Mesenchymal Stromal Cells Mitigate Experimental Colitis via Insulin-like Growth Factor Binding Protein 7-mediated Immunosuppression

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Mesenchymal stromal cells (MSCs) have shown great potential for treating inflammatory bowel disease, which is ameliorated through paracrine cross talk between MSCs and T-cells. Members of the insulin-like growth factor binding protein (IGFBP) family have important immunomodulatory functions in MSCs, but the underlying mechanisms behind these functions have not yet been clearly elucidated. In this study, we investigate whether MSC-produced IGFBP7 is involved in immune modulation using a mouse experimental colitis model. Gene expression profiling revealed that IGFBP7 was highly expressed in MSCs. Consistent with this findings, IGFBP7 knockdown in MSCs significantly decreased their immunomodulatory properties, decreasing the antiproliferative functions of MSCs against T-cells, while also having an effect on the proinflammatory cytokine production of the T-cells. Furthermore, in the mouse experimental colitis model, MSC-derived IGFBP7 ameliorated the clinical and histopathological severity of induced colonic inflammation and also restored the injured gastrointestinal mucosal tissues. In conclusion, IGFBP7 contributes significantly to MSC-mediated immune modulation, as is shown by the ability of IGFBP7 knockdown in MSCs to restore proliferation and cytokine production in T-cells. These results suggest that IGFBP7 may act as a novel MSC-secreted immunomodulatory factor.

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INTRODUCTION

Crohn's disease (CD) is one of two major types of inflammatory bowel disease. While the aetiology of CD is not well understood, recent studies have indicated that it may involve a complex interaction among genetic and environmental factors, which together give rise to an inappropriate and exaggerated intestinal inflammatory response. This response is primarily associated with the dysfunction of mucosal T-cells (including activated $CD4^+$ Th1 and $CD8^+$ CTL cells)[1,2] and altered cytokine production that together lead to damage of the intestinal mucosa.[3] No treatment is currently available for CD. The most effective therapies seek to control inflammation in the intestines, but they tend to produce side effects that can decrease significantly a patient's quality of life,[4,5] and are in any case ineffective in 33% of CD patients.[6] Recent findings regarding the pathophysiological mechanisms of CD suggest that the immunosuppressive effects of mesenchymal stromal cells (MSCs) and their ability to promote tissue repair represent a promising potential strategy for treating the condition.[7,8]

A number of soluble factors have been reported to be associated with the immunoregulatory functions of MSCs, including transforming growth factor (TGF)-β,[9] NO,[10,11] Indoleamine-pyrrole 2,3-dioxygenase (IDO),[12,13] tumor necrosis factor-stimulated gene 6 (TSG6),[14,15] prostaglandin E2 (PGE-2) (ref. 16) and the galectins.[17] However, blockage of any one of these molecules is insufficient to abolish completely the immunoregulatory functions of MSCs, indicating that several other important mediators may have not been identified yet. Gieseke *et al.* found that human MSCs exerted immunomodulatory functions in the absence of interferon (IFN)-γ receptor 1 signaling and IDO, and that this process might involve insulin-like growth factor binding proteins (IGFBPs).[18] However, no previous study has sought to identify an IGFBP family member that acts as a major effector in the process of MSC immunomodulation.

In a previous study, we reported that MSC4, one of the subpopulations in the MSC family, possesses trilineage differentiation abilities, exhibits particularly vigorous immunomodulatory properties and expresses the highest levels of IGFBP7,[19] all of which

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suggests that IGFBP7 may play an important role in the immunomodulatory functions of MSCs.

IGFBP7, also known as mac25 or IGFBP-related protein-1, is a secreted protein that belongs to the IGFBP family.[20] The IGFBPs (IGFBP1-16), IGF-1/IGF-2 and their receptors make up the IGF axis, and play a key role in the growth, differentiation, and proliferation of mammalian cells. IGFBP7 has been reported to act as a tumor-suppressor gene that influences cell proliferation, survival and apoptosis via the Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-actiavted protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) signaling pathways.[20–22] Recently, a novel role for IGFBP7 in the mouse uterus was revealed to be inhibition of IGFBP7 resulted in pregnancy failure by shifting uterine cytokines to Th1 type dominance and repressing uterine decidualization.[23] Thus, the role of IGFBP7 in the immunoregulation of MSCs needs to be clarified.

RESULTS

IGFBP7 is highly expressed in mouse MSCs

Mouse MSCs were isolated from bone-marrow aspirates by adherent culture. The characteristics of the MSCs were confirmed based on the expression of typical surface markers and trilineage differentiation potential. We then analyzed the expression of IGFBP members by quantitative polymerase chain reaction. IGFBP7 was found to be most abundant in MSCs. mRNA of IGFBP2 and IGFBP6 was detected to a lesser extent than IGFBP7, and for IGFBP1, IGFBP3, IGFBP4, and IGFBP5 there was only weak or no expression (**Figure 1a**).

To investigate the role of IGFBP7 within the immunomodulatory properties of MSCs contributes, we generated a knockdown of IGFBP7 in MSCs by RNA interference, which was designed as $MSC^{shIGFBP7}$. The knockdown of IGFBP7 was analyzed by quantitative polymerase chain reaction and showed that there was a decrease of 70% of IGFBP7 expression compared with MSCs transduced without target sequences (MSC^{con}) (**Figure 1b**). Furthermore, western blotting analysis demonstrated the expression of IGFBP7 was almost undetectable in whole-cell lysate of $MSC^{shIGFBP7}$ (**Figure 1c**). To study whether IGFBP7 knockdown could affect the characteristics of the MSC, we first used Fluorescence-activated cell sorting (FACS) to analyze the cell surface markers of $MSC^{shIGFBP7}$. Compared with MSC^{con}, transduced cells expressed the same panel of surface markers, including Sca-1, CD44, and CD106, and the absence of CD34, CD45, CD11b or c-kit (**Figure 1d**), which indicated that the transduced cells maintained the phenotype of MSCs. Cell counting showed that IGFBP7 knockdown did not alter the proliferative properties of MSCs ($P > 0.05$, **Figure 1e**). To demonstrate the multipotency of $MSC^{shIGFBP7}$, we cultured cells under conditions that promote differentiation into osteogenic, adipogenic, or chondrogenic lineages. As confirmed by Alizarin Red S staining, oil red O staining or Aggrecan staining, respectively, the $MSC^{shIGFBP7}$ cells have shown no change in osteogenic, adipogenic or chondrogenic differentiation capacity as compared with MSC^{con} ($P > 0.05$, **Figure 1f**).

MSCs inhibit the proliferation of T-cells through IGFBP7 *in vitro*

To investigate whether IGFBP7 contributes to the immunomodulatory properties of MSCs, the antiproliferative effects of MSC^{con} or $MSC^{shIGFBP7}$ on T-cells were analyzed. MSC^{con} significantly inhibited the proliferation of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, respectively. In contrast, inhibition of T-cell populations by $MSC^{shIGFBP7}$ was significantly reduced in $CD3^-$ (59.49 ± 4.53% versus 27.70 ± 8.24%), $CD4^-$ (49.74 ± 2.74% versus 29.68 ± 6.72%), and $CD8^-$ (62.28 ± 2.43% versus 49.56 ± 0.40%) T-cells compared with MSC^{con} (see **Supplementary Figure S1a** and **Figure 2a,b**). To further confirm these results and exclude the possible off-target effects, we used two small interfering RNAs (siRNAs) of different sequences targeting the same transcript of IGFBP7. Both of two siRNAs specific targeting IGFBP7 caused a significant downregulation of IGFBP7 at mRNA and protein levels in the transduced MSCs, thus demonstrating effective induction of IGFBP7 silencing (see **Supplementary Figure S2a,b**). Similar with the data above, the IGFBP7 knockdown in MSCs partially restored the proliferation of T-cells (see **Supplementary Figure S2c**).

IGFBP7 was reported to arrest the cell cycle in various tumor cells.[22,24–26] Therefore, we investigated whether IGFBP7 mediated the antiproliferative properties of MSCs through affecting the cell cycle of T-cells. After stimulated with anti-CD3/CD28 in the presence or absence of MSCs, the proportion of T-cells in the G1 phase was significantly increased in the presence of MSCs compared with the stimulated T-cells. The percentage of G1 phase in $CD3^-$, $CD4^-$, and $CD8^-$ T-cells was partially restored when IGFBP7 expression was silenced in MSCs (see **Supplementary Figure S1b** and **Figure 2c,d**). Both these results indicate that MSCs may suppress the proliferation of T-cells partially through IGFBP7.

Since IGFBP7 was reported to inhibit the cell cycle progression of AML cells via the PI3K/AKT and MAPK/ERK signaling pathways,[22] we studied whether these two signaling pathways were involved in the IGFBP7-mediated cell cycle arrest of T-cells. Indeed, compared with the activated T-cells, the protein levels of p-ERK and p-AKT in $CD3^+$ T-cells were significantly reduced in the presence of MSC^{con}. By contrast, the inhibition of ERK and AKT signaling pathways activation of the T-cells were restored to the same extent when cocultured with $MSC^{shIGFBP7}$, respectively (see **Supplementary Figure S3a,b**), indicating that PI3K/AKT and MAPK/ERK signaling may contribute to the IGFBP7-mediated cell cycle arrest of T-cells.

IGFBP7 knockdown in MSCs restores the proinflammatory cytokine production of T-cells

MSCs are known to decrease the release of proinflammatory cytokines by T-cells.[17,27] Therefore, we analyzed the possible role of MSC-derived IGFBP7 in suppressing the tumor necrosis factor (TNF)-α and IFN-γ expression in T-cells. Expectedly, MSC^{con} significantly inhibited the expression of proinflammatory cytokines by activated T-cells. Remarkably, the IGFBP7 knockdown in MSCs restored the percentages of TNF-α-producing and IFN-γ-producing T-cells, respectively (see **Supplementary Figure S4a,b** and **Figure 3a–d**). The similar phenomena was observed when the IGFBP7 knockdown in MSCs through siRNA approach (see **Supplementary Figure S2d,e**). The results indicated that IGFBP7 not only contributes to the antiproliferative functions of MSCs but is also involved in the modulation of the cytokine production of T-cells.

The NF-κB signaling pathway was previously reported to be involved in the secretion of proinflammatory cytokines by

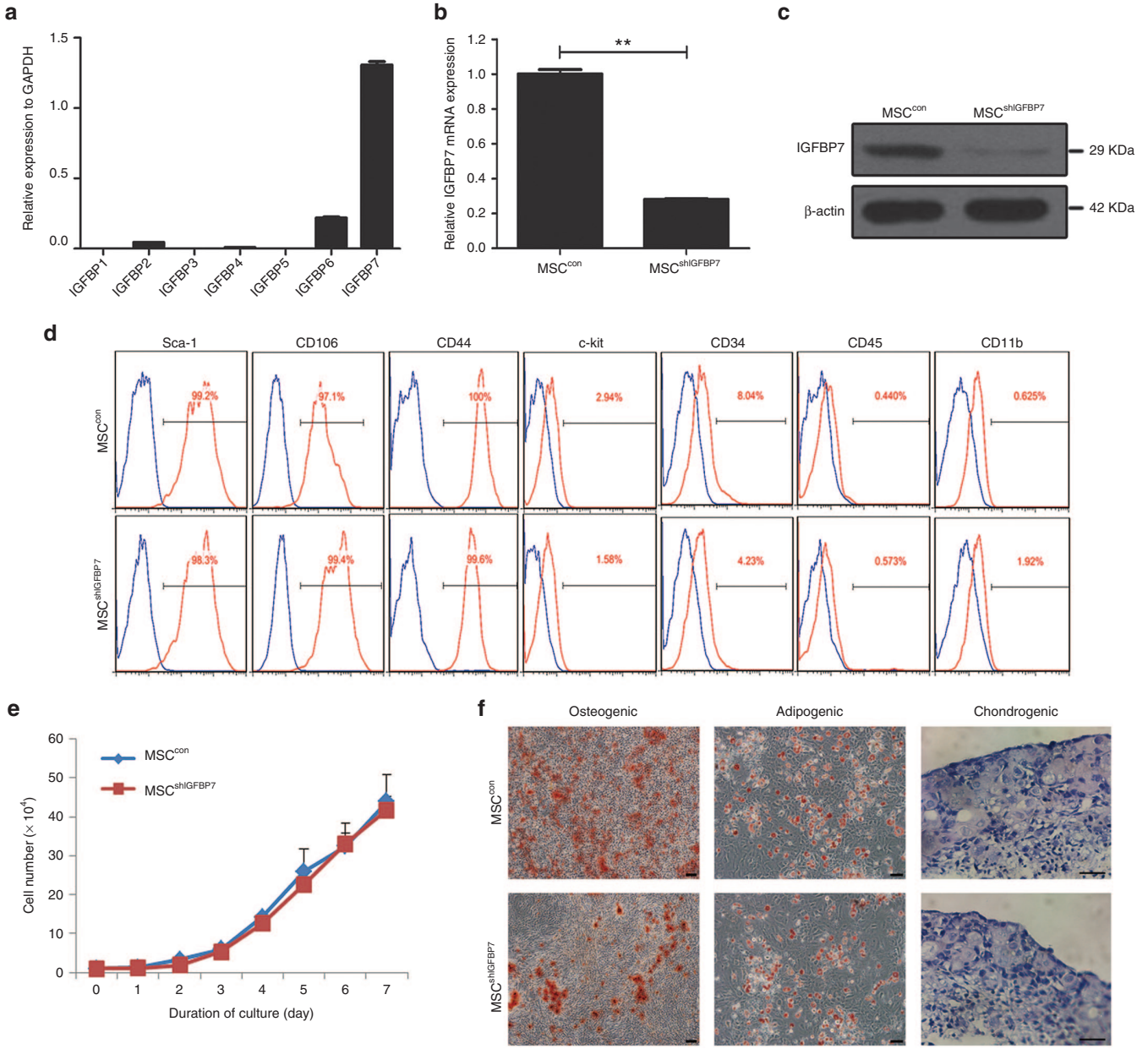


Figure 1 IGFBP7 is the only member of the IGFBP family that is highly expressed in mouse MSCs, the characteristics of which does not alter when IGFBP7 interference. (**a**) The relative mRNA expression levels of IGFBP1-7 were analyzed by quantitative polymerase chain reaction (qPCR) and normalized with respect to the expression of GAPDH. (**b**) The efficiency of shRNA-mediated down-regulation of IGFBP7 was assessed at the RNA level. The expression of IGFBP7 in MSC^{con} was regarded as 1. (**c**) The efficiency of shRNA-mediated down-regulation of IGFBP7 was assessed at the protein level. The expression of β-actin was used as a control. (**d**) Cell surface markers on MSC^{con} and $MSC^{shIGFBP7}$ were detected by flow cytometry. (**e**) Growth curves of MSC^{con} and $MSC^{shIGFBP7}$wereassessed by direct counting for 7 days. Three replicates were performed at each time point. (**f**) Adipogenic, osteogenic and chondrogenic differentiations of MSC^{con} and $MSC^{shIGFBP7}$. Scale bar = 100 μm. Data are shown as mean ± SEM ($n = 3$). $^{**}P < 0.01$. IGFBP, insulin-like growth factor binding protein; MSC, mesenchymal stromal cells; GAPDH, ; SEM, standard error of mean.

T-cells.[28] IGFBP3 and IGFBP5 were already known to suppress the secretion of proinflammatory cytokines by inhibiting the NF-κB signaling pathway.[29–32] Therefore, we studied whether this signaling pathway is involved in the IGFBP7-mediated suppression of cytokines. After T-cells were cocultured with MSC^{con}, their protein levels of p-p65 and p-IκBα in CD3- T-cells were significantly down-regulated. Compared with the experiments, the phosphorylation levels of p-p65 and p-IκBα were restored to the same extent when IGFBP7 expression was silenced in MSCs (see **Supplementary Figure S5**). These findings indicated that MSCs-derived IGFBP7 could inhibit the activation of the NF-κB signaling pathway.

Moreover, we investigate whether IGFBP7 play a similar role in the immunomodulatory function of human MSCs (hMSCs). Using five hMSCs samples from different donors, we identified that the expression of IGFBP7 is relatively high in hMSCs, but the expression level varies between donors (see **Supplementary Figure S6a,b**). Through silencing the IGFBP7 expression in human MSCs

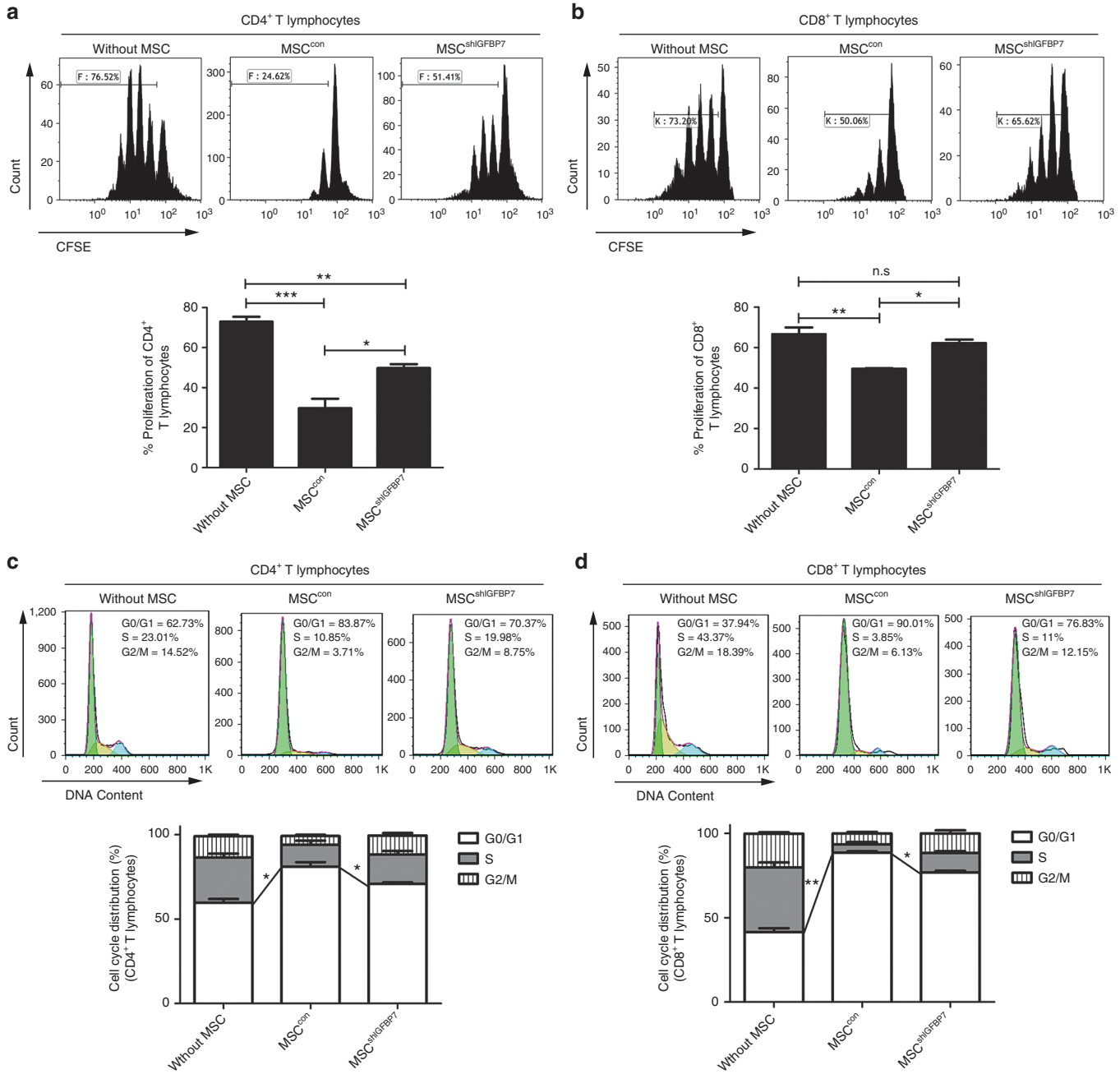


Figure 2 Mouse MSCs inhibit the *in vitro* proliferation of T-cells through IGFBP7 by arresting the cell cycle. The proliferation levels of mouse CD4⁺ T-cells (**a**) and CD8⁺ T-cells (**b**) were analyzed by flow cytometry; the change of CFSE fluorescence intensity indicates the growth ratio. The cell cycle distributions of mouse CD4⁺ T-cells (**c**) and CD8⁺ T-cells (**d**) were analyzed by flow cytometry. The percentages of cells in the G0/G1 (green peak), S (yellow peak) and G2/M (blue peak) phases were determined. Data are shown as mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and n.s. = not significant. IGFBP, insulin-like growth factor binding protein; MSC, mesenchymal stromal cells; CFSE, carboxyfluorescein succinimidyl ester; SEM, standard error of mean.

with siRNAs (see **Supplementary Figure S6c,d**), we found IGFBP7 knockdown significantly decreased their immunosuppressive properties, such as decreasing the antiproliferative functions of hMSCs against T-cells (see **Supplementary Figure S7**), restoring the proinflammatory production in T-cells (see **Supplementary Figure S8a,b**). Both of the results indicate IGFBP7 may also contribute to the immune modulation in hMSCs, which provide new clues to reveal novel mechanisms of action for MSCs.

MSC-derived IGFBP7 is negligible in MSC-mediated T-cell apoptosis or Treg cells increase

IGFBP7 was previously reported to induce apoptosis in tumor cells, including AML,[22] melanoma[21] and HCC[33]cells. Furthermore, MSCs are able to increase the percentage of regulatory T-cells both *in vitro* and *in vivo*.[34,35] To analyze of whether MSC-derived IGFPBP7 play a possible role in these processes, we monitored the apoptosis of T-cells and the percentage of

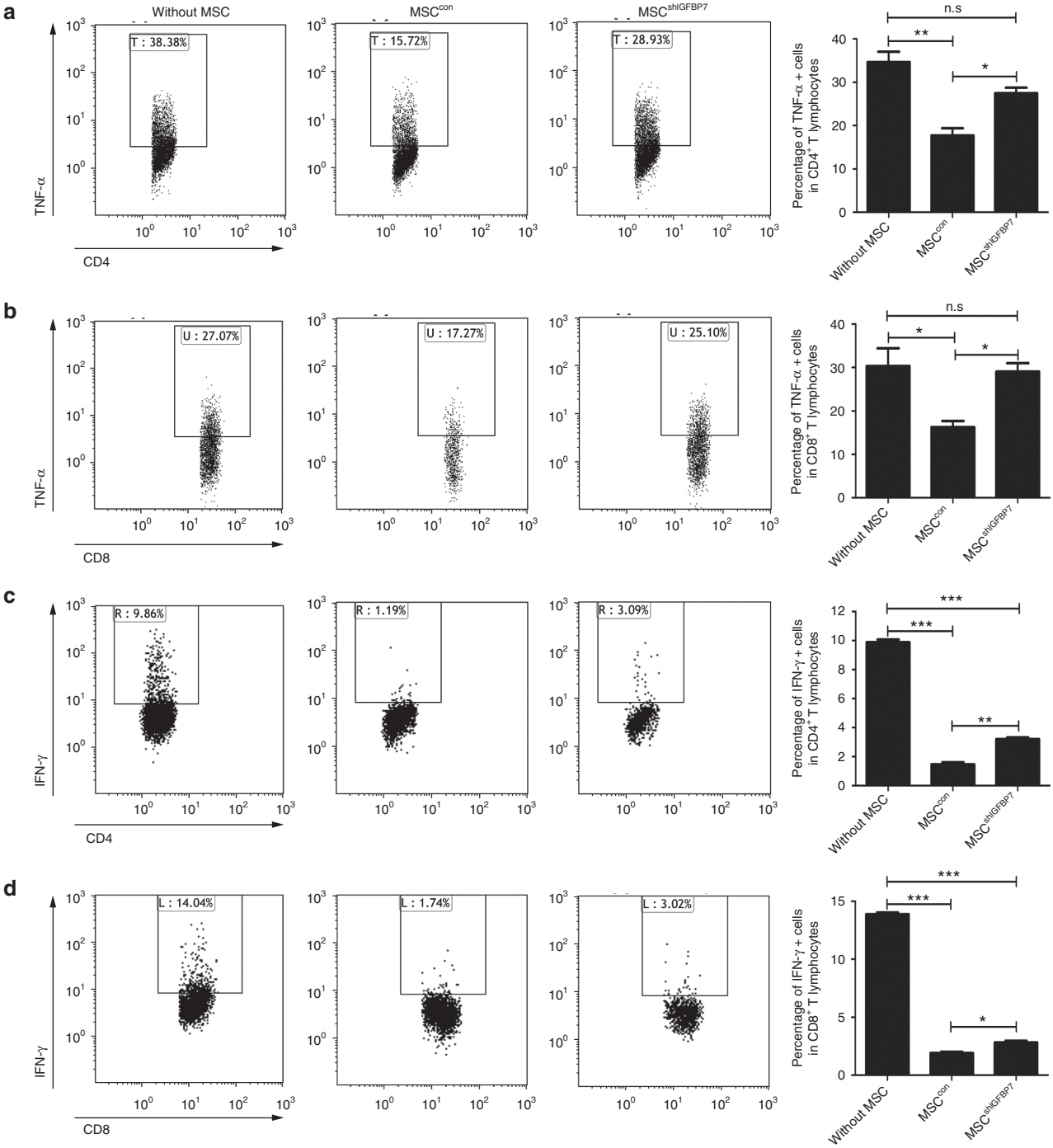


Figure 3 Mouse MSCs down-regulate the *in vitro* proinflammatory cytokine expression of T-cells through IGFBP7. The expression levels of TNF-α and IFN-γ in CD4⁺ T-cells (**a** and **c**, respectively) and CD8⁺ T-cells (**b** and **d**, respectively) were analyzed by flow cytometry after 2 days of coculture with MSCs. Data are shown as mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and n.s. = not significant. IGFBP, insulin-like growth factor binding protein; MSC, mesenchymal stromal cells; TNF, tumor necrosis factor; IFN, interferon; SEM, standard error of mean.

Treg cells in MSC/CD3 T-cell coculture experiments. The results showed that there was no difference in the proportion of Annexin V⁺ cells in CD3⁺ T-cells and their subpopulations between the MSC^{con} and MSC^{shIGFBP7} groups (see **Supplementary Figure S9a–c**). Similarly, although the proportion of CD4⁺CD25⁺Foxp3⁺ Tregs was significantly increased in the MSC^{con} compared with the group absence of MSCs, there was no difference between the MSC^{con} and MSC^{shIGFBP7}groups (see **Supplementary Figure S10**). These findings indicated that MSCs-derived IGFBP7 may not play a role in the apoptosis and Tregs induction of T-cells.

MSCs alleviate TNBS-induced experimental colitis through IGFBP7 *in vivo*

To examine the potential therapeutic relevance of MSC-produced IGFBP7 *in vivo*, we generated an experimental mouse model of colitis using 2,4,6-trinitrobenzene sulfonic acid (TNBS), and injected intraperitoneally (i.p.) either MSCs (MSC^{con} or $MSC^{shIGFBP7}$) or saline (no treatment; negative control, NC) 12 hours later. The results showed that the clinical parameters of colitic mice (including body weight, colitis score, survival rate, colon length, macroscopic and histological scores) were significantly improved in the MSC^{con} group, and were minimally improved in $MSC^{shIGFBP7}$ group compared with NC group (**Figure 4a–g**).

In detail, the body weights of colitic mice were increased quickly a day after TNBS injection in the MSC^{con} group, and were gained slowly 2 days after TNBS injection in $MSC^{shIGFBP7}$ and NC group (**Figure 4a**). The colitis score evaluated according to stool consistency and rectal bleeding were significantly relieved in MSC^{con} group (1.7 ± 0.9 points), and were not improved in $MSC^{shIGFBP7}$ group (3.00 ± 0.87 points) compared with NC group (3.44 ± 0.68 points) (**Figure 4b**). The similar phenomenon was observed in the survival rate (MSC^{con}: 90.00%, $MSC^{shIGFBP7}$: 50.00%, and NC: 40.00%) (**Figure 4c**), the degree of edema and hyperemia, colon length (MSC^{con}: 7.47 ± 0.12 cm, $MSC^{shIGFBP7}$: 6.60 ± 0.16 cm, and NC: 6.30 ± 0.16 cm), and macroscopic score (MSC^{con}: 2.33 ± 0.47 points, $MSC^{shIGFBP7}$: 5.00 ± 0.82 points, and NC: 7.33 ± 0.47 points) (**Figure 4d,e**), the status of inflammation cells infiltrating, and histological score (MSC^{con}: 1.25 ± 0.43 points, $MSC^{shIGFBP7}$: 3.00 ± 0.71 points, and NC: 3.75 ± 0.43 points) (**Figure 4f,g**), and the number of $CD4^+$ (MSC^{con}: 6.00 ± 2.97 cells/field, $MSC^{shIGFBP7}$: 25.60 ± 9.89 cells/field, and NC: 41.20 ± 2.40 cells/field) and $CD8^+$ lymphocytes (MSC^{con}: 3.80 ± 1.17 cells/field, $MSC^{shIGFBP7}$: 18.60 ± 6.15 cells/field, and NC: 29.0 ± 6.42 cells/field) of colon (see **Supplementary Figure S11a,b**).

We also analyzed the mRNA expression of proinflammatory cytokines (including TNF-α, interleukin (IL)-6, IL-1β, IL-17 and IL-12a), anti-inflammatory cytokines (IL-10), and chemokines (including MIP-2 and Rantes) in colon tissues. In comparison with the NC group, the expression of proinflammatory cytokines and chemokines decreased, while IL-10 increased in MSC^{con} group. In line with our other results, these effects were significantly attenuated in the $MSC^{shIGFBP7}$ group (see **Supplementary Figure S12**).To further compare the biodistribution and therapeutic effect of MSCs administration through i.p. and intravenous (i.v.) injection, the luciferase transduced MSCs were administrated with local i.p. and systemic i.v. injection 12 hours after TNBS injection, where the results showed that MSCs were sustained in peritoneum for 7 days after i.p. injection, and were distributed in lung for 4 days after i.v. injection and then were transferred to liver (see **Supplementary Figure S13a**). Moreover, MSCs through i.p. administration could significantly and quickly ameliorate experimental colitis symptoms, compared with the group of MSCs through i.v. injection (see **Supplementary Figure S13b–d**).

MSCs suppress the proliferation and proinflammatory cytokine secretions of mesenteric lymph node cells through IGFBP7

MSCs were found to ameliorate TNBS-induced colitis by reducing the response and migration of autoreactive T-cells to the colon. T-cells in mesenteric lymph node (MLN)s, which are the nearest immune organs to the colon, might be most likely affected.[7,14,36] Accordingly, we investigated whether MSC-produced IGFBP7 could affect the MLN cells of mice that were subjected to experimentally-induced colitis. Consistent with the *in vitro* and *in vivo* results presented above, the proliferation levels and the secretion of proinflammatory cytokines by MLN cells were strongly suppressed by MSC^{con} and were slightly suppressed by $MSC^{shIGFBP7}$ (**Figure 5a–c**).

In detail, the proliferation ratios of unstimulated MLN cells were 7.48 ± 0.32, 16.74 ± 1.09, and 19.28 ± 2.55% in the MSC^{con}, $MSC^{shIGFBP7}$, and NC groups respectively (**Figure 5a**). After being stimulated, the proliferation ratios of MLN cells were 63.12 ± 5.36, 78.79 ± 2.56, and 80.45 ± 1.56% (**Figure 5a**); the presence of TNF-α expressed MLN cells were 63.39 ± 0.80, 71.51 ± 1.00 , and 74.07 ± 1.11% in $CD4^+$ cells, and 8.27 ± 0.47, 54.94 ± 0.40, and 59.75 ± 0.63% in $CD8^+$ cells (**Figure 5b**); the presence of IFN-γ expressed MLN cells were 1.69 ± 0.17, 1.97 ± 0.17, and 1.99 ± 0.26% in $CD4^+$ cells, and 11.72 ± 0.25, 18.36 ± 1.08, and 20.56 ± 0.96% in $CD8^+$ cells (**Figure 5c**).

In addition, the proportion of $CD4^+CD25^+Foxp3^+$ Tregs in the MSC^{con}-treated group was significantly increased. However, the IGFBP7 knockdown in MSCs had no significant effect on Tregs percentage (see **Supplementary Figure S14**), the results indicating that MSC-secreted IGFBP7 might have no direct effects on Tregs.

MSCs, acting through IGFBP7, directly suppress the proliferation and proinflammatory cytokine secretion of MLN cells derived from mice subjected to experimental colitis

To strengthen the evidence that MSC-produced IGFBP7 could affect the MLN cells of mice subjected to experimental colitis, we cocultured the MLN cells derived from mice in NC group with MSC^{con} or $MSC^{shIGFBP7}$*in vitro*. The results showed that the proliferation levels and proinflammatory cytokine secretions of MLN cells were drastically suppressed by MSC^{con} and tenderly suppressed by $MSC^{shIGFBP7}$ (**Figure 6a–c**).

In detail, the proliferation ratios of MLN cells were 86.57 ± 0.98, 34.61 ± 4.84, and 59.17 ± 1.80% in blank control, MSC^{con} and $MSC^{shIGFBP7}$ group respectively (**Figure 6a**); the presence of TNF-α expressed MLN cells were 68.65 ± 0.62, 57.94 ± 1.98, and 64.46 ± 1.93% in $CD4^+$ cells, and 50.00 ± 3.64, 35.22 ± 0.71, and 40.96 ± 0.61% in $CD8^+$ cells (**Figure 6b**); the presence of IFN-γ expressed MLN cells were 1.24 ± 0.04, 0.39 ± 0.04, and 0.88 ± 0.18% in $CD4^+$ cells, and 11.57 ± 1.15, 3.04 ± 0.66, and 6.78 ± 0.51% in $CD8^+$ cells (**Figure 6c**). The results were similar to the data reported above regarding MLNs *in vivo*, which amounts to solid evidence that MSC-produced IGFBP7 may directly affect the MLN cells of mice subjected to experimental colitis.

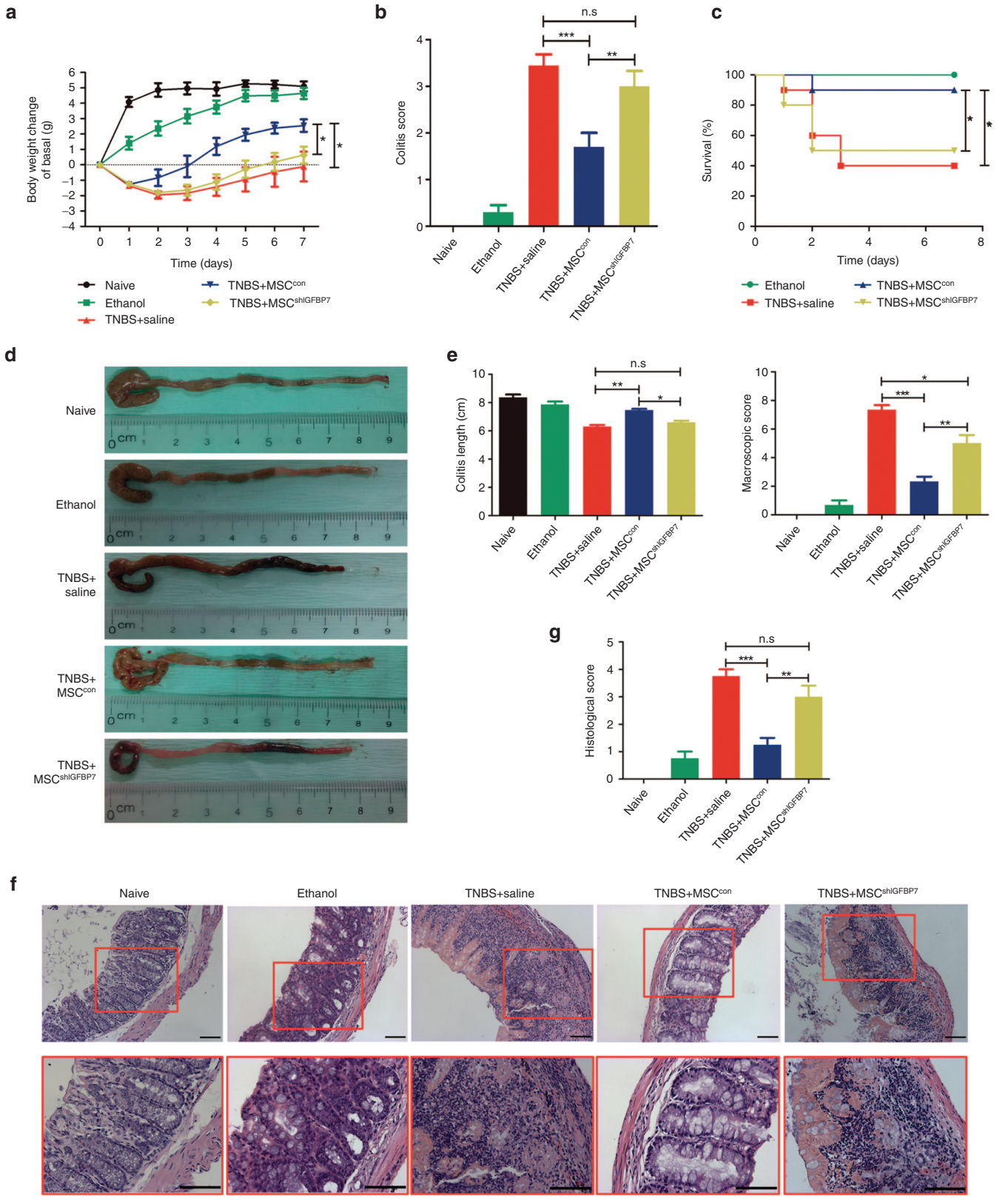


Figure 4 Mouse MSCs alleviate TNBS-induced experimental colitis through IGFBP7 in a mouse model. Colitis was induced by intracolonic administration of 2.5% TNBS or with 50% ethanol alone (control, ethanol group) or no solution (blank control, naive group). After 12 hours, mice (n = 10 per group) were treated i.p. with 2×10^6 MSC^{con}, $MSC^{shIGFBP7}$ cells or saline. Clinical evaluation of the disease was monitored based on body weight (**a**), colitis score (**b**) and survival rate (**c**). Colons were examined with respect to general form (**d**), length and macroscopic scores (**e**) on day 2 after TNBS intracolonic administration. Histopathologic analysis (H&E staining and histological score) (**f** and **g**) were determined 2 days after transplantation of the cells (n = 4 per group). Scale bar = 100 µm. Data are shown as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, and n.s. = not significant. IGFBP, insulin-like growth factor binding protein; MSC, mesenchymal stromal cells; SEM, standard error of mean; TNBS, 2,4,6-trinitrobenzene sulfonic acid.

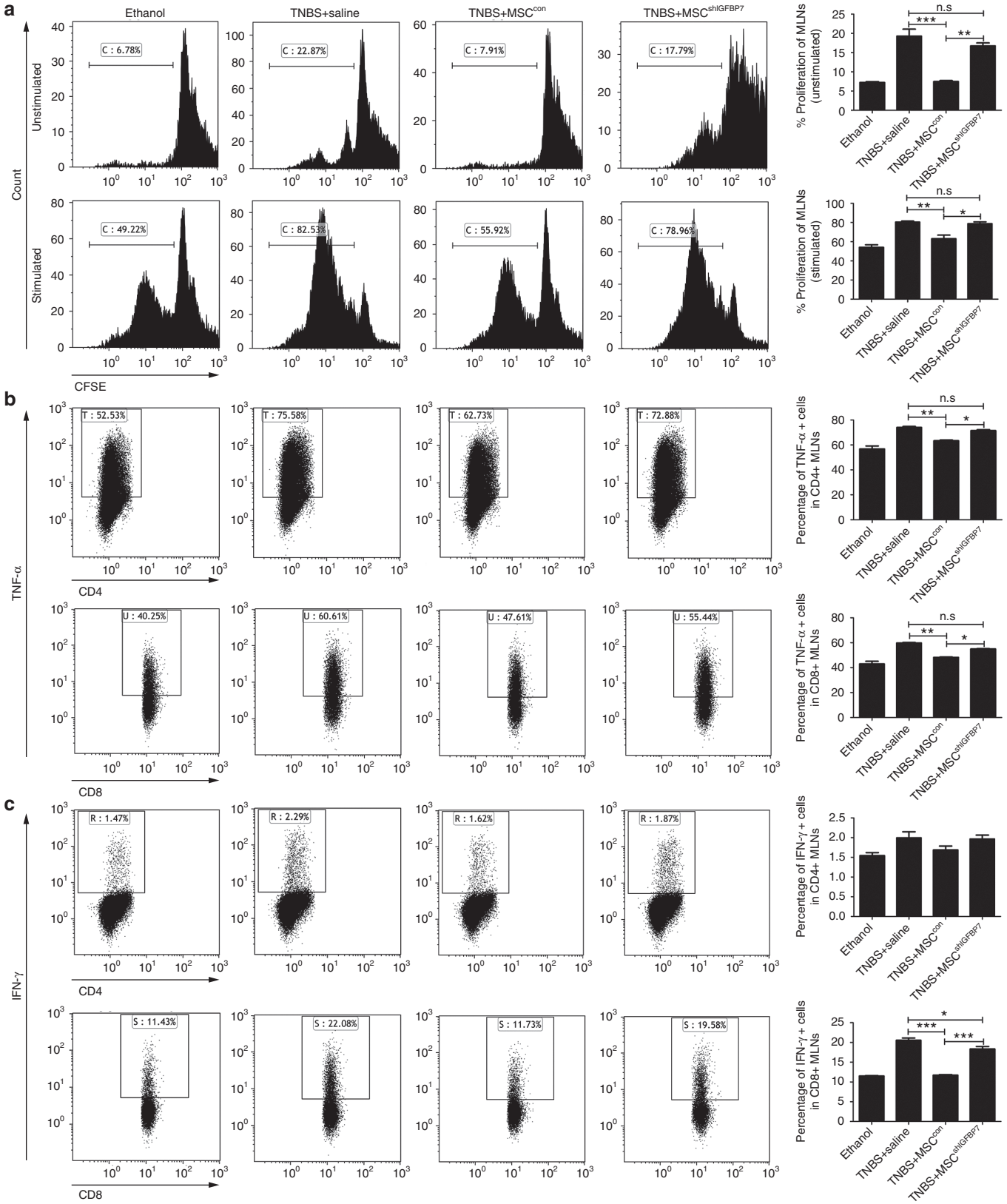


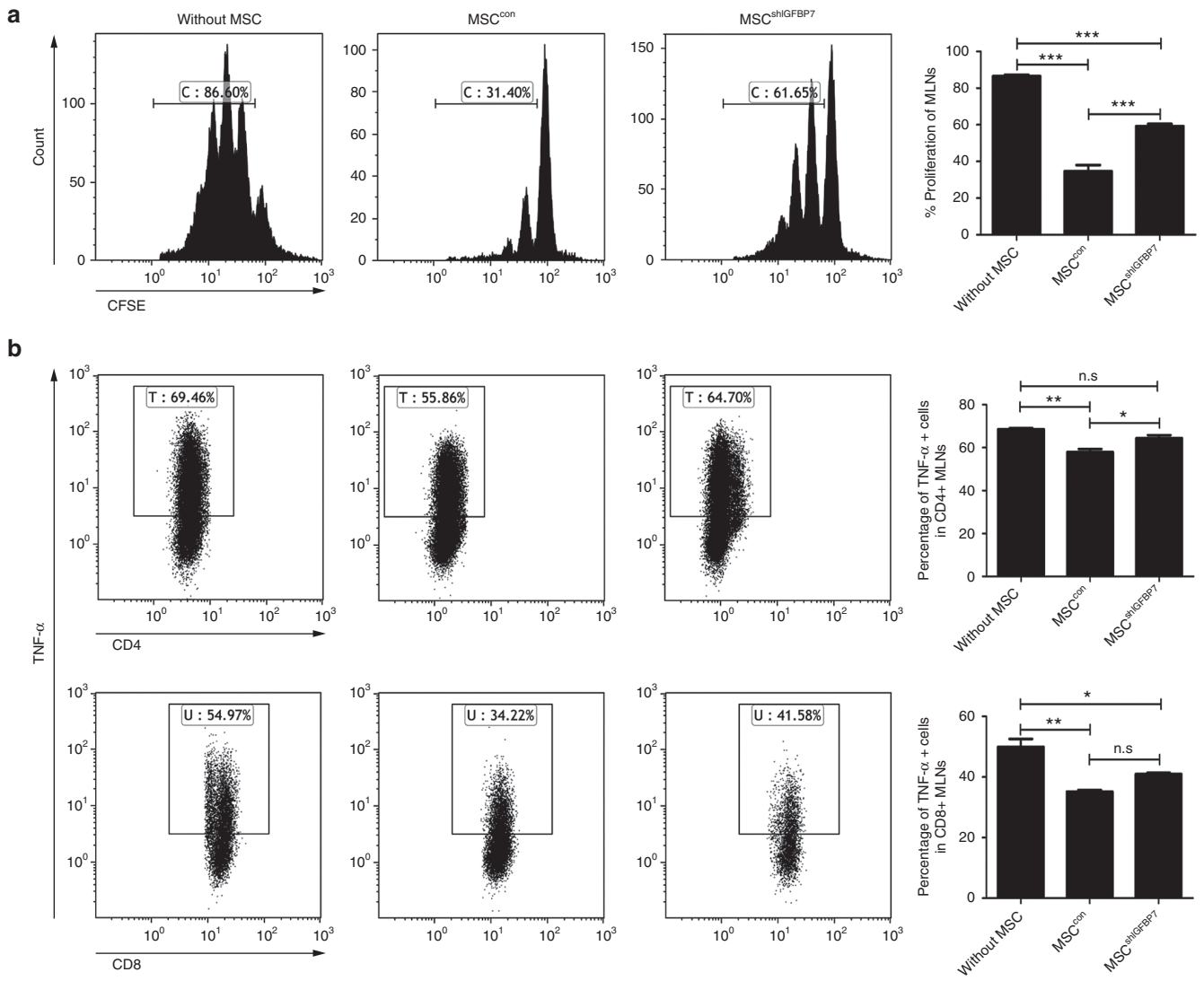
Figure 5 Mouse MSCs suppress the proliferation and proinflammatory cytokine secretion of mesenteric lymph node (MLN) cells through IGFBP7 in an experimental mouse model of colitis. (**a**) Changes in proliferation within each group of MLNs *in vitro* with or without stimulation. The proliferation ratios were reflected using CFSE and analyzed by flow cytometry. The production of TNF-α (**b**) and IFN-γ (**c**) was assessed in $CD4^+$ and $CD8^+$ MLN cells. These cells were stimulated immediately with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 hours after isolation from mouse. Data are shown as mean ± SEM ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and n.s. = not significant. IGFBP, insulin-like growth factor binding protein; MSC, mesenchymal stromal cells; SEM, standard error of mean; CFSE, ; PMA, phorbol ester; TNF, tumor necrosis factor; IFN, interferon.

The damage of intestinal barrier composed of intestinal epithelial cells (IECs) in inflammatory bowel diseases can lead to immune responses of intestinal mucosa and finally promotes the inflammatory process.[37] Previous study has demonstrated that MSCs could protect the damaged IECs via paracrine fashion.[38] To evaluate whether MSCs protect the IECs through IGFBP7, we performed a cell apoptosis assay induced by cytokines. Using MODE-K cells, a murine small IEC line, we found MSC^{con} could significantly inhibit the apoptosis of such cells treated with TNF-α and IFN-γ, but IGFBP7 knockdown has not impaired the cytoprotective function of MSCs. Both of the results indicate MSCs do have direct cytoprotective effects on intestinal epithelial cells, but IGFBP7 might not participate in this process (see **Supplementary Figure S15**).

DISCUSSION

As MSCs can modulate inflammation and contribute to tissue regeneration, it seems logical that MSC-based therapy might be a promising approach for treating CD. Among the immune regulatory factors, most of them are induced by inflammatory cytokines. Other factors believed to mediate the immunomodulatory effect of MSCs, such as galectin-1, are constitutively expressed at a high level.[17] We speculated that the factors involved and the underlying mechanisms differed based on the type of tissue, disease microenvironments and the species. In order to promote the clinical application of MSCs in CD, it is therefore necessary to identify the MSC-driven immunomodulators and related mechanisms that function in the CD microenvironment.

IGFBPs are important components of the IGF axis, playing a critical role in normal growth and development. IGFs bind to their specific receptors and exert biological functions via the PI3K/AKT and MAPK/ERK signaling pathways.[39,40] IGFBPs could transport IGFs, prolonging their half-lives and modulating their bioavailability and function. In addition, IGFBPs exert specific IGF-independent biological functions.[29,32] For example, Gieseke *et al*. reported that IGFBPs could contribute to the immunomodulatory functions of MSCs.[18] However, they did not examine which member of the IGFBP family would be acting as the major effector in this process. In the mouse MSCs used in the experiments reported here, IGFBP7 was the only member of the IGFBP family to show high-level expression. Since it was previously reported that senescent cells release more IGFBPs than



young cells,[41] we assessed MSCs in terms of their cell morphology, proliferation rate, differentiation potential, and immunomodulatory ability. The results confirmed that our MSCs were not senescent (**Figure 1e**,**f**).

The pathogenesis of CD is due to a dysfunctional interaction between the intestinal microflora and the mucosal immune system. In this context, the presence of overactive mucosal effector T-cells and/or underactive mucosal Treg cells leads to improper modulation of even normal effector T-cells.[32] The results of our *in vitro* and *in vivo* studies indicated that MSCs-secreted IGFBP7 could suppress the proliferation of $CD4^+$ and $CD8^+$ T-cells and thereby alleviate experimentally-induced colitis. The suppression of proliferation could have been the consequence of an IGFBP7-induced cell-cycle arrest in G1 phase. Previously, IGFBP7 was shown to act as a tumor-suppressor gene. Chen *et al.* showed that infection of the orthotopic xenograft mouse models of human HCC with an IGFBP7-expressing adenovirus (Ad.IGFBP7) profoundly inhibits primary tumor growth *in vivo*.[33]Wajapeyee *et al.* found that IGFBP7 secreted by primary melanocytes inhibits melanocyte proliferation and induce their senescence and apoptosis. Moreover, they showed that recombinant IGFBP7 blocks the proliferation of primary melanocytes in a dose-dependent fashion.[21] Verhagen *et al.* reported that the overexpression of IGFBP7 could induce the cell cycle arrest of AML cells in G2 phase.[22] Here, we report for the first time that IGFBP7 can affect immune cells in a manner similar to that seen in tumor cells.

CD is reportedly associated with excess production of IFN-γ and TNF-α, which could affect the small bowel and colon with discontinuous ulcerations.[42–45] T-cells are a major source of IFN-γ and TNF-α. It is therefore noteworthy that our results indicate that MSC-derived IGFBP7 can down-regulate the expression of TNF-α and IFN-γ in active T-cells, both *in vitro* and *in vivo*. Furthermore, in the colon tissues of colitis mice, MSC-generated IGFBP7 decreased the production of proinflammatory cytokines (TNF-α, IL-6, IL-1β, IL-17, and IL-12a) and chemokines (MIP-2 and Rantes) even as it increased the production of IL-10. These effects of IGFBP7 on cytokine production could contribute significantly to alleviating colitis. The literature contains little evidence that IGFBP7 influences cytokine expression. However, two other members of the IGFBP family are known to be associated with cytokine production, namely IGFBP3 and IGFBP5. The loss of IGFBP3 was previously shown to be associated with an increase in TNF-α expression,[30] and Ad.IGFBP-3 was found to down-regulate both local and systemic levels of NF-κB–targeted proinflammatory cytokines.[31] Moreover, Lee *et al.* reported that IGFBP3 degrades NF-κB regulatory molecules (IκBα and p65-NF-κB), thereby demonstrating that IGFBP-3R can mediate caspase activation via an IGF-independent mechanism.[29] In the context of IGFPB5, the L-domain was reported to inhibit TNF-α-induced NF-κB activity by binding to TNFR1.[46] Since the IGFBP family members have similar domains and structures, we hypothesize that IGFBP7 could inhibit NF-κB signaling by reducing the expression levels of p-P65 and p-IκBα.

In addition, although MSCs do have direct cytoprotective effects on IECs *in vitro* (see **Supplementary Figure S15**), indicating that MSCs might protect the integrity of intestinal barrier

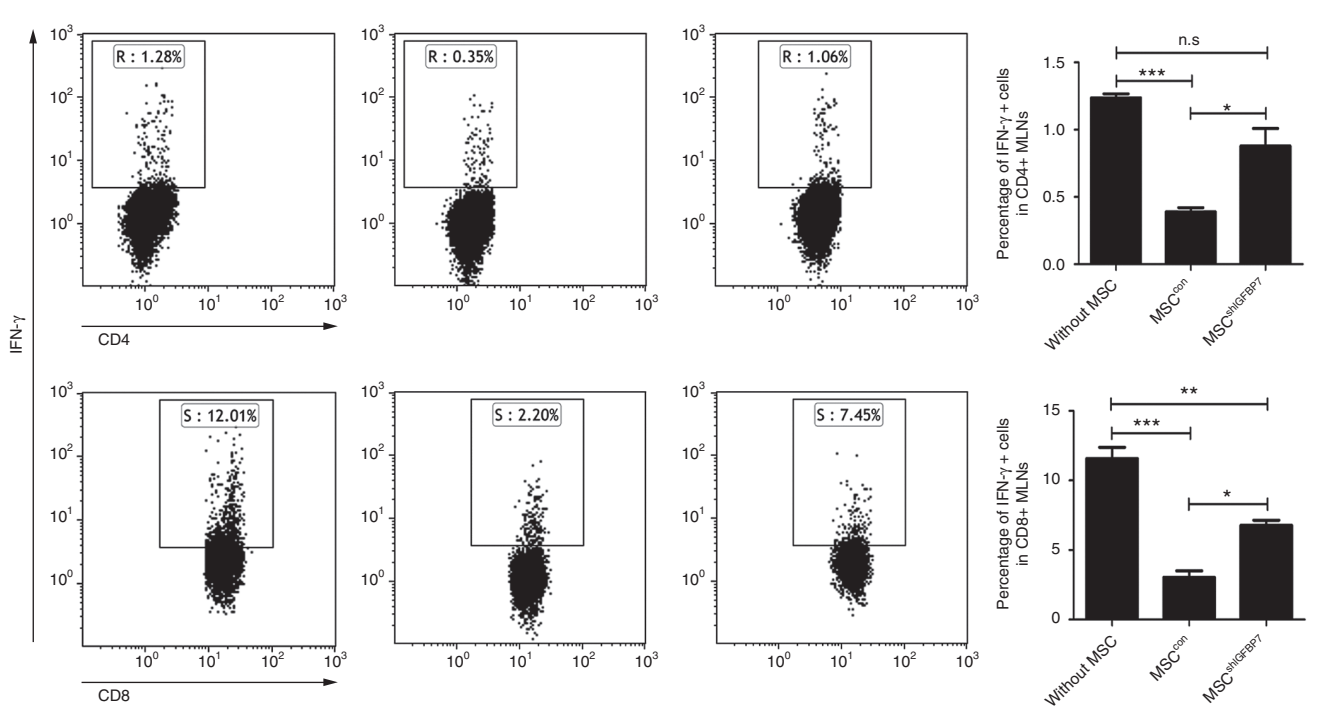


Figure 6 Mouse MSCs suppress the proliferation and proinflammatory cytokine secretion *in vitro* of MLN cells derived from an experimental mouse model of colitis through IGFBP7. MLN cells derived from mice of the TNBS + saline group were cocultured with or without MSC^{con} or $MSC^{shIGFBP7}$ for 3 days. (**a**) The proliferation of MLN cells was analyzed. The expression levels of TNF-α (**b**) and IFN-γ (**c**) in $CD4^+$ and $CD8^+$ MLN cells were analyzed by flow cytometry. Data are shown as mean ± SEM ($n = 3$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and n.s. = not significant. IGFBP, insulin-like growth factor binding protein; MSC, mesenchymal stromal cells; SEM, standard error of mean; MLN, mesenteric lymph node; TNBS, TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; IFN, interferon.

and then prevent the intestinal mucosa damage. Whereas IGFBP7 knockdown in MSCs did not affect the apoptosis of such cells, implying that IGFBP7 in MSCs ameliorate intestinal inflammation mainly through its immunomodulatory function.

In summary, we report that IGFBP7 is a novel immunosuppressive factor secreted by MSCs, and that it appears to alleviate intestinal inflammation in a mouse model of colitis. Mechanistically, this factor seems to inhibit T-cells proliferation by arresting the cell cycle at G0/G1 phase as a result of modulating the PI3K/AKT and MAPK/ERK signaling pathways, and it may suppress the expression of proinflammatory cytokines by altering NF-κB signaling, as shown *in vitro* and *in vivo*.

MATERIALS AND METHODS

Isolation and culture of mouse MSCs. All of the animal procedures were reviewed and approved by the Sun Yat-Sen University Institutional Animal Care and Use Committee. Mouse MSCs were isolated from the bone marrow of 8-week-old C57BL/6 mice according to the improved low-density culture method reported previously.[19,47] In brief, femurs and tibiae were removed and placed on ice in 5 ml L-Dulbecco's modified essential medium (DMEM) complete medium. Each bone marrow cavity was flushed with the medium, and individual cells were obtained by filtration through a 70-μm cell strainer. After red blood cells were removed by ammonium chloride lysis, the remaining cells were washed with Hanks balanced salt solution (HBSS), resuspended in L-DMEM complete medium, and plated in culture flasks at the low density of 5×10^4 cells/cm^2. The cells were then cultured for 3 days, and nonadherent cells were removed by a complete change of the medium, while the remaining adherent cells were cultured continuously.

RNA isolation and quantitative real-time PCR. Total RNA was extracted with the TRIzol reagent (Invitrogen, Carlsbad, CA), and reverse transcription (RT) was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania). The cDNA thus obtained was subjected to real-time PCR with the SYBR Green reagent (Roche, Indianapolis, IN) using the mouse primers listed in see **Supplementary Table S1**. The relative mRNA abundance was calculated using the ΔCt or ΔΔCt methods, and gene expression levels were normalized with respect to those of Glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western blot analysis. Total proteins were extracted, and the protein concentration was measured using a BCA protein assay kit (Thermo Scientific, Rockford, AL). Proteins were separated using 8% or 10% sulfate-polyacrylamide gel electrophoresis and were then transferred to a polyvinylidene fluoride (PVDF) membrane; the membrane was then blocked with Tris-buffered saline (TBS)/T containing 5% nonfat dry milk and analyzed for the target proteins. The specific antibodies that were used recognized IGFBP7 (sc-13095, Santa Cruz Biotechnology, Dallas, TX), pan-Akt, p-Akt (Thr308), MAPK (Erk1/2), p-MAPK (Erk1/2) (Thr202/Tyr 204), NF-κB p65, p-NF-κB p65, IκBα, and p-IκBα (Ser32) (all from Cell Signaling Technology, Danvers, MA).

Construction of the Lentivector for RNA silencing. The short hairpin RNA (shRNA) used here was designed in-house and synthesized by Sangon Biotech (Shanghai, China). The sequence is presented in **Supplementary Table S2**. The lentiviral vector, LentiLox 3.7(pLL3.7), was used for long-term interference with mouse MSCs. An insert-free vector was used as a negative control (designated "con").

Transfections with mouse and human siRNA specific targeting IGFBP7. Mouse or human MSCs (1×10^5cells) were plated to a 24-well plate (Corning, Corning, NY) and cultured overnight before transfection with 20 nmol/l siRNAs specific targeting mouse or human IGFBP7 and scrambled siRNA (negative control) using a commercial kit (Ribobio, Shanghai, China). Then, MSCs were incubating for 6 hours in serum-free medium before replacing with 1 ml per well of L-DMEM complete medium lacking antibiotics, and MSCs were used for the lymphocyte proliferation assay. The IGFBP7 siRNA sequence were presented in **Supplementary Table S3**.

Mouse MSC proliferation assay. MSCs were resuspended in L-DMEM complete medium and seeded to a 12-well plate at 10^4 cells per well. The cells were trypsinised at each indicated time point over 7days, and cell numbers were counted directly.

Flow cytometry. Flow cytometric analyses were performed with Influx (BD Bioscience, San Jose, CA) or Gallios (Beckman Coulter, Fullerton, CA) flow cytometers, and the data were analyzed with the FlowJo7.5 (Treestar, Ashland, OR) or Kaluza (Beckman Coulter) software packages. Antimouse Sca-1-APC (D7), CD106-eFluor 660 (429), CD44-PE (IM7), c-Kit-APC-eFluor 780 (2B8), CD34-eFluor 660 (RAM34), CD45-PE-Cyanine7 (30-F11), and CD11b-APC (M1/70) antibodies, along with the corresponding isotype control antibodies were purchased from eBioscience(San Diego, CA). CD3e-PE-CyTM7 (145-2C11), CD4-APC (RM4-5), and CD8a-Pacific Blue TM (53–6.7) were purchased from BD Pharmingen. Propidiumiodide (PI; BD Pharmingen, San Jose, CA) was used to stain dead cells.

Differentiation assays. For osteogenic, adipogenic and chondrogenic differentiations of MSCs *in vitro*, we used the methods described in our previous report.[19]

Mouse splenic lymphocyte proliferation assays. Mouse MSCs (1×10^5cells) were plated to a 24-well plate (Corning) and cultured for 24 hours before being used for the lymphocyte proliferation assay. Mouse splenocytes were washed twice with phosphate-buffered saline containing 3% fetal calf serum and then incubated with an antimouse CD3 antibody (BD Pharmingen) at 4°C for 30 minutes. Pure CD3$^+$ T-cells were sorted by flow cytometry (Influx), and 5,6-carboxyfluorescein diacetatesuccinimidyl ester (CFSE; Invitrogen) staining (5μmol/l) was used to assess CD3$^+$ T-cell proliferation. The cells were then suspended in Roswell Park Memorial Institute (RPMI)1640 at 2.5×10^6 cells/ml, and distributed to 24-well plates (1 ml/well) in the presence or absence of MSCs. In order to induce T-cell proliferation, anti-mouse CD3 and CD28 antibodies (BD Pharmingen; final concentration, 500 ng/ml) were added to the wells. After 3 days of coculture, the CD3$^+$ T-cells were collected and analyzed by flow cytometry. To investigate the ability of MSCs to inhibit the CD4$^+$ and CD8$^+$ T-cell subpopulations, the collected cells were also stained with an anti-CD4 and -CD8 antibody (eBioscience).

Cell cycle analysis. CD3$^+$ T-cells that had been stimulated with anti-CD3 and -CD28 were cocultured with MSCs for 3 days, and T-cells were collected and fixed overnight in 70% ethanol. The cells were then incubated for 30 minutes with RNase A (10 μg/ml; Sigma Aldrich, Darmstadt, Germany) at 37°C. Subsequently, cells were stained with PI (50 μg/ml, Sigma) and DNA content of T-cells was analyzed using flow cytometer (Beckman Coulter), and the results were analyzed using the FlowJo 7.6 software (Treestar).

Apoptosis detection of T-cells and IECs. After 3 days of co-culture mentioned above, the percentage of apoptotic CD3$^+$ T-cells was evaluated using an fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit I (BD Pharmingen) according to the manufacturer's instructions. MODE-K cells, a murine small intestinal epithelial cell line, were cocultured with or without MSCs in the transwell system and then were treated with 10 ng/ml TNF-α and IFN-γ for 24 hours. MODE-K cells were cultured alone in medium without cytokines act as negative control. After incubation, the percentage of apoptotic MODE-K cells was evaluated using above commercial kit.

Cytokine assays. For cytokine assays, mouse lymphocytes were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 6 hours; during this period, brefeldin A (BFA; 10 μg/ml) was used to inhibit the secretion of

cytokines (all from Sigma Aldrich). TNF-α and IFN-γ were analyzed by flow cytometry. Mouse splenocytes cultured with MSCs (ratio, 30:1) or alone for 2 days were used for the *in vitro* experiment. MLN cells obtained from the negative control, MSC^{con} and $MSC^{shIGFBP7}$ groups respectively at the peak of the disease (2 days after TNBS injection) were used for the *in vivo* experiment. Further, MLN cells obtained from the negative controls that were cultured either with MSCs (at a ratio of 30:1) or alone for 3 days were gathered to analyze.

Treg assays. The percentage of $CD4^+CD25^+Foxp3^+$ Tregs was evaluated using a Mouse Regulatory T-Cell Staining Kit 2 (eBioscience) according to the manufacturer's instructions. Mouse splenocytes and MLN cells were gathered for the experiments in the same manner in which the "cytokine assays" were performed, as described above.

Experimental colitis induced by TNBS. In order to induce colitis in 8-week-old male BALB/c mice, we referred to the previously published protocol.[48] In brief, on day 1, mice were smeared with 150 µl of presensitisation solution (TNBS; Sigma) on their backs. On day 7, the mice were divided into six groups (5 mice/group) and fasted (but allowed to drink ad libitum) for 24 hours. On day 8, mice were weighed and then treated intrarectally with 100 µl 2.5% TNBS in 50% ethanol, 50% ethanol alone (control, ethanol group) or no solution (blank control, naive group). At 12 hours post TNBS injection, animals were transplanted i.p. with 200 µl saline (negative control group), or 2×10^6 MSC^{con} or $MSC^{shIGFBP7}$cells suspended in 200 µl saline. The parameters of body weight loss, diarrhoea and survival were recorded for 7 days. Colons were collected from caecum to the anus 2 days after TNBS injection (the peak of the disease), and the colon length (taken as an indication of the degree of inflammation) was measured. Colons were evaluated for macroscopic damage according to the previous report.[7] Stool consistency and rectal bleeding were scored as previously described,[49] with minor modifications: 0 = normal stool; 1 = loose stool; 2 = soft stool; 3 = diarrhoea; and 4 = diarrhoea and anal bleeding. For histopathologic analysis, colon specimens were obtained and stained with H&E. Inflammation was graded as follows: 0 = no sign of inflammation; 1 = low leukocyte infiltration; 2 = moderate leukocyte infiltration; 3 = high leukocyte infiltration, moderate fibrosis, high vascular density, thickening of the colon wall, moderate goblet cell loss, and focal loss of crypts; and 4 = transmural infiltrations, massive loss of goblet cell, extensive fibrosis, and diffuse loss of crypts.[7] For immunohistologic analysis, sections were stained with antimouse CD4 (sc-7219, Santa Cruz) and CD8 (ab22378, Abcam, Cambridge, UK) primary antibodies. The numbers of $CD4^+$ and $CD8^+$ cells per high-power microscopic field (400×) were determined.

The biodistribution of mouse MSCs through different administration routes. In order to analyze the biodistribution of MSCs *in vivo*, 2×10^6 luciferase transduced MSC^{con} or $MSC^{shIGFBP7}$ cells were administrated through i.p. and i.v. injection 12 hours after TNBS injection. The *in vivo* tracing of cells at different time points (12 hours, 2 days, 4 days, and 7 days) after MSCs administration was detected by Xenogen IVIS In Vivo Imaging System Spectrum (Caliper Life Sciences, Hopkinton, MA).

MLN cell proliferation assay. MLN cells were obtained from colitic Balb/c mice at the peak of disease (2 days after TNBS injection). The proliferation of stimulated MLN cells was evaluated as described in "mouse splenic lymphocyte proliferation assays" above. The proliferation of MLN cells without stimulation was evaluated as well.

Statistical analysis. All results are expressed as mean ± SEM. Statistical comparisons were made using a two-tailed Student's *t*-test (between two groups) or a one-way analysis of variance (for multigroup comparisons). Changes in body weight were compared using a repeated measure analysis of variance. Survival was analyzed using the Kaplan–Meier log-rank test. $P < 0.05$ was considered to represent a significant difference. Analysis and graphing were performed using the Prism 5.01 software package (GraphPad, San Diego, CA).

SUPPLEMENTARY MATERIAL

Figure S1. Mouse MSCs inhibit the proliferation of $CD3^+$ T-cells *in vitro* through IGFBP7 by arresting the cell cycle.
Figure S2. Mouse MSC-derived IGFBP7 inhibits T-cells proliferation and proinflammatory cytokine expression.
Figure S3. Mouse MSC-derived IGFBP7 inhibits the PI3K/AKT and MAPK/ERK pathways in T-cells.
Figure S4. Mouse MSCs down-regulate the expression levels of pro-inflammatory cytokines in $CD3^+$ T-cells *in vitro* through IGFBP7.
Figure S5. Mouse MSC-derived IGFBP7 inhibits NF-κB signaling pathways in T-cells.
Figure S6. IGFBP7 expression in human MSCs.
Figure S7. IGFBP7 contributes to the antiproliferative effects of human MSCs against alloreactive T-cells.
Figure S8. The inhibition of the proinflammatory cytokine production of T-cells by human MSCs *in vitro* was restored after down-regulation of IGFBP7 in human MSCs.
Figure S9. Mouse MSCs do not influence the apoptosis of T-cells through IGFBP7 *in vitro.*
Figure S10. Mouse MSCs do not influence $CD4^+CD25^+FoxP3^+$ Tregs through IGFBP7 *in vitro*.
Figure S11. Mouse MSCs decrease $CD4^+$ and $CD8^+$ T-cells in colons of mouse experimental colitis model through IGFBP7.
Figure S12. Mouse MSCs act via IGFBP7 to modulate the cytokine secretion associated with the inflammation of MLN cells in an *in vivo* mouse model of experimental colitis.
Figure S13. The distribution and therapeutic effect of mouse MSCs administration through intraperitoneal (i.p.) and intravenous (i.v.) injection in mouse colitis model.
Figure S14. Mouse MSCs do not influence $CD4^+CD25^+FoxP3^+$ Tregs through IGFBP7 *in vivo.*
Figure S15. IGFBP7 do not contribute the cytoprotective effects of mouse MSCs on intestinal epithelial cells (IECs).
Table S1. Primers used for the amplification of mouse transcripts by real-time quantitative PCR.
Table S2. IGFBP7 shRNA sequence used to generate lentivirus plasmids for RNA silencing.
Table S3. IGFBP7 siRNA sequence used for RNA silencing. IGFBP7 siRNA sequence

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