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Soluble factors differ in platelets derived from separate niches: a pilot study comparing the secretome of peripheral blood and bone marrow platelets

Ryan C. Dregalla, PhD[1,2,*], Jessica A. Herrera, BS[1,2], Edward J. Donner, MD[1,2,3]

[1] Elite Regenerative Stem Cell Specialists, LLC, Johnstown, Colorado, USA
[2] R&D Regenerative Laboratory Resources, LLC, Johnstown, Colorado, USA
[3] Colorado Spine Institute, PLLC, Johnstown, Colorado, USA

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ABSTRACT

Background aims: Platelet-rich plasma (PRP) and bone marrow aspirate are commonly used in orthobiologics for their anti-inflammatory, anabolic/regenerative and immunomodulatory characteristics via platelet degranulation and cell secretions. Although platelets are derived from megakaryocytes in the bone marrow, no attention has been paid to the potential benefits of bone marrow platelets and whether their contents differ from aging platelets in peripheral blood.

Methods: In the present study, leukocyte-poor peripheral blood-derived platelets in plasma (LPP) and leukocyte-poor bone marrow platelets in plasma (BMP) were prepared from six donors, activated with calcium chloride, incubated and sampled at day 0, day 3 and day 6. LPP and BMP are platelet preparations intended to evaluate the respective platelet secretomes but are not classified as conventional PRPs, as they are not concentrated to the extent necessary to meet the qualifying criteria. At each time point, 15 growth and immunomodulatory factors were quantitated in LPP and BMP: platelet-derived growth factor AA, basic fibroblast growth factor/fibroblast growth factor 2, granulocyte-macrophage colony-stimulating factor, hepatocyte growth factor, macrophage colony-stimulating factor, stem cell factor, vascular endothelial growth factor, tumor necrosis factor alpha, IL-1β, interferon gamma, IL-4, IL-10, IL-1 receptor antagonist protein, IL-12p40 and arginase-1.

Results: The results illustrate that platelets derived from bone marrow have a unique secretome profile compared with those derived from peripheral blood, with significant differences in anti-inflammatory cytokines, which are associated with monocyte polarization.

Conclusions: Ultimately, bone marrow-derived platelets may be useful as a stand-alone orthobiologic or as an effective adjuvant to autologous cell therapies where anti-inflammatory and anabolic processes are desired, especially with respect to monocyte function.

Introduction

The field of orthobiologics relies heavily on autologous cell therapies (ACTs) derived from peripheral blood (PB) platelets, in the form of platelet-rich plasma (PRP), bone marrow concentrate (BMC) and micronized adipose tissue, as methods to resolve pain via mitigating inflammation [1–3]. In musculoskeletal (MSK) conditions, injuries associated with either chronic or acute inflammation result in pain and are often characterized by catabolic processes that must be addressed prior to resolution of the injury [4]. ACTs are thought to act as a source of cytokines and chemokines [5–7], collectively known as the secretome, which are suitable for reducing the production of inflammatory proteins created by infiltrating leukocytes, resident synoviocytes and chondrocytes [8,9]. With regard to PRP, platelets are used as a reservoir of various bioactive factors that serve to stimulate the proliferation and differentiation of resident and progenitor cells in diseased tissues [10], a key attribute for its use as a stand-alone orthobiologic and an adjunct to other ACTs. Although recent clinical and laboratory studies have highlighted the benefits of ACTs for various orthopedic/MSK conditions, a full understanding of the innate properties of each product has yet to be realized [11–13].

Platelets originate from megakaryocytes, which are large polyploid cells in the bone marrow (BM). Platelets form through a multistep process that begins with the extension of megakaryocyte pseudopodia, resulting in lengths ranging from 250 μm to 500 μm. These elongated processes create pro-platelets—with their

* Correspondence: Ryan C. Dregalla, PhD, Elite Regenerative Stem Cell Specialists, LLC, 4795 Larimer Pkwy, Johnstown, Colorado 80534, USA.
E-mail address: rdregalla@dremedtech.com (R.C. Dregalla).

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characteristic "beads on a string" appearance—which are loaded with platelet-specific cargo [14]. Pro-platelets are released from the megakaryocyte and enter the fenestrated vasculature as pre-platelets, ranging from 2 μm to 10 μm in size, which undergo fission, creating a platelet ranging from 1 μm to 3 μm in size [15].

During biogenesis, megakaryocytes package platelet granules with proteins from the BM niche and platelets continue to endocytose BM plasma proteins prior to entering PB [14,15]. While in circulation, platelets acquire PB plasma proteins via endocytosis [16] and continue translating messenger RNA packaged by the megakaryocyte [17]. Taken together, these features provide a rationale for investigating the potential differences in the secretome of BM-derived platelets and those in circulation. Any contrast may highlight the distinct therapeutic benefits of the respective platelet sources and expand the basis for the use of platelets in MSK injuries [18,19].

In the present pilot study, the authors aimed to determine whether there are innate differences in the secretome of BM- and PB-derived platelets. Here leukocyte-poor PB platelets in plasma (LPP) and leukocyte-poor BM platelets in plasma (BMP) were assayed for an array of growth factors and immunomodulatory cytokines known to be released by platelets over a 6-day time course. The factors assayed include platelet-derived growth factor AA (PDGF-AA), basic fibroblast growth factor/fibroblast growth factor 2 (bFGF/FGF-2), granulocyte-macrophage colony-stimulating factor (GM-CSF), hepatocyte growth factor (HGF), macrophage colony-stimulating factor (M-CSF), stem cell factor (SCF), vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), IL-1β, interferon gamma (IFN-γ), IL-4, IL-10, IL-1 receptor antagonist protein (IRAP), IL-12p40 and arginase-1. Based on the features of platelet biogenesis and the evolving changes in platelets while in peripheral circulation, the authors hypothesized that there would be a difference in the secretome of platelets from the two respective niches over the observed time course.

Methods

Donor demographic information

Six patients previously scheduled for a BMC procedure provided written consent to donate both unprocessed BM aspirate and whole blood. Donated specimens were not used for therapeutic purposes. Donors included four females aged 65, 65, 67 and 51 and two males aged 51 and 60. All donors were free of any blood or systemic condition/disease.

PB and BM aspirate processing

For each donor, 8.5 mL of PB and BM aspirate was collected in 1.5 mL of 4% sodium citrate 40 mg/mL (anticoagulant) (NDC 0942-9505-10; Fenwal, Lake Zurich, IL, USA) to create a final concentration of 15% anticoagulant v/v. Samples were centrifuged in separate 15-mL conical tubes (229411; CellTreat, Pepperell, MA, USA) at 1600 rpm for 8 min using a Sorvall ST16 (Thermo Fisher Scientific, Waltham, MA, USA). Following centrifugation, leukocyte-poor platelet suspensions were created by collecting the top half of the plasma portion (~2.5 mL) from each tube, resulting in LPP and BMP. These preparations are not PRPs by conventional criteria, as a therapeutic PRP is identified as having $\geq$1 million platelets/μL and/or approximately 4-fold over baseline (blood) counts [20,21]. Each donor was represented as a distinct symbol in all data sets.

Platelet and leukocyte quantification

All flow cytometry was performed using a CytoFLEX S (Beckman Coulter, Brea, CA, USA). After collecting LPP and BMP, 10 μL of each preparation was combined with 5 μL of anti-human CD45 conjugated to fluorescein isothiocyanate (304054; BioLegend, San Diego, CA, USA), 5 μL of anti-CD61 conjugated to allophycocyanin (336412; BioLegend) and 80 μL of phosphate-buffered saline (1010-023; Thermo Fisher Scientific). The samples were incubated at room temperature in the dark for 5 min. After staining, 10 μL was taken out and placed into a new tube containing 90 μL phosphate-buffered saline and run for analysis. The sample was run for 60 seconds on the slow setting to ensure at least 10 000 events were recorded. Regions were set separately for leukocytes and platelets based on size (forward scatter) and complexity (side scatter) to minimize background noise. Gating criteria for signal were based on samples prepared the same way with the respective isotypes, fluorescein isothiocyanate-conjugated mouse IgG1 (400108; BioLegend) and allophycocyanin-conjugated mouse IgG1 (400122; BioLegend).

Activation, sampling and storage of BMP and LPP preparations

A total of 600 μL of LPP and BMP was collected and placed in the respective microcentrifuge tube (229441; CellTreat), and 66.6 μL of 100 mg/mL calcium chloride stock (793639; Sigma-Aldrich, St Louis, MO, USA), 10 mg/mL (90.1 mM) final concentration, was added to each sample and incubated at 37°C over the 6-day time course to induce activation. Immediately after adding the calcium chloride to LPPs and BMPs, 150 μL of the sample was sampled (day 0). This was repeated at day 3 and day 6. At the time of each collection, to create cell-free plasma prior to freezing for storage, plasma was centrifuged at 10 000 rpm for 90 seconds. The supernatant was collected, placed in a fresh microcentrifuge tube and stored at −20°C until assayed.

Protein quantification

Protein quantification was determined using BioLegend LEGENDplex multi-analyte flow assay kit mix and match panels human macrophage/microglia and human growth factor. The human macrophage/microglia panel consisted of six analyte beads—TNF-α capture beads A5 (740513), IL-4 capture beads A7 (740515), IL-10 capture beads A8 (740516), IL-1β capture beads A10 (740517), IRAP capture beads B4 (740520) and IFN-γ capture beads B7 (740523)—for all six donors. In the latter four of the six donors, capture beads for arginase-1 (740518) and IL-12p40 (740521) were added to the panel. The human growth factor panel consisted of seven analyte beads—FGF-basic capture beads A7 (740184), GM-CSF capture beads A10 (740186), HGF capture beads B2 (740187), M-CSF capture beads B3 (740188), PDGF-AA capture beads B4 (740189), SCF capture beads B6 (740191) and VEGF capture beads B9 (740193)—which were used to assay all six donors. All samples were run in accordance with the manufacturer's instructions.

Data normalization

Platelet counts and protein concentrations were determined as described. If a sample in a data set reported below the detectable limit, to retain the same sample size between LPP and BMP, the value used was the minimum detectable concentration (e.g., a value <0.24 pg/mL was assigned 0.24 pg/mL) and was a conservative representation of the low value (i.e., did not falsely further distance the sample value from values at detectable levels). The number of μL required from each sample to achieve 1 million platelets was determined. The concentration of each protein in pg/mL (equivalent to fg/μL) was multiplied by this figure, normalizing the concentration in fg per 1 million platelets at each time point. Individual normalized values were used for statistical analysis and are represented in figures.

Statistical analysis

For each data set, values for LPP and BMP were analyzed via Prism 8.4.3 (GraphPad Software, San Diego, CA, USA) using a two-tailed

independent *t*-test with a 95% confidence interval. This statistical analysis was selected to appropriately test for differences between the two independent platelet populations.

Results

Platelet and leukocyte quantification and cytokine normalization to platelet count via PDGF-AA

Per μL, the mean platelet count was significantly higher in LPP (207 626 ± 38 442) compared with BMP (98 697 ± 25 637) ($P< 0.05$), and there was no significant difference in leukocyte content (9.0 ± 7.257 and 221.7 ± 168.8, respectively) (Figure 1A). Following normalization, PDGF-AA in fg/1 million platelets did not differ between LPP and BMP preparations, demonstrating appropriate cytokine normalization based on platelet count (Figure 1B).

Quantification of growth factors

bFGF/FGF-2, GM-CSF, SCF and VEGF

Compared with LPP, there was no significant difference in the concentration of cytokines bFGF/FGF-2, GM-CSF, SCF and VEGF compared with BMP (data not shown).

M-CSF

At day 0, there was no significant difference in HGF concentration between LPP and BMP. At day 3 and day 6, the mean M-CSF concentration in fg/1 million platelets was significantly elevated in BMP (2630.0 ± 567.8 and 2901.0 ± 623.1, respectively) compared with LPP (832.2 ± 263.8 and 919.0 ± 322.1, respectively) ($P< 0.05$ each day) (Figure 2A).

HGF

At day 0, HGF was elevated in plasma derived from BMP (4063 ± 1075.0 fg/1 million platelets) compared with LPP (305.3 ± 64.7 fg/1 million platelets) ($P< 0.01$) (Figure 2B). Concentrations of HGF increased in BMP plasma at day 3 (6509 ± 1130.0 fg/1 million platelets) and day 6 (6180 ± 1063.0) relative to day 0, differing significantly from LPP at both time points (274.7 ± 69.5 and 295.6 ± 67.7 fg/1 million platelets, respectively) ($P< 0.001$ at day 3 and day 6) (Figure 2B).

Quantification of immunomodulatory factors

TNF-α, IL-1β, IFN-γ and IL-12p40

Compared with LPP, there was no significant difference in the concentration of cytokines TNF-α, IL-1β, IFN-γ and IL-12p40 compared with BMP (data not shown).

IL-4

At day 0 and day 3, there was no significant difference between the LPP control and BMP. At day 6, levels of IL-4 in BMP (25.14 ± 5.34 fg/1 million platelets) were significantly elevated compared with LPP (8.612 ± 2.102) ($P< 0.05$) (Figure 3A).

IL-10

IL-10 was significantly increased in fg/1 million platelets in BMP compared with LPP at day 0 (8.53 ± 0.71 and 3.71 ± 0.72, respectively) ($P< 0.001$), day 3 (8.65 ± 1.56 and 2.65 ± 0.46, respectively) ($P< 0.01$) and day 6 (7.41 ± 0.73 and 2.48 ± 0.27, respectively) ($P< 0.001$) Figure 3B).

IRAP

At day 0, IRAP was significantly elevated in BMP compared with LPP (5472.0 ± 1073 fg/1 million platelets compared with 808.3 ± 107.4 fg/1 million platelets) ($P< 0.01$) (Figure 3C). At day 3, the average IRAP concentrations per 1 million platelets in both BMP and LPP were substantially reduced relative to day 0 and did not differ statistically from one another. At day 6, IRAP concentration dropped further in LPP plasma to 18.21 ± 6.51 fg/1 million platelets and was significantly lower compared with BMP IRAP levels (1381.0 ± 496.9 fg/1 million platelets) ($P< 0.05$) (Figure 3C).

Arginase-1

Despite appreciable differences in mean levels at day 0, there was no significant difference between BMP (7783.0 ± 4888.0 pg/1 million platelets) and LPP (20.83 ± 4.174 pg/1 million platelets) plasma levels of arginase-1. At day 3, levels of arginase-1 increased substantially in BMP plasma samples (12 059 ± 4180 pg/1 million platelets) and were significantly elevated compared with LPP plasma levels (52.91 ± 13.38 pg/1 million platelets) ($P< 0.05$) (Figure 3D). At day 6, arginase-1 concentrations in BMP plasma remained significantly elevated compared with concentrations in LPP plasma (12 932.0 ± 4716 and 102.1 ± 54.15 pg/1 million platelets, respectively) ($P< 0.05$) (Figure 3D). Overall, the average levels of arginase-1 were substantially higher in BMP compared with LPP but with considerable variability across BMP donor samples. It is important to note that the levels of arginase-1 are reported on a scale of pg per 1 million platelets, a concentration 1000-fold higher than all cytokines/proteins assayed (fg).

Discussion

PRP and BMC are commonly used in orthobiologics for their anti-inflammatory, anabolic/regenerative and immunomodulatory characteristics via platelet degranulation and cell secretions. Although

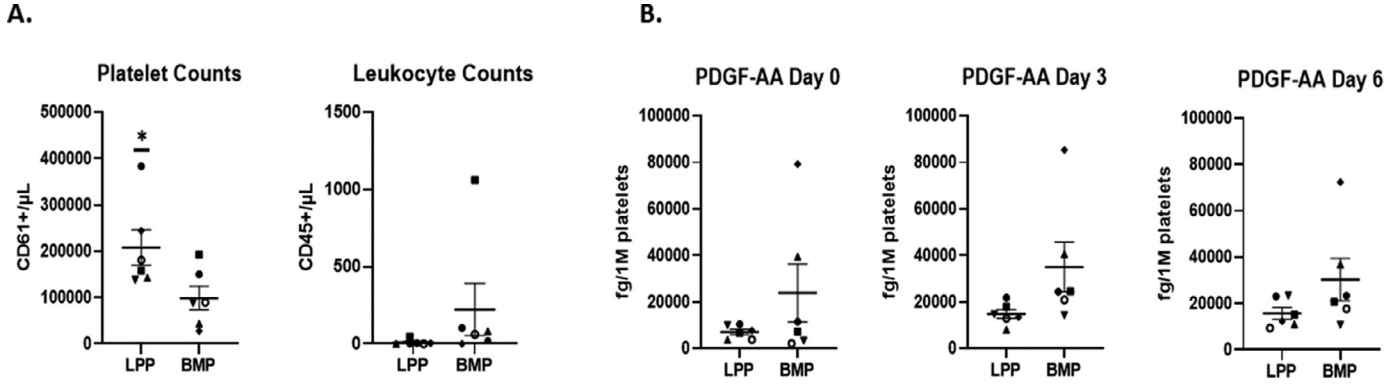

Fig. 1. (A) Platelet and leukocyte count per μL for preparations of LPP and BMP from six donors, each represented by a symbol. (B) Normalization of PDGF-AA levels per 1 million platelets. Graphs show mean and standard error of the mean. *$P < 0.05$. 1M, 1 million.

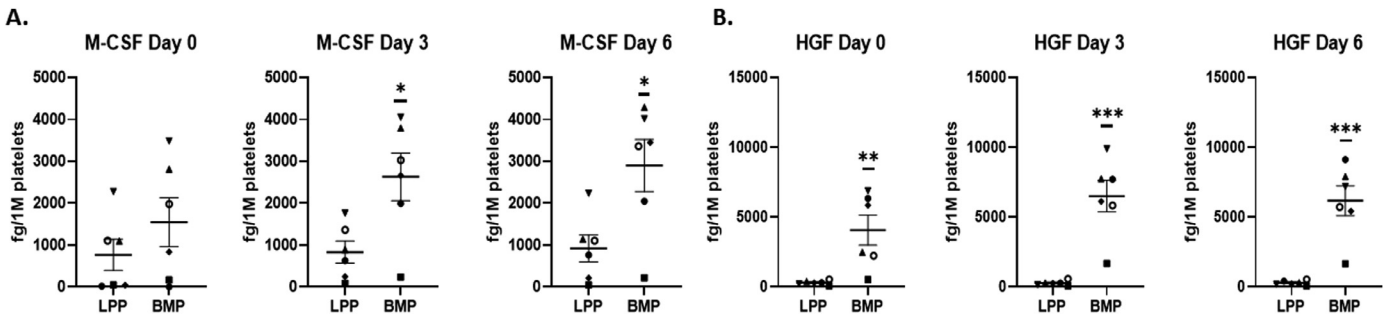

Fig. 2. Relative levels of the growth factors (A) M-CSF and (B) HGF from six donors over the 6-day time course. Symbols represent each donor. Graphs show mean and standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. 1M, 1 million.

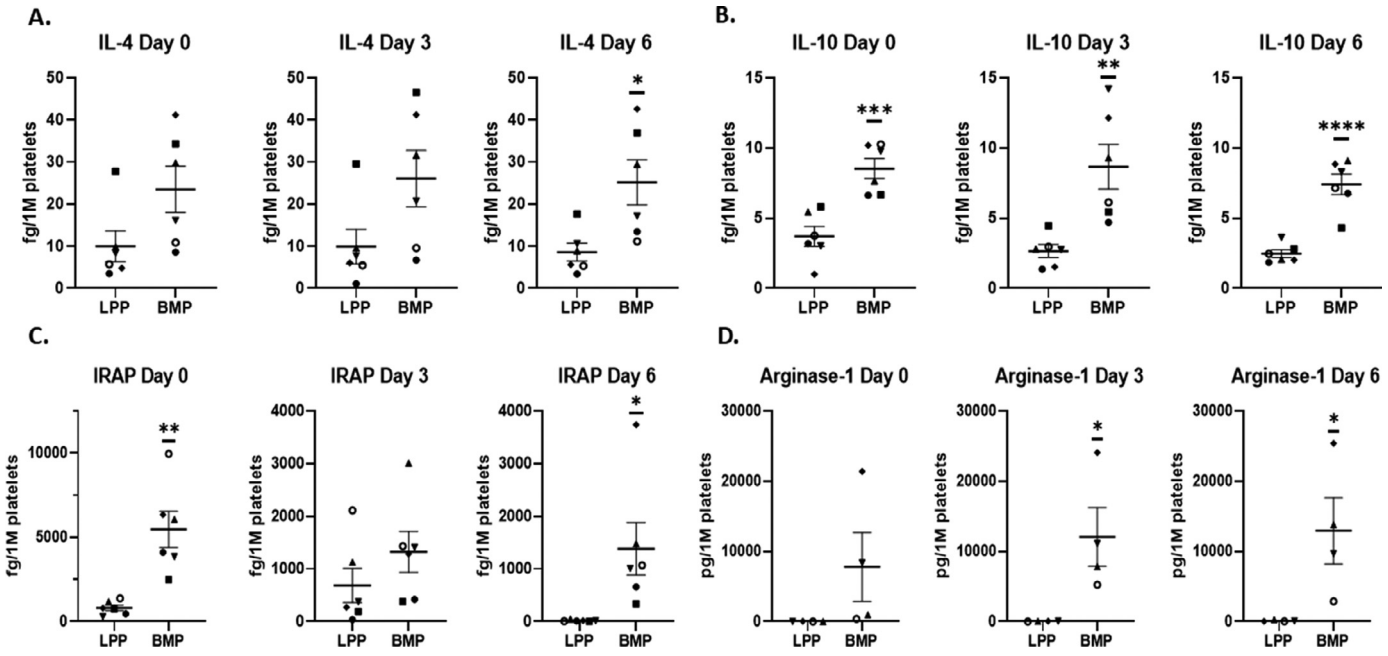

Fig. 3. Relative levels of the immunomodulatory factors (A) IL-4, (B) IL-10 and (C) IRAP from six donors and (D) arginase -1 from four donors over the 6-day time course. Each donor is represented by a symbol. The respective symbols used for arginase-1 detection pair with the donor symbols used throughout. Graphs show mean and standard error of the mean. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. 1M, 1 million.

considerable attention has recently been given to differences in PB PRP preparations, including hematocrit, platelet concentration over baseline and leukocyte content, little discussion has surrounded platelets derived from BM. In the present study, concentrations of 15 growth factors and immunomodulators were determined in (leukocyte-poor) LPP and BMP preparations over a 6-day time course, and factors were normalized to the respective platelets counts. Cytokine levels at day 0 represent innate levels in each niche. Cytokines inherently have a short half-life, ranging from minutes to hours [22–25], and dynamic changes over the time course are a balance between degradation and platelet-dependent release. Therefore, continued detection of the factors over 6 days and relative changes in cytokine levels in BMP compared with LPP provide insight into the differential secretome of platelets from the respective niches. Although the cell source of the cytokines assayed is variable and not exclusive to platelets, PDGF-AA, bFGF/FGF-2, GM-CSF, HGF, M-CSF, VEGF, TNF-α, IL-1β, IFN-γ, IL-12, IL-4, IL-10, IRAP/IL-1Ra and arginase-1 have all been reported to be released by platelets [25–31]. PDGF-AA, which is preferentially secreted by platelets, was used to normalize cytokines to platelet count.

The authors' initial platelet counts revealed an elevated number of platelets in LPP compared with BMP, which may be due to larger pro- and pre-platelets existing in the BM that reside in the (uncollected) buffy coat following centrifugation. As a validation for the authors' normalization approach, the platelet-secreted growth factor PDGF-AA was used over the time course, which in principle would show little variability between the samples per 1 million platelets. Indeed, through all time points there was no significant difference between the LPP and BMP groups with respect to PDGF-AA. Of the factors assayed, BMP did not result in a significant difference in bFGF/FGF-2, GM-CSF, SCF, VEGF, IL-1β, IFN-γ or IL-12p40 levels compared with the LPP control at any time point. By contrast, significant differences were seen in IRAP, M-CSF, HGF, IL-4, IL-10 and arginase-1. Cytokines detected in this study were classified as growth factors and immunomodulatory factors based on their conventional function; however, several of these growth factors have immunomodulatory functions as well [32–36].

The therapeutic benefit of IRAP as an immunomodulator is via antagonizing the IL-1 receptor, inhibiting intracellular signal transduction by IL-1β. The authors' results at day 0 mirrored the observations of Cassano *et al.* [37], in that BMP contained significantly higher levels of IRAP compared with LPP, demonstrating that IRAP levels in BM plasma are innately elevated. In the current study, the authors observed depleted IRAP levels at day 3 and day 6 in BMP relative to day 0, which was likely due to the short half-life of IRAP (on the order of hours) [38]. Importantly, IRAP concentrations per 1 million platelets in BMP differed from LPP at day 6, indicating a sustained release of IRAP in BM-derived platelets compared with PB-derived platelets.

There were significant differences in the relative levels of IL-4, IL-10, M-CSF and HGF, which were elevated in BMP compared with LPP over the 6-day time course. Importantly, each of these factors has key implications in the polarization of monocytes. Recently, monocytes/early macrophages have been found to be key regulators in the promotion of osteoarthritis [39], which involves polarization *in vivo* [40]. M1 polarization is catabolic and pro-inflammatory, whereas M2 polarization is anabolic, stimulating tissue regeneration and reducing inflammation [40–43]. *In vitro*, stimulation of monocytes with IL-4 and M-CSF has been shown to promote expression of CD206 [44] and, in conjunction with IL-10, M2 polarization [45,46]. With regard to HGF, this growth factor showed high levels relative to LPP at day 0 in BMP samples, with notable increases at the latter time points. This may provide therapeutic benefits that complement IL-4, IL-10 and M-CSF, as HGF is known to inhibit M1-polarized macrophage production of pro-inflammatory factors [47]. In addition, HGF has been noted to be a key factor in the anti-inflammatory effects of PRP [48]. Moreover, HGF is anti-fibrotic [49], which has significant implications in the treatment of diseased joints, and remarkably elevated in BMP relative to LPP.

Based on the role of immunomodulators and growth factors in monocyte polarization, both of which were elevated in the early phases of this study in BMPs, the M1-associated cytokine IL-12p40 and the M2-related enzyme arginase-1 were added to the authors' array beginning with the third donor. No significant differences were detected in IL-12p40 levels. In sharp contrast, arginase-1 levels in BMP showed the most remarkable differential compared with LPP. This enzyme is unique and known to be expressed by monocytes with an M2 phenotype and has a role in limiting nitric oxide production and promoting anti-inflammatory and anabolic processes [50]. Arginase-1 metabolizes L-arginine, which reduces the bioavailability of the amino acid extracellularly (and intracellularly when synthesized via monocytes), impairing nitric oxide production by monocytes/macrophages (M1 phenotype) [51]. In addition, the byproduct of L-arginine metabolism by arginase includes ornithine, which can be transported across the cell membrane from the extracellular environment [52] and serves as a precursor for the production of polyamines and proline, which are involved in cell proliferation, immunomodulation and collagen synthesis [51,53]. Taken together, the release of arginase-1 may be a valuable anti-inflammatory attribute of BM platelets.

In regenerative medicine, the use of orthobiologics is aimed at resolving imbalances in cellular activity, resulting in reduced pain and improved function. In this study, the trend observed across the cytokines evaluated characterized BMP as having a secretome related to monocyte function and an M2-promoting signature compared with LPP. The polarization and secretory activity of monocytes either promote or mitigate pain associated with an array of orthopedic conditions that are commonly treated with orthobiologics. Monocyte polarization toward an M1/pro-inflammatory phenotype or a deficiency in M2 phenotypes has been associated with low back pain [54], progression of intervertebral degenerative disc disease [55], neuropathic pain associated with spinal cord compression [56], tendinitis [57] and osteoarthritis progression [40] and severity [40,58]. Importantly, monocytes may have the unique ability to shift their polarization phenotype (i.e., from M1 to M2) [59]. The role of monocytes in pain and the progression of numerous orthopedic conditions suggests that targeting their polarization is a viable therapeutic approach. Based on the authors' results, BM-derived platelets may offer a unique benefit in mitigating M1 polarization.

Conclusions

The authors' results demonstrate that BM-derived platelets have a unique secretome compared with those in peripheral circulation and may be useful as a stand-alone therapy, when concentrated in BMC or as an adjuvant to micronized adipose tissue when treating chronic orthopedic conditions, especially those in which monocytes contribute to chronic inflammation.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: RCD and EJD. Acquisition of data: JAH. Analysis and interpretation of data: RCD and JAH. Drafting or revising the manuscript: RCD, JAH and EJD. All authors have approved the final article.

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