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Loss-of-Function CARD8 Mutation Causes NLRP3 Inflammasome Activation and Crohn’s Disease

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Abbreviations used in this paper:
CD: crohn’s disease; NLRP3: Nucleotide-Binding Oligomerization Domain, Leucine Rich Repeat And Pyrin Domain Containing 3; CARD8: Caspase Recruitment Domain Family Member 8; ASC: Apoptosis-Associated Speck-Like Protein Containing A CARD; PBMC: peripheral blood mononuclear cells

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Abstract:

In these studies we evaluated the contribution of the NLRP3 inflammasome to Crohn’s disease (CD) in a kindred containing individuals having a missense mutation in CARD8, a protein known to inhibit this inflammasome. Whole exome sequencing and PCR studies identified that the affected individuals had a V44I mutation in a single allele of the T60 isoform of CARD8. The serum levels of IL-1β in the affected individuals were increased compared with that in healthy controls and their peripheral monocytes produced increased amounts of IL-1β when stimulated by NLRP3 activators. Immunoblot studies probing the basis of these findings showed that mutated T60 CARD8 fails to down-regulate the NLRP3 inflammasome because it does not bind to NLRP3 and inhibit its oligomerization. In addition, these studies showed that mutated T60 CARD8 exerts a dominant negative effect by its capacity to bind to and form oligomers with unmutated T60 or T48 CARD8 that impede their binding to NLRP3. Finally, inflammasome activation studies revealed that intact but not mutated CARD8 prevents NLRP3 deubiquitination and serine dephosphorylation. CD due to a CARD8 mutation was not effectively treated by anti-TNF-α, but did respond to IL-1β inhibitors. Thus, patients with anti-TNF-α-resistant CD may respond to this treatment option.
Introduction

The influence of genetic factors in the pathogenesis of inflammatory bowel disease (IBD) was made dramatically evident by the identification of numerous IBD-associated risk polymorphisms, i.e., SNPs in various genetic regions that occur in greater frequency in patients with IBD than in the general population (1). These risk polymorphisms, in keeping with and supporting the hyper-responsive concept of IBD pathogenesis, quite frequently involve genes affecting inflammatory responses(1). In recent years several publications have appeared highlighting the possible pathogenetic importance of one such inflammation-associated gene, that encoding NLRP3(2, 3). NLRP3 is a pattern recognition receptor that initiates the activation of the NLRP3 inflammasome, a protein complex that regulates the generation of mature forms of IL-1β and IL-18 and the induction of inflammatory cell death known as pyroptosis(4). However, while factors that induce either excess or diminished NLRP3 inflammasome activity have indeed been shown to affect gastrointestinal (GI) inflammation, it has not been shown that the NLRP3 inflammasome can act on its own to induce such inflammation. As such, it appears that in most cases NLRP3 inflammasome activity is one of an array of pro-inflammatory factors causing GI inflammation in Crohn’s disease (CD, See further discussion below).

One characteristic of the NLRP3 inflammasome that might limit its effect on CD inflammation is that this source of pro-inflammatory cytokines is tightly controlled by several naturally occurring negative regulators. CARD8, is one such negative regulator expressed in humans and not in rodents is the focus of the present study. Thus, as shown by Ito et al, CARD8 binds to NLRP3 during inflammasome activation and in doing so, prevents its binding to its down-stream adaptor ASC and thereby the assembly of the NLRP3 inflammasome complex (5); in addition, these investigators showed that NLRP3 bearing mutations associated with Cryopyrin-Associated Periodic Syndromes (CAPS) and causing increased inflammasome activity, lost its ability to bind to CARD8. Additional evidence of
CARD8 inhibition of NLRP3 has come from studies by Cheung et al who reported that a frameshift mutation in CARD8 resulting in loss of binding to NLRP3 caused a form of CAPS marked by aphthous stomatitis, pharyngitis, and cervical adenitis (PFAPA)(6). These studies introduce the possibility that a loss-of-function mutation in CARD8 could be associated with increased NLRP3 inflammasome activity.

Previous studies relating to a possible CARD8 abnormality affecting the occurrence of CD have been concerned with a CARD8 polymorphism at rs2043211 that causes a stop codon in exon 5 of CARD8 (C10X) and therefore prevents transcription of the T48 isoform of CARD8 (the most prevalent isoform) is associated with increased risk for developing CD (7, 8). However, the association of this SNP with CD has not been corroborated in studies involving large numbers of patients (9). These more negative studies may be due to the fact that most patients bearing this polymorphism continue to express a slightly smaller but nevertheless fully-functional isoform (T47) as a result of transcription of a CARD8 isoform that initiates downstream of the C10X site (10).

In this study, we report a kindred containing several individuals carrying a CARD8 mutation at V44I up-stream of the T48 start site and therefore expressed in the T60 CARD8 isoform (See diagram in Figure 1). All three individuals carrying this mutation developed a CD-like intestinal inflammation associated with extensive evidence of NLRP3 inflammasome activation. The latter consisted of high levels of circulating IL-1β and the presence of peripheral monocytes exhibiting increased NLRP3 activation. The mutant CARD8 lacked the ability to interact with NLRP3 and inhibit NLRP3 oligomerization. More importantly, intestinal inflammation persisted despite a full range of biologic and immunosuppressive therapy until the Proband was treated with anakinra or an IL-1β neutralizing antibody. These data thus provide the first evidence that a mutation affecting a component of the NLRP3 inflammasome leading to its excessive activation can be a cause of CD in the absence of other abnormalities.
Results:

Clinical Courses of Patients in a Kindred with a CARD8 Mutation and CD-like Intestinal Inflammation

A kindred containing three members bearing a CARD8 mutation is depicted in Figure 1A. In all three of these members, the Proband, his mother and his maternal aunt, the mutation occurred in association with a CD-like intestinal inflammation (Figure 1B, C); see detailed description of the case histories of the Proband, his mother and maternal aunt in Supplemental Materials. The Proband’s father did not carry the CARD8 mutation and was free of GI disease. Of note, the Proband did not improve upon treatment with steroids plus anti-TNF-α and while initial histopathologic examination showed changes found in GvH disease, follow-up examination after some clinical improvement showed changes indicative of CD. He was therefore administered anakinra (IL-1 receptor antagonist), a treatment that led to decreased diarrhea and other GI symptoms, and was accompanied by a sharp decrease in the fecal calprotectin level (see Figure 1D). This suggested the presence of excessive NLRP3 inflammasome activity and led to the studies detailed below.

DNA Sequencing Data of Individuals in the Kindred

Whole exome sequencing identified a number of variants in the patients comprising the above kindred (Supplemental Figure 1). Among these, the variant chr19:48741719 C>T (hg19) in CARD8 stood out because of its role in the inflammasome (5, 6). This variant has an allele frequency of 0.0015% in the gnomAD database (2 heterozygous individuals), is predicted to be possibly damaging/deleterious by Polyphen and SIFT, and has a CADD-phred score of 11.3, which is above the mutation significance cutoff score (11) of 3.3 for CARD8. This variant overlaps multiple transcripts, so in the context of the gene, the variant could be in the intron, in the 5' UTR, or else a V44I missense mutation (Supplemental
Figure 2). However, we detected no difference in CARD8 expression at the mRNA and protein level in cells from the Proband compared to cells from a healthy control (Supplemental Figure 3A, B), so the only relevant annotation is a missense mutation at V44I.

In related studies in which DNA fragments generated by PCR using primers surrounding the CARD8 mutation site were sequenced the presence of a single allele V44I mutation in DNA from both the Proband and his mother (Supplemental Figure 4) was again observed; in addition, these studies also revealed the same CARD8 mutation in a maternal aunt who also has CD (Supplemental Figure 4). Finally, while these sequencing studies were performed on DNA obtained from peripheral cells, qPCR and Western blot studies of small and intestine and colonic tissues showed that CARD8 bearing the V44I mutation was expressed at a substantial levels in Proband gastrointestinal tissue (Supplemental Figure 5).

Additional analysis of the above sequencing data revealed another variant of potential interest, a missense R329H variant in the MEFV gene having a population allele frequency of 0.2%. The MEFV gene encodes Pyrin, a protein that forms the Pyrin inflammasome with ASC and caspase-1 that releases IL-1β when stimulated by its activator TcdB. However, we did not find excessive Pyrin inflammasome activity in the Proband (Supplemental Figure 6), the MEFV variant occurred only in the Proband and his mother, not in the Proband’s aunt with well-defined CD who also bears the CARD8 V44I mutation (Supplemental Figure 4). This suggests that the MEFV variant does not contribute to the development of the CD in this kindred.

Yet another fact to emerge from the above sequencing data was that DNA from neither the Proband nor his parents bear a SNP at rs2043211 in exon 6 (Genebank) of CARD8, a polymorphism that has been shown to occur at an increased frequency in patients with CD in some studies (7, 8).
CARD8 is known to be expressed as one of several isoforms with differing transcription start sites leading to differing molecular weights, this includes a T48 isoform with a start site in exon 6 (Genebank) and a larger T60 isoform with an upstream start site in exon 4 (Genebank) (See Figure 1E). The latter is the isoform that contains the Card8 mutation at V44I and therefore is the isoform that is potentially responsible for the functional effects of the mutation.

Finally, It should be noted that a gene equivalent to CARD8 is not found in mice. Thus, studies of the effects of mutations in the various isoforms of this gene are limited to in vitro studies of human cells that cannot be further elucidated by in vivo studies of similar CARD8 mutants in mice.

The CARD8 V44I Mutation is Associated with Enhanced IL-1β Production and Increased NLRP3 Inflammasome Activation

Recent studies have provided evidence that the CARD8 T48 isoform has an inhibitory effect on NLRP3 inflammasome activation(5). To examine if the CARD8 T60 isoform, i.e., the isoform that bears a V44 site capable of bearing a V44I mutation, has a similar effect on NLRP3 inflammasome regulation, we transfected plasmids expressing human NLRP3 together with plasmids expressing CARD8 T60, CARD T48 or empty vector into HEK293 cells and, 24 hours later, transfected the cells with plasmids expressing ASC, caspase-1 and pro-IL-1β to allow assembly of an NLRP3 inflammasome; then, after another 24 hours, the cells were stimulated with nigericin and the culture median of incubated cells were collected for assessment of IL-1β secretion. We found that cells transfected with plasmids expressing either CARD8 T60 or T48 isoforms produced significantly reduced levels of IL-1β compared with cells transfected with empty vector (Figure 2A). These data thus provided evidence that the intact (unmutated) CARD8 T60 isoform also plays an inhibitory role during NLRP3 inflammasome activation; in addition, they suggested that a loss-of-function CARD8 mutation in CARD8 T60 could be affecting this inhibitory role.
In initial studies to examine this latter possibility we determined concentrations of IL-1β and IL-6 in a random sample of Proband serum by ELISA and found that concentrations of both of these cytokines was clearly increased compared to levels in sex-matched control sera (Figure 2B). In related studies, we determined if Proband peripheral blood mononuclear cells (PBMCs) or monocytes produce increased amounts of IL-1β, as might be expected if the NLRP3 inflammasome was dysregulated by the mutation. Accordingly, we cultured PBMCs and purified Proband monocytes for 24 or 48 hours in the absence of stimulation and then determined the IL-1β concentration in the culture supernatants. We found IL-1β in the supernatants from both Proband cell cultures was significantly increased compared to supernatants of control cell cultures (Figure 2C).

With these data in hand we next conducted studies to formally determine if the CARD8 V44I mutation in the CARD8 T60 isoform affects NLRP3 inflammasome activation. We therefore examined IL-1β production by Proband and control peripheral monocyte derived dendritic cells (mDCs) stimulated by LPS alone or LPS in the presence of NLRP3 activators, ATP or nigericin. We found that cells from the Proband stimulated under these conditions produced significantly more IL-1β (Figure 2D) and IL-18 (Supplemental Figure 7) than cells from control individuals but produced similar amounts of IL-6 (Figure 2D). In addition, in Western blot studies we found that the cells from the Proband produced greatly increased amounts of mature IL-1β and mature caspase-1 than control cells (Figure 2E). Moreover, the Proband’s mother and maternal aunt carrying the CARD8 V44I mutation also displayed over-activity of the NLRP3 inflammasome (Supplemental Figure 8 and 9). During the study, peripheral blood from different healthy individuals were used as control and in all cases IL-1β production was lower than those observed in patients with CARD8 mutation. These data thus suggested that the V44I mutation negated the inhibitory role of CARD8 on NLRP3 inflammasome activation.
The CARD8 Mutation Does Not Affect Pyrin or NLRC4 Inflammasome Activation but has a Marginal Effect on AIM Inflammasome Activation

Since intact CARD8 may regulate one or more inflammasomes other than the NLRP3 inflammasome it was possible that a loss-of-function mutation in CARD8 causes increased IL-1β production via dysregulation of several inflammasomes. To investigate this possibility, we primed purified mDCs from Proband and control cells with LPS, and then stimulated with ATP, TcdB, transfected poly(dA:dT) or flagellin to activate the NLRP3, Pyrin, AIM2 or NLRC4 inflammasomes, respectively. Our results showed that, as expected, the mDCs from the Proband produced more IL-1β than those from control cells after NLRP3 activation with LPS/ATP stimulation (Figure 3A); in addition, Proband mDCs stimulated with the AIM inflammasome activator, polyAdT also produced a greater amount of IL-1β than control cells, although in this case the increase, while repeatable, was relatively small compared to that obtained by the activation of the NLRP3 inflammasome (Figure 3B). In contrast, the production of IL-1β was comparable between patient and control cells after TcdB, or flagellin stimulation (Figure 3C, D). These differential responses were specific to IL-1β as each of the stimuli resulted in equal IL-6 responses (Figure 3E). Together, these data demonstrated that the CARD8 V44I mutation mainly enhances NLRP3 inflammasome activation, and perhaps the AIM2 inflammasome, but does not affect the Pyrin or NLRC4 inflammasome. This is most likely attributable to the fact that CARD8 does not regulate these inflammasome even in the intact state.

CARD8 with a V44I Mutation Exhibits Reduced Binding to NLRP3 and Affects NLRP3 Inflammasome Assembly

To investigate the mechanism by which the CARD8 V44I mutation exhibits decreased inhibition of the NLRP3 inflammasome, we first asked if this mutation affects the transcription of the genes related with NLRP3 inflammasome and thus alters their expression. To examine this question we assessed expression levels
of various NLRP3 components in mDCs from the Proband as compared to that in mDCs from a healthy control individual. We found that Proband cells expressed comparable levels of ASC and caspase-1 (Figure 3F, G), but significantly increased levels of NLRP3 and IL-1β (Figure 3H, I). The increase in NLRP3 mRNA associated with the CARD8 V44I mutation is presently unexplained but may be due to the fact that the mutation negates known inhibitory effects of CARD8 on NF-κB activation and thereby causes an increase in NLRP3 transcription (12).

Next, we determined if the mutation in CARD8 affects its ability to bind to NLRP3. To this end, HEK293 cells transfected with plasmids expressing intact CARD8 T60 or CARD8 T60 bearing the V44I mutation along with a plasmid expressing NLRP3 were cultured for 48 hours after which cell lysates were prepared and subjected to immunoprecipitation with anti-CARD8 antibody followed by immunoblotting with anti-NLRP3 antibody. The results showed that the interaction between mutant CARD8 T60 and NLRP3 was remarkably reduced compared with the interaction between intact CARD8 T60 and NLRP3 (Figure 4A). In parallel studies, we determined if a similar effect can be observed under physiological conditions. In these studies, we stimulated mDCs from the Proband and a healthy control with LPS or LPS plus nigericin and then, after appropriate incubation period prepared cell lysates and subjected the latter to immunoblot studies as discussed above. We found that intact CARD8 bound to NLRP3 when stimulated with LPS, but not after LPS and nigericin treatment (Figure 4B). In contrast, the mutant CARD8 did not bind to NLRP3 in any circumstance (Figure 4B). These data indicated that CARD8 bearing the V44I mutation has a reduced capacity to bind to NLRP3 under physiologic conditions.

Despite the fact that the Proband does not express the CARD8 SNP at rs2043211 that has been associated with CD in some studies (7, 8) it was of interest to determine if this SNP also causes an abnormality of the capacity of CARD8 to bind to NLRP3 as does the V44I mutation. Whereas this SNP introduces a stop codon (C10X) in the T48 isoform of CARD8 (and therefore the likely formation of a
downstream transcriptional start site for this isoform) it causes a P102I mutation in the T60 isoform of CARD8 due to the upstream start site governing this isoform. Accordingly, to determine the effect of this SNP on CARD8 binding to NLRP3 we transfected plasmids expressing intact or P102I-mutated T60 CARD8 together with a plasmid expressing NLRP3 into HEK293 cells and then performed immunoblotting studies to examine CARD8/NLRP3 interactions. We found that the interaction between NLRP3 and CARD8 T60 P102I was comparable to that with intact CARD8 T60 (Figure 4C). In an additional study in which we determined the capacity of CARD8 T60 P102I or intact CARD8 T60 to inhibit an assembled NLRP3 inflammasome in HEK293 cells as described above we observed that P102I and intact CARD8 had comparable ability to inhibit the secretion of IL-1β by the NLRP3 inflammasome (Figure 4D). Thus, even in individuals carrying a homozygous SNP at rs2043211, the CARD8 isoform T60 still regulates NLRP3 inflammasome activity.

Finally, we investigated if the lack of CARD8 binding to NLRP3 associated with the mutation impacts NLRP3 inflammasome assembly. For this purpose, we determined if the mutation affects ASC polymerization and perinuclear speck formation, an obligate downstream effect of its binding to NLRP3 and inflammasome assembly and activation. We found that mDCs from the peripheral blood of the Proband with a CARD8 V44I mutation exhibited increased ASC oligomerization upon NLRP3 inflammasome activation with LPS and nigericin as compared to cells from a control individual (Figure 4E). In addition, oligomerization of NLRP3 was also increased in cells with the CARD8 V44I mutation (Figure 4F). These results indicated that reduced CARD8 function due to a V44I mutation is accompanied by increased NLRP3 assembly.

**CARD8 Bearing the V44I Mutation Has a Dominant Negative Effect**

As noted above, the CARD8 V44I mutation is found on only one allele of the Proband and the latter is therefore capable of producing intact CARD8 (albeit in
reduced amounts). It therefore seemed likely that the mutated CARD8 was exerting a dominant-negative effect and thereby inhibiting the function of the intact CARD8. To examine this possibility, we transfected plasmids expressing mutated and intact T60 isoforms of CARD8 alone or together into HEK293 cells and then performed immunoblot studies to determine CARD8 binding to NLRP3 as described above. We found that co-transfection of mutated CARD8 with intact CARD8 greatly decreased CARD8 and NLRP3 binding (Figure 5A).

Next, to determine if the interaction between CARD8 T48 and NLRP3 was affected by CARD8 T60 V44I mutation, we transfected HEK293 cells with a plasmid expressing the T48 isoform of CARD8 together with a plasmid expressing the intact or mutated T60 isoform of CARD8 and then performed immunoblot studies as described above. We found that the presence of mutated CARD8 T60 but not intact CARD8 T60 reduced T48 binding to NLRP3 (Figure 5B). This effect was confirmed by immunoprecipitation with anti-NLRP3 antibody (Figure 5C).

In a parallel experiment, we transfected NLRP3, ASC, caspase-1 and pro-IL-1β into HEK293 cells to allow assembly of a NLRP3 inflammasome, and then transfected intact CARD8 T60 or T48 alone or together with mutant CARD8 T60 to examine their effect on IL-1β production. We found that transfection of intact CARD8 T60 or T48 alone inhibited IL-1β production, whereas co-transfection of mutant CARD8 disrupted the inhibitory effect of the intact T60 or T48 on IL-1β production (Figure 5D). Taken together, these findings established that CARD8 T60 bearing a V44I mutation interferes with the function of intact CARD8 and thus acts in a dominant negative fashion.

Finally, in studies addressing the mechanism underlying this dominant negative effect, we investigated if the isoforms of CARD8 interact with one another. To this end, we transfected plasmids expressing flag-tagged CARD8 T48 together with myc-tagged intact or mutant CARD8 T60 into HEK293 cells and examined their interaction by immunoblotting. We found that whereas intact CARD8 T60 exhibited
barely discernable interaction with CARD8 T48, mutant CARD8 T60 exhibited robust interaction with CARD8 T48 (Figure 6A). Similarly, whereas intact CARD8 T60 interacted poorly with intact CARD8 T60, mutated CARD8 T60 interacted quite well with intact CARD8 T60 (Figure 6B). Along the same lines, electrophoresis of lysates of cells transfected with plasmids expressing intact CARD8 T60/T60 mixtures under non-reducing conditions revealed poor oligomer formation whereas lysates of cells transfected with intact CARD8 T60/mutated CARD8 T60 mixtures exhibited strong oligomer formation (Figures 6C and 6D). Similarly, intact CARD8 T48/T60 exhibited relatively poor oligomer formation whereas intact CARD8 T48/mutated CARD8 T60 exhibited robust oligomer formation (Figure 6E). These data thus suggest that mutated CARD8 exerts a dominant negative effect by binding to intact CARD8 isoforms and thereby disrupting the latter’s ability to bind to NLRP3 (See Model in Figure 6F).

The CARD8 V44I Mutation Results in Reduced NLRP3 Serine Phosphorylation, as well as Reduced K63 and K48 Polyubiquitination

Recent studies have provided evidence that serine phosphorylation of NLRP3 mediated by phosphokinase A (PKA) inhibits NLRP3 inflammasome activation and that such phosphorylation is disrupted in CAPS patients with increased NLRP3 inflammasome activity (13). In addition, it has been reported that NLRP3 polyubiquitination also inhibits NLRP3 inflammasome activation (16,17). It was therefore possible that the inhibitory effect of intact CARD8 and its loss in the presence of a V44I CARD8 mutation is due to effects on NLRP3 serine phosphorylation and NLRP3 polyubiquitination.

To examine this possibility, we transfected plasmids expressing human NLRP3, ASC, caspase-1, and either intact or mutant CARD8 into HEK 293 cells to determine the effect of CARD8 or mutant CARD8 on serine phosphorylation of NLRP3 during its activation in a re-assembled NLRP3 inflammasome. We found that serine phosphorylation of immunoprecipitated NLRP3 (detected with a
phosphoserine-specific antibody) in cells with expression of intact CARD8 was increased compared with cells without transfection of CARD8 (Figure 7A). In addition, the phosphorylation of NLRP3 in cells transfected with mutant CARD8 was reduced compared with cells transfected with intact CARD8 (Figure 7A). However, after treatment with nigericin, NLRP3 phosphorylation in both samples were reduced (Figure 7A). These data suggest that before activation, NLRP3 was in a serine-phosphorylated state, allowing the NLRP3 inflammasome to maintain an inactive status; however, when stimulated by its activators, the phosphorylation is reduced and thus allows activation. In contrast, the mutant CARD8, presumably due to its lack of binding, results in reduced phosphorylation of NLRP3 even prior to activation and therefore leads to enhanced activation.

In parallel studies to verify these finding under physiologic conditions we pre-treated mDCs from the Proband and a control individual with LPS and then activated the NLRP3 inflammasome with nigericin. Finally, we subjected cell lysates from these cells to immunoprecipitation with anti-NLRP3 antibody and then immunoblotting with anti-phosphoserine antibody as described above. In this case, we found that cells with the CARD8 mutation exhibited remarkably less NLRP3 serine phosphorylation than cells with intact CARD8 (Figure 7B). Taken together these studies show that CARD8 regulates NLRP3 inflammasome activation at the level of serine phosphorylation and the CARD8 mutation affects this regulation.

We next asked if intact or mutated CARD8 affects polyubiquitination of NLRP3. To this end, we transfected constructs carrying human NLRP3, ASC, Caspase-1, intact or mutant CARD8 T60 together with constructs expressing K63 or K48 ubiquitin chain into HEK293 cells. The cells were then incubated for 48 hours after which cell lysates were subjected to immunoprecipitation with anti-NLRP3 antibody and Western blot to examine the polyubiquitination of NLRP3. We found that the K63 and K48-linked polyubiquitination of NLRP3 in cells tranfected with CARD8 T60 V44I mutant was both reduced compared with those transfected with intact CARD8 T60 (Figure 7C). These studies therefore imply that CARD8 also
regulates NLRP3 inflammasome at the level of polyubiquitination and the CARD8 mutation affects this regulation.

Taken together, these results suggest that the overactivation of NLRP3 inflammasome in the CD patients bearing mutant CARD8 is caused by both reduced NLRP3 phosphorylation and K63 and K48-linked polyubiquitination of NLRP3.

**IL-1β Antibody Administration is an Effective Form of Treatment of CD in a Patient Bearing a CARD8 V44I Mutation**

As noted above, CD in the proband bearing the CARD8 mutation was greatly ameliorated by treatment targeting IL-1β (anakinra and canalkinumab). We therefore performed studies to determine the effect of this treatment on NLRP3 inflammasome function. We found that the antibody treatment reduced serum IL-1β as well as IL-6 and TNF-α to levels observed in control individuals (Figure 8A-8C). However, stimulation of circulating monocytes under NLRP3 activating conditions revealed that the antibody had no effect on inflammasome activation and the V44I mutation was still capable of inducing increased activation. The effect of antibody treatment was therefore directed at the output of the NLRP3 inflammasome activation rather than at its activation.

The above data showed that increased IL-1β secretion plays a pathogenic role in the Proband and that the concentration of IL-1β in the circulation is correlated with disease activity. This prompted us to ask how circulating IL-1β level in the patient with the CARD8 mutation compares with that in conventional CD patients without a CARD8 mutation. To this end, we collected serum samples from four patients with moderately active CD and compared the circulating IL-1β, IL-6 and TNF-α levels in these patients with those in the patient with the CARD8 mutation and healthy individuals. The results showed that the concentration of all three cytokines were significantly higher than that in healthy controls (Figure 8D, E, F) but lower
than in the patient with the CARD8 mutation. This suggests that whereas conventional CD patients have increased NLRP3 inflammasome activity, the latter is not as elevated as in the patient with the CARD8 mutation. This is probably one of the reasons why GI disease in the patient with the mutation was not responsive to treatment that does not address NLRP3 hyperactivity.
Discussion

CARD8 (also known as TUCAN or CARDINAL) is a component of the NLRP3 inflammasome that has been shown to negatively regulate its activation and capacity to process IL-1β (5). Here we report that a V44I mutation in the T60 isoform of CARD8 interferes with its regulatory function and causes a form of CD due to enhanced NLRP3 activity and its secretion of IL-1β. This is thus the first report that an abnormality of IL-1β secretion can be a primary cause of gastrointestinal inflammation.

The CARD8 mutation was present in three members of a kindred, all of whom had CD. Extensive studies of the Proband revealed that he exhibited high levels of circulating IL-1β associated with increased NLRP3 inflammasome activation. Mechanistically, this was caused by reduced capacity of the mutated CARD8 to interact with and inhibit NLRP3 oligomerization. Strong evidence that the mutation and its effect on NLRP3 activity was the cause of CD came from the fact that the Proband did not respond clinically to anti-TNF-α and/or immunosuppressive treatment but did respond to anakinra or anti-IL-1β antibody administration. Studies of the Proband’s mother and maternal aunt also revealed high circulating levels of IL-1β and increased NLRP3 inflammasome activity but in these cases possible clinical responses to anti-IL-1β administration have not been tested. These data on the relation of a CARD8 mutation to causation of CD is parallel to a recent finding that a frameshift mutation in CARD8 also leading to reduced binding of CARD8 with NLRP3 is a cause of periodic fever with aphthous stomatitis, pharyngitis and cervical adenitis (6).

The CARD8 gene is present on chromosome 19 and is capable of generating several isoforms of CARD8 that result from alternative N-terminal transcription start sites. The “canonical” T48 isoform has a start site in exon 6 and was shown initially to negatively regulate NF-κB and promote apoptosis (12, 14). Somewhat later it was shown to interact with NLRP3 via its FIIND domain and to negatively
regulate the NLRP3 inflammasome (5). Our studies show that a somewhat larger isoform of CARD8, the T60 isoform having a start site in exon 4, also binds to NLRP3 and inhibits its activity. As discussed further below, this is an important finding because the T60 isoform of CARD8, but not the T48 isoform of CARD8 bears the V44I mutation and therefore only this isoform can mediate the effect of the mutation on CARD8 regulatory function.

The V44I mutation is a single allele mutation and is therefore present with a normal allele capable of producing intact CARD8 isoforms with normal regulatory function. The T60 isoform of CARD8 bearing the mutation was therefore likely to be acting in a dominant negative fashion. In studies to explain this dominant negative effect we showed first that the co-transfection of mutated CARD8 T60 diminishes the binding of unmutated CARD8 T48 or T60 to bind to NLRP3. In addition, co-transfection of mutated CARD8 T60 with unmutated CARD8 T48 or T60 reduces the ability of either form of CARD8 to down-regulate NLRP3 inflammasome mediated secretion of IL-1β. Interestingly, this is associated with the fact that mutated CARD8 T60 has a much greater ability to bind to either CARD8 T60 or CARD8 T48 and to form oligomers with the unmutated isoforms. This supports the idea that mutated CARD8 T60 exerts a dominant negative effect by forming oligomers with intact CARD8 isoforms with a diminished ability to bind to NLRP3.

An understanding of how the above changes in CARD8 produced as a result of the mutation lead to the above changes in its binding patterns will require additional structure-based studies. The latter will have to first resolve the fact that both the T60 and T48 isoforms of CARD8 interact with NLRP3 via their N-terminal FIIND domains despite the fact that T60 FIIND domain is longer than the T48 FIIND domain. Perhaps more importantly, it will have to explain how the mutation of the T60 isoform at V44 causes a change in the FIIND region that produces a CARD8 that is unable to interact with NLRP3 as well as a change that produces a CARD8 more able to interact with unmutated T60 and T48 CARD8 isoforms present in the
heterozygous patients that thus block their ability to bind to NLRP3. We speculate that the structural changes affecting the inter-isoform binding involve C-terminal CARD-CARD interactions; thus, the same sequence variant that is unable to bind to NLRP3 via its FIIND domain achieves increased binding to unmutated CARD8 via its CARD domain.

Studies of patients with IBD have established that IL-1β secretion is an integral component of the cytokine milieu driving the colonic inflammation and, in fact, IL-1β levels correlate with disease severity (15-17). However, since IL-1β secretion is one among several pro-inflammatory factors driving gut inflammation it has not itself been clearly identified as a primary cause of disease. Studies of murine models of colitis addressing this question, usually DSS-colitis, are inconclusive since this form of colitis phenocopies human IBD rather poorly, particularly with regard to its dependence on loss of epithelial cell integrity. In any case, in utilizing these models it has usually been shown that loss of function abnormalities of the NLRP3 inflammasome, the main source of IL-1β, is associated with more rather than less intestinal inflammation (18-22). One possible explanation of this somewhat paradoxical result is that the NLRP3 inflammasome also generates protective factors such as anti-inflammatory cytokines and IL-18, the latter a cytokine that help maintain epithelial cell integrity and whose absence therefore leads to increased colitis, especially in the DSS-colitis model (19-21). This view is compatible with a recent study in which it was shown that a particular microRNA (miR-223) has an inhibitory effects on NLRP3 function and its absence in mice leads to increased DSS-colitis. Of interest, miR-223 levels are increased in CD patients (and in DSS-colitis), but this increase does not preclude the development of robust inflammation (23). These various studies of animal models in which decreased NLRP3 activity is shown not to lead to decreased intestinal inflammation are complemented by a study in which it was shown that increased NLRP3 activity does not lead to increased inflammation. Thus, in a study by Yao et al., it was shown that mice bearing a mutation in the NLRP3 gene resulting in NLRP3 with increased function similar to that occurring in the autoinflammatory
Muckle-Wells Syndrome leads to resistance to DSS-colitis. This was due to IL-1β induction of anti-bacterial peptides promoting the development of an intestinal microflora containing an organism (*L. marinus*) that induces regulatory T cells in the mucosa capable of suppressing inflammation (24).

The above studies of animal models suggesting that IL-1β is not normally the sole driver of gut inflammation do not rule out the possibility that this cytokine can play an increased pro-inflammatory role under circumstances in which it is produced in excess and is associated with other pro-inflammatory conditions. This is shown in studies of mice with abnormalities of IL-10 production or signaling since IL-10 has been shown to have a profound inhibitory effect on NLRP3 inflammasome activity and that therefore leads in its absence to increased IL-1β production (25). In these studies it has been shown that IL-10 KO or IL-10R KO mice exhibit increased NLRP3 inflammasome activity and that an NLRP3 inflammasome inhibitor such as glyburide or caspase1 inhibitor ameliorates colitis occurring in such mice (26, 27). In addition, in related studies it has been shown that blockade of IL-10 signaling causes colitis in TLR5 KO mice attributable to IL-1β since it does not occur in TLR5/IL-1βR double KO mice (28). Finally, and perhaps most importantly, it has been demonstrated that NLRP3 inflammasome hyperactivity and increased IL-1β secretion may mediate colitis in CD patients due to IL-10R deficiency can be successfully treated with the IL-1R blocker anakinra (29). This evidence of a negative relation between IL-10 and the NLRP3 inflammasome may have a direct relevance to the gut inflammation in patients with the CARD8 mutation reported here as it may be that the presence of a subtle IL-10 deficiency enhances the effect of the mutation on NLRP3 function and thus determines whether the mutation will cause inflammation.

Mutations leading to IL-10 or IL-10R deficiency are not the only genetic defects having possible effects on NLRP3 inflammasome activation. It has been shown, for instance, that several gene polymorphisms associated with increased risk for development of CD, most notably loss-of-function polymorphisms involving NOD2
and ATG16L1 have been associated with changes in NLRP3 activity and could thus be contributors to gut inflammation via their effect on inflammasome function (30). In addition, patients with intestinal inflammation associated with gene abnormalities leading to mevalonate kinase deficiency, and CGD have benefited from treatment with agents that block IL-1β (31, 32). Thus, increased production of IL-1β due to a variety of genetic abnormalities may contribute to gut inflammation. Finally, it should be mentioned that active NLRP3 inflammasome activity also results in secretion of IL-18 and while this cytokine may reduce inflammation by contributing to epithelial integrity as mentioned above, it also may have pro-inflammatory properties of its own. This is seen in patients with NLRC4 abnormalities that do not respond to IL-1β blockade but do respond to a combination of agents that block both IL-1β and IL-18(33).

The occurrence of a CD phenotype in the Proband and his relatives bearing the CARD8 mutation and manifesting increased NLRP3 activity but not in CAPS patients who bear NLRP3 mutations that also cause increased NLRP3 activity raises questions about the genetic and/or environmental factors necessary for disease appearance. One possible explanation is that these two genetically determined diseases are associated with one or more modifying genes that determine the capacity of IL-1β to mediate disease based on the downstream mechanisms of IL-1β activity. Another possible explanation is based on the presence or absence of an environmental factor such as a microbiome-related development of suppressor cells that prevent the development of GI disease. This possibility is supported by the recent finding already mentioned above(24), showing that mice bearing a CAPS-like mutation in NLRP3 leading to increased NLRP3 inflammasome activity results in the appearance of a gut organism capable of inducing the development of suppressor T cells. Further work will be necessary to establish these or other explanations of intestinal inflammation in the face of NLRP3 over-activity.
As discussed in the Introduction, a CARD8 polymorphism at rs2043211 has been associated with CD in some studies (7, 8) but not others (34, 35) and was not associated with CD in a large meta-analysis of many studies conducted by Zhang et al (9). This polymorphism, introduces a stop codon in CARD8 T48 that because of its proximity to the T48 start site would effectively result in loss of CARD8 expression. However, Bagnall et al., showed that individuals with the polymorphism express a functional CARD8 (T47) isoform presumably by utilizing a start site downstream of the polymorphism and that is not affected by the latter (5, 10). CARD8 T60 due to its alternative start site has a P102I substitution that does not cause a stop codon as a result of this polymorphism. Nevertheless, we conducted studies of CARD8 T60 containing this substitution to investigate its possible effect on CARD8 T60 function and showed that it had no effect of CARD8 T60 interaction with NLRP3 or NLRP3 regulatory function. Therefore, even in individuals carrying a homozygous SNP at rs2043211, the expression of CARD8 T60 and CARD8 T47 play a normal down-regulatory role in the NLRP3 inflammasome.

Several recent publications have implicated NLRP3 phosphorylation in the regulation of NLRP3 inflammasome activation. Thus, Stutz et al. and Mortimer et al. reported that dephosphorylation of serine (S5 or S295) is necessary for NLRP3 activation whereas Spalinger et al. reported that dephosphorylation of a tyrosine (Y861) has this function (13, 14, 36). It was therefore logical to assume that CARD8 regulates NLRP3 inflammasome by affecting NLRP3 dephosphorylation. We investigated this possibility by assessing the effect of unmutated and mutated CARD8 T60 on NLRP3 serine phosphorylation and showed that whereas unmutated CARD8 T60 was associated with NLRP3 having intact serine phosphorylation, mutated CARD8 T60 was associated with NLRP3 having diminished serine phosphorylation. Thus, loss of binding of CARD8 due to mutation was associated with loss of serine phosphorylation, both conditions in which the NLRP3 inflammasome is activated. These data suggest that CARD8 in an unmutated state retards serine dephosphorylation and thereby inhibits NLRP3
activation, however, additional studies will be necessary to demonstrate an actual cause and effect relation.

Data from several recent publications suggest that the NLRP3 inflammasome is regulated by polyubiquitination of NLRP3. Thus, Yan et al[37] reported that K48-linked ubiquitination negatively regulated NLRP3 inflammasome activity by promoting the degradation of NLRP3. This idea was supported by Han et al[38] and Song et al[39], although they identified different ubiquitinases were operative as compared to those identified by Yan et al. In addition, Py et al[40] showed that deubiquitination of both K63- and K48-linked polyubiquitin chains was required for NLRP3 inflammasome activation. A somewhat different view of the role of polyubiquitination in NLRP3 activation was suggested by Wei et al[41], who showed that the Yersinia-type III secretion effector YopM promoted K63-linked polyubiquitination and activation of NLRP3; thus, in this case K63-linked polyubiquitination was shown to be a positive regulator of the NLRP3 inflammasome. Our data showed that interaction of intact CARD8 with NLRP3 results in reduced K63 and K48-linked polyubiquitination of NLRP3; they thus support the idea that intact CARD8 is a negative regulator of NLRP3, at least in part, by its ability to promote NLRP3 K63 and K48-linked polyubiquitination; in contrast, mutant CARD8 does not maintain NLRP3 polyubiquitination and causes increased NLRP3 activation. It should be noted that serine phosphorylation and polyubiquitination of NLRP3 may be linked phenomena that operate in tandem to prevent NLRP3 activation.

In summary, these data provide the first evidence that enhanced production of IL-1β due to an intrinsic abnormality of the NLRP3 inflammasome activity, can be a primary cause of CD. This is thus contrary to the notion that IL-1β cannot in itself cause this form of GI inflammation and participates in the inflammatory process only in concert with other pro-inflammatory cytokines or absence of regulatory cytokines. With the discovery that enhanced NLRP3 inflammasome activity can be a cause of CD and that such patients require therapy (such as anti-IL-1β
administration) not ordinarily used in CD treatment, it is incumbent on gastroenterologists to consider the possibility that patients resistant to ordinary treatment may have a form of disease due to IL-1β and that can therefore be treated with anti-IL1β-based therapies.
**Methods**

**Human subjects**
Peripheral blood from the proband patient with CD, his mother, father and maternal aunt was drawn for whole exome sequencing and other laboratory examination. Control blood was obtained from gender matched healthy. Peripheral blood from another four patients with active CD was drawn for serum cytokine assay.

**Reagents and Constructs**
Ultrapure Lipopolysaccharide (LPS; L3012), ATP (tlrl-atpl), Nigericin (tlrl-nig), Poly (dA:dT) (tlrl-patn), flagellin (tlrl-epstfla-5), phorbol myristate acetate (PMA; P8139), MSU (tlrl-msu), were purchased from InvivoGen. TcdB toxin (6246-GT-020) was purchased from R&D systems. DSS crosslinker (Disuccinimidyl suberate, S1885) was purchased from Sigma-aldrich. Myc-DDK-tagged pCMV6 human CARD8 T60 construct, transcript variant 1, was purchased from Origene (RC230245). CARD8 V44I point mutation was introduced by using Q5 Site-Directed Mutagenesis Kit (E0554S, New England Biolabs). The primers used are as followings:
Forward: 5’- ATTGACAATAGCATAACGGGAA -3’ (forward);
Reverse: 5’- CAACAGTTTCCGTGATCCTTG -3’ (reverse).
Myc or Flag tag was deleted from pCMV6 constructs using Q5 Site-Directed Mutagenesis Kit, the primers used are as followings:
Myc deletion, forward: 5’-ATCCTGGATTACAAGGATGACGACGATAAGG-3’, reverse: 5’-CTCGAGCGGCCGCAGTACG-3’;
Flag deletion, forward: 5’-GTTTAAACGCGCAGCCGCG-3’, reverse: 5’-TGCCAGATCTCTCTTGCAGGAG-3’
pcDNA3.1-CARD8 T48 is provided by Dr. Tetsuo Kubota from Tokyo Medical and Dental University(5). CARD8 T48 was amplified by PCR and introduced into pCMV6 vector using Quick-Fusion Cloning kit (Biomake) with the primers as followings: forward: 5’-
AGATCTGCCGCGCGATCGCCATGATGACGACAGAGCGACGAG-3’, reverse: 5’-CTCGAGCGGCCGCAGTACGCTAAATTCTGCTGCTTAAAGATA-3’
**Human Cell Isolation**

PBMCs from both patients and healthy individuals were freshly isolated using LSM Lymphocytes separation medium (50494, MP Biomedicals). These Cells were subjected to human pan monocyte isolation kit (130-096-537, Miltenyi Biotec). The monocytes obtained were maintained in culture over night before use. All human cell isolation was approved by the Review Board of National Institutes of Health for human subject research.

**Whole Exome Sequencing**

We isolated DNA from whole blood using the salting-out method (Qiagen, Valencia, CA), followed by phenol chloroform extraction. We prepared whole genome libraries according to the KAPA HTP Library Construction Kit protocol (Kapa Biosystems), performed exome capture according to Nimblegen’s SeqCap EZ Library + UTR protocol, and sequenced with Illumina HiSeq 2500. We mapped reads for all samples to human GRCh37 reference with BWA (42) v0.7.13 using the default settings and marked duplicate reads with Picard (http://broadinstitute.github.io/picard). Next, we applied GATK (43) v3.5 base quality score recalibration, indel realignment, and performed SNP and INDEL discovery and genotyping across all samples simultaneously according to GATK Best Practices recommendations (44, 45). We normalized and decomposed variants with vt (46) v0.577, and annotated variants with variant effect predictor (47) v84. We filtered variants using GEMINI (48) v0.19.1 to obtain rare or novel variants with allele frequency < 0.1% according to 1000 Genomes, Exome Sequencing Project, and ExAC. While applying different genetic model filters with GEMINI, we also filtered variants to remove any where minimum heterozygous allelic balance was < 0.25 or with a “High” Gene Damage Index for general Mendelian diseases (http://lab.rockefeller.edu/casanova/GDI;(49)).

**Cell culture**
HEK293 cells were purchased from ATCC (ATCC® CRL-1573™) and were maintained in IMDM medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 1 mg/ml streptomycin. Human primary monocytes were maintained in RPMI 1640 medium containing 10% FBS, 100 IU/ml penicillin, 1 mg/ml streptomycin, and 50 μM β-mercaptoethanol. Human mDCs were allowed to differentiate in this RPMI 1640 medium mixture with recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/ml; Peprotech) and IL-4 (20 ng/ml; Peprotech) for 7 days. The cells were cultured in a humidified incubator with 5% CO₂ at 37°C. The purity of CD11C⁺ cells was higher than 80% when tested by flow cytometry (data not shown). These cells were used for inflammasome activation experiments.

**Inflammasome activation**

Human mDCs were primed for 6 hours with ultrapure LPS (100ng/ml, Invivogen) prior to inflammasome activation. The cells were then treated with ATP (5mM, 30 minutes), Nigericin (1μM, 30 minutes) or MSU (400ug/ml, 2 hours) to activate NLRP3 inflammasome. NLRC4 and AIM2 inflammasome were activated by transfection of flagellin (1ug/ml, 2 hours) or poly(dA:dT) (1ug/ml, 2 hours) using Lipofectamine LTX (15338100, ThermoFisher Scientific), respectively. TcdB toxin (1ug/ml, 2 hours) was added to culture medium to activate Pyrin inflammasome.

**Real time PCR**

Total RNA was extracted from the human primary mDCs by using RNaseq min kit (Qiagen, 74104). Reverse transcription of mRNA and synthesis of cDNA was performed using TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR was performed using the SYBR green quantitative PCR (qPCR) master mix (Toyobo) and the 7900HT Fast real-time PCR system (Applied Biosystems). Relative quantification of genes was achieved via normalization against β-actin. The primers used were the following:

GAPDH, 5’- ACGGATTTGGTCGTATTGGG-3’ (forward),
5’- CATGGGTGGAATCATATTGGGAAC-3’ (reverse);
Western Blots and Immunoprecipitation

HEK293 cells or human primary mDCs were incubated in lysis buffer containing 50 mM Tris (pH 7.5), 1% NP-40, 150 mM NaCl, and protease inhibitor cocktail. After centrifugation at 13,000 \( \times g \) for 15 min at 4°C, the supernatants were mixed with LDS loading buffer, heated at 100°C for 10 min and then separated via LDS electrophoresis. The proteins were transferred onto nitrocellulose membranes and blocked with 5% fat-free milk in 1× Tris-buffered saline containing 0.05% Tween 20 and then probed with the corresponding primary antibodies to detect mature IL-1β and pro-IL-1β (sc-7884; clone: H-153; Santa Cruz Biotechnology), NLRP3 (ALX-804-881; clone: cryo2; Enzo Life Sciences), ASC (sc-22514-R; clone: N-15; Santa Cruz Biotechnology), mature and pro-caspase-1 (sc-515; clone: C-20; Santa Cruz Biotechnology), CARD8 (sc-81213, clone: 2108C2a; Santa Cruz; NB100-56181, Novus biologicals), Myc tag(sc-40, clone: 9E10; Santa Cruz), Flag tag (F1804, clone: M2; Sigma-aldrich), phosphorylated NLRP3 (9621S, Cell signaling). For assay of mature caspase-1 and IL-1β assay, the cell culture supernatants were harvested and concentrated via the protein precipitation approach, with the harvested protein pellets mixed with SDS loading buffer for the detection of caspase-1 p10 and IL-1β p17. After incubation with appropriate
horseradish peroxidase-conjugated secondary antibodies, the immunoreactive bands were visualized using enhanced chemiluminescence reagent (K-12043-D10, Advansta).

**ASC, NLRP3 or CARD8 oligomerization detection**

The procedure for detecting ASC oligomerization was described previously (50). Briefly, human primary mDCs were primed with ultrp pure LPS (100 ng/ml) for 6 hours. The cells were then activated with ATP (5mM) for 30 minutes. The culture supernatants were removed and the cells were lysed with 0.2 ml of lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, and a protease inhibitor mixture. The cell lysates were allowed to go through a 21-gauge needle for 10 times and then centrifuged at 12,000 × g for 10 min at 4°C. The resultant insoluble cell debris was washed 3 times with PBS and resuspended in 500 μl of PBS. DSS crosslinker (0.8 mM, final concentration) was added to PBS containing the cell debris. The mixture was kept at 37°C for 30 minutes. The cross-linked cell debris was pelleted and solubilized in LDS loading buffer for Western blot detection of ASC oligomerization. To examine NLRP3 oligomerization, the mDCs were stimulated and lysed with above methods. The cell lysates were mixed with DSS crosslinker (0.8 mM, final concentration) and were incubated at 37°C for 30 minutes. Then, the cross-linked cell lysates were mixed with LDS loading buffer for Western blot. For detecting CARD8 oligomerization, CARD8 T60 or T48 was transfected into HEK293 cells. The cells were harvested 48 hours later, lysed and crosslinked using the method for NLRP3 crossling.

**ELISA.**

Serum samples were collected from CD patients and healthy controls, the IL-1β, IL-6 or TNF-α concentration was examined by enzyme-linked immunosorbent assay (ELISA; BD Biosciences). Supernatants of human PBMCs, primary monocytes, mDCs or HEK 293 cells were harvested, and IL-1β as well as IL-6 secretion were analyzed by ELISA. All the procedures were performed according to the manufacturers' instructions.
**Statistical Analysis.**

All the data are presented as mean ± SEM except where indicated otherwise. Statistical differences between two groups were analyzed using a two-tailed Students’ t test. The experiments with more than two groups of samples were analyzed using a one-way AVONA followed by Dunnett’s or Tukey’s post-hoc multiple comparisons. A P value of ≤0.05 was considered statistically significant.

**Study approval.**

Written Informed consents were obtained from all the patients and control individuals involved in this study. All procedures in this study were approved by the ethics committees of UH Cleveland medical center, the Childrens Hospital of Philadelphia, Stanford medical center and the National Institutes of Health.
Author Contributions: LM: Performance of studies and data analysis; writing of MS; AK: Planning and analysis of studies; MS and AO: Genetic analysis; LA, JK and AA: Clinical care of patients; MY: Clinical care of patients; KM: Collection of specimens and clinical care; IF: Clinical care of patients and planning/analysis of studies; MQ: Analysis of pathologic specimens; WS: Planning and analysis of studies; writing of MS.

Conflicts of interest: The authors disclose no conflicts.

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References


Figure 1. A Kindred with family members bearing a CARD8 Mutation and Crohn’s disease-like Intestinal Inflammation.

(A) A kindred containing three members bearing a CARD8 mutation; all these three members of the kindred, the Proband (Pt), his mother (Pt M) and his maternal aunt (Pt Aunt), the mutation occurred in association with a CD-like intestinal inflammation. (B) Macroscopic examination of the colon exhibited scattered areas of superficial erythema and ulceration having a lenticular pattern especially evident in the rectosigmoid region. N=3/group. (C) Biopsies from the terminal ileum (TI) and colon. a: Index patient colon 4X. Colitis with epithelial erosive changes and inflammation, significant crypt and goblet cell loss with regenerative changes; features consistent with GvHD; b, Index patient colon 20X. Colitis with rare residual gland showing goblet cell loss, repair changes and rare apoptotic bodies (arrow indicated); c, Index patient TI 4X. Ileitis with focal erosion, villi loss,
lymphocytic infiltrates, severe crypt drop-out and repair changes.; d, Index patient TI 10X. Chronic active ileitis with regenerative changes and poorly formed granulomas including giant cells; e, Index patient TI 20X. Poorly formed granuloma (arrow indicated) with giant cells; adjacent glands with repair/regenerative changes; f, Aunt TI 4X. Transmural lymphocyte infiltration with well formed granuloma present; g, Aunt TI 10X. Well formed granuloma (arrow indicated). N=3/group. (D) Anakinra therapy resulted in rapid clinical improvement marked by decreased fecal calprotectin levels. Data are representative of three independent experiments. (E) Whole exome sequencing revealed a CARD8 V44I mutation in one allele of chromosome 19 (see sequencing data in the text); the mutation site of V44I was present on the CARD8 T60 isoform, but not the “canonical” T48 isoform.
Figure 2. The CARD8 V44I Mutation is Associated with Enhanced IL-1β Production and Increased NLRP3 Inflammasome Activation.

(A) HEK293 cells were transfected with plasmids carrying human NLRP3 together with CARD8 T48 or T60 or empty vector (EV) and 24 hours later transfected with plasmids carrying ASC, caspase-1 and pro-IL-1β (to allow assembly of the NLRP3 inflammasome); 24 hours later the cells were stimulated with nigericin (NI) for 30 minutes following which culture supernatants were collected for IL-1β ELISA assay. Data are shown as the means ± SEM. **p<0.01 (One-way ANOVA with Dunnett’s post-hoc test) (B) Serum samples from the Proband (with a CARD8 V44I mutation, Pt) and age- and sex-matched healthy control individuals (H) were collected and subjected to IL-1β and IL-6 ELISA assays. (C) Proband (Pt) and Control (H) PBMCs and monocytes were cultured for 24 or 48 hours without stimulation after which supernatants were subjected to IL-1β ELISA assay. Data are shown as means ± SEM, n=3. **p<0.01; two-tailed student’s t-test. (D) Proband (Pt) and control (H) mDCs were primed with LPS (100ng/mL) for 6 hours and stimulated with ATP (5mM) or NI (1.2µM) for 30 minutes after which culture supernatants were collected and assayed for IL-1β and IL-6 by ELISA. Data are shown as means ± SEM, n=3. **p<0.01; two-tailed student’s t-test. (E) Western blot detection of...
mature IL-1β and mature caspase-1. Data for ELISA are representative of three independent experiments, data for Western blot are representative of two independent experiments.
Figure 3. The CARD8 Mutation Affects NLRP3 and AIM2 Inflammasome but not Pyrin and NLRC4 Inflammasome Activation

mDCs from the Proband (Pt) and a healthy control (H) were primed with LPS (100ng/ml, 6 hours) and then stimulated with ATP (5mM, A), poly(dA:dT) (1µg/ml, 2 hours, B), TcdB (1µg/ml, 2 hours, C) or flagellin (1µg/ml, 2 hours, D) to activate the NLRP3, AIM2, Pyrin or NLRC4 inflammasomes, respectively. Culture supernatants were collected and subjected to IL-1β (A-D) and IL-6 (E) assay by ELISA. Primary mDCs from Proband (Pt) and healthy control (H) were treated with or without LPS (100ng/mL) for 6 hours after which the cells were harvested and subjected to RNA extraction. qRT-PCR of the extracted RNA was performed to determine the expression of ASC (F), caspase-1 (G), NLRP3 (H) and pro-IL-1β (I). Data in are shown as means ± SEM, n=3. **p<0.01; *p<0.05. Two-tailed student's t-test. All the data are representative of three independent experiments.
Figure 4 CARD8 with a V44I Mutation Exhibits Reduced Binding to NLRP3 and Affects NLRP3 Inflammasome Assembly

(A) Plasmids expressing intact CARD8, CARD8 V44I mutation or empty vector (EV) were transfected into HEK293 cells along with a plasmid expressing NLRP3. At 48 hours after transfection, the cells were harvested and cell lysates were subjected to immunoprecipitation using anti-CARD8 antibody followed with immunoblotting. (B) mDCs from Proband (Pt) and healthy control (H) were stimulated with LPS (100ng/ml, 6 hours) or LPS plus nigericin (1.2µM, 30 minutes, NI); the cells were lysed and lysates were subjected to immunoprecipitation and immunoblotting. (C) HEK293 cells were transfected with a plasmid expressing NLRP3 along with myc-tagged intact CARD8 T60, CARD8 T60 P102I or empty vector; the cells were lysed at 48 hours and the lysates were subjected to immunoprecipitation using anti-myc antibody and followed by immunoblotting. (D) HEK293 cells were transfected with NLRP3 plasmid along with myc-tagged intact CARD8 T60, CARD8 T60 P102I or empty vector; 24 hours later the cells were then transfected with plasmids expressing ASC, caspase-1 and pro-IL-1β to allow assembly of the NLRP3 inflammasome. Another 24 hours later the cells were stimulated with nigericin (1.2µM, 30 minutes, NI); the cultural supernatants were examined for IL-1β concentration by ELISA. Data are shown as the means ± SEM. *p<0.05, ns: no significant (One-way ANOVA with Dunnett’s post-hoc test) (E) mDCs from Proband (Pt) and a healthy control (H) were stimulated with LPS (100ng/ml, 6 hour) plus nigericin (1.2µM, 30 minutes); the cells were treated with the method shown
in method section for Western blot. (F) The cell lysates in E were treated with the method shown in methods section for Western blot. Data for ELISA are representative of three independent experiments, data for Western blot are representative of two independent experiments.
Figure 5. Mutant CARD8 Exerts a Dominant Negative effect on the NLRP3 Inflammasome

(A) HEK293 cells were transfected with plasmids expressing intact and mutant CARD8 T60 alone or together (half of the amount per plasmids, to mimic the heterozygous status of the Proband); after 48 hours of incubation cell lysates were obtained and subjected to immunoprecipitation and immunoblotting to determine CARD8 binding to NLRP3.  

(B) HEK293 cells were transfected with NLRP3 and flag-tagged CARD8 T48 plasmids along with intact or mutant CARD8 T60 plasmids; after 48 hours cell lysates were obtained and subjected to immunoprecipitation (using anti-CARD8 antibody) and immunoblotting.  

(C) HEK293 cells were transfected with NLRP3- and CARD8 T48 plasmids along with intact or mutant CARD8 T60 plasmids; after 48 hours cell lysates were obtained and subjected to immunoprecipitation (using anti-NLRP3 antibody) and by immunoblotting.  

(D) HEK293 cells were transfected with NLRP3, intact CARD8 T48 or T60 alone or together with mutant CARD8 T60 plasmids, 24 hours later the cells were transfected with ASC, caspase-1 and pro-IL-1β plasmids to allow the assembly of
NLRP3 inflammasome. 24 hours later the cells were stimulated with nigericin (1.2μM) for 30 minutes. Cultural supernatants were collected for IL-1β ELISA assay. Data are shown as means ± SEM. *p<0.05. One-way ANOVA with Tukey’s post-hoc test. Data for ELISA are representative of three independent experiments, data for Western blot are representative of two independent experiments.
Figure 6. Mutant CARD8 T60 Disrupts Interaction Between T48 and NLRP3

(A) HEK293 cells were transfected with flag-tagged CARD8 T48 and with myc-tagged intact or mutant CARD8 T60; after 48 hours cell lysates were obtained and subjected to immunoprecipitation (with anti-flag antibody) followed by immunoblotting. (B) HEK293 cells were transfected with Myc-tagged CARD8 T60 along with flag-tagged intact or mutant CARD8 T60 plasmids; after 48 hours cell lysates were obtained and subjected to immunoprecipitation (using anti-myc antibody) followed by immunoblotting. (C) HEK293 cells were transfected with flag-tagged intact or mutant CARD8 T60 plasmids; after 48 hours cell lysates were obtained and treated with DSS crosslinker and then with LDS loading buffer for Western blot. (D) HEK293 cells were transfected with flag-tagged intact CARD8 T60 plasmid alone or together with a mutant CARD8 T60 plasmid; after 48 hours cell lysates were obtained and treated with DSS crosslinker and then with LDS loading buffer for Western blot. (E) HEK293 cells were transfected with a flag-tagged CARD8 T48 along with intact or mutant CARD8 T60 plasmids: after 48 hours cell lysates were obtained and treated with DSS crosslinker and then with LDS loading buffer for Western blot. (F) Diagram showing that mutant CARD8 T60 exhibits increased binding with intact T60 and T48 and this binding is thought to block interaction between intact CARD8 T60 or T48 with NLRP3. All the data are representative of two independent experiments.
Figure 7. The CARD8 V44I Mutation Results in Reduced NLRP3 Serine Phosphorylation, as well as Reduced K63 and K48 Polyubiquitination

(A) HEK293 cells were transfected with human NLRP3, ASC, caspase-1, and either intact or mutant CARD8 T60 plasmids. After 48 hours the cells were stimulated with or without nigericin (1.2µM, 30 minutes). The cell lysates were obtained and subjected to immunoprecipitation (with anti-NLRP3 antibody) and immunoblotting with anti-phosphoserine antibody. (B) mDCs from the Proband (Pt) and a control individual (H) were pre-treated with LPS (100ng/ml, 6 hours) and then activated with nigericin (1.2µM, 30 minutes, NI). The cell lysates from these cells were subjected to immunoprecipitation and immunoblotting as described above. (C) HEK293 cells were transfected with NLRP3, ASC, Caspase-1 plasmids as well as intact or mutant CARD8 T60 plasmids together with constructs expressing K63 or K48 ubiquitin chains; 48 hours later cell lysates were obtained and subjected to immunoprecipitation (with anti-NLRP3 antibody) and Western blot to examine the polyubiquitination of NLRP3. The top row image was spliced from images obtained from the same gel but different exposure time. All the data are representative of two independent experiments.
Figure 8. IL-1β Antibody Treatment reduced peripheral cytokine levels In Proband Bearing a CARD8 V44I Mutation

Seven serum samples from the proband at various time points were collected and subjected to assays of IL-1β (A), IL-6 (B) and TNF-α (C) concentrations by ELISA. Data in A, B and C are shown as means ± SEM. Serum samples from patients (Pt) with active CD without CARD8 V44I mutations and healthy individuals was collected and subjected to assays of the concentration of IL-1β (D), IL-6 (E) and TNF-α (F) by ELISA. CD patients, N=4; Control, N=5. Data in D, E and F are shown as means ± SEM **p<0.01. two-tailed Student’s t-test. All the data are representative of three independent experiments.
Supplemental Materials

Patients’ case histories

Proband:
The Proband is a 16-year-old male residing in Cleveland, OH who was admitted to UH Medical Center on 8/2014 with abdominal pain and bloody diarrhea with greater than 2 liters of liquid stool output/day of 2 months duration. EGD/colonoscopy disclosed macroscopic pancolitis and microscopic esophagitis, gastritis, duodenitis, and terminal ileitis. The patient exhibited a partial response to treatment with high dose steroids and 5-ASA with mild decrease in liquid stool output, but relapsed upon weaning of the steroids despite addition of imuran. Subsequent treatment with anti-TNF-α (Remicade) was also unable to bring about remission and the patient was transferred to Children’s Hospital of Philadelphia for further management. At this site, the patient’s abdominal pain and bloody diarrhea persisted despite an increase in the Remicade dose to 10 mg/kg every 4 weeks concomitant to high doses of IV steroids. In addition, patient had significant protein losing enteropathy as evident of Albumin levels of 1.5-2.5 requiring albumin infusions and TPN supplementation. Repeat EGD/colonoscopy at this point revealed markedly increased intestinal inflammation with evidence of pancolitis as well as macroscopic duodenitis and ileitis. Macroscopic examination of the colon exhibited scattered areas of superficial erythema and ulceration having a lenticular pattern especially evident in the rectosigmoid region; this was associated with colonic and ileal friability (Figure 1B). Biopsies from the terminal ileum (TI) and colon showed the presence of a severe inflammatory mononuclear infiltrate associated with loss of villous pattern and apoptosis of epithelial cells; this was thought to be similar to intestinal inflammation sometimes associated with GvHD (Figure 1C a-e). In view of continued refractory disease, additional opinion as to appropriate therapy was sought from NIH clinicians. Because of the presence of certain histologic features sugestive of GvHD, NIH clinicians advised treatment with anakinra, an IL-1β receptor antagonist and such treatment was subsequently instituted(1). However, prior to such therapy, the patient had been started on Vedolizumab and had received 2 induction doses without significant improvement. Anakinra therapy resulted in rapid
clinical improvement marked by decreased in stool output to < 500 cc/day and improved albumin level (no longer requiring albumin infusions). This led to shortly thereafter transfer back to UH Medical center where repeat histology examination revealed decrease in inflammatory process without evidence of GvH disease but that of routine Crohn’s disease (Figure 1 Cd-e). He was maintained on Anakinra and Vedolizumab with further improvement as correlated by improvement in albumin levels and change in stool output to semi-formed stools. Subsequent to discharge from hospital the patient was followed at NIH where he was transitioned from Anikinra to Canakinumab (anti-IL-1β monoclonal antibody). On this therapy stools remained formed and free of blood/mucous; in addition, abdominal symptoms of pain/cramping markedly improved. Patient remained on Canakinumab q 4 weeks and Vedolizumab q 7-week therapy. Of note the patient was recently diagnosed with avascular necrosis (AVN) of the hip after developing a limp upon ambulation. The latter was thought to be due to prior high dose steroid treatment. In follow-up a repeat colonoscopy in May, 2017, disclosed a left and transverse colon area of persistent microscopic inflammatory changes requiring an increase in Canakinumab dose from 3 to 4 mg/Kg/dose q 4 weeks and decrease interval dosing of Vedolizumab to q 4 weeks. At present the patient is predominately free of GI symptoms and enjoys normal growth. Laboratory correlates reveal a normalization of albumin levels (3.5-4.1) and as shown in Figure 1D fecal calprotectin and serum IL-1β levels have marked improved on corresponding therapy.

The Proband’s Mother:
The mother of the Proband is a 55-year-old female residing in Cleveland, OH. In 2014, she presented with a one month history of abdominal pain, weight loss and diarrhea. These symptoms prompted an abdominal CT scan which revealed marked circumferential wall thickening of small bowel corresponding to loop of ileum. However, she did not undergo colonoscopic evaluation due to concern that this might cause bowel perforation. She was then administered oral prednisone and on this treatment obtained cessation of abdominal pain and diarrhea symptoms over 2-3 month time period. Patient was then transitioned to budesonide therapy for one year duration with follow-up daily mesalamine treatment. Subsequent MRE studies demonstrate no acute GI illness.
The Proband’s Maternal Aunt:
The maternal aunt of the proband is a 48 y.o female who presented at age 21 with abdominal pain, vomiting, and mild diarrhea. She was found to have both proximal and distal ileal strictures suggesting the presence of CD, but had no colonic disease. She was treated with, but did not improve on, 5-ASA or prednisone therapy and subsequently underwent a distal ileal resection (resulting in removal of ≈30 cm of small bowel) and a stricturoplasty of the proximal stricture. The histology of the resected tissue revealed small intestinal mucosa with acute inflammation, mucosal erosions and occasional granuloma, all consistent with CD (Figure 1C f-g).

In 1993, she again developed recurrent obstructive GI symptoms and at this point was treated with 6-MP. However, no improvement was observed and underwent a second resection (resulting in loss of an additional ≈30 cm of bowel due to the occurrence of an ileal stricture. She remained on 6-MP post-operatively and concomitantly received IV iron infusions for severe iron deficiency anemia. She was not considered a candidate for anti-TNF-α therapy because of a history of a positive PPD in childhood that was treated with INH. Obstructive symptoms recurred in 2006 that once again led to a bowel resection secondary to stricture formation. Colonoscopy at that point revealed anastomotic recurrence but no colonic disease. Approximately 6 cm from the ileocecal valve there appeared to be a stricture 3-4 mm in diameter with ulcerations on the proximal side of the stricture. Ileal mucosa was extremely friable.

In between surgical procedures she has been relatively pain-free and maintained a stable weight; however, she has 4-5 loose watery stools/day. 6-MP was discontinued in 2008 while she was pregnant and breast feeding. She has not been on any medications since then but has been treated with acupuncture and probiotics.

In June 2016, she again experienced nausea and abdominal pain without emesis, or weight loss. Currently, she continues with 4-5 loose BMs/day (her baseline since first surgery) associated with occasional bloating. These symptoms improve with probiotics. Her weight remains stable and she has no extra-intestinal symptoms. Recent
colonoscopy revealed acute inflammation at the anastomosis site associated with histiocytic aggregates and mucosal erosions. No stricture formation noted.

Supplemental references:


Supplemental Figures:

Supplemental Figure 1. Whole Exome Sequencing of Proband DNA Identified CARD8 To Be a Potential Damaging Gene in The Proband with CD

Sequencing of proband DNA was initially performed at the Children’s Hospital of Philadelphia (CHOP) and revealed a single allele (heterozygote) CARD8 c.130G>A (V44I) mutation that was not predicted to be located in a functional domain. Subsequently, DNA from the blood of the patient and the patient’s mother and father were subjected to whole exome sequencing at NIH. 10x sequencing coverage for 89.9%, 90.2%, and 90.7% of coding regions in proband, mother and father was obtained, respectively. After applying various filters for variant quality, population allele frequency, variant impact and inheritance models, 87 variants in the proband were obtained. Of these, there was a single variant following the X-linked recessive model, 2 variants within the homozygous recessive model, and zero de novo variants passing filters. The X-linked recessive variant was a missense variant in DCAF12L1, predicted to be benign/tolerated by PolyPhen (2) and SIFT (3), with a low CADD-phred score (4) of 1.5. The two homozygous recessive variants were missense and inframe insertion in TROVE2, and LNP1 genes, respectively.
None of these three candidates were interesting based on predicted deleteriousness scores or gene function. Since the patient’s maternal aunt also carried a diagnosis of CD, 84 heterozygous variants common to the patient and the aunt were identified. Among these, the variant chr19:48741719 C>T (hg19) in CARD8 stood out because of its role in the inflammasome(5, 6). It was predicted to be possibly damaging/deleterious for CARD8.
Supplemental Figure 2. The CARD8 Variant chr19:48741719 C>T (hg19) Overlaps Multiple Transcripts of CARD8

Multiple transcripts overlapped by the CARD8 variant chr19:48741719 C>T (hg19) identified in whole exome sequencing data were shown. The possible outcome of the variant could be in the intron, in the 5' UTR, or else a V44I missense mutation.
Supplemental Figure 3. CARD8 Variant chr19:48741719 C>T Does Not Affect CARD8 Transcription and Protein Expression

Primary mDCs from Proband carrying the variant chr19:48741719 C>T (Pt) and healthy control (H) were treated with or without LPS (100ng/ml) for 6 hours and then were harvested for RNA or protein extraction. (A) qRT-PCR was performed to examine the expression of CARD8 T60 at the mRNA level. Data are shown as means ± SEM. (B) Western blot was performed to examine CARD8 T60 protein. Data in A are representative of three independent experiments, data in B are representative of two independent experiments.
Supplemental Figure 4. PCR Product Sequencing Confirmed CARD8 and MEFV Mutations in the Proband, His Mother and Aunt

mDCs from the Proband, his mother and aunt were treated with LPS (100ng/ml) for 6 hours and subjected to mRNA extraction. RT-PCR was performed to amplify CARD8 or MEFV. The PCR products were then subjected to DNA sequencing. The mutation sites in CARD8 T60 (upper panel) and MEFV (lower panel) were highlighted. Data are representative of three independent experiments.
Supplemental Figure 5. CARD8 Bearing the V44I Mutation was Expressed at a Substantial Level in the Proband’s Small Intestine and Colonic Tissues

Primary monocytes from Proband (Pt) and healthy control (H) were treated with or without LPS (100ng/ml) for 6 hours and then were harvested for mRNA or protein extraction. (A) Colon and duodenum biopsies from the patient was subjected to mRNA or protein extraction. qRT-PCR was performed to examine the expression of CARD8 T60. Data are shown as means ± SEM. (B) Western blot was performed to examine the protein expression of CARD8 T60 and T48. Data in A are representative of three independent experiments, data in B are representative of two independent experiments.
Supplemental Figure 6. The mDCs from the Proband Display Normal Pyrin Inflammasome Activation

(A) Primary mDCs from the Proband (Pt), the Proband's maternal aunt (Pt Aunt), both carrying CARD8 V44I mutation, and a healthy control individual (H) were treated with or without LPS (100ng/ml) for 6 hours and then were harvested for mRNA extraction; mRNA was then subjected to qRT-PCR to determine the transcription of pyrin. (B-E) LPS (100ng/ml, 6 hours) primed mDCs from the Proband, the Proband's aunt and H were stimulated with TcdB (1µg/ml) for 2 hours to activate pyrin inflammasome. The cultural supernatants were collected and subjected to assays of IL-1β (B, D) and IL-6 (C, E) by ELISA. Data are shown as means ± SEM. ns: not significant. Two-tailed student's t-test. All the data are representative of three independent experiments.
Supplemental Figure 7 Over production of IL-18 by peripheral monocytes from Proband patient with CARD8 mutation

mDCs from the Proband’s patient (Pt) and a healthy control individual (H) were treated with LPS (100ng/ml) for 6 hours and then stimulated with ATP (5mM, 30 minutes) or nigericin (1.2µM, 30 minutes, NI). The cultural supernatants were collected and then subjected to assays for IL-1β by ELISA. Data are shown as means ± SEM. **p<0.01. Two-tailed student's t-test. All the data are representative of three independent experiments.
Supplemental Figure 8. Over-Activation of the NLRP3 Inflammasome in mDCs from the Proband’s Aunt

mDCs from the Proband’s aunt (Pt aunt) carrying the CARD8 V44I mutation and a healthy control individual (H) were treated with LPS (100ng/ml) for 6 hours and then stimulated with ATP (5mM, 30 minutes) or nigericin (1.2µM, 30 minutes, NI), poly(dA:dT) (1µg/ml, 2 hours), flagellin (1µg/ml, 2 hours) or TcdB (1µg/ml, 2 hours) to activate the NLRP3, AIM2, NLRC4 or pyrin inflammasomes, respectively. The cultural supernatants were collected and then subjected to assays for IL-1β (A, B) and IL-6 (C, D) by ELISA. Data are shown as means ± SEM. *p<0.05; **p<0.01. Two-tailed student's t-test. All the data are representative of three independent experiments.
Supplemental Figure 9. Over-Activation of the NLRP3 Inflammasome in mDCs from the Proband’s Mother

mDCs from the Proband (Pt), his mother (Pt M) and a healthy control individual (H) were treated with LPS (100ng/ml) for 6 hours and then stimulated with ATP (5mM, 30 minutes) or nigericin (1.2µM, 30 minutes, NI), poly(dA:dT) (1µg/ml, 2 hours), flagellin (1µg/ml, 2 hours) or TcdB (1µg/ml, 2 hours) to activate the NLRP3, AIM2, NLRC4 or pyrin inflammasomes, respectively. The cultural supernatants were collected and then subject to assays of IL-1β (A, B) and IL-6 (C, D) by ELISA. Data are shown as means ± SEM. *p<0.05; **p<0.01. One-way ANOVA with Tukey’s post-hoc test. All the data are representative of three independent experiments.