Versican promotes T helper 17 cytotoxic inflammation and impedes oligodendrocyte precursor cell remyelination

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Remyelination failure in multiple sclerosis (MS) contributes to progression of disability. The deficient repair results from neuroinflammation and deposition of inhibitors including chondroitin sulfate proteoglycans (CSPGs). Which CSPG member is repair-inhibitory or alters local inflammation to exacerbate injury is unknown. Here, we correlate high versican-V1 expression in MS lesions with deficient premyelinating oligodendrocytes, and highlight its selective upregulation amongst CSPG members in experimental autoimmune encephalomyelitis (EAE) lesions modeling MS. In culture, purified versican-V1 inhibits oligodendrocyte precursor cells (OPCs) and promotes T helper 17 (Th17) polarization. Versican-V1-exposed Th17 cells are particularly toxic to OPCs. In NG2CreER:MAPTmGFP mice illuminating newly formed GFP+ oligodendrocytes/myelin, difluorosamine (peracetylated,4,4-difluoro-N-acetylglucosamine) treatment from peak EAE reduces lesional versican-V1 and Th17 frequency, while enhancing GFP+ profiles. We suggest that lesion-elevated versican-V1 directly impedes OPCs while it indirectly inhibits remyelination through elevating local Th17 cytotoxic neuroinflammation. We propose CSPG-lowering drugs as potential dual pronged repair and immunomodulatory therapeutics for MS.
Multiple sclerosis (MS) is a chronic inflammatory disorder characterized by infiltration of T lymphocytes into the central nervous system (CNS) resulting in demyelination and axonal injury. Interferon (IFN)-γ-producing T helper (Th1) and interleukin (IL)–17-expressing Th17 CD4+T lymphocytes are considered to be key promoters of neuroinflammation and myelin damage, as are CD8+ T cells and B lymphocytes1–4. Remyelination as a spontaneous repair response occurs extensively in many patients at early stages of MS but this process is not always efficient. Remyelination failure contributes to the axonal loss and progression of disability5,6. The failed repair process could be the consequence of ongoing toxic neuroinflammation and/or presence of inhibitors of oligodendrocyte lineage cells in lesions7,8.

Various extracellular matrix (ECM) molecules including chondroitin sulfate proteoglycans (CSPGs) contribute to the inhibitory microenvironment of MS lesions8–10. An important subgroup of CSPGs is the lecticans that include 4 members: brevican, neurocan, aggrecan and versican. Versican itself has at least 4 isoforms. The lectican CSPGs are upregulated in MS lesions11,12 and have been described to directly inhibit the differentiation of oligodendrocyte precursor cells (OPCs) into oligodendrocytes and to prevent remyelination13–16. The injection of the enzyme chondroitinase-ABC into the aged CNS promotes both OPC proliferation and differentiation17. In vivo inhibition of CSPG synthesis, deposition or signaling enhances remyelination post-injury14,16,18,19. However, which lectican CSPG member is particularly important in lesions is not clarified.

In addition to suppressing OPC differentiation, a commercially available CSPG mixture containing several lectican CSPGs enhances macrophage migration and production of pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-612,20,21. Thus, CSPGs deposited in demyelinated lesions may provide the fuel that exacerbates inflammatory responses, but this remains to be demonstrated.

Amongst the lectican CSPGs, versican-V1 is profoundly elevated in lesions of lyssolecithin-induced demyelination and at sites of infiltrating leukocytes in experimental autoimmune encephalomyelitis (EAE) and MS12. Not addressed is whether versican-V1 directly impairs OPCs, and whether it promotes neuroinflammation particularly Th17 immune responses capable of mediating CNS injury in MS22,23. Also unknown is whether impairing versican-V1 synthesis enhances remyelination in the EAE model, which is a crucial test as EAE has persisting innate and adaptive neuroinflammation typical of MS.

In the current study, we have sought to address critical gaps of knowledge on CSPGs: whether versican-V1 is associated in lesions of MS with reduced remyelination capacity, whether versican-V1 is directly inhibitory to OPCs, and whether CSPGs and versican-V1 locally regulate T cell plasticity particularly of Th17 cells that may then impair OPC function. T cells can be reprogrammed according to environmental cues at lesion sites24,25 and it is not known if CSPGs could be such a lesional factor. Finally, we have employed the NG2CreER:MAPTmGFP mice that report on newly formed, premyelinating oligodendrocytes that are distinct from OPCs and mature oligodendrocytes, even though the majority of BCAS1+ cells (76%) express a mature oligodendrocyte marker (CC1) and 16% of them are positive for an OPC marker (NG2)28. The first case had 2 demyelinated lesions in the same section (Fig. 1c) with markedly divergent amounts of versican-V1. The low versican-V1-containing lesion had higher number of BCAS1+ cells than the high versican-V1-containing plaque that did not have detectable BCAS1+ cells (Fig. 1c). Another case had 2 lesions where the versican-V1 expression was not as divergent. Here, the lower versican-V1-expressing lesion (diffuse stain, light brown, Fig. 1d upper panel) had more BCAS1+ cells than the higher versican-V1 immunoreactive area (darker brown, Fig. 1d lower panel).

Next, versican-V1 level of lesion was qualitatively divided into low versus high expression based on relative difference to versican-V1 immunoreactivity in NAWM in the small section. The comparison across 6 MS autopsy cases with 19 regions of interest (fields of view) shows that the expression of versican-V1 is not dependent on the type of lesion, with high or low versican-V1 being spread across active, chronic active, or inactive white matter lesions (Fig. 1e). However, the number of BCAS1+ remyelinating oligodendrocytes is inversely related to versican-V1 content, with high versican-V1 areas generally containing a lower number of BCAS1+ cells (Fig. 1e).

We conducted spatial RNA sequencing (spRNAseq) (Jain et al., in preparation) of post-mortem MS brain tissue section which showed elevated levels of versican mRNA in the inactive demyelinated lesion, and to a lesser extent in the active core, compared to normal appearing white matter (NAWM) or to non-MS controls (Supplementary Fig. 2a, b). Expression level of BCAS1 appears inversely correlated with versican mRNA transcripts in MS lesions. The relatively low sensitivity of spRNAseq did not result in the detection of IL-17, RORγT or instructive T cell markers, and we were thus unable to describe relationship of versican to Th17 inflammation through this technique.

Previous work documenting that CSPGs are inhibitory for oligodendrocytes in culture have utilized either a mixed CSPG preparation or purified aggrecan14,16. Since versican-V1 is prominently elevated in MS lesions with low BCAS1+ premyelinating oligodendrocytes, we plated murine (Fig. 2a) and human (Fig. 2f) OPCs onto versican-V1 in culture. Both mixed CSPG substrate (containing aggrecan, versican, phosphacan and neurocan according to the manufacturer) and purified versican-V1 reduced the number of attached OPCs; of those adhered, process extension was reduced from the earliest point examined (6 h) (Fig. 2a–g).

While process outgrowth in culture is indicative of the potential of an oligodendrocyte to extend elaborate processes to contact and enwrap multiple axons during myelination in vivo,
the maturation of OPCs into myelin basic protein (MBP)+ cells is another important feature of remyelination. Thus, we addressed the proportion of O4-expressing cells that matured into MBP+ oligodendrocytes at 72 h after plating, and this was decreased significantly on CSPGs or versican-V1 substrates (Fig.2h–l). Considering the effect of negatively charged CSPG surface on cell adhesion and subsequently on process outgrowth, positively charged poly-arginine peptide was used to counteract the negative charges of CSPGs. Although the neutralized substrate improved cell adhesion, the inhibitory effect of CSPGs on process outgrowth was not overcome (Supplementary Fig. 3b, c). Moreover, increasing the number of attached cells by high initial seeding density did not enhance the process growth on CSPGs, indicating suppressive role of CSPGs regardless of cell density (Supplementary Fig. 3d, e).

Collectively, the results highlight versican-V1 in MS lesions that is inversely correlated with the presence of BCAS1+ premyelinating oligodendrocytes. Moreover, in vitro, a versican-V1 substratum impairs OPC adhesion, morphological differentiation and their maturation into MBP+ oligodendrocytes.

Elevation of versican-V1 and particular ECM proteins in EAE lesions. The autopsy snapshot of MS cases did not permit a time course study. We thus examined the ECM in spinal cord white matter lesions of EAE mice (Fig. 3a, b) harvested at onset of clinical signs (day 12), peak (day 18) and post peak (day 40) of clinical severity. Elevation of versican-V1 was noted at the onset and peak of EAE followed by reduced but still detectable level at post peak EAE (Fig. 3c). Using Imaris 3D rendering, versican-V1
was found colocalized to CD45+ immune cells although not all versican-V1-positive cells were CD45+ (Fig. 3d). Moreover, versican-V1 was abundant in presumed extracellular spaces within the lesion and also associated within CD45+ immune cells. Intracellular versican staining may reflect cellular production, as we previously found versican-V1 transcript in CD45+ cells using in situ hybridization12.

We evaluated tissue sections at the peak of EAE clinical disability (day 18) to address the relative elevation of versican-V1 and other ECM members (Fig. 3e–j). Sections were also stained for CD45 as a marker of immune cells to define the lesion area. The percent area occupied by ECM proteins within the CD45+ lesions was compared to staining in normal appearing white matter (NAWM). We found increased levels of versican-V1,
fibronectin, thrombospondin and heparan sulfate proteoglycan in lesions while versican-V2 and aggrecan were unaltered (Fig. 3k–q). Together, these observations show that specific ECM members are deposited in the EAE lesions.

CSPG mixture and purified versican-V1 shift T cell polarization toward Th17 cells in culture. The presence of versican-V1 in MS and EAE lesions invites the assessment of whether it promotes pro-inflammatory T cell polarization that is a feature of MS. We addressed this by isolating naïve CD4+ T cells from mice and culturing them on ECM-coated plates (10 µg/ml) with cytokines/antibodies to generate different Th phenotypes (IFNγ+ Th1, IL-17+ Th17, FoxP3+ Treg). Among Th1, Th17 and Treg subsets, the mixed CSPGs preferentially promoted Th17 polarization (Fig. 4a, b) and this was reproduced by 20 µg/ml purified versican-V1 (Fig. 4c).

We used the mixed CSPGs as a surrogate of versican-V1, given the limited supply of the latter. Compared to other ECM molecules (fibronectin, thrombospondin, heparan sulfate proteoglycans) or trending towards an increase (fibronectin) in EAE (see Fig. 3), only CSPGs elevated Th17 differentiation as determined by flow cytometry or ELISA (Fig. 4d, e). T cell activation, proliferation and viability showed no difference in CSPG-exposed T cells compared to control (Supplementary Figure 4a–f).

T cells can express different receptors interacting with CSPGs such as protein tyrosine phosphatase Sigma (PTPσ), leukocyte common antigen (LAR) and integrins. Blocking the signaling pathway of PTP and LAR using intracellular sigma peptide (ISP) and intracellular LAR peptide (ILP) could not reverse the CSPG increase of Th17 polarization. Indeed, blocking these signaling pathways elevated the frequency of Th17 cells (Fig. 4f) which is in agreement with a previous study. We also used anti-integrin β1, β3, and β6 antibodies and determined that anti-integrin β3 neutralized the promoting effect of CSPGs on Th17 polarization (Fig. 4g).

Altogether, these results highlight the role of versican-V1 and CSPGs in Th17 differentiation, putatively through interaction with integrin β3 on T cells.

An inhibitor of CSPG production, difluorosamine, reduces Th17 cells in the spinal cord of EAE mice. We previously described difluorosamine to reduce the synthesis of CSPG in cultured cells; difluorosamine also lowered EAE severity in mice although levels of CSPGs in vivo were not determined. To address if difluorosamine impacts Th17 representation in EAE, we initiated its daily treatment for five days from three days post onset of EAE clinical signs. Despite our best efforts at randomizing mice into two groups at the beginning of their manifestation of clinical signs, mice appeared to be slightly divergent between the two groups although this was not statistically different (see Fig. 7 for another, longer term, experiment). This short-term treatment and prompt tissue harvest allowed us to study CNS T cell populations during peak clinical severity. Flow cytometry was then used to evaluate the composition of T cell subsets in the spinal cord and lymph nodes. The short period of treatment with difluorosamine reduced EAE severity marginally, which was associated with decreased CD4+ T cell frequency in the spinal cord (Fig. 5a–c). Among the CD4+ T cell population in the spinal cord, IL-17+ cells showed a decrease in treated mice while IFN-γ+ Th1 or FOXP3+ regulatory T cells (Treg) remained unaffected. There was no significant change in IL-17-producing CD8+ T cell (Fig. 5d). In contrast to the spinal cord, the frequency of different Th subsets in lymph nodes was not altered, ruling out a peripheral effect of difluorosamine on T cells that led to the spinal cord outcome. We excluded a direct effect of difluorosamine on Th17 polarization as noted in vitro (Supplementary Figure 4g).

These results link a potential inhibitor of injury-enhanced CSPG synthesis to locally decrease Th17 population in EAE, correspondent with less severe clinical disease.

Versican-V1-exposed Th17 cells are more toxic to myelinating oligodendrocytes. The increase of versican-V1 in MS and EAE lesions that locally elevates Th17 polarization raises the possibility that Th17 cellular activity could further be regulated by the CSPG microenvironment. Specifically, we addressed whether versican-V1-exposed Th17 cells could elevate OPC-killing activity to help account for the inverse relationship between premyelinating oligodendrocytes and versican-V1 in MS lesions (Fig. 1). 2D2 myelin oligodendrocyte glycoprotein (MOG)-reactive T cells were activated with MOG-loaded dendritic cells and differentiated to Th17 subset in the presence or absence of CSPGs (10 µg/ml) or versican-V1 (20 µg/ml) for 4 days. Th17 cells were then added to OPCs (Fig. 6a). Live imaging of calcine AM-labeled OPCs shows that few cells incorporate propidium iodide (PI), a small molecule that crosses the membrane of compromised cells, when exposed to soluble CSPGs (Fig. 6b). However, in the presence of Th17 cells, OPCs began to show PI incorporation from 2 h, signifying Th17 toxicity which became more apparent in the Th17 cells that were generated during CSPG or versican-V1 exposure (Fig. 6b, c). The proportion of PI-labeled OPCs was increased significantly following co-culture with CSPG- or versican-V1-exposed Th17 cells for 12 h (Fig. 6c).

In another experiment, the number of O4+ or O4+MBP+ mature oligodendrocytes (Fig. 6d) incubated with Th17 cells, or Th17 generated during CSPG and versican-V1 exposure, were enumerated. Figure 6e shows that CSPG- or versican-V1-exposed Th17 cells reduced O4+ and O4+MBP+ oligodendrocytes...
compared to Th17 cells alone. Process extension by oligodendrocytes was also markedly lowered by CSPG-Th17 cells (Fig. 6f), but not by Th17 lymphocytes generated during exposure to other ECM molecules.

We noted that the CSPG-enhanced Th17 toxicity was not non-specific to any T cell subset since CSPG-exposed Tregs did not overtly affect OPCs or oligodendrocytes (Supplementary Fig. 5). As well, we ruled out an effect of CSPGs on the inflammatory profile of MOG-loaded dendritic cells (see schematic in Fig. 6a) by culturing both immature and mature dendritic cells in control or CSPG-coated plates. In this context, the frequency, maturation state and cytokine secretion of dendritic cells remained unaffected following CSPG versus control exposure (Supplementary Fig. 6).
Overall, these results highlight that versican-V1 can modify Th17 cells during their polarization, resulting in the Th17 cells being more harmful to oligodendrocyte lineage cells.

CSPG-lowering drug improves presumed remyelination in EAE associated with decreased Th17 inflammation. It has been difficult to study remyelination in EAE as the lesions appear at unpredictable location and because of the uncertainty of whether the oligodendrocytes within lesions are spared or remyelinating oligodendrocytes. This challenge has been overcome by the use of tamoxifen-inducible NG2-CreER:MAPTmGFP mice where membrane-associated GFP expression localizes to newly formed oligodendrocytes and myelin (Fig. 7a, b).

EAE was induced in NG2-CreER:MAPTmGFP mice and spinal cords were isolated from tamoxifen injected control and EAE mice at peak (day 18) and post-peak of clinical severity (day 40); we refer to ‘peak’ as the period of pronounced clinical disability. To evaluate presumed remyelination during the course of EAE, immunofluorescence staining was performed on longitudinal tissue sections to allow evaluation of the entire thoracic spinal cord. All GFP+ cells were immunoreactive for Olig2+; a transcription factor specific to oligodendrocyte lineage cells (Fig. 7b). Few profiles of GFP+ positivity were evident at day 18 sample that has high accumulation of CD45+ cells but this increased in day 40 samples with reduced CD45+ cells (Fig. 7c, d). GFP expression was not detected in EAE mice without tamoxifen injection indicating that the Cre recombinase was not leaky (Supplementary Fig. 7a).

To help with subsequent EAE analyses, we tested the NG2-CreER:MAPTmGFP mice further using demyelination induced by the toxin lysolecithin (LPC, lysophosphatidylcholine), which provides discrete phases of de- and remyelination16,32 and therefore has been commonly used to study myelin repair. Indeed, in the NG2-CreER:MAPTmGFP mice with lyssolecithin demyelination of the ventral white matter of the spinal cord, GFP expression was progressively increased from 7 to 21 days after demyelination, correlating with myelin repair (Supplementary Figure 7c–f). These findings corroborate the NG2-CreER:-MAPTmGFP mice as a suitable model to study new oligodendrocytes and presumed remyelination in EAE.

Next, we tested difluorosamine in EAE. Difluorosamine was administered to NG2-CreER:MAPTmGFP mice daily starting from three days post onset of EAE clinical signs to exclude the effect of drug on immune cell infiltration. Mice were randomized into two groups according to their clinical scores to ensure similar starting clinical scores at the initiation of treatment. Figure 7e shows that difluorosamine progressively lowered disease severity compared to saline vehicle control.

At day 26, histological analyses of the spinal cord were performed. Given that treatment was started from peak clinical severity of EAE where substantial injury had already occurred, difluorosamine did not impact the extent of demyelination and axonal degeneration (Supplementary Figure 8a–c). Difluorosamine treated mice showed a non-significant increasing trend in CD45+ immune cells (Fig. 7f, i), no change to the number of Iba1+ microglia/macrophages and CD3+ T cells (Fig. 7g, j; Supplementary Figure 8d, e), while the number of CD4+ T cells was reduced (Fig. 7h, k).

Considering the increasing trend in number of CD45+ immune cells, we resorted to PCR analyses to compare selected phenotypic markers of microglia/macrophages and T cells. The expression of pro-inflammatory (IL-17, IL-1α) and pro-oxidative (iNOS) transcripts in lumbar cord was significantly reduced in difluorosamine-treated EAE mice whereas IL-10 mRNA level was elevated (Fig. 7j–s). These results indicate a shift in the balance of inflammatory and regulatory cells in difluorosamine treated mice.

We found that difluorosamine-treatment resulted in substantially higher area of the entire thoracic spinal cord white matter occupied by GFP+ myelinating oligodendrocytes and presumed new myelin sheaths (Fig. 8a–c). When we confined analysis to the well-delineated EAE lesions in thoracic cord, the increase in GFP+ processes by difluorosamine treatment was also noted (Fig. 8d). GFP+ oligodendrocytes in difluorosamine-treated mice had features of actively remyelinating cells with several processes suggestive of alignment along axons, while many GFP+ cells in control mice displayed short and irregularly arranged processes (Fig. 8e, f). In addition to increased number of oligodendrocyte lineage cells (Olig2+) and mature oligodendrocyte (olig2+CC1+), difluorosamine-treatment resulted in higher number of GFP+ mature oligodendrocytes within the lesion (Fig. 8g–j).

Moreover, in these lesional regions of interest, the immunoreactivity for total CSPGs and also versican-V1 was lowered by difluorosamine (Fig. 8k–m). Indeed, there was a significant negative correlation between GFP expression and CSPG immunoreactivity in lesional regions of interest across all mice (Fig. 8n). A slight effect of difluorosamine on reducing the levels of HSPGs has been shown previously by our group27; however, no change was found in the present study (Supplementary Fig. 8f, g).

These results highlight that by lowering CSPG content within lesions, difluorosamine reduced Th17 neuroinflammation and promoted GFP+ profiles indicative of remyelination in EAE.

**Discussion**

MS is a chronic and progressive inflammatory neurological disorder in which myelin sheaths and axons in the CNS are damaged by several types of immune cells including Th17 cells4. Findings from EAE studies have highlighted that Th17 cells can be reprogrammed at the site of inflammation1,24,25. However, local environmental cues and molecular mechanisms underlying T cell plasticity and perturbed T cell polarization at lesions have yet to be elucidated. Here, we provide evidence that the lectican CSPGs deposited in MS lesions11,12, and specifically versican-V1, have
this capacity as shown by increased Th17 generation in culture when CD4⁺ T cells are polarized in the presence of a mixed CSPG preparation and purified versican-V1. These findings are in accordance with a previous study, in which the chondroitin sulfate-A chain treatment before the onset of clinical signs exacerbated EAE severity accompanied by elevation of IL-17 in splenocytes. However, the direct effect of CSPGs on Th17 differentiation and whether it could change the balance of T cell subsets in the CNS remained unclear. We have now addressed these questions herein. When we employed difluorosamine that we have previously shown to reduce the cellular production of CSPGs in culture, and which we corroborate herein to diminish versican-V1 in the spinal cord of EAE mice (Fig. 8), the level of IL-17 expressing CD4⁺ cells was lowered locally in the spinal
Fig. 4 CSPGs and its prominent member, versican-V1, promote Th17 differentiation and IL-17 production. To assess the effect of ECM molecules on T cells, naive CD4+ T cells were cultured on ECM-coated wells (10 μg/ml) and were then activated and polarized to Th1, Th17 or Treg subset. The frequency of IFN-γ, IL-17 or FOXP3 expressing T cells was determined using flow cytometry 4 days later. a Representative histograms showing the frequency of IFN-γ, IL-17 or FOXP3 immunopositive T cells in control (green) or following CSPG treatment (red); isotype antibody control (blue) is also displayed. b The fold change of Th1 (71.7% Vs & 72.13%; n = 10 replicates), Th17 (18.64% Vs 23.61%; n = 11 replicates) and Treg (21.34% Vs 21.98%; n = 9 replicates) frequencies is shown as a bar graph across 3 experiments. Data are presented as mean ± SEM, two-way ANOVA - Bonferroni post hoc; ***p < 0.001. c, d Bar graph presenting the percentage of IL-17+ RORγT+ Th cells resulting from a commercial mixed CSPG preparation (10 μg/ml) compared to purified versican-V1 (20 μg/ml) (Control:9.6%, CSPG:12.31%, Versican-V1:13.53%) c or other ECM molecules (10 μg/ml) d, n = 9 replicates over three separate experiments. e Level of IL-17 in the conditioned medium of Th17 polarized cells measured by ELISA (Control: 1948 pg/ml, CSPG: 2800 pg/ml; n = 9 replicates over three separate experiments. f Frequency of Th17 cells after exposing cultures to signaling pathway inhibitors of CSPG receptors (PTPα and LAR) using intercellular peptide (ISP and ILP, respectively, 2.5 μM), n = 11 replicates for control and CSPGs, n = 9 replicates for other conditions, over three separate experiments. g Frequency of Th17 cells after exposing cultures to function blocking antibodies to the integrin β1, β3, or β6 (50 μg/ml); n = 9 replicates over three separate experiments. Data presented as mean ± SEM. One-way ANOVA - Bonferroni post hoc; “p = 0.01, ***p < 0.001. Source data are provided in the source data file.

While remyelination occurs in MS, the repair process is not efficient likely due to ongoing inflammation or inhibitors in lesions that prevent the recruitment and/or differentiation of OPCs. Overcoming inhibitors present within lesions has been proposed as a potential therapeutic approach for MS. We have implicated CSPGs as inhibitors of the differentiation of OPCs and remyelination, but the crucial role of versican-V1 on OPCs could not be addressed because of the previous unavailability of purified versican-V1 protein. We have rectified this herein, and demonstrate that purified versican-V1 in culture inhibits the adhesion, morphological differentiation and myelin production of OPCs. In MS lesions, high versican-V1-expressing regions do not have abundance of BCAS1+ cells which tend to reside in areas with low V1 content (Fig. 1). BCAS1 appears transiently in oligodendrocytes just before they myelinate axons. Our data are in line with a previous study showing lower levels of versican in the gray matter compared to the white matter region of leukocortical MS lesions, which was correlated with better remyelination in the gray matter region; the versican isoform was not defined in that study.

We note that while the mixed CSPG preparation is very inhibitory for OPC process outgrowth at 2.5 μg/ml, much higher concentration of purified versican-V1 (20 μg/ml) is required (Fig. 2). A possible reason for this is that the mixed CSPGs contain a variety of lecitanic CSPGs (aggrecan, brevican, neurocan and versican, according to the manufacturer) which may act in concert to inhibit OPCs. Moreover, aggrecan has extensive glycosaminoglycan chains, far exceeding that of versican, and the additional negative charges should more effectively impair adhesion since the attachment of OPCs onto CSPGs is neutralized by the positively charged poly-arginine peptide (Supplementary Fig. 3b, c).

Various T cell subsets impact remyelination differently. Regulatory T cells (Tregs) enhance remyelination whereas Th17 cells prevent myelin reformation. In vivo transfer of myelin-reactive Th17 cells reduces endogenous remyelination in a toxin-induced demyelination mouse model. A recent study shows the direct contact between CNS-infiltrating Th17 cells and oligodendrocytes in EAE and MS lesions results in oligodendrocyte death and impaired remyelination through release of glutamate. IL-17 from Th17 cells induces NOTCH1 (Notch homolog 1, translocation-associated) signaling in OPCs, resulting in apoptosis and reduced differentiation. We corroborate the toxicity of Th17 cells on OPCs, and further demonstrate that the exposure of Th17 cells to versican-V1 during their differentiation elevates their capacity to kill OPCs. Thus, the lesion content of versican-V1 not only affect Th17 polarization and OPC differentiation, but they endow the Th17 cells with greater capacity to kill OPCs. These results emphasize the importance of overcoming versican-V1 in lesions. The mechanisms underlying increased toxicity of versican-V1-exposed Th17 remain to be investigated in future studies.

Several means to reduce lesional CSPGs have been employed, and a common one is the local injection of the enzyme chondroitinase-ABC to remove the glycosaminoglycan chains of CSPGs. However, this leaves the core protein intact, with uncertain impact on immune and oligodendroglial cells, and the local injection would not be applicable to a multifocal lesion condition such as MS. We screened several glucosamine analogs and described difluorosamine as the most potent in reducing CSPG production by cells in culture. To test the overall therapeutic effects of difluorosamine, including on myelin formation, the EAE model with attendant adaptive and innate immunity, and histological hallmarks of MS would be important. Although the demonstration of thinner myelin sheaths by electron microscopy has become the gold standard for identifying sites of remyelination in localized toxin-induced demyelination models, it does not provide a ready means to study remyelination in EAE due to the uncertain location of lesions; thus, the microscale area of analysis in electron microscopy could easily miss a repairing lesion or under-report on remyelination across the spinal cord. Also, studies on myelin reformation in EAE have been hampered by the difficulty of assessing whether oligodendrocytes and myelin found in a lesion are spared or regenerated. The availability of the NG2CreER,MAPTmGFP mice has resolved these challenges, as newly formed oligodendrocytes from NG2 precursors prominently elevate microtubule associated protein tau (MAPT) which is required for process outgrowth and the transport of myelin basic protein mRNA to distal processes to form myelin. Thus, the GFP signal driven by the tau promoter in oligodendrocytes informs on newly formed oligodendrocytes and nascent myelin, as we validated in the lyssolecithin lesion with defined de- and remyelination phases (Fig. S7).

Using the tamoxifen-treated NG2CreER,MAPTmGFP mice induced for EAE, we found that mice administered difluorosamine had reduced EAE severity correspondent with lower versican-V1 content and frequency of Th17 cells in spinal cords, and more newly formed oligodendrocytes with elaborate processes (Figs. 7 and 8). While the GFP signal is associated with nascent myelin, we did not conduct electron microscopy analysis to definitively establish remyelination in the fluorosamine-treated mice. Thus, we have referred to the extensive GFP+ profiles as 'presumed' remyelination.
The association between T cell subsets and levels of versican-V1 within lesions may be studied further using RNAscope that provides better sensitivity and specificity (relative to spatial RNA sequencing) to measure low-abundance RNA biomarkers such as IL-17 and RORγT. In addition to T cells, the changes in innate immune responses and how they impact remyelination have yet to be explored thoroughly in the context of versican biology. Our future studies using versican conditional knock out mice would help to clarify the specific effect of versican rather than total CSPGs on immune cells during remyelination.

**Fig. 5** Difluorosamine reduces Th17 population in the CNS during EAE. 

a) A CSPG-lowering drug, difluorosamine (DIF), was administered at 25 mg/kg to mice daily starting three days post onset of EAE for 5 days. Data are presented as mean ± SEM of n = 10 mice per group pooled from 2 independent experiments. Results were analyzed using two-way repeated-measures ANOVA with Sidak’s post-hoc test; *p = 0.02. Average EAE clinical score of mice is shown with treatment period indicated by green box. 

b) The frequency of different T cell subsets was determined using flow cytometry. 

c) Bar graphs showing the frequency of CD3⁺, CD4⁺ and CD8⁺ cells; and d IFN-γ⁺, IL-17⁺ or FOXP3⁺ T cells within the CD4⁺ or CD8⁺ populations in the spinal cord and lymph nodes of DIF or vehicle treated mice. Data are presented as mean ± SEM. n = 4 mice per group for c, d, and results were reproduced in a second experiment (source data file). Two-tailed unpaired Student’s t test; **p = 0.003 for c, and *p = 0.025 or **p = 0.0017 for d. Source data are provided in the source data file.
We note that while versican-V1 appears to be the predominant lectican CSPG in EAE lesions, we did not evaluate if it is the sole or predominant CSPG in MS lesions. Other lectican CSPGs may play similar roles in MS as versican-V1, and this remains to be investigated in future studies.

In conclusion, we provide evidence for the role of CSPGs and versican-V1 in Th17 cell polarization including in EAE lesions. We found that mixed CSPGs and versican-V1 have multiple actions that would retard remyelination in MS, including directly inhibiting OPC differentiation, augmenting Th17 neuroinflammation, and elevating the propensity of Th17 cells to kill OPCs. Thus, the CSPG-reducing drug, difluorosamine, ameliorated clinical severity in EAE associated with lower Th17 neuroinflammation while enhancing oligodendrogenesis and remyelination.
presumed remyelination. We propose CSPG-lowering drugs as potential dual pronged repair therapeutics that directly affect OPCs, and that indirectly antagonize Th17 roles in neuroinflammation and oligodendrocyte injury.

Methods

All experiments were performed with ethics approval (protocol number AC21-0174) from the Animal Care Committee at the University of Calgary under regulations of the Canadian Council of Animal Care.

MS specimens. Postmortem frozen brain tissues from people with MS and healthy control brain tissue were obtained from The Multiple Sclerosis and Parkinson’s Tissue Bank at Imperial College, London (https://www.imperial.ac.uk/medicine/multiple-sclerosis-and-parkinsons-tissue-bank). This bank has been approved as a Research Tissue Bank by the Wales Research Ethics Committee (Ref. No. 18/WA/0238). Secondary progressive MS tissues used for spRNAseq were obtained from CRCHUM and University of Montreal research ethics committee. Paraffin-embedded sections from autopsied MS subjects were purchased from the Netherlands Brain Bank (https://www.brainbank.nl/). All samples at their local sites were collected with full informed consent for autopsy, and their use for research has been approved by local institutional ethics committee. The use of these human tissues in Calgary for research was approved by the Conjoint Health Research Ethics Board at the University of Calgary (Ethics ID REB15-0444).

MS sections were characterized using Lului fast blue (LFB) and Hematoxylin & Eosin (H&E) to identify lesions. In addition, a combination of CD45, MBP, and pan-neurofilament (NF) staining was used to determine the activity of lesions. The Amsterdam cases (Fig. 1c–e) were prosectioned in Holland for the presence or absence of BCAS1. To ascertain whether a given lesion has high or low versican immunoreactivity, we have compared the lesional staining intensity to the intensity of the normal appearing white matter within that tissue section. This avoids differences in staining intensity across slides, or the background stain in one slide versus another.

Mice. Female C57Bl/6 mice (6–8 weeks old) and litters from pregnant CD1 mice were obtained from Charles River and used for in vitro leukocyte or OPC cultures, respectively. N2G-CreER (JAX 088538) mice and Tau-MOG (JAX 021162) mice aged 6 to 8 weeks were acquired from Jackson Laboratories and bred in University of Calgary Animal facility to produce female N2G-CreER:MAPTmGFP mice; the 2D2 TCR (TCRMOG) transgenic mice (JAX 006912) were also from Jackson Laboratories. Mice were housed between 21 and 23 degrees Celsius, in low humidity, with 12 h light and 12 h dark cycle from 7 am light and starting 7 pm dark.

For EAE experiments or lyssolecithin demyelination, the N2G-CreER:MAPTmGFP mice at 8–10 weeks were injected intraperitoneally with 2 mg tamoxifen (100 mg/ml in corn oil). For RNA extraction, spinal cords were immediately fixed overnight in 4% PFA or Periodate-Lysine-Fluor-Formaldehyde (PLF) and RNA extraction, respectively. For FACS experiments, the whole spinal cord was used.

For lyssolecithin injection, mice were randomized into two groups of mice on day of MOG35–55 immunization. Intraperitoneal treatment with either lyssolecithin (25 mg/kg, dissolved in saline) or vehicle (saline) was started at the peak of disability and continued once a day until sacrifice.

All drug administration and EAE clinical scoring and analyses were done blinded to treatment. All procedures were performed according to the Canadian Council of Animal Care guidelines.

Lyssolecithin-induced demyelination. Lyssolecithin was injected into the ventral spinal cord to induce experimental demyelination24. Mice were anaesthetized with isoflurane and xylazine (100 mg/kg and 10 mg/kg, respectively) administered intraperitoneally. The analgesic buprenorphine (0.05 mg/kg) was injected subcutaneously prior to surgery and 12 h post-surgery. A 3 cm incision was made between the shoulder blades, and fat and muscle were separated using retractors. Connective tissue between the T3–T4 vertebrae were blunt disected to expose the dorsal spinal cord and the meninges were removed using a 30-gauge metal needle.

To induce focal demyelination in the ventrolateral white matter of the spinal cord, 0.5 ml of 1% lyssolecithin (LPC) (Sigma L1381) was injected at a rate of 0.25 μl/min over 2 min. A 10 μl Hamilton 34-gauge needle was inserted 1.3 mm into spinal cord to induce the injury in the ventrolateral side. Mice were then sutured and monitored until recovery. Mice were sacrificed at 7, 14, and 21 days post-injection of lyssolecithin with a lethal dose of ketamine/xylazine. Animals were then perfused with 10 ml of PBS and 10 ml of 4% paraformaldehyde (PFA). The lower cervical and upper thoracic section of the spinal cord was dissected.

Spinal cord tissue harvest. For RNA extraction, spinal cords were immediately flash frozen in liquid nitrogen and stored at −80 °C. For immunohistochemistry, spinal cords were fixed overnight in 4% PFA or Periodate-Lysine-Parafomaldehyde (PLP) at 4 °C, then transferred into 30% sucrose solution for 72 h. Tissues were then frozen in FSC 22 Frozen Section Media (Leica). Using a cryostat (ThermoFisher Scientific), spinal cord blocks were cut coronally or longitudinally into 20 μm sections, collected on to Superfrost Plus microscope slides (VWR) and stored at −20 °C prior to staining.

Immunofluorescence staining. Slides were thawed at room temperature for thirty min, then hydrated with PBS for five min. Blocking of tissue sections was performed using horse serum blocking solution (0.01 M PBS, 10% horse serum, 1% bovine serum albumin (BSA), 0.1% cold fish skin gelatin, 0.1% Triton-X100, and 0.05% Tween-20) for 1 h at room temperature. Slides were then incubated with diluted primary antibodies overnight at 4 °C. Next, slides were washed three times, 5 min each with PBS containing 0.25% Tween-20 and then incubated with fluorophore conjugated secondary antibodies (1:400) and 1 μg/ml of DAPI suspended in one site into each hind flank. On the day of immunization and 48 h later, animals received intraperitoneal injections of 300 ng of pertussis toxin (List Biological Laboratories).

Clinical signs of EAE were evaluated daily using a 0 to 15 point scoring scale44. For the tail, 0 signifies no signs, a score of 1 represents a half-paralyzed tail and a score of 2 reflects a fully paralyzed tail. For each of the hind- or forelimbs, a score of 1 is given to a mouse with a weak or altered gait, 2 represents paresis, while a score of 3 reflects a fully paralyzed limb. Mortality corresponds to a score of 15. Spinal cord tissues were dissected from EAE mice at three time points following MOG induction: onset (first day of the appearance of clinical signs, Day 12 post-immunization), at the peak of clinical severity (Day 18 post-immunization), and a late phase referred here as post-peak (Day 40 post-immunization). Mice were euthanized with ketamine (100 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally and then perfused with PBS through the left ventricle of the heart. Following PBS-perfusion, lumbar and thoracic spinal cord were dissected for immunohistochemistry and RNA extraction, respectively. For FACS experiments, the whole spinal cord was used.
the antibody dilution buffer for 1 h at room temperature. Antibodies were diluted in 0.01 M PBS containing 1% BSA, 0.1% cold fish skin gelatin, and 0.5% Triton-X100. The slides were then washed three more times and then mounted using Fluoromount G (SouthernBiotech). A sample slide stained with only the secondary antibodies and DAPI was used for each experiment as a control for non-specific secondary immunofluorescence.

For MBP staining, sections were also delipidated by sequential wash with 50%, 70%, 90%, 95%, 100%, 95%, 90%, 70%, and 50% ethanol. Then, samples were rehydrated with PBS for 10 min, and permeabilized with 0.2% Triton-X100 in PBS for 10 min. To remove the GAG chains so as to facilitate the binding of antibody to the core protein of CSPG members (versicans and aggrecan) and also to the stub chondroitin-4-sulfate, chondroitinase ABC (ChABC, Sigma) digestion was done before the blocking step. Slides were incubated with ChABC diluted in PBS (0.2 U/mL) at 37 °C for 30 min.

The following primary antibodies were used for immunofluorescence microscopy to identify specific targets: versican V0/V1 (Millipore, ab1033),}

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Fig. 7 Improved clinical EAE score following difluorosamine treatment in NG2CreER:MAPTmGFP mice is associated with the altered balance of immune cell subsets. A NG2CreER:MAPTmGFP mice were used where GFP expression denotes newly formed oligodendrocytes and myelin. B Representative images of new myelinating oligodendrocytes stained with GFP (green) and olig2 (red). C, D Representative images of longitudinal sections comparing control and peak (Day 18 post-inoculation) or post peak (Day 40) clinical severity of EAE. Stains were GFP (green), MBP for myelin sheaths (yellow) and CD45 (red). Dotted white lines indicate the lesion areas magnified in D. Similar results were noted in a separate experiment. E NG2CreER:MAPTmGFP mice were injected with difluorosamine (DIF, 25 mg/kg) starting three days post clinical onset of EAE, for 15 days (treatment period shown by the green box), and average EAE daily clinical score (mean ± SEM) is shown. n = 8 mice in each group from 2 independent experiments of 4 mice. Two-way repeated-measures ANOVA with Sidak’s post-hoc test: p = 0.03, ***p < 0.001. F Bar graphs (mean ± SEM) of number of CD45+ cells in the white matter or lesion ROI. Data are from one representative experiment of 4 mice in each group, where each dot represents one mouse where a mean of 10 lesions were analyzed per mouse. One-tailed unpaired Student’s t-test: p = 0.04. This data was reproduced in a second experiment (n = 4 mice). I-S mRNA expression (mean ± SEM) analyses of lumbar spinal cords comparing the levels of IL-17, IL-10, IL-17/IL-10 ratio, IL-12, arginase-1 (Arg1), inducible nitric oxide synthase (iNOS) and IL-1β between DIF and vehicle treated EAE mice. Data pooled from two independent experiments, n = 8 mice, two-tailed unpaired Student’s t-test: p = 0.01, ***p < 0.001. Source data are provided in the source data file.
with the Space Ranger software v.1.2, which uses STAR v.2.5.1 for genome alignment, against the GRCh38 human reference dataset. The count files generated for each library were then aggregated with normalization set to ‘Mapped’. The aggregated cloupe file was visualized in Loupe Browser. Spatial gene expression data from human MS tissue was visualized using the Loupe Browser 5.01 (10X Genomics). H&E stains were used to define areas of white matter and exclude gray matter in healthy control tissues. In the MS samples H&E was used to define the areas of the inactive core, active rim, and NAWM then the UMAP of the samples were used to define the exact boundaries. Heatmaps of DEGs between the Control NAWM, MS NAWM, active rim, and inactive core were generated for genes of interest.

Cell culture
Mouse oligodendrocyte precursor cells (OPC). Brains from postnatal day P0-2 mouse pups were isolated and meninges and choroid plexus tissue were removed.
Fig. 8 Diffusorpose reduces versican-V1 expression and promotes presumed remyelination in EAE. Longitudinal sections of the thoracic spinal cord from EAE NGZ2-cre:MAPTmGFP mice following DIF or vehicle treatment were analyzed. a Examples of slide scanner images showing lesions defined by CD45 + accumulation; the dotted rectangles are correspondant with high magnitude images in b acquired by confocal microscopy (Z-stack). c Bar graph (n = 4 mice per group) comparing the extent of GFP + in the white matter (WM), expressed as % of GFP immunoreactivity of the whole thoracic section. d Bar graph of GFP + in region of interest (ROI), where each ROI is a lesion defined by area occupied by CD45 + cells (n = 30 ROIs from 4 mice per group). e GFP - cells. f Bar graph comparing average particle size of GFP in each CD45 + lesion ROI (n = 41 ROIs from 4 mice per group). h Representative immunofluorescent images (z-stack) labeled for GFP (green), olig2 (white) and CC1 (red). g. i J Bar graphs comparing the number of olig2 +, mature oligodendrocytes (olig2+CC1+) and GFP + oligodendrocytes in the white matter as defined in c (n = 4 mice). k Representative images (z-stack) labeled with GFP (green), versican-V1 (red) and stub chondroitin-4-sulfate (gray). l, m Quantification of the percent area occupied by immunoreactive CSPGs or versican-V1 in lesional CD45 + ROI (n = 4 mice). Data presented as mean ± SEM from one experiment, n = 4 mice in each group, each dot represents one mouse where a mean of 8 lesions were analyzed per mouse, two-tailed unpaired Student’s t test. This data was reproduced in a second experiment. n Correlation analysis was performed between percent of GFP + and CSPGs + in the lesion ROI. Each dot represents one ROI from 4 mice per group. Pearson r -0.39; *p = 0.029. Source data are provided in the source data file.

using a digestion microscope. Cortices were removed and dissociated with a digestion of 

a cocktail containing papain (1.54 mg/ml, Worthington), DNase (60 μg/ml, Sigma), and L-cysteine (360 μg/ml, Sigma) in a 37 °C water bath for 30 min.

Following centrifugation at 300 g for 10 min, cells were resuspended in growth medium and plated in T-75 culture flasks pre-coated with poly-L-lysin (100 μg/ml, Sigma). The mixed glial culture was incubated in DMEM (Gibco) containing 10 mM N-acetyl-L-cysteine (Sigma), 50 ng/mL ciliary neurotrophic factor (PeProTech), 20 μg/ml Biotin (Sigma) and 0.01% (v/v) Trace Elements B (Fisher Scientific). The medium was switched to the same medium but lacking PDGF. Cultures were over 75% enriched as monitored by O4 marker. The use of the surgical material for the current study was approved by The Conjoint Health Research Ethics Board at the University of Calgary.

CD4 + T cell isolation and polarization. Naive CD4 + T Cells were isolated from single-cell suspensions of splenocytes using the EasySep Kit (STEMCELL) by negative selection. Unwanted cells were removed with biotinylated antibodies directed against non-naïve CD45 + CD4 + cells (CD8, CD11b, CD11c, CD19, CD24, CD28, CD3, CD45R, CD48, TCRyδ, TER119) and streptavidin-coated magnetic particles. Labeled cells were separated using an EasySep magnet (STEMCELL) without the use of columns. Purified cells were seeded in 24 well plates at a density of 1 × 106 cells in 1 ml of RPMI 1640 medium (Gibco) supplemented with 10% FBS (Gibco), 1% Glutamax, 1% sodium pyruvate, and 1% Penicillin-Streptomycin (all from Gibco) at 37 °C, 85% CO2 for 3 days. Medium was switched to the same medium but lacking PDGF. Cultures were over 75% enriched as monitored by O4 marker. The use of the surgical material for the current study was approved by The Conjoint Health Research Ethics Board at the University of Calgary.

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The correlation analysis (Pearson) of the levels of 2 different markers (GFP and CSPGs). Kolmogorov-Smirnov test was performed between two groups, significance was determined by two-tailed Student's t-tests. A p value smaller than 0.05 was considered statistically significant.

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Author contributions

S.G. designed the project, performed the majority of experiments, analyzed the results, and wrote the first draft of the paper. E.J. performed some of the oligodendrocyte culture studies. R.J. contributed SpRNAseq data. B.B. and C.L. helped with data analyses. B.L. performed lysolecithin surgeries. S.S. stained M.S. samples for BCAS and versican-V1. D.K.K. provided data of MS brain tissues. Y.D. helped with 2D T cell cultures. G.J.S., E.M.S., and J.G. characterized and provided BCAS-positive MS brain tissues. S.K. contributed to the blocking peptide experiments. T.W. purified and provided versican-V1. P.Z. and C.C.L. designed and synthesized difluorosamine. V.W.Y. supervised the study, provided operational support, and edited and finalized the manuscript. All authors reviewed and edited the manuscript.

Competing interests

P.Z., C.C.L. and VWY have a patent application (USA Patent application number 17/059,318) pending on the use of difluorosamine in multiple sclerosis. Other authors declare no competing interests.

Additional information

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