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Cellular targets and mechanistic strategies of remyelination-promoting IgMs as part of the naturally occurring autoantibody repertoire

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Abstract

Immunoglobulins with germline sequences occur in invertebrates and vertebrates and are named naturally occurring autoantibodies (NABs). NABs may target foreign antigens, self- or altered self-components and are part of the normal immunoglobulin repertoire. Accumulating evidence indicates that naturally occurring antibodies can act as systemic surveillance molecules, which tag, damaged or stressed cells, invading pathogens and toxic cellular debris for elimination by the immune system. In addition to acting as detecting molecules, certain types of NABs actively signal in different cell types with a broad range of responses from induction of apoptosis in cancer cells to stimulation of remyelination in glial cells. This review emphasizes functions and characteristics of NABs with focus on remyelination-promoting mouse and human antibodies. Human remyelination-promoting NABs are potential therapeutics to combat a wide spectrum of disease processes including demyelinating diseases like multiple sclerosis. We will highlight the identified glycosphingolipid (SL) antigens of polyreactive remyelination-promoting antibodies and their proposed mechanism(s) of action. The nature of the identified antigens suggests a lipid raft-based mechanism for remyelination-promoting antibodies with SLs as most essential raft components. However, accumulating evidence also suggests involvement of other antigens in stimulation of remyelination, which will be discussed in the text.

Keywords

autoantibodies; apoptosis; demyelination; multiple sclerosis; proliferation; TMEV animal model

Natural antibodies

Soon after natural antibodies were documented by Avrameas [1–3] and Notkins [4–7], it became evident that naturally occurring autoantibodies (NABs) are part of the human innate immunoglobulin repertoire [2,8].

NABs utilize germline-encoded genes directed against foreign antigens, self- and altered self-structures [1] and are present in newborns without stimulation by foreign antigens [9].

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NABs are polyreactive by definition with few or no somatic mutations in the VH and VL antibody regions, which are necessary for high affinity binding of a single antigen. NABs of the IgM isotype are found in invertebrates and vertebrates. High levels of IgG and lower amounts of IgM and IgA NABs are detected in higher vertebrates [10]. In general, NABs bind their antigen with rather low affinity but high avidity [11], which describes the combined synergistic strength of multiple bond interactions rather than the sum of bonds between antigen and antibody. In contrast, conventional antibodies, typically of the IgG isotype, undergo affinity maturation and contain somatic mutations to ensure high-affinity antigen binding.

Accumulating evidence categorize NABs as natural systemic surveillance molecules that tag damaged cells and foreign pathogens for elimination by the immune system through opsonization or antibody-dependent cytotoxicity. In addition to their molecular-tag function, some NABs can actively signal in different cell types including cancer and brain cells. The ability of certain NABs to detect and sometimes induce apoptosis in tumor cells may play an important function in tumor surveillance [12–15]. In mice, another class of NABs, termed remyelination-promoting antibodies, actively promotes repair in demyelinated spinal cord lesions [16–18].

Properties of B cells producing NABs

Natural antibodies are produced by different mature B-cell subsets. These include B1 cells that constitutively produce natural antibodies, most often of the IgM isotype followed by IgG and IgA, marginal zone-B lymphocytes and splenic follicular B2 cells [19,20]. B1 cells constitutively secrete antibodies without prior immune activation [21,22]. These immunoglobulins are termed naturally occurring antibodies because they may be present during fetal life without antigenic exposure. In contrast to mature follicular B cells (B2 cells), which are typically responsible for T-cell mediated immune responses, mature B1 cells and marginal-zone B cells mediate innate immune responses with rapid responses to non-protein antigens. Natural antibodies from B1 cells are often of germline origin and unaffected by somatic hypermutation [23].

Polyreactive NABs

Immunologists had some difficulties in accepting the existence of polyreactive monoclonal antibodies. The problem originates from the immunological paradigm that antibodies engage antigens with high-affinity in a monospecific manner [24]. Ever since, antibodies are associated with high affinity and (mono) specificity. This point of view is supported by the fact that polyreactive antibodies with germline origin are mostly ineffective as detecting agents in cell biological or biochemical settings where promiscuous binding of antibodies is commonly equalized with ‘non-specific binding’.

The establishment of natural antibody occurrence in physiological conditions by Avrameas [1–3] and Notkins [4–7] led to greater acceptance of the polyreactivity of antibodies in general. Antibodies are designated polyreactive when they bind sufficiently to more than one unrelated self or foreign antigen. Sufficient proof of monospecificity, on the other hand, is commonly accepted in arrays with $>10^4$ antigens. In the past, antibody monospecificity had not been tested to this degree. It remains to be seen to what extent previously classified monospecific antibodies would pass today’s criteria in antigen arrays of higher than 10^4 antigens.

Polyreactivity of a single immunoglobulin was formally proven in monoclonal antibodies produced by i) hybridoma technology, ii) Epstein-Barr transfected B lymphocytes and iii) from patients with B-cell malignancies [1,25,26] with polyreactive antibodies of the IgM,

IgG and IgA isotype [27]. Polyreactivity of antibodies has been highly conserved in evolution and can even be found in sharks [28]. The affinity of polyreactive NABs to their different antigens can vary by a factor 1000 and is, in general, lower ($K_d = 10^{-3}$ – 10^{-7} mol/l) than affinity-maturated monospecific antibodies ($K_d = 10^{-7}$ – 10^{-11} mol/l) (Table 1) [29].

The half-life of a polyreactive natural antibody *in vivo* is significantly shorter than a monospecific one (Table 1). The rapid serum clearance of polyreactive monoclonal antibodies is likely due to binding to multiple endogenous antigens.

Because linear amino acid sequence analysis could not explain differences in antigen specificity of polyreactive and monospecific antibodies, it became evident that 3D structure of the immunoglobulin is responsible for differences in specificity and affinity between both subtypes. The structural basis of polyreactivity relates to the properties of the variable domains, particularly of the heavy chain [30,31]. Accordingly, transferring the CDR3 region of the heavy Ig chain from a polyreactive to a monospecific antibody induces polyreactivity [32]. Supporting evidence comes from studies showing that single amino acid replacements in the CDRH3 region of a polyreactive antibody are sufficient to create a monospecific antibody [33,34]. Interestingly, so far no differences in the conformation, amino acid chain length or sequence can be detected in the CDRH3 regions of monospecific vs polyreactive antibodies [29]. In addition, a single amino acid replacement outside the antigen-binding pocket is sufficient to abolish polyreactivity [26]. This indicates that not only the paratope but the whole variable domain is essential for polyreactivity.

Of note, there is a higher degree in glycosylation of polyreactive monoclonal antibodies relative to monospecific monoclonal antibodies [35]. Bulky carbohydrate moieties attached to the variable regions of immunoglobulins contribute to the protein conformation [36]. Glycosylation of the immunoglobulin's variable domains can interfere with its ability to target its antigen [37,38]; this may be an alternative explanation for a higher degree of variability in the antigen-antibody interaction as seen with polyreactive antibodies compared with monoreactive antibodies.

Remyelination-promoting antibodies

The focus in the following section is on NABs that stimulate remyelination in different animal models of multiple sclerosis (MS).

Remyelination-promoting antibodies are a subclass of NABs—All identified remyelination-promoting antibodies were of germline origin or near germline with few somatic mutations, thus having the cardinal features of physiologic natural autoantibodies. So far, all identified remyelination-promoting antibodies with NAb features are of the IgM isotype (with the exception of high-affinity anti-Lingo IgG antibodies, which stimulate remyelination in rodents but do not have NAb features). In addition, all remyelination-promoting antibodies with known antigens are polyreactive, which is the result of their rather flexible antigen-binding site typical for NABs. Of note, all remyelination-promoting antibodies with identified antigens bind to at least one or multiple sphingolipids, which are glycosylated lipids with ceramide backbone and essential lipid-raft components. Only the hydrophilic carbohydrate moiety of the sphingolipids is exposed to the cell surface and, therefore, detectable by antibodies. This emphasizes the carbohydrate moiety and neglects the lipid backbone as the essential part of the antigen.

In summary, remyelination-promoting antibodies show all cardinal features of NABs and represent a subclass of NABs.

Discovery of remyelination-promoting antibodies—The first successful attempt to stimulate remyelination using NABs was performed in the Theiler's murine encephalomyelitis virus (TMEV)-induced model of demyelination [39]. We immunized TMEV-infected SJL mice with spinal cord homogenates (SCH) of normal mice to stimulate a polyclonal antibody response directed against a variety of CNS antigens including myelin components. Instead of an expected exacerbation of the disease course, mice immunized with SCH showed four-times' higher levels of remyelination than non-immunized mice. Whole antisera [40] or purified immunoglobulins [41] raised against CNS antigens increased remyelination to a similar extent in the same animal model. These findings demonstrated for the first-time a beneficial effect of antibodies in stimulating CNS remyelination. In order to raise a more specific (monoclonal) antibody response toward a single antigen, we screened hybridomas made from B-cells of SCH-immunized mice for their ability to induce remyelination in chronically demyelinated mice. Two monoclonal mouse antibodies of the IgM isotype (SCH79.08 and SCH94.03) proved effective in promoting remyelination [42]. Both antibodies (SCH79.08 and SCH94.03) were able to target mature oligodendrocytes (OLs) *in vitro*. In all following attempts to identify mouse or human remyelination-promoting antibodies, we used the antibodies ability to bind to OL-lineage cells as first selection criteria. This was a necessary but not sufficient criterion to identify remyelination-promoting antibodies. Very few antibodies stimulated remyelination *in vivo* without showing a cell-surface reactivity toward OLs [43]. Subsequent studies using four well-known mouse monoclonal IgMs (O4, HNK-1, A2B5 and O1) [44–46] resulted in their classification as promoters of CNS remyelination *in vivo* [47]. Based on this information, our laboratory employed a novel strategy to identify monoclonal remyelination-promoting antibodies of human origin from sera of patients with monoclonal gammopathies. Cerebellar slice cultures were used in addition to cultured OLs for preliminary screening [17]. This resulted in the identification of two serum-derived human remyelination-promoting antibodies (sHlgM22 and sHlgM46). To make a recombinant antibody (rHlgM22), we obtained the IgMs (HlgM22) variable region-coding DNA sequence through protein sequencing and cloned it into an IgM expression vector [48,49]. We confirmed identical properties of the recombinant IgM relative to its serum-derived counterpart to stimulate remyelination *in vivo* and to bind to OLs *in vitro*. rHlgM22 has recently been approved by the FDA for Phase I, multi-center, double-blind, randomized, placebo-controlled, dose-escalation study designed to evaluate safety, tolerability, pharmacokinetics, and immunogenicity of single IV administrations of rHlgM22 in patients with all clinical presentations of MS.

Identified mouse and human remyelination-promoting IgMs stimulate remyelination after TMEV- and lysolecithin-induced demyelination in mice [16–18,42,47,50–52]. A single bolus injection of monoclonal human IgM, rHlgM22, is sufficient to promote repair of demyelinated lesions [17,18]. Antibodystimulated remyelination was shown by electron microscopy and immunohistochemically [16,17,42,47].

Characterization of remyelination-promoting antibodies—The dissociation constants (K_d) of the remyelination-promoting IgMs O4 and O1 to sulfatide (O4) and galactosylceramide (O1) were recently determined (2.4×10^{-9} mol/l (O1) and 2.2×10^{-9} mol/l (O4)) and are unusually high for polyreactive natural antibodies (Table 1) [53]. A study by Paz Soldan *et al.* demonstrated that all tested remyelination-promoting IgMs induce a Ca^{2+} -influx in astrocytes (GFAP+), oligodendrocyte progenitor cells (OPCs) and immature OLs [43]. IgM-mediated effects in astrocytes and OLs were, however, independent from each other, based on different signaling mechanisms and based on different Ca^{2+} pools (ER-stored Ca^{2+} for astrocytes and extracellular Ca^{2+} for OLs) [43]. In summary, all well-characterized remyelination-promoting antibodies are of germline origin,

belong to the IgM isotype and induce calcium influx into astrocytes, OPCs and immature OLs.

Common antigens & mechanistic theories for remyelination-promoting antibodies

All remyelination-promoting antibodies with known antigens are polyreactive. The ganglioside-binding antibody A2B5 targets several sialylated glycosphingolipids (SLs) due to their similar carbohydrate epitope [44,54,55]. HNK-1 recognizes the SL 3-sulfoglucuronyl paragloboside (SGPG) [45,56,57] as well as the carbohydrate epitope of the glycoproteins MAG and P0 [57]. The mouse IgM O1 binds to galactocerebroside and similar SLs [58,59], whereas O4 targets sulfated galactocerebroside (sulfatide), seminolipid, the unknown prolignodendroblast antigen (POA) and cholesterol [58–61]. SLs and cholesterol are essential components of lipid rafts, which act as signaling platforms at the level of the plasma membrane in cells. Lipid rafts may enable or disable interactions between many different cell-surface receptors (proteins) to transduce extracellular signals over the plasma membrane into the cytoplasmic space (general lipid-raft concept). Pentameric IgM molecules can bind and cluster up to 10 antigens at a time on different cells and <10 antigens on a single cell due to steric hindrance/tension within the IgM molecule. We hypothesize that IgMs targeting SLs stabilize existing rafts or stimulate the formation of new lipid rafts at the plasma membrane, thereby enhancing the effects of extracellular stimuli via existing cellular signaling pathways (lipid-raft hypothesis).

Alternatively, remyelination-promoting IgMs may be involved in the opsonization of cellular debris and dead or apoptotic cells in a lesion site. Remyelination-promoting antibodies 94.03 and 79.08 [47], O1 and the human sulfatide-binding IgM DS1F8 [62] prominently stain filaments in astrocytes or HeLa cells, which are identified as microtubule-like structures [62]. Binding to intracellular filamentous structures seems to be common with antibodies targeting galactosylceramide and sulfatide [46,63,64]. This may indicate epitope similarities between OL-specific SLs present at the cell surface and in internal pools relative to intracellular cytoskeletal proteins detected by polyreactive antibodies. The ability of certain remyelination-promoting antibodies to target both membrane lipids and attached cytoskeletal proteins may significantly facilitate lesion clearance by the immune system and help to repopulate demyelinated areas.

A more recent study shifted our focus from SLs to glycoproteins as an alternative class of antigens for remyelination-promoting antibodies. As mentioned earlier, the carbohydrate, but not the lipid moiety of sphingolipids, is accessible to antibodies and may be sufficient for antibody binding when linked to either a lipid or protein backbone. The relatively simple carbohydrate structure on sphingolipids compared to the complex glycosylation found on many glycoproteins suggests that carbohydrate building blocks responsible for antibody binding can be found on glycoproteins as well. Inoko *et al.* (2010) demonstrated binding of the remyelination-promoting IgM A2B5 to a novel set of brain-derived glycoproteins as shown by Western blots [65]. It is well accepted that A2B5 binds to c-series gangliosides GT3 and GQ1c. However, c-series gangliosides and their O-acetyl derivatives are temporarily expressed in embryonic stages in the CNS but seldom during adulthood in vertebrates [66–70]. Cerebellar stellate neurons are the only known exception in the adult human CNS that express c-series gangliosides [71]. This raises the question whether remyelination-promotion in adult mice by A2B5 is mediated through binding to SLs or glycoproteins [47].

In summary, all remyelination-promoting IgM mAbs bind to SLs and potentially to glycoproteins at the cell surface of OPCs/OLs, and some bind to neurons. It remains elusive whether effects seen *in vivo* and *in vitro* are mediated through binding to cell-surface SLs, glycoproteins and/or internal cytoskeletal structures.

Responsive OL differentiation stages for IgM-stimulated lesion repair in brain & spinal cord—The remyelination-promoting mouse IgMs O4, O1 and A2B5 are commonly used markers for specific differentiation stages in OPCs and/or OLs [44–46,55–57]. However, the targeted differentiation stages vary strongly between different remyelination-promoting IgMs from OPCs (A2B5), late OPCs and immature OLs (O4), immature OLs (O1) to mature MBP-positive OLs (rHlgM22, 79.08). This would suggest that basically all OPC/OL-differentiation stages are responsive to IgM-stimulated repair in spinal-cord lesions. Overwhelming evidence demonstrates, however, that OPCs – but not surviving mature OLs – are responsible for remyelination *in vivo* [72–77]. Stimulation of remyelination by remyelination-promoting antibodies may be mediated by OPCs within the OL-lineage. This implies that: i) the SL antigens galactosylceramide (O1) and most likely sulfatide (O4) are not involved in antibody-stimulated remyelination due to the temporal mismatch between antigen expression and the responsive OPC stage involved in remyelination, ii) Identification of the late OL antigens for rHlgM22 (unknown) and 79.08 (MBP) may be unimportant for the same reason. With the latter possibility, the antigens are sufficiently expressed already at earlier stages during OL development but undetectable by immunocytochemistry. In support of these hypotheses, we demonstrated stimulation of OPC proliferation by rHlgM22 in a PDGF-dependent manner [78]. rHlgM22 had no detectable effects in mature (MBP⁺) OLs, which are strongly positive for rHlgM22 in immunocytochemistry. In addition, all remyelination-promoting antibodies stimulated calcium influx into OPCs but not into mature OLs [43]. The expression of SL sulfatide is associated with the immature OL stage but not with the late-OPC stage. However, expression of the unidentified pro-oligodendroblast antigen (POA) for O4 precedes the expression of sulfatide, which may explain the temporal mismatch between expression of the O4 antigen(s) and the fact that OPCs are mediators for remyelination. The POA antigen may be a sulfated glycoprotein.

In summary, there is a strong temporal mismatch between the expression of the antigens galactosylceramide (O1), sulfatide (O4), MBP (79.08), the unknown antigen for rHlgM22 and the OPC stage as the responsible developmental stage of OLs for remyelination. We conclude that other antigens on more immature cells other than the named myelin markers are responsible for remyelination *in vivo* and effects seen *in vitro*. The nature of those unidentified antigens remains unclear.

Low amounts of rHlgM22 are effective to stimulate remyelination in TMEV-infected animals—The effective dose of rHlgM22 to stimulate spinal-cord remyelination in TMEV-infected mice is as low as 500 ng per mouse when administered ip. in a single bolus injection. Remyelination was completed 5 weeks after the IgM injection. Surprisingly, higher antibody doses (up to 1000-fold) were not more effective in stimulating remyelination [18]. rHlgM22-mediated remyelination was detectable at 1 and 3 weeks after administration into chronically demyelinated mice; however, changes were not yet statistically significant [18]. In addition, multiple dosing of TMEV-infected mice with human antibody rHlgM22 was not more effective than a single dose of antibody. This may be the result of a possible immune response in mice toward human antibodies 5 weeks after the first exposure to rHlgM22.

The half-life of human rHlgM22 in infected and uninfected mice was ~15 h with no difference in serum clearance between both groups. This resulted in an 8.6-fold decrease of antibody detectable in serum at the 48 h timepoint [18].

In summary, rHlgM22 is a potent therapeutic in mice, which acts within a short window of time and causes longlasting tissue repair. It also suggests a molecular and cellular memory

effect (i.e., with newly synthesized cells or stable changes of cellular behavior) long after endocytosis and destruction of the antibody.

The integrity of the IgM molecule is required to stimulate remyelination—So far, all attempts to manipulate the integrity of the pentameric rHlgM22 molecule chemically or enzymatically have degraded its capability to remyelinate. Cleavage of the IgM molecule results in unstable pseudo-IgGs that spontaneously reassemble into a tetrameric isoform. The tetrameric isoform resulted in identical levels of (spontaneous) remyelination as PBS-treated mice, which was significantly lower when compared with animals receiving intact pentameric rHlgM22 molecule (unpublished data). This may either suggest that antigen clustering on a single living cell, clustering of multiple cells or antigen clustering of cellular debris by the IgM molecule is essential for its cellular effect.

Remyelination-promoting IgMs have access to demyelinated lesions—

Although debate continues concerning antibody access to the brain (i.e., limited brain access of high-molecular weight proteins; specific antibody-transporter systems via transferrin receptor-mediated transflux; age-related blood–brain barrier (BBB) changes [79], endogenous factors affecting BBB [80]), the concept has generated new potential therapeutics to treat demyelinating diseases including anti-Lingo IgGs and remyelination-promoting IgMs. Direct evidence of IgM access to the brain comes from a MRI study using antibodies labeled with ultra-small superparamagnetic iron oxide particles (USPIO). 3D T2-weighted imaging of spinal cords demonstrated co-localization of rHlgM22 with demyelinating lesions in mice spinal cords when injected into the tail vein of Theiler's virus-infected mice [81]. No IgM accumulation in the CNS was apparent in non-infected animals or animals without demyelination [81]. In addition, dysfunction of the BBB is a typical feature of MS [82,83] and has been demonstrated in different presentations of the disease including chronic-progressive MS [84–86] and acute MS plaques [85]. BBB breakdown in MS allows high molecular-weight serum proteins, including fibrinogen (340 kDa), vitronectin and fibronectin (~440 kDa), to enter and accumulate within the lesion area [87–93]. Therefore, it is plausible to accept IgM access to demyelinated brain lesions as well in the human situation.

Remyelination-promoting antibodies in EAE—The well-characterized IgMs O4 and O1 promote remyelination in the TMEV model [47,94] comparable to the degree induced by rHlgM22 [16–18], and polyclonal human IgM [17]. In stark contrast to results obtained from the TMEV model, multiple dosing with the mouse IgM O4 causes a more severe disease phenotype in acute PLP-induced experimental autoimmune encephalomyelitis (EAE) [95]. Confirming the discrepancy between different animal models of MS, we demonstrate rHlgM22-stimulated promotion of remyelination in TMEV- and lysolecithin-demyelinated mice [16–18], but not in EAE (unpublished data). Unlike O4, rHlgM22 does not exacerbate the disease course in EAE (unpublished data). The precise reason(s) for the opposite outcome in different animal models of MS are unknown. However, EAE is an animal model of brain inflammation with relatively little demyelination compared to the TMEV model. Drugs effective in EAE commonly target the immune system and modulate the inflammatory response. Remyelination-promoting antibodies have little or no effect on the immune response but directly target cells within the demyelinated lesion to stimulate remyelination. It is, therefore, not surprising that remyelination-promoting antibodies do not affect remyelination in EAE in the absence of substantial demyelination. Because these autoantibodies do not exacerbate an autoimmune disease in animals, they are unlikely to exacerbate autoimmune disease in humans.

Proposed mechanism(s) of action of remyelination-promoting IgMs—This section will concentrate on general mechanistic aspects of mouse and human remyelination-promoting IgMs with further focus on rHIgM22 in clinical trials.

Mouse and human remyelination-promoting IgMs stimulate repair in TMEV- and lyssolecithin-demyelinated mice [16–18,42,47,50–52]. To date, the precise mechanism of action is still unknown. Two main hypotheses have been proposed: i) the direct hypothesis proposes antibodies recognize OPCs and promote the synthesis of new myelin; ii) the indirect hypothesis proposes antibodies activate either immune cells or cell types other than cells of the OL lineage within the CNS, which, in turn, stimulate OPCs or OLs (i.e., by secreting remyelination-promoting factors).

Support exists for both hypotheses. Evidence supporting the direct hypothesis is derived from the observation that remyelination-promoting IgMs target OLs in culture, isolated myelin and myelin tracks in cerebellar slice cultures [16–18,42,43,47,50,94,96]. However, fluorescence microscopy, when used as an approach to detect rHIgM22 binding to glial cells qualitatively, detected binding only to mature OLs but not to OPCs, astrocytes or microglia. Biological effects were, however, detectable only in OPCs. Therefore, we conclude that fluorescence microscopy is not sufficiently conclusive to determine relevant cell types involved in rHIgM22-mediated effects.

All tested human and mouse remyelination-promoting IgMs induce calcium influx in cells of the OL-lineage, which suggests activation of intracellular signaling pathways potentially important for remyelination [43,94]. In addition, rHIgM22 inhibited OL differentiation and apoptotic signaling in enriched OPC cultures *in vitro* [97]. Inhibition of myelin-sheath formation in OLs and Schwann cells through IgMs *in vitro* has been previously shown with the mouse remyelination-promoting IgM O4 [98], the Ranscht-IgG targeting the sphingolipids galactosylceramide and sulfatide [60,99] and polyclonal IgM preparations [100].

Evidence supporting the indirect hypothesis is derived from the observation that remyelination-promoting IgMs stimulate Ca^{2+} signaling in GFAP-positive astrocytes [43]. The astrocytic response to IgMs is immediate and precedes the oligodendrocyte response [43]. However, the underlying mechanisms of calcium influxes into astrocytes and OPCs are very different and do not depend on each other [43]. The identification of an OPC-signaling complex responsible for rHIgM22-mediated actions, including PDGF α receptor [97], suggests involvement of the astroglial growth factor PDGF in rHIgM22-mediated actions in OPCs.

Most important, IgM-mediated OPC proliferation was detectable only in cultures containing substantial amounts of astrocytes, microglia and OPCs (mixed glial cultures) but not in highly enriched OPC populations [78]. Secreted astrocytic or microglial factors or direct cellular contact between OPCs and other glia seems to be essential for the proliferative response. In addition, we demonstrated rHIgM22-mediated activation of the PDGF receptor in oligodendrocytes. Because PDGF is secreted by astrocytes and possibly microglia [101,102] but not oligodendrocyte-lineage cells, it demonstrates an involvement of cells other than OLs in IgM-mediated OPC proliferation and, possibly, remyelination. In an analogy to rHIgM22, polyclonal IgM preparations inhibit OPC differentiation in mixed glial but not isolated OPC cultures [100].

It appears that three cell types (OPCs, microglia and astrocytes) are required for IgM-stimulated proliferation of OPCs *in vitro*. The growth factor PDGF and potentially other secreted microglial and astrocytic factors are important mediators for this effect. However, it

has not been determined whether rHIgM22 directly stimulates glial cells other than OPCs (i.e., to produce more growth factors) where IgM-binding to OPCs may not be essential for stimulation of OPC proliferation. Alternatively, rHIgM22-mediated calcium signaling in astrocytes may not affect OPC behavior with unchanged secreted astrocytic factors or cellular components necessary for physical contact with OPCs by rHIgM22. Instead, binding of rHIgM22 to OPCs only may be the necessary prerequisite for stimulation of OPC proliferation *in vitro* or lesion repair *in vivo*.

So far, supporting data exist for the latter hypothesis only. No IgM-mediated signal transductions other than calcium influx could be detected in astrocytic cultures [97] or in isolated microglia [97]. In contrast, remyelination-promoting IgMs, including rHIgM22, bind to OL-lineage cells, with rHIgM22 activating Lyn, ERK1 and ERK2 in OPCs leading to inhibition of OPC differentiation and reduced apoptotic signaling (Figure 1) [97]. We also identified the signaling complex in OPCs consisting of integrin $\alpha\beta3$, Lyn and the OPC marker PDGF α R responsible for rHIgM22-mediated effects [97]. Involvement of PDGF α R not only demonstrated that OPCs mediate the IgM effects but also suggested a central role for the astrocytic ligand, PDGF, in rHIgM22-stimulated OPC proliferation. Inhibition of the PDGFR kinase in OPCs prevented antibody-stimulated OPC proliferation in mixed glial cultures (Figure 1) [78]. In our most recent model (Figure 1), remyelination-promoting IgMs may directly act on OPCs by lowering the threshold for PDGF produced in low quantity in the *in vivo* situation [103]. This model is supported by the fact that rHIgM22 activates the PDGF receptor in OPCs [78].

It has not been determined whether PDGF is crucial for IgM-mediated remyelination *in vivo*. Detectable PDGF levels *in vivo* in the embryonic brain are below 1 ng/ml [103]. This amount of PDGF is not sufficient by itself to stimulate OPC survival [104,105] or proliferation [106] *in vitro*. We conclude additional factors are necessary to activate the PDGF response *in vivo* [107].

In addition, PDGF-concentrations in demyelinated lesions may be different from the surrounding tissue. In fact, evidence from a viral-demyelination model demonstrates up-regulated PDGF expression within the lesion and primarily associated with astrocytes but not neurons [108]. In a chemical-demyelination model, mice overexpressing PDGF have higher levels of proliferating OPCs and less OPC apoptosis in the lesion area [109]. This may indicate involvement of PDGF in OPC proliferation and survival to stimulate remyelination *in vivo*.

However, in order to make a definitive statement regarding the indirect or direct function of astrocytes in IgM-stimulated OPC proliferation *in vitro* and lesion repair *in vivo*, experiments involving IgM-stimulated remyelination in CST $^{-/-}$ (O4) or CGT $^{-/-}$ (O1) animals may be necessary to rule out whether IgM-binding to astrocytes, and potentially microglia, but not to OPCs is sufficient for lesion repair *in vivo*.

Remyelination-promoting antibodies & their potential beneficial or deleterious effects in the PNS—Elevated levels of certain immunoglobulins, termed autoantibodies targeting SLs and the myelin protein MAG, are sometimes found in peripheral neuropathies [110–114]. The constant association of anti-SL antibodies with PNS dysimmune neuropathies supports a pathogenic link between anti-SL antibodies and neuropathy (e.g., Guillain-Barre syndrome [GBS], Miller Fisher syndrome [MFS], neuropathy associated with IgM monoclonal gammopathy [PN+IgM], and chronic inflammatory demyelinating polyneuropathy [CIDP]). This includes immunoglobulins of the isotypes IgG, IgA and IgM. Antibodies to the glycoprotein MAG and >20 different SLs have been associated with chronic and acute peripheral neuropathic syndromes including GM1, GM1b, GD1a,

GalNAc-GD1a (acute motor axonal neuropathy), GQ1b, GD3, GD1b, GT1a (sensory variants GBS) [114] and sulfatide (GBS, CIDP) [115].

Interestingly, antibodies associated with peripheral neuropathies can bind to similar or identical antigens as remyelination-promoting antibodies (anti-sulfatide [O4] [112, 115–118]; anti-SGPG, anti-MAG [HNK1]) [113, 119–121]. Both categories of antibodies can also be of IgM isotype, which seems to be essential for the remyelination-promoting antibody rHlgM22. Some antibodies associated with peripheral neuropathies may act through complement fixation [122], which results in pore formation and cellular destruction after plasma-membrane binding. In contrast to these antibodies, the remyelination-promoting antibody rHlgM22 does not fix complement and does not target Schwann cells or peripheral nerves (unpublished data). Toxicology studies in primates and rodents using 1000-fold higher amounts of rHlgM22 than the therapeutic dose demonstrated no pathological effects in the PNS. Lack of antibody binding to peripheral nerves, however, suggests that rHlgM22 does not stimulate remyelination in the PNS.

Unlike rHlgM22, remyelination-promoting antibodies O4 (sulfatide), O1 (galactosylceramide) and HNK1 (anti-SGPG, MAG) do bind to cell-surface antigens on Schwann cells. It has not been determined whether these antibodies stimulate remyelination in the PNS. Surprisingly, antibodies associated with peripheral neuropathies target the same antigens as the mentioned promoters of remyelination (see above). The different outcome of various antibodies targeting identical antigens on myelinating OLs vs Schwann cells raises the question whether antibodies associated with peripheral neuropathies actively exacerbate the disease course or merely represent a bystander effect. Generation of anti-sphingolipid antibodies may occur after the axonal and myelin destruction due to increasing amounts of cellular debris.

In contrast to their suggested involvement in peripheral neuropathies, gangliosides promote neurite outgrowth and regeneration both *in vivo* and *in vitro* [123,124]. Clinical trials using purified gangliosides in neuromuscular disorders did not show sufficient efficacy but also did not give rise to an immune response followed by increased pathology [125,126]. Intramuscular doses of purified bovine ganglioside mixtures were used therapeutically for years throughout Europe, and it is widely believed that these protein-free sphingolipids are not antigenic and do not elicit immune-mediated side-effects. Experimentally, several studies failed to show that gangliosides enhance autoimmune demyelination in the PNS [127–130] and did not induce neurological signs of neuropathies or neuropathological changes [131–133]. In addition, passive transfer of anti-GM1 antibodies failed to transfer the disease [134–136].

Given the number of different diseases covered under the umbrella of ‘peripheral neuropathies’ and their individual complexity, it is extremely difficult to extrapolate which antibodies actively participate in the pathogenesis of different neuropathies based on clinical studies using immunosuppressive and immunomodulatory drugs. However, the efficacy of current immune therapies such as rituximab, prednisolone and cyclophosphamide in neuropathies with anti-MAG IgM antibodies remains unproven [137]. Plasma exchange (PE) seems to be effective in patients with paraproteinemic neuropathies associated with high IgG or IgA levels but not IgM levels [138]. Similarly, corticosteroids, when administered in monotherapy, were not effective in IgM-associated neuropathies [139]. In support of suggested differences between different antibody isotypes in paraproteinemic neuropathies, IgM-associated distal demyelinating symmetric neuropathies respond rather poorly to immunosuppressive therapy [140]. The reported differences between immunoglobulins of the IgM vs other isotypes in paraproteinemic neuropathies may be due to completely different pathophysiological mechanisms. Apparently, elimination or

suppression of the IgM molecule has little or no impact on the disease course and challenges direct involvement of the antibody in the pathophysiological mechanism.

All of this argues against the hypothesis that antibodies targeting SLs are involved in the pathogenesis of peripheral neuropathies.

Whether remyelination-promoting antibodies stimulate remyelination in the PNS remains undetermined. It seems unlikely, however, that remyelination-promoting antibodies O4, O1 and HNK-1 exacerbate the disease course in chronic inflammatory demyelinating polyneuropathies, which is an exclusion criterion in the rHIgM22 clinical trials. It is puzzling that, different IgM antibodies targeting identical SLs have very different outcomes on myelinating OLs compared to Schwann cells. They may lead either to remyelination in the CNS or cellular destruction in the PNS. Clinical results from IgM-associated neuropathies using immunomodulatory or immunosuppressive treatments suggest little or no direct involvement of IgMs in the pathological mechanism of the disease. It could be argued, however, that disease-associated anti-sulfatide IgMs and possibly other IgMs may stimulate remyelination in the CNS similar to the IgM O4 but are ineffective in the PNS. There is no direct evidence suggesting that disease-associated IgM antibodies differ from remyelination-promoting antibodies when targeting identical antigens and are of the same isotype. Mechanistic data indicates that rHIgM22 requires the astrocytic growth factors PDGF and likely FGF-2 to stimulate OPC proliferation and to reduce levels of apoptotic signaling *in vitro* [78,97]. No rHIgM22-mediated effects could be observed in isolated OPC cultures. This suggests that the cellular microenvironment (astrocytes and possibly microglia) is essential for rHIgM22-mediated effects in the CNS. It is unclear whether the microenvironment provided by Schwann cells and possibly other cells in the PNS support IgM-mediated stimulation of remyelination by rHIgM22 or possibly other remyelination-promoting antibodies.

In brief, it is very unclear whether remyelination-promoting antibodies have an impact on remyelination in the PNS. However, we tend to be rather skeptical because of the presence of disease-associated antibodies with potentially very similar properties compared to remyelination-promoting antibodies and lack of sufficient remyelination in the PNS in those cases. In addition, it remains elusive whether the cellular microenvironment present at the lesion site in the PNS resembles the microenvironment in CNS lesions, which is required for rHIgM22-mediated stimulation of remyelination.

Expert commentary & five-year view

The human-remyelination promoting IgM rHIgM22 recently entered Phase I clinical trial for MS patients. rHIgM22 shows efficacy in animal models of MS when administered as a single bolus injection. These results suggest a rather catalytic mechanism for rHIgM22 and possibly other remyelination-promoting antibodies *in vivo* with minimal amounts of antibodies required; this is in contrast to blocking or neutralizing antibodies, where stoichiometric ratios between antigen and antibody are necessary. BBB permeability is a necessity for all neurotherapeutics injected peripherally. Accumulating evidence suggests that <0.1% of the original amount of antibody administered crosses the BBB in different animal models of neurological diseases. No significant differences in BBB permeability were detected between antibodies of the IgG and IgM isotype. The low amount of antibody necessary for stimulation of remyelination (rHIgM22) favors NAb as a potential therapeutic for neurological and other disorders. Due to the complexity of MS, with multiple inhibitory factors present in demyelinated lesions, we expect combination therapies to be most successful. In order to find the most potent treatment, there clearly is a need to develop better pre-screening tests as well as animal models that more closely mimic human disease.

The choice of animal model will preselect certain classes of molecules or otherwise exclude potentially successful therapeutics from human trials. The progress made over the last years in the characterization of the lesion microenvironment, including inhibitory molecules on the one hand and the use of NABs in the treatment of human diseases on the other provides reasons for optimism.

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Key issues

- Naturally occurring autoantibodies (NAbs) are of germline origin with little or no somatic mutations. They are often polyreactive and bind with rather low affinity to one or multiple structurally unrelated antigens.
- NAbs are part of the innate immunity and involved in tissue homeostasis, defense against pathogens, tumor surveillance and stimulation of brain repair.
- NAbs often bind to carbohydrate epitopes on glycosphingolipids (SLs) and, potentially, proteins in normal, apoptotic or cancer cells.
- Remyelination-promoting antibodies belong to the group of natural occurring antibodies.
- Most known remyelination-promoting antibodies are of the IgM isotype and bind to myelin and oligodendrocytes. Their molecular targets are cell surface SLs and, to some extent, cytoskeletal proteins.
- Repair of brain lesions by remyelination-promoting antibodies may be mediated through increased lesion clearance with IgM-tagged myelin debris ready for opsonization or through lipid-raft formation in oligodendrocyte progenitor cells with higher levels of PDGF-receptor activation.
- Stimulation of oligodendrocyte progenitor proliferation *in vitro* by remyelination-promoting antibodies (rHIgM22) requires the presence of astrocytes and microglial cells.
- The remyelination-promoting antibody rHIgM22 stimulates remyelination in Theiler's murine encephalomyelitis virus-infected mice after a single minimal dose of 500 ng per animal.
- The integrity of the IgM molecule appears to be essential for stimulation of remyelination *in vivo*.
- Remyelination-promoting antibodies show efficacy in multiple sclerosis animal models with extensive (chronic) demyelination (Theiler's virus-induced demyelination) but not in primarily immune-mediated animal models (experimental autoimmune encephalomyelitis).

Figure 1. Proposed mechanism of action of rHIgM22 in oligodendrocyte progenitor cells

Evidence suggests that rHIgM22 targets and clusters SLs at the level of the plasma membrane in OL-lineage cells through its pentameric IgM structure. SLs are essential lipid raft components and IgM-mediated SL clustering may induce and stabilize certain cell surface domains. Membrane reorganization may bring together components of the identified signaling complex including integrin $\alpha\text{v}\beta\text{3}$, PDGF αR and Lyn kinase. IgM-stimulated activation of kinases Lyn and ERK1 and ERK2 is required for anti-apoptotic signaling and inhibition of OL differentiation. It is undetermined whether Lyn and ERK activation are essential for rHIgM22-stimulated OPC proliferation. Factors from cells other than OPCs are most likely essential for this effect (e.g., PDGF, FGF-2).

OL: Oligodendrocytes; OPCs: Oligodendrocyte progenitor cells; SLs: Glycosphingolipids.

Table 1

Properties of polyreactive versus monospecific monoclonal antibodies.

Property	Polyreactive monoclonal antibody	Monospecific monoclonal antibody
Antigen	Many structurally diverse and unrelated antigens	Single antigen
Affinity	Low ($K_d: 10^{-4}$ – 10^{-7})	High ($K_d: 10^{-7}$ – 10^{-11})
Sequence	Germline or near germline with few somatic mutations, no affinity maturation	Somatically mutated, affinity matured
Number of potentially allowed conformations of antigen-binding pocket	More than one (conformational selection) or (induced fit hypothesis)	Only one (lock and key fit mechanism)
Immunoglobulin subtype	Mainly IgM, also IgA and IgG	IgG, IgM, IgA
Half-life time	IgM: ~8 h; IgG: ~10 h; IgA: ~8 h	IgM: ~35 h; IgG: ~280 h; IgA: ~26 h

K_d : Dissociation constants.