Microglial Heparan Sulfate Proteoglycans Facilitate the Cluster-of-Differentiation 14 (CD14)/Toll-like Receptor 4 (TLR4)-Dependent Inflammatory Response*

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*Running Title: Microglial HSPGs facilitate inflammation

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Background: Microglia, CNS-resident macrophages, release TNFα and IL1β following TLR4 activation by the bacterial endotoxin LPS.

Results: LPS-induction of TNFα, IL1β and pro-CD14 is suppressed in heparanase-overexpressing primary microglia.

Conclusion: Microglial HSPGs facilitate a CD14-dependent pathway of TLR4 activation.

Significance: Heparanase remodeling of microglial HSPGs suppresses CD14-dependent TLR4 activation.

ABSTRACT

Microglia rapidly mount an inflammatory response to pathogens in the central nervous system (CNS). Heparan sulfate proteoglycans (HSPGs) have been attributed various roles in inflammation. To elucidate the relevance of microglial HSPGs in a pro-inflammatory response we isolated microglia from mice over-expressing heparanase (Hpa-tg), the HS-degrading endoglucuronidase, and challenged them with lipopolysaccharide (LPS), a bacterial endotoxin. Prior to LPS-stimulation, the LPS-receptor cluster-of-differentiation 14 (CD14) and toll-like receptor 4 (TLR4; essential for the LPS response) were similarly expressed in Ctrl and Hpa-tg microglia. However, compared to Ctrl microglia, Hpa-tg cells released significantly less tumor necrosis factor-α (TNFα), essentially failed to upregulate interleukin-1β (IL1β) and did not initiate synthesis of proCD14. Isolated primary astrocytes expressed TLR4, but notably lacked CD14 and in contrast to microglia, LPS challenge induced a similar TNFα response in Ctrl and Hpa-tg astrocytes, while neither released IL1β. The astrocyte TNFα-induction was thus attributed to CD14-independent TLR4 activation and was unaffected by the cells HS status. Equally, the suppressed LPS-response in Hpa-tg microglia indicated a loss of CD14-dependent TLR4 activation, suggesting that microglial HSPGs facilitate this process. Indeed, confocal microscopy confirmed interactions between microglial HS and CD14 in LPS-stimulated microglia and a potential HS-binding motif in CD14 was identified. We conclude that microglial HSPGs facilitate...
CD14-dependent TLR4 activation and that heparanase can modulate this mechanism.

Microglia, the resident macrophages in the central nervous system (CNS), are continually monitoring their surroundings for pathogens and tissue degeneration (1). Activated microglia assume a pro-inflammatory phenotype characterized by the release of cytokines, chemokines and reactive oxygen species (2,3). Many neurodegenerative disorders (e.g. Alzheimer’s disease) are accompanied by neuroinflammation and it has been proposed that impaired regulation of pro-inflammatory microglia aggravates the severity of these conditions (4,5). Astrocytes have multiple support functions in the CNS, but can also be triggered to produce pro-inflammatory cytokines (6,7). However, the astrocyte inflammatory response often occurs after an initial microglial response, suggesting differences in the reaction potential of these cell types (8).

Heparan sulfate proteoglycans (HSPGs) are present in the extracellular matrix and as membrane spanning syndecans or glycosphatidylinositol (GPI)-anchored glypicans on cell surfaces. They consist of a core protein with covalently attached heparan sulfate (HS) chains. HS is a linear polymer of alternating glucosamine and uronic (glucuronic or iduronic) acid residues that are sulfated at various positions (9). HSPGs often function as co-receptors to various cell-type specific receptors, for growth factors, morphogens and chemokines (9). HS chains are fragmented by heparanase, a mammalian endoglucuronidase. The active enzyme is a heterodimer consisting of a 50- and an 8 kDa subunit (10), that are cleaved from 65 kDa proheparanase by cathepsin-L (11).

HS has been implicated in various mechanisms central to inflammatory events (12,13). We recently demonstrated an essential role for HSPGs in macrophage-mediated CNS inflammation, using a transgenic mouse model that overexpresses heparanase (Hpa-tg) (14). The anti-inflammatory effect of heparanase overexpression was attributed to loss of vascular endothelial HS-chains, which are required to present chemokines from the inflamed tissue and in doing so direct transmigration of circulating monocytes into the CNS [see also (15,16)]. Shedding of the HSPG syndecan-1 prevents amplification of endotoxin-induced TNFα and IL6 production by disconnecting HS-bound chemokines from the cell surface (17). These studies support the concept that cell-surface HSPGs are important targets of inflammatory mediators.

The toll-like receptors (TLRs) are highly conserved pathogen detectors, with critical functions in innate immunity (18). The bacterial endotoxin lipopolysaccharide (LPS) is a well-established activator of TLR4. LPS first binds to cluster-of-differentiation 14 (CD14), which is GPI-anchored at the cell surface. LPS-bound CD14 interacts with TLR4, in complex with lymphocyte antigen 96 (MD2) (19). Subsequent NFκB-dependent signaling events induce IL1β and TNFα. CD14-dependent TLR4 activation is predicted in innate immune cells such as microglia. Importantly, LPS can also activate TLR4 independently of CD14 (20-22).

In the current study we explored whether LPS-driven inflammation in microglia and astrocytes is HSPG-dependent, using primary cell cultures established from Hpa-tg and Ctrl mice. Our results indicate that microglial HSPGs are integral to the CD14-dependent activation of TLR4, by which LPS induces upregulation of TNFα and IL1β. These findings suggest a pro-inflammatory function for microglial HSPGs that can be modulated by heparanase.

EXPERIMENTAL PROCEDURES

Heparanase transgenic (Hpa-tg) mice - The homozygous Hpa-tg mice express human heparanase preceded by a chicken heparanase signal peptide sequence, which promotes secretion of the enzyme (23). Constitutive expression of the heparanase construct is under control of the β-actin promoter (24). The chimeric enzyme construct was injected into fertilized eggs from C57BL/6 x Balb/c mice. The offspring were backcrossed at least 10 generations to a C57BL/6 background and C57BL/6 mice served as controls (Ctrl). All experiments involving mice were approved and carried out in accordance with the guidelines set by the local animal research ethics committee.

cDNA preparation and real-time quantitative polymerase chain reaction (qPCR) - RNA was isolated from mouse brain or primary cells using the E.Z.N.A. Total RNA kit (Omega bio-tek, Norcross, GA, USA). cDNA was prepared using the iScript cDNA synthesis kit (Bio-Rad). Real-time quantitative PCR (qPCR) analysis of transcript expression was performed using SsoFast EvaGreen Supermix (Bio-Rad, Stockholm, Sweden) and the primer pairs listed in Table 1. Primers were designed using Primer Blast NCBI software and produced by Life tech-
nologies (Invitrogen, Stockholm, Sweden). Target transcript expression was quantified relative to the expression of β-actin (Actb) or glyceraldehyde 3-phosphate dehydrogenase (Gapdh) mRNA transcript expression in the same sample. Equal concentrations of microglial and astrocyte RNA were used to prepare cDNA and to subsequently perform qPCR. The average and standard deviation of the house-keeping gene’s quantification cycle (Cq) for compared samples are stated in the figure legends.

Purification of primary microglia and astrocytes - Primary microglia were isolated as previously described (25,26). Briefly, Hpa-tg and Ctrl neonates (<4 days old) were sacrificed by decapitation and brains were isolated. The olfactory bulbs, hindbrain and meninges were removed in chilled phosphate buffered saline (PBS), under a dissecting microscope. One brain was triturated with a 1 ml pipette in 2 ml 0.25% trypsin, EDTA (Sigma-Aldrich AB, Stockholm, Sweden) and then incubated (10 mins) at 37°C. Trypsin was inactivated with 5 ml of Dulbecco’s modified Eagle’s medium F12 (Sigma-Aldrich AB, Stockholm, Sweden), 10% fetal bovine serum (Cambrex AB, Karlskoga, Sweden), 1% Primocin (Sigma-Aldrich AB), referred to hereafter as culture medium (CM), and the suspension was homogenized by pipetting. A suspension of two brains was centrifuged at 1500 x g for 5 mins at room temperature and the resulting pellet was resuspended in CM, filtered through a 40 µm cell strainer and transferred to a 75 cm² flask (Corning Life Sciences, Stockholm, Sweden); CM was changed every 24 h. After 10-14 days in vitro, when bright amoeboid cells appeared on the surface of the confluent cell culture and in the medium, flasks were shaken overnight or tapped firmly. The cell suspension was collected and centrifuged at 1500 x g for 5 mins; the resulting supernatant of glial-conditioned CM was filtered through a 0.2 µm syringe filter (VWR International AB, Stockholm, Sweden). The microglial pellet was resuspended and cultured in fresh CM substituted with 20% filtered glial-conditioned CM. These cultures were ~100% positive for the microglial marker Iba-1 confirming high microglial purity. Primary astrocytes were isolated according to an adapted protocol (27). Briefly, the initial brain-derived glial mix was cultured in CM for 24 h after which the flasks were vigorously tapped (15-20 sec) to remove easily detached cells and fresh CM was added. This was repeated every day for at least 5 days. On average, astrocytes isolated from 2 brains were confluent in a 75 cm² flask after 10 days in culture. Astrocytes were collected by trypsinization, counted and reseeded in CM. These cultures were >90% immunopositive for the astrocyte marker glial fibrillary acidic protein (GFAP; Abcam, Cambridge, UK).

Immunocytochemistry, microscopy and image analysis - Ctrl and Hpa-tg microglia or astrocytes were grown on poly-d-lysine coated (MatTek, Ashland, MA, USA) slides for 24 h, washed and then fixed (5 min) in 95% ethanol at -20°C. Fixed cells were permeabilized in 1 X PBS, 0.1% Tween, blocked (5-10 min) with Background sniper (Biocare medical, Concord, CA, USA) and then incubated overnight at 4°C or at room temperature for 1 h with primary antibodies against ionized calcium-binding adapter molecule 1 of microglia (Iba-1; Abcam, Cambridge, UK), glial fibrillary acidic protein of astrocytes (GFAP; Abcam, Cambridge, UK), or heparanase (733, gift from Israel Vlodavsky). LPS-stimulated microglia were fixed in 4% paraformaldehyde, permeabilized in 1 X PBS, 0.1% Tween, blocked in TNB blocking buffer (PerkinElmer Life Sciences, Waltham, MA, USA) and immunostained with antibodies against CD14 (M305; Santa Cruz Biotechnology, Dallas, TX, USA) and HS (10E4; Seikagaku Corporation, Kanagawa Japan). Antibodies were detected with species-specific fluorescein fluorescently conjugated secondary antibodies (Alexafluor, Molecular probes, Invitrogen). Fluorescence was visualized with a Nikon Eclipse 80i fluorescence microscope and captured with a Nikon DXM1200F digital camera operated with NIS 2.10 freeware. Confocal microscopy was carried out with a Zeiss LSM 710 instrument; images were acquired and analyzed using Zen imaging software. All images were processed with Photoshop software.

Characterization of heparan sulfate - Ctrl and Hpa-tg microglia (2 confluent 75 cm² flasks), and astrocytes (1 confluent 75 cm² flask) were cultured for 24 h in 8 ml CM/flask supplemented with 1000 µCi of Na₂³⁵SO₄. Cell medium was recovered and stored at -20°C. Cells were lysed in 4 M urea, 50 mM TrisHCl pH 7.4, 0.25 M NaCl, 1% Triton-X100. The supernatant resulting from 20 mins of 15,000-rpm centrifugation at 4°C was used for HS chain analysis. Total protein concentration analysis of lysate samples confirmed that medium from Ctrl and Hpa-tg flasks had been conditioned by a similar number of cells. For purification of the
lyophilized, dissolved in a minimal volume of water and desalted on a PD10 column (GE Healthcare). Lyophilized samples were then treated overnight at 37°C with 250 mU chondroitinase ABC (Seikagaku, Tokyo, Japan), followed by 2.5 h incubation with Benzonase nuclelease (Novagen®, Merck, Stockholm, Sweden) that was equilibrated with 30 ml of 50 mM NaAc pH 4.5, 2 M NaCl and subsequently eluted with 50 mM NaAc pH 4.5, 2 M NaCl. Eluted material was lyophilized, dissolved in a minimal volume of 0.15 M NaCl and subsequently eluted with 50 mM Tris HCl pH 7.4 followed by 50 mM NaAc pH 4.5, 0.1% Triton X-100. The eluates were collected as 0.5 ml fractions and 35S-HS/35S-HSPG radioactivity was analyzed by scintillation counting on a Beckman Coulter instrument. 

Enzymatic digests were subjected to gel chromatography on a Superose 12 column (GE Healthcare), equilibrated with 50 mM Tris HCl pH 7.4, 2 M NaCl, 0.1% Triton X-100. The eluates were collected as 0.5 ml fractions and 35S-HS/35S-HSPG radioactivity was analyzed by scintillation counting on a Beckman Coulter instrument. 

Inflammatory stimulation of microglia and astrocytes - 50 x 10^5 microglia (Ctrl and Hpa-tg) or astrocytes (Ctrl and Hpa-tg) were seeded per well of 96-well cell culture plates (Invitrogen, Stockholm, Sweden). Before treatment, cells were washed in serum free CM. The lipopolysaccharide (LPS) L2280, derived from E.coli O55:B5 derived from O55:B5 E.coli (Sigma-Aldrich), was diluted in serum-free CM to give the indicated final treatment concentrations. Cells were incubated with LPS for the indicated periods of time after which cell medium was collected and cells were lysed in CelLytic™ M lysis reagent (Sigma-Aldrich) containing Complete Protease Inhibitor Cocktail (Roche, Stockholm, Sweden). Prior to analysis medium and lysates were stored at -20°C in siliconized plasticware.

Enzyme linked immunosorbent assay (ELISA) for interleukin-1β (IL1β) and tumor necrosis factor-α (TNFα) - IL1β and TNFα concentrations in CM were quantified by Quantikine® ELISA kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer’s instructions. Optical density at 450 nm (minus optical density at 540 nm) was determined with a SpectraMAX 190 spectrophotometer and analyzed with SOFTMax Pro and Microsoft Excel software. Cytokine concentrations (pg/ml) were calculated from standard curves prepared with recombinant murine IL1β or TNFα (provided with the Quantikine® ELISA kits).

Western blotting - Cell lysates or brain homogenates were denatured and reduced in Laemmli buffer (100°C, 5 min) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-Tricine gels (Invitrogen, Stockholm, Sweden). Proteins were transferred to nitrocellulose or polyvinylidene difluoride membranes (BioRad, Hercules, CA, USA), which were then blocked (1 h) in 5% non-fat dry milk (BioRad), 1 X Tris buffered saline (TBS), 0.1% Tween-20 (Sigma Aldrich, St. Louis, MO, USA) or Odyssey blocking buffer (LI-COR, Bioscience, Lincoln, NE). Membranes were incubated overnight at 4°C with antibodies against heparanase (733 or 1453), IL1β (ab9722; Abcam, Cambridge England), CD14 (M305; Santa Cruz, Heidelberg, Germany), TLR4 (sc293072; Santa Cruz Biotechnology), MD2 (ab24182; Abcam), IkBα (sc-371; Santa Cruz Biotechnology), β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and were then washed with TBS, 0.1% Tween-20. After subsequent incubation with the appropriate secondary antibody, immunosignals for 733 (heparanase), IL1β and CD14 (Figure 3) were generated with SuperSignal West Dura Chemiluminescent Substrate (Thermo Scientific, Roskilde, Denmark), followed by exposure and development on ECL Hyperfilm (GE Healthcare, Uppsala, Sweden). CD14, TLR4, 1453 (heparanase), IkBα and MD2 immunosignals were detected with IRDye secondary antibodies (LI-COR, Lincoln, NE, USA) and visualized using an Odyssey two-channel infrared scanner (LI-COR). Fluorescent CD14 and IkBα Western blot images were processed with the ImageJ rolling-ball background subtraction application. Band intensities were determined using ImageJ. All Western blots were processed using Photoshop.

Sequence and structure analysis of CD14 - The mouse CD14 sequence (P10810) was retrieved from the Uniprot database. Potential heparin/HS-binding motifs bearing relevant
similarity to reported consensus sequences (13,28) were manually identified and alignment with the human CD14 sequence (P08571) was performed with the ClustalW2 tool available at EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Mouse CD14 three-dimensional conformation co-ordinates, deposited by (29) as 1WWL, were retrieved from the RCSB Protein Databank. The structure was viewed and the relevant features annotated using PyMol (PyMOL Molecular Graphics System, Version 1.3 Schrödinger, LLC).

**Statistical analyses** - Data points are presented with the mean and standard deviation. Significant differences between samples were determined using Student’s t-test and labeled as follows * = p < 0.05, ** = p < 0.01 and *** = p < 0.001.

**RESULTS**

We have previously reported that the recruitment of inflammatory macrophages across the blood-brain-barrier of LPS-stimulated Hpa-tg mice is impaired due to truncation of endothelial HS, which is required for transmigration (14). The LPS-induced cerebral IL1α response was also attenuated in Hpa-tg mice (14). The aim of the present study was to assess the involvement of Hpa-tg microglia and/or astrocytes in the impaired inflammatory response to LPS.

**Heparanase overexpression in microglia yields fragmented HS chains** - Primary microglia were isolated from Ctrl and Hpa-tg brain. Lysates were probed by Western blot with the anti-heparanase polyclonal antibody 733, which confirmed higher levels of the 50 kDa active subunit of heparanase in primary Hpa-tg microglia compared to Ctrl cells (Figure 1A). Size exclusion chromatography of metabolically 35S-labeled HS/HSPGs isolated from microglia lysates and 35S-labeled HS isolated from media revealed relative accumulation of HS fragments in Hpa-tg cultures (Figure 1B and C), indicative of truncated HS chains on cell-surface HSPGs.

**Lipopolysaccharide induction of TNFα and IL1β is impaired in Hpa-tg microglia** - Ctrl and Hpa-tg neonate brains showed similar basal mRNA expression of Cd14, Tlr4 and Mdi2, key components of the LPS-signaling complex (Figure 2A). Western blotting similarly revealed equal expression of TLR4 and MD2 proteins in Ctrl and Hpa-tg neonate brain tissue (Figure 2B). These data thus point to the availability of CD14, TLR4 and MD2 LPS-detectors in the Hpa-tg as well as the Ctrl mouse models.

To test whether microglial HSPGs are involved in pro-inflammatory signaling events primary microglia from Ctrl and Hpa-tg neonates were challenged over a 24 h period with LPS in serum free CM. LPS interacts with CD14, which activates the TLR4/MD2 inflammatory pathway, which culminates in the release of TNFα and IL1β (5,22). LPS essentially failed to induce IL1β and induced significantly less TNFα in Hpa-tg compared to Ctrl microglia as determined by cytokine-specific ELISAs (Figure 3A and E). According to recent reports TNFα release from rat microglia peaks after 6 h of LPS stimulation (30), and after 8 h in mouse microglia isolated through a protocol that selects adherent microglia (31) rather than the amoeboid microglia collected here (25). In our experiments TNFα release continued to increase up to 24 h of LPS stimulation (Figure 3E) suggesting that the dynamics of microglia cytokine release are influenced by species differences and cell isolation procedure. To address the mechanism behind the suppressed cytokine release in Hpa-tg microglia the expression of pro-IL1β in microglial lysates was assessed by Western blot. After 2.5 h LPS-stimulation, upregulation of pro-IL1β (31 kDa band) was detected in Ctrl microglia, along with additional 28 kDa (32) and 17 kDa components. These additional proteins are caspase-1 cleavage products, indicative of inflammasome activity (33), the latter being the mature active form of IL1β (Figure 3B). Pro-IL1β was also prominent after 8 and 24 h of LPS exposure in Ctrl microglia, whereas only a weak pro-IL1β band was detected in Hpa-tg microglia after 24 h (Figure 3B). The impaired induction of pro-IL1β in Hpa-tg microglia was separately confirmed following 24 h exposure to a higher LPS dose (Figure 3C). Additionally, qPCR analysis of IL1β and Tnfα mRNA expression confirmed that induction of cytokine transcripts was impaired in LPS-stimulated Hpa-tg microglia (Figure 3D and F). Together these results indicate that the diminished cytokine response in Hpa-tg microglia is due to a loss of function that precedes cytokine transcription.

LPS-stimulation of the TLR4 signaling pathway results in proteasomal degradation of IκBα (34). IκBα-bound NFκB is precluded from nuclear translocation, where it otherwise promotes cytokine expression. IκBα degradation was evident in LPS-stimulated (24 h) Ctrl as
well as Hpa-tg microglia (Figure 3G), suggesting that the NFκB pathway is at least partly viable in Hpa-tg microglia.

**Lipopolysaccharide fails to induce CD14 synthesis in Hpa-tg microglia** - The LPS-receptor CD14 is GPI-anchored to microglial membranes (mCD14) (35), but can also be released as a soluble form (36). Newly synthesized CD14 is evident on Western blot due to its increased molecular weight caused by the presence of a procID14 sequence (Figure 4A) (37,38). A mCD14 immunosignal at 55 kDa (35) was observed in Ctrl and Hpa-tg microglia at all early time points of LPS stimulation (Figure 4B, 0-2.5 h). After 8 and 24 h of LPS stimulation a strong 60 kDa pro-CD14 band appeared in Ctrl microglia, but not in Hpa-tg microglia blots (Figure 4B). LPS-induction of microglial CD14 is partly attributed to paracrine/autocrine signaling of released TNFα (39). Therefore, the failure of Hpa-tg microglia to upregulate CD14 is presumably secondary to their diminished TNFα response to LPS challenge. The impaired induction of C1d14 mRNA in Hpa-tg microglia was confirmed in a separate experiment, where CD14 expression by LPS-stimulated Hpa-tg cells remained as low as in unstimulated Ctrl or Hpa-tg microglia (Figure 4D). Importantly, Ctrl and Hpa-tg microglial lysates showed similar levels of TLR4, with and without LPS stimulation (Figure 4C). Tlr4 and Mdx2 mRNA expression in Ctrl and Hpa-tg microglia was similar in unstimulated cells (Figure 4E and F) and LPS induced the same changes in expression for both cell types, namely a slight decrease in Tlr4 mRNA and a slight increase in Mdx2 mRNA (Figure 4E and F). These findings suggest that the diminished LPS-induced TNFα response in Hpa-tg microglia results in a failure to upregulate CD14. Importantly, CD14, TLR4 and MD2 are similarly expressed in unstimulated Ctrl and Hpa-tg cells, confirming that these LPS-detectors should be equally available to Ctrl and Hpa-tg microglia.

**Astrocyte TNFα response is not affected by heparanase overexpression** - Astrogliosis is a prominent feature of numerous neurodegenerative disorders and reactive astrocytes are capable of a pro-inflammatory response (40,41). To assess whether the impaired inflammatory response in Hpa-tg microglia was replicated in astrocytes we again isolated primary cells from Ctrl and Hpa-tg neonates. Immunocytochemical analysis using the antibody 733 confirmed heparanase overexpression in Hpa-tg relative to Ctrl astrocytes (Figure 5A). Size-exclusion chromatography of metabolically 35S-labelled HS/HSPGs isolated from astrocyte lysates and 35S-labelled HS isolated from medium was indicative of an increased degradation of HS chains in Hpa-tg astrocytes (Figure 5B and C), similar to the findings for microglia (Figure 1B and C). Ctrl and Hpa-tg astrocytes were challenged with LPS for 24 h and TNFα and IL1β released into the medium were measured by ELISA. Unlike microglia, Ctrl and Hpa-tg astrocytes released comparable amounts of TNFα in response to LPS (Figure 5D; microglia data from Figure 3E inserted for comparison). LPS-stimulated astrocytes failed to produce any detectable IL1β (data not shown).

mCD14 is not detected in astrocyte lysates or medium - Ctrl astrocytes were incubated with (+) or without (-) LPS for 24 h. CD14 Western blot failed to reveal any mCD14 in Ctrl astrocyte lysates. Untreated Ctrl microglial lysates were included as positive controls for mCD14 expression (Figure 5E, CD14 blot). A weak procID14 band was detected in one of the LPS-treated astrocyte samples, suggesting that given sufficient stimulation CD14 synthesis can be induced in these cells (Figure 5E, CD14 blot lane 4). In contrast to CD14, the expression of TLR4 in astrocytes was similar to that observed in an equal number of microglia and was unaltered by LPS stimulation (Figure 5E, TLR4 blot). No CD14 immunosignals were detected in Ctrl astrocyte medium, irrespective of LPS administration (Figure 5F). These findings imply that the LPS-induced TNFα expression in astrocytes, which is substantially lower than that observed in an equal number of Ctrl microglia (Figure 5D), is the result of CD14-independent TLR4 activation.

To further explore the expression of the LPS detectors in Ctrl astrocytes we performed qPCR analysis of Cd14, Tlr4 and Mdx2 mRNA transcripts, with or without LPS stimulation. Compared to unstimulated Ctrl microglia (Mg-, set as 1.0), unstimulated Ctrl astrocytes expressed less Cd14, Tlr4 and Mdx2 (Figure 5G); however, while Tlr4 and Mdx2 transcript expression was ~3-fold lower, Cd14 was >20-fold lower. LPS stimulation increased Cd14 and to some extent Mdx2, but not Tlr4 expression in Ctrl astrocytes (Figure 5G). Finally, we established that the Cd14:Tlr4 ratio differs distinctly between microglia and astrocytes, in that Cd14 expression is 2-fold lower than Tlr4 in astrocytes, but 4-fold higher than Tlr4 in microglia (Figure 5H).
Together these findings are consistent with the concept that LPS-signaling in astrocytes occurs through CD14-independent TLR4/MD2 activation, while in microglia a greater cytokine response is observed due to additional CD14-dependent TLR4/MD2 activation. Given that the LPS-response is impaired in Hpa-tg microglia, but not in Hpa-tg astrocytes, we propose that microglial HSPGs facilitate CD14-dependent TLR4/MD2 signaling.

Heparan sulfate colocalizes with CD14 on LPS-stimulated microglia - Ctrl microglia were stimulated with LPS and immunostained with antibodies against HS (10E4) and CD14 (M305). Confocal microscopy revealed that HS associated with CD14-positive clusters (Figure 6A). Plotting the profile of HS and CD14 immunosignal intensities from single z-scans along a line revealed HS and CD14 co-localization within these clusters (Figure 6B a and b).

Identification of a potential HS-binding motif in CD14 - The microglia HS/CD14 interaction imaged in Figure 6 implies that CD14 carries a HS-binding site. However, CD14 was not detected in a recent bioinformatics screen for potential heparin/HS-binding motifs in murine immune proteins (13). Davis et al. applied the consensus heparin/HS-binding motifs XBBXXBX and XBBXBX (where X = alphabetic/aromatic amino acids and B = basic amino acids)(28), as well as truncated variants thereof, to detect potential HS-binding domains in 42 plasma membrane proteins, including TLR4, which has previously been reported from in vitro experiments [42]. By manually analyzing the murine CD14 sequence we identified the potential heparin/HS-binding motif XXBNXXBBX where N = neutral serine residues in the leucine-rich repeat 2 region (Figure 7A-C). This motif lies beneath the LPS-binding domain of CD14 (Figure 7A) and, based on available structural data (PDB ID: 1WWL (29), the basic residues protrude in a manner that could facilitate HS/heparin interactions (Figure 7A and B). Through sequence alignment we determined that human CD14 also contains a HS-binding motif (XBXXBXXBBX) in this region (Figure 7C).

DISCUSSION
Our results demonstrate that microglial HSPGs are required for a robust response to LPS via the CD14-dependent TLR4 pathway. The proposed function of pro-inflammatory microglial HSPGs and the rational supporting this model are illustrated in Figure 8. Briefly, LPS-induced IL1β and TNFα release were dramatically suppressed in Hpa-tg microglia (Figure 3), which carry shorter HS-chains than Ctrl cells. CD14, TLR4 and MD2 were equally expressed by unstimulated Ctrl and Hpa-tg microglia (Figure 4), and therefore not a limiting factor in the Hpa-tg response. However, LPS-induction of CD14 was completely abrogated in Hpa-tg microglia (Figure 4). In contrast to microglia, Ctrl and Hpa-tg astrocytes responded equally to LPS, as judged by release of TNFα (Figure 5D). Previous reports have offered conflicting conclusions regarding the expression of CD14 in astrocytes (42-45). In particular, Tarassishin et al. recently reported that mouse astrocytes express CD14 (46). Notably, however, Tarassishin et al. passaged primary mouse astrocytes for 6-weeks prior to experiment. In contrast we isolate astrocytes based on adherence, do not passage the cells and perform experiments typically within 10 days of isolation. It is conceivable that these different approaches result in astrocyte cultures that, despite being GFAP-positive, differ in their expression patterns for other proteins, including CD14. Interestingly, like Tarassishin et al. (46) and Lehnardt et al. (45) we observed that LPS increased astrocyte expression of CD14 mRNA (Figure 5G), albeit at low level compared to microglia (Fig. 5G). It thus seems reasonable to assume that under specific circumstances astrocytes are capable of expressing CD14. However, at the time-points investigated in this study neither mCD14 nor soluble CD14 could be detected in astrocytes (Figure 5E and F). LPS can induce inflammatory cytokines via CD14-dependent and CD14-independent TLR4 activation (20-22). Therefore, we attribute the LPS-induced TNFα response in astrocytes largely to CD14-independent TLR4 activation. Considering that heparanase overexpression had no effect on TNFα induction in astrocytes (Figure 5D), we propose that this CD14-independent TLR4 activation does not rely on cell surface HSPGs. Equally, due to the loss of cell surface HS in Hpa-tg microglia their CD14-dependent cytokine response is defective; therefore, as in astrocytes, the residual TNFα response detected in Hpa-tg microglia is attributed to CD14-independent TLR4 activation, which does not require HSPGs.

These considerations imply that HS on the microglial surface is required to facilitate CD14-dependent TLR4 activation, in partic-
ular, IL1β upregulation. In accord with this conclusion, microglial HS was found co-localized with CD14 in LPS-stimulated microglia (Figure 6), and a potential HS-binding motif was identified in the CD14 sequence (Figure 7).

Details of the downstream signaling pathway affected by heparanase overexpression remain unclear. IkBα degradation was observed in Hpa-tg as well as in Ctrl microglia following 24 h of LPS treatment (Fig. 3G). However, the interpretation of these results is complicated by the fact that NFkB activation initiates cycles of IkBα degradation, NFkB translocation, followed by NFkB induced upregulation of IkBα (47). The accumulated activation of the NFkB pathway is therefore not readily assessable. Moreover, the NFkB pathway may be intact in Hpa-tg microglia, while other transcription effectors (e.g. activator protein 1; AP1) may be affected by the loss of HS function. Further studies will be necessary to elucidate which specific signaling pathways are impaired in Hpa-tg microglia resulting in the diminished cytokine response.

Contrary to the anti-inflammatory effects of microglial heparanase overexpression presented here, several recent studies have implicated heparanase as a promoter of inflammation. In an experimental model of ulcerative colitis, the chronic inflammatory state was attributed to macrophage production of TNFα and cathepsin-L, which respectively induced and activated heparanase derived from the colon epithelium. Further, exogenous addition of recombinant heparanase to peritoneally derived macrophages increased their LPS-induced TNFα response (48). Importantly, this effect was dependent on catalytic activity of the enzyme, and Lerner et al. (48) proposed that heparanase relieves a HS-mediated inhibition of LPS-induced TLR4 signaling, supporting previous work by Brunn et al. (49). In accord with these findings Blich et al. reported loss of the pro-inflammatory activity of recombinant heparanase in peritoneal macrophages isolated from TLR2 and TLR4 knock-out mice (50). In a related context HS has also been considered an endogenous TLR4 agonist (51). Moreover, heparanase and soluble HS fragments were recently found to induce pro-inflammatory cytokines in human peripheral blood mononuclear cells and mouse splenocytes (52). It should be noted that contrary to the studies referenced above, where exogenous heparanase is added to cell cultures, we derived microglia from Hpa-tg mice that endogenously overexpress heparanase; therefore a direct comparison between the results is not readily made. Furthermore, the sulfation patterns of HS chains are cell-type specific (53) such that a microglial-specific HSPG may be specialized in supporting LPS-induced CD14-dependent TLR4 activation. Recently we reported lower levels of cerebral IL1β in Hpa-tg mice than in Ctrl mice in response to intraperitoneal LPS injection. The difference was primarily attributed to decreased recruitment of blood borne monocytes (14). The present study extends our understanding of these findings to include defective cytokine upregulation in Hpa-tg microglia. Clearly, heparanase can promote pro- and anti-inflammatory states.

We have previously reported that the Alzheimer’s disease (AD) amyloid-β peptide elevates the levels of specific cell surface microglia HSPGs (26). In light of the present study, it is conceivable that amyloid-β induction of microglia HSPGs would enhance a pro-inflammatory phenotype and sustain an inflammatory state in the diseased brain. Elucidating the context- and cell-specific inflammatory effects of heparanase in relation to cell-surface and soluble HS will increase our understanding of the various pathological conditions in which heparanase overexpression has been reported (48,54,55). The present study highlights the ability of heparanase to attenuate inflammatory states relevant to the CNS through its capacity to fragment microglial HSPGs.

REFERENCES

Microglial HSPGs facilitate inflammation


Microglial HSPGs facilitate inflammation


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FOOTNOTES

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The abbreviations used are: Alzheimer’s disease (AD), amyloid-β (Aβ), central nervous system (CNS), culture medium (CM), diethylaminoethyl (DEAE), enzyme linked immunosorbent assay (ELISA), glial fibrillary acidic protein (GFAP), heparanase transgenic (Hpa-tg), heparan sulfate (HS), heparan sulfate proteoglycans (HSPG), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), interleukin 1β (IL1β), interleukin-6 (IL6), ionized calcium binding adaptor molecule-1 (Iba-1), lipopolysaccharides (LPS), lymphocyte antigen 96 (MD2), membrane bound cluster of differentiation 14 (mCD14), nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), soluble cluster of differentiation 14 (sCD14), toll-like receptor 4 (TLR4), tumor necrosis factor-α (TNFα).

FIGURE LEGENDS

TABLE 1.

Primers used to perform quantitative PCR for mRNA transcripts.

FIGURE 1. Characterization of primary Hpa-tg microglia. A, Western blotting for the active 50 kDa subunit of heparanase (antibody 733) in Ctrl and Hpa-tg microglial lysates (A). B and C, Metabolically labeled 35S-HS/HSPGs were purified from Ctrl and Hpa-tg microglia lysates (B), while 35S-HS in the microglial medium was further dissociated from core proteins by alkaline treatment (C). Samples were analyzed by gel chromatography on Superose 12. Fractions were analyzed for 35S by scintillation counting. Arrow indicates elution position of a heparin 18-mer.

FIGURE 2. Characterization of key LPS-detectors in Ctrl and Hpa-tg neonatal brain. A, Relative expression of Cd14, Tlr4 and Md2 mRNA transcripts, corrected for the house-keeping gene Actb (β-actin), was assessed by real-time quantitative PCR (qPCR) in Ctrl and Hpa-tg neonate brains (n = 5) (A). B, Western blotting of Ctrl and Hpa-tg neonate brain homogenates (n = 5) with antibodies against heparanase (1453), TLR4, MD2 and β-actin (B).

FIGURE 3. Impaired inflammatory response in Hpa-tg microglia. A and E, Ctrl and Hpa-tg microglia (50 x 10^5 cells) were challenged with LPS (50 ng/250 µl) for the indicated periods of time. IL1β (A) and TNFα (E) in cell media were quantified by ELISA (pg/ml). Bars represent the mean and standard deviation from triplicate samples. B, Pro-IL1β in cell lysates was detected by Western blot (B). Ratios of pro-IL1β band intensities, corrected for GAPDH levels, were included for comparison; the 24 h Ctrl value is set to 1.0. C, Ctrl and Hpa-tg microglia were incubated (24 h) with (+) or without (-) LPS (100 µg/ml) and stained with anti-IL1β antibody. D, Ctrl and Hpa-tg microglia were incubated (24 h) with (+) or without (-) LPS (100 µg/ml) and stained with anti-TNFα antibody.
ng/250 µl), and lysates were subjected to IL1β Western blot (C). Band intensities relative to GAPDH levels from triplicate samples were quantified using ImageJ gel analysis software and presented as arbitrary units (AU). D, F and G, Ctrl and Hpa-tg microglia (100 x 10^3 cells) were incubated without (-) or with (+) LPS (50 ng/500 µl) for 24 h. qPCR analysis of Il1β (D) and Tnfα (F) mRNA transcript expression was corrected based on expression of the house-keeping gene Gapdh and presented relative to transcript expression in non-stimulated Ctrl microglia (set to 1.0). The average quantification cycle (Cq) for Gapdh in Figure 4D-F was 20.93 ± 0.36 (± SD). Bars represent the mean and standard deviation of biological duplicates. IkBα degradation in Ctrl and Hpa-tg microglial lysates was assessed by Western blot. Ratios represent IkBα band intensities corrected for β-actin levels, relative to the non-stimulated Ctrl microglia (G). Significant differences between LPS-stimulated Ctrl and Hpa-tg microglia was determined by Student’s t-test; ***p < 0.001, **p < 0.01.

**FIGURE 4.** Hpa-tg microglia fail to synthesize CD14. A and B. De novo CD14 synthesis is evident on Western blot as an increase in molecular weight due to the presence of a procCD14 sequence; this peptide is removed in the mature GPI-anchored (mCD14) and soluble (sCD14) forms of the protein, illustrated in (A). CD14 expression in Ctrl and Hpa-tg microglia after LPS stimulation (50 ng/250 µl) for the indicated time periods (B). Ratios represent the CD14 band intensities, adjusted for GAPDH levels. C-F, Ctrl and Hpa-tg microglia (100 x 10^3 cells) were incubated without (-) or with (+) LPS (50 ng/500 µl) for 24 h. TLR4 expression was detected by Western blot and ratios represent the TLR4 band intensities corrected for β-actin levels (C). qPCR analysis of Cd14 (D), Tlr4 (E) and Md2 (F) The mRNA transcript expression was corrected based on expression of the house-keeping gene Gapdh and presented relative to transcript expression in non-stimulated Ctrl microglia (set to 1.0). As in Figure 3 the average quantification cycle (Cq) for Gapdh in Figure 4D-F was 20.93 ± 0.36 (± SD). Bars represent the mean and standard deviation of biological duplicates.

**FIGURE 5.** The LPS-induced TNFα response in astrocytes is unaffected by heparanase overexpression. A-C. Elevated heparanase expression is detected in Hpa-tg astrocytes, compared to Ctrl, as demonstrated by immunostaining with antibody 733 (A). Nuclei are counterstained with DAPI (original magnification 200X). Heparanase immunostaining presents as granular inclusions in Hpa-tg astrocytes (A; right panel, original magnification 400X). Metabolically labeled 35S-HS/HSPGs were purified from Ctrl and Hpa-tg astrocyte lysates (B), while 35S-HS in the microglial medium was further dissociated from core proteins by alkaline treatment (C). Samples were analyzed by gel chromatography/Superose 12. 35S in effluent fractions was detected by scintillation counting. D, Ctrl and Hpa-tg astrocytes (50 x 10^3 cells) were incubated with 0 or 50 ng/250 µl of LPS for 24 h. TNFα (pg/ml) in the astrocyte medium was quantified by ELISA and compared with the values previously presented for Ctrl and Hpa-tg microglia (Figure 3D, 50 x 10^3 cells). Bars represent the mean and standard deviation of quadruplicate (astrocytes) and triplicate (microglia) samples; ***p < 0.001. E-H, Ctrl astrocytes (100 x 10^3 cells) were incubated with (+) or without (-) LPS (50 ng/500 µl) for 24 h. Lysates were immunoblotted for CD14 and TLR4; untreated Ctrl microglial lysates were included as positive controls, comparable loading was confirmed with β-actin (E). Medium from Ctrl astrocytes treated with (+) or without (-) LPS were subjected to CD14 Western blot; non-stimulated Ctrl microglial lysates (Mg Lys) were included as positive controls (F). The expression of Cd14, Tlr4 and Md2 mRNA transcripts in Ctrl astrocytes with (+) or without (-) LPS stimulation was assessed by qPCR. The expression levels were corrected for the expression of the house-keeping gene Actb (β-actin) (G) and presented relative to the equivalent expression in non-stimulated Ctrl microglia (Mg). The average quantification cycle (Cq) for Actb (β-actin) in Figure 5G was 20.23 ± 0.67 (± SD). Bars represent the mean and standard deviation of biological duplicates. Levels of Cd14, Tlr4, and Md2 mRNA expression in astrocytes and microglia are also represented relative to Tlr4 expression in each cell-type (H).

**FIGURE 6.** Microglial CD14 and HS co-localize. A and B, LPS-stimulated (50 ng/500 µl) Ctrl microglia were immunostained for CD14 and HS (antibody10E4) and imaged by confocal microscopy. HS accumulated in the periphery of the cell in areas that were positive for clusters of CD14 (A). The image in A represents a maximum intensity projection of eight z-planes collapsed together (A, z1-8). The spatial distribution of CD14 and HS signal intensities under the lines labeled a and b, from a single z-
plane (z5, indicated in the illustration), are presented as profile plots in a and b. (Original magnification, 630X; optical thickness of each z-plane = 1.2 µm, interval between z-planes = 1 µm).

**FIGURE 7.** A potential HS-binding motif of CD14. A-C, Based on comparisons with heparin/HS-binding consensus sequences a potential HS-binding motif was identified below the LPS-binding domain of murine CD14 (A). This motif is found in the leucine rich repeat 2 (LRR2) region. The basic, potentially HS-interacting, residues (B) are presented in blue, the aliphatic/aromatic residues (X) are in green and neutral residues (N) in the motif are represented by grey sticks (B). Similar HS-binding potential of the corresponding region of human CD14 was confirmed by sequence alignment. The structural representations were prepared in PyMol using the murine CD14 structure deposited at the RCSB Protein Databank as 1WWL.

**FIGURE 8.** Microglial HSPGs facilitate CD14-dependent TLR4 activation. LPS stimulates the maximal IL1β and TNFα response, and induces pro-CD14 via a combination of CD14-dependent and CD14-independent TLR4 signaling in Ctrl microglia. Heparanase overexpression truncates cell surface HS and impairs the ability of HSPGs to facilitate CD14-dependent TLR4 activation, thus suppressing the IL1β, TNFα and proCD14 response to LPS (Hpa-tg microglia). Astrocytes lack CD14; therefore the LPS-induced TNFα release is attributed to CD14-independent TLR4 activation, which does not require astrocyte HSPGs (Hpa-tg/Ctrl astrocytes).
Figure 1

A

B

Lysate

C

Medium

Microglial HSPGs facilitate inflammation
Microglial HSPGs facilitate inflammation

Figure 2

A

B

Relative mRNA expression

Ctrl  Hpa-tg  Ctrl  Hpa-tg  Ctrl  Hpa-tg

ns  ns  ns

Cd14  Tlr4  Md2

1.4  1.2  1.0  0.8  0.6  0.4  0.2

ns

ns

ns

Ctrl  Hpa-tg

0  1.2  1.0  0.8  0.6  0.4  0.2

1453

65 kD  50 kD

TLR4

95 kD

MD2

26 kD

β-actin

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Microglial HSPGs facilitate inflammation

Figure 3

A

B

C

D

E

F

G

IL-1β (pg/ml)

Ctrl
Hpa-tg

LPS (hr)

0 0.5 2.5 8 24

0 10 20 30 40 50 60 70 80 90

***

IL-1β (pg/ml)

Ctrl
Hpa-tg

LPS (hr)

0 0.5 2.5 8 24

0 10 20 30 40 50 60 70 80 90

***

LPS

GAPDH

0 0.5 2.5 8 24

GAPDH

0.2

0.1

0.1

0.2

- 0.2

- 0.1

- 0.1

- 0.0

- 0.0

- 0.0

- 0.0

IL-1β

mRNA expression

Ctrl
Hpa-tg

LPS

0 0.5 2.5 8 24

0 10 20 30 40 50 60 70 80 90

***

TNFα (pg/ml)

Ctrl
Hpa-tg

LPS (hr)

0 0.5 2.5 8 24

0 250 500 750 1000 1250 1500

***

Relative hIFN-γ

mRNA expression

Ctrl
Hpa-tg

LPS

- + - +

- + - +

LPS

- + - +

- + - +

LPS

- + - +

- + - +

LPS

- + - +

- + - +
Microglial HSPGs facilitate inflammation

Figure 4

A

B

C

D

E

F
Microglial HSPGs facilitate inflammation

Figure 5

A

B

C

D

E

F

G

H

Microglia Astrocytes

Ctrl

Hpa-tg

Lysate

Mg

Ctrl

Hpa-tg

Medium

Lysate

Mg

Ctrl

Hpa-tg

LPS

CD14

65 kD

CD14

65 kD

CD14

65 kD

Relative Cd14

mRNA expression

Relative Tlr4

mRNA expression

Relative M62

mRNA expression

Ctrl

Astrocytes

Medium

Mg

Relative to Tlr4

mRNA expression

Relative to Tlr4

mRNA expression

Relative to Tlr4

mRNA expression
Figure 6

Microglial HSPGs facilitate inflammation

(a) CD14 HS DAPI
(b) CD14 HS

Intensity (a.u.)
Distance (µm)

Intensity (a.u.)
Distance (µm)
Microglial HSPGs facilitate inflammation

Figure 7

A

LPS binding (16-82)

B

Potential HS binding (LRR2)

C

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Figure 8

Microglial HSPGs facilitate inflammation

Ctrl microglia

Hpa-tg/ Ctrl astrocytes

Hpa-tg microglia

LPS

MD2

TLR4

pro-CD14

NFXB

HSPG

LPS

HSPG

HSPG

HSPG

HSPG

HS

TGFβ

NFXB

TNFα

IL1β

(100%)

NFXB

TNFα

IL1β

HS

NFXB

NFXB

HSPG

NFXB

NFXB

NFXB

NFXB
Microglial heparan sulfate proteoglycans facilitate the cluster-of-differentiation 14 (CD14)/Toll-like receptor 4 (TLR4)-dependent inflammatory response
Paul O'Callaghan, Jin-Ping Li, Lars Lannfelt, Ulf Lindahl and Xiao Zhang

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