Published in final edited form as:

Biochim Biophys Acta. 2017 September ; 1861(9): 2228–2239. doi:10.1016/j.bbagen.2017.06.008.

Antibody blood-brain barrier efflux is modulated by glycan modification

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Abstract

BACKGROUND—Drug delivery to the brain is a major roadblock to treatment of Alzheimer's Disease. Recent results of the PRIME study indicate that increasing brain penetration of antibody drugs improves Alzheimer's treatment outcomes. New approaches are needed to better accomplish this goal. Based on prior evidence, the hypothesis that glycan modification alters antibody blood-brain barrier permeability was tested here.

METHODS—The blood-brain barrier permeability coefficient P_e of different glycosylated states of anti-amyloid IgG was measured using in vitro models of brain microvascular endothelial cells. Monoclonal antibodies 4G8, with sialic acid, and 6E10, lacking sialic acid, were studied. The amount of sialic acid was determined using quantitative and semi-quantitative surface plasmon resonance methods.

RESULTS—Influx of IgG was not saturable and was largely insensitive to IgG species and glycosylation state. By contrast, efflux of 4G8 was significantly lower than both albumin controls and 6E10. Removal of α 2,6-linked sialic acid group present on 12% of 4G8 completely restored efflux to that of 6E10 but increasing the α 2,6-sialylated fraction to 15% resulted in no change. Removal of the Fc glycan from 4G8 partially restored efflux. Alternate sialic acid groups with α 2,3 and α 2,8 linkages, nor on the Fc glycan, were not detected at significant levels on either 4G8 or 6E10.

CONCLUSIONS—These results support a model in which surface-sialylated 4G8 inhibits its own efflux and that of asialylated 4G8.

GENERAL SIGNIFICANCE—Glycan modification has the potential to increase antibody drug penetration into the brain through efflux inhibition.

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Keywords

SIALIC ACID; FAB FC ANTIBODY GLYCOSYLATION; ALZHEIMER'S DISEASE; BLOOD BRAIN BARRIER; SURFACE PLASMON RESONANCE

1. Introduction

The number of Alzheimer's disease (AD) patients, and the associated cost of care, is rising faster than many other diseases [1]. This is the consequence of both an aging population and lack of treatment [1]. Based on a correlation with AD, A β in the central nervous system (CNS) is a common target for new drugs [2, 3]. The most common biologic used to target A β are antibodies generated through active or passive immunotherapy [2]. The safety and efficacy of antibody-based immunotherapy has been established for non-CNS disorders [4–8]. Despite the impediment of the blood-brain barrier (BBB), antibody-based immunotherapy had suggested early promise for AD in pre-clinical studies with mice and small patient groups [9–12]. Unfortunately, these early treatments have not met their primary endpoints in larger clinical trials [13–16].

A recent Phase 1b clinical trial with the anti-A β antibody therapeutic aducanumab provides cautious optimism for AD treatment [17]. Although small (n=165), it is the largest trial to date that produced a reduction in cognitive decline [17]. Aducanumab differs from other anti-A β antibodies in that it has a >10,000-fold specificity of A β fibrils and oligomers over monomers, activates microglia to clear plaques, and also exhibits a 13-fold higher brain penetration [17]. While larger studies are needed to confirm this result, this trial highlights the importance of increased brain penetration as a means to improve the efficacy of antibody therapeutics for AD.

Increased brain penetration of a drug can be facilitated via improved influx and/or reduced efflux through the BBB [18, 19]. Most efforts to date have focused on enhancing antibody influx. Given that no specific influx transporter of antibodies exists [20], increasing antibody influx must involve altering either the antibody drug or possibly the BBB itself [21]. One option is to use antibody fragments as drugs, although high turnover is a limiting factor [22–26]. A second option is the “trojan horse” strategy, in which a second module is engineered into the antibody construct that binds a BBB transporter [19, 27, 28]. Traditional transporter targets are the receptors for insulin or transferrin [29, 30] although new transporter targets have been discovered more recently [31]. Also, small peptides have been designed to bind both antibodies and BBB components to facilitate entry [32]. While promising, these approaches are limited by toxicity and receptor selectivity [31] and toxicity [32]. As such, neither bispecific antibodies nor extrinsic trojan horse peptides have progressed from preclinical studies to AD clinical trials [32].

Compared with influx enhancement, efflux inhibition has been less actively pursued. Efflux inhibition can, in theory, increase the penetration of therapeutic antibodies into the brain. Prior studies have highlighted a role for the neonatal Fc receptor (FcRn) in IgG efflux, although the comparative rate of this efflux system to resorptive pathways is not consistent [33–35]. FcRn inhibitors could potentially result in increased IgG brain concentration [33,

36]. A complication here is that general FcRn blockade accelerates overall antibody turnover, which can offset any accumulation of therapeutic IgG in the brain [37, 38]. Alternative antibody efflux transport systems specific to the BBB are not presently known.

Glycosylation is a feature of therapeutic antibodies that has not been fully explored with respect to BBB penetration. A number of studies on non-IgG proteins highlights the importance of glycan targeting on the BBB for enhanced influx. Lectins *Ricinus communis* agglutinin I (RCAI) and wheat germ agglutinin (WGA) bind their specified glycans to facilitate Adsorptive-Mediated Transcytosis (AMT) while the FC5 llama V_HH “nanobody” binds a novel α 2,3-sialylated receptor to facilitate Receptor-Mediated Transcytosis (RMT) [31, 39–41]. Following a reverse strategy, high mannose glycans on the surface of HIV coat protein gp120 facilitate AMT after binding the mannose-6-phosphate receptor on ECs [42,43].

To date, no studies have directly investigated if and how IgG glycans modulate BBB influx and efflux. One notable study showed that full deglycosylation of anti-A β IgG did not impair brain amyloid reduction in AD mouse models but did not specifically address brain penetration [44]. However, lines of recent evidence have led to the study presented here.

An earlier study of human polyclonal anti-A β IgG indicated that both influx and efflux across the BBB were faster for mouse mAb 6E10 than mAb 4G8 and polyclonal human anti-A β IgG [45]. This finding is somewhat surprising given that both mAbs 6E10 and 4G8 exhibit a similar affinity towards the same target, although different epitopes and IgG subclasses could explain the difference [46]. A subsequent investigation of 4G8 and 6E10 glycosylation showed that 4G8 and human anti-A β IgG exhibited surface accessible sialic acid groups that could be removed with neuraminidase [47]. By contrast, surface sialic acid was largely absent from 6E10 [47]. Taken together, these studies suggested that surface-accessible sialic acid could play a role in BBB transport kinetics.

Here, we directly tested the effect of presence and absence of glycosylation and glycan sialic acid on BBB influx and efflux of IgG using *in vitro* methods. This *in vitro* method enables the two opposing processes of influx and efflux to be studied specifically for BBB ECs, facilitating direct measurement of the Permeability Coefficient in each direction. The mAb 4G8 provides a useful model of IgG surface glycans because it exhibits a single, highly processed, surface-accessible N-linked glycan in the Fab light chain [47]. While interpretation of complex glycoproteins can be problematic, the use of 4G8 in the present study enables the effect of a single surface glycan to be studied. For reference, mAb 6E10 is used for comparison as it does not exhibit any detectable surface sialic acid and is also reported to have minimal sialic acid at the Fc glycan [47, 48].

2. Materials and Methods

2.1 Materials for IgG preparation

Monoclonal antibodies 6E10 and 4G8 (1 mg/ml in PBS) were purchased from Covance. A β _{1–42} peptide was purchased from Anaspec (San Jose, CA). *Sambucus nigra* agglutinin (SNA), *Erythrina crystalgalli* lectin (ECL), *Maackia Amurensis* leucoagglutinin (*Maackia*

Amurensis Lectin I or MAL I), and *Maackia Amurensis* hemagglutinin (*Maackia Amurensis* Lectin II or MAL II) were obtained from Vector Laboratories (Burlingame, CA). A Siglec-11 – Fc fusion protein (Siglec-11) was purchased from Fisher. Enzyme α 2,6 sialyltransferase from *Photobacterium damsela* (2,6ST) and substrate Cytidine-5'-monophospho-N-acetylneuraminic acid (CNA) were obtained from Sigma-Aldrich (St. Louis, MO). Recombinant neuraminidase from *Clostridium perfringens* (NEU) was purchased from New England Biolabs (Ipswich, MA). EndoS enzyme IgGZERO was obtained from Genovis AB (Lund, Sweden). Biacore running buffer containing 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% v/v, pH 7.4 (HBS-EP) was obtained from a 10X stock (GE Life Sciences).

2.2 IgG Enzyme Treatments

Sialic acid was removed from IgG using NEU treatment consisting of a 100 μ L IgG stock solution (100 μ g, 667 pmol) with 4 μ L NEU solution (200 Units, ~0.9 μ g). The fraction of sialic acid in IgG was increased using 2,6ST treatment consisting of 100 μ L IgG stock solution (667 pmol) containing 10 μ L of 15 mM CNA (150 nmol) and 1 μ L 2,6ST (25 milliunits, < 5 μ g). Fc glycans were removed from IgG antibodies using EndoS treatment consisting of a 100 μ L IgG stock solution (667 pmol) and 5 μ L IgGZERO solution containing 100 Units (~2 μ g) of EndoS enzyme. All enzyme treatments involved incubation at 37 °C for a minimum of 3 hours, followed by a 15 minute exposure to 55 °C to inactivate the enzyme. These procedures ensured that all enzymes prepared for blood-brain barrier permeability studies were both inactive and less than 5% of IgG by mass. An aliquot from each preparation was removed for surface plasmon resonance (SPR) analysis and the remainder was exhaustively dialyzed (5X) into chlorine-free buffer suitable for iodination (250 mM phosphate, pH 7.5).

2.3 SDS PAGE and Native PAGE

ALL GELS BOTH USED 7.5% MTNI-PROTEAN TGX PRECAST GELS (BIO-RAD) and electrophoresis was performed with a MINIPROTEAN II (BioRad) using KALEIDOSCOPE PRESTAINED STANDARDS (BioRad 161-0324) run in the end lanes. FOR SDS-PAGE, SAMPLES WERE MIXED 1:1 WITH LAEMMLI SAMPLE BUFFER (BIO-RAD) WITH A LOADING CONCENTRATION OF 1 % SDS, HEATED TO 95 °C FOR 2 MINUTES and electrophoresis performed with Tris-Glycine-SDS (0.1% SDS) running buffer. FOR NATIVE PAGE, SAMPLES WERE MIXED 2:1 WITH TRTCINE SAMPLE BUFFER (BIO-RAD) and electrophoresis performed with Tris-Glycine running buffer. BANDS WERE DETECTED USING COOMASSIE BLUE STAINING, FOLLOWED BY FIXING AND DESTAIN.

2.4 SNA-affinity column enrichment and depletion of SNA-binding IgG

A SNA affinity column was prepared by packing 300 μ L of a SNA-agarose slurry (Vector Labs) into a 1.0 ml spin column (Pierce) and washed 5X under gravity flow with a full column of 250 mM phosphate pH 7.5. A 100 μ L sample of commercial 4G8 (1 mg/ml) was applied to the column and this eluate collected (aliquot 1). A series of 4 additional flow-through elution steps followed with 400 μ L, 400 μ L, 800 μ L, and 400 μ L of 250 mM phosphate pH 7.5. Upon completion of the flow through stage, a series of 6 elution steps for SNA-

binding IgG followed with 100 μ l, 100 μ l, 100 μ l, 400 μ l, and 100 ml of 250 mM phosphate, 0.5 M lactose, pH 7.5.

2.5 Production of Denatured Cysteine-Capped IgG Heavy and Light Chains

A 25 μ l sample of IgG at 833 μ g/ml, with or without enzyme treatment, was mixed with 75 μ l of 6 M Guanidinium Hydrochloride in Tris-HCl pH 8.6 and 1 μ l of beta-mercaptoethanol, sealed in a gas chromatography vial with a cap and septa insert, purged with nitrogen (2 min), and held under positive N₂ gas pressure at room temperature for 3 hours. In a darkroom, a volume of 40 μ l of freshly prepared 0.5 M iodoacetamide was added dropwise to the solution under a light vortexing, after which the sample was covered in foil and incubated in the dark for 30 minutes at 37 °C. Denatured IgG heavy and light chains were removed from reactants and exchanged into HBS-EP running buffer for SPR analysis using 7 kD MWCO Zeba spin columns (ThermoFisher). The recovery of both light and heavy chains was confirmed using SDS-PAGE (Section 2.3).

2.6 Fc production by Papain

A 16.2 μ l solution of 2 mg/ml papain was made from 10 μ l of 0.1 M sodium acetate, 1 mM EDTA, pH 5.5 stock, 5 μ l of 1M cysteine stock, and 1.2 μ l papain stock (28 mg/ml) and incubated prior to use at 35 °C for 15 minutes. Following this initial incubation, 6.4 μ l of the activated papain solution was added to 34 μ l of IgG at 1 mg/ml in PBS buffer (reaction conditions, 842 μ g/ml IgG, 317 μ g/ml papain) and incubated on a shaker table set to 200 RPM for 2.5 hours. The reaction was quenched with addition of 1.6 μ l of 0.5 M iodoacetamide, a subsequent 30 minute incubation at room temperature in the dark, followed by addition of 0.8 μ l of 1 M Tris pH 9.0. CLEAVAGE OF FAB AND FC FRAGMENTS BY THIS PAPAIN PROCEDURE, AND COMPARISON TO SHAM-CONTROLS OF IGG, WERE CONFIRMED USING BOTH SDS-PAGE AND NATIVE PAGE (SECTION 2.3).

2.7 SPR Sensor Chip Preparation and Regeneration

SPR flow cells with SNA, other lectins, Protein A (SpA), and A β ₁₋₄₂ oligomers (OAB) were prepared as described previously [47]. All SPR chip preparation and subsequent studies were performed using either a Biacore T200 system using Series S CM5 sensor chips (GE Healthcare) as described in previous work [47]. CM5 carboxyl group activation was performed using 7 min of a 1:1 mix of 50 mM N-hydroxysuccinimide (NHS) and 0.2 M N-ethyl-N-dimethylaminopropylcarbodiimide (EDC). Ligand immobilization was then conducted at 5 μ L/min in pH 4 10 mM acetate buffer for 7200 s with oligomeric A β (OAB) immobilization (described below) and 1200 s for SNA, SpA, and other lectins (1 mg/ml in all cases). After ligand immobilization, remaining activated groups were capped using 1.0 M ethanolamine (pH 8.5) for 7 min. After this procedure, flow cells exhibited between 6000–8000 RU of immobilized ligand.

While SNA, other lectins, and SpA immobilization solutions were prepared directly from commercial products, the OAB sensor design required additional procedures prior to and after immobilization. Briefly, commercial A β ₁₋₄₂ was disaggregated in 50:50 trifluoroacetic acid (TFA):hexafluoroisopropanol (HFIP) at a 1 mg:1 ml peptide:solvent ratio, bath sonicated for 1 h, dried under nitrogen, resuspended as monomeric peptide at 20 mg/ml in

dimethylsulfoxide (DMSO), diluted 1/40 in 100 mM phosphate, 100 mM NaCl, pH 7.4, centrifuged at $14,000 \times g$ for 10 min, filtered through a 10,000 MWCO Amicon filter [49, 50]. This $A\beta_{1-42}$ solution concentration was $104 \mu\text{M}$ by $e_{280} = 1200 \text{ cm}^{-1} \text{ M}^{-1}$ and close to the theoretical value of $114 \mu\text{M}$ [51]. This preparation was incubated at 25 deg C for a minimum of 3 weeks converting nearly all monomeric $A\beta_{1-42}$ into a roughly 2:1 transient equilibrium of soluble oligomer/insoluble fibrillary states. Immediately prior to immobilization, this $OA\beta$ preparation was centrifuged at $12000 \times g$, supernatant dialyzed into 10 mM acetate pH 4, and re-centrifuged again at $12000 \times g$. After immobilization, the $OA\beta$ sensor surface was treated with 0.2% SDS for a minimum of ten 60 sec pulses, after which these 0.2% SDS pulses were continued until the instrument response was stable within ± 0.5 Response Unit (RU) over 1 min. Using this procedures, the $OA\beta$ flow cell exhibited 2305 RU of immobilized ligand.

After each IgG binding measurement in the $OA\beta$ flow cell, regeneration was performed using one 30 s pulse of 0.2% SDS. After each IgG binding measurement in flow cells with immobilized SNA, SpA, or other lectin ligand, regeneration was performed using two 20 s pulses of 10 mM HCl.

2.8 Calibration-Free Concentration Analysis of IgG glycans

The concentration of total IgG and SNA-binding IgG in each sample was determined independently for each sample using calibration-free concentration analysis (CFCA) [52, 53]. CFCA was performed using a slightly modified SPR procedure of that used in previous work [47]. The slope of SPR binding from two sequential injections of IgG was measured for 60 s at different flow rates, 5 and 50 $\mu\text{L}/\text{min}$. The middle time points (20–55 s) of the binding response in standard SPR response units (RU) was used in determining the slope to avoid signal artifacts at the start and end of the injection period.

Once the two slopes (RU/sec) were calculated for low flow (m_1) and high flow (m_2) conditions, the molar concentration of IgG with specific binding to the immobilized ligand [IgG_{ligand}] was determined using Eq. 1 [47].

$$[\text{IgG}_{\text{ligand}}] = \frac{\beta}{\frac{1}{m_1} - \frac{1}{m_2}} \quad (1)$$

In Eq. 1, the coefficient $\beta = 5.5 \times 10^{-10} (\text{mol} \cdot \text{s})/(\text{L} \cdot \text{RU})$ incorporates all known variables of IgG properties and dimensions of the Biacore T200 flow cell [52, 53]. The SNA flow cell provides the concentration of IgG with surface accessible sialic acid- $\alpha 2,6$ -galactose linkage, denoted here as $[\text{IgG}_{\text{SNA}}]$ [54]. Since both 6E10 and 4G8 bind $OA\beta$, the $OA\beta$ flow cell provides the total IgG concentration, denoted here as $[\text{IgG}_{\text{OA}\beta}]$. After correcting concentration for any dilution prior to CFCA, the fraction of SNA-binding IgG in 4G8 and 6E10 (F_{SNA}) was determined as shown in Eq. 2.

$$F_{\text{SNA}} = \frac{[\text{IgG}_{\text{SNA}}]}{[\text{IgG}_{\text{OA}\beta}]} \quad (2)$$

Similar values were not determined for F_{ECL} , $F_{\text{MAL I}}$, $F_{\text{MAL II}}$, and $F_{\text{Siglec-11}}$ as no binding of [IgG] was observed with these sensors. For ECL, this is the result of its weak affinity for galactose, and would require $> 10 \mu\text{M}$ of IgG with surface galactose groups to produce a binding signal [55]. While sensor affinity of lectins MAL I, MAL II, and Siglec-11 may also preclude a signal on these sensors, it is also likely that their uncommon target glycan groups actually exist on 6E10 and 4G8.

2.9 On-Chip Analysis of IgG glycans

As a complementary method to CFCA, On-Chip “piggyback” method was used to determine the extent that intact IgG or Fc fragments present specific surface-accessible glycan groups containing sialic acid (Sia), galactose (Gal), and N-acetylglucosamine (GlcNAc) [47].

Lectins used to probe these specific configurations are SNA (Sia- α 2,6-Gal), ECL (Gal- β 1,4-GlcNAc), MAL I (Sia- α 2,3-Gal- β 1,4-GlcNAc), MAL II (Sia- α 2,3-Gal- β 1,3-GalNAc), and Siglec-11 (Sia- α 2,8-Sia) [54–57]. Using these lectin probes, the On-Chip method was used to determine the presence of surface glycans on 4G8 and 6E10 with their particular sialic acid target permutations.

The On-Chip method involves an initial “capture” of intact 4G8 and 6E10 IgG to the OA β sensor surface or 4G8 and 6E10 IgG and Fc fragments to the SpA sensor surface (Scheme 1). The capture step was followed with a second lectin-binding “probe” step using excess Siglec-11 (0.6 μM), MAL I (1 μM), MAL II (1 μM), ECL (11 μM), or SNA (1 μM). The lectin-binding response to sensor-bound IgG or Fc ligand, denoted inclusively as $R_{\text{L:IgG}}$, was determined using Eq. 3.

$$R_{\text{L:IgG}} = \frac{(RU_{\text{L+IgG}} - RU_{\text{L}})}{RU_{\text{IgG}}} \quad (3)$$

In Eq. 3, RU_{IgG} is RU of the first binding step involving IgG or Fc domain (500–1000 RU being ideal). $RU_{\text{L+IgG}}$ is RU produced in the second binding of lectin with IgG or Fc bound to the sensor. RU_{L} is a control of background lectin binding to the flow cell without IgG or Fc bound. RU_{L} is subtracted from $RU_{\text{L+IgG}}$ to determine the actual response due to lectin:IgG or lectin:Fc interaction. Representative SPR sensograms of these measurements are shown in Scheme 1.

For clarity, variations of this On-Chip method used in the present study are shown in Schemes 2–4. Schemes 2 and 3 show representative On-Chip experiments that determine $R_{\text{L:IgG}}$ for surface-exposed α 2,6 Sia-Gal groups on intact anti-OA β IgG (both 4G8 and 6E10) and SpA-binding IgG (4G8 only) respectively. Under these conditions, α 2,6 Sia-Gal groups on Fab glycans are detected while Fc glycans are not bound to a significant degree by

SNA lectin in Step 2. Scheme 4 shows a representative On-Chip experiment that determines $R_{L:Fc}$ for surface-exposed $\alpha 2,6$ Sia-Gal groups on Fc domains. Two differences between intact IgG On-Chip studies shown in Scheme 3 and Fc On-Chip studies shown in Scheme 4. First, both 4G8 and 6E10 Fc fragments derived from papain treatment were shown to bind the SpA sensor in Scheme 4 while only intact 4G8, but not 6E10, bound the SpA sensor in Scheme 3. Second, $\alpha 2,6$ Sia-Gal groups on Fc glycans were exposed for SNA binding to a greater degree in papain-generated Fc fragments (Scheme 4) than with intact IgG (Schemes 2 and 3).

An advantage of the On-Chip method is that multiple lectins can be tested for binding in a single experiment on a single capture sensor. Due to the high affinity of SNA for the Sia- $\alpha 2,6$ -Gal group, both CFCA and On-Chip are possible [47]. However, lower-affinity lectins, such as ECL, do not meet this requirement for CFCA and their binding to IgG can only be assessed under saturation conditions with the On-Chip method [47, 55]. A disadvantage of the On-Chip method is that the relative response of the On-Chip method depends on steric accessibility and can vary with different glycoproteins, lectin probes, and sensor ligands [47]. While lacking the accuracy of CFCA, the lectin probe binding of the On-Chip method does increase linearly with glycan availability on the captured target [47]. As such, it is useful to determine if a glycan is exposed on a glycoprotein and/or compare two glycoprotein variants such as IgG species.

2.10 SNA binding to Denatured IgG

Preparations of denatured IgG (Section 2.4) could not be analyzed with either CFCA, due to the high diffusion of the free chains, or the On-Chip method (no Step 1 capture). Thus, no reliable measure of total glycans per IgG chain, F_{SNA} or $R_{L:IgG}$, was possible. However, the relative fraction of total SNA-binding attributed to the Fab glycans versus Fc glycans (F_{SNA}^{Fab}) could be estimated. First, a measurement of the SPR binding response after 2 minutes of exposure to a SNA sensor by $\alpha 2,6$ sialylated Fab glycans in denatured IgG from which Fc glycans have been previously removed with EndoS treatment ($RU_{SNA}^{Denatured} \propto S_{EndoS}$ in Scheme 5). Next, the same measurement was made using denatured IgG with no prior enzymatic treatment ($RU_{SNA}^{Denatured} \propto S_{Untreated}$ in Scheme 6). Although mass transport limitations were not detected with denatured IgG, a high flow rate (50 μ l/min) was used to minimize any such complications. In the absence of mass transport, the intrinsic k_{on} and k_{off} of exposed $\alpha 2,6$ Sia-Gal groups in denatured IgG should be equal regardless of location within light or heavy chains. As such, the binding rate will depend only on concentration although the magnitude of the signal will differ for heavy and light chains.

Because $S_{Untreated}$ provides a measure of the total Fab and Fc $\alpha 2,6$ sialylated glycans, the ratio of S_{EndoS} to $S_{Untreated}$ provides an estimate of the fraction of SNA-binding sialic acid associated with Fab glycans (Eq. 4):

$$F_{TotalSNA}^{FabSNA} = \frac{S_{EndoS}}{S_{Untreated}} \quad (4)$$

Eq. 4 assumes the IgG concentrations in the EndoS and Untreated preparations are equal. Prior to denaturation, the concentrations of native [IgG] were measured using CFCA and confirmed to be equal. In the event that they differed by more than 5%, the concentration-corrected signal of SNA binding (S_C) to the denatured IgG chains was used for both S_{EndoS} and $S_{\text{Untreated}}$ (shown below in Eq.5).

$$S_C = \frac{RU_{\text{SNA}}^{\text{Denatured}}}{[\text{IgG}]_{\text{CFCA}}^{\text{Native}}} \quad (5)$$

A value of $F_{\text{TotalSNA}}^{\text{FabSNA}} = 1$ indicates that all $\alpha 2,6$ Sia-Gal groups are located on glycans in the Fab domains. A value of $F_{\text{TotalSNA}}^{\text{FabSNA}} = 0$ indicates that all $\alpha 2,6$ Sia-Gal groups are located on the conserved Fc glycan. Values of $F_{\text{TotalSNA}}^{\text{FabSNA}}$ between 0–1 estimated the relative fraction of $\alpha 2,6$ Sia-Gal groups found on the Fab glycans. Since SPR signal is proportional to analyte mass, Fc glycans are found exclusively on the heavy chains, and Fab glycans can be found on both heavy and light chains, the fractional value of $F_{\text{TotalSNA}}^{\text{FabSNA}}$ is a lower bound estimate of actual Fab sialylation. Lastly, it is emphasized here that a high fractional value of $F_{\text{TotalSNA}}^{\text{FabSNA}}$, i.e. a value of 1, does not indicate that all IgG is sialylated and only provides the relative ratio of Fab vs. Fc $\alpha 2,6$ -sialylation. The fraction of all IgG that is sialylated is best determined using the quantitative CFCA measurement of F_{SNA} in Eq. 2.

2.11 Animals

All mice were treated in accordance with NIH Guidelines for the Care and Use of Laboratory Animals in an AAALAC-accredited facility and approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Puget Sound Health Care System. Male CD-1 mice at 6–8 weeks of age were purchased from Charles River and kept on a 12/12-h light/dark cycle with ad libitum food and water.

2.12 Radioactive probes

IgG 4G8 and 6E10 were labeled with ^{125}I by the chloramine-T method, and the resulting ^{125}I -4G8 and ^{125}I -6E10 was purified on a G-10 Sephadex column [20]. BSA was labeled with $^{99\text{m}}\text{Tc}$ (technetium-labeled albumin, Tc-Alb) and purified on a G-10 Sephadex column [20].

2.13 Isolation of primary brain microvascular endothelial cells (BMECs)

Primary brain microvascular endothelial cells (BMECs) were isolated from 8-week-old CD-1 mice according to existing protocols with some modifications [58, 59]. BMECs were cultured in BMEC medium, consisting of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 20% plasma-derived fetal bovine serum (Animal Technologies), 1% GlutaMAX (Life Technologies), basic fibroblast growth factor (bFGF, 1 ng/ml; Roche Life Sciences), heparin (100 $\mu\text{g}/\text{ml}$), insulin (5 $\mu\text{g}/\text{ml}$), transferrin (5 $\mu\text{g}/\text{ml}$), selenium (5 ng/ml) (insulin-transferrin-selenium medium supplement; Life Technologies), and gentamicin (50 $\mu\text{g}/\text{ml}$; Sigma Aldrich). Puromycin (4 $\mu\text{g}/\text{ml}$; Sigma Aldrich) is added to

the BMEC medium for the first 48 h after plating to remove pericytes and increase endothelial cell purity [60]. Cultures were maintained at 37 °C in a humidified atmosphere of 5 % CO₂/95 % air. The medium is changed 24 h after plating to remove non-adhering cells, red blood cells, and debris. At 48 h after plating, the medium was changed again with a new medium containing all the components listed above, except puromycin. The purified primary BMECs were used to construct *in vitro* models when 80% confluent, typically the 5th day after isolation.

2.14 Construction of *in vitro* blood-brain barrier model

BMECs were cultured according to previously published methods [61]. BMECs were briefly trypsinized and seeded on the inside of the Transwell insert (0.33 cm², 0.4-μm pore size; Corning Inc.), which was coated with fibronectin and collagen type IV (0.1 mg/ml, each), at a density of 4 × 10⁴ cells per well. The inserts were then placed in a 24-well plate. The medium used to plate the cells in the 24-well Transwells contained all the components of the BMEC medium, listed above, with the addition of 500 nM hydrocortisone to reinforce tight junctions [62]. The medium in the luminal chamber (or the inside of the Transwell insert) was changed 24 h after seeding. BMEC monolayers were cultured for 3 days before use in experiments. An EVOM volt ohmmeter equipped with a STX-2 electrode (World Precision Instruments; Sarasota, FL) was used to measure transendothelial electrical resistance (TEER, in ohms per square centimeter) prior to treatment. The TEER of cell-free Transwell-clear inserts was subtracted from obtained values.

2.15 Transendothelial permeability

For permeability experiments, Transwell inserts (0.33 cm², 0.4-μm pore size; Corning Inc.) were washed with serum-free BMEC + HC medium + 0.1 % BSA (BHB medium). For influx experiments, 600 μl of BHB medium was added to the wells of a new 24-well plate (abluminal chamber), and the washed inserts were placed in this plate. To initiate influx transport experiments at time = 0 min, a 100 μl loading solution containing ¹²⁵I-IgG (10⁶ cpm/ml) and 99mTc-albumin (10⁶ cpm/ml) in BHB medium was added to the inner luminal (blood) insert chamber. Samples (500 μl) were collected from the outer abluminal (brain) chamber at 10, 20, 30, and 45 min. For efflux experiments, a 600 μl loading solution containing ¹²⁵I-IgG (10⁶ cpm/ml) and 99mTc-albumin (10⁶ cpm/ml) in BHB buffer was added to the outer abluminal (brain) chamber. To initiate efflux transport experiments at time = 0 min, Transwell inserts containing 100 μl of BHB buffer were placed over the plate containing the loading solution. Samples (50 μl) were collected from the abluminal (outer/ brain) chamber at 10, 20, 30, and 45 min. When samples were removed from either abluminal or luminal chambers, an equal volume of fresh BHB medium was immediately added to replace the volume removed chamber. Each sample was acid precipitated with an equal volume of 30% trichloroacetic acid and centrifuged at 5500×g for 10 min at 4 °C. The pellet was counted in a gamma counter for quantity of 99mTc and ¹²⁵I in the sample. The supernatant was removed by aspiration and discarded.

The permeability coefficient and transport of radioactivity was calculated according to previously published methods [63]. Transport was expressed as microliters of radioactive tracer diffusing from the luminal to abluminal chamber (influx) or abluminal to luminal

chamber (efflux). For a given measurement, the transported material in the collection chamber at a given measurement time point was calculated as shown in Eq. 6.

$$T(\mu l) = \frac{V_L * R_S}{\alpha * R_L} \quad (6)$$

In Eq. 5, R_L is the initial amount of radioactivity in the loading chamber, V_L is the volume of the loading chamber (in μl), R_S = the measured amount of radioactivity in the sample aliquot drawn from the collecting chamber, and $\alpha = V_S/V_C$ where V_C is the total volume of the collecting chamber and V_S is the volume of sample aliquot drawn from the collecting chamber ($V_S < V_C$).

If multiple collections were performed at consistently separated time points, Eq. 7 was used to determine the total cumulative transport (T_i) at collection iterations $i > 1$ beyond the initial collection event of $i = 1$:

$$T_i(\mu l) = \frac{i V_L R_i^S}{R_L (1 - (1 - \alpha)^i)} \quad (7)$$

In Eq. 6, R_i^S is the measured amount of radioactivity in the i^{th} sample aliquot drawn from the collecting chamber. If the time between collections was not constant, the cumulative amount of transported volume T_i was calculated numerically, accounting for material removed in the preceding sampling events.

The cumulative transport volumes were confirmed to increase linearly with time. The cumulative transport T_i was plotted versus time, and the slope was estimated by linear regression analysis. The slope of clearance curves for the BMEC monolayer plus Transwell membrane was denoted by PS_{app} , where PS is the permeability $\times$ surface area product (in microliters per minute). The control slope of transport with a Transwell membrane without BMECs was denoted by $PS_{membrane}$. The true PS value for the BMEC monolayer (PS_e) was calculated from Eq. 8 [64]:

$$\frac{1}{PS_e} = \frac{1}{PS_{app}} - \frac{1}{PS_{membrane}} \quad (8)$$

The PS_e values were divided by the surface area of the Transwell inserts (0.33 cm^2) to generate the endothelial permeability coefficient (P_e , in microliters per minute per square centimeter).

In the present work, the permeability coefficient of IgG is corrected by subtracting the permeability coefficient of the BSA internal control through Eq. 9.

$$\Delta P_e = P_e(IgG) - P_e(BSA) \quad (9)$$

BSA predominantly passes through the BBB via extracellular pathways, although some evidence exists for limited efflux transport of albumin by the neonatal Fc receptor [65]. A

$P_e > 0$ value indicates that a transport system of IgG is present. A $P_e = 0$ indicates that IgG predominantly passes through the BBB via extracellular pathways. A $P_e < 0$ indicates that a mechanism exists which interferes with the flow of IgG across the BBB.

2.16 Statistical analysis

For ^{125}I -4G8 influx and efflux, two experiments were performed consisting of five replicates each and three additional experiments were performed consisting of four replicates each ($n=22$). For ^{125}I -6E10 influx and efflux, one experiment was performed consisting of five replicates and a four experiments were performed consisting of four replicates ($n=21$). For both ^{125}I -4G8 and ^{125}I -6E10 influx and efflux in the presence of 67 nM IgG, two experiments were performed consisting of five replicates each and one additional experiment was performed consisting of four replicates ($n=14$). Influx and efflux of all enzyme-treated IgG was performed with three experiments consisting of four replicates each ($n=12$). All On-Chip SPR analysis was performed with two replicates ($n=2$) except for Papain-Treated 4G8 ($n=4$).

Statistical analysis was performed using the statistical functions and ANOVA toolpack of Microsoft Excel 2010. Prior to more intensive statistical analysis, single variable ANOVA was used on all data sets to avoid Type I errors and confirm statistically significant differences. If the ANOVA p-value was less than 0.05, a two-tailed student's t-test was performed on each data pair to confirm a $p < 0.05$. Figures denote different measurements that are not significantly different ($p > 0.05$) with the same letter (A,B,C,D). In qualitative analyses, an asterisk (*) was used to denote a value with a statistically significant positive value ($p > 0.05$).

3. Results

3.1 Antibody influx and efflux rates

Fig.1 shows the albumin-adjusted influx and efflux rates, expressed as IgG Permeability Coefficient P_e ($\mu\text{l}/\text{min}\cdot\text{cm}^2$) for both ^{125}I -4G8 and ^{125}I -6E10, determined with radioactively labeled antibody only and with addition of an excess of unlabeled antibody (67 nM). The P_e of both ^{125}I -4G8 and ^{125}I -6E10 influx was higher than that of albumin ($P_e=0$ designated for albumin). Addition of excess unlabeled antibody did not change the influx rate of either ^{125}I -4G8 and ^{125}I -6E10.

The efflux P_e of 4G8 was significantly lower than albumin ($P_e = -0.0041$) while the efflux P_e of ^{125}I -6E10 is significantly higher than albumin ($P_e = +0.0054$). The P_e of ^{125}I -6E10 efflux was statistically equivalent to ^{125}I -6E10 influx ($P_e = +0.0048$) but higher than ^{125}I -4G8 influx ($P_e = +0.0033$). Addition of excess of unlabeled antibody (67 nM) did not produce a change in either ^{125}I -4G8 or ^{125}I -6E10 influx and efflux P_e that was statistically significant at the level of $p < 0.05$.

3.2 Effect of glycosylation changes on 4G8 influx and efflux rates

Figures 2ABC show the impact of altering the glycosylation state of 4G8 on BBB influx and efflux. Figure 2A shows the fraction of 4G8 that exhibits the Sia- α 2,6-Gal linkage in a surface accessible position, as assessed by binding to SNA, a highly specific lectin to this glycoconjugation. Compared with untreated 4G8 ($F_{\text{SNA}} = 0.12$), neuraminidase treatment eliminated nearly all SNA binding. Treatment with α 2,6-sialyltransferase increased the fraction of SNA binding to $F_{\text{SNA}} = 0.15$. Removal of the Fc glycan with EndoS did not significantly alter 4G8 F_{SNA} . Both 6E10 ($F_{\text{SNA}} = 0.016$) nor EndoS-treated 6E10 ($F_{\text{SNA}} = 0.016$) exhibited low SNA binding. These results of the enzyme treatments in Figure 2A are consistent with a previous study [47]. The SNA binding to 4G8 was low ($F_{\text{SNA}} = 0.12$) but within the previously published lot-to-lot variation in commercial 4G8 ($F_{\text{SNA}} = 0.1\text{--}0.4$) [47].

Figure 2B shows the P_e of influx for 4G8 to be unchanged after the three enzyme treatments. However, EndoS treatment of 6E10 did produce a lowered influx rate ($P_e = +0.0014$) that was statistically significant relative to untreated 6E10. However, this difference was modest and was not statistically significant between EndoS-treated 6E10 and 4G8 treated with NEU or 2,6ST.

By contrast, the P_e of efflux in Figure 2C showed significant differences between untreated, neuraminidase-treated, and EndoS-treated 4G8. In particular, neuraminidase treated 4G8 resulted in an increased efflux ($P_e = 0.0076$) that equals 6E10 efflux within error. However, increasing the sialic acid content of 4G8 with 2,6ST did not produce a significant change from untreated 4G8. As such, further enhancement of sialic acid with additional β 1,4-galactotransferase co-treatment was not pursued at this time. Interestingly, the efflux of EndoS-treated 4G8 ($P_e = 0.0007$) was higher than untreated 4G8 but less than NEU-treated 4G8. This effect was not observed for EndoS-treated 6E10 ($P_e = 0.0043$) which showed an equivalent efflux rate as untreated 6E10.

3.3 Measurement of sialic acid on SNA-column depleted and enriched IgG

Figure 3 shows the CFCA-determined 4G8 concentration in eleven elution stages of SNA affinity chromatography. After integration of the flow through peaks in aliquots 1–5 and lactose elution aliquots 6–11, SNA-binding 4G8 was found to be a 0.07 fraction of total 4G8, with a small bleed of residual SNA-binding 4G8 continuing to elute after aliquot 11. Considering the additional 4G8 on the column, this fraction of 0.07 is consistent with the $F_{\text{SNA}} = 0.12$ found from direct 4G8 binding to the SNA sensor in Figure 2A. CFCA analysis of flow-through aliquot 2 ($F_{\text{SNA}} = 0.00 \pm 0.00$) and the highest elution peak in aliquot 9 ($F_{\text{SNA}} = 0.92 \pm 0.07$) produced expected values at or close to 0.0 and 1.0 respectively. Consistent with previous studies, the On-Chip method (shown in Schemes 1 and 2) scaled linearly with CFCA but with a less-than-expected value of $R_{\text{L:IgG}} = 0.59 \pm 0.08$ in 100% α 2,6-sialylated 4G8 [47]. This 1.5-fold difference has been shown to result from inherent steric hindrance of SNA access to the 4G8 glycan during the On-Chip analysis [47]. Having established this calibration factor both here and in previous work, the On-Chip method is a useful semi-quantitative method in identifying surface-accessible glycans with specific lectin probes on 4G8 and other IgG species. Also consistent with the $F_{\text{SNA}} = 0.01$ for 6E10 in

Figure 2A, SNA affinity chromatography could not produce a measurable amount of SNA-binding 6E10 in the lactose elution fractions (data not shown).

3.4 Measurement of α 2,3 and α 2,8 linked sialic acid on IgG

A semi-quantitative On-Chip SPR analysis of lectin affinity was performed on 4G8 and 6E10 to determine if a significant fraction of each IgG species contains carbohydrates that bind Siglec-11, Mal-I, Mal-II, ECL, and SNA (as per Schemes 1 and 2). Consistent with previous studies and Figure 2A in the present work, SNA, but not ECL, bound untreated, 2,6ST-treated, and EndoS-treated 4G8 (Fig. 4A). Also consistent with previous work, NEU-treated 4G8 bound ECL but no longer bound SNA (Fig. 4A). However, no significant binding of lectins specific to other sialoglycoforms, Siglec-11, Mal-I, and Mal-II, was found with any 4G8 preparation (Fig. 4A). Consistent with previous studies and Figure 2A in the present work, a small but significant level of SNA binding was found for untreated 6E10 (Fig. 4B) [47]. Also, a small but significant level of MAL II binding against EndoS-treated 6E10 but not the other 6E10 preparations.

3.5 Measurement of Fc sialic acid on IgG

An analysis of 4G8 and 6E10 Fc glycosylation is shown in Figures 5ABC. Figure 5A shows the fraction of total SNA binding attributed to the Fab domain ($F_{TotalSNA}^{FabSNA}$ in Eq. 4, Schemes 5 and 6). The value of $F_{TotalSNA}^{FabSNA}$ was 0.83 for 4G8 and 0.35 for 6E10. To confirm the accuracy of these measurements of EndoS and Untreated IgG in the denatured state, the relative binding of EndoS and Untreated 4G8 and 6E10 in the native state was measured with the same SNA sensor (Schemes 5 and 6 but using native IgG). These reference measurements with intact IgG showed a lack of EndoS effect on native 4G8 ($F_{TotalSNA}^{FabSNA}=1.04$) and marginal EndoS effect ($F_{TotalSNA}^{FabSNA}=0.80$) on native 6E10. The relative magnitudes of EndoS effect on native 4G8 and 6E10 reference measurements in Fig. 5A are consistent with those of previous work and of Fig. 2A and 4AB in the present study [47]. Also consistent with Fig. 4AB in the present work, no significant binding of either native or denatured 4G8 or 6E10 was detected against SPR sensors containing immobilized ECL, MAL I, MAL II, or Siglec-11 (data not shown). In light of these controls, values of $F_{TotalSNA}^{FabSNA}$ derived from SPR measurements of denatured IgG are expected to be accurate.

The On-Chip method (Eq. 3) was used with a SpA sensor to capture free Fc domains of 4G8 and 6E10 and probe these domains for SNA and ECL binding (Scheme 4). Prior to experiments with isolated Fc, a control experiment with intact 4G8 on the SpA sensor (Scheme 3, Fig. 5B) was compared with intact 4G8 on the OA β sensor (Scheme 2, Fig. 4A). Both SpA and OA β sensors in Fig. 5B and 4A showed similar results for untreated 4G8, with significant SNA binding and minimal ECL binding. A higher SNA binding was noted with 4G8 captured on the SpA sensor (Scheme 3, Fig. 5B) versus the OA β sensor (Scheme 2, Fig. 4A) which may reflect a different steric accessibility of SNA to the 4G8 Fab glycan between the two binding modes. Intact 6E10 was not studied because it did not bind the SpA sensor. However, the isolated Fc domain of 6E10 bound the SpA sensor at a comparable level as the 4G8 Fc domain and could be readily measured (Fig. 5C).

Figure 5C shows the fraction of free 4G8 and 6E10 Fc domains that bind SNA and ECL (represented in Scheme 4 for SNA). The complete cleavage of both 4G8 and 6E10 by papain is shown in the inset to Figure 5C. A significant level of both SNA ($R_{L:Fc} = 0.16$) and ECL ($R_{L:Fc} = 0.09$) binding was measured with the 4G8 Fc domain. As expected, both NEU and EndoS treatment of 4G8 reduced SNA binding significantly. By contrast, ECL binding to 4G8 Fc was not significantly altered by NEU or EndoS treatment. Given that ECL binding is readily modulated by NEU and EndoS treatment with intact 4G8 (Fig. 4A), the inability of these same enzymes to alter its binding to free 4G8 Fc suggests that ECL is not binding Gal residues on the free 4G8 Fc domain. A likely possibility is that weak nonspecific binding occurs under this On-Chip experiment conditions of immobilized papain-generated 4G8 Fc under high ECL concentrations.

Figure 5C also shows a relatively low level of SNA binding ($R_{L:Fc} = 0.03$) measured for the 6E10 Fc domain. Nonetheless, treatment with EndoS showed a significant reduction in SNA binding ($R_{L:Fc} = 0.007$) although NEU treatment did not show a significant effect. ECL binding to 6E10 Fc was low ($R_{L:Fc} = 0.016$) and not significantly altered by either NEU or EndoS treatment.

The fraction of Fc α 2,6-sialic acid for intact 4G8 and 6E10 (F_{SNA}^{Fc}) can be estimated from F_{SNA} in Figure 2A, $F_{TotalSNA}^{FabSNA}$ in Figure 5A with the assumption that Fc SNA binding is zero for intact IgG. A second estimate of F_{SNA}^{Fc} can be also be made using untreated and EndoS-treated $R_{L:Fc}$ values, the known Fc/SNA mass ratio of 0.36 for SPR scaling, and the assumption that SNA binding to Fc sialic acid is uninhibited in free Fc. Table 1 shows both estimates for 4G8 and 6E10.

All F_{SNA}^{Fc} estimates are low (<0.05) for both 4G8 and 6E10. These estimates are consistent with low Fc sialylation found with many IgG antibodies studied by mass spectroscopy [48, 66]. The F_{SNA}^{Fc} estimates of 0.009 and 0.026 for 6E10 are also consistent with a fraction of 0.022 reported from a mass spectrometry profiling of the 6E10 Fc glycan [48]. Given that other sialic acid-specific lectins did not bind denatured 4G8 (data not shown) or the free 4G8 Fc domain (Figure 5), there is strong evidence that the Fc domains of 4G8 and 6E10 are largely asialylated.

4. Discussion

By using a simple single-Fab glycan IgG model (4G8), quantitative glycan analysis methods (CFCA and On-Chip SPR), and *in vitro* BBB models, the present study was able to directly determine an inhibitory effect of α 2,6 linked sialic acid on IgG efflux through BBB endothelial cells. The *in vitro* BBB model was ideal for accurate measurement of the BBB

P_e for influx and efflux of anti-A β IgGs 4G8 and 6E10 in the absence of other complicating factors [43]. In so doing, changes to influx and efflux that may have been obscured during *in vivo* studies were more readily observed. In addition, the ability to accurately quantify the fraction of IgG with sialic acid by SPR methods was essential to revealing a new potentially translational discovery. The primary findings of the present study are summarized below:

1. The influx P_e is similar for 4G8 and 6E10 and is largely insensitive to changes in their glycosylation states (Fig. 1 and 2B).
2. The efflux P_e of 4G8 significantly lower for α 2,6-sialylated 4G8 than for predominantly asialylated 6E10 (Fig. 1).
3. Sialic acid is responsible for the lower efflux P_e of 4G8. Neuraminidase removes the sialic acid and equalizes the 4G8 efflux P_e to that of 6E10 (Fig. 2C).
4. Removal of the Fc glycan partially abrogates the inhibitory effect of sialic acid on 4G8 but has no effect on 6E10 efflux (Fig. 2C).
5. A significant reduction 4G8 efflux results from a relatively small fraction (12%) of 4G8 exhibiting surface α 2,6 sialic acid (Fig 2A and Fig. 3).
6. Increasing the 4G8 sialylation fraction to 15% produced equally low efflux as 12% (Fig. 2).
7. No sialic acid linkages other than α 2,6 exist on the surface of 4G8 (Fig. 4).
8. Fc sialylation of 4G8 is very low (1–4%) and is only α 2,6-linked (Fig. 5).

For gp120 BBB influx, removal of gp120 glycans reduced BBB influx [40, 42]. Given this precedent, the effect of IgG sialylation on BBB transport was expected to be an enhancement of influx. In this context, it was surprising that sialylation led to a reduction of efflux, with influx remaining largely unaffected. However, evidence for a reduced 4G8 efflux does exist in a prior *in vivo* study, which showed a longer residence time of 4G8 in the brain than 6E10 [45]. The present study provides an explanation for this observation, i.e that sialic acid on 4G8 impairs its efflux pathway.

An efflux-reducing role for IgG sialic acid is a noteworthy finding (Finding 3). However, it is equally significant that a majority of asialylated 4G8 was inhibited by a small fraction of sialylated 4G8 (Finding 5). Even if this 12% sialylated 4G8 population had a very low intrinsic efflux, the other 88% asialylated 4G8 should efflux at a rate comparable to 6E10, with only a minor change in the bulk 4G8 flow. Also, the increase in F_{SNA} to 0.15 after treatment of 4G8 with α 2,6-sialyltransferase did not result in a lower 4G8 efflux (Finding 6). This finding suggests that the sialic acid-sensitive efflux process is already maximally inhibited when the 4G8 F_{SNA} is 0.12. This observations warranted a test using the recovery and quantitative analysis of fully sialylated 4G8 purified from SNA affinity chromatography as confirmation that 100% sialylated 4G8 could be properly measured with CFCA (Figure 3). Since the methods were determined to be sound, it is concluded that 4G8 efflux does not follow a straightforward correlation with the surface sialic acid fraction.

While a number of explanations could account for these observations, two plausible hypotheses are discussed here. The first hypothesis is that all endogenous 4G8 exhibit reduced efflux because they all present sialic acid in a variety of linkage states and locations that are not detectable by surface SNA binding methods. In one version of this hypothesis, α 2,3 or α 2,8 sialylated glycans dominate 4G8 and that α 2,6 Sia-Gal linkages are a minor glycan component [54–57]. In a second version of this hypothesis, Fc sialylation or other

buried glycan groups with sialic acid are ubiquitous on all 4G8. With either case, this hypothesis accounts for the lack of effect of α 2,6-sialyltransferase on efflux since each 4G8 molecule already has sialic acid. This model also accounts for the effect of neuraminidase, which here would increase the intrinsic efflux of all 4G8 by removing all sialic acid groups from all 4G8 molecules.

A second hypothesis posits that only a minority of 4G8 is sialylated but that this minority can inhibit efflux of the remaining majority of asialylated 4G8. Since efflux is fully inhibited under the conditions here, excess unlabeled 4G8 (Fig. 1) or increased sialic level with α 2,6-sialyltransferase (Fig. 2C) would not be able to reduce it further. One possible mechanism of this second hypothesis could be that sialylated 4G8 directly outcompetes asialylated 4G8, i.e. binding to, but not being transported by, key transporters. If defined as an enzyme system, sialylated 4G8 would block the transporters as a competitive inhibitor of asialylated 4G8. The second possible mechanism of this hypothesis could be that sialylated 4G8 inhibits efflux through an indirect non-competitive process.

The hypothesis that all 4G8 possess sialic acid was directly tested in the present study. To test if α 2,3 and α 2,8 groups might be present, a panel of lectins were tested against both native and denatured 4G8: Siglec-11 for Sia- α 2,8-Sia bonds; MAL I for Sia(α 2,3)-Gal(β 1,4)GlcNAc conjugations found with N-linked glycans; and MAL II for Sia(α 2,3)-Gal(β 1,3)GalNAc conjugations found with O-linked glycans. As summarized in Finding 7 above, no binding of Siglec-11, MAL I, or MAL II was found for either native 4G8 (Fig 4AB) or for denatured 4G8 (data not shown).

To test an alternate version of this hypothesis, in which all 4G8 exhibited α 2,6 Fc sialic acid, Fc-specific endonuclease EndoS was used in two different experiments (Fig. 5). In the first experiment (Fig. 5A), the relative magnitude of EndoS cleavage on SNA sensor binding was low for denatured 4G8 and high for denatured 6E10. This result is consistent with the existing model, where the majority of sialic acid in 4G8 resides in the Fab domain and majority of sialic acid in 6E10 resides in the Fc domain. A second experiment (Fig. 5C) identified the sialic acid binding response to the free Fc domain of 4G8 and 6E10, in which the sialylated Fc glycan binding by SNA proved more readily accessible than with the Fab domain present (Scheme 4). This second experiment measured a low level of sialic acid on the 4G8 Fc domain (<5%) that cannot account for the global action of neuraminidase on 4G8.

These results in Figures 4 and 5 are consistent with previous work that found the majority of 4G8 sialic acid residing on a single N-linked glycoform on the light chain [47]. This glycosylation state was readily identified as a second heavier light chain band which bound SNA during lectin blotting and was eliminated after PNGase F treatment [47]. By contrast, 6E10 has only a single heavy and single light chain bands that remained relatively unchanged upon PNGase F treatment, with < 5% of IgG exhibiting sialic acid at the Fc glycan [47]. It is conceivable that other completely novel sialoglycoconjugates exist in 4G8 that cannot be detected with any known lectin. Unless such sialic acid forms can be identified, the results here support α 2,6-linked sialic acid as the primary sialic acid form relevant to 4G8 BBB efflux.

Given the evidence against the hypothesis that all 4G8 is sialylated, the alternate hypothesis that sialylated 4G8 inhibits asialylated 4G8 warrants further investigation. In Fig. 1 and 2, untreated 4G8 efflux P_e is negative and well below the BSA “zero” control. BSA is a protein known to have very slow efflux in MBECs and predominantly uses extracellular pathways [43, 64]. While BSA is a potential substrate for FcRn-mediated efflux, BSA binding with mouse FcRn is expected to be weak in the present case [65]. Given the negative 4G8 P_e in Fig. 1, sialylated 4G8 appears to engage in processes that reduce this efflux to a slower degree than even extracellular pathways.

While the raw BSA P_e value was highly variable for a given experiment, the average value over all 119 experiments was reasonable for an extracellular pathway with $P_e = 0.0080$. Assuming NEU-treated 4G8 ($P_e = 0.0076$) is the maximal efflux rate, the total efflux P_e would be expected to be $0.0080 + 0.0076 = 0.0156$. If so, this maximal efflux would be reduced 4-fold from 12% sialylation, assuming the endogenous 4G8 P_e is $0.0080 - 0.0041 = 0.0039$. Since additional sialic acid did not decrease this efflux rate further, inhibition by this sialic acid can be considered close to maximal. Thus the sialic-acid inhibited pathway roughly accounts for 75% of the total 4G8 efflux processes in BMECs.

Extracellular efflux is consistent with the observation that excess unlabeled 4G8 does not significantly reduce the P_e of ^{125}I -4G8 (Fig. 1). However, analysis of 6E10 efflux indicates that a more variable range of efflux processes exists. The 6E10 efflux P_e was greater than both albumin and 4G8 but was also not significantly reduced ($p = 0.25$) in the presence of excess unlabeled 6E10 (Fig. 1). Because 6E10 efflux was greater than 4G8, more active efflux pathways must exist in 6E10 that are not significantly limited under saturation conditions. A variety of efflux pathways might fit this description, including pinocytosis or a weak AMT/RMT pathway with low affinity between EC components and IgG [67–69].

FcRn is of particular interest here as it has been shown to enhance BBB efflux of 4G8 using *in vivo* AD mouse models [33]. Both the present study and a study by Deane et. al. support a model where 4G8 efflux can be inhibited [33]. One area of disagreement between the two studies is that active pathways of endogenous 4G8 efflux are intrinsically inhibited in the present study but appear to be active in the prior study [33]. Specifically, the previous study showed that endogenous radioactively labeled 4G8 efflux could be inhibited using excess unlabeled 4G8 (2 μM) and Fc (10 μM) [33].

These differences may result from varying experimental conditions in which 4G8 efflux is more inhibited under the present *in vitro* conditions than in prior *in vivo* conditions. First, the *in vitro* study here used picomolar ^{125}I -4G8 concentrations in the loading chamber while the previous *in vivo* study delivered nanomolar concentrations into the mouse CNS [33]. Second, the ratio of total moles of 4G8 to membrane surface area is approximately 100-fold greater for *in vitro* experiments than for *in vivo* conditions [70]. A third issue is that the commercial 4G8 supplier changed between the time the two studies were conducted. Because expression conditions alter IgG glycosylation and sialylation levels, it is possible that the prior study was performed with asialylated 4G8 [71]. Fourth, it is possible that other brain-to-blood pathways than BMEC efflux, such as those in the choroid plexus and also passive resorption, are involved in the *in vivo* study [72–74]. Lastly, it is possible that the

FcRn-mediated efflux mechanism proposed by Deane et. al. is indeed active and accounts for the ~25% residual efflux of sialylated 4G8 in the present study [33].

Here, sialylation is shown to reduce one or more pathways that dominate 4G8 efflux. Since FcRn is one of these pathways, a key question is if FcRn-mediated transcytosis is specifically inhibited by sialylated 4G8. Prior studies, including those with 4G8, have shown that FcRn is a saturable efflux transporter that can be inhibited with Fc-blockade [33]. As such, it is possible that sialylated 4G8 also competitively inhibits FcRn-mediated transport of asialylated 4G8. However, such a model requires that 12% sialylated 4G8 have a K_D with at least 10-fold higher affinity over the 88% asialylated 4G8 to outcompete and produce the efflux inhibition found in Figs. 1 and 2. This model cannot be ruled out at present. However, other studies argue against this model because sialic acid on IgG is reported to have either no effect or reduced affinity for FcR receptors [75].

An alternate interpretation is that sialylated 4G8 inhibits or redirects a step of the transport process other than FcRn binding in a noncompetitive manner. If an endocytotic mechanism is involved, sialylated 4G8 might interfere with initial endosome formation [67]. However, since FcRn is predominantly found in internalized endosomes and not at the abluminal membrane, it would seem unlikely that FcRn-4G8 inhibitory interactions occur at this initial step of the process [67]. Another possibility is that sialylated 4G8 interferes with the ability of recycling endosomes to direct other endocytosed 4G8 to the luminal (blood) membrane [67]. This is a promising mechanism and is the subject of future efforts. However, investigations into the inhibitory mechanism of FcRn efflux are outside the scope of the present study.

While sialylated 4G8 lowers BBB efflux in the present *in vitro* study, a clinically-relevant question is if sialylation logically increases BBB penetration of IgG *in vivo* when delivered intravenously at a therapeutic dose. BBB penetration has been low in prior clinical trials with passive antibody therapeutics that also failed to meet clinical endpoints [13–16]. The recent PRIME clinical trial using therapeutic antibody aducanumab showed a much higher BBB penetration than previous antibody treatments for AD and also showed positive clinical outcomes [17]. To this point, a previous study with mice suggest that 4G8 efflux from the brain is indeed slower than 6E10 but that influx rate is also an important consideration for the overall dose delivery to the CNS [45]. Thus, sialylation may offer a means to reduce IgG drug efflux from the brain in conjunction with other techniques and biochemical modifications that increase influx.

This study is important because IgG glycan modification may provide a useful method to increase the concentration of therapeutic antibodies in the brain of AD patients. The present study shows that modest alteration of IgG glycan composition with sialylated glycans can have significant effects on BBB pharmacokinetics. Furthermore, IgG glycosylation states studied here are all naturally occurring permutations and their composition changes are unlikely to increase the toxicity of an IgG drug. Sialylation may also confer other clinical benefits to AD patients, as sialylated IgG is reported to induce immune pathways with minimal inflammation [76–78].

Acknowledgments

This work was supported by the National Institutes of Health/NIA R03 AG050184 (J.M.F., W.A.B.), the Royalty Research Fund (J.M.F., W.A.B.), the Murdock Charitable Trust (J.M.F., H.A.H.), and the School of Interdisciplinary Arts and Sciences at the University of Washington Tacoma (J.M.F.).

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Highlights

1. The influx P_e is similar for 4G8 and 6E10 and is largely insensitive to changes in their glycosylation states.
2. The efflux P_e of 4G8 significantly lower for α 2,6-sialylated 4G8 than for predominantly asialylated 6E10.
3. Sialic acid is responsible for the lower efflux P_e of 4G8. Neuraminidase removes the sialic acid and equalizes the 4G8 efflux P_e to that of 6E10.
4. Removal of the Fc glycan partially abrogates the inhibitory effect of sialic acid on 4G8 but has no effect on 6E10 efflux.
5. A significant reduction 4G8 efflux results from a relatively small fraction (12%) of 4G8 exhibiting surface α 2,6 sialic acid.
6. Increasing the 4G8 sialylation fraction to 15% produced equally low efflux as 12%.
7. No sialic acid linkages other than α 2,6 exist on the surface of 4G8.
8. Fc sialylation of 4G8 is very low (1–4%) and is only α 2,6-linked
9. The results support a model in which sialylated 4G8 inhibits the efflux of asialylated 4G8.

Figure 1. 4G8 efflux is significantly lower than 6E10

In vitro influx and efflux P_e are shown for partially sialylated mAb 4G8 (left) and largely asialylated mAb 6E10 (n = 21, right). ^{125}I labeled IgG alone (black bars, 4G8 n=22, 6E10 n=21) and with 67 nM excess IgG (gray bars, n=14 for both) is shown with error bars indicating standard deviation. *Statistics:* Data bars with the same letter do not have statistically significant differences (p>0.05).

Figure 2. Removal of sialic acid and the Fc glycan increases 4G8 efflux

Effect of enzymatic glycan modification on *in vitro* influx and efflux for untreated 4G8 (4G8-U, n=22), neuraminidase-treated 4G8 (4G8-N, n=12), α 2,6-sialyltransferase-treated 4G8 (4G8-S, n=12), EndoS-treated 4G8 (4G8-E, n=12), untreated 6E10 (6E10-U, n=21), and EndoS-treated 6E10 (6E10-E, n=12). (A) The fraction of 4G8 exhibiting SNA binding F_{SNA} , (B) influx P_e and (C) efflux P_e for untreated 4G8 (None), and 4G8 treated with neuraminidase (NEU), α 2,6-sialyltransferase (2,6ST) and EndoS (EndoS). *Statistics*: Error

bars show standard deviations. Within each graph panel, data with the same letter do not have statistically significant differences ($p>0.05$).

Figure 3. CFCA confirms fully sialylated and asialylated 4G8 purified by SNA affinity chromatography

Elution aliquots from SNA-agarose affinity chromatography are shown by circles along with their concentration determined by CFCA. Aliquots 1–5 correspond to sample application and flow-through steps while aliquots 6–11 elute SNA-enriched 4G8 with 0.5 M lactose. The results of CFCA measurement of F_{SNA} and OAB On-Chip measurement of FL:IgG confirm the absence of α 2,6 sialic acid in flow-through aliquot 2 and the presence of fully α 2,6 sialylated in elution aliquot 9.

Figure 4. Only α 2,6 sialic acid is found on the surface of 4G8

OA β sensor On-Chip SPR measurement of the fraction of lectin binding $F_{L:IgG}$ by lectins Siglec-11, MAL I, MAL II, ECL, and SNA with mAbs 4G8 (A) and 6E10 (B) as untreated IgG (None) and IgG treated with neuraminidase (NEU), α 2,6-sialyltransferase (2,6ST) and EndoS (EndoS). *Statistics:* All measurements were made in duplicate (n=2). Positive values that are also statistically significant (p<0.05) above the baseline are indicated with an asterisk (*).

Figure 5. Fc glycans of 4G8 and 6E10 have a small fraction of sialic acid

(A) The fraction of total SNA binding in denatured IgG attributed to sialic acid in the Fab domain. (B) Control SpA sensor On-Chip analysis of intact 4G8. (C) SpA sensor On-Chip analysis of free Fc Glycans from 4G8 and 6E10 with and without NEU and EndoS glycosidase treatments. (Inset) SDS-PAGE gel of 4G8 and 6E10 cleavage by papain.

Statistics: Error bars show standard deviations if multiple measurements were performed (n=4 for Papain-only IgG, n=2 in all other cases). Lower panel: Data with the same letter

and do not have statistically significant differences ($p>0.05$). SNA (A,B,C) and ECL (A', B') were not compared as they exhibit different relative responses in this assay.

Scheme 1.

Representative surface plasmon resonance sensogram used in the On-Chip method. In Step 1, a particular IgG of interest is bound to immobilized ligands on the sensor chip and this binding signal is measured (RU_{IgG}). In Step 2, a glycan specific lectin is applied under saturating concentrations and this second binding signal measured (RU_{L+IgG}). A control to determine the degree of non-specific binding between lectin and the sensor surface is run prior to the measurement (RU_L).

Scheme 2.

Schematic of an On-Chip experiment using a OAβ sensor chip to capture anti-OAβ IgG and an SNA probe to detect surface accessible α2,6-Sia-Gal groups on these antibodies. Under these conditions, only α2,6-Sia-Gal groups on Fab glycans can be measured and Fc glycans are sterically inaccessible to SNA.

Scheme 3.

Schematic of an On-Chip experiment using a SpA sensor chip to capture SpA-binding IgG (human IgG, mouse IgG2b but not mouse IgG1) and an SNA probe to detect surface accessible α 2,6-Sia-Gal groups on these antibodies. Under these conditions, only α 2,6-Sia-Gal groups on Fab glycans can be measured and Fc glycans are sterically inaccessible to SNA.

Scheme 4.

Schematic of an On-Chip experiment using a SpA sensor chip to capture SpA-binding Fc generated with papain treatment (all human and all mouse IgG) and an SNA probe to detect α 2,6-Sia-Gal groups on these Fc domains. Under these conditions, α 2,6-Sia-Gal groups on the Fc glycan are accessible to SNA binding.

Scheme 5.

Schematic of an SPR experiment that measures only Fab $\alpha 2,6$ -Sia-Gal groups on denatured IgG glycans using a SNA sensor chip. In this experiment the IgG is treated with EndoS prior to denaturation to remove all Fc glycans. The SPR binding response of sialylated IgG

($RU_{SNA}^{\text{Denatured}}$) provides a measurement of Fab IgG sialylation (S_{EndoS}) that can be used in conjunction with $S_{\text{Untreated}}$ in Eq. 4 and Scheme 6 to estimate $F_{\text{TotalSNA}}^{\text{FabSNA}}$.

Scheme 6.

Schematic of an SPR experiment that measures all $\alpha 2,6$ -Sia-Gal groups on denatured IgG glycans using a SNA sensor chip. The SPR binding response of sialylated IgG ($RU_{SNA}^{\text{Denatured}}$) provides a measurement of total IgG sialylation ($S_{\text{Untreated}}$) that can be used in conjunction with S_{EndoS} in Eq. 4 and Scheme 5 to estimate $F_{\text{TotalSNA}}^{\text{FabSNA}}$.

Table 1

Estimate 1	F_{SNA}	$F_{TotalSNA}^{FabSNA}$	$F_{SNA}^{Fc} = F_{SNA} \left(\frac{1}{F_{TotalSNA}^{FabSNA}} - 1 \right)$
4G8	0.12	0.83	0.024
6E10	0.014	0.35	0.026
Estimate 2	$R_{L:Fc}^{Untreated}$	$R_{L:Fc}^{EndoS}$	$F_{SNA}^{Fc} = 0.36 \left(R_{L:Fc}^{Untreated} - R_{L:Fc}^{EndoS} \right)$
4G8	0.16	0.04	0.043
6E10	0.033	0.007	0.009