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LETTER TO THE EDITOR

Immunosuppressive exosomes from TGF-β1 gene-modified dendritic cells attenuate Th17-mediated inflammatory autoimmune disease by inducing regulatory T cells

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Dear Editor,

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is generally believed to originate from the complicated interactions between genetic factors and commensal flora. This leads to the breakdown of innate immunity and the aberrant activation of the immune system, which is largely responsible for tissue damages in patients and animal models [1]. A new $CD4^+$ T cell lineage, Th17, which predominantly produces IL-17, has been revealed recently to play a key role in the development of IBD. Studies in IL-17 receptor A (IL-17RA)-knockout mice have demonstrated that IL-17 is necessary for the development of IBD. Blockade of IL-17 signaling by an IL-17RA-IgG1 fusion protein significantly attenuated IBD [2]. It has also been shown that the $CD4^+$ T cells that express Foxp3 (Tregs) are the major regulatory T cells that help maintain intestinal homeostasis [3]. Despite the further understanding of the mechanisms of IBD, effective treatment is still lacking [4]. Therefore, it is necessary to explore new clues and targets for therapeutic interventions.

Exosomes are small vesicles released by various live cells with a structure of lipid bilayer and diameter ranging from 50 to 100 nm. They are formed by the fusion of multivesicular bodies with the plasma membrane followed by exocytosis [5]. It has been demonstrated that human tumor-derived exosomes can selectively impair lymphocyte responses to IL-2 and then impair their effector functions [6]. In addition, exosomes from immune inhibitory gene-modified bone marrow-derived dendritic cell (BMDC) have been found to suppress inflammation and collagen-induced arthritis [7]. Therefore, it is conceivable that exosomes may be used as vehicles for therapeutic intervention of autoimmune diseases.

In the present study, we were interested in whether exosomes derived from the TGF-β1 gene-modified BMDC (TGF-β1-EXO) have the immunosuppressive function and play a protective role during IBD development. First, we identified the profile of exosomes that we prepared. Under the electron microscope, the exosomes displayed typical characteristic of round morphology with a diameter of 50-100 nm. Immunoblotting results demonstrated that all types of exosomes contained the exosome-associated proteins Hsp70, Tsg101 and CD9, whereas ER-residing protein Grp94 was absent in exosomes (Supplementary information, Figure S1). We also examined the level of TGF-β1 in exosomes. The total amount of TGF-β1 in TGF-β1-EXO was about 15 pg/μg of exosomes and no TGF-β1 was expressed in exosomes derived from non-gene-modified BMDC (Control-EXO) or *LacZ* gene-modified BMDC (LacZ-EXO) (data not shown). When examined in mixed lymphocyte reaction system, Control-EXO and LacZ-EXO inhibited T cell proliferation at low-dose level. It was proposed that the inhibition was due to their low-level expression of costimulatory molecules. The T cell proliferation was promoted when dose was increased. Exosomes contain a large amount of allo-MHC molecules, which can be taken up by dendritic cells (DCs) and presented to T cells for allostimulation. This might be the reason why Control-EXO or LacZ-EXO did not inhibit, but promoted T-cell proliferation at a higher dose. On the other hand, TGF-β1-EXO showed a stronger inhibitory effect on T cell proliferation than on Control-EXO or LacZ-EXO via TGF-β1 and the effect was dose dependent (Supplementary information, Figure S2). We also analyzed the phenotype of exosomes by FACS (Supplementary information, Figure S3). In our results, there were no obvious difference in the expression level of costimulatory and adhesion molecules among Control-EXO, LacZ-EXO and except that TGF-β1-EXO express lower MHC class II molecules, which could explain that TGF-β1-EXO still showed stronger inhibitory effect than Control-EXO and LacZ-EXO after TGF-β1 was bloked. Moreover, TGF-β1-EXO plus anti-CD9 showed similar inhibitory effect to TGF-β1-EXO alone, excluding the possibility that anti-TGF-β1 can affect T cell modulatory properties of

TGF-β1-EXO through enhancing exosome capture by BMDC after antibody binding. These results further confirm that the inhibitory effect of TGF-β1-EXO is TGF-β1 dependent (Supplementary information, Figure S2).

Then we explored the effect of TGF-β1-EXO during IBD development. We found that *in vivo* administration of Control-EXO or LacZ-EXO did not prevent DSS-induced weight loss, whereas administration of TGF-β1-EXO significantly prevented the weight loss (Figure 1A). The disease activity index (DAI) scores and intestinal bleeding were also significantly reduced by the administration of TGF-β1-EXO, but not Control-EXO or LacZ-EXO (Figure 1B and 1C). Histological assessment of colonic damage revealed that the mice which were untreated or received Control-EXO or LacZ-EXO suffered severe diffuse inflammation involving the mucosa, submucosa, and in some cases extending through all intestinal layers (transmural inflammation). There was also pronounced disruption of the normal architecture and crypt loss. In contrast, DSS-mice treated with TGF-β1-EXO had much less damage in colon tissues, showing more conserved glandular structure and limited leukocyte infiltrations in mucosa and submucosa (Figure 1D). These results indicated that *in vivo* administration of TGF-β1-EXO could effectively inhibit the development of DSS-induced murine IBD. To further reveal the protective advantage of TGF-β1-EXO during the development of IBD, we compared the protective effect of TGF-β1-EXO and TGF-β1 cytokine. We found that 450 pg TGF-β1, the total amount in TGF-β1-EXO that we injected, showed no protective effect to IBD, and even if a higher dose of 4 500 pg TGF-β1 was injected, it showed only slight protective effect. The protective effect of TGF-β1-EXO was much better than TGF-β1 alone (Figure 1E). Although TGF-β1 is proved to play an important role in the protection of IBD, we could not detect the increase of TGF-β1 secretion at the inflammatory site (data not shown). The total TGF-β1 in exosomes that we injected into mice was about 450 pg; after diffusion *in vivo*, it was far from the detectable level. We supposed that the stability of TGF-β1 in TGF-β1-EXO would be favorable for the protective effect of TGF-β1-EXO, because we found that TGF-β1 presented in the form of exosomes showed stronger stability than TGF-β1 cytokine when digested by trypsin (Supplementary information, Figure S4). Moreover, the stability was related to the intact membrane structure of exosomes because disruption of the membrane structure of exosomes could decrease the ability of TGF-β1 and TSG101, another exosomal protein, in TGF-β1-EXO to resist the digestion of trypsin (Figure 1F). Inflammatory cells secret large amounts of proinflammatory cytokines such as TNF-α and IL-1, which increase the endotheliocytes to express ligands of adhesion molecule. Exosomes from BMDC are reported to express many kinds of adhesion molecules [8]. It is possible that exosomes will be recruited to an inflammatory site and cause the enriched effect of TGF-β1, which may be another factor that TGF-β1-EXO possess better protective effect than TGF-β1 alone.

Tregs were capable of suppressing colonic inflammation in IBD mice by downregulating Th17 responses via TGF-β [9]. We speculated that TGF-β1-EXO might induce the Tregs to exert protective effect. As expected, we found that TGF-β1-EXO could effectively induce $CD4^+Foxp3^+$ Tregs via TGF-β1 *in vitro* (Supplementary information, Figure S5), and we also found that TGF-β1-EXO induced $CD4^+Foxp3^+$ Tregs in lymphocytes from mesentery lymph nodes (mLNs) of inflammatory site (Figure 1G), but not in splenocytes (data not shown). It is known that the TCR signaling is required for the generation of Tregs, but not for TGF-β1 signaling [10]. Compared with lymphocytes of the inflammatory site, the possibility of splenocytes to contact intestinal antigen, which could provide the TCR signaling, was small. In addition, Tregs might transfer to spleen from mLNs and increase the Treg level in spleen. However, the increase of the transferred Tregs was too small to be detected in total $CD4^+$ T cells in the spleen. Therefore, TGF-β1-EXO-induced $CD4^+Foxp3^+$ Tregs were mainly from mLNs of the inflammatory site, but not in the remote splenocytes.

There is a delicate balance between Th17 and Treg. A signature transcription factor, RORγT for Th17 is also induced by TGF-β1. In the absence of IL-6, Foxp3 can inhibit RORγT function and drive Treg differentiation. However, when the cells also receive a signal from IL-6, Foxp3 function is inhibited and the Th17 differentiation pathway is induced [11]. Similar to $CD4^+Foxp3^+$ Tregs, we found that TGF-β1-EXO could decrease Th17 in lymphocytes from mLNs (Figure 1H), but not in splenocytes (data not shown). Furthermore, the levels of IL-17 and IL-6 in colon tissues were also inhibited by TGF-β1-EXO and results even showed that the serum level of IL-17 was decreased in TGF-β1-EXO-treated mice (Supplementary information, Figure S6), suggesting that TGF-β1-EXO could affect Th17 cells systemically.

To further confirm the protective effect of $CD4^+Foxp3^+$ Tregs, they were depleted by the pretreatment of mice with anti-CD25 monoclonal antibody (mAb). Then we found that the protective effect of TGF-β1-EXO against DSS-induced IBD was totally abrogated (Figure 1I) and the inhibitory effect of TGF-β1-EXO on IL-17 in colon tissues and serum also could not be observed (Figure 1J). These results further support the notion that $CD4^+Foxp3^+$ Tregs play a role in the prevention of DSS-induced IBD

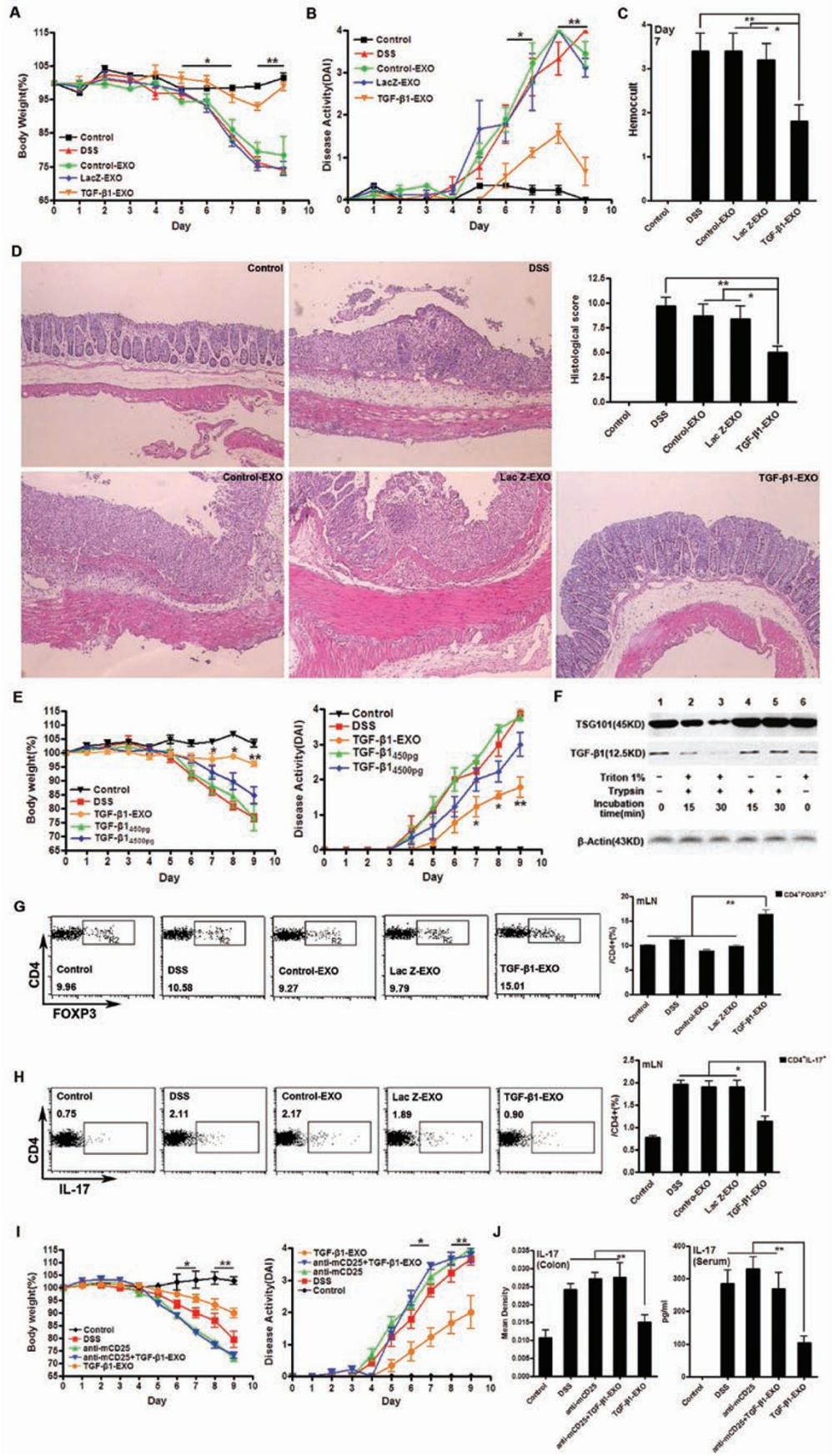
A
Body Weight(%)
Day
Control
DSS
Control-EXO
LacZ-EXO
TGF-β1-EXO
B
Disease Activity(DAI)
Day
C
Day 7
Hemoccult
D
Control
DSS
Control-EXO
Lac Z-EXO
TGF-β1-EXO
Histological score
E
Body weight(%)
Disease Activity(DAI)
TGF-β1450pg
TGF-β14500pg
F
TSG101(45KD)
TGF-β1(12.5KD)
Triton 1%
Trypsin
Incubation time(min)
β-Actin(43KD)
G
CD4
FOXP3
9.96
10.58
9.27
9.79
15.01
mLN
CD4+FOXP3+
H
IL-17
0.75
2.11
2.17
1.89
0.90
CD4+IL-17+
I
anti-mCD25
anti-mCD25+TGF-β1-EXO
J
IL-17 (Colon)
Mean Density
IL-17 (Serum)
pg/ml

Figure 1 TGF-β1-EXO delayed the development of the DSS-induced murine IBD by increasing $CD4^+Foxp3^+$ Tregs and inhibiting Th17. IBD was induced by administering 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 (10 μg exosomes/mouse/injection) on day−2, −3, and −5. **(A)** Body weight loss. Each point represents average weight data pooled from eight mice ± SD. **(B)** DAI (combined score of body weight, bleeding and stool consistency). Each point represents average DAI data pooled from eight mice. **(C)** Rectal bleeding was detected on day 7 by one step fecal occult blood test. Each column represents average score data pooled from eight mice. **(D)** Histological appearance 9 days after DSS induction. Representative colonic sections stained with H&E. Magnifications: 40×. **(E)** The protective effect of TGF-β1-EXO versus TGF-β1 cytokine in IBD. To evaluate the protective effect of TGF-β1 in IBD, mice were injected i.v. with TGF-β1 (150 pg or 1 500 pg/mouse/injection) on day−2, −3, and −5 (n = 8). **(F)** TGF-β1 in exosomes resists the hydrolysis of proteases. Exosomes were disrupted by 1% Triton X-100 for 30 min at 4 °C. Equal amount of disrupted and intact exosomes (30 μg) were then subjected to trypsin digestion at 37 °C with a concentration of 25 μg/ml for 15 and 30 min. After trypsin digestion, the samples were subjected to western blot analysis. Lane 1 (no triton, no trypsin); Lanes 2, 3 (triton lysis followed by trypsin digestion); Lanes 4, 5 (mock treatment followed by trypsin digestion); Lane 6 (triton lysis without trypsin digestion). β-actin control showed that equal amount of exosomes were used in each experiment. **(G)** The percentage of $CD4^+Foxp3^+$ Tregs from mLNs of each group of mice (n = 3). **(H)** The percentage of Th17 from mLNs of each group mice (n = 3). **(I)** Severity of DSS-induced IBD after $CD4^+Foxp3^+$ Treg depletion. $CD25^+$ T cells were depleted by i.p. injection of anti-mouse CD25 mAb (clone PC61.5.3). The anti-CD25 mAb (200 μg/mouse) was administered on day−8, −6 and −4. Control mice were injected with PBS alone without Ab (n = 5). **(J)** Level of IL-17 in inflammatory site and in murine sera after $CD4^+Foxp3^+$ Treg depletion (n = 5). All experiments were repeated three times. *P < 0.05, **P < 0.01, TGF-β1-EXO versus other groups except control.

by treatment with TGF-β1-EXO.

This is the first report about utilizing exosomes that were derived from TGF-β1 gene-modified BMDCs to delay DSS-induced IBD. TGF-β1-EXO induced $CD4^+Foxp3^+$ Tregs and decreased the proportion of Th17 in lymphocytes from mLNs of the inflammatory site. As acellular vesicles, exosomes can be preserved for a long time. Unlike DC, exosomes are derived from the DC and presumably reflect the function of the DC at the time of isolation. Moreover, the protective effect of TGF-β1-EXO in IBD was much better than TGF-β1 cytokine. All these specific characters make exosomes to be one of the attracting candidates for clinical application of autoimmune diseases. Experimental materials and methods are described in the Supplementary information, Data S1.

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(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)