# Appendix

**Appendix for**

**Exosome-like nanoparticles from Mulberry bark prevent DSS-induced colitis by AhR/COPS8 pathway**

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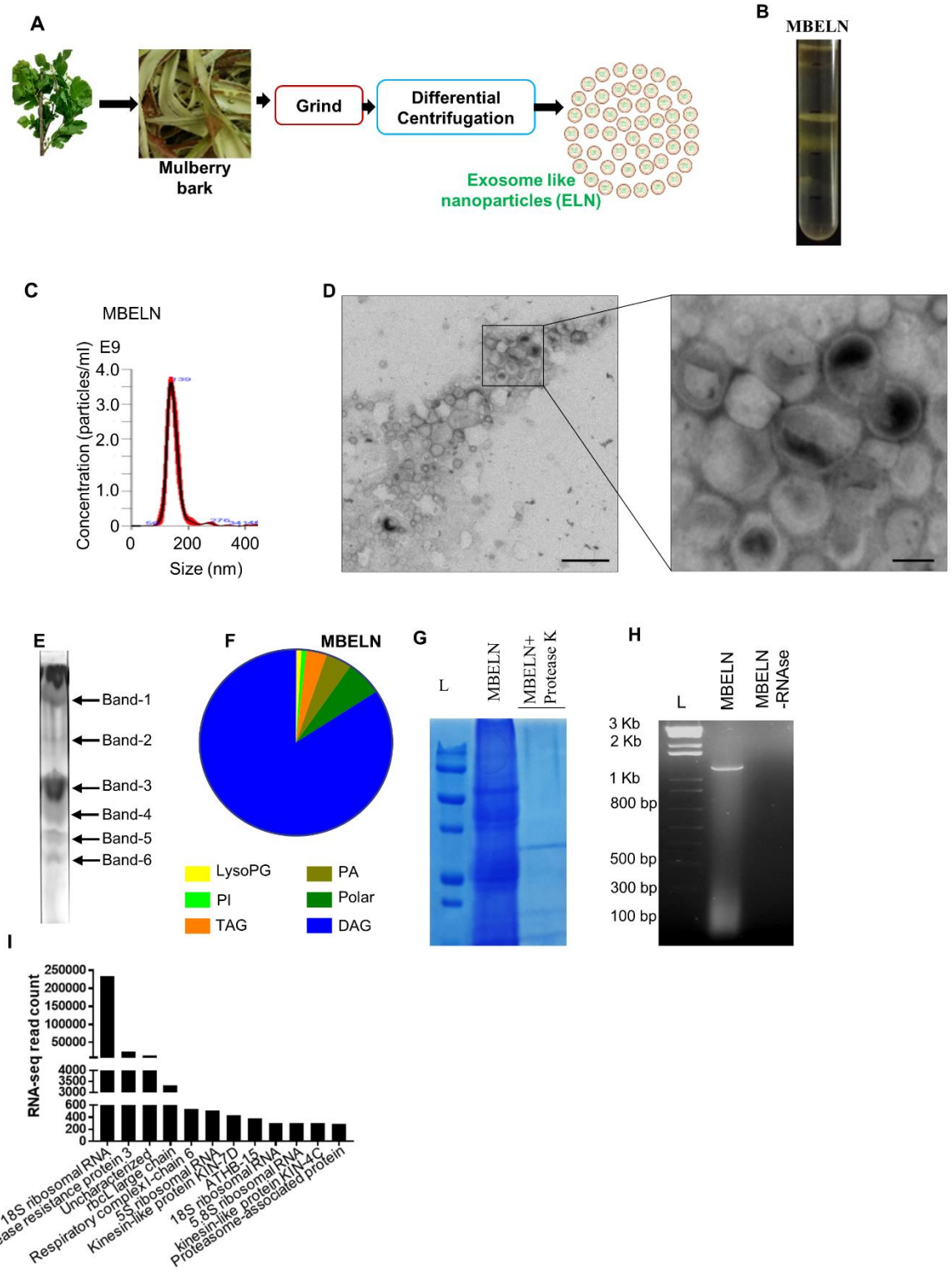
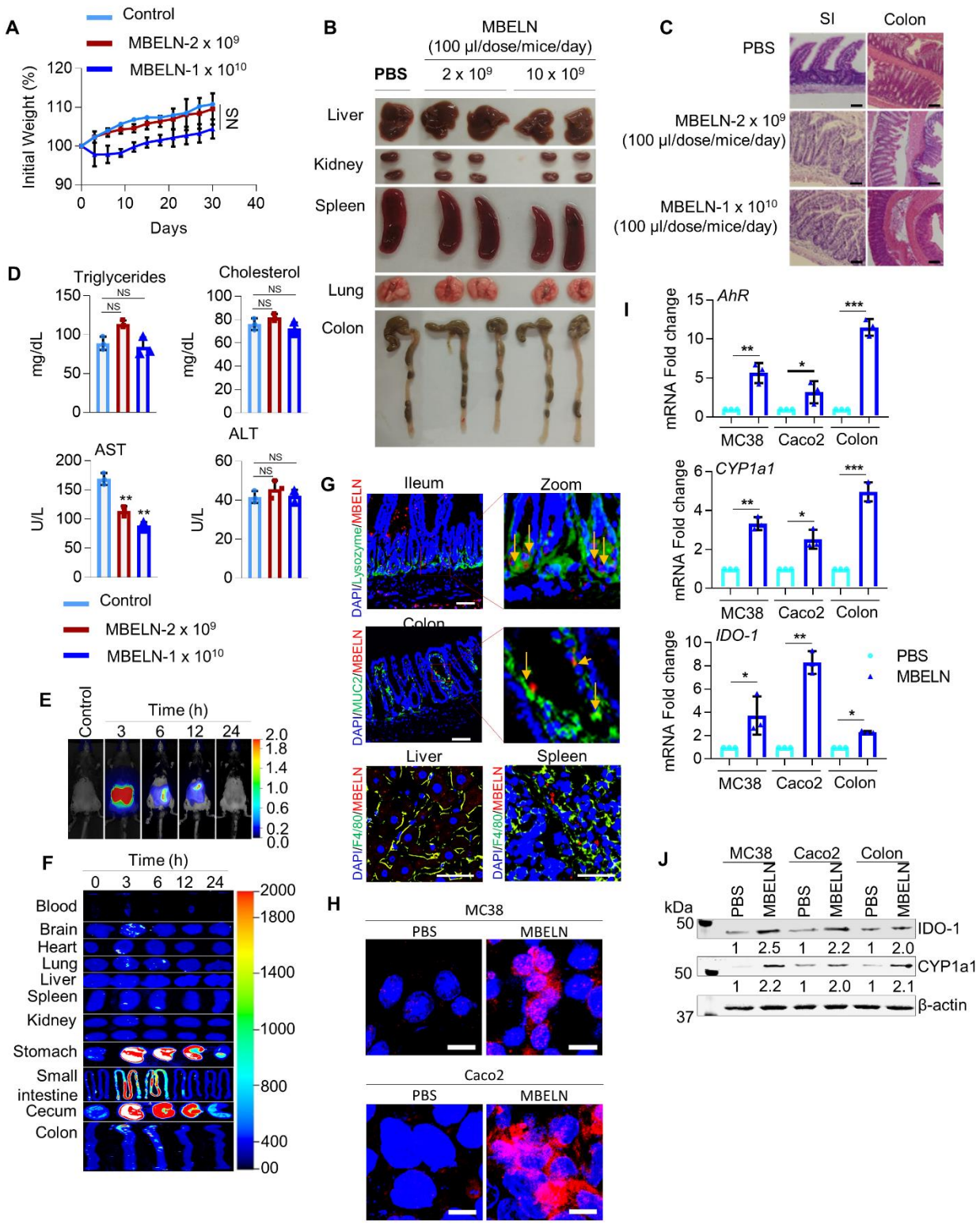
## Appendix figures

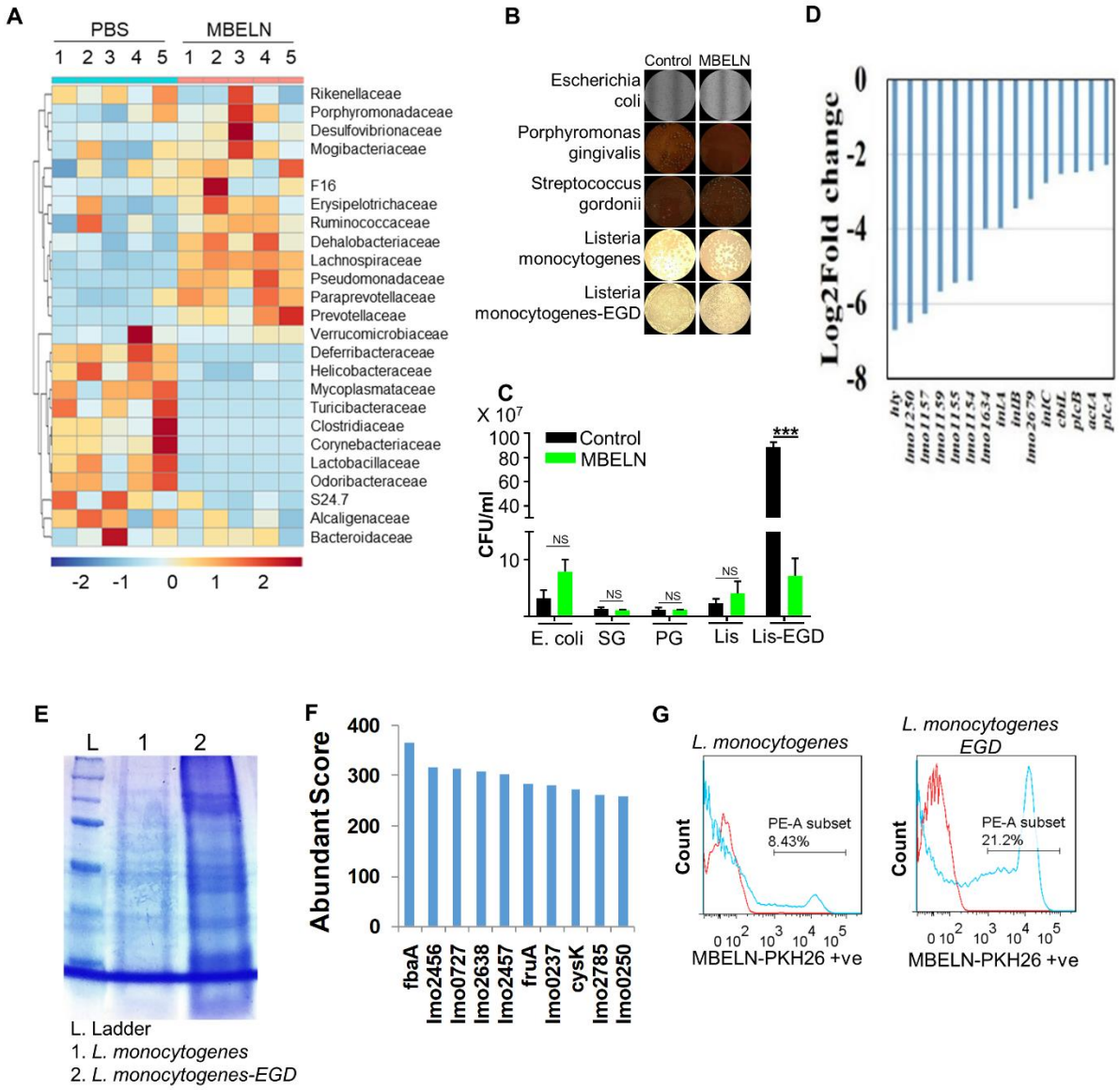



**Appendix fig S1. Preparation, characterization and phenotypic effect of mulberry bark derived exosome-like nanoparticles (MBELN).**
(A) Schematic diagrams of the MBELN preparation process.

(B) Size of MBELNs on the sucrose gradient. (n=3 biological replicates)
(C) Graph showing size of MBELNs using NS300 NanoSight. (n=3 biological replicates)
(D) Electron microscopy picture showing bilayer structure and size of MBELNs. Scale bar, 500μm (zoom scale bar 100 μm). (n=3 biological replicates)
(E) Thin layer liquid chromatography for lipid derived from MBELNs. (n=3 biological replicates)
(F) Lipid profile showing differential lipid composition in MBELNs. (n=3 biological replicates)
(G) Polyacrylamide gel electrophoresis showing presence of protein in MBELNs. (n=3 biological replicates)
(H) Visualization of total RNA on 1% agarose gels. (n=3 biological replicates)
(I) mRNA Seq analysis of MBELN derived RNA. (n=3 biological replicates)


A
Control
MBELN-2 x 10^9
MBELN-1 x 10^10
Initial Weight (%)
Days
NS
B
MBELN
(100 µl/dose/mice/day)
PBS
2 x 10^9
10 x 10^9
Liver
Kidney
Spleen
Lung
Colon
C
SI
Colon
PBS
MBELN-2 x 10^9
(100 µl/dose/mice/day)
MBELN-1 x 10^10
(100 µl/dose/mice/day)
D
Triglycerides
Cholesterol
AST
ALT
mg/dL
U/L
NS
Control
MBELN-2 x 10^9
MBELN-1 x 10^10
E
Control
Time (h)
3
6
12
24
F
Time (h)
Blood
Brain
Heart
Lung
Liver
Spleen
Kidney
Stomach
Small intestine
Cecum
Colon
G
Ileum
Zoom
Colon
Liver
Spleen
DAPI/Lysozyme/MBELN
DAPI/MUC2/MBELN
DAPI/F4/80/MBELN
H
MC38
PBS
MBELN
Caco2
I
AhR
CYP1a1
IDO-1
mRNA Fold change
MC38
Caco2
Colon
PBS
MBELN
J
MC38
Caco2
Colon
kDa
IDO-1
CYP1a1
β-actin

**Appendix fig S2.**
(A) Graph showing changes in initial weight of mice while treated with mulberry bark derived exosome-like nanoparticles (MBELN) at two different doses. Data are mean ± SEM of five biological replicates, NS- non-significant using Mann–Whitney test.
(B) Pictorial representation of morphological changes in internal organs while mice are treated with MBELN. (n=5 biological replicates)
(C) Hematoxylin and eosin (HE) staining to show histological changes due to MBELN administration. Scale bar 100μm, n=5 biological replicates.
(D) Graph showing plasma level of triglycerides, total cholesterol, aspartate transaminase (AST) and alanine aminotransferase (ALT) in mice treated with MBELN. Data are mean ± SEM of three biological replicates **P<0.01, NS- non-significant using one-way ANOVA.
(E) *In vivo* imaging showing trafficking of DiR labeled MBELN. (n=5 biological replicates)
(F) *Ex vivo* imaging showing localization of DiR labeled MBELNs in different organs. (n=5 biological replicates)
(G) Distribution of PKH26 labelled MBELNs *in vivo* in C57BL/6 mice ileum (Paneth cells-yellow arrow), colon, liver and spleen tissues following oral administration of MBELNs. Scale bar, 100μm (n=3 biological replicates).
(H) Confocal microscopy showing expression of total AhR in MC38 cells and Caco2 cells while being treated with MBELNs. Scale bar, 10μm (n=3 biological replicates).
(I) *mRNA* expression of aryl hydrocarbon receptor (*AhR), Cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1a1*) and Indoleamine 2,3-dioxygenase (*IDO-1*) in MC38, Caco2 cells and colon epithelial cells. Data are mean ± SEM. (n=3 biological replicates) *P<0.05, **P<0.01, ***P<0.001 using Student's t-test.
(J) Western blot analysis of CYP1a1 and IDO-1 in MC38 cells, Caco2 cells and mice colon epithelial cells following administration of MBELN. Data are mean ± SEM of three biological replicates, Student's t-test.




**Appendix figure S3.**

(A) Effect of Mulberry bark derived exosome-like nanoparticles (MBELN) on microbiome profile of fecal bacteria at family level. (n=5 biological replicates)

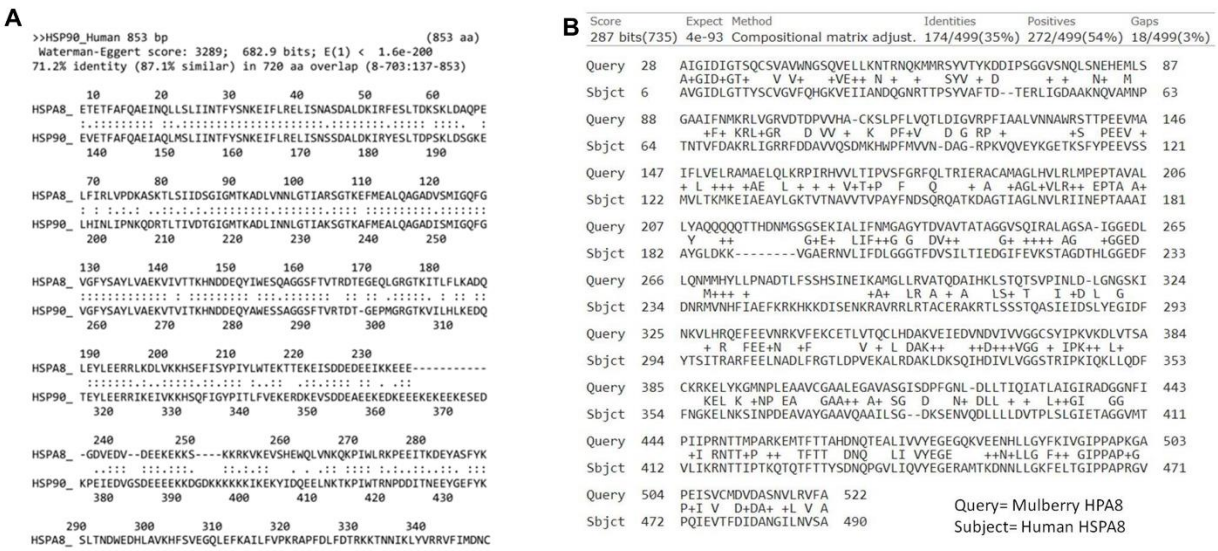
(B-C) Bacteriostatic effect of MBELNs evaluated on *Escherichia coli* (E. coli), *Porphyromonas gingivalis* (PG), *Streptococcus gordonii* (SG), *Listeria monocytogenes* (Lis) and *Listeria monocytogenes-EGD* (Lis-EGD). Data are mean ± SEM. ***p < 0.001, NS- non-significant using Student's t test.

(D) Change in mRNA level in *Listeria (L.) monocytogenes-EDG* compared to control (no treatment). (n=3 biological replicates)

(E) Virulent vs non-virulent Listeria proteins that bind with biotin labelled MBELNs and shown on an agarose gel. (n=3 biological replicates)

(F) Mass spectrometry (MS) analysis of protein samples: *Listeria (L.) monocytogenes* and *L. monocytogenes-EGD* total protein binding with biotin labelled MBELN protein. Top 10 most

abundant proteins from the virulent strain of *L. monocytogenes* shown interacting with MBELNs. (n=3 technical replicates)
(g) Uptake of PKH26 labelled MBELNs in the virulent and non-virulent strains of *L. monocytogenes*. (n=3 biological replicates)




**Appendix fig S4.**

(A) Comparison and alignment of amino acid sequence of mulberry heat shock protein family A (Hsp70) member 8 (HSPA8) protein and human heat shock protein HSP90aa1.

(B) Comparison and alignment of amino acid sequence of mulberry and human heat shock protein family A (Hsp70) member 8 (HSPA8) protein.

**Appendix Table S1: List of primers**

| Gene name | Forward sequence (5'-3') | Reverse sequence (5'-3') |
|---|---|---|
| **Real-time PCR primers sequence for Human** | | |
| AhR | CAAATCCTTCCAAGCGGCATA | CGCTGAGCCTAAGAACTGAAAG |
| CYP1a1 | ATCCTGGAGACCTTCCGACA | ACAAAGACACAACGCCCCTT |
| IDO1 | TTCAGTGCTTTGACGTCCTG | TGGAGGAACTGAGCAGCAT |
| **Real-time PCR primers sequence for Mouse** | | |
| AhR | GGCTTTCAGCAGTCTGATGTC | CATGAAAGAAGCGTTCTCTGG |
| CYP1a1 | CCTCATGTACCTGGTAACCA | AAGGATGAATGCCGGAAGGT |
| IDO1 | TGAGCATTGCAAGGAAAGTG | TATAGGCCATCAGGCAGTCC |
| Defa-rs1 | CACCACCCAAGCTCCAAATACACAG | ATCGTGAGGACCAAAAGCAAATGG |
| Defa1 | TGCCTGCTCATCCTAATCC | GCTCCTCAGTTTTAGTCTCTTC |
| Defa22 | TCCAAAACACAGATGAAGAGAC | GGCAGATCAGATCTCTCGAC |
| Defa21 | CCAGGGGAAGATGACCAGGCTG | TGCAGCGACGATTTCTACAAAGGC |
| CRS1C | CACCACCCAAGCTCCAAATACACAG | ATCGTGAGGACCAAAAGCAAATGG |
| CRS4C | TCGCAGCCATGAAGAAAC | CAAAAGAGACAGACACAGCC |
| Reg3b | TTCCTGTCCTCCATGATCAAAA | CATCCACCTCTGTTGGGTTCA |

| | | |
|---|---|---|
| REG3g | ATGCTGCTCTCCTGCCTGATG | CTAATGCGTGCGGAGGGTATATTC |
| Ang4 | ACTCTGGCTCAGAATGAAAGGT | TCACAGTATCTGTCGTCCCG |
| LYZ2 | TCAGCACGAGAGCAATTATAAC | TTGCCATCATTACACCAGTATC |
| COPS8 | AAGGAGACGCGCCTTTGCC | GTGGAAGAGGCTGTGAAAGGC |
| TNF-α | TCTATGGCCCAGACCCTCAC | GACGGCAGAGAGGAGGTTGA |
| IL-17A | TTTAACTCCCTTGGCGCAAAA | CTTTCCCTCCGCATTGACAC |
| IFN-γ | TCAGCAACAGCAAGGCGAAAAAGG | CCACCCCGAATCAGCAGCGA |
| **Gene cloning primers** | | |
| Human AhR | ATCATCATCACAGCAGCGGCGCTCTGAATGGCTTTGTATTAGTTG | AAGGTTCTTTAAGTAATCTGTCACGTATTTCGTTTTCGTAAATGC |
| HSPA8 (Mulberry) | AAAATCTATACTTCCAAGGAGACTACAAAGACGATGACGACAAGATGGCTGAACAAGCATACAC | TAGCAGCCTGTACTGAGGGACTATATTTTCTTCTGAACACTGACC |

AhR: aryl hydrocarbon receptor, CYP1a1: cytochrome P450 family 1 subfamily A member 1; Ahrr: aryl-hydrocarbon receptor repressor; Arnt: aryl hydrocarbon receptor nuclear translocator; IDO1: indoleamine 2,3-dioxygenase 1; Defa-rs1: defensin, alpha, related sequence 1; Defa: defensin, alpha; CRS: cryptdin-related sequence; Reg: regenerating islet-derived; Ang: angiogenin; LYZ: lysozymes; COPS8: COP9 constitutive photomorphogenic homolog subunit 8; HSPA8: heat shock protein family A (Hsp70) member 8; ZO-1: Zonula occludens-1; TNF-α: Tumor necrosis factor-α; IL-17A: Interleukin 17A; IFN-γ: Interferon gamma;.

**Appendix table S2: List of Antibodies and their details**

| S. No. | Antibody- | Application; | Company | (Catalog) |
|---|---|---|---|---|
| 1. | COPS8- | WB, IF, IP; | Abcam | ab77300 |
| 2. | COPS5- | WB; | BIOMOL | PW8365 |
| 3. | COPS6- | WB; | Santa Cruz | Sc-393023 |
| 4. | COPS7- | WB; | Santa Cruz | sc-398882 |
| 5. | Cullin 1- | WB; | EPITOMICS | 2436-1 |
| 6. | Cullin 3- | WB; | EPITOMICS | 2506-1 |
| 7. | CYP1a1- | WB; | Invitrogen | PA5-15213 |
| 8. | IDO-1- | WB; | Proteintech | 66528-1-IG |
| 9. | AhR- | WB, IF, IP; | Invitrogen | MA1-514 |
| 10. | Nf-kB- | WB; | BD Biosciences | 610869 |
| 11. | β-actin- | WB; | Santa Cruz | sc-47778 |
| 12. | α-tubulin- | WB; | Santa Cruz | sc-5286 |
| 13. | Zonula occludens-1 (ZO-1)- | WB; | Invitrogen | 33-9100 |
| 14. | IL-17a- | ELISA; | eBioscience™ | 14-7175-81 |
| 15. | IL-6- | ELISA; | eBioscience™ | 14-7069-81 |
| 16. | TNF-α- | ELISA; | eBioscience™ | 14-7325-81 |
| 17. | IL-10- | ELISA; | eBioscience™ | 14-7101-81 |
| 18. | IL-1b- | ELISA; | eBioscience™ | 14-7012-81 |
| 19. | IFN-γ- | ELISA; | eBioscience™ | 14-7313-81 |
| 20. | PE anti-mouse IL-17A- | FC; | BioLegend | 506904 |
| 21. | APC anti-mouse IL-17A- | FC; | BioLegend | 506916 |
| 22. | FITC anti-mouse IL-17A- | FC; | BioLegend | 506908 |
| 23. | FITC anti-human IFN-γ- | FC; | BioLegend | 502507 |
| 24. | APC anti-human IFN-γ- | FC; | BioLegend | 506510 |
| 25. | PE anti-mouse FOXP3- | FC; | BioLegend | 126404 |
| 26. | APC anti-CD11b- | FC; | BioLegend | 101212 |
| 27. | FITC anti-CD11b- | FC; | BioLegend | 101206 |
| 28. | PE anti-CD11b- | FC; | BioLegend | 101208 |
| 29. | APC anti-CD4- | FC; | BioLegend | 100412 |
| 30. | FITC anti-CD4- | FC; | BioLegend | 100406 |
| 31. | PE anti-CD4- | FC; | BioLegend | 100512 |
| 32. | PE/Cyanine7 anti-CD3- | FC; | BioLegend | 100220 |
| 33. | APC/Cyanine7 anti-CD3- | FC; | BioLegend | 100222 |
| 34. | PE/Cyanine7 anti-Gr-1- | FC; | BioLegend | 108416 |
| 35. | FITC anti-Ly-6G- | FC; | BioLegend | 127606 |
| 36. | PE anti-Ly-6G- | FC; | BioLegend | 127608 |
| Abbreviation: WB-Western blot; IF-Immunofluorescence; IP-Immuno-precipitation; FC- Flow cytometry | | | | |