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Cross-Tolerization between Nod1 and Nod2 Signaling Results in Reduced Refractoriness to Bacterial Infection in Nod2-Deficient Macrophages

Yun-Gi Kim,* Jong-Hwan Park,* Stephanie Daignault, † Koichi Fukase, ‡ and Gabriel Núñez2*

Nod2 is an intracellular innate immune receptor that plays a role in host defense and susceptibility to inflammatory disease. We show in this study that macrophages rendered refractory to TLR4 and Nod2 signaling by exposure to LPS and muramyl dipeptide (MDP) exhibit impaired TNF-α and IL-6 production in response to pathogenic Listeria monocytogenes and Yersinia pseudotuberculosis as well as commensal bacteria including Escherichia coli and Bacteroides fragilis. Surprisingly, Nod2 deficiency was associated with impaired tolerization in response to pathogenic and commensal bacteria. Mechanistically, reduced tolerization of Nod2-null macrophages was mediated by recognition of bacteria through Nod1 because it was abolished in macrophages deficient in Nod1 and Nod2. Consistently, Nod2-null macrophages tolerant to LPS and MDP showed enhanced production of TNF-α and IL-6 as well as increased NF-κB and MAPK activation in response to the dipeptide KF1B, the Nod1 agonist. Furthermore, reduced tolerization of Nod2-deficient macrophages in response to bacteria was abolished when mutant macrophages were also rendered tolerant to the Nod1 ligand. Finally, MDP stimulation induced refractoriness not only to MDP, but also to iE-DAP stimulation, providing a mechanism to explain the reduced tolerization of Nod2-deficient macrophages infected with bacteria. These results demonstrate that cross-tolerization between Nod1 and Nod2 leads to increase recognition of both pathogenic and commensal bacteria in Nod2-deficient macrophages pre-exposed to microbial ligands. The Journal of Immunology, 2008, 181: 4340–4346.

Detection of bacteria by host cells is mediated by the recognition of conserved and unique microbial structures by pattern-recognition molecules, such as the TLRs and nucleotide-binding oligomerization domain (NOD)3-like receptors (NLRs) (1, 2). TLRs mediate bacterial recognition of several molecules including LPS and microbial nucleic acids at the cell surface or endosomes and induce host immune responses (1). In contrast, NLRs induce innate immune responses through cytosolic recognition of bacterial components (2, 3). Two NLR family members, Nod1 and Nod2, sense molecules produced during the synthesis and/or degradation of bacterial peptidoglycan (4–7). Nod1 recognizes molecules containing the amino acid meso-diaminopimelic acid that are produced by most Gram-negative and certain Gram-positive bacteria (4, 5). In contrast, Nod2 is activated by muramyl dipeptide (MDP), which is present in both Gram-negative and Gram-positive bacteria (6, 7). Once activated, Nod1 and Nod2 induce transcription of immune response genes through the NF-κB transcription factor and the MAPK signaling pathways (4–8).

Previous studies have revealed an important role for Nod1 and Nod2 in host defense at mucosal surface (8, 9). Mice deficient in Nod2 are more susceptible to oral, but not intraperitoneal administration, of Listeria monocytogenes (8). Similarly, mice lacking Nod1 exhibit increased bacterial loads in the stomach than wild-type mice after oral infection with Helicobacter pylori (9). In addition, Nod1 and Nod2 have been shown to be important for bacterial recognition and host defense against L. monocytogenes after exposure of macrophages or animals to LPS or Escherichia coli (10). These observations suggest that the intracellular sensors Nod1 and Nod2 play a critical role in host defense when TLR signaling is reduced such as that found in intestinal tissue or after tolerization by exposure to TLR ligands. The importance of Nod2 in inflammatory homeostasis is underscored by the finding that specific mutations in the NOD2 gene are associated with increased susceptibility to Crohn’s disease, a disorder characterized by inappropriate bouts of inflammation in the intestinal wall (11–13). Such mutations are associated with Nod2 proteins that exhibit impaired MDP-induced NF-κB activation and cytokine production in human monocytes (7, 14, 15). Furthermore, MDP stimulation enhances the secretion of TNF-α, IL-18, and IL-1β induced by TLR ligands, an event that is abrogated in monocytes harboring Nod2 mutations (14, 15). There is also evidence that Nod2-deficient macrophages or dendritic cells exhibit enhanced TLR2-induced IL-12 production and that prestimulation with MDP inhibits cytokine responses in response to TLR ligands (16, 17). Thus, Nod2 appears to influence TLR-mediated responses in both a positive and negative manner depending on the experimental setting. In the current work, we studied the function of Nod2 in macrophages made hyposensitive to microbial stimulation by previous exposure to LPS and MDP. The analyses revealed an unexpected function for Nod2 in the regulation of proinflammatory responses to pathogenic and commensal bacteria in macrophages refractory to microbial stimulation.
Materials and Methods

Mice

Mice deficient in Nod1, Nod2, and Nod1 and Nod2 in C57BL/6 background have been described (18). C57BL/6 mice were purchased from The Jackson Laboratories. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals.

Reagents and bacterial infection

Ultrapure LPS from E. coli O55:B5 (Invivogen) and synthetic MDP (Bachem) were used to induce tolerization. Synthetic Nod1 ligand, KF1B, was synthesized previously (19). L. monocytogenes wild-type strain 10403S and E. coli strain K-12 were a gift from M. O’Riordan, the University of Michigan (Ann Arbor, MI). Yersinia pseudotuberculosis was a gift from J.B. Bliska, State University of New York and Bacteroides fragilis strain ATCC25285 was obtained from American Type Culture Collection. Bacteria were diluted to the desired concentration and used to infect macrophages at different bacterial/macrophage ratios. After 30 min of infection at 37°C, the macrophages were washed twice with PBS and IMDM containing 33 μg/ml gentamicin, which was added to limit the growth of extracellular bacteria.

Preparation and stimulation of murine macrophages

Bone marrow derived macrophages were prepared as previously described (20). For induction of macrophage tolerization, cells were cultured in 48-well plates at a concentration of 2 × 10^5/well or in 6-well plates at a concentration of 2 × 10^6 cells/well. The day after the plating, cells were treated with LPS (100 ng/ml), MDP (10 μg/ml), or KF1B (10 μg/ml) for 24 h.

Measurement of cytokines

Mouse cytokines were measured in culture supernatants using ELISA kits from R&D Systems.

Immunoblotting

Cells were lysed in buffer containing 1% Nonidet P-40 supplemented with complete protease inhibitor mixture (Roche) and 2 mM dithiothreitol. Lysate proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes by electro-blotting. Membranes were immunoblotted with Abs for mouse IκBα, phospho-IκBα, p38 and phospho-p38, phosphor-ERK and phospho-JNK (Cell Signaling Technology).
Statistical analysis

Differences in the levels of cytokines in three experiments for each of the bacteria were analyzed using generalized linear mixed models with repeated measures to account for the correlation in the triplicates that were performed. Fixed effects in the model include treatment (untreated vs LPS plus MDP), macrophage type (wild-type vs Nod2−/−), and the interaction of treatment and macrophage type. Pairwise comparisons between wild-type and Nod2−/− within each treatment were performed. Similarly, cytokine levels using other macrophage types (wild-type, Nod1−/−, Nod2−/−, and Nod1−/− Nod2−/−) were analyzed using the generalized linear mixed model with repeated measures. Pairwise comparisons were made within each treatment (untreated and LPS plus MDP). Experiments examining three treatments (untreated, LPS plus MDP, or LPS plus MDP plus KF1B) at different bacterial/macrophage ratio were also analyzed using a generalized linear mixed model with repeated measures. Pairwise comparisons between macrophage types were made within each different bacterial/macrophage ratio and Bonferroni corrections were used to adjust for the multiple comparisons within each model. Differences were considered significant when the p value was < 0.05 except when indicated otherwise. Comparisons of MDP compared with none and KF1B compared with none (see Fig. 5) were tested using a Wilcoxon rank-sum test. Differences were considered statistically significant when the p value was < 0.10. This significance level was chosen because this is the greatest significance level we can detect with three values per treatment group in each comparison. All statistical analysis was performed using SAS 9.1 (SAS Institute).

Results

Nod2 deficiency is associated with reduced tolerization in response to bacterial infection in macrophages pre-exposed to LPS and MDP

To assess the contribution of Nod2 to proinflammatory responses under conditions of microbial tolerization, bone marrow-derived macrophages were prestimulated with LPS and MDP, a stimulus that leads to unresponsiveness to a subsequent challenge with TLR ligands and MDP (10). In naive macrophages, Nod2 deficiency was associated with a slight reduction in TNF-α and IL-6 production after infection with L. monocytogenes whereas the

FIGURE 2. NF-κB and MAPK activation in Nod2-deficient and wild-type macrophages infected with bacteria. A and B, Bone marrow derived macrophages from wild-type and Nod2−/− mice were left untreated or stimulated with combination of LPS and MDP for 24 h and then infected with L. monocytogenes (A) or B. fragilis (B) at bacterial/macrophage ratio of 10 for the indicated times. Cell extracts were immunoblotted with Abs that detect total and phosphorylated (activated) forms of the indicated proteins. Results are representative of at least three separate experiments.

FIGURE 3. Nod1 is required for impaired tolerization in Nod2-deficient macrophages. A and B, Bone marrow derived macrophages from wild-type, Nod1−/−, Nod2−/−, and Nod1−/− Nod2−/− mice were stimulated with combination of LPS and MDP or left untreated for 24 h and then infected with live L. monocytogenes, at bacterial/macrophage ratio of 10. Cell-free supernatants were analyzed by ELISA for production of IL-6 (A) and TNF-α (B). The results represent combined values of at least three independent experiments ± SD. ** and *** indicate significant differences at p < 0.01 and p < 0.001, respectively.

Nod2 deficiency is associated with reduced tolerization in response to bacterial infection in macrophages pre-exposed to LPS and MDP

To assess the contribution of Nod2 to proinflammatory responses under conditions of microbial tolerization, bone marrow-derived macrophages were prestimulated with LPS and MDP, a stimulus that leads to unresponsiveness to a subsequent challenge with TLR ligands and MDP (10). In naive macrophages, Nod2 deficiency was associated with a slight reduction in TNF-α and IL-6 production after infection with L. monocytogenes whereas the
inflammatory response to *Yersinia pseudotuberculosis* was unimpaired (Fig. 1, A and B). Consistent with LPS- and MDP-induced tolerization, the amounts of IL-6 and TNF-α induced by *L. monocytogenes* and *Y. pseudotuberculosis* infection were reduced in wild-type macrophages prestimulated with LPS and MDP when compared with naïve macrophages (Fig. 1, A and B). Surprisingly, deficiency of Nod2 resulted in reduced and loss of tolerization in response to *L. monocytogenes* and *Y. pseudotuberculosis*, respectively (Fig. 1, A and B). The reduced tolerization of Nod2-deficient macrophages to live bacteria was not observed when the macrophages were prestimulated with MDP or LPS alone (data not shown). To determine whether the reduced tolerization of Nod2-deficient macrophages was specific to pathogenic bacteria, naïve and stimulated macrophages were infected with *E. coli* and *B. fragilis*, two commensal bacteria that are abundant in the human intestine. Similar to that observed with pathogenic bacteria, Nod2 deficiency resulted in reduced tolerization as they produced higher levels of TNF-α and IL-6 after infection with *E. coli* or *B. fragilis* (Fig. 1, C and D).

**FIGURE 4.** Cross-tolerization between Nod1 and Nod2 signaling. A and B, Bone marrow derived macrophages were left untreated (−) or pretreated with MDP or KF1B for 24 h to induce tolerization and then restimulated with MDP (A) or KF1B (B). Cell extracts were collected at the indicated times and immunoblotted with Abs that detect total and phosphorylated (activated) forms of the indicated proteins. Results are representative of at least three separate experiments.

**FIGURE 5.** Enhanced Nod1-induced cytokine production and signaling in Nod2-deficient macrophages pre-exposed to LPS and MDP. A and B, Bone marrow derived macrophages from wild-type and Nod2−/− mice were treated with combination of LPS and MDP or left untreated for 24 h. The macrophages were then restimulated with LPS in the presence of absence of MDP or KF1B. Cell-free supernatants were analyzed by ELISA for production of IL-6 (A) and TNF-α (B). *, Significant differences between cultures with and without MDP or KF1B at *p* < 0.10 (Wilcoxon rank-sum test) and *p* < 0.01 using *t* test. C, Bone marrow derived macrophages from wild-type and Nod2−/− mice were left untreated or pretreated with LPS or combination of LPS and MDP for 24 h to induce tolerization and then restimulated with KF1B. Cell extracts were collected at the indicated times and immunoblotted with Abs that detect total and phosphorylated (activated) forms of the indicated proteins. Results are representative of at least three separate experiments.

Nod2 deficiency is associated with increased bacterial-induced NF-κB and MAPK activation in tolerized macrophages

Bacteria induce the secretion of proinflammatory cytokines via NF-κB and MAPK activation (1, 21). To determine whether Nod2 deficiency is associated with alteration in the activation of these signaling pathways, macrophages prestimulated with MDP and LPS for 24 h, followed by infection with *L. monocytogenes* or *B. fragilis*, and extracts were immunobloted with Abs that recognize activated forms of NF-κB, ERK, JNK, and p38. *Listeria* infection resulted in phosphorylation and degradation of IκBα as well as phosphorylation of p38, ERK, and JNK in untreated macrophages which was unimpaired or slightly decreased in Nod2-deficient...
### Figure 6

Pre-exposure to Nod1 ligand inhibits bacterial-induced cytokine responses in Nod2-deficient macrophages stimulated with LPS and MDP. A–D, Bone marrow derived macrophages from wild-type and Nod2<sup>−/−</sup> mice were left untreated or stimulated with combination of LPS and MDP or combination of LPS, MDP, and KF1B for 24 h and then left untreated or infected with live L. monocytogenes (A and B) and Y. pseudotuberculosis (C and D) at bacterial/macrophage ratio of 1, 5, or 10. Cell-free supernatants were analyzed by ELISA for production of IL-6 and TNF-α. The results represent combined values of at least three independent experiments ± SD. * and **, Significant differences at p < 0.0125 and p < 0.001, respectively.

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macrophages (Fig. 2A). Such activation of NF-κB and MAPKs was inhibited in wild-type macrophages pretreated with LPS and MDP, which is consistent with tolerization (Fig. 2A). Notably, there was enhanced phosphorylation of ERK and p38 phosphorylation in Nod2-deficient macrophages pretreated with LPS and MDP when compared with wild-type macrophages (Fig. 2B). Similarly, the early phosphorylation of JNK and I-κB induced by Listeria was increased in tolerized Nod2-deficient macrophages (Fig. 2B). When naive macrophages were infected with B. fragilis, there was also phosphorylation of p38, ERK, JNK, and Iκ-B which was unimpaired in Nod2-deficient macrophages (Fig. 2B). Such activation of MAPKs and NF-κB induced by B. fragilis was reduced in wild-type and Nod2-deficient macrophages prestimulated with LPS and MDP (Fig. 2B). Furthermore, phosphorylation of p38 and Iκ-B was enhanced at 60 min and 90 min after infection in LPS and MDP-prestimulated Nod2-deficient macrophages when compared with similarly treated wild-type macrophages (Fig. 2B). These biochemical analyses indicate that Nod2 deficiency is associated with enhanced MAPK and/or NF-κB activation in macrophages pre-exposed to LPS and MDP, which is consistent with the cytokine responses shown in Fig. 1, A and D.

**Nod1 is required for reduced tolerization in Nod2-deficient macrophages prestimulated with LPS and MDP**

Nod1 is a NLR family member that, like Nod2, recognizes bacterial peptidoglycan-related products (4, 5). To test whether Nod1 contributes to the augmented TNF-α and IL-6 responses observed in Nod2-deficient macrophages, we assessed bacterial-induced responses in macrophages lacking Nod1, Nod2, or Nod1 and Nod2. In untreated macrophages, Nod1 or Nod2 deficiency resulted in a slight reduction in cytokine production in response to Listeria infection, which was further decreased in double mutant macrophages (Fig. 3, A and B). Importantly, the enhanced production of TNF-α and IL-6 observed in Nod2-deficient macrophages prestimulated with LPS and MDP was abrogated in double mutant Nod1 and Nod2 macrophages (Fig. 3, A and B). These results indicate that Nod1 is required for impaired tolerization which is associated with Nod2 deficiency in macrophages prestimulated with LPS and MDP.

**Nod1 and Nod2 agonists induce cross-tolerization**

To determine the relationship between Nod1 and Nod2 signaling, extracts were prepared from macrophages prestimulated with MDP or KF1B (a synthetic Nod1 agonist) for 24 h, followed by restimulation with MDP or KF1B for different times and immunoblotted with Abs that recognize the activated forms of NF-κB, ERK, JNK, and p38. By 30 min of MDP stimulation, phosphorylation, and degradation of Iκ-Bα as well as phosphorylation of p38, ERK and JNK was detected in naive macrophages (Fig. 4A). Such activation of NF-κB and MAPKs in response to MDP was greatly inhibited in macrophages prestimulated with MDP (Fig. 4A). Similarly, activation of JNK, ERK, p38, and NF-κB induced by MDP was reduced macrophages pretreated with KF1B (Fig. 4B). In reciprocal experiments, stimulation of naive macrophages with KF1B induced phosphorylation of Iκ-Bα, p38, ERK, and JNK, which was impaired in macrophages pretreated with KF1B or MDP (Fig. 4B). These results indicate that Nod1 and Nod2 agonists induce cross-tolerization in macrophages.

**Enhanced Nod1-induced signaling in Nod2-deficient macrophages pre-exposed to LPS and MDP**

To further assess the relationship between Nod1 and Nod2 signaling, macrophages were pretreated with LPS plus MDP or treated with medium alone for 24 h and then challenged with different amounts of LPS or medium in the presence and absence of MDP or KF1B. Stimulation of mouse macrophages with MDP or KF1B...
alone does not induce the production of cytokines, but costimulation with MDP or KF1B and TLR agonists enhances TLR-mediated responses (8, 18). In line with the latter studies, costimulation of wild-type macrophages with MDP or KF1B and LPS increased the production of IL-6 and TNF-α when compared with the response observed with LPS alone (Fig. 5, A and B). The cytokine response induced by stimulation with LPS was greatly diminished in macrophages pretreated with LPS and MDP, which is consistent with TLR4 tolerization (Fig. 5, A and B). As expected, MDP stimulation increased the amounts of IL-6 and TNF-α induced by LPS in naive macrophages from wild-type but not Nod2-null mice (Fig. 5, A and B). Notably, stimulation of macrophages tolerized to LPS and MDP with the Nod1 ligand, KF1B, induced enhanced production of IL-6 and TNF-α in Nod2-deficient macrophages when compared with wild-type macrophages (Fig. 5, A and B). Thus, the absence of Nod2 is associated with hypersensitiveness to Nod1 stimulation in macrophages pre-exposed to TLR4 and Nod2 agonists.

We next assessed NF-κB and MAPK activation induced by KF1B in wild-type and Nod2-deficient macrophages. In untreated cells and macrophages prestimulated with LPS, phosphorylation and degradation of Ik-Bα as well as phosphorylation of p38, ERK and JNK induced by KF1B was comparable in wild-type and Nod2-macrophages (Fig. 5C). Such activation of NF-κB and MAPKs in response to KF1B was greatly reduced in wild-type macrophages prestimulated with LPS and MDP which is consistent with cross-tolerization between MDP and KF1B (Fig. 5C). In contrast, activation of JNK, ERK, p38, and NF-κB induced by KF1B was unimpaired in Nod2-deficient macrophages pretreated with LPS and MDP (Fig. 5C). Thus, both secretion of proinflammatory cytokines and NF-κB and MAPK signaling induced by KF1B is increased in the absence of Nod2 in macrophages pre-exposed to LPS and MDP.

Pre-exposure to Nod1 agonist inhibits reduced tolerization observed in bacterial-infected Nod2-deficient macrophages

To further assess cross-tolerization between Nod1 and Nod2 signaling, macrophages were left untreated, stimulated with LPS plus MDP or LPS plus MDP and KF1B for 24 h before infection with intracellular bacteria. Production of TNF-α and IL-6 in response to infection with L. monocytogenes and Y. pseudotuberculosis, two bacteria that produce Nod1 and Nod2 agonists, were measured. Production of TNF-α and IL-6 was increased in Nod2-deficient macrophages pre-exposed to LPS and MDP. Although the mechanism that mediates MDP tolerance remains to be elucidated, it is likely that tolerization targets RICK or other molecules that are critical for both Nod1 and Nod2 signaling. Previous studies showed that TLR ligands such as LPS did not cross-tolerize to MDP in mouse macrophages (10). Consistently, some studies did not find cross-tolerization between MDP and TLR2 in human monocytes or TLR4 signaling in the production of IL-6 in human monocytes (26). In contrast, other studies found cross-tolerization between MDP and LPS in human monocytes (26, 27). The reason for the differences in results between the mouse and human system and among the human studies is unclear. One possibility is that the differences are related to differential signaling between mice and humans or to differences in the experimental systems. Further studies are needed to clarify the discrepancies observed among the different studies. The reduced tolerization to pathogenic and commensal bacteria associated with Nod2 deficiency may contribute to disease pathogenesis. Crohn’s disease-associated NOD2 variants defective in MDP recognition are present in up to 20% of healthy Caucasian individuals (28). Recent studies have shown an association between the loss-of-function Leu1007fsinsC Nod2 allele and increased sepsis-related mortality (29). Similarly, the Leu1007fsinsC variant has also been found associated with increased rate of transplant related mortality (30). Although the reason for the link between loss-of-function Nod2 variants and increased survival in sepsis and bone marrow transplantation remains unclear, one possibility is that such mutations are associated with deleterious inflammatory responses to enteric bacteria present in the gut. In the case of sepsis-associated mortality, there is evidence that increased production of proinflammatory cytokines such as TNF-α is a factor that promotes mortality in animal models (31, 32). Therefore, one possibility is that in individuals harboring loss-of-function Nod2 mutations, reduced or loss of tolerization to LPS and MDP may result in increased production of harmful cytokines in response to Nod1-producing bacteria.

Discussion

We show in this study that deficiency of Nod2 causes impaired tolerization to infection with pathogenic and commensal bacteria in macrophages that are rendered tolerant to LPS and MDP. The reduced refractoriness to bacteria associated with Nod2 deficiency was mediated through recognition of bacteria via Nod1. Mechanistically, the reduced tolerization of Nod2-deficient macrophages to bacterial infection is explained by our finding that there is cross-tolerization between Nod1 and Nod2 signaling. Thus, exposure of wild-type macrophages to MDP induced tolerization to both MDP and iE-DAP. However, Nod2-deficient macrophages that are insensitive to MDP-mediated recognition and tolerization remained responsive to Nod1 stimulation by bacteria.

Recognition of bacteria via TLRs and NLRs during infection induces the production of proinflammatory cytokines that are critical for the removal of invading bacteria. Although these cytokines are protective to the host, their excessive production induces organ damage and promotes mortality (22, 23). Thus, the induction of macrophage tolerance by LPS and other bacterial ligands is thought to reduce tissue damage and contribute to host survival upon bacterial infection. The reduced tolerization to bacterial infection in Nod2-deficient macrophages was observed with both pathogenic and commensal bacteria. However, there were differences in the responses observed with the different bacteria. For example, there was reduced refractoriness in response to L. monocytogenes whereas an absence of tolerization was observed when Nod2-deficient macrophages were stimulated with Y. pseudotuberculosis. We do not have an explanation that accounts for the differential response observed with Listeria and Yersinia. A possible explanation is that Y. pseudotuberculosis express virulence factors such as YopJ that are known to interfere with NF-κB and MAPK activation in host macrophages and such factors may impair LPS and/or MDP-induced tolerization (24, 25). We demonstrated in this study that stimulation with MDP led to refractoriness to both MDP and KF1B, the Nod1 agonist. In addition, KF1B induced tolerization not only to KF1B but also to MDP. The cross-tolerization between Nod1 and Nod2 signaling was associated with impaired NF-κB and MAPK activation in response to pretreatment of macrophages with MDP and KF1B. Although the mechanism that mediates MDP tolerance remains to be elucidated, it is likely that tolerization targets RICK or other molecules that are critical for both Nod1 and Nod2 signaling. Previous studies showed that TLR ligands such as LPS did not cross-tolerize to MDP in mouse macrophages (10). Consistently, some studies did not find cross-tolerization between MDP and TLR2 in human monocytes or TLR4 signaling in the production of IL-6 in human monocytes (26). In contrast, other studies found cross-tolerization between MDP and LPS in human monocytes (26, 27). The reason for the differences in results between the mouse and human system and among the human studies is unclear. One possibility is that the differences are related to differential signaling between mice and humans or to differences in the experimental systems. Further studies are needed to clarify the discrepancies observed among the different studies. The reduced tolerization to pathogenic and commensal bacteria associated with Nod2 deficiency may contribute to disease pathogenesis. Crohn’s disease-associated NOD2 variants defective in MDP recognition are present in up to 20% of healthy Caucasian individuals (28). Recent studies have shown an association between the loss-of-function Leu1007fsinsC Nod2 allele and increased sepsis-related mortality (29). Similarly, the Leu1007fsinsC variant has also been found associated with increased rate of transplant related mortality (30). Although the reason for the link between loss-of-function Nod2 variants and decreased survival in sepsis and bone marrow transplantation remains unclear, one possibility is that such mutations are associated with deleterious inflammatory responses to enteric bacteria present in the gut. In the case of sepsis-associated mortality, there is evidence that increased production of proinflammatory cytokines such as TNF-α is a factor that promotes mortality in animal models (31, 32). Therefore, one possibility is that in individuals harboring loss-of-function Nod2 mutations, reduced or loss of tolerization to LPS and MDP may result in increased production of harmful cytokines in response to Nod1-producing bacteria.
Disclosures

The authors have no financial conflict of interest.

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