NEURAL REGENERATION RESEARCH
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Polysialic acid-Siglec immune checkpoints of microglia and macrophages: Perspectives for therapeutic intervention

Hauke Thiesler, Herbert Hildebrandt*

Microglia are the resident macrophages of the central nervous system. They act as the first line of defense against pathogens and play essential roles in neuroinflammation and tissue repair after brain insult or in neurodegenerative and demyelinating diseases (Borst et al., 2021). Together with infiltrating monocyte-derived macrophages, microglia also play a critical role for brain tumor development, since immunosuppressive interactions between tumor cells and tumor-associated microglia and macrophages (TAM) are linked to malignant progression. This mechanism is of particular relevance in glioblastoma (GB), the deadliest form of brain cancer with a median overall survival of less than 15 months (Khan et al., 2023). Therefore, targeting microglia and macrophage activation is a promising strategy for therapeutic interference in brain disease.

In vertebrates, the glycan polymer polysialic acid (polySia) is long-known as a posttranslational modification of the neural cell adhesion molecule NCAM and as a prominent regulator of cell interactions during nervous system development (Thiesler et al., 2022). PolySia on NCAM is down-regulated during postnatal maturation of the brain and maintained only in regions of ongoing neurogenesis and synaptic plasticity. In several tumors, however, polySia-NCAM is re-expressed and has been linked to metastatic and invasive growth (Thiesler et al., 2022).

More recently, it has been shown that microglia and macrophages can produce polySia on two other protein carriers, neuropilin-2 and E-selectin ligand 1 (a.k.a. GLG1) (Werneburg et al., 2016). As elucidated in mice, inflammatory or injury-induced activation of microglia initiates the production of these polySia-presenting proteins, which first accumulate at the site of polySia biosynthesis in the Golgi compartment, before they translocate to the cell surface, where they are released by ectodomain shedding. Searching for possible functions of this release of polysialylated proteins by activated microglia it could be demonstrated that polySia, attached to these proteins or independent of its protein carrier, causes feedback inhibition of the inflammatory response by trans-activation of the inhibitory immune receptor Siglec-E (Werneburg et al., 2016; Thiesler et al., 2021). Siglec-E belongs to the CD33-related subgroup of sialic acid-binding immunoglobulin-like lectins (Siglecs). Their history of rapid evolution with five members in mice, but ten in humans makes it difficult to attribute functional equivalents (Macauley et al., 2014). Siglec-E binds to a range of sialylated glycans and Siglec-7 and Siglec-9 have been discussed as its human orthologues. PolySia-induced inhibition of microglia and macrophages, however, is mediated by human Siglec-11 (Karlstetter et al., 2017).

Like most of the CD33-related Siglecs, murine Siglec-E and human Siglec-11 possess an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their cytoplasmic domain, but some Siglecs lack the ITIM domain and instead possess a positively charged amino acid in their transmembrane region enabling interaction with the adaptor protein DAP12 (a.k.a. TYROBP) containing an ITAM. ITAM activation by Siglecs and numerous other immune receptors leads to the recruitment of signaling intermediates such as the spleen tyrosine kinase Syk, which is a regulatory target of ITIM signaling via tyrosine phosphatases such as the Src homology region 2 domain-containing tyrosine phosphatases SHP-1 and SHP-2 (Macauley et al., 2014). One of the ITAM-associating Siglecs is the human Siglec-16, a so-called paired receptor related to Siglec-11, because the two receptors show a high sequence similarity in the extracellular domain and recognize the same ligand, polySia, but lead to opposing signaling responses (Schwarz et al., 2017). In humans, Siglec-11 and Siglec-16 are exclusively expressed by macrophages and microglia and can occur in the same cell. As assumed for other paired receptors of the immune system, Siglec-16 may have evolved as activating counterpart to rebalance the Siglec-11-mediated inhibition by polySia-presenting pathogens, such as the neuroinvasive *E. coli* K1 or *Neisseria meningitides*, which both feature a polysialic acid capsular polysaccharide to escape the immune response by molecular mimicry (Schwarz et al., 2017). Strikingly, however, only about 40% of the human population have a *SIGLEC16* gene encoding the functional receptor, while roughly 60% carry an inactive pseudogene, *SIGLEC16P*, in which the open reading frame is disrupted by a four-nucleotide deletion (Schwarz et al., 2017).

As discussed in more detail elsewhere (Gretenkort et al., 2023), the only approach so far to assess the role of Siglec-11-mediated inhibition of neuroinflammation *in vivo* was performed by polySia application to a mouse model expressing human Siglec-11 in mononuclear phagocytes (Karlstetter et al., 2017), and until recently, virtually nothing was known about the possible relevance of polySia interacting with human Siglec-16 or murine Siglec-E for any neurological condition. Seeking proof of concept for the putative impact of the polySia-Siglec-16 axis, we took advantage of the disparate distribution of *SIGLEC16 versus SIGLEC16P* in the overall population on the one hand and the heterogeneity of polySia-NCAM in GB on the other. A retrospective study with a total of 170 tumor specimens from two cohorts of patients with GB revealed that about 84% of the tumor specimens were positive for polySia-NCAM on the tumor cells with a heterogeneous distribution within tumors (Thiesler et al., 2023). Genotyping indicated the expected allelic frequency and an approximately 40% penetrance of *SIGLEC16*, but not a single patient was homozygous for functional *SIGLEC16*, which significantly deviates from the 4.4% of individuals with this genotype in the overall population. Because the *SIGLEC16* status was independent of the presence or absence of polySia-NCAM on the tumor cells, and also independent from established prognostic factors for GB such as age, sex, extent of tumor resection, adjuvant chemotherapy, and MGMT promotor methylation, the two patient cohorts could be stratified for *SIGLEC16* and polySia-NCAM independently, and combined. Both the presence of a functional *SIGLEC16* allele and polySia-NCAM were linked to increased overall survival (OS) and the survival benefit was most pronounced in the group of *SIGLEC16*-carriers that were positive for polySia-NCAM on the tumor cells (**Figure 1A**). Moreover, among the 170 patients, the tumors of eight out of nine long-term survivors characterized by an OS of at least 36 months were *SIGLEC16* and polySia-NCAM double-positive. This disproportionately high number underscores the survival benefit of *SIGLEC16* and polySia double-positive GB patients. Together, these findings provide strong evidence for the prognostic relevance of the polySia-Siglec-16 axis.

Increased markers of pro- and reduced markers of anti-inflammatory TAM were associated with increased survival, and tumors with these features were significantly overrepresented in the group of *SIGLEC16* carriers with a polySia-positive GB. Elevated expression of tumor necrosis factor (TNF), a hallmark of inflammatory TAM activation, corroborated the shift towards a proinflammatory M1-like TAM profile in specifically this group of patients (**Figure 1B**). However, independent of the occurrence of polySia on tumor cells, perinuclear polySia signals were detected in a small population of TAM, resembling the transient accumulations of polysialylated proteins in the Golgi compartment of cultured and injury-induced microglia (Werneburg et al., 2016; Thiesler et al., 2021). Hence, polysialylated proteins might be released by TAM, raising the question if this might account for the moderate increase of TNF observed in tumors that were negative for polySia-NCAM on tumor cells but positive for Siglec-16 on TAM (**Figure 1B**). Furthermore, consistent with the recruitment of CD8 T cells by M1-like TAM, the small population of infiltrating T cells detected around blood vessels displayed increased ratios of CD8 T cells in specifically the tumors of *SIGLEC16*-positive patients with polySia-NCAM-positive tumor cells.

Two related cellular models were used to corroborate that the phenotypic shift of TAM depends on Siglec-16 activation by polySia-NCAM on tumor cells. Heterotypic spheroid cultures consisting of polySia-positive GB cells were interspersed with monocytes derived from either *SIGLEC16*-positive or negative blood donors or pure monocyte cultures were incubated with GB cell-conditioned medium. In both models, monocytes differentiated into TNF-producing macrophages (monocyte-derived macrophages, Md-MΦ) and, corresponding to their genotype, stained negative or positive for Siglec-16. As in heterotypic spheroids with Siglec-16-positive macrophages, the Md-MΦ with Siglec-16 produced higher levels of TNF when exposed to GB cell membrane fractions (GB-mf, **Figure 1C**). Further profiling revealed activating immune signaling in Siglec-16-positive and GB-mf stimulated Md-MΦ and an elevated release of cytokines and related factors that was largely compatible with the secretion profile of M1-like polarized macrophages.

Taken together, these data from the study by Thiesler et al. 2023 support a working model, in which polySia-NCAM on tumor cells activates Siglec-16 signaling in TAM to counteract the anti-inflammatory impact imposed by Siglec-11 as one out of the multiple factors, by which GB cells "educate" TAM to shape an immunosuppressive, pro-tumorigenic microenvironment (**Figure 1D**). Accordingly, the polySia-Siglec-16 axis leads to a "re-education" of TAM towards a proinflammatory, M1-like anti-tumorigenic phenotype, providing a mechanistic explanation for the better survival of the *SIGLEC16* and polySia-NCAM double-positive GB patients. In a broader context, overcoming the immunosuppressive tumor microenvironment is reminiscent of the strategy of immune checkpoint management and Siglec-16 can be considered a costimulatory immune checkpoint receptor. This raises hope that targeting the polySia-Siglec-16 axis or pharmaceutical activation of Siglec-16 signaling will help to improve the so far disappointing results of immunotherapy in the treatment of GB. Furthermore, the concept of TAM modulation by the polysialic acid-Siglec-16 axis may not only apply to glioblastoma, but also other polysialic acid-positive brain and peripheral tumors such astrocytoma, medulloblastoma, neuroblastoma, or small cell and non-small cell lung carcinomas, to name just a few (Thiesler et al. 2022).

As implicated by the working model outlined above, the Siglec-E mediated, exclusively inhibitory response of microglia towards polySia in mice mimics the situation in the majority of the human population lacking Siglec-16. In this context, another recent progress concerns the possible use of soluble, protein carrier-free polySia with defined degrees of polymerization (DP) in the targeted management of microglia polarization and the resolution of inflammation via engagement of Siglec-E. In contrast to a preferred binding of Siglec-E-Fc chimera to short oligomers with two or three sialic acid units on a glycan array, the Siglec-E-dependent attenuation of proinflammatory microglia activation could be pinpointed, and a DP of 24 was determined as the critical minimal chain length for efficient inhibition of an inflammatory microglia response (Schröder et al., 2023).

The potential of soluble polySia for Siglec-E-dependent immunomodulation in the mouse model was explored in the context of myelin repair. In the autoimmune disease multiple sclerosis (MS) the degradation of myelin in the central nervous system leads to axonal damage. Spontaneous remyelination can occur but the repair is often incomplete. Activating the intrinsic remyelination capacity has been identified as the key aspect of recovery and is considered the most promising therapeutic avenue (Franklin and Simons, 2022). Microglia and infiltrating monocyte-derived macrophages crucially contribute to de- and remyelination. During demyelination, activated, proinflammatory microglia engage in phagocytosis of myelin debris, whereas anti-inflammatory polarization seems beneficial for myelin repair.

Based on the inhibition of inflammatory microglia by the polySia-Siglec-E axis *in vitro*, the impact of soluble polySia on microglia activity and its consequences for myelin repair was studied after lysophosphatidylcholine-induced demyelination in the organotypic cerebellar slice culture (OSC) model (Schröder et al., 2023; **Figure 2A** and **B**). Application of a polySia pool with DPs between 24 and 30 (DP24–30), i.e., meeting the critical chain length for Siglec-E dependent microglia inhibition, efficiently improved remyelination in OSCs obtained from wildtype mice (*Siglece*$^{+/+}$). Importantly, this beneficial effect could not be achieved by polySia with DP8-14, or by application

of DP24–30 to OSCs from Siglec E deficient mice (*Siglece*$^{-/-}$). Furthermore, reduced production of nitric oxide and increased ratios of arginase-1-positive microglia were exclusively observed in *Siglece*$^{+/+}$ OSCs treated with DP24–30. Nitric oxide is a hallmark of inflammatory microglia activation, whereas arginase-1 opposes nitric oxide production and is a marker of anti-inflammatory, protective microglia. The data therefore indicate that the polySia-Siglec-E axis supports remyelination by attenuating inflammatory microglia activation, which promotes the polarization toward an anti-inflammatory, regeneration-supporting phenotype (**Figure 2C**). Together, the data of this study suggest that the application of polySia DP24–30 or activation of downstream signaling of the human inhibitory polySia receptor Siglec-11 could be a therapeutic strategy to improve myelin repair in demyelinating diseases.

In conclusion, the data so far indicate a relationship between polySia polymer size and effector functions and highlight the implications of polySia-Siglec interactions as novel innate immune checkpoints for microglia and macrophage regulation. Even though further studies will be needed to uncover in detail the regulatory mechanisms of the polySia-Siglec axes, this concept might be applicable not only in the context of myelin repair and glioblastoma progression, but may have much broader relevance for the challenging fields of cancer prognosis and therapy, neuroinflammation and neurodegenerative disease.

We apologize to all authors for papers omitted due to space limitations.

Deutsche Forschungsgemeinschaft (DFG, German Research Foundation), project numbers 324633948 and 409784463 (DFG grants Hi 678/9-3 and Hi 678/10-2, FOR2953) to HH, Bundesministerium für Bildung und Forschung – BMBF, project number 16LW0463K to HT.

HT and HH have a patent on "Polysialic acid and derivatives thereof, pharmaceutical composition and method of producing polysialic acid," WO2020025653A3, issued.

Hauke Thiesler, Herbert Hildebrandt*
Institute of Clinical Biochemistry, Hannover Medical School and Center for Systems Neuroscience Hannover, Hannover, Germany
***Correspondence to:** Herbert Hildebrandt, PhD, Hildebrandt.herbert@mh-hannover.de.
https://orcid.org/0000-0002-1044-0881 (Herbert Hildebrandt)

Date of submission: October 7, 2024
Date of decision: November 16, 2024
Date of acceptance: November 26, 2024
Date of web publication: December 16, 2024

https://doi.org/10.4103/NRR.NRR-D-24-01195
How to cite this article: *Thiesler H, Hildebrandt H (2026) Polysialic acid-Siglec immune checkpoints of microglia and macrophages: Perspectives for therapeutic intervention. Neural Regen Res 21(2):661-662.*

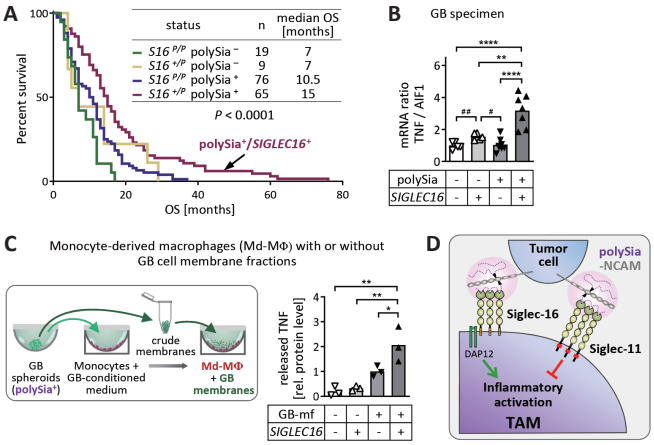

Figure 1 | **The polySia-Siglec-16 axis in glioblastoma is linked to increased survival and a shift towards a proinflammatory TAM profile.**
(A) Kaplan-Meier survival plot with log-rank test result and median overall survival (OS) of GB cases stratified for *SIGLEC16* genotype and polySia on tumor cells. (B) Ratios of TNF relative to AIF1 mRNA levels in GB specimen stratified for polySia and *SIGLEC16* status. (C) Left: Schematic of monocyte-to-macrophage differentiation by conditioned medium from GB cell spheroids and stimulation of monocyte-derived macrophages, (Md-MΦ) with crude membrane fractions from polySia-positive GB cells. Right: Relative amounts of TNF released into the supernatant of *SIGLEC16*-positive or -negative Md-MΦ stimulated with or without polySia-positive GB cell membrane fractions (GB-mf). (D) Simplified working model of the proposed regulation of TAM by interactions of polySia-NCAM on tumor cells with either Siglec-16, linked to activation signaling through the immunoreceptor tyrosine-based activation motif (ITAM) adaptor DAP12, or with Siglec-11, linked to inhibitory signaling by its immunoreceptor tyrosine-based inhibitory motif (ITIM) domains (red squares). Asterisks in B and C, indicate significant group differences by Tukey *post hoc* tests following two-way analysis of variance (for statistical assessment of data in B, square root transformation was performed to meet the assumption of equal variances). Hashes in B indicate significant differences by two-tailed *t*-tests ($^{*,\#}P < 0.05$, $^{**,\#\#}P < 0.01$, $^{****}P < 0.0001$). Graphs in A, B, and C were reprinted with permission from Thiesler et al. (2023). AIF1: Allograft inflammatory factor 1, a commonly used TAM marker gene; GB: glioblastoma; NCAM: neural cell adhesion molecule; TAM: tumor-associated microglia and macrophages; TNF: tumor necrosis factor.

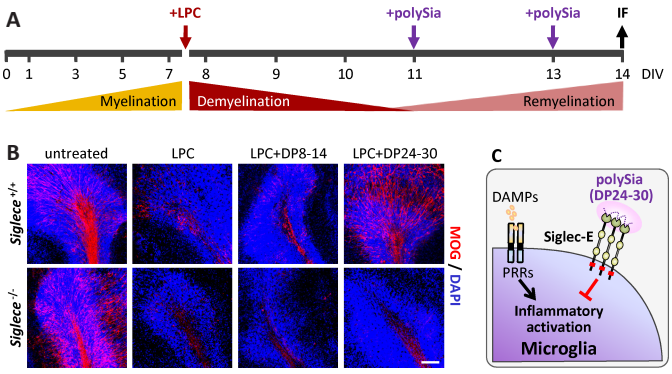

Figure 2 | **The polySia-Siglec-E axis promotes myelin repair by counteracting inflammatory microglia activation.**
(A) Experimental timeline. Organotypic cerebellar slice culture (OSCs) from 9 to 11 days old Siglec-E wildtype and knockout mice (*Siglece*$^{+/+}$, *Siglece*$^{-/-}$) were maintained for 7 days to complete developmental myelination before demyelination was induced by lysophosphatidylcholine (LPC; red arrow). To assess effects on remyelination, polySia with DP8–14 or DP24–30 was added at 11 and 13 days *in vitro* (DIV; purple arrows) and OSCs were analyzed by immunofluorescence staining (IF) at 14DIV. (B) Representative images of myelin staining of *Siglece*$^{+/+}$ and *Siglece*$^{-/-}$ OSCs after the indicated treatments. IF with myelin oligodendrocyte glycoprotein (MOG)-specific antibodies and nuclear counterstain with DAPI. Scale bar: 100 µm. (C) Simplified working model of the proposed anti-inflammatory impact of polySia DP24–30 interacting with Siglec-E to counteract proinflammatory activation of microglia by damage-associated molecular pattern (DAMPs)-induced signaling of pattern-recognition receptors (PRRs). Although direct evidence is lacking, Siglec-E clustering by polySia is proposed, because only long polySia chains can induce Siglec-E-dependent inhibition (see text), and high-avidity interactions of Siglecs usually require clustering (see Gretenkort et al., 2023 for detailed discussion). Panels A and B are reprinted with permission from Schröder et al. (2023). DAPI: 4′,6-Diamidino-2-phenylindole; DP: degrees of polymerization.

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C-Editors: Zhao M, Liu WJ, Qiu Y; T-Editor: Jia Y

小胶质细胞和巨噬细胞的多聚唾液酸-Siglec 免疫检查点：治疗干预的展望

一、文章重要性

1. 揭示新型免疫检查点机制：
   - 提出 polySia–Siglec 通路是小胶质细胞/巨噬细胞的新型先天免疫检查点，类似于 T 细胞中的 PD-1/PD-L1，具有广泛的免疫调节潜力。
2. 连接神经发育、肿瘤免疫与神经再生：
   - 将原本在神经发育中研究较多的 polySia，拓展至神经肿瘤、神经炎症与髓鞘修复等多个领域，体现了糖生物学与神经免疫的交叉融合。
3. 提供临床预后标志与治疗新靶点：
   - 在胶质母细胞瘤中，polySia-NCAM + SIGLEC16+ 患者生存期显著延长，提示该轴可作为预后标志；
   - 在脱髓鞘疾病中，外源性 polySia 可促进髓鞘修复，具有治疗潜力。

二、文章创新性特色

1. 提出“polySia–Siglec 轴”为新型免疫调节通路：
   - 不仅限于传统的 NCAM 介导的细胞黏附，更强调其作为可溶性免疫调节信号，通过 Siglec 受体调控炎症方向。
2. 揭示人类特异性受体配对机制：
   - Siglec-11（抑制性）与 Siglec-16（激活性）在人类中形成配对受体，且 Siglec-16 存在基因多态性，这为人群差异性免疫反应提供了解释。
3. 从基础机制到临床关联的转化研究：
   - 结合了：
     - 细胞与动物模型（如小胶质细胞激活、髓鞘修复）；
     - 人类肿瘤样本回顾性分析；
     - 基因型-表型关联研究。
4. 提出“糖链长度依赖性”功能差异：
   - 强调 polySia 链长（如 DP24–30）对其功能的影响，提示糖链结构特异性在治疗设计中至关重要。

三、对学科的启示

1. 拓展神经免疫学研究范式：
   - 将糖生物学引入神经免疫调控机制，提示糖链-受体相互作用是神经炎症、肿瘤微环境等领域的重要研究方向。
2. 为神经肿瘤免疫治疗提供新思路：
   - 胶质瘤微环境中 TAM 的极化状态影响肿瘤进展，polySia–Siglec 轴可作为重塑肿瘤免疫微环境的靶点。
3. 推动个体化医疗与生物标志物开发：
   - Siglec 基因多态性与 polySia 表达状态可作为患者分层工具，指导精准免疫干预。
4. 促进神经再生治疗策略创新：
   - 在脱髓鞘疾病中，通过调控小胶质细胞极化状态促进修复，为神经再生药物开发提供新靶点。

总结

该文章系统阐述了 polySia–Siglec 轴作为小胶质细胞和巨噬细胞的新型免疫检查点，在神经肿瘤、神经炎症与髓鞘修复中的关键作用。其创新性在于将糖生物学与神经免疫、肿瘤微环境、再生医学紧密结合，不仅深化了对神经免疫调控机制的理解，也为开发新型免疫治疗策略提供了理论依据与实验支持。该研究方向具有显著的跨学科特色与临床转化潜力，对未来神经科学、免疫学与肿瘤学的发展具有重要启示意义。