

# Ectosomes from neutrophil-like cells down-regulate nickel-induced dendritic cell maturation and promote Th2 polarization

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## ABSTRACT

DCs are the first immune cells to be exposed to allergens, including chemical sensitizers, such as nickel, a human TLR4 agonist that induces DC maturation. In ACD, DCs can interact with PMNs that are recruited and activated, leading, in particular, to ectosome release. The objective of this work was to characterize the effects of PMN-Ect on DC functions in an ACD context. We first developed a standardized protocol to produce, characterize, and quantify ectosomes by use of human PLB-985 cells, differentiated into mature PMN (PLB-Ect). We then studied the *in vitro* effects of these purified ectosomes on human moDC functions in response to NiSO<sub>4</sub> and to LPS, another TLR4 agonist. Confocal fluorescence microscopy showed that PLB-Ect was internalized by moDCs and localized in the lysosomal compartment. We then showed that PLB-Ect down-regulated NiSO<sub>4</sub>-induced moDC maturation, as witnessed by decreased expression of CD40, CD80, CD83, CD86, PDL-1, and HLA-DR and by decreased levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-12p40 mRNAs. These effects were related to p38MAPK and NF- $\kappa$ B down-regulation. However, no increase in pan-regulatory DC marker genes (GILZ, CATC, C1QA) was observed; rather, levels of effector DC markers (Mx1, NMES1) were increased. Finally, when these PLB-Ect + NiSO<sub>4</sub>-treated moDCs were cocultured with CD4<sup>+</sup> T cells, a Th2 cytokine profile seemed to be induced, as shown, in particular, by enhanced IL-13 production. Together, these results suggest that the PMN-Ect can modulate DC maturation in response to nickel, a common chemical

sensitizer responsible for ACD. *J. Leukoc. Biol.* 97: 000-000; 2015.

## Introduction

ACD is one of the most prevalent occupational cutaneous disorders, occurring after repeated contact with low molecular-weight chemicals, such as nickel. The sensitizing phase can take weeks to months of exposure before the onset of dermatitis (elicitation phase) [1, 2]. Nickel is a common metal sensitizer that can be sensed by DCs. Recently, the TLR4 was shown to be the key receptor for nickel-mediated inflammation in humans, independently of its coreceptor myeloid differentiation factor 2 [3, 4]. Indeed, nickel directly induces TLR4 dimerization, an event that requires the presence of 2 nonconserved histidine residues (H456 and H458). During this sensitization phase, previous *in vitro* studies from our group suggest that NF- $\kappa$ B, IRF1, and p38MAPK are activated in DCs, leading to phenotypic modifications, cytokine production, migration to draining lymph nodes, and finally, allergen presentation to naïve T lymphocytes [5, 6]. This leads to the generation of skin-homing CD8<sup>+</sup> Tc1/Tc17 and CD4<sup>+</sup> Th1/Th17 effector cells that enter the blood circulation. Upon re-exposure, the contact allergen triggers a cascade of events, resulting in the recruitment of monocytes, T cells, NK cells, and PMNs, which can thus interact with skin DCs [7–10].

PMNs have a major role in innate immunity, through functions, such as migration, phagocytosis, degranulation of preformed mediators, oxidative burst activity, cytokine production, and NET or ectosome release [11–13]. However, PMN can also regulate adaptive immunity, notably through interactions with APCs, such as DCs [14]. Indeed, PMN-derived elastase,

Abbreviations: ACD = allergic contact dermatitis, AMV = avian myeloblastosis virus, C1QA = complement component 1A, CATC = human dipeptidyl peptidase 1, CHOL = cholesterol, cMFI = corrected mean fluorescence intensity, Ct = comparative threshold, DC = dendritic cell, DEX = dexamethasone, DLS = dynamic light-scattering, DOPC = 1,2-dioleoyl-sn-glycero-3-phosphocholine, FSC = forward-scatter, GILZ = glucocorticoid-induced

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lactoferrin, TNF- $\alpha$ , and IL-8 can modulate DC activation, and PMN can also bind directly to DCs via  $\beta 2$  integrin [14–17]. Recently, our group reported that NETs are able to down-regulate DC activation [18]. Finally, it was shown that PMN-Ect down-regulate LPS-induced maturation of DCs and their capacity to induce T cell proliferation [19]. Thus, during nickel-related ACD, PMN-Ect might interact directly with skin DCs and modulate their function.

Long considered to be cellular debris or confused with exosomes and apoptotic bodies, ectosomes (also called microparticles) are now clearly identified as regulatory vectors of intercellular cross-talk [20–23]. They are released by budding from the cell membrane, spontaneously or in response to various stimuli, and are characterized by their size (diameter 0.1–1  $\mu$ m) and composition, pointing to their cellular origin [24–26]. Ectosomes contain a wide array of surface, cytoplasmic, and nuclear molecules that are incorporated into a membrane-bound structure, as particles leave the cell and are shed into extracellular space. They mediate cell–cell communication by transferring a cargo of components from the cell of origin to the target cell. Ectosomes may fuse with the target-cell plasma membrane or be engulfed. Activation of ectosome-producing cells is characterized by specific membrane changes, such as PS externalization, but also by cytoskeleton disruption and changes in the local concentrations of specific intracellular molecules [27]. Circulating ectosomes can originate from RBCs, leukocytes, platelets, and endothelial cells, among others. Their levels increase during various diseases, including cancer, heat stroke, cardiovascular disorders, and rheumatic diseases, suggesting that they may serve as biomarkers and disease mediators [25, 28–32]. Indeed, growing evidence suggests that ectosomes induce inflammation, stimulate coagulation, affect vascular functions, play a role in cell proliferation and differentiation, and even restrict bacterial growth and dissemination [33–36]. Interestingly, whereas ectosomes were initially considered to be a major source of proinflammatory effectors, it is now suggested that they might have anti-inflammatory and immunosuppressive properties, potentially helping to resolve local inflammation [26, 37–39]. Indeed, besides their down-regulating effect on some DC functions [19], PMN-Ect can also block endothelial cell activation via PS or Annexin-A1 binding [40] and also macrophage activation via several mechanisms (TGF- $\beta$  induction, Ca<sup>++</sup> influx, NF- $\kappa$ B inhibition, and MER proto-oncogene tyrosine kinase and PI3K/AKT activation) [41–43].

These newly described regulatory roles of PMN-Ect during inflammation prompted us to hypothesize an effect of ectosomes on DCs that could play a role in ACD. We addressed this question

by use of moDCs and NiSO<sub>4</sub>, one of the most prevalent contact sensitizers. We first developed a standardized model of ectosome production by PLB-985 cells differentiated into mature, neutrophil-like cells (PLB-Ect). These PLB-Ect were able to modulate NiSO<sub>4</sub>-induced moDC maturation, as witnessed by phenotypic changes and modulation of cytokine production, in a mechanism involving MAPK and NF- $\kappa$ B regulation. moDCs were also reprogrammed toward an effector phenotype that enhanced Th2 responses.

## MATERIALS AND METHODS

### PLB-985 culture and differentiation

The human myeloid leukemia cell line PLB-985 (ACC 139; DSMZ GmbH, Braunschweig, Germany) was cultured in RPMI-1640 medium containing 25 mM HEPES GlutaMAX (Gibco, Invitrogen, Saint Aubin, France) and supplemented with 10% FCS (Biowest, AbCys, Courtaboeuf, France), antibiotics [100 units/ml penicillin, 100  $\mu$ g/ml streptomycin (Gibco, Invitrogen)], and 1% pyruvate (Gibco, Invitrogen) at 37°C in humidified air containing 5% CO<sub>2</sub>. To induce PLB-985 cell granulocytic differentiation, exponentially growing cells at a starting density of  $2 \times 10^5$ /ml were cultured in RPMI medium, supplemented with 1% pyruvate, 0.5% dimethyl formamide (Carlo Erba, Rodano, Italy), and 5% FCS. On day 6, granulocytic differentiation was verified, as described previously [44].

### Isolation of human blood PMNs

PMNs were separated from fresh buffy coats prepared from healthy donor blood obtained from Etablissement Français du Sang (Rungis, France). After density centrifugation on a Ficoll gradient (Eurobio, Les Ulis, France), PMNs were separated from erythrocytes by sedimentation on a separating medium containing 5% Dextran T500 (Pharmacia, Uppsala, Sweden) in 0.9% NaCl. Contaminating erythrocytes were then removed by hypotonic lysis, and PMNs were resuspended in RPMI 1640, 25 mM HEPES GlutaMAX.

### Production and characterization of ectosomes (PLB-Ect and PMN-Ect)

Differentiated PLB-985 cells or PMNs ( $10^7$  cells/ml) were incubated for 30 min at 37°C in RPMI 1640, 25 mM HEPES GlutaMAX with 10 nM PMA (Sigma-Aldrich, St Quentin Fallavier, France). Intact cells were removed by centrifugation (15 min, 4000 g at 4°C), and the ectosome-rich supernatants were concentrated with Centriprep centrifugal filter devices (10 kDa MW cutoff; Millipore, Molsheim, France). Ectosomes were then pelleted by ultracentrifugation (40 min, 160,000 g at 4°C), washed in 0.9% NaCl, and stored in aliquots at –80°C until use. Endotoxin was quantified in the preparations by use of a colorimetric assay (ToxinSensor; Genscript, Paris, France), according to the manufacturer's instructions.

An amphiphilic cell linker dye kit (PKH67; Sigma-Aldrich) was used for membrane labeling of PLB-Ect and PMN-Ect. Ectosome preparation (10  $\mu$ l) was diluted in 400  $\mu$ l diluent C/dye solution and incubated with gentle shaking for 30 s at room temperature. Labeled ectosomes were separated from the remaining unbound dye by ultracentrifugation (40 min, 160,000 g at 4°C) and washed with 0.9% NaCl. Additional membrane labeling of ectosomes was then performed with Annexin V-PE (BD Biosciences, Le pont de Claix, France), which binds PS. Labeled PLB-Ect and PMN-Ect were observed with an LSR Fortessa flow cytometer (BD Biosciences), equipped with a FSC-PMT to increase sensitivity in size detection. Data were acquired with DIVA software and analyzed with FlowJo software. PLB-Ect and PMN-Ect were identified by their size, as assessed by the use of calibrated beads (0.5, 0.9, and 3  $\mu$ m diameter; Megamix; Biocytex, Marseille, France), amplification of their FSC and SSC signals, and PKH67 and PS staining. The absolute concentration of PLB-Ect and PMN-Ect/ $\mu$ l ectosome preparation was quantified by use of tubes preloaded with fluorescent TrueCount beads (BD Biosciences).

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leucine zipper, IRF1 = IFN regulatory factor 1, MFI = mean fluorescence intensity, moDC = monocyte-derived dendritic cell, Mx1 = myxovirus resistance 1, NET = neutrophil extracellular trap, NIMES1 = normal mucosa of esophagus-specific gene 1, NP-40 = Nonidet P-40, PDL-1 = programmed death-ligand 1, PLB-985 = human acute myeloid leukemia cell line, PLB-Ect = PLB-985-derived ectosomes, PMN = polymorphonuclear neutrophil, PMN-Ect = polymorphonuclear neutrophil-derived ectosomes, PMT = photomultiplier tube, PS = phosphatidylserine, PVDF = polyvinylidene difluoride, RFI = relative fluorescence intensity, SSC = side-scatter, TBP = TATA box binding protein

Finally, the size distribution of the ectosomes thus obtained was analyzed with the DLS method on a Zetasizer Nano analyzer (He-Ne laser 633 nm; Malvern Instruments, Malvern, United Kingdom). Measurements were carried out at 25°C, and intensity correlation functions were measured at a scattering angle of 173°. Ectosome size was obtained from the Stokes-Einstein relation. Before measurements, ectosome preparations were diluted 2-fold with 0.9% NaCl.

## Liposome preparation

Liposomes were prepared from DOPC (Sigma-Aldrich), CHOL (Sigma-Aldrich), and L- $\alpha$ -PS (Brain PS; Avanti Polar Lipid, Alabaster, AL, USA). The liposomal formulation DOPC/CHOL/PS (50:20:30 mol%) was prepared by the lipid hydration method, followed by extrusion [45]. The molar percentages of lipid components were chosen to resemble the cytoplasmic membrane composition [46]. In briefly, a lipid mixture containing 0.2 mmol total lipids was dissolved in 5 ml chloroform in a 100-ml round-bottomed flask. The solvent was evaporated to dryness under reduced pressure for 40 min at 45°C to form a thin lipid film. Multilamellar liposomes were formed by hydration of the lipid film at 36°C, under nitrogen, with 2.5 ml HEPES Buffer, pH 7.4 (10 mM HEPES, 145 mM NaCl; Sigma-Aldrich), filtered on a 0.22  $\mu$ m PVDF syringe filter (Millex-GV; Millipore), to yield a final lipid concentration of 80 mM. The resulting mixture was stirred vigorously, followed by extrusion 10 times. Extrusion was performed at 36°C, above the transition temperature of the lipid mixture [47], under nitrogen pressure, at 10 pounds per square inch, through a stack of 2, 400 nm polycarbonate filters (Isopore; Millipore). Liposomes were collected in sterilized flasks, flushed with nitrogen, and stored at 4°C.

The mean hydrodynamic diameter of the freshly prepared liposomes was determined by the DLS technique. Before measurements, liposomes were diluted in HBSS. They were quantified by flow cytometry after staining by use of the same protocol as described for ectosome preparations.

## Generation of human moDCs

PBMCs were purified from buffy coats obtained from Etablissement Français du Sang by centrifugation on a Ficoll density gradient. As described previously [5], monocytes were isolated from the mononuclear fraction by magnetic-positive selection with MiniMACS separation columns and anti-CD14 antibodies coated on magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocytes were cultured at  $1 \times 10^6$  cells/ml in the presence of GM-CSF (550 U/ml) and IL-4 (550 U/ml; Miltenyi Biotec) in RPMI 1640, 25 mM HEPES GlutaMAX, supplemented with 10% FCS, antibiotics, and 1% pyruvate at 37°C in humidified air containing 5% CO<sub>2</sub>. Within 5 days, monocytes differentiated into moDCs with an immature phenotype.

## moDC maturation and pretreatment with ectosomes or liposomes

On day 5, immature moDCs were harvested and washed in RPMI 1640, 25 mM HEPES GlutaMAX, supplemented with 10% FCS, antibiotics, and 1% pyruvate. PLB-Ect were then added at a proportion of 10 PLB-Ect for 1 moDC. Preliminary experiments showed that a ratio of 20:1 gave similar results (data not shown). In some experiments, instead of ectosomes, liposomes were added to cells at the same ratio. After 30 min of preincubation at 37°C, moDCs were treated with the chemical hapten NiSO<sub>4</sub> (500  $\mu$ M; Sigma-Aldrich), LPS (from *Escherichia coli* 055:B5 strain 20 ng/ml; Sigma-Aldrich) used as a positive control, or DEX (1  $\mu$ g/ml; Sigma-Aldrich) used as a tolerogenic DC inducer. The stimulation time at 37°C ranged from 30 min to 18 h, depending on the experiment.

## Confocal microscopic analysis of ectosome endocytosis by moDCs

PLB-Ect were stained with the amphiphilic cell linker dye PKH67, as described above, and resuspended in RPMI 1640. Ten labeled PLB-Ect per moDC were then incubated for 4 h at 37°C in humidified air containing 5% CO<sub>2</sub>. After

centrifugation, cells were incubated for 15 min at room temperature with a lysosomal compartment dye (50 nM; LysoTracker Red DND-99; Invitrogen, Life Technologies, Grand Island, NY, USA). Cell preparations were then analyzed by confocal microscopy (Laser Scanning Microscope 510 META; Zeiss, Le Pecq, France).

## Flow cytometric analysis of moDC maturation

After PLB-Ect pretreatment and 18 h of exposure to maturation agents (NiSO<sub>4</sub> or LPS), the moDC phenotype was analyzed by flow cytometry. The labeling procedure was as follows:  $2 \times 10^5$  cells/ml were washed in cold PBS, supplemented with 0.5% BSA, and stained with mAb in the dark on ice for 20 min. After washing with cold PBS, cells were fixed in 1% paraformaldehyde, diluted in PBS. The following mAb were used: PE-labeled mouse anti-human CD86 [2331 (FUN-1); BD Biosciences], PE-labeled mouse anti-human CD40 (MAB89; Beckman Coulter, Marseille, France), allophycocyanin-conjugated mouse anti-human CD83 (HB15e; BD Biosciences), FITC-labeled mouse anti-human CD80 (L307.4; Beckman Coulter), FITC-labeled mouse anti-human HLA-DR mAb (Immu-357; Beckman Coulter), and FITC-labeled mouse anti-human PDL-1 (CD274; MIH1; BD Biosciences). Appropriate isotype controls (mouse IgG1k; BD Biosciences) were used at the same concentration to determine nonspecific staining. A minimum of 10,000 cells was analyzed on a FACSCalibur cell analyzer by use of CellQuest software (Becton Dickinson). Living cells were identified on the basis of their physical characteristics (FSC and SSC). Results were expressed as the RFI by use of the cMFI, as follows: cMFI = MFI of cells/MFI of isotype control; RFI = cMFI of treated cells/cMFI of untreated cells.

## Cytokine quantification in moDC supernatants

TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, IL-10, and IL-12p70 were quantified in supernatants, collected 18 h after moDC activation by NiSO<sub>4</sub> or LPS, in the presence or absence of PLB-Ect by use of the BD Cytometric Bead Array system and a FACSCalibur cell analyzer (BD Biosciences). Data were analyzed with Flow Cytometry Data Analysis and Processing (FCAP) Array software.

## Quantitative real-time RT-PCR assay of cytokines in moDCs

Total RNA was extracted after lysis of 4 h-treated moDCs [ $10^6$  cells/ml, pretreated or not with PLB-Ect or liposomes in TRIzol reagent (Invitrogen, Life Technologies)]. Total RNA pellets were resuspended in RNase-free water and quantified by spectrophotometry.

First-strand cDNA was synthesized from total RNA by use of a thermocycler (Biomtra, Goettingen, Germany). The reaction used 1  $\mu$ g total RNA, a deoxynucleotide triphosphate mixture (containing 25 mM deoxy-adenosine triphosphate, deoxy-guanosine triphosphate, deoxy-cytidine triphosphate, and deoxy-thymidine triphosphate), and 50  $\mu$ M oligo (dT) primers (MWG Biotech, Ebersberg, Germany). Reverse transcription was carried out in 1 $\times$  AMV RT reaction buffer (Promega, Charbonnières-les-Bains, France) with RNase inhibitor (RNasine; Promega) at 40 U/ $\mu$ l, AMV RT (Promega) at 10 U/ $\mu$ l, and RNase-free water to a final volume of 10  $\mu$ l. A control without RT was used to confirm the absence of DNA contamination.

Real-time PCR was performed by use of the SYBR Green technology on a CFX96 system (Bio-Rad Laboratories, Marnes la Coquette, France). Each reaction mix consisted of 1:50 diluted cDNA in 4  $\mu$ l final of nuclease-free water; 0.5  $\mu$ M each forward and reverse primer for *IL-1 $\beta$* , *IL-6*, *IL-8*, *IL-10*, *IL-12p40*, *TNF- $\alpha$* , *TGF- $\beta$* , *GAPDH*, *TBP*, and 18S rRNA; and SsoAdvanced Supermix (Bio-Rad Laboratories) in a total reaction volume of 10  $\mu$ l. The following specific primers were used (forward and reverse, respectively): *IL-1 $\beta$* : 5'-ACA GAC CTT CCA GGA GAA TG-3' and 5'-GCA GTT CAG TGA TCG TAC AG-3'; *IL-6*: 5'-TCA ATG AGG AGA CTT GCC TG-3' and 5'-GAT GAG TTG TCA TGT CCT GC-3'; *IL-8*: 5'-TCT CTT GGC AGC CTT CCA TGA-3' and 5'-TGG GGT GGA AAG GTT TGG AG-3'; *IL-10*: 5'-GTG ATG CCC CAA GCT GAG A-3' and 5'-CAC GGC CTT GCT CTT GTT TT-3'; *IL-12p40*: 5'-TGG AGT GCG AGG AGG ACA GT-3' and 5'-TCT TGT GTG GGT CAG GTT TG-3'; *TNF- $\alpha$* : 5'-TCT TCT CGA ACC CCG AGT GA-3' and 5'-CCT CTG ATG

GCA CCA CCA G-3'; *TGF $\beta$* : 5'-CAG CAA CAA TTC CTG CGC ATA-3' and 5'-AAG GCG AAA GCC CTC AAT TT-3'; *GAPDH*: 5'-CAG CCT CAA GAT CAT CAG CA-3' and 5'-TGT GGT CAT GAG TCC TTC CA-3'; *TBP*: 5'-GAG AGC CAC GAA CCA CGG-3' and 5'-ACA TCA CAG CTC CCC ACC AT-3'; and *18S rRNA*: 5'-TAG GAA ACG GCT ACC ACA TC-3' and 5'-TAA CAG GGC CTC GAA AGA GT-3'. After 30 s at 95°C for Sso7d-fusion polymerase activation, amplification was allowed to proceed for 44 cycles, each consisting of denaturation at 95°C for 5 s and annealing/extension at 62°C for 5 s. Sevenfold serial dilutions of mixed cDNA (from different samples) were analyzed for each target gene, allowing us to construct linear standard curves from which the efficiency of each PCR run was evaluated. SYBR Green fluorescence was detected at the end of each elongation cycle, after which, a melting curve was constructed to confirm the specificity of the PCR products. Quantification was performed with Bio-Rad Laboratories CFX Manager software, and data were analyzed with the  $\Delta\Delta C_t$  method. Ratios were calculated as the geometrical mean of  $(1 + E)^{-\Delta\Delta C_t}$  values, where E is the efficiency, and  $\Delta\Delta C_t$  is the target gene expression of treated cells compared with normal levels in untreated cells, with correction for the expression of the reference genes *TBP*, *GAPDH*, and *18S rRNA*. Results were expressed as the fold-factor induction [i.e., ratio of  $(1 + E)^{-\Delta\Delta C_t}$  of treated cells /  $(1 + E)^{-\Delta\Delta C_t}$  of untreated cells].

### Quantification of effector and tolerogenic moDC gene expression

Total RNA was extracted from 24 h-treated moDCs by use of RNeasy mini kits and the QIAcube robot (Qiagen, Courtaboeuf, France); cDNAs were obtained by use of TaqMan RT reagents (Applied Biosystems, Carlsbad, CA, USA). mRNA expression was evaluated by quantitative PCR on a 7900HT real-time PCR system (Applied Biosystems) with predesigned TaqMan gene expression assays and reagents, according to the manufacturer's instructions. The expression of the following genes was assessed in moDCs, as described previously [48]: *GILZ* (Hs00608272\_m1), *C1QA* (Hs00381122\_m1), *CATC* (Hs00175188\_m1), *Mx1* (Hs00895608\_m1), and *NMES1* (Hs00260902\_m1).

Data were interpreted for each target gene by comparison with endogenous  $\beta$ -actin (Hs99999903\_m1) as a control. The relative amount of each target gene in each sample was calculated by comparison with the calibrator sample (untreated sample) by use of the  $\Delta\Delta C_t$  method. The magnitude of gene induction was calculated with the formula  $2^{-\Delta\Delta C_t} = 2^{-(\Delta C_t \text{ for stimulated cells} - \Delta C_t \text{ for unstimulated cells})}$ .

### Immunoblot analysis of signaling pathways in moDCs

After PLB-Ect pretreatment and 30 min incubation with  $\text{NiSO}_4$ , moDCs ( $10^6$  cells/ml) were harvested and washed in cold PBS before lysis in 40  $\mu$ l lysis buffer [20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA (pH 7.4), 2 mM sodium pyrophosphate, 1% Triton, 10% glycerol, 1 mM PMSF, 1 mM  $\text{Na}_3\text{VO}_4$ , 25 mM  $\beta$ -glycerophosphate, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, and 100  $\mu$ g/ml pepstatin]. After centrifugation at 15,000 g for 20 min at 4°C, 50  $\mu$ g denatured protein was loaded onto 12.5% SDS-PAGE gels and transferred to PVDF membranes, which were successively incubated with antibodies directed against the phosphorylated forms of p38MAPK (Thr180/Tyr182) and the NF- $\kappa$ B p65 subunit (Ser536) and an antibody against total p38MAPK as a loading control [all antibodies were from Cell Signaling Technology (Ozyme, St-Quentin en Yvelines, France)]. Immunoreactive bands were detected by their chemiluminescence by use of the ChemiDoc XRS+ System (Bio-Rad Laboratories). Bands were quantified with Image Lab software.

In some experiments, NF- $\kappa$ B activation was also evaluated by a pull-down assay, as described previously [49]. In brief, after PLB-Ect pretreatment and 30 min incubation with  $\text{NiSO}_4$ , moDCs ( $10^6$  cells/ml) were lysed in NP-40 lysis hypertonic buffer (0.2% NP-40, 20% glycerol, 20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 0.125  $\mu$ M okadaic acid, 62.5 mM EDTA, 40  $\mu$ M EGTA, 0.5 mM PMSF, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml aprotinin, and 1  $\mu$ g/ml pepstatin) and incubated with gentle agitation at 4°C for 30 min. Cell debris was removed by centrifugation at 4°C at 15,000 rpm for 20 min. The following 5'-biotin-labeled oligonucleotides (MWG Biotech) were hybridized: 5'-TTG AGG GGA

CTT TCC CAG G-3' and 5'-CCT GGG AAA GTC CCC TCA A-3', according to the human NF- $\kappa$ B promoter sequence. Mutated oligonucleotides were used as control for nonspecific binding (5'-TTG AGG CGA CTT TCC CAG G-3' and 5'-CCT GGG AAA GTC GCC TCA A-3'). Whole-cell extracts (150  $\mu$ g) were incubated at 4°C for 90 min with 2  $\mu$ g double-stranded 5'-biotinylated oligonucleotide coupled to 30  $\mu$ l streptavidin-agarose beads (Sigma-Aldrich). Complexes were washed in binding buffer and eluted by boiling in reducing sample buffer before protein separation by SDS-PAGE gel, followed by Western blot analysis of the NF- $\kappa$ B p65 subunit, as described above.

### Fluorescence analysis of NF- $\kappa$ B p65 subunit nuclear translocation in moDCs

moDCs ( $3 \times 10^6$  cells), treated with PLB-Ect, were stimulated with  $\text{NiSO}_4$  or LPS for 45 min at 37°C, then permeabilized with 0.1% Triton X-100, and then incubated with an anti-NF- $\kappa$ B p65 antibody (sc-109; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and then with a goat anti-rabbit antibody (Alexa 546). The cells were observed by fluorescence microscopy (Aristoplan; Zeiss).

### moDC/T cell coculture

To assess T cell polarization, PLB-Ect-pretreated moDCs were cocultured with allogeneic, naive  $\text{CD4}^+$  T cells at a 1:10 DC/T cell ratio for 5 days, as described previously [48]. In brief, naive  $\text{CD4}^+$  T cells were isolated from PBMC by negative selection with the MACS Naive  $\text{CD4}^+$  T cell Isolation Kit II (Miltenyi Biotec). These naive T cells were confirmed to have purity >95%, based on CD4 and CD45RA expression evaluated by flow cytometry. Supernatants were analyzed for their cytokine content: IFN- $\gamma$ , IL-13, IL-5, IL-4, and IL-9 production was quantified by use of a human cytokine bead kit (quantification range: 3.2–10,000 pg/ml; Merck Millipore, Darmstadt, Germany) and the Magpix system (Luminex, Austin, TX, USA). The results were analyzed according to the manufacturers' instructions.

### Statistical analysis

Nonparametric analysis (Wilcoxon test for paired data) was performed by use of GraphPad Prism software. Data are expressed as means  $\pm$  SEM.  $P < 0.05$  was considered to denote statistical significance.

## RESULTS

### Characterization of ectosome (PLB-Ect and PMN-Ect) and liposome preparations

Ectosomes were prepared from differentiated PLB-985 cultures (PLB-Ect) and from fresh human blood neutrophils (PMN-Ect). They were first identified by their size, as assessed by FSC and SSC signals (Fig. 1A). The FSC-SSC gate lower limit was set just above the background noise of the instrument. Then, we used Megamix submicrometer beads to adjust the upper side of the gate below the 0.9  $\mu$ m bead position; thus, the gate was set to detect particles below 900 nm. Finally, ectosomes were detected and discriminated from other particles though PKH67 dye and PS labeling with Annexin V-PE. Figure 1B shows that PLB-Ect were homogeneously double stained, although a small fraction of the population did not express PS. PMN-Ect were more heterogeneous, with far more unstained events and more ectosomes that did not express PS (Fig. 1C).

The size distribution of PLB-Ect was also analyzed by the DLS method, the results expressed as the intensity of scattered light, according to the particle diameter. This representation showed the presence of a moderate polydisperse ectosome population (polydispersity index = 0.33). However, most ectosomes had a diameter of  $\sim$ 200 nm (Fig. 1E).

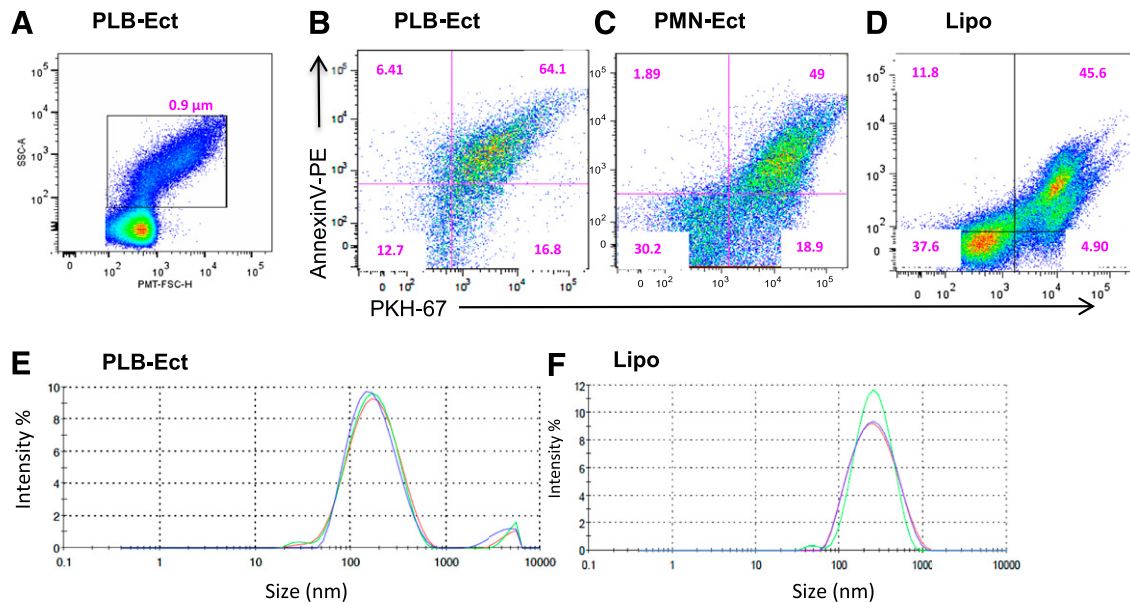


**Figure 1. Characterization of ectosome and liposome preparations.** Ectosomes prepared from PLB-985-differentiated cells (PLB-Ect) and fresh blood PMN (PMN-Ect), as well as liposomes were analyzed by flow cytometry with an LSR-Fortessa device (BD Biosciences). (A) Representative dot plots of PLB-Ect. SSC-A, SSC-area; PMT-FSC-H, PMT-FSC-height. (B) PLB-Ect staining with PKH67 and AnnexinV-PE. (C) PMN-Ect staining with PKH67 and AnnexinV-PE. (D) Liposome (Lipo) staining with PKH67 and AnnexinV-PE. (E and F) Evaluation of the size distribution of PLB-Ect and liposomes by DLS, showing diameter of  $\sim 200$  nm. This figure is representative of at least 3 independent experiments.

Together, these results showed that PLB-985 cells were a suitable model for producing homogenous ectosomes. Thus, PLB-Ect were used for subsequent experiments.

As a control, liposomes of similar sizes were also prepared. As shown in Fig1D and F, they also had a diameter of  $\sim 200$  nm with moderate polydispersion.

### PLB-Ect are internalized by immature moDCs

We first examined whether PLB-Ect interacted with immature moDCs. Thus, these cells were incubated for 4 h in the presence of PKH67-labeled PLB-Ect and then with LysoTracker Red, a lysosomal dye. Confocal microscopy showed green intracytoplasmic staining of all moDCs, suggesting ectosome internalization (Fig. 2A). Moreover, superposition of the 2 markers in immature moDCs (Fig. 2A and B) revealed their colocalization in  $\sim 50\%$  of cells, suggesting that after internalization, ectosomes could be routed to the lysosomal compartment.

### PLB-Ect prevent $\text{NiSO}_4$ -induced moDC maturation

In the next step, we evaluated the potential modulation of moDC maturation by PLB-Ect. After incubation with PLB-Ect, moDCs were treated for 18 h with  $\text{NiSO}_4$  or with LPS (positive control). Figure 3A shows representative histograms. In resting cells, PLB-Ect induced a small, nonsignificant increase in the expression of the studied surface markers (CD40, CD80, CD83, CD86, PDL-1, and HLA-DR; Fig. 3B). This was not a result of PLB-Ect endotoxin contamination, as levels were always  $<0.25$  IU/ml. As expected, LPS alone induced moDC maturation, as shown by increased expression of all markers (Fig. 3B). Preincubation with PLB-Ect significantly decreased LPS-induced up-regulation of all

surface markers, with the following percentage reductions: CD40 ( $27 \pm 5\%$ ), CD80 ( $12 \pm 2\%$ ), CD83 ( $19 \pm 2\%$ ), CD86 ( $20 \pm 5\%$ ), and PDL-1 ( $19 \pm 7\%$ ); HLA-DR expression was also reduced by  $10 \pm 3\%$  (Fig. 3B). Up-regulation of all of the markers was also observed when  $\text{NiSO}_4$  was used to induce moDC maturation, confirming the capacity of this chemical hapten to induce moDC maturation directly (Fig. 3B). Interestingly, PLB-Ect preincubation also significantly down-regulated this maturation-inducing effect, with the following percentage reductions: CD40 ( $24 \pm 5\%$ ), CD80 ( $22 \pm 6\%$ ), CD86 ( $26 \pm 9\%$ ), HLA-DR ( $18 \pm 5\%$ ), PDL-1 ( $22 \pm 5\%$ ), and to a lesser extent, CD83 ( $5 \pm 1\%$ ; Fig. 3B). Cell viability was determined by use of propidium iodide staining after 18 h and did not show any alteration in the presence of PLB-Ect (data not shown).

Together, these results suggested that based on membrane marker expression, PLB-Ect were able to prevent  $\text{NiSO}_4$ -induced moDC maturation as efficiently as they prevented LPS-induced maturation.

### PLB-Ect reduce $\text{NiSO}_4$ -induced cytokine production by moDCs

To document further the inhibitory effect of PLB-Ect on  $\text{NiSO}_4$ -induced moDC maturation, we first quantified the mRNA levels of several cytokines in moDCs by use of quantitative real-time PCR.

As shown in Fig. 4A, when PLB-Ect alone were incubated with moDCs in the absence of stimulus, no change was observed in any mRNA level. As expected, in the presence of  $\text{NiSO}_4$  or LPS (control), the mRNA levels of proinflammatory cytokines were up-regulated. Interestingly, when PLB-Ect were added, the

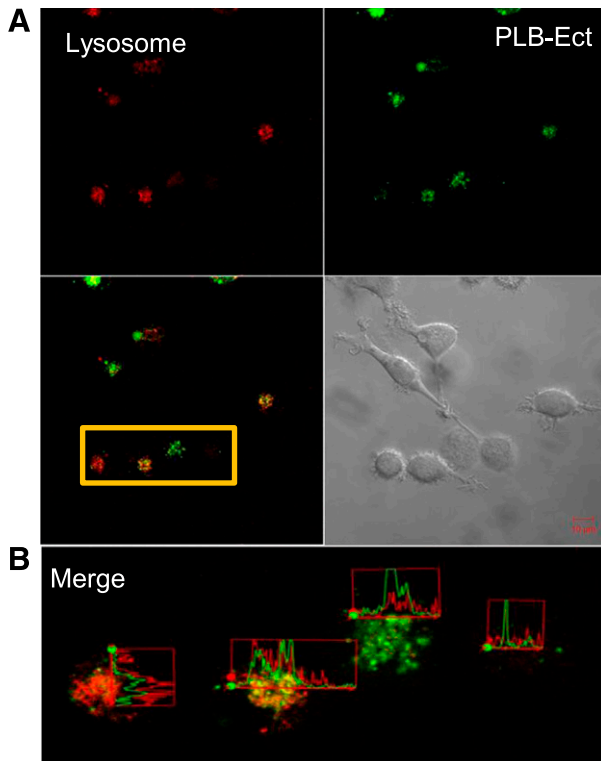


**Figure 2. PLB-Ect are internalized by immature human moDCs and can be targeted to the lysosomal compartment.** (A) moDCs were incubated with PLB-Ect labeled with PKH67 (green) for 4 h; then, DC lysosomes were stained with LysoTracker Red and analyzed by confocal microscopy. (B) Histograms of fluorescence intensity. Superposition of the 2 fluorescence signals in ~50% of the cells suggests that PLB-Ect can be routed to lysosomes. This figure is representative of 4 distinct experiments.

mRNA levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-12p40 fell significantly whether LPS or NiSO<sub>4</sub> was used as stimulus, with the following percentage reductions: with LPS as stimulus, IL-1 $\beta$  (27  $\pm$  2%), IL-6 (55  $\pm$  12%), TNF- $\alpha$  (63  $\pm$  5%), and IL-12p40 (68  $\pm$  7%); with NiSO<sub>4</sub> as stimulus, IL-1 $\beta$  (33  $\pm$  6%), IL-6 (42  $\pm$  7%), and TNF- $\alpha$  (40  $\pm$  9%). No significant change in the IL-8 mRNA level was observed (data not shown). NiSO<sub>4</sub> and LPS slightly reduced the mRNA levels of the regulatory mediators IL-10 and TGF- $\beta$ , neither of which was modified significantly in the presence of ectosomes (data not shown).

To test whether the effect of PLB-Ect was persistent or transient, moDC were preincubated for 30 min with PLB-Ect and then washed before adding NiSO<sub>4</sub> or LPS. We found that IL-6 and IL-12p40 mRNA levels were still down-regulated after cell washing, suggesting that the response was rapidly induced and persisted even when PLB-Ect were removed from the culture medium (data not shown).

To test whether ectosome effects were related to their internalization, we preincubated moDCs with cytochalasin D (0.5  $\mu$ g/ml) for 30 min before washing. PLB-Ect were then added for 30 min before LPS or NiSO<sub>4</sub> stimulation. We found that CytoD preincubation had no effect on PLB-Ect-induced down-regulation of IL-6 and IL-12 mRNA levels in moDCs, suggesting

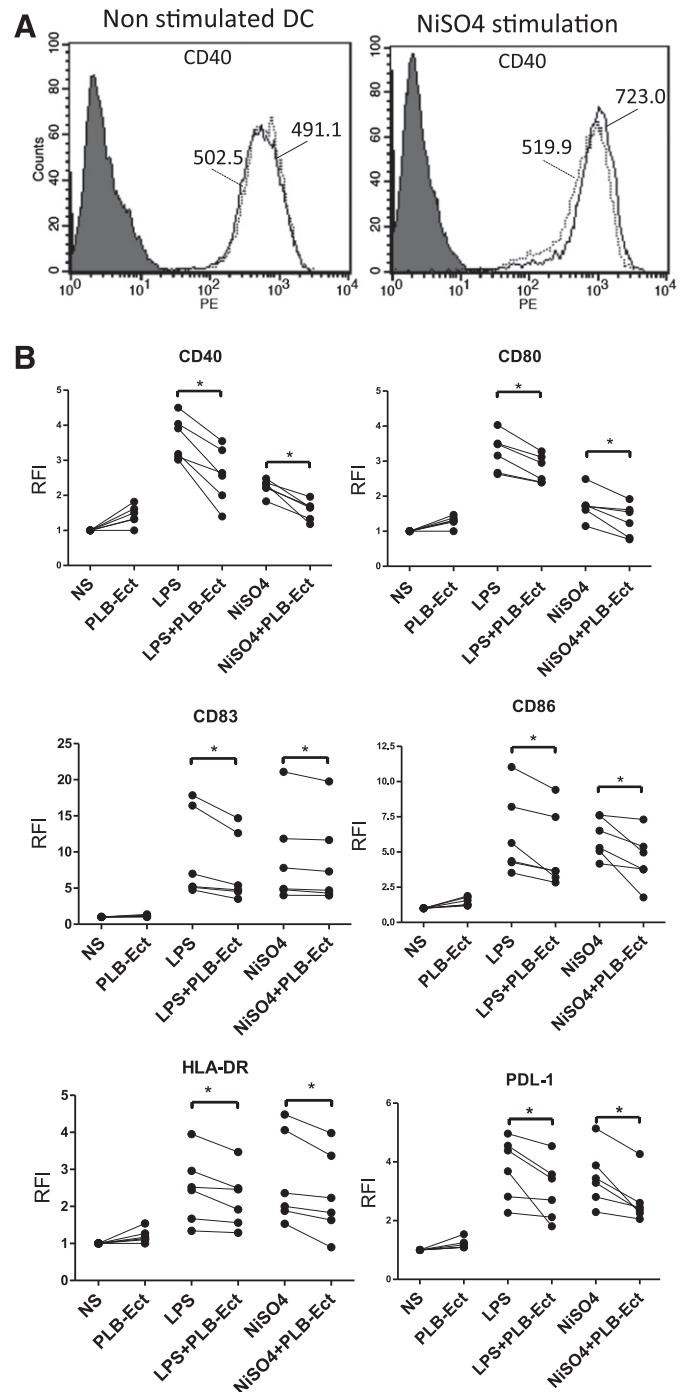


**Figure 3. PLB-Ect modulate the expression of key surface markers of moDC maturation.** moDCs were preincubated for 30 min with PLB-Ect or medium alone and then for 18 h with medium alone [nonstimulated (NS)], LPS (control stimulus), or NiSO<sub>4</sub>. moDCs were then collected, washed, and analyzed by flow cytometry for membrane expression of CD40, CD80, CD83, CD86, HLA-DR, and PDL-1. (A) Representative histograms of CD40 expression on nonstimulated DCs (left) and NiSO<sub>4</sub>-induced, stimulated DCs (right), without PLB-Ect (solid lines) or with PLB-Ect (dotted lines). Mean of fluorescence is indicated for each histogram. Isotype controls are in gray. (B) The results of 6 independent experiments are shown and expressed as the fold increase over nonstimulated. \**P* < 0.05.

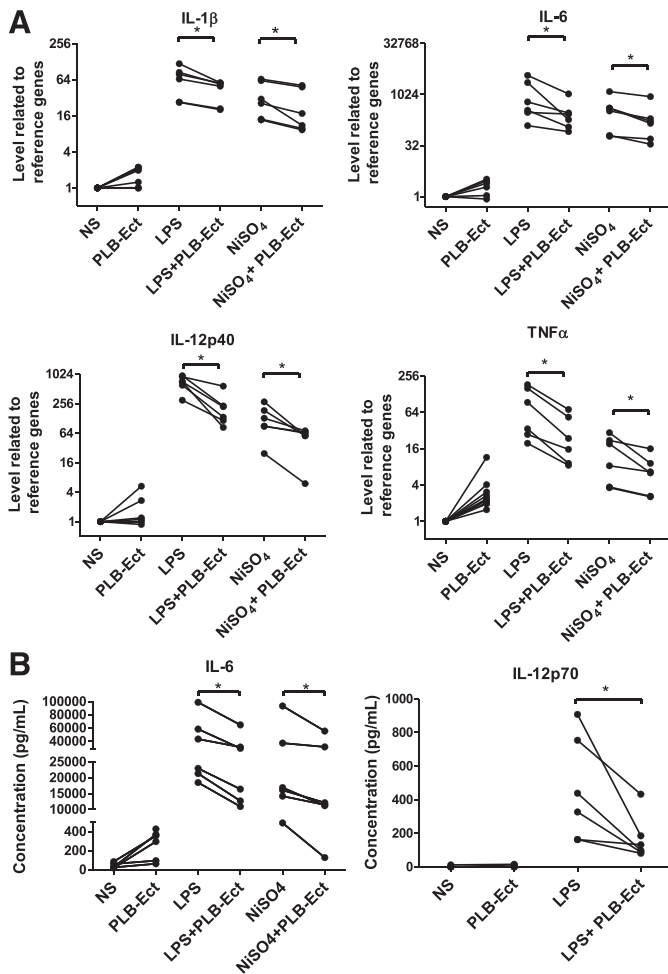


**Figure 4. PLB-Ect modulate NiSO<sub>4</sub>-induced proinflammatory cytokine production by moDCs at the mRNA and protein levels.** (A) Expression of cytokine mRNA. Immature moDCs were preincubated for 30 min with PLB-Ect or medium alone and then for 4 h with medium alone, LPS (control stimulus), or NiSO<sub>4</sub>. IL-1 $\beta$ , IL-6, IL-12p40, and TNF $\alpha$  mRNAs were quantified by real-time RT-PCR. Results were normalized to nonstimulated. The results of 6 independent experiments are shown. \* $P < 0.05$ . (B) Cytokine release in moDC supernatants. Immature moDCs were preincubated for 30 min with PLB-Ect or medium alone and then for 18 h with medium alone, LPS (control stimulus), or NiSO<sub>4</sub>. IL-6 and IL-12p70 levels are expressed in pg/ml. The results of 6 independent experiments are shown. \* $P < 0.05$ .

that external contact with cells was sufficient to induce the regulatory effect (data not shown).

Finally, to assess the proper effects of ectosomes, we also evaluated the effects of liposomes containing DOPC, CHOL, and PS on stimulated moDCs. As shown in Fig. 5, liposomes alone had no effect on moDC mRNA IL-6 and IL-12p40 levels. When preincubated before NiSO<sub>4</sub> or LPS activation, a decrease in IL-6 mRNA levels was observed, albeit significantly less marked than PLB-Ect-induced down-regulation. Similar but nonsignificant effects on IL-12 p40 mRNA levels were observed. These results suggest that ectosome lipids participate in the down-regulating effect but that other ectosome components are also involved.

We then examined whether these changes in gene expression could be observed at the protein level. Proinflammatory cytokine

levels were low or undetectable in the culture supernatants of moDCs, incubated or not incubated with PLB-Ect alone (Fig. 4B). As expected, NiSO<sub>4</sub> and LPS (control) both induced proinflammatory cytokine production (IL-6, IL-1 $\beta$ , IL-8, and TNF $\alpha$ ; data not shown). This production was down-regulated by PLB-Ect preincubation, but the effect was only significant for IL-6 ( $37 \pm 3\%$  and  $34 \pm 9\%$  with LPS and NiSO<sub>4</sub>, respectively; Fig. 4B). IL-12p70 release upon LPS activation was also strongly and significantly reduced in the presence of PLB-Ect ( $57 \pm 9\%$ ; Fig. 4B), but as expected, IL-12p70 was not released upon NiSO<sub>4</sub> activation [6]. These results confirmed that PLB-Ect modulated NiSO<sub>4</sub>-induced moDC maturation, as several proinflammatory cytokine genes were down-regulated, and IL-6 production was reduced.

### The NF- $\kappa$ B and MAPK signaling pathways are involved in the inhibitory effects of PLB-Ect on NiSO<sub>4</sub>-induced moDC maturation

The next step was to identify the intracellular signaling pathways that were modulated in the presence of PLB-Ect. As NiSO<sub>4</sub> and LPS are TLR4 ligands, they can induce NF- $\kappa$ B and p38MAPK

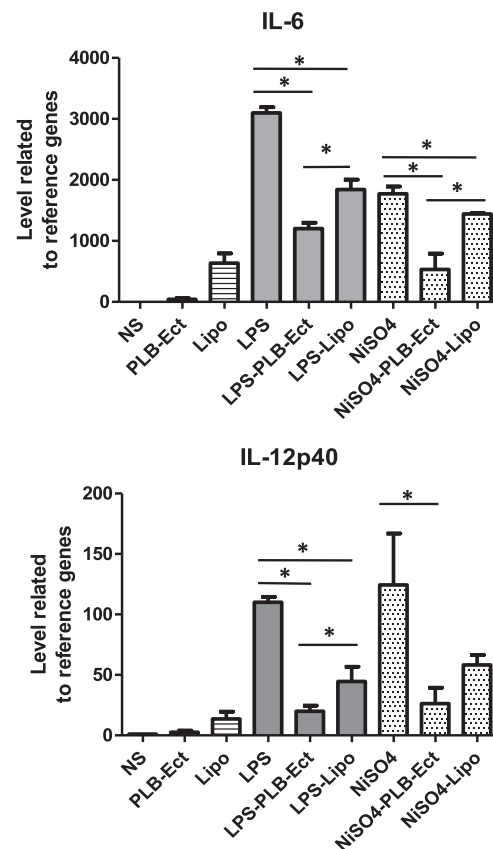


**Figure 5. Comparison of PLB-Ect and liposome preincubation on IL-6 and IL-12p40 mRNA levels in stimulated moDCs.** Immature moDCs were preincubated with PLB-Ect, liposomes, or medium alone for 30 min and then for 4 h with medium alone, LPS (control stimulus), or NiSO<sub>4</sub>. IL-6 and IL-12p40 mRNAs were quantified by real-time RT-PCR. Results were normalized to nonstimulated. The results of 3 independent experiments are shown. \* $P < 0.05$  compared with LPS or NiSO<sub>4</sub> alone.

activation in moDCs [6], in turn, modulating the expression of many proinflammatory genes. Thus, we investigated the impact of PLB-Ect on NiSO<sub>4</sub>- and LPS-induced phosphorylation of the NF- $\kappa$ B p65 subunit and p38MAPK. As shown in **Fig. 6A**, PLB-Ect alone had little or no effect on either phosphorylation, whereas NiSO<sub>4</sub> alone induced NF- $\kappa$ B p65 and p38MAPK phosphorylation.

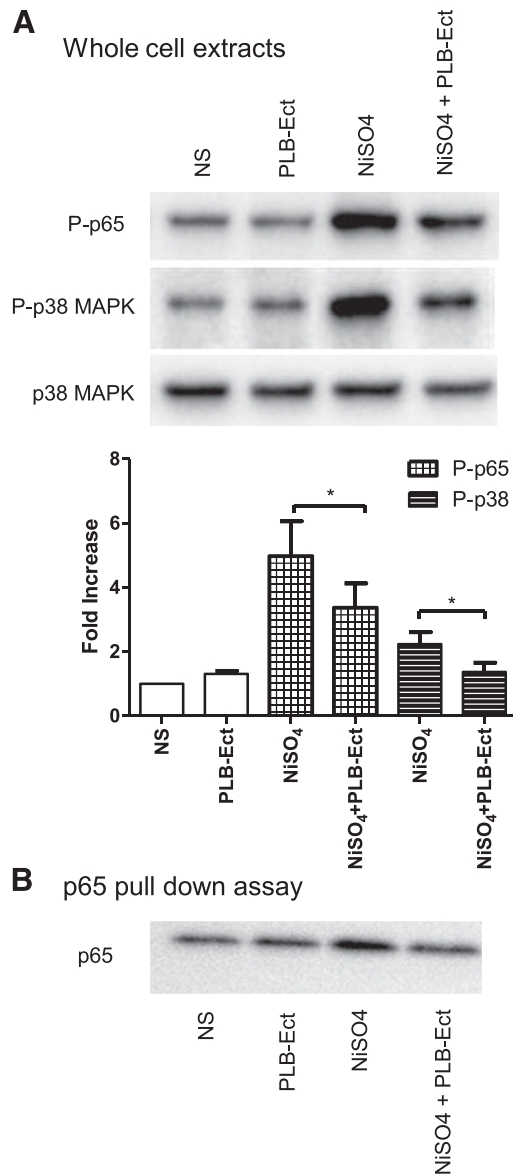


**Figure 6. PLB-Ect reduce NiSO<sub>4</sub>-induced phosphorylation of NF- $\kappa$ B p65 and p38MAPK in moDCs.** moDCs were preincubated with PLB-Ect or medium alone for 30 min and then for an additional 30 min with medium alone (NS) or NiSO<sub>4</sub>. (A) Immunoblotting of whole-cell extracts was used to quantify the phosphorylated forms of NF- $\kappa$ B p65 (P-p65) and p38MAPK (P-p38 MAPK). Bands were quantified by use of Image Lab software. Results are expressed as the fold induction, representing the normalized intensity of specific bands divided by the normalized intensity of untreated control cells. The results of 3 independent experiments are shown. \* $P < 0.05$ . (B) p65 DNA-binding activity was evaluated by use of an oligonucleotide pull-down assay. This figure is representative of 3 independent experiments.

Interestingly, both phosphorylations were decreased significantly in the presence of PLB-Ect. Reduction percentages were  $32.6 \pm 4.3\%$  for NF- $\kappa$ B p65 and  $39.7 \pm 2.7\%$  for p38MAPK. Moreover, with the use of a pull-down assay, we confirmed that ectosomes reduced NF- $\kappa$ B p65 DNA-binding activity in NiSO<sub>4</sub>-stimulated DCs (Fig. 6B).

We used immunofluorescence microscopy to investigate whether the decrease in the level of NF- $\kappa$ B phosphorylated p65 was related to dysregulation of NF- $\kappa$ B translocation to the nucleus. As shown in **Fig. 7**, NF- $\kappa$ B was cytoplasmic in most resting moDCs ( $90 \pm 5\%$  of cells; Fig. 7A) and in most moDCs incubated with PLB-Ect alone ( $86 \pm 6\%$  of cells; Fig. 7B). When moDCs were stimulated with NiSO<sub>4</sub>, NF- $\kappa$ B p65 translocated to the nucleus in  $65 \pm 6\%$  of cells (Fig. 7C). However, when PLB-Ect was added, this translocation was strongly and significantly inhibited, as only  $7 \pm 3\%$  of the cells exhibited nuclear staining (Fig. 7D).

These results showed that the NF- $\kappa$ B and p38MAPK signaling pathways were modulated by PLB-Ect, leading to down-regulation of proinflammatory cytokine production in response to NiSO<sub>4</sub>.

### PLB-Ect drive NiSO<sub>4</sub>-matured moDCs toward an effector phenotype

To understand better the functional consequences of DC modulation by ectosomes, we examined whether PLB-Ect were able to drive NiSO<sub>4</sub>-activated moDCs toward a tolerogenic or an effector phenotype. Thus, we quantified the expression of the NMES1 and Mx1 genes (associated with effector DCs) or the C1QA, CATC, and GILZ genes [associated with tolerogenic DCs (regulatory DCs)], as recently described by Zimmer et al. [48]. We found that PLB-Ect did not significantly modify the NiSO<sub>4</sub>-induced moDC effector phenotype, as shown by a trend toward an increase in NMES1 and Mx1 gene expression (**Fig. 8**). Similar effects were observed when cells were treated with LPS as a control. In contrast, a decrease in pan-regulatory DC markers (C1QA, CATC, and GILZ) was observed [48], whereas DEX (control) treatment of moDCs significantly up-regulated these genes (Fig. 8). Together, these data highlighted the capacity of PLB-Ect to drive effector rather than tolerogenic moDCs in the NiSO<sub>4</sub> and LPS maturation models.

### PLB-Ect drive NiSO<sub>4</sub>-matured moDCs to promote IL-13-producing CD4<sup>+</sup> T cells

To identify the type of effector DCs (i.e., DC1, DC2) induced by PLB-Ect, we analyzed the polarization of naïve, allogeneic CD4<sup>+</sup> T cells after coculture with treated moDCs, which were first incubated for 24 h with medium alone, PLB-Ect, LPS, LPS + PLB-Ect, NiSO<sub>4</sub>, or NiSO<sub>4</sub> + PLB-Ect and then washed and cocultured with CD4<sup>+</sup> T cells. Th polarization was analyzed after 5 days by evaluating cytokine levels in the culture supernatants (**Fig. 9**). PLB-Ect alone did not modify the CD4<sup>+</sup> T cell cytokine pattern compared with unstimulated moDCs. As expected, LPS-treated moDCs induced strong IFN- $\gamma$  secretion by T cells and a small IL-4, IL-5, IL-9, and IL-13 release. PLB-Ect + LPS-treated moDCs significantly reduced T cell production of IFN- $\gamma$ , whereas up-regulating IL-5, IL-9, and IL-13 production compared with CD4<sup>+</sup> T cells cocultured with LPS-treated moDCs in the absence of

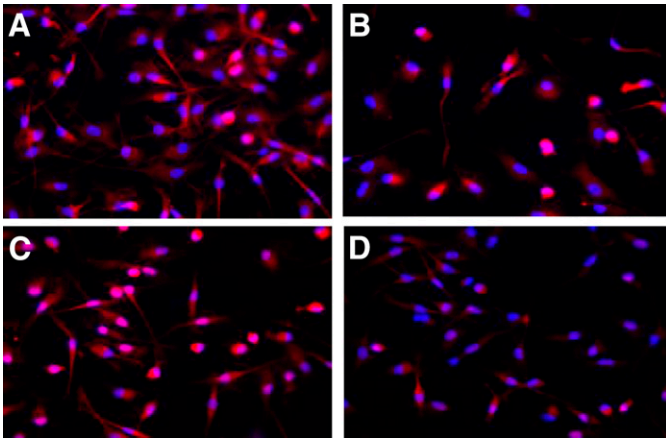


**Figure 7. PLB-Ect reduce NF- $\kappa$ B p65 nuclear translocation in NiSO<sub>4</sub>-treated moDCs.** moDCs were preincubated with PLB-Ect for 30 min or medium alone and then for 45 min with medium alone (A), medium + PLB-Ect (B), NiSO<sub>4</sub> alone (C), or NiSO<sub>4</sub> + PLB-Ect (D). Cells were then fixed, and their nuclei were stained with DAPI (blue), whereas NF- $\kappa$ B p65 was stained with an anti-p65 rabbit antibody, followed by an Alexa 546-labeled anti-rabbit antibody (red), with analysis by immunofluorescence microscopy. The figure is representative of 3 independent experiments.

PLB-Ect. Interestingly, NiSO<sub>4</sub>-treated moDCs decreased IFN- $\gamma$  production by T cells, while inducing moderate IL-4, IL-5, IL-9, and IL-13 production by comparison with nonstimulated moDCs. Moreover, a significant increase in IL-13 release was induced by PLB-Ect in the NiSO<sub>4</sub> model. No effect of PLB-Ect could be evidenced on Th17 polarization (data not shown).

This confirms the impact of PLB-Ect on LPS- or NiSO<sub>4</sub>-activated DCs and suggests that PLB-Ect might be critical for the induction of effector Th2 responses.

## DISCUSSION

The role of neutrophils in the control of DC activation is of growing interest. In particular, ectosomes have been shown to regulate DC functions in response to microbial products, such as LPS. In this study, we examined the offset of nickel, known to bind TLR4 and to cause ACD, a common skin disease. We first developed a model to produce, characterize, and quantify ectosomes by use of human PLB-985 cells differentiated into fully mature neutrophils. Then, with the use of this new biologic tool, we showed that ectosomes modulated nickel-induced moDC maturation in vitro through a mechanism involving MAPK and NF- $\kappa$ B regulation. These DCs exhibited an effector phenotype and promoted Th2 polarization.

The first step of this work was to develop a standardized method for ectosome production. We used the PLB-985 cell line, which can differentiate into fully mature, neutrophil-like cells. We and others [43, 45–47] have previously reported the ability of these differentiated PLB-985 cells to exhibit normal PMN functions, such as the oxidative burst, apoptosis, and netosis. Several authors have emphasized the importance of the preanalytic protocol for ex vivo ectosome production from fresh blood PMN: venopuncture, the time between blood collection and handling, as well as the conservation, centrifugation, and

washing steps are known to affect ectosome quality, whereas blood-derived preparations can contain contaminating micro-particles derived from other cell types [50–52]. Thus, we performed several experiments with the use of freshly isolated blood neutrophils from healthy volunteers in parallel to check the suitability of the PLB-985 model for ectosome production.

PLB-Ect were produced by use of a single, 40 min ultracentrifugation step to separate ectosomes from smaller vesicles, such as exosomes [53] [54]. These PLB-Ect were then characterized by use of 2 complementary methods: flow cytometry and DLS. Two markers were used for flow cytometric analysis, namely PKH67, an amphiphilic cell linker dye, and Annexin V, which binds to PS, highly expressed on ectosomes but not on exosomes

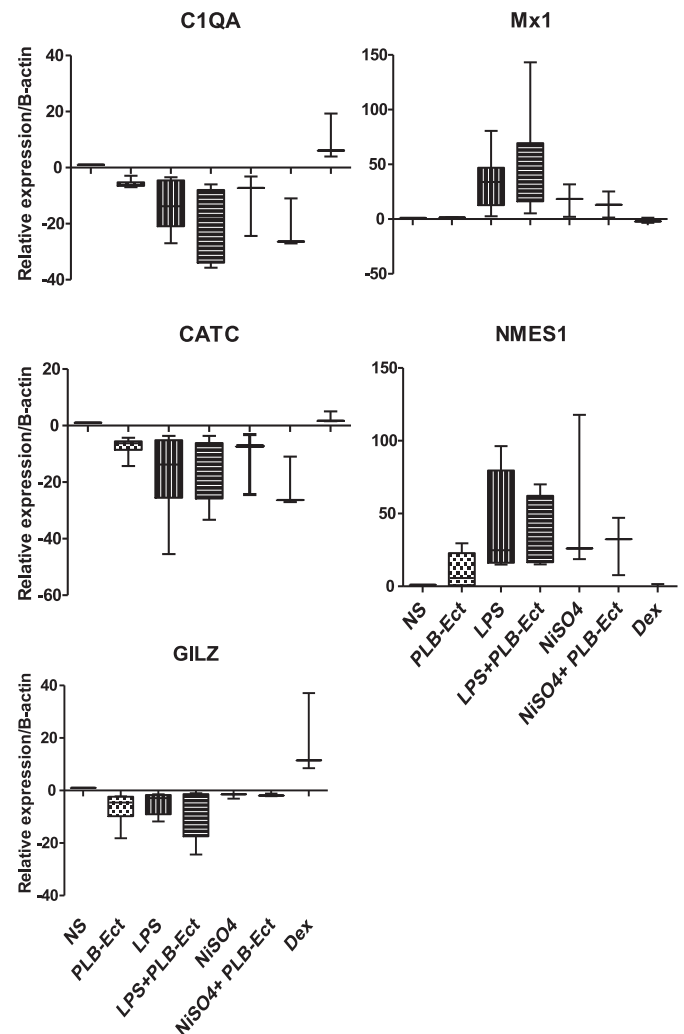


**Figure 8. PLB-Ect affect NiSO<sub>4</sub>-induced modulation of effector and tolerogenic gene expression in moDCs.** moDCs obtained from 4 healthy donors were preincubated with PLB-Ect or medium alone and then cultured with medium (NS), LPS, or NiSO<sub>4</sub> for 24 h. Total RNA was isolated, and *C1QA*, *CATC*, *GILZ*, *Mx1*, and *NMES1* gene expression was evaluated by real-time PCR. Data are expressed as relative amounts of mRNA in treated moDCs compared with moDCs incubated in medium alone. Data are normalized to the amount of  $\beta$ -actin. Box plots indicate data distribution: horizontal lines in the boxes show the minimum value, 25th percentile, median, 75th percentile, and maximum value.

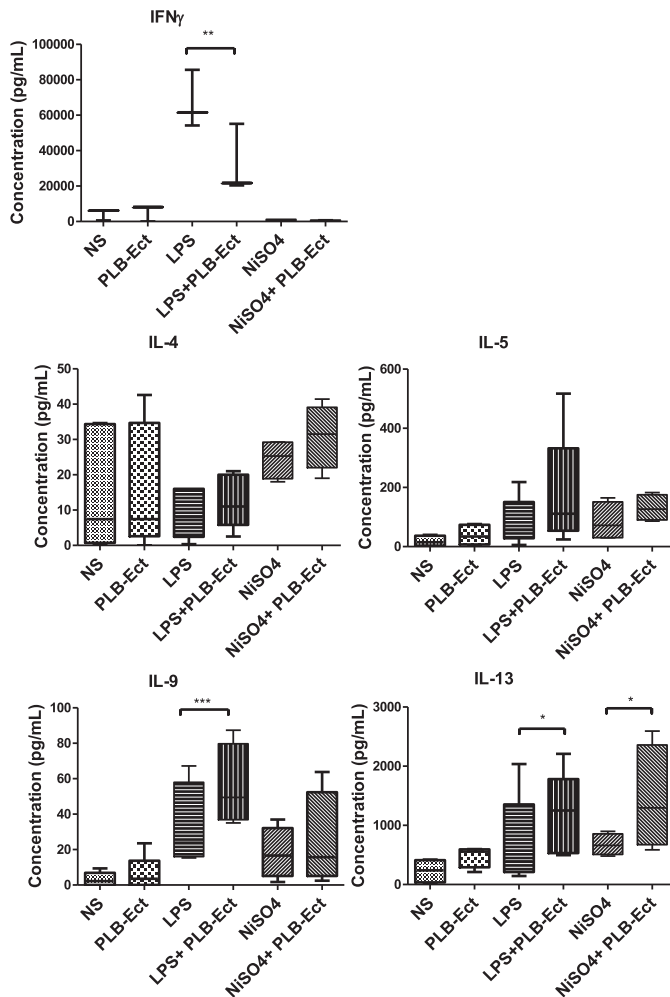


**Figure 9. CD4<sup>+</sup> T cells cocultured with PLB-Ect + NiSO<sub>4</sub>-treated moDCs exhibit Th2-type polarization.** moDCs left unstimulated (NS) or treated with PLB-Ect, LPS, LPS + PLB-Ect, NiSO<sub>4</sub>, or NiSO<sub>4</sub> + PLB-Ect were washed and incubated with purified, allogeneic, naive CD4<sup>+</sup> T cells for 5 days before cytokine measurement. Cytokine levels in supernatants were analyzed by use of a human cytokine bead kit. Box plots indicate data distribution: horizontal lines in the boxes show the minimum value, 25th percentile, median, 75th percentile, and maximum value (4 independent experiments). \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001.

[20]. The use of the PLB-985 cell line avoided the need for neutrophil-specific labeling. PLB-Ect were also identified by their size with the use of calibrated beads ranging from 0.5 to 3  $\mu$ m, as described previously [19, 55–57]. However, whereas most PLB-Ect were PKH67/Annexin V positive, some were Annexin V negative, suggesting the absence of PS on their surface. This was consistent with previous data showing that PS exposure on the outer membrane may be incomplete in some ectosomes, owing, in particular, to scramblase activity [58, 59]. Flow cytometry was also used to quantify PLB-Ect by use of a bead-based strategy, as recommended recently by the International Society on Thrombosis and Haemostasis Scientific Standardization Committee [56]. Indeed, flow cytometry can detect not only single ectosomes but also swarms, potentially underestimating actual concentrations [60, 61], and the detection of 100–400 nm

particles can sometimes be imperfect. In addition to flow cytometry, we determined the relative size distribution of PLB-Ect by use of DLS analysis, a method for analyzing particles ranging in size between 1 nm and 6  $\mu$ m [50]. We found that PLB-Ect exhibited a moderately polydisperse size distribution with a diameter of  $\sim$ 200 nm, in accordance with previous studies, thus excluding significant contamination by exosomes [29]. Thus, we show for the first time that differentiated PLB-985 cells are suitable for the production of large quantities of ectosomes with size and other characteristics similar to those derived from freshly isolated blood neutrophils. Moreover, control liposomes of similar size were specifically produced for the present study, with a lipid composition close to that of the plasmic membrane.

We then assessed the ability of PLB-Ect to modulate nickel-induced moDC maturation and function to better understand one of the mechanisms potentially involved in the interactions between PMN and DCs during the elicitation phase of ACD. We first examined the intracellular localization of PLB-Ect internalized by moDCs and found that most of them localized within the lysosomal compartment. This suggested that ectosomes were mainly internalized by endocytosis. This is in keeping with data from Gasser and Schifferli [62], who found that PMN-Ect internalization by macrophages was blocked by cytochalasin D, a phagocytosis inhibitor. Similar observations have been reported for platelet-derived microparticle uptake by endothelial cells [63]. PLB-Ect, routing to DC lysosomes, might be related to PS exposure, as PS is recognized by many receptors, including scavenger receptors expressed by phagocytic cells.

The next step was to document the effect of PLB-Ect on moDC maturation in response to nickel. In vivo, resident DCs are the first immune cells to be exposed to chemical sensitizers. We have shown previously that in vitro, a metallic compound, such as NiSO<sub>4</sub>, acts as danger signals, inducing DC maturation by activating the MAPK, NF- $\kappa$ B, and IRF1 pathways [5, 6, 64, 65]. More recently, it was shown that nickel and cobalt bind to TLR4 via 2 nonconserved histidine residues, leading to signal transduction and the production of proinflammatory cytokines [3, 4]. Thus, we used LPS, a major TLR4 ligand, as a positive control, comparing the effects of LPS and NiSO<sub>4</sub> on moDC maturation in the presence and absence of PLB-Ect. We confirmed that in response to NiSO<sub>4</sub> or LPS, moDCs undergo a maturation process, leading to up-regulation of costimulatory molecules (CD40, CD80, CD86), MHC class II molecules, and CD83. PLB-Ect alone did not modulate the expression of these molecules but down-regulated their NiSO<sub>4</sub>- and LPS-induced up-regulation. These results are in keeping with those of Eken et al. [19], who showed that blood PMN-Ect down-regulate LPS-induced moDC maturation. Similar changes in the moDC phenotype have been observed in the presence of ectosomes released by stored platelet concentrates [66]. We further analyzed DC phenotypic changes by studying PDL-1 expression. We evidenced LPS- and NiSO<sub>4</sub>-induced PDL-1 up-regulation at the moDC surface, confirming data from a recent study of Langerhans cells [67]. These findings are consistent with other works, showing that some chemical sensitizers, such as cinnamaldehyde, eugenol, and isogenol, can also induce PDL-1 up-regulation at the DC surface [68]. Moreover, we found, for

the first time, that PLB-Ect can decrease LPS- and NiSO<sub>4</sub>-induced PDL-1 up-regulation, suggesting that ectosomes might influence T cell activation and polarization during ACD.

Another major consequence of DC maturation is cytokine production. We have reported previously NiSO<sub>4</sub>-induced production of IL-6, IL-8, and IL-12p40 by moDCs [5, 65]. Here, we found that PLB-Ect alone did not induce cytokine production. In contrast, PLB-Ect down-regulated NiSO<sub>4</sub>-induced IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-12p40 mRNA and IL-6 protein production in moDCs. These results are in agreement with previous studies showing that neutrophil-derived ectosomes can hinder the release of some proinflammatory cytokines by zymosan A-stimulated macrophages [62] and by LPS-stimulated moDCs [19]. Platelet-derived ectosomes can also reduce TNF- $\alpha$  and IL-10 release by macrophages and inhibit LPS-induced maturation of moDCs [37]. Interestingly, we observed a decrease in IL-6 and IL-12p40 mRNA levels in the presence of liposomes, suggesting that lipids from ectosomes participate in the regulatory effect but that other ectosome components are also involved. These results are in accordance with those of Chen and coworkers [69, 70], who described PS-mediated IL-12 down-regulation in DCs.

We then examined the intracellular signaling pathways potentially modulated by PLB-Ect and resulting in cytokine down-regulation. We have shown previously that nickel can induce MAPK and NF- $\kappa$ B protein phosphorylation [5, 64, 65]. Here, we found that PLB-Ect reduced NF- $\kappa$ B p65 and p38MAPK phosphorylation and NF- $\kappa$ B p65 nuclear translocation in nickel-stimulated moDCs. These actions could explain the observed cytokine down-regulation, as both signaling pathways are strongly involved in TLR4 agonist-related cytokine release.

To understand better the functional consequences of the down-regulating effect of PLB-Ect on moDC maturation, we studied their ability to drive moDC and T cell polarization. Zimmer et al. [48] recently defined molecular signatures of effector and regulatory DCs, and we took advantage of this finding to study the impact of PLB-Ect on LPS- and NiSO<sub>4</sub>-activated moDCs. Although PLB-Ect down-regulated moDC maturation in response to both stimuli, the resulting DCs exhibited no increase in pan-regulatory DC markers (GILZ, CATC, and C1QA) but rather, expressed higher levels of DC effector markers (Mx1 and NMES1). To investigate whether these NiSO<sub>4</sub>-induced effector moDCs would affect T cell polarization, cocultures were performed by use of moDCs, treated or not with PLB-Ect. We report for the first time that in the absence of PLB-Ect, NiSO<sub>4</sub>-treated moDCs cannot trigger high IFN- $\gamma$  production by T cells, as can LPS-treated moDCs; conversely, Th2 cytokines and IL-9 are similarly produced by use of both DC stimuli. These new findings are in keeping with some clinical studies that documented the ex vivo production of Th2 cytokines (IL-4, IL-5, IL-13) by CD4<sup>+</sup> T cells from nickel-allergic patients [71–73]. In the presence of PLB-Ect, this Th2 response was maintained or even enhanced in our cocultures, depending on the cytokine; in particular, the IL-13 up-regulation was observed when moDCs were exposed to NiSO<sub>4</sub> but also to LPS. In addition, PLB-Ect decreased the IFN- $\gamma$  production in the LPS model, suggesting Th1 modulation too. Together, these

results highlight the modulatory function of ectosomes that could contribute to Th polarization driving, specifically in this in vitro model of ACD.

Therefore, we describe a new mechanism of DC modulation by neutrophils that may be involved in nickel-related ACD, a disorder in which both cell types are present in the skin. Indeed, in vitro, PLB-Ect (a model of neutrophil ectosomes) modulated nickel-induced moDC maturation and promoted a Th2 response. These observations further highlight the role of neutrophils in the regulation of DC activation, notably in response to NiSO<sub>4</sub>, a common danger signal.

## AUTHORSHIP

I.T., Y.G., A.G., and S.C.-M. conceived of and designed the experiments. I.T., Y.G., C.G., H.T., C.A.S., R.G., and H.G.-G. performed the experiments. I.T., Y.G., A.G., S.K.-R., M.P., L.M., and S.C.-M. analyzed the results. I.T., A.G., and S.C.-M. wrote the manuscript.

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## DISCLOSURES

The authors declare no conflict of interest.

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