Connections of Annexin A1 and Translocator Protein-18 KDa on Toll Like Receptor Stimulated BV2 Cells

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Abstract

Background: Annexin A1 (ANXA1) and the Translocator Protein-18KDa (TSPO) are markers for neuroinflammation. While ANXA1 exerts anti-inflammatory properties via formyl peptide receptor (FPR) stimulation, the mechanisms of TSPO action have not yet been elucidated. Hence, we investigated the connection of both proteins on activation of microglia mediated by Toll Like Receptor (TLR).

Methods: BV2, a murine microglial cell line, were stimulated by LPS (10 or 100 ng/mL) and treated with recombinant ANXA1 (rANXA1; 10 or 100 nM) to measure TSPO expression and inflammatory parameters. Anti-sense ANX1, TLR4 and TSPO, as well as pharmacological treatments were employed to detect the mechanisms involved.

Results: LPS-stimulated BV2 cells overexpressed TSPO, which was inhibited by the pharmacological blockade of TLR4 or TLR4 mRNA silencing; by ST 2825 treatment, an inhibitor of myeloid differentiation primary response gene 88 (MyD88) dimerization; and by PDTC, an inhibitor of nuclear factor κB activation. rANXA1 treatment impaired the LPS-induced TSPO upregulation by down modulating MyD88 and NF-κB signaling, and its effect was abolished by WRW4, an antagonist of the formyl peptide receptor 2 (FPR2). TSPO expression was not altered in knockdown ANXA1 BV2. rANXA1 treatment also downregulated the interleukin 1β (IL-1β) and tumor necrosis factor-α (TNFα) secretion in LPS-stimulated BV2 cells. Nonetheless, knockdown of TSPO in BV2 drastically augmented the secretion of TNFα induced by LPS and abolished the inhibitory effect of rANXA1 on TNFα secretion. Although TSPO knockdown did not modify the expression of FPR2.

Conclusions: Taken all together, our study shows an interaction between ANXA1 and TSPO signaling towards downmodulation of microglial activation, and indicates that the ANXA1/TSPO pathway is relevant to genesis of neuroinflammation

Key words: neuroinflammation; NF-κB; MyD88; LPS; FPR2; TNFα

Background

Neurodegenerative diseases, such as Parkinson's disease (PD), Alzheimer's (AD), multiple sclerosis (MS), and age-related dementia, are a major global cause of disability and premature death among older people [1, 2]. Moreover, prevalence of psychiatric syndromes has grown in young people [3]. It has been shown that persistent systemic and local inflammation plays a pivotal role in the genesis of neurodegenerative and psychiatric diseases [4-6]. Molecular mediators secreted in the peripheral inflammatory sites or in the central nervous system (CNS) enhance the permeability of the blood brain barrier (BBB), activate microglia and astrocytes, and lead to undesired migration of blood-activated leukocytes into the brain. Collectively with microglia and astrocytes, activated leukocytes produce free radicals, secrete granule contents and cytokines into the microenvironment, causing neuroinflammation [7, 8].

Inflammation is highly controlled by endogenous mediators to halt the inadequate exacerbation of the process. In this context, Annexin A1 (ANXA1), a 37-kDa protein positively regulated by glucocorticoids in different cells, has been pointed out as a pivotal modulator of innate immune system able to control inflammation. Once secreted, ANXA1 binds to plasma membrane phospholipids as well as to the formyl-peptide receptor 2 (FPR2), a G-protein coupled receptor that contributes to resolution of inflammation upon stimulation [9, 10]. In the CNS, endogenous ANXA1 is expressed by brain microvascular endothelial cells exhibiting a fundamental role in the integrity of BBB, by stabilizing tight and adherens junctions [11-13]. Reduced expression of ANXA1 by brain microvascular endothelial cells enhances BBB permeability and is

associated with postoperative cognitive decline and MS [11]. Moreover, ANXA1 is expressed in resident microglia and astrocytes localized in areas of autoimmune encephalomyelitis (EAE) in rats [14]. High levels of ANXA1 have also been reported in the brain tissue of patients with MS [15], AD [16] and PD [17]. The exact role and mechanisms of ANXA1 in the development of neurodegenerative diseases have not been completely elucidated; protective mechanism are likely related to stabilization of BBB tightness and triggering of efferocytosis of apoptotic neurons and inflammatory cells by microglia [11, 16, 18].

TSPO (Translocator protein 18-KDa) was initially termed as peripheral benzodiazepine receptor (PBR). It is mainly found as an outer mitochondrial membrane protein required for the translocation of cholesterol, which thus regulates the rate of steroid synthesis [19]. Moreover, it is well known that TSPO is expressed in CNS cells, especially on astrocytes and microglia, and exacerbated TSPO expression has been linked to brain cancer, neurodegeneration and neuropsychiatric disorders [20-22]. Accordingly, TSPO ligands have been used both as a diagnostic biomarker and therapeutic tools for different CNS diseases [23, 24]. Indeed, treatment with TSPO ligands present substantial *in vi*vo efficacy in animal model of neurodegeneration and anxiety, suggesting that the over expression of the protein is necessary for the actions of the ligands, triggering anti-inflammatory signaling and tissue regeneration [25-27]. Nevertheless, the exact role of TSPO on development or resolution of neuroinflammation, as well as the molecular basis of mechanism of actions, has not been yet clarified [19, 28, 29].

To elucidate the role of ANXA1 and TSPO in neuroinflammation, we investigated if the mechanism of action of TSPO could be mediated by the ANXA1 in LPS activated microglia. Our data show an interplay between ANXA1 and TSPO during LPS-induced cell activation, as LPS treatment enhances the expression of TSPO by activating TLR4/MyD88/NFκB pathways, which is down modulated by ANXA1/FPR2 interaction. Furthermore, TSPO is a down regulator and mediator of ANXA1 on LPS-induced TNFα secretion in BV2 cells.

Methods

Reagents

Human recombinant ANXA1 (rANXA1) was kindly provided by Dr. Chris Reutelingsperger (Maastrich University, NL), and used at the concentration of 10 or 100 nM [12]. Lipopolysaccharide of *E.coli* (LPS 055:B5, Sigma-Aldrich, St. Louis, MO, USA) was diluted in 3% bovine serum albumin (BSA) and used at 10 or 100 ng/mL. LPS from *Rhodobacter sphaeroides* (RS-LPS, InvivoGen, San Diego, CA, USA) was used at final concentration of 500 ng/mL. The formyl peptide receptor-2 antagonist (WRW4) (Tocris, Bristol, UK) was used at a final concentration of 1 mM. The inhibitor of MyD88 dimerization (ST 2825) (Apexbio, Houston, TX, USA) was first solubilized in DMSO and used at a final concentration of 20 μM. The Selective NF-κB inhibitor Ammonium pyrrolidinedithiocarbamate (PDCT, Sigma-Aldrich, Darmstadt, Germany) used at a final concentration of 50 μM. TNF-α and IL-1β (Sigma Chemical Co., St. Louis, MO) was used at a final concentration of 10 ng/mL. Elisa Kits to quantify IL-1β, IL-10, TNFα, TGFβ were purchased from BD Biosciences, Heidelberg,

Ge. NF-κB Elisa kit was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Anti-TSPO, secondary antibody FITC-donkey anti-rabbit IgG, anti-FPR2 FITC conjugated were purchased from Abcam (San Francisco, CA, USA), and primary antibody against MyD88 and horseradish peroxidase-linked secondary antibody from Abcam (Cambridge, UK).

Cell Culture

BV-2 murine microglial cells were obtained from the "*BCRJ (Banco de células do Rio de Janeiro*, Rio de Janeiro, Brazil*)"* and cultured at 37°C, 5% CO_2 in RPMI culture medium, with 10% fetal serum (FCS), 1% penicilin/streptomicin and 1% L-glutamine (Atená Biotecnologia, Campinas, Brazil).

Knockdown of TLR4, ANXA1 and TSPO in BV2 cells

The BV-2 cells (1 x 10^5 cells per well) were transfected with the anti-sense cDNA plasmid to ANXA1 containing 476 bp namely pRc/CMV ANXA1 AS [30], and it was used to knockdown ANXA1 expression in BV-2 cells ($ANXA1^{-/-}$). shRNA plasmids from the MISSION TRC shRNA collection (Sigma-Aldrich, St. Louis, MO, USA) targeting TSPO (shRNA-TSPO – $TSPO^{-/-}$) or TLR4 (shRNA-TLR4 – $TLR4^{-/-}$) receptors were used to knockdown the expression of each receptors respectively. BV-2 cells were transfected using FuGENE HD (Promega, Madison, Wisconsin, EUA) according to the manufacturer's instructions. After 48 hours transfection, cells were selected using geneticin (G-418, Promega, Madison, Wisconsin, EUA) for pRC/CMV plasmid and puromycin (Sigma-Aldrich, Darmstadt, Germany) for MISSIONs shRNA.

TSPO quantification

BV-2 cells ($1x10^6$) were stained for the intracellular marker TSPO (anti-TSPO 1:100) and secondary antibody FITC-donkey anti-rabbit IgG (1:200). Following O/N fixation and permeabilization (BD Pharmingen Technical, CA, EUA), cells were analyzed by flow cytometry (Accuri CSampler® flow cytometer; BD Biosciences, Frankin Lakes, NJ, USA). A total of 1×10^5 events were measured in the gates. The gate strategy for TSPO expression is shown in Supplementary Fig. 1a.

Enzyme-Linked Immunosorbent Assay

The secretion of IL-1β, IL-10, TNFα, TGFβ, as well as the nuclear translocation of the nuclear transcription factor NF-kB, was measured by ELISA (BD Biosciences, Heidelberg, Ge), and NF-κB (Cayman Chemical Company, Ann Arbor, MI, USA) respectively. All procedures were performed according to the manufacturer instructions. The protein quantification in samples was determined by Bradford assay. The concentrations of proteins and NF-κB were determined in a OD reader (Power Wave X340, BioTek Instruments, Winooski, VT).

Western Blotting

MyD88 expression was evaluated by Western Blot. Briefly, cell lysates (50 µg of protein) were separated on 12% acrylamide gel and transferred onto nitrocellulose membrane. Membranes were blocked in buffered saline

containing 0,1% Tween 20 (v/v) and 5% powdered milk, before probing overnight at 4ºC with primary antibody against MyD88 (polyclonal goat IgG, diluted 1:000 in TBS-T). Membrane was then probed with horseradish peroxidase-linked secondary antibody (polyclonal donkey IgG, diluted 1:3000 in TBS-T) for 1 hour at room temperature. Membranes were stripped and re-probed with anti-actin antibody for 1 hour at room temperature to evaluate protein load.

FPR2 analysis

BV-2 cells ($1x10^6$) were stained for membrane FPR2 expression (anti-FPR2 FITC conjugated 1:100), and cells were analysed by flow cytometry (Accuri CSampler® flow cytometer; BD Biosciences). A total of 1×10^5 events were measured in the gates. The gate strategy for FPR2 expression is shown in Supplementary Fig. 1b.

Statistical Analysis

All statistical analysis were performed with GraphPad Prism 5 software (Version 5.03; GraphPad software, Inc., San Diego, CA). Comparison among experimental groups were analyzed by one or two-way ANOVA followed by Bonferroni's post hoc test and statistical significance was set at $p<0.05$ for all tests. All values are presented as mean ± s.e.m.

Results

Exogenous ANXA1 impairs TSPO expression in LPS-stimulated BV2 cells via FPR2

To evaluate the role of ANXA1 in TSPO expression, BV2 cells were treated with rANXA1 and the expression of TSPO was evaluated at basal or LPS stimulated conditions**.** Data obtained showed that incubation with LPS augmented TSPO expression after 4 (Fig. 1a) and 12 hours (Fig. 1b) of stimulation. rANXA1 treatment did not alter the basal expression of TSPO, nevertheless it abolished the overexpression caused by LPS (Fig. 1a,b). Pre-incubation with the FPR2 antagonist WRW4 [31] reverted the ANXA1 inhibition on LPS-induced TSPO up-regulation (Fig. 1c). Anti-sense mRNA ANXA1 did not block the TSPO overexpression caused by 100 ng/mL of LPS (Fig. 1d). The efficacy of anti-sense ANXA1 strategy is shown in Supplementary Fig. 2**.**

Exogenous ANXA1 modulates LPS-induced TSPO overexpression via TLR-4, MyD88 and NF-κB signaling

To investigate the signaling pathways responsible for the LPS modulation of TSPO expression we used both a pharmacological approach, by blocking TLR4 using Lipopolysaccharide from the photosynthetic bacterium *Rhodobacter sphaeroides* (RS-LPS), as well as a molecular one, by using TLR-4 anti-sense transfected cells. Both experimental procedures abolished the ability of LPS to induce TSPO overexpression, as well as the ability of rANXA1 to reduce the expression of the receptor (Fig. 2a,b). TLR4 expression was reduced by 50% in siRNA transfected cells (Supplementary Fig. 3). Nevertheless, the expression of TSPO was enhanced in BV2 cells treated with RS-LPS when compared with cells treated with R10 medium (Fig. 2a). As LPS can also binds to TLR2

receptor, we suppose that TSPO expression may also be induced by TLR2 pathway when TLR4 is pharmacologically blocked.

Further experiments showed that LPS induces TSPO expression via MyD88 expression and NF-κB nuclear translocation, as expression of both were enhanced after LPS treatment (Fig. 2c,d), and respective pharmacological blockades reduced TSPO upregulation induced by LPS (Fig. 2e). Moreover, exogenous ANXA1 treatment impaired MyD88 expression and NF-κB nuclear translocation, suggesting that ANXA1 impairs LPS-induced TSPO overexpression by blocking these intracellular pathways evoked by LPS activation (Fig. 2c,d).

Exogenous ANXA1 leads to anti-inflammatory profile of cytokines secretion by BV2 activated by LPS

Data obtained showed that LPS incubation augmented the secretion of TNFα, IL-1β and IL-10 (Fig. 3a-f), and did not alter the levels of TGFβ (Fig. 3g-h). rANXA1 *per se* only enhanced the secretion of TGFβ that was suppressed in the presence of 100 ng/ml LPS (Fig. 3g-h). Co-incubation of rANXA1 with LPS reduced the secretion of TNFα evoked by 10 or 100 ng/mL of LPS and the secretion of IL-1β induced by 10ng/mL of LPS (Fig. 3a-d). rANXA1 treatment did not alter the secretion of IL-10 (Fig. 3e,f).

TSPO down modulates TNFα secretion in BV2 cells activated by LPS, and is mediator of ANXA1 action on LPS-induced TNFα secretion

LPS treatment enhanced TNFα and IL-1β secretion in BV2 cells, and TNFα levels were further enhanced in BV2 cells TSPO$^{-/-}$ (Fig. 4a,b), while IL-1β levels were not altered in TSPO$^{-/-}$ cells (Fig. 4c,d). Furthermore, LPS did not enhance TGFβ secretion and silencing of TSPO did not cause any modification on levels of the growth factor (Fig. 4e,f). The connection of TNFα on TSPO expression was further visualized by enhancement of TSPO expression preferentially by TNFα rather than IL-1β (Fig. 4g), suggesting a feedback between and TNFα and TSPO.

Furthermore, the rANXA1 treatments markedly reduced TNFα and IL-1β secretion elicited by LPS (Fig. 4a-d). On the contrary, rANXA1 treatment stimulated the expression of TGFβ (Fig. 4e) in BV2 cells. Moreover, the silencing of TSPO blocked the inhibitory effect of rANXA1 on LPS-induced TNFα over secretion (Fig. 4a-d). Such effect was not due to reduced expression of FPR2 in TSPO$^{-/-}$ BV2, as expression of the receptor was equivalent in both BV2 and TSPO$^{-/-}$ BV2 cells (Fig. 4h). Therefore, we concluded that inhibition of LPS-induced TNFα secretion elicited by ANXA1 involves the participation of TSPO signaling.

Discussion

Although overexpression of TSPO is a marker of neuroinflammation, and *in vivo* administration of its ligands leads to beneficial effects to neurodegeneration and psychiatric diseases, the role and mechanisms of action of TSPO on the CNS pathophysiology remain to be elucidated [28, 29]. Data presented here show the involvement of TLR4/MyD88/NF-κB pathway on

TSPO overexpression elicited by LPS, which is modulated by the binding of rANXA1 to FPR2. Moreover, we point out here the pivotal role of TSPO on TNFα secretion, by negatively modulating its secretion, and the involvement of TSPO on inhibitory action of ANXA1 on LPS-induced TNFα secretion. Hence, we show, for the first time, the pathways of LPS induced TSPO expression and the interplay network between TSPO and ANXA1 on LPS-induced inflammation in microglia (Figure 5).

Microglia is pivotal to the surveillance of cells in the brain, as it removes damaged cells and promotes tissue repair. Therefore, microglia show a degree of plasticity, acting as pro and anti-inflammatory cell [32]. LPS is a classical local or systemic activator of microglia, and here we confirm the direct action of LPS on expression of TSPO [31, 33, 34]. Furthermore, we depicted the intracellular pathway involved, by showing that the blockade of TLR4, MyD88 or NF-kB inhibited TSPO expression evoked by LPS. Moreover, we confirmed that TNFα stimulation induced TSPO expression [35], and for the first time we show the effect of IL-1β on TSPO expression. Hence, TLR4/MyD88/NF-κB/TNFα signaling may be pivotal to upregulate TSPO expression after LPS stimulation.

Furthermore, we showed that exogenous ANXA1 inhibits the expression of TSPO modulated by LPS via FPR2, and through the inhibition of the protein on MyD88 and NF-κB. ANXA1 is involved in a diversity of cell functions, and most of them, especially those related to anti-inflammatory properties, are mediated by FPR2 [13, 36]. Several intracellular proteins are phosphorylated by ANXA1/FPR2 pathway, such as signal transducer and activator of transcription 3 (Stat3) [37], mitogen-activated protein kinases (MAPKs), extracellular signal–regulated kinases (ERKs) [38] and cAMP response element binding protein

(CREB) [39]. To our knowledge, the effect of ANXA1 on MyD88 expression has not been previously shown. Furthermore conflicting results about ANXA1 actions on NF-κB translocation into nucleus have been described in different cell types. Constitutive augmented levels of ANXA1 on cancer cells induces NF-κB translocation [40], and we previously showed that the N-terminal peptide of ANXA1 (Ac-2-26), which binds to both FPR1 and FPR2, impaired the endotoxin-induced uveitis in rats, independently on inhibition of NF-κB translocation into nucleus of LPS stimulated ARPE-19 cells [41].

It is noteworthy to mention that exogenously added and endogenous ANXA1 seem to exert different control on TSPO expression, as intracellular silencing of ANXA1 does not directly affect the TSPO expression. Indeed, we have further evidences of differential actions of ANXA1 depending on its source, as ANXA1 silenced BV2 expressed lower membrane levels of CD36, which is not influenced by exogenous ANXA1 [42]. The pivotal role of FPR2 activation, extracellular levels of ANXA1 and its phosphorylation on TSPO expression will be investigated in the next future.

Further data here obtained show the main role of TSPO on TNFα secretion. TSPO-silenced BV2 cells secreted higher levels of TNFα, but not of IL-1β, suggesting that TSPO downmodulate TNFα secretion in response to TLR4 activation. The role of TSPO silencing on TNFα secretion has not been described before; conversely it has been demonstrated that activation of the TSPO in microglia by its ligands impairs the secretion of inflammatory chemical mediators, such as TNFα and IL1β [43, 44]. Meanwhile, data obtained here demonstrated an intriguing loop of TNFα and TSPO, in which TNFα stimulates TSPO expression and TSPO activation impairs LPS-induced TNFα secretion. It

is important to mention that TSPO ligands do not affect the secretion of TNFα by LPS–stimulated peripheral neutrophils and macrophages [45], showing that TSPO is a relevant receptor on LPS actions of inflammatory cells in the CNS.

The role of ANXA1 to inhibit and induce the secretion of pro-inflammatory and anti-inflammatory cytokines, respectively, has been fully described in different cells [46, 47]. In fact, ANXA1 treatment reduced the elevated concentration of IL-6, TNFα and IL-4 in BV2 cells stimulated by synthetic amyloid peptide $A\beta_{1-42}$ [36], and the treatment of rat primary culture microglia with Ac2-26 reduced the LPS-induced secretion of IL1β and TNFα [48]. Here we confirm the anti-inflammatory profile elicited by exogenous ANXA1, since it elicited a reduction on LPS-induced TNFα and IL1β over secretion in BV2 cells. Furthermore, exogenous ANXA1 caused a marked enhancement on the concentration of TGFβ, a cytokine which mediates the polarization of macrophages into M2 anti-inflammatory phenotype [49, 50], in the supernatant of BV2 even in basal condition. However, the most important outcome of our study is that TSPO knockdown blocked the ability of rANXA1 to inhibit the LPS-induced TNFα over secretion, without interfering with the effect of ANXA1 on the secretion of IL-1β and TGFα. In addition, its effect is not dependent on alterations on FPR2 expression in TSPO-silenced cells, showing an intracellular connection of FPR2/ANXA1 pathways and TSPO expression only on TNFα secretion.

All together, our data show a complex interaction between ANXA1 and TSPO on the modulation of LPS actions on microglia, and show that TSPO is a fundamental receptor on secretion of TNFα. Based on the relevance of both

ANXA1 and TSPO in neurodegenerative disease, data here presented open new directions to understand the fine-tuning balance of intracellular pathways on genesis of inflammatory process in CNS.

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Conflict of interest

Authors declare no conflict of interest.

Legends for Figures

Figure 1: Exogenous ANXA1 blocks TSPO expression evoked by LPS in BV-2 cells via FPR2. BV-2 cells were treated with ANXA1 (100 nM) or R10 medium 1 hour before LPS (10 or 100 ng/mL) or R10 medium treatments. TSPO was evaluated **(A)** 4 or **(B)** 12 hours after LPS or R10 treatments. **(C)** BV2 were treated with WRW4 (1 mM) 30 minutes before AnxA1 or R10 treatments and 1 hour later were stimulated with LPS (10 or 100 ng/mL) and TSPO was quantified 4 hours later **. (D)** ANXA1 silenced BV2 were treated with R10 culture medium or LPS (10 or 100 ng/mL) and TSPO expression quantified 4 hour later**.** TSPO expression was evaluated by flow cytometry. Data are expressed as mean ± s.e.m of 4-6 samples of cells in each group and were analyzed by Two Way ANOVA. $^{*}P<0.05$ and $^{**}P<0.01$ vs respective R10 medium treatment; $^{\#}P<0.05$ and $^{\#\#}P<0.01$ vs respective LPS treatments in R10 group.

Figure 2: Exogenous ANXA1 blocks the LPS-induced TSPO expression via TLR-4, MyD88 and NFκB pathways. (A) BV-2 cells were treated with R10 medium or RS-LPS (500ng/mL) 30 minutes before treatments with ANXA1 (100 µM) or R10 medium. LPS treatments (10 or 100 ng/mL) were carried out 1 hour after ANXA1 or R10 medium treatments and TSPO expression was evaluated 4 hours later**. (B)** TLR4 silenced BV2 cells were treated with LPS (10 or 100 ng/mL) or R10 medium and TSPO expression was quantified 4 hours later**.** BV-2 cells were treated with ANXA1 (100 nM) or R10 medium 1 hour before LPS treatments (10 or 100 ng/mL) or R10 culture medium, and **(C)** MyD88 and **(D)**

NF-κB were quantified 1 hour later. **(E)** BV-2 cells were treated with PDTC anti NF-κ (50 μM) or anti MyD88 (20 μM) 30 minutes before LPS stimulation (100 ng/mL) and TSPO expression was quantified 4 hour later**.** Data are expressed as mean ± s.e.m of 4-6 samples of cells in each group and were analysed by One Way ANOVA. *P<0.05 and ***P<0.001 vs respective R10 medium treatment; $^{\#}$P<0.05 vs respective R10 treated cells; @P<0.05 vs R10 medium treatment.

Figure 3: Exogenous ANXA1 blocks the LPS-induced inflammatory cytokine secretion. (A – D) BV-2 cells were treated with ANXA1 (100 nM) 1 hour before LPS (10 or 100 ng/mL) or R10 medium treatments and cytokine secretion was evaluated 4 or 12 hour later for normal BV-2 cells or **(E)** silenced for ANXA1 gene**.** Data are expressed as mean ± s.e.m of 5-6 samples of cells in each group and were analyzed by One Way ANOVA. *P<0.05; ***P<0.001 vs respective R10 medium treatment; $^{@}$P<0.05 vs respective R10 treated cells; $^{\#}$P<0.05 vs respective R10 treated cells.

Figure 4: TSPO down modulates TNFα secretion induced by LPS and mediates the inhibitory effect evoked by rAnxA1 (A-E) BV-2 cells were treated with ANXA1 (100 nM) 1 hour before LPS (100 ng/mL) or R10 medium treatments and cytokine secretion was evaluated 4 or 12 hour later for normal BV-2 cells or silenced for TSPO gene**. (F)** BV-2 cells were treated with TNFα, IL-1β or R10 medium and TSPO expression was quantified 4 hours later. **(G)** Expression of TSPO in BV2 cells 4 hours after TNFα or IL-1β stimulation**. (H)** Levels of FPR2 expression in BV2 or TSPO$^{-/-}$ BV2 cells treated or not with LPS

(100ng/mL during 1 hour). Data are expressed as mean ± s.e.m of 5-6 samples of cells in each group and were analysed by One Way ANOVA. $^{*}P< 0.05$ and $^{***}P<0.001$ vs respective R10 medium treatment; $^{@}P<0.05$ vs respective BV-2 treated cells; $^{\#}P<0.05$ vs LPS.

Figure 5 : ANXA1/TSPO connection on LPS activated BV2 cells. LPS/TLR4 activates MyD88, NF-κB and TSPO expression, which are inhibited by rANXA1. TSPO modulated LPS-induced TNFα secretion and the inhibitory effect of ANXA1

Supplementary Figure 1A: Characterization of TSPO expression in BV2 cells. **(a)** Representative dot plots and histograms showing gating strategy for analysis of TSPO expression in controls and samples. Doublets were excluded by plotting forward scatter area versus forward scatter height. Numbers indicate the percent of cells in each gate. **(b)** Typical histogram showing by flow cytometry analysis the difference between BV2 cells treated with TSPO or R10 media.

Supplementary Figure 1B. Characterization of FPR2 expression in BV2 cells. Representative dot plots and histograms showing gating strategy for analysis of FPR2 expression in controls and samples. Doublets were excluded by plotting forward scatter area versus forward scatter height. Numbers indicate the percent of cells in each gate.

Supplementary Figure 2: Workflow of transfection in BV-2 cells for silencing ANXA1 gene. **(a)** The cells were transfected with specific pRC/CMV plasmid

containing ANXA1 antisense sequence. **(b)** The gene sequence of the anti-sense plasmid is described with the designed primers based in the plasmid map. **(c)** Graphical representation of % of ANXA1 reversion. ANXA1 concentration was evaluated after selection by ELISA**. (d)** Growth curves of BV-2 cells normal or transfected over a 96 hours period. Data are expressed as mean ± s.e.m of 6 samples of cells in each group and were analysed by One Way ANOVA. $^{*}P< 0.05$ vs BV-2 cells.

Supplementary Figure 3: Workflow of transfection in BV-2 cells for silencing TSPO or TLR4 gene. **(a)** The cells were transfected with specific commercial shRNA plasmid containing TSPO or TLR4 antisense sequence. Graphical representation of % of **(b)** TLR4 or **(c)** TSPO reversion evaluated after selection by flow cytometry. **(d)** Growth curves of BV-2 cells normal or transfected over a 96 hours period. Data are expressed as mean ± s.e.m of 6 samples of cells in each group and were analysed by One Way ANOVA. $^{**}P< 0.01$ vs BV-2 cells.

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