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Nod2-Dependent Th2 Polarization of Antigen-Specific Immunity

Joao Gamelas Magalhaes,2* Jörg H. Fritz,2* Lionel Le Bourhis,* Gernot Sellge,† Leonardo H. Travassos,* Thirumahal Selvanantham,* Stephen E. Girardin,† Jennifer L. Gommerman,* and Dana J. Philpott3*

While a number of microbial-associated molecular patterns have been known for decades to act as adjuvants, the mechanisms and the signaling pathways underlying their action have remained elusive. Here, we examined the unfolding of the adaptive immune response induced by Nod2 in vivo upon activation by its specific ligand, muramyl dipeptide, a component of peptidoglycan. Our findings demonstrate that this bacterial sensor triggers a potent Ag-specific immune response with a Th2-type polarization profile, characterized by the induction of IL-4 and IL-5 by T cells and IgG1 Ab responses. Nod2 was also found to be critical for the induction of both Th1- and Th2-type responses following costimulation with TLR agonists. Importantly, the synergistic responses to Nod2 and TLR agonists seen in vivo were recapitulated by dendritic cells in vitro, suggesting that these cells likely play a central role in the integration of Nod2- and TLR-dependent signals for driving the adaptive immune response. Taken together, our results identify Nod2 as a critical mediator of microbial-induced potentiation and polarization of Ag-dependent immunity. Moreover, these findings affect our understanding of Crohn’s diseases pathogenesis, where lack of Nod2-dependent Th2 signaling in a subset of these patients might explain heightened Th1-mediated inflammation at the level of the intestinal mucosa. The Journal of Immunology, 2008, 181: 7925–7935.

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The repertoire of PRMs is composed of the TREM (triggering receptors expressed on myeloid cells) proteins (1), Siglec (sialic acid-binding Ig-like lectin) (2), C-type lectins (3), and TLR families (4), as well as the recently described families of RIG-like receptors (5) and Nod-like receptors (NLRs) (6).

NLRs represent a family of intracellular PRMs, which have in common a conserved tripartite domain structure, containing a central nucleotide-binding domain termed NACH domain (often also referred to as NOD domain), different N-terminal effector domains depending on the subfamily (PYRIN, caspase recruitment domain (CARD), or baculovirus inhibitior of apoptosis protein repeat (BIR) domain and a yet unclassified effector domain, X) (6), and a C-terminal leucine-rich-repeat domain. This leucine-rich repeat domain is thought to be involved in detection of microbial components and endogenous danger signals, although the exact mechanism is still unknown. The crucial step in NLR activation lies in the oligomerization of the NACH domain, thereby forming an active signaling platform that allows binding of adaptor molecules and effector proteins, ultimately leading to an inflammatory response characterized either by NF-κB-dependent signaling (Nod1 and Nod2) or the generation of IL-1, IL-18, and IL-33 by the action of caspase-1 (Nalps and IPAF) (7).

In addition to the role of NLR proteins in innate immune detection of microbes, polymorphisms in genes encoding certain NLR proteins have been associated with inflammatory barrier disorders. For example, alterations in the genes encoding Nod1 and Nod2 have been recently linked to asthma and Crohn’s disease, respectively, underscoring their relevance for the regulation of inflammatory responses and maintenance of local immune homeostasis (6).

While the key role of Nod proteins in innate immune defense has been established, few studies have examined their contribution to the adaptive immune response. This is despite the fact that the ligand of Nod2, a component of peptidoglycan termed muramyl dipeptide (MDP), has been known for decades for its adjuvant
potential (8). While many TLR agonists appear to favor the development of immune responses with a Th1 polarization profile (9), our recent findings showed that Nod1 activation drives a Th2-biased immune response (10). Because of its implication in Crohn’s disease, our aim was to understand the nature of the immune response generated by specific Nod2 stimulation. Interestingly, we show that similar to Nod1 stimulation, the Nod2-specific agonist MDP elicits priming of Ag-specific T and B cell immunity with a predominant Th2 cell polarization profile. Using Nod2-deficient mice, we were also able to show that the Nod1- and Nod2-mediated microbe-specific sensing specificities for distinct peptidoglycan (PGN)-derived muropeptides independently elicit the priming of Th2 polarized adaptive immunity. Furthermore, by using the broadly used immunopotentiator CFA, which contains a mixture of both TLR and Nod agonists, we demonstrated a critical role of Nod2 for the elicitation of both Th1 and Th2 immunity through synergistic activation of APCs with TLR and Nod agonists. Taken together, our findings show that Nod2 preferentially skews the immune response toward a Th2 profile and, together with TLR signaling, aids in shaping adaptive antimicrobial immunity in vivo. These findings have implications for our understanding of the pathogenesis of Crohn’s disease in those patients who carry Nod2 polymorphisms. Indeed, lack of Nod2-dependent Th2 signals at the level of the intestinal mucosa in these patients might contribute to the unbalanced Th1 response that characterizes this disease.

Materials and Methods

Mice

All animal experiments were approved by the Animal Ethics Review Committee of the University of Toronto. C57BL/6 mice were purchased from Charles River Laboratories. Nod2-deficient mice were obtained from Prof. Jean-Pierre Hugot, and have been backcrossed eight times into the C57BL/6 background (11). The animals were submitted to sanitary control tests and used at the age of 6–8 wk. All animal experiments were performed according to local guidelines.

Reagents

MDP (MurNAc-β-Ala-β-isoGln) was purchased from EMD Biosciences. Synthetic FK156 (D-lactyl-L-Ala-[H9253]-D-Glu-meso-DAP-Gly) was obtained from Fujisawa. PamCSK₄ was purchased from EMC Microcollections, Staphylococcus aureus PGN was obtained from Fluka, and highly purified Salmonella minnesota R595 LPS was obtained from Alexis Biochemicals. Specificity and purity of MDP, FK156, PamCSK₄, and LPS were ensured as previously described (12, 13). OVA devoid of TLR-, Nod1-, and Nod2-activating agonists was purchased from Worthington Biochemical, and the purity was ensured as recently reported (10). CFA and desiccated Mycobacterium butyricum fractions (regrouped as Mycobacterium smegmatis from American Type Culture Collection (ATCC) strains 19420 and 14468) were obtained from Difco.
Peptides were synthesized and purified as previously described (14). The following peptides were used: OVA377-393 (H-SIINFEKL-OH, H2-Kb) and OVA265-280 (HTETWSSNVMEERKIKV-OH, I-Ab), both derived from chicken OVA. Expression plasmids for Nod2 and TLR2 were purchased from Invitrogen. All reagents were free of endotoxin as determined by the Limulus amoebocyte lysate test (BioWhittaker).

Macrophage, dendritic cell (DC), and splenocyte preparations

Bone marrow-derived DCs (BMDCs) were obtained from wild-type (WT) and Nod2−/− mice as previously described (15, 16). In brief, total bone marrow cells depleted for RBC were seeded at 1.5 × 10^6 cells/well in 6-well plates (3 ml/well) in complete culture medium (DMEM supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 mM 2-ME (all purchased from Invitrogen), and 10% FCS (HyClone), supplemented with murine rGM-CSF (R&D Systems; 10 ng/ml). Cells were incubated for 7 days at 37°C and 5% CO2. On day 7, suspended cells and loosely adherent cells were harvested and enriched by magnetic cell sorting with CD11c microbeads (Miltenyi Biotec) according to the manufacturer’s protocol. CD1c-enriched BMDCs were seeded in complete culture medium containing 10 ng/ml of murine rIL-4 (R&D Systems) in 24-well plates at 2 × 10^5 cells/ml and used for stimulation.

Bone marrow-derived macrophages (BMM) were obtained from WT and Nod2−/− mice as described previously (17). In brief, total bone marrow cells were seeded at 5 × 10^5 cells/well in 10-cm dishes in 10 ml of complete culture medium (DMEM supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 50 mM 2-ME (all purchased from Invitrogen), and 10% FCS (HyClone) supplemented with murine rGM-CSF (R&D Systems; 10 ng/ml). Cells were incubated for 7 days at 37°C and 5% CO2. On day 7, suspended cells and loosely adherent cells were harvested and enriched by magnetic cell sorting with CD11c microbeads (Miltenyi Biotec) according to the manufacturer’s protocol. CD1c-enriched BMDCs were seeded in complete culture medium containing 10 ng/ml of murine rIL-4 (R&D Systems) in 24-well plates at 2 × 10^5 cells/ml and used for stimulation.

Freshly isolated splenocytes depleted for RBC were resuspended in complete medium (DMEM supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, and 50 mM 2-ME (all purchased from Invitrogen), and 10% FCS (HyClone) in 24-well plates at 2 × 10^6 cells/ml and used for stimulation. In cell depletion studies, splenocytes were depleted of CD11b+ APCs by negative selection with anti-CD11b microbeads (Miltenyi Biotec) according to the manufacturer’s protocol. The purity of the resulting cell subpopulation was determined by flow cytometry. Cells were seeded in complete medium in 24-well plates at 1 × 10^6 cells/ml and used for stimulation.

Cytokine and chemokine dosage

The concentrations of KC, MIP-2, MCP-1, IL-6, TNF-α, IL-10, IL-12p40, IP-10, and RANTES in sera or cell culture supernatants were determined by ELISA according to the manufacturer’s recommendations (DuoSet from R&D Systems).

Analysis of lymphocyte ratio and proliferation

To determine the ratio of lymphocyte populations of WT and Nod2−/− mice, single-cell suspensions of spleen and thymus of naive mice were prepared by collagenase type 4 digestion (purchased from Sigma-Aldrich; 1 mg/ml) and analyzed by flow cytometry. Cells were resuspended in FACS buffer (1 × PBS containing 0.05% of sodium azide) and incubated with cell culture supernatants of the hybridoma 2.4G2 (ATCC; αCD16/32 Ab) for 15 min on ice to block unspecific staining. Subsequently, the cells were incubated with Abs obtained from BD Biosciences: biotin-labeled Abs specific for CD80, CD86, CD44; PE-labeled Abs specific for CD8αa, B220, CD11b, MHC class II, CD62L, CD40; PerCP-Cy5.5-labeled Abs specific for CD3ε, Gr-1; allphycocyanin-labeled Abs specific for CD4, TCRβ, and CD11c. After staining, cells were washed twice with FACS buffer and analyzed by FACS (FACS Calibur flow cytometer; BD Biosciences). FlowJo software was used for the analysis of the results.

For lymphocyte proliferation, freshly isolated splenocytes (10^6 cells/ml) devoid of RBC were labeled with CFSE (purchased from Invitrogen; 2 μM CFSE with 10^6 cells/ml) for 10 min at 37°C. Cells were washed twice with 1 × PBS and seeded into 96-well plates (2 × 10^5 cells/well) for stimulation with Con A (10 μg/ml; Sigma-Aldrich) or LPS. Cells were stimulated for 4 days in duplicate for 96 h, and proliferation of T and B cells was analyzed by flow cytometry using PerCP-Cy5.5-labeled anti-CD3ε, allphycocyanin-conjugated anti-CD4 and anti-B220, and PE-labeled anti-CD8α Abs (all purchased from BD Biosciences).

Immunizations

For analysis of cytokine and chemokine responses, animals were injected i.p. with endotoxin-free 1 × PBS as control, MDP, or FK156 (both at 0.1 μg/mouse and diluted with 100 μl endotoxin-free PBS). At indicated time points, blood was collected and sera were analyzed by ELISA.

For analysis of Ag-specific T and B cell responses, mice were immunized with a prime-boost protocol at day 0 (prime) and day 28 (boost) s.c. into the left flank or i.p. with a mixture of OVA (100 μg/mouse) and adjuvant (MDP, FK156, or CFA) in a final volume of 100 μl sterile endotoxin-free PBS for both time points. Ten days after immunizations, splenocytes of immunization groups were restimulated ex vivo, and ELISPOT and intracellular FACS analysis were performed. Blood from tail veins was collected at 26 days after the prime and at 26 days after the boost immunization (54 days after the prime), and sera of individual mice were analyzed.
Analysis of T cell responses

For ELISPOT analysis, MultiScreen plates (MAHA S4510 for IFN-γ and MAIP S4510 for IL-4 and IL-5; Millipore) were coated (50 μl/well) with capture Abs (IFN-γ clone R4-6A2 at 1 mg/ml, IL-4 clone 11B11 at 2 mg/ml, and IL-5 clone TRFK5 at 2 mg/ml; all purchased from BD Biosciences) and the assay was conducted as described (14). In brief, freshly isolated splenocytes from immunized mice were resuspended in complete medium (DMEM supplemented with 2 mM l-glutamine, 1 mM sodium pyruvate, 1 x nonessential amino acids, and 50 mM 2-ME (all purchased from Invitrogen) and 10% FCS (HyClone) and plated in ELISPOT plates at 1 x 10^6, 5 x 10^5, and 3 x 10^5 cells per well in the presence of the indicated stimuli in triplicate: human serum albumin (50 mg/ml), OVA (50 mg/ml), OVA257–264 (10 mg/ml), OVA265–280 (10 mg/ml). As positive control, Con A (10 mg/ml) was applied (Sigma-Aldrich). Complete medium was used as negative control. The plates were incubated 20 h for IFN-γ and 48 h for IL-4 and IL-5) at 37°C/5% CO₂. Detection was performed with biotinylated secondary Abs (IFN-γ clone A18.17.24) at 1 mg/ml, kindly provided by K. Lingnau; IL-4 (clone BV26-24G2) at 1 mg/ml; and IL-5 (clone TRFK4) at 1 mg/ml; both obtained from BD Biosciences) and HRP-conjugated streptavidin (R&D Systems). Spots were visualized with 3,3′-diaminobenzidine and NiCl₂ as a substrate. Plates were analyzed with a Bioreader 2000 (BioSys). Results are expressed as the number of IFN-γ-, IL-4-, or IL-5-producing cells per 10⁶ splenocytes ± SD of triplicates. No detectable numbers of cytokine producing cells above medium control were observed when splenocytes were restimulated with a control protein, human serum albumin, or irrelevant epitopes for H2-Kb or I-A₅, ensuring the measurement of the Ag-specific response in all performed assays.

For intracellular FACS analysis, freshly isolated splenocytes from immunized mice were resuspended in complete DMEM medium and plated in 96-well plates at 1 x 10^⁶ and 3 x 10^⁵ cells per well in the presence of the above-indicated stimuli in triplicate. Intracellular FACS analysis was performed using the BD Cytofix/Cytoperm Plus kit (BD Biosciences) by adding the GolgiPlug after 24 h of stimulation for 10 h, before proceeding according to the manufacturer’s protocol. PE-Cy7-labeled Abs against IFN-γ and matching isotype controls were used for intracellular staining in combination with PerCP-Cy5.5-conjugated anti-CD-3e and allophycocyaninlabeled anti-CD4 or anti-CD8α Abs (all purchased from BD Biosciences).

Analysis of B cell responses

Ig isotypes were analyzed by sandwich ELISA comparing serially diluted serum samples with an assay-intrinsic isotype-specific standard (capture Ab: goat anti-mouse Ig (H+L), purchased from SouthernBiotech; IgG1 standard (15H6), obtained from SouthernBiotech; and IgG2c from Bethyl Laboratories; biotinylated detection Abs for IgG1 and IgG2c were purchased from SouthernBiotech). For analysis of Ag-specific Ig isotypes, plates were coated with 10 μg/ml OVA in PBS 0.1 M NaHCO₃ (pH 9.5).
ELISAs were performed by coating the standard capture Ab or OVA to 96-well plates (MaxiSorp; Nalgene Nunc). After washing and incubation with the isotype-control Abs for the standard or the serially diluted serum samples, detection was performed by sequential incubation with biotinylated secondary Abs, HRP-conjugated streptavidin (R&D Systems), and TMB (Sigma-Aldrich). Reaction was stopped by acidification with 2 N H₂SO₄ (Sigma-Aldrich) and absorbance was read at 450 nm.

**RT-PCR analysis**

Total RNA was prepared with the RNeasy Mini kit (Qiagen) according to the manufacturer’s protocol. Purified RNA was reverse-transcribed with SuperScript II RNase H (Invitrogen), and the obtained cDNAs of interest were amplified using primers for murine Nod2 (forward primer, 5'-CTC AGT CTC GCT TCC TCA GTA C-3', reverse primer, 5'-GTT TAA CAG GAC ACG TGC AGG-3') and β-actin (forward primer, 5'-CTT GGG CTC CCT AGG CAC CAA-3', reverse primer, 5'-CTT GAT GTC ACG CAC GAT TTC-3'). The cDNA samples were heat-denatured (95°C for 5 min) and then amplified with 35 cycles, with each comprising successive incubations at 95°C for 40 s, 55°C for 40 s, and 72°C for 60 s. A further extension step was done at 72°C for 5 min. The amplicons (Nod2, 450 bp; β-actin, 580 bp) were size-fractionated onto a 2% agarose gel and stained with ethidium bromide.

**Reporter assays for NF-κB activation**

Human embryonic kidney (HEK) 293T cells (ATCC) cultured in complete medium (DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, and 50 mM 2-ME (all purchased from Invitrogen) and 5% FCS (HyClone) were seeded into 24-well plates at a density of 10⁵ cells/ml, and transfections with various expression plasmids were conducted as described previously (13). In brief, cells were transfected overnight with 1% of FuGENE 6 transfection reagent (Roche Diagnostics) with 75 ng of the β-galactosidase expression plasmid and 75 ng of the reporter plasmid Igk-luciferase alone or plus the following vectors: 0.5 ng Nod2 or 50 ng TLR2. The pcDNA3.1 vector was used to balance the DNA concentration. Indicated stimuli were added shortly after transfection of cells. NF-κB-dependent luciferase expression was examined after 20 h of incubation and normalized as a ratio to β-galactosidase.
activity. Assays were performed in triplicate and data represent means ± SD of a representative experiment.

**Statistical analysis**

The results are given as means ± SD. Statistical analysis was performed with Graphpad Prism 5 software using either a Student's t test or a Mann-Whitney U test. A p-value of <0.05 was considered significant.

**Results**

**Induction of Th2 immunity by Nod2-specific stimulation**

To assess the impact of Nod2-specific activation for the induction of innate and adaptive immunity, we first analyzed the cytokine and chemokine profile indicative for Th1/Th2 polarization upon i.p. administration of the Nod2-specific agonist MDP in WT and Nod2−/− mice. We observed that MDP, in a Nod2-dependent manner, potently triggers the rapid release of proinflammatory cytokines and chemokines such as IL-6 and KC into the bloodstream 2 h after injection, which returned nearly to steady-state levels 24 h after application (Fig. 1). In contrast, i.p. injection of the Nod2 agonist failed to induce the systemic release of IL-12p70 as well as IP-10 and MIG (data not shown), mediators critical for Th1 polarization (18). However, injection of MDP triggered the substantial release of the Th2 immunity promoting chemokine MCP-1 (19) in a Nod2-dependent manner (Fig. 1).

To further investigate if Nod2-mediated PGN sensing elicits priming of polarized Ag-specific T and B cell immunity, WT and Nod2−/− mice were immunized with the model Ag OVA in combination with the Nod2 agonist MDP. Strikingly, WT mice immunized with a mixture of OVA and MDP elicited a profound Th2 response with high numbers of IL-4- and IL-5-producing T cells when restimulated with OVA protein or the MHC class II epitope OVA265–280, as compared with mice immunized with OVA only (Fig. 1). However, stimulation with the MHC class I epitope OVA257–264 did not yield substantial numbers of cytokine-producing CD8+ T cells over controls. Importantly, the Th2 response elicited upon coinjection of OVA + MDP was strictly Nod2-dependent, as no significant production of Ag-specific IL-4- and IL-5-producing cells over background levels could be detected in Nod2−/− mice. Moreover, we did not detect Ag-specific IFN-γ- and IL-17A-producing CD4+ and CD8+ T lymphocytes upon immunizations with OVA + MDP (data not shown).

Analysis of Ag-specific B cell immunity revealed the elicitation of OVA-specific Igs after immunizations with OVA + MDP in a Nod2-dependent manner (Fig. 3). After prime and boost immunizations, Nod2-specific stimulation led to the predominant elicitation of OVA-specific IgG1, as no significant OVA-specific IgG2c (Fig. 3), IgG2b, or IgG3 titters (data not shown) were detected, demonstrating that Nod2-mediated PGN recognition elicits Ag-specific T and B cell immunity with a predominant Th2 polarization profile. By comparing the recently described immunostimulatory capacity of the Nod1 agonist FK156 (10) (12) with the Nod2 agonist MDP, we revealed that the adjuvant activity of FK156 for priming Ag-specific immunity remained unaltered in Nod2-deficient mice (Figs. 2 and 3), demonstrating that the Nod1- and Nod2-mediated microbe-specific sensing for distinct PGN-derived mucopeptides independently elicits the priming of Th2-polarized adaptive immunity.

A thorough analysis of Nod2-deficient mice revealed that animals are outwardly healthy and that WT and Nod2−/− mice display equal amounts of basal serum Ig and IgG isotypes (data not shown). Additionally, FACS analysis of cell populations isolated from thymus and spleen revealed normal frequencies of B cells, dendritic cells (DCs), granulocytes, CD4+ T cells, and CD8+ T cells (data not shown). Moreover, since we observed expression of Nod2 mRNA in spleen-derived T, B, and dendritic cells, we ensured that Nod2 deficiency does not affect the activation potential of T and B cells (data not shown), suggesting that the altered onset of Ag-specific adaptive immunity in Nod2-deficient mice is not due to intrinsic defects of T and B cells.
Nod2 is required for optimal elicitation of T and B cell responses in a model of CFA immunization

Upon microbial intrusion, host cells recognize multiple MAMPs through a broad set of PRMs including TLRs and NLRs (20). Thus, to further study the impact of Nod2-mediated PGN recognition for the elicitation of Ag-specific host responses in vivo, we performed immunization experiments by utilizing the standard adjuvant CFA, containing heat-killed *M. butyricum*, which stimulates signaling downstream of TLR4, TLR9, and Nod1 (21), but also of TLR2 and Nod2 when tested in in vitro transfection assays (Fig. 4A). In WT animals, immunizations with OVA emulsified in CFA led to a mixed response, inducing OVA-specific T cells producing.

**FIGURE 6.** Activation of macrophages and DCs independent of Nod2. A, BMMs or (B) BMDCs from WT (filled bars) and Nod2−/− (open bars) animals were stimulated for 24 h with medium only, LPS (1 μg/ml) or Pam3CSK4 (100 ng/ml). Production of KC, MIP-2, IL-6, IL-12p40, IP-10, and RANTES was measured by ELISA. Data shown are representative of two independent experiments. The mean ± SD is given.
IFN-γ and IL-4 (Fig. 4B). In contrast, we observed that Nod2 deficiency resulted in a profound alteration of OVA-specific Th1 and Th2 immunity after immunization with OVA + CFA (Fig. 4, B and C). ELISPOT analysis revealed a markedly reduced frequency of IL-4- and IFN-γ-producing T cells upon restimulation with OVA protein, the MHC class I-restricted OVA-derived epitope OVA<sub>257–264</sub> or the MHC class II-restricted OVA-derived epitope OVA<sub>265–280</sub> (Fig. 4B). Additionally, intracellular FACS analysis revealed a significant reduction of OVA-specific IFN-γ-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells in Nod2<sup>−/−</sup> mice (Fig. 4C). To test whether the altered T cell priming correlates with reduced B cell immunity, we determined the Ag-specific Ab production in WT and Nod2-deficient mice after immunizations with OVA emulsified in CFA. In WT animals, prime-boost immunizations with OVA emulsified in CFA led to strong induction of OVA-specific IgG<sub>1</sub> and IgG<sub>2c</sub> Abs (Fig. 4D). However, a significantly lower production of OVA-specific IgG<sub>2c</sub> as well as IgG<sub>1</sub> Abs was observed in Nod2-deficient animals (Fig. 4D), demonstrating that the potent CFA-induced adjuvant effect mediated by mycobacterial cell wall constituents requires Nod2-mediated PGN recognition to induce a full-blown Ag-specific T and B cell response.

**Nod2 cooperates with TLRs for the increased production of Th1-polarizing mediators**

Activation of specialized APCs, such as DCs and macrophages, is thought to constitute the central platform that integrates microbe-derived signals and Ag specificity for the initiation of adaptive T and B cell immunity. Their immunoregulatory role relies on sensing of specific MAMPs by PRMs and/or receptors that bind mediators that are produced in response to microbial intrusion, which collectively modulate the function of APCs for the regulation of distinct immune effector arms (18). We therefore investigated whether Nod2 deficiency affects the capacity to directly activate DCs by comparing the stimulatory potential of various defined MAMPs, including the Nod2 agonist MDP, the TLR2 agonist Pam<sub>3</sub>C<sub>SK<sub>4</sub></sub>, the TLR4 agonist LPS, as well as a commercially available preparation of PGN from S. aureus, known to contain TLR2-activating molecules and Nod2-activating muropeptides (22).

Simultaneous stimulation of BMDCs from WT mice with MDP and Pam<sub>3</sub>C<sub>SK<sub>4</sub></sub> or LPS led to synergistic release of IL-6, as well as the Th1-promoting cytokine IL-12p40 (Fig. 5A). In contrast, the capacity of the Nod2 agonist MDP to synergize with TLR2 and TLR4 agonists for enhanced cytokine production was completely abolished in Nod2<sup>−/−</sup> BMDCs (Fig. 5), indicating that the stimulatory effects of MDP for the activation of BMDCs are abolished in Nod2-deficient animals. Importantly, equal amounts of the indicated cytokines were released upon stimulation of WT and Nod2<sup>−/−</sup> BMDCs with low doses of the TLR2 or TLR4 agonists (Fig. 5). Similarly, equal amounts of KC, MIP-2, IL-6, RANTES, IL-10, IL-12p40 (Fig. 6A), and IL-6, RANTES, IL-10, IP-10, and IL-12p40 (Fig. 6B) were produced when WT and Nod2-deficient BMMs or BMDCs, respectively, were stimulated with a high dose of the purified TLR2 agonist Pam<sub>3</sub>C<sub>SK<sub>4</sub></sub> or the TLR4 agonist LPS. These results indicate that deletion of Nod2 selectively abrogates MDP-mediated effects, but does not impact on the capacity of purified TLR2 and TLR4 agonists to activate BMDCs and BMMs.

To further investigate the role of Nod2 deficiency in APCs, we investigated the stimulatory effects of a commercially available preparation of PGN from S. aureus, containing Nod2-activating muropeptides, as well as lipopeptides and lipoteichoic acids activating TLR2 (22). Although, equal amounts of TNF-α were produced upon stimulation of BMDCs or total splenocytes from WT and Nod2-deficient animals, we observed a reduction of IL-10 release in Nod2<sup>−/−</sup> cells upon PGN stimulation (Fig.
Additionally, the release of the Th1 driving cytokine IL-12p40 by BMDCs upon stimulation by PGN was found to be significantly lower in Nod2<sup>−/−</sup> BMDCs (Fig. 7A). In accordance with this, we observed that total splenocytes from Nod2<sup>−/−</sup> animals have a markedly reduced capacity to produce IFN-γ secretion in response to PGN stimulation compared with cells from WT animals (Fig. 7B). Finally, we demonstrate that PGN induced production of IL-10 and IL-12p40 by total splenocytes was diminished to background levels when CD11b<sup>+</sup> cells have been depleted (Fig. 7C), suggesting that upon PGN stimulation, Nod2 expression in CD11b<sup>+</sup> DCs and macrophages among the total splenocyte population is critical for the induction of the Th1 polarizing cytokine IL-12. Taken together, these results indicate that in the presence of TLR and Nod2 agonists, ablation of Nod2 impairs the release of Th1-polarizing factors as well as the resulting IFN-γ production, demonstrating a key role of Nod2 for the elicitation of antimicrobial adaptive immunity.

**Discussion**

The PRM families of NLRs and TLRs are critical innate immune sensors of microbial products and danger signals that play a key role for the onset of first-line host responses to fend off microbial intrusion (6, 23). Recent evidence has suggested that innate immune detection of microbes by PRMs also provides essential signals for the elicitation and polarization of Ag-specific T and B cell responses (24). Therefore, we examined the role of the NLR family member, Nod2, in the generation of adaptive immune responses. Our observations clearly demonstrate that Nod2 instructs the onset of Ag-specific T and B cell immunity in vivo. We show herein that the Nod2 agonist MDP elicits priming of adaptive immunity with a predominant Th2 cell polarization profile, suggesting a critical role of Nod2 in the regulation of barrier immune responses. Moreover, we demonstrate that animals deficient in Nod2 mount severely altered adaptive immune responses to T and B cell-dependent Ag given in the standard adjuvant CFA. Finally, our study demonstrates a critical role of Nod2 to prime Th1 immune pathways by synergistic activation of DCs with TLR agonists, suggesting that Nod2 plays a central role in shaping systemic adaptive antimicrobial immunity in vivo.

The data presented herein demonstrate that specific stimulation of Nod2 elicits priming of Ag-specific T and B cell immunity with a predominant Th2 polarization profile. The specificity of this response is highlighted by the finding that Nod2-deficiency does not alter the recently described adjuvant effect of the Nod1 agonist FK156, demonstrating that Nod1 and Nod2 recognize distinct motifs from PGN for the induction of first-line host as well as Ag-specific immune responses. By performing a detailed characterization of the key mediators released upon specific Nod2 stimulation in vivo, we observed a substantial release of proinflammatory mediators such as IL-6, as well as KC, known to trigger recruitment of granulocytes (25). Additionally, we observed that Nod2-specific stimulation induces a rapid and marked release of MCP-1, known to promote Th2 immunity in infectious and allergic disease models (19), suggesting a key role for this inflammatory chemokine to prime Ag-specific Th2 immunity upon Nod2-mediated PGN detection.

Nod2-mediated PGN recognition elicits a Th2 response but fails to prime Th1 and Th17 immunity, which is similar to what we observed previously with Nod1-mediated adaptive immune responses (10). In contrast to Nod1 and Nod2, however, activation by most TLRs, such as TLR3, TLR5, TLR7, TLR8, TLR9, and TLR11, results in a dominant Th1-type response, while in the case of TLR4, a mixed Th1/2 polarization with a propensity for Th1 immunity is observed (26, 27). Among the TLR family, only TLR2 stimulation does not drive a prevailing Th1 response, but rather promotes polarization of Th2 immunity (28, 29). Therefore, the conclusion can be drawn that Nod1- and Nod2-mediated innate immune detection display complementary rather than overlapping functions with most TLRs for the polarization of adaptive immune responses. It will be of general interest to investigate if other NLR family members known to be involved in detection of microbial and danger signals, such as Nalp3 (30), can polarize adaptive immune responses similarly to Nod1 and Nod2. Indeed, recent reports suggest that the widely used Th2-polarizing vaccine adjuvant aluminum hydroxide instructs immune responses by signaling through Nalp3 (31). However, whether the Th2 polarizing activity of aluminum hydroxide solely relies on the activity of this NLR family member awaits further confirmation.

MDP has been identified as the minimal microbial-derived component of CFA required for exerting delayed-type hypersensitivity responses (32) long before its activity was assigned to rely on Nod2 function (33). Utilizing genetically modified animals deficient for Nod2, our data now indeed highlight that the classical adjuvant preparation CFA requires Nod2-mediated PGN recognition to instruct the onset of type 1 and type 2 immunity in vivo. Immunization of Nod2-deficient animals with T cell-dependent Ags given in CFA yielded markedly lower frequencies of Ag-specific IFN-γ-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cells as well as specific IgG2c and IgG1 Abs, which would likely contribute to the successful clearance of pathogens from the infected host. In support of these in vivo findings, our study further demonstrated that Nod2-mediated PGN recognition was essential to cooperate with TLRs for successful elicitation of Ag-specific Th1 immune responses. Cooperative stimulation of DCs or macrophages by Nod2 and TLRs for the production of the Th1-polarizing cytokine IL-12 was abrogated in Nod2-deficient cells, suggesting that synergistic priming of DCs and/or macrophages by Nod2 and TLRs is critical for the elicitation of Th1-promoting factors and the in vivo onset of Ag-specific T and B cell immunity. In this study, however, we cannot fully exclude a role of Nod2 for priming and regulating Ag-specific T and B cell immunity in cells other than DCs and/or macrophages (34), a possibility that in future experiments will be addressed by employing tissue-specific knockout animals.

Although the mycobacterial cell wall fraction used in the CFA preparation has also been shown to activate TLR2, TLR4, TLR9, and Nod1 (10, 21, 35), we observed that Nod2 deficiency cannot be overcome by stimulation of these additional PRMs. Similarly, we reported recently that Nod1 deficiency cannot be overcome by Nod2 stimulation both in terms of adaptive responses (10) but also in bacterial detection (36, 37). This observed lack of Nod2 to compensate for Nod1 and vice versa may indeed reflect a unique and complementary rather than overlapping functions of these individual molecules that come into play for the priming of the immune response. Furthermore, these observations are in accordance with findings that Nod1 cannot compensate for the Nod2 functional defects in Crohn’s disease patients.

Functional crosstalk between Nod2 and other PRMs such as TLRs is likely to be critical for the balance of immune effector arms for the regulation of systemic as well as local immune responses (34). Importantly, a key role in balancing local immune homeostasis has been assigned to Nod2, because polymorphisms in the gene encoding this PRM were shown to be linked to increased susceptibility of the inflammatory bowel...
disorder Crohn’s disease (38), which is characterized by dysregulated and exaggerated intestinal Th1 and Th17 immune responses (39). Although Nod2 stimulation has been shown to be critical for dampening intestinal TLR-driven Th1 immune responses (40, 41), the mechanisms behind these observations remain largely elusive. Moreover, our findings are in contrast to these results since deletion of Nod2 in our study selectively abrogated MDP-mediated effects, but did not affect the purity of purified TLR2 and TLR4 agonists to activate peripheral APCs such as macrophages and DCs. Importantly, these observations are in good agreement with reports analyzing peripheral DCs and macrophages derived from a different Nod2-deficient mouse strain (42, 43) as well as studies with APCs of human origin (13, 44, 45). Taken together, these findings suggest that, systemically, the cooperative stimulation of Nod2 and TLRs to synergize for cytokine production by peripheral DCs translates into enhanced systemic Ag-specific immune responses in vivo.

In conclusion, a growing body of evidence proposes that resident intestinal myeloid cells display an “anergy-like” phenotype, which is, at least in part, driven by the local production of the Th2-type mediators (34, 46, 47). Therefore, a defective Th1 type, which is, at least in part, driven by the local production of IL-10 to promote IL-12 production by dendritic cells. J. Exp. Med. 201: 1899–1903.


