The anti-inflammatory effects of platelet-derived microparticles in human plasmacytoid dendritic cells involve liver X receptor activation

Adam Ceroi,[1-4] Fanny Angelot Delettre,[1-4] Charline Marotel,[1-3] Thierry Gauthier,[1-4] Afag Asgarova,[1-3] Sabéha Biichlé,[1-4] Anne Duperrier,[1-3] Guillaume Mourey,[1-3] Sylvain Perruche,[1-4] Laurent Lagrost,[4-7] David Masson,[4-7] and Philippe Saas[1-4,8]

[1]INSERM, UMR1098, Besançon; [2]EFS Bourgogne Franche-Comté, UMR1098, Besançon; [3]Université Bourgogne Franche-Comté, UMR1098, Besançon; [4]LabEX LipSTIC, ANR-11-LABX-0021, Besançon/Dijon; [5]INSERM, U866, Dijon; [6]Université Bourgogne Franche-Comté, UMR866, Dijon; [7]CHRU Dijon; and [8]CHRU Besançon, INSERM CIC1431, FHU INCREASE, France

Correspondence: philippe.saas@efs.sante.fr
doi:10.3324/haematol.2015.135459

Supplemental Methods

Cell isolation. Blood samples were collected from healthy donors after regulatory approval of the French Ministry of Higher Education and Research (Ministère de l'Enseignement Supérieur et de la Recherche, agreement number #AC-2015-2408, May 22th 2015). Peripheral blood mononuclear cells (PBMC) from healthy donors (EFS B/FC, Besançon, France) were isolated by Ficoll gradient density centrifugation using Ficoll-Paque (Velizy-Villacoublay, France). Plasmacytoid DCs (PDC) were isolated using Diamond PDC Isolation Kit (Miltenyi Biotec, Paris, France) according to the manufacturer's recommendations. After isolation, PDC purity was controlled by cytometry after CD123/BDCA-2 monoclonal antibody (mAb) staining (Supplemental Table 1). The purity of isolated PDC was between 94 to 98%.

Cell culture. PDC isolated from healthy volunteers and the human leukemic PDC cell line CAL-1,[1] obtained from Dr Maeda (Nagasaki University, Japan), were cultured in complete medium consisting in RPMI 1640 Glutamax medium (InVitrogen, Cergy Pontoise, France) supplemented with 10% (vol/vol) heat inactivated fetal calf serum (FCS) (Invitrogen) and 1% Penicillin-Streptomycin (10000 U/mL, Life Technologies, Gaithersburg, MD). Culture medium was always supplemented with recombinant human IL-3 (10 ng/mL, PeproTech, London, UK). Cells from the human microvascular endothelial cell line (HMEC-1) were cultured in MCDB 131 medium (InVitrogen) supplemented with 10% (vol/vol) heat inactivated FCS, 1% Penicillin-Streptomycin (10000 U/mL), epidermal growth factor (EGF, 10 ng/mL, Sigma Aldrich, Saint-Quentin Fallavier, France) and Hydrocortisone (3 nM, Sigma Aldrich).

Microparticle production and isolation. Microparticles derived from HMEC-1 (referred as, EMP) were generated and isolated, as previously described.[2] Briefly, when HMEC-1 cells reached 80% of confluence, TNF-α (50 ng/mL, PeproTech) was added and culture was performed for 24 h. Cell culture supernatants were collected and cleared from detached cells and cell fragments by centrifugation at 1200*g* for 5 min. Supernatant was then centrifuged twice at 15000*g* for 90 min at 4°C. Pelleted EMP were resuspended in culture media and used immediately or stored until 3 weeks at 4°C before use, as previously described.[2] Platelet-derived microparticles (PMP) were isolated from platelet concentrates (EFS B/FC) obtained from healthy donors after written informed consent. Platelets were removed by two centrifugations at 2500*g* 15 min at room temperature (RT). Supernatants were collected and ultracentrifuged twice (15000*g*, 90 min, 4°C) to isolate PMP that were used or stored in same conditions than EMP.[2] Quantification of MP was performed by flow cytometry using a Navios cytometer (Beckman Coulter, Villepinte, France), Megamix plus™ (BioCytex, Marseille, France) to assess MP size and MP number was calculated on the basis of the known number of Cytocount® beads (Dako, Trappes, France) added to the sample. Identification of MP was performed after staining with fluorochrome-conjugated mAbs (Supplemental Table 1). Expression of phosphatidylserine by MP was assessed by cytometry using FITC-conjugated Annexin-V (AnxV, Beckmann Coulter).

Evaluation of plasmacytoid dendritic cell responses. Functional expression of LXR by human PDC was assessed by using both LXR agonists (T0901317, GW3965[3] or 22RHC [22-R-hydroxycholesterol]) or antagonist (22-S-hydroxycholesterol, 22SHC[4]) (Sigma Aldrich) and then, by analyzing induction of LXR target genes (see below) or expression of LXR (*lxra* and *lxrb*) isoform mRNA, assessed by qRT-PCR (Figure S1). ABCA-1 protein expression was assessed by confocal microscopy. Concentrations of LXR agonists or antagonist used were defined to avoid PDC death as assessed by AnxV and 7-Aminoactinomycin D (7-AAD, BD Bioscience) staining and flow cytometry. To assess the effects of LXR activation on innate PDC functions, freshly isolated PDC were stimulated with LXR agonist/antagonist for 24 h, and then, by either Toll-like receptor (TLR)7 ligand R848 (1 µg/ml, Invivogen, Toulouse, France), or TLR9 ligands, CpG-A (2 µmol/L, ODN2216, Invivogen) for additional 6 h (for mRNA analysis) or 18 h for (protein analysis). To assess the effects of MP on PDC functions, EMP or PMP were added to PDC at different PDC to MP ratio (1:10, 1:40, 1:80)

for 18 hours. Maturation of PDC was assessed by expression of maturation marker CD83 or costimulatory molecules by cytometry and cytokine production (at protein levels by ELISA, multiplex assay or intracellular staining/cytometry and at mRNA levels by transcript quantification). Internalization of MP by PDC was also assessed using labeled-MP (see below). Prevention of NF-kB activation in PDC was performed using JSH-23 (25 µM, Sigma Aldrich), a NF-kB p65 nuclear translocation inhibitor.[5]

Flow cytometry and antibodies. For PDC analysis, flow cytometry was performed with a CANTO II cytometer (BD Biosciences, Le Pont de Claix, France) using the DIVA 6.1 software (BD Biosciences). Fluorochrome-conjugated isotype control mAbs from the different mAb providers were used. Monoclonal antibodies used for cytometry and other cytometry reagents are described in supplemental table 1.

Cytokine production. Culture supernatants were collected from PDC after LXR ligand stimulation (24 h) followed or not by TLR ligand (18 h), or after stimulation by EMP or PMP (18 h). The multiplex DIAplex technology (Diaclone, Besançon, France) was used to measure the following cytokines: TNF-α, IL-10, IL-6 and IL-8. The human IFN-alpha Platinum ELISA kit (eBioscience SA, Paris, France) was used to measure IFN-α. The minimal detectable concentrations were as follows: 1.7 pg/mL, 1.3 pg/mL, 1.4 pg/mL, 9.8 pg/mL and 15 pg/ml for IL-10, IL-8, IL-6, TNF and IFN-α, respectively. Production of IFN-α, IL-6 and TNF-α by PDC was also determined by flow cytometry and intracellular staining using anti-IFN-α, anti-IL-6 and anti-TNF-α mAb, respectively (Supplemental Table 1). Brefeldin A (10 µg/ml, Sigma Aldrich) was added during the last 3 h before the end of activation with TLR ligands or MP. Staining was performed according to the manufacturer's instructions on fixed and permeabilized cells (cytofix/cytoperm Plus kit, BD Biosciences).

Microparticle internalization. Microparticle uptake was assessed by cytometry, as described.[2] Microparticles (EMP or PMP) were labeled with either CellTrace™ CFSE Cell Proliferation Kit (Thermo Scientific™, Fontenay-sous-Bois, France) or eFluor proliferation dye v450 (eBioscience) according to the manufacturers' instructions, and then, incubated at different ratio (see above) with freshly isolated PDC. Internalization was blocked before labeled-MP addition by pre-incubation (30 minutes) of MP with unlabeled AnxV (BD Biosciences), or PDC pre-incubation (1 hour) with either anti-BAI-1 mAb or anti-Mer-TK polyclonal antibody (R&D Systems, Lille, France), control isotype irrelevant mAb or polyclonal antibody (R&D Systems). Effects of LXR agonists on MP internalization were assessed by incubating PDC, with either T0901317 or GW3965 24 h prior addition of labeled-MP.

Quantitative RT-PCR analysis. Quantitative RT-PCR analysis was used to evaluate functionally LXR expression by measuring LXR-target gene expression *(i.e., lxra, abca1* and *srebp1c*) and confirm the effects of LXR agonists or MP on LXR target gene, endocytosis receptor or cytokine gene expression. Total RNA was extracted using RNeasy Mini Kit (Qiagen, Courtaboeuf, France). One to 2 µg of RNA was reverse-transcribed into cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Saint Aubin, France). Then, cDNA were quantified by real time RT-PCR using either the Power SYBR Green PCR Master Mix (Applied Biosystems) or the Taqman Universal Buffer II (Applied Biosystems) on a CFX96 Real-Time device (Biorad, Marnes-la-Coquette, France). Relative mRNA levels were determined using the ΔΔCt method. Values were expressed relative to *gapdh* levels. The sequences of the oligonucleotides used are described in supplemental table 2.

Confocal microscopy.

CAL-1 cells or freshly isolated PDC were incubated with LXR agonist for 24 h before CD123 and ABCA1 analysis. Phosphorylated NF-κB p65 (pRelA) analysis was performed after LXR treatment of PDC followed by R848 stimulation for 45 min. PDC stimulated with MP were directly stained for ABCA1 or pRelA. Cells were collected in cold PBS solution, washed and

immobilized onto glass microscope slides by Cytospin's centrifugation (1000 rpm, 5 min, RT). Then, cells were fixed and permeabilized in a methanol bath (20 min at -20°C), washed 3 times and incubated, overnight at +4°C, with the primary antibody (Supplementary Table 3). Slides were washed 3 times with 0.5% FCS/PBS solution, and incubated 1 h with Alexa fluor 555-conjugated Goat anti-mouse IgG (H+L) or Alexa fluor 488-conjugated Goat anti-Rabbit IgG (H+L) antibody (Supplementary Table 3). Slides were washed and mounted with Mounting Media with DAPI (Sigma Aldrich). Fluorescent images were acquired on an FV1000 confocal microscope (Olympus, Rungis, France) and analyzed with Olympus FV-viewer software.

Supplemental Tables

Supplemental Table 1. Reagents used for cytometry analysis

mAbs directed against:	Fluorochrome	Providers	Reference #
CD83	PE	BD	556855
CD80	APC-H7	BD	561134
HLA-DR	FITC	Diaclone	857.031.010
CD40	PerCPCy5.5	BioLegend	334316
CD123	PE-Cy7	Biolegend	306010
BDCA2	APC	Miltenyi Biotec	130-090-905
CD86	V450	BD	560357
IFN-α	PE	Miltenyi Biotec	130-092-601
IL-6	FITC	Biolegend	501104
TNF-α	PerCPCy5.5	eBioscience	45-7349-42
Navios Microparticle analysis			
CD41	PE-Cy7	Beckman Coulter	6607115
CD31	PE	Beckman Coulter	IM2409
Glycophorin A (CD235a)	PE-Cy7	Beckman Coulter	A71564
Annexin V	FITC	Beckman Coulter	100406-02
Binding Buffer		Beckman Coulter	10045-01
Megamix Plus™		BioCytex	7802
CYTOcount®		DAKO	S2366

Supplemental Table 2 Reagents for quantitative RT-PCR analysis.

SYBR Green Assay (Eurofins mwg/operon)	
Target	Sequence (5'->3')
abca1 forward	GCACTGAGAGATGCTGAAA
abca1 reverse	AGTTCCTGGAAGGTCTTCAC
srebp1c forward	GACATCGAAGACATGCTTCAGCT
srebp1c reverse	GACATCGAAGACATGCTTCAGCT
lxra forward	ACTCGAAGCCGGTCAGAAA
lxra reverse	GAAGAAACTGAAGCGGCAAGA
lxrb forward	AGATGTTGATGGCGATGAGCA
lxrb reverse	TGCCTGGTTTCCTGCAGCT
gapdh forward	ACATCATCCCTGCCTCTACTG
gapdh reverse	ACCACCTGGTGCTCACTGTA
Gene	Assay on demand reference # (Applied Biosystem)
il-6	Hs00985639_m1
tnfa	Hs01113624_g1
bai1	Hs00181777_m1
mertk	Hs01031973_m1
tim1	Hs00273334_m1
tim4	Hs00293316_m1
stabilin2	Hs00213948_m1
ifna1	Hs00356648_s1
gapdh	4352934E

Supplemental Table 3, Reagents used for confocal microscopy

Antibody against:	Reference #	Providers
CD123	555642	BD Biosciences
ABCA1	NB400-105	Novus
Phosphor-S536 p65 (RelA) NF-κB subunit	5583977	BD Biosciences
Alexa fluor 555-conjugated Goat anti-mouse IgG (H+L)	A21422	Life technologies
Alexa fluor 488-conjugated Goat anti-Rabbit IgG (H+L)	A11008	Life technologies

Supplemental Figures

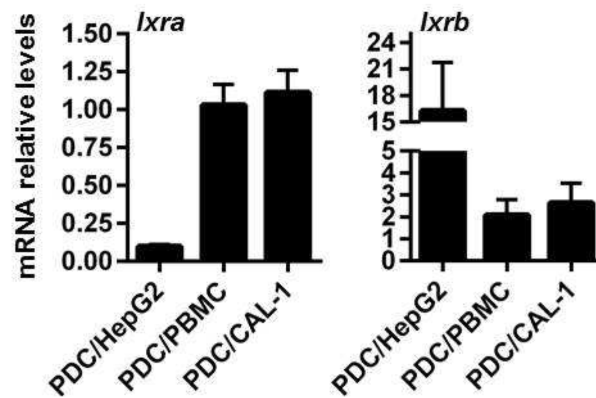

Figure S1. Freshly isolated plasmacytoid dendritic cells express preferentially the ubiquitous isoform *lxrb* mRNA.

Lxra and *lxrb* mRNA levels were evaluated in freshly isolated untreated PDC by qRT-PCR. The mRNA relative levels of PDC samples (n=24 and n=5 for *lxra* and *lxrb*, respectively) were compared to the mean of HepG2 hepatoma cell line (a control for *lxra*), PBMC or CAL-1 PDC cell line (n=4, n=4 and n=12, respectively). PDC express lower levels of lxra mRNA than HepG2 and similar levels of lxr isoforms than PBMC and CAL-1 cells.

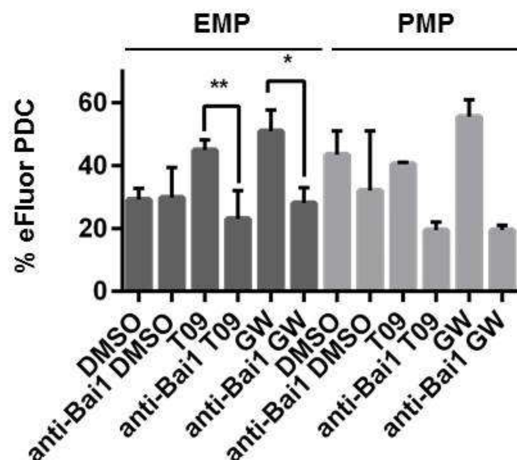

Figure S2: Stimulation of LXR pathway in freshly isolated plasmacytoid dendritic cells upregulates microparticle internalization *via* BAI-1, whatever the microparticle origin.

PDC were treated with 1 µM of T0901317 (T09) or GW3965 (GW) for 24 hours. Then, cells were cultured for 1 hour with neutralizing anti-BAI-1 antibody (α-BAI-1) or control antibody, before adding eFluor-labeled EMP or PMP for 4 h and analyzing by cytometry. (n=4 and 2 for EMP and PMP respectively). Cumulative data of 4 or 2 independent experiments expressed as mean ± S.E.M. of eFluor^{+} PDC are shown. * $p<0.05$, ** $p<0.01$, Mann-Whitney

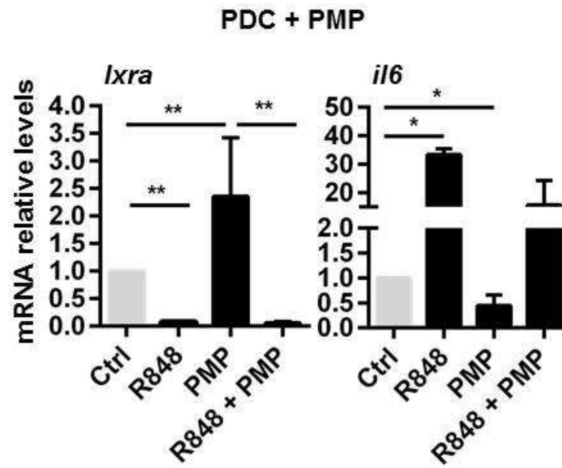

Figure S3: NF-κB triggering by TLR7 activation prevents platelet-derived microparticle-induced LXR activation in freshly isolated plasmacytoid dendritic cells.

Freshly isolated PDC were treated for 18 hours with R848 (1 µg/mL), PMP at a PDC:PMP ratio of 1:40, or with R848 plus PMP. Expression of LXR target gene (*lxra*) and inflammatory cytokine (*il6*) gene mRNA was quantified by qRT-PCR (n=3). Data were expressed as mean ± S.E.M. of 3 independent experiments. * $p<0.05$, ** $p<0.01$, Mann-Whitney.

Supplemental References

1. Maeda T, Murata K, Fukushima T, et al. A novel plasmacytoid dendritic cell line, CAL-1, established from a patient with blastic natural killer cell lymphoma. *Int. J. Hematol.* 2005;81:148–154.

2. Angelot F, Seilles E, Biichle S, et al. Endothelial cell-derived microparticles induce plasmacytoid dendritic cell maturation: potential implications in inflammatory diseases. *Haematologica*. 2009;94:1502–1512.

3. Bennett DJ, Cooke AJ, Edwards AS. Non-steroidal LXR agonists; an emerging therapeutic strategy for the treatment of atherosclerosis. *Recent Patents Cardiovasc. Drug Discov.* 2006;1:21–46.

4. Hessvik NP, Bakke SS, Smith R, et al. The liver X receptor modulator 22(S)-hydroxycholesterol exerts cell-type specific effects on lipid and glucose metabolism. *J. Steroid Biochem. Mol. Biol.* 2012;128:154–164.

5. Shin H-M, Kim M-H, Kim BH, et al. Inhibitory action of novel aromatic diamine compound on lipopolysaccharide-induced nuclear translocation of NF-kappaB without affecting IkappaB degradation. *FEBS Lett.* 2004;571:50–54.