



Immunological influence of serum-free manufactured umbilical cord-derived mesenchymal stromal cells for steroid-resistant acute graft-versus-host disease

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Abstract

This study investigated the safety, efficacy, and immunological influence of allogeneic umbilical cord-derived mesenchymal stromal cells (IMSUT-CORD) processed in serum-free medium and cryoprotectant, for treating steroid-resistant acute graft-versus-host disease (aGVHD). In a phase I dose-escalation trial, IMSUT-CORD were infused intravenously twice weekly over two cycles with up to two additional cycles. Four patients received a dose of 1×10^6 cells/kg, while three received 2×10^6 kg. Of 76 total adverse events, fourteen associated or possibly associated adverse events included 2 cases of a hot flash, headache, and peripheral neuropathy, 1 each of upper abdominal pain, hypoxia, increased γ -GTP, somnolence, peripheral vascular pain at the injection site, thrombocytopenia, hypertension, and decreased fibrinogen. At 16 weeks after the initial IMSUT-CORD infusion, three patients showed complete response (CR), two partial response (PR), one mixed response, and one no response. The overall response rate was 71.4%, and the continuous CR/PR rate was 100% for over 28 days after CR/PR. NK cell count significantly increased and correlated with treatment response, whereas IL-12, IL-17, and IL-33 levels decreased, but did not correlate with treatment response. CCL2 and CCL11 levels increased during IMSUT-CORD therapy. IMSUT-CORD are usable in patients with steroid-resistant aGVHD (UMIN000032819: <https://www.umin.ac.jp/ctr>).

Keywords Mesenchymal stromal cell · Umbilical cord · Acute GVHD · Clinical trial · Umbilical cord-derived mesenchymal stromal cells (UC-MSCs)

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Introduction

Acute graft-versus-host disease (aGVHD) occurs in 30–70% of patients undergoing allogeneic hematopoietic stem cell transplantation (HSCT) [1, 2], even after intensive prophylaxis with immunosuppressive reagents. Although severe aGVHD is first treated with steroids, steroid-resistant aGVHD represents a major cause of mortality after allogeneic HSCT; therefore, effective therapies remain warranted [3].

Although bone marrow (BM)-derived mesenchymal stromal cells (BM-MSCs) are beneficial as a salvage treatment against treatment-resistant severe aGVHD following allogeneic HSCT [4–8], BM-MSC harvesting is an invasive procedure that may cause hemorrhage, infection, or chronic pain. Furthermore, BM-MSCs exhibit accelerated senescence with increased donor age [9]. To overcome these limitations, perinatal tissue-derived MSCs, including those from cord blood (CB), placenta, and umbilical cord (UC) [10], have been investigated. We have previously examined the utilization of UC as an abundant source of MSCs for immunotherapy [11, 12]. We developed an efficient method to cryopreserve the whole UC tissue [13] and obtain master cells from frozen-thawed UC tissue using an improved explant method [14] and expanded the UC-MSCs (IMSUT-CORD) from master cells in GMP-grade processing. Processing and cryopreservation were conducted using reagents including serum-free medium and serum-free cryoprotectant. We provide a proof of concept using the serum-free manufactured UC-MSCs with an anti-inflammatory property illustrated using an allogeneic mixed lymphocyte reaction (MLR) and xenogeneic GVHD model [15]. UC-MSCs showed a rapid and high proliferative ability and HLA-DR negativity even in the presence of Interferon (IFN)- γ compared with BM-MSCs [12, 16].

Herein, we report the results of a clinical trial of serum-free manufactured IMSUT-CORD for patients with first-line treatment-resistant severe aGVHD following allogeneic HSCT, while considering safety, efficacy, and immunological influences.

Materials and methods

Manufacturing UC-MSCs (IMSUT-CORD)

UC collection, storage, and release including the safety and quality management were approved by the Ethics Committee of The Institute of Medical Science, The University of Tokyo (IMSUT) hospital (IRB#33-2, REC#29-28) and the individual collection hospitals (NTT

Medical Center Tokyo IRB#16-14 and Yamaguchi hospital IRB#33-2). After obtaining informed consent from the donor's mother, the UC was collected at birth, transferred in an in-house cool box to the Cord Blood and Cord Bank of the Research hospital, The Institute of Medical Science at The University of Tokyo (the bank name is IMSUT CORD) [10]. Infectious disease tests were conducted using the mother's blood and cord blood. The health assessment of both the infant and mother was followed for more than 6 months after birth to ensure the safety of UC. UC was manufactured into the product cells, designated IMSUT-CORD in accordance with the GMP standard [10]. Briefly, UC tissue was cut into 1 cm long and frozen in serum-free cryoprotectant (Stem-cellbanker; ZENOAQ Resource Co. Ltd., Fukushima, Japan) within 48 h after birth [13]. After comparative assessments of four donors derived UC, a UC derived from one donor with a relatively high growth rate and good immunosuppressive potency was selected as a master cell candidate. Then, we performed a viral safety evaluation of the master cells; the selected donor-derived master cells were sufficient to be expanded into the product cells (IMSUT-CORD) for implementation. Master cells were obtained from frozen-thawed UC tissues using an improved explant method [14], and cryopreserved again. The qualified master cells were thawed and further expanded in a large-scale Corning CellSTACK culture chambers (Corning Inc. NY) using a GMP-compatible in-house serum-free medium (Rohto Pharmaceutical Co. Ltd., Japan) [15]. Expanded product cells were suspended in an in-house, serum-free cryoprotectant comprising 10% DMSO (Cryoserv, Mylan GmbH, Switzerland), dextran 40 (Low molecular dextran D injection; Otsuka Pharmaceutical factory, Inc. (Otsuka), Japan), and bicarbonate solution (Bicanate injection; Otsuka, Japan), and were frozen using a controlled-rate freezer (Thermo Fisher Scientific, 7473, MA, USA) to suppress latent heat, and cryopreserved in a liquid nitrogen tank until use. The product cells (IMSUT-CORD), were packaged in 16 mL, which contained at least 64×10^6 viable MSCs after thawing. More than 2 weeks after the completion of the cryopreservation process, quality tests of frozen-thawed IMSUT-CORD were performed.

Analysis of surface markers in IMSUT-CORD

Flow cytometry was performed as described previously [15]. The cells were labeled with monoclonal antibodies listed in the Supplementary Table 1. The cells were acquired using BD™FACSCanto II flow cytometer (BD) and analyzed using FlowJo software (BD). To assess the influence of IFN- γ stimulation on IMSUT-CORD, the expression of HLA-DR, CD80, CD86, PD-L1, and PD-L2 of IMSUT-CORD were examined using flow cytometry in the absence or presence of IFN- γ at the dose of 100 ng/mL.

Analysis of inhibitory effect of allogeneic MLR and phytohemagglutinin-L (PHA-L) stimulation by IMSUT-CORD

Inhibitory effect of allogeneic MLR [12, 15] and PHA-L stimulation was analyzed by co-culture of UC-MSCs. In MLR, 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Vybrant CFDA SE Cell Tracer Kit; Invitrogen)-labeled peripheral blood-mononuclear cells (MNCs) were used as responder (R) cells. PMDC05 cells were kindly provided by Dr. Narita at the Faculty of Medicine, Niigata University [17] and used as stimulator (S) cells following irradiation. The principal of the CFSE-stained R cells in MLR assay and 10 µg/mL of PHA-L stimulation were conducted as previously described [12, 15]. On the day of MLR, R and S cells co-cultured with or without IMSUT-CORD were mixed. After 4 days of culture, the CFSE fluorescence intensities of R, T cells in the CD4+ and CD8+ gate were analyzed using FACS Canto II and FlowJo software (Supplementary table 1). The immunosuppressive effects of UC-MSCs were compared based on the blockade of the daughter cell peaks. Briefly, the parental intensity of the un-proliferated CFSE histogram gate was the standard line, which is indicated as M1, and subsequent gates (M2, M3, M3, ...) of the proliferated daughter cells were manually set to achieve approximately two-fold progressive reductions in CFSE intensity (Fig. 1C). The inhibition ratio (%) was indicated as the ratio of each division index; R + S + UC-MSCs and R + S (as 100%), or R + PHA-L + UC-MSCs and R + PHA-L (as 100%) as described previously [15, 16].

Analysis of IFN- γ , TNF- α , and prostaglandin E2 concentrations in the supernatant of allogeneic MLR co-cultured with IMSUT-CORD

Supernatant concentrations of IFN- γ , and TNF- α with or without IMSUT-CORD co-culture, were analyzed using the cytokine beads assay (BD CBA Human Th1/Th2/Th17 Cytokine Kit, BD) according to the manufacturer's instructions. The beads were acquired using FACS Canto II flow cytometer and analyzed using the FCAP Array v3.0 software (BD). Prostaglandin E2 expression was analyzed using ELISA (Prostaglandin E2 Parameter Assay Kit, R&D systems, MN) according to the manufacturer's instructions.

Quantitative transcription-polymerase chain reaction (RT-qPCR) analysis

Fold change in indoleamine 2,3-dioxygenase 1 (*IDO-1*) expression in IMSUT-CORD normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) expression

with or without IFN- γ stimulation at the dose of 100 ng/mL was determined using RT-qPCR ($n = 10$). Primers used were listed in supplementary Table 2. Relative quantification was performed using the comparative cycle threshold (Ct) ($2^{-\Delta\Delta Ct}$) method, where $\Delta\Delta Ct = \text{mean } \Delta Ct (\text{target gene}) - \text{mean } \Delta Ct (\text{housekeeping gene})$. Details of RT-qPCR conditions were previously reported [12].

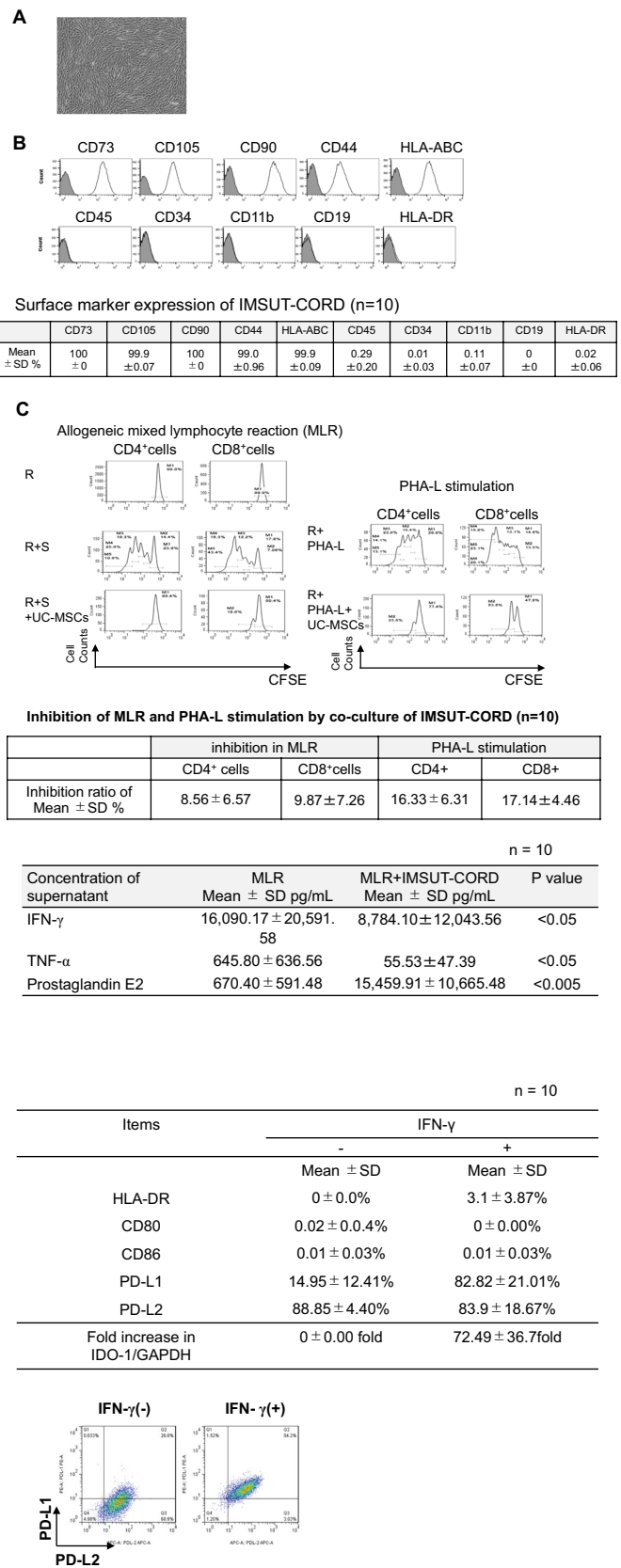
Trial design and patients

In this dose escalation, open-label, phase I clinical trial, patients were enrolled from July 2018 to June 2020 at three sites. This trial, registered as the Umin Clinical Trial Registry (UMIN-CTR: UMIN000032819), was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice guidelines after the approval of each Institutional Review Board (IRB #29-32, 30-21, 2020-1) and regulatory authority. Written informed consent was obtained from all patients.

aGVHD stage/grade was defined according to the 1994 Consensus Conference on Acute GVHD grading criteria [18]. In response to aGVHD, a complete response (CR) was defined as the complete resolution of aGVHD; partial response (PR) as a decrease in organ stage or grade; no response (NR) as no change in grade; progression (PG) as progressive worsening in grade; and mixed response (MR) as a mixture of a decrease and increase in organ stages of aGVHD. Steroid-refractory aGVHD was defined as the PG of aGVHD for 3 days with standard-dose steroid administration or NR after 5 days of therapy. Twenty- to seventy-year-old standard steroid therapy-resistant patients with Grade II to IV aGVHD after HSCT for hematological malignancies resistant to standard steroid-containing therapy were eligible. Patients not treated with standard steroid therapy for aGVHD, who were not in remission at HSCT except for myelodysplastic syndrome with or without leukemic transition and myeloproliferative diseases, with severe complications of vital organs tested positive for viral infections or were allergic to gentamicin and amphotericin B, utilized in the processing and culture, or DMSO, were excluded. The concentrations of gentamicin and amphotericin B are lower than the molecular weight in the final product because they were used during early processing and diluted; therefore, as instructed by the Japanese regulatory authorities, we used this as an exclusion criterion.

Cryopreserved IMSUT-CORD was rapidly thawed in 37 °C water bath and drawn into the syringe and slowly injected intravenously without washing and dilution over 10 min. Before IMSUT-CORD injection, hydrocortisone and chlorpheniramine, were infused to prevent infusion-related reactions. IMSUT-CORD was administered twice per week (designated as one cycle) in two cycles, and patients who showed PR or MR with no severe adverse

Fig. 1 Quality test results of frozen-thawed IMSUT-CORD for the clinical trial. **A** Morphological features of IMSUT-CORD before harvesting the cells. **B** Surface markers of IMSUT-CORD. A representative flow cytometry histogram image of 10 products, processed individually; the table shows mean \pm SD % of the products ($n = 10$). **C** Inhibitory effect of allogeneic mixed lymphocyte reaction (MLR) and phytohemagglutinin-L (PHA-L) stimulation by co-culture of UC-MSCs. Mean \pm SD % of inhibition of MLR and PHA-L stimulation by IMSUT-CORD is shown ($n = 10$). **D** Factors in the supernatant of allogeneic mixed lymphocyte reaction (MLR) with or without IMSUT-CORD. Supernatant concentration of IFN- γ , TNF- α , and prostaglandin E2, with or without IMSUT-CORD co-culture; mean \pm SD ($n = 10$). Statistical analysis is shown. **E** Influence of IFN- γ stimulation on IMSUT-CORD. Expression of HLA-DR, CD80, CD86, PD-L1, and PD-L2 of IMSUT-CORD examined using flow cytometry in the absence or presence of IFN- γ and shown as mean \pm SD of % positivity. Fold expression of indoleamine 2,3-dioxygenase 1 (IDO-1) of IMSUT-CORD normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) with or without IFN- γ stimulation, determined using quantitative real-time PCR ($n = 10$)



events were allowed to undergo two additional cycles. Dose escalation was conducted following the 3 + 3 dose-escalation scheme. Doses were as follows: cohort 1, 1×10^6 cells/kg body weight (BW); cohort 2, 2×10^6 cells/kg BW; cohort-1, 0.5×10^6 cells/kg BW. The initial dose was 1×10^6 cells/kg BW (cohort 1), while the subsequent dose was 2×10^6 cells/kg BW (cohort 2), when there was no Dose-limiting toxicity (DLT) in cohort 1.

To monitoring the adverse events associated with IMSUT-CORD injection, laboratory studies, viral tests, electrocardiogram (ECG), echocardiography were done according to the schedule. Chest X-ray, computed tomography, and bone marrow aspiration test were performed, if necessary. Vital signs, including ECG monitoring, percutaneous oxygen saturation concentration (SpO₂ levels), and blood pressure, were monitored from the IMSUT-CORD injection to two hours after injection.

The primary endpoint was safety, and the secondary endpoint was efficacy, including the overall response and change of stage/grade of organs damaged by aGVHD. Adverse events and laboratory test results were evaluated until 11 weeks after the final IMSUT-CORD administration. DLT was assessed within 7 days of the final injection of IMSUT-CORD according to the Common Terminology Criteria for Adverse Events (CTCAE) ver. 4.0-JCOG <http://www.jcog.jp/doctor/tool/ctcae5.html>: (1) CTCAE grade 4 neutropenia with < 5% blastic cells in the BM; (2) non-hematologic toxicity \geq grade 3 (controllable infusion reaction excluded); (3) and requirement of > 7 days for the next administration process due to adverse events. Chronic GVHD (cGVHD) was evaluated during a long-term follow-up according to NIH consensus criteria [19].

Patient blood test and immune cell analysis

Immunological studies were conducted, separate from the clinical trial, after obtaining written informed consent and approval from each Institutional Review Board.

The absolute immune cell counts, comprising CD3+T, CD3-CD56+natural killer (NK), CD3+CD4+T, CD3+CD8+T, CD19+B, and CD4+CD25+regulatory T (Treg) cells, in the peripheral blood were measured by counting white blood cells stained directly with antibodies in a BD Trucount™ tube containing beads for quantification (BD). Briefly, 100 μ L of blood, antibodies, and 7-AAD was added into a Trucount tube and red blood cells were lysed using 1 mL of Pharm Lyse (BD). After staining cells according to reagent instructions, the cells were acquired and analyzed using FACSCanto II flow cytometer to quantify immune cells.

Cytokine and chemokine assay in patient blood

We evaluated human cytokine and chemokine concentrations in frozen-thawed plasma collected on the day of IMSUT-CORD administration (before administration), and 1, 2, 3, 4, 6, 8, and 11 weeks after final administration. Cytokines and chemokines were analyzed using a Cytometric bead array; Human Inflammation Panel (13-plex; BioLegend, USA) and Human Proinflammatory Chemokine Panel (13-plex; BioLegend), respectively. All samples were analyzed in triplicate, according to the manufacturer's instructions. Bead fluorescence readings were obtained using FACS Canto II. Data were analyzed using LEGENDplex version 8.0 software (BioLegend).

We also measured C-C motif chemokines, such as chemokine (C-C motif) ligand 2 (CCL2) and CCL11 levels in the supernatant of MLR co-cultured with UC-MSCs ($n = 3$) using a Human Proinflammatory Chemokine Panel (13-plex; BioLegend). To exclude the FBS influence and attachment of them, we set the Transwell insert (Corning, MA, USA) between UC-MSCs and MLR and cultured without FBS supplement. The method for MLR setting was the same as described above.

Statistical analysis

The sample size was determined via the trial study design (early phase I safety study with 3 + 3 dose-escalation protocol). Data from patients who dropped out from the study due to additional treatments for aGVHD were included in any analysis when possible. Overall survival was analyzed using a Kaplan–Meier curve. A linear mixed model was used to assess time-dependent changes for some measurements while considering correlation with patients. Spearman's correlation coefficients were calculated to assess the association between immune cells, cytokines, and chemokines with the treatment response, including the aGVHD grade. Differences between groups were analyzed with JMP 10.0.2 software (SAS Institutes, Cary, NC, USA). Statistical analyses were performed with the Turkey–Kramer test or *T* test, and $P < 0.05$ was regarded as statistically significant.

Results

IMSUT-CORD characteristics

IMSUT-CORD were adherent with spindle-shaped morphology (Fig. 1A), positive for CD105, CD73, and CD90 and negative for CD45, CD34, CD11b, CD19, and HLA-DR (Fig. 1B). In an MLR, the triggering of responder T cell proliferation by allogeneic dendritic cells was efficiently inhibited to $8.56 \pm 6.57\%$ in CD4-positive cells and $9.87 \pm 7.26\%$

in CD8-positive cells via its co-culture with IMSUT-CORD (Fig. 1C). The proliferation of responder cells treated with phytohemagglutinin-L (PHA-L; Sigma-Aldrich) was also inhibited to $16.33 \pm 6.31\%$ in CD4-positive cells and $17.14 \pm 4.46\%$ in CD8-positive cells (Fig. 1C). Co-culture with IMSUT-CORD in the MLR system was accompanied by a significant reduction in IFN- γ and TNF- α levels as shown using cytokine beads assay and the induction of prostaglandin E2 using ELISA (Fig. 1D). Flow cytometry analysis showed that IMSUT-CORD cells were negative for HLA-DR, CD80, and CD86 even at high concentrations of IFN- γ (Fig. 1E). PD-L2 was constitutively expressed in UC-MSCs, whereas PD-L1 expression was induced upon IFN- γ addition (Fig. 1E, F). The abundant induction of *indoleamine 2,3-dioxygenase 1 (IDO-1)* in IMSUT-CORD, as one of the key immunoregulators in the presence of IFN- γ was confirmed using quantitative real-time PCR (Fig. 1E). No chromosomal abnormalities were observed in product cells after G-banding analysis.

Patient characteristics and GVHD response

Seven patients were enrolled in this study, and six agreed to participate in immunological studies. Four were treated in cohort 1 and three were treated in cohort 2 (Table 1). The median age was 43 years (range: 25–62 years); three patients were male and four were females. The prophylaxis

of aGVHD is also shown in Table 1. All patients were administered prednisolone or methylprednisolone as first-line therapy against aGVHD.

Two patients had grade III aGVHD, whereas five had grade II aGVHD (Table 2). Among the patients with grade III aGVHD, patient 2 demonstrated the following stages of organ involvement: skin, 3; liver, 1; gastrointestinal tract (GI tract), 2. Patient 3 demonstrated the following stages of organ involvement: skin, 1; liver, 3; GI tract, 0. Among patients with grade II aGVHD, patient 1 demonstrated skin, 1; liver, 0; GI tract 1, and the skin alone was affected in three patients, and the GI tract alone affected the remaining patient. In cohort 2, single organ involvement was observed. The median number of days from HSCT to the first administration of IMSUT-CORD was 57 days (range, 41–125 days). Three patients (patients 2, 3, and 6) received the first IMSUT-CORD injection after 100 days of HSCT, due to the late-onset aGVHD during the tapering of GVHD prophylaxis reagents. One patient underwent two cycles of IMSUT-CORD; two three cycles; and four four cycles. In cohort 1, one patient achieved CR, two PR, and one MR after 4 weeks from the first administration. In cohort 2, two patients achieved PR and one NR after 4 weeks from the first administration and the patients with PR reached CR after 12 weeks from the first administration (Table 2). Finally, overall, three patients achieved CR and two achieved PR possessing continuous duration for more than 28 days. All

Table 1 Characteristics of patients treated with IMSUT-CORD

Patient #	Sex	Age	Body weight	HSCT source	Disease status	HLA disparity (/6)	Conditioning	Prophylaxis for GVHD	First-line therapy for aGVHD
<i>Cohort 1</i>									
01	M	43	52.8	UCB	Ph1 ⁺ ALL, CR	2	AraC/BU/Flu/TBI 4 Gy	CsA	PSL/mPSL
02	M	49	48.9	rPSBC	GF after 1st UCBT, Overt leukemia from RAEB	3	Flu	Post CY/PSL/MMF/Tac	mPSL
03	M	35	49.4	rPBSC	Ph1 ⁻ ALL, CR	0	CY/TBI 12 Gy	CsA+sMTX	mPSL
04	F	25	46.9	UCB	AML with MRS from MDS RAEB-2	2	AraC/CY/TBI12Gy	CsA+sMTX	mPSL
<i>Cohort 2</i>									
05	F	62	46.8	UBM	Ph1 ⁺ ALL, CR	1	Flu/BU/TBI 4 Gy	Tac+sMTX	mPSL
06	F	37	44.5	UCB	AML with MRS from MDS → MDS	3	Flu/Mel/Bu/AraC/TBI	Tac	mPSL
07	F	59	41.7	rPBSC	Myelofibrosis, PR	0	FL/MEL/TBI 4 Gy	CsA+sMTX	mPSL

M male, *F* female, *UCB* unrelated cord blood, *rPBSC* related peripheral blood stem cells, *UBM* unrelated bone marrow, *ALL* acute lymphocytic leukemia, *AML* acute myeloid leukemia, *RAEB* refractory anemia with excess of blast, *MRS* myelodysplasia-related changes, *MDS* myelodysplastic syndrome, *GF* graft failure, *UCBT* unrelated CB transplantation, *CR* complete remission, *PR* partial remission, *HLA* human leukocyte antigen, *AraC* cytarabine, *BU* busulfan, *Flu* fludarabine phosphate, *TBI* total body irradiation, *CY* cyclophosphamide, *Mel* Melphalan, *CsA* Cyclosporin A, *Post CY* post-transplantation cyclophosphamide, *MMF* mycophenolate mofetil, *Tac* tacrolimus hydrate, *sTMA* short-term methotrexate, *PSL* prednisolone, *mPSL* methylprednisolone

Table 2 Results of treatment with IMSUT-CORD for severe acute GVHD

Patient #	Days after HSCT when 1st IMSUT-CORD infusion was administered	Overall aGVHD grade	GVHD stage			Cell dose ($\times 10^6/\text{kg}$)	No. of infusion cycles (2 infusions/cycle)	Response weeks after 1st infusion			Alive/dead after 1st IMSUT-CORD infusion
			Skin	Liver	GI-tract			4 W	12 W	16 W	
<i>Cohort 1</i>											
01	56	II	1	0	1	1	3	CR	CR	CR	Alive, day 712
02	114	III	3	1	2	1	3	MR	-	-	Dead, Day 46
03	124	III	1	3	0	1	4	PR	PR	PR	Alive, Day 615
04	57	II	3	0	0	1	2	PR	PR	PR	Alive, Day 382
<i>Cohort 2</i>											
05	41	II	3	0	0	2	4	PR	CR	CR	Alive, Day 272
06	125	II	0	0	1	2	4	NC	NC	NC	Alive, Day 244
07	41	II	3	0	0	2	4	PR	CR	CR	Alive, Day 104

HSCT; hematopoietic stem cells transplantation, GI-tract; gastro-intestinal tract, aGVHD; acute graft-versus-host disease, cGVHD; chronic GVHD, in response to aGVHD, CR; complete response, PR; partial response, NR; no response, MR; mixed response

patients except patient 2 were alive during the long-term follow-up period of 327 days (median; range: 104–712 days). The overall survival rate was 85.7% (90% CI 62.2–100) at day 100 (Supplementary Fig. 1).

Long-term follow-up revealed four of six evaluable patients developed cGVHD; one developed cGVHD within the monitoring period of adverse events and the remaining three developed after then. Patient 1 received cholecystectomy for cholecystitis due to gall stone and discontinued PSL after 37 weeks from the first administration of IMSUT-CORD, and liver dysfunction with elevated alkaline phosphatase developed after 49 weeks from the first administration, which improved without any immunosuppressant. Patient 3 had bronchiolitis obliterans (BO) after pneumonia and septic shock caused by *Streptococcus Pneumoniae* after 36 weeks from the first administration and developed into severe cGVHD with BO and dry eye and mouth controlled with tacrolimus and PSL. Patient 4 persisted skin aGVHD and transited into mild skin cGVHD treated with low-dose steroid therapy after 7 weeks from the first administration. Patient 7 showed cGVHD with dry eye treated after 27 weeks from the first administration.

Safety evaluations

No DLT was found in any patient. Adverse events until 11 weeks after the final IMSUT-CORD administration were observed in all patients and listed in Table 3. Fourteen out of total seventy-six adverse events were either associated or possibly associated with IMSUT-CORD administration. In 3/4 patients in cohort 1, one patient experienced hot flashes (grade 1) and upper abdominal pain (grade 1), one had a hypoxia (grade 1), and one had a headache (grade 1). Patients recovered from these symptoms without medication. In cohort 2, 2/3 patients showed either of the following adverse events: one event of increased γ -glutamine transferase levels (grade 1), hot flashes (grade 1), somnolence (grade 1), peripheral vascular pain at the injection site (grade 1), thrombocytopenia (grade 1), controllable hypertension (grade 3), fibrinogen decreased (grade 2), or headache (grade 2) and two events of peripheral neuropathies (grade 2). Patients recovered from all symptoms except peripheral neuropathy, although it could not be overlooked that this symptom may have been affected by other drugs. No serious adverse events related, or possibly related, to IMSUT-CORD administration were observed.

Immunological response to IMSUT-CORD

To assess the immunological influence of IMSUT-CORD, immunological properties during and after IMSUT-CORD therapy were monitored in six patients except patient 6. Blood samples were obtained on the day of IMSUT-CORD

Table 3 Summary of adverse events until 11 weeks after the final IMSUT-CORD administration

	Number of patients (%)			
	Cohort 1 (n=4)		Cohort 2 (n=3)	
	All grades	Grade 3/4	All grades	Grade 3/4
<i>Blood lymphatic system disorders</i>				
Anemia	0	0	1 (33.3%)	1 (33.3%)
<i>Cardiac disorders</i>				
Cardiomegaly	0	0	1 (33.3%)	0
Cardiac disorder	1 (25%)	0	0	0
Ventricular arrhythmia	0	0	1 (33.3%)	0
<i>Gastrointestinal disorders</i>				
Enterocolitis	1 (25%)	0	0	0
Gastrointestinal bleeding	0	0	1 (33.3%)	1 (33.3%)
Gastroesophageal reflux disease	1 (25%)	0	0	0
Nausea	1 (25%)	0	1 (33.3%)	0
Vomiting	1 (25%)	0	0	0
Diarrhea	1 (25%)	0	0	0
Upper abdominal pain	1 (25%)*	0	0	0
<i>General disorders and administration site conditions</i>				
Fever	2 (50%)	0	0	0
Injection site reaction (vascular pain)	0	0	1 (33.3%)*	0
<i>Hepatobiliary disorders</i>				
Cholecystitis (gallstones)	1 (25%)	1 (25%)	0	0
<i>Infections and infestations</i>				
Cytomegalovirus infection reactivation	0	0	1 (33.3%)	0
Gum infection	1 (25%)	0	0	0
Otitis externa	0	0	1 (33.3%)	0
Sinusitis	0	0	1 (33.3%)	0
<i>Injury, poisoning and procedural complications</i>				
Fall	1 (25%)	0	0	0
<i>Metabolism and nutrition disorders</i>				
Cholesterol high	1 (25%)	0	0	0
Hypoalbuminemia	1 (25%)	1 (25%)	0	0
Hypokalemia	1 (25%)	1 (25%)	1 (33.3%)	1 (33.3%)
Hypocalcemia	1 (25%)	0	0	0
Hypomagnesemia	1 (25%)	0	0	0
Hypophosphatemia	1 (25%)	0	0	0
Hyperlipidemia	3 (75%)	0	0	0
<i>Nervous system disorders</i>				
Headache	1 (25%)	0	2 (66.7%)*	0
Paresthesia	1 (25%)	0	0	0
Peripheral sensory neuropathy	0	0	2 (66.7%)*	0
Somnolence	0	0	1 (33.3%)*	0
<i>Psychiatric disorders</i>				
Insomnia	0	0	1 (33.3%)	0
<i>Respiratory, thoracic and mediastinal disorders</i>				
Allergic rhinitis	1 (25%)	0	0	0
Nasopharyngitis	1 (25%)	0	0	0
Upper respiratory infection	0	0	1 (33.3%)	0
Hypoxia	2 (50%)*	1 (25%)	0	0
Pleural effusion	1 (25%)	0	0	0
Chest discomfort	1 (25%)	0	0	0

Table 3 (continued)

	Number of patients (%)			
	Cohort 1 (n=4)		Cohort 2 (n=3)	
	All grades	Grade 3/4	All grades	Grade 3/4
Epistaxis	1 (25%)	0	0	0
Rhinorrhea	1 (25%)	0	0	0
<i>Skin and subcutaneous tissue disorders</i>				
Purpura	1 (25%)	0	0	0
<i>Vascular disorders</i>				
Hot flashes	1 (25%)*	0	1 (33.3%)*	0
Hypertension	0	0	1 (33.3%)	1 (33.3%)*
<i>Others</i>				
Chronic GVHD	1 (25%)	0	0	0
<i>Investigations</i>				
ALT increased	1 (25%)	1 (25%)	2 (66.7%)	1 (33.3%)
AST increased	1 (25%)	1 (25%)	0	0
Alkaline phosphatase increased	1 (25%)	1 (25%)	0	0
BNP increased	1 (25%)	0	0	0
Bloody stool	1 (25%)	0	0	0
C-reactive protein increased	0	0	1 (33.3%)	0
Fatty liver	1 (25%)	0	0	0
Fibrinogen decreased	1 (25%)	0	1 (33.3%)*	0
γ -globulin decreased	1 (25%)	0	1 (33.3%)	0
Fibrinolytic system activation	1 (25%)	0	0	0
GGT increased	0	0	2 (66.7%)*	1 (33.3%)
Hematuria	1 (25%)	0	1 (33.3%)	0
Hepatic transaminase increased	1 (25%)	0	0	0
Platelet count decreased	1 (25%)	1 (25%)	2 (66.7%)*	2 (66.7%)
Proteinuria	0	0	1 (33.3%)	0

AST aspartate aminotransferase, BNP brain natriuretic peptide, ALT alanine aminotransferase, CMV cytomegalovirus, GGT γ -glutamine transferase

*Indication of either associated or possibly associated adverse events

administration (before administration), and 1, 2, 3, 4, 6, 8, and 11 weeks after the final administration. Therefore, the data on the administration of IMSUT-CORD reflect the status before administration or the influence of the prior injections. CD14+ monocytes, CD3–CD56+ NK cells, CD19+ B cells, CD3+CD4+ T cells, and CD3+CD8+ T cells in the peripheral blood were analyzed by flow cytometry (Fig. 2A–E). In patients 1, 2, and 3, the absolute number of monocytes in peripheral blood showed an increasing tendency, whereas that in patients 4, 5, and 7 with skin GVHD showed no change or decreasing tendency during IMSUT-CORD administrations. Overall, there was no significant difference in monocytes change (Fig. 2A, Table 4). Absolute numbers of CD3–CD56+NK cells were significantly increased during IMSUT-CORD therapy in all patients except Patient 2, who developed liver GVHD (Fig. 2B, Table 4). NK cell numbers correlated with treatment response ($R^2 = 0.498$), whereas they were not correlated with aGVHD grade.

IL-12p70, IL-17A, and IL-33 levels were significantly suppressed during IMSUT-CORD therapy compared with those before injection, although they were not correlated with aGVHD grade or treatment response (Table 4). In these few patients, no significant correlation was observed between other inflammatory cytokines and aGVHD grades or therapeutic effects.

Levels of chemokines, such as CCL11 and CCL2, increased during IMSUT-CORD therapy, whereas MIP1 α levels decreased. Notably, CCL11 levels were inversely correlated with aGVHD and positively correlated with the treatment response (Table 4).

A clinical course of Patient 3 with grade III aGVHD (skin, 1; liver, 3; GI tract, 0), one of the representative responders to IMSUT-CORD was shown in Fig. 3. He was diagnosed with hepatic GVHD after pathological analysis, accompanied by an increase in liver transferase (ALT, AST, and γ -GTP) and a total bilirubin (Fig. 3A). The liver transferase was decreased after administration of IMSUT-CORD, and

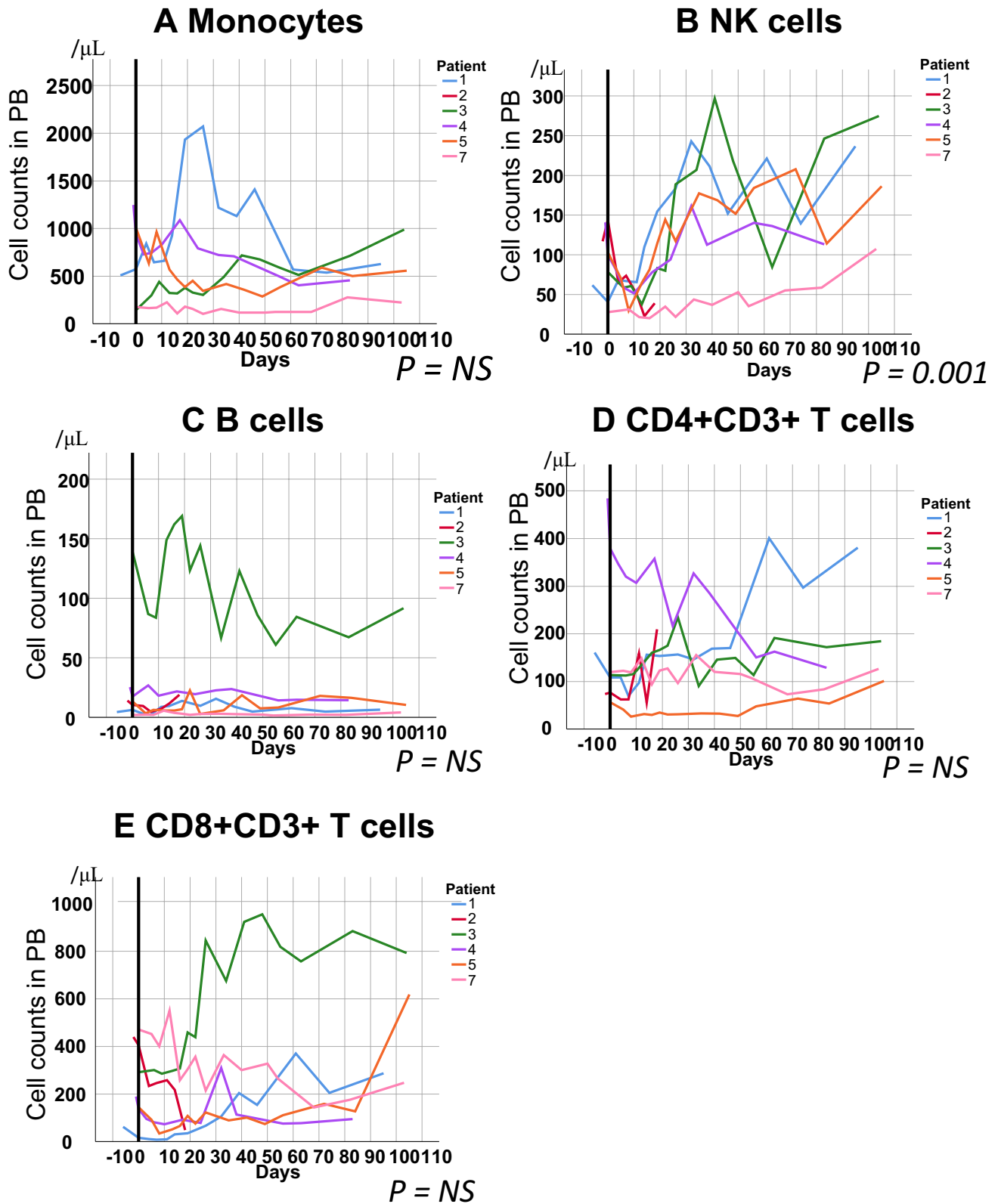


Fig. 2 Immune cells during and after UC-MSC administration in peripheral blood. **A** CD14+ monocytes, **B** CD3–CD56+ NK cells, **C** CD19+ B cells, **D** CD3+CD4+T cells, **E** CD3+CD8+T cells. Data

are shown in terms of the absolute number of peripheral blood cells in respective cases. PB, peripheral blood; ($n = 6$). NS, not significant

Table 4 Transition of immune cells, cytokines, and chemokines during IMSUT-CORD therapy

Tests	Change value per day*	P value	95% CI	Spearman's correlation to	
				aGVHD grade	Treatment response* ²
<i>Immune cells</i>					
CD3–CD56+ NK cells	2.24	0.001**	1.00 to 3.48	– 0.158	0.498**
CD19+ B cells	– 0.02	0.946	– 0.61 to 0.57	0.473	– 0.049
CD14+ monocytes	5.70	0.228	– 3.72 to 15.12	– 0.024	0.026
CD3+ CD4+ T cells	0.94	0.117	– 0.25 to 2.13	0.082	0.101
CD3+ CD8+ T cells	1.72	0.364	– 2.07 to 5.50	0.292	0.151
CD4+ CD25+ Tregs	0.00	0.963	– 0.13 to 0.13	– 0.426	0.188
<i>Cytokines</i>					
IL-1 β	– 0.01	0.897	– 0.20 to 0.17	0.007	– 0.087
IFN- α 2	0.37	0.661	– 1.34 to 2.09	– 0.219	– 0.078
IFN- γ	0.14	0.341	– 0.16 to 0.44	0.102	0.043
TNF α	– 0.01	0.910	– 0.26 to 0.23	– 0.046	– 0.078
IL-6	0.22	0.128	– 0.07 to 0.51	0.367	– 0.189
IL-10	– 0.17	0.692	– 1.02 to 0.69	0.288	– 0.115
IL-12p70	– 0.49	0.009**	– 0.86 to – 0.13	0.199	– 0.245
IL-17A	– 4.04	0.007**	– 6.90 to – 1.18	0.330	– 0.203
IL-18	1.14	0.822	– 9.06 to 11.35	0.556	– 0.299
IL-23	– 2.55	0.062	– 5.23 to 0.14	0.351	– 0.186
IL-33 (=ST-2)	– 3.08	0.021**	– 5.68 to – 0.49	– 0.136	– 0.116
<i>Chemokines</i>					
CCL2(MCP-1)	2.53	0.019**	0.44 to 4.61	0.423	0.055
CCL3(MIP1a)	– 0.51	0.044**	– 1.01 to – 0.01	0.444	0.055
CCL5(RANTES)	– 3.10	0.591	– 14.67 to 8.47	0.253	0.097
CCL11(Eotaxin-1)	0.90	0.014**	0.20 to 1.61	– 0.641	0.496**
CCL17(TARC)	– 0.94	0.102	– 2.08 to 0.19	– 0.180	0.010
CXCL-8(IL-8A)	0.55	0.325	– 0.57 to 1.67	0.509	– 0.259
CXCL-9(MIG)	8.30	0.098	– 1.61 to 18.21	– 0.026	– 0.100
CXCL-10(IP10)	20.33	0.324	– 20.78 to 61.43	0.012	– 0.179

n = 6, *CI* confidence interval, *CCL* chemokine (C–C motif) ligand, *CXCL* chemokine (C–X–C motif) ligand, *MCP-1* monocyte chemoattractant protein-1, *MIP1a* macrophage inflammatory protein 1-alpha, *RANTES* regulated on activation, normal T cell expressed and secreted, *TARC* thymus- and activation-regulated chemokine, *MIG* monokine induced by gamma interferon, *IP10* interferon gamma-induced protein 10, *Average change value for each additional day. ***P* < 0.05

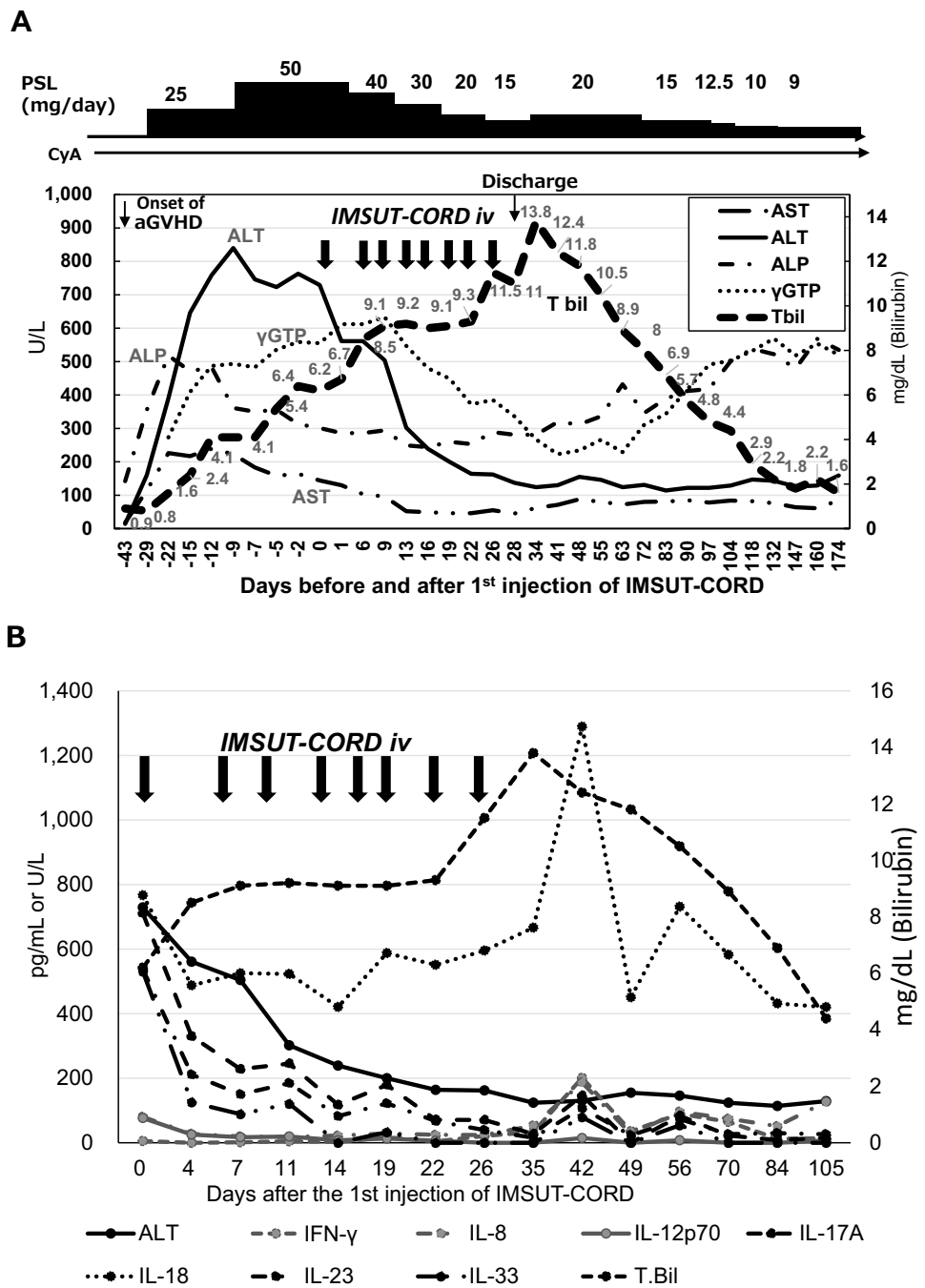
correspondingly levels of serum inflammatory cytokines, such as IL-18, IL-17A, IL-23, and IL-33 decreased rapidly after the first administration of IMSUT-CORD (Fig. 3B). Although his total bilirubin was delayed to be decreased after IMSUT-CORD and diagnosed as PR at the end of the observation period, he finally achieved CR with a normal range of total bilirubin on day 174.

CCL2 and CCL11 secretion in MLR with IMSUT-CORD

To understand the mechanism for the elevation in CCL2 and CCL11 levels in the peripheral blood (PB) of patients during IMSUT-CORD administration (Table 4), we measured the CCL2 and CCL11 levels in the supernatant of

MLR co-cultured with UC-MSCs (*n* = 3) using chemokine beads assay. UC-MSCs constitutively secreted CCL2, which was significantly induced in large quantities upon co-culture with MLR (Fig. 4A). The mean CCL2 levels in the supernatant of MNCs, UC-MSCs, MLR, and MLR co-cultured with UC-MSCs were 74.73, 519.7, 176.33, and 12,167.2 pg/mL, respectively. CCL11 was secreted from the activated MNCs in allogeneic MLR and increased significantly in the supernatant of MLR co-cultured with UC-MSCs (Fig. 4B). The mean CCL11 levels in the supernatant of MNCs, UC-MSCs, MLR, and MLR co-cultured with UC-MSCs were 0, 0, 68.94, and 108.66 pg/mL, respectively.

Fig. 3 Clinical course of patient 03. **A** Hepatic function test results, including results for AST, ALT, ALP, γ -GTP, and total bilirubin (T bil), and **B** Inflammatory cytokines, including IFN- γ , IL-8, IL-12p70, IL-17A, IL-18, IL-23, and IL-33, before and after the administration of UC-MSCs



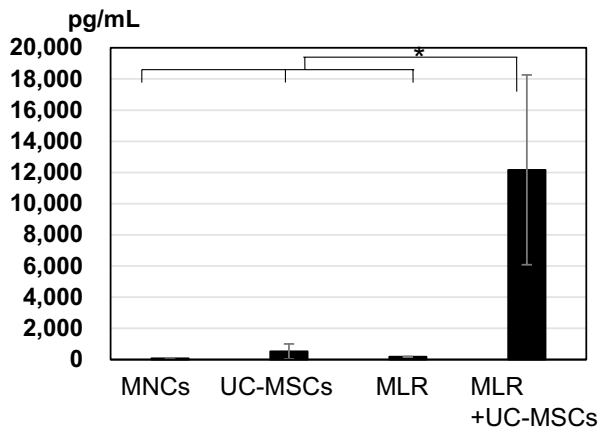
Discussion

Here, we conducted a phase I clinical trial for the treatment-resistant aGVHD with clinical-grade serum-free manufactured IMSUT-CORD. The immunosuppressive properties based on our previous report on UC-MSCs [12] were adopted to the quality tests for shipping the IMSUT-CORD. The demonstrated results showed that each lot of IMSUT-CORD were equivalent and functional. The IMSUT-CORD possessed the characteristics of UC-MSCs. They are

characterized by the absence of HLA-DR, CD80, and CD86 markers, even in the presence of IFN- γ , while HLA-DR is induced in BM-MSCs by IFN- γ stimulation as previously reported [12]. The IMSUT-CORD inhibited the proliferation of CD4- and CD8-positive T cells stimulated by allogeneic dendritic cells in a MLR and showed the significant induction of IDO-1 and PD-L1 [15, 16].

Certain BM-MSC-based drugs, such as Remestemcel-L[®] [20, 21], Prochymal[®] [20], and Temsel HS Inj[®] [22–24] have been approved in Canada, USA, and Japan, respectively,

A CCL2 (MCP -1)



B CCL11 (Eotaxin)

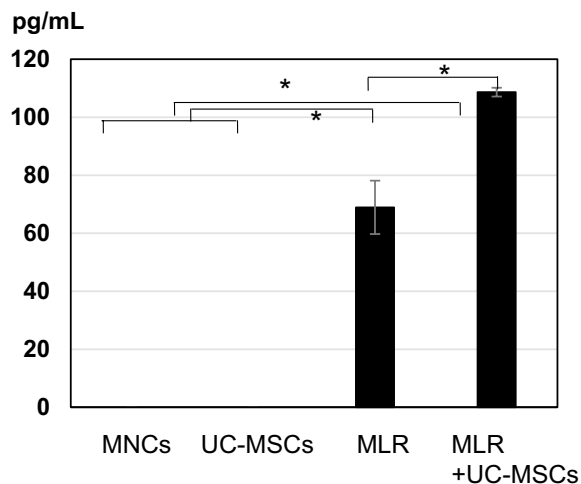


Fig. 4 CCL2 and CCL11 levels in the supernatant of MLR co-cultured with UC-MSCs. **A** CCL2 levels. **B** CCL11 levels. CCL2 and CCL11 levels in the supernatant of peripheral blood mononuclear cells (MNCs), umbilical cord-derived mesenchymal stromal cells (UC-MSCs), mixed lymphocyte reaction (MLR), and MLR co-cultured with UC-MSCs. Data are shown as the mean \pm SD (pg/mL) for three different donors with triplicate measurements. * $P < 0.05$

based on their safety and effectiveness in treatment-resistant aGVHD. Wu et al. [25] first reported that steroid-resistant gastrointestinal and hepatic aGVHD improved dramatically after each of the four infusions of UC-MSCs administered to two patients, without adverse effects. There are not many of clinical trials as the pure treatment with UC-MSCs against aGVHD among many clinical trials, including that for the prevention of graft failure and severe aGVHD in HSCT for patients with aplastic anemia [10, 26, 27]. However, most of MSCs were cultured with xenogeneic serum (FBS)-containing medium. No severe adverse events related, or possibly related, to IMSUT-CORD administration were observed and tolerable for the continuation of the IMSUT CORD

administration. To the best of our knowledge, this is the first study to report that serum-free manufactured UC-MSCs; IMSUT-CORD are safe and effective in some patients with steroid-resistant aGVHD.

Long-term follow-up showed that four out of 6 evaluable patients had cGVHD after reaching complete or partial response. One patient with skin aGVHD, which could not obtain CR developed cGVHD, while the remaining patients developed cGVHD after 6 months from IMSUT-CORD therapy when the effect of IMSUT-CORD might have already disappeared. Two of three patients (Patient 01 and 03) had inflammation (Cholecystitis) or infection (Pneumonia and sepsis) events prior development of cGVHD. The development of cGVHD in those patients seemed related to the prior inflammatory events rather than steroid or other immunosuppressant tapering. We cannot speculate on the influence of IMSUT-CORD treatment on the development of cGVHD, but we need to take care of the trigger episode of inflammation to prevent severe cGVHD.

In our study, Patient 2 presented stage 1 liver GVHD initially but progressed to liver dysfunction with elevated bilirubin during the administration of IMSUT-CORD. In contrast, Patient 3 with stage 3 liver aGVHD showed continuous elevation in bilirubin levels during the administration of IMSUT-CORD, then slow recovery to the normal range, while rapidly improving liver transferase levels together with a reduction of inflammatory cytokines from the first administration of IMSUT-CORD administration. The control of the liver GVHD remains unresolved without the suitable prognostic factors [24, 28]. Interestingly, the number of CD3⁺CD56⁺NK cells during IMSUT-CORD administration, significantly increased in all patients except Patient 2, who developed liver GVHD. NK cell number also correlated with IMSUT-CORD treatment rather than aGVHD grade. The influence of MSC on NK cells appears to be dependent on the surrounding environment. Boissel et al. showed that irradiated UC-MSCs feeder cells can increase the expansion of CB NK cells [29]. Vacca et al. reported that decidual MSCs (dMSCs) sustain the differentiation of CD34⁺ cell to CD56^{bright} CD16⁻ NK cells [30]. On the other hand, the same group demonstrate that dMSCs suppress IL-15-induced NK cell proliferation, inhibit the perforin and granzyme levels and NK cell cytotoxicity. Furthermore, TGF- β released by MSCs convert peripheral blood CD16⁺NK cells into CD16⁻ NK cells [31]. Like M1 and M2 macrophage, NK cells which are classified into type 1 and type 2 show different immunological functions [32], although the patient NK cells in this study remained unclassified. In the context of GVHD, Olson et al. [33] reported that NK cells suppress GVHD without causing GVHD. The authors showed that mice receiving donor NK and T cells exhibited improved survival and decreased GVHD scores compared with controls receiving donor T cells alone. Further, activated NK

cells mediate the direct lysis of re-isolated GVHD-inducing T cells in vitro. Yamazaki et al. also demonstrated that the CD56+ cell dose in peripheral blood stem cells allografts is significantly inversely correlated with the incidence of GVHD, suggesting that NK cells have a suppressive effect on aGVHD [34]. Therefore, NK cell transition might be an indicator of aGVHD response to IMSUT-CORD.

Th17 cells producing IL-17 contribute to the development of GVHD. Levels of inflammatory cytokines, including IL-12, IL-17, and IL-33 (ST2), were decreased during IMSUT-CORD therapy, although they showed no correlation with aGVHD grade or treatment in such a small sample set. IL-33, together with regenerating islet-derived protein 3 alpha are reportedly correlated with acute GVHD [35–37]. These cytokines might be vice-versa to activate MSCs and be suppressed by MSCs. The IMSUT-CORD can be activated upon the stimulation with IFN- γ resulting in the induction of IDO-1, while IFN- γ and TNF- α were suppressed after the co-culture of IMSUT-CORD in allogeneic MLR in vitro. High levels of inflammatory cytokines in patient 3 might activate IMSUT-CORD resulting in the rapid suppression inflammatory cytokines and improvement of liver function.

The importance of chemokines in aGVHD treatment has not been elucidated. Damaged tissues or activated lymphocytes secrete chemokines that recruit MSCs, some of which are secreted by MSCs to recruit other cells. We found that CCL2 known as monocyte chemoattractant protein-1 (MCP-1) and CCL11 (Eotaxin) levels were elevated during IMSUT-CORD administration and CCL11 was significantly correlated to the IMSUT-CORD therapy. However, the functions of chemokines are not fully understood in relation to MSC and MSC-associated immunotherapy. It is demonstrated that microvesicles from cytochalasin B-induced murine adipose-derived MSCs show a greater immunosuppressive potency after an increase in CCL11 levels than those from parental MSCs [38]. Additionally, CCL11 causes MSCs to traverse the BM endothelium, in a basal-to-apical and apical-to-basal direction [39]. Recently, Cruz-Barrera et al., through secretomic and transcriptomic analyses, demonstrated that the expression of CCL2 and CCL11 was upregulated by 2.29 folds in PHA-treated peripheral blood MNC/UC-MSC co-cultures compared to that in untreated UC-MSCs. They suggested that monocyte reprogramming from M1 to M2 triggered by UC-MSCs may be related to the induction of CCL2 expression [40]. Furthermore, Zhang et al. reported that CCL2 levels in supernatants of the co-culture comprising IL-2-activated T cells and allogeneic BM-MSCs were significantly higher than those in the MSC monoculture due to the upregulation of CCR2 expression in T cells. They concluded that

MSCs can enhance the chemotaxis of activated T cells through CCL2-CCR2 signaling in vitro [41], although the role of CCL11 remained unclear. To confirm which part secreted CCL2 and CCL11, not only CCL2 but also CCL11 levels (but less) were significantly increased in the supernatant of the allogeneic MLR co-cultured with UC-MSCs compared with those in the MLR alone, suggesting that IMSUT-CORD might have some influence on the elevation of these chemokines. In contrast, we could not mRNA of their representative receptors *CCR2*, *CCR3*, and *CCR5* in IMSUT-CORD, as analyzed by qRT-PCR (Supplementary table 3). We do not know how much CCL2 and CCL11 secreted from UC-MSCs product (i.e., IMSUT-CORD) influence on the increase of these chemokines in the plasma of the patients treated with IMSUT-CORD, but our data suggested these chemokines may have some function in aGVHD. Further study is necessary to elucidate the CCL2/CCL11-CCRs axis in the context of MSC and immunosuppression.

Although further studies are needed due to the limitations of a phase 1 trial with such a small patient population, monitoring of the immunological cell population and cytokine and chemokine profile is valuable to identify the prognostic biomarkers to determine the success of aGVHD treatment with UC-MSCs. In conclusion, IMSUT-CORD cultured and cryopreserved in a serum-free solution was safe for patients with treatment-resistant-aGVHD and can be used to conduct efficacy-confirmation clinical trials.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s12185-022-03408-7>.

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Data availability statement Deidentified individual participant data that underlie the reported results will be made available 3 months after publication for a period of 5 years after the publication date. Requests should be sent to tokikoni@ims.u-tokyo.ac.jp.

Declarations

Conflict of interest The study was partially supported by the joint research study of the University of Tokyo with Rohto Pharmaceutical Co. Ltd. in the development of culture medium and processing (December 2014, December to March 2020) and the joint study of the University of Tokyo with Human Life Cord Japan Inc. in the development of banking and clinical application (September 2017–). Nagamura-Inoue is the senior medical advisor of Human Life Cord Japan Inc.

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