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Original Article

Increasing robustness of *in vitro* assay for immnosuppressive effect of mesenchymal stromal/stem cells: The role of inflammatory cytokine production by peripheral blood mononuclear cells

Rumi Sawada [a], Shinji Kusakawa [a], Mika Kusuhara [a], Kazusa Tanaka [a], Takumi Miura [a], Satoshi Yasuda [a], Yoji Sato [b, *]

[a] *Division of Cell-Based Therapeutic Products, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki Ward, Kawasaki City, Kanagawa, 210-9501, Japan*

[b] *Division of Drugs, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki Ward, Kawasaki City, Kanagawa, 210-9501, Japan*

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ABSTRACT

Introduction: The Quality by Design (QbD) approach for developing cell therapy products using mesenchymal stromal/stem cells (MSCs) is a promising method for designing manufacturing processes to improve the quality of MSC products. It is crucial to ensure the reproducibility and robustness of the test system for evaluating critical quality attributes (CQAs) in the QbD approach for manufacturing of pharmaceutical products. In this study, we explored the key factors involved in establishing a robust evaluation system for the immunosuppressive effect of MSCs, which can be an example of a CQA in developing and manufacturing therapeutic MSCs for treating graft-versus-host disease, *etc*, and we have identified method attributes to increase the robustness of a simple *in vitro* assay to assess the immunosuppressive effects of MSCs.

Methods: We evaluated the performance of an assay system to examine the proliferation of peripheral blood mononuclear cells (PBMCs) activated with the mitogen phytohemagglutinin (PHA) when co-cultured with MSCs, the so-called one-way mixed lymphocyte reaction (MLR) assay. The MLR assay was performed on the same MSCs using 10 PBMC lots from different donors. In addition, 13 cytokine production levels in PHA-stimulated PBMCs were assessed.

Results: The PHA-stimulated proliferation response of PBMCs, the action of MSCs in the MLR test, and the cytokine release of the respective PBMCs significantly differed among the PBMC lots ($p < 0.05$). A correlation analysis between the amounts of cytokines released by PBMCs and the immunosuppressive potency of MSCs showed that IFNγ, TNFα, CXCL10, PD-L1, HGF, and CCL5 production in PBMCs was significantly correlated with the MSC-mediated inhibition of PBMC proliferation ($p < 0.05$). Therefore, we selected two PBMC lots with high PBMC proliferation and PHA-stimulated cytokine (such as IFNγ and TNFα) release for the subsequent one-way MLR assay. The robustness of the established test system was confirmed by repeating the assay several times on different days for the same MSCs (coefficient of variation <0.2).

Conclusions: To make robust the MSC immunosuppressive potency assay system, controlling the quality of PBMCs used for the assay is essential. Evaluating the inflammatory cytokine production capacity of PBMCs is effective in assessing the quality of the MLR assay system.

Abbreviations: ARDS, acute respiratory distress syndrome; CQAs, critical quality attributes; CV, coefficient of variation; GVHD, graft-versus-host disease; I-MSC, Immortalized human adipose tissue-derived MSCs; MIC, mitogen-induced cytokine; MLR, mixed lymphocyte reaction; MSCs, mesenchymal stromal/stem cells; PBMCs, peripheral blood mononuclear cells; PHA, phytohemagglutinin; QbD, Quality by Design.

* Corresponding author.
E-mail address: yoji@nihs.go.jp (Y. Sato).

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1. Introduction

Human mesenchymal stromal/stem cells (MSCs) perform various functions, such as exerting immunosuppressive effects, producing anti-inflammatory and humoral factors, and differentiating into various tissues, and are considered promising for various therapeutic applications [1]. Clinical trials have recently been conducted for Crohn's disease [2–5], graft-versus-host disease (GVHD) [6–10], and acute respiratory distress syndrome (ARDS) [11–15] caused by COVID-19 to demonstrate the immunosuppressive and anti-inflammatory effects of MSCs. Several clinical trials have been conducted; however, only a few studies have achieved the commercialization of the cell therapy products of MSCs [16]. For marketing authorization of a cell therapy product by regulatory authorities, a reproducible and robust manufacturing process, which ensures their efficacy, safety, and related quality, is required. Therefore, in the field of cell therapy product development, it is also important to accelerate the efficiency of highly reproducible cell manufacturing and the packaging of manufacturing technology through new methodologies such as the Quality by Design (QbD) approach, which has recently become common in the field of pharmaceutical manufacturing [17]. To achieve this, establishing a manufacturing process based on the quality of MSC products is essential. In addition, since the QbD approach requires that critical quality attributes (CQAs), which are essential for ensuring product efficacy and safety, be routinely monitored and maintained within normal ranges, it is critical to ensure the reproducibility and robustness of analytical methods for the CQAs.

While developing and manufacturing MSCs for treatment of abnormal immune responses, such as GVHD, a parameter associated with “the immunosuppressive action of MSCs” can be assumed as a CQA. As a potency assay to examine the immunosuppressive effects of MSCs *in vitro*, MSC functions need to be evaluated in a biological environment where *in vivo* immune and inflammatory responses can be mimicked. For example, the effects of MSCs on the proliferation of peripheral blood mononuclear cells (PBMCs) or T cells are typically evaluated following activation of these blood cells by mitogens, such as phytohemagglutinin (PHA), phorbol myristate acetate, or ionomycin [18,19].

In this study, we established a simple system to evaluate the immunosuppressive action of MSCs and analyzed its robustness. We set up an assay system to evaluate PBMC proliferation by co-culturing MSCs with PBMCs activated with mitogen PHA, the so-called one-way mixed lymphocyte reaction (MLR) assay. To establish an *in vitro* assay that mimics the immunosuppressive effects of MSCs *in vivo*, single donor-derived PBMC lots that show a certain proliferation response to PHA and a certain anti-proliferative effect by MSCs and secured abundant stocks of the lots are necessary. However, after obtaining a few PBMC lots for preliminary studies, the proliferation response of PBMCs to PHA and the proliferation inhibition by the same MSCs differed among PBMC lots (donors); therefore, we explored the responsible factors. First, we hypothesized that cytokine release from PBMCs after mitogen stimulation would have a significant influence on MSC function. Then, to examine the effect of different quality characteristics of PBMCs on the test performance of the MSC potency assay, we compared the immunosuppressive effects of MSCs across PBMC lots and focused on the cytokine release capacity of each PBMC. If it can be shown that monitoring quality characteristics such as cytokine release capacity of PBMCs makes robust the immunosuppressive assay system of MSCs, this could be one way to ensure the robustness of the CQA evaluation system as defined by the QbD approach in the manufacture of MSC processed products.

2. Methods

2.1. Cells and cell culture

Primary human bone marrow-derived MSCs (BM-MSC) were purchased from Lonza (Walkersville, MD, USA) (PT-2501). Immortalized human adipose tissue-derived MSCs (I-MSC; JCRB1555 HAdpc-26-E6-Bmi-1-TERT) were obtained from JCRB Cell Bank (Ibaraki, Osaka, Japan). Ten donor-derived human PBMCs were purchased from Lonza (Walkersville, MD, USA) (CC-2702, CC-2704, 4W-270A). Donor PBMC characteristics are presented in Supplementary Table S1. Culture of MSCs was performed based on Lonza's instructions for use of human mesenchymal stem cells. BM-MSCs and I-MSCs were cultured in MSCGM™ Mesenchymal Stem Cell Growth Medium BulletKit™ (Lonza Walkersville, MD; a culture system containing MSCGM™ Basal Media, MSCGM™ SingleQuots Supplement Kit, L-glutamine, and gentamycin/amphotericin-B). These MSCs were cultured in a 75 cm^2 U-Shaped Canted Neck Cell Culture Flask with Vent Cap (Corning, Glendale, AZ, USA) and maintained at 37 °C in a 5 % CO_2 atmosphere. The medium was replaced with a fresh medium every 3–4 days. These MSCs were detached through mild treatment with 0.025 % of trypsin/ethylenediaminetetraacetic acid solution (Lonza) and subcultured after reaching subconfluence (approximately 80–90 %). BM-MSCs within three passages were used for the experiments.

2.2. MLR assay using mitogen-stimulated PBMCs as an immunomodulatory potency assay for MSCs

Immunomodulatory effect of MSCs was determined as described [20] with some modifications as follows. BM-MSCs and I-MSCs were detached in the same manner as for the subculture described above and seeded into 96-well cell culture plates (2×10^4 cells/well, Falcon® 96-well Black/Clear Flat Bottom TC-treated Imaging Microplate, Corning Inc., NY, USA) (353219). The cells were cultured in the test medium RPMI 1640 Medium (Thermo Fisher Scientific Inc., Tokyo, Japan) (11835-030) supplemented with 10 % fetal bovine serum (NICHIREI BIOSCIENCES INC., Tokyo, Japan) (175012), 2 mmol/L L-glutamine (Thermo Fisher Scientific Inc.) (25030-149), 25 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) (Thermo Fisher Scientific Inc.) (15630-106), 0.1 mmol/L 2-mercaptoethanol (Thermo Fisher Scientific Inc.) (21985023), 1 % penicillin-streptomycin-mixed solution (Stabilized) (NACALAI TESQUE, INC., Kyoto, Japan) (09367-34) for 1 day, and the medium was replaced with a new medium containing Mitomycin C (MMC; 10 μg/mL, Wako, Osaka, Japan, 133–15931) and maintained for 2 h at 37 °C in a 5 % CO_2 atmosphere. PBMCs were thawed and suspended in the test medium with 20 U/mL DNase I (QIAGEN, MD, USA) (79254) for 10 min at 25 °C, followed by centrifugation at 200×*g* for 15 min at 25 °C. Subsequently, the cells were incubated in the test medium described above for 1 h (37 °C, 5 % CO_2) for recovery. Thereafter, 100 μL of the test medium containing 5×10^4 PBMCs was transferred to the MSCs (2×10^4 cells/well), which had been washed twice with HBSS(−) after MMC treatment, and PHA (PHA-P; 0, 0.2, 0.5, 1.0 μg/mL, Wako, 161–15251) was added and the cells were co-cultured for 2 days at 37 °C in a 5 % CO_2 atmosphere. Subsequently, BrdU Labeling Reagent of Cell Proliferation Enzyme-linked immunosorbent assay (ELISA), BrdU (chemiluminescent) (Sigma-Aldrich Co. LLC, Tokyo, Japan) (11669915001), was added and cultured for

another day. The plate was centrifuged (300×*g*, 10 min, 25 °C) to remove the supernatant and dried at 60 °C for 1 h. The plates were stored at 4 °C, and after 1 week, BrdU uptake by the cells was measured. First, the cells were fixed by adding 200 μL/well of FixDanat and allowed to stand at 25 °C for 30 min. After inverting the plate and removing the FixDenat, 100 μL/well of Anti-BrdU-POD working solution was added and allowed to stand at 25 °C for 90 min. Furthermore, the plates were inverted to remove the antibody reaction solution and washed thrice with a washing solution (200 μL added, 5 min static, removal). Similarly, 100 μL of a substrate solution was added, followed by placing it on a shaker for 3 min at 25 °C. Finally, luminescence was measured using a multimode plate reader (2030 ARVO X3, PerkinElmer, CT, USA) within 10 min (Fig. 1a).

2.3. Measurement of cytokine secretion by PBMCs

PBMCs were prepared as described in the lymphocyte proliferation assay and seeded at 5×10^4 cells/well in 96-well cell culture plates. PBMCs were stimulated with PHA (0, 0.2, 0.5, and 1.0 μg/mL, Wako, 161–15251) and maintained at 37 °C in a 5 % CO_2 atmosphere for 2 days. The plates were centrifuged (300×*g*, 10 min, 25 °C), and the supernatant was collected, stored at −80 °C, and thawed, followed by the evaluation of cytokine production. Cytokines produced by PHA-stimulated PBMCs were measured using a fully automated ELISA device, Ella (Bio-Techne Protein Simple, CA, USA). Thirteen cytokines (IFNγ, TNFα, IL-2, IL-6, CXCL8/IL-8, IL-10, CXCL5/ENA78, CXCL10/IP-10, B7–H1/PD-L1, CCL2/MCP-1, HGF, Galectin-3, and CCL5/RANTES) were measured using customized cartridges specifically for Ella (SPCKA–CS–003353 for IFNγ, IL-2, and TNFα; SPCKA–CS–009413 for Galectin-3 and CCL5/RANTES; SPCKA–CS–009410 for CXCL10/IP-10, IL-10, IL-6, and CXCL8/IL-8; SPCKA–CS–009411 for B7–H1/PD-L1, CCL2/MCP-1, CXCL5/ENA78, and HGF).

2.4. Statistical analysis

Statistical analyses were performed using the Prism 10 software (version 10.3.0; GraphPad Software Inc., CA, USA). One-way or two-way analysis of variance (ANOVA) was applied; it is mentioned accordingly. The correlation between the production of each cytokine when PBMCs were stimulated with 1.0 μg/mL PHA and the rate of PBMC proliferation inhibition by MSCs in the one-way MLR assay are indicated by the Spearman correlation coefficient. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of different PBMC donors on the in vitro immunomodulatory response of MSCs

As an *in vitro* immunomodulation study of MSCs, a one-way MLR assay was performed to evaluate the effect of MSCs on proliferation of mitogen-activated PBMCs. Ten PBMC lots derived from 10 donors (PBMCs 1–10) were used to compare PBMC proliferation and MSC action. The lots were numbered in descending order of the number of cell proliferation of PBMCs when stimulated with PHA 1.0 μg/mL. All PBMC lots showed a marked increase in their proliferation, assessed through BrdU incorporation, in response to PHA stimulation; however, PBMCs 6–10 exhibited remarkably higher proliferation than PBMCs 1–5 did (Fig. 1b). Primary BM-MSCs significantly promoted PBMCs 1–5 proliferation regardless of the PHA concentration. However, the proliferation of PBMCs 6–10 was significantly inhibited by BM-MSCs when stimulated with ≥0.5 μg/mL PHA (Fig. 1b). The rate of change due to MSC co-culture when the respective PHA-stimulated proliferation of PBMCs was set to 1 is shown in Fig. 1c. The y-axis shows the proliferation value in MSC co-culture relative to the proliferation value of PBMC alone as the rate of PBMC proliferation inhibition by MSCs; (PBMC + MSC)/PBMC. Lot differences in PBMCs significantly affected the immunomodulatory effect of MSCs, which were of two patterns: higher or lower than 1 (PBMCs 1–5 or 6–10, respectively) (Fig. 1c). BM-MSCs showed an immunosuppressive response in PBMCs 6–10 stimulated with PHA of ≥0.5 μg/mL; however, the opposite response (pro-inflammation) was observed for PBMCs 1–5 regardless of the PHA concentration (Fig. 1b and c). Thus, depending on the PBMC lots used in the study, the same MSCs may have opposite effects on PBMC proliferation. This indicates that despite the same MSCs, their immunomodulatory capacity tests varied markedly. These results were supported by similar experimental results with I-MSCs (Supplementary Fig. S1).

3.2. Cytokine production in PHA-stimulated PBMCs

To elucidate the cause of the differences in the immunomodulatory activities of MSCs among PBMC donors, we examined the profiles of cytokine released from PHA-treated PBMC lots. The production of 13 cytokines under PHA stimulation was compared for 10 donor-derived PBMC lots. The results showed that the ability to produce each cytokine and their dependence on PHA concentration differed among the PBMC lots (Fig. 2). Two-way ANOVA showed that the production of all cytokines was significantly affected by the stimulating PHA concentration and that the production of cytokines other than HGF was significantly affected by the PBMC donor, PHA concentration, and their interaction (Fig. 2). In the one-way MLR assay, following PHA stimulation, the production levels of certain cytokines seemed higher in PBMC lots with inhibited proliferation (induced by MSCs) than in those with proliferation promoted by MSCs (Fig. 1c and 2). These findings suggest that lot-to-lot differences in the cytokine production capacity of PBMCs may have influenced the one-way MLR assay, which tests the immunomodulatory potency of MSCs.

3.3. Correlation between the cytokines produced by PBMCs and the immunomodulatory potency of MSCs

To determine which cytokines produced by PBMCs are involved in the immunosuppressive potential of MSCs in the one-way MLR assay, we analyzed the correlation between the amounts of cytokines produced by PHA-stimulated PBMCs and the suppression rate of PBMC proliferation by MSCs. Spearman correlation analysis was performed to comprehensively examine the relationship between the production of each cytokine in 10 PBMC lots stimulated with 1.0 μg/mL PHA and the rate of proliferation suppression by BM-MSCs for each PBMC lot in the immunomodulatory potency assay (Fig. 3). The production levels of IFNγ, TNFα, CXCL10, PD-L1, HGF, and CCL5 by PBMCs was significantly correlated with the rate of PBMC proliferation suppression by BM-MSCs ($P < 0.05$). These results suggest that the levels of these cytokines (IFNγ, TNFα, CXCL10, PD-L1, HGF, and CCL5) produced by PBMCs are influential in the suppression of PBMC proliferation by MSCs. These results were supported by similar experimental results with I-MSCs (Supplementary Fig. S2).

We investigated whether the priming of MSCs with cytokines complemented their immunomodulatory effects on the PBMCs with low cytokine production. Of the six cytokines that were significantly correlated with the rate of PBMC proliferation inhibition by MSCs, IFNγ and TNFα, whose production levels were high, were used. In the immunomodulatory potency assay, BM-MSCs were primed with IFNγ and TNFα and co-cultured with two

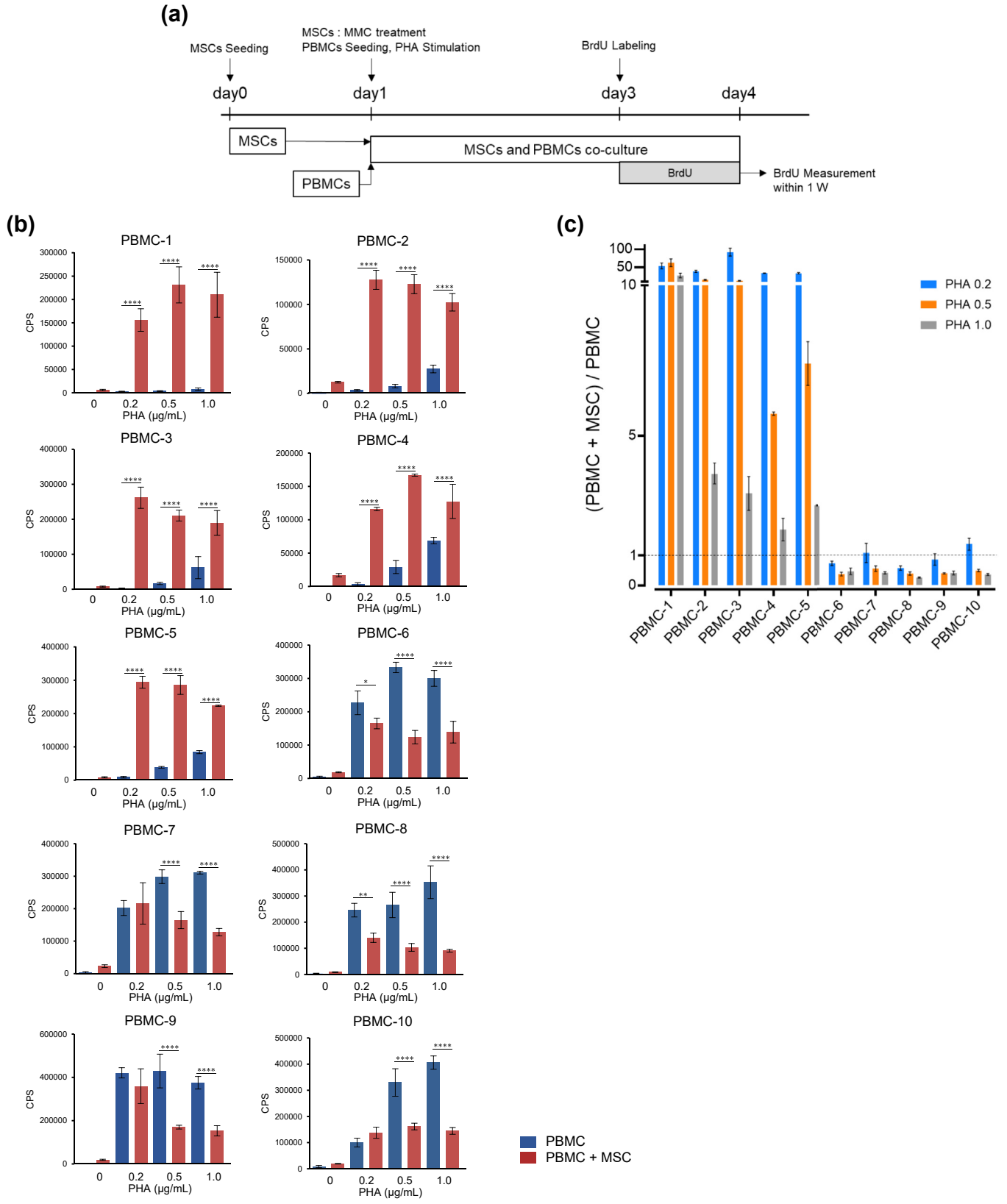

Fig. 1. Effect of different donor-derived human peripheral blood mononuclear cells (PBMCs) on the *in vitro* immunomodulatory response of bone marrow (BM)- mesenchymal stem cells (MSCs). (a) Schematic of the protocol for lymphocyte proliferation assays with mitogen as an immunomodulatory potency assay for MSCs. (b) Proliferation of 10 donor-derived PBMCs upon phytohemagglutinin (PHA; 0.2, 0.5, and 1.0 μg/mL) stimulation was assessed through BrdU uptake. BrdU uptake was indicated by luminescence signal (counts per second, CPS). Two-way ANOVA showed that PBMCs proliferation was significantly affected by the stimulating PHA concentration, the presence or absence of MSCs, and their interaction ($p < 0.0001$). All values indicate the mean ± standard deviation of mean of three biological replicates ($n = 3$) (*$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$, two-way analysis of variance, followed by Bonferroni's multiple comparison test). (c) Effect of PBMCs derived from 10 donors on the *in vitro* immunomodulatory response of BM-MSCs. The rate of suppression by BM-MSCs on PBMC proliferation stimulated with three PHA concentrations (0.2, 0.5, and 1.0 μg/mL) is shown as the immunosuppression rate; CPS(PBMC + MSC)/CPS(PBMC).

PBMC batches (PBMC-2 and 4) that produced low levels of both cytokines. The results showed that primed BM-MSCs suppressed PBMC proliferation, confirming that IFNγ and TNFα induced an immunosuppressive activity of MSCs in this assay (Supplementary Fig. S3).

3.4. Repeatability study of one-way MLR assay of the same MSCs with fixed PBMC lots

The results indicate that the MLR results vary depending on the PBMC lot used in the MLR test, and this can be attributed to the differences in the proliferation ability and some cytokines released by PBMCs stimulated with PHA. Therefore, we selected two PBMC lots with substantial PBMC proliferation and high release of cytokines, such as IFNγ and TNFα, upon PHA stimulation and repeated the one-way MLR assay for the same MSCs several times on different days to examine the robustness of the assay system. To repeat the assay using the same MSCs, immortalized MSCs were used in the assay but not primary MSCs, which were limited in cell number in the same lot. The one-way MLR test was repeated five and eight times using PBMC-8 and PBMC-10, respectively, to determine the coefficient of variation (CV) of the proliferation inhibition rate of PBMCs for each I-MSC. The CV of PBMC proliferation inhibition by MSCs was <0.2 for both PBMC lots, confirming that the variability between tests was small and that the results were stable (Table 1, Supplementary Fig. S4).

4. Discussion

MSCs have shown efficacy in treating fatal conditions such as ARDS and GVHD and are expected to be commercialized as cellular therapeutic products. To commercialize such innovative therapeutic cell-processed products, reproducibly manufacturing high-quality cell products is essential. Therefore, developing manufacturing processes using new approaches, such as QbD, is crucial [17]. Hence, ensuring the repeatability and robustness of the test system to evaluate CQAs in the QbD approach is important. In this study, we took the immunosuppressive effect of MSCs as an example of the CQAs in the development and manufacturing of therapeutic MSCs for treatment of abnormal immune responses such as GVHD, and investigated key factors for establishing a robust *in vitro* test method to evaluate this effect.

In this study, we set up a one-way MLR assay using single donor-derived PBMCs activated with mitogen as a simple system to evaluate the immunosuppressive effects of MSCs. Mitogen reportedly enhances PBMC proliferation and shortens the MLR assay period [19,21]. We used PHA as a mitogen, which is common in immunosuppressive potency assays of MSCs [19,22]. Ten lots of cryopreserved PBMCs from 10 donors were purchased and compared. We used commercially available PBMCs that had been cryopreserved for this study because we believe that the availability of abundant PBMCs of the same quality is crucial for repeatability of the test system. Cryopreservation has been reported to have little effect on the quality of PBMCs [23].

The PHA-stimulated proliferation response of PBMCs and the action of MSCs in the MLR assay significantly differed among the PBMC lots; that is, the MLR results significantly varied among the different PBMC lots. Furthermore, the cytokine release by each PBMC showed significant differences between lots. The amount of each cytokine release significantly depended on the PBMC lots and the PHA concentration. Furthermore, MSCs suppressed the proliferation of PBMCs with high mitogen-induced cytokine (MIC) release and promoted the proliferation of PBMCs with low MIC release. Therefore, a possible reason for the different MSC effects, that is, the variation in MLR results, was the difference in the proliferation response and cytokine production capacity of the PBMCs used in the study. The cytokines examined in this study were 13 cytokines, including IFNγ, TNFα, and IL-2. Many inflammatory factors reportedly play an important role in the immunoregulatory effect of MSCs [24–35]. Statistical analysis of the correlation between the amounts of cytokines released by PBMCs and the immunomodulatory potential of MSCs showed that IFNγ and TNFα production in PBMCs was strongly correlated with the rate of proliferation inhibition of PBMCs by MSCs. Similarly, the levels of CXCL10, PD-L1, HGF, and CCL5 production in PBMCs were significantly correlated with the rate of PBMC proliferation inhibition by MSCs. These cytokines have been reported to be interrelated, with IFNγ and TNFα increasing HGF expression [36], HGF increasing PD-L1 expression [28–30], and MSCs participating in immune responses via HGF [27]. CXCL10 reportedly participates in the activation of T lymphocytes by MSCs [31], and the CCL5 receptor, CCR5, is involved in the immunomodulatory effects of MSCs [32]. In addition, MSCs stimulated by proinflammatory factors reportedly enhance T-cell proliferation inhibition [26]. Low IFNγ concentrations stimulate antigen presentation by MSCs [37,38]. These findings indicate that the concentration of inflammatory cytokines influences the immunomodulatory response of MSCs, suggesting that differences in cytokine production by PBMCs used in the MLR assay significantly affected the immunomodulatory response of MSCs. Among the cytokines correlated in this study, the production of PD-L1 and HGF from PBMCs was very low, while the production of IFNγ and TNFα was considerably higher, and IFNγ and TNFα production was clearly higher in the PBMCs whose proliferation were suppressed by MSCs (PBMC6-10) than in the PBMCs whose proliferation were not suppressed by MSCs (PBMC1-5). Therefore, the effect of IFNγ and TNFα production on the variability of MLR assay results may be considerable. Notably, this study showed that MSCs produced immunosuppressive responses to PBMCs with high levels of inflammatory cytokine production, such as IFNγ and TNFα, and proinflammatory responses to PBMCs with low levels of inflammatory cytokine production, despite being the same MSCs (Fig. 4). Surrounded by activated immune cells, MSCs exerted an anti-inflammatory effect. This suggests that MSCs have varying effects, which are determined by their microenvironment. Clinical reactivity may depend on the interaction of the MSC product with host immune cells occurring in the recipient's inflammatory environment. As the immunomodulatory properties of MSCs as cell therapy products may also depend on the microenvironment of the patient to whom they are administered, understanding the state of the immune response of PBMCs used for MLR assays is essential to evaluate immunosuppressive effects as a CQA of MSCs. The cytokines released from PBMCs in response to PHA may have some effect on the physiological properties of the PBMCs themselves, but the immunosuppressive effect of MSCs was confirmed by prior priming of MSCs with IFNγ and TNFα in an MLR assay using PBMCs with low levels of inflammatory cytokine production, suggesting that the PBMC-derived cytokines influence to results of the assay for immunosuppressive ability of MSCs as well.

Based on the previous results, we hypothesized that the differences in the proliferation capacity of PBMCs and the proinflammatory cytokine-releasing capacity of PBMCs upon PHA stimulation could be responsible for the varying MLR results among the different PBMC lots used in the study. Hence, two PBMC lots with large PBMC proliferation and high release of proinflammatory cytokines such as IFNγ and TNFα by PHA stimulation were selected, and the one-way MLR test for the same MSCs was

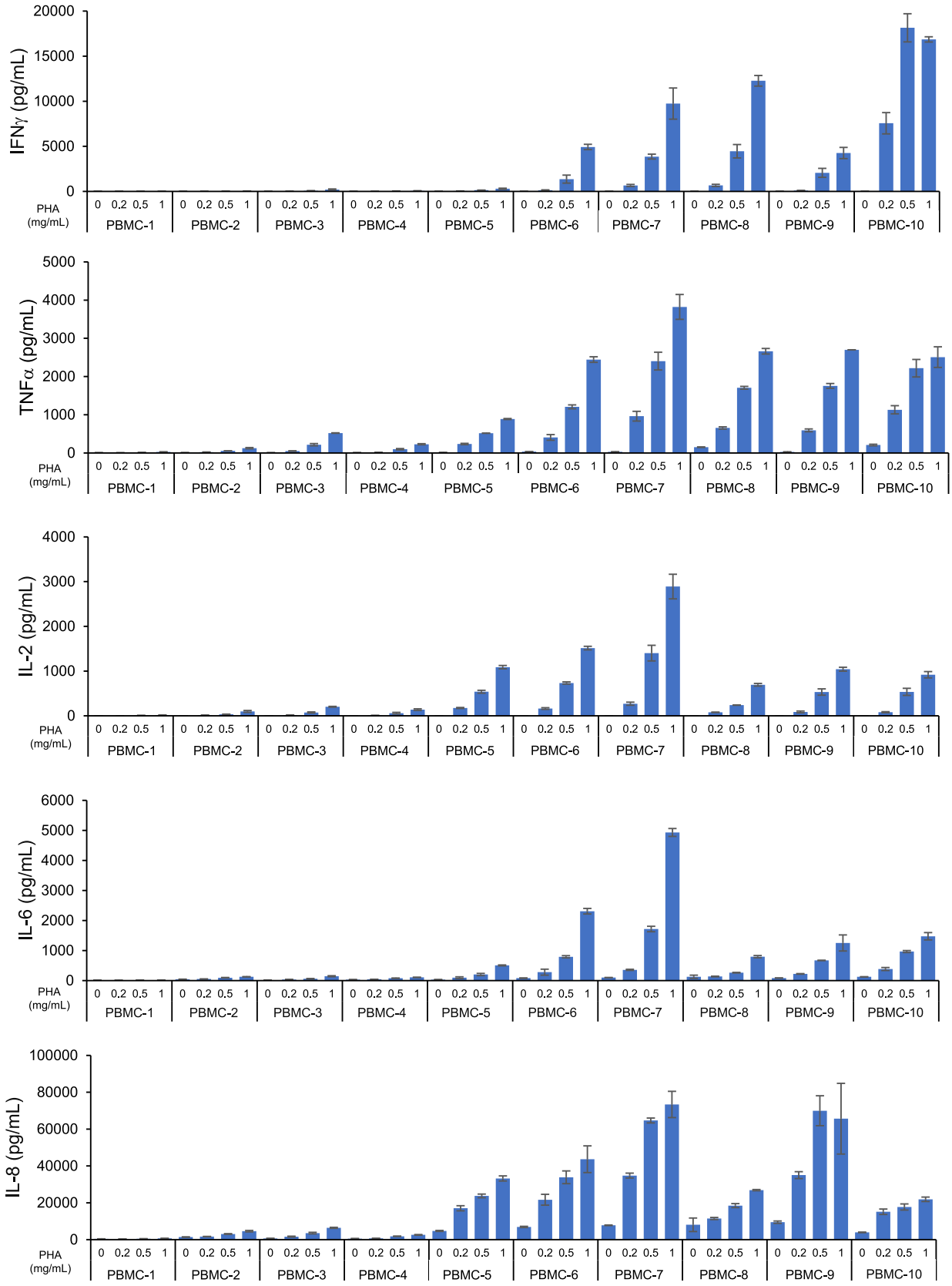

Fig. 2. Cytokine production in PHA-stimulated PBMCs. The production of 13 cytokines stimulated by three PHA concentrations (0.2, 0.5, and 1.0 μg/mL) was compared for 10 PBMC lots derived from 10 donors. The effects of different donors and PHA concentrations on the production of each cytokine by PBMCs were analyzed through a two-way analysis of variance. All values indicate the mean ± standard error of the mean of three biological replicates (n = 3).

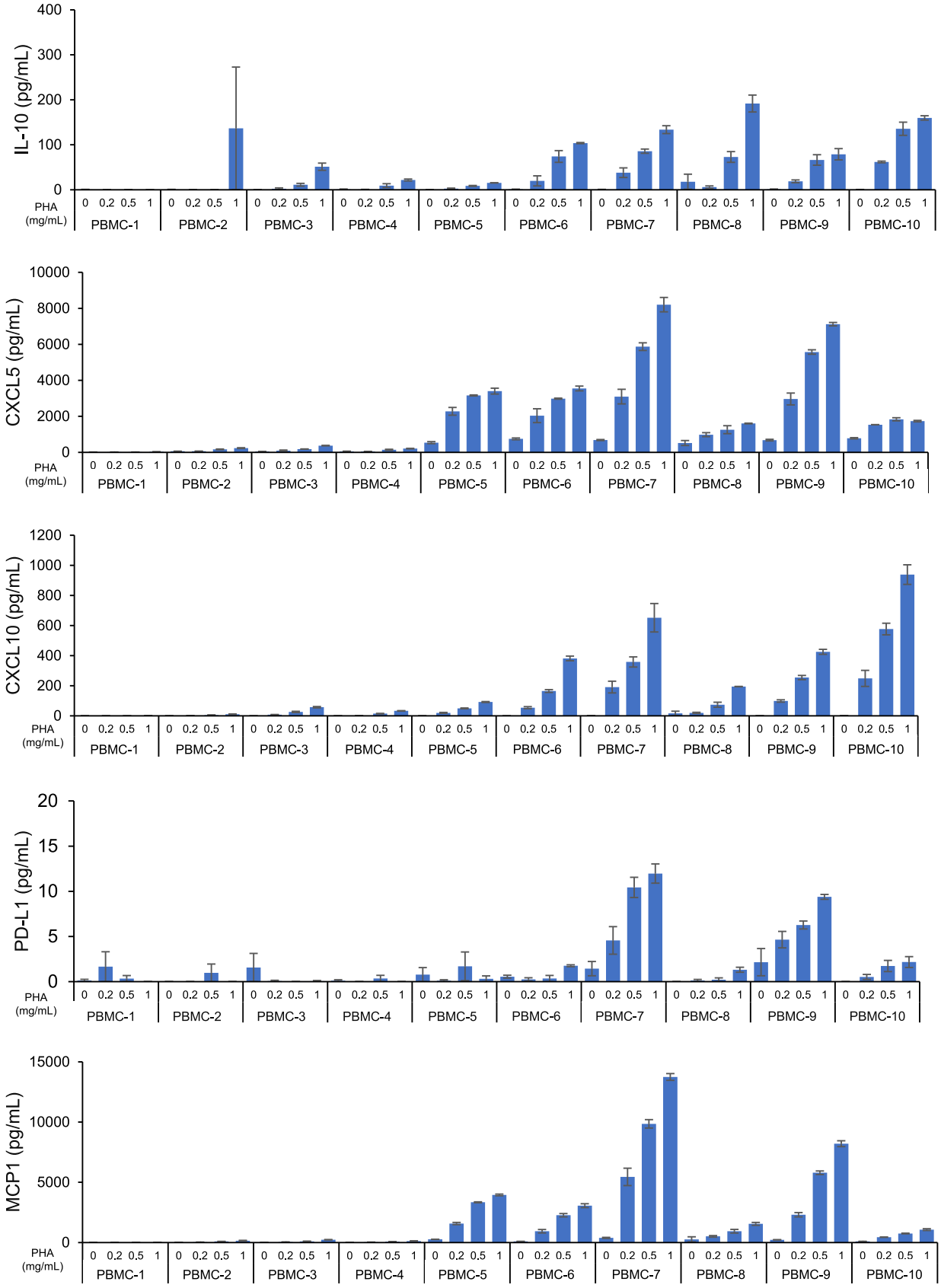

Fig. 2. (*continued*).

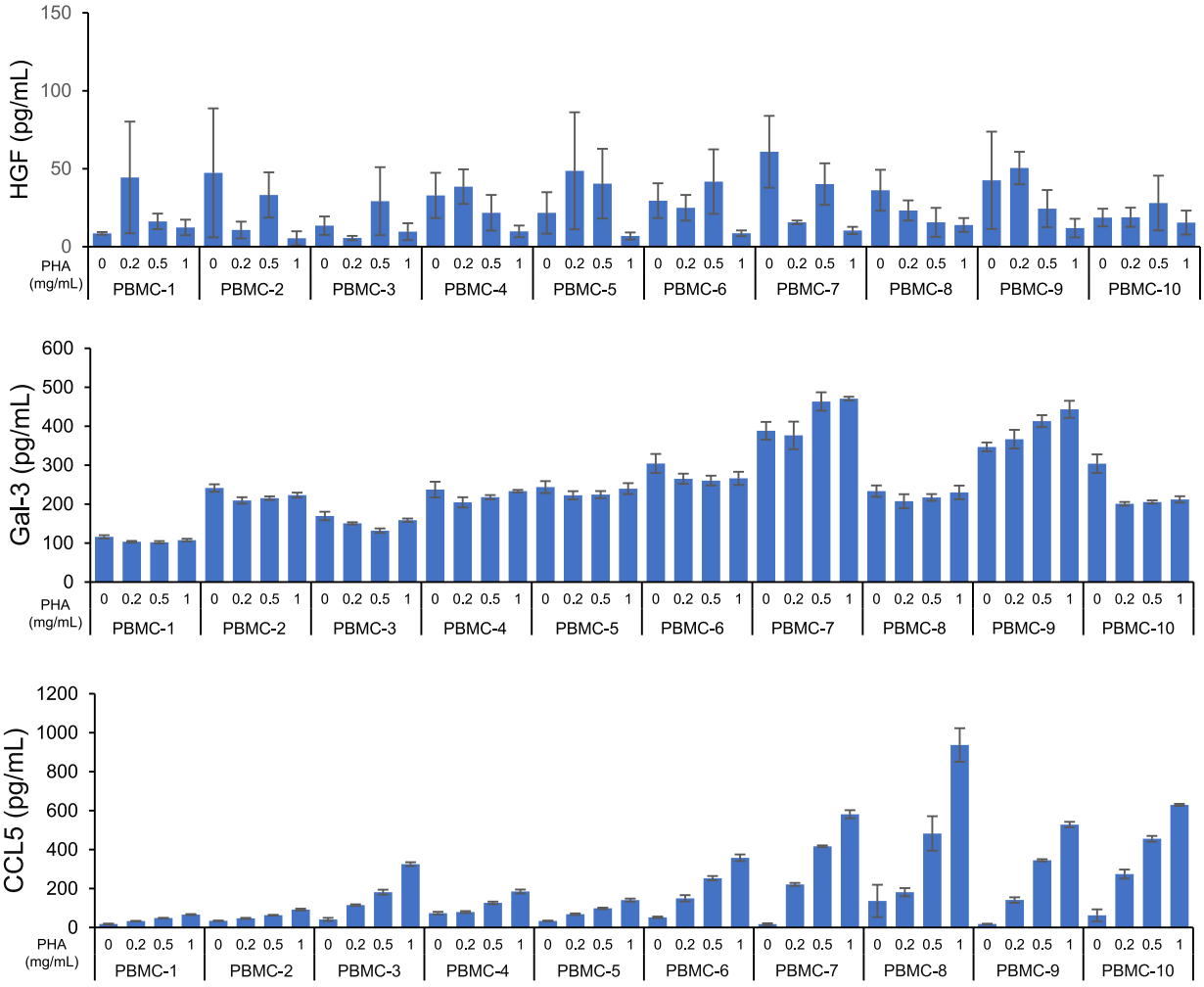

Fig. 2. (*continued*).

repeated several times on different days. PBMC proliferation was inhibited by MSCs in both PBMC lots, and the CV of the proliferation inhibition ratio was <0.2, showing a stable performance with little variation between tests. This implies that the MLR assay system was make robust using PBMCs with high inflammatory cytokine production induced by PHA stimulation. These results indicate that quality control of PBMCs in this one-way MLR study (i.e., the raw materials for the cell base assay) is crucial to establishing a robust evaluation system for immunosuppressive effects as a CQA as defined in the QbD approach. One possible method of quality control is by evaluating the levels of inflammatory cytokines such as IFNγ and TNFα produced by mitogen stimulation of PBMCs. This evaluation may be applicable to PBMCs derived from single and multiple donors. Thus, when selecting PBMCs for assay and evaluating the appropriateness of their use for the intended purpose, evaluating the inflammatory cytokine production capacity of PBMCs may be a criterion for selecting cells for cell-based assay. Several groups have reported protocols for priming MSCs with IFNγ prior to MLR in order to improve the robustness of the assay [39,40]. However, responsiveness to primed MSCs may not necessarily be the same between PBMCs with high and low MIC release, but there may be some difference. Therefore, even when performing an MLR assay in which MSCs are primed with cytokines such as IFNγ, it would be beneficial to assess the levels of mitogen-inducible cytokines released from the PBMCs used before starting the assay (or before banking the PBMCs) in order to further ensure the assay robustness.

Although this study was able to show a correlation between the production capacity of several inflammatory cytokines from PBMCs and the immunomodulatory capacity of MSCs, further detailed studies will be needed to determine the threshold for cytokine production by PBMCs in one-way MLR studies. In addition, the effects of various other characteristics of PBMC donors on immunosuppressive potency assays may also need to be investigated. In order to use cytokine production levels of PBMCs for a robust potency assay system in the manufacture of MSC processed products, the reference value should be determined based on clinical results from actual MSC treatment. Future work should examine the relationship between the clinical evaluation of MSCs and *in vitro* immunosuppressive potency assays.

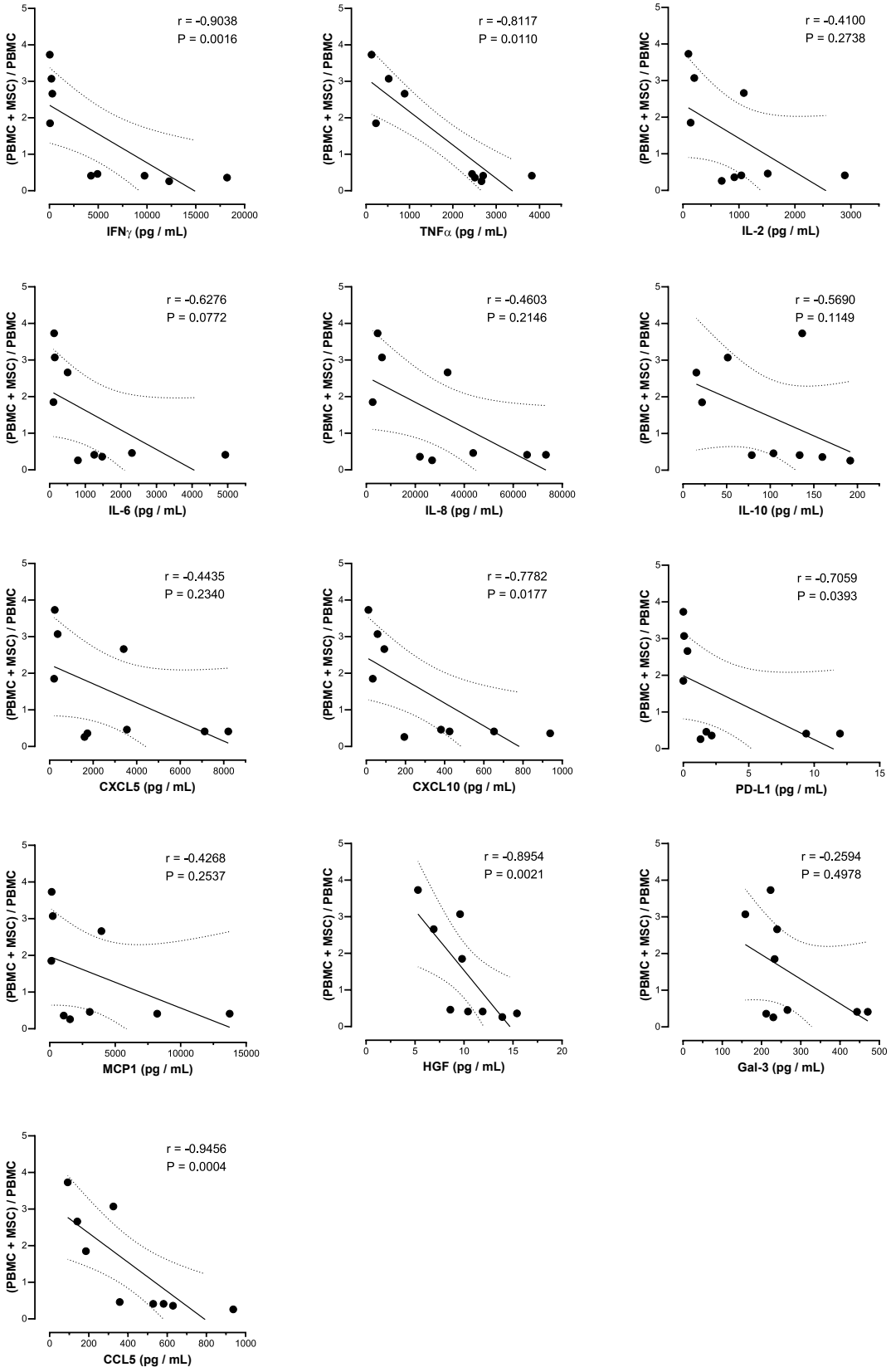

Fig. 3. Relationship between the production of cytokines by PHA-stimulated PBMCs and the suppression rate of PBMC proliferation by MSCs in the immunomodulatory potency assay. Spearman correlation analysis between the concentration of each cytokine in the culture supernatant when PBMCs were stimulated with 1.0 μg/mL PHA and the rate of proliferation inhibition by BM-MSCs in the immunomodulatory potency assay is shown with linear regression lines and their 95 % confidential intervals. r, correlation coefficient.

Table 1
Inter-test variability in the rate of proliferation inhibition of PBMCs by repeated one-way MLR assay.

MSCs	PBMC	n	One-way MLR assay (PBMCs proliferation rate[a])		
			Average	SD	CV[a]
I-MSCs	PBMC-8	5	0.50	0.09	0.18
I-MSCs	PBMC-10	8	0.49	0.06	0.11

[a] PBMCs proliferation rate: the proliferation value in MSC co-culture relative to the proliferation value of PBMC alone as the rate of PBMC proliferation inhibition by MSCs; (PBMC + MSC)/PBMC., CV: coefficient of variation.

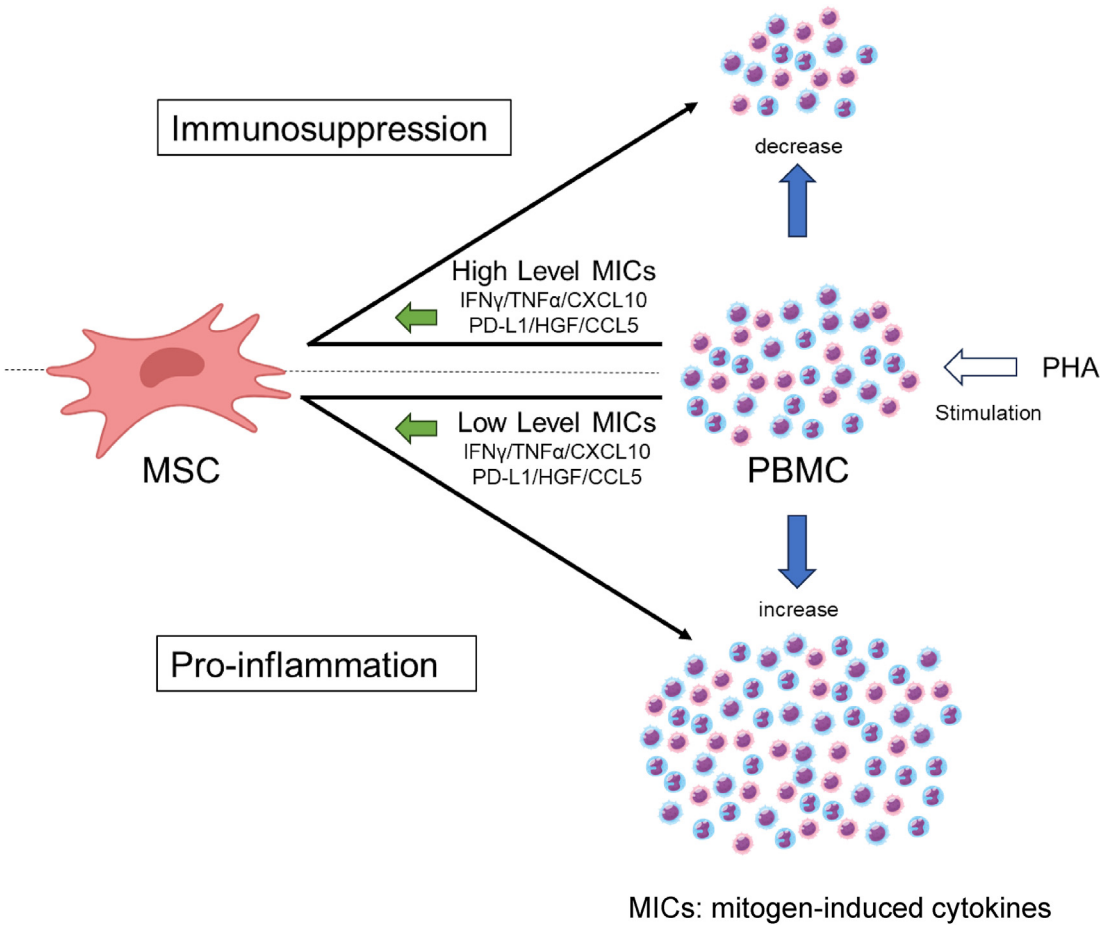

Fig. 4. Figure summarizing the flow of the reversed effects of MSCs depending on the cytokine production concentration of PHA-stimulated PBMCs in the immunomodulatory potency assay. The illustration of MSC in the figure was done using BioRender: scientific image and illustration software.

5. Conclusions

Factors to ensure reproducibility and robustness of the test system to evaluate CQAs necessary in the QbD approach in the manufacture of MSCs for treatment of abnormal immune responses were explored. The immunosuppressive function of MSCs was set as one of the CQAs. Monitoring the quality of the PBMCs used is essential to make robust the one-way MLR assay system, which is the evaluation method used for the assay. As a method to evaluate the quality of PBMCs, it is important to understand their ability to release inflammatory cytokines such as IFNγ and TNFα in response to a mitogen, which could be analytical procedure attributes as defined in ICH Q14 Guideline [41] for *in vitro* immunosuppressive function assays of MSCs. These results presented in this study will help to make the CQA evaluation system more robust in the manufacturing process to improve the quality of MSC products.

Author contributions

Conceptualization, R.S. and Y.S.; Methodology, S.K., K.T., M.K., and R.S.; Investigation, M.K., K.T., and S.K.; Writing-Original Draft, R.S.; Writing-Review and Editing, S.K., S.Y., T.M. and Y.S.

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Declaration of competing interest

The authors have no Conflict of Interest to disclose with respect to this sutudy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.12.016.

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