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The effect of platelet lysate fibrinogen on the functionality of MSCs in immunotherapy

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ABSTRACT

Human platelet lysate (PL) represents an attractive alternative to fetal bovine serum (FBS) for the *ex vivo* expansion of human mesenchymal stromal cells (MSCs). However, there is controversy whether MSCs propagated in unfractionated PL retain their immunosuppressive properties. Since fibrinogen can be a major component of PL, we hypothesized that the fibrinogen content in PL negatively affects the suppressor function of MSCs. Pools of outdated plateletpheresis products underwent a double freeze–thaw centrifugation and filtration to produce unfractionated platelet lysates (uPL), followed by a temperature controlled clotting procedure to produce a fibrinogen depleted platelet lysate (fdPL). Fibrinogen depletion affected neither the mitogenic properties of PL or growth factor content, however fdPL was less prone to develop precipitate over time. Functionally, fibrinogen interacted directly with MSCs, dose dependently increased IL-6, IL-8 and MCP-1 protein production, and compromised the ability of MSCs to up-regulate indoleamine dioxygenase (IDO), as well as, mitigate T-cell proliferation. Similarly uPL expanded MSCs showed a reduced capability of inducing IDO and suppressing T-cell proliferation compared to FBS expanded MSCs. Replacing uPL with fdPL largely restored the immune modulating effects of MSCs. Together these data suggest that fibrinogen negatively affects the immunomodulatory functions of MSCs and fdPL can serve as non-xenogenic mitogenic supplement for expansion of clinical grade MSCs for immune modulation.

1. Introduction

Despite the rarity of MSCs in the bone marrow, their pro-angiogeneic [1], immune modulatory features [2] and their expandability *ex vivo* makes them clinically useful [3]. Presently there have been over 200 registered clinical trials using MSCs worldwide (http://clinicaltrials.gov, search was performed using most known names of MSCs). Most of these studies targeted immune-related disorders (multiple sclerosis, graft versus host disease, Crohn's disease), cardiovascular conditions (myocardial infarction, ischemia) and orthopedic reconstruction, but pulmonary and neurological conditions are also under investigation.

In manufacturing MSCs, the majority of laboratories utilize serum-like supplements (whether human or animal origin) as a critical component for their *ex vivo* expansion. However with the potential for zoonotic transmission [4], reports of increased immunogenicity [5] and documented cases of anaphylactoid responses after implantation [6] the use of xenogeneic material, like fetal bovine serum (FBS), to clinically expand MSCs has spurred the development of alternatives. One such alternative is to expand human MSCs in human platelet lysate [7]. Platelets are small irregularly shaped enucleated (containing no DNA) cell fragments, derived from precursor megakaryocytes. Platelets are present in blood at high concentrations (150,000–50,000/μL, representing 0.1–0.25% (w/w) of the blood), normally persist approximately 5–9 days in the circulation and are primarily responsible for hemostasis. Platelets are also the primary source of a number of growth factors [8,9], attachment factors, and enzymes found in serum. In the past decade numerous studies have shown that platelet lysate is superior to FBS and serum for expanding MSCs *in vitro* [7,10–12],

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however there are conflicting data regarding the impact platelet lysate has on MSCs functionality. Several investigators have shown that MSCs grown in PL retain their immunosuppressive capabilities [11,13], whereas others have suggested that platelet lysate expanded MSCs have inferior immunosuppressive qualities [14,15]. In surveying the platelet lysate manufacturing protocols, we found that in studies where PL was generated from buffy coats and suspended in AB serum, PL expanded MSCs retained their immunosuppressive capabilities. Conversely in those MSCs where immunosuppressive capabilities were compromised, PL was derived from platelet concentrates which typically use donor plasma and 10% acid citrate dextrose (ACD) as part of the suspension medium.

Given that serum is essentially the liquid remnant of clotted plasma, we hypothesized that PL generated from platelet concentrates contain elements that are detrimental to MSCs. Based upon the literature [16] we reasoned that fibrinogen could be a candidate plasma component that might negatively affect the immunosuppressive properties of PL. Fibrinogen is a 340 kDa glycoprotein, primarily synthesized by hepatocytes. Fibrinogen circulates as a component of blood at a concentration of approximately 7 uM with a half-life of around 100 h [16]. Fibrinogen and its cleavage products have well described roles in hemostasis where fibrin forms a clot limiting blood loss and provides a key substrate of the provisional matrix which is vital for normal repair. Fibrinogen and its cleavage products are also recognized as being capable of altering vasoconstriction, angiogenesis, cell migration and proliferation in fibroblasts, smooth muscle cells and lymphocytes [17–21]. Therefore the presence of fibrinogen in platelet lysate generated from platelet concentrates has the potential to impact the behavior of *ex vivo* expanded hMSCs. In this study we hypothesized that depletion of fibrinogen from platelet lysate would yield a superior product for expanding human MSCs for use in immunomodulation therapy. To test this hypothesis we optimized a method to deplete fibrinogen from platelet concentrates/plateletpheresis products (fdPL) which restored the immunosuppressive activity of *ex vivo* expanded MSCs compared to non-fibrinogen depleted PL (uPL) and does so without compromising the desirable growth promoting characteristics of PL.

2. Materials and methods

2.1. Manufacturing of platelet lysate

At the Emory University Hospital blood bank plateletpheresis products are purchased from the American Red Cross (ARC) and meet all AABB & FDA regulatory requirements for sterility and infectious disease screening for transfusion products. Upon outdating of plateletpheresis products, for human infusion, we have obtained American Red Cross consent and an Emory IRB waiver to use these products to generate platelet lysate. To generate human phPL we employed a freeze–thaw procedure to ensure proper fracturing of platelet membranes for intracellular growth factor release. For each lot of PL, approximately five outdated plateletpheresis products were removed from the freezer and thawed at 4 °C. Each unit (approximately 200–250 mL) was then aliquoted into smaller volumes (approximately 20–25 mL) for re-freezing at −80 °C. Thawed platelet units were individually filtered through a 40 μm *PALL* blood transfusion filter (PALL BIOMEDICAL, INC USA) and allowed to pool into a collection bag. Pooled, filtered lysate was equally aliquoted into labeled 250 ml conical tubes (Corning® Lowell, MA USA) and centrifuged for 20 min at 4000 × *g* at room temperature. Spun lysate was again filtered (40 μm), aliquoted into 250 ml conical tubes, and either mixed with $CaCl_2 \pm 2$ U/ml heparin in their respective concentrations (fdPL) or progressively filtered using a 0.2 μm pore size (uPL). $CaCl_2 \pm$ heparin supplemented lysate was incubated for 1 h at 37 °C/CO_2/

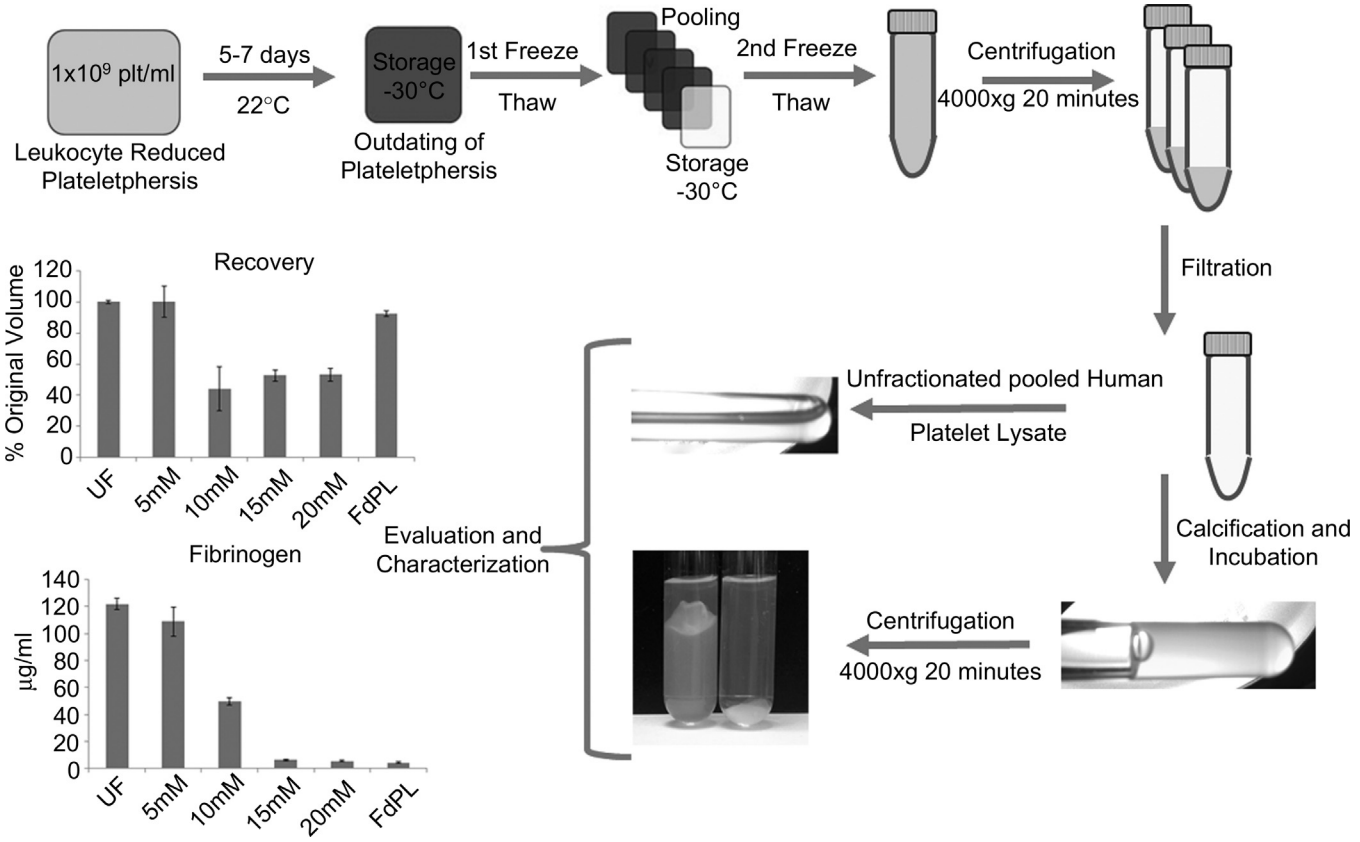

Fig. 1. Schematic representation of manufacturing process for generation of platelet lysate. Outdated plateletpheresis units are frozen at −30 °C thawed then pooled with 5–7 additional units and refrozen. After a second thawing, cell membranes and debris are removed by centrifugation and filtration to 0.2 μm. Unfractionated pooled human platelet lysate then underwent a series of calcium and temperature dependent clotting procedures which yielded varying degrees of supernatant recovery and fibrinogen depletion. $N = 4$ per group; $^{\#}p < 0.05$; NS = $p > 0.05$.

Table 1
Conditions evaluated to for maximal fibrinogen depletion and supernatant recovery.

Condition	2 h Temperature	12 h Temperature	Heparin	Recovery (%)	Standard deviation (SD)
5 mM	22			100	0
10 mM	22			58	3.11127
15 mM	22			52.6	3.394113
20 mM	22			52.95	10.53589
5 mM	37			100	0
10 mM	37			55.6	6.646804
15 mM	37			44.2	5.939697
20 mM	37			42.65	3.747666
5 mM		22		77.5	3.535534
10 mM		22		66.8	4.525483
15 mM		22		42.85	3.464823
20 mM		22		48	2.828427
5 mM		37		100	0
10 mM		37		53.05	3.606245
15 mM		37		50.2	7.353911
20 mM		37		55	7.071068
5 mM	37		2 U/ml	100	0
10 mM	37		2 U/ml	82.5	3.535534
15 mM	37		2 U/ml	79.4	0.848528
20 mM	37		2 U/ml	79.9	1.838478
5 mM	37	4	2 U/ml	100	0
10 mM	37	4	2 U/ml	88.75	1.767767
15 mM	37	4	2 U/ml	85	3.535534
20 mM	37	4	2 U/ml	86.25	5.303301

95% humidity then transferred and stored at 4 °C overnight to allow a fibrin clot to stabilize. The following day, fdPL samples were spun for 20 min at 4000 × *g* in room temperature, filtered to 0.2 μm, aliquoted and stored at −80 °C until use.

2.2. Preparation of human serum and platelet poor plasma

Human peripheral blood was collected from healthy volunteers in standard blood collection tubes. For serum preparation blood was collected in and placed in an upright position for 2 h at room temperature to ensure complete clotting. After clotting the serum was separated by centrifugation at 4000 × *g* for 10 min. For platelet poor plasma (PPP) blood was collected in heparinized tubes and immediately centrifuged at 2000 × *g* for 10 min to pellet all cells. The supernatant was collected and subjected to an additional spin of 4000 × *g* for 10 min to generate PPP.

2.3. Metabolic profiling

To compare the basic characteristics of serum, PPP, and PL, aliquots from each were prepared and sent to the Emory University Hospital core lab for a standard blood chemistry panel and osmolality test. Rapid, multichemistry profiles were performed by adding the media to reagent discs. A disc containing a general chemistry panel of analytes was used for these studies as was a disc providing specific liver function panel. Both of these discs provided measurements for AST, ALT, alkaline phosphatase (ALP), amylase (AMY), albumin (ALB), total protein (TP), and total bilirubin (TBIL), but only the general chemistry disc included blood urea nitrogen (BUN), creatinine (CRE), glucose (GLU), calcium (CA++), and cholesterol (CHOL) assays.

2.4. Analysis of protein content in platelet lysate

According to the manufacturer's protocol (RayBiotech® GA, USA) we incubated membranes with equal amounts of total protein (10 mg/ml) from normal human serum, platelet poor plasma, uPL and fdPL. Proteins were diluted in α-MEM then incubated with nylon membranes impregnated with antibodies specific to human cytokines (RayBio® Human Cytokine Antibody Array C Series 4000) for 2 h at room temperature with constant agitation. Membranes were then washed and incubated for an additional 2 h with array specific cocktail of biotinylated antibodies followed by incubation with an HRP-conjugate Streptavidin solution for 1 h. After washing the proteins were revealed by chemiluminescence using Kodax X-ray film. Films were then scanned at 600 dpi as Tiff images and analyzed using Image J to determine relative expression levels.

2.5. Human mesenchymal stromal cell culture

MSCs from normal volunteers were initially isolated from bone marrow aspiration as previously described [22], expanded and frozen as a master bank of cells at passage 1 and stored cryogenically at <−150 °C until needed. Upon thawing MSCs were seeded into plastic cultureware and allowed to recover for approximately 1 week before experimentation. After 7 days of growth hMSC were harvested, counted, and analyzed for viability and size using Invitrogen™ Countess™ automated cell counter (Invitrogen Grand Island, NY USA). Cells were reseeded at approximately 1000 cells/cm^2 using complete culture medium (CCM) which was comprised of HyClone® Minimum Essential Medium (MEM) Alpha Modification (1×) + L-glutamine, +ribonucleosides and deoxyribonucleosides; 0.1 μm sterile filtered (HyClone Laboratories, Inc. Logan, Utah USA), HyClone® L-glutamine 200 mM (100×) Solution; +29.2 mg/ml L-glutamine in 0.85% NaCl; 0.1 μm sterile filtered (HyClone Laboratories, Inc. Logan, Utah USA), and 10% PL or FBS.

2.6. MSC cell proliferation

MSCs were plated at 1000 cells/cm^2 and cultured for 3 days in either FBS or various formulations of PL medias then counted using Invitrogen™ Countess™ automated cell counter (Invitrogen Grand Island, NY USA). The size of the cells was also determined using both the Invitrogen™ Countess™ automated cell counter (Invitrogen Grand Island, NY USA) and by flow cytometry using forward and side scatter measurements.

2.7. Flow cytometry

For immunophenotyping MSCs were cultured for 7 days in FBS media or PL media, harvested and resuspended at a concentration of 1 × 10^6 cells/ml and analyzed by flow cytometry for the expression of CD45, CD34, CD44, CD73, CD90, CD105 and HLA-I (BD BioSciences). For analysis of fibrinogen binding and receptors, fluorescently conjugated fibrinogen CD29, CD41, CD51, CD49e, TRL4, CD61

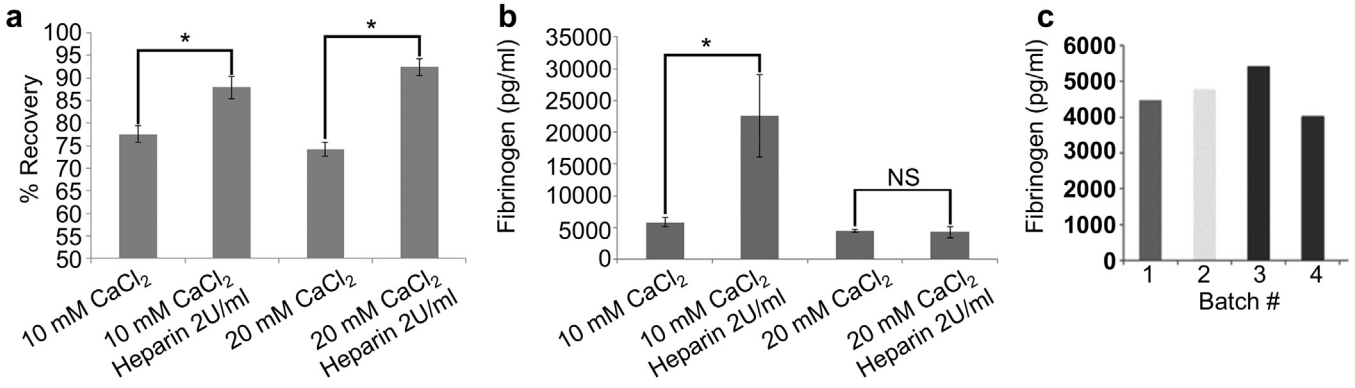

Fig. 2. Comparison of platelet lysate formulations. a) Platelet lysate supernatant percent recovery following 2 h incubation at 37 °C followed by incubation at 4 °C overnight with varying calcium and heparin concentrations. b) Fibrinogen content associated with platelet lysate supernatants recovered following 2 h incubation at 37 °C followed by incubation at 4 °C overnight with varying calcium and heparin concentrations. c) Comparison of fibrinogen content from four independent preparations of fibrinogen depleted platelet lysate using 20 mM $CaCl_2$, 2 U/ml heparin with incubations of 37 °C for 2 h and 4 °C overnight. $N = 4$ per group; $^*p < 0.05$; NS = $p > 0.05$.

Table 2
Metabolic profiling of serum platelet poor plasma, uPL and fdPL.

	Serum	PPP	uPL	fdPL
Osmolality	301	311	308 (2.08)	350 (4.9)
Sodium	140	141	160 (5.29)	154 (2.88)
Potassium	4.1	>15	4.5 (0.11)	4.6 (0.17)
Chloride	105	100	85 (2.64)	123 (3.6)
Glucose	92	101	246 (17.6)	216 (42.8)
Creatine	0.94	1.04	0.87 (0.09)	0.68 (0.03)
Total protein	7.1	7.1	5.4 (0.05)	5.3 (0.05)
Albumin	4.6	4.5	3.3 (0.11)	3.2 (0.1)
Calcium	9.6	<2.0	6.33 (0.05)	>20
Alkaline phosphates	45	<5	40.7 (5.5)	47.3 (5.5)

(Biolegend) were incubated with MSC in suspension at a concentration of 1×10^6 cells/ml. All samples were run on a Canto II flow cytometry with the appropriate isotype controls. Data are presented as either fold change (total mean fluorescent intensity (MFI) of marker/MFI) or as a histogram overlay of the marker and isotype.

2.8. Real time quantitative PCR

MSCs were cultured for 3 days and activated for 4 h with 5 ng/ml recombinant human interferon-gamma (rhIFN-γ). DNA-free total RNA was extracted and reverse transcribed as described [23]. Real-time qPCR assays were performed in duplicate on an ABI 7500 Fast Real-Time PCR system thermal cycler and SYBR Green Mastermix (Applied Biosystems) with human primer sequences for IDO, and B-actin. Primers were designed using the NCIB/Primer Blast designing tool. Data were analyzed using the relative quantification method [24].

2.9. T proliferation assay

Human peripheral blood mononuclear cells (PBMCs) were isolated from blood draws obtained from healthy volunteers using a Ficoll density gradient. Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen)-labeled PBMCs were cultured at 400,000 cells/well in a 24 well-plate with cultured 10% FBS RPMI. T lymphocytes were stimulated to proliferate using human CD3/CD28 Dynabeads (Invitrogen). T-cell proliferation was determined 4 days later by flow cytometry analysis of CFSE fluorescence intensity.

2.10. Statistical analysis

Data are reported as mean ± SD. All calculations were carried out using GraphPad Prism software (GraphPad Software). Comparisons between groups were made by ANOVA.

3. Results

3.1. Manufacturing of PL for maximal fibrinogen depletion and supernatant recovery

Standard plateletpheresis products manufactured by the American Red Cross (ARC) were re-suspended in donor plasma in which 10% v/v acid citrate dextrose (ACD) was added as a calcium chelator to prevent clotting. Typically upon dilution of PL into cell culture media the calcium content can overwhelm the citrate chelating capacity leading to conversion of free fibrinogen to fibrin. By inserting a calcification step into our PL manufacturing process we reasoned we could significantly reduce fibrinogen content

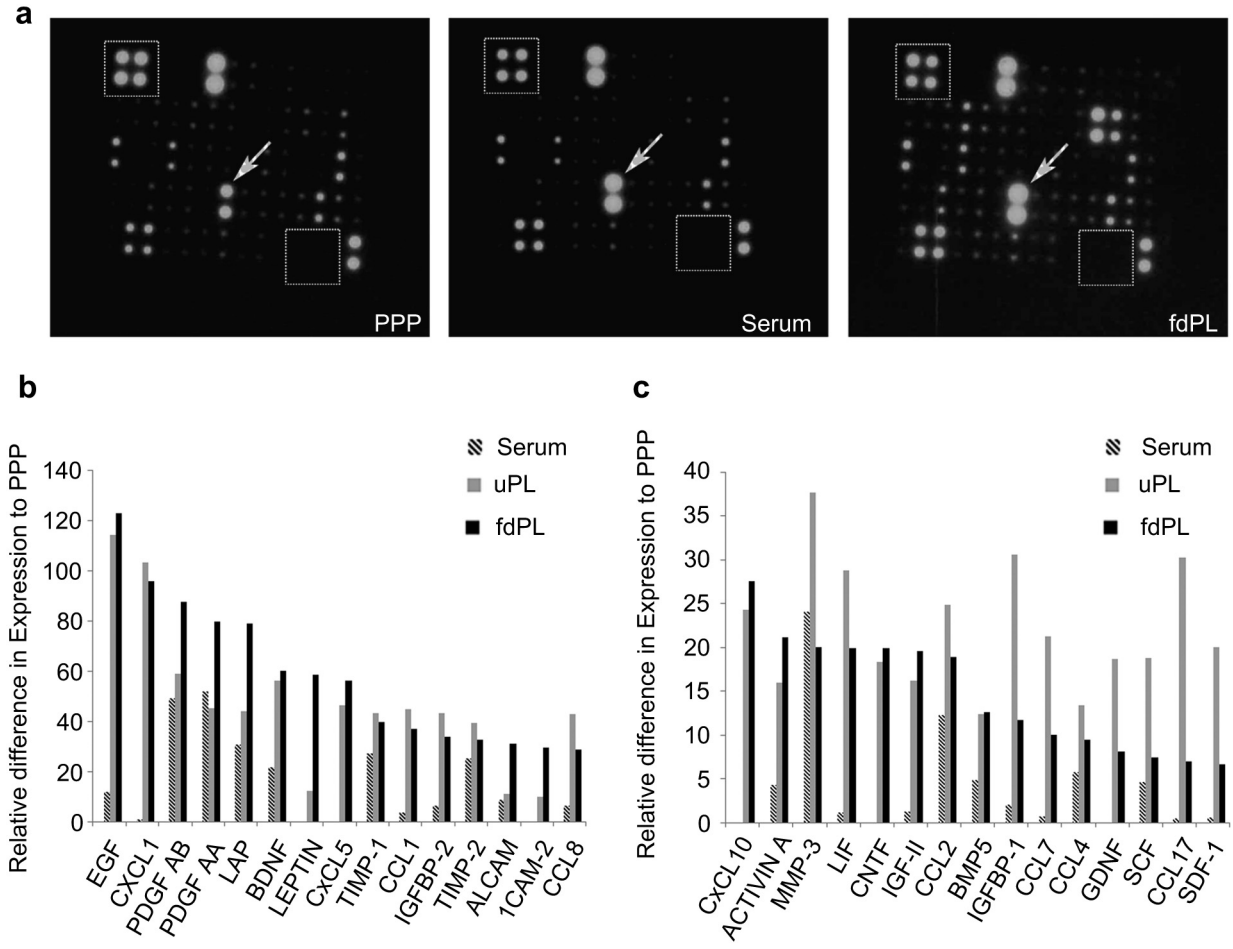

Fig. 3. Proteomic characterization of PL using Antibody arrays a) Representative images of antibody array profiling of proteins in platelet poor plasma (PPP), serum and fdPL. b, c) relative increased in expression of proteins in fdPL and uPL compared to platelet poor plasma (PPP) and serum.

leading to a more stable and uniform product. Commercial stock solutions of ACD-A used by ARC contain 3% citrate which equates to 112 mM citrate or 21.3 mg/ml [25] thus when added at 10% v/v ACD-A citrate concentration is approximately 11 mM or 2.13 mg/ml. As citrate does not bind calcium firmly [26] a ratio of three molecules of citrate to one of calcium is considered the minimum content necessary to prevent clotting. Thus raising the calcium content of a plateletpheresis product above its typical 2.5 mM concentration should promote the protease-driven conversion of fibrinogen to fibrin. In manufacturing platelet lysate (outlined in Fig. 1) we collected outdated plateletpheresis products and stored them at −30 °C for a minimum of 60 days. Products were thawed then pooled with 5–10 units and refrozen. Following a second thaw, product was centrifuged and filtered. At that point PL was separated into two lots (Fig. 1). One lot was frozen and stored as unfractionated platelet lysates (uPL) while the other half was subjected to increasing concentrations of calcium chloride ($CaCl_2$) to develop fibrinogen depleted platelet lysate (fdPL). Specifically the concentration of $CaCl_2$ in crude PL solutions was increased by 5 mM increments. Incubations were performed at various temperatures and times to determine the optimal strategy which would maximize both supernatant recover and fibrinogen depletion. The results of this work are outlined in Table 1.

Addition of 5 mM $CaCl_2$ to uPL was insufficient to overcome the chelating effects of ACD-A at either temperature following 2 h of incubation or overnight at 37 °C. However, a clot did form after adding 5 mM $CaCl_2$ to uPL following overnight incubation at room temperature (RT ≈ 22 °C). Upon centrifugation >75% of the supernatant could be recovered. For uPL solutions with $CaCl_2$ concentration of 10 mM or higher, incubations for as little as 2 h at either RT or 37 °C yielded solid clots where supernatant recovery was generally less than 60% (Fig. 1). Consistent with this observation analysis of fibrinogen content showed that increasing $CaCl_2$ concentration by 5 mM only reduced fibrinogen content by 11%, 10 mM $CaCl_2$ reduced fibrinogen by 60%, while both 15 mM and 20 mM $CaCl_2$ could reduce fibrinogen content by >95% (Fig. 1).

In an attempt to increase our recovery of platelet lysate following fibrinogen depletion, we added heparin at a concentration of 2 U/ml during the calcification procedure to modulate clot formation and clot strength [27]. At 5 mM $CaCl_2$, the addition of heparin had no effect. However, at the 10, 15 and 20 mM $CaCl_2$ concentrations addition of heparin increased recovery of supernatant by 48%, 79% and 87% respectively. Finally, the effect of cold storage (4 °C) overnight was shown to further increase the recovery of platelet lysate supernatant following calcification.

Directly comparing the fibrinogen content and percent recovery of uPL incubated with 10 mM or 20 mM $CaCl_2$ ± 2 U/ml heparin for 2 h at 37 °C followed by overnight incubation at 4 °C led to similar levels of recovery in all conditions. However, at 10 mM $CaCl_2$, fibrinogen content was significantly higher when heparin was added (Fig. 2a) negating the beneficial increase in % supernatant recovery (Fig. 2b). Conversely at 20 mM $CaCl_2$ fibrinogen content was no different (Fig. 2a) yet % recovery was significantly higher with the addition of heparin (Fig. 2b).

Based upon these experiments we determined that re-calcification with 20 mM $CaCl_2$ in the presence of 2 U/ml heparin consistently resulted in removal of >95% of fibrinogen with recovery of >85% of the platelet lysate supernatant. Subsequently we found that we could reliably produce a fibrinogen depleted platelet lysate product with a fibrinogen concentration of 4 μg/ml (Fig. 2c) whereas uPL has fibrinogen content typically in the range of 120 μg/ml. Metabolic profiling of three lots of uPL and fdPL in comparison to serum and PPP (Table 2) demonstrated that fdPL had a higher osmolality than uPL, serum and PPP and this is can be directly attributed with increased calcium concentration. Both uPL and fdPL had higher glucose concentrations as well as lower total protein and albumin content compared to serum and PPP. These differences are likely due to addition of ACD-A.

To assess cytokines and growth factors present in uPL and fdPL we employed protein array screening technology. Fig. 3a shows representative images from one of these experiments in which the right panel shows an antibody array blot for PPP, the middle panel represents serum and the left panel fdPL. Using both positive and negative control spots (hashed boxes in Fig. 3a) we could normalize the expressions of each membrane to one another and also correct for differences in background signal. Based upon a minimum

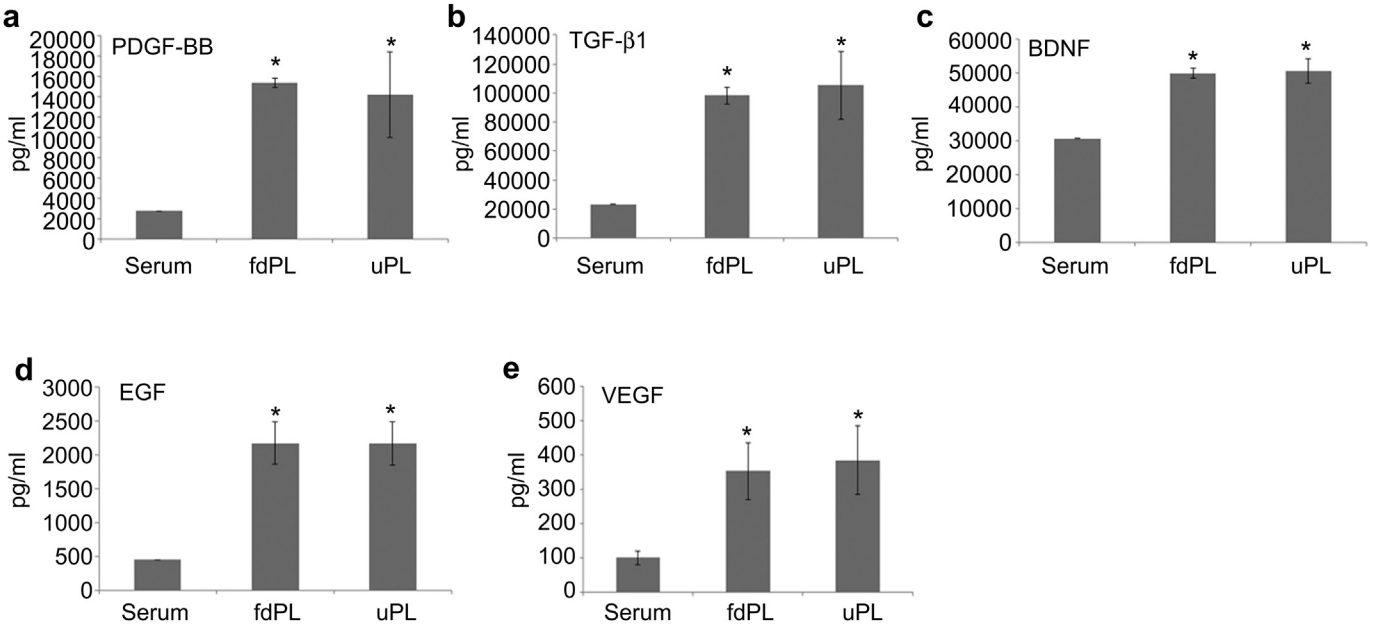

Fig. 4. Quantification of proteins in serum, fdPL and uPL. Elisa quantification of, a) PDGF-BB, b) TGF-β1, c) BDNF, d)EGF and e) VEGF levels in serum, fdPL and uPL. $N = 3$ per group; $^{*}p < 0.05$ vs serum.

relative expression unit of 15 as determined by image analysis (Image J) we could reliably detect 116 proteins in at least one of the four solutions (human serum, platelet poor plasma, uPL and fdPL). Of these 116 proteins, 87 could be detected in fdPL, 113 in uPL 113, 66 in serum and 60 PPP (see Supplemental Table 1) and could identify changes in relative protein expression for a number of proteins (arrows in Fig. 3a). The most abundant proteins in all samples were typically MSP-alpha, ACRP30, Angiogennin and Rantes (see Supplemental Table 1) and were not notably different from one another, however when comparing relative an uPL and fdPL had enhanced levels for numerous proteins compared to both serum and PPP. Of note growth factors like EGF and PDGF, neurotrophic factors like BDNF, CTNF, and GDNF as well as several chemokines like CxCL1, CxCL5 and CXCL12/SDF-1 were highly enriched in uPL and fdPL (Fig. 2b and c).

Unlike the genomics large scale proteomic detection and analysis is complicate by the dynamic range of protein expression which can span seven or eight orders on magnitude in complex solutions like serum. As a consequence, antibody arrays are note particularly reliable at showing changes in protein expression at the outer boundaries of the dynamic range. Furthermore, typically antibody arrays utilize antibodies that identify the active form of proteins and therefore are not particularly good at measuring proteins that are coupled to latency binding proteins as is often the case for the TGF-β proteins. As such and to confirm that PL is enriched for specific protein factors of interest relative to serum we performed a series of ELISAs (Fig. 4). Based on the literature we chose to evaluate the levels of PDGF-BB,TGF-β1 and VEGF [7] and based on our array data we further analyzed EGF and BDNF. In all cases we were able confirm that these proteins were significantly elevated in our PL formulations compared to serum. Comparatively there was no statistical difference between uPL and fdPL in terms of overall PDGF-BB (15 ng/ml uPL vs. 14 ng/ml fdPL), TGF-β 1(105 ng/ml uPL vs. 98 ng/ml fdPL), VEGF (0.38 ng/ml uPL vs. 0.35 ng/ml), EGF (2.3 ng/ml uPL vs. 2.1 ng/ml fdPL) or BDNF (50 ng/ml uPL vs. 51 ng/ml fdPL) content.

3.2. Stability of GMP-PL

When stored at 4, 22 or 37 °C for up to 4 weeks the stability of PDGF-BB and EGF in uPL and fdPL showed similar degradation profiles (Supplemental Fig. 1) such that levels of PDGF-BB and EGF did not change noticeably when uPL or fdPL were stored at 4 or 22 °C, whereas at 37 °C there was a progressive decline in PDGF-BB while EGF remained stable. Despite similar protein degradation profiles there was clear difference in terms of particle formation. Despite removal of particles of >0.2 μm or larger, only fdPL was resistant to secondary precipitate formation. Platelet lysate was processed into uPL and fdPL and stored at 4 °C for up to 7 days. Immediately after filtration (day 0), at day 1 and day 7 we performed particle analyses using a flow cytometer where the forward and side scatter signals could estimate particle size, abundance and granularity. Immediately following filtration both uPL and fdPL both had very little debris (Fig. 5a – lower panel), however after 24 h at 4 °C there was a dramatic accumulation of debris in uPL which had increased in size by day 7. This debris formation could be easily observed microscopically in uPL at day 7 (Fig. 5c), but was not readily apparent in fdPL (Fig. 5b). Flow cytometry analysis further confirmed that storage of fdPL at 4 °C for 1 and 7 days did not lead to a significant accumulation of debris (Fig. 5a – upper panel).

3.3. Impact of fibrinogen-depleted PL on MSC

Using 4 separate MSC populations we found that compared to FBS both uPL and fdPL significantly reduced doubling time (FBS-MSC $dt = 44 \pm 6$ h, uPL-MSCs $dt = 25 \pm 1.8$ h, fdPL $dt = 21 \pm 1.3$ h; $p < 0.05$)

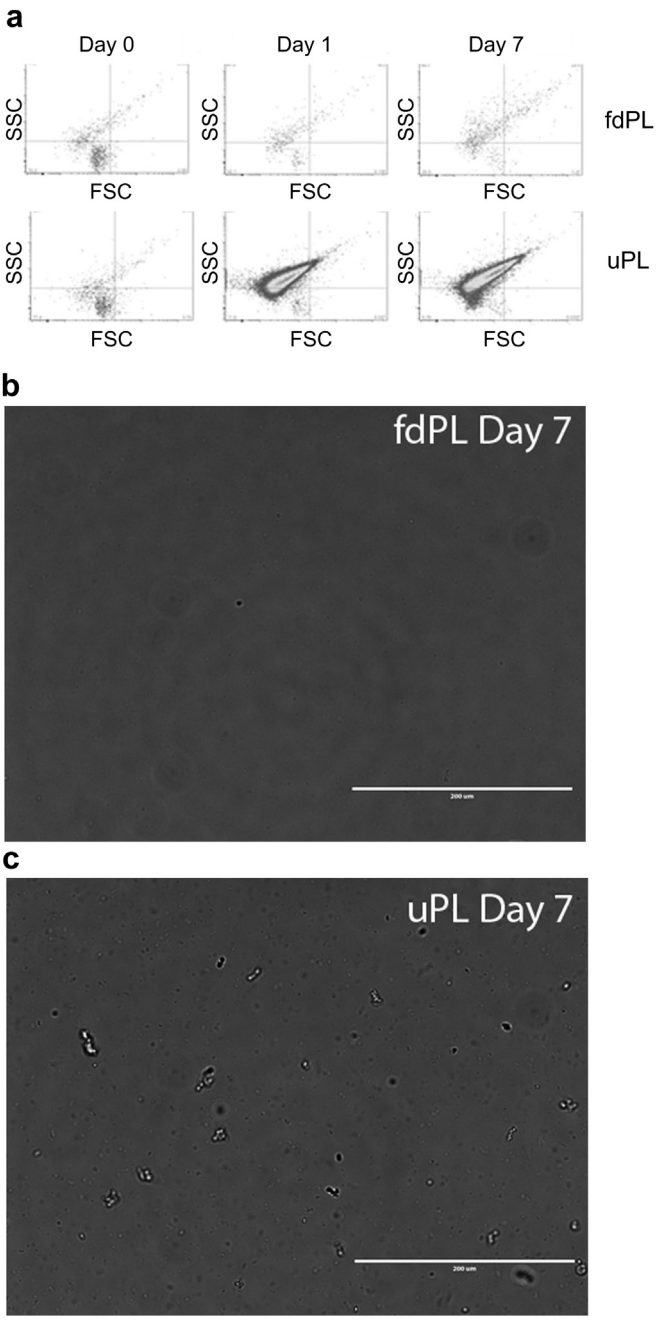

Fig. 5. Evaluation of debris formation in fdPL and uPL. a) Flow cytometry plots of forward and side scatter of fdPL (upper panel) and uPL (lower panel) and immediately after filtration to 0.2 μm (day 0) and following 1 and 7 days storage at 4 °C. b) Visual representation of debris accumulation in fdPL after 7 days storage at 4 °C. b) Visual representation of debris accumulation in uPL after 7 days storage at 4 °C.

of bone marrow derived MSCs (Fig. 6a) and consistently resulted in MSC populations that were significantly smaller based on radial measurement (Fig. 6b) and by forward/side scatter (Fig. 6c). When directly compared to one another MSC expanded in fdPL had a significantly lower doubling time compared to MSCs in uPL (Fig. 6a). Phenotypically, MSCs grown in FBS, uPL or fdPL all expressed the typical markers of MSCs including CD44, CD90, CD73, HLA-I, CD105, and lacked expression of CD45 and CD34 (Fig. 6d). When expression levels were compared based upon MFI fold change over isotype there was a significant increase in overall expression of CD44, CD90, CD73 and HLA-I in MSCs expanded in both uPL and fdPL compared to FBS expanded counterparts (Fig. 6d).

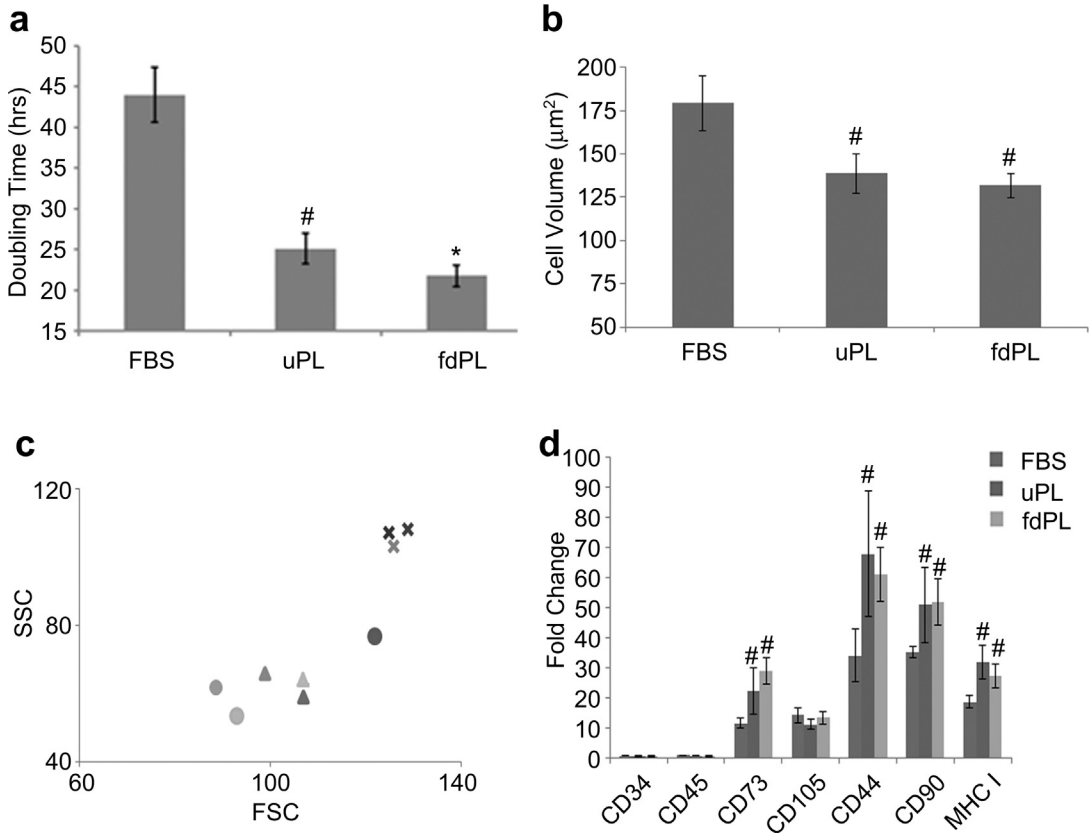

Fig. 6. Impact of platelet lysate formulations on MSCs. a) Population doubling time (hours) of low passage MSCs (P2–4) seeded at 1000 cells/cm^2 in 10% FBS, uPL or fdPL for 3 days. b) Cell volume calculated from cell diameter digital measurement of low passage MSCs cultured in 10% FBS, uPL or fdPL. c) Flow cytometry plots forward and side scatter (FSC, SSC) of low passage MSCs expanded in 10% FBS (×) uPL (●) and fdPL (△). d) Flow cytometry analysis immunophenotype of MSCs expanded in 10% FBS, uPL or fdPL expressed as fold change over isotype control mean fluorescent intensity. $N = 3–5$ unique MSC populations per group; $^{\#}p < 0.05$ vs FBS; $^{*}p < 0.05$ vs all groups; NS = $p > 0.05$.

To evaluate whether MSCs could bind fibrinogen and whether this binding could evoke a biologic response, MSCs in suspension were incubated with increasing concentrations of fluorescently conjugated fibrinogen and subjected to FACS analysis. After 1 h of incubation there was a well-defined dose dependent increase in fibrinogen binding up to the maximum concentration of 1 mg/ml evaluated (Fig. 7a). Using flow cytometry we next evaluated the presence of several receptor complexes on MSCs that have been shown to bind fibrinogen [16]. This included the αiibβ3 (CD41/CD61), αvβ3 (CD51/CD61) and α5β1 (CD49e/CD29) integrin complexes and the non-integrin complexes CD44 and TRL4 [28]. Of the three integrin complexes, α5β1 had the highest overall expression while αiibβ3 was the lowest as αiib (CD41) was undetectable (Fig. 7b). CD44 is highly expressed on all MSC populations, while TLR4 expression was detectable, but low. Confirming that MSC can not only bind fibrinogen, but this binding can evoke a physiologic response, MSCs were exposed to either 0.5 or 1 mg/ml fibrinogen, under serum free conditions, for 24 h. After the incubation period, conditioned media were collected and the content of IL-8, MCP-1 and IL-6 determined (Fig. 7c). In all case this short term exposure of MSCs to fibrinogen caused significant increases in expression of all three cytokines, such that IL-6 levels increased from 34 pg/ml to 281 pg/ml and 508 pg/m following exposure to0, 0.5 and 1 mg/ml fibrinogen respectively. In the absence of fibrinogen IL-8 was undetectable when cultured in serum free conditions for 24 h however fibrinogen at 0.5 and 1 mg/ml increase IL-8 levels to 94 and 360 pg/ml. Finally the most dramatic change was observed in MCP-1 secretion which was 5 pg/ml in serum free media, but increased to 616 and 929 pg/ml following 24 h exposure to 0.5 and 1 mg/ml fibrinogen.

3.4. Immunomodulatory action of PL

MSCs have been shown to be have a profound effect on T cell activation and proliferation *in vitro* (Fig. 8a) Recently, our group has established that the ability of MSCs to up-regulate IDO expression correlates to their ability to suppress T-cell proliferation [29]. To evaluate whether fibrinogen could influence the ability of MSCs to up-regulate IDO we cultured MSCs in FBS for a minimum of 1 week then under serum free conditions exposed MSCs to either 0, 10 or 100 μg/ml of fibrinogen for 24 h and then stimulated the MSCs with the IDO inducer cytokine INF-γ (5 ng/ml). After 4 h RNA was isolated from the cells and IDO gene expression evaluated by real-time PCR. Based upon these analyses we found that a short term exposure of fibrinogen could dose dependently reduce the amplitude to which MSCs can up-regulated IDO expression following exposure to INF-γ (Fig. 8b). Consistent with these data when MSCs were cultured in either FBS, uPL or fdPL for a minimum of 7 days then similarly exposed to INF-γ, uPL expanded MSCs had a significantly blunted IDO response compared to FBS expanded MSCs, however depletion of fibrinogen in the fdPL almost completely restored the IDO response in MSCs (Fig. 8c). Together these data suggest that fibrinogen content in the media used to expand MSCs could impact their immunosuppressive capabilities.

To support this idea we performed a series of *in vitro* T-cell proliferation assays using stimulation with anti-CD3/anti-CD28 Dynabeads. Following a 4-day exposure of peripheral blood mononuclear cells (PBMCs) to CD3/CD28 activation, T-cells proliferated robustly in the absence of MSCs, whereas MSCs at a 1:10 dilution could significantly reduce the proliferation (Fig. 8a). If

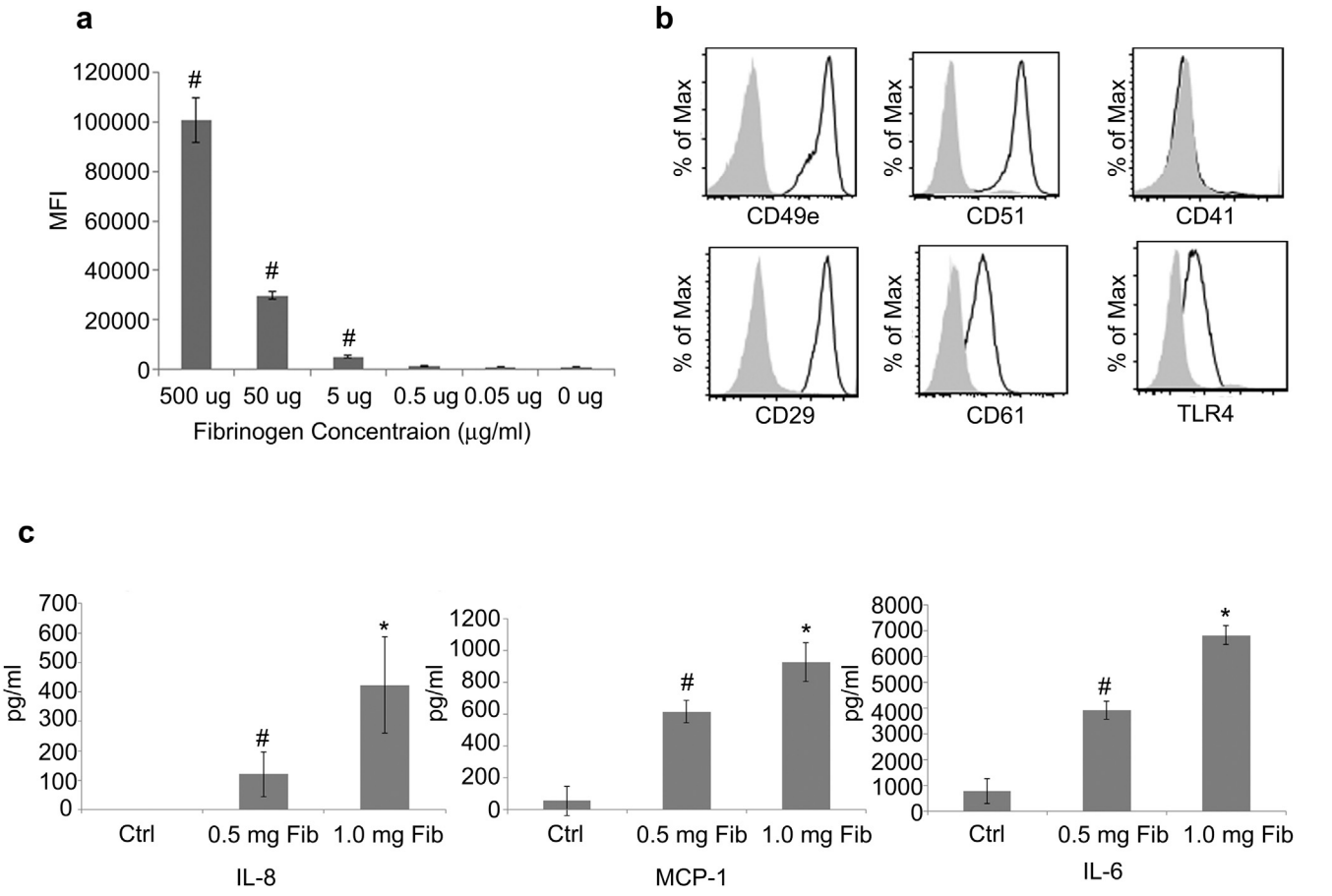

Fig. 7. Fibrinogen binding to MSCs and functional response. a) Binding capacity of different concentrations of FITC labeled fibrinogen to hMSC analyzed by measuring MFI using flow cytometry following 1 h incubation at 37 °C. b) Relative expression of fibrinogen binding receptors αiiβ/β3, αvβ3, α5β1 and TLR4 analyzed by flow cytometry and compared to isotype controls. c) Elisa quantification of MSC MCP-1, IL-8 and IL-6, secretion following 24 h exposure to different concentrations of fibrinogen under serum free conditions. $N = 4$ unique MSC populations per group; $^{\#}p < 0.05$ vs ctrl or 0 ug; $^{*}p < 0.05$ vs all groups.

MSCs at a similar concentration were pre-incubated with fibrinogen (100 μg/ml) for 16 h, washed then co-cultured with PBMCs and stimulated with CD3/CD28 immunosuppression was significantly reduced (Fig. 8d). Consistent with this observation when MSCs were grown in PL containing high levels of fibrinogen (uPL) their ability to suppress T-cell proliferation was altered compared to those MSCs expanded in FBS (Fig. 8e). Specifically when expanded in FBS at a concentration of 1:10 MSCs could significantly suppress T-cell proliferation as could MSCs expanded in fdPL to a less degree, however when expanded in uPL MSCs were no longer immunosuppressive, but rather promoted PBMC expansion suggestive of a pro-inflammatory phenotype.

4. Discussion

To date, the majority of cell therapy trials involving *ex vivo* cell manipulation rely on fetal bovine serum (FBS) to help maintain cell viability and function. However as FBS is xenogeneic, cells exposed to it have the potential to become immunogenic, and as a result, the host can rapidly reject even autologous cells [5]. To this end, MSCs for clinical evaluation are increasingly being expanded in human-derived alternatives including human serum, platelet-rich plasma and platelet lysate.

In 2004, two separate studies showed that PDGF released from platelets was able to promote the proliferation of mesenchymal stromal cells [30,31], while the following year Doucet et al. [32], empirically demonstrated that platelet lysate was not only superior to fetal bovine serum in expanding MSCs, but also showed that freeze fracturing platelets were more efficient than either platelet adhesion or platelet aggregation at recovering mitogenic factors. Subsequently Doucet et al., demonstrated that bone marrow mononuclear cells grown in either FBS or platelet lysate were not different in regards to their differentiation potential or immunophenotype as characterized by CD90, CD73, CD105 positivity, and CD45, CD14 negativity. Our data are consistent with these data such that at concentrations of 10% our PL could reduce doubling time by approximately 50% compared to FBS expanded cells. The overall immunophenotype of our PL expanded MSCs were similar to FBS expanded MSCs, however our platelet lysate formulations yielded MSCs, which were not only significantly smaller, but on a per cell basis had significantly higher expressions of CD73, CD90, CD44 and MHCI. Whether higher expression of markers alters the biological activity of MSC was not determined, however, the ability to generate an MSC product comprised of small cells may be of clinical significance.

While platelet lysate appears to be an ideal alternative to FBS to expand MSCs for clinical evaluation, platelet lysates are relatively crude products and thus can be challenging to work with. In particular the potential for residual clotting and formation of fibrin precipitates are common, but rarely discussed issues that must be delt with when working with platelet lysate. Furthermore variability between groups regarding the manufacturing process has

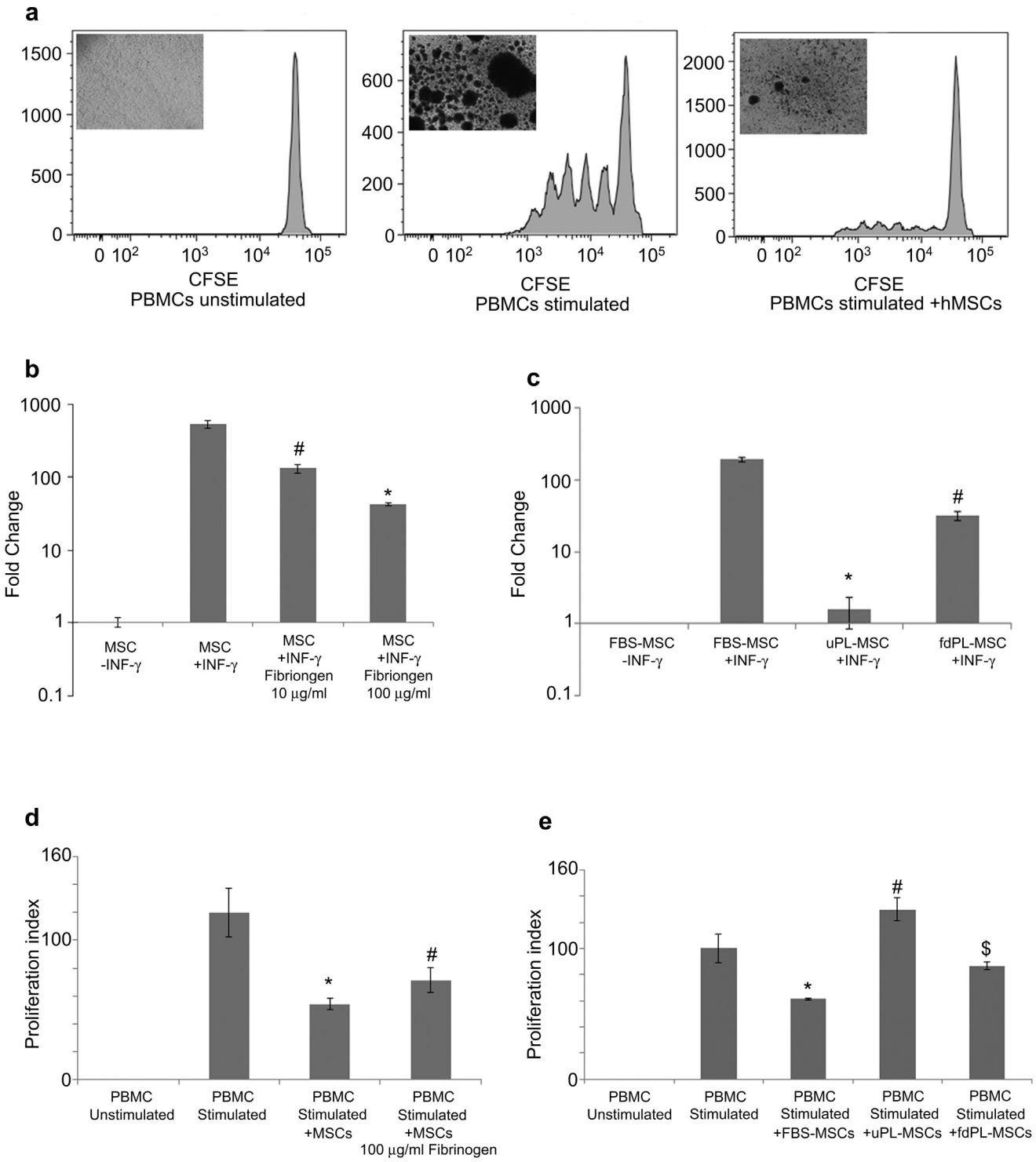

Fig. 8. Altered immunosuppressive potential of MSCs exposed to fibrinogen. a) Representative images of T cell proliferation assays performed using CFSE-labeled human PBMCs cultured for 4 days un-stimulated (left), activated with CD3/CD28 Dynabeads (middle) or activated with CD3/CD28 Dynabeads and co-cultured (5:1 ratio) with MSCs. b) Relative IDO gene expression was analyzed by real-time PCR on 3 human MSC donors after 24 h exposure to fibrinogen at 10 and 100 μg/ml followed stimulation with 5 ng/ml of rhIFN-γ for 4 h. c) Relative IDO gene expression was analyzed by real-time PCR on 3 human MSC donors expanded in FBS, uPL and fdPL for a minimum of 4 days followed by stimulation with 5 ng/ml of rhIFN-γ for 4 h under serum free conditions. d) T cell proliferation index of un-stimulated CFSE-labeled human PBMCs, and CD3/CD28 stimulated PBMCs alone or co-cultured for 4 days with MSCs (MSC:PBMC ratio 1:10) following 24 h incubation under serum-free conditions with or with fibrinogen (100 ug/ml). e) T cell proliferation index of un-stimulated CFSE-labeled human PBMCs, and CD3/CD28 stimulated PBMCs alone or co-cultured for 4 days with MSCs (MSC:PBMC ratio 1:10) following expansion in either FBS, uPL or fdPL. $N = 3$ unique MSC populations per group; $^{\#}p < 0.05$ vs positive control; $^{*}p < 0.05$ vs all groups, $^{\$}p < 0.05$ vs PBMC stimulated + uPL MSCs. Positive controls = MSC + INF-γ, FBS-MSC + INF-γ, and PBMC stimulated.

led to some discrepancies in the field regarding the utility of PL for expanding MSCs as a therapeutic for immune modulation [11,13–15]. In this work we sought to refine the methodology for preparing PL from platelet concentrates. In doing so we have developed a simple and reliable process which maximizes both fibrinogen depletion and recovery of platelet lysate supernatant without compromising the growth factor composition profile associated with unfractionated platelet lysate (uPL). Functionally we demonstrate that fibrinogen depleted PL (fdPL) is more stable than uPL with regards to fibrin flocculation and enhances MSC proliferation over both FBS and uPL. We further demonstrate that uPL expanded MSCs have inferior *in vitro* immunosuppressive capabilities compared to FBS expanded MSCs and this reduction is associated with reduced ability to up-regulate IDO. By performing a controlled clotting procedure which significantly depletes fibrinogen we can largely restore the IDO responsiveness of PL expanded MSCs and enhance their immunomodulatory capabilities.

Previously Ode et al., demonstrated that MSCs could bind to fibrinogen *in vitro* [33], which we confirmed by flow cytometric analysis and then established that this binding produces a biologically significant response. Specifically we show that fibrinogen can dose dependently increase MSCs secretion of the pro-inflammatory cytokines, MCP-1, IL-8 and IL-6, and can also reduce the ability of MSCs to up-regulate IDO following INF-γ stimulation. In endothelial cells, fibrinogen was shown to increased synthesis of MCP-1 and IL-8 by binding to the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins which triggers nuclear translocation of NF-κB [17,18]. Stellate cells also bind to fibrinogen via the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins to up-regulate not only MCP-1 and IL-8, but also IL-6 [20] and do so via increased phosphorylation of the MAP kinases (Erk, p38 and JNK), and nuclear translocation of NF-κB. Macrophages and monocytes can evoke an MCP-1, IL-6 and IL-8 response to fibrinogen, but do so via the TLR4 receptor, which produces phosphorylation of the MAP kinases as well as NF-κB, signaling [19,34–36]. On MSCs our flow cytometric data suggest fibrinogen can potentially bind to both the $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrin complexes, as well asTLR4. Together these data suggest that despite fibrinogen binding to multiple receptor complexes, the net effect is an up-regulation of cytokines that polarize a cell toward a pro-inflammatory phenotype.

The concept that fibrinogen can polarize MSCs toward a pro-inflammatory phenotype is consistent work by several groups demonstrating that when the TLR4 receptor is activated in MSCs these cells not only increase their secretion of IL-6, MCP-1 and IL-8, but also have reduced immunomodulatory properties [24,37,38]. This modulation appears to be ligand specific because activation of the TLR3 receptor polarizes MSCs to a more immunosuppressive phenotype. These data have led to an extrapolation of a concept from monocytes/macrophage biology that naïve MSCs can be polarized toward either an M1 (pro-inflammatory) or an M2 (immunosuppressive) state by binding to specific ligands [38]. Further supporting this concept is our observation that MSCs exposed to fibrinogen have a reduced capability to up-regulate indoleamine 2,3-dioxygenase (IDO). In numerous cell types (including MSCs) IDO is induced by IFN-γ in the course of an inflammatory response and this induction activity leads to a nearly complete depletion of the essential amino acid tryptophan which in turn leads to inhibition of T-cell proliferation, T-cell anergy and apoptosis [39]. Recently Francois et al., demonstrated that alterations in the ability of individual MSCs populations to up-regulate IDO correlates with their ability to suppress T-cell proliferation *in vitro* [29]. These data are consistent with our observation that MSCs exposed to different concentrations of fibrinogen either alone or in platelet lysate not only have a reduced capacity to up-regulate IDO following INF-γ exposure, but also have an reduced ability to influence T-cell proliferation. Within the literature there is no documentation that fibrinogen can affect the induction of IDO in any cells type, however other signaling molecules like IL-4 [40] and arachidonic acid [41] can antagonize the ability of INF-γ to up-regulate IDO in monocytes, while molecules like nitric oxide can accelerate IDO degradation [42].

5. Conclusion

The field of regenerative medicine and in particular adoptive cellular immunotherapy has shown tremendous growth over the last decade and several cellular products are now available for clinical trials. However increasing concerns regarding the use of xenogeneic growth supplements has led to increased use of human blood derived growth supplements. The generation of a lysate from human platelet concentrates is considered a promising alternative for the clinical expansion of MSCs, however our data indicate that fibrinogen content in platelet lysate can adversely affect their immunosuppressive capabilities. Thus, when expanding MSCs for use in the treatment of auto and allo-immune disorders, minimizing the fibrinogen content in the expansion media should be a priority.

Conflict of interest

None reported.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.06.050.

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