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Original Article

Xenotransplantation of cryopreserved human clumps of mesenchymal stem cells/extracellular matrix complexes pretreated with IFN-γ induces rat calvarial bone regeneration

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ABSTRACT

Introduction: Three-dimensional (3D) clumps of mesenchymal stem cells (MSCs)/extracellular matrix (ECM) complexes, composed with cells and self-produced intact ECM, can be grafted into defect areas without artificial scaffold to induce successful bone regeneration. Moreover, C-MSCs pretreated with IFN-γ (C-MSCsγ) increased the immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) expression and thereby inhibited T cell activity. Xenotransplantation of human C-MSCsγ suppressed host T cell immune rejection and induced bone regeneration in mice. Besides, we have also reported that C-MSCs retain the 3D structure and bone regenerative property even after cryopreservation. To develop the "off-the-shelf" cell preparation for bone regenerative therapy that is promptly provided when needed, we investigated whether C-MSCsγ can retain the immunosuppressive and osteogenic properties after cryopreservation.

Methods: Confluent human MSCs that had formed on the cellular sheet were scratched using a micropipette tip and then torn off. The sheet was rolled to make a round clump of cells. The round cell clumps were incubated with a growth medium for 3 days, and then C-MSCs were obtained. To generate C-MSCsγ, after 2 days' culture, C-MSCs were stimulated with 50 ng/ml of IFN-γ. Both C-MSCs and C-MSCsγ were cryopreserved for 2 days and then thawed to obtain Cryo-C-MSCs and Cryo-C-MSCsγ, respectively. The biological properties of those cell clumps were assessed *in vitro*. In addition, to test whether human Cryo-C-MSCsγ attenuates immune rejection to induce bone regeneration, a xenograft study using a rat calvarial defect was performed.

Results: Both IFN-γ pretreatment and cryopreservation process did not affect the 3D structure and cell viability in all human cell clumps. Interestingly, Cryo-C-MSCsγ showed significantly increased IDO mRNA expression equivalent to C-MSCsγ. More importantly, xenotransplantation of human C-MSCsγ and Cryo-C-MSCsγ induced rat calvarial bone regeneration by suppressing rat T cells infiltration and the grafted human cells reduction in the grafted area. Finally, there were no human donor cells in the newly formed bone, implying that the bone reconstruction by C-MSCsγ and Cryo-C-MSCsγ can be due to indirect host osteogenesis.

Conclusion: These findings implied that Cryo-C-MSCsγ can be a promising bone regenerative allograft therapy that can be certainly and promptly supplied on demand.

1. Introduction

Bone plays a pivotal role in shielding the organs, supporting the body structure, and maintaining homeostasis by providing

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minerals, growth factors, and blood cells [1,2]. Bones are relatively regenerative tissues due to their unique remodeling system. However, there are severe bone defect cases plunged into irreversible conditions due to pathological fractures, infection, inflammation, tumor resection, and aging [3,4]. Thus, developing a novel promising bone regenerative therapy is still in demand.

Mesenchymal stem cells (MSCs), a class of adult stem cells, have attracted much attention for tissue regenerative cell therapy because of their multipotency, trophic effects, and self-renewing capacity [5–8]. Especially, bone marrow-derived MSCs, which can be comparatively easily isolated, have been well-studied stem cells for bone tissue engineering applications, both in the basic research and clinical practice [9–11]. Indeed, several clinical trials that graft the autologous bone marrow-derived MSCs by using artificial scaffolds have reported promising results [12]. However, to apply MSCs as an established bone regenerative medicine in clinical settings, there still remain several obstacles to be overcome.

One of the problems is due to patient age. Bone fractures prevalence is increasing in an ever-aging society [13], and the difficulty of fractured bone treatment in aged patients is widely accepted. Importantly, it is well reported that aging disrupts the self-renewal capacity and multipotency of MSCs [14,15]. Thus, it is difficult for elderly patients to isolate sufficient numbers of functional MSCs from their bone marrow for bone regenerative therapy. To solve this problem, MSCs allograft therapy applying the cells from a healthy donor will be utilized, though it needs to avoid the host immune rejection.

The second problem is the graft preparation process. Even using the allogeneic donor cells preserved at the cell bank, combining the MSCs with an artificial scaffold requires an extended culture period and adequate quality control examination. This graft preparation process is inevitably time-consuming. Notably, for irreversible bone defect diseases, such as severe bone fracture, prompt treatment is a key to achieving successful bone regeneration. In other words, a stockpiling system of MSCs grafts, which can be promptly supplied at the time when the patient needs them, should be developed.

Recently, we have developed three-dimensional (3D) clumps of MSCs/ECM complexes (C-MSCs), which consisted of cells and self-produced ECM [16]. C-MSCs can be grafted into the defect areas with no artificial scaffold to induce successful bone and periodontal tissue regeneration [16,17]. Moreover, we have reported that C-MSCs pretreated with IFN-γ (C-MSCsγ) increased a robust immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) expression and thereby inhibited T cell activity. Xenotransplantation of human C-MSCsγ suppressed host T cell immune rejection and induced bone regeneration in mice calvarial defect model [18]. These findings indicated a possibility that C-MSCsγ can be applied for allograft bone regenerative cell therapy. Besides, we have also revealed that rat C-MSCs can retain the 3D structure and osteogenic capacity even after cryopreservation. Indeed, transplantation of rat C-MSCs cryopreserved for 6 months induced successful bone regeneration equivalent to normal C-MSCs in the rat calvarial defect model [19].

Interestingly, a recent study unveiled that cryopreserved MSCs that are pretreated with IFN-γ before the cryopreservation showed higher IDO expression and immunomodulatory property than that of cryopreserved MSCs with no pretreatment [20]. Based on these accumulating lines of evidence, we hypothesized that C-MSCsγ, which exerts immunomodulatory property by up-regulated IDO expression, can retain the cellular function through the cryopreservation process. If so, cryopreserved C-MSCsγ generated from the allogenic donor cells can be reliable “off-the-shelf” cell preparation for bone regenerative therapy that is promptly provided as a standardized material when needed. Thus, in this present study, we assessed the effect of cryopreservation on human C-MSCsγ *in vitro*. Besides, the bone regenerative capacity of human cryopreserved C-MSCsγ xenotransplantation was tested in a rat calvarial defect model.

2. Methods

2.1. Preparation of human C-MSCs and C-MSCsγ

Human bone marrow-derived MSCs (MSC-R37 and -R14) were provided from RIKEN BioResource Center (Ibaragi, Tsukuba, Japan). The cells were cultured with Dulbecco's modified Eagle's Medium (DMEM, Sigma–Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, Sigma–Aldrich), 100 U/mL penicillin (Sigma–Aldrich), and 100 μg/mL streptomycin (Sigma–Aldrich). The cells at the third passage were employed to generate C-MSCs as previously reported with minor modifications [18]. Briefly, MSCs were seeded at a density of 1.0×10^5 cells/well in 48-well plates (Corning, Corning, NY) and maintained in high-glucose DMEM (Sigma–Aldrich) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (growth medium), and 50 μg/ml L-ascorbic acid (Sigma–Aldrich) for 4 days. To obtain three-dimensional C-MSCs, confluent cells that had formed on the cellular sheet, consisting of the ECM proteins, were scratched using a micropipette tip and then torn off. The sheet shape MSCs/ECM complexes detached from the bottom of the culture plate were transferred to a 24-well ultra-low-binding plate (Corning) and rolled up to make a round clump of cells. The round cell clumps were incubated with a growth medium for 3 days, and then C-MSCs were obtained. To generate C-MSCsγ, after 2 days' culture, C-MSCs were stimulated with 50 ng/ml of IFN-γ (Peprotech, Rocky Hill, NJ, USA) for 24 h. The C-MSCs exposed to IFN-γ are hereafter called C-MSCsγ. Several C-MSCs and C-MSCsγ were cryopreserved as described below.

2.2. Cryopreservation procedure

The C-MSCs or C-MSCsγ were cryopreserved as previously reported with minor modification [19]. This study employed STEMCELLBANKER® DMSO free GMP grade (Takara, Tokyo, Japan) for the cryopreservation solution. Briefly, one C-MSCs or C-MSCsγ was soaked in 500 μL cryopreservation solution and transferred into a cryotube vial (Nunc cryotube®, Thermo Scientific, Waltham, MA). Then, the samples were placed into a deep-freezer set at −80 °C. After 2 days of cryopreservation, the samples were rapidly thawed in a 37 °C water bath until no ice was detectable. The cell clumps were washed thoroughly to remove cryomedium and then incubated with a growth medium in a 24-well ultra-low-binding plate. The C-MSCs or C-MSCsγ recovered from this cryopreservation process hereafter are referred to as Cryo-C-MSCs or Cryo-C-MSCsγ, respectively.

2.3. Histological analysis for C-MSCs

Cultured cell clumps were fixed with 4% paraformaldehyde and embedded in paraffin. Five-micrometer-thick semi-serial sections were prepared. The samples were then stained with hematoxylin and eosin (H&E) and observed using NIKON ECLIPSE E600 microscope (NIKON, Tokyo, Japan). To detect apoptotic cells, the sectioned samples were assessed using a DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI) according to the manufacturer's instruction. Fluorescence signals were detected by using the Olympus FV1000D laser scanning confocal microscopy (Olympus, Tokyo, Japan).

2.4. Real-time polymerase chain reaction (PCR)

Total RNA from each cultured C-MSCs, C-MSCsγ, Cryo-C-MSCs, and Cryo-C-MSCsγ was extracted using RNA-iso (Takara) and quantified by spectrometry at 260 and 280 nm. First-strand complementary DNA was synthesized with 1 μg of total RNA using ReveTraAce (Toyobo, Osaka, Japan). Then, real-time PCR was performed in a StepOne™ system (Applied Biosystems, Waltham, MA) using SYBR green (Roche Applied Science, Mannheim, Germany) to determine the relative mRNA expression of *IDO*, alkaline phosphatase (*ALP*), osteopontin (*OPN*), and bone morphogenetic protein 2 (*BMP-2*). Fold changes in these genes of interest were calculated with ΔΔCt method using *18S* as a reference control. The sequences of the primes are lites in Table 1.

2.5. Surgical procedures

Male eight-week-old F344 rats (Charles River Laboratories Japan, Yokohama, Japan) were employed as a calvarial defect model after obtaining approval from the Animal Care Committee of Hiroshima University (protocol number: A14-147). Animals were anesthetized with an intraperitoneal injection of medetomidine (0.3 mg/kg), midazolam (4 m/kg), and butorphanol tartrate (5 mg/kg). The skin at the surgical region was shaved and disinfected, and a sagittal skin incision was made from the occipital to the frontal bone. Avoiding the cranial suture, calvarial defects of 1.6 mm diameter was created in parietal bones by using rotatory instruments. One human C-MSCs, C-MSCsγ, Cryo-C-MSCs, or Cryo-C-MSCsγ was directly grafted into the defect with no artificial scaffold, respectively (n = 6/each group). Then, the skin was closed using 4-0 silk sutures (Mani, Tochigi, Japan).

2.6. Micro-CT analysis

Rats were sacrificed at 8 weeks after surgery, and the cranial region was imaged using a SkyScan1176 *in vivo* μCT with the following conditions: 50 kV, 0.5 mA, 8 μm pixel size, and 0.5° rotation step with 230 ms exposure time. Three-dimensional reconstructions were generated using CTVOL software (Bruker). The region of interest (ROI) for bone volume measurement was the 1.6-mm circle of the bony lesion that consists of 30 2D slices (approximately 0.6 mm thickness). Segmentation of the ROI and following bone volume measurement were performed by CT-An software (Bruker) with a threshold range of 80–255.

2.7. Tissue preparation and histological analysis

Animals were sacrificed at 1, 3, and 8 weeks after surgery. Calvarial bones were collected and fixed with 4% paraformaldehyde overnight. The samples were then decalcified with 10% ethylenediaminetetraacetic acid (pH 7.4) for 10 days. After decalcification, the specimens obtained from 8 weeks observation groups were embedded in paraffin. Serial sections (5 μm) were cut in the frontal plane. These sections, representing the central portion of the bony lesion, were stained with H&E and observed using the NIKON ECLIPSE E600 microscope. To visualize the rat CD3 T cells or human vimentin-positive cells by immunofluorescence analysis, the fixed and decalcified samples obtained from 3 weeks observation groups were embedded in Tissue-TEC OCT compound (Sakura, Torrance, CA). Semi-serial sections (20 μm) were cut using a cryostat. The sections were washed with PBS, and then non-specific binding was blocked with Blocking one Histo (Nacalai Tesque, Kyoto, Japan). These sections were incubated with a mouse anti-rat CD3 IgG antibody (clone G4.18; 1:100; Novus Biologicals, Littleton, CO) or a rabbit anti-human vimentin IgG antibody (clone SP20, 1:100, Abcam, Cambridge, MA) at 4 °C overnight. After being washed 3 times with PBS, samples were treated with an Alexa Fluor 594® goat anti-mouse IgG antibody (1:100; Invitrogen, Carlsbad, CA) or an Alexa Fluor 488® goat anti-rabbit IgG antibody (1:100; Invitrogen) for 2 h at room temperature. Nuclei were counterstained with DAPI (5 μg/mL; Invitrogen). After washing the samples with PBS, fluorescence signals were detected using the Olympus FV1000D laser scanning confocal microscope (Olympus, Tokyo, Japan).

Table 1
Sense primers and antisense primers for real-time PCR.

Target gene		*Primer sequence*
IDO	Forward	5′-CAAAGGTCATGGAGATGTCC-3′
	Reverse	5′-CCACCAATAGAGAGACCAGG-3′
ALPase	Forward	5′-GCGGTGAACGAGAGAATG-3′
	Reverse	5′-CGTAGTTCTGCTCGTGCAC-3′
BMP-2	Forward	5′-CTGTATCGCAGGCACTCA-3′
	Reverse	5′-CTCCGTGGGGATAGAACTT-3′
OC	Forward	5′-GCAGCGAGGTAGTGAAGAGAC-3′
	Reverse	5′-GGTCAGCCAACTCGTCACAG-3′
OPN	Forward	5′-GATGGCCGAGGTGATAGTGT-3′
	Reverse	5′-CCATTCAACTCCTCGCTTTC-3′
18S	Forward	5′- GTAACCCGTTGAACCCCATT-3′
	Reverse	5′- CCATCCAATCGGTAGTAGCG -3′

2.8. Statistical analysis

One-way ANOVA with Tukey–Kramer post hoc test was conducted. Any p values less than 0.05 were considered to be significant.

3. Results

3.1. Cryo-C-MSCγ retains the 3D structure and high IDO expression

Three-dimensional (3D) C-MSCs and C-MSCsγ, pretreated with IFN-γ for 24 h, were generated from human bone marrow-derived MSCs as described in the Materials and Methods section. Then, both cell clumps were cryopreserved for 48 h to generate Cryo-C-MSCs and Cryo-C-MSCsγ as shown in the schematic Fig. 1A. IFN-γ stimulation did not affect the 3D structure evidenced by HE staining image of C-MSCsγ (Fig. 1B). Notably, both Cryo-C-MSCs and Cryo-C-MSCsγ also retained the 3D round shape composed of cells and self-produced intact ECM proteins (Fig. 1B). Besides, there was no significant difference in the number of TUNEL-positive apoptotic cells among all cultured cell clumps, suggesting that IFN-γ exposure and cryopreservation did not affect the cell viability in C-MSCs (Fig. 1C and D).

We have previously reported that C-MSCs pretreated with IFN-γ, i.e., C-MSCsγ, the high expression level of IDO [18], which is a robust immunomodulatory enzyme. Thus, we have next investigated whether Cyro-C-MSCγ also retains the IDO expression level. In addition, to apply the Cryo-C-MSCsγ for a bone regenerative therapy, osteogenic marker expressions were also tested. Consistent with our previous report, compared to C-MSCs, C-MSCsγ, pretreated with IFN-γ for 24 h, showed drastically increased IDO mRNA expression level (Fig. 2A). More importantly, Cryo-C-MSCsγ also expressed high IDO mRNA expression equivalent to C-MSCsγ, whereas its expression level in Cryo-C-MSCs was not increased (Fig. 2A), suggesting that the high IDO mRNA expression in C-MSCsγ can be maintained after cryopreservation. On the other hand, neither IFN-γ stimulation nor cryopreservation did not reduce osteogenic marker genes expression, including *OPN*, *ALP*, *OCN*, and *BMP*-2 mRNA (Fig. 2B–E), implying that C-MSCsγ and

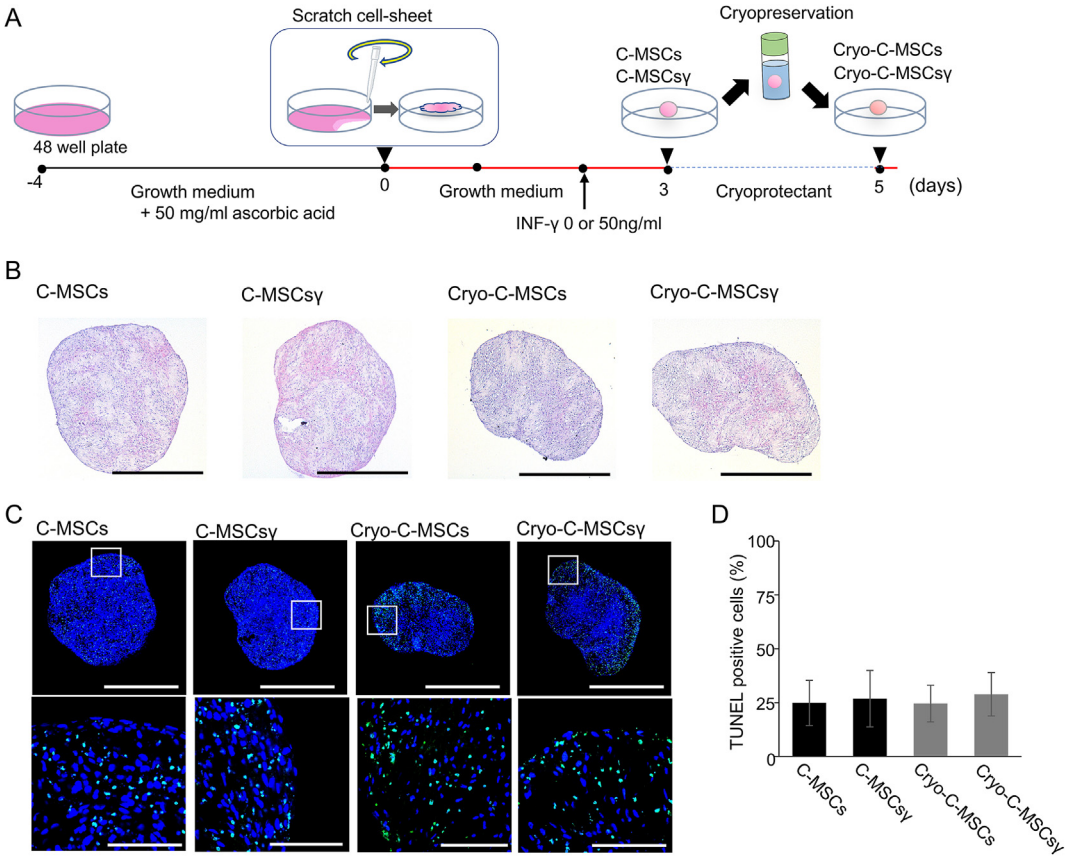

Fig. 1. C-MSCs and C-MSCsγretain the 3D structure and cell viability after cryopreservation. (A) Schematic images of C-MSCs, C-MSCsγ, Cryo-C-MSCs, and Cryo-C-MSCsγ. (B and C) All generated human cell clumps as described in A and semi-serial sections were stained with HE (B) and TUNEL (C). (B) Bar = 500 μm. (C) The upper panel indicates the lower magnification images. Bar = 500 μm. Higher magnified images in the boxed regions are shown in the lower panels. Bar = 50 μm. (D) The graph shows the percentage of TUNEL-positive apoptotic cells. Values represent means ± S.D. of four cultured samples. Images and graphs are representative of three independent experiments.

Cryo-C-MSCsγ may possess the comparable osteogenic capacity to normal C-MSCs. Based on the findings in Figs. 1 and 2, it is demonstrated that Cryo-C-MSCsγ retained the 3D structure consisting of ECM protein, cell viability, and immunomodulatory property even after the cryopreservation process.

3.2. Xenotransplantation of human C-MSCsγ and Cryo-C-MSCsγ into a rat calvarial defect model induces bone regeneration

To assess the possibility of human cell clumps allograft bone regenerative therapy, in this present study, we tested the effect of human C-MSCs, C-MSCsγ, Cryo-C-MSCs, and Cryo-C-MSCsγ xenografts into a rat calvarial bone defect model. Micro-CT image showed unsuccessful bone regeneration in the human C-MSCs grafted group at 8 weeks after surgery (Fig. 3A). Of note, both human C-MSCsγ and Cryo-C-MSCsγ transplantation significantly induced bone regeneration in the rat bony lesion area, whereas Cyro-C-MSCs caused only a slight one (Fig. 3A and B). To confirm these findings from micro-CT images, histological analysis using HE staining was also performed. Most defect areas were filled with granulation-like tissue in human C-MSCs and Cryo-C-MSCs implantation groups (Fig. 3C). On the other hand, consistent with the micro-CT images, C-MSCsγ and Cryo-C-MSCsγ induced the new lamellar bone to form the periphery of the defects (Fig. 3C).

3.3. C-MSCsγ and Cryo-C-MSCsγ suppresses rat T cell xenoreactivity in grafted regions to induce host indirect osteogenesis

Based on the findings in Fig. 3, we speculated that both C-MSCs and Cryo-C-MSCs caused rat T cells immune response, which could be responsible for a failure of bone regeneration, though C-MSCsγ and Cryo-C-MSCsγ ameliorated such xeno-immune rejection. To test this tentative hypothesis, we assessed the early-stage of human cell clumps xenotransplantation into rat calvarial defects. Among all tested groups, few rat CD3 positive T cells were infiltrated into the defect area after 1-week human cells transplantation (Supplementary Fig. 1). Three weeks after surgery, HE staining demonstrated the agglomerated thick connective tissue in all tested defect areas that could be descended from grafted human cell clumps (Fig. 4A). Importantly, CD3 positive T cells were apparently observed in the human C-MSCs and Cryo-C-MSCs grafted area (Fig. 4B and C). However, the number of CD3 positive T cells was significantly reduced in the human C-MSCsγ xenotransplantation group (Fig. 4B and C). More importantly, there were also few CD3 positive T cells in the human Cryo-C-MSCsγ grafted group (Fig. 4B and C), suggesting that C-MSCsγ and Cryo-C-MSCsγ may attenuate the host immune rejection.

Then, to assess the fate of human donor cells in the process of xeno-immune rejection and bone regeneration in the rat

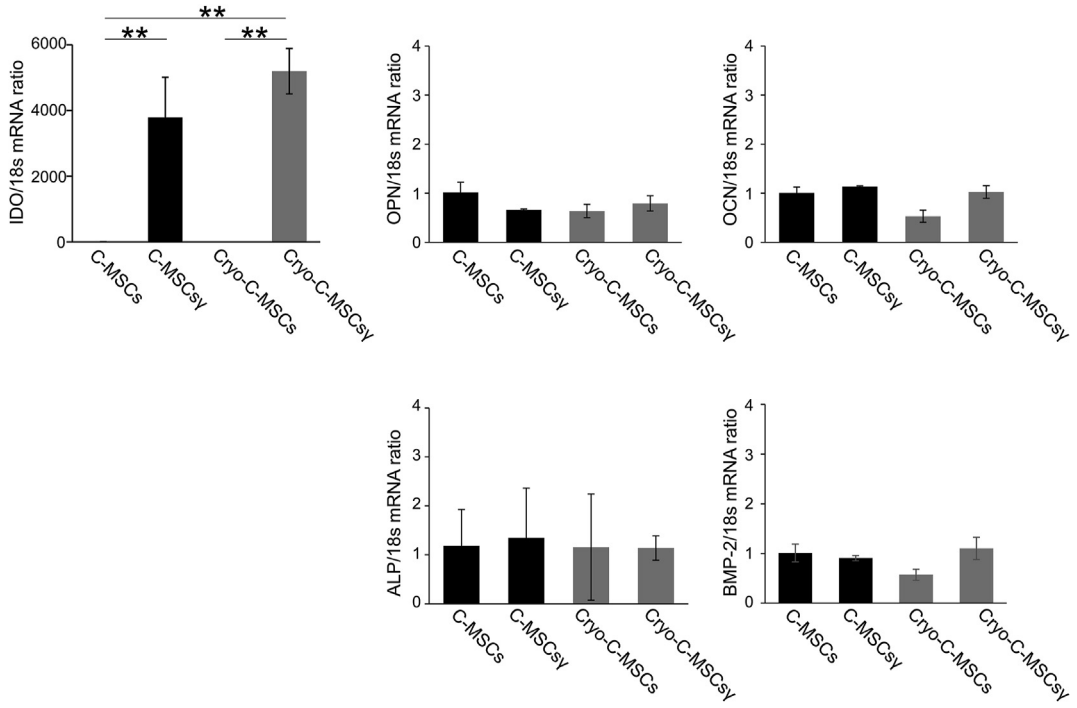

Fig. 2. Immunomodulatory enzyme IDO and osteogenic marker genes expression in generated cell clumps. C-MSCs, C-MSCsγ, Cryo-C-MSCs, and Cryo-C-MSCsγ were generated as described in the Methods section. The expression levels of IDO, OPN, ALP, OCN, and BMP-2 were analyzed by real-time CPR with ΔΔCt method by using 18S as a reference control. Data were normalized to the values of C-MSCs. Values represent means ± S.D. of three cultured samples. $^{**}p < 0.01$ (ANOVA). All graphs are representative of four independent experiments.

calvarial defects, immunofluorescence analysis using an anti-human vimentin antibody was conducted. At 1 week after transplantation, when there were few host T cells infiltration, the equivalent number of human vimentin-positive cells were observed among all tested groups (Fig. 5A and B). However, compared to C-MSCsγ or Cryo-C-MSCsγ grafted groups, the numbers of human vimentin-positive cells were drastically reduced in the human C-MSCs or Cryo-C-MSCs transplanted area after 3 weeks of surgery (Fig. 5C and D) accompanied by the increased number of rat CD3-positive T cells (Fig. 4), implying the rejection of human donor cells by host rat immune cells. Notably, even in the human C-MSCsγ or Cryo-C-MSCsγ implanted groups, very few human vimentin-positive cells were detected in the region where the new bone formation was observed 8 weeks after surgery (Fig. 5E and F). These findings may suggest that bone regeneration caused by C-MSCsγ or Cryo-C-MSCsγ is due to the indirect osteogenesis of host rat cells.

4. Discussion

In this present study, we demonstrated that human C-MSCsγ could retain the 3D structure, cell viability, and increased IDO mRNA expression even after cryopreservation. More importantly, xenotransplantation of human Cryo-C-MSCsγ ameliorated rat T cells infiltration and held the human donor cells in the grafted region to induce bone regeneration, as well as the human C-MSCsγ.

Cryopreservation is one of the essential factors for reliable tissue regenerative cell therapy using MSCs. Great scientific efforts have developed promising cryoprotectants or cooling systems that do not affect the MSCs cellular function, though those techniques mainly targeted the cell single suspension [21]. Despite these advanced cryopreservation studies, it is still challenging to cryopreserve the cells-artificial scaffold constructs, which should be an ideal approach for regenerative cell therapy to provide the cell constructs with ready-to-use characteristics. The difficulty may be attributed to cell-substrate contact being more sensitive to cryoinjury, followed by cell detachment and death [22–24]. As far as we know, only a few studies have succeeded in cryopreserving the adherent vital MSCs on artificial scaffolds [25,26], whereas those tissue regenerative capacities were not assessed. In this context, it is noteworthy that cryopreserved rat C-MSCs retain the 3D structure, composed of the cells and ECM proteins, and bone regenerative property [19]. Moreover, we have previously demonstrated that the ECM proteins, including type I collagen, exert the cytoprotective role against cryoinjury [19]. Consistent with our previous reports, since human C-MSCs and C-MSCsγ are also maintained their fundamental characteristics after cryopreservation, there might be a similar cytoprotective mechanism by ECM proteins. Taken together, the C-MSCs culture technique, which can utilize reliable cryopreservation, will be a novel promising tissue engineering approach.

In this decade, MSCs have attracted much scientific attention due to their immunomodulatory property in the immunological study field. Especially, the studies controlling such immune-suppressive function of MSCs have been well conducted. Krampera et al., initially reported that MSCs primed by IFN-γ (MSCsγ) significantly inhibited T cell proliferation, and its molecular mechanism was dependent on the increased IDO expression [27]. Following this excellent former report, nowadays, many studies have demonstrated the effectiveness of MSCsγ highly expressing IDO for immunological disorders [28–30]. Diverting this concept, we have previously generated C-MSCsγ to achieve effective bone regenerative cell therapy, which can avoid the host immune rejection. Besides, in this present study, we developed human Cryo-C-MSCsγ that can retain the up-regulated IDO mRNA expression *in vitro*. More importantly, we have demonstrated that human C-

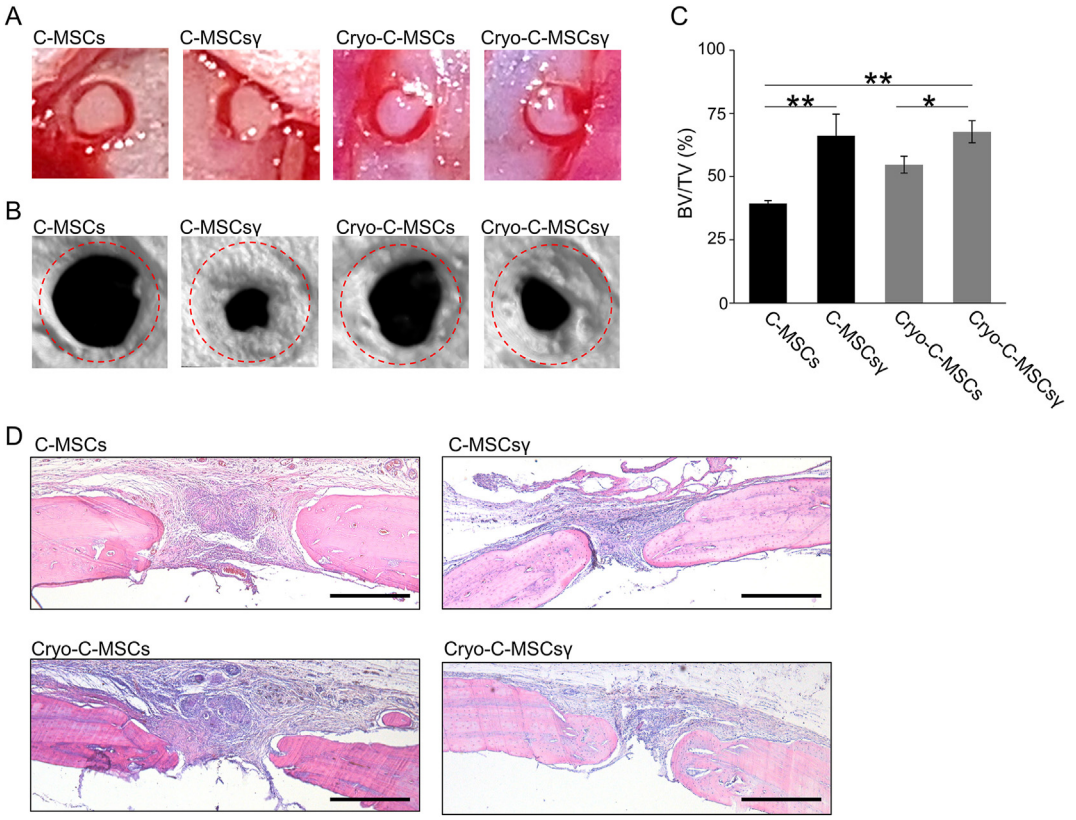

Fig. 3. Xenotransplantation of human C-MSCsγ and Cryo-C-MSCsγ induced bone regeneration in a rat calvarial defect model. (A) Cultured human C-MSCs, C-MSCsγ, Cryo-C-MSCs, Cryo-C-MSCsγ were transplanted into a rat calvarial defect 1.6 mm in diameter with no artificial scaffold. Macroscopic images are shown. (B) Representative micro-CT images of six samples at 8 weeks of surgery. (C) The graph shows the ratio of the segmented bone volume (BV) to the total volume (TV) of the defect region. Values are mean ± S.D. of six mice per group. *$p < 0.05$ (ANOVA). (D) Animals were sacrificed at 8 weeks after surgery, and the calvarial bones were fixed. Coronal sections were obtained and stained with HE. Bar = 500 μm.

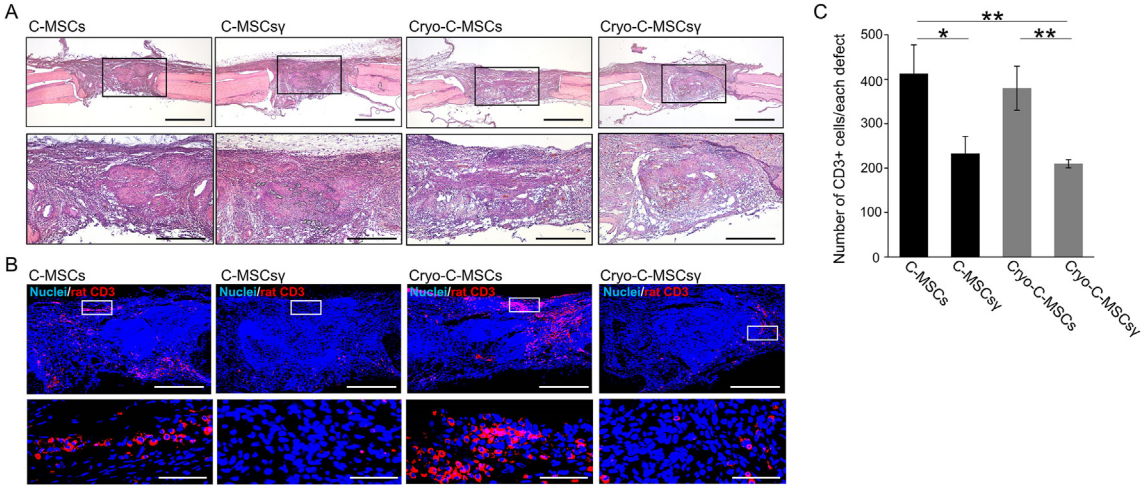

Fig. 4. Human C-MSCsγ and Cryo-C-MSCsγ suppressed rat T cells infiltration in a rat calvarial defect model. (A and B) Cultured human C-MSCs, C-MSCsγ, Cryo-C-MSCs, Cryo-C-MSCsγ were transplanted into a rat calvarial defect 1.6 mm in diameter with no artificial scaffold. Animals were sacrificed at 3 weeks after surgery, and the calvarial bones were fixed. Coronal serial sections were obtained and stained with HE (A) or immunostained with anti-rat CD3 antibody (B). Nuclei were counterstained with DAPI. The upper panel indicates the lower magnification images. Higher magnified images in the boxed regions are shown in the lower panels. (A) Bar = 500 μm (upper panels) and 250 μm (lower panels). (B) Bar = 250 μm (upper panels) and 50 μm (lower panels). (C) Graph shows the number of rat CD3-positive cells in the total defect area. Values are mean ± S.D. of six mice per group. **$p < 0.01$ (ANOVA).

MSCsγ or Cryo-C-MSCsγ showed a reduced number of rat CD3-positive T cells accompanied by the increased human donor cells survival in the xeno-graft region in rats (Figs. 4 and 5), suggesting that both these two cell clumps can ameliorate xeno-immune rejection. Unfortunately, the precise molecular mechanism of how C-MSCsγ and Cryo-C-MSCsγ attenuated the T cell response *in vivo* is still unclear. Since human C-MSCsγ inhibited human T cell proliferation via the increased IDO *in vitro* [18], the inhibition of T

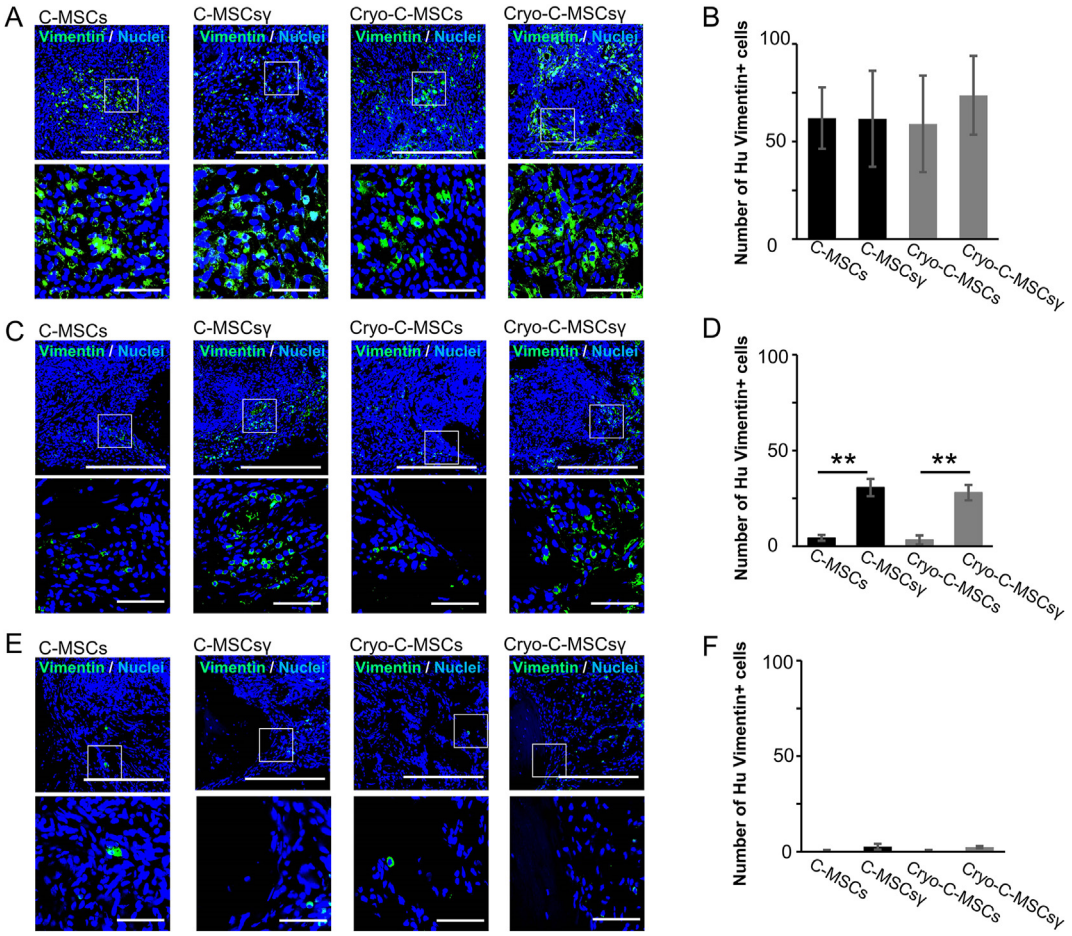

Fig. 5. The fate of grafted human donor cells in the process of xeno-immune response and bone regeneration in rat calvarial defect model. Cultured human C-MSCs, C-MSCsγ, Cryo-C-MSCs, Cryo-C-MSCsγ were transplanted into a rat calvarial defect 1.6 mm in diameter with no artificial scaffold. Animals were sacrificed at 1 (A and B), 3 (C and D), and 8 weeks (E and F) after surgery, and the calvarial bones were fixed. Coronal serial sections were obtained and immunostained with anti-human vimentin antibodies (A, C, and E). Nuclei were counterstained with DAPI. The upper panel indicates the lower magnification images. Higher magnified images in the boxed regions are shown in the lower panels. Bar = 250 μm (upper panels) and 50 μm (lower panels). (B, D, and F) Graphs show the number of human vimentin-positive cells in the higher magnified images. Values are mean ± S.D. of six mice per group. $^{**}p < 0.01$ (ANOVA).

cell infiltration by C-MSCsγ and Cryo-C-MSCsγ might be explained by IDO. However, this present study lacked the loss of function assay by using IDO inhibitor. Besides, emerging evidence discovered the other responsible molecules for the immunomodulatory property of MSCγ. For instance, Zhang et al. reported that interleukin 10 (IL-10) plays a role in the immunosuppressive function of MSCγ [31]. Otherwise, IFN-γ treatment up-regulates programmed cell death ligand 1 (PD-L1), which binds to programmed cell death 1 (PD-1) in immune cells to attenuate their activity [32]. Accordingly, not only IDO but also the other cytokines or cell surface proteins, including IL-10 or PD-L1, may be candidates explaining the immunosuppressive property of human C-MSCsγ and Cryo-C-MSCsγ in our xenograft system.

Previous studies reported the high concentration of IFN-γ ameliorates MSCs multipotency, especially osteogenic capacity [33,34]. Thus, based on our previous report [18] and the preliminary study, we employed 50 ng/ml of IFN-γ as the appropriate concentration to develop Cryo-C-MSCsγ for xenograft bone regenerative study. Indeed, C-MSCsγ and Cryo-C-MSCsγ expressed similar levels of osteogenic genes compared to untreated C-MSCs (Fig. 2). However, it is suspicious whether the bone regeneration by C-MSCsγ and Cryo-C-MSCsγ was due to the donor cells' direct osteogenesis. We have previously reported that C-MSCs pretreated with osteoinductive medium (OIM) increased osteogenic capacity *in vitro* [35], and caused more effective bone and periodontal tissue regeneration than that of normal C-MSCs [16,17]. In addition, a part of the bone regeneration by such highly osteogenic C-MSCs generated with OIM was accomplished by donor cells' direct osteogenesis [36]. It is plausible that the osteogenic property of C-MSCsγ and Cryo-C-MSCsγ could be lower compared to the C-MSCs treated with OIM. Thus, we speculate that the newly formed bone by C-MSCsγ and Cryo-C-MSCsγ might be due to different ways from OIM-treated C-MSCs: regulating the host indirect osteogenesis. Importantly, supporting this speculation, we demonstrated that very few human donor cells were detectable in the newly formed bone at 8 weeks after C-MSCsγ and Cryo-C-MSCsγ implantation (Fig. 5). These findings clearly suggested that the host rat cells contributed to the new bone reconstruction in our experimental model. It is well known that MSCs exert tissue regenerative capacity via not their multipotency but their paracrine effects, such as immunomodulation and cytoprotection [37–39]. For instance, IFN-γ-regulated IDO expression in MSCs induces the monocytes into immunesuppressive and tissue-reparative M2 macrophage [40]. Accordingly, the molecular mechanism underlying the bone regeneration caused by C-MSCsγ and Cryo-C-MSCsγ may be mainly associated with an indirect paracrine effect, such as IDO activity, but not direct osteogenesis, although additional study is needed.

As described above, we have previously assessed the immunomodulatory property of human C-MSCsγ using mice xenograft models [18]. Considering the future clinical application of our cell clumps for bone regenerative allograft therapy, this present study employed a rat xenograft model because the immune system of rats should be more potent than that of mice. However, for the human cells, the rodents' immune rejection systems should be relatively weak. This fact indicates the limitations of our study. Only human cells xenograft studies are not enough to guarantee the safety and effectiveness of Cryo-C-MSCs allograft bone regenerative therapy. A basic study testing the human cells allograft is a bit difficult theoretically. In addition, our rodent calvarial defect size was obviously small that can be easily treated with bone regenerative materials. In clinical practice, we will treat severe irreversible bone defects, such as a segmental tibial fracture with a cm order gap. It is unclear whether allogenic Cryo-C-MSCsγ can induce successful bone regeneration in such severe defect cases, avoiding the robust human immune system. Thus, a highly extrapolate preclinical study investigating the bone regenerative property of Cryo-C-MSCsγ allotransplantation will be needed. More specifically, an allograft study using a dog can be a good candidate. Indeed, several preclinical studies using the canine periodontal defect model reported the effectiveness and safety of allogenic MSCs transplantation therapy [41,42]. More importantly, since we have already generated dog C-MSCs, we will conduct the preclinical dog study for Cryo-C-MSCsγ allograft bone regenerative therapy.

As well as this non-clinical proof of concept study using beagle dogs, establishing a standard operating procedure based on the reliable quality control system is indispensable for the doctor-initiated clinical trial. Regarding this point, monitoring the IDO mRNA expression level in the cell clumps for its immunomodulatory property should be cumbersome. Ideally, assessing the components in culture supernatant will be an easy and reasonable way for quality control. Here, it is of note that IDO catalyzes the conversion from tryptophan to kynurenine, which is a responsible amino acid for the immunomodulatory function of IDO [43]. Indeed, we have previously showed that kynurenine activity is increased in the culture supernatant of human C-MSCsγ [18]. Thus, monitoring the level of kynurenine in Cryo-C-MSCsγ will develop good quality control that guarantees the safety and effectiveness of Cryo-C-MSCsγ allograft cell therapy.

Taken together, after conducting the beagled dog study and establishing reasonable quality control marker using kynurenine, through the well-designed clinical trial, Cryo-C-MSCsγ can be a promising bone regenerative allograft therapy in the clinical practice.

5. Conclusions

Cryopreserved human C-MSCs, pretreated with IFN-γ before the cryopreservation, retain the 3D structure, cell viability, and elevated IDO mRNA expression. The human Cryo-C-MSCsγ xenotransplantation into a rat calvarial defect model induce bone regeneration suppressing the host T cell immune response. Accordingly, if allogeneic MSCs can be utilized, Cryo-C-MSCsγ can be a reliable “off-the-shelf” cell preparation for bone regenerative therapy that is promptly provided as a standardized material when patients needed.

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Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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