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Alterations in *Nucleotide-Binding Oligomerization Domain 2* expression, pathway activation, and cytokine production in Yao Syndrome

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Abstract

Yao syndrome (YAOS) is a systemic autoinflammatory disease (SAID), formerly termed *nucleotide-binding oligomerization domain 2 (NOD2)*-associated autoinflammatory disease. Due to the recent identification of YAOS, the molecular mechanisms underlying its disease pathogenesis are unclear. With specific *NOD2* variants as characteristic genotypic features of YAOS, our study examined *NOD2* expression, transcript splicing, signaling pathway activation, and cytokine profiles in peripheral blood mononuclear cells (PBMCs) from 10 YAOS patients and 6 healthy individuals. All participants were genotyped for *NOD2* variants; all YAOS patients were heterozygous for the *NOD2* IVS8⁺¹⁵⁸ variant (IVS8⁺¹⁵⁸) and four patients also carried a

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Conflict of Interest

The authors report no conflicts of interest.

concurrent *NOD2* R702W variant (IVS8⁺¹⁵⁸/R702W haplotype). Resembling other SAIDs, plasma levels of TNF α , IL-1 β , IL-6, IFN γ , and S100A12 were unaltered in YAOS patients. Intron 8 splicing of *NOD2* transcripts was unaffected by carriage of *NOD2* IVS8⁺¹⁵⁸. However, *NOD2* transcript level and basal p38 mitogen-activated protein kinase (MAPK) activity were significantly elevated in PBMCs from IVS8⁺¹⁵⁸ YAOS patients. Moreover, these patients' cells had elevated basal IL-6 secretion that was enhanced by muramyl dipeptide (MDP) stimulation. Tocilizumab treatment of a YAOS IVS8⁺¹⁵⁸ patient resulted in marked clinical improvement. In contrast, MDP-stimulated NF- κ B activity was uniquely suppressed in haplotype IVS8⁺¹⁵⁸/R702W patients, as was TNF α secretion. Our study demonstrates for the first time that *NOD2* expression and pathway activation are aberrant in YAOS, and specific *NOD2* genotypes result in distinct *NOD2* expression and cytokine profiles. These findings may also help select therapeutic strategies in the future.

Keywords

autoinflammatory disease; NOD2; Yao Syndrome; anti-cytokine therapy; genetics

Introduction

Nucleotide-binding, oligomerization domain 2 (NOD2) is an intracellular bacterial sensor protein of the NOD-like receptor (NLR) family (1). NOD2 detects a component of the bacterial cell wall, muramyl dipeptide (MDP) and stimulates signal transduction cascades that activate NF- κ B and mitogen-activated kinases (MAPKs), which in turn result in the secretion of pro-inflammatory cytokines. The NOD2 protein is comprised of three main regions that include amino-terminal caspase recruitment domains (CARDs), a central nucleotide-binding, oligomerization domain (NOD), and carboxyl-terminal leucine-rich repeats (LRRs). The *NOD2* gene is mapped to chromosome 16q12–21, and several genetic variants are known to predispose individuals to the development of inflammatory diseases (2). For example, specific genetic variants in the LRR domain are associated with increased Crohn disease (CD) susceptibility (2), while distinct genetic variants in the NOD are proposed to drive the development of Blau syndrome (BS) (3).

More recently, specific *NOD2* variants have been linked to Yao syndrome (YAOS, OMIM#617321), a systemic autoinflammatory disease (SAID) characterized by recurrent episodes of fever, dermatitis, and inflammatory arthritis (**Figure 1**) (4–6). All YAOS patients carry *NOD2* variants (6), and nearly all patients are heterozygous for a C>T single nucleotide polymorphism (SNP) in intron 8 of *NOD2* (rs5743289, IVS8⁺¹⁵⁸) (6, 7). Approximately 20% of patients carry both IVS8⁺¹⁵⁸ and a nonsynonymous C>T SNP that results in an arginine to tryptophan substitution at amino acid 702 (rs2066844, R702W; haplotype IVS8⁺¹⁵⁸/R702W) (4, 6, 8). Other rarer *NOD2* variants have also been identified at a low frequency in the disease (6). Several of these *NOD2* variants are shared with CD and have been demonstrated to impair NOD2 function *in vitro* (6, 9); however, the function of NOD2 in YAOS patients has not been reported yet.

YAOS was initially reported in 2011 and originally designated as *NOD2*-associated autoinflammatory disease (NAID) (5, 10, 11). YAOS patients are predominantly white adults

with a female to male ratio of 2:1 at an estimated disease prevalence of 1–10/100,000(6). YAOS was initially identified in the United States (4, 12) and reports are now appearing in Europe (13). Disease occurrence is primarily sporadic, although a minority of patients can have a positive family history (4, 14). Apart from the characteristic clinical phenotype described above, patients can also have distal lower extremity swelling, gastrointestinal, and sicca-like symptoms (10, 15). YAOS possesses clinical features of SAID with episodic disease flares in the absence/low titers of autoantibodies or antigen-specific T cells (6), but it is distinct from CD, BS, and monogenic hereditary SAIDs phenotypically and genotypically (6).

Approximately 50% of YAOS patients experience frequent and severe flares despite treatment with glucocorticoids and sulfasalazine, which are more effective compared with other disease modifying anti-rheumatic drugs (4). To meet the outstanding therapeutic challenge, a search for more effective, targeted therapies is warranted. For this purpose, we examined *NOD2* expression, pathway activation, and resultant cytokine production in primary cells from YAOS patients to determine if this molecular pathway is altered in patients to gain insights into candidate mechanisms underlying the disease.

Materials and Methods

Study participants

Ten patients and six healthy individuals gave informed consent prior to enrolment in the study that was approved by the Institutional Review Board of the Cleveland Clinic (IRB#09–367 and IRB#13–758). YAOS patients were selected for recruitment based on established diagnostic criteria (4, 6). Patients were genotyped for *NOD2* variants by DNA sequencing in the Center for Genetic Testing at Saint Francis, Tulsa, Oklahoma. Healthy individuals were genotyped using TaqMan probes to the three major *NOD2* variants (rs2066844/R702W, rs2066845/G908R, and rs2066847/L1007fs) and rs5743289/IVS8⁺¹⁵⁸ (Affymetrix). The white blood cell counts of all patients were within a normal range (3,700–11,000/ μ L). The demographic, genotypic and therapeutic data of the patients are summarized (**Table 1**).

Isolation and culture of peripheral blood mononuclear cells (PBMCs)

Venous blood samples were collected from study participants by trained phlebotomists. Cells were separated from plasma by density gradient centrifugation as previously described (16) and cultured in Roswell Park Memorial Institute media with 10% fetal bovine serum (Life Technologies). For cytokine assays, 2×10^5 PBMCs were stimulated in triplicate for 18h with MDP (1 μ g/mL; Bachem) and cell-free supernatants analyzed. For immunoblot analyses, 4×10^6 cells were stimulated for 30 min with MDP (10 μ g/mL).

Polymerase chain reaction (PCR) analysis of *NOD2* transcripts

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and cDNA synthesized by random-primed reverse transcription using the iScript cDNA Synthesis Kit (BioRad) according to manufacturers' instructions. *NOD2* transcript levels were quantified by real-time PCR using the 2^{-CT} method (17) and SYBR Green Supermix with ROX (BioRad) with the following primer sets: *NOD2* (5' –AAATCAGGTTGCCGATCTTCA–3' and 5' –

CAGCCAATCCATTGCTTTC-3') and *GAPDH* (5'-CATGAGAAGTATGACAACAGCCT-3' and 5'-AGTCCTTCCACGATACCAAGGT-3'). The splicing of intron 8 in *NOD2* transcripts was determined by PCR amplification of cDNA using the primers: 5'-ATTCTGGGGCAACAGAGTGG-3' and 5'-TTCTTTGCCAGCATCAGTGC-3'. PCR products were separated on a 1.5% agarose/TBE gel and visualized by SYBR green. Amplicon sequence was confirmed by capillary sequencing on an ABI 3730xl DNA Analyzer.

Immunoblot Analysis

PBMCs were lysed in 20mM Tris pH7.5, 150mM sodium chloride, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM β -glycerophosphate, 1mM sodium orthovanadate, 1 μ g/mL leupeptin, 1 $\times$ Phosphatase Inhibitor Cocktail 2 (Sigma). Proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes (iBlot; Life Technologies). Membranes were blocked in 5% milk/Tris buffered saline, 0.1% Tween20 (TBST). Primary antibodies (Cell Signaling Technologies) to phosphorylated NF- κ B p65 (Serine 536, clone 93H1), phosphorylated p38 (Threonine 180/Tyrosine 182), or GAPDH (clone 14C10) were incubated in 5% milk/TBST overnight at 4°C. Membranes were washed in TBST and probed with donkey anti-rabbit-HRP (Jackson ImmunoResearch). Washed membranes were developed using ECL (Millipore) and quantitated using Image J software (<http://imagej.nih.gov/ij/>).

Enzyme-Linked Immunosorbent Assay

Cytokine levels in plasma or cell culture supernatants were measured in duplicate according to manufacturers' instruction for TNF α , IL-1 β , IL-4, IL-6, IL-17, IL-23, IFN γ (BioLegend) and S100A12/EN-RAGE (MBL International).

Statistical Analysis

Significance determined by 1-way ANOVA with Tukey's multiple comparison test using Prism6.0 (GraphPad Software). For PBMC cytokine analyses, paired T-test or its nonparametric equivalent, the Wilcoxon signed rank test was calculated using SAS 9.3 software (SAS Institute, Cary, NC). Results were considered significant when $p < 0.05$.

Results

Circulating markers of inflammation are not elevated in YAOS patients.

Certain pro-inflammatory cytokines in cell cultures or tissues, such as IL-1 β and IL-6, are involved in the pathogenesis of several SAIDs, thus therapies blocking these inflammatory mediators have shown clinical benefit (18, 19). Similarly, we assessed the levels of a panel of cytokines (TNF α , IL-1 β , IFN γ , IL-4, IL-6, IL-10 and IL-17) and the inflammation marker, S100A12, in the plasma of YAOS patients by ELISA and compared with those of healthy controls, but did not observe any significant differences between the two groups (Figure 2).

NOD2 genotype influences NOD2 transcript levels, but does not affect transcript splicing.

Nearly all YAOS patients (85%) carry at least one IVS8⁺¹⁵⁸ *NOD2* variant allele (6) and of these patients, over 90% are heterozygous for this intronic variant. This contrasts the analysis of a similar ethnic group by the 1000 Genomes Project indicating a frequency of heterozygous carriage in a general European population to be 26.2% (20). Since intronic variants can affect both mRNA splicing and gene expression (21), we examined the splicing of intron 8 from *NOD2* transcripts and compared *NOD2* transcript levels between YAOS patients and healthy subjects using total RNA isolated from PBMCs. In all study participants, intron 8 was spliced out of *NOD2* mRNA transcripts correctly as demonstrated by amplification of the exon 8–9 junction resulting in the same size product in all samples and confirmed by sequence analysis of the exon ligation junction (**Figure 3A**). Overall, there were no significant differences in *NOD2* transcript levels between healthy donors and all YAOS patients (**Figure 3B**). However, further subanalysis of the data suggested that *NOD2* transcript levels were clustered into two subgroups within the YAOS patients. When the data was analysed in view of *NOD2* genotype, it was noted that YAOS patients with only the IVS8⁺¹⁵⁸ variant had significantly higher levels of *NOD2* expression than haplotype IVS8⁺¹⁵⁸/R702W YAOS patients or healthy subjects (2.47±0.27 in IVS8⁺¹⁵⁸ YAOS vs. 0.90±0.14 in IVS8⁺¹⁵⁸/R702W YAOS vs. 0.87±0.28 in healthy controls; **Figure 3C**). Interestingly, this type of change was not observed in a healthy donor heterozygous for IVS8⁺¹⁵⁸ (relative expression of 0.62). Taken together, these findings indicate that there may be functional subtypes of YAOS patients depending on *NOD2* genotype.

Haplotype IVS8⁺¹⁵⁸/R702W YAOS patients have impaired NOD2-dependent NF-κB activity and decreased TNFα secretion.

The *NOD2* variant R702W is known to increase susceptibility to CD development and reduce NOD2 function (2, 9). In our study, NOD2 function was assessed by the phosphorylation level of NF-κB and MAPK p38 in cultured PBMCs in response to the NOD2 ligand, MDP, in immunoblots. In haplotype IVS8⁺¹⁵⁸/R702W YAOS patients, basal activation of these signaling pathways was not different from healthy controls (**Figure 4**). Intriguingly, MDP treatment increased the phosphorylation of the p65 subunit of NF-κB and MAPK p38 approximately 2-fold in healthy controls, whereas PBMCs from haplotype IVS8⁺¹⁵⁸/R702W YAOS patients did not show any activation of NF-κB after MDP stimulation (**Figure 4A**). We also observed that MDP-stimulated phosphorylation of MAPK p38 was similarly induced in cells from both groups (**Figure 4B**). These data suggest impairment of *NOD2*-dependent NF-κB activation in patients with haplotype IVS8⁺¹⁵⁸/R702W, and this deficiency paralleled a loss of MDP-induced TNFα secretion (**Figure 4C**), while IL-6 secretion from these patient cells was unaltered (**Figure 4D**).

PBMCs from YAOS patients with only IVS8⁺¹⁵⁸ have increased basal p38 kinase activation and IL-6 secretion, which is further increased after MDP stimulation.

In contrast to haplotype IVS8⁺¹⁵⁸/R702W YAOS patients, patients with IVS8⁺¹⁵⁸ only had lower basal phosphorylation of p65 NF-κB than healthy controls, but similar MDP-induced NF-κB activation (**Figure 5A**). The level of TNFα secretion from PBMCs of these YAOS patients was not different from healthy controls either basally or in response to MDP

(**Figure 5B**). Yet, the basal phosphorylation of p38 MAPK was significantly elevated in the cells of IVS8⁺¹⁵⁸ only YAOS patients, and it was further increased upon MDP stimulation (**Figure 5C**). The increase of p38 kinase activity correlated with an elevated basal secretion of IL-6 and a higher MDP-induced IL-6 secretion from these patient PBMCs (**Figures 5D**). This type of change was not observed in PBMCs from the IVS8⁺¹⁵⁸ heterozygous healthy individual, in whom both basal and MDP-induced IL-6 levels were similar to those in healthy controls with wild type (WT) *NOD2* (basal: 6,055pg/mL in IVS8⁺¹⁵⁸ only healthy subject vs. 9,797±3,639pg/mL in WT healthy subjects; MDP-induced: 14,897pg/mL in IVS8⁺¹⁵⁸ only healthy subject vs. 15,500±5,211pg/mL in WT healthy subject). These results indicate that while YAOS patients with IVS8⁺¹⁵⁸ only showed elevated levels of MAPK signaling and IL-6 production, IVS8⁺¹⁵⁸ alone may not lead to disease onset.

IL-6 receptor blockade is effective treatment for an IVS8⁺¹⁵⁸ only YAOS patient.

A 67-year-old white male presented with 13 years of recurrent high fever, erythematous patches/plaques on his trunk and extremities, inflammatory polyarthritis, and abdominal pain. His sedimentation rate was elevated and serum antinuclear antibodies were negative. Symptomatic flares resulted in frequent hospitalizations that required high doses of prednisone (60 mg daily) to control symptoms, followed by a minimum daily dose of prednisone (20 mg). He failed various immunosuppressive agents (methotrexate, azathioprine, mycophenolate, leflunomide, and cyclosporine). Despite trials of multiple biologics (etanercept, adalimumab, infliximab, anakinra, abatacept, and rituximab), the patient still required high doses of prednisone and had frequent emergency room visits. Genetic testing confirmed heterozygous carriage of the IVS8⁺¹⁵⁸ *NOD2* variant and the diagnosis of YAOS. After receiving tocilizumab (560mg intravenously every 3 weeks), he began to notice symptomatic improvement approximately 4 weeks later, resulting in 85% symptomatic improvement, decreased frequency of disease flares, and improved physical functionality. This case study suggests that elevated IL-6 may be pathogenic for YAOS patients with the IVS8⁺¹⁵⁸ haplotype, as blockade of IL-6 signaling significantly resolved disease activity in this patient.

Discussion and Conclusions

YAOS is a SAID associated with specific *NOD2* variants (6), and the disease is relatively common when compared with monogenic SAIDs in the American adult patient population (14). The etiopathogenesis of YAOS is elusive due to its recent discovery. A better understanding of the disease mechanisms underlying YAOS undoubtedly will enhance the development and selection of more efficacious therapeutic agents. This study is the first to examine the cytokine profile and functional status of *NOD2* in YAOS patients to gain insights into potential molecules involved in disease pathogenesis.

Circulatory cytokine profiles are often different between SAIDs, such as cryopyrin-associated periodic syndrome (CAPS), and autoimmune disorders like rheumatoid arthritis (19, 22). For example, IL-1 β is undetectable in the plasma of CAPS patients (23), whereas IL-1 β is elevated in rheumatoid arthritis patients (24). When we measured several cytokines

associated with SAIDs in YAOS patients, we observed no significant increases in these cytokines in the blood circulation. This finding is in line with those reported in other SAIDs.

Unlike systemic profiles, cytokines secreted from cells are elevated in some SAIDs. These increased cytokines often contribute to disease pathology, thus therapeutically targeting them has been effective for SAIDs patients. For example, IL-1 β production is elevated from cells of CAPS patients due to heightened activation of the inflammasome (25). CAPS patients are successfully treated with IL-1 β inhibitors, such as anakinra, canakinumab, and rilonacept (25). The cytokine secretion profile from cultured PBMCs of all YAOS patients did not show alterations in basal TNF α or IL-1 β levels; however, spontaneous IL-6 secretion from the cells isolated from IVS8⁺¹⁵⁸ only patients was significantly higher than that in healthy controls, and it further increased with NOD2 stimulation. Interestingly, this did not correlate with changes in activation of signal transducer and activator of transcription 3 (STAT3), the production of IL-23, or IL-17 (data not shown) that YAOS patients may not have an elevated Th17 response in blood cells. We believe that the difference in IL-6 production is unlikely to result from alterations in mononuclear cell populations, because all patients had normal white blood cell counts. In the future, further analysis of the PBMC cell subpopulations responsible for producing the elevated IL-6 levels in the IVS8⁺¹⁵⁸ patients may provide additional insights into disease mechanism. Excitingly, therapeutically targeting IL-6 responses using tocilizumab in the IVS8⁺¹⁵⁸ YAOS patient resulted in significant clinical improvement, suggesting that IL-6 may be a pivotal mediator of disease pathology in this majority subtype which accounts for approximately 80% of YAOS patients. Further clinical trials are necessary to determine if this therapeutic approach is effective in a larger group of IVS8⁺¹⁵⁸ YAOS patients.

As stated earlier, specific *NOD2* variants are considered as predisposing factors for CD, BS, and YAOS (1, 14, 26). In CD, the three main disease-associated *NOD2* variants are associated with decreased NOD2 function, reflective of a loss-of-function genotype (2, 9). In contrast, BS is an autosomal dominant disorder associated with distinct *NOD2* variants that result in overactive NOD2 signaling *in vitro* (3, 9). These highlight that the change of a homeostatic level of NOD2 function contributes to the development of inflammatory disease. YAOS patients carry *NOD2* variants too, but their functional role in the disease was not characterized previously.

Our current data indicate that NOD2 is altered in YAOS, dependent on *NOD2* haplotype. NOD2 function is aberrant in haplotype IVS8⁺¹⁵⁸/R702W patients that account for approximately 20% of all YAOS patients in our clinical practice. This aberration is characterized by decreased activation of NF- κ B and lower production of TNF α upon NOD2 stimulation, reflective of loss-of-function. This mirrors the functional characteristics of R702W *NOD2* variant activity described in CD patients (27–29). However, due to the distinct clinical phenotype of YAOS, we predict that the contribution of the R702W variant to YAOS pathogenesis may be distinct from that in CD (6, 27). In contrast, IVS8⁺¹⁵⁸ patients appear to have intact NOD2 responses, but an increased *NOD2* expression that may amplify IL-6 production. Based upon the normal result obtained in the IVS8⁺¹⁵⁸ healthy individual, we postulate that the elevated spontaneous IL-6 secretion and *NOD2* expression level in patients may not be a direct consequence of the IVS8⁺¹⁵⁸ *NOD2* variant. As

proinflammatory cytokines, such as IL-6, TNF α , and IL-1 β , are known to increase *NOD2* expression (30), we predict that the increased *NOD2* expression in the IVS8⁺¹⁵⁸ patient PBMCs is due to higher levels of autocrine IL-6 in these cultures. These findings suggest that there are differential subtypes of YAOS.

YAOS is a genetically complex, multifactorial disease. In YAOS, there is a genetic component that confers susceptibility to disease; however, as observed in CD, there is not a strong familial clustering of YAOS. Sole carriage of specific *NOD2* variants is usually not sufficient to drive disease onset, as these *NOD2* variants are present in healthy populations (8). Specifically, the 1000 Genomes Project reports that 26.2% of a general population with a similar ethnic background to our YAOS patients are heterozygous carriers of IVS8⁺¹⁵⁸ (rs5743289), while 9.7% are heterozygous for R702W (rs2066844) (20). Instead, these *NOD2* variants represent predisposing genetic risk factors for these diseases (31). Functional defects in *NOD2* signaling have been demonstrated in experimental and human CD as a result of the carriage of *NOD2* variants (9, 27, 28, 32). In YAOS, almost all patients carry *NOD2* IVS8⁺¹⁵⁸, with concurrent carriage of R702W in nearly one fourth of patients (14). Our current data have demonstrated the existence of the abnormal expression and activation of *NOD2* pathway in YAOS, supporting dysregulation of the innate immune response as a hallmark of this SAID. Extensive study and analyses of CD patients with *NOD2* variants have provided future avenues for an in-depth investigation in YAOS. The currently proposed hypotheses for *NOD2* variant-associated CD include: decreased intestinal barrier function, loss of antimicrobial responses, dysbiosis of the intestinal microbiota, and enhanced pro-inflammatory cytokine production (33–35). In the context of YAOS, approximately 60% of patients have gastrointestinal symptoms without inflammatory bowel disease. Given that *NOD2* IVS8⁺¹⁵⁸ (rs5743289) can share haplotype with R702W (rs2066844), G908R (rs2066845), or L1007fs (rs2066847), it is reasonable to speculate that the IVS8⁺¹⁵⁸ variant may be in linkage disequilibrium with another unidentified SNP that may drive the disease via interactions with environmental factors or triggers.

NOD2 is a bacterial sensor protein that modulates multiple immune responses to promote an inflammatory and antimicrobial response (2), as well as shapes the immune response in concert with other immune receptors, such as Toll-like receptors (TLRs) (32, 36–39). Depending on the cell type involved and the timing of the stimuli, *NOD2* can either synergize with TLRs to amplify inflammation (37, 39), or tolerize cells to a subsequent TLR stimulation to prevent excessive inflammation and subsequent tissue damage (32, 36). Disease-associated *NOD2* variants may result in either increased or decreased function, indicating a proper balance of *NOD2* activity is critical to maintain human health. We hypothesize that the role of *NOD2* dysfunction in YAOS is an amplifier or modifier of inflammation and not necessarily a direct initiator of disease. A future study of broader profile of immune function in these patients may provide deeper insights into the disease mechanisms and possible targets of therapeutic intervention.

YAOS is a polygenic SAID, and the presence of specific *NOD2* variants confers susceptibility to the disease. *NOD2* expression and pathway activation are aberrant in YAOS, and specific *NOD2* genotypes result in distinct *NOD2* expression and cytokine profiles.

These data underscore the role of innate immune response in YAOS as a SAID. These findings may also help identify more effective therapeutic targets.

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Figure 1: Clinical features of YAOS patients.

(A) Patchy erythema on upper chest. (B) Erythematous plaques on face. (C) Subacute spongiotic dermatitis. (D) Distal lower extremity swelling. Reprinted with permission from: Yao Q, et al. NOD2-associated autoinflammatory disease: a large cohort study. *Rheumatology* (2015) 54(10): 1904–12.

Figure 2: Circulating markers of inflammation are not elevated in YAOS patients. Levels of TNF α (A), IL-1 β (B), IFN γ (C), and S100A12 (D) were measured in plasma samples from healthy controls (HC) and YAOS patients (YAOS) by ELISA. IL-4, IL-6, IL-10, and IL-17 were also measured but values were below the level of detection for all samples. Values from individual donors shown by symbols, mean $\pm$ SEM indicated by bars, and the limits of assay detection indicated by the dashed line.

Figure 3: NOD2 genotype influences NOD2 transcript levels but does not affect transcript splicing.

(A) *NOD2* transcript splicing of intron 8 determined by PCR using primers flanking the intron (expected amplicon sizes shown in diagram). All PCR products were sequenced to confirm exon junction integrity. (B) Comparison of *NOD2* transcript levels in PBMCs from healthy controls and YAOS patients by qRT-PCR. (C) Comparison of *NOD2* transcript levels between healthy controls, YAOS patients with IVS8⁺¹⁵⁸ only, and YAOS patients carrying haplotype IVS8⁺¹⁵⁸/R702W. Values from individual donors shown by symbols, mean $\pm$ SEM indicated by bars.

Figure 4: Haplotype IVS8⁺¹⁵⁸/R702W YAOS patients have impaired NOD2-dependent NF-κB activity and decreased TNFα secretion.

(A) Immunoblot analysis of NF-κB activation as measured by phosphorylation state of the p65 subunit (p-p65) in lysates from PBMCs treated with or without MDP (10μg/mL) for 30 min. Membranes were also probed for GAPDH protein levels as a loading control. Levels of phosphorylated NF-κB p65 were quantified by densitometry and normalized to GAPDH levels. (B) Immunoblot analysis of MAPK p38 activation as measured by phosphorylation of p38 (p-p38) as described in A. (C) Measurement of secreted TNFα from PBMCs treated with or without MDP (1μg/mL) for 18h by ELISA. (D) Measurement of secreted IL-6 from PBMCs as described in B. Values from individual donors shown by symbols, mean ±SEM indicated by bars. HC=healthy control; IVS8⁺¹⁵⁸/R702W YAOS=YAOS patients compound heterozygous for IVS8⁺¹⁵⁸ and R702W *NOD2* variants.

Figure 5: PBMCs from YAOS patients with only IVS8⁺¹⁵⁸ have increased basal p38 MAPK activation and IL-6 secretion that is further increased by MDP stimulation.

(A) Immunoblot analysis of NF-κB activation as measured by phosphorylation state of the p65 subunit (p-p65) in lysates from PBMCs treated with or without MDP (10μg/mL) for 30min. Membranes were also probed for GAPDH protein levels as a loading control. Levels of phosphorylated NF-κB p65 were quantified by densitometry and normalized to GAPDH levels. (B) Measurement of secreted TNFα from PBMCs treated with or without MDP (1μg/mL) for 18h by ELISA. (C) Immunoblot analysis of MAPK p38 activation measured by phosphorylation of p38 (p-p38) as described in A. (D) Measurement of secreted IL-6 from PBMCs as described in B. Values from individual donors shown by symbols, mean ±SEM indicated by bars. HC= healthy control; IVS8⁺¹⁵⁸ YAOS= YAOS patients heterozygous for the IVS8⁺¹⁵⁸ *NOD2* variant.

Table 1:

Demographics of study subjects

Variable	Yao Syndrome			Healthy Individuals (n=6)
	All Patients (n=10)	IVS8 ⁺¹⁵⁸ Haplotype (n=6)	IVS8 ⁺¹⁵⁸ /R702W Haplotype (n=4)	
Age				
Mean (SD)	43.7 (15.7)	47 (17.9)	38.8 (12.1)	38 (7.5)
Range	22–63	25–63	22–51	24–44
Gender				
Male	3	1	2	2
Female	7	5	2	4
NOD2 variants				
IVS8 ⁺¹⁵⁸	6	6	0	1
IVS8 ⁺¹⁵⁸ + R702W	4	0	4	0
Medications	None	None	None	None