Milk-derived extracellular vesicles alleviate ulcerative colitis by regulating the gut immunity and reshaping the gut microbiota

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Figure S1. Gene Ontology analysis of the differentially expressed proteins in mEVs.
Figure S2. The signaling pathways in ulcerative colitis potentially targeted by mEV miRNAs.
Figure S3. Uptake of mEVs by RAW264.7 cells. The nucleus was labeled with DAPI (blue), actin filaments were labeled with FITC Phalloidin (green), and mEVs were labeled with PKH26 (red). (A) PKH26-labeled mEVs (500 μg/mL) were added into confocal dishes (5 × 10^4 cells/dish) and incubated at 37 °C for 4 h; PBS-PKH26 (free...
dye) was used as the control. (B) The effect of mEV dose (0 - 500 μg/mL) on uptake of mEVs. (C) The effect of incubation time (1, 2, 4, 8 and 16 h) on uptake of mEVs (200 μg/mL). (D) The dose and time-dependent curves of mEV uptake by RAW264.7 cells. The fluorescent intensity of PKH26 (mEV labeling) was quantified using Image J (n = 3).
Figure S4. Immunomodulatory effects of mEVs in vitro. (A) A schematic diagram illustrating the experimental steps in vitro. (B) The cell viability assessed by MTT assay. (C, D) The effects of mEVs on the production of NO and PGE2. (E-G) mRNA expression of immune cytokines. GAPDH was used as the reference gene. (H-J) Secreted protein levels of cytokines IL-1β, IL-6 and TNF-α in the culture medium. Data were obtained from three independent experiments and presented as mean ± SD (n = 3). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. LPS model group.
Figure S5. Representative images showing changes in the morphology of LPS-stimulated RAW264.7 cells. Cells were incubated in the presence or absence of mEVs for 8 h. Images were taken under fluorescent inverted microscope. Scale bars represent 100 μm.
Figure S6. *In vivo* biodistribution of mEVs after oral administration. (A) Accumulation of mEVs in different organs at different time points. Mice were gavage-administered DiR–labeled mEVs (0.5 mg/mouse) and imaged over 12 h by IVIS imaging system. To visualize the various amounts of mEVs, different scale bars were used at different time points. (B) Accumulation of mEVs in different organs (the duodenum, colon and liver) at different time points following a gavage of DiR-labeled mEVs or DiR-labeled PBS (free dye control). The same fluorescence intensity scale bar was applied to all images for easy comparison. (C) Quantitative analysis of fluorescence intensity of mEVs accumulated in different organs using Image J software. \( N = 4 \) mice per time point per treatment group. *\( p < 0.05 \) compared with free dye (DiR-PBS) group.
Figure S7. *In vivo* biodistribution of mEVs after intravenous administration. DiR-labelled mEVs (0.5 mg/mouse) or free dye in PBS (DiR-PBS) were injected through the tail vein. Accumulation of mEVs or free dye in different organs at different time points was analyzed by IVIS imaging system over 12 h.
Figure S8. mEVs inhibit production of immune cytokines in DSS-induced colitis mice. (A-C) Serum levels of IL-1β, IL-6 and TNF-α measured by ELISA kits. Data were presented as mean ± SD (n = 7). (D) MPO activity in colon tissue determined by an ELISA kit. Data were presented as mean ± SD (n = 7). (E, F) Gene expression levels of IL-1β, IL-6, IL-2, IL-22 and TNF-α in the colon determined by qPCR. Data were presented as mean ± SD (n = 5). *p < 0.05, #p < 0.01 and ‡p < 0.001 vs. DSS group.
Figure S9. Correlation analysis of the gut microbiota and immune inflammatory factors. (A) Differentially enriched gut microbiota in each group of mice at the family level by linear discriminant analysis (LDA). (B) Correlation matrix showing the strength of correlation between the gut microbiota (at the family level)-immune inflammatory factors in the colon. Values in cells are Spearman correlation coefficient (Spearman r). Statistical significance was determined for all pairwise comparisons using Spearman’s method. *p < 0.05. Spearman r values range from -0.5 (blue) to 0.5 (red).
## Table S1. EV-associated proteins identified in mEVs.

| Family                        | Protein name                                      |
|-------------------------------|--------------------------------------------------|
| Tetraspanins                  | CD63, CD81, CD82                                 |
| MHC class I                   | MHC class I antigen, MHC class I heavy chain      |
| Complement-binding proteins   | CD59                                             |
| EMMPRIN                       | BSG                                              |
| ESCRT-I/II/III                | TSG101, CHMP*                                    |
| Rab proteins                  | Rab-25, RAB14, Rab18, etc.                       |
| Heat shock proteins           | HSP90, HSP70                                     |
| Annexin                       | Annexin A1, A4, A5, A7, etc.                     |

* The Charged Multivesicular Body Protein (CHMP).
Table S2. Number of mEV proteins involved in the immune inflammation pathways.

| Map_ID   | Map Name                                      | Number of proteins |
|----------|-----------------------------------------------|--------------------|
| bta04014 | Ras signaling pathway                         | 23                 |
| bta05163 | Human cytomegalovirus infection               | 21                 |
| bta04151 | PI3K-Akt signaling pathway                    | 19                 |
| bta04062 | Chemokine signaling pathway                   | 19                 |
| bta04015 | Rap1 signaling pathway                        | 18                 |
| bta04145 | Phagosome                                     | 17                 |
| bta04010 | MAPK signaling pathway                        | 15                 |
| bta04722 | Neurotrophin signaling pathway                | 13                 |
| bta04024 | cAMP signaling pathway                        | 12                 |
| bta04152 | AMPK signaling pathway                        | 10                 |
| bta04621 | NOD-like receptor signaling pathway           | 8                  |
| bta04130 | SNARE interactions in vesicular transport     | 6                  |
| bta04660 | T cell receptor signaling pathway             | 6                  |
| bta04662 | B cell receptor signaling pathway             | 6                  |
| bta04630 | JAK-STAT signaling pathway                    | 5                  |
| bta04620 | Toll-like receptor signaling pathway          | 5                  |
| bta04750 | Inflammatory mediator regulation of TRP channels | 4              |
| bta04370 | VEGF signaling pathway                        | 4                  |
| bta04064 | NF-kappa B signaling pathway                  | 3                  |
| bta04657 | IL-17 signaling pathway                       | 3                  |
| bta04672 | Intestinal immune network for IgA production  | 2                  |
| bta05321 | Inflammatory bowel disease (IBD)              | 2                  |
| bta05320 | Autoimmune thyroid disease                    | 2                  |
| **Total**|                                               | **223**            |
Table S3. Number of mEV miRNAs (Top 100 miRNAs) targeting the immune inflammation pathways/inflammatory bowel disease (IBD).

| Number | miRNA       | Targeting inflammation pathway | Targeting IBD |
|--------|-------------|---------------------------------|---------------|
| 1      | miR-148a    | √                               | √             |
| 2      | miR-186     | √                               |               |
| 3      | miR-27b     | √                               | √             |
| 4      | miR-141     | √                               |               |
| 5      | miR-339b    | √                               |               |
| 6      | miR-125b    | √                               |               |
| 7      | miR-2285t   | √                               |               |
| 8      | miR-151-3p  | √                               |               |
| 9      | miR-423-5p  | √                               |               |
| 10     | miR-375     | √                               |               |
| 11     | miR-152     | √                               | √             |
| 12     | miR-10174-3p| √                               | √             |
| 13     | miR-185     | √                               |               |
| 14     | miR-2478    | √                               |               |
| 15     | miR-660     | √                               |               |
| 16     | miR-429     | √                               |               |
| 17     | miR-182     | √                               | √             |
| 18     | miR-652     | √                               |               |
| 19     | miR-19b     | √                               |               |
| 20     | miR-1839    | √                               |               |
| 21     | let-7a-3p   | √                               |               |
| 22     | miR-106b    | √                               | √             |
| 23     | miR-194     | √                               |               |
| 24     | miR-2284x   | √                               | √             |
| 25     | miR-484     | √                               |               |
| 26     | miR-1260b   | √                               |               |
| 27     | miR-374b    | √                               |               |
| 28     | miR-342     | √                               |               |
| 29     | miR-28      | √                               | √             |
| 30     | miR-6524    | √                               |               |
| 31     | miR-22-5p   | √                               |               |
| 32     | miR-11986b  | √                               | √             |
| 33     | miR-2285bf  | √                               |               |
| 34     | bta-miR-143 | √                               |               |
| 35     | miR-7       | √                               |               |
| 36     | miR-142-5p  | √                               | √             |
Table S4. Primer sequences for qRT-PCR analysis.

| Gene      | Forward Primer Sequence | Reverse Primer Sequence |
|-----------|-------------------------|-------------------------|
| TNF-α     | GCGACGTGGAACTGGCAGAAG   | GCCACAAGCAGGAATGAGAAGAGG|
| IL-1β     | TCGCAGCAGCACATCAAAGAG   | TGCTCATGCTCTCATCTGGAAGG |
| IL-2      | GCAGCTCGCATCCTGTGTCAC   | CTGCTGTGCTCGGTAGAG      |
| IL-6      | ACTTCCATCCAGTTCTTCTTG   | TTAAGCCTCCGACTTTGAAGTG  |
| IL-22     | TCCAACCTCCAGCCAGCATAC   | GCACTGATCCCTTAGCACTGACTCC|
| IL-10     | GAGGATCAAGCAGGGCCAGTAC  | AAGGCAGTCGAGAGCTTAG     |
| TLR-4     | ACAAGGCATGGCATGCTTACAC  | TGTCTCCACAGCCACAGTTTAC  |
| MyD88     | GCTAGAGCTGCTGGCCTTGTTAG | TCTCGGACTCTGTTGCTG      |
| iNOS      | TGCCACGGACGAGACGGATAG   | CTCTTCAAGCACCTCCAGGAAC  |
| NLRP3     | GAGCTGGACCTCAGTGCAATGC  | ACCAAATGCGAGATCCGGACAAC  |
| GAPDH     | GGTGTCTCCTCGGACTTCA     | TGGTCCAGGTTTCTTACTCC    |
Table S5. The mouse dietary ingredients.

| Numbers | Component                  |
|---------|----------------------------|
| 1       | corn                       |
| 2       | soybean meal               |
| 3       | flour                      |
| 4       | wheat middlings            |
| 5       | fish meal                  |
| 6       | plant oil                  |
| 7       | dicalcium phosphate        |
| 8       | limestone                  |
| 9       | salt                       |
| 10      | vitamins                   |
| 11      | mineral elements           |