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Regulation of IL-8 and IL-1β expression in Crohn's disease associated *NOD2/CARD15* mutations

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Crohn's disease (CD) is a chronic inflammation affecting the gastrointestinal tract. Three mutations (Arg702Trp, Gly908Arg and Leu1007fsinsC) within the *NOD2/CARD15* gene increase CD susceptibility. Here, we define cytokine regulation in primary human mononuclear cells, with muramyl dipeptide (MDP), the minimal *NOD2/CARD15* activating component of peptidoglycan. By microarray, MDP induces a broad array of transcripts, including interleukin 1β (IL-1β) and interleukin 8 (IL-8). Leu1007fsinsC homozygotes demonstrated decreased transcriptional response to MDP. Electromobility shift assay demonstrated that MDP-induced NF-κB activation is mediated via p50 and p65 subunits, but not RelB or c-Rel. In wild-type individuals, MDP-induced IL-8 protein expression with a greater response to high dose (1 μg/ml) compared with low-dose (10 ng/ml) MDP. At low MDP doses, in all homozygotes, we observed no induction of IL-8 protein. With high doses of MDP, Leu1007fsinsC homozygotes showed no induction. Modest induction of IL-8 protein was observed in Gly908Arg and Arg702Trp homozygotes, indicating varying MDP sensitivity of the CD-associated mutations. In wild-type healthy control, CD and ulcerative colitis individuals, low-dose MDP and TNFα alone results in only modest IL-1β protein induction. With MDP plus TNFα, there is a synergistic induction of IL-1β secretion. In Leu1007fsinsC homozygotes, there is a profound defect in IL-1β secretion, despite marked induction of IL-1β mRNA. These findings demonstrate post-transcriptional dependency on the *NOD2/CARD15* pathway for IL-1β secretion with MDP and TNFα treatment. Taken together, these studies suggest that a signaling defect of innate immunity to MDP may be an essential underlying defect in the pathogenesis of some CD patients.

INTRODUCTION

Crohn's disease (CD) is a chronic, relapsing inflammation affecting the gastrointestinal tract, most commonly the terminal ileum (1). It is characterized by chronic, intermittent inflammation of the intestines resulting in abdominal pain, diarrhea and, in children, growth retardation. Its prevalence is between 100 and 200 per 100 000 persons in the US (2,3) and the peak age of onset is between 15 and 30 years of age. CD results from a complex interplay of genetic and environmental factors (1,4), and three coding region polymorphisms (Arg702Trp, Gly908Arg and Leu1007fsinsC) within the *NOD2/CARD15* gene have been definitively associated with increased CD susceptibility (5,6). Heterozygous carriage of the risk alleles confers a 2–4-fold increased risk, and double-dose carriage (homozygotes or compound heterozygotes) confers a 20–40-fold increased risk (7,8).

NOD2/CARD15 is expressed in the cytoplasm of a variety of cell types, including monocytes and monocyte-derived cells (9), and is comprised of N-terminus caspase-activation recruitment domains (CARD), a central nucleotide-binding domain, and a C-terminus leucine-rich repeat (LRR) domain (10). The LRR domain is required for *NOD2/CARD15* signaling in response to muramyl dipeptide (MDP) (11,12), the minimally active component of bacterial cell wall peptidoglycan present in the cell walls of Gram-negative and Gram-positive bacteria. Oligomerization of *NOD2/CARD15* following exposure to MDP results, via CARD–CARD interactions, in the recruitment of the CARD-containing protein, RICK/RIP2/CARDIAK (10,13,14). The intermediate domain of

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RICK interacts with the regulatory component, IKKγ, of the IKK complex to activate the NF-κB transcription factors. The frameshift mutation, Leu1007fsinsC, is associated with a complete loss of MDP-mediated NF-κB activation (5,11,15,16), whereas Arg702Trp and Gly908Arg are associated with modest decreases in HEK293T cell transfectants, despite the fact that all three mutations confer comparable increased risk. These data indicate a role for altered bacterial sensing in contributing to CD susceptibility (17).

Comprehensive studies in primary mononuclear cells in CD patients comparing the functional effects of the individual disease-associated mutations, as well as comparing functional consequences in heterozygous carriers of established risk alleles have not been reported. Cytokines involved in the immediate innate immune responses include interleukin-8 (IL-8) and interleukin-1β (IL-1β). IL-8, a member of the CXC chemokine family, is an important activator and chemoattractant for neutrophils and has been implicated in the pathogenesis of a variety of inflammatory diseases, including asthma, sepsis and inflammatory bowel disease (18,19). IL-8 is primarily regulated at the transcriptional level (20). In contrast, IL-1β is regulated at the transcriptional, translational and post-translational levels (21–24). In particular, proteolytic maturation of the inactive, 33 kDa IL-1β precursor (proIL-1β) into the 17 kDa mature, secreted, biologically functional form requires caspase-1 activity (25–27).

In this study, we examine the effects of MDP-mediated activation of primary peripheral mononuclear cells stratified on *NOD2/CARD15* genotype. Studies in mononuclear cells have the advantage of evaluating MDP-mediated effects on cytokine gene regulation. Such genotype–phenotype correlations provide a means of identifying key functional outcomes using primary cells that provide insight into likely mechanisms of disease pathogenesis. Furthermore, as we establish that double-dose Leu1007fsinsC primary cells represent a complete loss of function with respect to MDP responsiveness, such studies have the capacity to identify those pathways which are *NOD2/CARD15*-dependent, such as the post-transcriptional activation of IL-1β expression with MDP and TNFα treatment. Such studies provide a basis for more precisely defining functional outcomes contributing to the genetic basis of NOD2/CARD15 variants in contributing to CD susceptibility.

RESULTS

Monocyte-derived macrophage (MDM) response to MDP is impaired in Leu1007fsinsC homozygotes

To define the broad transcriptional responses of wild-type and mutant *NOD2/CARD15* in response to MDP, we performed microarray analyses using an *in vitro* model of MDMs derived from three *NOD2/CARD15* wild-type healthy controls and two CD patients homozygous for the Leu1007fsinsC mutation. Whereas MDP treatment results in significant alterations in a number of transcripts in wild-type MDMs (Fig. 1A), there is a globally blunted transcriptional response in Leu1007fsinsC MDMs (Fig. 1B). Specifically, in all three wild-type MDMs, MDP treatment results in a clearly greater transcriptional response (Fig. 1C) compared with MDMs from Leu1007fsinsC homozygotes.

Similar to other bacterial components, MDP treatment of control MDMs, but not Leu1007fsinsC homozygotes, results in a marked induction of abundant chemokines including IL-8, RANTES, SCYA3 and SCYA4 which are well-known to be rapidly transcriptionally activated (Table 1). In addition, activated macrophages are known to induce a potent mix of broadly active pro-inflammatory cytokines including IL-1β, TNFα and IL-6 (28). All of these cytokines are induced by MDP, similar to that of other known activators of macrophage function (29), including LPS (28). In contrast, although LPS is well-known to strongly induce Th1-polarizing cytokines (28,30,31), MDP treatment of macrophages modestly induces IL-18, and does not induce IL-12 (p35 and p40 subunits) at all. Finally, MDP treatment neither significantly induce TGFβ nor IL-10, cytokines produced by suppressor T cell subsets, which ameliorate Th1-mediated intestinal inflammation (32).

MDP stimulation of primary peripheral blood mononuclear cells (PBMCs) demonstrates activation of p50 and p65 NF-κB subunits

NOD2/CARD15 has been reported to activate NF-κB activity in cell transfectants, and the activation of NF-κB is instrumental in the regulation of many of the cytokines and chemokines induced by MDP. Therefore, we sought to define the effects of MDP on specific NF-κB family members in primary cells. In wild-type healthy controls ($n = 3$), compared with untreated (U) nuclear extracts (Fig. 2A and B), MDP-treated (M) extracts demonstrated a significant increase in a DNA–protein complex. Co-incubation with NF-κB transcription factor antibodies demonstrated a supershift of this DNA-binding complex with antibodies against p65 (S1, Fig. 2A and B) and p50 (S2, Fig. 2A and B), indicating that MDP-mediated transcriptional activation of NF-κB can occur through the p50–p65 classical pathway (33). Of note, incubation with antibodies against c-Rel (S3, Fig. 2A) and RelB (S4, Fig. 2A) had no effect. Similar to *NOD2/CARD15* wild-type healthy controls, MDP-induced binding of the p50–p65 complex in wild-type CD patients ($n = 4$) (Fig. 2C). In contrast, in CD patients homozygous for the Leu1007fsinsC ($n = 2$) (Fig. 2D) and Gly908Arg ($n = 2$) (Fig. 2E) mutations, no induction of NF-κB-binding complex is observed. These data establish that the altered transcriptional response of double-dose carriers to MDP treatment is due in part to impaired activation of NF-κB.

MDP and TNFα induction of IL-8 and IL-1β mRNA in wild-type healthy controls

The dose-dependent effects in NF-κB consensus element binding observed by electrophoretic mobility shift assay (EMSA) are also observed with respect to increased IL-8 and IL-1β mRNA as measured by real-time polymerase chain reaction (PCR) (Fig. 3). MDP treatment (1 μg/ml) results in cytokine induction comparable to that observed with TNFα (5 ng/ml) treatment. For both IL-8 and IL-1β, further inductions in mRNA levels are observed with combined MDP (10 ng/ml) and TNFα treatment.

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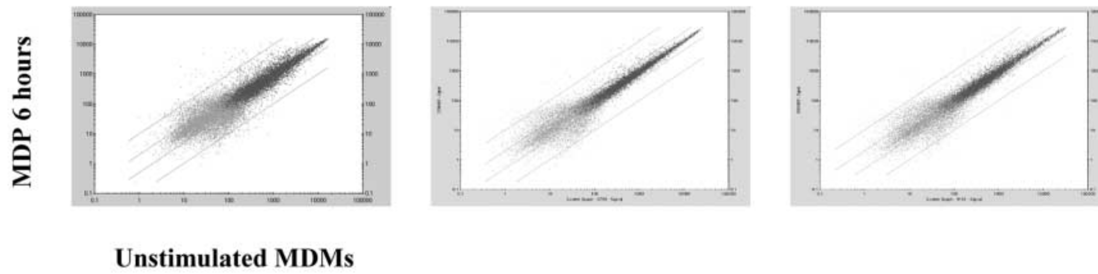
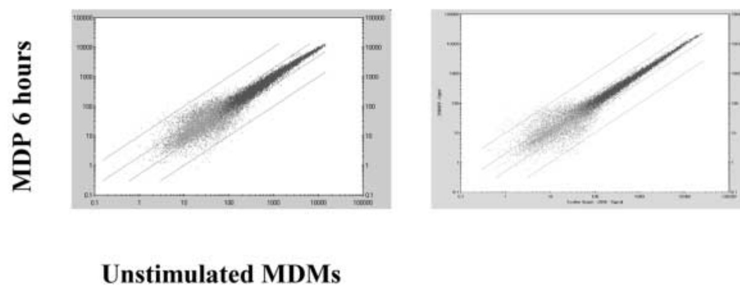
A NOD2/CARD15 wild types



B Leu1007fsinsC homozygotes



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Individual	Increased genes	Decreased genes
Wild-type 1	1667	1952
Wild-type 2	715	1123
Wild-type 3	1096	1001
Leu1007fsinsC1	130	19
Leu1007fsinsC2	164	17

Figure 1. Microarray analysis of MDP-treated MDMs stratified on *CARD15* genotype. Scatter plots comparing hybridization intensities for unstimulated and MDP-treated (10 ng/ml) MDMs from (**A**) three wild-type healthy controls and (**B**) two Leu1007fsinsC homozygotes. The dark and light dots demonstrate transcripts which are present and absent, respectively. The inner two parallel lines represent a 2-fold increase or decrease, whereas the outer two lines reflect a 10-fold change. (**C**) Number of increased and decreased genes from wild-type and Leu1007fsinsC homozygotes.

MDP dose-dependent regulation of IL-8 secretion in *NOD2/CARD15* healthy wild-type, CD wild-type and mutant PBMCs

Next, we sought to more broadly define MDP regulation of IL-8 in wild-type and mutant PBMCs. Because it is primarily transcriptionally regulated and known to be activated by NF-κB, protein measurements of secreted IL-8 from MDP-treated PBMCs represent a straightforward measurement of *NOD2/CARD15* genotype-dependent alterations. In initial dose-ranging experiments, we did not observe an induction in wild-type cells at the 1 ng/ml dose, and saw a plateauing of effects compared with the 1 and 10 μg/ml doses (data not shown). In healthy controls (Fig. 4), we observed a significant induction of IL-8 protein with low-dose (10 ng/ml) MDP treatment alone ($P = 2 \times 10^{-4}$). We observed a significantly greater induction of IL-8 protein expression with 1 μg/ml of MDP compared with the 10 ng/ml dose ($P = 0.016$), which was consistent with the greater induction with high-dose MDP by EMSA (Fig. 2B), and by mRNA quantification using real-time PCR ($P = 0.034$).

In CD and ulcerative colitis (UC) patients wild-type for *CARD15/NOD2*, we observed similar results, with significant induction of IL-8 observed with low-dose MDP, and with a significantly greater induction observed with high-dose MDP. The data on wild-type PBMCs from either control or identical by descent patients establish a primarily intact MDP-mediated signaling pathway, with marked induction at the 10 ng/ml of MDP, and a dose-response effect at 1 μg/ml.

Figure 5 illustrates the MDP dose-dependent IL-8 absolute protein measurements for healthy control (Fig. 5A) and CD wild-type (Fig. 5B), CD heterozygous (Fig. 5C) and CD double-dose mutant (Fig. 5D) PBMCs. Note that for CD wild-type and heterozygous individuals, the absolute levels of IL-8 secretion were significantly higher compared with wild-type healthy controls. In contrast, in all double-dose carriers tested, no induction of IL-8 was observed with low-dose MDP treatment, with only modest inductions observed with high-dose MDP in the Arg702Trp and Gly908Arg homozygotes. Of note is that for the Leu1007fsinsC double-dose carriers, no induction is observed even with high doses of MDP, indicating that this mutation uniquely represents a complete loss of responsiveness to MDP induction.

As *NOD2/CARD15* heterozygotes demonstrate increased disease risk, though lower than that of homozygotes (7,8), we hypothesized that PBMCs from heterozygous CD patients would demonstrate a range of IL-8 induction with MDP treatment, reflecting functional heterogeneity in this group. We examined MDP-mediated IL-8 induction of PBMCs from heterozygous CD patients and for whom no other rare amino acid polymorphisms implicated in disease association were identified by sequencing. Although a range of IL-8 induction was observed (Fig. 5), no significant differences were observed in this cohort compared with wild-type CD patients.

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Table 1. Regulation of cytokines in wild-type and mutant macrophages stimulated with MDP

mRNA transcript	Healthy wild-type 1						Healthy wild-type 2						Healthy wild-type 3					
	US		MDP		MDP versus US		US		MDP		MDP versus US		US		MDP		MDP versus US	
	Signal	Detect	Signal	Detect	Change	*P*-value	Signal	Detect	Signal	Detect	Change	*P*-value	Signal	Detect	Signal	Detect	Change	*P*-value
Chemokines																		
Interleukin 8 (IL8) NM 000584.1	927.1	P	11319	P	I	0.00002	1015.8	P	11517.8	P	I	0.000244	814.1	P	14190.9	P	I	0.000244
(RANTES) NM 002985.1	175.6	P	5151.5	P	I	0.00002	608.7	P	3304.6	P	I	0.000491	1879.3	P	3617.9	P	I	0.000491
Small inducible cytokine A4 (SCYA4) NM 002984.1	3267.1	P	11350.8	P	I	0.00002	751.7	P	15774.7	P	I	0.000244	616.1	P	17434.6	P	I	0.000244
Small inducible cytokine A3 (SCYA3) NM 002983.1	2307.1	P	8027.7	P	I	0.00002	2235.6	P	7759.2	P	I	0.000244	1205.9	P	11132.2	P	I	0.000244
Inflammatory cytokines																		
Interleukin 1, beta (1L1B), NM 000576.1	822.9	P	11072.6	P	I	0.00002	444.7	P	7497.1	P	I	0.000219	480.1	P	10107.6	P	I	0.000219
Tumor necrosis factor (TNF) NM 000594.1	286.4	P	1067.8	P	I	0.000068	92.9	P	532.5	P	I	0.000244	139	P	809.3	P	I	0.000244
Interleukin 6 (IL6) NM 000600.1	87.8	A	237.4	P	I	0.00225	74	P	314.7	P	I	0.001221	157.7	P	520.2	P	I	0.000732
Th1-polarizing cytokines																		
Interleukin 18 (1L18) NM 001562.1	938.2	P	1657.6	P	I	0.000088	760.2	P	1019.3	P	I	0.000244	513.7	P	744.1	P	I	0.000732
Interleukin 12A, p35 (IL12A) NM 000882.1	15.9	A	27.4	A	NC	–	22.6	A	16.6	A	NC	0.366211	19.4	A	15.3	A	NC	0.398926
Interleukin 12B, p40 (IL12B) NM 002187.1	79.4	P	92	A	NC	–	31.1	M	43.9	P	NC	0.030273	31	A	45.6	A	NC	0.095215
Down-regulatory cytokines																		
Transforming growth factor, beta 1 (TGFB1) NM 000660.1	25.2	A	17	A	NC	–	23.6	A	36.5	A	NC	0.219482	40.3	A	69.7	A	NC	0.246094
Interleukin 10(IL10) NM 000572.1	195.1	P	151.5	A	NC	–	86.3	A	162.6	P	I	0.354442	167	P	239.1	P	NC	0.354442

mRNA transcript	Leu1007fsinsC homozygote 1						Leu1007fsinsC homozygote 2					
	US		MDP		MDP versus US		US		MDP		MDP versus US	
	Signal	Detect	Signal	Detect	Change	*P*-value	Signal	Detect	Signal	Detect	Change	*P*-value
Chemokines												
Interleukin 8 (IL8) NM 000584.1	1803.3	P	2535.2	P	NC	–	1472.2	P	1077.8	P	NC	–
(RANTES) NM 002985.1	447.3	P	509.5	P	NC	–	479.6	P	313.7	P	NC	–
Small inducible cytokine A4 (SCYA4) NM 002984.1	2401.8	P	2214.2	P	NC	–	410.3	P	235.5	A	NC	–
Small inducible cytokine A3 (SCYA3) NM 002983.1	2136.3	P	1972.4	P	NC	–	1325	P	993.8	P	NC	–
Inflammatory cytokines												
Interleukin 1, beta (1L1B), NM 000576.1	2134.4	P	1916.4	P	NC	–	169.9	A	171.9	A	NC	–
Tumor necrosis factor (TNF) NM 000594.1	280.9	P	287.1	P	NC	–	43.4	A	92.3	A	NC	–
Interleukin 6 (IL6) NM 000600.1	121.6	P	144.9	P	NC	–	91.7	P	73.9	P	NC	–
Th1-polarizing cytokines												
Interleukin 18 (1L18) NM 001562.1	461.9	P	635.8	P	NC	–	780.9	P	880.1	P	NC	–
Interleukin 12A, p35 (IL12A) NM 000882.1	68.2	A	40.3	A	NC	–	37.3	A	19.3	A	NC	–
Interleukin 12B, p40 (IL12B) NM 002187.1	45.4	A	15.3	A	NC	–	19.3	A	19.3	A	NC	–
Down-regulatory cytokines												
Transforming growth factor, beta 1 (TGFB1) NM 000660.1	19.9	A	47.9	A	NC	–	29	A	41.3	A	NC	–
Interleukin 10(IL10) NM 000572.1	151.9	P	212.7	P	NC	–	290.7	P	228.8	P	NC	–

US, unstimulated; P, present; A, absent; I, increase; NC, no change.

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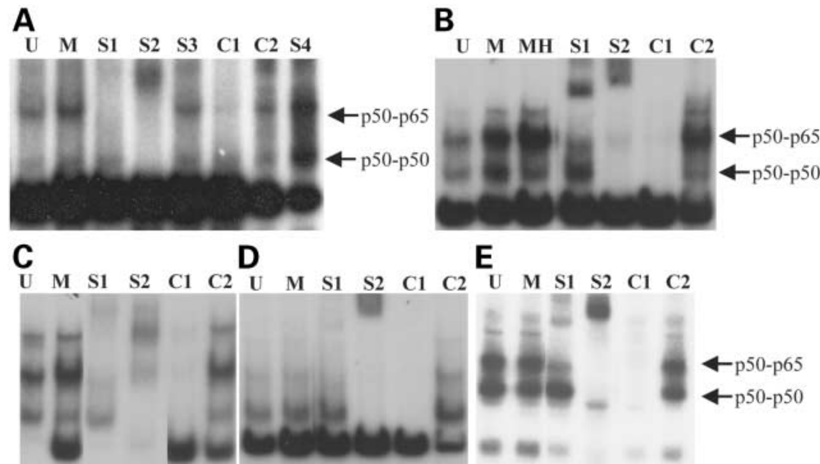

Figure 2. MDP-stimulated electromobility shift assays from PBMCs stratified on NOD2/CARD15 genotype. NF-κB EMSA of (**A** and **B**) healthy control and (**C**) CD wild-type individuals were compared with CD individuals homozygous [(**D**) Leu1007fsinsC homozygote; (**E**) Gly908Arg homozygote] for established NOD2/CARD15 risk alleles. Results for wild-type healthy control and CD individuals are representative of three separate experiments. Results for homozygotes are representative of two separate experiments. U, unstimulated PBMC; M, MDP (10 ng/ml); MH, MDP (100 ng/ml); S1, supershift, MDP (10 ng/ml)/p65 antibody; S2, supershift, MDP (10 ng/ml)/p50 antibody; S3, supershift, MDP (10 ng/ml)/cRel antibody; S4, supershift, MDP (10 ng/ml)/RelB antibody; C1, MDP (10 ng/ml) plus cold NF-κB oligo, C2, MDP (10 ng/ml) plus cold AP-1 oligo.

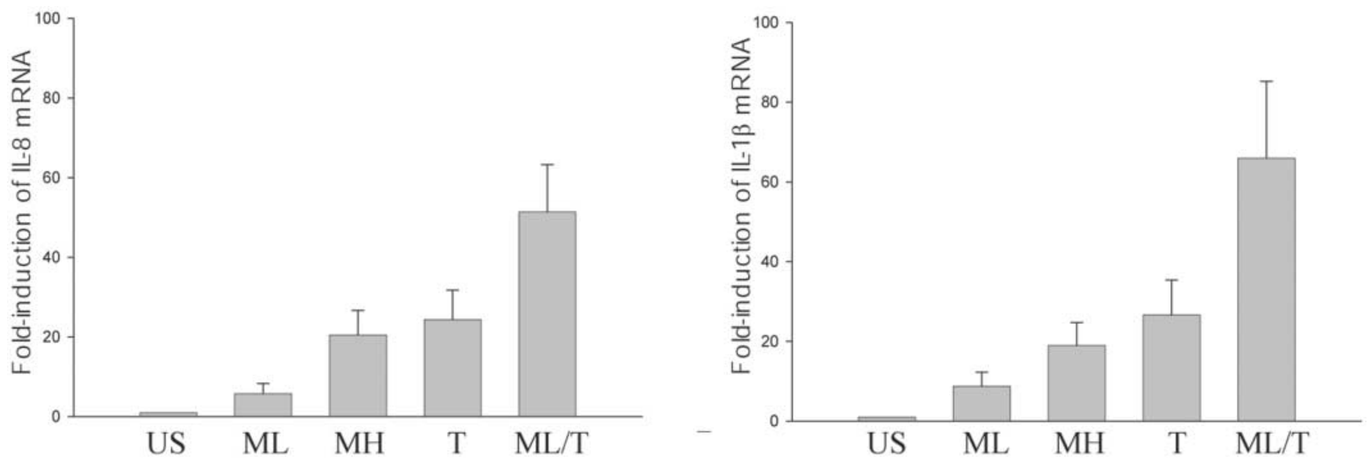

Figure 3. MDP and TNFα regulation of IL-8 and IL-1β mRNA from wild-type PBMCs. For each individual, induction of mRNA levels was measured in triplicate relative to unstimulated (US) mRNA levels. Average results between individuals ($n = 5$) are plotted ±SEM. ML, MDP 10 ng/ml; MH, MDP 1 μg/ml, T, TNFα 5 ng/ml; ML/T, MDP 10 ng/ml plus TNFα 5 ng/ml.

Marked synergistic effect of MDP and TNFα on IL-1β, but not on IL-8 protein secretion in healthy wild-type PBMCs

Whereas IL-8 is largely transcriptionally regulated, IL-1β expression is regulated transcriptionally and post-transcriptionally. Like MDP, TNFα treatment is known to induce IL-8 secretion, in part via activation of NF-κB. At these doses, in wild-type healthy controls and CD patients, combined MDP and TNFα treatment did not result in a synergistic effect in IL-8 secretion compared with either treatment alone (Fig. 4, confidence intervals for ML/T−ML, ML/T−T cross zero). In marked contrast (Fig. 4), for wild-type controls, CD and UC patients, whereas IL-1β production with MDP or TNFα alone results in modest absolute inductions of IL-1β protein, a highly significant, synergistic effect is observed with combined treatment compared with either MDP (ML/T−ML) or TNFα alone (ML/T−T). Studies in NOD2/CARD15 mutants may be utilized to further define the regulatory levels at which this synergistic effect is mediated.

Leu1007fsinsC double-dose carriers demonstrate a complete loss of MDP and TNFα-mediated IL-1β secretion

In four CD patients double-dose for the Leu1007fsinsC mutations (three homozygotes and one compound heterozygote), we observed no significant induction of IL-1β secretion

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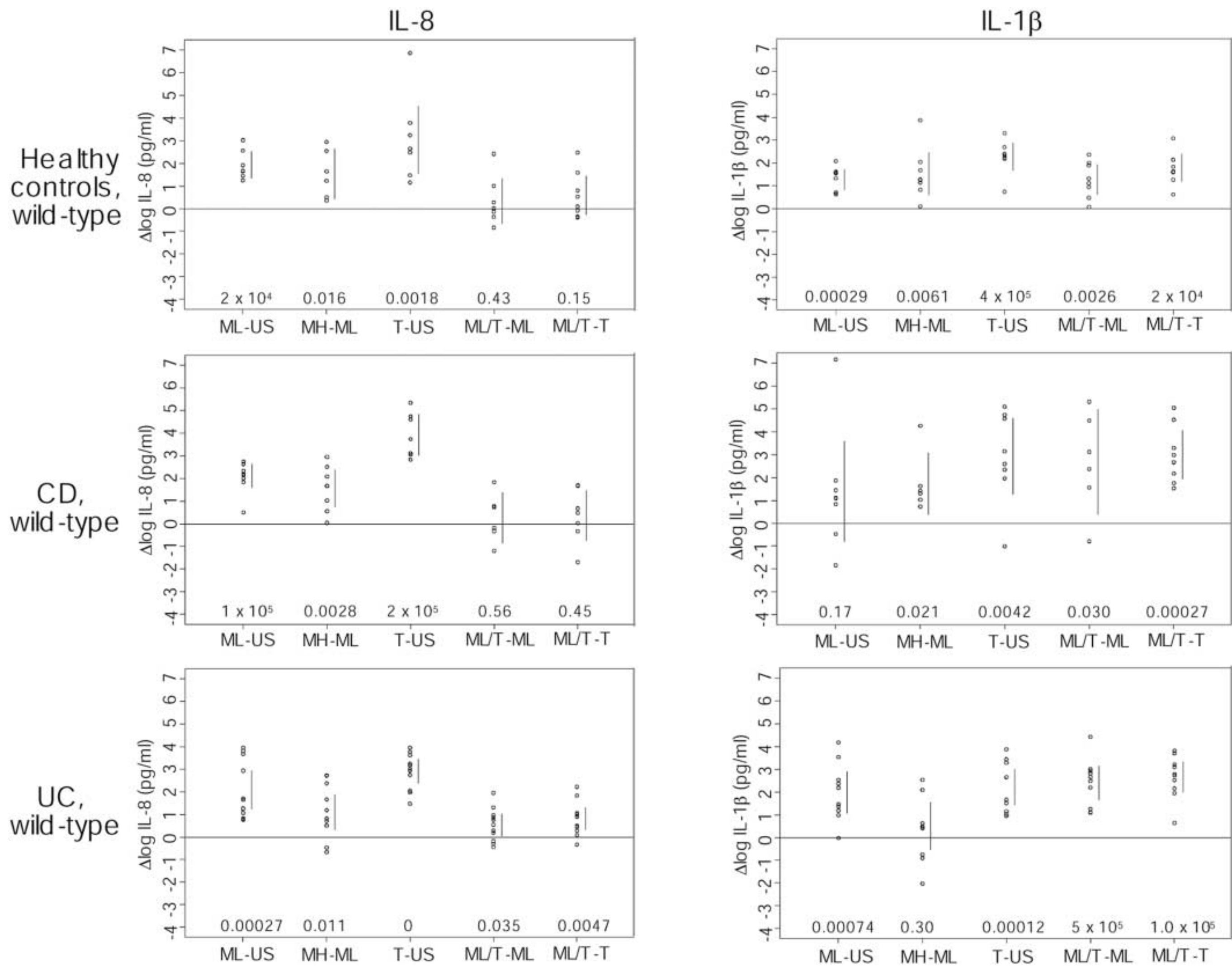

Figure 4. MDP and TNFα regulation of IL-8 and IL-1β protein secretion from wild-type PBMCs. PBMCs were isolated from wild-type healthy control ($n = 8$), CD ($n = 9$) and UC ($n = 10$) individuals wild-type for NOD2/CARD15 and plated at 3×10^6/ml. Following MDP (ML, 10 ng/ml, MH; 1 μg/ml) and/or TNFα (T) exposure for 16 h, IL-8 and IL-1β were measured in the media by ELISA in duplicate. US, unstimulated; ML/T, MDP (10 ng/ml) plus TNFα (5 ng/ml). Absolute ELISA values were logarithmically transformed and confidence intervals (vertical lines) for the difference in the mean protein levels between two conditions were constructed. *P*-values for testing that two conditions have the same effect on the mean protein levels were calculated using normal approximations. The 'MH−ML' data represent the differences in the log values between high- and low-dose MDP. Similarly, the 'ML/T−ML' data represent the difference between combined MDP and TNFα treatment compared with low-dose MDP alone.

with MDP and TNFα treatment alone or in combination (Table 2). As expected, no induction of IL-1β mRNA was observed with MDP treatment alone. In addition, TNFα treatment alone induced a marked increase (>50-fold) in IL-1β mRNA, indicating an intact transcriptional response to TNFα signaling. However, no induction of IL-1β protein secretion was observed with MDP and TNFα treatment together. As double-dose Leu1007fsinsC cells represent an MDP-mediated loss of function mutant, this would indicate that the MDP-*NOD2/CARD15* pathway is involved in post-transcriptional induction of IL-1β expression. Not unexpectedly, in all Leu1007fsinsC homozygotes, LPS alone significantly induced IL-1β secretion, indicating that the post-transcriptional activation of IL-1β expression mediated via the LPS pathway is intact.

DISCUSSION

The capacity to define genotype–phenotype relationships is a central goal of human genetics. Defining the host response of primary monocytic cells to peptidoglycan components (MDP) in CD provides an ideal opportunity to define such relationships. Although murine studies with the *NOD2/CARD15* knockout have confirmed the importance of the MDP signaling pathway (34), key species differences exist between human and murine responses to innate signals (35), highlighting the importance of studies in primary human tissues. Furthermore, studies with human cells from CD and UC patients also have the potential advantage of defining shared and distinct altered functional outcomes stratified on *NOD2/CARD15* wild-type.

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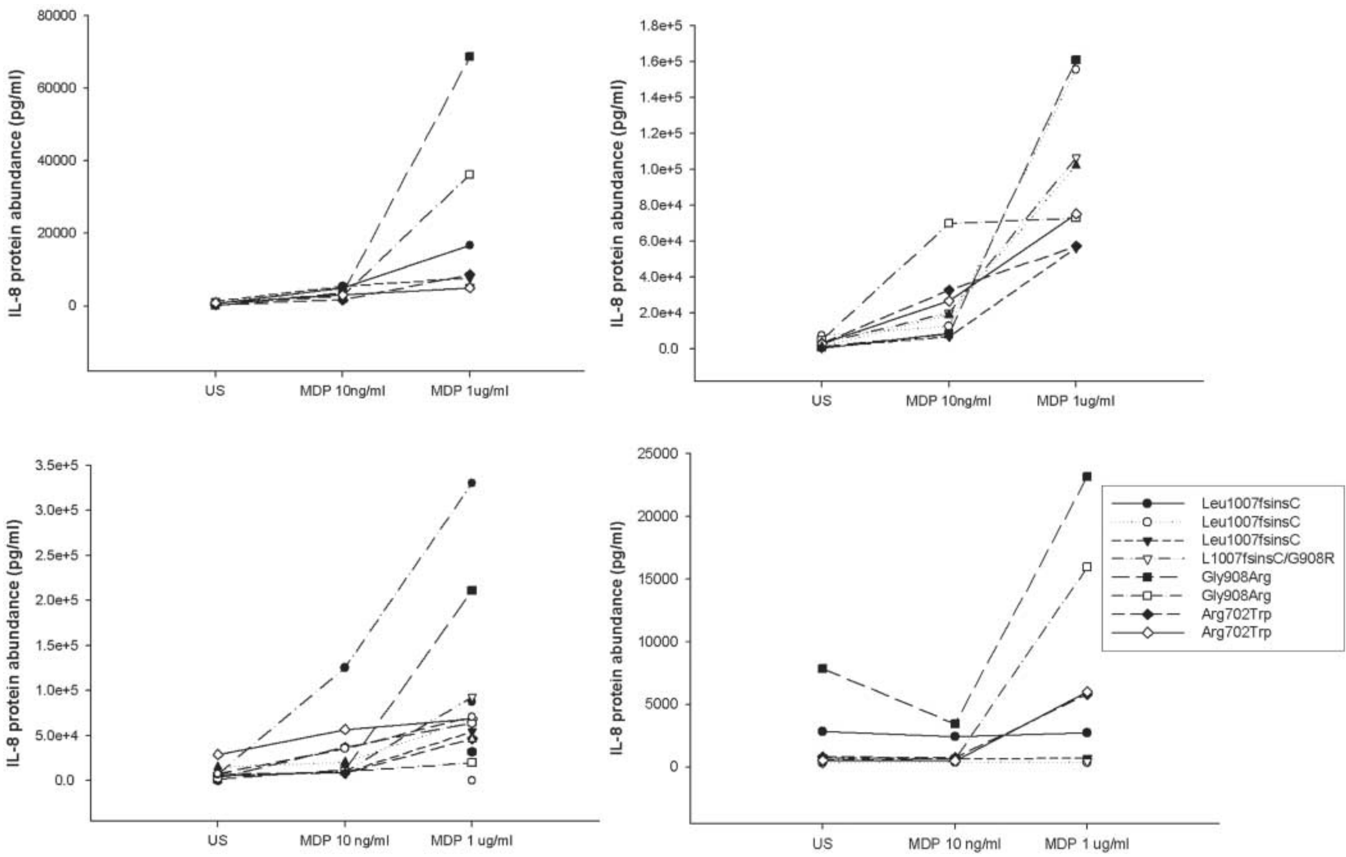

Figure 5. MDP dose-dependent IL-8 induction in PBMCs from wild-type healthy control and CD patients wild-type, heterozygous and double-dose for the Leu1007fsinsC, Gly908Arg and Arg702Trp mutations. PBMCs from wild-type healthy controls ($n = 8$) and CD patients wild-type ($n = 9$), heterozygous ($n = 15$) and double-dose for NOD2/CARD15 mutations ($n = 8$) were plated at 3×10^6/ml and treated with MDP for 16 h. Note the scale differences between cohorts.

In this study, we demonstrated that primary MDMs from Leu1007fsinsC homozygotes compared with wild-type controls have a globally blunted transcriptional response (Fig. 1) to MDP. Whereas it has been previously established that MDP is capable of inducing *NOD2/CARD15*-dependent activation of NF-κB (11,12), these results would indicate that in MDMs, there are likely to be no signaling pathways (e.g. via toll-like receptors) for MDP that can function independently of *NOD2/CARD15*. As such, treatment of primary cells with MDP provides a powerful means of defining *NOD2/CARD15* genotype-dependent phenotypic outcomes.

Prior studies have demonstrated that all three of the major risk alleles have comparable allele frequencies and genetic risk for heterozygotes (2–4-fold) and homozygotes (20–40-fold) (7,8). Despite genetic equivalence among the three major variants, only the Leu1007fsinsC has a complete loss of function in prior cellular transfectants studies (5,11,15,16). In contrast, although the Gly908Arg and Arg702Trp variants have a significant decrease in NF-κB activity compared with wild-type *NOD2/CARD15*, some induction of NF-κB activity was observed. The differences among the three major mutations in prior cellular transfectant studies are mirrored by the present findings with respect to IL-8 regulation. IL-8 is regulated via both NF-κB and the MAP kinase pathways (36). Because it is primarily transcriptionally regulated, protein measurements of secreted IL-8 from MDP-treated PBMCs of patients represent a simple, clear-cut means of defining *NOD2/CARD15* genotype-dependent alterations. All double-dose carriers demonstrate no induction of IL-8 protein with low-dose (10 ng/ml) MDP treatment. However, at higher MDP doses (1 μg/ml), significant IL-8 induction is observed in Arg702Trp and Gly908Arg homozygotes, although the absolute IL-8 levels even at 1 μg/ml are quite low compared with CD wild-type PBMCs (Fig. 5) and might be predicted to result in impairment of neutrophil recruitment. In contrast, the frameshift mutation acts like a complete loss of function even at the higher MDP doses (1 μg/ml). We speculate this may indicate that the lower MDP doses are more physiologically relevant, although the precise means by which the intracellular *NOD2/CARD15* pathway is stimulated is largely unknown.

These results establish that the Leu1007fsinsC mutation most clearly defines those altered outcomes resulting from a failure of *NOD2/CARD15* to appropriately sense peptidoglycan components via its C-terminus LRR domain.

In our cohort, 20% of healthy control European Americans are heterozygous for the CARD15 risk alleles, compared with 28% of CD patients. Heterozygous carriage of

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Table 2. Quantitative RT-PCR and ELISA results implicate a post-transcriptional defect in IL-1β secretion in Leu1007fsinsC double-dose carriers

	IL-Iβ CT	GAPDH CT	ΔCT (IL-Iβ CT-GAPDH CT)	ΔΔCT	Estimated fold mRNA change	IL-Iβ protein (pg/ml)
Leu1007fsinsC homozygote #1						
Unstimulated	27.5	18.5	9	0	1	2.8
MDP 10 ng/ml	27	19.5	7.5	−1.5	2.5	1.4
TNFα 5 ng/ml	19.7	20.1	−0.4	−9.4	>50 fold	19
MDP 10 ng/ml + TNFα 5 ng/ml	19.5	19.8	−0.3	−9.3	>50 fold	3.4
LPS	15.1	19.6	−4.5	−13.5	>50 fold	1526
Leu1007fsinsC homozygote #2						
Unstimulated	31.3	18.5	12.8	0	1	Below range
MDP 10 ng/ml	31.2	20.1	11.1	−1.7	3.2	Below range
TNFα 5 ng/ml	24.4	20.7	3.7	−9.1	>50 fold	24
MDP 10 ng/ml + TNFα 5 ng/ml	24.6	19.6	5	−7.8	>50 fold	2.2
LPS	24.2	19.5	4.7	−8.1	>50 fold	2113
Leu1007fsinsC homozygote #3						
Unstimulated	23	18.7	4.3	0	1	Below range
MDP 10 ng/ml	23.4	19.1	4.3	0	1	Below range
TNFα 5 ng/ml	17.7	18.1	−0.4	−4.7	30	21
MDP 10 ng/ml + TNFα 5 ng/ml	17.2	18.3	−1.1	−5.4	42	Below range
LPS	16.2	19.2	−3	−7.3	147	1604
Leu1007fsinsC/Gly908Arg compound heterozygote						
Unstimulated	23.8	18.4	5.4	0	1.0	4.1
MDP 1 μg/ml	25.2	19.8	5.4	0	1.0	5.4
TNFα 5 ng/ml	18.4	18.7	−0.3	−5.7	>50 fold	5.5
MDP 1 μg/ml + TNFα 5 ng/ml	18.6	19.0	−0.4	−5.8	>50 fold	5.7

CT, cycle threshold; ΔΔCT, difference for that condition compared with unstimulated. Fold mRNA change compared with unstimulated estimated by $2^{\Delta\Delta CT}$.

NOD2/CARD15 risk alleles increases CD susceptibility 2–4-fold, whereas double-dose carriage increases risk 20–40-fold (7,8). These findings are not consistent with an additive model of disease risk and/or gene-dose effect on functional alteration, where the risk to heterozygotes would be precisely half-way between that for double-dose and wild-type carriers (37). Rather, it may be speculated that the genetic data are consistent with an autosomal recessive model where a subset of heterozygous *NOD2/CARD15* carriers are actually misclassified double-dose carriers, carrying a second, currently unidentified, disease-associated mutation contributing to reduced peptidoglycan and/or MDP-responsiveness. In the present cohort, we did not observe a significant difference in MDP dose-dependent IL-8 induction in heterozygous compared with wild-type CD, however, it may be that only a modest subset of *NOD2/CARD15* heterozygotes carry additional risk alleles contributing to altered MDP or peptidoglycan responses.

Whereas MDP induces a number of rapid-response, transcriptionally regulated chemokines such as IL-8, it also regulates other members of the innate immune response having more complex levels of gene regulation. Like IL-8, IL-1β expression is induced by NF-κB, and IL-1β mRNA levels are induced with MDP treatment. In addition to transcriptional activation (21), IL-1β mRNA stability and translational activation are mediated via the p38 and JNK pathways (22). Finally, IL-1β is also regulated post-translationally. IL-1β is produced by activated macrophages as a preprotein, which is proteolytically processed to its active form by caspase-1. Caspase-1 is itself proteolyzed from an inactive, CARD-containing holoenzyme (45 kDa) to form an active heterodimer of p20 (20 kDa) and 10 kDa subunits (23,24). RICK is one of a limited number of proteins demonstrated to bind to caspase-1 via CARD–CARD interactions and results in its proteolytic activation (38–40). The induction with MDP treatment of IL-1β mRNA but not secreted protein demonstrates the presence of additional levels of post-transcriptional regulation. Because RICK/RIP2 is not known to be activated by TNFα signaling and TNFα alone has not been reported to activate caspase-1, we hypothesize that the marked synergistic effect of MDP and TNFα on IL-1β secretion results in part from a *NOD2/CARD15*-mediated activation of caspase-1 (Table 2). The failure to significantly induce IL-1β secretion with MDP and TNFα in Leu1007fsinsC double-dose carriers, therefore, results from a significant defect in caspase-1 activation mediated via *NOD2/CARD15*. Taken together, these data demonstrate an additional function of *NOD2/CARD15*, namely, MDP-mediated activation of caspase-1. These findings extend previous observations that NOD1/CARD4 contributes to activation of caspase-1 (40,41). As such, the defects in IL-8 and IL-1β secretion in *NOD2/CARD15* mutants highlight multiple defects in the immediate response of innate immune cells to peptidoglycan products. This suggests that a signaling defect of innate immunity to MDP may be an essential underlying defect in the pathogenesis of a subset of CD patients.

The concept that CD results in part from a defect in innate immunity is supported by a combination of clinical and

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therapeutic experience, as well as from murine models. A variety of uncommon human immunodeficiency disorders (chronic granulomatous disease, glycogen storage disease Ib, leukocyte adhesion deficiency) characterized by defects in neutrophil or monocyte function can manifest a Crohn's like intestinal phenotype (42). Under normal conditions, rapid neutrophil migration to sites of foreign object invasion leads normally to rapid digestion and eradication. Failure of this process (43,44) may result in the engulfment of foreign objects by macrophages leading to chronic inflammation, specifically, granuloma formation (17). This may well explain the findings that granulomas are typically observed uniquely with CD, and not UC.

NF-κB-deficient mice (p50−/−p65+/−) have increased susceptibility to *Helicobacter hepaticus*-induced colitis, suggesting a role for NF-κB in inhibiting intestinal inflammation (45). Adoptive transfer studies in RAG-deficient mice (deficient or sufficient in NF-κB) implicate a defect within innate cells of NF-κB-deficient animals that abrogates the ability of regulatory lymphocytes to suppress *H. hepaticus*-induced intestinal inflammation (46). Taken together, these data support the concept that defects in innate cell NF-κB activation in response to bacterial exposure can result in increased intestinal inflammation. It may be speculated that defects in the initial innate response and subsequent instruction of the adaptive immune response results in increased susceptibility to inflammation.

The importance of fully understanding mechanisms of *NOD2/CARD15* CD pathogenesis will include identifying unique functional responses of the *NOD2/CARD15* pathway, especially in comparison with other innate pathways, such as the plasma membrane toll-like receptors. Whereas it was previously known that *NOD2/CARD15* activates NF-κB, here we establish that MDP-mediated *NOD2/CARD15* activation specifically involves activation of the p50 and p65 NF-κB subunits, and not RelB and c-Rel. This pattern of NF-κB activation is in contrast with TLR4-mediated LPS activation, which involves activation of p65, c-Rel, p50 and RelB (47). It has been speculated that various members of the NF-κB family mediate distinct functions of monocytic and dendritic cell physiology (47), and dissecting unique features of these interacting pathways is of highest importance.

MATERIALS AND METHODS

Patient recruitment

In all cases, informed consent for genetic and expression studies was obtained in a protocol approved by the institutional review board at the University of Chicago. Diagnoses for CD or UC were obtained from primary review of endoscopic, radiologic and pathologic data.

Patient genotyping

The Arg702Trp, Gly908Arg and Leu1007fsinsC variants were typed using allele-specific PCR as described previously (5,15). In all cases, confirmation of NOD2/CARD15 genotypes was performed by sequencing. All heterozygotes were sequenced throughout C-terminus to the nucleotide-binding domain in order to test for the presence of additional amino-acid polymorphisms (48).

Primary mononuclear cell culture

PBMCs were isolated by Ficoll-Hypaque centrifugation (Axis-Shield Poc AS, Oslo, Norway) of peripheral blood of control and CD individuals stratified by the three major CARD15 mutations. Cells were grown in RPMI supplemented with 10% fetal borine serum, 10 mM HEPES and 20 μg/ml gentamycin in 37°C incubator with 95% O_2 and 5% CO_2. For the microarray analysis, PBMCs were isolated from a CD individual homozygous for the Leu1007fsinsC variant, followed by negative selection of monocytes through a Stemsep column (StemCell, Vancouver, Canada). Monocytes were cultured in M-CSF (R & D System Inc. Minneapolis, MI, USA) for 7 days to differentiate them to macrophages, and treated with MDP (Sigma, St Louis, MO) for 6 h.

Microarray

The target preparation protocol followed the Affymetrix GeneChip Expression Analysis Manual (Santa Clara, CA, USA) with minor modifications. Briefly, 10 μg of total RNA was used to synthesize double-stranded cDNA using the Superscript Choice System (Invitrogen Corporation, Carlsbad, CA). First strand cDNA synthesis was primed with a T7-(dT_{24}) oligonucleotide. From 3 μg of phase-log gel-purified cDNA, biotin-labeled antisense cRNA was synthesized using BioArray High Yield RNA Transcript Labeling Kit. After precipitation with 4 M lithium chloride, 20 μg of cRNA was fragmented in fragmentation buffer (40 mM Tris–Acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc) for 35 min at 94°C and then hybridized to Affymetrix Arrays for 16 h at 45°C and 60 rpm in an Affymetrix Hybridization Oven 640. The arrays were washed and stained with streptavidin phycoerythrin in Affymetrix Fluidics Station 400 using the Affymetrix GeneChip protocol and then scanned using the Affymetrix Agilent GeneArray Scanner. Data analysis was performed using GeneSpring software (Silicon Genetics).

Electrophoretic mobility shift assays

EMSAs were performed using nuclear extracts (2 μg) and binding buffer containing 5 mM Tris (pH 7.5), 37.5 mM KCl, 0.5 mM EDTA, 2% Ficoll, 50 μg/ml poly (dI–dC) (Sigma, St Louis, MO) and 5000 cpm of [γ-^{32}P]-ATP labeled probe, and incubated on ice for 15 min. After 1 h of MDP (Sigma, St Louis, MO, USA) stimulation, nuclear extracts were isolated. For the supershift studies, antibodies against p65, p50, RelB and cRel of the NF-κB (Santa Cruz Biotechnology Inc., Santa Cruz, CA) family of transcription factors, were added. The DNA–protein complexes were analyzed by electrophoresis through a 5% polyacrylamide gel. The gels were dried and exposed to radiographic film.

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IL-1β and IL-8 protein abundance

Freshly isolated PBMCs from healthy controls, wild-type CD patients, CD heterozygotes or CD homozygotes were grown at 3×10^6/ml in 6-well plates. Cells were treated with either MDP, TNFα (R & D system Inc., Minneapolis, MN) alone, or in combination of MDP and TNFα for 16–18 h at the doses indicated. LPS was purified by phenol extraction and contains up to 60% RNA and <1% protein contaminations. Conditioned media were collected, and IL-8 and IL-1β protein were measured by enzyme-linked immunosoxbent assay (ELISA) (Pierce Biotechnology Inc., Rockford, IL).

RT-PCR

PBMCs were stimulated with human TNFα and MDP for 16 h. Total RNA was isolated by RNAeasy mini kit. RNA was treated by DNase. Each RT reaction was carried out by combining 5–10 μg of total RNA mix with 500 ng of random primer, 1 μl of 10 mM dNTPs (65°C, 5 min), 4 μl of 5× first strand buffer, 2 μl of 0.1 M dithiothreital and 1 μl of RNase out (25°C, 10 min), 1 μl of Superscript II (42°C, 50 min and 70°C, 15 min). The quantitative PCR reaction was performed by using Sybr Green PCR core reagents kit (Applied Biosystems, Foster City, CA, USA). For each gene tested, a master mix combining amplitaq gold, 20 mM Tris–HCl, 50 mM KCl, 3 mM $MgCl_2$, 200 mM dGTP, 200 mM dATP, 200 mM dCTP, 400 mM dUTP, 1 U UDG, 200 nM forward primer, 200 nM reverse primer was added to cDNA. The BioRad iCycler was carried out following thermal protocol 50°C for 2 min, 95°C for 7 min and 45 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were run in iCycler iQ PCR plates and measured in triplicate. The specificity of primers was confirmed by melt curve analysis. IL-1β forward primer sequence: ACAGATGAAGTGCTCCTTCCA; IL-1β reverse primer sequence: GTCGGAGATTCGTAGC-TGGAT; IL-8 forward primer sequence: ATGACTTCCAA-GCTGGCCGTGGCT; and IL-8 reverse primer sequence: TCTCAGCCCTCTTCAAAAACTTCTC.

Statistical analysis

The data were logarithmically transformed to bring the measurements on a scale where their distribution is close to a normal distribution. Confidence intervals for the difference in the mean protein or mRNA levels between two stimuli were constructed using normal approximations to the distribution of the differences. *P*-values for testing the two stimuli have the same effect on the mean protein or mRNA were calculated using normal approximations and using a permutation procedure. The results were similar in magnitude, and *P*-values based on the normal approximations are reported.

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REFERENCES

1. Podolsky, D.K. (2002) Inflammatory bowel disease. *N. Engl. J. Med.*, **347**, 417–429.
2. Loftus, E.V., Jr, Silverstein, M.D., Sandborn, W.J., Tremaine, W.J., Harmsen, W.S. and Zinsmeister, A.R. (1998) Crohn's disease in Olmsted County, Minnesota, 1940–1993: incidence, prevalence, and survival. *Gastroenterology*, **114**, 1161–1168.
3. Loftus, E.V., Jr and Sandborn, W.J. (2002) Epidemiology of inflammatory bowel disease. *Gastroenterol. Clin. North Am.*, **31**, 1–20.
4. Bonen, D.K. and Cho, J.H. (2003) The genetics of inflammatory bowel disease. *Gastroenterology*, **124**, 521–536.
5. Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., Britton, H., Moran, T., Karaliuskas, R., Duerr, R.H. *et al.* (2001) A frameshift mutation in *NOD2* associated with susceptibility to Crohn's disease. *Nature*, **411**, 603–606.
6. Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., Almer, S., Tysk, C., O'Morain, C.A., Gassull, M. *et al.* (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*, **411**, 599–603.
7. Cavanaugh, J.A., Adams, K.E., Quak, E.J., Bryce, M.E., O'Callaghan, N.J., Rodgers, H.J., Magarry, G.R., Butler, W.J., Eaden, J.A., Roberts-Thomson, I.C. *et al.* (2003) *CARD15/NOD2* risk alleles in the development of Crohn's disease in the Australian population. *Ann. Hum. Genet.*, **67**, 35–41.
8. Cuthbert, A.P., Fisher, S.A., Mirza, M.M., King, K., Hampe, J., Croucher, P.J., Mascheretti, S., Sanderson, J., Forbes, A., Mansfield, J. *et al.* (2002) The contribution of *NOD2* gene mutations to the risk and site of disease in inflammatory bowel disease. *Gastroenterology*, **122**, 867–874.
9. Gutierrez, O., Pipaon, C., Inohara, N., Fontalba, A., Ogura, Y., Prosper, F., Nunez, G. and Fernandez-Luna, J.L. (2002) Induction of *Nod2* in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J. Biol. Chem.*, **277**, 41701–41705.
10. Ogura, Y., Inohara, N., Benito, A., Chen, F.F., Yamaoka, S. and Nunez, G. (2001) Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB. *J. Biol. Chem.*, **276**, 4812–4818.
11. Inohara, N., Ogura, Y., Fontalba, A., Gutierrez, O., Pons, F., Crespo, J., Fukase, K., Inamura, S., Kusumoto, S., Hashimoto, M. *et al.* (2003) Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease. *J. Biol. Chem.*, **278**, 5509–5512.
12. Girardin, S.E., Boneca, I.G., Viala, J., Chamaillard, M., Labigne, A., Thomas, G., Philpott, D.J. and Sansonetti, P.J. (2003) Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection. *J. Biol. Chem.*, **278**, 8869–8872.
13. Inohara, N., del Peso, L., Koseki, T., Chen, S. and Nunez, G. (1998) RICK, a novel protein kinase containing a caspase recruitment domain, interacts with CLARP and regulates CD95-mediated apoptosis. *J. Biol. Chem.*, **273**, 12296–12300.
14. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J. *et al.* (1999) Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB. *J. Biol. Chem.*, **274**, 14560–14567.
15. Bonen, D.K., Ogura, Y., Nicolae, D.L., Inohara, N., Saab, L., Tanabe, T., Chen, F.F., Foster, S.J., Duerr, R.H., Brant, S.R. *et al.* (2003) Crohn's disease-associated NOD2 variants share a signaling defect in response to lipopolysaccharide and peptidoglycan. *Gastroenterology*, **124**, 140–146.
16. Chamaillard, M., Philpott, D., Girardin, S.E., Zouali, H., Lesage, S., Chareyre, F., Bui, T.H., Giovannini, M., Zaehringer, U., Penard-Lacronique, V. *et al.* (2003) Gene–environment interaction modulated by allelic heterogeneity in inflammatory diseases. *Proc. Natl Acad. Sci. USA*, **100**, 3455–3460.
17. Mitsuyama, K., Toyonaga, A., Sasaki, E., Watanabe, K., Tateishi, H., Nishiyama, T., Saiki, T., Ikeda, H., Tsuruta, O. and Tanikawa, K. (1994) IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease. *Clin. Exp. Immunol.*, **96**, 432–436.
18. Standiford, T.J., Kunkel, S.L., Basha, M.A., Chensue, S.W., Lynch, J.P., 3rd, Toews, G.B., Westwick, J. and Strieter, R.M. (1990) Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. *J. Clin. Invest.*, **86**, 1945–1953.
19. Yoshimura, T., Matsushima, K., Oppenheim, J.J. and Leonard, E.J. (1987) Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). *J. Immunol.*, **139**, 788–793.

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20. Matsusaka, T., Fujikawa, K., Nishio, Y., Mukaida, N., Matsushima, K., Kishimoto, T. and Akira, S. (1993) Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8. *Proc. Natl Acad. Sci. USA*, **90**, 10193–10197.
21. Lorenz, J.J., Furdon, P.J., Taylor, J.D., Verghese, M.W., Chandra, G., Kost, T.A., Haneline, S.A., Roner, L.A. and Gray, J.G. (1995) A cyclic adenosine 3′,5′-monophosphate signal is required for the induction of IL-1 beta by TNF-alpha in human monocytes. *J. Immunol.*, **155**, 836–844.
22. Sirenko, O.I., Lofquist, A.K., DeMaria, C.T., Morris, J.S., Brewer, G. and Haskill, J.S. (1997) Adhesion-dependent regulation of an A+U-rich element-binding activity associated with AUF1. *Mol. Cell. Biol.*, **17**, 3898–3906.
23. Gu, Y., Wu, J., Faucheu, C., Lalanne, J.L., Diu, A., Livingston, D.J. and Su, M.S. (1995) Interleukin-1 beta converting enzyme requires oligomerization for activity of processed forms *in vivo*. *EMBO J.*, **14**, 1923–1931.
24. Wilson, K.P., Black, J.A., Thomson, J.A., Kim, E.E., Griffith, J.P., Navia, M.A., Murcko, M.A., Chambers, S.P., Aldape, R.A., Raybuck, S.A. *et al.* (1994) Structure and mechanism of interleukin-1 beta converting enzyme. *Nature*, **370**, 270–275.
25. Howard, A.D., Kostura, M.J., Thornberry, N., Ding, G.J., Limjuco, G., Weidner, J., Salley, J.P., Hogquist, K.A., Chaplin, D.D., Mumford, R.A. *et al.* (1991) IL-1-converting enzyme requires aspartic acid residues for processing of the IL-1 beta precursor at two distinct sites and does not cleave 31-kDa IL-1 alpha. *J. Immunol. (Baltimore)*, **147**, 2964–2969.
26. Cerretti, D.P., Kozlosky, C.J., Mosley, B., Nelson, N., Van Ness, K., Greenstreet, T.A., March, C.J., Kronheim, S.R., Druck, T., Cannizzaro, L.A. *et al.* (1992) Molecular cloning of the interleukin-1 beta converting enzyme. *Science*, **256**, 97–100.
27. Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J. *et al.* (1992) A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature*, **356**, 768–774.
28. Nau, G.J., Richmond, J.F., Schlesinger, A., Jennings, E.G., Lander, E.S. and Young, R.A. (2002) Human macrophage activation programs induced by bacterial pathogens. *Proc. Natl Acad. Sci. USA*, **99**, 1503–1508.
29. Vidal, V.F., Casteran, N., Riendeau, C.J., Kornfeld, H., Darcissac, E.C., Capron, A. and Bahr, G.M. (2001) Macrophage stimulation with Murabutide, an HIV-suppressive muramyl peptide derivative, selectively activates extracellular signal-regulated kinases 1 and 2, C/EBPbeta and STAT1: role of CD14 and toll-like receptors 2 and 4. *Eur. J. Immunol.*, **31**, 1962–1971.
30. Takeda, K., Tsutsui, H., Yoshimoto, T., Adachi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K. and Akira, S. (1998) Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity*, **8**, 383–390.
31. Morelli, A.E., Zahorchak, A.F., Larregina, A.T., Colvin, B.L., Logar, A.J., Takayama, T., Falo, L.D. and Thomson, A.W. (2001) Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. *Blood*, **98**, 1512–1523.
32. Toms, C. and Powrie, F. (2001) Control of intestinal inflammation by regulatory T cells. *Microbes Infect.*, **3**, 929–935.
33. Ghosh, S. and Karin, M. (2002) Missing pieces in the NF-kappaB puzzle. *Cell*, **109** (suppl.), S81–S96.
34. Pauleau, A.L. and Murray, P.J. (2003) Role of nod2 in the response of macrophages to toll-like receptor agonists. *Mol. Cell. Biol.*, **23**, 7531–7539.
35. Rehli, M. (2002) Of mice and men: species variations of toll-like receptor expression. *Trends Immunol.*, **23**, 375–378.
36. Wang, J., Guan, E., Roderiquez, G. and Norcross, M.A. (1999) Inhibition of CCRS expression by IL-12 through induction of beta-chemokines in human T lymphocytes. *J. Immunol.*, **163**, 5763–5769.
37. Ranade, K., Jorgenson, E., Sheu, W.H., Pei, D., Hsiung, C.A., Chiang, F.T., Chen, Y.D., Pratt, R., Olshen, R.A., Curb, D. *et al.* (2002) A polymorphism in the beta1 adrenergic receptor is associated with resting heart rate. *Am. J. Hum. Genet.*, **70**, 935–942.
38. Thome, M., Hofmann, K., Burns, K., Martinon, F., Bodmer, J.L., Mattmann, C. and Tschopp, J. (1998) Identification of CARDIAK, a RIP-like kinase that associates with caspase-1. *Curr. Biol.*, **8**, 885–888.
39. Zhang, W.H., Wang, X., Narayanan, M., Zhang, Y., Huo, C., Reed, J.C. and Friedlander, R.M. (2003) Fundamental role of the Rip2/caspase-1 pathway in hypoxia and ischemia-induced neuronal cell death. *Proc. Natl Acad. Sci. USA*, **100**, 16012–16017.
40. Yoo, N.J., Park, W.S., Kim, S.Y., Reed, J.C., Son, S.G., Lee, J.Y. and Lee, S.H. (2002) Nod1, a CARD protein, enhances pro-interleukin-1beta processing through the interaction with pro-caspase-1. *Biochem. Biophys. Res. Commun.*, **299**, 652–658.
41. Stehlik, C., Hayashi, H., Pio, F., Godzik, A. and Reed, J.C. (2003) CARD6 is a modulator of NF-kappa B activation by Nod1- and Cardiak-mediated pathways. *J. Biol. Chem.*, **278**, 31 941–31 949.
42. Korzenik, J.R. and Dieckgraefe, B.K. (2000) Is Crohn's disease an immunodeficiency? A hypothesis suggesting possible early events in the pathogenesis of Crohn's disease. *Dig. Dis. Sci.*, **45**, 1121–1129.
43. Worsaae, N., Staehr Johansen, K. and Christensen, K.C. (1982) Impaired *in vitro* function of neutrophils in Crohn's disease. *Scand. J. Gastroenterol.*, **17**, 91–96.
44. Wandall, J.H. and Binder, V. (1982) Leucocyte function in Crohn's disease. Studies on mobilisation using a quantitative skin window technique and on the function of circulating polymorphonuclear leucocytes *in vitro*. *Gut*, **23**, 173–180.
45. Erdman, S., Fox, J.G., Dangler, C.A., Feldman, D. and Horwitz, B.H. (2001) Typhlocolitis in NF-kappa B-deficient mice. *J. Immunol.*, **166**, 1443–1447.
46. Tomczak, M.F., Erdman, S.E., Poutahidis, T., Rogers, A.B., Holcombe, H., Plank, B., Fox, J.G. and Horwitz, B.H. (2003) NF-kappa B is required within the innate immune system to inhibit microflora-induced colitis and expression of IL-12 p40. *J. Immunol.*, **171**, 1484–1492.
47. Hofer, S., Rescigno, M., Granucci, F., Citterio, S., Francolini, M. and Ricciardi-Castagnoli, P. (2001) Differential activation of NF-kappa B subunits in dendritic cells in response to Gram-negative bacteria and to lipopolysaccharide. *Microbes Infect.*, **3**, 259–265.
48. Lesage, S., Zouali, H., Cezard, J.P., Colombel, J.F., Belaiche, J., Almer, S., Tysk, C., O'Morain, C., Gassull, M., Binder, V. *et al.* (2002) *CARD15/NOD2* mutational analysis and genotype–phenotype correlation in 612 patients with inflammatory bowel disease. *Am. J. Hum. Genet.*, **70**, 845–857.

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