# Fc galactosylation of anti-platelet human IgG1 alloantibodies enhances complement activation on platelets

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**Received:** December 13, 2021.
**Accepted**: March 22, 2022.
**Prepublished**: March 31, 2022.

**https://doi.org/10.3324/haematol.2021.280493**

**Supplementary Data**

**Supplemental Material and Methods:**

## Generation of mAbs from single B-cells

Peripheral blood was obtained from a pregnant woman hyperimmunized for both HPA-1a and HLA, with a history of fetal and neonatal alloimmune thrombocytopenia (FNAIT), after informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated and enriched for $CD19^+$ B-cells with CD19 beads (Dynabeads, Thermo Scientific). In parallel, HPA-$1a^+$ platelets were obtained from platelet-rich plasma and labeled with either CellTrace Violet or CellTrace Far Red (Thermo Scientific). To enrich for HPA-1a specific B cells, $CD19^+$ B cells were incubated with a mix of labeled platelets (1:10 B-cell/platelet ratio) followed by analysis using flow cytometry. B cells, double positive for the platelet dyes ($Violet^+$ Far $Red^+$), were single-cell sorted onto feeder cells (irradiated EL4.B5) to promote antibody production, as described previously [1]. After 10 days of culture, supernatants were analyzed for the presence of antibodies by ELISA. mRNA of B cells corresponding to positive wells was isolated (RNeasy Plus Micro Kit, Qiagen) and cDNA was subsequently synthesized using SuperScript III cDNA synthesis kit according manufacturer's instructions (Thermo Scientific). IgH and Igκ V gene transcripts were amplified by nested PCR using primers as previously described [2] and the identified V genes were synthesized from GeneArt (Life Technologies). IgH V gene was designed with compatible restriction sites for expression in pcDNA3.1 vectors containing the constant regions for IgG1.

## Expression Vectors

The protein sequences of the variable regions of all mAbs were used to assemble pcDNA3.1 expression vectors encoding for full human IgG1. Sequences of anti-HLA mAbs SN230G6 and SN607D8 were provided by the Department of Immunology, LUMC, Leiden, the Netherlands, and the sequences of W6/32, B2G1 and 26.4 were described previously [3–5]. Linear DNA fragments, coding for the VH and VL were ordered from Integrated DNA Technologies. The DNA fragments were inserted in anterior to the hKappa or the hIgG1*03 sequence in pcDNA3.1 expression vectors. The pcDNA3.1 vectors coding for W6/32 VL + hKappa and W6/32 VH + hIgG1*03 were designed and ordered fully cloned via the GeneArt Gene Synthesis Portal of Thermo Fisher Scientific. The vectors were used for the transformation of DH5α competent bacteria (Thermo Fisher Scientific). Plasmids were isolated using a NucleoBond Xtra Maxi Kit (Macherey-Nagel) according to manufacturer's instructions. For glycoengineering, the pEE6.4 or pEE14.4 expression vector, encoding for β-1,4 galactosyltransferase 1 (B4GALT1) or β-galactoside alpha-2,6-sialyltransferase 1 (ST6GALT), respectively, were used as described previously [6,7]. To enhance IgG production, vectors encoding for p21 (Invivogen, porf-hp21), p27 (Invivogen, pORF-hp27 v02), and SV40 adenovirus large –T antigen (pSVLT) were used[8].

## Production of recombinant mAbs

The production of recombinant mAbs was described previously [9,10]. In brief, human embryonic kidney (HEK) 293F cells (Thermo Fisher Scientific) were cultured in vented Erlenmeyer flasks (Corning) with FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific) at 37°C and 8% $CO_2$ on an orbital shaker with a rotation speed of 125 rpm. The cell concentration was adjusted to $1x10^6$ cells/mL prior to transfection, in fresh medium. A DNA mix, containing pcDNA3.1 expression vectors coding for the H- and L-chain of the anti-HLA/HPA-1a antibodies and a pSVLT/p21/p27-mix [8], was added to Opti-MEM (Thermo Fisher Scientific) containing 45 mg/ml linear polyethyleneimine HCl (PEI) MAX (Polysciences) and incubated

for 20 min at RT. The transfection mixture was added to the cell cultures and incubated at 37°C and 8% $CO_2$ on an orbital shaker with a speed of 125 rpm. 100 U/ml penicillin and 100 µg/ml streptomycin (Thermo Fisher Scientific) were added 4 hours post transfection. After six days, the cells were centrifuged and the supernatant was collected and filtered with a 0.45-µm syringe filter (Whatman).

**Glycoengineering of mAbs**

In order to increase Fc galactosylation, 1 hour prior to the transfection 5 mM D-galactose (Sigma-Aldrich) was added to the cell culture [6]. Furthermore, 1% of the pEE6.4 expression vector, encoding for β-1,4 galactosyltransferase 1 (B4GALT1), was added to the DNA mix for transfection. This percentage was calculated based on the total amount of DNA used. To increase Fc-sialylation, 5 mM D-galactose, 1% pEE6.4 B4GALT1 and 2.5% of the pEE14.4 expression vector, encoding for ST6GALT, were added to the DNA mix for transfection [6].

**Purification of mAbs**

Antibody purification was performed on the AKTA Prime Plus (GE HealthCare) equipped with a 5 mL protein A HiTrap HP column (GE HealthCare) and elution was done with a low pH buffer (0.08 M Citric Acid/0.04 M $Na_2HPO_4$, pH 3). After elution the pH was neutralized with 1 M Tris-HCL pH 9 and the obtained fractions were concentrated using the 2-6 mL Pierce™ Protein Concentrator PES, 10K MWCO (Thermo Fisher Scientific). The antibodies were dialyzed O/N to 5 mM sodium acetate buffer (pH 4.5) to avoid aggregation.

**Digestion of IgG for mass spectrometry**

100 µL of 100 mM formic acid was incubated with 5 µg antibody for 30 min. at RT while shaking. Hereafter, the sample was dried for 2 hours at 50ºC in a centrifugal vacuum concentrator and dissolved in 20 µL 50 mM ammonium bicarbonate. 20 µL of 10 ng/µL sequencing grade modified trypsin (Promega) was added to digest the antibodies O/N at 37ºC.

**Mass spectrometric glycosylation analysis**

The glycopeptides were analyzed by nano-RP-LC-ESI-qTOF-MS on an Ultimate 3000 RSLCnano system (Thermo Scientific) coupled to an Impact quadrupole-time-of-flight-MS using a CaptiveSpray and nanoBooster (Bruker Daltonics) [11]. 200 nL of sample was injected on a trap column (Pepmap100 C18 5 µm 0.3 mm x 5 mm (Thermo Scientific) and washed at 25 µl/min with 0.1% TFA (Merck) for 1 min before separation on a BEH C18 column (nanoEase M/Z peptide, 1.7 µm particle size, 75 µm x 100 mm (Waters). A binary linear gradient was applied at 600 nl/min with 0.1% TFA (solvent A) in 95% acetonitrile (LC-MS grade, Biosolve; solvent B): gradient from 3% B to 21.7% B in 0–4.5 min, to 50% B in 4.5-5.5 min, 50% B for 2.5 min and in 1 min to 3% B and re-equilibration for 2.5 min. ESI was performed with an end plate offset of -500 V, capillary voltage 1200 V, nanoBooster pressure 0.2 bar using acetonitrile-enriched nitrogen, dry gas flow 3.0 L/min, dry temperature 180°C. Mass spectra were recorded from *m/z* 550-1800 at 1 Hz with active focus setting, using 5eV collision cell voltage, 110 µs transfer time, and 21 µs pre-pulse storage. The data analysis was performed in Skyline 19.1.0.193 or DataAnalysis 5.0. The first isotopic peak of triple protonated glycopeptides signals were manually integrated and the relative abundance was calculated. The glycosylation traits were calculated with the following formulas as detailed previously[6]: fucosylation = G0F+G0FN+G1F+G1FN+G1FS+G2F+G2FN+G2FS+G2FS2, bisection = G0FN+G1FN+G2FN, galactosylation = (G1F+G1FN+G1FS)/2+(G2+G2F+G2FS+G2FS2) and sialylation = (G1FS+G2FS)/2 + G2FS2

**Production of HLA-monomers**

Recombinant HLA-A*02:01 heavy chains were produced in Escherichia coli ( E. coli). Peptide-HLA (pHLA) class I complexes pHLA-A*02:01 were generated through *in vitro* refolding reactions with E. coli-derived β2M, as described [12], in the presence of EBV peptide GLCTLVAML (JPT Peptide Technologies GmbH). All pHLA class I complexes were, enzymatically biotinylated using BirA, purified by gel-filtration HPLC in PBS (pH 7.4) and stored at -70°C as reported elsewhere [12].

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**Supplemental table/figure legends:**

**Supplemental Table 1: HLA-typing of all platelet donors**

**Supplemental Figure 1 | A)** Representative mass spectra of IgG1 glycopeptides at N297 positioned in the Fc-region of the glycoengineered SN230G6 monoclonal antibodies. The 3+ charged glycopeptides with relative abundance of >1% are annotated. **B)** Fc-glycosylation profiles of produced anti-HLA mAbs using different glycoengineering techniques to increase Fc galactosylation and sialylation, analyzed by mass spectrometry.

**Supplemental Figure 2 |** Flow cytometric gating strategy and histograms of the binding of anti-platelet antibodies and their complement activation properties. **A-B)** The platelets were gated based on the FSC-A/SSC-A and the single cells were selected (FSC-H/FSC-A). **C-E)** The geometric-mean fluorescence intensities (gMFI) of all parameters were calculated. Platelets were incubated with 20 µg/mL Isotype Ctrl (Blue) or SN230G6 + SN607D8 Sial. (Red).

**Supplemental Figure 3 | A-F)** Complement activating properties of unmodified anti-HLA and anti-HPA-1a mAbs on the surface of matched platelets analyzed by flow cytometry at 20 µg/mL. C3b-deposition was normalized to the maximum C3b-deposition per experiment. Data represents the mean and SEM of three independent experiments using different platelet donors with the following HLA-typing: HLA-A01/A02/B07/B08 and HLA-A02/A31/B40/B44. For the statistical analysis an ordinary One-way ANOVA with Tukey's multi-comparison test was performed.

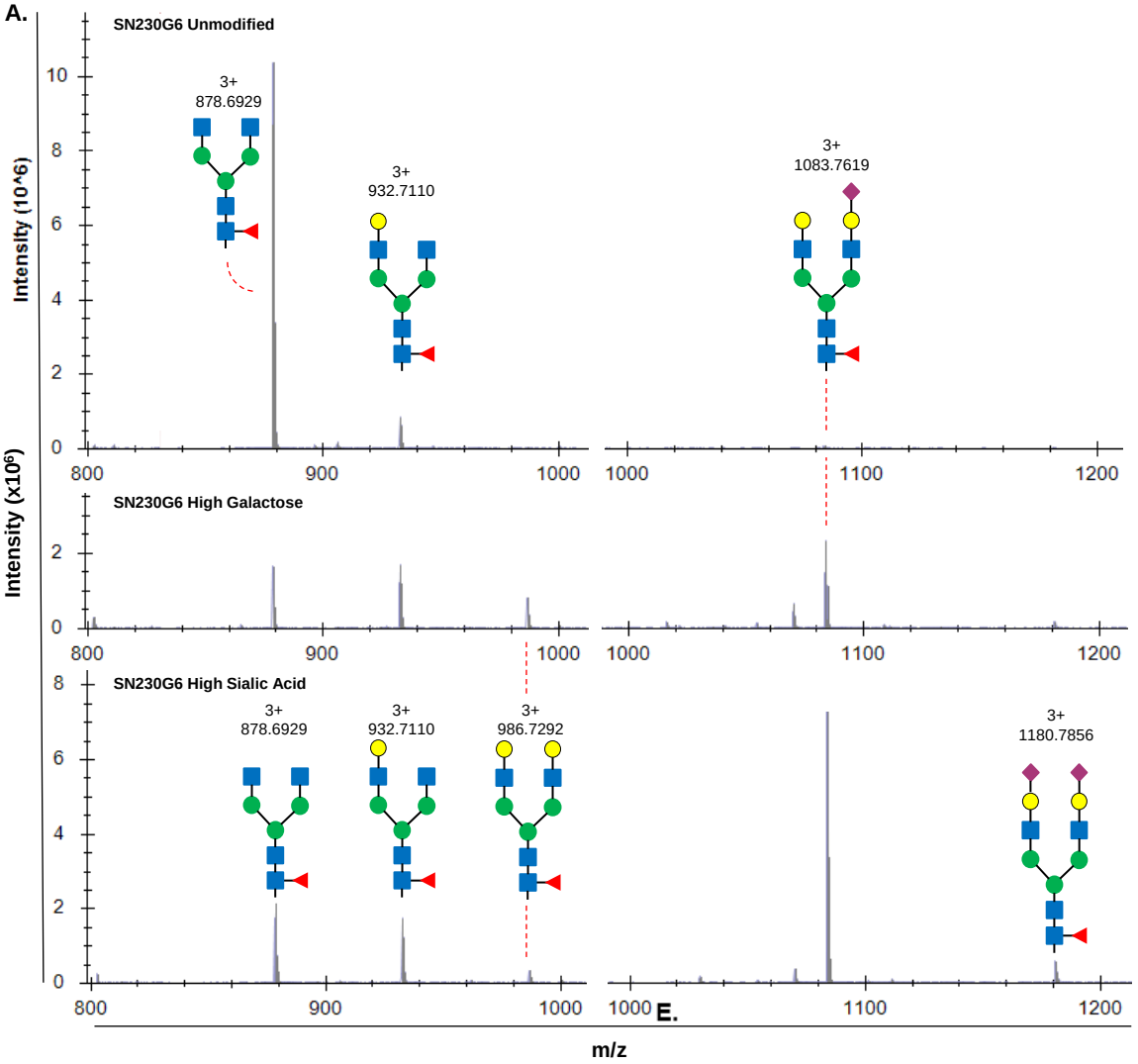
**Supplemental Figure 4 | Complement activating properties of glycoengineered anti-HLA and anti-HPA-1a mAbs on the surface of matched platelets analyzed by flow cytometry at 20 µg/mL. A-E)** C1q-binding and **F-J)** C3b-deposition were normalized to the complement activating properties of the unmodified antibodies. Data represents the mean and SEM of 3-5 independent experiments, for the statistical analysis an ordinary One-way ANOVA with Tukey's multi-comparison test was performed.

**Supplemental Figure 5 | Complement Dependent Cell Death, caused by anti-HLA mAbs, of non-fixed platelets. A-K)** Non fixed platelets with HLA-typing: A01/A02/B07/B08 were incubated with individual or combinations of glycoengineered anti-HLA mAbs (20 µg/mL) in the presence of complement rich serum. Samples were analyzed by flow cytometry and differences between SSC-A/FSC-A were visualized to show complement dependent cell death. Abbreviations: Unm. = Unmodified, Gal. = High Galactose and Sial. = High Sialic Acid.

**Supplemental Table 1: HLA-typing of all platelet donors**

| Donor # | HLA-Typing | | | | |
|---|---|---|---|---|---|
| 3527 | HLA | A02 | A26 | B35 | B44 |
| 0968 | HLA | A01 | A02 | B07 | B08 |
| 6244 | HLA | A02 | A31 | B40 | B44 |
| 6775 | HLA | A02 | - | B35 | B40 |
| 2469 | HLA | A02 | A24 | B07 | B35 |
| 7470 | HLA | A01 | A02 | B07 | B08 |

A.

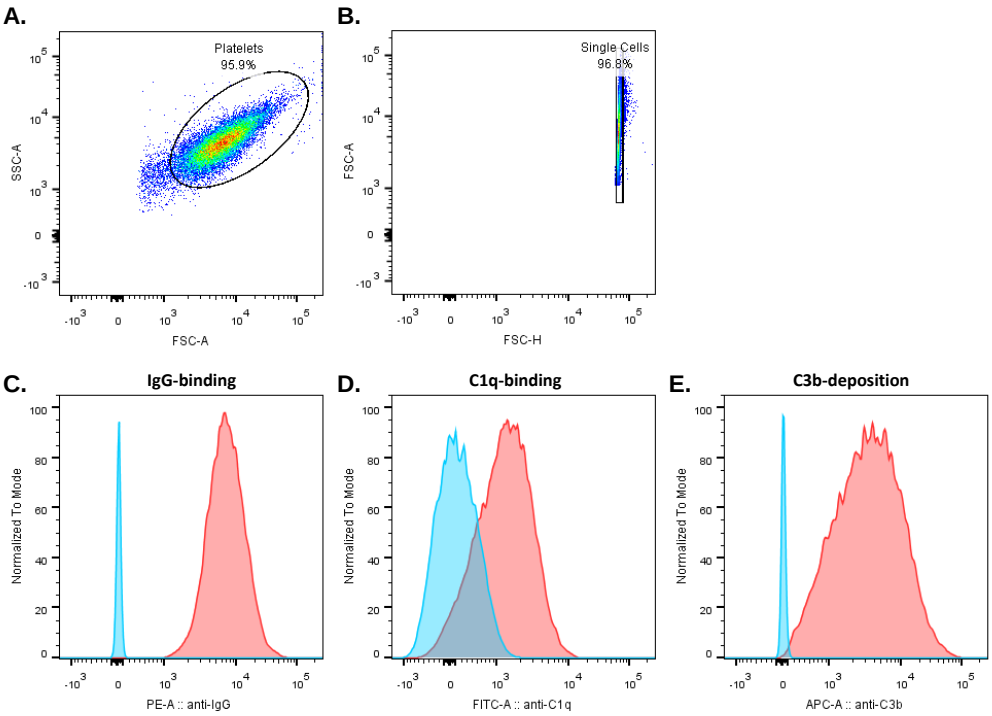



B.

| | Unmodified | | | High Galactose | | | High Sialic Acid | | |
|---|---|---|---|---|---|---|---|---|---|
| H3N4 (G0) | 0.39 | 0.42 | 0.31 | 0.34 | 0.17 | 0.06 | 0.18 | 0.18 | 0.05 |
| H3N4F1 (G0F) | 65.14 | 61.33 | 41.62 | 12.75 | 8.05 | 6.82 | 12.67 | 6.54 | 7.39 |
| H3N5F1 (G0FN) | 2.44 | 3.54 | 1.23 | 0.25 | 0.28 | 0.19 | 0.22 | 0.17 | 0.17 |
| H4N4F1 (G1F) | 25.25 | 26.44 | 39.58 | 35.38 | 12.89 | 13.67 | 28.73 | 12.83 | 15.36 |
| H4N5F1 (G1FN) | 1.30 | 1.61 | 1.42 | 1.13 | 1.02 | 0.86 | 0.88 | 0.61 | 0.73 |
| H4N4F1S1 (G1FS) | 0.78 | 0.51 | 0.42 | 0.47 | 0.14 | 0.10 | 8.84 | 2.99 | 1.58 |
| H5N4 (G2) | 0.10 | 0.14 | 0.04 | 0.21 | 0.27 | 0.25 | 0.15 | 0.15 | 0.08 |
| H5N4F1 (G2F) | 3.18 | 4.74 | 13.45 | 37.45 | 60.12 | 63.41 | 13.45 | 24.35 | 23.97 |
| H5N5F1 (G2FN) | 0.57 | 0.58 | 0.24 | 1.07 | 1.26 | 0.64 | 0.59 | 0.57 | 0.34 |
| H5N4F1S1 (G2FS) | 0.65 | 0.58 | 1.37 | 8.14 | 12.19 | 11.45 | 26.13 | 38.58 | 39.18 |
| H5N4F1S2 (G2FS2) | 0.21 | 0.12 | 0.32 | 2.83 | 3.62 | 2.55 | 8.15 | 13.04 | 11.14 |
| Fucosylation | 95.77 | 94.29 | 97.00 | 98.07 | 98.26 | 98.63 | 98.57 | 98.89 | 98.96 |
| Galactosylation | 18.37 | 20.44 | 36.12 | 68.17 | 84.48 | 85.61 | 67.71 | 84.90 | 83.55 |
| Sialylation | 0.92 | 0.67 | 1.21 | 7.13 | 9.78 | 8.33 | 25.64 | 33.82 | 31.52 |
| Bisection | 4.31 | 5.73 | 2.89 | 2.45 | 2.56 | 1.69 | 1.69 | 1.35 | 1.25 |
| | SN230G6 U | SN607D8 U | W6/32 U | SN230G6 Gal | SN607D8 Gal | W6/32 Gal | SN230G6 Sial | SN607D8 Sial | W6/32 Sial |



**Supplemental Figure 1** | A) Representative mass spectra of IgG1 glycopeptides at N297 positioned in the Fc-region of the glycoengineered SN230G6 monoclonal antibodies. The 3+ charged glycopeptides with relative abundance of >1% are annotated. B) Fc-glycosylation profiles of produced anti-HLA mAbs using different glycoengineering techniques to increase Fc galactosylation and sialylation, analyzed by mass spectrometry.

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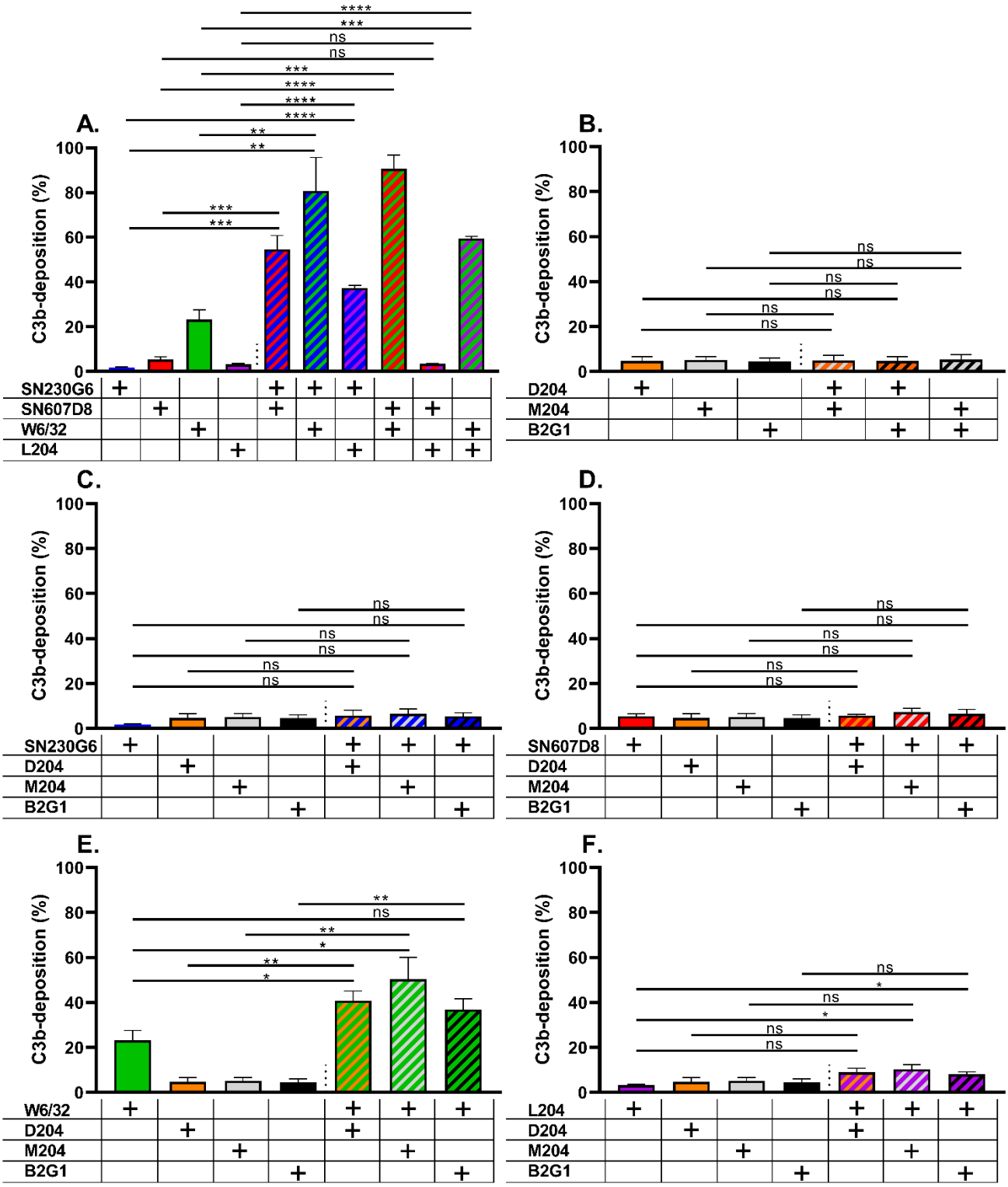


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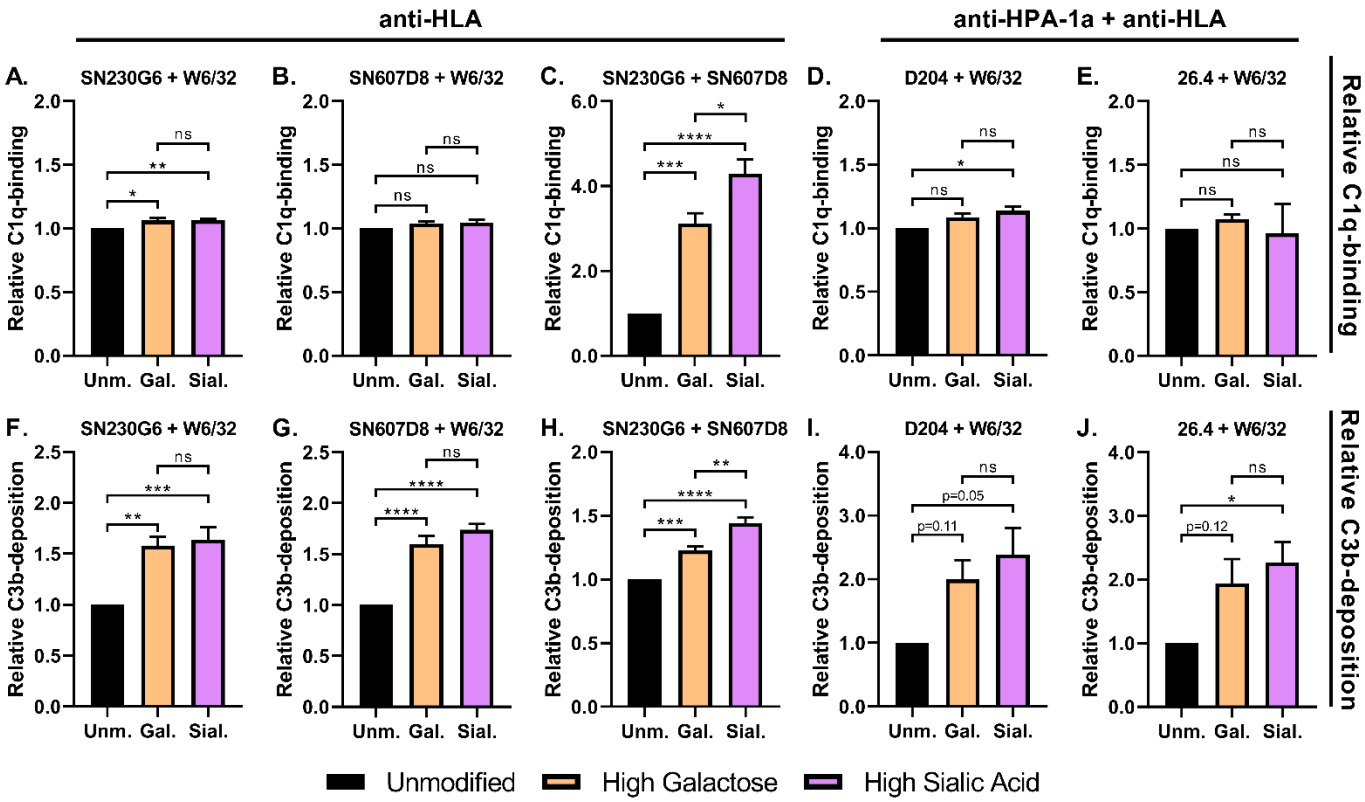


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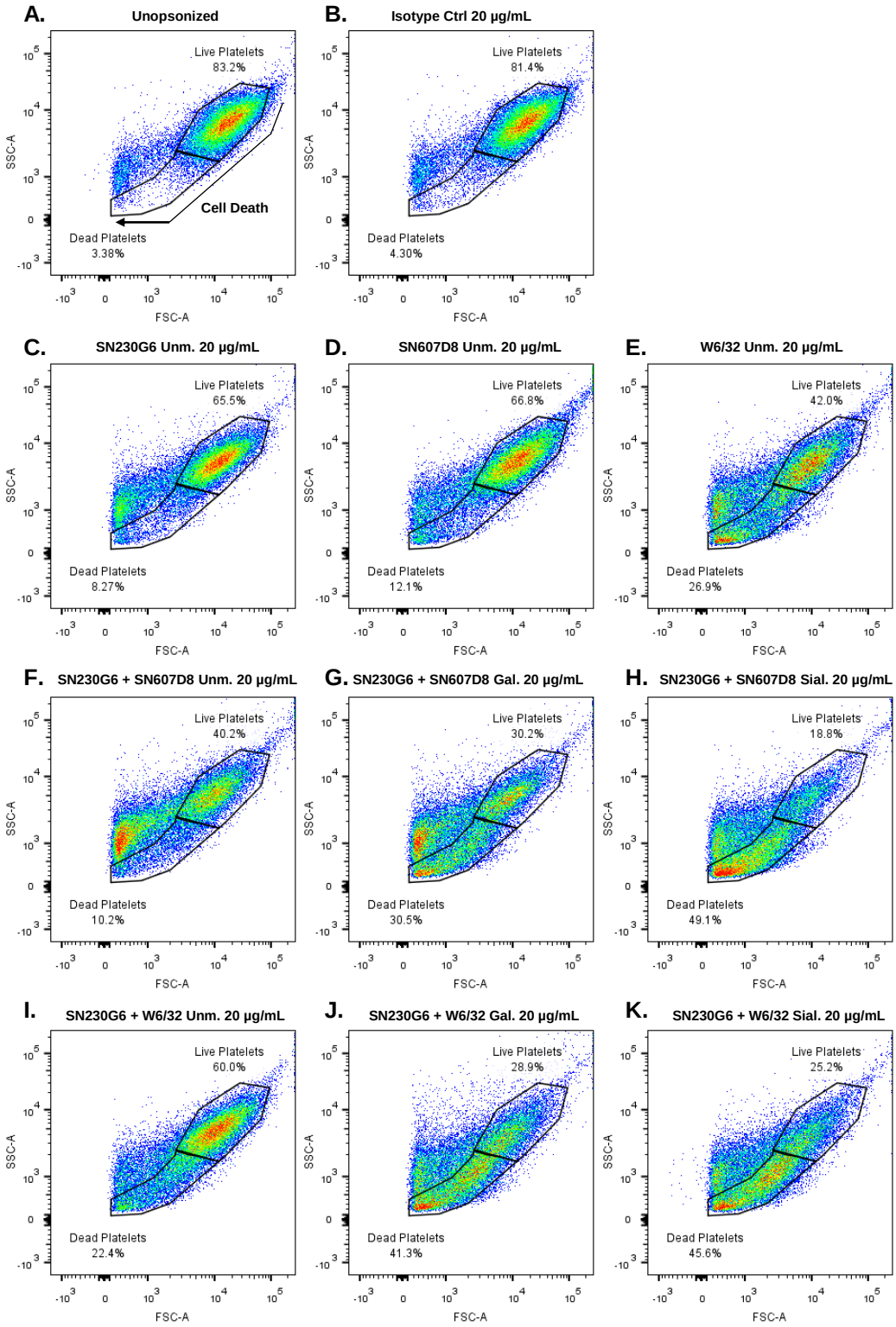


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