

## The role of platelet microparticles in the production of antibodies from B lymphocytes against HLA-DR antigen in vitro

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### Abstract

**Background:** Platelets can activate B cells and stimulate them for the production of antibodies. Since platelet microparticles (PMPs) originate from platelets, they may have the same virtue. In the present study, the effect of PMPs was investigated on the production of human leukocyte antigen (HLA)-specific antibody from B cells in vitro.

**Materials and Methods:** In this experimental study, HLA-DR antigen was solubilized from the immortalized B lymphocytes (Daudi cell line) and purified using the affinity chromatography. Antigen properties were determined by the ELISA technique. PMPs were isolated from platelet concentrate bags by centrifugation. Fresh blood products were prepared from the Innovation Center of Iranian Blood Transfusion Organization (IBTO) and B lymphocytes were purified by the MACS method. B cells were exposed with PMPs and HLA-DR antigens in the culture medium. On the third day of culture, the culture supernatant was examined in terms of antibody production using the ELISA test. The results were analyzed using paired sample T-test and P-value <0.05 was considered statistically significant.

**Results:** The specificity of the purified HLA-DR antigen was confirmed using the anti-HLA-DR antibody and ELISA technique in the presence of appropriate controls. The results showed that PMPs could stimulate the production of antibodies from B cells. The difference between the case and control was significant (P-value=0.001). Although total immunoglobulin (IgG) was higher in HLA-DR-treated wells, HLA-DR-specific antibodies were not identified by ELISA technique.

**Conclusion:** PMPs have the capability to induce IgG antibodies from B cells. In order to ensure the production of specific antibodies, further testing is required with high sensitivity.

**Keywords:** Antibody, HLA-DR, Immunization, Microparticles, Platelet.

### Introduction

Studies have shown that platelets play an important role in the development of innate and adaptive immune responses in addition to their role in coagulation and hemostasis (1). In recent studies, the role of platelets in modulating the adaptive immune responses has been investigated. Accordingly, the role of platelets in regulating the activation of dendritic cells, increasing the response of T cells, inducing B cells to produce immunoglobulin (IgG) antibodies, and increasing the germinal center (GC) formation has been demonstrated (2). It

was reported that platelets contain the CD40L molecule, a co-stimulatory molecule previously thought to be limited to T lymphocytes (3). Platelets are currently considered to be the main source of soluble CD40L (sCD154). In addition to platelets, other cells, including T lymphocytes, mast cells, monocytes, and endothelial cells, express CD40L on their surface (3-6). The role of CD40L in the activation of B cells, antibody production, isotype switching, and the formation of memory cells, has been recognized (4). The B lymphocyte is responsible for the production of antibodies in the primary

and secondary immune response during infection and immunization. Considering the T lymphocyte-mediated humoral immunity, the interactions between B cells and CD4 + T cells via CD154 are the most important event in the antibody production (7). Additionally, platelet-derived microparticles (MPs) express CD40L on their surface (8). MPs are different from the exosomes that germinate from the intracellular membranes because they are directly created from the cell membrane (9). Although almost all body cells produce MP, PMPs are the most abundant circulating MPs (70-90%) in the blood (10).

CD40L level was increased in platelets or platelet-derived MPs after 3 days of storage (11). Since MP can express platelet membrane surface receptors, it is considered as the carrier of cell-cell or cell-platelet interactions. It is also used as a carrier for bioactive molecules such as growth factors, signal molecules, and microRNAs (11, 12).

In addition to glycoproteins, other molecules that are important for the function of MPs can be found in their membranes such as platelet-activating factor, B-amyloid precursor protein, calcium ion-dependent protease calpain, arachidonic acid, and other phospholipids (13). Flow cytometry is the most common method for studying PMPs and their surface antigens (12). MPs are involved in transmitting platelet-derived signals to the components of the humoral immune system, which are often far away from the place of the activated platelets. In addition, MPs, especially PMPs, are able to interact with the membrane surface molecules of other cells like B cells, resulting in the increased production of antigen-specific IgG antibodies and the formation of germinal centers (14). Previous studies have demonstrated that the platelet activation leads to the modification of the immune system through B and T cells (15, 16). Platelet-derived membrane vesicles are available to carry platelet-derived

signals to other cells under physiological conditions (17-19). Additionally, it has been recognized that the platelets and PMPs affect on the activity of other cells, such as monocytes, neutrophils and endothelial cells (20,21). Unlike PMPs, exosomes do not obtain the surface markers of the activated platelet membranes (22, 23). Membrane vesicles released by DC increase the antigen-specific immune responses (24).

Limited studies have been conducted on the interaction between the PMPs and B cells in the presence of an antigen in vitro. Therefore, the effect of the PMPs on the peripheral blood B lymphocytes in the presence of a specific antigen (HLA-DR) has been investigated in this research as a preliminary study. For this purpose, platelet-derived MPs were exposed to peripheral blood B lymphocytes and the related antigen in vitro, and the antibody produced from the B cells against the antigen was surveyed at a given time.

## Materials and Methods

### Preparation of a cell line containing HLA-DR antigen

The human Burkitt's lymphoma cell line was purchased from the Pasteur Institute of Iran. It was then cultured in the RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Gibco, Germany), 1.0% L-glutamine (Merck, Germany), 100 U/ ml penicillin (Sigma, USA), and 100 ug / ml streptomycin (Sigma, USA) in a cell culture flask and incubated at 37 ° C in 95% humidity and 5% CO<sub>2</sub>. The cell suspension was collected and centrifuged for 10 minutes at 2500 RPM, and the cellular precipitate was washed with phosphate buffered saline (PBS) solution and stored in a freezer at -70°C until the solubilization of cell membrane was performed (24).

### Solubilization of cell membrane antigens

To prepare cell membrane antigens, 1 ml cell lysis buffer containing 150 mM NaCl, 25 mM Tris-HCl (Merck, Germany), 1

mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, USA), pH 7.5, 1.0% NP-40, 10 mM EDTA was added to the cellular precipitate prepared in previous stage and it was placed on the rotator for 2 hours. Additionally, it was centrifuged at 1000g for 10 min to remove the insoluble materials and finally, the supernatant was transferred into the dialysis bag with the cut-off of 3.5 KD and dialyzed in the PBS solution.

#### **Purification of HLA-DR antigen from cell membrane using an affinity chromatography**

After covalent binding of the purified mouse anti-human HLA-DR monoclonal antibody (OriGene Technologies, USA) to Cyanogene bromide-activated sepharose 4B according to the manufacturer's instructions and preparing the chromatography column, the cell suspension dialyzed was passed through the column previously washed with PBS buffer. The isolation of HLA-DR antigens from the column was performed by glycine buffer (0.2 M, pH 2.8) and collected in tubes containing Tris buffer (pH 8.0), and then the samples with an absorbance at 280 nm were collected. To condense the protein antigens collected, the tubular membranes with 10000 MVCO were used and the concentration of HLA-DR antigen obtained was determined using the Bradford technique (24).

#### **Investigating the specificity of HLA-DR antigen using ELISA technique**

For this purpose, given the concentration of antigen that was calculated in the previous stage, the antigen obtained was diluted with PBS and 50 µl of it was coated in the wells of a polystyrene microtiter plate (Nunc, Denmark), as well as 50 µl of HCG protein and PBS solution as negative controls were coated in other wells, and they were incubated in the refrigerator at 4°C for 18-24 hours. The plate was then incubated with a 2.0% BSA solution for 3 hours at room temperature. In the next stage, Mouse anti-human HLA-DR antibody was added to the plate and

incubated for 1 hour at room temperature. Then, HRP-conjugated anti-mouse IgG was used. Between each stage, the wells were washed with a PBS buffer containing 0.06% Tween 20 (Sigma, USA) to remove the unbound molecules. Next, by adding the TMB substrate (Trinity Biotech, USA), the HRP- conjugated anti-mouse IgG led to the production of a colored product of TMB. In the final stage, by adding sulfuric acid as stopping solution, the blue color resulted from the reaction was converted to yellow. In the final stage, readings were taken at 450 nm.

#### **Isolation of PMPs**

For this purpose, after receiving informed consent from the blood donors, platelet concentrate bags were prepared from Tehran Blood Transfusion Center. There was no choice in terms of the blood type, volume, and appearance of the bags. Before the start of the work, the platelet bags were stored in an incubator under agitation at 20-24°C. Each platelet bag was then separately poured into a 50 ml sterilized flask tube and centrifuged at 1200g for 15 minutes to precipitate all cells, including red blood cells, white blood cells, and platelets. Then, the supernatant of each tube containing plasma and MPs was transferred to another 50 ml Falcon tube and then again centrifuged with the same rounds and time until all the platelets and cells were completely precipitated. Then, the supernatant of each tube was poured separately into another 50 ml falcon tube and centrifuged at 16000g for 20 minutes. To ensure that the plasma proteins were not mixed with MPs, twice wash with sterile PBS and centrifugation at 16000g for 20 minutes were performed. In the final stage, the supernatant of the falcon tubes was discarded and the PMPs at the bottom of the tube were suspended by PBS or RPMI 1640 medium. Bradford technique was also used to determine the concentration of the MPs (25,26).

#### **Investigating the platelet origin of MPs**

Initially, 5 µl of the FITC-conjugated anti-CD41 antibody was added to the MP

sample. The sample was incubated in the refrigerator at 4°C for 40 minutes. An isotype control was also used to ensure the specificity of the reactions. Then, the sample was examined for CD41 expression by flow cytometry (25).

#### **Determination of the molecular size of MPs**

Firstly, 10 µL of a 500-fold diluted fluorescent 1-µm microbeads; FluoSpheres® microspheres (molecular probes, USA) was added to a MP sample at a concentration of 30 µg/ml in a 1.5 ml microtube, and the sample was increased to 100 µl by PBS buffer. Then, the sample was used to estimate the size of the MPs using the flow cytometry (25).

#### **Isolation of B lymphocytes from peripheral blood**

For each exposure of B lymphocytes to the MP and antigen, a complete fresh blood bag (one day old) containing citrate phosphate dextrose adenine (CPDA) as an anticoagulant solution was prepared from the Innovation Center of IBTO. The selection of the complete blood bag was done randomly. Then, the fresh blood sample was diluted with the sterile PBS buffer in a 1-1 ratio in a 50-ml falcon tube and the mononuclear blood cells were separated by a ficoll method. Finally, the cell suspension was washed twice with PBS to remove the toxic effect of ficoll on the cells.

The magnetic activated cell sorting (MACS) method was used to remove NK, T and monocyte cells. 10 µL of cell suspension prepared in the previous stage were removed and diluted with trypan blue 0.4% solution in a 1:1 ratio and the cell counting was performed. According to the number of cells counted, anti CD14 (Dako Cytomation, Denmark), anti-CD3 (HIT3a) (Abbiotec, New York), and anti-CD16 (Abcam, Cambridge) was added to the falcon tube containing 1 ml of peripheral blood mononuclear cell suspension and was incubated in the refrigerator at 4°C for 1 hour. At intervals of 10 min, the falcon tube containing cell suspension was slowly

shaken to allow antibodies to be bound to the target cells. After the completion of the incubation period, the cell suspension was washed with sterile PBS buffer and centrifuged at 2500 rpm for 10 minutes. Then, in proportion to the number of cells counted, Dyna beads goat anti-mouse IgG (Invitrogen, USA) were added to the cell suspension and incubated in the refrigerator at 4°C for 24 hours. Then, the falcon tube containing mononuclear cell suspension was transferred to the MPC field. At this stage, magnetic beads attached to the antibodies of T, NK, and monocyte cell surface were absorbed into the wall of the falcon tube under the magnetic field. The cell suspension remained in the middle of the falcon tube was mainly B lymphocytes. Finally, the cell suspension was slowly removed and transferred to another falcon tube. The whole process was done in sterile conditions. For effective and desirable exposure of B lymphocytes to MPs and HLA-DR antigen in RPMI 1640 medium, the number of the given cells should be sufficient. Hence, cell counting was performed on mononuclear cell suspensions. 10 µl of cell suspension was removed and diluted with trypan blue 0.4% in a 1: 1 ratio. After mixing, cell counting was performed using neubauer lam and optical microscope.

#### **Investigating the purity of B lymphocytes**

According to the number of cells counted, 100,000 B lymphocytes were added to a 1.5 ml microtube and suspended with 100 µl of PBS. 50 µl of the PE-conjugated anti-CD19 antibody was added to the cellular suspension. Microtube was incubated at 4°C for 35 minutes. Isotype control was used to ensure the specificity of the reactions. Then, the CD19 expression was investigated and the purity of lymphocyte B was determined using flow cytometry.

#### **The exposure of B lymphocytes with HLA Antigen in the presence of PMPs in the culture medium**

B lymphocytes were exposed to PMPs and HLA-DR antigen in 24-well plates and RPMI 1640 medium containing 10% fetal bovine serum (FBS), 1.0% L-glutamine, 100 u / ml penicillin, and 100ug / ml streptomycin. The number of B cells ( $1 \times 10^5$ ), MPs (at concentrations of 100  $\mu\text{g/ml}$  and 500  $\mu\text{g/ml}$ ), HLA-DR antigens (at concentrations of 200 ng/ml, 500 ng/ml) and RPMI medium were selected so that the volume of each plate well was 1 ml. The wells in the bottom row of the plate were considered as negative controls, and lymphocyte B, lymphocyte B with antigen, and lymphocyte B with MPs were added respectively to each of them. At the end of this step, the plate was incubated at 37°C in 5% CO<sub>2</sub> for 3 days (25,26).

#### **Total IgG measurement in the supernatant of the culture medium**

Sandwich ELISA was used to measure total IgG. Initially, anti-human immunoglobulin (Ig) was diluted with PBS buffer and coated in each of the wells of the 96-well microplate. After the completion of the incubation period, the solutions in wells were drained and 2% BSA solution was added to each well (blocking stage) and placed at room temperature for two and a half hours. The solutions in the wells were drained and the culture supernatant samples were added to each of the wells. The plate was again incubated at room temperature for one hour. After washing the plate, the HRP-conjugated anti-Human IgG was added to each of the wells as detection antibody and the plate was incubated for 45 minutes at room temperature. After the incubation period, the plate was washed and dried. Then, the TMB substrate solution was added to determine the enzyme activity. The plate was covered with an aluminum foil and incubated for 15-30 minutes at room temperature in the dark. Then, the sulfuric acid solution was used to stop the enzyme activity. In the last stage, the optical density (OD) of the wells was read using an ELISA Reader at 450 nm (26).

#### **Statistical test**

SPSS software was used for statistical analysis. Data were entered into the software and the mean of the variables considered in the samples was calculated. Paired sample T-test was used to compare the means. The P value <0.05 were considered statistically significant.

#### **Results**

##### **Investigating the specificity of HLA-DR antigen purified from Human Burkitt's lymphoma cell line**

The specificity of the purified HLA-DR antigen was determined by ELISA technique after reaction with HLA-DR-specific antibody. The corresponding results have been shown in Figure 1.

##### **Investigating the purity of B lymphocytes isolated from peripheral blood**

CD19 expression on the B cells surface was determined 88.64% after the examination of the samples containing B lymphocytes by the flow cytometry. Figure 2 shows the results obtained by flow cytometry in relation to the CD19 expression on the surface of B cells isolated from peripheral blood.

##### **Determination of the platelet origin of MPs**

The CD41 expression on the surface of MPs was examined by flow cytometry. The expression was determined as  $92.2 \pm 6$ . Figure 3 shows the result of flow cytometry for CD41 expression in a MP sample isolated from a 5-day platelet concentrate sample.

##### **Determination of the molecular size of PMPs by the fluorescent-conjugated microbeads**

By the flow cytometry analysis, mean fluorescence intensity (MFI) for the distribution of the size of the reference population (R2) and for the MP population (R1) was determined as equal to 37.61 and 14.79, respectively, indicating that the PMPs had a size smaller than 1000 nm (Figure 4).

### Investigating the effect of PMPs on the production of IgG antibody in the presence of HLA-DR antigen

After examining the total IgG antibody in the culture supernatant of the test samples at different concentrations and control samples at the third day of culture using ELISA technique and comparing them with each other, it was determined that the mean optical density of the IgG antibody has been increased in the test samples compared to each of the control samples. Mean and standard deviation ( $\pm$  SD) of the test and control samples at two concentrations of HLA-DR antigen (200

ng/ml, 500 ng/ml) and MP (100  $\mu$ g/ ml, 500  $\mu$ g/ ml) have been presented in the comparative Figures 5 and 6.

The differences between the mean values of IgG between B-MP-HLA and B-HLA were significant at two concentrations of MP (100 $\mu$ g/ ml, 500  $\mu$ g/ ml) with a P-value of 0.001. The results showed a considerable IgG production from B cells after exposure with PMPs. Although, the results displayed higher IgG production in the wells containing HLA-DR antigen (Figures 5 and 6), but the HLA-DR-specific antibody was not detected by ELISA technique.

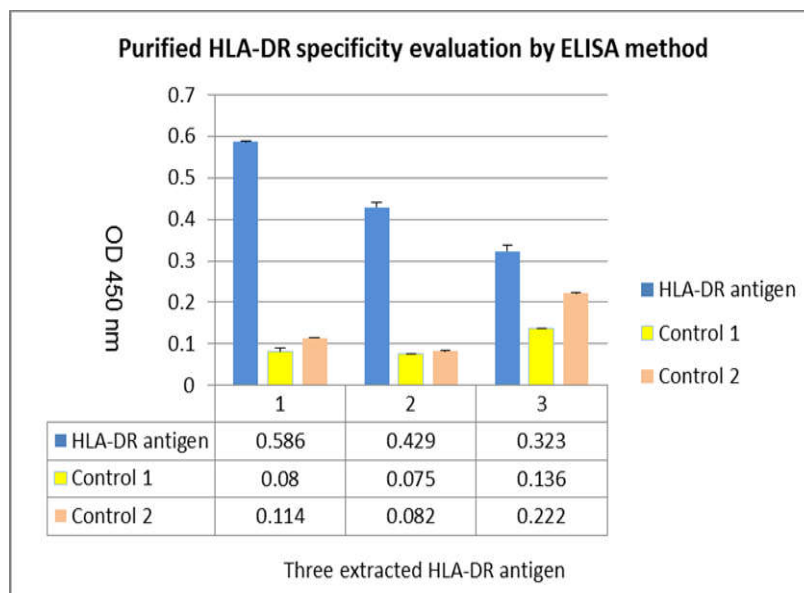


Figure 1: the results related to the coated HLA-DR antigen specificity Control 1: HCG - Control 2: PBS.

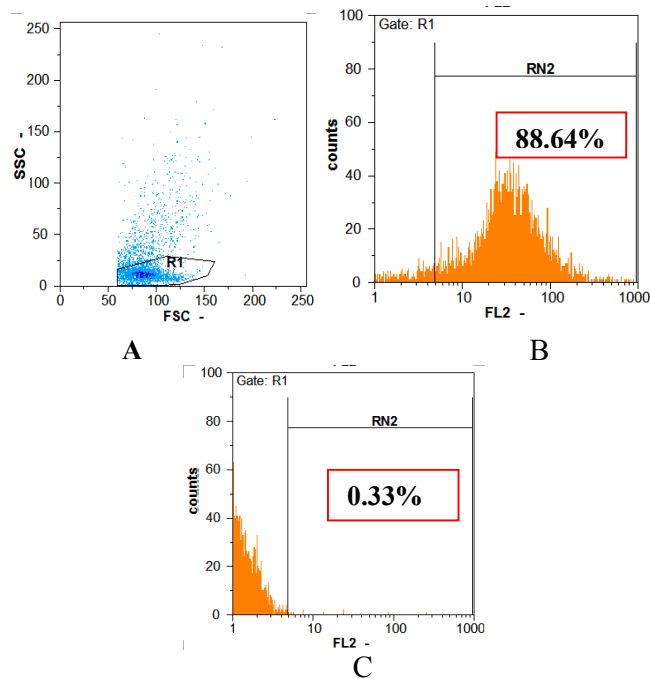


Figure 2: CD19 expression levels on the surface of isolated B cells, A. Gate of the B cells population; B. CD19 expression on the B cells surface; and C. Isotype control.

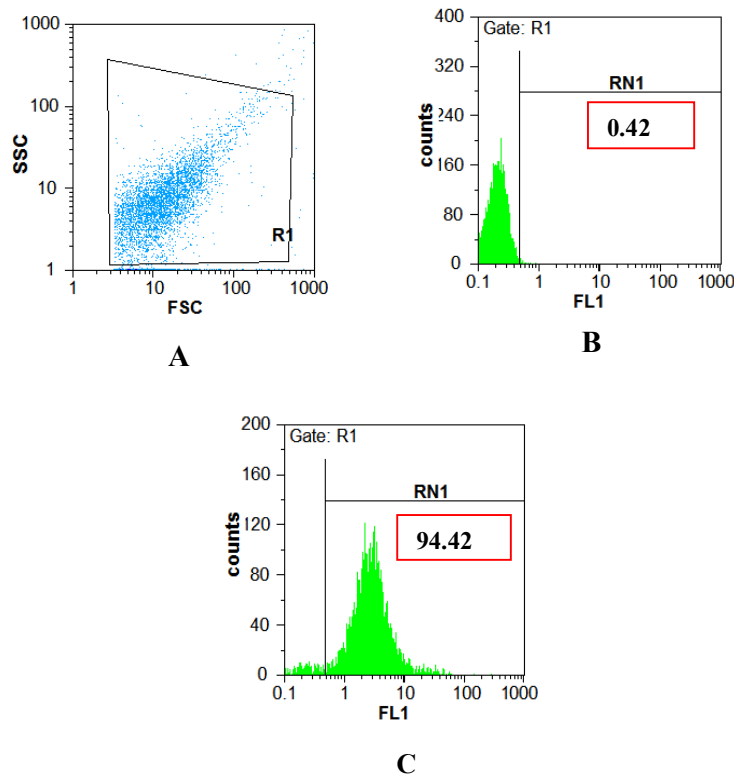


Figure 3: CD41 expression levels on the surface of MPs obtained from a 5-day platelet concentrate. A. Gate of the MP population; B. Isotype control and C. CD41 expression on the MPs surface

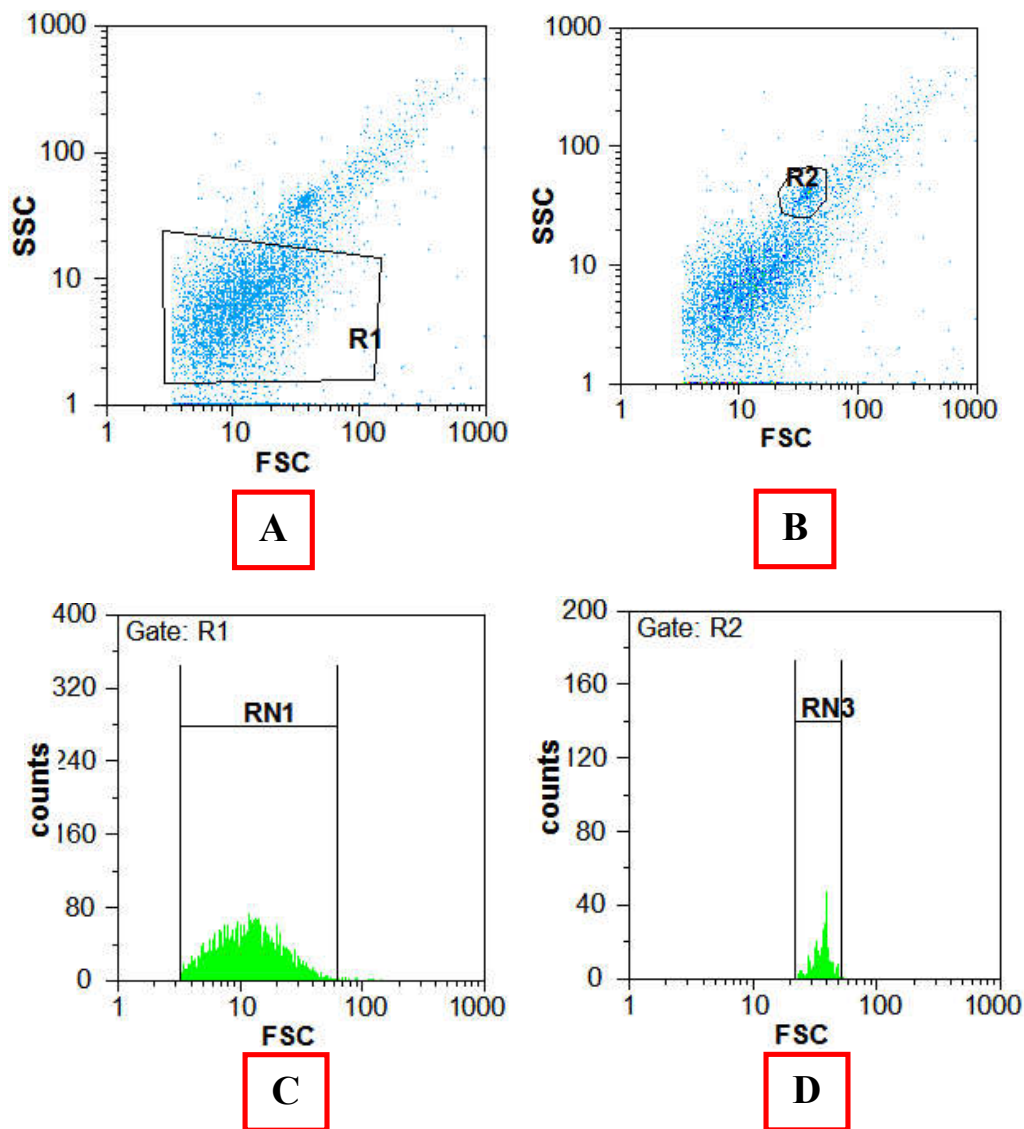
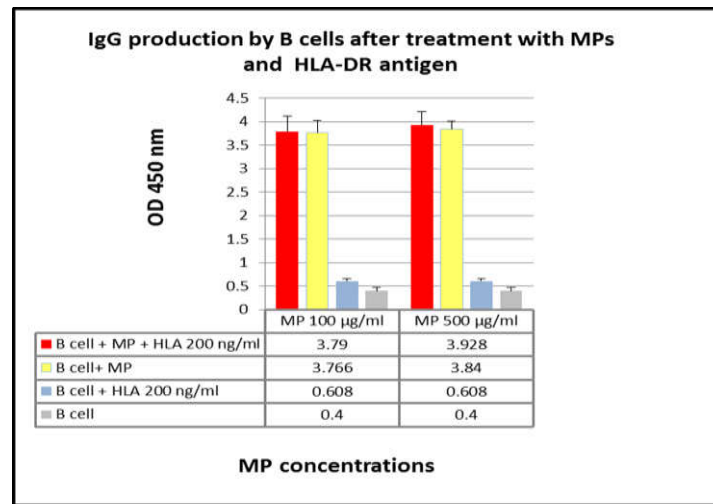
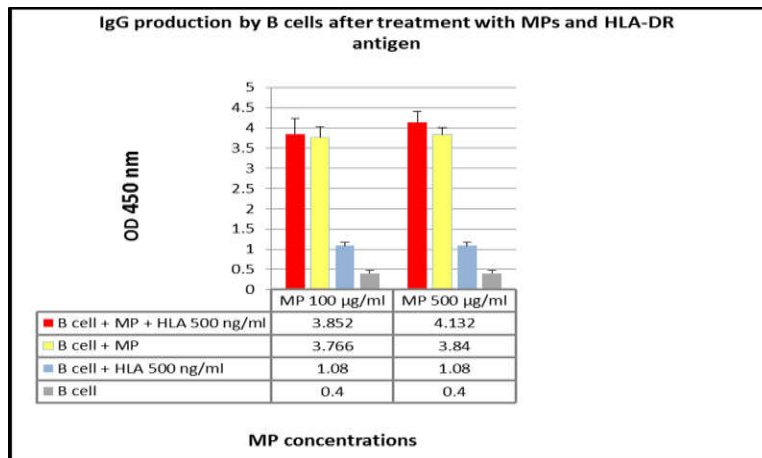


Figure 4: Comparison of the size of MPs and microbeads in flow cytometry analysis, A. Gate of the MP population (R1); B. Gate of the 1-μm microbeads (R2); C. A heterozygous population of microparticles has been shown (RN1); D. A population with a finite size (1 micrometer) is shown for standard fluorescent beads (RN3).



**Figure 5:** Diagram of the IgG antibody production from B cells after exposure to PMPs at two concentrations (100 µg / ml, 500 µg/ ml) in the presence of 200 ng/ ml HLA-DR antigen. The figure shows the efficient effect of MPs in the production of antibodies from B cells. Total antibody production was increased after treatment of B cells with HLA-DR antigen. The differences between the mean values of IgG between B-MP-HLA and B-HLA were significant at two concentrations of MP (100µg/ ml, 500 µg/ ml) with a P-value of 0.001.



**Figure 6:** The diagram of the IgG antibody production from B cells after exposure to PMPs at two concentrations (100 µg/ ml, 500 µg/ ml) in the presence of 500 ng/ml HLA-DR antigen. Total antibody production was increased after treatment of B cells with HLA-DR antigen.

## Discussion

The present study tried to investigate the effect of platelet-derived MPs on the production of antibodies from B lymphocytes in the presence of an antigen in vitro. The data from our study showed that PMPs stimulated the production of antibodies from B cells. In this study, two concentrations of HLA-DR antigen (200 ng/ ml, 500 ng/ ml) were used. There was a significant increase in IgG production in the test (antigen-MP-B cells) in comparison to the control sample (B cells-antigen). The data obtained from the comparison of two different concentrations of MPs and antigens showed that the MPs at the concentration of 500 µg/ml and the antigen at the concentration of 500 ng/ml produced higher levels of IgG antibody. There was a significant difference between the mean value of IgG produced in the test (B+MP+HLA) and control samples (B+HLA) (P-value: 0.001). Additionally, the mean value of IgG was higher in the test samples (B-MP-HLA) than the control sample (B-MP). Also, the mean value of IgG produced was higher in the test (B+HLA) than control samples (B).

Our results were correlated with the research conducted in 2007 by Cognass (27) about the effects of platelets on the production of antibodies from B cells. Their research showed that the co-culture of platelets with B lymphocytes leads to the activation of B cells in vitro. It was recognized by their observations that the platelet reaction with B lymphocytes was due to the interaction between the surface markers of CD40L and CD40. Although their study investigated the effect of platelets on antibody production from B cells and this study investigated the effect of PMPs on the antibody production, both studies have similar results. In 2008, Sprague et al assumed that the relationship between the platelet and the adaptive immune system was mediated by PMPs (2). The data from their study proved that PMPs were sufficient to induce IgG production from B cells, increase the

formation of germinal centers, and induce the proliferation of B cells and activation of endothelial cells in a CD40L-dependent fashion in vitro. Sprague and co-workers investigated the effect of PMPs on the production of antibodies in vivo. In a recent study, we showed the effects of PMPs on the production of antibodies from Daudi cell line in vitro (26).

Additionally, in the present study, an in vitro immunization method was also evaluated against HLA-DR antigen in the presence of PMPs. The effective role of PMPs for induction of specific antibodies in vitro needs further investigation using more sensitive methods for detection of small amounts of specific antibodies.

## Conclusion

PMPs have the ability to induce IgG antibodies from B cells. In order to ensure the production of specific antibodies for example against HLA antigen, further studies are required by using more sensitive detection methods.

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## Conflict of interest

The authors declare no conflict of interest.

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