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T cell intrinsic role of Nod2 in promoting type I immunity against *Toxoplasma gondii*

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Abstract

Nod2 belongs to the (NOD)-like receptor (NLR) family of proteins, which function as intracellular pathogen sensors in innate immune cells. Nod2-deficiency results in an impaired immune response against bacterial pathogens. However, our understanding of how this protein promotes host defense against intracellular parasites is unknown. Here we found that *Nod2*$^{-/-}$ mice showed reduced clearance of *Toxoplasma gondii* and decreased interferon-γ production. Reconstitution of T-cell deficient mice with *Nod2*$^{-/-}$ T cells followed by *T. gondii* infection revealed a T cell-intrinsic defect. *Nod2*$^{-/-}$ CD4^{+} T cells displayed poor helper T cell differentiation, which was associated with impaired IL-2 production and nuclear accumulation of c-Rel. These data revealed a T cell-intrinsic role of Nod2 signaling that is critical for host defense against *T. gondii*.

Introduction

The mammalian nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins function as intracellular pattern recognition receptors and as regulators of host immunity by ‘detecting’ microbial products1. The prototypic NLR members, Nod1 and Nod2, sense fragments of peptidoglycan (PGN) from bacteria. Nod1 activity is triggered by γ-D-glutamyl-*meso*-diaminopimelic acid (*meso*-DAP), which is unique to PGN structures in all Gram-negative bacteria and certain Gram-positive bacteria, including the genus *Listeria* and *Bacillus*1. In contrast, Nod2 is activated by muramyl dipeptide (MDP), a PGN motif present in all Gram-positive and Gram-negative bacteria1. The importance of NLRs in detecting specific microbial products is underscored by the susceptibility of mice with targeted deletion of various NLR genes2. However, the role of NLRs in sensing intracellular parasites is currently unclear.

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Toxoplasma gondii is an obligate intracellular protozoan pathogen capable of infecting various animal species. Infection with *T. gondii* can cause severe disease, such as pneumonia and encephalitis, in immunocompromised hosts. One major determinant of the outcome of *T. gondii* infection is the ability of the host to elicit robust cellular immunity against the parasite. This protective immunity is mediated primarily by interleukin 12 (IL-12)-induced interferon (IFN)-γ derived from natural killer (NK) cells and T helper type 1 (T_H1) polarized T cells3, 4.

Host recognition of extracellular *T. gondii* by the toll-like receptors (TLRs) expressed on dendritic cells (DCs) promotes secretion of the pro-inflammatory cytokine IL-12. *T. gondii*-induced IL-12 production and optimal resistance to infection is dependent on TLR11, which recognizes a profilin-like protein from *T. gondii*5. Others have implicated TLR2 in parasite-induced responses6, suggesting that the parasite expresses more than one type of TLR ligand. In addition to TLRs, DC-derived IL-12 can also be induced by parasite-derived cyclophilin-18 via CCR5, a $G_{i\alpha}$ protein-coupled chemokine receptor7. From these studies, it is evident the host is endowed with extracellular sensing mechanisms that elicit immune response against *T. gondii*. However, given that *T. gondii* resides and replicates in a nonfusogenic vacuole in the cytoplasm, we sought to determine if the host employs an intracellular detection system, such as the mammalian NLRs, to sense intracellular parasites. We found that Nod2 provides a T cell-intrinsic signal that is necessary not only for generating protective T_H1 immunity against *T. gondii* but also for driving T cell-mediated colitis.

Results

Nod2 promotes resistance to *T. gondii*

To assess whether NLRs participate in host defense against *T. gondii*, mice genetically deficient in various NLR family members were inoculated intraperitoneally (i.p.) with the avirulent ME49 strain of *T. gondii*, and the survival of the animals was monitored. Among the mutant mice strains tested, mice lacking Nod1, Nlrc4, ASC, Nlrp6 and Nlrp12 exhibited normal resistance to *T. gondii* infection; only Nod2-deficient mice exhibited impaired survival after *T. gondii* infection (Figure 1a; Supplementary Figure 1). The enhanced susceptibility of Nod2-null animals was clearly distinct from that of mice lacking IL-12p35 or IFN-γ (Figure 1a). Unexpectedly, mice lacking RICK (also called Rip2), a caspase-recruitment domain (CARD)-containing kinase that has been implicated in Nod1 and Nod2 signaling8, were resistant to parasite infection (Figure 1a). The resistance of the Nod2-deficient animals to acute infection was confirmed by examining the peritoneal exudate cells (PECs) harvested from the peritoneum cavity on day 7 post-infection. In both $Nod2^{-/-}$ and wild-type animals, less than 1% of the cells were infected with the parasite; in contrast greater than 20% of the PECs recovered at the same time point from $Ifng^{-/-}$ mice were infected (Figure 1b). However, examination of PECs harvested from infected Nod2-deficient mice on day 12 revealed high numbers of infiltrating mononuclear cells harboring parasites as well as numerous extracellular parasites (Figure 1c). Furthermore, in contrast to wild-type mice, which contained few (<1%) infected cells and lower concentrations of serum IFN-γ, there was a 20-fold increase in the number of cells infected with tachyzoites

and high concentrations of circulating IFN-γ in *Nod2*$^{-/-}$ mice (Figure 1d and data not shown). Together, these results suggest that the susceptibility of *Nod2*$^{-/-}$ animals following parasite challenge appears to be due to a delayed impairment in the ability to control parasite replication at the site of infection.

Nod2$^{-/-}$ mice exhibit impaired T_H1 responses

As protective immunity against *T. gondii* is critically dependent on IL-12- induced IFN-γ production, we measured the concentrations of these two cytokines in the serum of *Nod2*$^{-/-}$ mice during infection. During the course of infection, wild-type and *Nod2*$^{-/-}$ mice secreted similar amounts of IL-12p40 (Figure 2a). In contrast, *Nod2*$^{-/-}$ animals produced lower amounts of IFN-γ than wild-type mice on day 7 post-infection (Figure 2b). Thus, the delayed failure to control parasite growth in the peritoneal cavity of *Nod2*$^{-/-}$ mice could be explained by insufficient quantities of IFN-γ produced following infection.

The major cellular sources of IFN-γ during *T. gondii* infection are NK cells9 and T_H1 lymphocytes4. However, we observed no discernible difference in IFN-γ production by IL-12-stimulated wild-type and *Nod2*$^{-/-}$ NK cells (data not shown) or by day 5 *T. gondii*-infected mice (Supplementary Figure 2). Furthermore, *in vivo* antibody-mediated depletion of NK cells during *T. gondii* challenge induced a comparable reduction in the serum concentrations of IFN-γ on day 5 post-infection in wild-type and *Nod2*$^{-/-}$ animals (Supplementary Figure 2). These experiments suggest that Nod2-deficient NK cells show no defect in IFN-γ production, and are consistent with reports that NK cells are an early source of IFN-γ during *T. gondii* infection3.

To address whether Nod2 plays a role in promoting the generation of T_H1 responses, wild-type and *Nod2*$^{-/-}$ mice were immunized with a γ-irradiated live uracil auxotrophic strain of *T. gondii* (CPS), which is non-replicative yet capable of infecting cells and inducing protective immunity10. This strategy permits the analysis of T cell activation and T_H1-differentiation under conditions of similar antigen exposure and yet circumvents host mortality. Using this approach, we detected a high frequency of effector T_H1 $CD4^+$ and $CD8^+$ T cells—defined by intracellular expression of IFN-γ after *in vitro* re-stimulation—in the peritoneal cavity of wild-type animals on day 9 post-immunization (peak of T cell response) (Figure 3). In contrast, Nod2-deficient animals following immunization exhibited a reduced percentage of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells (Figure 3). However, we detected no significant differences in the total number of $CD4^+$ and $CD8^+$ cells in the peritoneal cavity of wild-type and *Nod2*$^{-/-}$ mice (Supplementary Figure 3a). In agreement with the reduced frequency of IFN-γ$^+$ effector T cells, the amount of IFN-gamma; secreted by *Nod2*$^{-/-}$ PECs *ex vivo* was also impaired (Supplementary Figure 3b).

DCs play a major role in T cell priming and promoting IL-12-driven T_H1 immunity against *T. gondii*11. Therefore, the defective T cell response seen in Nod2-deficient mice could be due to impaired DC function. To evaluate whether *Nod2*$^{-/-}$ DCs have functional defects in processing and presenting *T. gondii* antigen, *T. gondii*-infected wild-type and Nod2-deficient bone marrow-derived DCs (BMDCs) were used to stimulate FACS purified peritoneal $CD4^+$ T lymphocytes derived from day 9 CPS immunized wild-type or *Nod2*$^{-/-}$ animals. Both *Nod2*$^{-/-}$ and wild-type BMDCs efficiently induced IFN-γ expression in wild-type $CD4^+$ T

cells (Fig. 4a). Interestingly, IFN-γ production by *Nod2*$^{-/-}$ CD4^{+} T cells remained attenuated, even in the presence of wild-type DCs. Consistent with the flow cytometry analysis, *ex vivo* cultures containing Nod2-deficient CD4^{+} cells secreted reduced quantities of IFN-γ when stimulated with parasite-infected wild-type or *Nod2*$^{-/-}$ BMDCs (Figure 4b). To confirm that the blunted T cell response observed in *Nod2*$^{-/-}$ mice is not due to impaired antigen presentation and/or T cell priming by *Nod2*$^{-/-}$ DCs, wild-type (Thy-1.1^{+}) CD4^{+} T cells were adoptively transferred into wild-type or *Nod2*$^{-/-}$ recipient mice. These chimaeras were then immunized with *T. gondii*. Similar frequencies of donor (Thy-1.1^{+}) IFN-γ^{+} CD4^{+} T cells were observed in wild-type and *Nod2*$^{-/-}$ recipients of wild-type T cells (Fig. 4c). Collectively, these findings argue that *Nod2*$^{-/-}$ DCs are functionally normal during *T. gondii* infection and suggest that Nod2 functions in a T cell- intrinsic manner to promote optimal T_H1 immunity against *T. gondii*.

T cell-intrinsic role for Nod2

To directly assess the functional role of Nod2 in T cells, wild-type and *Nod2*$^{-/-}$ CD4^{+} T cells were adoptively transferred into *Tcrb*$^{-/-}$ or *Tcrb*$^{-/-}$ *Nod2*$^{-/-}$ recipients. The reconstituted mice were then immunized with CPS and the frequency of effector T cells was assessed 9 days later. *Tcrb*$^{-/-}$ and *Tcrb*$^{-/-}$*Nod2*$^{-/-}$ recipient mice reconstituted with wild-type CD4^{+} T cells exhibit high frequencies of IFN-γ^{+} CD4^{+} T lymphocytes following parasite challenge (Figure 5a). However, there was a significant reduction in the percentage of IFN-γ^{+} CD4^{+} T lymphocytes in mice reconstituted with Nod2-deficient CD4^{+} T cells regardless of the genotype of the recipient mice (Figure 5a). The impaired CD4^{+} T cell response is consistent with the lower IFN-γ production by PECs harvested from *Tcrb*$^{-/-}$ and *Tcrb*$^{-/-}$ *Nod2*$^{-/-}$ mice reconstituted with *Nod2*$^{-/-}$ CD4^{+} T cells (Figure 5b). Lastly, the resistance of *Nod2*$^{-/-}$ mice following *T. gondii* infection was enhanced by the presence of wild-type T cells (Supplementary Figure 4). Taken together, these findings indicate that the impaired T cell response observed in *Nod2*$^{-/-}$ mice during infection is associated with an intrinsic defect in Nod2-deficient T cells.

Nod2 in T_H cell differentiation and IL-2 production

To better understand the functional role of Nod2 in an *in vitro* antigen-specific T cell response, naïve *Nod2*$^{-/-}$ ovalbumin (OVA)-specific OT-II TCR transgenic cells were stimulated with OVA-pulsed BMDCs. Wild-type and *Nod2*$^{-/-}$ OT-II cells stimulated with OVA expressed similar amounts of early activation markers, such CD25 (Figure 6a) and CD69 (data not shown). This unimpaired T cell activation was also reflected in the similar frequency of activated CD44^{high}CD62L^{low} CD4^{+} lymphocytes (Figure 6a). However, unexpectedly, *Nod2*$^{-/-}$ OT-II cells stimulated in the presence of wild-type or *Nod2*$^{-/-}$ DCs exhibited impaired IL-2 production. (Figure 6b).

IL-2 plays a critical role in T cell priming for both IFN-γ and IL-4 production11. To investigate whether the impaired IL-2 production by *Nod2*$^{-/-}$ T cells results in attenuated T_H1 or T_H2 cell development, naïve wild-type OT-II and *Nod2*$^{-/-}$ OT-II cells were cultured under T_H1 or T_H2 polarizing conditions. Under T_H1 conditions, wild-type T cells produced high concentrations of IFN-γ. In contrast, differentiation of naïve *Nod2*$^{-/-}$ T cells into T_H1 cells was severely impaired (Figure 6c). IL-4 production by Nod2-deficient cells cultured

under T_H2 polarizing conditions was also significantly reduced (Figure 6c). To determine whether the reduced IL-2 production by *Nod2*$^{-/-}$ T cells was accountable for the impaired T_H cell differentiation, exogenous IL-2 was added to the cultures; exogenous IL-2 was sufficient to rescue the impaired T_H1 and T_H2 differentiation of *Nod2*$^{-/-}$ T cells (Figure 6d).

General role of Nod2 in T cell responses

Our finding that Nod2 functions in a T cell-autonomous fashion raises the question of whether other T cell responses would be adversely affected by Nod2-deficiency. To address this possibility, we utilized various adoptive transfer systems where the innate immune cells in recipient animals are Nod2-sufficient while the donor T cells are either Nod2-sufficient or Nod2-deficient. First, we probed whether *Nod2*$^{-/-}$ T cells are capable of undergoing homeostatic proliferation or 'lymphopenia-induced proliferation' (LIP), a space-driven expansion of T cell populations that acts as a compensatory mechanism to restore the peripheral T lymphocyte pool under conditions of lymphopenia12. *Nod2*$^{-/-}$ donor T cells, if transferred alone, persisted in a lymphopenic host (Supplementary Figure 4a). However, when co-transferred with wild-type $CD4^+$ T cells into the same hosts, Nod2-deficient T cells underwent little LIP (Supplemental Figure 4b). This result suggests that *Nod2*$^{-/-}$ cells are unsuccessful at competing for the limited resources required for LIP.

As an additional test to evaluate the role of Nod2 in regulating T cell function, we utilized a mouse model of colitis whereby the transfer of purified $CD4^+CD25^-CD45RB^{hi}$ T cells into *Rag1*$^{-/-}$ mice leads to colitis driven by T_H1 cytokines13, 14. Wild-type $CD4^+CD25^-CD45RB^{hi}$ T cells predictably induced wasting disease (Figure 7a). In contrast, *Rag1*$^{-/-}$ mice reconstituted with *Nod2*$^{-/-}$ $CD4^+CD25^-CD45RB^{hi}$ T cells remained healthy and none exhibited wasting disease, as evidenced by the increase in body weight (Figure 7a). At gross examination, the large intestine was thickened in *Rag1*$^{-/-}$ recipients of wild-type, but not *Nod2*$^{-/-}$ $CD4^+CD25^-CD45RB^{hi}$ cells (Figure 7b). Microscopic examination of the small and large intestine isolated from *Rag1*$^{-/-}$ mice reconstituted with wild-type $CD4^+CD25^-CD45RB^{hi}$ cells revealed an increase in inflammatory cell accumulation (Figure 7c). In contrast, sections from the intestines of *Rag1*$^{-/-}$ recipients of *Nod2*$^{-/-}$ $CD4^+CD25^-CD45RB^{hi}$ cells revealed substantially less severe histological lesions (Figure 7c). Consistent with the microscopic examination, *Rag1*$^{-/-}$ recipients of Nod2-deficient T cells had lower histologic scores (Figure 7c), and T cells in the mesenteric lymph nodes of these mice produced less IFN-γ (Figure 7d). Taken together, these data support a direct role of Nod2 in regulating T cell function.

Nod2 binds c-Rel and enhances *Il2* transcription

The blunted LIP, T cell-driven colitis and IL-2 secretion by *Nod2*$^{-/-}$ T cells suggests that Nod2 may function downstream of either TCR or co-stimulatory signals. As TCR-driven phosphorylation of Jnk, p38 and Erk MAP kinases was similar in wild-type and Nod2$^{-/-}$ T cells (Supplementary Figure 5), we investigated whether Nod2 is involved in the CD28 costimulatory signaling pathway. The production of IL-2 in response to CD28 co-stimulation depends on the activation of the NF-κB subunit, c-Rel, which interacts with the NF-κB binding sites as well as CD28-responsive elements (CD28RE) in the proximal *Il2* promoter region15, 16. The relevance of c-Rel for IL-2 production is underscored by the

observation that T cells from c-Rel-deficient mice exhibit a profound deficiency in IL-2 production17. The ability of c-Rel to interact with the CD28RE to induce IL-2 production is regulated by NF-κB inducing kinase, NIK18 (also called MAP3KI4). T cells from alymphoplasia (*aly/aly*) mice that bear a NIK mutation also exhibit defects in IL-2 secretion in response to CD3 and CD28 stimulation19. In light of these findings, we hypothesized that Nod2 may interact with c-Rel and/or NIK in T lymphocytes to promote IL-2 production. To determine if Nod2 can interact with c-Rel or NIK, HEK293T cells were transiently transfected with expression vectors encoding c-Rel, NIK and/or Nod2. As previously reported, NIK coimmunoprecipitated with Nod2 (Figure 8a)20. Interestingly, c-Rel was also present in Nod2 immunoprecipitates (Figure 8a). When Nod2, NIK and c-Rel were simultaneously coexpressed, both NIK and c-Rel coimmunoprecipitated with Nod2 (Figure 8a), suggesting that these three proteins can form a tertiary complex. As a specificity control, Bcl10, which is known to play a role in T cell receptor signaling, associated with CARMA1 as previously reported21, 22; however, no interaction was observed between Bcl10 and Nod2 (Figure 8a)

Having established the existence of an interaction between Nod2, NIK and c-Rel, we next assessed whether Nod2 plays a functional role in regulating IL-2 transcription induced by CD28 co-stimulation in Jurkat T cells. Cells were transiently transfected with a CD28RE-AP1 reporter construct along with different combinations of NIK, c-Rel and/or Nod2 expression vectors. As expected, transfection of c-Rel significantly upregulated CD28RE-AP1 reporter transcription, and the presence of NIK synergistically enhanced the ability of c-Rel to upregulate CD28RE-AP1 reporter transcription18. Notably, Nod2 synergistically enhanced c-Rel-driven CD28RE-AP1 reporter activity, and co-expression of c-Rel, NIK and Nod2 resulted in maximal activation of the CD28RE-AP1 element, despite the fact that Nod2 by itself or in the presence of NIK did not induce any significant reporter activity (Figure 8b). These results are consistent with a positive role for Nod2 in the transcriptional regulation of *Il2*.

Finally, the finding that Nod2 interacts with both NIK and c-Rel prompted us to assess whether Nod2 deficiency could impact c-Rel nuclear accumulation, an event critical for *Il2* transcription. Nuclear extracts from CD28-costimulated wild-type and *Nod2*$^{-/-}$ T cells were prepared and the presence of nuclear c-Rel was examined. Consistent with previous studies16, substantial nuclear accumulation of c-Rel was observed in wild-type cells after 6–9 hours of stimulation (Figure 8c). In contrast, *Nod2*$^{-/-}$ cells exhibited less than half the amount of nuclear c-Rel at the same time points after stimulation. The impaired nuclear accumulation of c-Rel was not a general defect affecting all NF-κB subunits induced following co-stimulation, as similar amounts of phosphorylated-p65 were observed in the nuclei of wild-type and *Nod2*$^{-/-}$ cells (Figure 8c). Taken together these data suggest that the Nod2 is required for optimal c-Rel nuclear accumulation and *Il2* gene transcription.

Discussion

The present study demonstrated that Nod2 is required for host resistance against *T. gondii*. The increased susceptibility of *Nod2*$^{-/-}$ mice following *T. gondii* infection was unexpected, given that Nod2 was initially identified as a sensor for intracellular bacteria. To our

knowledge, *T. gondii* does not contain motifs analogous to the Nod2 agonist MDP. It is possible that Nod2 promotes host immune responses against *T. gondii* by 'sensing' other parasite-derived protein(s) injected into the host cytosol during the invasion process23, 24. However, this appears unlikely given that *T. gondii* infected *Nod2*$^{-/-}$ DCs and macrophages exhibited unimpaired cytokine secretion, as well as MAP kinase activation (data not shown). Thus, the decreased resistance of *Nod2*$^{-/-}$ mice following *T. gondii* infection is not associated with defects in pathogen recognition.

Our *in vivo* transfer experiments establish that Nod2 plays a T cell-intrinsic role in the generation of an effective T_H1 response. This work is the first to demonstrate that Nod2 is required for optimal IL-2 production by activated T cells. IL-2 plays a multi-faceted role in T cell biology including T cell differentiation25, 26, 27. Accordingly, the attenuated IL-2 secretion by Nod2-deficient T cells manifests as a profound impairment in T_H cell differentiation. Addition of exogenous IL-2 during *in vitro* polarization was sufficient to fully rescue the impaired T_H2 differentiation of naïve *Nod2*$^{-/-}$ CD4$^+$ T cells, and to partially (>65%) rescue the impaired T_H1 differentiation. This finding suggests that Nod2 promotes T_H1 differentiation via IL-2-dependent and IL-2-independent pathways. Furthermore, the presence of wild-type CD4$^+$ T cells *in vivo* was able to partially rescue *Nod2*$^{-/-}$ animals following parasite challenge, presumably due to the paracrine action of IL-2 produced by wild-type CD4$^+$ T lymphocytes28. Thus, the impaired IL-2 production by *Nod2*$^{-/-}$ T cells during activation is sufficient to alter T_H cell differentiation thereby resulting in a decreased resistance of *Nod2*$^{-/-}$ animals against *T. gondii*, a pathogen known to induce a vigorous T_H1 response.

The observed impaired T cell function in the absence of Nod2 is not limited to IFN-γ production following *T. gondii* infection, but also to lymphopenia induced T cell expansion and T cell driven colitis, two models systems that are positively driven by the interaction of the TCR and CD28 with peptide-MHC complexes and B7 molecules on accessory cells, respectively29–33. Given that TCR signaling appeared to be largely intact in the absence of Nod2, we hypothesized that Nod2 may impinge on the CD28 signaling pathway, which results in the activation of genes that depend on c-Rel or NF-κB p50-p65 complexes33. Genes belonging to the former category include IL-217. Our results demonstrating that Nod2 can interact with c-Rel and NIK, and that the presence of Nod2 boosts *Il2* reporter element transcription induced by c-Rel and NIK, support our hypothesis that Nod2 acts downstream of CD28 signaling pathway. Furthermore, nuclear accumulation of c-Rel was reduced in the absence of Nod2. Taken together, our data suggests that not only can Nod2 interact with c-Rel, but this interaction also facilitates nuclear entry of c-Rel required for *Il2* transcription.

It is conceivable that Nod2, which contains two N-terminal CARD-domains, is functions as a scaffolding protein that promotes interaction with other CARD-containing proteins, such as Bcl10 and CARD11, which have been demonstrated to promote NF-κB signaling following T cell activation34. However, this appears unlikely given that interaction between Nod2 and Bcl10 was undetectable, and that *Nod2*$^{-/-}$ T cells are phenotypically distinct from T cells lacking Bcl10 or CARD1135, 36. In addition to the CARD-domains, Nod2 contains a series of C-terminal leucine-rich repeats that may mediate protein-protein interactions37.

Thus in light of our findings, we propose that Nod2, in addition to sensing microbial products in innate immune cells such as macrophages and DCs, can also operate as a molecular scaffold integrating costimulatory signals necessary for proper T cell function.

Nod2 activation in macrophages following MDP stimulation results in the recruitment and activation of RICK, a serine-threonine kinase essential for the activation NF-κB and MAPKs, resulting in the release of pro-inflammatory cytokines and chemokines8. In our studies, Nod2 activation in T cells appears to trigger the induction of a distinct signaling pathway not shared with MDP-stimulated macrophages. During *T. gondii* infection, RICK was dispensable for the induction of an effective type 1 immune response; this finding is consistent with previous results demonstrating that type I immune responses are normal in the absence of RICK38, 39. Taken together, our data suggests that, at least in T cells, Nod2 has a functional role independent of MDP. Therefore, the two divergent signaling pathways induced by Nod2 may be dependent on the context of the stimulus, as well as the responding cell type.

The role of Nod2 in regulating T cell function has been the focus of recent studies. The results from these studies suggest a differential requirement for Nod2 to promote optimal antibody responses under various experimental conditions. For example, Nod2-deficient mice exhibited normal OVA-specific T_H2 responses after immunization with a Nod1 agonist40 and normal HSA-specific IgG production following treatment with a TLR7 agonist41. In contrast, antigen-specific antibody production was reduced in response to Incomplete Freund's adjuvant and antigen (without the addition of either TLR or NLR agonists) in the absence of Nod242. The latter finding would be consistent with our current observation that Nod2 regulates adaptive immunity independent of its innate role in 'sensing' MDP. The discrepancy in the requirements for Nod2 in generating effective adaptive immunity in these studies may be due to the use of different adjuvants, which could differentially influence the expression of other B7-related co-stimulatory molecules43 and/or tumor necrosis factor receptor superfamily molecules44 (which are involved in T cell activation) on antigen-presenting cells. Thus, the influence of Nod2 on adaptive immune responses *in vivo* may be determined by whether or not CD28 signaling is preferentially required. Although the precise mechanistic role of Nod2 in bridging c-Rel nuclear accumulation and *Il2* transcription in T cells remains to be fully elucidated, the *in vivo* relevance of Nod2 function in T cells was clearly demonstrated by the challenge of *Nod2*$^{-/-}$ mice with *T. gondii*. Our results are in accord with published reports demonstrating the requirement for IL-245 and T cell-intrinsic expression of c-Rel46 in host resistance against *T. gondii*. Furthermore, the impaired ability of *Nod2*$^{-/-}$ T cells to proliferate homeostatically and drive colitis suggests a more global impairment in the ability of the host to mount an effective immune response in the absence of Nod2. Finally, our finding that Nod2 directly participates in the generation of adaptive immune responses underscores the importance of this protein in regulating both innate and adaptive immunity.

Online Methods

Mice

All mice were purchased from the Jackson Laboratory. *Nod2*$^{-/-}$ mice have been described 41. All mice (B6 background) were bred and maintained under specific pathogen-free condition at the University of Michigan animal facility. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals.

T. gondii Infection

Cysts of the avirulent ME49 strain of *T. gondii* were obtained from the brains of chronically infected mice. Mice were challenged with 20 *T. gondii* cysts by i.p. injection. To assess parasite burden, cytospin preparations were made from peritoneal lavage collected on day 7 and 12 post-infection and the number of infected cells enumerated by microscopy. Vaccinations consisted of a single i.p. dose of 1×10^6 of the CPS strain of parasites that had been irradiated at 15 krad (dose rate of 2Gy/min).

Cell culture and cytokine assays

Single-cell suspensions were prepared from individual peritoneum from each mouse following infection. Cell suspensions were cultured in complete RPMI 1640 (Invitrogen Life Technologies) with live CPS tachyzoites for 24 h. For *in vitro* restimulation of PEC $CD4^+$ T cells with *T. gondi*i-infected DC (Toxo-DC), peritoneal $CD4^+$ T lymphocytes from day 9 CPS primed wild-type or *Nod2*$^{-/-}$ mice were FACS sorted (10^5) and subsequently co-incubated with 5×10^4 Toxo-DC in 96-well plate and the culture supernatants were harvested at 24 h for cytokine analysis. For *in vitro* T cell activation and IL-2 production, splenic $CD4^+$ T cells from wild-type OT-II or *Nod2*$^{-/-}$ OT-II transgenic mice were purified using MACs microbeads (Miltenyi Biotec). Purified (>95%) $CD4^+$ T cells were then co-cultured with OVA loaded wild-type or *Nod2*$^{-/-}$ BMDC (1:1 ratio). At the indicated time points, the culture supernatants were harvested and the cells were subjected to flow cytometric analysis for various T cell activation markers. For T cell polarization experiments, $CD4^+$ cells were incubated with an equal number of OVA-loaded BMDCs with or without exogenous IL-2 (10 ng/ml). For T_H1 conditions, 10 μg/ml of α-IL-4 (clone 11B11) and 5 ng/ml of exogenous IL-12 were added. For T_H2 conditions, 10 μg/ml of α-IL-12, 10 μg/ml of α-IFN-γ (clone XMG1.2), and 10 ng/ml of exogenous IL-4 were added (all from eBiosciences). Cells were cultured for 3 days and rested for 3 additional days. Polarized T_H1 or T_H2 cells were harvested after 6 days of culture, counted, and restimulated for 24 h with plate-bound α-CD3 (1 μg/ml) and IFN-γ and IL-4 secretion was measured by ELISA. The Jurkat human leukemic T cell line was cultured in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine and antibiotics

Flow cytometry

PECs from day 9 CPS primed animals were cultured in RPMI 1640 complete medium in the presence or absence of live CPS tachyzoites for 6 h, with the addition of brefeldin A (10 μg/mL) during the last 2 h. Surface staining was performed with directly conjugated mAbs

from eBiosciences. Following surface staining, cells were fixed and permeabilized with Cytofix/Cytoperm and Perm/Wash (BD Biosciences). For staining of intracellular cytokine, allophycocyanin-conjugated anti-IFN-γ (BD Biosciences) was added to permeabilized cells. For detection of intracellular IFN-γ from Toxo-DC $CD4^+$ T cells co-cultures, peritoneal $CD4^+$ T lymphocytes from individual day 9 CPS primed mice were FACS sorted and incubated with Toxo-DC at a 1:1 ratio for 24 h, with the addition of brefeldin A during the last 4 h; staining was performed as described above. Flow cytometry analysis was performed with a FACSCalibur on 10^5 cells. Data analysis was performed using FlowJo software (Tree Star, Inc.).

Preparation of APCs

For Toxo-DC preparation, mouse BMDCs from wild-type or *Nod2*$^{-/-}$ animals were obtained as previously described 47 and infected with irradiated *T. gondii* tachyzoites at four parasites per DC for 12 h. After incubation, free parasites were removed by low speed centrifugation for 10 min. For OVA-DC preparation, BMDCs were incubated with 2.5 μg/mL CpG oligonucleotide (InvivoGen) in the presence or absence of 20 μg/mL ovalbumin (Worthington) for 2 h. After incubation, DCs were washed three times in complete media and used to stimulate wild-type OT-II or *Nod2*$^{-/-}$ OT-II transgenic $CD4^+$ T cells.

Adoptive transfer

Total splenic $CD4^+$ T cells from wild-type $Thy1.1^+$ and *Nod2*$^{-/-}$ $Thy1.1^+$ mice were isolated as described above. $CD4^+$ T cells (5×10^5) were injected intravenously through the tail veins of either *Tcrb*$^{-/-}$ or *Nod2*$^{-/-}$*Tcrb*$^{-/-}$ recipient mice. Reconstituted mice were then challenged on the same day with 1×10^6 irradiated CPS. Day 9 PECs were then isolated for analysis as described above. For *Rag1*$^{-/-}$ reconstitution, FACs purified $CD4^+CD25^-CD45RB^{hi}$ cells were intravenously injected into *Rag1*$^{-/-}$ recipients.

In vitro biochemical assays

Purified $CD4^+$ cells (4×10^6) were stimulated in 24-well plates (Corning) coated with α-CD3 (1 μg/ml) and α-CD28 (10 μg/ml) (BD Biosciences) for the indicated times. Nuclear and cytoplasmic fractions from stimulated cells were extracted using NE-PER reagents (Pierce Biotechology). For immunoblotting, 1μg of both nuclear and cytoplasmic extracts was used in subsequent immunoblotting with antibodies specific for c-Rel (Santa Cruz Biotechology) and p65 (Cell Signaling).

Transfection and luciferase assays

Transcriptional activity in Jurkat cells was measured using pCD28RE/AP-1luc reporter plasmid (gift from A. Weiss, University of California San Francisco, CA) after transient transfection of exponentially growing cells (2×10^6 cells/ml in OPTIMEM medium), with the Lipofectamine PLUS reagent (Life Technologies Inc). The total amount of DNA in each transfection was kept constant by using the corresponding empty expression vectors. After 4 h of incubation, RPMI medium containing 5% FBS was added to cells and the incubation was continued for 16 h to complete transfection. Cells were harvested, lysed and luminiscence was measured for 10 seconds in a luminometer using the Dual-luciferase

Assay System Kit (Promega). Data are expressed in relative firefly luciferase units (RLUs) normalized by the relative renilla luciferase units obtained in the control samples of every transfection and μg of protein.

Statistics

Prism software (GraphPad) was used to determine the statistical significance of differences in the means of experimental groups using unpaired, two-tailed Student's *t* tests, unless otherwise specified. A *P* value of <0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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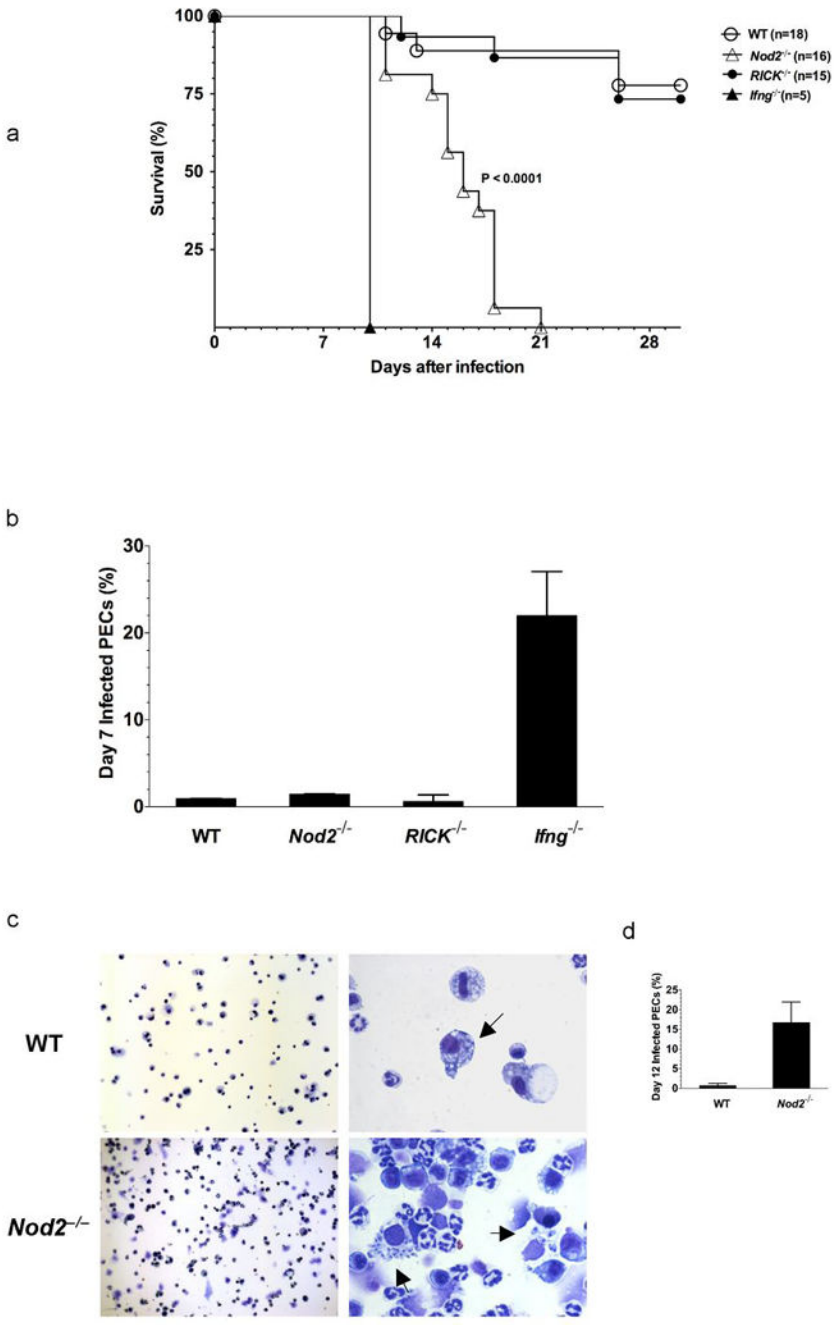

Figure 1.

Nod2 in host defense against *T. gondii* infection. Wild-type (WT), *Nod2*$^{-/-}$, *RICK*$^{-/-}$ and *Ifng*$^{-/-}$ mice were challenged i.p. with 20 cysts of the ME49 strain of *T. gondii*. (a) Survival was monitored over 30 days. (b) The percentage of tachyzoite-infected peritoneal exudate cells (PEC) was enumerated on day 7. (c) Cytospins of PECs from day 12 infected wild-type and *Nod2*$^{-/-}$ mice were prepared and stained to visualize intracellular parasites (left panels 20× magnification; right panles 100× magnification; arrows indicate parasites). (d) On day 12 post-challenge, the percentage of infected PECs in infected wild-type and *Nod2*$^{-/-}$ mice

was determined. In (a) the *P* value was determined using the Mantel-Cox log-rank test and is representative of at least four similar experiments with 5–20 mice per group. In (b-d) values shown are the mean ± s.d. of infected PECs and are representative of at least three experiments with 3–5 mice per group.

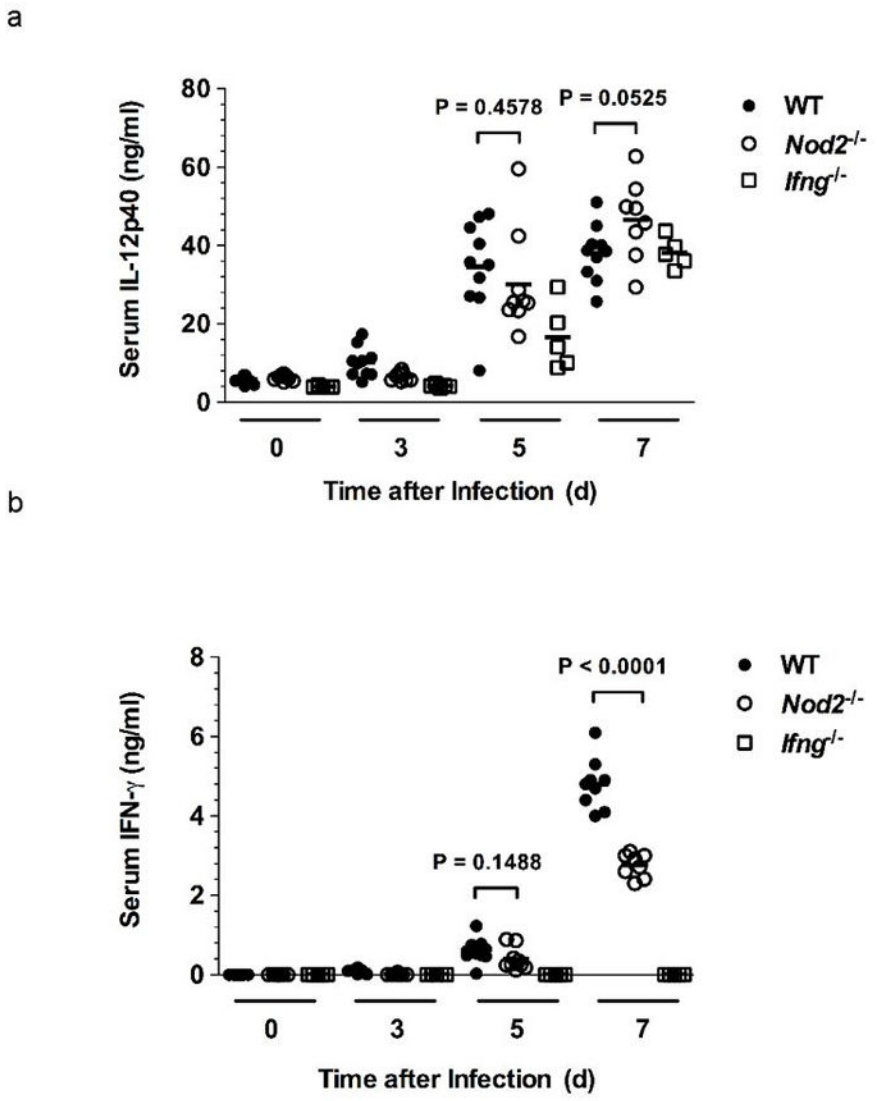

Figure 2.

Nod2-deficient mice exhibit impaired IFN-γ production despite normal IL-12p40 production. Serum from *T. gondii*-infected wild-type, *Nod2*$^{-/-}$, and *Ifng*$^{-/-}$ mice were collected on day 0, 3, 5 and 7 post-inoculation and the concentrations of (a) IL-12p40 and (b) IFN-γ were determined by ELISA. The horizontal bars represent the mean of 5–10 mice per group and are representative of at least three independent experiments.

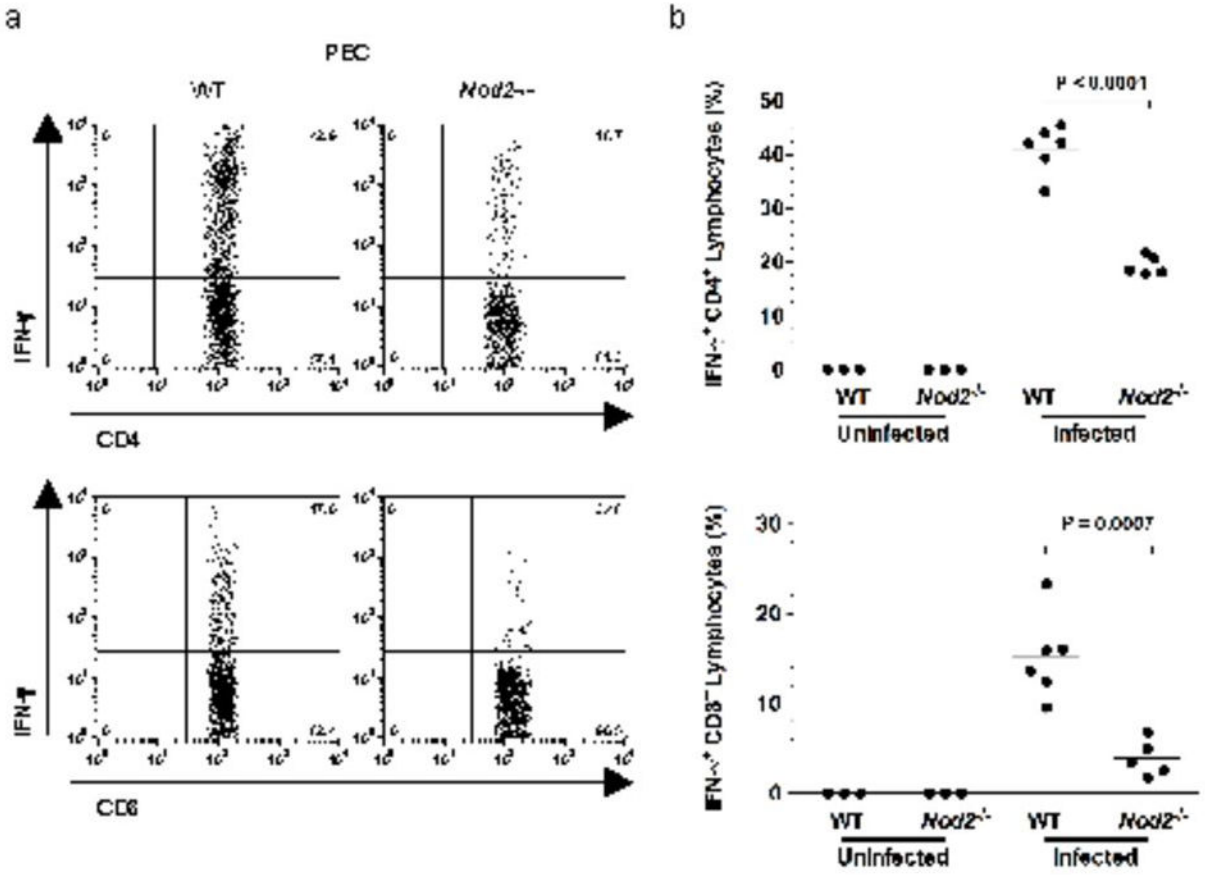

Figure 3.

Nod2-deficient mice exhibit impaired type I immune responses during *T. gondii* infection. (a) $CD4^+$ and $CD8^+$ lymphocytes among PECs from CPS-immunized wild-type and $Nod2^{-/-}$ mice were stimulated for 6 h *in vitro* with live CPS tachyzoites. IFN-γ production was measured by flow cytometry. Each dot-plot is gated on $CD4^+TCR\text{-}\beta^+$ cells and is from one representative mouse per group (n = 3–6 mice per group). (b) Data shown in (a) were pooled; each dot represents one mouse and horizontal bars represent the mean. All data shown are representative of at least three experiments with 3–6 mice per group and all mice were analyzed individually by flow cytometry.

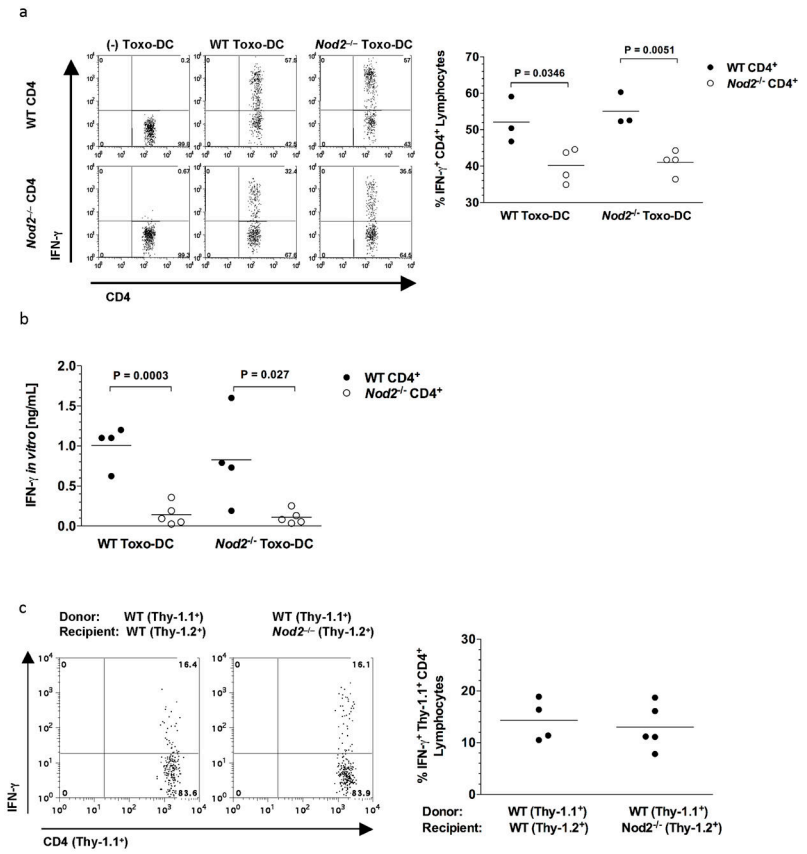

Figure 4.

DC function during *T. gondii* infection is unaffected in the absence of Nod2. (a) Left, FACs sorted $CD4^+$ T cells from the peritoneal cavity of day 9 CPS-immunized wild-type and *Nod2*$^{-/-}$ mice were stimulated for 6 h with or without wild-type or *Nod2*$^{-/-}$ BMDCs that were infected with *T. gondii*. IFN-γ production was measured by flow cytometry. Each dot-plot is gated on $CD4^+TCR\text{-}\beta^+$ cells and is from one representative mouse per group (n = 3–4 mice per group). Right, data were pooled; each dot represents one mouse and horizontal bars represent the mean. (b) IFN-γ secretion by peritoneal $CD4^+$ cells was determined as described in (a), but cells were restimulated for 24 h *in vitro*. (c) Left, MACS-sorted splenic $CD4^+$ cells from naïve wild-type (Thy-1.1) mice were injected intravenously into wild-type (Thy-1.2) or *Nod2*$^{-/-}$ (Thy-1.2) recipients, which were then challenged i.p. with irradiated CPS parasite. On day 9 post-infection, PECs were isolated and IFN-γ production by donor CD4 cells was determined by intracellular cytokine staining (left; dot plots were gated on $CD4^+TCR\beta^+Thy\text{-}1.1^+$ cells). Right, the data were pooled; each dot represents one mouse and horizontal bars represent the mean.

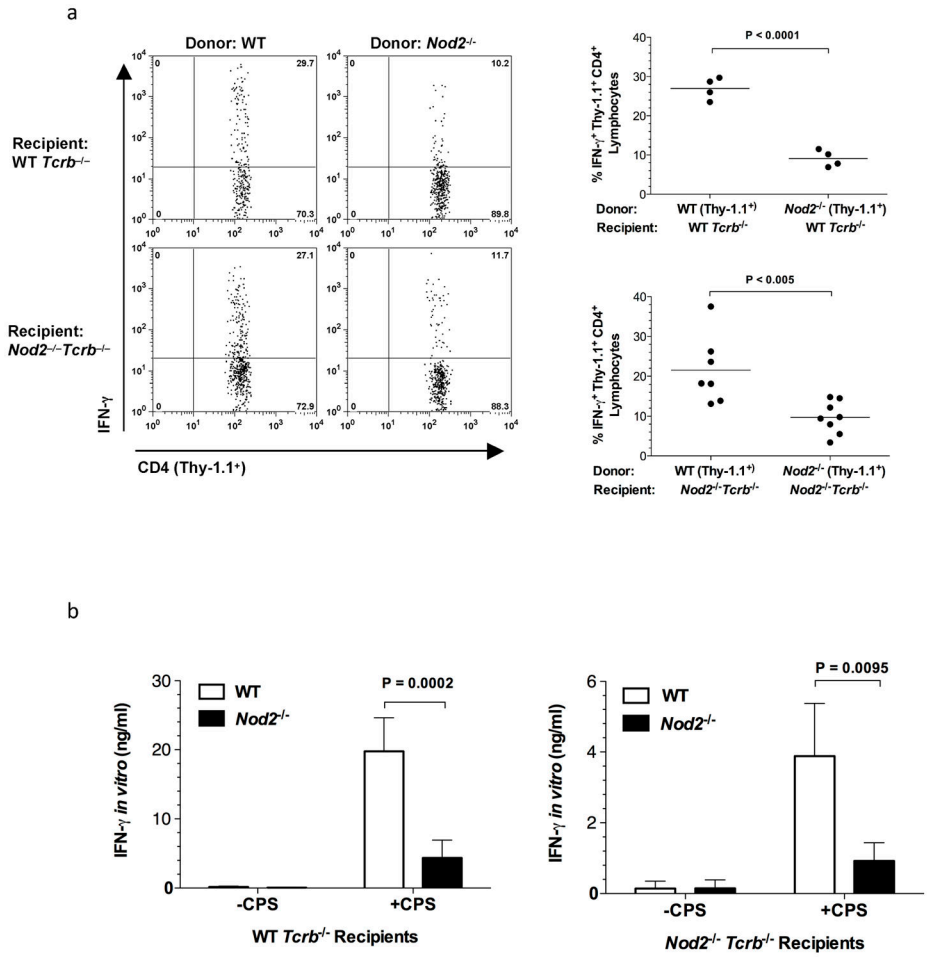

Figure 5.

Nod2$^{-/-}$ CD4 cells have an intrinsic defect in IFN-γ production. (a) Left, MACS-sorted splenic CD4^{+} cells from wild-type (Thy-1.1) and *Nod2*$^{-/-}$ (Thy-1.1) mice were injected intravenously into wild-type *Tcrb*$^{-/-}$ (Thy-1.2) or *Nod2*$^{-/-}$*Tcrb*$^{-/-}$ (Thy-1.2) recipients, which were then challenged with irradiated CPS parasites. On day 9 post challenge, PECs were harvested from reconstituted mice and IFN-γ production was determined by intracellular cytokine staining following *in vitro* stimulation with live parasite. Each dot plots is representative of 4–8 mice per group and is gated on CD4^{+}TCR-β^{+}Thy-1.1^{+} cells. Right, the data were pooled; each dot represents one mouse and horizontal bars represent the mean. (b) PECs of reconstituted mice as described in (a) were co-cultured with live parasite for 24 h and IFN-γ in the supernatants was determined by ELISA.

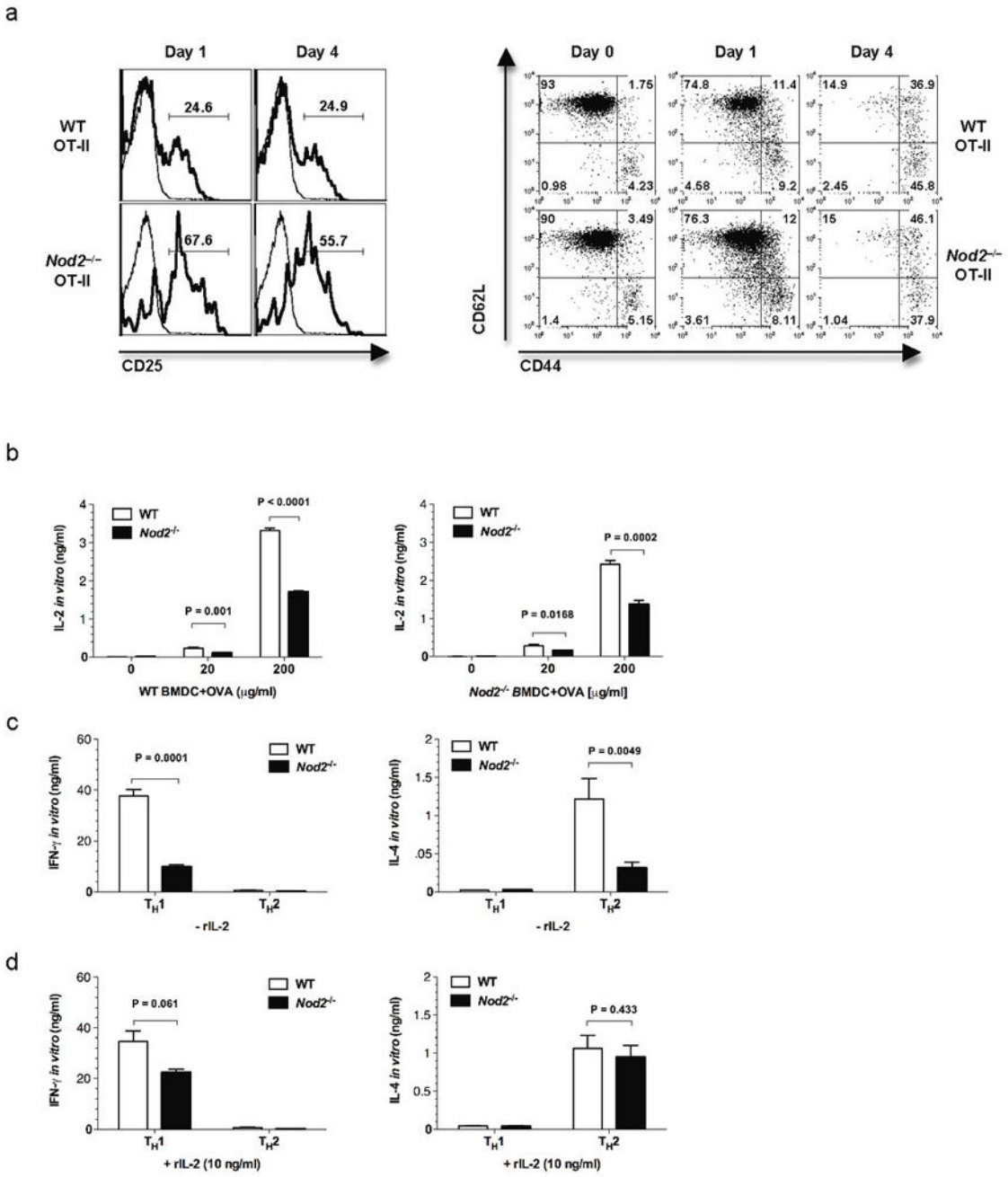

Figure 6.

Impaired T_H cell differentiation and IL-2 production by *Nod2*$^{-/-}$ T cells. (a) Purified wild-type OT-II and *Nod2*$^{-/-}$ OT-II cells were co-incubated *in vitro* with OVA-loaded BMDCs. At the indicated time points, the expression of CD25, CD44 and CD62L was determined by flow cytometry. The numbers in (a) represent the percentage of T cells in each gate or quadrant. (b) Wild-type OT-II and *Nod2*$^{-/-}$ OT-II cells were co-cultured with wild-type or *Nod2*$^{-/-}$ BMDCs loaded with varying doses of OVA for 3 days. IL-2 in the culture supernatant was measured by ELISA. (c,d) Splenic wild-type OT-II and *Nod2*$^{-/-}$ OT-II cells

were purified and cultured with OVA-loaded wild-type BMDCs in T_H1 or T_H2 polarizing conditions in the presence or absence of exogenous IL-2. Efficiency of polarization was determined on day 6 based on IFN-γ (T_H1) and IL-4 (T_H2) production following α-CD3 restimulation for 24 h. All data shown are representative of at least three experiments with 3–4 mice per group. (b-d) Values shown are the mean ± s.d. from three mice pre-group.

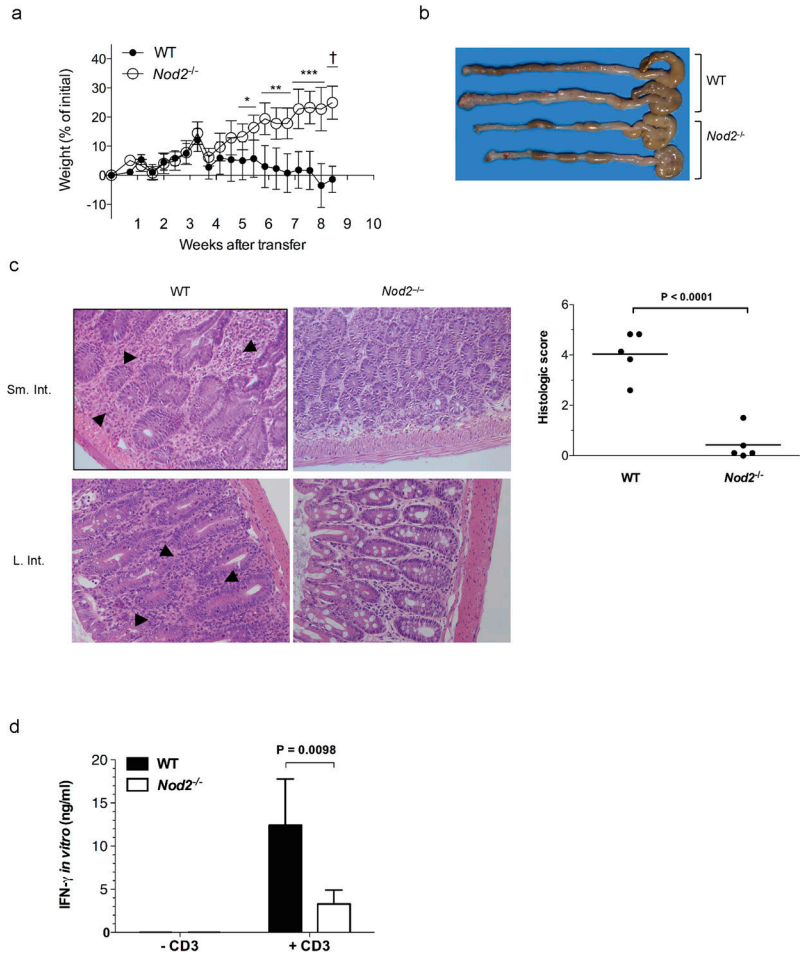

Figure 7.

Role for Nod2 in T cell-induced colitis. (a) Body weights of *Rag1*$^{-/-}$ recipients of purified CD4^{+}CD25^{-}CD45RB^{hi} T cells from *Nod2*$^{-/-}$ or wild-type mice, represented as percent of initial (day 0) weight. (b) Gross morphology of the colon from the recipient mice in (a) at eight weeks post reconstitution. (c) Left, histology of small intestine and colonic tissues from the mice in (a). Right, severity of intestinal inflammation in the mice in (a) was assessed by histologic scoring on three major categories (extent of epithelial damage, level of involvement, and inflammatory cell infiltration). (d) Mesenteric lymph nodes were harvested from the mice in (a) and restimulated with α-CD3 for 24 h. IFN-γ concentrations in the culture supernatants were determined by ELISA. Data shown are representative of two experiments with n=5 mice per group. * P< 0.05; ** P < 0.005; *** P < 0.0005; † P < 0.0001.

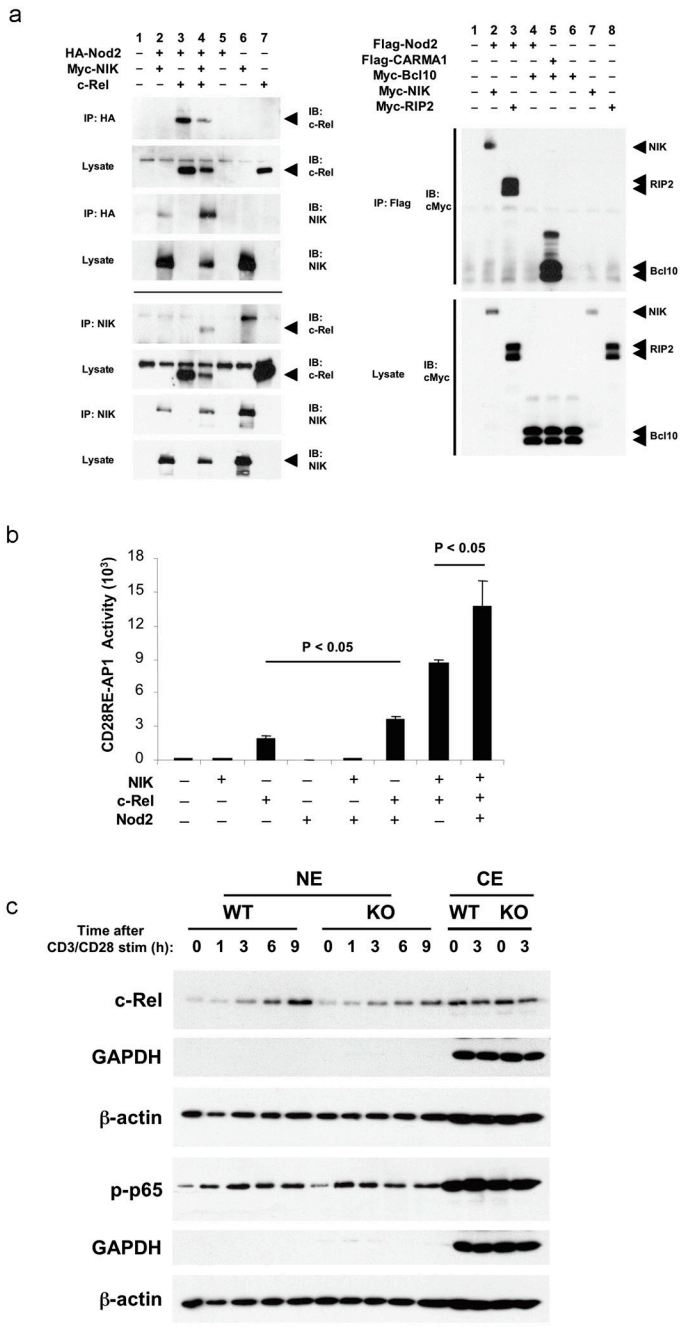

Figure 8. Nod2 interacts with c-Rel and enhances *Il2* transcription

(a) HEK293T cells were transiently transfected with 1μg of the indicated expression plasmids. Cells were harvested after 48 h and whole cell lysates were immunoprecipitated with antibodies against NIK or HA (Nod2) (left) or Flag (Nod2 or CARMA1) (right). Bcl10 was used as a specificity control. The immunoprecipitates were subjected to SDS-PAGE and immunoblotting with the indicated antibodies. (b) Jurkat cells were transiently transfected with a luciferase reporter driven by a CD28 response element, along with equivalent amounts of Nod2, NIK, and c-Rel expression constructs as indicated. The cells were stimulated with anti-CD3 and anti-CD28 and the average relative luciferase activity is

shown (±s.d.). A *renilla* luciferase reporter construct was used to normalize for transfection efficiency and the results are representative of three independent experiments. (c) Nuclear and cytosolic fractions from purified T cells stimulated with plate-bound α-CD3 and α-CD28 for 1–9 h were subjected to SDS-PAGE, followed by immunoblotting with the indicated antibodies. The results are representative of three similar experiments.