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Functional defects in NOD2 signaling in experimental and human Crohn disease

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Increasing evidence suggests that a deficit in innate immunity may play a causative role in the pathogenesis of inflammatory bowel disease. The most compelling support for this hypothesis comes from the genetic association of Crohn disease (CD) with carriage of polymorphisms within the *NOD2* gene, which represent the most frequent genetic defect in CD. Our findings suggest that SAMP1/YitFc mice, which develop CD-like ileitis in the absence of NOD2 genetic mutations, fail to respond to MDP administration by displaying decreased innate cytokine production and impaired bacterial clearance before the onset of disease. This provides evidence that dysregulated NOD2 signaling, genetic or functional in nature, predisposes to chronic intestinal inflammation, and supports a new paradigm that CD may occur from a deficit in innate immunity as opposed to an overly aggressive immune response. This new paradigm could lead to potential development of new preventative or therapeutic modalities for patients with CD.

Keywords: innate immunity, nucleotide-binding oligomerization domain 2, inflammatory bowel disease, Crohn disease, SAMP/YitFc

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The gastrointestinal tract is a unique environment wherein a huge burden of bacteria exists in close proximity to the richest immunological structure of the human body. The ability of the host immune system to distinguish between commensals and pathogens is required to maintain the appropriate immune responses to pathogens and avoid the development of responses to self vs. environmental antigens.[1-3] In this context the innate immune system remains a pivotal player in controlling host resistance and maintaining the mucosal immune balance in the gut.[4] The innate immune system provides a primary host response to bacterial invasion by using pattern recognition receptors (PRRs) to recognize microbial agents.[5] PRRs, including Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain receptors (NLRs), sense evolutionarily conserved pathogen-associated molecular patterns (PAMPs) of microorganisms, and trigger sequential activation of intracellular signaling pathways leading to induction of a range of cytokines that drive the primary host resistance to pathogens.[6,7]

The critical importance of maintaining a finely balanced immune response in the intestine is highlighted when functional or genetic deficiencies in some components of the innate immune system exist, and this balance is lost. An example of such failure in maintaining mucosal homeostatic mechanisms is during the development of chronic intestinal inflammation, such as that observed in inflammatory bowel disease (IBD).[8,9] Over the past years, the pathogenesis of IBD has been primarily attributed to an overly aggressive adaptive immune response against luminal antigens. However, several studies have evidenced that this hypothesis is insufficient to explain the immunobiology of IBD and, importantly, may not consider the early events that take place during the initiation of inflammation that substantially differ from the late stages of disease.[10] Consequently, a substantial shift has occurred in understanding the etiopathogenesis of IBD, which supports

the novel hypothesis that this chronic, relapsing inflammatory disease of the gut actually results from a primary defect in intestinal innate immunity.[11] The most compelling support for this hypothesis comes from the clear genetic association of Crohn disease (CD) with carriage of polymorphisms within the *NOD2* gene, which represent the most frequently altered genetic defect in CD.[12,13] Nucleotide binding oligomerization domain containing 2 (NOD2) is a PRR and member of the NLR protein family that is mainly expressed in monocyte-derived cells[14,15] Upon induction by inflammatory agonists, like TNF-α or IFN-γ, NOD2 expression can be upregulated in epithelial cells including those of the gastrointestinal tract and skin.[15] NOD2 has the essential role of initiating innate immune responses upon intracellular exposure to muramyl-dipeptide (MDP), a breakdown product of peptidoglycan that is present in the cell wall of both Gram-negative and Gram-positive bacteria. The NOD2 protein is composed of two N-terminal CARD domains for interacting with other CARD containing proteins, a central NACHT domain for self-oligomerization, and ten C-terminal leucine-rich repeats (LRRs). NOD2 is thought to exist in an auto-inhibited state with the LRRs folded back onto the NACHT domain. Exposure to MDP causes a conformational change in NOD2, allowing both NOD2 oligomerization through the NACHT domain and binding of the dual specificity kinase RIP2 through homotypic CARD-CARD interactions.[16] Binding to NOD2 promotes RIP2 kinase activation, and the active NOD2:RIP2 complex then initiates a number of intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK) and the NFκB pathway.[14,17-19] NOD2 also has recently been shown to influence MHC cross-presentation,[20] autophagy induction, and resistance to intracellular bacterial infection.[21,22] Thus, while most well-known for its acute signaling effects, NOD2 activation causes a variety of cellular changes in vivo that are also likely important for immunologic homeostasis.

The major CD-associated *NOD2* polymorphisms (Leu1007fsinsC, Gly908Arg, and Arg702Trp), located within the LRR region of the protein, are considered loss-of-function phenotypes.[23,24] The associated risk is dose-dependent, with heterozygous carriers of the *NOD2* gene polymorphisms harboring a 2- to 4-fold increased risk of CD and homozygous or compound heterozygous carriers having a 20- to 40-fold increased risk. To date, the exact mechanism explaining the effects of the loss-of-function polymorphisms on downstream NOD2 signaling and the pathogenesis of CD have yet to be fully elucidated. A generally accepted explanation is that decreased NOD2 function manifests itself in a failure to respond to pathogens, causing an increased bacterial burden and abnormal interactions between the gut mucosal immune system and luminal antigens that ultimately culminate in chronic intestinal inflammation characteristic of CD. Although *NOD2* polymorphisms represent the first genetic risk factor associated with CD, they account for only approximately 15–20% of CD cases.[25] The majority of patients who develop CD, nearly 85% of cases, actually have wild-type NOD2. Therefore, it is possible that in these patients the defect might manifest itself in some other functional component of the NOD2 signaling pathway, resulting in the same end result of dysregulated initiation, propagation, or termination of NOD2-mediated innate intestinal immune responses. Remarkably, a large number of the genes identified as affecting the pathogenesis of IBD function in several innate immune signaling pathways. For example, some, like ATG16L1, IRGM, or LRRK2, are responsible for influencing bacterial uptake, killing, and cross-presentation through their effects on autophagy.[26] Others, like DLG5, are responsible for limiting exposure to luminal bacterial through epithelial integrity. Still others, like the IL-23R, allow the coupling of innate to adaptive immunity.[26] It is not unlikely, therefore, that the nearly 85% of CD patients lacking the *NOD2* polymorphisms may display combined or separate functional defects in innate immunity, possibly influencing and influenced by the NOD2 signaling pathway, which, like the genetic mutation, renders patients unable to mount effective innate immune responses.

In our study we determined the functional role of NOD2 in the SAMP1/YitFc (SAMP) murine model of CD-like ileitis.[27] These mice spontaneously develop a severe and progressive chronic ileitis without chemical, genetic, or immunological manipulation. Furthermore, the resulting ileitis in these mice bears remarkable phenotypic similarities to CD with regard to disease location, histological features, extra-intestinal manifestations, and response to therapies that are effective in treating the human disease.[28] Notably, these mice, like 85% of CD patients, lack *NOD2* mutations. Interestingly, we provided evidence that SAMP mice, despite their wild-type *NOD2* genotype, fail to respond to MDP administration by displaying decreased innate cytokine production and dysregulated NOD2 signaling before the onset of disease. We showed that, unlike in other mouse models of colitis,[29] in vivo administration of MDP does not prevent dextran sulfate sodium (DSS)-induced colitis in SAMP mice and that the abnormal NOD2 response is specific to the hematopoietic cellular component.[27] Moreover, we demonstrated that MDP fails to enhance intracellular bacterial killing in SAMP mice, a feature common with NOD2 dysfunction.[22,27] The end result is an ineffective maintenance of immunologic mucosal homeostasis due to dysregulation of NOD2-induced bacterial clearance with concomitant inflammatory disease susceptibility in the presence of a wild-type *NOD2* genotype. In line with our discoveries, recent studies have identified a subset of CD patients who do not carry NOD2 mutations but fail to produce innate cytokine in response to MDP stimulation (personal communication, Nuñez et al.). These data further underscore the relevance and similarities of the SAMP model with the human condition and raise the possibility that the functional defect in NOD2 signaling and MDP response present in our model may represent a disease process underlying CD pathogenesis, at least in a subgroup of patients with CD.

Our study supports the hypothesis that, although CD is principally localized to the gut, it may be the result of a primary immunodeficiency localized within the hematopoietic compartment.[30] In line with this concept, Marks et al. showed that patients with CD had significantly lower accumulation of neutrophils and suppressed pro-inflammatory (IL-8 and IL-1) cytokine expression 6 h post-trauma. Cultured macrophages from CD patients displayed diminished IL-8 secretion after stimulation with various inflammatory stimuli. Finally, inoculation with *E. coli* induced attenuated local responses in patients with CD.[31] In separate studies Smith et al. showed that macrophages derived from blood monocytes of CD patients fail to secrete pro-inflammatory cytokines and chemokines in response to bacteria or bacterial products. Interestingly, this phenotype was shared by all CD patients tested, regardless of their NOD2 genotype, and was markedly distinct from healthy controls.[32] This parallels our findings that macrophages from SAMP mice (which have a wild-type NOD2 genotype) are refractory to MDP-stimulated cytokine production and MDP-enhanced *Salmonella* clearance and suggests that NOD2 dysfunction in hematopoietic cells plays a critical role in disease pathogenesis.[27] Moreover, because NOD2 is tightly associated to autophagy,[22] it is possible that the contribution of autophagic mechanisms to the intestinal disease of SAMP mice might be also involved. This hypothesis is actively under investigation in our laboratory. We have preliminary data showing that the autophagic targeting of intracellular bacteria is defective in SAMP mice, which may contribute to persistence of intra-ileal bacteria and chronic inflammation (personal communication, Christine McDonald et al.).

NOD2 is well known to synergize with other TLR pathways and is thought to provide a rheostat-like mechanism by which innate immune responses can be tailored and measured.[33,34] The previously described synergistic effects of concomitant stimulation of immune cells with MDP and TLR ligands were not observed in macrophages from SAMP mice. The abnormal cytokine response to MDP stimulation observed in SAMP mice does not appear to be associated with induction of bacterial tolerance.[27] Under normal conditions, the gastrointestinal tract maintains a delicate balance between bacterial tolerance and inflammation through tight regulation of the innate immune system. Any perturbations in bacterial recognition pathways could disrupt this homeostatic balance. In general, recognition of bacteria via TLRs and NLRs induces the production of physiologic amounts of cytokines that are critical for the removal of invading bacteria.[35] Although these cytokines are protective to the host, their excessive production induces harmful effects in tissues and can lead to severe immunopathology, such as that seen in patients with CD.[36] Thus, induction of bacterial tolerance is a physiological mechanism used by the gut innate immune system to decrease the negative inflammatory effects of prolonged exposure to bacterial infection or commensal bacteria. It has been well described that stimulation with MDP induces tolerance to MDP, but not to other TLR agonists; conversely, macrophages tolerant to LPS or to other TLRs ligands remain fully responsive to MDP.[37] To rule out the possibility that induction of tolerance is the potential cause of the reduced cytokine response observed in SAMP mice, we tested the functional effect of MDP stimulation in cells isolated from pre-inflamed SAMP mice (4–6 wk). In addition, we found that LPS elicits a normal response in terms of cytokine production, suggesting that the hypo-responsiveness induced by MDP does not involve other innate immune pathways but is specifically associated to NOD2 signaling.

SAMP mice develop full spontaneous CD-like ileitis at approximately 20 wk of age, allowing us to study both the pre-inflamed and inflamed phases of disease.[28] In our study we were able to mechanistically determine the early, immunodeficient pathogenic events leading to the development of spontaneous ileitis in the absence of *NOD2* genetic mutations. The fact that a deficit in NOD2 function predisposes SAMP mice to ileitis suggests that NOD2 has a physiologic protective role against the onset of disease. However, it appears that wild-type NOD2 may play a dichotomous role during different stages of disease such that any deviation, either positively or negatively, could cause an immunologic dysfunction that lead to chronic intestinal inflammation.[38-40] This is a recurrent concept seen with other immune mediators such as "innate cytokines" that play a dichotomous role during homeostatic conditions and chronic intestinal inflammation.[41] These molecules generate acute inflammatory responses, which result in the elimination of excessive numbers of bacteria. At the same time certain cytokines, such as TNF-α, IL-18, and IL-33 for example, facilitate the repair process, which re-establishes the integrity of the epithelial monolayer.[42-45] In contrast, during the chronic intestinal inflammation, one or more of the homeostatic mechanisms are dysfunctional and several deficiencies may occur. The antimicrobial and epithelial barriers may be inadequate to hold the commensal bacteria separate from the gut-associated immune system of the lamina propria, and the intracellular processing of bacteria may be impaired or the secretion of pro-inflammatory factors may be dysregulated. The end result of these defects is the setting of the mucosal immunostat on "inflammation," which results in the continuous release of pro-inflammatory factors.[41] In this context, the NOD2 signaling pathway may have a critical role in determining the production of pro-inflammatory cytokine and perpetuation of chronic intestinal inflammation. Notably, as NOD2 and RIP2 are both NFκB regulated genes, upon inflammatory stimulation the expression of both molecules increases considerably.[15] For example, in pediatric CD, it has been shown that wild-type NOD2 and its obligate kinase RIP2 are overexpressed and hyperactive in ileitis.[46] Moreover, a recent study from Jamontt et al. demonstrated that mice double deficient in IL-10 and NOD2 are protected from developing severe chronic colitis; they provide evidence that NOD2 contributes to enhanced pro-inflammatory activity of macrophages from IL-10$^{-/-}$ mice and that the synergistic

activity of MDP with TLR ligands in the absence of IL-10 renders macrophages intrinsically hyperresponsive to bacterial stimulus, thus contributing to the dysregulated immune environment that ultimately results in the development of colitis in IL-10$^{-/-}$ mice.[47] In this context it has been postulated that inhibition of NOD2:RIP2 signaling in the presence of a wild-type NOD2 may be efficacious in CD.[38] Therefore, it is possible that NOD2 signaling plays a double-edged role in the pathogenesis of chronic intestinal inflammation by protecting the intestinal mucosa in physiological conditions and mediating the production of proinflammatory cytokines during chronic gut inflammation.

Taken together, our studies provide compelling evidence that SAMP mice, in the absence of *NOD2* genetic mutations, fail to respond to MDP, the natural ligand for NOD2, and display impaired bacterial clearance. These results support the concept that a dysfunctional NOD2 response in SAMP mice predisposes them to chronic intestinal inflammation. We believe that these results provide a paradigm shift by demonstrating that CD-like ileitis is caused by a deficit in some components of the mucosal innate immune response and that this deficit leads to impaired bacterial clearance and exaggerated activation of the adaptive immune system with chronic relapse of intestinal inflammation. Therefore, new treatment modalities aimed at stimulating the innate immune system and enhancing NOD2 signaling may prove effective in treatment of early disease, including preventative therapies.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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