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ORIGINAL ARTICLE

# TNFα-induced M-MDSCs promote transplant immune tolerance via nitric oxide

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**Abstract**
Efficient induction of functional competent myeloid-derived suppressor cells (MDSCs) will be critical for the clinical application of MDSCs to treat autoimmune diseases and to induce transplantation immune tolerance. In the present study, we tried to establish the MDSC induction system with M-CSF and tumor necrosis factor α (TNFα) and investigated the immunosuppressive function of M-CSF + TNFα-induced MDSCs in transplant mouse models. Monocytic MDSCs (M-MDSCs) were induced by culture of the non-adherent mouse bone marrow cells with M-CSF or M-CSF + TNFα, respectively, for 7 days. Phenotype analysis revealed that the majority of M-CSF- and M-CSF + TNFα-induced MDSCs express F4/80. The addition of TNFα in the induction period increased Gr-1, Ly6C, CD80, and CD274 expressions on these cells. M-CSF + TNFα-induced M-MDSCs showed poor TNFα, IL-12, and IL-6 expressions after lipopolysaccharide (LPS) stimulation and decreased arginase 1 (Arg-1) and Fizz expressions after IL-4 stimulation compared with M-CSF-induced M-MDSCs. M-CSF + TNFα-induced M-MDSCs showed enhanced ability to suppress T cell proliferation and cytokine production than M-CSF-induced M-MDSCs. M-CSF + TNFα-induced M-MDSCs express high levels of inducing nitric oxide synthase (iNOS) and blocking iNOS activity by a chemical inhibitor or gene deficiency significantly reversed the inhibitory effects of M-CSF + TNFα-induced M-MDSCs on T cells. Adoptive transfer of M-CSF + TNFα-induced M-MDSCs promoted immune tolerance in a male-to-female skin-grafted mice, but M-CSF + TNFα-induced iNOS-deficient M-MDSCs failed to do so. Thus, M-CSF + TNFα-induced M-MDSCs have powerful immunosuppressive activity, which is mediated by an iNOS-dependent pathway. M-CSF + TNFα-induced M-MDSCs can promote immune tolerance to donor antigens in a transplant mouse model.

**Key message**

- The combination of M-CSF and TNFα efficiently induces functional M-MDSCs in vitro.
- M-CSF + TNFα-induced M-MDSCs promote immune tolerance in a transplant mouse model.
- The immunosuppressive ability of M-CSF + TNFα-induced M-MDSCs is dependent on iNOS.

**Keywords** Myeloid-derived suppressor cells · Immune tolerance · Nitric oxide · Transplantation

Fan Yang, Yang Li and Tingting Wu contributed equally to this work.

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## Introduction

Myeloid-derived suppressor cells (MDSCs) were first reported in the 1970s as natural suppressor cells with the ability to suppress T cell proliferation and function [1]. In the early reports, MDSCs can be induced by Bacillus Calmette-Guerin- or total body irradiation-induced systemic inflammation [2, 3], and most commonly in different tumor models [4, 5]. The phenotypic markers for MDSCs were CD11b (also known as integrin-αM) and granulocyte differentiation antigen-1 (Gr-1). MDSCs can be divided into two major subpopulations, monocytic MDSCs (M-MDSCs) with $CD11b^{+}Ly6C^{high}Ly6G^{-}$ phenotype and granulocytic MDSCs (G-MDSCs) with $CD11b^{+}Ly6C^{low}Ly6G^{+}$ phenotype, respectively [6, 7]. These two MDSC subsets used different mechanisms to suppress T cell function [8]. M-MDSCs are considered to be more immunosuppressive, have long life-span, and can differentiate into mature macrophages [9]. The immunosuppressive mechanism of M-MDSCs were mainly through inducing nitric oxide synthase (iNOS), arginase 1 (Arg-1), or indoleamine 2, 3-dioxygenase 1 (IDO) expression which can consume the essential nutrients for T cell activation or through secreting large amounts of nitric oxide (NO) to disrupt the Jak3/stat5 signaling in T cells [10–13].

MDSCs are closely involved in the prevention of graft rejection and can be induced in vitro for preventing allograft rejection [7, 14]. MDSCs can be induced from bone marrow progenitors in vitro by granulocyte macrophage colony stimulating factor (GM-CSF) at high concentrations for 4 days or low for 8–10 days [15]. MDSCs induced by GM-CSF and IL-6 from bone marrow cells significantly prolonged the survival of islet allografts [16]. The in vitro-induced MDSCs by GM-CSF, G-CSF, and IL-13 can prevent the graft versus host disease [17]. Except for GM-CSF, macrophage colony stimulating factor (M-CSF) was also an effective cytokine for bone marrow progenitor differentiation into immunosuppressive M-MDSCs [10]. Hematopoietic stem cells cultured with a cocktail of cytokines like IL-3, IL-6, c-kit ligand, TPO, FLT3L, VEGF, and M-CSF induced the differentiation of MDSCs capable of inhibiting T cell activation in vitro and in a mouse model of graft versus host disease [18]. It has been reported that tumor necrosis factor α (TNFα) was required for MDSCs to gain immunosuppressive function in chronic inflammation [19]. Thus, we proposed that co-application of TNFα with M-CSF should promote differentiation of M-MDSCs with enhanced immunosuppressive ability, which could be potentially used for preventing graft rejection and transplantation immune tolerance induction in the future.

## Materials and methods

**Mice** BALB/c, C57BL/6 (B6), and $CD45.1^{+}$ B6 mice were purchased from the Beijing Laboratory Animal Research Center (Beijing, China). iNOS knockout (KO) mice were kindly provided by the Key Laboratory of Human Diseases Comparative Medicine, the Ministry of Public Health (Beijing, China). Mice were used between 6 and 12 weeks of age. Experimental protocols were approved by the Animal Ethics Committee of the Institute of Zoology (Beijing, China).

**Cell preparation and culture** Tibias and femurs from BALB/c or B6 mice were removed using sterile techniques, and bone marrow was flushed with PBS. Red blood cells were lysed with ammonium chloride. Total bone marrow cells were planted into 100-mm dishes (Corning, USA) for 2 h, and the non-adherent cells were collected to exclude macrophages [20]. For MDSCs induction, $1.5 \times 10^6$ non-adherent bone marrow cells were cultured with 50 ng/ml M-CSF or 50 ng/ml M-CSF + 100 ng/ml TNFα, respectively, in dishes with 60-mm-diameter dish (Corning, USA) in 4 ml of complete RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 μM 2-ME, 150 U/ml streptomycin, 200 U/ml penicillin, and 10 % heat-inactivated FBS for 7 days at 37 °C, 5 % $CO_2$. Half of the medium was changed on day 5. Bone marrow-derived macrophages (BMDMs) were induced from bone marrow cells as previously described [21]. Bone marrow-derived dendritic cells (BMDCs) were induced in 60-mm-diameter dish with 4 ml of complete RPMI 1640 medium seeding $10 \times 10^6$ non-adherent bone marrow cells with 20 ng/ml GM-CSF. The culture medium was entirely discarded at days 3 and 5, replaced by fresh medium with GM-CSF (20 ng/ml). On day 6, cells were stimulated with 1 μg/ml lipopolysaccharide (LPS) for 24 h to induce DC maturation. Cells in the culture supernatant and loosely adherent cells were harvested by gentle washing with PBS and were used for subsequent experiments.

For T cell proliferation assay, single cell suspension was prepared from the spleen as described previously [22]. Cells were labeled with 2 μM CFSE for 5 min in PBS at 37 °C and washed twice with RPMI 1640. The labeled cells were then cultured with ConA in the presence of different doses of MDSCs as indicated. The cell proliferation was determined by a flow cytometry (FCM) after staining with anti-CD4 or anti-CD8 mAb, respectively. For the mixed lymphocyte reaction (MLR), $2 \times 10^5$ CFSE-labeled lymph node cells were co-cultured with $2 \times 10^5$ allogeneic BMDCs for 5 days in 200 μl complete RPMI 1640 medium in 96-well round bottom plates. MDSCs were added at different ratios for suppressing alloreactive T cell proliferation.

**Abs and reagents** Anti-mCD11b-PE-Cy5, anti-mF4/80-PE, anti-mGr1-PE, anti-mLy6C-FITC, anti-mCD11c-PE, anti-mCD86-FITC, anti-mCD80-PE, anti-mI-Ab-PE, anti-mCD120b-PE, anti-mCD115-PE, anti-mCD124-PE, anti-mCD274-PE, anti-mCD31-PE, and anti-mTNF-α-PE were purchased from BD Biosciences Pharmingen (San Diego,

CA). Anti-CD4-FITC, anti-mCD4-PE, anti-mCD8-PE-Cy5, and anti-mLy6G-PE were purchased from eBioscience (San Diego, CA). Anti-mCD120a-PE, anti-mIL-12p40-PE, anti-mCD45.1-PE, and anti-mIL-6-PE were purchased from Biolegend (San Diego, CA). Bacterial LPS (*Escherichia coli* 055:B5) was purchased from Sigma-Aldrich. Recombinant mouse IL-4 and TNFα were purchased from PeproTech (Rocky Hill, NJ).

**Quantitative PCR** The inflammatory response of MDSCs was induced by LPS (100 ng/ml) at different time points. The type 2 macrophage (M2) response of MDSCs was induced by IL-4 (1000 U/ml) treatment for 0, 24, and 48 h [21]. Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) and reverse transcription was performed with M-MLV superscript reverse transcriptase according to the manufacturer's instructions. Real-time PCR was performed using multiple kits (SYBR Premix Ex TaqTM, DRR041A, Takara Bio) on CFX96 (Bio-Rad). The primers used in the present study are listed in Table 1. To determine the relative induction of cytokine messenger RNA (mRNA) in response to various stimuli, the mRNA expression levels of each gene were normalized to the expression level of the housekeeping gene hypoxanthine phosphoribosyl transferase.

**Table 1** Primers used for qRT–PCR analysis

| Genes | | Primer sequence (5'–3') |
|---|---|---|
| HPRT | Forward primer: | AGTACAGCCCCAAAATGGTTAAG |
| | Reverse primer: | CTTAGGCTTTGTATTTGGCTTTTC |
| TNFα | Forward primer: | GAGTGACAAGCCTGTAGCC |
| | Reverse primer: | CTCCTGGTATGAGATAGCAAA |
| IL-1β | Forward primer: | TGGGAAACAACAGTGGTCAGG |
| | Reverse primer: | CCATCAGAGGCAAGGAGGAA |
| IL-6 | Forward primer: | AACCGCTATGAAGTTCCTCTC |
| | Reverse primer: | AATTAAGCCTCCGACTTGTGAA |
| IL-12b | Forward primer: | CACGGCAGCAGAATAAATA |
| | Reverse primer: | CTTGAGGGAGAAGTAGGAATG |
| Fizz1 | Forward primer: | CTGCCCTGCTGGGATGACT |
| | Reverse primer: | CATCATATCAAAGCTGGGTTCTCC |
| Ym1 | Forward primer: | CAAGTTGAAGGCTCAGTGGCTC |
| | Reverse primer: | CAAATCATTGTGTAAAGCTCCTCTC |
| Arg-1 | Forward primer: | CCAGAAGAATGGAAGAGTCAGTGT |
| | Reverse primer: | GCAGATATGCAGGGAGTCACC |
| iNOS | Forward primer: | CACCAAGCTGAACTTGAGCG |
| | Reverse primer: | CGTGGCTTTGGGCTCCTC |
| COX2 | Forward primer: | CCTTCTCCAACCTCTCCTACT |
| | Reverse primer: | ACCTTTTCCAGCACTTCTTTTG |
| IDO1 | Forward primer: | CAATCAAAGCAATCCCCACTG |
| | Reverse primer: | AAAACGTGTCTGGGTCCAC |

*HPRT* hypoxanthine phosphoribosyl transferase; *TNFα* tumor necrosis factor alpha; *IL* interleukin; *iNOS* inducible nitric oxide synthase; *IDO* indoleamine-2, 3-dioxygenase-1; *COX2* cyclooxygenase 2; *qRT–PCR* quantitative PCR

**Cell staining and FCM** For surface marker staining, the induced MDSCs or ConA-stimulated splenocytes were incubated with the appropriate antibody as described previously [22]. For intracellular cytokine staining, stimulated splenocytes were collected and re-stimulated for 6 h with PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (750 ng/ml; Calbiochem) in the presence of Golgi Plug at the recommended concentrations (BD Pharmingen). Cells were first incubated with anti-CD4-FITC, and anti-mCD8-PE-Cy5 for cell surface staining. Then, after wash, they were fixed and permeabilized with Cytofix/Cytoperm solution (BD Pharmingen); anti-mIFN-γ-PE (XMG1.2) or anti-mIL-2-PE (JES6-5H4) mAb were used for intracellular staining [23]. Samples were analyzed on a Beckman Coulter Epics XL benchtop FCM (Beckman Coulter) with FCS express software (De Novo Software, Thornhill, Canada).

**NO production assay** After incubating equal volumes of culture supernatants (100 μl) with Greiss reagent (1 % sulfanilamide in 5 % phosphoric acid and 0.1 % N-1-naphthylethylenediamine dihydrochloride in double-distilled water) at room temperature for 10 min, the absorbance at 550 nm was measured using a microplate reader (Bio-Rad) [21]. Nitrite concentrations were determined by comparing the absorbance values for the test samples to a standard curve generated by serial dilution of 0.25 mM sodium nitrite.

**Arginase assay** The arginase activity assay was performed as previously described [24]. Briefly, the cells were lysed in 100 μl 0.1 % Triton X-100. After 30 min on a shaker, 100 μl 25 mM Tris–HCl was added to the cell lysates at a final concentration of 12.5 mM, and 20 μl 10 mM $MnCl_2$ was added to obtain a 1-mM final concentration. The arginase was activated by heating for 10 min at 56 °C, and 100 μl of 0.5 M L-arginine (pH 9.7) substrate was added at a final concentration of 250 mM. The reactions were incubated at 37 °C for 30 min and stopped with 800 μl $H_2SO_4$(96 %)/$H_3PO_4$(85 %)/$H_2O$ (1/3/7, *v*/*v*/*v*). After the addition of a-isonitrosopropiophenone (dissolved in 100 % ethanol) and heating for 30 min at 95 °C, the urea production was measured as the absorbance at 540 nm, and the data were normalized to the total protein content. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μM of urea per min.

**Skin transplantation and histological examination** Recipient B6 mice were injected with $5 \times 10^6$ M-CSF- or M-CSF + TNFα-induced M-MDSCs intravenously on day 1. The skin grafting was performed on day 0. For acute rejection, full-thickness tail skin tissue of BALB/c mice was placed on

the dorsal part of the B6 recipients, according to the procedure as described previously [22]. Skin graft survival was followed by daily visual inspection for acute rejection. For chronic rejection, male B6 tail skin was placed on the dorsal part of the female B6 recipients. Chronic skin graft rejection was followed once every 2 days. Grafts were defined as rejected when <10 % of the graft remained viable.

**Statistical analysis** All data are presented as the mean ± SD. A two-tailed unpaired Student's *t* test was used to compare two different groups. One-way or two-way ANOVA analysis was used for comparison among multiple groups with GraphPad Prism software (GraphPad Software, La Jolla, CA) according to the type of data. *P* values for comparison between graft survivals were determined using Log-rank tests. A *P* value less than 0.05 was considered to be statistically significant.

## Results

### TNFα altered the phenotype and inflammatory ability of M-CSF-induced M-MDSCs

To understand whether the presence of inflammatory cytokine TNFα during myeloid cell development alters the phenotype of the induced M-MDSCs, we determined the cell surface markers including F4/80, Gr-1, Ly6C, Ly6G, CD11c, CD86, CD80, I-Ab, CD115, CD124, CD274, and CD31 by flow cytometry after bone marrow cells were cultured with M-CSF alone or M-CSF + TNFα for 7 days using BMDMs and BMDCs as control. As shown in Fig. 1, almost all MDSCs induced by M-CSF alone or M-CSF + TNFα expressed CD11b and F4/80 molecules but did not express Ly6G and CD11c, indicating these induced cells display monocytic phenotype. Comparing with BMDMs and BMDCs, the

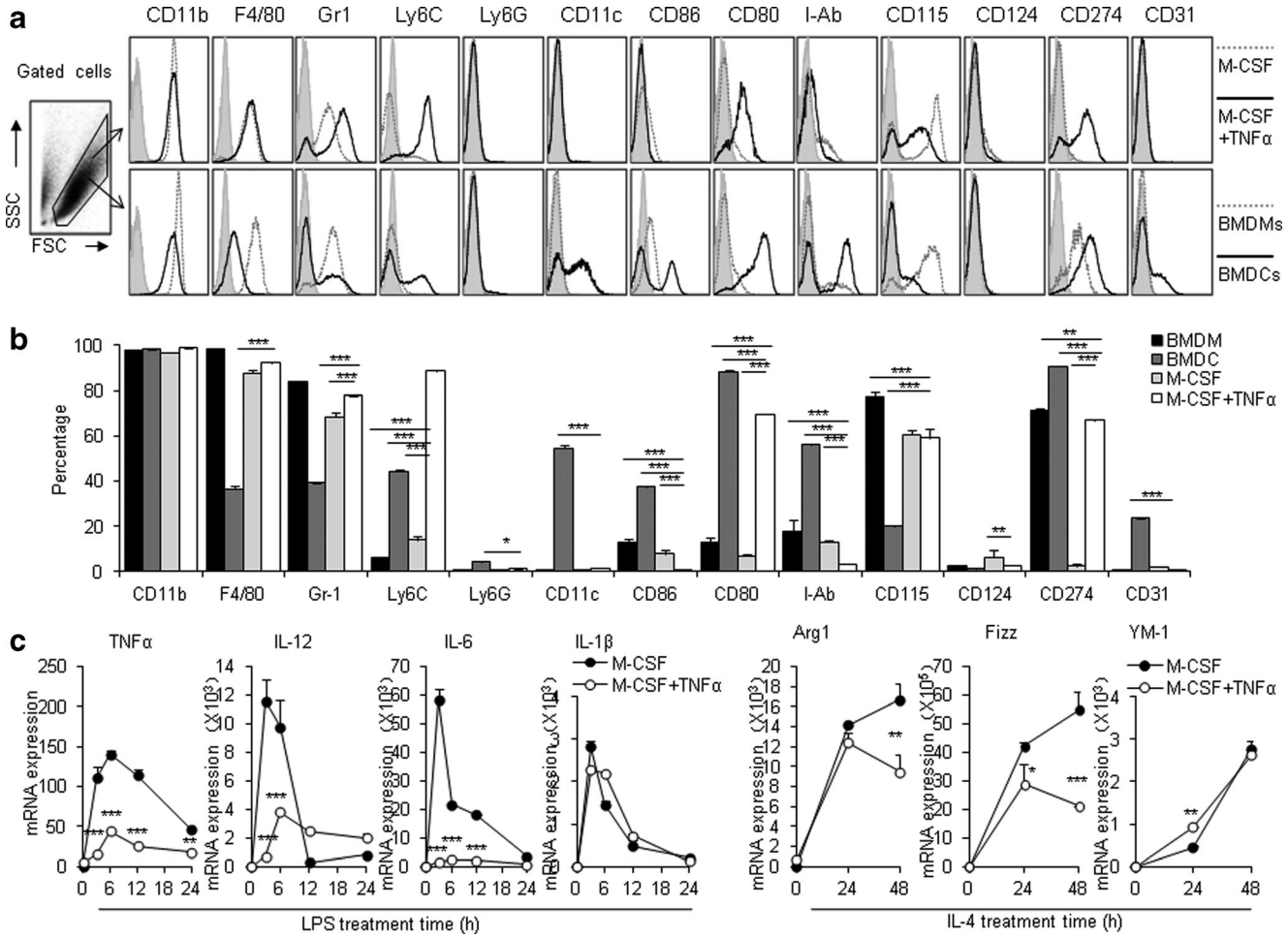


**Fig. 1** The phenotypes and inflammatory characteristics of M-CSF- and M-CSF + TNFα-induced MDSCs. **a** Bone marrow cells were cultured in M-CSF or M-CSF + TNFα as described in "Materials and Methods" for 7 days. The cells were then stained with PE, FITC, or PE-cy5-labeled anti-F4/80, CD11b, CD11c, Gr-1, Ly6C, Ly6G, I-Ab, CD80, CD86, CD31, CD115, CD124, or CD274 mAb. $5 \times 10^5$ induced cells were analyzed by FCM. **b** The percentages of the indicated molecules were summarized. **c** M-CSF- or M-CSF + TNFα-induced MDSCs were stimulated with either LPS (100 ng/m) or IL-4 (1000 U/ml) for the indicated periods, and the mRNA expression of TNFα, IL-12, IL-6, IL-1β, Arg1, YM-1, and Fizz was determined by real-time PCR. Data are shown as mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared between the indicated groups

phenotypes of induced M-MDSCs were closer to BMDMs, including the expression of CD115, Gr-1, and CD124 in addition to F4/80. TNFα treatment during induction increased the expressions of Ly6C and CD274 meanwhile decreased I-Ab and CD86 expressions compared with M-CSF-induced MDSCs ($P<0.001$, Fig. 1a, b). Less cells were harvested when MDSCs were induced by M-CSF + TNFα compared with MDSCs induced by M-CSF alone, suggesting that TNFα may affect cell viability and/or cell proliferation during induction period (Suppl. Fig. 1a). The expressions of TNF receptor-1 (TNFR-1, CD120a) and TNFR-2 (CD120b) on MDSCs induced by M-CSF + TNFα were higher than those on MDSCs induced by M-CSF (Suppl. Fig. 1b). To demonstrate the potential inflammatory response ability of these induced $CD11b^{+}Gr\text{-}1^{+}$ M-MDSCs by M-CSF or M-CSF + TNFα, we detected the marker molecules for classic M1 macrophage activation induced by LPS and anti-inflammatory M2 macrophage polarization induced by IL-4 stimulation respectively with real-time PCR. M-CSF + TNFα-induced M-MDSCs expressed significantly lower TNFα, IL-12, and IL-6 compared with M-CSF-induced M-MDSCs after LPS stimulation, although these cells expressed similar levels of IL-1β (Fig. 1c). The decreased protein expression of TNFα, IL-12, and IL-6 in M-CSF + TNFα-induced M-MDSCs was further confirmed by flow cytometry analyses ($P<0.001$, Suppl. Fig. 2). M-CSF + TNFα-induced M-MDSCs expressed less Arg-1 and Fizz than M-CSF-induced M-MDSCs after IL-4 stimulation. M-CSF- and M-CSF + TNFα-induced M-MDSCs expressed identical levels of YM1 after IL-4 stimulation ($P<0.01$, Fig. 1c). These data suggest that the M-CSF + TNFα-induced M-MDSCs displayed distinctive phenotype and inflammatory gene expression compared with M-CSF-induced M-MDSCs.

## TNFα significantly enhanced the immunosuppressive ability of M-CSF-induced M-MDSCs

To determine the immune regulatory effect of M-CSF + TNFα-induced M-MDSCs on T cell proliferation and activation, we detected cell proliferation and cytokine productions of T cells stimulated by mitogen ConA in the presence of the induced MDSCs at different ratios. Addition of M-CSF-induced M-MDSCs markedly inhibited the proliferative response of $CD4^{+}$ and $CD8^{+}$ T cells in a dose-dependent manner

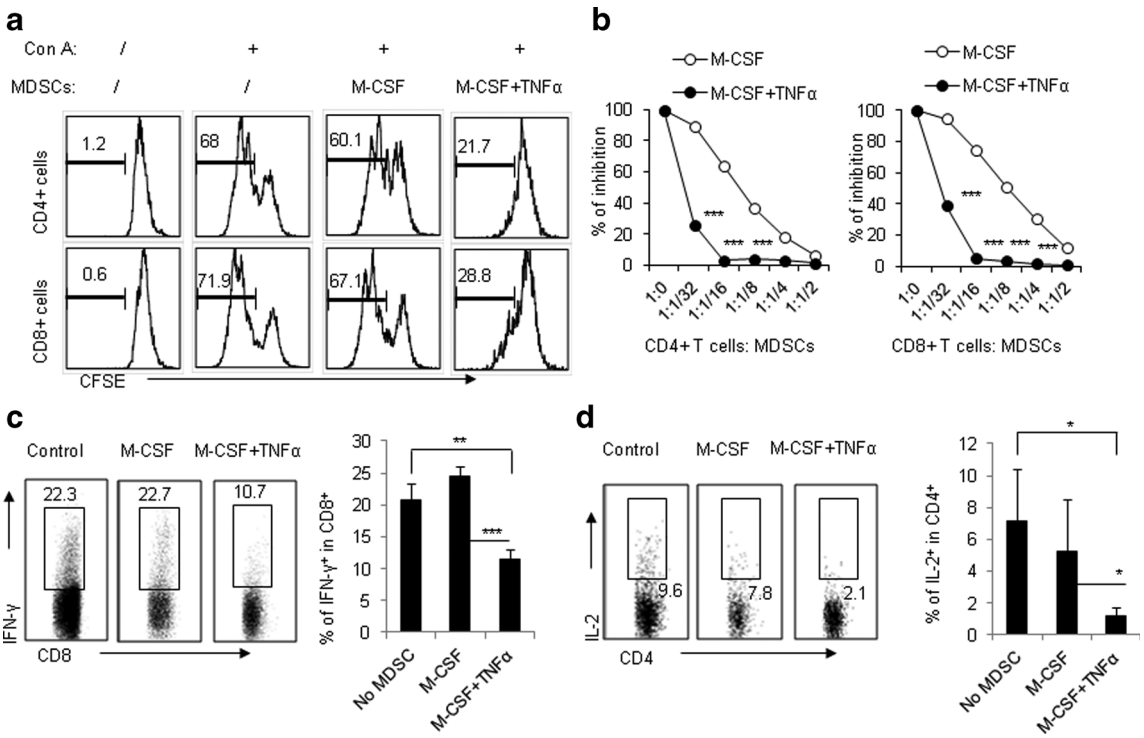


**Fig. 2** M-CSF + TNFα-induced M-MDSCs suppressed T cell activation and cytokines production more efficiently than M-CSF-induced M-MDSCs in vitro. **a** CFSE-labeled T cells ($2\times10^{5}$ per well) were stimulated by ConA (2 μg/ml), and M-MDSCs were added at different ratios between T cells and M-MDSCs and co-cultured for 72 h. The pattern of proliferation was examined by flow cytometric analysis of CFSE dilution in the gated $CD4^{+}$ and $CD8^{+}$ T cells. The percentages indicate CFSE dilution in the gated T cell population. **b** The inhibitory rates were calculated based on the proliferation rates determined by FCM analysis. Data are representative of three independent experiments with similar results. Splenocytes were cultured as described in **a** for 48 h; the cells were stimulated by PMA/ionomycin in the presence of Golgi plug for an additional 6 h. Then, cells were harvested for intracellular staining of IFN-γ (**c**) and IL-2 (**d**) in $CD8^{+}$ and $CD4^{+}$ T cells, respectively. Experiments were done more than two times. Data are shown as mean ± SD ($n=3$). *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared between the indicated groups

(Fig. 2a, b). However, M-CSF + TNFα-induced M-MDSCs showed significantly stronger immunosuppressive ability on T cell proliferation than M-CSF-induced M-MDSCs, as these cells efficiently inhibited T cell proliferation even in lower doses (Fig. 2a, b). In an allogeneic proliferation assay, T cell proliferation was stimulated by allogeneic BMDCs for 5 days and MDSCs were added at different ratios to compare the ability to suppress alloreactive T cell proliferation. M-CSF + TNFα-induced M-MDSCs also showed significantly stronger immunosuppressive ability on alloreactive T cell proliferation than M-CSF-induced M-MDSCs (Suppl. Fig. 3). Furthermore, M-CSF + TNFα-induced M-MDSCs significantly inhibited IFN-γ production by $CD8^+$ T cells and IL-2 production by $CD4^+$ T cells at a low ratio (1:1/8) between T cells and M-MDSCs (Fig. 2c, d), in which M-CSF-induced M-MDSCs did not show detectable inhibition on these cytokine expressions. Thus, the in vitro M-CSF + TNFα-induced M-MDSCs had more powerful immunosuppressive function than M-CSF-induced M-MDSCs.

## The immunosuppressive function of the induced M-MDSCs is mediated by iNOS

To investigate the mechanisms for the immunosuppressive activity mediated by M-CSF + TNFα-induced M-MDSCs, we detect the mRNA expressions of the potential relevant genes by real-time PCR. M-CSF-induced M-MDSCs express low levels of iNOS, Arg-1, IDO, and cyclooxygenase 2

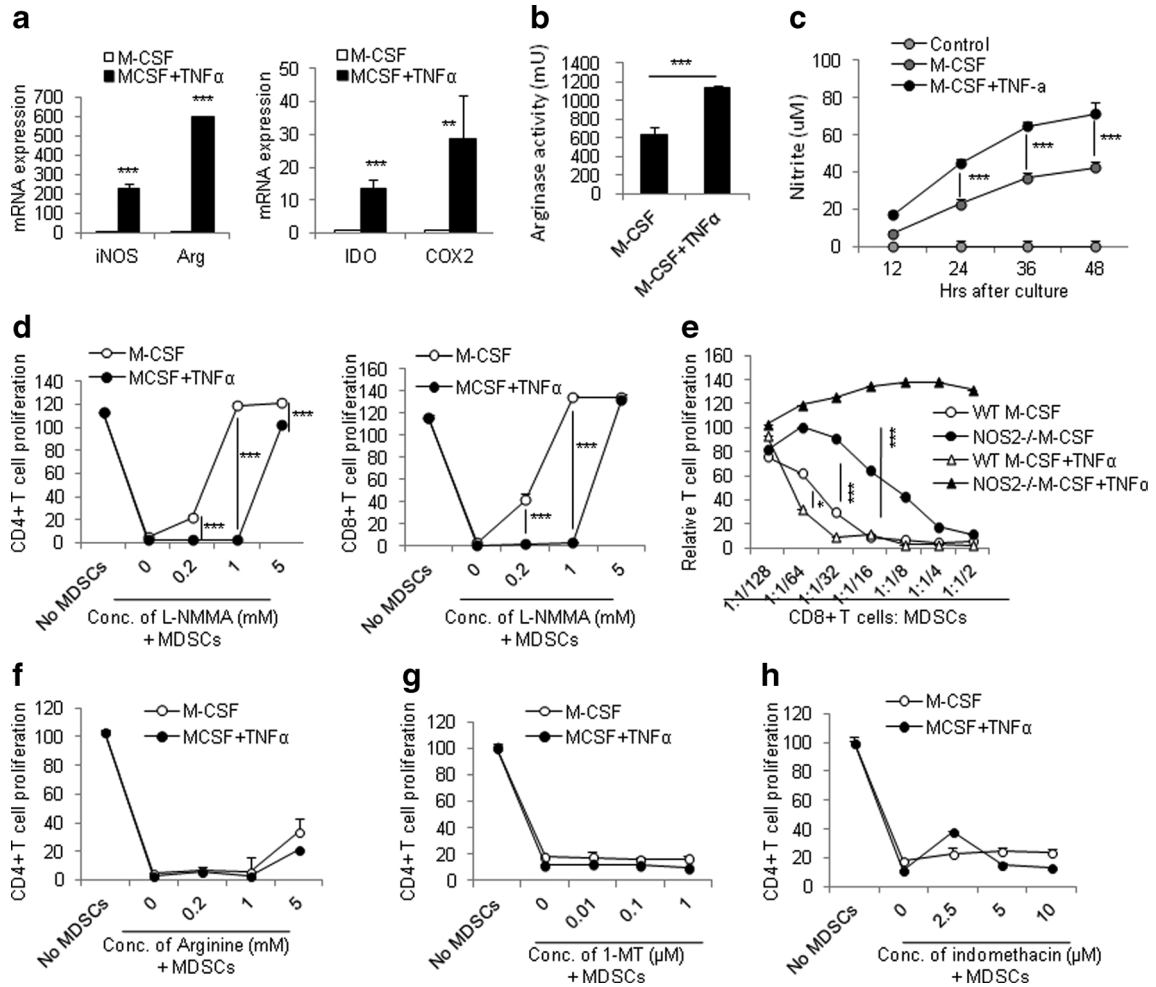


**Fig. 3** The inhibitory effect on T cells of the induced M-MDSCs is mediated by NO. **a** iNOS, Arg-1, COX2, and IDO mRNA expressions in M-CSF- and M-CSF + TNFα-induced M-MDSCs were determined by real-time PCR with no stimulation. **b** The in vitro induced M-MDSCs were stimulated with culture supernatant from PMA/ionomycin activated T cells for 72 h; arginase activity was detected as described in "Materials and Methods." **c** The induced M-MDSCs were co-cultured with T cells stimulated by ConA (T cells-to-MDSC ratio = 1:4), and the NO levels in the culture medium were detected. **d** Blocking NO production by iNOS inhibitor L-NMMA significantly reversed the inhibitory ability of M-CSF- and M-CSF + TNFα-induced M-MDSCs. **e** Bone marrow cells from iNOS KO mice were used for M-MDSC induction in vitro. The induced WT and iNOS-deficient M-MDSCs were added to CFSE-labeled T cell proliferation assay. iNOS deficiency significantly reversed the inhibitory effect of M-CSF + TNFα-induced M-MDSCs. Additional Arginine (**f**), and inhibitors of IDO (**g**) and Cox2 (**h**) failed to reverse the inhibitory ability of the induced M-MDSCs. Data were shown as mean ± SD ($n = 3$). Experiments were done more than two times. Data are shown as mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ compared between the indicated groups

(Cox2). However, the expression of iNOS, Arg-1, IDO, and Cox2 in M-CSF + TNFα-induced M-MDSCs was much higher than M-CSF-induced M-MDSCs ($P < 0.001$, Fig. 3a). The arginase activity of M-CSF + TNFα-induced M-MDSCs was significantly higher than M-CSF-induced M-MDSCs (Fig. 3b). In the immunosuppressive assay system in which T cells were activated by ConA in the presence of M-MDSCs, the NO production by M-CSF + TNFα-induced M-MDSCs was significantly higher than M-CSF-induced M-MDSCs (Fig. 3c). To see whether NO is involved in M-MDSCs-mediated immunosuppression, we used the inhibitor L-NMMA to specifically block NO production in the in vitro assays for MDSC immunosuppressive function on T cells. Addition of L-NMMA significantly reversed the inhibitory effects on T cells of M-CSF- and M-CSF + TNFα-induced M-MDSCs in a dose-dependent manner (Fig. 3d). Furthermore, M-CSF-induced iNOS-deficient M-MDSCs showed significantly less efficiency to inhibit T cell proliferation (Fig. 3e). More strikingly different to the efficient immunosuppressive ability of M-CSF + TNFα-induced M-MDSCs, M-CSF + TNFα-induced iNOS-deficient M-MDSCs failed to show any detectable immunosuppressive function on T cells (Fig. 3e). In addition, the phenotypes of M-CSF + TNFα-induced M-MDSCs induced from bone marrow cells of WT and iNOS-deficient mice were compared. M-CSF + TNFα-induced iNOS KO M-MDSCs expressed lower levels of F4/80, Gr-1, Ly6C, CD115, and CD274 compared with M-CSF + TNFα-induced WT M-MDSCs, supporting the

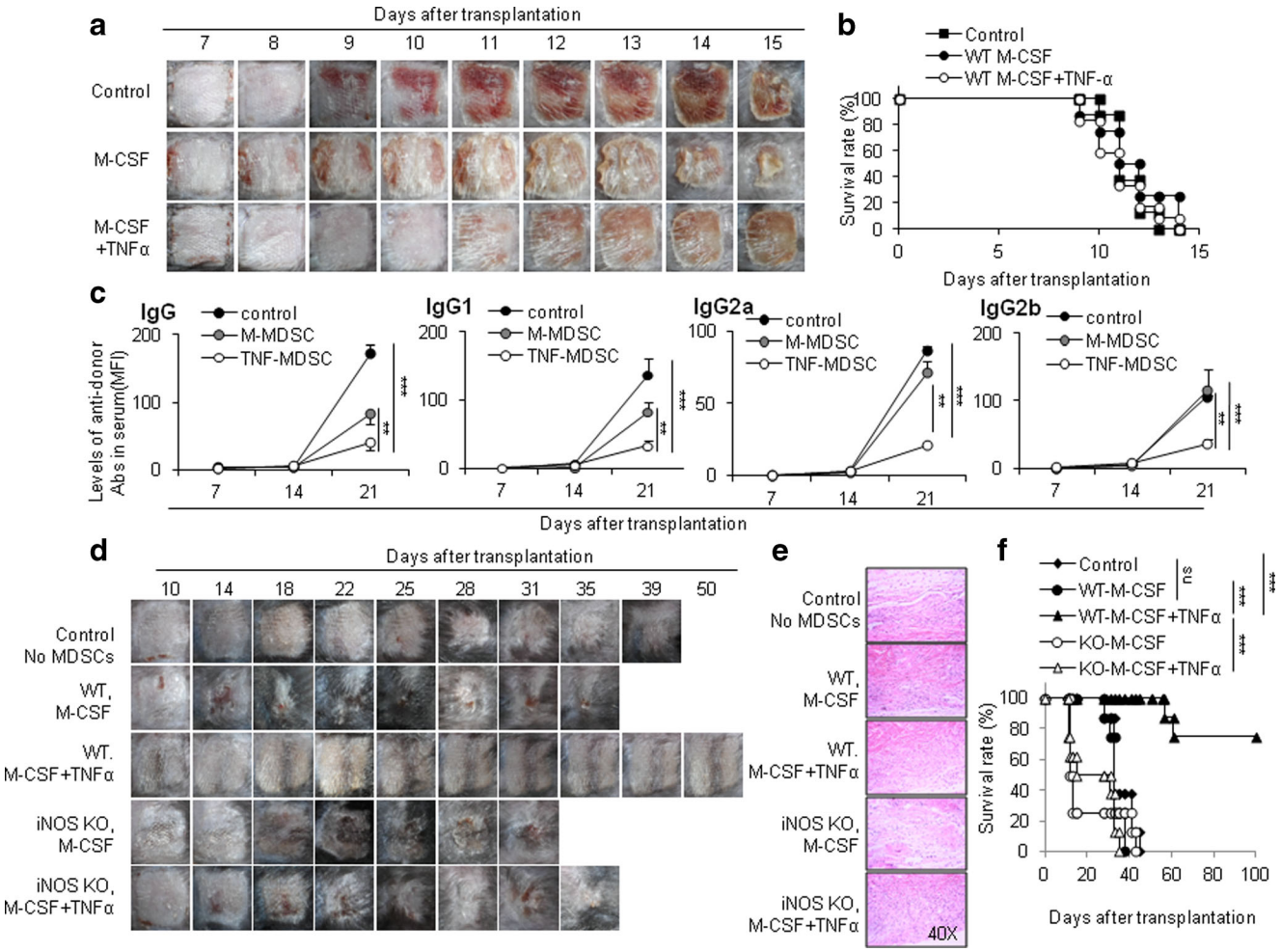


**Fig. 4** The inhibitory effect of the induced M-MDSCs in alloskin-grafted and male-to-female mouse models. B6 mice were injected i.v. with the induced M-MDSCs ($5 \times 10^6$ cells) on day 1 and BALB/c skin allograft transplantation on day 0. **a** The digital photo representatives of graft rejection were shown. **b** The percentages of graft survival over time after transplantation were summarized ($n = 8$). **c** Anti-donor Abs, including IgG, IgG1, IgG2a, and IgG2b in the sera of B6 recipients injected with M-CSF + TNFα-induced M-MDSCs were decreased compared to recipients with M-CSF-induced M-MDSCs or received no cells control recipients. Data were shown as mean ± SD ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control or mice with M-CSF-induced M-MDSCs. **d** Female B6 or iNOS KO mice were injected i.v. with the induced M-MDSCs ($5 \times 10^6$) on day 1 and day 7, and male B6 skin was grafted on day 0. Representative digital photos were shown. **e** H&E staining of skin grafts on day 30 were presented (×40 objective). **f** The percentages of graft survival over time after transplantation for chronic rejection were shown ($n = 8$). *P* values were determined using Log-rank tests (control versus WT-M-CSF, $P = 0.1033$; control versus WT-M-CSF-TNFα, $P = 0.0149$; WT-M-CSF-TNFα versus KO-M-CSF-TNFα, $P = 0.0036$; control versus KO-M-CSF-TNFα, $P = 0.3655$)

important role of iNOS in maintaining the function of M-MDSCs (Suppl. Fig. 4a, b). Additional arginine supplement failed to efficiently rescue the poor T cell proliferation caused by the induced M-MDSCs, indicating that the consumption of arginine by the induced M-MDSCs is not the major reason for the poor T cell proliferation in this system (Fig. 3f). On the other hand, inhibition of IDO and Cox2 with their corresponding inhibitors, respectively, failed to recover the T cell proliferation in the presence of M-CSF- or M-CSF + TNFα-induced M-MDSCs (Fig. 3g, h). Therefore, iNOS but not Arg-1, IDO, and Cox2 expression in M-MDSCs is responsible for the enhanced immunosuppression of M-CSF + TNFα-induced M-MDSCs.

### The M-CSF + TNFα-induced M-MDSCs induced immune tolerance to donor antigens in male-to-female skin-grafted mice via iNOS

To determine the role of immune regulation by M-CSF + TNFα-induced M-MDSCs in vivo, we adaptively transferred the in vitro M-CSF + TNFα-induced M-MDSCs intravenously into B6 or CD45.1 B6 recipients, on which fully allogeneic BALB/c skin were grafted on the following day. B6 mice transferred with M-CSF- or M-CSF + TNFα-induced M-MDSCs rejected these alloskin grafts as efficiently as B6 mice without receiving M-MDSCs (Fig. 4a, b). But recipients transferred with M-CSF + TNFα-induced M-MDSCs produced less anti-donor Abs as determined by the levels of anti-donor IgG, IgG1, IgG2a, and IgG2b in the sera (Fig. 4c), and the infiltration of immune cells was also reduced in the allografts in these recipients (Suppl. Fig. 5). Furthermore, $CD8^+$ T cells from draining lymph nodes of recipient mice transferred with M-CSF + TNFα-induced M-MDSCs produced less IFN-γ after stimulation with allogeneic BALB/c splenocytes for 24 h and activated by PMA/Iono for 6 h comparing with recipients receiving M-MDSCs and mice without receiving M-MDSCs (Suppl. Fig. 6). In addition, we used a chronic rejection mouse model in which male skin was grafted to female mice. B6 mice transferred with M-CSF + TNFα-induced M-MDSCs significantly prolonged male skin graft survival compared with B6 recipients with receiving no MDSCs and the M-CSF-induced M-MDSCs group. Interestingly, female B6 recipients with M-CSF- or M-CSF + TNFα-induced iNOS-deficient rejected male skin grafts in an accelerated pattern compared with B6 recipients who received no or wild-type M-MDSCs (Fig. 4d, f). H&E staining of skin grafts on day 30 after transplantation also showed reduced cell infiltration in the grafts from mice receiving M-CSF + TNFα-induced M-MDSCs, and iNOS deficiency reversed this phenomenon (Fig. 4e). These data indicate that M-CSF + TNFα-induced M-MDSCs had improved immunosuppressive ability, and its immunosuppression is mainly mediated by iNOS in vivo.

## Discussion

MDSCs are recognized for their robust immunosuppressive activity on immune response and are certainly involved in the delayed graft rejection and transplantation immune tolerance induction [7]. Thus, MDSCs may be potentially used for clinical therapy to control the graft rejection by recipient's immune system and to induce immune tolerance to donor antigens. However, up to now, the current systems for the ex vivo differentiation of MDSCs are inefficient and fail to achieve significant levels of amplification [25]. Previous study found that the major subset of M-MDSCs derived from the GM-CSF induction system were unstable immature myeloid cells which could rapidly differentiate into macrophages in vivo [22]. Thus, we tried to build a more stable M-MDSCs induction system with a prolonged cell culture period. M-CSF acted as an important regulator of monocyte/macrophage differentiation and polarization [26]. It can predispose cells toward an M2 phenotype in cell culture experiments involving both human monocytes and mouse bone marrow-derived macrophages [27, 28]. Additionally, M-CSF combined with IL-4, IL-10, and TGF-β can induce a population of macrophages with dominant immunosuppressive phenotype from human blood monocytes [29]. Recently, several studies have demonstrated that M-CSF exerts a prominent role in MDSCs-related disease states [30]. In our system, we found that the majority of M-CSF-induced MDSCs were macrophage-like M-MDSCs. They expressed more mature macrophage markers and costimulatory molecules such as F4/80 and CD80 but almost did not express Ly6G and CD11c. TNFα treatment inhibited the expression I-Ab and CD86, which were important for activation of adaptive immune response [23], and increased Ly6C and CD274 expression which were recognized as markers for M-MDSCs in tumor models. Additionally, after polarization with LPS or IL-4, we found that M-CSF + TNFα-induced M-MDSCs could not efficiently polarize into M1 or M2 macrophages as indicated by the poor expression of M1 and M2 macrophage-related molecules like TNFα, IL-12, IL-6, Arg-1, and Fizz expressions. These results indicated that M-CSF + TNFα induced a population of macrophage-like cells with immunosuppression property which is different from M1 and M2 macrophages.

In the present M-MDSCs-inducing system, the M-CSF + TNFα-induced M-MDSCs displayed significantly enhanced immunosuppressive function in vitro and in vivo compared with M-CSF-induced M-MDSCs. M-CSF + TNFα-induced M-MDSCs efficiently inhibited T cell proliferation and inflammatory cytokine productions in vitro. The inhibitory effects of these cells were significantly stronger than M-CSF-induced M-MDSCs. M-CSF + TNFα-induced M-MDSCs inhibited anti-donor antibody productions and lymphocyte infiltration into grafts in an alloskin-transplanted mouse model

and significantly reduced the cytokine IFN-γ expression of $CD8^+$ T cell function in draining lymph nodes. Importantly, M-CSF + TNFα-induced M-MDSCs promoted immune tolerance to donor antigens in about 80 % male-to-female transplant recipients as indicated by the long-term graft survival. Thus, TNFα has the ability to enhance the immunosuppressive function of M-MDSCs during the differentiation of M-MDSCs from bone marrow precursors driven by M-CSF.

TNFα is a multifunctional immunomodulating cytokine and harmful in many pathologies such as chronic inflammation, autoimmunity, and cancer [31, 32]. However, the mechanism by which TNFα inhibits host immune response is still unclear. More recently, it is reported that "the dark side" of TNFα was correlated with the immunosuppressive cells-MDSCs [19] and TNFα signal pathway was important for regulating survival/apoptosis of MDSCs in tumor models [22, 33]. M-CSF + TNFα-induced M-MDSCs exerted their immunosuppressive functions via an iNOS-dependent pathway, but not via Arg-1, IDO, and Cox2 pathways. We found that M-CSF + TNFα-induced M-MDSCs produced large amounts of NO than M-CSF-induced cells even when these cells were not subsequently stimulated with LPS or others. Inhibiting NO production by chemical inhibitor or iNOS gene deficiency greatly blocked the immunosuppressive ability of these M-MDSCs in vitro and in vivo. These effects may be due to TNFα-triggered activation of the NF-KB pathway, which is well known for its close involvement in the regulation of iNOS expression [34]. The detailed mechanisms for the effects of TNFα on M-MDSC function need to be studied.

In summary, M-CSF + TNFα-induced M-MDSCs have more potential immunosuppressive function in vitro and in vivo. M-CSF + TNFα-induced M-MDSCs can promote immune tolerance to donor antigens in a male-to-female mouse model. The immunosuppressive activity of M-CSF + TNFα-induced M-MDSCs is mainly mediated by the iNOS-dependent pathway.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no competing financial interests.

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