View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Archivio istituzionale della ricerca - Università di Padova

Facci et al. Journal of Neuroinflammation (2018) 15:164
https://doi.org/10.1186/s12974-018-1205-6

Journal of Neuroinflammation

RESEARCH Open Access

Serum amyloid A primes microglia for ATP-dependent interleukin-1β release

Laura Facci, Massimo Barbierato, Morena Zusso, Stephen D. Skaper* and Pietro Giusti

Abstract

Background: Acute-phase response is a systemic reaction to environmental/inflammatory insults and involves production of acute-phase proteins, including serum amyloid A (SAA). Interleukin-1β (IL-1β), a master regulator of neuroinflammation produced by activated inflammatory cells of the myeloid lineage, in particular microglia, plays a key role in the pathogenesis of acute and chronic diseases of the peripheral nervous system and CNS. IL-1β release is promoted by ATP acting at the purinergic $P2X_7$ receptor ($P2X_7R$) in cells primed with toll-like receptor (TLR) ligands.

Methods: Purified (> 99%) microglia cultured from neonatal rat cortex and cerebellum were first primed with the putative TLR4/TLR2 agonist SAA (recombinant human Apo-SAA) or the established TLR4 agonist lipopolysaccharide (LPS) followed by addition of ATP. Expression of genes for the NLRP3 inflammasome, IL-1β, tumor necrosis factor-α (TNF-α), and SAA1 was measured by quantitative real-time polymerase chain reaction (q-PCR). Intracellular and extracellular amounts of IL-1β were determined by ELISA.

Results: Apo-SAA stimulated, in a time-dependent manner, the expression of NLRP3, IL-1β, and TNF-α in cortical microglia, and produced a concentration-dependent increase in the intracellular content of IL-1β in these cells. A 2-h 'priming' of the microglia with Apo-SAA followed by addition of ATP for 1 h, resulting in a robust release of IL-1β into the culture medium, with a concomitant reduction in its intracellular content. The selective $P2X_7R$ antagonist A740003 blocked ATP-dependent release of IL-1β. Microglia prepared from rat cerebellum displayed similar behaviors. As with LPS, Apo-SAA upregulated SAA1 and TLR2 mRNA, and downregulated that of TLR4. LPS was less efficacious than Apo-SAA, perhaps reflecting an action of the latter at TLR4 and TLR2. The TLR4 antagonist CLI-095 fully blocked the action of LPS, but only partially that of Apo-SAA. Although the TLR2 antagonist CU-CPT22 was inactive against Apo-SAA, it also failed to block the TLR2 agonist Pam_3CSK_4.

Conclusions: Microglia are central to the inflammatory process and a major source of IL-1β when activated. $P2X_7R$-triggered IL-1β maturation and export is thus likely to represent an important contributor to this cytokine pool. Given that SAA is detected in Alzheimer disease and multiple sclerosis brain, together with IL-1β-immunopositive microglia, these findings propose a link between $P2X_7R$, SAA, and IL-1β in CNS pathophysiology.

Keywords: Microglia, Serum amyloid A, Interleukin-1β, Toll-like receptor, P2X purinoceptor 7, NLRP3 inflammasome, Neuroinflammation

Background

Inflammatory conditions are marked by the production of mediators such as cytokines, chemokines, reactive oxygen species, and acute phase proteins that are key elements of the accompanying physiological and metabolic changes. C-reactive protein, complement proteins, and serum amyloid A protein (SAA) are some of the principal acute phase proteins, mainly generated in the liver and released into the systemic circulation in response to inflammation [1, 2]. SAA is the generic name of a family of proteins that share high levels of sequence homology but are encoded by different genes [3]. Humans possess four SAA genes (SAA1, SAA2, SAA3, and SAA4) mapped in a 150-kb region of chromosome 11p15.115. Mice also have four SAA genes whose protein products are highly homologous to their human counterparts [3]. Inducible expression is characteristic of

* Correspondence: stephen.skaper@unipd.it
Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Largo "E. Meneghetti" 2, 35131 Padua, Italy

© The Author(s). 2018 **Open Access** This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated.

all acute-phase SAAs including SAA1 and SAA2. Extra-hepatic expression of SAA has been reported as well [4].

Central nervous system (CNS) disorders are characterized by central activation of innate immunity and activation of a potent peripheral acute phase response that influences central inflammation and contributes to poor outcome [5]. Syrian hamsters injected systemically with lipopolysaccharide (LPS) had elevated levels of mRNA for Apo-SAA in all tissues examined, including brain [6]. While not detectable in normal brain, SAA protein has been found in Alzheimer disease (AD) brain, along with SAA gene expression in multiple sclerosis (MS) brain tissue [7]. Elevated SAA concentration was described in cerebrospinal fluid of AD subjects [8], as well as SAA immunoreactivity that co-localized with amyloid β-peptide deposits in AD brain [9]. Induction of a systemic acute phase response in SAA transgenic mice enhanced amyloid β-peptide deposition [10]. Further, Chung et al. [11] reported a much stronger immunostaining of SAA in brain of patients with neurologically confirmed AD and MS in comparison to unaffected regions and non-AD/MS brain. Barbierato et al. [12] recently demonstrated that cortical glia responds to pro-inflammatory agents (LPS, tumor necrosis factor alpha (TNF-α), Apo-SAA) by upregulating their expression of *Saa1*.

Interleukin-1β (IL-1β), a master regulator of neuroinflammation [13] mainly produced by activated inflammatory cells of the myeloid lineage [14], contributes importantly to cellular activation and cytokine production. IL-1β plays a key role in the pathogenesis of acute and chronic diseases of both the peripheral nervous system and CNS [15–17]. LPS, a potent stimulus for IL-1β synthesis by microglia is rather inefficient, given that most of the secreted cytokine remains in the immature (inactive) pro-form [18]. One of the molecules mainly involved in IL-1β maturation is the purinoceptor $P2X_7$ ($P2X_7R$), an ATP-gated ion channel that chiefly acts through the recruitment of the NLRP3 inflammasome-caspase-1 complex [14, 19]. This activation process involves first recognition by toll-like receptors (TLRs, a sub-family of pattern recognition receptors) of exogenous (e.g., bacterial- and virus-derived pathogens) or endogenous (e.g., components of cell damage) stimuli to induce transcription and translation of IL-1β ('priming'). This is followed by a secondary signal such as ATP to trigger formation of the inflammasome complex that leads to caspase 1 activation and cleavage/release of IL-1β [20–22]. $P2X_7R$-triggered IL-1β maturation and export may thus represent a major contributor to this cytokine pool [20, 23].

SAA appears to be an endogenous ligand for both TLR4 [24–27] and TLR2 [28–31], despite having little structural resemblance to the bacteria-derived ligands of either receptor. Although SAA can upregulate the NLRP3 inflammasome in peripheral immune cells [25] and provoke mediator production in a variety of non-neural cells, nothing is known about its ability to stimulate IL-1β release from CNS glia in the presence of ATP, a multi-target danger signal in the brain [32] in a $P2X_7R$-dependent manner. This is especially important, given the growing body of data indicating that genetic or pharmacological manipulation of $P2X_7Rs$ alters responsiveness in animal models of CNS neurological disorders [33]. Recent studies suggest also that $P2X_7Rs$ regulate the pathophysiology of psychiatric disorders, including mood disorders [33]. The present study was undertaken to examine the ability of ATP to promote the intracellular production, and release, of IL-1β from cortical microglia stimulated with Apo-SAA, and the involvement of $P2X_7R$, TLR4, and TLR2.

Methods

Tissue culture media, antibiotics, fetal calf serum (FCS), and NP40 cell lysis buffer (10×) were purchased from Life Technologies (San Giuliano Milanese, Italy); lipopolysaccaride (LPS) (Ultra-Pure LPS-EB from *E. coli* 0111:B4 strain; only activates TLR4), Pam_3CSK_4, and ethyl-(6R)-6-(N-(2-chloro-4--fluorophenyl)sulfamoyl)cyclohex-1-ene-1-carboxylate (CLI-095 or TAK 242) were from InvivoGen (Cayla-Invivogen Europe, Toulouse, France); A740003 from Tocris Bioscience, Pittsburgh, PA, USA); poly-L-lysine hydrobromide (mol wt 70,000–150,000), papain, DNase I (bovine pancreas), trypsin inhibitor, L-leucyl-L-leucine methyl ester (L-LME), protease inhibitor cocktail, Pefabloc® SC (100 mM), CU-CPT22, and all other biochemicals were purchased from Sigma-Aldrich (Milan, Italy) unless noted otherwise; recombinant human Apo-SAA (consensus SAA molecule corresponding to human Apo-SAA1α, except for the presence of an N-terminal methionine, the substitution of asparagine for aspartic acid at position 60, and arginine for histidine at position 71) from Peprotech (endotoxin level < 0.1 ng/μg protein; London, UK); QIAzol from Qiagen (Milan, Italy. Falcon tissue culture plastic-ware were purchased from BD Biosciences. Sterilin Petri plastic dishes (10 cm Ø) were obtained from Sarstedt (Verona, Italy).

Cell culture

Microglia were isolated from mixed glial cell cultures as previously described [34]. All experiments were conducted in compliance with Italian Ministry of Health (art. 31, D.L. 26/2014) guidelines for the care

and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the University of Padua (958/2016-PR). In brief, cells dissociated from postnatal day 1 rat pups (Charles River, Calco, Italy; strain: CD) cerebral cortices were plated in 75 cm^2 poly-L-lysine-coated tissue culture flasks (1.5 brains per flask) and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) with 2 mM glutamine, 50 units/ml penicillin/50 μg/ml streptomycin, 50 μg/ml gentamycin, and 10% FCS ('growth medium'). Culture medium was changed after 24 h. The cultures reached confluence by 7 days at which time microglia were recovered by shaking the flasks on an orbital shaker at 200 rpm for 1 h (37 °C). The remaining cell monolayers were highly enriched in astrocytes (< 5% microglia, as determined by flow cytometry using cell type-specific antibodies) [35]. The culture supernatant containing microglia was transferred to Sterilin plastic Petri dishes and incubated for 45 min at 37 °C (5% CO_2, 95% air) to allow adhesion of microglia. The adherent microglial cells (> 99% pure, as determined by flow cytometry using cell type-specific antibodies) [35] were detached by mechanically scraping into growth medium and re-plated in this same medium, on poly-L-lysine-coated 24-well or 96-well culture plates (250,000 and 50,000 cells per well for mRNA and cytokine analysis, respectively). For some experiments, the astrocyte monolayers were depleted of residual microglia using a 60-min exposure (50 mM) to the lysosomotropic agent L-LME [36], as described previously [37, 38]. Astrocyte plating densities were the same as used for microglia.

Quantitative real-time polymerase chain reaction (q-PCR)

Total RNA was extracted from cells by QIAzol, according to the manufacturer's instructions. RNA integrity and quantity were determined by RNA 6000 Nano assay in an Agilent BioAnalyser ($A_{260/280}$ ratio > 1.8). Reverse transcription was performed with Superscript III reverse transcriptase (Invitrogen). The q-PCR reaction was performed as described previously [37]. Primer sequences are listed in Table 1. Amounts of each gene product were calculated using linear regression analysis from standard curves, demonstrating amplification efficiencies ranging from 90 to 100%. Dissociation curves were generated for each primer pair, showing single product amplification. Data are normalized to β-actin mRNA level.

IL-1β production and release

Purified microglia and enriched astrocytes were plated in poly-L-lysine coated 96-well plates (50,000 cells per well) in growth medium and allowed to adhere overnight. These plating densities do not affect glial cell vitality/function [39, 40]. Cells were primed by pre-treating with 0.1 μg/ml LPS (optimal concentration chosen from preliminary experiments) [18, 37, 41] or different concentrations of recombinant human Apo-SAA (as indicated in the experiment) for 2 h in serum-free culture medium prior to stimulation with 5 mM ATP [41] for 1 h. None of the treatments, at the concentrations tested, affected cell viability (data not shown; see also [12, 42–44]). Cell supernatants were collected and stored at −20 °C until the day of assay (avoiding repeated freeze-thaw cycles). Cell lysates were prepared by adding to each 96-well culture 100 μl lysis solution containing: 89 μl NP40 lysis buffer, 10 μl of 10× protease inhibitor cocktail, and 1 μl of 100 mM Pefabloc SC. IL-1β content of culture medium and cell lysates was analyzed using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Antigenix America, Huntington Station, NY, USA). Standards with known amounts of IL-1β and TNF-α were used to convert values into absolute concentrations of the cytokine in pg/ml.

Table 1 PCR primers used in this study

Gene target	Direction	Sequence
β-ACT	F	5′-CCCCATTGAACACGGCATTGTCA-3′
	R	5′-ACCCTCATAGATGGGCACAGTGT-3′
IL-1β	F	5′-TGTGGCAGCTACCTATGTCT-3′
	R	5′-GGGAACATCACACACTAGCA-3′
NLRP3	F	5′-TGATGCATGCACGTCTAATCTC-3′
	R	5′-CAAATCGAGATGCGGGAGAG-3′
SAA1	F	5′-ACACGGAGCAGAGGACTCAAG-3′
	R	5′-GGTCGAAAGTGGTTGGGGTC-3′
TNF-α	F	5′-CATCTTCTCAAAACTCGAGTGACAA-3′
	R	5′-TGGGAGTAGATAAGGTACAGCCC-3′
TLR2	F	5′-TCCATGTCCTGGTTGACTGG-3′
	R	5′-AGGAGAAGGGCACAGCAGAC-3′
TLR4	F	5′-GATTGCTCAGACATGGCAGTTTC-3′
	R	5′-CACTCGAGGTAGGTGTTTCTGCTAA-3′

β-ACT β-actin, *SAA* serum amyloid A, *IL-1β* intereukin-1β, *TNF-α* tumor necrosis factor α, *NLRP3* inflammasome, *TLR* toll-like receptor. *F* forward, *R* reverse

Statistics

Data are given as mean ± sem, unless stated otherwise. Statistical analyses to determine group differences were performed either by two-sample equal variance Student's *t* test, or by one-way analysis of variance followed by Dunnett's or Bonferroni's post hoc tests for comparisons involving more than two data groups. Significance was taken at $p < 0.05$.

Results

SAA upregulates NLRP3 and IL-1β mRNA in rat primary microglia

SAA mediates cytokine production by a variety of non-neural cell types [45], as well as primary microglia [12]. However, it is not known if SAA is able also to up-regulate expression of the NLRP3 inflammasome gene in CNS-derived glia. Incubation of rat primary cortical microglia with Apo-SAA resulted in a time-dependent rise in *Nlrp3* expression that peaked at 3 h (Fig. 1, solid bars); a similar time-course was observed when microglia were stimulated with LPS (Fig. 1, shaded bars). Relative expression levels appeared higher for Apo-SAA than for LPS-treated cells (optimal concentrations chosen for Apo-SAA and LPS were based on preliminary experiments; see also [12, 35]). The kinetics of *Tnf-α* expression in Apo-SAA- and LPS-stimulated microglia mirrored that seen for NLRP3 mRNA (Additional file 1: Figure S1). Interestingly, the expression level of *Il-1β* in both Apo-SAA- and LPS-treated cells peaked already at 3 h (Fig. 2, solid and shaded bars, respectively) and remained at plateau through 24 h for Apo-SAA. In contrast, intracellular content of IL-1β peaked at 6 h in both Apo-SAA- and LPS-treated microglia and then declined, although remained above basal up to 24 h (Additional file 2: Figure S2). Exposure of microglia to either Apo-SAA or LPS led to a gradual up-regulation of *Saa1* over the 24-h study period (Fig. 3, solid and shaded bars, respectively), suggesting a potential autocrine/paracrine effect of SAA. Again, the effect of Apo-SAA was more robust than that of LPS in terms of relative expression levels for *Saa1*.

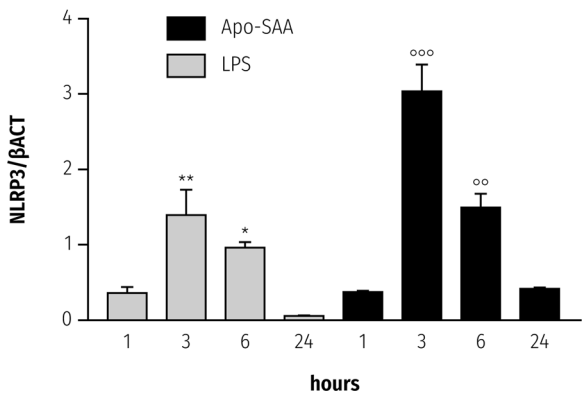

Fig. 1 Treatment of rat cortical microglia with Apo-SAA or LPS upregulates in a time-dependent manner mRNA for NLRP3. Cultures were treated the day after plating with 0.5 μg/ml recombinant human Apo-SAA or 0.1 μg/ml LPS and processed 1, 3, 6, and 24 h later for q-PCR, as detailed in the "Methods" section. Data are presented as relative expression level (normalized with respect to β-actin (βACT)) at each time point and are mean ± sem, $n = 6$. Control values (which were at the limit of detection) were omitted for clarity. Apo-SAA (■); LPS (▒). For LPS, $*p < 0.05$ and $**p < 0.01$ vs 1 h; for Apo-SAA, $^{\circ\circ}p < 0.01$ and $^{\circ\circ\circ}p < 0.001$ vs 1 h

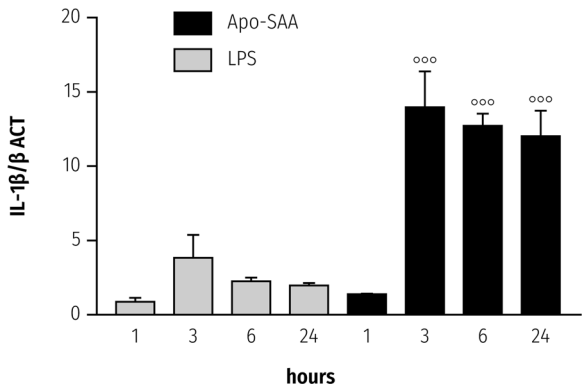

Fig. 2 Treatment of rat cortical microglia with Apo-SAA or LPS upregulates in a time-dependent manner mRNA for IL-1β. Cultures were treated the day after plating with 0.5 μg/ml recombinant human Apo-SAA or 0.1 μg/ml LPS and processed 1, 3, 6, and 24 h later for q-PCR, as detailed in the "Methods" section. Data are presented as relative expression level (normalized with respect to β-actin (βACT)) at each time point and are mean ± sem, $n = 3$. Control values (which were at the limit of detection) were omitted for clarity. Apo-SAA (■); LPS (▒). For Apo-SAA, $^{\circ\circ\circ}p < 0.001$ vs 1 h

SAA primes cortical and cerebellar microglia for ATP- and P2X$_7$R-dependent IL-1β release

In the brain, IL-1β is mainly produced by activated microglia [46], and ATP-dependent/P2X$_7$R-triggered IL-1β maturation and export is believed to contribute significantly to this cytokine pool in nervous system

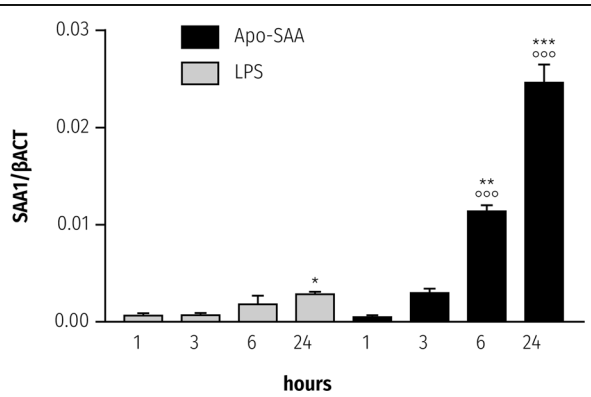

Fig. 3 Treatment of rat cortical microglia with Apo-SAA or LPS upregulates in a time-dependent manner mRNA for SAA1. Cultures were treated the day after plating with 0.5 μg/ml recombinant human Apo-SAA or 0.1 μg/ml LPS and processed 1, 3, 6, and 24 h later for q-PCR, as detailed in the "Methods" section. Data are presented as relative expression level (normalized with respect to β-actin (βACT)) at each time point and are mean ± sem, $n = 6$. Control values (which were at the limit of detection) were omitted for clarity. Apo-SAA (■); LPS (▒). For LPS, $*p < 0.05$ vs 1 h; for Apo-SAA, $^{\circ\circ\circ}p < 0.001$ vs 1 h, $**p < 0.01$ vs 1 h, and $***p < 0.001$ vs 6 h

pathologies [20, 23]. TLR4/TLR2 signaling pathways may be involved in neurodegenerative disorders including motor neuron disease [47], cerebral hypoxia-ischemia [48, 49] and blood-spinal cord barrier dysfunction after ischemia/reperfusion injury [50], and neuropathic pain [51]. Using a protocol established for TLR agonist priming of primary CNS glia [38], cortical microglia were first incubated with different concentrations of Apo-SAA for 2 h in serum-free medium followed by addition of ATP to a final concentration of 5 mM. After a further 60 min of incubation, cell culture medium and cell lysates were collected for measurement of IL-1β by ELISA. Incubation with Apo-SAA only (no ATP) led to a concentration-dependent accumulation of intracellular IL-1β that was already maximal at 0.5 μg/ml Apo-SAA, with very little IL-1β in the culture medium (left half of Fig. 4a, b, respectively). Conversely, incubation with Apo-SAA/ATP produced a concentration-dependent accumulation of IL-1β in the culture medium (again maximal at 0.5 μg/ml Apo-SAA), with a concomitant loss of intracellular IL-1β (right half of Fig. 4a and b, respectively, solid bars). Qualitatively similar effects were seen with 0.1 μg/ml LPS (maximal effective concentration), although once again the magnitude of cytokine production was greater for Apo-SAA.

Routinely used methods for preparing rodent primary astrocyte cell cultures generally contain variable, small percentages (up to 5%) of contaminating microglia [52]. A number of studies have demonstrated that inflammatory mediator output from enriched astrocytes is dependent on the presence of residual microglia [12, 37, 38, 53, 54]. Utilizing the lysosomotropic agent L-LME [55] to remove any remaining microglia [36–38, 56–58], purified cortical astrocytes were unresponsive to priming by Apo-SAA, as observed earlier when using LPS [38] (data not shown).

To interrogate a role for $P2X_7R$ in the Apo-SAA/ATP-mediated IL-1β release, microglia were pretreated 30 min with the selective $P2X_7R$ antagonist A740003 [59], then primed 2 h with Apo-SAA followed by a further 1-h incubation with ATP. The expected rise in intracellular IL-1β caused by exposure to either Apo-SAA or LPS was unaffected by A740003, while the fall in intracellular IL-1β in cells treated with Apo-SAA or LPS and ATP did not occur (Fig. 5a). In contrast, A740003 prevented the increase in extracellular IL-1β in Apo-SAA- or LPS-primed rat cortical microglia treated with ATP (Fig. 5b). These observations most likely are a consequence of blocking $P2X_7R$-dependent cleavage of IL-1β precursor to the releasable mature form of the cytokine.

Purified microglia cultured from rat cerebellum also respond to ATP-dependent IL-1β release upon priming with TLR2, TLR3, and TLR4 isoform agonists, in a $P2X_7R$-dependent manner [38].

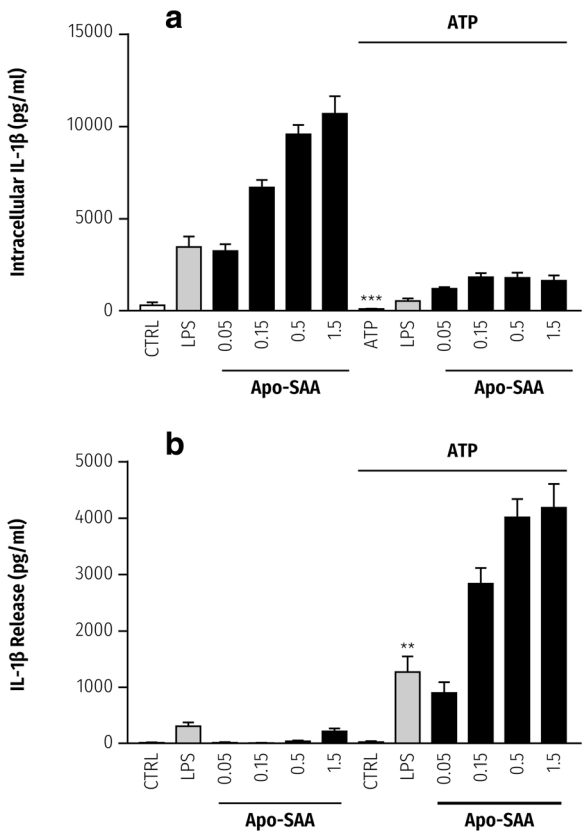

Fig. 4 Extracellular ATP causes release of IL-1β from rat cortical microglia primed with Apo-SAA or LPS. Cortical microglia cultured in 96-well plates were first incubated 2 h with 0.05–1.5 μg/ml recombinant human Apo-SAA or 0.1 μg/ml LPS. ATP was then added to a final concentration of 5 mM as indicated by upper horizontal bar. After a further 60-min incubation, cell lysates (**a**) and culture medium (**b**) were collected for IL-1β analysis by ELISA. Note that Apo-SAA was again a more efficacious priming stimulus than was LPS. Control (CTRL). Apo-SAA (■); LPS (▩). Data are mean ± sem ($n = 6$). **a** $^{**}p < 0.01$ vs LPS only (no ATP). Apo-SAA at all concentrations differed significantly with added ATP ($p < 0.001$) compared to the same concentration with no ATP. **b** $^{***}p < 0.001$ vs LPS only (no ATP). Apo-SAA at all concentrations differed significantly with added ATP ($p < 0.001$) compared to the same concentration with no ATP

To determine if this is also the case for priming with Apo-SAA, cerebellar microglia were incubated for 2 h with 0.5 μg/ml recombinant human Apo-SAA followed by addition of ATP to 5 mM or culture medium only. As Fig. 6a shows, Apo-SAA caused a clear rise in the intracellular content of IL-1β, which was markedly diminished in the presence of ATP. Conversely, IL-1β in the culture medium increased for Apo-SAA + ATP-treated cells. Qualitatively analogous results were obtained using LPS (Fig. 6b), as seen previously [38].

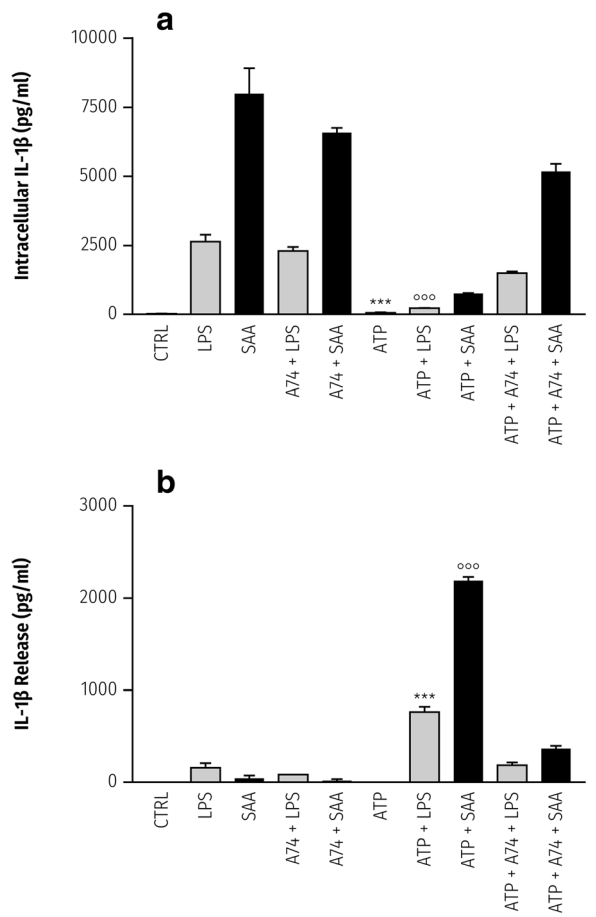

Fig. 5 The P2X$_7$R antagonist A740003 prevents the ATP-dependent fall in intracellular IL-1β (**a**) and rise in extracellular IL-1β (**b**) in Apo-SAA- or LPS-primed rat cortical microglia. Microglia cultured in 96-well plates were pretreated for 30 min with 10 μM A740003 ('A74') followed by a 2-h incubation with 0.5 μg/ml recombinant human Apo-SAA ('SAA') or 0.1 μg/ml LPS. ATP was then added to a final concentration of 5 mM. After a further 60-min incubation, culture medium (**b**) and cell lysates (**a**) were collected for IL-1β analysis by ELISA. Apo-SAA (▒); LPS (▬) Data are mean ± sem ($n = 3$). ***$p < 0.001$ vs LPS or LPS + ATP + A740003; °°°$p < 0.001$ vs Apo-SAA or Apo-SAA + ATP + A740003. There were no statistically significant differences between the LPS/LPS + A740003/LPS + ATP + A740003 and Apo-SAA/Apo-SAA + A740003/Apo-SAA + ATP + A740003 groups

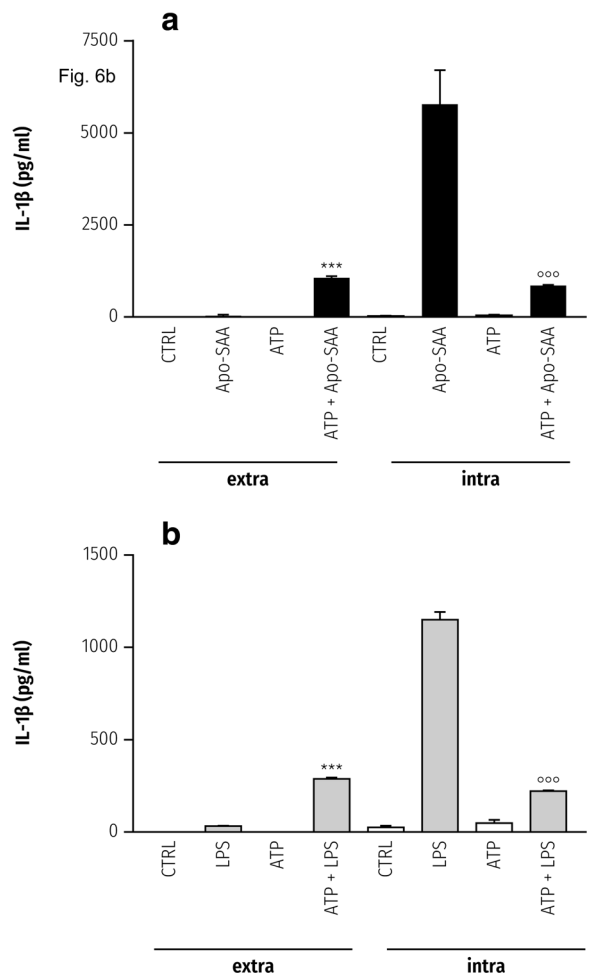

Fig. 6 Extracellular ATP causes release of IL-1β from purified rat cerebellar microglia primed with Apo-SAA or LPS. Microglia cultured in 96-well plates were first incubated 2 h with 0.5 μg/ml recombinant human Apo-SAA (**a**) or 0.1 μg/ml LPS (**b**). ATP was then added to a final concentration of 5 mM. After a further 60-min incubation, culture medium ('extra') and cell lysates ('intra') were collected for IL-1β analysis by ELISA. As for cortical microglia, here also Apo-SAA was a more efficacious stimulus than was LPS (note the difference in y-axis scales). Control (CTRL). Data are mean ± sem ($n = 6$). ***$p < 0.001$: Apo-SAA vs Apo-SAA + ATP; or LPS vs LPS + ATP (extracellular). °°°$p < 0.001$ vs Apo-SAA vs Apo-SAA + ATP; or LPS vs LPS + ATP (intracellular)

Apo-SAA modulates TLR2 and TLR4 gene expression in cortical microglia

In purified primary rat cortical microglia, TLR4 and TLR2 agonists have been reported to upregulate TLR2 mRNA expression while downregulating that of TLR4 [35]. Given that SAA is a putative agonist for both TLR4 and TLR2, we asked whether SAA could also affect *Tlr2* and *Tlr4* expression in cortical microglia. Apo-SAA treatment of microglia time-dependently increased *Tlr2*, with a peak at 3 h and a decline over 24 h (Fig. 7a). In contrast, *Tlr4* expression (normalized to β-actin) was significantly reduced at 3, 6, and 24 h (Fig. 7b). In confirmation of previous findings [35], LPS also raised *Tlr2* expression at 3 h, while downregulating that of *Tlr4* eat all time points (Additional file 3: Figure S3).

Several studies suggest that SAA is capable of activating TLR4 [24–27] and TLR2 [28–31]. To explore this in the present setting, cortical microglia were first pre-incubated for 30 min with either the TLR4 antagonist CLI-095 [60] or the TLR2 antagonist CU-CPT22 [61]. The former (also known as TAK-242) suppresses specifically TLR4 signaling, inhibiting the production of pro-inflammatory cytokines [62]. CLI-095 acts by

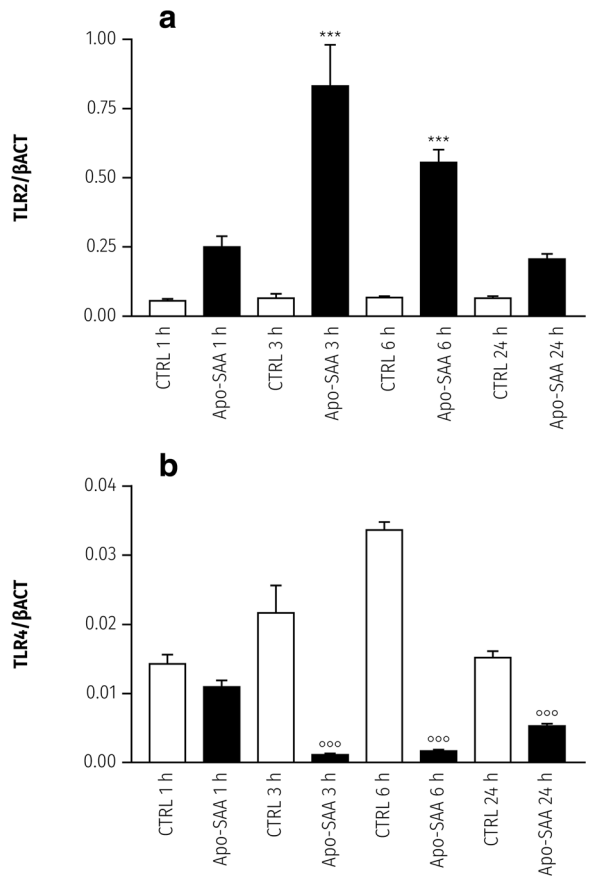

Fig. 7 Treatment of rat cortical microglia with Apo-SAA, in a time-dependent manner, upregulates mRNA for TLR2 and downregulates that for TLR4. Cultures were treated the day after plating with 0.5 μg/ml recombinant human Apo-SAA and processed 1, 3, 6, and 24 h later for q-PCR, as detailed in the "Methods" section. **a** TLR2. **b** TLR4. Data are presented as relative expression level (normalized with respect to β-actin (βACT)) at each time point and are mean ± sem, $n = 3$ ($n = 9$ for 3 h). Control (CTRL; untreated) cultures. ***$p < 0.001$ vs CTRL for that time point; °°°$p < 0.001$ vs CTRL for that time point. Note the difference in expression levels between TLR2 and TLR4 mRNA

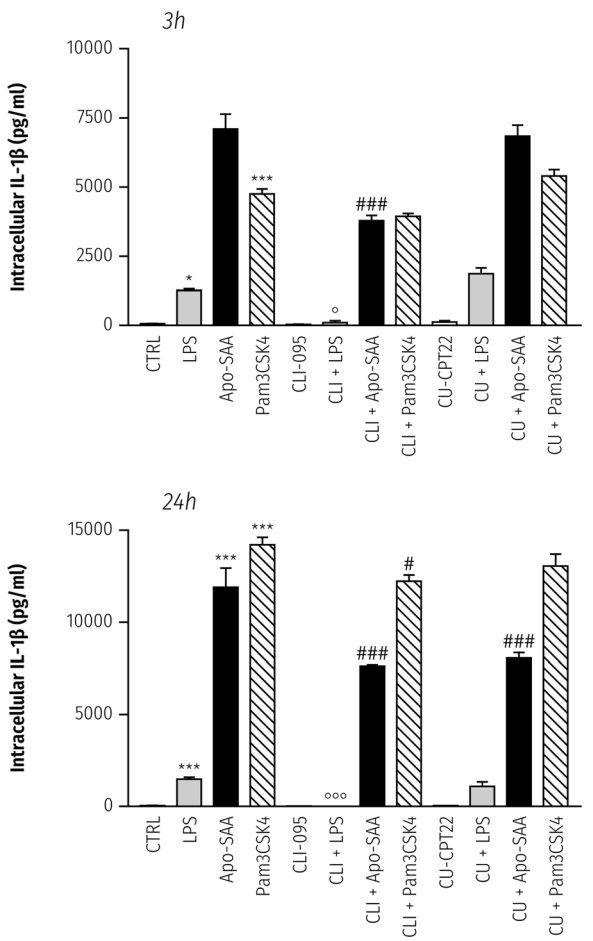

Fig. 8 Effect of the TLR4 antagonist CLI-095 and the TLR1/2 antagonist CU-CPT22 on intracellular IL-1β content in rat cortical microglia treated with LPS, Apo-SAA, or Pam_3CSK_4. The day after plating culture medium was replaced with 50 μl/well of serum-free medium containing 1 μg/ml CLI-095 ('CLI') or 20 μM CU-CPT ('CU'). After 30-min incubation, equal volume of medium was added containing 0.2 μg/ml LPS, 1 μg/ml recombinant human Apo-SAA, or 0.6 μg/ml Pam_3CSK_4. Final concentrations of all added agents were thus one-half of the values indicated. After a further incubation of 3 and 24 h cell lysates were collected for IL-1β analysis by ELISA, as detailed in the "Methods" section. (upper panel) 3 h; (lower panel) 24 h. Data are mean ± sem, $n = 4$. Control (CTRL; untreated) cultures. *$p < 0.05$ and ***$p < 0.001$ vs CTRL; °$p < 0.05$ and °°°$p < 0.001$ vs LPS; ###$p < 0.001$ vs Apo-SAA; #$p < 0.05$ and vs Pam_3CSK_4

blocking signaling mediated by the intracellular domain of TLR4, but not the extracellular domain, and suppresses both ligand-dependent and ligand-independent signaling of TLR4 [62]. CU-CPT22 is reported to compete with the synthetic triacylated lipoprotein (Pam_3CSK_4) binding to TLR1/2 and repress downstream signaling from IL-1β and TNF-α [61].

Subsequent addition of LPS (0.1 μg/ml), Apo-SAA (0.5 μg/ml), or the TLR2 agonist Pam_3CSK_4 (0.3 μg/ml) produced significant increases in the intracellular content of IL-1β after both 3 and 24 h (Fig. 8, upper and lower panels, respectively). CLI-095 pre-treatment fully blocked the effect of LPS, and partially (but significantly, $p < 0.001$) that of Apo-SAA at both time points. However, the stimulatory effect of Pam_3CSK_4 was unaffected by the TLR4 antagonist at 3 h, and modestly so at 24 h. CU-CPT22 failed to alter the stimulatory effect of Apo-SAA or LPS; surprisingly, this TLR1/2 antagonist also was ineffective when tested on cells challenged with the TLR1/2 agonist Pam_3CSK_4—in contrast to earlier findings [61]. Similar results were obtained for TNF-α release from the same cells (data (pg/ml) are mean ± sem, 3-h point): control, CLI-095, and CU-CPT22-treated cells, 0; LPS, 311 ± 24; Apo-SAA, 840 ± 102; Pam_3CSK_4, 322 ± 9; LPS + CLI-095, 0; Apo-SAA + CLI-095, 224 ± 3; Pam_3CSK_4 + CLI-095, 242 ± 12; LPS + CU-CPT22, 399 ± 23; Apo-SAA +

CU-CPT22, 822 ± 76; 420 ± 3; CLI-095 fully eliminated the effect of LPS and partially, but significantly, that of Apo-SAA ($p < 0.001$).

Discussion

Growing evidence indicates that CNS disorders are characterized by central activation of innate immunity, as well as activation of a potent peripheral acute phase response that influences central inflammation and leads to poor disease outcome [5]. The acute phase response plays a critical role in the innate immune response to tissue injury [63]. Among acute phase proteins such as C-reactive protein, complement proteins, and SAA, the last one can be considered a "danger signal" that influences the inflammation process [64]. Its low basal level and high inducibility are in keeping with danger signal molecules [65], being produced in response to potentially harmful environmental cues, including trauma, infection, surgery, and severe stress. A number of studies imply a role for SAA in inflammation-associated neuropathologies [7–11], although the underlying molecular processes remain to be fully explored. Here, we show that in neonatal cortical microglia, Apo-SAA time-dependently upregulates NLRP3 inflammasome and IL-1β mRNA expression and intracellular production of IL-1β and stimulates release of IL-1β in the presence of ATP, a multi-target danger signal in the brain [32] in a $P2X_7R$-dependent manner. The rise in extracellular release of IL-1β in the presence of ATP was accompanied by a fall in the intracellular content, consistent with NLRP3/caspase 1-complex 1 activation and cleavage of the pro-form of IL-1β to the mature, active secreted species [20–22]. The action of Apo-SAA was not limited to cortical microglia, as similar ATP-dependent release of IL-1β was also seen for cerebellar microglia. IL-1β is viewed as a master regulator of neuroinflammation [13] that contributes importantly to cellular activation and cytokine production. This cytokine plays a key role in the pathogenesis of acute and chronic diseases of both the peripheral nervous system and CNS [15–17]. It merits mention that the effects of Apo-SAA on gene expression and cytokine production were of a far greater magnitude than those obtained using the optimal concentration of LPS as benchmark, thus highlighting the pro-inflammatory potency of this acute phase protein. Whether adult microglia would respond differently was not tested.

A comparison of the SAA concentrations used in the present investigation with levels of SAA previously detected in human cerebrospinal fluid and plasma suggests the potential for physiological relevance to the in vivo setting. In one report, SAA levels in cerebrospinal fluid of AD subjects were found to be much higher than in normal controls [8], and generally within the range of the highest concentration used here. Serum concentrations of SAA in relapsing-remitting MS patients have been reported elevated with a mean level of 12.1 ± 8.7 μg/ml [66], and significantly increased (mean value 10 μg/ml, $p = 0.030$ vs. control) in neuromyelitis optica patients [67]. These values contrast with active concentrations of 0.15–1.5 μg/ml in the present in vitro study.

An expanding body of data demonstrates that pharmacological or genetic manipulation of $P2X_7Rs$ alters their responsiveness in animal models of CNS neurological disorders [33, 68]. The $P2X_7R$ has been suggested to also regulate the pathophysiology of psychiatric disorders, including mood disorders [33]. $P2X_7R$-triggered IL-1β maturation and export is thus likely to represent an important contributor to this cytokine pool [20, 23]. SAA is not detectable in normal brain but has been reported in AD brain, together with its gene in MS brain [7]. Miida et al. [8] described a raised SAA concentration in cerebrospinal fluid of AD. SAA immunoreactivity was reported to co-localize with amyloid β-peptide deposits in AD brain [9]. $P2X_7R$-positive microglia surrounded amyloid plaques in a mouse transgenic AD model [69], and microglia around amyloid plaques in AD brain are immunopositive for IL-1β [70]. Collectively, these findings propose a link between $P2X_7R$, Apo-SAA, and IL-1β in AD pathophysiology. In addition, the ability of Apo-SAA to regulate its own gene expression suggests the potential for autocrine/paracrine effects of SAA. Since microglia in the AD brain adopt distinct functional and molecular phenotypes, it is conceivable that the response of "AD microglia" to SAA would differ from that of wild-type microglia.

A number of reports indicate the capability of SAA to act as an agonist for both TLR4 [24–27] and TLR2 [28–31, 71]. Ligand engagement of TLR4 by LPS and TLR2 by Pam_3CSK_4 leads to the upregulation of *Tlr2* and downregulation of *Tlr4* in cortical microglia [35]. Consistent with its putative action as a ligand for both TLR2 and TLR4, Apo-SAA produced a time-dependent robust and significant increase in *Tlr2* expression in cortical microglia, with a concomitant reduction in the relative level of *Tlr4*. Conceivably, this action of SAA could result in a 'feed-forward' mechanism, whereby SAA increases expression of its receptor and amplification of a priming response. Attempts at using pharmacological tools to dissect participation of TLR4 and TLR2 in the actions of Apo-SAA were equivocal. The selective TLR4 antagonist CLI-095 completed blocked the ability of LPS to synthesize/release IL-1β and partially, but significantly, that of Apo-SAA. While the TLR2 antagonist CU-CPT22 failed to alter the stimulatory effect of Apo-SAA or LPS, it also proved ineffective on microglia treated with the TLR1/2 agonist Pam_3CSK_4. Our inability to confirm the earlier report

for CU-CPT22 action against Pam_3CSK_4 [61] could be due to differences in cell type used (primary microglia vs RAW264.7 macrophages) or treatment times (not specified in [61]), even though we used two incubation times and the same concentrations of ligand and antagonist as in [61]. Another consideration is that CU-CPT22 was designed to compete with Pam_3CSK_4 binding to TLR1/2, thus disrupting formation of the TLR1/TLR2 heterodimer [61]. Although outside the scope of the present study, the use of microglia from $TLR2^{-/-}$ animals could provide a tool to address this question.

A failure of remyelination is responsible, in large part, for the long-term neurologic consequences of MS. An intriguing study by Sloane et al. [72] described upregulated TLR2 expression by oligodendrocytes in MS lesions, with pathogen-derived TLR2 agonists, but not agonists for other TLRs, inhibiting oligodendrocyte precursor cell (OPC) maturation in vitro. Ablated expression of TLR2 also enhanced remyelination in a lysolecithin animal model of MS [72]. Intense immunohistochemical staining of SAA has been detected in the brains of patients with neurologically confirmed MS in comparison to an unaffected region and non-MS brains, with the major site of staining being the myelin sheaths of axons in affected cortex [11]. Pro-inflammatory cytokines such as IL-1β and TNF-α [73] may play important roles in expression of SAA1 and SAA2. The pathophysiology of a variety of neurological disorders, including MS, is associated with TNF-α [74, 75], a master pro-inflammatory product of activated microglia and peripheral macrophages implicated in the pathogenesis of CNS demyelination [76, 77]. Apo-SAA treatment of rat cortical microglia increased production of TNF-α and IL-1β, while TNF-α time-dependently raised *Saa1* expression in cultured OPCs [12]. Our findings in the context of the above considerations, together with evidence for $P2X_7R$ in the development of experimental autoimmune encephalomyelitis [78] and microglia-oligodendrocyte crosstalk [79], propose a vicious cycle of Apo-SAA, IL-1β, and TLR2 leading to the demise of OPCs.

Conclusions

CNS disorders are characterized by central activation of innate immunity and activation of a potent peripheral acute phase response that influences central inflammation and contributes to poor outcome. Our data show that in microglia, the acute phase protein Apo-SAA upregulates NLRP3 inflammasome and IL-1β mRNA expression and intracellular production of IL-1β and stimulates release of IL-1β in the presence of ATP in a $P2X_7R$-dependent manner. Apo-SAA upregulated expression of its own gene and that of TLR2, suggesting a potential 'feed-forward' mechanism, whereby SAA increases expression of its receptor and amplification of a priming response. The effects of Apo-SAA on gene expression and cytokine production were of a far greater magnitude than those observed with the classical TLR4 agonist lipopolysaccharide, highlighting the pro-inflammatory potency of this acute phase protein. Given the evidence for $P2X_7Rs$ involvement in CNS neurological disorders and expression of SAA in AD and MS brain, the findings presented here propose a link between $P2X_7R$, SAA, and IL-1β in CNS pathophysiology.

Additional files

Additional file 1: Figure S1. Treatment of rat cortical microglia with Apo-SAA or LPS upregulates, in a time-dependent manner mRNA for TNF-α. Cultures were treated the day after plating with 0.5 μg/ml recombinant human Apo-SAA or 0.1 μg/ml LPS and processed 1, 3, 6, and 24 h later for q-PCR, as detailed in the "Methods" section. Data are presented as relative expression level (normalized with respect to β-actin (βACT)) at each time point and are mean ± sem, $n = 3$. Control values (which were at the limit of detection) were omitted for clarity. Apo-SAA (■); LPS (▓). For LPS, $*p < 0.05$ vs 1 and 24 h; for Apo-SAA: $^{\circ\circ\circ}p < 0.001$ vs 1, 6, and 24 h. (TIF 468 kb)

Additional file 2: Figure S2. Treatment of rat cortical microglia with Apo-SAA or LPS upregulates, in a time-dependent manner, intracellular content of IL-1β. Cultures were treated the day after plating with 0.5 μg/ml recombinant human Apo-SAA or 0.1 μg/ml LPS and processed 1, 3, 6, and 24 h later for measurement of intracellular IL-1β, as detailed in the "Methods" section. Apo-SAA (■); LPS (▓). Data are expressed as mean ± sem, $n = 3$. The quantity of intracellular IL-1β in unstimulated cells was below the detection limit of the ELISA assay kit. For LPS, $**p < 0.01$ vs 3 and 6 h; for Apo-SAA, $^{\circ\circ\circ}p < 0.001$ vs 1 h, $***p < 0.001$ vs 3 and 24 h. (TIF 485 kb)

Additional file 3: Figure S3. Treatment of rat cortical microglia with LPS, in a time-dependent manner, upregulates mRNA for TLR2 and downregulates that for TLR4. Cultures were treated the day after plating with 0.1 μg/ml LPS and processed 1, 3, 6, and 24 h later for q-PCR, as detailed in the "Methods" section. (a) TLR2. (b) TLR4. Data are presented as relative expression level (normalized with respect to β-actin (βACT)) at each time point and are mean + sem, $n = 3$. Control (CTRL; untreated) cultures. Data are expressed as mean ± sem, $n = 3$ ($n = 9$ for 3 h). (a) $**p < 0.01$ vs CTRL (3 h) and $***p < 0.001$ vs CTRL (6 h). (b) $*p < 0.05$ vs CTRL (3 h), $***p < 0.001$ vs CTRL for that time point. Note the difference in expression levels between TLR2 and TLR4 mRNA. (TIF 957 kb)

Abbreviations

AD: Alzheimer's disease; DMEM: Dulbecco's modified Eagle's medium; FCS: Fetal calf serum; IL-1β: Interleukin-1β; L-LME: L-leucyl-L-leucine methyl ester; LPS: Lipopolysaccaride; MS: Multiple sclerosis; q-PCR: Quantitative real-time-PCR; TLR: Toll-like receptor; TNF-α: Tumor necrosis factor-α

Acknowledgements

The authors wish to thank Luca Di Giacomo for assistance in preparing the figures which accompany this manuscript.

Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MB and LF carried out the q-PCR analyses and data elaboration. LF participated in the preparation of the cultures, experimental design and culture treatments, cytokine assays, and in the statistical analysis. PG and MZ participated in drafting the manuscript. SDS conceived and coordinated the study and drafted the manuscript. All authors critically revised and approved the final manuscript.

Ethics approval
All experiments were conducted in compliance with Italian Ministry of Health (art. 31, D.L. 26/2014) guidelines for the care and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee of the University of Padua (958/2016-PR).

Competing interests
The authors declare that they have no competing interests.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 20 March 2018 Accepted: 15 May 2018
Published online: 26 May 2018

References
1. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340:448–54.
2. Gruys E, Toussaint MJ, Niewold TA, Koopmans SJ. Acute phase reaction and acute phase proteins. J Zhejiang Univ Sci B. 2005;6:1045–56.
3. Sun L, Ye RD. Serum amyloid A1: structure, function and gene polymorphism. Gene. 2016;583:48–57.
4. Kumon Y, Sipe JD, Brinckerhoff CE, Schreiber BM. Regulation of extrahepatic apolipoprotein serum amyloid A (ApoSAA) gene expression by interleukin-1 alpha alone: synthesis and secretion of ApoSAA by cultured aortic smooth muscle cells. Scand J Immunol. 1997;46:284–91.
5. Rajkovic I, Denes A, Allan SM, Pinteaux E. Emerging roles of the acute phase protein pentraxin-3 during central nervous system disorders. J Neuroimmunol. 2016;292:27–33.
6. Hardardóttir I, Sipe J, Moser AH, Fielding CJ, Feingold KR, Grünfeld C. LPS and cytokines regulate extra hepatic mRNA levels of apolipoproteins during the acute phase response in Syrian hamsters. Biochim Biophys Acta. 1997; 1344:210–20.
7. Liang JS, Sloane JA, Wells JM, Abraham CR, Fine RE, Sipe JD. Evidence for local production of acute phase response apolipoprotein serum amyloid A in Alzheimer's disease brain. Neurosci Lett. 1997;225:73–6.
8. Miida T, Yamada T, Seino U, Ito M, Fueki Y, Takahashi A, Kosuge K, Soda S, Hanyu O, Obayashi K, Miyazaki O, Okada M. Serum amyloid A (SAA)-induced remodeling of CSF-HDL. Biochim Biophys Acta. 2006;1761:424–33.
9. Kindy MS, Yu J, Guo JT, Zhu H. Apolipoprotein serum amyloid A in Alzheimer's disease. J Alzheimers Dis. 1999;1:155–67.
10. Guo JT, Yu J, Grass D, de Beer FC, Kindy MS. Inflammation-dependent cerebral deposition of serum amyloid a protein in a mouse model of amyloidosis. J Neurosci. 2002;22:5900–9.
11. Chung TF, Sipe JD, McKee A, Fine RE, Schreiber BM, Liang JS, Johnson RJ. Serum amyloid A in Alzheimer's disease brain is predominantly localized to myelin sheaths and axonal membrane. Amyloid. 2000;7:105–10.
12. Barbierato M, Borri M, Facci L, Zusso M, Skaper SD, Giusti P. Expression and differential responsiveness of central nervous system glial cell populations to the acute phase protein serum amyloid A. Sci Rep. 2017;7:1. https://doi.org/10.1038/s41598-017-12529-7.
13. Basu A, Krady JK, Levison SW. Interleukin-1: a master regulator of neuroinflammation. J Neurosci Res. 2004;78:151–6.
14. Giuliani AL, Sarti AC, Falzoni S, Di Virgilio F. The P2X7 receptor-interleukin-1 liaison. Front Pharmacol. 2017;8:123. https://doi.org/10.3389/fphar.2017.00123.
15. Allan SM, Tyrrell PJ, Rothwell NJ. Interleukin-1 and neuronal injury. Nat Rev Immunol. 2005;5:629–40.
16. Dinarello CA, Simon A, van der Meer JW. Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. Nat Rev Drug Discov. 2012;11:633–52.
17. Tanaka S, Ishii A, Ohtaki H, Shioda S, Yoshida T, Numazawa S. Activation of microglia induces symptoms of Parkinson's disease in wild-type, but not in IL-1 knockout mice. J Neuroinflammation. 2013;10:143. https://doi.org/10.1186/1742-2094-10-143.
18. Chauvet N, Palin K, Verrier D, Poole S, Dantzer R, Lestage J. Rat microglial cells secrete predominantly the precursor of interleukin-1β in response to lipopolysaccharide. Eur J Neurosci. 2001;14:609–17.
19. Franceschini A, Capece M, Chiozzi P, Falzoni S, Sanz JM, Sarti AC, Bonora M, Pinton P, Di Virgilio F. The P2X7 receptor directly interacts with the NLRP3 inflammasome scaffold protein. FASEB J. 2015;29:2450–61.
20. Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, Di Virgilio F. The P2X7 receptor: a key player in IL-1 processing and release. J Immunol. 2006;176:3877–83.
21. Idzko M, Ferrari D, Eltzschig HK. Nucleotide signalling during inflammation. Nature. 2014;509:310–7.
22. Venereau E, Ceriotti C, Bianchi ME. DAMPs from cell death to new life. Front Immunol. 2015;6:422. https://doi.org/10.3389/fimmu.2015.00422.
23. Volonté C, Apolloni S, Skaper SD, Burnstock G. P2X7 receptors: channels, pores and more. CNS Neurol Disord Drug Targets. 2012;11:705–21.
24. Sandri S, Rodriguez D, Gomes E, Monteiro HP, Russo M, Campa A. Is serum amyloid A an endogenous TLR4 agonist? J Leukoc Biol. 2008;83:1174–80.
25. Niemi K, Teirilä L, Lappalainen J, Rajamäki K, Baumann MH, Öörni K, Wolff H, Kovanen PT, Matikainen S, Eklund KK. Serum amyloid A activates the NLRP3 inflammasome via P2X7 receptor and a cathepsin B-sensitive pathway. J Immunol. 2011;186:6119–28.
26. Tamamoto T, Ohno K, Goto-Koshino Y, Tsujimoto H. Feline serum amyloid A protein as an endogenous toll-like receptor 4 agonist. Vet Immunol Immunopathol. 2013;155:190–6.
27. Ebert R, Benisch P, Krug M, Zeck S, Meißner-Weigl J, Steinert A, Rauner M, Hofbauer L, Jakob F. Acute phase serum amyloid A induces proinflammatory cytokines and mineralization via toll-like receptor 4 in mesenchymal stem cells. Stem Cell Res. 2015;15:231–9.
28. Cheng N, He R, Tian J, Ye PP, Ye RD. Cutting edge: TLR2 is a functional receptor for acute-phase serum amyloid A. J Immunol. 2008;181:22–6.
29. Connolly M, Rooney PR, McGarry T, Maratha AX, McCormick J, Miggin SM, Veale DJ, Fearon U. Acute serum amyloid A is an endogenous TLR2 ligand that mediates inflammatory and angiogenic mechanisms. Ann Rheum Dis. 2016;75:1392–8.
30. Nishida E, Aino M, Kobayashi SI, Okada K, Ohno T, Kikuchi T, Hayashi JI, Yamamoto G, Hasegawa Y, Mitani A. Serum amyloid A promotes E-selectin expression via toll-like receptor 2 in human aortic endothelial cells. Mediat Inflamm. 2016;2016:7150509. https://doi.org/10.1155/2016/7150509.
31. Passey SL, Bozinovski S, Vlahos R, Anderson GP, Hansen MJ. Serum amyloid A induces toll-like receptor 2-dependent inflammatory cytokine expression and atrophy in C2C12 skeletal muscle myotubes. PLoS One. 2016;11:1. https://doi.org/10.1371/journal.pone.0146882.
32. Rodrigues RJ, Tomé AR, Cunha RA. ATP as a multi-target danger signal in the brain. Front Neurosci. 2015;9:148. https://doi.org/10.3389/fnins.2015.00148.
33. Sperlágh B, Illes P. P2X7 receptor: an emerging target in central nervous system diseases. Trends Pharmacol Sci. 2014;35:537–47.
34. Skaper SD, Facci L. Culture of neonatal rodent microglia, astrocytes, and oligodendrocytes from the cortex, spinal cord, and cerebellum. Methods Mol Biol. 2018;1727:49–61.
35. Marinelli C, Di Liddo R, Facci L, Bertalot T, Conconi MT, Zusso M, Skaper SD, Giusti P. Ligand engagement of toll-like receptors regulates their expression in cortical microglia and astrocytes. J Neuroinflammation. 2015;12:244. https://doi.org/10.1186/s12974-015-0458-6.
36. Hamby ME, Uliasz TF, Hewett SJ, Hewett JA. Characterization of an improved procedure for the removal of microglia from confluent monolayers of primary astrocytes. J Neurosci Methods. 2006;150:128–37.
37. Barbierato M, Facci L, Argentini C, Marinelli C, Skaper SD, Giusti P. Astrocyte-microglia cooperation in the expression of a pro-inflammatory phenotype. CNS Neurol Disord Drug Targets. 2013;12:608–18.
38. Facci L, Barbierato M, Marinelli C, Argentini C, Skaper SD, Giusti P. Toll-like receptors 2, -3 and -4 prime microglia but not astrocytes across central nervous system regions for ATP-dependent interleukin-1β release. Sci Rep. 2014;4:6824. https://doi.org/10.1038/srep06824.
39. Culbert AA, Skaper SD, Howlett DR, Evans NA, Facci L, Soden PE, Seymour ZM, Guillot F, Gaestel M, Richardson JC. MAPKAP kinase 2 deficiency in microglia inhibits pro-inflammatory mediator release and resultant neurotoxicity: relevance to neuroinflammation in a transgenic mouse model of Alzheimer's disease. J Biol Chem. 2006;281:23658–67.
40. Skaper SD, Facci L, Culbert AA, Evans NA, Chessell I, Davis JB, Richardson JC. $P2X_7$ receptors on microglial cells mediate injury to cortical neurons in vitro. Glia. 2006;54:234–42.
41. Sanz M, Di Virgilio F. Kinetics and mechanism of ATP-dependent IL-1β release from microglial cells. J Immunol. 2000;164:4893–8.
42. Facci L, Skaper SD. Cell enumeration assays: application of the MTT and sulforhodamine B assays to lipopolysaccharide-stimulated neonatal rodent microglia. Methods Mol Biol. 2018;1727:167–78.

43. Yu Y, Liu J, Li SQ, Peng L, Ye RD. Serum amyloid a differentially activates microglia and astrocytes via the PI3K pathway. J Alzheimers Dis. 2014;38:133–44.
44. Holm TH, Draeby D, Owens T. Microglia are required for astroglial toll-like receptor 4 response and for optimal TLR2 and TLR3 response. Glia. 2012;60:630–8.
45. De Buck M, Gouwy M, Wang JM, Van Snick J, Proost P, Struyf S, Van Damme J. The cytokine-serum amyloid A-chemokine network. Cytokine Growth Factor Rev. 2016;30:55–69.
46. Van Dam AM, Bauer J, Tilders FJ, Berkenbosch F. Endotoxin-induced appearance of immunoreactive interleukin-1 beta in ramified microglia in rat brain: a light and electron microscopic study. Neuroscience. 1995;65:815–26.
47. Casula M, Iyer AM, Spliet WG, Anink JJ, Steentjes K, Sta M, Troost D, Aronica E. Toll-like receptor signaling in amyotrophic lateral sclerosis spinal cord tissue. Neuroscience. 2011;179:233–43.
48. Yao L, Kan EM, Lu J, Hao A, Dheen ST, Kaur C, Ling EA. Toll-like receptor 4 mediates microglial activation and production of inflammatory mediators in neonatal rat brain following hypoxia: role of TLR4 in hypoxic microglia. J Neuroinflammation. 2013;10:23. https://doi.org/10.1186/1742-2094-10-23.
49. Wang Y, Ge P, Yang L, Wu C, Zha H, Luo T, Y Z. Protection of ischemic post conditioning against transient focal ischemia-induced brain damage is associated with inhibition of neuroinflammation via modulation of TLR2 and TLR4 pathways. J Neuroinflammation. 2014;11:15. https://doi.org/10.1186/1742-2094-11-15.
50. Li XQ, Lv HW, Tan WF, Fang B, Wang H, Ma H. Role of the TLR4 pathway in blood-spinal cord barrier dysfunction during the bimodal stage after ischemia/reperfusion injury in rats. J Neuroinflammation. 2014;11:62. https://doi.org/10.1186/1742-2094-11-62.
51. Kim D, Kim MA, Cho IH, Kim MS, Lee S, Jo EK, Choi SY, Park K, Kim JS, Akira S, Na HS, Oh SB, Lee SJ. A critical role of toll-like receptor 2 in nerve injury-induced spinal cord glial cell activation and pain hypersensitivity. J Biol Chem. 2007;282:14975–83.
52. Saura J. Microglial cells in astroglial cultures: a cautionary note. J Neuroinflammation. 2007;4:26. https://doi.org/10.1186/1742-2094-4-26.
53. Solà C, Casal C, Tusell JM, Serratosa J. Astrocytes enhance lipopolysaccharide-induced nitric oxide production by microglial cells. Eur J Neurosci. 2002;16:1275–83.
54. Crocker SJ, Frausto RF, Whitton JL, Milner R. A novel method to establish microglia-free astrocyte cultures: comparison of matrix metalloproteinase expression profiles in pure cultures of astrocytes and microglia. Glia. 2008;56:1187–98.
55. Thiele DL, Kurosaka M, Lipsky PE. Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: delineation by its sensitivity to the lysosomotropic agent, l-leucine methyl ester. J Immunol. 1983;131:2282–90.
56. Guillemin G, Boussin FD, Croitoru J, Franck-Duchenne M, Le Grand R, Lazarini F, Dormont D. Obtention and characterization of primary astrocyte and microglial cultures from adult monkey brains. J Neurosci Res. 1997;49:576–91.
57. Hewett SJ. Interferon-gamma reduces cyclooxygenase-2-mediated prostaglandin E2 production from primary mouse astrocytes independent of nitric oxide formation. J Neuroimmunol. 1999;94:134–43.
58. Kim S, Steelman AJ, Koito H, Li J. Astrocytes promote TNF-mediated toxicity to oligodendrocyte precursors. J Neurochem. 2011;116:53–66.
59. Honore P, Donnelly-Roberts D, Namovic MT, Hsieh G, Zhu CZ, Mikusa JP, Hernandez G, Zhong C, Gauvin DM, Chandran P, Harris R, Medrano AP, Carroll W, Marsh K, Sullivan JP, Faltynek CR, Jarvis MF. A-740003 [N-(1-{[(cyanoimino)(5-quinolinylamino) methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl)acetamide], a novel and selective P2X7 receptor antagonist, dose-dependently reduces neuropathic pain in the rat. J Pharmacol Exp Ther. 2006;319:1376–85.
60. Li M, Matsunaga N, Hazeki K, Nakamura K, Takashima K, Seya T, Hazeki O, Kitazaki T, Iizawa Y. A novel cyclohexene derivative, ethyl (6R)-6-[N-(2-chloro-4- fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits toll-like receptor 4-mediated cytokine production through suppression of intracellular signalling. Mol Pharmacol. 2006;69:1288–95.
61. Chang K, Wang X, Zhang S, Yin H. Discovery of small molecule inhibitors of the TLR1-TLR2 complex. Angew Chem Int Ed Engl. 2013;51:12246–9.
62. Kawamoto T, Ii M, Kitazaki T, Iizawa Y, Kimura H. TAK-242 selectively suppresses toll-like receptor 4-signalling mediated by the intracellular domain. Eur J Pharmacol. 2008;584:40–8.
63. Kushner I, Rzewnicki D. Acute phase response. In: JGallin JI, Snyderman R, editors. Inflammation: basic principles and clinical correlates. Philadelphia: Lippincott Willams & Wilkins; 1999. p. 317–29.
64. Matzinger P. Friendly and dangerous signals: is the tissue in control? Nat Immunol. 2007;8:11–3.
65. Seong SY, Matzinger P. Hydrophobicity: an ancient damage-associated molecular pattern that initiates innate immune responses. Nat Rev Immunol. 2004;4:469–78.
66. Ristori G, Laurenti F, Stacchini P, Gasperini C, Buttinelli C, Pozzilli C, Salvetti M. Serum amyloid A protein is elevated in relapsing-remitting multiple sclerosis. J Neuroimmunol. 1998;88:9–12.
67. Yokote H, Yagi Y, Watanabe Y, Amino T, Kamata T, Mizusawa H. Serum amyloid A level is increased in neuromyelitis optica and atypical multiple sclerosis with smaller T2 lesion volume in brain MRI. J Neuroimmunol. 2013;259:92–5.
68. Skaper SD, Debetto P, Giusti P. The P2X7 purinergic receptor: from physiology to neurological disorders. FASEB J. 2010;24:337–45.
69. Parvathenani LK, Tertyshnikova S, Greco CR, Roberts SB, Robertson B, Posmantur R. P2X7 mediates superoxide production in primary microglia and is up-regulated in a transgenic mouse model of Alzheimer's disease. J Biol Chem. 2003;278:13309–17.
70. Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron F, Feinstein DL, et al. Neuroinflammation in Alzheimer's disease. Lancet Neurol. 2015;14:388–405.
71. Ji YR, Kim HJ, Bae KB, Lee S, Kim MO, Ryoo ZY. Hepatic serum amyloid A1 aggravates T cell-mediated hepatitis by inducing chemokines via toll-like receptor 2 in mice. J Biol Chem. 2015;290:12804–11.
72. Sloane JA, Batt C, Ma Y, Harris ZM, Trapp B, Vartanian T. Hyaluronan blocks oligodendrocyte progenitor maturation and remyelination through TLR2. Proc Natl Acad Sci U S A. 2010;107:11555–60.
73. Uhlar CM, Whitehead AS. Serum amyloid A, the major vertebrate acute-phase reactant. Eur J Biochem. 1999;265:501–23.
74. McCoy MK, Tansey MG. TNF signalling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J Neuroinflammation. 2008;5:45. https://doi.org/10.1186/1742-2094-5-45.
75. Selmaj K, Raine CS, Cannella B, Brosnan CF. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. J Clin Invest. 1991;87:949–54.
76. Dal Canto RA, Shaw MK, Nolan GP, Steinman L, Fathman CG. Local delivery of TNF by retrovirus-transduced T lymphocytes exacerbates experimental autoimmune encephalomyelitis. Clin Immunol. 1999;90:10–4.
77. Probert L, Akassoglou K, Pasparakis M, Kontogeorgos G, Kollias G. Spontaneous inflammatory demyelinating disease in transgenic mice showing central nervous system-specific expression of tumor necrosis factor alpha. Proc Natl Acad Sci U S A. 1995;92:11294–8.
78. Sharp AJ, Polak PE, Simonini V, Lin SX, Richardson JC, Bongarzone ER, Feinstein DL. P2X7 deficiency suppresses development of experimental autoimmune encephalomyelitis. J Neuroinflammation. 2008;5:33. https://doi.org/10.1186/1742-2094-5-33.
79. Peferoen L, Kipp M, van der Valk P, van Noort JM. Amor S. Oligodendrocyte-microglia cross-talk in the central nervous system. Immunology. 2014;141:302–13.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

BMC