Intestine epithelial cell-derived extracellular vesicles alleviate inflammation induced by *Clostridioides difficile* TcdB through the activity of TGF-β1

Shuangshuang Wan
Hangzhou Medical College
Guangzhong Song
Hangzhou Medical College
Hui Hu
Hangzhou Medical College
Yaqing Xu
Hangzhou Medical College
Peng Zeng
Hangzhou Medical College
Shan Lin
Hangzhou Medical College
Xiaojun Song
Zhejiang Provincial People's Hospital
Dazhi Jin (jind@hmc.edu.cn)
Hangzhou Medical College

Research Article

Keywords: extracellular vesicles, Clostridioides difficile, TGF-β1, TcdB, regulatory T cells, inflammatory cytokines, immunotherapy

Posted Date: February 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-240505/v1

Abstract

Objective: *Clostridioides difficile* infection (CDI) has been primarily associated with the toxin B (TcdB), which can activate the intestinal immune system and lead to pathological damage. Even though the biological functions of intestine epithelial cell-derived extracellular vesicles (I-Evs) have been well documented, the role of I-Evs in the process of CDI is still unknown.

Methods: I-Evs were isolated from mouse intestine tissues by ultracentrifugation protocol, identified by electron microscopy, nanoparticle tracking, sucrose density gradient centrifugation, and western blotting. Intestinal pathological damage was measured after intraperitoneal injection of TcdB into mice.

Results: We isolated I-Evs ranging from 100–200 nm in mean diameter, with a density of 1.09-1.17 g/mL. These I-Evs expressed the extracellular vesicle-associated specific surface markers, CD63 and TSG101. *In vitro,* 50 μg I-Evs decreased the expression of IL-6, TNF-a, IL-1β, and IL-22 induced by 0.8 ng/mL *C. difficile* TcdB, and increased expression of TGF-b1. *In vivo*, I-Evs also promoted regulatory T cell induction, which improved the survival rate of mice up to 80% relative to *C. difficile* TcdB mice, dependent on the TGF-b1 signalling pathway.

Conclusion: As an emerging immunotherapy, I-Evs can reduce the intraperitoneal infection induced by *C. difficile* TcdB and improve survival in mice.

Background

In recent decades, with the excessive application of broad-spectrum antibiotics, diseases related to intestinal flora disorders have precipitously increased. *Clostridioides difficile* (*C. difficile*) is one of the main pathogens of antibiotic-associated diarrhoea and hospital-acquired infections in the United States and other developed countries[1]. Toxin A (TcdA) and B (TcdB) are the major pathogenic factors leading to diarrhoea, pseudomembranous colitis, toxic megacolon, and other intestinal symptoms[2]. The mechanism lies in the inactivation in the host epithelial cells of proteins from the Rho family of GTPases-including Rho, Rac, or Cdc42 by glycosylation, and upregulation of a series of pro-inflammatory cytokines such as interleukin IL-1, IL-6, and TNF-α[3]. Meanwhile, toxins recruit neutrophils and other inflammatory immune cells to induce intestinal mucosal cell apoptosis, necrosis, shedding, and increased permeability, triggering a widespread loss of intestinal barrier function, and initiating imbalance of flora and intestinal epithelial damage. According to the American Infectious Society, and the European Society of Clinical Microbiological Infections, in addition to other practical guidelines, oral metronidazole or vancomycin are the best methods to treat *Clostridioides difficile* infection (CDI)[4]. In addition, some new narrow-spectrum antibiotics such as fidaxomicin[5] and rifaximin have little impact on the intestinal flora and reduce the risk of drug resistance; however prohibitively high prices limit their clinical applications. In recent years, a number of immune-based agents[6] have entered clinical trials; however, their efficacy needs to be further validated. Faecal microbiota transplantation (FMT) has been recognised in the United States as a treatment method to restore normal intestinal flora and prevent recurrent attacks. However, in a meta-

analysis of randomised clinical trials in 2019, the cure rate of FMT was only 76.1%. Furthermore, there are still many unanswered questions about FMT, including the optimal timing, preparation methods, and the patients who are likely to benefit most from this procedure. As its standard protocol is relatively complicated and involves ethical review, FMT has not yet been widely used in our country.

Extracellular vesicles (Evs) are small vesicle-like substances secreted by cells, which possess various biological activities when released outside of the cell. They have a diameter ranging from approximately 30 nm-1 μm, and are generally classified into exosomes, microvesicles, and apoptotic bodies based on their size, biogenesis, and mechanism of secretion[7]. It is difficult to determine the functional differences between these three types of Evs, due to the lack of specific markers with which to distinguish them. Although once thought to be cellular debris, Evs are now recognised as vital vehicles involved in the communication between cells. Research has confirmed that Evs contain a wide range of biologically active components, and their corresponding functions depend on the source tissue or cell type. Evs also exist in body fluids such as serum, alveolar lavage fluid, and breast milk, carry messenger RNAs, microRNAs, and DNA[8,9]; this suggests potential applications as biomarkers for the diagnosis of diseases, as part of a liquid biopsy technology[10]. Recently, it has been reported that Evs can be designed to function as effective carriers in the treatment of various diseases, including in the delivery of long non-coding RNAs[11,12]. In addition, Evs play a significant therapeutic role in regulating complex intracellular pathways in certain diseases, such as inflammatory bowel disease (IBD)[13,14], and osteoarthritis[15]. Furthermore, it has been discovered that Evs derived from mesenchymal stem cells possess important immunomodulatory effects in areas such as neurodegenerative diseases, ageing, and inflammation[16-18]. Previously, we have reported that $CD8\alpha^{+}CD11c^{+}$ Evs derived from lungs reduce the allergic reaction of asthmatic mice through TGF-β1 and IL-10, thereby maintaining the immune balance of the respiratory tract[19]. In the context of the recent outbreaks of COVID-19 around the world, mesenchymal stem cells and their Evs could be used as potential drug candidates for the treatment of severe cases, mainly through the induction of anti-inflammatory macrophages, regulatory T and B cells, and regulatory dendritic cells[20].

Strikingly, infection with TcdB-producing strains alone, but not $TcdA^{+}B^{-}$ strains, can cause severe CDI symptoms[21]. Our work using purified *C. difficile* TcdB, together with cell lines and mice, confirmed that TcdB can induce expression of the inflammatory genes IL-6, TNF-a, IL-22, and IL-1b, and upregulation of TGF-b1 *in vitro*. Intestine epithelial cell-derived extracellular vesicles (I-Evs) rescue this phenomenon *in vivo* by inducing proliferation of regulatory T cells, dependent on TGF-β1 and the corresponding downstream molecules Smad2/3. Here, we established an animal model of intraperitoneal infection induced by *C. difficile* TcdB and proved that I-Evs can improve the survival rate of mice.

Results

Isolation and identification of intestine epithelial cell-derived extracellular vesicles. We used electron microscopy to visualise the morphology of the purified intestine epithelial cell-derived extracellular vesicles (I-Evs); combined with Nanoparticle tracking analysis this showed that the isolated I-Evs had a

mean diameter of 100–200 nm (Figure 1A, B). To further explore the I-Evs, sucrose density gradient centrifugation was used to detect the density range of I-Evs, which was 1.09–1.17 g/mL (Figure 1C). Protein analysis identified that I-Evs were positive for universal surface markers of extracellular vesicles, including CD63 and TSG101, and the intestinal epithelial cell-specific protein A33, but negative for GRP94, as detected by western blot (Figure 1D). In addition, high levels of TGF-β1 were expressed in I-Evs, implying a role in immunoregulation. The results showed that we successfully isolated and identified I-Evs.

I-Evs attenuated the down-regulation of TGF-b1 induced by purified *C. difficile* TcdB *in vitro*. Real-time PCR results showed that, compared to the control group, the expression of pro-inflammatory genes (IL-6, TNF-a, IL-1β, and IL-22) was increased in the 0.4 ng/mL *C. difficile* TcdB group, but significantly decreased in the 0.8 ng/mL I-Evs group. In contrast, the expression of the anti-inflammatory genes TGF-b1 and IL-10 was statistically increased in the I-Evs group compared to the TcdB groups (Figure 2A). Western blot results showed that protein levels of the immunosuppressive cytokine TGF-b1 were decreased in MC38 murine colon carcinoma cells, and LOVO human colon carcinoma cells, stimulated by *C. difficile* TcdB (Figure 2B). The concentration of *C. difficile* TcdB used was 0.1 ng/mL, 0.2 ng/mL, 0.4 ng/mL, or 0.8 ng/mL. This decrease could be rescued by I-Evs (Figure 2C). Altogether, these results indicate that the I-Evs containing TGF-b1 had anti-inflammatory effects *in vitro*.

I-Evs alleviate *C. difficile* TcdB-induced intraperitoneal inflammation in mice. Intestinal epithelial damage, caused by CDI, and leading to intraperitoneal infection, is a severe inflammatory complication. We sought to explore whether I-Evs can be applied in this condition as a type of anti-inflammatory immunotherapy. I-Evs contain more TGF-b1 than intestinal lysates as determined by western blot, which indicated a likely strong immunosuppressive effect. Next, we established a murine intraperitoneal infection model to investigate the treatment effect of I-Evs (Figure 3A). As shown in Figure 3B, the survival rate of mice after *C. difficile* TcdB injection was only 50%, while I-Evs increased the survival rate of mice up to 80%. The intestinal tissues displayed marked leukocyte infiltration and sections of glandular structure damage; consistently, histopathological analysis showed only slight leukocyte infiltration and epithelial cell damage after application of I-Evs (Figure 3C). Moreover, intestinal epithelial damage, congestion and mucosal oedema were significantly increased in the *C. difficile* TcdB mice when compared with the control mice (Figure 3D, E), however, less intestinal damage and limited leukocyte infiltration were observed when mice were treated with I-Evs. These findings implied that I-Evs attenuated pathological changes occurring as a result of *C. difficile* TcdB-induced intraperitoneal inflammation, thereby protecting mice from acute lethal inflammation.

Induction of regulatory T cells by I-Evs alleviated intraperitoneal infection caused by *C. difficile* TcdB through a TGF-β1-dependent mechanism. A previous study showed that EpCAM-dependent I-Evs alleviated IBD by inducing regulatory T cells[22]. I-Evs induced an increase in the proportion of $CD4^+Foxp3^+$Tregs *in vitro* and *in vivo* (Figure 4A–D); these immunoregulatory cells exhibit immunosuppressive effects in the development of disease. When the activity of TGF-β1, a potent immunosuppressive cytokine, was blocked (using the protocol described in the Materials and Methods), I-

Evs immediately lost the ability to induce $CD4^+Foxp3^+$Tregs in the spleen. Concurrently, I-Evs were not able to increase the survival rate of mice, and the improvement of pathological effects previously seen was also undetectable (Figure 4E, F). Together, these results suggest that immunosuppressive regulatory T cells induced by I-Evs attenuated *C. difficile* TcdB- induced intraperitoneal inflammation in a mechanism dependent on TGF-β1. Smad2/3 are the main downstream proteins involved in the TGF-b1 signalling pathway. The phosphorylation levels of Smad2/3 were decreased after stimulation with *C. difficile* TcdB, although protein levels of Smad2/3 remained the same; treatment with I-Evs promoted phosphorylation of Smad2/3, and thereby upregulation of TGF-b1 (Figure 4G). These results suggest that Smad2/3 is inhibited by *C. difficile* TcdB, leading to the down-regulation of TGF-b1 expression. Conversely, I-Evs with high expression of TGF-b1 activate Smad2/3 and contribute to the upregulation of TGF-b1, thereby alleviating *C. difficile* TcdB-induced acute lethal inflammation in mice.

Discussion

A severe comorbidity of CDI is intraperitoneal infection, which may arise due to intestinal perforation after either infection or intestinal surgery, particularly in high-risk populations, such as patients with IBD; respiratory insufficiency; heart and renal failure; ages over 60 years; and several other underlying diseases. The majority of CDI can be treated with metronidazole and fidaxomicin, in addition to other antibiotics. Surgical removal of necrotic intestinal tissue can reduce mortality rates with severe explosive colitis. Nevertheless, postoperative bleeding, and intestinal stenosis and obstruction, are extremely distressing to the patient. Prevention, management, and non-surgical treatment are the fundamental principles of CDI. However, the most severe toxic colitis cases are unable to benefit from drugs and surgery, and there is an urgent need to establish an effective treatment programme based on immunotherapy. Evs participate in a variety of physiological and pathological processes, including neurological disorders[23], osteoarthritis[24], infection[25], and tumours[26]. Evs have been proven to be involved in immune regulation and antigen presentation, and our research group demonstrated that Evs derived from intestinal epithelial cells alleviate IBD in mice by inhibiting dendritic cell activation and inducing Tregs[22]. In this study, I-Evs isolated from the intestine, with mean diameters of 100–200 nm as detected by electron microscopy scanning and Nanoparticle tracking analysis, expressed the characteristic protein markers of Evs, CD63 and TSG101. Enrichment of the immunosuppressive cytokine, TGF-b1, in I-Evs inspired us to hypothesise an immunomodulatory function for I-Evs. A recent study verified that Evs derived from human mesenchymal stem cells can relieve colitis by reducing pro-inflammatory responses and increasing anti-inflammatory responses[27]. As is well-established, colitis caused by *C. difficile* relies on a series of virulence factors, including toxins, which initially target intestinal epithelial cells and subsequently destroys the intestinal membrane integrity. Hosts exposed to intestinal microorganisms trigger immune inflammatory responses. The dominance of either TcdA or TcdB was still controversial in this research field, despite a multi-laboratory follow-up research study pronouncing that TcdB acts as a critical toxin in colonic epithelial injury and mortality *in vivo*, whereas TcdA caused inflammation in mice to a small extent[28]. In the work presented here, TcdB induced increased gene expression of IL-6, TNF-a, IL-1β, and IL-22. I-Evs were able to rescue this phenomenon, and interestingly, TGF-b1 and IL-10 gene

actually increased upon co-incubation with I-Evs. Moreover, I-Evs could reverse the decreased expression of TGF-β1 stimulated by *C. difficile* TcdB, as detected by western blot analysis of MC38 and LOVOlysates. We also report for the first time that I-Evs can improve survival of mice with intraperitoneal inflammation induced by *C. difficile* TcdB. The mechanism lies in the induction of $CD4^+Foxp3^+$Tregs, which play an important role in maintaining immune tolerance and homeostasis; the decline or dysfunction of Tregs has previously been shown to increase intestinal inflammation in IBD mice[29]. Similarly, $CD4^+CD25^+$ Treg cells transferred into hosts ameliorated colitis symptoms.

The I-Evs in this study contained TGF-b1; IL-10 is also known to be an important immunosuppressive cytokine, but we could barely detect the presence of IL-10 in our isolated I-Evs. Whether IL-10 still performs an important function is unknown. Furthermore, proinflammatory cytokines were undetectable following stimulation with *C. difficile* TcdB. Indeed, we improved various experimental schemes to optimise the experimental conditions, unfortunately, the corresponding data were still not available. We speculate that the effect of *C. difficile* TcdB on cells *in vitro* was different from that *in vivo*. As for the animal challenge experiment, in order to induce chronic inflammatory intestinal infection, five antibiotic mixtures were fed to mice, in addition to intraperitoneal injection with clindamycin and *Clostridioides difficile* TcdB. I-Evs improved both the survival of mice, and intestinal tissue pathological scores, when transferred into mice.

In summary, we isolated I-Evs and confirmed that they improve survival of mice with intraperitoneal inflammation induced by *C. difficile* TcdB. This was dependent on the TGF-b1 signalling pathway and the downstream Smad2/3 proteins. As an emerging immunotherapy, though further research is still required, I-Evs may have potential to be a novel treatment target.

Materials And Methods

Toxins, antibodies, and reagents. *C. difficile* TcdB was gifted from the Tao Liang research group (West Lake University, Hangzhou, China)[30]. Primary antibodies against CD63 (ab213090), TGF-b1 (ab8227), GRP94 (ab238126), TSG101 (ab125011), and b-Actin (ab8227) were from Abcam (Cambridge, MA, USA). PRMT1 (A33) (#2449), Smad2 (D43B4), Smad3 (C67H9), phospho-Smad2 (Ser465/Ser467) (E8F3R), and phospho-Smad3 (Ser423/425) were from Cell Signaling Technology (Danvers, MA, USA), and the corresponding secondary antibodies were from BBI (Shanghai, China). Fluorescent-labelled antibodies against CD4 (GK1.5) and Foxp3 (PCH101) were from eBioscience (San Diego, CA, USA).

Real-time fluorescence quantification PCR. The classic TRIzol (Gibco, USA) method was used to extract RNA, using a reverse transcription kit (TOYOBO) to acquire cDNA. Real-time, fluorescence quantification PCR (qRT-PCR) was performed in a Step One Plus Real Time PCR System (Roche) to detect gene expression. The mouse-specific primers used are shown in Appendix A.

Mouse and cell lines. The MC38 cell line was purchased from Wuhan Fine Biotech Co., Ltd. (Wuhan, China). The cells were negative for mycoplasma as detected by fluorescence and culture methods.

Human LOVO cells were kindly provided by Jia Jing (Hangzhou Medical College, Hangzhou, China). Male C57BL/6J mice (6–8 weeks old) were purchased from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). The mice were housed in a specific pathogen-free animal facility, and experimental protocols were approved by the Animal Care and Use Committee of Hangzhou Medical College, all animals were treated according to the guidelines for animal experimentation of Hangzhou Medical College in Hangzhou, China. The animal experiments were also performed in accordance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines [31].

Isolation and quantification of I-Evs. Mouse large intestines were surgically extracted and ground in a sufficient volume of PBS. They were then digested with 1 mg/mL collagenase type Ⅷ from *Clostridium histolyticum* (Gibco) for 2 h at 37 ˚C. The resulting suspension of intestinal tissue fragments was centrifuged at 400 *g* for 10 min, and the supernatant carefully removed for further centrifugation at 10,000 *g* for 30 min, to remove larger vesicles. The resulting supernatant was then filtered by a 0.22-μm screening and ultracentrifuged at 100,000 *g* for 1 h. Crude pellets of I-Evs were washed in sterile PBS and centrifuged at the same speed for an additional 1 h. The harvested I-Evs were resuspended in PBS. A BCA assay was used to detect the concentration of I-Evs (ThermoFisher, Waltham, MA, USA).

Electron microscopy scanning and Nanoparticletracking analysis. Suspensions of I-Evs were loaded onto a coated copper grid, and a drop of 2% phosphotungstic acid added as a negative staining method. The sample was then allowed to dry at room temperature and transferred to a transmission electron microscope (Hitachi H7650, Hitachi, China) to take pictures and record at a voltage of 80 kV. To detect size distribution, I-Evs were diluted with PBS, and 0.3 mL analysed by NanoSight Nano instruments (Malvern, UK).

Western blot and flow cytometry analysis. For western blot analysis, 40 μg I-Evs or protein lysates extracted from intestinal tissues were separated by 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were blocked with 5% milk in phosphate buffered solution-Tween 20 (PBS-T) and incubated with the corresponding primary antibodies at 4 ˚C overnight. The next day, membranes were incubated with an HRP-coupled secondary antibody for 1 h at room temperature and scanned using a Canon 4500 imaging system (Shanghai, China). For cytometry analysis, cells were washed with cold PBS and incubated with a fluorescent antibody for 30 min at 4 ˚C in the dark. Cells were analysed by fluorescence-activated cell sorting (BD, Franklin Lakes, NJ, USA).

$CD4^+Foxp3^+$Tregs induction assay. Murine $CD4^+$ T cells were isolated with the EasySep Mouse CD4+ T Cell Isolation Kit (Stemcell), and labelled with an anti-CD62L antibody for flow cytometry. Magnetic sorting was then performed using the EasySep Mouse Biotin Positive Selection Kit (Stemcell). Cells were then incubated with 1 μl anti-CD3/CD28-coated beads for 72 h (2 x 10^5 cells/well), with or without 50 μg/mL I-Evs. To block the TGF-b1 signal, 0.6 μg/mL TGF-b1 inhibitor was applied to cells (*in vitro*), or 15 μg/mL anti–TGF-b1–neutralising antibody was injected into mice (*in vivo*). The percentage of $CD4^+Foxp3^+$Tregs was analysed by flow cytometry.

Induction and treatment of murine intraperitoneal inflammation induced by *C. difficile* TcdB. C57BL/6J male mice were randomised into groups and given antibiotics through their drinking water for 5 days. The antibiotic mixture consisted of gentamicin (0.035 mg/mL), kanamycin (0.4 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA). The following day, mice were injected with clindamycin (10 mg/kg). After this, purified TcdB was injected intraperitoneally into mice (0.5 μg/kg); this was noted as day 0. Functional I-Evs (50 μg/100 μL PBS) were injected after 5 h, and on day 1. After sacrificing the animals, the intestinal tissue in different groups was collected and prepared for H&E staining.

Statistical analysis. Data are presented as the mean ± SEM. Data were compared using a Student's *t*-test with GraphPad Prism 8 (San Diego, CA, USA). P 0.05 was considered statistically significant.

Declarations

Acknowledgements

This work was supported by the Zhejiang Medicine and Health Technology Program (2021KY639), the General Research Project of Zhejiang Provincial Department of Education (Y202045376), the Major Science and Technology Medicine and Healthcare in Zhejiang (WKJ-ZJ-2107). We would also like to thank Editage [www.editage.cn] for English language editing.

Author contributions

S.W. and D.J. conceived and designed the experiments. S.W., G.S., H.H., and X.J. performed the experiments. S.W., G.S., and H.H. wrote the manuscript. Y.X., P.Z., and S.L. assisted with experiments. All authors read and approved the manuscript.

Competing interests

All authors declare no competing interests.

References

1. Lessa, F. C. *et al.* Burden of Clostridium difficile infection in the United States. *N Engl J Med* **372**, 825-834, doi:10.1056/NEJMoa1408913 (2015).
2. Buonomo, E. L. & Petri, W. A., Jr. The microbiota and immune response during Clostridium difficile infection. *Anaerobe* **41**, 79-84, doi:10.1016/j.anaerobe.2016.05.009 (2016).
3. Ng, J. *et al.* Clostridium difficile toxin-induced inflammation and intestinal injury are mediated by the inflammasome. *Gastroenterology* **139**, 542-552, 552.e541-543, doi:10.1053/j.gastro.2010.04.005 (2010).
4. Kociolek, L. K. & Gerding, D. N. Breakthroughs in the treatment and prevention of Clostridium difficile infection. *Nat Rev Gastroenterol Hepatol* **13**, 150-160, doi:10.1038/nrgastro.2015.220 (2016).

5. Louie, T. J. *et al.* Fidaxomicin versus vancomycin for Clostridium difficile infection. *N Engl J Med***364**, 422-431, doi:10.1056/NEJMoa0910812 (2011).
6. Yang, Z. *et al.* A novel multivalent, single-domain antibody targeting TcdA and TcdB prevents fulminant Clostridium difficile infection in mice. *J Infect Dis***210**, 964-972, doi:10.1093/infdis/jiu196 (2014).
7. Raposo, G. & Stoorvogel, W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol***200**, 373-383, doi:10.1083/jcb.201211138 (2013).
8. Colombo, M., Raposo, G. & Théry, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol***30**, 255-289, doi:10.1146/annurev-cellbio-101512-122326 (2014).
9. Miyake, H. *et al.* Human breast milk exosomes attenuate intestinal damage. *Pediatr Surg Int***36**, 155-163, doi:10.1007/s00383-019-04599-7 (2020).
10. Yang, D. *et al.* Progress, opportunity, and perspective on exosome isolation - efforts for efficient exosome-based theranostics. *Theranostics***10**, 3684-3707, doi:10.7150/thno.41580 (2020).
11. Babuta, M. *et al.* Dysregulated Autophagy and Lysosome Function Are Linked to Exosome Production by Micro-RNA 155 in Alcoholic Liver Disease. *Hepatology***70**, 2123-2141, doi:10.1002/hep.30766 (2019).
12. Cao, S. Q. *et al.* Long non-coding RNA highly up-regulated in liver cancer promotes exosome secretion. *World J Gastroenterol***25**, 5283-5299, doi:10.3748/wjg.v25.i35.5283 (2019).
13. Wang, G. *et al.* HucMSC-exosomes carrying miR-326 inhibit neddylation to relieve inflammatory bowel disease in mice. *Clin Transl Med***10**, e113, doi:10.1002/ctm2.113 (2020).
14. Wu, H. *et al.* Extracellular vesicles containing miR-146a attenuate experimental colitis by targeting TRAF6 and IRAK1. *Int Immunopharmacol***68**, 204-212, doi:10.1016/j.intimp.2018.12.043 (2019).
15. Liu, X. *et al.* Exosomes derived from platelet-rich plasma present a novel potential in alleviating knee osteoarthritis by promoting proliferation and inhibiting apoptosis of chondrocyte via Wnt/β-catenin signaling pathway. *J Orthop Surg Res***14**, 470, doi:10.1186/s13018-019-1529-7 (2019).
16. Williams, A. M. *et al.* Mesenchymal Stem Cell-Derived Exosomes Provide Neuroprotection and Improve Long-Term Neurologic Outcomes in a Swine Model of Traumatic Brain Injury and Hemorrhagic Shock. *J Neurotrauma***36**, 54-60, doi:10.1089/neu.2018.5711 (2019).
17. Boulestreau, J., Maumus, M., Rozier, P., Jorgensen, C. & Noël, D. Mesenchymal Stem Cell Derived Extracellular Vesicles in Aging. *Front Cell Dev Biol***8**, 107, doi:10.3389/fcell.2020.00107 (2020).
18. Harrell, C. R. *et al.* Molecular and Cellular Mechanisms Responsible for Beneficial Effects of Mesenchymal Stem Cell-Derived Product "Exo-d-MAPPS" in Attenuation of Chronic Airway Inflammation. *Anal Cell Pathol (Amst)***2020**, 3153891, doi:10.1155/2020/3153891 (2020).
19. Wan, S. *et al.* CD8α(+)CD11c(+) Extracellular Vesicles in the Lungs Control Immune Homeostasis of the Respiratory Tract via TGF-β1 and IL-10. *J Immunol***200**, 1651-1660, doi:10.4049/jimmunol.1701447 (2018).

20. Allan, D., Tieu, A., Lalu, M. & Burger, D. Mesenchymal stromal cell-derived extracellular vesicles for regenerative therapy and immune modulation: Progress and challenges toward clinical application. *Stem Cells Transl Med***9**, 39-46, doi:10.1002/sctm.19-0114 (2020).
21. Mileto, S. J. *et al.* Clostridioides difficile infection damages colonic stem cells via TcdB, impairing epithelial repair and recovery from disease. *Proc Natl Acad Sci U S A***117**, 8064-8073, doi:10.1073/pnas.1915255117 (2020).
22. Jiang, L. *et al.* EpCAM-dependent extracellular vesicles from intestinal epithelial cells maintain intestinal tract immune balance. *Nat Commun***7**, 13045, doi:10.1038/ncomms13045 (2016).
23. Chung, I. M., Rajakumar, G., Venkidasamy, B., Subramanian, U. & Thiruvengadam, M. Exosomes: Current use and future applications. *Clin Chim Acta***500**, 226-232, doi:10.1016/j.cca.2019.10.022 (2020).
24. Ni, Z. *et al.* Exosomes: roles and therapeutic potential in osteoarthritis. *Bone Res***8**, 25, doi:10.1038/s41413-020-0100-9 (2020).
25. Kumar, S., Zhi, K., Mukherji, A. & Gerth, K. Repurposing Antiviral Protease Inhibitors Using Extracellular Vesicles for Potential Therapy of COVID-19. *Viruses***12**, doi:10.3390/v12050486 (2020).
26. Scavo, M. P. *et al.* Exosomes for Diagnosis and Therapy in Gastrointestinal Cancers. *Int J Mol Sci***21**, doi:10.3390/ijms21010367 (2020).
27. Ocansey, D. K. W. *et al.* Exosome-mediated effects and applications in inflammatory bowel disease. *Biol Rev Camb Philos Soc***95**, 1287-1307, doi:10.1111/brv.12608 (2020).
28. Carter, G. P. *et al.* Defining the Roles of TcdA and TcdB in Localized Gastrointestinal Disease, Systemic Organ Damage, and the Host Response during Clostridium difficile Infections. *mBio***6**, e00551, doi:10.1128/mBio.00551-15 (2015).
29. Yamada, A. *et al.* Role of regulatory T cell in the pathogenesis of inflammatory bowel disease. *World J Gastroenterol***22**, 2195-2205, doi:10.3748/wjg.v22.i7.2195 (2016).
30. Shen, E. *et al.* Subtyping analysis reveals new variants and accelerated evolution of Clostridioides difficile toxin B. *Commun Biol***3**, 347, doi:10.1038/s42003-020-1078-y (2020).
31. Percie du Sert, N. *et al.* The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLoS Biol***18(7)**, e3000410, https://doi.org/10.1371/journal.pbio.3000410 (2020).

Appendix

Appendix. A Primer sequence for real-time PCR

Gene name	F□R	Primer sequence□5’ to 3’□
b-actin	Forward	CACAGCTGAGAGGGAAATCGT
	Reverse	GCCATCTCCTGCTCGAAGTCTA
IL-6	Forward	AGTTGCCTTCTTGGGACTGA
	Reverse	TCCACGATTTCCCAGAGAAC
TNF-a	Forward	CTGGGACAGTGACCTGGACT
	Reverse	GCACCTCAGGGAAGAGTCTG
IL-1b	Forward	CCAAAAGATGAAGGGCTGCT
	Reverse	ACAGAGGATGGGCTCTTCT
IL-22	Forward	ATGAGTTTTTCCCTTATGGGGAC
	Reverse	GCTGGAAGTTGGACACCTCAA
TGF-b	Forward	CACCGGAGAGCCCTGGATA
	Reverse	GCCGCACACAGCAGTTCTT
IL-10	Forward	GGTTGCCAAGCCTTATCGGA
	Reverse	ACCTGCTCCACTGCCTTGCT

Figures

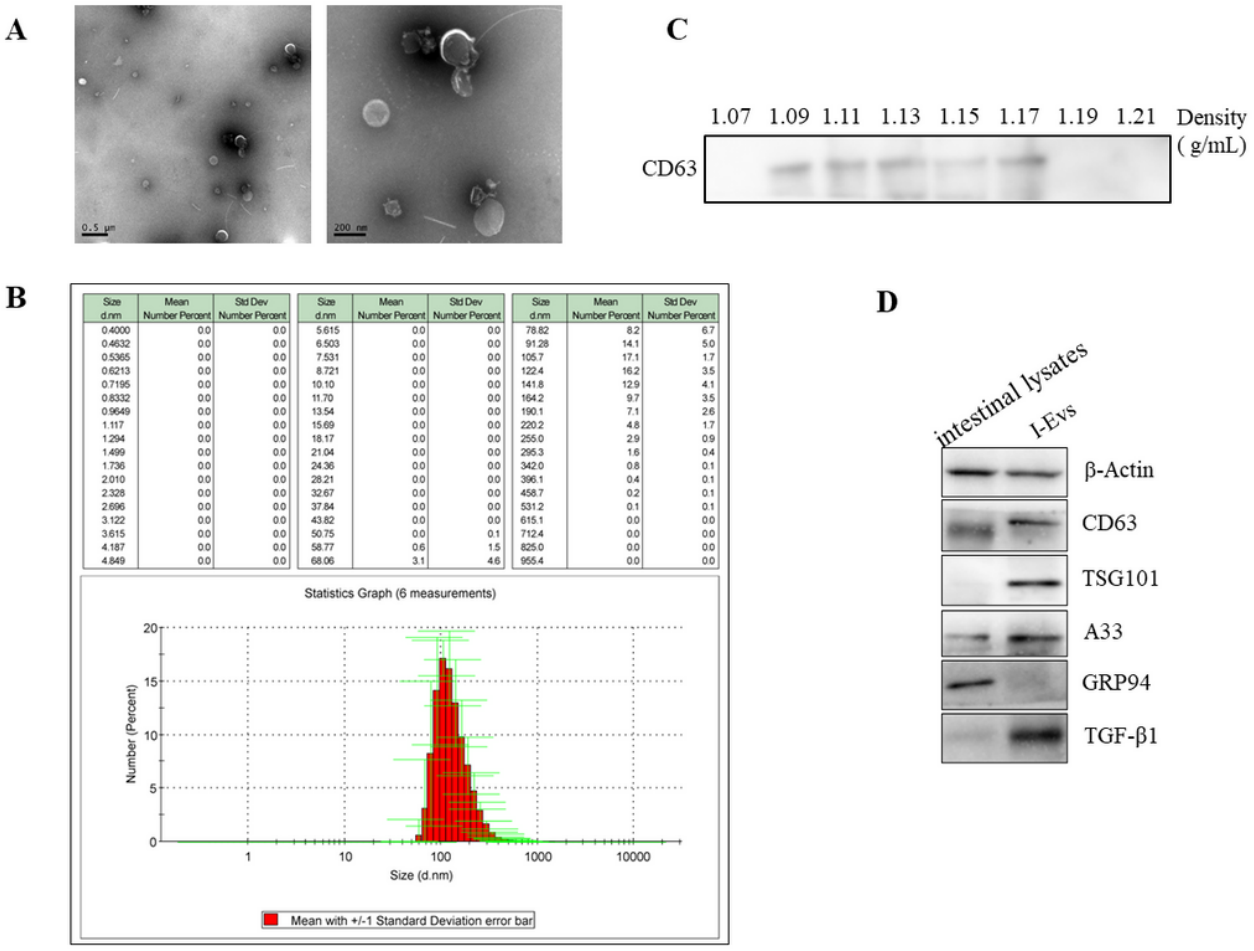

Figure 1

Identification of intestine epithelial cell-derived Evs (I-Evs). Extracellular vesicles were isolated from murine intestinal tissues and digested by standard procedures. (A, B) The morphology and diameter of I-Evs were analysed by electron microscopy and Nanoparticle tracking analysis. (C) 200 μg I-Evs were placed onto different concentrations of sucrose solution, and analysed by western blot with an anti-CD63 antibody. (D) 40 μg of intestinal lysates and I-Evs were separated by SDS-PAGE and transferred to a PVDF membrane. β-Actin, CD63, TSG101, A33, GRP94, and TGF-β1 were detected using antibodies. All data were verified by three independent experiments.

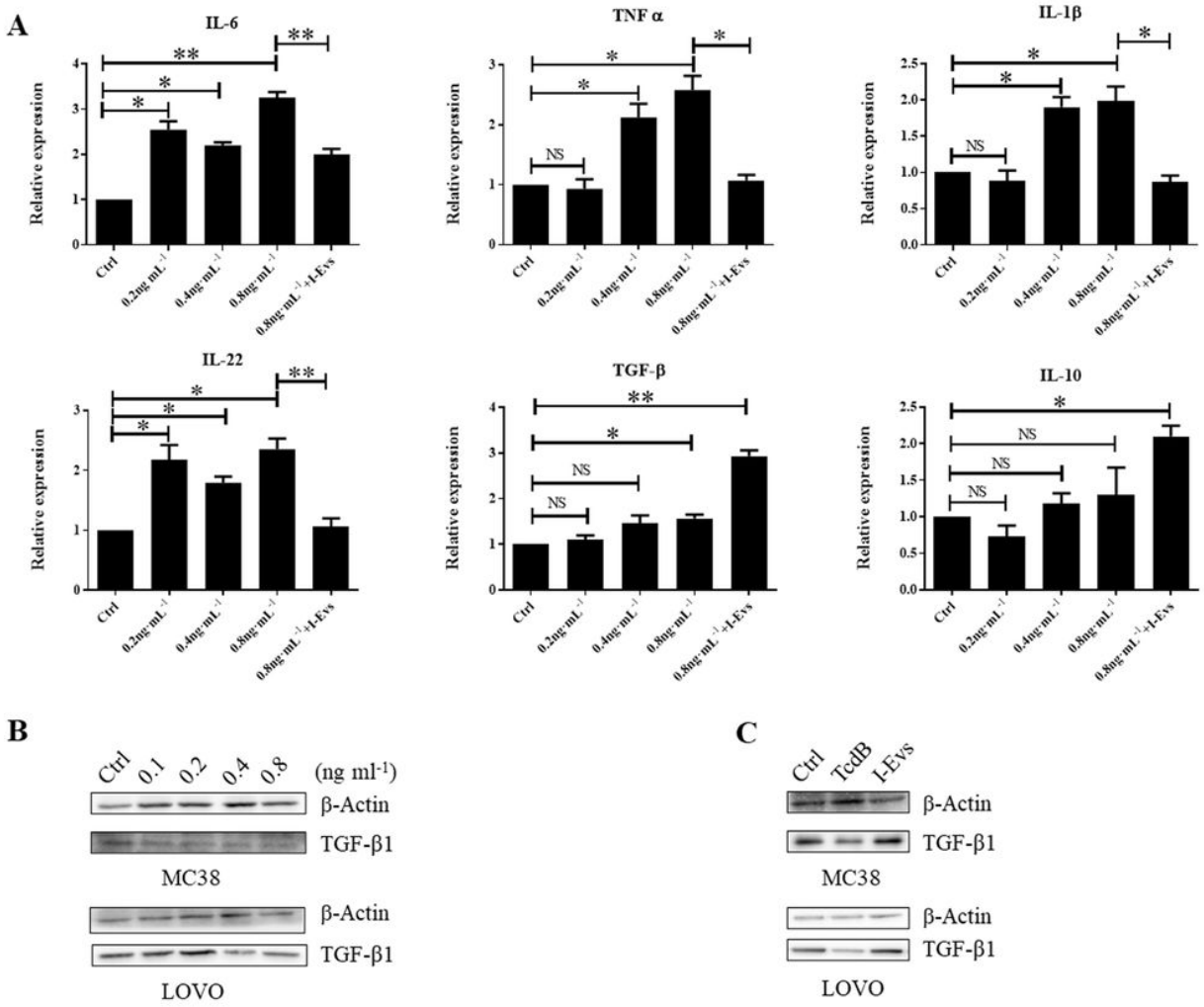

Figure 2

I-Evs attenuated the downregulation of TGF-β1 induced by purified C. difficile TcdB in vitro. (A) MC38 cells were exposed to different concentrations of TcdB (0.2 ng/mL, 0.4 ng/mL, or 0.8 ng/mL) for 5 h, or simultaneously treated with 50 μg I-Evs. Real-time PCR was used to detect gene expression levels of IL-6, TNF-α, IL-1β, IL-22, TGF-β1, and IL-10. (B) MC38 and LOVO cells were stimulated with C. difficile TcdB (0.1 ng/mL, 0.2 ng/mL, 0.4 ng/mL, or 0.8 ng/mL), before cell lysates were analysed by western blot. (C) Similarly, cells were treated with C. difficile TcdB and I-Evs, and the TGF-β1 protein levels analysed by western blot. All data were verified by three independent experiments. P values were calculated by one-way analysis of variance (ANOVA), versus control conditions (*: P<0.05, **: P<0.01, ***: P<0.001, NS: not significant).

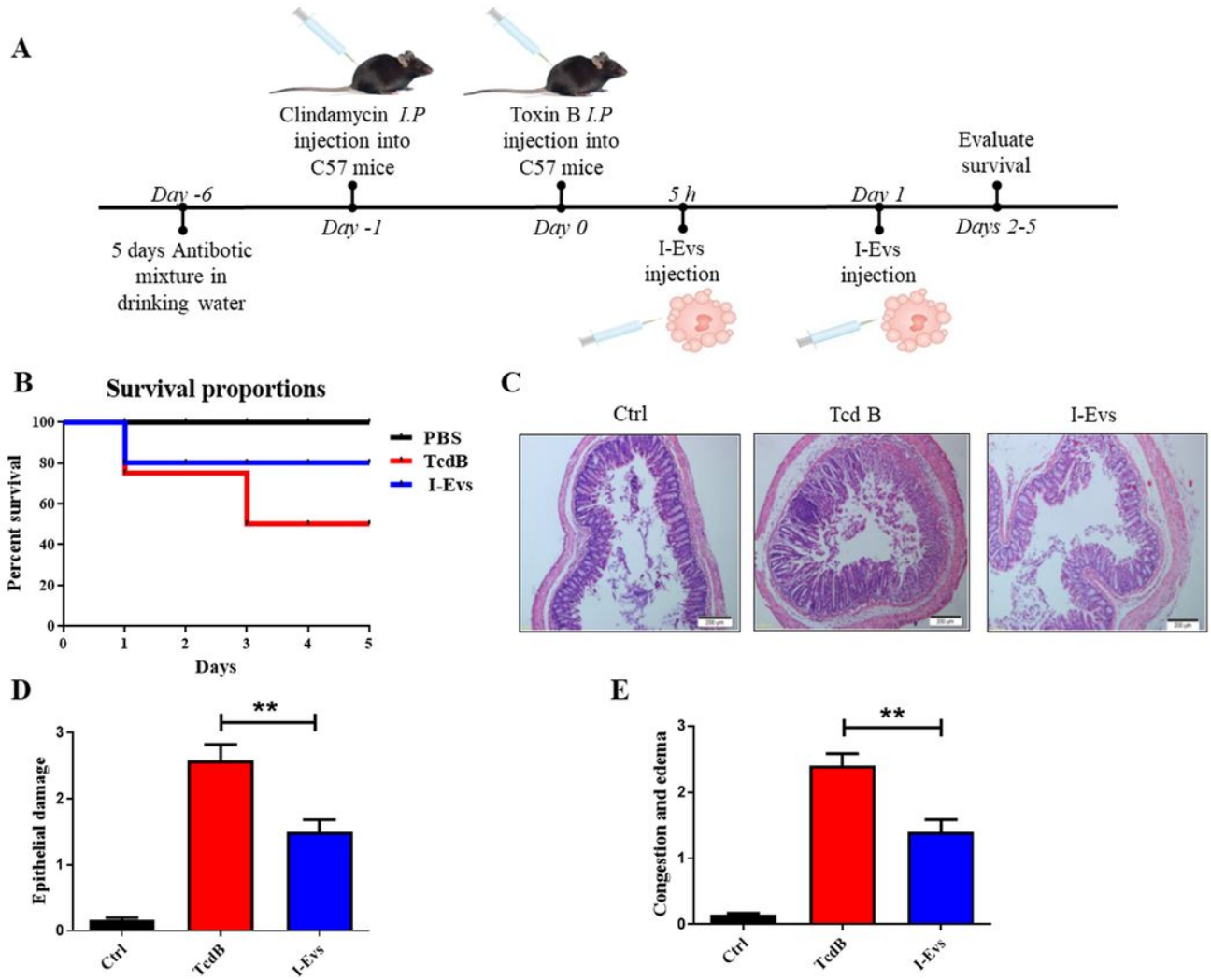

Figure 3

I-Evs alleviate murine C. difficile TcdB-induced intraperitoneal inflammation. (A) C57BL/6J mice received antibiotics for 5 days through their drinking water. The antibiotic mixture consisted of gentamicin (0.035 mg/mL), kanamycin (0.4 mg/mL), colistin (850 U/mL), metronidazole (0.215 mg/mL), and vancomycin (0.045 mg/mL). The following day, mice were injected with clindamycin (10 mg/kg), followed by C. difficile TcdB (0.5 µg/kg) via intraperitoneal injection. This was noted as day 0. Functional I-Evs (50 µg/100 µL PBS) were injected after 5 h and on day 1. (B) The survival rate of mice. (C) Intestinal tissue was collected and prepared for H&E staining. Epithelial damage (D) and congestion (E) were scored as histopathological severity. Images are representative of results from five animals, at the indicated time points after the TcdB challenge. All data were verified by three independent experiments. Values represent the mean ± SEM (n=5 animals) versus control animals (*: P□0.05, **: P□0.01, ***: P□0.001, NS: not significant).

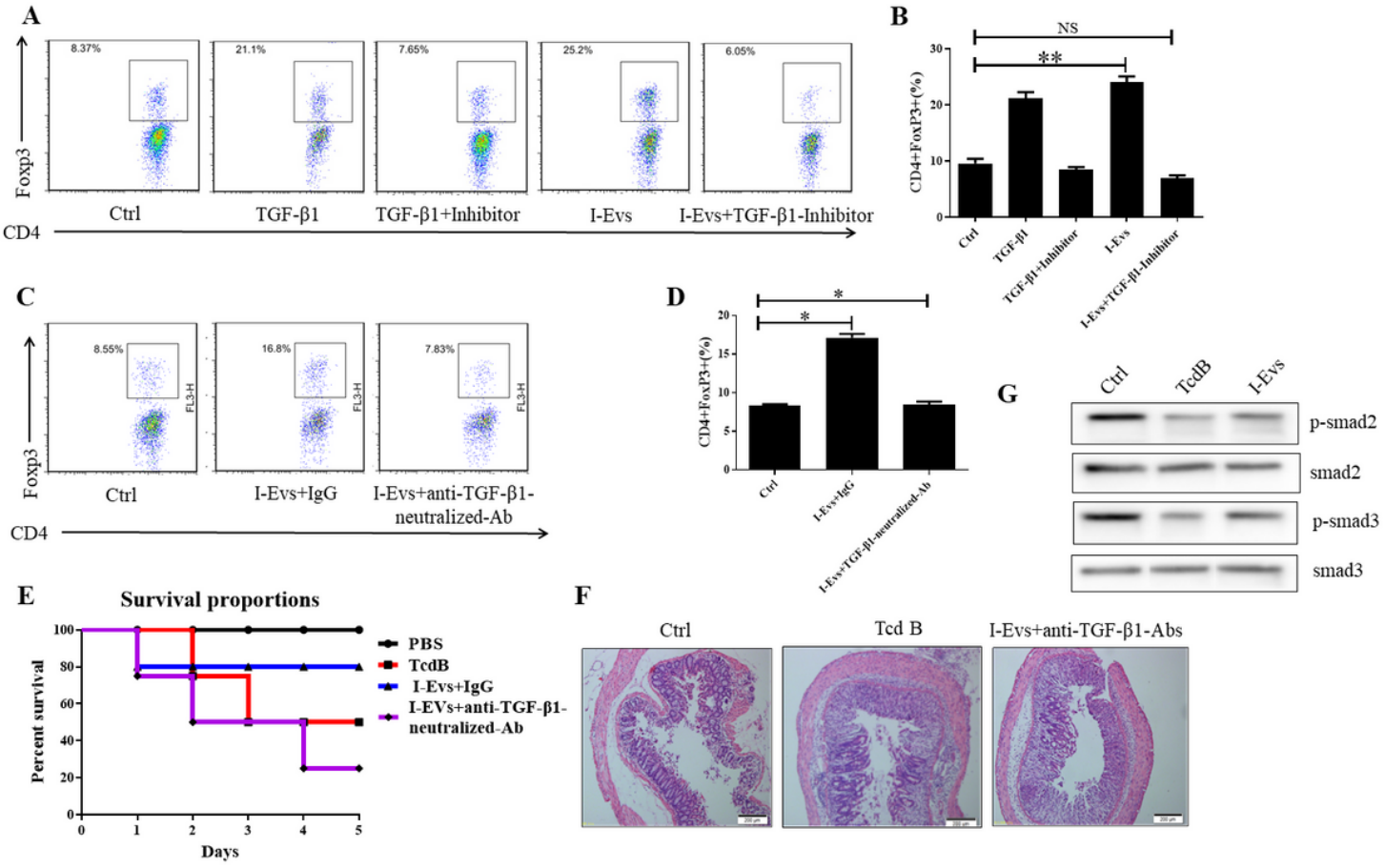

Figure 4

Induction of regulatory T cells by I-Evs alleviated intraperitoneal infection caused by C. difficile TcdB through a TGF-β1-dependent mechanism. (A) A lymphocyte suspension was obtained by grinding and filtering the spleen and lymph nodes of mice. Naïve CD4+ T cells were magnetically separated with the EasySep Mouse CD4+ T Cell Isolation Kit, and incubated with 1 μL anti-CD3/CD28-coated beads for 72 h (2 x 105 cells/well), and separately treated with 3 ng/mL TGF-β1, 0.6 μg/mL TGF-β1 inhibitor, or 50 μg I-Evs. (B). Statistical analysis of (A) (n=9). (C) To block the TGF-β1 signal in vivo, 15 μg/mL anti-TGF-β1-neutralising antibodies were injected into mice and I-Evs were transfused three days later. The percentage of CD4+Foxp3+Tregs was analysed by flow cytometry. (D) Statistical analysis of (C) (n=9). (E) The survival rate of mice. (F) The infiltration of neutrophils and destruction of intestinal cells. (G) Western blot analysis of Smad2/3, and phosphorylated Smad2/3, in MC38 cells stimulated with C. difficile TcdB. All data were verified by three independent experiments. P values were calculated by one-way analysis of variance (ANOVA), versus control animals (*: P□0.05, **: P□0.01, ***: P□0.001, NS: not significant).