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Microparticles (MP) shed by platelets (PLT) during storage have procoagulant activities, but little is known about their properties to modify inflammation or immunity. In this study, we studied the capacity of MP present in PLT concentrates to alter the function of macrophages and dendritic cells (DC). The size of the purified MP was between 100 and 1000 nm, and they expressed phosphatidylserine; surface proteins of PLT (CD61, CD36, CD47), including complement inhibitors (CD55, CD59), but not CD63; and proteins acquired from plasma (C1q, C3 fragments, factor H). These characteristics suggest that the MP shed by PLT are formed by budding from the cell surface, corresponding to ectosomes. The purified PLT ectosomes (PLT-Ect) reduced the release of TNF-α and IL-10 by macrophages activated with LPS or zymosan A. In addition, PLT-Ect induced the immediate release of TGF-β from macrophages, a release that was not modified by LPS or zymosan A. Macrophages had a reduced TNF-α release even 24 h after their exposure to PLT-Ect, suggesting that PLT-Ect induced a modification of the differentiation of macrophages. Similarly, the conventional 6-d differentiation of monocytes to immature DC by IL-4 and GM-CSF was modified by the presence of PLT-Ect during the first 2 d. Immature DC expressed less HLA-DP DQ DR and CD80 and lost part of their phagocytic activity, and their LPS-induced maturation was downmodulated when exposed to PLT-Ect. These data indicate that PLT-Ect shed by stored PLT have intrinsic properties that modify macrophage and DC differentiation toward less reactive states. ***The Journal of Immunology*****, 2011, 186: 6543–6552.**

Platelets (PLT) are anucleate discoid-shaped cells that circulate at a level of 150–400 $\times$ 10^9 platelets/l. They are key players in initial stages of the blood coagulation process, leading to the arrest of bleeding at sites of vascular injury. In vivo, PLT release microparticles (PMP). The first report about PMP is from 1967, when Wolf (1) described the presence of fragments derived from PLT in human plasma. PMP constitute ~70–90% of circulating microparticles (MP) in healthy individuals (2–4). PMP are multitalented, participate in blood coagulation (5), and are involved in fundamental processes of vascular biology, such as inflammation, angiogenesis, tissue regeneration, and tumor metastasis (6, 7). In many disease states, the finding of high levels of PMP correlates with platelet activation or consumption (8) and/or with the severity of the disease, for example, infection versus sepsis (9). PMP are routinely formed in vitro after exposure to thrombin, collagen, or complement (10–12); they may also be generated by chemical agents (calcium ionophore), as well as by exerting mechanical stress (13) or by extreme temperatures. In vivo platelet vesiculation could be part of normal wear and tear in a maturing PLT.

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Abbreviations used in this article: AnV, annexin V; DC, dendritic cells; FH, factor H; FSC, forward scatter; GPA, glycophorin A; HMDM, human monocyte-derived macrophages; iDC, immature dendritic cells; mDC, mature dendritic cells; MFI, mean fluorescence intensity; MP, microparticles; NHS, normal human serum; PLT, platelets; PLT-Ect, PLT ectosomes; PMN, polymorphonuclear leukocytes; PMP, PLT release microparticles; PS, phosphatidylserine; RT, room temperature; SN, supernatants; SSC, side scatter; TF, tissue factor.

Two main types of PMP are released by PLT (14). The first are shed from the plasma membrane by ectocytosis; they are relatively large, >100 nm in diameter with an outer membrane leaflet enriched in phosphatidylserine (PS), and do not express CD63 (14, 15). In contrast, the second type of PMP is smaller, <100 nm, and expresses CD63, thus corresponding to exosomes (14). They interact poorly with annexin V (AnV), and thus express little or no PS, do not bind prothrombin and factor X, and therefore have probably no coagulation function. These platelet exosomes are formed in multivesicular bodies and released when the multivesicular bodies fuse with the plasma membrane (14).

Platelet concentrates are transfused to patients to treat or prevent bleeding (16). During storage, PLT release PMP, the number of which increases significantly with time, so that large quantities are transfused together with PLT (17). The generation of PMP from stored PLT is only partially blocked by inhibitors of PLT activation (17). The biological properties of these PMP merit attention because they are transfused in large amount to patients who are often in critical immunosuppressed situations (18). It has been suggested that they may increase thrombosis, but their properties to regulate immune cells have received little attention. In the present work, we studied the larger PMP shed from stored platelets to see whether they would modify the functional properties of macrophages and dendritic cells (DC) because they express PS. Indeed, PS is known for its procoagulant function, but also for its immunosuppressive activity when expressed by apoptotic cells and ectosomes released by erythrocytes (19) and polymorphonuclear leukocytes (PMN) (20).

Materials and Methods

Abs and reagents

All Abs used were mouse anti-human mAbs; the following mAbs directed against CD14 (M5E2), CD36 (CB38), CD40 (5C3), CD47 (B6H12), CD61 (VI-PL2), CD63 (H5C6), CD80 (L307.4), CD83 (HB15e), CD86 (FUN-1),

and CD154 (TRAP1), as well as their isotype controls IgG1 and IgG2a, were FITC coupled, and were all purchased from BD Biosciences/BD Pharmingen. AnV FITC and anti-glycophorin A (GPA) (GA-R2 [HIR2]) PE were from BD Biosciences/BD Pharmingen as well. The anti–HLA-DP DQ DR (WR18) FITC and the anti-CD59 (MCA1054) PE were from Serotec. We used the monoclonals to CD45 (MEM-28) PerCP from Abcam. The anti-tissue factor (4508cj) FITC was from American Diagnostic; the anti-C1q (CL7611F) and factor H (FH; CL7672F) FITC were from Cedarlane Laboratories; CD55 (143-30) FITC was from US Biological; and FITC-conjugated rabbit anti-C3d Ab was from DakoCytomation. Rabbit polyclonal pan anti–TGF-β (sc-7892) (Santa Cruz Biotechnology), followed by Cy5 donkey anti-rabbit secondary Ab (Jackson ImmunoResearch Laboratories), was used for confocal fluorescence microscopy.

Zymosan A from *Saccharomyces cerevisiae* was from Sigma-Aldrich (St. Louis, MO); LPS and human C1q were from Calbiochem (San Diego, CA); OptEIA ELISA kits for IL-6, IL-10, TGF-β1, and TNF (TNF-α) were from BD Biosciences. When stated, Multiplex measurements of cytokines in high-density formats using Multi-Array Technology from Meso Scale Discovery, a division of Meso Scale Diagnostics, were used for IL-1β, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, and TNF-α simultaneous determination.

PLT supernatants

PLT concentrates were obtained by apheresis procedure; the blood from the donor is processed with a cell separator with an in-line centrifuge for PLT collection (Blood Transfusion Center Beider Basel). The PLT are then transferred to a collection bag, whereas the other blood cells and most of the plasma are returned to the donor. PLT concentrates were stored at room temperature (RT) for up to 5 d. Several RT centrifugations were assessed to clear all residual cells. To remove the high-density cells (RBCs and leukocytes), the centrifugation was performed for 15 min ($300 \times g$). Any residual RBC and leukocytes were removed by recentrifugation for 15 min ($500 \times g$). The PLT were pelleted by centrifugation of the suspension for 20 min ($800 \times g$). Any residual PLT and low-density debris were removed by centrifugation for 20 min ($3000 \times g$), and the supernatants (SN) were stored in aliquots at −80°C until use. The SN containing PLT ectosomes (PLT-Ect) were ultracentrifuged for 1 h at 200,000 × *g* at RT, and the pellet was washed in 0.9% NaCl before use.

Transmission electron microscopy of ectosomes/negative staining

PLT-Ect were resuspended in PBS, and then fixed in 1% glutaraldehyde (final concentration) for 20 min at RT. PLT-Ect were then adsorbed to parlodion-coated copper grids. After washing, samples were stained with 2% uranylacetate before being observed in a Philips Morgani 268 D transmission electron microscope operated at 80 kV.

Staining and flow cytometric analysis

PLT-derived ectosomes. Analysis of PLT-Ect was performed using a FACSCalibur flow cytometer (BD Biosciences). Data from 30,000 events were acquired and analyzed with the use of CellQuest software (BD Biosciences). PLT-Ect and TruCount beads were identified by their size, as assessed by the logarithmic amplification of their forward scatter (FSC) and side scatter (SSC) signals. PLT-Ect were localized within R2 region. The TruCount beads were localized in R1 region (Fig. 1*Ba*).

PLT-Ect were identified in SN of PLT concentrates using an anti-CD61 PLT marker. The absolute concentration of PLT-Ect per microliter of ultracentrifuged SN was quantified by using tubes preloaded with a known density of fluorescent TruCount bead lyophilized pellets (BD Biosciences). A total of 50 μl PLT-Ect sample was incubated with 10 μl FITC-conjugated anti-CD61 and 10 μl PerCP-conjugated anti-CD45 for leukocyte-derived MP or PE-conjugated anti-GPA for erythrocyte-derived MP for 30 min at RT in the dark. After staining, the ectosome suspension was then diluted with 430 μl PBS to make a final volume of 500 μl. The absolute numbers of PLT-Ect were then calculated.

In a separate experiment, 50 μl PLT-Ect were incubated for 30 min at RT in the dark with 5 μl FITC-conjugated AnV in the absence or presence of 2.5 mM $CaCl_2$. The mixture was then diluted in their respective buffer, PBS containing $CaCl_2$ or not, and analyzed by flow cytometry within 30 min.

Proteins expressed and acquired by PLT-Ect. Following the previously described procedure, PLT-Ect were incubated after gentle resuspension for 30 min at RT in 10 μl labeled Abs against CD36, CD47, CD55, CD59, CD63, CD154, tissue factor (TF), C1q, C3d, or FH. The protein expression was analyzed by flow cytometry within 30 min. The controls were done with the appropriate Abs.

Dendritic cells. Flow cytometric analyses of cell surface markers were performed using the following FITC-conjugated mAbs: CD14, CD40, CD80, CD83, CD86, and HLA-DP DQ DR. In each experiment, parallel stainings with isotype-matched controls IgG1-FITC and IgG2a-FITC were performed. After each incubation, cells were spun down, resuspended in PBS/1% BSA, and labeled for 30–45 min at 4°C with appropriate Abs. After labeling, cells were washed twice in PBS/1% BSA, and data were acquired with a FACSCalibur flow cytometer (BD Biosciences) and analyzed using Summit software (DakoCytomation). A minimum of 10,000 events was collected per dataset.

Membrane labeling of PLT-Ect

An amphiphilic cell linker dye kit (PKH67; Sigma-Aldrich) was used according to the manufacturer's instructions. Briefly, PLT-Ect resuspended in 200 μl diluent C/dye solution (dye diluted 1/200) were incubated with gentle shaking for 1 min at RT. Then RPMI 1640 (1 ml; without phenol red) was added to stop the reaction. Labeled PLT-Ect were separated from the unbound dye by ultracentrifugation (20 min, 200,000 × *g* at 4°C), and washed with 0.9% NaCl.

Binding of PLT-Ect to erythrocytes

Whole hirudine-anticoagulated blood was incubated with fluorescently labeled PLT-Ect in the presence or absence of 10 mM EDTA for 30 min at 37°C. Erythrocytes were washed three times (5 min, 500 × *g*, RT) with PBS. Samples were then labeled with anti-GPA PE Ab for 15 min at RT in the dark. The samples were analyzed with a FACSCalibur flow cytometer. The settings were chosen to allow detection of cells, but not of PLT-Ect. The fluorescence channels were set at logarithmic gain. We then assessed the fraction of erythrocytes having bound PLT-Ect.

Isolation and culture of human monocyte-derived macrophages and DC

Monocytes were isolated from fresh buffy coats, as previously described (21). Briefly, a buffy coat was diluted 1/1 (v/v) with HBSS, layered over Histopaque-1077 (Sigma-Aldrich), and centrifuged for 30 min at 350 × g. PBMC were recovered, washed twice in HBSS, and layered over a Percoll gradient. Percoll was prepared by mixing 1 vol 1.5 M NaCl with 9 vol Percoll (Sigma-Aldrich). The Percoll gradient was done by mixing 1.5:1 (v/v) isosmotic Percoll with PBS/citrate (1.49 mM NaH_2PO_4, 9.15 mM Na_2HPO_4, 139.97 mM NaCl, 13 mM $C6H5Na_3O7.2H_2O$ [pH 7.2]). Isolated monocytes were resuspended at 2×10^6 cells/ml in DMEM supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, and 2 mM L-glutamine. Monocytes were then allowed to adhere for 1 h at 37°C on culture plates. Adherent monocytes were washed three times with prewarmed DMEM and finally incubated for 7 d in DMEM supplemented with 10% normal human serum (NHS; pooled from 40 healthy donors). The culture was maintained in 5% CO_2 at 37°C, and the medium was changed at days 3 and 7. Macrophages were used between days 7 and 10.

For DC generation, after Histopaque-1077 density gradient centrifugation, PBMC were washed and cultured in complete medium (RPMI 1640, 1% L-glutamine, 1% penicillin/streptomycin, and 10% FCS) for 1 h at 37°C in 6-well plates or in 75-ml flasks. After incubation, nonadherent cells were removed by washing twice with prewarmed RPMI 1640. The remaining adherent cells were then cultured in complete medium supplemented with 50 ng/ml GM-CSF and 50 ng/ml IL-4 (ImmunoTools) in presence or absence of PLT-Ect (Fig. 6*A*). On days 2 and 5, the media, including GM-CSF and IL-4, were replaced, whereas no further PLT-Ect were added (Fig. 6*A*). On day 6, nonadherent immature DC (iDC) were harvested, counted, and plated in 6- or 24-well plates (1×10^5 cells/ml) in fresh medium (22, 23). On day 6, LPS (10 ng/ml final concentration; Sigma-Aldrich) and/or PLT-Ect were added (Fig. 6*A*). DC and SN were collected 15 h later.

Endocytic activity

Endocytic activity of DC was measured by assessing uptake of FITC-conjugated dextran (molecular mass, 40,000 kDa; Molecular Probes) (22). To that end, cells were incubated with 0.5 mg/ml FITC-conjugated dextran in complete medium for 15, 30, or 45 min at 37°C and 4°C to measure specific uptake versus nonspecific binding, respectively. DC were then washed three times and analyzed by flow cytometry.

Activation of human monocyte-derived macrophages

Human monocyte-derived macrophages were washed several times with prewarmed DMEM without NHS. Subsequently, each well was filled with 250 μl (final volume) fresh DMEM without NHS. Zymosan A (5 μg/ml

final concentration), LPS (10 ng/ml final concentration), and/or PLT-Ect were added, and SN were collected 15 h later. In some experiments, before coincubation with macrophages, PLT-Ect were preincubated for 30 min at 4°C with rAnV (50 μg/ml final concentration), washed, and added to macrophages, as previously described, and SN were collected after 15 h.

The number of PLT-Ect used in our experiments was quantified by FACS, as previously described, and a ratio of 100 PLT-Ect per cell was used in the experiments, unless otherwise stated. Assays were performed in triplicate. Results are representative of four independent experiments.

Collection of SN and analysis of cytokines

Human monocyte-derived macrophage and DC SN were collected, and spun for 1 min at 14,000 × *g* at 4°C (Mikro 24-48R centrifuge; Hettich, Bäch, Switzerland) to remove cell debris. The cytokines TNF-α, IL-10, and TGF-β1 released in the SN of human monocyte-derived macrophages were determined by ELISA, according to manufacturer's instructions. To analyze the cytokine released in the SN of DC, we used Multiplex simultaneous measurements of cytokine concentrations (IL-1β, IL-6, IL-8, IL-10, IL-12p70, IFN-γ, and TNF-α).

Confocal fluorescence microscopy

Human monocyte-derived macrophages were generated on 8-well culture slides (Falcon; BD Biosciences). After 7–10 d of culture, macrophages were washed several times with serum-free DMEM, and incubated with membrane dye-labeled PLT-Ect for 30 min. Labeling was performed according to standard procedures. Briefly, slides were fixed 15 min with PBS/4% platelet-activating factor, permeabilized with 0.1% Triton X-100, blocked for 30 min with PBS/3% BSA, and incubated for 1 h with a rabbit polyclonal pan anti–TGF-β (Santa Cruz Biotechnology), followed by an incubation with anti–rabbit-Cy5 (Jackson ImmunoResearch Laboratories) for 1 h. Cells were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA). All images were acquired with a 710 LSM confocal microscope (Carl Zeiss) with ×63 Plan Apo objective.

Statistical analysis

For statistical analysis, we used two-tailed paired Student *t* test using GraphPad Prism software. Data are expressed as mean ± SEM. A p value ≤ 0.05 was considered statistically significant.

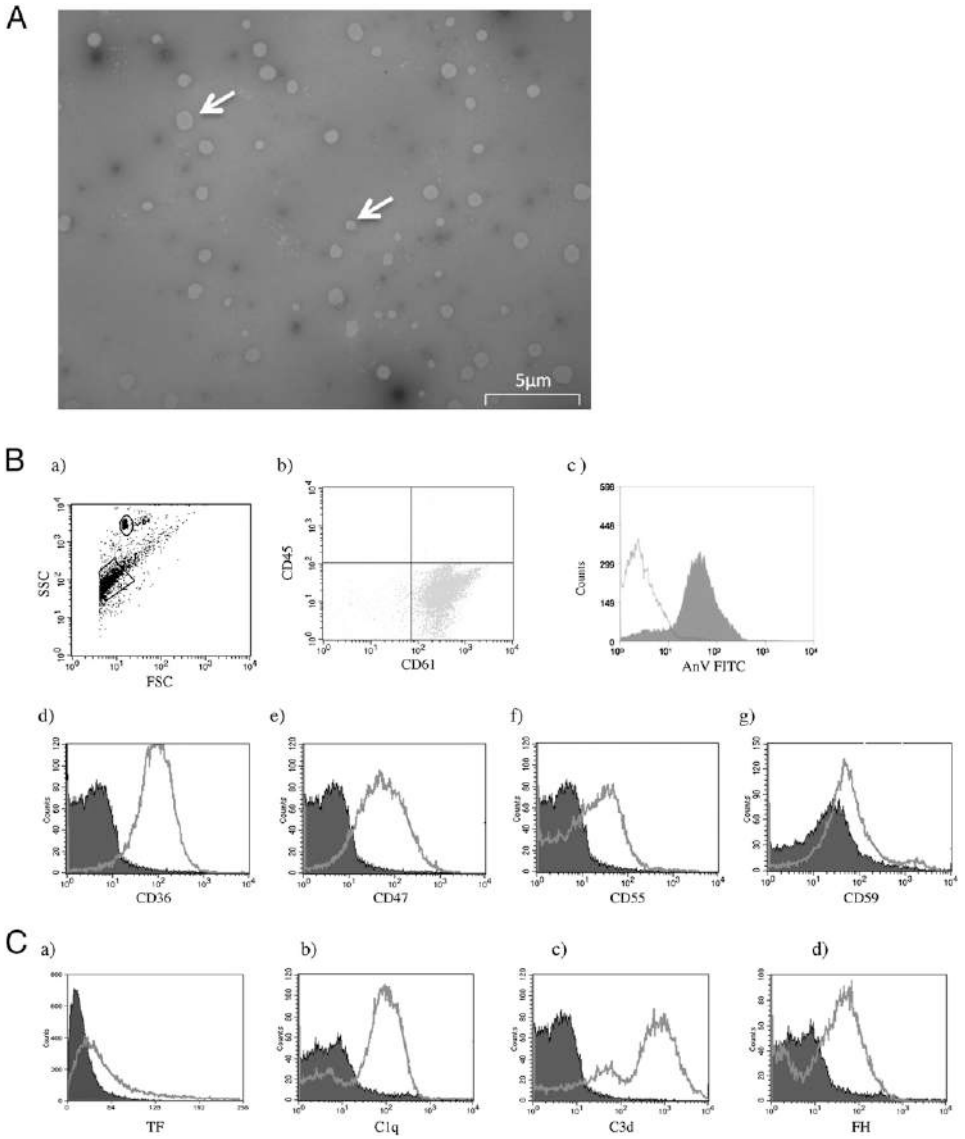

FIGURE 1. Electron microscopy and FACS analysis of PLT-Ect. *A*, Picture of a PLT-Ect preparation showing heterogeneity in size (arrows), which ranged from 100 to 1000 nm. Size scale bar, 5 μm. *Ba*, Representative flow cytometric dot plot of PLT-Ect. The region R2 represents FSC/SSC light scatter gate of PLT-Ect. The region R1 represents the known density TruCount beads. *Bb*, Double staining with anti-CD61 PLT marker and anti-CD45 leukocyte marker. *Bc*, Histogram of PLT-Ect stained with AnV-FITC. In presence of binding buffer (filled histograms), in calcium-depleted buffer (open line). *Bd–g*, PLT-Ect expression of thrombospondin receptor CD36, receptor molecule CD47, and complement regulatory proteins decay-accelerating factor (CD55) and CD59 is represented by open lines. Filled histograms represent staining with isotype control Abs. *C*, Plasma protein binding to PLT-Ect shown by FACScan histogram of PLT-Ect incubated for 30 min with mAbs against the following: *a*, TF; *b*, C1q; *c*, C3d; and *d*, FH. The binding is depicted by the open lines. The filled histograms represent staining with isotype control Abs.

Results

MP were isolated by differential centrifugation of PLT concentrates stored for 5 d, as described in *Materials and Methods*. When observed by electron microscopy, they had a round shape and a size between 100 nm and 1 μm (Fig. 1*A*). This size corresponds to PMP shed by ectocytosis from the cell surface of activated PLT and not to exosomes, which are smaller (14). We will therefore refer to them as PLT-Ect.

FACS analysis of PLT-Ect released during conventional storage of human PLT

The identification of MP was assessed by flow cytometry based on size and membrane marker expression. The size was assessed by the logarithmic amplification of FSC and SSC signals (Fig. 1*Ba*). Ectosomes were localized within the (R2) region. Flow cytometry analysis demonstrated that they were derived from PLT because >95% reacted with an Ab against glycoprotein IIIa (CD61) known to be a specific marker of PLT (Fig. 1*Bb*). There was no contamination with leukocyte-derived or erythrocyte-derived ectosomes as assessed by the absence of reaction with CD45 (Fig. 1*Bb*) and GPA, respectively (data not shown). As expected, PLT-Ect expressed PS, as demonstrated by binding of AnV. In presence of calcium, >80% of the PLT-Ect bound AnV (Fig. 1*Bc*), whereas no binding was seen in the absence of calcium. PLT-Ect were negative for the PLT lysosomal protein CD63 (data not shown), excluding an intracellular origin. Integral membrane proteins of PLT were present on PLT-Ect, including thrombospondin receptor CD36 and CD47 (Fig. 1*Bd*, 1*Be*). PLT-Ect expressed weakly CD154 (data not shown).

PMP released by stimulated PLT have been shown to express complement regulatory proteins CD55 and CD59 (24); we found that PLT-Ect released during storage expressed both as well (Fig. 1*Bf*, 1*Bg*).

PLT-Ect acquire plasma proteins during storage

Ectosomes of PMN are known to acquire proteins from plasma (25, 26). Because PLT are stored in a medium containing 30% plasma, we expected that many proteins could be adsorbed by or specifically bound to PLT-Ect. MP of PLT origin purified from plasma bear TF, and PLT-Ect expressed TF as well (Fig. 1*Ca*). In addition, complement protein C1q and the C3d fragment of C3 were detected by FACS, indicating a possible activation of complement during storage (Fig. 1*Cb*, 1*Cc*). Mnjoyan et al. (27) have shown that FH, the strongest regulator of the alternative pathway of complement, binds to PLT integrin $\alpha_{IIb}\beta_3$. FH was also found on PLT-Ect, where it might inhibit amplification of complement (Fig. 1*Cd*). Whether purified PLT-Ect were still capable of activating complement despite the presence of CD55 and FH was tested in an immune adherence assay. We have previously found that ectosomes of PMN activate complement and bind C3b, which allows them to bind to the CR1/CD35 receptor of erythrocytes (26). Purified PLT-Ect were fluorescently labeled, and added to whole blood anticoagulated with hirudine at 37°C, so as to keep complement activity intact. After 30 min, erythrocytes were labeled with an anti-GPA mAb, and the whole blood was analyzed by FACS to see whether PLT-Ect would adhere to erythrocytes when complement is intact. A significant amount of erythrocytes was bearing PLT-Ect (15% of erythrocytes were double positive; Fig. 2*C*), but not when complement was blocked by adding EDTA in hirudine blood (2%; Fig. 2*D*).

Having defined some of the central structural characteristics of PLT-Ect, we were interested to know whether they would be able to modulate macrophages and DC because in many experimental systems PMP enhance coagulation and inflammation. However, the expression of PS may have opposing effects, as suggested by multiple experiments using apoptotic cells and ectosomes of other origins.

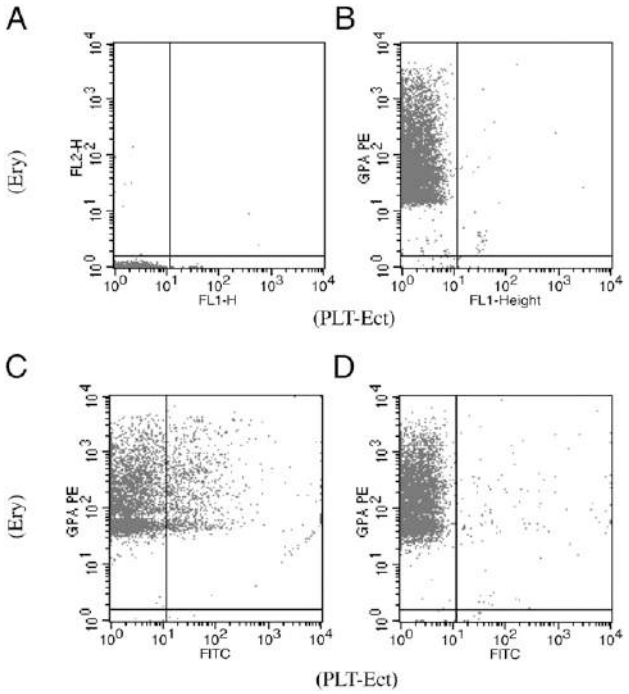

FIGURE 2. Adherence of PLT-Ect to erythrocytes in whole blood. Representative FACScan dot plots showing the binding of PLT-Ect to GPA-PE–labeled erythrocytes (Ery) in hirudine whole blood (n = 3). *A*, Control fluorescence of whole blood in the absence of PLT-Ect. *B*, GPA-PE–labeled whole blood in the absence of PLT-Ect. *C*, GPA-PE–labeled whole blood incubated with fluorescent PLT-Ect. *D*, GPA-PE–labeled whole blood incubated with fluorescent PLT-Ect in the presence of EDTA.

PLT-Ect downregulate the inflammatory response of human macrophages

We coincubated PLT-Ect with macrophages in the presence or absence of zymosan A or LPS for 15 h. SN were analyzed for TNF-α, IL-10, and TGF-β1. In the absence of stimuli, we could detect no or minimal amount of TNF-α and IL-10 release by macrophages. The addition of PLT-Ect did not modify this profile, as shown for TNF-α and IL-10 (Fig. 3*A*, 3*B*). Under the same conditions, the addition of PLT-Ect on macrophages alone induced an increase in TGF-β1 release (Fig. 3*C*). As expected, in the control experiments, zymosan A induced the release of TNF-α and IL-10, but had almost no effect on TGF-β1 (Fig. 3*D–F*). In the presence of PLT-Ect, the activation of macrophages by zymosan A was inhibited. Indeed, the release of TNF-α and IL-10 induced by zymosan A was almost blocked in the presence of PLT-Ect even at the lower ratio of PLT-Ect to macrophages (Fig. 3*D*, 3*E*). In addition, at high concentration PLT-Ect induced a 3- to 4-fold increase in the secretion of TGF-β1, as shown in Fig. 3*F*. The biological effects of PLT-Ect were almost identical when macrophages were stimulated with LPS instead of zymosan A (data not shown). Next, we analyzed the kinetics of the release of TNF-α and IL-10 by macrophages exposed to zymosan A and/or PLT-Ect during the first 20 h. The suppressive effect of PLT-Ect on TNF-α release was already apparent at 2 h, whereas suppression of IL-10 occurred only after 6 h, suggesting different regulatory mechanisms (Fig. 3*G*, 3*H*). The kinetic analysis of TGF-β1 confirmed a rapid release already at 30 min and a sustained liberation along the 20 h as well, suggesting an immediate release from a storage pool, followed by production of TGF-β1 (Fig. 3*I*).

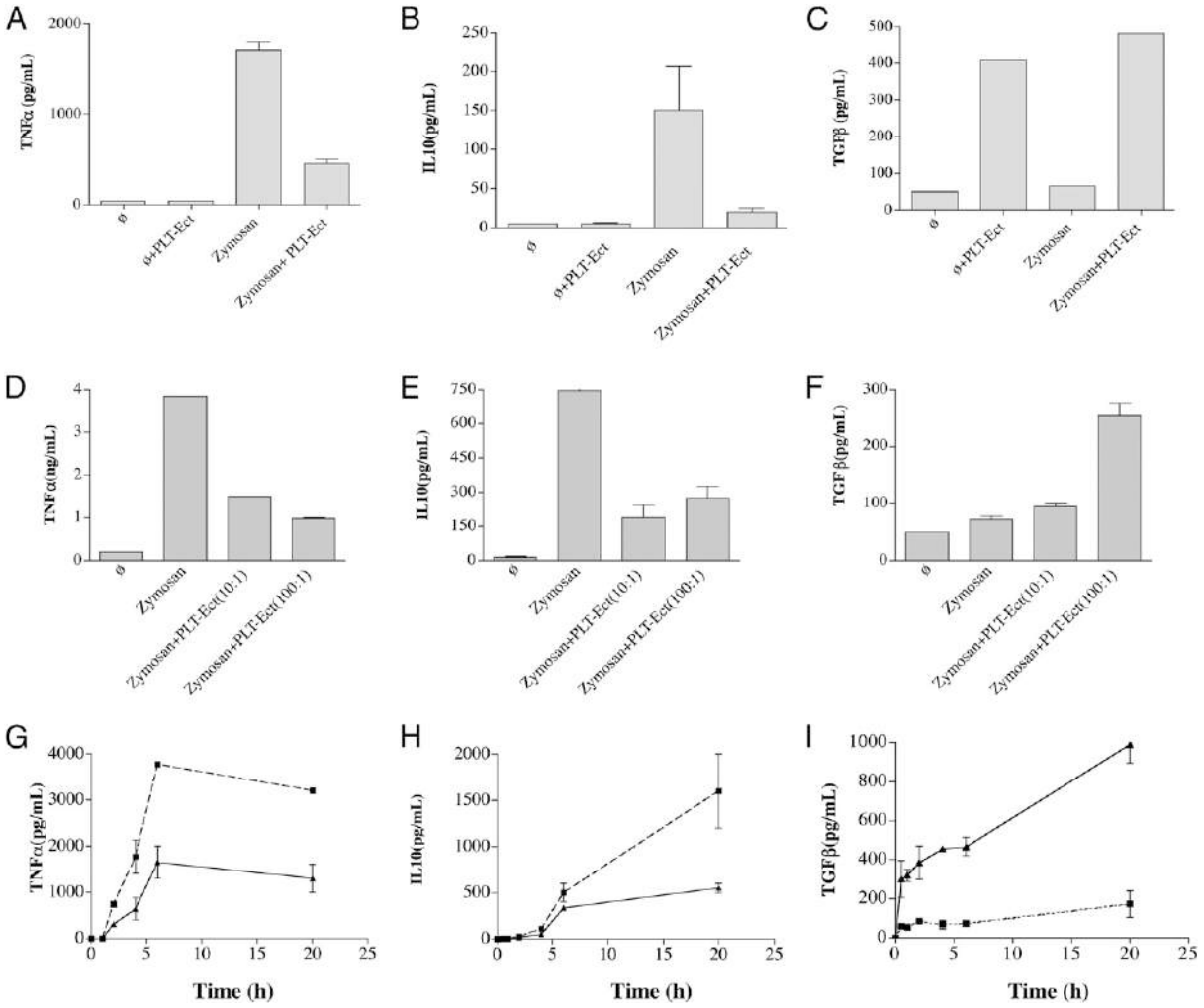

FIGURE 3. Cytokine release by macrophages exposed to PLT-Ect. *A–C*, Macrophages were incubated overnight in medium alone (Ø), medium with PLT-Ect, medium with zymosan A, and medium with zymosan A and PLT-Ect. SN were analyzed for the following: *A*, TNF-α; *B*, IL-10; and *C*, TGF-β1. Assays were performed in triplicate. Results are representative of four independent experiments. Error bar indicates ± SEM. *D–F*, Macrophages were incubated overnight in medium alone (Ø), medium with zymosan A, and medium with zymosan A and different ratios of PLT-Ect to macrophage (10:1, 100:1). SN were analyzed for the following: *D*, TNF-α; *E*, IL-10; and *F*, TGF-β1. Assays were performed in triplicate. Results are representative of four independent experiments. Error bar indicates ±SEM. In the forthcoming experiments, we used the (100:1) ratio PLT-Ect to macrophages. *G–I*, Macrophages incubated in medium with zymosan A, dotted line (—), medium with zymosan A and PLT-Ect, plain line (—), for up to 20 h. SN were harvested immediately, after 30 min, 1 h, 2 h, 4 h, 6 h, and 20 h, and analyzed for the following: *G*, TNF-α; *H*, IL-10; and *I*, TGF-β1. Assays were performed in duplicate. Results are representative of three independent experiments. Error bar indicates ±SEM.

To analyze the first phase of TGF-β release, we carried out immunofluorescence microscopy of macrophages in presence or absence of PLT-Ect under resting conditions or zymosan A activation (Fig. 4). Interestingly, TGF-β was found stored in an undefined compartment in resting, but also in zymosan A-stimulated macrophages. These pools disappeared when the cells were exposed to and had ingested PLT-Ect, independently of the presence of zymosan A. Of note in Fig. 4*d*, macrophages that have ingested PLT-Ect released TGF-β, as attested by the decrease in TGF-β staining as opposed to some macrophages that did not encounter PLT-Ect and in which TGF-β staining remained intact.

These results were confirmed by measuring a rapid release TGF-β1 in the SN of macrophages exposed only to PLT-Ect (after 30 min the SN concentration was 300 pg/ml).

The role of PS in inducing these effects was explored by adding AnV to PLT-Ect before using them in the different assays. As shown in Fig. 5*A*, AnV inhibited the functional activity of PLT-Ect on TNF-α (Fig. 5*Aa*) and TGF-β1 (Fig. 5*Ab*).

To see whether the exposure of macrophages to PLT-Ect would produce a modification in their behavior even for forthcoming inflammatory stimuli, we preincubated macrophages with PLT-Ect for 60 min, then removed the SN containing the uningested PLT-Ect. The macrophages were left to recover (1, 24, or 48 h) before overnight stimulation with zymosan A. The release of TNF-α by zymosan A-activated macrophages was inhibited for up to 24 h after the contact between PLT-Ect and macrophages, indicating a lasting alteration of the macrophage-signaling machinery. However, this effect was not maintained at 48 h, indicating a clear time dependency with return to no inhibition (Fig. 5*B*). When we studied the lasting effect of PLT-Ect on TGF-β1 released by human monocyte-derived macrophages (HMDMs), we preincubated HMDMs with PLT-Ect for 1 h, and then washed the cells to remove the remaining uningested PLT-Ect. We then rechallenged HMDMs by adding PLT-Ect 1, 24, or 48 h after the washing. Although TGF-β1 was released from the storage pools from macrophages at their first encounter with PLT-Ect (pre-exposure), there was significant

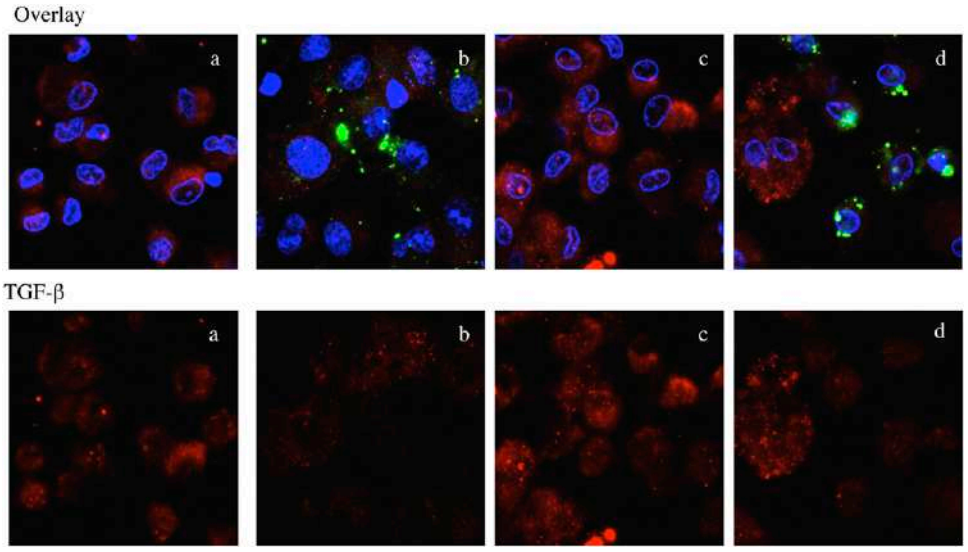

FIGURE 4. PLT-Ect induced TGF-β release by macrophages. Macrophages were incubated 30 min with labeled PLT-Ect in the presence or not of zymosan A. *a*, Nonstimulated; *b*, nonstimulated in presence of PLT-Ect; *c*, zymosan A-stimulated macrophages; *d*, zymosan A-stimulated macrophages in presence of PLT-Ect. Cells were triple immunofluorescence stained with Ab against TGF-β, followed by secondary anti-rabbit Ab coupled to Cy5 (red), with PKH67 fluorescent cell linker for PLT-Ect (green), and with DAPI for nuclei (blue). *Upper panels*, Overlay visualization. *Lower panels*, Visualization of TGF-β alone. Original magnification ×63.

amount of TGF-β1 24 h after each challenge, indicating that TGF-β1 production and release were maintained (Fig. 5*C*).

On apoptotic cells, PS has been identified as a major factor influencing monocyte-derived DC maturation and function (28–31). PLT-Ect might have similar effect because they express PS as well. Having shown that PLT-Ect have long lasting effects on macrophages, we first investigated whether the differentiation of monocytes toward DC with an immature phenotype (iDC) would

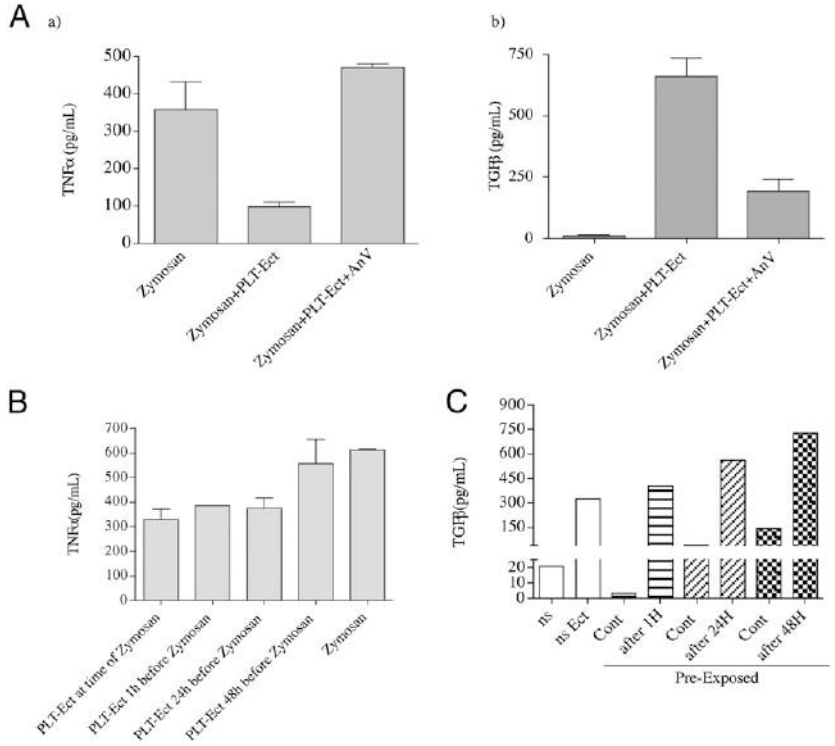

FIGURE 5. Blockade of PLT-Ect by AnV and their lasting effects. *A*, Macrophages were incubated overnight with zymosan A in presence or absence of PLT-Ect. In some experiments, zymosan A medium was supplemented with PLT-Ect that were preincubated with AnV. SN were analyzed for TNF-α (*Aa*) and TGF-β1 (*Ab*). Assays were performed in duplicate. Results are representative of three independent experiments. Error bars indicate ±SEM. *B*, Macrophages were incubated for 1 h with PLT-Ect; unbound PLT-Ect were washed; and 1, 24, or 48 h after PLT-Ect exposure, macrophages were exposed to zymosan A overnight. SN were analyzed for TNF-α. Assays were performed in duplicate. Results are representative of three independent experiments. Error bar indicates ±SEM. *C*, Macrophages were incubated for 1 h with PLT-Ect; unbound PLT-Ect were washed; and 1, 24, or 48 h after PLT-Ect exposure, macrophages were re-exposed to PLT-Ect overnight. SN were analyzed for TGF-β1. Assays were performed in duplicate. Results are representative of five independent experiments.

be modified when monocytes were exposed to PLT-Ect during the initial 2 d of the 6-d culture to achieve differentiation (Fig. 6*A*; Monocytes→iDC). We will refer to them as PLT-Ect–modified iDC as compared with conventional iDC. We then looked at whether PLT-Ect–modified iDC matured by LPS would be altered as compared with maturation of conventional iDC exposed to LPS (Fig. 6*A*; iDC→mature DC [mDC]).

PLT-Ect modify the differentiation of monocytes toward iDC as well as their maturation

We compared the phenotypes of iDC differentiated from monocytes with GM-CSF and IL-4 to PLT-Ect–modified iDC. First, both types of iDC lost CD14, indicating that PLT-Ect did not freeze the differentiation process. PLT-Ect–modified iDC showed a significant drop in the expression of HLA-DP DQ DR ($p < 0.05$) and the costimulatory molecule CD80 ($p < 0.004$) when compared with conventionally developed iDC (Fig. 6*Ba*, 6*Bb*). The modifications of the phenotype observed in the presence of PLT-Ect might influence the Ag presentation. However, CD83 and CD86 showed no consistent modifications overall (data not shown).

Second, the phagocytic activity of PLT-Ect–modified iDC was investigated. The data obtained indicated that PLT-Ect–modified iDC had a reduced endocytic capacity as compared with conventional iDC, a modification that would reduce their capacity to take up Ags (Fig. 6*Bc*).

The early and transient exposure to PLT-Ect not only impaired generation of conventional iDC, but also modified their maturation induced by LPS. These PLT-Ect–modified mDC released significantly less cytokines (Fig. 7*A*), except for IL-8 (data not shown): TNF-α (1801 ± 478 versus 730 ± 432 pg/ml; $p \leq 0.008$)(Fig. 7*Aa*), IL-6 (2833 ± 692 versus 722 ± 193 pg/ml; $p \leq 0.01$)(Fig. 7*Ab*), IL-12p70 (43.5 ± 10 versus 17 ± 3 pg/ml; $p \leq 0.025$) (Fig. 7*Ac*), IL-1β (12.2 ± 3 versus 5 ± 1 pg/ml; $p \leq 0.05$)(Fig. 7*Ad*), IFN-γ (518 ± 108 versus 241 ± 36 pg/ml; $p \leq 0.035$) (Fig. 7*Ae*), and IL-10 (83 ± 25 versus 25 ± 5 pg/ml; $p \leq 0.05$) (Fig. 7*Af*).

In addition, PLT-Ect–modified mDC expressed significantly less HLA-DP DQ DR molecules than conventional mDC ($p \leq 0.003$) (Fig. 7*Ba*). The costimulatory molecule CD80 was also decreased (Fig. 7*Bb*). CD83 and CD86 showed no consistent modifications overall (data not shown). Finally, the phagocytic activity dropped further for PLT-Ect–modified mDC than for conventional mDC (Fig. 7*Bc*). Once monocytes were exposed to PLT-Ect, both of the differentiation and maturation characteristics were modified.

In summary, the exposure of monocytes to PLT-Ect modified the differentiation of DC with a reduced potential for inflammation and Ag presentation.

Effect of PLT-Ect on conventional iDC

We wanted then to explore the direct effect of PLT-Ect on conventionally developed iDC and on their LPS-induced maturation. However, we were surprised by their limited effects. At this later stage, conventional iDC were prepared and their maturation achieved by adding LPS overnight in presence or absence of PLT-Ect. The presence of PLT-Ect during the maturation process did not modify the phenotypic marker HLA-DP DQ DR of mDC (data not shown); only slightly and not significantly reduced the phagocytic capacity of mDC (data not shown); downmodulated the release of only TNF-α (1801 ± 478 versus 570 ± 370 pg/ml; $p \leq 0.005$) and IL-6 (2833 ± 692 versus 2068 ± 493 pg/ml; $p \leq 0.05$); had a minimal and not significant effect on the release of IL-12p70, IL-1β, IFN-γ, and IL-10; and no effect on IL-8 (data not shown).

Discussion

In the current study, we have highlighted novel aspects of PLT MP/ectosomes biology. We could demonstrate that ectosomes shed by PLT during storage downmodulate macrophage activation, indicating that they are active players in the regulation of inflammation. They also modified the monocyte differentiation toward iDC and their subsequent maturation to DC, thus interfering with the normal immune response. These anti-inflammatory properties are in apparent conflict with many reports, which illustrate the prothrombotic and proinflammatory activities of MP released by activated PLT in vitro and in vivo (32, 33). The major differences are probably that the ectosomes analyzed in this work

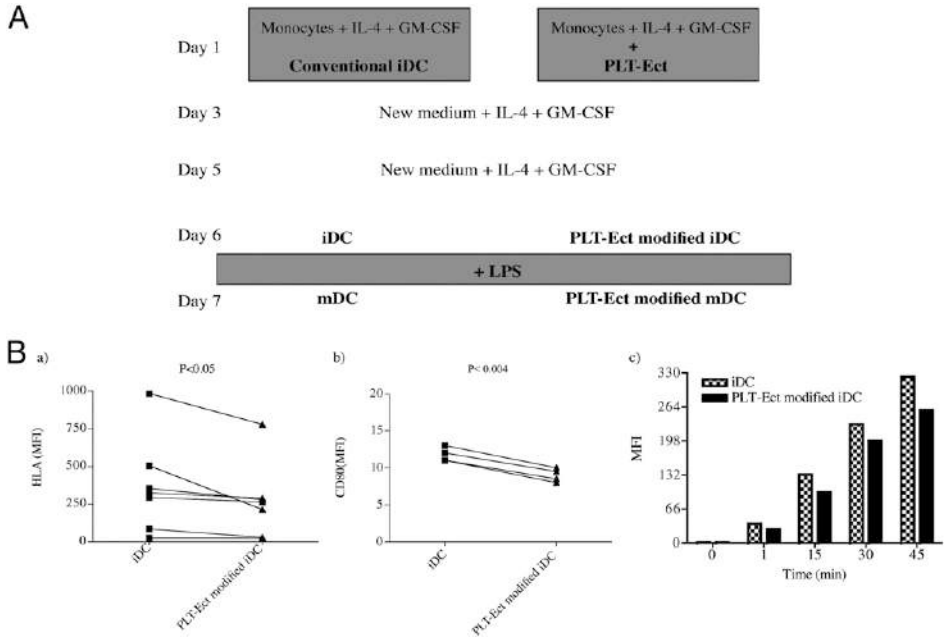

FIGURE 6. The differentiation of monocytes to iDC in the presence of PLT-Ect. *A*, Scheme representing the in vitro model for DC preparation. *B*, iDC were developed in absence or presence of PLT-Ect. *Ba*, HLA-DP DQ DR expression. The mean fluorescence intensity (MFI) of seven independent experiments are shown. *Bb*, CD80 expression. The MFI of four independent experiments are shown. *Bc*, The capacity of FITC-conjugated dextran phagocytosis of both iDC was measured at different time points by flow cytometry. The results shown are from one representative experiment.

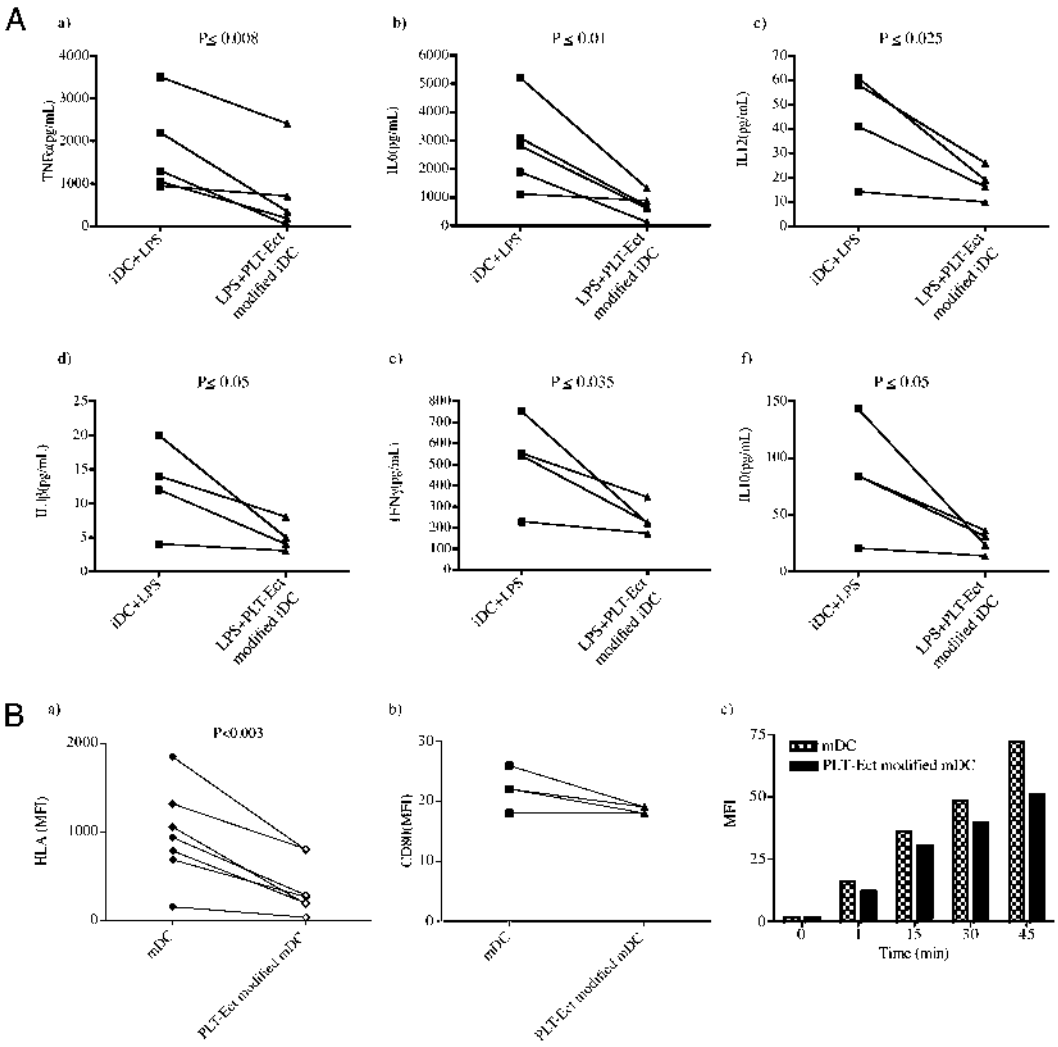

FIGURE 7. The maturation of PLT-Ect–modified iDC: cytokine release, HLA expression, and phagocytosis. mDC were developed after an overnight LPS stimulation of conventional iDC and PLT-Ect–modified iDC. *A*, The release of proinflammatory cytokines TNF-α (*a*), IL-6 (*b*), IL-12p70 (*c*), IL-1β (*d*), IFN-γ (*e*), and IL-10 (*f*) was analyzed in the SN of mDC and PLT-Ect–modified mDC. The results of five experiments done in duplicates are shown. *Ba*, HLA-DP DQ DR expression of both of mDC and PLT-Ect–modified mDC was analyzed by flow cytometry. The MFI of seven independent experiments are shown. *Bb*, CD80 expression. The MFI of four independent experiments are shown. *Bc*, The capacity of FITC-conjugated dextran phagocytosis of both mDC was examined at different time points by flow cytometry. The results shown are from one representative experiment.

were released by stored PLT, which show low signs of activation before transfusion, and the experiments performed were done in vitro, and thus might not describe the overall properties that ectosomes express in vivo.

We have chosen to describe these MP as PLT-Ect based on many structural aspects. The ectosomes were originating from PLT ($CD61^+$), with little contamination by other MP; they were of a large size (100–1000 nm), bound AnV efficiently, and did not express CD63, but many proteins found on PLT cell surface including proteins acquired from plasma such as C1q, C3 fragments, and FH. These characteristics contrast with the vesicles released from multivesicular bodies defined as exosomes. Exosomes are smaller, bind AnV poorly, and express CD63, a late endocytic marker (14). Whereas activated PLT release both type of vesicles, it appears that those formed by stored PLT are mainly ectosomes. The presence of some exosomes can evidently not be excluded because they are too small to be measurable by FACS (14, 34, 35). Thus, vesicles having the typical "little AnV but high CD63 bindings" could not be identified. However, the size analysis by electron microscopy showed only few vesicles <100 μm. PLT-Ect expressed the "don't eat me signal" CD47 molecule; however, this was not sufficient to block the phagocytosis of PLT-Ect by macrophages. In addition, PLT-Ect expressed CD154. However, the global effect of PLT-Ect was inhibitory as opposed to what would be expected for CD154 (36–38), with a decrease in the costimulatory molecule expression CD80 and cytokine production. These results would suggest that the presence of a specific molecule does not guarantee function.

Microvesicles released by activated PLT in vitro activate the classical pathway of complement with binding of C1q to the gC1q receptor (or PS?) and further deposition of C4 and C3 fragments as well as C5b-C9 complexes (24, 39, 40). These MP appear to be equivalent in size and AnV expression to the ectosomes described in this work. We confirmed and extended this observation by demonstrating immune adherence of PLT-Ect to erythrocytes in a whole blood system. Evidently, the many molecules capable of blocking complement activation present on PLT-Ect [C1inh (41), FH (27), CD55 (24)] are insufficient to inhibit efficient C3b deposition on PLT-Ect and its binding to CR1 on erythrocytes. The CD35/CR1 receptor for C3b of erythrocytes has already been shown to bind complement-activating bacteria, viruses, and immune complexes, and for immune complexes to deliver them to

the fixed macrophages of liver and spleen (42). Whether the same phenomenon might occur in vivo for PLT-Ect remains to be demonstrated; however, this mechanism might be one way by which PLT-Ect are removed from blood. This clearance mechanism is evidently not sufficient in many diseases in which a high amount of PMP is found in plasma (43).

AnV binding is a marker for PS expression at the surface of PLT-Ect. PS plays a central role in the procoagulant properties of many different cells and vesicles, which are in contact with blood. However, PS expression induces other reactions as well. On apoptotic cells, the exposure of PS on the outer leaflet of the cell plays a major role in downregulating immunity and inflammation. PS expressed on ectosomes of PMNs (20, 25) and erythrocytes (19) as well as on liposomes appears to have similar functions when tested in vitro using macrophages and/or DC (19, 20, 28, 44). Thus, it seemed logical to see whether PLT-Ect were also capable of interfering with the activities of these cells involved in inflammation and immunity. Indeed, PLT-Ect showed several downmodulatory activities on human macrophages. First, PLT-Ect tested in an in vitro model of macrophage activation exhibited an evident capacity to inhibit the release of TNF-α and IL-10 by macrophages activated by LPS or zymosan A, known to react with TLR-4 and TLR-2, respectively. These results were similar to those observed for PMN and erythrocyte ectosomes (19, 20). The kinetics analysis of the cytokine releases showed that the release of IL-10 occurs after that of TNF-α, and may represent another mechanism to limit inflammation that may have been induced by the proinflammatory cytokines. If that is the case, it would not be surprising that lower release of TNF-α in the presence of PLT-Ect is by itself directly responsible for less IL-10 release. Like ectosomes of PMN and vesicles shed by different tumor cells (45), PLT-Ect enhanced the release of TGF-β1 by macrophages directly, that is, even in the absence of TLR stimulation. Noteworthy was that this release was very rapid, in minutes, from an intracellular storage compartment, as shown by immuno-imaging. This immediate release was followed by a steady state of further TGF-β1 release over hours, which might be due to synthesis of the protein. This observation indicates that PLT-Ect have a specific function not only when macrophages are activated, but also to modify the biology of the macrophages directly, even when they are resting. Whether this is due to the presence of PS on the PLT-Ect remains uncertain because those shed by erythrocytes express PS as well, but do not induce the immediate release of TGF-β1 (19). AnV, by binding to PS, inhibits the uptake of ectosomes by macrophages (20). Thus, that AnV interfered with the activities of PLT-Ect indicated only that a direct contact/uptake between PLT-Ect and macrophages was necessary, but cannot be taken as evidence that PS is directly involved in the downregulatory functions of PLT-Ect. Indeed, PS might be essential for the bridging of PLT-Ect to macrophages, but not for all downstream signaling events. Finally, the exposure of macrophages to PLT-Ect induced a long lasting anti-inflammatory phenotype to the cells because once exposed for 1 h to PLT-Ect, macrophages showed a reduced release of cytokines for up to 24 h, whereas TGF-β1 was continuously released.

Because PLT-Ect modified the properties of macrophages, it was of interest to follow the development of monocytes to iDC in the presence of PLT-Ect, and see whether such iDC would be modified as well. Indeed, PLT-Ect interfered with this differentiation producing modified iDC, with altered characteristics, as follows: a reduced expression in HLA surface markers, costimulatory molecule CD80, and phagocytosis of dextran particles. In addition, the maturation of PLT-Ect–modified iDC was damped, as evidenced by the reduced release of cytokines, HLA class II and CD80, as well as by the loss of phagocytic activity, indicating changes in their intrinsic properties. These observations might be more directly relevant to in vivo situations, in which monocytes, before leaving the circulation, may meet PLT and PLT-Ect. Locally, at a site of injury, such monocytes are thus less likely to develop to full-blown proinflammatory DC. Of particular interest is that in humans PLT transfusions do not lead to much inflammation and thrombosis despite the presence of PLT-Ect in large numbers, suggesting that our results correspond to some biological reality: PLT-Ect do not induce pathological reactions, but might have properties comparable to apoptotic cells, in particular their silent anti-inflammatory elimination.

In summary, few studies have been made explicitly with ectosomes released by stored PLT, and the experiments were mostly centered on coagulation. The properties of such ectosomes might be very different from those found in vivo under different pathological conditions. In addition, the methods used to produce PLT for transfusion, the storage time of PLT, as well as the purification steps used to separate the ectosomes from the PLT may all influence the final properties of ectosomes. Furthermore, the in vitro systems used in this study looked at only at one specific aspect, which is different from what occurs in the full biological milieu. Despite these restrictions, it is of interest to realize that PLT-Ect have many faces, including activities, that may dampen inflammation and immunity under specific circumstances.

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Disclosures

The authors have no financial conflicts of interest.

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