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Blau Syndrome–Associated Nod2 Mutation Alters Expression of Full-Length NOD2 and Limits Responses to Muramyl Dipeptide in Knock-in Mice

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The biochemical mechanism by which mutations in nucleotide-binding oligomerization domain containing 2 (NOD2) cause Blau syndrome is unknown. Several studies have examined the effect of mutations associated with Blau syndrome in vitro, but none has looked at the implication of the mutations in vivo. To test the hypothesis that mutated NOD2 causes alterations in signaling pathways downstream of NOD2, we created a Nod2 knock-in mouse carrying the most common mutation seen in Blau syndrome, R314Q (corresponding to R334Q in humans). The endogenous regulatory elements of mouse Nod2 were unaltered. R314Q mice showed reduced cytokine production in response to i.p. and intravitreal muramyl dipeptide (MDP). Macrophages from R314Q mice showed reduced NF-κB and IL-6 responses, blunted phosphorylation of MAPKs, and deficient ubiquitilation of receptor-interacting protein 2 in response to MDP. R314Q mice expressed a truncated 80-kDa form of NOD2 that was most likely generated by a posttranslational event because there was no evidence for a stop codon or alternative splicing event. Human macrophages from two patients with Blau syndrome also showed a reduction of both cytokine production and phosphorylation of p38 in response to MDP, indicating that both R314Q mice and cells from patients with Blau syndrome showed reduced responses to MDP. These data indicate that the R314Q mutation when studied with the Nod2 endogenous regulatory elements left intact is associated with marked structural and biochemical changes that are significantly different from those observed from studies of the mutation using overexpression, transient transfection systems.

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dominant disorder characterized by granulomatous inflammatory arthritis, dermatitis, and uveitis (12). Mutations associated with Blau syndrome are located in the NOD domain of NOD2, and at least 17 different mutations have been identified (12, 13). Tran- sient transfection assays performed in vitro using plasmids with powerful promoters that overexpress NOD2 have found that mutations associated with Blau syndrome cause excessive NF-κB and MAPK activation compared with the wild-type (WT) form of NOD2, and this has led to the hypothesis that Blau syndrome is the result of a gain of function of Nod2 (14). However, this gain-of- function hypothesis has not been supported when clinical specimens from patients with Blau syndrome were analyzed (12, 15–17). PBMCs were not found to have spontaneous release of cytokines, nor were inflammatory responses observed when PBMCs were stimu- lated with MDP. Moreover, the degree of enhanced Nod2 function seen in vitro does not correlate with the severity of the disease in patients (16). To clarify the mechanism by which Blau syndrome– associated mutations alter the function of Nod2, we sought to de- velop a model not based on overexpression of the gene in vitro.

In this study, we created a Nod2 knock-in (KI) mouse by ho- logomologous recombination. The mice will be referred to as R314Q mice because they carry one of the most common genetic variants (R314Q, the ortholog of R334Q in humans) associated with Blau syndrome. The regulatory elements for Nod2 expression were not altered by this approach. In this article, we show that the R314Q mutation does not lead to a gain of function of NOD2. Rather, the mutation leads to a truncated form of NOD2 and altered cytokine and intracellular signaling responses to MDP both in vivo and in vitro. Macrophages from patients with Blau syndrome also showed reduced cytokine production and intracellular signaling in response to MDP.

Materials and Methods

Construction of the R314Q-Nod2 KI mice

A bacterial artificial chromosome (Children’s Hospital Oakland Research Institute, Oakland, CA) containing the murine Nod2 genomic locus was modified by recombineering to create the specific point mutation (G-to-A) change within codon 314 of Nod2 leading to an arginine (R)-to-glutamine (Q) amino acid change (codon number as per UniProtKB/Swiss-Prot: Q8K3Z0.1, the 1020-aa-long isoform of murine NOD2). A second round of recombineering was performed to insert a neomycin resistance gene (Neo) flanked by FLIPPASE recognition target (FRT) sequences in a region of intron 3 showing low homology across several species. A BglII restriction site was placed 5′ to the first FRT site for use in screening. Subcloning from the mutated BAC clone was performed in pBluescript to create a construct with the point mutation, neomycin resistance, and “homology arms” 4 kb on either side of the mutation (Fig. 1A, targeting vector). Using the University of Michigan Transgenic Animal Model Core Facility, the construct was electroporated into embryonic stem cells and cloned on soft agar containing the selection agent, G418. Successful ho- logomologous recombination creating an intermediate locus (5.1 kb) was distinguished from the WT locus (3.8 kb) by Southern blotting with BglII digestion and the indicated probe (Δ) (Fig. 1A). A clone containing the mutation was used for homologous injection and embryonic stem cell–mouse chimeras were produced. After additional breeding to ensure germline transmission, offspring were bred on an Flp recombinase strain to remove the Neo gene detected by the presence of a 3.1-kb BglII fragment (Fig. 1A, final locus), and then for 10 generations on C57BL/6 (Jackson Laboratory, Bar Harbor, ME) to create R314Q heterozygous mice (+/m). WT (+/+) litter mates were used as controls for all studies. Brother-sister mating was used to create homozygous R314Q mice (m/m). The genotypes of the final lines were confirmed by Southern blot analysis (Fig. 1B) and sequence analysis of genomic DNA (Fig. 1C). Genotyping of +/+ vs. m/m, and m/m lines was routinely performed by PCR screening of genomic DNA amplified by PCR (Taq DNA Polymerase with Thermopol Buffer; New England Biolabs) using primers: CATCAGAATTTGCACTGGAGA and primer; GCAGGAATATAGCCGGGAAAC. The primers span intron 3, giv- ing a WT amplicon that results in a 221-bp fragment and a mutant amplicon that results in a 254-bp fragment because of the presence of the FRT se- quence remaining after FLP-FRT recombination. Amplicons were analyzed by electrophoresis in a 3% agarose gel. Genotypes were also confirmed every third generation by Southern blot analysis of genomic DNA digested with BglII, electrophoresed in 1% agarose, transferred to a nylon membrane (Roche), cross-linked, and then hybridized with a digoxigenin-labeled probe and developed as per the protocol recommended by the manufac- turer (Roche). The digoxigenin-labeled probe corresponds to a region of exon 4 in Nod2 (Fig. 1A, top) and was generated by PCR (primer sequence available upon request). The R314Q locus yields a 3.1-kb restriction frag- ment, whereas the WT locus produces a 3.8-kb restriction fragment (Fig. 1B). NOD2-deficient mice (−/−) were purchased from Jackson Laboratory. Mice were maintained under specific pathogen-free conditions at the Portland VA Medical Center. All animal studies were performed under protocols approved by the Institutional Animal Care and Use Commit- tee of the Portland VA Medical Center.

Real-time and RT-PCR

RNA was isolated (RNaseasy; Qiagen, Valencia, CA) from bone marrow– derived macrophages (BMDMs) and cDNA was synthesized (Superscript VILO; Invitrogen, Grand Island, NY). Real-time PCR for Nod2 and β-actin (Actb) mRNA levels were then performed with iQ Sybr-Green Supermix (BioRad, Hercules, CA) and analyzed according to comparative cycle threshold method using Actb as the endogenous control plus comparing the range of expression to untreated cells from WT mice. RT-PCR to screen for splice variants was also performed by analyzing cDNA from +/+ vs. +/m, and m/m macrophages, and looking for changes in PCR size indicating a de- rection-based recognition sequence (Fig. 2). SDS-PAGE and protein transfer to PVDF membrane was performed using the manufacturer’s recommended proto- cols (Invitrogen, Grand Island, NY). Immunoblotting was performed with Abs to mouse IκBα, p-ERK, p-JNK, mouse p38, mouse p-p38 (Cell Sig- naling, Danvers, MA), mouse β-actin (Abcam, Cambridge, MA), ubiquitin, RIP2 (Santa Cruz Biotechnology, Santa Cruz CA), and mouse NOD2 (clone 26nNOD2; ebioscience, San Diego, CA) in PBS plus 5% nonfat milk. HRP-conjugated secondary Abs (Santa Cruz Biotechnology) were detected by ECL (Pierce, Rockford, IL).

Macrophage generation and stimulation

Murine macrophages were generated from bone marrow cells with 30% L929 conditioned media as previously described (18). After 7 d, BMDMs were treated overnight with either 100 μg/ml polyinosinic-polycytidylic acid (poly(I:C); Invivogen, San Diego, CA), 10 μg/ml MDP (Bachem), or for 6 h with 10 ng/ml IFN-γ. Human macrophages were generated from monocytes from PBMCs (obtained under a protocol approved by the Institu- tional Review Board of the DuPont Hospital for Children because viably frozen until use) from two patients with familial Blau syndrome (a mother and son carrying an R334W mutation). The mother (Blau-1), age 42 y, and son (Blau-2), age 15 y, had onset of disease at ages 2 and 1 y, respectively. Blau-1 had been treated with infliximab in the past, and was taking methotrexate and alendramin to control eye disease and arthritis. Blau-2 had been on anakinra in the past and was on daily methylprednisolone, azathioprine, and cyclosporine to control active eye disease. His arthritis was not active. Control macrophages were from viably frozen PBMCs from healthy individuals. PBMCs were incubated in RPMI 1640 containing 20% type AB Normal Human Serum (Atlanta Biologicals, Flowery Branch, GA) and 100 ng/ml GM-CSF (R&D Systems) for 5 d. After 5 d, nonadherent cells were discarded and the attached cells were treated with poly(I:C) (100 μg/ml), MDP (100 μg/ml), or LPS (100 ng/ml, Escherichia coli O55:B5 [Sigma-Aldrich]) for cytokine studies or MDP alone (100 μg/ml) for MAPK activation as described in Fig. 5.

Short interfering RNA

Approximately 4 × 10⁸ BMDMs from +/+, +/m, and −/− mice were resuspended in 100 μl sterile OptiMem (Invitrogen) plus 200 pmol ON-TARGETplus Mouse NOD2 SiRNA (257632; Thermo Scientific) or SiGenome Non-Targeting SiRNA Pool 1 (Thermo Scientific). After gently mixing, the cells were pulsed in a Gene Pulser II electroporation apparatus (400 V and 150 μF resistance; BioRad) and cultured for 2 d. BMDMs were then treated with poly(I:C) for 24 h, lysed, and then analyzed by Western blot for levels of NOD2 and β-actin protein.
Results

R314Q-KI mice are refractive to MDP challenge in vivo

To better understand the mechanism by which mutations of NOD2 cause alterations in NOD2-signaling pathways, we constructed a KI mouse wherein the most frequent mutation observed in Blau syndrome (R334Q) was inserted into the Nod2 locus of C57BL/6J mice at the orthologous location (R314Q) in exon 4. An important aspect of the homologous recombination approach taken in this study was that this allowed the endogenous regulatory elements of the mouse Nod2 gene to remain intact and unaltered (Fig. 1A). Southern blot analysis of genomic DNA confirmed the successful recombination of the WT allele (“+”) and mutated (“m”) allele as detected by the 3.8- and 3.1-kb fragments, respectively (Fig. 1B). As shown in Fig. 1C, genomic sequencing further identified the introduction of the point mutation (G to A) within codon 314 of the Nod2 gene, which led to an arginine (R)-to-glutamine (Q) amino acid substitution. The KI mice were backcrossed 10 generations onto C57BL/6J background and survived normally and showed the expected Mendelian inheritance pattern for the mutation. We did not observe any gross abnormalities of the skin, inflammatory changes in the eyes, or cathespin activation indicative of arthritis in mice that were housed in specific pathogen-free conditions and examined up to 36 wk of age (Supplemental Fig. 1). The cellularity of peripheral blood (WBCs, lymphocytes, mononuclear cells, granulocytes), as well as albumin, alkaline phosphatase, alanine aminotransferase, amylase, blood urea nitrogen, calcium, creatinine, globulin, glucose, potassium, sodium, phosphorus, total bilirubin, and total protein were comparable in +/+ and m/m mice (data not shown).

To study the effect of the R314Q mutation on systemic responses, we tested the in vivo inflammatory response of KI mice to an MDP challenge. Compared with WT mice that responded to MDP with a 3-fold increase in IL-6, the addition of MDP to KI mice did not alter basal IL-6 levels (Fig. 2A). NOD2 knockout (KO) mice also did not respond to MDP. A similar pattern was observed for KC (murine IL-8 homolog) and MIP2α, other commonly measured indicators of MDP-induced NOD2 activation (27, 28). We did not observe any basal changes in the levels of IL-6, MIP2α, and KC among the three genotypes, which argues against a gain of function induced by R314Q as measured by a systemic response in vivo. These results indicate that the R314Q-NOD2 mutation results in a loss of function of NOD2 to respond to MDP in vivo.

To further examine the consequences of the R314Q mutation on inflammatory responses in vivo, we administered mice an intracocular injection of MDP (Fig. 2B), which we had previously shown elicits an acute ocular inflammation and measurable cytokine response (26). In WT (+/+ ) mice, MDP injection results in a significant increase in KC production compared with control, saline-injected eyes (Fig. 2B). In contrast with the WT mice, +/m and m/m mice failed to respond to MDP through production of KC (or IL-6, data not shown), thereby indicating an inability to respond to MDP.

R314Q-NOD2 mutation results in loss of macrophage responsiveness to MDP challenge

We further sought to investigate whether the loss of MDP responsiveness at the systemic level coincides with functional changes at the cellular level. Poly(I:C) primed BMDMs were stimulated with MDP, and the production of IL-6 was measured among the three genotypes and KO mice (Fig. 3A). Because the level of NOD2 expression is relatively low in BMDM, poly(I:C) treatment was used in these and subsequent studies to enhance NOD2 and RIP2 expression, and augment NOD2 signaling (29). In response to MDP, a significant increase in IL-6 production was observed in...
WT cells, and this was absent in negative control NOD2 KO cells, thereby indicating the NOD2 specificity for the MDP-induced IL-6 response. Interestingly, MDP-triggered IL-6 production was significantly reduced in a gene dosage-dependent effect. Indeed, the MDP-induced IL-6 production in m/m cells was completely abrogated and was indistinguishable from the cellular response of NOD2 KO cells.

To assess whether the decrease in cytokine production in KI cells was due to impaired intracellular signaling responses, we examined MDP induction of the NF-κB and MAPK pathways in BMDMs (Fig. 3B). Western blot analysis detected diminished expression of IκBα in the WT cells within 30 min, which ultimately recovered 120 min later. Consistent with the heterogenous expression of Nod2, a disappearance of IκBα was observed in +/- cells, albeit it was slightly delayed (i.e., it took 45 min). BMDMs of m/m mice did not respond to MDP as indicated by the unaltered expression of IκBα. The lack of responsiveness to MDP in m/m cells was further observed for MDP induction of the MAPK pathway. MDP stimulation resulted in increased expression level of the active, phosphorylated forms of the signaling mediators ERK, JNK, and p38, which occurred within 30 min in WT cells. This response was impaired and absent in m/+ and m/m cells, respectively.

One of the most immediate and measurable intracellular responses of MDP-induced NOD2 activity is the ubiquitination of the kinase RIP2, which is necessary for downstream signal transduction events in the NF-κB and MAPK pathways (30). Using TUBEs, which are protein complexes that bind cellular polyubiquitinated proteins and protect them from deubiquitination or proteasomal degradation (31), we evaluated whether the R314Q mutation altered MDP-induced RIP2 ubiquitination. As shown in Fig. 3C, antiubiquitin immunoblotting showed the abundance of ubiquitinated proteins in all three genotypes, indicating that the TUBE protein complexes were successful in isolating polyubiquitinated proteins from the BMDMs (Fig. 3C, top). However, immunodetection specifically for the ubiquitinated form of RIP2 showed different amounts of polyubiquitination among the three different genotypes (Fig. 3C, middle). RIP2 is detected over a broad m.w. range because the diverse array of ubiquitin linkages that can occur creates a modified population of RIP2 protein of varying sizes (32). The amount of RIP2 in the lysates of all genotypes before pull down with TUBEs was comparable (Fig. 3C, bottom). The level of polyubiquitinated RIP2 was the highest in BMDMs from +/+ mice, reduced in lysates prepared from +/m mice, and not observed in lysates from m/m, even at the
later time points. These results show that NOD2 in BMDMs prepared from +/+ and m/m mice have an impaired ability to activate RIP2 in response to MDP and the degree of impairment is a function of the copy number of the mutation. Collectively, these data indicate that introduction of the Blau-point mutation results in impaired cellular responses to MDP involving RIP2-initiated signal transduction and/or translational control of NF-κB. 

R314Q mutation results in the conversion of NOD2 from full-length protein to a smaller form

To assess whether the abnormal response to MDP observed both in vivo and in vitro for R314Q mice was the result of alterations in transcriptional and/or translational control of Nod2 at the cellular level, we performed studies on BMDMs prepared from WT and KI mice. To determine whether the point mutation alters Nod2 transcription, we analyzed the levels of mRNA expression by quantitative real-time PCR. As shown in Fig. 4A, the expression levels of Nod2 in the baseline state were comparable among all three genotypes. Stimulation with poly(I:C) elicited a robust induction of Nod2 mRNA expression, and this gene induction was not significantly altered by the point mutation. As expected, the mRNA expression of Nod2 was nondetectable in BMDMs prepared from NOD2 KO mice. These data indicate that the introduction of the R314Q mutation does not impair the transcriptional control of Nod2.

To investigate whether a change in protein expression occurs as a consequence of the R314Q mutation, we measured NOD2 protein levels in BMDMs in the resting state or upon induction with poly(I:C). Immunoblotting revealed the expected full-length 116-kDa form of NOD2 in cells from WT (+/+; Fig. 4B), which was further increased upon stimulation with poly(I:C). NOD2 expression was appropriately absent in cells prepared from control NOD2 KO mice (−/−), indicating the Ab specificity of this assay. In contrast, BMDMs from KI mice (+/+ or m/m) did not show constitutive NOD2 expression in the naive state. Indeed, the m/m cells lacked the full-length NOD2 and instead showed the appearance of a smaller, 80-kDa protein that reacted with the anti-NOD2 Ab. The smaller 80-kDa band was further increased by stimulation with poly(I:C). Consistent with their heterozygous genotype, the BMDMs from +/+ mice showed expression of both the 116- and 80-kDa forms of putative NOD2 upon induction with poly(I:C). In line with prior reports that NOD2 is upregulated by IFN-γ (33), we found increased expression of a 116-kDa form of NOD2 in +/+ BMDMs when stimulated with IFN-γ (Supplemental Fig. 2A). Again, the full-length form of NOD2 was absent from both m/m cells and NOD2 KO cells, and instead generation of the 80-kDa band in m/m or m/+ cells was confirmed. Of note, a longer exposure of the blot revealed a faint 80-kDa form of NOD2 in WT cells. This raises the possibility that the 80-kDa form may be generated normally when NOD2 is upregulated, and that the R314Q mutation promotes the conversion to the 80-kDa form of NOD2. We have further confirmed the specificity of the anti-NOD2 Ab used in these studies using HEK293 cells transiently transfected with a plasmid carrying mouse Nod2 cDNA (Supplemental Fig. 2B). These data indicate that despite normal transcription, the point mutation results in loss of full-length NOD2 protein.

To further investigate generation of the putative 80-kDa form of NOD2 that occurs in KI mice, we performed knockdown experiments in BMDMs prepared from WT (+/+ versus mutant mice (m/m) using short interfering RNA (siRNA). Compared with the scrambled, control siRNA, the specific siRNA treatment resulted in significant reduction in full-length WT-NOD2 (Fig. 4C, left) and also the 80-kDa form in the mutant BMDCs (Fig 4C, right). These data demonstrate that posttranscriptional gene silencing by siRNA effectively knocked down both forms of NOD2, thereby indicating that introduction of R314Q mutation results in a unique and smaller form of the NOD2 protein.

The identity of the mutant 80-kDa form of NOD2 was further evaluated by mass spectrometry (Fig. 4D). Analysis of tryptic digests from gel slices cut from the 116-kDa region of lysates from BMDMs of +/+ mice identified two peptides, GFSEEIQQLYLRK (aa 426–438) and SLYEMQEEQLQEAVR (aa 724–739), of NOD2 defined by mass-to-charge ratios and fragment ions created from the reference library of overexpressed NOD2. The peptide SLYEMQEEQLQEAVR was also identified in gel slices from the 80-kDa region of lysates of BMDMs isolated from m/m mice, thereby identifying NOD2 in the 80-kDa region. Other spectra matched too few ions to be able to identify their source. Although the mass spectrometry results indicate that the 80-kDa region contained a modified form of the full-length 116-kDa NOD2 protein, the yield of tryptic fragments was insufficient to generate spectra throughout
FIGURE 4. R314Q mutation results in the conversion of NOD2 from full-length protein to a smaller form. (A) Nod2 mRNA levels present in BMDMs from +/+/+ , +/+/- , m/m, and /-/- mice cultured in media alone (no treatment) or media supplemented with poly(I:C) for 24 h. Transcript levels were determined in cDNA by quantitative RT-PCR and expressed as fold induction compared with β-actin for each condition. Error bars represent SD. Results are representative of three separate experiments. (B) Western blot analysis with anti-NOD2 Ab on lysates from BMDMs of +/+ , +/+/- , m/m, and /-/- mice cultured in media alone (-) or media supplemented (+) with poly(I:C) for 24 h. Two examples (different BMDM preparations) are shown for m/m mice. Results are representative of three separate experiments. (C) siRNA knockdown of NOD2. Immunoblot of lysates from poly(I:C)-primed BMDMs of +/+ or m/m mice, which were electroporated with either nonspecific scrambled control siRNA (Scr) or NOD2-specific siRNA. Blots were developed with anti-NOD2 antisera or anti-β-actin as a loading control. (D) Mass spectrometry analysis of proteins eluted from gel slices of the 80-kDa region after electrophoresis of lysates from BMDMs of m/m mice. Tryptic digests were analyzed by targeted LC-MS2 for the presence of NOD2 peptides. A peptide corresponding to residues 724–739 of mouse NOD2 in the 80-kDa band is shown. Each line on the chromatogram represents the intensity of one of the fragment ions also observed when a digest of NOD2 standard was analyzed. +/+ , heterozygous mutant; m/m, homozygous mutant; +/+ , WT.
cytokine production systemically and locally within the eye was absent. In contrast with previous studies based on transient transfection assays, we did not find any evidence for the spontaneous activation of NF-κB or enhanced responses to MDP. PBDMs from two different patients with Blau syndrome exhibited the same phenotype in that MDP-induced cytokine and intracellular signaling responses involving MAPK and NF-κB were impaired. These data are all consistent with the possibility that Blau syndrome, like Crohn’s disease, is also a disease mediated by loss of function of NOD2. Although Blau syndrome is not associated with gut inflammation, arthritis and uveitis can occur in Crohn’s disease, and both diseases are associated with granulomatous inflammation.

Even though macrophages from R314Q mouse showed a comparable biochemical response to challenge with MDP as did macrophages from patients, the mice did not develop a spontaneous clinical phenotype. This is comparable with other genetic manipulations of Nod2, including Nod2 KO mice (35) and mice engineered to contain a mutation found in Crohn’s disease (28). It is possible that the transcriptome response in humans that causes Blau syndrome does not trigger the same orthologs in B6 mice, and the mice cannot respond to the NOD2 mutation in the same way as a human carrying the mutation (36). Human diseases caused by loss-of-function mutations in NLR family members may be more difficult to model in mice than gain-of-function mutations. Our R314Q KI model showing a loss of function of NOD2 and no spontaneous disease is in contrast with well-understood gain-of-function genetic disorder involving NLRP3. Mutations in NLRP3 cause cryopyrin-associated periodic syndrome (CAPS), a gain-of-function autosomal dominant disorder associated with spontaneous activation of the NLRP3 inflammasome, excessive IL-1β release, and a uniform response in patients to IL-1β inhibitors (37). This phenotype has readily been reproduced in KI mouse models where autoactivation of the inflammasome was demonstrated (38, 39). Because mutations in Blau syndrome and CAPS are found at analogous positions in their respective NLR family member genes, it has been inferred that they would have a comparable mechanism of action (40, 41). However, in contrast with patients with CAPS, patients with Blau syndrome do not spontaneously release cytokines from PBMCs. Our R314Q model, in contrast with NLRP3 KI models, also does not show spontaneous activation of cytokines triggered by the NOD2 pathway.

We have expressed WT and murine R314Q mutated NOD2 in HEK cells using expression vectors and in murine J774 macrophages using retroviral vectors to produce stable transfectants (data not shown). Western blot analysis of lysates from these cells does not show the 80-kDa truncated form of NOD2. It is possible in artificial overexpression systems that the cellular processes responsible for the 80-kDa form of NOD2 are not functional or are only expressed in cell types different from these cell lines used because of their ease of in vitro culture and manipulation. The lack of NOD2 processing when mutant NOD2 is overexpressed in HEK cells may also account for the discrepancy between the gain of function attributed to mutant NOD2 in HEK cells and the lack of response to MDP observed in patients with Blau syndrome. We have initiated studies of PBDMs to investigate whether the conversion process of full-length Nod2 to the 80-kDa form is observed in patients with Blau syndrome. However, as previously described by others (42), interpreting these studies is difficult because of the quality and specificity of antisera or Abs currently available. Using a reagent from ProSci that had been used by others to detect human Nod2 (43), we identified multiple irresolvable bands in the 80- to 120-kDa section of Western blots from PBDMs of healthy control subjects, as well as patients Blau-1 and -2 (data not shown), such that a definitive conclusion about the disappearance of full-length Nod2 or appearance of an 80-kDa truncated form could not be made and will require further study. The heterozygous nature of Blau syndrome creates a further challenge for experiments using Western blot analysis. Because patients express a normal 116-kDa form of Nod2, detecting the disappearance of full-length Nod2 on Western blots would require interpreting relative intensities of bands compared with healthy individuals, unlike the homozygous R314Q mutant mice created by breeding where the disappearance of full-length NOD2 is readily appreciated.

One mechanism by which we believe that mutant NOD2 results in loss of responsiveness to MDP is through the posttranslational modification and conversion of the full-length NOD2 to a smaller form of NOD2 that lacks the ability to sense MDP. The structure of the 80-kDa form of NOD2 is under further study. The commercial anti-mouse NOD2 mAb used in this study was created by immunizing rats with two separate large peptide fragments (NOD2 aa 150–450 and 481–750), but the exact epitope was not elucidated. Furthermore, this Ab did not work in our hands for immunoprecipitation studies requiring us to analyze whole-cell lysates to study the structure. Although our work with mass spectrometry did identify one peptide that allowed us to conclude that the 80-kDa region of electrophoresis gels of lysates from BMDMs from m/m did contain NOD2, the approach did not yield enough material to be quantitative and provide information of which residues or domain of the NOD2 protein was altered. The structure of the 80-kDa mutant and its conversion from a full-length to a truncated form is the subject of further study.

Control (NL) and Blau patient 2 (Blau-2) pretreated overnight in poly(I:C) followed by treatment for an additional 24 h in media alone, or media supplemented with LPS or MDP. (B) IL-6 and IL-8 levels in supernatants of MDMs from healthy control (NL) and Blau patient 2 (Blau-2) pretreated overnight in media alone (for subsequent study with LPS) or media supplemented with poly(I:C) followed by treatment for an additional 24 h in media alone, or media supplemented with LPS or MDP. (C) MDMs from a healthy control and Blau patients 1 and 2 were treated with MDP from 0–60 min (indicated at the top of each lane), and lysates were analyzed by immunoblot with Abs for the phosphorylated and nonphosphorylated forms of p38. Blau-1 and Blau-2 were analyzed in different experiments. Densitometry readings of p-p38 and p38 bands are indicated below each lane.

FIGURE 5. Monocyte-derived macrophages (MDMs) from patients with Blau syndrome have reduced responses to MDP. (A) IL-6 and IL-8 levels in supernatants of MDMs from a healthy control (NL) and Blau patient 1 (Blau-1) pretreated overnight in poly(I:C) followed by treatment for an additional 24 h in media alone or media supplemented with MDP. (B) IL-6 and IL-8 levels in supernatants of MDMs from a healthy control (NL) and Blau patient 2 (Blau-2) pretreated overnight in media alone (for subsequent study with LPS) or media supplemented with poly(I:C) followed by treatment for an additional 24 h in media alone, or media supplemented with LPS or MDP. (C) MDMs from a healthy control and Blau patients 1 and 2 were treated with MDP from 0–60 min (indicated at the top of each lane), and lysates were analyzed by immunoblot with Abs for the phosphorylated and nonphosphorylated forms of p38. Blau-1 and Blau-2 were analyzed in different experiments. Densitometry readings of p-p38 and p38 bands are indicated below each lane.
form of NOD2 is currently under further study. Nonetheless, it is interesting to consider whether the 80-kDa form has a yet-to-be-determined function. For example, if the 80-kDa form showed autoinhibitory capability for NOD2 oligomerization, like NOD2-S34), or antagonism for MDP-induced activation of NOD2, like NOD2-C2 (42), this could explain the loss of response to systemic administration of MDP seen in R314Q +/-m mice and the absent response to MDP seen in macrophages from Blau syndrome patients who still carry a normal copy of NOD2. It has been shown that the truncated form of NOD2 caused by the 302insC mutation found in Crohn’s disease does result in a new function that may be pathogenic: the inhibition of basal levels of IL-10 release by interfering with phosphorylation of hnRNP-A1 (44). Interestingly, this new function was observed only in humans, not mice, indicating an important difference between mouse and human orthologs of NOD2.

Another topic of interest uncovered by our studies is the investigation of the mechanism by which the full-length form of NOD2 is converted to the mutant 80-kDa form. We have rules out a premature stop codon in the mRNA and a splice variant. Several proteins have been shown to bind to Nod2 and negatively regulate downstream signaling events, including Erbin, CENTB1, AAMP, CAD, and JNKBP1 (45–50). These negative regulators are not thought to act by cleavage of Nod2. More recently, Nod2-binding partners were identified that regulated Nod2 pathways by controlling proteasome-mediated turnover of Nod2, including Hsp90, Socs3, and TRIM27 (33, 51). Nod2 degradation did not occur by these pathways in the presence of proteasomal inhibitors. We have conducted studies to investigate whether full-length NOD2 could be recovered in BMDM from +/-m and +/-m mice by a variety of inhibitors, including the calpain inhibitors ALLN and calpastatin, the proteasome inhibitors bortezomib and MG132, the pan caspase inhibitor z-vad.fmk, the caspase inhibitors L-006235, chymostatin, and pepstatin A, and NH4Cl as an inhibitor of phagosome-lysosomal fusion (data not shown). All of these treatments, including some in combination, failed to restore the 116-kDa form of NOD2. If a specific enzyme is responsible for generating the 80-kDa form in the setting of the mutation, it remains to be identified. A recent study using a genome-wide siRNA screen for proteins regulating the NF-κB pathway was

**References**


Supplemental Figure 1: (A) Photograph showing mice of each genotype and their indistinguishable phenotypic appearance at 10 weeks of age. (B) Representative histological image of the anterior eye segment (left) and clinical fundus image (right) from mice 36 weeks of age. TEFI, topical endoscopic fundus imaging (C) Representative NIR fluorescence images of mouse knee joints and coinciding histology. Colored bar on right indicates intensity of fluorescence, with dark blue being no fluorescence and red indicating maximal fluorescent intensity.
Supplemental Figure 2: (A) NOD2 protein in BMDM treated with IFN-γ from R314Q KI mice is reduced in size compared to +/+ mice. Western blot analysis as in Fig. 4B using BMDM cultured in media alone (-) or media supplemented (+) with IFN-γ for 6 hours. A short (top panel) and long (bottom panel) exposure are shown. Note the presence of low levels of the 80 kDa form of NOD2 in +/+ mice. (B) Anti-mouse NOD2 mAb (25mNOD2) is specific for mouse Nod2. Immunoblot with anti-mouse NOD2 antibody of lysates from HEK293T cells transiently transfected with either empty plasmid only (Lane 1) or plasmid containing subcloned mouse NOD2 cDNA (Lane 2). Numbers on right indicate kDa. (C) RT-PCR analysis of NOD2 mRNA does not show the presence of splice variants. BMDM (2 mice per genotype) were treated overnight with poly(I:C) (100 μg/ml), RNA was extracted, converted to cDNA, amplified using primer pairs spanning the open reading frame and analyzed by agarose gel electrophoresis. The positions of the primers used are shown on the diagram and numbers correspond to nucleotide positions in the mRNA from the 5’ end. The RT-PCR results from +/+, +/m and m/m mice show the presence of a single band at the predicted size in all genotypes and no evidence of a smaller band that would indicate a splice variant. cDNA from +/ mouse served as a negative control.