Nod2 protects the gut from spreading experimental colitis to small intestine
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Abstract

Background and Aims: Nucleotide Oligomerization Domain 2 (*NOD2*) mutations are key risk factors for Crohn's disease (CD). *NOD2* contributes to intestinal homeostasis by regulating innate and adaptive immunity together with intestinal epithelial function. However, the exact roles of *NOD2* in CD and other *NOD2*-associated disorders remain poorly known.

Methods: We initially observed that *NOD2* expression was increased in epithelial cells away from inflamed areas in CD patients. To explore this finding, *Nod2* mRNA expression, inflammation and cytokines expression were examined in the small bowel of wild-type (WT), *Nod2* knockout and *Nod2* mutant mice after rectal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS).

Results: In WT mice, *Nod2* upregulation upstream to rectal injury was associated with pro-inflammatory cytokine expression but no overt histological inflammatory lesions. At the opposite, in *Nod2* deficient mice, the inflammation spread from colitis to ileum and duodenum.

Conclusions: *Nod2* protects the gut from spreading colitis to small intestine.

Key words: *Nod2*/Crohn's disease/CD4⁺ T cells/inflammatory cytokines/gut barrier.

Abbreviations: CD: Crohn's disease; MDP: Muramyl dipeptide; *NOD2*: nucleotide oligomerization domain 2; TNBS: 2,4,6-trinitrobenzene sulfonic acid; WT: wild-type.

Introduction

Crohn's Disease (CD) is an inflammatory bowel disease (IBD) that can affect any part of the gastrointestinal tract. Genetic and epidemiological studies indicate that CD is a complex, multifactorial disorder. Interplay between genetics and the environment promotes gut abnormalities of autophagy, reticulum endoplasmic stress, innate and adaptive immune responses, Th-1 and Th-17 polarization, intestinal barrier and microbial composition.¹⁻⁴

Nucleotide oligomerization domain 2 (*NOD2*, also known as NLR-C2 and CARD15) is the most prominent susceptibility gene for CD.^{5, 6} One-third to one-half of CD patients display one or more *NOD2* mutations.⁷ Wild-type (WT) *NOD2* is activated by muramyl dipeptides (MDP) which are components of the bacterial cell wall⁸ while CD-associated *NOD2* mutations prevent MDP responses.⁹ CD can therefore be considered as an immune deficiency associated to insufficient responses to bacteria. Nevertheless, the exact mechanisms involved in *NOD2* mutations contribution to CD pathogenesis remain a matter of debate.¹⁰⁻¹³

NOD2 regulates the homeostasis of the intestinal epithelium.¹⁴⁻¹⁸ *Nod2* ablation in mice leads to an increased bacterial translocation¹⁶ across the small intestinal epithelium. In Human, it increases the production of inflammatory cytokines by Th17 oriented T-cells.¹⁵ As a whole, *Nod2* deficiency is characterized by an impaired crosstalk between inflammatory cytokine-secreting CD4⁺ T-cells and epithelial cells.^{16, 19} Similarly, increased CD4⁺ T-numbers and altered homeostasis of epithelial cells have been reported in the intestinal mucosa of CD patients.^{20, 21} Furthermore, CD therapies like anti-TNF- α antibodies restore the intestinal barrier in treated patients.²² Impaired epithelial functions may therefore be an early event in the progression of CD lesions.

Here, we show that *NOD2* expression in CD patients is not only increased in inflammatory lesions but also at sites distant from inflammatory areas. In mice, *Nod2* expression limits the inflammation to the colon in a model of colitis induced by 2,4,6 trinitrobenzene sulfonic acid (TNBS).

Material and Methods

Patients and biopsies. Intestinal biopsies were obtained from 17 untreated children during routine endoscopies performed to establish CD diagnosis. Clinical and endoscopic data of included patients are summarized in Supplementary Table 1. Controls were histologically normal digestive biopsies obtained from 5 children without IBD. For each participant, one or two biopsies from the ileum and/or caecum were sampled. Biopsies were either immediately frozen and later stained with toluidine blue or fixed in 4%-phosphate-buffered formalin and stained with hematoxylin and eosin. NOD2 immunostaining was performed as previously described with two different rabbit polyclonal antibodies (Cayman Chemical and a gift from G Thomas CEPH).²³ Laser microdissection was performed on 7µm sections obtained from the frozen biopsies. After verification of the quality of the tissues, and the absence of ulcers, surface epithelial cells and lamina propria cells were laser-microdissected using a Leica^R AS LMD system (Leica microsystems) in less than one hour. A mean of 500 cells were microdissected from each of the specimens (range 100-1000 cells) and stored in Trizol^R reagent (Invitrogen, Groningen, The Netherlands). The study was approved by the national ethical committee (Saint Louis Hospital, Paris, France) and all the parents of participants provided a signed informed consent.

Animal models. Housing and experiments were conducted according to institutional animal healthcare guidelines and were approved by the local ethical committee for animal experimentation (Comité Régional d'Ethique en matière d'Expérimentation Animale no. 4, Paris, France). C57BL/6 wild-type (WT), *Nod2* null allele (*Nod2*^{KO}) and *Nod2*^{2939insC} mice (homozygotes for a mutation homologous to the Human 3020insC variant) were generated or hosted in a pathogen free animal facility.^{15, 16} The animal facility was monitored every six months in accordance with the full set of FELASA high standard recommendations.

CD4⁺ T-cells were depleted by two intra-peritoneal T(i.p.) injections of 100µg purified GK1.5 (anti-L3T4 (CD4⁺) monoclonal antibody (Pharmingen, Germany), 96 and 24 hours before experimentation and 24hours after TNBS administration.¹⁶

Colitis induction. Colitis was induced in 12 weeks old mice by a single intra-rectal administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS, Sigma, France), which was dissolved in ethanol (50:50 vol/vol) at a dose of 120 mg/kg body weight under anaesthesia.¹⁵ Groups used as controls (vehicle) received an equal volume of PBS and ethanol (50:50 vol/vol) intra-rectally. A 100 µl aliquot of the freshly prepared solution was injected into the colon, 4 cm from the anus, using a 3.5 F polyethylene catheter as previously described.¹⁵ Body weight loss and disease activity index (DAI) were monitored before and 72h after TNBS

administration. Mice were sacrificed by cervical dislocation. Colonic length and macroscopic damage Wallace score were recorded.²⁴

Duodenal and ileal samples were collected at respectively 4 cm downstream the stomach and 4 cm upstream the caecum. They were fixed in 4%-phosphate-buffered formalin and embedded in paraffin. 5µm sections were cut and stained with hematoxylin and eosin. Grading of the inflammatory scores were performed in blind fashion according the following criteria²⁵: 0, no sign of inflammation; 1, very low level of leukocyte infiltration; 2, low level of leukocyte infiltration; 3, high level of leukocyte infiltration, high vascular density, and thickening of the colon wall; 4, transmural infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall in less than half of circumference ; 5 necrosis of more than half the circumference and transmural inflammation.

MDP localization. Mice were injected intraperitoneally with 300µg of rhodamine-labeled MDP (InvivoGen, San Diego, CA). Two hours later, mice were anesthetized with isofurane (Centre Spécialités Pharmaceutiques, Moussey-le-Neuf, France) and sacrificed. Ileal and duodenal samples were collected and rinsed with ice-cold PBS (ThermoFisher, Waltham, MA). Tissue was frozen in liquid nitrogen using HistoLab OCT cryomount (Histolab, Gothengurg, Sweden), 10µm-thick cryosections were cut and then fixed in 4%-phosphate-buffered formalin. MDP-rhodamine localization was detected by fluorescence confocal microscopy (confocal sp8, Leica, Frankfurt am Main, Germany).

ELISA. Biopsies of duodenum, ileum and colon from different Mouse models were collected and washed with cold PBS.¹⁵ Biopsies were then homogenized using an ultra-thorax in 1 ml of PBS1X and protein concentration was determined using a commercial kit (Biorad, Marnes la Coquette, France).²⁶ IFN-γ, IL-1β, IL-12 and TNF-α protein levels were determined by ELISA according to manufacturer's instructions (BD Biosciences).²⁷

DNA extraction and real time quantitative PCR. After extraction by the NucleoSpin RNA II Kit (Macherey-Nagel, France), total RNAs were converted to cDNA using random hexonucleotides and then used for RT-PCR (Invitrogen). We conducted qPCR with QuantiTect SYBR Green PCR Kit (Applied, France) using sense and antisense primers specific for *g3pdh*, *Ifn-γ*, *Il-1β*, *Il-12*, *Nod2*, and *Tnf-α* (primers used are available in table 1). The cycle threshold (Ct) was defined as the number of cycles at which the normalized fluorescent intensity passed the level of 10 times the standard deviations of the baseline emission calculated on the first 10 PCR cycles. Results are expressed as $2^{-\Delta\Delta Ct}$ as previously described.²⁸ For RNA samples obtained by laser microdissection, NOD2 expression was measured in triplicate and normalized

using the Abelson housekeeping gene. To derive a relative number of mRNA molecules, a titration curve was established with NOD2 plasmids (from 1 to 10^6 copy/microliters).

Statistical analysis. For all analysis, multigroup comparisons were performed using one-way ANOVA statistics with Bonferroni correction for multiple comparisons where an unpaired t-test assuming the Gaussian distribution was applied. The Gaussian distribution was tested by the Kolmogorov-Smirnov test. Statistical analyzes were performed using GraphPad Prism 7.00 (GraphPad Software). A two-sided P-value < 0.05 was considered statistically significant.

All authors reviewed the data and approved the final manuscript.

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Results

Epithelial NOD2 expression is increased in uninflamed mucosae of CD patients.

Looking at NOD2 expression in CD patients, we were surprised to see that epithelial NOD2 expression is increased in mildly inflamed areas of the digestive tract, away from the sites of injury. To confirm NOD2 upregulation in these areas, we examined expression in ileal and/or caecal biopsies from 17 treatment-naïve pediatric CD cases and five non-inflammatory controls. Although nine CD patients had heterozygous mutations in NOD2 (1007fs n=3, R702W n=5 and R373C n=1), no histological differences or changes of NOD2 expression were seen between patients with WT or mutant NOD2. Immunostaining using two different antibodies showed that NOD2 was weakly expressed by villi enterocytes and rare mononuclear cells immediately under the epithelium in control ileum (Figure 1A). In contrast, NOD2 expression was increased in the ileum of CD patients (Figure 1B and C). While NOD2 expression was upregulated in lamina propria mononuclear cells within inflammatory areas, the most prominent increases were observed in epithelial cells in uninflamed areas (Figure 1B and C). Analysis of caecal biopsies provided similar conclusions (Figure 1D-F). Semi-quantifying methods of inflammation and immunostaining further supported that epithelial NOD2 expression was mainly observed in uninflamed intestinal areas (Table 2, Spearman correlation test $r_s = -0.95$; $P < 0.0001$).

To confirm these findings, we quantified by qPCR NOD2 mRNA expression within the epithelial and lamina propria compartments of the same biopsy. Biopsies from eight active patients and four controls were studied after laser microdissection (Figure 1G). In accordance with immunostaining data, NOD2 expression was low in both compartments in controls. In patients, we observed that NOD2 mRNA expression was inversely correlated in the epithelial and lamina propria compartments of the same biopsy. In the lamina propria, the average NOD2 copy number was 43.1 in controls (normalized arbitrary units). In CD patients, similar values (43.6) were observed in uninflamed areas whereas NOD2 expression was increased by 5-fold (205.907) in the inflamed ileum. On the contrary, the mean values were 4.91 in epithelial cells of controls and 4.6 in inflamed ileal areas while a 100-fold increase in NOD2 expression (660) was detected in uninflamed ileum. Noteworthy, normal Paneth cells had low NOD2 expression in controls (1.43). This expression was increased by the inflammation and in heterotopic colonic Paneth cells, NOD2 being mostly expressed by the enterocytes. We thus concluded that NOD2 expression is markedly increased in epithelial cells distant from inflammatory lesions in CD patients.

Colitis increases epithelial Nod2 and cytokine expressions in small intestine by a mechanism involving CD4⁺ T-cells.

To explore the expression of epithelial Nod2 in healthy areas distant from intestinal lesions, we turned to mouse models. We treated *Nod2* wild-type (*Nod2*^{WT}) mice by an intra-rectal administration of TNBS. Instillation of TNBS in mice is known to induce a severe inflammation in the distal colon.¹⁵ In small bowel (*i.e.* at a significant distance from the gut injury), TNBS has been shown to alter the biochemical activity of brush border enzymes (sucrase isomaltase and aminopeptidase), mucins and cytokines levels²⁹ but without overt histological lesions. Three days after instillation, mice were sacrificed and the severity of inflammation was assessed (Figure 2). As expected, in the distal colon, TNBS administration induced a robust colonic inflammation as evidenced by decreased body weight, increased DAI, reduced colon length and high macroscopic Wallace damage scores (Figure 2A-D). Consistent with this phenotype, expression levels of TNF- α , IFN- γ and IL-12 were increased (Figure 2E) at the site of colonic inflammation.

We next examined the small intestine. As expected, we did not find any overt inflammatory lesion in the duodenum or ileum despite higher mononuclear cells infiltration (Figure 2F) and increased TNF- α , IFN- γ and IL-12 proteins (Figure 2H) and mRNA (Figure 2I) levels. Myeloperoxidase staining did not show frank infiltration by neutrophils in the small bowel compared to the inflamed colon (figure 2G). As observed in CD patients, expression of *Nod2* was increased in the duodenum, ileum and the uninflamed part of the large intestine remote from rectal injury (Figure 2I). We hypothesized that this effect was consecutive to the recirculation of CD4⁺ pro-inflammatory T-cells in the gut mucosa. We therefore treated TNBS-challenged mice with anti-CD4⁺ monoclonal antibodies. This treatment only partially improved the colitis but restored normal levels of *Nod2* and inflammatory cytokines in the duodenum and ileum (Figure 2A-I).

Of note, given the abundance of immune cells in inflamed areas, higher levels of epithelial NOD2 would be expected in the inflamed bowel of CD patients if the expression of epithelial *NOD2* was under the control of CD4⁺ T-cells. We therefore determined the populations of immune cells present in the lamina propria of CD patients by immunostaining. Lamina propria CD4⁺ T-cell numbers were not increased in areas with the highest grades of inflammation. Most immune cells present in the lamina propria at these sites within the ileum (Figures 3A and B) and the colon (Figure 3C and D) were CD163⁺ macrophages. Consistently, limited in the ileum, CD4⁺ T-cells predominated in areas with mild grade inflammation.

NOD2 activation maintains the barrier integrity on remote small bowel.

To check the role of Nod2 in the maintenance of the gut barrier, *Nod2*^{WT} mice were treated with MDP for two consecutive days before experimentation.¹⁹ Intraperitoneal injection of rhodamine-labelled MDP confirmed the ability of MDP to enter the enterocytes (Figures 4A-C). NOD2 stimulation slightly reduced DAI, colonic length, Wallace damage scores and pro-inflammatory cytokine expressions without any effects on body weight loss after rectal TNBS infusion (Figures 4D-H). In small bowel, MDP treatment normalized mRNA (Figure 4I) and protein (Figure 4J) levels of pro-inflammatory cytokines. As expected, MDP did not affect *Nod2* expression (Figure 4I).

These data suggested that Nod2 activation may prevent an inflammation in the small bowel in case of inflammation. We thus looked at Nod2 deficient mice. Ablation of *Nod2* in mice (*Nod2*^{KO}) resulted in an increased susceptibility to TNBS-induced colitis (Figures 5A-D).¹⁵ More importantly, while TNBS-treated *Nod2*^{WT} mice exhibited no lesion in the small bowel (Figure 5E), two thirds of *Nod2*^{KO} mice showed overt duodenal inflammatory lesions as shown by a slight infiltration of scattered neutrophils in the *lamina propria* (Figures 5E and F). In the ileum, we observed a marked inflammation in 5/8 *Nod2*^{KO} mice, an infiltration of neutrophils and mononuclear cells in the villi and the crypts and a loss of muco-secretion. In addition, *Nod2*^{KO} mice exhibited an increased expression of pro-inflammatory cytokines in the duodenum and the ileum (Figure 5G). As expected, treatment with MDP did not correct the expression of pro-inflammatory cytokines in the intestine of *Nod2*^{KO} mice (Figures 6A-F).

In humans, among the *NOD2* genetic polymorphisms associated with CD, the 3020insC mutation encoding for a truncated (1007fs) protein has the strongest effect. We thus studied *Nod2*^{2939insC} mice which carry a mutation homologous to the Human 3020insC variant.¹³ As for *Nod2*^{KO} mice, they developed a slightly more severe colitis after TNBS administration (Figure 5A-D). Here too, we observed inflammatory lesions in the duodenum and ileum of respectively 4/5 and 6/8 *Nod2*^{2939insC} mice after TNBS instillation (Figures 5E and F). *Nod2*^{2939insC} mice exhibited an increased expression of pro-inflammatory cytokines in the duodenum and the ileum (Figure 5G). Finally, and as expected, treatment of *Nod2*^{2939insC} mice with MDP did not reduce the expression of pro-inflammatory cytokines in the small intestine (Figure 6A-E). We thus concluded that mice carrying a CD-associated mutation of *NOD2* are not able to restrain the intestinal inflammation at the site of the primitive inflammatory lesions.

Discussion

Here we show that Nod2 protects the gut from the inflammation spreading from the colon to the small intestine.

We first observed that NOD2 expression was increased remote from primary inflammatory lesions in naïve pediatric CD patients. Interestingly, the increase in NOD2 expression was not restricted to immune cells in inflammatory areas as it was also detected in epithelial cells distant from CD lesions. We therefore hypothesized that epithelial NOD2 may have a specific role in healthy intestinal areas and explored the intestine tissue at distance from local injuries in mice.

The TNBS-induced colitis is a well-known model of self-limited inflammation. In rats, the rectal administration of TNBS was found to induce some limited changes in the small intestine but without overt histological lesions.³⁰ In mice, we also observed an increase of pro-inflammatory cytokines (TNF- α and IFN- γ) in the small intestine with no overt duodenitis or ileitis. We also confirmed the overexpression of epithelial *Nod2* as in CD patients, indicating that the TNBS induced-colitis model was relevant to further explore the question.

Since Nod2 is known to protect the gut barrier, we supposed that its overexpression remote to the inflammatory area could limit the extension of the inflammation. In accordance with this hypothesis, *Nod2* ablation or *Nod2* mutations induced obvious inflammatory lesions in the small intestine. Of note *Nod2* deficiency slightly increased the severity of the colitis itself. The intensity of the colitis may thus contribute at least in part to this effect. Indeed, a correlation between the intensity of inflammatory lesions in both colon and small intestine is likely expected in case of a cross-talk between the small and large bowels. However, the frank effect of Nod2 activation in WT mice strongly supports the role of NOD2 in limiting the gut inflammation.

The mechanisms involved in gut inflammation in Nod2 deficient mice have been studied in details previously.^{15,16,18} Proinflammatory Th1 and Th17-oriented T-cells present in the lamina propria produce high levels of TNF- α and IFN- γ . These proinflammatory cytokines increase the gut permeability via myosin-like chain kinase activation which opens the tight junctions.¹⁵ As a result, there is an increased translocation of small molecules and bacteria which promotes the inflammatory response locally. In WT mice, this vicious circle is limited by Nod2 activation which limits both Th17 T-cells orientation¹⁶ and tight junction opening.¹⁵ Of note, Nod2 expression by both epithelial and immune cells are important in WT mice.¹⁸

The data presented here are concordant with this general model. Activated recirculating CD4⁺ T-cells export their effect remote to the initial lesion. Epithelial Nod2 is overexpressed

under the stimulation by TNF- α and IFN- γ .¹⁵ It reduces the gut permeability, limits bacterial translocation and controls the inflammation. However, if Nod2 is absent or ineffective, epithelial Nod2 is no more able to prevent the inflammation and inflammatory lesions are exported to the small intestine.

The role of NOD2 in the development of small intestinal lesions is well established in CD patients. Indeed, people carrying NOD2 mutations most often exhibit ileal (L1) or ileocolonic (L3) phenotypes.⁷ The data presented here suggest that this finding may be in part explained by the defective role of Nod2 in the small intestine. If true, time and space self-limited intestinal inflammations may be sufficient to induce chronic small bowel lesions.

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Figures legend.

Figure 1: NOD2 expression is increased in intestinal mucosa of Crohn disease patients.

(A-F) Biopsies from controls (A and D) and naïve pediatric CD patients (B, C, E and F) were immunostained with anti-NOD2 antibodies. Ileal (A-C) or cecal (D-F) biopsies were obtained from inflamed (C and F) or uninfamed areas (B and E). Data shown are representative of 5 controls and 17 CD patients. (G) Number of *NOD2* mRNA copies were normalized by the expression of *Abelson* gene and expressed as arbitrary units. mRNA levels were calculated for the epithelial monolayer (in blue) and the lamina propria (in red) from the same biopsy after laser microdissection. Biopsies from 8 CD patients were obtained from inflamed or uninfamed intestinal areas and referenced by an arbitrary number. “C” denote biopsies from 4 controls.

Figure 2: Increased pro-inflammatory cytokines expression in small intestine are mediated by inflammatory CD4⁺ T-cells induced by colonic TNBS infusion.

(A-I) C57BL/6 wild-type mice (*Nod2*^{WT}) were instilled intra-rectally with TNBS. Vehicle control group was challenged by PBS-Ethanol. Mice were treated with anti-CD4⁺ antibodies where indicated. 3 days after instillation, the intensity of the colitis was monitored with the following parameters: (A) Weight loss; (B) Disease activity index; (C) Colonic length (cm); (D) Colonic macroscopic score (Wallace score); (E) Pro-inflammatory cytokine levels in inflamed colons. In parallel, intestinal samples were explored by microscopic examination after hematoxylin-eosin staining (F, original magnification x20) and myeloperoxidase staining (G, scale bars 100µm); and by proteins (H) and mRNA expression (I) levels of pro-inflammatory cytokines. Each point = one mice ; mean±s.e.m; 3 independent experiments; *P<0.05, *P<0.01 and ***P<0.001 vs. vehicle control group or indicated group; ++P<0.01 vs. TNBS group.

Figure 3: CD4 and CD163 immunostaining of ileal and colonic biopsies from CD patients.

(A-B) Ileal and (C-D) colonic biopsies were collected from uninfamed or inflamed locations in CD patients. (A and C) Grading of the inflammation was confirmed by coloration by hematoxylin-eosin (HES) and CD4⁺ or CD163⁺ positive cells were assessed by immunostaining. (B and D) CD4⁺ or CD163⁺ positive cells were counted in the lamina propria. (At least n=6 fields per patients; mean ± SEM; *P<0.05 vs. uninfamed CD4⁺ T-cells; +P<0.05 and +++P<0.001 vs. uninfamed CD163⁺ T-cells). Areas with lymphoid follicles were excluded.

Figure 4: NOD2 activation reverses the remote effects of TNBS-induced colitis.

(A-C) Localization of muramyl dipeptide (MDP) in the small intestine after intraperitoneal injection. Rhodamine-labeled MDP is detected in epithelial cells of the small intestine two hours after IP injection. Fluorescence (red) was detected in epithelial cells of the (B) duodenum and (C) ileum. Nuclei were stained with DAPI (blue). (A) The ileum of a mouse injected with distilled water was used as a negative control. Original magnification, X40. Scale bars: 100µM. (D-H) C57BL/6 wild-type mice were instilled intra-rectally with TNBS. Vehicle control group was challenged with PBS-Ethanol. Mice were treated with MDP where indicated. 3 days after induction, the colitis was monitored by the following parameters: (D) Disease activity index; (E) Colonic length; (F) Wallace score; (G) levels of pro-inflammatory cytokines in inflamed colon; (H) Weight loss. In parallel, the following measures were made in the duodenum and ileum: (I) mRNA expression of pro-inflammatory cytokines and *Nod2*; (J) protein expression of pro-inflammatory cytokines. (Each point = one mouse; mean $\pm$ s.e.m; 3 independent experiments; *P<0.05, **P<0.01 and ***P<0.001 vs. vehicle group or indicated group; +P<0.05, ++P<0.01 and +++P<0.001 vs. TNBS vehicle group).

Figure 5: TNBS induced colitis leads to small bowel inflammation in *Nod2* deficient or mutated mice.

(A-F) *Nod2*^{WT}, *Nod2*^{KO} and *Nod2*^{2939insC} mice were challenged by intra-rectal instillation of TNBS. 3 days after induction, the colitis was monitored with the following parameters: (A) Weight loss; (B) Disease activity index; (C) Colonic length; (D) Wallace score. (E) In parallel, microscopic examination after hematoxylin-eosin staining showed a slight infiltrate by mononuclear cells of the villous axis in the duodenum of mutated and invalidated mice. The infiltrate was more pronounced in the ileal samples with villous injuries in *Nod2* deficient mice. (F) protein levels of pro-inflammatory cytokines. (One point = one mouse; mean $\pm$ s.e.m; 3 independent experiments; **P<0.01 and ***P<0.001 vs. Vehicle group; ++P<0.01 vs. indicated group; ns=non-significant).

Figure 6: The small bowel is not protected by muramyl dipeptide in Nod2 deficient or mutated mice.

(A-E) *Nod2*^{WT}, *Nod2*^{KO} and *Nod2*^{2939insC} mice were challenged by intra-rectal instillation of TNBS. Mice were treated with muramyl dipeptide (MDP) or PBS (vehicle) where indicated. (A) Disease activity index; (B) Colonic length; (C) Wallace score; Levels of pro-inflammatory cytokine in the (D) colon or (E) small bowel. (One point = one mouse; mean $\pm$ s.e.m; 3 independent experiments; *P<0.05 and **P<0.01 vs. indicated group; +P<0.05, ++P<0.05 and +++P<0.001 vs. instilled TNBS *Nod2*^{WT} group).

Table 1. List of primers used for qPCR analyses in mice.

mRNA	Sense	Antisense
IL-12	5'-ACGAGAGTTGCCTGGCTACTAG-3'	5'-CCTCATAGATGCTACCAAGGCAC-3'
IFN- γ	5'-CAGCAACAGCAAGGCGAAAAAGG-3'	5'-TTTCCGCTTCCTGAGGCTGGAT-3'
TNF- α	5'-CATCTTCTCAAAATTCGAGTGACAA-3'	5'-TGGGAGTAGACAAGGTACAACCC-3'
Nod2	5'-GCCAGTACGAGTGTGAGGAG -3'	5'-CCCTGACGTGCTGTAGAAGG-3'

Table 2: NOD2 expression measured by immunohistochemistry and histological grading of the inflammation in biopsies from ileum (I) and caecum (C) of CD patients.

Location I/C	NOD2 Epithelium	NOD2 Lamina propria	Histological score of inflammation*
I	+	+++	6
I	+++	+/-	0
I	++	++	3
I	+++	+/-	0
I	+	+++	6
	+++	+/-	0
I	+++	-	0
I	+++	-	0
I	+	+++	6
C	++	++	3
C	+	+++	6
C	+	+++	4
C	+	+++	7
C	+	+++	5
C	+++	+	0
C	+++	+/-	0
C	+++	+	0
C	++	+++	5
C	++	+++	4

*according to D'Haens G et al. Endoscopic and histological healing with infliximab anti-tumor necrosis factor antibodies in Crohn's disease: A European multicenter trial. *Gastroenterology* 1999; 116:1029 - 34. Intensity of NOD2 staining : +/-: very low; +: low; ++: moderate; +++ high. Correlations between NOD2 expression in the epithelium and the lamina propria (columns 1 or 2) and the inflammatory score (column 3) were highly significant (Spearman test $r_s = -0.95$ and $r_s = 0.91$ respectively; $P < 0.0001$ for each test).

Figure 1

G

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6