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Intrinsic expression of Nod2 in CD4+ T lymphocytes is not necessary for the development of cell-mediated immunity and host resistance to *Toxoplasma gondii*

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Summary

Nod2 belongs to the NLR family of proteins and senses bacterial cell wall components to initiate innate immune responses against various pathogens. Recently it has been reported that T cell-intrinsic expression of Nod2 promotes host defense against *Toxoplasma gondii* infection by inducing type 1 immunity. Here we present results that demonstrate that Nod2 does not play a role in the defense against *T. gondii* infection. Nod2-deficient mice were fully capable of inducing Th1 immune responses and did not show enhanced susceptibility to infection. Upon T cell receptor stimulation *in vitro*, Nod2-deficient CD4+ T cells showed normal activation, IL-2 production, proliferation and Th1/2 differentiation. Nod2 mRNA and protein are expressed in CD4+ T and CD8+ T cells at substantial levels. Therefore Nod2, although expressed in CD4+ T cells, does not have an intrinsic function in T cell activation and differentiation.

Keywords

Toxoplasma gondii; Nod2; NLR proteins

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Conflict of interests:

The authors have no financial or commercial conflict of interest.

Introduction

Nucleotide binding domain-leucine rich repeats (NLR) proteins are a diverse set of cytoplasmic pattern recognition receptors that detect microbial products [1, 2]. The NLR family member Nod2 recognizes muramyl dipeptide (MDP), an active moiety of bacterial cell wall peptidoglycan [3–5]. Since most bacterial cell walls utilize peptidoglycan as a cell wall component, Nod2 functions as a sensor of a wide variety of bacterial strains [6, 7]. Consistent with its role in the first line of defense against pathogens, Nod2 is preferentially expressed in myeloid cells such as macrophages and epithelial cells, particularly in Paneth cells in the ileum [8–11].

It has recently been proposed that Nod2 may be crucial to the host defense against infection by *Toxoplasma gondii* [12]. *T. gondii* is an obligate intracellular protozoan parasite that can cause congenital toxoplasmosis in the fetuses of infected pregnant women and lethal encephalitis in immunosuppressed individuals [13]. An appropriate response to *T. gondii* infection involves IFN- γ mediated immune responses provided by Th1 CD4⁺ T cells and NK cells [14]. *Nod2*-deficient mice were shown to be highly susceptible to *T. gondii* due to a T cell-intrinsic defect in proliferation and Th1 differentiation [12]. This observation is highly intriguing because it shows an important role for Nod2 in T cells, a feature that has been previously unexplored in both the T cell and NLR fields. In this study, we infected *Nod2*-deficient mice and found that Nod2 does not play a role in protection against *T. gondii*. Furthermore, intrinsic expression of Nod2 in CD4⁺ T cells, was not required for proliferation, cytokine production and differentiation of Th1 lymphocytes.

Results and Discussion

Nod2 has no major role in the host defense and Th1 immune responses against *Toxoplasma gondii* infection

To investigate the role of Nod2 in the immune response to *Toxoplasma gondii*, wild-type and *Nod2*-deficient mice were intraperitoneally challenged with the ME49 strain of *T. gondii* and examined for survival. *Nod2*-deficient mice did not show any susceptibility to *T. gondii* infection (Fig. 1A). Furthermore, the numbers of *T. gondii* brain cysts were similar between wild-type and *Nod2*-deficient mice (Fig. 1A). It has been well established that IFN- γ and Th1 effector T cells are required for optimal resistance against *T. gondii* infection [14]. We therefore examined the levels of IFN- γ and IL-12, which are required for the Th1 differentiation of CD4⁺ T cells, in the *T. gondii* infected wild-type and *Nod2*-deficient mice. *T. gondii* infection increased levels of IFN- γ and IL-12p40 in peritoneal lavage as reported previously, but the levels of those cytokines in *Nod2*-deficient mice were comparable to those in wild-type control mice (Fig. 1B) [14]. Furthermore, splenocytes from *T. gondii* infected mice were cultured and assessed for cytokine production. Although there was a slight reduction in IL-12p40 production in the *Nod2*-deficient splenocytes (Fig. 1C), IFN- γ production from *Nod2*-deficient splenocytes restimulated with TLA or concanavalin A was comparable to that of splenocytes from wild-type mice. In summary, these results indicate that like wild-type mice, *Nod2*-deficient mice are resistant against *T. gondii*, and have normal Th1 responses and IFN- γ production *in vivo* during *T. gondii* infection.

Nod2-deficient CD4⁺ T cells have intact proliferation and Th1 differentiation capability

Next, to directly assess if T cell-intrinsic expression of Nod2 plays any role in T cell activation and differentiation, we investigated CD4⁺ T cells isolated from *Nod2*-deficient mice. CD4⁺ T cells were isolated from wild-type and *Nod2*-deficient mice and stimulated with anti-CD3 antibody alone or in combination with anti-CD28 antibody. The proliferation of *Nod2*-deficient CD4⁺ T cells as assessed by MTT assay and CFSE staining was similar to that of wild-type CD4⁺ T cells (Fig. 2A and B). *Nod2*-deficient CD4⁺ T cells were fully

capable of producing IL-2, also suggesting that *Nod2*-deficient CD4⁺ T cells can undergo activation upon TCR stimulation (Fig. 2C). Production of IFN- γ and IL-4 was indistinguishable between wild-type and *Nod2*-deficient CD4⁺ T cells under neutral, Th1, or Th2-skewing conditions, indicating that *Nod2*-deficient CD4⁺ T cells were fully capable of differentiating into Th1 effector cells (Fig. 2C and D). Taken together, T cell-intrinsic Nod2 does not have any role in the activation, proliferation and Th1 differentiation.

Nod2 expression in T cells

These observations led us to investigate if Nod2 is indeed expressed in CD4⁺ T cells. Transcripts from CD4⁺ and CD8⁺ T cells were quantified by qRT-PCR using Nod2-specific primers. Both CD4⁺ and CD8⁺ cells express substantial levels of Nod2 mRNA, though still lower than that of bone marrow derived macrophages (Fig. 3A). Expression of Nod2 protein was also examined by Western blot analysis; Nod2 protein was detected in sorted CD4⁺ T cells, purified either by flow cytometry (FACS CD4: purity >99%) or magnetic beads (MACS CD4: purity $\approx$ 95%), at quantities slightly less than the amount found in bone marrow derived macrophages (Fig. 3B). No Nod2 protein was detected in CD4⁺ T cells or in bone marrow derived macrophages from *Nod2*-deficient mice. As reported previously, the expression of Rip2, a kinase downstream of Nod2, was detected in both bone marrow derived macrophages and CD4⁺ T cells (Fig. 3B) [15]. Taken together, Nod2 is expressed in T cells although it does not contribute to the activation and differentiation of CD4⁺ T cells in response to anti-CD3 with or without anti-CD28 antibody, or in response to *T. gondii* infection.

In conclusion, experiments performed in three independent laboratories indicate that *Nod2*-deficient mice are not compromised in their protection against *T. gondii*. Both *T. gondii* infection studies using ME-49 strain and T cell function analysis were performed in two different laboratories independently with similar results, supporting our conclusion. *Nod2*-deficient mice are able to induce normal Th1 immune responses upon *T. gondii* infection. Moreover, *Nod2*-deficient CD4⁺ T cells have no intrinsic defect in activation, proliferation and Th1 differentiation. Our data is inconsistent with an earlier report [12] showing higher susceptibility of *Nod2*-deficient mice to *T. gondii* infection due to impaired Th1 responses. To demonstrate a T-cell intrinsic defect in Th1 differentiation, they showed that *Nod2*-deficient CD4⁺ T cells have reduced ability to differentiate into Th1 effector cells and produce IFN- γ . It was proposed that the direct association of Nod2 with c-Rel in CD4⁺ T cells is important for Th1 differentiation. However, various reports have not provided support for a T cell-intrinsic function for Nod2. In mixed leukocyte reactions (MLRs), both wild-type and *Nod2*-deficient T cells proliferate similarly [16]. In experimental autoimmune encephalomyelitis (EAE), Nod2 does not play a role in Th1 or Th17 cell differentiation, as the development of antigen-specific IFN- γ ⁺ and IL-17⁺ CD4⁺ T cells is similar to that in wild-type mice [17]. Furthermore, *Helicobacter hepaticus* colonization in *Nod2*-deficient mice leads to Th1-dominant granulomatous ileal inflammation [18]. It is unclear how to reconcile these findings with the data provided by Shaw et al. One possibility is that the earlier findings may have been due to differences in the genetic background of the mice. We backcrossed *Nod2*-deficient mice to C57BL/6 mice for 12 generations and the genetic background has been confirmed by the genome scan. The former reports also indicated that the mice were backcrossed to C57BL/6 and incomplete backcrossing may have resulted in the differences observed in T cell activation and proliferation. Another possibility would be differences in intestinal microflora. It has been shown that *Nod2*-deficient mice have altered commensal bacteria flora due to impaired bacterial killing activity in the ileum even between littermate controls with the same congenic background and diet [19]. Depending on the caging conditions of mice, the difference in intestinal microflora may be exacerbated, affecting susceptibility to *T. gondii* *in vivo*. Interestingly, although our data show that

substantial levels of Nod2 are expressed in CD4⁺ T cells, the protein has no role in the activation and Th1 differentiation of those cells. Further studies are required to uncover a functional role for Nod2 in T cells.

Materials and Methods

Mice

Nod2-deficient mice (kindly provided by Dr. Richard Flavell, Yale University) were backcrossed to C57BL/6 for 12 generations and a C57BL/6 congenic background was confirmed by the genome scan (Taconic). The mice were re-derived into specific pathogen-free, *Helicobacter*-free conditions and maintained in isolated barrier units thereafter at Taconic Farms. C57BL/6 mice were purchased from Taconic Farms and used as controls.

T. gondii infection

Mice were inoculated intraperitoneally (*i.p.*) with 25 cysts of the ME-49 strain obtained from brain homogenates, monitored for survival or sacrificed at either 8 days post-infection to collect fluid from the peritoneal cavity and spleen, or at 60 days post-infection to harvest the brain. Samples from each mouse were individually processed and analyzed. Spleens were homogenized, red blood cells were lysed (Reed Blood Cell Lysis buffer, Sigma), and splenocytes were resuspended in complete RPMI medium. Cells were cultured at 5×10^6 /well in 24-well tissue culture plates in the absence or presence of *Toxoplasma* tachyzoite lysate antigens (TLA) or concanavalin A (ConA), and supernatants were collected 48 h later for IL-12p40 and IFN γ measurement with the DuoSet ELISA kit from R&D Systems. We employed an optical microscope to count ME-49 strain cysts in brain homogenates from C57BL/6 or *Nod2*-deficient mice at 60 days post-infection. *T. gondii* infection studies were conducted in the animal facility at the University of Massachusetts Medical School and University of São Paulo, School of Medicine of Ribeirão Preto in compliance with the national and institutional guidelines with approved protocols.

Purification, proliferation, activation and polarization of splenic T cells

Splenic CD4⁺ T cells from wild-type or *Nod2*-deficient mice were purified by MACS MicroBeads (Miltenyi Biotec). Purified CD4⁺ T cells (purity $\cong$ 95%) were stimulated with plate-bound anti-mouse CD3 ϵ Ab and soluble anti-mouse CD28 Ab (eBioscience). Proliferation was assessed after 72h by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Sigma) assay. Briefly, cells were treated with MTT for the last 4h of culture and formazon crystal were dissolved in DMSO. Absorbances were recorded at 595nm using a 96 well plate reader. To analyze cell division, purified CD4⁺ T cells were stained with 1 μ M CFSE (carboxyfluorescein diacetate succinimidyl ester) (Invitrogen) and cultured for 72h. Cell division was analyzed by flow cytometry. IL-2 and IFN- γ levels in supernatants were measured by ELISA (eBioscience). For T cell-polarization experiments, splenic CD4⁺ T cells were purified using a FACSaria (BD) and stimulated with plate-bound anti-mouse CD3 ϵ Ab (5 μ g/ml) and soluble anti-mouse CD28 Ab (1 μ g/ml) for 72 h. For Th1 polarizing conditions, 5 μ g/ml of anti-IL-4 and 5 ng/ml of exogenous IL-12 were added, and for Th2 polarizing conditions, 5 μ g/ml of anti-IFN- γ and 1000U/ml of exogenous IL-4 were added into the culture medium (all Abs eBiosciences). After 72h, live cells were collected by spinning cells over Ficoll-Paque. Cells were washed and cultured in the presence of plate bound anti-mouse CD3 ϵ Ab (5 μ g/ml) for 24h and IL-4 and IFN- γ secretion were measured by ELISA.

PCR and Western blots

Splenic CD4⁺ T cells from wild-type or *Nod2*-deficient mice were purified using a FACSARIA (BD). Total RNA and protein from the cells were isolated using the RNeasy mini kit (Qiagen) and cell lysis buffer (Cell Signaling), respectively. cDNA synthesis was performed using the qScript cDNA synthesis kit (Quanta Biosciences) as per manufacturer's instructions. cDNA was used to perform real-time quantitative PCR analysis using the Quanta Biosciences Sybr Green qPCR mix (Quanta Biosciences) on an Applied Biosciences ABI 7700 real time machine. Primer pairs used in the qPCR analysis were as follows: 5'-CGACATCTCCACAGAGTTGTAATCC-3 (forward), 5'-GGCACCTGAAGTTGACATTTTGC-3 (reverse).

Protein were resolved by 4–12% gradient SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membranes. The membranes were blocked with non-fat milk, incubated overnight at 4 °C with primary antibodies and for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies. The membranes were developed using the SuperSignal West Pico chemiluminescent kit (Thermo Scientific).

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Abbreviations

NLR	Nucleotide binding domain-leucine rich repeats
TLA	Toxoplasma lysate antigen

References

1. Ting JP, Lovering RC, Alnemri ES, Bertin J, Boss JM, Davis BK, Flavell RA, Girardin SE, Godzik A, Harton JA, Hoffman HM, Hugot JP, Inohara N, Mackenzie A, Maltais LJ, Nunez G, Ogura Y, Otten LA, Philpott D, Reed JC, Reith W, Schreiber S, Steimle V, Ward PA. The NLR gene family: a standard nomenclature. *Immunity*. 2008; 28:285–287. [PubMed: 18341998]
2. Wilmanski JM, Petnicki-Ocwieja T, Kobayashi KS. NLR proteins: integral members of innate immunity and mediators of inflammatory diseases. *J Leukoc Biol*. 2008; 83:13–30. [PubMed: 17875812]
3. Girardin SE, Boneca IG, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott DJ, Sansonetti PJ. Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J Biol Chem*. 2003; 278:8869–8872. [PubMed: 12527755]
4. Inohara N, Ogura Y, Fontalba A, Gutierrez O, Pons F, Crespo J, Fukase K, Inamura S, Kusumoto S, Hashimoto M, Foster SJ, Moran AP, Fernandez-Luna JL, Nunez G. Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J Biol Chem*. 2003; 278:5509–5512. [PubMed: 12514169]
5. Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science*. 2005; 307:731–734. [PubMed: 15692051]
6. Girardin SE, Travassos LH, Herve M, Blanot D, Boneca IG, Philpott DJ, Sansonetti PJ, Mengin-Lecreulx D. Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2. *J Biol Chem*. 2003

7. Hasegawa M, Yang K, Hashimoto M, Park JH, Kim YG, Fujimoto Y, Nunez G, Fukase K, Inohara N. Differential release and distribution of Nod1 and Nod2 immunostimulatory molecules among bacterial species and environments. *J Biol Chem.* 2006; 281:29054–29063. [PubMed: 16870615]
8. Lala S, Ogura Y, Osborne C, Hor SY, Bromfield A, Davies S, Ogunbiyi O, Nunez G, Keshav S. Crohn's disease and the NOD2 gene: a role for paneth cells. *Gastroenterology.* 2003; 125:47–57. [PubMed: 12851870]
9. Ogura Y, Lala S, Xin W, Smith E, Dowds TA, Chen FF, Zimmermann E, Tretiakova M, Cho JH, Hart J, Greenson JK, Keshav S, Nunez G. Expression of NOD2 in Paneth cells: a possible link to Crohn's ileitis. *Gut.* 2003; 52:1591–1597. [PubMed: 14570728]
10. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature.* 2007; 448:427–434. [PubMed: 17653185]
11. Coombes JL, Powrie F. Dendritic cells in intestinal immune regulation. *Nat Rev Immunol.* 2008; 8:435–446. [PubMed: 18500229]
12. Shaw MH, Reimer T, Sanchez-Valdepenas C, Warner N, Kim YG, Fresno M, Nunez G. T cell-intrinsic role of Nod2 in promoting type 1 immunity to *Toxoplasma gondii*. *Nat Immunol.* 2009; 10:1267–1274. [PubMed: 19881508]
13. Elsheikha HM. Congenital toxoplasmosis: priorities for further health promotion action. *Public Health.* 2008; 122:335–353. [PubMed: 17964621]
14. Denkers EY, Gazzinelli RT. Regulation and function of T-cell-mediated immunity during *Toxoplasma gondii* infection. *Clin Microbiol Rev.* 1998; 11:569–588. [PubMed: 9767056]
15. Kobayashi K, Inohara N, Hernandez LD, Galan JE, Nunez G, Janeway CA, Medzhitov R, Flavell RA. RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature.* 2002; 416:194–199. [PubMed: 11894098]
16. Penack O, Smith OM, Cunningham-Bussell A, Liu X, Rao U, Yim N, Na IK, Holland AM, Ghosh A, Lu SX, Jenq RR, Liu C, Murphy GF, Brandl K, van den Brink MR. NOD2 regulates hematopoietic cell function during graft-versus-host disease. *J Exp Med.* 2009; 206:2101–2110. [PubMed: 19737867]
17. Shaw PJ, Barr MJ, Lukens JR, McGargill MA, Chi H, Mak TW, Kanneganti TD. Signaling via the RIP2 adaptor protein in central nervous system-infiltrating dendritic cells promotes inflammation and autoimmunity. *Immunity.* 34:75–84. [PubMed: 21236705]
18. Biswas A, Liu YJ, Hao L, Mizoguchi A, Salzman NH, Bevins CL, Kobayashi KS. Induction and rescue of Nod2-dependent Th1-driven granulomatous inflammation of the ileum. *Proc Natl Acad Sci U S A.* 107:14739–14744. [PubMed: 20679225]
19. Petnicki-Ocwieja T, Hrcir T, Liu YJ, Biswas A, Hudcovic T, Tlaskalova-Hogenova H, Kobayashi KS. Nod2 is required for the regulation of commensal microbiota in the intestine. *Proc Natl Acad Sci U S A.* 2009; 106:15813–15818. [PubMed: 19805227]

Figure 1. *Nod2* deletion does not affect resistance against *T. gondii*

(A) Survival curve (left panel) and cyst counts in the brain (right panel) of wild-type and *Nod2*-deficient mice infected i.p. with 25 cysts of the ME-49 strain of *T. gondii*. Survival was followed for 60 days (wild-type mice $n=7$; *Nod2*-deficient mice $n=7$) and at the end of the experiment animals were sacrificed for cyst counts in the brain (wild-type mice $n=4$; *Nod2*-deficient mice $n=5$). The data is representative of four independent experiments. (B) Wild-type and *Nod2*-deficient mice were infected as described above and the fluid from the peritoneal cavity was collected at 8 days post-infection for measurement of IL-12p40 (left panel) and IFN- γ (right panel). (C) Wild-type and *Nod2*-deficient mice were infected as described above; spleens were harvested at 8 days post-infection, and splenocytes were cultured in the absence of exogenous stimuli, and in the presence of TLA (20 μ g/ml) or ConA (5 μ g/ml) as indicated. IL-12p40 (left panel) and IFN- γ (right panel) levels were measured in the supernatant of spleen cells cultured for 48 h. (B–C) The data are representative of two independent experiments. Each circle and square represents data from an individual mouse. Mean values are indicated by the horizontal lines. No significant difference was observed in the results obtained from *Nod2*-deficient as compared to wild-type control mice using Student's *t*-test.

Figure 2. *Nod2* deletion has no effect on the proliferation, activation and differentiation of CD4+ T cells upon TCR engagement

(A) Proliferation of splenic CD4+ T cells from wild-type and *Nod2*-deficient mice in the presence of plate-bound anti-CD3 Ab alone or in combination with soluble anti-CD28 Ab was examined by MTT assay. (B) CFSE-stained wild-type (black line) and *Nod2*-deficient (grey line) CD4+ T cells were stimulated with plate bound anti-CD3 Ab (2 μ g/ml) and soluble anti-CD28 Ab (1 μ g/ml) for 48h and cell division was assessed by flow cytometry. Unstimulated wild-type cells are indicated by the shaded area. The data is representative of three independent experiments. (C) Wild-type and *Nod2*-deficient CD4+ T cells were stimulated with plate-bound anti-CD3 Ab alone or in combination with soluble anti-CD28 Ab at the indicated concentrations. Release of IL-2 and IFN- γ was measured by ELISA, 24 and 72 hours after stimulation, respectively in triplicate. The data is representative of four independent experiments. (D) Wild-type and *Nod2*-deficient CD4+ T cell were cultured in Th1 and Th2 polarization conditions as described in materials and methods. Cells were restimulated with plate-bound anti-CD3 Ab (5 μ g/ml) and the release of IL-4 and IFN- γ was examined by ELISA in triplicate. The data is representative of two independent experiments. Each circle and square represents data from an individual mouse. Mean values are indicated by bars.

Figure 3. Nod2 expression in splenic CD4 + T cells

Splenic CD4 + T cells were purified by FACSARIA sorting, and total RNA and protein were isolated from the cells. (A) The expression of *Nod2* in purified CD4+ or CD8+ T cells and bone marrow derived macrophages (BMDM) with/without LPS stimulation (10 ng/ml) for 6 hours was examined by quantitative real-time PCR. The data is representative of two independent experiments. (B) Western blot analysis of Nod2 and Rip2 expression in CD4+ T cells sorted by FACSARIA (purity >99%) or MACS (purity $\approx$ 95%), or bone marrow derived macrophages with or without LPS stimulation (10 ng/ml) for 16 hours. Macrophages and CD4+ T cells from *Nod2*-deficient mice were used as negative controls. β -actin was used as a loading control.