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Scavenger receptor class A ligands induce secretion of IL1β and exert a modulatory effect on the inflammatory activation of astrocytes in culture

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Abstract

Class-A Scavenger receptor (SR-A) is expressed by microglia, and we show here that it is also expressed by astrocytes, where it participates on their inflammatory activation. Astrocytes play a key role on the inflammatory response of the central nervous system, secreting several soluble mediators like cytokines and radical species. Exposure to SRs ligands activated MAPKs and NF-κB signaling and increase production of IL1β and nitric oxide (NO). IL1β classically an inflammatory cytokine surprisingly did not increase but inhibited LPS+IFNγ-induced NO production by astrocytes. Our results suggest that SRs expressed by astrocytes participate in the modulation of inflammatory activation.

Keywords

ERK; glia; IL1β; JNK; MAPK; Neuroinflammation; NFκB; NO; SR-A

1. Introduction

Neuroinflammation is a complex pathophysiological response involving soluble factors and glial cell activation, in response to infection and tissue damage induced by ischemic, traumatic, immune or other injuries, such as toxic, and heat/cold, radiation. The inflammatory process normally leads to recovery and healing. However, inflammation can lose its repair function and enhance tissue damage if the process is not properly regulated (Nathan, 2002). Inflammation is recognized as a major contributor to many acute and chronic central nervous system (CNS) disorders, playing an important role in their pathogenesis. For chronic neurodegenerative disorders like Alzheimer's disease and Parkinson's disease, neuroinflammation depends on the activation of the brain innate immune response (Rivest, 2009; Glass et al., 2010; von Bernhardi et al., 2010) involving dysregulation of glial cells (von Bernhardi, 2007).

Astrocytes and microglia participate in the immune inflammatory response, representing the first line of defense of the CNS. They produce inflammatory cytokines like IL1β and release

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short-lived cytotoxic mediators such as nitric oxide (NO) and reactive oxygen species (ROS) (Griffiths et al., 2009; von Bernhardi and Eugenín, 2012). In fact, astrocytes stimulated with brain-derived neurotrophic factor (BDNF) release NO, and conditioned medium from activated astrocytes can be detrimental for neurons. A process that is amplified by NO (Colombo et al., 2012) and could be relevant for the neuroinflammatory response in neurodegenerative diseases like multiple sclerosis (Liu et al., 2001; Colombo et al., 2012). On the other hand, astrocytes provide trophic support to the CNS and establish an intimate interaction with neurons, microglia and endothelial cells. They also play a regulatory and protective role by modulating microglial cell activity by secreting modulating cytokines like transforming growth factor-β (TGFβ) (von Bernhardi and Eugenín, 2004; Ramírez et al., 2005; Herrera-Molina and von Bernhardi, 2005).

Astrocytes and microglia express several receptors that participate in the inflammatory response. One of these receptor groups are the Scavenger Receptors (SRs). Scavenger Receptor A (SR-A) was described for the first time in 1979 by Goldstein et al., who described a binding site mediating the uptake and degradation of acetylated LDL (acLDL) by macrophages (Goldstein et al., 1979; de Winther et al., 2000), which was initially referred to as the acLDL receptor. Currently, it is known that SR-A belongs to a large family of SR denominated Class A SR, which also includes the Scavenger Macrophage Receptor with Collagenous Structure (SR-MARCO) (Krieger, 1997; de Winther et al., 2000).

SRs recognize apoptotic cells, secreted pattern recognition molecules and several different microbial structures including lipopolysaccharide (LPS) (Mukhopadhyay and Gordon, 2004; Areschoug and Gordon, 2009). In addition to binding and uptake of ligands, SRs also could play an important role in innate immune defense acting as regulators of cytokines production (Ozeki et al., 2006). Macrophages stimulated with SR-A ligands increase production of inflammatory cytokines, like tumor necrosis factor-α (TNFα), in a time- and dose-dependent manner. In the macrophage cell line J774A.1, SR-A ligands also induce TNFα and Interleukin-1β (IL1β) production, effects that appear to be mediated by the activation of mitogen activated protein kinases (MAPKs) JNK, p38 and ERK1/2 signaling pathways (Hsu et al., 2001). Similarly, activation of SR-A ligands mediated signaling induce NO production on RAW 264.7 cells (a mouse leukaemic monocyte macrophage cell line) as well as activation of JNK, p38 and ERK1/2 MAPK, and NF-κB (Campa et al., 2005). Furthermore, IL1β release induced by the activation of p38 and ERK1/2 pathways in murine peritoneal macrophages depended on the presence of SR-A ligands (Kwon et al., 2007).

Up to now, the expression of SR-A was thought to be mainly confined to cells belonging to the monocyte macrophage family (Naito et al., 1991; Hughes et al., 1995). In the CNS, SR-A has been observed in resident macrophage cells, the microglia (Alarcón et al., 2005, Farina et al., 2007). In contrast, only a few SRs have been described in astrocytes, which include SR-B1, SR-CL and SR-MARCO, recently described by our group (Alarcón et al., 2005; Farina et al., 2007).

Expression of SR-A and its capability for inducing signaling pathways and release of inflammatory molecules has not been described in astrocytes. Because astrocytes are a major player in the regulation of cytotoxic neuroinflammatory activation, we are interested in evaluating the presence of SR-A in astrocytes, and the effect of SRs ligands like fucoidan and Poly I on the activation of ERK1/2, JNK1/2 and IκB/NFκB signaling pathways, all of which are associated with inflammatory cellular responses including IL1β synthesis and NO secretion. Furthermore, we evaluated if IL1β plays a role on the regulation of NO secretion by inflammatory activation of astrocytes.

2. Materials and methods

Materials

Lipopolysaccharide (LPS; from *Escherichia coli* 0111:B4), fucoidan, polyinosinic acid (Poly I) and polycytidylic acid (Poly C) were purchased from Sigma (USA); IFNγ and IL1β were purchased from R&D (USA). Cell culture media, antibiotics and serum were purchase from Gibco (Life Technologies, USA). Animals were obtained from the institutional animal facility. All procedures were performed following the animal handling and bioethical requirements defined by the Pontificia Universidad Católica de Chile School of Medicine Ethics Committee. All animals were anaesthetized before sacrifice.

Glial Cultures

Mixed glial cell cultures, containing astrocytes and microglia, were obtained from the cerebral cortex of 1–2 day old Sprague-Dawley rats as described by Giulian and Baker (1986). Cortices were rinsed with Ca^{2+}/Mg^{2+}- free Hank's balanced salt solution (HBSS); meninges were removed, and tissue was minced and incubated with 0.25% trypsin-EDTA in HBSS at 37°C for 10 min. The tissue was mechanically dissociated and cells were seeded in 75 cm^2 cell culture flasks (one brain per flask) in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cultures were incubated in water saturated, 5% CO_2 atmosphere at 37°C.

After 14 days in culture, microglial cells were obtained by shaking the mixed glial culture at 110 rpm in an orbital shaker (Unimax 1010, Heidolph, Germany) at 37°C for 15–20 min. Astrocytes were purified after microglial cell purification by trypsinization of attached cells. This procedure yields a highly enriched astrocyte (95% or more astrocytes) and microglial cell (over 99% microglia) cultures. Cell identity was evaluated on cultures by labeling with fluorescein isothiocyanate (FITC)-conjugated lectin from *Griffonnia simplicifolia* (1:200; Sigma), which recognizes microglia, and glial fibrillary acidic protein immunocitochemistry (antibodies α-GFAP; 1:200; Dako, Denmark) to identify astrocytes (data not shown). Glial cells were seeded in 96-well plates at a density of $3X10^4$ cells per well for nitrites (NO^{2-}) determination and plated at a density of $5X10^5$ cells per 35 mm diameters Petri dishes for western blot assays.

Cell line culture

The DI-TNC1 (ATCC® Number: CRL-2005™) rat astrocytes cell line, was cultured in DMEM/F12 supplemented with 10% FBS, and antibiotics. The mouse microglia N9 cell line was cultured in DMEM/F12 supplemented with 5% FBS and antibiotics. Cultures were maintained in a water saturated, 5% CO_2 atmosphere at 37°C.

Determination of Nitrites (NO^{2-})

Nitrites (NO^{2-}), is a stable downstream product of the NO released by cells, was determined by the Griess assay (Pfeiffer et al., 1997). For NO^{2-} determination, cells were maintained under control culture conditions or were incubated with either inflammatory molecules (1 μg/mL LPS + 10 ng/mL INFγ) or with various concentrations of SRs ligands (1 to 100 μg/ml fucoidan; 10 to 200 μg/ml Poly I; 10 to 200 μg/ml Dextran Sulphate; 200 μg/ml Poly C). For the determination of NO^{2-}, 50 μl of medium was mixed with 10 μl EDTA:H_2O 1:1 (0.5 M, pH 8.0) and 60 μl of freshly prepared Griess reagent (20 mg *N*-[1-naphtyl]-ethylendiamine and 0.2 g sulphanilamide dissolved in 20 ml of 5% phosphoric acid, w/v). Standard curves were established with 1–80 μM $NaNO_2$. Absorbency was measured at 570 nm in a microplate auto reader (ANTHOS 2010, Anthos Labtec Instrument).

Western Blot Analysis

Astrocytes ($5x10^5$ cells per 35 mm diameter petri dish), after incubation with the various experimental conditions, were lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and protease inhibitors). Protein concentration was determined by the BCA assay. Cell samples (70 μg protein) were electrophoretically separated on 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked with 0.1% Tween 20, 5% milk in phosphate buffer saline (PBS) for 1 h, and then incubated with the primary antibody in blocking buffer: goat α-IL1β (1:1,000; R&D), rabbit αpERK and α-ERK (1:500; Santa Cruz Biotechnology), rabbit α-pJNK and α-JNK (1:500; Cell signal), rabbit α-IκB 1:500; Santa Cruz Biotechnology) or mouse α-βTub I+II (1:1,000; Chemicon). Primary antibodies were rinsed and membranes were incubated with horseradish peroxidase-labeled donkey α-goat, α-rabbit or α-mouse depending on the primary antibody previously used, as secondary antibody. Signals were detected by enhanced chemiluminescence (Amersham Biosciences) in accordance with the manufacturer's instructions. Densitometry was done with the ImageJ program.

Statistical Analysis

In vitro data correspond to at least 3–5 independent experiments in triplicate and were expressed as mean ± SEM. Statistical analysis was performed with the Kruskal Wallis one-way ANOVA and the Wilcoxon Rank Sum/Mann-Whitney U-test. A Dunn's comparison was used for multiple comparisons. Evaluation was performed using the GBstat statistical software (Dynamic Microsystems, Inc). For all statistical analysis, a value of $p < 0.05$ was considered significant.

3. Results

Astrocytes express SR-MARCO and SR-A

Expression of SR-A at the protein level by astrocytes in culture was evaluated by western blot assay using reductive conditions (Fig. 1A) and by immunofluorescence (Fig. 1B). The blot showed a band of 60 kDa corresponding to the SR-A monomer, which was observed both in astrocytes and microglial cells. Furthermore, expression of SR-A by the astrocyte cell line DI-TNC1 (Fig. 1) demonstrated that SR-A was effectively present in astrocytes and it was not the result of the microglial cell contamination that is unavoidable in astrocyte cultures. SR-MARCO monomeric isoform (50 kDa) was expressed both by microglia and astrocytes, as previously described (Alarcón et al., 2005). Figure 1B shows that SR-A was expressed by both astrocytes (arrows) and microglia (arrow heads).

SR ligands induced NO production by astrocytes and microglia

Once the presence of SR-A and SR-MARCO on astrocytes and microglia was demonstrated, we evaluated the stimulation of glial cell cultures with different SRs ligands, assessing the production of NO, a marker of inflammatory cell activation (Table 1). We used classically described SRs ligands, fucoidan and Polyinosinic acid (Poly I) (Goldstein et al., 1979; Krieger, 1997; Shechter et al., 1981; Zhang et al., 1993; Nishikawa et al., 1990; de Winther et al., 2000). Polycytidylic acid C (Poly C) a polyanion that does not bind to SR class A, and Dextran sulfate (DS) a ligand described as not being able to bind to SRs but unable to induce a transduction signaling response (Nakamura et al., 2006), were used as negative controls. As positive control we used inflammatory activation with LPS plus INFγ (LI).

Increasing concentrations of fucoidan (1 to 200 μg/ml) (Fig. 2A) and increasing duration (24 to 96 h) of stimulation (Fig. 3A) were unable to induce NO production by astrocytes (Fig. 2A and Fig. 3A, respectively). On the other hand, astrocytes that were exposed to Poly I showed induction of NO production in a concentration- (10 to 400 μg/ml) and time-

dependent manner (Dunn's Multiple comparisons, $p<0.001$). Treatment with 200 μg/ml of Poly I (Fig. 2B) for 48 h and 96 h (Fig. 3B) were the concentration and time at which production of NO reached a plateau (180 μM NO^{2-}). Higher concentration of Poly I and longer exposition maintained this same level of NO^{2-}. In contrast, astrocytes exposed to increasing concentrations of DS (10, 50 and 200 ng/ml) (Fig. 2C), did not produce detectable levels of NO. However, we observed that DS was cytotoxic for astrocytes at all of the concentrations tested (data not shown). Astrocytes exposed to LI (Fig. 3C) for increasing times (24 to 96 h) produced high amounts of NO in a time dependent manner (Fig. 3C), which was similar to that obtained by Poly I. Astrocytes exposed to Poly C at increasing times (24 to 96 h) failed showing induction of NO production (Fig. 2C).

Microglia exposed to fucoidan increased NO production by 2.2-fold at 48 h and 2.0-fold at 96 h compared with the control condition (Fig. 3D). Similarly Poly I induced a 3.0-fold increase of NO production at 48 h and a 6.0-fold increase at 96 h compared with control cells. LI induced a 9.6-fold increase of NO production at 48 h and a 10-fold increase at 96 h compared with its control.

Our results indicate that SRs ligands induced a differential effect on astrocytes and microglia. Only Poly I and LI were capable to inducing NO production by astrocytes (Table 1), whereas microglia also showed activation when exposed to fucoidan.

SRs ligands activated MAPKs and NFκB signaling pathways of astrocytes

Knowing that binding of specific SR-A and SR-MARCO ligands can induce production of NO by astrocytes, as a next step we evaluated the activation of downstream signaling pathways, JNK, ERK and IκB/NF-κB, which could be involved on the induction of inducible NO synthase (iNOS) by fucoidan, Poly I or LI (Fig. 4; Table 1). Astrocytes exposed to Poly I induced a 3.0-fold increase of the phosphorylation of ERK; induction that reached a 5.0-fold increase after stimulation with LI (Fig. 4A). In contrast, fucoidan was unable to induce activation of ERK signaling at 24 h (Fig. 4A). Similarly, treatment with Poly I and LI, but not fucoidan, induced a 2.5-fold and 4.3-fold increment of JNK phosphorylation respectively (Fig. 4B). For the assessment of the activation of IκB/NFκB signaling pathway, we evaluated the levels of IκB. Astrocytes exposed to fucoidan and Poly I ligands maintained unaffected IκB levels (Fig. 4C). In contrast, LI induced a 2.0-fold decrease of IκB compared with the control (Fig. 4C) indicating the activation of this pathway at 24 h of stimulation.

SRs ligands induced IL1β production by astrocytes

IL1β is a cytokine secreted early during the inflammatory response of glial cells. We evaluated by western blot the *de novo* production and by ELISA the secretion of this cytokine by astrocytes stimulated with SRs ligands (Fig. 5, Table 1). Astrocytes treated with fucoidan, Poly I and LI for 24 h, showed an increment on the presence of IL1β by 8.2-fold, 11.4-fold, and 17.7-fold, respectively, compared with astrocytes under control condition (Fig. 5, Table 1). These results indicate that regardless of the different patterns of inflammatory activation induced by SRs ligands, both fucoidan and Poly I induced the production of IL1β by astrocytes.

IL1β inhibited NO production in astrocytes

Astrocytes stimulated with increasing concentrations of IL1β (0.1 to 200 ng/ml) did not increase their production of NO (Fig. 6A). Furthermore, IL1β inhibited LI-induced production of NO by astrocytes. We stimulated astrocytes with IL1β at increasing concentrations (0.1-to-200 ng/ml), with and without co-stimulation with LI for 96 h (Fig. 6B). Astrocytes simultaneously stimulated with IL1β and LI produced levels of NO that

were approximately 2.2-fold lower than astrocytes exposed to LI, which showed a 20-fold induction of NO production compared with the control conditions. However, IL1β at high concentration (200 ng/ml) was less effective reducing NO induction.

These data suggest that IL1β, depending on the stimulation condition, can both potentiate and decrease inflammatory response on astrocytes.

4. Discussion

One of the main findings of this work is that it shows for the first time the presence of SR-A in rat astrocytes, indicating that in addition to the SRs previously described, including SR-MARCO (Alarcón et al., 2005; Farina et al., 2007), astrocytes also express SR-A, capable of binding ligands like fucoidan, Poly I, and LPS. Stimulation with these SRs-ligand showed a differential effect on the activation of ERK, JNK and IκB/NF-κB signaling pathways, as well as on the induction of NO and IL1β production, being both markers of inflammatory activation for glial cells.

SRs ligand binding is characterized by high affinity and broad specificity (Krieger and Herz, 1994). Class A SRs contain a cluster of positive charged residues in their collagenous domain that are highly conserved and appears to provide a sticky surface that function as “molecular flypaper” for several different ligands (Krieger and Herz, 1994). The signaling determinants that allow different SR ligands to induce different cell responses have not been identified. SR-A and SR-MARCO appear to be unable to initiate inflammatory signaling pathways by themselves (Fong and Le., 1999; Kosswig et al., 2003). Therefore, the existence of multiple binding sites on a single SR, or the interaction of SR, with other SRs or with other types of receptors, could differentially activate signaling pathways and allow for the diversity of cellular responses.

There are also differences on ligand binding depending on cell type. Microglia, in contrast to astrocytes, responded to all ligands including Poly C showing an increased NO production. Microglia could have more SRs at their surface or express other co-receptors like TLRs (Farina et al., 2007), a group of pattern recognition receptors that as mentioned, have ligands shared with class A SRs, for example LPS, which also binds to TLR-4, or Poly I and Poly C, which also bind to TLR-3 (Chang, 2010). Our data supports the existence of differences on SR-A ligand differences between microglia and astrocytes. Microglial cell activation appears to be more robust and broader in terms of ligands.

The presence of SR-A on astrocytes is especially relevant because SR-A is involved in inflammatory diseases (Sun et al., 2007; Hickman et al., 2008; Manning-Tobin et al., 2009) and astrocytes are key regulator of the inflammatory response in the CNS. Astrocytes modulate the inflammatory response of microglial cells, inhibiting NO production in presence of ligands such as IL1β and TNFα (Tichauer et al., 2007). Furthermore, binding of SR-A ligands induce the production of cytokines such as IL1β, a master regulator of neuroinflammation, which is produced by astrocytes in the CNS (Campa et al., 2005; Basu et al., 2004; Hsu et el., 2001; Palkama, 1991). Therefore, the presence of this scavenger receptor on astrocytes is an exciting premise.

In this work, we used the classical ligands fucoidan and Poly I (Takakura et al., 1999), and LPS, a ligand binding both Toll like receptor-4 (TLR-4) and SRs (Amiel et al., 2009). The various ligands generated different responses on astrocytes. Poly I and LI but not fucoidan induced production of NO in a concentration- and time-dependent manner. Coherently, Poly I and LI, but not fucoidan induced MAPKs and NFκB signaling pathways, classical activators of iNOS (Saha and Pahan, 2006). In contrast, all three ligands induced IL1β production by astrocytes.

SR-A can internalize Aβ peptide in animal models of AD (Husemann et al., 2002; Hickman et al., 2008). Association of Aβ with SR-A, have also been associated with inflammatory activation in the CNS (Hsu et al., 2001; Kim et al., 2003) and potentiation of the activation induced other SR ligands *in vitro* (Murgas et al., 2012). Increased inflammatory activation can facilitate cytotoxicity and neurodegeneration (von Bernhardi et al., 2007; Ramírez et al., 2008). There are reports on the detrimental effects of absence of both SR-MARCO and SR-A on antibacterial defenses in mice. A defect in the uptake or killing of bacteria as well as dysregulation of inflammatory responses may contribute to defective antibacterial defense in both SR-MARCO- and SR-A-deficient mice, being one of the mechanisms responsible for decreased survival of receptor KO mice during bacterial infections (Józefowski et al., 2005; Chen et al., 2010). However, although there is an important wealth of information that SR-A and SR-MARCO trigger intracellular signaling, modulating inflammatory, phagocytic and microbicidal activities of macrophages (Józefowski et al., 2005; Chen et al., 2010), it is less known their participation in brain parenchyma processes.

IL1β is a classical inflammatory cytokine that promotes glial activation, inducing production of other inflammatory cytokines (Viviani et al., 2004; Mrak and Griffin, 2005). However, we have previously found that acting with different timing, both TGF-β1 and IL-1β modulate the amplitude and duration of glial activation in response to inflammatory activation, suggesting they can be key for the understanding of inflammatory mechanisms involved in the pathogenesis of neurodegenerative diseases (Ramírez et al., 2005). Here, we detected that IL1β did not induce NO production by astrocytes. Furthermore, co-stimulation with LI and IL1β resulted in the inhibition of LI-induced NO production, suggesting that IL1β has a modulatory function on astrocytes exposed to inflammatory conditions. Production of IL1β is induced by the stimulation of glial cells by LPS (Rankine et al., 2006; Harvath et al., 2008), as well as by the binding of SR ligands (Palkama, 1991). IL1β is an early cytokine on the neuroinflammatory process (Basu et al., 2004). It is upregulated in several neurological diseases and neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis (Blum-Degen et al., 1995; Basu et al., 2004). Increase of IL1β plus TNFα has been related to the induction of iNOS in astrocytes (Marcus et al., 2003). Furthermore, IL1β induces the activation of the transcriptional factor NFκB in astrocytes, promoting transcription of genes encoding for adhesion molecules, chemokines and cytokines (John et al., 1986; John et al., 2004; Xia and Zhai, 2010). However, as mentioned, we have also found that IL1β can also decrease NO production by glial cells exposed to inflammatory molecules, inhibiting ERK phosphorylation (Saud et al., 2005; Tichauer et al., 2007). There are also reports that IL1β can generate tolerance to LPS *in vivo*, and down regulate the presence of TLR-4, a receptor that is crucial in the signaling of LPS (Alves-Rosa et al., 2002). IL1β appears to exert beneficial effects, particularly when released at low concentrations. Astrocytes activated by IL1β have an enhanced capacity to sustain neuronal survival, reestablishing CNS homeostasis and blood brain barrier function (Liberto et al., 2004). Astroglial activation is delayed in mice lacking IL1β as well as in mice lacking the IL1β type 1 receptor (Herx et al., 2000). Altogether, these findings suggest that IL1β promotes the adaptive responses of astrocytes to injury, and probably could exert a protector effect regulatory the inflammatory response.

Conclusion

We demonstrated the presence of SR-A in astrocytes. We showed that SRs ligands can induce the activation of MAPKs and IκB/NFκB signaling pathways inducing NO and IL1β production, indicating that astrocytes expressed functional SRs. On the other hand, although IL1β can be a potent inflammatory cytokine, it inhibited NO production by astrocytes stimulated with LI at the present stimulation conditions; indicating that IL1β appears to exercise a novel effect on astrocytes response as a modulator of neuroinflammation.

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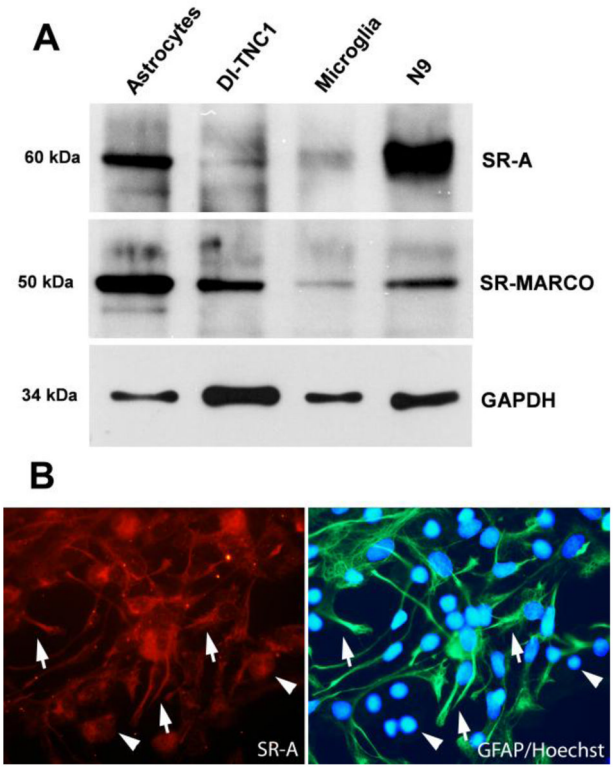

Figure 1. Presence of SR-A in primary astroglial and microglia cells

A) Western blot of monomeric SR-A (60 kDa) and SR-MARCO (50 kDa). Both proteins were detected in rat astrocytes, the astrocytes cell line DI-TNC1 of rat origin, microglia and the mice microglial cell line N9. Immune detection of GAPDH was used as loading control. B) Immunofluorescence labeling of SR-A (Rho) and GFAP as astrocyte identity marker (Alexa 488), and Hoechst stain for nuclea (blue). Both astrocytes (arrows) and microglia (arrow heads: GFAP(-) cells) are labeled by anti-SR-A antibodies.

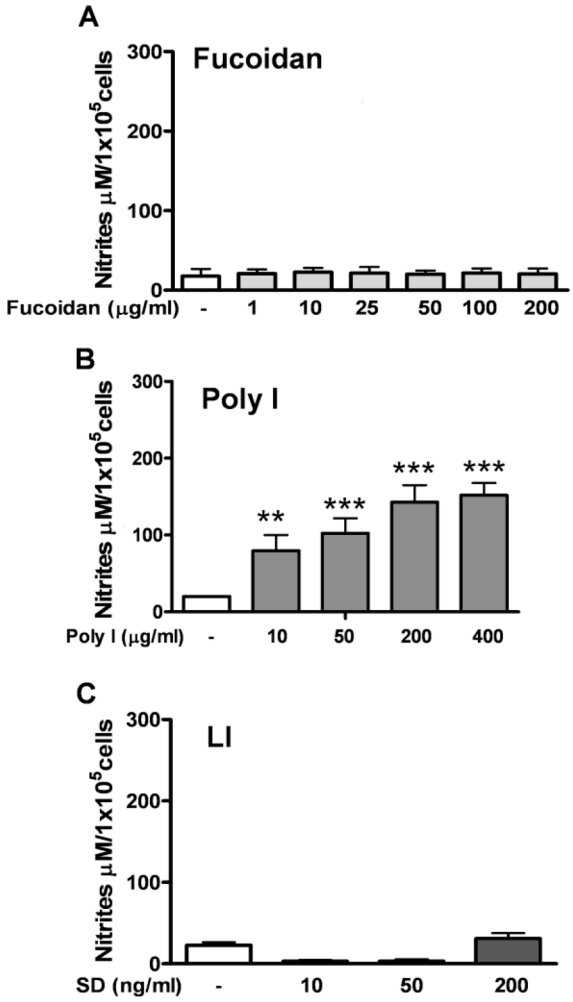

Figure 2. Astrocytes exposed to SR ligands increased production of NO

Production of NO by astrocytes exposed for 48 h to increasing concentrations of A) fucoidan, B) Poly I and C) Dextran Sulphate (DS). The results correspond to the mean ± SEM of 2–4 independent experiments performed in quadruplicate. * $p<0.05$; ** $p<0.01$; *** $p<0.001$, of SR-A ligand stimulated astrocytes vs. the control condition.

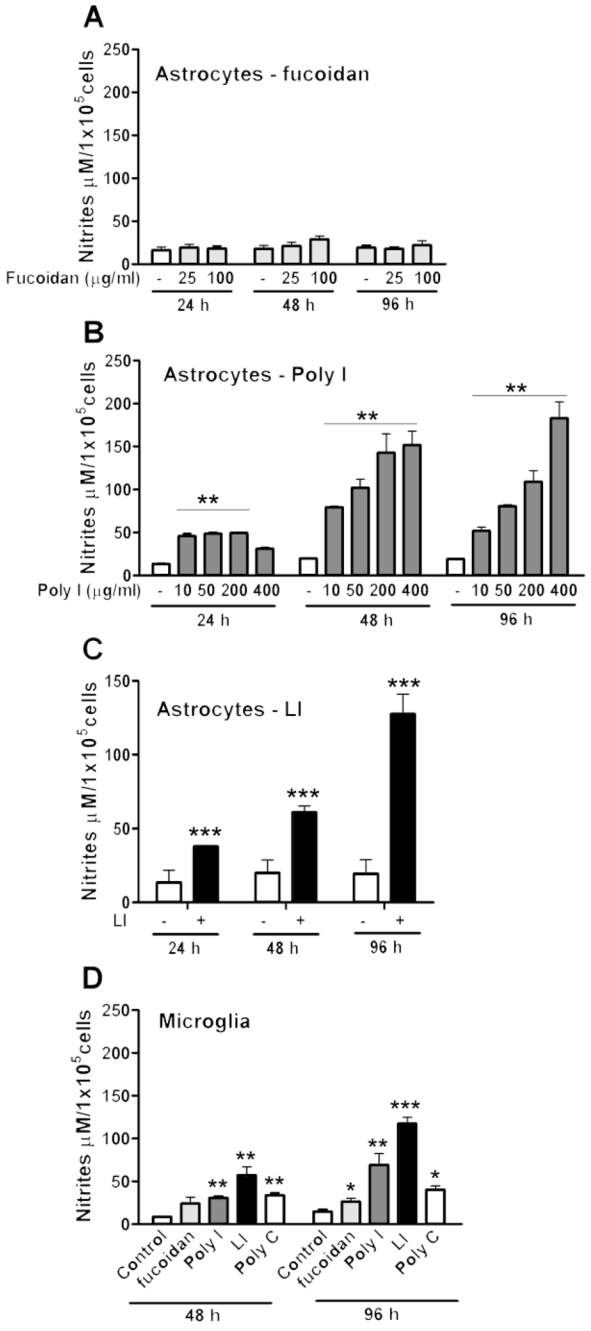

Figure 3. NO production by astrocytes exposed to Poly I and LI increased with time

Production of NO by astrocytes (A-C) exposed for increasing concentrations and times to A) fucoidan, B) Poly I and C) LI; and microglial cells exposed to various stimulus D), 200 μg/ml fucoidan, 200 μg/ml Poly I, 1μg/ml LPS plus 10 ng/ml INFγ (LI) and 200 μg/ml Poly C for 48 and 96 h of stimulation. Results correspond to the mean ± SEM of 3–5 independent experiments, performed in quadruplicate. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ of SR-A ligand stimulated cell vs. the control condition.

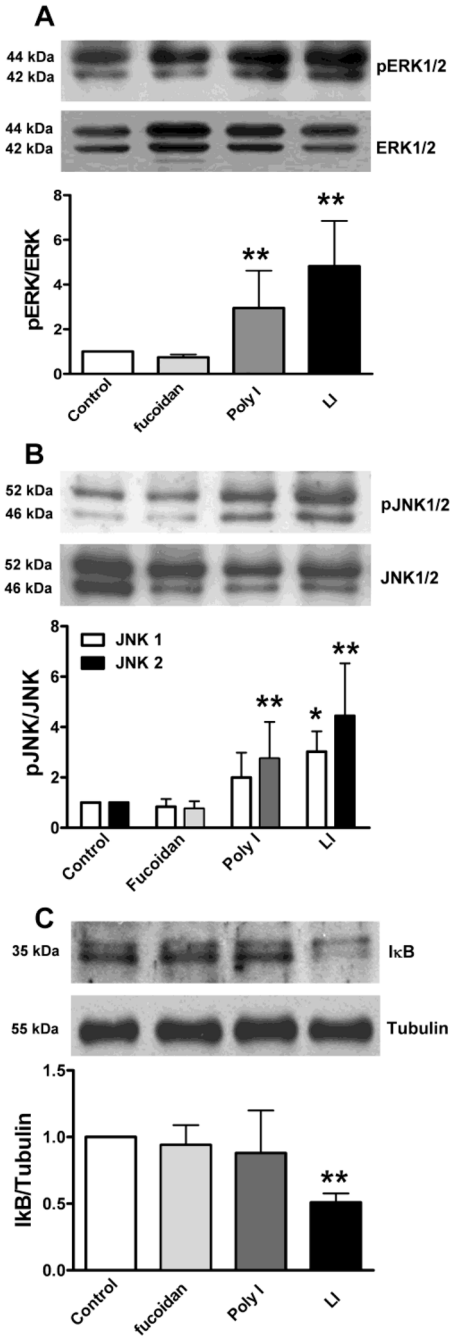

Figure 4. SR ligands differentially activated ERK, JNK and IκB/NFκB signaling pathways in astrocytes

Astrocytes were stimulated with 200 μg/ml fucoidan, 200 μg/ml Poly I and 1μg/ml LPS and 10 ng/ml INFγ (LI) for 24 h. The activation of A) ERK, and B) JNK, expressed as the increased ratio of phosphorylated to total ERK or JNK (pERK/ERK and pJNK/JNK respectively) and C) decrease of IκB as a measure of the activation of NFκB pathway was evaluated. Results correspond to the mean ± SEM of 3–4 independent experiments performed in quadruplicate. *p<0.05, for SR-A ligand stimulated cells vs. the control condition.

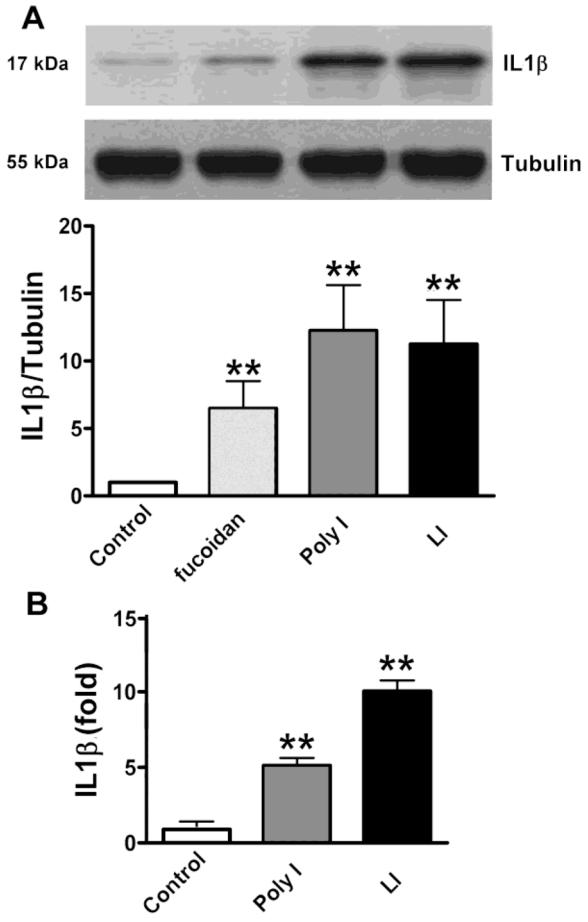

Figure 5. Astrocytes exposed to SRs ligands produced increased levels of IL1β

Astrocytes were stimulated with 200 μg/ml fucoidan, 200 μg/ml Poly I and LI (1μg/ml LPS and 10 ng/ml INFγ) for 24 h. IL1β was evaluated by A) Western blot and quantification by densitometry, which was normalized by tubulin and B) ELISA. Data represent the mean ± SEM of 3–4 independent experiments performed in quadruplicate.*$p<0.05$, **$p<0.01$, for stimulated cells vs. the control condition.

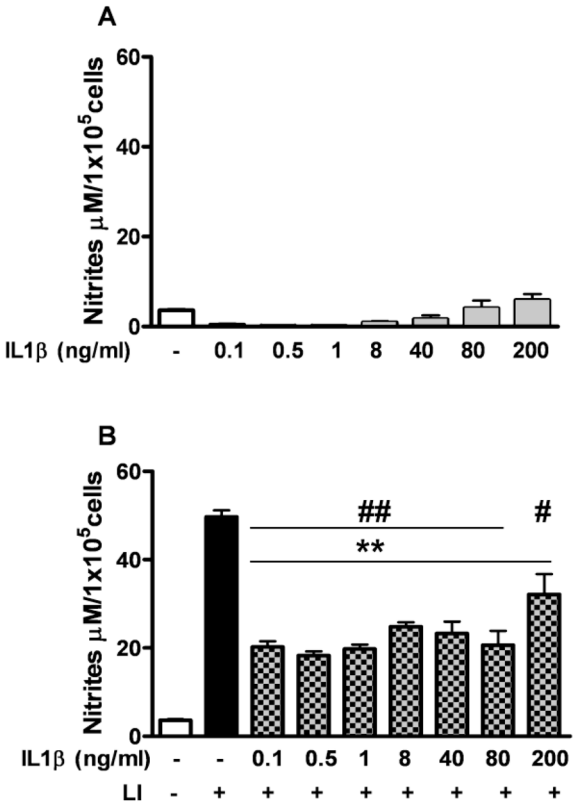

Figure 6. IL1β inhibits LI-induced NO production by astrocytes

NO production by astrocytes exposed for 24 h to A) increasing concentrations of IL1β and B) LI with or without co-stimulation with increasing concentration of IL1β. The results correspond to the mean ± SEM of 4 independent experiments performed in quadruplicate. ** $p<0.001$, for LI or LI+IL1β stimulated cells vs. the control condition. # $p<0.05$, ## $p<0.001$ for LI+IL1β stimulated cells vs. LI stimulated cells.

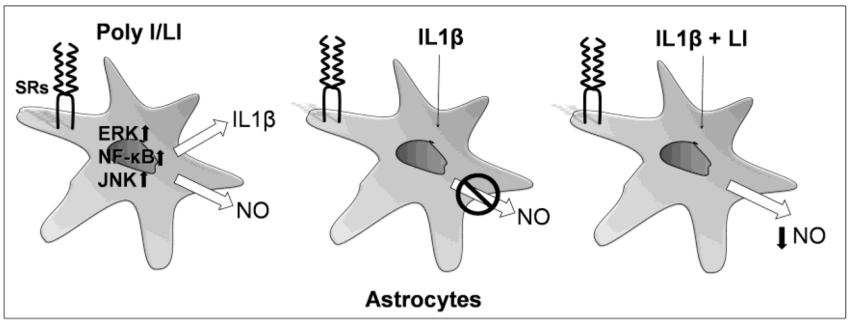

Figure 7. Scavenger receptors class A ligands induce secretion of IL1β and exert a modulatory effect on the inflammatory activation of astrocytes in culture

Astrocytes exposed to Poly I and LI release NO and produced increased levels of IL1β in a concentration and time dependent manner. Astrocytes exposed to increased concentrations of IL1β did not produce detectable levels of NO. Furthermore, astrocytes exposed to the co-stimulation with LI and IL1β LI produced at least 50% less NO compared with astrocytes stimulated solely with LI, indicating that IL1β inhibited NO production induced by inflammatory stimuli.

Table 1

Astrocytes - Inflammatory activation

Exp. stimuli	Fucoidan	Poly I	LPS+IFNγ
IL1β	↑	↑↑	↑↑
NO	-	↑↑	↑↑
ERK $_{activ}$	-	↑	↑↑
JNK $_{activ}$	-	↑	↑
NFκB $_{activ}$	-	-	↑

Effect of SR-A ligands on the Inflammatory activation of astrocytes *in culture.*

ERK$_{activ}$, JNK$_{activ}$ and NFκB $_{active}$ indicates the activation of the signaling pathways.

(↑) increases, and (-) no change.