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NOD2-Deficient Mice Have Impaired Resistance to Mycobacterium tuberculosis Infection through Defective Innate and Adaptive Immunity

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NOD2/CARD15 mediates innate immune responses to mycobacterial infection. However, its role in the regulation of adaptive immunity has remained unknown. In this study, we examined host defense, T cell responses, and tissue pathology in two models of pulmonary mycobacterial infection, using wild-type and Nod2-deficient mice. During the early phase of aerosol infection with Mycobacterium tuberculosis, Nod2−/− mice had similar bacterial counts but reduced inflammatory response on histopathology at 4 and 8 wk postchallenge compared with wild-type animals. These findings were confirmed upon intratracheal infection of mice with attenuated Mycobacterium bovis bacillus Calmette-Guérin. Analysis of the lungs 4 wk after bacillus Calmette-Guérin infection demonstrated that Nod2−/− mice had decreased production of type 1 cytokines and reduced recruitment of CD8+ and CD4+ T cells. Ag-specific T cell responses in both the spleens and thoracic lymph nodes were diminished in Nod2−/− mice, indicating impaired adaptive antimycobacterial immunity. The immune regulatory role of NOD2 was not restricted to the lung since Nod2 disruption also led to reduced type 1 T cell activation following i.m. bacillus Calmette-Guérin infection. To determine the importance of diminished innate and adaptive immunity, we measured bacterial burden 6 mo after aerosol infection with M. tuberculosis and followed a second infected group for assessment of survival. Nod2−/− mice had a higher bacterial burden in the lungs 6 mo after infection and succumbed sooner than did wild-type controls. Taken together, these data indicate that NOD2 mediates resistance to mycobacterial infection via both innate and adaptive immunity. The Journal of Immunology, 2008, 181: 7157–7165.

Mycobacteria are extremely successful intracellular pathogens that infect and cause disease by manipulating the host immune response (1, 2). En route to their intracellular niche in macrophages, mycobacteria trigger an innate immune response mediated by pattern recognition molecules (PRMs)3 (3). Studies using gene knockout mice have shown a role for each of TLR2 (3–5), TLR4 (3, 6, 7), TLR6 (4), and TLR9 (5) in protective immunity to mycobacterial infection. Moreover, absence of the common adaptor for TLRs, MyD88, results in profound susceptibility to mycobacterial infection (5, 8), suggesting that multiple rather than single TLRs are required for the innate defense against mycobacterial infection. Remarkably, adaptive immunity is not impaired in MyD88-deficient mice during the course of Mycobacterium tuberculosis infection (9, 10). These findings suggest that other PRMs, signaling through a MyD88-independent pathway, are involved in shaping adaptive immunity to mycobacterial infection.

Among the PRMs, NOD-like receptors present as attractive candidates for MyD88-independent mycobacterial recognition. NOD-like receptor proteins are localized in the cytoplasm and have been implicated in the recognition of intracellular pathogens (11–13). NOD2, also known as CARD15, activates innate immunity in response to peptidoglycan-derived muramyl dipeptide (MDP) (14, 15). Mycobacterial sensing by NOD2 has been the subject of study by several groups. Consistently, these studies have shown that defects in NOD2 signaling lead to impaired in vitro mycobacterial recognition by human- or murine-derived macrophages (16–19). Furthermore, Gandotra and colleagues reported that Nod2-deficient mice exhibit decreased innate antimycobacterial immunity following M. tuberculosis infection; however, the bacterial burden in the first months after challenge did not differ as a function of Nod2 status (17). More recently, human NOD2 variants were found to be associated with susceptibility to tuberculosis in an African American population (20). In light of these findings, the importance of NOD2-mediated immunity during a chronic mycobacterial infection requires further study.

Relatively little is known about the role of NOD2 in the regulation of adaptive immunity in general, and its function in instructing the T cell response to mycobacterial infection is unknown. To investigate the role of the NOD2 sensor molecule in antimycobacterial immunity,
we examined host defense, T cell responses, and tissue immunopathology in two models of pulmonary mycobacterial infection and one model of i.m. bacillus Calmette-Guérin (BCG) infection. Our findings provide evidence that NOD2 plays a critical role in both innate and adaptive immunity to pulmonary mycobacterial infection.

Materials and Methods

Mice

Nod2<sup>−/−</sup> males backcrossed on a C57BL/6 background were obtained from the Congenics Facility at Yale University and bred with C57BL/6 mice purchased from Harlan Laboratories to establish a Nod2<sup>−/−</sup> breeding colony at the McGill University Health Centre. All study mice were 8–12 wk old and experiments were conducted in accordance with the guidelines of the animal research ethics board of McGill University.

Bacterial strains, growth conditions, and bacteria-derived reagents

*M. tuberculosis* H37Rv and *Mycobacterium bovis* BCG Russia were grown at 37°C in Middlebrook 7H9 medium (Difco Laboratories) containing 0.05% Tween 80 (Sigma-Aldrich) and 10% albumin-dextrose-catalase (BD Biosciences) supplement on a rotating platform. For solid media, Middlebrook 7H10 (without Tween) supplemented with oleic acid-albumin-dextrose catalase was used. Pure LPS from *Escherichia coli* 055:B5 and N-acetyl MDP were purchased from Sigma-Aldrich.

Cell purification and ex vivo culture and stimulation of macrophages

Pulmonary alveolar macrophages were purified from the airway of naive *Nod2<sup>+/+</sup>* and *Nod2<sup>−/−</sup>* mice by bronchoalveolar lavage (BAL). In some experiments lungs were digested. Briefly, lungs were perfused through the left ventricle with Hanks’ buffer (Invitrogen). The lungs were then cut into small pieces (<1 × 1 mm) and incubated with 150 U/ml of collagenase type 1 (Sigma-Aldrich) for 1 h at 37°C. Lung fragments were then crushed through a 100-μm pore size filter (BD Biosciences). After single-cell suspensions were generated, samples were incubated in RBC lysis buffer for 2 min. Then, cells were washed and enumerated. CD11b<sup>+</sup> cells were purified by a MACS column purification procedure by positive selection from lung of *Nod2<sup>+/+</sup>* and *Nod2<sup>−/−</sup>* mice. The purity of purified CD11b<sup>+</sup> cells was always >90%.

Cells (1 × 10<sup>5</sup>/well) were cultured in 96-well plates with or without live bacterial infection (2 CFU/cell) or stimulation with *M. tuberculosis* culture filtrate (*M.tb*-CF, 8 μg/ml), LPS (10 ng/ml), and N-acetyl MDP (10 μg/ml). Stimulation with *M.tb*-CF, LPS, and MDP was performed by adding these bacteria-derived reagents directly to wells containing adherent macrophages (3 wells/condition). The culture supernatants were collected at designated intervals and stored at −20°C until cytokine measurements. Cytokine production by macrophages was assayed using the ELISA kit (R&D Systems) to measure IL-12 p40, IFN-α, IL-10, and IL-4 in culture supernatants. NO production was assayed using the Measure-iT high-sensitivity nitrite assay kit (Invitrogen).

**FIGURE 1.** NOD2 deficiency leads to decreased type 1 cytokines without affecting the internalization of mycobacteria. A, Naive alveolar macrophages from WT and NOD2-deficient mice were stimulated with MDP, LPS, and LPS plus MDP for 24 h. B, Naive alveolar macrophages from WT and NOD2-deficient mice were infected with *M. bovis* BCG for 48 h and the levels of TNF-α, IL-12 p40, and IL-10 in supernatant were measured by ELISA. C, Using confocal microscopy, intracellular IL-12 p70 was visualized in infected WT and *Nod2<sup>−/−</sup>* macrophages (labeled green). Nucleic acids were stained with DAPI (labeled blue, ×1000 magnification). D, Naive alveolar macrophages from WT or *Nod2*-disrupted mice were infected with *M. bovis* BCG or *M. tuberculosis* and the uptake of mycobacteria was evaluated after 5 or 48 h postinfection. One representative experiment out of three is shown. *, p < 0.05 compared with WT.
Confocal microscopy

For total IL-12 determination, a commercially available anti-IL-12 p70 Ab (R&D Systems) was used to localize IL-12 p70 with rat anti-mouse IL-12 p70 IgG1 followed by FITC-conjugated mouse anti-rat IgG1. Nonspecific binding was blocked using 0.5% BSA for 1 h at room temperature. Nucleic acids were labeled with mounting media containing DAPI (Vector Laboratories). Labeled alveolar macrophages were analyzed on an Olympus FluoView confocal laser scanning microscope.

In vitro infection of alveolar macrophages

Alveolar macrophages harvested from Nod2+/+ and Nod2−/− mice were allowed to adhere in a 6-well culture plate (0.5 × 10⁶/well) for 24 h. Adherent cells were washed and infected with mycobacteria (2 CFU/cell) for varying time periods (6 wells/condition). At different time points, cells were washed extensively with sterilized PBS and lysed in 1% Triton X-100 (Sigma-Aldrich) for 5 min. Mycobacterial CFU were enumerated 28 days after plating cell lysates on Middlebrook 7H10 agar plates incubated at 37°C.

Pulmonary infection, BAL, and histopathology

To assess the role of NOD2 during infection with fully virulent M. tuberculosis, 100 bacteria of M. tuberculosis strain H37Rv were delivered by aerosol and adequacy of infection was ascertained by enumeration of bacteria from the lungs of four animals 24 h after infection. For immunological studies, we used M. bovis BCG Russia, an attenuated vaccine strain, permitting experimentation in level 2 facilities. BCG Russia was grown and quantified as previously described to elicit an intratracheal infection with 0.5 × 10⁶ live bacilli (21). For both infections (M. tuberculosis and BCG), bacterial burden was measured at different time points by serial plating or auramine/rhodamine staining (TB fluorescent stain kit T, BD Biosciences) and tissue was also fixed in 10% formalin for H&E staining. Representative slides were read by a pathologist blinded to mouse genotype and were also semiquantitatively assessed by two readers that ranked the extent of pathology blinded to Nod2 status.

Assessment of adaptive immune responses via FACS and ELISPOT

At day 28 post-BCG infection, splenocytes and digested lung cells were harvested and enumerated, and 1 × 10⁶ cells were seeded in 96-well U-bottom plates. Cells were washed and blocked with CD16/CD32 in 0.5% BSA/PBS for 15 min on ice, then stained with appropriate Abs against cell surface markers according to the manufacturer’s instructions to estimate the frequency of T cells, using the FACSCalibur (BD Pharmingen) with analysis by FlowJo software. ELISPOT assay was conducted according to the manufacturer’s instructions (R&D Systems). Briefly, isolated splenocytes or lymph node cells (0.5 × 10⁶ cells/well) were seeded into the 96-well plate precoated with mouse IFN-γ capture Abs. Cells were incubated for 24 h with or without antigenic stimulation (M.tb-CF, 8 μg/ml). The plate was then developed by using standardized streptavidin-conjugated alkaline phosphatase and chromogen method.

FIGURE 2. NOD2 deficiency reduces histopathologic responses to pulmonary M. tuberculosis infection. A, Infected WT and NOD2-deficient mice were sacrificed at 4 and 8 wk, and the levels of mycobacterial infection in lungs and spleens were evaluated by measuring CFU. CFU are presented as means ± SEM from five mice per group and are representative of two independent experiments. B, Lung tissue sections prepared from infected mice were fixed, processed, and stained with H&E. The lungs of WT mice mounted an earlier inflammatory response at 4 wk, which was intensified by 8 wk; this response was remarkably less intense in NOD2-deficient lungs. The microhistographs are representative of the lungs from four mice per group.
Mice were i.m. immunized with BCG (1 × 10^6 CFU). At day 14 postimmunization, mice were challenged with heat-killed BCG in the footpad of the hindfeet. Popliteal lymph nodes and spleens were removed after 5 days, and IFN-γ ELISPOT as well as ELISA was conducted according to the manufacturer’s instructions (R&D Systems).

Long-term M. tuberculosis infection

To assess the role of NOD2 in resistance to M. tuberculosis infection, we delivered an elevated dose (400 bacteria) of M. tuberculosis strain H37Rv by aerosol in two independent experiments. To ensure a fully virulent challenge agent, we verified production of phthiocerol dimycocerosate before challenge by lipid extraction and thin-layer chromatography, as strains of M. tuberculosis that have lost the capability of producing phthiocerol dimycocerosate have attenuated virulence in animal models (22). For one experiment, mice were sacrificed 6 mo after pulmonary infection to measure bacterial burden as described above. For the second experiment, mice were monitored biweekly for change in health status and sacrificed when they had lost 15% of their body mass from the maximum weight attained during the experiment. Duration to sacrifice was plotted using GraphPad Prism and compared as a function of Nod2 status. Necropsies were performed at the time of sacrifice to document the cause of death.

Results

Nod2-deficient alveolar macrophages have impaired type 1 cytokine production upon mycobacterial infection

Because natural infection with M. tuberculosis occurs via the pulmonary route, we assessed the cytokine response of alveolar macrophages from naive Nod2^+/+ and Nod2^−/− mice. To confirm the validity of this model system, cells were initially stimulated with MDP alone, LPS alone, and MDP combined with LPS. As expected, MDP alone failed to elicit significant TNF-α production, and the combination of MDP and LPS resulted in synergistic production of TNF-α, which was reduced in Nod2^−/− alveolar macrophages (Fig. 1A).

Upon infection with M. bovis BCG, Nod2^−/− alveolar macrophages released significantly less TNF-α than did Nod2^+/+ cells (Fig. 1B, left panel). Since control of mycobacterial infection is known to depend on components of the IL-12/IFN-γ axis (23), we also tested for IL-12 response and included IL-10 and IL-4 as prototypical type 2 cytokines. As shown in Fig. 1B (middle and
right panels), IL-12 p40 secretion during *M. bovis* BCG infection depended significantly on the presence of NOD2, while IL-10 levels were not affected and IL-4 was not detected (data not shown). Since IL-12 p70 is the bioactive form of IL-12, we also assessed the levels of IL-12 p70 under the same conditions. Because there is a tight regulation in release of IL-12 p70 by macrophages (24), we used confocal microscopy to visualize this cytokine intracellularly (25). The IL-12 p70-labeled green fluorescent cells were only visualized in infected wild-type (WT) alveolar macrophages, while they were undetectable in infected Nod2−/− macrophages (Fig. 1C).

To verify that these findings were not confounded by altered bacterial uptake, survival, or replication in Nod2-deficient cells, we measured bacterial counts at 5 and 48 h postmycobacterial infection. As shown in Fig. 1D, the bacterial burden was similar in Nod2+/+ and Nod2−/− macrophages whether infected with *M. bovis* BCG or virulent *M. tuberculosis*. Thus, NOD2 mediates the ex vivo induction of type 1 cytokines by alveolar macrophages upon mycobacterial infection but does not affect replication of attenuated and virulent mycobacteria.

**Nod2 deficiency leads to reduced inflammatory responses during pulmonary mycobacterial infection**

To determine the consequence of Nod2 disruption in vivo, we infected Nod2−/− and Nod2+/+ mice with virulent *M. tuberculosis*. Consistent with findings reported by Gandotra and colleagues (17), the mycobacterial burden in both the lungs and spleen was unaffected by Nod2 status at both 4 and 8 wk after *M. tuberculosis* pulmonary infection (Fig. 2A). Upon examination of histopathology, we observed that Nod2−/− mice had reduced inflammatory responses at both time points (Fig. 2B). Differences were noted across all animals per group, with WT mice being assigned a higher pathology score than Nod2−/− mice by two readers blinded to genotype (*p* < 0.05 at each time point by rank-sum test).

To determine whether this altered immunopathology could be attributed to a reduced type 1 immune response, we infected mice intratracheally with attenuated *M. bovis* BCG. Again, bacterial burden was unaffected by Nod2 status (Fig. 3A), but the histopathologic response was reduced in Nod2-deficient mice (Fig. 3B). Because T cells play a major role in granuloma formation and antimycobacterial host defense (26), we next examined the extent of T cell infiltration in the lung by FACS analysis (Fig. 3C). As shown in Fig. 3D, mycobacterial infection resulted in increased frequency of CD8^+^ and CD4^+^ T cells compared with uninfected controls. Consistent with histopathology, this response was significantly reduced in Nod2−/− compared with Nod2+/+ mice (*p* < 0.05 for both cell types). Therefore, although mycobacterial replication was similar during short-term pulmonary infection of WT and Nod2-deficient mice, the latter feature decreased immunopathology and T cell recruitment in the lungs.

**Nod2 deficiency leads to reduced type 1 cytokine responses to pulmonary mycobacterial infection**

The observed alteration in immunopathology and T cell recruitment in Nod2-deficient mice prompted us to investigate whether cytokine production was also affected in these mice. Analysis of BAL fluid 28 days after pulmonary mycobacterial infection revealed that the total number of inflammatory cells within the airways was similar between the two groups of mice (data not shown). In contrast, direct cytokine analysis of BAL fluid showed that the levels of TNF-α and IFN-γ were reduced in the lungs of Nod2−/− mice (Fig. 4A). Levels of IL-12 p40 and of type 2 cytokines, IL-10 and IL-4 (undetectable), were not significantly affected (Fig. 4A and data not shown).

To further evaluate the functionality of alveolar macrophages during the course of mycobacterial infection as a function of Nod2 status, APCs derived from the BAL fluid of 4 wk BCG-infected Nod2+/+ and Nod2−/− mice were left unstimulated or were stimulated with either mycobacterial culture filtrate (*M.tb*-CF), live mycobacteria, or MDP. As shown in Fig. 4B, levels of TNF-α and IL-10 were unaffected by Nod2 status after ex vivo restimulation. Interestingly, the levels of IL-12 p40 were lower in macrophages from Nod2−/− mice and reached statistical significance in the case of *M.tb*-CF or live mycobacterial stimulation (Fig. 4B). Because of the critical link between type 1 cytokines and NO production in antimycobacterial immunity, we also measured NO levels. Consistent with findings reported by Gandotra and colleagues, Nod2 deficiency was associated with significantly reduced NO production in unstimulated and stimulated cells (17).

Since immune responses measured in alveolar macrophages may differ from lung interstitial macrophages, we purified CD11b^+^ cells from 4 wk BCG-infected lungs and restimulated them ex vivo. Similar to alveolar macrophages, CD11b^+^ lung macrophages from Nod2-deficient mice produced lower levels of TNF-α and IL-12 p40 after stimulation with live BCG (Fig. 4C). Altogether, these results demonstrate that reduced lung cellular
responses in Nod2-deficient mice were closely associated with decreased production of TNF-α, IL-12, IFN-γ, and NO at the site of infection.

**Reduced activation of Ag-specific T cell response in Nod2-deficient mice during mycobacterial infection**

It was recently shown that NOD1-mediated innate immunity is required for optimal generation of Ag-specific T cell response (27). To test the hypothesis that NOD2 might also bridge innate and adaptive immunity, we measured mycobacterial Ag-specific T cell responses in the spleen and thoracic lymph nodes of Nod2-deficient and WT mice (Fig. 5A). The number of Ag-specific T cells was significantly lower in draining lymph nodes (Fig. 5E), which was associated with decreased levels of IFN-γ production (Fig. 5F). A similar trend was observed in the spleen, but it did not reach statistical significance. Taken together, these findings indicate that NOD2-mediated signaling serves as a bridge between innate and adaptive immune response during mycobacterial infection, which is not restricted to the lung.

**Nod2 deficiency causes increased bacterial burden and reduced survival in the chronic phase of pulmonary M. tuberculosis infection**

It is well documented that mice disrupted for IL-12 p40 or for IFN-γ have severely reduced survival after mycobacterial challenge (28, 29). Because our data indicated reduced, but not ablated, production of these cytokines in BCG-infected Nod2−/− mice, we tested whether there was an effect of NOD2 on bacterial growth and survival during the chronic phase after aerosol infection with M. tuberculosis. Six months after M. tuberculosis infection, the bacterial burden was significantly higher in the lung of Nod2−/− mice compared with WT mice (Fig. 6A). In an independent experiment, survival of Nod2−/− animals was significantly shorter
than their WT counterparts, with the Nod2−/− group reaching its median survival (198 days) before the first fatality in the WT controls (Fig. 6B). At necropsy, lungs were affected with a severe diffuse chronic active histiocytic pneumonitis (Fig. 6C), with large numbers of stainable mycobacterial organisms seen (Fig. 6D).

Discussion

Innate immune responses are critical for controlling mycobacterial infection and initiating adaptive immunity (30). However, pathogenic mycobacteria produce molecules that subvert innate immunity, tipping the balance between host and pathogen in favor of a chronic mycobacterial infection (31). Since APCs are a key determinant of the initiation and instruction of the immune response (32), we set out to determine whether NOD2-mediated sensing by macrophages affects host recognition and control of mycobacterial infection. In the present study, we initially showed that alveolar macrophages derived from Nod2-deficient mice feature impaired innate immune recognition of mycobacterial infection. We extended this observation to demonstrate for the first time that Nod2-disrupted mice manifest altered adaptive immunity during in vivo mycobacterial infection. Additionally, we showed that Nod2 deficiency ultimately leads to decreased host resistance to chronic mycobacterial infection.

Although NOD2 contributes to innate antimycobacterial immunity, Nod2−/− macrophages still respond to live mycobacterial infection. This observation highlights the existence of other pathogen-associated molecular pattern (PAMP)-recognition pathways, including TLR-mediated recognition. While the potential importance of TLR-mediated immunity in mycobacterial infection was suggested by the profound susceptibility of MyD88−/− mice, it was recently shown that Tlr2/4/9-deficient mice control bacterial burden in infected organs as well as do WT animals (10). This differential outcome of M. tuberculosis infection in Tlr2/4/9−/− vs MyD88−/− mice may be explained by the involvement of MyD88 in IL-1 receptor (10, 33) and/or IFN-γ receptor signaling (34).

Importantly, MyD88−/− mice have intact adaptive immune responses to mycobacterial infection but reduced survival during the early phase (4 wk) of infection (9). In contrast, Nod2−/− mice have impaired adaptive immunity with reduced survival in the chronic phase of infection. Collectively, these findings suggest that MyD88-dependent and -independent pathways are both important but lead to different outcomes during mycobacterial infection.

While our conclusions differ from those of Gandotra and colleagues (17), our data on innate immune responses and bacterial growth are comparable. In our study, we also observed that during the early phase of infection, Nod2 deficiency did not affect bacterial burden, but was associated with reduced production of components of the IL-12/IFN-γ axis and diminished induction of NO. In contrast to Gandotra et al.’s study, we observed reproducible differences in tissue inflammatory responses, stimulating further in vivo experiments. Through in vivo study, we demonstrated that Nod2-deficient mice had reduced mycobacterial Ag-specific T cell responses, signifying that NOD2 plays a role in driving adaptive immunity during mycobacterial infection. Consistent with this impaired generation of adaptive immunity, Nod2−/− mice revealed higher pulmonary bacterial burden and succumbed to death at an earlier time point than did WT controls. Thus, the ultimate effect of Nod2 disruption on mycobacterial resistance may manifest itself long after the initial interaction of host and pathogen, reflecting an ongoing defect in the ability of the host to recognize mycobacterial organisms during the course of infection.

The fact that NOD2 signaling has been associated in different studies with either decreased or increased inflammatory outcomes (16, 35) may reflect the importance of different bacteria used in experimental infections. Studies on NOD2 have employed a variety of different organisms, including classical extracellular pathogens (29), intracellular pathogens that escape to the cytosol (16), and intracellular organisms that remain confined in a phagosomal compartment (13). Peptidoglycan from these various organisms...
may vary in quantity, location, and structure. An interesting ob-
servation is that mycobacteria and related actinomycetes encode

7164 NOD2 AND MYCOBACTERIAL INFECTION

and survival. Work by Chackerian et al. (37) and Wolf et al. (38)
has shown that the timing of initial T cell activation is critical in

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