Tenascin C regulates multiple microglial functions involving TLR4 signaling and HDAC1

Verena Haage\textsuperscript{a}, Nirmeen Elmadanya\textsuperscript{b}, Lars Roll\textsuperscript{b}, Andreas Faissner\textsuperscript{b}, David H. Gutmann\textsuperscript{a,c}, Marcus Selmter\textsuperscript{a,1}, Helmut Kettenmann\textsuperscript{a,1,}\textsuperscript{•}

\textsuperscript{a} Cellular Neurosciences, Max-Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin 13125, Germany
\textsuperscript{b} Zellmorphologie und Molekulare Neurobiologie, Fakultät für Biologie und Biotechnologie, Ruhr-Universität Bochum, Bochum, Nordrhein-Westfalen 44801, Germany
\textsuperscript{c} Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110, USA

\textsuperscript{1} Authors contributed equally.

\textsuperscript{•} Corresponding author at: Cellular Neurosciences, Max-Delbrück Center for Molecular Medicine in the Helmholtz Association, Robert-Rössle-Str. 10, 13125 Berlin, Germany.

E-mail address: kettenmann@mdc-berlin.de (H. Kettenmann).

Abbreviations: AU, arbitrary unit; μg, microgram; μl, microliter; μM, micromolar; μm, micrometer; g, gram; h, hour; hrs, hours; l, liter; LPS, Lipopolysaccharide; M, molar; min, minute/s; ml, milliliter; mM, millimolar; mg, milligram; ng, nanogram; nM, nanomolar; nm, nanometer; rpm, rounds per minute; SEM, standard error of mean; Tnc, Tenascin C; WT, wildtype

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Tenascin C (Tnc) is an extracellular matrix glycoprotein, expressed in the CNS during development, as well as in the setting of inflammation, fibrosis and cancer, which operates as an activator of Toll-like receptor 4 (TLR4). Although TLR4 is highly expressed in microglia, the effect of Tnc on microglia has not been elucidated to date. Herein, we demonstrate that Tnc regulates microglial phagocytic activity at an early postnatal age (P4), and that this process is partially dependent on microglial TLR4 expression. We further show that Tnc regulates proinflammatory cytokine/chemokine production, chemotaxis and phagocytosis in primary microglia in a TLR4-dependent fashion. Moreover, Tnc induces histone-deacetylase 1 (HDAC1) expression in microglia, such that HDAC1 inhibition by MS-275 decreases Tnc-induced microglial IL-6 and TNF-α production. Finally, Tnc‘/−’ cortical microglia have reduced HDAC1 expression levels at P4. Taken together, these findings establish Tnc as a regulator of microglia function during early postnatal development.

1. Introduction

Microglial cells are important regulators of brain homeostasis and sensors for pathological events in the central nervous system (CNS) (Hanisch and Kettenmann, 2007). As the immunocompetent cells of the CNS, microglia express pattern recognition receptors (PRRs), including toll-like receptors (TLRs). TLRs recognize endogenous and exogenous ligands to orchestrate innate and adaptive immune responses to infection, inflammation and tissue injury (Garantziotis et al., 2008). Upon ligand binding, TLR signaling requires receptor dimerization and the recruitment of adaptor proteins that initiate downstream effector signaling. TLR activation most commonly leads to the translocation of nuclear factor kappa B (NFκB) to the nucleus or activation of the MAP kinase pathway, initiating the transcription of proinflammatory mediators, such as cytokines and cyclooxygenase-2 (Broad et al., 2007; Kawai and Akira, 2010; Takeda and Akira, 2004; Zhang et al., 1999). To date, ten human and thirteen murine TLRs have been reported, which are all expressed in microglia (Bsibsi et al., 2002; Olson and Miller, 2004).

Of these TLR family members, Toll-like receptor 4 (TLR4) is critical for the recognition of gram-negative bacterial components, such that the bacterial cell wall component lipopolysaccharide (LPS) has become the canonical ligand for TLR4 activation (Beutler et al., 2001; Poltorak et al., 1998). In addition to its role in brain bacterial infections (Weber and Tuomanen, 2007), TLR4 is also important in the setting of stroke or spinal cord injury (Caso et al., 2008, 2007; Lehnardt et al., 2003), as well as in neurodegenerative diseases, including Alzheimer’s disease and Parkinson’s disease (Song et al., 2011; Zhao et al., 2016). The involvement of TLR4 signaling in the pathogenesis of non-infectious brain diseases suggests that TLR4 can recognize endogenous ligands. To this end, various TLR4 ligands have been reported, including Tenascin C (Tnc) (Midwood et al., 2009; Park et al., 2004; Smiley et al., 2001; Termeer et al., 2002; Vabulas et al., 2002).

Tnc is a highly conserved hexameric extracellular matrix (ECM)
protein, expressed during brain development as well as under pathological conditions (Carey et al., 2010; Faissner et al., 2017; Midwood et al., 2016). In the CNS, astrocytes are the main source of Tnc (Faissner and Steindler, 1995; Gates et al., 1995). Several ECM binding partners and receptors are described for Tnc, and Tnc is involved in the regulation of diverse cellular processes, including cell adhesion, cell signaling and gene expression (Midwood et al., 2016). As an endogenous ligand of Toll-like receptor 4, Tnc regulates cytokine synthesis in primary human macrophages (Midwood et al., 2009). Moreover, we recently demonstrated that Tnc is a glioma stem cell-derived factor that specifically controls IL-6 secretion in glioma-associated microglia in a TLR4-dependent manner (Dzaye et al., 2016). In addition, Tnc has been suggested to affect microglia in other brain diseases, including globoid cell leukodystrophy and Alzheimer’s disease (Claycomb et al., 2014; Xie et al., 2013). Although the expression levels of Tnc correlate with states of elevated microglia activity in the course of CNS development and inflammatory processes, the effects of Tnc on microglia function in this context have not been studied to date.

Histone deacetylases (HDACs) have been shown to control several functions of monocytes/macrophages (Das Gupta et al., 2016). As such, stimulation of microglia with LPS has been linked to the induction of HDACs, such that HDAC1 or HDAC2 inhibition or knockdown suppresses LPS-induced release of Interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) in microglia (Durham et al., 2017; Kannan et al., 2013). In addition, regulator of G protein signaling 10 (RGS10), a negative regulator of NFκB is transcriptionally silenced by HDAC1 following LPS stimulation in microglia (Alqinyah et al., 2017; Lee et al., 2011). For these reasons, we analyzed the effect of Tnc on microglia biology with respect to both, TLR4 dependence and HDAC expression. We found that Tnc regulates microglial phagocytosis and HDAC1 expression during early postnatal brain development. This is relevant since microglia have been shown to regulate many neurodevelopmental processes, including neurogenesis, gliogenesis, neuronal migration in addition to axonal outgrowth, myelination, as well as synaptic pruning by phagocytosis (Kettenmann et al., 2013; Salter and Stevens, 2017; Schafer et al., 2012; Wu et al., 2015). Furthermore, as astrocytes are the major source of Tnc, these findings uncover a novel communication pathway between these two glial cell types in development and inflammation.

2. Material and methods

2.1. Ethics statement

All procedures involving the handling of living animals were performed in strict accordance with the German Animal Protection Law, and were approved by the Regional Office for Health and Social Services in Berlin (Landesamt für Gesundheit und Soziales, Berlin, Germany, Permit Number T0014/08, X0923/12) and by the State Agency for Nature, Environment and Consumer Protection (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, D-45659 Recklinghausen, Germany, Permit Number 84-02.05.40.16.A051). Adult mice were euthanized by cervical dislocation or by intraperitoneal injection of pentobarbital (Narcoren, Merial GmbH, Hallbergmoos, Germany). All efforts were made to minimize suffering.

2.2. Animals

Experiments were performed using C57BL/6J WT mice (Charles River Laboratories, Sulzfeld, Germany) or Tlr4 knockout mice (Tlr4−/−) on a C57BL/6J background. Tlr4−/− mice were generated by Dr. Shizuo Akira (Osaka University, Japan), and obtained from Oriental BioServices Inc., Kyoto, Japan. For the in situ phagocytosis assay and immunohistochemical staining with Tnc knockout mice (Tnc−/−), we used 129/Sv mice as controls, since Tnc−/− mice were crossed on a 129/Sv background (Forsberg et al., 1996). A colony was established in the animal facility of the Faculty of Biology and Biotechnology of the Ruhr-University Bochum (Czopka et al., 2009). This study was executed in accordance with the European Council Directive of September 22, 2010 (2010/63/EU) for care of laboratory animals and approved by the animal care committee of North Rhine-Westphalia, Germany, based at the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz, Nordrhein-Westfalen, D-45659 Recklinghausen, Germany). The study was supervised by the animal welfare commissioner of the Ruhr-University Bochum. Animals were kept according to the German law for animal protection under a 12 hrs/12 hrs dark-light cycle with food and water supply ad libitum.

2.3. Purification of Tenascin C

Tnc proteins were isolated from early postnatal mice by immunoadfinity chromatography as previously described (Faissner and Kruse, 1990), and detailed in the Supplementary Material.

2.4. Preparation of primary neonatal microglia

Neonatal primary microglial cultures from WT and Tlr4−/− mice were prepared as previously described (Prinz et al., 1999), and detailed in the Supplementary Material.

2.5. Preparation of primary neonatal astrocytes

Following microglia harvest from neonatal primary microglia cultures (see 2.4, for details see Supplementary Material), astrocytes were harvested by trypsinization and subsequently used for downstream analysis.

2.6. Flow cytometry analysis

Primary microglia and astrocytes were harvested, processed as previously published (Turaç et al., 2013), and analyzed with a LSRII flow cytometer. For detailed descriptions, see Supplementary Material.

2.7. Cytokine array

WT or Tlr4−/− microglia were stimulated with Tnc (55 nM), LPS (100 ng/ml) or PBS for 24 hrs, and cytokine screening performed using the Proteome Profiler Mouse Cytokine Array Panel A (R&D Systems, Minneapolis, Minnesota, USA). Quantification of the pixel intensity of the induced cytokine spots was performed using Image J software.

2.8. Enzyme-linked immunosorbent assay (ELISA)

WT or Tlr4−/− microglia were treated with Tnc (55 nM), LPS (100 ng/ml) or PBS for 24 hrs, supernatants collected, and IL-1β, IL-6 and TNF-α secretion was assessed by ELISA, as detailed in Supplementary Material.

2.9. Griess assay (NO release)

WT or Tlr4−/− microglia were treated in a 96-well plate (0.1 × 10^6 cells per well) with Tnc (55 nM), LPS (100 ng/ml) or PBS in triplicates per biological replicate. After 24 hrs, the supernatant was collected, 100 µl was transferred into a new 96 well plate, and 100 µl freshly mixed Griess reagents were added. Griess reagent was composed of reagent A (100 mg Naphthylethylenediamel in 50 ml aqua dest.) and reagent B (1 g Sulfanilamide, 61 H3PO4 (85%) in 44 ml aqua dest.), mixed 1:1. The solution was mixed, and the absorbance at a wavelength of 550 nm was determined. Dissolved sodium nitrite in plain medium served as a
Table 1

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2.10. qRT-PCR analysis

Total RNA was extracted from treated primary neonatal microglia, acutely isolated macrophages by MACS or whole brain samples using ReliaPrep RNA Miniprep System (Promega, Madison, Wisconsin, USA). cDNA synthesis was performed using the PrimeScript™ RT reagent Kit (Takara, Shiga, Japan). Quantitative real-time PCR reactions to amplify 1 ng of total cDNA were performed in a 7500 Fast Real-Time thermocycler (Applied Biosystems, Carlsbad, USA) using the SYBR Select Master Mix (Applied Biosystems). CT values were normalized using TATA-binding protein (Tbp) as housekeeping gene. Primers were tested for their efficiency beforehand, and the ΔΔCt-method was applied for analysis of relative expression. To ensure the specificity of the PCR product, we analyzed the melting curves of each product. For primer sequences, see Table 1.

2.11. Agarose spot assay

Agarose spot assay was performed, as described previously (Wiggins and Rappoport, 2010), and detailed in Supplementary Material.

2.12. In vitro phagocytosis assay

Primary WT or Tim4+/− microglia were incubated with opsonized yellow-green fluorescent microspheres, simultaneously stimulated either with Tnc (55 nM), LPS (100 ng/ml) or control (PBS), subsequently fixed stained, imaged, and the number of engulfed beads per cell was quantified. For detailed description, see Supplementary Material.

2.13. Western blot analysis

WT or Tim4+/− microglia were treated with Tnc (55 nM), LPS (100 ng/ml) or PBS for 24 hrs, washed with cold PBS, scraped into ice-cold RIPA buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing protease inhibitor cocktail (Roche, Basel, Switzerland) followed by centrifugation at 15,000 rpm for 10 min to remove insoluble cell debris. Protein concentration was measured by the BCA protein assay kit (Thermo Fisher Scientific) and a total of 6–12 µg protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). PVDF membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences GmbH, Bad Homburg, Germany) for 1 h at RT and incubated with primary antibodies against HDAC1 (Cell Signaling Technology, Danvers, Massachusetts, USA) or GAPDH (abcam) overnight at 4 °C. After washing, membranes were then incubated with secondary antibodies (IRDye®800CW or 680CW, LI-COR Biosciences GmbH) for 3 hrs at RT, washed and detected with Odyssey® Imaging System. Quantification of the bands was performed using ImageJ software.

2.14. Magnetic Activated Cell Sorting (MACS) of microglia

For a detailed description of microglia isolation using magnetic beads, refer to Supplementary Material.

2.15. in situ phagocytosis assay

The in situ phagocytosis assay was performed as described previously (Wendt et al., 2017), and detailed in Supplementary Material.

2.16. Immunohistochemistry

Cortical slices of C57BL/6, 129/Sv, Tim4+/− and Tnc+/− mice were fixed in 4% PFA and IHC using primary and secondary antibodies was performed. For detailed description, see Supplementary Material.

2.17. Statistics

All data are represented as mean ± SEM. Statistical significance between multiple samples (e.g. within WT and Tim4+/− samples) was tested in IGOR 6.37 using one-way ANOVA following Tukey’s multiple comparisons test, while differences between two samples were assessed by the Student’s t-test. Significance in the figures is indicated as: n.s. (not significant) = p > 0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.

3. Results

3.1. Tnc controls phagocytic activity of cortical microglia during early postnatal development

Tenascin C has been previously suggested to affect microglia in the context of disease (Claycomb et al., 2014; Xie et al., 2013), however, its effects on microglia function during development have not been studied to date. Microglia play a major role in early postnatal development through a process called synaptic pruning (Paolicelli et al., 2011). At the same time, Tnc expression in the CNS is increased during embryonic and early postnatal stages (Supplementary Fig. 1A). We therefore sought to determine whether Tnc is involved in the regulation of microglial phagocytic activity during early postnatal development. We assessed microglial phagocytic activity in the cortex of WT and Tnc−/− microglia using magnetic beads, refer to Supplementary Material.

3.1. Tnc controls phagocytic activity of cortical microglia during early postnatal development

WT and Tim4−/− microglia were treated with Tnc (55 nM), LPS (100 ng/ml) or PBS for 24 hrs, washed with cold PBS, scraped into ice-cold RIPA buffer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing protease inhibitor cocktail (Roche, Basel, Switzerland) followed by centrifugation at 15,000 rpm for 10 min to remove insoluble cell debris. Protein concentration was measured by the BCA protein assay kit (Thermo Fisher Scientific) and a total of 6–12 µg protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). PVDF membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences GmbH, Bad Homburg, Germany) for 1 h at RT and incubated with primary antibodies against HDAC1 (Cell Signaling Technology, Danvers, Massachusetts, USA) or GAPDH (abcam) overnight at 4 °C. After washing, membranes were then incubated with secondary antibodies (IRDye®800CW or 680CW, LI-COR Biosciences GmbH) for 3 hrs at RT, washed and detected with Odyssey® Imaging System. Quantification of the bands was performed using ImageJ software.

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All data are represented as mean ± SEM. Statistical significance between multiple samples (e.g. within WT and Tim4−/− samples) was tested in IGOR 6.37 using one-way ANOVA following Tukey’s multiple comparisons test, while differences between two samples were assessed by the Student’s t-test. Significance in the figures is indicated as: n.s. (not significant) = p > 0.05; * = p < 0.05; ** = p < 0.01; *** = p < 0.001.
**Figure A**

WT P4 | Tnc<sup>−/−</sup> P4 | WT P28-P31 | Tnc<sup>−/−</sup> P28-P31

![Images of cell cultures at 5μm and 30μm scales](image_url)

**Figure B**

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*Significance levels: *** indicates p < 0.001, n.s. indicates non-significant difference.*

**Figure C**

WT P4 | Tlr4<sup>−/−</sup> P4 | WT P28-P31 | Tlr4<sup>−/−</sup> P28-P31

![Images of cell cultures at 30μm scale](image_url)

**Figure D**

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*Significance levels: *** indicates p < 0.001, n.s. indicates non-significant difference.*
knockout (Tnc<−/−>mice at P4 (physiologically high Tnc levels) and P28-P31 (physiologically low Tnc levels) using an in situ phagocytosis assay. Acute brain slices from P4 and P28-P31 male WT and Tnc−/−mice were incubated with fluorescent microbeads and subsequently, microglia were stained for the microglia marker ionized calcium binding adapter molecule 1 (Iba1). Confocal imaging followed by Imaris software-based 3D reconstruction was performed for quantification of the number of beads incorporated into a 3D-rendered Iba1-labelled volume, allowing us to calculate the phagocytic index of microglia (Fig. 1A, B). Consistent with a previous report (Lenz and Nelson, 2018), we found that microglial phagocytic activity in WT mice was decreased from 1.69 ± 0.2 at P4 (n = 57) to 0.43 ± 0.09 at P28-P31 (n = 61, p < 0.0001). Strikingly, the phagocytic activity of Tnc−/−microglia at P4 was reduced to similar levels (0.46 ± 0.06, n = 50, p < 0.0001 compared to WT P4), as found in P28-P31 WT and P28-P31 Tnc−/−microglia (0.43 ± 0.09m = 61 for P28-P31 WT, p < 0.0001 compared to Tnc<−/−> microglia; 0.52 ± 0.07, n = 51 for P28-P31 Tnc−/−, p < 0.0001 compared to P4 Tnc−/−). These findings support a role for Tnc in controlling microglial phagocytosis during early postnatal CNS development.

Since Tnc is an endogenous TLR4 ligand, and TLR4 has been previously shown to regulate HDAC1 expression (Prinz et al., 1999) we sought to determine whether Tnc might exert its effects on microglial phagocytosis during early postnatal development through TLR4. We determined cortical microglial phagocytic activity in P4 and P28-P31 WT and Tlr4−/−mice (Fig. 1C, D). Both, WT and Tlr4−/−microglia exerted lower phagocytic activity at P28-P31 (2.08 ± 0.19, n = 53 and 1.22 ± 0.09, n = 80 for WT and Tlr4−/−, respectively) compared to P4 (7.74 ± 0.39, n = 57, p < 0.0001 and 4.77 ± 0.46, n = 65, p < 0.0001 for WT and Tlr4−/−, respectively). Microglia from Tlr4−/−mice showed a decrease in phagocytic activity compared to WT at P4 (p < 0.0001), but not at P28-P31 (p = 0.1955). The decrease in microglial phagocytic activity in Tlr4−/−mice supports a role for TLR4 in regulating microglial phagocytosis, however also suggests that additional receptors might be involved in mediating this process.

3.2. Tnc<−/−> mice show reduced HDAC1 protein levels in P4 cortical microglia in situ

In brain development, expression of Tnc peaks between embryonic day E18.5 and postnatal day P4 and subsequently decreases to nearly undetectable levels in early adulthood (after P28; Allen Developing Mouse Brain Atlas; Supplementary Fig. 1A). Similarly, HDAC1 and HDAC2 were recently shown to be highly expressed in microglia during embryonic development, after which their expression gradually decreases to lowest expression levels in the adult stage (Datta et al., 2018). Moreover, inhibition of HDACs has been previously shown to impair phagocytosis in macrophages (Mombelli et al., 2011). We therefore compared HDAC1 protein levels in cortical microglia from WT and Tnc−/−mice at P4 (physiologically high Tnc levels) and P28-P31 (physiologically low Tnc levels). We prepared cortical slices of P4 and P28-P31 WT and Tnc−/−mice and identified microglia with Iba1, HDAC1 protein with a specific antibody, as well as nuclei with DAPI staining. Following confocal imaging, we performed 3D rendering of Iba1 microglia and their nuclei, and quantified the mean intensity of the HDAC1 signal, reflecting HDAC1 protein expression. HDAC1 protein levels were clearly abundant in the nuclei of P4 microglia (Fig. 2A) and exerted a robust fluorescent signal of 937 ± 81 AU (n = 36 cells) (Fig. 2B). Intriguingly, HDAC1 signals in microglia from P4 Tnc−/−mice were significantly reduced compared to WT mice (145 ± 24 AU, n = 36 cells, p < 0.0001). At P28-P31, when physiological Tnc levels are low, microglia from WT (719 ± 65 AU, n = 44 cells) and Tnc−/−mice (925 ± 72 AU, n = 44 cells) displayed similar HDAC1 protein levels (p = 0.0937). These results indicate that extracellular Tnc might influence HDAC1 levels in microglia during early postnatal development.

To further assess which other CNS cell types express HDAC1 during early postnatal development, we leveraged a published RNA sequencing dataset generated from P7 mice and extracted data on HDAC1 expression (Supplementary Fig. 5A; Zhang et al., 2014). HDAC1 is expressed across all brain cell types, including astrocytes, neurons, oligodendrocyte precursor cells (OPCs), newly formed as well as myelinating oligodendrocytes, endothelial cells and microglia/macrophages. Due to its expression in all CNS cell types in the early postnatal brain, we additionally quantified HDAC1 intensity in 3D-rendered nuclei of all cells in cortical slices of WT and Tnc−/−mice at age P4 (Supplementary Fig. 5B). Interestingly, HDAC1 intensity was decreased in all 3D-rendered nuclei in P4 Tnc−/−cortical slices compared to P4 WT mice, indicating that Tnc might affect HDAC1 expression in other CNS cell types in addition to microglia.

Lastly, we also tested whether TLR4 is involved in the regulation of Tnc-dependent HDAC1 expression during early postnatal development. For these studies, we used the same experimental setup as previously described by preparing cortical slices from P4 and P28-P31 WT and Tlr4−/−mice (Fig. 2C). P4 microglia of WT and Tlr4−/−mice showed no differences in HDAC1 protein levels, implying that Tnc-regulated changes in HDAC1 protein levels during early postnatal development are not mediated by TLR4 signaling.

3.3. Tnc regulates HDAC1 expression in primary microglia via TLR4

In order to confirm that Tnc regulates HDAC1 expression in microglia, we stimulated primary neonatal microglia with Tenascin C purified from early postnatal mice (see Section 2.3). Primary neonatal microglia, prepared as previously described (Prinz et al., 1999) were characterized and tested for purity (see Supplementary Fig. 2 and 3) and stimulated with Tnc (55 nM, for concentration see Section 3.4 and Supplementary Fig. 4A). In addition to HDAC1 expression, we also assessed expression levels of HDACs 2–11 by qRT-PCR (Fig. 3A). HDAC1 (4.9 ± 0.3 fold, n = 5, p = 0.0002), HDAC2 (1.1 ± 0.0 fold, n = 5, p = 0.0463) and HDAC9 (1.5 ± 0.1 fold, n = 5, p = 0.0297) expression levels were significantly increased, whereas we found a reduction of HDAC4, HDAC5, HDAC8, HDAC10 and HDAC11 expression after stimulation with Tnc. We also tested LPS (100 ng/ml)-dependent changes in HDAC expression as the exogenous ligand of TLR4 has previously been reported to transiently change the expression of multiple histone deacetylases in microglia (Kannan et al., 2013; Midwood et al., 2009). Strikingly, Tnc and the TLR4 agonist LPS affected microglial HDAC expression in an identical fashion (Fig. 3A). We next tested if Tnc- or LPS-induced HDAC1 expression is dependent...
on TLR4 (Fig. 3B). The LPS-induced increase in HDAC1 mRNA levels (4.7 ± 0.5 fold, n = 4, p = 0.0017 for WT microglia) was completely abolished in Tlr4−/− microglia (1.0 ± 0.2 fold, n = 3, p = 0.8239). Interestingly, Tnc still induced HDAC1 expression in Tlr4−/− microglia (2.3 ± 0.2 fold, n = 3, p = 0.0246 to PBS), however, to lower levels than WT microglia (4.9 ± 0.7 fold, n = 4, p = 0.0316 wt vs Tlr4−/−). While HDAC1 expression in microglia was independent of TLR4 during early postnatal development, we now observed a TLR4-dependency of HDAC1 expression during early postnatal development.
microglial HDAC1 expression. These data suggest that microglial HDAC1 expression is regulated by Tnc, however the engagement of TLR4 in this process might be context-dependent. While TLR4 might not be involved in Tnc-mediated microglial phagocytosis during early postnatal development (see Fig. 2C), TLR4 might rather play a role under inflammatory conditions when Tnc levels rise more abruptly as mimicked by our in vitro experiments. However, as HDAC1 levels were still increased in Tlr4−/− microglia, Tnc might affect HDAC1 expression also using alternative signaling pathways.

Additionally, we assessed HDAC1 protein levels in WT microglia by Western Blot 24 hrs after stimulation with Tnc or LPS (55 nM Tnc; 100 ng/ml LPS; Fig. 3C). HDAC1 protein levels were increased similarly upon Tnc (1.5 ± 0.1 fold, n = 3, p = 0.0069) and LPS (1.6 ± 0.1 fold, n = 3, p = 0.0018) stimulation compared to PBS.
3.4. Inhibition of HDAC1 decreases Tenascin C-induced TNF-α and IL-6 release

A recent study reported that inhibition of HDAC1 or HDAC2 suppresses IL-6 and TNF-α expression in LPS-stimulated BV-2 microglia (Durham et al., 2017). In order to test whether Tnc is able to induce microglial cytokine release and to assess whether HDAC1 is involved in this process, we first tested a concentration range of Tnc previously shown to be physiologically relevant in experiments with primary human macrophages, since physiological concentrations of Tnc in the mouse or human brain have not been determined to date (Midwood et al., 2009). We compared the release of TNF-α and IL-6 24 hrs after stimulation of primary microglia with increasing concentrations of purified mouse Tnc (11 nM to 110 nM) as well as with LPS (100 ng/ml) by ELISA (Supplementary Fig. 4A). Indeed, Tnc induced microglial TNF-α and IL-6 secretion, and we determined 55 nM of Tnc as working concentration. Heat-inactivated Tnc or LPS neutralized by Polymyxin B did not induce TNF-α or IL-6 release (Supplementary Fig. 4B), excluding the possibility of unspecific effects.

We sought to determine whether HDAC1 activity controls Tnc-induced cytokine production in microglia. MS-275 (Entinostat) selectively inhibits HDAC1, 2, and 3 and was shown to reduce LPS-induced nuclear translocation of NFκB p65 and secretion of inflammatory cytokines (Choo et al., 2010; Zhang and Schluesener, 2012). At 300 nM, MS-275 should specifically inhibit HDAC1, whereas 1 μM MS-275 acts on HDAC1, HDAC2 and HDAC3. Primary microglia were pretreated with MS-275 in DMSO or DMSO alone (control) for 24 hrs prior to stimulation with LPS or Tnc for 24 hrs, and TNF-α and IL-6 levels were subsequently measured by ELISA (Fig. 3D). Pretreatment with MS-275 led to significant decreases in Tnc-induced TNF-α release from microglia (from 3612 ± 702 pg/ml to 1884 ± 259 pg/ml, n = 5, p = 0.0406 with 300 nM MS-275 and to 1532 ± 128 pg/ml, n = 5, p = 0.0147 with 1 μM MS-275). Similarly, the Tnc-induced release of IL-6 was decreased by 300 nM MS-275 from 2336 ± 422 pg/ml (control, n = 7) to 1098 ± 374 pg/ml (MS-275; n = 5, p = 0.0492) and by 1 μM MS-275 to 296 ± 57 pg/ml (n = 5, p = 0.0023). Following LPS stimulation of MS-275 pretreated microglia, we only observed significant decreases in TNF-α and IL-6 secretion at concentrations of 1 μM and 3 μM MS-275 (see Supplementary Fig. 4C). In addition, we assessed Il6 and Tnf-α mRNA expression in primary microglia 6 hrs after Tnc stimulation following 24 hrs pretreatment with MS-275 (300 nM and 1 μM) (Supplementary Fig. 4D). Pretreatment of primary microglia with 300 nM MS-275 caused a significant reduction of Tnc-induced stimulation of Il6 mRNA expression, while Tnf-α expression was only reduced following pretreatment with 1 μM MS-275. Overall, these results suggest that HDAC1 regulates Tnc-induced cytokine synthesis in primary microglia.

3.5. Tnc regulates cytokine release via TLR4 signaling in primary microglia

In order to study the effect of Tnc on the synthesis of a broader pattern of microglial cytokines, we performed a cytokine array assessing the production of 40 different cytokines following stimulation with Tnc. To determine the dependency on TLR4 signaling, we compared the responses in WT and Tlr4−/− microglia. Primary cultures were stimulated with Tnc or PBS as control for 24 hrs, and cytokine profiles from collected supernatants were assessed (Fig. 4B). In addition, we also compared the microglial cytokine profile after LPS stimulation (100 ng/ml). In unstimulated WT microglia, we detected low levels of M-CSF, TNF-α, CCL2, CCL3, CCL4, CXCL10, CXCL12, and CXCL13 and slightly higher levels of IL-27 and CXCL9, while CCL2, CCL4 and CXCL10 were the only detectable cytokines in unstimulated Tlr4−/− microglia (data not shown). All other cytokines were undetectable in WT or Tlr4−/− unstimulated microglia.

Tnc induced the expression of fifteen different cytokines in WT microglia with G-CSF, TNF-α, IL-1ra, IL-6, CCL2, CCL3, CCL4, CCL5, CCL12, CXCL1, CXCL2, CXCL10 and ICAM-1 being strongly up-regulated while IL-10 and TIMP-1 were mildly induced. Expression of IL-27 and CXCL9 was abolished after Tnc-stimulation of WT microglia. Tnc-induced microglial secretion of G-CSF, TNF-α, IL-1ra, IL-6, IL-10, CCL2, CXCL1 and TIMP-1 was dependent on microglial TLR4 expression as they were undetectable in Tnc-stimulated Tlr4−/− microglia. The induction of CCL5, CXCL2, CXCL10 and ICAM-1 release by Tnc was reduced in Tlr4−/− microglia compared to WT microglia. Interestingly, Tnc-induced expression of CCL2, CCL3 and CCL4 was independent of microglial TLR4 expression, as quantified intensities did not differ between WT and Tlr4−/− microglia, suggesting that Tnc might use an alternative pathway to control the synthesis of these cytokines.

In addition, we compared the Tnc-induced microglial cytokine pattern with the cytokine profile of microglia stimulated with LPS. All cytokines induced by Tnc in microglia were mimicked by LPS stimulation. Hereby, LPS-induced IL1-ra and IL-27 levels were higher, while CCL2 levels were lower compared to Tnc-stimulated microglia.

3.6. Tnc regulates expression of COX2, iNOS and NO release via TLR4 signaling in primary microglia

Previous studies showed that TLR4 signaling regulates the expression of Cyclooxygenase-2 (COX2) (Fukata et al., 2006), as well as the expression of inducible nitric oxide synthase (iNOS) resulting in the release of nitric oxide (NO) (Itsumi et al., 2016). We therefore tested whether Tnc might also affect microglial COX2 and iNOS expression via TLR4 signaling. Primary WT or Tlr4−/− microglia were treated with Tnc or LPS for 6 hrs and COX2 and iNOS expression levels determined by RT-qPCR (Fig. 4C). Tnc induced COX2 expression as potently as LPS in WT microglia (10459 ± 2647 fold and 7813 ± 1370 fold for Tnc and LPS, respectively; n = 4; p = 0.4090 comparing Tnc and LPS) while the Tnc induced increase was much lower in Tlr4−/− microglia (309 ± 33 fold n = 4; p = 0.0086 compared to WT). However, Tlr4−/− microglia showed a significant increase in COX2 expression after Tnc (p < 0.001), but not after LPS stimulation (p = 0.3645), compared to untreated controls. Expression of iNOS was induced by Tnc (9821 ± 2121 fold; n = 4; p = 0.0036 to PBS) and LPS (12396 ± 2027 fold; n = 4; p = 0.0009 to PBS) in WT microglia 6 hrs after stimulation, but this induction was abrogated in Tlr4−/− microglia (Tnc: 138 ± 101 fold; n = 4; p = 0.2661 to PBS; LPS: 2 ± 1 fold; n = 4; p = 0.3626 to PBS). In addition, we examined the NO release of WT and Tlr4−/− microglia 24 hrs after stimulation with Tnc or LPS using the Griess assay. NO release was induced in WT microglia after Tnc (12396 ± 2027 fold; n = 4; p = 0.0036 to PBS) and more potently after LPS stimulation (100 ng/ml; n = 4; p = 0.0009 comparing Tnc and LPS). Overall, these results suggest that HDAC1 regulates Tnc-induced cytokine synthesis in primary microglia.

To test whether the observed Tnc-induced release of TNF-α and IL-6 is dependent on microglial TLR4 expression, we stimulated primary Tlr4−/− microglia with Tnc or LPS for 24 hrs and measured TNF-α and IL-6 release (Fig. 4A). As also shown in Supplementary Fig. 4A, 55 nM Tnc induced a robust release of TNF-α and IL-6 in WT microglia compared to PBS (p = 0.0004 and p = 0.0001, respectively). This Tnc-induced stimulation was reduced in Tlr4−/− microglia (p = 0.0022 for TNF-α; p = 0.0001 for IL-6, WT vs. Tlr4−/−, respectively), providing strong evidence that Tnc-induced production of IL-6 and TNF-α is dependent on TLR4. Interestingly, Tnc (p = 0.0122), but not LPS (p = 0.9925), still induced a residual release of TNF-α from Tlr4−/− microglia relative to PBS, implying that Tnc acts through additional, TLR4-independent pathways.
3.7. Tnc stimulates chemotaxis and phagocytosis of primary microglia via TLR4

Chemotaxis towards sites of CNS injury is a hallmark of microglia physiology. Tnc promotes migration in several cell types (Cai et al., 2017; Sun et al., 2018; Zagzag et al., 2002), while TLR4 signaling was shown to augment chemotaxis in monocytes (Liu et al., 2013; Tajima et al., 2008). In order to determine whether Tnc regulates microglial chemotaxis via TLR4 signaling, we performed an agarose spot assay. WT or Tlr4^−/− microglia were seeded in dishes with control agarose spots containing PBS and agarose spots containing either Tnc (55 nM), LPS (100 ng/ml) or the TLR7 agonist Loxoribin (Lox, 1 mM) as a
Tnc regulates secretion of cytokines, COX2, iNOS and NO in primary cultured microglia via TLR4. A. Cytokine release of TNF-α and IL-6 by WT (red) or Tlr4−/− (black) primary microglia 24 hrs after treatment with Tnc (55 nM) or LPS (100 ng/ml) measured by ELISA (n = 4). For statistical analysis, WT or Tbr4−/− samples were tested using one-way ANOVA followed by Tukey’s multiple comparisons test; for statistical analysis between WT or Tbr4−/− samples for each condition, Student’s t-test was applied. B. On the left, representative images of cytokine and chemokine profiles of WT microglia after PBS, Tnc or LPS stimulation and Tnc stimulation in Tbr4−/− microglia. Microglia were treated for 24 hrs, and harvested supernatants were subjected to proteome profiling (Fig. 2B). These data provide further evidence that Tnc-induced Tbr4−/− microglia was not completely reduced to PBS control levels. Together, these findings indicate that TLR4 is not the only receptor by which Tnc controls microglial function. Indeed, Tnc is known to act through several integrin receptors (Tucker and Chiquet-Ehrismann, 2015; Yoshida et al., 2015), as well as the epidermal growth factor (EGF) receptor (Swindle et al., 2001). In this regard, the EGF receptor is highly expressed in the developing brain at E18.5 (The Allen Developing Mouse Brain Atlas), and has been previously linked to microglial migration and phagocytosis in a human neutrophil cell line (Li et al., 2014). Moreover, integrin-dependent phagocytosis has been described in microglia (Koenigsknecht and Landreth, 2004). Other receptors associated with microglial phagocytosis in development include complement receptor 3 (CR3) and Interleukin-1 receptor like-1 (IL1RL1) (Schafer et al., 2012; Vainchtein et al., 2018). Further studies will be required to identify the additional receptors utilized by Tnc to regulate microglial function.

Our findings on Tnc have relevance to CNS disease states, including inflammation, fibrosis and cancer (Faisser et al., 2017). Acting as the first line of immune defense, microglia are activated by pathologic events or disturbances in homeostasis, where they can orchestrate the immunological response in many neuropathological states (Wolf et al., 2017). While its expression in the adult, healthy brain is restricted to astrocytes within neurogenic niches, it is upregulated upon injury by reactive astrocytes (Brodky et al., 1995; Karus et al., 2011; Laywell et al., 1992; Steinbier et al., 1995). Moreover, stromal cells, leukocytes and cancer cells are also sources of Tnc in pathology (Claycomb et al., 2014; Dzaye et al., 2016; Goh et al., 2010; O’Connell et al., 2011; Xie et al., 2013). In the present study, we demonstrate Tnc regulation of microglial activity, suggesting that Tnc acts as a modulator of microglial function in CNS pathology (Faisser et al., 2017).

During CNS development, Tnc is transiently expressed by radial glia (Czopka et al., 2009; Garcia et al., 2004; Götz et al., 1998; Stoykova et al., 1997) as well as oligodendrocyte precursor cells (OPCs) (Czopka et al., 2009; Garwood et al., 2004). Cytokines including Tnf-α and IL-6 participate in cell genesis or support cell health, in addition to their ability to stimulate spine and synapse formation through IL-10 and PGE2 (Hagemeyer et al., 2017; Lenz et al., 2013; Lenz and Nelson, 2018; Lim et al., 2013; Parkhurst et al., 2013; Shigemoto-Mogami et al., 2014; Włodarczyk et al., 2017). The impact of Tnc on microglial cytokine release might therefore not only affect CNS neuropathobiology, but might also play a role in central nervous system development by modulation of microglial function. In the developing quail retina, Tnc has been reported to negatively regulate radial migration of microglia and to induce microglial ramification (Sánchez-López et al., 2004). However, the effects of Tnc on cell migration might be dependent on the spatial as well as the temporal context and Tnc has been previously

In order to confirm our findings on Tnc regulating microglial phagocytosis during early postnatal development as well as to further study its dependence on TLR4 in a simplified setup, we employed an in vitro phagocytosis assay. We incubated primary WT or Tbr4−/− microglia with fluorescent microbeads (YFP; white) along with simultaneous stimulation with purified mouse Tnc (55 nM) or LPS (100 ng/ml). LPS was used as a positive control since it has previously been shown to increase phagocytic activity in primary microglia (Kobayashi et al., 2016). After fixation, cells were stained for the microglia marker Iba1 (red) (Fig. 5B), and phagocytosed beads per cell were quantified to calculate the phagocytic index (see Materials and Methods 2.12). Phagocytic activity of WT microglia (19.0 ± 1.2, n = 7) was significantly increased after stimulation with Tnc (40.5 ± 7.1, n = 4, p = 0.0083) or LPS (35.9 ± 11.2, n = 4, p = 0.0339). Unstimulated Tbr4−/− microglia had a significantly lower phagocytic activity (10.8 ± 1.4, n = 3, p = 0.0063) than WT microglia. LPS did not alter the phagocytic activity of Tbr4−/− microglia (8.0 ± 1.2, n = 3, p = 0.3861), while there was still an increase in phagocytosis in Tnc-stimulated Tbr4−/− microglia (17.2 ± 1.5, n = 3, p = 0.0400) as previously seen in P4 Tbr4−/−/Tbp−/− microglia in situ (Fig. 2B). These data provide further evidence for Tnc regulation of microglial phagocytic activity as well as a role for TLR4 in mediating this process.

4. Discussion

In the present study, we demonstrate that Tnc is an important regulator of microglial function during postnatal development. Tnc also induces the production of pro-inflammatory cytokines and chemokines, including the inflammatory enzymes COX2 and iNOS, as well as increases chemotaxis and phagocytosis. Moreover, the impact of Tnc on these microglial functions was reduced in Tbr4−/− microglia, indicating that Tnc activates the TLR4 pathway in microglia, as previously reported for human macrophages (Midwood et al., 2009). In addition, we report that Tnc increases HDAC1 expression in microglia and that this induction is reduced in Tbr4−/− microglia, indicating that Tnc-induced TLR4 signaling in microglia requires HDAC1. As such, Tnc induction of the cytokines IL-6 and TNF-α was mediated by HDAC1, and was blocked by the HDAC inhibitor MS-275.

Tbr4−/− microglia treated with Tnc induce COX2 and iNOS expression, chemotaxis and phagocytosis. Similarly, HDAC1 expression of Tnc-stimulated Tbr4−/− microglia was not completely reduced to PBS control levels. Together, these findings indicate that TLR4 is not the only receptor by which Tnc controls microglial function. Indeed, Tnc is known to act through several integrin receptors (Tucker and Chiquet-Ehrismann, 2015; Yoshida et al., 2015), as well as the epidermal growth factor (EGF) receptor (Swindle et al., 2001). In this regard, the EGF receptor is highly expressed in the developing brain at E18.5 (The Allen Developing Mouse Brain Atlas), and has been previously linked to microglial migration and phagocytosis in a human neutrophil cell line (Li et al., 2014). Moreover, integrin-dependent phagocytosis has been described in microglia (Koenigsknecht and Landreth, 2004). Other receptors associated with microglial phagocytosis in development include complement receptor 3 (CR3) and Interleukin-1 receptor like-1 (IL1RL1) (Schafer et al., 2012; Vainchtein et al., 2018). Further studies will be required to identify the additional receptors utilized by Tnc to regulate microglial function.

Our findings on Tnc have relevance to CNS disease states, including inflammation, fibrosis and cancer (Faisser et al., 2017). Acting as the first line of immune defense, microglia are activated by pathologic events or disturbances in homeostasis, where they can orchestrate the immunological response in many neuropathological states (Wolf et al., 2017). While its expression in the adult, healthy brain is restricted to astrocytes within neurogenic niches, it is upregulated upon injury by reactive astrocytes (Brodky et al., 1995; Karus et al., 2011; Laywell et al., 1992; Steinbier et al., 1995). Moreover, stromal cells, leukocytes and cancer cells are also sources of Tnc in pathology (Claycomb et al., 2014; Dzaye et al., 2016; Goh et al., 2010; O’Connell et al., 2011; Xie et al., 2013). In the present study, we demonstrate Tnc regulation of microglial activity, suggesting that Tnc acts as a modulator of microglial function in CNS pathology (Faisser et al., 2017).
reported to inhibit, as well as stimulate, cell migration (Faisstner, 1997; Sánchez-López et al., 2004).

As an important developmental function of microglia, we examined phagocytosis in the presence and absence of Tnc. Microglial phagocytic activity in brain development has been observed between embryonic day 9.5 (E9.5) and P4 (Lenz and Nelson, 2018). Our data show that the phagocytic activity of microglia is compromised in Tnc−/− mice at P4, suggesting that Tnc is a regulator of this essential microglial property. Since Tnc expression and microglial phagocytosis peak at the same time during late embryonic development (E18.5; the Allen Developing Mouse Brain Atlas), one might speculate that the observed differences in phagocytic activity during early postnatal development might be even more pronounced at earlier stages (E18.5). Interestingly, abnormal behavior of Tnc−/− mice has been reported previously, including abnormal circadian rhythm, hyperlocomotion as well as poor swimming capacity (Mackie and Tucker, 1999). In vivo experiments testing the direct effect of Tnc−/− on microglial synaptic pruning capacity will be necessary to clarify whether abnormal behavior in Tnc−/− mice can be explained by disruption of this essential developmental process.

Microglial phagocytosis at P4 was also reduced in Tlr4−/− mice compared to P4 wild type controls, suggesting that TLR4 plays a role in regulating microglial phagocytic activity. However, in P4 Tnc−/− mice, microglial phagocytosis was reduced to the level of P28-P31 WT, indicating that the difference in phagocytosis activity between these two age ranges depends entirely on Tnc. In contrast, in Tlr4−/− mice at P4 the level of phagocytosis was still higher than at P28-P31 WT indicating the existence of an alternative Tnc-dependent, TLR4-independent pathway that controls microglial phagocytosis during early postnatal development.

We further show that Tnc controls HDAC1 expression in microglia during early postnatal development. Microglial HDAC1 expression levels were decreased in Tnc−/− mice at postnatal day 4 (P4) compared to P4 WT mice. The observed disparity in HDAC1 expression levels between WT and Tnc−/− mice at P4 was abrogated at day P28-P31 when physiological Tnc levels are low (Supplementary Fig. 2A; The Allen Developing Brain Atlas). Interestingly, P4 WT mice displayed a rather heterogeneous distribution of HDAC1 expression levels compared to P28-P31 WT mice, which was also observed in the case of P4 WT and Tlr4−/− mice. The heterogeneity in HDAC1 levels might be explained by a rather heterogeneous expression of Tnc in the developing CNS, since it is strictly regulated by its microenvironment including local growth factors, cytokines and mechanical stress (Imanaka-yoshida and Aoki, 2014). Our finding of Tnc controlling microglial HDAC1 during early postnatal development is particularly interesting in the light of HDAC1 and HDAC2 emerging as important control points in CNS development (Jaworska et al., 2015). In line with our findings is a recent publication showing that HDAC1 is highly enriched in microglia between mouse embryonic days E8.0 and E14.5 and subsequently downregulated to low levels in adult microglia, correlating with Tnc expression levels in the developing mouse brain (Datta et al., 2018). HDAC1 protein expression changes in microglia observed in the presence and absence of Tnc in early postnatal development were independent of TLR4 expression, further supporting the idea that Tnc might - in addition to TLR4 - act through alternative receptors to control microglial function as well as HDAC1 expression.

4.1. Conclusion

The ECM protein Tenascin C controls key functions of microglia, including cytokine/chemokine secretion, iNOS and COX2 expression, as well as chemotaxis and phagocytosis. TLR4 is an important, but not the only, receptor for Tnc signaling in microglia. Moreover, Tnc induces HDAC1 expression in primary microglia, and inhibition of HDAC1 reduces Tnc induced secretion of cytokines. During early postnatal development, Tnc controls microglial phagocytic activity and regulates HDAC1 expression in microglia independent of microglial TLR4 expression. Taken together, we show that Tnc is a novel regulator of microglial properties, which could be important during brain development and in the setting of CNS pathology.

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Author Contributions

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Declaration of Competing Interests

The authors declare no competing interests.
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