



## Clinical Cellular Therapeutics Accelerate Clot Formation

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### ABSTRACT

Clinical cellular therapeutics (CCTs) have shown preliminary efficacy in reducing inflammation after trauma, preserving cardiac function after myocardial infarction, and improving functional recovery after stroke. However, most clinically available cell lines express tissue factor (TF) which stimulates coagulation. We sought to define the degree of procoagulant activity of CCTs as related to TF expression. CCT samples from bone marrow, adipose, amniotic fluid, umbilical cord, multi-potent adult progenitor cell donors, and bone marrow mononuclear cells were tested. TF expression and phenotype were quantified using flow cytometry. Procoagulant activity of the CCTs was measured *in vitro* with thromboelastography and calibrated thrombogram. Fluorescence-activated cell sorting (FACS) separated samples into high- and low-TF expressing populations to isolate the contribution of TF to coagulation. A TF neutralizing antibody was incubated with samples to demonstrate loss of procoagulant function. All CCTs tested expressed procoagulant activity that correlated with expression of tissue factor. Time to clot and thrombin formation decreased with increasing TF expression. High-TF expressing cells decreased clotting time more than low-TF expressing cells when isolated from a single donor using FACS. A TF neutralizing antibody restored clotting time to control values in some, but not all, CCT samples. CCTs demonstrate wide variability in procoagulant activity related to TF expression. Time to clot and thrombin formation decreases as TF load increases and this procoagulant effect is neutralized by a TF blocking antibody. Clinical trials using CCTs are in progress and TF expression may emerge as a safety release criterion. *STEM CELLS TRANSLATIONAL MEDICINE* 2018;7:731–739

### SIGNIFICANCE STATEMENT

Clinical cellular therapeutics express different amounts of tissue factor. As their tissue factor load increases, time to clot formation relative to control decreases. Adipose and amniotic fluid MSCs express high levels of tissue factor and accelerate clot formation the most. Tissue factor expression of clinical cellular therapeutics should be considered as a safety release criterion for clinical use.

### INTRODUCTION

There are at least 780 active clinical trials involving clinical cellular therapeutics (CCTs) in the U.S. according to a conservative estimate using common search terms on clinicaltrials.gov. CCTs often express varying amounts of tissue factor (TF), a potent activator of the coagulation cascade. TF is typically expressed by perivascular cells to form a hemostatic envelope around blood vessels [1]. The vascular endothelium separates TF from its circulating clotting factor FVII/VIIa. Extravascular TF is exposed to intravascular FVII/VIIa after disruption of the endothelial barrier, for example after traumatic injury. Intravenous infusion of CCTs is a common route of treatment delivery as their effects are thought to be mediated via

interactions with the lung and spleen [2–6]; however, their effect on coagulation *in vivo* is not fully understood.

*In vitro* studies of the CCT procoagulant effect in humans has only recently been described. Moll et al. [7,8] identified TF to be responsible for the procoagulant effect of mesenchymal stromal cells (MSCs). Coagulation was absent after MSCs were exposed to FVII and FX deficient plasma, but did occur with FXII deficient plasma. Christy et al. [9] demonstrated the procoagulant effect of human adipose and bone marrow MSCs using the functional coagulation tests thromboelastography (TEG) and calibrated thrombogram (CAT). MSC-associated TF accelerated thrombin formation and hastened the clotting process. The

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percent of MSCs expressing TF correlated with this procoagulant activity. In addition, TF expression on MSCs was dependent on their tissue source and increased as time in culture progressed.

Acute side effects of systemic cellular therapy in humans are rare; however, their effect on the coagulation and complement cascade can be measured. Moll et al. [8] reported significantly elevated levels of thrombin and the complement activation marker C3a after infusion of bone marrow MSCs in a cohort of 44 patients treated for graft versus host disease related to hematopoietic stem cell transplantation. In a similar but smaller ( $N = 8$ ) follow-up study, Moll et al. [7] reported significantly elevated D-dimer levels 3 hours after infusion of decidual stromal cells.

Animal studies demonstrate that the procoagulant effect of MSCs as well. Gleeson et al. [10] demonstrated in a porcine myocardial infarction model microvascular obstruction and intramyocardial hemorrhage after intracoronary delivery of bone marrow derived MSCs. This adverse event was not present when MSCs were delivered with heparin. Compared with heparin alone, MSCs delivered with heparin demonstrated improved infarct size and left ventricular remodeling after 6 weeks. Gleeson et al. demonstrate the beneficial therapeutic effects of MSCs after myocardial infarction while also highlighting the need to control procoagulant effects from MSC associated TF.

The purpose of this study was to broadly survey the procoagulant activity of CCTs derived from various source tissues. We hypothesize that procoagulant activity from CCTs correlates with their TF expression. We present evidence demonstrating that TF expression by CCTs predicts procoagulant activity using clinical and experimental clotting assays.

## METHODS

### Clinical Cellular Therapeutic Sources and Preparation

CCTs were sourced from six different human tissues including amniotic fluid derived MSCs, adipose derived MSCs, bone marrow derived MSCs, umbilical cord derived MSCs, multi-potent adult progenitor cells, and bone marrow mononuclear cells. All tissues were acquired either from commercial sources or under IRB-approved protocols.

Processing of the amniotic fluid derived MSCs (AF MSCs) was carried out in an ISO Class 7 human cell production facility in compliance with current Good Manufacturing Practice (cGMP) guidelines of the FDA. Amniotic fluid samples were collected through the approved IRB protocol HSC-MS-11-0593. All reagents used were GMP-grade and risk analysis of the manufacturing process was performed as previously described [11]. In brief, amniotic fluid was centrifuged at 400g for 15 minutes and the pellet was resuspended in sterile-filtered complete TheraPEAK XenoFree chemically defined mesenchymal stromal cell growth medium (Lonza, Walkersville, MD) supplemented with 20% allogeneic pooled human AB serum (Valley Biomedical, Winchester, PA) and 5 ng/ml basic fibroblast growth factor (CellGenix, Freiburg, Germany). Cells were plated on Corning (Corning, NY) CellBIND surface and incubated at 37°C in a 5% CO<sub>2</sub> and 95% RH environment. Non-adherent cells were removed after 48 hours and growth medium was changed every 3–5 days. Upon reaching 70%

confluence, cells were rinsed with calcium- and magnesium-free PBS, detached with TryPLE Express XenoFree reagent (Thermo Fisher Scientific, Waltham, MA) and progressively passed and transferred to the scale-appropriate cell-culture platform for expansion. Cells were frozen in CryoStor CS10 (Biolife Solutions, Bothell, WA) animal protein-free, defined cryopreservation medium and stored in a liquid nitrogen vapor freezer.

Primary adipose biopsy samples were kindly provided by Dr Saverio LaFrancesca and designated for research use only. Adipose MSCs (ADP MSCs) were isolated by washing the tissue three times in cold alpha-MEM (Sigma Aldrich, St. Louis, MO) containing 50 µg/ml gentamicin and mincing tissue into 5 mm pieces. Tissue was digested in buffer containing alpha-MEM, 300 IU/ml of Collagenase Type II (Worthington Biochemicals, Lake wood, NJ), 50 µg/ml gentamicin, and 1% bovine serum albumin 7.5% (Fraction, Gibco, Grand Island, NY) for 55 minutes at 37°C/5% CO<sub>2</sub>. For every 3 g of tissue, 10 ml of digestion buffer was used. After incubation, the tubes were centrifuged at 400g for 15 minutes at room temperature. The cell pellet was plated at a density of 9 g tissue/225 cm<sup>2</sup> Flasks (Thermo, Pittsburgh, PA). Cells were expanded in 5% platelet lysate (Gulf Coast Blood Bank, Houston, TX) in alpha-MEM, 1,000 U/ml heparin and 10 µg/ml gentamicin. Passage 0 was maintained at 37°C/5% CO<sub>2</sub>, fed every 3rd day until confluency reached 70%. Upon reaching desired confluency, the medium was discarded; the cultures were washed with PBS and the adherent cells harvested with 0.25% trypsin/1 mM EDTA for 5 minutes at 37°C and frozen at 10<sup>6</sup> cells per ml in a cryosolution containing 10% DMSO (Cryostor CS10, Stem Cell Technologies, Vancouver, BC, Canada) for subsequent experiments.

Bone marrow derived MSCs (BM MSCs) were isolated from commercially available fresh human bone marrow aspirates (AllCells, Emeryville, CA) or obtained from fresh bone marrow through the approved IRB protocol HSC-MS-08-0393 and expanded following established procedures [11]. Briefly, BM MSCs were cultured in complete culture medium that consisted of alpha-minimal essential medium (Life Technologies, Grand Island, NY), 17% fetal bovine serum (FBS; lot-selected for rapid growth of MSC; Atlanta Biologicals, Norcross, GA), 100 units/ml penicillin (Thermo Fisher Scientific, Waltham, MA), 100 mg/ml streptomycin (Life Technologies), and 2 mM L-glutamine (Thermo Fisher Scientific, Waltham, MA). BM MSC were incubated with medium replaced every 2 days until 70% confluent. Medium was then discarded and cultures were washed with phosphate-buffered saline (PBS) and adherent cells were harvested with 0.25% trypsin/1 mM EDTA (Thermo Fisher Scientific) for 5 minutes at 37°C and frozen at 10<sup>6</sup> cells per milliliter for subsequent experiments.

Umbilical cord derived MSCs (UMB MSCs) were obtained through the approved IRB protocol HSC-MS-11-0601. UMB MSC isolation was performed using the explant migration method. Briefly, 3–5 mm long cords were placed on a 100 mm cell culture dish 5 mm apart in 5% platelet lysate expansion medium. MSCs were allowed to migrate out of the explant into the culture dish for 7 days. Explant was then removed and cells were expanded until confluency reached 70%. Upon reaching desired confluency, the cells were harvested and frozen.

Bone marrow mononuclear cells (BM MNCs) were isolated from fresh whole bone marrow from a commercial source (AllCells, Emeryville, CA) according to common protocols using

density centrifugation. Briefly, bone marrow from healthy donors was diluted 1:2 with PBS and layered on top of Ficoll-Paque (GE Healthcare, Chicago, IL) and centrifuged at 400g for 30 minutes at room temperature. The mononuclear cell layer was carefully collected and rinsed twice with PBS and BM MNCs were suspended in RPMI media supplemented with 10% heat inactivated FBS (AtlantaBio, Atlanta, GA). Aliquots of  $1 \times 10^7$  cells/ml were cryopreserved by the addition of 10% DMSO, followed by freezing and storage in the vapor phase of a liquid nitrogen freezer for future use.

Multipotent adult progenitor cells (MAPC) are a distinctive adult, adherent cell type isolated from bone marrow and other tissues and unique from bone marrow mononuclear cells and MSC based on size [12], transcriptome [13], secretome [14], miRNA profile [15,16], and differentiation capability [13]. MAPC samples were obtained from Athersys, Inc. through a material transfer agreement. A proprietary modification of the Verfaillie method similar to that described by Jiang et al. [17] was used to expand and culture MAPCs prior to transfer.

Cell viability of at least 70% was insured using Trypan blue staining for all samples. For experiments described below, cells were counted using a hemocytometer and resuspended in PBS to a working concentration of  $10^6$  cells/ml immediately before use.

### Flow Cytometry

Cells were evaluated for the percent cells expressing mesenchymal markers CD29, CD44, CD73, CD90, CD105, CD31, CD34, CD45, and HLA-DR using multi-parametric flow cytometry panels (BD Biosciences, San Diego, CA). Mouse anti-human CD142 (BD Biosciences) was included in the panel to determine the level of tissue factor expression on the cell surface. Cells were washed and suspended in staining buffer (Biolegend, San Diego, CA) at a concentration of  $0.5 \times 10^6$  cells per 100  $\mu$ l. Antibodies were added followed by 20-minute incubation at room temperature. 7AAD was added to exclude dead cells and diluted to a total volume of 1 ml. Flow analysis was performed on a Gallios (Beckman Coulter, Brea, CA) and analyzed using Kaluza vr. 1.5a analysis software (Beckman Coulter). Results presented are percent of expression relative to controls of unstained cells, isotype controls, and fluorescence minus one (FMO) controls (Table 1).

### Calculation of Tissue Factor Load

To estimate a load of TF present in CCT samples, the product of the mean fluorescent intensity (MFI) and percent cells expressing the anti-human CD142 antibody, or TF, from flow cytometry was calculated as relative units. MFI reflects the density of antibody detected on a single cell. The MFI is

calculated from the arithmetic mean for all cells expressing TF. Expressing CCT TF in this manner reflects both the percentage of cells expressing TF in a sample and their average density of expressed TF on the cell surface.

### Thromboelastography

TEG is a viscoelastic assay that measures coagulation kinetics of whole blood or platelet rich plasma. Primary metrics include R time which reflects time to initial fibrin formation, maximal amplitude (MA) which reflects the contribution of fibrin and platelets to ultimate clot strength, alpha angle which reflects the rate of fibrin formation, and lysis (LY30) which reflects the degree of fibrinolysis 30 minutes after the MA is reached.

TEG was performed using the Haemoscope TEG 5000 Coagulation Analyzer (Haemoscope Corp., Niles, IL). Whole blood was collected through approved IRB protocol HSC-MS-10-0190 from a healthy volunteer through venipuncture in the antecubital fossa and stored in 3.2% citrate at room temperature. Final assay volume in the TEG cup was 360  $\mu$ l which included 20  $\mu$ l of 0.2 molar  $\text{CaCl}_2$ , 36  $\mu$ l of CCTs suspended in PBS at concentration  $10^6$  cells per milliliter, and 304  $\mu$ l of citrated whole blood.  $\text{CaCl}_2$  was added to the TEG cup first followed by the CCTs and blood was added last. The sample was mixed twice using a pipette before starting the assay. The final molarity of  $\text{CaCl}_2$  was 0.01 molar and concentration of CCTs was  $10^5$  cells per milliliter. Control assays had an identical volume of PBS vehicle instead of CCT suspension added to each sample. Each sample was run in duplicate and values for TEG parameters were averaged. For dilution experiments, stock preparations at  $10^6$  cells per milliliter were diluted with PBS to concentrations of  $10^5$  and  $10^4$  cells per milliliter for final concentrations of  $10^4$  and  $10^3$  cells per milliliter, respectively.

### Calibrated Thrombogram

CAT is a micro-well assay performed in 96-well plates that measures thrombin generation in platelet poor plasma (PPP). Primary metrics include lag time which reflects time to initial thrombin production, peak height which reflects the maximum amount of thrombin produced, time to peak which reflects the time to peak height, and endogenous thrombin potential which reflects the total amount of thrombin produced.

To prepare PPP, whole blood was collected through approved IRB protocol HSC-MS-09-0314 from eight healthy volunteers into 4.5 ml vacutainers containing 3.2% sodium citrate. Samples were centrifuged for 10 minutes at 200g and platelet rich plasma supernatant was centrifuged again for 10 minutes at 2,000g to obtain PPP. Samples from each donor were pooled and aliquoted. CAT was performed using a

**Table 1.** Mesenchymal markers of AF MSC, ADP MSC, BM MSC, UMB MSC, and BM MNC

	7AAD	CD105	CD73	CD34	CD45	CD90	HLA DR	CD44	CD29	CD31	CD142	APN
AF MSC	88	++	+++	-	-	+++	-	+++	+++	-	+++	3
ADP MSC	99	++	+++	-	-	+++	-	+++	+++	-	+++	1
BM MSC	86	+++	+++	-	-	+++	-	+++	+++	-	++	3
UMB MSC	89	++	+++	-	-	+++	-	+++	+++	-	++	1
BM MNC	87	-	-	-	-	+	-	+++	++	++	+	1

“+++” indicates the marker is highly expressed, “++” moderately expressed, “+” expressed and “-” indicates not detected. Abbreviation: APN, average passage number.

Calibrated Automated Thrombogram (Diagnostica Stago, Parsippany NJ). In a 96-well plate, 20  $\mu$ l of phospholipid reagent was added to 72  $\mu$ l of PPP and either 8  $\mu$ l of CCTs suspended in PBS at concentration  $10^6$  cells per milliliter or PBS vehicle. Thrombin generation was initiated with 20  $\mu$ l of 0.1 M calcium chloride and fluorogenic substrate solution. Samples were performed in duplicate. Reactions were measured using a Fluoroskan Ascent plate reader (ThermoFisher, Waltham, MA).

### Fluorescence-Activated Cell Sorting

AF and BM MSC samples were thawed and resuspended in PBS as above. Staining for CD142 (TF) was performed in an identical manner as preparation for flow cytometry and 7AAD was added to measure cell viability. Cells were sorted into different vials based on TF expression using a BD FACS Aria II (BD Biosciences, San Jose, CA). Sorting was gated for the top and bottom 8% of TF expressing cells; creating populations of the lowest and highest TF expressing cells from the same sample. Sorted cells were resuspended and delivered at the standard experimental concentration of  $10^5$  cells per milliliter for functional coagulation testing.

### Inhibition of Clinical Cellular Therapeutic Associated Tissue Factor

TF and factor VIIa together form a proteolytically active complex on cell surfaces that activates factors IX and X. The monoclonal antibody (MAb) TF8-5G9 binds to the TF/VIIa complex and prevents its catalytic function [18]. Increasing concentrations of this MAb were added to AF, ADP, BM MSCs, and BM MNCs and incubated at room temperature for 20 minutes then added as described above to TEG. The MAb was not washed away before the addition of the cell suspensions to TEG.

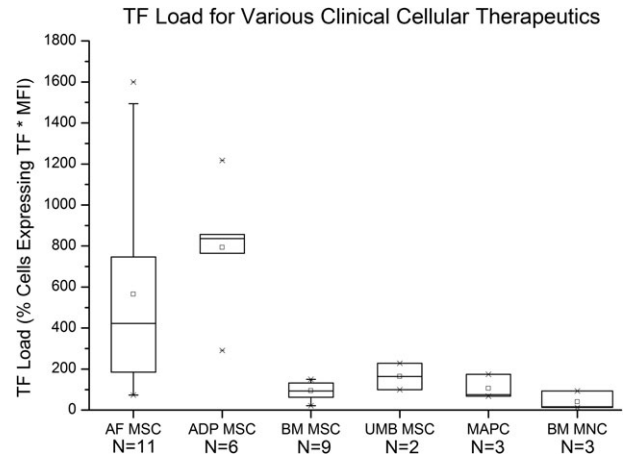
### Data Analysis

TF load and percentage change in TEG R time are presented as box-and-whisker plots demonstrating the median, the 25th and 75th percentile and outliers for each cell type. Statistical differences between TF load of CCTs were determined using analysis of variance (ANOVA) with Tukey post hoc analysis. Differences in clotting times after FACS and treatment with the neutralizing MAb were determined using a Student's *t* test. Correlation between TF load and functional coagulation tests was determined with Pearson product moment correlations. Statistical significance was set to  $p < .05$ . Analysis was performed using Stata 14.2 (StataCorp LLC, College Station, TX) and graphically presented using Origin 8 (OriginLab Corp., Northampton, MA).

## RESULTS

### Clinical Cellular Therapeutic Tissue Factor Load Varies with Tissue Source

TF load was calculated for 33 CCT donors from six different tissue sources. TF load is the product of cells expressing CD142 (TF) and the MFI from flow cytometry as an approximation for the total amount of TF present in a reaction (Fig. 1). Representative histogram plots of flow cytometry for each tissue source are included as a Supporting Information Figure S1. In general, TF load for the 11 AF MSC samples was highly variable with a mean and standard deviation of  $565 \pm 526$  relative units (RU), despite the MSCs displaying similar expression profiles of



**Figure 1.** TF load varies for each tissue source. TF load is the product of percent cells expressing TF and the mean fluorescent intensity of the sample measured by flow cytometry. Abbreviations: AF MSC, amniotic fluid mesenchymal stromal cell; ADP MSC, adipose mesenchymal stromal cell; BM MSC, bone marrow mesenchymal stromal cell; UMB MSC, umbilical cord mesenchymal stromal cell; MAPC, multipotent adult progenitor cell. BM MNC, bone marrow mono-nuclear cell.

common surface markers (Table 1). TF load for the five ADP MSC samples was less variable with one high and low extreme. ADP MSC TF load mean and standard deviation was  $793 \pm 331$  relative units. The remaining four tissue sources (BM MSC, UMB MSC, MAPC, and BM MNC) demonstrated consistently lower TF load without any outliers. All cell samples were from third passage or earlier of cell culture. TF load of BM MSC, MAPC, and BM MNC were significantly different from AF and ADP MSC.

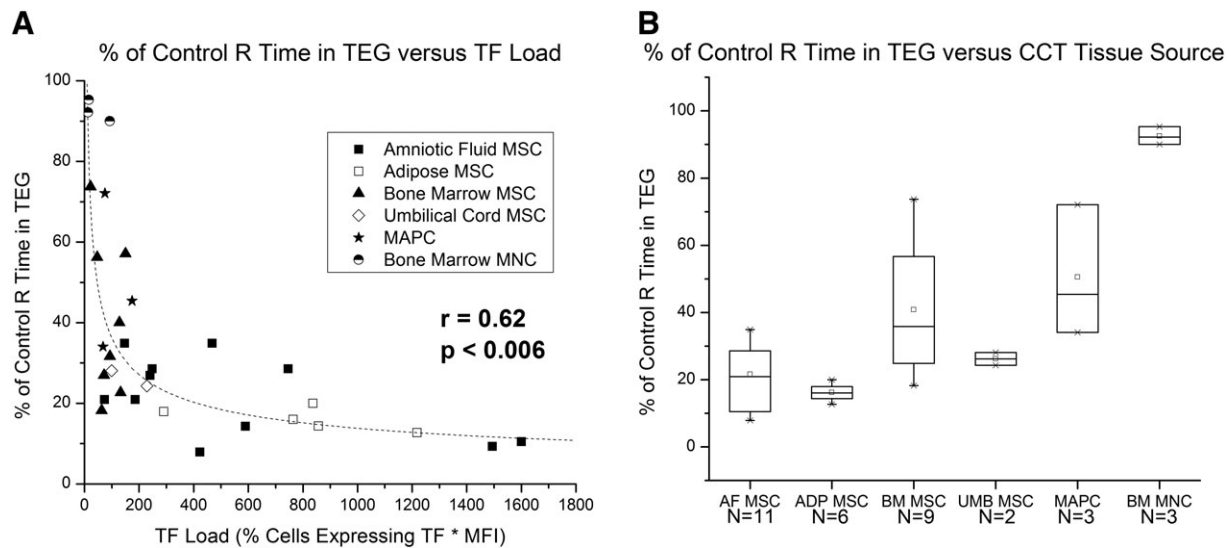
### Clinical Cellular Therapeutic TF Load Correlates with Accelerated Clot Formation and Thrombin Production

TEG R time, a measure of when fibrin formation begins, decreased as TF load of the MSC sample increased (Fig. 2A). For samples with very low TF load like BM MNCs, R time is very near control values. However for samples with high TF load like AF or ADP MSC, R time is as low as 10% of control values. A Pearson's product-moment correlation between TEG R time and TF load calculated an *r* value of .62 with  $p < .006$ . ADP MSC, UMB MSC, and BM MNC demonstrate small variation in their procoagulant effect compared with AF MSC, BM MSC, and MAPC (Fig. 2B).

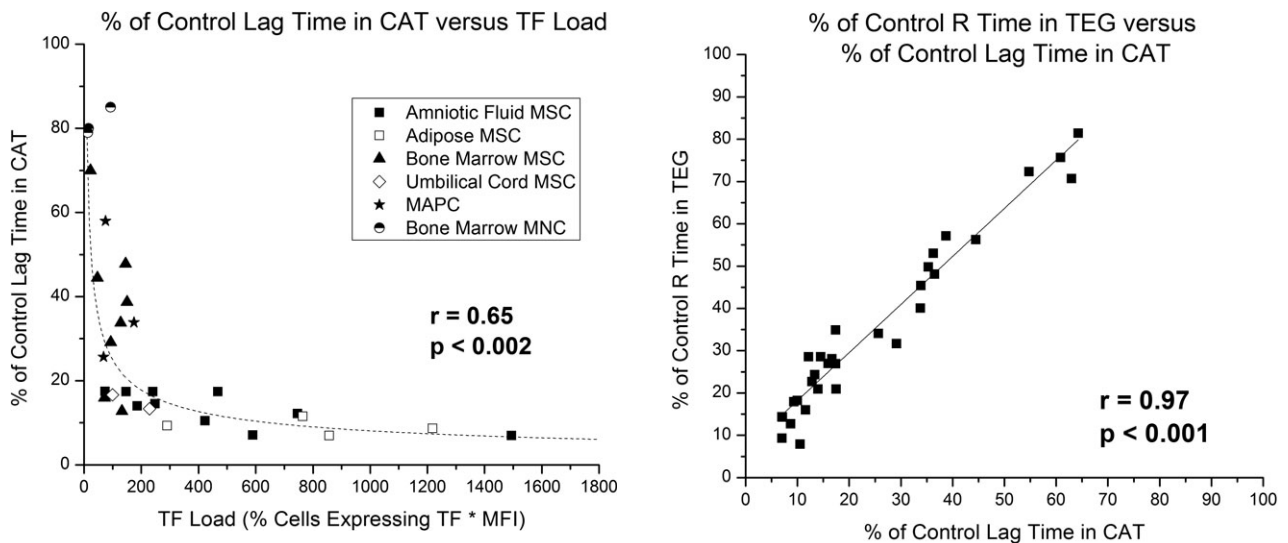
Consistent with our observations in TEG, CAT lag time, a measure of when thrombin formation is first detected, decreased as TF load increased (Fig. 3). When the results of the TEG and CAT assays are directly compared, R time in TEG and lag time in CAT directly correlate linearly (Fig. 4) across all the CCTs, cross-validating the results of the different assays.

### Clinical Cellular Therapeutic TF Expression is an Independent Determinant of the Procoagulant Effect

CCTs sorted based on their TF expression using FACS have different effects on coagulation parameters in TEG. In a sample of BM MSCs, the lower 8% of cells expressing TF decreased R time to 81% of control while the upper 8% of cells expressing TF decreased R time to 72% of control. In a sample of AF MSCs, the corresponding R time decrease was 64% of control



**Figure 2.** Panel represents Pearson correlation between TEG R time and TF load (A). R time is expressed as percent of control. (B): Box-and-whisker plots demonstrating percent of control R time after treatment with different CCTs.



**Figure 3.** Pearson correlation between CAT lag time and TF load. Lag time is expressed as percent of control.

**Figure 4.** TEG R time and CAT lag time correlate closely when varying CCT associated TF. Both metrics are expressed as percent of controls.

for low TF expressing cells and 51% of control for high TF expressing cells (Fig. 5). Changes in R time after FACS between specific cell treatments were all significantly different from each other. Of note, the antibody used to sort the cells using FACS partially inhibits the procoagulant effect of the MSC TF. Without antibody stain the unsorted BM MSC sample demonstrated an R time that was 56% of control and the unsorted AF MSC sample was 24% of control. These results demonstrate the direct correlation of TF with coagulation.

### The Procoagulant Effect of Clinical Cellular Therapeutic Associated TF Is Reversible

A subset of the cell samples were chosen for further investigation based on availability and to assay a wide range of TF expression. ADP MSCs and AF MSCs were chosen because they demonstrate high TF load while BM MSC and BM MNC were chosen because they demonstrate lower TF load. Samples were

incubated with a TF neutralizing monoclonal antibody (Mab) to confirm that CCT-associated TF activity was directly contributing to the procoagulant effect. Increasing concentrations of the neutralizing Mab inhibited the procoagulant effect of all four MSC tissue types in a dose-dependent manner. The effect of the neutralizing Mab reaches a steady state for all specimens at 200  $\mu\text{g}/\text{ml}$  (Fig. 6A). At this concentration of Mab, TEG R time returned to control values in assays run with ADP MSCs and BM MNCs. In contrast, assays run with AF or BM MSCs at this concentration of Mab in TEG demonstrated an R time restored to only 45% and 65% of controls, respectively (Fig. 6B).

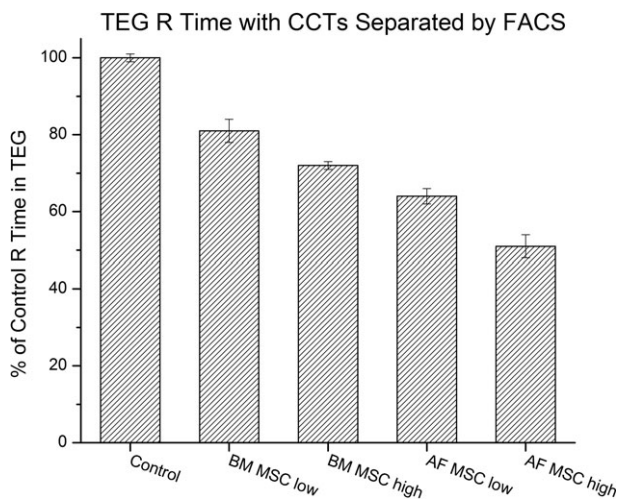
### Diluting Clinical Cellular Therapeutic Concentration Decreases the Procoagulant Effect

All previously described assays utilized a standardized CCT assay concentration of  $10^5$  cells per milliliter. To investigate the

procoagulant effect as a function of cell number, select samples of ADP MSC, AF MSC, and BM MSC were assayed in TEG as previously described at concentrations of  $10^5$ ,  $10^4$ , and  $10^3$  cells per milliliter (Fig. 7). These specific cell types were chosen to demonstrate a broad range of TF expression. The TF load for the ADP MSC sample was 1,218, for the AF MSC was 240, and for the BM MSC 150. As CCT preparations were diluted, the procoagulant effect decreased in a logarithmic, dose-dependent fashion. For each order of magnitude dilution, relative R time in TEG increased on average by 20% regardless of tissue type.

## DISCUSSION

Our data demonstrate a causal relationship of CCT associated TF with clot formation and acceleration of thrombin

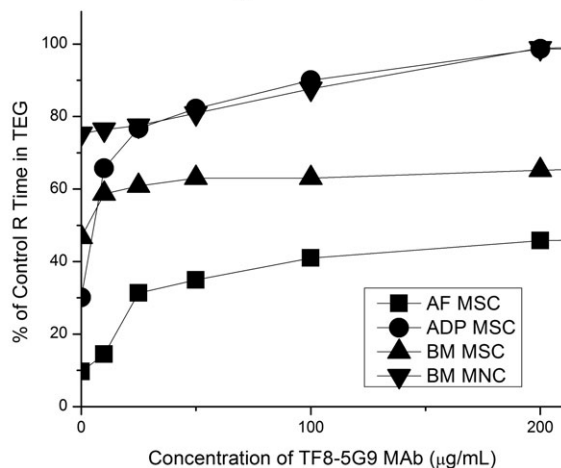


**Figure 5.** The procoagulant effect of MSCs is variable when cells are sorted based on TF expression. High TF expressing populations will have a larger decrease in TEG R time than lower TF expressing populations compared with controls.

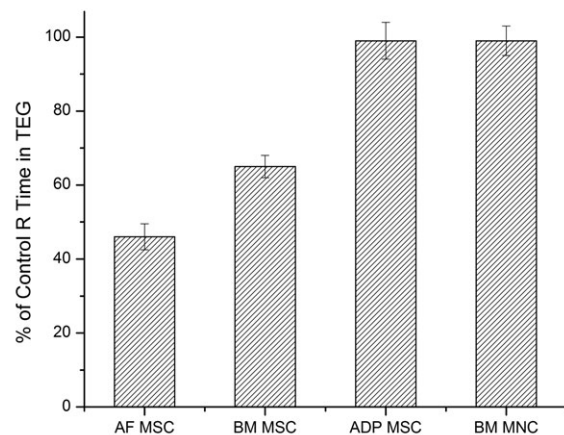
production in human blood. CCTs are functionally procoagulant depending upon the density of TF on their surface and the percentage of cells expressing TF in a given cell preparation. In TEG, R time was decreased compared with controls as TF dose increased, regardless of CCT tissue type. In CAT, time to thrombin formation relative to controls was similarly decreased as TF dose increased. These methods and findings are similar to previous study by Christy et al. [9] and we expand upon their findings by broadly surveying different tissue sources of CCTs. In addition, we demonstrate that high TF expressing cells will speed time to clot formation more than low TF cells when sort from single CCT donors. CCT TF expression was also demonstrated to be directly involved in coagulation, as a TF blocking antibody significantly reduced their procoagulant effects. In some cases, blocking TF alone was sufficient to completely reverse the effects of the cells, while other CCTs demonstrated signs of additional mechanisms contributing to coagulation. Finally, diluting concentration of CCT treatments decreased the procoagulant effect in a dose-dependent fashion regardless of tissue type.

The procoagulant effect from MSCs must be considered when treating patients and designing clinical trials. MSC associated TF has the potential to create a hypercoagulable state and lead to adverse events like venous-thromboembolism during or after systemic therapy. Certain tissue sources of CCTs express more TF than others in our study—AF and ADP MSC specifically express much higher loads of TF compared with other tissue sources, such as BM MSC, UMB MSC, MAPC, or BM MNCs. Heparin has been shown as an effective method of preventing the CCT procoagulant effect in *in vitro* human studies [7] and *in vivo* animal studies [10,19,20]. In an *in vivo* human study, Baygan et al. [21] demonstrated a successful anticoagulation protocol using heparin for the delivery of decidual stromal cells for hemorrhagic cystitis and graft versus host disease after allogeneic hematopoietic stem cell transplantation. We demonstrate that simple dilution of CCT treatments will decrease the procoagulant effect regardless of tissue type.

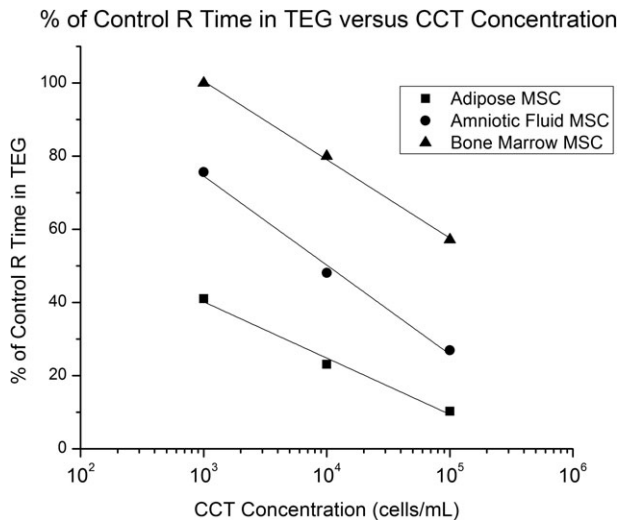
**A** Effect of TF Neutralizing MAb on CCT Procoagulant Activity



**B** Restoration to Control R Time in TEG with TF MAb



**Figure 6.** The procoagulant effect of MSCs is reversible with a TF neutralizing MAb. **(A):** As concentration of the TF8-5G9 MAb increases, the procoagulant effect of all MSCs decreases and eventually reaches a steady-state. **(B):** The procoagulant effect of ADP and BM Derived MSC is reversed at 200 µg/ml of the MAb. The procoagulant effect of AF and BM MSC is only partially reversed.



**Figure 7.** The procoagulant effect of MSCs is decreased when cell concentration is diluted. Logarithmic curves applied to each sample demonstrate excellent fit with  $R^2 > 0.96$ .

Decreased R time in TEG has established clinical relevance in multiple patient populations. These studies demonstrate that functional coagulation assays are predictors of VTE in multiple patient populations. In a cohort of 61 trauma patients, Van et al. [22] demonstrated a significantly lower R time (6.1 vs. 7.6 minutes,  $p < .01$ ) in patients that developed a deep vein thrombus (DVT) despite anticoagulation with low molecular weight heparin. In a population of 49 ischemic stroke patients, Elliot et al. [23] demonstrated a significantly lower R time (4.8 vs. 6.0 minutes,  $p < .0004$ ) in the stroke cohort compared with normal controls. In a population of 24 burn patients, Van Haren et al. [24] demonstrated a significantly lower R time upon admission (4.4 vs. 12.3 minutes,  $p < .022$ ) in those patients that developed a DVT. Rotational thromboelastometry (ROTEM) is a widely used functional coagulation assay of clinical importance similar to TEG. Hincker et al. demonstrated faster clot formation time (CFT) measured by ROTEM in major non-cardiac surgery patients who developed a VTE. Ten out of 313 enrolled patients developed a thromboembolic complication before discharge [25]. The primary finding of our study, that MSC treatments decrease TEG R time dependent on their TF load, must be considered relevant in the overall context of clinical VTE risk.

Time to thrombin generation and time to thrombin peak in CAT is a predictor of venous thromboembolism after trauma. Park et al. identified CAT lag time and time to thrombin peak as risk factors for development of venous thromboembolism in patients after acute trauma, HR 1.66 (1.06, 2.60)  $p < .028$  and HR 2.02 (1.45, 2.80)  $p < .001$ , respectively. They conclude that CAT is potentially a useful clinical tool in predicting the occurrence of VTE after injury [26]. CAT is advantageous because it is not confounded by cellular components of blood and is specific for thrombin production. This is in contrast to TEG, which is typically an assay performed with whole blood reflecting parallel coagulation processes like fibrin formation, platelet contraction and fibrinolysis [27]. However, CAT is not a standard clinical testing platform compared with an assay like TEG. In our study, we demonstrate that CAT lag time correlates linearly with TEG R time when varying TF dose. CAT lag time marks the moment thrombin

production begins and TEG R time marks the moment fibrin formation begins. Thus, TEG R time is a potential surrogate for measuring kinetics of thrombin production for the purposes of VTE prediction.

Certain MSC tissue sources like AF and BM express procoagulant activity that is not reversed with a TF neutralizing antibody. The cause of this residual procoagulant activity is unknown but may be related to activation of the complement system, inhibition of fibrinolysis, negatively charged extra-cellular membrane components like collagen, or incomplete neutralization of TF by the TF neutralizing antibody. MSC exposure to whole blood leads to activation of the complement system and generation of the chemotactic anaphylatoxins C3a and C5a [28,29]. Proinflammatory cytokines like C3a and C5a have been proposed to inactivate native anticoagulants protein C and S [30,31]. MSCs also express plasminogen activator inhibitor-1 (PAI-1). PAI-1 inhibits serine proteases like tissue or urokinase plasminogen activator which ultimately blocks formation of plasmin and reduces clot fibrinolysis. BM MSCs have been shown to express high levels of PAI-1; however, correlation with a procoagulant effect is not yet established [32]. Collagen is an important mediator of the FXII dependent contact activation pathway. BM MSCs express this procoagulant protein in addition to TF. Moll et al. [7,8] demonstrated high levels of collagen on BM MSC, especially with prolonged ex vivo expansion. Finally, there are a number of other agents to experimentally block the procoagulant activity of TF. For example, active site-inhibited factor VIIa (FVIIai) binds to TF and blocks formation of active FVIIa [8]. This protein could potentially be more efficacious than the neutralizing antibody used in this study, however comparative studies are still needed.

A counter argument to the potential clinical significance of these data is that thousands of patients have had MSCs infused without notable adverse events. One possible explanation is the variability of TF expression. Another possibility is that many patients in the large clinical trials of myocardial infarction, stroke, and acute respiratory distress syndrome (ARDS) are often already systemically anticoagulated. Similarly, beneficial effects of MSCs may be negated by procoagulant properties when delivered intravenously. These data document an augmentation of the procoagulant response ex vivo. A limitation is that we are not testing patient TEG and CAT data after infusing various MSC products. However, due to the similarity to data published by Moll et al. [7,8], we believe these data are representative of the in vivo circumstances. It has yet to be determined if TF expression can be dissociated from MSC immunomodulatory properties and paracrine effects, but the variability in expression across cell types that have proven efficacious in preclinical models suggest that TF is not required for MSC immunomodulation.

Areas in need of further study include the effects of cell culture conditions and cell passage number on CCT procoagulant potential. In addition, repeating this study with blood samples from patient populations that actually receive CCTs like stroke or trauma patients is needed to fully understand the clinical relevance of the procoagulant effect.

## CONCLUSION

Previous studies have established a relationship between CCT TF expression and a procoagulant response. Our study

confirms these findings in six different CCT sources. In addition, we found evidence of an additional procoagulant mechanism other than TF in amniotic fluid and bone marrow MSC samples. TF expression should be considered as a safety release criterion for systemic cellular therapies.

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#### AUTHOR CONTRIBUTIONS

M.J.G.: Conception and design, collection of data, data analysis, manuscript writing; K.P. and N.E.T.: Collection of data, data analysis, manuscript writing; Y.W.: Collection of data, data analysis; B.S.G., C.E.W., and S.D.O.: Conception and design,

data analysis, final approval of manuscript; C.S.C.: Conception and design, final approval of manuscript, provision of study material.

#### DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

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#### REFERENCES

- Drake TA, Morrissey JH, Edgington TS. Selective cellular expression of tissue factor in human tissues. Implications for disorders of hemostasis and thrombosis. *Am J Pathol* 1989;134:1087–1097.
- Walker PA, Shah SK, Jimenez F et al. Intravenous multipotent adult progenitor cell therapy for traumatic brain injury: Preserving the blood brain barrier via an interaction with splenocytes. *Exp Neurol* 2010;225:341–352.
- Pati S, Gerber MH, Menge TD et al. Bone marrow derived mesenchymal stem cells inhibit inflammation and preserve vascular endothelial integrity in the lungs after hemorrhagic shock. *PLoS One* 2011;6:e25171.
- Mezey E, Chandross KJ, Harta G et al. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* 2000;290:1779–1782.
- Weiss DJ, Bertonecello I, Borok Z et al. Stem cells and cell therapies in lung biology and lung diseases. *Proc Am Thorac Soc* 2011;8:223–229.
- Walker PA, Shah SK, Jimenez F et al. Bone marrow-derived stromal cell therapy for traumatic brain injury is neuroprotective via stimulation of non-neurologic organ systems. *Surgery* 2012;152:790–793.
- Moll G, Ignatowicz L, Catar R et al. Different procoagulant activity of therapeutic mesenchymal stromal cells derived from bone marrow and placental decidua. *STEM CELLS DEV* 2015;24:2269–2279.
- Moll G, Rasmusson-Duprez I, von Bahr L et al. Are therapeutic human mesenchymal stromal cells compatible with human blood? *STEM CELLS* 2012;30:1565–1574.
- Christy BA, Herzig MC, Montgomery RK et al. Pro-coagulant activity of human mesenchymal stem cells. *J Trauma Acute Care Surg* 2017;83:S164–S169.
- Gleeson BM, Martin K, Ali MT et al. Bone marrow-derived mesenchymal stem cells have innate procoagulant activity and cause microvascular obstruction following intracoronary delivery: Amelioration by antithrombin therapy. *STEM CELLS* 2015;33:2726–2737.
- Kota DJ, Prabhakara KS, Toledano-Furman N et al. Prostaglandin E2 indicates therapeutic efficacy of mesenchymal stem cells in experimental traumatic brain injury. *STEM CELLS* 2017;35:1416–1430.
- Fischer UM, Harting MT, Jimenez F et al. Pulmonary passage is a major obstacle for intravenous stem cell delivery: The pulmonary first-pass effect. *STEM CELLS DEV* 2009;18:683–692.
- Roobrouck VD, Clavel C, Jacobs SA et al. Differentiation potential of human postnatal mesenchymal stem cells, mesoangioblasts, and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions. *STEM CELLS* 2011;29:871–882.
- Burrows GG, Van't Hof W, Newell LF et al. Dissection of the human multipotent adult progenitor cell secretome by proteomic analysis. *STEM CELLS TRANSLATIONAL MEDICINE* 2013;2:745–757.
- Booser S, Lehman N, Lakshminpathy U et al. Global characterization and genomic stability of human pluripotent stem cells. *J Stem Cells* 2009;4:17–28.
- Aranda P, Agirre X, Ballestar E et al. Epigenetic signatures associated with different levels of differentiation potential in human stem cells. *PLoS One* 2009;4:e7809.
- Jiang Y, Vaessen B, Lenvik T et al. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol* 2002;30:896–904.
- Ruf W, Edgington TS. An anti-tissue factor monoclonal antibody which inhibits TF.VIIa complex is a potent anticoagulant in plasma. *Thromb Haemost* 1991;66:529–533.
- Stephenne X, Nicastro E, Eeckhoudt S et al. Bivalirudin in combination with heparin to control mesenchymal cell procoagulant activity. *PLoS One* 2012;7:e42819.
- Liao L, Shi B, Chang H et al. Heparin improves BMSC cell therapy: Anticoagulant treatment by heparin improves the safety and therapeutic effect of bone marrow-derived mesenchymal stem cell cytotrophy. *Theranostics* 2017;7:106–116.
- Baygan A, Aronsson-Kurttila W, Moretti G et al. Safety and side effects of using placenta-derived decidual stromal cells for graft-versus-host disease and hemorrhagic cystitis. *Front Immunol* 2017;8:795.
- Van PY, Cho SD, Underwood SJ et al. Thrombelastography versus AntiFactor Xa levels in the assessment of prophylactic-dose enoxaparin in critically ill patients. *J Trauma* 2009;66:1509–1515. discussion 1515–1517.
- Elliott A, Wetzel J, Roper T et al. Thromboelastography in patients with acute ischemic stroke. *Int J Stroke* 2015;10:194–201.
- Van Haren RM, Thorson CM, Valle EJ et al. Hypercoagulability after burn injury. *J Trauma Acute Care Surg* 2013;75:37–43. discussion 43.
- Hincker A, Feit J, Sladen RN et al. Rotational thromboelastometry predicts thromboembolic complications after major non-cardiac surgery. *Crit Care* 2014;18:549.
- Park MS, Spears GM, Bailey KR et al. Thrombin generation profiles as predictors of symptomatic venous thromboembolism after trauma: A prospective cohort study. *J Trauma Acute Care Surg* 2017;83:381–387.
- Katori N, Tanaka KA, Szlam F et al. The effects of platelet count on clot retraction and tissue plasminogen activator-induced fibrinolysis on thrombelastography. *Anesth Analg* 2005;100:1781–1785.
- Moll G, Jitschin R, von Bahr L et al. Mesenchymal stromal cells engage complement and complement receptor bearing innate effector cells to modulate immune responses. *PLoS One* 2011;6:e21703.



**29** Moll G, Alm JJ, Davies LC et al. Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *STEM CELLS* 2014;32:2430–2442.

**30** Taylor FB Jr. Studies on the inflammatory-coagulant axis in the baboon response

to *E. coli*: Regulatory roles of proteins C, S, C4bBP and of inhibitors of tissue factor. *Prog Clin Biol Res* 1994;388:175–194.

**31** Levi M, van der Poll T, ten Cate H et al. The cytokine-mediated imbalance between coagulant and anticoagulant mechanisms in

sepsis and endotoxaemia. *Eur J Clin Invest* 1997;27:3–9.

**32** Hogan NM, Joyce MR, Murphy JM et al. Impact of mesenchymal stem cell secreted PAI-1 on colon cancer cell migration and proliferation. *Biochem Biophys Res Commun* 2013;435:574–579.



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