Defcr-related sequence 10 (Defcr-rs10) was very low in Nod2−/− mice and was further reduced after infection in Nod2−/− animals relative to wild-type mice (Fig. 3, G and H). Cryptdins are antimicrobial peptides that are preferentially produced in intestinal Paneth cells, and their antimicrobial activity is important in suppressing infection with pathogenic bacteria, including L. monocytogenes (21) and Mycobacterium paratuberculosis, an organism implicated in CD (22, 23). Of the cryptdins, Defcr4 has the most potent bactericidal activity (24), with its expression being highest in the lower ileum, in contrast to other cryptdins (25, 26). By comparison, Defcr5 was expressed normally in Nod2−/− mice both before and after infection (Fig. 3I) (25).

Our results indicate that Nod2 is essential in the detection of bacterial MDP and is capable of activating the adaptive immune system by acting as an adjuvant receptor for antibody production, either directly or by enhancing the production of α-defensins (27, 28) or other immunostimulatory molecules. Therefore, Nod2 is critical in protecting the host from intestinal bacterial infection. More specifically, we reveal an important role for Nod2 in the regulation of a subgroup of cryptdins, offering a plausible mechanism to explain the association between Nod2 and susceptibility to CD. Murine cryptdins represent a more diverse family than those of human α-defensins and are already known to be critical in the innate immune responses to bacterial infection (29). CD-associated Nod2 mutations predispose primarily to ileal lesions (30–33), corresponding to the location of Paneth cells. Recent reports suggest that the expression of α-defensins is diminished in human CD patients, particularly those who have Nod2 gene mutations (34, 35). However, it remains to be established whether a defect in Paneth cell function is the only possible mechanism by which Nod2 mutations might associate with the development of CD in humans. Nevertheless, it seems reasonable to suggest that mutations in Nod2 might promote CD through defective regulation of responses to commensal and/or pathogenic bacteria, rather than acting as an initiating factor for disease. Further studies may resolve this issue and may lead to the development of more rational therapeutic approaches for treating CD.

References and Notes
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Nod2 Mutation in Crohn’s Disease Potentiates NF-κB Activity and IL-1β Processing
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Variants of NOD2, an intracellular sensor of bacteria-derived muramyl dipeptide (MDP), increase susceptibility to Crohn’s disease (CD). These variants are thought to be defective in activation of nuclear factor κB (NF-κB) and antibacterial defenses, but CD clinical specimens display elevated NF-κB activity. To illuminate the pathophysiological function of NOD2, we introduced such a variant to the mouse Nod2 locus. Mutant mice exhibited elevated NF-κB activation in response to MDP and more efficient processing and secretion of the cytokine interleukin-1β (IL-1β). These effects are linked to increased susceptibility to bacterial-induced intestinal inflammation and identify NOD2 as a positive regulator of NF-κB activation and IL-1β secretion.

Crohn’s disease (CD) is a chronic inflammatory bowel disease (IBD) thought to be caused by genetic and environmental factors that affect host-microbe interactions and production of inflammatory mediators (1, 2). Mutations that increase susceptibility to CD up to 40 times were mapped to the NOD2/CARD15 locus (3, 4). The NOD2 protein contains two N-terminal caspase recruitment domains (CARDs), a nucleotide-binding domain (NBD), and 10 C-terminal leucine-rich repeats (LRRs), and it is expressed mainly by macrophages and dendritic cells (5). NOD2 mediates intracellular recognition of MDP, a building block for bacterial cell walls (6, 7), and can activate NF-κB (5). Macrophages within the intestinal lamina propria of CD patients overproduce NF-κB targets, including the proinflammatory cytokine tumor necrosis factor–α (TNFα) and the interleukins IL-1β and IL-6 (2, 8). Many of the anti-inflammatory drugs used to treat CD inhibit NF-κB activation, which suggests it is a key pathogenic factor (8, 9). However, paradoxically, transient transfection experiments indicate that CD-associated

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Figs. S1 to S7
Tables S1 and S2
References
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734 4 FEBRUARY 2005 VOL 307 SCIENCE www.sciencemag.org
NOD2 variants no longer activate NF-κB in response to MDP (6, 7), which suggests that defective NF-κB activation in macrophages facilitates infection of the lamina propria by enteric bacteria. However, macrophages can activate NF-κB in response to bacteria independently of NOD2 (10), and Nod2 gene ablation did not cause spontaneous intestinal infections or colonic inflammation (11).

To find an explanation for these quandaries and to illuminate the mechanism by which CD-associated NOD2 variants act, we generated mice whose Nod2 locus harbors the homolog of the most common CD susceptibility allele, 3020insC, which encodes a truncated protein lacking the last 33 amino acids (3, 4). This was done through insertion of cytosome at position 2939 (corresponding to 3020 in human NOD2) of the Nod2 open reading frame (Fig. 1, A and B). Homozygous Nod22939ic mice were obtained at the expected mendelian ratio and did not show abnormalities of the gastrointestinal tract (fig. S1) or other organs; they were healthy (12). The mutation had no effect on Nod2 mRNA or protein amounts in bone marrow–derived macrophages (BMDMs) (Fig. 1, C and D).

We examined the effect of the Nod22939ic mutation on NF-κB activation in BMDM cultures. The activity of IKK, which is the inhibitor of κB (IκB) kinase, the degradation of IκBα, and NF-κB DNA binding activity were higher in MDP-stimulated Nod22939ic macrophages than in wild-type (WT) cells (Fig. 2A). Only marginal differences in mitogen-activated protein kinases (MAPKs) were observed (fig. S2). No genotype-specific differences in NF-κB activation were observed after macrophage treatment with other microbial components that activate Toll-like receptors (TLRs) (10), including the TLR2 agonists Pam3Cys (tripalmitoyl-S-glyceryl-Cys-Ser-4(Lys)) and peptidoglycan (PGN), the TLR4 agonist lipopolysaccharide (LPS), and the TLR9 agonist unmethylated CpG-containing DNA (Fig. 2B) (12). Expression of several NF-κB target genes was increased in MDP-treated Nod22939ic macrophages relative to WT counterparts (Fig. 2C). Only minor differences in expression of these genes were observed when macrophages were stimulated with LPS or PGN. Although MDP-induced gene expression of several cytokine genes was increased in Nod22939ic macrophages, only IL-1β secretion was significantly elevated in these cells relative to WT counterparts (Fig. 2, D and E, fig. S3). Secretion of IL-1α was modestly elevated, and neither IL-6 nor TNFα were secreted in response to MDP. The only microbial product that stimulated IL-1β secretion by Nod22939ic macrophages was MDP (Fig. 2E).

Macrophages involved in CD most likely reside in the lamina propria (2). To expose these cells to enteric bacteria, mice were treated with dextran sodium sulfate (DSS), an agent that kills mucosal epithelial cells and disrupts their barrier function, causing bacterial invasion (13). WT and homozygous Nod22939ic mice (8 to 12 weeks old) were given 3% DSS in drinking water for 6 days and monitored for weight loss, a characteristic of severe intestinal inflammation. After 8 days, body weight loss was greater in Nod22939ic mice relative to WT mice (Fig. 3A). Nod22939ic mice also exhibited increased mortality relative to WT mice (37.5% versus 0%) (fig. S4). Surviving mice of both genotypes regained body weight after day 11 and returned to normal 30 days after DSS administration (12). Histological analyses revealed that the severity and extent of inflammatory lesions in the colons of Nod22939ic mice were significantly (P < 0.05) greater than in WT controls, with larger areas of ulceration and increased infiltration of F4/80-positive macrophages (Fig. 3B, fig. S5).

After DSS exposure, Nod22939ic homozygotes expressed greater amounts of mRNAs encoding proinflammatory cytokines and che-
mokines in their colons relative to WT mice (Fig. 3C). The amounts of IL-1β, IL-6, and cyclooxygenase-2 (COX-2) protein were significantly higher in colons of DSS-treated Nod2<sup>2939C</sup> mice relative to WT counterparts (Fig. 3D). IL-6 and COX-2 were predominantly expressed in F4/80-positive macrophages within inflammatory lesions (Fig. 3E, fig. S6) (12). IKK and NF-κB activities and RelA(p65) nuclear staining were also higher in colons of Nod2<sup>2939C</sup> mice than in the WT (Fig. 3F, fig. S7). MAPK activation, however, was only marginally affected by the genotype (fig. S8).

The intestinal inflammatory response to DSS is dramatically reduced by oral antibiotics, which supports involvement of enteric bacteria (14). When given a high dose of DSS (6%) without oral antibiotics, WT and Nod2<sup>2939C</sup> mice died within 9 days after DSS administration (12), but mice that received oral antibiotics survived and developed mild inflammation and weight loss, without any genotype-linked differences (fig. S9). Thus, enteric bacteria elicit the inflammatory response to DSS, and without bacterial exposure, Nod2<sup>2939C</sup> mice have the same reaction as WT counterparts.

Exposure of macrophages to bacteria activates inflammatory and apoptotic caspases (15). More apoptotic cells, most of which were positive for the F4/80 macrophage marker, were found in the lamina propria of DSS-treated Nod2<sup>2939C</sup> mice than in WT counterparts (Fig. 4, A and B). Increased macrophage apoptosis is associated with activation of caspase-1 (16), an enzyme required for secretion of mature IL-1β (17, 18). Congruently, only background levels of secreted IL-1β were present in colons of untreated mice, but IL-1β concentrations were elevated after DSS treatment, particularly in Nod2<sup>2939C</sup> mice (Fig. 3D). Macrophage activation with LPS induces pro–IL-1β, but its processing and release require activation of caspase-1 by a different signal (16). LPS did not induce secretion of mature IL-1β in either Nod2<sup>2939C</sup> or WT macrophages, although it stimulated TNFα release (Fig. 2, D and E). In contrast, MDP stimulated release of mature IL-1β but not TNFα by Nod2<sup>2939C</sup> macrophages. To determine whether IL-1β secretion may be involved in the increased inflammatory response to DSS in Nod2<sup>2939C</sup> mice, mice were injected once daily with IL-1 receptor antagonist (IL-1Ra) from the start of DSS exposure. Average body weight loss and histological score were improved in IL-1Ra-treated mice, and differences in weight loss (Fig. 4C) and inflammatory score (Fig. 4D, fig. S10) between the genotypes were abolished.

By contrast to the Nod2<sup>2939C</sup> mutation, deletion of Ikkβ in hematopoietic and myeloid cells reduced the inflammatory response to DSS (fig. S11). However, its deletion in enterocytes increased the inflammatory response to DSS (19).

Collectively, our results suggest that Nod2<sup>2939C</sup> is a gain-of-function allele, whose product induces elevated IKK and caspase-1 activation in response to MDP. Although Nod2 was suggested to be a negative regulator of TLR2 (20), we found no effect of the Nod2<sup>2939C</sup> mutation on signaling by TLR2,

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**Figure 2.** Nod2<sup>2939C</sup> macrophages exhibit elevated NF-κB activation and IL-1β secretion in response to MDP. (A) BMDMs from WT and Nod2<sup>2939C</sup> (m/m) mice were incubated with MDP (1 μg/ml). Where indicated, cytosolic and nuclear extracts were prepared and used to analyze IKK activation (KA), IκBα degradation, and NF-κB DNA binding activity, respectively. Nuclear extract quality was monitored by measuring nuclear factor-Y (NF-Y) DNA binding. (B) BMDMs were stimulated with PamCys (1 μg/ml), LPS (100 ng/ml), or CpG DNA (1 μM) to activate TLR2, 4, and 9, respectively. Where indicated, nuclear extracts were prepared and NF-κB DNA binding activity was analyzed. (C) Expression of NF-κB target genes was examined in Nod2<sup>2939C</sup> and WT macrophages stimulated with MDP, LPS, or peptidoglycan (PGN from Staphylococcus aureus, 10 μg/ml). After 4 hours, cells were collected, total cytoplasmic RNA was prepared, and gene expression was analyzed by real-time polymerase chain reaction (PCR). Data are presented as the fold increase in mRNA expression in Nod2<sup>2939C</sup> macrophages relative to WT macrophages, which was given an arbitrary level of 1.0 for each gene. Results are means ± SEM of three independent experiments. (D) Elevated IL-1β secretion in MDP-stimulated Nod2<sup>2939C</sup> macrophages. WT and Nod2<sup>2939C</sup> (m/m) BMDMs were stimulated as indicated. After 24 hours, culture supernatants were collected and secreted cytokines were measured. (E) MDP induces IL-1β release by Nod2<sup>2939C</sup> (m/m) BMDMs. Macrophages were treated with MDP or LPS for 24 hours. Culture supernatants were collected and analyzed by immunoblotting with antibodies against IL-1β and TNFα.
as coinoculation of macrophages with MDP plus a TLR2 agonist (PGN) did not reduce the response to PGN (Fig. 2D). The inhibitory function hypothesis is also inconsistent with in vivo findings in Nod2 knockout mice, which did not show increased inflammation (11). The gain-of-function hypothesis is consistent with clinical observations made in CD patients (8,21).

The NF-κB signaling pathway induces many proinflammatory genes coding for cytokines and chemokines, including IL-1β, TNFα, and IL-6 (22,23), and may therefore be an important pathogenic factor in CD (8).

Although increased transcription of many

Fig. 3. Enhanced NF-κB activation and inflammation in DSS-treated Nod22939C mice. (A) Increased body weight loss in DSS-exposed Nod22939C mice. Mice of either genotype were given 3% DSS in drinking water for 6 days and weighed daily. Data are means ± SEM. Significant differences, *P < 0.05. (B) Typical colon appearance (upper panels) and histology (bottom panels) 11 days after initiation of DSS administration. Nod22939C mice exhibit more inflammation and ulceration. Arrowheads, borders of ulcers. Magnification, 100×. (C) Induction of inflammation-associated genes in colons of DSS-treated mice. Colonic RNA isolated 11 days after initiation of DSS treatment was analyzed by real-time PCR. Results are means ± SEM of fold increase in normalized (relative to glyceraldehyde-3-phosphate dehydrogenase mRNA) mRNA amounts in DSS-treated mice over untreated mice of the same genotype (n = 4 per group). (D) Elevated IL-1β and IL-6 in colons of DSS-treated Nod22939C mice. The indicated cytokines were measured in colonic extracts prepared 0 or 11 days after DSS exposure. Results are means ± SD (n = 4 to 8). Significant difference, *P < 0.05. (E) Immunohistochemical detection of IL-6 and Cox-2.

Colon sections prepared 11 days after initiation of DSS treatment were analyzed by indirect immunoperoxidase staining for IL-6 and Cox-2. Magnification, 100×. (F) Colonic NF-κB and IKK activities. Nuclear and cytosolic extracts of colonic mucosa prepared 0 and 11 days after initiation of DSS administration were analyzed for NF-κB DNA binding and IKK kinase (KA) activities. Protein recovery in nuclear extracts was determined by immunoblotting with antibody against histone deacetylase (HDAC), and IK recovery was determined with antibody against IKK (IKKa).
NF-kB targets was observed, the results with IL-1β were unique, as it was the only pro-inflammatory cytokine whose secretion in response to MDP was markedly elevated in Nod2<sup>2939iC</sup> macrophages relative to WT counterparts. Our results suggest that IL-1β is indeed an important contributor to the increased colonic inflammation in Nod2<sup>2939iC</sup> mice, as previously suggested for CD patients (2).

Although NF-kB was thought to be the major effector for NOD2, it should be noted that NF-kB is more effectively activated by bacterial products through TLRs (see Fig. 2). Thus NF-kB activation is not unique to NOD2, and its loss may not compromise NF-kB signaling in response to bacterial infection. Recently, TLR signaling and a certain amount of enteric bacteria were shown to be critical for maintenance of the intestinal barrier function (24), a function that was suggested to deteriorate in CD patients (2). However, maintenance of barrier function is unlikely to involve NOD2. By contrast, a unique function of NOD2, not provided by TLRs, is induction of IL-1β processing and release. This function may be mediated through the N-terminal CARD domains of NOD2, which may directly interact with caspase-1 or upstream caspases. Given the importance of IL-1β for the pathology of DSS-induced colitis in Nod2<sup>2939iC</sup> mice and the imbalance between IL-1β and IL-1-RA in CD patients (2), it would be of interest to critically evaluate its role in CD pathogenesis.

References and Notes
12. S. Maeda et al., unpublished observation.
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Materials and Methods Figs. 51 to 511
References and Notes
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Reports: "Nod2 mutation in Crohn's disease potentiates NF-κB activity and IL-1β processing" by S. Maeda et al. (4 Feb. 2005, p. 734). It was brought to the authors’ attention that the two control panels in Fig. 4A look alike. After examining the issue, it was realized that during preparation of the figure, one of the control panels was mistakenly inserted twice, and the other control was omitted. The correct figure is shown here. The authors apologize for any possible confusion and inconvenience that may have been created. The results and conclusions remain as before, i.e., increased macrophage apoptosis in the lamina propria of Nod2<sup>2998nt/m</sup> (m/m) mice after DSS treatment.