Supporting Information

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SI Materials and Methods

IVIg. IVIg (Gammagard) was purchased from Baxter Healthcare Corporation. For all biological studies, s4-IVIg and IVIg were derived from the same IVIg lot.

B4GalT and ST6Gal1. Human B4GalT1 was cloned from a human cDNA (matching NM_001497). Human ST6Gal1 was synthesized and codon-optimized by GeneArt (Life Technologies). Both were cloned into an expression vector that contained an N-terminal fusion of a Dual Strep-Tag (IBA LifeSciences) followed by an enhanced histidine affinity tag that can bind an Ni-NTA (nickel-nitrilotriacetic acid) column. Mammalian expression was accomplished using a BacMam (Life Technologies) virus approach (1). Gateway cloning methods were used to shuttle the insert cassette into the bacmid-based mammalian secretion vector, pJif1a. This vector allows making a bacmid in PP2 cells, which can be transfected into Sf9 cells to make amplifiable BacMam. P2 virus was concentrated, sterile-filtered, and added to 10- to 40-L bioreactors of 1×10^6 cells/mL HEK293 cells grown in Freestyle-293 Media (Life Technologies) for 2–4 d at 37 °C. The cells were spun down, and the spent media were concentrated to 10 L by way of tangential flow filtration ultrafiltration using a 30-kDa membrane. The enzymes were captured on a Poros HS-50 (Applied Biosystems) column and eluted with a sodium chloride gradient. The pooled enzyme fractions were loaded onto a second column for the final purification step. B4GalT1 was purified on a StrepTactin-Sepharose FastFlow (IBA GmbH) column and eluted with desthiobiotin. ST6Gal1 was purified with an Ni-NTA column and eluted with imidazole. Both enzymes were buffer-exchanged into PBS, concentrated to ∼10 mg/mL, and sterile-filtered. They were frozen in aliquots at −30 °C until needed. Both were found to be highly purified of host cell proteins using a variety of methods, including ELISA and MS.

Recombinant Human Fc. A stable Chinese hamster ovary cell line was generated that expressed the Fc domain of human IgG1 (residues 234–461) by the Kabat numbering system (2). The secreted recombinant human IgG1 Fc was captured from spent media and purified by protein A affinity chromatography followed by cation exchange chromatography.

Sialylation Reaction Time-Course Analysis. Galactosylation–sialylation reactions were set up as follows: 3.3 mg recombinant human IgG1 Fc was incubated for 48 h at 37 °C with 0.4 U B4GalT in a 0.8-mL solution containing 25 mM Mops-HCl (pH 7.4), 10 mM $MnCl_2$, and 50 mM UDP-Gal. For sialylation, Mops-HCl (pH 7.4) was increased to 50 mM, CMP-NANA was added to 20 mM, and 3 U ST6-GalT1 was added in a final volume of 1 mL. The reaction mixture was incubated at 37 °C. Aliquots were extracted at the indicated times and frozen at −20 °C until analyses were performed.

Endotoxin Measurement. Endotoxin levels were determined using an Endosafe-PTS (point-of-use test system) (Charles River Laboratories), PTS cartridges, and pyrogen-free, sterile pipet tips and vials. Aliquots of IVIg and s4-IVIg were diluted with limulus amebocyte lysate reagent water before they were loaded onto the cartridges.

Size Exclusion Chromatography Coupled to MS. In total, 90 μg IVIg was diluted to a concentration of 3 μg/μL in 30 μL 100 mM ammonium acetate (pH 6.7). Papain (Worthington Biochemical) was added at a 1:200 (wt/wt) ratio. Digestion was carried out at 37 °C for 15 min and quenched by cooling on ice and adding iodoacetamide (IAM) to a concentration of 50 mM. Size exclusion chromatography separation was performed on a Zenix-C SEC-300 (2.1 × 350 mm; Sepax Technologies) using a mobile phase consisting of 78.98% (vol/vol) water, 20% (vol/vol) acetonitrile, 1% formic acid (FA), and 0.02% trifluoroacetic acid at a flow rate of 80 μL/min. Mass spectra were acquired on an QSTAR Elite (Applied Biosystems) Q-ToF Mass Spectrometer operated in positive mode. Bayesian peak deconvolution was applied to determine the neutral mass of the polypeptides.

Peptide and Glycopeptide Liquid Chromatography–MS/MS. IVIg/s4-IVIg at 100 mg/mL was diluted in 6 M guanidine (Sigma) to a final concentration of 2 mg/mL. DTT was added to a concentration of 10 mM, and the protein was denatured by reducing the disulfide bonds at 65 °C for 30 min. After cooling on ice, the samples were incubated with 30 mM IAM for 1 h in the dark to cause carbamidomethylation of the cysteine residues. Guanidine, DTT, and IAM were all purchased from Sigma. The protein was then dialyzed across a 10-kDa membrane into 25 mM ammonium bicarbonate buffer (pH 7.8). Trypsin (Promega) was added to the sample for proteolysis, which was carried out in a Barocycler (NEP 2320; Pressure Biosciences, Inc.). The pressure was cycled between 20,000 psi and ambient pressure at 37 °C for a total of 30 cycles in 1 h.

Liquid chromatography (LC)–tandem MS analysis of the tryptic digests was performed on an Ultimate 3000 (Dionex) Chromatography System and an LTQ XL (Thermo Fisher Scientific) Mass Spectrometer. Peptides were separated on a BEH PepMap (Waters) Column using 0.1% FA in water and 0.1% FA in acetonitrile as the mobile phases.

Glycosylation was quantified for the different isotypes by the area from the extracted ion chromatogram for the tryptic glycopeptides as described by Zauner et al. (3). IgG1 and IgG4 isotypes have unique glycopeptide sequences that can be quantified individually (IgG1, EEQYNSTYR; IgG4, EEQFNSTYR). IgG2 and IgG3 share a glycopeptide sequence and were quantified together (IgG2/3, EEQFNSTFR).

Posttranslational modifications (PTMs) were quantified from the same tryptic digest. Monitored PTMs included asparagine deamidation, methionine oxidation, proteolytic fragments, and glycol oxidation products (i.e., AGEs). Quantitation of the PTMs was achieved by extracting and integrating the signal from the most abundant charge state for the modified and unmodified peptides; the relative signal values were used to estimate the relative abundances. Heavy-chain isotype distribution was determined based on the relative abundance of similar but unique peptides from the CH2 domain of each isotype. The relative abundance of IgG1 and IgG3 was based on the area of the peptide ALPAPIEK, which is common to both isotypes. IgG2 was quantified based on the area of the peptide GLPAPIEK, and IgG4 was quantified based on the area of the peptide GLPSSIEK. The abundance of IgG1 relative to IgG3 was based on the area of the peptide FNWYVDGVEVHNAK relative to the area of the peptide WYVDGVEVHNAK. The light-chain isotypes were quantified similarly using peptides from the constant light domain of the κ-light chain and the λ1- and λ2-light chains.

IVIg Background Proteome Analysis. Protein G HP SpinTrap (GE Healthcare) columns were used for the removal of IgG and the enrichment of non-IgG proteins. Briefly, the IVIg sample (500 μg) was added onto the spin column for binding of IgGs. After 10 min of binding, the spin columns were washed, and the wash was then

collected for the non-IgG proteins. The wash sample was buffer-exchanged into 50 mM ammonium bicarbonate solution using Zeba Spin Desalting Columns (Thermo Scientific). A very low amount of protein was present in the wash sample; hence, the entire sample was taken for reduction [20 mM DTT/8 mM Tris(2-carboxyethyl) phosphine for 30 min at 50 °C followed by alkylation using 40 mM IAM in darkness for 20 min. Samples were digested using trypsin at a 50:1 (wt/wt) protein-to-enzyme ratio overnight at 37 °C with shaking at 400 rpm. A total of 1 μg of each digested sample was injected for nano–LC-MS/MS analysis. Separations were carried out using an Ultimate 3000 RSLC (Dionex) Nanosystem. Chromatography was carried out using an analytical EASY-Spray PepMap RSLC (25 cm × 75 μm i.d., C18 2 μm, 100 Å; Thermo Scientific) nanocolumn thermostatically controlled at 50 °C and 300 nL/min with a linear gradient from 1% to 38% acetonitrile/water both containing 0.1% (vol/vol) FA for a total duration of 150 min. The separation step was followed by a 30-min washing step with 99% acetonitrile/water, which was followed by a 20-min equilibration step with 99% water/acetonitrile, both containing 0.1% (vol/vol) FA. Data-dependent MS/MS was performed on the top 25 precursor ions from the full MS scan on the Orbi-Velos Mass Spectrometer (Thermo Scientific). Proteome discoverer with the SequestHT search engine (Thermo Scientific) was used for database searching of each sample against the Uniprot human database (UniProtKB).

Fab Glycosylation Analysis: Papain Digestion. IVIg was digested using papain enzyme at a 1:200 enzyme-to-substrate ratio under denaturing conditions at 37 °C for 30 min. The reaction was quenched using IAM.

Fab Glycosylation Analysis: Affinity Purification. Papain-digested IVIg was further separated using protein A on a Profinia (Bio-Rad) system. Citrate buffer was used to elute the Fc, and the Fab was collected in the flow through and wash. Additional Fab (flow through and wash combined) was purified on the Protein L column and eluted using a glycine buffer. Purified Fc and Fab fragments were buffer-exchanged into 1× PBS and lyophilized for glycan analysis.

Fab Glycosylation Analysis: Glycan Release and Analysis. Resultant Fab and Fc postaffinity separation was subjected to glycan release. Denaturing conditions were used for the Fab and Fc deglycosylation, which was performed under native conditions. PNGase F enzyme was used with the deglycosylation buffer (ProZyme) for ∼3.5 h at 37 °C in a water bath. The samples were then cleaned using Hypercarb (Thermo Scientific) cartridges on a vacuum manifold to remove the protein pellet. The released glycans were labeled with 2-aminobenzamide (2AB) for ∼2.5 h at 65 °C followed by hydrophilic interaction LC-based cleanup to remove excess label. Both Fab and Fc glycans were analyzed with the use of hydrophilic interaction LC-LC-MS. A BEH glycan column was used for separation using 10 mM ammonium formate buffer at pH 4.5 (buffer A). The glycans were loaded with 90% acetonitrile in 10 mM ammonium formate buffer (buffer B) and then, eluted at 50% of buffer A after 35 min. Fluorescence detection was used at excitation of 420 nm and emission of 330 nm for 2AB-labeled glycans.

Antigen-Binding Specificity ELISA. Sandwich ELISAs were used that included the corresponding antigens and antibodies: human anti-*Haemophilus influenzae* B IgG (980–100-PHG; Alpha Diagnostic International), human anti-CMV IgG antibody (Qayee-Bio), human varicella zoster virus IgG (Qayee-Bio), human parvovirus B19 IgG (Qayee-Bio), human antirubella virus IgG (Qayee-Bio), human measles virus IgG (Qayee-Bio), human tetanus antibody (Qayee-Bio), and human anti-EBV antibody (Qayee-Bio). The assays consisted of a sandwich ELISA, in which samples and standards were added to a 96-well plate precoated with a capture antibody for the target of interest. An HRP-labeled detection antibody was added to the samples/standards, and the assay plate was incubated for 1 h. Plates were then washed, a chromogenic substrate was added, and color was developed by the enzymatic reaction of HRP on the substrate. The reaction was terminated, and absorbance at 450 nm was measured with a plate reader.

Pharmacokinetic Analysis. The pharmacokinetic study was carried out using 8-wk-old C57BL6/J female mice (20 ± 2 g) purchased from The Jackson Laboratory. Animals were housed in standard cages and given ad libitum access to food and water. They were acclimatized for at least 3 d before the experiment. One group of animals was administered IVIg at 0.6 g/kg in a volume of 200 μL i.v., and the other group was injected with s4-IVIg at 0.6 g/kg in the same volume. Blood samples (50 μL) were collected from saphenous veins of two mice per time point from the groups at 0.5, 4, 8, and 24 h on day 1 and 2, 4, 6, 8, 12, 14, 21, 26, 30, 36, 41, 50, 56, 61, and 68 d postdose. A staggered sampling strategy was used, such that each mouse was bled a total of three times followed by terminal cardiac bleed. Serum concentrations of IVIg and s4-IVIg were determined using ELISA. In brief, $F(ab')_2$ goat anti-human IgG (H+L) at 1 μg/mL was coated onto 96-well Nunc MaxiSorp (Sigma) microplates, and HRP-conjugated $F(ab')_2$ goat anti-human IgG (H+L) at 1:50,000 dilution was used for detection. The detection range in the standard curve was 5–500 ng/mL. For the assays, serum matrix controls of analytes within the standard curve range were assessed for accuracy and precision during assay development. Pharmacokinetic parameters, including half-life, area under the curve, maximum concentration (C_{max}), and clearance, were determined by noncompartmental analysis in WinNonlin and expressed as means ± SDs. Data are presented as means ± SDs of samples from two individual mice.

Biodistribution Analysis. Female BALB/c mice were injected i.v. with 0.2 mL of a 50-mg/mL mixture containing a mixture of unlabeled and 594-nm labeled (70:30 wt/wt) IVIg or s4-IVIg at time 0. Mice were imaged in an in vivo imaging system spectrum after 4 h, 24 h, 48 h, 4 d, and 10 d. At each time point, two mice from each group were humanely killed for ex vivo imaging as follows. Immediately after in vivo imaging, mice were humanely killed by CO_2 asphyxiation before perfusion with 10 mL saline. Heart, thymus, lungs, kidneys, spleen, brain, and liver lobes were arranged in 24-well black wall plates for ex vivo imaging. Intestines were imaged on weigh boats. All images were captured using a 570/620-excitation/emission filter set. Regions of interest were drawn around the periphery of each organ. Fluorescence measurements were normalized to regions of interest areas. Ex vivo data are presented as a percentage relative to mean 4-h fluorescence of each tissue for each group (mean ± SEM; $n = 2$).

Animals for Efficacy Studies. Age-matched mice at 8–16 wk of age on the C57BL/6 background obtained from Janvier were used in all experiments. KRN TCR transgenic mice on a C57BL/6 background (K/B) were gifts from Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA) and bred with NOD mice to generate K/BxN mice. All mice were maintained under specific pathogen-free conditions. All experiments were performed with the approval of the local ethics committees and according to the guidelines of the National Institutes of Health and the legal requirements of Germany and the United States.

CAIA Model. On study day 1, the C57B/6 mice were dosed i.v. with vehicle control, and 0.1 g/kg and 1 g/kg of IVIg and s4-IVIg 15–20 min before they were given i.p. injection (8 mg) of an arthritogenic mAb mixture (ArthritoMab; MD Biosciences). On study day 4, the animals received i.p. injection (100 μg) of LPSs to boost disease induction/progression. Animals were given a clinical score

based on the sum of all disease severity in all paws (each paw was scored on a scale of 1–4), and body weights were recorded daily.

ITP Induction and Therapeutic Intervention. 6A6-IgG2a antibodies were produced by transient transfection of 293T cells followed by purification of recombinant antibodies from serum-free cell culture supernatants with protein G beads (GE Healthcare) as suggested by the manufacturer. IVIg preparations were diluted in glycine buffer (saline) for experiments. Chronic ITP was induced by daily injections of 0.1 μg/g 6A6-IgG2a antiplatelet antibody. Mice were rendered thrombocytopenic until the end of the experiment (day 3). Platelet counts were determined before and 4 h after daily antibody injection of a 1:4 dilution in PBS in a hematology system (Advia 120; Bayer HealthCare). Platelet counts before antibody injection were set to 100%. For intervention, either IVIg preparation or saline was injected 48 h after the first injection of antiplatelet antibody.

Serum Transfer Arthritis and Therapeutic Intervention. Sera of K/BxN mice collected over several weeks were pooled and frozen in aliquots to be used in all experiments. Inflammatory arthritis was induced by one 14-μL/g injection of pooled sera of K/BxN mice. For intervention, either IVIg preparation or saline was injected 3 d after transfer of K/BxN serum. Arthritis was scored by clinical examination, and the index of all four paws was added (0 = unaffected, 1 = swelling of one joint, 2 = swelling of more than one joint, 3 = severe swelling of the entire paw) as described (4).

Epidermolysis Bullosa Acquisita Induction and Therapeutic Intervention. IgG serum antibodies containing antibodies specific for mouse type VII collagen were purified from rabbit hyperimmune serum. Induction of epidermolysis bullosa acquisita in C57BL/6 mice was performed as described (5). Briefly, 2 mg purified total rabbit IgG serum antibodies containing antibodies specific for murine type VII collagen were injected s.c. into the backs of adult mice every second day over a period of 12 d. Blisters or erosions were counted, and the extent of skin disease was evaluated as a percentage of the body surface area affected by blisters/erosions. For intervention, either IVIg preparation or saline (vehicle) was injected 2 h before the first passive transfer of collagen VII-specific antibodies.

Histopathology and Immunofluorescence Microscopy. For histopathology of epidermolysis bullosa acquisita-diseased mice, biopsy specimens of lesional and perilesional skin (at day 12) were fixed in 4% (vol/vol) buffered formalin and embedded in paraffin. Five-micrometer sections from paraffin-embedded tissues were stained with H&E. Histologic evaluation was performed in at least four different areas of each biopsy specimen.

Statistical Analysis. All statistical analyses were performed in Prism (GraphPad) software. For calculation of statistical significance, one-way ANOVA with Bonferroni correction (posthoc test) was used in Figs. 4 and 5 and Fig. S3. $P < 0.05$ was considered significant.

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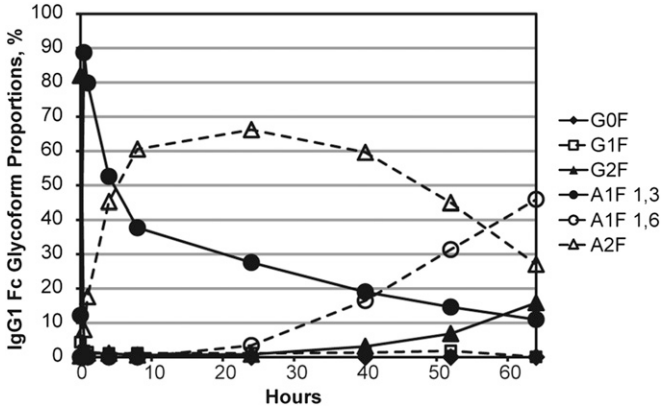

Fig. S1. Time course of IVIg Fc glycoform proportions in the presence of CMP-NANA and ST6Gal1. Galactosylated IVIg was incubated with 20 mM CMP-NANA and 0.3 U/mg ST6Gal1 at 37 °C. Aliquots were removed at different time points, and the relative proportions of IVIg glycoforms were determined by glycopeptide LC-MS/MS analyses.

AGE	Site	rhFc, %	S-rhFc (high-purity reagents), %	S-rhFc (low-purity reagents), %
Carboxymethyl arginine (CMA)	R292, R255, R355, R416	Not detected	≤1%	1-3%
Carboxyethyl arginine (CEA)	R292, R255, R355, R416	Not detected	≤1%	1-3%
5-methylimidazol-4-one (MG-H1)	R292, R255, R355, R416	Not detected	≤1%	1-10%
Imidazolidine	R292, R255, R355, R416	Not detected	≤0.1%	0.5-2%
Fructoselysine	K326, K344, K348	≤0.5%	≤0.5%	1.5%
Carboxymethyllysine	K326, K344, K348	Not detected	≤0.5%	1.5-3%

Fig. S2. Site-specific AGE modifications identified in recombinant human Fc (rhFC) after sialylation reaction. S-rhFc, sialylated-recombinant Fc.

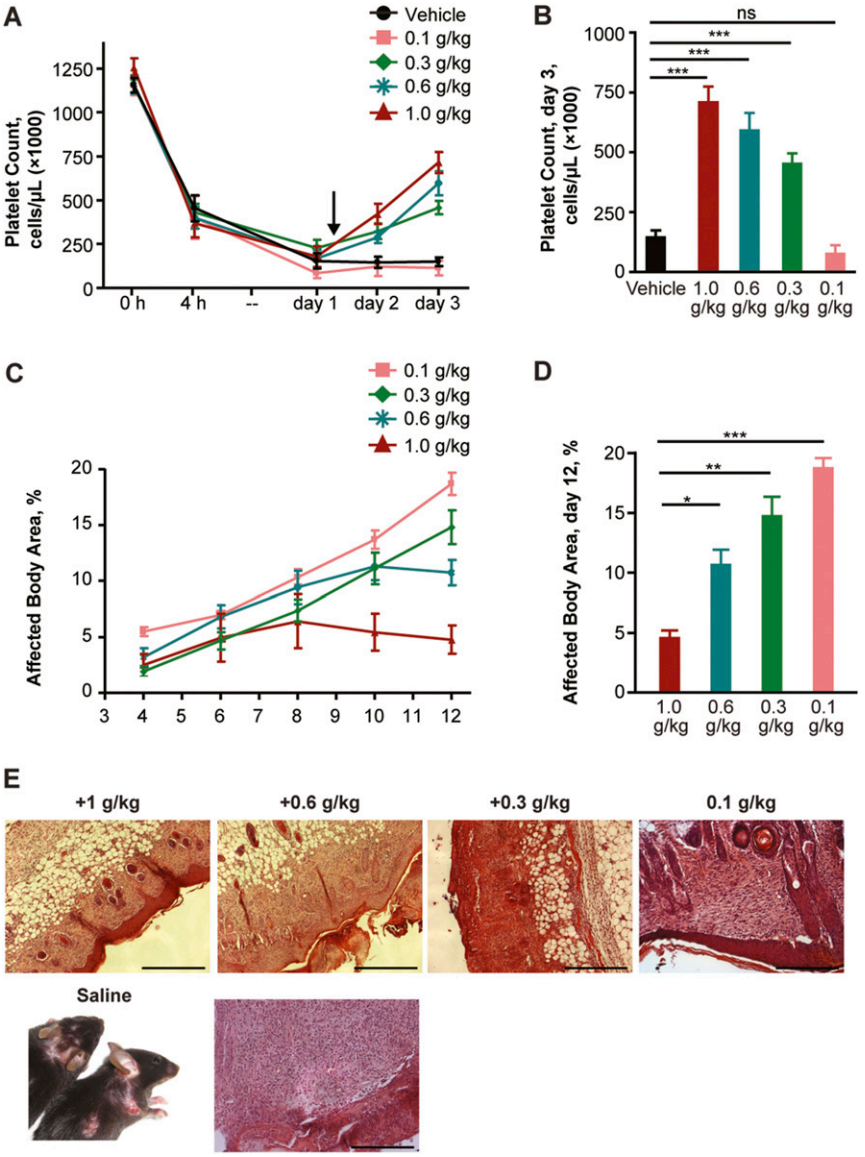

Fig. S3. IVIg dose–response in (*A* and *B*) ITP and (*C* and *D*) epidermolysis bullosa acquisita pemphigoid mouse models. (*E*) Representative histology analysis after treatment with different doses of IVIg. ns, Not significant. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Table S1. Glycolytic pathway enzymes identified in sialyltransferases of different purity

Enzyme	Detected in impure ST6Gal1	Detected in highly purified ST6Gal1
α-Enolase	Yes	No
Fructose-bisphosphate aldolase A isoform 1	Yes	No
Transketolase-like	Yes	No
Glyceraldehyde-3-phosphate dehydrogenase	Yes	No
Phosphoglycerate mutase-1	Yes	No
Phosphoglycerate kinase-1	Yes	No