The Role of Mouse Mesenchymal Stem Cells in Differentiation of Naive T-Cells into Anti-Inflammatory Regulatory T-Cell or Proinflammatory Helper T-Cell 17 Population

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Bone marrow-derived mesenchymal stem cells (MSCs) modulate immune response and can produce significant levels of transforming growth factor- β (TGF- β) and interleukin-6 (IL-6). These 2 cytokines represent the key factors that reciprocally regulate the development and polarization of naive T-cells into regulatory T-cell (Treg) population or proinflammatory T helper 17 (Th17) cells. In the present study we demonstrate that MSCs and their products effectively regulate expression of transcription factors Foxp3 and ROR γ t and control the development of Tregs and Th17 cells in a population of alloantigen-activated mouse spleen cells or purified CD4⁺ CD25⁻ T-cells. The immunomodulatory effects of MSCs were more pronounced when these cells were stimulated to secrete TGF- β alone or TGF- β together with IL-6. Unstimulated MSCs produce TGF- β , but not IL-6, and the production of TGF- β can be further enhanced by the anti-inflammatory cytokines IL-10 or TGF- β . In the presence of proinflammatory cytokines, MSCs secrete significant levels of IL-6, in addition to a spontaneous production of TGF- β . MSCs producing TGF- β induced preferentially expression of Foxp3 and activation of Tregs, whereas MSC supernatants containing TGF- β together with IL-6 supported ROR γ t expression and development of Th17 cells. The effects of MSC supernatants were blocked by the inclusion of neutralization monoclonal antibody anti-TGF- β or anti-IL-6 into the culture system. The results showed that MSCs represent important players that reciprocally regulate the development and differentiation of uncommitted naive T-cells into anti-inflammatory Foxp3⁺ Tregs or proinflammatory ROR γ t⁺ Th17 cell population and thereby can modulate autoimmune, immunopathological, and transplantation reactions.

Introduction

THE ABILITY OF MESENCHYMAL stem cells (MSCs) to modulate immune response has been well documented in numerous models. MSCs inhibit mitogen- or antigen-induced cell proliferation, cytokine production, and generation of cytotoxic cells in vitro [1–4]. Various types of immune response, such as autoimmune, antitumor, or transplantation reactions, have been attenuated by the transfer of MSCs [5–8]. However, in some models, MSCs stimulate immune reactions as opposed to the immunosuppressive effects of MSCs usually observed [9–11]. So far, cell-to-cell contact, production of inhibitory molecules, or induction of regulatory T-cells (Tregs) has been implicated in the mechanism of immunomodulation mediated by MSCs [6,12–14]. However, the manifestation of individual mechanisms of MSC-mediated suppression differs among various models or between species.

MSCs are also potent producers of various cytokines. After mitogen stimulation or upon a contact with lymphocytes, they produce numerous cytokines; especially interleukin-6 (IL-6) and transforming growth factor- β (TGF- β) can be regularly found in MSC supernatants [15,16]. IL-6 and TGF- β have been shown to be the main factors reciprocally regulating the development of proinflammatory ROR γ t⁺ T helper 17 (Th17) cells and anti-inflammatory Foxp3⁺ Treg population [17,18]. In naive CD4⁺ T-cells, TGF- β rapidly induces expression of both Foxp3 and ROR γ t regardless of the presence of IL-6 [19,20]. However, in the presence of IL-6 expression of Foxp3 is suppressed, expression of ROR γ t predominates, and the production of IL-17 is favored [19]. The crucial role in differentiation of T-cell subsets play concentrations of TGF- β . In low TGF- β concentrations IL-6 suppresses Foxp3 expression, and the development of Th17 cells prevails. On the other hand,

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higher concentrations of TGF- β favor the development of Foxp3⁺ Tregs [21]. Although other cytokines can also modulate the development and the activity of Tregs and Th17 cells, TGF- β and IL-6 remain the most important cytokines that, in a concentration-dependent manner, can orchestrate the differentiation of Tregs and Th17 cells. As MSCs are a potent source of both IL-6 and TGF- β , they represent a candidate cell population that could significantly influence differentiation of Th17 cells and Tregs and in this way to modulate autoimmune, immunopathological, and protective immune reactions.

Here we show that MSCs produce variable levels of TGF- β and IL-6 depending on the presence of proinflammatory IL-1, IL-2, interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), or anti-inflammatory (TGF- β , IL-10) cytokines and thus skew differentiation of naive CD4⁺ T lymphocytes into Treg or Th17 direction. Although MSCs can also contribute to immunoregulation by other mechanisms involving production of inhibitory molecules [11,22,23] or a direct cell–cell contact [1,12], the cytokine-regulated production of TGF- β and IL-6 by MSCs offers explanation for the different levels and patterns of immunoregulatory properties of MSCs. The regulation of differentiation and development of T-cell subsets represents a novel property of mouse bone marrow (BM)-derived MSCs.

Materials and Methods

Mice

BALB/c and C57BL/6J (B6) mice of both sexes were obtained at the age of 8–12 weeks from the breeding unit of the Institute of Molecular Genetics, Prague, Czech Republic. The use of animals was approved by the local Animal Ethics Committee.

Isolation and culture of MSCs

MSCs were isolated from the BM of BALB/c mice. The BM from the femurs and tibias was flushed out, washed, and cultured in Dulbecco's modified Eagle's medium (DMEM; PAA Laboratories, Pasching, Austria) supplemented with 10% fetal calf serum (FCS; Sigma Co., St. Louis, MO), antibiotics (100 μ g/mL of streptomycin and 100 U/mL of penicillin), and 10 mM HEPES buffer (hereafter referred to as complete DMEM). The cells were cultured at a concentration of 4×10^6 cells/mL in 6 mL of the culture medium in 25-cm² tissue culture flasks (Nunc, Roskilde, Denmark). After 24 h of incubation, the nonadherent cells were removed by washing and the remaining adherent cells were cultured with regular exchange of the culture medium and passaging of the cells to maintain an optimal cell concentration. After ~3 weeks of culture (2–3 passages), the cells were harvested by gentle scraping and immunodepleted of CD11b⁺ and CD45⁺ cells by magnetic activated cell sorting (MACS). In brief, cells were incubated for 15 min with CD11b MicroBeads and CD45 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and CD11b[−]CD45[−] MSCs were isolated using the AutoMACS magnetic separation system (Miltenyi Biotec). The purity of sorted cells (>98% of cells were CD11b[−]CD45[−]) was confirmed by flow cytometry using an LSRII cytometer (BD Biosciences, Franklin Lakes, NJ).

Characterization of MSCs by flow cytometry

MSCs isolated using MACS were washed in PBS containing 0.5% bovine serum albumin and incubated for 30 min on ice with allophycocyanine (APC)-labeled monoclonal antibody (mAb) anti-CD44 (clone IM7; BD PharMingen, San Jose, CA), fluorescein isothiocyanate (FITC)-labeled anti-CD90.2 (clone 30-H12; BioLegend, San Diego, CA), phycoerythrin (PE)-labeled anti-CD105 (clone MJ7/18; eBioscience, San Diego, CA), APC-labeled anti-CD11b (clone M1/70; BioLegend), PE-labeled anti-CD31 (clone MEC 13.3; BD PharMingen), or FITC-labeled anti-CD45 (clone 30-F11; BioLegend). Dead cells were stained using Hoechst 33258 dye (Invitrogen, Carlsbad, CA) added to the samples 15 min before flow cytometry analysis. Data were collected using an LSRII cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

Differentiation of MSCs to adipocytes and osteoblasts

MSCs growing for 2–3 weeks and separated by magnetic cell sorting were cultured in a complete DMEM medium supplemented with specific adipogenic (containing 0.1 μ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, 0.1 mM indomethacin, and 0.5 μ g/mL of insulin) or osteogenic (0.1 μ M dexamethasone, 0.1 mM L-ascorbic acid, and 10 mM β -glycerolphosphate disodium salt pentahydrate) reagents [24]. Differentiation of cells was confirmed by staining with Oil Red O or Alizarin Red S.

Immunosuppressive properties of MSCs

Spleen cells (0.5×10^6 /mL) from BALB/c mice were stimulated in a volume of 0.2 mL of RPMI 1640 medium (Sigma Co.) supplemented with 10% FCS, antibiotics, HEPES buffer, and 5×10^{-5} M 2-mercaptoethanol (hereafter a complete RPMI 1640 medium) in 96-well tissue culture plates (Corning Co., Corning, NY) with 10 μ g/mL of mAb anti-CD3 [25]. Purified MSCs were added to these cultures at the ratios of spleen cells to MSCs 5:1, 10:1, and 20:1. Cell proliferation was determined by adding ³H-thymidine (0.5 μ Ci/well, Nuclear Research Institute, Rez, Czech Republic) for the last 6 h of the 72-h incubation period.

Preparation of MSC supernatants

MSCs purified by MACS were incubated at a concentration of 1×10^5 cells/mL in a volume of 1 mL of a complete RPMI 1640 medium in 24-well tissue culture plates (Nunc). The cells were cultured unstimulated or stimulated with 10 ng/mL of mouse recombinant IL-1 α , IL-2, IL-6, IL-10, IFN- γ , or TNF- α (all cytokines were purchased from Immunotools, Friesoythe, Germany), 1 ng/mL of human TGF- β 1 (PeproTech, Rocky Hill, NJ), or with 1 μ g/mL of lipopolysaccharide (LPS; Difco Laboratories, Detroit, MI). After 24 h of incubation period the adherent cells were washed several times with excess of medium to remove added cytokines. The cells were then incubated in fresh medium without cytokines. Since the presence of TGF- β in FCS could interfere with the TGF- β detection by ELISA, MSCs used for TGF- β production were first transferred into serum-free medium containing 200 μ g/mL of bovine serum albumin

and then stimulated with cytokines. All supernatants were harvested after another 72-h period and the production of cytokines or NO was assessed.

Cytokine detection

The production of IL-1 β , IL-4, IL-6, IL-10, IL-12, IFN- γ , and TGF- β by MSCs was quantified by ELISA. For the detection of IL-4, IL-6, IL-10, and IFN- γ , the cytokine-specific capture and detection mAb purchased from PharMingen was used. IL-1 β , IL-12, and TGF- β were measured using ELISA kits purchased from R&D Systems (Minneapolis, MN). To test the production of IL-17, spleen cells (0.8×10^6 /mL) from BALB/c mice were stimulated for 96 h with irradiated ($3,000$ R) spleen cells (0.8×10^6 /mL) from B6 mice in the presence of TGF- β , IL-6, or MSC supernatants. The concentrations of IL-17 in the supernatants were assessed using an ELISA kit (R&D Systems).

NO determination

Nitrite concentrations in MSC supernatants were measured using the Griess reaction [26]. In brief, 100 μ L of the tested supernatant was incubated with 50 μ L of 1% sulfanilamide (in 2.5% H_2SO_4) and 50 μ L of 0.3% *N*-1-naphthylethylenediamine dihydrochloride (in 2.5% H_2SO_4). Nitrite was quantified by spectrophotometry at 540 nm using sodium nitrite as a standard.

Regulation of IL-17 production and Foxp3 or ROR γ t expression

Spleen cells (0.8×10^6 /mL) from BALB/c mice were stimulated in a volume of 1 mL of a complete RPMI 1640 medium (Sigma Co.) in 48-well tissue culture plates (Corning Co.) with irradiated ($3,000$ R) spleen cells (0.8×10^6 /mL) from B6 mice. Recombinant human TGF- β (1 ng/mL; PeproTech), TGF- β (1 ng/mL) together with IL-6 (1 ng/mL; Immunotools), or the supernatants (30% of the volume) obtained after a 72-h incubation of MSCs were added to the cultures of stimulated spleen cells. The proportion of CD4 $^+$ cells expressing Foxp3 or ROR γ t was determined after a 96-h incubation period by flow cytometry. The concentrations of IL-17 in the supernatants were determined by ELISA. In some experiments, purified CD4 $^+$ CD25 $^-$ cells were used as responder cells. This subpopulation was isolated from spleen single-cell suspensions using a CD4 $^+$ CD25 $^+$ Regulatory T-cell isolation kit (Miltenyi Biotec) and the AutoMACS magnetic separation system (Miltenyi Biotec), as we have described [27]. This procedure yielded >96% pure CD4 $^+$ CD25 $^-$ cell population. Over 99% of these cells were Foxp3 $^-$ and ROR γ t $^-$, and >86% of them expressed CD62L, a marker of naive T-cells (data not shown).

To determine the MSC-derived factors responsible for the regulation of T-cell differentiation, neutralization mAb anti-IL-6 (clone MP5-20F3; BioLegend) or anti-TGF- β 1 (clone ab64715; Abcam, Cambridge, United Kingdom) at a concentration of 2 μ g/mL was added to the cultures of spleen cells stimulated with alloantigens in the presence of MSC supernatants.

Detection of TGF- β and IL-6 gene expression by real-time polymerase chain reaction

MSCs were cultured untreated or were stimulated with IL-1 α (10 ng/mL), TGF- β (1 ng/mL), or LPS (1 μ g/mL). Total

RNA was extracted from the cells using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. One μ g of total RNA was treated with deoxyribonuclease I (Promega, Madison, WI) and used for subsequent reverse transcription. The first-strand cDNA was synthesized using random hexamers (Promega) in a total reaction volume of 25 μ L using M-MLV Reverse Transcriptase (Promega).

Quantitative real-time polymerase chain reaction (PCR) was performed on an iCycler (BioRad, Hercules, CA) as we have previously described [28,29]. The primers used for amplification were as follows:

GAPDH: 5'-AGAACATCATCCCTGCATCC-3' (sense), 5'-ACATTGGGGGTAGGAACAC-3' (antisense);

TGF- β : 5'-TGGAGCAACATGTGGAAGTC-3' (sense), 5'-CAGCAGCCGGTTACCAAG-3' (antisense);

IL-6: 5'-GCTACCAAACTGGATATAATCAGGA-3' (sense), 5'-CCAGGTAGCTATGGTACTCCAGAA-3' (antisense).

The PCR parameters included denaturation at 95°C for 3 min, 40 cycles at 95°C for 20 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The CT values were within the range 25–28, 21–24, and 17–20 for *IL-6*, *TGF- β* , and *GAPDH*, respectively. Each single experiment was done in triplicate. Fluorescence data were collected at each cycle after an elongation step at 80°C for 5 s, and the data were analyzed on the iCycler Detection system.

Intracellular staining of Foxp3 and ROR γ t

The cultured spleen cells were harvested and washed with PBS containing 0.5% bovine serum albumin. Before intracellular staining, the cells were incubated for 30 min on ice with Alexa Fluor 700-labeled mAb anti-CD4 (clone GK1.5; BioLegend) and Live/Dead Fixable Violet Dead Cell Stain Kit (Molecular Probes, Eugene, OR) for staining of dead cells. Cells were washed in PBS/0.5% bovine serum albumin, fixed, and permeabilized using eBioscience Foxp3 buffer staining set according to the manufacturer's instructions. For intracellular detection of Foxp3 and ROR γ t, the cells were stained for 30 min with PE-labeled mAb anti-mouse/rat Foxp3 (clone FJK-16s, eBioscience) or PE-conjugated mAb anti-mouse/human ROR γ t (clone AFKJS-9, eBioscience). Data were collected using an LSRII cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Detection of suppressor activity of Tregs

CD4 $^+$ CD25 $^-$ spleen cells (0.6×10^6 /mL) from BALB/c mice were incubated in 48-well tissue culture plates (Nunc) in a volume of 1 mL of complete RPMI 1640 medium unstimulated, with irradiated B6 spleen cells (0.8×10^6 /mL) or with irradiated B6 cells in the presence of supernatants (30% of the volume) from MSCs that were either untreated or preincubated with IL-1 α or TGF- β . After a 96-h incubation period, the cells were harvested and tested for their ability to inhibit mixed leukocyte reaction (MLR) BALB/c anti-B6 [27]. In this assay, BALB/c spleen cells (0.75×10^6 /mL) were incubated with irradiated B6 cells (0.75×10^6 /mL) in a volume of 200 μ L of a complete RPMI 1640 medium, and the cells from the primary cultures were added to this MLR at a reactive cell to preincubated cell ratio of 4:1. Cell proliferation was determined after 96 h according to the 3H -thymidine incorporation.

Statistical analysis

The results are expressed as the mean $\pm$ SD. Comparisons between 2 groups were analyzed by Student's *t*-test, and multiple comparisons were calculated by analysis of variance. A value of $P < 0.05$ was considered statistically significant.

Results

Characterization of MSCs

BM-derived adherent cells growing for 3 weeks in culture represent a heterogeneous cell population with a variable cell size and morphology. After the MACS separation, the purified cells had a uniform spindle-shaped morphology. Over 98% of cells were negative for CD45, CD11b, and CD31 markers, but were positive for CD44, CD90, and CD105, the molecules attributed to murine MSCs (Fig. 1A). The MSCs were further characterized by their ability to undergo specific adipogenic and osteogenic differentiation (Fig. 1B) and by their capability to inhibit anti-CD3 induced T-cell proliferation (Fig. 1C), consistent with our previous results [27,28]. In addition, we confirmed a specific adipogenic and osteogenic differentiation of MSCs by expression of adipocyte- and osteoblast-specific markers by real-time PCR (data not shown). Thus, these MACS-separated BM-derived adherent cells possess the phenotype, differentiation, and functional properties associated with MSCs.

Cytokine production by MSCs

Unstimulated MSCs did not produce detectable levels of IL-6 or any of the tested cytokines (IL-1 β , IL-4, IL-10, IL-12, or IFN- γ). After stimulation with IL-1 α or LPS, MSCs produced significant ($P < 0.001$) levels of IL-6, but not the other tested cytokines (Fig. 2A and unpublished observations). In contrast, MSCs spontaneously produced a high level of TGF- β and this production was decreased ($P < 0.05$) in the presence of IL-1 α or LPS (Fig. 2A). Unstimulated MSCs also produce no detectable levels of NO. NO production remained very low even after stimulation with IFN- γ or LPS. However, significant NO production ($P < 0.001$) was detected if the MSCs were stimulated with LPS together with IFN- γ (Fig. 2B). Next we evaluated the effects of a panel of selected cytokines on IL-6 and TGF- β production by MSCs. We found that proinflammatory cytokines IL-1 α , IL-2, IFN- γ , and TNF- α (and LPS) significantly stimulated IL-6 production, but anti-inflammatory cytokines IL-10 and TGF- β did not induce detectable levels of IL-6 (Fig. 2C). On the contrary, TGF- β , but not proinflammatory cytokines, enhanced ($P < 0.05$) production of TGF- β (Fig. 2C). Since the presence of TGF- β in the FCS could interfere with TGF- β detection by ELISA, the cultures for TGF- β production were performed in serum-free medium. To confirm the results from ELISA, MSCs were cultured in the serum-containing RPMI 1640 medium and the levels of TGF- β mRNA and IL-6 mRNA were determined by real-time PCR. As shown in Fig. 2D, unstimulated MSCs

FIG. 1. Characterization of purified MSCs. **(A)** Expression of CD44, CD90.2, CD105, CD11b, CD31, and CD45 markers was assessed by flow cytometry. One of 5 similar experiments is shown. **(B)** The ability of MSCs to undergo **(a)** adipogenic and **(b)** osteogenic differentiation. The cultures without (*upper panel*) or with (*lower panel*) addition of differentiation agents were stained with Oil Red O for adipocytes or Alizarin Red S for osteoblasts. Original magnification: **(a)** 200 $\times$, **(b)** 40 $\times$. **(C)** Immunosuppressive properties of MSCs. Spleen cells were stimulated with mAb anti-CD3 in the absence or presence of MSCs (at the ratios 5:1, 10:1, and 20:1) and cell proliferation was determined. Each bar represents the mean $\pm$ SD from 4 individual experiments. Values with asterisk are significantly different ($P < 0.05$) from the positive control. MSC, mesenchymal stem cell; mAb, monoclonal antibody.

FIG. 2. Production of selected cytokines and NO by MSCs. MSCs were cultured unstimulated or in the presence of 10 ng/mL of IL-1 α , IL-2, IL-10, IFN- γ , or TNF- α , 1 ng/mL of TGF- β , or 1 μ g/mL of LPS. After a 24-h preincubation the cells were washed and cultured for another 72 h. **(A)** The presence of IL-6, TGF- β , IL-1 β , and IL-4 in the supernatants was detected by ELISA. **(B)** The concentrations of NO were measured by spectrophotometry. **(C)** The effects of proinflammatory and anti-inflammatory cytokines on IL-6 and TGF- β production were assessed by ELISA. **(D)** Effects of IL-1 α , TGF- β , and LPS on TGF- β or IL-6 gene expression were detected by a real-time polymerase chain reaction. Each bar represents the mean $\pm$ SD from 4 to 6 independent experiments. TGF- β , transforming growth factor- β ; IL, interleukin; IFN, interferon; LPS, lipopolysaccharide.

expressed a significant level of TGF- β mRNA, but not IL-6 mRNA. Expression of TGF- β mRNA was significantly ($P < 0.01$) decreased in the presence of IL-1 α or LPS and slightly enhanced by exogenous TGF- β . Strong expression of IL-6 gene was detected after stimulation of MSCs with IL-1 α and LPS (Fig. 2D).

The effects of TGF- β and IL-6 on Foxp3 and ROR γ t expression and IL-17 production

Since MSCs can produce significant levels of IL-6 and TGF- β , we first tested the effects of recombinant IL-6 and TGF- β on IL-17 production by alloantigen-activated spleen cells. As shown in Fig. 3A, alloantigen-stimulated spleen cells produced a significant amount of IL-17 and this production was inhibited by TGF- β and considerably enhanced in the presence of TGF- β together with IL-6. These modulatory effects of IL-6 and TGF- β were confirmed when highly purified CD4 $^{+}$ CD25 $^{-}$ cells were used as responder cells (Fig. 3B). To demonstrate that IL-6 and TGF- β also regulate expression of transcription factors Foxp3 and ROR γ t, which determine the development of Tregs and Th17 cells, respectively, we cultured spleen cells alone, with irradiated allogeneic cells or with allogeneic cells in the presence of TGF- β or TGF- β together with IL-6, and the percentage of CD4 $^{+}$ Foxp3 $^{+}$ and CD4 $^{+}$ ROR γ t $^{+}$ cells was determined by flow

cytometry. As shown in Fig. 3C, the percentage of CD4 $^{+}$ Foxp3 $^{+}$ cells was significantly enhanced in the presence of TGF- β and this increase was reversed by IL-6. On the contrary, the percentage of CD4 $^{+}$ ROR γ t $^{+}$ cells was highest in the cultures containing TGF- β together with IL-6 (Fig. 3D).

Regulation of IL-17 production and Treg activity by MSC supernatants

Spleen cells were stimulated with irradiated allogeneic cells alone or in the presence of MSC supernatants. As demonstrated in Fig. 4A, supernatants obtained from the cultures of untreated MSCs significantly inhibited IL-17 production. This suppression was even more pronounced, when MSCs were pretreated with TGF- β or IL-10. On the contrary, the supernatants prepared from MSCs that were pretreated with IL-1 α , IL-2, or LPS to produce IL-6 significantly enhanced the IL-17 production (Fig. 4A).

To demonstrate the effects of MSC supernatants on development of Treg activity, purified naive CD4 $^{+}$ CD25 $^{-}$ spleen cells were activated with allogeneic cells in the presence of MSC supernatants. It was observed that supernatants from untreated MSCs or from MSCs preincubated with TGF- β activated Tregs with more pronounced suppressive activity than the supernatants from MSCs preincubated with IL-1 (Fig. 4B).

FIG. 3. The regulatory effects of TGF- β and IL-6 on IL-17 production and Foxp3 or ROR γ t expression in spleen cells stimulated with alloantigens. Spleen cells (A) or purified CD4⁺CD25⁻ cells (B) from BALB/c mice were cultured unstimulated (a), stimulated with irradiated B6 spleen cells (b), or were stimulated with B6 cells in the presence of 1 ng/mL of TGF- β (c) or TGF- β together with 1 ng/mL of IL-6 (d). The concentrations of IL-17 (A and B) in the supernatants were measured after a 96-h incubation period by ELISA. Each bar represents the mean $\pm$ SD from 4 determinations. The percentage of CD4⁺Foxp3⁺ (C) or CD4⁺ROR γ t⁺ (D) spleen cells was determined by flow cytometry. One typical experiment of 5 determinations is shown.

Modulation of Foxp3 and ROR γ t expression by MSC supernatants

The supernatants containing TGF- β or TGF- β together with IL-6 were prepared by a 72-h incubation of untreated MSCs or MSCs that were preincubated for 24 h with IL-1 α or TGF- β and then carefully washed to remove exogenous cytokines before preparation of MSC supernatants. The supernatants were added to the cultures of spleen cells stimulated with irradiated allogeneic cells, and the percentage of CD4⁺Foxp3⁺ and CD4⁺ROR γ t⁺ cells was determined. As demonstrated in Fig. 5A and B, the supernatants from untreated MSCs slightly increased the proportion of CD4⁺Foxp3⁺ cells. This increase was more apparent if the supernatants from MSCs pretreated with TGF- β (and containing endogenous TGF- β produced by MSCs) were used. On the contrary, supernatants from MSCs pretreated with IL-1 α (which contain TGF- β and IL-6 produced by MSCs) rather decreased the percentage of CD4⁺Foxp3⁺ cells, but significantly enhanced the proportion of CD4⁺ROR γ t⁺ cells (Fig. 5C and D).

Effects of mAb anti-IL-6 and anti-TGF- β on the immunomodulatory properties of MSC supernatants

Supernatants from untreated MSCs (which contain TGF- β , but not IL-6) inhibited IL-17 production in cultures of alloantigen-stimulated spleen cells. Inclusion of neutralization mAb anti-TGF- β into these cultures abrogated the inhibitory effect of MSC supernatants (Fig. 6). On the contrary, supernatants from MSCs pretreated with IL-1 α (which con-

tain TGF- β and IL-6) significantly elevated IL-17 production. This enhancing effect of MSC supernatants was inhibited by mAb anti-IL-6 (Fig. 6). Since irrelevant, isotype-matched mAb added to these cultures did not abrogate immunomodulatory effects of MSC supernatants (data not shown), TGF- β and IL-6 were identified as the main factors produced by MSCs and responsible for regulation of IL-17 production.

Discussion

BM-derived adherent cells represent a morphologically and phenotypically heterogeneous cell population. Especially in the mouse, a significant proportion of in vitro grown adherent BM cells express markers of hematopoietic precursors. To enrich MSCs in cultures of BM-derived cells, we performed depletion of CD11b⁺ and CD45⁺ cells by magnetic separation. The residual population of MSCs was characterized by a uniform spindle-shaped morphology, by the absence of CD11b⁺ and CD45⁺ cells and by expression of phenotypic markers associated with murine MSCs. These purified MSCs had the ability to specifically differentiate into adipocytes and osteoblasts and effectively inhibit T-cell proliferation, as it has been described for MSCs [6,29].

The purified MSCs spontaneously produced TGF- β , but without stimulation they produce no IL-6, IL-1, IL-4, IL-10, IFN- γ , or NO. After stimulation with proinflammatory cytokines (IL-1 α , IL-2, IFN- γ , and TNF- α) or LPS, these MSCs secreted significant levels of IL-6, but not other tested cytokines (ie, IL-1 β , IL-4, IL-10, IL-12, or IFN- γ). Anti-inflammatory cytokines TGF- β and IL-10 did not induce IL-6

FIG. 4. Regulation of regulatory T-cell and T helper 17 cells development by supernatants from MSCs. **(A)** Spleen cells from BALB/c mice were cultured for 96 h unstimulated (–), stimulated with irradiated allogeneic cells (B6), or were stimulated with allogeneic cells in the presence of supernatants (30% of a total volume) from MSCs. For preparation of supernatants, MSCs were cultured untreated (unst.) or were preincubated with IL-1 α , IL-2, IL-10, TGF- β , or LPS. Each bar represents the mean $\pm$ SD from 4 to 5 determinations. Values with asterisks are significantly different (* P < 0.05, ** P < 0.01) from the control values in cultures without MSC supernatants. **(B)** The MLR BALB/c anti-B6 was set up, and purified CD4⁺CD25[–] cells that were preincubated for 96 h with MSC supernatants were added into this culture. The reactive cells were cultured unstimulated (–) or were stimulated with irradiated allogeneic cells (B6). The purified CD4⁺CD25[–] cells were preincubated for 96 h unstimulated (a), stimulated with irradiated B6 cells (b), or were stimulated with B6 cells in the presence of supernatants from untreated MSCs (c) or from MSCs which were pretreated with TGF- β (d) or IL-1 α (e). Preincubated cells were added to MLR at a ratio of 4 reactive cells to 1 preincubated cell, and cell proliferation was determined. Values with asterisks are significantly different (* P < 0.05, ** P < 0.01) from the control values (cultures without preincubated cells). Each bar represents the mean $\pm$ SD from 3 independent experiments. MLR, mixed leukocyte reaction.

production, but enhanced production of TGF- β . This cytokine production profile of purified mouse MSCs is similar to that described by Oh et al. [15] for human MSCs. Human MSCs produced spontaneously TGF- β , but not IL-6. IL-6 was induced by co-culture of MSCs with peripheral blood

mononuclear cells, but other tested cytokines (IL-10, TNF- α , and IFN- γ) were not produced by human MSCs [15]. Similarly, increased expression of the gene for IL-6 and for some other growth factors was detected in human MSCs during culture with peripheral blood leukocytes [30]. Our results have shown that the production of TGF- β and IL-6 by purified MSCs is strictly regulated by cytokine environment.

It has been demonstrated in various models that TGF- β induces Foxp3 expression and plays a crucial role in the development of Tregs, whereas a combination of TGF- β and IL-6 leads to the activation of ROR γ t⁺ Th17 cells and production of IL-17 [19–21]. To test the effects of MSCs and their supernatants on the differentiation of naive T-cells, we used a model of alloantigen-stimulated spleen cells. We confirmed that the production of IL-17 and expression of transcription factors Foxp3 and ROR γ t are reciprocally regulated by TGF- β and IL-6. Exogenous TGF- β added to the cultures of alloantigen-stimulated spleen cells induced Foxp3 expression, supported development of Tregs, and inhibited IL-17 production. On the contrary, the combination of TGF- β and IL-6 induced ROR γ t expression and enhanced production of IL-17. The addition of supernatants from untreated MSCs or from MSCs preincubated with TGF- β to the cultures of alloantigen-stimulated spleen cells induced Foxp3 expression and activation of Tregs, and resulted in an inhibition of IL-17 production. This inhibition of IL-17 production may be due to a negative effect of TGF- β on the development of Th17 cells and due to the effects of Tregs that are activated in the presence of TGF- β [21,27]. Kimura et al. [31] showed that IL-6 inhibits the activation of Foxp3 expression and supports expression of ROR γ t, which is a crucial factor responsible for the development of Th17 cells. An alternative explanation for the effects of TGF- β and other cytokines on the IL-17 expression in human naive T cells was presented by Manel et al. [32]. These authors showed that TGF- β upregulated ROR γ t expression, but at the same time inhibited its ability to induce IL-17 production. This inhibition was relieved by proinflammatory cytokines [32]. In accordance with the above observations, the supernatants from MSCs stimulated by proinflammatory cytokines induced lower levels of Foxp3 than supernatants from unstimulated MSCs, supported ROR γ t expression, and enhanced IL-17 production. Thus, MSCs and their supernatants can suppress or enhance Treg or Th17 cell development according to their activation status and their cytokine production. These observations are in agreement with the immunosuppressive properties of MSCs described in numerous models [1–4,6], but can also explain immunostimulatory effects of MSCs observed in other cases [9–11].

It has been shown that the immunomodulatory effects of MSCs are dose-dependent. At high ratios of MSCs to lymphocytes prevails suppression of lymphocyte proliferation, while lower concentrations of MSCs rather enhance cell proliferation [3,6]. Similarly, the switch between Foxp3 and ROR γ t expression is dependent on the concentrations of TGF- β and IL-6 [21]. Since the immunomodulatory effects of MSCs are mediated by factors present in MSC supernatants, the variable concentrations of TGF- β and IL-6 could explain these effects. We observed that TGF- β used at a wide range of concentrations (0.2–10 ng/mL) enhanced Foxp3 expression in alloantigen-activated T-cells, and that low concentrations of IL-6 (<0.2 ng/mL) can redirect development of TGF- β -induced Foxp3⁺ cells into IL-17-producing cells (data

FIG. 5. Effects of MSC supernatants on Foxp3 and RORγt expression. The supernatants were prepared by a 72-h incubation of MSCs that were untreated or were preincubated with IL-1α or TGF-β, and were added to the cultures of BALB/c spleen cells stimulated with irradiated B6 cells. The percentage of CD4⁺Foxp3⁺ (A, B) or CD4⁺RORγt⁺ (C, D) cells was determined by flow cytometry after a 96-h incubation. A and C show representative flow cytometry dot plots of (a) unstimulated spleen cells, (b) spleen cells stimulated with alloantigens, (c) spleen cells stimulated with alloantigens in the presence of supernatant from untreated MSCs, (d) supernatants from MSCs pretreated with IL-1α, or (e) supernatants from MSCs pretreated with TGF-β. B and D show the mean ± SD of CD4⁺Foxp3⁺ or CD4⁺RORγt⁺ cells from 6 independent determinations. Asterisks indicate a significant difference (**P* < 0.05) from the control values (cultures without supernatants).

FIG. 6. Effect of neutralization mAb anti-TGF-β or anti-IL-6 on the ability of MSC supernatants to modulate IL-17 production. The supernatants containing TGF-β or TGF-β together with IL-6 were prepared by a 72-h incubation of untreated MSCs (unst.) or MSCs preincubated with IL-1α. These supernatants were added to the cultures of BALB/c spleen cells that were stimulated with irradiated B6 cells. Neutralization mAb anti-TGF-β or anti-IL-6 were added to these cultures at a concentration of 2 μg/mL. The levels of IL-17 in the culture supernatants were determined after a 96-h incubation by ELISA. Each bar represents the mean ± SD from 4 determinations. Asterisks indicate statistically significant difference (**P* < 0.05, ***P* < 0.01).

not shown). However, the conditions in vivo, where cell-cell contact, membrane-bound cytokine molecules, and distinct local cytokine concentrations can be involved, may be quite different from those in vitro, and the immunoregulatory role of MSCs must be carefully controlled. Using neutralization mAb anti-TGF-β and anti-IL-6, we showed that TGF-β and IL-6 present in MSC supernatants are the main cytokines responsible for the reciprocal regulation of Treg or Th17 cell development by MSCs. Similarly, Liu et al. [16] showed that rat MSCs can inhibit or stimulate proliferation of myelin basic protein-specific T lymphocytes in a dose-dependent manner and that cytokines produced by MSCs are responsible for these immunomodulatory effects.

So far, various mechanisms have been proposed to explain the immunomodulatory effects of MSCs. Different cytokines and factors produced by MSCs were suggested as molecules responsible for MSC-mediated suppression [6,12,13,23]. Sato et al. [33] proposed that NO produced by MSCs plays a critical role in suppression of T-cell proliferation. A possibility that MSCs activate Tregs has also been suggested [14,22,34]. Our results have shown that TGF-β and IL-6 are the principal molecules responsible for the ability of MSCs to reciprocally activate or inhibit the development and functions of Tregs and Th17 cells.

The ability of MSCs to inhibit T-cell-mediated immune reactions has been used to prevent or treat autoimmune diseases [35,36], to suppress transplantation reactions [2,7,14,37], or to attenuate GVHD in humans and mice [38,39]. However, the results remain variable and the suppression was not achieved in all models [40,41]. The

suppression could be more pronounced if MSCs are modified with cytokines [36,40] or the treatment is combined with immunosuppressive drugs [37,41]. However, some discrepancies in efficiency of cytokine-treated MSCs still exist among individual models. While in our *in vitro* experiments IFN- γ -pretreated mouse MSCs produced TGF- β and IL-6, which stimulate IL-17 production, Polchert et al. [39] observed that IFN- γ -treated MSCs were more effective in the prevention of graft-versus-host reaction *in vivo*. These differences might reflect different models used and/or the involvement of distinct cell populations in proinflammatory reactions and in the graft-versus-host reaction. Altogether, the data indicate that the ongoing immune response and cytokine environment may significantly modify the effectiveness of MSC treatment.

It has recently been shown that human MSCs inhibit effector functions of Th17 cells and suppress IL-17 production [42]. Our results demonstrate that MSCs and their supernatants also inhibit the differentiation and development of Th17 cells from naive CD4⁺CD25⁻ precursors. Thus, MSCs inhibit proinflammatory Th17 cells on both the level of their activation and their effector function. These observations may explain the immunosuppressive effects of MSCs observed in models, such as transplantation or autoimmune reactions, where Th17 cells play an important role. However, the immunosuppressive function of MSCs can be reversed by IL-6 and, in some situations, MSCs can induce an extensive expansion of T-cell clones [30]. These interplays between MSCs, Tregs, and Th17 cells have to be taken into account when MSCs are used to treat autoimmune, immunopathological, or transplantation reactions.

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Author Disclosure Statement

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References

- Di Nicola M, C Carlo-Stella, M Magni, M Milanese, PD Longoni, P Matteucci, S Grisanti and AM Gianni. (2002). Human bone marrow stroma cells suppress T-lymphocyte proliferation induced by cellular and nonspecific mitogenic stimuli. *Blood* 99:3838–3843.
- Bartholomew A, C Sturgeon, M Siatskas, K Ferrer, K McIntosh, S Patil, W Hardy, S Devine, D Ucker, R Deans, A Moseley and R Hoffman. (2002). Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 30:42–48.
- Le Blanc K, L Tammik, B Sundberg, SE Haynesworth and O Ringdén. (2003). Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* 57:11–20.
- Angoulvant D, A Clerc, S Benchalal, C Galambrun, A Farre, Y Bertrand and A Eljaafari. (2004). Human mesenchymal stem cells suppress induction of cytotoxic response to alloantigens. *Biorheology* 41:469–476.
- Djouad F, P Plence, C Bony, P Tropel, F Apparailly, J Sany, D Noël and C Jorgensen. (2003). Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 102:3837–3844.
- Le Blanc K and O Ringdén. (2007). Immunomodulation by mesenchymal stem cells and clinical experience. *J Intern Med* 262:509–525.
- Casiraghi F, N Azzollini, P Cassis, B Imberti, M Morigi, D Cugini, RA Cavinato, M Todeschini, S Solini, A Sonzogni, N Perico, G Remuzzi and M Noris. (2008). Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. *J Immunol* 181:3933–3946.
- Parekkadan B, AW Tilles and ML Yarmush. (2008). Bone marrow-derived mesenchymal stem cells ameliorate autoimmune enteropathy independently of regulatory T cells. *Stem Cells* 26:1913–1919.
- Fang L, C Lange, M Engel, AR Zander and B Febse. (2006). Sensitive balance of suppressing and activating effects of mesenchymal stem cells on T-cell proliferation. *Transplantation* 82:1370–1373.
- Bocelli-Tyndall C, L Bracci, S Schaeren, C Feder-Mengus, A Barbero, A Tyndall and GC Spagnoli. (2009). Human bone marrow stem cells and chondrocytes promote and/or suppress the *in vitro* proliferation of lymphocytes stimulated with the cytokines IL 2, IL 7 and IL 15. *Ann Rheum Dis* 8:1352–1359.
- Le Blanc K. (2009). Immunomodulatory effects of fetal and adult mesenchymal stem cells. *Cytotherapy* 5:485–489.
- Rasmusson I, O Ringdén, B Sundberg and K Le Blanc. (2005). Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. *Exp Cell Res* 305:33–41.
- Caplan AI. (2009). Why are MSC therapeutic? New data: new insight. *J Pathol* 217:318–324.
- Ge W, J Jiang, J Arp, W Liu, B Garcia and H Wang. (2010). Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation* 90:1312–1320.
- Oh JY, MK Kim, MS Shin, WR Wee and JH Lee. (2009). Cytokine secretion by human mesenchymal stem cells cocultured with damaged corneal epithelial cells. *Cytokine* 46:100–103.
- Liu X-J, J-F Zhang, B Sun, H-S Peng, Q-F Kong, S-S Bai, Y-M Liu, G-Y Wang, J-H Wang and H-L Li. (2009). Reciprocal effects of mesenchymal stem cells on experimental autoimmune encephalomyelitis is mediated by transforming growth factor- β and interleukin-6. *Clin Exp Immunol* 158:37–44.
- Bettelli E, Y Carrier, W Gao, T Korn, TN Strom, M Gukka, HL Wiener and VK Kuchroo. (2006). Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235–238.
- Weaver CT, RD Hatton, PR Mangan and IE Harrington. (2007). IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25:821–852.
- Zhou L, II Ivanov, R Spolski, R Min, K Shenderov, T Egawa, DE Levy, WJ Leonard and DR Littman. (2007). IL-6 programs TH17 cell differentiation by promoting sequential

- engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 8:967–974.
20. Ichiyama K, H Yoshida, Y Wakayabashi, T Chinen, K Saeki, M Nakaya, G Takaesu, S Hori, A Yoshimura and T Kobayashi. (2008). Foxp3 inhibits ROR γ t-mediated IL-17A mRNA transcription through direct interaction with ROR γ t. *J Biol Sci* 283:17003–17008.
 21. Zhou L, JE Lopes, MMW Chong, II Ivanov, R Min, GD Victora, Y Shen, J Du, YP Rubtsov, AY Rudensky, SF Ziegler and DR Littman. (2008). TGF- β -induced Foxp3 inhibits T_H17 cell differentiation by antagonizing ROR γ t function. *Nature* 453:236–241.
 22. Aggarwal S and MF Pittenger. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 105:1815–1822.
 23. Meisel R, A Zibert, M Laryea, U Gobel, W Daubener and D Dilloo. (2004). Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 103:4619–4621.
 24. Tropel P, D Noël, N Platet, P Legrand, A-L Benabid and F Berger. (2004). Isolation and characterization of mesenchymal stem cells from adult mouse bone marrow. *Exp Cell Res* 295:395–406.
 25. Tomonari K. (1988). A rat antibody against a structure functionally related to the mouse T-cell receptor/T3 complex. *Immunogenetics* 28:455–458.
 26. Green LC, DA Wagner, J Glogowski, PL Skipper, JS Wishnok and SR Tannenbaum. (1982). Analysis of nitrate, nitrite, and [¹⁵N] nitrate in biological fluids. *Anal Biochem* 126:131–138.
 27. Prochazkova J, J Fric, K Pokorna, A Neuwirth, M Krulova, A Zajicova and V Holan. (2009). Distinct regulatory roles of TGF- β and IL-4 in development and maintenance of natural and induced CD4⁺CD25⁺Foxp3⁺ regulatory T cells. *Immunology* 128:670–678.
 28. Holan V, K Pokorna, J Prochazkova, M Krulova and A Zajicova. (2010). Immunoregulatory properties of mouse limbal stem cells. *J Immunol* 184:2124–2129.
 29. Zajicova A, K Pokorna, A Lencova, M Krulova, E Svobodova, S Kubinova, E Sykova, M Pradny, J Michalek, J Svobodova, M Munzarova and V Holan. (2010). Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. *Cell Transplant* 19:1281–1290.
 30. Crop MJ, CC Baan, SS Korevaar, JNM Ijzermans, W Weimar and MJ Hoogduijn. (2010). Human adipose tissue-derived mesenchymal stem cells induce explosive T-cell proliferation. *Stem Cells Dev* 19:1843–1853.
 31. Kimura A, T Naka and T Kishimoto. (2007). IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc Natl Acad Sci USA* 104:12099–12104.
 32. Manel N, D Unutmaz and DR Littman (2008). The differentiation of human T_H17 cells requires transforming growth factor- β and induction of the nuclear receptor ROR γ t. *Nat Immunol* 9:641–649.
 33. Sato K, K Ozaki, I Oh, A Meguro, K Hatanaka, T Nagai, K Muroi and K Ozawa. (2007). Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* 109:228–234.
 34. Maccario R, M Podesta, A Moretta, A Cometa, P Comoli, D Montagna, L Daut, A Ibatici, G Piaggio, S Pozzi, F Frassoni and F Locatelli. (2005). Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4⁺ T-cell subsets expressing a regulatory/suppressive phenotype. *Haematologica* 90:516–525.
 35. Rafei M, PM Campeau, A Aguilar-Mahecha, M Buchanan, P Williams, E Birman, S Yuan, YK Young, MN Boivin, K Forner, M Basik and J Galipeau. (2009). Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17 T cells in a CC chemokine ligand 2-dependent manner. *J Immunol* 182:5994–6002.
 36. Choi JJ, SA Yoo, SJ Park, YJ Kang, WU Kim, IH Oh and CS Cho. (2008). Mesenchymal stem cells overexpressing interleukin-10 attenuate collagen-induced arthritis in mice. *Clin Exp Immunol* 153:269–276.
 37. Ge W, J Jiang, ML Baroja, J Arp, R Zassoko, W Liu, A Bartholomew, B Garcia and H Wang. (2009). Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance. *Am J Transplant* 9:1760–1772.
 38. Le Blanc K, I Rasmusson, B Sundberg, C Götherström, M Hassan, M Uzunel and O Ringdén. (2004). Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 363:1439–1441.
 39. Polchert D, J Sobinsky, GW Douglas, M Kidd, A Moadsiri, E Reina, K Genrich, S Mehrotra, S Setty, B Smith and A Bartholomew. (2008). IFN- γ activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. *Eur J Immunol* 38:1745–1755.
 40. Sudres M, F Norol, A Trenado, S Grégoire, F Charlotte, B Levacher, JJ Lataillade, P Bourin, X Holy, JP Vernant, D Klatzmann and JL Cohen. (2006). Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J Immunol* 176:7761–7767.
 41. Sbano P, A Cuccia, B Mazzanti, S Urbani, B Giusti, I Lapini, L Rossi, R Abbate, G Marseglia, G Nannetti, F Torricelli, C Miracco, A Bosi, M Fimiani and R Saccardi. (2008). Use of donor bone marrow mesenchymal stem cells for treatment of skin allograft rejection in a preclinical rat model. *Arch Dermatol Res* 300:115–124.
 42. Ghanman S, J Pene, G Torcy-Moquet, C Jorgensen and H Yssel. (2010). Mesenchymal stem cells inhibit human Th17 cell differentiation and function and induce a T regulatory cell phenotype. *J Immunol* 185:302–312.

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