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Nod1 and Nod2 Enhance TLR-Mediated Invariant NKT Cell Activation during Bacterial Infection

Thirumahal Selvanantham,* Nichole K. Escalante,* Mayra Cruz Tleugabulova,* Stephanie Fiévé,* Stephen E. Girardin,† Dana J. Philpott,* and Thierry Mallevaey*

Invariant NKT (iNKT) cells act at the crossroad between innate and adaptive immunity and are important players in the defense against microbial pathogens. iNKT cells can detect pathogens that trigger innate receptors (e.g., TLRs, Rig-I, Dectin-1) within APCs, with the consequential induction of CD1d-mediated Ag presentation and release of proinflammatory cytokines. We show that the cytosolic peptidoglycan-sensing receptors Nod1 and Nod2 are necessary for optimal IFN-γ production by iNKT cells, as well as NK cells. In the absence of Nod1 and Nod2, iNKT cells had a blunted IFN-γ response following infection by Salmonella enterica serovar Typhimurium and Listeria monocytogenes. For Gram-negative bacteria, we reveal a synergy between Nod1/2 and TLR4 in dendritic cells that potentiates IL-12 production and, ultimately, activates iNKT cells. These findings suggest that multiple innate pathways can cooperate to regulate iNKT cell activation during bacterial infection. The Journal of Immunology, 2013, 191: 5646–5654.

Although the vast majority of αβ T cells found in humans and mice recognize peptide Ags in the context of MHC molecules, lipid-reactive αβ T cells have emerged as a prevalent and critical component of the mammalian immune system. Invariant NKT (iNKT) cells, the best characterized lipid-reactive T cells, represent an evolutionarily conserved T cell subset that recognizes microbe-derived lipids, as well as self-lipids, in the context of the MHC-Ib molecule CD1d (1–3). iNKT cells express TCRs with restricted diversity compared with that of classical αβ T cells. These TCRs are formed by a unique and invariant TCRαβ-chain (Vα24-Jα18 in humans, Vα14-Jα18 in mice) and TCRβ-chains with limited TCR β-chain usage (mainly Vβ11 in humans; Vβ8s, Vβ7, and Vβ2 in mice), yet with quite extensive CDR3β functional diversity (1, 2). iNKT TCRs recognize a variety of structurally diverse lipid Ags, mainly ceramide-based and diacylglycerol-based glycolipids, as well as phospholipids (4–6). These cells can be best identified using CD1d tetramers loaded with lipid Ags, including α-galactosylceramide (α-GC) or analogs thereof. iNKT cells can release copious amounts of functionally diverse cytokines and chemokines, including both Th1 and Th2 cytokines, within only minutes to hours following Ag exposure (3). These unique properties allow iNKT cells to exert protective or deleterious functions in numerous diseases.

iNKT cells play important roles in responses against bacteria (7–11) and viruses (12–14). Their capacity to detect pathogens depends on several pathways. The first mechanism involves the direct recognition of microbe-derived glycolipids presented by CD1d. These lipids are mainly α-linked glycosylceramides or diacylglycerols, are structurally distinct from mammalian glycolipids, and can be considered microbial signature molecules. Such lipids have been isolated from a few bacterial species, such as Sphingomonas spp. (15), Borrelia burgdorferi (16), and Streptococcus pneumoniae (17). However, direct evidence (e.g., genetic ablation of enzymes responsible for their synthesis) for the involvement of these lipids during infection is lacking. In a second mechanism, pathogens trigger innate receptors within APCs, such as dendritic cells (DCs), which, in turn, activate iNKT cells through the combined presentation of self-lipid species by CD1d and production of proinflammatory cytokines, such as IL-12 and type I IFN (18, 19). A large number of bacteria, including Salmonella typhimurium and Listeria monocytogenes, activate iNKT cells through this indirect mechanism. The pattern recognition receptors (PRRs) involved include TLR4 (20, 21), TLR9 (19) (TLR8 in humans) (22), TLR7 (13), Rig-I (13), and Dectin-1 (23). Although the self-lipid species implicated remain poorly characterized, they may include isoglobotrihexosylceramide (20, 24) and β-glucosylceramide (25). Finally, iNKT cells can be activated in a CD1d-independent, but IL-12–dependent, manner (26), suggesting that this cytokine can bypass the need for TCR engagement in certain circumstances.

The innate immune system serves as the first line of defense against invading pathogens, conferring active immunity and pathogen clearance, as well as providing important cues to T and B cells. The mammalian immune system consists of a broad diversity of innate receptors, including TLRs, Nod-like receptors (NLRs), RIG-I–like receptors (RLRs), and C-type lectin-like receptors (CLR) (27–30). Nod1 and Nod2, two members of the NLR family, are cytosolic PRRs that recognize particular motifs found in bacterial peptidoglycan (31–33). Nod1 detects di-glycyl-d-meso-diaminopimelic acid, found mainly in Gram-negative bacteria, and Nod2 senses muramyl dipeptide (MDP), found in both Gram-negative and Gram-positive bacteria (34–37). Nod1 and Nod2 stimulation ultimately...
leads to NF-κB activation and the subsequent production of proinflamatory cytokines. Nod1/2 activation polarizes humoral responses toward Th2 yet enhances TLR-mediated Th1 immunity (38–40). These two receptors play critical functions at mucosal surfaces. Mutations in Nod1 and Nod2 in humans have been associated with inflammatory disorders, such as asthma and Crohn’s disease, respectively (41, 42). Nod1 and Nod2 are key bacteria-sensing receptors and are important in the early response against enteric pathogens, including S. typhimurium and Citrobacter rodentium (43, 44).

Given the importance of Nod receptors in microbial immunity, we investigated the contribution of Nod1 and Nod2 receptors in iNKT cell activation during bacterial infection. We show that Nod1 and Nod2 synergize with TLR4 in DCs to increase IL-12 production and enhance iNKT cell activation. We further demonstrate that, although Nod1/2 do not influence iNKT cell development and intrinsic functions, they are important regulators of the IFN-γ response by iNKT cells during S. typhimurium and L. monocytogenes infections.

Materials and Methods

Mice

C57BL/6 mice were purchased from Charles River (Wilmington, MA). Nod−/− and Nod2−/− mice were generated by Millennium Pharmaceuti- cals and by Marco Giovaninni (Centre d’Etude du Polymorphisme Humain, Fondation Jean Dausset, Paris, France) and Jean-Pierre Hugot (Hôpital Robert-Debré, Paris, France), respectively. Nod1/2−/− mice were generated by crossing of Nod1−/− and Nod2−/− mice. V14 Tg mice were a generous gift from Dr. Albert Bendelac (University of Chicago, Chicago, IL). All animals were housed under specific pathogen–free conditions at the Centre for Cellular and Biomolecular Research, Division of Comparative Medicine. All procedures were approved by the Faculty of Medicine and Pharmacy Animal Care Committee at the University of Toronto (20009158 and 20009741 to T.M. and 20008651, 20009337, and 20009889 to D.J.P.). Age- and sex-matched mice were used in all experimental procedures.

Reagents

αGC was purchased from Diagnocine (Hackensack, NJ). Standard and ultrapure preparations of Escherichia coli O111:B4 LPS were purchased from InvivoGen (San Diego, CA). For in vitro experiments, the Nod2 ligand MDP was purchased from EMD Biosciences (San Diego, CA), and the Nod1 ligand FK565 was purchased from Astellas Pharma (Tokyo, Japan). For in vitro experiments, C12-iE-DAP (Nod1 ligand) and L18-MDP (Nod2 ligand) were purchased from InvivoGen, because these acylated ligands were better able to stimulate cells in culture (45–48). Unloaded and PBS57-loaded CD1d tetramers were obtained from the National Institutes of Health Tetrramer Core Facility (Atlanta, GA). Alternatively, unloaded and PBS57-loaded CD1d monomers obtained from the National Institutes of Health Tetrramer Core Facility were tetramerized using allophycocyanin- or PE-labeled streptavidin (eBioscience, San Diego, CA).

Bacterial infection

Mice were injected i.v. with 2×10^7 CFU Salmonella enterica serovar Typhimurium SL1344 or 5×10^7 CFU L. monocytogenes 10335 in 200 μl PBS or with PBS alone. Bacterial load was determined by homogenizing fecal matter or spleens in PBS containing 1% Triton X-100 and plating on MacConkey Agar plates containing 50 μg/ml streptomycin. For orogastric infections, mice were fasted for 2 h prior to oral administration of 20 mg streptomycin. After 24 h, mice were fasted again for 2 h and then infected via oral gavage with 5×10^7 CFU streptomycin-resistant S. typhimurium SL1344.

Bone-marrow–derived DC generation and coculture assay

Bone marrow–derived DCs (BMDCs) were grown from bone marrow cells, lysed of RBCs, in complete RPMI 1640 (10% FCS) plus 5% culture su- pernatant of the GM-CSF–producing B78 hi-hybridoma for 8 d. BMDCs were stimulated for 4 h and washed extensively. A total of 5×10^5 BMDCs was cultured alone for a period of 18 h or together with 10^5 spleen iNKT cells for 36 h. Spleen iNKT cells were cultured following a method adapted from Chiba et al. (49). Briefly, iNKT cells were sorted from the spleen of V14 Tg mice using TCRβ and PBS57-CD1d tetramers. Sorted iNKT cells were seeded on CD1d-coated plates loaded with αGC in the presence of IL-2 (10 ng/ml) and IL-7 (10 ng/ml). iNKT cells were expanded as needed and used after 9 d of culture.

Flow cytometry

Liver mononuclear cells were isolated as previously described. Briefly, PBS-perfused mouse livers were homogenized through 70-μm cell strainers, washed twice in PBS, and resuspended in 33% Percoll (Sigma-Aldrich, St. Louis, MO). After centrifugation at 1000×g for 20 min with minimal brake, the pellet was recovered and washed in PBS. RBCs were lysed using RBC Lysis buffer (Sigma-Aldrich). To isolate splenocytes and thymocytes, spleens and thymi were homogenized through 70-μm cell strainers in complete RPMI 1640, followed by RBC lysis. Lamina propria lymphocytes were isolated as previously described (43). All Abs were purchased from eBioscience. Anti-IL-4 Abs were purchased from BD Biosciences (San Jose, CA) or BioLegend (San Diego, CA). For intracellular staining of cytokines, cells were incubated for 2–4 h in Protein Transport Inhibitor Cocktail (eBioscience), unless otherwise stated. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm Kit (BD Biosciences). Viability was assessed using LIVE/DEAD Cell Viability Assays (Life Technologies, Carlsbad, CA). Samples were analyzed using a FACSCan II or LSR II flow cytometer (BD Biosciences) and FlowJo software (TreeStar, Ashland, OR).

ELISA

IFN-γ, IL-4, and IL-12p40 were analyzed by ELISA using Ab pairs pur- chased from eBioscience, following standard procedures. DuoSet ELISA kits for IL-12p70 and IL-23 were purchased from R&D Systems (Minneapolis, MN). The ABTS peroxidase substrate was obtained from KPL (Gaithersburg, MD).

Statistical analysis

Two-tailed Student t tests or Mann–Whitney tests were performed using GraphPad Prism software for a confidence interval of 95%.

Results

TLR4, Nod1, and Nod2 synergize to enhance IFN-γ production by iNKT cells

iNKT cells detect bacteria predominantly through indirect path- ways that involve the triggering of innate receptors within APCs. In agreement, culture of LPS-sensitized BMDCs with iNKT cells led to the production of IFN-γ, but not IL-4, by iNKT cells (Fig. 1A) (21). It is known that standard preparations of LPS are often contaminated with other pathogen-associated molecular patterns, including peptidoglycan that contains motifs that activate TLR2, as well as the cytosolic sensors Nod1 and Nod2 (36, 50). To test whether Nod1 and Nod2 participated in iNKT cell activation, we first compared iNKT cell activation by E. coli O111:K58:H2 (O111:B4) standard LPS and ultrapure LPS purified with addi- tional hydrolysis and phenol triethylamine-deoxycholate sodium to reduce peptidoglycan and lipoprotein contamination. The ul- trapure LPS-sensitized BMDCs were less efficient at inducing iNKT cell activation, as measured by IFN-γ secretion, compared with the standard LPS (Fig. 1A). Moreover, iNKT cells showed reduced activation by LPS-treated Nod1/2-deficient (Nod1/2−/−) BMDCs compared with wild-type (WT) BMDCs, suggesting that Nod1 and Nod2 receptors played a potent role in iNKT cell ac- tivation. Treatment of BMDCs with the specific Nod1 and Nod2 agonists C12-iE-DAP and L18-MDP induced little to no iNKT cell activation. However, cotreatment of WT, but not Nod1/2−/−, BMDCs with C12-iE-DAP and L18-MDP, together with LPS, greatly enhanced iNKT cell activation, as measured by IFN-γ release. Of note, iNKT cells produced no detectable IL-17A in any of the conditions tested (data not shown). Direct stimulation of iNKT cells by αGC, in the absence of BMDCs, resulted in their production of both IFN-γ and IL-4 (data not shown), in agreement with previous findings (51). However, stimulation of iNKT cells in

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the absence of BMDCs with LPS or Nod1/2 agonists, alone or in combination, did not induce detectable IFN-γ secretion. This result is in contrast with previous findings where iNKT cells were activated by LPS through direct TLR4 engagement (52).

Indirect iNKT activation generally requires the CD1d-mediated presentation of self-lipid species, as well as IL-12 or type I IFN production by DCs. Ab-mediated neutralization of CD1d reduced iNKT cell activation in all conditions tested (Fig. 1B). FACS analysis of BMDCs revealed that CD1d was only marginally increased upon treatment with TLR4 and Nod ligands, alone or in combination (Fig. 1C). Importantly, C12-iE-DAP and L18-MDP had no major additive effect on CD1d, CD80/86, or MHC class II expression compared with LPS alone in the conditions used (Fig. 1C). However, Nod agonists increased IL-12/23p40 secretion by WT, but not Nod1/2−/−, BMDCs compared with LPS alone (Fig. 1D). Of note, the slight reduction in IL-12/23p40 secretion by

**FIGURE 1.** iNKT cell activation following TLR4 and Nod1/2 stimulation in vitro. (A) WT and Nod1/2−/− BMDCs were stimulated for 4 h with vehicle, αGC (100 ng/ml), *E. coli* 0111:B4 standard purified (Std) or ultrapurified (UP) LPS (−) (250 ng/ml), the Nod1 and Nod2 ligands C12-iE-DAP and L18-MDP (50 μM each), or a combination of UP LPS and Nod1/2 ligands and subsequently cultured with in vitro–expanded iNKT cells for 36 h. IFN-γ and IL-4 production was assessed by ELISA. (B) WT BMDCs were stimulated as in (A) and cultured with iNKT cells for 36 h in the presence of the anti-CD1d blocking Ab 1B1 (10 μg/ml) or an IgG2b isotype control (10 μg/ml). IFN-γ production was assessed by ELISA. (C) BMDCs were stimulated as in (A), and expression of CD1d, CD80, CD86, and MHC class II on CD11b+CD11c+ BMDCs was assessed by flow cytometry. (D) WT and Nod1/2−/− BMDCs were stimulated for 4 h, washed extensively, and cultured for 16 h. IL-12p40, IL-12p70, and IL-23 production was assessed by ELISA. (E) WT BMDCs were stimulated as in (A) and cultured with iNKT cells for 36 h in the presence of the anti–IL-12p40 or anti–IL-23p19 blocking Abs (10 μg/ml) or isotype controls (10 μg/ml). Data represent the mean ± SEM of three or four independent experiments performed in triplicate conditions (A, B, D, E), or one representative experiment of three (C). *p < 0.05, **p < 0.01, ***p < 0.001, two-tailed Student t test.
Nod1/2−/− BMDCs in response to ultrapure LPS suggests that this LPS preparation may still contain traces of contaminating peptidoglycan. Increased amounts of bioactive IL-12 (p70) and IL-23, which both share the IL-12/23p40 subunit, were also detected when Nod agonists were used in combination with LPS compared with LPS alone (Fig. 1D). Ab-mediated neutralization of IL-12/23p40, but not IL-23p19, abrogated IFN-γ production by iNKT cells, confirming the critical role of IL-12 in iNKT cell activation (Fig. 1E). Taken together, these findings indicate that Nod receptors potentiate TLR4-mediated CD1d-dependent iNKT cell activation by enhancing IL-12 secretion by DCs.

Synergistic iNKT and NK cell activation in vivo by TLR4, Nod1, and Nod2

We next investigated whether the synergy between TLR4 and Nod1/2 occurred in vivo. Immunization of mice with ultrapure LPS consistently induced lower frequencies (>2-fold reduction) of IFN-γ+ iNKT cells in the spleen compared with standard LPS (Fig. 2A). Interestingly, we found that immunization with ultrapure LPS also induced lower frequencies of activated IFN-γ+ NK cells compared with standard LPS (Fig. 2B). Additionally, coimmunization with ultrapure LPS and the Nod1 and Nod2 agonists FK565 and MDP increased the frequency of IFN-γ+ iNKT and NK cells in the spleen ~2-fold compared with ultrapure LPS alone (Fig. 2C, 2D). Immunization of mice with Nod1/2 agonists in the absence of LPS did not lead to detectable iNKT or NK cell activation in the conditions tested (data not shown), which contrasts with a previously reported role for Nod2 in human NK cell activation (53, 54). These results demonstrate that Nod1/2 potentiate TLR4-mediated iNKT and NK cell activation in vivo.

Unaltered iNKT cell phenotype and functional properties in Nod1/2-deficient mice

Analysis of various specific pathogen–free and germ-free mice indicated that signals from the gut microbiota influence iNKT cell development and function (55, 56), which begged the question whether Nod receptors are involved in these phenomena. Analysis of iNKT cell numbers in the thymus, spleen, and liver of Nod1−/−, Nod2−/−, and Nod1/2−/− mice revealed no major difference compared with WT mice, with the exception of higher iNKT cell frequency (data not shown) and absolute numbers in the spleen of Nod1/2−/− mice (Fig. 3A). Further phenotypic characterization of iNKT cells in Nod1/2−/− mice revealed no difference in the expression of the activating receptors NK1.1 and NKG2D, as well as the inhibitory receptors Ly49A and Ly49C, in all tissues tested (Fig. 3B), indicating that Nod1/2 deficiency does not impact central or peripheral iNKT cell maturation.
We next analyzed iNKT activation in Nod1/2−/− mice in response to strong TCR ligation. Immunization of WT and Nod1/2−/− mice with a GC induced similar frequencies of IFN-γ+ and IL-4+ splenic and hepatic iNKT cells in both groups of mice (Fig. 3C, 3D). Taken together, our analysis revealed that Nod receptors do not impact iNKT cell development or intrinsic functional properties.

Blunted IFN-γ production by iNKT cells, but not NK cells, in Nod1/2−/− mice during bacterial infection

Intact TLR signaling is essential to mediate iNKT cell activation during bacterial infection (18, 24). Specifically, deficiency in the adaptor molecule MyD88 abrogates iNKT cell response against S. typhimurium and L. monocytogenes (18, 24). S. typhimurium and L. monocytogenes are known to activate Nod1 and Nod2; in light of the findings described above, we assessed whether Nod1/2 could synergize with TLRs to potentiate iNKT cell activation during infection. First, WT and Nod1/2−/− mice were injected i.v. with 2 × 10⁴ CFU of S. typhimurium SL1344, and splenic iNKT cells were analyzed 3 d postinfection by flow cytometry using CD1d tetramers. CD69 expression by iNKT cells was elevated in S. typhimurium–infected mice versus uninfected mice, but it was equivalent in WT and Nod1/2−/− mice (data not shown). However, iNKT cells in Nod1/2−/− mice had blunted IFN-γ production (∼50% reduction) compared with WT mice (Fig. 4A).
Concomitant analysis of NK cells revealed that a sizeable fraction of NK cells produce IFN-γ in response to *S. typhimurium* infection, but this response was unaffected in Nod1/2−/− mice (Fig. 4B). This result indicates that, although not absolutely required, Nod1 and Nod2 participate in iNKT cell activation and their secretion of the Th1 cytokine IFN-γ during *S. typhimurium* infection.

In addition to *S. typhimurium*, WT mice and Nod1/2−/− mice were infected i.v. with Gram-positive *L. monocytogenes* at a dose of 5 × 10^4 CFU. Twenty-four hours postinfection, splenic iNKT cells in WT and Nod1/2−/− mice expressed similarly elevated levels of the activation marker CD69 (data not shown). Intracellular cytokine staining revealed a blunted (30% reduction) iNKT cell IFN-γ response in Nod1/2−/− mice versus WT mice (Fig. 4C). In addition, WT and Nod1/2−/− mice had similar frequencies of splenic IFN-γ+ NK cells (Fig. 4D). Together, these results show that activation of the cytosolic sensors Nod1 and Nod2 during *S. typhimurium* and *L. monocytogenes* infections contributes to IFN-γ production by iNKT cells but not NK cells.

**iNKT cell activation during oral infection by *S. enterica serovar Typhimurium***

*S. typhimurium* is a food-borne pathogen that causes acute colitis in infected humans. Oral infection of mice causes a systemic disease with limited intestinal inflammation. However, pretreatment of mice with streptomycin leads to acute colitis upon *S. typhimurium* infection (57). Using this model, we first found that, although only few iNKT cells could be detected in the cecum lamina propria of uninfected mice, *Salmonella*-infected mice had higher iNKT cell frequencies, indicating that these cells may be recruited to and/or proliferate at the site of infection (data not shown). In addition, iNKT cells became activated and produced IFN-γ in the spleen, liver, and cecum lamina propria starting 2 d postinfection (data not shown), with a peak response at day 3 (Fig. 5). Splenic iNKT cells had reduced IFN-γ response (50% reduction) in Nod1/2−/− mice compared with WT mice. Nod1/2 deficiency did not affect iNKT cell activation in the liver or in the cecum lamina propria. Together with the above results, these observations demonstrate the importance of Nod1 and Nod2 in iNKT cell activation during *S. typhimurium* infection.

**Discussion**

iNKT cells are important regulators of antimicrobial and inflammatory responses. iNKT cells detect pathogens either through the direct recognition of microbe-derived lipid Ags presented by CD1d or through indirect pathways involving self-lipid species and proinflammatory cytokines, which are induced upon engagement of innate receptors within APCs. In this study, we showed that, although the engagement of the cytosolic peptidoglycan-sensing receptors Nod1 and Nod2 during *S. typhimurium* and *L. monocytogenes* infections contributes to IFN-γ production by iNKT cells but not NK cells.

Indirect innate and cytokine-driven pathways appear to dominate iNKT cell activation in response to multiple bacteria over the direct recognition of microbe-derived Ags presented by CD1d or through indirect pathways involving self-lipid species and proinflammatory cytokines, which are induced upon engagement of innate receptors within APCs. In this study, we showed that, although the engagement of the cytosolic peptidoglycan-sensing receptors Nod1 and Nod2 in DCs failed to induce iNKT cell activation, it potentiates TLR4-mediated iNKT cell activation and release of IFN-γ during bacterial infection.

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**FIGURE 4.** Blunted iNKT cell activation in Nod1/2-deficient mice during *S. typhimurium* and *L. monocytogenes* infection. WT and Nod1/2−/− mice were injected i.v. with 2 × 10^6 CFU of *S. typhimurium* (A, B), 5 × 10^4 CFU of *L. monocytogenes* (C, D), or vehicle. FACS analysis of IFN-γ in spleen iNKT cells [TCRβ⁺ PBS57/CD1d tetramer⁺] (A, C) and NK cells [TCRβ⁺ PBS57/CD1d tetramer⁻ NK1.1⁺] (B, D) 72 or 24 h after *Salmonella* or *Listeria* infection, respectively. Data show representative dot plots, as well as the mean + SEM of three independent experiments (n = 7–12 mice/group). **p < 0.01, two-tailed Student t test.
ment of Nod receptors alone does not induce IL-12 secretion by DCs, but it potentiates TLR4-induced IL-12 production by DCs, in agreement with previous reports (38, 39). In addition, blocking experiments revealed that both CD1d–TCR interactions and IL-12, but not IL-23, are important in TLR4/Nod1/2 synergistic activation. The self-glycosphingolipids isoglobotrihexosylceramide (20, 24) and, more recently, β-glucosylceramide (25) were shown to be involved in TLR4-mediated iNKT cell activation. Although we provide evidence that cooperative engagement of Nods and TLR4 increases IL-12 production, it remains to be elucidated whether Nod receptor activation affects the lipid repertoire presented by CD1d to iNKT cells.

Innate receptors, such as TLRs and NLRs, are important to protect from infectious microorganisms, and they relay important cues from the indigenous microbiota to educate the immune system, especially at the level of the intestine (59, 60). In this regard, Blumberg’s group (56) recently reported that early-life microbial exposure impacted the recruitment of iNKT cells in the gut and lung mucosa, which had important consequences on the development of inflammatory disorders. Wingender et al. (55) further demonstrated that splenic iNKT cells in germ-free mice have severely blunted cytokine responses following antigenic stimulation, a phenomenon that is independent of TLR signaling because MyD88−/− Trif−/− mice remain unaffected. In the current study, we found that iNKT cell development, maturation, and response to antigenic stimulation are essentially unaffected in Nod-deficient mice. In line with the previous studies, this suggests that unidentified signals from the intestinal microbiota and host receptors can greatly influence iNKT cell development and function.

Genetic ablation of the adaptor molecule MyD88 revealed the crucial role of TLR signaling during bacterial infection, including Salmonella and Listeria (18, 20, 21, 24). Using Nod1/2-deficient mice, we report that Nod receptors greatly contribute to iNKT cell production of IFN-γ during S. typhimurium and L. monocytogenes infections. This does not reflect a general state of hyporesponsiveness, because upregulation of the early activation marker CD69 was unaffected in Nod1/2-deficient mice. Importantly, we show that iNKT cells are activated in the cecum lamina propria, as well as spleen and liver, upon S. typhimurium oral infection and likely contribute to the IFN-γ response against this pathogen. However, the contribution of Nod1/2 receptors in this model could only be evidenced in the spleen. It is possible that the local triggering of multiple other innate pathways by S. typhimurium, particularly those leading to IL-12 production, compensates for the lack of Nod receptors. In addition, the fact that L. monocytogenes is devoid of the TLR4 ligand LPS suggests that Nod1/2 can synergize with other TLRs, such as TLR2 or TLR5, or potentially other innate receptors to activate iNKT cells during this infection. Because most bacteria express multiple pathogen-associated molecular patterns that are capable of activating various PRRs, synergistic activation of iNKT cells through the coordinated engagement of multiple innate receptors, including the TLR4/Nods synergy described in this article, is likely to be relevant in most bacterial infections.

The data provided in this article demonstrate that coengagement of Nod1/2 and TLR4 in DCs potentiates IFN-γ production by iNKT cells, as well as shows a contribution of Nod1/2 in the release of this cytokine by iNKT cells during bacterial S. typhimurium and L. monocytogenes infection. IFN-γ is often critical to antibacterial immunity (61, 62), and iNKT cells, which act at the cross-road between innate and adaptive responses, are among the earliest IFN-γ producers. In addition, they rapidly activate NK cells (63, 64) and γδ T cells (65) to enhance the overall IFN-γ response. Therefore, it is tempting to speculate that the ability to concomitantly “integrate” multiple microbial signals allows...
iNKT cells to regulate the magnitude of antimicrobial responses (66), such as in sepsis (67).

NK cells provide substantial antimicrobial immunity. In addition to their cytotoxic functions, these cells are potent and early producers of IFN-γ during infection. In this study, using a reductionist immunization approach, we show that LPS induced IFN-γ production by NK cells, which was potentiated by coimmunization with Nod1/2 ligands. However, the production of IFN-γ by these cells upon S. typhimurium infection was largely unaffected in Nod1/2-deficient mice. NK cell activation is regulated by a myriad of activating and inhibitory signals, as well as cytokines, which could compensate for the lack of Nod1/2 during microbial infection (68). LPS is known to activate human NK cells either directly or indirectly via APCs (69, 70). Additionally, human NK cells were shown to express Nod2, and its ligand NDP can activate NK cells directly, in synergy with IL-12 or IFN-α, to potentiate IFN-γ production (53). We hypothesize that a similar two-hit mechanism may be at play here, whereby LPS targets APCs to produce IL-12 and/or type I IFN, and Nod1/2 ligands target NK cells directly.

In summary, our findings indicate that the innate receptors Nod1 and Nod2 synergize with TLR4, and potentially other PRRs, in DCs to fully activate iNKT cells. We propose that this integration of multiple innate pathways regulates iNKT cell activation and their production of key antibacterial cytokines.

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Disclosures

The authors have no financial conflicts of interest.

References

SYNERGISTIC NOD/TLR-MEDIATED iNKT CELL ACTIVATION


