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Nucleotide Binding Oligomerization Domain 2 Deficiency Leads to Dysregulated TLR2 Signaling and Induction of Antigen-Specific Colitis

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Summary

In this study, we determined conditions leading to the development of colitis in mice with nucleotide binding oligomerization domain 2 (NOD2) deficiency, a susceptibility factor in Crohn's disease. We found that NOD2-deficient antigen-presenting cells (APCs) produced increased amounts of interleukin (IL)-12 in the presence of ovalbumin (OVA) peptide and peptidoglycan or recombinant *E. coli* that express OVA peptide (ECOVA). Furthermore, these APCs elicited heightened interferon-γ (IFN-γ) responses from cocultured OVA-specific CD4$^+$ T cells. We then demonstrated that NOD2-deficient mice adoptively transferred OVA-specific CD4$^+$ T cells and that administered intrarectal ECOVA developed colitis associated with the expansion of OVA-specific CD4$^+$ T cells producing IFN-γ. Importantly, this colitis was highly dependent on Toll-like receptor 2 (TLR2) function since it was suppressed in NOD2 and TLR2 double-deficient mice. Thus, NOD2-deficient mice become susceptible to colitis as a result of increased TLR2 responses when they have the capacity to respond to an antigen expressed by mucosal bacteria.

Introduction

A subgroup of patients with Crohn's disease bear mutations of *CARD15*, a gene that encodes nucleotide binding oligomerization domain 2 (NOD2) (Hugot et al., 2001; Ogura et al., 2001; Lesage et al., 2002). NOD2 belongs to the NOD-LRR (leucine-rich-repeat) family of proteins, whose members are characterized by a tripartite domain structure consisting of a carboxy-terminal recognition LRR domain, a central NOD domain, and an amino-terminal domain composed of protein-protein interaction cassettes, such as caspase recruitment domains (CARDs) or pyrin domains (Strober et al., 2006; Watanabe et al., 2005a). NOD2 is expressed in the cytoplasmic region of antigen-presenting cells (APCs) (Gutierrez et al., 2002) and epithelial cells (Hisamatsu et al., 2003), and its LRR domain serves as a receptor for muramyl dipeptide (MDP), a small molecule derived from bacterial cell wall peptidoglycan (PGN) (Girardin et al., 2003; Inohara et al., 2003). Stimulation of NOD2 with MDP leads to activation of the serine-threonine kinase, Receptor-interacting protein (RIP)-like interacting caspase-like apoptosis regulatory protein kinase (RICK) via a CARD-CARD interaction that, in turn, results in ubiquitinylation of NF-κB essential Modulator (NEMO) and translocation of nuclear factor-kappa B (NF-κB) subunit into the nucleus (Abbott et al., 2004; Ogura et al., 2001). Thus, one outcome of NOD2 activation by MDP is the activation of the NF-κB pathway.

It is not at all clear, however, that this function of NOD2 is responsible for the susceptibility to Crohn's disease in patients with NOD2 mutations. On the one hand, human embryonic kidney 293 cells expressing the mutated NOD2 associated with Crohn's disease exhibit defective NF-κB activation after stimulation with MDP (Inohara et al., 2003) and, on the other, increased production of NF-κB-related Th1 cytokines such as IL-12 and IFN-γ plays an indispensable role in the development of intestinal inflammation in Crohn's disease (Bouma and Strober, 2003; Strober et al., 2002). In a previous study, we attempted to resolve this paradox with the use of murine APCs lacking NOD2 or expressing the mutated NOD2 associated with Crohn's disease (Watanabe et al., 2004). We found that PGN induction of IL-12 is normally downregulated by concomitant stimulation of NOD2 via the PGN component, MDP. Thus, in the absence of NOD2 or in the presence of Crohn's disease-associated mutated NOD2, PGN elicits an excessive NF-κB-dependent IL-12 response (Watanabe et al., 2004), suggesting that an unregulated innate immune response to a ubiquitous component of the intestinal microflora could at least partially account for the robust Th1 response causing inflammatory bowel disease (IBD) in patients with NOD2 mutations.

The above studies, however, left unanswered the question of whether the dysregulated innate IL-12 response resulting from NOD2 deficiency was sufficient for the occurrence of IBD or whether adaptive responses mediated by T cells also played an essential role (Bouma and Strober, 2003; Strober et al., 2002). Therefore, in the current studies we addressed the question of whether the excessive IL-12 response to PGN occurring in the absence of NOD2 signaling also supports a strong adaptive IFN-γ response by antigen (Ag)-specific CD4$^+$ T cells and whether such an adaptive response could lead to mucosal inflammation if the stimulating Ag were a component of a mucosal organism.

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Results

PGN-Stimulated NOD2-Deficient APCs Enhanced IFN-γ Responses by Ag-Specific CD4+ T Cells

In a previous paper, we reported that PGN-stimulated splenic CD11b+ cells from NOD2-deficient (*Card15*$^{-/-}$) mice produce increased amounts of IL-12 as compared to cells from NOD2-intact (*Card15*$^{+/+}$) (littermate control) mice (Watanabe et al., 2004). Since IL-12 plays a pivotal role in the differentiation of Th1 cells, we addressed in the present study whether this enhanced production of IL-12 by NOD2-deficient APCs affects production of cytokines by naive Ag-specific CD4+ T cells. In initial studies to explore this question, CD4+ T cells recognizing the 323–339 peptide fragment of ovalbumin ($OVA_{323-339}$ peptide) in the context of IA^b were isolated from OT-II transgenic mice (Kondrack et al., 2003) and then stimulated in vitro with $OVA_{323-339}$ peptide presented by splenic CD11b+ cells from NOD2-deficient or NOD2-intact mice (Pauleau and Murray, 2003) in the presence of various TLR or NOD ligands: lipopolysaccharide (LPS), a TLR4 ligand; PGN from *Staphylococcus aureus* (PGN S), PGN from *Escherichia coli* (PGN E), and Pam_3CSK4 (Pam), TLR2 ligands; double stranded RNA (dsRNA), a TLR3 ligand; Flagellin, a TLR5 ligand; Loxoribine (Lox), a TLR7 ligand; CpG, a TLR9 ligand; and muramyl dipeptide (MDP), a NOD2 ligand.

As mentioned above, our previous studies had shown that PGN-stimulated IL-12 production via TLR2 signaling is negatively regulated by MDP-mediated activation of NOD2 signaling, and this negative regulation is released in the absence of intact NOD2 signaling. Consistent with this, IL-12p70 production is enhanced in cultures of PGN-stimulated splenic CD11b+ cells from NOD2-deficient mice compared with cells from littermate control mice (Figure 1A). In addition, such production is further enhanced when OVA-specific CD4+ T cells and $OVA_{323-339}$ peptide are added to cultures containing NOD2-deficient APCs but not to cultures containing NOD2-intact APCs. As suggested by studies of CD40 ligand-deficient mice, this further enhancement may be due to the fact that CD40 and CD40 ligand interaction between Ag-activated T cells and APCs plays a critical role in IL-12 production by the latter cells (Campbell et al., 1996; Kamanaka et al., 1996). Although addition of $OVA_{323-339}$ peptide and OVA-specific CD4+ T cells to LPS (TLR4)-stimulated cultures and, to a lesser extent, to CpG (TLR9)-stimulated cultures led to enhanced IL-12 secretion, in this case, no difference in IL-12 secretion between cultures containing NOD2-deficient and NOD2-intact APCs was seen.

To address the specificity of NOD2 signaling in the regulation of PGN-mediated TLR2 responses, we stimulated CD11b+ cells with Pam_3CSK4, a TLR2 ligand that unlike PGN does not contain MDP and therefore does not result in simultaneous TLR2 and NOD2 signaling. We found that whereas CD11b+ cells from NOD2-intact and NOD2-deficient mice produced equivalent amounts of IL-12p70 when stimulated with Pam_3CSK4 alone, cells from NOD2-intact mice produced significantly less IL-12p70 than cells from NOD2-deficient mice when stimulated with 1 μg/ml of Pam_3CSK4 plus 100 μg/ml of MDP (IL-12p70 production in pg/ml by NOD2-intact cells versus NOD2-deficient cells, Pam_3CSK4 stimulation alone: 136.0 ± 10.1 versus 113.0 ± 15.0; Pam_3CSK4 plus MDP stimulation: 53.0 ± 3.1 versus 107.0 ± 6.7, $p < 0.01$). These studies show that TLR2 signaling is regulated by a ligand specific for NOD2, MDP. In related studies, we showed that PGN (10 μg/ml) stimulation of CD11b+ cells from NOD1-deficient mice (Viala et al., 2004), unlike those from NOD2-deficient mice, exhibit equivalent IL-12p70 production compared to littermate control mice (IL-12p70 production in pg/ml by NOD1-intact cells versus NOD1-deficient cells, 92.5 ± 5.9 versus 94.6 ± 2.9). Thus, the regulation of TLR2 stimulation does not occur in relation to a NOD protein (NOD1) with a similar structure to NOD2, further supporting the specificity of NOD2 for such regulation.

Having defined the cytokine responses of NOD2-deficient APCs to PGN and other TLR agonists during presentation of Ag to cocultured Ag-specific CD4+ T cells, we were in a position to evaluate the responses of the cocultured T cells. Not surprisingly, IFN-γ production by the latter cells, regardless of the type of stimulating TLR ligand present, occurred only in cultures containing $OVA_{323-339}$ peptide, i.e., the stimulant of the Ag-specific T cells was present (Figure 1B). However, in PGN-stimulated cultures, NOD2-deficient APCs led to markedly greater IFN-γ production than NOD2-intact APCs, whereas in cultures stimulated by other TLR ligands, no difference between cultures containing NOD2-deficient and NOD2-intact APCs was seen. In addition, as shown in Figure 1C, in cultures of PGN-stimulated NOD2-deficient APCs containing $OVA_{323-339}$ peptide, Ag-specific CD4+ T cells produced significantly less IL-13 than in equivalent cultures containing PGN-stimulated NOD2-intact APCs, whereas cultures containing NOD2-deficient and NOD2-intact APCs produced equivalent amounts of IL-18, IL-10, and TNF (see Figure S1 in the Supplemental Data available online). Taken together, these data show that PGN stimulation of NOD2-deficient APCs mediated by TLR2 signaling results in enhanced production of IL-12p70 and, in turn, leads to enhanced differentiation of naive Ag-specific CD4+ T cells into IFN-γ-producing CD4+ T cells. It should be noted that the relatively greater enhancement of IL-12p70 as compared to IFN-γ in the cultures containing NOD2-deficient versus NOD2-intact APCs was probably due to the fact that costimulation via CD40 ligand-CD40 is more important for IL-12p70 production than for IFN-γ under these circumstances.

PGN Is Acting as a TLR2 Agonist in the Studies of NOD2-Deficient APCs

Some investigators have suggested that contaminants present in semipurified (commercially available) PGN (such as lipoteichoic acid) rather than PGN itself accounts for the TLR2 stimulation by such PGN (Travassos et al., 2004). However, other investigators claim that contaminant-free PGN can be obtained that retains TLR2 stimulating activity (Dziarski and Gupta, 2005). In view of this uncertainty, we next conducted studies to verify that the NOD2 regulation of in vitro PGN responses shown above was in fact related to TLR2 signaling. In an initial set of studies addressing this issue, we stimulated CD11b+ cells from littermate control, NOD2-deficient, and NOD2 and TLR2 double-deficient (NOD2-TLR2-deficient) mice with commercially available

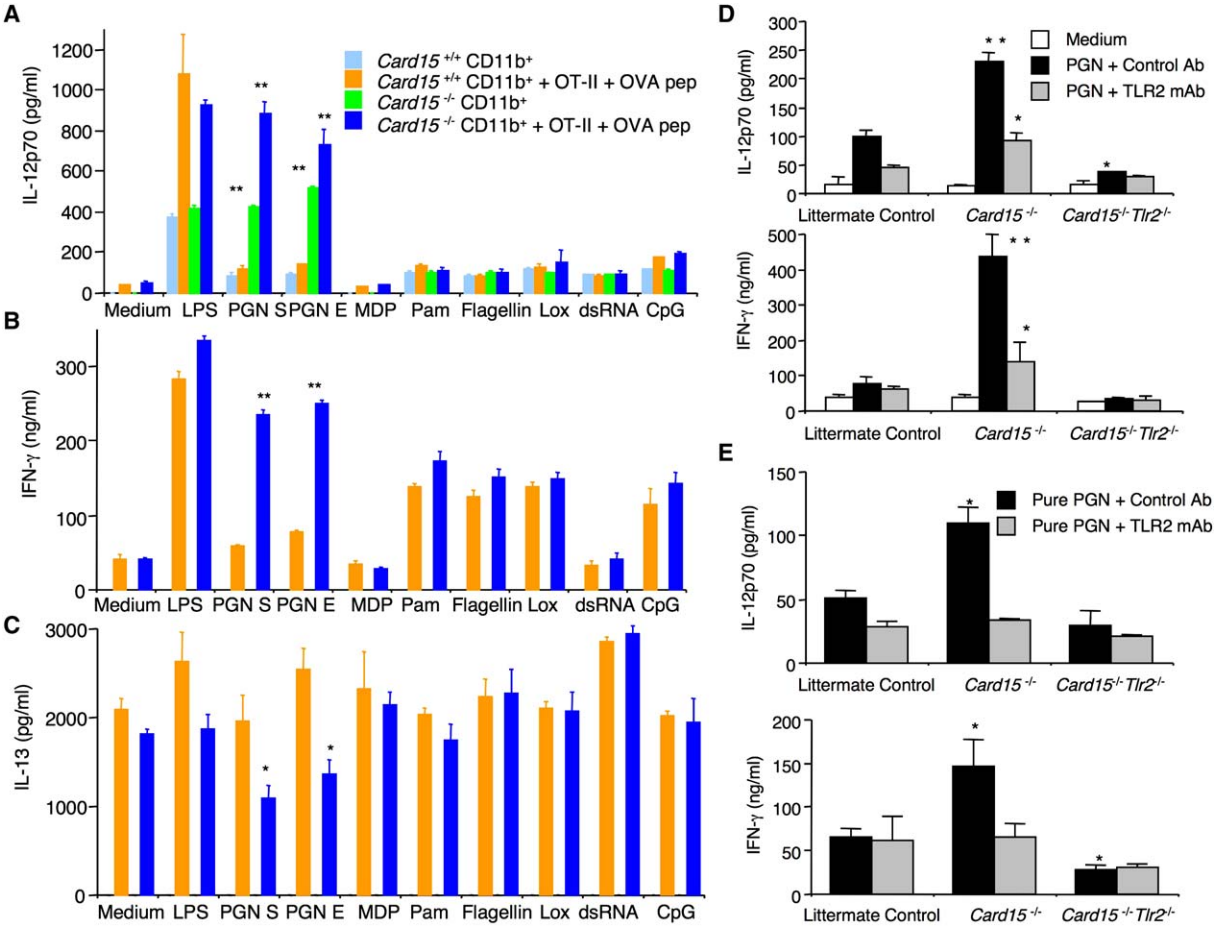

Figure 1. Increased IL-12 Production by NOD2-Deficient APCs Stimulated with PGN Leads to Enhanced Differentiation of Naive Ag-Specific $CD4^+$ T Cells into Th1 Cells

(A–C) Splenic $CD4^+$ T cells (1×10^6/ml) recognizing the 323–339 peptide fragment of ovalbumin ($OVA_{323-339}$ peptide) in the context of IA^b were isolated from OT-II transgenic mice. The cells were then cultured in vitro with 0.5 μM of $OVA_{323-339}$ peptide presented by splenic $CD11b^+$ cells (1×10^6/ml) from NOD2-intact or NOD2-deficient mice. These cultures were carried out in the presence of various TLR or NOD ligands including: LPS, a TLR4 ligand; PGN from *Staphylococcus aureus* (PGN S), PGN from *Escherichia coli* (PGN E), and Pam_3CSK4 (Pam), TLR2 ligands; dsRNA, a TLR3 ligand; Flagellin, a TLR5 ligand; Loxoribine (Lox), a TLR7 ligand; CpG, a TLR9 ligand; and MDP, a NOD2 ligand. Culture supernatants were harvested at 48 hr for IL-12p70 (A) assay and at 72 hr for IFN-γ (B) and IL-13 (C) assays.

(D and E) Splenic $CD11b^+$ cells (1×10^6/ml) from littermate control, NOD2-deficient, and NOD2 and TLR2 double-deficient mice were stimulated in vitro with commercial PGN (10 μg/ml) ([D], top) or purified PGN (50 μg/ml) ([E], top) in the presence of control Ab or neutralizing TLR2 Ab (100 μg/ml). Culture supernatants were harvested at 48 hr for assay of IL-12p70. Splenic $CD4^+$ T cells (1×10^6/ml) isolated from OT-II transgenic mice were stimulated in vitro with 0.5 μM of $OVA_{323-339}$ peptide presented by splenic $CD11b^+$ cells (1×10^6/ml) from littermate control, NOD2-deficient, and NOD2 and TLR2 double-deficient mice in the presence of commercial PGN (10 μg/ml) ([D], bottom), purified PGN (50 μg/ml) ([E], bottom), control Ab or neutralizing anti-TLR2 Ab (100 μg/ml). Culture supernatants were harvested at 72 hr for assay of IFN-γ. *$p < 0.05$, **$p < 0.01$, compared with the results of cytokine concentrations of the culture containing APCs from littermate control mice. Data shown are representative of two separate experiments. Data are expressed as mean ± SD.

PGN (as in the experiments described above). We found that whereas the NOD2-deficient cells produced increased amounts of IL-12p70 as compared to NOD2-intact cells, NOD2-TLR2-deficient cells produced even less IL-12p70 than NOD2-intact cells (Figure 1D, top). In addition, a similar result was obtained with cultures in $CD11b^+$ cells containing $OVA_{323-339}$-specific T cells and $OVA_{323-339}$ peptide in relation to IFN-γ production (Figure 1D, bottom): again, enhancement of such production seen in cultures containing NOD2-deficient $CD11b^+$ cells was abolished in cultures containing NOD2-TLR2-deficient cells. Finally, we inhibited TLR2 signaling with a monoclonal antibody (mAb) recognizing TLR2, and again the enhancement of either IL-12p70 or IFN-γ production was inhibited in the two-culture systems (Figure 1D). These results show that in the absence of TLR2 signaling, PGN (or contaminants contained therein) cannot induce an IL-12 response that can be regulated by NOD2 signaling.

In a complementary approach to this issue, we stimulated $CD11b^+$ cells (or $CD11b^+$ cells plus $OVA_{323-339}$-specific T cells and $OVA_{323-339}$ peptide) with a preparation of purified PGN that was free of lipoteichoic acid (Dziarski and Gupta, 2005), and again cultures containing NOD2-deficient $CD11b^+$ cells manifested enhanced IL-12p70 or IFN-γ production compared to those containing NOD2-intact cells, and the enhancing effect was abolished in cultures containing NOD2-TLR2-deficient cells (Figure 1E). Furthermore, addition of TLR2 mAb again prevented the enhancement in cultures containing

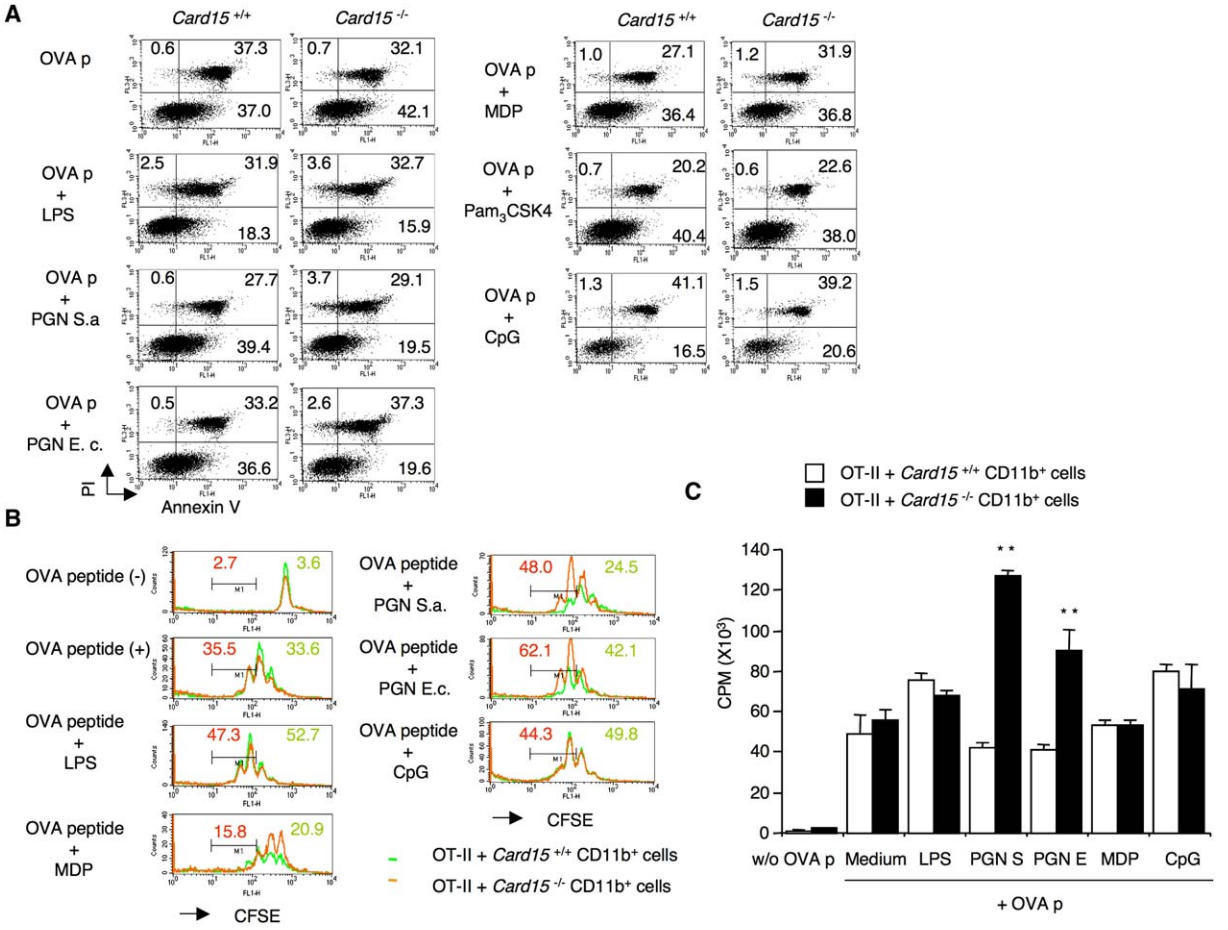

Figure 2. Apoptosis and Cell Division of OVA-Specific CD4+ T Cells Stimulated in the Presence of TLR or NOD Ligands

Naive $OVA_{323-339}$ peptide-specific CD4+ T cells were isolated from OT-II transgenic mice and then stimulated in vitro with 0.5 μM of $OVA_{323-339}$ peptide presented by splenic CD11b+ cells from NOD2-intact or NOD2-deficient mice in the presence of various TLR or NOD ligands as described in Figure 1.

(A) Flow cytometric analysis of Annexin V versus propidium iodide (PI) staining of naive OVA-specific CD4+ T cells. Cells were cultured for 48 hr and then stained with FITC-conjugated Annexin V, PE-conjugated Vβ5 mAb, and PI; gate was set on CD4+Vβ5+ T cells; the number in each figure indicates the percentage of cells in the corresponding quadrant.

(B) Flow cytometric analysis of $OVA_{323-339}$ peptide-specific CD4+ T cells. T cells were incubated with 2 μM of CFSE and cultured for 72 hr as described in (A). Green indicates T cells in cell cultures containing NOD2-intact CD11b+ cells; orange indicates T cells in cultures containing NOD2-deficient CD11b+ cells. The number in each figure indicates the percentage of cells in the gate M1.

(C) Proliferative responses of naive $OVA_{323-339}$ peptide-specific CD4+ T cells evaluated by ^3H thymidine incorporation. T cell cultured as described in (A) were incubated for 72 hr with addition of 1 μCi of ^3H thymidine during the final 16 hr. **$p < 0.01$, T cell proliferation in cultures containing NOD2-deficient APCs compared with T cells in cultures containing NOD2-intact APCs. Data shown are representative of two separate experiments. Data are expressed as mean ± SD.

NOD2-deficient cells. Thus, even with purified PGN, TLR2 signaling was necessary to produce a response that could be regulated by NOD2.

PGN-Stimulated NOD2-Deficient APCs Is Associated with Decreased Apoptosis and Increased Proliferation of CD4+ T Cells

Previous studies have shown that IL-12 not only drives Th1 differentiation but also inhibits T cell apoptosis of newly differentiated cells and thereby enhances their survival (Fuss et al., 1999; Watanabe et al., 2003). We therefore asked whether presentation of OVA peptide by PGN-stimulated NOD2-deficient APCs (CD11b+ cells) to OVA-specific CD4+ T cells (OT-II T cells) affects the apoptosis of the T cells. Accordingly, we cultured OVA-specific CD4+ T cells with PGN-stimulated NOD2-deficient or NOD2-intact APCs in the presence of $OVA_{323-339}$ peptide for 2 days and then measured the percentage of Annexin V+ and propidium iodide− (PI^-) CD4+ T cells in the cell culture by flow cytometry with the Vβ5+ marker to limit the analysis to responding OVA-specific CD4+ T cells. T cells cultured with NOD2-deficient APCs exhibited markedly less apoptosis than T cells cultured with NOD2-intact APCs in the presence of PGN (Figure 2A). In addition, there was no difference in the percentage of apoptotic OVA-specific CD4+ T cells when the latter were cultured with LPS-MDP-Pam_3CSK4- or CpG-stimulated NOD2-deficient versus NOD2-intact APCs.

An additional possible effect of increased IL-12 production by PGN-stimulated NOD2-deficient APCs presenting an Ag to CD4+ T cells is that the latter manifest

increased proliferation. To explore this possibility, we assessed proliferation of OVA-specific $CD4^+$ T cells presented $OVA_{323-339}$ peptide by TLR-stimulated NOD2-deficient or NOD2-intact APCs with carboxyfluorescein diacetate succinimidyl ester (CFSE) to assess by flow cytometry the number of cell divisions occurring in the cultures after 3 days. Addition of OVA peptide to the cultures led to greater number of cell divisions of OVA-specific $CD4^+$ T cells presented Ag by PGN-stimulated NOD2-deficient APCs than T cells presented Ag by PGN-stimulated NOD2-intact APCs (Figure 2B). In contrast, LPS or CpG stimulation of NOD2-deficient or NOD2-intact APCs led to equal levels of proliferation of OVA-specific $CD4^+$ T cells. These results were confirmed by ^{3}H-thymidine incorporation assays. Thus, OVA-specific $CD4^+$ T cells exhibited increased proliferation when presented OVA peptide by NOD2-deficient APCs in the presence of PGN as compared to T cells presented peptide by NOD2-intact APCs (Figure 2C).

Finally, to show that these effects on $CD4^+$ T cell proliferation and IFN-γ production occur as a result of enhanced IL-12 production, we added IL-12p40 mAb to cultures containing NOD2-deficient APCs, OVA-specific $CD4^+$ T cells, and OVA peptide and showed that such addition led to substantially reduced IFN-γ production (Figure S2A), increase in the percentage of Annexin V^+PI^- apoptotic cells (Figure S2B), and decreased T cell proliferation (Figure S2C). It should be noted that while the addition of IL-12p40 mAb to cultures containing NOD2-deficient APCs did not lead to complete inhibition of IFN-γ production, the level of inhibition obtained was equal to that seen in cultures containing NOD2-intact APCs, suggesting that it was not related to NOD2 presence or absence. It may instead be due to the fact that in this in vitro system, the cells are under a strong activation stimulus involving both TLR ligand and Ag signaling and thus are difficult to completely inhibit in a competitive assay. In summary of this series of studies, we conclude that the enhanced IL-12 production by PGN-treated NOD2-deficient APCs noted above increases not only IFN-γ production by responding $CD4^+$ T cells, but also the survival and proliferation of the latter cells.

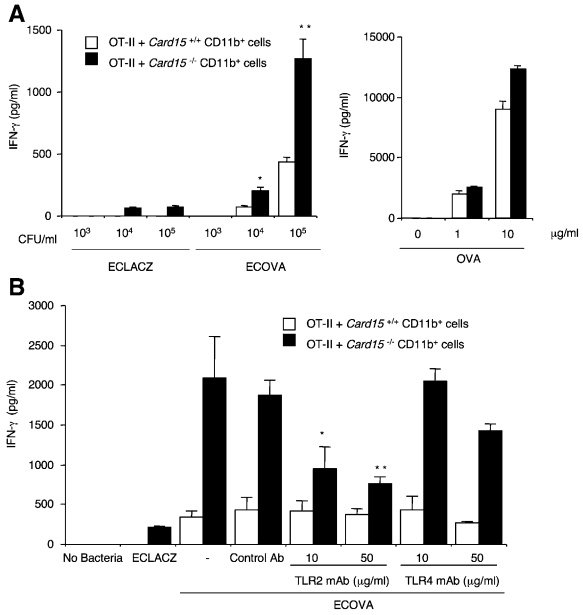

Figure 3. IFN-γ Production by OVA-Specific $CD4^+$ T Cells Stimulated with ECOVA in the Presence of Splenic $CD11b^+$ Cells

$OVA_{323-339}$ peptide-specific $CD4^+$ T cells were isolated from OT-II transgenic mice and then stimulated in vitro with various doses of ECOVA or ECLACZ in the presence of splenic $CD11b^+$ cells from NOD2-intact or NOD2-deficient mice. As controls, OT-II $CD4^+$ T cells were stimulated with OVA protein (1 or 10 μg/ml) in the presence of splenic $CD11b^+$ cells from NOD2-intact or NOD2-deficient mice.

(A) IFN-γ secretion into culture supernatants collected at 96 hr and measured by ELISA. $^{**}p < 0.01$, $^{*}p < 0.05$, compared with the results of cytokine concentrations of the culture containing NOD2-intact APCs.

(B) IFN-γ secretion into culture supernatants of cells cultured as above, but in the presence of neutralizing antibodies specific for TLR2 or TLR4. $^{**}p < 0.01$, $^{*}p < 0.05$, compared with the results of cytokine concentrations of the culture containing control Ab. Data shown are representative of two separate experiments. Data are expressed as mean ± SD.

NOD2-Deficient APCs Stimulated with *E. coli* Expressing OVA Induce Enhanced IFN-γ Production

The above studies revealing enhanced adaptive responses in T cells presented an Ag by PGN-stimulated APCs lacking NOD2 could apply to Ags associated with the intestinal microflora. As a first step in the exploration of this possibility, we asked whether NOD2-intact and NOD2-deficient APCs differ in their capacity to respond to Ags (proteins) associated with bacteria. For this purpose, we stimulated NOD2-intact and NOD2-deficient APCs with recombinant *E. coli* expressing either OVA (ECOVA) or a control antigen, LACZ (ECLACZ) (Yoshida et al., 2001, 2002), in the presence of OVA-specific $CD4^+$ T cells. OVA-specific $CD4^+$ T cells stimulated with ECOVA and NOD2-deficient APCs produced markedly more IFN-γ than those stimulated with ECOVA and NOD2-intact cells (Figure 3A). In contrast, no difference was seen in IFN-γ production when the cells were stimulated with OVA protein and NOD2-deficient or NOD2-intact APCs, suggesting that enhancement of IFN-γ production with stimulation by NOD2-deficient APCs requires the stimulating Ag to be part of a bacteria that is capable of concomitant stimulation of the APCs via TLRs. On the other hand, the latter stimulation alone was not sufficient for IFN-γ production, as indicated by the fact that little production was seen in the presence of bacteria expressing an irrelevant antigen (LACZ). Thus, these results indicate that NOD2-deficient APCs can induce an Ag-specific Th1 response to a bacterial Ag in vitro when exposed to the intestinal microflora.

In a final set of in vitro studies involving stimulation by whole organisms, we asked whether stimulation of NOD2-deficient APCs by ECOVA requires an intact TLR2 signaling pathway. To this end, we determined whether neutralizing TLR2 mAb (Meng et al., 2004) or TLR4 mAb (Poltorak et al., 2000) blocks ECOVA stimulation of $CD11b^+$ cells. We found that addition of TLR2 mAb to the culture reduced IFN-γ production in a dose-dependent manner, whereas addition of TLR4 Ab had only a marginal effect that lacked statistical significance (Figure 3B). These data therefore indicate that the enhanced Th1 responses obtained in cultures of

ECOVA-stimulated NOD2-deficient APCs does in fact require TLR2 signaling.

NOD2-Deficient Mice Are Susceptible to the Development of ECOVA Colitis

The enhanced Th1 responses of Ag-specific $CD4^+$ T cells stimulated by NOD2-deficient APCs in the presence of recombinant *E. coli* expressing the Ag led us to determine whether Ag-specific responses to a bacterial antigen could serve as the basis for colitis in NOD2-deficient mice. To explore this possibility, we turned to a murine model of colitis in which the colonic inflammation is induced in recipient mice adoptively transferred OVA-specific $CD4^+$ T cells from OVA-T cell receptor transgenic mice and then administered ECOVA (Watanabe et al., 2005b; Yoshida et al., 2001, 2002).

In the studies performed here, NOD2-deficient or NOD2-intact mice were adoptively transferred OVA peptide-specific $CD4^+$ T cells from OT-II mice (OVA-specific T cells) and then immunized subcutaneously with OVA+CFA (complete Freund's adjuvant) to expand the population of OVA-specific T cells (a necessary step in these nonimmunodeficient recipient mice). 3 days after immunization, the mice were administered ECOVA or ECLACZ per rectum (Figure S3A).

In preliminary studies we assessed whether subcutaneous immunization with OVA+CFA to expand the transferred OVA-specific $CD4^+$ T cell population has a differential effect on the expansion of OVA-specific $CD4^+$ T cells in the draining lymph nodes in NOD2-deficient versus NOD2-intact mice. OVA+CFA treatment greatly expands the percentage of $CD4^+V\beta5^+$ T cells (i.e., T cells specific to $OVA_{323-339}$ peptide) in draining lymph nodes of both NOD2-deficient and NOD2-intact mice, and this expansion is equivalent in the two types of mice (Figure S3B). In addition, the expanded cell population produced equivalent amounts of IFN-γ and IL-4 (Figure S3C). Thus, the use of OVA+CFA to expand the OVA-specific $CD4^+$ did not lead to a differential increase in OVA-specific $CD4^+$ T cells in NOD2-deficient mice.

NOD2-deficient mice adoptively transferred OVA-specific T cells and challenged with ECOVA but not ECLACZ developed significant colitis, whereas littermate control mice did not develop colitis when challenged by either recombinant bacterium. First, NOD2-deficient mice exhibited significantly more weight loss in the 3 days after ECOVA challenge than NOD2-intact mice after a similar type of challenge, whereas neither types of mice lost a significant amount of weight after ECLACZ challenge ($p < 0.05$, Figure 4A). Similarly, histologic examination of colon tissue harvested on day 3 or 5 after ECOVA or ECLACZ administration revealed obvious and severe inflammation in the colons of NOD2-deficient mice administered ECOVA but not in those administered ECLACZ; in contrast, administration of these recombinant organisms to NOD2-intact mice led to only a small increase in the number of infiltrating mononuclear cells in the colons of mice administered ECOVA and no increase in infiltration cells in the colons of mice administered ECLACZ (Figure 4B). In severe lesions of NOD2-deficient mice challenged with ECOVA, the inflammation was characterized by massive infiltration of mononuclear cells and destruction of crypt architecture. Of interest, however, these inflammatory lesions were discontinuous, "skip" lesions, as noted previously in ECOVA colitis (Yoshida et al., 2001, 2002). To obtain semiquantitative estimates of these histologic changes, we derived colitis scores for each experimental group based on crypt loss, cellular infiltration, and the depth of the inflammation (Watanabe et al., 2005b; Yoshida et al., 2001, 2002). Colons harvested on day 3 or 5 from NOD2-deficient mice challenged with ECOVA exhibited a significantly higher score compared with those of the other three groups ($p < 0.01$, Figure 4C).

Second, we measured the clonal expansion of OVA-specific $CD4^+$ T cells in the mesenteric lymph node (MLN) and colonic lamina propria (LP) of the adoptively transferred NOD2-deficient or NOD2-intact mice challenged with ECOVA and ECLACZ. The percentage of OVA-reactive $CD4^+V\beta5^+$ T cells was markedly increased only in the MLN and LP of NOD2-deficient mice challenged with ECOVA (Figure 5A). Since the total number of MLN cells and LP lymphocytes were also increased in these mice (data not shown), these data strongly suggest that ECOVA induces a massive expansion of OVA-specific $CD4^+$ T cells in the intestine of NOD2-deficient mice.

Third and finally, we assessed the production of cytokines in the adoptively transferred NOD2-deficient and NOD2-intact mice after being challenged with ECOVA and ECLACZ. In these studies, MLN or LP cells were isolated from tissues of NOD2-deficient and NOD2-intact mice on day 3 after ECOVA or ECLACZ challenge and then restimulated ex vivo with OVA peptide and PGN to induce IFN-γ or IL-12 production. LP cells obtained from ECOVA-challenged NOD2-deficient mice exhibited a great increase in IL-12p70 production after stimulation with OVA peptide and PGN, as compared to LP cells obtained from the various control mice (Figure 5B). Despite the fact that the cells were cultured only 3 days after the initiation of the inflammation, this increase was accompanied by a small increase in IL-23 secretion (data not shown). Furthermore, both MLN and LP cells from NOD2-deficient mice exhibited a massive increase in IFN-γ secretion after ECOVA challenge as compared to cells from NOD2-intact mice or cells from either type of mice challenged with ECLACZ (Figure 5C, left). In contrast, there was no significant difference in the production of IL-10 or TNF at this time point (data not shown). IL-4 was below the detection limit in every group. In contrast, stimulation of the same cells with a polyclonal stimulant, CD3 mAb, did not lead to significant differences in IFN-γ production by cells from the various mouse groups, suggesting that only OVA-specific Th1 cells played a role in the development of the colitis (Figure 5C, right). Taken together, these data provide strong support for the idea that NOD2-deficient mice are indeed more susceptible to colitis than their wild-type counterparts and, surprisingly, the colitis can be induced by a microbial Ag provided that the mouse is responsive to that Ag.

The marked increase of Th1 cytokines such as IL-12 and IFN-γ associated with the development of ECOVA colitis in NOD2-deficient mice suggested to us that this colitis was driven by IL-12. To explore this question, we determined whether neutralization of these cytokines by an IL-12p40 mAb could prevent the development of the colitis. In these studies, NOD2-deficient

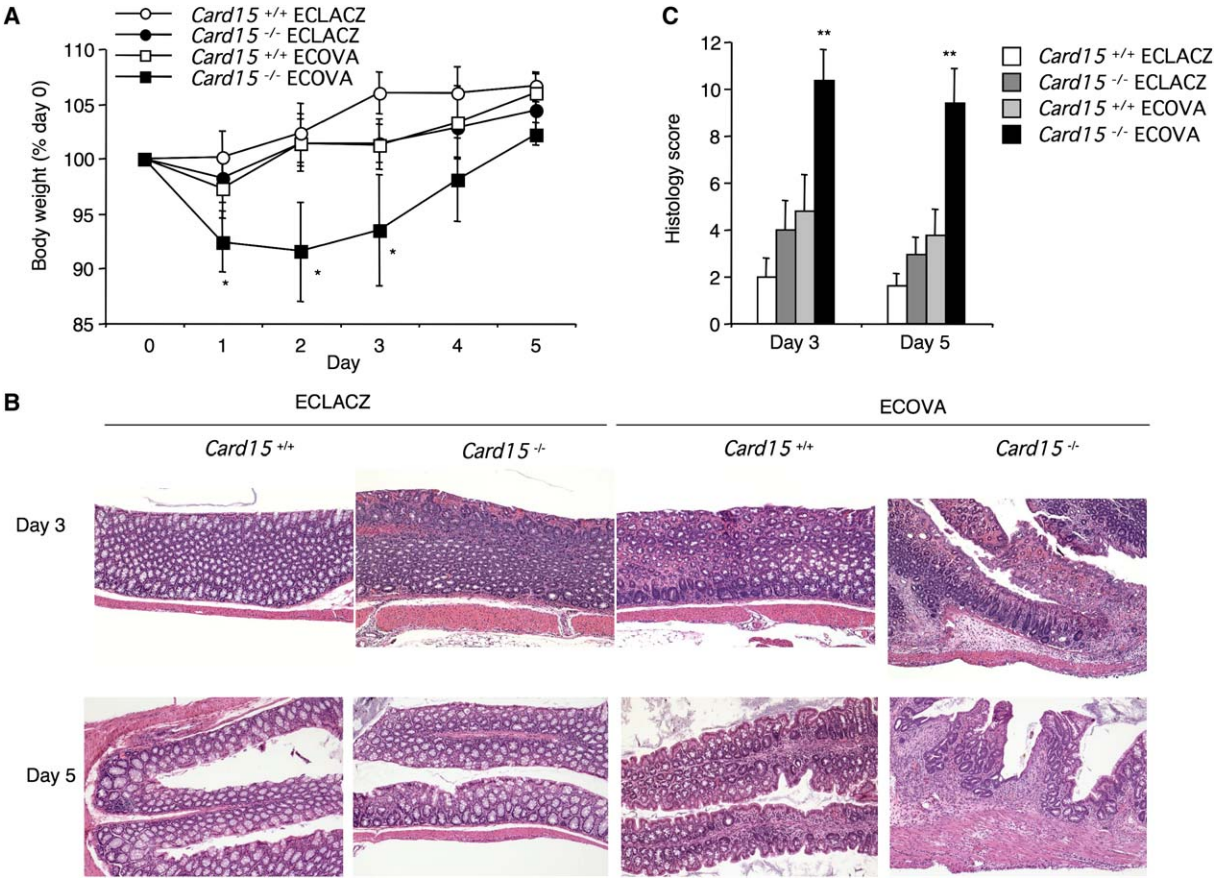

Figure 4. NOD2-Deficient Mice Are Susceptible to Colitis Induced by an Ag Expressed by a Microbial Organism

NOD2-intact or NOD2-deficient mice were adoptively transferred OVA-specific $CD4^+$ T cells (5×10^6/mouse) (day −4) and on the next day subjected to subcutaneous immunization with OVA+CFA (day −3). 3 days later (day 0), the mice were administered intrarectal ECOVA or ECLACZ after intrarectal treatment with 50% ethanol. The mice were then administered intrarectal recombinant *E. coli* every day during the subsequent observation period.

(A) Changes of body weights in the mice of each group. *$p < 0.05$, compared with the results of body weights of the mice in other groups. Data shown are the combined results of two separate experiments (n = 5 in each group) and are expressed means ± SD.

(B) Representative H&E-stained colonic tissue of the mice on day 3 and 5.

(C) Colitis scores derived from colons harvested on day 3 and day 5. **$p < 0.01$, comparing colitis score of NOD2-deficient mice with colitis scores of mice in any of other groups. Data are expressed as mean ± SD.

mice were administered a neutralizing IL-12p40 mAb that recognizes both IL-12 and IL-23 or control Ab at the indicated time points (see Experimental Procedures). While marked body weight loss was seen in NOD2-deficient mice treated with control Ab and challenged with ECOVA, little body weight loss was seen in NOD2-deficient mice treated with IL-12p40 mAb and challenged with ECOVA (Figure 6A). Furthermore, IL-12p40 treatment prevented the development of histologic evidence of colitis (Figure 6B). IL-12p40 mAb treatment was associated with a level of IFN-γ production by MLN cells from ECOVA-challenged mice equivalent to that seen in ECLACZ-challenged mice (Figure 6C). Finally, IL-12p40 mAb treatment effectively reduced production of both IL-12p70 and IL-23 by MLN cells stimulated with OVA peptide and PGN (data not shown). Taken together, these data provide strong evidence that development of ECOVA colitis in NOD2-deficient mice depends upon IL-12 and possibly IL-23 production by APCs.

NOD2-TLR2 Double-Deficient Mice Are Resistant to the Development of ECOVA Colitis

Based on the in vitro studies described above and studies described previously (Watanabe et al., 2004), an underlying premise of the above findings in ECOVA colitis is that NOD2-deficient mice with such colitis are mounting a dysreglated response to a TLR2 ligand associated with the recombinant *E. coli*, namely, PGN. To address this issue, in a final series of studies we determined whether ECOVA colitis can be induced in NOD2-TLR2 double-deficient (NOD2-TLR2-deficient) mice. Accordingly, we examined colitis induction as described above in four groups of mice administered either intrarectal ECLACZ or ECOVA: littermate control, NOD2-deficient, TLR2-deficient, and NOD2-TLR2-deficient mice. As before, OVA-specific $CD4^+$ T cells (OT II T cells) were transferred to each mouse prior to intrarectal recombinant *E. coli* administration. ECOVA colitis was indicated by the occurrence of weight loss, developed in NOD2-deficient mice as before, whereas NOD2-TLR2-deficient mice

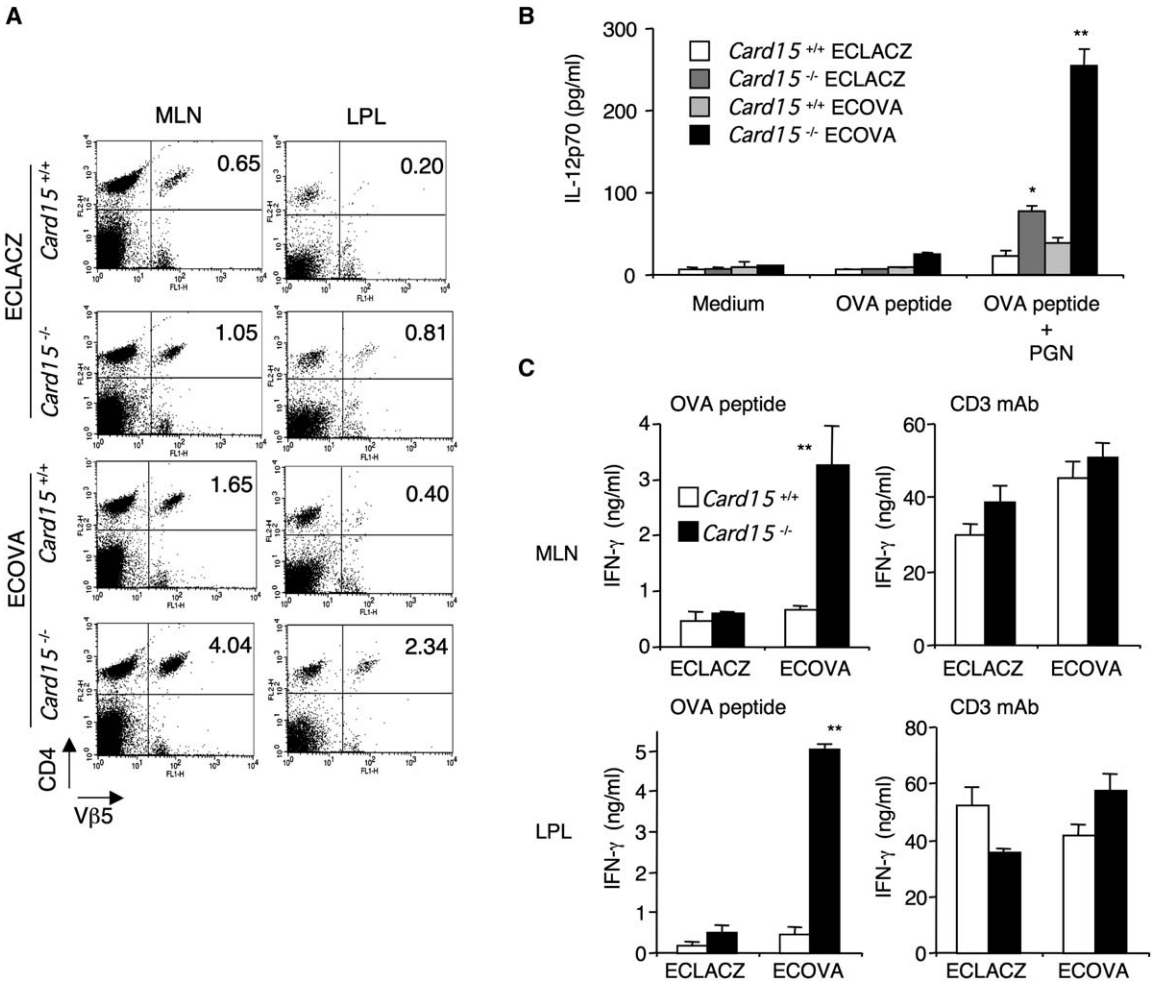

Figure 5. Clonal Expansion of OVA-Specific CD4$^+$ T Cells Producing IFN-γ in the Mesenteric Lymph Nodes and Colonic Lamina Propria

Mesenteric lymph node cells and colonic lamina propria cells were isolated from mice administered ECOVA or ECLACZ on day 3 after administration.

(A) Flow cytometric analysis of mesenteric lymph node cells and colonic lamina propria lymphocytes stained with PE-conjugated CD4 mAb and FITC-conjugated Vβ5 mAb. Dead cells were excluded by PI-staining. The number in each figure shows the percentage of CD4$^+$Vβ5$^+$ T cells (OVA-specific CD4$^+$ T cells).

(B) Concentrations of IL-12p70 in supernatants of cultures of colonic lamina propria cells stimulated with $OVA_{323-339}$ peptide (0.5 μM) together with PGN (10 μg/ml) for 48 hr. Data are expressed as mean ± SD. *p < 0.05, **p < 0.01, compared with the results of NOD2-intact mice treated with ECOVA.

(C) Concentrations of IFN-γ in supernatants of cultures of mesenteric lymph node cells and colonic lamina propria cells stimulated with CD3 mAb (2 μg/ml) or $OVA_{323-339}$ peptide (0.5 μM) for 60 hr. Results are representative one of two separate experiments (n = 5 in each group). Data are expressed as mean ± SD. **p < 0.01, NOD2-deficient mice compared with the NOD2-intact mice.

exhibited only minor weight loss (Figure 7A). In addition, upon histological analysis, severe colitis characterized by massive infiltration of inflammatory cells along with loss of mucosal architecture was seen in NOD2-deficient mice administered ECOVA but not in the other mouse groups including the NOD2-TLR2-deficient group (Figure 7B). In particular, in the NOD2-TLR2-deficient mice, cellular infiltration was greatly decreased compared to the NOD2-deficient mice and manifested itself only as an occasional and isolated island of inflammation; loss of mucosal architecture was not seen. These clinical findings correlated with immunologic features. Thus, clonal expansion of OVA-specific CD4$^+$ T cells was greatly reduced in the MLN of NOD2-TLR2-deficient mice challenged with ECOVA as compared with that of NOD2-deficient mice (Figure 7C). In addition, these differences were corroborated by studies showing that MLN cells from NOD2-deficient mice produced increased amounts of IFN-γ ex vivo upon stimulation with OVA peptide, whereas cells from NOD2-TLR2-deficient did not (Figure 7D). This lack of significant ECOVA colitis in the double-deficient mice strongly suggests that TLR2 stimulation via PGN in the ECOVA is necessary for the development of ECOVA colitis in NOD2-deficient mice.

Discussion

In previous studies, we showed that NOD2-deficient APCs manifest enhanced PGN-induced IL-12p70 responses as compared to NOD2-intact APCs (Watanabe et al., 2004). In studies that enlarge on this finding, we

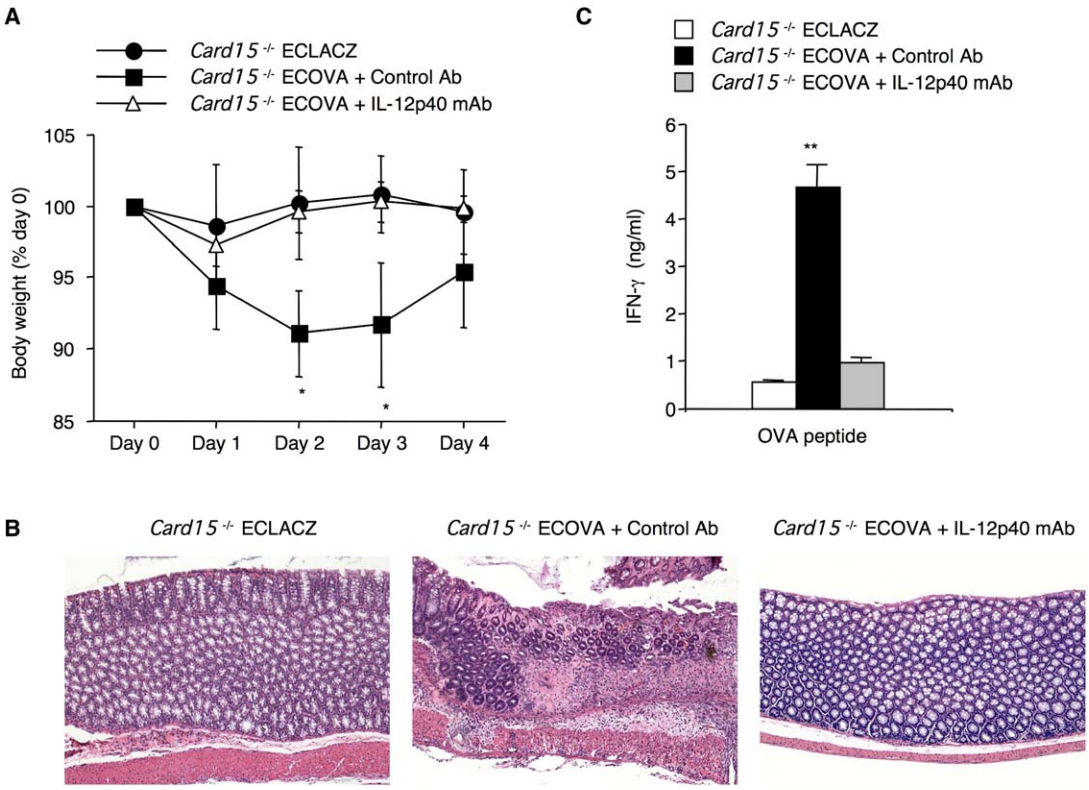

Figure 6. Development of Colitis in NOD2-Deficient Mice Challenged with ECOVA Depends upon IL-12

NOD2-deficient mice were administered ECOVA or ECLACZ as described in Figure 4. Mice received intraperitoneal injection of IL-12p40 mAb or control Ab (1 mg) on day −1 and day 2.

(A) Changes of body weight of the mice in each group (n = 6). Results are expressed mean ± SD. *$p < 0.05$, compared with the results of body weights of the mice in other groups.

(B) H&E-stained colonic tissue of the mice administered ECOVA or ECLACZ harvested on day 4.

(C) Concentrations of IFN-γ in supernatants of cultures of mesenteric lymph node cells stimulated with $OVA_{323-339}$ peptide (0.5 μM) for 60 hr. Results are expressed as mean ± SD. **$p < 0.01$, compared with the results of NOD2-deficient mice treated with ECLACZ.

now show that naive OVA-specific $CD4^+$ T cells presented OVA peptide by PGN-stimulated APCs from NOD2-deficient mice exhibit enhanced Th1 (IFN-γ) responses. Similarly, we show that naive OVA-specific $CD4^+$ T cells also exhibit enhanced Th1 responses when cultured with NOD2-deficient APCs in the presence of a recombinant *E. coli* expressing OVA (ECOVA). In this case, the *E. coli* organisms were the source of the stimulating PGN as well as the OVA peptide being presented. These in vitro studies set the stage for in vivo studies relating to the induction of colitis in NOD2-deficient mice. Here we demonstrated that NOD2-deficient mice but not NOD2-intact mice adoptively transferred with OVA-specific $CD4^+$ T cells and then administered recombinant *E. coli* expressing OVA (ECOVA) per rectum develop colitis. In addition, development of this type of colitis is virtually absent in NOD2-TLR2 double-deficient mice or NOD2-deficient mice treated with IL-12p40 mAb. Thus, these in vivo studies show that a dysregulated innate immune response to PGN caused by NOD2 deficiency can act via a specific adaptive immune response to an Ag associated with a gut commensal to cause mucosal inflammation.

The concept that both innate and adaptive immune responses may be necessary for the induction of mucosal inflammation as demonstrated here is presaged by previous studies of experimental models of colitis. It has been shown, for instance, that hapten-induced (TNBS) colitis mediated by Th1 responses is readily induced in mouse strains with a genetic susceptibility locus on chromosome 11 that also encodes a gene that gives rise to an enhanced LPS-induced IL-12 response. It thus appears that an innate IL-12 response to an TLR4 agonist, or possibly some other TLR response to an as yet undefined bacterial component, sets the stage for, and, indeed, is a necessary accompaniment of colitis induced by a specific hapten Ag, TNBS (Bouma et al., 2002). Similarly, mice with Stat3 deficiency and therefore with impaired IL-10 responses manifest an IL-12-driven colitis probably due to the faulty immunoregulation that accompanies the IL-10 defect (Kobayashi et al., 2003). Of interest to the present discussion, the mice with Stat3 deficiency did not develop colitis if they were interbred with mice lacking TLR4 expression; thus, in this model, an innate immune response again mediated by LPS is required to drive the development of colitis. Furthermore, in this model, development of colitis was not seen in mice lacking T cells, suggesting the importance of adaptive responses in addition to excessive IL-12 production in innate responses. Taken together, these models indicate that innate responses play a critical role in mucosal inflammation, both in situations in which

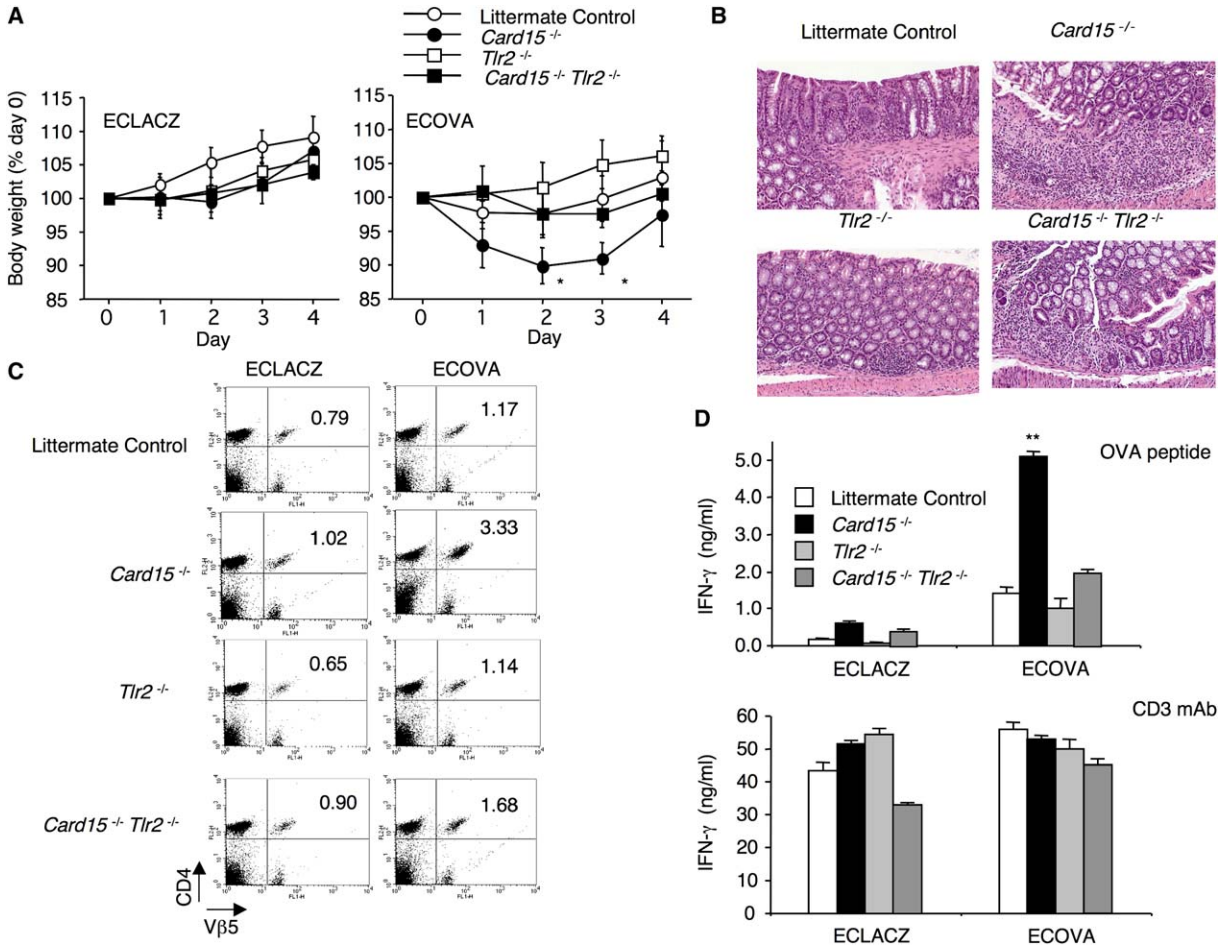

Figure 7. NOD2-TLR2 Double-Deficient Mice Are Protected from the Development of ECOVA Colitis

Littermate control, NOD2-deficient, TLR2-deficient, and NOD2-TLR2-deficient mice were administered ECOVA or ECLACZ as described in Figure 4.

(A) Changes of body weight of the mice in each group (n = 5). Results are expressed as mean ± SD. *p < 0.05, compared with the results of body weights of the mice in other groups.

(B) H&E-stained colonic tissue of the mice administered ECOVA harvested on day 4. Histology of NOD2-deficient mice with colitis highly indicative of inflammation seen throughout the colon tissue; histology of NOD2-TLR2-deficient mice indicating occasional island of cellular infiltration; most of the tissue was free of inflammation.

(C) Flow cytometric analysis of mesenteric lymph node cells stained with PE-conjugated CD4 mAb and FITC-conjugated Vβ5 mAb. Dead cells were excluded by PI staining. The number in each figure shows the percentage of $CD4^+V\beta5^+$ T cells (OVA-specific $CD4^+$ T cells).

(D) Concentrations of IFN-γ in supernatants of cultures of mesenteric lymph node cells stimulated with CD3 mAb (2 μg/ml) or $OVA_{323-339}$ peptide (0.5 μM) for 60 hr. Results are expressed as mean ± SD. **p < 0.01, compared with the results of littermate control mice treated with ECOVA.

disease is caused by an excessive effector Th1 response (mice with TNBS colitis) as well as in situations in which disease is due to lack of regulatory cytokine responses such as IL-10 (*Stat3*$^{-/-}$-*Tlr4*$^{-/-}$ mice).

A notable feature of ECOVA colitis in NOD2-deficient mice is that the colitis does not occur or is greatly decreased in NOD2-TLR2 double-deficient mice, i.e., in the absence of TLR2 signaling. This has several implications. First, it indicates that the generation of IL-12 and other features of the Th1 response in ECOVA colitis is not due to a defect in NOD2 signaling that is independent of PGN interaction and signaling through TLR2. Instead, it adds support to the idea that the colitis is in fact due to a dysregulated TLR2 response related to defective NOD2 signaling. Such dysregulation could be due to the possibility that intact NOD2 signaling results in the elaboration of one or more factors that normally regulate TLR2 signaling and thereby downmodulate the IL-12 response that results from such signaling. Second, it shows that a gram-negative organism such as *E. coli* that contains a relatively small amount of the TLR2 ligand, PGN (or TLR2 ligands associated with PGN), in its coat (as compared to gram-positive organisms) is sufficient to cause colitis in NOD2-deficient mice. This can be due to the fact that the amount of PGN in the ECOVA, although relatively small, is sufficient for the induction of inflammation or that the inflammation is driven by PGN derived from other organisms as well. Third and finally, the attenuation of ECOVA colitis in the absence of TLR2 implies that an innate immune response of mucosal APCs to PGN (such as their production of IL-12) is sufficient for the induction of colitis specific to an Ag associated with the commensal microflora. By the same token, dysregulation of the response of

other TLRs as a result of the absence of NOD2 is not necessary for the occurrence of colitis in this model. One caveat to this last point is that the ECOVA colitis induced here was a transient colitis (see Discussion below), and it may be that in a more prolonged colitis due to deficient NOD2 function, dysregulation of other TLR responses will be seen as well.

The ECOVA-induced colitis in NOD2-deficient mice described here can be considered a model of human Crohn's disease associated with NOD2 abnormalities, inasmuch as the latter occurs not only in patients with disease of the terminal ileum, but also those with ileocolonic disease or even colonic disease alone (Lesage et al., 2002). In addition, the model resembles the human disease on several immunologic grounds. First, the experimental model is associated with a genetic defect that, as discussed above, leads to an abnormal innate immune response characterized by the development of APCs overproducing IL-12. Second, the model is characterized by an IL-12-driven Th1 T cell-mediated inflammation associated with increased Ag-specific T cell proliferation and survival (the latter due to decreased apoptosis). This was shown by the fact that increased numbers of OVA-specific T cells could be identified in LP and MLN of NOD2-deficient mice after ECOVA challenge and, as shown previously, is traceable in part to the known antiapoptotic effect of IL-12 (Fuss et al., 1999; Watanabe et al., 2003). Third and perhaps most importantly, the model is associated with an inflammation induced by a nonpathogenic (commensal) organism, i.e., an *E. coli* that expressed an Ag relevant to the adoptively transferred $CD4^+$ T cells. These characteristics are mirrored in Crohn's disease in that the human inflammation is also marked by overproduction of IL-12 by APCs in the lamina propria (Monteleone et al., 1997; Parronchi et al., 1997) as well as the presence of Th1 effector cells producing IFN-γ (Fuss et al., 1996). In addition, as in the model, T cells in the lesions are resistant to apoptosis (Boirivant et al., 1999). Finally, in recent years, Crohn's disease has also been linked to abnormal responses to one or more Ags in the commensal organisms of the mucosal microflora (Bouma and Strober, 2003; Strober et al., 2002). This is highlighted by emerging data that flagellins and perhaps other Ags associated with mucosal microflora may be involved in the development of Crohn's disease (Cong et al., 1998; Lodes et al., 2004). These parallel findings in the model and the human inflammatory bowel disease suggest that Crohn's disease associated with NOD2 mutations (and perhaps other patients as well) is due to an abnormal innate immune response leading to increased IL-12 responses of APCs coupled with an abnormal and excessive IFN-γ response to an Ag associated with a commensal organism. In this formulation of disease pathogenesis, while the mutations are necessary for the occurrence of disease, they are not sufficient: a second genetic or environmental factor that allows expansion of $CD4^+$ T cells recognizing a commensal Ag must also be present for development of disease.

While ECOVA colitis in NOD2-deficient mice bears many similarities to human Crohn's disease, it also contains some notable differences. One such difference concerns the fact that TNF production was not increased in NOD2-deficient mice with ECOVA colitis, reflecting previous data that PGN-stimulated APCs from such mice do not produce increased amounts of this cytokine (Watanabe et al., 2004). This lack of TNF overproduction contrasts with the massive TNF production seen in Crohn's disease and the response of patients to treatment with TNF Ab therapy (Targan et al., 1997). One possible resolution of this discrepancy is that the ECOVA colitis in NOD2-deficient mice represents an early or primordial form of Crohn's disease and reflects the latter at an early stage of disease development that is dependent on IL-12 production but not TNF production. In this view, it is only later when the disease results in additional pathologic changes that one sees the TNF response.

A second difference between ECOVA colitis in NOD2-deficient mice and Crohn's disease is the transience of the former and the persistence of the latter. One factor that could explain this discordance is that the ECOVA (the inciting organism) had only a narrow window of access to potentially reactive mucosal cells in this model. It should be noted, in this regard, that the ECOVA was administered with ethanol only on day 0 of colitis induction and therefore mucosal barrier function after the first or second day may have been sufficient to exclude this organism from the internal milieu. In humans with Crohn's disease, the inciting organism may be both more persistent than ECOVA and more capable of penetrating the mucosal barrier such as the enteroadherent *E. coli* said to be associated in some patients with the disease (Martin et al., 2004).

In summary, the data described here provide insight into the conditions necessary for the development of Th1-mediated colitis in mice with an abnormality of NOD2 function. In essence they show that a NOD2 abnormality leads to a defective innate immune response involving APCs that gives rise to disease only in the presence of T cells that mount an adaptive immune response to an Ag in the mucosal microflora. While the colitis induced in NOD2-deficient mice by administration of ECOVA requires provision of exogenous T cells specific for OVA, it is conceivable that humans with NOD2 mutations spontaneously develop T cells that react with specific Ags in the bacterial microflora because of environmental factors or additional genetic factors. Finally, in relating a genetically determined Th1 response and subsequent inflammation to an Ag in the bacterial microflora, these data provide additional support for the concept that Crohn's disease results from an abnormality in mucosal unresponsiveness to microfloral components.

Experimental Procedures

Mice

6- to 10-week-old male *Card15*$^{+/+}$ and *Card15*$^{-/-}$ mice (Pauleau and Murray, 2003) were used. *Tlr2*$^{-/-}$ mice were kindly provided by Dr. S. Akira and crossed to *Card15*$^{-/-}$ mice to generate *Card15*$^{-/-}$*Tlr2*$^{-/-}$ mice. OT-II mice were purchased from Jackson Laboratory and have a transgenic Vα2Vβ5 TCR specific for the $OVA_{323–339}$ epitope in the context of IA^b. Animal use adhered to National Institutes of Health Animal Care Guidelines.

Cell Isolation and Stimulation

Total splenocytes were isolated as described previously (Watanabe et al., 2002). $CD11b^+$ cells were isolated from the spleen of NOD2-deficient or NOD2-intact mice by positive selection by means of anti-mouse CD11b microbeads (Miltenyi BioTech). $CD4^+$ T cells were isolated from the spleen of OT-II mice by positive selection

with anti-mouse CD4 microbeads (Miltenyi BioTech). The purity of each fraction was >90% by flow cytometry. OT-II $CD4^+$ T cells (1×10^6/ml) were stimulated with $OVA_{323-339}$ peptide (0.5 μM) presented by splenic $CD11b^+$ cells (1×10^6/ml) from NOD2-deficient and NOD2-intact mice. LPS (1 μg/ml, Sigma), PGN from *Staphylococcus aureus* (10 μg/ml, Fluka), PGN from *Escherichia coli* (10 μg/ml, InvivoGen), Pam_3CSK4 (1 μg/ml, InvivoGen), dsRNA (50 μg/ml, InvivoGen), Flagellin (1 μg/ml, InvivoGen), Loxoribine (100 μM, InvivoGen), CpG (1 μM, InvivoGen), or MDP (10 μg/ml, Sigma) was added to this culture. Purified PGN from *Staphylococcus aureus* was kindly provided by Dr. R. Dziarski (Dziarski and Gupta, 2005). Cells were incubated in RPMI1640 medium containing 10% FCS for 48 or 72 hr. Unless specified, the concentrations described above were used throughout the study. Culture supernatants were harvested at 72 hr for IFN-γ, IL-4, IL-10, and IL-13 and 48 hr for TNF, IL-18, and IL-12p70. Concentrations of cytokines were determined with the use of ELISA kits purchased from Pharmingen (IFN-γ, IL-4, IL-10, IL-18, and TNF) and R&D Systems (IL-13 and IL-12p70). In this study, apoptotic cell death and cell division of responder OT-II $CD4^+$ T cells were assessed by Annexin V binding assay and CFSE staining, respectively, as described before (Watanabe et al., 2003). For Annexin V and CFSE staining, cells were incubated for 48 and 72 hr, respectively.

Flow Cytometry

PE-CD4 and FITC-Vβ5 mAbs were purchased from Pharmingen. Nonspecific binding of Abs was blocked by Fc-block mAb (2.4G2, Pharmingen) and dead cells were excluded by PI staining. The analysis was performed on a Becton Dickinson FACS Caliber with CELLQuest II software.

Stimulation of Cells with ECOVA or ECLACZ

ECOVA and ECLACZ were prepared as described previously (Watanabe et al., 2005b; Yoshida et al., 2001, 2002). OVA-specific $CD4^+$ T cells isolated from the spleen of OT-II mice were stimulated with several doses of gentamycin-killed ECOVA or ECLACZ in the presence of splenic $CD11b^+$ cells from NOD2-deficient or NOD2-intact mice. In some experiments, neutralizing Ab for TLR2 (eBioScience) (Meng et al., 2004) or TLR4 (Imgenex) (Poltorak et al., 2000) was used at the indicated dose.

Induction of Colitis

Littermate control, *Card15*$^{-/-}$ (NOD2-deficient), *Tlr2*$^{-/-}$, and *Card15*$^{-/-}$*Tlr2*$^{-/-}$ (NOD2-TLR2-deficient) mice were adoptively transferred with 5×10^6 $CD4^+$ T cells purified from the spleen of OT-II mice followed by subcutaneous immunization of OVA+CFA in the footpad the next day. 3 days after the immunization, recipient mice were challenged with intrarectal administration of ECOVA or ECLACZ (1×10^9 colony-forming units) after pretreatment with intrarectal injection of 100 μl of 50% ethanol (day 0, Figure S3A). Mice were challenged with intrarectal administration of the bacteria every day and were treated with antibiotics in the drinking water containing 1.0 mg/ml of ampicillin (Sigma) and kanamycin (Sigma) (Watanabe et al., 2005b; Yoshida et al., 2001, 2002). In some experiments, mice were intraperitonealy injected with neutralizing Ab to murine IL-12p40 (C17.8, 1 mg/mouse) or control Rat IgG (ICN) on day 1 and day 2. At the indicated time points, colon tissues were taken and fixed in 10% buffered formalin for histological analysis via hematoxylin and eosin (H&E) staining. Histology score based on cell infiltration, depth, and crypt loss was evaluated as previously described (Watanabe et al., 2005b; Yoshida et al., 2001, 2002). At the indicated time points, mesenteric lymph node cells and colonic lamina propria cells were isolated as described previously (Watanabe et al., 2005b; Yoshida et al., 2001, 2002). These cells (1×10^6/ml) were stimulated with 0.5 μM of $OVA_{323-339}$ peptide, 10 μg/ml of PGN, or 2 μg/ml of CD3 mAb (Pharmingen) for 48 or 60 hr to measure the concentration of IFN-γ, IL-4, IL-10, IL-12p70, and TNF in the supernatants. Mesenteric lymph node cells and lamina propria cells were stained with PE-CD4 mAb and FITC-Vβ5 mAb for visualizing the population of OVA-specific $CD4^+$ T cells by flow cytometry. In some experiments, mononuclear cells were isolated from the popliteal lymph nodes 3 days after the subcutaneous immunization of OVA+CFA at the footpad. These lymph node cells (1×10^6/ml) were stimulated with 0.5 μM of $OVA_{323-339}$ peptide for IFN-γ and IL-4 assay.

Statistical Analysis

Student's t test was used to evaluate the significance of the differences. Statistical analysis was performed with the StatView v.4.5 program (Abacus Concepts, Berkeley, CA). A value of $p < 0.05$ was regarded as statistically significant.

Supplemental Data

Supplemental Data include three figures and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/25/3/473/DC1/.

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