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PLTP regulates STAT3 and NFκB in differentiated THP1 cells and human monocyte-derived macrophages

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ABSTRACT

Phospholipid transfer protein (PLTP) plays an important role in regulation of inflammation. Previously published studies have shown that PLTP binds, transfers and neutralizes bacterial lipopolysaccharides. In the current study we tested the hypothesis that PLTP can also regulate anti-inflammatory pathways in macrophages. Incubation of macrophage-like differentiated THP1 cells and human monocyte-derived macrophages with wild-type PLTP in the presence or absence of tumor necrosis factor alpha (TNFα) or interferon gamma (IFNγ) significantly increased nuclear levels of active signal transducer and activator of transcription 3, $pSTAT3_{Tyr705}$ ($p<0.01$). Similar results were obtained in the presence of a PLTP mutant without lipid transfer activity ($PLTP_{M159E}$), suggesting that PLTP-mediated lipid transfer is not required for activation of the STAT3 pathway. Inhibition of ABCA1 by chemical inhibitor, glyburide, as well as ABCA1 RNA inhibition, reversed the observed PLTP-mediated activation of STAT3. In addition, PLTP reduced nuclear levels of active nuclear factor kappa-B (NFκB) p65 and secretion of pro-inflammatory cytokines in conditioned media of differentiated THP1 cells and human monocyte-derived macrophages. Our data suggest that PLTP has anti-inflammatory capabilities in macrophages.

1. Introduction

Macrophages are the key immune cells in organs and tissues, are involved in the innate immune response, and play a critical role in both initiation and resolution of inflammation. Activation of the macrophage-dependent immune response is of decisive importance in acute infections. However, prolonged activation of macrophages associated with chronic disease is often detrimental, and contributes to the pathophysiological processes of numerous chronic diseases, including atherosclerosis, chronic kidney disease and diabetes. Therefore, understanding the physiological and pathophysiological processes that regulate the macrophage-dependent immune response is a significant element in the formulation of novel prophylactic and therapeutic approaches for many chronic diseases.

Phospholipid transfer protein (PLTP) is a versatile, widely expressed protein involved in transport of lipids and vitamin E among lipoproteins, and between lipoproteins and cells. Reported functional roles of PLTP include participation in reverse cholesterol transfer, lipoprotein metabolism, inflammatory processes, signal transduction, cell differentiation and apoptosis [1–8]. PLTP levels and activity are significantly altered in numerous human diseases and conditions with a strong inflammatory component, such as atherosclerosis, diabetes, and neuroinflammatory diseases [9–16]. These reports suggest that PLTP may be playing an important role in the regulation of inflammatory processes, although the mechanisms of PLTP actions are currently poorly understood.

PLTP is expressed in macrophages in both normal and atherogenic blood vessels [9,17,18]. Recent reports suggest that macrophage-derived PLTP plays an important role in atherosclerosis [19,20]. However, the interplay between macrophages and PLTP is inadequately understood. The macrophage-dependent immune response is regulated by signal transduction pathways dependent on extracellular ligands and other pathway components that activate or inactivate signal transduction processes. We previously reported that PLTP interacts with the ATP-binding cassette A1 (ABCA1) to enhance cholesterol efflux from cells, and that PLTP's interaction with ABCA1 activates Janus kinase-2, JAK2 [21,22]. In this study we provide evidence that PLTP activates STAT3, and inhibits NFκB in macrophage-like THP1 cells and in human monocyte-derived macrophages.

Abbreviations: ABCA1, ATP-binding cassette transporter A1; IFNγ, interferon gamma; IL1α, interleukin 1 alpha; JAK2, Janus kinase-2; LPS, lipopolysaccharides; MIP1α, macrophage inflammatory protein 1 alpha; NFκB, nuclear factor kappa-B; PLTP, phospholipid transfer protein; PMA, phorbol 12-myristate 13-acetate; STAT3, signal transducer and activator of transcription 3; TBP, TATA binding protein; TGFβ, tumor growth factor beta; TLR, Toll-like receptor; TNFα, tumor necrosis factor alpha

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2. Materials and methods

2.1. Antibodies, cells, and materials

Antibodies against phosphorylated JAK2 (Cat. No. 3771), STAT3 (Cat. No. 4904), STAT3 phosphorylated at tyrosine 705 (Cat. No. 9131), NFκB p65 total and phosphorylated at serine 536 (Cat. Nos. 3034 and 3033, respectively), IL1β (Cat. No. 2022), TGFβ (Cat. No. 3711), and human TNFα (Cat. No. 2169) were obtained from Cell Signaling Technology (Danvers, MA). Antibody against JAK2 was obtained from Santa Cruz Biotechnologies (Cat. No. SC-34479-R) TATA-binding protein (TBP) and MIP1α antibodies (Cat. Nos. 62125; and ab0381, respectively) were from Abcam (Cambridge, MA). Anti-IL6 (Cat. No. AF-206-NA) and anti-IL10 antibodies (Cat. No. AF-217-NA), as well as IL1β, MIP1α and TGFβ ELISA plates (Cat. Nos. HSLB00C, DMA00 and DB100B, respectively) were purchased from R&D Systems (Minneapolis, MN). Antibody against β-actin (Cat. No. A2066), interferon gamma (IFNγ; Cat. No. I32265), PMA (Cat. No. P8139) and glyburide (Cat. No. G2569) were from Sigma-Aldrich (St. Louis, MO). Secondary antibodies, anti-rabbit, anti-mouse and anti-goat TrueBlot (Cat. Nos. 18-8816; 18-8817; and 18-8814, respectively), and recombinant human M-CSF and GM-CSF (Cat. Nos. 14-8789 and 14-8339, respectively) were from eBioscience Inc. (San Diego, CA). Human ABCA1 shRNA Mission lentiviral transduction particles and control Mission pLKO.1-puro transduction particles were from Sigma-Aldrich St. Louis, MO (Cat. Nos. SHCLNV-NM_005502 and SHC001V, respectively), as well as puromycin (Cat. No. P9620). Protein isolation kit, NE-PER, HALT protease inhibitor cocktail, Detoxi-Gel endotoxin removing column (Cat. No. 20344) and SuperSignal West Femto maximum sensitivity substrate were obtained from Thermo Scientific/Pierce Biotechnology (Rockford, IL). Phosphatase inhibitor cocktail (Cat. No. 524627) and PhosphoSafe protein isolation solution were purchased from Calbiochem-EMD BioSci (La Jolla, CA). Criterion XT gels, MOPS buffer and XT sample buffer were obtained from Bio-Rad (Hercules, CA). PhosphoBlocker blocking reagent (Cat. No. AKR-104) was from Cell Biolabs (San Diego, CA). Human mononuclear cells PBMC (Cat. No. 1001) were procured from Astarte Biologics (Redmond, WA). Specialized macrophage medium, macrophage-SFM, was purchased from Invitrogen (Carlsbad, CA). RPMI-1640 media, L-glutamine, non-essential amino-acids, and trypsin–versene were obtained from Lonza Biosciences (Walkersville, MD). Nutridoma-SP (Cat. No. 11011375001) was from Roche Applied Science (Indianapolis, IN). Characterized fetal bovine serum with endotoxin levels below biologically significant concentration was acquired from Thermo Fisher Scientific/Hyclone (Logan, UT). [C^{14}]-labeled phosphatidylcholine was purchased from Perkin Elmer Life Sci. Pyrochrome *Limulus polyphemus* Amoebocyte Lysate (LAL) kit (Cat. No. C1500), endotoxin standards (*E. coli* O113-H10; Cat. No. EC010), LAL reagent water, Pyroplate microplates, endotoxin-free pipette tips and borosilicate glass tubes were from the Associates of Cape Cod Inc. (East Falmouth, MA). Multiplex kits for human cytokines and chemokines were purchased from Linco Research (St. Charles, MO) and Bio-Rad (Hercules, CA). Recombinant wild-type and mutant PLTP were expressed and isolated as previously reported [1,23].

2.2. Cell culture

The human monocytic leukemia cell line THP1 (ATCC Cat. No. TIB-202) was grown in RPMI media supplemented with 10% fetal bovine serum or Nutridoma, at 37 °C in 5% CO_2. No differences were observed in cells grown with Nutridoma compared to serum supplementation (not shown). THP1 cells in suspension were seeded at equal density into multiwell plates, differentiated into macrophage-like cells by addition of PMA (1.6×10^{-7} M) for 72 h, and then used in experiments. These THP1 cells were pre-incubated with PLTP (5 μg/ml) for 4 h, extensively washed with PBS, and then exposed to either 100 ng/ml LPS, 10 ng/ml IFNγ, or 100 ng/ml TNFα for up to 24 h. Similar studies were performed using co-incubation protocols. In time-dependent studies for both modalities (pre- and co-incubation) the optimal time for our experiments was evaluated. Chosen protocols were confirmed by experiments under identical conditions using fully developed human monocyte-derived macrophages 8 days after attachment, which had been grown in Macrophage-SFM medium, supplemented with GM-CSF. The cells were pre-incubated with PLTP in SFM medium for 4 h, extensively washed and then incubated for up to 24 h with TNFα. The resulting conditioned media had reduced IL-1β levels, indicating that pre-incubation of human monocyte-derived macrophages with PLTP reduces secretion of pro-inflammatory cytokines similar to THP1 cells (see Results), thus validating our cell culture data obtained in the THP1 cell line. All cell culture experiments were performed in triplicate for each condition, and repeated at least three times to confirm our results.

2.3. ABCA1 RNA inhibition

Inhibition of ABCA1 expression in THP1 cells was performed using human ABCA1 shRNA Mission lentiviral transduction particles (five constructs: TRCN0000029089, TRCN0000029090, TRCN0000029091, TRCN0000029092 and TRCN0000029093) and control Mission pLKO.1-puro transduction particles (Sigma-Aldrich, St. Louis, MO). We initially established the concentration of the selection agent (puromycin) required to kill 100% of THP1 cells. Cells were incubated for 48 h in growth medium without (control) or with puromycin (0.1–1 μg/ml). Cell survival was assessed by Trypan blue. All cells were dead in the presence of 0.6 μg/ml of puromycin, and this concentration of puromycin was used in the selection protocol.

Transduction of THP1 cells was performed by spinoculation (transduction by centrifugation; modified from [24]). THP1 cells (3×10^5 cells/ml) grown in suspension in RPMI medium supplemented with 10% FBS were placed in sterile conical 15 ml tubes (6 tubes; 2 ml of cells in each tube). Lentiviral particles (11 μl/ml of cells) were added to the cells and centrifuged at 1000 ×*g*, 28 °C for 1 h. Medium containing lentiviral particles was aspirated, cells mixed with fresh growth medium (RPMI, 10% FBS) and placed in a 6-well plate. Cells were incubated at 37 °C, 5% CO_2, 95% humidity for 48 h. Following incubation, the spinoculation was repeated and cells placed in puromycin-containing medium (0.6 μg/ml) for selection of clones containing puromycin-resistant constructs. Control THP1 cells were subjected to the same procedure, but without exposure to the virus or puromycin. Cell viability was tested after 48 h, and all samples contained viable cells. Clones were expanded in the presence of puromycin, and tested for ABCA1 by qRT-PCR and Western blotting. Based on these studies, THP1 cells in which ABCA1 was inhibited with construct TRCN0000029089 were selected for analysis.

Following successful transduction, spinoculation control THP1 cells, THP1 cells containing control (mock) or ABCA1 shRNA construct were incubated with PMA for 72 h in the presence of puromycin. Adherent THP1 macrophages were used for evaluation of the PLTP effect on activation of STAT3.

2.4. Protein isolation

Cytoplasmic and nuclear proteins were isolated using the NE-PER kit according to the manufacturer's instructions, with addition of phosphatase inhibitor cocktail to preserve phosphorylated proteins. Membrane proteins were isolated using MEM-PER kit according to the manufacturer's instructions. Whole cell lysates were prepared using PhosphoSafe protein isolation solution, according to the manufacturer's instructions. Samples were evaluated for total protein concentration, and aliquots stored at −70 °C until analysis.

2.5. JAK2 immunoprecipitation and Western blotting

THP1 cells were lysed in Tris–HCl buffer (50 mM Tris–HCl, pH 7.4, 120 mM NaCl, 1% Nonidet P40) supplemented with protease inhibitors (Complete mini; Roche) and phosphatase inhibitors (phosphatase inhibitor cocktail II; Calbiochem) and then centrifuged at 15,000 ×*g* for 10 min at 4 °C. Protein concentration was measured using the Bio-Rad protein assay reagent as instructed. For immunoprecipitation, 100 μg of lysates was incubated with the indicated JAK2 antibody (2–4 μg) overnight at 4 °C followed by incubation for 1 h with Protein A-magnetic beads (Invitrogen, Dynabeads protein A). Immunocomplexes were resolved by SDS-PAGE and immunoblotted with phospho-JAK2 (Cell Signaling), then stripped and re-probed with JAK2 antibodies.

2.6. Electrophoresis, Western blot

Proteins were isolated using PhosphoSafe protein isolation solution, which preserves phosphorylated sites in the presence of protease inhibitors (Halt protease inhibitor cocktail, Invitrogen), according to the manufacturer's instructions. Cell proteins were subjected to denaturing sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis as follows: samples were loaded on 4–12% gel (Bis–Tris Criterion XT), and following electrophoresis, transferred to nitrocellulose or PVDF membrane using Bio-Rad wet or semi-dry transfer. Blots were incubated overnight with primary antibodies at 4 °C with agitation in PBS with 5% PhosphoBlocker, a specialized blocker that preserves availability of phosphorylated sites for antibody binding, or with dry milk (the same blocker was used in all incubation steps). Protein loading was controlled by using β-actin (cytoplasmic) or TBP (nuclear proteins). The membranes were incubated with the appropriate TrueBlot secondary antibody for 2 h at room temperature. The blots were developed using West Femto SuperSignal chemiluminescent kit, scanned on a Kodak CF400 imaging system, and evaluated by densitometry scanning.

2.7. PLTP activity assay

Phospholipid transfer activity of PLTP was performed as reported [25]. One hundred microliters of conditioned cell culture medium per sample was used to measure transfer of radioactively-labeled phosphatidylcholine ([C^{14}]PC) to a high density lipoprotein (HDL) acceptor. Triplicate samples were analyzed in triple technical replicates, and counted in Beckman-Coulter LS6500 scintillation counter three times. Samples were held frozen at −70 °C until analysis, and each sample was thawed only once.

2.8. Limulus Amoebocyte Lysate, LAL assay

All materials employed in our experiments, including rPLTP, media and sera, were tested with the LAL assay to confirm that the observed findings are not due to the known PLTP-mediated inactivation of bacterial lipopolysaccharides, LPS [1,26]. The assay was performed according to the manufacturer's instructions. The endotoxin levels in all of the above were below biologically relevant LPS levels (<0.05 EU/ml or <0.03 EU/μg of rPLTP).

2.9. Multiplex analyses

Levels of secreted cytokines in conditioned media were assessed by Bio-Plex System Luminex xMAP technology (Bio-Rad) using Bio-Rad and Linco plates. Two hundred microliters of conditioned media were incubated in wells containing beads labeled with antibodies against multiple pro-inflammatory markers, including IL1α, IL1β, IL1ra, IL6, GM-CSF, TGFα, TNFα, MIP1α, interferon gamma (IFNγ), and VEGF. Triplicate samples for each condition were assayed in triple replicates, and multiple analyses were performed using the same samples. Positive results were confirmed by Western blotting or ELISA.

2.10. ELISA

Levels of IL1β, MIP1α and TGFβ in conditioned media were assessed by commercial ELISA kits according to the manufacturer's instructions (R&D Systems).

2.11. Statistical analyses

Statistical analyses were performed with Statistica for Windows (StatSoft Inc., 2000, Tulsa, OK), using *t*-test, Mann–Whitney *U* test and Wilcoxon matched pair test, and p-values<0.05 were considered statistically significant.

3. Results

3.1. PLTP induces activation of STAT3 in macrophage-like THP1 cells and primary human monocyte-derived macrophages

We have previously shown that PLTP activates JAK2 in J774 macrophages [22]. In this study we confirm that PLTP activates JAK2 in THP1 cells as well (Fig. 1).

To test whether PLTP induces activation of STAT3 in macrophages, we incubated THP1 cells without or with PLTP, and evaluated nuclear levels of STAT3 phosphorylated at tyrosine 705 ($pSTAT3_{Tyr705}$), the active form of STAT3 (Fig. 2A) [27–29]. Addition of PLTP significantly increased levels of $pSTAT3_{Tyr705}$ in nuclei of THP1 cells, suggesting that PLTP induces activation of STAT3. Similar effects were observed in human monocyte-derived macrophages (Fig. 2B).

3.2. PLTP reverses pro-inflammatory action of IFNγ and TNFα on nuclear levels of $pSTAT3_{Tyr705}$ in differentiated THP1 cells and human monocyte-derived macrophages

Following the above findings, we evaluated effects of PLTP on IFNγ-induced inactivation of STAT3. Addition of PLTP reversed IFNγ-induced reduction of $pSTAT3_{Tyr705}$ nuclear levels (Fig. 2C). Similar results were obtained in human monocyte-derived macrophages incubated with tumor necrosis factor alpha (TNFα) (Fig. 2D).

3.3. PLTP effect on intranuclear levels of active STAT3 does not depend on PLTP phospholipid transfer ability

We evaluated whether PLTP-mediated lipid transfer is required for the observed increase in levels of active STAT3 in the nucleus. We

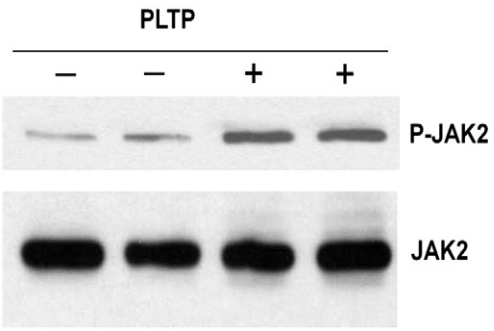

Fig. 1. PLTP activates Janus kinase 2 (JAK2) in THP1 macrophages. THP1 cells were differentiated using PMA for 72 h and incubated without or with PLTP (5 μg/ml) for 30 min. Isolated protein was immunoprecipitated using antibody against JAK2 and blotted against phosphorylated and total JAK2.

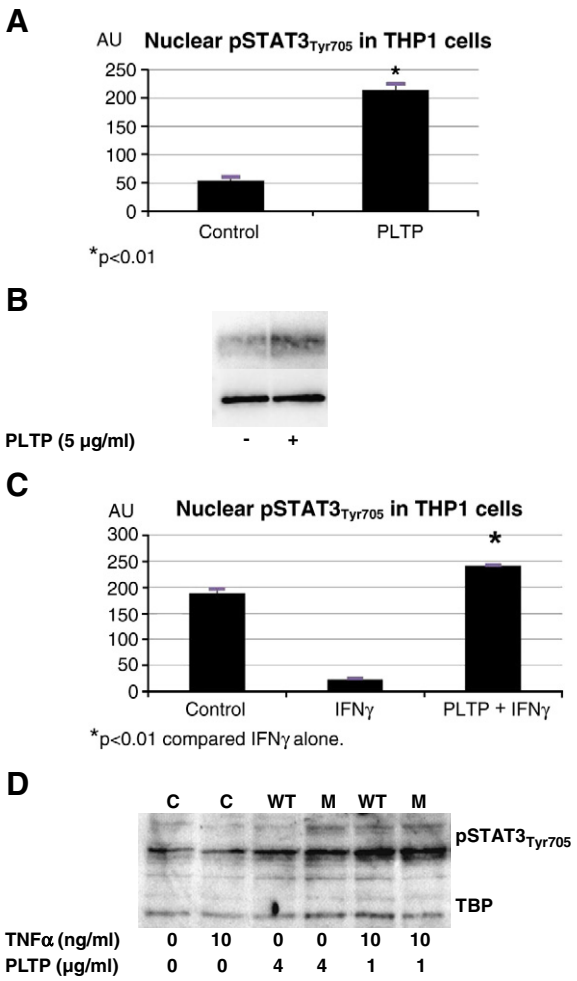

Fig. 2. PLTP increases levels of nuclear pSTAT3$_{Tyr705}$ and reverses IFNγ- and TNFα-mediated reduction of nuclear levels of pSTAT3$_{Tyr705}$ in THP and human monocyte-derived macrophages. A) THP1 cells were differentiated into macrophages through PMA exposure for 72 h, and incubated without (control) or with PLTP (5 μg/ml) for 3 h; proteins were isolated using NE-PER kit (Pierce); pSTAT3$_{Tyr705}$ levels in nuclear proteins were assessed by Western blotting and densitometry measurements adjusted for TATA-binding protein, TBP. Bars represent mean and SD. B) Mature human monocyte-derived macrophages were incubated in the presence or absence of PLTP for 3 h; proteins were isolated using NE-PER kit (Pierce); pSTAT3$_{Tyr705}$ levels in nuclear fraction were assessed by Western blotting. C) Following PMA-mediated differentiation, THP1 cells were incubated without (control) or with interferon gamma (IFNγ, 100 ng/ml), alone or with PLTP (5 μg/ml). Nuclear levels of pSTAT3$_{Tyr705}$ were assessed by Western blotting and densitometry measurements adjusted for TBP. Bars represent mean, SD. D) PLTP wild type (WT) or mutant (M = PLTP$_{M159E}$) have similar effect on intranuclear levels of pSTAT3$_{Tyr705}$, suggesting that PLTP-mediated increase of nuclear pSTAT3$_{Tyr705}$ is not dependent on PLTP's lipid transfer ability. PMA-differentiated THP1 cells were incubated without or with TNFα, in the presence or absence of WT or mutant (without phospholipid transfer activity) PLTP (PLTP$_{M159E}$). Nuclear pSTAT3$_{Tyr705}$ levels were assessed by Western blotting. TATA-binding protein (TBP) was used as loading control.

incubated THP1 macrophages with wild type PLTP and mutant PLTP without phospholipid transfer activity (PLTP$_{M159E}$) [22]. Our results indicate that the PLTP-mediated increase in nuclear pSTAT3$_{Tyr705}$ is not dependent on PLTP's phospholipid transfer activity (Fig. 2D).

3.4. Inhibition of ABCA1 with glyburide reverses the PLTP-mediated increase in nuclear pSTAT3$_{Tyr705}$ levels in THP1 and primary human monocyte-derived macrophages

To confirm that the observed increase in active STAT3 in the nucleus is dependent on ABCA1, we inhibited ABCA1 with glyburide, a commonly used ABCA1 chemical inhibitor [30–33]. Glyburide reversed the PLTP-mediated increase in nuclear pSTAT$_{Tyr705}$ in TNFα-stimulated, differentiated THP1 cells (Fig. 3A) and human monocyte-derived macrophages (Fig. 3B). Similar effects were observed using wild-type PLTP or mutant PLTP without phospholipid transfer activity, PLTP$_{M159E}$ (Fig. 3B), confirming previously published findings that both wild-type and mutant PLTP interact with ABCA1 [22], and indicating that inhibition of ABCA1 reverses the PLTP-dependent induction of pSTAT3$_{Tyr705}$. Furthermore, glyburide inhibited the PLTP-dependent reduction of cytokines, such as MIP1α and IL-1β, in conditioned media of THP1 macrophages (Fig. 3C).

3.5. ABCA1 RNA inhibition attenuates PLTP-mediated activation of STAT3 in THP1 cells

Chemical inhibition data were further confirmed using ABCA1 RNA inhibition. PLTP increased levels of pSTAT3$_{Tyr705}$ in cells transduced with mock viral construct, but not in cells with ABCA1 RNAi (Fig. 4). Spinoculation procedure did not interfere with PLTP-mediated increase in pSTAT3$_{Tyr705}$ (not shown).

3.6. PLTP reduces levels of secreted pro-inflammatory cytokines in THP1 cells and human monocyte-derived macrophages

Activation of STAT3 in macrophages inhibits transcription of pro-inflammatory cytokines [34]. Therefore, we tested levels of secreted cytokines in conditioned media of LPS-, TNFα- and IFNγ-activated THP1 macrophages in the presence or absence of PLTP, using both pre-incubation and co-incubation modalities. PLTP significantly reduced levels of pro-inflammatory cytokines under both experimental conditions (Fig. 5). These findings were confirmed in conditioned media derived from human monocyte-derived macrophages by blotting for selected pro-inflammatory cytokines (see Fig. 3C for an example). Consequently, PLTP significantly reduces secretion of pro-inflammatory cytokines in macrophages. Interestingly, exposure of cells to PLTP prior to exposure to LPS, TNFα or IFNγ was sufficient to reduce secretion of pro-inflammatory cytokines, supporting our hypothesis that PLTP-mediated regulation of signal transduction may be involved.

3.7. PLTP reduces nuclear levels of active NFκB p65 in THP1 cells and human monocyte-derived macrophages

NFκB-mediated regulation of the inflammatory response in macrophages is one of the main mechanisms involved in the initiation of the inflammatory response [35]. Thus, we also tested the effect of PLTP on NFκB in human monocyte-derived macrophages and differentiated THP1 cells. Addition of PLTP to cells significantly reduced levels of active p65, phospho-p65$_{Ser536}$, in nuclei of human monocyte-derived and THP1 macrophages, as well as in cells stimulated with TNFα (Fig. 6).

4. Discussion

In our recent studies we identified proteins associated with PLTP in human plasma, and reported that the majority of the PLTP-associated proteins play a role in the innate immunity and the inflammatory response [36]. We have previously shown that PLTP binds, transfers, and neutralizes LPS, but does not present it to CD14 [1,26]. These observations have been confirmed by in vivo studies, which show that PLTP is critical for survival in endotoxemia, as PLTP deficiency enhanced LPS effects and caused significant mortality [37]. In severe endotoxemia, PLTP−/− mice had significantly higher levels of pro-inflammatory cytokines in plasma and tissues, and isolated splenocytes from PLTP−/− mice exposed to LPS had an enhanced inflammatory response and higher rates of cell death compared to those isolated from WT mice [37]. These data are in line with clinical observations that PLTP activity is increased in patients with bacterial

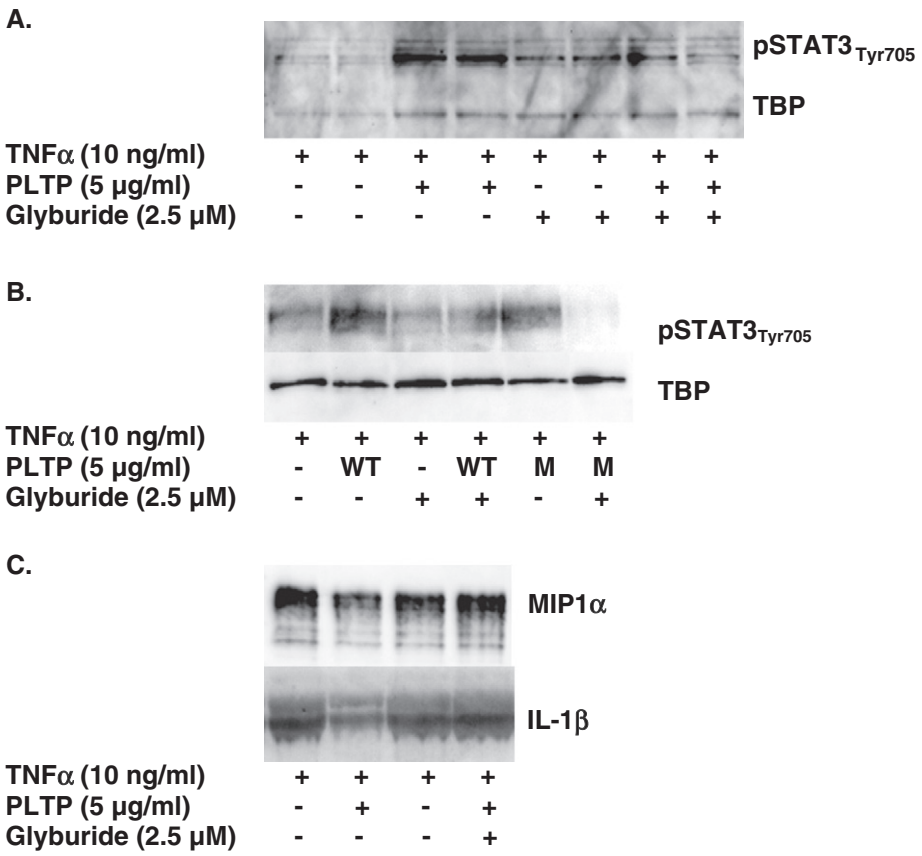

Fig. 3. PLTP-mediated increase in nuclear pSTAT3$_{Tyr705}$ is reversed in the presence of the ABCA1 inhibitor, glyburide. A) Nuclear pSTAT3$_{Tyr705}$ in PMA-differentiated THP1 cells incubated with TNFα (10 ng/ml) in the presence or absence of glyburide (2.5 μM) and/or PLTP (5 μg/ml). B) Nuclear pSTAT3$_{Tyr705}$ in human monocyte-derived macrophages incubated without or with TNFα (10 ng/ml), PLTP WT or mutant PLTP without lipid transfer activity (PLTP$_{M159E}$; 5 μg/ml), and glyburide (2.5 μM). C) Glyburide counters PLTP-mediated reduction in levels of secreted pro-inflammatory cytokines in conditioned media of human monocyte-derived macrophages. The amount of conditioned medium used in Western analyses was standardized based on total cell protein.

infection and systemic inflammation, and may be a compensatory change that is vital for survival [3,38]. Moreover, these findings unequivocally confirm that the role of PLTP in acute bacterial infection is anti-inflammatory, and critical for a successful resolution of the inflammatory processes and survival.

Findings that the increase of PLTP activity in chronic periodontal disease is associated with a reduction in the periodontal tissue damage suggest that PLTP activity plays an important role in the resolution of the inflammatory process in chronic inflammatory diseases [39]. Hence, the observed increases in PLTP levels and activity

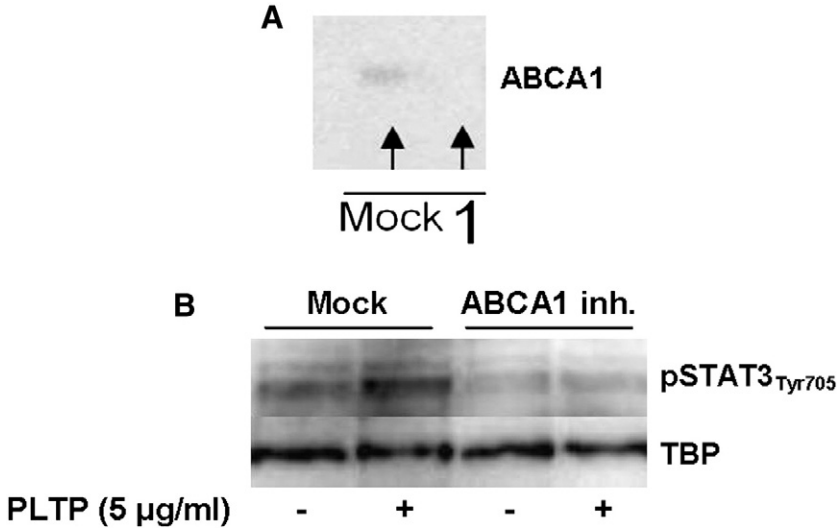

Fig. 4. ABCA1 RNA inhibition counters PLTP-mediated increase in levels of pSTAT3$_{Tyr705}$ in THP1 cells. THP1 cells were transduced with viral vectors containing control (Mock) or ABCA1 shRNA constructs. Following successful transduction by spinoculation, cells were selected using puromycin (0.6 μg/ml) and differentiated with PMA for 72 h. A) Levels of ABCA1 in cells transduced with mock and ABCA1 shRNA construct TRCN0000029089 (1). B) THP1 macrophages were then incubated without (control) or with rPLTP (5 μg/ml) for 3 h. Nuclear proteins were isolated using NE-PER and resolved by SDS-PAGE and blotted using antibodies against TBP (loading control) and pSTAT3$_{Tyr705}$.

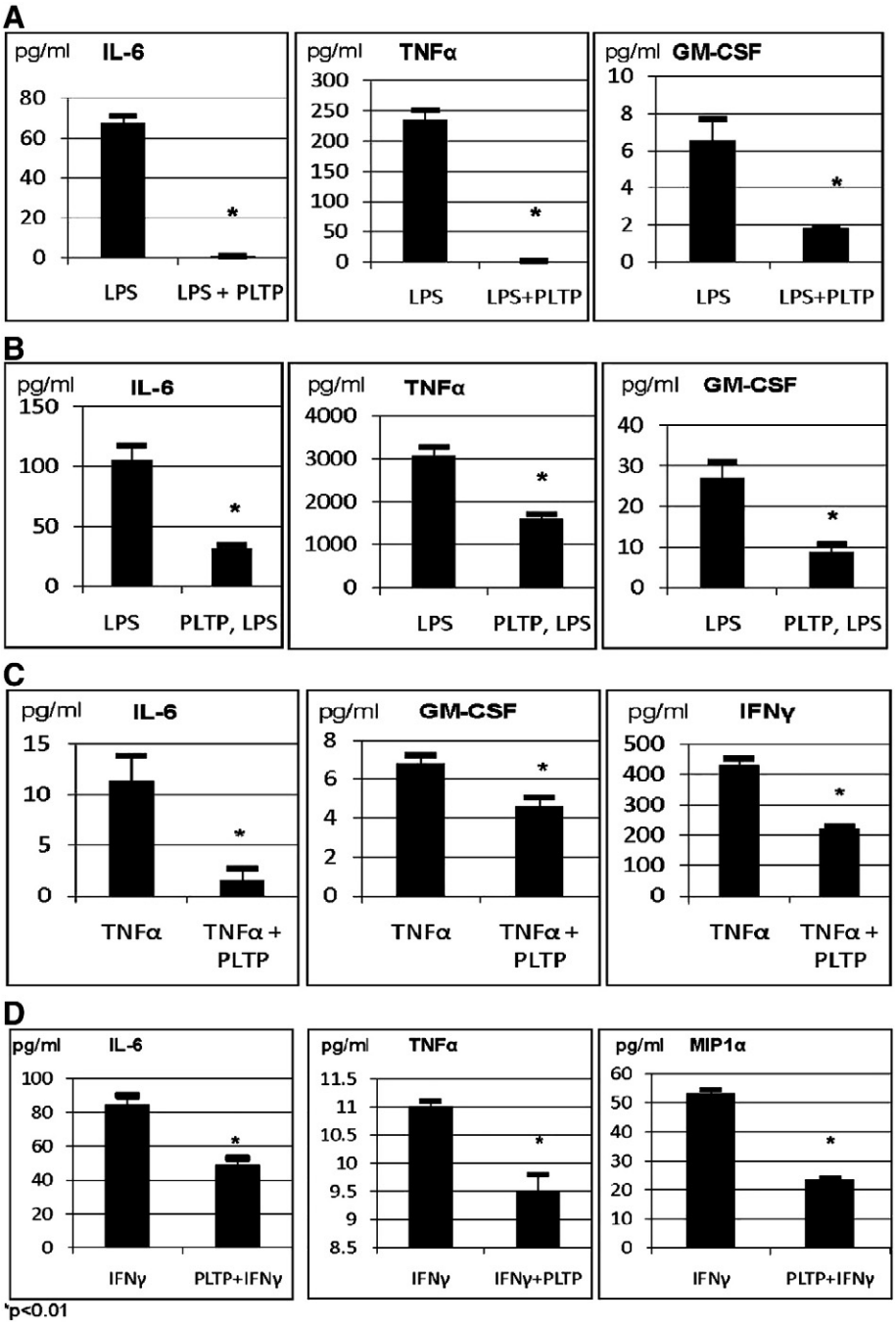

Fig. 5. PLTP reduces secretion of pro-inflammatory cytokines by THP1 macrophages. A) Co-incubation of PLTP and lipopolysaccharides (LPS) reduces levels of cytokines in conditioned media of THP1 cells. Differentiated THP1 cells were incubated with PLTP (5 μg/ml) and LPS (100 ng/ml) for 6 h. Cytokines in conditioned media were measured using Bio-Rad MultiPlex plates. B) Pre-incubation of THP1 macrophages with PLTP significantly reduced LPS-induced secretion of pro-inflammatory molecules. THP1 macrophages were pre-incubated with PLTP (5 μg/ml) for 4 h, extensively washed and then exposed to LPS (100 ng/ml) for 4 h. Cytokines in conditioned media were measured utilizing Bio-Rad MultiPlex plates. C) PLTP reduces levels of cytokines in conditioned media of THP1 cells incubated with interferon gamma (IFNγ). Conditioned media of THP1 cells incubated with IFNγ (100 ng/ml) with or without PLTP (5 μg/ml) were analyzed for MIP1α, TNFα and IL-6. Cytokines were measured using Bio-Rad MultiPlex plates or R&D Systems ELISA kit (for IL-6). D) PLTP reduces levels of cytokines in THP1 conditioned media of cells incubated with tumor necrosis factor α (TNFα). Conditioned media of THP1 cells incubated with TNFα (10 ng/ml) with or without PLTP (5 μg/ml) were analyzed for IL-6, GM-CSF and IFNγ. All presented data are representative of at least three separate experiments, and are expressed as mean ± SD.

in diseases with a strong inflammatory component may be a relevant protective or compensatory mechanism that limits tissue and organ damage induced by the inflammatory response. This idea is further supported by the fact that reduction of PLTP activity in multiple sclerosis is associated with active disease, while measures of recovery correlate with an increase in PLTP activity [16]. Additionally, reduced plasma PLTP levels and activity have been reported as a risk factor for peripheral artery disease, which has a significant inflammatory component [13]. These findings support the idea that PLTP has an anti-inflammatory role in both acute and chronic inflammatory diseases.

Previously published studies suggest that macrophage PLTP plays an anti-atherogenic role [19,20]. However, different results were also reported using the same mouse model under somewhat different conditions [40]. The inflammatory component of the atherogenic process has long been recognized as one of the most important

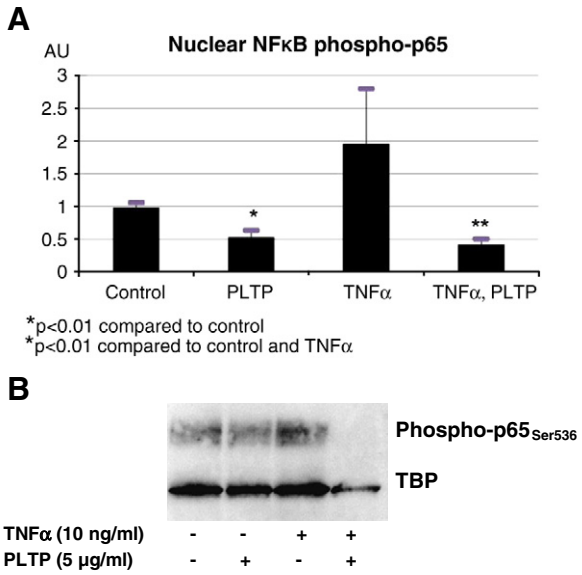

Fig. 6. PLTP reduces levels of p65 phosphorylated at serine 536 in nuclei of macrophages incubated with TNFα. A) PLTP reduces levels of p65 phosphorylated at serine 536 in nuclei of differentiated THP1 cells incubated with TNFα. Differentiated THP1 cells were incubated alone (control), with PLTP (5 μg/ml), TNFα (10 ng/ml), or TNFα and PLTP together for 24 h. Nuclear proteins were isolated, resolved on SDS-PAGE, transferred, and blotted, employing antibodies against phospho-p65 Ser536. TATA-binding protein (TBP) was used as loading control. Resulting blots were scanned, evaluated by densitometry, and adjusted for TBP. Bars represent mean, SD. B) Human monocyte-derived macrophages were incubated with or without TNFα and PLTP, and levels of nuclear NFκB p65 phosphorylated at serine 536 were evaluated by Western blotting. TBP was used as loading control.

pathological processes contributing to the development of atherosclerosis [41–44]. Due to the pivotal role macrophages play in inflammation, determining whether PLTP affects macrophages in a pro- or anti-inflammatory manner is relevant for the overall understanding of its physiological and pathophysiological roles. Findings reported in this study strongly suggest that PLTP induces an anti-inflammatory response through activation of the ABCA1/STAT3 pathway.

Recent studies identified ABCA1 as one of the critical intersections in the regulation of inflammation and atherosclerosis [45]. PLTP interacts with ABCA1, stabilizes it, and its interaction with ABCA1 activates JAK2 in a manner similar to apoA-I [21,22]. Tang and colleagues showed that binding of apoA-I to ABCA1 activates JAK2 and STAT3, potently preventing LPS-challenged macrophages from inducing the inflammatory cytokines, IL-1β, IL-6, and TNF-α [46]. Additionally, a recent study has shown that the ABCA1-dependent lipid efflux plays a role in regulating the lipid raft-mediated activity of Toll-like receptors (TLR) [47]. Thus, these findings suggest that ABCA1 in macrophages functions as an anti-inflammatory receptor.

Activation of the STAT3 pathway in macrophages has an anti-inflammatory role [34]. The pathway may be physiologically relevant because the loss of STAT3 in mouse macrophages increases susceptibility to endotoxic shock and promotes chronic enterocolitis, indicating its importance in protection against both acute and chronic inflammation [48,49]. Moreover, constitutive expression of active STAT3 in cultured macrophages nearly abolishes inflammatory cytokine induction [34]. Therefore, the PLTP-dependent increase in nuclear $pSTAT3_{Tyr705}$, the active form of STAT3, and the concomitant reduction in secreted pro-inflammatory cytokines indicate that PLTP activates STAT3, thereby inhibiting the transcription of inflammatory cytokines. Our findings that glyburide abolishes the observed PLTP-mediated induction of STAT3, which were confirmed by ABCA1 RNA inhibition experiments, further suggest that PLTP-dependent upregulation of the STAT3 pathway occurs through PLTP's interaction with ABCA1. These pivotal new findings present a possible mechanism that may be responsible for the observed reduction in atherosclerosis levels in mice expressing macrophage PLTP [19,20]. Furthermore, our findings are in concordance with a study by Wehinger and colleagues showing that in the presence of toxic lipids, macrophage PLTP does not induce inflammation, but rather shifts the cellular response to toxic lipids towards apoptosis [6]. Consequently, it is possible that PLTP acts as a switch between inflammatory and apoptotic response in macrophages, and that its effects depend on the type, and possibly duration of the stimulus.

Our findings indicate that the PLTP-mediated anti-inflammatory response related to its interaction with the ABCA1-dependent activation of the JAK2/STAT3 pathway is not dependent on PLTP lipid transfer, since both wild type and mutant PLTP (without phospholipid transfer ability) caused similar pathway activation. Therefore, the ABCA1-dependent anti-inflammatory response is likely related to the direct interaction between PLTP and ABCA1, in which PLTP regulates activation of the STAT3 pathway through ligand binding to the ABCA1 receptor. Furthermore, PLTP reduced levels of active NFκB in the nuclei of human monocyte-derived and THP1 macrophages, indicating that PLTP acts on at least two different pathways. The NFκB activity in macrophages is predominantly regulated by TLRs, suggesting that the PLTP anti-inflammatory action is exerted through several signal transduction pathways in macrophages.

4.1. Conclusions

In macrophages, PLTP activates the ABCA1/STAT3 anti-inflammatory pathway, as well as reduces activation of NFκB. Further evaluation of the mechanisms responsible for the observed PLTP-mediated anti-inflammatory response in macrophages is essential for understanding the roles PLTP plays in inflammation, and in diseases with a strong inflammatory component.

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