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Title: Exosomes from mesenchymal stromal cells reduce murine colonic inflammation via a macrophage-dependent mechanism.

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Conflict of interest statement

The authors have declared that no conflict of interest exists.
Abstract
Conventional treatments for inflammatory bowel disease (IBD) have multiple potential side effects. Therefore, alternative treatments are desperately needed. This work demonstrated that systemic administration of exosomes from human bone marrow-derived mesenchymal stromal cells (MSC-Exos) significantly mitigated colitis in various models of IBD. MSC-Exos treatment downregulated inflammatory responses, maintained intestinal barrier integrity and polarized M2b macrophages, but did not favor intestinal fibrosis. Mechanistically, infused MSC-Exos mainly acted on colonic macrophages and macrophages from colitic colons acquired obvious resistance to inflammatory re-stimulation when prepared from mice treated with MSC-Exos versus untreated mice. The beneficial effect of MSC-Exos was blocked by macrophage depletion. Besides, the induction of IL-10 production from macrophages was partially involved in the beneficial effect of MSC-Exos. MSC-Exos were enriched in proteins involved in regulating multiple biological processes associated with the anti-colitic benefit of MSC-Exos. Particularly, metallothionein-2 in MSC-Exos was required for the suppression of inflammatory responses. Taken together, MSC-Exos are critical regulators of inflammatory responses and may be promising candidates for IBD treatment.
**Introduction**

Inflammatory bowel disease (IBD) is a family of chronic, idiopathic, relapsing and tissue-destructive disorders including Crohn’s disease and ulcerative colitis, that has emerged as a worldwide public health challenge (1). It is characterized by an abnormal immune response to the gut microflora in genetically susceptible individuals (2). Despite advances in anti-tumor necrosis factor therapy, management of IBD in current clinical practice remains a daunting challenge (3). Traditional therapies for IBD frequently result in multiple serious side effects and treatment resistance (4), highlighting the unmet need for alternative treatment options for IBD.

Mesenchymal stromal cells (MSCs) have recently shown great potential as a feasible and effective biological treatment for IBD (5-7). The anti-inflammatory and immunomodulatory activities of MSCs provide the theoretical grounds for MSC transplantation in IBD patients and as a potential novel cellular therapy for IBD (8). Several preclinical studies demonstrate that implanted MSCs can home to the injured tissue and differentiate as functional cells in situ to replace damaged cells (9). However, only a low percentage of implanted MSCs survive in vivo and engraft into injured tissues (10). This strongly suggests that therapeutic effects mediated by MSCs might be attributed to their secretory pathways rather than their tissue-homing capacity. Indeed, the cell-free conditioned media of MSCs and the secretome of MSCs (containing extracellular vesicles and exosomes) afford beneficial effects in various diseases (11-13). Despite numerous therapeutic vectors in the MSC secretome with anti-inflammatory and immunosuppressive properties, no specific mediators have been identified as responsible for the beneficial effects of MSC-based therapy.
Moreover, the risk of malignant transformation in human recipients, though not yet definitely confirmed, remains a critical problem impeding progress in the clinical translation of MSCs (5, 14).

In contrast, exosomes derived from MSCs (MSC-Exos), providing immune regulatory and tissue repair properties like MSCs, have recently emerged as a powerful component in the “secretory pathways” of MSCs and have aroused great enthusiasm (15). Indeed, MSC-Exos evoke MSC-like protective effects in several diseases (16-18). Mechanistically, Zhao et al. recently reported that exosomes from adipose-derived stem cells attenuate adipose inflammation and obesity through polarizing M2 macrophages (19). In addition, Willis et al. demonstrated that MSC-Exos blunt hyperoxia-induced inflammation, partially by modulating the lung macrophage phenotype (11). MSC-Exos based cell-free treatment is more than a compensation for MSC-based treatment. MSC-Exos possess certain desirable features including immunosilence, non-oncogenicity, high stability, cell/tissue-specific homing, no vascular obstructive propensity (20, 21). Therefore, MSC-Exos offer a therapeutic advantage over MSC-based treatment and it is of increasing interest to explore the potential roles of MSC-Exos in the management of IBD and their related mechanisms. Accordingly, we confirm that human borrow derived MSC-Exos have protective effects on experimental colitis. Furthermore, we characterize the biological mechanism underlying the therapeutic value of MSC-Exos. Our findings present a new therapeutic tool for the treatment of IBD.
Results

Isolation and purification of MSC-Exos. We isolated MSC-Exos from the supernatants of human bone marrow-derived MSCs. Prior to exosome extraction, flow cytometric analysis for MSC-related markers revealed that the MSCs were positive (≥ 95%) for CD105, CD90, and CD73, but were negative (≤ 2%) for CD45, CD79a, CD19, CD34, CD14, CD11b, and HLA-DR (Figure 1A). Propidium iodide (PI) and Annexin V analysis showed a good in vitro culture condition of the MSCs (Figure 1B), ruling out apoptotic bodies and random cell debris. Particle metrix (PMX) (Figure 1C) and transmission electron microscopy (TEM) (Figure 1D) confirmed their size distribution and shape were consistent with exosomes. Furthermore, western blot confirmed the exosomal identity markers TSG101 and CD9 (Figure 1E). Together, these data are indicative of successful isolation and purification of MSC-Exos.

MSC-Exos inhibit chemically-induced colitis in mice. Next, we explored the potential protective effects of MSC-Exos in murine models of IBD induced by oral dextran sodium sulfate (DSS) administration or intrarectal infusion of 2, 4, 6-trinitrobenzenesulfonic acid solution (TNBS), both of which exhibit clinical, histopathological and immunological phenotypes that closely parallel human IBD. By 7 days, mice receiving oral administration of 5% DSS displayed significantly elevated disease activity indexes (DAI) (Figure 2A), with continuous body weight loss (Figure 2B). In striking contrast, intravenous injection of MSC-Exos reduced the clinical activity compared with that in DSS plus phosphate-buffered saline (PBS) treated mice (Figure 2, A and B). Simultaneously, DSS administration led to significant colon shortenings, whereas MSC-Exos treatment significantly improved colonic
inflammation, as shown by an increased colon length (Figure 2C). Histological examination indicated that MSC-Exos treatment maintained intestinal structural integrity and significantly reduced disruptions of the architecture, crypt loss and infiltration of inflammatory cells, with lower histological scores compared with corresponding controls (Figure 2, D and E). Moreover, MSC-Exos treatment afforded a comparable decrease in colonic myeloperoxidase (MPO) activity, suggesting less neutrophil infiltration (Figure 2F).

We further examined the anti-colitic benefit of MSC-Exos in chronic and recurrent colitis. Oral treatment of 3% DSS in two cycles resulted in two peaks of colitis characterized by continuous weight loss, diarrhea and bloody stools and causing ~80% mortality (Figure 3, A and B). MSC-Exos infusion at the first acute peak of colitis (day 7) markedly ameliorated the disease severity by day 9, as demonstrated by improved survival and stool consistency, less rectal bleeding, decreased colon shortening, reduced MPO activity, and amelioration of colon damage (Figure 3, A-F). Noteworthily, mice with a single MSC-Exos injection at the first disease peak were endowed with clear resistance to disease activity during the second cycle of DSS treatment. A second MSC-Exos treatment at the second onset of disease (day 16) effectively prevented the recurrence of colitis (Figure 3, A-F).

The anti-colitic benefit of MSC-Exos is not limited to DSS-induced colitis. Analogous to results using the DSS-induced colitis model, MSC-Exos were proven to be effective in inhibiting TNBS-induced colitis (Supplemental Figure 1, A-F). Moreover, the therapeutic effects of MSC-Exos on colitis seemed to be dose- and
frequency-dependent (Supplemental Figure 2, A and B). However, pretreatment with
MSC-Exos 2 days before DSS administration did not provide prophylactic effects for
DSS-induced colitis (Supplemental Figure 2, C and D).

Effect of MSC-Exos on mucosal inflammation and intestinal barrier integrity.
Dysregulation of inflammatory mediators has been well recognized in murine
experimental colitis and also in IBD patients (22). Thus, we determined the in vivo
effect of MSC-Exos on the production of inflammatory cytokines mechanistically
associated with IBD. Compared with untreated DSS-colitic mice, MSC-Exos
administration decreased expression of several pro-inflammatory cytokines including
IFN-γ, IL-1β, IL-6 and TNF-α, whereas expression of the anti-inflammatory cytokine
IL-10 was significantly increased (Figure 4, A-E).

Since it is a salient feature of IBD (23, 24), we further investigated the effects of
MSC-Exos on intestinal barrier dysfunction. DSS administration increased the serum
levels of FITC-dextran, whereas MSC-Exos treatment significantly reduced the
DSS-induced increase of intestinal permeability, as shown by significantly decreased
levels of FITC-dextran (Figure 4F). An abnormal intestinal barrier could result in
increased infiltration of intestinal microbes into intestinal lamina propria(23). Indeed,
DSS challenge increased the invasion of colonic tissues by microbes and this was
significantly reduced in mice treated with MSC-Exos (Figure 4G). In accordance with
that, DSS administration decreased the mRNA levels of antimicrobial peptides in
colons, including lysozyme 1 (Lyz1), defensin, alpha, 20 (Defa20), defensin, alpha, 29
(Defa29) and angiogenin, ribonuclease A family, member 4 (Ang4), whereas
MSC-Exos treatment reversed these trends (Figure 4H). Together, these data indicated that MSC-Exos contribute to the maintenance of gut homeostasis.

The anti-colicit benefit of MSC-Exos is macrophage dependent. Subsequently, to elucidate the cell type targeted by implanted MSC-Exos in colitis, we intravenously infused PKH26-labeled MSC-Exos into DSS-colicit mice. By analyzing the frequency of exosome-positive cells (PKH26+) in a million cells from each colon, we found that MSC-Exos were recruited by the inflamed colon, but not by non-inflamed intestine (Figure 5, A and B). In addition, about 50% of PKH26+ cells were F4/80+CD11b+ as analyzed by flow cytometry, a phenotype consistent with colonic macrophages (Figure 5, A and C). In contrast, labeled MSC-Exos were not efficiently engulfed by colonic macrophages in DSS-untreated mice (Figure 5, A-C). Together, these data suggest that macrophages are the predominant cell type taking up MSC-Exos in colitis and are probably responsible for the anti-colicit benefit of MSC-Exos. To substantiate this supposition, we deleted colonic macrophages in mice with intraperitoneal injection of clodronate-liposomes (Clod-lipo) (PBS-liposomes (PBS-lipo) as negative controls). As anticipated, administration of Clod-lipo effectively deleted colonic macrophages (Supplemental Figure 3, A and B). MSC-Exos were no longer effective in DSS-colicit mice lacking macrophages, providing no significant improvement in DAI, colon length, histopathological examination and colonic MPO activity (Figure 5, D-G). Taken together, these findings indicate that colonic macrophages are essential for the anti-colicit benefit of MSC-Exos treatment.

MSC-Exos ameliorate mucosal inflammation by remodeling macrophage...
phenotypes. Colonic macrophage mediated mucosal inflammatory responses play a central role in the onset and severity of IBD. To delineate the mechanism of the anti-colitic benefit of MSC-Exos, we investigated the in vivo effects of MSC-Exos on colonic macrophages. Despite no significant change in the percent of F4/80⁺CD11b⁺ cells in the lamina propria of colons, the expression levels of CD206 and arginase-1 in colonic macrophages were significantly elevated upon MSC-Exos treatment (Figure 6, A-D). As anticipated, the colonic expression of markers indicative of M2 macrophages was also significantly upregulated upon transfer of MSC-Exos (Supplemental Figure 4, A and B). More excitingly, macrophages isolated from mice treated with MSC-Exos produced less IL-1β, IL-6 and TNF-α, but more IL-10 on ex vivo culture (Figure 6E), revealing that the reduction of mucosal inflammatory responses upon MSC-Exos treatment might be a consequence of direct action on colonic macrophages. Importantly, macrophages from colitic colons acquired obvious resistance to lipopolysaccharide (LPS) re-stimulation when prepared from mice treated with MSC-Exos versus untreated mice (Figure 6E). Altogether, these data suggest that MSC-Exos attenuate mucosal inflammation by polarizing M2b macrophages.

The effect of MSC-Exos on intestinal fibrosis was also analyzed. ELISA assays (Supplemental Figure 4C) and RT-PCR gels (Supplemental Figure 4D) revealed no significant differences in the levels of collagen in MSC-Exos treated mice compared with controls. As shown by Mason’s trichrome staining, DSS administration led to increased collagen in the colons of chronic DSS-colitic mice, whereas MSC-Exos
treatment did not result in increased colonic collagen (Supplemental Figure 4E).

Collectively, these data indicate that MSC-Exos treatment does not favor intestinal fibrosis.

Macrophage-derived IL-10 is key for the beneficial effect of MSC-Exos. Because MSC-Exos treatment increases colonic expression of the immunosuppressive cytokine IL-10 (Figure 4E), we asked whether IL-10 is important for the anti-colitic benefit of MSC-Exos in DSS-colitic mice. Administration of an antibody to IL-10 in mice treated with MSC-Exos and 5% DSS yielded intermediate DAI and colonic MPO activity compared to time-matched DSS and MSC-Exo plus DSS treated mice (Figure 7A). Therefore, IL-10 is an important component of the anti-colitic benefit of MSC-Exos. We next set out to understand the source of IL-10 elevation in colons upon MSC-Exos treatment. It is unlikely that the injected MSC-Exos are the source of the elevated IL-10, because the protein cargo contained in MSC-Exos does not include IL-10 as determined by western blot and ELISA assay (data not shown).

Large amounts of IL-10 are mainly produced by type 2 helper T (Th2) cells, T regulatory cells and macrophages (25, 26). To assess the involvement of T cells in elevating IL-10, we isolated CD4+ T cells from the mesenteric lymph nodes (MLNs) of DSS-colitic mice. On ex vivo culture, CD4+ T cells with MSC-Exos treatment did not upregulate IL-10 expression, even after re-stimulation with phytohaemagglutinin (PHA) (Figure 7B). In addition, the type 1 helper T (Th1) and Th2 cell profile of CD4+ T cells was not significantly influenced upon MSC-Exos treatment (Figure 7C and Supplemental Figure 4F). Macrophage depletion impaired the anti-colitic effect
of MSC-Exos, supporting the notion that macrophages might be the source of the elevated IL-10 in colons upon MSC-Exos treatment. Further confirming this hypothesis, F4/80+ macrophages isolated from mice treated with MSC-Exos produced more IL-10 on ex vivo culture (Figure 6E). Together, these data suggest that the elevated IL-10 in colons upon MSC-Exos treatment is at least partially derived from colonic macrophages, and is probably not associated with Th2 or T regulatory cells.

To further confirm the involvement of macrophage-derived IL-10 in the beneficial effects of MSC-Exos, we analyzed the regulatory potential of macrophages educated by MSC-Exos on CD4+ T cells. In contrast to control macrophages, those educated by MSC-Exos and IL-4 significantly inhibited the proliferation of CD4+ T cells at ratios as low as 1:10 (Figure 7, E and F), and significantly suppressed the production of the inflammatory cytokines TNF-α and IFN-γ (Figure 7, G and H). The IL-10 blockade partially reversed the inhibitory activity of macrophages educated by MSC-Exos on activation of CD4+ T cells (Figure 7, E-H).

**MSC-Exos inhibit the inflammatory response of macrophages via transporting metallothionein-2.** To understand the molecular basis mediating the effects of MSC-Exos, we performed proteomic analysis to analyze the protein expression profiles in MSC-Exos and the corresponding supernatants from their parent MSCs (PRIDE Archive, Project PXD015386). We identified 1315 protein groups, among which 992 proteins were quantified. All the identified proteins were biologically interpreted and their involved biological processes were explored. MSC-Exos were enriched in proteins involved in regulating multiple biological processes related to the
anti-colic benefit of MSC-Exos, such as immune modulation, cytokine secretion, NF-κB signaling pathway, acute inflammatory response regulation, and collagen catabolic process (Figure 8A). We selected 389 differentially expressed proteins by volcano plot filtering between MSC-Exos and the corresponding supernatants (fold change ≥ 2 and $P$ value ≤ 0.05) (Figure 8B). Among them, metallothionein-2, a highly conservative protein between *Homo sapiens* and *Mus musculus*, was the most highly upregulated by more than 9-fold in MSC-Exos compared to the supernatants (Figure 8B). ELISA assays confirmed the significant enrichment of metallothionein-2 in MSC-Exos (Figure 8C) and metallothionein-2 appeared to be uniquely upregulated in MSC-Exos (Supplemental Figure 5A). In addition, the presence of metallothionein-2 in exosomes was necessary to have uptake into macrophages (Supplemental Figure 5B), which at least partly explained why free metallothionein-2 had no activity on the polarization of macrophages (Supplemental Figure 5, C and D).

To investigate the role of metallothionein-2 in the beneficial effect of MSC-Exos in macrophages, three siRNAs (siMT2A#1, siMT2A#2 and siMT2A#3) were used to knockdown metallothionein-2 expression in MSC-Exos. The inhibitory efficiency of these siRNAs was tested by ELISA assays. Results from ELISA assays showed the down-regulation of metallothionein-2 in MSC-Exos from MT2A-silenced MSCs (MSC-Exos$^\text{siMT2A#2}$) (Figure 8C). As expected, the reduction of metallothionein-2 in MSC-Exos interfered with the anti-inflammatory effects of MSC-Exos both in vitro (Figure 8D) and in vivo (Supplemental Figure 6, A-C). Previous study revealed that metallothionein-2 could attenuate NF-κB activity (27). Indeed, treatment with
MSC-Exos dose-dependently resulted in reduced level of phosphorylated IκB-α in macrophages, but increased total IκB-α level at both protein and mRNA (Figure 8E and Supplemental Figure 6D). A reduction of NF-κB p65 subunit nuclear translocation was also observed (Figure 8E). Furthermore, knockdown of metallothionein-2 significantly reversed the increase of IκB-α mRNA levels induced by MSC-Exos (Supplemental Figure 6D). As expected, the inhibitory effect of MSC-Exos on NF-κB activation was compromised as well (Figure 8E). It has been reported that metallothionein-2 could enhance IκB-α transcription via interacting with myeloid zinc finger 1 (MZF1) which directly bound to IκB-α promoter (28). In agreement with this, MSC-Exos failed to enhance IκB-α transcription in macrophages when MZF1 was knocked down using siRNA (Supplemental Figure 6E), implying a vital role of MZF1 on the increase of IκB-α transcription. Taken together, these findings revealed that metallothionein-2 transmitted by MSC-Exos was likely to suppress NF-κB activation via MZF1 by enhancing IκB-α transcription and inhibiting the phosphorylation of IκB-α.
Discussion

Current available medical therapeutic options towards IBD have not been able to completely halt the progression of IBD or to change its natural history. Therefore, designing optimal therapeutic strategies has been the subject of intense investigation. We propose a new therapeutic tool for IBD treatment in murine pre-clinical models using MSC-Exos, pivoting from MSC-based cell transplantation to cell-free therapeutics. Systemic infusion of MSC-Exos at the disease onset was sufficient to reduce the severity of acute colitis, and administration of MSC-Exos in mice significantly ameliorated the severity of chronic colitis. An initial MSC-Exos infusion also prevented the recurrence of the disease after subsequent DSS administration. In addition, the anti-colitic effect of MSC-Exos is mouse strain independent. MSC-Exos treatment efficiently ameliorated colitis in both C57BL/6 and Balb/c strains. These results are potentially relevant with respect to disease intervention.

A critical concern is whether the use of human exosomes in animal models, a xenogeneic material systemic administration, would elicit immune rejection. Theoretically, exosomes are non-immune rejective (20). Indeed, when using human exosomes in animal models, numerous studies have revealed significant therapeutic effects in several diseases and no immune rejection until now has been observed in the xenogeneic system (11, 16, 20, 29). In line with the previous reports (11, 16, 20, 29), no significant immunological rejection was detected when MSC-Exos were administrated to xenogeneic host in this study (Supplemental Figure 7, A-D). Additionally, class I and II major histocompatibility complex, key mediators of potential immune rejection, are absent on MSC-Exos as determined by western blot
These findings suggest that the immunosuppressive action of MSC-Exos is non-major histocompatibility complex-restricted and that the infused MSC-Exos are immunotolerated by the host, which is convenient for a future clinical application of MSC-Exos in IBD.

Dysregulation of inflammatory mediators and disruption of intestinal barrier integrity in IBD patients and in experimental colitis often interact in a positive feedback loop, together forming a vicious cycle (30-32). MSC-Exos treatment could serve dual roles to break this cycle, by reducing a panel of inflammatory mediators and maintaining intestinal homeostasis. Regarding the potential mechanisms, it is interesting that infused MSC-Exos are mainly targeted to macrophages in DSS-elicited inflamed colons. In addition, MSC-Exos treatment increases markers indicative of M2 macrophages in the colons of DSS-colitic mice. Therefore, intestinal macrophages may mediate the anti-colitic benefit of MSC-Exos. Indeed, this benefit of MSC-Exos was abolished in macrophage-depleted mice, which further supports our hypothesis.

Subsequently, we sought to understand why pretreatment with MSC-Exos failed to exert prophylactic effects on experimental colitis. By in vivo trafficking MSC-Exos, we found that non-inflamed intestines failed to recruit infused MSC-Exos and macrophages were only 5%-10% of all the cells that incorporated MSC-Exos, indicating an equal distribution in the body. This may provide a straightforward biological explanation for the failure of pretreatment. Furthermore, infused MSC-Exos were detected and maintained in the inflamed colons for no less than 5
days after injection, a time lag long enough to exert long-lasting protective actions. In striking contrast, the presence of transplanted MSCs decreased dramatically in the recipient at 2 days after infusion (33). This may endow MSC-Exos with a therapeutic advantage over MSCs. Noteworthy for MSC-Exos treatment is the possibility that the increase of M2 macrophage activity may lead to intestinal fibrosis, a severe and distressing complication of IBD (34, 35). On this front, although our data negate that MSC-Exos treatment favors intestinal fibrosis, optimization research establishing an ideal model for experimental research of intestinal fibrosis should be performed to monitor the possibility of fibrosis after MSC-Exos transplantation.

Regulatory cytokines can inhibit murine experimental colitis (36, 37). We found that colonic levels of IL-10 were significantly increased in mice treated with DSS plus MSC-Exos compared to DSS plus PBS treated control mice. Moreover, protection from colitis in MSC-Exos treated mice is dependent on regulatory cytokine IL-10, as administration of MSC-Exos was less effective in DSS-colitic mice treated with mAb against IL-10. Subsets of T cells are a possible source of the elevated IL-10 (25, 26). However, we observed that MSC-Exos treatment failed to increase the production of IL-10 on ex vivo culture, which preliminarily negates the hypothesis that subsets of T cells produce the elevated IL-10. In contrast, macrophages isolated from treated DSS-colitic mice produced significantly higher levels of IL-10 than those from non-treated mice. These results suggest that colonic macrophages are at least one source of the increased IL-10 and reinforce the notion that macrophage-derived IL-10 is a critical mediator of the anti-colitic benefit of MSC-Exos. Nevertheless,
macrophage-derived IL-7 may be involved in ameliorating murine experimental colitis when treated with exosomes from human umbilical cord MSCs (38). Therefore, we cannot exclude the possibility that additional factors after transfer of MSC-Exos in DSS-colitic mice may also play a role in protection from colitis, since neutralization of IL-10 alone did not fully block the anti-colitic effect of MSC-Exos.

Infiltration and activation of neutrophils in the intestinal mucosa are essential to the pathogenesis of IBD (39, 40). Neutrophils migrate into the lamina propria of DSS-induced colitis, where they eliminate invading bacteria using MPO, concomitantly eliciting oxidative organ damage, an undesirable effect of MPO (41-43). As such, MPO activity has become a widely accepted indicator of neutrophil activity. We observed that colonic MPO activity was significantly decreased in DSS-colitic mice treated with MSC-Exos, indicating that MSC-Exos treatment inhibits neutrophil invasion. IL-10 is a potent regulator of the migration and invasion of neutrophils. It is possible that the decreased MPO activity was a consequence of the elevated IL-10 in MSC-Exos treated DSS-colitic mice. This is primitively validated by the fact that the neutralizing anti-IL-10 antibody reverses the reduction of colonic MPO activity following intravenous injection of MSC-Exos.

To assess the molecular basis mediating the effects of MSC-Exos, we performed a proteomic analysis of MSC-Exos, which indicated that immunosuppressive or anti-inflammatory activity is a salient feature of MSC-Exos. Specifically, our findings revealed that levels of metallothionein-2 were extremely high in MSC-Exos. When metallothionein-2 was inhibited in the parent MSCs, the anti-inflammatory effects of
MSC-Exos on macrophages in vitro were significantly suppressed. NF-κB signaling is a downstream target of metallothionein-2 (27, 44). Consistent with this, MSC-Exos induced a significant decrease in NF-κB activity, whereas this effect was compromised when the expression of metallothionein-2 in MSC-Exos was inhibited. Our findings suggest that metallothionein-2 in MSC-Exos acts as a critical negative regulator of the inflammatory response in macrophages. Of note, metallothionein-2 possesses high conservation between *Homo sapiens* and *Mus musculus*, indicating that human metallothionein-2 in MSC-Exos could work in mice. However, the anti-inflammatory effects of MSC-Exos were not fully blocked by metallothionein-2 inhibition both in vitro and in vivo, suggesting that other components of MSC-Exos might also serve as active mediators.

Metallothionein-2, a member of metallothionein family, is a low-molecular-weight cysteine-rich protein (45, 46). Metallothioneins are present in multiple tissues and mainly consist of 4 groups: metallothionein-1A, metallothionein-2, metallothionein-3, and metallothionein-4. It has been reported that metallothionein-2 serves as a critical regulator of numerous cell activities, including differentiation, apoptosis, autophagy, immune response, carcinogenesis, and drug resistance (28, 47-51). The effects of metallothionein-2 on pathophysiological processes, particularly inflammation, are subjects of many studies (52). A study by Liu et al. showed that metallothionein-2 knock-down increased the lipopolysaccharide-induced IL-6 production in endothelial cells (50), indicating a protective role against inflammatory responses. Takano and colleagues demonstrated that transgenic mice with metallothioneins overexpression
were more resistant to neutrophilic lung inflammation and lung edema induced by intratracheal challenge with lipopolysaccharide (53). Metallothionein-2 has also been found to be a critical regulator of NF-κB signaling since its upregulation attenuates NF-κB activity in tumor cells, keloid fibroblastic cells as well as cardiomyocyte cells (54-57). In line with these studies, our results demonstrated the anti-inflammatory effects of metallothionein-2 on both macrophages and mouse colitis models. It is noteworthy that the presence of this protein in exosomes may offer a therapeutic advantage over its free form since free proteins are unlikely to interact with cells or have uptake into cells (58, 59). Furthermore, the role of metallothionein-2 might be not unique for colitis. In contrast, it should be applicable to other disorders, especially those with macrophages involved in the pathogenesis.

In summary, we propose a novel therapeutic strategy for the treatment of IBD based on the use of MSC-Exos, and of obvious therapeutic significance is that generation of a clinically effective dosage of MSC-Exos under good manufacturing practice is feasible and rapid (60), and MSC-Exos can be stored frozen and ready to use in any clinical scenario. Once the benefits and risks related to the infusion of MSC-Exos are well evaluated, this cell-free therapy will provide new perspectives for IBD therapy.
Methods

Detailed procedures are provided in Supplemental Experimental Procedures.

Exosome preparation. Exosomes were isolated from the supernatant of human bone marrow-derived MSCs (herein referred to as MSC-Exos) by standard ultracentrifugation methods, as previously described (61).

Induction of experimental colitis. IBD mouse models of experimental colitis were induced by oral DSS administration and by intrarectal infusion of TNBS. Exosomes were intravenously administrated to mice at the indicated time and macrophage depletion using Clod-lipo was performed as previously described (62-64). Mice were sacrificed at the indicated time and the entire colon was removed from the caecum to the anus, and colon length was measured as an indirect marker of inflammation. Colon segments were processed for histopathological or flow cytometry analysis or frozen in liquid nitrogen for protein and RNA extraction. Cytokines and myeloperoxidase (MPO) activities were measured according to the manufacturer’s protocol. To track injected exosomes in vivo, MSC-Exos were labelled with PKH26 before infusion and analyzed via flow cytometry analysis.

Histopathological evaluation. Collected colon samples were fixed with 10% formalin and embedded in paraffin. After paraffin embedding, 5μm thick sections were made and stained with haematoxylin and eosin (HE). Histopathology scores were determined in colonic sections by two blinded trained pathologists (unaware of group identity), with a combined score for inflammatory cell infiltration (score, 0-3) and tissue injury (score, 0-3), according to our previous report (2).
*Isolation of macrophages from the lamina propria of mouse colons.* Colonic macrophages were isolated using immunomagnetic separation with anti-F4/80 mAb (Miltenyi Biotec), determined by F4/80 and CD11b positive staining and then used for the analysis of cytokine secretion.

*Measurement of colonic tissue bacterial load.* Colon tissues were collected and homogenized in sterile physiological saline. After centrifuging at 13,000 g for 10 min at 4 °C to remove insoluble material, 100 μl of the tissue lysate supernatants containing 100 μg of colonic tissue extracts were plated in duplicate on blood agar plates and incubated at 37 °C for 2 days. Bacterial loads were defined as the average number of bacterial colonies forming units per gram of colonic tissues (wet weight).

*Statistics.* Results from the duplicate reproducible experiments were combined for statistical analysis. SPSS16.0 was used for all the statistical analyses. Data represent mean ± SD. To determine the statistical significance, the 2-tailed Student’s t test or 1-way ANOVA was applied for the continuous variables with normal distributions, whereas Mann-Whitney or Kruskal-Wallis test was used when distributions were skewed. *P* values ≤ 0.05 were considered statistically significant.

*Study approval.* All samples from human tissues were collected with informed written consent from donors, and all procedures were performed with the approval of the Institutional Review Board of the Sixth Affiliated Hospital of Sun Yat-sen University. Animal experiments were approved by the Institutional Animal Care and Use Committee of the Sun Yat-sen University and conformed to the “Guide for the Care and Use of Laboratory Animals” of the National Institute of Health in China.
Author contributions

HSL, ZXL, FWW, XRW and PL conceptualized the study. HSL, XWH, XRW and PL developed the experimental methods. HSL, ZXL, FWW, CZ, XBZ, TH, XRW and PL performed the investigations and data analyses. HSL and XRW wrote the original draft of the manuscript. HSL, ZXL, FWW, CZ, XBZ, TH, XWH, XRW and PL reviewed and edited the manuscript. PL and XRW acquired funding. PL and XRW supervised the study. HSL, ZXL and FWW contributed equally to this work. HSL, ZXL and FWW initiated and completed the work. This determined the authorship order.
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**Figures and Figure legends**

**Figure 1. Characterization of MSC-Exos.** 3 independent experiments were performed and yielded similar results. (A) Flow cytometry analysis showing the phenotypic markers of passage 5 MSCs cultured in medium supplemented with 10% exosome-depleted fetal bovine sera (FBS). Numerical values denote the percentage of positive cells. (B) Flow cytometry analysis for PI and Annexin V of MSCs cultured under exosome-depleted FBS media. The apoptosis of MSCs was induced by 10 µM etoposide as a positive control. Numerical values denote the percentage of apoptotic cells (Annexin V+). (C) Size profile of MSC-Exos by PMX. (D) TEM analysis of MSC-Exos. Left and right panels represent TEM using negative staining with uranyl acetate and tungstophosphoric acid, respectively. Left panel, scale bar: 200nm; right panel, scale bar: 50nm. (E) Western blot analysis of TSG101 and CD9; MW, molecular weight. Extracts of MSC-Exos were exposed to Triton plus Proteinase K (PK) or PK alone.
Figure 2. MSC-Exos protect against DSS-induced colitis. Male C57BL/6 mice at 6-8 weeks of age (n = 12-20 mice per group from two independent but reproducible experiments) were subjected to 5% DSS in the drinking water for 7 days and MSC-Exos (200 μg per mouse) were infused intravenously on day 2 (arrow in panel A). DAI scored from body weight loss, stool consistency and bleeding (A) and body weight (B) were recorded daily. (C) Measurements of colon length from mice on day 7. (D) Histopathological changes in colon tissues analyzed by hematoxylin and eosin (HE) staining on day 7. Upper panel, magnification: 100x; lower panel, magnification: 200x. (E) Semiquantitative scoring of histopathology performed as described in the supplemental experimental procedures. (F) Neutrophil infiltration determined by measuring colonic MPO activity on day 7. The number of animals studied is shown in each figure. ***P≤ 0.001, by Mann-Whitney test (A and E) or 1-way ANOVA (B, C and F).
Figure 3. Anti-colitic benefit of MSC-Exos in DSS-induced chronic and recurrent colitis.

Male C57BL/6 mice at 6-8 weeks of age (n = 20 mice per group) were subjected to 3% DSS in the drinking water in a cyclic manner. Each cycle consisted of 7 days of DSS followed by a 7-day phase without DSS supplementation. MSC-Exos (200 μg per mouse) were infused intravenously on day 7 or on days 7 and 16 (arrows in panel A). DAI (A) and mortality (B) were recorded. (C) Measurements of colon lengths. (D) Histopathological changes. Left panel, magnification: 100x; right panel, magnification: 200x. (E) Semiquantitative scoring of histopathology. (F) Neutrophil infiltration determined by measuring colonic MPO activity on day 28. The number of animals studied is shown in each figure. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, by Kruskal-Wallis test (A, E and F) or Log-rank test (B) or 1-way ANOVA (C).
Figure 4. MSC-Exos reduce mucosal inflammatory responses and contribute to the maintenance of intestinal barrier integrity. (A-E) ELISA assays for IFN-γ, IL-1β, IL-6, TNF-α and IL-10 in colonic tissues (n = 5 mice per group). For panels F-H, 5% DSS-colitic mice were killed on day 7 for next detections. (F) FITC-dextran levels in serum 4 h after oral gavage with FITC-dextran (50 mg/100g body weight) (n = 6 mice per group). (G) Bacterial counts in colons from mice with or without MSC-Exos treatment (n = 6 mice per group). (H) qRT-PCR showing expression of antimicrobial peptides in colon samples, including Lyz1, Defa20, Defa29 and Ang4, with Actb as a housekeeping gene (n = 6 mice per group). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, by 2-tailed Student’s t test (A, B, C, D and E) or 1-way ANOVA (F, G and H).
Figure 5. The anti-colitic benefit of MSC-Exos in DSS-colitic mice is macrophage dependent.

PKH26-labeled MSC-Exos (200 μg per mouse) were intravenously administrated to mice on day 2 during the 7-day 5% DSS administration. Mice were sacrificed on days 3, 5 and 7 for tracking analysis. (A, upper panel) The frequency of PKH26-positive intestinal cells dissociated from DSS treated or untreated mice (one million cells were detected in each colon). Numerical values denote the mean percentage of exosome positive (PKH26⁺) intestinal cells. (A, lower panel) Flow cytometric profiles of F4/80 and CD11b expression in PKH26⁺ cells. Numerical values denote the mean percentage of PKH26⁺ cells expressing CD11b and F4/80. (B) The quantification of PKH26⁺ cells. (C) The quantification of PKH26⁺ cells expressing F4/80 and CD11b. n = 3-4 mice per group for panels A-C. For panels D-G, mice received Clod-lipo or PBS-lipo according to the
schematic flowchart (see Supplemental Figure 3A), and MSC-Exos (200 μg per mouse) were
infused intravenously on day 2 (arrow in panel D). Colitis was assessed by DAI (D) daily. On day
7, mice were sacrificed and colon lengths (E), histopathological scores (F) and colonic MPO
activity (G) were determined. n = 12-15 mice per group for panels D-F and n = 5 for panel G. *P
≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ns indicates P > 0.05, by 2-tailed Student’s t test (B, C, E
and G) or Mann-Whitney test (D and F).
Figure 6. MSC-Exos reduce mucosal inflammation by polarizing M2b macrophages. (A, upper panel) Percent of cells expressing F4/80+CD11b+ in lamina propria of colons at the indicated time. Numerical values denote the mean percent of F4/80+CD11b+ macrophages (Mφ) in lamina propria of colons. (A, median and lower panels) Expression of CD206 (median panel) and arginase-1 (lower panel) in macrophages. Numerical values denote the relative mean fluorescence intensity (RelMFI) normalized to fluorescence minus one control. Quantification of macrophages (B) and of macrophages expressing CD206 (C) and arginase-1 (D) in the lamina propria of colons. n = 3 mice/group in panels A-D. (E) The cytokine contents in culture supernatants of F4/80+ macrophages isolated at day 7 from 5% DSS-colitic mice with or without MSC-Exos treatment on ex vivo 24 h culture with or without 100 ng/ml LPS re-stimulation (n = 4 mice/group). *P ≤ 0.05, **P ≤ 0.01 and ns indicates P > 0.05, by 2-tailed Student’s t test (B, C, D and E).
Figure 7. The anti-colitic benefit of MSC-Exos is partially dependent on macrophage-derived IL-10. (A) Intermediate DAI and colonic MPO activity in mice receiving MSC-Exos plus an antibody to IL-10 compared to DSS and MSC-Exo plus DSS treated mice (n = 8 mice per group). (B) CD4+ T cells isolated from MLNs of 5% DSS-colitic mice at day 7, were ex vivo culture with or without MSC-Exos (30 μg/ml) for 2 days. After re-stimulation with or without 5 μg/ml PHA for 24 h, the cytokine IL-10 was determined in culture supernatants (n = 4 mice per group). (C) CD4+ T cells isolated from human peripheral blood mononuclear cells were treated with or without MSC-Exos (30 μg/ml) for 2 days, and Th1 and Th2 flowcytometric profile were determined (n = 4 independent experiments). (D-H) Human peripheral blood monocyte-derived macrophages were cultured with MSC-Exos (30 μg/ml) or IL-4 (20 ng/ml) for 2 days. Cells were harvested and co-cultured with human peripheral blood-derived CD4+ T cells at a ratio of 1:10 stimulated with PHA (5 μg/ml) using a transwell system in the presence or absence of anti-IL-10 antibodies (10 μg/ml) or an isotype-matched IgG control. (D) Schematic drawing of the study design for the coculture experiments. (E) Proliferation of 5, 6-carboxyfluorescein...
diacetatesuccinimidyl ester (CFSE)-labeled CD4+ T cells assessed after 3-day coculture by flow cytometry. Numbers denote the percentage of cells undergoing at least one cellular division (mean ± SD, n = 4 independent experiments). (F) Quantification of proliferation of CFSE-labeled CD4+ T cells in panel E. (G-H) After 3-day coculture, CD4+ T cells were harvested and re-stimulated with PHA (5µg/ml) for 24 h and then TNF-α and IFN-γ contents were measured in supernatants by ELISA (n = 5 independent experiments). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 and ns indicates P > 0.05, by Kruskal-Wallis test (A) or 2-tailed Student’s t test (B and C) or 1-way ANOVA (F, G and H).
Figure 8. MSC-Exos reduce inflammatory responses in macrophages by transporting metallothionein-2. (A) Biological process (GO term) related to anti-colitic benefit. (B) Volcano plot showing the differential abundance of protein expression in MSC-Exos compared with the corresponding supernatants of exosome depletion. The vertical dotted lines correspond to 2-fold increase and decrease, respectively, and the horizontal dotted line represents a P-value of 0.05. Metallothionein-2 is annotated on the volcano plot by the red point. n = 3 in panels A and B. (C) The levels of metallothionein-2. Supernatants of MSCs were depleted of exosomes by ultracentrifugation and all samples were adjusted to have an equal total protein concentration, and then ELISA assays were performed. n = 4 independent experiments. (D) mRNA levels of TNF,
IL6 and IL1B in macrophages derived from human peripheral blood monocytes treated with 30 μg/ml MSC-Exos or MSC-ExosMock or MSC-ExosMT2A#2 for 2 days, and then were stimulated with LPS (100 ng/ml) during the last 4 h, with 18S rRNA as a housekeeping gene. n = 3 independent experiments. (E) Representative immunoblot for phosphorylated IκB-α (p-IκB-α), total IκB-α (t-IκB-α), and p65 subunit from whole-cell, nuclear and cytoplasmic extracts in macrophages which were treated for 2 days, with the indicated dose of MSC-Exos from MSCs that were that were untransfected (Un), mock transfected (Mock-si), or transfected with MT2A-siRNAs (MT2A-si). 3 independent experiments were performed and yielded similar results. MW, molecular weight. β-actin and Lamin A serve as loading controls. **P ≤ 0.01, ***P ≤ 0.001 and ns indicates P > 0.05, by Kruskal-Wallis test (C and D).