

BACTERIAL INTERACTIONS WITH CELLS OF THE INTESTINAL MUCOSA: TOLL-LIKE RECEPTORS AND NOD2

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SUMMARY

Toll-like receptors (TLR) and NOD2 are emerging as key mediators of innate host defence in the intestinal mucosa, crucially involved in maintaining mucosal as well as commensal homeostasis. Recent observations suggest new (patho-) physiological mechanisms of how functional versus dysfunctional TLRx/NOD2 pathways may oppose or favour inflammatory bowel disease (IBD). In health, TLRx signalling protects the intestinal epithelial barrier and confers commensal tolerance whereas NOD2 signalling exerts antimicrobial activity and prevents pathogenic invasion. In disease, aberrant TLRx and/or NOD2 signalling may stimulate diverse inflammatory responses leading to acute and chronic intestinal inflammation with many different clinical phenotypes.

INTRODUCTION

The intestinal mucosa must rapidly recognise detrimental pathogenic threats to the lumen to initiate controlled immune responses but maintain hyporesponsiveness to omnipresent harmless commensals. Charles Janeway Jr first suggested that so-called pattern recognition receptors (PRRs) may play an essential role in allowing innate immune cells to discriminate between “self” and microbial “non-self” based on the recognition of broadly conserved molecular patterns.¹ Toll-like receptors (TLRs) which comprise a class of transmembrane PRRs play a key role in microbial recognition, induction of antimicrobial genes, and the control of adaptive immune responses. NODs (NOD1 and NOD2) are a structurally distinct family of intracellular PRRs which presumably in the context of microbial invasion subservise similar functions (fig 1). TLRs and NOD2 are widely expressed on various cell types of the gastrointestinal mucosa, participating in host defence against microbial pathogens in at least four ways:

- (1) recognition of molecular patterns present on pathogens;
- (2) expression at the interface with the “environment” of the gastrointestinal lumen;
- (3) induction of secretion of pro/anti-inflammatory cyto- and chemokines that link to the adaptive immune system; and
- (4) induction of antimicrobial effector pathways.

Recent studies have greatly advanced our understanding of these mechanisms through which the gastrointestinal innate immune system can mediate differential host-microbial interactions in recognition and sorting of the broad luminal spectrum of diverse microbial products. Furthermore, related findings propose that mammalian TLRx and NOD2 dysfunctions play a key role in the pathophysiology of IBD.

MOLECULAR BASIS OF BACTERIAL-MUCOSAL INTERACTIONS

Toll-like receptors (TLRs)

Structure

Mammalian TLRs comprise a family of (so far) 11 individual type I transmembrane receptors which are characterised by three common structural features (fig 1A): a divergent ligand binding extracellular domain with leucine rich repeats (LRR), a short transmembrane region, and a highly homologous cytoplasmic Toll/interleukin 1 receptor (TIR) domain, similar to that of the interleukin 1 receptor family and essential for initiation of downstream signalling cascades.²

Expression pattern

TLRs are differentially (inducibly or constitutively) expressed by many distinct cell types throughout the whole gastrointestinal tract in vitro and in vivo, including (mature and immature) epithelial cells of the stomach, small intestine, and colon,^{3–14} as well as intestinal monocytes/macrophages^{15–16} and dendritic cells (Stagg AJ, personal communication, 2005) of the lamina propria and myofibroblasts,¹⁷ endothelial cells,¹⁸ and adipocytes (Siegmond B, personal communication, 2005) of the intestinal submucosa.

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Some inter cell line and inter laboratory differences in detection levels of constitutive TLRx expression have been described for various intestinal epithelial tumour cell lines in vitro.^{3 8 10 11 19–21} It is a common, if not expected, problem that epigenetic drifts from the original progenitor lineage may occur in such cell lines with each cell culture passage. Different phenotypes of TLRx expression may also derive from distinct cell culture conditions which alter differentiation state in vitro.²² Regarding TLR expression in primary intestinal epithelial cells (IEC), there is field wide consensus that TLR2 and TLR4 are present only in small amounts on IEC in vivo, thus minimising recognition of luminal bacteria in the healthy intestine.^{4 7 9 11 14 16} In contrast, TLR4 is significantly increased in primary IEC throughout the lower gastrointestinal tract in active disease of both Crohn's disease (CD) and ulcerative colitis (UC)⁴ and murine colitis.^{9 14}

Ligand specificity

Different pathogen associated molecular patterns selectively activate different TLRs (that is, each TLR binds specific "molecular signatures" of different classes of microorganisms or individual features present on diverse commensals or

pathogens) (fig 2). TLR2, for example, recognises bacterial lipopeptides and lipoteichoic acid which are found abundantly in cell walls of Gram positive bacteria.²³ TLR2 may cooperate with TLR6 and TLR1, suggesting an essential mechanism for diversifying the repertoire of TLR mediated responses.²⁴ RNA from double stranded and "sense" single stranded viruses activates TLR3,^{25 26} whereas RNA from "antisense" single stranded viruses activates TLR7 and TLR8.^{27 28} TLR4 is the major receptor for lipopolysaccharide (LPS) activation²⁹ which may require the presence of accessory proteins, such as MD-2, CD14 and LPS binding protein. Flagellin and flagellated bacteria have been identified as specific ligands for TLR5.³⁰ Unmethylated CpG DNA found in prokaryotic genomes and DNA viruses modulates TLR9³¹ and TLR11 is activated by uropathogenic bacteria,³² but the specific ligand has yet to be determined. Importantly, species specific differences in distinct TLRx-ligand recognition appear to exist.³³

Furthermore, endogenous mediators may regulate various TLRs, such as heat shock proteins³⁴ or fibronectin³⁵ but it is controversial as to whether such bona fide ligands may directly activate TLRs under physiological conditions, or rather potential contaminants in the preparations.^{36 37} Nevertheless, TLRs may indeed be activated by endogenous ligands under pathophysiological conditions, such as "self"-DNA complexes. CpG sequences in "self"-DNA are an important potential trigger for autoantibody secretion in systemic autoimmune disorders. In rheumatoid arthritis, autoantibodies may complex to chromatin, leading to exaggerated B cell activation via TLR9.³⁸ In systemic lupus erythematosus, DNA containing immune complexes within lupus serum stimulate plasmacytoid dendritic cells to produce cytokines and chemokines via TLR9 and CD32.³⁹ In primary biliary cirrhosis, CpG DNA induced IgM production by activated B cells via TLR9⁴⁰ may drive IgG autoantibody responses and complex formation in a similarly relentless autodysregulatory loop. Thus impaired "self" versus "non-self" discrimination by TLRs in autoimmune disease (including IBD) may lead to self directed immune responses contributing to exaggerated production of proinflammatory cytokines and subsequent tissue damage.

Some reports suggest that TLR4 ligand binding might trigger assembly of a multi-receptor complex in which several components apart from the central ligand binding receptor contribute to LPS signalling. It is widely accepted that TLR4 forms a functional LPS recognition complex together with MD-2 and CD14 (see Gangloff and Gay⁴¹ for review) but whether several additional molecules (for example, an LPS binding complex of Hsp70-Hsp90-GDF5-CXCR4⁴² or CD55⁴³) which reside in lipid rafts next to TLR4 play a direct or, if at all, an auxiliary role in LPS binding and downstream signalling is not yet clear. Similarly, it has been proposed that CD36 may act as a facilitator or co-receptor to TLR2/6 for selective diacylglyceride recognition.⁴⁴ Further studies aimed at defining more complete models of functional interactions between these (and possibly other) molecules and the TLR4/MD-2/CD14 (or TLR1/2/6) cores, respectively, will hopefully soon elucidate potential fine tuning mechanisms of ligand recognition by such cooperative or competitive multi-receptor complexes.

Subcellular distribution

Subcellular compartmentalisation of TLRs appears to be a critical determinant of immune responsiveness. Thus based

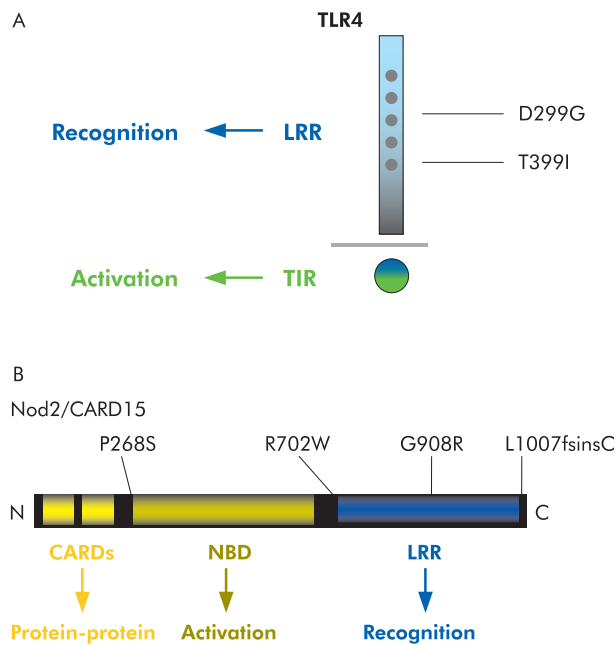


Figure 1 Toll-like receptor 4/nucleotide binding oligomerisation domain 2 (TLR4/NOD2) structure and inflammatory bowel disease associated common variants. (A) Mammalian TLRs are a family of type I transmembrane receptors which are all characterised by three common structural features, as exemplified for TLR4 here: a divergent ligand binding extracellular domain with multiple leucine rich repeats (LRR), a short transmembrane region and a highly homologous cytoplasmic Toll-interleukin 1 receptor (TIR) domain. Two common cosegregating missense mutations (D299G and T399I) that affect the extracellular ligand recognition domain of the TLR4 receptor are associated with deregulated immune responses to lipopolysaccharide in humans. (B) NOD1 and NOD2 are members of a family of intracellular proteins that contain an N terminal caspase recruitment domain (CARD), a centrally located nucleotide binding domain (NBD), and a C terminal regulatory domain. As shown here, NOD2 protein contains two N terminal CARDs fused to a central NBD domain followed by 10 tandem LRRs at the C terminus. Three main NOD2 variants (R702W, G908R, and L1007fsinsC) were confirmed to be associated with susceptibility to some types of Crohn's disease.

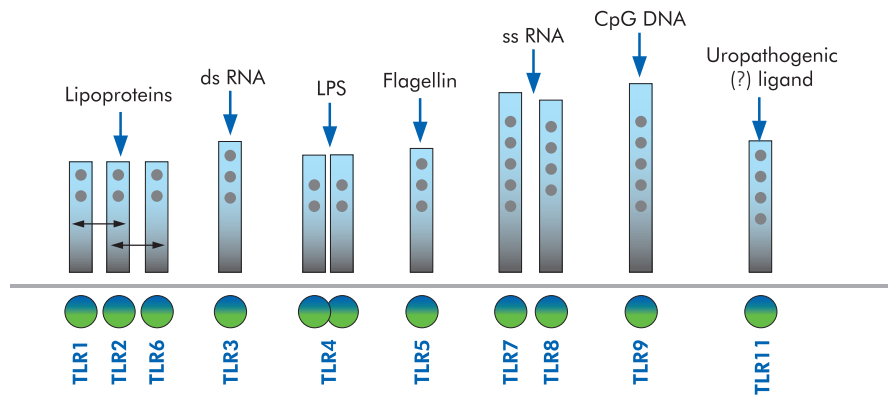


Figure 2 TLRx ligand diversity. Different pathogen associated molecular patterns (PAMPs) selectively activate different Toll-like receptors (TLRs) (that is, each TLR binds specific “molecular signatures” of different classes of microorganisms or individual features present on diverse commensals or pathogens). A short list of the main ligands is presented.

on the optimal site of bacterial ligand interaction, TLRs are strategically localised on the cell surface as well as in different subcellular compartments. Dynamic redistribution may be regulated by a state of differentiation as well as ligand exposure. TLR2 and TLR4 are mainly expressed at the apical pole of differentiated IEC *in vitro*, thus well positioned to monitor the luminal frontline of bacterial components whereas both receptors are present mostly in cytoplasmic compartments in non-differentiated IEC.^{4, 22} There are several reports suggesting that LPS-TLR4 redistributes between plasma membrane and endosomal structures which have been identified as part of the Golgi apparatus.^{22, 45–47} Yet the functional consequence of recycling and Golgi recruitment of TLR4 on LPS recognition and signalling seems to be ambiguous,^{48, 49} possibly reflecting cell type or species specific differences. TLR9 is retained in the endoplasmic reticulum⁵⁰ but readily cycles to sites of CpG DNA after cellular uptake.⁵¹ In contrast, TLR5 is stably expressed at the basolateral pole of

intestinal epithelia *in vitro*,⁵ the major scene of action of *Salmonella* translocated flagellin but *in vivo*, it seems that TLR5 is expressed on both poles of IEC.^{4, 52}

Signalling

Individual TLRs differentially activate distinct signalling events via diverse cofactors and adaptor proteins mediating specific immune responses. To date, at least five different adaptor proteins have been identified in humans: MyD88, Mal/TIRAP, TRIF/TICAM-1, TRAM/Tirp/TICAM-2, and SARM (for review see O’Neill and colleagues⁵³). The first identified so-called “classical” pathway mediated by the conserved TIR cytoplasmic domain in TLRs involves recruitment of the adaptor molecule MyD88, activation of the serine/threonine kinases of the interleukin 1 receptor associated kinase (IRAK) family, subsequently leading to degradation of inhibitor κ B (I κ B) and translocation of nuclear factor κ B (NF κ B) to the nucleus (fig 3). Downstream, different

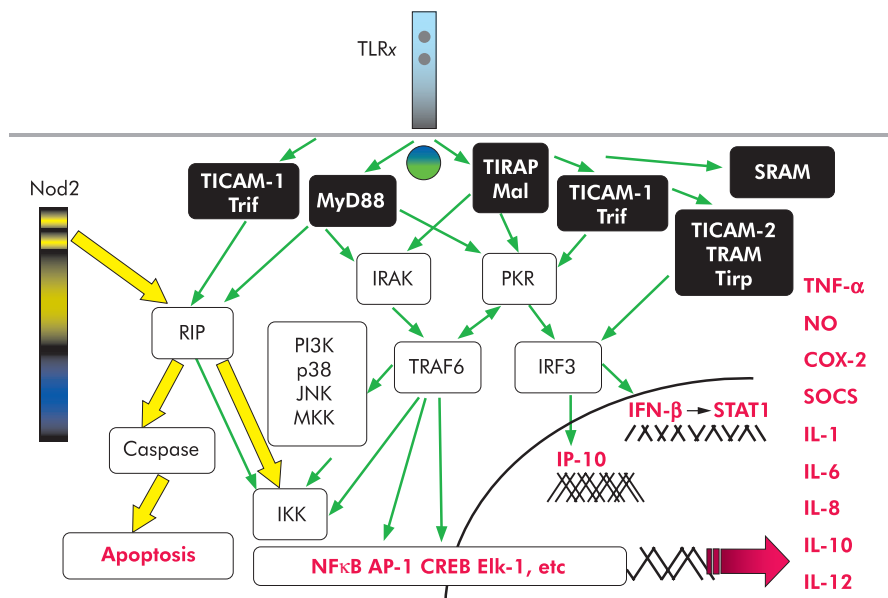


Figure 3 TLRx/NOD2 signalling pathways. Toll-like receptor (TLR) signalling is mediated by a complexity of various selective pathways. Rip2 is a direct downstream signal transducer of both TLRx and nucleotide binding oligomerisation domain (NOD2), thus possibly allowing regulatory cross talk between these two distinct pathways. IFN- γ , interferon γ ; IRAK, interleukin 1 receptor associated kinase; IL, interleukin; TNF- α , tumour necrosis factor α ; NF κ B, nuclear factor κ B.

signalling modules and partially interacting complexes result in activation of several transcription factors, including NFκB, AP-1, Elk-1, CREB, STATs, and the subsequent transcriptional activation of genes encoding pro- and anti-inflammatory cytokines and chemokines as well as induction of costimulatory molecules. All of these various downstream effects are critically involved in the control of pathogen elimination, commensal homeostasis, and linkage to the adaptive immunity. The mechanisms of agonist recognition and engagement with cell signalling may show considerable variation between TLRs and for each TLR perhaps also show variations between cell types and organ origin. Thus analysis of cytokine gene expression to distinct ligands in macrophages has identified important differences in biological responses induced by individual TLRs. Signalling through different TLRs can result in considerable qualitative differences in TH dependent immune responses by differential modulation of MAPKs and the transcription factor c-FOS.⁵⁴ However, the precise molecular mechanisms that differentially influence cell type dependent outcome of TLRx specific signalling effects are not yet fully understood.

NODs

Structure

The NOD (nucleotide binding oligomerisation domain) family (also called the CATERPILLAR family) comprises at present more than 20 different mammalian NOD-LRR proteins which mostly contain three distinct functional domains (fig 1B): a carboxy terminal ligand recognition (LRR) domain, a centrally located nucleotide binding domain (NBD), and a structurally variable amino terminal effector binding domain which consists of protein-protein interaction domains, such as caspase recruitment domains (CARDs) or pyrin domains (reviewed by Inohara and Nunez⁵⁵ and Chamillard and colleagues⁵⁶). Recent research has mostly focused on two cytosolic receptors of this family, NOD1 (also designated CARD4) and NOD2 (CARD15), which both play a major role in intestinal regulation of proinflammatory signalling through NFκB in response to distinct bacterial ligands. NOD2 shares significant homology with NOD1, but contains two, instead of one, CARD domains at its N terminus.

Toll-like receptors (TLRs)

- ▶ 11 mammalian, type I transmembrane receptors with divergent LRR-extracellular domain, homologous TIR.
- ▶ Each TLR binds distinct "molecular signatures" present on diverse commensals/pathogens.

NODs

- ▶ 20 mammalian intracellular proteins with C terminal LRR, central NBD, and N terminal CARD(s).
- ▶ PGN derived ligands: NOD2—muramyl dipeptide; NOD1—iE-DAP

Both

- ▶ Constitutively or inducibly expressed by many different cells throughout the gastrointestinal tract.
- ▶ Downstream activation of pro/anti-inflammatory cytokine secretion and/or apoptotic cascades.

Expression pattern

NOD2 has been shown to be constitutively or inducibly expressed in monocytes, macrophages, T and B cells, dendritic cells,⁵⁷ as well as IEC,^{58–61} including Paneth cells.^{62–63} NOD1 is ubiquitously expressed in many tissues and cells.

Ligand specificity

A specific motif of peptidoglycan (PGN), muramyl dipeptide (MDP), has been identified as (so far) the sole ligand of NOD2,^{64–65} whereas NOD1 senses a Gram negative PGN derivative, γ -D-glutamyl-meso-diamino-pimelic acid (iE-DAP).^{66–68} Based on these studies, it has become evident that neither NOD1 nor NOD2 sense LPS directly but rather detected contaminations of these PGN motifs within non-purified LPS preparations in earlier studies.^{69–70} Initially it was also believed that PGN is co-recognised, independently of its MDP or iE-DAP components, by TLR2. But TLR2 mediated cell activation induced by commercially available *Staphylococcus aureus* PGN preparations results from significant impurities.⁷¹

It remains to be determined whether MDP or iE-DAP directly bind to NODs through LRRs or rather through—as yet unidentified—interim co-mediators. Another essential question is how muropeptides from non-invasive bacteria gain entry into the cytosol and thus access to intracellular NODs. A recent study provided the first answer by demonstrating that non-invasive *Helicobacter pylori* delivers PGN to intracellular NOD1 in epithelial cells only in the presence of a functional *cag* pathogenicity island encoding a bacterial type IV secretion system.⁷² In addition, enzymes present in the lumen or produced from phagocytic cells in the lamina propria may possibly digest bacterial cell wall PGN, resulting in release and subsequent cellular uptake of muropeptides.

Subcellular distribution

NOD2 protein has been detected throughout the cytoplasm of IEC in vivo³⁹ but the exact subcellular organelle localisation of NOD1/NOD2 as well as the place of interaction with PGN fragments have not yet been identified.

Signalling

Despite enormous research developments and advances in this field during the last few years, current knowledge and understanding regarding detailed NOD1/2 signalling pathways is still limited (fig 3). On ligand stimulation, both NOD1 and NOD2 enter into CARD-CARD interactions with the serine-threonine kinase Rip2/RICK/CARDIAK which leads to NFκB activation⁷³ and augmentation of caspase induced apoptotic cascades. An intermediate region that is located between CARD and the kinase domain of Rip2 interacts with IKK γ (also called NEMO), thereby linking NOD1 and NOD2 to the regulatory subunit of the IKK complex which initiates its ubiquitinylation.^{74–75} Functional consequences deriving from NOD2 (and mutant variants) signalling events are discussed below.

PHYSIOLOGICAL IMPACT OF BACTERIAL-MUCOSAL INTERACTIONS: BENEFICIAL EFFECTS FOR THE HOST—LINK TO MUCOSAL HOMEOSTASIS

Tolerance

In the intestine, tolerance is an essential mucosal defence mechanism maintaining hyporesponsiveness to harmless luminal commensals and their products. Exaggerated

inflammatory responses in the absence of pathogenic bacteria would be otherwise deleterious. Several molecular immune mechanisms that ensure tolerance via TLRs in IEC have recently been described (see detailed review by Cario and Podolsky⁷⁶): decreased surface receptor expression which limits frontline recognition,^{4, 19} high expression levels of the downstream signalling suppressor Tollip which inhibits IRAK activation,¹¹ ligand induced activation of peroxisome proliferator activated receptor γ (PPAR γ) which uncouples NF κ B dependent target genes in a negative feedback loop,^{77, 78} and external regulators which may suppress TLR mediated signalling pathways. Additional tolerising mediators negatively modulating immune responses by interference with the TLR signalling complex have recently been identified in other cell systems, such as SIGIRR (single immunoglobulin interleukin 1R related molecule; also known as TIR8),⁷⁹ TRIAD3A,⁸⁰ the zinc finger protein A20,⁸¹ and ST2.⁸² Interestingly, TIR8 deficient mice are more susceptible to developing intestinal inflammation, suggesting a crucial role for TIR8 in tuning mucosal tolerance towards commensals.⁸³ The ubiquitin ligase TRIAD3A directly enhances proteolytic degradation of TLR4 and TLR9.⁸⁰ A20 also downregulates the TLR4 pathway by deubiquitination of TRAF6 and blocking NF κ B activation⁸⁴ as well as the TLR3 pathway by inhibition of interferon (IFN)- β gene expression via TRIF.⁸⁵

Commensal bacteria may assist the host in maintaining mucosal homeostasis by suppressing inflammatory responses and inhibiting specific intracellular signal transduction pathways,⁸⁶ potentially directly via TLR4 through elevation of PPAR γ expression, uncoupling NF κ B dependent target genes in a negative feedback loop⁷⁷ which may lead to attenuation of colonic inflammation.⁷⁸ Administration of CpG-DNA ameliorates the severity of dextran sodium sulphate (DSS) induced colitis^{87, 88} via TLR9⁸⁹ and limits cytokine derived intestinal epithelial proinflammatory immune responses.⁹⁰

Commensals may also play a key role in preventing allergic sensitisation via TLR activation (reviewed by Prioult and Nagler-Anderson⁹¹). TLR4 dependent signals by the intestinal microflora protect the host by inhibiting allergic responses to food antigens, while TLR4 mutant/knockout mice are highly susceptible to develop food allergy, which correlates with high levels of TH2 cytokines, interleukin (IL)-4 and IL-13.⁹² Thus LPS stimulation may prevent allergen induced TH2-type inflammation by upregulation of TH1 responses via TLR4 in regulatory T cells.⁹³

Intestinal mucosal "intolerance" (that is, exaggerated immune responsiveness towards commensals) may occur as a consequence of endogenously or exogenously induced disturbance of any of the TLR dependent signalling mechanisms of tolerance described above. Further mechanistic understanding of such host beneficial TLR/NOD signalling mechanisms of commensal mediated suppression of intestinal inflammation and how imbalance in such signalling events may lead to intestinal disease could potentially provide an efficient immunoadjuvant therapeutic approach in IBD (and atopic diseases).

Mucosal barrier protection

Commensals are able to not only suppress but also actively induce expression of host genes that participate in important physiological functions, including cell differentiation and maturation.⁹⁴ In order to maintain mucosal homeostasis against tissue damage, commensal induced host modulatory

effects seem to require functional TLRx and NOD.^{95, 96} "Healthy" gut conditions appear to demand constant exposure of the intestinal surface to commensal derived TLRx ligands and basal state of activation of downstream signalling pathways, thus ensuring rapid restitution and limited inflammatory responses.⁹⁵ Deficient TLRx or NOD signalling may imbalance commensal dependent mucosal homeostasis, facilitating injury and leading to disease. Emerging evidence exists that commensals directly assist the host to strengthen intestinal epithelial barrier resistance.^{94, 97, 98} Our group has recently described an important role for commensal induced TLR2 signalling in enhancement of intestinal epithelial barrier function, which correlated with distinct tight junction associated morphological changes.⁹⁹ However, our results also suggested that impaired function of TLR2 itself did not lead to increased intestinal epithelial permeability. In this context, further studies are needed to investigate whether lack of host protective TLR2 (or any other TLRx) ligands in the lumen that may be present in the resident microflora could lead to loss of barrier protection, facilitating invasion of pathogenic bacteria in disease.

Bacterial clearance and antimicrobial activity

NOD2 has been found to exert antibacterial activity in intestinal epithelial cells limiting survival of enteric bacteria after invasion. Bacterial clearance of *Salmonella typhimurium* is strongly accelerated in IEC expressing a functional NOD2 protein, whereas L1007fsinsC mutant expressing IEC are virtually unable to clear the pathogen in vitro.⁶¹ NOD2, as well as RIP2 (as downstream component of the NOD2 signalling pathway) knockout mice, exhibit a profoundly decreased ability to clear intracellular *Listeria monocytogenes*,^{73, 96} inducing persistent immune activation by combined loss of antibacterial activity, dysregulation of cytokine production, and imbalance of T cell activation. Although the NOD2 induced gene targets that lead to elimination of invasive intracellular bacteria are not yet identified in detail, recent reports suggest that TLR^{100, 101} as well as NOD^{96, 102} signalling pathways may critically be involved in commensal induced antimicrobial peptide production, such as defensins. These natural antibiotic peptides represent an important mechanism of host defence in innate immunity by efficiently killing phagocytosed microbes, thus helping to prevent pathogenic bacteria from crossing the mucosal barrier (reviewed by Ganz¹⁰³). Interestingly, β -defensin 2 may directly act as an endogenous ligand for TLR4,¹⁰⁴ thus possibly exponentiating bactericidal activity in a reverse autocrine loop.

However, the full spectrum of host beneficial signalling pathways activated by commensal derived TLR/NOD ligands which may balance responsiveness and survival and confer

Physiological effects of TLRs and NODs in the healthy gastrointestinal tract

- ▶ Tolerance (that is, maintaining hyporesponsiveness to harmless luminal commensals).
- ▶ Inhibition of allergic responses to food antigens.
- ▶ Protection of intestinal epithelial barrier function.
- ▶ Bacterial clearance by induction of antimicrobial peptide production.
- ▶ Maintenance of commensal and mucosal homeostasis.

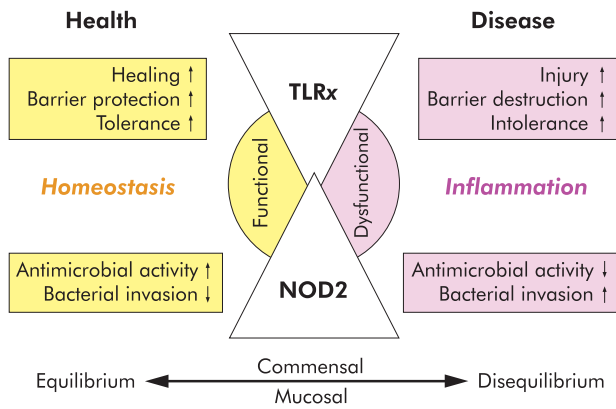


Figure 4 TLRx/NOD2 physiology and pathophysiology. In the healthy gut, both Toll-like receptors (TLRx) and nucleotide binding oligomerisation domain 2 (NOD2) are majorly involved in host defence and tissue repair responses, thus maintaining mucosal as well as commensal homeostasis. TLRx signalling protects the intestinal epithelial barrier, confers tolerance, and promotes healing. NOD2 exerts antimicrobial activity through defensin production and prevents intracellular bacterial invasion. When commensal and/or mucosal homeostasis are impaired due to genetic and/or environmental triggers, disease may develop: bacterial dysrecognition and intolerance through aberrant TLR and/or NOD signalling stimulates exaggerated proinflammatory responses leading to chronic inflammation via cytokine and chemokine production. TLR dysfunction induces tissue damage and barrier destruction by loss of commensal mediated colonic epithelial progenitor responses. NOD2 dysfunction leads to defective sensing of microbial threats and loss of bactericidal responses, thus allowing bacterial invasion into the host.

integrity of the healthy intestinal mucosa (fig 4) remains to be identified in depth.

PATHOPHYSIOLOGICAL IMPACT OF BACTERIAL-MUCOSAL INTERACTIONS: GENETIC ABERRATIONS—LINK TO MUCOSAL DISEASE

Toll-like receptor polymorphisms associated with IBD
Chronic recurrent intestinal inflammation in IBD may result from undue stimulation of the mucosal immune system by the resident microflora. “Healthy” intestinal mucosa expresses low concentrations of TLR2 or TLR4 protein *in vivo*. However, TLR4 expression is significantly increased in IEC and lamina propria mononuclear cells throughout the lower gastrointestinal tract in association with IBD⁴ and murine colitis.^{9–14} TLR4 upregulation could also result from ligands other than luminal LPS. T cell derived cytokines, such as IFN- γ and tumour necrosis factor α (TNF- α), which play significant pathophysiological roles in triggering IBD, have been found to upregulate intestinal epithelial TLR4 expression *in vitro*.^{7–10} It remains to be shown whether upregulated TLR4 confers functional hyperresponsiveness of the intestinal epithelium to LPS or rather reflects a loss of response.

The TLR4 gene is localised on chromosome 9 (q32–33),¹⁰⁵ a genomic region in which a CD susceptibility gene has been implicated.¹⁰⁶ In active IBD, variant alleles in the TLR4 gene could induce functional dysregulation of the LPS receptor. “Gain of function” mutations could functionally exhibit proinflammatory effects in response to physiological concentrations of LPS. Two common mutations in the human TLR4 gene, D299G and T399I, have been observed to occur at a general frequency of between 6% and 10% in Caucasian

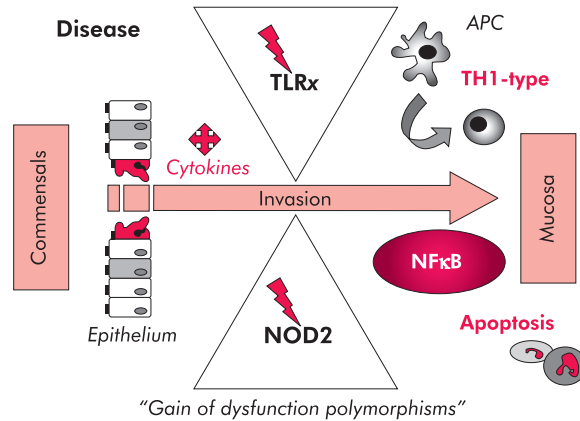


Figure 5 Current concept of how TLRx/NOD2 dysfunctions may contribute to the pathophysiology of Crohn's disease. Toll-like receptor (TLRs) and nucleotide binding oligomerisation domains (NODs) are present in the intestinal epithelium, the frontline of the mucosal immune system. “Gain of dysfunction” receptor variants may allow commensals to cross the intestinal epithelial barrier and gain access to the antigen presenting cells (APC) of the underlying mucosa. Subsequent commensal mediated stimulation of APC signalling may induce nuclear factor κ B (NF κ B) dysregulation leading to exaggerated TH1 responses and perpetuation of apoptosis.

populations.¹⁰⁷ D299G polymorphism has been associated with CD as well as UC in a Belgian population,¹⁰⁸ but no association was found in Scottish¹⁰⁹ or German¹¹⁰ populations. Increased susceptibility to IBD has been associated with coexistence of TLR4 and/or CD14 and NOD2 mutated alleles in a Greek population.¹¹¹

Although the D299G mutation (but not the T399I mutation) has been shown to interrupt TLR4 mediated LPS signalling *in vitro*,¹¹² the functional phenotypic consequence remains unresolved in IBD. Highly variable TLR4 gene mutations have been identified in various mice strains which exhibit a broad distribution of different phenotypic responses to LPS, ranging from hyper- to hyposensitivity,¹¹³ suggesting that additional gene interactions of the diverse strain backgrounds were involved. TLR4 knockout mice exhibit diminished TH1 driven immune responses and are therefore highly resistant to develop chronic nematode infections of the gut.¹¹⁴ Spontaneous colitis occurring in STAT3 knockout mice does not develop when these mice are crossed with TLR4 knockout mice, suggesting that aberrant TLR4 signalling in response to the indigenous intestinal flora contributes to the development of intestinal inflammation through the TH1 pathway.¹¹⁵ The role of TLR4 in TH1 dominant TNBS colitis has not yet been examined. These studies so far suggest that commensal mediated TLR4 signalling of mucosal T cells can be detrimental, leading to some forms of murine mucosal inflammation associated with excess TH1 responses (fig 5). Of note, TH1 type shifts appear to have a significant pathogenetic role in CD. However, other data from a different murine colitis model also support the fact that TLR4 signalling may exert cytoprotective characteristics, at least against chemically induced tissue injury in the intestinal mucosa. TLR4 mutant (C3H/HeJBir) and TLR4 deficient mice show increased susceptibility to DSS colitis in comparison with wild-type mice,^{95–116} suggesting that commensal mediated protection of the intestinal epithelial barrier (together with other mucosal cells) might be significantly impaired in TLR4 dysfunction after toxic DSS damage.

Appropriate regeneration and restitution following epithelial wounding with DSS may require commensal mediated amplification of colonic epithelial progenitor responses which are specifically impaired in mice lacking MyD88,¹¹⁷ downstream of TLR4.

“Loss of function” TLR4 mutations diminish sensitivity to LPS in C3H/HeJ mice²⁹ but paradoxically predispose to Gram negative infections, such as live *Salmonella typhimurium*.¹¹⁸ Thus D299G mutation could confer hypersensitivity to *Escherichia coli* or any other Gram negative commensal or pathogenic ligand causing unwanted inflammation in IBD. Flagellin, the structural component of bacterial flagella, is secreted by several commensal and pathogenic bacteria, including *Salmonella typhimurium*, and induces intestinal epithelial chemokine secretion via TLR5 which will, in turn, initiate dendritic cell migration and recruitment to the mucosal site of inflammation.⁵ Interestingly, dominant antigens in sera from colitic C3H/HeJBir (TLR4 mutant) mice seem to be flagellins.¹¹⁹ Similar hyperreactivity to flagellins was also observed in sera from patients with CD.^{119, 120} Although it is unclear whether there was any significant correlation with TLR4 (or NOD2) mutations in these CD patients, one may speculate that TLR4 mutations (similar to NOD2) could lead to impaired bacterial clearance and thus greater presence of bacterial antigens in the lumen, including flagellins. It is also possible that TLR4 mutation may induce genetic disequilibrium of TLR5 (or other TLRx) leading to unwanted inflammation through flagellin (or other TLRx specific ligand) dysrecognition.

Results from a preliminary report suggest that CD is also associated with TLR9 promoter polymorphisms in a single German cohort.¹²¹ Any association between IBD and TLR2 R753Q polymorphism which has recently been identified in septic patients has not yet been investigated. Although MyD88 deficient mice exhibit increased susceptibility to DSS induced colitis and tissue damage,^{95, 122} mutations within TLR efferent signalling proteins have not yet been described for IBD (or other intestinal diseases).

NOD2—a major susceptibility gene for Crohn’s disease

In 2001, NOD2 was mapped via linkage disequilibrium to chromosome 16q12 as an excellent candidate gene for CD (*IBD1*). Consequently, certain genetic variations have been associated with increased susceptibility to some types of CD.^{123, 124} Three major variants of the LRR region (within or nearby) include one frameshift mutation (L1007fsinsC) and two missense mutations (R702W and G908R), suggesting that a defect in bacterial recognition may be associated with CD. Initial studies demonstrated that up to 8% of Caucasian CD patients possessed at least one allele of the most common mutation, L1007fsinsC, causing a truncated NOD2 protein

lacking the last 33 amino acids. In CD, NOD2 expression in Paneth cells is increased, possibly secondarily induced by proinflammatory cytokines.^{60, 61, 125}

Previous studies have started to reveal the molecular mechanisms involved by which NOD2 may influence innate immune responses in the intestinal mucosa. It seems that different NOD2 mutations may span a spectrum of diverse phenotypes, ranging from complete “loss of function” to maximal “gain of function”. NOD2 mutations within NBD lead to constitutive ligand independent NFκB activation, causing a chronic systemic inflammatory disorder known as “Blau syndrome”.⁵⁵ Conversely, it has been suggested that CD associated NOD2 mutants which are predominantly found in the microbial ligand dependent LRR domain rather reflect “loss of function” phenotypes. Several in vitro transfection studies showed that human CD associated NOD2 mutants significantly abolish NFκB activation in response to MDP.^{64, 65, 126} However, paradoxically, macrophages within the intestinal lamina propria of CD patients overproduce NFκB targets, including exaggerated production of pro-inflammatory cytokines, such as TNF-α and IL-1β (reviewed by Podolsky¹²⁷). Accordingly, a recent in vivo study now demonstrates that MDP stimulated macrophages isolated from mice generated with a murine NOD2^{2932iC} variant, homologous to the human NOD2^{3020iinsC} (= L1007fsinsC) variant, exhibit enhanced NFκB activation, increased apoptosis, and elevated IL-1β secretion,¹²⁸ possibly implying an important mechanism of how dysfunctional NOD2 may trigger intestinal inflammation in some types of CD (fig 5). Thus this murine “gain of function” NOD2 frameshift mutation in the LRR region may imbalance functions of both terminal parts of the whole protein: bacterial dysrecognition through the impaired LRR domain, ligand independent NFκB activation, as well as uncontrolled apoptosis and subsequent induction of IL-1β processing and release through the hyperactive CARD domains.

In another in vivo study which was simultaneously published by a different group, it was shown that mice lacking full length NOD2 protein (NOD2 knockout) are more susceptible to oral infection with the bacterial pathogen *Listeria monocytogenes*.⁹⁶ The NOD2 gene product is most abundant in ileal Paneth cells^{62, 63} which express a diverse population of microbicidal defensins restricting colonisation or invasion of small intestinal epithelium by bacteria.¹²⁹ Stimulation with the NOD2 ligand MDP elicits cryptidin secretion from murine NOD2 expressing Paneth cells.¹³⁰ Production of a subgroup of mucosal antimicrobial peptides, known as cryptidins, is significantly diminished in NOD2 knockout animals,⁹⁶ potentially supporting the recently

TLR4 alteration associated with IBD

- ▶ Upregulation of mucosal TLR4 expression in active human IBD.
- ▶ Association of TLR4^{D299G}-polymorphism with IBD in selective populations.
- ▶ Murine TLR4 “gain of function”: spontaneous intestinal inflammation with TH1 excess.
- ▶ Murine TLR4 “loss of function”: increased susceptibility to DSS colitis and Gram infections.

NOD2 mutations associated with some forms of Crohn’s disease or colitis

- ▶ Upregulation of NOD2 expression in Paneth cells in active CD.
- ▶ Worldwide genotypic and phenotypic heterogeneity (major mutations: L1007fsinsC, R702W, G908R).
- ▶ CD associated mutant transfectants abolish NFκB activation in response to MDP in vitro.
- ▶ Murine NOD2^{2932iC}: “gain of function” (induction of NFκB and IL-1β, uncontrolled apoptosis).
- ▶ Murine NOD2-/-: “loss of function” (decreased bacterial clearance due to lack of cryptidin production).

proposed concept of a “defensin deficiency” defect in human IBD.¹³¹ Expression of defensin related cryptidin 4, which is mostly secreted from Paneth cells in the distal small intestine,¹³² is particularly decreased in NOD2 knockout mice⁹⁶ whereas ileal expression of two human α -defensins (HD-5 and HD-6) is significantly diminished in NOD2 mutant CD patients.¹⁰² Interestingly, human mutations in the NOD2 gene are strongly associated with distinct phenotypic expressions of ileal disease (corresponding to the location of Paneth cells), mainly involving fibrostenosing complications,^{133–136} but not formation of epithelioid granulomas.¹³⁷ However, in heterogeneous CD, it is likely that Paneth cell dysfunction is only one pathophysiological mechanism which is specifically associated with a certain phenotypical subtype of inflammatory disease with severe hyperproliferative changes in the lower ileum in response to, yet unidentified, pathogenic or commensal organisms.

It is important to note that neither NOD2 knockout nor NOD2 frameshift mutant mice develop intestinal inflammation spontaneously.^{57 96 128} However, in contrast with NOD2^{2932iC} mutant mice, NOD2 knockout mice do not demonstrate significantly enhanced susceptibility to colitis in the DSS induced model, implying possibly variable mechanistic phenotypes of innate host defence. Genotypic heterogeneity at the single NOD2 locus could not only lead to intraspecies but also interspecies variability of phenotypes. Thus direct proof is needed of whether the described signalling targets and effects of murine NOD2 dysfunctions indeed represent phenotypic consequences of NOD2^{L1007fsinsC} (and other human NOD2 mutants (in association with or without TLRx mutations)) which directly contribute to the complex pathophysiology of IBD in humans.

TLRx/NOD2 synergy?

From a physiological point of view, it is conceivable that TLR and NOD pathways may cooperate in (positive and/or negative) regulatory feedback loops to modulate immunological responses. Synergistic effects between different sensing events could be advantageous for the host by exhibiting more controlled and immediate innate immune responses to potential threats. On the other hand, non-synergistic states of such interactions could lead to disease by uncontrolled actions of the innate immune system. Ligand dysrecognition and subsequent disequilibrium of signalling events could thus induce imbalance of pro- versus antiapoptosis and disturbance of bacterial clearance in the intestinal mucosa. TAK-1^{138 139} and Rip-2^{73 140} have previously been identified as common regulatory checkpoints of NOD2 and TLRx signalling pathways (fig 3) which could combine multiple signalling pathways (including TNFR/IL1R/IL18R) of both the innate and adaptive immune systems. In addition, negative cross talk between TLR2 and NOD2 has recently been proposed. Stimulation with the NOD2 ligand MDP inhibited TLR2 driven TH1 cytokine production by tackling NF κ B. Conversely, in NOD2 knockout mice, stimulation with synthetic lipopeptide was observed to result in NF κ B dysregulation and subsequent imbalance of cytokine production (increase in IL-12, decrease in IL-10) via TLR2.^{141 142} Thus NOD2 may exhibit anti-inflammatory activities limiting NF κ B activation after bacterial stimulation of the TLR2 pathway, suggesting that the hyperinflammatory mucosal response in CD may be due to lack of the inhibitory function of NOD2 on proinflammatory TLR2 signalling to bacterial ligands in the lumen. However, two other recent studies

failed to observe a similar negative regulatory effect of NOD2 on TLR2.^{96 128} These contrasting results may imply ambiguous interrelations between these two pathways under different experimental conditions which remain to be clarified. In this context, one may also consider that PGN was used as a presumed TLR2 ligand in some of these studies but, when highly purified, PGN sensing does not seem to occur via TLR2.⁷¹ Taken together, discussion of potential TLRx-NOD2 interrelations and their functional impact remains unresolved at this point of the investigation and requires further study.

Genetic and phenotypic heterogeneity of TLR4/NOD2 carriers

Mutations of PRRs may modify detection of pathogens, leading to altered susceptibility to disease. As outlined above, certain mutations of TLR4 and NOD2 have been strongly associated with CD, implying that PRR dysfunction may be involved in the pathogenesis of some types of CD (fig 5). NOD2 mutants have been associated with fibrostenosing ileal disease in CD but at this point in the investigation it remains elusive whether NOD2 genotyping would be helpful in clinical practice to predict the severity of the disease course or treatment response.¹⁴³ Compelling evidence emerges that genetic heterogeneity between ethnic populations exists for TLR4 and NOD2 variant alleles, which may reflect phenotypic heterogeneity in IBD worldwide.^{144 145} Interestingly, no common NOD2 variant was found in Japanese CD patients.¹⁴⁶ Therefore, absence of a mutation in a healthy individual may not exclude development of intestinal disease later on, while on the other hand, a healthy individual may carry NOD2 variants, without ever developing intestinal disease. But protective gene-environment or gene-gene interactions at this locus remain to be identified. Therefore, recognition that the contribution of NOD2 mutations to disease susceptibility may significantly vary in different racial and ethnic populations in geographical areas has led to increased efforts towards identifying potential genetic, environmental, and immunological cofactors that may determine manifestation or resistance to disease in susceptible individuals. Phenotypic alterations of the innate immune response in different individuals may be caused by diverse mutational pressure and selectivity within imbalanced interplays of several gene combinations, but not all genes that may modulate innate immune responsiveness have yet been functionally identified. Thus future studies will need to extend “forward genetic” approaches in this field (that is, starting with the immunological difference of healthy versus diseased NOD2/TLR4-mutant carriers and then proceed from the top down to identify the causative gene complex(es)). It will be important to dissect the multi-factor causes of the detrimental shift from host-beneficial to host-threatening innate immune recognition of commensals via TLRx/NOD2 in human relevant studies *in vivo*.

Role of TLRx/NOD2 in gastrointestinal mucosal diseases other than IBD?

Celiac disease is a HLA linked inflammatory disorder of the small intestine that is triggered by gluten peptides and strongly associated with circulating mucosal IgA autoantibodies to tissue transglutaminase. Twin studies led to the conclusion that celiac disease is strongly linked to genetic factors but it does not necessarily strike both twins of an identical pair,¹⁴⁷ suggesting that environmental factors may

be involved in the onset of the disease. Commensals or pathogens could potentially provide such required cofactors for the development of this T cell mediated disease.¹⁴⁸ A recent study provided first evidence of attachment of rod shaped (not further characterised) bacteria to the intestinal epithelium in the upper small bowel of some untreated and treated coeliac disease patients, but not in healthy controls, suggesting that bacteria may be involved in the pathogenesis of coeliac disease.¹⁴⁹ It is possible that bacterial products may sensitise CD4+ T cells to gluten via TLRx.¹⁵⁰ Interestingly, certain gluten fragments elicit a direct innate response, while others preferentially drive adaptive responses.¹⁵¹ Thus one may also speculate that dysfunctional TLRx (or other PRRs) could directly recognise dietary gluten peptides as “non-self” leading to release of IL-15 (see comment by Schuppan and colleagues¹⁵²). But no study has yet clearly examined the potential role of TLRs and their specific ligands in the pathophysiology of coeliac disease.

Helicobacter pylori induces NFκB activation in gastric epithelial and monocytic cells but it is controversial as to which specific TLR(s) may be involved in this immune response—TLR2,¹⁵³ TLR4,^{12 153–155} or TLR5,^{155 156} whereas others have suggested that NOD1 may act as a sensor.⁷²

Further evaluation of potential involvement of TLRx/NODx in other pathogenic infections of the intestine will be of clinical importance.

CONCLUSIONS AND PERSPECTIVES

Recent studies have begun to define the mechanisms through which crucial PRRs may regulate intestinal innate immunity. Cooperative as well as competitive interactions may occur between different bacterial and non-bacterial ligands via TLRs and NODs or other components of the innate immune system leading to differential pro- as well as anti-inflammatory immune responses in different cell types. Both TLRx and NOD2 are significantly involved in host defence and tissue repair responses, thus crucially maintaining mucosal homeostasis. TLRx signalling protects intestinal epithelial barrier and maintains tolerance while NOD2 signalling exerts antimicrobial activity and prevents bacterial invasion. Thus both receptors collectively exhibit distinct features that ensure commensal as well as mucosal homeostasis. Imbalance of the complex interrelations between commensals and PRRs may result in tissue injury and subsequent inflammation of the intestinal mucosa (fig 4). Aberrant TLR and/or NOD signalling may stimulate diverse inflammatory responses leading to chronic intestinal inflammation with many different clinical phenotypes.

Perspectives of current and future studies

- ▶ Characterisation of the distinct phenotypic consequences of NOD2^{L1007fsinsC} and TLR4^{D299G} mutations, possibly resulting in specific subforms of IBD.
- ▶ Identification of genetic, environmental, and immunological cofactors that determine manifestation or resistance to IBD in susceptible individuals.
- ▶ Clarification of potential positive/negative TLRx-NOD2 synergy.
- ▶ Investigation of PRR involvement in other gastrointestinal disorders, such as coeliac disease or enteric infections.
- ▶ Evaluation of the potential value of PRRs (activation versus inactivation) as therapeutic targets.

Further studies of the physiological and pathophysiological mechanisms within this network of possible cell-cell, ligand-ligand, and PRR-PRR signalling interactions that may favour or prevent inflammatory disease could lead to promising novel approaches that may differentially exploit the TLR/NOD pathways as a means of inducing salutary immune responses for treatment of IBD. It is likely that differential therapeutic strategies will need to include agonists as well as antagonists of PRRs, taking into account differences in PRR pathophysiology at different stages of disease as well as phenotypic and genotypic heterogeneity between distinct subgroups of IBD patients. Prophylactic application of selective TLR/NOD2 ligands could enhance desired commensal mediated tissue protective processes in order to prevent disease. Once an acute inflammatory episode has broken out, some of the untoward effects of intestinal inflammation could be abrogated by blocking uncontrolled signal transduction by specific TLR/NOD2 inhibitors, thus dampening tissue destructive effects. One key element to this disease modifying approach might be to blunt, rather than entirely eliminate, the dysregulated innate responses in acute IBD. In this context, careful assessment of adverse effects will be critical when modulating such fundamental host defence pathways of innate immunity. Given the rapid and exciting advancements of research in this field over the last few years, it is reasonable to presume that more immunological evidence and concrete directions for the potential value of these PRRs as therapeutic targets in IBD (and possibly other intestinal diseases) will emerge in the near future.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 1180

The histological features of the gastric mucosa, infiltrated by epitheloid and spindle cells, were suggestive of a metastatic malignant melanoma. This was confirmed with HMB45, MART-1, and tyrosinase (pan-melanoma cocktail) positivity. His computed tomography scan had shown evidence of widespread metastatic disease with hepatic, mesenteric, and retroperitoneal lesions. He was managed conservatively and died two months after discharge.

The incidence of melanoma is rising and now represents 3% of all newly diagnosed cancers and is the leading fatal illness arising in the skin. While excision of early disease is potentially curative, it is an aggressive cancer which frequently metastasises. The gastrointestinal tract is a common site of metastasis. Fifty per cent of patients with metastatic melanoma will have a gastrointestinal tract metastasis, although only 9% of these are detected during life. The most common sites for metastases are the small bowel (60%), colon (20%), and stomach (20%). Symptoms from malignant deposits in the gastrointestinal tract are rare and frequently present late in the course of the disease. Common modes of presentation are anaemia and intestinal haemorrhage, although intussusception and acute bowel obstruction can also occur. While treatment options exist for primary disease, the prognosis of metastatic melanoma remains poor, with an average prognosis for those with stage IV disease of less than six months. Recent research has suggested a possible improved prognosis and quality of life among patients with early diagnosis and surgical resection of gastrointestinal melanoma metastases.

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