EFFECTS OF DIFFERENTIAL GLYCOSYLATION OF GLYCODELINS ON LYMPHOCYTE SURVIVAL

Cheuk-Lun Lee[1,2#], Poh-Choo Pang[3#], William S.B. Yeung[1], Bérangère Tissot[3], Maria Panico[3], Terence T.H. Lao[4], Ivan K. Chu[2], Kai-Fai Lee[1], Man-Kin Chung[1], Kevin K.W. Lam[1], Riitta Koistinen[5,6], Hannu Koistinen[6], Markku Seppälä[6], Howard R. Morris[3,7], Anne Dell[3*] and Philip C.N. Chiu[1*]

[1] Department of Obstetrics and Gynaecology, [2] Department of Chemistry, University of Hong Kong, Pokfulam Road, Hong Kong, China.

[3] Division of Molecular Biosciences, Faculty of Natural Sciences, Imperial College London, SW7 2AZ, United Kingdom.

[4] Department of Obstetrics and Gynaecology, Chinese University of Hong Kong, Shatin, Hong Kong, China.

[5] Department of Obstetrics and Gynaecology, [6] Clinical Chemistry, Helsinki University Central Hospital, 00029 HUS Helsinki, Finland.

[7] M-SCAN Ltd, Wokingham, Berks RG41 2TZ, UK.

[#] The two authors contributed equally to this study.

[*] To whom correspondence should be addressed: Dr. P.C.N. Chiu, Tel: 852-28199378. Fax: 852-28161947, email: pchiucn@hkucc.hku.hk; or Professor A. Dell, Tel: (+44-207) 594-5219. Fax: (+44-207) 225-0458. email: a.dell@imperial.ac.uk.

1   **Glycodelin is a human glycoprotein with**
2 **four reported glycoforms, namely glycodelin-A**
3 **(GdA), glycodelin-F (GdF), glycodelin-C (GdC)**
4 **and glycodelin-S (GdS). These glycoforms have**
5 **the same protein core and appear to differ in**
6 **their N-glycosylation. The glycosylation of GdA is**
7 **completely different from that of GdS. GdA**
8 **inhibits proliferation and induces cell death of**
9 **T-cells. However, the glycosylation and**
10 **immuno-modulating activities of GdF and GdC**
11 **are not known. This study aimed to use ultra-high**
12 **sensitivity mass spectrometry to compare the**
13 **glycomes of GdA, GdC and GdF, and to study the**
14 **relationship between the immunological activity**
15 **and glycosylation pattern among glycodelin**
16 **glycoforms. Using MALDI-TOF strategies, the**
17 **glycoforms were shown to contain an enormous**
18 **diversity of bi-, tri- and tetra-antennary**
19 **complex-type glycans carrying Galβ1-4GlcNAc**
20 **(lacNAc) and/or GalNAcβ1-4GlcNAc (lacdiNAc)**
21 **antennae backbones with varying levels of fucose**
22 **and sialic acid substitution. Interestingly, they all**
23 **carried a family of Sda**
24 **(NeuAcα2-3(GalNAcβ1-4)Gal)-containing glycans**
25 **which were not identified in the earlier study**
26 **because of less sensitive methodologies used.**
27 **Among the three glycodelins, GdA is most heavily**
28 **sialylated. Virtually all the sialic acid on GdC**
29 **occurs on the Sda antennae. With the exception**
30 **of the Sda epitope, the GdC N-glycome appears**
31 **to be the asialylated counterpart of the GdA/GdF**
32 **glycomes. Sialidase activity, which may be**
33 **responsible for transforming GdA/GdF to GdC,**
34 **was detected in cumulus cells. Both GdA and GdF**
35 **inhibited the proliferation, induced cell death and**
36 **suppressed IL-2 secretion of Jurkat cells and**
37 **peripheral blood mononuclear cells. In contrast,**
38 **no immunosuppressive effect was observed for**
39 **GdS and GdC.**

40   Glycodelin is a member of the lipocalin family.
41 It consists of 180 amino acid residues (1) with two
42 sites of N-linked glycosylation. There are four
43 reported glycodelin isoforms, namely glycodelin-A
44 (amniotic fluid isoform, GdA), glycodelin-F
45 (follicular fluid, GdF), glycodelin-C (cumulus
46 matrix, GdC) and glycodelin-S (seminal plasma,
47 GdS) (2-5). Among the four glycodelin isoforms,
48 only the N-glycan structures of GdA and GdS have
49 been previously determined. This was achieved
50 using fast atom bombardment mass spectrometry
51 (6,7). The glycan structures of GdA and GdS are
52 completely different. In GdA, the Asn-28 site carries
53 high mannose, hybrid and complex-type structures,
54 whereas the second Asn-63 site is exclusively
55 occupied by complex-type glycans (6). The major
56 non-reducing epitopes characterised in the
57 complex-type glycans are Galβ1-4GlcNAc (lacNAc),
58 GalNAcβ1-4GlcNAc (lacdiNAc),
59 NeuAcα2-6Galβ1-4GlcNAc (sialylated lacNAc),
60 NeuAcα2-6GalNAcβ1-4GlcNAc (sialylated
61 lacdiNAc), Galβ1-4(Fucα1-3)GlcNAc (Lewis-x) and
62 GalNAcβ1-4(Fucα1-3)GlcNAc (lacdiNAc analog of
63 the blood group substance Lewis-x) (6). Many of
64 these oligosaccharides are rare in other human
65 glycoproteins. GdS glycans are unusually fucose
66 rich, and the major complex type glycan structures
67 are bi-antennary glycans with Lewis-x and Lewis-y
68 antennae. Glycosylation of GdS is highly
69 site-specific. Asn-28 contains only high mannose
70

structures, while Asn-63 contains only complex type glycans. More than 80% of the complex glycans have 3-5 fucose residues/glycan, and none of the glycans is sialylated, which is unusual for a secreted human glycoprotein (7). The glycan structures of GdF and GdC are not known, though they differ in lectin binding properties and isoelectric point from the other two glycodelin isoforms (5).

Glycans are involved in various intracellular, intercellular and cell-matrix recognition events (8,9). Glycosylation determines the biological activities of the glycodelin isoforms (2,10). For example, both GdA and GdF inhibit the spermatozoa-zona pellucida binding (11) via fucosyltransferase-5 (12), but only the latter inhibits progesterone-induced acrosome reaction, thus preventing a premature acrosome reaction of the spermatozoa. There is evidence that cumulus cells can convert exogenous GdA and -F to GdC, the physicochemical properties of which suggest that it is differently glycosylated compared with GdA/F (5). Moreover, GdC stimulated spermatozoa-zona pellucida binding in a dose-dependent manner and it effectively displaced sperm-bound GdA and -F (4,5). GdS suppresses capacitation probably via its inhibitory activity on cholesterol efflux from spermatozoa (13).

Except for the effects on fertilization, GdA is involved in feto-maternal defense. This glycodelin isoform suppresses proliferation and induces apoptosis of T-cells (2), and also inhibits natural killer cell (14) and B-cell activities (15). Glycosylation is involved in the binding of GdA to receptors on T-cells (16). The sialic acid of GdA contributes to the apoptotic activity in T-cells (17,18) and binding to CD45, a potential GdA receptor (16). The importance of glycosylation in glycodelin is further shown by the absence of immunosuppressive activities in GdS with different glycosylation (18). The immuno-modulating activities of GdF and GdC are unknown.

Our previous work showed that glycans are indispensable for the different glycodelins to exhibit their binding activities and biological effect (13,19,20). The present study aims at identifying the effect of all four glycodelin isoforms on lymphocyte viability, cell death, and interleukin-2 (IL-2) secretion and to correlate these bioactivities with their glycosylation patterns determined by mass spectrometry.

EXPERIMENTAL PROCEDURES

Preparation of peripheral blood mononuclear cells (PBMCs)- Human peripheral blood from healthy female donors was obtained from the Hong Kong Red Cross blood transfusion service. PBMCs were isolated from the buffy coat by Ficoll-Paque density gradient centrifugation (GE Healthcare, Uppsala, Sweden). In brief, diluted buffy coat (1:1 with phosphate buffer saline, PBS, pH 7.2) was layered on the Ficoll and centrifuged at 400 g for 40 minutes at room temperature. The remaining red blood cells were removed using red blood cell lysing buffer (150mM NH_4Cl, 10mM $NaHCO_3$, 0.1mM EDTA). The PBMCs were then washed twice with PBS and resuspended in RPMI medium containing 10% fetal bovine serum (FBS), 50 units/ml of penicillin and 50 µg/ml of streptomycin. Monocytes were removed by cell adhesion in plastic culture flask for 2 hours.

Cell cultures- PBMCs and human cell lines including oviductal cells (OE E6/E7), natural killer cells (NK92mi), cells from a chronic myelogenous leukaemia line (K562), T-lymphoma cells (Jurkat), hepato-carcinoma cells (Hela), trophoblast choriocarcinoma cells (BeWo) and endometrial cells (RL95) were cultured in RPMI 1640 (K562, Jurkat, PBMCs), DMEM (Hela, OE E6/E7, BeWo and RL95) or MEM (NK92mi) supplemented with 10% fetal bovine serum, 50 units/ml of penicillin and 50 µg/ml of streptomycin at 37°C in an atmosphere of 5% CO_2 in air.

Purification of glycodelins- The study protocol was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. GdA, -S, -F and -C were purified as described from first trimester amniotic fluid, seminal plasma, follicular fluid, and cumulus matrix of human, respectively (5,12,19) at Queen Mary Hospital, Hong Kong. The collected samples were diluted with tris-buffered saline (TBS, pH 7.4) and 0.1% Triton X-100 in a ratio of 1:3-1:5 was added. GdA, -S and -C were purified by monoclonal anti-glycodelin (clone F43-7F9) chromatography. In brief, the diluted amniotic fluid, seminal plasma or cumulus matrix were loaded onto anti-glycodelin column, which was then washed successively by TBS, 1M NaCl with 1% isopropanol, 10 mM ammonium acetate with 0.1% isoproponal, pH 5 and TBS. Glycodelin was eluted by 20 mM $CaCl_2$ with 0.1% trifluoroacetic acid. The eluted GdS and -C were further purified with anion-exchange Mono-Q (GE Healthcare) column by AKTA purifier 10 (GE Healthcare). The purification of GdF involved the successively use of several chormatographic columns including Hi-trap blue, protein-G, lectin affinity, Mono-Q and gel filtration as described (21). The purified glycodelins were dialyzed in 2 mM Tris-HCl, pH 7.5 and concentrated by Amicon-10 concentrator (Amicon Inc., Billerica, USA). Deglycosylated glycodelin was prepared by denaturation of GdA in 0.1% β-mecaptoethanol before incubation with 0.5 mU PNGase F at 37°C for 24 hours. The digest was boiled for 5 minutes to inactivate the PNGase F and dialyzed in 2 mM

Tris-HCl, pH 7.5. Desiaylation of GdA and GdF was performed using sialidase coated agarose beads (Sigma, MO, USA) in 1M Tris-HCl (pH 7) at 37 °C for 18 hours. The free sialic acid was removed by dialysis with 2 mM Tris-HCl, pH 7.5 at 4 °C overnight. The success of desiaylation was verified by the decreased binding of the treated glycodelin to the lectin, *wheat germ agglutinin* which binds strongly to sialylated glycans and weakly to other glycoconjugates (22). The concentrations of the purified glycodelins were determined by a commercial protein assay kit (Bio-Rad, Hercules, USA). The purified glycodelins showed single bands in 12% gel sodium dodecyl sulfate polycryamide gel (Figure 1).

XTT cell viability/proliferation assay- Cell viability was determined by a colorimetric assay (Roche Diagnostics Co., Basel, Switerland) which measured the production of a color formazan end-product from a tetrazolium salt sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT) from the viable cells. In this assay, XTT labeling mixture was freshly prepared by mixing XTT labeling reagent with electron coupling reagent at a ratio of 50:1. Fifty micro-litres of the labeling mixture was added to the cell culture 12 hours before the end of the incubation period. The absorbance was measured at 450 nm with λ correction at 595 nm (Infinite F200, Tecan, Männedorf, Switzerland). The cell suppression was expressed by the following equation:

Suppression Index (%) = (Absorbance of glycodelin treated cells - Absorbance of blank) / (Absorbcance of control cells – Absorbance of blank) × 100%

Cell death analysis by flow cytometry- After treatment the cells were labeled with Yo-Pro®-1 and propidium iodide (PI) for cell death analysis according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Briefly, 3×10^5 cells were washed twice with PBS. Yo-Pro®-1 dye (1 μl) and PI (1 μl) in 0.5 ml of PBS was added into the cell suspension. After 15 minutes of incubation, the cells were analyzed immediately with a Coulter Epic Elite ESP flow cytometer (Beckman Coulter, Int. Fullerton, CA, USA) equipped with an argon laser of 488 nm. Fluorescence signal was measured using the 525 nm and 610 nm band pass filters, and was analyzed by the Winlist software (Verity Software House, Topsham, ME, USA). Cells that were not stained were counted as viable. Cells labeled with Yo-Pro®-1 only were counted as apoptotic cells. Cells labeled with both Yo-Pro®-1 and PI were counted as necrotic cells. It should be noted that this cell population may also contained late apoptotic cells that were propidium iodide positive.

Determination of IL-2 production by ELISA- The

levels of IL-2 was measured from the culture supernatant of the treated cells according to the manufacturer protocol (BD Biosciences Pharmingen, San Diego, USA). In brief, capture antibody in 0.1 M sodium carbonate buffer (pH 9.5) was coated on a 96-well ELISA plate overnight at 4 °C. The wells were blocked with 200 μl of 10% FBS in PBS for 1 hour and were incubated with 100 μl of culture medium overnight at 4 °C. Then 100 μl of biotinylated detection antibody and avidin horseradish peroxidase conjugate mixture was added and incubated for 1 hour. Color development was achieved by enzymatic reaction using 3.3',5.5'-tetramethylbenzidine as substrate. The reaction was terminated by the addition of 50 μl of 2M H_2SO_4. The absorbance was measured at 450 nm with λ correction of 595 nm. The wells were washed five times with 0.05% Tween-20 in PBS between each step.

Lectin binding assays- Lectin binding assay (19) was used to study the glycosylation of glycodelins. Briefly, the wells in 96-well plate were coated with various lectins (10 μg/ml) overnight. Uncoated sites in the well were blocked by incubation with 100 μl of 5% casein in PBS for 3 hours with slow shaking. Glycodelins (7.5 pmol in 250 μl) were then added and incubated overnight at 4 °C. After washing the wells twice with 0.05% Tween 20-TBS, monoclonal anti-glycodelin antibody (clone F43-7F9, 2.5 μg in 200 μl) was added and the mixture was incubated for 2 hours. The wells were washed 4 times and 100 μl of HRP conjugated anti-mouse IgG at a dilution of 1:5000 (Sigma, St. Louis, MO, USA) was added. After 2 hours of incubation, the wells were washed and 100 μl per well of o-phenylenediamine (Sigma) was added. The OD_{490} was determined with a microplate reader (MR5000, Dynatech, Embrach, Switzerland). The assay was done at room temperature. The control wells contained BSA instead of glycodelin.

Processing of the glycodelin N-glycans- Preparations of purified GdA, GdF and GdC (25-50 μg each) were used for all MALDI experiments and GC-MS analyses. Each of the 50 μg of GdA preparations was purified from separate pools of 500 ml of midtrimester amniotic fluid from 20 pregnant women whereas each of the 50 μg of GdF preparations was purified from separate pools of 2.5 liters of follicular fluid from around 270 women enrolled in the assisted reproduction program. Preparations of 50 μg of GdC were purified from 500 ml of cumulus matrix pooled from around 165 cases of oocyte retrieval.

The purified glycodelins were initially subjected to reduction, carboxymethylation, and tryptic digestion before the N-glycans were released by PNGase-F (Roche Applied Science, Burgess Hill,

UK) digestion. After that, the released N-glycans were purified by using a Sep-Pak C18 cartridge (Waters Corp, Hertfordshire, UK). Prior to mass spectrometric analyses, the purified native N-glycans were derivatised using the sodium hydroxide permethylation procedure. All of the above procedures were carried out as described previously (23).

MS and MS/MS data acquisition of permethylated glycodelin N-glycans- MALDI- MS and MS/MS data were acquired using a 4800 MALDI-TOF/TOF (Applied Biosystems, Darmstadt, Germany) mass spectrometer. The collision energy was set to 1 kV, and argon was used as collision gas. Samples were dissolved in 10 μl of methanol and 1 μl was mixed at a 1:1 ratio (v/v) with 2,5-dihydrobenzoic acid (20 mg/ml in 70% methanol in water) as matrix.

Analyses of MALDI data- The MS and MS/MS data were processed using Data Explorer 4.9 Software (Applied Biosystems, Warrington, UK). The mass spectra were baseline corrected (default settings) and noise filtered (with correction factor of 0.7), and then converted to ASCII format. The processed spectra were then subjected to manual assignment and annotation with the aid of a glycobioinformatics tool, GlycoWorkBench (24). Peak picking was done manually, and proposed assignments for the selected peaks were based on ^{12}C isotopic composition together with knowledge of the biosynthetic pathways. The proposed structures were then confirmed by data obtained from MS/MS and linkage analysis experiments.

GC-MS linkage analysis- Partially methylated alditol acetates were prepared and analysed by GC-MS as previously described (23).

Biological characterization of different glycodelin isoforms- XTT assay and flow cytometry was used to study the effect of glycodelin on cell viability/proliferation and cell death, respectively. In brief, PBMC and cell lines including OE E6/E7 (25), NK92mi, K562, Jurkat, Hela, BeWo and RL95 were incubated with 0, 0.001, 0.01, 0.1 and 1 μg/ml of glycodelins or deglycosylated glycodelin in 100 μl of culture medium for 36 hours before XTT viability assay. Cell death analysis by flow cytometry was performed as described above in cells treated with 1 μg/ml of glycodelins in 500 μl of culture medium for 48 hours. For IL-2 analysis, cells were incubated with 1 μg/ml of glycodelins and stimulated by 5 μg/ml of phytohaemagglutinin (PHA) in 500 μl of culture medium for 16 hours. The media after incubation were then collected. The cell debris in the conditioned media was removed by centrifugation at 500 g for 5 minutes. The IL-2 production was determined by ELISA as described above. The viability of the cells at 16 hours was also determined by flow cytometry as described above.

Determination of sialidase activity on cumulus cells- The Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster approved the protocol for collection and use of cumulus oophorus in this study. Cumulus-oocyte complexes were obtained from women who underwent treatment with intracytoplasmic sperm injection for male infertility. Human menopausal gonadotropin (Serono, Geneva, Switzerland) was used for ovarian stimulation after down-regulation with buserelin (26). The cumulus-oocyte complexes were dispersed in 1 ml of 0.1% hyalurondidase in human serum albumin supplemented G-MOPS medium (Vitrolife, Kungsbacka, Sweden) at 37°C. After digestion, cumulus cells were pelleted at 300 g for 5 minutes. The dispersed cumulus cells were then washed twice in TC-199 medium (Sigma) and resuspended to a final concentration of 4×10^6 viable cells/ml. Trypan blue exclusion test was employed to determine the viability of the cells. They were then cultured in 2 ml of TC-199 medium supplemented with 20% fetal bovine serum as described (27).

After 48 hours, sialidase activity on the cell surface of cumulus cells was determined using 4-methylumbelliferyl *N*-acetylneuraminic acid (4MU-NANA; Sigma-Aldrich, St. Louis, MO, USA) as a substrate, according to previously described method (28). Briefly, after washing with fresh medium, the cells were incubated with 50 μmol/l of 4MU-NANA in 1 ml of culture medium for 2-8 hours at 37°C. The conditioned medium was then collected, centrifuged at 500 g for 15 minutes, and the liberated 4-methylumbelliferone in the supernatant was measured using a fluorometer with excitation at 340 nm and emission at 505 nm (Infinite F200, Tecan, Männedorf, Switzerland).

Data analyses - All values were expressed as mean ± S.E.M. (standard error mean). The data were compared by student's t-test or ANOVA followed by a post-hoc test (Tukey's test) to discern differences between individual groups. The data were analysed using SigmaStat 2.03 (Jandel Scientific, San Rafael, CA, USA). A P value below 0.05 was taken as significant.

RESULTS

Differential lectin binding properties of glycodelins - Glycodelin-A, -F, -S and -C were purified from amniotic fluid, follicular fluid, seminal plasma and cumulus matrix, respectively (Figure 1), and were analyzed for lectin binding. The lectin binding properties of glycodelins are shown in Table 1. Consistent with our previous data (5), GdF was characterized by significantly lower affinity to UAEI (P<0.05) and higher affinity to S-WGA (P<0.05), while GdS was characterized by a low affinity to

WFA (P<0.05) when compared with other isoforms. GdA and GdF had significantly higher affinities to WGA and SNA than GdC and GdS (P<0.05). No significantly differences were found in ConA, PNA and RCA$_{120}$ affinity between the glycodelin isoforms.

Characterisation of the N-glycomes of GdA, GdC and GdF - Glycomic profiling was carried out using well documented methodologies (29,30). Briefly, samples were reduced, carboxymethylated, digested with trypsin and the *N*-glycans were released by peptide *N*-glycosidase F digestion and permethylated prior to MALDI-TOF profiling. When sample amounts permitted, MALDI-TOF/TOF sequencing and gas chromatography-MS linkage analyses were additionally carried out. Analyses were done on two preparations of each of the purified glycodelins to allow assessment of reproducibility. The MALDI-TOF profiles for one of each of the GdA, GdC and GdF samples are shown in Figures 2 to 4 respectively. Spectra from the replicate batches are presented in Supplementary Figures S1 to S3, respectively. For clarity, not all components are given their *m/z* values and annotations in Figures 2 to 4; complete assignments are presented in the expanded Supplementary Figures. The structures shown in the annotations were assigned from compositional information provided by the MALDI MS data, complemented by MALDI MS/MS and linkage data where available, taking into account biosynthetic considerations plus results of the earlier GdA study (6). Because of the limited amount of material, only a selection of the molecular ions observed in the MALDI-TOF spectra could be subjected to MS/MS experiments. Molecular ions analysed by MS/MS are flagged in the Supplementary Figures.

Important characteristics of these data are: (i) All of the glycodelin glycomes are immensely complex with many components of the same *m/z* value being mixtures of more than one type of structure. This trait is observed throughout the whole detectable mass range of *m/z* 1500-5200 (Supplementary Figures S1 to S3): from the lower mass region (e.g. *m/z* 1836, 2040, 2070, 2244, 2285, 2459, and 2592) to the middle mass region (e.g. *m/z* 2646, 2663, 2775, 2861, 3095) and high mass region (e.g. *m/z* 4267, 4512); (ii) Both lacNAc and lacdiNAc are present as antenna backbones in all three glycodelins and in many cases are substituted with fucose or sialic acid; (iii) Some of the glycans have compositions consistent with bisected structures and this was confirmed for GdA and GdC by the presence of 3,4,6-linked mannose in their linkage analysis data (Table 2); (iv) Biantennary glycans are the most abundant family in all glycodelins but a great diversity of tri- and

tetraantennary structures are also present; (v) Sialylation levels differ markedly in the three isoforms. GdA is the most heavily sialylated, GdF has a similar glycan composition as GdA but with much lower sialylation, whilst GdC has the smallest repertoire of glycans carrying sialic acid. The duplicate batches gave broadly similar data, albeit with quantitative differences in some components. Whether these quantitative differences are an experimental artefact arising during sample handling prior to glycomic analysis or are due to differences between individual women remains to be established. With respect to the latter, it is significant that quantitative differences have previously been observed between individual GdA samples (31). Also it should be noted that GdC and GdF are low abundance glycodelins requiring the pooling of follicular fluid from almost 300 women for the preparation of a batch of 50 μg. (vi) Consistent with earlier observations (6), low levels of high mannose structures were observed in GdA and they were also detected as minor components of the GdC and GdF glycomes; (vii) All of the molecular ions observed in the original Fast Atom Bombardment (FAB) data for GdA ((6), Figure 2) are recapitulated in the current GdA data and abundances are comparable once the poor sensitivity at high mass of the FAB-MS methods of the 1990's is taken into account. The MALDI experiments have additionally revealed a wealth of components above *m/z* 3000 which were not detected using the FAB ionization. Many of these glycans were unexpectedly found to carry the Sda epitope (NeuAcα2-3(GalNAcβ1-4)Gal) which gives characteristic fragment ions in MS/MS analysis (Figure 5 and (32), Figure 2B) and 3,4-linked Gal in the linkage analysis (Supplementary Figure S4). The structures of Sda-containing glycans are shown in Supplementary Table S1; (ix) Sda containing glycans were also found in GdC and GdF (see Table S1). Interestingly those present in GdC are characterised by the absence of sialylation on other antennae. Indeed, careful scrutiny of the MALDI profiles allows us to conclude that the sialic acid on GdC is almost exclusively associated with the Sda epitope. Moreover, with the exception of this epitope, the GdC N-glycome appears to be the asialylated counterpart of the GdA glycome (see Discussion).

Glycodelin-A and -F reduced viability/proliferation of lymphocytes - The effects of glycodelin treatment for 48 hours on the viability/proliferation of various cell lines are shown in Table 3. GdA and GdF at concentrations of ≥0.1 μg/ml significantly decreased (P<0.05) the viability of Jurkat and PBMCs, whereas GdS, GdC and deglycosylated glycodelin had no effect. At 1 μg/ml, GdA decreased the viability/proliferation of Jurkat cells and PBMC to

30.4±3.8% and 44.3±7.3% respectively. GdF at the same concentration reduced lymphocyte viability to a similar extent (Jurkat: 37.6±3.3%, p=0.002; PBMC 37.7±5.1%, P<0.001). None of the glycodelin glycoforms did affect the viability/proliferation of OE E6/E7, NK92mi, K562, Hela, BeWo and RL95 cells at the tested concentrations (data not shown).

Glycodelin-A and -F induced apoptotic and necrotic cell death of lymphocytes - The YoPro-PI assay was used to determine the proportion of viable, apoptotic and necrotic cells simultaneously in a sample (Figure 7 and Table 4). Treatment with 1 µg/ml of GdA significantly increased the apoptotic population of Jurkat cells from 6.4±0.3% to 33.4±5.3%, and the necrotic population from 6.4±0.3% to 22.7±12.1%. The corresponding values for GdF were 27.9±5.4% and 37.5±8.1%, respectively.

GdA and GdF also caused cell death of PBMCs (Figure 7 and Table 4). GdA and GdF treatment for 48 hours significantly increased the percentage of necrotic cells from 7.6±0.4% to 73.9±2.7% and 75.8±1.9% and of apoptotic cells from 3.8±0.5% to 8.9±0.9% and 8.7±0.9%, respectively. On the other hand, GdS, GdC and deglycosylated glycodelin had no significant effects on apoptosis and necrosis of Jurkat cells and PBMCs.

Sialyation is important for activity of glycodelin-A on cell death of lymphocytes - Sialidase treatment decreased the sialic acid content of GdA and GdF as shown by a decrease in binding to wheat germ agglutinin (Supplementary Table S2). Desialyation abolished the ability of GdA and GdF in inducing cell death (Table 4).

Glycodelin-A and -F suppress IL-2 secretion by lymphocytes- The effects of glycodelin treatment for 16 hours on cytokine secretion of Jurkat cells and PHA-induced PBMCs were studied. None of the glycodelin isoforms affected cell viability within the treatment period (Figure 8). GdA and GdF significantly inhibited IL-2 secretion by Jurkat cells from 851.6±228.3 pg/ml (untreated control) to 339.1±58.9 pg/ml (P<0.01) and 187.5±44.5 pg/ml (P<0.05), respectively. Other glycodelin isoforms and deglycosylated glycodelin had no effect. PBMCs were less sensitive to the inhibitory activity of GdA and GdF. The corresponding levels of IL-2 were 925.9±53.1 pg/ml (P<0.01) and 857.1±117.0 pg/ml, which were significantly lower (P<0.05) than the control (1355.8±102.3 pg/ml). GdC had a slight but significant inhibitory effect (P<0.05) on IL-2 secretion in PBMCs.

Cumulus cells possess sialidase activity - The sialidase activity in cumulus cells is shown in Figure 9. The sialidase activity of the cumulus cells increased the production of the 4-methylumbelliferone in a time-dependent manner and was significantly (P<0.05) higher than the corresponding medium control after 4 hours of incubation.

DISCUSSION

This is the first study to investigate the immunosuppressive activity of the two recently discovered glycodelin family members, GdF and GdC. Like GdA, GdF reduced cell viability/proliferation, induced cell death and reduced PHA-induced production of IL-2 from lymphocytes. The reduction in IL-2 production may be partly responsible for the decrease in cell viability/proliferation as IL-2 modulates proliferation of stimulated T cells (33). Our data on the immunosuppressive effects of GdA agree with previous reports that GdA inhibits lymphocyte growth by induction of cell death and reduction of IL-2 synthesis (18,34,35). Importantly, the immunosuppressive activity of GdA and GdF was glycosylation dependent and was abolished after deglycosylation and desialyation.

Comparing the MALDI data for all three glycodelins, it is clear that there are both similarities and differences in their glycomes. The similarities lie in shared antennae sequences and branching patterns, whilst the differences are associated with variations in relative glycan abundances plus the absence of α2-6 linked sialic acid in GdC. GdA is the most heavily sialylated glycodelin as exemplified by the most abundant biantennary glycans observed in the MALDI profile (m/z 2646, 3007 and 3211, Figure 2) all of which are sialylated (Figure 6C). In contrast, GdF and GdC are both relatively poorly sialylated and their most abundant biantennary glycans are non-sialylated (Figure 6C). However, it is important to note that, like GdA, GdF carries α2-6 linked sialic acid on a portion of its glycans (Supplementary Figure S3), whilst GdC is unique in lacking this type of sialylation.

The shared outer arm α2-6 sialylation of GdA and GdF could explain the comparable immunosuppressive activities found in the present study, assuming that a small amount of an active glycoform is sufficient for conferring function. Similar observations have been reported for another glycoprotein with immunomodulatory properties, α1-acid glycoprotein (36). However, these observations do not exclude the possibility that GdA and GdF may suppress the lymphocyte activities by different glycans. Whether the similar immunosuppressive activities of GdA and GdF are due to the same or different glycan chain(s) is still an open question.

In some other assays GdA and GdF have been found to behave differently. This may be a reflection of the lower degree of sialylation of GdF and/or differences in abundance of other sequences such as

fucosylated lacdiNAc. For example, only GdF but not GdA suppresses the progesterone-induced acrosome reaction (19). GdF also has higher sperm binding affinity compared to GdA (12,19,20).

The advancement of MS technologies has led to the interesting discovery that there is an additional family of glycans in all the female glycodelins which was not characterised earlier, namely the Sda-capped family. The molecular ions of the most abundant members of this family are well above m/z 3000, and were therefore refractory to the mass spectrometry of the 1990's; Even though smaller Sda-containing N-glycans are observed at m/z lower than 3000, they are of low abundance and are isobaric to other, more abundant, glycans. Moreover, the MS/MS methods equivalent to the TOF-TOF technology employed in the present work were not available when GdA was first characterised. Thus, the minor biantennary Sda-containing components now observed in the MALDI spectra (Figure 2A) were impossible to detect in the earlier FAB experiments (6).

Cumulus cells can transform GdA and GdF to GdC (5). One of the important findings in this report is that the structures of the N-glycans of GdC are consistent with desialylation of the α2-6 sialylated antennae in GdA/GdF. This observation suggests that GdC may be a product of remodelling of GdA/GdF by the cumulus cells and that this process might be dominated by desialylation. Furthermore, the remodelling of GdA/GdF to GdC hypothesis is consistent with the present finding that cumulus cells possess sialidiase activity. Our unpublished observation has also shown that desialyation of GdA and GdF is associated with loss of sperm-zona pellucida binding inhibitory activity thereby resulting in comparable behaviour to GdC.

The sialidase of the cumulus cells is likely to have similar activity to the sialidase from *Vibrio cholera* (37) because it does not remove the sialic acid on the Sda epitope. To date, not much is known about the type of sialidase expressed on the surface of cumulus cells. Plasma membrane-associated sialidase (Neu3) is localized mainly on the cell surface, and has shown to be involved in the regulation of transmembrane signalling (38). However, since Neu3 is a glycolipid-specific sialidase which acts preferentially on ganglioside substrates, but have no activity against sialylated glycoproteins (Ha et al., 2004), glycodelins would be less likely the substrates of Neu3. Alternatively, the sialidase could be secreted by the cumulus cells as a soluble enzyme into the cumulus matrix. In this case it would be similar to the glycan modifying enzymes in luminal fluid of rat epididymis that are known to be involved in sperm maturation (39). The recent discovery that a cell surface tumor suppressor called Klotho is a 2,6 specific sialidase and is known to be

expressed in mouse cumulus (data accessible at NCBI GEO database (40), accession GSE4260 (41)) makes this molecule a possible candidate for our observed sialidase activity (42).

The putative liberation of the sialic acid residues from GdA/GdF to form GdC may result in greater exposure of the Sda epitope. However to date, no receptor for this epitope has been identified. The Sda epitope is relatively rare in human and very little is known about its function. Nevertheless it is of interest that the Sda epitope in bovine pregnancy associated glycoproteins appears to be hormonally regulated (31).

The new structural discoveries of the present work allow refinement of the hypothesis of the roles of glycodelin isoforms in human fertilisation (43). Glycodelin is synthesized in the granulosa cells of the late secondary follicle. During the periovulatory period, the sialidase present in cumulus cells use GdA and -F in the follicular fluid as substrate for the production of the GdC, which is then released into the extracellular matrix during cumulus expansion or shortly after ovulation. During cumulus penetration, GdC in the cumulus matrix displaces sperm-bound glycodelin isoforms and promotes the zona binding capacity of the penetrated spermatozoa.

Carbohydrate interactions induce lymphocyte differentiation, maturation, activation, migration and responsiveness (44-46). In this study, both GdF and GdA were shown to possess a diversity of sialylated glycans and are immunosuppressive, while the non-sialylated GdS as well as GdC, which has Sda-restricted sialylation, lacked equivalent immunosuppressive activity. Importantly, the ability of GdA and GdF to induce lymphocyte cell death was abolished after desialyation, consistent with the reported crucial role of sialylation in the immunosuppressive activity of glycodelin (47). Sialic acid is an acidic monosaccharide that is usually the terminal sugar residue of N-glycan chains (48). The presence of sialic acid receptors on leukocytes is well recognized (49). Therefore, it is tempting to speculate that GdA and GdF might mediate their immunosuppressive effect through this type of receptor on lymphocytes.

The immunosuppressive activity of glycodelin was abolished after deglycosylation in the present study. It has also been proposed that the apoptotic activity of glycodelin is associated with its protein backbone (50) based on the observation that GdA glycopeptide produced by trypsin digestion did not induce apoptosis (18) and mutation of both glycosylation sites (Asn-28 and Asn-68) of glycodelin yielded recombinant non-glycosylated glycodelin which retained anti-proliferative activity (50). The discrepancy in the immunosuppressive activity of deglycosylated glycodelin in the two

studies could be due to the deglycosylation method used; enzymatic deglycosylation in the present study and site-direct mutagenesis in the previous report. The former involved a denaturing step while the latter may change the protein configuration of the resulting molecule. Experiments using recombinant glycodelin from prokaryotes may help to solve the discrepancy. Recombinant glycodelin produced in *E. coli* has been shown to bind to monocytes, but not to T-cells and B-cells (51). However, glycosylation is essential for proper folding of glycoproteins (52-53), and deglycosylation strategies may therefore affect the binding and bioactivity of the deglycosylated molecules.

The glycosylation of glycodelins is also important for their influence on human sperm behaviour. Deglycosylation abolishes the actions of glycodelins on capacitation, the acrosome reaction and zona pellucida binding ability (4,10,54). It has also been demonstrated that the glycans derived from GdA can modulate hormone production from trophoblast cells (55).

In conclusion, the glycosylation of GdA, GdF and GdC show many similarities but there are some important differences, most notably in the level and type of sialylation. Evidence is presented that the glycans, particularly the sialic acid residues, are important in the immunosuppressive activities of glycodelin. Further understanding of the glycosylation of glycodelin isoforms, particularly their actions on lymphocytes may assist in the rational design of novel therapeutic strategies for immune-based disorders in reproduction.

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FOOTNOTES

Abbreviations used: **MALDI**, matrix assisted laser desorption/ionization; **TOF**, time of flight; **MS**, mass spectrometry; **MS/MS**, tandem mass spectrometry; **GC**, gas chromatography; **PBMC**, peripheral blood mononuclear cell; **Sda epitope**, (NeuAcα2-3(GalNAcβ1-4)Gal); **XTT**, sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate; **Gd,** glycodelin; **PHA,** phytohemagglutinin;

FIGURE LEGENDS

Figure 1. Purity of purified glycodelins. The purity of 0.5 μg GdA, -F, -S, -C and deglycosylated glycodelin (De-Gd) were determined in a 12% sodium dodecyl sulfate polyacryamide gel electrophoresis and visualized by silver staining..

Figure 2. MALDI-TOF mass spectrum of GdA N-glycans: Panel A, *m/z* 1500-3500; Panel B, *m/z* 3500-5200. The N-glycans from purified glycodelin preparations were released by PNGase-F and permethylated ("Experimental Procedures"). A representative spectrum of GdA N-glycans is shown [batch (i)] and an additional sample analysed yielded similar data (Supplementary Figure S1). Data were acquired in the positive ion mode [M+Na]$^{+}$. The figure on each right hand axis gives the total ion count for that panel of the overall spectrum. Peak assignments are based on ^{12}C isotopic composition together with knowledge of the biosynthetic pathways, and structures were confirmed by MS/MS and linkage analyses. The sugar symbols are those employed by the Consortium for Functional Glycomics for the representation of glycan structures. Structures shown outside a bracket have not been unequivocally defined. For simplicity, specific linkages are not assigned in the mass spectra and only one branching pattern for tri-antennary structures is shown. Therefore, the position of an antenna in a cartoon does not imply designation of a specific arm. MS/MS experiments showed that the biantennary glycans have the usual α3 and α6 arms as indicated in the cartoons. However we cannot rule out minor structures carrying both antennae on the α3 arm. Note the high level of sialylation and the presence of N-glycans with Sda antennae.

Figure 3. MALDI-TOF mass spectrum of GdC N-glycans: Panel A, *m/z* 1500-2800; Panel B, *m/z* 2800-4500. A representative spectrum of GdC N-glycans is shown [batch (i)] and an additional sample analysed yielded similar data (Supplementary Figure S2). Data acquisition and peak assignments were carried out as for GdA (Figure 2). Note the lack of sialylation and the presence of N-glycans with Sda antennae.

Figure 4. MALDI-TOF mass spectrum of GdF N-glycans: Panel A, *m/z* 1500-2800; Panel B, *m/z* 2800-4500. A representative spectrum GdF is shown [batch (i)] and an additional sample analysed yielded similar data (Supplementary Figure S3). Data acquisition and peak assignments were carried out as for GdA and GdC (Figures 2 and 3).

Figure 5. Representative MALDI-TOF/TOF tandem mass spectra of Sda-containing glycodelin N-glycans: Panel A, MS/MS of the *m/z* 3253 component of GdA; Panel B, MS/MS of the *m/z* 3545 component of GdC. Signals present in Figures 2 and 3 were subjected to tandem MS and the resulting MS/MS data are shown. The fragment ions are consistent with the sequences shown in the inset. Fragmentation is usually favoured on the reducing side of HexNAc residues. The peaks which are labeled with an "x" are due to contaminating molecular or fragment ions from neighbouring peaks. The horizontal arrows on the spectra indicate losses from the molecular ion of the designated glycan moieties. Note the

characteristic fragment ion for the Sda epitope at *m/z* 1092 and the fragment ions at *m/z* 2184 (in Panel A), *m/z* 2476, 2013 and 1823 (in Panel B) for the loss of Sda-containing antenna. Other signals with potential Sda arrangements (Supplementary Figures S1 to S3) were analysed for GdA, GdC and GdF, and they yielded similar data.

Figure 6. **Major N-glycans of GdA/F and GdC: Panel A, antennae and cores of GdA and GdF; Panel B, antennae and cores of GdC; Panel C, structures of the major components corresponding to the three most abundant molecular ions in the biantennary regions of the MALDI profiles of GdA, GdC and GdF.** The +/- annotations in the composite structures in A and B indicate that not all glycans carry the associated structural feature.

Figure 7. **Dot plot of glycodelin on PBMCs and Jurkat cells death after 48 hours treatment.** Viable, necrotic and apoptotic cells were identified and quantified by bivariate Yo-Pro®-1/PI flow cytometry. Cells without stain were counted as   (green). Cells labed with Yo-Pro®-1 only were counted as apoptotic cells (blue). Cells labeled with both Yo-Pro®-1 and PI were counted as necrotic cells (Red).

Figure 8. **Effect of glycodelin on IL-2 secretion and viability of PBMCs and Jurkat cells after 16 hours treatment.** PBMCs primed by PHA (5 μg/ml) and Jurkat cells were incubated with 1 μg/ml of glycodelin for 16 hours. IL-2 secretion and Cell viability were quantified by ELISA and flow cytometry respectively. Data are mean ± S.E.M., N=4, * and ** $P<0.05$ and 0.01 vs corresponding control, respectively

Figure 9. **Sialidase activity on the intact cumulus cells.** Sialidase activity of intact cumulus cells was determined by incubation with 4MU-NANA at pH 7.4 for 2-8 hours at 37°C. The results represent the mean of three independent experiments ± S.E.M and are expressed as percentage of activity at time 0. *$P<0.05$ when compared to the corresponding control at the same time point.

Table 1. Binding of lectins with glycodelin-C, -A, -S and -F. Data are presented as means ± S.E.M. (N=5). The control coated with 10000 ng/ml BSA instead of lectins had OD between 0.07-0.13. [a-b, c-d, e-f] $P<0.05$ within the same row (ANOVA on rank).

Table 2. GC-MS linkage analyses of partially methylated alditol acetates obtained from the PNGase F released N-glycans of glycodelin-A, glycodelin-F and glycodelin-C. Permethylated N-glycans were hydrolyzed, reduced, acetylated and analyzed by GC-MS ("Experimental Procedures"). Note the presence of the 3,4-linked galactose which provides evidence for the Sda epitope, and 3,4,6-linked mannose for bisecting GlcNAc.

Table 3. Effect of Glycodelins on viability of Jurkat and PBMCs by XTT assay. Cells of 3×10^4 were incubated with 0.001, 0.01, 0.1 and 1 μg/ml of glycodelins for 36 hours, XTT labeling mixture was added 12 hours before measurement. Percentage stimulation (%) = (Abs Gd - Abs blank) / (Abs control–Abs blank) x 100%. Data are mean ± S.E.M, N=8. P values are shown for significant differences as compared to control without treatment (One-way ANOVA).

Table 4. Effect of deglycosylation and desialyation of GdA on cell death of Jurkat and PBMCs. Jurkat cells and PBMCs were incubated with 1 μg/ml of glycodelin A, F, S, C and deglycosylated glycodelin (De-Gd). Viable, necrotic and apoptotic cells were identified and quantified by bivariate Yo-Pro®-1/PI flow cytometry. Cells without stain were counted as viable cell. Cells labeled with Yo-Pro®-1 only were counted as apoptotic cells. Cells labeled with both Yo-Pro®-1 and PI was counted as necrotic cells. Data are mean ± S.E.M, N=4, * and ** $P<0.01$ and 0.001, P values are shown for significant differences as compared to

control without treatment (student's t-test).

Supplementary Figure S1. MALDI-TOF mass spectra of N-glycans from two GdA preparations. Regions from the MALDI MS spectra for both batches are expanded for clarity. Data are presented in sets of spectra with the upper panel of each subfigures being the first batch of GdA [GdA - batch (i); 50 µg starting material] and the lower panel, the second batch [GdA - batch (ii); 50 µg starting material]. Subfigure S1-A, *m/z* 1560-2000;   Subfigure S1-B, *m/z* 2000-2300; Subfigure S1-C, *m/z* 2300-2600; Subfigure S1-D, *m/z* 2600-2900; Subfigure S1-E, *m/z* 2900-3200; Subfigure S1-F, *m/z* 3200-3500; Subfigure S1-G, *m/z* 3500-3800; Subfigure S1-H, *m/z* 3800-4090; Subfigure S1-I, *m/z* 4090-4550; Subfigure S1-J, *m/z* 4500-5200 (GdA - batch (i) only because batch (ii) appeared to contain a little less sample and signal to noise was poor above *m/z* 4500).

The glycodelin N-glycans were released by PNGase F, permethylated, and subsequently subjected to Sep-Pak cleanup ("Experimental Procedures"). Data were acquired in the positive ion mode [M+Na]$^{+}$. Peak assignments are based on ^{12}C isotopic composition together with knowledge of the biosynthetic pathways, and structures were confirmed by MS/MS and linkage analyses. The assignments for the peaks with boxed *m/z* values were confirmed by MS/MS. The sugar symbols are those employed by the Consortium for Functional Glycomics for the representation of glycan structures. Structures shown outside a bracket have not been unequivocally defined. For simplicity, specific linkages are not assigned in the mass spectra and only one branching pattern for tri-antennary structures is shown. Therefore, the position of an antenna in a cartoon does not imply designation of a specific arm. Peaks which are labelled with an "x" are due to known contaminants.

Supplementary Figure S2. MALDI-TOF mass spectra of N-glycans from two GdC preparations. Regions from the MALDI MS spectra for both batches are expanded for clarity. Data are presented in sets of spectra with the upper panel of each subfigures being the first batch of GdC [GdC - batch (i); 50 µg starting material] and the lower panel, the second batch [GdC - batch (ii); 50 µg starting material]. Subfigure S2-A, *m/z* 1560-2000;   Subfigure S2-B, *m/z* 2000-2300; Subfigure S2-C, *m/z* 2300-2600; Subfigure S2-D, *m/z* 2600-2900; Subfigure S2-E, *m/z* 2900-3200; Subfigure S2-F, *m/z* 3200-3500; Subfigure S2-G, *m/z* 3500-3800; Subfigures S2-H (*m/z* 3800-4090) and S2-I (*m/z* 4090-4500) are data only from GdC - batch (i) because batch (ii) appeared to contain a little less sample and signal to noise was poor above *m/z* 3600. For experimental details see Figure S1.

Supplementary Figure S3. MALDI-TOF mass spectra of N-glycans from two GdF preparations. Regions from the MALDI MS spectra for both batches are expanded for clarity. Data are presented in sets of spectra with the upper panel of each subfigures being the first batch of GdF [GdF - batch (i); 50 µg starting material] and the lower panel, the second batch [GdF - batch (ii); 25 µg starting material]. Subfigure S3-A, *m/z* 1560-2000;   Subfigure S3-B, *m/z* 2000-2300; Subfigure S3-C, *m/z* 2300-2600; Subfigure S3-D, *m/z* 2600-2900; Subfigure S3-E, *m/z* 2900-3200; Subfigure S3-F, *m/z* 3200-3500; Subfigures S3-G (*m/z* 3500-3800), S3-H (*m/z* 3800-4090) and S3-I (*m/z* 4090-4500) are data only from GdF - batch (i) because the batch (ii) had half the amount of sample compared to batch (i) and signal to noise was poor above *m/z* 3500. For experimental details see Figure S1.

Supplementary Figure S4. Electron impact mass spectra (EI-MS) of the partially methylated alditol acetate (PMAA) derived from 3,4-linked galactose, which provides the evidence for the Sda epitope: Upper Panel, GdA; Lower Panel, GdC. GC-MS linkage analyses of partially methylated alditol acetates obtained from the PNGase-F released N-glycans of GdA and GdC. Permethylated N-glycans were hydrolyzed, reduced, acetylated and analyzed by GC-MS ("Experimental Procedures"). The retention time on the capillary GC column and the fragment ions observed in the EI-MS spectra are used to identify the 3,4-linked galactose. Characteristic fragment ions of 3,4-linked galactose PMAA shown in the inset.

Supplementary Table 1. **The structures of the Sda-containing glycans.** The table is tabulated from the signals observed in the mass spectra (Supplementary Figures S1 to S3). The *m/z* values are rounded up to whole numbers for the ease of comparison. Values which are more than *m/z* 3200 are adjusted by the addition of *m/z* 1 to make the rounding more accurate.

Supplementary Table S2. Binding of GdA with or without sialidase treatment to wheat germ agglutinin (WGA).
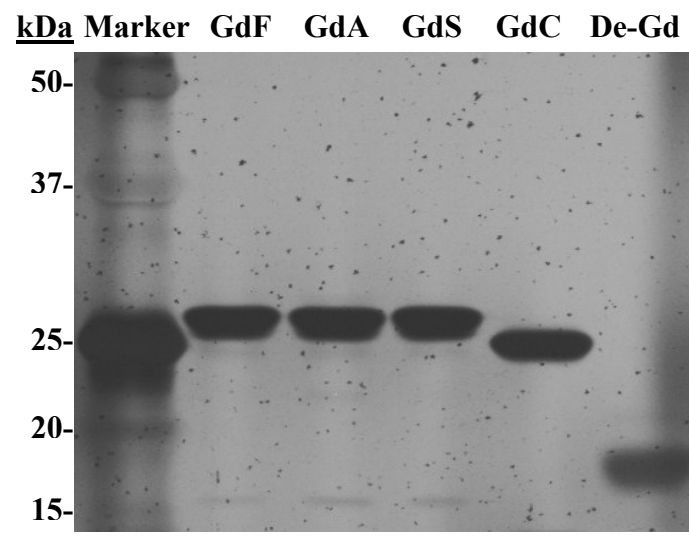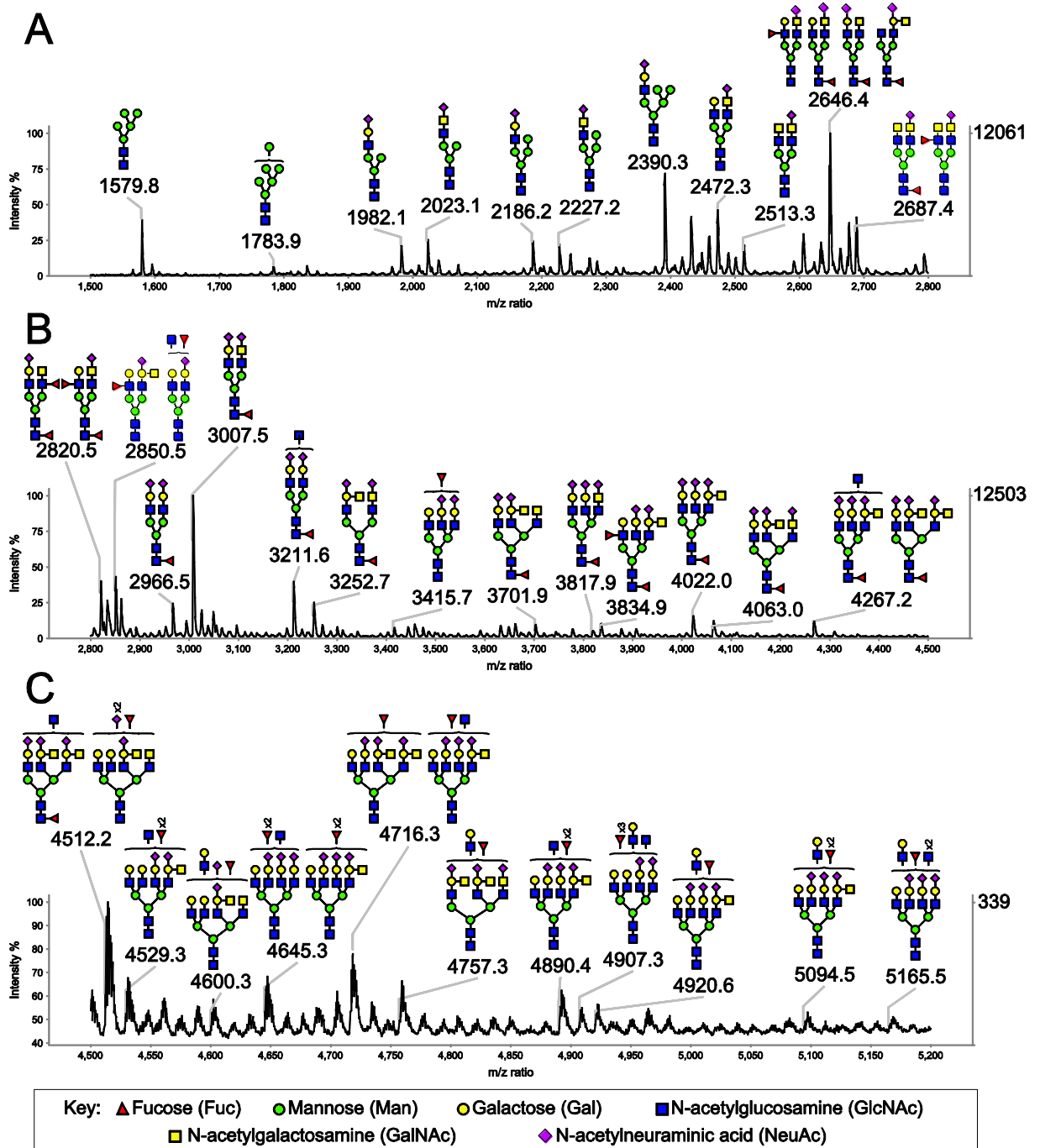
Figure 1

Figure 2

Figure 3

Key: ▲ Fucose (Fuc)   ● Mannose (Man)   ○ Galactose (Gal)   ■ N-acetylglucosamine (GlcNAc)
□ N-acetylgalactosamine (GalNAc)   ◆ N-acetylneuraminic acid (NeuAc)

Figure 4

Key: ▲ Fucose (Fuc)   ● Mannose (Man)   ○ Galactose (Gal)   ■ N-acetylglucosamine (GlcNAc)
     □ N-acetylgalactosamine (GalNAc)   ◆ N-acetylneuraminic acid (NeuAc)

Figure 5

Figure 6

A (GdA/F)

B (GdC)

C GdA
m/z 2646   m/z 3007   m/z 3211

GdC
m/z 2285   m/z 2489   m/z 2592

GdF
m/z 2040   m/z 2244   m/z 2489

Key:
▲ Fucose (Fuc)
● Mannose (Man)
○ Galactose (Gal)
■ N-acetylglucosamine (GlcNAc)
□ N-acetylgalactosamine (GalNAc)
◆ N-acetylneuraminic acid (NeuAc)

Figure 7

Figure 8

Figure 9
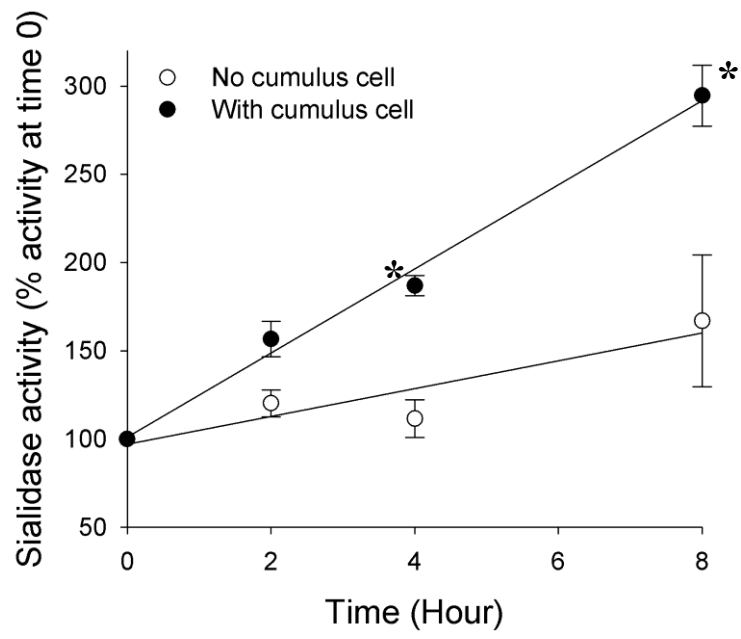
Table 1

Lectin [specificity]	Lectin-immunoassay at OD_{490} (Mean ± SEM)			
	GdC	GdF	GdA	GdS
Peanut agglutinin (PNA) [-Gal(1-3)GalNAc]	0.18 ± 0.02	0.12 ± 0.01	0.07 ± 0.01	0.10 ± 0.01
Succinylated wheat germ agglutinin (S-WGA) [GlcNAc or its oligomer]	0.62 ± 0.05[a]	1.34 ± 0.06[b,c]	0.48 ± 0.04[d]	0.32 ± 0.04[d]
Concanavalin A (ConA) [-Man, -Glc]	1.45 ± 0.08	1.20 ±0.16	1.19 ±0.18	1.33 ± 0.15
Wisteria floribunda agglutinin (WFA) [GalNAc]	1.47 ± 0.15[a]	0.92 ± 0.05[b,c]	1.04 ± 0.08[b,e]	0.29 ± 0.02[b,d,f]
Ricinus communis agglutinin (RCA$_{120}$) [-Gal]	1.16 ± 0.12	0.80 ± 0.12	0.98 ±0.15	1.20 ± 0.17
Sambucus nigra bark agglutinin (SNA) [-NeuNAc(2-6)Gal/GalNAc]	0.52 ± 0.04[a]	1.65 ± 0.18[b,c]	1.71 ± 0.18[b,e]	0.39 ± 0.07[d,f]
Wheat germ agglutinin (WGA) [(GlcNAc)$_2$, NeuNAc]	0.48 ± 0.09[a]	1.26 ± 0.15[b,c]	1.02 ± 0.14[b,e]	0.27 ± 0.03[d,f]
Ulex europaeus agglutinin (UEAI) [-L-fuc]	1.56 ± 0.10[a]	0.90 ± 0.12[b,c]	1.28 ± 0.07[d]	1.48 ± 0.13[d]

Data are presented as means ± S.E.M. (N=5).

The control coated with 10000 ng/ml BSA instead of lectins had OD between 0.07-0.13.

[a-b, c-d, e-f] $P<0.05$ within the same row (ANOVA on rank).

Table 2

Characteristic Fragment Ions	Assignment	Elution Time (Mins)	
		GdA	GdC
115, 118, 131, 162, 175	terminal fucose	17.47	16.97
102, 118, 129, 145, 161, 162, 205	terminal mannose	18.98	18.47
102, 118, 129, 145, 161, 162, 205	terminal galactose	19.23	18.73
129, 130, 161, 190	2-linked mannose	20.14	19.62
118, 129, 161, 234	3-linked mannose	20.32	19.79
118, 129, 161, 234	3-linked galactose	20.43	19.89
99, 102, 118, 129, 162, 189, 233	6-linked galactose	20.95	Not detected
118, 305	3,4-linked galactose	21.10	20.56
130, 190, 233	2,4-linked mannose	21.34	20.78
129, 130, 189, 190	2,6-linked mannose	21.74	21.17
118, 129, 189, 234	3,6-linked mannose	21.89	21.33
118, 333	3,4,6-linked mannose	22.35	21.78
117, 159, 203, 205	terminal GlcNAc	22.83	22.50
117, 159, 203, 205	terminal GalNAc	23.27	22.62
117, 159, 233	4-linked GlcNAc	23.72	23.12
117, 159, 346	3,4-linked GlcNAc	24.58	23.98
117, 159, 203, 233	6-linked GalNAc	24.71	Not detected
117, 159, 261	4,6-linked GlcNAc	25.02	24.40

Table 3

	Glycodelin (µg/ml)	Suppression Index (S.I. ± SEM)				
		GdA	GdF	GdS	GdC	De-Gd
Jurkat	**0.001**	102.1 ± 2.3	108.5 ± 3.1	100.0 ±2.2	98.9 ± 0.4	99.9 ± 0.7
	0.01	97.3 ± 5.6	94.5 ± 6.3	99.6 ±3.2	99.2 ± 1.6	98.3 ± 1.0
	0.1	59.6 ± 3.8 p<0.001	66.5 ± 10.2 p<0.001	101.0 ±3.0	94.4 ± 3.9	101.3 ± 0.8
	1	30.4 ± 3.8 p=0.026	37.6± 3.3 p=0.002	97.0 ±2.3	99.7 ± 3.3	100.9 ± 1.3
PBMCs	**0.001**	99.2 ± 3.5	104.0 ± 1.8	104. ± 2.3	106.6 ± 4.6	106.6 ± 5.0
	0.01	97.6 ± 5.8	99.9 ± 5.6	108.2 ± 5.4	110.1 ± 6.1	103.2 ± 1.2
	0.1	73.2 ± 8.0 p=0.017	75.4 ± 6.1 p<0.001	108.0 ± 5.3	111.3 ± 5.4	100.6 ± 4.7
	1	44.3 ± 7.3 p<0.001	37.7 ± 5.1 p<0.001	96.5 ± 3.4	101.9 ± 3.8	105.6 ± 3.6

Data are mean ± S.E.M (N=8).

Suppression Index (%) = (Abs Gd - Abs blank) / (Abs control–Abs blank) x 100%

P values were shown for significant differences as compared to control without treatment (One-way ANOVA).

Table 4

	Control	GdA	GdF	GdS	GdC	Deglycosylated Gd	Desialyated GdA	De-sialyated GdF
Jurkat								
Viable	87.3 ± 0.4%	22.7 ± 12.1% *	34.6 ± 13.5% *	86.3 ± 1.0%	86.3 ± 1.3%	84.1 ± 1.9%	82.9 ± 1.6	87.9 ± 0.7
Apoptosis	6.4 ± 0.3%	33.4 ± 5.3% *	27.9 ± 5.4% *	6.5 ± 0.5%	6.6 ± 0.7%	7.5 ± 1.1%	9.3 ± 1.4	6.3 ± 1.0
Necrosis	6.4 ± 0.3%	22.7 ± 12.1% *	37.5 ± 8.1% *	7.2 ± 0.5%	7.1 ± 0.7%	8.4 ± 0.9%	7.7 ± 1.0	5.6 ± 1.6
PBMCs								
Viable	88.3 ± 0.9%	15.6 ± 1.9% **	14.2 ± 1.2% **	87.6 ± 0.3%	83.2 ± 2.5%	82.5 ± 2.9%	83.4 ± 1.6	82.9 ± 1.6
Apoptosis	3.8 ± 0.5%	8.9 ± 0.9% **	8.7 ± 0.9% **	3.0 ± 0.2%	4.1 ± 0.7%	4.9 ± 1.1%	9.8 ± 1.7	9.3 ± 1.4
Necrosis	7.6 ± 0.4%	73.9 ± 2.7% **	75.8 ± 1.9% **	8.9 ± 0.3%	12.0 ± 1.8%	11.8 ± 1.6%	6.8 ± 1.0	7.7 ± 1.0

Data are mean ± S.E.M (N=4).
* and ** $p < 0.01$ and 0.001, P values were shown for significant differences as compared to control without treatment (Student's t-test).