

Interleukin-17 enhances immunosuppression by mesenchymal stem cells

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IL-17 is one of the most potent and most actively investigated proinflammatory cytokines. In this study, we examined the effect of IL-17 on mesenchymal stem cells (MSCs) under the influence of inflammatory cytokines. Ironically, IL-17 dramatically enhanced the immunosuppressive effect of MSCs induced by IFN γ and TNF α , revealing a novel role of IL-17 in immunosuppression. Interestingly, we found that this action of IL-17 was dependent on the promoted expression of a key immune suppressive molecule, inducible nitric oxide synthase (iNOS), in MSCs. In a concanavalin A (ConA)-induced hepatitis mouse model, we found that IL-17 also enhanced the *in vivo* immunosuppressive effect of MSCs in an iNOS-dependent manner. Moreover, this promoting effect of IL-17 was found to be exerted through enhancing mRNA stability by modulating the protein level of ARE/poly(U)-binding/degradation factor 1 (AUF1), a well-known factor that promotes mRNA decay. In *auf1*^{-/-} MSCs, IFN γ and TNF α could induce maximal immunosuppressive effect, both *in vitro* and *in vivo*, without the need for IL-17. Thus, our studies demonstrated that in the presence of MSCs, IL-17 promotes immunosuppression.

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Interleukin 17A (hereafter referred to as IL-17) is the first member discovered of the IL-17 cytokine family.¹ It is one of the key inflammatory cytokines during immune responses, primarily produced by Th17 cells.^{2,3} IL-17 is well-known for its proinflammatory functions in infectious, inflammatory, and autoimmune responses.^{4–6} IL-17 signals through a heteromeric receptor complex composed of IL-17RA and IL-17RC.^{7,8} Upon IL-17 binding, IL-17RA recruits Act1, a critical downstream mediator of the IL-17-induced signaling process.^{9,10} Although much is known about the IL-17-induced signaling pathways, its cellular targets and mode of action during inflammatory processes and autoimmune diseases remain elusive.

Mesenchymal stem cells (MSCs) exist in various tissues, including bone marrow, muscle, teeth, skin, and fat.¹¹ Upon stimulation by cytokines in an inflammatory environment, MSCs can secrete a variety of chemokines, immune-regulatory molecules, and growth factors. These products can further recruit other cells including inflammatory cells, modulate immune responses, participate in tissue repair, and affect pathological progression.^{12–15} Although we have reported that among all the cytokines produced by activated T cells, interferon- γ (IFN γ), tumor necrosis factor- α (TNF α),

and likely IL-1 are the key initiating cytokines in inducing immunosuppression by MSCs;¹³ it is not known whether other cytokines accompanying an inflammatory response can also affect the immunosuppressive effects of MSCs. In this study, we found that the otherwise most potent inflammatory cytokine, IL-17, could in fact effectively synergize with IFN γ and TNF α in inducing immunosuppression by promoting inducible nitric oxide synthase (iNOS) expression in MSCs.

Besides transcription, the abundance of mRNAs is also regulated by their decay rate.¹⁶ Regulation of mRNA decay is executed through interactions between specific mRNA sequences and the trans-acting factors that bind them.^{16,17} The ARE-binding protein ARE/poly(U)-binding/degradation factor 1 (AUF1) is abundant in lymphoid organs,¹⁸ regulates various immune-related molecules, including IL-2, GM-CSF, TNF α , iNOS, and IL-6 mRNAs, and thus controls expression of these inflammatory proteins during immune responses.^{19,20} In our study, we showed that IL-17 could enhance iNOS mRNA stability through reducing AUF1 protein levels in MSCs treated with IFN γ and TNF α . Thus, AUF1 serves as a control point through which IL-17 must act to exert its immunosuppressive function in MSCs in an inflammatory environment.

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Abbreviations: Act.D, actinomycin D; ALT, alanine aminotransferase; AUF1, ARE/poly(U)-binding/degradation factor 1; ConA, concanavalin A; IFN γ , interferon- γ ; IL-17, interleukin-17; iNOS, inducible nitric oxide synthase; MNCs, mononuclear cells; MSCs, mesenchymal stem cells; TNF α , tumor necrosis factor- α

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Because of their powerful immunomodulatory functions, MSCs are being investigated for their therapeutic potential in many diseases. Although various studies have demonstrated the therapeutic effect of MSCs on chronic diseases, including graft-versus-host disease, Crohn's disease, liver cirrhosis, systemic lupus erythematosus, and multiple sclerosis (MS),^{21–25} little is known about the effectiveness of MSCs in treating acute inflammatory disorders such as acute liver injury. Here, we showed that MSCs pretreated with inflammatory cytokines together with IL-17 could effectively cure concanavalin A (ConA)-induced liver injury in mice in an iNOS-dependent manner. Consistent with our *in vitro* findings, *auf1*^{-/-} MSCs treated with inflammatory cytokines alone demonstrated similar effects and IL-17 supplementation was not needed. We believe that our findings not only revealed a novel role of IL-17 in enhancing immunosuppression, but also provided novel findings of the action of AUF1 in the IL-17 signaling pathway, and better understanding of the therapeutic effects of MSCs.

Results

IL-17 enhances the immunosuppressive effect of MSCs.

Our previous studies have shown that a combination of IFN γ with TNF α is required to elicit the immunosuppressive effect of MSCs. We examined whether IL-17 could affect IFN γ and TNF α -induced immunosuppression of MSCs. We found that T-cell proliferation was reduced in the presence of IFN γ and TNF α , and surprisingly, this suppression was markedly enhanced by IL-17 (Figure 1a). As IL-17 signals through IL-17RA and IL-17RC, the expression of these receptors in MSCs was determined by quantitative RT-PCR (Figure 1b), in which a mouse macrophage cell line Raw 264.7 was used as a positive control. Cell surface expression of the receptor was also confirmed by flow cytometric analysis (Figure 1c).

As the concentrations of inflammatory cytokines vary at different stages of an inflammatory response, we examined the concentration dependence of IFN γ and TNF α on IL-17-enhanced immunosuppression. IL-17 was able to enhance

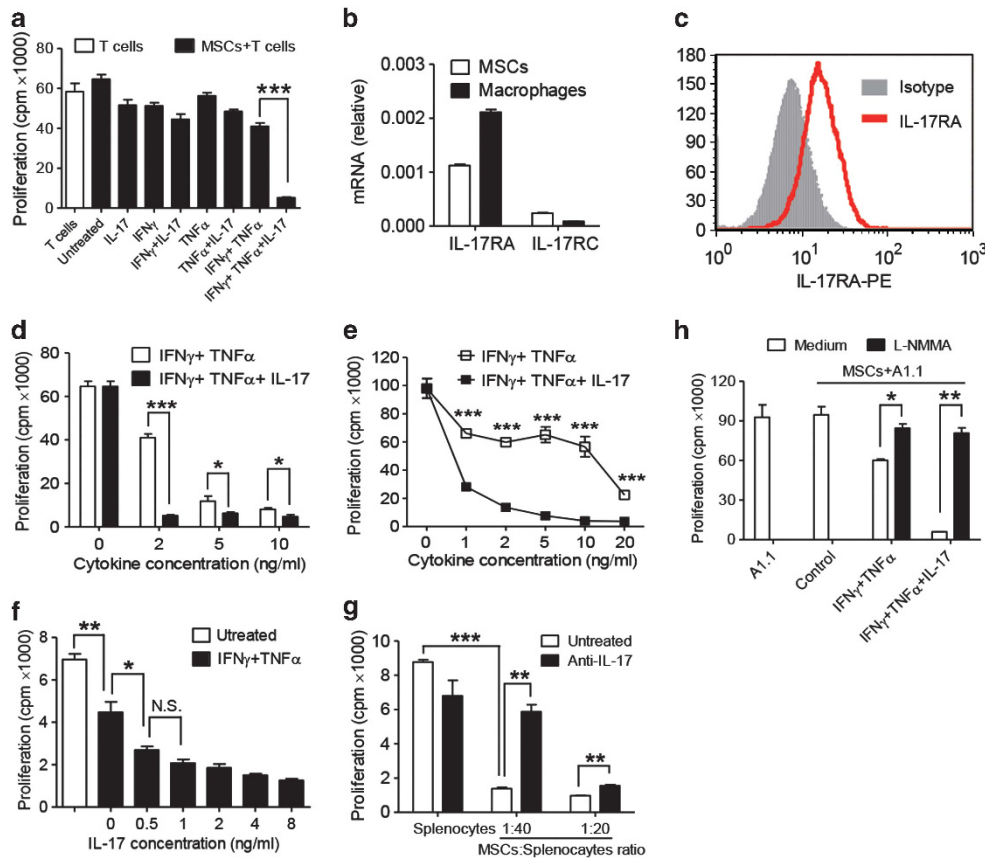


Figure 1 IL-17 enhances the immunosuppressive effect of MSCs. (a) Cloned MSCs were first treated with the indicated combinations of recombinant cytokines IFN γ , TNF α , and IL-17 (2 ng/ml each) for 12 h, then co-cultured with T-cell blasts at a 1 : 20 ratio (MSC: T cells), and proliferation was assessed by ³H-thymidine incorporation after an additional 12 h. (b) The mRNA expression of IL-17 receptor family members in MSCs or cell line Raw 264.7 were examined by quantitative RT-PCR. (c) Surface expression of IL-17RA was detected by flow cytometry with cloned MSCs. (d and e) MSCs were first treated with IFN γ and TNF α , with or without IL-17 (10 ng/ml). IFN γ and TNF α were supplemented at different cytokine concentrations, for 12 h, and then co-cultured with T-cell blasts (d) or T-cell hybridoma A1.1 cells (e) at a ratio of 1 : 20 for 12 h. T-cell proliferation was measured by ³H-thymidine incorporation. (f) MSCs were first treated with IFN γ and TNF α (2 ng/ml) with graded concentrations of IL-17 for 12 h, and then co-cultured with T-cell hybridoma A1.1 cells at a ratio of 1 : 10 for 12 h. T-cell proliferation was measured by ³H-thymidine incorporation. (g) MSCs were co-cultured with fresh C57BL/6 splenocytes activated with anti-CD3 and anti-CD28, or in the presence of antibodies against IL-17A, at a 1 : 20 or 1 : 40 ratio (MSCs: splenocytes), for 48 h, and then cell proliferation was assessed by ³H-thymidine incorporation. (h) MSCs were first treated with IFN γ and TNF α (2 ng/ml), or together with IL-17 for 12 h, and then co-cultured with T-cell hybridoma A1.1 cells at a ratio of 1 : 10, in the presence or absence of L-NMMA, for 12 h. T-cell proliferation was measured by ³H-thymidine incorporation. Proliferation values represent means \pm S.E.M. of three wells from a representative of three independent experiments

the immunosuppressive effect of MSCs on T cells at IFN γ and TNF α concentrations as low as 1–2 ng/ml each (Figures 1d and e). Even at higher concentrations of IFN γ and TNF α (10–20 ng/ml), IL-17 still improved immunosuppression by MSCs though the effect was less pronounced. Nonetheless, in the presence of MSCs, as little as 0.5 ng/ml IL-17 was sufficient to elicit a dramatic decrease in T-cell proliferation (Figure 1f). We also examined the role of T-cell death in this effect of IL-17. However, our results showed that the strongly inhibited T-cell proliferation by MSCs pretreated with IFN γ + TNF α + IL-17 was not due to T-cell death (Supplementary Figure S1).

To verify that IL-17 contributes to the immunosuppressive effect of MSCs during a T-cell immune response, antibody against IL-17 was used to neutralize it in a co-culture system with MSC-activated splenocytes. While proliferation of activated splenocytes was markedly inhibited by MSCs, this inhibition was partially reversed upon addition of antibody against IL-17 (Figure 1g). The optimal effect of anti-IL-17 occurred with a MSC/splenocyte ratio of 1:40. At a MSC/splenocyte ratio of 1:20, the reversal effect was less pronounced, but still statistically significant. Furthermore, this enhancing effect of IL-17 on immunosuppression of MSCs was completely reversed by iNOS inhibitor, L-NMMA (Figure 1h), suggesting that iNOS has a crucial role in this action of IL-17.

To expand our studies, we compared the function of IL-17 between bone marrow-derived MSCs and adipose-derived MSCs. IL-17 could also dramatically enhance IFN γ and TNF α -induced immunosuppression in adipose-derived MSCs (Supplementary Figure S2A), indicating that this effect of IL-17 may be independent of the source of MSCs. As the self-renewal capacity and plasticity may be altered after several passages, we examined whether the responsiveness of MSCs to IL-17 treatment was compromised as passage number increased. IL-17 still enhanced the immunosuppressive effect of MSCs at higher passage number, even though IFN γ and TNF α can induce modestly more immunosuppression in this situation (Supplementary Figure S2B).

IL-17 synergizes with inflammatory cytokines to induce the expression of immune modulatory genes in MSCs.

We have shown that IL-17 can enhance the immunosuppressive effect of MSCs. To reveal the mechanism, we initially hypothesized that IL-17 affects the proliferation of MSCs, especially considering that IL-17 has been reported to promote the proliferation of human MSCs.²⁶ However, we found that in the presence of IFN γ and TNF α , the addition of IL-17 modestly inhibited the proliferation of MSCs (Supplementary Figure S2C). Thus, the promoting effect of IL-17 on immunosuppression by MSCs is not through influencing the proliferation of MSCs.

We previously showed that NO and chemokines, acting in concert, are the key molecules mediating the immunosuppressive effects of MSCs. The induction of iNOS and some chemokine genes in MSCs is initiated by IFN γ and TNF α .²⁷ As IL-17 enhanced immunosuppression by MSCs cultured with IFN γ and TNF α (Figures 1d and e), and iNOS was essential for this effect of IL-17 (Figure 1h), we hypothesized that IL-17 synergizes with IFN γ and TNF α to induce the expression of

iNOS or chemokines in MSCs. To test this hypothesis, MSCs were cultured with various combinations of IFN γ , TNF α , and/or IL-17, and the expression levels of some key immune modulatory genes were assessed. Compared with MSCs cultured with single or double combinations of cytokines, addition of IFN γ , TNF α , and IL-17 dramatically increased the expression of iNOS mRNA (Figure 2a). Western blotting analysis and assay of nitrate in supernatant also confirmed an increase of iNOS protein (Figure 2b, Supplementary Figure S2D). However, the expression of other chemokines such as CCL2, CCL5, CXCL9, and CXCL10, which have pivotal roles in the immunosuppressive effects of MSCs, were all unaffected by addition of IL-17 (Figure 2c), indicating a specific effect of IL-17 on iNOS expression. The addition of IL-17 can induce MSCs to produce large amounts of NO, which can further inhibit the proliferation of T cells. Excessive NO can also exhibit a cytotoxic effect on MSCs, resulting in suppressed proliferation of IFN γ + TNF α + IL-17 treated MSCs (Supplementary Figure S2C).

To verify that the effects on gene expression were due to IL-17, MSCs were cultured with supernatant from anti-CD3 and anti-CD28-activated splenocytes in the presence or absence of neutralizing antibody against IL-17. Addition of anti-IL-17 to supernatant diminished its ability to induce iNOS gene expression without affecting CCL2, CCL5, CXCL9, or CXCL10 (Figures 2d and e). Furthermore, we also verified our findings by blocking signal transduction of IL-17 in MSCs by knocking down Act1, a key component in the signal transduction pathway from the IL-17 receptor. As has been shown in other cells,¹⁰ the phosphorylation of I κ B α , ERK, p65, and JNK, key molecules in the IL-17 signaling pathway, were impaired in these Act1 knockdown MSCs (Supplementary Figure S3A). As expected, IL-17 cannot upregulate IFN γ and TNF α -induced iNOS expression in Act1 knockdown MSCs (Supplementary Figures S3B, C). At the cellular level, the immunosuppressive effect of IFN γ and TNF α could not be further enhanced by IL-17 in the absence of Act1 (Supplementary Figure S3D).

IL-17 together with IFN γ and TNF α -pretreated MSCs alleviate ConA-induced liver injury in an iNOS-dependent manner.

To investigate the promoting effect of IL-17 on IFN γ and TNF α -induced immunosuppression of MSCs *in vivo*, we employed the ConA-induced liver injury mouse model. ConA-induced liver injury is a well-established *in vivo* model of hepatocyte apoptosis induced by acute immune responses, in which T lymphocytes are identified as the major effector cells.²⁸ Wild-type MSCs were pretreated or not with IFN γ and TNF α in the presence or absence of IL-17 for 12 h. After ConA administration for 30 min, these differently pretreated MSCs were intravenously injected into the mice. The immunosuppression mediated by MSCs is exerted locally. Thus, GFP-transgenic MSCs were transfused into mice that received ConA injection 30 min earlier. Localization of administrated MSCs in the damaged liver was indeed observed (Supplementary Figure S4). Interestingly, liver injury was not inhibited in mice administrated with wild-type MSCs or wild-type MSCs pretreated with IFN γ and TNF α . However, MSCs pretreated with IFN γ , TNF α , and IL-17 almost completely inhibited liver damage, exhibited as

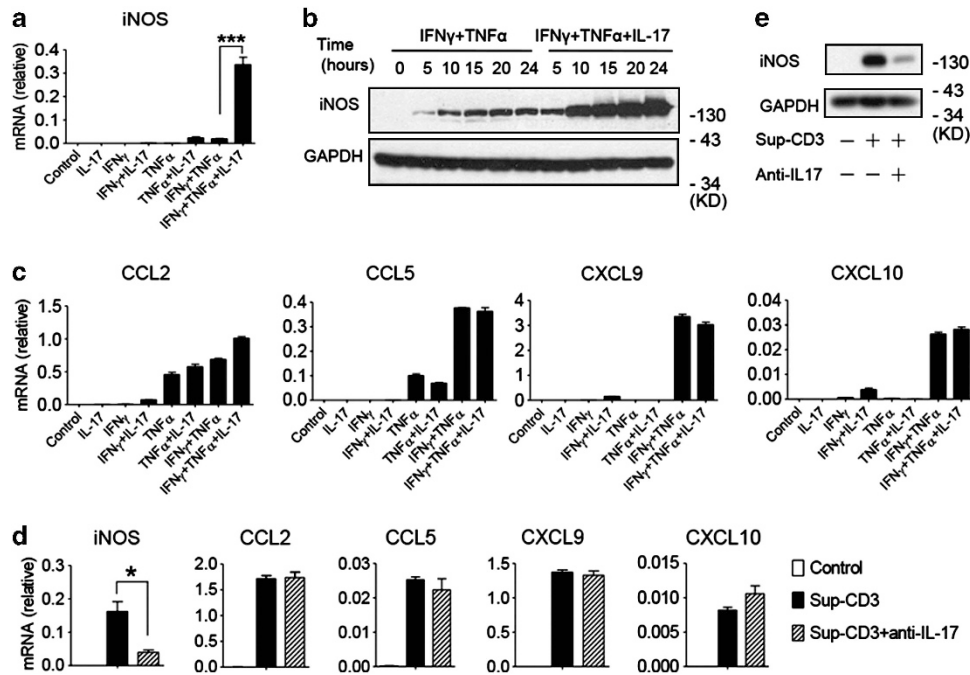


Figure 2 IL-17 synergizes with inflammatory cytokines to induce the expression of immune modulatory genes in MSCs. (a and c) MSCs were cultured with different combinations of inflammatory cytokines IFN γ , TNF α , and IL-17 (10 ng/ml) for 12 h, and then cells were harvested for total RNA extraction. The mRNA expression levels of iNOS (a) and chemokines CCL2, CCL5, CXCL9, CXCL10 (c) were detected by quantitative RT-PCR. (b) MSCs were cultured with IFN γ and TNF α , or together with IL-17 (10 ng/ml) for the indicated times, and the protein levels of iNOS were detected by western blotting analysis. (d) MSCs were supplemented with Sup-CD3 or Sup-CD3 pretreated with antibodies against IL-17, and cells were collected for RNA extraction after 12 h (Sup-CD3: supernatant from splenocytes activated by anti-CD3 and anti-CD28). The expression of the iNOS, CCL2, CCL5, CXCL9 and CXCL10 genes was measured by quantitative RT-PCR. (e) MSCs were treated with Sup-CD3 or Sup-CD3 pretreated with antibodies against IL-17, and the protein level of iNOS was assessed by western blotting analysis after 24 h. mRNA expression values are means \pm S.E.M. of three wells from a representative of three independent experiments. Western blotting data are from a representative of three independent experiments

a dramatic reduction of serum alanine aminotransferase (ALT) level, centrilobular necrosis, and inflammatory infiltration (Figures 3a, c and d). Detailed analyses of immune cells in the liver showed that the infiltration of mononuclear cells (MNCs), CD3⁺CD4⁺, and CD3⁺CD8⁺ T cells were dramatically decreased in mice administered with wild-type MSCs pretreated with IFN γ and TNF α , together with IL-17 (Figure 3b). However, the ameliorated effects of MSCs pretreated with IFN γ , TNF α , and IL-17 in liver injury was not observed in iNOS^{-/-} MSCs (Figures 3a, c and d), indicating that iNOS is a key mediator in the promoting effect of IL-17 on immunosuppression by MSCs *in vivo*.

IL-17 reverses the suppression of gene expression imposed by RNA-binding protein AUF1. Messenger RNAs encoding iNOS and many cytokines/chemokines are rapidly degraded after interaction with mRNA decay factors, which provides a mechanism to limit the abundance of these molecules during an immune response. Activation of signaling pathways, particularly during immune responses, stabilizes many of these mRNAs to increase their expression. Indeed, a major mechanism by which IL-17 influences the expression of many inflammatory mediator genes is by stabilizing their mRNAs.^{29,30} AUF1 has been shown to promote the degradation of iNOS mRNA.²⁰ We thus hypothesized that AUF1 may act to limit the expression of iNOS mRNA and that IL-17 may block this activity of AUF1 (thereby increasing gene expression).

To test this hypothesis, cytokine-induced gene expression was compared between MSCs derived from bone marrow of wild-type and *auf1*^{-/-} mice.³¹ Cells were cultured as before with IFN γ and TNF α , with or without IL-17. Addition of IL-17 (together with IFN γ and TNF α) dramatically further increased iNOS mRNA in wild-type MSCs (Figure 4a). In contrast, iNOS mRNA was much higher in *auf1*^{-/-} MSCs cultured with IFN γ and TNF α ; interestingly, addition of IL-17 had little effect (i.e., IL-17 increased iNOS mRNA abundance less than twofold). Likewise, IFN γ and TNF α were sufficient to maximally induce iNOS protein in *auf1*^{-/-} MSCs without the need for IL-17, in contrast to wild-type MSCs (Figure 4b, compare lanes 7 and 8 with lanes 2 and 5). To verify these findings, we knocked down AUF1 in wild-type MSCs (Figure 4c), and treated these cells with IFN γ and TNF α , with or without IL-17. Consistent with *auf1*^{-/-} MSCs, IFN γ and TNF α induced maximal iNOS mRNA and protein expression in AUF1-knockdown MSCs (Figures 4d and e), and the enhancing effect of IL-17 was abated. Moreover, IL-17 further increased the suppressive activity of IFN γ and TNF α -stimulated wild-type MSCs on T-cell proliferation; however, IFN γ and TNF α were sufficient to induce maximal immunosuppression in *auf1*^{-/-} MSCs and IL-17 did not further enhance the inhibitory effect on T-cell proliferation (Figure 4f).

IL-17 enhances gene expression through modulating AUF1-mediated mRNA decay in MSCs. Given the effects of AUF1 and IL-17 on gene expression and

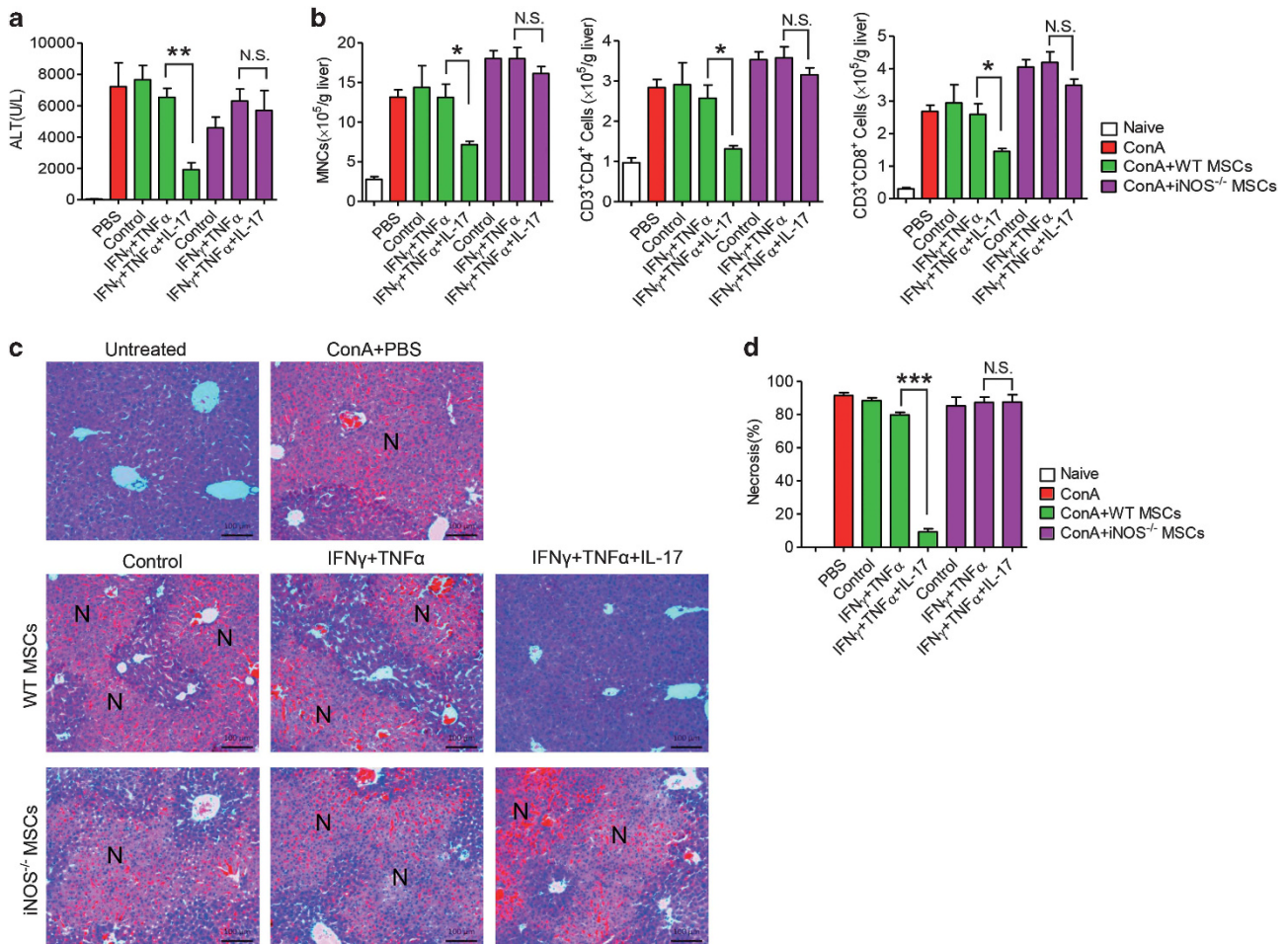


Figure 3 MSCs pretreated with IL-17 together with IFN γ and TNF α alleviate ConA-induced liver injury in an iNOS-dependent manner. Mice were intravenously injected with ConA (15 mg/kg); 30 min later, untreated or cytokine-pretreated wild-type or iNOS^{-/-} MSCs were transfused. After 7.5 h, serum and livers were sampled. (a) Serum levels of ALT were measured. (b) Absolute numbers of MNCs in liver tissues were calculated ($n = 5$ mice per group). Absolute numbers of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells were determined by flow cytometry. (c) Hematoxylin & eosin staining of liver sections at 8 h after ConA administration. N: necrosis area. (d) Percentages of necrosis were calculated

immunosuppression by MSCs, we considered the possibility that knockout of AUF1 alone would be sufficient to provide the degree of mRNA stabilization that would normally require IL-17. mRNA decay assays were performed with wild-type and *auf1*^{-/-} MSCs, cultured with IFN γ and TNF α , with or without IL-17. In wild-type MSCs, iNOS mRNA was relatively unstable with a half-life of 4.3 ± 1.4 h; IL-17 led to a twofold stabilization of iNOS mRNA (Figure 5a); $P < 0.05$. The CCL2 and CXCL10 mRNAs, which did not respond to IL-17 (see Figure 2c), were not stabilized by IL-17, as would be expected (Figure 5a; $t_{1/2} = \sim 2$ h for both mRNAs, with or without IL-17). In contrast to wild-type MSCs, knockout of AUF1 strongly stabilized iNOS mRNA (Figure 5b; $t_{1/2} > 10$ h). In these *auf1*^{-/-} MSCs, IL-17 had no effect on the half-life of iNOS mRNA (Figure 5b). We also verified these findings in AUF1-knockdown MSCs (Figure 5c).

To assess the interaction of IL-17 and AUF1 on the enhancing effect of IL-17 on MSC immunosuppression, the protein level of AUF1 was analyzed in MSCs treated with IFN γ and TNF α , in the presence or absence of IL-17, as a function of time. We found that the addition of IL-17 could reduce AUF1 protein levels in IFN γ +TNF α -treated MSCs (Figure 5d).

Therefore, IL-17 enhances iNOS mRNA stability by reducing AUF1 protein levels. Thus, AUF1 is one key factor mediating the enhancing effect of IL-17 on IFN γ and TNF α -induced iNOS expression by MSCs. Further experiments were employed to assess potential interactions between AUF1 and IL-17 downstream molecules. As either knockdown of Act1 or knockout of AUF1 in MSCs abolished the ability of IL-17 to enhance their immunosuppressive effects (Supplementary Figure S3 and Figure 4), we assessed interaction of Act1 and AUF1 in MSCs upon IL-17 stimulation using co-immunoprecipitation. IL-17 stimulation increased the interaction of endogenous Act1 with AUF1 (Figure 5e). These data support the ideas that AUF1 downregulation and Act1–AUF1 interactions are key steps in the enhancing effect of IL-17 on both gene expression and immunosuppression in MSCs.

Critical roles of AUF1 in IL-17-enhanced therapeutic effect of MSCs on ConA-induced acute liver injury. Given the crucial role of AUF1 in the enhancing effect of IL-17 on immunosuppression by MSCs *in vitro*, we next examined the effects of IL-17 on immunosuppression *in vivo* by wild-type and *auf1*^{-/-} MSCs. Wild-type and *auf1*^{-/-}

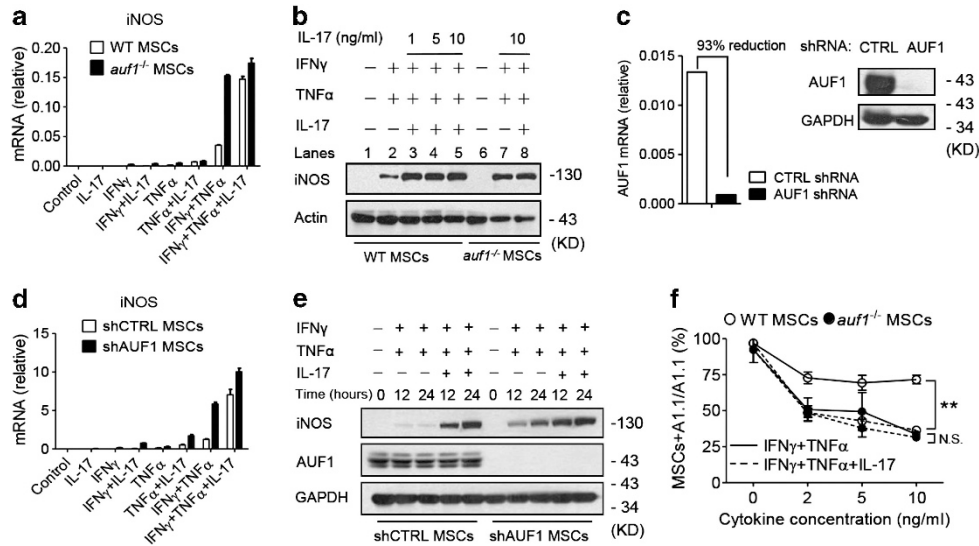


Figure 4 IL-17 reverses the suppression of gene expression imposed by RNA-binding protein AUF1. (a and d) WT MSCs and *aif1*^{-/-} MSCs (a) or shCTRL MSCs and shAUF1 MSCs (d) were treated with IFN γ and TNF α , or together with IL-17 (all cytokines supplemented at 10 ng/ml) for 12 h, and iNOS mRNA expression was measured by quantitative RT-PCR. (b) WT MSCs were treated with IFN γ and TNF α (10 ng/ml), or together with different concentrations of IL-17; *aif1*^{-/-} MSCs were treated with IFN γ and TNF α (10 ng/ml), or together with 10 ng/ml of IL-17. After 24 h, cells were harvested for detection of iNOS by western blotting analysis. (c) MSCs were stably transfected with plasmid expressing shRNA against AUF1 or plasmid encoding scrambled sequences (CTRL), and cells were collected for identification of AUF1 by quantitative RT-PCR and Western Blot. These cells are referred to as shAUF1 MSCs or shCTRL MSCs. (e) shCTRL MSCs or shAUF1 MSCs were stimulated with IFN γ and TNF α , or together with IL-17 (all cytokines supplemented at 10 ng/ml) for 12 or 24 h. iNOS expression was detected by western blot. (f) WT MSCs or *aif1*^{-/-} MSCs were first treated with IFN γ and TNF α , with or without IL-17 (10 ng/ml), IFN γ and TNF α were supplemented at different concentrations, for 12 h, and then co-cultured with T-cell hybridoma A1.1 cells at a ratio of 1:10 for 12 h. T-cell proliferation was measured by ³H-thymidine incorporation, taking the proliferation level of A1.1 alone as 100%. mRNA expression values are means \pm S.E.M. of three wells from a representative of three independent experiments. Western blotting data are representative of three independent experiments. Proliferation values are means \pm S.E.M. of three wells from a representative of three independent experiments

MSCs were first pretreated with or without IFN γ and TNF α , in the presence or absence of IL-17 for 12 h, and then these cells were transfused into mice that received ConA injection 30 min earlier. Consistent with the results in Figure 3, wild-type MSCs pretreated with IFN γ , TNF α , and IL-17 showed the best therapeutic effect on liver injury, in which liver injury was almost completely alleviated with sharply reduced serum ALT activity, liver necrosis, and lymphocyte infiltration (Figures 6a, c and d). However, maximal therapeutic effects on ConA-induced liver injury were also achieved with *aif1*^{-/-} MSCs pretreated with only IFN γ and TNF α , without the need for IL-17 (Figures 6a, c and d). Infiltration of MNCs and CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in liver was also dramatically decreased in mice administered with wild-type MSCs pretreated with IFN γ , TNF α , and IL-17, or in mice administered *aif1*^{-/-} MSCs pretreated with IFN γ and TNF α , either with or without IL-17 (Figure 6b). The percentages of CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells, Th1, Th17, and Treg subpopulations were analyzed in liver-infiltrated cells among the different groups. However, no significant changes were observed among them (Supplementary Figures S5A, B and C). Thus, the therapeutic effect of MSCs pretreated with IFN γ , TNF α , and IL-17 on ConA-induced liver injury was mainly dependent on their robust suppression of T-cell proliferation, but not by affecting differentiation of T-cell subpopulations.

Discussion

The interactions between MSCs and the inflammatory environment have been actively studied in the last few years

in both *in vitro* and *in vivo* models. We have previously elucidated critical roles of NO and chemokines in the immunosuppressive effect of MSCs, with the co-presence of IFN γ and TNF α , or IL-1 as a requisite.¹³ As IL-17 is one of the most potent inflammatory cytokines and is often elevated in the serum and tissues of patients with inflammatory or autoimmune diseases,³² it is important to examine the influence of this key inflammatory cytokine in modulating the immunosuppressive properties of MSCs. In addition, to elicit the immunosuppressive property of MSCs, critical levels of inflammatory cytokines are required.³³ It is possible that the presence of IL-17 can further change the dynamics of the interaction between MSCs and inflammatory cytokines. In our study, we found that IL-17 could enhance the immunosuppressive function of MSCs, even in the presence of low doses of inflammatory cytokines IFN γ and TNF α .

The identification of the role of IL-17 in enhancing immunosuppression by MSCs is a novel concept. Since its discovery, IL-17 has been widely reported to exacerbate disease progress in multiple autoimmune diseases, including rheumatoid arthritis, MS, and inflammatory bowel disease, in which the IL-17 level is dramatically elevated.³² In addition, disease progression slows when IL-17 is genetically ablated or IL-17 blocking antibody is administered.³⁴ Thus, it is well accepted that IL-17 is critical in promoting inflammation and autoimmunity. However, IL-17 not always promotes immune responses, as it has been reported that IL-17 has a protective function in gut inflammatory disorders. Genetic ablation or neutralization of IL-17 can actually aggravate disease progress in dextran-sulphate-sodium-induced colitis in

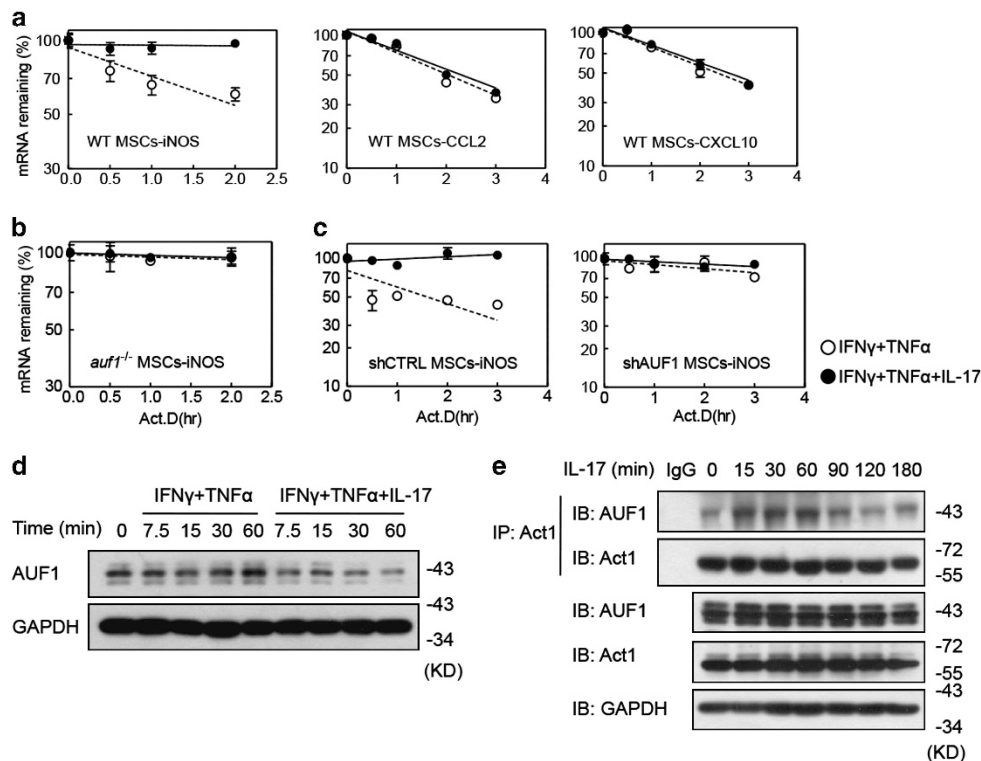


Figure 5 IL-17 enhances gene expression by modulating AUF1-mediated mRNA decay in MSCs. (a–c) Wild-type (a) or *aurf1*^{-/-} MSCs (b), shCTRL MSCs or shAUF1 MSCs (c) were treated with IFN γ and TNF α , with or without IL-17 (all cytokines supplemented at 10 ng/ml) for 6 h, and then actinomycin D (5 μ g/ml) was added to stop transcription. At the indicated time points, mRNA levels were assayed by quantitative RT-PCR, taking the expression level at the time of actinomycin D addition as 100%. mRNA expression values are means \pm S.E.M. of three wells from a representative of three independent experiments. (d) Wild-type MSCs were treated with IFN γ and TNF α , with or without IL-17 (all cytokines supplemented at 10 ng/ml). At different time points, cells were collected for analysis of AUF1 protein level by western blot. (e) Wild-type MSCs were treated with IL-17 (10 ng/ml) for different time, and cells were harvested for analysis of interaction of Act1 and AUF1 by immunoprecipitation. Western blotting data is from a representative of two independent experiments

mice.^{35,36} However, the molecular mechanisms underlying this apparently opposite effect of IL-17 is not known. Our study shows that IL-17 can enhance the immunosuppressive property of MSCs, and it is possible that MSCs may not suppress immune responses effectively without IL-17. Thus, these findings provide novel information for a better understanding of the pathophysiological roles of IL-17. We also examined the function of human IL-17 in human MSCs. As inhibition on T-cell responses by human MSCs is induced by IFN γ ,³⁷ we tested if human IL-17 could affect IFN γ -induced immunosuppression in human MSCs. We co-cultured human MSCs with anti-CD3 and anti-CD28-activated human peripheral blood mononuclear cells (PBMCs), in the presence of IFN γ , or together with IL-17, and detected the proliferation of PBMCs. Our results showed that the addition of IL-17 could weakly promote IFN γ -induced immunosuppression by human MSCs, although not as significant as in the mouse system (data not shown). More detailed investigations are necessary to understand the role of various cytokine combinations in the context of IL-17-regulated immunosuppression of human MSCs.

ConA-induced liver injury in mice is a well-established model for investigating the pathophysiological process of autoimmune or viral fulminant hepatitis, in which acute immune responses have a pivotal role in mediating liver damage.²⁸ It is well established that suppression of immune responses can

dramatically attenuate ConA-induced liver injury.³⁸ Adipose tissue-derived stromal cells have been shown to reduce liver damage in this model.^{39,40} In the present investigation, we used bone marrow-derived MSCs and investigated the role of IL-17 in modulating MSC-mediated therapy of liver injury. We found that in this acute inflammatory disease model, MSCs only marginally affected disease progression. We reasoned that the immunosuppressive capacity of MSCs requires stimulation by inflammatory cytokines. Although many cytokines can be produced after ConA administration *in vivo*, these cytokines remain at high levels for only a short time²⁸ and thus may not be able to endow MSCs with immunosuppressive capacity when administered at a later time. To circumvent this scenario, we pretreated MSCs with inflammatory cytokines IFN γ and TNF α for 12 h. Clearly, the therapeutic effect was improved to some extent (see Figures 3 and 6). Interestingly, when we pretreated MSCs with inflammatory cytokines IFN γ and TNF α together with IL-17, there was a dramatic enhancement of the therapeutic effect. This enhancement is likely related to a robust induction of iNOS by the combination with IL-17 (Figure 2), as iNOS^{-/-} MSCs didn't present any therapeutic effect on this liver injury model. Thus, this experiment revealed a novel *in vivo* role of IL-17 in enhancing immunosuppression.

AUF1 is a well-known RNA-binding protein that promotes degradation of ARE-containing mRNAs encoding numerous

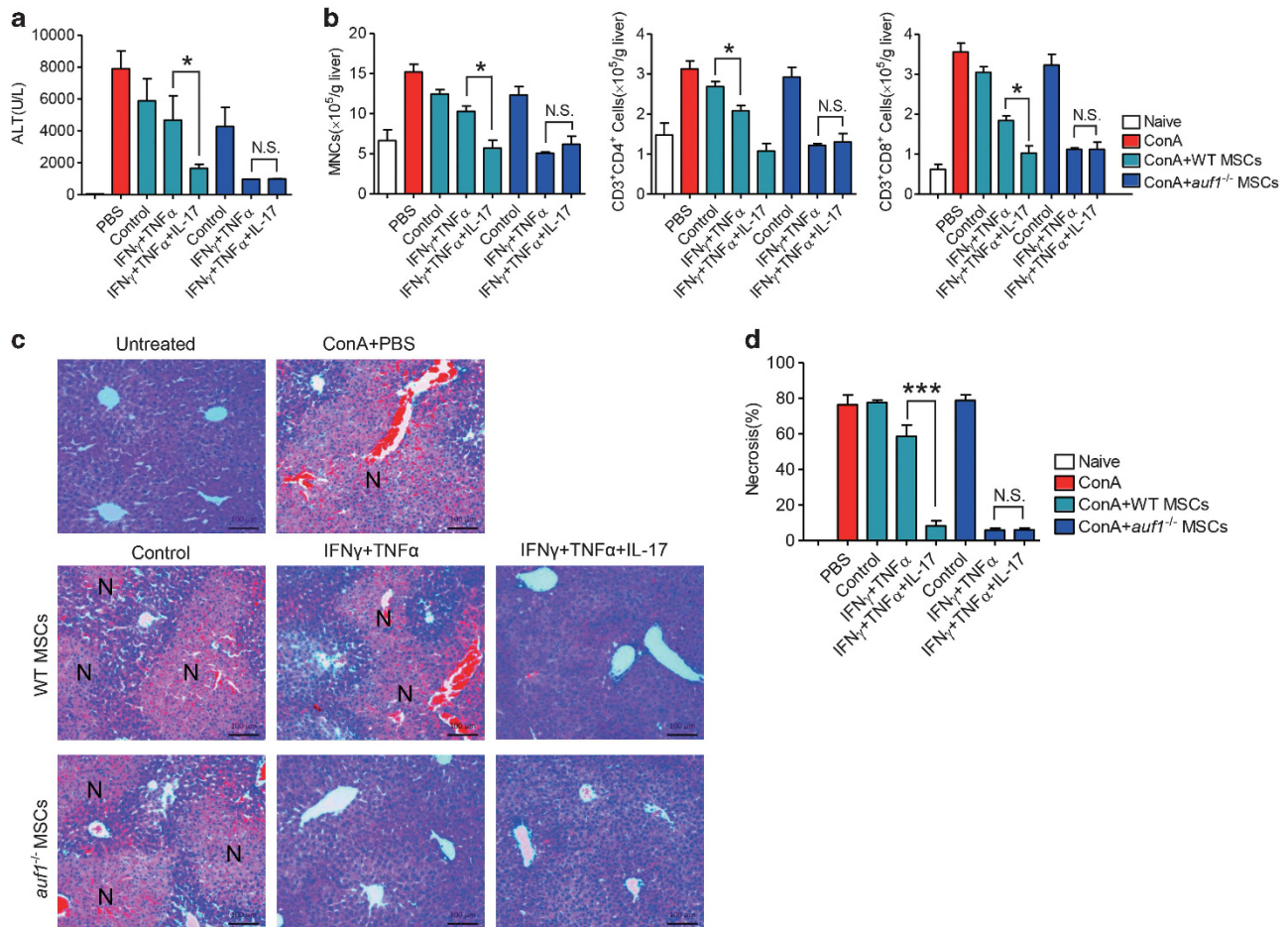


Figure 6 Critical roles of AUF1 in IL-17-enhanced therapeutic effect of MSCs on ConA-induced acute liver injury. Mice were intravenously injected with ConA (15 mg/kg); 30 min later, untreated or cytokine-pretreated wild-type or *auf1*^{-/-} MSCs were transfused. After 7.5 h, serum and livers were sampled. (a) Serum levels of ALT were measured. (b) Absolute numbers of MNCs in liver tissues were calculated ($n=3-5$ mice per group). Absolute numbers of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells were determined by flow cytometry. (c) Hematoxylin & eosin staining of liver sections at 8 h after ConA administration. N: necrosis area. (d) Percentages of necrosis were calculated

cytokines, cell cycle regulators, and other inflammatory molecules.^{20,41,42} Indeed, IL-17 induces cytokine/chemokine gene expression via mRNA stabilization.²⁹ Knockout of AUF1 in MSCs led to induction and stabilization of iNOS and IL-6 mRNAs in MSCs treated with IFN γ and TNF α (Figure 4 and data not shown), indicating that AUF1 critically controls expression of these genes in MSCs. Moreover, AUF1 knockout eliminated the requirement for IL-17 to modulate gene expression, mRNA stabilization, and immunosuppression by MSCs. Further investigations revealed AUF1-Act1 interactions and the action of IL-17 on AUF1 in MSCs. We found that addition of IL-17 reduced AUF1 protein levels in IFN γ + TNF α treated MSCs, and this effect led to stabilization of iNOS and IL-6 mRNAs (Figure 5 and data not shown). Our results also showed that Act1, a crucial adaptor protein that binds the IL-17 receptor, interacted with AUF1 upon IL-17 stimulation of MSCs. In addition, IL-17 signal transduction was defective in MSCs in the absence of AUF1 (data not shown). These data suggest that AUF1 must have dual roles in the enhancing effect of IL-17: AUF1 is required to initiate IL-17 signaling; AUF1 also serves as a target of IL-17 and is downregulated by

IL-17 in MSCs treated with IFN γ and TNF α . Thus, we have uncovered novel roles of AUF1 in the enhancing effect of IL-17 on immunosuppression.

In conclusion, we have demonstrated that IL-17 can enhance immunosuppression in the presence of MSCs. This novel effect of IL-17 is exerted in part by reversing the suppression of gene expression normally conferred by mRNA decay factor AUF1. It would be of great interest to uncover the detailed molecular mechanisms underlying the control of AUF1 by IL-17 in MSCs. In addition, much needs to be learned about the kinetics of IL-17 production during various inflammation processes and how such kinetics affects the therapeutic effect of MSCs. Nevertheless, we believe that further investigations in this direction will lead to better application of MSCs in clinical settings.

Materials and Methods

Reagents and mice. Recombinant mouse IFN γ , TNF α , IL-17A, and antibodies against IL-17A were from eBiosciences (La Jolla, CA, USA). Recombinant mouse IL-2 was from R&D Systems (Minneapolis, MN, USA). Antibodies against β -actin, GAPDH, iNOS, p65, p-I κ B α , p-p65, p-JNK, and p-ERK1/2 were from Cell Signaling Technology (Danvers, MA, USA).

Antibody against Act1 was from Santa Cruz Biotechnology (Dallas, TX, USA). L-NMMA, propidium iodide, and actinomycin D were purchased from Sigma-Aldrich (St Louis, MO, USA). Concanavaline A (ConA) was from Vector Labs (Burlingame, CA, USA).

C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences (Shanghai, China) and maintained under specific pathogen-free conditions in the vivarium of Shanghai Jiao Tong University School of Medicine with water and food provided *ad libitum*. All animal protocols are approved by our Institutional Animal Care and Use Committee.

Cells. MSCs were generated using our previously described protocol.¹³ Briefly, tibia and femur bone marrow of wild-type, *iNOS*^{-/-}, or *auf1*^{-/-} mice was harvested. Cells were cultured in DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin (complete medium, all from Invitrogen, Carlsbad, CA, USA). All nonadherent cells were removed after 24 h (hr), and adherent cells were maintained. Medium was changed every 2–3 days. To obtain MSC clones, cells at confluence were harvested and seeded into 96-well plates by limited dilution. Individual clones were then picked and expanded. These MSCs were capable of differentiating into adipocytes and osteocytes under the respective differentiation conditions. Cells were used before the 20th passage.

The generation of T-cell blasts was done as previously described.^{13,43}

Proliferation assay. To assay cell proliferation, 0.5 μ Ci of ³H-thymidine (Tdr, Shanghai Institute of Applied Physics, Chinese Academy of Sciences, China) was added to each well of 96-well plates 6 h before termination of the cultures by freezing. Plates were then thawed, cells were harvested, and incorporated ³H-Tdr was assessed with a Wallac Microbeta scintillation counter (Perkin-Elmer, Waltham, MA, USA).

DNA content analysis. DNA content analysis was done as previously described.⁴⁴ Briefly, A1.1 cells were co-cultured with MSCs for 12 h, harvested, and washed once with PBS. The cells were then resuspended in staining buffer (PBS supplemented with 50 μ g/ml propidium iodide, 0.2% saponin, 10 μ g/ml RNase A). After 30 min incubation in dark, samples were analyzed by flow cytometry on a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

RNA isolation and gene expression assay. Total RNA was isolated with the RNeasy Pure Cell/Bacteria Kit (TIANGEN Biotech, Beijing, China). First-strand cDNA synthesis was performed with a cDNA synthesis kit (TIANGEN Biotech). The levels of mRNAs were measured by quantitative RT-PCR (7900 HT; Applied Biosystems, Foster City, CA, USA) with SYBR Green Master Mix (TaKaRa Biotech, Dalian, China) and normalized to the level of β -actin mRNA. Sequences of forward and reverse primer pairs are as follows:

AUF1: forward 5'-CAGCAGAGTGGTTATGGGAAAGTATCC-3' and reverse 5'-GACAGGAGCCACCTTCAATGAATC-3';
 β -actin: forward 5'-CCACGAGCGGTTCCGATG-3' and reverse 5'-GCCACAGGATTCCATACCCA-3';
 CCL2: forward 5'-TCTCTCTTCTCCACCACCATG-3' and reverse 5'-GCGTAACTGCATCTGGCTGA-3';
 CCL5: forward 5'-TTTCTACACCAGCAGCAAGTGC-3' and reverse 5'-CCTTCGTGTGACAAACACGAC-3';
 CXCL9: forward 5'-AGTGTGGAGTTCGAGGAACCC-3' and reverse 5'-TGCAGGAGCATCGTCATT-3';
 CXCL10: forward 5'-TAGCTCAGGCTCGTCAGTTCT-3' and reverse 5'-GATGGTGGTTAAGTTCGTGCT-3';
 IL-6: forward 5'-GAGGATACCACTCCCAACAGACC-3' and reverse 5'-AAGTGCATCATCGTTGTCATACA-3';
 IL-17RA: forward 5'-AGTGTTCCTCTACCCAGCAC-3' and reverse 5'-GAAAACCGCCACCGCTTAC-3';
 IL-17RC: forward 5'-GCTGCCTGATGGTGACAATGT-3' and reverse 5'-TGGACGCGAGTACAGTAAGAAG-3';
 iNOS: forward 5'-CAGCTGGGCTGTACAAACCTT-3' and reverse 5'-CATTGGAAGTGAAGCGTTTCG-3'.

Messenger RNA decay assays. Messenger RNA decay assays were performed essentially as described.^{45–47} Briefly, MSCs were incubated with cytokine combinations for 6 h. Actinomycin D (Act.D) was added into the medium

at a final concentration of 5 μ g/ml to stop transcription. At various time points after addition of Act.D, cells were harvested for the extraction of total RNA. Levels of iNOS, IL-6, CCL2, and CXCL10 mRNAs were measured at each time point by quantitative RT-PCR. Percentages of mRNA remaining at each time point were plotted versus time after Act.D addition. First-order decay constants, *k*, were determined by nonlinear regression. The associated mRNA half-lives, *t*_{1/2}, were calculated with the equation *t*_{1/2} = ln2/*k*.

RNA interference. Plasmids bearing shRNA targeting AUF1 mRNA (5'-GTTGTAGACTGCACTCTGA-3') or Act1 mRNA (5'-GACAGTGATGATAA TCGTA-3') were constructed by the insertion of annealed oligonucleotides into the Hind III and BamH I sites of pSILENCER 2.0-U6 (Ambion, Austin, TX, USA). Plasmids bearing scrambled sequences (shCTRL), shRNA targeting AUF1 mRNA (shAUF1) or Act1 mRNA (shAct1) were introduced into MSCs (1.5 \times 10⁶ cells) using Amaxa Nucleofector, with program U-023, and the Nucleofector kit V (Amaxa, Cologne, Germany). Cells were seeded in DMEM complete medium in six-well plates. After 2–3 days, the medium was changed, and stably transfected clones were picked and expanded using DMEM complete medium containing 250 μ g/ml hygromycin B (Invitrogen) for 3–4 weeks.

Western blotting analysis. Cells were washed twice with ice-cold PBS, harvested and lysed in the RIPA buffer (Millipore, Temecular, CA, USA) containing a cocktail of protease inhibitors (Roche, Nutley, NJ, USA) and PMSF (Sigma) for 30 min on ice. Lysates were clarified by centrifugation at 16 000 \times *g* for 15 min. Protein concentration of the supernatant was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). Protein samples were diluted in 5 \times SDS loading buffer and fractionated in a 10% SDS-polyacrylamide gel. Proteins were electroblotted onto a nitrocellulose membrane (Whatman Inc., Clifton, NJ, USA) and incubated for 1 h in 5% nonfat dry milk dissolved in TBST (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.05% Tween 20) at room temperature. The blotting membranes were incubated with primary antibodies overnight at 4 $^{\circ}$ C, extensively washed in TBST, incubated with hP-conjugated secondary antibody (Cell Signaling) for 1.5 h at room temperature, and washed again with TBST. The blotting membranes were developed with chemiluminescent reagents (Millipore, Billerica, MA, USA) according to the instructions provide by the manufacturer.

Immunoprecipitation. Cells were lysed in RIPA buffer (Millipore, Temecular, CA, USA) containing a cocktail of protease inhibitors (Roche) and PMSF (Sigma) for 30 min on ice. Lysates were clarified by centrifugation at 16 000 \times *g* for 15 min. Protein concentration of the supernatant was determined by the Bradford assay (Bio-Rad). After precleared with protein G sepharose beads, cell extracts were incubated with 20 μ l protein G sepharose beads in the presence of 1 μ g Act1 antibody or nonspecific IgG overnight at 4 $^{\circ}$ C. After washing four times with RIPA buffer, beads were suspended in 20 μ l 2 \times SDS loading buffer, boiled at 100 $^{\circ}$ C for 10 min, and analyzed by western blot.

ConA-induced liver injury in mice. C57BL/6 mice (8–10 week old) were intravenously injected with ConA in PBS at 15 mg/kg to induce acute inflammatory liver injury. MSCs (5 \times 10⁵) derived from wild-type mice, *iNOS*^{-/-} mice or *auf1*^{-/-} mice were treated with or without IFN γ and TNF α in the presence or absence of IL-17 (10 ng/ml for each cytokine) for 12 h, and then intravenously administrated into mice that have been treated with ConA for 30 min. Mice were killed and serum and liver tissues were sampled after another 7.5 h. Serum ALT activity was determined by an ALT detection kit (Yihua Medical Scientific, Shanghai, China). Formalin-fixed liver histological sections were stained with hematoxylin & eosin.

Localization of MSCs in mouse liver. Mice were transfused with GFP-transgenic mouse MSCs 30 min after ConA injection. After another 7.5 h, livers were harvested. Frozen sections were stained with anti-GFP antibody (Abcam, New Territories, Hong Kong) and labeled with Alexa Fluor 594 conjugated donkey anti-rabbit secondary antibody (Invitrogen) and DAPI (Sigma-Aldrich) to reveal localization of MSCs.

Isolation of liver MNCs and flow cytometric analysis. Liver mononuclear cells (MNCs) were purified by a 40%/70% percoll gradient⁴⁸ and stained with anti-CD3-PE, anti-CD4-PerCP/Cy5.5, anti-CD8a-APC, or anti-CD25-FITC (eBiosciences) for 30 min at 4 $^{\circ}$ C in staining buffer (PBS, 3% FCS). For detection of intracellular proteins, cells were first stimulated with PMA (Sigma)

andinomycin (Sigma) for 4 h and stopped by addition of Brefeldin A (Sigma). After cell surface staining, cells were fixed and permeabilized (eBiosciences), and then stained respectively with anti-IFN γ -FITC, anti-IL-17A-PE, or anti-Foxp3-PE. For detection of surface expression of IL-17RA in cloned MSCs, cells were stained with anti-IL-17RA-PE (eBiosciences). All samples were analyzed by flow cytometry on a FACS Calibur flow cytometer (Becton Dickinson).

Statistical analysis. Nonlinear regression and statistical analyses were performed with PRISM v5 software (GraphPad Software, Inc., La Jolla, CA, USA). Comparisons between samples were performed with the unpaired *t*-test. Differences with $P < 0.05$ were considered significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$); N.S., not significant.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

XH, YW, GB, and YFS designed research; XH, QY, LL, CX, CZ, and YH performed research and collected data; XH, YS, GB, YW, XC, ML, WC, KC, and QC analyzed data; GX, YZ, JZ, and RJS provided studied material; and XH, YW, YQ, GB, and YS wrote the paper.

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