Inflammation Research (2019) 68:545–555
https://doi.org/10.1007/s00011-019-01237-9

Inflammation Research

ORIGINAL RESEARCH PAPER

Adoptive transfer of IFN-γ-induced M-MDSCs promotes immune tolerance to allografts through iNOS pathway

Fan Yang[1] · Yang Li[1] · Weilong Zou[2] · Yanan Xu[1] · Hao Wang[3] · Wei Wang[4] · Yong Zhao[1,5]

Received: 1 August 2018 / Revised: 19 March 2019 / Accepted: 22 April 2019 / Published online: 4 May 2019

Abstract

Aim and objective Efficient production of monocytic myeloid-derived suppressor cells (M-MDSCs) with stable immunosuppressive function is crucial for immunomodulatory cell therapy for many diseases such as transplant rejection, graft-versus-host disease and autoimmune diseases.

Methods We used M-CSF as growth factor for myeloid progenitor cell differentiation and activated them with IFN-γ during early stage in vitro to produce M-MDSCs. The cell phenotypes were determined using flow cytometry, the immunosuppressive function and mechanisms were determined by skin grafted mouse models and genetic modified mice.

Results IFN-γ treatment endows these cell strong immunosuppressive function by inhibition of T cell proliferation and cytokine productions. The phenotype of these cells also changed towards M-MDSCs. IFN-γ significantly upregulated iNOS expression in these M-MDSCs and inhibition of this molecule significantly reversed their immune regulatory function. The functional stability of induced M-MDSCs by IFN-γ was tested in vivo by transferring them to alloskin-grafted mice. Adoptive transfer of these cells significantly prolonged allograft survival and promoted immune tolerance, whereas iNOS deficiency in these cells reversed this effect.

Conclusions We established one M-MDSCs-inducting protocol with the combination of M-CSF and IFN-γ in vitro. M-CSF+IFN-γ-induced M-MDSCs are promising to prevent graft rejection by immune regulation.

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

Introduction

Myeloid-derived suppressor cells (MDSCs) are the major immune regulators in many pathologic conditions such as cancer, infection, trauma and transplantation induced by emergency myelopoiesis, which was known as accelerated myeloid cell differentiation [1]. Two signals are

Responsible Editor: Mauro Teixeira.

Fan Yang, Yang Li and Weilong Zou equally contributed as the first authors.

✉ Wei Wang
zico73@medmail.com.cn

✉ Yong Zhao
zhaoy@ioz.ac.cn

[1] Transplantation Biology Research Division, State Key Laboratory of Membrane Biology, Institute of Zoology, Chinese Academy of Sciences, University of Chinese Academy of Sciences, Beichen West Road 1-5, Chaoyang District, Beijing 100101, China

[2] Surgery of Transplant and Hepatopancrobiliary, The General Hospital of Chinese People's Armed Police Forces, Beijing, China

[3] Department of Radiation Oncology, Peking University Third Hospital, Beijing, China

[4] Department of Urology, Beijing Chaoyang Hospital, Capital Medical University, 8 Gong Ti Nan Road, Chaoyang District, Beijing 100020, China

[5] Institute for Stem Cell and Regeneration, Chinese Academy of Sciences, Beijing 100101, China

needed for MDSC development and accumulation. One signal for MDSC expansion is provided by growth factors and cytokines, and the another signal for MDSC activation is provided by inflammatory molecules [2]. In mice, MDSCs can be divided into two subsets as monocytic (M)- and polymorphonuclear (PMN)-MDSCs with phenotype definition as $CD11b^{+}Ly6G^{-}Ly6C^{high}$ and $CD11b^{+}Ly6G^{+}Ly6C^{low}$, respectively [3]. Human MDSCs also have M- and PMN-MDSC two subsets defined by $CD11b^{+}CD14^{+}HLA\text{-}DR^{low/-}CD15^{-}$ and $CD11b^{+}CD14^{-}CD15^{+}$ (or $CD66b^{+}$). Early-stage MDSCs (eMDSCs) are defined as $Lin(CD3/14/15/19/56)^{-}HLA\text{-}DR^{-}CD33^{+}$ in humans which had no counterpart defined in mice [4]. After activation, different subsets of MDSCs use distinct mechanisms to mediate immune suppression. Usually, M-MDSCs upregulate their expression of nitric oxide synthase 2 (NOS2 or iNOS) and PMN-MDSCs have higher levels of reactive oxygen species (ROS) which are mediators of immune suppression [5]. Arginase 1 (Arg1) can be employed by both two subsets for immune regulation by depletion of L-arginine in lymphoid and non-lymphoid tissues to suppress the translation of CD3ζ in T cells [6]. Indoleamine 2,3 dioxygenase (IDO), heme oxygenase-1 (HO-1), prostaglandin E2 (PGE2) produced by cyclooxygenase-2 (COX2), TGF-β and IL-10 are also reported for MDSCs-mediated immunosuppression [7, 8].

MDSCs are important immunomodulatory cells in pathological condition like transplantation and autoimmune diseases [9, 10]. MDSCs can be generated by hematopoietic stem cells or progenitor cells in mice and humans with growth factor and cytokines in vitro [11, 12]. Healthy donor peripheral blood mononuclear cells (PBMCs) were also available for MDSC induction [13]. These suggest that MDSCs have broad progenitor source for large scale in vitro induction for cell therapy. Nowadays immune regulatory cells-based cell therapy for transplantation in clinical trials included Treg, DCreg, Mreg and mesenchymal stem cells (MSCs), but there were no reports on MDSCs (see http://clinicaltrials.gov). In mice, the in vitro induced MDSCs often cultured with factor GM-CSF for expansion, resulting in the accumulation of both MDSC subsets [14]. GM-CSF alone induced functional MDSCs from bone marrow cells prolonged alloskin and heart graft survival [15–17]. GM-CSF+IL-6-induced MDSCs from bone marrow cells prolonged pancreas and skin allograft survival [18, 19]. But for this combination, the induced MDSCs-mediated suppression was due to over-activation-induced anergy of T cells, LPS treatment or repeated injection of MDSCs further enhanced this effect [19]. MDSCs induced by GM-CSF+G-CSF+IL-13 from bone marrow cells inhibited GVHD via Arg-1 [20]. GM-CSF+G-CSF-induced MDSCs prevents GVHD by skewing helper T cell differentiation to Th2 [21]. Combination of LPS plus IFN-γ with GM-CSF promoted development of bone marrow cells towards MDSCs but not DCs with suppressive function [22]. In human, stable MDSCs can be induced from PBMCs or monocytes isolated from PBMCs by GM-CSF in combination with IL-6 or IL-4+PGE2 [13, 23]. M-CSF was another colony-stimulating factor for myeloid cell differentiation at homeostatic conditions [24]. M-CSF alone can induce suppressive macrophages promoting alloskin graft survival [25]. It has been reported that M-CSF combined with IL-3, IL-6, c-kit ligand, TPO, FLT3L and VEGF induce MDSCs from HSCs with suppressive function in vitro and in vivo [11]. We recently reported that the treatment with M-CSF with TNF-α generated functional MDSCs which can greatly prolong allograft survival [26]. IFN-γ was an important cytokine for immunomodulation with mechanism different from TNF-α in MSCs [27]. IFN-γ upregulated expression of IDO in DC for immune suppression [28]. In the present study, we successfully induced MDSCs by combination of M-CSF with IFN-γ, which may be promising for immunomodulatory cell therapy especially in transplantation.

Materials and methods

Mice

BALB/c, C57BL/6 (B6) and CD45.1 B6 mice were purchased from Beijing University Experimental Animal Center (Beijing, China). iNOS knockout (KO) mice were provided by Dr. Lianfeng Zhang, the Key Laboratory of Human Diseases Comparative Medicine, Ministry of Public Health, Beijing, China. All mice were bred and maintained under specific pathogen-free conditions in the animal facility of the Institute of Zoology, Chinese Academy of Science. Sex-matched littermate mice of 6–12 weeks old were used. All animal assays were approved by the Animal Ethics Committee of the Institute of Zoology (Beijing, China) (Table 1).

Induction of M-MDSCs in vitro

Bone marrow cells were prepared by flushing tibias and femurs of B6 mice with PBS. Red blood cells were lysed with ammonium chloride. Then cells were planted in 100-mm dishes (Corning, USA) for 2 h and adherent cells were discarded to exclude bone marrow macrophage. For M-MDSC induction, 4×10^5/ml non-adherent bone marrow cells were cultured with 50 ng/ml M-CSF with 50 ng/ml IFN-γ in RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 μM 2-ME, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 10% heat inactivated FBS for 7 days at 37 °C, 5% CO_2. IFN-γ were added on day 3 and half of the medium was changed on day 3 and 5.

Table 1 Primers used for qRT–PCR analysis

Genes	Primer sequence (5′–3′)
HPRT	
Forward primer:	AGTACAGCCCCAAAATGGTTAAG
Reverse primer:	CTTAGGCTTTGTATTTGGCTTTTC
Arginase1	
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
NOX2	
Forward primer:	GACCCAGATGCAGGAAAGGAA
Reverse primer:	TCATGGTGCACAGCAAAGTGAT
TGF-β	
Forward primer:	GGCGGTGCTCGCTTTGTA
Reverse primer:	TCCCGAATGTCTGACGTATTGA
IL-10	
Forward primer:	GGAGCAGGTGAAGAGTGATT
Reverse primer:	CCCAAGGAGTTGTTTCCGTTA
IL-4	
Forward primer:	TTGTCATCCTGCTCTTCTTTCTC
Reverse primer:	CAGGAAGTCTTTCAGTGATGTGG

HPRT hypoxanthine phosphoribosyl transferase, *TGFβ* transforming growth factor beta, *IL* interleukin;, *iNOS* inducible nitric oxide synthase, *IDO1* Indoleamine-2,3-dioxygenase-1, *COX2* cyclooxygenase 2, *NOX2* NADPH oxidase 2, *qRT–PCR* quantitative PCR

T cell inhibition assays

For T cell proliferation assay, single cell suspension was prepared from lymph node of B6 mice and labeled with CFSE. Bone marrow-derived DCs (BMDCs) were induced by bone marrow cells at 2×10^6/ml with 40 ng/ml GM-CSF for 7 days and activated with LPS for 24 h. 2×10^5 CFSE labeled lymphocytes were co-cultured with 5×10^4 allogenic BALB/c BMDC in 200 µl complete RPMI 1640 medium in 96-well round bottom plates for 5 days. For suppression assay, MDSCs were added at different ratios. For rescue assay, iNOS inhibitor L-NMMA was added at the beginning of the co-culture. For cytokine production, similar procedure was employed with non-labeled LN cells stimulated by allogeneic BMDC for 3 days. Then cells were re-stimulated by PMA (50 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Sigma-Aldrich, St Louis, MO, USA) for 5 h with GolgiStop (BD Pharmingen, San Diego, CA, USA). After the simulation, cells were incubated with anti-mCD4-FITC or anti-mCD8-PE-Cy5 for surface staining. Then after fixation and membrane permeabilization with Cytofix/Cyoperm solution (BD Pharmingen), intracellular cytokine staining was performed using anti-mIFN-γ-PE or anti-mTNF-α-PE. Samples were analyzed by flow cytometry.

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-mCD115-PE, anti-mCD124-PE, anti-mCD274-PE, anti-mCD31-PE, anti-mCD40-PE, anti-mTNF-α-PE, anti-mIFN-γ-PE were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Anti-CD4-FITC, anti-mCD4-PE, anti-mCD8-PE-Cy5, and anti-mLy6G-PE were purchased from eBioscience (San Diego, CA, USA). Recombinant mouse M-CSF, GM-CSF and IFN-γ were purchased from PeproTech (Rocky Hill, NJ, USA).

Flow cytometry

For surface marker staining of MDSCs, induced cells at 5×10^5/tube in 100 µl PBSA (0.1%) were incubated with the appropriate antibody at 4 °C in the dark for 30 min. Then cells were washed with PBSA and analyzed by flow cytometry. Assays were performed on a Beckman Coulter Epics XL benchtop flow cytometer (Beckman Coulter, Brea, CA, USA) and data were analyzed by FCS express software (De Novo Software, Canada). A minimum of 50,000 events was collected for each sample.

Quantitative PCR

Total RNA was isolated with TRIzol (Invitrogen, USA) and reverse transcription was performed with M-MLV superscript reverse transcriptase according to the manufacturer's instructions (Takara Bio, Japan). Quantitative real-time PCR was performed on the CFX96 Real-Time System (Bio-Rad Laboratories, USA) with Power SYBR PCR Master Mix (Takara Bio, Japan).

Skin transplantation

Male B6 tail skin was grafted on the dorsal part of the female B6 recipients. 5×10^6 cells induced M-MDSCs per mouse were transferred to recipient mice by intravenous injection the day before transplantation. Skin graft survival was monitored by daily observations. Graft rejection was determined when less than 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 ANOVA analysis was used for comparison among multiple groups with GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

Results

IFN-γ switched the differentiation of bone marrow progenitor cells toward M-MDSCs

To investigate the effect of IFN-γ on bone marrow cell differentiation with M-CSF as growth factor, we first determined the phenotype of differentiated myeloid cells induced by M-CSF alone or M-CSF+IFN-γ by flow cytometry. Cell surface molecules including F4/80, Gr-1, Ly6C, Ly6G, CD11c, CD86, CD80, CD40, I-Ab, CD115, CD124 (IL-4Rα), CD274 (PD-L1, B7-H1), and CD31 were stained and analyzed. As shown in Fig. 1, addition of IFN-γ reduced the expression of CD115 and macrophage marker F4/80, but MDSC markers including Gr-1 and Ly6C were significantly upregulated ($P < 0.001$, Fig. 1b, c). The expression of PD-L1 was significantly upregulated suggesting an immune regulatory role of IFN-γ-induced myeloid cells. The expression levels costimulatory molecules including CD86 and CD40 were upregulated ($P < 0.001$, Fig. 1b), but CD80 which had more affinity to CTLA-4 on T cells for immunomodulation were also significantly upregulated. The expression of IL-4Rα, a marker for suppressive myeloid cells, was also upregulated on IFN-γ-induced myeloid cells. It should be noticed that the expression of CD11c and I-A^b which were related to the function of antigen presentation were also upregulated. However, the addition of IFN-γ reduced the cell number of $CD11b^+F4/80^+$ macrophage-like cells compared with M-CSF-induced cells (Fig. 1d). Combining these

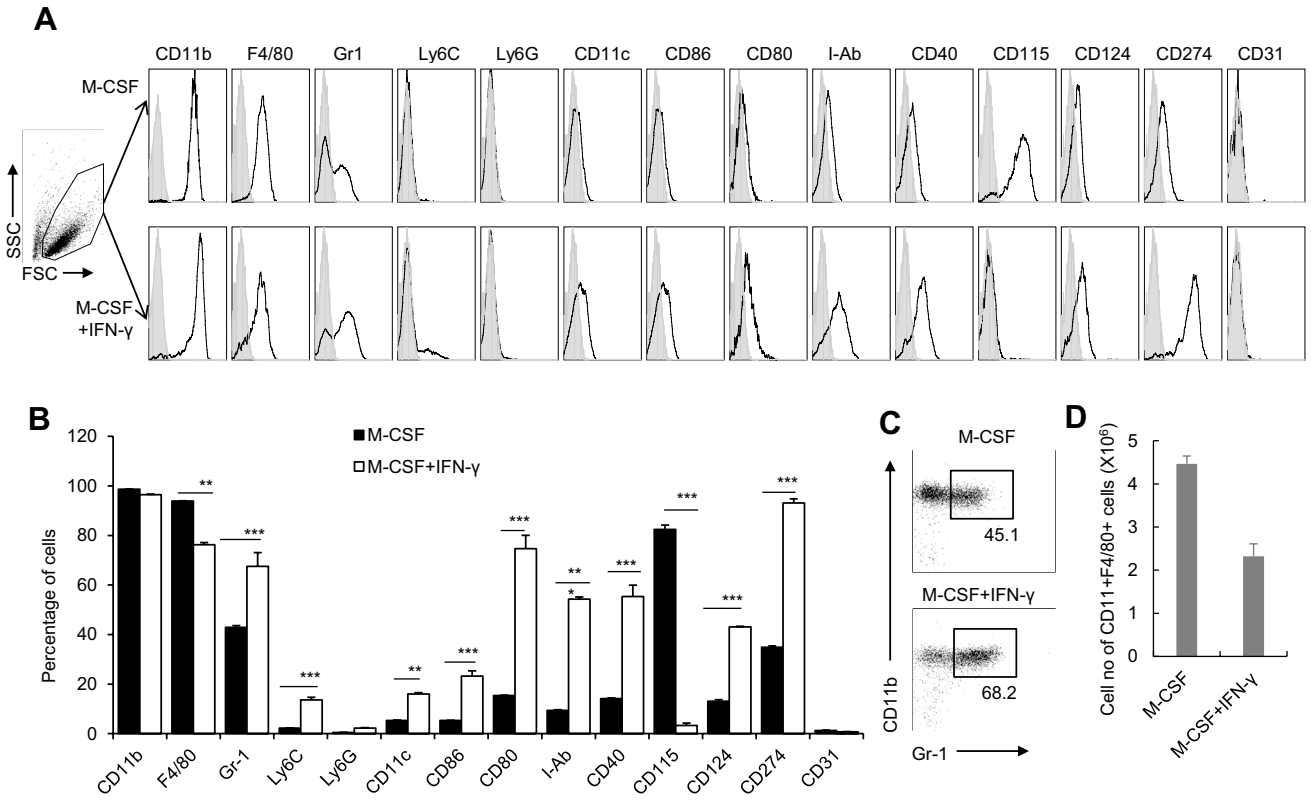

Fig. 1 The phenotypes of M-CSF- and M-CSF+IFN-γ-induced MDSCs. **a** Bone marrow cells were cultured in M-CSF or M-CSF+IFN-γ as described in Materials and Methods for 7 days. The cells were then stained with PE, FITC or PE-cy5-labeled anti-F4/80, CD11b, CD11c, Gr-1, Ly6C, Ly6G, I-Ab, CD80, CD86, CD40, CD115, CD124, CD274 or CD31 mAb. 5×10^5 induced cells were analyzed by flow cytometric (FCM) assay. **b** The percentages of the indicated molecules were summarized. **c** Typical example of flow cytometry analysis showed the percentage of $CD11b^+Gr\text{-}1^+$ MDSCs after 7 days induction. **d** The total cell numbers of $CD11b^+F4/80^+$ cells after 7 days induction with either M-CSF alone or M-CSF+IFN-γ. Data are shown as mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared between the indicated groups

results, we defined these cells as M-MDSCs according to the phenotype. We then determined the suppressive function of these induced cells in vitro and in vivo in the following studies.

M-CSF+IFN-γ-induced M-MDSCs suppressed T cell function in vitro

To determine the immunosuppressive function of M-CSF+IFN-γ-induced M-MDSCs, we performed mixed leukocyte reaction (MLR) using BALB/c bone marrow cells-derived BMDCs as stimulators and B6 lymphocytes as responder cells. M-CSF+IFN-γ-induced M-MDSCs from B6 bone marrow cells were added at different ratio with M-CSF alone induced myeloid cells as control. Proliferation of CFSE-labeled responder $CD4^+$ or $CD8^+$ T cells was significantly inhibited by M-CSF+IFN-γ-induced M-MDSCs comparing to controls by flow cytometry in a dose-dependent manner ($P < 0.001$, Fig. 2). Cytokine productions for IFN-γ and TNF-α in both $CD4^+$ and $CD8^+$ T cells were decreased by addition of M-CSF+IFN-γ-induced M-MDSCs ($P < 0.001$, Fig. 3a–f). The expression of T cell activation marker CD25 was determined in this assay too. The CD25 expression on responder $CD8^+$ but not $CD4^+$ T cells was downregulated by the addition of M-CSF+IFN-γ-induced M-MDSCs comparing to M-CSF alone induced M-MDSCs ($P < 0.001$, Fig. 3g–i).

M-CSF+IFN-γ-induced M-MDSCs mediated immunosuppression through iNOS

To determine the mechanism employed by M-CSF+IFN-γ-induced M-MDSCs to mediate immunomodulation, mRNA expressions of potential effector molecules used by myeloid cells for immunosuppression were investigated by real-time PCR. The expressions of iNOS and Arg-1 in M-CSF+IFN-γ-induced M-MDSCs were much higher than M-CSF alone-induced control myeloid cells ($P < 0.001$, Fig. 4a). Expressions of IDO and COX2 were slightly enhanced. Expressions of NOX2, HO-1, TGF-β, IL-10 and IL-4 in M-CSF+IFN-γ-induced M-MDSCs were not changed comparing to control (Fig. 4a). To investigate whether iNOS pathway in M-CSF+IFN-γ-induced M-MDSCs plays a central role for their T cell suppression, iNOS inhibitor L-NMMA was added in MLR assays with M-CSF+IFN-γ-induced M-MDSCs or control myeloid cells induced by M-CSF alone. This inhibitor totally reversed the suppressive effect of M-CSF+IFN-γ-induced M-MDSCs on T cell proliferation in a dose-dependent pattern (Fig. 4b–d), suggesting a

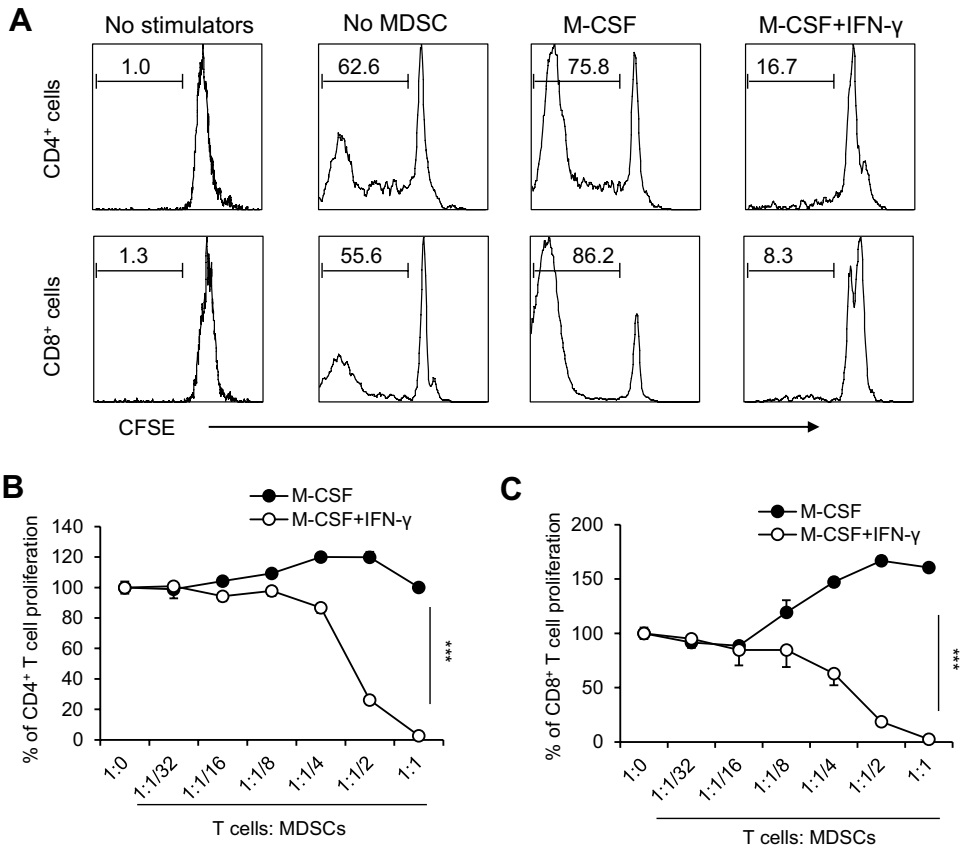

Fig. 2 M-CSF+IFN-γ-induced M-MDSCs suppress allogeneic DCs-stimulated T cell proliferation. M-CSF and M-CSF+IFN-γ-induced M-MDSCs were added at different ratios in allogeneic DCs-stimulated T cell proliferation system for 5 days. **a** Typical example of flow cytometry analysis for T cell proliferation. Percentage of $CD4^+$ (**b**) and $CD8^+$ (**c**) T cell proliferation measured by CFSE dilution was summarized (T cells-to-MDSC ratio = 1:1/2). Data are shown as mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared between the indicated groups

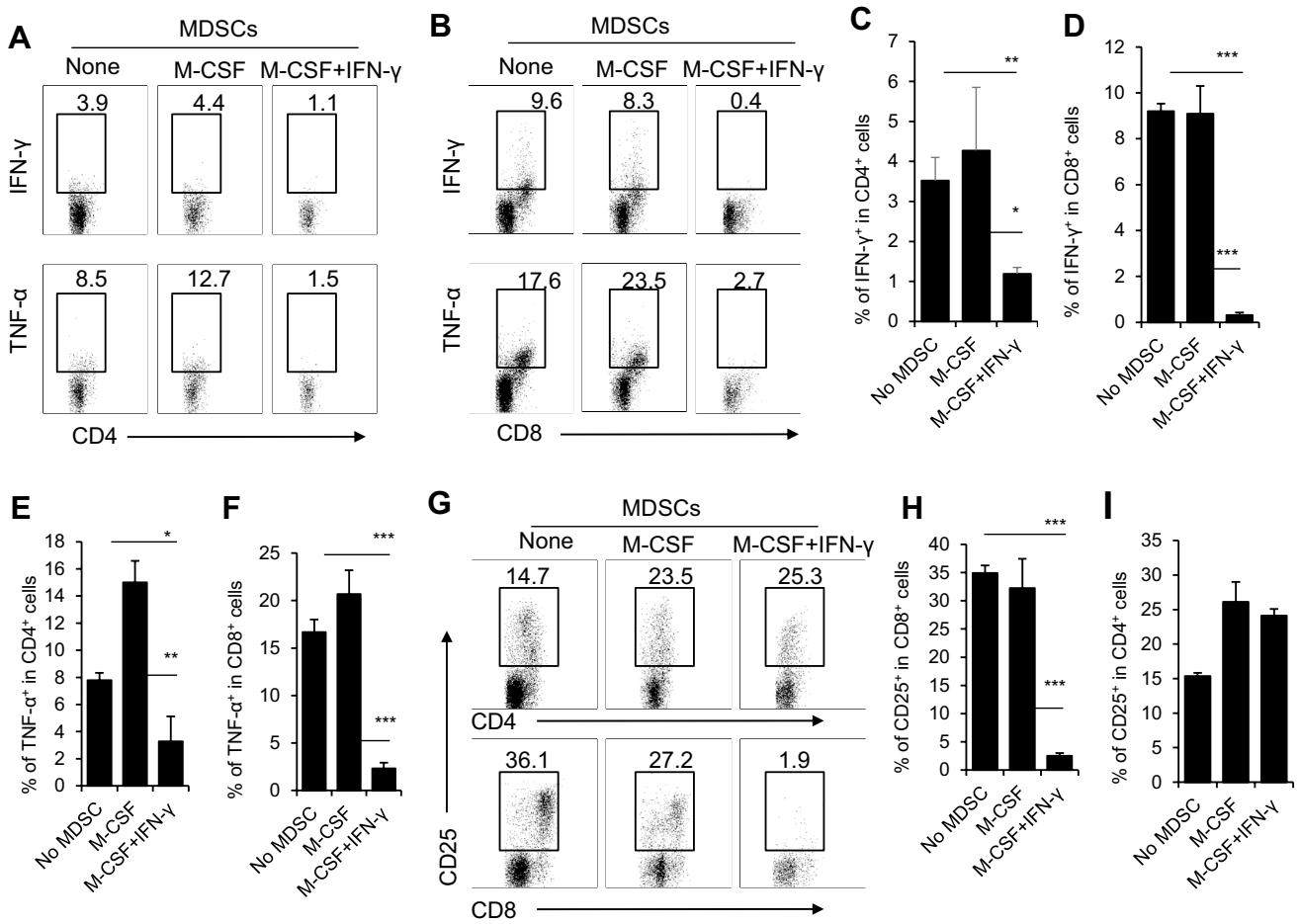

Fig. 3 M-CSF+IFN-γ-induced M-MDSCs suppress allogeneic DCs-stimulated T cell activation and cytokine productions. M-CSF and M-CSF+IFN-γ-induced M-MDSCs were added in allogeneic DCs-stimulated T cell proliferation system for 3 days followed by stimulation with PMA and Ionomycin for 5 h. **a**, **b** Typical example of flow cytometry analysis for T cell cytokine production. IFN-γ (**c**, **d**) and TNF-α (**e**, **f**) cytokine productions by $CD4^+$ and $CD8^+$ T cells stimulated by allo-DCs in the presence or absence of M-MDSCs were summarized. **g** Typical example of flow cytometry analysis for CD25 expression on T cells co-cultured with the induced M-MDSCs. CD25 expression on $CD4^+$ (**h**) and $CD8^+$ (**i**) T cells were determined by FCM and summarized. Data are shown as mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared between the indicated groups

key role of iNOS for M-CSF+IFN-γ-induced M-MDSCs to mediate immunosuppression.

Both donor- and recipient-derived M-MDSCs prolonged alloskin graft survival efficiently

To address whether M-CSF+IFN-γ-induced M-MDSCs had functional stability to mediate immune regulatory effect in vivo, we adoptively transferred M-CSF+IFN-γ-induced M-MDSCs or control differentiated myeloid cells to female recipient B6 mice. The following day, male B6 tail skin grafts were transplanted on recipient mice of all groups. To see the immunosuppressive ability of MDSCs derived from either donor or recipient origin, recipient and donor bone marrow cells from naïve female or male B6 mice were treated by M-CSF alone or M-CSF+IFN-γ for MDSC induction, respectively. The induced cells from female or male B6 mice were adoptively transferred to recipient female B6 mice at 5×10^6 per mouse and male skin transplantations were performed for all groups. The results showed that both donor and recipient M-CSF+IFN-γ-induced M-MDSCs significantly prolonged male skin allograft survival, but M-CSF-induced control cells failed to prolong skin graft survival (Fig. 5a, b). Importantly, donor-derived MDSCs have been shown to be more effective in protecting skin graft rejection than recipient-derived MDSCs in this model.

M-CSF+IFN-γ-induced M-MDSCs prolong alloskin graft survival via iNOS

To test the role of iNOS in M-MDSCs-mediated immunomodulation in vivo, M-CSF+IFN-γ-induced donor

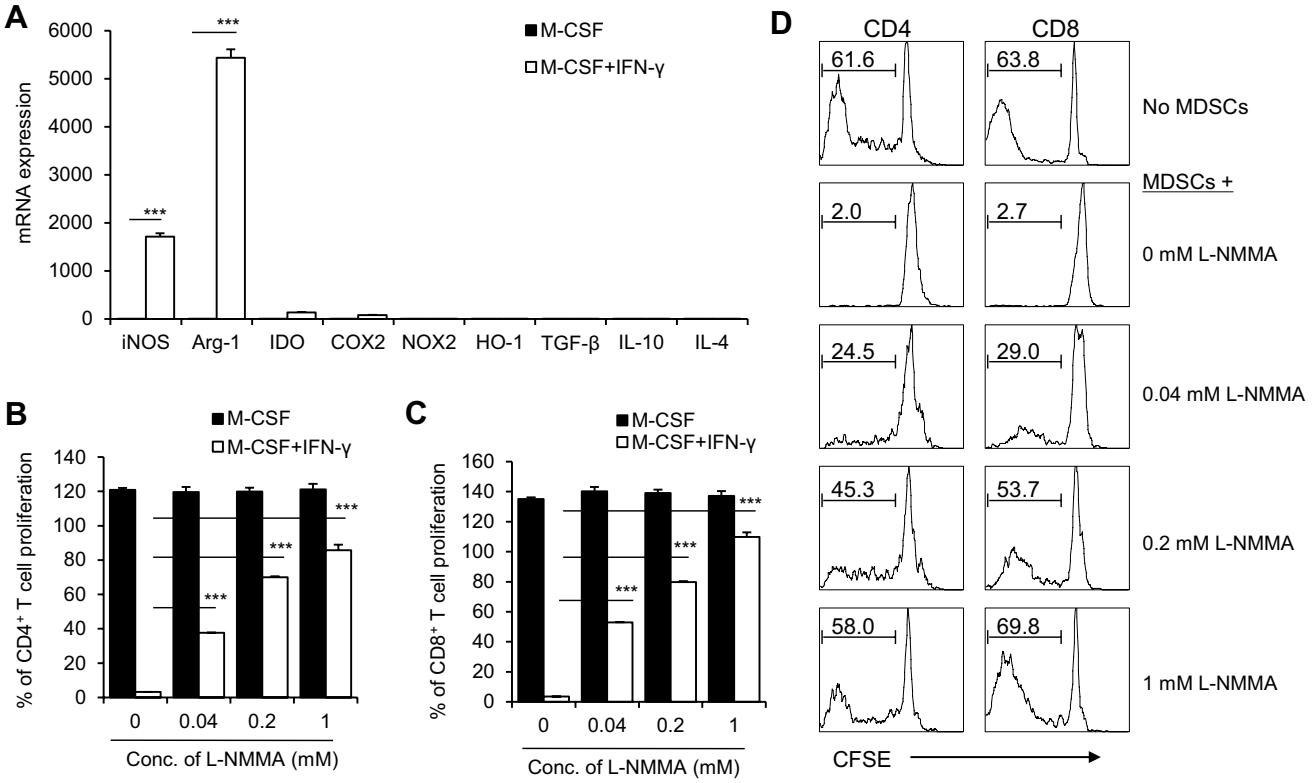

Fig. 4 The inhibitory effect on T cells of the induced M-MDSCs is mediated by iNOS. iNOS, Arg1, IDO, COX2, NOX2, HO-1, TGFβ, IL-10 and IL-4 mRNA expressions in M-CSF- and M-CSF+IFN-γ-induced M-MDSCs were determined by real-time PCR, data were summarized in **a**. Administration of iNOS inhibitor L-NMMA in T cell proliferation assays reversed the inhibitory effects of M-CSF+IFN-γ-induced M-MDSCs on $CD8^+$ (**b**) and $CD4^+$ (**c**) T cell proliferation (T cells-to-MDSC ratio = 1:1/2). **d** Typical example of flow cytometry analysis for T cell proliferation suppressed by M-CSF+IFN-γ-induced M-MDSCs and rescued by L-NMMA treatment (T cells-to-MDSC ratio = 1:1/2). 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

iNOS-deficient or WT M-MDSCs were transferred to B6 female recipient mice, respectively. Next day, donor skin grafts from male B6 mice were transplanted on female recipient mice with different kinds of transferred cells. The results showed that WT donor M-MDSCs induced by M-CSF+IFN-γ significantly prolonged the survival of allo-skin grafts. Some grafts survived even longer than 100 days (Fig. 6a, b). The iNOS-deficient M-MDSCs greatly reversed the regulatory effect of transferred M-MDSCs (Fig. 6a, b), suggesting a dominant role of iNOS for M-CSF+IFN-γ-induced M-MDSC function in vivo.

Discussion

In our present study, M-CSF+IFN-γ-induced M-MDSCs showed the ability to suppress T cell proliferation via iNOS. Based on our previous study, M-CSF was used as colony-stimulating factors but not GM-CSF with IFN-γ to extend MDSC induction system in vitro. Unlike GM-CSF which usually required stimulation for generation, M-CSF constitutively produced by many types of cells [29]. Since MDSCs mainly induced in emergency hematopoiesis, this growth factor was not paid enough attention by researchers for MDSC induction. Actually early research showed that M-CSF-induced macrophages co-cultured with T cells upregulated IDO for inhibiting T cell proliferation through activating T cells-derived IFN-γ [30]. M-CSF alone with low concentration also induced suppressive macrophages for immunomodulation [31]. These studies suggest that M-CSF can be used for M-MDSC induction. So we choose M-CSF for M-MDSC induction to get the pure monocyte-macrophage lineage with longer life span and survival time after adoptive transfer to recipients.

IFN-γ treatment significantly enhanced the immunosuppressive function of MDSC in our study. IFN-γ has long been considered to play paradoxical roles on immunity. In IFN-γ or its receptor deficient mice, increased susceptibility and severity were found after autoimmune disease induction such as EAE and CIA [32–34]. Immune regulatory effect mediated by IFN-γ was through acting on immune or non-immune cells. IFN-γ directly acted on T cell to mediate

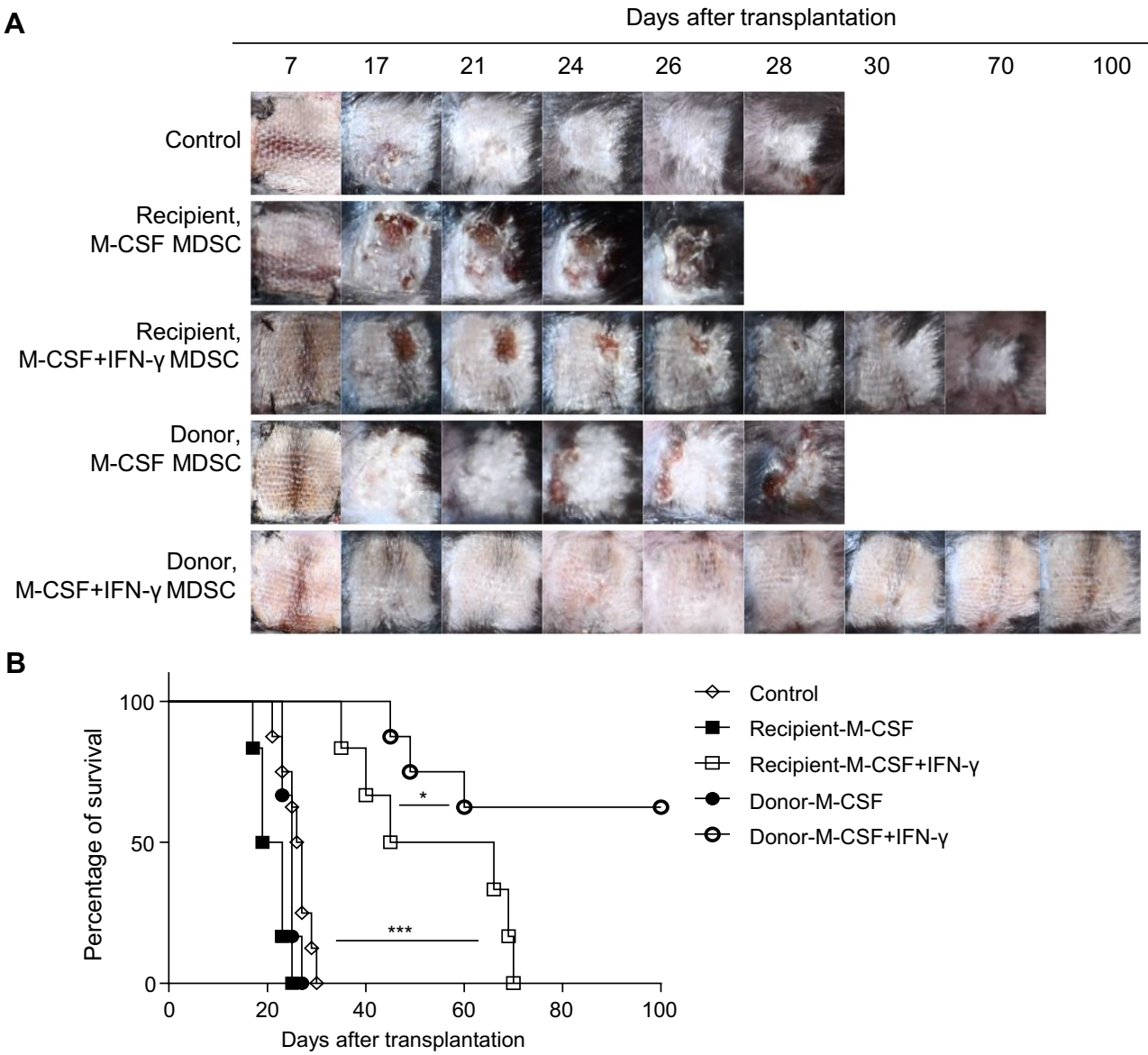

Fig. 5 Either donor or host M-MDSCs prolonged alloskin graft survival. Recipient female B6 mice were injected i.v. with recipient or donor bone marrow-derived M-MDSCs (5×10^6) induced by M-CSF or M-CSF+IFN-γ, respectively, on day 1 and donor male B6 skin allografts were transplanted on day 0. Recipient female mice in a control group were injected with vehicle PBS. **a** The digit photo representatives of graft rejection were shown. **b** The percentages of graft survival over time after transplantation were summarized ($n=6$). *P* values were determined using Log-rank tests. $^*P<0.05$, $^{***}P<0.001$ compared between the indicated groups

activation-induced cell death by promoting caspase-8-dependent apoptosis for expansion restriction [35]. During type-1 inflammation, IFN-γ endowed Treg functions for their recruitment and accumulation by upregulating T-bet [36]. Either exogenous or endogenous IFN-γ induced monocytes differentiated to tolerant DCs which promoted Treg induction [37]. In our results, IFN-γ also increased expression of iNOS in M-MDSCs. However, iNOS inhibition or iNOS deficiency remarkably reversed their immunosuppressive function in vitro and in vivo, indicating the involvement of iNOS in the immunosuppression of M-CSF+IFN-γ-induced MDSCs. NO produced by iNOS may inhibit alloreactive T cell function in vivo by inhibiting JAK3-STAT5 signaling, induction of T cell apoptosis and generation of Treg cells [38]. Consistent with our results, IFN-γ and M-CSF-induced regulatory macrophages from mouse monocytes almost completely dependent on iNOS for mediating immunosuppression [39]. In this paper, donor-derived regulatory macrophages significantly prolonged allo-skin graft survival. Our results also showed that donor-derived M-MDSCs prolong skin graft survival in a male skin-grafted female mouse model more efficiently than recipient-derived M-MDSCs.

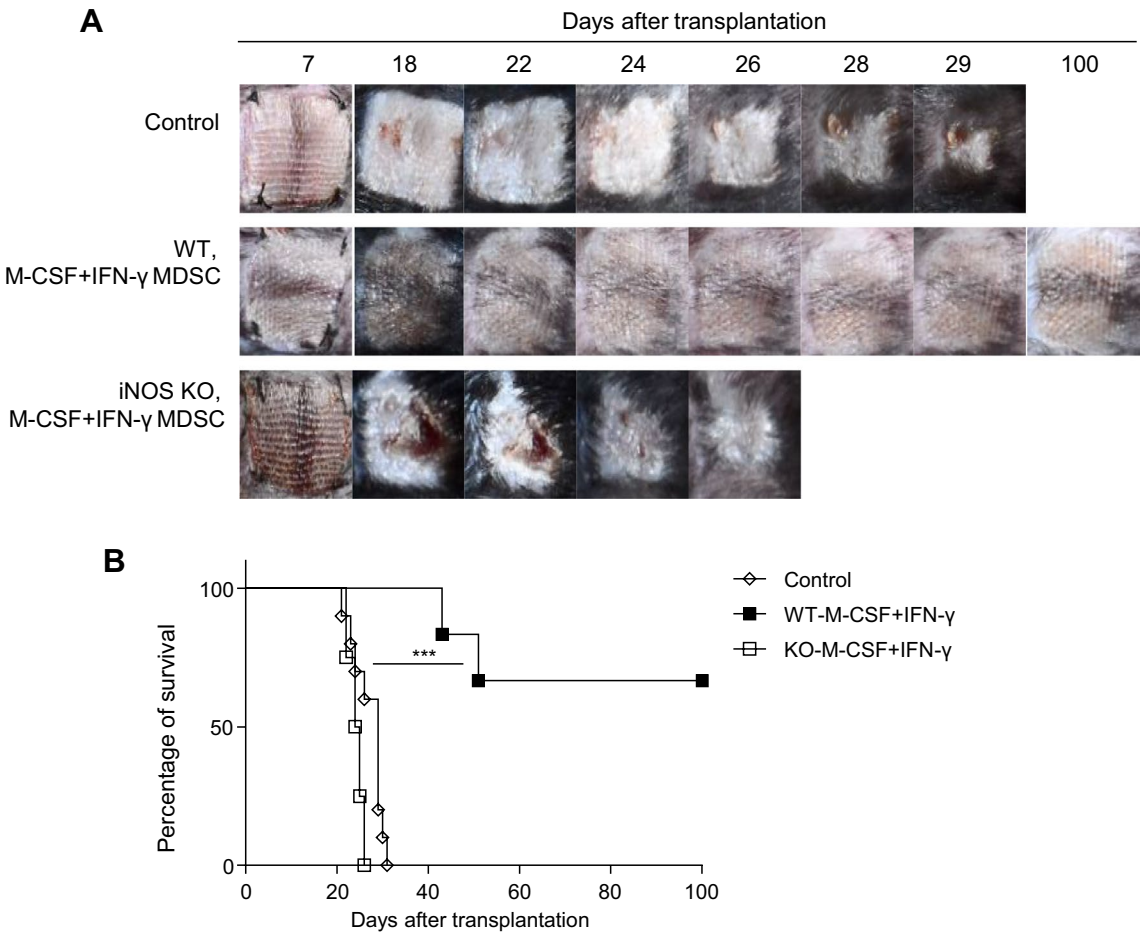

Fig. 6 Induced M-MDSCs prolonged alloskin graft survival via iNOS. Recipient female B6 mice were injected i.v. with WT or iNOS KO donor-derived M-MDSCs (5×10^6) on day-1 and donor male B6 skin allografts were transplanted on day 0. **a** The digit photo representatives of graft rejection were shown. **b** The percentages of graft survival after transplantation transferred with WT or iNOS KO M-MDSCs were summarized ($n=6$). *P* values were determined using Log-rank tests. ***$P<0.001$ compared between the indicated groups

This may be due to the fact that apoptotic donor cells themselves can promote graft survival [40]. Another possibility is that donor-derived MDSCs can induce more donor alloantigen-specific Treg cells. This issue should be addressed in the future.

The phenotype changes were also found. Enhanced expression of M-MDSC markers by IFN-γ treatment suggested that this signal partially blocked differentiation of bone marrow progenitors to macrophages. Previous results also showed that IFN-γ blocked monocyte differentiation to DCs induced by GM-CSF+IL-4 and switched the differentiation direction to macrophages [41]. Though M-CSF+IFN-γ-induced MDSCs showed immunosuppressive function with upregulated costimulatory molecule expressions in our study. This was similar to the reports of IFN-γ conditioned regulatory DCs [42]. It was reported that IFN-γ increased the expression of PD-L1 on MSCs, placenta-derived multipotent cells and tumor cells which then mediated immunomodulation by these cells [27, 43–45]. In our study, PD-L1 expression on M-CSF+IFN-γ-induced M-MDSCs also significantly increased. But their role for M-CSF+IFN-γ-induced M-MDSCs in transplant tolerance needs to be clarified.

In summary, the combination of M-CSF and IFN-γ can efficiently induce functional M-MDSCs which suppress T cell proliferation and cytokine productions. Adoptive transfer of these induced cells significantly prolong allograft survival via iNOS. These results lay the foundation for the potential clinical application of M-CSF+IFN-γ-induced M-MDSCs for immunomodulation, providing a promising regulatory cell induction system for cell therapy in transplantation.

Acknowledgements The authors appreciated Drs. Yuzhu Hou and Peng Wang for their critical reviewing our manuscript, and Mrs. Ling Li for her excellent laboratory management. This work was supported by Grants from the National Natural Science Foundation for General and Key Programs (81530049, U1738111, Y.Z.), the National Key Research and Development Program of China (2017YFA0105002,

2017YFA0104402, Y.Z.), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDA16030301, XDA16030300, Y.Z.), and The China Manned Space Flight Technology Project (TZ-1).

Compliance with ethical standards

Conflict of interest The authors herein declare that all authors have no competing financial interests.

References

1. Yang F, et al. The effect of immunosuppressive drugs on MDSCs in transplantation. J Immunol Res. 2018;2018:16.
2. Condamine T, Gabrilovich DI. Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. Trends Immunol. 2011;32(1):19–25.
3. Peranzoni E, et al. Myeloid-derived suppressor cell heterogeneity and subset definition. Curr Opin Immunol. 2010;22(2):238–44.
4. Bronte V, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun. 2016;7:12150.
5. Lu T, Gabrilovich DI. Molecular pathways: tumor infiltrating myeloid cells and reactive oxygen species in regulation of tumor microenvironment. Clin Cancer Res. 2012;18:4882.
6. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol. 2009;9(3):162–74.
7. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012;12(4):253–68.
8. De Wilde V, et al. Endotoxin-induced myeloid-derived suppressor cells inhibit alloimmune responses via heme oxygenase-1. Am J Transplant. 2009;9(9):2034–47.
9. Scalea JR, et al. Myeloid-derived suppressor cells and their potential application in transplantation. Transplantation. 2018;102(3):359–67.
10. Zhang Q, et al. The role and potential therapeutic application of myeloid-derived suppressor cells in allo- and autoimmunity. Mediators Inflamm. 2015;2015:421927.
11. Zhou Z, et al. Development and function of myeloid-derived suppressor cells generated from mouse embryonic and hematopoietic stem cells. Stem Cells. 2010;28(3):620–32.
12. Wu WC, et al. Circulating hematopoietic stem and progenitor cells are myeloid-biased in cancer patients. Proc Natl Acad Sci USA. 2014;111(11):4221–6.
13. Lechner MG, Liebertz DJ, Epstein AL. Characterization of cytokine-induced myeloid-derived suppressor cells from normal human peripheral blood mononuclear cells. J Immunol. 2010;185(4):2273–84.
14. Dolcetti L, et al. Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. Eur J Immunol. 2010;40(1):22–35.
15. Wu T, et al. mTOR masters monocytic myeloid-derived suppressor cells in mice with allografts or tumors. Sci Rep. 2016;6:20250.
16. Han C, et al. The effect of immunosuppressive drug cyclosporine A on myeloid-derived suppressor cells in transplanted mice. Inflamm Res. 2016;65(9):679–88.
17. Zhao Y, et al. Dexamethasone-induced myeloid-derived suppressor cells prolong allo cardiac graft survival through iNOS- and glucocorticoid receptor-dependent mechanism. Front Immunol. 2018;9:282.
18. Marigo I, et al. Tumor-induced tolerance and immune suppression depend on the C/EBP beta transcription factor. Immunity. 2010;32(6):790–802.
19. Drujont L, et al. Evaluation of the therapeutic potential of bone marrow-derived myeloid suppressor cell (MDSC) adoptive transfer in mouse models of autoimmunity and allograft rejection. PLoS One. 2014;9:6.
20. Highfill SL, et al. Bone marrow myeloid-derived suppressor cells (MDSCs) inhibit graft-versus-host disease (GVHD) via an arginase-1-dependent mechanism that is up-regulated by interleukin-13. Blood. 2010;116(25):5738–47.
21. Messmann JJ, et al. In vitro-generated MDSCs prevent murine GVHD by inducing type 2 T cells without disabling antitumor cytotoxicity. Blood. 2015;126(9):1138–48.
22. Greifenberg V, et al. Myeloid-derived suppressor cell activation by combined LPS and IFN-γ treatment impairs DC development. Eur J Immunol. 2009;39(10):2865–76.
23. Obermajer N, et al. PGE2-induced CXCL12 production and CXCR7 expression controls the accumulation of human MDSCs in ovarian cancer environment. Can Res. 2011;71(24):7463–70.
24. Ushach I, Zlotnik A. Biological role of granulocyte macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) on cells of the myeloid lineage. J Leukoc Biol. 2016;100(3):481–9.
25. Carretero-Iglesia L, et al. Comparative study of the immunoregulatory capacity of in vitro generated tolerogenic dendritic cells, suppressor macrophages, and myeloid-derived suppressor cells. Transplantation. 2016;100(10):2079–89.
26. Yang F, et al. TNFα-induced M-MDSCs promote transplant immune tolerance via nitric oxide. J Mol Med. 2016;94:1–10.
27. English K, et al. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. Immunol Lett. 2007;110(2):91–100.
28. Hwu P, et al. Indoleamine 2, 3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J Immunol. 2000;164(7):3596–9.
29. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol. 2008;8(7):533.
30. Munn DH, et al. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J Exp Med. 1999;189(9):1363–72.
31. Carretero-Iglesia L, Hill M, Cuturi MC. Generation and characterization of mouse regulatory macrophages. In: Cuturi CM, Anegon I, editors. Suppression and regulation of immune responses: methods and protocols, vol. II. New York: Springer; 2016. p. 89–100.
32. Ferber IA, et al. Mice with a disrupted IFN-gamma gene are susceptible to the induction of experimental autoimmune encephalomyelitis (EAE). J Immunol. 1996;156(1):5–7.
33. Manoury-Schwartz B, et al. High susceptibility to collagen-induced arthritis in mice lacking IFN-gamma receptors. J Immunol. 1997;158(11):5501–6.
34. Vermeire K, et al. Accelerated collagen-induced arthritis in IFN-gamma receptor-deficient mice. J Immunol. 1997;158(11):5507–13.
35. Refaeli Y, et al. Interferon γ is required for activation-induced death of T lymphocytes. J Exp Med. 2002;196(7):999–1005.
36. Koch MA, et al. T-bet controls regulatory T cell homeostasis and function during type-1 inflammation. Nat Immunol. 2009;10(6):595–602.
37. Eljaafari A, Li Y-P, Miossec P. IFN-gamma, as secreted during an alloresponse, induces differentiation of monocytes into tolerogenic dendritic cells, resulting in FoxP3+regulatory T cell promotion. J Immunol. 2009;183(5):2932.
38. Bogdan C, Röllinghoff M, Diefenbach A. Reactive oxygen and reactive nitrogen intermediates in innate and specific immunity. Curr Opin Immunol. 2000;12(1):64–76.

39. Riquelme P, et al. IFN-gamma-induced iNOS expression in mouse regulatory macrophages prolongs allograft survival in fully immunocompetent recipients. Mol Ther. 2013;21(2):409–22.
40. Ravishankar B, et al. The amino acid sensor GCN2 inhibits inflammatory responses to apoptotic cells promoting tolerance and suppressing systemic autoimmunity. Proc Natl Acad Sci USA. 2015;112(34):10774–9.
41. Delneste Y, et al. Interferon-γ switches monocyte differentiation from dendritic cells to macrophages. Blood. 2003;101(1):143–50.
42. Jurgens B, et al. Interferon-gamma-triggered indoleamine 2,3-dioxygenase competence in human monocyte-derived dendritic cells induces regulatory activity in allogeneic T cells. Blood. 2009;114(15):3235–43.
43. Spranger S, et al. Up-regulation of PD-L1, IDO, and T(regs) in the melanoma tumor microenvironment is driven by CD8(+) T cells. Sci Transl Med. 2013;5(200):200ra116.
44. Abiko K, et al. IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer. Br J Cancer. 2015;112:1501.
45. Chang CJ, et al. Placenta-derived multipotent cells exhibit immunosuppressive properties that are enhanced in the presence of interferon-γ. Stem Cells. 2006;24(11):2466–77.