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Platelet lysate enhances synovial fluid multipotential stromal cells functions, implications for therapeutic use.

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Objective: Whilst intra-articular injection of platelet products is increasingly being used for joint regenerative approaches, there is limited data on their biological effects on joint resident multipotential stromal cells (MSCs) which are directly exposed to the effects of these therapeutic strategies. Therefore, this study investigated the effect of platelet lysate (PL) on synovial fluid-derived MSCs (SF-MSCs), which *in vivo* have direct access to sites of cartilage injury.

Methods: SF-MSCs were obtained during knee arthroscopic procedures (N=7). Colony forming unit–fibroblastic (CFU-F), flow-cytometric phenotyping, CFSE based immunomodulation for T cells and trilineage differentiation assays were performed using PL and compared to standard conditions.

Results: PL enhanced SF-MSCs proliferation as CFU-F colonies were 1.4-fold larger and growing cultures had shorter population doubling-times. PL-MSCs and FCS-MSCs had the same immunophenotype and similar immunomodulation activities. In chondrogenic and osteogenic differentiation assays, MSCs grown in PL or "PL-MSCs" produced 10 % more sulphated-glycosaminoglycan (sGAG) and 45 % less Ca^{++} compared to FCS-MSCs, respectively. Replacing chondrogenic media TGF-β3 with 20 % or 50 % PL further increased sGAG production of PL-MSCs by 69 % and 95 % respectively, compared to complete chondrogenic media. Also, DMEM-(high-glucose) plus 50 % PL induced more chondrogenesis compared to DMEM-(high-glucose) plus 10 % FCS and was comparable to complete chondrogenic media.

Conclusion: This is the first study to assess SF-MSCs responses to PL and provides biological support that PL may be capable of modulating multiple functional aspects of joint resident MSCs which have direct access to injured cartilage.

Key words: Synovial fluid multipotential stromal cells, platelet lysate and chondrogenesis.

Introduction:

Osteoarthritis (OA) is the most common musculoskeletal disease that affects an increasing proportion of the population and is a major cause of global healthcare expenditure [1]. The OA process can start in multiple locations within the joint including the cartilage, bone, ligaments, synovium and meniscus, with eventual whole joint failure [2]. To date, there are limited therapeutic options for the treatment of joint dysfunction in OA. For early OA emanating in cartilage or "chondrogenic" OA, symptomatic treatments with analgesics, non-steroid medications, steroid injection and physical therapy, rather than regenerative approaches are invariably offered [3, 4]. In more advanced disease, total knee replacement is generally considered in patients over 65 years and more than 160,000 of these are carried out annually in the UK alone (according to National Joint Registry, 2017). However, in younger patients with small sized chondral lesions, regenerative therapies including microfracture and autologous chondrocyte implantation (ACI) may be used. Although these approaches are associated with good short term pain relief, they mostly lead to formation of fibrocartilaginous tissue which is not capable of withstanding mechanical stresses over time [5-7].

There is an urgent need for novel regenerative treatment options for isolated articular cartilage defects before they lead to advanced cartilage loss. One experimental strategy is the use of bone marrow-derived MSCs (BM-MSCs) [8, 9], or adipose tissue-derived MSCs (AT-MSC) [10, 11], with pre-clinical and clinical results showed promising results. However, due to the expense and time-consuming nature of these procedures, as they require clinical grade facilities and times to expand the cells, there is an increasing interest in harnessing the power of endogenous joint resident MSCs in conjunction with scaffolds or growth factors or a combination as part of a one stage procedure.

One potential source of endogenous MSCs that could play a role in OA joint repair are synovial fluid-derived MSCs (SF-MSCs) that are present in OA, rheumatoid arthritis (RA) and non-arthritic joint fluid [12]. Elevation in SF-MSC numbers has been reported in early OA, and following meniscal injury, suggesting their potential role in physiological joint repair [13, 14]. Furthermore, SF-MSCs are easy to isolate by aspiration and they are thought to be derived from the adjacent synovium, which is a tissue rich in MSCs with strong chondrogenic potential [15, 16]. Finally, animal model studies showed that synovial origin MSCs facilitated cartilage repair following injection directly to the defect area with or without scaffold [17, 18]. Since both the topography and high chondrogenic potential of SF-MSCs indicate their potential role in endogenous joint repair, the question arises as to whether this process could be exploited or further enhanced.

Platelet Lysate (PL) is a biological platelet derivative rich in growth factors and cytokines that encourage tissue repair, which is driven by a multitude of growth factors including basic fibroblast-derived growth factor (bFGF), transforming growth factor beta (TGF-β), platelet derived growth factor-AA (PDGF-AA), PDGF-AB, PDGF-BB, [19, 20]. Increasingly, PL is being injected directly into OA knee joints with some encouraging results [21] but the mechanisms of its action remain unknown. In 2005, Doucet proposed the use of PL as a substitute to FCS for MSC expansion intended for cellular therapy [22]. Subsequent studies have shown the capacity of PL to promote cell proliferation while maintaining immunophenotype and trilineage differentiation of BM-MSCs, AT-MSCs and umbilical cord blood MSCs [20, 23-26]. The potential interactions between PL and SF-MSCs have not thus far been explored, but could be key towards enhancing the endogenous MSC repair responses.

The aim of this study was therefore to evaluate the impact of PL on SF-MSCs immunophenotype, proliferation, immunomodulation, and trilineage differentiation in comparison to FCS containing standard expansion media, with a particular focus of the capacity of PL to act as chondrogenic inducer. We studied commercial PL ((Stemulate) from Cook Regentec) as it has a more consistent growth factor content as it is produced in large batches to avoid lot to lot variation and is available off the shelf in large quantities.

Patients and Methods:

Isolation and culture of SF-MSCs

Approval for the study was obtained from the national research ethics committee (Rec reference:14/YH/0087). Samples were collected after informed written consent from all study participants who were undergoing elective knee diagnostic or therapeutic arthroscopy. No effusion was present at time of arthroscopy and patients with inflamed synovium were not included as inflammation might have an impact on chondrogenesis [15]. To ensure collection of all SF-MSCs, SF was collected after an initial injection of saline (up to 50ml) (N=7 patients). The aspirated fluid was centrifuged at 500rcf for 5 minutes and cells were re-suspended in 10ml DMEM media (with no FCS). For expansion, 2ml of cell suspension were cultured in PL-media containing DMEM (Gibco Paisley-UK) supplemented with 5% PL-fibrinogen depleted (Stemulate, Cook Regentec-US) (referred to as PL-MSCs) or standard FCS-media (StemMACS media, Miltenyi Biotec-UK) (referred to as FCS-MSCs); each containing 100 units/ml penicillin, 100mg/ml streptomycin (all from Gibco). Cells were incubated in a humidified atmosphere at 37°C and 5 % CO_2, with a full media change after 48 hours, followed by media changes twice weekly. Once the cells reached 80 % confluence, the adherent cells were harvested with 0.25 % trypsin/1 mM ethylenediaminetetraacetic acid (EDTA) solution (Sigma-Aldrich, Dorset, UK) and passaged at a seeding density of 5–7 x 10^3 cells/cm^2. Donor-

matched cultures were used in all experiments when they reached passage 3. SF-MSCs doubling time was calculated from the number of population doublings up to passage 0 as previously described [27]. According to the following formula: days in culture till the end of passage 0/population doubling number up to passage 0. The latter (population doubling number) was calculated as = $\log_2$ (N total cell count at p0/ Total CFU-F number).

Characterisation of the surface marker expression

The following antibodies were used to compare immunophenotype of PL- and FCS- expanded SF-MSCs: CD90-PE-Cy7, CD45-PE-Cy7 and CD19-PE (BD Biosciences Pharmigen-UK), CD105-PE (AbD Serotec-UK), PE-CD73, CD34-APC and CD14-FITC (Miltenyi biotec-UK), with appropriate isotype controls. DAPI was used to gate out dead cells. Samples were acquired using a three-laser flow cytometer, LSRII (BD Biosciences) and analysed by FacsDiva version 8.

Colony-forming unit–fibroblast (CFU-F) assay:

Freshly obtained SF cells were plated in duplicate 60 mm diameter petri dishes (Greiner bio-UK) either with FCS- or PL-media. Cells were incubated in a humidified atmosphere at 37°C and 5 % CO_2, with a full media change after 48 hours, followed by half media changes three times a week. Colonies were stained with 1 % methylene blue after 2 weeks of culture for counting and measuring colony size using ImageJ version 2.0.0.

To ensure that any potential differences in colony counts were due to SF-MSCs proliferation in the different media rather than differential adhesion, SF-MSCs adhesion to the plastic under both conditions was assessed. After the initial seeding and attachment phase, a full media change was performed after 48 hours and cells were washed with Phosphate Buffer Saline (PBS). PL-media was added to the cells which were originally plated with FCS-media, and vice versa. After 14 days, colonies were fixed and stained with 1 % methylene blue.

Standard trilineage differentiation assays:

Trilineage differentiation potential of PL-MSCs was initially examined in comparison to FCS-MSCs. For chondrogenic differentiation, donor-matched replicate cultures of FCS-MSCs and PL-MSCs (5 replicates of $2.5x10^5$ cells each) were placed in screw-capped Eppendorf tubes (BDH Chemicals-UK) in 0.5 ml of complete chondrogenic media consisting of DMEM (high glucose), 50 μg/ml Ascorbic-2-phosphate (As2P), 100 μg/ml Sodium Pyruvate, 40 μg/ml Proline, 1.25 mg/ml bovine serum albumin, 1:100 of mixture of recombinant human insulin, human transferrin, and sodium selenite (ITS+), 10 nM dexamethasone (DEX) and 10 ng/ml transforming growth factor-beta (3) (TGF-β3), all from Sigma [14]. Half media changes were performed 3 times a week. After 3 weeks, sGAG production was visualized on 5 μm frozen sections stained with 1 % toluidine blue (Sigma). Production of sGAG was further quantified using an Alcian blue binding assay (Immunodiagnostic Systems, Boldon, UK) following digestion in 100 μl of papain solution [14]. For osteogenic differentiation, 5 replicates of donor-matched FCS-MSCs and PL-MSCs (10^4 cell/well) were seeded in 12 well tissue culture plates with freshly prepared complete osteogenic media (DMEM, 10 % FCS, 100 μM As2P, 10 mM β glycerophosphate and 100 nM DEX, all from Sigma) [28]. Media was changed twice a week and after 21 days, cells were washed with PBS, fixed and stained with 1 % Alizarin red (Sigma). For calcium quantification, cells were incubated in 0.5 N HCl for 5 minutes and gently scraped off the surface before agitating for 4 hours at 4^oC. Quantification was performed using the Ca^{++} liquid assay according to manufactures instructions (Sentinel Diagnostics-UK) [14]. For adipogenesis, 24 well plates were seeded with 5 replicate cultures of donor matched FCS-MSCs and PL-MSCs ($5x10^4$ cells/well) in freshly prepared complete adipogenic media; DMEM (low glucose) supplemented with 10 % FCS, 10 % horse serum, 0.5 mM isobutylmethylxantine, 60 μM indomethacine and 0.5 mM hydrocortisone (all from sigma)

[14]. Media was changed twice a week and after 3 weeks quantitative analysis was performed by initially staining cells with DAPI (8 μg/ml) and measuring fluorescence from excitation and emission at 355/460 nm. Cells were further stained with Nile red (1 μg/ml) and fluorescence measured from excitation and emission at 485/535 nm using Berthold plate reader [29]. The ratio of Nile red to DAPI was then calculated [29]. For Oil Red staining, cells were fixed in 10 % (v/v) formalin (Biostain Ready Reagents, Manchester, UK) and stained with oil red solution 0.5 % oil red (w/v) (Sigma) in isopropanol, as previously described [29].

PL as a trilineage differentiation inducer:

To investigate whether PL can act as chondrogenic inducer, different chondrogenic media were prepared as follows. First, to examine if PL can replace TGF-β3, basal chondrogenic media was prepared which contained DMEM (high glucose), 50 μg/ml As2P, 100 μg/ml Sodium Pyruvate, 40 μg/ml Proline, 1.25 mg/ml bovine serum albumin, 1:100 of mixture of recombinant human insulin, human transferrin, and ITS+ and 10 nM DEX). Instead of TGF-β3, this basal media was supplemented with 20 % PL and 50 % PL (referred to as basal chondro-20 % PL and -50 % PL respectively). Secondly, to investigate if PL alone can induce chondrogenesis without addition of any other inducer molecules present in basal chondrogenic media, DMEM (high glucose) media was supplemented with 50 % PL alone (referred to as DMEM, 50 % PL). DMEM with 10 % FCS and no inducers was used a negative control, chondrogenesis was performed as described earlier. In osteogenic and adipogenic complete media, 10 % FCS was replaced with 10 % PL (referred to as Osteo-PL and Adipo-PL), assays were performed as described earlier.

Immunomodulation assay:

Peripheral blood mononuclear cells (PBMCs) were obtained using Lymphoprep reagent (Axis-Shield, Oslo, Norway) from heathy donors, with informed consent. Prior to co-culture, donor-matched PL-MSCs or FCS-MSCs were irradiated (30 Gy) to prevent further proliferation of MSCs. PBMCs were stained with Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen-UK) according to the manufacturer's protocol. Co-cultures were performed in 24 well plates with following ratios of PBMCs to SF-MSCs 1:1, 2:1, 5:1 and 10:1. Wells containing no MSCs were used as positive controls. Human CD3/CD28 Dynabeads (Invitrogen-UK) were used to stimulate T-Lymphocyte proliferation [30]. Non-stimulated PBMCs in the absence of SF-MSCs were used as negative controls. Glutamax™ RPMI 1640 media (Invitrogen-UK) supplemented with 10 % FCS was added to all wells. Flow cytometry analysis was performed five days after stimulation. $CD4^+$ T cells were gated based on viability (using 7-AAD), followed by gating on the $CD90^-CD45^+$ population (to exclude any MSCs), and finally, on the $CD14^-CD4^+$ population. T-lymphocyte proliferation was analysed using ModFit software version 5.0.9 (Verity Software House, Topsham, ME. USA). T-lymphocyte proliferation was calculated using a proliferation index, the ratio of the starting population plus daughter cells divided by the starting population cell count [31].

Statistics:

All statistical analyses were carried out using GraphPad Prism software (GraphPad software). Wilcoxon matched-pairs signed rank test was used to compare proliferation and standard trilineage differentiation of PL-MSCs to FCS-MSCs. Two-Way ANOVA–Bonferroni's multiple comparisons tests for group analysis were performed to compare immunosuppression of SF-MSCs at different co-culture ratios and immunosuppression of PL-MSCs to FCS-MSCs, trilineage differentiation of PL-MSCs and FCS-MSCs with different induction media.

Results:

PL maintains the SF-MSCs phenotype

Flow cytometric analysis showed FCS-MSCs lacked expression of CD14, CD19, CD34 and CD45 cell surface markers and positively expressed CD73 (95.6 ± 3.0 %), CD90 (96.33 ± 1.0 %) and CD105 (92 ± 2.8 %) (Figure 1). Similarly, PL-MSCs lacked expression of CD14, CD19, CD34 and expressed positive for CD73 (98.5 ± 1.1 %), CD90 (97.6 ± 2.8 %) and CD105 (89.6 ± 10 %). Thus, the immunophenotype of PL-MSCs was consistent with International Society for Cell Therapy (ISCT) basic requirements for expanded MSCs as they were compared to donor-matched FCS-MSCs.

PL enhancement of SF-MSCs proliferation

Using the CFU-F assay, comparisons were made to investigate the impact of PL on SF-MSCs proliferation compared to FCS containing media. PL significantly increased SF-MSCs proliferation, with 1.4-fold change compared to FCS ($p<0.05$, N=7, Figure 2A-C). In agreement with the CFU-F results, PL significantly shortened the doubling time of SF-MSC cultures as compared to FCS expanded cells at the end of passage 0 ($p<0.05$, N=7, Figure 2D). There was no significant difference in colony numbers of SF-MSCs plated with PL or FCS (Figure 2E). To confirm that PL had no impact on SF-MSCs adhesion to the plastic, while affecting their proliferation, colony numbers were compared in different attachment conditions. Results showed that cells plated with either PL in the first 48 hours (PL48) or with FCS in the first 48 hours (FCS48), had no significant difference in the colony numbers (Figure 2F). In conclusion PL, significantly increased proliferation of SF-MSCs and had no significant impact on their adhesion to plastic.

Comparisons of PL-MSCs and FCS-MSCs trilineage differentiation

To assess the differentiation potential of PL-MSCs towards osteoblasts, adipocytes and chondrocytes compared to FCS-MSCs, MSC differentiation assays were performed using donor matched cultures grown in PL or under standard conditions. PL-MSCs showed significant potential to undergo chondrogenesis in complete chondrogenic media when compared to FCS-MSCs. After 21 days in culture with complete chondrogenic media, PL-MSCs and FCS-MSCs pellets were obtained, and positively stained for glycosaminoglycans (Figure 3A). The sGAG production quantified after pellet digestion revealed that PL-MSCs produced on average 10 % more sGAG compared to FCS-MSCs ($p<0.05$, N=5, Figure 3A). However, PL-MSCs cultured with complete osteogenic media for 21 days deposited on average 45 % less Ca^{++} compared to FCS-MSCs ($p<0.05$, N=5, Figure 3B). Alizarin red staining of PL-MSCs confirmed less Ca^{++} deposition compared to FCS-MSCs (Figure 3B). Finally, using complete adipogenic media, PL-MSCs showed no difference in the accumulated lipid vacuoles and the Nile red/DAPI ratio was comparable to FCS-MSCs (Figure 3C).

PL has the potential to act as chondrogenic inducer

PL was also evaluated as a chondrogenic inducer by either replacing TGF-β3 in the complete media or as chondrogenic inducer alone. These data showed that replacing 10 ng TGF-β3 with 20 % or 50 % PL in the basal chondrogenic media led to 69 % and 95 % increases in sGAG production by PL-MSCs, respectively, ($p\leq0.0001$ N=5, Figure 4A). Additionally, 20 % and 50 % of PL increased the sGAG produced FCS-MSCs 50 % and 84 % ($p<0.05$, N=5, Figure 4A). Furthermore, using high glucose DMEM with 50 % PL alone induced chondrogenesis in both cultures compared to the negative control (DMEM high glucose with 10 % FCS) with sGAG production comparable to that of complete chondrogenic media, ($p<0.0001$, N=5, Figure

4A). In conclusion replacing TGF-β3 with 20 % or 50 % PL has enhanced chondrogenic induction, while PL alone was sufficient to act as a chondrogenic inducer.

In osteogenesis, replacing FCS with PL in the complete osteogenic media significantly enhanced Ca^{++} deposition in PL-MSCs (by 135 %) and FCS-MSCs (by 96 %), ($p \leq 0.0002$, Figure 4B). Although, the replacement of FCS with PL in the complete osteogenic media enhanced Ca^{++} deposition, PL-MSCs showed less potential for osteogenesis compared to FCS-MSCs with Osteo–PL media ($p<0.01$, Figure 4B). Similarly, replacing FCS with PL in adipogenic media significantly increased the adipogenic induction by 40 % for PL-MSCs and 30 % for FCS-MSCs compared to the complete adipogenic media ($p<0.005$, Figure 4C).

PL maintains SF-MSCs immunomodulatory function

In order to assess whether the immunomodulatory function of SF-MSCs is maintained in the presence of PL, PL-MSCs and FCS-MSCs were co-cultured with activated PBMCs for 5 days (Figure 5A). Clusters of lymphocytes in stimulated cultures lacking SF-MSCs were clearly visible, indicating lymphocyte proliferation (Figure 5B). However, in co-cultures with PL-MSCs or FCS-MSCs, stimulated lymphocytes did not cluster, indicating the immunomodulatory effects of the SF-MSCs (Figure 5B). The proliferation index of viable $CD14^{-}CD4^{+}CFSE^{+}$ cells significantly decreased with increases in PL-MSCs or FCS-MSCs numbers ($p<0.0001$ for both co-cultures, N=3, Figure 5C-D). This indicated that SF-MSCs had the capacity to inhibit the $CD4^{+}$ T cells proliferation in a dose-dependent manner and that PL-MSCs maintained this immunosuppression capacity at all ratios. No significant difference in the inhibition of $CD4^{+}$ T cells proliferation between either FCS-MSCs or PL-MSCs was observed (Figure 5D).

Other studies have reported the immunosuppression capacity of the BM-MSCs and AT-MSC which is comparable to SF-MSCs, as 1:10 ratio of T-cells: MSCs was capable of suppressing

stimulated T-cells proliferation and PL did not alter immunosuppression capacity of the BM-MSCs [32] and AT-MSC cells [33].

Discussion:

Although it was historically thought that cartilage repair was the remit of BM-MSCs, there is increasing evidence from animal models for a pivotal or even dominant role for joint cavity MSCs including synovial fluid MSCs in cartilage repair [17, 18, 34, 35]. While there is a large amount of literature on the effect of PL on BM-MSCs, to the best of our knowledge this is the first study to investigate the effect of PL on SF-MSCs which are joint resident MSCs that have direct access to injured superficial cartilage. In this study, low number SF-MSCs were obtained from joint cavity and culture expanded before effects were studied. Given that SF-MSCs are likely to derive from synovium where native MSCs appear to be abundant *in vivo*[35],it is possible that the endogenous immunomodulatory activity within the joint is actually quite high. Our *ex vivo* findings indicate that PL has multiple effects on SF-MSCs including beneficial effects on their proliferation and chondrogenesis. These findings provide biological support for the concept that intra articular PL may have the potential to augment cartilage regeneration. Our findings of SF-MSCs immunomodulatory activity raise the possibility that allogenic SF-MSCs could be used in conjunction with PL for therapy development. These findings provide the impetus for further *in vivo* clinical research to optimally define the use of this strategy.

Since SF-MSCs have the advantage of direct access to the site of injury without the need to breach the subchondral plate [36], combining PL with SF-MSCs might be relevant for early cartilage repair. Our results showed that PL maintained the *in vitro* phenotype in agreement with the recommendation of the ISCT [37]. Moreover, PL shortened SF-MSCs doubling times and increased SF-MSCs proliferation as they displayed bigger colony sizes. Similar observations have been reported in several studies with other types of MSCs, which promoted

PL as the gold standard for cell proliferation [22, 38]. There is data to show that activated platelet rich plasma PRP injected into subchondral bone with full thickness cartilage defects can profoundly affect SF-MSC numbers [39].

The trilineage differentiation potential of SF-MSCs expanded in PL revealed that PL preconditioned SF-MSCs towards chondrogenesis compared to FCS-MSCs. The PL expansion of MSCs according to their sources (bone marrow or synovial derived) influencing *ex vivo* differentiation is intriguing. It has been shown that BM-MSCs expanded in PL have a greater capacity towards osteogenesis, while adipose-derived MSCs have more potential towards adipogenesis [24, 40, 41]. In this current work, we showed for the first time that PL favorably influenced SF-MSCs chondrogenesis (Figure 4). Taking these findings together, PL might modulate BM-MSCs towards osteogenesis and SF-MSCs towards chondrogenesis, with the latter capability being a potentially advantageous for intra-articular MSC manipulation, especially if PL were also to suppress SF-MSCs osteogenesis in the repair of partial thickness cartilage defects. A proposed mechanism of PL priming BM-MSCs toward osteogenesis suggests elevation of osteoblastic genes expression alkaline phosphatase (ALP) and osteopontin (OP), as ALP and OP expression were significantly high compared to FCS expanded cells [24] and future study is warranted to investigate the molecular mechanisms of chondrogenic induction by PL in SF-MSCs.

There is an interest in the use of third party "off the shelf" allogeneic MSCs for convenient intra articular injection or in combination with scaffolds for one stage cartilage repair strategies. In this work, we demonstrated for the first time that SF-MSCs has immunosuppression capacity which could support use of third party allogeneic SF-MSCs in addition to their enhanced chondrogenic differentiation capacity. A similar observation was reported for BM-MSCs and AT-MSC expanded in PL [32, 33, 42]. However, two previous studies have reported that PL expansion might in fact reduce MSCs immune suppression capability [43, 44], yet in these

studies different methods of PL preparation were used, compared to the PL used in this study. Copland et al. suggested this suppression of MSCs immunomodulation was due to the presence of fibrinogen, since a direct interaction between MSCs and fibrinogen lead to increased levels of IL-6, IL-8 and MCP-1 protein production and reduced the MSCs ability to up-regulate indoleamine dioxygenase (IDO) [43]. Although other studies have used fibrinogen rich PL, pooled from group O-typed and AB plasma donors, which showed that PL maintained immunomodulation of MSCs [20, 45]. Therefore, the presence of fibrinogen and ABO blood donor pooling might impact PL immunomodulation capacity. In this study, we used fibrinogen-depleted PL in our cultures; which may explain our observations that PL had no detrimental effect of SF-MSCs ability to suppress $CD4^+$ T cells proliferation. This could indicate that the introduction of PL in the joint cavity unlikely to alter the SF-MSCs capacity for immunosuppression. Further studies are required to investigate the impact of fibrinogen in PL on SF-MSCs immunomodulation.

TGF-β is an important factor for cartilage maintenance *in vivo* and it is a major inducer of chondrogenesis *in vitro* [46]. Moreover, TGF-β reduces activity of IL-β1, degradation of type II collagen, and proteoglycan in OA [47]. Complete chondrogenic media is composed of several inducers combined with TGF-β and it was shown that the combination of TGF-β and IGF-I enhances chondrogenic differentiation [48, 49]. To the best of our knowledge, our strategy of incremental TGF-β substitution with PL and looking at ensuing degrees of *in vitro* chondrogenesis is novel since there was no data on PL as chondrogenic inducer and as replacement of TGF-β in complete chondrogenic media. There is also only limited data of the use of PRP concentrate (as a gel) with complete chondrogenic media, consistent with our study it showed enhanced chondrogenesis [50]. The results presented here showed that PL has a potential to act as chondrogenic inducer, as chondrogenic media containing 20 % PL was

sufficient to replace 10 ng TGF-β and induce chondrogenesis. The amount of TGF-β in 100 % PL (Stemulate) is approximately 75 ng, according to the manufacturer, in this study we use 20 % PL which contains approximately 14.4 ng TGF-β to successfully induce chondrogenesis of SF-MSCs. As the increase of sGAG production with 20 % PL was more than 69 % compared to complete chondrogenic media, this suggests there are other inducers in PL which aided in the chondrogenic enhancement. Interestingly, our findings showed that PL alone can also induce chondrogenesis similar to complete chondrogenic media as indicated by sGAG production.

On the other hand, in osteogenesis replacing FCS with PL enhanced Ca^{++} deposition for both PL-MSCs and FCS-MSCs. However, PL-MSCs showed less Ca^{++} deposition compared FCS-MSCs which collectively supports that PL-MSCs are per-conditioned toward chondrogenesis. Taking the above findings our data supports the concept of using PL and SF-MSCs as tool for cartilage repair.

In summary, PL has the potential to promote SF-MSCs proliferation, maintain immunophenotype and immunosuppression, and pre-conditions SF-MSCs towards chondrogenesis. These findings suggest that PL introduced into the joint cavity could influence local, resident MSCs and that this might be relevant for OA therapy development. Our findings provide a basis toward the further exploration of PL and SF-MSCs therapy cellular therapy for early OA.

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Disclosure of Interest:

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Figure Legends

Figure 1. Immunophenotype of cultured FCS-MSC and PL-MSC analyzed by flow cytometry. Isotype control (dark histograms) and cells stained with antibody against the surface proteins (light histogram). The results presented are Mean percentage expression ± SD, N=3 donors-matched.

Figure 2. SF-MSCs proliferation**. (**A) A representative CFU-F plate seeded in FCS media and single colony under 100x magnification. (B) CFU-F plate seeded in PL media and single colony under 100x magnification. (C) Average colony diameter in mm. (D) Cultured SF-MSCs doubling time at p0. (E) Average colony numbers in CFU-F/ml. (F) Impact of PL on SF-MSCs adhesion; cells were divided into 2 groups: FCS (48h): cells seeded in FCS media for 48 hours before exchanging to 5% PL media for the next 12 days. PL (48h): cells seeded with 5 % PL media for 48 hours before exchanging to FCS media for the next 12 days. Wilcoxon matched-pairs test, *p<0.01, N=7 donor-matched samples.

Figure 3. SF-MSCs expanded with PL have greater chondrogenic capacity (A) PL-MSCs and FCS-MSCs pellets after 21 days culture in complete chondrogenic media, scale bar represents 1 mm. Insert, stained section of the chondrogenic pellet with 1 % toluidine blue and sGAG production from donor matched cultures. (B) Alizarin Red staining of PL-MSCs and FCS-MSCs after 21 days culture in complete osteogenic media and Ca^{++} deposition from donor matched cultures. (C) Oil Red staining of PL-MSCs and FCS-MSCs after 21 days culture in complete adipogenic media (complete Adipo) and lipid production quantification using the Nile Red/DAPI fluorescent ratio. Wilcoxon matched-pairs test, *p<0.05, N=5 donors.

Figure 4. PL as trilineage differentiation inducer. (A) sGAG production of PL-MSCs and FCS-MSCs with different chondrogenic media: Complete chondro: complete chondrogenic media; basal-20 % PL: basal chondrogenic media supplemented with 20 % PL; basal-50 % PL: basal chondrogenic media supplemented with 50 % PL; DMEM-50 % PL: high glucose DMEM with 50 % PL only, control; DMEM high glucose with 20 % FCS only. (B) Ca^{++} deposition of PL-MSCs and FCS-MSCs with complete osteogenic media and PL-Osteo: osteogenic media with 10 % PL instead of FCS. (C) Nile Red/DAPI fluorescent ratio of PL-MSCs and FCS-MSCs with complete adipogenic media (complete Adipo) and PL-Adipo: Adipogenic media used 10 % PL instead of FCS, two-way ANOVA–Bonferroni's multiple comparisons test, ****p<0.0001, ***p<0.0005, **p<0.005, *P<0.01, N=5 donors.

Figure 5. Immunomodulation of PL-MSCs and FCS-MSCs. PMBCs co-cultured for 5 days with either with FCS- MSCs or PL-MSCs at the following ratios 1:1, 2:1, 5:1, and 10:1, Stimulated (positive control): PMBCs with no SF-MSCs. CD3/CD28 beads were added to all wells except non-stimulated (negative control) 1:0. (A) illustration of immunomodulation

experiment steps. (B) Representative light microscopic image at day 5. (C) Representative Modfit graphs showing $CD4^+$ T cells populations, blue peak represents parent population with subsequent generations represented in multiple colour. (D) Proliferation index of T-lymphocyte. n=3, two-way ANOVA, Bonferroni post t test, ****p<0.0001, N=3 donors.

Figures:

Figure 1

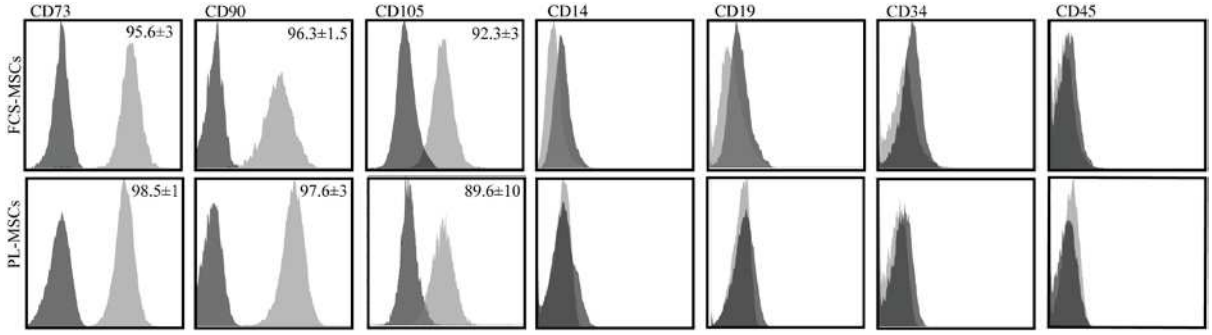


Figure 2

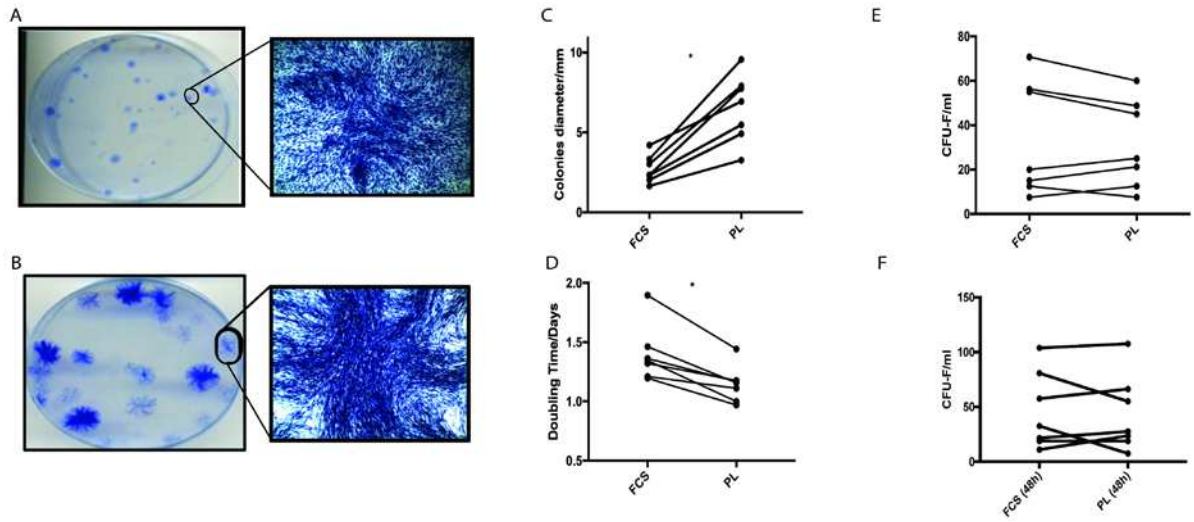


Figure 3

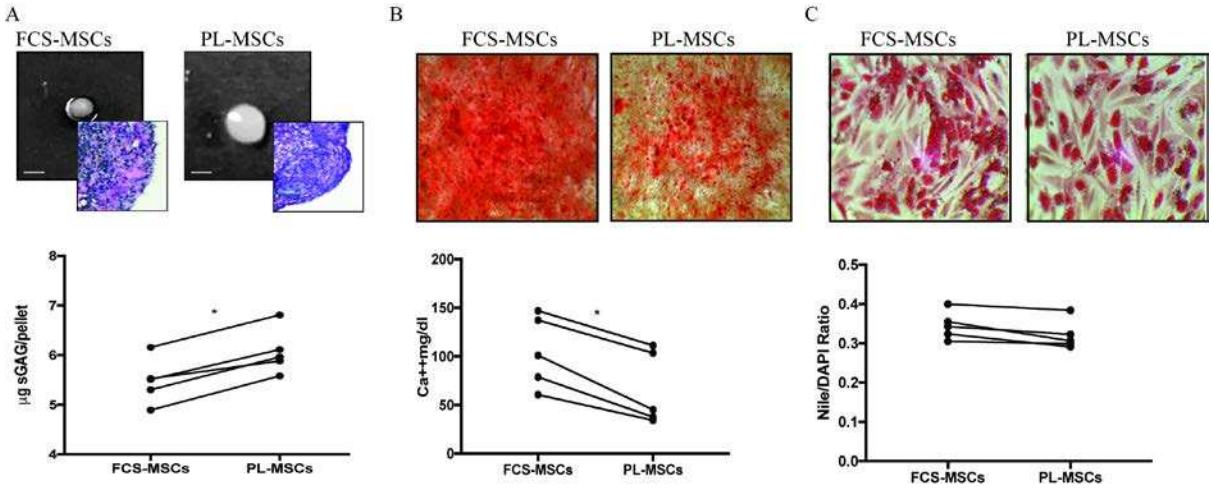


Figure 4

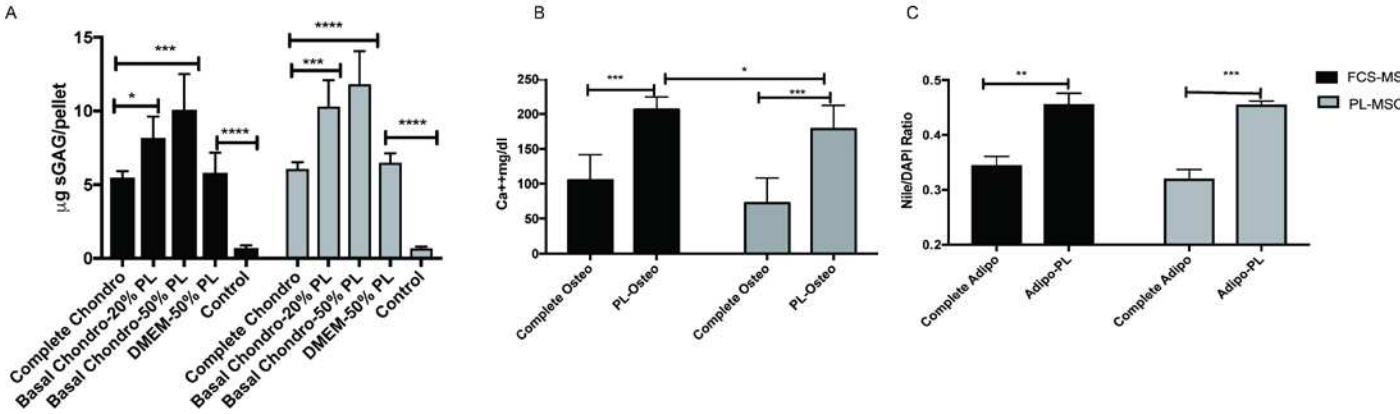
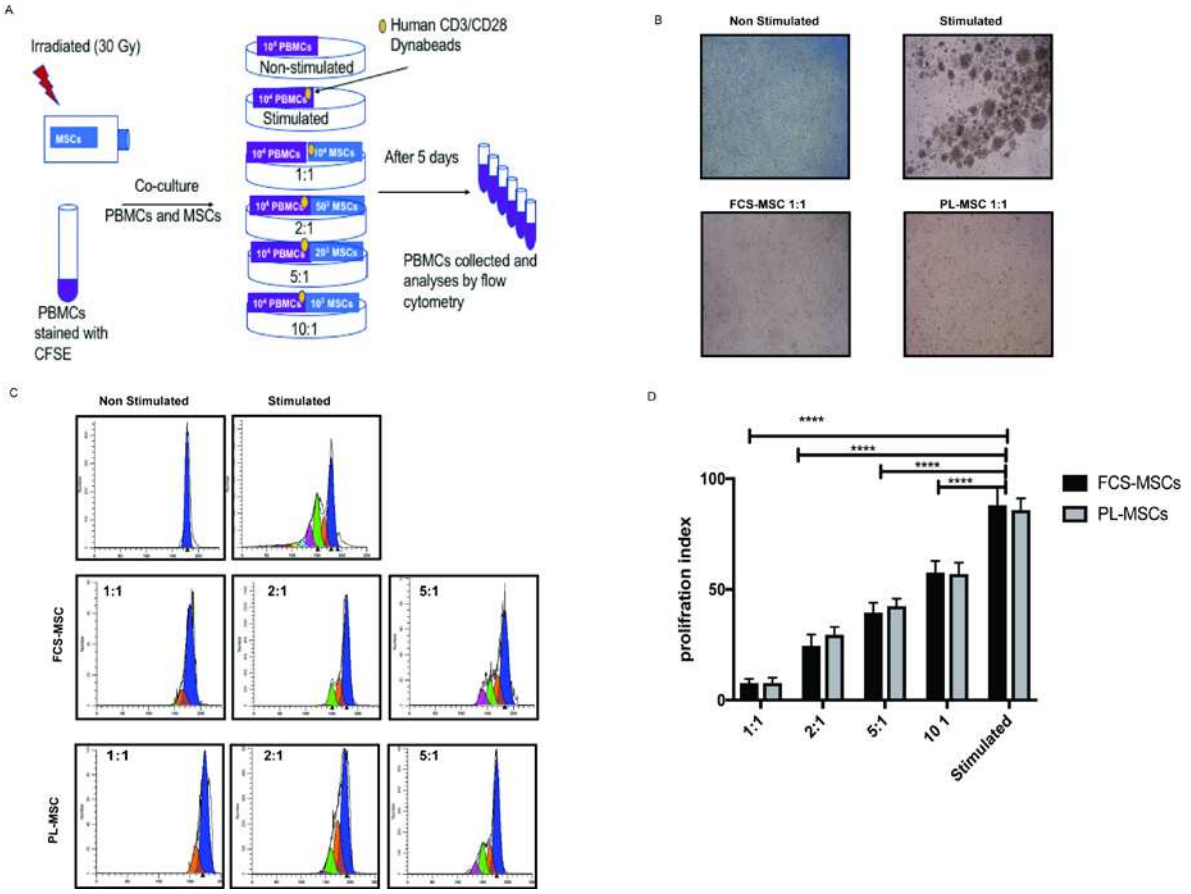


Figure 5


A
Irradiated (30 Gy)
MSCs
Co-culture
PBMCs and MSCs
PBMCs stained with CFSE
Human CD3/CD28 Dynabeads
Non-stimulated
Stimulated
1:1
2:1
5:1
10:1
After 5 days
PBMCs collected and analyses by flow cytometry
B
Non Stimulated
Stimulated
FCS-MSC 1:1
PL-MSC 1:1
C
Non Stimulated
Stimulated
FCS-MSC
PL-MSC
1:1
2:1
5:1
D
proliferation index
FCS-MSCs
PL-MSCs
Stimulated