial signal from Toll-like receptors and intracytoplasmic Nod proteins is well describe, members of the formyl peptide receptor family have only recently been implicated in epithelial response to bacteria. [21, 22] Originally described and characterized in mammalian phagocytes, we recently reported that epithelial cell can perceive commensal bacterial via epithelial FPRs, and activated the non proinflammatory MAPK ERK, [14] in contradistinction to the highly inflammatory and proapoptotic NF-κB and JNK pathways typically activated by TLR on Nod signaling. Because of the FPR dependence of commensal mediated ERK signaling, we asked if other aspect of FPR dependant phagocyte function, i.e. ROS generation, was also present in epithelial cells.

FPRs and FPR signaling

Formyl peptide receptors (FPRs) are seven transmembrane receptors first described in neutrophils and macrophages. [10-12] The best characterized FPR ligands are formyl peptides, prokaryotic translation products modified with N-formyl-methionine, though

FPRs are known to be promiscuous in ligand recognition. FPRs are represented in humans by the originally characterized FPR1 and the closely related FPR2/ALX. FPR1 is the high affinity receptor for its best characterized ligand, N-formyl-Met-Leu-Phe (fMLF) and FPR2/ALX the low affinity receptor. [12] Upon ligand binding in phagocytes, FPRs undergo a conformational change that causes the recruitment of G proteins of the G_i family. This signaling initiates 1) changes in actin dynamics and initiation of chemotaxis, 2) transcriptional upregulation of inflammatory effectors and cytokines, and 3) the activation of NADPH enzymes and ROS generation (respiratory burst). FPR activity is inhibited by pertussis toxin, a 105 kDa exotoxin from *Bordetella pertussis* that catalyzes the ADP-ribosylation of $G\alpha_i$ subunits of GPCRs and blocks downstream cellular signaling stimulated by this class of receptor, [23] and Boc2, a fMLF peptidomimetic, which binds both FPR1 and FPR2/ALX with high affinity and has been shown to specifically block fMLF/FPR binding in neutrophils [24, 25]. Intestinal epithelial cells have been shown to express both FPR1 and FPR2, which are restricted mainly to the apical aspect of intestinal epithelial cells and have been shown to be involved in beneficial wound healing and pro-proliferative responses in response to commensal bacteria and bacterial N-formyl translation products. [13, 14] Specifically, we previously showed fMLF activates PI3K signaling and subsequent Ca+ flux in a wound healing model, resulting in activation of cytoskeletal rearrangements. Additionally, we showed commensal bacteria and fMLF activated ERK signaling and resultant transcriptional response causing an increase in cell proliferation.

Induction of cellular ROS by bacteria

We have found ROS generated within epithelial cells stimulated by exogenous agents such as commensal bacteria, soluble fermentation products, sterile components of bacterial cell walls and N-formyl peptides. [6, 26] This process is likely highly analogous to the activation of high level of ROS (respiratory burst) in profession phagocytes. These events are also highly conserved, since ROS generation as a response to environmental stressors, including bacteria, is widely present in plants and lower metazoans. [27-30] Specifically, plants purposefully generate ROS as signaling molecules to control various processes including pathogen defense, programmed cell death, and stomatal behavior. Interestingly, in plant and lower metazoans, not only are ROS producing enzymes conserved, but ROS generation also appears to be regulated by GTPases, indicating the possibility of a conserved manner of receptor mediated ROS generation. Since mammals utilize FPRs for ROS production, it would be interesting to investigate the conservation of FPR expression and activity in other species.

ROS include superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals, and a variety of their reaction products. Several eukaryotic signaling proteins, including growth factors, hormones, and cytokines, are capable of stimulating ROS generation in various cell types including epithelial cells. [20, 31-34] Physiological generation of these molecules can derive from mitochondrial sources, by 5′-lipoxygenase, or dedicated enzymes homologous to the phagocytic NADP(H) oxidase (Phox). [35] As mentioned, the FPR dependant respiratory burst in phagocytes in mediated via the gp91phox complex (NOX2) in response to exposure to microorganisms or inflammatory mediators. NOX2 consists of the catalytic

subunit gp91phox, together with the regulatory subunits p22phox, p47phox, p40phox, p67phox and the GPTase Rac. Along with the phagocytic NADP(H) oxidase, there are seven human homologues, NADP(H) oxidases 1-5 and dual oxidases (DUOX) 1 and 2, expressed in a variety of tissues and activated by numerous stimuli. Specifically, in colonic epithelial cells highly express NOX1 and both DUOXs. [35] Experiments to implicate these non phagocytic NADPH oxidases are currently in progress.

Redox signaling

ROS are short-lived, highly localized molecules, with a small radius of action, properties that allow for specificity of signaling effects. ROS signaling can be transduced by a subset of sensor enzymes that are transiently inactivated by reversible oxidation of catalytic cysteine residues within the active sites. [36] Such enzymes include a variety of tyrosine phosphatases such as PTEN, antioxidants such as thioredoxins and peroxiredoxins, and, as we and others have shown members of the Ubc family of proteins, DUSP family of MAPK phosphatases. [6] Specifically, commensal induced ROS reduces inflammatory signaling both *in vivo* and *in vitro* through oxidative inactivation of Ubc12, which normally neddylates the IκB-α subunit of the NF-κB complex and targets IκB-α for proteosomal degradation. With IκB-α no longer targeted for degradation, NF-κB remains trapped in the cytosol by IκB-α, unable to translocate to the nucleus to activate transcription of inflammatory mediators. Additionally, commensal generated ROS aids in cell motility by oxidative inactivation of tyrosine phosphatases, LMW-PTP and SHP-2, responsible for desphosphorylation of FAK, allowing sustained activation of FAK, formation of focal adhesions, and subsequent *in vivo* and *in vitro* wound healing.

(unpublished data) Here, we show commensal induced ROS aids in sustaining ERK signaling and subsequent cell proliferative events through oxidative inactivation of DUSP3. There are sixteen mammalian DUSPs that show dephosphorylation activity toward MAPK, having a variable tissue distribution, of which ten target the ERK phosphorylation, indicative of a great deal of redundancy within the group. DUSP3 is also known as the human *Vaccinia* H1-related (VHR) phosphatase. [37, 38] DUSP3 is 185-amino acid (20 kDa) long protein and was cloned based on its homology with the *Vaccinia* virus H1 open reading frame, the first identified dual-specific phosphatase. Recently, DUSP3 has been shown to be important for regulating cell growth and differentiation. [39]

In humans, probiotics have been shown to greatly benefit gastrointestinal health. Specifically, probiotic therapy decreases inflammatory responses, [7] and are now being used in IBD clinical trials and have been clinically indicated in the remission of UC and pouchitis. [40, 41] By determining FPRs as receptors by which commensal bacteria use to signal directly to intestinal epithelial cells, as well as the mechanism used to decrease inflammation and augment barrier function in intestinal epithelial cells, may allow better therapies in fighting intestinal inflammatory diseases.

Materials and Methods

Reagents

H_2O_2 and NAC were purchased from Sigma-Aldrich. MG-262 was purchased from Biomol. Pertussis toxin (PTx) was purchased from Calbiochem (Darmstadt, Germany) and FPR antagonist *N-tert*-butoxycarbonyl-Met-Leu-Phe (Boc2) through MP Biomedicals (Aurora, OH).

Cell culture

Human intestinal epithelial cell line SK-CO15 was grown in high glucose (4.5 g/L) DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 15 mM HEPES (pH 7.4), 2 mM L-glutamine, and 1% nonessential amino acids as previously described. [13]

ROS detection

Epithelial cells colonized with *Lactobacillus rhamnosus* GG [5 x 10^7 cfu/ml] or fMLF [500 nM] for the indicated time were washed with HBSS and incubated in the dark with HBSS containing 5mM of CM-H2DCF-DA (Molecular Probes) for an additional 5 min as previously described. [6] To evaluate antioxidant or inhibitors of GPCRs or FPRs could block observed ROS generation, cells were pretreated with NAC (20mM), DPI (40mM), PTx (1µg/ml) or Boc2 (100ng/ml) 30 min prior to *L. rhamnosus* GG or fMLF treatment. All images were acquired using a confocal laser scanning microscope (Zeiss LSM 510) through an x20 objective. For DCF, images were captured using 488nM excitation and

515-540nM emission. Quantification of fluorescence intensity was determined using ImageJ software with treatments at 30 min.

Immunoblotting

Antibodies were obtained as follows: anti DUSP3, anti-myc, and phospho-ERK (Cell Signaling), β-actin (Sigma-Aldrich), and HRP-conjugated donkey anti-rabbit or sheep anti-mouse secondary antibody (GE Healthcare). Immunoblot was performed as previously described. [42] Nuclei were stained with To-Pro-3 iodide (Molecular Probes). Fluorescent images acquired by laser confocal microscopy through an x 63 objective (Zeiss).

Reporter gene assays

SK-CO15 cells were transiently transfected using Lipofectamine 2000 (Invitrogen Life Technologies) according to manufacturer's instructions. For luciferase reporter assays, cells were transfected with ERK dependent Elk1 reporter plasmids (Luciferase Trans-Reporting Systems, Stratagene) according to manufacturer's instructions. Following cell treatment, cells were lysed in reporter lysis buffer (Promega) and activity determined using the Dual Luciferase Reporter Assay System (Promega).

Quantitative RT-PCR analysis

Total RNA was extracted from either cultured SK-CO15 cells or colonic epithelial scrapings from 3 mice using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription reactions were done using QuantiTect Reverse Transcription kit (Qiagen,

Valencia CA) and PCR reactions undertaken using QuantiTect SYBR Green PCR kit (Qiagen). Primers used for PCR reactions include 18S (forward) 5'-gtaacccgttgaaccccatt-3', 18S (reverse) 5'-ccatccaatcggtagtagcg-3', hDUSP3 (forward) 5'-taaaaaccccaccatttgga-3' and hDUSP3 (reverse) 5'-cttcctgcttgtcttctgg-3'. DUSP3 gene expression were standardized against 18S transcript levels in the same sample, and experimental results recorded as fold increase relative to measurements PBS treated samples. PCR reactions were done in triplicate using two separate RNA preparations for each data point.

Plasmids and constructs

The coding sequence for DUSP3 was purchased from Open Biosystems (Huntsville, AL). The DUSP3 open reading frame was cloned into pCMV-myc to create pCMV-myc-*DUSP3*. A catalytically inactive form of DUSP3 was created (mDUSP3) where the cysteine residue at position 124 is substituted to an alanine; a mutation reported to render DUSP3 catalytically inactive.

Analysis of protein oxidation

DUSP3 redox state was monitored by electrophoretic mobility shift on SDS-PAGE under nonreducing conditions. Cell lysates were prepared using a buffer containing 20mM Tris-HCl (pH 7.4), 1% Triton X-100, 10mM N-ethylmaleimide (NEM), and Complete Mini protease inhibitor cocktail (Roche). The lysates were separated by SDS-PAGE in the presence or absence of βME and immunoblotted.

Mice

All murine experimental procedures were undertaken according to Emory University

guidelines for ethical treatment of animals. For analysis of colonic tissue by intrarectal (i.r.) treatment, 6- to 8-week-old B6 mice were anesthetized prior to administration of PBS, *L. rhamnosus* GG, or fMLF for 7 or 30 min. Subjects were euthanized, and tissues removed for analysis. The colon was opened along the mesenteric border, placed in 4% paraformaldehyde 20 min, and subsequent colon whole mount preparation performed as described below.

Colon whole mount preparation

Dissected murine tissues were fixed for 20 min in 4% paraformaldehyde, washed in PBS, permeabilized with 0.1% Triton X-100 for 5 min, and washed again. Samples were blocked in 5% normal goat serum for 1 hr before incubation with Rabbit anti-phospho-ERK (Cell Signaling) for 1hr at 37°C (or o/n at 4°C), and then with FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch). Cellular actin was stained with Alexa Flour® Phallodin-633 (Molecular Probes). Tissue was cut into 2 mm to 5 mm small pieces, mounted on slides and visualized by Laser Confocal Microscopy at 63X.

Reproducibility and data presentation

Differences of $p \leq 0.05$ using the student's t-test were considered significant. The results of statistical analyses are given in the figure legends.

Acknowledgements

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Figure Legends

Figure 1. fMLF induces the generation of ROS in cultured epithelial cells in an FPR dependent manner. A: CM-H_2DCF-DA [5 μM] detection of ROS in SK-CO15 cells treated with *fMLF [500 nM] over 45 min*. **B:** CM-H_2DCF-DA [5 μM] detection of *ROS* in SK-CO15 cells colonized with *L. rhamnosus GG [5 x* 10^7 *cfu/ml] over 45 min*. For A and B, fluorescence was measured at 40x by confocal laser scanning microscopy (Zeiss). **C:** Quantitative representation of ROS production in A at 30 min. **B:** Quantitative representation of ROS production in B at 30 min. For C and D, data is representative of three independent assays quantified with ImageJ software and is expressed in units of fluorescence.

Figure 2. Dampening of cellular ROS levels attenuates *L. rhamnosus* GG or fMLF induced ERK pathway activation and cellular proliferation. **A:** Immunoblot analysis for phospho-ERK in cultured SK-CO15 cells treated with NAC [20 μM] 30 min prior to stimulation with *L. rhamnosus* GG [5x10^7 cfu/ml] or fMLF [500 nM] up to 45 min. **B:** ERK pathway specific luciferase reporter gene assay from transfected SK-CO15 cells treated with NAC [20 μM] 30 min prior to *L. rhamnosus* GG [5x10^7 cfu/ml], fMLF [500 nM] or H_2O_2 [1 mM] stimulation.* = P< 0.05 ** = P< 0.001 **C:** EdU incorporation into cultured SK-CO15 cells treated with NAC [20 μM] prior to incubation for 12 h with *L. rhamnosus* GG [5x10^7 cfu/ml] or fMLF [500 nM]. To-Pro3 (blue), EdU (red). Confocal microscope magnification x 63 **D:** Quantitative representation of EdU positive cells in C. number of EdU positive cells per 10 fields of view at x20 for 3 replicates per treatment. * = P<0.05.

Figure 3. *L. rhamnosus* GG or fMLF upregulate DUSP3 mRNA and protein. **A:** Quantitative RT-PCR analysis of DUSP3 gene expression in cultured SK-CO15 cells stimulated with *L. rhamnosus* GG [5 x 10^7 cfu/ml] or fMLF [500 nM] for 30 min. PCR reactions were done in triplicate using two separate RNA preparations for each data point. Error bars represent SEM. SK DUSP3 qPCR. **B:** Immunoblot analysis for total DUSP3 in cultured SK-CO15 cells stimulated with *L. rhamnosus* GG [5x10^7 cfu/ml] or fMLF [500 nM] up to 1 hr. **C:** Quantitative RT-PCR analysis of DUSP3 gene expression in mouse colonic epithelial scrapings treated *in vivo* with 100 µl *L. rhamnosus* GG [10^7 cfu/ml] or fMLF [500 nM] for 30 min. PCR reactions were done in triplicate using two separate RNA preparations for each data point. Error bars represent SEM. **D:** Immunoblot analysis for total Duap3 in mouse colonic epithelial cell scrapings treated *in vivo* with 100 µl *L. rhamnosus* GG [10^7 cfu/ml] or fMLF [500 nM] for 30 min.

Figure 4. *L. rhamnosus* GG or fMLF- induced generation of ROS oxidizes DUSP3. **A:** SK-CO15 cultured cells transfected with vector control or plasmids harboring DUSP3 or mDUSP3 were assayed by immunoblot for tonic levels of phospho-ERK. **B:** ERK pathway specific luciferase reporter gene assay for tonic levels of ERK stimulation from SK-CO15 cells transfected with vector control or plasmids harboring DUSP3 or mDUSP3.* = P< 0.05 ** = P< 0.001. **C:** SK-CO15 cultured cells transfected with vector control or plasmids harboring DUSP3 or mDUSP3 or DUSP3 treated with NAC [20 µM] 30 min prior to stimulation with *L. rhamnosus* GG [5x10^7 cfu/ml] or fMLF [500 nM] up to 30 min and assayed for DUSP3 oxidation status by immunoblot for myc in non-

reducing conditions or phospho-ERK by immunoblot in reducing conditions. **D:** SK-CO15 cultured cells transfected with plasmid harboring DUSP3 treated with H_2O_2 [1 mM] for 30 min, then media removed and replaced with non-H_2O_2 containing media and assayed for DUSP3 oxidation status by myc immunoblot in non-reducing conditions or phospho-ERK by immunoblot in reducing conditions. In C and D, all cells were lysed in a buffer containing 10 mM NEM to prevent oxidation of cysteines during sample preparation. DUSP3 oxidation was monitored by changes in electrophoretic mobility. Ox-oxidized DUSP3, myc-reduced DUSP3.

Figure 5. Antioxidant pretreatment inhibits *L. rhamnosus* GG or fMLF induced phosphorylation of ERK in murine enterocytes. **A:** Immunofluorescence of phospho-ERK of intestinal whole mount preparations (as described in material and methods) in either baseline intestinal mucosa or pretreated in vivo with NAC [20 µM] 30 min prior to 100 µl *L. rhamnosus* GG [10^7 cfu/ml] or fMLF [500 nM] for 7 min. **B:** Immunoblot analysis for phospho-ERK in mouse colonic epithelial cell scrapings treated with NAC [20 µM] 30 min prior to *in vivo* with 100 µl *L. rhamnosus* GG [10^7 cfu/ml] or fMLF [500 nM] for 7 min.

FIGURES

Figure 1

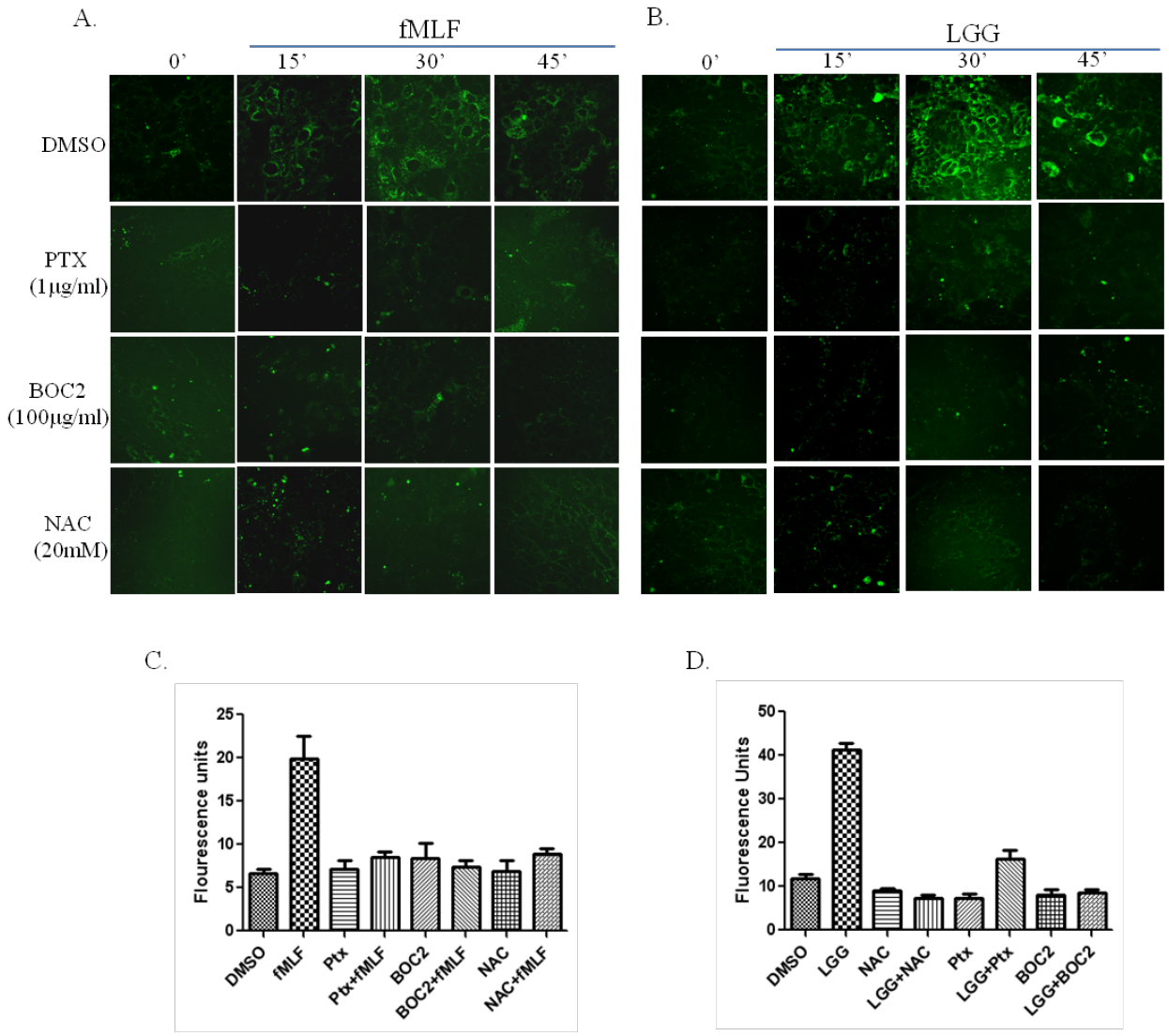


Figure 2

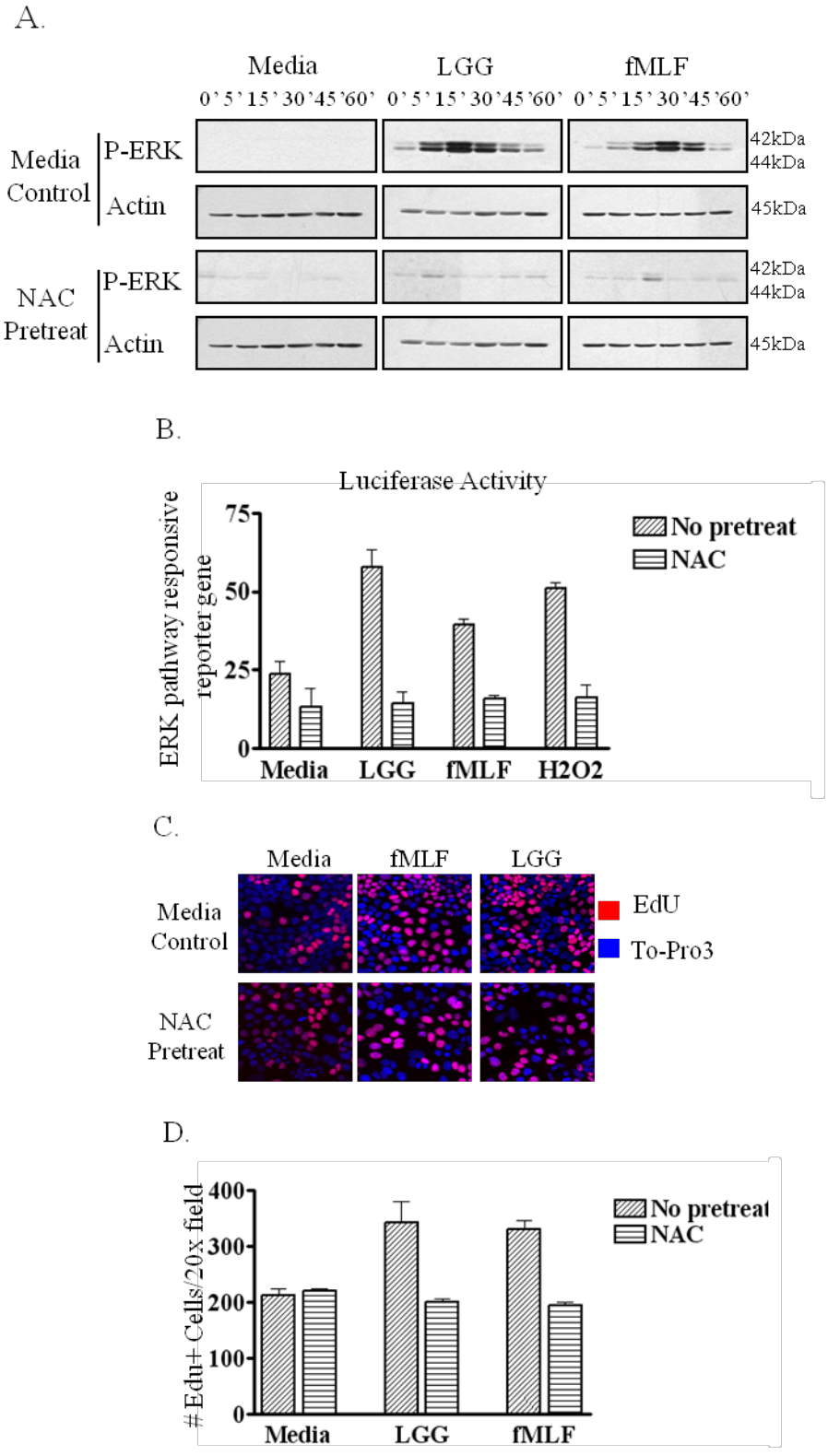
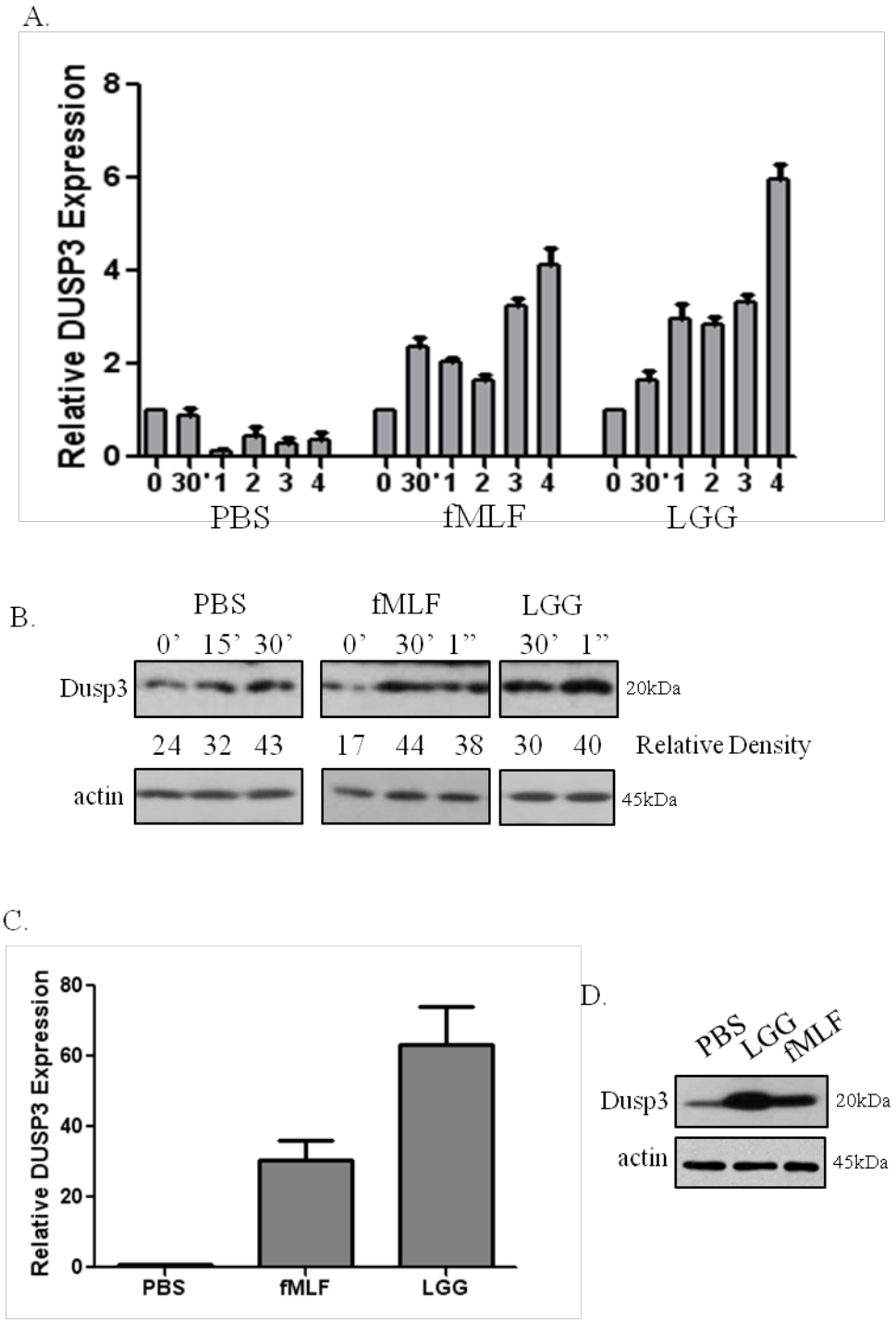

A.
Media
LGG
fMLF
0' 5' 15' 30' 45' 60'
Media Control
P-ERK
Actin
NAC Pretreat
42kDa
44kDa
45kDa
B.
Luciferase Activity
ERK pathway responsive reporter gene
No pretreat
NAC
Media
LGG
fMLF
H2O2
C.
EdU
To-Pro3
D.
Edu+ Cells/20x field

Figure 3


A.
Relative DUSP3 Expression
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4
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8
0 30' 1 2 3 4
0 30' 1 2 3 4
0 30' 1 2 3 4
PBS
fMLF
LGG
B.
PBS
fMLF
LGG
0' 15' 30'
0' 30' 1"
30' 1"
Dusp3
20kDa
24 32 43
17 44 38
30 40
Relative Density
actin
45kDa
C.
Relative DUSP3 Expression
0
20
40
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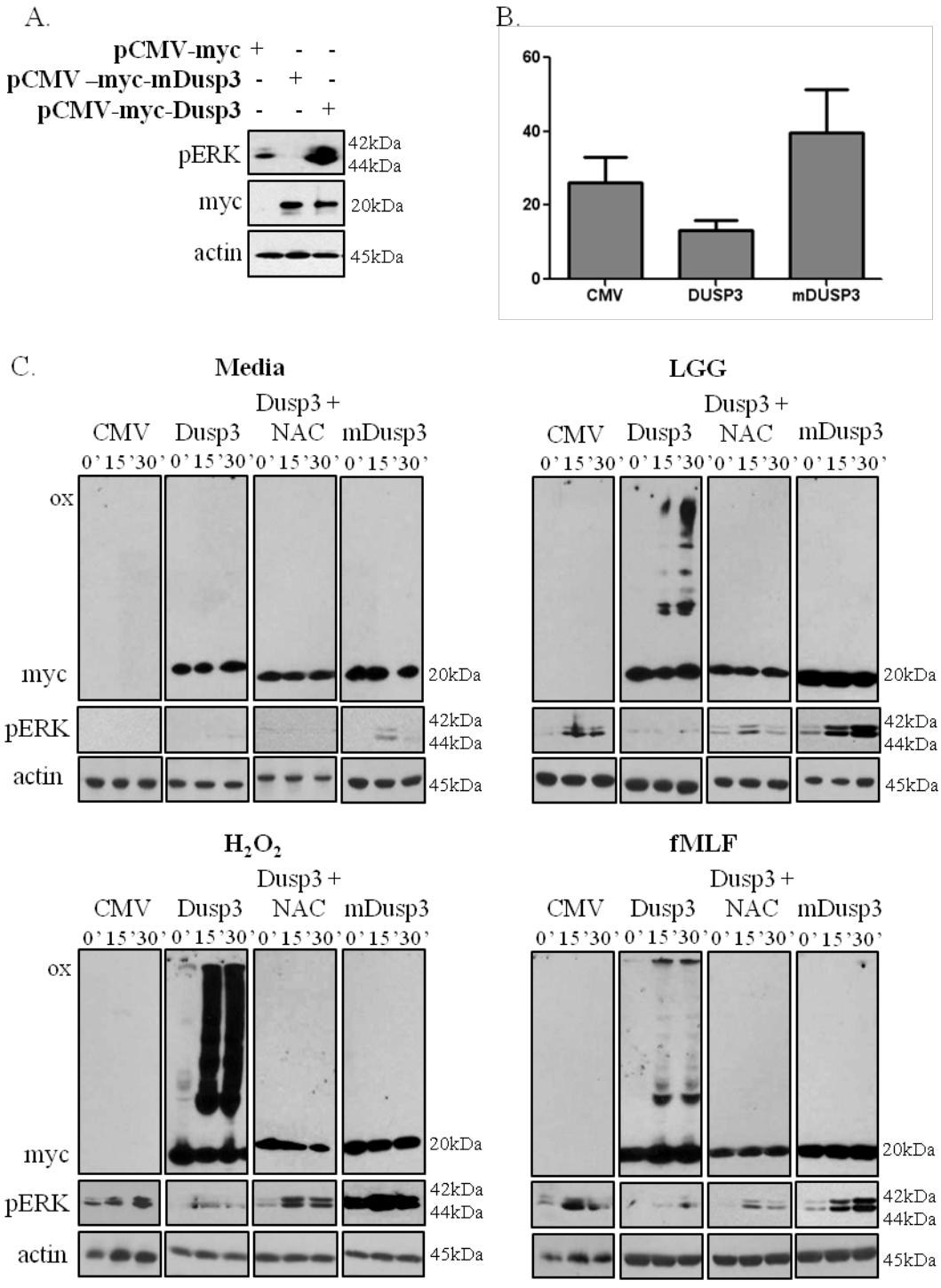
80
PBS
fMLF
LGG
D.
PBS
LGG
fMLF
Dusp3
20kDa
actin
45kDa

Figure 4


A.
pCMV-myc + - -
pCMV –myc-mDusp3 - + -
pCMV-myc-Dusp3 - - +
pERK
myc
actin
42kDa
44kDa
20kDa
45kDa
B.
60
40
20
0
CMV
DUSP3
mDUSP3
C.
Media
LGG
H2O2
fMLF
Dusp3 +
CMV Dusp3 NAC mDusp3
0' 15' 30' 0' 15' 30' 0' 15' 30' 0' 15' 30'
ox
myc
pERK
actin
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45kDa

Figure 4 cont.

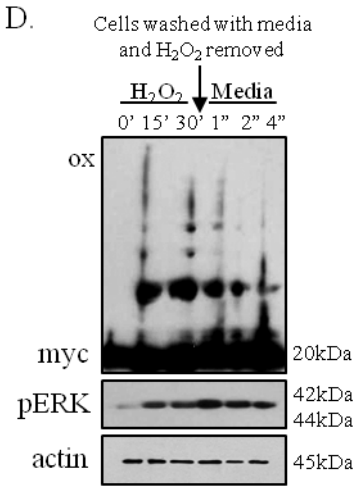
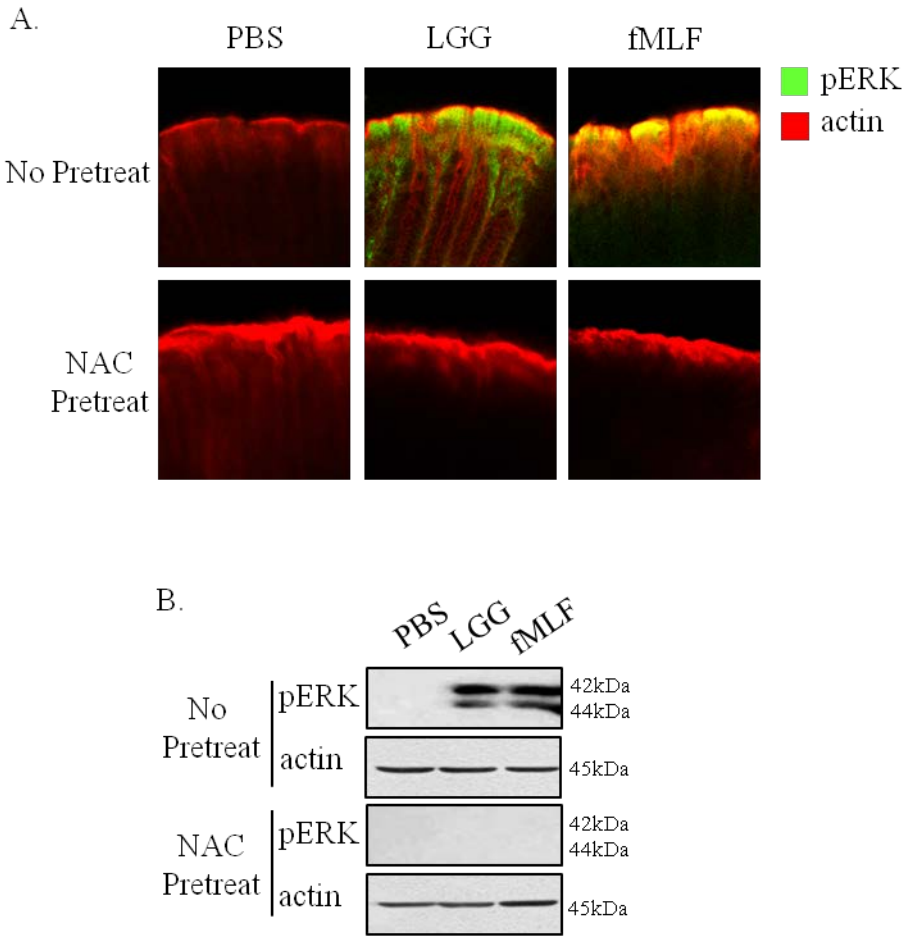


Figure 5


A.
PBS
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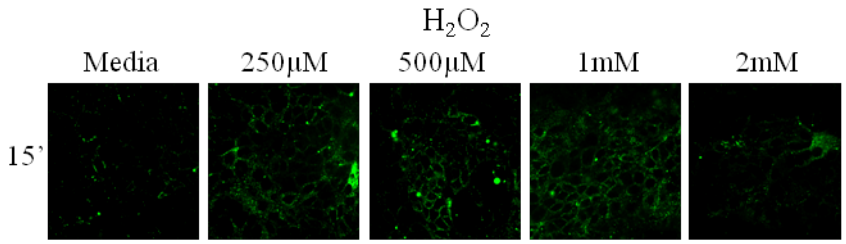
Supplemental Figure 1



Supplemental Figure Legend: *L. rhamnosus* GG or fMLF ROS production is approximate to 250 µM of H_2O_2. CM-H_2DCF-DA [5 µM] detection of ROS in SK-CO15 cells treated with H_2O_2 *at indicated concentrations.*

References

1. Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE: Metagenomic analysis of the human distal gut microbiome. Science. 2006, 312:1355-1359

2. Xu J, Mahowald MA, Ley RE, Lozupone CA, Hamady M, Martens EC, Henrissat B, Coutinho PM, Minx P, Latreille P, Cordum H, Van Brunt A, Kim K, Fulton RS, Fulton LA, Clifton SW, Wilson RK, Knight RD, Gordon JI: Evolution of symbiotic bacteria in the distal human intestine. PLoS Biol. 2007, 5:e156

3. Hooper LV, Bry L, Falk PG, Gordon JI: Host-microbial symbiosis in the mammalian intestine: exploring an internal ecosystem. Bioessays. 1998, 20:336-343

4. Neish AS: Microbes in gastrointestinal health and disease. Gastroenterology. 2009, 136:65-80

5. Park J, Floch MH: Prebiotics, probiotics, and dietary fiber in gastrointestinal disease. Gastroenterol Clin North Am. 2007, 36:47-63, v

6. Kumar A, Wu H, Collier-Hyams LS, Hansen JM, Li T, Yamoah K, Pan ZQ, Jones DP, Neish AS: Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. EMBO J. 2007, 26:4457-4466

7. Lin PW, Myers LE, Ray L, Song SC, Nasr TR, Berardinelli AJ, Kundu K, Murthy N, Hansen JM, Neish AS: Lactobacillus rhamnosus blocks inflammatory signaling in vivo via reactive oxygen species generation. Free Radic Biol Med. 2009, 47:1205-1211

8. Hooper LV, Gordon JI: Commensal host-bacterial relationships in the gut. Science. 2001, 292:1115-1118

9. Pull SL, Doherty JM, Mills JC, Gordon JI, Stappenbeck TS: Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. Proc Natl Acad Sci U S A. 2005, 102:99-104

10. Migeotte I, Communi D, Parmentier M: Formyl peptide receptors: a promiscuous subfamily of G protein-coupled receptors controlling immune responses. Cytokine Growth Factor Rev. 2006, 17:501-519

11. Rabiet MJ, Huet E, Boulay F: The N-formyl peptide receptors and the anaphylatoxin C5a receptors: an overview. Biochimie. 2007, 89:1089-1106

12. Ye RD, Boulay F, Wang JM, Dahlgren C, Gerard C, Parmentier M, Serhan CN, Murphy PM: International Union of Basic and Clinical Pharmacology. LXXIII. Nomenclature for the formyl peptide receptor (FPR) family. Pharmacol Rev. 2009, 61:119-161

13. Babbin BA, Jesaitis AJ, Ivanov AI, Kelly D, Laukoetter M, Nava P, Parkos CA, Nusrat A: Formyl peptide receptor-1 activation enhances intestinal epithelial cell restitution through phosphatidylinositol 3-kinase-dependent activation of Rac1 and Cdc42. J Immunol. 2007, 179:8112-8121

14. Wentworth CC, Jones RM, Kwon YM, Nusrat A, Neish AS: Commensal-epithelial signaling mediated via formyl peptide receptors. Am J Pathol. 2010, 177:2782-2790

15. Forman HJ, Maiorino M, Ursini F: Signaling functions of reactive oxygen species. Biochemistry. 2010, 49:835-842

16. Janssen-Heininger YM, Mossman BT, Heintz NH, Forman HJ, Kalyanaraman B, Finkel T, Stamler JS, Rhee SG, van der Vliet A: Redox-based regulation of signal transduction: principles, pitfalls, and promises. Free Radic Biol Med. 2008, 45:1-17

17. Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L: Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature. 2003, 422:442-446

18. Cerignoli F, Rahmouni S, Ronai Z, Mustelin T: Regulation of MAP kinases by the VHR dual-specific phosphatase: implications for cell growth and differentiation. Cell Cycle. 2006, 5:2210-2215

19. Lin PW, Nasr TR, Berardinelli AJ, Kumar A, Neish AS: The probiotic Lactobacillus GG may augment intestinal host defense by regulating apoptosis and promoting cytoprotective responses in the developing murine gut. Pediatr Res. 2008, 64:511-516

20. Kamata H, Honda S, Maeda S, Chang L, Hirata H, Karin M: Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell. 2005, 120:649-661

21. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E:

Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol. 2006, 8:1327-1336

22. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R: Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004, 118:229-241

23. Bokoch GM, Gilman AG: Inhibition of receptor-mediated release of arachidonic acid by pertussis toxin. Cell. 1984, 39:301-308

24. Gavins FN, Yona S, Kamal AM, Flower RJ, Perretti M: Leukocyte antiadhesive actions of annexin 1: ALXR- and FPR-related anti-inflammatory mechanisms. Blood. 2003, 101:4140-4147

25. Babbin BA, Lee WY, Parkos CA, Winfree LM, Akyildiz A, Perretti M, Nusrat A: Annexin I regulates SKCO-15 cell invasion by signaling through formyl peptide receptors. The Journal of biological chemistry. 2006, 281:19588-19599

26. Kumar A, Wu H, Collier-Hyams LS, Kwon YM, Hanson JM, Neish AS: The bacterial fermentation product butyrate influences epithelial signaling via reactive oxygen species-mediated changes in cullin-1 neddylation. J Immunol. 2009, 182:538-546

27. Ha EM, Oh CT, Ryu JH, Bae YS, Kang SW, Jang IH, Brey PT, Lee WJ: An antioxidant system required for host protection against gut infection in Drosophila. Dev Cell. 2005, 8:125-132

28. Kotchoni SO, Gachomo EW: The reactive oxygen species network pathways:an essential prerequisite for perception of pathogen attack and the acquired disease resistance in plants. J Biosci. 2006, 31:389-404

29. Pauly N, Pucciariello C, Mandon K, Innocenti G, Jamet A, Baudouin E, Herouart D, Frendo P, Puppo A: Reactive oxygen and nitrogen species and glutathione: key players in the legume-Rhizobium symbiosis. J Exp Bot. 2006, 57:1769-1776

30. Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B: Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic interaction. Plant Cell. 2006, 18:1052-1066

31. Mahadev K, Wu X, Zilbering A, Zhu L, Lawrence JT, Goldstein BJ: Hydrogen peroxide generated during cellular insulin stimulation is integral to activation of the distal insulin signaling cascade in 3T3-L1 adipocytes. J Biol Chem. 2001, 276:48662-48669

32. Mahadev K, Zilbering A, Zhu L, Goldstein BJ: Insulin-stimulated hydrogen peroxide reversibly inhibits protein-tyrosine phosphatase 1b in vivo and enhances the early insulin action cascade. J Biol Chem. 2001, 276:21938-21942

33. Chen KC, Zhou Y, Xing K, Krysan K, Lou MF: Platelet derived growth factor (PDGF)-induced reactive oxygen species in the lens epithelial cells: the redox signaling. Exp Eye Res. 2004, 78:1057-1067

34. Goldstein BJ, Mahadev K, Wu X, Zhu L, Motoshima H: Role of insulin-induced reactive oxygen species in the insulin signaling pathway. Antioxid Redox Signal. 2005, 7:1021-1031

35. Lambeth JD: NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol. 2004, 4:181-189

36. Reddie KG, Carroll KS: Expanding the functional diversity of proteins through cysteine oxidation. Curr Opin Chem Biol. 2008, 12:746-754

37. Guan KL, Broyles SS, Dixon JE: A Tyr/Ser protein phosphatase encoded by vaccinia virus. Nature. 1991, 350:359-362

38. Liu K, Lemon B, Traktman P: The dual-specificity phosphatase encoded by vaccinia virus, VH1, is essential for viral transcription in vivo and in vitro. J Virol. 1995, 69:7823-7834

39. Rahmouni S, Cerignoli F, Alonso A, Tsutji T, Henkens R, Zhu C, Louis-dit-Sully C, Moutschen M, Jiang W, Mustelin T: Loss of the VHR dual-specific phosphatase causes cell-cycle arrest and senescence. Nat Cell Biol. 2006, 8:524-531

40. Hart AL, Stagg AJ, Kamm MA: Use of probiotics in the treatment of inflammatory bowel disease. J Clin Gastroenterol. 2003, 36:111-119

41. Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, Kamm MA, Weismueller J, Beglinger C, Stolte M, Wolff C, Schulze J: Maintaining remission of ulcerative colitis with the probiotic Escherichia coli Nissle 1917 is as effective as with standard mesalazine. Gut. 2004, 53:1617-1623

42. Collier-Hyams LS, Zeng H, Sun J, Tomlinson AD, Bao ZQ, Chen H, Madara JL, Orth K, Neish AS: Cutting edge: Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. J Immunol. 2002, 169:2846-2850

CHAPTER IV: CONCLUSIONS

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The mammalian gastrointestinal tract has co-evolved with bacteria over millennia, and as a result has developed a mutually beneficial relationship where the host offers a nutrient rich environment for bacteria to flourish, and in return the commensal bacteria provide colonic energy sources, vitamin and micronutrient synthesis, augmented immunity, enhanced barrier function, and exclusion of pathogens from access to the epithelial surface. Recently, commensal bacteria have also been shown to stimulate physiological cellular turnover, attenuate inflammation, and enhance wound healing. Furthermore, commensal bacteria are utilized as probiotics in therapy for inflammatory diseases and aiding in premature intestinal development. How commensal bacteria signal to the intestinal epithelium to engender these effects is unknown. The aim of these studies was to elucidate a mechanism by which commensal bacteria directly interact and signal to the intestinal epithelium.

Chapter 2 of this study describes the identification of formyl peptide receptors (FPRs) as novel PRRs used by commensal bacteria to directly signal to the intestinal epithelia. FPRs were initially discovered in neutrophils and macrophages to sense N-formyl peptides. Activation of FPRs in neutrophils results in increased mobility (chemotaxis), transcriptional activation of cytokines, and ROS generation (respiratory burst). Recent investigations have demonstrated that FPRs are situated mainly on the apical surface of intestinal epithelial cells, directly in contact with the luminal contents. [1] Consistent with known effect of fMLF on neutrophil motility, contact of N-formyl peptides with the apical side of epithelial cells caused upregulation of cytoskeletal remodeling pathways

and an increase in the rate of monolayer migration *in vitro*. [2] In describing FPR as a novel PRR, we observed that commensal bacteria and N-formyl peptides directly activated ERK and Akt pathway signaling, and subsequent transcriptional responses, resulting in an increase in FPR dependent cellular proliferation. We confirmed direct commensal signaling through FPRs when stimulated cells were exposed to pertussis toxin and Boc2 and resulted in attenuation of ERK activity.

As a follow up, chapter 3 describes FPR dependent ROS generation as a mechanism for ERK activation. As previously stated, FPR initiated signaling in neutrophils results in the generation of ROS (respiratory burst). In intestinal epithelia, ROS can also be generated in response to commensal bacteria, though to a much lesser degree. ROS induction by commensal bacteria in intestinal epithelia aids in augmenting barrier function, wound healing, and attenuating inflammation, though microbicidal effects have not been reported. In this study, we observed that the generation of ROS in epithelia can also be induced by purified N-formyl peptides, suggesting FPR involvement in ROS generation in this cell type. When exposed to pertussis toxin or Boc2, ROS activity is greatly diminished indicating FPR involvement in inducing ROS generation in intestinal epithelial cells.

Chapter 3 also proposes a mechanism by which ROS modulated DUSP3 regulates MAPK ERK signaling. ROS modulation of signaling occurs by oxidation of critical sensor catalytic cysteine residues within certain enzymes. ROS has been shown to inhibit NF-κB inflammatory signaling in a manner in which enzymes that affect the IκB complex are

oxidatively inactivated. [3] We observed a decrease in ERK activity in the presence of ROS inhibitors, suggesting ROS redox regulation of ERK signaling. Intestinal cells treated with LGG or fMLF showed a significant increase in DUSP3, an ERK phosphatase, expression over four hours. Like the IκB modified enzymes, DUSP3 can be oxidatively inactivated by ROS modulation. We found that the DUSP3 was indeed oxidatively inactivated by the ROS induction by commensal bacteria. This indicates that though DUSP3 is being made, it is being oxidatively inactivated by ROS generation, thus allowing ERK activation to go unabated. Together, these data elucidate a mechanism of signal regulation so bacteria both repress any possible inflammatory response, as well as maintain the beneficial signaling to intestinal epithelia.

Overall, these studies show commensal bacteria signal to the intestinal epithelia via circuitry likely analogous to phagocyte signaling by FPR activation and subsequent downstream events (Figure 1), though there are several distinguishing features. Epithelial induced ROS generation is several fold lower compared to neutrophils and has a signaling aspect as opposed to a solely bactericidal effect. This study identifies a mechanism by which commensal bacteria regulate signaling in intestinal epithelial cells through ROS modulation of DUSP proteins (Figure 2). Epithelial FPR signaling does not induce transcription of proinflammatory cytokines or cellular inflammation, but rather induces ERK signaling, and subsequent cell proliferative responses. Significantly, as compared to other PRRs, such as TLRs, epithelial FPRs only activate proproliferative ERK/MAPK signaling without activating the typical proapoptotic p38 and JNK/MAPK

or proinflammatory NF-κB signaling. In all, our data are consistent with FPR acting as novel PRR in the intestinal epithelia.

FPR signaling is largely restricted to the apical surface of polarized intestinal epithelia, unlike TLR signaling which occurs mainly basolaterally or within the cytoplasmic vesicle. While PRR mediated signaling clearly has a central role in initiating cellular inflammation during infection by pathogens, it has also been determined that basal tonic TLR (and likely other PRR) mediated signaling in response to the commensal bacteria and their products are necessary for mucosal health. As such, supplementation of TLR ligands, such as CpG DNA and flagellin, has shown to provide cytoprotective effects in the intestine. [4, 5] Importantly, these effects underscore the importance of intestinal-bacterial interaction as a beneficial and necessary relationship. Consistently, the FPR related receptor GPR43 has recently been identified as a receptor for microbial short chain fatty acids and has a clear role in the resolution of intestinal inflammation. [6] Indeed, FPR2/ALX was initially characterized by its anti-inflammatory and pro-resolving actions, where germline mutations resulted in enhanced inflammation in a variety of models. [7] Thus, apical FRP mediated activation of ERK signaling and ROS generation may represent a novel pathway by which microbial signals are transduced to facilitate normal epithelial physiology as opposed to pathogen mediated inflammatory pathology mediated through intracytoplasmic and basolateral TLRs. As the normal microbiota (and therapeutic probiotic administration) is associated with epithelial growth and wound healing, therapeutic stimulation of the FPRs may be relevant to treating intestinal inflammation/injury.

The completion of these studies has generated a body of data in a previously understudied field. The potential impact of the knowledge generated here is substantial because identifying the molecular mechanisms of the beneficial influence of commensal bacteria on health is the next logical step in the research for the development of probiotics for the prevention or treatment of disease. This study advances the field by identifying the requirement of fMLF and ROS as signaling molecules in intestinal epithelial homeostasis. Current irrefutable data show overt ROS has a negative impact on tissue health. However, overuse of antioxidants and probiotics either as supplements to a preventative dietary regime, or as part of therapy practiced on patients recovering from gut injury, potentially interferes with physiological ROS and ERK levels that contribute positive influences on gut homeostasis. The knowledge generated from these studies will potentially direct practitioners to employ a more balanced approach to the use of antioxidants and probiotics.

Future work will be to identify other bacterial products that induce ROS generation and/or ERK signaling in epithelial cells. I have been involved in investigations that have identified purified components of bacterial peptidoglycan that when applied apically to polarized cultured cells induced the generation physiological ROS (Young-Man Kwon, Ph.D., personal communication). The identification of beneficial bacterial products will be the basis for the development of more focused preventive or therapeutic agents, as it would avoid the potential deleterious effects of microbial overgrowth following supplementation with live probiotics. In regards to the cellular signaling events induced

by commensal bacteria-generated ROS, recent studies has significantly advanced the field by identification of hyper-reactive cysteine residues within several hundred proteins, including MKK7, JNK2, ERK1, Myd88, and SOD1. [8] These intermediaries of signaling pathways govern cell fate, including proliferation/differentiation or apoptosis, and thus are candidate molecules to be modulated by commensal bacteria-induced generation of ROS. Additionally, we are currently investigating the NADP(H) oxidase (NOX) enzyme responsible for ROS generation. Both NOX1 and DUOX2 have been shown to be expressed in intestinal epithelial cells, specifically in the colon. [9, 10] Whether NOX1 alone is responsible for intestinal ROS production or acts in concert with DUOX2 is also unknown.

The current investigations are a springboard to attaining the long-term goal to identify the cellular signaling pathways, the cellular receptors, and the bacterial proteins or products that mediate commensal bacteria-facilitated promotion of gut epithelial homeostasis. Collectively these investigations will continue to grow and evolve in important directions because they directly relate to the prevention or treatment of disease.

In future directions, we speculate that understanding how commensal bacteria interact with the human body will be imperative to maintenance of human health. The human body has coevolved in constant contact with trillions of bacteria, with only a fraction of those being pathogenic. In determining that bacteria directly signal to the host epithelia to elicit beneficial effect, this study has a profound discovery that is important for understanding basic human functions. In elucidating commensal interactions with the

human host, a whole new field, with commensal bacteria being just as necessary for human survival as humans are for bacterial survival, has opened.

Figure Legend

Figure 1: Neutrophil v. Intestinal epithelial FPR signaling.

Figure 2: Intestinal epithelial FPR model.

Figures

Figure 1

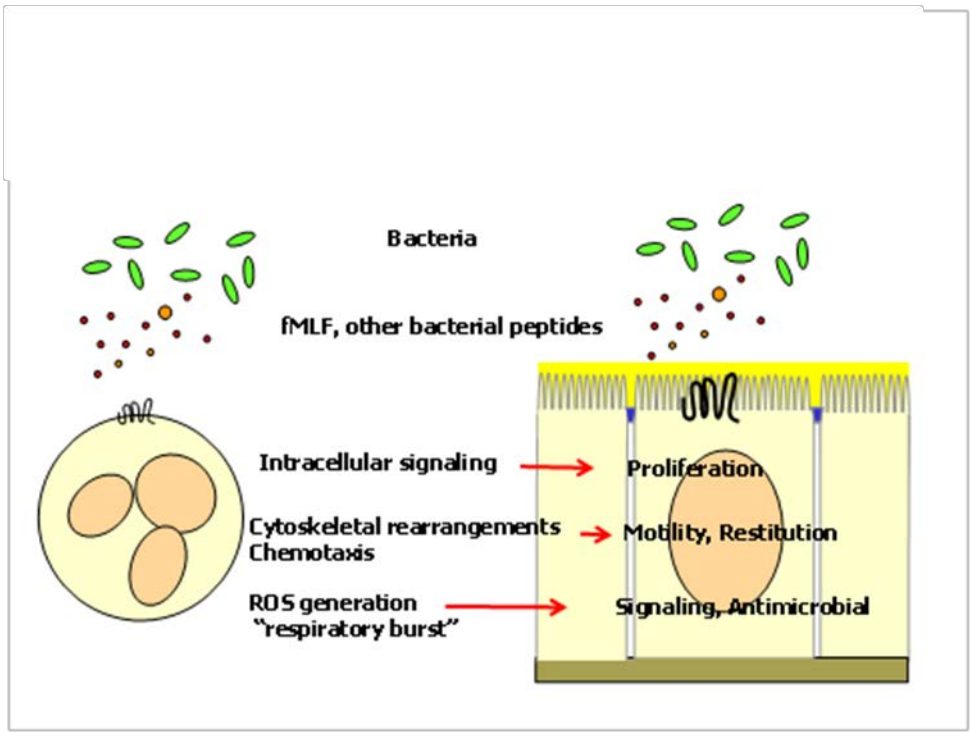
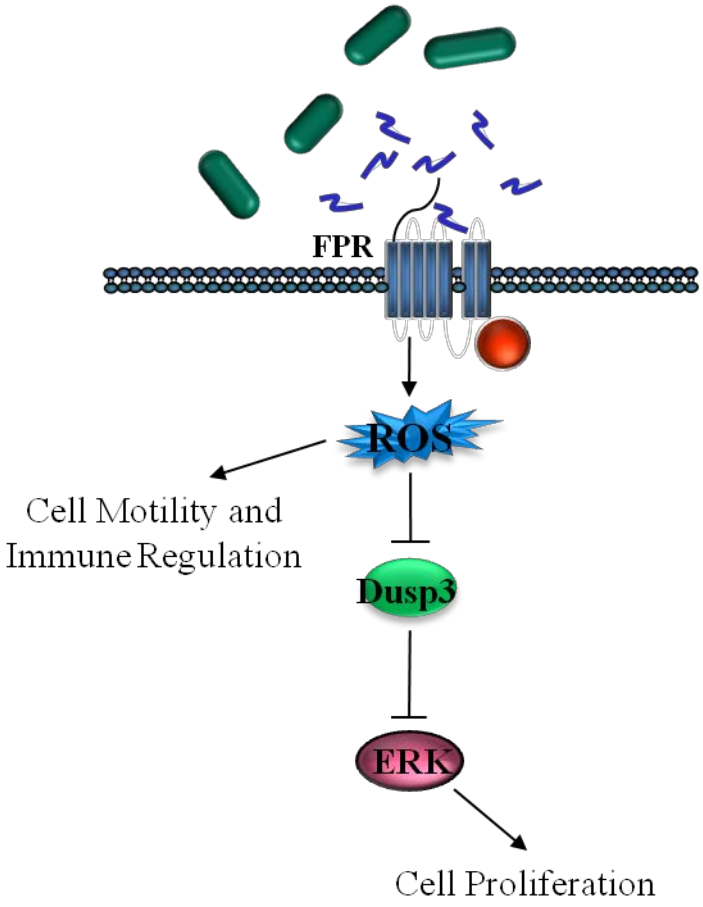


Figure 2


FPR
ROS
Cell Motility and
Immune Regulation
Dusp3
ERK
Cell Proliferation

References

1. Babbin BA, Lee WY, Parkos CA, Winfree LM, Akyildiz A, Perretti M, Nusrat A: Annexin I regulates SKCO-15 cell invasion by signaling through formyl peptide receptors. J Biol Chem. 2006, 281:19588-19599
2. Babbin BA, Jesaitis AJ, Ivanov AI, Kelly D, Laukoetter M, Nava P, Parkos CA, Nusrat A: Formyl peptide receptor-1 activation enhances intestinal epithelial cell restitution through phosphatidylinositol 3-kinase-dependent activation of Rac1 and Cdc42. J Immunol. 2007, 179:8112-8121
3. Kumar A, Wu H, Collier-Hyams LS, Hansen JM, Li T, Yamoah K, Pan ZQ, Jones DP, Neish AS: Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. EMBO J. 2007, 26:4457-4466
4. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R: Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. Cell. 2004, 118:229-241
5. Lee J, Mo JH, Katakura K, Alkalay I, Rucker AN, Liu YT, Lee HK, Shen C, Cojocaru G, Shenouda S, Kagnoff M, Eckmann L, Ben-Neriah Y, Raz E: Maintenance of colonic homeostasis by distinctive apical TLR9 signalling in intestinal epithelial cells. Nat Cell Biol. 2006, 8:1327-1336
6. Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D, Schilter HC, Rolph MS, Mackay F, Artis D, Xavier RJ, Teixeira MM, Mackay CR: Regulation of inflammatory responses by gut microbiota and chemoattractant receptor GPR43. Nature. 2009, 461:1282-1286

7. Serhan CN, Chiang N, Van Dyke TE: Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol. 2008, 8:349-361

8. Weerapana E, Wang C, Simon GM, Richter F, Khare S, Dillon MB, Bachovchin DA, Mowen K, Baker D, Cravatt BF: Quantitative reactivity profiling predicts functional cysteines in proteomes. Nature. 2010, 468:790-795

9. Cheng G, Lambeth JD: Alternative mRNA splice forms of NOXO1: differential tissue expression and regulation of Nox1 and Nox3. Gene. 2005, 356:118-126

10. El Hassani RA, Benfares N, Caillou B, Talbot M, Sabourin JC, Belotte V, Morand S, Gnidehou S, Agnandji D, Ohayon R, Kaniewski J, Noel-Hudson MS, Bidart JM, Schlumberger M, Virion A, Dupuy C: Dual oxidase2 is expressed all along the digestive tract. Am J Physiol Gastrointest Liver Physiol. 2005, 288:G933-942

APPENDIX I:

Salmonella AvrA coordinates suppression of host immune and apoptotic defenses via JNK pathway blockade

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SUMMARY

Salmonellae have evolved sophisticated evasion strategies to escape host immune responses via the secretion of preformed effector proteins into mammalian cells. These effector proteins usurp innate immune and apoptotic signaling pathways thus allowing *Salmonellae* to avoid elimination. We show that the secreted *Salmonella typhimurium* effector protein AvrA possesses acetyltransferase activity toward MAPKKs and can mediate repressive effects on conserved innate immune and pro-apoptotic responses. Using both transgenic *Drosophila* and murine models, AvrA mediated potent inhibition of JNK and NF-κB pathway signaling. Furthermore, we show *Salmonella* AvrA suppresses both the epithelial innate immunity phase of *Salmonella* interaction with the intestinal mucosa, while simultaneously preventing the apoptotic "fail safe' cells have evolved to respond to irreversible threats. This activity is consistent with the natural history of *Salmonella* in mammalian host, where the bacteria pass through but do not destroy epithelial cells, and may suggest other intracellular pathogens target JNK signaling to dampen apoptosis.

INTRODUCTION

All multi-cellular organisms possess the ability to respond to and manage microbial threats. This process involves transmembrane pattern recognition receptors that perceive the macromolecular signatures of microbes and set in motion cytoplasmic signaling cascades, which subsequently activate nuclear transcription factors and ultimately induce the transcriptional synthesis of anti-microbial, pro-inflammatory and cyto-protective genes that serve to eradicate microbes and limit/repair damage. Alternatively, and often in parallel, microbial stresses induce apoptotic signaling, which in certain short lived cell types -such as intestinal epithelia- also serve to eradicate invasive pathogens. This framework of recognition, response and repair, or focal apoptotic cell loss, has examples in plants, invertebrates and mammals.

Key signaling pathways involved in these defenses against microbes include the mitogen activated protein kinase (MAPK) pathways. The MAPKs function in a triple kinase sequence that involves the rapid and controlled relay of phosphorylation events to convey “alarm” signals (Roux and Blenis, 2004). Perception of diverse threats induces the upstream activators, kinases of the MAPKKK class. These proteins serve to activate the MAPKKs (or MEKKs), which subsequently activate the MAPKs and a downstream battery of immune and cell survival effector systems. MAPKKs such as MKK6 and MKK3 phosphorylate and activate members of the ERK and p38 MAPKs respectively, which mediate primarily proliferative and cytoprotective responses. In contrast, the MAPKK MKK4/7, activates Jun N-terminal kinase (JNK), which is proinflammatory or during prolonged activation, potently pro-apoptotic (Weston and Davis, 2007). In parallel,

kinases of the IκB kinase family (IKKs) -which are closely structurally related to the MAPKKs- set in motion the NF-κB/Rel pathway by phosphorylation of IκB. This leads to proteasomal mediated degradation of IκB and subsequent activation of NF-κB by nuclear translocation and induction of innate immune and anti-apoptotic gene products. The involvement of MAPK family members in cellular responses to microbes are highly conserved across eukaryotic life, including animal and plants. Recognizable orthologs of the mammalian MAPKs, IKKs and Rel factors are well described in vertebrates and arthropods (Hoffmann, 2003; Lemaitre and Hoffmann, 2007; Silverman and Maniatis, 2001).

Given the central role MAPKs and Rel pathways play in antimicrobial signaling, it is not surprising that some bacterial pathogens have evolved mechanisms to surmount their effects. Bacterial inhibition of NF-κB has been described at the level of IκB phosphorylation (Ruckdeschel et al., 1998), nuclear translocation (Kelly et al., 2004), and in past data from our laboratory, at the level of IκB ubiquitination (Neish et al., 2000). Inhibition of MAPKs has been described in *Yersinia*, *Shigella* and anthrax infections (Duesbery et al., 1998; Li et al., 2007; Orth et al., 1999). It is generally assumed that bacterial pathogens evolved these capacities to prevent or reduce the upregulation of the inflammatory response, thus blocking the influx of phagocytic leukocytes, and/or to activate or augment apoptotic pathways in immunoregulatory cells such as macrophages– both events likely permissive for bacterial proliferation and dissemination.

Bacteria can influence host cell signaling pathways via soluble effector proteins that are often translocated into the host cells or the environment via a “type III secretion apparatus” (Staskawicz et al., 2001). The effector proteins are generally assumed to usurp host cellular functions for the benefit of the invading organism, and consequently are often designated as toxins or “virulence factors”. One family of secreted effector proteins with inhibitory effects on MAPKs is the YopJ/AvrA family. These proteins have been detected in a variety of bacteria that associate intimately with eukaryotic hosts. YopJ of *Yersinia enterocolitica* was shown to inhibit both multiple MAPK and NF-κB pathways, inducing rapid apoptotic death in infected macrophages (Orth et al., 1999; Ruckdeschel et al., 1998). Orthologs of this protein are found in other enteric pathogens such as *Vibrio parahemalyticus*, VopA (Trosky et al., 2004) and *Aeromonas salmonicida*, AopP (Fehr et al., 2006), and in a spectrum of plant pathogens (Ellis et al., 2007). Intriguingly, an ortholog is present in Rhizobia sp, a nitrogen-fixing plant symbiont (Freiberg et al., 1997), suggesting that members of this class of proteins are found in a wide variety of bacteria that mediate both pathogenic and symbiotic relationships – presumably by manipulating host innate immune signaling pathways.

Another member of this family is AvrA, a *Salmonella* protein that is translocated into intestinal epithelial cells during the initial stages of invasion (Hardt and Galan, 1997). We have shown that AvrA overexpressed in transfected cells blocked NF-κB translocation and transcriptional activation of inflammatory effector genes (Collier-Hyams et al., 2002). To further understand its effects on cellular signaling pathways in vivo, we have exploited directed expression of AvrA in *Drosophila melanogaster*.

Unlike experiments previously carried out with cultured mammalian cells, *Drosophila* allows investigation of the physiological effects of AvrA in an intact animal, through well-defined genetic approaches using tissue specific and inducible expression systems. We show that AvrA is a potent inhibitor of the *Drosophila* IMD pathway in response to Gram negative infection, but only marginally affects the Toll pathway, which is activated in response to Gram positive or fungal infection. Remarkably, immune blockade occurs without induction of apoptotic cell death characteristically seen during inhibition of host stress signaling pathways. We show that AvrA expression resulted in a potent blockade of JNK activation at the level of the MAPKK MEKK4/7 in both flies and human cells, and inhibited JNK mediated apoptosis. Consistently, we show that AvrA$^-$ *Salmonella* induced higher levels of inflammation, JNK activation and epithelial apoptosis in infected murine intestine. These data suggests that the AvrA effector can inhibit innate immune responses in a eukaryotic host, without the induction of apoptosis, and indicates how an intracellular pathogen can “quiet” both inflammatory and apoptotic defenses.

EXPERIMENTAL PROCEDURES

Plasmids and constructs

pCMV-myc-*avrA* and pCMV-myc-m*avrA* are described previously (Collier-Hyams et al., 2002). The mAvrA mutant is a catalytically inactive form of AvrA, in which the cysteine residue at position 186 is substituted to an alanine. A plasmid harboring the *aopP* coding sequence was a gift from Joachim Frey. A catalytically inactive form of AopP (mAopP) was created by overlap extension PCR where the cysteine residue at position 177 was substituted to an alanine. The *aopP* and m*aopP* coding sequences were cloned into pCMV-myc, creating pCMV-myc-*aopP* and pCMV-myc-m*aopP* respectively. Plasmids harboring *vopA* or m*vopA* coding sequences were a gift from Kim Orth. The *vopA* and m*vopA* coding sequences were cloned into pCMV-myc, creating pCMV-myc-*vopA* and pCMV-myc-m*vopA* respectively. pcDNAflag-*JNK1* and pcDNAflag-*MKK7* were a gift from Prof. Roger Davis. The JNK1 coding sequence was cloned into pCMV-myc to facilitate simultaneous detection of JNK1 and MKK7 expression in lysates of transfected cells. DNA amplicons of myc-*avrA*, myc-m*avrA*, myc-*aopP*, myc-m*aopP*, myc-*vopA* or myc-m*vopA* were cloned into pP[UAST] (a gift from Kevin Moses) creating pP[UAST]-myc-*avrA*, pP[UAST]-myc-m*avrA*, pP[UAST]-myc-*aopP*, pP[UAST]-myc-m*aopP*, pP[UAST]-myc-*vopA* or pP[UAST]-myc-m*vopA* respectively.

Drosophila lines.

The vectors pP[UAST]-myc-*avrA*, pP[UAST]-myc-m*avrA*, pP[UAST]-myc-*aopP*, pP[UAST]-myc-m*aopP*, pP[UAST]-myc-*vopA* and pP[UAST]-myc-m*vopA* were micro-injected into W^{1118} embryos, creating fly lines harboring UAS-myc-*avrA*, UAS-myc-

m*avrA*, UAS-myc-*aopP* or UAS-myc-m*aopP* UAS-myc-*vopA* or UAS-myc-m*vopA* respectively. Other fly stocks used include rel^{E20}, spz^{rm7}, DD1 (*y, w, P(ry+, Dpt-lacZ), P(w+, Drs-GFP)*), UAS-d*TAK* and UAS-*eiger*.

Experimental infection

Infections were performed by stabbing adult *Drosophila* or third instar larva with a thin tungsten needle dipped in a concentrated pellet of *Erwinia carotovora*, *Eschericia coli*, or *Micrococcus luteus*. Fungal spore infections were done using the natural fly pathogen *Bauvaria bassiana. Cultures of B. bassiana were propagated in fly vials, whereupon* anesthetized flies were passaged into the vial and covered in fungal spores by shaking the vial for 2 min. Survival rate were determined by counting survivors at time intervals. Larval infection was undertaken by parenteral infection with microbially infected needle. Infected larvae were immediately placed in a Petri dish containing water saturated tissue paper to avoid desiccation. The fat bodies of 10 larvae were dissected and combined in 50μl of SDS PAGE loading buffer before immunoblot analysis.

Quantitative RT-PCR analysis

Total RNA was extracted from the abdomens of 10 adult *Drosophila* using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription reactions were done using QuantiTect Reverse Transcription kit (Qiagen, Valencia CA) and PCR reactions undertaken using QuantiTect SYBR Green PCR kit (Qiagen). Primers used for PCR reactions include rpl32(forward) 5'-cagtcggatcgatatgctaagctg-3', rpl32(reverse) 5'-taaccgatgttgggcatcagatac-3', Dipt(forward) 5'-ttcaccattgccgtcgccttactt-3', Dipt(reverse)

5’-ccaccgccgcctccctgaa-3’, Dros(forward) 5’-gccgactgcctgtccggaagatacaag-3’ and Dros(reverse) 5’-ttagcatccttcgcaccagcacttcagact-3’. Dipterecin and Drosomycin gene expression were standardized against rpl32 transcript levels in the same sample, and experimental results recorded as fold increase relative to measurements in uninfected *Drosophila*. PCR reactions were done in triplicate using two separate RNA preparations for each data point.

LacZ reporter assay and GFP determination

The abdomens of 10 four day old gram-negative or gram positive bacteria infected adult female *Drosophila* harboring DD1 (y, w, P(ry+, Dpt-lacZ), P(w+, Drs-GFP)); *yolk*-GAL4; UAS-*avrA* were homogenized in assay buffer. GFP determination was done by measuring fluorescence using a Hitachi Fluorescence spectrophotometer F-4500 (Hitachi, Danbury, CT). Values for β-galactosidase activity and GFP determinations were normalized with respect to protein concentration. Assays were done in triplicate using two separate extract preparations for each data point.

Cell culture and Transfection

Human 293T or HeLa cells were used for transfections. Cells were cultured in Dulbecco’s modified Eagle media with 10% heat inactivated calf serum at 37ºC and 5% CO_2. Plasmids were transfected using Lipofectamine^{TM} 2000 Reagent (Invitrogen, Carlsbad, CA). Luciferase reporter gene assays measuring activation of JNK, p38 or ERK signaling pathways were done using the PathDetect c-Jun *trans*-Reporting System for JNK, the PathDetect CHOP *trans*-Reporting System for p38 and the PathDetect Elk1

trans-Reporting System for the ERK according to manufacturer's instructions (Stratagene, La Jolla CA).

Antibodies and Reagents

Antibodies against phospho-JNK, phospho-p38, phospho-ERK, phospho-IκB, phospho MKK4, JNK, IκB and actin were purchased from Cell Signaling Technology (Danvers, MA) and anti-myc was purchased from Clontech (Mountain View, CA). Active Caspase-3 for larval eye disk and histological staining was purchased from Cell Signaling whilst active Caspase-3 for immunoblot was obtained from Active Motif (Carlsbad CA). Antibodies against *Drosophila* tubulin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-cactus 3H12 antibody was obtained from the Developmental Studies Hybridoma Bank developed under the support of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. The anti-Relish N-terminus antibody used for both immuno staining was a gift from Svenja Stöven, Umea University, Sweden. The anti-DIF antibody was a gift from Ylva Engström and Gunnel Björklund, Stockholm University, Sweden. DNA was stained with SYTO24 (Molecular Probes). Secondary antibody incubations were done using goat anti-rabbit Cy5 or goat anti-mouse FITC (Jackson ImmunoReserach, West Grove PA).

Immuno blot assays and Immunohistochemistry.

For Cactus immunoblot analysis, the fat body of 10 third instar larvae were dissected and placed in 50µl cell lysis buffer. Immunoreactive species were detected using anti-rabbit HRP or anti-mouse HRP followed by visualization with ECL chemiluminescence

detection reagent (GE Healthcare Biosciences Piscataway NJ). For immuno-staining procedures, third instar larvae fat bodies, or immaginal eye disks were dissected in PBS and fixed in 4% paraformaldehyde for 20 min. The tissues were washed 3 x 10 min in 0.1% Triton X-100, and then placed in blocking solution (1% Goat serum in 0.1% Triton X-100) for 30min. The tissues were incubated in primary antibody for 1 h at 37°C with gentle rocking, before being washed 3 x 10 min in 0.1% Triton X-100, and incubated in the secondary antibody at 4°C overnight.

Mass Spectrometry analysis.

HeLa cultured cells were transfected with pCMV-mycMKK4 and pcDNA-flagAvrA. Forty-eight hours after transfection, cells were lysed, and MKK4 immunoprecipitated with anti-myc agarose beads. The purified MKK4 preparations were resolved by SDS/PAGE and the gel stained by Imperial^{TM} protein stain (Pierce). Gel slices containing MKK4 were excised, washed and digested with trypsin. Peptides were separated by nanoLC-MS/MS using an Ultimate 3000 nanoHPLC system (Dionex) on a 0.3 x 5 mm C18 PepMap cartridge with flow rate set at 300 nL/min (Dionex). The eluate was directly sprayed into a Q-STAR XL (LC-MS/MS) and an MS spectrum acquired followed by up to 4 MS/MS spectra based upon observed ions in the MS spectrum. The data was processed by ProteinPilot v2.0 (Applied Biosystems) using the SWISSPROT database as the protein database source with user defined modifications for O-acetylated serine and threonine programmed into the search. Critical MS/MS spectra were manually examined and confirmed. For the inclusion of non-activity data for lysates of pCMV-mycMKK4 alone, or pCMV-mycMKK4 co-transfected with pcDNA-flag<u>m</u>AvrA, we encouraged

missed-cleavages (by diluting trypsin 20-fold combined with a 2-h digestion) to include the theoretical $T^{261}R^{262}$ tryptic dipeptide such that we obtain an amino acid fragment of sufficient length for MS analysis. No such problems were encountered with obtaining a fragment of sufficient length over this area when MKK4 was acetylated at residues K^{260} and T^{261}.

Murine Infections

The creation of an *avrA* null of *Salmonella* SL3201 mutant is previously described (Collier-Hyams et al., 2002). Pathogen-free female BALB/cj mice (6 to 8 weeks) were procured from Jackson Laboratories (Bar Harbor, ME). Animals were deprived of food and water for 4 hours before treatment with 7.5 mg of streptomycin by oral gavage. Afterward, animals were supplied with food and water *ad libitum*. At 20 hours post streptomycin treatment, water and food were withdrawn again for 4 hours before the mice were infected with 10^8 CFU wild type *Salmonella* SL3201, the isogenic *avrA*$^-$ mutant form of SL3201, or the mutant complemented with plasmid expressed AvrA. Construction of these mutants are as described in (Collier-Hyams et al., 2002). At 6h post infection, blood samples were collected and serum KC levels were determined using a mouse CXC chemokine KC DuoSet kit according to manufacturer's instructions (R&D Systems, Minneapolis, MN). Thereafter, ceca were removed and neutrophil infiltration into tissue quantified by measuring MPO enzyme activity (a marker for neutrophils) by biochemical assay. Histological sections of ceca were prepared from 5 infected animals per treatment. Sections were assessed for active caspase-3 activity by numeration by the average number of positive cells in forty 200X fields per infected animal. Animal

experiments were approved by the Emory University institutional ethical committee and performed according to the legal requirements.

For ex-vivo analysis of phospho-JNK activation in murine gut, small intestines were removed from 8-week-old BALB/cj mice, and 2cm segments immediately placed in Hanks buffer at 37°C. The segments were then infected with *Salmonella* cultures (10^9 CFU/ml) for time periods up to 1hour. Infected and control segments were opened along the mesenteric border, and epithelial tissue scraped and lysed by homogenization in RIPA buffer (100 mg tissue per milliliter of buffer) and centrifuged at 16 000 r.p.m. for 20 min at 4°C. Protein concentrations of supernatants were determined by protein assay (Bio-Rad) and equal amounts of protein were diluted with denaturing SDS–PAGE loading buffer and assayed by immunoblot.

RESULTS

AvrA expression in the Drosophila *fat body suppresses Imd pathway activation*

To investigate AvrA function in the context of a whole animal, we generated transgenic *Drosophila* harboring either wild type *Salmonella typhimurium* protein AvrA or a catalytically inactive mutant form of AvrA (mAvrA) (C186A transition) under the transcriptional control of the UAS promoter, allowing tissue specific expression by crossing to GAL4 driver lines (Brand, 1994) Appropriate expression was determined by immunoblot analysis of fly tissue (Figure S1A in the Supplemental Data available with this article online). No toxicity was observed with AvrA expression in larval or adult animals. We examined whether expression of AvrA in the *Drosophila* fat body (the site of fly systemic immune response) could suppress innate immunity. Flies expressing AvrA had a greater mortality rate compared to flies expressing mAvrA following parenteral Gram-negative infections with *Escherichia coli* or *Erwinia carotovora* (Figure 1A), while control and mAvrA expressing flies had similar mortality rates. Flies expressing AvrA infected with either the Gram-positive bacteria *Micrococcus luteus* or *Bauvaria basiana* fungal spores did not result in a detectable reduction in survival rates (Figure 1A), suggesting specificity of immunosuppression toward Gram-negative bacteria.

Fat body expression of AvrA inhibited the *Erwinia carotovora* stimulated upregulation of transcripts encoding the anti-Gram negative specific peptide Diptericin (Figure 1B), and also inhibited a Diptericin promoter driven reporter construct (Figure 1C) relative to the increased expression of Diptericin measured in control or mAvrA expressing flies. AvrA

expression had a minimal inhibitory effect on the *Micrococcus luteus* stimulated upregulation of the anti-Gram positive-peptide Drosomycin (Figure 1B) or a Drosomycin reporter construct (Figure 1C).

To directly implicate AvrA activity on *Drosophila* immune signaling, we evaluated the activation of the Toll pathway transcription factor Dif and the *imd* transcription factor Relish in response to immune challenge. Upon activation, Dif and Relish are translocated to the nucleus where they initiate the upregulation of anti-microbial peptides production (Diagrammed in Figure S2) (Lemaitre and Hoffmann, 2007). Larvae expressing either AvrA or mAvrA in their fat bodies were parenterally infected with either Gram negative or Gram-positive bacteria. Relish translocation was completely inhibited in AvrA expressing tissue following Gram-negative infection (Figure 1D) while AvrA had no inhibitory effect on Dif translocation (Figure 1E). Collectively, these data show that AvrA expression in the fat body of adult *Drosophila* selectively interferes with responses to Gram-negative infection (IMD pathway).

AvrA expression is not pro-apoptotic and inhibits JNK/Bsk phosphorylation and Caspase-3/Drice activation in response to dTAK or Eiger over expression.

The IMD/Relish pathway is tightly intertwined with apoptotic signaling intermediates such as the c-Jun N-terminal Kinase (JNK) (Liu and Lin, 2007). Furthermore, inhibition of Rel pathways is known to potently stimulate pro-apoptotic pathways in many systems and cell types. *Drosophila* offers an exquisitely sensitive system for assay of direct pro-apoptotic potential of expressed proteins by ectopic expression to the developing retina.

We thus used the GMR-Gal4 driver to express AvrA immediately posterior to the morphogenetic furrow, allowing a spatial and temporal evaluation of apoptotic effects on the epithelial cells of the developing retina (Figure S3A). Surprisingly AvrA (and the mutant form) had no discernable effects on gross appearance of the adult eye, or any evidence of cell death (Figure 2A). This is in contrast to the "rough eye" phenotype induced as a result of the expression of two paralogs of AvrA; AopP from *Aeromonas* (Figure S3B and Figure 2A) which has been reported to exhibit inhibitory effects on NF-κB signaling but having no effect on MAPK signaling (Fehr et al., 2006), and the *Vibrio* protein VopA (Figure S3C and Figure 2B), reported to block ERK and JNK MAPK signaling while having no effect on NF-κB signaling (Trosky et al., 2007; Trosky et al., 2004). Importantly, the rough eye phenotype exhibited by AopP could be reversed by overexpression of *Drosophila* Inhibitor of Apoptosis (dIAP) and the IAP-like molecule p35, confirming this protein is directly pro-apoptotic (Figure 2A). Additionally, the VopA mediated rough eye was reversed upon co-expression of activated Rolled (*Drosophila* ERK), confirming the ERK inhibitory role of VopA and showing that AvrA does not have inhibitory affects on the ERK MAPK pathway (Figure 2B). We also found that AvrA had no direct apoptotic effect when transfected in mammalian cell culture, even when cells were treated with TNF-α, whereas consistent with previous reports, cells transfected with YopJ exhibited an increased number of cells with an apoptotic phenotype (Figure S4A), TUNEL (Figure S4B and S4C) and annexin V positivity (Figure S4D). Thus, we conclude that AvrA does not possess any direct pro-apoptotic activity, in striking contrast to paralogs encoded by other pathogens.

In flies and mammals, a key regulator of both inflammatory and apoptotic signaling is the MAPKKK TAK-1 (Diagrammed in Figure S2) (Sato et al., 2005; Vidal et al., 2001). In flies, TAK-1 is a central regulator of the IMD immune pathway, and constitutive expression of TAK-1 signaling is pro-apoptotic (Takatsu et al., 2000). Immune mediated (and TAK-1 dependant) apoptosis in flies and mammals can be mediated by prolonged and unopposed JNK signaling, events that can be physiologically antagonized by phosphatases induced via simultaneous and parallel Rel pathway activation, to allow immune responses in the absence of apoptosis (Kamata et al., 2005; Park et al., 2004). Third instar retinal imaginal disk constitutive expression of dTAK-1 resulted in a strong small eye phenotype consistent with past reports (Leulier et al., 2002) (Figure 2C). Strikingly, AvrA coexpressed with dTAK-1 nearly completely suppressed the small eye phenotype, while mAvrA had no effect (Figure 2C). *Drosophila* Eiger/Wengen, the ortholog of mammalian TNF/TNF-R, is a potent and specific activator of JNK signaling and apoptosis (Moreno et al., 2002). Constitutive expression of Eiger in the retina resulted in an extreme small eye phenotype, consistent with past reports (Igaki et al., 2002) (Figure 2E). Constitutive expression of Eiger, an extra cellular cytokine, has the advantage of activating signaling without potential artifacts caused by intracellular constitutive expression of a cytoplasmic kinase. Again, AvrA expression was able to totally suppress this phenotype, while the mutant form did not. TAK-1 is a MAPKKK that functions as a JNK-KK. Hemipterous (MKK4/7 in mammals) is the subsequent MAPKK (JNK-K) in the JNK pathway (Diagrammed in Figure S2). Constitutive expression of Hemipterous (UAS-hep) or Basket (UAS-bsk) in the *Drosophila* eye did not activate the JNK pathway and did not result in a small eye phenotype. However,

concurrent constitutive expression of UAS-hep and UAS-bsk under GMR-GAL4 strongly activates the JNK pathway and is lethal in *Drosophila* at the pupal stage of development. AvrA expression in the GMR-GAL4 UAS-hep UAS-bsk genetic background resulted in a viable adult *Drosophila* exhibiting a mild rough eye phenotype (Fig 2G), whereas expression of mAvrA could not rescue the lethal phenotype. Finally, a constitutively active allele of Hemipterous (UAS-hep(act)) (Amino acid replacement: S326D and T330D in the kinase activation loop) also mediated a rough eye phenotype when driven by GMR-GAL4. Importantly, this phenotype could not be reversed by AvrA expression (Figure 2G). Together, these epistatic data indicate that AvrA inhibits the JNK pathway at the level of Hemipterous (MKK4/7) in *Drosophila*.

To confirm that apoptotic inhibitory effects were due to JNK pathway blockade, we subjected third instar eye imaginal disks to immunostaining with anti-phospho-JNK antibodies. As expected, both eye disk specific TAK-1 and Eiger constitutive expression resulted in a strong activation of phospho-JNK immediately distal to the morphogenetic furrow that was concurrent with appearance of active capase-3 (Figure 2C-F). Both phospho-JNK and active caspase-3 activity was suppressed by AvrA expression, but not mutant AvrA. Finally, levels of phosphorylated JNK in flies expressing AvrA in the fat body following Gram-negative infection were analyzed by immunoblot of fat body lysates. There was only a trace increase in phosphorylated JNK 30 minutes post infection in flies expressing AvrA in the fat body compared to wild type flies or those expressing mAvrA (Figure 2H), indicating AvrA could suppress microbially stimulated JNK signaling.

AvrA expression represses the JNK pathway in mammalian cell culture.

To determine if our findings showing inhibition of JNK signaling extended to a mammalian system, we expressed AvrA and mAvrA in human 293T cells. Transfection efficiency was estimated at >80% by transfecting a plasmid harboring GFP coding sequence. Cells transfected with plasmids harboring AvrA or mAvrA, as well as YopJ (which served as positive control) were stimulated with TNF-α and cell lysates were analyzed by immunoblot for various endogenous signaling intermediates. While JNK was phosphorylated within 15 minutes of TNF-α addition in mAvrA and vector only control, a complete blockade of JNK phosphorylation occurred in cells transfected with AvrA (Figure 3A). Note that unphosphorylated JNK increased in cells transfected with AvrA but not mutant, and JNK immunoreactivity accumulated upon TNF-α treatment. In contrast, AvrA had no effect on TNF-α induced phosphorylation of ERK or p38. Inhibition of TNF-α induced phosphorylation of ERK, JNK and to a lesser extent p38 occurred in cells transfected with YopJ (Figure 3A), whereas cells transfected with mYopJ had similar profiles to mAvrA transfected cells (data not shown). Additionally, AvrA had no affect on TAK-1 phosphorylation in response to TNF-α stimulation (Figure 3B) indicating that AvrA inhibits the JNK pathway in mammalian cells distal to TAK-1.

Also, AvrA expression resulted in partial stabilization of IκB-α (Figure 3C), while having little or no effect on TNF-α induced IκB-α phosphorylation consistent with past experiments (Collier-Hyams et al., 2002). Finally, AvrA, but not mAvrA expression could repress JNK dependant reporter gene expression while having no measurable effect

on p38 or ERK pathway signaling. In comparison, YopJ exhibited the expected inhibition of the JNK, p38 and ERK pathways (Figure 3D). Thus, AvrA is a potent inhibitor of the JNK pathway, with partial effects on the NF-κB pathway.

AvrA inhibits JNK pathway activation at the level of MKK4/7 by acetylation.

In *Drosophila*, AvrA rescued dTAK1 mediated rough eye phenotype, but not the small eye phenotype caused by overexpression of an activated form of Hemipterous (*Drosophila* MKK4/7). Additionally, AvrA did not inhibit TAK1 phosphorylation. This suggests AvrA inhibits the JNK pathway at the level of the MAPKK. Thus, we probed TNFα stimulated cell lysates with an antibody against phospho-MKK4 and found that activation of this JNK kinase was significantly reduced (Figure 3A). Furthermore, we activated the JNK pathway in human cultured cells with plasmids harboring JNK1 and MKK4 or MKK7 respectively. Previous reports have shown co-transfection of JNK1 and MKK7 induced JNK phosphorylation (Lei et al., 2002) AvrA cotransfection, but not mAvrA could totally abolish both MKK4 or MKK7 mediated JNK phosphorylation (Figures 4A and 4B) thus indicating that AvrA mediates JNK pathway inhibition at the level of MKK4 and MKK7.

Previous reports have showed that the AvrA homologue YopJ mediates inhibition of the MAPK pathway by acting as an acetyltransferase, using acetyl–coenzyme A (CoA) to modify both Ser and Thr residues in the activation loop of MKK6 and IKKβ thereby blocking phosphorylation (Mittal et al., 2006; Mukherjee et al., 2006). In order to determine whether AvrA has similar acetyltransferase activity on MKK4, we analyzed

tryptic digests from lysates obtained by the co-transfection of pCMV-mycMKK4 with pcDNA-flagAvrA by mass spectrometry. Data revealed an increase of 42 amu at K^{260} and Thr^{261} active site residue of the MKK4 consistent with an O-acetylation of an amino acid residue (Figure 4C and Figure 4D). We found no evidence of acetylation at the Ser^{257} active site residue in tryptic digests including this residue. Control experiments where MKK4 was transfected with vector alone or catalytically inactive mAvrA showed no active site residue modification (Figure 4E and Figure 4F). Thus, AvrA can act as an acetyltransferase in the manner described for other members of this family (Mittal et al., 2006; Mukherjee et al., 2006). Also note the constitutively active form of *Drosophila* Hemipterous (MKK4/7) could not be suppressed by AvrA (Figure 2G), presumably because the T to D transition could not be O-acetylated.

Salmonella *AvrA mediates suppression of epithelial innate immunity and apoptosis during natural infection*

To evaluate the effect of AvrA in natural infection in mammals, we turned to a recently developed model of intestinal Salmonellosis using streptomycin pretreated mice. This model has been widely validated and has been used by ourselves and others as a system that faithfully replicates the acute inflammatory events on enteric Salmonellosis (Barthel et al., 2003; Vijay-Kumar et al., 2006). We used wild-type *Salmonella* SL3201, an $AvrA^-$ isogenic *Salmonella* mutant bearing a non-polar insertional mutation in the chromosomal *avrA* gene, and the $AvrA^-$ mutant complemented with wild-type $avrA^+$ ($AvrA^-$ /pAvrA). Murine infection with the $AvrA^-$ *Salmonella* resulted in elevated levels of serum KC (the

murine ortholog of the neutrophil specific chemoattractant IL-8) (Figure 5A), relative to infection with isogenic wild type *Salmonella* or the *avrA*$^{+}$ complemented mutant, again consistent with AvrA mediating a repression of innate immune signaling. Additionally, increased neutrophil influx into the intestinal mucosa was seen in mice infected with AvrA^{-} *Salmonella* as measured by myeloperioxidase (MPO) (Figure 5B), a histochemical marker of neutrophils.

We next sought to determine effects of AvrA on epithelial apoptosis in the context of natural infection. Infection with wild-type *Salmonella* or the *avrA*$^{+}$ complemented AvrA^{-} mutant resulted in scattered apical caspase-3 and TUNEL positive cells, consistent with our previous observations (Vijay-Kumar et al., 2006) (Figure 5C and Figure 5D). Strikingly, the isogenic AvrA^{-} mutant elicited a marked increase in both caspase-3 and TUNEL positive cells at this time point. From this data, we conclude that *Salmonella* AvrA serves to suppress both epithelial innate immunity and apoptosis during the initial phase of *Salmonella* interaction with the intestinal mucosa.

Finally, we evaluated the effects of AvrA on JNK pathway activation during initial events of infection using a murine mucosal ex vivo model. Intestinal segments were infected for 5 minutes with either wild-type *Salmonella*, AvrA^{-} mutant *Salmonella*, or the AvrA^{-} mutant complemented with wild-type *avrA*$^{+}$. Infection with the AvrA^{-} *Salmonella* resulted in elevated phospho-JNK levels in cells at the apical regions of the intestinal villi whereas infection with the other bacteria did not (Figure 6A). Additionally, immunoblot analysis of scrapings from intestinal mucosa showed increased levels of phospho-JNK

following infection by AvrA$^-$ mutant bacteria, compared to the wild type or the *avrA*$^+$ complemented AvrA$^-$ mutant (Figure 6B). Collectively, these data indicate AvrA suppresses phospho-JNK activation during the initial phase of *Salmonella* contact with intact epithelia.

DISCUSSION

Drosophila has been a workhorse of modern biology for nearly a century. The ease of working with this species, and the "genetic tractability" developed over years has led to the use of *Drosophila* in many areas of experimental biology, including development, cell biology, neuro- and behavioral biology. However, only recently has this organism been recognized to possess an anti-microbial defense system with striking functional and structural homology to mammalian innate immune networks, thus allowing this model system to be applied to problems in innate immunology (Lemaitre and Hoffmann, 2007). Recently, genetic approaches to the study individual prokaryotic or viral effector molecules have been attempted (Guichard et al., 2006; Jia et al., 2006; Leulier et al., 2003) and have shown utility in elucidating effects of non-eukaryotic proteins on an intact animal.

In this report, we examined AvrA in a transgenic *Drosophila* genetic model. Our experiments revealed that AvrA mediated the selective inhibition of the IMD pathway in *Drosophila*. *Drosophila* expressing AvrA were highly susceptible to Gram negative infection, did not upregulate the Gram negative specific peptide Diptericin, and failed to activate the Gram negative specific Relish factor (assayed by cytoplasmic to nuclear translocation) upon bacterial challenge. The selective inhibition of the IMD pathway, with its specificity toward Gram negative bacteria, is consistent with the hypothesized function of a *Salmonella* effector protein, itself a Gram negative pathogen. The IMD pathway, unlike the Toll pathway, requires activation of MAPKK signaling intermediates, including Ird5 (*Drosophila* IKK-β) (Silverman et al., 2000) and Kenny

(*Drosophila* IKK-β) (Rutschmann et al., 2000). IMD pathway inhibition by AvrA is highly consistent with the proposed activities of the AvrA/YopJ effectors as inhibitors of the MAPKK class of enzymes. Additionally, our data supports previous reports where infection of *Drosophila* mbn-2 cultured cells with *Salmonella typhimurium* dampened AMP activation via inhibition of the Imd/Relish pathway (Lindmark et al., 2001).

The effects of the *Salmonella typhimurium* protein AvrA on cellular innate immune signaling has been studied using mammalian cell based assays (Collier-Hyams et al., 2002). These experiments showed that overexpressed AvrA suppressed the canonical NF-κB pathway by inhibiting nuclear translocation of the p50/p65 Rel proteins resulting in blockade of NF-κB dependant promoter activation. These observations are concordant with the AvrA mediated inhibition of Relish translocation and suppression of Rel dependant diptercin activation in the fat body of *Drosophila*, illustrating the conservation of AvrA mediated effects on this pathway. In both our past manuscript (Collier-Hyams et al., 2002) and the current work, we did not see inhibition of IκB-α phosphorylation but did observe stabilization of IκB-α. This is similar to data obtained with over expressed YopJ, which while mediating marked inhibition of IKK-β kinase activation and subsequent stabilization of the IκB-α protein, showed with only minimal effects on endogenous IκB-α phosphorylation (Mittal et al., 2006), possibly suggesting that IKKs have functional targets in the NF-κB pathway distinct from the classic dual serine motif of IκB-α.

There are three orthologs of AvrA with described activities toward the MAPKK family and IKKs, and all serve to repress innate immune responses. YopJ of Yersinia has a wide range of inhibitory activities against ERK, p38, JNK, and IKK (Orth et al., 1999), VopA of *Vibrio parahemalyticus*, which has been shown to inhibit ERK, p38 and JNK, but not IKK (Trosky et al., 2007; Trosky et al., 2004), and AopP of *Aeromonas salmonicida*, which is apparently specific to IKK (Fehr et al., 2006). Our data is highly supportive of the concept that these proteins can differentially and variably inhibit members of the MAPKKs and IKKs. In biochemical and functional analysis directly comparing the ability of YopJ and AvrA to repress the spectrum of MAPK pathways in mammalian cells, we showed that AvrA was especially inhibitory to JNK, with lesser activity toward IKK, while YopJ repressed all three MAPK pathways and IKKs. These observations indicate that bacteria can impinge on innate immune pathways by targeting multiple different MAPKKs, highlighting the interrelated role these pathways have on immune signaling.

Our most unexpected and striking finding was the ability of AvrA, unique among this class of effectors, to suppress apoptosis. AvrA did not result in apoptosis in *Drosophila* fat body constitutively or during systemic infection (data not shown). AvrA did not induce apoptosis during constitutive or induced expression in *Drosophila* eye or wing discs, in striking contrast to the expression of AopP which was directly and potently pro-apoptotic in these tissues and in transfection experiments in mammalian cell culture. (YopJ has been shown to have a similar effect to AopP in publicly shown but unpublished data). We show YopJ to be potently pro-apoptotic in cultured mammalian

cells in the presence of TNF-α, again in contrast to AvrA, which had no pro-apoptotic activity even in the presence of this cytokine. We previously had reported that infection of cultured HeLa cells with AvrA mutants in the background of *Salmonella* strains/species that are non-proinflammatory in human cells (*Salmonella typhimurium* PhoP^{c}, and *S. pullorum*) resulted in an increase in the apoptotic cell population (Collier-Hyams et al., 2002). However, Ye et al described reduced apoptosis in murine intestine infected with these strains (Ye et al., 2007). While these in vivo experiments did not use a proinflammatory pathogenic wild type host strain, the data is consistent with AvrA mediating an anti-apoptotic role and suggests results obtained in cell culture represent a situation idiosyncratic to in vitro systems. In the context of proinflammatory enteropathogenic Salmonellosis, our results suggest AvrA represses apoptosis during the epithelial step of the infectious process.

As mentioned, members of the YopJ-like family of proteins are well described as having differential inhibitory effects on substrates at the MAPKK level (Mittal et al., 2006; Mukherjee et al., 2006; Trosky et al., 2007). This selectivity of substrate specificity likely accounted for the striking differential effects on apoptosis. In general, JNK is thought of as a pro-apoptotic pathway, while ERK and p38 (and NF-κB) are understood as mediating survival signals (Weston and Davis, 2007). Our data is supportive of this concept, with AvrA as a JNK inhibiter acting as a survival (or non-pro-apoptotic) effector protein. Furthermore, we show AvrA is able to repress apoptotic signaling from constitutively active Eiger and the JNK-KK TAK1, consistent with the known pro-apoptotic role of sustained JNK activation (Kamata et al., 2005).

Recent biochemical studies showed YopJ and VopA acts as an acetyltransferase, using acetyl–coenzyme A (CoA) to modify critical serine and threonine residues of target MAPKKs and thereby blocking phosphorylation and activation of the protein (Mittal et al., 2006; Mukherjee et al., 2006; Trosky et al., 2007). The reported biochemical target of VopA coincides with functional repression exhibited in our genetic data showing VopA mediated ERK pathway inhibition at the level of dSor (*Drosophila* MKK6). Our molecular data strongly suggested that AvrA inhibits JNK signaling at the level of the JNK-K (MKK4/7 in mammals, Hemipterous in *Drosophila*). Consistently, we used mass spectroscopy to evaluate recombinant MKK4 when coexpressed with AvrA, demonstrating the novel O-acetylation on the Thr residue of the MKK4 activation loop, as expected for this class of bacterial proteins. That a modification of the activation loop is responsible for the apoptotic repressive action of AvrA is also strongly supported by genetic data showing a constitutively active JNK-K (Hemipterous) –which is defined by a phospho-mimetic mutation of the Thr in the activation loop -causes a rough eye that cannot be suppressed by AvrA, while JNK-KK (TAK) mediated rough eye is suppressed (Figure 2G). Other reports have concluded that the YopJ/AvrA proteins function as deubiquitinating enzymes (Sweet et al., 2007; Ye et al., 2007; Zhou et al., 2005). Such a biochemical activity may occur in parallel with acetyltranferase mediated inhibition, and is not inconsistent with our observed effects on signaling pathways.

The overall paradigm of defensive response or altruistic cell loss –as well as the associated biochemical signaling pathways- is highly conserved across metazoans, including mammals and invertebrates (Hoffmann, 2003; Silverman and Maniatis, 2001;

Weinrauch and Zychlinsky, 1999). Collectively, our data show complete concordance of the ability of AvrA to block specific MAPKK mediated signaling, immune activation and apoptosis in widely disparate eukaryotic in vivo systems. The AvrA protein apparently allows the invading bacterium to dampen innate immune signaling, but also preventing the apoptotic elimination in cells that have perceived microbial compromise. This ability would be expected from an intracellular pathogen that does not result in marked epithelial damage characteristic of other enteric pathogens such as *Shigella* and EHEC (Sansonetti and Di Santo, 2007).

Conversely, the greater pro-apoptotic activity of the AvrA ortholog in *Yersinia*, YopJ (Mukherjee et al., 2006) (ascribed to its more promiscuous range of MAPKK inhibitory activity) is consistent with that organism's ability to rapidly kill phagocytic cells and mediate a more virulent infection. It appears *Salmonella* has refined a similar biochemical activity to mediate a more benign cellular consequence. Indeed the existence of related biochemical effectors in plant symbionts (Staskawicz et al., 2001) illustrates the exquisite fine tuning of host responses that bacteria can engender.

Supplemental data

The supplemental data include Supplemental figures and can be found with this article at www.cellhostandmicrobe.com

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FIGURE LEGEND

Figure 1. AvrA expression in the *Drosophila* fat body suppresses innate immunity in response to Gram-negative, but not Gram-positive infection. (A) Survival rates of the flies expressing AvrA (*c564*-GAL4 UAS-*avrA*, or of the isogenic fly harboring UAS-m*avrA*) following parenteral Gram-negative *E. carotovora* or *E. coli* infection, and Gram-positive infection *Micrococcus luteus* or natural *Bauvaria bassiana* fungal infection. Relish mutant flies (rel^{E20}) susceptible to Gram-negative and *spatzle* (*spz*) null flies susceptible to fungal infections were used as controls. Results represent the average of triplicate studies and error bars represent standard deviation. (B) Quantitative RT-PCR analysis of *diptericin* and *drosomycin* gene expression in flies infected with *E. carotovora* or *M. luteus*. Error bars represent standard errors of mean (S.E.M.). (C) β-galactosidase assays and GFP measurements of *E. carotovora* and *M. luteus* infected flies harboring DD1 (*y, w, P(ry+, Dpt-lacZ), P(w+, Drs-GFP)*); *yolk*-GAL4*;*UAS-*avrA*, or of the isogenic fly harboring UAS-m*avrA*. Error bars represent S.E.M. (D) Fat body tissue from *M. luteus* infected third–instar larvae expressing AvrA or mAvrA was fixed and analyzed for Dif protein distribution by immunostain using an anti-Dif antibody. (E) Fat body tissue from *E. caratavora* infected larval was analysed for Relish distribution by immunostaining using an antibody against the N-terminal domain of Relish.

Figure 2. AvrA expression is not pro-apoptotic and inhibits JNK/Bsk phosphorylation and Caspase-3/Drice activation in response to dTAK or Eiger over expression. (A) Phenotypes of adult *Drosophila* expressing AvrA, mAvrA, AopP, or mAopP under the eye specific driver GMR-GAL4 respectively. Suppression of AopP

mediated small eye phenotypes by p35 or dIAP confirm the phenotype is a result of apoptotic events. (B) Phenotypes of adult *Drosophila* expressing VopA or mVopA under the eye specific driver GMR-GAL4 respectively. Suppression of VopA mediated small eye phenotypes by overexpression of activated Rolled (*Drosophila* ERK) confirms the VopA rough eye phenotype is because of ERK pathway inhibition. (C) Phenotypes of adult *Drosophila* eyes resulting from dTAK over-expression and from a combination of dTAK1 and AvrA over-expression, with genotypes indicated. Third instar larval eye disks were immunostained for p-JNK and active caspase-3. (D) Immunoblot analysis of larval eye imaginal disks from genotypes examined in figure 2c were analyzed for p-JNK levels. (E) Phenotypes of adult eyes resulting from the Eiger over-expression and from a combination of Eiger and AvrA over-expression, with genotypes indicated. Third instar larval eye disks were immunostained as above. (F) Larval eye imaginal disks from figure 2E were analyzed for p-JNK. (G) Phenotypes of adult *Drosophila* eyes resulting from activated hep over-expression and from a combination of activated hep and AvrA over-expression in the *Drosophila* eye. (H) Parenteral *E. caratavora* infection of third instar larva expressing AvrA in the fat body. Larval fat body was dissected at time points up to 1 hour post infection and analyzed for p-JNK by immunoblot.

Figure 3. AvrA expression represses the JNK pathway in mammalian cell culture. (A) 293T cultures cells transfected with vector control, or plasmids harboring AvrA, mAvrA or YopJ were stimulated with 10 ng/ml TNF-α over 1h and assayed by immunoblot with the indicated antibodies. (B) 293T cultured cells transfected with vector control or plasmids harboring AvrA or mAvrA were stimulated with 10 ng/ml TNF-α

over 10 min in the presence of 10 ng/ml Calyculin A and assayed by immunoblot using anti-phospho TAK1 antibody. (C) 293T cultured cells transfected with Vector control, or plasmids harboring AvrA or mAvrA were stimulated with 10 ng/ml TNF-α over 1h and assayed for IκB or phospho IκB. (D) JNK, p38 or ERK signaling pathways activation levels in 293T cells were measured by PathDetect (Stratagene) luciferase reporter gene assay in the presence of plasmids harboring AvrA, mAvrA, YopJ or mYopJ respectively. Error bars indicate S.E.M.

Figure 4. AvrA inhibits JNK pathway activation at the level of MKK4/7 by acetylation. (A) Immunoblot analysis for phospho-JNK activity in lystaes of HeLa cultured cells transfected with the indicated plasmids. Myc antibody detects JNK at 45kDa and AvrA/mAvrA at 34kDa. (B) Immunoblot analysis for phospho-JNK activity in lystaes of HeLa cultured cells transfected with the indicated plasmids. Myc antibody detects JNK at 45kDa and MKK4 42kDa flag detects AvrA/mAvrA at 34kDa. (C-F)**,** Electrospray ionization (ESI) tandem mass spectrometry (MS/MS) spectra of modified tryptic peptide C [mass-to-charge ratio (m/z) of 693.37 (z = 3)] and peptide D [m/z of 579.27 (z=3)] from MKK4-AvrA, peptide E [mass-to-charge ratio (m/z) of 565.27 (z = 3)] from rMKK4, and peptide F [mass-to-charge ratio (m/z) of 605.79 (z = 4)] from MKK4-mAvrA. The b and y ions are marked on the MS/MS spectra. The amino acid sequence for each peptide is shown. Putatively acetylated residues are designated by Ac in the sequence. The abbreviation ppa (proprionamide) is for alkylation of the cysteine residues by acrylamide from the gel.

Figure 5. ***Salmonella* AvrA mediates suppression of epithelial innate immunity and apoptosis during natural infection.** Streptomycin pretreated mice were inoculated with 10^8 CFU WT *Salmonella* or isogenic AvrA$^-$ mutant, or a AvrA$^-$ mutant complemented with wild-type *avrA*$^+$ (AvrA$^-$/pAvrA) for six hours. (A) KC ELISA from peripheral serum and (B) Myeloperoxidase (MPO) assay for colonic mucosal lysates. (C) Immunohistochemical staining for active caspase-3 and TUNEL stain in colonic mucosa 6 hours post oral infection with the indicated strain. Five mice were analyzed in each experimental group. (D) Quantification of active-caspase-3 positive cells from (C). * = $P<0.05$, ** = $P<0.005$, and *** = $P<0.001$.

Figure 6. *Salmonella* AvrA mediates suppression of phospho-JNK activation in the murine intestine. Murine intestine was dissected and infected with 10^9 CFU of WT *Salmonella*, isogenic AvrA$^-$ mutant, or an AvrA$^-$ mutant complemented with wild-type *avrA*$^+$ (AvrA$^-$ /pAvrA) for 5min. (A) Immunohistochemical staining for p-JNK in intestinal mucosa 5min post infection with the indicated strain. (B) Immunoblot analysis of mouse intestinal epithelial cells infected for 5min with the indicated *Salmonella* strain, with a p-JNK antibody. Relative intensities of p-JNK band at 46kDa were measured using Scion Image beta analysis program (Scion Corporation).

FIGURES

Figure 1

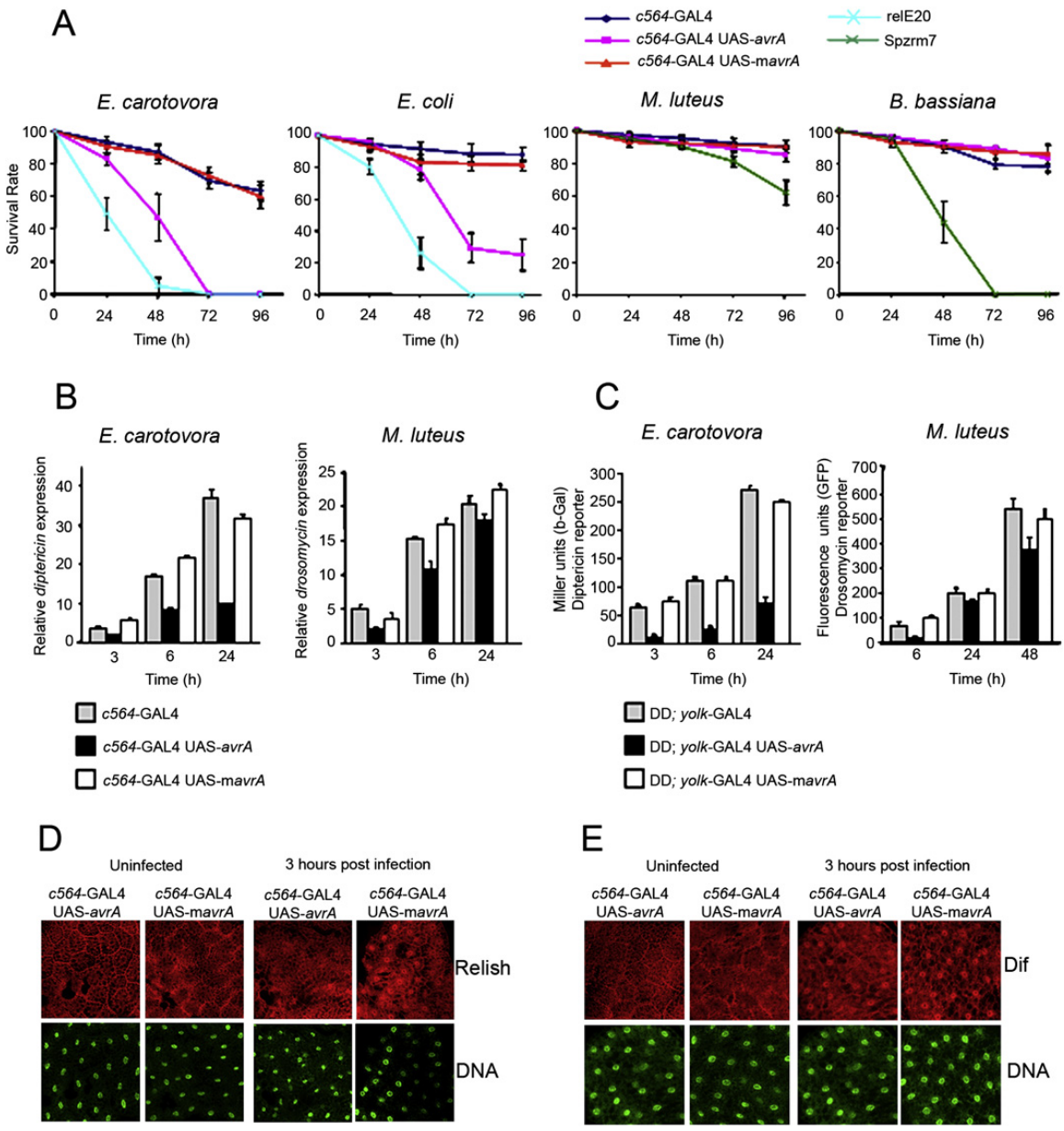
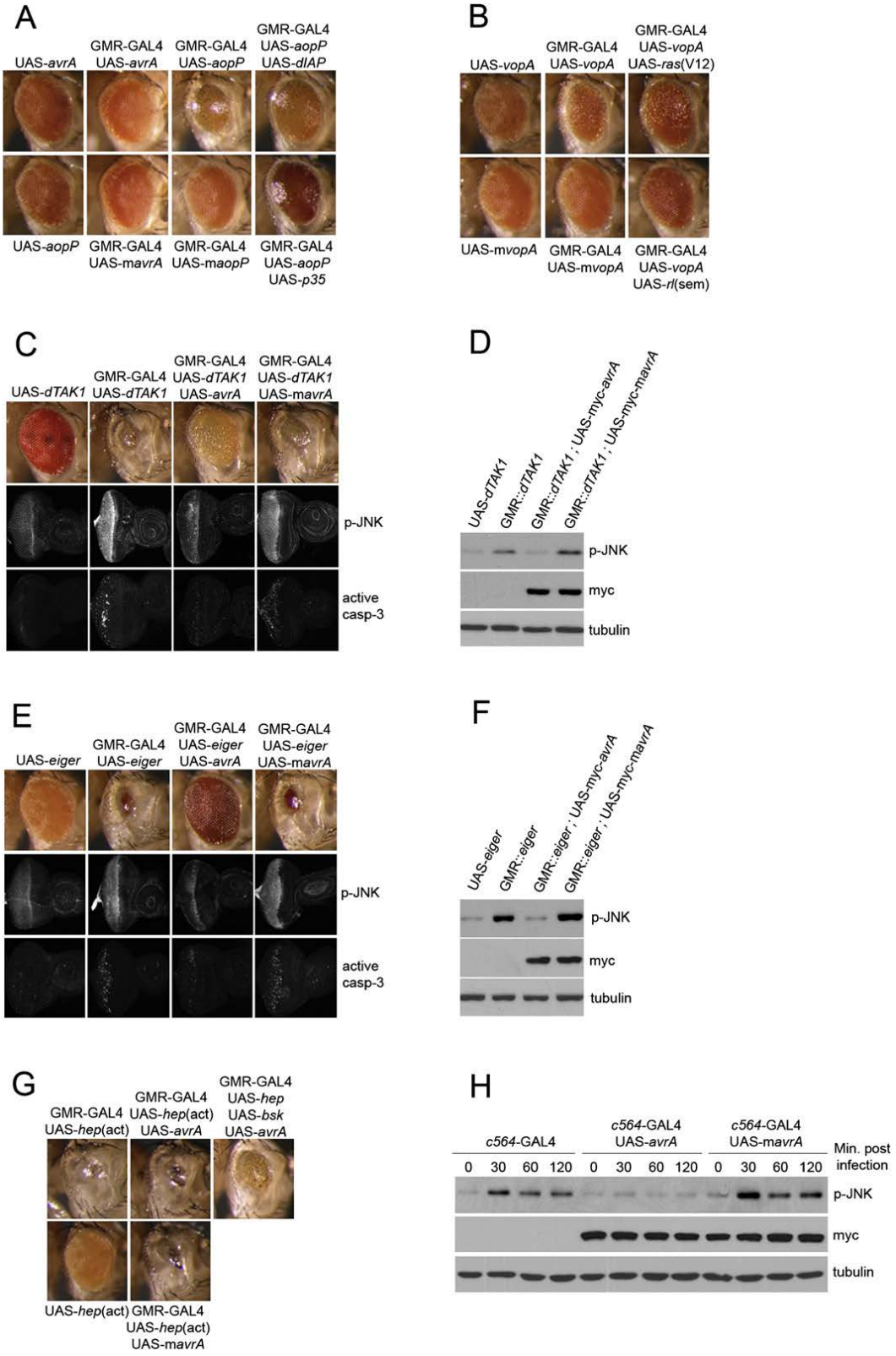


Figure 2


A
UAS-avrA
GMR-GAL4 UAS-avrA
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GMR-GAL4 UAS-aopP UAS-dIAP
UAS-aopP
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GMR-GAL4 UAS-maopP
GMR-GAL4 UAS-aopP UAS-p35
B
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GMR-GAL4 UAS-vopA
GMR-GAL4 UAS-vopA UAS-ras(V12)
UAS-mvopA
GMR-GAL4 UAS-mvopA
GMR-GAL4 UAS-vopA UAS-rl(sem)
C
UAS-dTAK1
GMR-GAL4 UAS-dTAK1
GMR-GAL4 UAS-dTAK1 UAS-avrA
GMR-GAL4 UAS-dTAK1 UAS-mavrA
p-JNK
active casp-3
D
UAS-dTAK1
GMR::dTAK1
GMR::dTAK1 ; UAS-myc-avrA
GMR::dTAK1 ; UAS-myc-mavrA
p-JNK
myc
tubulin
E
UAS-eiger
GMR-GAL4 UAS-eiger
GMR-GAL4 UAS-eiger UAS-avrA
GMR-GAL4 UAS-eiger UAS-mavrA
p-JNK
active casp-3
F
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tubulin
G
GMR-GAL4 UAS-hep(act)
GMR-GAL4 UAS-hep(act) UAS-avrA
GMR-GAL4 UAS-hep UAS-bsk UAS-avrA
UAS-hep(act)
GMR-GAL4 UAS-hep(act) UAS-mavrA
H
c564-GAL4
c564-GAL4 UAS-avrA
c564-GAL4 UAS-mavrA
Min. post infection
0 30 60 120 0 30 60 120 0 30 60 120
p-JNK
myc
tubulin

Figure 3

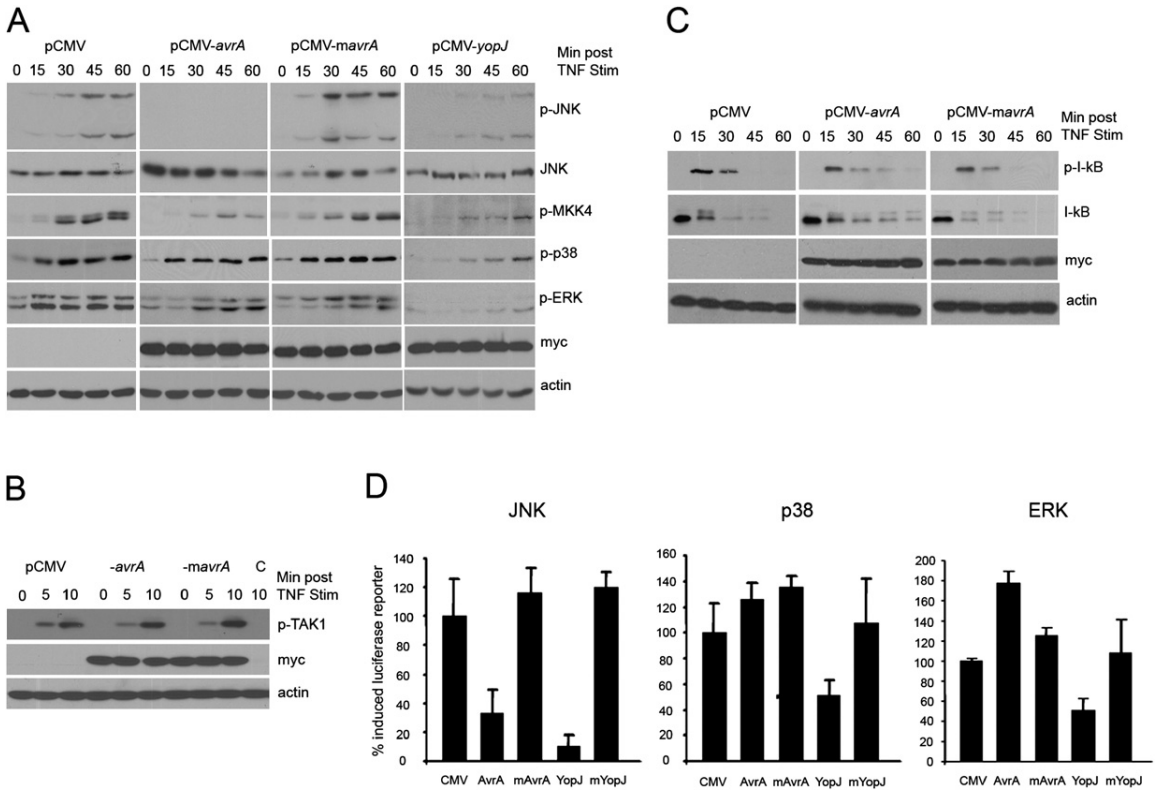

A
pCMV
pCMV-avrA
pCMV-mavrA
pCMV-yopJ
0 15 30 45 60
Min post TNF Stim
p-JNK
JNK
p-MKK4
p-p38
p-ERK
myc
actin
C
pCMV
pCMV-avrA
pCMV-mavrA
Min post TNF Stim
p-I-kB
I-kB
myc
actin
B
pCMV
-avrA
-mavrA
C
0 5 10
Min post TNF Stim
p-TAK1
myc
actin
D
JNK
p38
ERK
% induced luciferase reporter
CMV AvrA mAvrA YopJ mYopJ

Figure 4

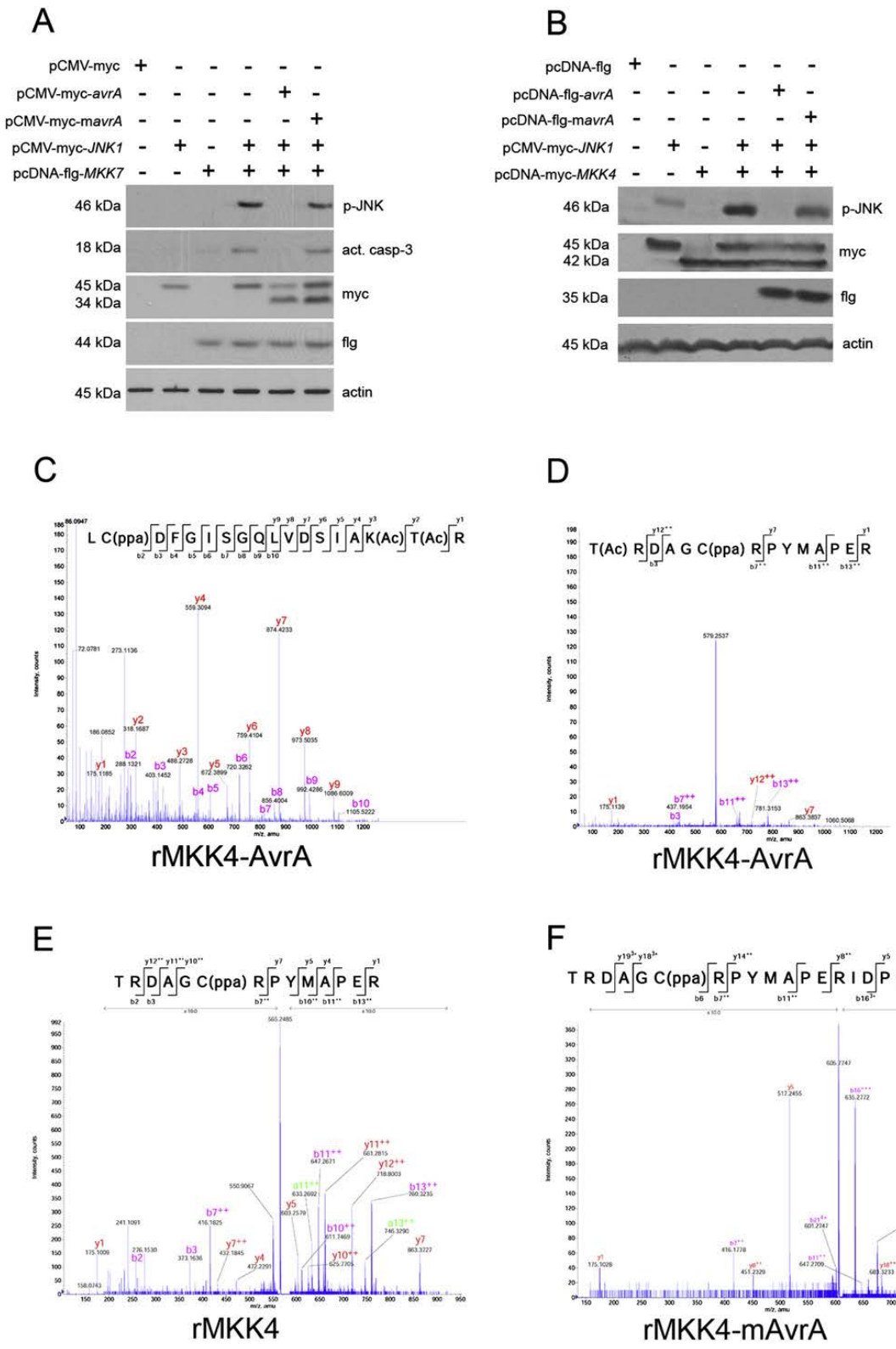

A
pCMV-myc
pCMV-myc-avrA
pCMV-myc-mavrA
pCMV-myc-JNK1
pcDNA-flg-MKK7
46 kDa
p-JNK
18 kDa
act. casp-3
45 kDa
34 kDa
myc
44 kDa
flg
45 kDa
actin
B
pcDNA-flg
pcDNA-flg-avrA
pcDNA-flg-mavrA
pCMV-myc-JNK1
pcDNA-myc-MKK4
46 kDa
p-JNK
45 kDa
42 kDa
myc
35 kDa
flg
45 kDa
actin
C
L C(ppa) D F G I S G Q L V D S I A K(Ac) T(Ac) R
rMKK4-AvrA
D
T(Ac) R D A G C(ppa) R P Y M A P E R
rMKK4-AvrA
E
T R D A G C(ppa) R P Y M A P E R
rMKK4
F
T R D A G C(ppa) R P Y M A P E R I D P S A S R
rMKK4-mAvrA

Figure 5

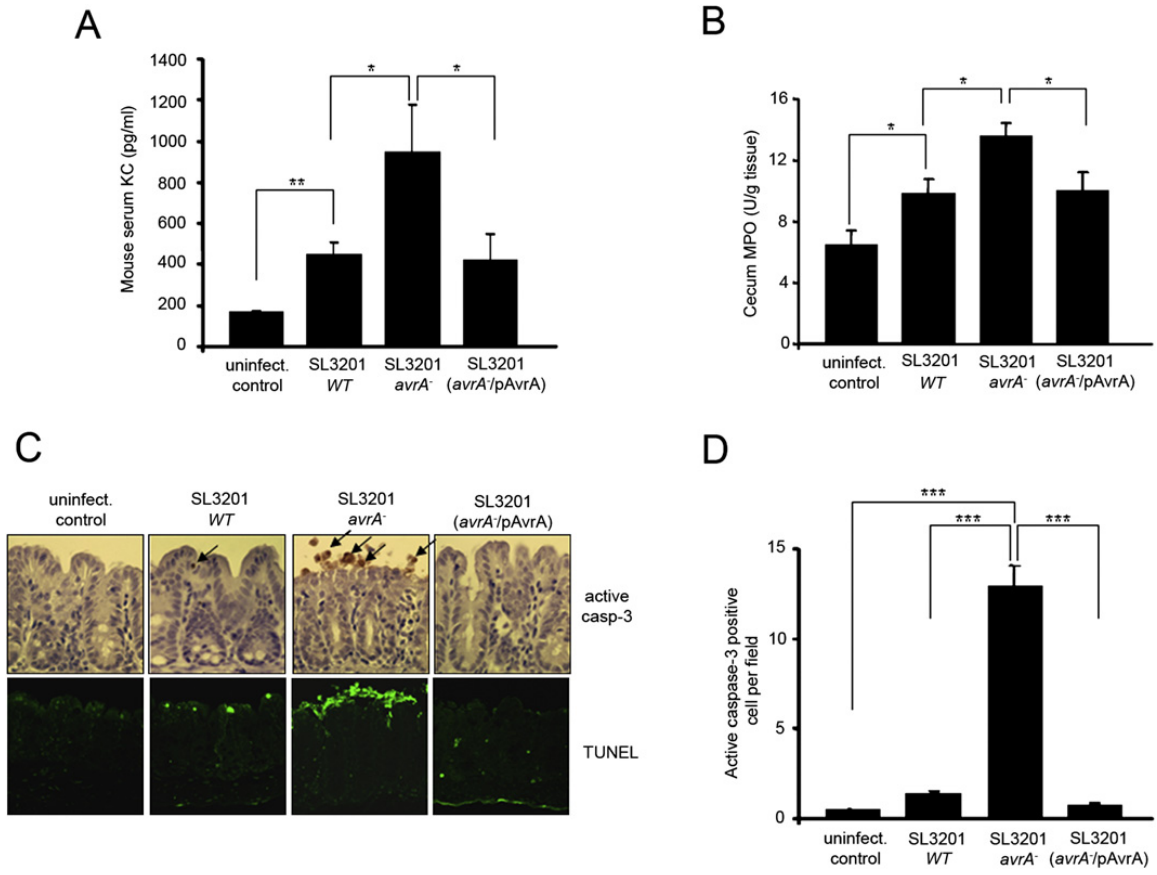
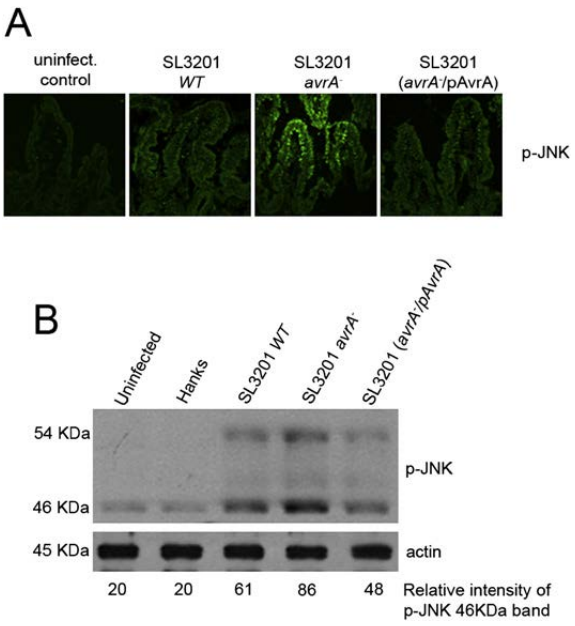

A
Mouse serum KC (pg/ml)
1400
1200
1000
800
600
400
200
0
*
*
**
uninfect. control
SL3201 WT
SL3201 avrA-
SL3201 (avrA-/pAvrA)
B
Cecum MPO (U/g tissue)
16
12
8
4
0
*
*
*
uninfect. control
SL3201 WT
SL3201 avrA-
SL3201 (avrA-/pAvrA)
C
uninfect. control
SL3201 WT
SL3201 avrA-
SL3201 (avrA-/pAvrA)
active casp-3
TUNEL
D
Active caspase-3 positive cell per field
15
10
5
0
***
***
***
uninfect. control
SL3201 WT
SL3201 avrA-
SL3201 (avrA-/pAvrA)

Figure 6


A
uninfect. control
SL3201 WT
SL3201 avrA-
SL3201 (avrA-/pAvrA)
p-JNK
B
Uninfected
Hanks
SL3201 WT
SL3201 avrA-
SL3201 (avrA-/pAvrA)
54 KDa
46 KDa
p-JNK
45 KDa
actin
20
20
61
86
48
Relative intensity of p-JNK 46KDa band

REFERENCES

Barthel, M., Hapfelmeier, S., Quintanilla-Martinez, L., Kremer, M., Rohde, M., Hogardt, M., Pfeffer, K., Russmann, H., and Hardt, W.D. (2003). Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infection and immunity *71*, 2839-2858.

Brand, A. (1994). Ectopic expression in Drosophila. . Methods in Cell Biology *44*, 635-654.

Collier-Hyams, L.S., Zeng, H., Sun, J., Tomlinson, A.D., Bao, Z.Q., Chen, H., Madara, J.L., Orth, K., and Neish, A.S. (2002). Cutting edge: Salmonella AvrA effector inhibits the key proinflammatory, anti-apoptotic NF-kappa B pathway. J Immunol *169*, 2846-2850.

Duesbery, N.S., Webb, C.P., Leppla, S.H., Gordon, V.M., Klimpel, K.R., Copeland, T.D., Ahn, N.G., Oskarsson, M.K., Fukasawa, K., Paull, K.D., *et al.* (1998). Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science (New York, NY *280*, 734-737.

Ellis, J.G., Dodds, P.N., and Lawrence, G.J. (2007). The role of secreted proteins in diseases of plants caused by rust, powdery mildew and smut fungi. Current opinion in microbiology *10*, 326-331.

Fehr, D., Casanova, C., Liverman, A., Blazkova, H., Orth, K., Dobbelaere, D., Frey, J., and Burr, S.E. (2006). AopP, a type III effector protein of Aeromonas salmonicida,

inhibits the NF-kappaB signalling pathway. Microbiology (Reading, England) *152*, 2809-2818.

Freiberg, C., Fellay, R., Bairoch, A., Broughton, W.J., Rosenthal, A., and Perret, X. (1997). Molecular basis of symbiosis between Rhizobium and legumes. Nature *387*, 394-401.

Guichard, A., Park, J.M., Cruz-Moreno, B., Karin, M., and Bier, E. (2006). Anthrax lethal factor and edema factor act on conserved targets in Drosophila. Proceedings of the National Academy of Sciences of the United States of America *103*, 3244-3249.

Hardt, W.D., and Galan, J.E. (1997). A secreted Salmonella protein with homology to an avirulence determinant of plant pathogenic bacteria. Proceedings of the National Academy of Sciences of the United States of America *94*, 9887-9892.

Hoffmann, J.A. (2003). The immune response of Drosophila. Nature *426*, 33-38.

Igaki, T., Kanda, H., Yamamoto-Goto, Y., Kanuka, H., Kuranaga, E., Aigaki, T., and Miura, M. (2002). Eiger, a TNF superfamily ligand that triggers the Drosophila JNK pathway. The EMBO journal *21*, 3009-3018.

Jia, J., Wang, Y., Zhou, L., and Jin, S. (2006). Expression of Pseudomonas aeruginosa toxin ExoS effectively induces apoptosis in host cells. Infection and immunity *74*, 6557-6570.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. Cell *120*, 649-661.

Kelly, D., Campbell, J.I., King, T.P., Grant, G., Jansson, E.A., Coutts, A.G., Pettersson, S., and Conway, S. (2004). Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. Nature immunology *5*, 104-112.

Lei, K., Nimnual, A., Zong, W.X., Kennedy, N.J., Flavell, R.A., Thompson, C.B., Bar-Sagi, D., and Davis, R.J. (2002). The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. Molecular and cellular biology *22*, 4929-4942.

Lemaitre, B., and Hoffmann, J. (2007). The host defense of Drosophila melanogaster. Annual review of immunology *25*, 697-743.

Leulier, F., Marchal, C., Miletich, I., Limbourg-Bouchon, B., Benarous, R., and Lemaitre, B. (2003). Directed expression of the HIV-1 accessory protein Vpu in Drosophila fat-body cells inhibits Toll-dependent immune responses. EMBO reports *4*, 976-981.

Leulier, F., Vidal, S., Saigo, K., Ueda, R., and Lemaitre, B. (2002). Inducible expression of double-stranded RNA reveals a role for dFADD in the regulation of the antibacterial response in Drosophila adults. Curr Biol *12*, 996-1000.

Li, H., Xu, H., Zhou, Y., Zhang, J., Long, C., Li, S., Chen, S., Zhou, J.M., and Shao, F. (2007). The phosphothreonine lyase activity of a bacterial type III effector family. Science (New York, NY *315*, 1000-1003.

Lindmark, H., Johansson, K.C., Stoven, S., Hultmark, D., Engstrom, Y., and Soderhall, K. (2001). Enteric bacteria counteract lipopolysaccharide induction of antimicrobial peptide genes. J Immunol *167*, 6920-6923.

Liu, J., and Lin, A. (2007). Wiring the cell signaling circuitry by the NF-kappa B and JNK1 crosstalk and its applications in human diseases. Oncogene *26*, 3267-3278.

Mittal, R., Peak-Chew, S.Y., and McMahon, H.T. (2006). Acetylation of MEK2 and I kappa B kinase (IKK) activation loop residues by YopJ inhibits signaling. Proceedings of the National Academy of Sciences of the United States of America *103*, 18574-18579.

Moreno, E., Yan, M., and Basler, K. (2002). Evolution of TNF signaling mechanisms: JNK-dependent apoptosis triggered by Eiger, the Drosophila homolog of the TNF superfamily. Curr Biol *12*, 1263-1268.

Mukherjee, S., Keitany, G., Li, Y., Wang, Y., Ball, H.L., Goldsmith, E.J., and Orth, K. (2006). Yersinia YopJ acetylates and inhibits kinase activation by blocking phosphorylation. Science (New York, NY *312*, 1211-1214.

Neish, A.S., Gewirtz, A.T., Zeng, H., Young, A.N., Hobert, M.E., Karmali, V., Rao, A.S., and Madara, J.L. (2000). Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. Science (New York, NY *289*, 1560-1563.

Orth, K., Palmer, L.E., Bao, Z.Q., Stewart, S., Rudolph, A.E., Bliska, J.B., and Dixon, J.E. (1999). Inhibition of the mitogen-activated protein kinase kinase superfamily by a Yersinia effector. Science (New York, NY *285*, 1920-1923.

Park, J.M., Brady, H., Ruocco, M.G., Sun, H., Williams, D., Lee, S.J., Kato, T., Jr., Richards, N., Chan, K., Mercurio, F., *et al.* (2004). Targeting of TAK1 by the NF-kappa B protein Relish regulates the JNK-mediated immune response in Drosophila. Genes & development *18*, 584-594.

Roux, P.P., and Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev *68*, 320-344.

Ruckdeschel, K., Harb, S., Roggenkamp, A., Hornef, M., Zumbihl, R., Kohler, S., Heesemann, J., and Rouot, B. (1998). Yersinia enterocolitica impairs activation of transcription factor NF-kappaB: involvement in the induction of programmed cell death and in the suppression of the macrophage tumor necrosis factor alpha production. The Journal of experimental medicine *187*, 1069-1079.

Rutschmann, S., Jung, A.C., Zhou, R., Silverman, N., Hoffmann, J.A., and Ferrandon, D. (2000). Role of Drosophila IKK gamma in a toll-independent antibacterial immune response. Nature immunology *1*, 342-347.

Sansonetti, P.J., and Di Santo, J.P. (2007). Debugging how bacteria manipulate the immune response. Immunity *26*, 149-161.

Sato, S., Sanjo, H., Takeda, K., Ninomiya-Tsuji, J., Yamamoto, M., Kawai, T., Matsumoto, K., Takeuchi, O., and Akira, S. (2005). Essential function for the kinase TAK1 in innate and adaptive immune responses. Nature immunology *6*, 1087-1095.

Silverman, N., and Maniatis, T. (2001). NF-kappaB signaling pathways in mammalian and insect innate immunity. Genes & development *15*, 2321-2342.

Silverman, N., Zhou, R., Stoven, S., Pandey, N., Hultmark, D., and Maniatis, T. (2000). A Drosophila IkappaB kinase complex required for Relish cleavage and antibacterial immunity. Genes & development *14*, 2461-2471.

Staskawicz, B.J., Mudgett, M.B., Dangl, J.L., and Galan, J.E. (2001). Common and contrasting themes of plant and animal diseases. Science (New York, NY *292*, 2285-2289.

Sweet, C.R., Conlon, J., Golenbock, D.T., Goguen, J., and Silverman, N. (2007). YopJ targets TRAF proteins to inhibit TLR-mediated NF-kappaB, MAPK and IRF3 signal transduction. Cellular microbiology *9*, 2700-2715.

Takatsu, Y., Nakamura, M., Stapleton, M., Danos, M.C., Matsumoto, K., O'Connor, M.B., Shibuya, H., and Ueno, N. (2000). TAK1 participates in c-Jun N-terminal kinase signaling during Drosophila development. Molecular and cellular biology *20*, 3015-3026.

Trosky, J.E., Li, Y., Mukherjee, S., Keitany, G., Ball, H., and Orth, K. (2007). VopA inhibits ATP binding by acetylating the catalytic loop of MKKs. The Journal of biological chemistry.

Trosky, J.E., Mukherjee, S., Burdette, D.L., Roberts, M., McCarter, L., Siegel, R.M., and Orth, K. (2004). Inhibition of MAPK signaling pathways by VopA from Vibrio parahaemolyticus. The Journal of biological chemistry *279*, 51953-51957.

Vidal, S., Khush, R.S., Leulier, F., Tzou, P., Nakamura, M., and Lemaitre, B. (2001). Mutations in the Drosophila dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. Genes & development *15*, 1900-1912.

Vijay-Kumar, M., Wu, H., Jones, R., Grant, G., Babbin, B., King, T.P., Kelly, D., Gewirtz, A.T., and Neish, A.S. (2006). Flagellin suppresses epithelial apoptosis and limits disease during enteric infection. The American journal of pathology *169*, 1686-1700.

Weinrauch, Y., and Zychlinsky, A. (1999). The induction of apoptosis by bacterial pathogens. Annual review of microbiology *53*, 155-187.

Weston, C.R., and Davis, R.J. (2007). The JNK signal transduction pathway. Current opinion in cell biology *19*, 142-149.

Ye, Z., Petrof, E.O., Boone, D., Claud, E.C., and Sun, J. (2007). Salmonella effector AvrA regulation of colonic epithelial cell inflammation by deubiquitination. The American journal of pathology *171*, 882-892.

Zhou, H., Monack, D.M., Kayagaki, N., Wertz, I., Yin, J., Wolf, B., and Dixit, V.M. (2005). Yersinia virulence factor YopJ acts as a deubiquitinase to inhibit NF-kappa B activation. The Journal of experimental medicine *202*, 1327-1332.