Supplementary Data

Crohn's disease-associated NOD2 mutation suppresses human *IL10* transcription by inhibiting the activity of heterogeneous nuclear ribonucleoprotein-A1

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Materials and Methods

Cell culture Human monocytes were isolated from peripheral blood of healthy donors and cultured with RPMI 1640 supplemented with 10% heat-inactivated serum (Hyclone), L-glutamine, and penicillin-streptomycin in 100 mm^2 dishes for 18h.

Cytokine measurement Human IL-12p40, mouse IL-12p40, human IL-12p70, human IL-10, mouse IL-10 and human IL-1β were measured by ELISA kits, according to protocols provided by BD Pharmingen.

Reporter plasmids The human *IL10* promoter-luciferase construct (pIL-10 (–1044/+30)-luc) was described previously [1]. Substitution mutants were generated using pairs of overlapping internal primers that contain the mutant sequences. All constructs are relative to the transcriptional initiation site and were confirmed by sequencing against the human *IL10* promoter (GenBank accession no. Z30175). The mouse *Il10* promoter-luciferase construct (-1538/+64) [2] was generously provided by S. Cao (University of Maryland, MD). The human IL-12p40 promoter-reporter construct was described previously [3].

Nuclear extract preparation Nuclear extracts for immunoblotting and EMSA assays were prepared as described previously [4].

Immunoblot and immunoprecipitation Phospho-hnRNP-A1 was immmunoprecipitated from pre-cleared extracts of an equivalent number of viable cells with an hnRNP-A1 polyclonal antibody (Santa Cruz Biotechnologies). Immunoprecipitates were washed with PBS before resolving by SDS-PAGE and transfer to PVDF for immunoblot analysis.

Chromatin immunoprecipitation assay The chromatin immunoprecipitation (ChIP) procedure was performed using an assay kit following the manufacturer's instructions (Upstate Biotechnology), with 1×10^7 human monocytes in each condition. The input DNA was diluted 200 times before PCR amplification. The input and precipitated DNA were amplified with primers encompassing the NRE site in the human *IL10* promoter (forward primer: 5'-CTGTGCCGGGAAACCTTGATTGTG-3' and reverse primer: 5'- GCTGAGCTGTGCATGCCTTCTTTT-3'). The PCR conditions were as follows: 95°C for 3 min, 60°C for 40 s, and 72°C for 30 s, 1 cycle; 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, 33 cycles; and 72°C for 7 min, 1 cycle. The PCR products were analyzed by electrophoresis on a 1.2% agarose gel. As a negative control, a separate region of the human *IL10* promoter located between –3158 and –2947 upstream of the NRE region was amplified using the primers: forward primer, 5'-AGTGAGAAGGCAGGCACCTA-3', and reverse primer, 5'-ATCCCCCACTGGAAAAATTC-3'. The relative binding in each sample measured by real time PCR was normalized against its genomic input and calculated as $2^{-(Cti-Ctinput)}$ where Ct*i* represents the individual Ct of a given ChIP sample, and Ct*input* is the Ct of its corresponding input DNA.

siRNA Four siRNAs designed to be specific for human hnRNP-A1 were purchased from Dharmacon. 1.5 μg of siRNA was transfected into 9×10^6 cells primary human monocytes with the Amaxa Nucleofector device set to program Y-001 with the Human Monocyte Nucleofector kit (Amaxa). The sequences of these siRNAs are as follows: #1 Sense: 5' cggaaaccttggtgtagtttt 3' (nucleotides 1525-1543); Antisense: 5'Paactacaccaaggtttccgtt 3'; #2, Sense: 5' gggaatgaagcttgtgtattt 3' (nucleotides 1689-1710); Antisense: 5'Patactcttgcttcattccctt 3'; #3, Sense: 5' caacttcggtcgtggaggatt 3' (nucleotides 727-745); Antisense: 5'Ptcctccacgaccgaagttgtt; #4, Sense: 5' tagaattccttcagggtgatt 3' (nucleotides 1448-1466); Antisense: 5' Ptcagggtgaaggaattctatt 3'.

nLC-MS/MS analysis Nanoflow liquid chromatography-tandem mass spectrometry analysis (nLC-MS/MS) was performed using an 1100 series LC/MSD *Ultra Plus* ion trap mass spectrometer (Agilent Technologies). The system was equipped with an Agilent Chip Cube interface and a silicon wafer "chip-column" that integrates a C18 enrichment column, C18 resolving column and nanospray emitter. Samples were loaded on the enrichment column at a flow rate of 5 μL/min and then resolved at at a flow rate of 0.3 μl/min on 40mm × 75μM of ZORBAX 300 C18 resin (5μM particle size). The LC gradient was 10% to 40% solvent B for 30 min, followed by 40% to 90% solvent B for 20 min. Solvent A contained 0.1% formic acid in 3% ACN and solvent B contained 0.1% formic acid in 90% ACN. ESI conditions included a needle voltage of 2 KV, nitrogen gas flow rate of 4 L/min and a capillary temperature of 300° C. MS spectra were acquired at a scan speed of 20,000 m/z/sec and the four most intense precursor ions at intervals of 0.5 sec were selected for MS/MS fragmentation. The fragmentation amplitude was 1.15V and the skimmer voltage was 30 V. This method was adapted from reference Hao and Gross [5].

Database search of MS/MS data for peptide sequence identification Analysis of MS/MS spectra for peptide identification was performed by protein database searching using *Spectrum Mill* software (Agilent Technologies). Raw MS/MS spectra were first processed to extract MS/MS spectra that could be assigned to at least two y- or b-series ions, and only those spectra were searched against the mouse SwissProt protein database. Key search parameters were a minimum matched peak intensity of 50%, a precursor mass tolerance of 2.0 Da and a product mass tolerance of 0.6 Da. The maximum number of missed cleavages allowed was one. The threshold used for peptide identification was a Spectrum Mill score greater than 13.0 and SPI% (the percentage of assigned spectrum intensity of total spectrum intensity) greater than 70%. All MS/MS spectra were validated by manual inspection.

PCR primers The PCR primers used to amplify the human hnRNP-A1 cDNA: forward primer, 5′-CCCTGCCGTCATGTCTAAGT-3’; reverse primer, 5’-CTGGCTCTCCTCTCCTA-3’, based on the GenBank sequence X06747. The PCR primers used to amplify the mouse hnRNP-A1 were: forward primer, 5’ ATGAGAGATCCAAACACCAAGAG 3’; reverse primer, 5’-TTAGAA CCTCCTGCCACTG-3’. The following primers were used for PCR amplification/detection: 1) human IL-10, forward: 5’-AATAAGGTTTCTCAAGGGGCT-3’, reverse 5’ AGAACCAAGACCCAGACATCAA-3’; 2) human NOD2, forward 5'-GCGCGATAACAATATCTCAGA-3', Reverse 5'- CAGAGTTCTTCTAGCATGACG-3'; 3) human hnRNP A1, forward 5'-AAGCAATTTTGGAGGTGGTG-3‘, reverse: 5'-ATAGCCACCTTGGTTTCGTG-3’; 4) human β-actin, forward: 5’-CGCCCCAGGCACCAGGGC-3’, reverse: 5’-GCTGGGGTGTTGAAGGT-3’; 5) mouse GAPDH, forward: 5’-ATGGGAAGCTTGTCATCAACG-3’, reverse: 5’-GGC AGT GAT GGC ATG GAC TG-3’.

Fig 1. Expression of transfected NOD2 and *3020insC* Mrna in RAW264.7 cells

The human *IL10* promoter-reporter was transfected together with empty vector (pCDNA3) or vector encoding wild-type or *3020insC* NOD2 constructs into RAW264.7 cells at an effector to reporter molar ratio of 0.3:1. One day later, expression of the transfected NOD2 and *3020insC* was verified by RT-PCR using primers selective for human but not mouse NOD2.

Fig 1

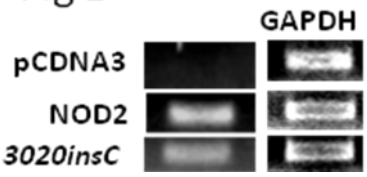

Fig 2

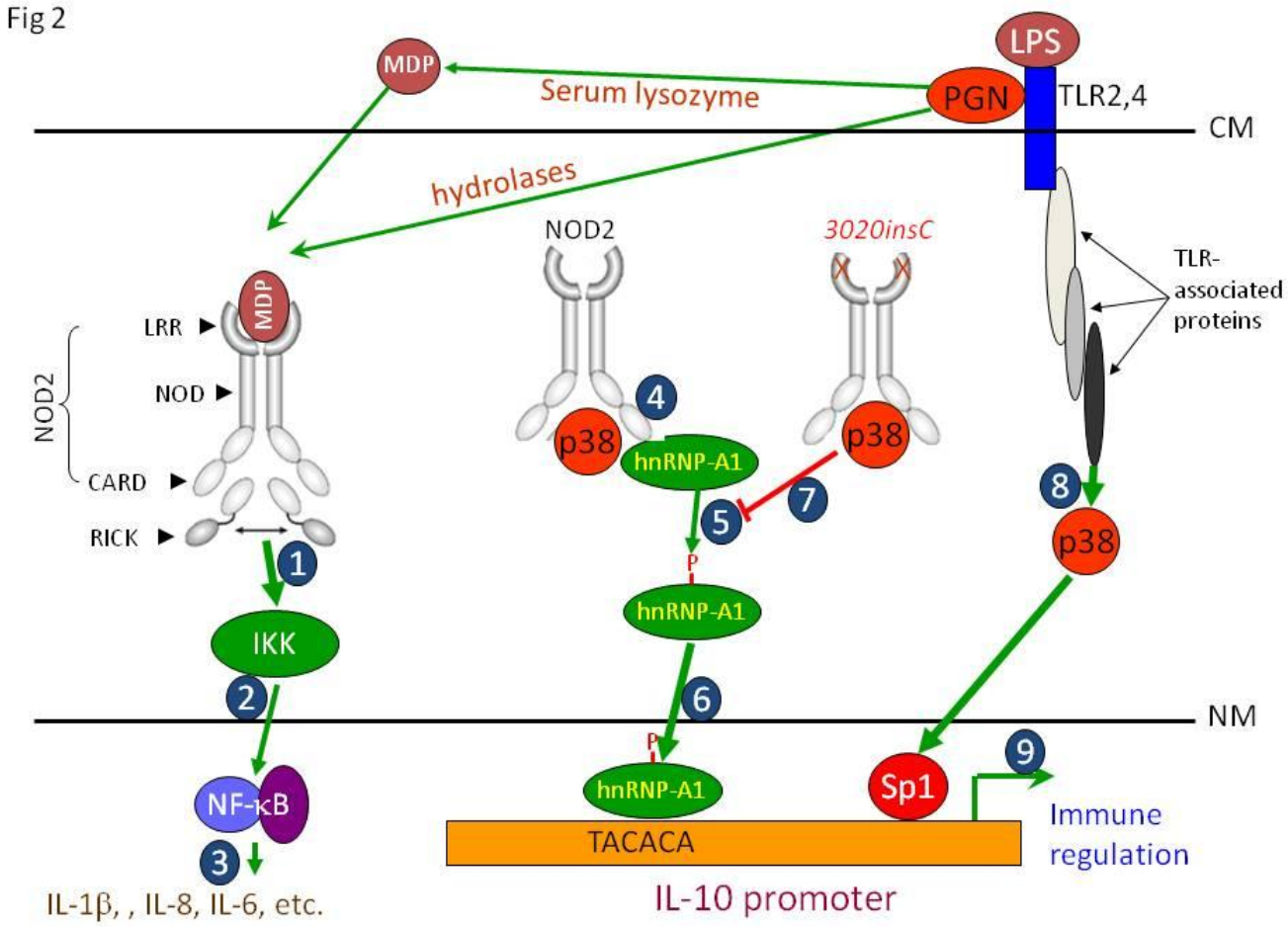

Fig 2. Model of *3020insC*-mediated inhibition of IL-10 expression

MDP can be generated from PGN by serum lysozyme digestion extracellularly, or by hydrolases intracellularly. MDP then binds to the leucine-rich repeats (LRR) of NOD2 homodimers. MDP binding activates NOD2 and results in recruitment of the serine-threonine kinase RICK (also known as RIP2 or CARDIAK) through CARD-CARD domain interactions (Step 1). This triggers a cascade of downstream events that lead to the activation of IKK-NF-κB (Step 2) and expression of proinflammatory cytokine and induction of anti-microbial responses (Step 3). Wild-type NOD2 also interacts with hnRNP-A1 and withactivated p38 MAPK in the cytosol, forming a tri-molecular complex (Step 4). This interaction is perhaps important for the phosphorylation of hnRNP-A1 (Step 5), which may be important for the cleavage of hnRNP-A1 into the transcriptionally active 26 kDa cleavage product. Cleaved hnRNP-A1 then moves into the nucleus, binds the *IL10* TACACA element (NRE) and drives basal *IL10* transcription (Step 6). The *3020insC* mutant, however, interferes with the interaction between wild-type NOD2, p38, and hnRNP-A1 (Step 7). By doing so, it reduces p38-mediated phosphorylation of hnRNP-A1, as well as hnRNP-A1 cleavage, nuclear translocation and binding to the *IL10* NRE. Independently, TLR-mediated signaling triggered by the engagement by microbial pathogens (Step 8), also stimulates *IL10* expression via activation of Sp1 (Step 9). P, phosphorylated serine residues; CM, cell membrane; NM, nuclear membrane.

Fig 3. Expression of transfected NOD2 and *3020insC* mRNA in NOD2-KO macrophages

BMDM derived from three *Nod2*$^{-/-}$ mice were infected two times with empty retrovirus (EV), or with retrovirus encoding wild-type (WT) or *3020insC* NOD2. On day 5 post-infection, equivalent mRNA expression of the transduced human NOD2 and *3020insC* in non-stimulated *Nod2*$^{-/-}$ BMDM was ensured by RT-PCR using primers selective for human but not mouse NOD2.

Fig 3

Table 1. PCR primers used for genotyping NOD status in CD patients

Primer name and concentration used	Primer sequence 5'-3'	Annealing temp. (°C)	PCR product size (bp)
3020insC for (1 pmol)	GACGGGACACCGCTGATCGTTTATT TC-CAGGTTGTCCAATAACT	59.8	123
3020insC univ (9 pmol)	fluorophore-GGGACACCGCTGATCGTTTA		
3020insC rev (10 pmol)	CAGGGGCCTTACCAGACT		

References

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