

Anti-β2 Glycoprotein I Antibodies Cause Inflammation and Recruit Dendritic Cells in Platelet Clearance

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Keywords

Antiphospholipid antibodies, platelets, macrophages, dendritic cells, tumor necrosis factor-α

Summary

Scavenger phagocytes are mostly responsible for the in vivo clearance of activated or senescent platelets. In contrast to other particulate substrates, the phagocytosis of platelets does not incite proinflammatory responses in vivo. This study assessed the contribution of macrophages and dendritic cells (DCs) to the clearance of activated platelets. Furthermore, we verified whether antibodies against the β2 Glycoprotein I (β2GPI), which bind to activated platelets, influence the phenomenon. DCs did not per se internalise activated platelets. In contrast, macrophages efficiently phagocytosed platelets. In agreement with the uneventful nature of the clearance of platelets in vivo, phagocytosing macrophages did not release IL-1β, TNF-α or IL-10. β2GPI bound to activated platelets and was required for their recognition by anti-β2GPI antibodies. DCs internalised platelets opsonised by anti-β2GPI antibodies. The phagocytosis of opsonised platelets determined the release of TNF-α and IL-1β by DCs and macrophages. Phagocytosing macrophages, but not DCs, secreted the antiinflammatory cytokine IL-10. We conclude that anti-β2GPI antibodies cause inflammation during platelet clearance and shuttle platelet antigens to antigen presenting DCs.

Introduction

The equilibrium between platelet production in the bone marrow and platelet clearance in catheretic organs warrants the homeostasis of circulating platelets. Removal of platelets mainly involves the monocyte/macrophage system. It is increased in vivo by administration of macrophage colony-stimulating factor (1), and depends on the macrophage scavenger receptor A (2). In normal conditions activated or senescent platelets are safely eliminated and this event fails to elicit inflammatory responses from the ingesting phagocyte. Furthermore, platelet clearance does not associate to antiplatelet immunity.

Little is known about the signals involved in the physiological recognition and phagocytosis of platelets. Perturbation of platelet membrane due to activation or senescence leads to changes in the expression of membrane glycoproteins (3) and in the expression of anionic phospholipids such as phosphatidylserine (PS) (4-6). PS exposure is involved in the clearance of apoptotic cells by macrophages (7) and in the coagulation cascade (8). PS exposure by circulating cells is buffered by plasma cationic cofactors, including Annexin V and β2 Glycoprotein I (β2GPI) (9). Recognition of PS-expressing platelets by β2GPI allows their recognition by anti-β2GPI antibodies (10, 11). The association of antibodies recognising β2GPI with recurrent spontaneous abortions and/or venous and arterial thrombosis defines the antiphospholipid syndrome (aPS) (12). This syndrome can occur isolated, the Primary antiphospholipid Syndrome (PaPS), or in association with other autoimmune diseases, mainly Systemic Lupus Erythematosus (SLE).

Anti-β2GPI antibodies are highly heterogeneous (13-15) and sustain the phagocytosis of platelets by scavenger phagocytes (16). Furthermore, platelet opsonisation is involved in the pathogenesis of thrombosis occurring in aPS (17). Suppression of the tissue factor pathway inhibitor-mediated control on tissue factor induced coagulation (18), or the enhanced generation of tissue factor by monocytes (19), contribute to facilitate thrombotic events in the presence of anti-β2GPI antibodies. The molecular events linking anti-β2GPI antibodies to enhanced procoagulant activity of monocytes are still poorly understood. Proinflammatory cytokines may be involved. Accordingly, patients with anti-β2GPI antibodies have higher levels of circulating tumor necrosis factor-α (20).

In this study we show that anti-β2GPI antibodies biases the in vitro clearance of platelets, causing the local release of proinflammatory cytokines by phagocytes. Furthermore anti-β2GPI antibodies are required to endow with the ability to phagocytose platelets immature dendritic cells (DC), i. e. the most potent cells capable to initiate immune and autoimmune responses (21).

Materials and Methods

Reagents. Human β2GPI was purified by normal human serum by perchloric acid precipitation and affinity chromatography on heparin-Sepharose (HiTrap Heparin, Pharmacia Biotech Europe, Brussels, Belgium) followed by cationic exchange chromatography (Resource S, Pharmacia Biotech Europe) as described (22, 23). The purity of each preparation was routinely controlled by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Human immunoglobulins G (IgGs) were purified from the sera of patients with SLE or primary APS (diagnosed according to the criteria reported in 24 and 25 respectively) by mean of chromatography on protein G-Sepharose (HiTrap Protein G, Pharmacia), further purified by human β2GPI-affinity chromatography. Anti-β2GPI IgGs were retrieved by acid elution in a 0.1 M glycine, pH 2.8, 500 mM NaCl buffer, and immediately extensively dialyzed against PBS, pH 7.4. The

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Kd of anti-β2GPI IgGs for their antigen was not influenced by this procedure (22) indicating that affinity purification does not cause major conformational alterations influencing the specificity of the antibodies. The protein concentration was evaluated using both chemical (BCA protein assay reagent, Pierce Europe, Amsterdam, The Netherlands) and immunologic assays (LC-partigen IgG; Istituto Boehring, L'Aquila, Italy). β2GPI-affinity purified IgGs maintain the ability to recognize the cofactor as well as cardiolipin, as detected by ELISA assays. Cytochalasin D (CCD) was purchased from Sigma.

Cells. Mononuclear cells were obtained from buffy-coats (kindly provided by the Blood Transfusion Department of our Institution) after density gradient centrifugation. The adherent/monocyte enriched population was retrieved and differentiated in vitro to macrophages and immature DCs. Macrophages were obtained by plastic adherence and culture for 7-10 days in DMEM containing 5% heat inactivated pooled human serum (Sigma) (26). Immature human DCs were derived in RPMI containing 3% heat inactivated pooled human serum in the presence of recombinant human GM-CSF and IL-4 as described (27). The membrane phenotype of the two populations was routinely monitored by mean of flow cytometry as described. Briefly, the expression of the membrane receptors CD1a, CD14, CD40, CD80, CD86, HLA-DR, CD32 and CD36 was verified incubating the cells for 30 min at 4° C in the presence of the FITC- or PE-conjugated specific monoclonal antibodies (all from Pharmingen). Samples were analysed after extensive washing using a FACScan apparatus (Becton Dickinson).

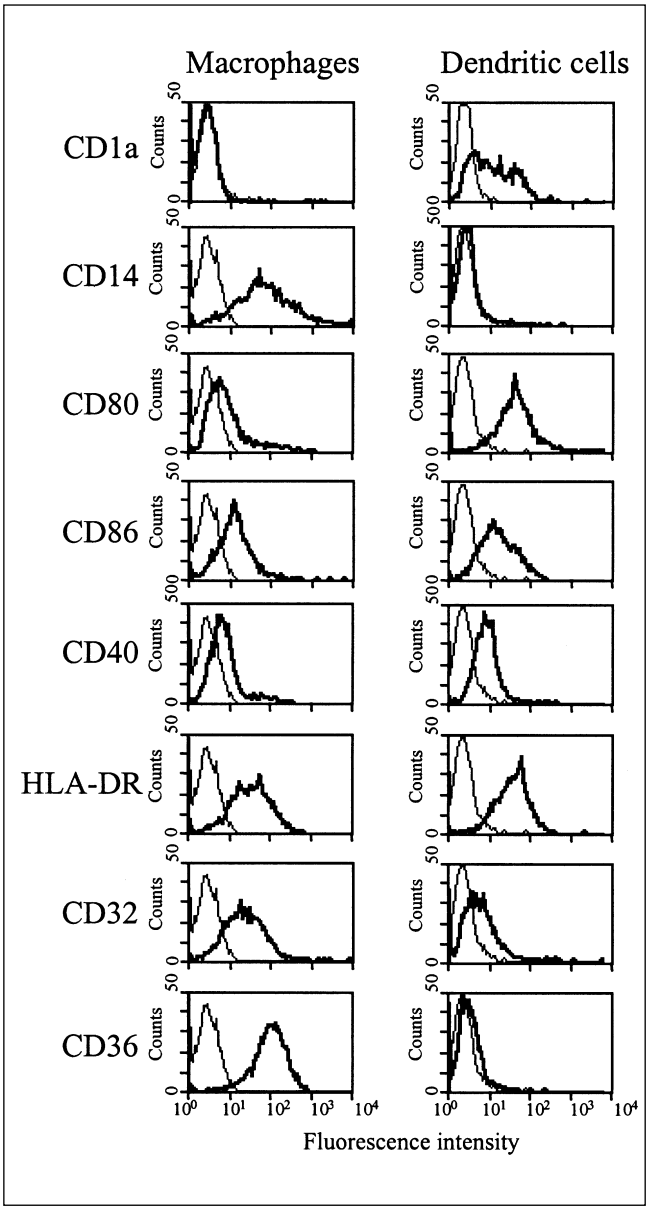

Fig. 1 Monocyte-derived DCs and macrophages express MHC class II molecules and costimulatory molecules. DCs and macrophages (Mϕ) were derived from peripheral blood monocytes, as described in the Materials and Methods section. The membrane expression of membrane receptors was detected by flow cytometry after staining with fluorochrome-conjugated monoclonal antibodies. Results are expressed as Fluorescence Intensity (arbitrary units, x axis, solid lines). Each panel also reports the relative fluorescence background (arbitrary units, x axis, thin lines). Cell numbers are reported on the y axis. Results are representative of n = 9 separate experiments

Platelets. Blood was collected by venipuncture from the ante-cubital vein of normal healthy volunteers on the day of the assay. No subject had taken any antiplatelet drugs for the previous 7 days. Blood was collected into plastic tubes with anticoagulant (6 part blood, 1 part citric acid 65 mM, sodium citrate 85 mM, D-(+) glucose monohydrate 2%) and centrifuged at room temperature at 200 g for 15 min. Platelet rich plasma (PRP) was retrieved and centrifuged at 700 g for 15 min. The platelet pellet was re-suspended in Tyrode's solution (HEPES 10 mM, KCl 5 mM, $MgSO_4$ 1 mM, pH 7.38, NaCl 145 mM, glucose 10 mM). Platelets were then counted in a haemocytometer and their concentration adjusted to 50×10^6/ml. Platelets were activated with bovine thrombin (0.05-0.5 U/ml, Sigma, St. Louis, MO) for 10 min at room temperature. $CaCl_2$ (2 mM final concentration) was added just before activation. Optimal conditions for platelet activation were defined as described (13). Briefly, light transmission after the addition of thrombin was recorded by dual-channel aggregometry (ELVI 5-80). Platelets were loaded with Fura 2 (Calbiochem, San Diego, CA), administered as acetoxymethyl-ester for 30 min at room temperature. Analysis was performed after the addition of sulfinpyrazone (250 mM, Sigma) in a Perkin-Elmer LS-5B fluorimeter (Perkin-Elmer, Norwalk, CT) (13). Exposure of anionic phospholipid by freshly derived and thrombin-activated (0.05 U/ml) platelets was monitored using FITC-annexin V (20 ng/10^6 platelets, Bender Med Systems, Vienna, Austria). When indicated platelets were incubated with purified β2GPI for 20 min at 4° C. Binding was revealed using a polyclonal rabbit antihuman β2GPI serum, followed by an antirabbit FITC-conjugated second step reagent (Sigma) (28). Fluorochrome-conjugated monoclonal antibodies to platelet antigen CD42a and to CD62P (P-Selectin) were purchased from Becton Dickinson (Sunnyvale, CA). Samples were analysed on a FACScan (Becton Dickinson). The ability of the IgGs eluted from patients' sera (see above) to bind to resting and thrombin-activated platelets was evaluated after 30 min at 4° C in the presence of human purified β2GPI. Binding of purified anti-β2GPI IgGs to resting platelets or to platelets recently activated or chased for up to 6 h after removal of thrombin was performed in the presence (final concentration 20 μg/ml) or in the absence of the purified plasma cofactor. Bound IgGs were revealed by means of a FITC-conjugated antihuman second step reagent (Sigma). Results are expressed as Relative Fluorescence Intensity (R.F.I.) calculated as follows: mean fluorescence intensity (m.f.i.) of platelets in the presence of the relevant antibody and the second step reagent/ m.f.i. in the presence of the second step reagent only. As a negative control, platelets were stained with the second step reagent only. Samples were analysed on a FACScan (Becton Dickinson).

Phagocytosis. Macrophages and immature DCs were washed, counted and plated the day before the assay in the appropriate medium containing Nutridoma (1%, Roche Diagnostics, Milan, Italy). Washed platelets were labelled with ^{51}Cr as described (29) and activated with 0.05 U/ml thrombin. The incorporated radioactivity ranged in different experiments from 0.099 to 0.135 c.p.m./platelet. Platelets were preincubated with IgGs purified from healthy individuals, washed and then incubated with or without anti-β2GPI IgGs (0.5 μg/sample) for 20 min at 4° C, in the presence or absence of purified β2GPI (20 μg/ml final concentration). After extensive washing, ^{51}Cr labelled platelets, opsonised or not with anti-β2GPI IgGs, were coincubated with the appropriate phagocyte at 37° C (platelet:phagocyte ratio = 20:1). All experiments were also run in parallel at 4° C, i.e. at a temperature that does not allow actin-based cytoskeleton reorganisation and active phagocytosis, to evaluate the relevant background values. After 60, 180 or 300 min of coincubation, macrophages were treated with trypsin/EDTA for 5 min at 37° C and then

washed with 5 mM EDTA PBS at 4° C, to remove bound but not internalised platelets. Actual removal of non-internalised platelets was routinely verified by microscopy. DCs were treated with trypsin/EDTA for 5 min at 37° C in suspension and then retrieved by means of magnetic beads conjugated with antihuman HLA-DR monoclonal antibodies (MiniMACS, Miltenyi Biotech, Bologna, Italy). Phagocytes were then lysed in PBS containing HCl (0.75 N). Radioactivity was measured after liquid scintillation in a β-counter. The number of internalised platelets per phagocyte was calculated according to the formula: incorporated platelet per phagocyte = (radioactivity associated to internalising phagocytes/radioactivity incorporated by each platelet)/phagocyte number.

Cytokine production and measurement. Phagocytes were cultured for the last 24 h in the appropriate medium containing Nutridoma (1%, Roche). Phagocytes were then incubated with platelets, opsonised or not with anti-β2GPI IgGs in the presence of the purified β2GPI plasma cofactor (20 μg/ml) as above. In parallel experiments CCD (final concentration 10 μg/ml, Sigma) was added 60 min before platelet addition and maintained throughout the assay. After 18 h supernatants were collected, cleared by centrifugation at 2000 rpm and stored at –30° C. We also assessed in parallel the cytokine production induced by lipopolysaccharide (LPS) stimulation (1 ng/ml, Sigma) for 18 h. IL-1β and IL-10 concentrations were determined by ELISA, according the manufacturers instructions (Quantikine immunoassays, R & D Systems, Minneapolis, MN). Bioactive TNF-α concentration was calculated comparing the death of TNF-α sensitive WEHI 13 clone 164 cells in the presence of the supernatants with the death in the presence of increasing concentration of recombinant human TNF-α.

Statistical analysis. The results are expressed as mean ± S.E.M. n represents the number of different experiments. Statistical analysis was performed by the Mann.Whitney U test.

Results

DCs Fail to Recognise and Clear Activated Platelets

We derived immature DCs and macrophages from peripheral blood monocytes of healthy donors. Immature DCs, propagated in the presence of recombinant GM-CSF and IL-4, grew as loosely adherent aggrega-

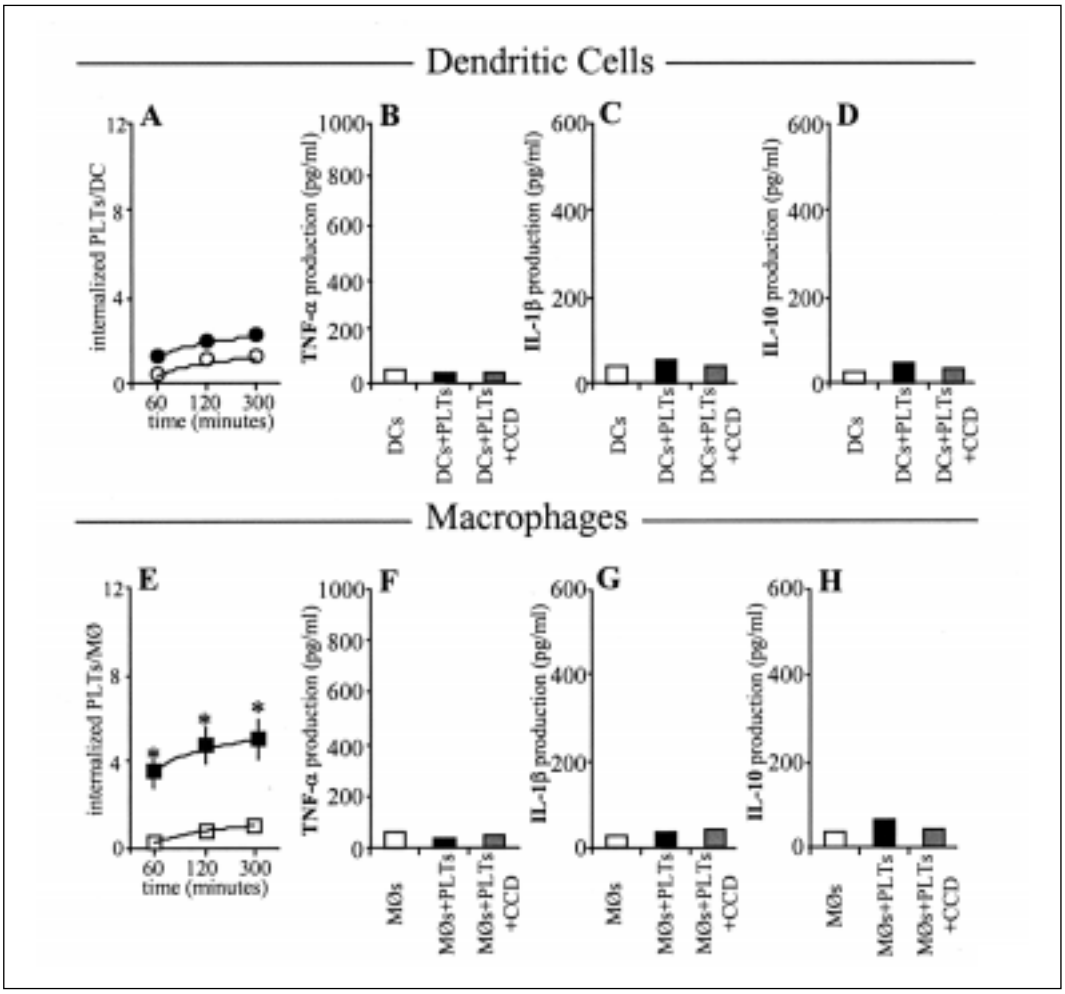

Fig. 2 DCs ignore platelets while macrophages internalise them without secreting IL-10, IL-1β or TNF-α. **A:** Immature DCs did not phagocytose thrombin-activated ^{51}Cr-labelled platelets (PLTs) (PLTs:DCs ratio = 20:1) neither at 4° C (open circles) or at 37° C (filled circles). Incubation times are reported along the x axis. The number of PLTs internalised by each DC is reported along the y axis (see Materials and Methods). Coincubation did not elicit the release of TNF-α (black columns, **B**), IL-1β (black columns, **C**) or IL-10 (black columns, **D**) above the background level (white columns, **B-D**). Treatment with CCD did not influence the extent of the secretion (grey columns, **B-D**). **E:** Mϕ from the same donors efficiently phagocytosed at 37° C thrombin-activated ^{51}Cr-labelled platelets (PLTs:M ratio = 20:1) (filled squares). Phagocytosis abated when the coincubation was performed at 4° C (open squares). The difference between phagocytosis at 37° C and at 4° C was statistically significant (*, p <0,05). Mϕ phagocytosing PLTs did not release TNF-α (black columns, **F**), IL-1β (black columns, **G**) or IL-10 (black columns, **H**) above the background level (white columns, **F-H**). Treatment with CCD did not influence the extent of the secretion (grey columns, **F-H**). Results shown are mean S.E.M of n = 4 independent experiments run in triplicates

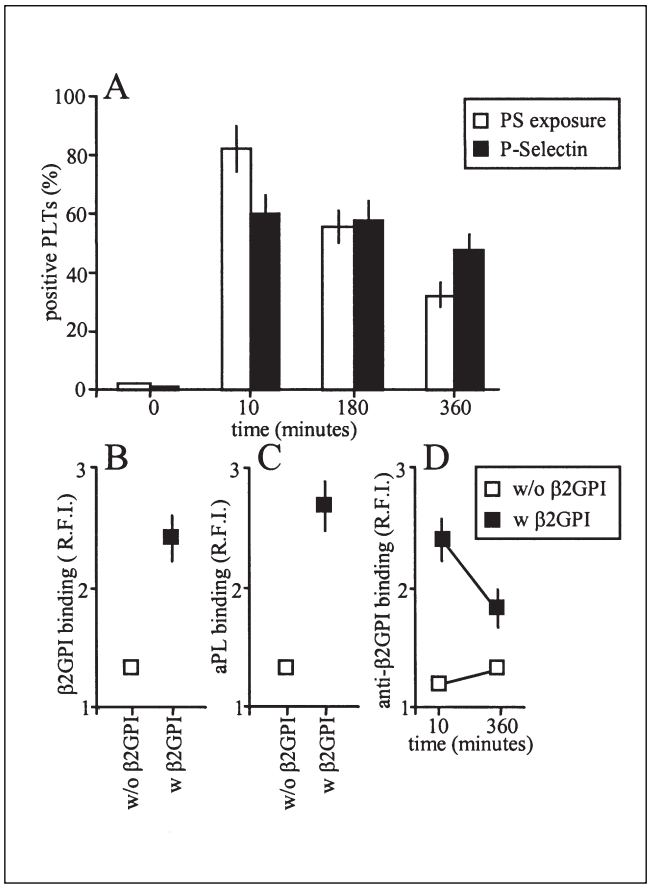

Fig. 3 Thrombin activation of platelets determines exposure of PS, binding of β2GPI and recognition by β2GPI-dependent human autoantibodies. **A:** The exposure of the anionic phospholipid PS (open columns) was detected by flow cytometry using FITC-labeled annexin V (see Material and Methods). The expression of the activation molecule P-Selectin (filled columns) was monitored using a specific mAb. PLTs were analyzed at different time points before (time: 0) and after 10 min exposure to thrombin (min, x axis). The results are expressed as % of positive PLTs (y axis). **B:** Thrombin-activated PLTs bind to the plasma cofactor β2GPI (y axis). The experiment was performed in the absence (open squares) or in the presence (filled squares) of affinity purified β2GPI (see Materials and Methods for details). **C:** Thrombin activated PLTs are recognized by aPL antibodies purified by affinity chromatography from the sera of autoimmune patients (y axis) in the presence (filled squares) but not in the absence of β2GPI (open squares). Seven independent preparations from autoimmune patients were tested. **D:** The recognition of activated PLTs by anti-β2GPI antibodies (y axis) is time-dependent: the optimal binding was achieved after 10 min treatment with thrombin in the presence (filled squares) but not in the absence (open squares) or β2GPI. The binding decreased when PLTs were chased after thrombin removal (min, x axis). Results shown are mean ± S.E.M of n = 5 independent experiments, run with four independent preparations of affinity purified antibodies derived from three autoimmune patients

tes, expressed the CD1a marker and high levels of HLA-DR, B7.1/CD80, B7.2/CD86 and CD40 molecules (Fig. 1). In contrast, macrophages expressed the GPI-linked lipopolysaccharide receptor CD14, a marker of the monocyte-macrophage lineage, CD36, a receptor involved in apoptotic cell scavenging, and lower levels of MHC class II (HLA-DR) molecules and of B7.1/CD80, B7.2/CD86 and CD40 molecules (Fig. 1). Both populations of phagocytes expressed, even to a different extent, receptors for the Fc fraction of immunoglobulins (CD32). Immature DCs ignored platelets: the coincubation with resting (not shown) or thrombin-activated platelets did not result in phagocytosis either (Fig. 2, panel A) and did not elicit the release of TNF-α, IL-1β or IL-10 (Fig. 2, panels B-D). In contrast, macrophages from the same donors cleared after 60 min at 37° C a substantial fraction of the platelets they were incubated with, corresponding to an average of 3,6 platelets per macrophage (Fig. 2, panel E). Phagocytosis abated when the coincubation was performed at 4° C, a temperature that does not allow actin-based cytoskeleton reorganisation or in the presence of CCD (not shown). Longer incubations did not substantially enhance the phagocytic ability of the macrophages. Macrophages phagocytosing platelets, in contrast with those that phagocytosed other particulate substrates, like bacteria or dying cells, did not release substantial amounts of TNF-α, IL-1β or IL-10 (Fig. 2, panels F-H).

β2GPI Binds to Activated Platelets and allows their Recognition by Anti-β2GPI Antibodies

The treatment of platelets with low concentrations of thrombin results in their activation without determining their aggregation. Fig. 3, panel A, shows that short treatments (10 min) with thrombin cause a dramatic reorganisation of the platelet plasma membrane. This comprises the exposure of P-Selectin and the swift externalisation of PS. Fig. 3, panel B shows that the plasma cofactor β2GPI selectively binds to PS exposing activated platelets. Protein G-purified immunoglobulins from sera of antiphospholipid positive patients bound, in the presence of β2GPI, to activated platelets (Fig. 3, panel C). Immunoglobulins from autoimmune patients further purified by β22GPI affinity chromatography retained the ability to recognise activated platelets (Fig. 3, panel D). Total immunoglobulins and β2GPI-specific IgG bound to platelets only when experiments were performed in the presence of affinity purified β2GPI. In contrast, they failed to recognise platelets in the absence of the cofactor (Fig. 3, panels B-D). The optimal binding of anti-β2GPI antibodies to activated platelets was achieved after 10 min treatment with thrombin (Fig. 3, panel D).

Anti-β2GPI Antibodies Endow DCs with the Ability to Clear Activated Platelets and Prompt the Secretion of TNF-α and IL-1β

Immature DCs efficiently internalised platelets opsonised in vitro, with an efficiency ranging from 3,9 platelets per DC after 60 min to 5,7 after 300 min (Fig. 4). Fig. 5 shows the confocal imaging of immature DCs co-incubated with anti β2GPI antibody-opsonised platelets. CD42 positive platelets were revealed into phalloidin counter-stained DCs (Fig. 5, panel A). Antibodies still covered platelets into intracellular vesicles of DCs (Fig. 5, panel B). Phagocytosing DCs secreted substantial amounts of TNF-α and IL-1β, which promote DC migration to lymph nodes and function (21) while they released only limited amounts of IL-10: the difference in IL-10 secretion was not statistically significant when compared with background levels. The treatment of DCs with CCD, which disrupts the actin-based cytoskeleton, prevented the internalisation of platelets by DCs and the secretion of cytokines (Fig. 4, panels B-D). Macrophages internalised with higher efficiency antibody opsonised platelets (Fig. 4, panel E). The clearance was not only more efficient, but also accompanied by the release of cytokines, which includes both proinflammatory signals (TNF-α, IL-1β) and the antiinflammatory cytokine IL-10 (Fig. 4, panel F-H).

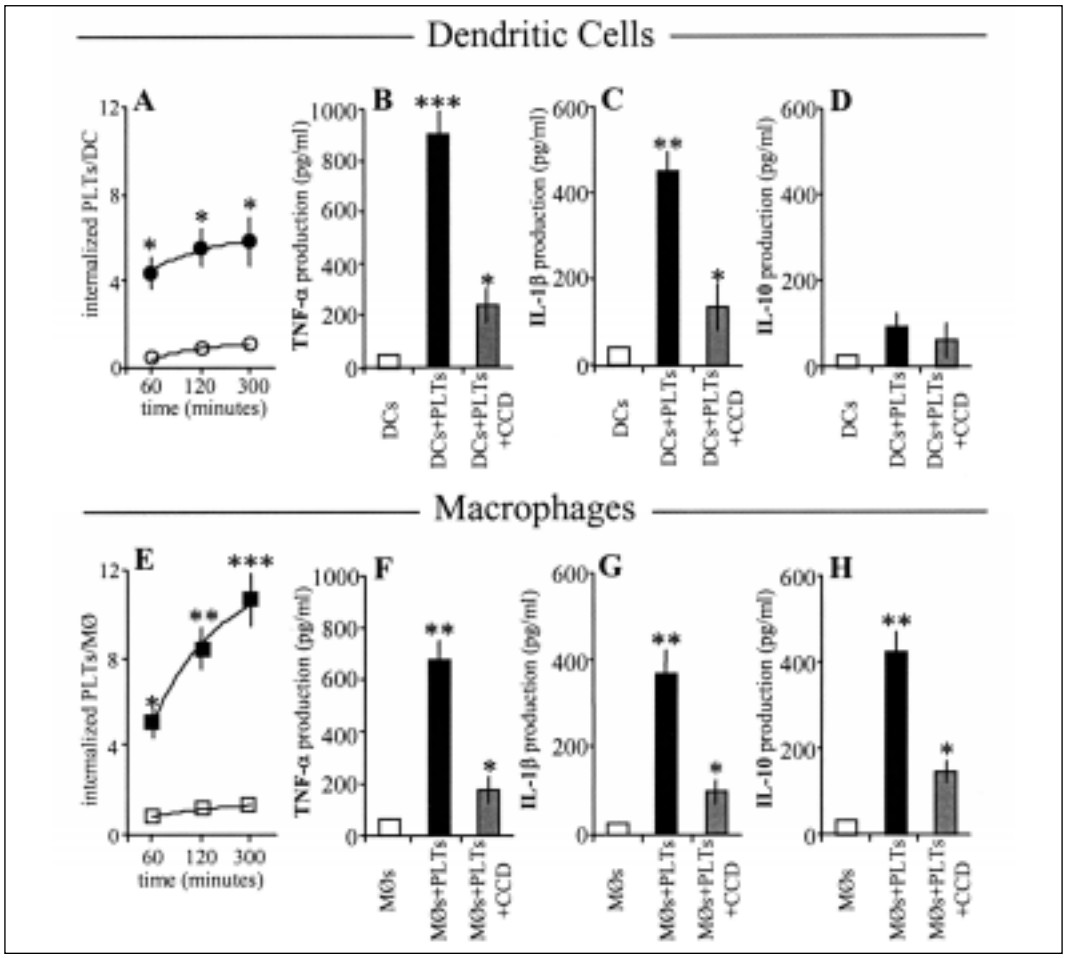

Fig. 4 DCs and macrophages internalise anti-β2GPI antibody-opsonised platelets, secreting different arrays of cytokines. **A:** Immature DCs efficiently phagocytosed anti-β2GPI antibody-opsonised ^{51}Cr-labelled PLTs (PLTs:DC = 20:1) at 37° C (filled circles). Internalisation abated when the coincubation was performed at 4° C (open circles). Incubation times are reported along the x axis. The number of PLTs internalised by each DC is reported along the y axis (see Materials and Methods). The difference between phagocytosis at 37° C and at 4° C was statistically significant (*, p <0,05). Phagocytosis elicited the release of TNF-α (black columns, **B**) and IL-1β (white columns, **C**). The difference between TNF-α and IL-1β released in the presence or in the absence of anti-β2GPI antibody-opsonised PLTs was statistically significant (**, p <0,01; ***, p <0,005). DCs did not produce IL-10 (black columns, **D**) above the background level (white columns, **D**). Treatment with CCD abolished the secretion of TNF-α and IL-1β (grey columns, **B-D**) (*, p <0,05). **E:** Macrophages phagocytosed with higher efficiency at 37° C anti-β2GPI antibody-opsonised ^{51}Cr-labelled PLTs (PLTs:M ratio = 20:1) (filled squares). Internalisation abated when the co-incubation was performed at 4° C (open squares). The difference between phagocytosis at 37° C and at 4° C was always statistically significant (min, x axis), (**, p <0,01; ***, p <0,005). Macrophages phagocytosing anti-β2GPI antibody-opsonised platelets released TNF-α (filled columns, **F**), IL-1β (filled columns, **G**) or IL-10 (filled columns, **H**) above the background level (open columns, **F-H**) (**, p <0,01). Treatment with CCD influenced the extent of the secretion (grey columns, **F-H**) (*, p <0,05). Results shown are mean ± S.E.M of n = 3 independent experiments, each run in triplicate with four independent preparations of affinity purified antibodies derived from three autoimmune patients

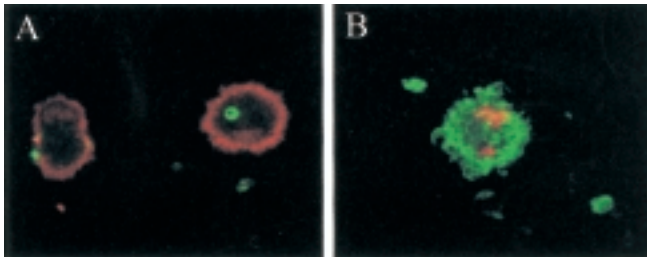

Fig. 5 Opsonised platelets reach intracellular compartments of immature DCs. **A:** CD42 positive PLTs (green color) were revealed into phalloidin counter-stained DCs (red color) by confocal laser scanning microscopy (see Materials and Methods). **B:** Internalised PLTs were still covered with antibodies, as demonstrated by the staining with Texas red-labelled antihuman IgG (red color). Immature DCs were counterstained using a FITC-conjugated antihuman HLA-DR monoclonal antibody (green color)

Discussion

The enhanced procoagulant activity of monocytes plays a crucial role in the pathogenesis of thrombotic events associated with the aPS (19, 30-34). Several mechanisms are involved, including the antibody-mediated interference with the tissue factor pathway inhibitor activity in autoimmune patients (18, 33). Cytokines also influence the procoagulant activity of circulating monocytes (discussed in 35). Patients with aPS have raised plasma levels of TNF-α, a cytokine endowed with inflammatory and prothrombotic activities (20, 35). The responsible mechanism(s) are still not completely elucidated, especially since no direct correlation was observed with the genetic polymorphism of TNF-α promoters (20). Here we describe a novel molecular link between the clearance of platelets in the presence of anti-β2GPI antibodies and the production of TNF-α and other cytokines.

Human macrophages efficiently internalise platelets without secreting TNF-α (Fig. 3). The scenario may change in subjects with circulating anti-β2GPI antibodies. These antibodies indeed recognise β2GPI on the surface of PS-exposing platelets (10, 11, 16 and Fig. 2). Antibody binding results in the efficient opsonisation of platelets, with enhanced phagocytic clearance by human macrophages (16 and Fig. 4). Macrophages phagocytosing antibody-opsonised platelets secrete substantial amounts of TNF-α, IL1-β, and IL-10 (Fig. 4). This event influences the final outcome of platelet clearance, shifting from a relatively uneventful process to a signal promoting local inflammation and contributing to thrombosis.

We investigated in parallel the involvement in the clearance of platelets of the most potent antigen presenting cells, DCs. Immature DCs in peripheral tissues are endowed with the ability to capture particulate antigens. For example they efficiently internalise bacteria and PS expressing dying cells (21). They however, mainly rely on PS-independent signals recognised by integrins, like the $\alpha_V\beta5$ receptor (36). In our system, immature DCs ignored PS exposing platelets and had no part in their clearance (Fig. 2). DCs are specialised in presenting antigens to T cells (21). Therefore, platelets ignored by DCs may represent privileged substrates and fail to elicit T cell dependent antiplatelet immune responses, like those occurring during thrombocytopenic autoimmune purpura (37).

The opsonisation by anti-β2GPI antibodies was necessary for platelets' access to antigen presenting DCs (Fig. 4 and 5). To our knowledge, this is the first demonstration of naturally occurring auto-antibodies that cause the recruitment of DCs in the uptake of platelets. Uptake by DCs is required for the initiation of immune and autoimmune responses (21) and influences the immunogenicity of dying cells (38, 39). Of importance, here we show that the internalisation of antibody-opsonised platelets occurs in a proinflammatory context (Fig. 4), which promotes the functional maturation and the antigen presenting function of DCs (21, 38). Further studies are warranted to evaluate the in vivo relevance of these phenomena to the pathogenesis of thrombosis and to the spreading of antiplatelet autoimmunity in aPS patients.

Acknowledgments

We thank Prof. C. Rugarli for discussions and support, Dr. F. Pellegatta for helpful advice, Dr. U. Fascio for help in confocal imaging. This work was supported by the Ministero della Sanità, the MURST (cofinanziamento 2000) and the AIRC. VSZ was supported by the Fondation pour la Recherche Médicale (Ref. nr. SE000760-01). PRQ was the recipient of an award from the Società Italiana di Medicina Interna-Fondazione Aventis.

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Received June 1, 2001 Accepted after revision July 31, 2001