Bacterial TLR4 and NOD2 signaling linked to reduced mitochondrial energy function in active inflammatory bowel disease

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\textbf{ABSTRACT}

Inflammatory bowel disease (IBD) has been linked to active signaling with bacterial components and reduced mitochondrial ATP production; however, synergism between both of these disease characteristics remains unclear. We aimed to determine in human IBD transcriptomes the link between a transcriptional signature unique to intestinal cells (ICs) with reduced mitochondrial ATP function (Mito-0) and bacteria triggered signaling using a bioinformatics approach. We generated an IC Mito-0 panel comprised of 199 differentially expressed (DE) transcripts mediated by reduced mitochondrial ATP function (DEGseq, log\textsubscript{2} fold-change > |2|, p < .001). Transcripts from this panel were involved in diverse biological functions including regulation of mitochondrial energy (lower ATP), extracellular matrix, cell–cell contact, cytoskeleton, growth, metabolism, and inflammation. Next, unsupervised hierarchical clustering showed that the Mito-0 panel distinctly separated inflamed IBD from non-inflamed transcriptomes, which was also supported by principal component analysis (PCA) revealing distinct variation between sample types based on presence of the Mito-0 signature (PCA, \( p = 8.77 \times 10^{-09} \)). Utilizing three independent IBD cohorts, we validated that 60 novel transcripts from the Mito-0 panel were significantly increased in inflamed tissue. Subsequently, KEGG generated bacterial TLR4 and NOD2 transcriptional signatures strongly associated with inflamed IBD transcriptomes and with the Mito-0 signature as determined by Spearman’s analysis (coefficient of correlation, \( r = 0.92, p < .05 \)). Herein, using a comprehensive analysis we demonstrated existence of an axis between bacteria triggered signaling and reduced mitochondrial energy function. Furthermore, we identified and validated novel transcripts within this axis as potential drivers and therapeutic targets for human IBD.

\textbf{ARTICLE HISTORY}

Received 31 January 2019
Revised 12 April 2019
Accepted 18 April 2019

\textbf{KEYWORDS}

TLR4; NOD2; mitochondria; inflammatory bowel disease; bioinformatics

\textbf{Introduction}

Intestinal inflammation associated with inflammatory bowel disease (IBD) is driven by genetic susceptibility, exacerbated immune responses, impaired intestinal barrier function,\textsuperscript{1,2} and signaling stimulated by bacterial components.\textsuperscript{3} Emerging findings have also linked intestinal inflammation to metabolic changes associated with reduced energy production from intracellular organelles known as mitochondria.\textsuperscript{4–7} However, mechanisms linking reduced mitochondrial energy function to other drivers of IBD pathophysiology are not well understood or explored.

Intestinal homeostasis is maintained by complex cross-talk between gut bacteria and intestinal tissue.\textsuperscript{8} Alterations in this cross-talk could lead to activation of signaling conferring increased susceptibility to intestinal inflammation.\textsuperscript{8} Specifically, intestinal cells (ICs) are exposed to luminal bacterial components known as pathogen-associated molecular patterns (PAMPs) including endotoxins, flagellin, lipoteichoic acid, unmethylated CpG motifs, peptidoglycan, muramyl-peptide (MDP), and lipopolysaccharide (LPS).\textsuperscript{9–12} Intestinal tissue is also equipped with specific receptors recognizing select PAMPs including the family of cell surface proteins called toll-like receptors (TLRs) and the cytoplasmic receptor known as nucleotide-binding oligomerization domain-containing protein 2 (NOD2). Overstimulation of TLR4 and NOD2 is implicated in the development of intestinal inflammation through activation of...
downstream signaling leading to release of pro-inflammatory mediators. However, potential links between bacteria-triggered TLR4/NOD2 and metabolic reprogramming in mediating intestinal inflammation is unexplored.

Mitochondria play complex roles in diverse cell functions including energy production, regulation of reactive oxygen species (ROS) and metabolites, and cell signaling linked to mitophagy and cell death. While certain mitochondrial processes such as increased ROS levels have been shown to foster inflammation, a role of reduced mitochondrial energy function linked to ATP production is poorly understood. It has been suggested that gut bacteria and mitochondrial energy function in most tissues could be co-dependent; gut bacterial signaling could affect mitochondrial function, while in return, mediators released from tissues (e.g., endocrine, immune) that are regulated by mitochondria could affect gut microbiota. Also, in mice polymorphisms in two mitochondrial genes linked to low intestinal ATP levels exacerbate local inflammation, while a polymorphism resulting in increased ATP levels is thought to be protective. We have demonstrated that ICs with select reduction in mitochondrial ATP levels exhibit strong inflammatory responses and their global transcriptomic changes resemble those observed in human IBD. Moreover, these ICs also have activated bacterial signaling; however, whether in IBD this signaling is connected to reduced mitochondrial energy function in driving and/or in sustaining disease is unclear.

Here we aimed to determine in IBD tissue if bacterial signaling is linked to reduced mitochondrial energy function using a bioinformatics approach. In inflamed IBD transcriptomes, we found an abundance of the IC transcriptional signature mediated by reduced mitochondrial function that strongly corresponded with bacterial TLR4 and NOD2 signaling. Utilizing three independent patient cohorts, we validated that 60 novel transcripts were significantly increased in inflamed IBD tissue. From this signature, we identified individual transcripts involved in regulation of diverse biological functions including generation of mitochondrial energy (decreased ATP production) (MT-CO, MT-ND, MT-RNR1, MT-CY, MT-ATP), extracellular matrix (VCAM, MUC13, COL13,17, FGF9), cell–cell contact (TM4SF, FLRT3, THBS, NRP), cytoskeleton (VIM, MID2, MYO15, TINAGl1), growth (IGFBP6, GDF1, GDF15, CDK1A, CDK6), metabolism (PRKAA2, CPA4, TGM2, SGK1, SLC7A11), and inflammatory response (TNFR, IL17RD, IFIT2,3, NTSE, TLR6).

Results

Generating an intestinal cell transcriptional panel mediated by deficient mitochondrial energy production

Intestinal inflammation associated with IBD is linked to low ATP levels. We and others have shown in animal models that reduced intestinal ATP due to deficient mitochondrial energy (ATP) production facilitates intestinal inflammation; however, how this drives disease pathobiology is unclear. We previously generated ICs with reduced mitochondrial energy function by selective targeting of mitochondrial but not nuclear DNA polymerase with low-dose ethidium bromide in parental cells. Consequently, in these cells we confirmed their viability, loss of mitochondrial DNA, and reduced ATP synthase activity. Following RNAseq we utilized their transcriptomes (SRP093357) to generate a specific transcriptional panel (i.e., Mito-0 transcriptional panel). Initially, DE transcripts of IC with reduced mitochondrial energy function relative to control were analyzed by DEGseq to calculate fold change between the two groups meeting stringent differential expression and statistical thresholds of log2 fold-change > |2| and an adjusted \( p < 0.001 \). This IC Mito-0 panel was comprised of 199 transcripts among which 164 were increased and 35 decreased relative to control as shown by scatter plot in which green dots represent transcripts with an adjusted \( p < 0.001 \) and an absolute value of log2 fold change > |2| (Figure 1(a,b)). Additionally, unsupervised hierarchical clustering of DE transcripts between IC with reduced mitochondrial energy function relative to control were analyzed by DEGseq to calculate fold change between the two groups meeting stringent differential expression and statistical thresholds of log2 fold-change > |2| and an adjusted \( p < 0.001 \). From this signature, we identified individual transcripts involved in regulation of diverse biological functions including generation of mitochondrial energy (decreased ATP production) (MT-CO, MT-ND, MT-RNR1, MT-CY, MT-ATP), extracellular matrix (VCAM, MUC13, COL13,17, FGF9), cell–cell contact (TM4SF, FLRT3, THBS, NRP), cytoskeleton (VIM, MID2, MYO15, TINAGl1), growth (IGFBP6, GDF1, GDF15, CDK1A, CDK6), metabolism (PRKAA2, CPA4, TGM2, SGK1, SLC7A11), and inflammatory response (TNFR, IL17RD, IFIT2,3, NTSE, TLR6).
Also in this panel, several transcripts were encoded by unannotated genes with uncharacterized function. These data represent an IC specific transcriptional signature mediated by aberrant mitochondrial energy function and include transcripts with established and undetermined functions in IBD.

**Human inflamed IBD tissue transcriptomes contains a high abundance of the IC Mito-0 signature**

Next, we assessed transcriptomes from tissue of IBD patients for the presence of the Mito-0 signature. We utilized publicly available transcriptomic data from human inflamed IBD and matched non-inflamed control tissue biopsies (GSE107593). Analysis of these IBD transcriptomes indicated 184 out of 199 transcripts in the Mito-0 panel were distinctly present in human inflamed IBD tissue relative to matched non-inflamed control (Table 1 (asterisk)). Unsupervised hierarchical clustering revealed that the Mito-0 signature separated two distinct clusters visibly differentiating inflamed IBD from non-inflamed matched control (Figure 2(a)). Moreover, this analysis also outlined three groups of transcripts from the Mito-0 panel that were altered in inflamed IBD, those significantly decreased (Group 1), increased (Group 3), or with a mixed response (Group 2) (Figure 2(a)). In order to determine if these transcripts were universally altered in IBD affected tissue we assessed their levels in three independent patient cohorts (GSE4183, GSE14580, GSE38713). We initially determined statistically meaningful differences in transcript levels from a large number of inflamed IBD tissue samples compared to healthy control using the robust multi-array
Table 1. Intestinal cell Mito-0 panel comprised of 199 genes differentially expressed (DE) due to reduced mitochondrial energy function (DESeq R studio; absolute value of Log2(fold change) normalized > |2|, \( p < 0.001 \) (*Denotes transcripts in both the Mito-0 panel and inflamed IBD transcriptomes; prefix “MT” indicates mitochondrial genome encoded transcript; prefix “ENSG” represents uncharacterized transcripts; †Denotes transcripts found in the Mito-0 panel but not in inflamed IBD transcriptomes). (Continued)

| Gene symbol | Gene name | log(2) (fold-change): Mito-0 vs control |
|-------------|-----------|----------------------------------------|
| 1†         | ENSG00000279576 | 8.4 |
| 2†         | ENSG00000280527 | 8.0 |
| 3†         | ENSG00000265569 | 7.2 |
| 4*         | MAGEB1 | MAGE B1 | 6.3 |
| 5*         | VIM-AS1 | VIM antisense RNA 1 | 6.2 |
| 6†         | ENSG00000278203 | 6.2 |
| 7*         | TM4SF18 | Transmembrane 4 L six family member 18 | 6.0 |
| 8*         | MUC13 | Mucin 13 | 5.7 |
| 9*         | VIM | Vimentin | 5.6 |
| 10*        | RNU6-833P | RNA, U6 small nuclear 833, pseudogene | 5.3 |
| 11†        | IER3-AS1 | IER3 antisense RNA1 | 5.3 |
| 12†        | ENSG00000280573 | 5.2 |
| 13*        | RNU6-118P | RNA, U6 small nuclear 118, pseudogene | 5.0 |
| 14*        | CDH12P2 | Cadherin 12 pseudogene 2 | 5.0 |
| 15*        | RNU6-199P | RNA, U6 small nuclear 199, pseudogene | 5.0 |
| 16*        | TM4SF1 | Transmembrane 4 L six family member 1 | 4.9 |
| 17*        | FLRT3 | Fibronectin leucine rich transmembrane protein 3 | 4.9 |
| 18†        | ENSG00000266229 | 4.9 |
| 19*        | ARL4C | ADP ribosylation factor like GTPase 4C | 4.8 |
| 20*        | RNU6-388P | RNA, U6 small nuclear 388, pseudogene | 4.7 |
| 21*        | GLI5 | GLI family zinc finger 3 | 4.7 |
| 22†        | ACO78993 | 4.4 |
| 23*        | SLCA7A1 | Solute carrier family 7 member 11 | 4.2 |
| 24*        | SEMA3D | Semaphorin 3D | 4.2 |
| 25*        | BCL2L15 | Bcl2 like 15 | 4.2 |
| 26*        | TENM3 | Teneurin transmembrane protein 3 | 4.2 |
| 27*        | CALB2 | Calbindin 2 | 4.1 |
| 28*        | CV1P1A | Cytochrome P450 family 1 subfamily A member 1 | 4.1 |
| 29*        | VCAN | Versican | 3.9 |
| 30*        | SNTB1 | Syntrophin beta 1 | 3.9 |
| 31*        | LURAP1L | Leucine rich adaptor protein 1 like | 3.8 |
| 32*        | PRKAA2 | Protein kinase, AMP-activated alpha 2 catalytic subunit | 3.8 |
| 33*        | LINP1 | IncRNA in non-homologous end joining pathway 1 | 3.7 |
| 34*        | LUCAT1 | Lung cancer associated transcript 1 | 3.7 |
| 35*        | MIR22HG | mir22 host gene | 3.7 |
| 36*        | AQP3 | Aquaporin 3 | 3.7 |
| 37*        | ZBTB18 | Zinc finger and BTB domain containing 18 | 3.6 |
| 38*        | NCAN-AS1 | NCAN antisense RNA 1 | 3.6 |
| 39*        | THBS1 | Thrombospondin 1 | 3.5 |
| 40*        | COL13A1 | Collagen type XIII alpha 1 chain | 3.5 |
| 41*        | FAT4 | FAT atypical cadherin 4 | 3.4 |
| 42*        | CRAA | Carboxypeptidase A4 | 3.4 |
| 43*        | IGBP4 | Insulin like growth factor binding protein 6 | 3.4 |
| 44*        | NRP1 | Neuropilin 1 | 3.3 |
| 45*        | MAMLD2 | Mastermind transcriptional coactivator 2 | 3.3 |
| 46*        | RBM24 | RNA binding motif protein 24 | 3.3 |
| 47*        | PIK3R3 | Phosphoinositide-3-kinase regulatory subunit 3 | 3.3 |
| 48*        | PRF1 | Perforin 1 | 3.2 |
| 49*        | GDF15 | Growth differentiation factor 15 | 3.2 |
| 50*        | INE2 | Inactivation escape 2 | 3.1 |
| 51*        | SERpine2 | Serpin family E member 2 | 3.1 |
| 52*        | MAMDC2 | MAM domain containing 2 | 3.1 |
| 53*        | DHRS3 | Dehydrogenase/reductase 3 | 3.1 |
| 54*        | CEMIP2 | Cell migration inducing hyaluronidase 2 | 3.1 |
| 55*        | ALDH1A3 | Aldehyde dehydrogenase 1 family member A3 | 3.1 |
| 56*        | GDF1 | Growth differentiation factor 1 | 3.1 |
| 57*        | HIST1H2BD | Histone cluster 1 H2B family member d | 3.0 |
| 58*        | OSGIN1 | Oxidative stress induced growth inhibitor 1 | 3.0 |
| Gene symbol | Gene name | log₂ (fold-change): Mito-0 vs control |
|------------|----------|-------------------------------------|
| NTSE       | S80-nucleotidase ecto | 2.9 |
| MILR1      | Mast cell immunoglobulin like receptor 1 | 2.9 |
| PLPP3      | Phospholipid phosphatase 3 | 2.9 |
| MAP1B      | Microtubule-associated protein 1b | 2.9 |
| ARL4D      | ADP ribosylation factor like GTPase 4D | 2.9 |
| TSC2D3     | TSC22 domain family member 3 | 2.9 |
| COL17A1    | Collagen type XVII alpha 1 chain | 2.9 |
| PLK2       | Polo like kinase 2 | 2.8 |
| TGM2       | Transglutaminase | 2.8 |
| TP53N1P1   | Tumor protein p53 inducible nuclear protein 1 | 2.8 |
| TMEM158    | Transmembrane protein 158 | 2.8 |
| FTH1P3     | Ferritin heavy chain 1 pseudogene 3 | 2.7 |
| ZBED2      | Zinc finger BED-type containing 2 | 2.7 |
| SYTL2      | Synaptotagmin like 2 | 2.7 |
| HIST1H2AC  | Histone cluster 1 H2A family member c | 2.6 |
| CDKN1A     | Cyclin dependent kinase inhibitor 1 | 2.6 |
| FGF9       | Fibroblast growth factor 9 | 2.6 |
| SLC30A1    | Solute carrier family 30 member 1 | 2.6 |
| SERPIN1    | Serpin family I member 1 | 2.6 |
| ETS1       | ETS proto-oncogene 1, transcription factor | 2.5 |
| IGKV1OR-2  | Immunoglobulin kappa variable 1/OR-2 | 2.5 |
| TESC       | Tescalcin | 2.5 |
| ZCCHC24    | Zinc finger CCHC-type containing 24 | 2.5 |
| RNU6-216P  | RNA, U6 small nuclear 216, pseudogene | 2.5 |
| KRT15      | Keratin 15 | 2.5 |
| IFIT3      | Interferon induced protein with tetratricopeptide repeats 3 | 2.5 |
| TINAG      | Tubulointerstitial nephritis antigen | 2.5 |
| TINAGL1    | Tubulointerstitial nephritis antigen like 1 | 2.5 |
| SH3TC2     | SH3 domain and tetratricopeptide repeats 2 | 2.5 |
| FTH1P15    | Ferritin heavy chain 1 pseudogene 15 | 2.5 |
| LCN2       | Lipocalin2 | 2.5 |
| DUSP4      | Dual specificity phosphatase 4 | 2.5 |
| CAVIN3     | Caveolae associated protein 3 | 2.5 |
| CEMP       | Cell migration inducing hyaluronidase 1 | 2.5 |
| WDFC3      | WAP four-disulfide core domain 3 | 2.5 |
| SGK1       | Serum/glucocorticoid regulated kinase 1 | 2.5 |
| PMAIP1     | Phorbol-12-myristate-13-acetate-induced protein 1 | 2.5 |
| BCA3       | BCA3, NSP family adaptor protein | 2.4 |
| AC105460   | | 2.4 |
| NR4A2      | Nuclear receptor subfamily 4, group A, member 2 | 2.4 |
| RNU6-516P  | RNA, U6 small nuclear 516, pseudogene | 2.4 |
| DDX3Y      | DEAD-box helicase 3 Y-linked | 2.4 |
| PLCDX2     | Phosphatidylinositol specific phospholipase C X domain containing 2 | 2.3 |
| RASSF8     | Ras association domain family member 8 | 2.3 |
| Ctcf        | Chromosome 6 open reading frame 141 | 2.3 |
| MID2       | Midline 2 | 2.3 |
| NPAS2      | Neuronal PAS domain protein 2 | 2.3 |
| DDIT4L     | DNA damage inducible transcription 4 like | 2.3 |
| CLC4       | Chloride intracellular channel 4 | 2.3 |
| PLAU       | Plasminogen activator, urokinase | 2.3 |
| SLC7A1     | Solute carrier family 7 member 1 | 2.3 |
| PDGFC      | Platelet derived growth factor c | 2.3 |
| GALNT5     | Polypeptide N-acetylgalactosaminyltransferase 5 | 2.3 |
| TL6        | Toll-like receptor 6 | 2.3 |
| ASAP2      | ArfGAP with SH3 domain, ankyrin repeat and PH domain 2 | 2.3 |
| TMEM200A   | Transmembrane protein 200A | 2.3 |
| ATP1B1     | ATPase Na+/K+ transporting subunit beta 1 | 2.2 |
| EML6       | EMAP like 6 | 2.2 |
| IFIT2      | Interferon induced protein with tetratricopeptide repeats 2 | 2.2 |
| SAMD4A     | Sterile alpha motif domain containing 4A | 2.2 |
| TNFRSF10D  | TNF receptor superfamily member 10d | 2.2 |
| Gene symbol | Gene name                                      | log$_2$ (fold-change): Mito-0 vs control |
|-------------|-----------------------------------------------|-----------------------------------------|
| TIMP2       | TIMP metallopeptidase inhibitor 2             | 2.2                                     |
| ANXA3       | Annexin A3                                   | 2.2                                     |
| SH3BP4      | SH3 domain binding protein 4                 | 2.2                                     |
| CTSO        | Cathepsin O                                  | 2.2                                     |
| SAMD5       | Sterile alpha motif domain containing 5      | 2.2                                     |
| EHF         | ETS homologous factor                        | 2.2                                     |
| CDK6        | Cyclin dependent kinase 6                    | 2.2                                     |
| TMC2        | Transmembrane and tetratricopeptide repeat containing 2 | 2.2                                     |
| SEMA3A      | Semaphoring 3A                               | 2.2                                     |
| DHR52       | Dehydrogenase/reductase 2                    | 2.2                                     |
| LOXL2       | Lysyl oxidase like 2                         | 2.2                                     |
| CPPED1      | Calcineulin like phosphoesterase domain containing 1 | 2.2                                     |
| IL17RD      | Interleukin receptor D                       | 2.2                                     |
| CD59        | CD59 molecule                                | 2.2                                     |
| RNF182      | Ring finger protein 182                      | 2.2                                     |
| GCNT3       | Glucosaminyl transferase 3, mucin type       | 2.2                                     |
| MYO15B      | Myosin XVB                                   | 2.2                                     |
| STK17A      | Serine/threonine kinase 17a                  | 2.2                                     |
| TMX4        | Thioredoxin related transmembrane protein 4  | 2.1                                     |
| EPHA4       | EPH receptor A4                              | 2.1                                     |
| TNS4        | Tensin 4                                     | 2.1                                     |
| YPEL5       | Yippee like 5                                | 2.1                                     |
| AADACP1     | Arylacetamide deacetylase pseudogene 1       | 2.1                                     |
| CLIP4       | CAP-Gly domain containing linker protein family member 4 | 2.1                                     |
| RGS2        | Regulator of G protein signaling 2           | 2.1                                     |
| NCEH1       | Neutral cholesterol ester hydrolase 1        | 2.1                                     |
| SYBU        | Syntabulin                                   | 2.1                                     |
| WWTR1       | WW domain containing transcription regulator 1 | 2.1                                     |
| LAMA3       | Laminin subunit alpha 3                      | 2.1                                     |
| POPDC3      | Popeye domain containing 3                   | 2.1                                     |
| AL392023    |                                              | 2.1                                     |
| RPS4Y1      | Ribosomal protein S4 Y-linked 1              | 2.1                                     |
| ANTXR2      | ANTXR cell adhesion molecule 2               | 2.1                                     |
| TOB1        | Transducer of ERBB2, 1                       | 2.1                                     |
| ALCAM       | Activated leukocyte cell adhesion molecule   | 2.0                                     |
| LAMB1       | Laminin subunit beta 1                       | 2.0                                     |
| PSTK        | Phosphoseryl-tRNA kinase                     | 2.0                                     |
| PIK3R1      | Phosphoinositide-3-kinase regulatory subunit 1 | 2.0                                     |
| CUEDC1      | CUE domain containing 1                     | 2.0                                     |
| ZSCAN31     | Zinc finger and SCAN domain containing 31    | 2.0                                     |
| PAPSS2      | 30-phosphoadenosine-50-phosphosulfate synthase 2 | 2.0                                     |
| FHL1        | Four and a half LIM domains 1                | 2.0                                     |
| DCUN1D3     | Defective cullin neddylation 1 containing 3  | 2.0                                     |
| AL009031    |                                              | 2.0                                     |
| UCP2        | Uncoupling protein 2                         | 2.0                                     |
| CDH7        | Cadherin 7                                   | −2.1                                    |
| PCDH7       | Protocadherin 7                              | −2.1                                    |
| TAT         | Tyrosine aminotransferase                    | −2.2                                    |
| AC025419    |                                              | −2.3                                    |
| SPTSSB      | Serine palmitoyltransferase small subunit B  | −2.3                                    |
| H3F3AP4     | H3 histone, family 3A, pseudogene 4          | −2.6                                    |
| MTCO3P39    | MT-CO3 pseudogene 39                         | −3.4                                    |
| LINC02458   | Long intergenic non-protein coding RNA 2458  | −3.7                                    |
| ENSG00000277201  |                                        | −4.4                                    |
| RPL23AP42   | Ribosomal protein L23a pseudogene            | −4.5                                    |
| LSM12P1     | LSM12 pseudogene 1                           | −5.1                                    |
| MIR1282     | MicroRNA 1282                                | −5.3                                    |
| ANXA10      | Annexin A10                                  | −5.6                                    |
| MTRNR2L3    | MT-RNR2 like 3                               | −7.1                                    |
| MTCO1P12    | MT-CO1 pseudogene 12                         | −8.3                                    |
| MTCO1P1     | MT-CO1 pseudogene 12                         | −8.6                                    |

(Continued)
Table 1. (Continued).

| Gene symbol | Gene name                                      | log$_2$ (fold-change): Mito-0 vs control |
|-------------|------------------------------------------------|-----------------------------------------|
| 181* MTND2P2 | MT-ND2 pseudogene 2                            | −9.3                                    |
| 182* MTND2P28| MT-ND2 pseudogene 28                           | −10.2                                   |
| 183* MT-RNR1 | Mitochondrially encoded 12S RNA                 | −10.4                                   |
| 184* AC132825|                                               | −11.1                                   |
| 185* MT-ND6  | Mitochondrially encoded NADH dehydrogenase 6   | −13.7                                   |
| 186* MT-ND5  | Mitochondrially encoded NADH dehydrogenase 5   | −14.0                                   |
| 187* MT-ND1 | Mitochondrially encoded NADH dehydrogenase 1   | −14.2                                   |
| 188* MT-ND3 | Mitochondrially encoded NADH dehydrogenase 3   | −14.4                                   |
| 189* MT-CYB | Mitochondrially encoded cytochrome b           | −14.8                                   |
| 190* MT-ND2 | Mitochondrially encoded NADH dehydrogenase 2   | −14.8                                   |
| 191* MTATP6P1| MT-ATP6 pseudogene 1                           | −14.8                                   |
| 192* MT-ND4L| Mitochondrially encoded NADH dehydrogenase mitochondrially encoded NADH 4L | −15.3                                   |
| 193* MT-ND4  | Mitochondrially encoded NADH dehydrogenase 4   | −15.5                                   |
| 194* MT-ATP8 | Mitochondrially encoded ATP synthase 8         | −15.5                                   |
| 195* MT-ATP6 | Mitochondrially encoded ATP synthase 6         | −16.2                                   |
| 196* MT-CO2 | Mitochondrially encoded cytochrome c oxidase II| −16.4                                   |
| 197* MT-CO3 | Mitochondrially encoded cytochrome c oxidase III| −16.7                                  |
| 198* MT-RNR2 | Mitochondrially encoded 16S RNA                | −18.0                                   |
| 199* MT-CO1 | Mitochondrially encoded cytochrome c oxidase I | −21.0                                   |

Figure 2. Inflamed IBD transcriptomes reveal high abundance of the Mito-0 signature. (a) Hierarchical clustering, as shown by representative heatmap, revealed two distinct clusters of human IBD samples separated by the Mito-0 signature differentiating between inflamed IBD and matched non-inflamed control transcriptomes (GSE107593). (b) Principal component analysis (PCA) of inflamed IBD and matched non-inflamed control transcriptomes with the Mito-0 signature was performed to estimate variation between samples. Two axis values of the PCA showed the Mito-0 signature significantly differentiated inflamed IBD from matched non-inflamed control (PCA, $p = 8.77 \times 10^{-9}$). (c) Presence of the Mito-0 signature represented as a score in inflamed IBD compared to non-inflamed matched control transcriptomes ($p = 4.14 \times 10^{-8}$).
average method and differential expression testing package.\(^{20,21}\) We found that most of the transcripts with elevated expression from Group 3 (Figure 2(a)) were also upregulated in inflamed IBD tissue in these three cohorts with strong statistical differences evident by \(p\)-values ranging from \(4.14\times10^{-02}\) and \(2.96\times10^{-34}\) (Table 2). These findings demonstrate independent validation and universal alteration of these transcripts across IBD patient tissues. Several of these transcripts have been identified in IBD disease activity including LCN2, ALCAM, and TLR6,\(^{22–24}\) however, the majority of these transcripts were novel and not yet described in intestinal inflammation. Moreover, the abundance of the Mito-0 signature in inflamed IBD tissue transcriptomes was further supported by Principal Component Analysis (PCA) and Mito-0 scoring. Precisely, PCA of IBD samples with the Mito-0 panel showed distinct grouping based on inflammation status (i.e., inflamed vs non-inflamed) (Figure 2(b); \(p = 8.77\times10^{-09}\)). Also, a score that summarizes the expression of the Mito-0 signature into one value was significantly higher in inflamed IBD compared to non-inflamed control (Figure 2(c); \(p = 4.14\times10^{-08}\)). These data demonstrate a high abundance of the IC Mito-0 signature in actively inflamed IBD tissue and identified individual transcripts considerably upregulated in inflamed IBD across multiple cohorts.

In inflamed IBD tissue transcriptomes, the Mito-0 signature is strongly linked to active bacterial TLR4 and NOD2 signaling

Previously, we demonstrated that intestinal inflammatory responses driven by TNF, an established player in IBD pathobiology, is mediated in part, by reducing mitochondrial energy function.\(^{16}\) However, potential links between signaling triggered by bacterial components such as TLR4 and NOD2 receptors, both critical in IBD pathobiology,\(^{9,25,26}\) and reduced mitochondrial function is not well understood. Initially, using KEGG we generated transcriptional panels specific for TLR4 and NOD2 signaling pathways and Kruskal–Wallis testing revealed their strong association within inflamed IBD transcriptomes compared to non-inflamed controls (Figure 3(a,b); TLR4: \(p = 1.81\times10^{-08}\) and NOD2: \(p = 1.12\times10^{-08}\)). Moreover, PCA differentiated inflamed IBD and non-inflamed matched control by the presence of TLR4 and NOD2 signatures (Figure 3(c,d); TLR4: \(p = 3.28\times10^{-08}\) and NOD2: \(p = 9.91\times10^{-09}\)). Finally, in inflamed IBD transcriptomes we assessed potential associations between bacterial TLR4 and NOD2 pathways and the Mito-0 signature. Using Spearman’s correlation, we found that the IC Mito-0 score showed a strong correlation with both TLR4 and NOD2 pathway scores; coefficient of correlation, \(r = 0.92\) for both TLR4 and NOD2, \(p < .05\) (Figure 4(a,b)). Taken together, these data revealed in human inflamed IBD intestine a strong link between bacterial TLR4 and NOD2 signaling and a reduction in mitochondrial function.

Discussion

Emerging evidence has revealed a strong association between intestinal inflammation and reduced mitochondrial energy function; yet, how this could drive IBD pathophysiology remains unknown. Thus, we created a distinct IC transcriptional signature characteristic for reduced mitochondrial energy function (IC-Mito-0) and found its high abundance in inflamed IBD transcriptomes. We also showed that in inflamed IBD this signature was considerably linked to active bacterial TLR4 and NOD2 signaling. Furthermore, we identified that novel IC Mito-0 transcripts were significantly altered in inflamed IBD tissue across additional independent patient cohorts, revealing their universal alterations. These data support that in human inflamed IBD this axis including bacteria triggered signaling and reduced mitochondrial function could be a hallmark of disease activity and potential target for treatment options.

We demonstrated transcripts included in the Mito-0 signature are DE in human inflamed IBD tissue. Reduced ATP levels in IBD-affected tissue have been linked to mitochondrial changes that occur before the clinical appearance of disease,\(^{4,17,27}\) however, the effects of this were not well understood. More direct evidence indicating lower mitochondrial ATP production in intestinal inflammation is shown by Bar et al. utilizing mice with the same nuclear but different mitochondrial genomes.\(^{7}\) Mice with polymorphisms in mitochondrial mt-Cox3 and mt-Nd3 genes, leading to elevated ATP production, are protected from chemically induced intestinal inflammation. Bar et al. suggest that increased mitochondrial ATP production
Table 2. Transcripts from the Mito-0 panel differentially expressed in inflamed IBD compared to matched control. Transcripts were sorted by fold-change across three combined cohorts (n=81 patients; GSE4183, GSE14580, GSE38713) (*Denotes transcripts with reported alterations in inflamed IBD).

| Gene   | Gene name                                      | Fold change | Adjusted p-value |
|--------|-----------------------------------------------|-------------|------------------|
| 1*     | LCN2 Lipocalin 2                               | 13.7        | 2.13E−25         |
| 2      | CEMIP Cell migration inducing hyaluronidase 1  | 7.9         | 1.53E−14         |
| 3      | TMEM158 Transmembrane protein 15B (gene/pseudogene) | 5.2  | 6.39E−20         |
| 4      | SLC7A11 Solute carrier family 7 member 11      | 4.5         | 2.96E−34         |
| 5      | PLAU Plasminogen activator, urokinase          | 4.4         | 5.99E−14         |
| 6      | IFIT3 Interferon induced protein with tetratricopeptide repeats 3 | 4.3 | 2.26E−16         |
| 7      | LOXL2 Lysyl oxidase like 2                    | 4.0         | 2.07E−16         |
| 8      | DUSP4 Dual specificity phosphatase 4           | 3.3         | 3.22E−13         |
| 9      | RGS2 Regulator of G protein signaling 2        | 2.8         | 1.93E−09         |
| 10     | TM4SF1 Transmembrane 4 L six family member 1   | 2.7         | 1.65E−09         |
| 11     | VCAN Versican                                  | 2.7         | 2.41E−10         |
| 12*    | AQP3 Aquaporin 3                               | 2.5         | 9.25E−15         |
| 13     | NR4A2 Nuclear receptor subfamily 4, group A, member 2 | 2.5 | 2.08E−05         |
| 14     | TMG2 Transglutaminase 2                        | 2.4         | 1.66E−16         |
| 15     | SERPIN2 Serpin family E member 2               | 2.4         | 2.52E−10         |
| 16     | PIK3R3 Phosphoinositide-3-kinase regulatory subunit 3 | 2.3 | 2.04E−20         |
| 17     | ARL4C ADP ribosylation factor like GTPase 4C   | 2.2         | 7.12E−08         |
| 18     | ANXA3 Annexin A3                               | 2.0         | 1.81E−05         |
| 19     | FLRT3 Fibronectin leucine rich transmembrane protein 3 | 2.0 | 1.07E−04         |
| 20     | LAMA3 Laminin subunit alpha 3                  | 2.0         | 1.14E−05         |
| 21     | PMAIP1 Phorbol-12-myristate-13-acetate-induced protein 1 | 2.0 | 4.90E−06         |
| 22     | TM4SF18 Transmembrane 4 L six family member    | 2.0         | 1.90E−09         |
| 23     | SERPINI1 Serpin family member I member 1       | 1.9         | 2.84E−07         |
| 24     | SLC7A1 Solute carrier family 7 member 1        | 1.9         | 8.67E−16         |
| 25     | TESC Tescalcin                                 | 1.9         | 5.43E−12         |
| 26     | TSC22D3 TSC22domain family member 3            | 1.9         | 8.92E−06         |
| 27     | UCP2 Uncoupling protein 2                      | 1.9         | 2.75E−05         |
| 28     | ZBED2 Zinc finger BED-type containing 2        | 1.9         | 2.51E−06         |
| 29*    | ALCAM Activated leukocyte cell adhesion molecule | 1.8 | 1.30E−11         |
| 30     | ETS1 ETS proto-oncogene 1, transcription factor | 1.8 | 1.43E−07         |
| 31     | NRP1 Neurulin 1                                | 1.8         | 5.61E−05         |
| 32     | VIM Vimentin                                   | 1.8         | 3.30E−04         |
| 33     | CD59 CD59 molecule                             | 1.7         | 9.87E−08         |
| 34     | PLK2 Polo like kinase 2                        | 1.7         | 4.06E−05         |
| 35     | WWTR1 WW domain containing transcription regulator | 1.7 | 1.13E−05         |
| 36     | IFIT2 Interferon induced protein with tetratricopeptide repeats 2 | 1.6 | 4.74E−03         |
| 37     | LAMB1 Laminin subunit alpha 3                  | 1.6         | 1.35E−07         |
| 38     | PRF1 Perforin                                  | 1.6         | 3.60E−05         |
| 39     | RASSF8 Ras association domain family member 8  | 1.6         | 1.00E−03         |
| 40     | TNS4 Tensin 4                                  | 1.6         | 9.30E−07         |
| 41     | TIMP2 TIMP metalloproteinase inhibitor 2       | 1.6         | 5.24E−07         |
| 42     | ZCCHC24 Zinc finger CCHC-type containing 24    | 1.6         | 2.85E−05         |
| 43     | IGFBP6 Insulin like growth factor binding protein 6 | 1.5 | 1.32E−04         |
| 44     | ALDH1A3 Aldehyde dehydrogenase 1 family member A3 | 1.4 | 1.94E−03         |
| 45     | CLIP4 CAP-Gly domain containing link protein member 4 | 1.4 | 9.41E−04         |
| 46     | GLIS3 GLIS family zinc finger 3                | 1.4         | 8.16E−04         |
| 47     | FAT4 FAT atypical cadherin 4                   | 1.4         | 9.53E−04         |
| 48     | MAP1B Microtubule-associated protein 1b        | 1.4         | 6.80E−03         |
| 49     | MUC13 Mucin 13                                 | 1.4         | 9.91E−03         |
| 50     | SAMD4A Sterile alpha motif domain containing 4A | 1.4 | 1.35E−05         |
| 51     | ANTXR2 ANTXR cell adhesion molecule 2          | 1.3         | 1.33E−04         |
| 52     | LURAP1L Leucine rich adaptor protein 1 like    | 1.3         | 1.93E−02         |
| 53     | TMTC2 Transmembrane and tetratricopeptide repeat containing 2 | 1.3 | 6.46E−03         |
| 54     | EML6 EMAP like 6                               | 1.2         | 1.68E−02         |
| 55     | EPHA4 EPH receptor A4                          | 1.2         | 1.21E−02         |
| 56     | MILR1 Mast cell immunoglobulin like receptor 1 | 1.2         | 3.72E−03         |
| 57     | SH3TC2 SH3 domain and tetratricopeptide repeats 2 | 1.2 | 4.77E−04         |
| 58*    | TLR6 Toll-like receptor 6                      | 1.2         | 8.79E−04         |
| 59     | ZBTB18 Zinc finger and BTB domain containing 18 | 1.2 | 1.67E−03         |
| 60     | CALB2 Calbindin 2                              | 1.1         | 4.14E−02         |
Figure 3. Bioinformatics assessment of bacterial TLR4 and NOD2 signaling pathway in human IBD inflamed transcriptomes. (a,b) Presence of the TLR4 and NOD2 signature (comprised of 98 and 65 specific transcripts, respectively) scores in inflamed IBD compared to non-inflamed matched control transcriptomes ($p = 1.81 \times 10^{-8}$ TLR4; $p = 1.12 \times 10^{-8}$ NOD2). (c,d) Principal component analysis (PCA) of inflamed IBD and matched non-inflamed control transcriptomes with the TLR4 and NOD2 signatures was performed to estimate variation between samples. Two axis values of the PCA showed that both the TLR4 and NOD2 signatures could significantly differentiate inflamed IBD from matched non-inflamed control (PCA, $p = 3.28 \times 10^{-8}$ TLR4; $p = 9.91 \times 10^{-8}$ NOD2).

Figure 4. Bacterial TLR4 and NOD2 signaling strongly correlate with mitochondrial dysfunction in IBD transcriptomes. (a, b) Scatterplots representing the TLR4 and NOD2 signature scores according to the Mito-0 signature score and distribution of inflamed IBD and matched non-inflamed control transcriptomes; Spearman's coefficient of correlation, $r = 0.92$ for both TLR4 and NOD2, $p < .05$. 
could provide metabolic energy for IC proliferation resulting in efficient restitution of barrier function. Similar suggestions were made by Cunningham et al. after finding that mitochondrial Peroxisome Proliferator-activated Receptor γ Coactivator 1α (PGC1α), a nuclear encoded protein critical for mitochondrial biogenesis, was protective in a mouse model of intestinal inflammation. We demonstrated that transcripts from the Mito-0 panel are critical for regulation of not only proliferation but also diverse cellular function such as extracellular matrix, cell–cell contact, and cytoskeleton regulation. Moreover, among transcripts validated in three different IBD cohorts a few have recently been implicated in intestinal inflammation including activated leukocyte cell adhesion molecule (ALCAM) and toll-like receptor 6 (TLR6), most likely acting through Th1/Th17 immune responses. However, the majority of validated transcripts from the Mito-0 panel were novel with unidentified roles in IBD. Thus, it is plausible that dysfunctional mitochondrial energy production have systemic effects on tissue that could trigger and drive IBD pathobiology.

Currently, we showed that in inflamed IBD the IC Mito-0 signature significantly correlated with active bacterial TLR4 and NOD2 signaling. The effect of gut bacteria triggered signaling on IC mitochondrial function and possible roles in intestinal homeostasis and disease development is understudied. Gut pathogens are capable of destabilizing host cell mitochondrial functions related to calcium signaling for their survival and transmission. Similarly, under normal circumstances select gut pathobionts tend to control mitochondrial activity in favor of infection and inflammation through the production of hydrogen sulfide (H2S) and nitrogen oxide (NO). Also, gut commensal bacteria metabolites, including short-chain fatty acids (SCFA) and secondary bile acids, could affect host IC mitochondrial energy functions in homeostasis and during inflammation. Furthermore, bacterial LPS along with induced inflammatory TNF and IL-6 also have been shown to impair mitochondria ATP production. On the other hand, mitochondrial functions might modify the gut microbiota composition due to alterations in mitochondrial innate immune responses. For example, polymorphisms in mt-ND5 and mt-CYTB have been shown to induce specific gut microbiota compositions, which could play a role in inducing inflammation. Also, altered mitochondria function could influence intestinal immune cells, which in turn can affect gut microbiota composition. We demonstrated before that reduced mitochondrial energy function leads to increased inflammatory mediators and elevated TLRs signaling. Additionally, recent work has demonstrated that loss of barrier function to commensal bacteria triggered by dysfunctional mitochondrial activity included aberrant NOD2 function, suggesting an important role for mitochondrial-NOD2 regulation in maintaining IC homeostasis and restricting inflammatory processes. Nevertheless, our findings reveal that in human inflamed IBD reduced mitochondrial energy function is strongly linked not only to TNF signaling, but also to signaling triggered by bacterial components, highlighting a direct role for mitochondria in sensitizing the intestine to commensal bacteria and exacerbating inflammation.

Reduced mitochondrial function has been implicated in driving disease activity in a number of inflammatory disorders including multiple sclerosis, rheumatoid arthritis, and IBD. We determined in human IBD tissue transcriptomes the significant presence of a mitochondrial signature mediated by aberrant energy function that highly corresponds with activation of bacterial pattern recognition receptors, TLR4 and NOD2. These comprehensive analyses describe a new bacterial-mitochondrial-inflammatory axis and identified novel transcripts altered in inflamed IBD tissue across independent patient cohorts. Collectively this furthers our understanding of the complex pathophysiology underlying IBD and provides the basis for new targeted approaches for therapeutic intervention.

**Materials and methods**

**Patient cohorts**

We accessed publicly available transcriptomes (Illumina, RNAseq) from inflamed or matched non-inflamed control intestinal tissue from patients diagnosed with IBD from the NCBI GEO data repository (GSE107593). In total, 48 patient samples were utilized for analysis. Also, for validation of transcript expression, publicly available transcriptomes from an additional 81 IBD tissue samples (Affymetrix, microarray) from
three independent cohorts were utilized (NCBI GEO: GSE4183, GSE14580, GSE38713).

Microarray processing and differential expression

To validate transcript expression in additional IBD patient transcriptomes, we generated a single list of DE transcripts using microarray data from three independent IBD cohorts (GSE4183, GSE38713, GSE14580). To reduce for batch effect and inconsistencies among probe identifiers, only cohorts utilizing the same microarray platform (Affymetrix Human Genome U133 Plus 2.0 Array platform) were used. Data were initially processed and normalized using the robust multi-array average method (justRMA) from the affy Bioconductor package (v. 1.60.0). Differential expression testing between IBD and control samples was performed using the limma package (v. 3.38.2). Briefly, linear models for each gene were fit using the lmFit function and empirical Bayes smoothing was applied to standard errors using the eBayes function. Duplicate genes were removed and only those genes meeting an adjusted $p < 0.05$ were included in analysis.

RNAseq processing and differential expression testing

Data analysis was performed in the Tulane Cancer Center Next Generation Sequence Analysis Core using core computational resources (www.tulane.edu/som/cancer/research/core-facilities/cancer-crusaders). Raw RNAseq reads (GSE107593) were mapped to an index containing the human haploid genome sequence (Genome Reference Consortium homo sapiens genome build 38, GRCh38). The software program, RSEM (v.1.2.25) was utilized to quantify RNAseq data. Transcript reads as measured by Fragments Per Kilobase of transcript per Million mapped reads (FPKM) were utilized for subsequent transcriptome analysis.

Generation of the transcriptional signature representing reduced mitochondrial function

For generating the transcriptional signature specific for ICs with reduced mitochondrial energy function (Mito-0) we utilized the DEGseq R package to calculate differential expression and fold change differences between the two conditions; Sequence Read Archive (SRA) SRP093357. The Bonferroni correction was applied to adjust for multiple hypothesis testing. The created signature contains select transcripts with an adjusted $p < 0.001$ and an absolute value of log$_2$ fold-change $> 2$.

Hierarchical clustering

Hierarchical clustering of transcriptomes from tissue samples was performed with an uncentered correlation as a symmetric matrix and Pearson correlation as the similarity measure. Complete linkage method was performed utilizing Cluster3 software and the corresponding heatmaps were visualized with JavaTree software.

Principal component analysis (PCA)

PCA of inflamed and non-inflamed control IBD transcriptomes were performed using the FactoMineR R package with PCA function. The first two coordinates of samples and their percentage of variation were extracted and plotted.

Gene signature score

For summarizing gene expression of the Mito-0 signature into a single score, the following formulation was applied:

$$Score = \text{mean} \left[ \log_2 \left( \frac{x + 1}{m} \right) \right]$$

with $x$ representing the expression value of the transcript and $m$ representing the median of the transcripts among all samples according to the approach employed by Agrawal et al.

Spearman’s correlation and statistical tests

Spearman’s correlation test was performed with the cor.test function in ‘R’ with “spearman” method. As a non-parametric statistical test, a Kruskal–Wallis test was utilized with the kruskal.test function in ‘R’ for all the statistical comparisons.
Conflict of Interest

Suzana D. Savkovic wishes to disclose ownership in Pegasus Biosolution, LLC. No other conflicts of interest, financial or otherwise, are declared by the authors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work is supported by a NIH RO1 National Cancer Institute [CA160809]; National Institutes of Health [P01CA214091];National Institutes of Health (US) [STLTR001418-04].

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