Supplementary information, Data S1 Materials and Methods

Mice, Ad vectors and reagents

Female C57BL/6 mice, 8-10 weeks of age, were purchased from Joint Ventures Sipper BK Experimental Animal Co. (Shanghai, China) and bred in a specific pathogen-free environment. The animal study protocol was approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China). Replication-defective recombinant adenovirus (Ad) encoding human TGF-β1 was constructed and conserved in our lab. FITC-conjugated anti-mouse CD4; PE-conjugated anti-mouse CD4, IL-17A were from BD PharMingen (San Diego, CA). PE-conjugated anti-mouse MHC-II, CD80, CD86, ICAM-1 and CD11c, PE-Cy5-conjugated anti-mouse Foxp3, Foxp3 staining buffer set, cell stimulation cocktail were from eBioscience (San Diego, CA). Rabbit anti-mouse HSP70, CD9, TGF-β1 and IL-17, goat anti-mouse TSG101, GRP94 and IL-6, HRP-conjugated secondary Abs were from Santa Cruz Biotechnology (Santa Cruz,CA). Anti-human LAP (TGF-β1) antibody was from R&D Systems (Minneapolis, MN). CD25 neutralized antibody (clone PC61.5.3.) was from Abcam (Cambridge, MA). Recombinant human TGF-β1, murine granulocyte/macrophage colony-stimulating factor (rmGM-CSF) and IL-4 were from PeproTech (Rocky Hill, NJ); ELISA kits for TGF-β1, IL-17 and IL-6 were from R&D Systems; Dextran sulfate sodium (mol wt. 36,000-50,000) was from MP Biomedicals (Solon, OH).

DC generation

Murine BM-derived DCs were prepared as described previously[1] with minor modification. Briefly, BM mononuclear cells were prepared from mouse tibia and

femur suspensions by depletion of red cells and cultured at a density of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, 10ng/ml rmGM-CSF and 1ng/ml IL-4. Non-adherent cells were gently washed out after 48 h of culture; the remaining loosely adherent clusters were cultured for another 48 h and harvested for Ad transduction. For Ad infection, 1×10^6 DCs were mixed with 5×10^7 PFU of the viruses in a total volume of 1 ml of serum-free medium. After incubation for 24 h, DCs were washed intensively five times and incubated with fresh medium supplemented with rmGM-CSF and IL-4 for 48 h. On day 8, culture supernatant was collected for exosomes purification.

Exosomes isolation

Exosomes were isolated as previously described[2]. Collected culture supernatants were centrifuged at 300×g for 10 min, 1,200×g for 20 min, and 10,000×g for 30 min sequentially. The supernatant was then ultracentrifuged at 100,000×g for 1 h. The exosome pellet was washed in large volume of PBS and centrifuged at 100,000×g for additional 1 h. Exosomes in the final pellet were resuspended in PBS.

Electron microscopy

Exosomes pellets were fixed in 4% paraformaldehyde, and then loaded onto electro-microscopy grids coated with formvar carbon, contrasted and embedded in a mixture of uranyl acetate and methylcellulose. Sections were observed with a Philips Tecnai-10 transmission electron microscope operating at 80 kV (Phillips Electronic Instruments, Mahway, NJ).

FACS analysis

For FACS analysis of exosomes, 20μg exosomes were incubated with 5μl of 4-μm-diameter aldehyde/sulfate latex beads for 15 minutes at room temperature in a 20-μl final volume followed by gentle shaking for 1 hour in 1ml PBS and then centrifuged. The pellet was blocked by incubation with 20μl FCS for 30 minutes. Exosomes-coated beads were washed thrice in PBS and resuspended in 50μl PBS. Beads were incubated for 1 h with corresponding fluorescent Abs. Cells and beads were analyzed by flow cytometry using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson, Mountain View, CA).

Test for resistance of TGF-β1 contained in exosomes to trypsin digestion

6ng TGF-β1 cytokine and 40μg TGF-β1-EXO containing the same amount of TGF-β1 were digested by trypsin at 37 °C with concentration of 25μg/ml for 15 and 30 min. To access the relationship between the integrality of exosomal membrane and stability of exosomeal TGF-β1, exosomes were disrupted by 1% Triton X-100 for 30 min at 4 °C. Equal amount of disrupted and intact exosomes (30μg) were then subjected to trypsin digestion. After trypsin digestion, the samples were subjected to Western blot analysis. β-Actin was the equal amount exosomes treated without triton and trypsin, which was detected as a loading control.

Western blot analysis

Western blot was preformed as described previously[2]. Briefly, 10μg exosomal or cellular lysate proteins were separated by 10% SDS-polyacryamide gel, and transferred onto polyvinylidene difluoride membrane. After incubating with primary antibody and HRP-coupled secondary antibody sequentially, specific band on the

membrane was visualized with chemiluminescence kit (ECL detection Kit, Amersham Bioscience).

Cytokine assay

The level of TGF-β1 contained in exosomes and murine IL-17 and IL-6 production in serum of the mouse models were detected by ELISA kits according to the manufacturer's instructions.

Mixed lymphocyte reaction

The serial concentrations of exosomes were added into culture of T cells isolated from BALB/c spleen and Control DCs from C57BL/6 at 10:1 ratio. In TGF-β1 blocking assay, TGF-β1-EXO was co-incubated with 1μg/ml TGF-β1 neutralized antibodies (anti-TGF-β1), 1μg/ml anti-CD9 antibodies (anti-CD9) or 1μg/ml isotype antibodies (ISO). [^{3}H]-Thymidine (0.5μCi/well) was added 96 h later, and cells were incubated for further 18 h. [^{3}H]-Thymidine incorporation was assayed by liquid scintillation counting.

Induction and experimental treatment of IBD

Mice were randomized into groups with identical average body weight. IBD was induced by giving DSS in acidified drinking water, 1.5% (w/v) for 9 days. The day that mice started to drink DSS was regarded as day 0. For the treatment of IBD, mice were injected i.v. with exosomes (10μg exosomes/mouse/injection) on days -2, 3, and 5. As control, two groups of mice were fed with DSS only or normal water, respectively. To evaluate the protective effect of TGF-β1 in IBD, mice were injected i.v. with TGF-β1 (150pg or 1500pg/mouse/injection) on days -2, 3, and 5. In some

experiments, $CD25^+$ T cells were depleted by i.p. injection of anti-mouse CD25 mAb (clone PC61.5.3). In brief, the anti-CD25 mAb (200μg/mouse) was administrated on days -8, -6, and -4. Depletion efficacy was verified by staining lymphocytes and then analyzing by FACS.

Assessment of IBD

For all animals, body weight, rectal bleeding and stool consistency were monitored daily. Intestinal bleeding was determined by using one step fecal occult blood test (W.H.P.M. Bioresearch & Technology Co., Ltd, El Monte, CA), as well as by observation of bleeding signs on the anus or gross bleeding. The daily disease activity index (DAI) was calculated by grading on a scale of 0–4 the following parameters: change in weight (0, ≤1%; 1, 1–5%; 2, 5–10%; 3, 10–15%; 4, >15%), intestinal bleeding (0, negative; 4, positive) and stool consistency (0, normal; 2, loose stools; 4, diarrhea). The combined scores were then divided by 3 to obtain the final DAI. Mice were sacrificed nine days following the disease induction and the colon was collected and evaluated for microscopic damage.

Microscopic scoring

Proximal, medial, and distal portions of colon were fixed in 10% phosphate-buffered formalin. Paraffin-embedded sections were stained with hematoxylin and eosin. The evaluation system was used as described previously[3]. The degree of histological damage and inflammation was graded in a blinded fashion. The following parameters were scored: (a) amount of inflammation (0, none; 1, mild; 2, moderate; 3, severe; 4, accumulation of inflammatory cells in the gut lumen); (b)

distribution of lesions (0, none; 1, focal; 2, multifocal; 3, nearly diffuse; 4, diffuse); (c) depth of inflammation and layers involved (0, none; 1, mucosa only; 2, mucosa and submucosa; 3, limited transmural involvement; 4, transmural). The overall histological score was the sum of the three parameters (maximum score: 12).

Analysis of $CD4^+Foxp3^+$Tregs and Th17

To test the function of TGF-β1-EXO induce $CD4^+Foxp3^+$Tregs in vitro, splenocytes were isolated from the spleens of C57 mice and 1×10^6/ml splenocytes were were cocultured with 10μg/ml TGF-β1-EXO, TGF-β1-EXO plus 1μg/ml TGF-β1 neutralized antibodies or 1μg/ml anti-CD9 antibodies in the presence of 0.5μg/ml of anti-CD3 and 1μg/ml of anti-CD28 for 48 h. To analysis of $CD4^+Foxp3^+$ Tregs in vivo, lymphocytes isolated from murine spleens or mesentery lymph nodes (mLNs) were first stained with PE-conjugated anti-mouse CD4 Abs and then the Foxp3 intracellular staining was carried out according to the manufacturer's protocol. To analysis of Th17, lymphocytes were first stimulated with cell stimulation cocktail for 6 h and then stained with anti-mouse CD4 and IL-17 Abs according to the manufacturer's protocol. The percentages of $Foxp3^+$ Tregs or Th17 were determined by FACS analysis.

Statistical analysis

Results were compared using one-way ANOVA and Student's *t*-test. Values of $p < 0.05$ were considered to be statistically significant.

Supplemental References

1 Zhang M, Tang H, Guo Z *et al.* Splenic stroma drives mature dendritic cells to differentiate into regulatory dendritic cells. *Nat Immunol* 2004; **5**:1124-1133.

2 Chen W, Wang J, Shao C *et al.* Efficient induction of antitumor T cell immunity by exosomes derived from heat-shocked lymphoma cells. *Eur J Immunol* 2006; **36**:1598-1607.

3 Aharoni R, Sonego H, Brenner O, Eilam R, Arnon R. The therapeutic effect of glatiramer acetate in a murine model of inflammatory bowel disease is mediated by anti-inflammatory T-cells. *Immunol Lett* 2007; **112**:110-119.