Neuroscience

A human anti-polysialic acid antibody as a potential treatment to improve function in multiple sclerosis patients

Jens O. Watzlawik[1], Meghan M. Painter[2], Bharath Wootla[1], and Moses Rodriguez[1,*]

[1]Department of Neurology, [2]Mayo Graduate School, Clinic College of Medicine, Rochester, Minnesota, USA

We previously identified a human monoclonal antibody, termed HIgM12 that stimulates spontaneous locomotor activity in a chronically demyelinating mouse model of multiple sclerosis. When tested as a molecular substrate, HIgM12 stimulated neurite outgrowth *in vitro*. We recently reported that polysialic acid (PSA) attached to the neural cell adhesion molecule (NCAM) is one of the cellular antigens for HIgM12. Fluorescent double-labeling of astrocytes using HIgM12 and commercially available anti-PSA antibody showed dramatic co-localization. Neural tissue homogenates and primary CNS cultures from mice lacking the three major NCAM splice variants NCAM180, NCAM140 and NCAM120 (NCAM KO) were no longer able to bind HIgM12. Furthermore, enzymatic digestion of PSA on wild type (WT) glia abolished HIgM12-binding. Moreover, neurons and glia from NCAM KO animals did not attach to HIgM12-coated nitrocellulose in neurite outgrowth assays. We conclude that HIgM12 targets PSA attached to NCAM, and that the PSA moiety mediates neuronal and glial adhesion and subsequent neurite outgrowth in our *in vitro* assay. Therefore, this anti-PSA antibody may serve as a future therapeutic to stimulate functional improvement in multiple sclerosis patients and other neurodegenerative diseases. *Journal of Nature and Science, 1(8):e141, 2015*

polysialic acid | multiple sclerosis | antibody | motor function

Our group has identified human antibodies from patients with Waldenstrom's macroglobulinemia that possess therapeutic potential for multiple sclerosis (MS) and other neurodegenerative diseases (1, 2). Candidate antibodies were preselected for cell-surface-binding to oligodendrocyte-lineage cells or neurons. This cohort was subsequently tested for therapeutic effects in the Theiler's murine encephalomyelitis virus (TMEV)-induced chronic demyelination and the lysolecithin mouse model of MS (2). A few of the antibodies that targeted mature and immature oligodendrocyte-lineage cells were capable of stimulating substantial remyelination in demyelinated spinal cord lesions. One of these antibodies, termed rHIgM22, is currently in clinical trials for MS patients (3, 4). Another human antibody that bound to CNS cells, termed HIgM12, did not affect remyelination. However, this antibody was still able to stimulate spontaneous nocturnal activity in TMEV-induced chronically demyelinated mice over a period of eight weeks following a single intraperitoneal injection (5). Furthermore, when used as a molecular substrate *in vitro*, HIgM12 stimulated significant neurite extension (6). It is clear that both HIgM22 and HIgM12 possess protective, neuromodulatory capabilities, albeit by different mechanisms. In order to investigate the mechanism by which HIgM12 exerts neuroprotection *in vitro* and *in vivo,* we first sought out to determine the cellular antigen responsible for mediating functional effects.

Recently, we identified polysialic acid (PSA) attached to the neural cell adhesion molecule (NCAM) as the cellular antigen for HIgM12 (7). Within the CNS, NCAM is the major (> 95%) polysialylated molecule with long, negatively charged α2'-8'-linked sialic acid homopolymers (n > 10 sialic acid residues). PSA is abundant in the developing brain and its early expression is tightly linked to critical developmental events including neuronal precursor migration (neuroblasts), axonal sprouting and oligodendrocyte progenitor proliferation. PSA is down regulated during postnatal development (8, 9), and in the adult brain, its expression is restricted to those regions undergoing self-renewal or exhibiting plasticity (such as the SVZ, suprachiasmatic nucleus, hippocampus, hypothalamus, and specific spinal cord nuclei) (10-15). While the precise mechanism of PSA-mediated molecular effects is still under investigation, PSA is thought to act as a negative regulator of NCAM function (16-19).

We initially discovered (PSA)-NCAM as a potential candidate antigen for HIgM12 using immunoprecipitation assays of adult murine CNS tissue with subsequent mass spectroscopic analysis. The interaction between HIgM12 and NCAM was further confirmed by Western blot analysis of immunoprecipitated proteins using two different NCAM-specific antibodies. Corroborative data that NCAM is the sole protein antigen was shown in Western blots using brain lysates from mice lacking the three major CNS NCAM splice variants NCAM180, 140 and 120 (NCAM KO) (20, 21); when compared to wild type (WT) littermate controls, HIgM12 prominently targeted protein(s) present in WT but not in NCAM KO tissue lysates. Furthermore, via immunocytochemistry, we demonstrated that HIgM12 bound to cerebellar granular cells and glia from WT mice but not to NCAM KO littermates.

Next, we sought to identify the specific epitope for HIgM12-binding on NCAM. The highly antigenic character of (bulky) carbohydrates attached to the NCAM protein core made it a likely target for this human antibody. Fluorescent cell surface labeling of live mixed glial cultures using HIgM12 and anti-PSA antibody (clone 2-2B) was performed on ice to prevent endocytosis and demonstrated dramatic overlap between both antibodies (Fig. 1). Enzymatic digestion of PSA in CNS lysates using endo-neuraminidases abolished HIgM12- and anti-PSA binding in Western blots. However, anti-NCAM (protein core) binding was unaffected. These data support the hypothesis that PSA, but not the NCAM protein core, serves as the molecular epitope for HIgM12 binding. *In vitro,* a subset of glial fibrillary acidic protein (GFAP)-positive astrocytes express PSA-NCAM (7), and this moiety can be cleaved in cell culture through enzymatic digestion. For this set of experiments, endoneuraminidase-NF was added to mixed glial cultures which resulted in abrogation of HIgM12 binding (Fig. 2).

Here we further characterized the cell type specificity of HIgM12 under live conditions (on ice) to mouse mixed glial cultures containing astrocytes, oligodendrocyte-lineage cells and microglia (Fig. 3). Markers A2B5 and NG2 (chondroitin sulfate proteoglycan) were used to identify oligodendrocyte progenitor cells (OPCs), the ionized calcium-binding adapter molecule 1 (IBA-1) was used for microglial cells, and glial fibrillary acid protein (GFAP) for reactive astrocytes. Confirming our previous observations in mouse and rat glia and neurons (7) we show binding of HIgM12 to a subset of A2B5-positive oligodendrocyte-lineage cells and to a subset of GFAP-positive reactive astrocytes (Fig. 1, 3). Little or no overlap could be detected between HIgM12 and NG2-positive cells or between HIgM12 and IBA-1-positive microglia. Interestingly, a substantial proportion of HIgM12-positive cells did not co-label with GFAP, IBA-1, A2B5 or NG2 (Fig. 3). Many of these HIgM12-positive cells revealed a wide-spread morphology combined with large DAPI-positive nuclei typical for cultured astrocytes (see also Fig. 1). Despite its use as a prototypical marker for the identification of reactive astrocytes, GFAP is not an absolute marker of all non-reactive astrocytes and is often not immunohistochemically detectable in astrocytes in

Conflict of interest: No conflicts declared.
* Corresponding Author. Email: rodriguez.moses@mayo.edu

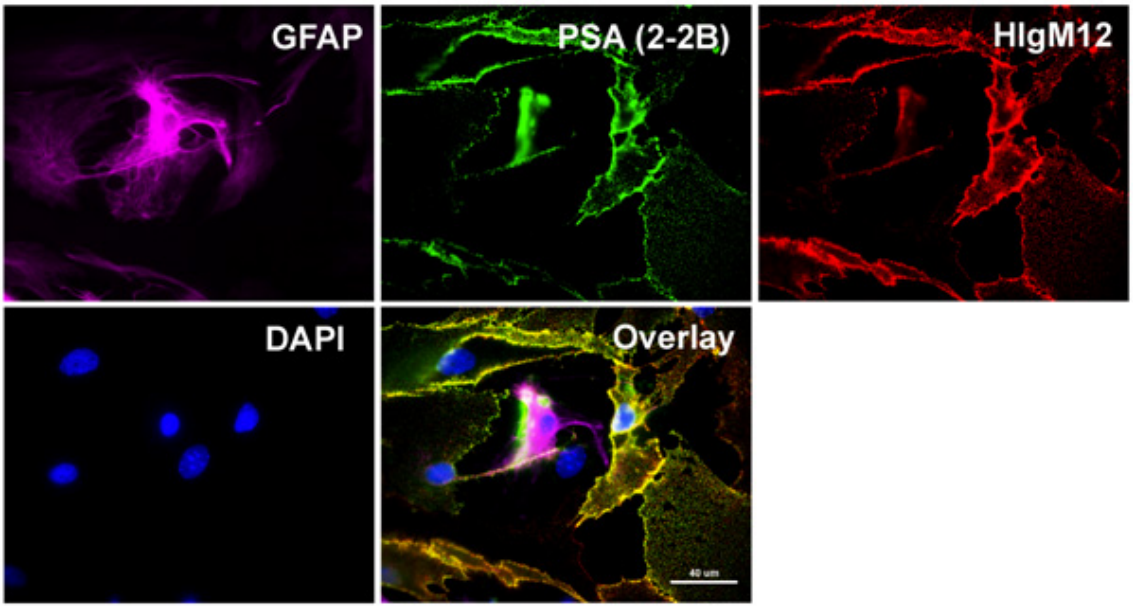

Figure 1. HIgM12 and anti-PSA antibody co-localize in mouse glial cells. A. Glial cells from P7 WT mice were plated on poly-D-lysine coated glass coverslips and cultured overnight in FBS-containing (10 %) B27-supplemented Neurobasal A followed by three days in FBS-free B27-supplemented Neurobasal A. Cells were labeled live with HIgM12 (red) and anti-PSA antibody (green) (clone 2-2B) (10 µg/mL each) on ice for 30 minutes, fixed, permeabilized, labeled with GFAP (purple) and processed for immunocytochemistry. Mounting medium contained nuclear marker 4',6-diamidino-2-phenylindole (DAPI) (blue) for cell counting. All images were taken at 60x magnification (Olympus AX70) under identical conditions in each channel and processed identically. In the overlay, HIgM12 and anti-PSA resulted in a virtually identical cell surface pattern shown in yellow, while red and green colors would indicate separate localizations of both labels. The scale bar in the overlay is 40µm.

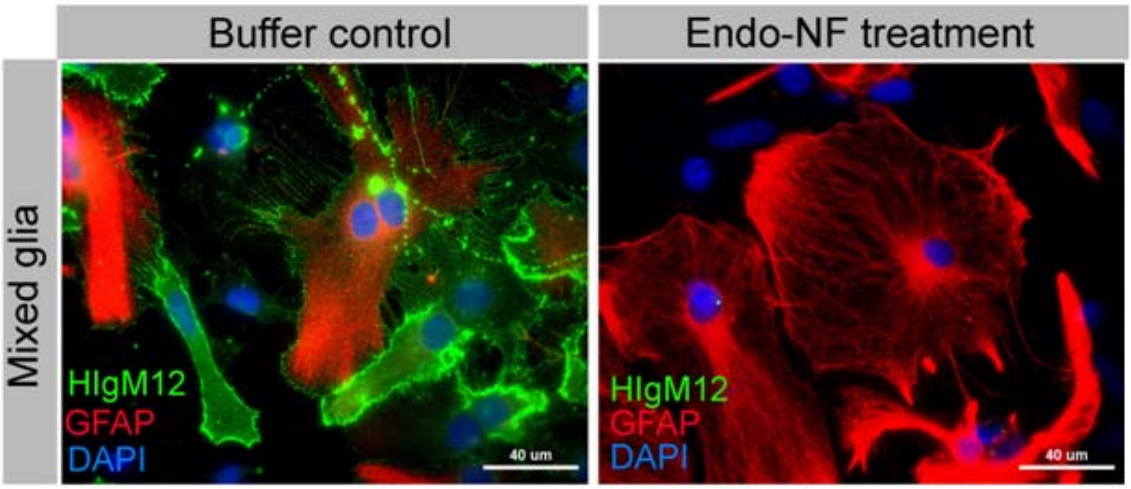

Figure 2. Endoneuraminidase-NF treatment abolishes HIgM12 binding in mouse mixed glial cultures. Mouse mixed glial cells from WT animals were cultured for 3 days *in vitro* in Neurobasal A supplemented with 10% FBS and B27 (1 : 50). On day 3 Neuraminidase-NF (30 µg/mL) was added over night at 37°C to the culture medium. Immunofluorescence with HIgM12 (10 µg/mL) was performed live on ice (30 min); cells were subsequently fixed, permeabilized and stained for internal marker glial fibrillary acidic protein (GFAP). In the overlay cell type-specific markers GFAP is shown in red, HIgM12 in green and nuclear marker DAPI in blue. Fluorescent images were taken at 60x magnification (Olympus AX70). All images compared were obtained using the same instrument settings and processed identically. Scale bars are 40µm.

healthy CNS tissue or remote from CNS lesions (22, 23). GFAP expression by astrocytes *in vivo* exhibits regional and local variability that is dynamically regulated by a large number of inter- and intra-cellular signaling molecules (22).

Given the morphological differences between the three major glial cell types including nuclear sizes (DAPI staining), the relatively low number of IBA-1 positive microglia (<10 %) and the large number of HIgM12-positive cells (>50 %) present in our culture system we suggest that the majority of HIgM12-positive cells are likely GFAP-negative astrocytes.

Next, we tested whether PSA-NCAM is a functionally-relevant protein antigen for HIgM12-stimulated neurite outgrowth *in vitro.* When supplied as a cellular substrate, antibody A2B5 targets cell surface gangliosides on neurons and promotes neurite outgrowth (6, 24), and we have previously reported similar stimulatory growth effects in CNS cell cultures using HIgM12 as substrate (6). However, when cerebellar neurons from NCAM KO and WT mice were plated on HIgM12- or A2B5-coated nitrocellulose, only cerebellar neurons from WT, but not NCAM KO animals, attached to HIgM12-coated nitrocellulose. Importantly, cellular attachment, neurite outgrowth, viability and overall physical appearance were identical between NCAM KO and WT cultures of cerebellar neurons when grown on A2B5-coated nitrocellulose. Collectively, these results demonstrate the importance of PSA-NCAM for HIgM12-mediated neuronal attachment and subsequent neurite outgrowth.

Another important question was whether other polysialylated CNS proteins, such as SynCAM 1 (25), are targeted by HIgM12. Since the literature neither reports differences in chain length nor conformation of conjugated PSA to NCAM and SynCAM, we hypothesized that HIgM12 would also bind PSA-SynCAM. We were able to detect SynCAM via Western blot in our CNS tissue lysates from WT mice using two different anti-SynCAM antibodies.

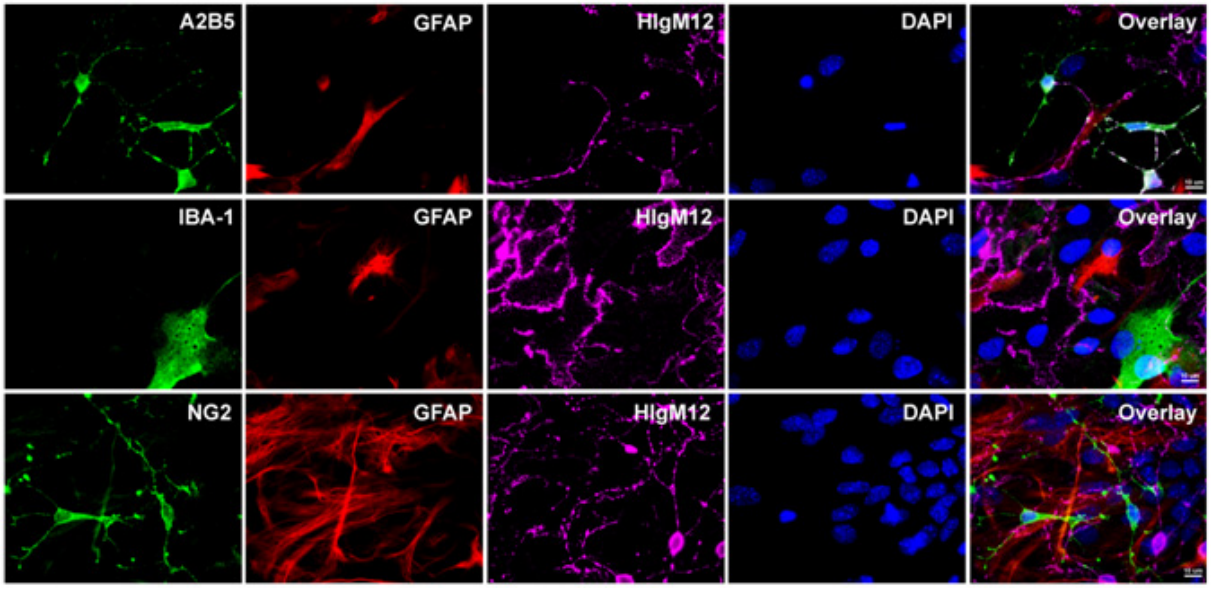

Figure 3. Characterization of HIgM12-positive cell types present in mouse mixed glia. Glial cells from P0 WT mice were plated on poly-D-lysine coated glass coverslips and cultured in FBS-containing (10 %) B27-supplemented Neurobasal A. After 4 days in culture cells were labeled live for 30 minutes at 4 ºC with HIgM12 (10 µg/mL) (purple), A2B5 (10 µg/mL) (green) or anti-NG2 antibody (1 µg/mL) (green), fixed, permeabilized, labeled for internal antigen GFAP (red) and processed for immunocytochemistry. Mounting medium contained nuclear marker DAPI (blue) for cell counting. All images were taken at 100x magnification (Olympus AX70) under identical conditions in each channel and processed identically. Scale bars in the overlays are 10µm.

However, neither HIgM12 nor the commercially available anti-PSA antibody (clone 2-2B) were able to detect PSA-SynCAM at different embryonic stages in the CNS of WT and NCAM KO mice. Immunoprecipitation assays using HIgM12 as the pull-down agent resulted in the detection of PSA-NCAM but not PSA-SynCAM in embryonic CNS tissue homogenates of WT and NCAM KO mice. Thus, our unexpected outcome in immunoprecipitation assays using highly enriched antigen concentrations could not simply be explained by different detection limits/affinities of anti-PSA antibodies. Instead, our results suggest at least some differences in PSA chain length or structure between NCAM and SynCAM. Importantly, our results indicate that PSA-SynCAM is not involved in HIgM12-mediated effects.

Now that we have identified a CNS glycoprotein antigen to which HIgM12 readily binds, a future direction in our lab is the study of molecular signaling mechanisms by which HIgM12 exerts neuroprotective effects in animal models of MS. A working hypothesis is that HIgM12 directly binds PSA-NCAM-positive CNS-resident cells in disease states with disrupted blood brain barrier (BBB). PSA-NCAM-positive neuronal and oligodendrocyte progenitor cells do not ubiquitously populate the healthy adult brain and spinal cord; rather these specific cell types predominate in structurally-isolated neural niches undergoing self-renewal. Furthermore, PSA-NCAM-positive cell numbers are increased in human MS lesions (26, 27) and experimental traumatic brain injury models (28, 29) which may link PSA-NCAM expression to CNS repair and regeneration. Further support for the hypothesis that an IgM antibody is capable of directly stimulating CNS cells comes from human trials using the recombinant antibody rHIgM22 (3). In this study, rHIgM22 was detected in the cerebral spinal fluid (CSF) two days after intravenous injection (i.e., ≥0.05 ng/ml) in all rHIgM22-treated patients at two dose levels. Even 29 days after treatment, rHIgM22 was measurable in the CSF of 5 out of 12 patients (4). This human data demonstrates that IgM antibodies are able to cross the BBB and persist in the CSF (>40 % of patients) for almost a month after treatment. Previous reports in animal studies have shown that less than 0.1% of peripherally-administered IgM-antibodies cross the BBB (30-33). Since we have reported that two human IgM antibodies are capable of binding CNS antigens (1, 7), these data collectively imply that even at low CNS concentrations, IgM antibodies are capable of stimulating neuroprotective effects *in vivo*.

In summary, we have identified PSA (NCAM) as the antigen for HIgM12. Furthermore, binding of HIgM12 to PSA causes CNS-cell attachment and subsequent neurite extension *in vitro* and most likely underlies the stimulation of neurological function *in vivo*.

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