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Low immunogenicity of allogeneic human umbilical cord blood-derived mesenchymal stem cells *in vitro* and *in vivo*

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ABSTRACT

Evaluation of the immunogenicity of human mesenchymal stem cells (MSCs) in an allogeneic setting during therapy has been hampered by lack of suitable models due to technical and ethical limitations. Here, we show that allogeneic human umbilical cord blood derived-MSCs (hUCB-MSCs) maintained low immunogenicity even after immune challenge *in vitro*. To confirm these properties *in vivo*, a humanized mouse model was established by injecting isolated hUCB-derived CD34+ cells intravenously into immunocompromised NOD/SCID IL2γnull (NSG) mice. After repeated intravenous injection of human peripheral blood mononuclear cells (hPBMCs) or MRC5 cells into these mice, immunological alterations including T cell proliferation and increased IFN-γ, TNF-α, and human IgG levels, were observed. In contrast, hUCB-MSC injection did not elicit these responses. While lymphocyte infiltration in the lung and small intestine and reduced survival rates were observed after hPBMC or MRC5 transplantation, no adverse events were observed following hUCB-MSC introduction. In conclusion, our data suggest that allogeneic hUCB-MSCs have low immunogenicity *in vitro* and *in vivo*, and are therefore "immunologically safe" for use in allogeneic clinical applications.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells found in various tissues, including the bone marrow, adipose, and umbilical cord blood (UCB). The therapeutic efficacy of MSCs has been proved in preclinical studies using several disease-relevant animal models [1]. Allogeneic MSCs are considered "immunoprivileged" as they have immunomodulatory effects [2,3] and do not express major histocompatibility complex class II (MHC-II) antigen or costimulatory factors including CD40, CD80, and CD86 [4]. Due to these therapeutic benefits, MSCs are increasingly being applied in a clinical setting. Human UCB-derived mesenchymal stem cells (hUCB-MSCs) are an alternative MSC therapy with several advantages including noninvasive collection, hypo-immunogenicity, superior tropism, and differentiation potential [5]. Furthermore, the secretome of hUCB-MSCs contains therapeutically important molecules, including thrombospondin-2 (TSP-2) [6], galectin-3 [7], and soluble intracellular adhesion molecule-1 (sICAM-1) [8]. Based on these biological advantages, our group has initiated clinical trials for cartilage deficiency treatment under the approval of the Food and Drug Administration (Clinical Trials Gov Identifier: NCT01733186) and Alzheimer's disease therapy under Korean Food and Drug Administration regulation (Clinical Trials Gov Identifier: NCT01696591). Despite numerous clinical trials with allogeneic MSCs, more studies on their immunogenicity under clinical conditions are necessary, since these cells could elicit a deleterious antidonor immune response [9,10]. However, technical and ethical limitations have precluded the development of *in vivo* model systems for addressing the immune interactions between transplanted MSCs and cells in the host tissues.

Humanized mice, in which animals with a genetically defined immunodeficiency are engrafted with human hematopoietic stem cells (HSCs) or peripheral blood mononuclear cells (PBMCs) are powerful preclinical tools that have narrowed the gaps between human and animal systems [11]. Recently, a new immunodeficient animal, the NOD/SCID IL2γnull (NSG) mouse, was generated; this mouse lacks an IL-2 gamma receptor chain required for the development of lymphocyte subsets. The lack of lymphocytes, NK activity, and dendritic cell function in NSG mice facilitates high

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engraftment efficiency, full-lineage differentiation to immune cells, and confers longevity [12,13]. Based on these properties, NSG mice are most suited for establishing humanized mouse models and have been used in many translational studies [14–16].

To our knowledge, this study is the first to evaluate the *in vivo* immunogenicity of hUCB-MSCs in a humanized mouse model. The negligible immunogenicity and lack of deleterious side effects upon engraftment make these cells a promising source for allogeneic therapies.

2. Materials and methods

2.1. Isolation of hUCB-derived CD34+ cells

This study was approved by the Institutional Review Board of MEDIPOST Co. Ltd., Seoul, Korea. hUCBs were collected from umbilical veins following neonatal delivery, after obtaining informed consent. Mononuclear cells (MNCs) were isolated from hUCBs using Ficoll–Hypaque (Sigma, St. Louis, MO, USA) gradient centrifugation. After MNC isolation, CD34+ cells were positively selected using a magnetic cell-sorting MACS CD34 isolation kit (Miltenyi Biotec Inc., Auburn, CA). CD34+ cell purity was estimated by flow cytometry before use for generating humanized mice.

2.2. hUCB-MSC culture

hUCB-MSCs were isolated by separating MNCs using a Ficoll–Hypaque solution ($d = 1.077$ g/cm^3; Sigma, St. Louis, MO); then, they were suspended in α-minimum essential medium (α-MEM, Gibco, Carlsbad, CA) supplemented with 10% FBS (Gibco, CA) and seeded at 5×10^3 cells/cm^2. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2; the medium was changed twice per week. The cultured hUCB-MSCs were HLA-mismatched unrelated with isolated CD34+ cells from hUCBs.

2.3. Mice

NSG mice were purchased from the Jackson Laboratory (Bar Harbor, ME; Stock #005557) and maintained in the animal facilities of the Hanyang University under specific pathogen-free conditions. The animal study was approved by the Institutional Animal Care and Use Committee of Hanyang University (HY-IACUC-10-030, 046).

2.4. The generation of humanized mice

As previously reported [17], NSG mice were irradiated (240 cGy, total body, GammaCell 1000 irradiator containing ^{137}Cs (MDS Nordion, Ontario, Canada)) at 7 weeks of age. Irradiated mice were transplanted intravenously with highly purified CD34+ cells (1×10^5 cells/mouse) from hUCB. After transplantation, the mice were maintained for 24 weeks, and engraftment was monitored by flow cytometry analysis of peripheral blood for the presence of human CD45+ cells.

2.5. Evaluation of engrafted human immune cells

The presence of human cells in the bone marrow or peripheral blood cells of engrafted mice was assessed at 4–24 weeks after transplantation, and human cell engraftment was evaluated. Engraftment of human hematopoietic cells (CD45), MHC class II cells (HLA-DR), monocyte cells (CD14), B cells (CD19), NK cells (CD56), T cells (CD3, CD4, and CD8) and murine-CD45+ cells was determined using antibodies labeled either with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) (BD Pharmingen, San Jose, CA) and flow cytometry (FACS Caliber, Becton Dickinson, San Jose, CA).

2.6. Mixed lymphocyte reaction (MLR) assay

Stimulator hPBMCs (Allcells, LLC, CA, USA) and hUCB-MSCs were inactivated by treatment with 10 μg/ml mitomycin-C (Sigma, St. Louis, MO) for 1 h at 37 °C. Inactivated hPBMCs (1×10^5 in 100 μl) or hUCB-MSCs (1×10^4) were added to each well of a 96-well culture plate. Responder cells (1×10^5 in 100 μl) from single cell suspensions of spleens from humanized mice or naïve hPBMCs were added to triplicate wells containing stimulator cells or phytohemagglutinin (PHA) (5 μg/ml, Sigma), before 5-days incubation at 37 °C in a humidified 5% CO_2 atmosphere. Bromodeoxyuridine (BrdU, Roche, Mannheim, Germany; final concentration, 10 μM) was added to the culture plates and incubated at 37 °C in 5% CO_2 for an additional 18 h. Subsequently, the plates were centrifuged at 470×*g* for 30 min, and the substrate was added per the manufacturer's instructions. Cells were subjected to a cell proliferation BrdU ELISA assay (Roche) for alloantigen-induced proliferation. The MLR supernatants were analyzed for the human IFN-γ level by ELISA (R&D Systems, Minneapolis, MN, USA).

2.7. Evaluation for the immunogenicity of hUCB-MSCs in humanized mice

Mice were injected intravenously with PBS, HLA-mismatched allogeneic hPBMCs (1×10^7 cells/mouse), MRC5 (2.5×10^5 cells/mouse) or hUCB-MSCs (2.5×10^5 cells/mouse) 24 weeks post-transplantation. At 26 weeks, mice were again injected with human cells. From the second day of injection, mice were fed with water containing BrdU (1 mg/ml, Sigma) for 1 week, and then spleen, plasma, small intestine and lung tissues were harvested. Splenocytes were stained with human CD4 and CD8 antibodies, fixed, permeabilized, treated with DNase I, and stained with anti-BrdU (BrdU Flow kit, BD Pharmingen) before flow analysis. The plasma samples were analyzed using the following kits: human IgG ELISA (RayBiotech, Norcross, GA), human IFN-γ ELISA, and human TNF-α ELISA (R&D Systems) kits. Small intestine and lung tissues were fixed in 10% formalin, embedded in paraffin, and cut into 4-μm-thick sections for hematoxylin and eosin (H&E) staining.

2.8. Survival rate assessment in humanized mice

Mouse survival was monitored daily and Kaplan–Meier survival estimation was performed using SPSS software, version 16.0 (IBM Corporation, Armonk, NY).

2.9. Statistical analyses

Statistical analyses were performed using the nonparametric (Mann–Whitney *U* test) method and one-way ANOVA, with post hoc Turkey HSD (SPSS software, version 16.0). $P < 0.05$ was considered statistically significant. Values are given as mean ± SD (standard deviation).

3. Results

3.1. Low immunogenicity of hUCB-MSCs in vitro

We examined hUCB-MSCs immunogenicity *in vitro* using an allogeneic MLR. hUCB-MSCs did not elicit the proliferative response of allogeneic hPBMCs, in contrast to the responses stimulated by PHA or allogeneic hPBMCs (Fig. 1A). Pro-inflammatory cytokines in the MLR cultured supernatants were measured by

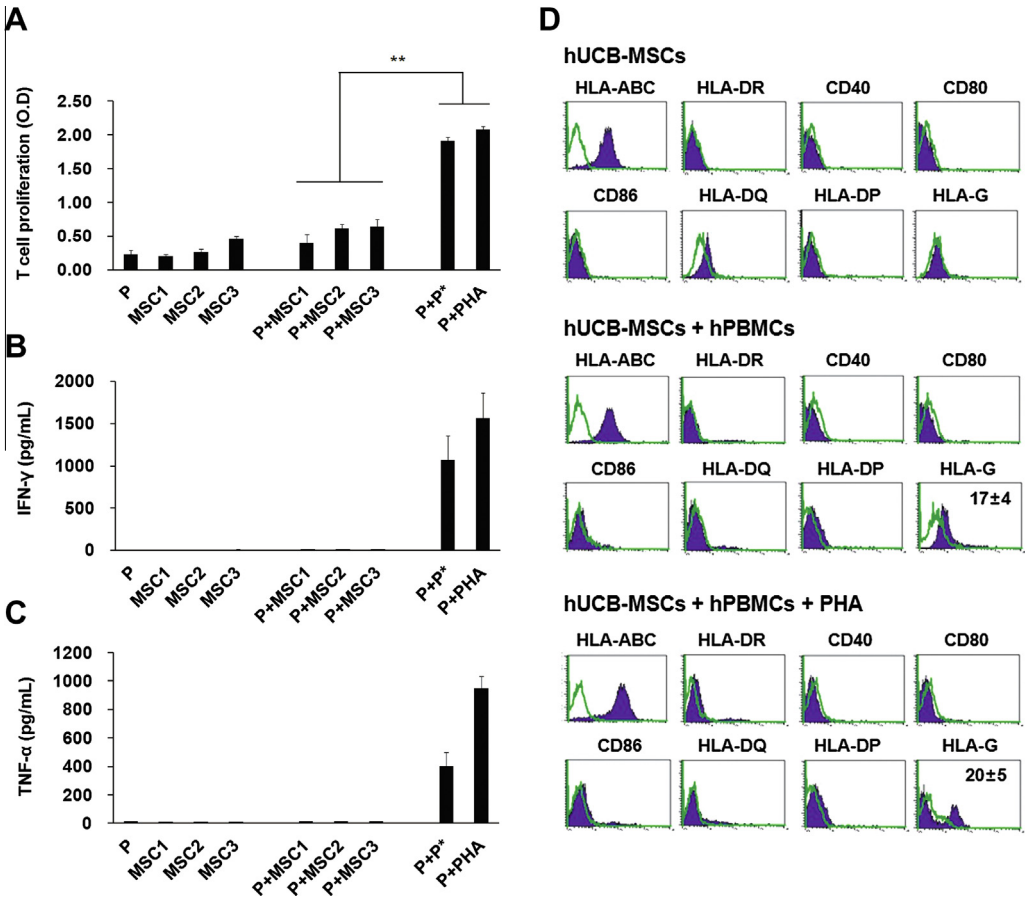

Fig. 1. Immunological characterization of hUCB-MSCs *in vitro*. (A) Evaluation of hUCB-MSC immunogenicity using the MLR assay. Allogeneic hPBMCs (1×10^5) were cocultured with hUCB-MSCs (1×10^4) from 3 different donors. The proliferation of responding cells was assessed using the MLR assay. To further monitor the immunogenic influence on lymphocyte proliferation, IFN-γ (B) and TNF-α (C) levels were examined using ELISA. (D) Immunophenotypic analysis of naïve hUCB-MSCs and stimulated hUCB-MSCs. Stimulated hUCB-MSCs were cocultured with hPBMCs or PHA treated with hPBMCs for 4 days. Each viable hUCB-MSC was analyzed by flow cytometry for HLA-ABC, HLA-DR, CD40, CD80, CD86, HLA-DQ, HLA-DP, and HLA-G expression. The data of 3 independent experiments, performed in triplicate, are expressed as mean ± SD. $^{**}P < 0.01$. *Abbreviations:* P, human PBMCs; P*, allogeneic human PBMCs as stimulator; MSC1, MSC2, and MSC3, human UCB-MSCs.

ELISA. IFN-γ and TNF-α were not detected in hUCB-MSCs cocultured with allogeneic hPBMCs (Fig. 1B and C). Consistent with previous reports [4], naïve hUCB-MSCs did not express HLA-class II molecules, including HLA-DQ, DP, DR, and costimulatory molecules containing CD40, CD80, and CD86 (Fig. 1D). Expression of these markers was not observed even when hUCB-MSCs were cocultured with allogeneic hPBMCs or PHA-stimulated allogeneic hPBMCs (Fig. 1D). However, hUCB-MSCs cocultured with allogeneic hPBMCs or PHA-stimulated allogeneic hPBMCs caused the induction of HLA-G, an immune tolerogenic antigen [18], with a frequency of 17 ± 4% and 20 ± 5%, respectively, while naïve hUCB-MSCs were negative for HLA-G (Fig. 1D).

3.2. Generation of humanized NSG mice using human CD34+ cells

To generate a humanized mouse, we injected hUCB-derived CD34+ cells into NSG mice. Engraftment of human CD45+ cells in the peripheral blood was evaluated at 4, 9, and 24 weeks after transplantation. While the proportion of human CD45+ cells increased in a time-dependent manner (mean values, 4%, 14%, and 46% at 4, 9, and 24 weeks, respectively, after transplantation), that of mouse CD45+ cells decreased in this timeframe (Fig. 2A). Human myeloid and lymphoid subsets including CD3+(T cell), CD19+(B cells), CD14+(monocytes), CD56+(NK cells), and HLA-DR+(MHC-II) cells were detected in the peripheral blood and bone marrow at 24 weeks (Fig. 2B). To further evaluate the subpopulation of T cells, peripheral blood cells and splenocytes from humanized mice were subjected to FACS analysis; this revealed that CD4+ or CD8+ T cells subsets were also present (Fig. 2C).

3.3. Evaluation of engrafted human cell immune function in humanized NSG mice

To examine the functionality of engrafted human immune cells in NSG mice, MLR and ELISA analyses were performed. Splenocytes from humanized NSG mice were harvested and stimulated with PHA or allogeneic hPBMCs. When these splenocytes were cocultured with allogeneic hPBMCs or PHA, the cluster formation of T cells was induced compared to that of the non-humanized mice (Fig. 3A). Although the responses of hPBMCs were higher than those in splenocytes after immune activation, the proliferative response of splenocytes from humanized mice against allogeneic hPBMCs or PHA stimulation was induced (Fig. 3A and B). Similarly, IFN-γ was detected in the MLR supernatant from splenocytes of humanized mice in response to allogeneic hPBMCs or PHA stimulation (Fig. 3C). However, there were no responses in non-engrafted NSG mice. Human IgG was also detected in the plasma of humanized NSG mice, but was absent from their non-engrafted counterparts (Fig. 3D).

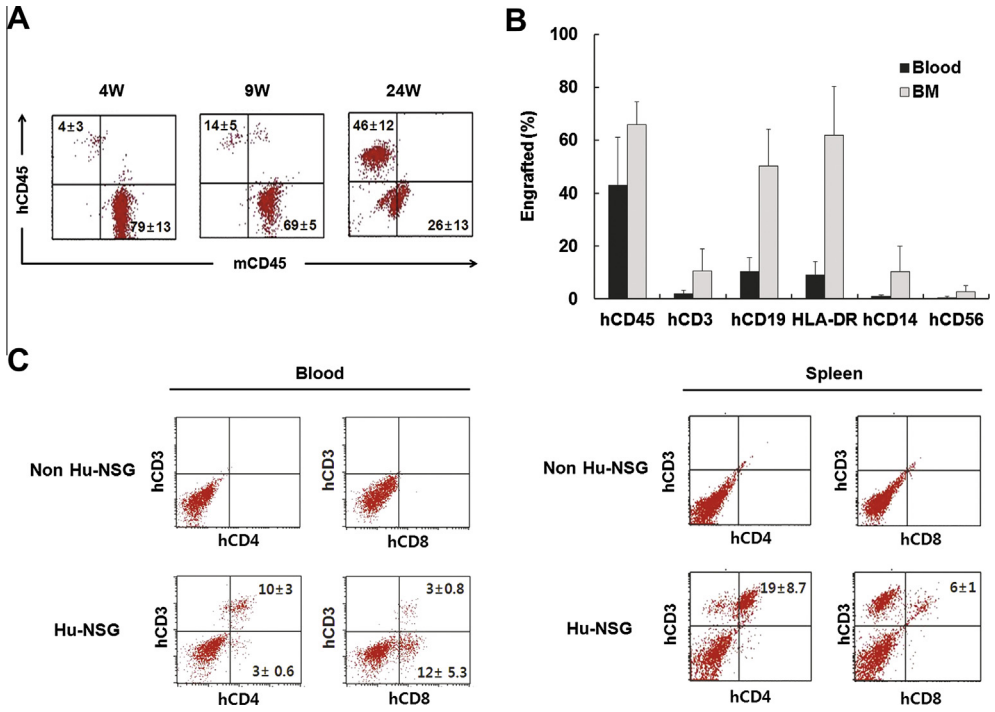

Fig. 2. Evaluation of engrafted human cells from generated humanized mice. (A) Kinetic analysis of human CD45+ cell reconstitution in humanized NSG mice. PBMCs were sampled from the tail of each group of humanized NSG mice at different times as indicated. PBMCs were isolated and stained with an antibody specific for human CD45. (B) Bone marrow (BM) and peripheral blood cells were isolated from humanized NSG mice at 24 weeks post-transplantation with human CD34+ cells. The cells were stained with antibodies specific for hCD45, hCD3, hCD19, hCD14, hCD56, and HLA-DR. (C) Analysis of the T cell sub-population in humanized NSG mice. Peripheral blood and spleen cells were stained with antibodies specific for hCD3, hCD4, and hCD8. The results were analyzed by flow cytometry. The data of at least 3 independent experiments are expressed as mean ± SD.

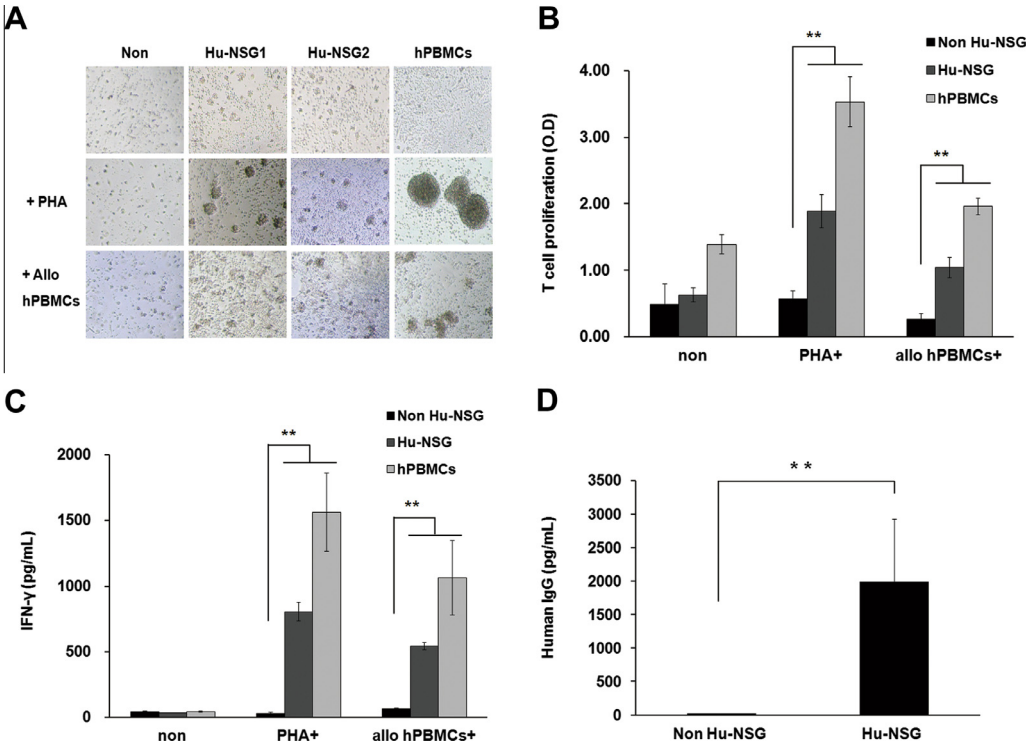

Fig. 3. Functional analysis of engrafted human immune cells. (A and B) The proliferation of engrafted human T cells in response to allogeneic hPBMCs and PHA. Spleens were isolated from humanized NSG mice at 24 weeks post-transplantation with human CD34+ cells. hPBMCs and splenocytes from humanized NSG mice were cocultured for 6 days. The proliferative response of splenocyte T cells was examined using the MLR assay. (C) IFN-γ levels in the MLR culture supernatants were measured by ELISA. (D) Production of human immunoglobulins. Twenty-four weeks after human CD34+ cell injection, plasma samples from humanized NSG mice were collected and evaluated for human IgG concentration with ELISA. Plasma samples of non-humanized NSG mice were used as negative controls. The data of 3 independent experiments, performed in triplicate, are expressed as mean ± SD. $^{**}P < 0.01$. *Abbreviations:* Hu-NSG, humanized NSG mouse.

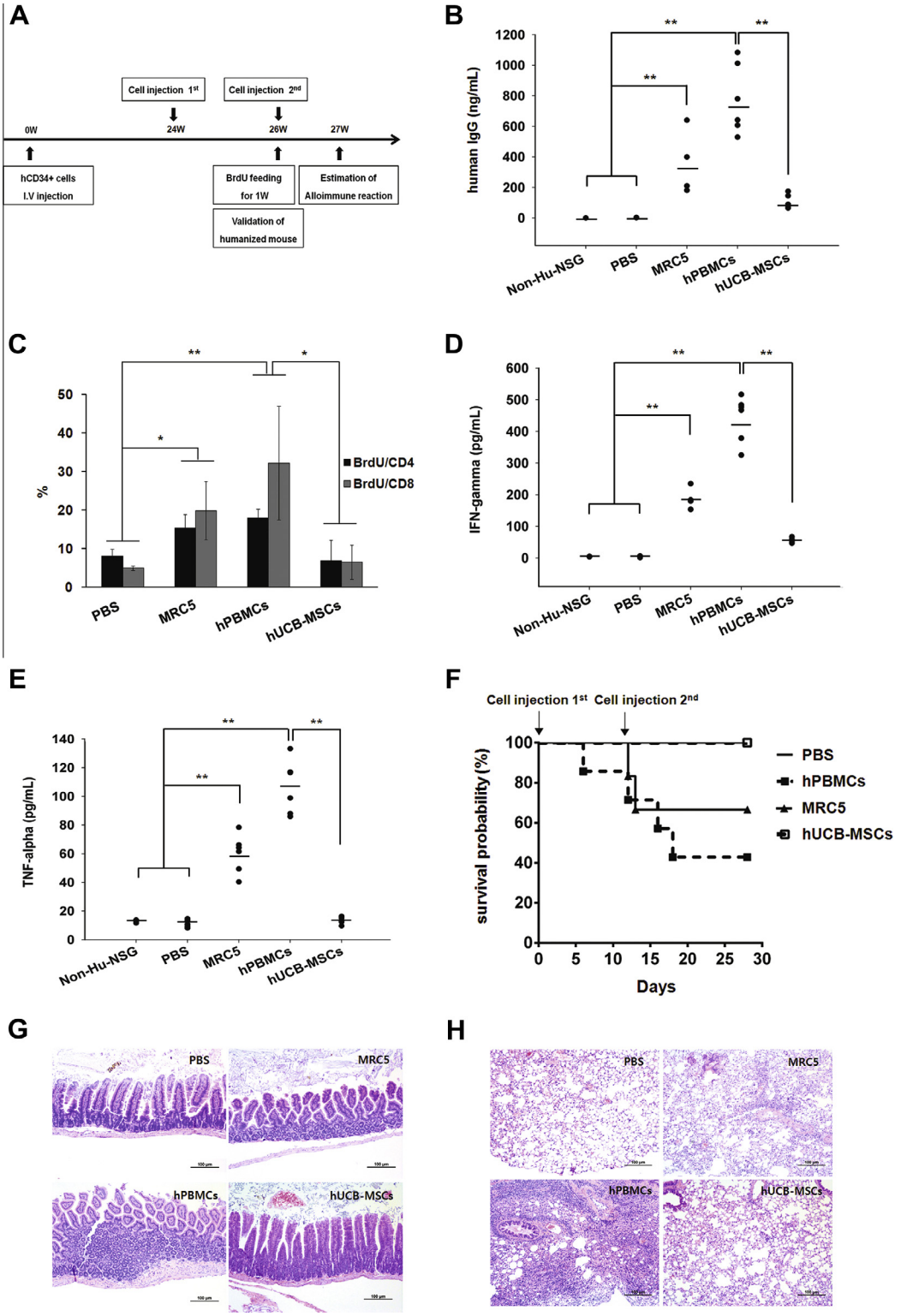

Fig. 4. Evaluation of immunogenicity of hUCB-MSCs in humanized mice. (A) Scheme for an experiment to evaluate the immunogenicity of hUCB-MSCs in humanized mice. NSG mice conditioned by total body irradiation at 240 cGy were injected via the tail vein with 1×10^5 human CD34+ cells isolated from umbilical cord blood. The humanized mice were repeatedly injected (days 0 and 14) for 3 weeks with PBS, HLA-mismatched allogeneic hPBMCs, MRC5, or hUCB-MSCs. From the second day of injection (day 14), mice were fed with BrdU (1 mg/ml)-containing water for 1 week; then, the spleens, plasma, small intestines, and lungs from humanized mice were harvested. (B) Human immunoglobulin production. The plasma of humanized NSG mice was sampled, and evaluated for the human IgG concentration by ELISA. (C) Analysis of splenocyte T cell activation in humanized NSG mice. Splenocytes were stained with antibodies specific for hCD4, hCD8, and BrdU. Pro-inflammatory cytokines such as IFN-γ (D) and TNF-α (E) in the plasma of humanized NSG mice were examined using ELISA. (F) Kaplan–Meier analysis of humanized mice. The survival events of humanized mice with PBS ($n = 7$), hPBMCs ($n = 6$), hMRC5 ($n = 5$), and hUCB-MSC ($n = 7$) were monitored daily for 30 days after cell injection. Histological analysis. Small intestines (G) and lungs (H) were isolated from humanized NSG mice. The sections were stained with hematoxylin and eosin (H&E). The data of 3 independent experiments, performed in triplicate, are expressed as mean ± SD. $^*P < 0.05$ or $^{**}P < 0.01$. Scale bar: 10 μm.

3.4. Low immunogenicity of hUCB-MSCs after intravenous injection into humanized NSG mice

We tested whether the low immunogenicity of hUCB-MSCs was maintained after intravenous injection into humanized mice (Fig. 4A). The level of secreted human IgG in peripheral blood was significantly higher in allogeneic MRC5 or hPBMC injected groups than in those injected with the PBS control. The IgG levels in humanized NSG mice injected with hUCB-MSCs were significantly lower than those of the groups injected with the other two cell types (MRC5: 357 ± 211 ng/ml, hPBMCs: 775 ± 227 ng/ml, and hUCB-MSCs: 110 ± 46 ng/ml; Fig. 4B). The proportion of human activated T cells from splenocytes was higher in mice injected with MRC5 or hPBMCs than in those injected with hUCB-MSCs (Fig. 4C). The IFN-γ and TNF-α levels in humanized NSG mice injected with hUCB-MSCs were lower than those observed following injection of the other two cell types (MRC5: 188 ± 34 pg/ml, hPBMCs: 420 ± 97 pg/ml, and hUCB-MSCs: 54 ± 7 pg/ml, Fig. 4D; MRC5: 60 ± 13 pg/ml, hPBMCs: 106 ± 17 pg/ml, and hUCB-MSCs:13 ± 2 pg/ml, Fig. 4E).

Kaplan–Meier survival analysis revealed that, after the second injection at 26 weeks, the surviving fraction decreased to 42% and 66% for mice injected with MRC5 or hPBMCs, respectively. In contrast, no dead mice were found in the PBS- or hUCB-MSCs-injected group (Fig. 4F).

Histology (Fig. 4G and H) revealed that hPBMC injection resulted in high amounts of lymphocyte infiltration in the small intestine and lungs. The lung alveoli were also larger, and mice exhibited focal airspace enlargement and heterogeneous alveolar size because of lymphocyte infiltration and inflammation. The structure of the small intestine villi was stunted and associated with lymphocyte infiltration at the basement regions. The MRC5-injected group had moderate lymphocyte infiltration and inflammatory lesions in the small intestine and lungs. In contrast, systemic hUCB-MSC injection did not lead to histopathological changes, and the tissues were similar to those in PBS-injected mice.

4. Discussion

Based on the immune-privileged properties of MSCs, these cells can be used as universal donors in allogeneic clinical applications. An advantage of allogeneic approaches is their use as "off-the-shelf" therapeutics, which allows for timely treatment for most acute diseases. Although allogeneic MSCs therapy appears immunologically safe [19], it remains uncertain to what degree the cells trigger immune responses *in vivo*. Indeed, the low immunogenic or immunosuppressive properties of allogeneic MSCs *in vitro* are not always maintained *in vivo* [10,20]. Intracardiac injection of allogeneic porcine MSCs elicits an immune response *in vivo*, but the same cells were found to have low immunogenicity *in vitro* [21]. Another study demonstrated that BM-MSCs sustain colitis via IL-7 secretion after transplantation [22].

More suitable models are required to evaluate the immunological issues associated with allogeneic MSC therapy; this has been attempted with allogeneic animal models in rodents, pigs, and non-human primates [9,10]. Although these studies provided valuable information, their common limitation is that they do not recapitulate key elements of human immunological system [23]. Here, to more closely reflect a human allogeneic setting, we used a humanized NSG mouse model to provide evidence that the low immunogenicity of allogeneic hUCB-MSCs can be maintained *in vivo*.

Under inflammatory conditions, MHC-II antigens such as HLA-DR are induced in BM-MSCs [24] and adipose-MSCs [25]. However, we observed no HLA-DR expression in hUCB-MSCs, even after immune stimulation with IFN-γ and TNF-α (Fig. 1D). MSCs derived from Wharton's jelly of the human umbilical cord do not express HLA-DR during inflammation [26]. Furthermore, we found that hUCB-MSCs induced HLA-G expression by interaction with naïve or activated hPBMCs. Placentally expressed HLA-G plays a role in immune tolerance in pregnancy by inhibiting the cytotoxic T lymphocyte (CTL) response or the activity of NK cells [18]. These results imply that hUCB-MSCs can have low immunogenicity even when injected into inflammation sites in patients, which would reduce the chances of immune rejection. These immunological features were maintained in humanized mice since hUCB-MSC injection resulted in low-level T cell proliferation and IFN-γ, TNF-α and IgG production, reflecting the absence of alloimmune responses. Furthermore, the injection of allogeneic hUCB-MSCs did not affect survival rate or provoke lymphocyte infiltration (Fig. 4F–H). Taken together, we suggest that systemic injection of hUCB-MSCs is immunologically stable and safe, and offers an alternative strategy for allogeneic MSC therapy.

We found that long-term engraftment of human CD34+ cells enhanced human immune cell differentiation and that the NSG mice harbored functional T cells and generated human IgG antibody and pro-inflammatory cytokines. However, the MLR responses of splenocytes were lower than those of hPBMCs (Fig. 3A–C), suggesting that a small proportion of mature T cells is present. Consistent with this, a majority of immature T cells is seen in humanized NSG mouse models due to abnormal thymic selection [27,28], whereas normal B cell functionality is retained [28]. Therefore, further studies using improved humanized mouse models are needed to reconfirm present results. In this regard, improved T cell maturation in humanized mice is possible by cotransplanting human bone marrow, liver, and thymus, and autologous CD34+ cells [29], or by using humanized transgenic immunodeficient mice that express the human MHC-II antigen gene, HLA-A2 [30].

In conclusion, our study demonstrates that allogeneic hUCB-MSCs have low immunogenicity *in vitro* and *in vivo*. We suggest that the immunological safety of hUCB-MSCs makes them suitable for allogeneic clinical applications.

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