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Nod1/Nod2-Mediated Recognition Plays a Critical Role in Induction of Adaptive Immunity to Anthrax after Aerosol Exposure▽

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Toll-like receptors and Nod-like receptors (NLR) play an important role in sensing invading microorganisms for pathogen clearance and eliciting adaptive immunity for protection against rechallenge. Nod1 and Nod2, members of the NLR family, are capable of detecting bacterial peptidoglycan motifs in the host cytosol for triggering proinflammatory cytokine production. In the current study, we sought to determine if Nod1/Nod2 are involved in sensing *Bacillus anthracis* infection and eliciting protective immune responses. Using mice deficient in both Nod1 and Nod2 proteins, we showed that Nod1/Nod2 are involved in detecting *B. anthracis* for production of tumor necrosis factor alpha, interleukin-1α (IL-1α), IL-1β, CCL5, IL-6, and KC. Proinflammatory responses were higher when cells were exposed to viable spores than when they were exposed to irradiated spores, indicating that recognition of vegetative bacilli through Nod1/Nod2 is significant. We also identify a critical role for Nod1/Nod2 in priming responses after *B. anthracis* aerosol exposure, as mice deficient in Nod1/Nod2 were impaired in their ability to mount an anamnestic antibody response and were more susceptible to secondary lethal challenge than wild-type mice.

Mammalian innate immune receptors, such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), recognize pathogen-associated molecular patterns conserved among various pathogens. Sensing of microbial components by TLRs and NLRs initiates multiple proinflammatory pathways for controlling infection and initiating antigen-specific immune responses (14). TLRs are expressed on the host cell surface and endosomal compartment, whereas NLRs are expressed in the host cytosol. Two described NLRs, Nod1 and Nod2, initiate NF-κB and mitogen-activated protein kinase signaling upon sensing molecules produced during the synthesis and degradation of bacterial peptidoglycan. Nod1 recognizes a dipeptide, γ-D-glutamyl-*meso*-diaminopimelic acid, produced by most gram-negative bacteria (6, 16, 17) and some gram-positive bacteria, including several *Bacillus* species (22). Nod2 senses the peptidoglycan motif muramyl dipeptide (MDP), which is present in nearly all gram-negative and gram-positive bacteria (17, 26). Peptidoglycan is also recognized by cell surface-expressed TLR2 (20, 51). Recent studies, using mostly in vitro techniques, have implicated Nod1 and/or Nod2 as an innate receptor for a variety of bacterial species (13, 28). For example, *Listeria monocytogenes*, an intracellular bacterial pathogen, is sensed by both Nod1 and Nod2 (22, 29, 44).

The prominent role of Nod proteins in innate immunity has been known for some time, but recent work indicates a role for Nod proteins in polarizing adaptive immune responses as well. Thus, innate immune recognition by Nod proteins is important for controlling early pathogen replication and spread, in addition to eliciting and polarizing adaptive immunity (52). Recent work has shown that recognition of MDP via Nod2 or of peptidoglycan via Nod1 (bacterially derived constituents) results in a Th2 polarization of the adaptive immune response (15, 36). However, there are few studies investigating the involvement of Nod proteins in polarizing adaptive responses and subsequent protection in microbial infection (15).

Bacillus anthracis is a gram-positive, spore-forming bacterium and the etiologic agent of anthrax. Spores are the primary infectious agent, germinating into vegetative bacilli in favorable environments (39). The most deadly form of the disease, inhalational anthrax, occurs after spores are inhaled into the lungs (2). Spores in the alveolar space are taken up by pulmonary phagocytes, such as macrophages and dendritic cells (DC) (4, 8, 19, 50). Once inside the cell, spores germinate, and unless controlled by cell defenses, bacilli escape the endosome and bacterial replication occurs in the cytosol (9, 24, 27). Thus, cytosolic sensing of vegetative *B. anthracis* is likely the first point in the infection at which bacilli can be detected by the host. Bacilli eventually escape the host cell and replicate extracellularly, exposing bacterial motifs sensed by surface-expressed TLRs (25).

After the anthrax mail attacks in the fall of 2001, emphasis has been placed on understanding *B. anthracis* pathogenesis for development of therapeutics and vaccines. *B. anthracis* produces two toxins, lethal toxin, a combination of protective antigen (PA) with lethal factor (LF), and edema toxin, a combination of PA with edema factor (EF) (58). PA is named for

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its ability to elicit protective immunity to anthrax infection, and PA is the primary component of the current U.S. licensed vaccine (anthrax vaccine absorbed) (3). The toxin complex, a combination of PA and LF/EF, inserts into the host phagosome membrane, and LF or EF is translocated into the cytoplasm of the host cell (58). LF is a zinc-dependent metalloprotease capable of cleaving host cell mitogen-activated protein kinase kinases in the cell cytosol (10, 54). EF is an adenylate cyclase that increases intracellular concentrations of cyclic AMP, which also disrupts host cell responses (23, 31, 53).

The current study was aimed at understanding host immune activation after spore exposure and identifying the role of Nod1/Nod2 in sensing and responding to *B. anthracis*. Using in vitro techniques, we show that irradiated spores do not induce a strong proinflammatory response by macrophages or lung cells, but instead, viable spores are required for optimal production of various cytokines and chemokines. Likewise, Nod1/Nod2 was required for optimal production of interleukin-12 p70 (IL-12p70) after exposure to viable spores. Taking the results together, we identify Nod1/Nod2 as intracellular receptors of germinating spores for cytokine responses. In the context of in vivo spore exposure, our results indicate that Nod1/Nod2 are important in priming responses after aerosol *B. anthracis* spore exposure, as mice deficient in Nod1/Nod2 exhibited impaired expansion of T cells in the draining lymph nodes (LNs), impaired anamnestic antibody responses, and enhanced susceptibility to lethal rechallenge.

MATERIALS AND METHODS

Mice. C57BL/6 and A/J mice were purchased from The Jackson Laboratory. TLR2 knockout (KO) mice and Nod1/Nod2 KO mice in the C57BL6/J background have been described previously (25, 43). *B. anthracis* aerosol challenges and spore preparations were completed as previously described (34). Briefly, mice were exposed to aerosolized spores for 70 min using a nose-only exposure system (CH Technologies, Westwood, NJ), with fresh air supplied for 8 min before and after the spore challenge. The spore inoculum for each challenge contained 12 ml of 5×10^9 spores/ml in distilled water with 0.01% Tween 80. The 70-min challenge results in approximately 2×10^6 to 4×10^6 spores being retained in the lung, which is approximately 10 times the 50% lethal dose calculated for strain 7702 (34). Generation and purification of *B. anthracis* Sterne spores (strain 7702) were carried out as previously described (12, 48). For intraperitoneal (i.p.) challenge, mice were given 1×10^9 spores in 100 μl. All mice were housed at the Center for Biologics Evaluation and Research under the approval of the Institutional Animal Care and Use Committee.

In vitro studies. J774A.1 macrophage stimulation with *B. anthracis* was performed as previously described with minor modifications (47). Briefly, J774A.1 macrophages were seeded into 96-well flat-bottom plates and left untreated or treated with 3 μg cytochalasin D (CytD) for 45 min. Cells were subsequently infected with Sterne spores at a multiplicity of infection of 50 in antibiotic-free medium for 1 h. The medium was removed, and cells were incubated for 1 h with 200 μl of medium containing 30 μg/ml of gentamicin to kill any extracellular bacteria. Gentamicin-containing medium was removed and replaced with antibiotic-free medium, and this was considered time zero. At 0, 2.5, 5, 7.5, and 10 h, supernatant was removed and stored at −80°C. Cells were washed once and lysed, and the lysate was serially diluted and plated to determine intracellular bacteria levels.

Total adherent pulmonary cells were collected from healthy mice for in vitro stimulation as follows. Total lung cells were isolated using previously published methods (30). Briefly, mice were humanely euthanized using avertin and subsequently exsanguinated. The descending aorta was severed, and the pulmonary vasculature was flushed with sterile phosphate-buffered saline (PBS) with 0.5 mM EDTA to remove peripheral blood from the lungs. The lungs were removed from the body and the lobes detached from the bronchi and trachea. The lungs were cut into small pieces and incubated with liberase blenzyme 3 (0.3 U/ml) (Roche, Indianapolis, IN) and DNase I (50 μg/ml) (Sigma, St. Louis, MO) in 6 ml of PBS for 1 h at 37°C in an orbital shaker. Released cells were collected by running the homogenate solution over a 45-μm filter. Fetal bovine serum (FBS) was added to a final concentration of 20%, and the cells were centrifuged at $400 \times g$ for 15 min. Cells were washed once with RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FBS. Cells were subsequently counted and plated at 2×10^6 cells per well in a 48-well flat-bottom plate. Cells were plated for 1 h at 37°C. For in vitro stimulation, nonadherent cells were washed away and 2×10^6 Sterne strain spores (irradiated or viable) were added in medium without antibiotics. After 45 min, the medium was removed, cells were washed once, and cell culture medium with 10 μg/ml of gentamicin was added; this was considered time zero. For flow cytometric analysis, cells were collected after the 1-h adherence step described above or cultured for 24 h before being collected as follows. Cell culture plates were put on ice for 10 min to aid in cell release from the plate. A pipette tip was used to gently scrape any remaining adherent cells from the bottom of the plate. Cells were collected, washed once with PBS plus 10% FBS, and subsequently stained with antibodies for phenotyping.

BMDMs. Bone marrow-derived macrophages (BMDMs) were prepared as previously described (5). Briefly, bone marrow cells were cultured for 5 days with Dulbecco's modified Eagle medium supplemented with 30% L929 supernatant containing macrophage-stimulating factor, nonessential amino acids, 10% heat-inactivated FBS (Invitrogen Life Technologies), and antibiotics. The cells were stimulated with 10 ng/ml lipopolysaccharide (LPS) in the presence or absence of 10 μg/ml MDP for 24 h. Ultrapure LPS was from *Escherichia coli* O55:B5 (Invivogen), and synthetic MDP was from Bachem.

Flow cytometric analysis. The following antibodies were used for phenotypic analysis: anti-CD11b–allophycocyanin (clone M1/70), anti-CD11c–phycoerythrin (clone HL3), anti-CD8–Alexa 647 (clone 53-6.7), and anti-CD4–biotin (clone GK1.5) (BD Biosciences). Streptavidin-fluorescein isothiocyanate was used in conjunction with biotinylated antibody. Data were acquired using FACS Diva software (BD Biosciences) on an LSR II flow cytometer (Becton Dickinson) and analyzed using FlowJo software (TreeStar, Ashland, OR).

Real-time PCR. SYBR green-based real-time PCR was carried out for various mRNA targets according to the manufacturer's recommendations (SYBR green master mix; Applied Biosystems, Foster City, CA). The amount of mRNA was calculated using the $2^{-\Delta\Delta CT}$ method as previously described (33). The sequences of the PCR primers (5′ to 3′) were as follows: IL-1α-Forward, GCCATTGAC CATCTCTCTCTGAATC; IL-1α-Reverse, GGTGAAGTTGGACATCTTTG ACG; KC-Forward, GGCTGGGATTCACCTCAAGAAC; KC-Reverse, TGG CTATGACTTCGGTTTGGG; CCL5-Forward, GTGCCAACCCAGAGAAGA AGTG; and CCL5-Reverse, AGAGCAAGCGATGACAGGGAAG. Primers for IL-1β, IL-6, and β-actin were previously described (40, 41).

Cytokine measurement. Levels of tumor necrosis factor alpha (TNF-α) (Biosource), IL-6, IL-10, and IL-12p70 (R&D Systems) in cell culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) run according to the recommendations of the kit manufacturers.

Serum antibody titers. Titers of anti-PA immunoglobulin G (IgG) were determined by ELISA as previously described (35). Samples were assayed in triplicate, and the end point antibody titers were expressed as the maximum dilution giving an absorbance (at 405 nm) of greater than 0.2. The results are presented as the reciprocal of the dilution multiplied by the absorbance value.

Statistical analysis. GraphPad Prism software (version 4.00; GraphPad Software, San Diego, CA) was used for all statistical analysis. A log rank test was used to analyze differences in survival after aerosol and i.p. challenge. A Student *t* test was used for analysis of real-time PCR, cytokine ELISA, and cell population data. A one-way analysis of variance and Tukey's multiple-comparison test were used for analyzing antibody titer data.

RESULTS

Cytokine secretion by J774A.1 macrophages after exposure to Sterne strain *B. anthracis* spores. In order to determine if spore uptake and germination are required for stimulation of early cytokine production by phagocytic cells, an assay utilizing J774A.1 macrophages was used. Cells were exposed to viable spores for 1 h to allow for phagocytic uptake before washing away extracellular spores. Cells were harvested for enumeration of intracellular bacteria to identify the time during which the number of intracellular bacteria increased. Supernatant collected from cells was assayed for TNF-α levels. The results indicate that intracellular levels of bacteria increased between 5 and 7.5 h (Fig. 1A), indicating germination and an increase of

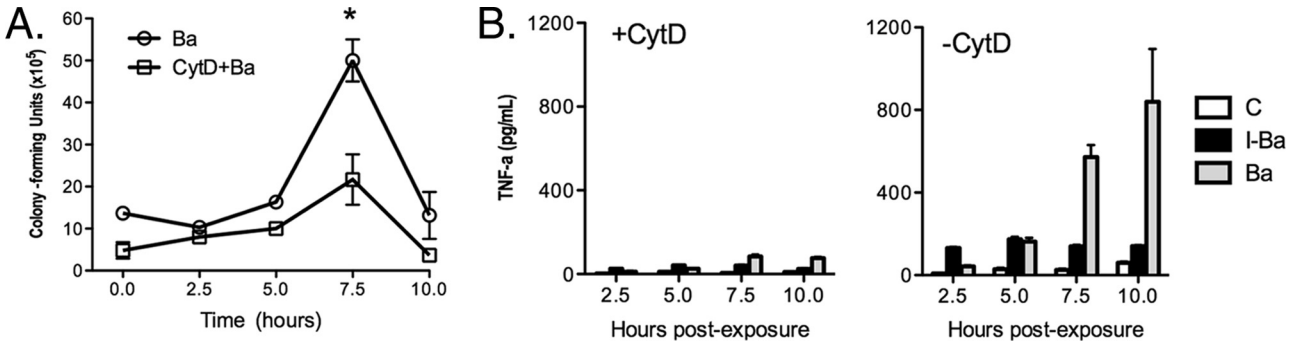

FIG. 1. TNF-α secretion by J774A.1 macrophages after exposure to *B. anthracis* spores. (A) J774A.1 macrophages were untreated (circles) or treated with 3 μg CytD (squares) for 45 min and subsequently exposed to Sterne spores as described in Materials and Methods. Intracellular bacteria were enumerated at 0, 2.5, 5, 7.5, and 10 h after spore exposure. (B) Supernatants collected from J774A.1 treated with (+CytD) or without (−CytD) CytD after exposure to medium alone (C), irradiated spores (I-Ba), or viable spores (Ba) and assayed for TNF-α. Data are expressed as the mean ± standard error of the mean from three samples. Data are representative of two independent experiments. A Student *t* test was used for statistical analysis, and *P* values of <0.05 are indicated (*).

intracellular vegetative cells. However, by 10 h, the number of intracellular bacteria decreased, indicating that bacteria had released from the cells. At 7.5 h after spore exposure, TNF-α levels were elevated in the supernatant, indicating that intracellular recognition of germinating spores likely occurred (Fig. 1B). If irradiated spores were used, TNF-α levels remained low, indicating that germination was important for pathogen recognition and TNF-α production (Fig. 1B). Cells were also exposed to CytD, an inhibitor of phagocytosis, to determine if spore uptake was required for TNF-α production. In cells treated with CytD, TNF-α production following exposure to both viable and irradiated spores was low, indicating that phagocytic uptake of spores was required for increased production of TNF-α (Fig. 1B).

Nod proteins are involved in recognition of live *B. anthracis* for induction of proinflammatory mediators. Nod1 and Nod2 proteins are expressed in the cell cytosol and serve as host pattern recognition receptors for peptidoglycan substructures of bacteria, including *B. anthracis* (17). *B. anthracis* has been shown to replicate in the host cytosol, where Nod1 and Nod2 are expressed (9). The data in Fig. 1 provided evidence that intracellular recognition of bacilli resulted in increased TNF-α production, indicating involvement of an intracellular pattern recognition receptor in sensing *B. anthracis* infection. To determine if host Nod1/Nod2 are involved in recognition of *B. anthracis* after spore infection, pulmonary cells from wild-type (WT) and Nod1/Nod2 KO mice were exposed to viable and irradiated Sterne strain spores. The response of pulmonary cells was examined because they are likely to be one of the first cell types to engulf *B. anthracis* spores during inhalational infection. The pulmonary cells used for this study are described as a mixture of interstitial macrophages, alveolar macrophages (AMac), and DC based on CD11c and CD11b expression, as previously described (7). The phenotype of the adherent population of pulmonary cells changed between 1 h and 24 h of culture (Fig. 2). Thus, we decided to use the total adherent population for these studies instead of sorting a single antigen-presenting cell from the mixture.

After stimulation of pulmonary cells with viable spores, TNF-α levels, measured by ELISA, were significantly higher in WT pulmonary cells than in Nod1/Nod2 KO cells (Fig. 3A). In addition to enhanced TNF-α cytokine production, levels of IL-1α, IL-1β, CCL5, IL-6, and KC mRNAs were significantly higher in WT cells than in cells from Nod1/Nod2 KO mice 8 h after spore stimulation (Fig. 3B). IL-6 and KC mRNA levels did not increase as much as IL-1α, IL-1β, and CCL5 levels, though all cytokines tested were affected by the lack of Nod1/Nod2 (Fig. 3B). IL-6 and KC mRNA levels in WT cells stim-

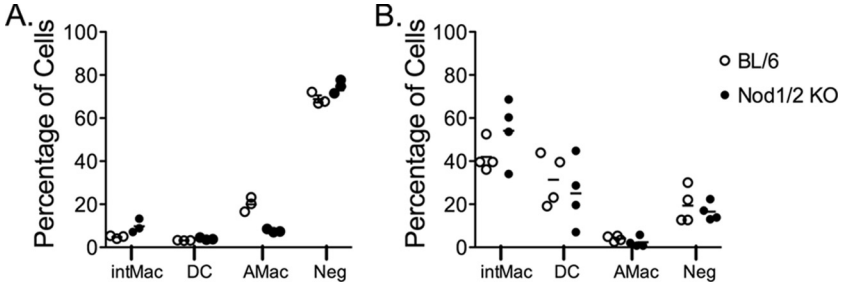

FIG. 2. Characterization of pulmonary adherent cells. Pulmonary adherent cells were collected from BL/6 and Nod1/Nod 2 KO mice as described in Materials and Methods and subsequently stained for CD11b and CD11c markers. Three lung cell populations, i.e., interstitial macrophages (intMac) ($CD1lc^{low}/CD11b^{high}$), DC ($CD1lc^{high}/CD11b^{high}$), and AMac ($CD1lc^{high}/CD11b^{low}$), were identified, and the percentage of each population was different at 1 h (A) and 24 h (B) following culture. Cells that did not stain for either CD11b or CD11c at each time point are also indicated (Neg). Each dot represents a single animal, and a bar is drawn at the mean. A Student *t* test was used for statistical analysis. No statistically significant difference was observed between BL/6 and Nod/Nod2 cells for any cell population except for AMac at 1 h ($P = 0.003$).

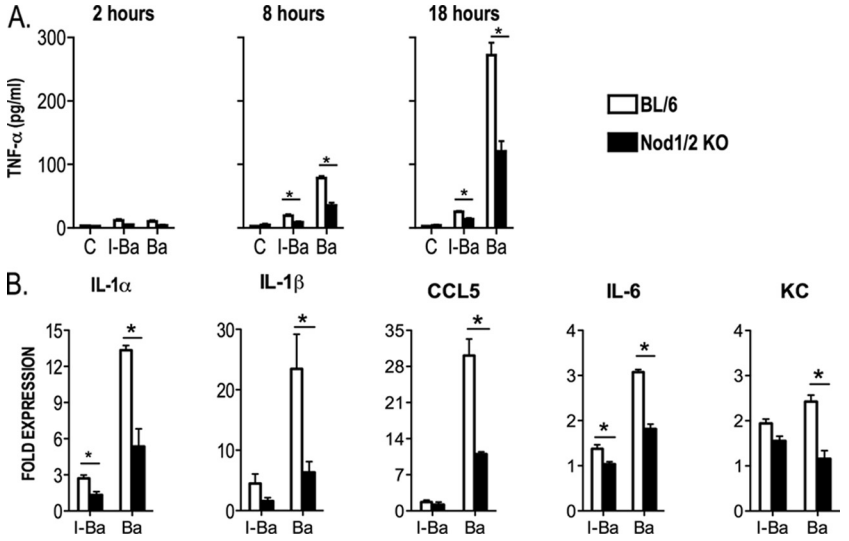

FIG. 3. Nod1/Nod2 proteins are involved in recognition of live *B. anthracis* for induction of proinflammatory mediators. Pulmonary cells from WT BL/6 and Nod1/Nod2 KO mice were collected and exposed to medium alone (C), irradiated Sterne strain spores (I-Ba), or viable Sterne strain spores (Ba). (A) At 2, 8, and 18 h postexposure, supernatant was collected and assayed for TNF-α. (B) At 8 h after spore exposure, mRNA levels of the indicated proinflammatory mediators were measured. Real-time data are expressed as a fold increase in expression over the result for cells treated with medium alone after normalizing to β-actin using the $2^{-\Delta\Delta CT}$ method. Data are representative of two independent experiments and are presented as the mean ± standard deviation. A Student *t* test was used for statistical analysis, and *P* values of <0.05 are indicated (*).

ulated with viable spores were only about twofold higher than levels in Nod1/Nod2 cells. However, mRNA levels of CCL5 were approximately threefold greater and those of IL-1α and IL-1β were nearly fourfold greater in WT than in Nod1/Nod2 KO cells. Spores that were able to germinate induced significantly higher levels of proinflammatory mediators compared to after stimulation with irradiated spores, indicating that vegetative bacilli contribute significantly to stimulation through Nod1/Nod2. In order to eliminate the possibility that Nod1/Nod2 KO cells were generally deficient in their ability to produce cytokines, BMDMs were tested for their ability to respond to LPS. Recognition of LPS is mediated by TLR4 and is independent of Nod1 or Nod2. The response of BMDMs to LPS from Nod1/Nod2 KO mice was indistinguishable from the response of BL/6 BMDMs as measured by IL-6 and TNF-α production (Fig. 4). As expected, MDP-induced cytokine responses in BMDMs costimulated with LPS were abrogated in Nod1/Nod2-deficient cells (Fig. 4), which was used an internal control.

Nod1/Nod2 plays a significant role in induction of protective immunity after primary aerosol exposure to *B. anthracis*. Our laboratory utilizes a murine aerosol challenge model to investigate *B. anthracis* pathogenesis (34). Previous work utilizing this model has shown an important role for MyD88-mediated events in survival after aerosol challenge, though TLR2, which recognizes extracellular peptidoglycan, was not required (25). To determine if Nod1/Nod2 is essential for survival after aerosol challenge, groups of Nod1/Nod2 KO mice were exposed to aerosols of Sterne strain *B. anthracis* spores. Nod1/Nod2 KO mice survived primary aerosol challenge, as did WT BL/6 mice (Fig. 5A). A/J mice, which are susceptible to aerosol challenge due to a C5 deficiency, were used as a control of the challenge (21, 55, 56). As all of the Nod1/Nod2 KO mice survived primary challenge, we were able to investigate the contribution of

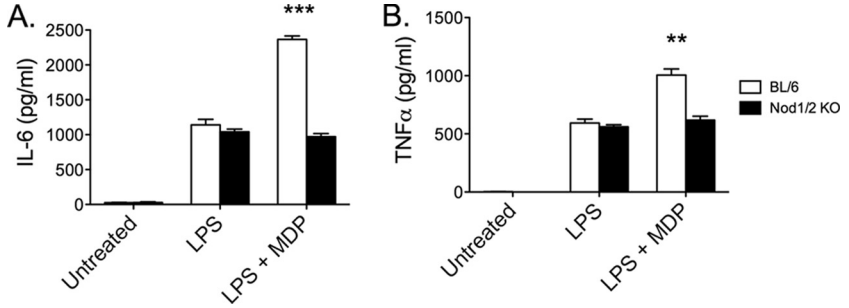

FIG. 4. BMDMs from WT and Nod1/Nod2 KO mice were stimulated with 10 ng/ml LPS in the presence or absence of 10 μg/ml MDP for 24 h. Cell-free supernatants were analyzed by ELISA for production of IL-6 (A) and TNF-α (B). ** and *** indicate significant differences between cultures with and without MDP at *P* values of <0.01 and <0.001, respectively. The statistical significance of differences between two groups was determined by a two-tailed *t* test with unequal variance (Aspin-Welch's *t* test; Microsoft Excel). Error bars indicate standard deviations.

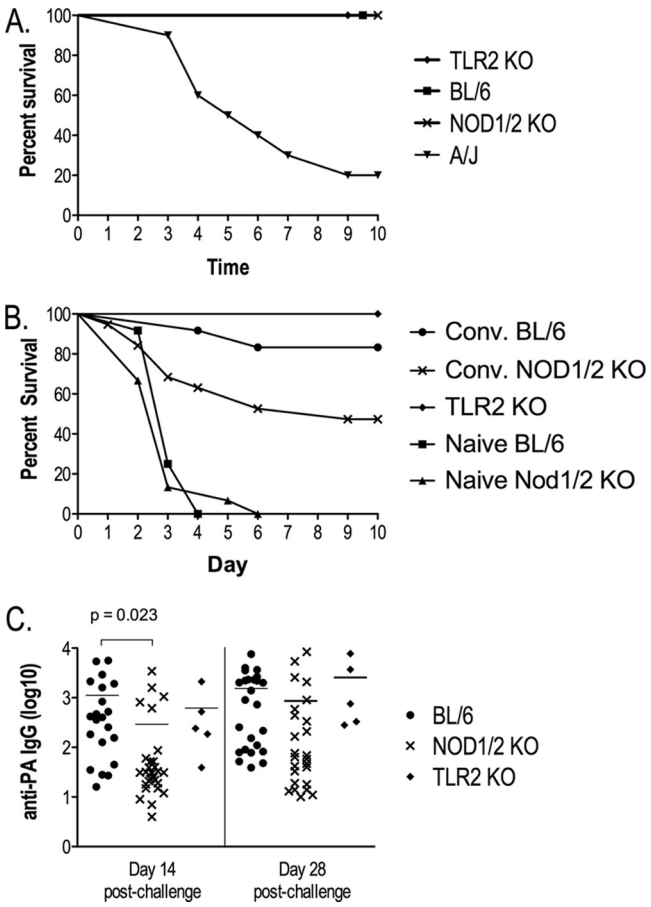

FIG. 5. Nod1/Nod2 plays a significant role in induction of protective immunity after primary aerosol exposure to *B. anthracis*. (A) BL/6, TLR2 KO, Nod1/Nod2 KO, and A/J mice were exposed to aerosolized spores and survival followed for 10 days. (B) BL/6, TLR2 KO, and Nod1/Nod2 KO mice that survived aerosol challenge were rechallenged with a lethal dose of Sterne strain spores 30 days after primary challenge and survival followed for 10 days. Naïve BL/6 mice were included as controls. (C) Sera were collected from BL/6 and Nod1/Nod2 KO mice 14 and 28 days after primary aerosol challenge and assayed for anti-PA IgG titers. Results are pooled from three independent experiments.

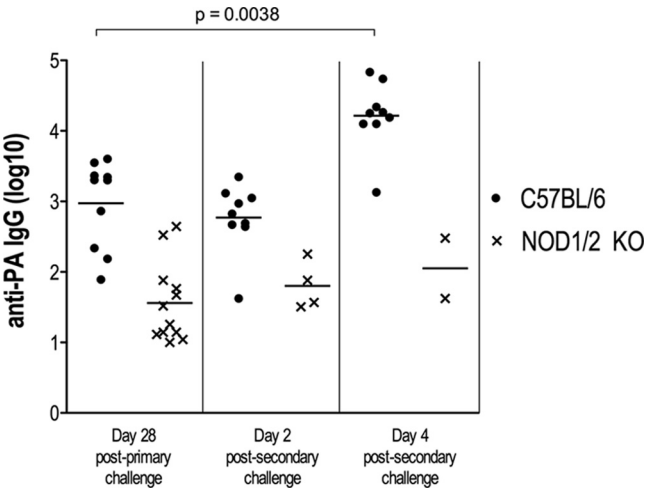

FIG. 6. Nod1/Nod2 plays a significant role in induction of anamnestic immunity after primary aerosol exposure to *B. anthracis*. BL/6 and Nod1/Nod2 KO mice were exposed to aerosolized spores. Mice that survived aerosol challenge were rechallenged with a lethal dose of Sterne strain spores 30 days after primary challenge. Sera were collected from BL/6 and Nod1/Nod2 KO mice 28 days after primary aerosol challenge and 2 and 4 days after the secondary lethal challenge. Sera were assayed for anti-PA IgG titers. Results are pooled from two independent challenges. A Student *t* test was used for statistical analysis. For the BL/6 mice and the Nod1/Nod2 mice, titers at day 2 postchallenge and day 4 postchallenge were compared to titers on day 28. Only the BL/6 titers at day 4 were significantly different from those at day 28.

these pathogen recognition receptors to induction of protective immunity. Thirty days after primary aerosol challenge, mice were rechallenged with a lethal dose of Sterne strain spores by the i.p. route. Figure 5B indicates that 83% of convalescent BL/6 KO mice survived rechallenge but only 47% of convalescent Nod1/Nod2 KO mice survived. Thus, even with the expression of all TLRs in Nod1/Nod2 KO mice, recognition through Nod1/Nod2 was required for optimal induction of adaptive immunity for subsequent protection. There was no significant difference in the susceptibilities of naïve BL/6 and naïve Nod1/Nod2 KO mice to the i.p. challenge (Fig. 5B).

Antibody titers to PA have been described as a correlate of protection after anthrax vaccination (32, 49). To determine if anti-PA IgG titers correlated to susceptibility exhibited by Nod1/Nod2 KO mice after rechallenge, serum antibody titers were measured at 14 and 28 days after primary aerosol challenge. Figure 5C indicates that anti-PA IgG titers differed significantly between BL/6 and Nod1/Nod2 KO mice at 14 days after exposure. At 28 days after primary exposure there was a wide range in anti-PA IgG titers among mice of the same strain, and individual mice deficient in Nod1/Nod2 had titers as high as those in WT BL/6 mice, even 14 days after aerosol challenge (Fig. 5C). In order to assess the ability of Nod1/Nod2 mice to mount an anamnestic response following reexposure to anthrax, an additional experiment was performed to measure anti-PA IgG titers in BL/6 and Nod1/Nod2 KO mice after primary and secondary exposure to *B. anthracis*. First, mice were exposed to aerosols of Sterne strain *B. anthracis* spores and anti-PA IgG titers measured 28 days later. Thirty days after primary aerosol challenge, mice were rechallenged with a lethal dose of Sterne strain spores by the i.p. route. Serum antibody titers were measured on days 2 and 4 after secondary challenge (Fig. 6). Following secondary challenge, antibody titers rose significantly relative to prechallenge (day 28) titers in BL/6 mice but did not appear to rise in Nod1/Nod2 KO mice (Fig. 6). Although the antibody titers in the Nod1/Nod2 mice surviving at day 4 postchallenge were not higher than the titers seen in the same mice at day 28, there were not enough survivors to make a statistically significant comparison.

Impaired expansion of lymphocytes in draining LNs of Nod1/Nod2 KO mice after aerosol anthrax challenge. We examined changes in lymphocyte numbers in draining cervical LNs of WT BL/6 and Nod1/Nod2 KO mice to begin identifying a potential mechanism for the deficient adaptive immune response in Nod1/Nod2 KO mice. WT BL/6 and Nod1/Nod2 KO mice were exposed to aerosols of *B. anthracis* Sterne strain and cervical LNs collected 7 days later for examining total LN

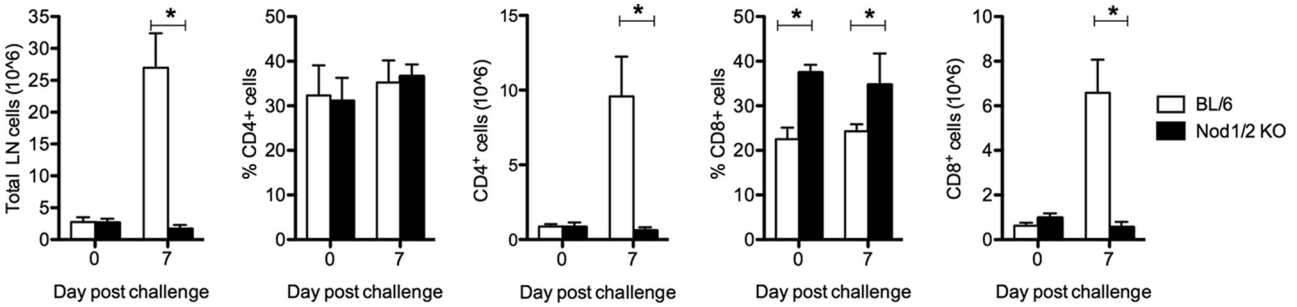

FIG. 7. Impaired expansion of lymphocytes in draining LNs of Nod1/Nod2 KO mice after aerosol challenge with Sterne strain *B. anthracis*. Seven days after primary aerosol challenge, cervical LNs were collected from mice and assessed for total LN cell numbers. Values for unchallenged mice are also included (day 0). Using flow cytometric analysis, the percentage and numbers of $CD4^+$ T cells and $CD8^+$ T cells were also measured. Data are representative of two independent experiments. A Student *t* test was used for statistical analysis, and *P* values of <0.05 are indicated (*). Error bars indicate standard deviations.

numbers as well as $CD4^+$ and $CD8^+$ T-cell numbers. The percentage of $CD4^+$ cells was not significantly different between mouse strains, whereas Nod1/Nod2 KO mice had a higher percentage of $CD8^+$ T cells than WT BL/6 mice (Fig. 7). This difference was also observed in noninfected mice, indicating a baseline difference in the percentage of $CD8^+$ cells in the cervical LNs of Nod1/Nod2 KO mice. Overall, following aerosol challenge Nod1/Nod2 KO mice had significantly fewer LN cells than WT BL/6 mice, indicating an aberrant activation of the immune response. This could be due to decreased proliferation of antigen-specific cells in the draining LNs, as well as decreased infiltration of other immune cells.

Impaired IL-12p70 production by pulmonary cells following exposure to *B. anthracis* spores. Antigen-presenting cell activation, through recognition of pathogen-associated molecular patterns, is involved in initiation of the adaptive immune response. IL-12p70 and IL-10 play roles in the adaptive immune response, promoting either a Th1 or a Th2 response, respectively. To further investigate the role of Nod1/Nod2 recognition of *B. anthracis* and its subsequent role in the adaptive immune response, we examined the production of IL-12p70 and IL-10 from pulmonary cells after spore exposure. The data presented in Fig. 8 indicate that cells from Nod1/Nod2 KO mice produced less IL-12p70 following spore exposure than cells from WT BL/6 mice. The lack of IL-12p70 produced by pulmonary antigen-presenting cells following spore exposure may be involved in the decreased expansion of T-cell populations in the draining LNs observed after in vivo spore challenge (Fig. 7).

DISCUSSION

The current study demonstrates an important role for Nod1/Nod2 recognition of *B. anthracis* for induction of both innate and adaptive immune responses. We show that Nod1/Nod2 was involved in detection of *B. anthracis* after spore infection for the production of proinflammatory mediators, such as IL-1 and CCL5 (Fig. 3), as well as IL-12p70 (Fig. 8). In addition, Nod1/Nod2 was required for optimal induction of protective immunity after aerosol exposure (Fig. 5). Overall, our results illustrate the involvement of Nod1/Nod2 in activation of the immune responses to anthrax infection.

Nod1 and Nod2 are cytosolic host proteins involved in recognition of peptidoglycan substructures of bacteria, including *Bacillus* (17). *B. anthracis* has been shown to replicate in the host cytosol, where Nod1 and Nod2 proteins are expressed (9). After aerosol exposure, *B. anthracis* spores are taken up by

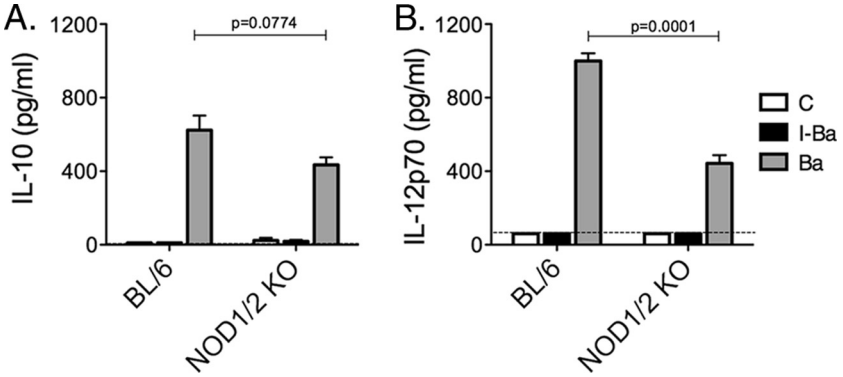

FIG. 8. Nod1/Nod2 recognition of spores by pulmonary cells is required for optimal IL-12p70 production. Pulmonary cells from WT BL/6 and Nod1/2 KO mice were collected and exposed to medium alone (C), irradiated Sterne strain spores (I-Ba), or viable Sterne strain spores (Ba). Twenty-four hours following stimulation, supernatants were collected and assayed for IL-10 (A) and IL-12p70 (B). A Student *t* test was used for statistical analysis, and *P* values are indicated. Error bars indicate standard deviations.

phagocytes in the lung, which become the first defenders against anthrax infection (4, 8, 19, 50). The results obtained in the current study show that macrophages and pulmonary cells responded to spore exposure with the production of proinflammatory cytokines, and responses were significantly reduced in Nod1/Nod2-deficient cells (Fig. 3). The percentage of AMac in Nod1/Nod2 KO mice is lower in 1-h pulmonary adherent cells than in BL/6 mice; however, the total percentage of phagocytes (interstitial macrophages, DC, and AMac) is not significantly different between WT and KO mice. As phagocytic cells other than AMac have been shown to phagocytose spores, it is likely that all cultured phagocytes produced cytokines in response to spore infection. Thus, the difference in the percentage of AMac between WT and Nod1/Nod2 KO mice was unlikely to affect the observed cellular responses. In addition, after 24 h of culture, there was no significant difference in the phenotype of the pulmonary adherent cells (Fig. 2B). The differences between the phenotypes observed at 1 h and 24 h is likely due to the proteolytic cleavage of surface proteins during the lung dissociation step.

Spores that were able to germinate induced significantly higher levels of proinflammatory mediators than after stimulation with irradiated spores, indicating that vegetative bacilli contribute significantly to stimulation through Nod1/Nod2. This was also observed with the production of IL-12p70, a cytokine involved in adaptive immune responses (Fig. 8). Using a different system, it was previously shown that inactivated spores induce NF-κB activation dependent on Nod1/Nod2, although the relevance of these findings was not explored (18). Though spore antigens may be able to induce activation through Nod1/Nod2, our results indicate that intracellular vegetative bacilli induce much higher levels of proinflammatory mediators than inactivated spores (Fig. 1 and 3). The studies presented here were performed using a nonencapsulated, toxigenic strain, though it is unlikely that the spore coat differs significantly from that of the fully virulent strains of *B. anthracis*. As Nod1/Nod2 recognize peptidoglycan motifs on the surface of *B. anthracis*, it is possible that the capsule shields these motifs from recognition by host innate receptors. Future work using an encapsulated strain with in vitro assays is aimed at addressing this issue.

Previous work has implicated anthrax toxins in subverting host immune responses. The majority of these studies have been done using purified toxins with in vitro assays (1, 11, 42, 45, 46). In the current study, in vivo challenges were performed with a toxigenic strain of *B. anthracis*. In these experiments WT BL/6 mice were able to mount a protective immune response, but Nod1/Nod2 KO mice were not. This indicates the important contribution of Nod1/Nod2 signaling during the course of infection to the induction of protective immune responses, even in the face of toxin expression. TLR2 expression was not required during priming responses, as TLR2 KO mice were not susceptible to secondary lethal challenge.

It has previously been shown that murine macrophages produce CCL5 in response to Nod1/Nod2 agonists (57), and in our studies, CCL5 was one of several cytokines affected by the loss of Nod1/Nod2 in response to *B. anthracis* spore exposure (Fig. 3). CCL5 is involved in recruitment of immune cells, aside from neutrophils, to sites of infection (37, 57). Although we examined responses of lung antigen-presenting cells to spore exposure, during inhalational anthrax infection it is likely that proinflammatory cytokine secretion occurs primarily in draining LNs, as phagocytes migrate to the draining LNs after uptake and recognition of *B. anthracis* spores (4, 8). Vegetative bacilli are found in the draining LNs, but not the lungs, early after aerosol spore exposure, and TNF-α levels in draining LNs of infected mice are significantly higher than lung TNF-α levels (34). Further evidence supporting the unlikelihood of chemokine secretion in the lungs is provided by the lack of inflammation and cell infiltration into the lungs of moribund animals suffering from inhalational anthrax (34).

To date, few data are available on the mechanistic involvement of Nod1/Nod2 in induction of adaptive immunity. Previous work by Fritz et al. has shown altered levels of antigen-specific Ig production in Nod1 KO mice after ovalbumin immunization as well as *Helicobacter pylori* infection (15). Our results indicate an impaired induction of antibody responses following pulmonary infection with *B. anthracis* in Nod1/Nod2 KO mice. The susceptibility of Nod1/Nod2 KO mice to secondary challenge was likely due to a failure to mount a rapid anamnestic antibody response (Fig. 5 and 6). Cellular expansion in the draining LNs of Nod1/Nod2 KO mice was severely decreased after primary aerosol challenge (Fig. 7). This could be due to decreased proliferation of antigen-specific cells in the draining LN, as well as decreased infiltration of other immune cells. Proinflammatory cytokine levels after spore infection were significantly decreased in cells from Nod1/Nod2 KO mice, including the chemokines KC and CCL5 (Fig. 1, 3, and 8). CCL5 expression, which can recruit macrophages, T cells, and immature DC to sites of infection, occurs after Nod1/Nod2 signaling (37, 57), and KC is involved in neutrophil recruitment. IL-12p70 induces the differentiation of $CD4^+$ T cells into Th1 cells, and the deficiency of Nod1/Nod2 KO cells in producing IL-12p70 may be involved in the enhanced susceptibility of these mice to secondary challenge. Thus, the decrease in cell numbers in the draining LNs of Nod1/Nod2 KO mice may be attributed to decreased chemokine production and subsequent cell infiltration after aerosol exposure or to the decreased expansion of T cells during the primary stage of infection. Further work is warranted to determine if either of these defective responses is involved in the enhanced susceptibility of Nod1/Nod2 KO mice to secondary anthrax exposure. Additional approaches, such as adoptive transfer of cells, are required due to possible congenital defects in Nod1/Nod2 KO mice, namely, differences in the percentage of basal $CD8^+$ T cells in the cervical LNs.

There is mounting evidence that innate immune recognition of invading pathogens instructs antigen-specific immunity, though the precise mechanisms by which this occurs have not been completely defined (38). Our results indicate that the susceptibility of Nod1/Nod2 KO mice to rechallenge appears to be due to inadequate activation of adaptive immune defenses after primary aerosol exposure. This is likely due to the impaired activation of the innate response, which typically triggers proper activation of the adaptive response. Future and ongoing work is directed toward identifying and characterizing the mechanisms by which Nod1/Nod2 recognition leads to induction of adaptive immune responses.

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