Different expression and subcellular localization of Phosphoinositide-specific Phospholipase C enzymes in differently polarized macrophages

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Abstract Macrophages' phenotypic and functional diversity depends on differentiating programs related to local environmental factors. Recent interest was deserved to the signal transduction pathways acting in macrophage polarization, including the phosphoinositide (PI) system and related phospholipase C (PLC) family of enzymes. The expression panel of PLCs and the subcellular localization differs in quiescent cells compared to the pathological counterpart. We analyzed the expression of PLC enzymes in unpolarized (M0), as well as in M1 and M2 macrophages to list the expressed isoforms and their subcellular localization. Furthermore, we investigated whether inflammatory stimulation modified the basal panel of PLCs' expression and subcellular localization. All PLC enzymes were detected within both M1 and M2 cells, but not in M0 cells. M0, as well as M1 and M2 cells own a specific panel of expression, different for both genes' mRNA expression and intracellular localization of PLC enzymes. The panel of PLC genes' expression and PLC proteins' presence slightly changes after inflammatory stimulation. PLC enzymes might play a complex role in macrophages during inflammation and probably also during polarization.

Keywords Signal transduction · Macrophage polarization · M0 · M1 · M2 · PLC · Gene expression · LPS · Inflammation · Podosome · Nanotube · Post-transcriptional regulation

Introduction

Macrophages derive from blood circulating monocytes. Once differentiated into macrophages, they develop specialised functions, exhibiting a certain grade of heterogeneity. Macrophages belong to the cascade of innate immunity and play a central role in organ development, as well as in tissue turnover and regeneration (Chang 2009; Pollard 2009; Osborn and Olefsky 2012).

Phenotypic and functional diversity depends on differentiating programs being strictly related to local environmental factors (Gordon 2007; Mosser and Edwards 2008; Daigneault et al 2010). Distinct state of polarized activation allowed categorize macrophages into M1 and M2 on the basis of the transcriptional profile and cytokine production (Martinez et al. 2006a, b; Zhang et al 2015; Röszer 2015).

M1 activation is induced by intracellular pathogens or bacterial components, lipoproteins and some cytokines. The M2 activation is induced by fungal elements, parasites, immune complexes, complement activation, apoptotic cells and various cytokines (Benoit et al 2008). M1 cells secrete inflammatory cytokines and produce nitric oxide (NO) (Martinez and Gordon 2014a, b; Akira et al 2013). M2 show enhanced phagocytic activity and produce extracellular matrix components, anti-inflammatory cytokines as well as angiogenic factors. In fact, beside the role in immunity, M2 cells clear apoptotic debris, promote tissue regeneration and can mitigate the inflammatory response (Forbes and Rosenthal 2014; Sica and Mantovani 2012).

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However, many issues remain to be addressed regarding the specific role of M1 and M2, as well as the events leading to macrophage polarization. Recent interest was deserved to the signal transduction pathways acting in macrophage polarization, including the phosphoinositide (PI) system (Tuosto et al 2015; Zhao et al 2014; Liu et al. 2007a, b; Botelho et al 2000; Holian 1986).

PIs are minority acidic phospholipids in cell membranes. Beside instructional roles, PIs contribute an important intracellular signaling system involved in a variety of cell functions such as hormone secretion, neurotransmitter signal transduction, cell growth, membrane trafficking, ion-channel activity, cytoskeleton regulation, cell-cycle control and apoptosis (Suh et al 2008), as well as in cell and tissue polarity (Comer and Parent 2007; Suh et al. 2008). A combination of compartmentalized and temporal changes in the expression of PI signaling molecules elicits different cellular responses, including gene expression regulation, DNA replication, and chromatin degradation. In the PI pathway crucial events are related to the regulation of phosphatidyl inositol 4,5-bisphosphate (PIP₂), mainly located in the inner half membrane. PIP₂ is hydrolyzed by enzymes belonging to the PI-specific phospholipase C (PLC) family in response to a wide panel of stimuli, including growth factors, hormones, and neurotransmitters, that act on specific receptors localized at the plasma membrane. Once activated, PLC cleaves the membrane PIP₂ into inositol trisphosphate (IP₃) and diacylglycerol (DAG). IP₃, a small water-soluble molecule, diffuses rapidly to the cytoplasm. IP₃ induces calcium release from the endoplasmic reticulum (ER) by binding to IP₃-gated calcium-release channels (Berridge 1981; Berridge and Irvine 1984; Rhee et al. 1991; Suh et al 2008; Berridge 2009; Bunney and Katan 2011). DAG can be further cleaved to release arachidonic acid, which either acts as a messenger or is used in the synthesis of eicosanoids or can activate serine/threonine calcium-dependent protein kinase C enzymes (PKC), also influenced by the IP₃-induced calcium increase (Tang et al. 2005).

The mammalian PLC family comprises a related group of complex, modular, multi-domain enzymes which cover a broad spectrum of regulatory interactions, including direct binding to G protein subunits, small GTPases from Rho and Ras families, receptor and non-receptor tyrosine kinases and lipid components of cellular membranes (Rhee et al. 1991). PLC enzymes are 13 isoforms classified on the basis of amino acid sequence, domain structure and mechanism of recruitment into six subfamilies: β (1–4), γ (1–2), δ (1, 3, 4), ϵ , ζ , and η (1–2) (Suh et al. 2008).

The panel of expression of PLCs in tissues is critical for any kind of study, as they are strictly tissue specific (Suh et al. 2008; Bunney and Katan 2011) and the expression panel is different in quiescent cells compared to the pathological counterpart (Lo Vasco et al. 2007, 2013, 2014a, b, c, d; 2010a, b). Moreover, the subcellular localization of PLC enzymes differs depending on the isoform and may vary under different

conditions (Suh et al 2008; Lo Vasco et al. 2007; Lo Vasco et al. 2007, 2010b, 2012, 2014a, b, 2015).

In the present experiments, we analyzed the expression of PLC enzymes in unpolarized (M0), M1 and M2 macrophages to list the isoforms expressed in the polarized macrophages and their subcellular localization. Furthermore, we investigated whether inflammatory stimulation modified the basal panel of PLCs' expression and subcellular localization.

Materials and methods

All the experiments were conducted three times in triplicate.

Cell differentiation and culture

Macrophages were differentiated from peripheral blood mononuclear cells (PBMC) obtained from healthy blood donors as previously described (Beyer et al 2012). Briefly, PBMC were isolated by density gradient centrifugation from buffy coats of healthy donors using Ficoll-Paque. CD14⁺ monocytes were purified by incubating with anti CD-14 coated microbeads (Miltenyi Biotec) followed by magnetic sorting, according to the manufacturers protocol. CD14⁺ monocytes were cultured in RPMI1640 medium containing 10% FCS and differentiated into macrophages using GM-CSF (500 U/ml). or M-CSF (100 U/ml). for 3 days. Growth-factor (GF). containing medium was exchanged on day 3 and cells were polarized for 3 days with the following stimuli: IFN- γ (200 U/ml), TNF- α (800 U/ml), ultrapure LPS (LPSu, 10 μ g/ml), IL-4 (1,000 U/ml), IL-13 (100 U/ml). (Beyer et al. (all the reagents were purchased from Sigma-Aldrich, Germany)).).

To assess M1 or M2 polarization macrophages were stained with phycoerythrin- or allophycocyanin-conjugated anti-CD64 and anti-CD86 for M1-associated phenotype or anti-CD23 and anti-CD163 for M2-associated phenotype and analyzed by FACs as previously described (Mantovani et al. 2002a, b). 1×10^6 cells were stimulated with 200 ng/ml LPS (strain 0111:B4 E. Coli) (Sigma-Aldrich). Molecular biology

1.5×10^6 macrophages were used for each experiment. Cells were detached and suspended using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA was extracted with a SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration and purity of the obtained RNA was checked using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Inc. USA).

RNA was reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Foster City, CA, USA) following manufacturer's indications for 10 min at 25 °C, 120 min at 37 °C and 5 min at 85 °C in a Gene Amp® PCR System 9700 thermocycler (Applied Biosystems Inc, Foster City, CA).

Glyceraldehyde 3 phosphate dehydrogenase (*GAPDH*) was used as positive control (Bio Basic Inc, Amherst, New York, USA). The primer pairs (Bio Basic Inc, Amherst, New York, USA) for each PLC isoform and for *GAPDH* gene are listed in Table 1. The specificity of the primers was verified by searching in the NCBI database for possible homology to cDNAs of unrelated proteins. RNA samples were also amplified by PCR without RT to exclude possible contamination. Human osteosarcoma cells 143B and MG63 were used as positive controls to check the efficiency of primers (data not shown). Standard analytical PCR reaction was performed with GoTaq Master Mix (Promega) following manufacturer's instructions. Cycling conditions were performed with 95 °C initial denaturation step for 1 min was followed by 40 cycles consisting of 95 °C denaturation (30s), annealing (30 s) at the appropriate temperature for each primer pair and 72 °C extension (1 min) in Gene Amp® PCR System 9700 (Applied Biosystems) thermocycler. Amplified PCR products were analysed by 1.5% TAE ethidium bromide-stained agarose gel electrophoresis (Agarose Gel Unit, Bio-Rad Laboratories S.r.l., Segrate, IT). A PC-assisted CCD camera (GelDoc 2000 System/Quantity One Software; Bio-Rad) was used for gel documentation and quantification. Optical densities were normalized to the mRNA content of *GAPDH*.

RNA samples were amplified by PCR without reverse transcription. No band was observed, excluding DNA contamination

during the procedure (data not shown). Experiments were independently repeated at least 3 times for each isoform.

Immunofluorescence localization of PLC enzymes

Cells were washed three times with PBS and fixed with 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS) for 10 min at 4 °C, followed by three washes with PBS. Cells were incubated with primary antibodies diluted in PBS for 1 h at room temperature. The primary anti-human PLC antibodies and the appropriated fluorescence conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cover-slips were then incubated with the specific secondary antibody Texas Red or fluorescein-conjugated for 1 h at room temperature. Cells were washed twice with 1X PBS 5 min, then counterstained with 4',6-diamidino-2-phenylindole (DAPI) fluorescent staining. The slides (Ibidi, Germany) were visualized images were visualized and captured with an Olympus IX50 inverted fluorescence microscope (Olympus, Tokyo, Japan) and processed using Adobe Photoshop 7.0 software.

Immunohistochemistry of breast samples

Paraffin embedded samples of surgically excised adenosis affected breast tissue were cut into sections of 5 µm, mounted on

Table 1 List of the PCR primers used for PLC isoforms' amplification

PLC β1 (PLCB1; OMIM *607120)	forward 5'-AGCTCTCAGAACAAGCCTCCAACA-3' reverse 5'-ATCATCGTCGTCGTCACCTTTCCGT-3'
PLC β2 (PLCB2; OMIM *604114)	forward 5'-AAGGTGAAGGCCTATCTGAGCCAA-3' reverse 5'-CTTGCAAACCTCCCAAAGCGAGT-3'
PLC β3 (PLCB3; OMIM *600230)	forward 5'-TATCTTCTTGACCTGCTGACCGT-3' reverse 5'-TGTGCCCTCATCTGTAGTTGGCTT-3'
PLC β4 (PLCB4; OMIM *600810)	forward 5'-GCACAGCACACAAAGGAATGGTCA-3' reverse 5'-CGCATTTCTTCTGCTTTCCCTGTCA-3'
PLC γ1 (PLCG1; OMIM *172420)	forward 5'-TCTACCTGGAGGACCCTGTGAA-3' reverse 5'-CCAGAAAGAGAG CGTGTAGTCG-3'
PLC γ2 (PLCG2; OMIM *600220)	forward 5'-AGTACATGCAGATGAATCACGC-3' reverse 5'-ACCTGAATCCTGATTGACTGC-3'
PLC δ1 (PLCD1; OMIM *602142)	forward 5'-CTGAGCGTGTGGTTCCAGC-3' reverse 5'-CAGGCCCTCGGACTGGT-3'
PLC δ3 (PLCD3; OMIM *608795)	forward 5'-CCAGAACCCTCTCAGCATCCA-3' reverse 5'-GCCA TTGTTGAGCACGTCAGTCA-3'
PLC δ4 (PLCD4; OMIM *605939)	forward 5'-AGACACGTCCCAGTCTGGAACC-3' reverse 5'-CTGCTTCTCTTCTCATATTC-3'
PLC ε (PLCE; OMIM *608414)	forward 5'-GGGGCCACGGTCATCCAC-3' reverse 5'-GGGCCTTCATACCGTCCATCCTC-3'
PLC η1 (PLCH1; OMIM *612835)	forward 5'-CTTTGGTTCGGTTCTTGTGTGG-3' reverse 5'-GGATGCTTCTGTCACTCCTTCC-3'
PLC η2 (PLCH2; OMIM *612836)	forward 5'-GAAACTGGCCTCCAAACACTGCCCGCCG-3' reverse 5'-GTCTTGTGGAGATGCACGTGCCCTTGC-3'

silanized charged slides and allowed to dry for 1 h at RT, followed by 1 h in an incubator at 60 °C. After deparaffinization and rehydration, slides were incubated with proteinase K solution for 5 min. After washing in distilled water, sections were covered for 5 min with 3% H₂O₂ to block endogenous peroxidase, followed by an additional washing procedure with the supplied buffer. For each PLC isoform, slides were incubated for 30 min with the primary antibody directed to the specific isoforms in a humid chamber. After rinses, slides were incubated with the following secondary antibodies: DAKO LSAB Universal LSAB2 Kit/HRP, Rabbit/Mouse, for or Goat-on-Rodent HRP-Polymer (Biocare Medical Products, CA USA). Tissue staining was visualized with a DAB substrate chromogen solution. Slides were counterstained with hematoxylin, dehydrated, and mounted.

Results

Molecular biology

GAPDH transcript was always detected as expected (Fig. 1).

M0. The transcripts of the following isoforms' genes were detected: PLCB2, PLCB3, PLCG1, PLCG2, PLCD1, PLCH1 and PLCH2. PLCB1 and PLCB4 were detected, although the intensity of the gel electrophoresis band was very weak. After treatment with LPS, no transcript was visualized for PLCB1, PLCB3, PLCG1 and PLCD1. By contrast, PLCD3 was detected. The intensity of the gel electrophoresis band for PLCG2 mRNA was increased (Fig. 1) Quantification of the band with ImageJ program indicates a mean 50% increase with compared to untreated cells.

M1. The transcripts of the following isoforms' genes were detected: PLCB1, PLCB2, PLCB3, PLCG1, PLCG2, PLCD1, PLCD3, PLCH1 and PLCH2. After treatment with LPS, PLCB4 transcript was detected and PLCD1 transcript was not detected (Fig. 1). The intensity of the gel electrophoresis band for PLCG2 mRNA was increased. Quantification of the band with ImageJ program indicates a 43% increase with compared to untreated cells.

M2. The transcripts of the following isoforms' genes were detected: PLCB1, PLCB2, PLCB3, PLCG1, PLCG2, PLCD3, PLCH1 and PLCH2. After treatment with LPS, PLCD1 was detected (Fig. 1).

Immunofluorescence localization of PLC enzymes

M0: PLC β1, PLC β2, PLC δ4, and PLC η1 proteins were detected in the cytoplasm. Also PLC β3 was detected in the cytoplasm, although the signal appeared weak. PLC β4 and PLC ε were detected in vesicles located in the cytoplasm. PLC β4 also showed an inconstant nuclear localization and was observed in long, thin protrusions from the

Fig. 1 Gel electrophoresis of RT-PCR results. For PLCB4 line, please note that although the image was cut the experiment and gel were the same

membrane. PLC γ2 and PLC γ1 were detected in a subtle perinuclear halo. PLC γ1 was also observed in long, thin protrusions from the membrane. PLC η2 was detected in the cytoplasm with a punctuate distribution, corresponding to the Golgi apparatus (Fig. 2), and in membrane protrusions. The remaining PLC isoforms were not detected within the cells.

After LPS treatment PLC β1 was distributed as an evident perinuclear halo. PLC δ1, absent in untreated cells, was detected in the cytoplasm. PLC η2, present in untreated cells, was not detected (Fig. 3).

M1: PLC β1, PLC β2, PLC β4, PLC δ1 and PLC ε were detected in the cytoplasm. Also PLC β3 and PLC δ3 were detected in the cytoplasm, although the signal appeared weak. PLC β4 was observed in long, thin protrusions from the membrane. PLC γ1, PLC γ2 were detected in the cytoplasm. PLC d4 was detected in the cytoplasm and also observed in thin membrane

Fig. 2 Immunofluorescence analysis of PLC isoforms in M0, M1 and M2 macrophages (40X). PLC isoforms are detected with fluorescence antibodies (red or green) and nuclei are marked with DAPI counterstain (blue)

protrusions extending among cells. PLC $\eta 1$ was located around the nucleus. PLC $\eta 2$ was localized in the Golgi apparatus (Fig. 2).

After LPS treatment, a stronger signal was detected for PLC $\beta 1$ distributed in the cytoplasm. No localization or gross visual intensity differences were identified for PLC $\delta 1$ and PLC $\delta 3$. PLC $\eta 1$ was localized in the cytoplasm; in some cells it was also detected in the nucleus. PLC $\eta 2$ was localized in the cytoplasm (Fig. 3).

The CD68 co-localization experiment confirmed the perinuclear localization of PLC $\eta 1$, as well as confirmed the distribution of PLC $\gamma 2$ in the Golgi apparatus and the presence

in nanotubes. The CD-68 co-localization experiment confirmed the presence of PLC $\gamma 1$ in the perinuclear region; the enzyme was detected in vesicles co-localizing with CD-68. PLC $\eta 2$ resulted polarized in one side of the cytoplasm and localized also in the cell membrane (Fig. 4).

M2: PLC $\beta 1$ was strongly expressed and localized in the cytoplasm. Also PLC $\beta 2$, PLC $\beta 3$, PLC $\gamma 1$, and PLC $\delta 4$ were localized in the cytoplasm, as well as PLC $\delta 3$ was weakly visualized in the cytoplasm. PLC $\beta 4$ was localized in granules in the cytoplasm; it was located in the nuclei of giant cells and PLC $\beta 4$ also showed an inconstant nuclear localization and was observed

Fig. 3 Immunofluorescence analysis of PLC isoforms in M0, M1 and M2 macrophages cultured with LPS (40X)

in long, thin protrusions from the membrane. PLC γ 1 was located in the cytoplasm and focally in the Golgi apparatus. PLC δ 1 was located in the perinuclear region, both diffusely and located in perinuclear vesicles. PLC ϵ was weakly detected both in the nucleus and cytoplasm. PLC η 1 was detected as a perinuclear halo. PLC η 2 was detected in selected areas of the cytoplasm (Fig. 2).

After LPS treatment, no localization or gross visual intensity differences were identified for PLC β 1 and PLC δ 3. PLC δ 3 was localized in the cytoplasm. PLC η 1 was weakly visible in the cytoplasm. PLC η 2 was localized in the cytoplasm and in the Golgi apparatus (Fig. 3).

Immunohistochemistry of breast samples

Fat necrosis was evident and foamy cells were observed. PLC β 1, PLC β 3, PLC γ 1, PLC δ 1, PLC δ 4, PLC ϵ , PLC η 2 were expressed with a strong signal; signal was evident within the cells and less in the stromal component (Fig. 5).

Fig. 4 Immunohistochemistry analysis of PLC isoforms in adenosis affected breast tissue (10X). Foam cells are visible in necrosis areas

PLC β 2 was detected with a weak signal, reinforced in the stromal component; it was evident in the cytoplasm of multinucleated cells. PLC β 4 was detected with a strong signal, almost absent in the stromal component; it was detected within the cytoplasm or as membrane reinforcement (Figs. 5 and 6).

PLC γ 2 was detected with a weak signal; it was detected within the cytoplasm or as membrane reinforcement. PLC δ 3 was not detected. PLC η 1 was detected with a strong signal, although it was almost absent in the stromal component; it was detected within the cytoplasm or as membrane reinforcement; it was not detected in multinucleated cells (Fig. 5).

Discussion

The present results demonstrate that M0, as well as M1 and M2 cells own a specific panel of expression, different for both genes' mRNA expression and intracellular localization of

Fig. 5 Giant cells in breast tissue (left, immunohistochemistry, 40X) and in macrophage culture (right, immunofluorescence, nuclei are identified with DAPI counterstain, 40X)

PLC enzymes. All PLC enzymes were detected within both M1 and M2 cells, but not in M0 cells.

Our results also indicate that the panel of PLC genes' expression and PLC proteins' presence slightly changes after inflammatory stimulation. The three analyzed macrophages cell lines share some changes after inflammatory stimulation. However, changes of selected PLC isoforms after inflammatory stimulation differed in M0, M1 and M2 macrophage lines. That might accord to previous observations indicating that in murine

peritoneal macrophages PLCB1 transcription and PLC β 1 levels were reduced by LPS stimulation (Grinberg et al 2009), probably following post-transcriptional regulation.

In M0 transcripts for many PLC isoforms were detected (Table 2). The LPS inflammatory stimulation induced some differences in the expression of PLC genes (Table 2). PLC enzymes were detected within the cells, excepting for PLC δ 1 and PLC δ 3 (see Table 3). Therefore, the transcript of PLCD1 was detected, no PLC δ 1 enzyme was within the cells, probably indicating a complex post-transcriptional regulation. Accordingly to previous literature data (Aki et al 2008), inflammatory stimulation increased the expression of PLCG2. Interestingly, peculiar localization of selected PLC isoforms was identified. PLC β 1, PLC β 2, PLC β 3 PLC δ 4, and PLC η 1 enzymes localized in the cytoplasm. PLC β 4 and PLC ϵ were located in cytoplasmic vesicles. PLC γ 1 and PLC γ 2 were detected in a subtle perinuclear halo. PLC η 2 was detected in the cytoplasm with a punctuate distribution, corresponding to the Golgi apparatus. The LPS inflammatory stimulation induced some differences in PLCs' localization in M0 cells. PLC β 1 enzyme was distributed as a strong perinuclear halo. PLC δ 1, absent in untreated cells, was detected in the cytoplasm. PLC η 2, present in untreated cells, was not detected.

In M1 transcripts for many PLC genes were detected (Table 2). The LPS inflammatory stimulation induced some differences. (Table 2). Surprisingly, all PLC enzymes were detected within the cells (Table 3), most in the cytoplasm.

Fig. 6 Colocalization of CD-68 (TRITC conjugated antibody, green) and PLC isoforms (Texas red conjugated antibody, red). Nuclei are counterstained with DAPI (blue) [40X]

Table 2 PCR results of transcript analyses in M0, M1 and M2 macrophages, untreated (nt) and after LPS treatment (LPS). On the left column, the analyzed genes. (+): transcript visualized as a band with gel electrophoresis. (+/-): a weak band was visualized. (-): absent transcript. (>): the band intensity was increased compared to the corresponding untreated band [for description please refer to the text]

	M0		M1		M2	
	nt	LPS	nt	LPS	nt	LPS
GAPDH	+	+	+	+	+	+
PLCB1	+/-	-	+	+	+	+
PLCB2	+	+	+	+	+	+
PLCB3	+	-	+	+	+	+
PLCB4	+/-	+/-	-	+	-	-
PLCG1	+	-	+	+	+	+
PLCG2	+	+(>)	+	+(>)	+	+
PLCD1	+	-	+	-	-	+
PLCD3	-	+	+	+	+	+
PLCD4	-	-	-	-	-	-
PLCE	-	-	-	-	-	-
PLCH1	+	+	+	+	+	+
PLCH2	+	+	+	+	+	+

On the left column, the analyzed genes. (+): transcript visualized as a band with gel electrophoresis. (+/-): a weak band was visualized. (-): absent transcript. (>): the band intensity was increased compared to the corresponding untreated band [for intensity description please refer to the text]

Table 3 Differences in the detection of mRNA of PLC genes and corresponding PLC proteins. *GAPDH* gene was used as a control. CD68 antibody was used to mark macrophages

	M0		M1		M2	
	mRNA	protein	mRNA	protein	mRNA	protein
<i>GAPDH</i>	+	Na	+		+	
CD68	Na	+	Na	+	Na	+
PLC β 1	+/-	+	+	+	+	+
PLC β 2	+	+	+	+	+	+
PLC β 3	+	+/-	+	+/-	+	+/-
PLC β 4	+/-	+	-	+	-	+
PLC γ 1	+	+/-	+	+	+	+
PLC γ 2	+	+	+	+	+	+
PLC δ 1	+	-	+	+	-	+
PLC δ 3	-	-	+	+/-	+	+/-
PLC δ 4	-	+	-	+	-	+
PLC ϵ	-	+	-	+	-	+/-
PLC η 1	+	+	+	+	+	+
PLC η 2	+	+	+	+	+	+

(+) presence of mRNA and/or protein. (-) absence of mRNA or protein. . (+/-): a weak band was visualized analyzing the mRNA content with gel electrophoresis. Na = not applicable

PLC γ 1, PLC γ 2 and PLC δ 4 were detected in the cytoplasm and in nanotubes extending among cells. PLC η 1 was strongly evident and located around the nucleus. PLC η 2 was localized in the Golgi apparatus.

The LPS inflammatory stimulation induced slight differences in the localization of PLC enzymes. A strong signal was detected for PLC β 1 enzyme in the cytoplasm. PLC η 1 was localized in the cytoplasm; in some cells it was also detected within the nucleus. PLC η 2 was localized in the cytoplasm.

In M2 transcripts for a number of PLC genes were detected (Table 2). The LPS inflammatory stimulation induced slight differences in PLC genes' expression of M2 cells (Table 2). All PLC enzymes were detected within the cells, most in the cytoplasm. Some PLC enzymes had peculiar cytoplasmic location. PLC β 4 was localized in granules in the cytoplasm and in the nuclei of giant cells. PLC γ 1 was located in the cytoplasm focally in the Golgi apparatus. PLC δ 1 was located in the perinuclear area and in the Golgi apparatus. PLC ϵ was weakly detected both in the nucleus and cytoplasm. PLC η 1 was detected as a perinuclear halo. PLC η 2 was detected in selected areas of the cytoplasm, with diffusely complex localization pattern, highly concentrated close to the plasma membrane, in fibres and in punctuate accumulations polarized at one side of the cytoplasm, probably a podosome location. Slight differences were observed in the enzymes' distribution after LPS treatment. PLC δ 3 was localized in the cytoplasm. PLC η 1 was weakly detected in the cytoplasm. PLC η 2 was localized in the cytoplasm and in the Golgi apparatus.

Treating cells with inflammatory stimuli induced variations in the panel of expression of selected PLC genes which differed depending on the polarization. The most relevant effects of inflammatory stimulation were observed in M0 cells. In fact, inflammatory stimulation produces relevant variations of gene expression in M0 cells, probably related to differentiation induction. The variations of PLC genes' expression might be related to differentiation changes. Differently from M1 and M2, in M0 the low transcription of PLCB1 and the transcriptions of PLCB3 and PLCG1 were stopped. By contrast, the transcription of PLCD3, unexpressed in untreated M0 cells, was observed.

PLC β 4 was low expressed in M0 and not expressed in untreated M1 and M2. That accords to literature data indicating specific nervous tissue specificity for this enzyme. However, after inflammatory stimulation, PLCB4 transcription was identified in M1 cells. That might indicate a specific role for the PLC β 4 enzyme during inflammation related to the pro-inflammatory arm of the macrophage differentiation.

The inflammatory stimulation induced the most interesting differences regarding the PLC δ 1 enzyme. We identified the PLC δ 1 protein within the cell, in the peri-nuclear region, both diffusely and located in vesicles. After inflammatory stimulation, PLCD1 was transcribed and the PLC δ 1 protein was located at the perinuclear area, although the vesicles were

reduced and the distribution was more uniformly distributed around the nuclei. The observation that PLCD1 transcription started after inflammatory stimulation might indicate that the store of PLC $\delta 1$ enzyme needed to be replaced or increased during inflammation, suggesting a specific role of this enzyme. By absolute contrast, the same enzyme behaved oppositely in M0, where the PLCD1 transcript was detected and the corresponding enzyme was not present within the cell. After inflammatory stimulation, the transcription of PLCD1 stopped. However, the corresponding enzyme was detected within the cells, indicating that the protein translation was completed. In M1, both the PLCD1 transcript and the corresponding enzyme were detected and inflammatory stimulation did not induce differences. All those observations suggest that recruitment of PLC $\delta 1$ enzyme might play a different and specific role in M0, M1 and M2, especially during inflammation, more probably related to the anti-inflammatory arm of the macrophage activity. Recently, Kudo et al. demonstrated that in mouse macrophages PLC $\delta 1$ negatively regulates the LPS induced production of pro-inflammatory cytokines, such as IL-1 β , and Fc γ receptor-mediated phagocytosis (Kudo et al. 2015). In mouse keratinocytes, the absence of *plcd1* leads the cells to overproduce interleukin (IL)-23, an inflammatory cytokine inducing a psoriasis-like inflammation of the skin (Kanemaru et al. 2012). It was suggested that PLC $\delta 1$ might be related to inflammation involved molecules playing a sort of preventive role. Our present results might accord to this hypothesis. In fact, quiescent M0 and M1 cells own both PLC $\delta 1$ enzyme and transcript. After inflammatory activation both M0 and M1 do not express PLCD1. By contrast, quiescent M2 cells do not express PLCD1, while after LPS activation the gene was transcribed. Interestingly, previous findings indicated that PLCD1 transcription was suppressed after Fibroblast Growth Factor stimulation in human endothelial cells (Lo Vasco et al. 2014a, b, c, d). Moreover, the non linear correspondence between the mRNA and the enzyme suggest a complex regulation of the expression of PLCD1 gene, probably at a post-transcriptional level (Table 3).

Both PLC $\eta 1$ and PLC $\eta 2$ are expressed in macrophages. Treating the three analyzed cell lines with inflammatory stimuli did not seem to induce differences in the expression of *PLCH1* or *PLCH2* genes (Table 2). However, the sub-cellular distribution of PLC η enzymes deserved attention. PLC $\eta 1$ was detected in M0 cells' cytoplasm. PLC $\eta 2$ was diffusely localized in the cytoplasm with a complex localization pattern, highly concentrated close to the plasma membrane, in fibres and in punctuate accumulations. Marking the cytoplasm with CD-68 antibody, we observed that PLC $\eta 2$ seems also to be polarized at one side of the cytoplasm. Interestingly, the side portion of cytoplasmic PLC $\eta 2$ localization resembles the structure of podosome, a specialized region of the F-actin cytoskeleton that mediate local adhesion, invasion, and migration

in specific cell types, including macrophages, (Miyauchi et al. 1990; Evans and Matsudaira 2006; Calle et al 2006; Tsuboi 2007; Carman et al 2007).

However, after LPS treatment PLC $\eta 2$ was not detected, probably indicating depletion of the cell store. That suggests stored PLC $\eta 2$ was actively acting and worn out during the activation of cells. In M1 cells, after LPS treatment, PLC $\eta 2$ was diffusely localized in the cytoplasm and the accumulations were more evidently reorganizing. PLC η enzymes are the most recent class of mammalian PLC enzyme to be identified, owning a peculiar structure (Popovics et al 2014). Isoenzymes belonging to the η subfamily are the closest to the PLC δ subfamily (Hwang et al. 2005; Nakahara et al. 2005; Stewart et al. 2005; Zhou et al., 2005). However, the function of these enzymes is not entirely clear. PLC η enzymes are sensitive to changes in $[Ca^{2+}]_i$ within the physiological range (Kim et al. 2011; Popovics et al. 2011; Hwang et al. 2005; Nakahara et al. 2005). That might be related to the presence of specific EF domain features (Popovics et al 2014). PLC η enzymes can modulate further (or sustained) release of Ca^{2+} from intracellular stores through IP_3 production. Notably, our present results indicate that more than other PLC enzymes PLC η show a polarized distribution. In fact, beside the membrane localization, CD-68 co-localization experiment also detected PLC $\eta 2$ side-polarized in the cytoplasm. The observation that a complex reorganization of the regulation pattern of enzymes belonging to η and δ subfamilies, which are known to be the most sensitive to calcium levels, occurs might deserve attention.

Finally, we observed the presence of selected PLC isoforms, namely PLC $\beta 4$, $\gamma 1$, $\delta 4$ and PLC $\eta 1$, in thin and long cell protrusions originating from the plasma membrane probably involved in the long-distance intercellular connectivity (Fig. 2).

Our data overall indicate that PLC enzymes might play a complex role in macrophages during inflammation and probably also in polarization. The different expression of selected PLC genes, as well as the different cell localization of the corresponding enzymes might suggest that changes in the PLC enzymes' might be related to the differentiation stages of macrophages. Our results also suggest that PLC enzymes might be involved in the long distance protrusions of macrophages and in podosome structures.

Further studies are required in order to verify the relationship among the PLC family, the macrophage differentiation and inflammation, highlighting the role of each PLC enzyme. It remains to be clarified whether the observed PLCs' expression differences might be causally related to the macrophage differentiation or simply might follow this event. Further investigations will also allow indicate whether PLCs' expression might be useful in characterizing the differentiation stages of macrophages.

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