Selectivity induction of anti-inflammatory monocyte-platelet aggregates in a model of pulsatile blood flow at low shear rates

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Abstract

In patients with cardiovascular abnormalities or immunological disorders, an increased number of circulating leukocyte-platelet aggregates is observed. Leukocyte-platelet aggregates play an essential role in linking the hemostatic and immune systems. High shear stress and pro-coagulant and pro-inflammatory stimuli are known to activate platelets and promote the formation of aggregates. Pulsatile blood flow under low shear stress can also induce platelet activation in comparatively mild conditions. However, the effect of such events on leukocyte-platelet aggregates has not yet been investigated. To determine whether low shear stress affects the formation of aggregates, we established a simple "inverting rotation" method of inducing periodic changes in the direction of blood flow in combination with low shear stress. We demonstrated that after the inverting rotation treatment for 10–20 min more than 70% of monocytes selectively aggregated with platelets. The formation of monocyte-platelet complexes was inhibited by an anti-CD162 (PSGL-1) monoclonal antibody or a Ca²⁺ chelator. The phagocytic activity of monocytes was augmented by inverting rotation, whereas phagocytosis mediated by granulocytes remained unaffected. Interestingly, the formation of monocyte-platelet complexes suppressed the production of pro-inflammatory cytokines such as interleukin (IL)-1β. At the same time, monocyte-platelet complexes augmented the expression of the anti-inflammatory cytokine IL-10. Our results suggest that platelet-bound monocytes show an anti-inflammatory phenotype under low shear stress conditions. Thus, our method provided new insights into the mechanisms of monocyte-platelet aggregate formation and regulation.

Introduction

Platelets play an essential role in primary homeostasis and are important for immune responses [1–3]. In patients with myocardial infarction or ischemic stroke, an increase in the number of circulating leukocyte-platelet aggregates is observed [4–6]. The formation of leukocyte-platelet complexes results in an excessive adhesion of leukocytes to endothelial cells, which may enhance vascular inflammation [7–9]. Furthermore, an increase in the number of leukocyte-platelet aggregates has been observed in various inflammatory disorders, such as Behçet disease, psoriatic arthritis, rheumatoid arthritis, diabetic microangiopathy, ulcerative colitis, antiphospholipid syndrome, and systemic lupus erythematosus [10–14]. Therefore, understanding the mechanisms of the formation of leukocyte-platelet aggregates is critically important for elucidating pathological consequences of both cardiovascular and immunological diseases.

High shear stress, and pro-coagulant and pro-inflammatory stimulations are known to be potent inducers of leukocyte-platelet aggregates [15–17]. However, the formation of leukocyte-platelet aggregates under a low-pulsatile shear stress in the absence of pro-coagulants or pro-inflammatory stimulation has never been demonstrated. Investigation of low shear stress conditions helps to elucidate several important aspects of platelet function, because preferential region of atherosclerosis, such as the vascular region at arterial branch points and curvatures, leads to disturbances in flow pattern with a low shear stress (<0.4 Pa) [18]. Furthermore, low-pulsatile shear stress (0.01–0.5 Pa), observed, for example, in the recirculation zone of stenotic arteries, induces platelet activation [19, 20].

Numerous studies revealed that the formation of neutrophil extracellular traps requires the interaction of neutrophils with platelets in the bloodstream, which also induces phosphotyrosine signaling in neutrophils and enhances phagocytosis [2, 3]. However, complexes of monocytes and platelets form more rapidly and are more stable than other leukocyte-platelet aggregates [2, 21].

Previous studies have demonstrated that platelet-activated monocytes are recruited more efficiently at inflammation sites. Such monocytes tend to have a pro-inflammatory phenotype and usually differentiate into dendritic cells in the tissue [2, 21]. In contrast to observations of these pro-inflammatory properties, several recent studies have pointed to anti-inflammatory actions of platelets [2, 22, 23]. Mechanisms underlying such opposite effects of platelets remain controversial.

In this study, we devised a method to induce low shear stress with a change in blood flow pattern in vitro, which we termed the

Keywords

Inflammatory cytokine, leukocyte-platelet aggregates, low shear stress, monocytes, PSGL-1

History

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“inverting rotation” (IR) method. Using this approach, we showed that IR selectively induced the formation of monocyte-platelet complexes and a functional shift to the anti-inflammatory phenotype in monocytes. Since IR can be applied to a small amount of blood in a micro-tube, it is suitable for flow cytometry-based high-throughput screening for drugs, which regulate the interaction between monocytes and platelets and biological activity of corresponding aggregates.

Methods

Blood collection

Healthy volunteers provided informed consent under institutional guidelines of the Hyogo College of Medicine and according to principles of the Declaration of Helsinki. Venous blood samples were collected into blood collection vacuum tubes containing 3.13% sodium citrate (Venoject II, VP-C050K, Terumo, Tokyo, Japan) by using 21G needles (Terumo). Next, 0.3 mL of blood was transferred into a 1.5-mL sampling polypropylene tube (Sarstedt, Nümbrecht, Germany) with a maximum inner diameter of 8 mm and long axis length of 40 mm and gently mixed with 1.5 μL of 0.1 M calcium chloride (final concentration 0.5 mM), as described previously [24, 25]. In several experiments, platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were collected by using sodium citrate as described previously [24, 25].

Inverting flow induction in 1.5-mL tubes

Blood samples in 1.5-mL tubes were incubated with IR using the Rotator RT-50 (TAITEC, Koshigaya, Japan) in a 37°C incubator. Blood was stagnated in the tubes when it was rotated from 0° (top of the spinning disk) to 90° (see Supplementary Figure 1A). As the tube passed the 90° angle mark, the blood inside rapidly and completely migrated from one end of the tube to the other (cap side). Then, the blood stagnated again until it passed the 270° angle mark. Thus, during a complete 360° rotation of the spinning disc, the blood migrated twice (see Supplementary Figure 1B). The blood migrated from one end of the tube to the other in approximately 1.5 s. The blood was stagnant for 5 s during 5 IRs per min (i-rpm). Since blood migration speed in the tube depended upon its viscosity and gravity, it did not change in the range of rotation frequencies from 5 to 20 i-rpm. Migration frequencies of 5, 10, or 20 i-rpm corresponded to 10, 20, or 40 blood migrations per minute, respectively. During repeated cycles of stagnation and migration, the average distance of blood movement in the tube was 25 mm.

When 0.3 mL of blood moves in the tube for 1.0–1.5 s, the average velocity (v), Reynolds number (Re), average shear rate (γ), shear stress (τ), and change of the pressure (∆p) can be expressed as follows:

\[
u = 0.3 \times 10^{-6}\text{m}^3/(3.14 \times (4 \times 10^{-3})^2)\text{(m}^2\times 1.5\text{s})\]
\[= 4.0 \times 10^{-3}\text{m/s}\]
\[Re = \frac{\rho d u}{\eta}\]
\[= 1000\frac{(kg/m^3) \times 0.004 - 0.006(m/s) \times 0.008(m)/(0.003(Pa \cdot s))}{1000}\]
\[= 11.0\]

where ρ is blood density, u is average velocity, d is tube diameter, and η is blood viscosity.

\[v = 4Q/(\pi d^3)\]
\[= 4 \times 0.3 \times 10^{-6}\text{m}^3/1.0\]
\[= 1.5\text{s}/(3.14 \times (0.004 \text{ m})^2) = 4.0\text{s}^{-1}\]
\[\tau = \eta v = 0.003 \times 4.0 = 1.2 \times 10^{-2}(Pa)\]
\[\Delta p = 0.5\rho u^2\]
\[= 0.5 \times 1000\frac{(kg/m^3) \times (0.004 \text{ m/s})^2}{8.0 \times 10^{-3}(Pa)\text{m}}\]

This velocity corresponds to the flow in arterioles and the Reynolds number corresponds to the flow in small arteries <1 mm in diameter. Values of shear stress and pressure changes are very low.

The total number of leukocytes and relative fractions of monocytes, lymphocytes, and granulocytes were not significantly changed by IR (Supplementary Figure 2). However, the number of platelets was significantly decreased (Supplementary Figure 2). The extent of platelet number decrease was not significantly associated with IR frequency. After IR, blood coagulation did not occur for at least 18 h.

Reagents and antibodies

The following reagents and antibodies were used: SB203580 (Calbiochem, Darmstadt, Germany); U0126 (Promega, Madison, WI); BD Phosflow Lyse/Fix buffer, peridinin chlorophyll protein-conjugated streptavidin, fluorescein isothiocyanate (FITC)-

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Figure 1. Platelet activation during inverting rotation. Whole blood (A) or PRP (B) was treated by 0, 5, 10, and 20 i-rpm for 0, 5, 10, and 20 min at 37°C. After the treatment, cells in whole blood or platelets in PRP were stained with an anti-CD62P mAb. Samples were analyzed by flow cytometry. Asterisks denote significant differences of respective data points from measurements taken after the control (0 i-rpm) treatment as follows: *P < 0.05; **P < 0.01; ***P < 0.001 (ANOVA with Bonferroni test; n = 4).

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Figure 2. Analysis of leukocyte-platelet aggregates induced by inverting rotation. (A) Flow cytometry analysis of leukocyte-platelet aggregates after inverting rotation. Whole blood was treated by 0 or 5 i-rpm for 20 min at 37°C. After the treatment, leukocytes were stained with an isotype-matched control antibody (Ab, background) or a combination of anti-CD61 and anti-CD14 mAbs. Each leukocyte fraction was gated on a dot plot and CD61-positive cells were analyzed using histograms. FSC, forward scatter; SSC, side scatter. (B) Kinetic analysis of leukocyte-platelet aggregates induced by inverting rotation. Whole blood was treated by 0, 5, 10, and 20 i-rpm for 0, 5, 10, and 20 min at 37°C. After the incubation, leukocytes in whole blood were stained with specific antibodies. The percentages of CD62P-, CD42a-, or CD61-positive cells in each leukocyte fraction were measured by flow cytometry. Monocyte population was confirmed by counterstaining using the monocyte lineage marker CD14 (data not shown). Asterisks denote significant differences of respective data points from measurements taken after the control (0 i-rpm) treatment as follows: *P < 0.05; **P < 0.01; ***P < 0.001 (ANOVA with Bonferroni test; n = 4).

Whole blood cell-surface staining

To avoid platelet aggregation in cold conditions, the blood was aliquoted into 1.5-mL tubes (50 μL/tube) and immediately mixed with the Lyse/Fix buffer (1 mL/tube) for 10 min at 37°C. Red blood cells hemolyzed by the buffer during fixation were removed by washing with phosphate-buffered saline (PBS) at 2000 × g for 1 min at 25°C. The remaining leukocytes were suspended in PBS and allowed to react with antibodies at 25°C for 45 min. The cells were washed with PBS and their fluorescence intensity was measured by flow cytometry (FACSCalibur and FACSCanto II, BD Biosciences). The data for individual cells were then gated using forward- and side-scatter values to separate the data for different cell populations (granulocytes, monocytes, and lymphocytes). The fluorescence intensity of each population was analyzed using CellQuest Pro software, version 4.0.2 (BD Biosciences), and FlowJo software, version 6.2 (TreeStar, Ashland, OR). In several experiments, each leukocyte fraction was sorted by using FACSAria (BD Biosciences).

Confocal microscopy observation

The blood was immediately fixed using the Lyse/Fix buffer after IR. Monocytes in whole leukocytes were sorted by FACSAria. The cytospin was prepared after staining with a combination of anti-CD14 mAb, anti-CD61 mAb, and 4′,6-diamidino-2-phenylindole (Merck, Darmstadt, Germany). It was then mounted with the PermaFluor Aqueous Medium (Thermo Scientific, Waltham, MA) as described previously [26]. The cells were observed by confocal microscopy (LSM 700, Carl Zeiss, Jena, Germany) and analyzed by ZEN software, version 2012.

Phagocytosis assay

The blood (0.3 mL) was mixed with pHrodo-labeled Escherichia coli BioParticles (0.05 mg/mL; Life Technologies, Eugene, OR) and incubated for 30 min at 37°C in 1.5-mL microtubes. After the incubation, a 50-μL sample was transferred into the Lyse/Fix buffer (0.5 mL) and incubated at 37°C for 10 min. The cells were washed with PBS and then the mean fluorescence intensity of each sample was measured by flow cytometry.

Cytokine measurements

Following the treatment with or without IR, the blood was diluted 10-fold with the RPMI 1640 medium supplemented with L-glutamine (Sigma, St. Louis, MO) containing low-molecular-weight heparin (5 U/mL). Diluted blood samples were then incubated in the presence or absence of lipopolysaccharide (LPS, 2 μg/mL, E. coli 0127:B8, Sigma) in a 24-well culture plate (final reaction volume 1 mL/well) for 18 h in an atmosphere of 5% carbon dioxide. After incubation, the conditioned medium was collected by centrifugation at 2000 × g for 1 min at 4°C and stored at −80°C until each measurement. The BD Cytometric Bead Array System, a human inflammatory cytokine kit, and monocyte chemotractant protein-1 (MCP-1, CCL2) flex beads (BD Biosciences) were used for multiplex measurements of cytokines in the medium.

IL-10-secreting cell assay

IL-10-secreting cells were detected using an IL-10 Secretion Assay Detection Kit (Miltenyi Biotec, Gladbach, Germany). Following the manufacturer’s protocol, whole blood was diluted with RPMI 1640 medium after treatment with or without IR. The blood cells were incubated with 1 μg/mL of LPS for 12 h, and
then the cells were labeled with IL-10 catch reagent. The labeled cells were reincubated in RPMI 1640 containing 10% autologous plasma for 45 min at 37°C and then stained with IL-10 detection antibody and lineage marker antibodies (CD3, CD14, CD16, CD19, and CD61).

Statistics

Data are expressed as the mean ± standard error. Data sets were compared by the analysis of variance (ANOVA) with a subsequent post hoc Bonferroni’s test or by paired Student’s t-test by using Prism Software, version 5.03 (GraphPad Software, San Diego, CA). Differences were considered statistically significant if P values were less than 0.05.

Results

Platelet activation in the whole blood or PRP by IR

Platelet expression of CD62P (P-selectin, LECAM-3) initiated by the activation-dependent degranulation plays a key role in the interaction of platelets with leukocytes [27]. To confirm the induction of platelet activation by IR, CD62P levels were measured (Figure 1). In whole blood, the fraction of CD62P-positive (CD62P+) platelets was significantly increased by IR at 20 i-rpm (Figure 1A). In PRP, the number of CD62P+ platelets was also enhanced by IR depending on the inversion frequency and incubation time (Figure 1B). However, no significant changes in CD62P+ were found after 5 i-rpm IR in either whole blood or PRP. Thus, 5 i-rpm IR treatment barely induced platelet activation.

Analysis of leukocyte-platelet aggregates induced by IR treatment using flow cytometry

To verify the formation of leukocyte-platelet complexes, the expression of the platelet-specific antigen CD61 (GP IIa, β3 integrin) was measured. Representative results are shown in Figure 2A. Each leukocyte population in the whole blood was gated by forward-scatter and side-scatter parameters (left side, dot plot) and then analyzed on a CD61 histogram (right side, histograms). “Background” represents isotype-matched control mAb staining data. Results of the control “0 i-rpm” treatment are represented by histograms with two peaks corresponding to CD61-positive and CD61-negative subsets in each leukocyte population. After 5 i-rpm IR treatment, a reduction of the CD61-positive peak was observed in lymphocyte and granulocyte populations. In contrast, in the monocyte population, CD61-negative cells disappeared and a shift toward CD61-positive cells was observed.

Table I. Effects of heparin and rotation angle on monocyte–platelet aggregation evaluated by CD61% expression on monocytes.

| Rotation(1)       | No anticoagulant | Heparin(2) | P value(3) |
|-------------------|------------------|------------|------------|
| No rotation       | 35.6 ± 1.7 (a)   | 33.3 ± 3.0 (b) | NS (a vs. b) |
| (0 i-rpm)         | (n = 5)          | (n = 3)    |            |
| Invertible        | 76.8 ± 7.7 (c)   | 79.8 ± 3.9 (d) | NS (c vs. d) |
| (5 i-rpm)         | (n = 5)          | (n = 3)    |            |
| Horizontal        | 43.3 ± 1.9 (e)   | ND         | <0.01 (e vs. c) |
| (5 h-rpm)         | (n = 3)          |            |            |

ND, not done; NS, not significant; h-rpm, horizontal rotations per minute.

(1)Invertible indicates that the long axis of the tube is 90° to the rotation axis; horizontal indicates that the long axis of the tube is parallel to the rotation axis.

(2)The low-molecular-weight heparin concentration was 5 U/mL.

(3)One-way analysis of variance with a post hoc test (Bonferroni).

Selective induction of the formation of monocyte-platelet complexes by IR

The proportion of CD42a+ or CD61+ monocytes was ~40% before IR, suggesting that monocytes, similarly to lymphocytes and granulocytes, spontaneously bound to platelets (Figure 2B, right-top and right-middle panels). In contrast to lymphocytes and granulocytes, monocytes markedly bound to platelets after IR depending on the rotation time. The fraction of monocyte-platelet aggregates significantly increased in 5–10 min after IR and then comprised over 70% at 20 min after IR. The lowest IR frequency used, 5 i-rpm, was sufficient to induce the formation of monocyte-platelet aggregates. Interestingly, 5 i-rpm treatment induced CD62P expression in monocyte-platelet aggregates (Figure 2B, right-bottom panel), but not in platelets from the whole blood (Figure 1A). These results indicated that moderate IR treatment (5 i-rpm) activated platelets and induced their aggregation with monocytes.

To examine the relationship between the frequency of blood contact with the inside tube walls and the formation of monocyte-platelet complexes, we investigated effects of horizontal rotation (Supplementary Figure 1A). Horizontal rotation induced a laminar flow when the blood made contact with the inside wall. The number of monocyte-platelet aggregates was not increased by horizontal rotation (Table I). Furthermore, to investigate the effect of untraceable blood coagulation caused by the contact with the inside wall, low-molecular-weight heparin (5 U/mL) was added to blood samples during IR. The addition of heparin did not inhibit the formation of monocyte-platelet complexes (Table I). These observations clearly indicated that tubes are biocompatible with the blood and that changes in the blood flow caused by IR induced monocyte-platelet aggregates in the absence of artificial coagulation.

We had to exclude the possibility that monocytes expressed CD61 on their membrane after IR. To address this issue, we conducted a reconstitution experiment using purified peripheral blood mononuclear cells (PBMCs), PRP, and PPP. CD61 expression in monocytes was upregulated after 5 i-rpm IR treatment in the presence of PRP, but not of PPP (Supplementary Figure 3, 5 i-rpm). Furthermore, CD61 expression in monocytes was not upregulated after 0 i-rpm treatment, even in the presence of PRP.

The effect of IR on lymphocyte-platelet and granulocyte-platelet aggregates

The presence of lymphocyte-platelet aggregates was evaluated by analyzing the expression of platelet-specific antigens CD42a (GPIX, von Willebrand factor receptor), CD61, and CD62P on lymphocytes after IR (Figure 2B, left panels). Before IR, about 40% of lymphocytes were positive for CD42a or CD61, suggesting that this fraction of the total lymphocyte population bound to platelets spontaneously (Figure 2B, left-top and left-middle panels). The number of these spontaneous aggregates was slightly reduced after IR, while CD62P expression was not observed (Figure 2B, left-bottom). These results suggested that IR treatment did not induce binding of platelets to lymphocytes.

Spontaneous granulocyte-platelet aggregates were also detected and proved to be slightly downregulated by IR. Patterns of CD42a and CD61 expression (Figure 2B, central-top and central-middle panels) and a lack of CD62P expression in granulocyte-platelet aggregates after IR (Figure 2B, central-bottom panel) were similar to the phenomena observed in lymphocyte-platelet aggregates. These results indicated that IR did not induce the formation of granulocyte-platelet complexes.
PRP (Supplementary Figure 3, +PRP). These observations demonstrated that CD61 upregulation in monocytes was required for both platelet and IR.

**Morphological observations of IR-induced monocyte-platelet aggregates**

Morphological observations of monocyte-platelet aggregates are illustrated in Figure 3. The monocyte population was sorted from the whole blood after treatments with 0 i-rpm (left-side panels) or 5 i-rpm (right-side panels) and then confirmed by staining for CD61 and CD14, which are markers of platelets and monocytes, respectively. Large homotypic platelet aggregates were not observed on monocytes. However, platelets were detected on monocyte surfaces following 5 i-rpm treatment. Furthermore, monocytes exhibited extended pseudopods after this treatment. These observations suggested that IR induced the formation of monocyte-platelet complexes and resulted in monocyte activation.

**Augmentation of monocyte phagocytic activity after IR**

We performed a phagocytosis assay with the IR-treated whole blood after using BioParticles, which allowed visualizing phagosomes by the bright red fluorescence (Figure 4). There was no difference in the phagocytic activity of control granulocytes and granulocytes treated by 5 i-rpm IR. In contrast, monocyte phagocytic activity was significantly enhanced after IR at 5 i-rpm. These results suggested that IR selectively activated monocytes.

**Involvement of calcium ions in the formation of leukocyte-platelet complexes**

To elucidate mechanisms of leukocyte-platelet complex formation following IR treatment, we tested the effects of calcium-signaling inhibitors on the formation of leukocyte-platelet complexes (Figure 5A). Ethylene glycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid (EGTA) was added to the whole blood during the leukocyte-platelet aggregates assay. U0126 (an inhibitor of both MEK1 and MEK2 with an IC50 of 72 nM and 58 nM, respectively) or SB203850 (a p38MAPK inhibitor with an IC50 of 0.3-
0.5 μM) were also added to the blood. EGTA significantly inhibited the formation of monocyte-platelet complexes induced by IR, while MEK1/2 or p38MAPK inhibitors were without effect. These results suggested that the formation of monocyte-platelet complexes depended on the presence of free calcium ions, but not on the activity of MAPK signaling pathways.

EGTA also prevented downregulation of the number of spontaneous lymphocyte- and granulocyte-platelet aggregates induced by IR (Figure 5A). Lymphocyte- and granulocyte-bound platelets did not express CD62P (Figure 2). These observations indicated that IR inhibited aggregate formation via calcium-signaling mechanisms, which are likely to be implicated in platelet activation.

**Formation of monocyte-platelet complexes requires PSGL-1**

CD62P expressed on the surface of platelets has been reported to bind to PSGL-1 on monocytes [28, 29]. A blocking antibody against PSGL-1 was used to establish whether IR induced binding of platelets to monocytes. This PSGL-1 blocking antibody significantly inhibited the formation of monocyte-platelet complexes induced by 5 i-rpm IR treatment (Figure 5B). On the other hand, the antibody did not affect spontaneous formation of lymphocyte- or granulocyte-platelet aggregates (0 i-rpm) or IR-induced decrease in the number of lymphocyte- or granulocyte-platelet aggregates (5 i-rpm; Figure 5B). These results suggested that the interaction between PSGL-1 and CD62P was required for the IR-induced formation of monocyte-platelet complexes, but not for the generation of spontaneous lymphocyte-, granulocyte-, or monocyte-platelet aggregates.

**Effects of monocyte-platelet aggregates on cytokine production induced by LPS**

We investigated the effects of monocyte-platelet aggregates on cytokine production. Unexpectedly, IR did not trigger the production of inflammatory cytokines, such as IL-1β, IL-6, IL-8, IL-10, IL-12p70, tumor necrosis factor (TNF)-α, or MCP-1 in the absence of LPS stimulation (data not shown) as levels of these cytokines were almost undetectable.

After LPS stimulation, these cytokines could be detected in the conditioned medium. The levels of IL-1β and IL-12p70 in the blood treated with IR were significantly lower than those in the blood exposed to the control treatment (IR(-); Figure 6). Reduced levels of IL-1β and IL-12p70 were restored to control levels in the presence of anti-PSGL-1 antibody. These results suggested that monocyte-platelet aggregates inhibited the production of pro-inflammatory cytokines induced by LPS in whole blood. In contrast, the levels of IL-10 and MCP-1 in the blood treated with IR at 5 i-rpm were significantly higher than those in the untreated blood (IR(-); Figure 6). Augmented production of MCP-1 was significantly inhibited by anti-PSGL-1 antibody. These results indicated that the formation of monocyte-platelet complexes induced by IR did not increase the production of pro-inflammatory cytokines; however, surprisingly, it enhanced the production of chemokines and the anti-inflammatory cytokine IL-10 after LPS stimulation.

**Monocytes secrete IL-10 in whole blood after IR**

IL-10-producing cells in whole blood were measured by flow cytometry after incubating diluted whole blood incubation in the presence of LPS for 12 h. CD61⁺ leukocytes were detected in granulocytes (CD16⁺), monocytes (CD14⁺), T cells (CD3⁺), and B cell (CD19⁺) fractions after this incubation in the presence of LPS. However, IL-10-secreting cells were mainly detected in the CD61⁺ monocyte subset (Figure 7A), and IL-10-secreting monocytes were significantly augmented after IR treatment (Figure 7B). These observations indicated that IL-10 secretion from monocytes was accelerated by the formation of complexes with platelets induced by IR.
Figure 6. Inverting rotation downregulates LPS-induced production of IL-1β and IL-12p70, but enhances the generation of IL-10 and MCP-1. Whole blood was treated with (closed column) or without (open column) IR at 5 i-rpm for 20 min at 37°C in the presence (control Ab or anti-PSGL1 Ab) or absence (vehicle) of antibodies. After the treatment, the blood was diluted 10-fold in RPMI 1640 medium and incubated in the presence of LPS (2 μg/mL) for 18 h. Asterisks denote significant differences between the data points as follows: NS, no significant difference; *P < 0.05; **P < 0.01 (ANOVA with Bonferroni test; n = 3–4).

Figure 7. Inverting rotation selectively enhances the generation of IL-10+ monocytes in whole blood. Whole blood was treated with (5 i-rpm, IR+) or without (0 i-rpm, IR-) IR for 20 min at 37°C in the presence of LPS (2 μg/mL) for 12 h. These blood cells were analyzed by IL-10 Secretion Assay Detection Kit and stained with antibodies to CD14, CD16, and CD61. Each leukocyte lineage markers (CD3, CD14, CD16, and CD19), and IL-10-producing cells were measured in each leukocyte fraction. (A) Flow cytometry analysis of IL-10-producing cells in each leukocyte population after incubation with LPS (representative data from four independent experiments). (B) The percentages of IL-10-producing cells in each leukocyte fraction were analyzed in whole blood after incubation in the presence or absence of LPS. Asterisks denote significant differences between (−) and (+): *P < 0.05 (Student’s t-test; n = 4).
Discussion

To the best of our knowledge, this is the first study demonstrating the effects of low shear stress induced by alterations in blood flow pattern on leukocyte-platelet aggregates in vitro using IR method. IR selectively induced the formation of monocyte-platelet aggregates and suppressed inflammatory cytokine production. We demonstrate that IR approach is useful for the analysis of modifications of monocyte functions following platelet binding.

The shift toward increased numbers of pro- or anti-inflammatory monocytes is a critical step in the control of the intestinal immune system [30, 31]. A recent study showed that a constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. Circulating monocytes reach the intestine and differentiate locally into mature, anti-inflammatory macrophages in steady-state conditions [30]. The functional shift of monocytes to anti-inflammatory phenotype was induced by IR in the absence of potent stimulants, suggesting that platelets play an important role in immune homeostasis via the formation of monocyte-platelet complexes.

To validate the IR method, we calculated the shear stress induced by IR and compared the stress with that induced by the previously reported reperfusion blood flow model using a “cone and plate shearing device”. This model is also known to induce monocyte-platelet aggregates under low shear stress [32]. The maximum shear stress during IR is $1.8 \times 10^{-2}$ Pa (0.18 dynes/cm$^2$), while the minimum shear stress with the cone and plate shearing device was $2.4 \times 10^{-1}$ Pa (2.4 dynes/cm$^2$). Therefore, the shear stress induced by the IR method is much less than that generated by the cone and plate shearing method.

The shear stress of pulsatile blood flow changes is commonly measured at the carotid artery using an ultrasound probe. The shear stress at the proximal internal carotid artery is oscillated from 0.4 to $-0.7$ Pa (4 to $-7$ dynes/cm$^2$); during early systole to mid systole, and the average change in shear stress is $-5 \times 10^{-2}$ Pa ($-0.5$ dynes/cm$^2$) [33]. The shear stress of IR was calculated as $1.8 \times 10^{-2}$ Pa with a maximum $\Delta$Pa of $1.2 \times 10^{-3}$ Pa, as described in the methods. Therefore, this lower shear stress condition created by IR is similar to the stress in the small artery (diameter $<1$ mm). These very low shear stress conditions ($<2 \times 10^{-2}$ Pa) are found throughout the body.

In our experiments, we assessed the importance of the blood–air interface during IR treatment. To elucidate consequences of the blood–air interaction in the tube, IR was performed after exchanging air for nitrogen or oxygen in the micro-tube (Supplemental Table 1). Neither bubbling nor replacing air with nitrogen or oxygen affected the formation of monocyte-platelet complexes (Supplemental Table 1).

The fraction of leukocyte-platelet aggregates detected in this study (~40%) is larger than the proportion of similar conjugates detected in the peripheral circulation of healthy subjects according to published literature. However, neutrophils from healthy volunteers were not activated in peripheral blood because augmented CD16 expression and increased granularity were not observed before IR treatment (data not shown). Furthermore, we checked the expression of CD62P, a platelet activation marker, at time 0, but no significant CD62P expression was detected in any leukocyte subsets (Figure 2B). These observations suggested that platelets and neutrophils were not activated by the blood preparation. A possible reason of the discrepancy between our study and previous reports is the fixation method. Our previous study demonstrated that the whole blood assay can be used for direct observation of intact cell surfaces [34]. Thus, in this study, we immediately fixed the whole blood with the Lyse/Fix buffer (BD Bioscience) at 37°C. This manipulation is also recommended for the analysis of phosphorylated proteins in single cells using flow cytometry; thus, artificial cell activation during fixation is not likely to have occurred. We speculate that this prefixing of blood may be one reason for the large fraction of leukocyte-platelet aggregates in the peripheral circulation of healthy subjects seen in this study. Indeed, continuous collisions between leukocytes and circulating platelets were observed by intravital microscopy, while interaction of platelets with other cells was usually only transient [16].

In Supplemental Figure 3, we show that >20% of monocytes were CD61$^+$ monocytes (aggregated with platelets) in PBMCs isolated using the Ficoll-Paque method. These data suggest that some monocytes may spontaneously form complexes with platelets in the peripheral blood of healthy volunteers. Alternatively, during the separation of PBMCs from peripheral blood, in experimental operations such as centrifugation or treatment with Lyse/Fix buffer, these complexes may be formed artificially. Although it is very difficult to exclude the possibility of artificial complex formation before IR, the monocyte-platelet aggregates under IR are almost certainly generated by these experimental conditions. We speculate that constitutive production of monocyte-platelet aggregates in normal circulation may be a message for tissues to “stay on standby” to maintain homeostasis in an immunological response.

To clarify the effect of Lyse/Fix buffer on the formation of monocyte-platelet aggregates, we compared the Lyse/Fix buffer method with standard method (Ab stain→fix) under gentle centrifugation (400 × g, 5 min). As we show in Supplementary Figure 4, there were no significant differences between the rate of monocyte-platelet aggregates using “Lyse/Fix buffer” method and “Ab stain→fix” method (Supplementary Figure 4A). Several reports demonstrated that the baseline percentage of monocyte-platelet aggregates were 20–40% in the whole blood samples obtained from healthy volunteers [29, 35]. Especially, Rinder and colleagues have performed the pre-fix and post-staining method, which is similar to Lyse/Fix buffer method, and reported that the baseline percentage of the monocyte-platelet aggregates was 45.25% ± 3.2% from healthy volunteers [35]. Furthermore, purification of monocytes using magnetic beads from the Lyse/Fix buffer-fixed cells (Lyse/Fix buffer→IMag) reduced the percentage of monocyte-platelet aggregates (Supplementary Figure 4A). This result shows that, in some cases, the loss of platelet antigens on monocytes happens in the step of monocyte separation even after the fixation. On the contrary, morphology of each leukocyte subset in the whole blood sample was better preserved when the Lyse/Fix buffer method was used (Supplementary Figure 4B). Therefore, these observations suggest that a method using Lyse/Fix buffer is one of the suitable methods to detect the baseline percentage of monocyte-platelet aggregates correctly in whole blood.

Unlike the monocyte-platelet aggregates, the lymphocyte- and granulocyte-platelet aggregates were reduced after IR. One possibility is a break in weak cell–cell adhesion following shear forces during IR. Phagocytosis of platelets by activated granulocytes may be another possibility. However, the reduction of the number of lymphocyte-platelet aggregates cannot be readily explained by phagocytosis. In fact, mechanisms mediating down-regulation of lymphocyte-platelet aggregates remain unclear and further experiments will be necessary to clarify them.

To verify the effect of platelets binding to monocytes on phagocytosis, anti-PSGL1 Ab was added into the whole blood sample before IR. As expected, the addition of anti-PSGL1 Ab partially inhibited phagocytosis of monocytes. However, the inhibition of phagocytosis by the addition of anti-PSGL1 Ab was also observed in the blood sample without IR treatment (data not shown). Furthermore, the inhibition of phagocytosis after the
addition of anti-PSGL1 Ab depends on the individual blood sample (data not shown). These observations suggest that the platelet-monocyte binding may assist the augmentation of phagocytosis, but is not essential. Further investigation using tools other than anti-PSGL-1 Abs is required for elucidating the relationship between augmentation of phagocytosis by IR and formation of platelet-monocyte aggregates.

All Toll-like receptors (TLR1–9) are expressed on platelets. This expression is known to induce binding between bacteria and platelets [1]. In this study, the phagocytic activity of monocytes was augmented by IR. After IR, BioParticles (E. coli) may be trapped not only on monocytes but also platelets through the TLRs, inevitably increasing the number of BioParticles in the monocyte-platelet aggregates compared to single monocytes.

Previous studies have shown that platelet-monocyte binding initiates the production of pro-inflammatory molecules, such as IL-8, MCP-1, TNF-α, tissue factor, and cyclooxygenase 2 [21, 28, 36, 37]. Binding of PSGL-1 to CD62P is not sufficient to induce the production of pro-inflammatory factors; additional activation of G protein-linked family proteins induced by the RANTES (regulated on activation normal T cell expressed and secreted) is required [38]. In this study, IR did not enhance, but reduced, pro-inflammatory cytokine production by promoting platelet–monocyte interactions via PSGL-1 binding to CD62P. In contrast, IR augmented the production of the anti-inflammatory cytokine IL-10. The enhancement of IL-10 secretion and reduction of TNF-α secretion by monocytes following platelet activation has also been described previously [22]. Interestingly, blocking the interaction between PSGL-1 and CD62P was not sufficient to inhibit the production of pro-inflammatory cytokines, as reported previously [2, 39]. Therefore, simple activation via PSGL-1 binding to CD62P may induce monocytes with an anti-inflammatory phenotype.

We chose a whole blood assay to analyze cytokine production in order to avoid modifications to the leukocyte-platelet aggregates by purification of leukocytes. Therefore, we had to consider cytokine production from granulocytes (mainly neutrophils) and lymphocytes (T cells and B cells), in addition to monocytes. In the case of IL-10, monocytes were the main producer and IL-10 production was augmented by IR (Figure 7). Furthermore, treatment with anti-PSGL1 mAb inhibited monocyte-platelet complex formation by IR, as well as the recovery of IL-1β production that had been inhibited by IR. These observations allowed us to speculate that cytokine production may be related to the formation of monocytes-platelet complexes. However, we cannot exclude the possible effect of granulocytes and lymphocytes on the downregulation of cytokine production by IR. Further investigations using the IR method are required to evaluate these effects on cytokine production.

The production of IL-12p70 and TNF-α was significantly augmented by the addition of anti-PSGL1 Ab compared to the addition of control Ab, with IR treatment (IR(+); Figure 6 and Supplementary Figure 5). These results strongly suggested that the formation of monocyte-platelet aggregates via PSGL-1 was associated with the inhibition of IL-12p70 and TNF-α. However, the production of IL-12p70 was not recovered to the level in the treatment without IR by the addition of anti-PSGL-1 Ab. Furthermore, independently of IR treatment (IR (-)), anti-PSGL1 Ab augmented TNF-α production compared to the control Ab. Thus, the effects of complex formation on the production of TNF-α and IL-12p70 are not clear. The augmentation of IL-8 production induced by IR treatment was not inhibited by the addition of anti-PSGL1 Ab, and the modulation of IL-6 production by IR treatment was poor (Supplementary Figure 5). Nevertheless, IR treatment never induced pro-inflammatory cytokines, only chemokines, and IL-10-secreting cells were mainly found in the CD61+ monocyte subset. These results suggest that platelet-bound monocytes induced by IR show an anti-inflammatory phenotype.

In conclusion, we demonstrated that our IR method selectively induced the formation of monocyte-platelet aggregates, which exhibited anti-inflammatory properties. This method may therefore help to elucidate the role of platelets in the regulation of immune homeostasis.

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Declaration of interest
The authors report no declarations of interest.

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