Original Article

# Conjugates of B cells and platelet microparticles in the human peripheral blood

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## Abstract

**Background:** Activated platelets shed microparticles (MPs) in vivo and certainly in vitro under storage. Like platelets, platelet-derived MPs contribute to hemostatic and inflammatory responses. We sought to determine the interactions between platelet MPs and peripheral B lymphocytes in the healthy blood circulation to propose a possible role for platelet MPs in the functioning of B cells.

**Materials and Methods:** An enzyme-linked immunosorbent assay (ELISA) was established to determine the normal interactions between human peripheral blood B lymphocytes and platelet MPs. B cells were isolated and bound to the wells of microtiter plates using coated anti-CD19. Then the presence of attached MPs was surveyed. Also, platelet MPs were separated from human platelet concentrates and applied to confirm the new binding capacities of B cells for these microvesicles.

**Results:** Platelet MPs were recognized in the wells of ELISA in which only B cells were isolated. So MPs were bound with peripheral blood B cells. Furthermore, using this method, the role of CD40/ CD40L interaction was displayed for the binding.

**Conclusion:** It seemed that the binding of platelet MPs to B cells normally took place in vivo and a percent of B cells circulate in blood in connection with platelet MPs.

**Keywords:** ELISA, Platelet microparticles (Platelet MPs), B cells, CD40L

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## Introduction

Platelets are enucleated cellular fragments produced by megakaryocytes and are best known for their role in hemostasis (1). These cellular elements participate in inflammation by releasing various proinflammatory substances that interact with circulating leukocytes and endothelial cells lining vessels (2, 3). However, recent studies indicate an additional role in modulating adaptive immune responses. In this regard, platelets have been shown to modulate dendritic cell activation (4-6), enhance T-cell responses (4, 7), induce B-cell production of IgG antibodies (4, 8), and enhance germinal center (GC) formation in cooperation with T cells (1, 9). Many reports show that CD40L is expressed on platelets, and soluble CD40L (sCD40L) is released from platelets after activation. The high concentrations of sCD40L in platelet preparations might explain the febrile or adverse responses associated with platelet transfusion. The CD40/CD40L interaction is important for many steps of B-cell differentiation (2, 10-12). The interaction of CD40 and CD40L (CD154) is critical for formation of germinal centers (GCs) (13-16). The

pivotal role of CD40-CD40L in T cell-dependent B cell responses was proven by the finding that patients suffering from the X-linked hyper-IgM syndrome (HIGM), were characterized by mutations in their CD40L gene (17, 18).

MPs (MPs), microvesicles or ectosomes are shed from several cell types including leukocytes, erythrocytes, endothelial cells and various cancer cells (19). Existence of MPs was first noticed by Wolf in 1967 that described for the first time platelet's membrane fragments originated from activated platelet called platelet dust (20). MPs expose phosphatidylserine and display surface plasma membrane markers from their parental cells (21, 22). MPs derived from platelets may transfer many platelet antigens to the surfaces of hematopoietic stem-progenitor cells (23). They contribute to haemostatic and inflammatory responses, vascular remodeling, angiogenesis, cell survival, and apoptosis (22, 24, 25). MPs are also present, and accumulate, in blood products such as erythrocyte and platelet concentrates during storage (26). It has long been thought that 70–90% of MPs in plasma are derived from activated platelets in comparison to other MP generating cells which contribute to the total pool. Typically, MPs range in size from 0.1 μm to 1 μm (19, 21, 27). PMP surface is approximately 50- to 100-fold more procoagulant than the surface of activated platelets (28).

We examined the capacity of platelet MPs for binding to human peripheral blood B cells and the presence of platelet MP/B cell aggregates in human healthy blood. Also, we designed an ELISA method to obtain different information about the platelet MP/B cell interactions.

# Methods

**Sample preparation.** Informed consent was obtained from the blood candidates by Iranian Blood Transfusion Organization (IBTO). Five whole blood and five single donor platelet concentrates (PC, JMS Singapore Pte Ltd.) were prepared from IBTO (24 hours after PC preparation and completion of the viral safety tests).

**B cells isolation from human whole blood using panning method (positive selection).** Briefly, microtiter plates (Greiner bio-one, Frickenhausen, Germany) were coated with 100 μL of a 5 μg/ml solution of anti-CD19 (Abcam, UK) per well. The phosphate-buffered saline (PBS), pH 7.8 was used, and the plates were left 2 hours at 37ºC. Blocking of the plates was performed using a 2 % solution of BSA in PBS (3 h, RT), followed by incubation with 100 μL of the ficol-isolated buffy coats and incubation at 4ºC for overnight. After washing step, the adhered B cells were used to screen for the presence of platelet MPs.

**Isolation of platelet-derived MPs from platelet concentrate.** For preparing platelet-derived MPs from platelet concentrates, platelets were transferred into 15 ml falcon tubes. The tubes were centrifuged at 150 g for 15 min to remove remaining white and red blood cells. The supernatant were separated and then depleted from platelets using two times centrifugation at 1200 g for 15 min. Finally MPs were isolated using centrifugation at 16000 g for 15 min.

**Evaluation of the binding of B cells and platelet-derived MPs using ELISA method.** Paraformaldehyde (0.5%) was used to fix absorbed B cells in the ELISA wells. In the next step, 0.1 M glycine in phosphate buffer was employed to react with free aldehyde groups and prevent unspecific binding during ELISA procedure. Then after washing step, in screening for platelet MPs and their binding to B cells, biotin-conjugated anti-human gpIbα (Gentaur Europe BVBA, Brussels, Belgium) was added. Again, 45 minutes incubation at RT was chosen. Subsequent to routine washing step, addition of streptoavidin-HRP (Antigenix America, Huntington Station, NY, USA) was accomplished (Fig.1). Once more, 40 minutes incubation at RT was chosen. After washing step, the enzymatic activity was determined using tetramethyl–benzidine (Usb, USA) as substrate. Notably, the wells devoid of the coated antigen or the specific antibody to gpIbα were used as negative controls.

Alternatively, isolated MPs were used to study the additional binding sites on B cells for MPs. 100 μL of anti-CD40L (4 μg/ml, Abcam, UK) treated or untreated MPs were added to the wells containing B cells for 45 min/RT. The experiment was continued as mentioned above to complete ELISA protocol.

Instead, in an independent experiment, mouse IgG (1 μg/ml) was added to all wells of ELISA plate for 30 min before addition of anti-gpIbα for being

certain of the specificity of the binding of anti-human gpIbα to MPs and to demonstrate that the binding was taken place via antigen binding site and not by FC receptors.

**B cells/ MPs Aggregates in the culture medium.** Buffy coats were obtained from ficol-treated human whole blood. B cells were isolated by negative selection method using anti-CD16 (Abcam, UK), anti-CD3 (Abcam, UK), anti-CD14 (Dako, Denmark), and Dynabeads® goat anti-mouse IgG (invitrogen Dynal AS, Oslo, Norway). The cells (106/ml) were co-cultured with platelet MPs (500 μg/ml). After 7 days samples were taken and stained with hematoxylin-eosin method.

# Results

**Evaluation of B cell binding to platelet MPs using ELISA method.** As it could be seen in Table 1, the mean OD450 for uncoated wells was determined 0.19±0.014 whereas it was determined for B cells (alone); 0.508±0.001. This absorbance could demonstrate the presence of MPs in the wells of ELISA plate in attachment with B cells (Fig.1). Treatment of B cells with MPs changed the OD450 to 1.273±0.165. So the increasing of OD450 could be attributed to binding of MPs to non-saturated sites of B cells for MPs. Additionally, previous treatment of MPs with anti-CD40L and addition to B cells decreased OD450 to 0.805±0.052. This result implied that the CD40-CD40L molecules have a role in the interaction of B cells and platelet MPs so application of antibodies against CD40L could affect the interaction (Table 1).

**Table 1:** OD450 (mean±SD) obtained in ELISA method for the screening of platelet MPs attached to B cells. Details were explained under Materials and Methods section.

| Wells condition | Absorbance (OD450 nm) |
|---|---|
| **Negative control (Without anti-CD19 coated antibody)** | 0.19±0.014 |
| **B cell** | 0.508±0.001 |
| **B cell + MPs** | 1.273±0.165 |
| **B cell + (anti-CD40L treated) MPs** | 0.805±0.052 |

**Table 2:** OD450 (mean±SD) obtained in ELISA method for the screening of platelet MPs in attachment with B cells. Mouse IgG was used before addition of anti-gpIbα as mentioned under Materials and Methods.

| Introducing the wells | Absorbance (OD450nm) |
|---|---|
| **Negative control (Without anti-CD19 coated antibody)** | 0.175±0.007 |
| **B cell** | 0.552±0.12 |
| **B cell +MPs** | 1.522±0.02 |
| **B cell + (anti-CD40L treated) MPs** | 0.867±0.02 |

Alternatively, in the ELISA protocol, we also used mouse IgG before addition of anti-gpIbα to be certain of the specific binding of anti-gpIbα antibody to gpIbα and not to FC receptors. As it could be seen in Table 2, the mean OD450 for B cells alone was 0.552±0.12. Whereas treatment of B cells with MPs changed the OD450 to 1.522±0.02. So again, the increasing of OD450 could be also attributed to the additional binding capacity of B cells for MPs. It is worth mentioning that previous treatment of MPs with anti-CD40L decreased OD450 to 0.867±0.02. This result showed that the CD40 and CD40L molecules have a role in the interaction of B cells and platelet MPs so application of anti-CD40L could affect the interaction (Table 2).

**B cells/ MPs Aggregate in the culture medium.** As it could be seen in the simultaneous culture of human B cells and platelet-derived microparticles, aggregates were formed between them (Fig.2). It seemed that MPs could bridge between the

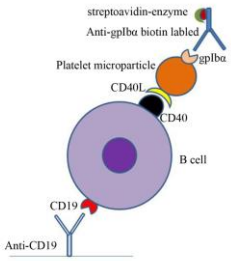


**Figure 1.** A schematic representation for different stages in an established ELISA method

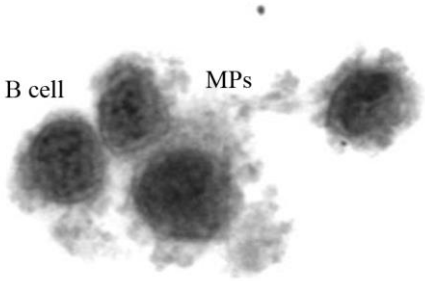


**Figure 2.** Microscopic examination of the interaction of B/platelet MPs in the culture medium after 3 days co-culture (after staining with hematoxylin and eosin)

B cells.

## Discussion

Platelet MPs comprised most of the MPs in human circulation. The result of this study showed that platelet MPs like platelets could bind to human B cells. Additionally, a proportion of B cells adhered to MPs normally and created aggregates that circulate in the healthy blood.

Cognasse in 2007 showed that in co-culture, platelets and B lymphocytes were bound and mutually activated, as demonstrated by the increased expression of platelet CD62P and B-cell CD86. Platelet/B-cell interactions were accompanied by changes in membrane expression of CD40 and CD40L by both platelets and B lymphocytes (2). In accordance, our study demonstrated the role of CD40L for the binding of platelet MPs to B cells in vitro.

In 2003, Héloire and coworkers reported the presence of aggregates of endothelial MPs and platelets in the healthy human blood. In this study, it showed that activated endothelial cells MPs can interact with platelets and form aggregates in vitro and in vivo (29). We obtained similar results for platelet MPs and B cells.

## Conclusion

Our study introduced a novel application of ELISA method for studying the interactions between MPs of different sources and the cells. Additionally, new binding capabilities could also be studied using this method with addition of the MPs to the interest target cells in the wells of ELISA plate. Alternatively, the molecules that were engaged in the interaction could be investigated and recognized using this technique as it was described previously.

Although there were some studies about application of platelet MPs in the mouse models to show their effects on the production of antibodies from B cells, we didn't find any report about the existence of aggregates of B cells and platelet MPs in the human peripheral blood. This finding may imply a role for platelet MPs in the regulation of B cells function in vivo. Further studies could be carried out to reveal the potential role of platelets MPs in the adaptive immune response.

## Conflicts of Interest

None of the authors have any conflicts of interest to declare.

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