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Endothelial cell-derived microparticles induce plasmacytoid dendritic cell maturation: potential implications in inflammatory diseases

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ABSTRACT

Background
Increased circulating endothelial microparticles, resulting from vascular endothelium dysfunction, and plasmacytoid dendritic cell activation are both encountered in common inflammatory disorders. The aim of our study was to determine whether interactions between endothelial microparticles and plasmacytoid dendritic cells could contribute to such pathologies.

Design and Methods
Microparticles generated from endothelial cell lines, platelets or activated T cells were incubated with human plasmacytoid dendritic cells sorted from healthy donor blood or with monocyte-derived dendritic cells. Dendritic cell maturation was evaluated by flow cytometry, cytokine secretion as well as naive T-cell activation and polarization. Labeled microparticles were also used to study cellular interactions.

Results
Endothelial microparticles induced plasmacytoid dendritic cell maturation. In contrast, conventional dendritic cells were resistant to endothelial microparticle-induced maturation. In addition to upregulation of co-stimulatory molecules, endothelial microparticle-matured plasmacytoid dendritic cells secreted inflammatory cytokines (interleukins 6 and 8, but no interferon-α) and also induced allogeneic naive $CD4^+$ T cells to proliferate and to produce type 1 cytokines such as interferon-γ and tumor necrosis factor-α. Endothelial microparticle endocytosis by plasmacytoid dendritic cells appeared to be required for plasmacytoid dendritic cell maturation. Importantly, the ability of endothelial microparticles to induce plasmacytoid dendritic cells to mature was specific as microparticles derived from activated T cells or platelets (the major source of circulating microparticules in healthy subjects) did not induce such plasmacytoid dendritic cell maturation.

Conclusions
Our data show that endothelial microparticles specifically induce plasmacytoid dendritic cell maturation and production of inflammatory cytokines. This novel activation pathway may be implicated in various inflammatory disorders and endothelial microparticles could be an important immunmodulatory therapeutic target.

Key words: endothelial cell-derived microparticles, plasmacytoid, inflammatory diseases, dendritic cells.

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Introduction

Membrane vesiculation is a general physiological process that leads to the release of cell plasma fragments, called microparticles.[1-3] These microparticles are defined by their size (0.1-1 μm) and can be generated from nearly every type of cell during activation, injury or apoptotic processes.[1] All microparticles, whatever their cell origin, have negatively charged phospholipids – such as phosphatidylserine – in their outer membrane leaflet, accounting for their procoagulant properties.[1-3] They also express proteins, characteristic of their cellular origin, on their surface and carry proteins packaged from numerous cellular compartments,[4,5] as well as mRNA.[6] Microparticles are different from exosomes, since these latter are smaller (30-90 nm) and derived from endocytic compartments leading to an enrichment of tetraspanin molecules.[7] Microparticles can participate in the maintenance of homeostasis under physiological conditions. Among the various circulating microparticles, platelet-derived microparticles are the most abundant in the bloodstream, representing between 70 to 90% of circulating microparticles in healthy subjects.[8] As shown for apoptotic bodies[9] – another form of *cell dust* or debris, tumor- or fibroblast-derived microparticles captured by monocytes may induce regulatory cytokine secretion.[10] In addition, microparticles from activated T cells can deliver differentiation signals to stem cells.[11] In contrast, microparticles can initiate deleterious processes if produced in excess[1] or when carrying pathogenic constituents or inflammatory signals.[12,13] Vascular endothelium aggression may lead to the vesiculation and shedding of endothelial microparticles (EMP). Increased levels of EMP have been reported in a variety of pathological situations including thrombosis,[3,8] atherosclerosis,[14] renal failure,[15,16] diabetes,[17] graft-versus-host disease after hematopoietic cell transplantation[18,19] and systemic lupus erythematosus.[1,20,21] These data emphasize the link between endothelial damage, the release of EMP and the modulation of inflammatory and/or immune responses.

Dendritic cells play a major role in immune responses. They are specialized to capture and present antigens to T cells.[22] Two major subsets of dendritic cells have been described in humans: conventional dendritic cells (previously called myeloid dendritic cells) and plasmacytoid dendritic cells (PDC).[23] These latter represent a particular population of dendritic cells that were first identified as the principal cells secreting interferon α (IFN-α) in response to viral or bacterial stimulation.[23] As such, PDC contribute to the innate anti-viral and anti-bacterial defense system. Alterations of PDC homeostasis and function with increased production of IFN-α have been implicated in various autoimmune or inflammatory diseases including type I diabetes, psoriasis, multiple sclerosis, and systemic lupus erythematosus.[23-25] In addition, PDC have been found in the atherosclerotic plaques.[26-28] However, a growing body of data shows that PDC can also be involved in tolerance induction.[29-31] On the other hand *in vitro* activation of immature human PDC with ligands for Toll-like receptor (TLR) 7 or TLR9 (e.g., R848 or type A CpG motifs, respectively) leads to proinflammatory cytokine production and to increased co-stimulatory molecule expression, subsequently inducing naive T-cell activation.[23] Other factors, such as damage-associated molecular patterns, also induce PDC activation.[23,32] Nothing is known about the capacity of EMP to activate PDC. The common finding of endothelial damage – associated with increased EMP release – and PDC activation in several inflammatory diseases[32] led us to investigate whether EMP could provide signals that promote phenotypic and functional maturation of PDC *in vitro*.

Design and Methods

Flow cytometry and antibodies

Flow cytometry was performed with a CANTO A cytometer (BD Biosciences, Le Pont de Claix, France) using DIVA 6.1 software (BD Biosciences). The following monoclonal antibodies were used: fluorescein isothiocyanate-conjugated BDCA-2 (AC144, Miltenyi Biotech, Paris, France), CD14 (MYA-4), HLA-DR (B-F1), phycoerythrin-conjugated CD40 (mAb89), CD31 (1F11), phycoerythrin-Texas red-x (ECD)-conjugated CD41 (P2), phycoerythrin-cyanin-5 (PC5) CD146 (TEA 1/34), phycoerythrin cyanin-7-conjugated CD3 (UCHT1), CD4 (SFCI12T4D11) (Beckman Coulter Immunotech, Villepinte, France), phycoerythrin-conjugated CD123 (9F5), CCR7 (CD197, clone 2H4), CD83 (HB15e), fluorescein isothiocyanate-conjugated CD80 (MAB104), CD86 (2331FUN1) and allophycocyanin-conjugated CD1a (HI149), CD62E (68-5H11) (BD Biosciences). Fluorescent-conjugated isotype control monoclonal antibodies from the different monoclonal antibody providers were used. The mean fluorescence intensity ratio was obtained by dividing the mean fluorescence intensity for a given marker by the mean fluorescence intensity of the respective isotype control monoclonal antibody.

Generation and flow cytometry quantification of microparticles

EMP were prepared from a human microvascular dermal endothelial cell line (HMEC-1) as previously described.[33,34] Briefly, confluent HMEC-1 cells were incubated for 24 h with 50 ng/mL TNF-α (Sigma Aldrich, Saint-Quentin Fallavier, France). Culture supernatants were collected and cleared from detached cells and cell fragments by centrifugation at 1200g for 5 min. The supernatant was then centrifuged twice at 15000g for 90 min (Cambrex, Verviers, Belgium) at 4°C. Pelleted EMP were resuspended in culture media and used immediately. The absence of residual TNF-α in EMP samples was confirmed using ELISA (Diaclone). EMP were also prepared from quiescent HMEC-1 (without TNF-α) as well as from the human umbilical vein cell line, EAY926 (ATCC CRL-2922) using the same protocol. EMP preparations were checked for endotoxin contamination using the *Limulus amebocyte* lysate assay. Endotoxin content was always less than 0.05 ng/mL. In some experiments, the supernatant resulting from the last wash was used as a control. This supernatant was free of EMP, as demon-

strated by cytometric analysis. The absence of mycoplasma was confirmed for all cultures using the Mycolasma PCR detection kit (Venor® GeM, BioValley, Marne la Vallée, France).

Platelet-derived microparticles were isolated from acid-citrate-dextrose anticoagulated human blood from healthy volunteers obtained after written informed consent (Etablissement Français du Sang Bourgogne Franche-Comté, EFS B/FC, Besançon, France). The platelet-rich plasma was obtained by centrifugation at 100g for 15 min. This plasma was spun at 1000g for 10 min, the supernatant was then collected and the platelet-derived microparticles were pelleted by centrifugation at 20000g for 2 h at 4°C.[12] The purity of platelet-derived microparticle isolation (~95%) was assessed using CD31 and CD41 monoclonal antibody staining. Microparticles derived from activated T cells were prepared, using the same protocol as that for EMP, from CD3/CD28 activated peripheral blood mononuclear cells isolated from healthy donors. After isolation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) centrifugation, the peripheral blood mononuclear cells were activated with CD3/CD28 Dynabeads (Invitrogen, Cergy Pontoise, France) and 600 UI/mL of interleukin (IL)-2 (Proleukine, Sanofi, Paris, France) for 48 h. Supernatants were collected and T-cell-microparticles were isolated.

In order to quantify the microparticles, an aliquot of 10 µL of microparticles was labeled using fluorescein isothiocyanate-conjugated annexin V (Annexin V apoptosis detection kit I, BD Biosciences) according to manufacturer's instructions. In each experiment, control labeling was performed by incubating annexin V with the supernatant resulting from the last wash of the microparticle preparation. A defined number of calibrated 3-µm latex beads (Flowcount™ beads, Beckman Coulter) was added to the sample, as an internal standard. Consequently, microparticles derived from endothelial cells, platelets and activated T cells were defined as elements with a size comprised between 0.1-1 µm and positively labeled by fluorescein isothiocyanate-annexin V.

Generation and isolation of dendritic cells

The GEN 2.2 cell line derived from a PDC leukemia patient was cultured on MS-5 irradiated feeder cells in RPMI 1640 Glutamax 1 (InVitrogen, Cergy Pontoise, France) containing 1 mM sodium pyruvate (Sigma), 20 µg/mL gentamicin (InVitrogen), non-essential amino acids (InVitrogen) (hereafter referred to as RPMI complete medium) and 10% heat inactivated fetal calf serum (Life Technologies, Gaithersburg, MD, USA). Peripheral blood mononuclear cells from healthy donors were obtained after written informed consent and isolated by Ficoll-Hypaque (Pharmacia) centrifugation. Circulating normal PDC were isolated using immunomagnetic cell sorting (BDCA-4 cell isolation Kit, Miltenyi Biotec) according to the manufacturer's recommendations. After isolation, PDC purity was controlled by cytometry using CD123/BDCA-2 monoclonal antibody staining and was between 94-98%. Monocyte-derived dendritic cells (Mo-DC) were generated from blood monocytes – isolated by negative depletion (Negative Monocyte Isolation Kit, Dynal, InVitrogen) – by a 6-day culture in RPMI 1640, containing 10% heat inactivated fetal calf serum, 50 ng/mL granulocyte-monocyte colony-stimulating factor and 20 ng/mL IL-4 (PeproTech, London, UK). At the end of the culture, the generation of Mo-DC was attested by a $CD1a^+$ $CD14^-$ phenotype.

Evaluation of dendritic cell maturation

Maturation of freshly isolated PDC was induced by culture for 18 h in the presence of 10 ng/mL IL-3 (PeproTech) and R848 (1 µg/mL, Invivogen, Toulouse, France), or CpGA (2 µmol/L, ODN2216, Invivogen), or recombinant CD40L (1 µg/L, COGER, Paris, France) or by addition of microparticles (ratio 10 microparticles:1 dendritic cell; this corresponds to 5000 microparticles/µL, an EMP concentration found *in vivo* in several pathological situations including myocardial infarction[35] and thrombocytopenic purpura[36]). Mo-DC (6-day culture) were incubated with either lipopolysaccharide from *Escherichia coli* (LPS, 2 µg/mL, Sigma) or EMP (10 EMP:1 Mo-DC) for 48 h. To assess the involvement of phosphatidylserine-mediated signaling in Mo-DC maturation, cells were incubated with EMP or apoptotic cells, as a positive control, (corresponding to murine thymocytes submitted to 40 Gray γ-irradiation- with a ratio of 5 apoptotic cells:1 dendritic cell), and stimulated 24 h later by lipopolysaccharide. IL-12(p70) was measured in supernatants (ELISA, BD OptEIA, BD Biosciences).

Confocal microscopy analyses

PDC were incubated with anti-BDCA-2 monoclonal antibody (AC144 Miltenyi Biotech) for 30 min at +4°C, washed and labeled with cyanin 5-conjugated goat anti-mouse Ig (BD Biosciences). PDC were incubated with EMP previously labeled with CFSE (5 mM) (Molecular Probes, Leiden, Netherland) for 2 h at 37°C in a Labtek II culture chamber (VWR International, Fontenay-sous-Bois, France). After nuclear staining using DAPI (Molecular Probes), cells were washed in phosphate-buffered saline, fixed in 4% paraformaldehyde and mounted (Fluorescent Mounting Medium, Dako, Trappes, France). Fluorescent images were acquired on an FV1000 confocal microscope (Olympus, Rungis, France).

Cytokine production

Culture supernatants were collected from PDC or Mo-DC incubated with or without TLR ligands or EMP for 18 h. The following cytokines: IL-12p70 (only for Mo-DC), TNF-α, IL1-β, IL-10, IL-6, IL-8 and transforming growth factor (TGF)-β were measured in the culture supernatants using Luminex Technology kits (HCYTO-60 K, Linco, Millipore, Saint-Quentin en Yvelines, France) on a Luminex 100® analyzer (Luminex, Austin, TX, USA), according to the manufacturer's instructions. Interferon (IFN)-α was measured by using a Luminex Technology kit (Biosource, Clinisciences, Montrouge, France).The minimal detectable concentrations were as follows: 1 pg/mL for IL-12(p70), TNF-α and IL-1β; 2 pg/mL for IL-10, IL-6 and IL-8; 15 pg/mL for IFN-α; 20 pg/mL for TGF-β. Intracellular expression of IFN-α by PDC was determined by flow cytometry using anti-IFN-α monoclonal antibody (clone LT27:295, Miltenyi Biotec). Brefeldin A (10 µg/mL, Sigma) was added 3 h before the end of the

activation with TLR ligands or EMP. Staining was performed according to the manufacturer's recommendations for fixed and permeabilized cells (Cytofix/cytoperm Plus kit, BD Biosciences).

Capacity of dendritic cells to induce naive CD4+ T-cell proliferation and cytokine production

Mixed leukocyte reactions were performed by culturing naive CD4+ T cells as responding cells and allogeneic dendritic cells stimulated with or without TLR ligands or EMP as stimulating cells. CD4+ CD45RA+ naive T cells were isolated from peripheral blood by negative selection using two sorting kits (CD4 Negative Isolation Kit, Dynal, InVitrogen and CD45 microbeads, Miltenyi Biotec) in two steps according to the manufacturers' instruction. CD4+ CD45RA+ T-cell purity evaluated by cytometry was between 90 to 94%. Naive CD4+ T cells were then incubated with allogeneic dendritic cells in RPMI complete medium containing 10% human serum (EFS BFC, Besançon, France) for 6 days. T-cell proliferation was measured using the DELFIA Cell Proliferation Kit (AD0077P-1, Perkin Elmer, Boston, MA, USA) on a DELFIA-type reader (DELFIA Envision, 2102 Multilabel Reader, Perkin Elmer). Five-bromo-2-deoxyuridine (10 μL/mL, Perkin Elmer) was added 18 h before the end of the mixed lymphocyte reaction. Cytokine secretion by T cells after mixed lymphocyte reactions was assayed with a Luminex Kit (HCYTO-60 K, Linco, Millipore). After 7 days, culture supernatants were collected and cytokines (IFN-γ, IL-5, IL-4, IL-2, IL-10, IL-17, TNF-α and TGF-β) of primed T cells were evaluated. CD45RA+ CD4+ naive T cells cultured in complete medium alone were used as controls. The minimal detectable concentrations were the same as described above plus as follows: 1 pg/mL for IL-5, and IL-17; 2 pg/mL for IFN-γ and IL-2; 4 pg/mL for IL-4.

Analysis of endothelial microparticle-plasmacytoid dendritic cell interactions

EMP were added to PDC and incubated at 37°C or 4°C for 15 to 90 min. After incubation, PDC were washed three times to remove non-adherent EMP. Then, transfer of phosphatidylserine or endothelial-specific antigens (CD146 and CD62E) to PDC was analyzed by cytometry. Alternatively, CFSE-labeled EMP were incubated at different EMP:PDC ratios (1:1, 10:1, 100:1) for 4 h and PDC were analyzed by cytometry. Sorted CD4+ T cells were used as a negative control for endocytosis. The role of sodium-proton exchange and an intact PDC cytoskeleton for EMP capture was evaluated by pretreatment of PDC for 1 h with either dimethyl amiloride (100 μM) or cytochaline D (20 μM) (Sigma-Aldrich).[37]

Statistical analysis

Statistical analyses were performed using Sigma Stat 2.0 software (SPSS Inc., Jandel Scientific, Erkrath, Germany). Group comparisons of parametric or non-parametric data were performed using the Student's *t* test or Mann Whitney test, respectively. Data were tested for mean ± S.E.M. A p value less than 0.05 was considered statistically significant.

Results

Endothelial microparticles increase co-stimulatory molecule expression on plasmacytoid dendritic cells, but not on conventional dendritic cells

Interactions between PDC and EMP *in vitro* have never been evaluated to date. Since PDC activation and endothelial damage associated with EMP production are encountered in similar diseases, we decided to explore these interactions, as well as the capacity of EMP to modulate PDC maturation. We first used TNF-α-induced EMP as representative of inflammatory conditions. Expression of co-stimulatory (CD80, CD86, CD40) and activation (HLA-DR, CD83, CCR7) molecules on freshly isolated PDC or a PDC cell line (GEN2.2) incubated with EMP was compared to expression of these molecules on immature PDC or GEN2.2. Incubation of TNF-induced EMP with freshly isolated PDC for 18 h, induced PDC maturation, as evidenced by upregulation of the expression of co-stimulatory molecules, the increase of HLA-DR molecules, as well as of CD83 and CCR7 as compared to levels in unstimulated immature PDC (Figure 1A-C). The effects of EMP on PDC maturation were comparable to those induced by classical maturation agents such as the TLR7 ligand, R848 (Figure 1A-C) and the TLR9 ligand, CpGA (*data not shown*). Similar results were obtained using the GEN2.2 PDC cell line (Figure 1D) or using the endothelial cell line EAY926 as the source of EMP (*data not shown*). As shown in Figure 1E, PDC maturation was still observed for a lower (1:80) PDC:EMP ratio. In contrast, PDC incubated with the supernatant resulting from the last wash of EMP, but not containing EMP, did not induce PDC or GEN2.2 maturation (Figure 1C-D). Moreover, we checked for the absence of residual TNF-α, bacterial endotoxins or mycoplasms which could affect dendritic cells maturation. These factors were not detected in any of the culture conditions (*data not shown*). We also verified that EMP derived from TNF-α-stimulated HMEC-1 did not express the membrane markers HLA-DR, CD83 or CD123 on their surface and that the increase of such markers on PDC did not result from EMP uptake by PDC (*data not shown*). PDC maturation was also confirmed by morphological analysis, since EMP-stimulated PDC acquired dendrites (Figure 1F), a morphological feature of mature dendritic cells.

To determine whether EMP-induced maturation was restricted to PDC or was also observed for conventional DC, we studied the interactions between EMP and Mo-DC using the same design experiment as described for PDC. We did not observe a significant increase of expression of co-stimulatory molecules on the surface of Mo-DC exposed to EMP at a ratio of 1 Mo-DC to 10 EMP (Figure 1G). Only a slight increase of HLA-DR on the surface of Mo-DC was observed in the presence of EMP (Figure 1G). The same results were observed with a higher number of EMP (Mo-DC:EMP ratio 1:80, *data not shown*). The TLR4 ligand, lipopolysaccharide, was used as a control for Mo-DC maturation (Figure 1G). Altogether, these data showed that EMP generated in the presence of TNF-α induced PDC maturation, but not

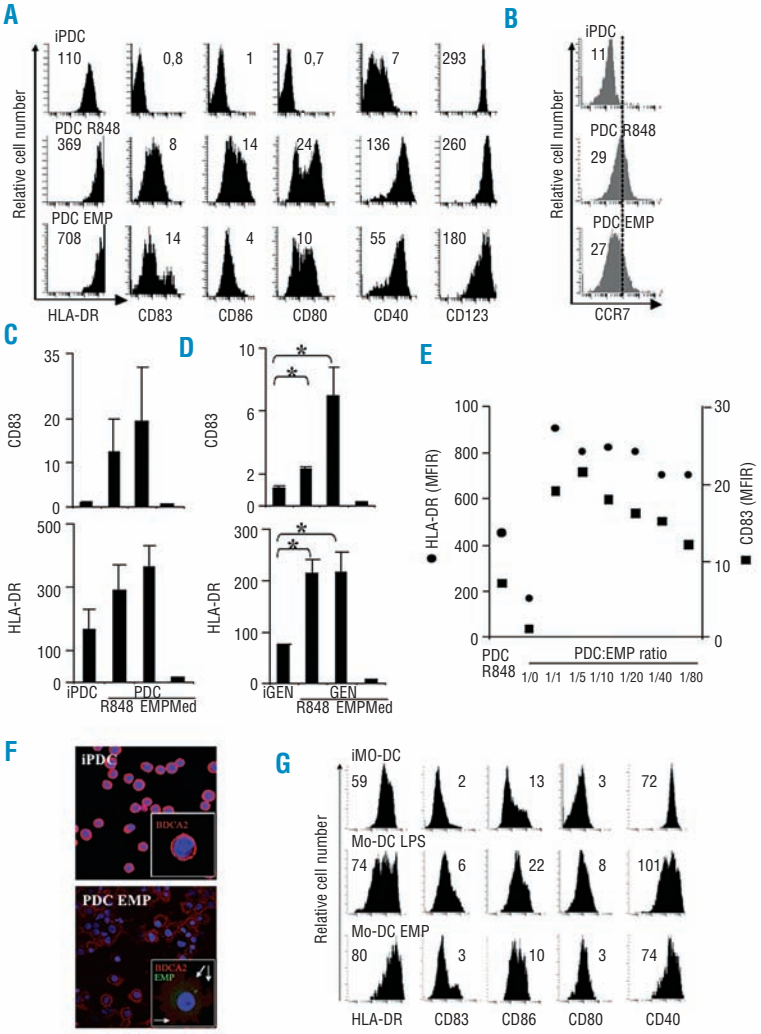

Figure 1. EMP induce morphological and phenotypic maturation of PDC but not of conventional dendritic cells. PDC or the GEN 2.2 PDC cell line (GEN) were incubated with classical maturation agents R848 (PDC R848, GEN R848) or with medium used to generate EMP but not containing EMP (Med) or with TNF-induced EMP (PDC EMP, GEN EMP) at the ratio of 1 dendritic cell to 10 EMP (corresponding to 5000 EMP/μL *in vivo*)[35,36] for 18 h. Co-stimulatory and maturation marker expression was then determined by flow cytometry and compared with the immature unstimulated PDC (iPDC) or GEN2.2 cell (iGEN) phenotype. Expression of the PDC-specific marker CD123 did not vary in any of the conditions tested (A). (A) Histograms from one representative experiment out of eight are shown. (B) Histograms from one representative experiment out of two are shown. On each histogram (A,B), values represent the mean fluorescent intensity (MFI) ratio (MFIR) obtained by dividing the MFI for a given marker by the MFI of the respective isotype control monoclonal antibody. (C,D) Histograms represent the mean±S.E.M of HLA-DR and CD83 expression (expressed as MFIR) from three independent experiments. *$p<0.05$. (E) PDC were incubated for 18 h with TNF-induced EMP at different PDC:EMP ratios: 1 PDC for 0 (corresponding to medium stimulated PDC), 1, 5, 10, 20, 40, and 80 EMP and maturation was determined by cytometry. HLA-DR (●) and CD83 (■) expression (assessed by MFIR) on PDC incubated with EMP was compared with R848-activated PDC (PDC R848). Results from two independent experiments are shown. (F) Morphological analysis of PDC incubated with EMP (PDC EMP) or not (iPDC) confirmed PDC maturation as attested by dendrite acquisition (white arrows) using confocal microscopy, which also enabled the purity of the PDC after immunomagnetic cell sorting to be assessed. PDC are identified here by BDCA-2 staining (red fluorescence) and nuclear staining (blue fluorescence). Before incubation with PDC, EMP were labeled with CSFE (green fluorescence). Results from one experiment out of two are shown. (G) Conventional dendritic cells were activated with the TLR4 ligand lipopolysaccharide (Mo-DC LPS) or incubated with EMP (Mo-DC EMP) at the ratio of 1 Mo-DC to 10 EMP for 48 h. Co-stimulation and maturation marker expression was then determined by flow cytometry and compared with the immature unstimulated Mo-DC (iMo-DC) phenotype. Expression of CD83, CD86, CD80, CD40 and HLA-DR molecules increased only in response to lipopolysaccharide stimulation (Mo-DC LPS), but not after EMP incubation (Mo-DC EMP). Histograms from one representative experiment out of five are shown. Values on each histogram represent MFIR.

Mo-DC maturation.

Endothelial microparticle-induced mature plasmacytoid dendritic cells are functional and secrete inflammatory cytokines

In order to confirm PDC maturation after EMP interactions using a functional assay, we determined cytokines secreted in the supernatants after culture of PDC with EMP and by comparison with PDC cultures in the presence of the TLR7 ligand, R848 or the TLR9 ligand, CpGA. Immature PDC alone did not produce significant levels of IL-1β, IL-6, IL-10 or TNF-α (Figure 2A). As expected, PDC in the presence of R848 or CpGA secreted high levels of IL-6 and IL-8, and low levels of IL-10 (Figure 2A), while TGF-β, IL-1α and TNF-β were not detected (*data not shown*). In the presence of EMP, PDC also secreted IL-6 and IL-8, although at lower levels than in the presence of R848 or of CpGA (Figure 2A). The same experiments were performed using Mo-DC matured in the presence of lipopolysaccharide or incubated with EMP. In the presence of EMP, Mo-DC did not secrete significant levels of IL-6 and exhibited a slight secretion of IL-8 that did not reach statistical significance (Figure 2B). Lipopolysaccharide induced the secretion of IL-12 by Mo-DC (Figure 2B). In contrast, incubation of Mo-DC with EMP did not increase IL-12 secretion (Figure 2B). These functional data further confirm that EMP induced PDC maturation, but not Mo-DC maturation.

PDC are the major cells producing IFN-α, as confirmed *in vitro* after stimulation with the TLR9 ligand, CpGA (Figure 2C). However, PDC did not secrete IFN-α when stimulated by EMP, nor in the presence of R848 (Figure

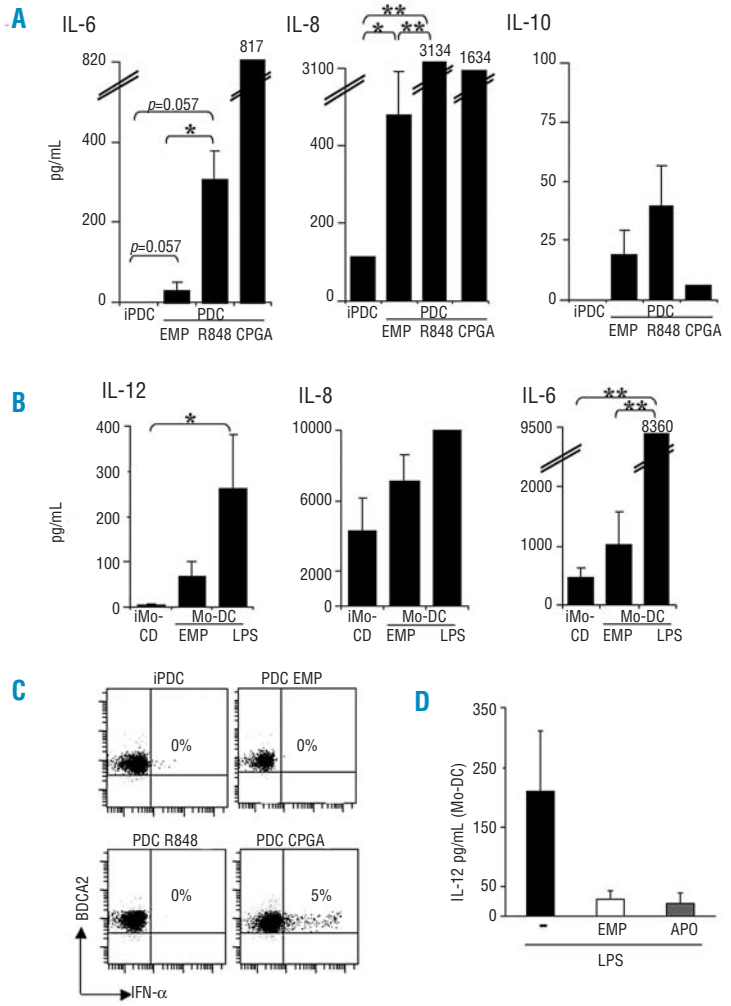

Figure 2. EMP induce cytokine secretion by PDC, but not by conventional dendritic cells. (A) Culture supernatants collected from immature PDC (iPDC), PDC stimulated with the TLR7 ligand R848 (PDC R848), the TLR9 ligand CpGA (PDC CPGA) or with TNF-induced EMP (PDC EMP, ratio of 1 PDC to 10 EMP) for 18 h were assayed for IL-6, IL-8, and IL-10 using Luminex Technology. EMP-activated PDC secreted the same cytokine profile as PDC R848 or PDC CpGA. Data, expressed in pg/mL, represent the mean±S.E.M of four independent experiments; *$p<0.05$; **$p<0.001$. Data with PDC CPGA are from one experiment. (B) Culture supernatants collected from immature Mo-DC (iMo-DC), Mo-DC stimulated with lipopolysaccharide (Mo-DC LPS) or with EMP (Mo-DC EMP, ratio 1:10) for 18 h were assayed for IL-12, IL-6 and IL-8 using Luminex Technology. Data, expressed in pg/mL, represent the mean±S.E.M of five experiments: *$p<0.05$; **$p<0.001$. (C) Percentage of IFN-α^+ cells after stimulation of PDC with R848 (PDC R848), CpGA (PDC CPGA) or with EMP (PDC EMP, ratio 1:10) for 18 h were assayed for intracellular IFN-α measured by flow cytometry. Data were compared with unstimulated immature PDC (iPDC). Dot plot histograms represent expression of IFN-α on BDCA2$^+$ CD123$^+$ cells. Histograms from one representative experiment out of three are shown. Similar data were obtained in cell supernatants (*data not shown*). (D) Culture supernatants collected from immature (iMo-DC), Mo-DC stimulated with TNF-induced EMP (Mo-DC EMP, ratio of 1 Mo-DC to 10 EMP) or with apoptotic cells (APO) (Mo-DC APO, ratio: 1 Mo-DC to 5 APO) for 24 h and then stimulated for 24 h with the TLR4 ligand lipopolysaccharide (LPS), were assayed for IL-12(p70) using ELISA. Data, expressed in pg/mL, represent the mean±S.E.M. of three independent experiments.

2C). In addition, significant amounts of IFN-α were not found in the supernatant of EMP- or R848-stimulated PDC (*data not shown*). The same results were observed whatever the PDC:EMP ratio used (*data not shown*). Overall, these data demonstrate that exposure of PDC to TNF-α-induced EMP increases the secretion of inflammatory cytokines by PDC with one major exception, IFN-α.

EMP express high levels of phosphatidylserine[33] and phosphatidylserine signaling in conventional dendritic cells after interactions with apoptotic cells is known to interfere with dendritic cell maturation.[38] We, therefore, incubated Mo-DC with EMP or apoptotic cells then stimulated with lipopolysaccharide for 24 h. Supernatants were collected and IL-12p70 was measured. Pre-incubation of EMP with Mo-DC reduced lipopolysaccharide-induced IL-12p70 secretion, such as observed with apoptotic cell pre-incubation (Figure 2D). This suggests that Mo-DC respond to an EMP-induced inhibitory signal.

Endothelial microparticle-induced mature plasmacytoid dendritic cells stimulate naive CD4 T-cell proliferation

To further explore the maturation of PDC by EMP, EMP-stimulated PDC were cultured with naive allogeneic CD45RA$^+$ CD4$^+$ T cells. As shown in Figure 3A, naive CD4$^+$ T cells proliferated significantly in the presence of EMP-induced mature PDC whereas no proliferation was observed using unstimulated immature PDC (p=0.025). The rate of proliferation in the presence of EMP-induced mature PDC was similar to that observed in the presence of R848-induced mature PDC (p=0.72) (Figure 3A). Similar results were obtained using the GEN2.2 cell line as stimulating cells (Figure 3B). In contrast, Mo-DC stimulated by EMP did not induce significant naive CD4$^+$ T-cell proliferation in comparison to that induced by unstimulated Mo-DC (Figure 3B). IL-2 secretion at the end of the mixed leukocyte reaction was also assessed as a marker of naive CD4$^+$ T-cell proliferation. While significant amounts of IL-2 were not detected in CD4$^+$ T cells co-cultured with immature PDC (mean + S.E.M, 7±4 pg/mL, n=5), increased levels of IL-2 were found in CD4$^+$ T cells cultured with R848-stimulated PDC (1574±436 pg/mL, n=5, p=0.007 *vs.* immature PDC) or EMP-incubated PDC (590±362 pg/mL, n=5, p=0.008 *vs.* immature PDC).

Endothelial microparticle-induced mature plasmacytoid dendritic cells stimulate naive

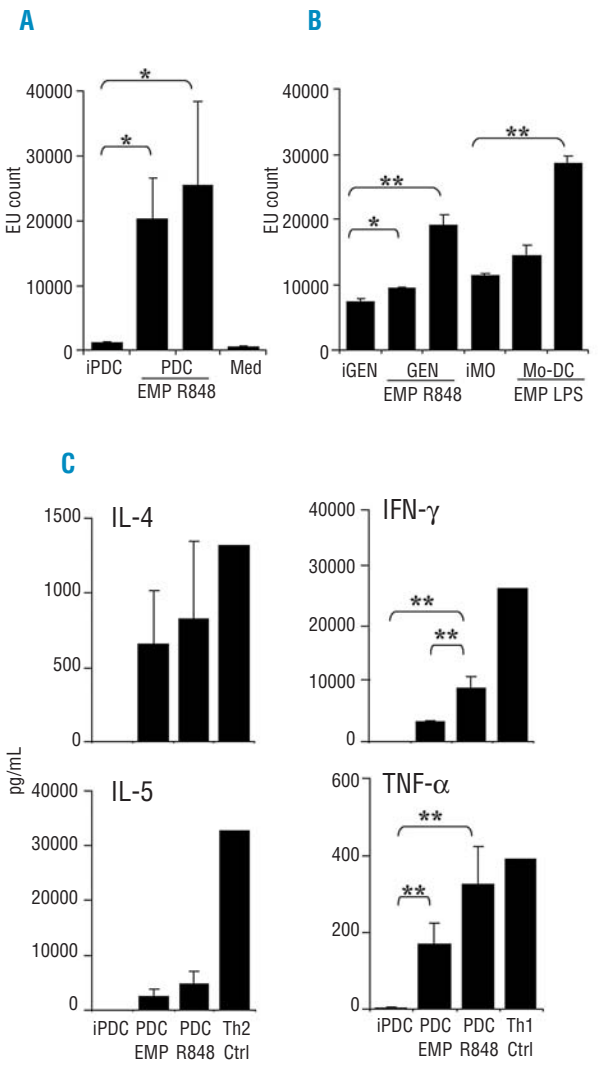

Figure 3. EMP-induced mature PDC induce allogeneic naive CD4 T-cell proliferation and Th1 cytokine secretion. (A) Proliferation of naive CD4+ CD45RA+ T cells in response to unstimulated immature PDC (iPDC), PDC activated by R848 (PDC R848) or to PDC incubated with TNF-induced EMP at a ratio of 1:10 (PDC EMP) was analyzed as described in the Design and Methods section. Co-culture conditions were defined by preliminary experiments and the adequate ratio is 20,000 PDC to 50,000 naive CD4+ T cells. Basal CD4+ T-cell proliferation was also measured (Med). Histograms represent the mean±S.E.M of the four independent experiments. Results are expressed in europium count (EU count). *$p<0.05$. (B) Proliferation of naive CD4+ CD45RA+ T cells in response to unstimulated immature Mo-DC (iMo-DC), Mo-DC activated by lipopolysaccharide (MO-DC LPS), Mo-DC incubated with EMP at a ratio of 1:10 (Mo-DC EMP), or to GEN2.2 PDC cell line (unstimulated, iGEN; activated with R848, GEN R848 or incubated with EMP, GEN EMP) was analyzed as described in the *Design and Methods* section. Data are from one representative experiment out of three for GEN2.2 and of two for Mo-DC. Results are expressed in europium counts (EU count, mean±S.E.M.). *$p<0.05$; **$p<0.001$. (C) Culture supernatants collected from CD4+ T cells co-cultured with immature (iPDC), with R848-activated PDC (PDC R848), or with PDC incubated with TNF-induced EMP (PDC EMP) were assessed for Th2-related cytokines (IL-4 and IL-5, left panels) and for Th1-related cytokines (IFN-γ and TNF-α, right panels) using Luminex Technology. Histograms represent the mean±S.E.M. of five independent experiments. Results are expressed in pg/mL for each of the cytokines analyzed. In order to help data interpretation, additional controls were performed consisting in Th2 control conditions (using PDC stimulated with CD40L and IL-3, as described)[49] and Th1 control conditions (using Mo-DC stimulated with lipopolysaccharide). *$p<0.05$; **$p<0.001$.

CD4 T cells to produce Th1 cytokines

In order to explore CD4+ T-cell polarization after activation by EMP-induced mature PDC, cytokine secretion by co-cultured CD4+ T cells was examined and compared with that of CD4+ T cells incubated with immature PDC and TLR7-stimulated PDC as well as with Th1 or Th2 well-defined conditions (lipopolysaccharide-stimulated Mo-DC and CD40 plus IL-3 stimulated-PDC, respectively). Cytokine production was measured directly in the supernatants after the mixed lymphocyte reactions. As shown in Figure 3C, naive CD4+ T cells stimulated by EMP-induced mature PDC secreted Th2-related cytokines, IL-5 and IL-4, but at non-significant and variable levels according to the experiments considered. However, CD4+ T cells primed by EMP-induced PDC secreted significant levels of Th1-related cytokines, IFN-γ and TNF-α (Figure 3C). Despite the high levels of IL-6 produced by PDC after EMP interactions and the role of IL-6 in Th17 polarization, we did not observe either IL-17-secreting CD4+ T cells after co-culture with EMP-induced mature PDC or TGF-β production in any of the culture conditions (*data not shown*). The cytokine profile observed after EMP stimulation was similar to that of CD4+ T cells stimulated with TLR7-activated PDC (Figure 3C). Naive CD4+ T cells stimulated by immature PDC did not secrete these cytokines (Figure 3C). None of these cytokines was detected in the supernatants from EMP or T cells cultured in medium alone (*data not shown*). Comparison with Th1 or Th2 control conditions (Figure 3C) suggests that EMP-induced mature PDC favor Th1 polarization, as evidenced by IFN-γ and TNF-α secretion.

In contrast to endothelial microparticles, plasmacytoid dendritic cells do not respond to platelet- or T-cell-derived microparticles

In order to appreciate the relevance of EMP-induced PDC maturation, we compared the effects of TNF-α-induced EMP to the effects of quiescent EMP (i.e., EMP produced in the absence of TNF-α), of platelet-derived microparticles (the major form of microparticles found in the bloodstream of healthy subjects) and of normal T-cell-derived microparticles. As shown in Figure 4A, quiescent EMP were as potent as TNF-induced EMP at inducing PDC maturation, as assessed by co-stimulatory molecule expression (Figure 4A) and naive CD4+ T-cell activation (*data not shown*). In contrast, microparticles derived from platelets or activated T cells did not significantly induce PDC maturation (Figure 4A). We also studied different ratios of PDC to platelet-derived microparticles, but whatever the ratio used, the platelet-derived microparticles did not induce PDC maturation (Figure 4B-C). This suggests that the origin of microparticles – and particularly the endothelial origin – influences PDC maturation.

Concerning the effect of TNF on EMP production, we observed that TNF-α stimulation of endothelial cells significantly increased the number of EMP produced (Figure 4D). We, therefore, analyzed whether a dose-effect relationship exists for PDC maturation. As shown in Figure 4E, we observed a dose-effect of EMP on PDC maturation. Altogether, this suggests that PDC respond

to a given number of EMP rather than to *inflammatory* EMP (i.e., TNF-induced EMP) and, therefore *in vivo* PDC may be activated in pathological conditions in which EMP production is increased, such as injury to the vascular endothelium.

Endothelial microparticle-plasmacytoid dendritic cell interactions are temperature-dependent and require sodium-proton exchanges as well as an intact cytoskeleton

To define the mechanism involved in the maturation process of PDC induced by EMP, we first determined whether EMP adhere to PDC and, if so, whether a fusion between EMP and PDC membranes occurs. In these conditions, a transfer of endothelial antigen to PDC would occur. Incubation of EMP with PDC did not lead to endothelial antigen (CD62E or CD146) or phosphatidylserine transfer to PDC (*data not shown*). To further evaluate a role of an uptake of EMP by PDC, EMP were labeled with CFSE before their incubation with PDC. As shown in Figure 5A-B, significant numbers of CFSE$^+$ PDC were identified when cells were incubated with EMP at 37°C whereas incubation at 4°C significantly decreased the percentage of CFSE$^+$ PDC. In contrast, significant amounts of CFSE were not detected in CD4$^+$ T cells whatever the incubation temperature used (Figure 5A). This suggests that EMP uptake by PDC is temperature-dependent. To go further, we treated PDC with an inhibitor of sodium-proton exchange, dimethyl amiloride or the F-actin depolymerizing agent, cytocha-

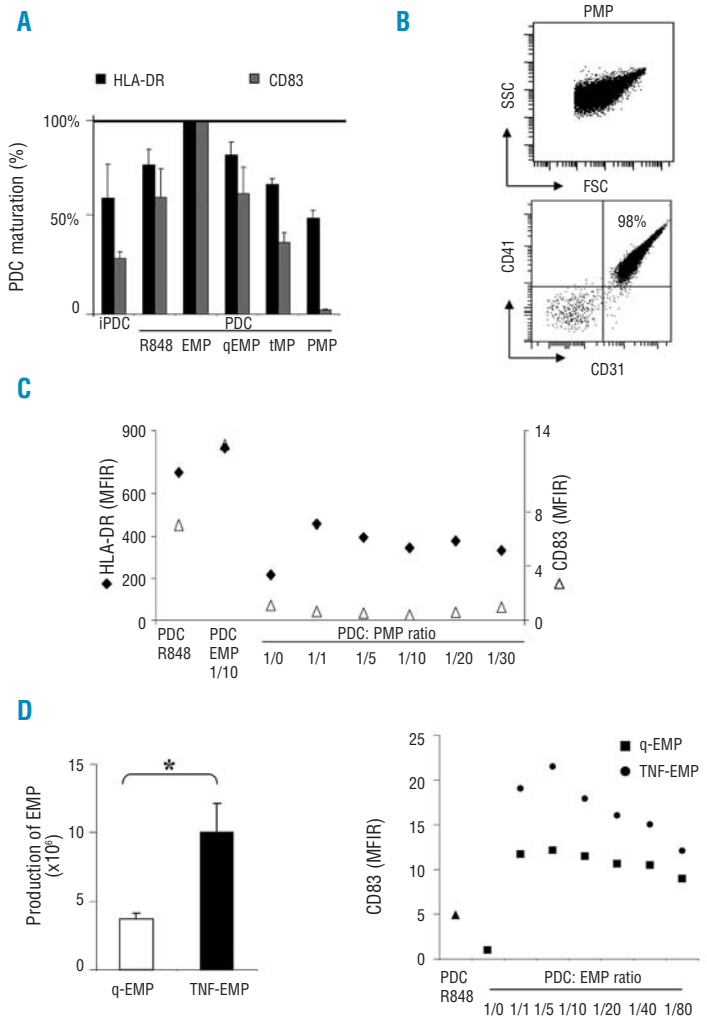

Figure 4. PDC respond to EMP but not to activated T-cell- or platelet-derived microparticles. (A) The effects of TNF-α-stimulated EMP (EMP) on PDC maturation were compared to those of quiescent EMP (qEMP), T-cell-derived microparticles (tMP) or platelet-derived microparticles (PMP). PDC were activated by R848 (PDC R848) or incubated with TNF-α induced EMP (PDC EMP) or quiescent EMP (PDC qEMP) at a ratio of 1 PDC to 10 EMP for 18 h. PDC were also incubated with tMP and PMP at the same ratio and in the same experimental conditions. Maturation marker expression of HLA-DR and CD83 on stimulated PDC – analyzed by cytometry – was then compared with the expression on immature unstimulated PDC (iPDC). Histograms represent the percentage of PDC maturation based on HLA-DR and CD83 expression with expression on PDC EMP arbitrarily considered to indicate 100% maturation. Pooled results of four independent experiments are shown. (B) Platelet-derived microparticle were isolated and analyzed by flow cytometry, identified in the FSC *vs.* SSC gate based on expression of CD41 and CD31. A representative experiment out of four is shown. (C) PDC were incubated for 18 h with PMP at different PDC:PMP ratios: 1 PDC to 0 (corresponding to medium-stimulated PDC), 1, 5, 10, 20, and 30 PMP and maturation was determined by cytometry. HLA-DR (◆) and CD83 (△) expression (assessed by MFIR) on PDC incubated with PMP was compared with expression on R848-activated PDC (PDC R848). Results from one out of two independent experiments are shown. (D) TNF-α exposure increases EMP production by the dermal endothelial cell line HMEC-1. EMP were produced as described in the Design and Methods section. The numbers of EMP produced per culture flask (50 mL) in basal conditions (q-EMP) or after TNF-α (50 ng/mL, TNF-EMP) stimulation were quantified by cytometry. Histograms represent the mean + S.E.M of the absolute number of q-EMP or TNF-EMP in a 50 mL culture flask from six independent productions. *p=0.03. (E) PDC were incubated for 18 h with TNF-induced EMP (TNF-EMP, ●) or quiescent EMP (qEMP, ■) at different PDC:EMP ratios: 1 PDC to 0 (corresponding to medium-stimulated PDC), 1, 5, 10, 20, 40, and 80 EMP and maturation was determined by cytometry. CD83 expression (assessed by MFIR) on PDC incubated with EMP was compared with expression on R848-activated PDC (PDC R848). Results from two independent experiments are shown. Of note, 1 EMP:1 PDC corresponds to 500 EMP/μL; a higher concentration than that in healthy donors (10 + 10 EMP/μL, *personal data*).

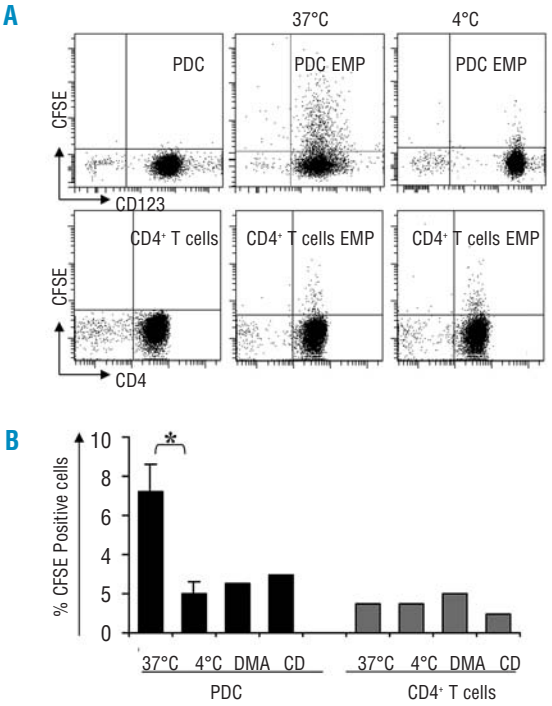

Figure 5. Interaction between EMP and PDC is an active process dependent on temperature, sodium-proton exchanges and on an intact actin cytoskeleton. (A) EMP were labeled with CFSE and incubated with PDC or CD4+ T cells at 37°C or 4°C at the ratio of 1 PDC (or 1 CD4+ T cell) to 100 EMP for 4 h. PDC were identified based on high expression of CD123. CD4+ T cells were identified by CD4 expression. After extensive washes, cells were analyzed by cytometry. Dot plot histograms show the data for one representative experiment out of three. (B) Histograms represent the mean of the percentage of PDC (black) or CD4+ T cells (gray) positive for CFSE after different incubation conditions: 37°C, 4°C, dimethyl amiloride (DMA), or cytochalasin D (CD) treatment. DMA, an inhibitor of sodium-proton exchange, or CD, an F-actin depolymerizing agent, was incubated with PDC or CD4+ T cells for 1 h before addition of EMP. Histograms from three or two independent experiments are shown. Results are expressed as mean ± SEM of the percentage of CFSE+ cells.

lasin D 1 h prior to the addition of EMP. This treatment significantly reduced the number of CFSE+ PDC (Figure 5B) and prevented PDC maturation (assessed by co-stimulatory marker expression, *data not shown*). Taken together, these data indicate that PDC activation by EMP is dependent on temperature and requires sodium-proton exchange and an intact cytoskeleton.

Discussion

Increased circulating EMP related to vascular endothelial dysfunction and PDC activation are encountered in similar pathological situations, including vascular diseases as well as inflammatory diseases.[3,8,14-21,23-28] We, therefore, hypothesized that EMP could trigger PDC maturation and that such an interaction could contribute to the above mentioned inflammatory disorders. Here, we demonstrate that EMP can indeed induce PDC maturation, as shown by co-stimulatory molecule upregulation, inflammatory cytokine secretion, and by allogeneic naive CD4+ T-cell proliferation. Moreover, naive CD4+ T cells primed in the presence of EMP-matured PDC produced mainly type 1 cytokines. Despite the high levels of IL-6 produce by PDC after EMP interactions and the role of IL-6 in Th17 polarization,[39] IL-17-secreting CD4+ T cells were not observed after co-culture with EMP-induced mature PDC. This could be due to the absence of TGF-β – another critical cytokine for Th17 differentiation[39] – in PDC cultures after EMP incubation. In contrast to PDC isolated from healthy volunteers or to the GEN2.2 PDC cell line, EMP did not induce significant Mo-DC maturation and, in this setting, reduced lipopolysaccharide-induced IL-12 secretion, as observed after incubation with apoptotic cells.[9,38] Furthermore, microparticles derived from activated T cells or platelets did not induce PDC maturation. Lastly, PDC maturation induced by EMP required an active uptake mechanism. Overall, these data suggest a link between vascular endothelial damage and PDC activation. Maturation of PDC by EMP released following damage to the endothelium may, therefore, be implicated in the physiopathology of different inflammatory and vascular diseases.

The *in vivo* relevance of our observations remains to be explored further. The PDC:EMP ratio used here (1:10) corresponds to 5000 EMP/μL, an EMP concentration found *in vivo* in pathological situations.[35,36] Infiltration of mature PDC in injured tissues (such as in atherosclerotic plaques,[26-28] in the skin in psoriasis[23,32] or systemic lupus erythematosus)[24,25] has been observed in several chronic inflammatory diseases. Increased expression of the chemokine receptor CCR7[40] – as observed here – has also been reported in trapped dendritic cells found in atherosclerotic plaques.[26] Thus, elevated concentrations of EMP in response to vascular endothelial damage and/or endothelial dysfunction[15,41] may initiate PDC migration and subsequent maturation.

Chronic inflammatory diseases are often associated with uncontrolled IFN-α production by PDC. Such IFN-α secretion is notably found in systemic lupus erythematosus,[42,43] psoriasis[23,32] and in atherosclerotic plaques.[27,28] In our study, EMP did not cause PDC to secrete IFN-α. A first hypothesis to explain this is that PDC activation by EMP may be insufficient to induce IFN-α secretion. Preliminary data show that EMP are able to activate nuclear factor-κB (*data not shown*). Thus, EMP can be considered, similarly to necrotic cells, as a cell byproduct that may alert the innate immune system (i.e., a damage-associated molecular pattern). Necrotic cells have been reported to activate conventional dendritic cell maturation through the TLR2 signaling pathway[44] or CLEC9A.[45] Since nucleic acids can be packed into microparticles,[6] one can speculate that EMP-induced PDC maturation may involve endosomal TLR7 or TLR9. These receptors are specialized in sensing *foreign* nucleic acids. Production of IFN-α is dependent on localization of TLR9-CpGA interactions in endosomal compartments; interactions in the transferrin+ early endosomes lead to IFN-α secretion while interactions in the LAMP1+ late endosomes (i.e., lysosomes) are responsible for IL-6 and IL-8 production as well as increased expression of co-stimulatory molecules.[46,47] This may be an explana-

tion for our observations. In our hands, R848 (a TLR7 ligand) had the same effects as EMP on PDC (namely, increased expression of co-stimulatory molecules, IL-6 and IL-8 secretion, Th1 $CD4^+$ T-cell polarization and no secretion of IFN-α). However, pretreatment of EMP by DNAse I did not prevent PDC maturation (*data not shown*), excluding a role of nucleic acids packed into EMP in PDC maturation. Alternatively, EMP-induced PDC maturation may use a distinct mechanism that does not lead to IFN- α secretion. Some C-type lectin receptors, such as DCIR, on the PDC surface have been shown to inhibit IFN-α secretion despite co-stimulatory molecule upregulation.[48] EMP contain fragments of the endothelial cell plasma membrane and may thus express several potential ligands at their surface that could induce PDC maturation without IFN-α secretion. Nevertheless, the release of IL-8 by EMP-stimulated PDC may lead to neutrophil infiltration and participate in such inflammation. One may also speculate that EMP-induced PDC activation occurs early in chronic inflammatory diseases and that other IFN-α- inducing agents (such as host defense peptide LL-37/DNA complexes in psoriasis)[32] act in parallel to induce IFN-α secretion. The molecular mechanisms used by EMP to induce PDC maturation remain to be identified. However, we observed here that PDC maturation induced by EMP is an active mechanism resembling endocytosis. Indeed, like endocytosis,[37] EMP/PDC interactions are temperature-dependent and inhibited by dimethyl amiloride or cytochalasin D treatment.

Microparticles can be released during several physiological or pathological processes from nearly every type of cell. These microparticles contain membrane, cytoplasmic as well as nuclear components, related to their origin. Circulating microparticles constitute a heterogeneous population, differing in cellular origin, antigenic composition and functional properties.[2] Here we demonstrated that EMP (generated from two different endothelial cell lines) differed from microparticles derived from platelets or activated T cells in their capacity to induce increased expression of co-stimulatory molecules on PDC. Data obtained with platelet-derived microparticles are particularly relevant, since such microparticles represent the major form of microparticles in the blood stream in healthy subjects. Therefore the endothelial origin of microparticles influences PDC activation.

PDC maturation is not the hallmark of TNF-induced EMP, since similar maturation was obtained with EMP derived from unstimulated endothelial cells. This suggests that factors involved in PDC activation are shared by EMP and quiescent EMP and are not induced by TNF-α. A recent proteomic study comparing EMP generated by different agonists identified 432 common proteins in quiescent EMP, plasminogen activator inhibitor type 1-induced EMP and TNF-induced EMP.[5] Variations in protein abundance in these different EMP were found.[5] Our data are rather in favor of an increase of EMP in inflammatory conditions (as observed here after TNF-α stimulation of endothelial cells) being sufficient to induce PDC maturation. Indeed, increased concentrations of circulating EMP in comparison with levels in healthy controls have been observed in many inflammatory and vascular diseases,[35,36] suggesting a dose-effect relationship for *in vitro* PDC maturation.

In summary, we show here for the first time that EMP are able to specifically activate PDC. EMP, resulting from increased membrane vesiculation of endothelial cells, could represent a new activating factor for PDC and thus contribute to inflammation. Control of EMP production in inflammatory disorders may be beneficial in order to avoid excessive and inappropriate PDC activation. EMP may be an important immunmodulatory therapeutic target.

Authorship and Disclosures

FA performed experiments, analyzed data, prepared the figures and tables and drafted the manuscript; SB performed experiments; YB and FDG provided expertise on endothelial microparticles, the HMEC-1 cell line and reviewed the manuscript; BG analyzed data and reviewed the manuscript; JP and LC provided the plasmacytoid dendritic cell line GEN2.2 that enabled the initiation of this work; PT reviewed the manuscript; PS supervised the study and wrote the manuscript, FGO and ES designed and supervised the study and contributed to writing the manuscript.

The authors reported no potential conflicts of interest.

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