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A RECOMBINANT IgG Fc THAT RECAPITULATES THE ANTI-INFLAMMATORY ACTIVITY OF IVIG

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Abstract

High doses of monomeric IgG purified from pooled human plasma confer anti-inflammatory activity for a wide variety of autoimmune diseases. The heterogeneity of IVIG, derived from its Fab specificity, IgG subclass distribution and variable glycosylation have confounded efforts to develop a recombinant substitute for this blood-derived product. Recent studies have demonstrated that this paradoxical anti-inflammatory activity of IgG is completely dependent on sialylation of the N-linked glycan of the IgG Fc fragment. Determining the precise glycan requirements for this anti-inflammatory activity allowed appropriate glycan engineering of an IgG1 Fc fragment, leading to the generation of a fully recombinant, sialylated IgG1 Fc with greatly enhanced potency.

The complex, bi-antennary N-linked glycan found at Asn 297 of the IgG Fc consists of a heptasaccharide which can be variably modified by the addition of fucose or GlcNAc to the $Man_3GlcNAc_2$ core, and galactose and sialic acid to the outer arms (Figure 1A)(1). The fully processed N-linked glycan is present on 2–4% of the total IgG in IVIG(2). The anti-inflammatory activity of IVIG has been demonstrated in a variety of animal models of autoimmunity, including autoantibody induced thrombocytopenia(3), serum transfer arthritis (4) and nephrotoxic nephritis(5) and is a property of the Fc fragment and its associated glycan (2, 3, 6). Removal of the terminal sialic acid of IVIG or its papain-derived Fc fragment abrogates the anti-inflammatory activity in these animal models. Conversely, enrichment of the sialylated fraction of IVIG enhances this activity(2). Since sialic acid can be found in either a 2,3 or 2,6 linkage to the penultimate galactose, we set out to determine which linkage type was present in IVIG by analyzing the PNGase F released glycan from Fc fragments derived from IVIG (Figure 1B–D). Sequential mass spectrometric based glycan analysis revealed a preferential 2,6 linkage in the IVIG preparations that conferred anti-inflammatory activity (Figure 1B–D). To confirm this association, we next analyzed IVIG activity following treatment with either 2,3 or 2,3/2,6 sialidase (SA) in the K/BxN serum transfer arthritis model (Figure 1E and supplemental Figure 1). Consistent with the glycan analysis, we observed complete abrogation of the anti-inflammatory activity when the 2,6 linkages were removed. In contrast, no 2,3 linkages were detected on intact IVIG and no attenuation of the anti-inflammatory activity was observed following 2,3 SA treatment (Figure 1E and supplemental figure 1B). These results are in agreement with previous studies that noted a preferential

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utilization of the 2,6 linkage of sialic acid-galactose in human IgG preparations, a requirement of 2,6 but not 2,3 sialyltransferase (ST) for B cell responses, the over expression of the 2,6 sialyltransferase in B cells, and general absence of 2,3 linkages of sialic acid in Fc attached glycans for all isotypes(7–11).

To determine if the preferential 2,6 sialic acid-galactose linkage was a necessary and sufficient property of the anti-inflammatory activity of the sialylated, N-linked glycan at Asn 297, we generated IVIG derived Fc fragments terminating in either the 2,3 or 2,6 sialic acid linkage. N-linked glycans attached at Asn-297 released from human plasma derived IgG are variously galactosylated, as has been reported (2) and was observed for IVIG preparations by MALDI-TOF analysis(2). The majority of the glycans released by PNGase F from IVIG are found either in the G0 (no galactose) or G1 (monogalactosylated) forms, as has been observed previously for human IgG(12,13). Therefore, to efficiently sialylate the Fc fragment derived from IVIG, we first converted the population of glycans to the G2 (digalactosylated) form by *in vitro* treatment with β1,4 galactosyltransferase (β1,4GT), and next the galactosylated IVIG Fc was targeted with either 2,3 or 2,6 sialyltransferases capable of adding sialic acid to N-linked glycans. (Figure 2A). The amount of terminal galactose following galactosylation was increased two-fold by this procedure as determined by ECL binding, shifting the population of glycans to the G2 form (Figure 2B). This galactosylated substrate was then reacted either with 2,3 sialyltransferase (2,3ST) or 2,6 sialyltransferase (2,6ST) (supplemental Figure 2A). *In vitro* sialylation was evaluated directly by lectin blotting for 2,3 and 2,6 linkages (Figure 2C), and indirectly by assaying for reduced ECL binding (supplemental Figure 2A, B). The 2,6 sialyltransferase appeared to quantitatively convert the G2 glycan to a fully sialylated form, as demonstrated by the absence of ECL reactivity (Supplemental Figure 2). The efficiency of the 2,3 sialyltransferase was estimated to be approximately 50%, based on residual ECL binding to these reacted Fc linked glycans (Supplemental Figure 2). By comparison, ECL binding was also observed to the SNA-enriched Fc derived from IVIG, which contain approximately 30% fully sialylated Fc glycans (Supplemental Figure 2), yet represent a 10 fold enrichment in sialic acid as compared to unfractionated IVIG derived Fc fragments. As reported previously, the level of sialylation observed in the SNA-enriched IVIG Fc fragments is sufficient to induce an anti-inflammatory response at a 10-fold reduced dose as compared to unfractionated IVIG, and been demonstrated to confer enhanced anti-inflammatory activity (2).

These preparations where then administered to mice (supplemental Table 1) in the K/BxN serum induced arthritis model. Joint inflammation was effectively reduced by administering either the SNA^+ IVIG Fc fragments, as was observed previously(2), or the *in vitro* 2,6 sialyltransferase treated IVIG Fc fragments (Figure 3A). Suppression of inflammation was comparable with these two preparations and was 10-fold more active than Fc fragments derived from IVIG that had neither been fractionated nor *in vitro* sialylated. In contrast, IVIG Fc fragments sialylated *in vitro* with 2,3 sialyltransferase were ineffective at reducing the joint inflammation induced by K/BxN serum (Figure 3A), despite the greater degree of sialylation observed in these preparations as compared to the SNA-enriched Fc preparations (Supplemental Figure 2).

We had previously demonstrated that sialylation of the N-linked glycan associated with the Fc domain of IgG resulted in reduced FcR binding, leading to a reduction in the A/I ratio(2), a value derived from the affinity constants for an IgG Fc binding to individual activating (A) or inhibitory (I) IgG Fc receptors. This ratio has been shown to be predictive of the *in vivo* cytotoxicity for a specific IgG Fc(14). Fc sialylation thus reduced the cytotoxicity of IgG antibodies in the induced thrombocytopenia model as well as in *in vitro* models of ADCC(2, 15). We therefore set out to determine if this reduction in FcR binding and cytotoxicity was influenced by the 2,3/2,6 linkage between sialic acid and galactose. A monoclonal anti-platelet

IgG2b antibody previously shown to lead to platelet consumption was sialylated *in vitro* as described above and tested for *in vivo* activity. Both terminal 2,3 and 2,6 *in vitro* sialylated IgG Fc reduced the cytotoxicity of this anti-platelet antibody, 6A6-IgG2b, in an *in vivo* model of thrombocytopenia (Figure 3B), consistent with previous studies(2,15). Thus, the effect of Fc sialylation on the cytotoxicity of an IgG antibody is not dependent on the specificity of the linkage to the penultimate galactose. In contrast, the anti-inflammatory activity of the sialylated IgG Fc fragment (a property we have previously demonstrated to be independent of the canonical IgG Fc receptors(2,6)) displayed a clear preference for the 2,6 sialic acid-galactose linkage, as seen in Figure 3A (and supplemental figure 2B). These results further support our previous observations that the anti-inflammatory property of IVIG is mediated through a distinct pathway(2,6) that does not involve binding to canonical FcγRs, as has been suggested by others(16,17).

To fully demonstrate that the *in vivo* anti-inflammatory activity of the 2,6 sialylated IgG Fc is solely a property of the IgG Fc glycan and not the result of other components that might be found in the heterogeneous, IVIG Fc preparations, we set out to recapitulate the anti-inflammatory activity of sialylated IVIG Fc using a homogeneous, recombinant human IgG1 Fc substrate (rFc), derived from a cDNA expressed in 293T cells. The purified recombinant human IgG1 Fc fragment was glycan engineered *in vitro*, as described above, by β1,4 galactosylation, followed by 2,6 sialylation (Figure 4A). The preparation was purified and characterized by lectin blotting and MALDI-TOF analysis (Figure 4A and supplemental figure XXX) before *in vivo* analysis. As seen in Figure 4B, the 2,6 sialylated recombinant human IgG1 Fc fragment demonstrated comparable anti- inflammatory activity to that obtained with either IVIG-derived sialic-enriched Fc fragments (SNA^+ IVIG Fc) or *in vitro* 2,6 sialylated IVIG-derived Fc fragments (2,6ST IVIG Fc). Each of these preparations was active at 30mg/kg, as compared to the 1,000–2,000 mg/kg required for native IVIG (supplemental Table 1).

The exquisite specificity of glycan structures has long been appreciated as providing the structural basis for discrete biological responses. Although their functions are unknown, lectins purified from plants readily distinguish sialic acid linkage types(18). Additionally, the haemagglutinin expressed by the Spanish flu (H1N1) strain preferentially binds 2,6 linkages whereas an avian flu strain (H5N1) displays a preference for 2,3 linkages(19–21). This specificity holds true for other pathogens, including bacteria (19,22) and parasites (23,24). Sialic acid specificity is a well-characterized feature of the innate immune response, as observed in the ligand specificity of the selectin adhesion receptors(18,25), and sialic acid binding Ig-like lectins (siglecs)(26,27). The results reported here demonstrate that the sialic acid – galactose linkage specificity for the N-linked glycan of the IgG Fc confers anti-inflammatory activity on the IgG, either through its ability to engage a lectin receptor with the appropriate specificity or by inducing a specific conformation that is recognized by an anti-inflammatory receptor(2,6,18). IgG can thus exist in three distinct states, controlled by sialylation of the IgG Fc. In its desialylated state, IgG confers cytotoxic activity through binding to activation Fc receptors. IgG can lose its cytotoxic capability by sialylation of the Fc-linked glycan, thereby converting IgG into a unreactive, non-inflammatory state by reducing FcR binding, a effect that is insensitive to the nature of the sialic acid-galactose linkage since it is true for both 2,3 and 2,6 linkages. Finally IgG Fc can be anti-inflammatory, i.e. actively able to suppress inflammatory responses, mediated through the 2,6 sialic acid linkage binding to a cognate receptor. Recognition of these three potential states for the IgG Fc is a significant consideration in the design of IgG based therapeutics.

The observation that the anti-inflammatory activity of IVIG is dependent on a precise glycan structure on the Fc further supports the model we have previously advanced(2,6) that a specific receptor for the sialylated Fc, and not a canonical Fc receptor, is involved in this pathway. Our data support a model in which binding of the 2,6 sialylated Fc to its cognate receptor expressed

on a population of regulatory myeloid cells results in the trans upregulation of the inhibitory IgG Fc on effector macrophages, located at sites of inflammation, such as the inflamed joint, thus raising the threshold required for cytotoxic IgGs to engage activation FcRs and trigger inflammatory responses(14).

For over 30 years high dose IVIG has been successfully employed as an anti-inflammatory therapeutic for the treatment of a wide variety of autoimmune disorders. While its use as an anti-inflammatory has been steadily increasing, it has been limited by availability and the exceedingly high dose requirement for efficacy to be observed. The studies described here demonstrating our ability to recapitulate this anti-inflammatory property of IVIG in a recombinant molecule at a much reduced dose should provide for the development of this recombinant alternative as a broadly acting anti-inflammatory drug for the treatment of autoimmune disorders. This recombinant molecule will also facilitate the further characterization of its mechanism of action by providing a homogeneous compound for further structure-function studies of this novel ligand-receptor system.

Materials and Methods

Mice

C57BL/6 and NOD mice were purchased from the Jackson Laboratory (Bar Harbor, ME). KRN TCR transgenic mice on a C57BL/6 background (K/B) were gifts from D. Mathis and C. Benoist (Harvard Medical School, Boston, MA) and were bred to NOD mice to generate K/BxN mice. Serum is prepared as described previously(4). Briefly, serum is separated from blood collected from the K/BxN mice (6–12 weeks old). Several weeks of serum collection are pooled together and frozen in aliquots to be used in all the experiments described here. One intravenous injection of 1.5X diluted K/BxN serum (4μl of pooled K/BxN serum per gram of mouse) induced arthritis. Arthritis was scored by clinical examination. Index of all four paws are added: 0 [unaffected], 1 [swelling of one joint], 2 [swelling of more than one joint], and 3 [severe swelling of the entire paw]. IVIG is injected 1 hr before K/BxN serum injection. Some mice received 5μg of platelet depleting 6A6-IgG2b antibody, and platelet counts were determined 0, 4, and 24 hours post treatment using an Advia 120 haematology system (Bayer). Age-matched female mice at 6–9 weeks of age were used for all experiments and maintained at the Rockefeller University animal facility. All experiments were done in compliance with federal laws and institutional guidelines and have been approved by the Rockefeller University (New York, NY).

Sequential Mass Spectrometry Analysis

For sialic acid linkage analysis, oligosaccharides were released from SNA-enriched IVIG Fc fragments with PNGaseF, purified by C18 solid-phase extraction followed by graphitized carbon solid-phase extraction, reduced with sodium borohydride, and permethylated in DMSO with iodomethane and sodium hydroxide(28). Reduced and permethylated oligosaccharides were directly infused into an ion trap mass spectrometer with a nanoelectrospray source (LTQ, ThermoFisher, San Jose, CA). Fragments were successively isolated and subjected to collision-induced dissociation until the B/Y type monomer fragments of the antennal galactose moieties were isolated and fragmented (29). These monomer fragment spectra were compared to the analogous fragments from 3′-sialyl-lactose and 6′-sialyl-lactose standards (V-labs, Covington, LA). Oligosaccharides were examined as lithium ion adducts.

Sialidase Digestions

Initial digestions using 2,3/6 and 2,3 sialidases (QA Bio) were performed according to manufacturers instructions at 37°C on fetuin (Sigma) at different intervals. 4 hour incubates

proved to be the most efficient, and these conditions were then applied to IVIG. All digestions were confirmed by lectin blotting.

IVIG Fractionation

Human intravenous immune globulin (IVIG, 5% in 10% maltose, chromatography purified, Octapharma) digestion was performed as described (20). Briefly, IVIG was digested by 0.5 mg/ml papain for 1 hr at 37°C, and stopped by the addition of 2.5 mg/ml iodoacetamide. Fab and Fc resulting fragments 2 were separated from non-digested IVIG on a HiPrep 26/60 S-200HR column (GE Healthcare), followed by purification of Fc and Fab fragments with a Protein G column (GE Healthcare) and a Protein L column (Pierce). Fragment purity was checked by SDS-PAGE, followed by coomassie brilliant blue staining and immunoblotting using anti-human IgG Fab or Fc-specific antibodies (Jackson ImmunoResearch). Purity was judged to be greater than 90%. Sialic acid enriched IVIG Fc fragments were isolated by lectin affinity chromatography with *Sambucus nigra* agglutinin (SNA) agarose (Vector Laboratories), and was verified by lectin blotting (see below). 6A6-IgG2b and 4-4-20-human IgG1 were produced by transient transfection of 293T cells followed by purification via protein G as described (6,15). Fc fragments were generated from 4-4-20-human IgG1(30) by papain digestion as described above.

Chromatography

SNA-agarose (Vector Laboratories) was used to enrich IVIG Fcs with terminal 2,6 sialic acid as described previously(2). Briefly, four milligrams of Fc fragments in 4 ml of Tris-buffered saline (TBS, pH 7.5) with 0.1 M $CaCl_2$ are applied into a 4ml column, and incubated at room temperature for 10 minutes. The column was washed with 8 ml of TBS and flow-through fraction collected as the non binding fraction. IgG binding to the column are eluted with 4 ml of 0.5 M lactose in TBS for 10 min, followed by the same amount of 0.5 M lactose in 0.2 M acetic acid for another 10 min. The negative fraction is reapplied to the column and purified in the same way. Purification will be verified by lectin blotting. Anion exchange chromatography was performed using a ATKA HPLC (GE Healthcare) fitted with a HiTrap DEAE column (GE Healthcare) by binding 2mg of sialylated Fcs to the column in 0.005M phosphate buffer, pH8 and eluted by linear gradient with 0.2M phosphate buffer, pH7.4 over 40 column volumes.

Western and lectin blots

5μg of IgG were resolved on SDS-PAGE gel under non-reducing conditions, transferred to polyvinylidene difluoride membranes, blocked with Western Blocking Reagent (Roche), and incubated with alkaline phosphatase (ALP)-conjugated goat anti-human IgG Fc-specific antibodies (Jackson ImmunoResearch) for Western blotting, biotinylated SNA lectin (2μg/ml, Vector Laboratories) for 2,6 sialic acid linkages, biotinylated MAL I lectin (4μg/ml, Vector Laboratories) for 2,3 sialic acid linkages, followed by incubation with ALP-conjugated goat anti-biotin antibody. The immune complex was visualized with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche).

In vitro glycosylation

IVIG or recombinant Fc fragments were treated with 2,3/6 sialidase to remove sialic acid residues. In 50mM MOPS, pH7.2, 20mM MnCl2, 30mg/ml Fc fragments were incubated with 10μM UDP-galactose and 25mU/10mg substrate of β1–4 galactosyltransferase for 48 hours at 37°C(31). Galactosylation was confirmed by ECL blot. Next, portions of the galactosylated Fc fragments (XX mg/ml) were sialylated(32) in 50mM sodium cacodylate-HCl, pH6.0, 10mM MnCl2, 10μM CMP-sialic acid at 37°C for 48 hours with xx mU/ml α2–3 (Calbiochem) or xx

mU/ml α2–6 sialyltransferase(33). The reaction was confirmed by lectin blotting with α2–3 specific or α2–6 specific lectins (MAL or SNA, respectively).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Takahashi N, et al. Biochemistry 1987;26:1137. [PubMed: 3567160]
2. Kaneko Y, Nimmerjahn F, Ravetch JV. Science 2006;313:670. [PubMed: 16888140]
3. Samuelsson A, Towers TL, Ravetch JV. Science 2001;291:484. [PubMed: 11161202]
4. Bruhns P, Samuelsson A, Pollard JW, Ravetch JV. Immunity 2003;18:573. [PubMed: 12705859]
5. Kaneko Y, Nimmerjahn F, Madaio MP, Ravetch JV. J Exp Med 2006;203:789. [PubMed: 16520389]
6. Nimmerjahn F, Ravetch JV. J Exp Med 2007;204:11. [PubMed: 17227911]
7. Dalziel M, McFarlane I, Axford JS. Glycoconj J 1999;16:801. [PubMed: 11133020]
8. Hennet T, Chui D, Paulson JC, Marth JD. Proc Natl Acad Sci U S A 1998;95:4504. [PubMed: 9539767]
9. Arnold JN, et al. J Immunol 2004;173:6831. [PubMed: 15557177]
10. Arnold JN, Wormald MR, Sim RB, Rudd PM, Dwek RA. Annu Rev Immunol 2007;25:21. [PubMed: 17029568]
11. Kitagawa H, Paulson JC. J Biol Chem 1994;269:17872. [PubMed: 8027041]
12. Wormald MR, et al. Biochemistry 1997;36:1370. [PubMed: 9063885]
13. Jefferis R, et al. Biochem J 1990;268:529. [PubMed: 2363690]
14. Nimmerjahn F, Ravetch JV. Science 2005;310:1510. [PubMed: 16322460]
15. Scallon BJ, Tam SH, McCarthy SG, Cai AN, Raju TS. Mol Immunol 2007;44:1524. [PubMed: 17045339]
16. Park-Min KH, et al. Immunity 2007;26:67. [PubMed: 17239631]
17. Siragam V, et al. Nat Med 2006;12:688. [PubMed: 16715090]
18. Varki A. Nature 2007;446:1023. [PubMed: 17460663]
19. Lehmann F, Tiralongo E, Tiralongo J. Cell Mol Life Sci 2006;63:1331. [PubMed: 16596337]
20. Tumpey TM, et al. Science 2007;315:655. [PubMed: 17272724]
21. Ibricevic A, et al. J Virol 2006;80:7469. [PubMed: 16840327]
22. Hanisch FG, Hacker J, Schroten H. Infect Immun 1993;61:2108. [PubMed: 8097494]
23. Mayer DC, Kaneko O, Hudson-Taylor DE, Reid ME, Miller LH. Proc Natl Acad Sci U S A 2001;98:5222. [PubMed: 11309486]
24. Orlandi PA, Klotz FW, Haynes JD. J Cell Biol 1992;116:901. [PubMed: 1310320]
25. Sperandio M, et al. Eur J Immunol 2006;36:3207. [PubMed: 17111351]
26. Crocker PR, Paulson JC, Varki A. Nat Rev Immunol 2007;7:255. [PubMed: 17380156]
27. Crocker PR, Werb Z, Gordon S, Bainton DF. Blood 1990;76:1131. [PubMed: 2205308]
28. Ashline DJ, et al. Anal Chem 2007;79:3830. [PubMed: 17397137]
29. Ashline D, Singh S, Hanneman A, Reinhold V. Anal Chem 2005;77:6250. [PubMed: 16194086]
30. Kranz DM, Voss EW Jr. Proc Natl Acad Sci U S A 1981;78:5807. [PubMed: 6795637]
31. Warnock D, et al. Biotechnol Bioeng 2005;92:831. [PubMed: 16187338]
32. Raju TS, Briggs JB, Chamow SM, Winkler ME, Jones AJ. Biochemistry 2001;40:8868. [PubMed: 11467948]
33. Williams MA, Kitagawa H, Datta AK, Paulson JC, Jamieson JC. Glycoconj J 1995;12:755. [PubMed: 8748151]

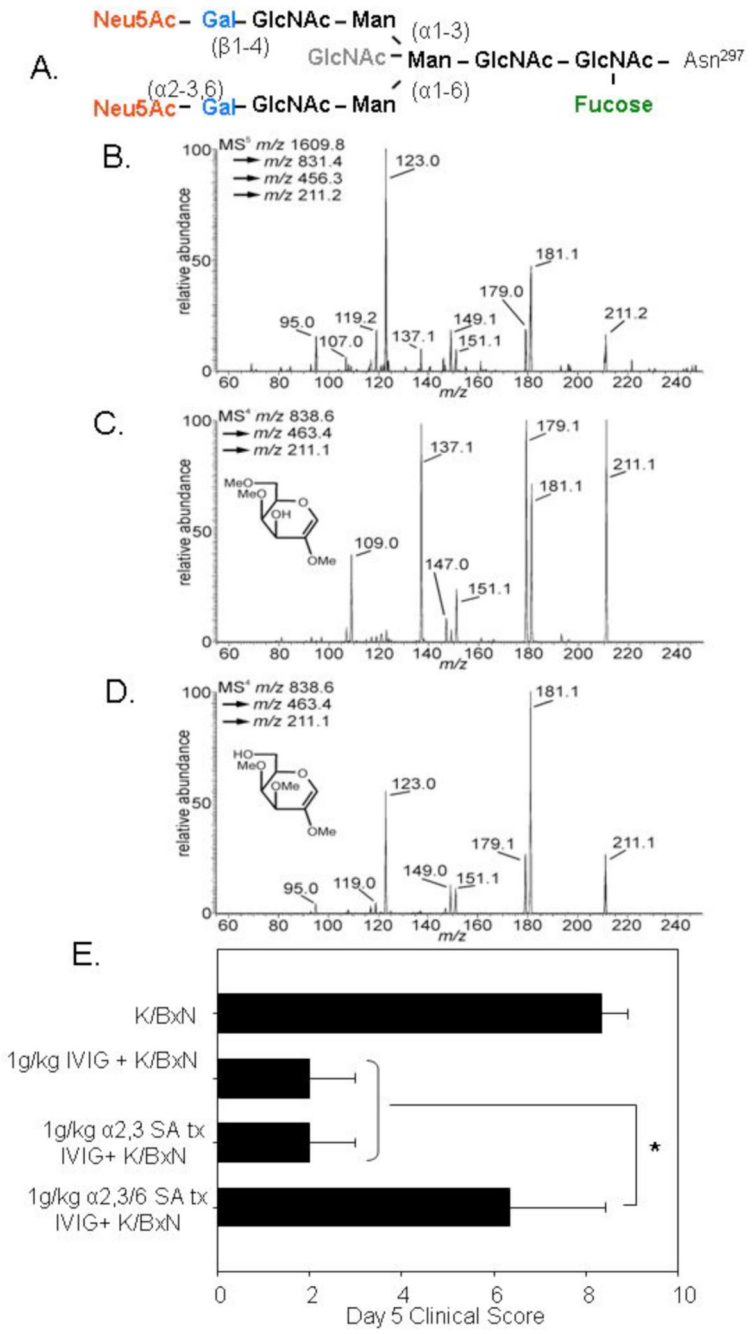

Figure 1.

α2,6 linkages are the predominant sialic acid linkage on IVIG Fc glycans. A. The IgG Fc glycan is a bisecting core of seven sugars (black), and can vary at a number of positions by the addition of fucose (green) to the core, a bisecting GlcNAc (gray), or by addition of galactose (blue) and sometimes sialic acid (red) to the arms. B. Sequential mass spectrometry analysis of SNA-enriched IVIG Fc glycans was performed to determine the sialic acid linkage type and relative proportion of the linkages in the active component of IVIG. The resulting footprint of the B/Y galactosyl fragment monomer derived from the SNA-enriched Fc glycan was compared to the analogous B/Y fragments from (C) 2,3 sialyllactose and (D) 2,6 sialyllactose standards. The spectrum generated from the SNA^+ IVIG Fc glycan (B) most closely matches that of 2,6

sialyllactose (D), particularly with respect to the *m/z* 123 and 95 fragments, and the much smaller abundances of *m/z* 137 and 109 ions. Next, IVIG was treated with linkage-specific sialidases to remove only 2,3 (α2,3 SA) or both 2,3 and 2,6 (α2,3/6 SA) sialic acids. E. The sialidase-treated IVIG preparations were administered to mice prior to K/BxN sera, and footpad swelling was monitored over the next seven days and recorded as clinical scores. Mean and standard deviation of 5 mice per group 5 days post treatment are plotted; *denotes $p<0.05$ as determined by an Anova test followed by Tukey post hoc test.

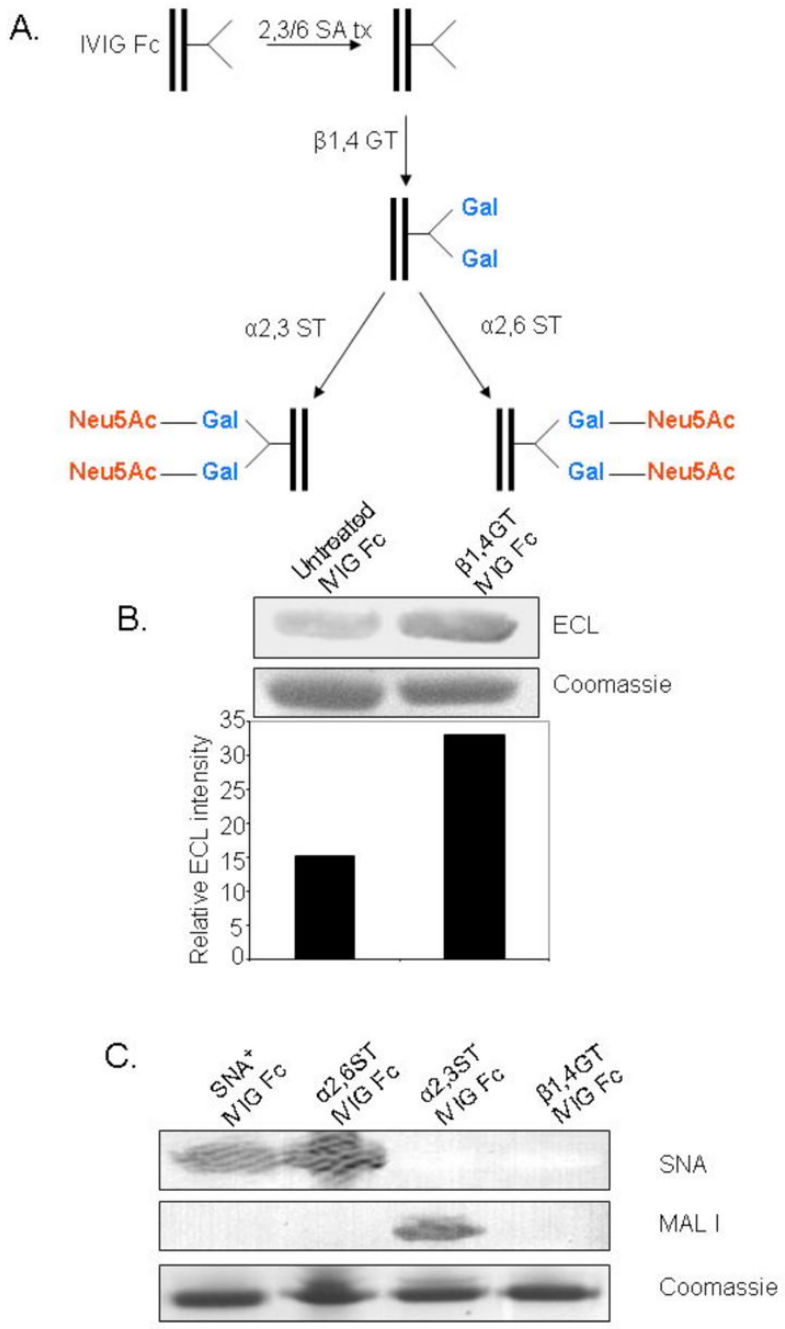

Figure 2.

In vitro sialylation of IVIG Fcs. A. As shown in the schematic diagram of the sialylation strategy, IVIG Fc fragments were initially treated with 2,3/6 sialidase (2,3/6 SA) to remove all sialic acid residues, galactosylated (β1,4 GT), and finally sialylated with either 2,3 or 2,6 sialyltransferases (α2,3 ST and α2,6 ST, respectively). B. Galactosylation was verified by lectin blotting with ECL (top panel), which recognizes terminal galactose residues. Relative band intensity ratios of ECL to coomassie loading controls are plotted below. The galactosylated Fcs were then sialylated with 2,3 or 2,6 sialyltransferases, and (C) each sialylation reaction was confirmed by lectin blotting for 2,6 linkages with SNA (top panel) and 2,3 linkages with MAL I (middle panel). Coomassie stained loading controls are show below.

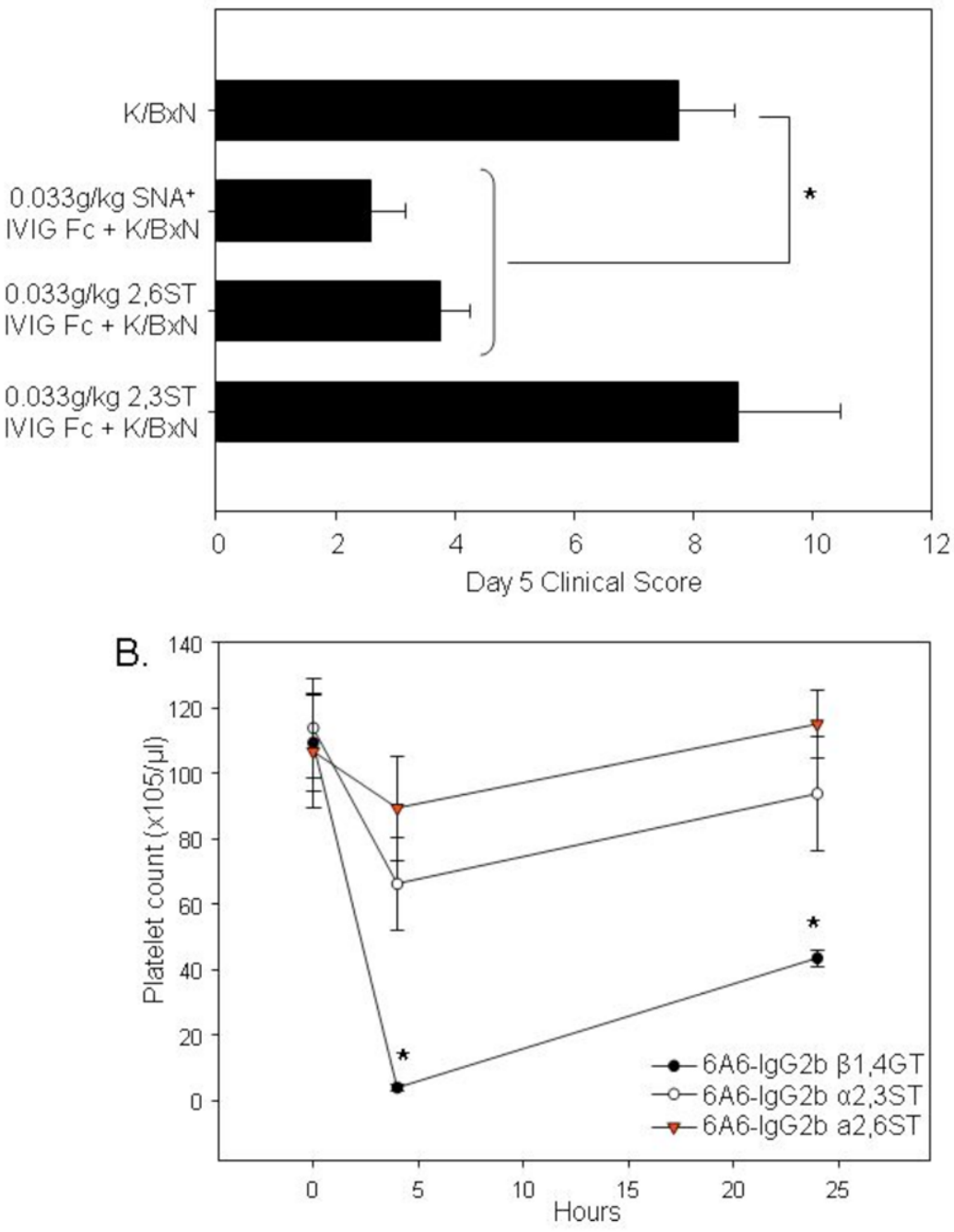

Figure 3.

Sialic acid-dependent IgG anti-inflammatory activity is linkage specific, although the reduced antibody dependent cytotoxicity is not. A. Anion exchange purified sialylated IVIG Fcs were administered to mice 1 hour prior to K/BxN sera, and paw swelling monitored over the next several days. Means and standard deviations of 4 mice per group are plotted; *denotes $p<0.05$ as determined by Anova followed by Tukey's post hoc. To determine whether the reduced ADCC of sialylated antibodies was also dependent on specific linkages, platelet-depleting 6A6-IgG2b antibodies were sialylated as described above. (B) Sialylated 6A6-IgG2b antibodies were administered to mice and platelet counts determined 0, 4, and 24 hours

following treatment. Mean and standard deviation of 5 mice per group are plotted; *$p<0.05$ as determined by Anova followed by Tukey's post hoc.

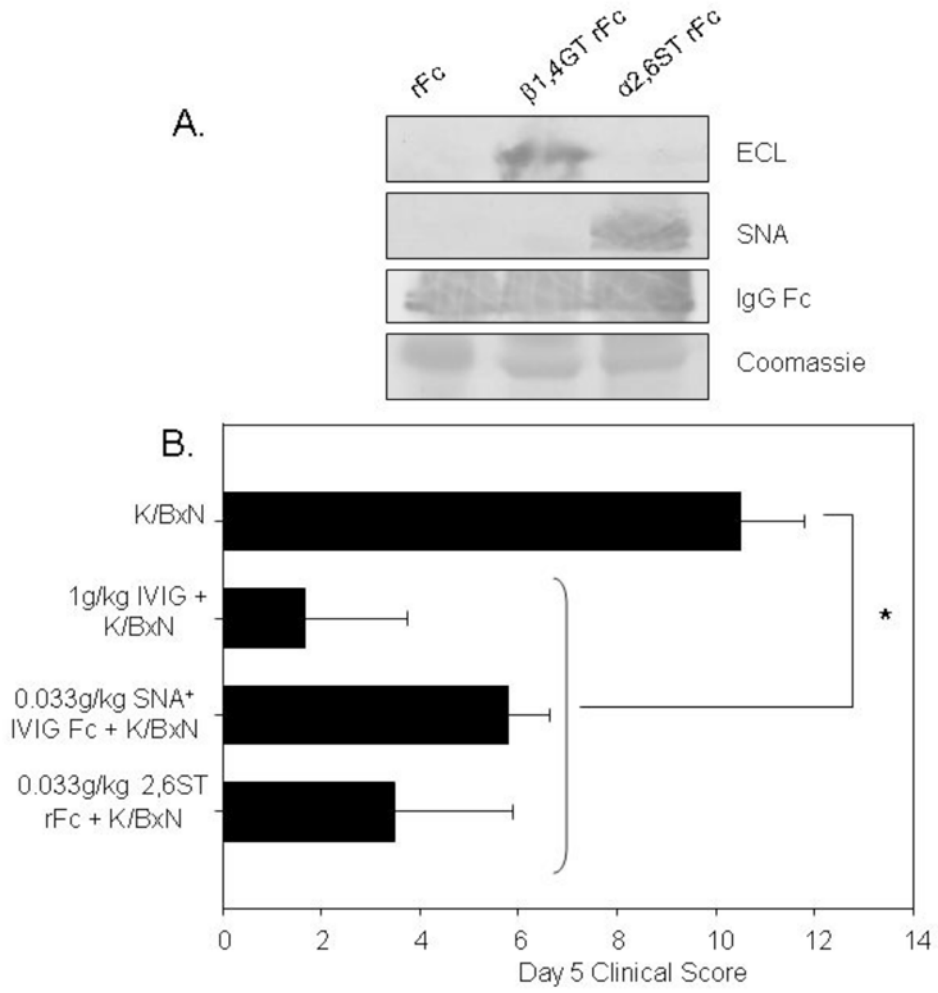

Figure 4.

Recombinant, sialylated IgG Fc fragments are anti-inflammatory. Recombinant human IgG1 was digested with papain and Fcs were purified by HPLC followed by protein G purification. The recombinant Fcs (rFc) were galactosylated and sialylated *in vitro* with α2,6 sialyltransferase. A. Glycosylation was confirmed by lectin blotting for terminal galactose with ECL (top panel), α2,6 sialic acid with SNA (middle panel), and coomassie loading controls are shown in the bottom panel. B. Mice were administered 1g/kg IVIG, 0.033g/kg SNA^{+} IVIG Fcs, or 0.33g/kg sialylated rFc (2,6ST rFc) 1 hour prior to K/BxN sera, and footpad swelling was monitored over the next several days. Mean and standard deviation of clinical scores of 4–5 mice per group are plotted; *denotes $p<0.05$ as determined by Kruskal-Wallis Anova followed by Dunn's post hoc.