View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Elsevier - Publisher Connector

Nod1-Mediated Innate Immune Recognition of Peptidoglycan Contributes to the Onset of Adaptive Immunity

Jörg H. Fritz,[1] Lionel Le Bourhis,[1] Gernot Sellge,[4] Joao Gamelas Magalhaes,[1] Hafida Fsihi,[5] Thomas A. Kufer,[4] Cathy Collins,[2] Jérôme Viala,[6] Richard L. Ferrero,[7] Stephen E. Girardin,[3] and Dana J. Philpott[1,*]
[1] Department of Immunology
[2] Department of Molecular Genetics
[3] Department of Laboratory Medicine and Pathobiology
Medical Sciences Building, University of Toronto, 1 King's College Circle, M5S 1A8 Toronto, Ontario, Canada
[4] Unité de Pathogénie Microbienne Moléculaire, Institut Pasteur and INSERM U786, Paris 75724 Cedex 15, France
[5] Unité de Recherche de Mycologie Moléculaire, Institut Pasteur, 25-28 Rue du Dr Roux, 75724 Paris, France
[6] Department of Paediatric Gastroenterology and Nutrition, Assistance Publique Hôpitaux de Paris, Hôpital Robert Debré, 75935 Paris Cedex 19, France
[7] Department of Microbiology, Monash University, Clayton 3800, Victoria, Australia
*Correspondence: dana.philpott@utoronto.ca
DOI 10.1016/j.immuni.2007.03.009

SUMMARY

Recent evidence has suggested that signals other than those from Toll-like receptors (TLRs) could contribute to the elicitation of antigen-specific immunity. Therefore, we examined the role of the Nod-like receptor (NLR) family member, Nod1, in the generation of adaptive immune responses. Our findings show that innate immune sensing of peptidoglycan by Nod1 is key for priming antigen-specific T cell immunity and subsequent antibody responses in vivo. Nod1 stimulation alone was sufficient to drive antigen-specific immunity with a predominant Th2 polarization profile. In conjunction with TLR stimulation, however, Nod1 triggering was required to instruct the onset of Th1 and Th2 as well as Th17 immune pathways. Cells outside of the hematopoietic lineage provided the early signals necessary to orchestrate the development of Nod1-dependent immune responses. These findings highlight Nod1 as a key innate immune trigger in the local tissue microenvironment that drives the development of adaptive immunity.

INTRODUCTION

Engagement of pattern recognition molecules (PRMs) with their microbial ligands induces specific downstream signaling events and thereby provides immediate first-line protection of the host from invading pathogens. This is mediated by a number of innate systems including the activation of the complement pathway, phagocytosis of microbes, the release of direct antimicrobial mediators, and the production of cytokines, chemokines, and interferons that collectively instruct mechanisms to combat infection. Several PRMs have been characterized in a number of different hosts, such as pathogen-resistance proteins (R-proteins) in plants, the *Drosophila* Toll protein, and Toll-like receptors (TLRs) in *Caenorhabditis elegans* and mammals. During the last decade, many microbial motifs sensed by TLRs have been deciphered, and their impact on the induction of first-line host responses has been demonstrated (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2005).

Depending on the cell type and molecule, the localization of TLRs is restricted to the cell surface or specialized cell organelles, especially the endolysosomal compartment. Conversely, bacteria use a variety of strategies to escape the endolysosomal compartment (Cossart and Sansonetti, 2004), which suggests that the cytosolic location of a pathogen may provide optimal protection from immune recognition and response. Recent findings now indicate that vertebrates have also evolved strategies to recognize these bacteria inside the cell via members of the Nod-like receptor (NLR) protein family (Fritz et al., 2006; Inohara et al., 2004; Ting and Davis, 2005), Nod1 and Nod2, in particular, representing a means of cytosolic surveillance. The relevance of Nod proteins for antimicrobial immunity has recently been demonstrated by studies with Nod-deficient animals. Nod1-deficient mice have a higher susceptibility toward *Helicobacter pylori* infection (Viala et al., 2004), and diminished clearance of *Listeria monocytogenes* is observed in Nod2-deficient mice (Kobayashi et al., 2005). Increasingly, studies implicate Nod1 and/or Nod2 in the antimicrobial response to a variety of different bacteria through the recognition of distinct peptidoglycan (PGN) motifs, leading to subsequent downstream signaling events by activation of transcription factors such as nuclear factor κB (NF-κB) and activator protein (AP)-1 (Fritz et al., 2006; Kufer et al., 2005).

The concept that successful antigen-specific immunity is dependent on innate immune recognition systems has been supported by the fact that PRM-mediated detection

of microbe-associated molecular patterns (MAMPs), such as stimulation of TLRs by their cognate agonists, provides signals for priming antigen-specific Th1 and Th2 immune responses (Dabbagh and Lewis, 2003; Johnson et al., 2003). In addition, animals deficient in MyD88, a common TLR signaling protein, have altered antigen-specific T and B cell immunity (Pasare and Medzhitov, 2005; Schnare et al., 2001). More recently, however, these findings were challenged because mice genetically deficient in both MyD88 and TRIF, and thereby completely lacking the ability to signal downstream of TLRs (Hoebe et al., 2003; Janssen et al., 2006; Yamamoto et al., 2003), mount unaltered antibody responses to T cell-dependent antigens, given in the context of adjuvant preparations (Gavin et al., 2006; Nemazee et al., 2006). These intriguing findings suggest, therefore, that innate immune recognition systems other than TLRs contribute to the onset of antigen-specific immune responses (Wickelgren, 2006). Following this idea, we set out to investigate the instructive potential of Nod1-mediated PGN recognition on priming of adaptive immune responses.

Our findings uncover a key role for Nod1 in priming antigen-specific responses in vivo. By using the common adjuvant, complete Freund's adjuvant (CFA), in typical immunization experiments with T cell-dependent antigen, we show the critical role of Nod1 triggering, acting in synergy with TLRs, to prime Th1 as well as T helper 17 cell (Th17) immune pathways in vivo. These results are further highlighted by the observation that the antigen-specific response to *H. pylori*, a bacterium that was shown to trigger Nod1 signaling in gastric epithelial cells (ECs) (Viala et al., 2004), is diminished in Nod1-deficient animals. When acting alone, Nod1 agonist elicits priming of antigen-specific T and B cell immunity with a predominant Th2 cell polarization profile. Even though dendritic cells (DCs) are thought to be key players in integrating microbial and antigen signals to instruct adaptive immune responses, wild-type DCs and hematopoietic cells in general could not compensate for Nod1 deficiency as revealed by reconstitution experiments. Taken together, these findings highlight Nod1 as a key component of innate immune signaling responsible for priming of antigen-specific responses in vivo. Consistent with recent findings (Kapsenberg, 2003; Stetson et al., 2004), our results also underscore the importance of the tissue microenvironment, which includes nonhematopoietic cells such as ECs, fibroblasts, and stromal cells, in conditioning DCs and thereby impacting on the generation of the antigen-specific adaptive immune response.

RESULTS

Nod1 Is Required for Optimal Generation of T Cell Responses

To analyze the role of Nod1-mediated PGN recognition for the onset of adaptive immunity, we first applied the standard immunopotentiator CFA, containing heat-killed *Mycobacteria*, which are sensed by TLRs as well as Nod1 (see Figures S1A–S1C in the Supplemental Data available online). As expected in wild-type (WT) animals, immunizations with the T cell-dependent antigen ovalbumin (OVA) and CFA led to a mixed response, inducing antigen-specific T cells producing IFN-γ, IL-4, and IL-17A. In contrast, markedly altered Ag-specific immunity was observed in spleen and draining lymph nodes of Nod1-deficient mice after immunization with CFA (Figures 1A–1C). Intracellular FACS and ELISPOT analysis revealed a significant reduction of OVA-specific IFN-γ-producing $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in Nod1-deficient mice (Figures 1A and 1B). In addition, reduced numbers of IL-4-producing T cells upon restimulation with OVA protein, OVA(257-264), or OVA(265-280) were observed in Nod1-deficient mice after immunization with OVA and CFA (Figure 1B), demonstrating that the elicitation of antigen-specific IFN-γ- and IL-4-producing $CD4^+$ and $CD8^+$ T cells is severely diminished in Nod1-deficient animals. Moreover, markedly less IFN-γ and IL-17A was found in supernatants from splenocytes and cells of draining lymph nodes restimulated with OVA or OVA(265-280) of Nod1-deficient animals (Figures 1B and 1C), indicating a defect in priming of $CD4^+$ Th17 cells. Taken together, these results revealed that the potent CFA-induced adjuvant effect mediated by mycobacterial cell-wall constituents required Nod1-mediated PGN recognition to instruct the onset of Th1 and Th2 as well as Th17 immune pathways.

Optimal Antibody Responses Require Nod1 Triggering

To test whether the altered T cell priming correlated with impaired B cell immunity, we determined the antigen-specific antibody production in WT and Nod1-deficient mice after subcutaneous immunizations with OVA emulsified in CFA. Animals immunized with OVA and CFA showed that Nod1-deficient mice produced markedly lower amounts of OVA-specific antibodies than the WT counterparts. Although slightly less antigen-specific IgG1 was observed, the amount of IgG2b, IgG2c, and IgG3 were found to be significantly lower in Nod1-deficient animals (Figure 2B), demonstrating that Nod1-mediated PGN recognition of mycobacteria present in CFA is critical for the elicitation of antigen-specific T and B cell immunity. Importantly, Nod deficiency did not impact on the general ability of the mice to prime adaptive immune responses, as shown by the fact that immunizations with OVA in the sole oil-in-water emulsion incomplete Freund's adjuvant (IFA) led to similar amounts of antigen-specific IgG1, IgG2b, IgG2c, and IgG3 as WT mice (Figure 2A). Similar results were obtained by adsorbing OVA to aluminium hydroxide (data not shown).

Because we demonstrated that Nod1-deficient mice have a higher susceptibility toward *H. pylori* infection (Viala et al., 2004), we hypothesized that the altered bacterial clearance in these mice, at least in part, might result from a diminished antigen-specific response. WT and Nod1-deficient mice were challenged orally with *H. pylori*, and sera were collected 1 month after infection. Analysis of the antibody response of mice to *H. pylori* urease, a major protein of the bacterium, revealed equivalent amounts

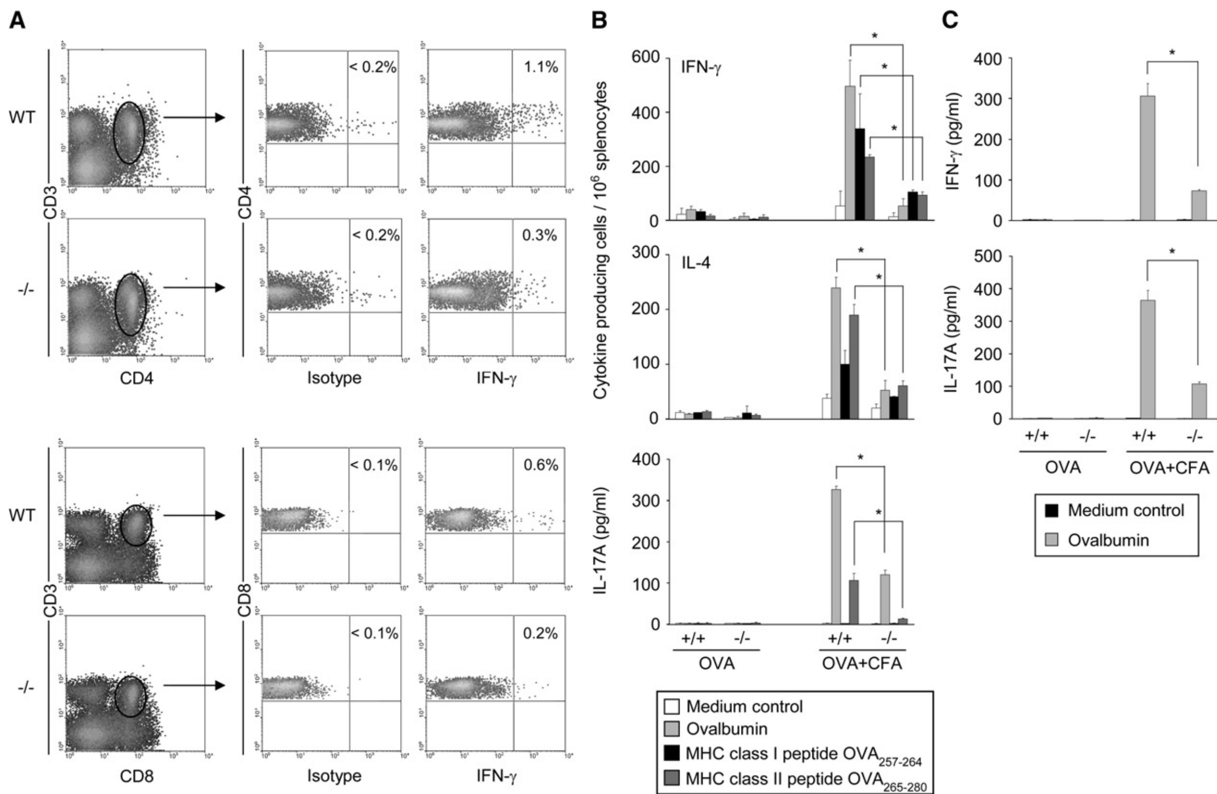

Figure 1. Altered Antigen-Specific T Cell Responses in Nod1-Deficient Mice Immunized with CFA

Immunizations of wild-type (+/+) and Nod1-deficient mice were carried out with ovalbumin (OVA) or OVA emulsified in complete Freund's adjuvant (CFA). On day 10 after each immunization, pools of splenocytes (A and B) or draining lymph nodes (C) from immunization groups (n = 4) were restimulated with medium as control, OVA, an MHC class I-restricted ($H\text{-}2K^b$, OVA(257-264)) or an MHC class II-restricted ($I\text{-}A^b$, OVA(265-280)) epitope derived from OVA.

(A) The frequencies of Ag-specific $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells producing IFN-γ after restimulation with OVA protein were determined by intracellular FACS analysis. The percentages of gated IFN-γ-positive cells are indicated. Data shown are representative of two independent experiments. A representative experiment is shown.

(B) The numbers of Ag-specific $CD4^+$ and $CD8^+$ T cells producing IFN-γ or IL-4 per million splenocytes were determined by ELISPOT analysis, and production of IL-17A in supernatants was determined by ELISA. Data shown are representative of three independent experiments. The mean ± standard deviation of a representative experiment is given. The asterisk indicates a significant difference ($p < 0.05$).

(C) Ag-specific IFN-γ and IL-17A production of total draining lymph node cells from immunized mice was analyzed by ELISA. The mean ± standard deviation of triplicates is given.

of IgG1 but markedly diminished IgG2c titers in Nod1-deficient animals compared to WT mice, indicating a diminished type 1 immune response (Figure 2C) in Nod1-deficient animals. Taken together, these data indicate that in the context of bacterial challenge, Nod1-mediated PGN recognition is critical for instructing adaptive immune responses, which likely contribute to successful bacterial clearance from the infected host.

Induction of Adaptive Immunity by Nod1-Specific Stimulation

Mycobacterial extracts of CFA as well *H. pylori* are recognized by multiple innate recognition systems including TLRs and Nod1. Thus, to investigate how Nod1-mediated PGN sensing elicits priming of antigen-specific immunity, we next tested whether Nod1 stimulation alone is able to prime adaptive immunity in vivo. WT and Nod1-deficient mice were immunized subcutaneously with the Nod1-specific agonist FK156 in conjunction with OVA. We ascertained the purity of all compounds used for immunization experiments to ensure that the observed responses are solely due to specific stimulation of Nod1 (Figures S2A–S2F). Compared to the effect of immunizations with OVA only, WT mice given a mixture of OVA and FK156 elicited markedly higher numbers of IL-4- and IL-5-producing $CD4^+$ T cells upon restimulation with OVA protein or OVA(265-280) (Figure 3A). Interestingly, stimulation with OVA(257-264) did not yield substantial numbers of cytokine-producing $CD8^+$ T cells over controls. Moreover, immunizations with OVA and the Nod2-specific agonist muramyl-dipeptide (MDP) or OVA and FK156, respectively, did not induce antigen-specific $CD4^+$ and $CD8^+$ T lymphocytes producing IFN-γ or IL-17A (data not shown). Comparable results were obtained when animals were immunized intraperitoneally (Figure 3B). As for priming of antigen-specific T cells, we observed that

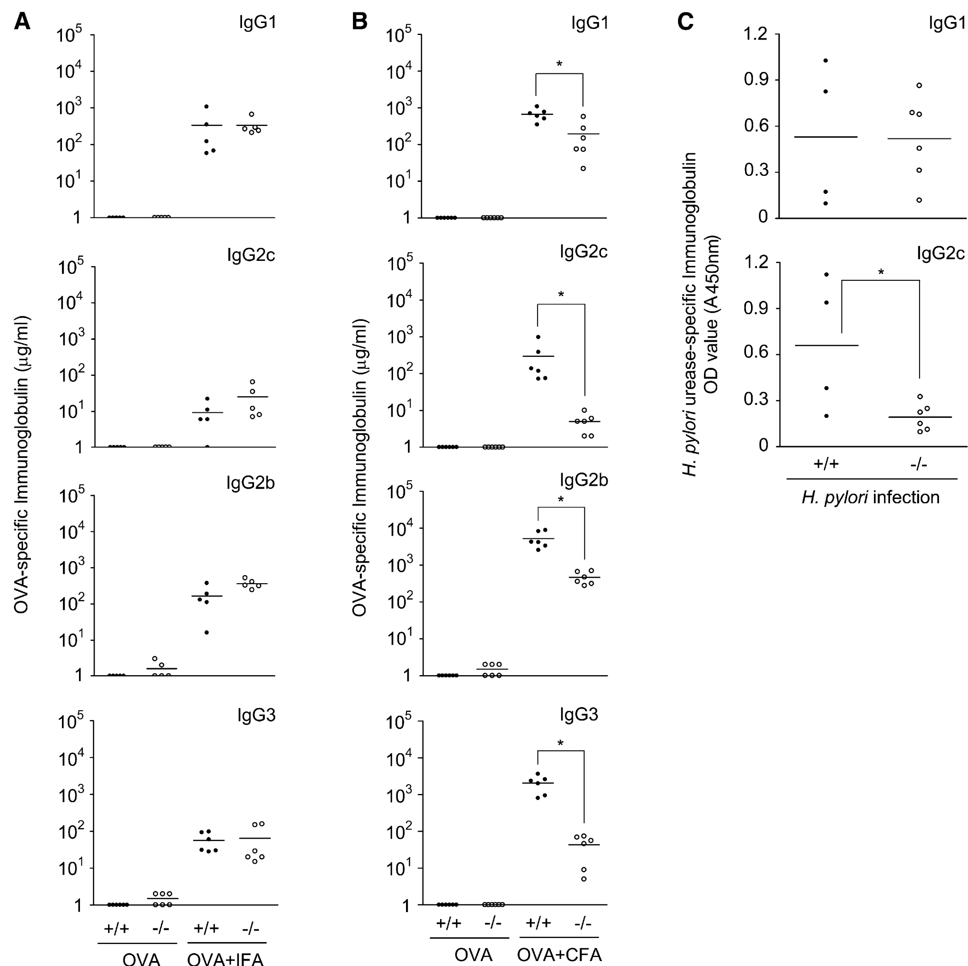

Figure 2. Altered Antigen-Specific Immunoglobulin Production in Nod1-Deficient Mice upon Immunization with CFA and Bacterial Infection

Wild-type (+/+, represented by filled circles) and Nod1-deficient (−/−, represented by empty circles) mice were immunized with ovalbumin (OVA) or OVA emulsified in incomplete Freund's adjuvant (IFA) (A) or complete Freund's adjuvant (CFA) (B), or infected with *H. pylori* (C). Serum from single mice was taken on day 26 after immunization or infection, and Ag-specific IgG1, IgG2b, IgG2c, and IgG3 production was analyzed by ELISA. The bar represents the mean of each cohort (n = 4–6). The asterisk indicates a significant difference ($p < 0.05$).

in WT mice, OVA-specific immunoglobulins (Ig) were induced after immunizations with FK156. Nod1-deficient animals displayed a severe deficiency in the production of antigen-specific IgG1, which was the predominant isotype induced (Figure 3C). Although a second injection of WT animals with OVA and FK156 further enhanced the antigen-specific antibody production, this mixture still failed to do so in Nod1-deficient mice (Figure 3C), demonstrating that FK156 elicits priming of a $CD4^+$ T helper cell immune response with a Th2 polarization profile and subsequent IgG1 production in a Nod1-dependent manner. By comparing the immunostimulatory capacity of FK156 with the Nod2 agonist MDP, we observed the induction of similar antigen-specific IL-4- and IL-5-producing T cells and IgG1 specific for OVA (Figures 3A and 3C), revealing that the Nod2-mediated T cell priming by MDP remains unaltered in Nod1-deficient animals. No significant OVA-specific IgG2b, IgG2c, or IgG3 titers were detected after prime-boost immunizations with OVA in conjunction with the Nod1 agonist FK156 or the elicitor of Nod2, MDP (data not shown), suggesting that both PGN fragments induce a predominant type 2 immune response. In addition, it was observed that the adjuvant activity of the Nod1-specific agonist FK156 remained unaltered in Nod2-deficient mice (data not shown), indicating that Nod1 and Nod2 recognize distinct PGN structures for elicitation of adaptive immune responses.

A detailed analysis of Nod1-deficient mice revealed that animals were outwardly healthy and that WT and *Nod1*$^{-/-}$ mice displayed equal amounts of basal Ig and IgG isotypes (Figures S3A and S3B). In addition, FACS analysis of cells isolated from thymus and spleen showed normal development of B cells, DCs, $CD4^+$ T cells, and $CD8^+$ T cells (Figures S3C–S3H). Because Nod1 is also expressed in T and B cells (Figure S4A), we investigated whether Nod1 deficiency alters the intrinsic stimulatory potential

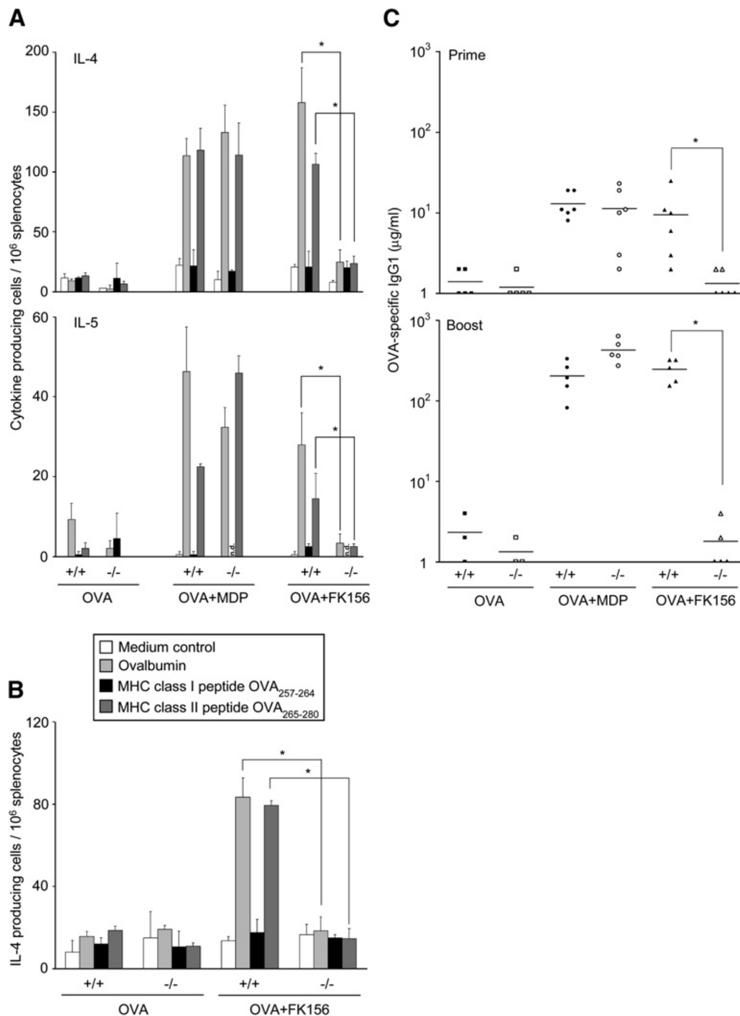

Figure 3. Impaired Antigen-Specific T and B Cell Immunity in Nod1-Deficient Mice Immunized with FK156

Subcutaneous prime-boost (A and C) (day 0 and day 28) or intraperitoneal one-shot immunizations (B) of wild-type (+/+, represented by filled circles) and Nod1-deficient (−/−, represented by empty circles) animals with ovalbumin (OVA) only or in conjunction with MDP or FK156 were carried out.

(A) On day 10 after immunizations, pools of splenocytes from immunization groups (n = 4) were restimulated ex vivo with medium as control, OVA, an MHC class I-restricted (H-2K^b, OVA(257-264) or an MHC class II-restricted (I-A^b, OVA(265-280)) epitope. The frequencies of OVA-specific CD4$^+$ and CD8$^+$ T cells producing IL-4 or IL-5 per million splenocytes were determined by ELISPOT analysis. Data shown are representative of three independent experiments. The mean ± standard deviation of a representative experiment is given.

(C) Serum of each mouse was taken on day 26 (prime) and day 54 (boost), and OVA-specific IgG1 production was analyzed by ELISA. Data shown are representative of three independent experiments. The bar represents the mean of each cohort (n = 3–6). The asterisk indicates a significant difference ($p < 0.05$).

of T and B cells. In vitro stimulation of naive splenic CD4$^+$ and CD8$^+$ T cell as well as CD19$^+$ B cells revealed a normal proliferative response of Nod1-deficient cells (Figures S4B and S4C). Moreover, comparable amounts of IFN-γ, IL-2, IL-4, and IL-17A were produced by WT and Nod1-deficient CD4$^+$ T cells (Figure S4D). In addition, no differences of the homeostatic proliferation potential of T and B cells (Figure S4E) as well as unaltered expression levels of maturation markers of T cells (data not shown) were observed by comparing WT and Nod1-deficient animals, indicating that the altered onset of antigen-specific adaptive immunity in Nod1-deficient mice is not due to intrinsic defects of T and B cells.

TLRs and Nod1 Synergize to Elicit Adaptive Immune Responses

The orchestration of the various forms of antigen-specific immunity is regulated by professional antigen-presenting cells, predominantly DCs. Their immunoregulatory role in the infected host relies mainly on sensing specific MAMPs by receptors including PRMs and/or receptors that bind mediators that are produced in response to infection, which collectively initiate and modulate DC function to drive different immune effector arms (Kapsenberg, 2003). Thus, we investigated whether Nod1 deficiency impacts on the capacity to directly activate DCs. We did so by comparing the stimulatory potential of various defined MAMPs, including the murine Nod1 elicitor FK156 (Magalhaes et al., 2005), and various TLR agonists on WT and Nod1-deficient DCs. The activity of FK156 for IL-1β, IL-6, TNF-α, and IL-12p40 production by bone marrow-derived dendritic cells (BMDCs) isolated from WT mice was found to be completely abolished in cells from Nod1-deficient animals (Figure 4A). However, FK156 stimulation of BMDCs did not induce the release of IL-10, IL-12p70, IL-23, TGF-β, KC, MIP-2, or MCP-1 or change the cell-surface expression of costimulatory molecules CD80, CD86, and CD40 (data not shown). Equal amounts of IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-23, TNF-α (Figures 4B and 4C), as well as TGF-β (data not shown), were produced when Nod1-deficient or WT BMDCs were treated with the purified TLR2 agonist Pam$_3$CSK$_4$ or the TLR4 elicitor LPS. Moreover, TLR2 and TLR4 stimulation induced similar amounts of cell-surface expression of costimulatory molecules CD80, CD86, and CD40 on BMDCs from WT and Nod1-deficient mice (data

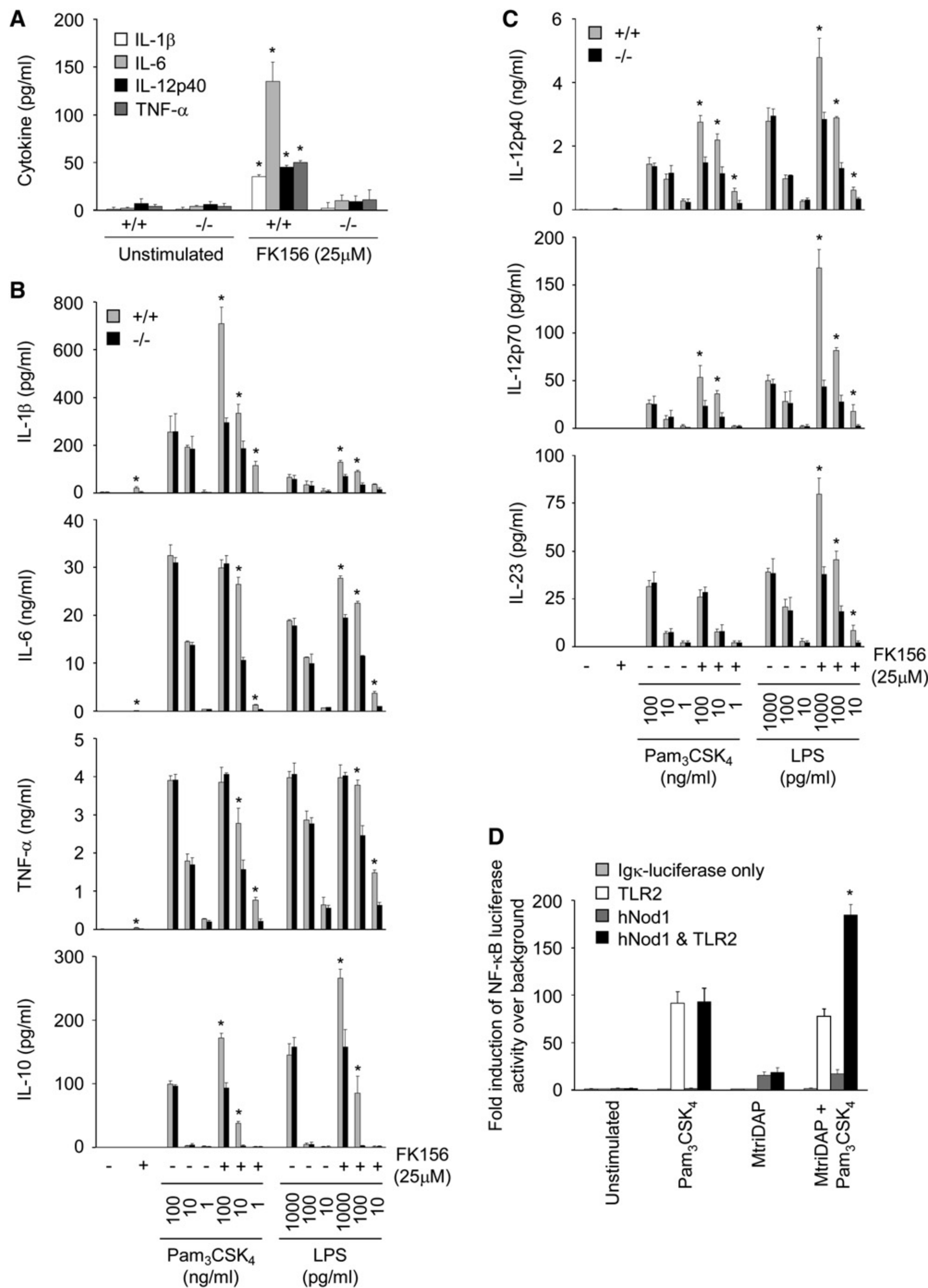

Figure 4. TLRs and Nod1 Synergize for Dendritic Cell Cytokine Production and NF-κB Signaling

(A–C) Bone marrow-derived dendritic cells were stimulated for 40 hr with FK156 only (A) or combinations of FK156 and Pam_3CSK_4 or LPS (B and C), and the amounts of IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, IL-23, and TNF-α in supernatants was determined by ELISA. Data shown are representative of three independent experiments. The mean ± standard deviation of a representative assay performed in triplicate is shown. The asterisk indicates a significant difference ($p < 0.05$).

(D) HEK293T cells were transiently cotransfected with combinations of TLR2 and hNod1 together with a NF-κB-luciferase reporter construct. Agonists for hNod1 (MtriDAP, 250 nM), TLR2 (Pam_3CSK_4, 100 nM) were used to analyze the NF-κB-dependent luciferase activation. The fold induction of NF-κB-dependent luciferase activity over background stimulation showing mean ± standard deviation is given. A representative assay performed in triplicate is shown. The asterisk indicates a significant difference ($p < 0.05$).

not shown), indicating that Nod1 deletion abolishes FK156-mediated effects but does not alter the capacity of TLR agonists to stimulate cytokine release in BMDCs.

In addition, comparable numbers of $CD11c^{+}CCR7^{+}$ DCs were found in draining lymph nodes of FK156 (data not shown) or CFA-injected WT and Nod1-deficient mice

Cell PRESS

(Figure S5). Furthermore, similar cell-surface expression of the costimulatory molecules CD80, CD86, and CD40 were observed in draining lymph nodes of WT and Nod1-deficient mice, indicating that Nod1 deficiency does not alter the migratory capacity of DCs (Figure S5 and data not shown). Interestingly, simultaneous stimulation of BMDCs from WT mice with FK156 and Pam_3CSK_4 or LPS led to synergistic production of the mediators IL-1β, IL-6, TNF-α, and IL-12p40 as well as the anti-inflammatory cytokine IL-10 (Figures 4B and 4C), whereas no significant changes for TGF-β were observed (data not shown). Moreover, costimulation of DCs by FK156 with Pam_3CSK_4 or LPS led to a significant increase in the production of the Th1 immunity promoting cytokine IL-12p70 (Figure 4C). In addition, synergistic activation by FK156 and LPS was observed for the production of the Th17-promoting cytokine IL-23 (Figure 4C). However, the capacity of FK156 to cooperate with TLR agonists for enhanced cytokine production was completely absent in Nod1-deficient animals (Figures 4B and 4C), indicating that the BMDC potentiating effects of FK156 are abolished in Nod1-deficient mice.

HEK293T cells cotransfected with Nod1 and TLR2 and synergistically stimulated by their respective agonists revealed enhanced activation of NF-κB compared to stimulations of Nod1 or TLR2 alone (Figure 4D). This suggests that cooperative Nod1 and TLR activation enhances signaling through NF-κB, leading to the synergistic production of Th1- and Th17-promoting factors such as IL-12 and IL-23. To investigate whether these observations are of relevance for the initiation and development of adaptive immunity in vivo, we analyzed the antigen-specific immune response after coinjections with FK156 and TLR agonists. When compared to injections with the sole compounds, simultaneous application of FK156 and LPS lead to a significant increase of IL-12p40 into the bloodstream of animals (Figure 5A). In addition, coinjections of OVA with Pam_3CSK_4 and FK156 or LPS and FK156 significantly increased the magnitude of Ag-specific T cells compared to immunization with single TLR2 or TLR4 agonists (Figures 5B–5D). Although comparable numbers of OVA-specific IL-4-producing $CD4^+$ T cells were found, a marked increase in Ag-specific release of IFN-γ and IL-17A was observed by ELISPOT, ELISA, and intracellular FACS analysis (Figures 5B–5D), demonstrating that Nod1-mediated PGN recognition synergizes with TLR agonists to prime Ag-specific Th1 as well as Th17 immune responses.

Stimulation of Nod1 in Nonhematopoietic Cells Is Required for Priming Antigen-Specific Immunity

To reveal the mechanisms involved leading to the Th2 immune response induced by sole Nod1 stimulation, we further analyzed in vivo the key mediators released upon FK156 injection. We observed that the Nod1-specific agonist FK156 induced a substantial release of the chemokines KC and MIP-2 in the bloodstream 2 hr after injection, which returned nearly to steady-state levels 22 hr after application (Figures 6A and 6C). Moreover, FK156 induced a marked release of the Th2 immunity promoting chemokine MCP-1 (Figure 6B). This response was strictly Nod1 dependent, because no significant induction of cytokines and chemokines was found in Nod1-deficient mice (Figures 6A–6C), suggesting that the release of these inflammatory mediators contributes to the elicitation of Nod1-mediated adaptive immune responses. Because we observed that Nod1 is not only expressed in ECs and cells of myeloid origin such as splenic $CD11c^+$ DCs, but also by $CD4^+$ and $CD8^+$ T cells as well as $CD19^+$ B cells (Figure S4A), we investigated whether the potent and fast production of cytokines and chemokines by FK156 was dependent on T and B cells. Injection of FK156 into *Rag1*$^{-/-}$ animals did not alter the release of KC, MCP-1, MIP-2 (Figures 6A–6C), IL-6, and TNF-α (data not shown), indicating that the rapid innate immune response induced by Nod1-specific PGN sensing is independent of T and B cells.

To address the mechanisms that cause defective innate and adaptive immune responses in Nod1-deficient mice, we performed add-back and reconstitution experiments. By adding back WT BMDC to Nod1-deficient mice, we first tested in vivo whether the defect for chemokine production after FK156 application could be restored. Although WT animals injected with FK156 were fully capable of producing MCP-1 (Figure 7A) and KC (data not shown), a markedly reduced chemokine production was observed in Nod1-deficient animals receiving WT BMDCs. In contrast, both WT and Nod1-deficient animals were capable of producing MCP-1 upon injection of LPS (Figure 7A). This suggests that in vivo DCs were not required for the rapid cytokine and chemokine release induced upon Nod1-mediated PGN sensing. Comparable results were obtained with bone-marrow reconstitution experiments. Upon injection of FK156, a rapid release of MCP-1 (Figure 7B) and KC (data not shown) was observed in WT animals reconstituted with WT bone marrow but not in Nod1-deficient animals receiving WT bone marrow. Importantly, we observed that Nod1-deficient animals reconstituted with WT bone marrow failed to induce Nod1-mediated Th2 immune responses. WT animals as well as WT animals reconstituted with WT bone marrow, injected with OVA and FK156, elicited antigen-specific IL-4- and IL-5-producing $CD4^+$ T cells upon restimulation with OVA protein or OVA(265-280) (Figure 7C). In contrast, severely diminished T cell frequencies were observed in Nod1-deficient mice as well as Nod1-deficient animals reconstituted with WT bone marrow, strongly suggesting that nonhematopoietic cells are key in triggering the Nod1-mediated Th2 immune responses.

DISCUSSION

The concept that successful antigen-specific immunity is instructed by innate immune recognition systems has been put forward by numerous reports, purporting that direct stimulation of TLRs by cognate agonists activate DCs to provide signals for priming adaptive immunity (Iwasaki and Medzhitov, 2004; Kawai and Akira, 2005). However, recent evidence points to the idea that innate

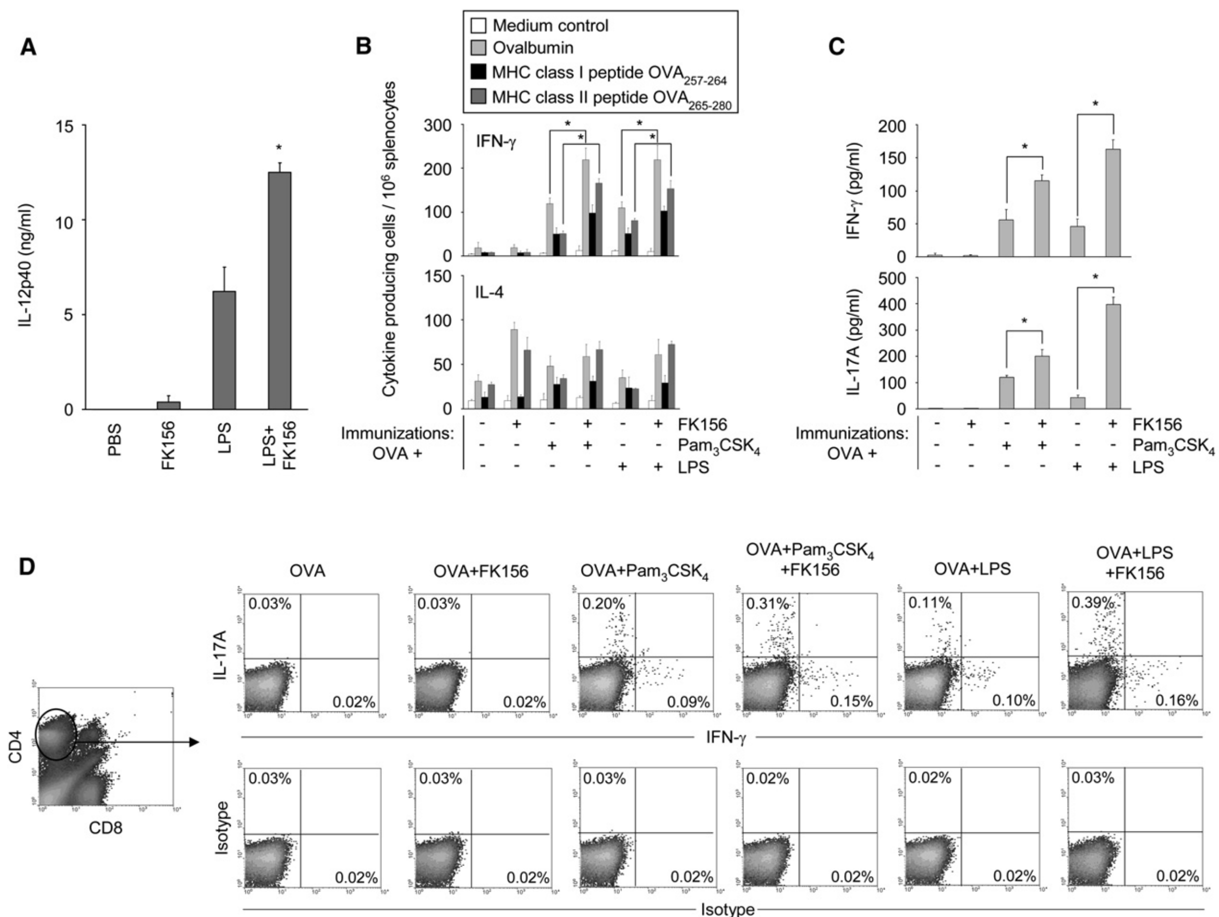

Figure 5. The Nod1 Agonist FK156 Synergizes with TLR Agonists for Priming of Antigen-Specific Immunity

(A) Blood of wild-type (WT) animals was collected 2 hr after intraperitoneal injection of PBS only, FK156, LPS, or LPS and FK156, and the content of IL-12p40 in the serum of each mouse was analyzed by ELISA. The bar represents the mean of each cohort (n = 2–3). The asterisk indicates a significant difference ($p < 0.05$).

(B–D) WT animals were immunized with ovalbumin (OVA) only, or in conjunction with the indicated stimuli and on day 10 after each immunization, pools of splenocytes from immunization groups (n = 4) were restimulated with medium as control, OVA, or an MHC class I-restricted (H-2K^b, OVA(257-264)) or an MHC class II-restricted (I-A^b, OVA(265-280)) epitope derived from OVA.

(B and C) The numbers of Ag-specific CD4$^+$ and CD8$^+$ T cells producing IFN-γ or IL-4 per million splenocytes were determined by ELISPOT analysis (B) and the Ag-specific production of IFN-γ and IL-17A in supernatants was determined by ELISA (C). Data shown are representative of two independent experiments. The mean ± standard deviation of a representative experiment is given. The asterisk indicates a significant difference ($p < 0.05$).

(D) The frequencies of Ag-specific CD4$^+$ T cells producing IFN-γ or IL-17A after restimulation with OVA protein were determined by intracellular FACS analysis. The percentages of gated positive cells are indicated. Data shown are representative of two independent experiments.

signals distinct from those generated downstream of TLRs are required for driving the adaptive immune response (Gavin et al., 2006; Nemazee et al., 2006). Our observations now clearly demonstrate that the NLR protein family member Nod1 contributes to the onset of adaptive immunity. Animals deficient in Nod1 mount altered T and B cell immunity to T cell-dependent antigen given in the standard immunopotentiator, CFA. Moreover, we observed that Nod1-mediated PGN recognition is crucial to instruct antigen-specific immunity upon infection with a live pathogen. Finally, our study suggests that, in the context of driving adaptive immunity, Nod1 plays a predominant role in nonhematopoietic cells.

Our data demonstrate that classical adjuvant preparations require Nod1-mediated PGN recognition to instruct the onset of Th1 and Th2 as well as Th17 cell responses. Immunizations of Nod1-deficient animals with T cell-dependent antigens given in CFA yielded markedly lower frequencies of antigen-specific IFN-γ-, IL-17A-, and IL-4-producing CD4$^+$ and CD8$^+$ T cells as well as diminished IgG1, IgG2b, IgG2c, and IgG3 antibody titers compared to WT controls. Moreover, *H. pylori*-infected Nod1-deficient mice showed diminished antigen-specific titers. Nod1-mediated PGN recognition, therefore, appears to be essential for the elicitation of antigen-specific responses during immunization as well as in bacterial

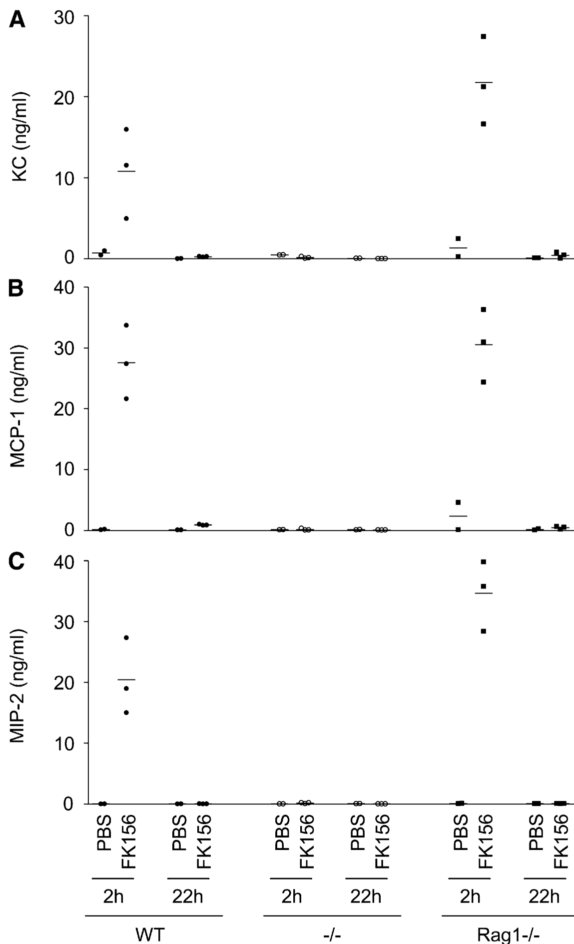

Figure 6. Impaired FK156-Mediated Chemokine Production in Nod1-Deficient Mice

Blood of wild-type (+/+, represented by filled circles), Nod1-deficient (−/−, represented by empty circles), and *Rag1*$^{-/-}$ (represented by filled squares) animals was collected 2 and 22 hr after intraperitoneal injection of PBS or FK156, and the content of KC (A), MCP-1 (B), and MIP-2 (C) in the serum of each mouse was analyzed by ELISA. Data shown are representative of two independent experiments. The bar represents the mean of each cohort (n = 2–3).

infection, which likely contributes to the successful clearance of pathogens from the infected host.

Our study demonstrates that Nod1-mediated PGN recognition is essential to cooperate with TLRs for successful elicitation of adaptive immune responses. Nod1-mediated PGN sensing markedly enhances TLR-mediated IL-12 release and priming of Th1 and Th17 immune responses in vivo. Because *H. pylori* (reviewed in Ferrero, 2005) as well as the *mycobacterial* cell-wall constituents used for the preparation of CFA activate both TLRs and Nod1, our findings suggest that the severely reduced antigen-specific T and B cell responses observed in immunizations with CFA and during infection with *H. pylori* in Nod1-deficient animals result from an impaired Nod1-TLR crosstalk.

In support of these in vivo findings, we demonstrate that cooperative stimulation of Nod1 and TLRs synergizes for NF-κB activation and the production of cytokines by DCs such as IL-12, IL-1β, IL-6, and IL-23, known to be essential for priming of Th1 and Th17 immune responses (Bettelli et al., 2006; Langrish et al., 2005; Mangan et al., 2006; Sutton et al., 2006). In accordance with what has been reported for DCs of human origin (Fritz et al., 2005; Tada et al., 2005; van Heel et al., 2005), these data suggest that the cooperative stimulation of Nod1 and TLRs to synergize for cytokine production by DCs translates into enhanced antigen-specific immune responses in vivo. As reported recently (Ferwerda et al., 2005), we also observed that the *mycobacterial* cell-wall constituents used in the CFA preparation stimulate Nod2. Despite this, however, our data suggest that in vivo, Nod1 deficiency cannot be overcome by Nod2 stimulation. The effect of Nod2 deletion on the onset of adaptive immunity upon immunization with CFA remains to be studied.

Despite the fact that Nod1 stimulation synergizes with TLRs to prime Th1 and Th17 immune responses, we observed that the Nod1-specific agonist FK156, when applied alone, elicits priming of antigen-specific T and B cell immunity with a predominant Th2 cell polarization profile. The specificity of this response is highlighted by the observation that Nod1 deficiency does not alter the adjuvant effect of the Nod2 agonist muramyl dipeptide, which was shown previously to also generate antigen-specific Th2 responses (Kobayashi et al., 2005). These findings underscore the fact that Nod1 and Nod2 recognize distinct PGN motifs for the induction of innate as well as adaptive immune responses. By investigating key mediators released upon specific Nod1 stimulation in vivo, we observed that FK156 induced a substantial release of IL-6, TNF-α (Magalhaes et al., 2005), as well as the chemokines KC and MIP-2, known to trigger the recruitment of neutrophils (Mantovani et al., 2004). Moreover, we found that FK156 induced, in a Nod1-dependent manner, a marked release of the inflammatory chemokine MCP-1, which promotes homing of monocytes and immature DCs to inflamed tissues via CCR2 (Mantovani et al., 2004). Several independent observations describe a predominant role for MCP-1 in promoting Th2 immunity in both infectious and allergic disease models (reviewed in Daly and Rollins, 2003). Indeed, the application of MCP-1 to naive T cells in the presence of antigen drives the differentiation to Th2 cells (Karpus et al., 1997; Lukacs et al., 1997). Moreover, immunization of MCP-1-deficient mice yields normal levels of Ag-specific T cells producing IFN-γ but markedly alters levels of Th2 cytokines and reduces IgG1 production (Gu et al., 2000). In light of these observations, our data suggest that the strong induction of MCP-1 by Nod1-mediated PGN sensing is key for its ability to prime Ag-specific immunity with a predominant Th2 polarization profile.

We further aimed to investigate which cell population is essential for driving Nod1-specific priming of adaptive immune responses. Surprisingly, even though DCs are thought to be key players in integrating microbial and antigen signals to instruct adaptive immune responses,

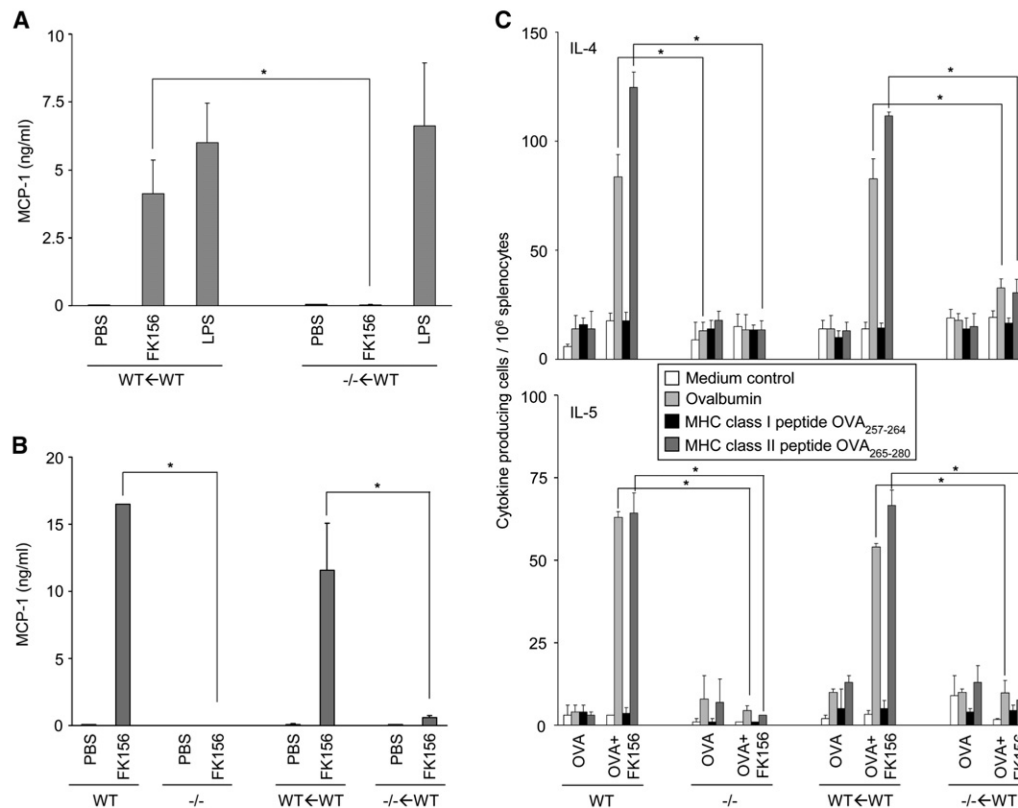

Figure 7. Stimulation of Nod1 in Nonhematopoietic Cells Is Required for Priming Antigen-Specific Immunity

(A) Blood of wild-type (WT) and Nod1-deficient animals who received WT BMDCs (WT←WT and *Nod1*$^{-/-}$←WT, respectively) was collected 2 hr after intraperitoneal injection of PBS, FK156, or LPS, and the content of MCP-1 in the serum of animals was analyzed by ELISA. The mean ± standard deviation of (n = 2–3) is given. The asterisk indicates a considered significance ($p < 0.05$).

(B) Wild-type (WT), Nod1-deficient, and bone-marrow chimera animals (WT reconstituted with WT bone-marrow [WT←WT] and Nod1-deficient reconstituted with WT bone marrow [*Nod1*$^{-/-}$←WT]) were injected intraperitoneally with PBS only or FK156. Blood of animals was collected 2 hr after intraperitoneal injection, and the content of MCP-1 in the serum of each mouse was analyzed by ELISA. The mean ± standard deviation of (n = 2–3) is given. The asterisk indicates a considered significance ($p < 0.05$).

(C) Wild-type (WT), Nod1-deficient, and bone-marrow chimera animals (WT reconstituted with WT bone-marrow [WT←WT] and Nod1-deficient reconstituted with WT bone marrow [*Nod1*$^{-/-}$←WT]) were injected intraperitoneally OVA only or OVA+FK156. 10 days after immunizations, splenocytes from immunized animals were restimulated ex vivo with medium as control, OVA, an MHC class I-restricted (H-2K^b, OVA(257-264)), or an MHC class II-restricted (I-A^b, OVA(265-280)) epitope. The frequencies of OVA-specific CD4$^+$ and CD8$^+$ T cells producing IL-4 or IL-5 per million splenocytes were determined by ELISPOT analysis. The mean ± standard deviation (n = 2–6) is given. The asterisk indicates a considered significance ($p < 0.05$).

WT DCs and hematopoietic cells in general could not compensate for Nod1 deficiency. Thus, our data clearly demonstrate that nonhematopoietic cells are key for Nod1-mediated innate immune recognition of PGN to instruct antigen-specific Th2 immune responses.

Th2 immunity requires orchestration of innate and adaptive immune effector mechanisms to protect internal and external host sites from pathogens and is collectively described as barrier immunity (Stetson et al., 2004). Th2 cells that stably secrete IL-4 are central in the orchestration of Th2 immunity (Stetson et al., 2004), involving the coordinated concentration of activated eosinophils, mast cells, basophils, and a recently described yet ill-defined cell population (Fallon et al., 2006; Owyang et al., 2006). With the propensity for Th2 immune responses to develop at epithelial barriers, there is the suggestion that the instructive signals and possibly also the terminal differentiation of Th2 immune responses in vivo occurs in tissue and that signals that mediate this occur predominantly in epithelia (Stetson et al., 2004). Although we cannot exclude the possibility that cells of myeloid origin are required to further translate signals initiated by Nod1-mediated PGN recognition into a full-blown Th2 response, our data clearly highlight that myeloid cells are not required to trigger these first signals. Indeed, our findings point to the idea that innate immune priming by Nod1 is carried by cells within the tissue microenvironment of the barrier, including nonhematopoietic cells such as ECs, fibroblasts, and stromal cells. Signals emanating from the barrier are then crucial for the conditioning of DCs (Rimoldi et al., 2005) and B cells (Xu et al., 2007) and thereby impact on the generation of the antigen-specific adaptive immune response. Future experiments will attempt to delineate which innate and/or adaptive

Cell PRESS

immune cells and mediators are involved to translate the barrier signals given upon Nod1-mediated PGN sensing to instruct antigen-specific Th2 immune responses.

Although ECs provide the first line of defense, bacteria can gain access across barrier surfaces, where they encounter DCs, which constitutively sample the tissue environment (Kelsall and Rescigno, 2004). Thus, multiple engagements of PRMs on DCs, such as cooperative stimulation by TLRs and Nod1, might play a key role instructing innate as well as adaptive immune responses, once pathogens breach the barrier surface. Hence, based on our observations demonstrating that Nod1 stimulation significantly enhanced Th1 immune responses elicited by TLRs, we suggest that these two innate immune recognition systems likely cooperate to fight enteroinvasive bacteria. Consistent with this idea, it has been shown that IL-17 produced by Th17 cells is implicated in regulating host defense mechanisms such as recruitment of neutrophils (Schwarzenberger et al., 2000; Ye et al., 2001) and production of antimicrobial peptides (Kao et al., 2004; Liang et al., 2006). Thus, the marked enhancement of antigen-specific Th17 immunity by the cooperative activity of TLRs and Nod1 suggests that these Nod1-mediated responses constitute an important mechanism to fight bacterial infection.

Crosstalk between TLRs and NLRs is likely to be crucial for the balance of immune effector arms, with alterations in this balance tipping the response toward chronic inflammatory disorders and autoimmune diseases. Indeed, a key role in balancing host immune responses has recently been assigned to Nod1, because polymorphisms in the gene encoding this protein were shown to be linked to increased susceptibility to asthma (Hysi et al., 2005) and atopic eczema (Weidinger et al., 2005), all of which represent inflammatory barrier diseases associated with dysregulated innate and adaptive immune responses. Taken together, our recent report highlighting the role of Nod1 in bacterial clearance (Viala et al., 2004) and the observations provided here stress the importance of Nod1-mediated PGN recognition in balancing innate as well as adaptive effector immune responses, and thereby contributing to host defense. These studies also open up possible avenues of research in order to explore the specific role of Nod1 and its crosstalk with TLRs in the control of specific disease models of inflammatory disorders.

EXPERIMENTAL PROCEDURES

Reagents

Muramyl-dipeptide (MDP, MurNAc-L-Ala-D-isoGln) was purchased from EMD Biosciences (San Diego, CA). MtriDAP (MurNAc-L-Ala-γ-D-Glu-*meso*-DAP) was prepared as described previously (Girardin et al., 2003). Synthetic FK156 (D-lactyl-L-Ala-γ-D-Glu-*meso*-DAP-Gly) was obtained from Fujisawa Inc. (Japan). Pam_3CSK_4 was purchased from EMC Microcollections (Tübingen, Germany), and highly purified LPS was from *Salmonella minnesota* R595 from ALEXIS Corp. (Lausen, Switzerland). Specificity and purity of MDP, MtriDAP, FK156, Pam_3CSK_4, and LPS was ensured as previously described (Fritz et al., 2005; Magalhaes et al., 2005). Human serum albumin (HSA) and ovalbumin ("OVA-crude") were obtained from Sigma-Aldrich (St. Louis, MO). "OVA-clean" was purchased from Worthington-Biochemical Corporation (Lakewood, NJ). Purified recombinant *H. pylori* urease was kindly provided by T.H. Ermak (Acambis, Inc., MA). The enzyme was produced in an inactive but fully assembled form (Lee et al., 1995). Complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), and desiccated *Mycobacterium butyricum* fractions (regrouped as *Mycobacterium smegmatis* from ATCC strains 19420 and 14468) were obtained from Difco (Detroit, MI). Peptides were synthesized and purified as previously reported (Fritz et al., 2004). The following peptides were used: $OVA_{257\text{-}264}$ (H-SIINFEKL-OH, H2-K^b) and $OVA_{265\text{-}280}$ (H-TEWTSSNVMEERKIKV-OH, I-A^b), both derived from chicken ovalbumin; mTRP-$2_{181\text{-}188}$ (H-VYDFFVWL-OH, H2-K^b) derived from mouse tyrosinase related protein 2; and $NBVc_{128\text{-}140}$ (H-TPPAYRPPNAPIL-OH, I-A^b) derived from the core protein of hepatitis B virus. Expression plasmids for mNod1 and hTLR2 were purchased from InVivogen (San Diego, CA). hNod1-K208R was generated by site-directed mutagenesis. Expression plasmids for hNod1 and hNod2 were described previously (Girardin et al., 2003). Fluorescein-(FITC)-labeled antibodies specific for CD4 (RM4-4), CD11c (HL3), phycoerythrin-(PE)-labeled anti-CD8α (53-6.7), CD80 (16-10A1), CD86 (16-10A1), and matching isotype controls were purchased from Becton Dickinson (San Diego, CA). Biotin anti-mouse CCR7 (4B14), allophycocyanin (APC) anti-mouse CD3e (145-2C11), APC-Cy7 anti-mouse CD8α (53-6.7), matching isotype controls, and Streptavidin-PE were obtained from eBioscience (San Diego, CA).

Reporter Assay for NF-κB Activation

Human embryonic kidney (HEK) 293T cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids (all purchased from Invitrogen, Carlsbad, CA), and 5% fetal calf serum (FCS) (Hyclone, Logan, UT). HEK293T cells were seeded into 24-well plates at a density of 10^5 cells/ml, and transfections with various expression plasmids were carried out as described previously (Fritz et al., 2005). In brief, cells were transfected overnight with 1% of FuGENE 6 transfection reagent (Roche Diagnostics, Germany) with 75 ng of the β-galactosidase expression plasmid and 75 ng of the reporter plasmid Igκ-luciferase alone or plus the following vectors: 0.5 ng mNod1, hNod1, hNod1-K208R or hNod2, 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 hr of incubation and normalized as a ratio to β-galactosidase activity. Assays were performed in triplicate and data represent at least three independent experiments. Data show mean ± SEM.

Cytokine Dosage

The concentrations of IFN-γ, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-17A, KC, MCP-1, MIP1α, MIP1β, MIP-2, TGF-β, TNF-α (DuoSet; R&D Systems, Minneapolis, MN), and IL-23 (eBioscience) in culture supernatants were determined by ELISA as specified by the manufacturer.

Flow Cytometry

Cells were resuspended in FACS buffer (ice-cold phosphate buffered saline [PBS] supplemented with 0.1% FCS and 0.2% NaN_3 [Sigma-Aldrich]) and incubated with cell-culture supernatants of the hybridoma 2.4G2 (ATCC; anti-CD16/32 antibody) for 15 min on ice to block nonspecific staining. Subsequently, specific antibodies or isotype controls were added and incubated for 30 min. Cells were washed extensively and resuspended in PBS/0.1% FCS and analyzed by FACS (FACScan or LSR Benchtop, Becton Dickinson). Dead cells were excluded based on propidium iodide (Sigma-Aldrich) staining. Data were analyzed with WinMDI 2.8 or FlowJo software.

Mice, Immunizations, and Infections

All animal experiments were approved by the Animal Ethics Review Committee of the University of Toronto. C57BL/6 mice were purchased from Janvier (Le Genest, France) or Charles River Laboratories

(Wilmington, MA). *Rag1*$^{-/-}$ mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Nod1-deficient mice (Millenium Pharmaceuticals) have been backcrossed to the seventh generation into the C57BL/6 background. Mice were submitted to sanitary control tests and used at the age of 6–14 weeks. All animal experiments were performed according to local guidelines. WT and Nod1-deficient mice were tested *Helicobacter*-free by bacteriological and serological assays (data not shown).

For the generation of chimeras, recipient mice (WT or Nod1-deficient) were γ-irradiated twice with 5 Gy with a MDS Nordion Gammacell 200 irradiator and were reconstituted with 1.5 × 10^6 T cell-depleted bone-marrow cells from CD45.1 WT donor mice (6–8 weeks of age). Donor marrow was depleted of T lymphocytes with CD4 and CD8 microbeads (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. At 6–8 weeks after reconstitution, mice were tested for chimerism.

DC add-back experiments were carried out as described previously (Hanada et al., 2003). In brief, 1.2 × 10^6 BMDCs from CD45.1 were resuspended in endotoxin-free PBS and applied intraperitoneally into WT or Nod1-deficient mice (6–8 weeks of age). Mice were injected with PBS, 0.1 μmol FK156 or LPS (2 μg) 24 hr after cell transfer.

To determine the ratio of lymphocyte populations, single-cell suspensions of spleen and thymus of naive mice were prepared and analyzed by flow cytometry. Basal serum immunglobulin levels were analyzed by ELISA from blood of naive mice drawn from tail veins.

For analysis of dendritic cell migration, mice were injected subcutaneously into the footpads with sterile endotoxin-free PBS (BioWhittaker, Apen, Germany) or CFA in a total volume of 100 μl (50 μl per footpad). Popliteal lymph nodes were removed at indicated time-points, single-cell suspensions were prepared, and cells were stained and analyzed by FACS acquiring 2 × 10^4 cells per sample.

For analysis of cytokine and chemokine responses, animals were injected intraperotoneally with 0.1 μmol FK156 in endotoxin-free PBS, LPS (2 μg), a combination of LPS (2 μg) and 0.1 μmol FK156, or endotoxin-free PBS only in a total volume of 100 μl. At indicated time points, blood was collected and sera were analyzed by ELISA.

For responses of thymus-dependent antigens, mice were immunized subcutaneously into the left flank or intraperitoneally with a mixture of 100 μg "OVA-clean" and adjuvant (0.1 μmol MDP or FK156, CFA, or IFA) in a total volume of 100 μl sterile endotoxin-free PBS at intervals of 28 days. For analysis of the immune response induced by FK156 in synergy with the TLR agonists Pam_3CSK_4 or LPS, mice were immunized with a mixture of 100 μg ovalbumin emulsified in IFA with or without 0.1 μmol FK156, 25 μg Pam_3CSK_4, or 25 μg LPS. 10 days after immunizations, pools of spleen cells or draining inguinal lymph nodes of immunization groups were restimulated ex vivo, and ELISPOT, intracellular FACS analysis, and ELISA of supernatants were performed as described below. Blood from tail veins was drawn 26 days after immunization and sera of individual mice were analyzed.

For analysis of homeostatic proliferation of T and B cells in vivo, age-matched (14 weeks) WT and Nod1-deficient animals were supplied with BrdU (Sigma, San Diego, CA)-containing drinking water (1 mg/ml) for 10 days (BrdU-containing drinking water was shielded from light and replaced every 2 days). BrdU incorporation of spleen and inguinal lymph node $CD3^+CD4^+$, $CD3^+CD8^+$, and $B220^{high}$ cells were analyzed with the FITC BrdU Flow Kit (BD Bioscience, San Diego, CA).

The *H. pylori* strain B128 was routinely subcultured on blood agar medium containing 10% horse blood and an antibiotic supplement. Bacterial suspensions for mouse inoculation were prepared directly in peptone trypsin broth from 36 hr plate cultures (Viala et al., 2004). WT and Nod1-deficient mice were each administered a single 0.1 ml aliquot of these suspensions (equivalent to 10^8 colony-forming units) via a teflon catheter as described previously (Ferrero et al., 1998). The presence of gastric *Helicobacter* infection in mice was determined 1 month after inoculation, by quantitative culture of gastric tissue fragments containing both the antrum and corpus and by ELISA of serum antibodies (Ferrero et al., 1998). Viable counts were performed by serial dilution of samples in sterile peptone trypsin broth (Ferrero et al., 1998). Diluted samples from gastric biopsy samples were plated onto blood plates supplemented with bacitracin and nalidixic acid (Ferrero et al., 1998).

Analysis of T Cell Responses

For intracellular FACS analysis and determination of T cell-derived cytokines in cell-culture supernatants, freshly isolated splenocytes from immunized mice were resuspended in complete medium (DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids [all purchased from Invitrogen], and 5% fetal calf serum [FCS; Hyclone]) and plated in 96-well tissue culture plates at 1 × 10^6 per well, or in ELISPOT plates at 1 × 10^6, 5 × 10^5, 3 × 10^5, or 1 × 10^5 cells per well in the presence of the indicated stimuli in triplicate: OVA (50 μg/ml), HSA (50 μg/ml), OVA(257-264) (10 μg/ml), OVA(265-280) (10 μg/ml), irrelevant H2-K^b (mTRP-$2_{181-188}$), and I-A^b ($NBVc_{128-140}$) restricted peptides (10 μg/ml). As positive controls, concanavalin A (10 μg/ml) or PMA/ionomycin (2 × 10^{-8} M/7.5 × 10^{-7} M) were applied (all purchased from Sigma-Aldrich). Complete medium was used as negative control.

Supernatants of tissue-culture plates were collected after 48 hr of stimulation and analyzed for the presence of IFN-γ or IL-17A by ELISA. Intracellular FACS analysis was performed with the BD Cytofix/Cytoperm Plus kit (BD Biosciences) by adding the Golgi-Plug after 24 hr of stimulation for 10 hr before proceeding according to the manufacturer's protocol. Cells were analyzed by intracellular FACS after staining with a PE-Cy7-labeled antibody against IFN-γ (Becton Dickinson), a ALEXA-647 antibody specific for IL-17A (eBioscience) and matching isotype controls.

For analysis by ELISPOT, Multiscreen plates (MAHA S4510 for IFN-γ and MAIP S4510 for IL-4 and IL-5; Millipore, Molsheim, France) were coated (50 μl/well) with capture antibodies (IFN-γ [R4-6A2] at 1 μg/ml; IL-4 [11B11] at 2 μg/ml; IL-5 [TRFK5] at 2 μg/ml; all purchased from Becton Dickinson, San Diego, CA) and the assay was carried out as described (Fritz et al., 2004). In brief, freshly isolated splenocytes were plated and incubated (IFN-γ for 20 hr, IL-4 and IL-5 for 40 hr) at 37°C/5% CO_2 in triplicate in the presence of the indicated stimuli. Detection was performed with biotinylated secondary antibodies (IFN-γ [AN18.17.24] at 1 μg/ml, kindly provided by K. Lingnau; IL-4 [BVD6-24G2] at 1 μg/ml; IL-5 [TRFK4] at 1 μg/ml; both obtained from Becton Dickinson) and horseradish peroxidase-conjugated streptavidin (R&D Systems). Spots were visualized with 3,3′-Diaminobenzidine and $NiCl_2$ as a substrate. Plates were analyzed with a BIOREADER 2000 (BioSys, Karben, Germany). Results are expressed as the number of IFN-γ-, IL-4-, or IL-5-producing cells per 1 × 10^6 splenocytes ± standard deviation 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 MHC class I (H2-K^b) or MHC class II (I-A^b), ensuring the measurement of the antigen-specific response in all performed assays.

Analysis of B Cell Responses

Basal levels of Ig isotypes were analyzed by sandwich ELISA on serially diluted serum samples. Primary antibodies were goat anti-mouse Ig for total IgG, IgG1, IgG3, IgM, and IgA purchased from Southern Biotechnologies (Birmingham, AL); and rat anti-mouse IgG2b (R9-91) obtained from Becton Dickinson. Isotype controls were IgM (11E10), IgA (S107), IgG1 (15H6), IgG2b (A-1), IgG3 (B10), and total IgG, all purchased from Southern Biotechnologies; biotinylated detection antibodies for total IgG, IgG1, IgG3, IgA, IgM, and IgE were purchased from Southern Biotechnologies; biotinylated rat anti-mouse IgG2b (R12-3) was obtained from Becton Dickinson. Inbred mouse strains with the *Igh1-b* allele, such as C57BL/6, do not have the gene for IgG2a and instead express the IgG2c isotype (Martin et al., 1998). Because commercial IgG2a antibodies crossreact inadequately against IgG2c, antibodies specific for IgG2c purchased from Bethyl Laboratories Inc. (Montgomery, TX) were used. For analysis of antigen-specific Ig isotypes, plates were coated with 10 μg/ml OVA in PBS or with

250 μg *H. pylori* urease in 0.1 M $NaHCO_3$ (pH.9.5). ELISAs were performed by coating antibodies or antigens to 96-well plates (MaxiSorp; Nalgene Nunc, Roskilde, Denmark). Bound antibodies of plated sera samples were detected by sequential incubation with biotinylated secondary antibodies, horseradish peroxidase-conjugated streptavidin (R&D Systems), and TMB (Sigma-Aldrich). Reaction was stopped by acidification with 0.5 M H_2SO_4 (Sigma-Aldrich) and absorbance was read at 450 nm.

Bone Marrow-Derived Dendritic Cell Preparations

BMDCs were grown from WT and *Nod1*$^{-/-}$ mice as described previously (Lutz et al., 1999; Yao et al., 2005). In brief, total bone marrow cells depleted for red blood cells were seeded at 1.5 × 10^6 cells per well in 6-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) in 3 ml of complete culture medium (DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acids, 50 mM 2-mercaptoethanol, all purchased from Invitrogen), 10% fetal calf serum (FCS; Hyclone), and 10 ng/ml murine rGM-CSF (R&D Systems). Cells were incubated for 7 days at 37°C in a humidified atmosphere with 5% CO_2. On day 7, suspended cells and loosely adherent cells were harvested and cells were enriched by magnetic cell sorting with CD11c microbeads (Miltenyi Biotech) according to the manufacturer's protocol. CD11c-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 × 10^6 cells/ml and used for stimulation.

Lymphocyte Proliferation, Cytokine Production, and RT-PCR Analysis

Various cell populations from total spleen cells were enriched by MACS. Dendritic cells were purified with CD11c microbeads while $CD4^+$ and $CD8^+$ T cells as well as $CD19^+$ B cells depleted for $CD11c^+$ cells were enriched with CD4, CD8, or CD19 mirobeads, respectively. All microbeads were purchased from Miltenyi Biotech and used according to the manufacturer's protocol. As analyzed by flow cytometry, purity of sorted cell populations was above 98%. Purified T cells or B cells were placed into 96-well plates (4 × 10^5/well) and stimulated with either plate-bound anti-CD3ε (145-2C11, BD) and/or anti-CD28 (37.51, BD) or Concanavalin A (Sigma), anti-IgM F(ab')2 fragment (Jackson Immuno Research), or LPS (from *Salmonella minnesota* R595 from ALEXIS Corp.). Cells were stimulated in triplicate for 72 hr at indicated concentrations followed by a 12 hr pulse with 0.25 μCi per well of ^{3}H-thymidine (Sigma). Culture supernatants were assayed for the production of IL-2, IL-4, IL-17A, and IFN-γ by ELISA.

Total RNA was prepared with the RNeasy Mini Kit (Qiagen, Valencia, CA). Purified RNA was reverse-transcribed with Superscript II RNase H (Invitrogen) according to the manufacturer's protocol. The generated cDNA was amplified by using primers for murine Nod1 (forward primer: 5′-AGG AGG CCA ACA GAC GCC-3′, reverse primer: 5′-CTG ACC TAG AGG GTA TCG-3′), and β-actin (forward primer: 5′-GTG GGC CGC TCT AGG CAC CAA-3′, reverse primer: 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′). The cDNA samples were heat-denatured (95°C for 5 min) and then amplified with 35 cycles, 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 (Nod1, 310 bp; β-actin, 250 bp) were size fractionated onto a 2% agarose gel and stained with ethidium bromide.

Statistical Analysis

The results are given as mean ± SEM. Statistical analysis was performed with Graphpad Prism software with either a Student's t test (Figures 1, 2, and 7) or a Mann-Whitney test. A $p < 0.05$ was considered significant.

Supplemental Data

Five figures and Results are available at http://www.immunity.com/cgi/content/full/26/4/445/DC1/.

ACKNOWLEDGMENTS

We thank W. Zauner, K. Lingnau, L. Bougneres, D. Rueff-Juy, and V. Soulard for supplying peptides and antibodies and O. Otava and S. Brunner for help with ELISAs. We are grateful to J. Bertin and A. Cole from Millennium Pharmaceuticals for providing the Nod1-deficient mice. We thank M.A. Nahori, C. Werts-Larzilliere, M. Jehanno, and I. Tattoli for continuous support throughout the study and M. Parmier for excellent technical help and plasmid preparations. We acknowledge L. Rogge and P. Vieira for helpful discussions on the manuscript. J.H.F. acknowledges support from a fellowship from the "Programme Transversal de Recherche 153" from the Institut Pasteur followed by an Erwin Schrödinger Research Fellowship (J2630-B13) of the Austrian Science Fund (FWF). L.L.B. acknowledges a fellowship by the Association François Aupetit (AFA) followed by a "Bourse fin de thèse" from the French Fondation Recherche Médicale (FRM). Work in the laboratory of R.L.F. is supported by the National Health and Medical Research Council of Australia. D.J.P. is an EMBO Young Investigator and Howard Hughes International Research Scholar. Work in the laboratories of D.J.P. and S.E.G. is supported by grants from the Canadian Institutes of Health Research.

Received: May 23, 2006
Revised: February 22, 2007
Accepted: March 5, 2007
Published online: April 12, 2007

REFERENCES

Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T.B., Oukka, M., Weiner, H.L., and Kuchroo, V.K. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature *441*, 235–238.

Cossart, P., and Sansonetti, P.J. (2004). Bacterial invasion: the paradigms of enteroinvasive pathogens. Science *304*, 242–248.

Dabbagh, K., and Lewis, D.B. (2003). Toll-like receptors and T-helper-1/T-helper-2 responses. Curr. Opin. Infect. Dis. *16*, 199–204.

Daly, C., and Rollins, B.J. (2003). Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. Microcirculation *10*, 247–257.

Fallon, P.G., Ballantyne, S.J., Mangan, N.E., Barlow, J.L., Dasvarma, A., Hewett, D.R., McIlgorm, A., Jolin, H.E., and McKenzie, A.N. (2006). Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. J. Exp. Med. *203*, 1105–1116.

Ferrero, R.L. (2005). Innate immune recognition of the extracellular mucosal pathogen, *Helicobacter pylori*. Mol. Immunol. *42*, 879–885.

Ferrero, R.L., Thiberge, J.M., Huerre, M., and Labigne, A. (1998). Immune responses of specific-pathogen-free mice to chronic *Helicobacter pylori* (strain SS1) infection. Infect. Immun. *66*, 1349–1355.

Ferwerda, G., Girardin, S.E., Kullberg, B.J., Le Bourhis, L., de Jong, D.J., Langenberg, D.M., van Crevel, R., Adema, G.J., Ottenhoff, T.H., Van der Meer, J.W., and Netea, M.G. (2005). NOD2 and toll-like receptors are nonredundant recognition systems of *Mycobacterium tuberculosis*. PLoS Pathog. *1*, 279–285. 10.1371/journal.ppat.0010034.

Fritz, J.H., Brunner, S., Birnstiel, M.L., Buschle, M., Gabain Av, A., Mattner, F., and Zauner, W. (2004). The artificial antimicrobial peptide KLKLLLLLKLK induces predominantly a T(H)2-type immune response to co-injected antigens. Vaccine *22*, 3274–3284.

Fritz, J.H., Girardin, S.E., Fitting, C., Werts, C., Mengin-Lecreulx, D., Caroff, M., Cavaillon, J.M., Philpott, D.J., and Adib-Conquy, M. (2005). Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. Eur. J. Immunol. *35*, 2459–2470.

Fritz, J.H., Ferrero, R.L., Philpott, D.J., and Girardin, S.E. (2006). Nod-like proteins in immunity, inflammation and disease. Nat. Immunol. *7*, 1250–1257.

Gavin, A.L., Hoebe, K., Duong, B., Ota, T., Martin, C., Beutler, B., and Nemazee, D. (2006). Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science *314*, 1936–1938.

Girardin, S.E., Travassos, L.H., Herve, M., Blanot, D., Boneca, I.G., Philpott, D.J., Sansonetti, P.J., and Mengin-Lecreulx, D. (2003). Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. J. Biol. Chem. *278*, 41702–41708.

Gu, L., Tseng, S., Horner, R.M., Tam, C., Loda, M., and Rollins, B.J. (2000). Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. Nature *404*, 407–411.

Hanada, T., Yoshida, H., Kato, S., Tanaka, K., Masutani, K., Tsukada, J., Nomura, Y., Mimata, H., Kubo, M., and Yoshimura, A. (2003). Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. Immunity *19*, 437–450.

Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S.O., Goode, J., Lin, P., Mann, N., Mudd, S., et al. (2003). Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature *424*, 743–748.

Hysi, P., Kabesch, M., Moffatt, M.F., Schedel, M., Carr, D., Zhang, Y., Boardman, B., von Mutius, E., Weiland, S.K., Leupold, W., et al. (2005). NOD1 variation, immunoglobulin E and asthma. Hum. Mol. Genet. *14*, 935–941.

Inohara, N., Chamaillard, M., McDonald, C., and Nunez, G. (2004). NOD-LRR proteins: role in host-microbial interactions and inflammatory disease. Annu. Rev. Biochem. *74*, 355–383.

Iwasaki, A., and Medzhitov, R. (2004). Toll-like receptor control of the adaptive immune responses. Nat. Immunol. *5*, 987–995.

Janssen, E., Tabeta, K., Barnes, M.J., Rutschmann, S., McBride, S., Bahjat, K.S., Schoenberger, S.P., Theofilopoulos, A.N., Beutler, B., and Hoebe, K. (2006). Efficient T cell activation via a Toll-Interleukin 1 Receptor-independent pathway. Immunity *24*, 787–799.

Johnson, G.B., Brunn, G.J., Tang, A.H., and Platt, J.L. (2003). Evolutionary clues to the functions of the Toll-like family as surveillance receptors. Trends Immunol. *24*, 19–24.

Kao, C.Y., Chen, Y., Thai, P., Wachi, S., Huang, F., Kim, C., Harper, R.W., and Wu, R. (2004). IL-17 markedly up-regulates beta-defensin-2 expression in human airway epithelium via JAK and NF-kappaB signaling pathways. J. Immunol. *173*, 3482–3491.

Kapsenberg, M.L. (2003). Dendritic-cell control of pathogen-driven T-cell polarization. Nat. Rev. Immunol. *3*, 984–993.

Karpus, W.J., Lukacs, N.W., Kennedy, K.J., Smith, W.S., Hurst, S.D., and Barrett, T.A. (1997). Differential CC chemokine-induced enhancement of T helper cell cytokine production. J. Immunol. *158*, 4129–4136.

Kawai, T., and Akira, S. (2005). Pathogen recognition with Toll-like receptors. Curr. Opin. Immunol. *17*, 338–344.

Kelsall, B.L., and Rescigno, M. (2004). Mucosal dendritic cells in immunity and inflammation. Nat. Immunol. *5*, 1091–1095.

Kobayashi, K.S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., and Flavell, R.A. (2005). Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science *307*, 731–734.

Kufer, T.A., Fritz, J.H., and Philpott, D.J. (2005). NACHT-LRR proteins (NLRs) in bacterial infection and immunity. Trends Microbiol. *13*, 381–388.

Langrish, C.L., Chen, Y., Blumenschein, W.M., Mattson, J., Basham, B., Sedgwick, J.D., McClanahan, T., Kastelein, R.A., and Cua, D.J. (2005). IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J. Exp. Med. *201*, 233–240.

Lee, C.K., Weltzin, R., Thomas, W.D., Jr., Kleanthous, H., Ermak, T.H., Soman, G., Hill, J.E., Ackerman, S.K., and Monath, T.P. (1995). Oral immunization with recombinant *Helicobacter pylori* urease induces secretory IgA antibodies and protects mice from challenge with *Helicobacter felis*. J. Infect. Dis. *172*, 161–172.

Liang, S.C., Tan, X.Y., Luxenberg, D.P., Karim, R., Dunussi-Joannopoulos, K., Collins, M., and Fouser, L.A. (2006). Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J. Exp. Med. *203*, 2271–2279.

Lukacs, N.W., Chensue, S.W., Karpus, W.J., Lincoln, P., Keefer, C., Strieter, R.M., and Kunkel, S.L. (1997). C-C chemokines differentially alter interleukin-4 production from lymphocytes. Am. J. Pathol. *150*, 1861–1868.

Lutz, M.B., Kukutsch, N., Ogilvie, A.L., Rossner, S., Koch, F., Romani, N., and Schuler, G. (1999). An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. J. Immunol. Methods *223*, 77–92.

Magalhaes, J.G., Philpott, D.J., Nahori, M.A., Jehanno, M., Fritz, J., Bourhis, L.L., Viala, J., Hugot, J.P., Giovannini, M., Bertin, J., et al. (2005). Murine Nod1 but not its human orthologue mediates innate immune detection of tracheal cytotoxin. EMBO Rep. *6*, 1201–1207.

Mangan, P.R., Harrington, L.E., O'Quinn, D.B., Helms, W.S., Bullard, D.C., Elson, C.O., Hatton, R.D., Wahl, S.M., Schoeb, T.R., and Weaver, C.T. (2006). Transforming growth factor-beta induces development of the T(H)17 lineage. Nature *441*, 231–234.

Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M. (2004). The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. *25*, 677–686.

Martin, R.M., Brady, J.L., and Lew, A.M. (1998). The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. J. Immunol. Methods *212*, 187–192.

Nemazee, D., Gavin, A., Hoebe, K., and Beutler, B. (2006). Immunology: Toll-like receptors and antibody responses. Nature *441*, E4.

Owyang, A.M., Zaph, C., Wilson, E.H., Guild, K.J., McClanahan, T., Miller, H.R., Cua, D.J., Goldschmidt, M., Hunter, C.A., Kastelein, R.A., and Artis, D. (2006). Interleukin 25 regulates type 2 cytokine-dependent immunity and limits chronic inflammation in the gastrointestinal tract. J. Exp. Med. *203*, 843–849.

Pasare, C., and Medzhitov, R. (2005). Control of B-cell responses by Toll-like receptors. Nature *438*, 364–368.

Rimoldi, M., Chieppa, M., Salucci, V., Avogadri, F., Sonzogni, A., Sampietro, G.M., Nespoli, A., Viale, G., Allavena, P., and Rescigno, M. (2005). Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat. Immunol. *6*, 507–514.

Schnare, M., Barton, G.M., Holt, A.C., Takeda, K., Akira, S., and Medzhitov, R. (2001). Toll-like receptors control activation of adaptive immune responses. Nat. Immunol. *2*, 947–950.

Schwarzenberger, P., Huang, W., Ye, P., Oliver, P., Manuel, M., Zhang, Z., Bagby, G., Nelson, S., and Kolls, J.K. (2000). Requirement of endogenous stem cell factor and granulocyte-colony-stimulating factor for IL-17-mediated granulopoiesis. J. Immunol. *164*, 4783–4789.

Stetson, D.B., Voehringer, D., Grogan, J.L., Xu, M., Reinhardt, R.L., Scheu, S., Kelly, B.L., and Locksley, R.M. (2004). Th2 cells: orchestrating barrier immunity. Adv. Immunol. *83*, 163–189.

Sutton, C., Brereton, C., Keogh, B., Mills, K.H., and Lavelle, E.C. (2006). A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. J. Exp. Med. *203*, 1685–1691.

Tada, H., Aiba, S., Shibata, K., Ohteki, T., and Takada, H. (2005). Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. Infect. Immun. *73*, 7967–7976.

Ting, J.P., and Davis, B.K. (2005). CATERPILLER: a novel gene family important in immunity, cell death, and diseases. Annu. Rev. Immunol. *23*, 387–414.

van Heel, D.A., Ghosh, S., Butler, M., Hunt, K., Foxwell, B.M., Mengin-Lecreulx, D., and Playford, R.J. (2005). Synergistic enhancement of

Toll-like receptor responses by NOD1 activation. Eur. J. Immunol. *35*, 2471–2476.

Viala, J., Chaput, C., Boneca, I.G., Cardona, A., Girardin, S.E., Moran, A.P., Athman, R., Memet, S., Huerre, M.R., Coyle, A.J., et al. (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. Nat. Immunol. *5*, 1166–1174.

Weidinger, S., Klopp, N., Rummler, L., Wagenpfeil, S., Novak, N., Baurecht, H.J., Groer, W., Darsow, U., Heinrich, J., Gauger, A., et al. (2005). Association of NOD1 polymorphisms with atopic eczema and related phenotypes. J. Allergy Clin. Immunol. *116*, 177–184.

Wickelgren, I. (2006). Immunology. Mouse studies question importance of toll-like receptors to vaccines. Science *314*, 1859–1860.

Xu, W., He, B., Chiu, A., Chadburn, A., Shan, M., Buldys, M., Ding, A., Knowles, D.M., Santini, P.A., and Cerutti, A. (2007). Epithelial cells trigger frontline immunoglobulin class switching through a pathway regulated by the inhibitor SLPI. Nat. Immunol. *8*, 294–303.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., Takeuchi, O., Sugiyama, M., Okabe, M., Takeda, K., and Akira, S. (2003). Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science *301*, 640–643.

Yao, Y., Li, W., Kaplan, M.H., and Chang, C.H. (2005). Interleukin (IL)-4 inhibits IL-10 to promote IL-12 production by dendritic cells. J. Exp. Med. *201*, 1899–1903.

Ye, P., Rodriguez, F.H., Kanaly, S., Stocking, K.L., Schurr, J., Schwarzenberger, P., Oliver, P., Huang, W., Zhang, P., Zhang, J., et al. (2001). Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J. Exp. Med. *194*, 519–527.