

## Do Cryopreserved Mesenchymal Stromal Cells Display Impaired Immunomodulatory and Therapeutic Properties?

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**Key Words.** Bone marrow stromal cells • Cellular therapy • Clinical translation • Cryopreservation • Immunotherapy • Engraftment • Instant blood-mediated inflammatory reaction

### ABSTRACT

We have recently reported that therapeutic mesenchymal stromal cells (MSCs) have low engraftment and trigger the instant blood mediated inflammatory reaction (IBMIR) after systemic delivery to patients, resulting in compromised cell function. In order to optimize the product, we compared the immunomodulatory, blood regulatory, and therapeutic properties of freeze-thawed and freshly harvested cells. We found that freeze-thawed MSCs, as opposed to cells harvested from continuous cultures, have impaired immunomodulatory and blood regulatory properties. Freeze-thawed MSCs demonstrated reduced responsiveness to proinflammatory stimuli, an impaired production of anti-inflammatory mediators, increased triggering of the IBMIR, and a strong activation of the complement cascade compared to fresh cells. This resulted in twice the efficiency in lysis of thawed MSCs after 1 hour of serum exposure. We found a 50% and 80% reduction in viable cells with freshly detached as opposed to thawed *in vitro* cells, indicating a small benefit for fresh cells. In evaluation of clinical response, we report a trend that fresh cells, and cells of low passage, demonstrate improved clinical outcome. Patients treated with freshly harvested cells in low passage had a 100% response rate, twice the response rate of 50% observed in a comparable group of patients treated with freeze-thawed cells at higher passage. We conclude that cryobanked MSCs have reduced immunomodulatory and blood regulatory properties directly after thawing, resulting in faster complement-mediated elimination after blood exposure. These changes seem to be paired by differences in therapeutic efficacy in treatment of immune ailments after hematopoietic stem cell transplantation. *STEM CELLS* 2014;32:2430–2442

### INTRODUCTION

Multipotent mesenchymal stromal cells (MSCs) harbor great potential for regenerative and immunomodulatory therapies and are currently under investigation in numerous clinical trials, the majority thereof relying on systemic infusion. A major drawback in MSC therapy appears to be the incomplete understanding of fate and function of MSCs following systemic administration [1–5]. Recent attention has been attributed to the fact that many MSC therapeutics are cryobanked for immediate “off-the-shelf” availability in the clinic [5–7]. It was suggested that freeze-thawed cells have impaired therapeutic properties compared to culture-derived fresh MSCs, which are used in many experimental and preclinical studies to demonstrate efficacy [5–7]. Here we summarize our preclinical and clinical findings

on comparing freeze-thawed and freshly harvested MSCs to deepen our understanding about the fate and efficacy of therapeutic MSCs after systemic infusion, in order to improve this novel treatment.

Analysis of tissues following MSC therapy in humans indicates very limited long-term engraftment [8, 9]. MSCs and other stromal cells trigger an innate immune attack, termed instant blood mediated inflammatory reaction (IBMIR) [10–13], which has previously been shown to compromise survival, engraftment, and function of cellular therapeutics, such as islet cells and hepatocytes, after systemic administration [14]. The IBMIR elicits deleterious effects on cellular therapeutics through a cascade of events; first initiated by triggering of the innate immune cascades and then followed by subsequent effector cell infiltration and graft destruction. Within this process both

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MSCs procoagulant [10–12] and complement activating properties [15–17] have been implied to hamper graft performance [3, 4, 7, 18]. Known risk factors attributed to recognition of MSCs by IBMIR are induction of tissue factor expression during culture [10–12], long-term expansion *ex vivo* [10, 12], and potentially allogeneic mismatch [16, 18, 19].

We have previously reported on the complement activating nature of MSCs [15]. A recent report further demonstrated that MSCs activate indeed all three pathways of the complement system [16], eliciting cell membrane damage and cytoplasmic leakage. The clinical relevance of this phenomenon is unclear. It was not clarified if cell survival is compromised and not specified if freshly harvested or freeze-thawed MSCs were tested. Animal studies indicate that high doses of freeze-thawed cells are less well-tolerated than fresh cells [7]. Not only does complement injury itself poses a risk for graft survival, freeze-thaw associated microdamage to the cell surface and subsequent binding of complement activation products may also promote graft clearance by various types of phagocytes, such as polymorphonuclear leukocytes, macrophages, monocytes, dendritic cells, and natural killer cells [20–22]. These innate immune cells recognize abnormal cells and cell debris via receptors with specificity for particles displaying non-self/apoptotic features [22, 23]. Complement activation factors are key elements in tuning the resulting inflammatory response [22, 24], as previously demonstrated for MSCs [15]. It is of particular interest that phagocytes, which ingest apoptotic cells and cell debris, can be turned into immunosuppressive type 2 cells [22, 25, 26]. These anti-inflammatory type 2 effector cells secrete a vast array of immunosuppressive mediators, a principle potentially exploitable in the clinic when combating various immune ailments [27].

Based on findings by Galipeau and coworkers [5, 6] that the storage and reconstitution procedure could potentially affect the therapeutic efficacy of MSCs, we specifically tested this new parameter within our established assays and conducted retrospective analysis of our patient data. This study reports that cryostorage and the subsequent freeze-thaw procedure impair MSCs immunomodulatory properties and promotes activation of innate immune cascades after contact of MSCs with human serum/blood, potentially hampering cell graft performance in patients.

## SUBJECTS, MATERIALS, AND METHODS

### Ethics Statement, MSC Treatment, Clinical Response, and Engraftment Analysis

This study was approved by the review board at Karolinska University Hospital, Huddinge with donors and patients, or their legal guardians, providing informed written consent. MSCs were isolated, expanded, and characterized as described previously [10, 28, 29] in line with the guidelines of the MSC Consortium of the European Blood and Marrow Transplantation Group and approved by the Swedish National Board of Health and Welfare [29, 30]. A total of 160 infusions, given for various complications associated with hematopoietic stem cell transplantation (HSCT) at Karolinska University Hospital between 2002 and 2012 were evaluated and 28 patients receiving a total of 44 MSC infusions until 2007 were included

in the comparison of fresh and freeze-thawed MSCs. All fresh infusions were given prior to 2007. Treatments after 2007 were excluded in the comparison as patient selection and supportive care of HSCT patients has changed in recent years. The patient cohort and clinical outcome has been reported previously in part [10, 29]. Briefly, MSCs were obtained from bone marrow aspirates of healthy volunteer donors ( $n = 22$ ; median age 38; range 22–66) and expanded in medium containing 10% fetal calf serum (Hyclone, Logan, UT, <http://www.hyclone.com>) for up to four passages and infused at a median dose of  $1.6 \times 10^6$  cells per kg (range  $0.7$ – $3.6 \times 10^6$ ). The predominant indications for fresh MSC treatment were acute graft versus host disease (GvHD) and tissue injury in hemorrhagic cystitis; infusions given for other indications were excluded. The majority of patients received a single MSC infusion, but 11 received multiple infusions (2–5, median 2). The MSCs were from unmatched third party donors ( $n = 31$ ), haploidentical related donors ( $n = 11$ ), or human leukocyte antigen (HLA)-identical siblings ( $n = 2$ ).

A total of 44 MSC infusions, of which 9 were fresh MSC and the remaining freeze-thawed, were evaluated regarding clinical response. Response was classified as complete response (CR), partial response, stable disease, or progressive disease, as defined previously [10, 29]. Twenty-two infusions, 6 of which were freshly harvested MSCs, were evaluated regarding engraftment. Tissue samples ( $n = 108$ ) taken at autopsy or colonoscopy from 15 of the patients have been analyzed for engraftment using polymerase chain reaction (PCR) for MSC donor DNA, as reported previously [3].

### Freeze-Thawing of MSCs, Cell Viability Assessment, Complement Activation Studies After Serum Treatment, and Triggering of the IBMIR After Whole Blood Exposure

MSCs for cell viability, serum and whole blood exposure experiments were obtained either from frozen cryostocks or from subconfluent cell layers detached with trypsin/EDTA. For donor-matched comparison of fresh or freeze-thawed clinical MSCs, cells were adjusted to  $1$ – $2 \times 10^6$  cells per milliliter in phosphate buffered saline (PBS)/EDTA containing 5%–10% human blood type AB plasma (ABP) and split into two equal fractions. One fraction was kept at  $4^\circ\text{C}$  to simulate waiting time in bag before infusion, the other reconstituted in  $4^\circ\text{C}$  cold ABP containing 10% dimethyl sulfoxide (DMSO) and frozen at  $-80^\circ\text{C}$  with a rate controlled cell freezing device (Cool-Cell; BioCision, Larkspur, CA, <http://www.biocision.com>). Immediately prior to experimentation, cryopreserved MSCs were thawed and washed twice with PBS containing 5% ABP, reconstituted, and counted in fresh PBS containing 5% ABP.

**Incubation of MSCs with Human Serum.** Serum preparation and cell treatment were conducted as described previously [15]. Here a pool of five AB-serum donors was used to obtain an averaged complement lysing activity and a longer serum incubation time was chosen (60 minutes at  $37^\circ\text{C}$  instead of 20 minutes). In all experiments using human serum, the final concentration of complement active normal human AB-serum (NHS), or EDTA-inactivated NHS (NHS/EDTA) was 50% (v/v). Complement activity was stopped by the addition of 10 mM EDTA. Non-serum-treated cells and cells treated with NHS/

EDTA served as controls [15]. Complement binding, viability, and total number of MSCs were assessed before and after serum treatment.

**Time Lapse Imaging, CASY Counter, and Flow Cytometry Analysis.** Fresh or freeze-thawed MSCs were seeded at a density of  $1 \times 10^6$  cells per milliliter in 24-well flat bottom plates modified with an ultra-low attachment surface (Corning, Tewksbury, MA, <http://www.corning.com>) and exposed to NHS or NHS/EDTA serum. Imaging was performed at 37°C on a Leica DMI6000 wide field microscope with an EM-CCD 16-bit camera (Evolve; Andor Technology PLC, Belfast, Northern Ireland, <http://www.andor.com>) and exposure time of 10 milliseconds. Transmitted light images were obtained every 2 minutes for 1 hour with a 20× extra long working distance objective. Visible cell counts were quantified with Image-J v1.46r. Total cell number and viability of cells in suspension was assessed with the Cell Counter and Analyser System Model TT (CASY-TT; Roche Diagnostics GmbH, Penzberg, Germany, <http://www.roche-applied-science.ch>). Flow cytometry was conducted on cells labeled with the antibodies (Ab's) outlined in Supporting Information Table S1, fixed with 1% paraformaldehyde, and analyzed on a LSR-II Fortessa (Becton Dickinson, Franklin Lakes, NJ, <https://www.bd.com>). Percentage of excluded debris and 2,000–5,000 gated events were quantified and analyzed with Summit v.4.1 (Dako, Glostrup, Denmark, <http://www.dako.com>).

Apoptotic and dead cell analysis was conducted with flow cytometry, according to the manufacturer's instructions (Fluorescein isothiocyanate (FITC) Annexin-V Apoptosis Detection Kit II, BD) [31]. Viable cells are Annexin V (AV)<sup>-</sup> Propidium iodide (PI)<sup>-</sup>, cells that are in early apoptosis are AV<sup>+</sup> PI<sup>-</sup> and cells in late apoptosis/dead are AV<sup>+</sup> PI<sup>+</sup>.

**Chandler Whole Blood Loop Experiments.** Fresh or freeze-thawed MSCs were exposed to human blood using the Chandler loop system (Corline Systems AB, Uppsala, Sweden, <http://www.corline.com>) [10, 32]. Fresh non-anticoagulated human blood was obtained from healthy volunteers who had received no medication for at least 10 days. Briefly, tubing containing 7 ml of human blood was prepared [32] and supplemented with 100 μl PBS containing 5%–10% ABP +/- fresh or freeze-thawed MSCs (15,000 cells per milliliter). One milliliter samples from each tube were collected before and 5, 15, and 30 minutes after cell addition. Reactions were stopped by addition of 10 mM EDTA (pH 7.4). Platelet and cell counts were obtained using a cell counter (Beckman Coulter, Brea, CA, <https://www.beckmancoulter.com>). The remaining sample volume was centrifuged at 3,000g for 20 minutes at 4°C. Plasma was collected, stored at -80°C and formation of thrombin-anti-thrombin complex, complement C3 activation fragment a (C3a), and soluble C5b-9 complex (sC5b-9) was measured by ELISA [10].

### Immunomodulatory Activity of Fresh and Freeze-Thawed MSCs

Fresh and freeze-thawed MSCs were cultured with or without 100 U/ml recombinant human interferon gamma (rhIFN $\gamma$ ; Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>) for 1–7 days, with media changes at day 3 and 24 hours before harvesting. Conditioned culture media (CM), cell

lysates, and total RNA were harvested either after 24 hours or 7 days IFN $\gamma$ -stimulation.

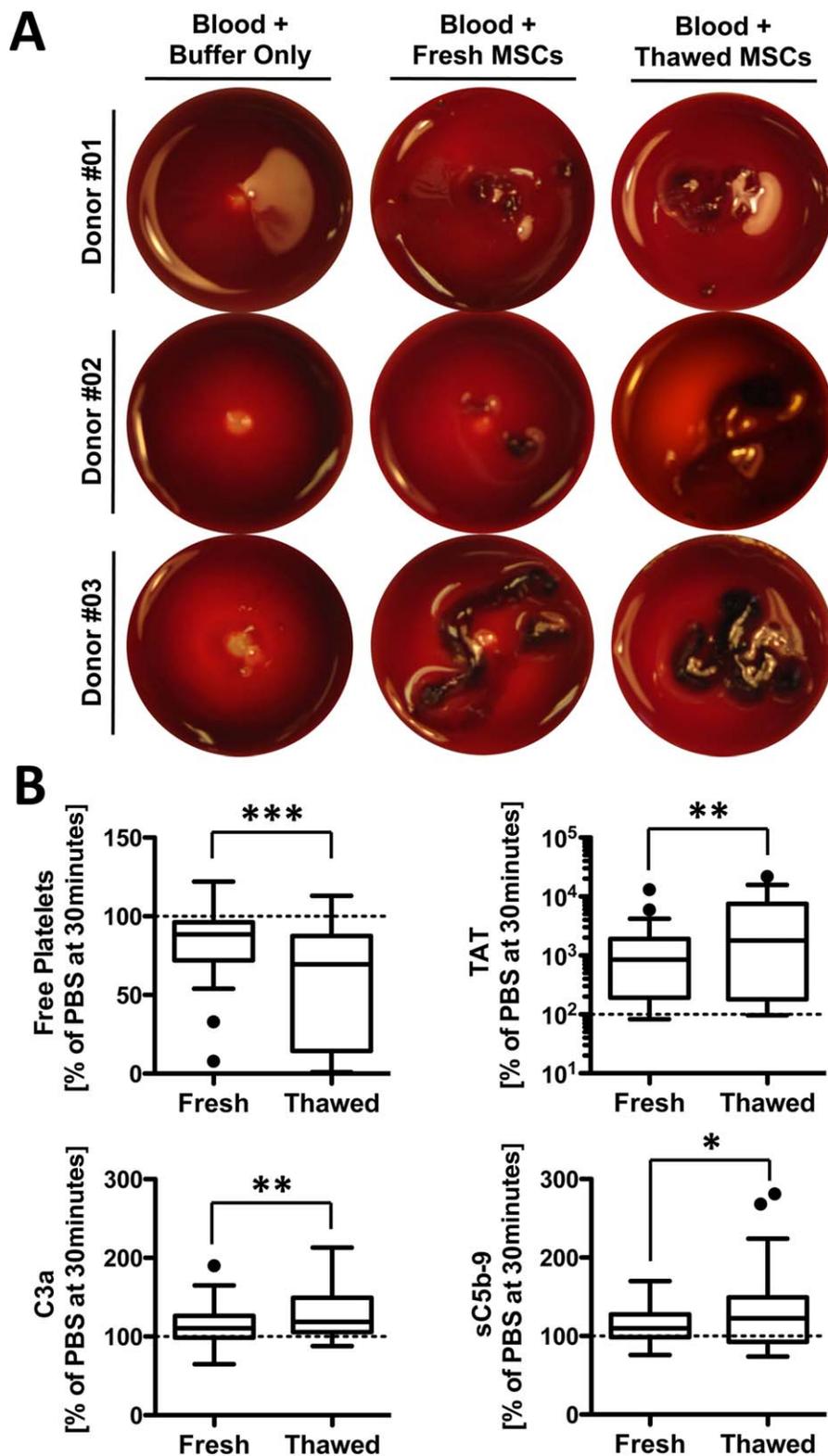
**Quantitative Reverse Transcriptase PCR.** mRNA levels of indoleamine 2,3-dioxygenase (IDO) and interleukin 6 (IL6) were quantified with quantitative reverse transcriptase PCR (QRT-PCR). Total RNA was isolated and cDNA was generated as described previously [10, 13]. Concentration of RNA was determined with a Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA, <http://www.thermoscientific.com>). QRT-PCR analysis was performed with BioRad CFX384 Real-Time, C1000 touch Thermal Cycler system (BioRad, Hercules, CA, <http://www.bio-rad.com>), and expression was calculated using  $\beta$ -actin as the internal standard (for primers see Supporting Information Table S2).

**IDO and Cytokine Analyses.** Western blotting of IDO within MSC cell lysates was quantified as previously described [33], with 10 μg of total protein per sample as assessed by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, <http://www.piercenet.com>). Membranes were probed with rabbit anti-human IDO Ab (H-110; Santa Cruz Biotechnology, Santa Cruz, CA, <http://www.scbt.com>; 2 μg/ml) or mouse anti-human beta-tubulin Ab (TUB2.1; Sigma, 2.6 μg/ml), followed by incubation with respective peroxidase conjugated secondary Ab (IDO 1:2,000 of goat-anti-rabbit antibody; beta-tubulin 1:3,000 of goat-anti-mouse from Dako). Positive signal was quantified with densitometry after subtraction of background (Image-J v1.46r). The enzymatic activity of IDO within MSC and mixed lymphocyte reaction (see below) CM was determined by measuring the concentration of the tryptophan metabolite, L-kynurenine, as outlined previously [33]. Absorbance was read at 492 nm and concentrations of L-kynurenine in the samples were calculated using a standard curve of defined L-kynurenine concentrations (0–100 μM). Levels of IL6 within CM samples were quantified at days 1 and 7 by ELISA (Life Technologies Europe BV) according to the manufacturer's instructions.

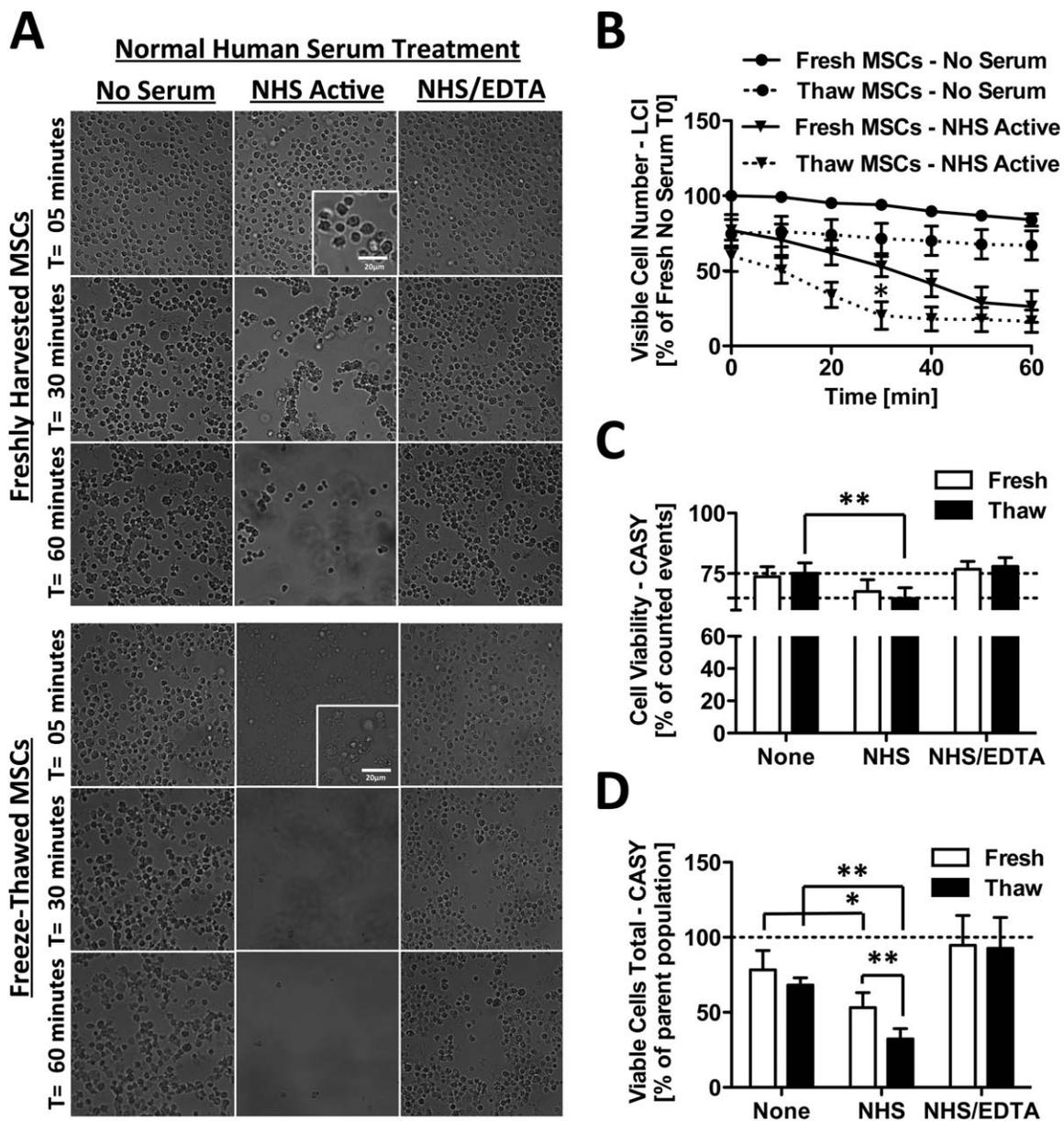
**Mixed Lymphocyte Reactions.** Mixed lymphocyte reactions (MLRs) were performed as described earlier [28]. Responder peripheral blood mononuclear cells (PBMCs) were stimulated with phytohaemagglutinin (PHA)-mitogen (3 days) or alloantigen (5 days;  $n = 5$ ). Freeze-thawed or fresh third-party MSCs (P2-4) were irradiated (20 Gy) and added at a 1:10 ratio to PBMCs. MSC-mediated suppression of PBMC proliferation was assessed with <sup>3</sup>H thymidine incorporation (18 hours).

### Statistical Analysis

Comparisons between MSCs were analyzed using dependent samples statistics (two-tailed). Paired  $t$  test was used for pairwise comparisons and repeated measures ANOVA in case of more than two comparisons. If data did not fit a normal distribution or equal variances nonparametric related samples Wilcoxon signed rank test or Friedman test were used. Normality and equal variances were checked with Shapiro-Wilk and Levene's test, respectively.  $p < .05$  was considered statistically significant (Prism 5.0; GraphPad Software Inc., La Jolla, CA, <http://www.graphpad.com> and IBM SPSS 19). For clinical response, differences between responders and non-responders were evaluated using Fisher's exact test (IBM SPSS 21).



**Figure 1.** Freeze-thawed MSCs elicit an increased triggering of the instant blood mediated inflammatory reaction (IBMIR). Donor-matched freshly harvested or freeze-thawed MSCs (15,000 cells per milliliter) were tested for triggering of the IBMIR by exposing them to non-anticoagulated whole blood in the Chandler blood loop model. **(A):** Representative photographs of clot formation after a 60-minute blood exposure of either freshly harvested or thawed MSCs, or buffer as negative control. **(B):** Percentage (relative to PBS, 30 minutes time point) of coagulation and complement activation markers after blood exposure with fresh or thawed MSCs ( $n = 30$ ): free platelets and ELISA quantification of TAT, complement C3 activation fragment a (C3a), and soluble C5b-9 complex (sC5b-9). The dotted line corresponds to values obtained for PBS-treated blood. Boxplot whiskers 1.5 interquartile range, \*,  $p < .05$ , \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ , paired  $t$  test. Abbreviations: MSCs, mesenchymal stromal cells; PBS, phosphate buffered saline; TAT, thrombin-anti-thrombin complex.



**Figure 2.** Freeze-thawed MSCs exhibit increased sensitivity to complement lysis. Time lapse live cell imaging (A, B) and electrical-impedance based CASY cell counter (C, D) were used to study the cell morphology and viability of fresh or freeze-thawed MSCs after exposure to either PBS (no serum), complement active NHS, or EDTA-inactivated NHS/EDTA without complement lysing activity. (A): Transmitted light images (5, 30, and 60 minutes) for fresh or thawed MSCs within focal plane for one representative MSC donor after respective serum treatments. (B): Quantification of the average visible cell number within focal plane of microscope ( $n = 6$  MSC donors, data are presented as percentage of fresh non-serum-treated cells at time point zero set to be 100%, corresponding videos can be found in supplement). (C): Cell viability of cells in suspension (% of counted cells,  $n = 9$  MSC donors) and (D) total viable cell number in suspension (% of parent population at start,  $n = 9$  MSC donors). Mean  $\pm$  SD, \*,  $p < .05$ ; \*\*,  $p < .01$ ,  $p$  values from paired  $t$  test. Abbreviations: MSC, mesenchymal stromal cells; NHS, normal human serum.

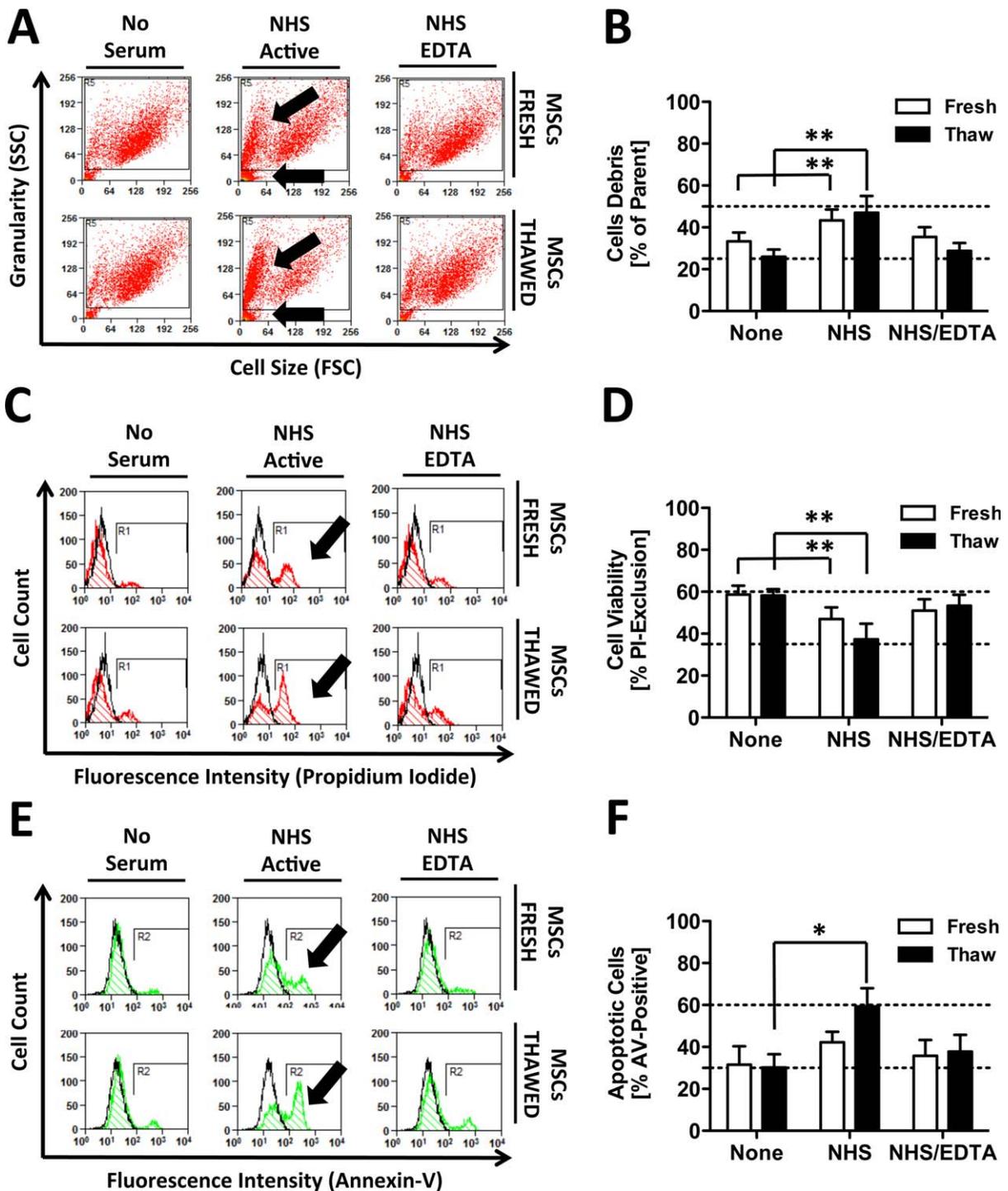
## RESULTS

### Freeze-Thawed MSCs Show Increased Triggering of the IBMIR and Complement-Mediated Cell Lysis In Vitro

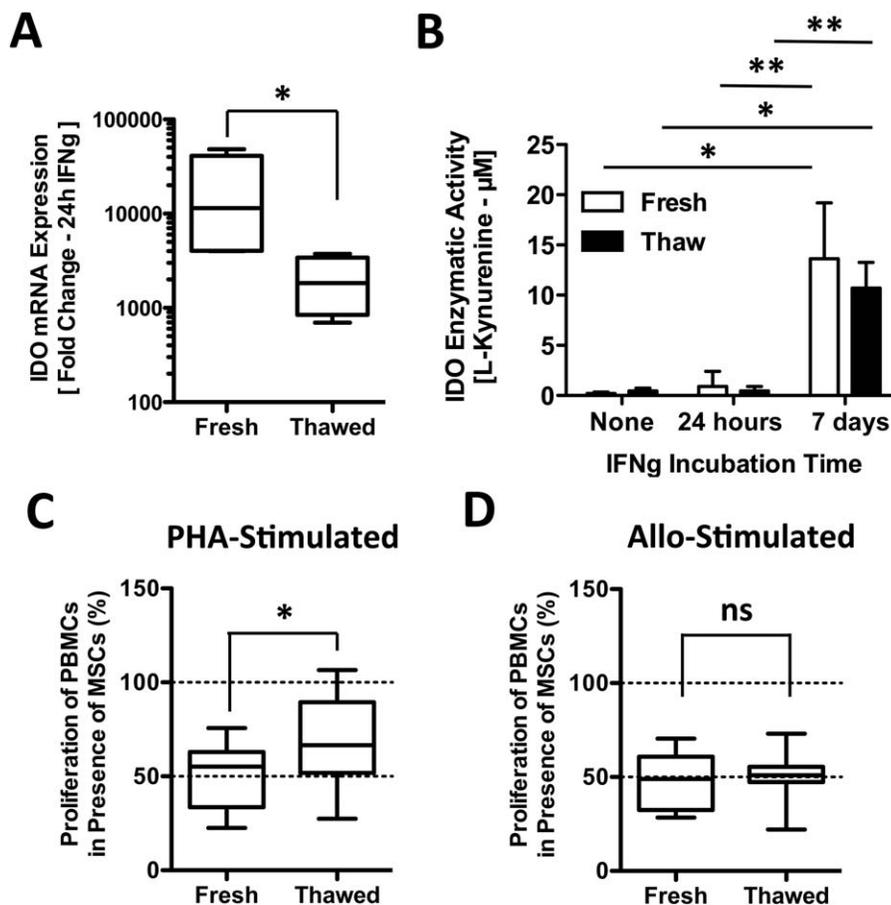
MSCs are often stored cryobanked, thawed, washed repeatedly, and reconstituted in transfusion bags containing saline and either 5%–10% human ABP or human serum albumin. We therefore simulated exposure of fresh or freeze-thawed low passage MSCs (P2-4) to human whole blood and NHS in order to investigate if freeze-thawed cells are more prone

to triggering of IBMIR and complement lysis than fresh cells.

When exposing low passage MSCs (P2-4) to whole blood in vitro (Fig. 1), triggering of the IBMIR was weak and showed donor variation as described in an earlier study [10]. Despite this, IBMIR was stronger with freeze-thawed MSCs compared to fresh cells. With fresh MSCs, we found an average 20% drop in free platelets, a 10-fold increase in thrombin and very low production of complement activation products C3a and sC5b-9 in comparison to controls (Fig. 1B). These responses were all



**Figure 3.** Freeze-thawed MSCs show increased cell shrinkage, debris formation, propidium iodide (PI)-membrane permeabilization, and AV-binding after serum exposure. Flow cytometry study of cell morphology and viability of freshly harvested or freeze-thawed MSCs after exposure to either PBS (none), complement active NHS, or EDTA-inactivated NHS/EDTA. **(A, B):** Typical dot plots indicating cell size (FSC) and granularity (SSC) of freshly harvested and freeze-thawed MSCs within gate R0 before and after serum treatment, with indication of cell shrinkage (upper arrow) and formation of cell debris (lower arrow); quantitative results ( $n = 9$  experiments) are shown in panel (B). **(C, D):** Histogram overlays for detection of PI incorporating cells (gate R1, PI-labelled cells shown as red histograms) within gated cell population (gate R0) versus unlabeled negative control (grey histogram), with indication of increased PI-incorporation (black arrows) in NHS-treated cells ( $n = 9$  MSC donors, with 5 technical replicates in each experiment). Cell viability presented in panel (D) was calculated based on PI-exclusion. If cell count stopped before set threshold (2,000 counts) missing counts were counted as dead cells. **(E, F):** Histogram overlays for AV binding to MSCs (shown as green histograms) versus negative control (shown as grey histograms) and quantification of AV-positive cells (gate R2), with indication of increased AV-binding to NHS-treated cells (black arrows). MSCs were subjected to similar treatment as described above ( $n = 6$ , one sample each), and the percentage of AV-positive cells (apoptotic cells) is shown in panel (F). The data in (B, D, and F) are presented as the mean  $\pm$  SD. \*,  $p < .05$ ; \*\*,  $p < .01$ ,  $p$  values from paired  $t$  test. Abbreviations: AV, Annexin V; FSC, forward scatter; MSC, mesenchymal stromal cell; NHS, normal human serum; SSC, sideward scatter.



**Figure 4.** Freeze-thawed MSCs have impaired immunomodulatory properties. **(A):** IDO mRNA expression (fold change) in freshly detached or freeze-thawed MSCs ( $n = 4$  each) was studied with quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) analysis after 24-hour stimulation with or without IFN- $\gamma$  (100 U/ml), boxplot whiskers min to max,  $p$  value from Wilcoxon signed rank test. **(B):** Quantification of IDO enzymatic activity in MSC conditioned medium ( $n = 4$ ). IDO activity was detected as tryptophan metabolite L-Kynurenine ( $\mu$ M). Means  $\pm$  SD,  $p$  values from paired  $t$  test. **(C, D):** Suppression of peripheral blood mononuclear cell (PBMC) proliferation (% proliferation of PBMCs in presence of MSCs) by freeze-thawed or fresh MSCs ( $n = 12$  tests in total, with  $n = 6$  MSC donors, and  $n = 2$  PBMCs) is shown relative to dividing control PBMCs without MSCs (100% proliferation). The suppressive effect of MSCs was studied either with phytohaemagglutinin (PHA)-mitogen- (C; readout day 3–4), or alloantigen-stimulated (D; readout day 5–6) mixed lymphocyte reactions. Boxplots whiskers min to max, \*,  $p < .05$ ; \*\*,  $p < .01$ , paired  $t$  test. Abbreviations: IDO, indoleamine 2,3-dioxygenase; IFN $\gamma$ , interferon gamma; MSC, mesenchymal stromal cell; PBMC, peripheral blood mononuclear cell; PHA, phytohaemagglutinin.

significantly stronger with freeze-thawed MSCs, indicating activation of coagulation and particularly complement cascades.

We subsequently studied how freeze-thawing may affect the interaction of MSCs with the complement system. Live cell imaging (LCI) revealed rapid aggregation of fresh and particularly freeze-thawed MSCs after exposure to NHS (Fig. 2A; Supporting Information video). This was not apparent with non-serum-treated or inactive NHS/EDTA-treated cells, which moved as loose cell clusters over the surface of the culture plate (cells remain in focal plane). Quantification of the visible cell numbers demonstrated that 95% of fresh, non-serum-treated cells remain within focus throughout the imaging period (Fig. 2B). Exposure to NHS led to a visible cell count of just 20% at the end of the imaging period. The mean reduction was two-fold stronger with freeze-thawed, compared to fresh MSCs, with an average visible cell count of 10% after 30 minutes of active serum exposure ( $p < .05$ ).

CASY counter analysis indicated a cell viability of 75% for both fresh and thawed MSCs before initiation of serum treatment (Fig. 2C). Treatment with active serum led to a small

decrease in viability (8%–10% reduction,  $p = .078$  [fresh] and  $p < .01$  [freeze-thawed]), compared to non-serum- or NHS/EDTA-treated cells. The absolute viable cell number in suspension after serum treatment was also recorded, allowing expression of viable cells relative to viable cell number at the start of the experiment and assessment of cell loss due to complement lysis and assay procedure (Fig. 2D). We found a mean 10%–20% reduction of viable cells in suspension for non-serum- or NHS/EDTA-treated cells, irrespective of the freeze-thaw procedure. This may be attributed to assay handling. The NHS-treatment led to a 50% reduction in viable cells for fresh MSCs and >80% reduction in viable cell number for freeze-thawed MSCs (Fig. 2D,  $p < .05$  and  $p < .01$ , respectively), amounting to a twofold difference in remaining viable cells ( $p < .05$ ). This indicates that complement lysis of freeze-thawed MSCs has taken place, which did not occur when the complement system in serum was inactivated with EDTA. As noted earlier by Galipeau [6], viability assessment is strongly dependent on the method used (Supporting Information Fig. S3A), possibly leading to overestimation in viability

**Table 1.** Clinical response to MSC-treatment comparing fresh and thawed cells

	Thawed cells (35 infusions)	Fresh cells (9 infusions)	<i>p</i> value
MSC recipients			
Sex: male/female	24/11	7/2	0.6
Age: median (range)	45 (0.5–65)	48 (1.5–64)	0.8
Age: children/adults	12/23	03/06	1.0
Indication for MSC treatment			
Acute Graft-versus-host disease	29 (83%)	7 (78%)	0.7
Hemorrhagic cystitis	6 (17%)	2 (22%)	
HLA match MSC with recipient	7/35 (20%)	6/9 (67%)	0.006**
Third party unrelated donor	28 (80%)	3 (33%)	
Haploidentical related donor	6 (17%)	5 (56%)	
HLA-identical sibling	1 (3%)	1 (11%)	
Patient response to treatment			
CR, Complete response	19/35 (54%)	7/9 (70%)	0.3
PR, Partial response	13 (40%)	3 (33%)	
SD, Stable disease	6 (26%)	4 (44%)	
PD, Progressive disease	7 (11%)	1 (11%)	
9 (23%)	1 (11%)		
MSC donors			
Sex: male/female	16/19	4/5	0.9
Age: median (range)	38 (22–66)	38 (24–66)	0.8
Cell dose: median × 10 <sup>6</sup> /kg (range)	1.6 (0.7–3.6)	1.4 (0.7–2.0)	0.9
(CR+PR) Responders	1.5 (0.7–3.0)	1.4 (0.7–2.0)	
(SD+PD) Nonresponders	1.6 (0.7–3.6)	1.5 (1.0–2.0)	
Cell passage: median (range)	3.0 (2.0–4.0)	2.0 (1.0–3.0)	0.08
(CR+PR) Responders	3.0 (2.0–3.0)	2.0 (1.0–3.0)	
(SD+PD) Nonresponders	3.0 (2.0–4.0)	3.0 (3.0–3.0)	
Cell viability: %, median (range)	93% (73–98)	91% (89–99)	0.8
(CR+PR) Responders	90% (82–98)	91% (89–99)	
(SD+PD) Nonresponders	93% (73–98)	95% (91–99)	

All data are per infusion. *p* value is calculated using Mann-Whitney rank-sum test (for continuous variables), Fisher's exact *t* test (comparing two categorical variables), or  $\chi^2$  test (comparing more than two categorical variables).

\*\**p* < .01.

Abbreviations: HLA, human leukocyte antigen; MSC, mesenchymal stromal cell.

when using the Trypan method. Furthermore, effects of holding time, temperature, different parenteral solutions, and DMSO concentration on cell viability and functionality should be considered [34–37].

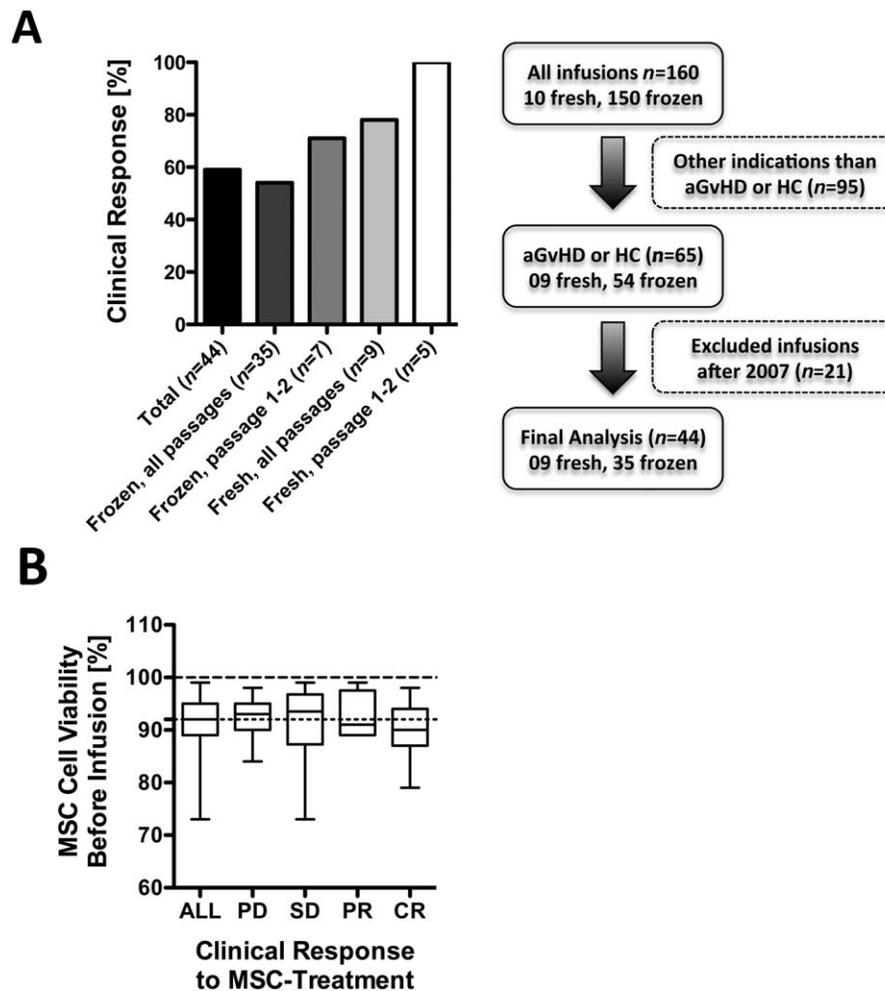
To verify our results obtained with LCI and CASY counter, we repeated the analysis with a flow cytometric-based readout (Fig. 3). Both fresh and freeze-thawed cells displayed a similar degree of expression for typical MSC surface antigens and complement/coagulation cell surface regulatory molecules (Supporting Information Figs. S1A, S1B). As described earlier [15], cell exposure to NHS, but not NHS/EDTA, led to an increased cell surface binding of complement pathway initiators C1q, MBL, and C3-activation fragments to MSCs (Supporting Information Fig. S2A); although positivity was weak for low passage MSCs compared to earlier findings [15–17]. Binding of pattern recognition molecules C1q and MBL was more evident for freeze-thawed than fresh MSCs (Supporting Information Figs. S2A, S2B), while surface binding of Ficolins was low or undetectable in both groups. Furthermore, activation and binding of C3-activation fragments was abrogated in the presence of C3-antagonistic peptide Compstatin, while binding of C1q and MBL remained detectable.

We observed a change in MSC morphology particularly after treatment with NHS, which did not occur with NHS/EDTA (Fig. 3A). Both fresh and particularly freeze-thawed MSCs demonstrated cell shrinkage and a twofold increase in cell debris within the sample (Fig. 3A, 3B; *p* < .01; Supporting Information Fig. S3B), potentially indicating membrane damage and cell lysis. Forward scatter-based analysis demon-

strated an average 25% and 50% reduction in cell size for fresh and freeze-thawed MSCs, respectively when treated with NHS serum (Supporting Information Fig. S3C, *p* < .01 and *p* < .001), suggesting damage to the plasma membrane and cytoplasmic leakage. Side scatter analysis of cell granularity did not demonstrate any significant changes (Supporting Information Fig. S3D).

Detection of PI incorporation within the gated cell population (excluding debris) showed a weak increase in PI-positive cells after NHS treatment (black arrows; Fig. 3E) compared to non-serum-treated cells (Fig. 3C; Supporting Information Fig. S3E), most evident in freeze-thawed cells (lower arrow). Cell viability based on PI-exclusion was approximately 60%, with a 10% or 20% reduction in viable cells observed when fresh or freeze-thawed MSCs were treated with NHS (Fig. 3D, *p* < .01). The reduction in viability was less for fresh MSCs, although sample acquisition took longer and often yielded insufficient counts (accounted as dead cells), suggesting a cell loss after NHS-treatment.

Apoptosis, as assessed by AV staining, indicated a shift in AV-binding particularly after treatment with NHS (black arrows). Approximately 30% of the non-serum-treated MSCs bound AV irrespective of the freeze-thaw procedure (Fig. 3F), indicating that trypsin-detached cells may display a relatively high background for binding of AV and that freeze-thawing alone may not necessarily increase phosphatidylserine (PS)-exposure. However, the binding of AV increased for both fresh MSCs (45% positive, *p* = .06) and particularly thawed MSCs (60% positive, *p* < .05) after treatment with NHS. AV staining only showed a weak increase (5%–10%) after treatment with NHS/EDTA. It was noted



**Figure 5.** Analysis of patient response to fresh and thawed MSCs. **(A):** Between 2002 and 2007, nine infusions of fresh MSCs were given to patients with treatment indication aGvHD and HC, which was paralleled by application of 35 freeze-thawed cells to patients with similar indication in the same time frame. The average response to all 44 infusions was 60%. Early passage cells (P1–2) yielded a 71% response when given thawed ( $n = 7$ ) and a 100% response when given fresh ( $n = 5$ ). **(B):** Evaluation of clinical response to MSCs for stratified patient cohort regarding cell viability at infusion (%). MSCs given to complete and partial responders (CR and PR,  $n = 13$ ) showed similar viability (TRYPAN, median viability 93% vs. 90%) as cells given to patients with stable and progressive disease (SD and PD,  $n = 23$ ). Boxplot whiskers min to max. Abbreviations: aGvHD, acute graft versus host disease; CR, complete responder; HC, hemorrhagic cystitis; MSC, mesenchymal stromal cell; PD, progressive disease; PR, partial responders; SD, stable disease.

that in some experiments also NHS/EDTA-treated MSCs showed a weakly increased PI-uptake and AV-binding, most likely accountable to EDTA-related cell toxicity, but no lysis was detected.

### Freshly Thawed MSCs Display Impaired Immunomodulatory Properties In Vitro

Previous findings comparing the immunomodulatory properties of fresh and freeze-thawed MSCs appear to be inconclusive. We therefore conducted a donor-matched comparison between fresh and freeze-thawed MSCs. Fresh MSCs demonstrated significantly higher IDO mRNA expression after 24 hours of IFN $\gamma$  exposure compared to freeze-thawed cells (Fig. 4A;  $p < .05$ ); however very low levels of IDO protein were detected within the CM (Fig. 4B) at this time point. After 7 days of IFN $\gamma$  stimulation, significant increases in IDO secretion ( $p < .05$ ; Fig. 4B) were detected compared to untreated controls, with comparable protein levels between MSC treatment groups being recorded. Similar effects were seen by Western

blotting for IDO protein (Supporting Information Fig. S4A, S4B). Strong IDO activity was evident in CM from MLRs with fresh and freeze-thawed MSCs, most likely due to the release of IFN $\gamma$  by the responder lymphocytes. No difference in IDO activity between the fresh and freeze-thawed MSCs was demonstrated. Furthermore, no increase in IDO activity was seen when licensing the MSCs with IFN $\gamma$  for 7 days before MLR (Supporting Information Fig. S4C).

mRNA and protein levels of IL6 were measured within IFN $\gamma$ -exposed fresh and freeze-thawed MSCs. IFN $\gamma$  treatment induced IL6 secretion within both treatment groups ( $p < .01$  [fresh],  $p < .05$  [freeze-thawed]), with a further significant increase between 24 hours and 7 days seen only in the fresh cells ( $p < .01$ ). However, overall IL6 expression levels were comparable between fresh and freeze-thawed cells at both the mRNA and protein level (Supporting Information Fig. S4D, S4E). Freeze-thawed MSCs were inferior to fresh MSCs in suppression of PHA-stimulated MLRs ( $p < .05$ , Fig. 4C), while no difference was noted in allo-stimulated MLRs (Fig. 4D).

**Table 2.** MSC-donor DNA detection in tissues comparing fresh and thawed cells

	Thawed cells (16 infusions)	Fresh cells (6 infusions)	<i>p</i> value
MSC recipients			
Sex: male/female	11/05	5/1	0.5
Age: median (range)	50.5 (8–67)	53.5 (8–64)	0.7
Age: children/adults	1/15	1/5	0.4
Indication for MSC treatment			
Acute Graft-versus-host disease	9 (56%)	4 (67%)	0.7
Hemorrhagic cystitis	5 (31%)	2 (33%)	
HLH	2 (13%)	0 (0%)	
HLA match with recipient			
Haploidentical related donor	3/16 (19%)	4/6 (67%)	0.05
Third party unrelated donor	3 (19%)	4 (67%)	
Third party unrelated donor	13 (81%)	2 (33%)	
MSC donors			
Sex: male/female	5/10 <sup>a</sup>	2/4	1.0
Age: median (range)	38 (22–66)	38 (27–39)	0.5
Cell dose: median × 10 <sup>6</sup> /kg (range)	1.4 (0.6–2.2)	1.0 (0.7–2.0)	0.6
Cell passage: median (range)	3.0 (2.0–3.0)	2.0 (1.0–3.0)	0.02*
Sample data			
Tissues sampled: median (range)	7 (1–10)	6 (4–14)	0.7
Sampling time: days, median (range)	26 (6–408)	34 (9–505)	0.7
Infusions with positive samples:	8/16 (50%)	3/6 (50%)	1.0
Positive MSC-DNA detection	18/98 (18%)	5/40 (13%)	0.5
Lung	4/13 (31%)	0/4 (0%)	
Kidney	3/10 (30%)	1/3 (33%)	
Lymph node	3/11 (27%)	1/4 (25%)	
Intestine	3/14 (23%)	1/5 (20%)	
Bladder	1/7 (14%)	1/3 (33%)	
Liver	1/11 (8%)	1/5 (20%)	
Other	2/29 (7%)	0/14 (0%)	

All data are per infusion. *p* value is calculated using Mann-Whitney rank-sum test (for continuous variables), or  $\chi^2$ -test (comparing categorical variables).

\*, *p* < .05.

<sup>a</sup>Two infusions, one male and one female, have been calculated as one as the PCR-probe could not separate the two.

Abbreviations: HLA, human leukocyte antigen; HLH, hemophagocytic lymphohistiocytosis; MSC, mesenchymal stromal cells.

### Freeze-Thawing of MSCs Potentially Influences Their Therapeutic Efficacy

The clinical response to fresh MSCs compared to freeze-thawed MSCs was analyzed retrospectively in a comparable patient cohort (Table 1; Fig. 5A). As the number of fresh infusions was limited, no multivariate analysis could be performed. Confounding variables (MSC passage, cell dose, HLA-match, and patient age) were analyzed in univariate models. No individual factor was shown to be significantly associated with improved outcome, but we found a trend toward better response in patients receiving early passage (P1–2; *p* = .08) and fresh MSCs (*p* = .3). Patients infused with fresh MSCs of very early passage (P1–2; *n* = 5) demonstrated a 100% response rate, compared to 50% in patients receiving freeze-thawed MSCs at a higher passage (P3–4; *n* = 28). This difference was not statistically significant (*p* = .06, Fig. 5A) but indicates potential additive positive effects for both of these factors regarding therapeutic value. Both fresh and thawed MSCs had similar viability before infusion (Table 1) and viability did not differ between responders and nonresponders (Table 1; Fig. 5B).

We also revisited the retrospective evaluation for long-term engraftment in MSC-treated patients previously published [4] and re-evaluated the data comparing the use of freshly harvested to freeze-thawed cells; however no major difference in engraftment could be observed between the two groups (Table 2). For both fresh and thawed MSCs, approximately half of the patients demonstrated a positive signal for MSC donor DNA in one or more tissues, with higher positivity for earlier sampling [4]. Overall, 13% of the samples

analyzed for fresh MSCs were donor DNA positive, compared to 18% of samples analyzed for thawed MSCs. In the lungs, 4 of 13 samples were positive for thawed MSCs but none for fresh cells. Four of the patients had been infused with both fresh and thawed MSC, two of them with positive samples in one or three tissues, respectively. Within these positive samples DNA from both MSC donors could be detected, indicating that factors in the patient, rather than MSC-specific differences, affected the engraftment.

### DISCUSSION

The immunomodulatory and regenerative properties of MSCs have been extensively studied. Still, in depth analysis on the best therapeutic product and its exact preparation for clinical use is scarce [5]. Translational studies with closer resemblance to the clinical practice are essential for a better understanding of fate and function of the therapeutic cells in vivo. A multitude of product parameters, such as donor-recipient-match, cell culture media supplementation, culture time, cell preparation, and delivery method [5, 7, 18, 19, 34–38], all potentially affect MSCs therapeutic efficacy. There is a practical need for storage/banking of therapeutic cells, allowing for an “off the shelf” readily available product [5]. Although frozen cells are commonly used in the clinic, animal studies indicate that fresh cells are potentially better tolerated than thawed cells when used at high doses [7]. Galipeau et al. recently reported that cryopreserved MSCs are of lower therapeutic value than fresh MSCs due to an increased display of proapoptotic

features and impaired immunosuppressive activity [5, 6]. These results imply that therapeutic MSCs should be recovered in culture before clinical usage.

### Studies on Cell Viability and Complement Injury of Fresh and Thawed MSCs

Freeze-thawing of therapeutic cells could potentially lead to increased PS-exposure and reduced cell viability [34–37]. However, the reported viability of 50% [6] appears to be rather low for thawed MSCs. In our hands, thawed MSCs display a viability of 70% and up to 90% for up to 30 months of cryostorage [39] depending on the assessment method used [34–37]. We therefore undertook experiments aiming to validate earlier findings on viability and study the interactions of fresh and freeze-thawed MSCs with human blood, as many products are applied via systemic infusion. We observed a significantly increased triggering of the IBMIR after blood exposure of thawed MSCs but to a lesser degree with freshly detached low passage cells. We and others have previously shown that MSCs elicit triggering of the IBMIR [10–13], which was in our hands more evident with higher passage and MLR-activated MSCs [10]. Our present finding that low passage resting MSCs show increased triggering of IBMIR when being subjected to freeze-thawing before infusion adds additional relevance to this topic.

A recent report demonstrated that MSCs are injured after exposure to active complement [16]. We thus assessed viability and total cell number of fresh and freeze-thawed MSCs before and after complement exposure. Here we report a 80% decrease in total viable cell number in freeze-thawed cells, whilst fresh MSCs demonstrated only a 50% decrease on exposure to NHS. Addition of EDTA to serum abrogated this effect, indicating that complement lysis occurred in a calcium-dependent fashion.

We were previously unable to document complement mediated cell injury or even lysis of MSCs with our flow cytometry readout [15]. Within this study an increased incubation time and reduced cell number per Fluorescence-activated cell sorting (FACS) tube allowed us to confirm freeze-thawed MSCs are lysed after complement exposure, a phenomena less evident with fresh cells, and abrogated when adding EDTA. Complement activation and subsequent cell lysis went in hand with binding of C3-fragments, cell shrinkage, cell debris formation, increased PI-uptake, and AV-binding. In agreement with Galipeau's findings [6], we detected a higher degree of dead cells with both CASY counter and flow cytometry, as opposed to trypan blue staining (Supporting Information Fig. S3A), which potentially overestimates viable cell number, since "dying" cells are not detected with this method. For both CASY and flow cytometric measurements we noted a low viability for fresh MSCs (75% and 60%, respectively), which is often reported to be higher than 90% (Trypan). In addition to the viability assessment method, this discrepancy may also be related to the assay procedure, necessitating multiple washing and centrifugation steps over several hours. We estimate an average 5% reduction in viable cell number for each hour of assay-related handling. Importantly, neither method detected a reduction in viable cells, increased PI-uptake or AV-binding after one freeze-thaw cycle. However, all indicators worsened after a 60-minute treatment with NHS, with a particularly bad outcome for freeze-thawed

cells, indicating that thawed MSCs are more sensitive to complement recognition and lysis.

### Studies on the Immunomodulatory Properties of Fresh and Freeze-Thawed MSCs

Galipeau et al. [6] reported no suppressive activity of freeze-thawed MSCs, while freshly harvested MSCs showed a weak suppressive activity against CD3/CD28-driven T-cell proliferation, mainly via IDO-activity. Our retrospective analysis (2003–2011) on the immunosuppressive activity of MSCs, obtained from more than 60 donors, indicated an average 60% inhibitory activity of fresh or thawed MSCs in alloantigen and PHA-mitogen stimulated MLRs [15]. Most MSCs were thawed from cryostorage immediately prior to MLR. We also reported in an earlier study that the suppressive activity of MSCs in MLRs appeared to be enhanced after undergoing freeze-thawing and cryostorage [39]. To obtain more conclusive results we performed a donor matched-paired analysis of fresh and freeze-thawed MSCs. Our QRT-PCR results support Galipeau's findings [6] that freeze-thawed MSCs show lower IFN $\gamma$ -responsiveness, with IDO mRNA levels significantly higher in fresh than freeze-thawed MSCs in response to 24 hours of IFN $\gamma$  stimulation. This suggests a potential impairment of MSCs immunosuppressive phenotype directly after thawing. These differences were not evident at the protein level at 24 hours; however we hypothesize a delay in translation of these transcriptional level differences to detectable protein expression. Significant upregulation of IDO protein levels were evident within day 7 IFN $\gamma$  stimulated samples. These levels were comparable between the fresh and freeze-thawed treatment groups at this time point, suggesting that the freeze-thawed cells have stabilized and are functionally comparable in terms of IFN $\gamma$  responsiveness and immunomodulatory activity. These findings are supported by results of the MLR assays with differences between the two cell types most evident with an early readout (PHA), but comparable with prolonged in vitro assay time (Allo-MLRs); further confounded by the comparable levels of IDO activity between fresh and freeze-thawed MSCs post-Allo-MLR.

It has been extensively reported that the inherent immunosuppressive effects of MSCs are multifactorial [40, 41]. We hypothesized therefore that the suppressive activity/effect of MSCs may not be restricted to IDO alone. We therefore investigated IL6 expression, a cytokine widely reported to be upregulated by MSCs in response to IFN $\gamma$  licensing. Our results indicate no significant differences in transcription or secretion of this cytokine between fresh and freeze-thawed treatment groups after IFN $\gamma$  licensing at either time point.

Complement activation and lysis of freeze-thawed MSCs may not necessarily be a negative aspect. As described by Galipeau [6], phagocytes may recognize apoptotic/complement opsonized MSCs, which could tune these immune effector cells into a regulatory type 2 (M2) phenotype, producing anti-inflammatory mediators. This phenomenon is well-described in the literature and may potentiate MSC anti-inflammatory/trophic properties. We have shown that MSC immunosuppressive effect in vitro can be reduced by blocking complement activation at its central step (C3) or by removing myeloid cells from MLRs [15], suggesting a role of complement activation for triggering the immunosuppressive effect of these cells via engagement of myeloid cells.

## Studies on Clinical Response and Engraftment of Fresh and Freeze-Thawed MSCs

Patient analysis of clinical response to fresh or thawed MSCs is limited by the scarcity of comparable infusions. A power analysis aiming at 80% power at the 0.05 significance level shows that we can only expect statistical significance for differences in clinical effect of more than 50%, meaning that we might ignore clinically relevant differences. We must therefore also consider trends, even though this could be considered speculative. Conclusion of our analysis is clearly limited by the small number of infusions using fresh cells ( $n = 9$ ) but indicates a potentially favourable therapeutic value for fresh cells, in addition to the previously reported advantage for very early passage cells [9, 10, 42]. Fresh cells were given more often from HLA-haploidentical relatives, which could possibly confound our analysis. However, HLA-matching was not associated with better clinical outcome previously [29] or in this study ( $p = .5$ ). Among fresh infusions, the two nonresponders received cells from a haploidentical relative whereas the three receiving third party cells all responded, indicating that HLA-matching is not the explanation behind our findings. This potentially improved outcome for giving fresh cells at very early passage is an important finding, which needs further investigation.

Analysis of MSC-donor DNA in patient tissue indicated equally low engraftment for fresh and frozen cell sources. The absence of positive findings for fresh MSCs in the lungs might potentially indicate improved cell passage through the lung capillaries, in consistency with our *in vitro* findings of generally improved blood compatibility for fresh cells. Studies on the biodistribution of fresh and freeze-thawed cells after systemic infusion could therefore be of interest. Importantly, any form of licensing protocols for cell therapeutics should be designed with consideration to the anticipated delivery method, for example, if cells are applied via systemic delivery, studies of cell interaction with blood should be conducted.

### SUMMARY AND CONCLUSION

Our *in vitro* data indicate that freeze-thawed MSCs are more prone to activate the IBMIR and display increased sensitivity to complement lysis, therefore compromising therapeutic MSC survival. The stronger recognition of thawed cells by innate

immune defence cascades resulted from an increase in direct recognition by pattern binding molecules C1q and MBL. In turn, complement activation led to increased apoptotic and necrotic cell features and consequently MSC lysis. These *in vitro* findings are paralleled by indications of better therapeutic efficacy for fresh MSCs, especially when given at a very early passage, although long-term engraftment was not affected. The ready availability of cryopreserved cells is a prerequisite for a practical treatment of acute diseases such as acute GvHD and hemorrhagic cystitis. Thus, we believe that the best way forward for practically exploiting these novel findings is to improve the design of future studies, aiming to improve the therapeutic efficacy and delivery mode of cryopreserved cells.

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### AUTHOR CONTRIBUTIONS

G.M., B.N. and K.L.B.: designed the study and wrote the manuscript; K.L.B.: led the clinical study; G.M., L.V.B., J.J.A., and L.D.: performed the research and analyzed the data; N.H., L.F., O.H., R.H., L.I., M.L., H.L., J.D.L., Y.T., and K.N.E.: assisted experiments. J.J.A. and L.C.D. contributed equally to this work.

### DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

The authors indicate no potential conflicts of interest.

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