Old Herborn University Seminar Monograph 24:
Development of strategies to overcome barriers to effective
mucosal immunization of infants in developing countries.
Editors: Peter J. Heidt, Richard I. Walker, and Volker Rusch.
Old Herborn University Foundation, Herborn-Dill, Germany: 35-50 (2010).

IMPACT OF THE INTESTINAL MICROBIOTA ON THE DEVELOPMENT OF MUCOSAL DEFENCE

ANDREW S. NEISH

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

SUMMARY

The resident microbiota of the mammalian intestine influences diverse homeostatic functions of the gut, including regulation of cellular growth, restitution after injury, maintenance of barrier function, and modulation of immune responses. Normal acquisition of the microbiota in early infancy has inductive effects on these processes. However, it is unknown how commensal prokaryotic organisms mechanistically influence gut biology. We have shown that epithelia contacted by enteric commensal bacteria *in vitro* and *in vivo* rapidly generate reactive oxygen species (ROS), and distinct microbial taxa have markedly different potencies in stimulating this response. This physiologically generated ROS is known to participate in a variety of cellular signal pathways via the rapid and transient oxidative inactivation of a spectrum of regulatory enzymes. We show that these oxidant sensitive enzymes include key control points in the pro-inflammatory NF-κB pathway, regulation of cytoskeletal dynamics and activation of proliferative signals. Accordingly, we demonstrate various commensal bacteria have the ability to suppress inflammatory signalling and stimulate cell motility both in cell culture and in animal models. These events are consistent with known effects of the microbiota and selected probiotics. Collectively, our studies outline a molecular mechanism that may account for aspects of microbial-host cross-talk in the intestine in normal physiology and during therapeutic intervention with probiotics. These data illustrate that the normal flora, particularly in its initial acquisition in the neonatal period, can influence innate and structural defences and have consequences in adaptive immune development.

EUKARYOTIC/PROKARYOTIC INTERACTIONS IN THE GASTROINTESTINAL TRACT

Commensal host-microbe interactions have coevolved over millennia in many animals, with the human luminal ecosystem representing a highly medically relevant example (*Neish*, 2009). The vast majority of the human microbiota is represented by about 500 genera of bacteria, broadly grouped into two taxonomic divisions, the Bacteroidetes and Firmicutes. An accurate census of the microbiota is not practical by culture based microbiological techniques. However, recent high-throughput sequencing and molecular taxonomic

methodologies have greatly increased our understanding of the population composition, dynamics, and ecology of the gut microflora (reviewed in: *Hooper* and *Gordon*, 2001; *Xu* et al., 2007; *Dethlefsen* et al., 2007; *Gill* et al., 2006; *Backhed* et al., 2005). The gut is sterile *in utero* and is colonized immediately after birth, rapidly developing into a diverse and stable community, though marked variations in microbial composition between individuals is typical (*Eckburg* et al., 2005). Total numbers vary from 10^{11} cells/ gram luminal content in the ascending colon, 10^{7-8} in the distal ileum, and 10^{2-3} in proximal ileum and jejunum. Most members of the microbiota are autochthonous, meaning indigenous and stable, though allochthonous, or transient members are known (certainly most enteric pathogens fall into this category).

The microbiota is separated from the systemic compartment of the host by only a single layer of epithelial cells (or epithelial derived component, e.g. mucus layer). Impressively, epithelia and the complete mucosa perform vital fluid and nutrient absorptive functions, and must do so in presence of the microbiota and their products. Epithelial cells, by definition, act as interfaces between the host and the environment, and are equipped with apical surface specializations (microvilli, mucus production, vectorial ion secretion, intercellular junctions) to permit physiological function while contacting the microbiota -thus comprising a barrier. However, studies with germ-free mice have revealed that the microbiota is not functionally insulated from the mucosa, but in contrast, gut bacteria can fundamentally influence epithelial metabolism, proliferation and survival, and barrier function (*Ismail* and *Hooper*, 2005; *Madsen* et al., 2001; *Smith* et al., 2007; *Hooper* and *Gordon*, 2001; *Hooper* et al., 2001). For example, the small intestinal villi of the germ-free gut are elongated, while crypts are atrophic, show a slower turnover of the epithelial cells (*Pull* et al., 2005) and defective angiogenesis (*Stappenbeck* et al., 2002). Such mice mono-colonized with a single gut symbiont species (*Bacteriodes thetaiotaomicron*) exhibit robust host transcriptional responses, indicating that host perception of the microbiota occurs (*Hooper* et al., 2001).

Intestinal bacteria thrive in a stable, nutrient rich environment but also serve beneficial functions to the host including energy salvage of otherwise indigestible complex carbohydrates, vitamin and micronutrient syntheses, stimulation of immune development and competitive exclusion of pathogenic microorganisms (*Hooper* and *Gordon*, 2001; *Marchesi* and *Shanahan*, 2007). Thus there is a dynamic interaction between the microbiota and the host, where the epithelia form the major interface, allowing for the most part a mutually beneficial relationship. However, in other cases, the normal flora of the intestine may be sufficient to provoke intestinal inflammation, such as that seen in IBD [which includes Ulcerative colitis (UC) and Crohn's disease (CD)] (*Sartor*, 2008). There is much current interest in quantitative and/or qualitative abnormalities of the flora that may be associated with other systemic metabolic, infectious and particularly, immune and allergic disorders (*Wills-Karp* et al., 2001; *Noverr* and *Huffnagle*, 2004). The microbiota is clearly involved in the anatomic and functional development of mucosal immunity (*Slack* et al., 2009). Peyer's patches are grossly hypoplastic, and IgA responses are reduced in germ-free animals. It is also known that germ-free animals have reduced total CD4 T-cell populations and an inap-

propriate balance of T_H-cell subsets (*Macpherson* and *Harris*, 2004), which can be moderated within weeks upon colonization with a representative member of the normal flora (*Bacteroides fragilis*) (*Mazmanian* et al., 2005) via dendritic cell recognition of a specific polysaccharide (Polysaccharide A) component of *B. fragilis* (*Mazmanian* et al., 2008).

There is also increasing interest in potential therapeutic benefits of supplementing the normal flora with exogenous viable bacteria. This approach, termed probiotics, has been reported to dampen inflammation, improve barrier function, and augment adaptive immune processes and has shown promise as therapy in several inflammatory and developmental disorders of the intestinal tract (*Park* and *Floch*, 2007; *Hord*, 2008). Thus, there is increasing and compelling evidence that the gut flora beneficially affects intestinal -and systemic- homeostasis and thus health. However, little is known of how the host perceives non-pathogenic bacteria, or how the microbiota mechanistically influences gut biology.

PATTERN RECOGNITION RECEPTORS AND EPITHELIAL PERCEPTION OF BACTERIA

All eukaryotic cells have the ability to respond to and manage threats from bacterial pathogens -and by extrapolation, respond to and manage commensals. Transmembrane and intracytoplasmic receptors, such as the now well-studied Toll-like receptors and related Nod proteins, are designated "pattern recognition receptors" or PRRs. PRRs recognize and bind to conserved structural motifs present on the surface of a wide range of microbes, which are termed MAMPs, or "microbe associated molecular patterns". For example, TLR4 recognizes lipopolysaccharide and TLR2 binds specific peptidoglycans -both components of bacterial cell walls (*Sansonetti*, 2006). TLR5 detects the bacterial protein flagellin (*Zeng* et al., 2003). The now well known association of Crohn's disease with mutant forms of Nod2 clearly underscores the importance of PRR monitoring in intestinal health (*Sartor*, 2008).

PRRs are expressed in most cells; however, given the vast microflora, the dominant interaction of bacteria with host cells occurs in the intestine, especially the epithelia. PRRs and their downstream signalling pathways, such as the MAPK and NF-κB systems, have an ancient lineage, exhibiting impressive structural and functional homology even at the level of invertebrates and plants. These systems represent entwined cytoplasmic information relays, which when activated employ rapid post translational events (covalent protein modifications and regulated protein degradation) to transduce PRR binding into well defined inflammatory and apoptotic tissue responses that evolved to eliminate pathogenic threats (*Neish*, 2009; *Sansonetti*, 2004; *Abreu* et al., 2005). However, while PRR mediated signalling clearly has a central and dominant role in initiating cellular inflammation during infection, it is now also apparent that basal tonic TLR (and possibly other PRR) mediated signalling in response to the normal flora and their products is necessary for mucosal health. Murine models with defective PRR signalling 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 (*Rakoff-Nahoum* et al., 2004; *Burdelya* et al., 2008). Regenerative responses to colonic injury are markedly attenuated in germ-free animals, indicating a discernable role of the flora in stimulation of epithelial proliferation and response to injury, and restitution is reduced in MyD88 (a signalling intermediate required by multiple TLRs) null mice, reinforcing the notion that PRR mediated signalling is necessary for trophic/restitutive effects (*Pull* et al., 2005). These and related observations with mice null in epithelial NF-κB pathway components (*Zaph* et al., 2007; *Nenci* et al., 2007; *Ben-Neriah* and *Schmidt-Supprian*, 2007; *Chen* et al., 2003) support the hypothesis that a constitutive degree of PRR signalling is necessary for normal gut homeostasis, presumably because of the tonic upregulation of cytoprotective genes in either epithelial cells or lamina propria macrophages (gene products with anti-apoptotic, chaperone/stress response, and antioxidant effects) (*Zaph* et al., 2007) and underscores the importance of gut-prokaryotic interaction as a beneficial and necessary relationship.

FORMYLATED PEPTIDE RECEPTORS

Another type of PRRs are the formylated peptide receptors (FPR). Classically, the FPRs are seven membrane pass, G-protein linked surface receptors expressed on neutrophils and macrophages, where they perceive bacterial cell wall products and stimulate phagocyte function (*Migeotte* et al., 2006). The best characterized ligands are formylated peptides, which are modified prokaryotic translation products tagged with a bacterial specific amino acid N-formyl-methionyl-leucyl-phenylalanine (fMLP). Upon ligand recognition in phagocytes, the FPR receptors undergo a conformation change that allows binding of pertussis toxin sensitive G proteins of the Gi family. Subsequent signalling trifurcates to PI3K MAPK signalling pathways, calcium release, and GTPase activation which eventuate in:

1) changes in actin dynamics and initiation of chemotaxis,
2) transcriptional upregulation of inflammatory effectors and cytokines, and
3) the activation of NADPH dependant oxidase enzymes and ROS generation (respiratory burst).

Thus, the FPRs are a key PRR that controls the biological response of professional phagocytes to bacterial ligands.

The formylated peptide receptors are represented in humans by the originally characterized FPR and the closely related FPRL1 and FPRL2. FPR has been characterized as high affinity with an ED_{50} for fMLP in the nanomolar range, while the low affinity FPRL1/FPRL2 responds to the same agonist at micromolar ranges (*Le* et al., 2002). Importantly, immunohistochemical staining has shown the formylated peptide receptors are expressed on the apical surface of the intestinal epithelia, prompting interest that this and related epithelial receptors may mediate physiological responses in the gut (*Babbin* et al., 2007). We have found that live commensal contact mediated activation of the ERK MAPK signalling pathway in gut epithelial cells *in vitro* and *in vivo*. A range of commensal bacteria tested potently induced ERK phosphorylation without stimulating pro-inflammatory phospho-IκB or phospho-JNK. Interestingly, this pattern of signalling activation was

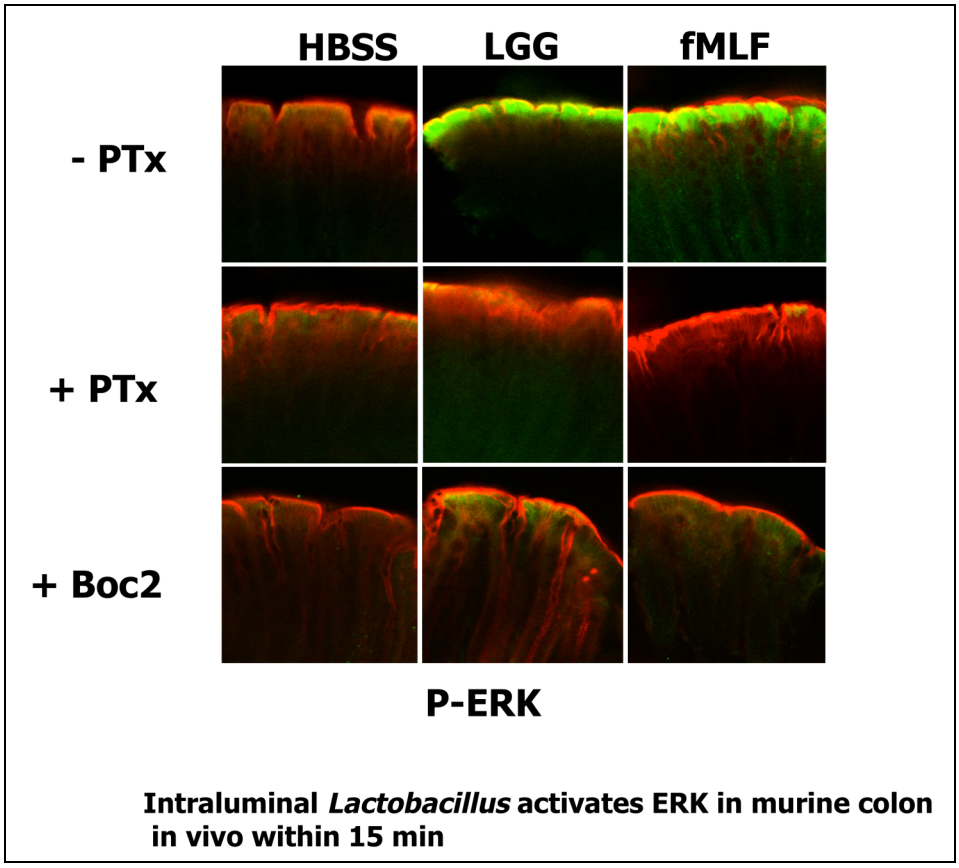

Figure 1: Commensal bacteria activate ERK MAPK *in vivo*. Immunostaining of murine intestine stimulated with addition of commensal bacteria by intra rectal instillation. tBOC and PTX and extracellular and intracellular inhibitors of FPR signaling, respectively. Activated ERK represents an example of non-inflammatory signaling stimulated by commensal bacteria.

recapitulated using the peptide, N-formyl-Met-Leu-Phe (fMLF), consistent with a role for formyl peptide receptors in 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 (Figure 1). 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 signalling in gut epithelia.

PHYSIOLOGICAL GENERATION OF REACTIVE OXYGEN SPECIES

The rapid generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), hydroxyl radicals and a variety of their degradation products are a result of excitation or incomplete reduction of molecular oxygen. ROS are short-lived reactive molecules and at high levels are considered potently microbiocidal, necessary for the killing of engulfed organ-

isms. ROS production in response to FPR stimulation is a cardinal feature of the cellular response of phagocytes to both pathogenic and symbiotic bacteria. Phagocytes generate ROS via a very well studied enzymatic apparatus. The neutrophil NADPH oxidase, Nox2 (formerly gp120phox), is a constitutively inactive multi-subunit complex comprised of a membrane bound dimer of p22phox and gp91phox (*Lambeth*, 2004). The *in vivo* role of this enzyme in host defence is vividly illustrated by the fact that the genetic absence of Nox2 function results in chronic granulomatous disease (CGD), a condition where phagocytes fail to induce ROS and patients are predisposed to recurrent pyogenic infections. Invertebrate phagocytes stimulated by formylated peptides generate ROS (MAMPs) in the same manner as mammalian neutrophils, and plants also utilize induced ROS in response to bacterial pathogens and symbionts, continuing the theme of conversion of basic machinery of microbial perception and effector pathways (*Kotchoni* and *Gachomo*, 2006; *Pauly* et al., 2006; *Tanaka* et al., 2006; *Schneeweiss* and *Renwrantz*, 1993; *Lambeth*, 2004). Drosophila requires commensal microbe-induced hydrogen peroxide (H_2O_2) to maintain gut epithelial homeostasis (*Ha* et al., 2005a,b; *Pull* et al., 2005; *Abreu* et al., 2005). However, in the case of the fly, the ROS generation occurs in the epithelia, and is necessary for control of the luminal flora. This latter observation suggests a conserved role for epithelial ROS (as opposed to strictly phagocyte) generation in gut homeostasis and microbial control. Additionally, it is now apparent that the ROS generating enzymes activated by FPRs in neutrophils (Nox2) have functional paralogous enzymatic complexes in non-phagocytic cells (*Lambeth*, 2004). Indeed, a family of NADPH oxidase enzymes, the Nox's and Duox's is seen in many non-phagocytic tissues, with two, Nox1 and Duox2, strongly expressed in the intestinal epithelia (the inducible ROS observed in Drosophila intestine is produced by the fly ortholog of Duox). In general, the non-phagocytic NADPH oxidases exhibit similar, but not identical organization to the phagocyte enzyme.

Recently, we have shown that several species of normal human gut bacteria can induce rapid, "deliberate" generation of ROS within epithelial cells (*Kumar* et alo., 2007). Furthermore, these cells immediately show increased oxidation of soluble redox sinks, such as glutathione and thioredoxin, and exhibit an increase in redox stimulated transcriptional activation, both reflecting a cellular reaction to increased ROS. Interestingly, different strains of commensal bacteria can elect marked differences in ROS levels in contacted cells. We have found that the Lactobacilli are especially potent in ROS production in cultured cells and *in vivo*, though all bacterial tested have some ability to alter the redox environment of the cell. This is not surprising given that phagocytes can induce a respiratory burst regardless of whether they encounter nominal pathogens or stray commensals. As mentioned, Nox enzymes play a central role in ROS generation in phagocytes; whether the Nox's or Duox's are involved in the generation of ROS in mammalian epithelia or if this ROS also has microbiostatic functions is not known.

High ROS stimulating bacteria, such as Lactobacilli, may possess specific membrane components or even secreted factors that activate cellular ROS production. For instance Yan reported soluble factors of Lactobacilli that mediated beneficial effects in *in*

vivo inflammatory models (*Yan* et al., 2007). Alternatively, high ROS stimulating bacteria may simply possess enhanced adhesion or ability to penetrate mucin layers and gain more proximal access to cellular receptors such as TLRs and FPRs. As the FPRs are expressed on apical surfaces and are known to directly stimuli ROS production in phagocytes, these are interesting candidates for this function. Alternative possibilities include endogenous production of ROS from prokaryotic enzymes, though experiments showing potent ROS stimulation with non-viable and denatured bacterial components make this notion less likely. Additional sources of cellular ROS generation could include 5-lipoxygenase, xanthine oxidase and mitochondrial respiratory chain enzymes. Clearly, bacteria, unlike individual peptides and cytokines, are multifaceted biological stimuli and clearly would be expected to elicit a complex range of cellular receptors and influence diverse processes.

ROS MEDIATED SIGNALLING

ROS also have functions beyond microbial killing. Controlled generation of ROS by activation of receptors for various hormones, cytokines and growth factors mediate critical roles in the modulation of signal transduction pathways seen in all multi-cellular life, plants and animals alike (*Terada*, 2006; *Ogier-Denis* et al., 2008; *Kotchoni* and *Gachomo*, 2006; *Pauly* et al., 2006; *Tanaka* et al., 2006). The specificity of biological responses to altered levels of ROS can be modulated by the specific molecular species of ROS, the intensity/duration of the signal, the subcellular sites of production and the developmental stage of the cell (*Terada*, 2006; *Ogier-Denis* et al., 2008). ROS are short-lived molecules and can have a very small functional radius of action, which contributes to the selectivity of action. Indeed certain receptors physically interact with a ROS generating Nox enzyme, presumably to limit ROS mediated influences to the immediate vicinity of effector proteins (*Karrasch* et al., 2007).

A major mechanism by which ROS are thought to exert their effects on signal transduction pathways is by their ability to reversibly oxidize cysteine residues in specific target proteins (*Barford*, 2004). Only a subset of proteins can be modified by this reaction as oxidation of cysteine requires this amino acid to be present in the thiolate anion form (Cys-S-), whereas most cysteines (pK_a~8.5) are protonated (Cys-SH) at physiological pH. Only some cysteine residues exist as a thiolate anion at neutral pH as result of lowering of their pKa value by vicinal charged amino acids (*Rhee* et al., 2005). Specific examples of such oxidant sensitive proteins include protein tyrosine phosphatases (PTPs), the lipid phosphatase (PTEN), MAP kinase phosphatases (MAPK-P or DUSPs), and low-molecular-weight protein tyrosine phosphatases (LMW-PTPs) (*Tonks*, 2005; *Kamata* et al., 2005; *Chiarugi* and *Buricchi*, 2007). More recently examples of ROS mediated inactivation of enzymes have come from studies by Bossis and Melchior (*Bossis* and *Melchior*, 2006) and from our own laboratory (*Kumar* et al., 2007) with the sumoylation and the neddylation enzymes, respectively. Sumoylation and neddylation are the conjugation of ubiquitin-like proteins, Sumo or Nedd8, to target lysine resi-

dues of substrate proteins. The latter, Nedd8, plays a role in the control of the key inflammatory transcription factor, NF-κB, as is discussed next.

MICROBIAL EFFECTS ON INFLAMMATORY SIGNALLING

While it is obvious that the host must defend against threats posed by bacterial pathogens, the benefits conferred by the microbiota require that immune and inflammatory systems not eliminate them entirely. The epithelia can suppress TLR signalling or reduce TLR expression to moderate immuno-inflammatory signaling (*Sansonetti*, 2006; *Abreu* et al., 2005). Additionally, individual members of the microbiota are able to actively modulate signalling intensity (*Kelly* et al., 2005; *Iyer* et al., 2008; *Neish*, 2003). A variety of reports have described commensals -many employed as probiotics- are able to suppress eukaryotic inflammatory signalling pathways such as NF-κB and block inflammatory effector functions (*Yan* et al., 2007; *Menard* et al., 2004; *Pena* and *Versalovic*, 2003; *Madsen* et al., 1999). Several mechanisms have been described. The gut symbiont *Bacteroides thetaiotaomicron* has been elegantly shown to inhibit NF-κB pathways by regulating cytoplasmic to nuclear translocation of the p65 NF-κB subunit (*Kelly* et al., 2004). Several laboratories have demonstrated that intestinal bacteria are able to influence inflammatory pathways, and very likely other cellular regulatory processes, by manipulating the ubiquitin system (*Neish* et al., 2000; *Tien* et al., 2006; *Petrof* et al., 2004; *Iyer* et al., 2008). Ubiquitination is a covalent modification increasingly recognized to play a regulatory role in a wide spectrum of biochemical events, generally by targeting modified proteins for controlled degradation via the proteasome organelle. An example of a signalling component regulated by ubiquitination is the inhibitory component of the NF-κB pathway, IκB (*Karin* and *Ben-Neriah*, 2000), and there are numerous examples of pathogens that utilize preformed effector proteins to influence IκB ubiquitination and thus innate immunity (*Kim* et al., 2005; *Angot* et al., 2007; *Rytkonen* and *Holden*, 2007). Members of the microbiota interacting with epithelial cells *in vitro* are capable of blocking IκB ubiquitination and thus NF-κB activation by interference with the function of the IκB ubiquitination ligase, $SCF^{\beta TrCP}$(Skp1, Cdc53/Cullin, F box receptor) (Neish et al., 2000; *Collier-Hyams* et al., 2005; *Lee*, 2008). This enzymatic complex is activated by a second covalent modification, neddylation, on the regulatory subunit of the complex, cullin-1. Neddylation is the covalent modification of the SCF ubiquitin ligases by the ubiquitin-like protein Nedd8. The event is emerging as a central regulatory event in cellular processes that are controlled by protein degradation, including NF-κB and β-catenin. Neddylation occurs by an enzymatic series analogous to the ubiquitination reaction, specifically catalyzed by a Nedd8 ligase called Ubc12. We have shown that contact of commensal bacteria with epithelia *in vitro* and *in vivo* resulted in the rapid and reversible loss of the Nedd8 modification, accounting for the loss of overall SCF ubiquitin ligase function and consequent blockade of NF-κB activation (*Collier-Hyams* et al., 2005). Prompted by observations that other enzymes involved in modification of regulatory proteins by ubiquitin-like enzymes (the SUMOylation process) were controlled by transient oxidative inactivation, we

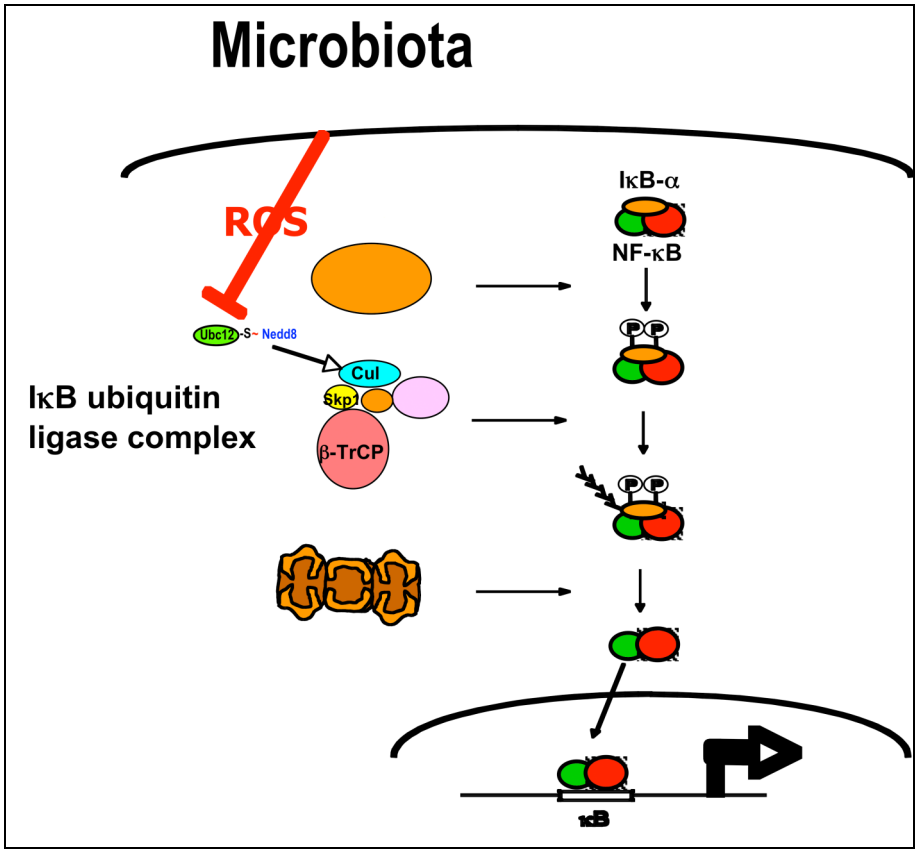

Figure 2: Diagram of the NF-κB pathway. NF-κB is activated by sequential modifications of IκB; phosphorylation (by IKK), ubiquitination (by the SCF complex) and degradation (by the proteasome). Free NF-κB dimer can then translocate to the nucleus and activate transcription. The SCF ubiquitin ligase (cullin subunit) must be modified by the ubiquitin-like protein Nedd8 for activity, and the neddylation reaction is mediated by the oxidant sensitive ligase Ubc12. Intracellular ROS from bacterial contact transiently inactivates Ubc12 and thus blocks activity of downstream functions, including IκB ubiquitination/degradation and NF-κB mediated signaling.

investigated if the neddylation reaction was influenced by oxidative signalling. We demonstrated that both endogenous ROS (H_2O_2) and ROS generation by bacterial contact was able to transiently inactive the Nedd8 ligase, Ubc12 (*Kumar* et al., 2007). These results demonstrated that commensal bacteria directly modulate a critical control point of the ubiquitin-proteasome system and is the first example of a eukaryotic signalling pathway influenced via bacterially stimulated ROS, and furthermore provides a detailed molecular mechanism for bacterial suppression of a key host inflammatory pathway (Figure 2). When considering the defences of the immature intestine, one must bear in mind that the gut is totally naive to bacteria and their products while *in utero*, and is instantly challenged by their presence at birth with the introduction of the normal flora. Potentially, an immature microbiota may be inadequate to modulate innate immune pathways with consequences on downstream events, including contribution to adaptive immunity.

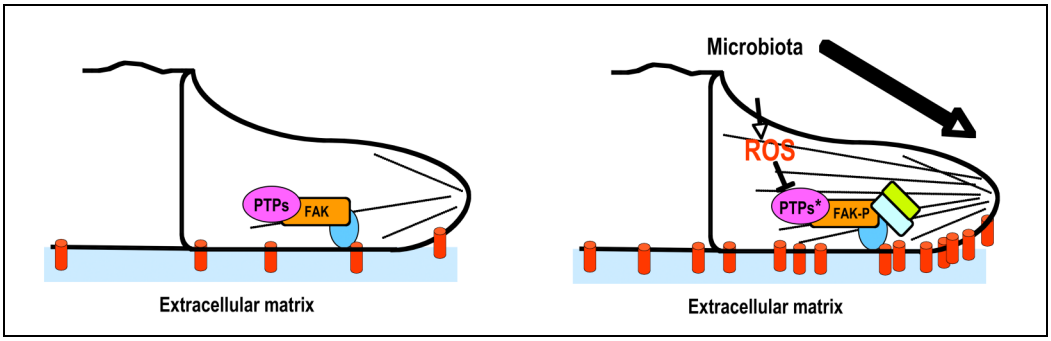

Figure 3: Diagram of the epithelial motility. In a resting state, oxidant sensitive protein tyrosine phosphatases (PTPases including LMW-PTPase) maintains focal adhesion kinase FAK in a dephosphorylated inactive state. Intracellular ROS from bacterial contact transiently inactivates PTPases and permits the autophosphorylation of FAK. Activated FAK acts as a nidus, recruiting other regulatory proteins and stimulating assembly of the actin cytoskeleton, eventuating in initiation of cellular movement.

MICROBIAL EFFECTS ON EPITHELIAL CELL FUNCTION, GROWTH AND SURVIVAL

As previously discussed, germ-free mice show defective epithelial proliferation and wound healing, indicating that commensal enteric bacteria are able to stimulate epithelial cell migration post-injury and during development, suggesting a mechanism by which the microflora could affect physical epithelia defences, such as barrier function. The single cell layer epithelium reconstitutes itself every 5 days from the crypt stem cell pool. Maintenance of this intestinal epithelial lining requires tight regulation of cell proliferation and migration. Epithelial cell migration depends on coordinated changes in actin cytoskeleton involving spatial and temporal changes in adhesion of the protruding membrane edge to the cell extracellular matrix at specialized signalling nidus points called focal adhesions (FA). FA assembly is regulated by focal adhesion kinase, a 125 kDa protein that is maintained in an inactive dephosphorylated form by the constitutive action of redox sensitive tyrosine phosphatases, LMW-PTPase and SHP-2 (*Mitra* et al., 2005). Past reports have shown that endogenous physiological stimuli, such as growth factors and integrin engagement with the epithelial basement membrane induced local ROS production via activation Nox1, resulting in rapid oxidative inactivation of these PTPase's, and consequent phosphorylation of FAK and initiation of cellular motility (*Chiarugi* et al., 2003). Accordingly, we have shown that interaction(s) of wounded intestinal epithelia with natural commensal bacterial strains is associated with rapid accumulation of ROS, especially at the leading edge of the migrating monolayer. Elicitation of ROS results in reversible oxidation of target low pKa cysteines in LMW-PTP and SHP-2, and thereby a consequent increase in phosphorylation of focal adhesion kinase (FAK). Concomitantly, commensal bacteria mediate an increase in number of FA at the migrating edge of the monolayer, and increased cell adhesion and velocity of epithelial migration (Figure 3). Functionally, commensal bacteria mediate enhanced wound closure in an *in vitro*

model of injury and enhanced resolution of dextran sodium sulfate-induced mucosal damage in a mouse model. Thus ROS production associated with commensal-epithelial contact can stimulate epithelial motility and likely contribute to epithelial barrier function. This data suggests another means for how the microbiota mediates physical defences in the gut.

Finally, the DUSPs are redox sensitive PTPases that serve as negative regulators of various MAPKinases, including ERK. Plausibly, FPR dependant activation of the ERK MAP-Kinase pathways may also be regulated by microbial induced redox events inactivating DUSPs. Experiments to address this hypothesis are in progress.

DISCUSSION

We have shown that epithelia exhibit increased ROS generation in response to commensal bacteria, in a manner similar to the events induced in phagocytic cells, suggesting a deep functional conservation. Indeed, recent data in invertebrates suggest that ROS generation for signalling and microbiocidal functions in the gut epithelia may represent the ancestral form of response to bacteria (*Ha* et al., 2005). We have shown ROS generated in epithelial in response to bacteria serves a signalling function (as in many non epithelial cells), and likely there are numerous ROS sensitive enzymes that could be influenced by changes in cellular redox status. As mentioned, reversible oxidative inactivation of a wide range of regulatory enzymes is an increasingly recognized mechanism of signal transduction (*Terada*, 2006; *Chiarugi* and *Buricchi*, 2007). Current protoeomic approaches that exploit reactive cysteines to label individual peptides may be employed as a high throughput system to screen for oxidant sensitive regulatory proteins (*Sethuraman* et al., 2004). Alternatively (but not contradictory), an epithelial antimicrobial function (as in phagocytes and the Drosophila gut) of bacterial elicited ROS, especially in limited locations such as the intestinal crypt is also plausible, and are questions to be resolved.

The source of ROS is an intriguing topic. Clearly the Nox enzymes, especially Nox1 and Duox2 are prime candidates given their pattern of tissue expression, but other sources such as mitochondria respiration chain enzymes, lipoxigenases and others could contribute to redox control in the cell. FPRs are attractive candidates for receptor stimulated ROS production, given that many of the same mechanisms that mediate FPR signalling in professional phagocytes are conserved in epithelial cells. Additionally, it is also unclear whether certain commensals could generate ROS by their own enzymatic machinery and influence eukaryotic signalling by exogenous ROS (conversely, some bacteria could achieve this result by producing anti-oxidants).

ROS mediated signalling may occur during rapid quantitative changes in microbial populations or qualitative changes in the composition in the gut, during development, or with probiotic therapy. The observation that different taxa of bacteria exhibit markedly different potencies in the ability to elicit/provide ROS supports the idea that qualitative changes in community composition can affect host biology. This notion may be relevant to the development and optimization of probiotics, and may explain a parameter that defines a healthy vs. “dysbiotic” mi-

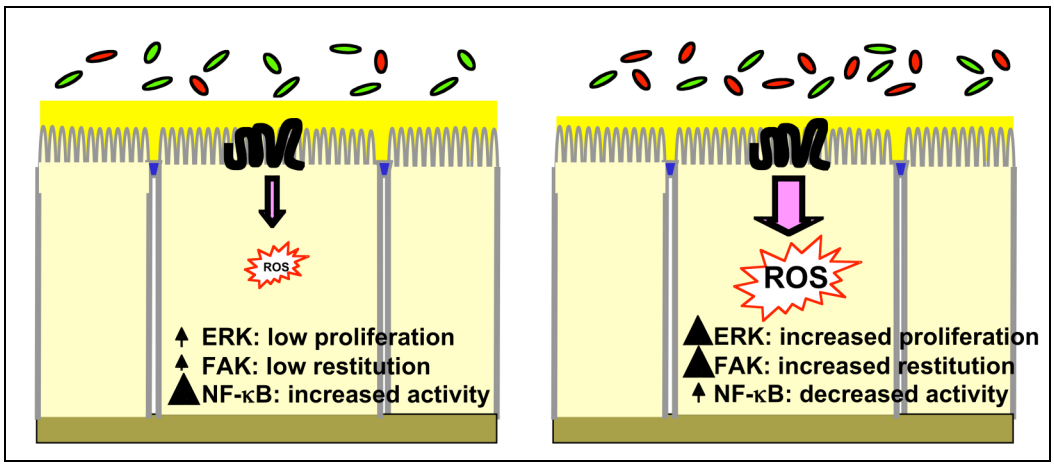

Figure 4: Possible scheme for ROS signaling in the gut. In conditions with low ROS generation, whether decreased total bacterial numbers or relative scarcity of high ROS stimulating bacteria, the NF-κB system is fully active while FAK dependant motility and ERK signaling pathway is relatively inactive. With increasing ROS signaling, inactivation of relevant enzymes leads to suppression of NF-κB and augmentation of motility and ERK. The long term consequences of these events are unknown, and clearly, other ROS sensitive enzymes could be influenced by ROS.

crobiota. Long-term biochemical accommodation to tonic bacterial presence, as in the colon, may affect different aspects of redox biology.

In conclusion, cellular ROS by microbe-epithelial contact is a conserved processes with many known, expected and plausible consequences, making this mechanism attractive as a general and non-species selective means by which a complex floral community could influence a wide range of host signalling and homeostatic processes (*Lee*, 2008). It is hoped that a fuller understanding of this mechanism may advance our understanding of the natural microbiota and exploitation of probiotic organisms.

LITERATURE

Abreu, M.T., Fukata, M., and Arditi, M.: TLR signaling in the gut in health and disease. J. Immunol. 174, 4453-4460 (2005).

Angot, A., Vergunst, A., Genin, S., and Peeters, N.: Exploitation of eukaryotic ubiquitin signaling pathways by effectors translocated by bacterial type III and type IV secretion systems. PLoS Pathog. 3, e3 (2007).

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

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., and Gordon, J.I.: Host-Bacterial Mutualism in the Human Intestine. Science 307, 1915-1920 (2005).

Barford, D.: The role of cysteine residues as redox-sensitive regulatory switches. Curr. Opin. Struct. Biol. 14, 679-686 (2004).

Ben-Neriah, Y. and Schmidt-Supprian, M.: Epithelial NF-kappaB maintains host gut microflora homeostasis. Nat. Immunol. 8, 479-481 (2007).

Bossis, G. and Melchior, F.: Regulation of SUMOylation by reversible oxidation of

SUMO conjugating enzymes. Mol. Cell 21, 349-357 (2006).

Burdelya, L.G., Krivokrysenko, V.I., Tallant, T.C., Strom, E., Gleiberman, A.S., Gupta, D., Kurnasov, O.V., Fort, F.L., Osterman, A.L., DiDonato, J.A., Feinstein, E., and Gudkov, A.V.: An agonist of Toll-like receptor 5 has radioprotective activity in mouse and primate models. Science 320, 226-230 (2008).

Chen, L.W., Egan, L., Li, Z.W., Greten, F.R., Kagnoff, M.F., and Karin, M.: The two faces of IKK and NF-kappaB inhibition: Prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. Nat. Med. 9, 575-81 (2003).

Chiarugi, P., Pani, G., Giannoni, E., Taddei, L., Colavitti, R., Raugei, G., Symons, M., Borrello, S., Galeotti, T., and Ramponi, G.: Reactive oxygen species as essential mediators of cell adhesion: The oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion. J. Cell. Biol. 161, 933-944 (2003).

Chiarugi, P. and Buricchi, F.: Protein tyrosine phosphorylation and reversible oxidation: Two cross-talking posttranslation modifications. Antioxid. Redox Signal. 9, 1-24 (2007).

Collier-Hyams, L.S., Sloane, V., Batten, B.C., and Neish, A.S.: Cutting edge: Bacterial modulation of epithelial signaling via changes in neddylation of cullin-1. J. Immunol. 175, 4194-4198 (2005).

Dethlefsen, L., McFall-Ngai, M., and Relman, D.A.: An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449, 811-818 (2007).

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., and Relman, D.A.: Diversity of the human intestinal microbial flora. Science 308, 1635-1638 (2005).

Gill, S.R., Pop, M., Deboy, R.T., Eckburg, P.B., Turnbaugh, P.J., Samuel, B.S., Gordon, J.I., Relman, D.A., Fraser-Liggett, C.M., and Nelson, K.E.: Metagenomic analysis of the human distal gut microbiome. Science 312, 1355-1359 (2006).

Ha, E.-M., Oh, C.-T., Ryu, J.-H., Bae, Y.-S., Kang, S.-W., Jang, I.-h., Brey, P.T., and Lee, W.-J.: An antioxidant system required for host protection against gut infection in Drosophila. Dev. Cell 8, 125-132 (2005a).

Ha, E.-M., Oh, C.-T., Bae, Y.S., and Lee, W.-J.: A direct role for dual oxidase in Drosophila gut immunity. Science 310, 847-850 (2005b).

Hooper, L.V. and Gordon, J.I.: Commensal host-bacterial relationships in the gut. Science 292, 1115-1118 (2001).

Hooper, L.V., Wong, M.H., Thelin, A., Hansson, L., Falk, P.G., and Gordon, J.I.: Molecular analysis of commensal host-microbial relationships in the intestine. Science 291, 881-884 (2001).

Hord, N.G.: Eukaryotic-microbiota crosstalk: Potential mechanisms for health benefits of prebiotics and probiotics. Annu. Rev. Nutr. 28, 1-17 (2008).

Ismail, A.S. and Hooper, L.V.: Epithelial cells and their neighbors. IV. Bacterial contributions to intestinal epithelial barrier integrity. Am. J. Physiol. Gastrointest. Liver Physiol. 289, G779-G784 (2005).

Iyer, C., Kosters, A., Sethi, G., Kunnumakkara, A.B., Aggarwal, B.B., and Versalovic, J.: Probiotic Lactobacillus reuteri promotes TNF-induced apoptosis in human myeloid leukemia-derived cells by modulation of NF-kB and MAPK signalling. Cell. Microbiol. 10,1442-1452 (2008).

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

Karin, M. and Ben-Neriah, Y.: Phosphorylation meets ubiquitination: The control of NF-[kappa]B activity. Annu. Rev. Immunol. 18, 621-663 (2000).

Karrasch, T., Kim, J.S., Muhlbauer, M., Magness, S.T., and Jobin, C.: Gnotobiotic IL-10-/-;NF-kappa B(EGFP) mice reveal the critical role of TLR/NF-kappa B signaling

in commensal bacteria-induced colitis. J. Immunol. 178, 6522-6532 (2007).

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

Kelly, D., Conway, S., and Aminov, R.: Commensal gut bacteria: Mechanisms of immune modulation. Trends Immunol. 26, 326-333 (2005).

Kim, D.W., Lenzen, G., Page, A.L., Legrain, P., Sansonetti, P.J., and Parsot, C.: The Shigella flexneri effector OspG interferes with innate immune responses by targeting ubiquitin-conjugating enzymes. Proc. Natl. Acad. Sci. USA 102, 14046-14051 (2005).

Kotchoni, S.O. and Gachomo, E.W.: The reactive oxygen species network pathways: An essential prerequisite for perception of pathogen attack and the acquired disease resistance in plants. J. Biosci. 31, 389-404 (2006).

Kumar, A., Wu, H., Collier-Hyams, L.S., Hansen, J.M., Li, T., Yamoah, K., Pan, Z.Q., Jones, D.P., Neish, A.S.: Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. EMBO J. 26, 4457-4466 (2007).

Lambeth, J.D.: NOX enzymes and the biology of reactive oxygen. Nat. Rev. Immunol. 4, 181-189 (2004).

Le, Y., Murphy, P.M., and Wang, J.M.: Formyl-peptide receptors revisited. Trends Immunol. 23, 541-548 (2002).

Lee, W.J.: Bacterial-modulated signaling pathways in gut homeostasis. Sci. Signal. 1, pe24 (2008).

Macpherson, A.J. and Harris, N.L.: Interactions between commensal intestinal bacteria and the immune system. Nat. Rev. Immunol. 4, 478-485 (2004).

Madsen, K.L., Doyle, J.S., Jewell, L.D., Tavernini, M.M., and Fedorak, R.N.: Lactobacillus species prevents colitis in interleukin 10 gene-deficient mice. Gastroenterology 116, 1107-1114 (1999).

Madsen, K., Cornish, A., Soper, P., McKaigney, C., Jijon, H., Yachimec, C., Doyle, J., Jewell, L., and De Simone, C.: Probiotic bacteria enhance murine and human intestinal epithelial barrier function. Gastroenterology 121, 580-591 (2001).

Marchesi, J. and Shanahan, F.: The normal intestinal microbiota. Curr. Opin. Infect. Dis. 20, 508-513 (2007).

Mazmanian, S.K., Liu, C.H., Tzianabos, A.O., and Kasper, D.L.: An immunomodulatory molecule of symbiotic bacteria directs maturation of the host immune system. Cell 122, 107-118 (2005).

Mazmanian, S.K., Round, J.L., and Kasper, D.L.: A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453, 620-625 (2008).

Menard, S., Candalh, C., Bambou, J.C., Terpend, K., Cerf-Bensussan, N., and Heyman, M.: Lactic acid bacteria secrete metabolites retaining anti-inflammatory properties after intestinal transport. Gut 53, 821-828 (2004).

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

Mitra, S., Hanson, D., and Schlaepfer, D.: Focal adhesion kinase: In command and control of cell motility. Nat. Rev. Mol. Cell. Biol. 6, 56-68 (2005).

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

Neish, A.S.: Microbial interference with host inflammatory responses. In: Microbial pathogenesis and the intestinal epithelial cell (Hecht, G., Ed.). ASM Press, Washington, DC, 175-190 (2003).

Neish, A.S.: Microbes in gastrointestinal health and disease. Gastroenterology 136, 65-80 (2009).

Nenci, A., Becker, C., Wullaert, A., Gareus, R., van Loo, G., Danese, S., Huth, M., Nikolaev, A., Neufert, C., Madison, B., Gumucio, D., Neurath, M.F., and Pasparakis, M.: Epithelial NEMO links innate immunity to chronic intestinal inflammation. Nature 446, 557-561 (2007).

Noverr, M.C. and Huffnagle, G.B.: Does the microbiota regulate immune responses outside the gut? Trends Microbiol. 12, 562-568 (2004).

Ogier-Denis, E., Mkaddem, S.B., and Vandewalle, A.: NOX enzymes and Toll-like receptor signaling. Semin. Immunopathol. 30, 291-300 (2008).

Park, J. and Floch, M.H.: Prebiotics, probiotics, and dietary fiber in gastrointestinal disease. Gastroenterol. Clin. North Am. 36, 47-63 (2007).

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

Pena, J.A. and Versalovic, J.: Lactobacillus rhamnosus GG decreases TNF-alpha production in lipopolysaccharide-activated murine macrophages by a contact-independent mechanism. Cell. Microbiol. 5, 277-285 (2003).

Petrof, E.O., Kojima, K., Ropeleski, M.J., Musch, M.W., Tao, Y., De Simone, C., and Chang, E.B.: Probiotics inhibit nuclear factor-kappaB and induce heat shock proteins in colonic epithelial cells through proteasome inhibition. Gastroenterology 127, 1474-1487 (2004).

Pull, S.L., Doherty, J.M., Mills, J.C., Gordon, J.I., and Stappenbeck, T.S.: Activated macrophages are an adaptive element of the colonic epithelial progenitor niche necessary for regenerative responses to injury. Proc, Natl. Acad. Sci. USA 102, 99-104 (2005).

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., and Medzhitov, R.: Recognition of commensal cicroflora by Toll-like receptors is required for intestinal homeostasis. Cell 118, 229-241 (2004).

Rhee, S.G., Kang, S.W., Jeong, W., Chang, T.S., Yang, K.S., and Woo, H.A.: Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. Curr. Opin. Cell. Biol. 17, 183-189 (2005).

Rytkonen, A. and Holden, D.W.: Bacterial interference of ubiquitination and deubiquitination. Cell Host Microbe 1, 13-22 (2007).

Sansonetti, P.J.: War and peace at mucosal surfaces. Nat. Rev. Immunol. 4, 953-964 2004.

Sansonetti, P.J.: The innate signaling of dangers and the dangers of innate signaling. Nat. Immunol. 7, 1237-1242 (2006).

Sartor, R.B.: Microbial influences in inflammatory bowel diseases. Gastroenterology 134, 577-594 (2008).

Schneeweiss, H. and Renwrantz, L.: Analysis of the attraction of haemocytes from Mytilus edulis by molecules of bacterial origin. Dev. Comp. Immunol. 17, 377-387 (1993).

Sethuraman, M., McComb, M.E., Huang, H., Huang, S., Heibeck, T., Costello, C.E., and Cohen, R.A.: Isotope-coded affinity tag (ICAT) approach to redox proteomics: Identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. J. Proteome Res. 3, 1228-1233 (2004).

Slack, E., Hapfelmeier, S., Stecher, B., Velykoredko, Y., Stoel, M., Lawson, M.A., Geuking, M.B., Beutler, B., Tedder, T.F., Hardt, W.D., Bercik, P., Verdu, E.F., McCoy, K.D., and Macpherson, A.J.: Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. Science 325, 617-620 (2009).

Smith, K., McCoy, K.D., and Macpherson, A.J.: Use of axenic animals in studying the adaptation of mammals to their commensal intestinal microbiota. Semin. Immunol. 19, 59-69 (2007).

Stappenbeck, T.S., Hooper, L.V., and Gordon, J.I.: Developmental regulation of intestinal

angiogenesis by indigenous microbes via Paneth cells. Proc, Natl. Acad. Sci. USA 99, 15451-15455 (2002).

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

Terada, L.S.: Specificity in reactive oxidant signaling: Think globally, act locally. J. Cell. Biol. 174, 615-623 2006.

Tien, M.-T., Girardin, S.E., Regnault, B., Le Bourhis, L., Dillies, M.-A., Coppee, J.-Y., Bourdet-Sicard, R., Sansonetti, P.J., and Pedron, T.: Anti-inflammatory effect of Lactobacillus casei on Shigella-infected human intestinal epithelial cells. J. Immunol. 176, 1228-1237 (2006).

Tonks, N.K.: Redox redux: Revisiting PTPs and the control of cell signaling. Cell 121, 667-670 (2005).

Wills-Karp, M., Santeliz, J., and Karp, C.L.: The germless theory of allergic disease: Revisiting the hygiene hypothesis. Nat. Rev. Immunol. 1, 69-75 (2001).

Xu, J., Mahowald, M.A., Ley, R.E., Lozupone, C.A., Hamady, M., Martens, E.C., Henrissat, B., Coutinho, P.M., Minx, P., Latreille, P., Cordum, H., Van Brunt, A., Kim, K., Fulton, R.S., Fulton, L.A., Clifton, S.W., Wilson, R.K., Knight, R.D., and Gordon, J.I.: Evolution of symbiotic bacteria in the distal human intestine. PLoS Biol. 5, e156 (2007).

Yan, F., Cao, H., Cover, T.L., Whitehead, R., Washington, M.K., and Polk, D.B.: Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. Gastroenterology 132, 562-575 (2007).

Zaph, C., Troy, A.E., Taylor, B.C., Berman-Booty, L.D., Guild, K.J., Du, Y., Yost, E.A., Gruber, A.D., May, M.J., Greten, F.R., Eckmann, L., Karin, M., and Artis, D.: Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. Nature 446, 552-556 (2007).

Zeng, H., Carlson, A.Q., Guo, Y., Yu, Y., Collier-Hyams, L.S., Madara, J.L., Gewirtz, A.T., and Neish, A.S.: Flagellin is the major proinflammatory determinant of enteropathogenic Salmonella. J. Immunol. 171, 3668-3674 (2003).