Original Article

Novel Human Umbilical Di-Chimeric (HUDC) cell therapy for transplantation without life-long immunosuppression

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Background: Cell-based therapies are promising for tolerance induction in bone marrow (BM), solid organs, and vascularized composite allotransplantation (VCA). The toxicity of bone marrow transplantation (BMT) protocols precludes this approach from routine clinical applications. To address this problem, we developed a new therapy of Human Umbilical Di-Chimeric (HUDC) cells for tolerance induction in transplantation. This study established *in vitro* characterization of the created HUDC cells.

Methods: We performed sixteen *ex vivo* polyethylene glycol (PEG)-mediated fusions of human umbilical cord blood (UCB) cells from two unrelated donors. Fusion feasibility was confirmed *in vitro* by flow cytometry (FC) and confocal microscopy (CM). The HUDC cells' genotype was assessed by lymphocytotoxicity test and short tandem repeat-polymerase chain reaction (STR-PCR) analysis, phenotype by FC, viability by LIVE/DEAD® assay, and apoptosis level by Annexin V staining. We used COMET assay to assess HUDC cells' genotoxicity after the fusion procedure. Clonogenic properties of HUDC cells were evaluated by colony forming unit (CFU) assay. Mixed lymphocyte reaction (MLR) assay assessed immunogenic and tolerogenic properties of HUDC cells.

Results: We confirmed the creation of HUDC cells from two unrelated human donors of UCB cells by FC and CM. Human leukocyte antigen (HLA) class I and II typing, and STR-PCR analysis of HUDC cells confirmed the presence of alleles and loci from both unrelated UCB donors (donor chimerism: 49%±8.3%, n=4). FC confirmed the hematopoietic phenotype of HUDC cells. We confirmed high HUDC cells' viability (0.47% of dead cells) and a low apoptosis level of fused HUDC cells (15.9%) compared to positive control of PKH-stained UCB cells (20.4%) before fusion. COMET assay of HUDC cells revealed a lack of DNA damage. CFU assay confirmed clonogenic properties of HUDC cells, and MLR assay revealed a low immunogenicity of HUDC cells.

Conclusions: This study confirmed creation of a novel HUDC cell line by *ex vivo* PEG-mediated fusion of UCB cells from two unrelated donors. The unique concept of creating a HUDC cell line, representing the genotype and phenotype of both, transplant donor and the recipient, introduces a promising approach for tolerance induction in BM, solid organs, and VCA transplantation.

Keywords: Chimerism; Human Umbilical Di-Chimeric cell therapy (HUDC cell therapy); stem cells; tolerance induction; vascularized composite allotransplantation (VCA)

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Introduction

Remarkable advances in surgical techniques have allowed further development of the vascularized composite allotransplantation (VCA) field. However, there are limitations, such as the need for life-long immunosuppression to prevent allograft rejection, that preclude routine application of VCA in clinical practice. Over the past decade, different approaches were tested to induce tolerance and reduce side effects of immunosuppressive therapies (1-3).

Cell-based therapies are regarded as the most promising approach for tolerance induction in the VCA field (4-7). Thus, different sources of hematopoietic stem cells (HSCs), including bone marrow (BM), peripheral blood (PB), and umbilical cord blood (UCB)-derived progenitor cells have been tested to assess the impact of different cell lineage origins, dosages, and routes of cell delivery on the efficacy of cells engraftment, homing, regenerative quality, and tolerogenicity (8-10). Cell-based therapies were found to be more applicable for tolerance induction through chimerism and regulation of the alloreactive responses when compared to other alternative treatments (11).

UCB became a feasible option for pediatric and adult patients requiring allogeneic HSC transplantation when no suitable donor is available. UCB gained a status of an attractive stem cell source due to the accessibility and a lower immunogenicity, which may be associated with a higher number of immature lymphocytes as well as the presence of suppressor cells, such as mesenchymal stem cells (12).

Some preclinical studies confirmed improvement in the organ recovery using UCB-derived cells in large animal models of myocardial infarction (13-15). Furthermore, encouraging results of clinical application of UCB in bone marrow transplantation (BMT) opened new indications for UCB-derived cell therapies in the field of regenerative medicine including VCA transplants (16-20). One of the advantages of the UCB transplants (UCBTs) in VCA is the lower number of required human leukocyte antigen (HLA) matches, such as 5/6 or 6/6, compared to 10/10 required for the BMT match. Infusions of 4/6 or 5/8 HLA-matched UCB-derived cells are regularly performed in clinical cases where there is no access to the matched unrelated donor (21). Moreover, UCB transplantation has been associated with decreased risk of graft-versus-host disease (GvHD) compared to BMT (12). Retrospective published data demonstrate viable results regarding the use of unrelated UCBTs for hematological malignancies (22), inherited metabolic storage diseases (23), and primary immunodeficiency diseases (24) in the pediatric population. A cohort study, based on the American and European data records, confirmed that survival rates after the UCBT in the adult population were equivalent across the registries (25). Therefore, considering the unique properties of UCB, the creation of a novel UCB-based therapy would introduce a promising approach for the field of BMT, solid organ transplants (SOTs), and VCA transplants, due to the immunomodulatory properties of the UCBT when compared with the BMT (26).

Based on our twenty years of experience in studies on chimerism and tolerance induction in transplantation (27-35), we have recently reported the new generation of BM-derived $CD34^+$ human hematopoietic chimeric cell (HHCC) via *ex vivo* polyethylene glycol (PEG)-mediated fusion (36). Moreover, we confirmed HHCC safety by the confirmation of the viability by Trypan Blue staining and LIVE/DEAD® assay, a low apoptosis profile by Annexin V staining and TUNEL assay, and the donor-specific genotype by lymphocytotoxicity test and STR-PCR analyses, as well as the proliferative properties by colony forming unit (CFU) assay and the immunogenicity by mixed lymphocyte reaction (MLR) of this new hematopoietic cell line.

To further assess the role of cells of hematopoietic origin in tolerance induction in transplantation, in the current study, we have successfully adapted our well-established *ex vivo* PEG-mediated fusion protocol to create Human Umbilical Di-Chimeric (HUDC) cells from two unrelated human UCB donors and tested *in vitro* HUDC cells' properties as a potential novel supportive cell-based therapy for BMT, SOT, and VCA transplants. First, the successful creation of the HUDC cell line by *ex vivo* PEG-mediated fusion was confirmed. Next, we characterized the HUDC cells' genotype by HLA class I, HLA class II, and short tandem repeat (STR) typings, and confirmed the presence of the alleles and loci specific for both human UCB donors. We have also confirmed the safety, high viability, and low apoptosis level of the HUDC cell therapy. Finally, the phenotype analysis confirmed the hematopoietic origin of HUDC cells, while the clonogenic properties were confirmed by CFU assay and the tolerogenic properties by MLR assay.

The creation of HUDC cell-based therapy will introduce the unique concept of personalized immunomodulatory supportive therapy to the transplantation field, thus opening a new era of immunosuppression free transplantation for BMT, SOT, and VCA transplants. We present this article in accordance with the MDAR reporting checklist (available at

https://sci.amegroups.com/article/view/10.21037/sci-2023-024/rc).

Methods

Creation of the new HUDC cell line from UCB cells

UCB cells isolation

For each experiment, the human UCB units were purchased from Cleveland Cord Blood Bank. The UIC Office for the Protection of Research Subjects has determined that this activity does not meet the definition of human subjects' research as defined by the 45 Code of Federal Regulations (CFR) 46.102(f). No ethical approval or informed consent was required due to the nature of this study. The human UCB cells were isolated from UCB derived from unrelated male and female donors using density gradients (Lymphoprep™, StemCell Technologies, Vancouver, Canada). The UCB samples were centrifuged for 25 min at 300 g, and the cells in interphase were collected. Next, the UCB cells were purified using anti-human CD235a (glycophorin A) MicroBeads and magnetic-activated cell sorting (MACS) separation (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's instructions. Then, the isolated UCB cells were washed in RPMI medium containing 10% fetal bovine serum (FBS) and 1× antibiotic/antimycotic solution and resuspended for counting.

PEG-mediated cell fusion procedure

The HUDC cell line was created from human UCB cells derived from two unrelated donors (donors 1 and 2) (*Figure 1A*). The unstained parent UCB cells were collected, isolated, and fluorescently stained separately for each donor with PKH26-red or PKH67-green traceable cell membrane dyes (MiliporeSigma, Burlington, MA, USA). First, the pellets with the UCB parent cells were suspended in diluent C (Sigma, St. Louis, MO, USA), and 6 µL of each PKH dye was added into 1 mL of total volume. The PKH staining was performed for 3 min. Next, the PKH26- and PKH67-stained UCB cells were washed and suspended in serum-free RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA), mixed in a ratio of 1:1, and fused using 500 µL of PEG 4000 solution (EMD, Burlington, MA, USA). The cell fusion procedure was performed sixteen times as previously reported (37,38). Fused HUDC cells were centrifuged, and suspended in DPBS-based fluorescence-activated cell sorting (FACS) buffer containing 25 mM HEPES, 2 mM ethylenediaminetetraacetic acid (EDTA) and 1% FBS in preparation for sorting (BD FACSAria™ II cell sorter, Becton Dickinson, Franklin Lakes, NJ, USA) based on PKH26-red and PKH67-green fluorescent cell labeling. Double-stained PKH26/PKH67 cells, representing the HUDC cells, were selected and subjected to further analysis. The purity of the created HUDC cells (1×10^5 cells, n=3) was assessed by BD LSRFortessaTM cell analyzer (BD Biosciences, San Jose, CA, USA). The samples were collected for flow cytometry (FC) and confocal microscopy (CM) analyses. Finally, the sorted HUDC cells were seeded in low-adhesion culture T25 or T75 flasks at a minimum density of 10^5 cells/mL in an optimized medium (H3000 + CD34 supplement + 20% FBS, StemCell Technologies, Vancouver, Canada).

FC and CM analysis for confirmation of HUDC cell fusion

The creation of the HUDC cells by *ex vivo* PEG-mediated cell fusion of human UCB from two unrelated donors was confirmed by the presence of the double-stained cells (orange), characteristic for the overlap of PKH26-red and PKH67-green fluorescent dyes.

For CM assessment, all collected samples of the PKH26- and PKH67-stained human UCB donor cells (donors 1 and 2, respectively), the mixed unfused PKH-stained human UCB cells, and the fused HUDC cells were spun onto positively charged lysine coated microscope slides, fixed in 4% paraformaldehyde (EMS, Hatfield, PA, USA) for 15 min in room temperature, mounted with VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA), and examined on a Leica TCS SP upright confocal microscope with Retiga 2000R camera (True Confocal Scanner Leica, Wetzlar, Germany) and ImagePro Plus (Media Cybernetics, Rockville, MD, USA).

Genotype analysis of HUDC cells by lymphocytotoxicity test for HLA class I and II typing and STR-polymerase chain reaction (STR-PCR)

The HLA class I and II typing was performed using the UCB fusion parent cells and HUDC cells immediately after the fusion procedure. The UCB and HUDC cell suspensions were incubated for 20 min with Lympho-Kwik T Lymphocyte reagent (One Lambda Inc., Canoga Park, CA, USA) to isolate T lymphocytes, while B lymphocytes were purified using Dynabead HLA Cell Prep II (Invitrogen,

Waltham, MA, USA) according to the manufacturer instructions. Next, T and B lymphocytes were applied on commercially available class I and II HLA typing trays (Jena Bioscience GmbH, One Lambda Inc.). Trays were examined under an inverted fluorescence microscope (Leica, Germany).

The STR-PCR analysis was performed after completion of the fusion procedure to determine the presence of the loci for all STRs specific for both human UCB parents cell donors, evaluate genetic identity, and confirm chimerism in the created HUDC population. The DNA samples from the UCB parent cells and HUDC cells were extracted using the DNeasy Blood and Tissue Isolation kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The STR-PCR analysis (n=4) was performed as previously described (36). The extracted DNA was amplified using Biosystems ABI 3730 DNA Analyzer (Thermo Fisher Scientific) and AmpFLSTR™ Identifiler™ PCR Amplification kit (Thermo Fisher Scientific). Appropriate negative and positive controls were used. Data was obtained for the following STR loci (genetic markers): D8S1179, D21S11, D7S820, CFS1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, AMEL, D5S818, FGA. The raw data were uploaded to the GeneMapper™ 5.0 analysis software (Thermo Fisher Scientific) and the allelic profiles were created according to the analysis conditions supplied by Promega (Madison, WI, USA). The STR-based chimerism assessment was performed using Excel application to compute the ratio of donor specific alleles present in the DNA isolated from the HUDC cells.

FC analysis for assessment of HUDC cells' phenotype

To assess the phenotype of the fused HUDC cells, immunostaining was performed at 7 days after fusion to characterize the cell surface marker expression. Assessments of the following hematopoietic markers were evaluated for the expression of T lymphocytes (CD3, CD4, CD8), B lymphocytes (CD5, CD19), and stem cells (CD34). The following anti-human monoclonal antibodies were used: CD3 (APC, 300311, RRID AB_314047, BioLegend, San Diego, CA, USA), CD4 (APC VioBlue® REAfinity™, Miltenyi Biotec, 130-114-725, Gaithersburg, MD, USA), CD5 (APC/Cyanine7, 300629, RRID AB_2566472, BioLegend), CD8 (Brilliant Violet 421™, 344747, RRID AB_2629583, BioLegend), CD19 (Pacific Blue™, 115526, RRID AB_493341, BioLegend), and CD34 (BD Pharmingen™ APC, 561209, RRID AB_10683161, BD Biosciences). The UCB controls and HUDC cells samples (1×10^6, n=3/group) were blocked with human BD Fc BlockTM Reagent (BD Biosciences) for 5 min and incubated with the previously mentioned antibodies, added at saturating concentrations into PBS solution containing 1% bovine serum albumin (BSA). Assessment was performed using BD® LSR II Flow cytometer (BD Biosciences) and Flowjo™ (Becton Dickinson).

LIVE/DEAD® assay for assessment of HUDC cells' viability and Annexin V staining for apoptosis level

The viability of the UCB donor cells before fusion as well as the viability of the created HUDC cells at 3 hours after fusion (n=3) was assessed by LIVE/DEAD® Fixable Dead Cell staining (Thermo Fisher Scientific) according to the manufacturer's instructions. The apoptosis assessment of the unstained human UCB cells, PKH-stained UCB cells, and HUDC cells (1×10^6, n=3/group) was performed using Annexin V-APC staining (BioLegend) according to the manufacturer's instructions (BioLegend). The results were evaluated by LSRFortessa™ (BD Biosciences) and Flowjo™ software (Becton Dickinson). Gating strategy for Annexin V/Sytox Blue staining was completed as previously described (35).

Single cell gel electrophoresis (SCGE) COMET assay for assessment of HUDC cell fusion safety

The SCGE COMET assay (Abcam, Cambridge, United Kingdom) was performed to assess the DNA damage after cell fusion (n=3). The UCB donor cells and HUDC cells samples were processed according to the manufacturer's instructions. The UCB donor cells and HUDC cells samples were visualized immediately using Vista Green fluorescent dye diluted in Tris-EDTA (TE) buffer (1:10,000), and examined under the MZ16FA stereomicroscope (Leica) equipped with a Retiga 2000R camera (True Confocal Scanner Leica). The DNA damage was confirmed by the visual presence of the 'comet'-like tail which refers to the pattern of damaged DNA migrating through the electrophoresis gel. With the visual scoring system, a total of fifty cells were evaluated on each electrophoresis gel, and the DNA damage was classified into five categories from 0 (no tail) to 4 (all DNA in tail) with the average minimal score of 0 arbitrary unit and average maximal score of 200 arbitrary units (39).

CFU assay for assessment of HUDC cells' clonogenic properties, proliferation, and differentiation

To confirm maintenance of the clonogenic properties, proliferation, and differentiation of the created HUDC cells, the samples of the UCB donor cells, the PKH26- and PKH67-stained mixed UCB cells, and the fused HUDC cells were analyzed after 14 days of cell culture. First, the UCB cells and the fused HUDC cells were cultured separately, and seeded in 35 mm dishes according to the MethoCult® manufacturer's instructions (StemCell Technologies). Next, 1×10^3 cells were plated on a methylcellulose-based medium (MethoCult® H4034, StemCell Technologies). Following CFU assay, cell colony numbers were evaluated under the light microscope (Leica MZ16FA stereomicroscope, Leica) equipped with a Leica DFC290 HD Color Digital Camera, and presented in a chart for further analysis. Photographs of the cell colonies were taken with the high objective lens (×40).

MLR assay for assessment of HUDC cells' tolerogenic properties, and immunogenicity

PB T-cells derived from one of the UCB donor cells (n=3/assay) were labeled with 3 µM CellTrace™ Violet (Thermo Fisher Scientific) and applied as responder cells (1×10^6/assay). The UCB cells and the created HUDC cells were irradiated (25 Gy) using a 137Cs source (Gammacell® 3000, Ottawa, ON, Canada) and used as stimulator cells. The HUDC cells were seeded at concentration of 0.5×10^6, 1×10^6 and 2×10^6 cells/assay. The mixed responder and stimulator cells were cultured in 200 µL of "complete" RPMI 1640 medium containing 10% FBA and 1X antibiotic/antimycotic solution (1XAAS, Thermo Fisher Scientific) for 5 days.

Statistical analysis

Statistical analysis was performed using Minitab software (OriginLab Corp. Northampton, MA, USA). Assays were performed in independent experiments with isolated unstained and/or PKH-stained human UCB donor cells as reference controls. Values are presented as mean ± standard deviation. Statistical differences between respective groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Statistical significance was considered at $P<0.05$.

Results

Confirmation of a new HUDC cell line creation from two unrelated UCB donors

The study design of HUDC cells creation by *ex vivo* PEG-mediated cell fusion of the human UCB cells derived from two unrelated donors (donors 1 and 2) is presented on *Figure 1A*. The creation of the HUDC cell line was confirmed by FC (*Figure 1B*,*1C*) and CM (*Figure 1D*). The average fusion efficacy assessed as presented on *Figure 1C* (right image, red gate) was at 67.4%±3.4% (n=5). Additionally, the UCB cells of donor 1 stained with PKH26-red fluorescent dye and the UCB cells of donor 2 stained with PKH67-green fluorescent dye were analyzed by CM. Before the fusion procedure, the donor cells were mixed and CM assessments revealed the absence of PKH fluorescent dyes overlap. The double-stained PKH26/PKH67 HUDC cells of yellow color (overlapping of PKH26-red and PKH67-green traceable cell membrane dyes) confirmed the efficacy of the fusion procedure of the human UCB cells from two unrelated donors and the chimeric state of the created HUDC cells. We confirmed the successful creation of HUDC cells from two unrelated human UCB donor cells by FC and CM analyses.

Confirmation of the donor-specific genotype in the HUDC cell line

The genotype of HUDC cells was assessed by lymphocytotoxicity test, confirming the presence of HLA class I and II antigens (A, B, Cw, DR, DRB3, DRB4, DQA, DQB) specific for both unrelated human UCB donor cells (donors 1 and 2) after completion of cell fusion (*Figure 2A*). The STR-PCR assessment of HUDC cells confirmed the presence of the STR's loci (genetic markers: D8S1179, D21S11, D7S820, CFS1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, AMEL, D5S818, FGA) specific for both unrelated human UCB donor cells (donors 1 and 2) after completion of cell fusion (*Figure 2B*). An overall donor chimerism was achieved at 49%±8.3% (n=4).

Confirmation of hematopoietic phenotype of HUDC cells by FC

The phenotype of the created HUDC cells was assessed by

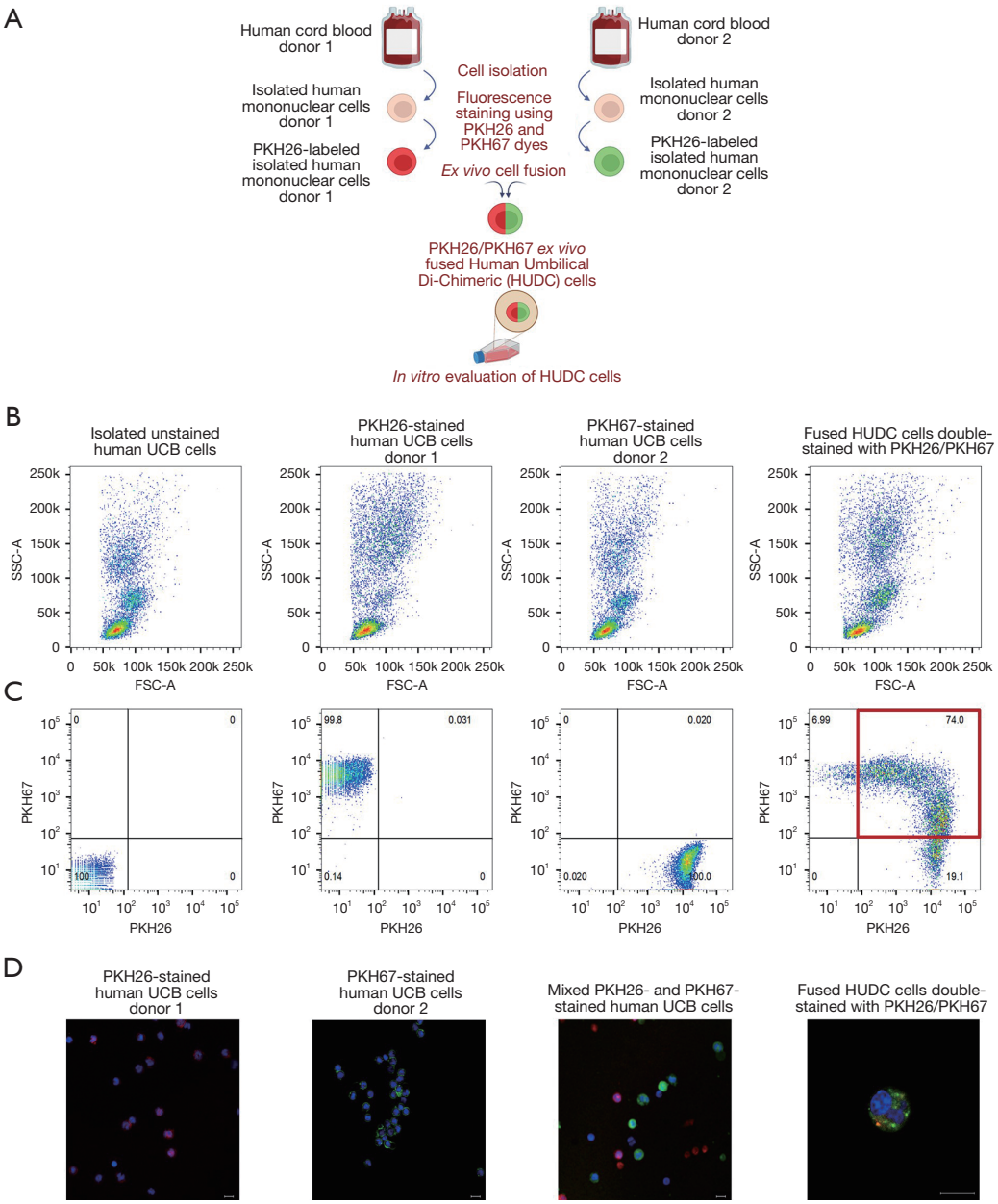

Figure 1 Confirmation of the creation of the HUDC cells via PEG-mediated *ex vivo* cell fusion procedure. (A) The study design of the creation process of a novel hematopoietic cell line from UCB cells of two unrelated human donors (donors 1 and 2), as well as the *in vitro* assessments after cell fusion. (B) Representative flow cytometry forwards *vs.* side scatter dot plots of (from left): isolated unstained human UCB cells; PKH26-stained human UCB cells of donor 1; PKH67-stained human UCB cells of donor 2; and fused HUDC cells double-stained with PKH26/PKH67 fluorescent dyes. (C) Representative flow cytometry PKH26 *vs.* PKH67 fluorescent labeling dot plots of (from left): isolated unstained human UCB cells; PKH26-stained human UCB cells of donor 1; PKH67-stained human UCB cells of donor 2; and fused HUDC cells double-stained with PKH26/PKH67 fluorescent dyes (red gate), revealing a fusion efficacy of 74%. (D) Representative immunofluorescence images of (from left): PKH26-stained human UCB cells of donor 1 (red); PKH67-stained human UCB cells of donor 2 (green); human UCB cells of donor 1 (stained with PKH26) and donor 2 (stained with PKH67) mixed before the fusion procedure; and fused HUDC cells (yellow color), represented by overlapping PKH26/PKH67 fluorescent dyes, confirming the chimeric state of the created HUDC cells. Original magnification: ×630, scale bar: 10 µm. HUDC, Human Umbilical Di-Chimeric; UCB, umbilical cord blood; SSC-A, side scatter-area; FSC-A, forward scatter-area; PEG, polyethylene glycol.

A

Sample	HLA alleles distribution after completion of HUDC cells fusion							
	HLA class I			HLA class II				
	A	B	Cw	DR	DRB3	DRB4	DQA	DQB
Human UCB cells (Donor 1)	2, 23, 29, 31	35, 51	4, 7, 14	11, 15	–	53	02:01, 04:01	2, 4, 7
Human UCB cells (Donor 2)	2, 11, 28, 32	18, 44, 57	4, 6, 10	4, 7	51, 52	–	01:02, 02:01	6, 8, 9
Fused HUDC cells	2, 11, 23, 28, 29, 31, 32	18, 35, 44, 51, 57	4, 6, 7, 10, 14	4, 7, 11, 15	51, 52	53	01:02, 02:01, 04:01	2, 4, 6, 7, 8, 9

B

Sample	STR loci distribution after completion of HUDC cells fusion														
	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	AMEL	D5S818	FGA
Human UCB cells (Donor 1)	11, 13	30, 31.2	11, 12	10, 12	14, 16	8, 9	10, 11	11	22, 23	13, 16.2	16, 19	10, 11	12, 15	XY	12	23, 24
Human UCB cells (Donor 2)	12, 14	30, 33.2	9, 10	10, 11	15, 16	6, 9.3	9, 11	9, 11	24, 25	15, 16.2	17	9, 11	14, 16	XX	11	23, 26
Fused HUDC cells	11, 12, 13, 14	30, 31.2, 33.2	9, 10, 11, 12	10, 11, 12	14, 15, 16	6, 8, 9, 9.3	9, 10, 11	9, 11	22, 23, 24, 25	13, 15, 16.2	16, 17, 19	9, 10, 11	12, 14, 15, 16	XY	11, 12	23, 24, 26

C

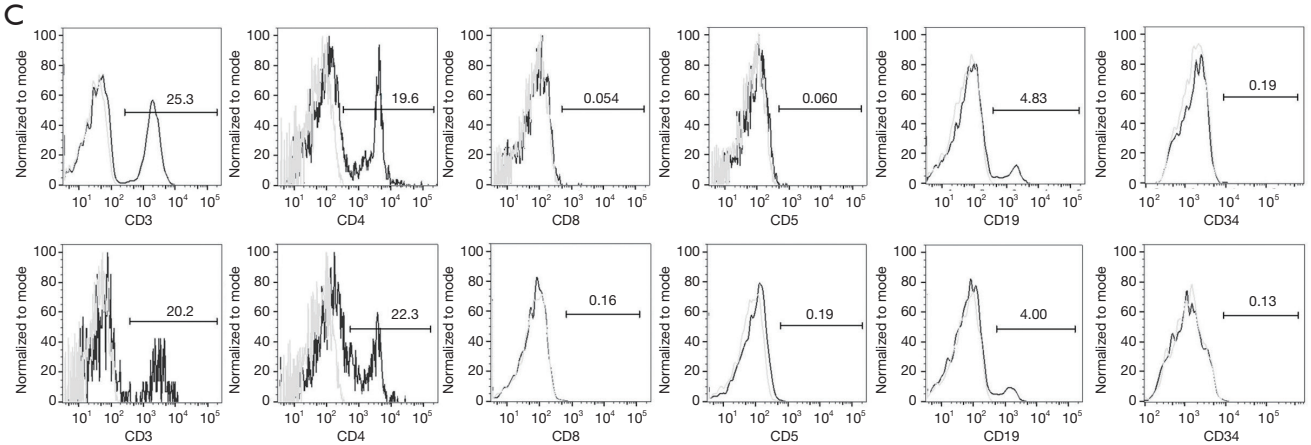

Figure 2 Confirmation of the donor-specific genotype of the created HUDC cells (A,B) from two unrelated human UCB donor cells and evaluation of HUDC cells' phenotype by assessment of the hematopoietic markers expression (C) after completion of the fusion procedure. (A) Lymphocytotoxicity test analysis for HLA classes I and II typing confirmed the presence of the alleles specific for both unrelated human UCB donors (donors 1 and 2; green and red respectively). (B) STR-PCR analysis confirmed the presence of specific STRs loci derived from each unrelated human donor (donors 1 and 2; green and red respectively) in the DNA isolated from HUDC cells. Results show an overall donor chimerism achieved at 49%±8.3% (n=4). (C) Representative flow cytometry histograms at 7 days after cell fusion comparing the expression of hematopoietic markers specific for T lymphocytes (CD3, CD4, CD8), B lymphocytes (CD5, CD19), and stem cells (CD34) on the surface of: (upper row) untreated human UCB cells, and (lower row) HUDC cells. There was no significant change in expression of the assessed hematopoietic markers on the UCB cells before and HUDC cells after the cell fusion procedure. HLA, human leukocyte antigen; UCB, umbilical cord blood; HUDC, Human Umbilical Di-Chimeric; STR, short tandem repeat; PCR, polymerase chain reaction.

FC analysis at 7 days after cell fusion procedure (*Figure 2C*), and revealed a low number of CD8, CD5 and CD34 (<1%) positive cells within the untreated UCB control cells and HUDC cell population. The HUDC cells presented 20.2% of CD3 and 22.3% of CD4 positive cells which was comparable to the 25.3% of CD3 and 19.6% of CD4 expression on the surface of untreated UCB control cells. The expression of CD19, a marker specific for B lymphocytes lineage, was 4–5% on the surface of the isolated UCB control cells and HUDC cells. There was no

significant change in expression of the assessed hematopoietic markers on the untreated UCB control cells before fusion procedure and on the created HUDC cells after the fusion procedure, further confirming that the *ex vivo* PEG-mediated fusion procedure has not introduced significant changes in the expression patterns of the assessed hematopoietic cell surface markers between the UCB control cells and HUDC cells.

Confirmation of high viability and low apoptosis in the created HUDC cell line

The viability of the HUDC cells was assessed by LIVE/DEAD® assay (*Figure 3A*). The results showed that, when compared to the negative control of unstained UCB cells presenting with 0% of dead cells (*Figure 3A*, left image), only 0.47% of the cells were dead in the HUDC cell population (*Figure 3A*, middle image) at 3 hours after cell fusion. The positive control of PKH-stained UCB cells treated with 500 µM of H_2O_2 (*Figure 3A*, right image) showed 97.4% of dead cells, indicating that the cell fusion procedure did not affect the viability of HUDC cells.

The assessment of apoptosis level in the HUDC cell population was performed by Annexin V/Sytox Blue assay (*Figure 3B,3C*). The results showed that, when compared to the negative control of unstained UCB cells presenting with 16.3% of apoptotic cells (*Figure 3B*, left image), only 15.9% of the cells were apoptotic in the HUDC cell population (*Figure 3B*, middle image) at 3 hours after cell fusion. The positive control of PKH-stained UCB cells before the fusion procedure (*Figure 3B*, right image) showed 20.4% of apoptotic cells, indicating that the staining procedure did not affect the apoptosis level of the fused HUDC cells. Annexin V^+/Sytox $Blue^+$ staining showed an increase in the average number of early apoptotic cells in the PKH26-stained UCB cells (16%±4.6%) and HUDC population (22.2%±5%) compared to isolated UCB controls (12.1%±5.2%); however, it was not statistically significant (P>0.05, *Figure 3C*). Annexin V^+/Sytox $Blue^+$ staining showed an increase in the number of late apoptotic HUDC cells after fusion (6.8%±2.1%), compared to isolated UCB cells and PKH-stained UCB cells (2.16%±0.4% and 2.3%±0.4%, respectively; P<0.05 *vs.* HUDC cells, *Figure 3C*).

Confirmation of fusion safety by lack of genotoxicity in the created HUDC cell line

The safety of the cell fusion procedure was evaluated by the COMET assay (*Figure 4*) after the fusion procedure. The COMET assay analysis revealed the absence of DNA damage for the negative control of untreated UCB donor cells (*Figure 4A*) and fused HUDC cells (*Figure 4B*), as confirmed by the lack of a 'comet'-like tail. Assessment of the positive control of HUDC cells treated with 100 µM of H_2O_2 (*Figure 4C*) revealed the presence of DNA damage, confirmed by the presence of a 'comet'-like tail. These results confirm the absence of DNA damage in the created HUDC cells and the safety of the cell fusion procedure, as determined by the COMET assay. Visual scoring of COMET assay of the negative control of UCB cells, PKH26-stained UCB cells, PKH67-stained UCB cells, and HUDC cells showed negligible number of cells scored as 1 and 2 (0.3±0.7, 0.2±0.4, 0.6±0.9 and 0.3±0.5, respectively; P>0.05) compared to the positive control of UCB cells treated with 100 µM of H_2O_2 (158±37, P<0.05) (*Figure 4D*).

Confirmation of clonogenic properties of HUDC cell line by CFU assay

The clonogenic properties of the created HUDC cells were assessed by CFU assay at 3 hours after fusion procedure and 14 days of cell culture post-fusion. CFU assay confirmed that the fused HUDC cells produce the same types (*Figure 5A*) and comparable numbers of burst forming unit-erythroid (BFU-E)/colony forming unit-erythroid (CFU-E) and colony forming unit-granulocyte, macrophage (CFU-GM) colonies as the human UCB control cells (*Figure 5B*). The average total number of BFU-E/CFU-E colonies produced by: the isolated unstained UCB cells was 29.1±7.3, the mixed PKH-stained UCB cells was 33.9±14.3, and the fused HUDC cells was 21.4±8 (P>0.05). The average total number of CFU-GM colonies produced by the isolated unstained UCB cells was 25.4±4.7, the mixed PKH-stained UCB cells was 28.3±9.4, and the fused HUDC cells was 23.7±4.5 (P>0.05). Evaluation of culture of the isolated unstained UCB cells, the mixed PKH-stained UCB cells, and the created HUDC cells revealed a lack of statistically significant difference in the average number of BFU-E/CFU-E and CFU-GM among the analyzed groups. The comparison of the average number of colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) colonies produced by the mixed PKH-stained UCB cells and the HUDC cells was not statistically significant (6.5±3.1 and 5.4±2.4, respectively; P>0.05); however a decrease in the number of CFU-GEMM colonies was observed in the PKH-stained UCB cells and the fused

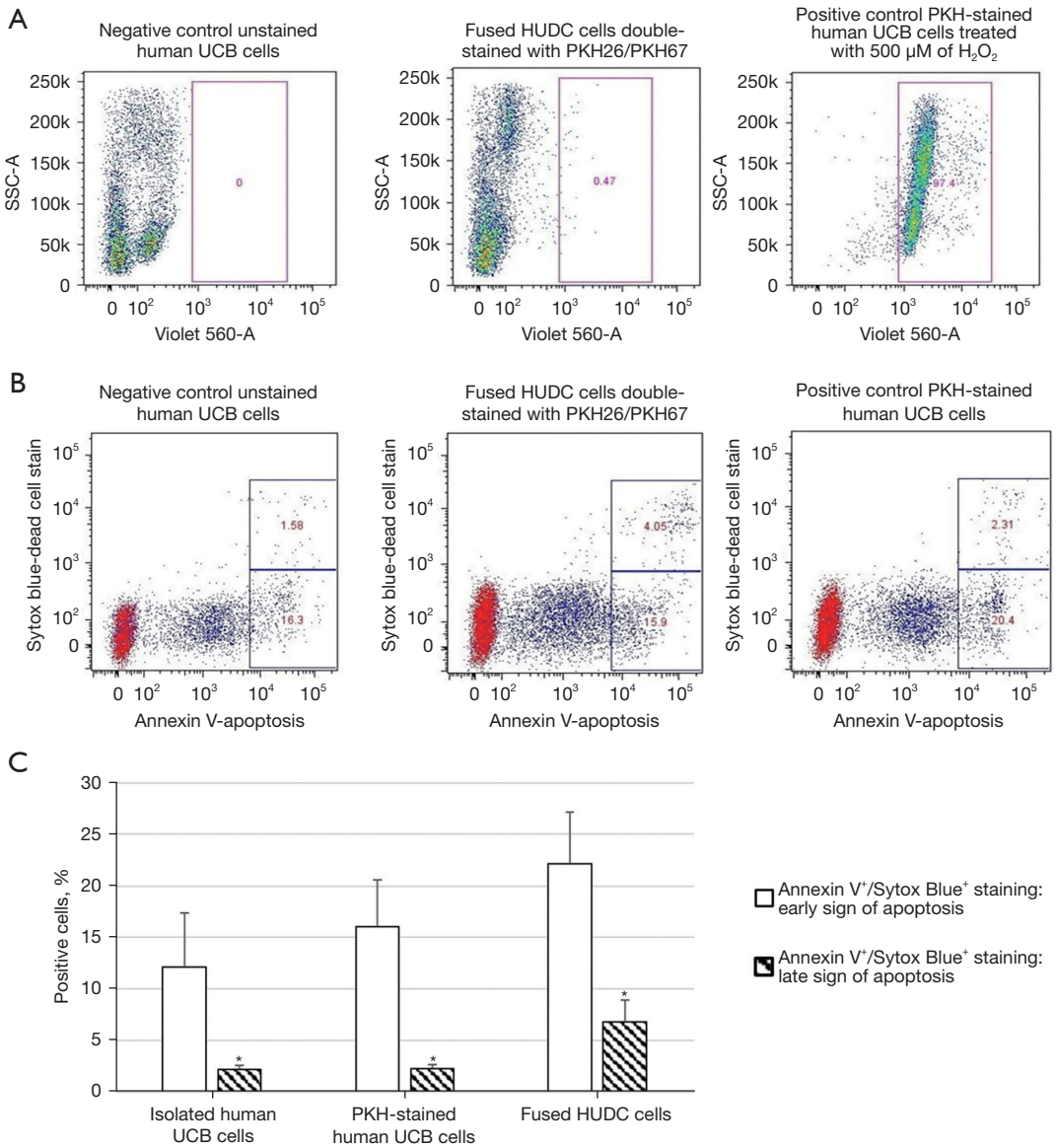

Figure 3 Confirmation of high viability and low apoptosis level in the UCB donor cells and the created HUDC cells at 3 hours after cell fusion. (A) Viability assessment of the created HUDC cells assessed by LIVE/DEAD® assay and analyzed by FC for: (from left) negative control of unstained human UCB cells showing 0% of dead cells; fused HUDC cells double-stained with PKH26/PKH67 revealing 0.47% of dead cells; and positive control of PKH-stained human UCB cells treated with 500 µM of H_2O_2 showing 97.4% of dead cells. (B,C) Qualitative and quantitative evaluation of HUDC cells for early and late signs of apoptosis by Annexin V/Sytox Blue staining. (B) Representative flow cytometry Annexin V-APC *vs.* Sytox Blue staining dot plots of (from left): negative control of unstained human UCB cells; fused HUDC cells double-stained with PKH26/PKH67; and positive control of PKH-stained human UCB cells before fusion. (C) The average number of early apoptotic cells increased in the PKH-stained human UCB control cells (16%±4.6%) and fused HUDC cells (22.2%±5%) compared to the isolated human UCB control cells (12.1%±5.2%); however, it is not statistically significant (P>0.05). Annexin V^+/Sytox $Blue^+$ staining showed an increase in the number of late apoptotic HUDC cells after fusion (6.8%±2.1%), compared to the isolated human UCB cells and PKH-stained human UCB cells (2.16%±0.4% and 2.3%±0.4%, respectively; *, P<0.05 *vs.* HUDC cells). UCB, umbilical cord blood; HUDC, Human Umbilical Di-Chimeric; SSC-A, side scatter-area; FC, flow cytometry.

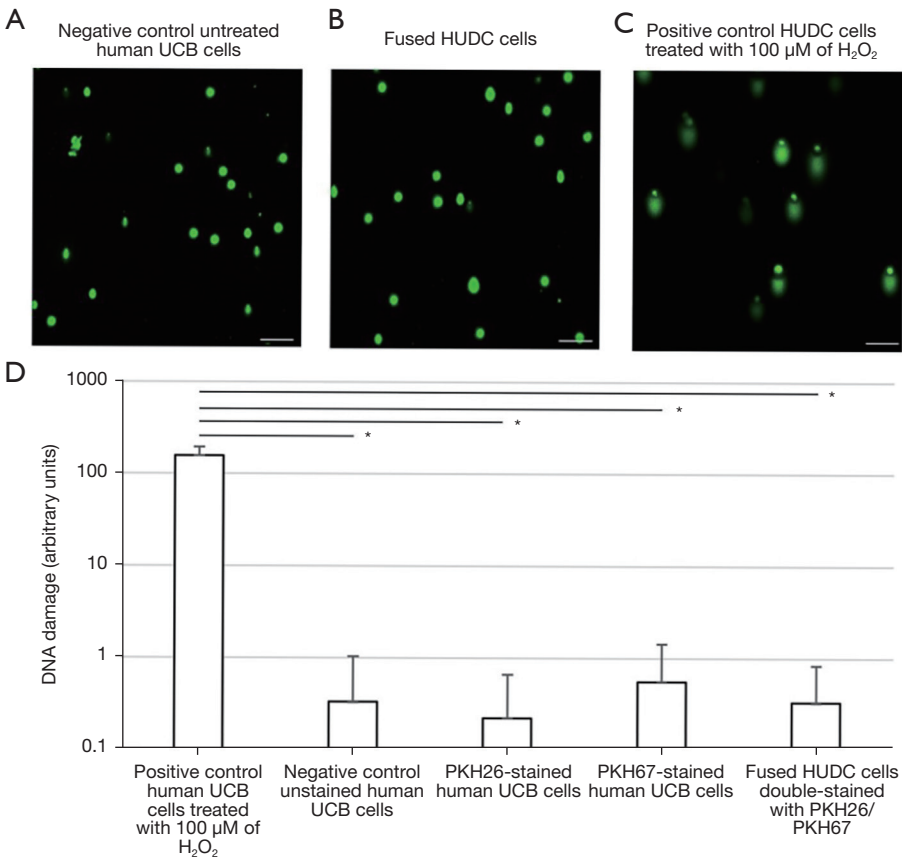

Figure 4 Qualitative and quantitative evaluation of DNA damage in HUDC cells after fusion procedure using a SCGE COMET assay. Representative fluorescent images of COMET assay at 7 days after cell fusion after two consecutive passages of: (A) negative control of untreated human UCB cells; (B) fused HUDC cells without presence of 'comet'-like tail, confirming DNA stability of the created HUDC cells; and (C) positive control of HUDC cells treated with 100 µM H_2O_2 presenting 'comet'-like tail revealing DNA damage. Green: Vista Green DNA Staining Solution (nucleus stain). Images were captured using LSM 710 fluorescence microscope Meta (Zeiss), magnification: ×200, scale bars: 100 µm. (D) Visual scoring of COMET assay of positive control of human UCB cells treated with 100 µM H_2O_2, negative control of unstained human UCB cells, PKH26-stained human UCB cells, PKH67-stained human UCB cells, and fused HUDC cells double-stained with PKH26/PKH67. Data are presented as mean ± SD, *, P<0.05. UCB, umbilical cord blood; HUDC, Human Umbilical Di-Chimeric; SCGE, single cell gel electrophoresis; SD, standard deviation.

HUDC cells when compared to the isolated unstained UCB cells (P<0.01, *Figure 5B*).

Confirmation of tolerogenic properties of HUDC cell line by MRL assay

The results showed that when compared to the negative control of unstimulated CellTrace™ Violet-labeled T-cell responders presenting with a proliferation rate of 2.27%±0.5% (*Figure 5C*, left image), the proliferative response of CellTrace™ Violet-labeled T-cell responder cells stimulated with irradiated allogeneic UCB cells was at 34.2%±6.9%, P<0.05 (*Figure 5C*, middle image). MLR assay showed significantly decreased response of CellTrace™ Violet-labeled T-cell to irradiated HUDC cells (13.2%±2.4%, P<0.05) (*Figure 5C*, right image) when compared to the allogeneic control of UCB stimulated responder T-cells.

Discussion

VCA is a pioneering and emerging field of reconstructive surgery, providing promising solutions for traumatic injuries in civilian and military patients. As we approach the

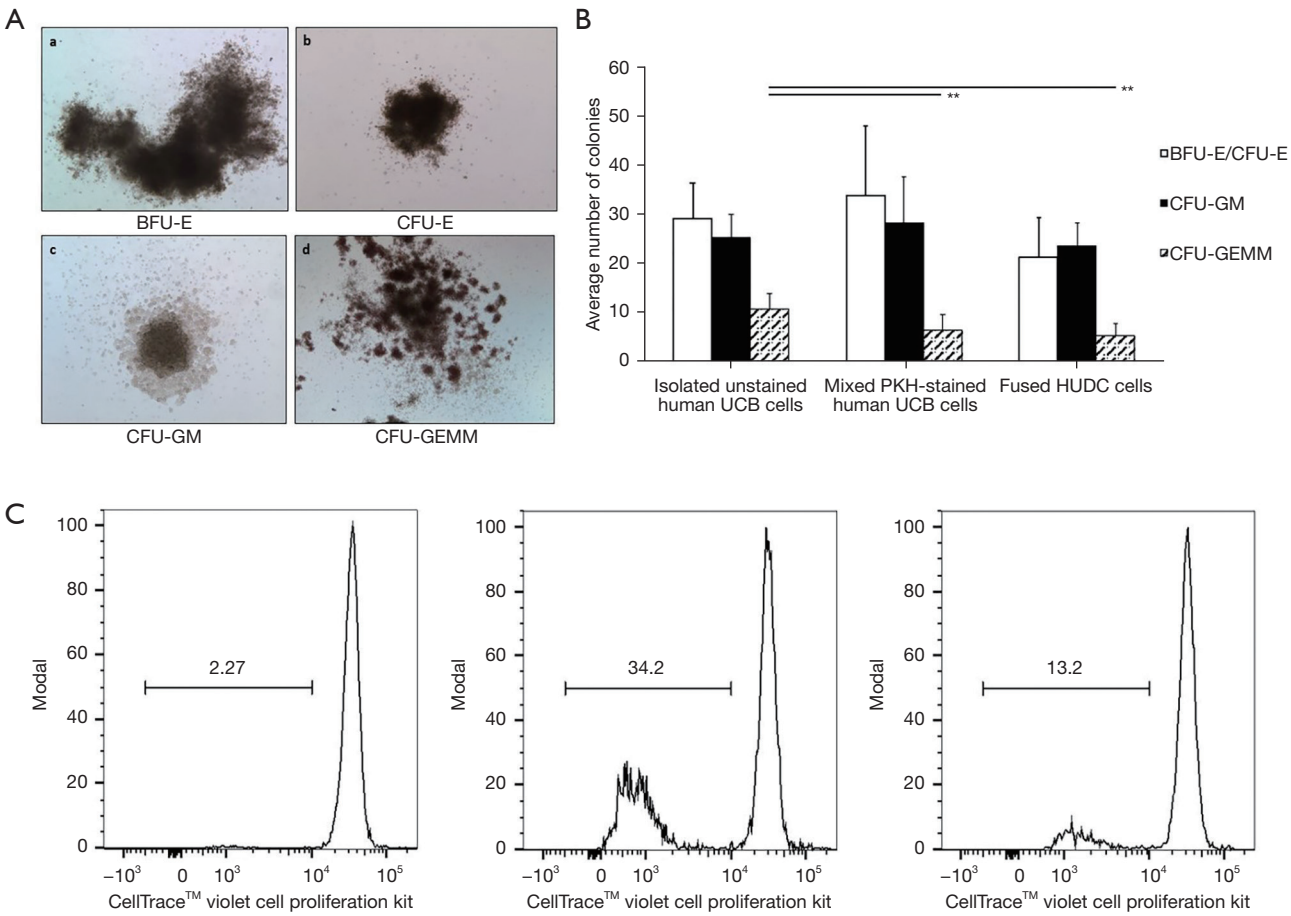

Figure 5 Confirmation of clonogenic (A,B) and tolerogenic properties (C) of the created HUDC cells. (A,B) Assessment of clonogenic properties of HUDC cells collected at 3 hours after cell fusion and analyzed after 14 days of cell culture by CFU assay. (A) Representative images of: BFU-E, burst forming unit-erythroid; CFU-E, colony forming unit-erythroid; CFU-GM, colony forming unit-granulocyte, macrophage; and CFU-GEMM, colony forming unit-granulocyte, erythroid, macrophage, megakaryocyte, taken from cell culture under a light microscope. Original magnification: ×40. (B) Comparative analysis of the average number of colonies created by: isolated human UCB cells, mixed PKH-stained human UCB cells, and fused HUDC cells revealed a decrease in the number of CFU-GEMM colonies in mixed PKH-stained human UCB cells and fused HUDC cells when compared to isolated untrained human UCB cells at 14 days of cell culture after fusion procedure, **, P<0.01. (C) Assessment of tolerogenic properties of HUDC cells collected after 5 days of cell culture using one-way MLR assay. Representative histograms assessing lymphocyte proliferation rate of: from left: (left image) negative control of non-proliferating responder T-cells, (middle image) allogeneic control of UCB stimulated responder T-cells, and (right image) HUDC cells stimulated with responder T-cells. UCB, umbilical cord blood; HUDC, Human Umbilical Di-Chimeric; CFU, colony forming unit.

fifteen-year anniversary marking the first near-total face transplant in the United States performed by our group (40), remarkable advances in surgical techniques have outpaced consistent and reproducible solutions to the challenge of inducing long-term recipient immune tolerance to the allogeneic transplants. Thus, VCA are considered as the life-enhancing rather than life-saving procedures due to the potential risk of life-long immunosuppression and related side effects. Potential candidates that could greatly benefit from VCA are currently disfavored by the prevailing necessity and associated morbidity of a life-long immunosuppression, known for severe side effects including: malignancy, metabolic disorders, opportunistic infections, as well as chronic rejection (1-3). The introduction of new, easily available cell-based therapies presenting a low immunogenic profile is critical to advance stem cell

transplantation and successfully treat hematopoietic disorders and malignancies as well as facilitate tolerance induction in the setting of SOT, and VCA transplantation.

The most common approach to improve the efficacy of UCB transplantation is to increase the number of HSC via *in vitro* cell cultures (41); however, long-term culturing may decrease the expression of homing markers at the cells' surface as well as capability of HSC to differentiate after transplant.

Decades of investigational work into the intentional immune system reprogramming to recognize allogeneic tissues as "self", well known as the paradigmatic "Holy Grail" in immunology and transplantology, have elucidated the importance of introducing donor-specific hematopoietic chimerism as a foundational mechanism and a means to perform VCA without the need for life-long immunosuppression (42). As clinically observed, VCA graft rejection occurs by cytotoxic immune-cell mediated processes against HLA class I cell markers (43). Introduction of cell-based therapies, such as donor-recipient chimeric cells (DRCCs) of hematopoietic lineage origin, is regarded as the most promising approach towards induction of donor-specific immune tolerance.

In different animal models, immune tolerance induction was already successful via hematopoietic chimerism and was confirmed across the HLA barrier in VCA (5,44) and SOTs (45). Although tolerance induction in human allogeneic transplantation was not yet achieved, the examples of donor BM transplant supporting graft acceptance in human VCA (4,45) and SOT (46-49) have been encouraging and should be considered in the future trials.

The toxicity of the recipient conditioning required for marrow transplant limits the routine use of BMT in the clinical setting. Therefore, the myelosuppressive conditioning regimens should balance the potency required to sufficiently overcome the mismatched-HLA barrier, and at the same time, prevent severe impairment of the hematopoiesis. It is estimated that half of patients on the transplant list do not have the HLA-matched donor available despite the large number of over 13 million registered BM donors worldwide (50). This is exemplified by the fact that there are approximately 10 to 15 thousand patients per year that have trouble finding an unrelated adult donor due to the rapid disease progression and subsequent death (51).

Moreover, match disparities most significantly and disproportionately affect racial and ethnic minorities (52). To overcome the lack of matched BM donors, the use of different HSCs sources could greatly benefit patients seeking for different alternatives. Previous research into HSC sources, which include BM, PB and UCB-derived progenitors, illustrates the diversity of the hematopoietic lineage profiles including different sources of the cell origin (8-10).

The UCB-derived hematopoietic progenitors are very appealing to expand the donor pool. Since the advent of UCB banking in the late 1990s, it has been recorded that over 55 thousands cord blood units have been provided for transplantation purposes (53). The UCB is an attractive potential option over the sources of the HSCs derived from the unrelated donors of BM or PB for a variety of reasons including: a greater proportion of highly proliferative progenitor cells (54); a lower severity and incidence of acute GvHD compared to the other graft sources (50,55); a faster bank procurement with a 3-week waiting period for UCB units compared to 3-month for matched unrelated BM donor (56); a lower immune reactivity with immunomodulatory properties (57,58); and a lower stringency for HLA matching compared to BMT, which is currently clinically accepted at the threshold of 4 of 8 HLA antigens (HLA-A, -B, -C, and -DRB1) (59).

Over the past 20 years, our Microsurgery Laboratory developed different chimeric cell lines to overcome these limitations. First, the DRCCs were created by *ex vivo* PEG-mediated fusion of BM cells derived from two unrelated August Copenhagen Irish (ACI) and Lewis rats (34,35). The pro-tolerogenic properties of the created DRCCs were confirmed, facilitating DRCCs engraftment, prolonging VCA survival, and inducing donor-specific immune tolerance. After successful testing of DRCCs *in vivo* in the rat VCA model, the next step was to create the $CD34^+$ HHCC for potential clinical applications in transplantation.

Inspired by the potential of the encouraging outcomes of the rat DRCCs, our Microsurgery Laboratory successfully created HHCC from the BM-derived $CD34^+$ cells originating from two unrelated human donors, according to the previously described protocol (36). The properties of the HHCC originating from human $CD34^+$ cells were characterized *in vitro*, confirming the tolerogenic properties of HHCC and the potential application of HHCC therapy for tolerance induction in BM, solid organs, and VCA transplantation (36).

Therefore, the fusion of HSCs across HLA barriers represents a novel approach for transplant tolerance induction with a great clinical potential. Considering the need to expand the application of HSCs in the search of

the "universal donor" and based on the attractive qualities of low immunogenicity of UCB cells, we propose a new cellular therapy based on *ex vivo* created chimeric cells derived from UCB cells from two unrelated donors as an alternative approach to the BM-based therapies for the BM, solid organs, and VCA support.

The current study was designed to create human HUDC cells via fusion of UCB cells derived from two unrelated human donors, as a novel alternative therapy for tolerance induction in BMT, SOT, and VCA transplantation. We confirmed the feasibility, reproducibility, and safety of the *ex vivo* PEG-mediated fusion protocol applied for creation of HUDC cells. Next, we confirmed *in vitro* the hematopoietic phenotype as well as high viability, proliferative and clonogenic properties of HUDC cells.

The creation of HUDC cells relies on the fusion of the genetic material from the two unrelated human UCB donor cells through *ex vivo* PEG-mediated fusion procedure. Therefore, the created donor-recipient HUDC cells are carrying the HLA class I and II antigens and the STR loci specific for each of the human parent UCB donor cells, as confirmed by the lymphocytotoxicity test and STR-PCR analysis, respectively. Phenotype analysis at 7 days after cell fusion confirmed hematopoietic origin and stability of the HUDC cells' phenotype. Since HUDC cells represent an example of the Di-Chimera created from the two unrelated donors, HUDC cells may reduce immune response towards the recipient and the need for immunosuppression.

The high viability of HUDC cells after cell fusion was comparable to the viability of the unstained human UCB cells before the fusion procedure. Additionally, the level of late signs of apoptosis of the fused HUDC cells was low when compared to the positive control of PKH-stained UCB cells before the fusion procedure performed by the Annexin V and Sytox Blue assays, further supported the stability of the new HUDC cell line.

To ensure the long-term safety of the HUDC cells therapy and the lack of genotoxicity of the *ex vivo* cell fusion procedure, the COMET assay was performed on the UCB donor cells before fusion and on the created HUDC cells after fusion, and revealed the absence of the 'comet'-like tail, characteristic of the DNA damage, in the created HUDC cells, thus confirming further the safety of the *ex vivo* PEG-mediated fusion procedure.

The clonogenic properties were confirmed by the differentiation of HUDC cells into the granulocyte, erythroid, macrophage, and megakaryocyte progenitor cells after 14 days of cell culture. The formation of colonies in the cell culture further confirmed the hematopoietic origin of the created HUDC cell line. Moreover, the proliferative rate of the HUDC cells was comparable to the rate of the unfused human UCB cells after 14 days of incubation on the methylcellulose-based medium, confirming the proliferative properties of the fused HUDC cells. Finally, the MLR assay revealed a decreased immune response of the HUDC cells compared to allogeneic control, further verifying the tolerogenic properties of HUDC cells.

There are several similarities between our two hematopoietic lines—the HHCC and HUDC cell line of created human chimeric cells. Both lines represent cells of the human hematopoietic lineage origin and both contain rich sources of the HSCs applicable for transplantation (36). When comparing the hematopoietic markers, the UCB cells have been shown to contain a high percentage of primitive stem cells, including very small embryonic-like stem cells, and a lower immunogenicity compared to other sources of stem cells, such as BM and PB (60,61). Moreover, HSCs derived from BM or PB have been extensively studied and characterized for the presence of hematopoietic markers, including expression of CD34, CD38, and CD133. It is also well established that HSC are capable of self-renewal and differentiation into various blood cell lineages (8-10). Regarding comparison of safety, it is well-known that the transplantation of UCB cells has been associated with a decreased risk of GvHD compared to BMT in addition to the significantly better immune profile when considering the donor match which is acceptable at the 4–6/10 for UCB and 10/10 for BM transplant (21). The safety of HHCC, which represent a new generation of BM-derived $CD34^+$ HSC created via *ex vivo* PEG-mediated fusion, has been confirmed in our recently published study reporting the lack of DNA damage confirmed by COMET assay as well as a low apoptosis profile and a lack of tumor formation assessed by magnetic resonance imaging (MRI) (36).

In summary, the current study confirmed the feasibility and reproducibility of creation of a new hematopoietic HUDC cell line from human UCB cells derived from two unrelated donors via *ex vivo* PEG-mediated cell fusion procedure. Moreover, the chimeric state of HUDC cells was confirmed by the presence of HLA class I alleles, class II alleles, and the STR loci from both UCB donors whereas absence of genotoxicity was confirmed by the COMET assay. Furthermore, we demonstrated preservation of the hematopoietic phenotype and clonogenic properties of the HUDC cells under standard *ex vivo* cell culture conditions after cell fusion.

To the best of our knowledge, this is the first report on the successful creation of the new HUDC cell line via *ex vivo* PEG-mediated fusion of UCB cells derived from two unrelated human donors. This study characterized and confirmed *in vitro* the safety of *ex vivo* fused HUDC cells. This study suggests that HUDC cell line-based therapy may be a promising alternative approach to the BM-based therapies applied for tolerance induction in transplantation. The potential of donor-specific tolerance induction has promising implications for reducing the side effects of immunosuppressive protocols and addressing the problem of donor shortage in BMT. Furthermore, the possibility of creating personalized HUDC cell therapy that closely matches the HLA of the donor and the recipient offers a potential solution to the issue of the donor HLA incompatibility and the donor shortage.

In order to advance this therapy to clinical trials and facilitate potential therapeutic applications, our Microsurgery Laboratory aimed to characterize and confirm *in vivo* the safety and efficacy of the created HUDC cell therapy.

Conclusions

In this study, we successfully established the *ex vivo* fusion protocol to create a novel HUDC hematopoietic cell line from the UCB cells derived from two unrelated human donors. Creation of HUDC cells was confirmed by FC and CM. COMET assay confirmed lack of DNA damage and safety of *ex vivo* PEG-mediated cell fusion procedure. The unique concept of HUDC cell therapy represents a new promising approach for tolerance induction and immunosuppression for free transplants of solid organs, BM, and VCA.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://sci.amegroups.com/article/view/10.21037/sci-2023-024/rc

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://sci.amegroups.com/article/view/10.21037/sci-2023-024/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The UIC Office for the Protection of Research Subjects has determined that this activity does not meet the definition of human subjects' research as defined by 45 CFR 46.102(f). No ethical approval or informed consent is required because of the nature of this study.

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