Research Article

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Regenerative Medicine

Pure platelet-rich plasma and supernatant of calcium-activated P-PRP induce different phenotypes of human macrophages

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Aim: This study aimed to evaluate the effect of two platelet preparations used in the clinic, pure platelet-rich plasma (P-PRP) and the supernatant of calcium-activated P-PRP (S-PRP), on the phenotype of human macrophages. **Materials & methods:** Surface markers and cytokine production of human monocyte-derived macrophages were analyzed after 24 h stimulation with P-PRP or S-PRP. **Results:** P-PRP and S-PRP present no difference in the expression of CD206, a M2 tissue-repair macrophage-related marker. However, these same macrophages presented different levels of CD163, CD86 as well as different IL-10 secretion capacities after 24 h incubation. **Conclusion:** These platelet preparations do not have an equivalent biological effect over macrophages, which suggest that they may present different clinical regenerative potentials.

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Keywords: growth factors • IL-10 • macrophages • mannose receptor CD206 • M2 macrophages • platelet-rich plasma • regenerative medicine • scavenger receptor CD163 • supernatant of activated platelet-rich plasma

Presently, platelet-rich plasma (PRP) and derived preparations are used in regenerative medicine to facilitate tissue and organ repair [1]. Products, such as PRP, autologous platelet lysates or activated PRP, have been reported to accelerate tissue regenerations of diabetic ulcers, as well as cutaneous, retinal and bone tissue injuries [1,2]. Nonetheless, extensive use of platelet preparations is still controversial, due to the great variability observed in clinical trials [3–5]. The diversity of existing protocols and the lack of a consensus terminology for platelet preparations have complicated the comparison of tissue regenerative effects [6–10]. Even now, many platelet products available for regenerative medicine are still considered to have an equivalent biological effect, and used indistinctively in the clinic [10,11].

Part of the beneficial effects of PRP are mediated by growth factors contained in platelets, such as the PDGF, TGF-β1, IGF-I, FGF-2, HGF and VEGF-A. All these growth factors and cytokines are stored in cytoplasmic granules and liberated during platelets activation induced by thrombin, collagen, PAF or calcium [12]. In some cases, clot formed after PRP activation can be discarded and the supernatant itself can be used as a platelet product for tissue-repair therapies, as it contains the growth factors released by activated platelets [13,14]. A large number of studies indicate that platelets and their released content are capable to modify the function, proliferation and migration of various types of cells involved in tissue repair, such as smooth muscle cells, mesenchymal stem cells, fibroblasts and macrophages [1,15,16].

Macrophages are immune cells that also participate of tissue repair and remodeling [17,18]. In response to diverse stimuli, macrophages are capable to polarize into different phenotypic profiles, traditionally simplified as proin-

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flammatory macrophages (M1) and reparatory-type macrophages (M2) [19]. Proinflammatory M1 macrophages can be generated by stimulation with bacterial lipopolysaccharides (LPS) in combination with IFN-γ [20]. This type of stimulation also increases secretion of TNF-α, which correspondingly inhibits macrophage polarization toward M2 reparatory macrophages [19]. M2 macrophages are associated with tissue repair and remodeling, are more heterogeneous and can be generated by various stimuli [21,22]. Commonly, M2 macrophages are identified by the expression of surface markers as the scavenger receptor CD163, the mannose receptor CD206 and the production of the cytokine IL-10 [20].

Platelets contain pro- and anti-inflammatory factors that participate in macrophage polarization [20,23]. Furthermore, platelet activation produces microparticles with immunoregulatory properties that are capable to polarize monocytes to tissue-resident macrophages type-M2 [24]. As different platelet preparations are used as equivalent biological effect in the clinic, it is not clear how these could distinctively affect immune cells that participate in tissue regeneration. To address this, in this study we characterized two commonly used platelet preparations: leukocyte-depleted 'pure platelet-rich plasma' (P-PRP), as defined by Dohan *et al.* [10], and the supernatant released from the calcium-activated P-PRP clot (S-PRP). We then compared their effects over the phenotype of human monocyte-derived macrophages.

Materials & methods

P-PRP preparation

Peripheral blood of 15 healthy donors between 18 and 60 years old, excluding patients with chronical diseases, sexually transmitted infections or a hemoglobin concentration under 12 g/l, was obtained from the Blood Bank of the Clinical Hospital of University of Chile in Santiago, Chile, after signed an informed consent. Blood samples were retrieved in 10 ml vacutainer tubes with sodium citrate, and centrifuged at 100 ×*g* for 5 min at room temperature. After centrifugation, three fractions are formed in the tube: red blood cells at the bottom; a platelet-enriched fraction corresponding to PRP in the middle; and a third fraction with less number of platelets in the upper fraction, here named platelet-low plasma (PLP). Carefully avoiding red blood cells, PRP and PLP fractions were retrieved from the tube, and transferred to a new vacutainer without anticoagulant. PLP was used as a control to compare the platelet and cytokine concentration with the PRP fraction. To prepare P-PRP, the PRP fraction was passed through a leukocyte filter (high-efficiency leukocyte reduction filtration system for platelets; Hemonetics, CA, USA) that retained residual leukocytes and microaggregates (PLP fraction was also filtered). Platelets and leukocyte concentrations in P-PRP and PLP from each donor were determined with a Hematologic Counter Machine (ADVIA 2120i; Siemens, CA, USA). Purity and activation degree of P-PRP were assessed by flow cytometry using a BD FACSCalibur flow cytometer (Becton-Dickinson, NJ, USA), after staining with antihuman monoclonal antibodies CD61-FITC (clone VI-PL2 conjugated with fluorescein isothiocyanate; BD Bioscience, NJ, USA) and CD62P-PE (clone AK-4 conjugated with phycoerythrin; BD Bioscience).

P-PRP activation & S-PRP preparation

P-PRP and PLP were activated with 50 µl/ml of 10% $CaCl_2$, and incubated at 37°C. After 10 min, a clot is formed. Activated P-PRP and PLP were incubated for 20 min more at room temperature to liberate the activated-platelet content during the clot retraction. Preparations were centrifuged at 2000× g for 10 min and S-PRP and activated PLP (S-PLP) were retrieved. Supernatants were passed by a 0.2-µm pore size filter to ensure elimination of any remaining platelets. Both S-PRP and S-PLP preparations cytokine levels of TGF-β, IL-1β, IL-10 and TNF-α were measured by ELISA (ELISA Ready-SET-Go!; eBioscience, CA, USA).

Generation of monocyte-derived macrophages

Buffy coats of 15 healthy donors between 18 and 60 years old, excluding patients with chronical diseases, sexually transmitted infections or a hemoglobin concentration under 12 g/l, were obtained from the Blood Bank of the Hospital del Salvador, Santiago, Chile, after signed an informed consent. Human monocytes were isolated and cultured, as previously described, with minor modifications [25]. Briefly, monocytes were isolated using the RosetteSep™ Human Monocyte Enrichment Cocktail (STEMCELL Technologies, Vancouver, Canada) according to the manufacturer's instructions. Residual erythrocytes and platelets were removed by ammonium-chloride-potassium lysing buffer and low-speed centrifugation (200 ×*g*), respectively. Monocyte-derived macrophages were obtained by culturing monocytes for 7 days at 37°C in RPMI 1640 (Gibco, Invitrogen Corporation, CA, USA) supplemented with 10% fetal bovine serum (HyClone, IL, USA), 50 U/ml penicillin, 50 µg/ml streptomycin

(Gibco, Invitrogen Corporation) and 50 ng/ml of M-CSF (MiltenyiBiotec, Bergisch Gladbach, Alemania) in 6-well plates at a density of 3×10^6 cells per well (2 ml total volume). Monocyte-derived macrophages were collected by a soft-cell scraping, and morphological features were evaluated by hematoxylin and eosin staining. Same monocyte-derived macrophages were stained with the antihuman monoclonal antibodies CD14-PE (clone M5E2; BD Biosciences, NJ, USA), CD32-APC (clone FLI8.26 conjugated with allophycocyanin; BD Biosciences) and CD11c-PE (clone B-ly6; BD Biosciences), and analyzed by flow cytometry. Macrophages incubated for an additional 24 h in conditioned medium were compared with proinflammatory-polarized macrophages. Proinflammatory macrophages were induced by replacing the culture medium for RPMI 1640 supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 µg/ml streptomycin and 100 ng/ml LPS (Sigma-Aldrich, MO, USA) plus 20 ng/ml IFN-γ (MiltenyiBiotec) for an additional 24 h at 37°C [25]. Phenotypic markers CD206-PECy5 (clone 1502 conjugated with phycoerythrin-cyanine 5; BioLegend, CA, USA) and CD163-PE (clone GHI/61; eBioscience) were measured by flow cytometry. TNF-α production was also measured in the culture supernatant by ELISA, to confirm a proinflammatory macrophage profile. Experimental n corresponds to different macrophage donors.

Macrophages stimulation with different dose of P-PRP

3×10^5 macrophagesof six individuals were stimulated with different amount of P-PRP (1 macrophage for 125–1000 platelets). These were coincubated in a 24-well plates for 24 h at 37°C. Expression of CD206 (antihuman monoclonal antibody CD206-FITC clone 19.2; eBioscience) on macrophages was analyzed by flow cytometry.

Macrophages stimulation with P-PRP or S-PRP

Aliquots of 300 µl of P-PRP with a concentration of 1×10^6 platelets/µl or 300 µl of S-PRP – obtained from P-PRP with an equal number of platelets – were added to 500 µl of culture medium with 3×10^5 macrophages (final proportion of 1 macrophage per 1000 platelets or platelet supernatant). Dilutions of 125–1000 platelets/ml were generated by adding phosphate-buffered saline (PBS). Coculture were incubated in 24-well plates at 37°C and 5% CO_2 for 24 h. As experimental controls, 300 µl culture medium alone or containing 17 µl of 10% $CaCl_2$ was added to additional macrophage cultures. After incubation, coculture supernatants were recovered and production of IL-10 and TNF-α was measured by ELISA. Macrophages were harvested and stained with antihuman monoclonal antibodies CD206-FITC (clone 19.2; eBioscience), CD163-PE (clone GHI/61; eBioscience) and CD86-PECy5 (clone IT2.2; eBioscience), and analyzed by flow cytometry.

Cytokine production on macrophages

IL-10 and TNF-α were measured by ELISA Ready-SET-Go! (eBioscience), using capture and detection antibodies and following manufacturer instructions. Cytokines were measured in triplicates.

Scanning electron microscopy analysis

Aliquots of 25 µl of P-PRP (3×10^5 platelets/µl) were added to 3×10^5 macrophages (1:1 ratio) in a 24-well plate, with glass cover slips for scanning electron microscopy (SEM) analysis. Platelets and macrophages were coincubated for 45 min at 37°C. Cocultures were washed once with PBS and fixed in 2% paraformaldehyde and 0.5% glutaraldehyde. Then, samples were processed as previously described [26], and examined utilizing the Jeol JSM-IT300LV scanning electron microscope (JEOL, CA, USA). Macrophages or platelets alone were also analyzed by SEM.

Statistical analysis

Data were analyzed by two-tailed Student's *t*-test or analysis of variance (ANOVA), as applicable, to determinate significant differences between treatments. Statistical significance was considered at p-values < 0.05. All statistical analyses were done in GraphPad Prism software.

Results

P-PRP & S-PRP preparation & properties

There are many protocols to generate platelet preparations for regenerative medicine. Some of these preparations use whole platelets, like PRP, while other studies use the growth factor-enriched content released by activated platelets. However, the mechanisms of action of these preparations are poorly understood and the equivalence

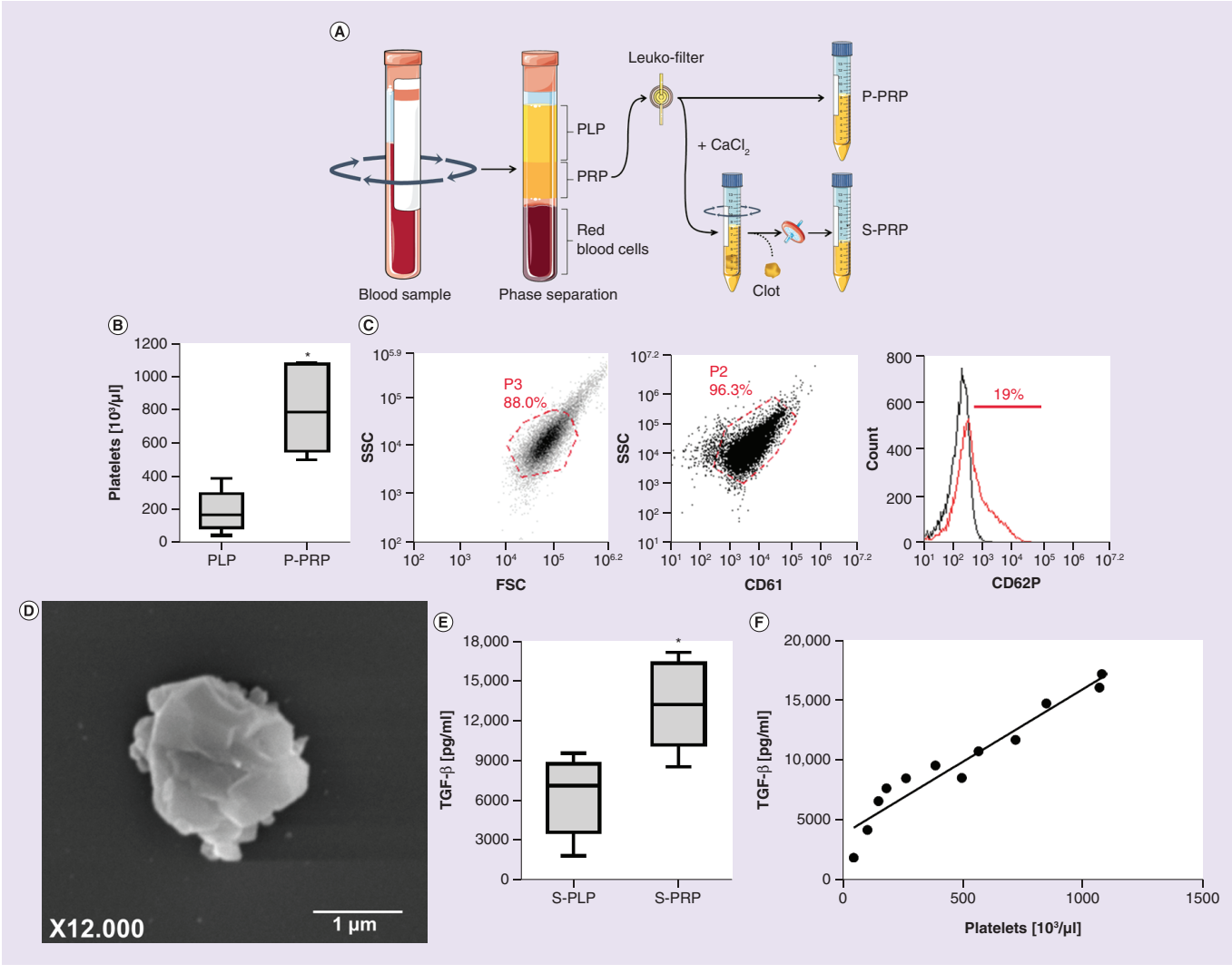

Figure 1. Pure platelet-rich plasma and supernatant of calcium-activated pure platelet-rich plasma preparation and properties. (A) Protocol of P-PRP and S-PRP preparation from whole blood samples. P-PRP purity was increased by using a leukocyte filter to retain residual leukocytes before further use. **(B)** Platelet concentration in P-PRP and PLP were measured by a Hematologic Counter Machine. Bars represent SE. n = 6, *p < 0.05 (paired *t*-test). **(C)** Expression of CD61-FITC and CD62P-PE surface markers on platelets, analyzed by flow cytometry. The gate (red) on the dot plot on the right shows the population selected by size (FSC) and complexity (SSC), the gate on the dot plot on the left indicate the population of CD61-positive cells (platelets) and the histogram shows the percentage of activate platelets in this gated population. **(D)** Platelet morphology in P-PRP analyzed by SEM at 12,000× magnification. No pseudopods were observed. **(E)** Concentration of TGF-β in S-PRP and the S-PLP measured by ELISA. Bars represent SE. n = 6. *p < 0.05 (paired *t*-test). **(F)** Relationship between TGF-β concentration and the number of platelets. The graphic shows a Pearson correlation coefficient r of approximately 0.96 with a p-value < 0.0001.

FITC: Fluorescein isothiocyanate; FSC: Forward-scattered light; PE: Phycoerythrin; PLP: Platelet-low plasma; P-PRP: Pure platelet-rich plasma; SE: Standard error; SEM: Scanning electron microscopy; S-PLP: Supernatant of calcium-activated PLP; S-PRP: Supernatant of calcium-activated P-PRP; SSC: Side-scattered light.

of their biological effect has not been proven. To compare the biological effects between whole platelets and the growth factor-enriched supernatants, two different platelet preparations were generated, P-PRP and S-PRP.

Platelets were separated from whole blood samples by centrifugation, and two different plasma phases with different platelet concentrations were generated, PRP and PLP. PRP phase was recovered and passed through a leukocyte filter to obtain P-PRP, a PRP depleted of leukocytes (Figure 1A). Means of approximately 200,000 and 800,000 platelets per microliter were obtained for PLP and P-PRP, respectively, between six donors. As expected, the

platelet concentration in P-PRP was fourfold higher than PLP (Figure 1B). Leukocyte number was between 20 and 50 cells per microliter, less than 0.01% of P-PRP (data not shown). Platelets in P-PRP were further characterized by analyzing different platelet markers by flow cytometry, proving that purity was almost 100% and that only 20% of the platelets were activated (Figure 1C). Platelets nonactivated morphology, which does not present pseudopodia, was confirmed by SEM (Figure 1D).

To generate S-PRP, P-PRP was activated with $CaCl_2$. After clot retraction, the supernatant of activated P-PRP were collected and filtered to obtain S-PRP (Figure 1A). Platelets in P-PRP contain different growth factors and cytokines that are released once activated. Consequently, we evaluate the cytokine content in S-PRP, using S-PLP as a control (Supplementary Table 1). We confirmed that S-PRP contains more TGF-β than S-PLP (Figure 1E), and a direct correlation between platelet concentration and TGF-β secretion was determined (Figure 1F).

Preparation & characterization of monocyte-derived macrophages

To study the effect of different platelet preparation on the innate immune cells, human macrophages were generated. $CD14^+$ monocytes were purified from whole blood using a negative depletion cocktail followed by a density gradient. The monocyte enriched fraction was recovered and cultured with M-CSF for macrophage differentiation (Figure 2A). After 7 days, typical macrophage morphology was observed (Figure 2B & C). Macrophage populations were characterized by flow cytometry, showing increased expression of CD11c, while CD14 expression decreased and CD32 expression did not change when compared with monocytes (Figure 2D).

Some studies indicate that M-CSF induces a differentiation of monocytes toward an immature M2 macrophage phenotype [27], therefore, M2 macrophages-associated surface markers, CD206 and CD163, were also evaluated. Monocyte-derived macrophages incubated for an additional 24 h in conditioned medium, or M-CSF-differentiated macrophages, were compared with monocyte-derived macrophages stimulated with IFN-γ and LPS for an additional 24 h to induce an M1 phenotype. The results showed that both CD206 and CD163 were expressed in M-CSF-differentiated macrophages, however, their expression decreased on macrophages stimulated with IFN-γ and LPS (Figure 2E). CD206 and CD163 mean expression in M-CSF-differentiated macrophages were twofold higher than M1 macrophages (Figure 2F). Additionally, M1 macrophage phenotype was confirmed by high production of TNF-α only in macrophages stimulated with IFN-γ and LPS (data not shown).

Interaction between macrophages & platelets

Macrophages are capable to activate and phagocytose platelets [16]. P-PRP contains high concentrations of whole platelets able to directly interact with macrophages. To confirm this, macrophages were coincubated with P-PRP for 15 and 45 min and analyzed by SEM. After 15 min, an interaction between platelets and macrophages through macrophage prolongations was observed (Figure 3A). Activated platelet morphology was evidenced by pseudopodia emission [28]. As shown in Figure 3B & C, after 45 min of coincubation, interactions between macrophage filopodia and platelets were observed.

The platelets effect on CD206 marker expression

M-CSF-differentiated macrophages express high levels of CD206, which is associated with tissue-repair activity. Platelets could induce even higher levels of expression of this macrophage surface marker, which has been associated with higher levels of phagocytosis [29]. To prove this, macrophages were coincubated with increasing doses of platelets in P-PRP for 24 h. Macrophages were then stained for CD206 and analyzed by flow cytometry. The expression levels of CD206 increased in a dose-dependent manner with a maximum in 1000 platelets per macrophage (Figure 4A). Compared with macrophages without platelets, a ratio of 1:1000 macrophages per platelets induces threefold higher levels of CD206 expression (Figure 4B). It should be noted that higher concentrations of platelets were not included as aggregates formed in the cell culture wells. For further assays, a ratio of 1000 platelets per macrophage was used.

Effect of P-PRP & S-PRP in the macrophage phenotype

Although platelet preparations are used to promote tissue repair, the immunological mechanisms involved in this process remain poorly understood. Particularly, macrophages are present in large numbers surrounding damaged tissues [30], thus the importance to evaluate the effect of different platelet preparations over these phagocytes and their phenotype. To examine this effect, macrophages were stimulated with either P-PRP or S-PRP preparations for 24 h after which surface markers and cytokine production were analyzed by flow cytometry and ELISA, respectively. Since S-PRP preparations contain calcium, macrophages stimulated with $CaCl_2$ were used as a control.

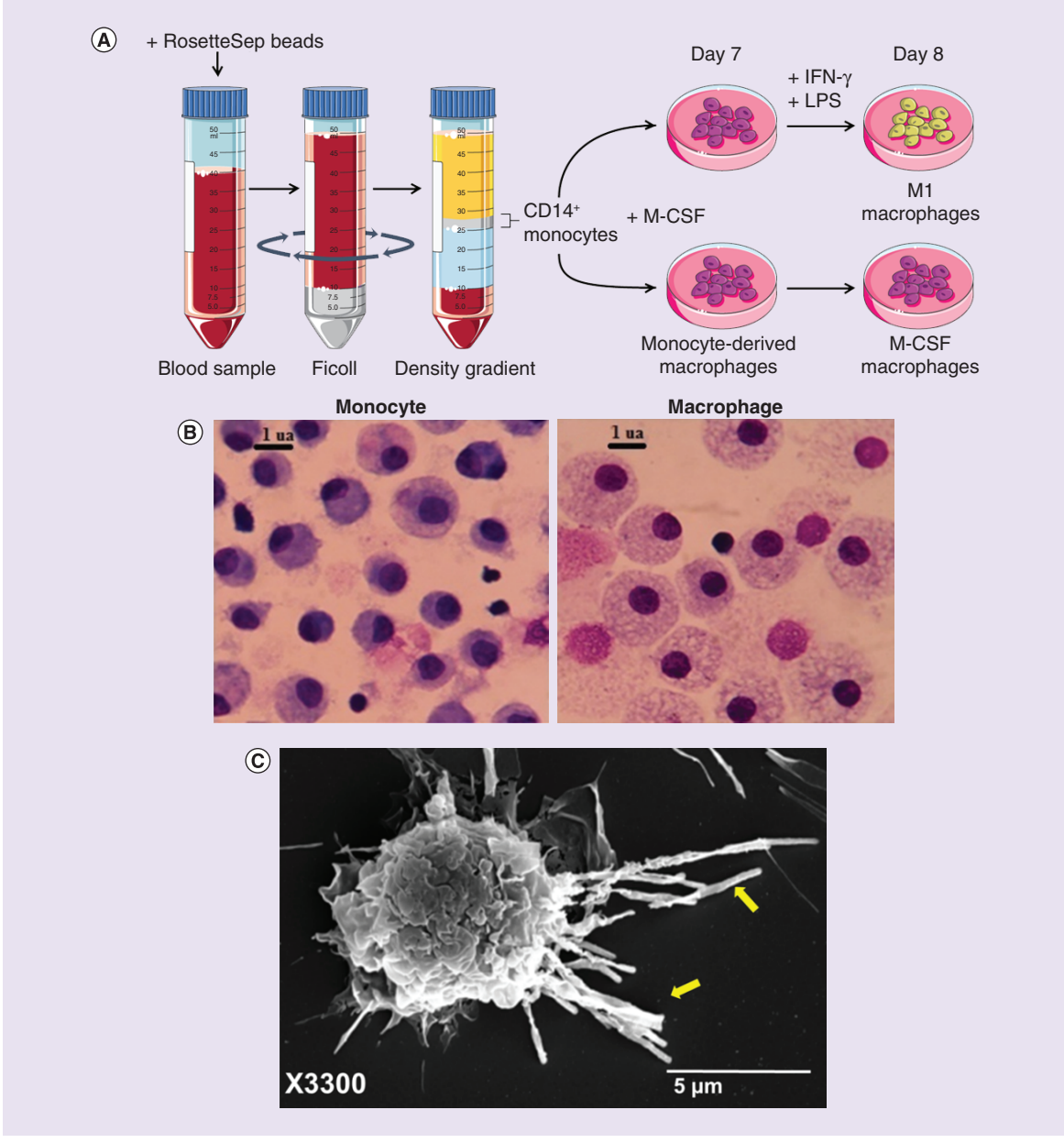

Figure 2. Preparation and characterization of human monocyte-derived macrophages. (A) Protocol for the generation of monocyte-derived macrophages. CD14-positive monocytes were obtained from whole blood samples using RossetteSep Beads followed by centrifugation on a density gradient. Macrophage differentiation was induced by stimulating monocytes with M-CSF at 37°C and 5% CO_2 for 7 days. **(B)** Hematoxylin and eosin staining of monocyte-derived macrophages and monocytes. The macrophage morphology was examined after 7 days of incubation with or without M-CSF. Scale bar in arbitrary units (au). **(C)** Scanning electron microscopy analysis of macrophage morphology at 3300× magnification. Arrows indicate macrophage prolongations. **(D)** Histograms of CD14-PE, CD32-APC and CD11c-PE expression on monocyte-derived macrophages (green) and monocytes (blue). The histograms were generated from a CD14-positive population analyzed by flow cytometry. The red curve indicates basal fluorescence. **(E)** Histograms of CD206-PECy5 and CD163-PE expression levels on M-CSF-differentiated macrophages and LPS- and IFN-γ-stimulated macrophages. Macrophages stimulated with M-CSF for 7 days were then stimulated with IFN-γ and LPS or conditioned medium for an additional 24 h **(A)**. **(F)** The mean fluorescence intensity of CD206 PECy5 and CD163-PE molecules expression on M-CSF-differentiated macrophages and LPS- and IFN-γ-stimulated macrophages determined by flow cytometer analysis. Bars represent standard error. n = 3. *$p < 0.05$ (paired *t*-test).
APC: Allophycocyanin; LPS: Lipopolysaccharides; PE: Phycoerythrin; PECy5: Phycoerythrin-cyanine 5.
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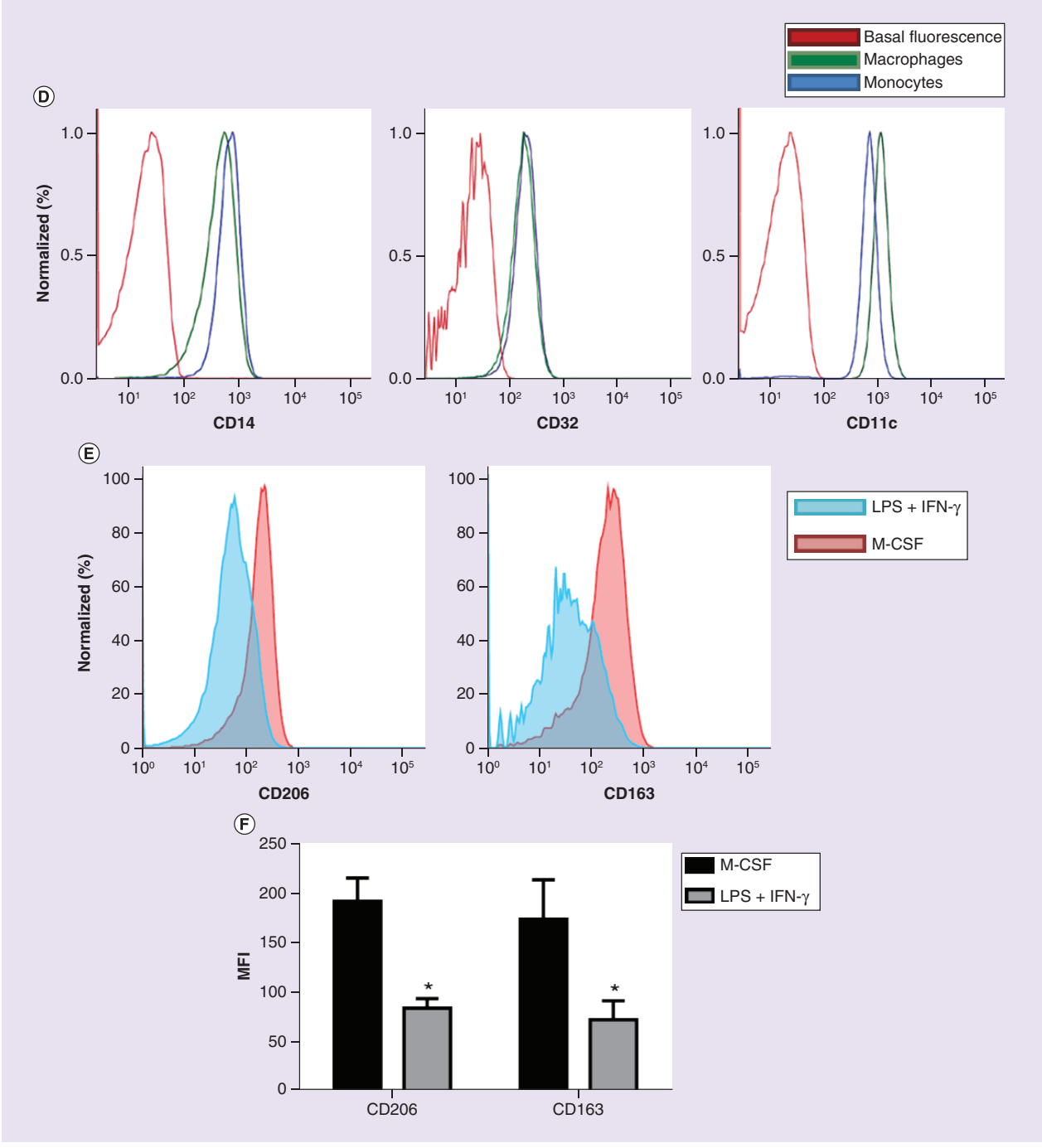

Figure 2. Preparation and characterization of human monocyte-derived macrophages (cont.). (A) Protocol for the generation of monocyte-derived macrophages. CD14-positive monocytes were obtained from whole blood samples using RosetteSep Beads followed by centrifugation on a density gradient. Macrophage differentiation was induced by stimulating monocytes with M-CSF at 37°C and 5% CO_2 for 7 days. **(B)** Hematoxylin and eosin staining of monocyte-derived macrophages and monocytes. The macrophage morphology was examined after 7 days of incubation with or without M-CSF. Scale bar in arbitrary units (au). **(C)** Scanning electron microscopy analysis of macrophage morphology at 3300× magnification. Arrows indicate macrophage prolongations. **(D)** Histograms of CD14-PE, CD32-APC and CD11c-PE expression on monocyte-derived macrophages (green) and monocytes (blue). The histograms were generated from a CD14-positive population analyzed by flow cytometry. The red curve indicates basal fluorescence. **(E)** Histograms of CD206-PECy5 and CD163-PE expression levels on M-CSF-differentiated macrophages and LPS- and IFN-γ-stimulated macrophages. Macrophages stimulated with M-CSF for 7 days were then stimulated with IFN-γ and LPS or conditioned medium for an additional 24 h **(A)**. **(F)** The mean fluorescence intensity of CD206 PECy5 and CD163-PE molecules expression on M-CSF-differentiated macrophages and LPS- and IFN-γ-stimulated macrophages determined by flow cytometer analysis. Bars represent standard error. n = 3. *$p < 0.05$ (paired *t*-test).
APC: Allophycocyanin; LPS: Lipopolysaccharides; PE: Phycoerythrin; PECy5: Phycoerythrin-cyanine 5.
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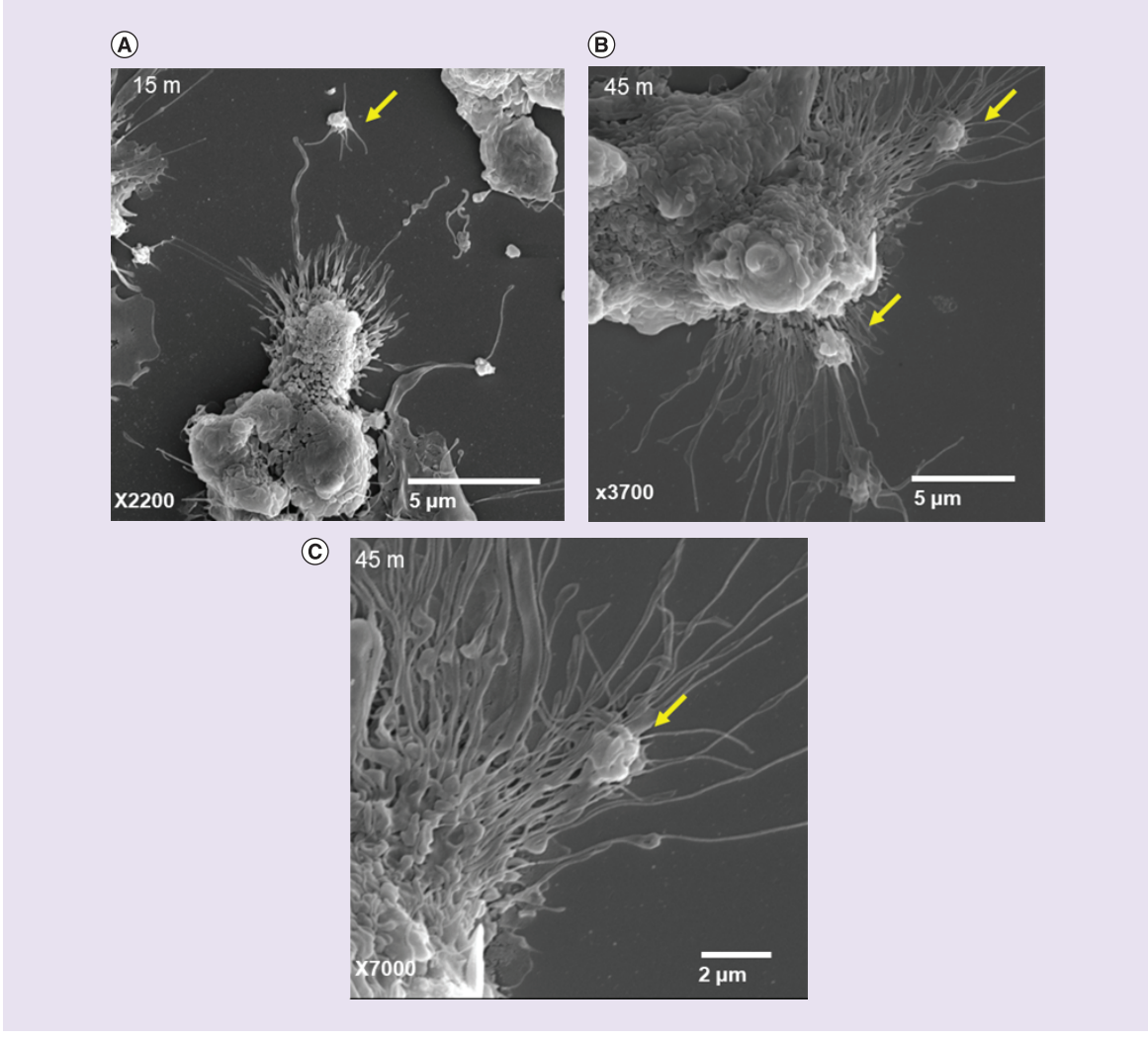

Figure 3. Interaction between macrophages and platelets analyzed by electron microscopy. Monocyte-derived macrophages were coincubated with platelet for 15 **(A)** or 45 **(B)** min before processing for scanning electron microscopy analysis at 2200× and 3700× magnification, respectively. Scanning electron microscopy analysis at 3700× magnification was made to examine more closely the interaction **(C)**. Yellow arrows indicate a platelet–macrophage interaction.

Macrophages stimulated with either P-PRP or S-PRP presented no differences in CD206 expression, however, both CD163 and CD86 presented higher expression levels when macrophages were stimulated with S-PRP instead of P-PRP (Figure 5A). Similarly, CD86 expression also increased when macrophages were stimulated with $CaCl_2$. Interestingly, nonsignificant differences in the macrophage surface markers were observed by comparing unstimulated macrophages with macrophages stimulated with S-PRP (Supplementary Figure 1), however, $CaCl_2$-stimulated macrophages reduce both CD206 and CD163 expression. The IL-10 production in P-PRP-stimulated macrophages was higher than S-PRP-stimulated macrophages, however, no differences were obtained in TNF-α production (Figure 5B). Notably, macrophages stimulated with P-PRP produced tenfold higher levels of IL-10 when compared with unstimulated macrophages (Supplementary Figure 2). Furthermore, IL-10/TNF-α production ratio was sevenfold higher in P-PRP-stimulated macrophages than S-PRP-stimulated macrophages (Figure 5C). No difference in cytokine production was observed between macrophages stimulated with S-PRP or $CaCl_2$.

Discussion

In this study, we evaluated the effect of two platelet preparations used in the clinic over human monocyte-derived macrophages. Our results showed that P-PRP and S-PRP generate different macrophage phenotypes. There are

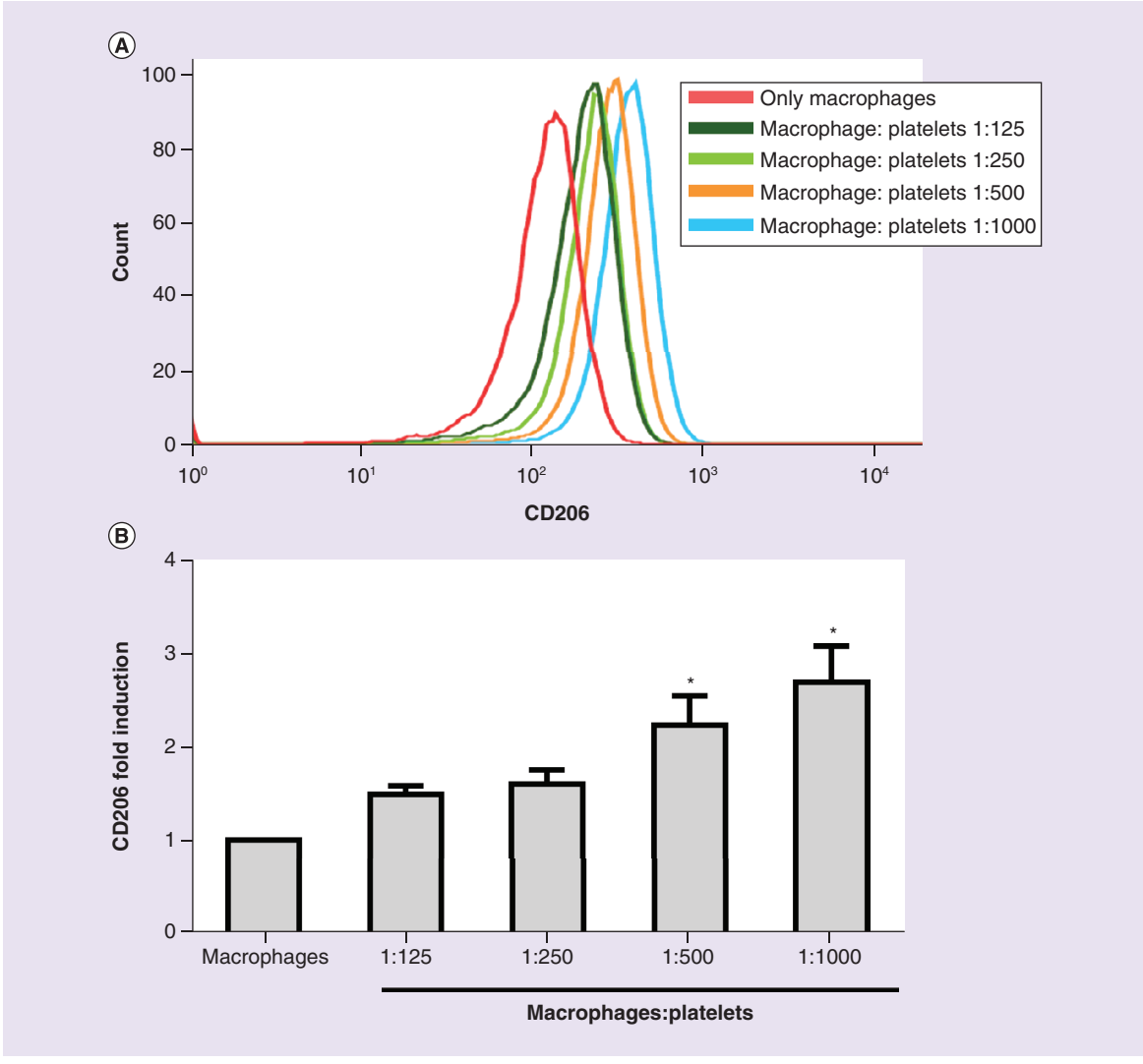

Figure 4. The platelets effect on CD206 marker expression on macrophages. Different macrophage/platelets ratios were coincubated for 24 h before analysis of CD206-fluorescein isothiocyanate expression by flow cytometry. **(A)** Representative histogram of CD206 expression levels on monocyte-derived macrophages stimulated with platelets from pure platelet-rich plasma. **(B)** Fold induction of CD206 marker expression on monocyte-derived macrophages coincubated with increasing amounts of platelets. CD206 expression levels are expressed relative to monocyte-derived macrophages without platelets (macrophages). Each bar represents standard error. n = 6. *$p < 0.05$ (one-way analysis of variance).

no significant differences on CD206 surface expression, a M2-macrophage marker. However, S-PRP-stimulated macrophages showed higher levels of expression of CD163 and CD86 than P-PRP-stimulated macrophages, and on the other hand, P-PRP-stimulated macrophages secreted more IL-10. These results indicate that these preparations do not have an equivalent biological effect on macrophages, and suggest that they could present a different clinical tissue-repair potential.

Comparison between platelet preparations has been complicated due to the different nomenclature and techniques utilized to generate platelet concentrates, thus increasing the difficulties to compare different contents and preparation methods [10,31]. This study compares two well-described platelet preparation effects on immune cells. It is important to notice that these preparations utilized have different compositions. For example, fibrins and fibrinogens are present in P-PRP but not in S-PRP, as S-PRP was obtained from the supernatant after clot retraction, and these molecules are in the clot [13]. Fibrins and fibrinogens enhance IL-10 production while reducing TNF-α production in macrophages [32], which could explain the results we observed with P-PRP. Second, P-PRP contains

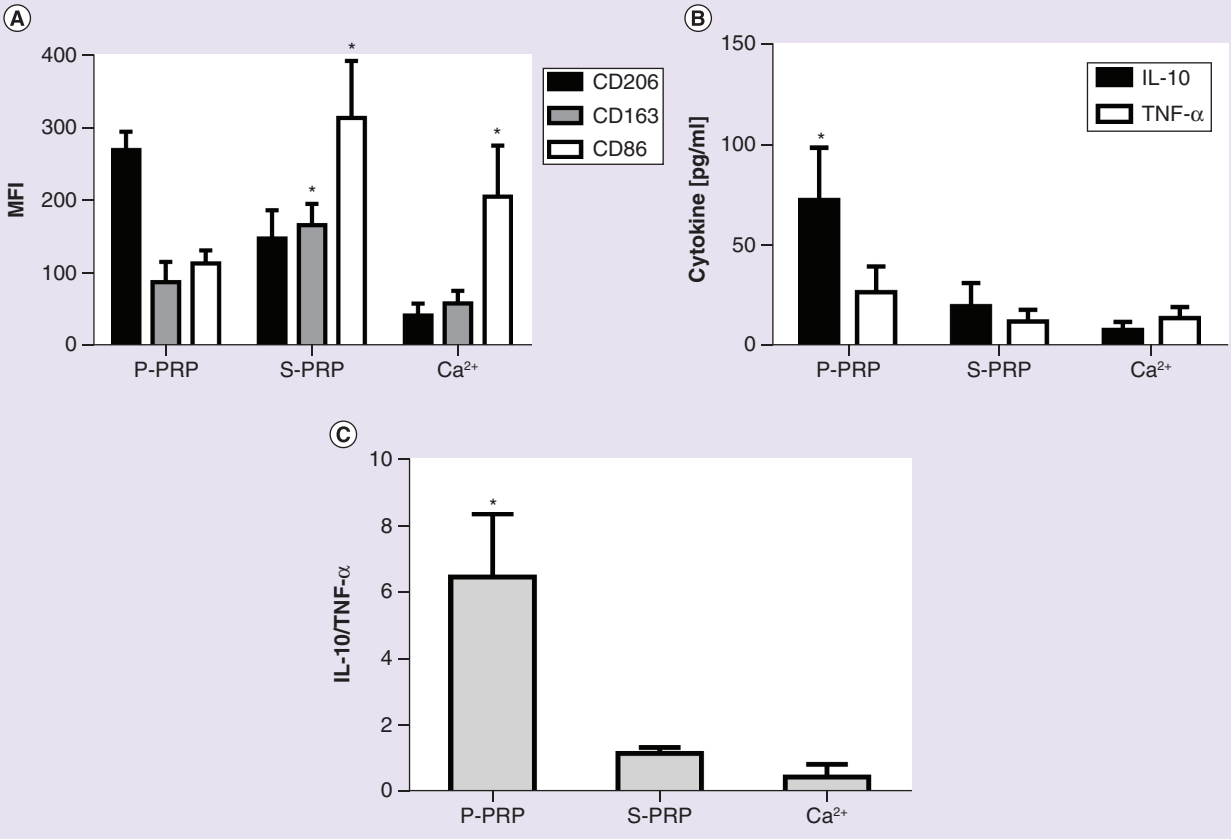

Figure 5. Comparative effect of pure platelet-rich plasma and supernatant of calcium-activated pure platelet-rich plasma on macrophages phenotype. (A) MFI of CD206-FITC, CD163-PE and CD86-PECy5 molecule expression on monocyte-derived macrophages stimulated with P-PRP, S-PRP or $CaCl_2$ (Ca^{2+}) for 24 h determined by flow cytometry. Bars represent SE. n = 5. *$p < 0.05$ (paired t-test). **(B)** Cytokine production of IL-10 and TNF-α by monocyte-derived macrophages stimulated with P-PRP, S-PRP or $CaCl_2$ after 24 h, determined by ELISA. **(C)** Relationship between IL-10 and TNF-α production by macrophages. Each bar represents SE. n = 3. *$p < 0.05$ (two-way analysis of variance).
FITC: Fluorescein isothiocyanate; MFI: Mean fluorescence intensity; PE: Phycoerythrin; PECy5: Phycoerythrin-cyanine 5; P-PRP: Pure platelet-rich plasma; SE: Standard error; S-PRP: Supernatant of calcium-activated P-PRP.

whole platelets while S-PRP, which passed through a microbiological filter of 0.2 μm, only contains serum and the molecules released during the platelet activation. Activated platelets and platelet-derived microparticles interact with leukocytes through CD62P, inducing activation and rapid local cytokine liberation [16]. In P-PRP generated in this study, 19% of platelets were $CD62P^+$, a marker of activated platelets, and interaction between platelets and macrophages could be observed after 15 min of coincubation. This interaction may induce platelets activation and subsequent liberations of their cytoplasmic content. Therefore, growth factors and TGF-β, which could enhance tissue-repair macrophage functions [33], are presented in both P-PRP and S-PRP preparations. Another difference between these platelet preparations is the presence of $CaCl_2$ in S-PRP. This molecule is capable to increase macrophages adhesion to plastic, which favors phagocytosis and antigen presentation [34]. Macrophages stimulated with $CaCl_2$ or S-PRP presented higher levels of CD86, an inflammatory M1-associated marker, in comparison to P-PRP-stimulated macrophages. This protein is expressed in activated antigen-presenting cells and acts as a costimulatory signal during lymphocytes activation. $CaCl_2$-stimulated macrophages also decreased expression of CD206 and CD163 when compared with unstimulated macrophages (Supplementary Figure 1), which could be understood as a proinflammatory polarization. Interestingly, proinflammatory macrophages with enhanced bactericide capabilities can be generated with a calcium ionophore [35]. Recently, Anitua's group has described a new protocol to obtain supernatant of calcium-activated P-PRP – or PRGF supernatant as they called it [14] – that uses a lower amount of anticoagulant and $CaCl_2$ closer to physiological concentrations than our protocol. One possible

advantage of this new supernatant preparation could be lower levels of CD86, as this preparation protocol uses less $CaCl_2$.

High plasticity and the lack of specific markers have made it difficult for researchers to precisely describe macrophage populations. Thus, it is recommended detailing the characterization strategy and the stimuli used, as a way to avoid confusion and reach common understanding [22]. In this study, monocytes stimulated with M-CSF for 7 days presented a macrophage-like morphology when analyzed hematoxylin and eosin staining and these same cells also presented long pseudopodium characteristic of macrophages when analyzed by SEM. Flow cytometry analysis demonstrated that these cells also presented common monocyte/macrophage linage markers as CD14, CD32 and CD11c, but also M2-associated markers as CD206 and CD163. After treating these macrophages with LPS and IFN-γ, the expression of CD206 and CD163 were reduced, while macrophages treated with P-PRP showed a doses-dependent increase of CD206 surface expression (Figures 2F & 4A). CD206 is a mannose receptor, a C-type lectin responsible of eliminating pathogenic cellular debris, as well as eliminating inflammatory agents and components of the cellular matrix such as collagen [36,37]. Therefore, increased levels of CD206 on macrophages, as obtained with P-PRP (Figure 4), could be a key factor to reducing inflammation, as this marker is also related to macrophages' phagocytic activity [38].

As previously mentioned, P-PRP-stimulated macrophages presented higher levels of IL-10 production than S-PRP-stimulated macrophages. IL-10 is a potent anti-inflammatory cytokine that promotes production of IL-4 by type 2 T-helper cells, as well as wound healing, as it activates arginase 1, contributing to extracellular matrix production [39]. Although P-PRP enhanced IL-10 production and CD206 expression, it decreased expression of CD163, a haptoglobin–hemoglobin scavenger receptor related to M2 macrophages, compared with macrophages without stimulation (Supplementary Figure 1). A study by Porcheray *et al.* has proven that CD206 and CD163 present mutually excluding induction patterns [40]. These researchers observed that TGF-β stimulation increases CD206 expression while decreases CD163 expression on macrophages, phenotype similar to what is observed in P-PRP-stimulated macrophages. On the other hand, S-PRP-stimulated macrophages presented higher levels of CD163 compared with P-PRP. Even though S-PRP contains TGF-β, which is liberated during $CaCl_2$ platelet activation, its concentration may not be enough to produce this effect. In fact, to generate S-PRP, activated P-PRP was incubated 10 min at 37°C for clotting and 20 min more at room temperature for clot retraction, a procedure standardized and performed by the Clinical Hospital of University of Chile. Correspondingly, even though TGF-β concentration was twofold higher in S-PRP than S-PLP, it is likely that more TGF-β could be released after more clot retraction time, as used in Anitua *et al.*, since PRP contains four-times more platelets than PLP [41]. Optimal clot retraction time is an interesting point to be evaluated in a further study. Furthermore, it is important to notice that part of the TGF-β secreted by S-PRP is in an inactive form as it is activated by serine protease and other factors liberated by platelets and other cells [42]. A hypothesis is that during P-PRP stimulation of macrophages, cell-cell interactions could allow the activation of TGF-β released by platelets, generating high local concentrations of this cytokine, favoring rapid macrophage activation.

The PRP and PRP-derived products' preparation procedures differ in centrifugation times and force, activation methods, platelet concentrations, presence of fibrin/fibrinogen or leukocytes, among other [10,31,43]. Protocol variations for platelet preparations and the lack of an established nomenclature have generated contradictory results of the efficacy of platelet-related therapies [10,11,44]. In the same way, defining standard operating procedures is indispensable to obtain a standard composition of these platelet preparations and to able to reliably compare their effectiveness [31,45]. In this study, we used a nomenclature according to the content of the platelet preparation, P-PRP – for PRP without leukocytes – and S-PRP – for the supernatant of calcium-activated P-PRP – to be able to describe in detail the procedure to obtain both products. Lastly, some clinically used platelet preparations are compared in Table 1. These preparations were classified in three groups: nonactivated platelet products, gel products and supernatants. PRP with or without leukocytes was indicated as L-PRP or P-PRP, respectively, as classified by Dohan *et al.* [10]. PRP gel is activated PRP with the clot mixed with the plasma, and L-PRP gel and P-PRP gel correspond to gels generated from PRP with or without leukocytes, as classified by Dohan *et al.* [10]. The supernatants correspond to S-PRP, which contains serum, and S-PL, a supernatant obtained by centrifugation of repeated freeze and thawed washed platelets [46].Table 1 summarizes some of the different contents which would explain the different biological effects of these preparations.

Table 1. Platelet preparations contents.

	Nonactivated PRP		PRP gel		PRP supernatants	
	P-PRP	L-PRP	P-PRP Gel	L-PRP Gel	S-PRP	S-PL
Citations	†	†	‡	§	¶	#
Nonactivated platelets	+	+				
Activated platelets			+	+		
GFs and cytokines	+	+	+	+	+	+
Fibrinogen	+	+				
Fibrin			+	+		
Coagulation factors	+	+	+	+		
Leukocytes		+		+		
Activating agent			+	+	+	

Presence (+) in different platelets preparations.
†Fitzpatrick *et al.* [5].
‡PRGF of Anitua *et al.* [2].
§PLG of Jia *et al.* [49].
¶S-PRP Clinical Hospital of University of Chile in Santiago, Chile; PRGF supernatant of Anitua *et al.* [14].
#PL supernatant of Bernardi *et al.* [46].
GF: Growth factor; L-PRP: Leukocyte and platelet-rich plasma; PL: Platelet lysates; PLG: Platelet-leukocyte gel; P-PRP: Pure platelet-rich plasma; PRGF: Plasma rich in growth factors; S-PL: Supernatant of repeated freeze and thawed washed platelet; S-PRP: Supernatant of calcium-activated P-PRP.

Conclusion

In this study we demonstrated that macrophages stimulated *in vitro* for 24 h with platelet preparations, P-PRP and S-PRP, favored the generation of different profiles of tissue-repair macrophages. While both preparations increased the expression of CD206 mannose receptor, they induced different levels expression of CD163 and CD86, as well as IL-10 production. However, our study has limitations, as cocultures between platelets and macrophages were allogeneic and some results have a low number of samples. Further studies are necessary to corroborate these results and evaluate if the same effects are detected in autologous cultures.

Translational perspective

Despite the aforementioned limitations, this study reinforces the idea that different platelet preparations do not possess the same biological effect [47]. This is an interesting finding considering that controversial results have emerged from the implementation of platelet-related therapies in regenerative medicine. In this regard, platelet preparations should be evaluated *in vitro* to understand and identify their optimal clinical use. The therapy of choice should be defined according to the effects on cells involved in inflammation and tissue-remodeling. We have discussed that one of the main problems between different preparations are their different contents. We demonstrated that leukocyte-depleted P-PRP improves tissue-repair macrophage phenotype and functions including IL-10 production. P-PRP could be used in tissue damage areas to promote tissue regeneration, partially mediated by platelets interaction with resident macrophages. On the other hand, S-PRP-stimulated macrophages increased CD163 expression. These macrophages could play a key role in the removal of hemoglobin–haptoglobin complex at sites of physiologic or pathologic intravascular hemolysis, but by itself is not able to promote tissue repair [48]. Finally, proinflammatory macrophages are presented in the initial states of the healing process and in several chronical inflammatory diseases, the use of P-PRP to repolarize *in vitro* these cells into tissue-repair macrophages is an interesting proposal to further studies.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/suppl/10.2217/rme-2017-0122

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Summary points

- Because of variations in the methods of preparation and nomenclature of platelets products, different studies present contradictory data about platelets' therapeutic efficacy.
- The effect of pure platelet-rich plasma (P-PRP) and supernatant of calcium-activated P-PRP (S-PRP) over innate immune cells could shed some light on the clinical significance of their differences.
- S-PRP contains growth factors such as TGF-β1 released by activated platelets as well as $CaCl_2$.
- P-PRP contains whole platelets that can directly interact with macrophages.
- M-CSF-differentiated macrophages present CD206 expression marker, a M2 macrophage-related marker, which is enhanced after stimulation with P-PRP.
- S-PRP-stimulated macrophages present higher levels of the expression markers CD163 and CD86 compared with macrophages stimulated with P-PRP. While CD163 is a M2 macrophage-related marker, CD86 is a proinflammatory marker that could have been enhanced by the presence of $CaCl_2$ in S-PRP preparations.
- Only P-PRP induced macrophages to have a higher IL-10 production, a potent anti-inflammatory cytokine that promotes tissue regeneration.
- Although many researchers consider that different platelet preparation have an equivalent biological effect, outcomes of the present study provide evidence that they may present different clinical regenerative potentials.

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Ethical conduct of research

The authors state that they have obtained an informed consent from the participants involved.

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