The nucleotide-binding oligomerization domain (NOD) proteins NOD1 and NOD2, the founding members of the intracellular NOD-like receptor family, sense conserved motifs in bacterial peptidoglycan and induce proinflammatory and antimicrobial responses. Here, we discuss recent developments about the mechanisms by which NOD1 and NOD2 are activated by bacterial ligands, the regulation of their signaling pathways, and their role in host defense and inflammatory disease. Several routes for the entry of peptidoglycan ligands to the host cytosol to trigger activation of NOD1 and NOD2 have been elucidated. Furthermore, genetic screens and biochemical analyses have revealed mechanisms that regulate NOD1 and NOD2 signaling. Finally, recent studies have suggested several mechanisms to account for the link between NOD2 variants and susceptibility to Crohn’s disease. Further understanding of NOD1 and NOD2 should provide new insight into the pathogenesis of disease and the development of new strategies to treat inflammatory and infectious disorders.

Introduction
The innate immune system carries out important functions, including the recognition and clearance of infectious organisms through the detection of microorganisms by germline-encoded pathogen recognition receptors (PRRs). The nucleotide-binding oligomerization domain (NOD) proteins NOD1 and NOD2 represent two well-characterized PRRs of the NOD-like receptor (NLR) family; they sense conserved fragments found in the cell wall of many types of bacteria and activate intracellular signaling pathways that drive proinflammatory and antimicrobial responses. In contrast to PRRs such as Toll-like receptors (TLRs), which recognize microbial ligands at the cell surface or within endosomes, NOD1 and NOD2 sense bacterial products in the host cytosol to provide another level of microbial surveillance that is often associated with pathogen invasion. Here, we focus on recent discoveries of the molecular mechanisms that regulate NOD1 and NOD2 activation and signaling, the disruption of these pathways in inflammatory diseases, and efforts to develop small molecules capable of modulating these pathways for therapeutic applications. Specifically, we highlight recent knowledge about the mechanism of recognition and activation of NOD receptors, regulation of NOD1 and NOD2 signaling pathways, and new evidence linking NOD2 to the development of Crohn’s disease (CD).

NOD1 and NOD2: Ligand Recognition and Activation
Sequence-homology searches identified NOD1 as the first NLR member (Bertin et al., 1999; Inohara et al., 1999). NOD1 encodes an intracellular multidomain scaffolding protein consisting of a caspase activation and recruitment domain (CARD), a NOD, and multiple leucine-rich repeats (LRRs). This led to the discovery of NOD2, a closely related protein with an additional CARD (Ogura et al., 2001b). Early work showed that NOD1 and NOD2 mediate activation of the nuclear factor kappa B (NF-κB) family of transcriptional regulators in response to distinct peptido-

glycan (PGN) fragments (Inohara et al., 2001). Subsequent studies demonstrated that NOD1 can be activated by γ-D-glutamyl-meso-diaminopimelic acid (E-DAP), a motif present in many Gram− bacteria and certain Gram+ bacteria (Chamaillard et al., 2003a; Girardin et al., 2003a; Hasegawa et al., 2006). Additional work revealed that muramyl dipeptide (MDP), a PGN motif widely distributed among both Gram+ and Gram− bacteria, is sufficient to trigger NOD2 activity (Girardin et al., 2003b; Inohara et al., 2003). Ultimately, NOD1 and NOD2 signaling contributes to host defense via the production of proinflammatory cytokines and antimicrobial molecules (Kobayashi et al., 2005; Masumoto et al., 2006).

The mechanisms by which bacterial PGN enters cells and activates NOD1 and NOD2 receptors remain poorly understood, but multiple routes of entry have been reported (Figure 1). Two recent studies have revealed a role for endosomes in the activation of NOD1 and NOD2 signaling (Irving et al., 2014; Nakamura et al., 2014). In one study, two peptide transporters, SLC15A3 and SLC15A4, were shown to transport MDP across endosome membranes of phagosomes that had internalized bacteria via phagocytosis, and NOD1 and NOD2 localized to these membranes (Nakamura et al., 2014). This study is consistent with previous reports showing decreased NOD1-mediated cytokine production in SLC15A4−/− mice (Sasawatari et al., 2011) and reduced NF-κB activation after NOD1 stimulation upon SLC15A4 downregulation (Lee et al., 2009). Notably, SLC15A3 is present near a susceptibility locus associated with CD by a genome-wide association study (Jostins et al., 2012). Although the subcellular localization and expression of NOD1 and NOD2 vary depending on the cell type, the source and route of entry of the ligand also influence NOD1 and NOD2 complex formation. Indeed, outer membrane vesicles (OMVs) from a variety of bacteria (Irving et al., 2014; Thay et al., 2014) activate NOD1 and NOD2 signaling, revealing an important role for vesicle internalization in the delivery of ligands to cytosolic NOD1 and NOD2.
receptors. Given that soluble NOD1 and NOD2 ligands are sufficient to stimulate host cells, there are clearly multiple routes for these molecules to enter the cell.

Although the role of NOD1 and NOD2 in sensing bacterial PGN fragments has been reported for over a decade, it remains unclear whether these receptors respond directly to bacterially derived molecules or indirectly through some other intermediate. Recent experimental evidence supports a direct interaction between bacterial L-Ala–D-Glu-mesoDAP (Tri-DAP) and NOD1 by surface plasmon resonance (SPR) and atomic force microscopy (Laroui et al., 2011) and between MDP and NOD2 by SPR (Grimes et al., 2012) and biotin-labeled MDP pull-downs (Mo et al., 2012). Although these studies agree on a direct interaction between receptor and ligand, the SPR data suggest a role for the LRRs of both NOD1 and NOD2 in mediating the interaction, whereas the pull-down experiments implicate the NOD region. Notably, SPR analysis between NOD2 and MDP revealed a relatively high-affinity interaction ($K_d \sim 50 \, \text{nM}$), whereas the NOD1-Tri-DAP interaction was significantly weaker ($K_d \sim 30 \, \mu\text{M}$), which might reflect differences in the ligand used or in assay conditions. A direct interaction between labeled PGN fragments and NOD1 is also supported by fluorescence energy transfer (Irving et al., 2014). Together, these studies support a direct interaction between NOD1 and NOD2 and their bacterial ligands, but they do not rule out a role for accessory molecules that might facilitate ligand recognition, similar to the function of MD-2 as a TLR4 coreceptor for bacterial lipopolysaccharide (LPS) (Park et al., 2009). Further studies to define the molecular details of these interactions will require structural data.

Existing structural analyses of the NLR family member NLRC4 and Apaf-1, an NLR-related protein that regulates apoptosis, have provided insight into potential mechanisms of NOD1 and NOD2 activation (Hu et al., 2013; Park et al., 2009). NOD1 and NOD2 share a NOD module composed of a nucleotide-binding domain (NBD), a winged helix (WH), and helix domains (HD1 and HD2). The ADP-mediated interaction between the NBD and the WH is important for stabilizing the closed conformation, whereas the LRRs occlude these domains to render NLR proteins in a monomeric state (Hu et al., 2013). Upon ligand binding to the LRRs, HD2 mediates conformational changes of the NBD, WH, and HD1 to allow ADP-ATP exchange, self-oligomerization, and downstream signaling (Lechtenberg et al., 2014; Riedl et al., 2005). Consistently, HD2 variants in NOD2 result in either gain or loss of function (Hu et al., 2013; Tanabe et al., 2004). Furthermore, similar gain- and loss-of-function NOD2 variants are associated with early-onset sarcoidosis (EOS) or Blau syndrome and CD, respectively (Caso et al., 2014; Franchi et al., 2009). Complementation between NOD2 variants in HD2 and LRRs further supports this mechanism of NOD2 activation (Tanabe et al., 2004). Collectively, available evidence suggests that NOD1 and NOD2 reside in an autoinhibited monomeric state in the cytosol, and upon ligand recognition, they undergo

Figure 1. Potential Mechanisms for Bacterial Recognition by NOD1 and NOD2
NOD1 and NOD2 sense intracellular PGN fragments from bacteria. Host cells can internalize PGNs by multiple routes, such as (1) phagocytosis of bacteria and subsequent bacterial degradation, (2) uptake of PGN fragments from bacteria-derived extracellular OMVs, (3) transport across host membranes via channels, pore-forming molecules, or bacterial secretion systems, (4) endocytosis, or (5) from neighboring cells. Once inside the cell, NOD1 and NOD2 activation typically involves their relocalization to various intracellular locations, such as the plasma and endosomal membranes, via different adaptor molecules that are differentially expressed in host cells. For example, the activation of NOD2 by intracellular pathogens induces the formation of an autophagosome, which is mediated by ATG16L1.
conformational changes that promote their activation. However, further studies, including structural data to elucidate the regulation of NOD1 and NOD2 activation, are needed.

**NOD1 and NOD2 Signaling and Regulation**

Once the conformation of NOD1 and NOD2 is open, the proteins self-oligomerize and recruit receptor-interacting serine/threonine-protein kinase 2 (RIPK2) through homotypic CARD-CARD interactions, resulting in close proximity of RIPK2-IκB kinase (IKK) complexes (Inohara et al., 1999). RIPK2 then mediates the recruitment and activation of the serine/threonine kinase TAK1, which is a prerequisite for activation of the IKK complex and MAPK pathway (Figure 2). IKK-mediated phosphorylation of the NF-κB inhibitor IκBα leads to its polyubiquitination (pUb) and subsequent degradation through the proteasome, allowing NF-κB to translocate to the nucleus and influence the expression of downstream target genes. The posttranslation modification of proteins with ubiquitin (Ub) affects many steps in the NF-κB pathway. For example, NOD1 and NOD2 signaling is regulated via Lys63-linked pUb of RIPK2 on Lys209, which is necessary for the recruitment of downstream serine/threonine kinases (S/T kinases) RIPK2 occurs through CARD-CARD interactions. Subsequent activation of the NF-κB and MAPK pathways results in the transcriptional upregulation of proinflammatory and host-defense genes. Multiple steps in the pathway are regulated either positively or negatively by posttranslational modifications, such as phosphorylation and pUb events. Multiple regulatory genes act to influence various steps in the pathway, often in a cell-type-dependent manner. LUBAC stands for linear pUb chain assembly complex.

**Figure 2. NOD2 Signaling Pathways for Gene Activation**

NOD2 interacts directly with intracellular bacterial PGN fragments containing the MDP motif. Ligand recognition relieves intramolecular autoinhibitory interactions, leading to NOD oligomerization. Recruitment of the downstream serine/threonine kinase (S/T kinase) RIPK2 occurs through CARD-CARD interactions. Subsequent activation of the NF-κB and MAPK pathways results in the transcriptional upregulation of proinflammatory and host-defense genes. Multiple steps in the pathway are regulated either positively or negatively by posttranslational modifications, such as phosphorylation and pUb events. Multiple regulatory genes act to influence various steps in the pathway, often in a cell-type-dependent manner. LUBAC stands for linear pUb chain assembly complex.
for the recruitment of the TAK1 complex (Hasegawa et al., 2008; Ogura et al., 2001b). Multiple groups have identified several different E3 ligases capable of binding and catalyzing Lys48-linked RIPK2 pUb, including cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2 (Bertrand et al., 2009), ITOH (Tao et al., 2009), and Pellino3 (Yang et al., 2013), suggesting a role for these proteins in regulating NOD1 and NOD2 signaling. Notably, like variants in NOD2, variants in X-linked inhibitor of apoptosis protein (XIAP), an E3 ligase that binds and polyubiquitinates RIPK2, are associated with CD (Zeissig et al., 2014) and impaired NOD1 and NOD2 signaling (Damgaard et al., 2013). Additional E3 ligases that influence NOD1 and NOD2 signaling, including TNF-receptor-associated factor 2 (TRAF2) and TRAF5 (Hasegawa et al., 2008), TRAF4 (Marinis et al., 2011), and RNF34 (Zhang et al., 2014), have been identified. The linear Ub chain assembly complex, which consists of the E3 ligase RNF31 (also known as HOIP) and directs the linear pUb of NF-κB essential modulator (NEMO), also plays a positive role in NOD2-induced NF-κB signaling (Damgaard et al., 2012).

The removal of Ub by proteases can further fine tune NOD1 and NOD2 signaling. For instance, A20 was the first Ub protease identified to negatively regulate NOD2 signaling (Hitotsumatsu et al., 2008). More recently, the deubiquitinating OTULIN, which was shown to dampen NOD2 signaling (Fil et al., 2013), and inhibition of the Ub-specific protease USP8 and several of its interacting partners also negatively regulates NOD2-induced interleukin-8 (CXCL8) production (Warner et al., 2014). Other roles for Ub in the regulation of NOD1 and NOD2 signaling have been revealed. For example, NOD2 is destabilized by Lys48-linked pUb and subsequent degradation via the E3 ligase TRIM27 (Lee et al., 2012; Zurek et al., 2012). This might partially account for the phenomenon of MDP tolerance, where MDP pretreatment significantly decreases subsequent NOD2 activation upon restimulation with MDP in both human cells (Hedi et al., 2007) and mouse cells (Kim et al., 2008a). Similarly, direct pUb of NOD1 has been reported (Zhang et al., 2014). Structural details of the interaction between the CARD of NOD1 and Ub have also been resolved by nuclear magnetic resonance (Ver Heul et al., 2013) and X-ray crystallography (Ver Heul et al., 2014). Interestingly, analogous to variants in the Ub-interacting interface of NOD1, variants in the CARD of NOD2 result in loss of Ub binding and increased NOD2-induced CXCL8 secretion.

NOD1 and NOD2 Function in Pathogen Recognition and Immunity

Consistent with a role for NOD1 and NOD2 in host responses against bacterial infection, Nod1−/− and Nod2−/− mice show enhanced susceptibility to several pathogens (Franchi et al., 2009; Philpott et al., 2014). Because bacteria can be sensed by multiple PRRs, it is not surprising that in most cases, deficiency of NOD1 and/or NOD2 has only modest effects on pathogen clearance in vivo (Philpott et al., 2014). Consistently, NOD1 and NOD2 play redundant roles with TLRs in the detection of bacteria and the production of proinflammatory molecules, given that both signaling pathways lead to NF-κB and MAPK activation (Park et al., 2007; Tada et al., 2005). A cooperative role for TLR4 and NOD1 was also revealed in the mobilization of mature hematopoietic stem cells to the spleen upon Escherichia coli infection (Burberry et al., 2014). Furthermore, the activity of NOD1 and NOD2 becomes important when TLR signaling is absent or reduced in vivo (Kim et al., 2008b). In line with these observations, TLR-ligand-insensitive epithelial cells still respond to NOD1 ligands and NOD1-stimulatory bacteria (Kim et al., 2004). Additionally, NOD1 and NOD2 signaling is enhanced by pretreatment with TLR ligands, such as LPS or viral infection through a type-I-interferon (IFN)-dependent mechanism both in vitro and in vivo (Kim et al., 2014).

NOD1 is widely expressed by a variety of cell types, such as epithelial cells, stromal cells, and endothelial cells (Inohara et al., 1999; Park et al., 2007). Stimulation of intestinal epithelial cells with NOD1-activating molecules induces the production of chemokines and the recruitment of acute inflammatory cells in vivo (Masumoto et al., 2006). NOD1 contributes to the activation of immune responses in epithelial cells infected with several Gram− bacteria, including Shigella flexneri (Girardin et al., 2003a; Kim et al., 2010) and Helicobacter pylori (Allison et al., 2009). In both infection models, either intracellular delivery of S. flexneri LPS-containing NOD1 ligand or injection of H. pylori PGN fragments via a type IV secretion system promotes NOD1-dependent activation of NF-κB in epithelial cells (Allison et al., 2009; Girardin et al., 2001). H. pylori can also induce the synthesis of type I IFN via NOD1 (Watanabe et al., 2010), and consequently, Nod1−/− mice are more susceptible to H. pylori infection, demonstrating the importance of NOD1 in pathogen recognition in vivo (Viala et al., 2004).

NOD1 is also involved in the sensing of enteroinvasive E. coli (Kim et al., 2004), Pseudomonas aeruginosa (Travassos et al., 2005), Campylobacter jejuni (Zibauer et al., 2007), Pasteurellaceae Ni1060 (Jiao et al., 2013), and Gram+ Clostridium difficile (Hasegawa et al., 2011). Consistently, Nod1−/− mice are more susceptible to C. difficile infection, which is associated with impaired C. difficile clearance, increased commensal translocation, and defective recruitment of neutrophils to the infected site (Hasegawa et al., 2011). Similarly, NOD1 is important for neutrophil recruitment in response to accumulation of the pathobiont Ni1060 at damaged gingival sites, which results in mice periodontitis (Jiao et al., 2013). Furthermore, Nod1−/− mice show increased susceptibility to lung infection with Legionella pneumophila, which is associated with reduced neutrophil recruitment to the lungs (Fruitouso et al., 2010).

In contrast to NOD1 expression, NOD2 expression is limited to certain cell types, such as hematopoietic cells (Ogura et al., 2003). In the intestine, NOD2 is expressed in Paneth cells and stem cells (Barnich et al., 2005; Nigro et al., 2014), where it senses many types of PGNs, which vary in their level of NOD2-stimulatory activity across bacterial species (Hasegawa et al., 2006), and activates NF-κB and MAPK signaling in the context of Streptococcus pneumoniae (Opitz et al., 2004) and E. coli (Theivanthiran et al., 2012) infections. NOD2 is also involved in sensing intracellular Listeria monocytogenes (Kobayashi et al., 2005), Salmonella typhimurium (Hisamatsu et al., 2003), S. flexneri (Kufer et al., 2006), and Mycobacterium tuberculosis (Ferwerda et al., 2005). Several studies have reported a role for NOD2 in host defense in vivo. For instance, S. pneumoniae recognition by NOD2 induces the production of CC-chemokine ligand 2 (CCL2), leading to the recruitment of inflammatory macrophages that are necessary for bacteria clearance in the lung (Davis et al., 2011). Similarly, NOD2 promotes the clearance of

Immunity 41, December 18, 2014 ©2014 Elsevier Inc. 901
Citrobacter rodentium by the induction of CCL2 and T helper 1 (Th1) cell immune responses in the intestine (Kim et al., 2011a) and of Staphylococcus aureus in the skin (Hruz et al., 2009).

Given the role of both NOD1 and NOD2 in the innate immune response against bacterial pathogens, it is not surprising that pathogens have evolved mechanisms to evade detection and clearance by the host. Indeed, L. monocytogenes can escape NOD1-mediated detection by modifying its PGN via N-deacetylation (Boneca et al., 2007). A similar evasion mechanism, through the modification of cell-wall PGNs, has also been described for H. pylori to avoid NOD1-mediated detection (Chaput et al., 2006).

In addition to evidence of the role of NOD proteins in innate immune responses to bacterial infections, there is mounting evidence that NOD1 and NOD2 signaling influences adaptive immune responses. PGN derivatives have been identified as adjuvants of antigen-specific immunoglobulin G (IgG) production (Ellouz et al., 1974). As expected, the adjuvant activity of MDP, including antigen-specific IgG responses, was mediated by NOD2 in vivo (Girardin et al., 2003b; Inohara et al., 2003; Kobayashi et al., 2005). NOD2 also regulates Th17 cell responses (Brain et al., 2013; van Beelen et al., 2007). In mice, stimulation with either NOD1 or NOD2 alone leads to predominantly Th2-cell-dependent adaptive immune responses, whereas costimulation with TLR agonists promotes the priming of Th1, Th2, and Th17 cell immune responses (Fritz et al., 2007; Magalhaes et al., 2008). Furthermore, NOD1 and NOD2 stimulation induces OX40 ligand, which is important for Th2-cell-oriented acquired immunity (Duan et al., 2010; Magalhaes et al., 2011). Both NOD1 and NOD2 contribute to IL-6-dependent induction of mucosal Th17 cell responses during early stages of intestinal infection with C. rodentium and S. typhimurium (Geeddes et al., 2011). Notably, radiation-resistant nonhematopoietic cells play a role in triggering NOD1-mediated Th2 cell immune responses (Fritz et al., 2007), although the mechanisms involved remain unknown. Together, these studies provide evidence of a role for NOD1 and NOD2 signaling in regulating adaptive immune responses.

NOD2 is required for the “training” of monocytes induced by tuberculosis vaccination. Indeed, monocytes can be functionally reprogrammed to exhibit enhanced and lasting protective functions not only against tuberculosis but also against secondary mycobacterial challenges through a NOD2-dependent mechanism (Kleinnijenhuis et al., 2012). Overall, this work suggests a role for NOD2 in modulating the adaptive features of innate immunity.

NOD2 Function and CD
Polymorphisms in NOD2 are the strongest known genetic risk factors in the development of CD (Hugot et al., 2001; Ogura et al., 2001a). Three common NOD2 variants (R702W, G908R, and L1007insC) and multiple minor variants in the C-terminal LRR region and HD2 are linked to the development of CD (Chamaillard et al., 2003b). Individuals with just one NOD2 variant have an only mildly increased risk of developing CD, whereas those homozygous or compound heterozygous for NOD2 variants exhibit a 20- to 40-fold increased risk (Hugot et al., 2007). However, the majority of individuals homozygous for NOD2 variants do not develop CD, indicating that environmental factors, altered immune regulation, and/or the gut microbiota are critical for disease development. Consistently, no spontaneous intestinal inflammation occurs in Nod2−/− mice or knockin mice homozygous for the CD-associated L1007insC NOD2 variant and housed under specific-pathogen-free facilities (Kim et al., 2011b; Kobayashi et al., 2005; Pauleau and Murray, 2003).

The mechanisms by which NOD2 variants contribute to CD pathogenesis remain unclear, but several hypotheses have been proposed (Figure 3). The first suggests that NOD2 variants lead to impaired NOD2 activation in response to MDP stimulation and thus result in a loss-of-function phenotype (Inohara et al., 2003). Consistently, induction of inflammatory cytokines after MDP stimulation is impaired in monocytes homozygous for CD-associated NOD2 variants (Inohara et al., 2003; van Heel et al., 2005). Overall, this hypothesis suggests that NOD2 variants impair bacterial recognition and clearance and thus lead to aberrant inflammation through NOD2-independent pathways. Because monocytes and neutrophils express NOD2, defects in bacterial recognition could involve phagocytic cells recruited to intestinal sites (Ogura et al., 2001b). Furthermore, some intestinal bacteria rely on NOD2 recognition for activation of innate immune responses, but such recognition is impaired in macrophages harboring CD-associated NOD2 variants (Kim et al., 2011b). Paneth cells that are located in the crypts of the small intestine and secrete antibacterial proteins and peptides such as α-defensins express NOD2 (Lala et al., 2003; Ogura et al., 2003). Notably, CD-associated NOD2 variants confer susceptibility to the development of ileal, but not colonic, lesions, corresponding to the location of Paneth cells (Gasche and Grundtner, 2005). Furthermore, altered expression of α-defensins was observed in patients with ileal CD (Wehkamp et al., 2005). However, whether the reduced expression of α-defensins is a primary pathogenic event or an epiphenomenon of inflammation reflecting Paneth cell loss due to mucosal damage remains controversial (Simms et al., 2008). In mice, NOD2 regulates the expression of a subgroup of Paneth-cell-expressed α-defensins (Kobayashi et al., 2005) and the accumulation of both commensal and pathogenic bacteria in the terminal ileum (Petnicki-Ocwieja et al., 2009). However, other studies have shown no difference in the expression of antimicrobial molecules between Nod2−/− and wild-type (WT) mice (Robertson et al., 2013; Shanahan et al., 2014). So, it is still controversial whether CD-related inflammation is triggered by impaired antimicrobial activity within the intestinal crypts and abnormal accumulation of pathogenic bacteria and/or pathobionts.

Initial work showed that compared to WT mice, Nod2−/− mice have an altered composition of the gut microbiota with a net increase in the abundance of Bacteroidetes and Firmicutes phyla in the feces and terminal ileum (Petnicki-Ocwieja et al., 2009; Rehman et al., 2011). However, other studies did not identify significant differences in the composition of the gut microbial community in Nod2−/− mice (Robertson et al., 2013; Shanahan et al., 2014), raising the possibility that the imbalance in bacterial communities found in Nod2−/− mice might be due to maternal transmission of the microbiota and/or other environmental factors independent of genotype. However, the controversy of whether NOD2 deficiency promotes dysbiosis requires further study given that small intestinal abnormalities, including goblet cell alterations (which are associated with an increased number of
interferon-γ-producing intraepithelial lymphocytes), have been reported in Nod2−/− mice (Ramanan et al., 2014). Notably, these abnormalities correlate with the selective expansion of the commensal Bacteroides vulgatus, which can exacerbate intestinal inflammation in Nod2−/− mice treated with the NSAID piroxicam. However, the biological relevance of these intestinal alterations observed in Nod2−/− mice and the relative abundance of B. vulgatus in patients with CD need to be further investigated.

Another hypothesis that links NOD2 variants to the development of CD involves the recently recognized role of NOD2 in the autophagy pathway. NOD2 interacts with and recruits the CD-associated autophagy protein ATG16L1 to the plasma membrane at bacterial entry sites (Travassos et al., 2010). Furthermore, NOD2 stimulation promotes host cell autophagosome formation, which is associated with an increase in either killing of S. typhimurium or antigen presentation (Cooney et al., 2010; Homer et al., 2010). Notably, CD-associated NOD2 variants are defective in ATG16L1 recruitment and exhibit impaired bacteria-induced autophagy in a cell-type-specific manner (Homer et al., 2010). Furthermore, patients with ileal CD have impaired secretion of Paneth-cell-derived α-defensins, a defect that can arise from mutations in NOD2 or autophagy genes (Cadwell et al., 2008; Wehkamp et al., 2004; Wehkamp et al., 2005). Loss of function of ATG16L1 has also been associated with Paneth cell abnormalities in mice, although pathology associated with Paneth cell function relies on prior exposure to mouse norovirus (Cadwell et al., 2008; Cadwell et al., 2010). However, no defects in autophagy have been reported in Nod2−/− mice in vivo. Therefore, further work is needed to ascertain the functional interplay between CD-associated NOD2 and ATG16L1.

Finally, another hypothesis suggests that NOD2 negatively regulates TLR signaling and that NOD2 variants result in deregulated TLR signaling and enhanced inflammation. Consistently, TLR-induced IL-12 production was increased in macrophages...
and dendritic cells (DCs) deficient in NOD2 (Watanabe et al., 2006). This model proposes that NOD2 acts as a brake to dampen immune responses and that NOD2 variants lead to a deregulated TLR-mediated Th1 cell response in the intestine. However, the bulk of studies have failed to reveal an increase in the production of cytokines, such as IL-12, in macrophages or human DCs deficient in NOD2 after TLR stimulation (Kim et al., 2008b; Kramer et al., 2006; Park et al., 2007; Salucci et al., 2008). Furthermore, NOD2 positively regulates the production of IL-10 in response to microbial stimulation, and CD-associated NOD2 variants inhibit MDP-induced IL-10 transcription in human monocytes (Noguchi et al., 2009; Wagener et al., 2014). The latter most likely reflects, in part, the overall impairment of immune responses to MDP in cells homozygous for CD-associated NOD2 mutations.

Early evidence showed that macrophages isolated from knockin mice expressing the CD-associated L1007insC NOD2 variant exhibit increased NF-κB activation and IL-1β secretion after MDP stimulation (Maeda et al., 2005). However, the interpretation of the latter studies is difficult because subsequent examination of this mouse model showed a duplication of the 3’ end of the WT Nod2 locus, which was targeted by the mutation (Maeda et al., 2005). Furthermore, an independently generated knockin L1007insC NOD2 mouse model revealed that the variant acts as a loss of function (Kim et al., 2011b), consistent with human studies.

**Potential for the Therapeutic Modulation of NOD1 and NOD2 Signaling**

Several groups are attempting to develop small molecules capable of modulating NOD1 and NOD2 signaling in order to influence pathogen clearance and inflammation (Seddes et al., 2009; Jakopin, 2014; Maisonneuve et al., 2014). For instance, molecules capable of enhancing or blocking NOD2 signaling would have application in diseases such as CD and EOS, which have been associated with NOD2 loss- and gain-of-function variants, respectively. The development of small molecules capable of modulating NOD1 and NOD2 signaling might also have potential applications in other infectious and inflammatory diseases, such as asthma, arthritis, leprosy, graft versus host disease, and periodontitis, which have each been associated with deregulated NOD1 and/or NOD2 signaling (Correa et al., 2012; Jiao et al., 2014; Philpott et al., 2014) but await further functional validation. One of the first successful approaches to modulating NOD2 signaling with small molecules came from the development of kinase inhibitors capable of blocking RIPK2 (Jun et al., 2013; Tigno-Aranjuez et al., 2010; Tigno-Aranjuez et al., 2014), and others have identified anti-inflammatory activities for diterpene-based molecules via their ability to specifically block NOD2 signaling (Bielig et al., 2010). More recently, libraries of small molecules were screened, identifying lead compounds capable of influencing NOD1 (Khan et al., 2011; Rickard et al., 2013) and NOD2 signaling (Correa et al., 2011; Rickard et al., 2013). In most cases, the molecular mechanisms by which these small molecules act to regulate NOD1 and NOD2 signaling remain poorly understood. It is possible that these small molecules act directly to affect NOD protein stability, ligand recognition, and/or ATP binding and hydrolysis activity. Alternatively, they might target any one of the numerous regulatory proteins revealed by recent small interfering RNA screens to participate in the NOD1 and NOD2 pathway (Lipinski et al., 2012; Warner et al., 2014).

Although most of the small-molecule screens have identified NOD1 and NOD2 inhibitors, some investigators have attempted to identify NOD1 and NOD2 immunostimulatory molecules with potential applications as vaccine adjuvants or antimicrobial agents. Early efforts in this area involved the chemical modification of core PGN ligands to enhance NOD1 and NOD2 activity. Experiments on the relationship between structure and activity revealed chemical modifications that either attenuate or enhance the stimulatory activity of the NOD1 ligand iE-DAP (Agnihotri et al., 2011; Jakopin et al., 2013) or MDP (Jakopin et al., 2012; Rubino et al., 2013). These studies suggest that new ligands can be developed to enhance NOD1 and NOD2 immunity.

In addition to evidence of the role of NOD1 and NOD2 in regulating gut homeostasis and protection against colitis, there is evidence that NOD1 and NOD2 regulate colitis-associated cancer. For example, mice lacking NOD1 (Chen et al., 2008), RIPK2, or NOD2 (Couturier-Maillard et al., 2013) develop more tumors in colitis-associated colon cancer models. There is also data suggesting a role for NOD1 and NOD2 in tumors outside of the intestine (da Silva Correia et al., 2006). Together, these studies support the modulation of NOD1 and NOD2 signaling as a therapeutic strategy in the treatment of proinflammatory diseases and cancers; however, additional studies will be required for determining the negative and positive effects of targeting the NOD1 and NOD2 pathway in order to balance the inhibition of detrimental inflammation against immune suppression and susceptibility to infection.

**Concluding Remarks**

Compelling evidence indicates that NOD1 and NOD2 are involved in the recognition of highly conserved PGN molecules that enter the host cytosol through phagocytosis, endocytosis, endosomal membrane transporters, bacterial secretion systems, pore-forming toxins, or OMVs. NOD1 and NOD2 directly recognize PGN molecules, leading to their activation and induction of proinflammatory and antimicrobial molecules. Biochemical analyses and genetic screens have revealed complex mechanisms that regulate NOD1 and NOD2 signaling. NOD1 and NOD2 play a role in the clearance of invading pathogens and act redundantly and cooperatively with TLRs in the detection of bacteria and production of proinflammatory molecules. Experimental evidence suggests several mechanisms by which NOD2 variants regulate the susceptibility to CD. Although our knowledge about NOD1 and NOD2 has increased, several key questions—including the precise mechanism of NOD1 and NOD2 activation by PGN molecules and how NOD2 acts in the intestine to regulate susceptibility to CD—remain. Undoubtedly, elucidating these questions will provide new insights into the mechanisms of host defense and the pathogenesis of inflammatory disease.

**ACKNOWLEDGMENTS**

We thank Grace Chen for critically reviewing the manuscript. We acknowledge funding from the NIH, the Crohn’s & Colitis Foundation of America, the Broad
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