Fluorinated per-acetylated GalNAc metabolically alters glycan structures on leukocyte PSGL-1 and reduces cell binding to selectins

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ABSTRACT

Novel strategies to control the binding of adhesion molecules belonging to the selectin family are required for the treatment of inflammatory diseases. We tested the possibility that synthetic monosaccharide analogs can compete with naturally occurring sugars to alter the O-glycan content on human leukocyte cell surface selectin-ligand, P-selectin glycoprotein ligand-1 (PSGL-1). Resulting reduction in the sialyl Lewis-X (sLe\(^X\)) bearing epitopes on this ligand may then reduce cell adhesion. Consistent with this hypothesis, 50\(\mu\)M per-acetylated 4F-GalNAc added to the growth media of promyelocytic HL-60 cells reduced the expression of the cutaneous lymphocyte associated-antigen (CLA/HECA-452 epitope) by 82% within two cell doubling cycles. Cell binding to all three selectins (L-, E- and P-selectin) was reduced \textit{in vitro}. 4F-GalNAc was metabolically incorporated into PSGL-1 and this was accompanied by a ~20-25% reduction in PSGL-1 glycan content. A 70-85% reduction in CLA and N-acetyl lactosamine content in PSGL-1 was also noted upon 4F-GalNAc addition. Intravenous 4F-GalNAc infusion reduced leukocyte migration to the peritoneum in a murine model of thioglycolate-induced peritonitis. Thus the compound has pharmacological activity. Overall, the data suggest that 4F-GalNAc may be applied as a metabolic inhibitor to reduce O-linked glycosylation, sLe\(^X\) formation, and leukocyte adhesion via the selectins.
INTRODUCTION

The binding of adhesion molecules belonging to the selectin family to carbohydrate ligands facilitate the adhesion of blood leukocytes to activated endothelial cells, platelets and other leukocytes in the human vasculature. Such molecular interactions play an important role in regulating leukocyte recruitment at sites of inflammation, cancer metastasis and various cardiovascular disorders. While numerous glycoproteins and glycolipids participate in selectin mediated cell adhesion, interactions with carbohydrate epitopes expressed on the leukocyte glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1, CD162) are particularly important since this ligand binds all three members of the selectin family (E-, P- and L-selectin) with high affinity and under fluid flow conditions. Structural analysis of the glycans of PSGL-1 expressed on human promyelocytic leukemia HL-60 cells reveal that PSGL-1 is predominantly composed of core-2 based O-linked glycans. The prototypic selectin-binding carbohydrate structure sialyl Lewis-X (NeuAcα2,3Galβ1,4(Fucα1,3)GlcNAc-, sLeX, Fig. 1a) is expressed on 2-14% of these O-glycans.

There is active interest in developing antagonists that control/block selectin mediated cell adhesion using either competitive inhibitors or metabolic inhibitors. Competitive inhibitors attempt to block cell adhesion by regulating the ligand-binding epitope of either the selectin or its primary counter-receptor PSGL-1. Antagonists used for such inhibition include the tetrasaccharide sialyl Lewis-X and its glycomimetics, humanized antibodies directed against selectins, and soluble recombinant PSGL-1-Ig fusion protein. Only limited clinical success has been reported with these molecules, thus far. While the use of competitive inhibitors is conceptually straightforward, in practice this is complicated by the overlapping functional redundancies among the members of the selectin family and their carbohydrate ligands, the multiple roles of selectins in both ligand binding and signaling, and the limited half-life in circulation of some classes of inhibitors.

The use of metabolic inhibitors is more recent and less well-developed. These are mostly designed based on the growing knowledge of cellular glycosylation reactions and pathways. This approach employs small molecules that penetrate the cell to divert/block metabolic pathways that
normally lead to the formation of selectin-binding carbohydrate epitopes. This strategy targets a group of related cellular reactions as opposed to a single pathway. Preliminary success has been noted using this approach. First, surrogate-acceptors or decoys that act as unnatural substrates for glycosyltransferases have been introduced into cells. Glycosyltransferases act on such artificial substrates. This results in incomplete glycosylation of the natural glycoconjugates. While an early approach showed that aryl-N-acetyl-α-galactosaminides (Benzl, Phenyl, p-nitrophenyl-α-GalNAc) added to cell culture media can alter glycans on mucinous glycoproteins, these reagents were applied at high concentrations (1-7.5mM). Later, it was demonstrated that per-acetylated forms of Galβ1,4GlcNAc-β-O-napthalenemethanol (NAP) and GlcNAcb1,3Gal-β-O-NAP at 50μM can act as decoys/primers that block selectin-ligand formation. Second, glycosyltransferase inhibitors are also in development based on the structure of the sugar-nucleotide transition-state analogs and high throughput screens, though testing of these reagents has largely been performed in cell-free enzymatic assays. Third, per-acetylated, modified monosaccharides have been applied to cells as these may compete with the natural monosaccharides. Here, unnatural monosaccharides are incorporated into cellular glycoconjugates. While analogs of Galactose, GlcNAc, GalNAc and Mannose have been synthesized, only limited studies have been conducted in cellular assays. 4F-GlcNAc is an example of this class of inhibitors. This molecule reduces selectin mediated CLA+ (cutaneous lymphocyte-associated antigen) T-cell adhesion in vitro and in vivo models of skin inflammation.

Since O-linked glycans linked to PSGL-1 and other glycoproteins participate as important selectin-ligands and since the attachment of GalNAc to serine/threonine residues on the peptide backbone is critical for the initiation of O-glycan assembly, we tested the hypothesis that modified monosaccharides based on GalNAc may be used to disrupt/alter the pattern of O-linked glycosylation. This can result in reduced selectin binding function. In this regard, unlike GlcNAc which plays a major role in modifying both N- and O-linked glycans, heparan sulfates and glycolipids, GalNAc is primarily important for the initiation of O-linked glycosylation, and chondroitin sulfates. Here, we tested the effect of a synthetic analog of the natural GalNAc monosaccharide called per-acetylated 4F-GalNAc (2-acetamido-1,3,6-tri-
O-acetyl-2,4-dideoxy-4-fluoro-D-galactopyranose; Fig. 1b). This is an unnatural monosaccharide where fluorine replaced the hydroxyl group at the C-4 position of GalNAc. For simplicity, this per-acetylated molecule is referred to as ‘4F-GalNAc’ while its de-acetylated analog 2-acetamido-2,4-dideoxy-4-fluoro-D-galactopyranose is called ‘non-acetylated 4F-GalNAc’. Results demonstrate that 50μM 4F-GalNAc added in cell culture media reduced selectin mediated cell adhesion function in vitro. 4F-GalNAc also impaired leukocyte recruitment in vivo in a mouse inflammation model.
MATERIALS AND METHODS

Supplemental Methods

Supplemental Material contains details on many procedures used in this work. Chemical synthesis of acetylated monosaccharides, fluorinated analogs and radioactive [14C]4F-GalNAc (Fig. 1b) is described here.

Cell culture

Human promyelocytic leukemia HL-60 cells were cultured in Iscove’s Modified Dulbecco’s Media (IMDM; Invitrogen, Grand Island, NY) with 20% FBS as recommended by ATCC (Manassas, VA). In many runs, per-acetylated forms of the natural monosaccharide GalNAc, monosaccharide analog (4F-GalNAc, 4F-GlcNAc) or radiolabeled [14C]4F-GalNAc at various doses (0-100µM) were added to cells at 0.2×10⁶ cells/ml, while they were in their exponential growth phase. The time when monosaccharide was added is designated \( t=0 \)h. In some cases, cells were treated with 0.05U/ml Vibrio Cholerae neuraminidase/sialidase (Sigma) for 1h at 37°C in 30mM Hepes buffer (pH=6.9) containing 10% FBS. This enzyme cleaves \( \alpha 2,3/6/8 \)-linked sialic acids. Residual neuraminidase was removed by washing prior to experimentation.

Flow cytometry

Flow cytometry measured cellular antigen expression (Lewis-X/Le^X/CD15, sialyl Lewis-X/Le^X/CD15s/CSLEX-1, CLA/HECA-452, PSGL-1/CD162, Galβ1,4GlcNAc-/LacNAc/DSA lectin binding and \( \alpha 1,3/4/6 \)-linked Fucose/AAL lectin binding) and selectin (human P-/L-/E-selectin) fusion protein binding to leukocytes. Details are part of Supplemental Material.
Cell adhesion under fluid flow

Parallel-plate flow chamber experiments were performed as described previously. Flow chamber substrate contained either a confluent monolayer of mouse fibroblast L-cells that stably expressed human E-selectin and ICAM-1 (L-E/I) or recombinant P-selectin IgG fusion protein. HL-60 cells cultured for 38h either in the presence of 50µM 4F-GalNAc or vehicle control were perfused over the selectin bearing substrate at 0.4×10^6/ml. The number of rolling cells, adherent cells and cell rolling velocities were quantified as described in Supplemental Material.

Metabolic labeling studies performed with [14C]4F-GalNAc

HL-60 were cultured with per-acetylated [14C]4F-GalNAc (0.5µCi/ml, 100µM) for 38h. The fate of the radioactive monosaccharide was analyzed by measuring: i) total radioactivity in various cell fractions, ii) monosaccharide status in cell culture supernatant, and iii) metabolic incorporation of [14C]4F-GalNAc into leukocyte PSGL-1. Supplemental Material provides detailed procedures.

Cytometry-bead assay and western blotting

The carbohydrate structure(s) on PSGL-1 was assessed by immunoprecipitating this protein from HL-60 lysates. Quantitative cytometry-bead assays and qualitative western blot analysis was performed (Supplemental Material and reference).

Briefly, in each cytometry-bead experiment, PSGL-1 from 200-400µg cell lysate was immunoprecipitated onto “TB5-beads” (6µm polystyrene beads with covalently immobilized anti-PSGL-1 mAb TB5). The binding of fluorescent reagents to these beads was measured. These includes FITC conjugated HECA-452, FITC-DSA lectin, and unconjugated anti-PSGL-1 antibody (H-300, rabbit pAb) followed by FITC-conjugated F(ab’)2 mouse-anti-rabbit IgG. Mean fluorescence intensity from control experiments performed in the absence of cell lysate was subtracted from all data presented in figures.
For western blots, PSGL-1 immunoprecipitated using 7µg/ml anti-PSGL-1 (H-300) and protein A/G beads was separated using either 8% or 4-20% gradient Tris-SDS-PAGE gel. In experiments with radiolabeled compounds, protein blotted onto nitrocellulose membrane was visualized using phosphorimaging. In other studies, the membrane was probed with anti-PSGL-1 mAb (KPL-1) or HECA-452 followed by appropriate HRP-conjugated secondary Abs, and chemiluminescence detection.

**Mouse peritonitis model**

Two types of experiments were performed (see Supplemental Material). In some cases, mouse bone marrow cells (BMCs) recovered from donor C57BL/6 mice were cultured *ex vivo* for 2-days with acetylated monosaccharides or vehicle control. Antigen levels and selectin/IgG binding were measured using flow cytometry. BMCs were then re-injected into recipient mice and peritonitis was induced by intraperitoneal injection of thioglycollate\(^\text{36}\). In other cases, 100mg/kg/day 4F-GalNAc or vehicle control was tail-vein injected into mice for 4-days prior to induction of peritonitis. Leukocyte counts in peritoneal lavage were measured in both experiments. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Roswell Park Cancer Institute.

**Statistics**

Data are presented as mean ± standard error mean (SEM) for ≥3 independent experiments. In cases where results are presented in % form, normalization was performed with respect to vehicle control (<0.25% DMSO) runs conducted in parallel. Student’s t-test (two-tailed) was performed for dual comparisons with respect to vehicle control. ANOVA was applied for comparison between multiple treatments. * \( p < 0.05 \) was considered significant.
RESULTS

We evaluated the ability of synthetic monosaccharide analogs, primarily 4F-GalNAc and also 4F-GlcNAc (Fig. 1b), to alter carbohydrate structures on PSGL-1 and the selectin binding function of leukocytes. All carbohydrates were acetylated to enhance their cell-permeability. Acetyl groups are hydrolysed by intracellular esterases13.

4F-GalNAc reduces leukocyte P-selectin binding and the expression of cell-surface CLA and sLeX epitopes

Two types of experiments were performed. Similar to a recent publication28, in some cases, HL-60 were treated with neuraminidase to remove cell-surface sialic acid prior to addition of acetylated monosaccharide (Fig. 2a-h). Neuraminidase reduced P-selectin binding to HL-60 by 85% (Fig. 2a, data at \( t=0 \)h) and cell-surface CLA/HECA expression by 90% (Fig. 2b). The anti-CLA mAb HECA-452 binds both CD15s/sLeX and other sLeX-like antigens37 (Fig. 1a). No change in PSGL-1 levels was noted (Fig. 2c), though LeX expression (data not shown) and DSA-lectin binding (Fig. 2d) increased by 900% and 20% respectively. Since DSA-lectin recognizes Gal\( \beta \)1,4GlcNAc structures (Fig. 1a), the data suggest that removal of sialic acid exposes new terminal LacNAc structures. In other runs, monosaccharides were added without prior sialidase treatment (Fig. 2i-l). While the first set of experiments examine CLA and sLeX expression, and P-selectin binding function of newly synthesized PSGL-1 only, the latter also account for the recycling of PSGL-1 and the effects of cell division.

Following neuraminidase removal at \( t=0 \)h, cells were allowed to grow in normal culture media containing either acetylated monosaccharides at indicated dose (Fig. 2e-h) or vehicle control (Fig. 2a-d). In vehicle control, P-selectin fusion protein binding returned to baseline levels by 14h (Fig. 2a), while CLA/HECA took 36h (Fig. 2b). Addition of 4F-GalNAc reduced P-selectin binding in a dose-dependent manner (Fig. 2e). 50\( \mu \)M 4F-GalNAc prevented the formation of any new CLA/HECA in HL-60 (Fig. 2f), and reduced DSA-lectin (Fig. 2h) and P-selectin binding by \( \sim \)70% compared to vehicle/GalNAc control at 38h. 4F-GalNAc treatment of HL-60 also abrogated anti-sLeX mAb CSLEX-1 binding to cells. P-selectin
and DSA-lectin binding to 4F-GalNAc treated cells was higher at 19h (one cell division) compared to 38h (two cell divisions) suggesting that the effects of acetylated monosaccharides are more prominent after cell division. While PSGL-1 expression was not affected by any treatment (Fig. 2g), a 10-15% reduction in cell growth rate at 38h was noted compared to the vehicle/untreated control. 4F-GalNAc was a superior inhibitor of selectin binding compared to 4F-GlcNAc in our experimental system (Fig. 2e-h). Vehicle control runs with \(< 0.25\%\) DMSO behaved identically to cells grown in normal cell culture medium in all assays described in this manuscript. The specificity of the P-selectin binding assay was confirmed in control studies (Supplemental Fig. S2, ref.\textsuperscript{34,38}).

Similar observations as Fig. 2e-h were made in runs where cells were not treated with neuraminidase prior to the addition of acetylated monosaccharide (Fig. 2i-l). Here too, 50µM 4F-GalNAc was more effective compared to 50µM 4F-GlcNAc at reducing P-selectin binding (66% vs. 22% at 38h, Fig. 2i), CLA/HECA (82% vs. 20% at 38h, Fig. 2j) and LacNAc (65% vs. 22% at 38h, Fig. 2l) epitope expression. No significant changes were observed in either GalNAc supplemented cells or vehicle controls. Overall, 4F-GalNAc reduced both P-selectin binding to HL-60 and the expression of CLA/HECA and sLe\textsuperscript{X} epitopes.

**4F-GalNAc reduces L- and E-selectin fusion protein binding to HL-60**

We tested if E- and L-selectin IgG binding to monosaccharide treated cells is also reduced (Fig. 3a). Control runs verified the specificity of the measured interaction by demonstrating that selectin binding was blocked by either 5mM EDTA, 2mM sLe\textsuperscript{X} analog TBC1269, or blocking antibodies against individual selectins. Addition of 50µM 4F-GalNAc to growth medium resulted in 80% and 60% reduction in E- and L-selectin binding respectively to HL-60 cells at 38h, compared to vehicle control. Thus, 4F-GalNAc functions as a pan-selectin inhibitor.

Reduction in CLA and sLe\textsuperscript{X} expression upon 4F-GalNAc treatment could be due to alteration in the extent of fucosylation in addition to the extent of sialylation. To test this possibility, cytometry studies
were performed with antibodies and AAL, an α1,3/4/6-fucose binding lectin (Fig. 1a). As seen, Le\textsuperscript{X} epitope expression and AAL binding decreased by 73% and 40% respectively in 4F-GalNAc supplemented cells compared to vehicle control (Fig. 3b). AAL lectin binding specificity for fucose was verified using 100mM L-Fucose, which abrogated lectin binding interaction. Thus, reduction in the extent of fucosylation may partly account for the reduced selectin binding.

**Treatment with 4F-GalNAc increases leukocyte rolling velocity**

The ability of 4F-GalNAc to reduce selectin mediated cell adhesion under fluid flow was measured using parallel-plate flow chambers (Fig. 4). HL-60 were pretreated with neuraminidase prior to culture with either vehicle control or 4F-GalNAc for 38h. Thereafter, the binding of these cells to E-selectin bearing cell monolayers (Fig. 4a-c) or immobilized P-selectin fusion protein (Fig. 4d-f) was measured.

Cell rolling velocity, number of rolling cells and adherent cell density were quantified. The rolling cell density of HL-60 cultured with 4F-GalNAc was similar to vehicle control both in the case of E-selectin (~110 cells/mm\textsuperscript{2}) and P-selectin (~145 cells/mm\textsuperscript{2}) mediated cell adhesion. Cell rolling velocities were 2.4-fold higher for cells cultured with 4F-GalNAc compared to vehicle control for E-selectin mediated rolling (Fig. 4a-c). Similar results were observed for P-selectin mediated rolling, where the rolling velocity for 4F-GalNAc treated cells ranged from 4-20μm/s compared to 2-10μm/s for vehicle and GalNAc control. While firmly attached cells were observed in vehicle control runs (77±9 cells/mm\textsuperscript{2} at 3 dyn/cm\textsuperscript{2}) in the E-selectin adhesion assay, adherent cell number was reduced to 14±8 cells/mm\textsuperscript{2} when HL-60 were cultured with 4F-GalNAc. The firmly attached cells were likely due to the high density of E-selectin on the L-E/I monolayer since most of these cells could be detached upon increasing the shear stress to 6 dyn/cm\textsuperscript{2}. This interaction could also be blocked by anti-E-selectin mAb.

In control runs, cell rolling via both E- and P-selectin was absent upon chelating calcium with 5mM EDTA. HL-60 also did not interact with substrates bearing rabbit-anti-mouse F(ab')\textsubscript{2} antibody alone in the absence of P-selectin-IgG. Further, HL-60 rolling/adhesive interactions to either P-selectin or E-
selectin bearing substrates could be completely blocked upon incubation of substrates with anti-P-selectin mAb G1 or anti-E-selectin mAb EP5C7. Experiments performed with control per-acetylated GalNAc resulted in cell rolling phenotype that was similar to vehicle control. Overall, 4F-GalNAc substantially increased E- and P-selectin mediated cell rolling velocities. This may be due to alterations in the carbohydrate structure of PSGL-1.

4F-GalNAc reduces the molecular weight of leukocyte PSGL-1 and the expression of CLA/HECA and LacNAc epitopes on this glycoprotein

We determined the effect of 4F-GalNAc on PSGL-1 glycosylation (Fig. 5). With the goal of obtaining quantitative results and time-course data rapidly, polystyrene beads covalently linked with anti-PSGL-1 mAb TB5 (“TB5-beads”) and cytometry based assays were developed (Supplemental Fig. S3). In these assays, PSGL-1 from cell lysate was first captured onto TB5-beads via immuno-affinity. Following this, the beads were probed with either: i) FITC-conjugated anti-CLA mAb HECA-452, ii) FITC-labeled DSA-lectin to detect LacNAc structures, or iii) anti-PSGL-1 antibody H-300 (rabbit IgG) along with FITC-labeled mouse-anti-rabbit secondary Ab. Competitive binding assays, blocking studies and isotype-control beads described in Fig. S3, confirm that this assay specifically probes changes in glycan structures associated with PSGL-1.

Our experiments monitored PSGL-1 in cells cultured in the presence of 50µM GalNAc, 4F-GalNAc or 4F-GlcNAc. (Fig. 5a,b). After 36h, CLA/HECA expression and DSA lectin binding was reduced by 86% and 73% respectively upon 50µM 4F-GalNAc treatment, compared to 10% and 53% for cells cultured with 4F-GlcNAc. Time course studies demonstrated reduced CLA/HECA and LacNAc (detected by DSA-lectin) expression on PSGL-1 in 4F-GalNAc treated cells beginning at 9-18h. No change was noted in GalNAc control (Fig. 5c-d).

Immunoprecipitation of PSGL-1 followed by western blot analysis verified results from cytometry-bead assays. CLA/HECA antigen associated with PSGL-1 was reduced by 4F-GalNAc, but not GalNAc or 4F-GlcNAc (Fig. 5e). Gels run for extended duration show that the apparent molecular weight
of PSGL-1 is reduced by 12.5% upon 4F-GalNAc addition (Fig. 5f). After accounting for the molecular weight of the peptide backbone, this translates to a ~20% reduction in the total glycan content of PSGL-1. Overall, the culture of cells with 4F-GalNAc results in a reduction in the LacNAc epitope, CLA/HECA expression and molecular weight of PSGL-1.

**4F-GalNAc is metabolically incorporated into PSGL-1**

HL-60 cells were cultured with per-acetylated [14C]4F-GalNAc for 38h under standard conditions, and the fate of the radioactive compound was followed. 2-3% of the radioactivity was associated with the cellular glycoproteins while a majority of the compound remained in the culture media (Table 1). With regard to this observation, since HL-60 cells (0.7×10^6 cells/ml, ~15.6 μm diameter) constitute only 0.14% of the volume of the experimental well (1ml), 3% of 4F-GalNAc associated with the cells represents a relatively high intra-cellular concentration of the modified monosaccharide (1.1mM).

Since 97% of the total radioactivity was associated with the culture media (Table 1), additional studies followed the fate of [14C]4F-GalNAc in the growth media. Here, media containing [14C]4F-GalNAc was placed under standard growth conditions for 38h either in the presence or absence of HL-60 cells. Media recovered at 38h were separated using a Biogel P-2 column (Fig. 6a, 6b). Negligible radioactivity was measured at the void volume (position 1) indicating that secreted proteins from HL-60 had negligible [14C]4F-GalNAc. Two major peaks were evident when HL-60 cells were present in culture (positions 2, 3, Fig. 6a) while only one peak appeared in the absence of cells (position 5, Fig. 6b). Eluates corresponding to these peaks were separated using thin layer chromatography/TLC (Fig. 6c) and their migration was compared to non-radiolabeled per-acetylated 4F-GalNAc and non-acetylated 4F-GalNAc (~10μg) under identical conditions. As seen, the first peak (position 2 and 5) that is common between both runs contains primarily per-acetylated, 4F-GalNAc (Fig. 6c, lanes 2 and 5). Some product also appeared at intermediate distances between the per-acetylated and de-acetylated 4F-GalNAc and this likely represents 4F-GalNAc that was partially hydrolyzed in culture media. In this regard, the original
[\textsuperscript{14}C]4F-GalNAc diluted and kept in DMSO under the same culture conditions appeared as a unique spot that migrated identically to fully-acetylated 4F-GalNAc shown in lane 7 (Fig. 6c). The second peak (position 3, lane 3), that is unique to experiments containing HL-60, migrated identically to non-acetylated 4F-GalNAc. This may represent 4F-GalNAc that was de-acetylated inside cells prior to being secreted/diffusing out from the cells.

In another assay, radioactivity associated with PSGL-1 was determined by immunoprecipitating the protein using Ab H-300, separation on 4-20% gradient gel and transfer to nitrocellulose membrane. Phosphorimaging of membrane was subsequently performed. Supernatant depleted of PSGL-1 was also analyzed in parallel. This study demonstrates the direct incorporation of 4F-GalNAc into leukocyte PSGL-1 and also other glycoproteins (Fig. 6d). Radioactivity associated with leukocyte glycoproteins could be partially removed by $\alpha$-N-acetylgalactosaminidase (Supplemental Fig. S4) and this confirms that the radioactivity measured is associated with O-glycans. Overall, 4F-GalNAc is metabolically active and it can be incorporated into multiple cellular glycoproteins including PSGL-1.

**4F-GalNAc reduced leukocyte recruitment to inflamed peritoneum**

We determined if 4F-GalNAc can reduce selectin mediated leukocyte recruitment at sites of inflammation (Figure 7). Bone marrow cells (BMCs) from C57BL/6 mouse femur were cultured ex vivo with either vehicle control or per-acetylated monosaccharides. These BMCs consist predominantly of functional neutrophils\textsuperscript{39-41}. Consistent with these reports we also observed, following 2 days of ex vivo culture in the presence of 20ng/ml G-CSF and 1ng/ml IL-3, that a majority of LDS-751$^+$ nucleated cells were positive for both granulocytic markers Gr-1 and 1A8/Ly-6-G (Supplemental Fig. S5). These cells, which appear in region R1 in the flow cytometry dot plot (Fig. 7a), expressed mouse PSGL-1 and CD45, and they bound human P-selectin fusion protein (Fig. 7b, and Supplemental Fig. S5). Cells in region R2 represent 28% of the LDS-751$^+$ cells and these did not stain for granulocyte markers. Culture of BMCs with 25$\mu$M 4F-GalNAc, but not 25$\mu$M GalNAc or vehicle control, led to a 62% decrease in P-selectin-IgG binding to granulocytes (Fig. 7b). While CD45 expression was not altered under any of the growth
conditions, a 22% and 14% reduction in PSGL-1 was noted upon culture with per-acetylated 4F-GalNAc and GalNAc respectively. In control runs, P-selectin-IgG binding to mouse cells could be abrogated by 10µg/mL mouse anti-human P-selectin/CD62P blocking antibody G1, 3mM sLe\(^X\) analog TBC1269 and 5mM EDTA.

We tested the hypothesis that 4F-GalNAc may reduce selectin mediated leukocyte adhesion in mice. Thus, BMCs cultured ex vivo for two days in the presence of 25µM 4F-GalNAc or vehicle-control were differentially labeled with lipophilic tracers, DiL (red fluorescence) or DiO (green fluorescence). These cells were mixed in 1:1 proportion and introduced into recipient C57BL/6 mice via tail-vein injection. Peritonitis was immediately induced by intraperitoneal injection of thioglycollate\(^36\). In this model of inflammation, granulocytes constitute >70% of the cells in the peritoneal lavage 5h after induction of peritonitis\(^36\). Flow cytometry analysis of granulocytes (Gr-1\(^+\)) just after labeling (Fig. 7c) and from the peritoneal lavage (Fig. 7d,e) demonstrate that cells labeled with DiO (region R4, Fig. 7c) and DiL (region R5) have distinct fluorescence compared to unlabeled native cells (region R3). The forward-side-scatter profile of the cultured cells and extravasated cells was similar. Fig. 7e presents a representative experiment where cells cultured with 4F-GalNAc were labeled with DiO while vehicle control cells were DiL-labeled. Here, the number of DiL-labeled cells exceeds the DiO-labeled population. Overall, a 48% reduction in the number of migrated leukocytes was observed with respect to vehicle control when 4F-GalNAc treated cells were stained with DiL and vehicle controls were DiO-labeled (Fig. 7f). % reduction in cell migration was 60% when the labeling protocol was reversed, i.e. 4F-GalNAc treated cells were stained with DiO.

In other studies, 4F-GalNAc or vehicle control was tail-vein injected at 100mg/kg/day into mice for 4 days (Fig. 7g). There was no evidence of toxicity, outward signs of distress, or alteration in the alertness/movement of the animals treated with 4F-GalNAc. Pharmacological activity of the monosaccharide analog was confirmed since there was a 40-50% reduction in total leukocyte, neutrophil, macrophage and eosinophil count in the peritoneum in 4F-GalNAc treated animals 5h after induction of peritonitis.
DISCUSSION

Experiments were performed to test the possibility that 4F-GalNAc may alter O-linked glycosylation and reduce selectin mediated cell adhesion. Consistent with this proposition, 4F-GalNAc addition to leukocytes reduced selectin fusion protein binding to HL-60 cells (Fig. 2, 3), increased HL-60 rolling velocity under fluid shear conditions (Fig. 4), reduced the CLA/HECA epitope expression on the glycoprotein PSGL-1 (Fig. 5), and inhibited leukocyte migration in a murine model of inflammation (Fig. 7). In the last experiments, BMCs cultured ex vivo with 4F-GalNAc displayed reduced migration into the peritoneum during inflammation. Direct intravenous infusion of 4F-GalNAc also reduced leukocyte recruitment and thus the compound is pharmacologically active.

While rolling velocity doubled upon 4F-GalNAc treatment compared to vehicle control in the flow-chamber assays and there was a ~75-80% reduction in the number of adherent cells, the total number of cells interacting with the selectin bearing substrate was not statistically different. In contrast, in the static assay, 4F-GalNAc reduced the binding of all three members of the selectin family (L-, E- and P-selectin) to HL-60 cells by ~70%. One explanation for the more prominent reduction in fusion protein binding compared to cell adhesion under flow is that sLeX on other glycoproteins besides PSGL-1 may co-operate to stabilize soluble-selectin binding to leukocytes. While blocking PSGL-1 alone is sufficient to abrogate molecular interactions under both static and shear-flow conditions, blocking sLeX/CLA formation with 4F-GalNAc is more effective under static conditions. Alternatively, the application of 4F-GalNAc may result in the appearance or exposure of other glycan structures that compensate for the loss of the sLeX/CLA epitope on PSGL-1. This change in the selectin binding epitope may then lead to an increase in leukocyte rolling velocity. As shown by Jung et al, increasing cell rolling velocity reduces firm cell adhesion at sites of inflammation by reducing the time available for the leukocytes to integrate local chemoattractant signals. In our animal studies, also, we observed that 4F-GalNAc treatment reduced leukocyte migration into the inflamed peritoneum. Taken together, the data demonstrate that 4F-GalNAc can reduce selectin binding and leukocyte adhesion in disease models.
In addition to reducing cell adhesion, 4F-GalNAc dramatically reduced cell surface sLeX expression on HL-60 by ~82% and it partially reduced the incorporation of fucose into cellular glycoconjugates. The reduction in sLeX expression was consistently observed using antibodies against CLA (HECA-452) and CD15/sLeX (CSLEX-1). This inhibition was more prominent upon addition of 50μM 4F-GalNAc compared to 50μM 4F-GlcNAc, or even 100U/mL chymotrypsin\(^{35}\). SLeX on HL-60 is primarily expressed on glycoproteins\(^{44}\), though the exact protein scaffolds and glycan structures that bear this epitope remains unknown. Taken together, the data suggest that 4F-GalNAc may be a useful reagent in studies that examine the functional effects of sLeX/CLA located on glycoproteins. Given the absence of efficient tools to block O-glycosylation in cells, this reagent may also find utility in studies that examine the role of O-linked glycosylation in not only disease, but also immunity\(^{45}\) and development\(^{46}\).

Quantitative cytometry-bead assays coupled with qualitative western blot analysis demonstrate that the glycans of PSGL-1 are modified upon 4F-GalNAc treatment. In this regard, a 12.5% reduction in the apparent molecular weight of PSGL-1 was noted, along with a decrease in the expression of the CLA/HECA and Galβ1,4GlcNAc- (LacNAc) epitopes on this glycoprotein. These glycan changes are likely to occur in O-glycan linkages since PSGL-1 is a mucinous protein with 71 Ser/Thr sites for O-linked glycosylation and only 3-sites for N-glycosylation\(^2\). GalNAc is also only seldom a part of N-glycans\(^{32}\). A similar 14.5-16% reduction in the apparent molecular weight of PSGL-1 has been reported by others\(^{47}\), who quantified PSGL-1 molecular weight in bone marrow neutrophils of Core 2-GlcNAcT-I deficient mice compared to littermate controls. Core 2 O-glycans are absent in these transgenic mice and selectin binding function is reduced. Thus, in both this published study and our work, the total glycan content of PSGL-1 is reduced by 20-25%. Alteration in PSGL-1 molecular weight is not noted in a study that examines the effect of 4F-GlcNAc on selectin function\(^{28}\). Together, the data are consistent with the notion that 4F-GalNAc modifies critical O-linked glycans that are required for selectin recognition. Further, 4F-GalNAc and 4F-GlcNAc may reduce selectin-ligand interaction via different mechanisms.

Studies performed with radioactive, per-acetylated 4F-GalNAc demonstrate the direct incorporation of the modified monosaccharide into cellular glycoproteins including PSGL-1. This
suggests that 4F-GalNAc is metabolically active and it may undergo several metabolic transformations. First, acetylation increases molecular hydrophobicity and this enhances cell permeability\textsuperscript{13}. Once in the cytoplasm, esterases hydrolyze O-acetyl groups\textsuperscript{13}. 4F-GalNAc likely undergoes activation by UDP-transfer to form UDP-4F-GalNAc. Such activation may allow facilitated transport of sugar nucleotide into the Golgi lumen via amino-sugar transporters\textsuperscript{32}. The efficiency of such transport is high due to the low $K_M$ (1-10\textmu M) of these transporters and the relatively higher concentration (~mM) of modified sugar nucleotides in the cytoplasm. Unlike activated sugar-nucleotides, which may be transported into the Golgi via facilitated transport, passive diffusion into the Golgi may limit the efficacy of aryl-GalNAc molecules\textsuperscript{12} and decoys\textsuperscript{14,15} used by others. Finally since the 4-OH moiety of GalNAc is important for the reversible conversion of GalNAc analogs to GlcNAc analogs via UDP-Gal/GalNAc-4-epimerase, it is possible that 4F-GalNAc cannot be transformed into other monosaccharides\textsuperscript{32}. Thus the effect of 4F-GalNAc may be localized to a relatively fewer metabolic steps compared to 4F-GlcNAc. The later molecule may also interact with the ManNAc and sialic acid biosynthesis pathways\textsuperscript{32}.

Besides the direct incorporation of 4F-GalNAc into the glycans of PSGL-1, other mechanisms may also contribute to the observed reduction in selectin-binding function. First, the attachment of 4F-GalNAc may reduce the extent of Core 2 glycan formation. In support of this, Brockhausen et al.\textsuperscript{48,49} have shown that Core-2 forming enzyme $\beta$1,6GlcNAcT recognizes many of the –OH groups in the glycan core 1 (Gal$\beta$1,3GalNAc) structure. Hydroxyl groups at the C-4 and C-6 positions of both Gal and GalNAc along with the C-2 N-acetyl group are shown to be essential for full activity of this enzyme. Second, it may be possible that monosaccharide analogs may act as inhibitors of specific glycosyltransferases, like fucosyltransferases. In this regard, native 4F-GalNAc, its nucleotide form UDP-4F-GalNAc or specific glycoconjugates formed due to 4F-GalNAc incorporation may display enzyme inhibition function. Third, besides the initial steps that regulate 4F-GalNAc incorporation into glycoconjugates, later steps like the time course of 4F-GalNAc accumulation in cells and cellular recycling/salvage pathways may also be important at later times following the first cell division step. While each of the aspects above is not
specifically examined in this study, these could be the subject of future investigations.

While 4F-GalNAc was well tolerated by cells in our study, we noted that the compound was also incorporated into other proteins besides PSGL-1. 4F-GalNAc, like 4F-GlcNAc, also altered the glycosaminoglycan content of cells (S.N., unpublished data). In this regard, 4F-GalNAc reduces chondroitin sulfate biosynthesis. Others have shown that 4F-GlcNAc reduces both heparan sulfate and chondroitin sulfate biosynthesis. Thus, the pathways engaged are not specific to PSGL-1 alone, and the functional consequence of this remains to be studied. We also noted a 10-15% reduction in cell growth rate at 38h compared to the vehicle/untreated control. Such non-specific effects are common to many other studies that use metabolic approaches to target cell adhesion. For example, Sharma et al demonstrate that both 4F-GalNAc and 4F-GlcNAc can reduce cell growth to a similar extent. Similar 10-15% reduction in cell growth is also reported in studies that employ decoys Galβ1,4GlcNAc-NM and GlcNAcβ1,3Gal-NM to reduce selectin binding. While reduction in cell growth is noted in these studies, metabolic labeling studies that employ radioactive carbohydrate and amino acid precursors show that protein synthesis is only reduced at extracellular concentrations in the 0.3-1mM range. Another study also notes that 100μM 4F-GalNAc and 4F-GlcNAc have no adverse effects on cellular protein biosynthesis.

Overall, the manuscript introduces 4F-GalNAc as a potential modifier of O-linked glycosylation. The in vitro and in vivo studies suggest that the compound can reduce the expression of the sialyl Lewis-X epitope on glycoproteins, and diminish selectin-mediated cell adhesion.
ACKNOWLEDGMENTS

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AUTHORSHIP AND CONFLICT OF INTEREST STATEMENT

D.D.M. designed research, performed experiments and wrote the paper. A.B.Jr., E.V.C., J.X., R.D.L. and M.N. performed experiments. J.T.Y.L. designed research. K.L.M. provided key carbohydrate reagents and designed research. S.N. designed research, performed experiments, coordinated project activities and wrote the paper.

The authors declare no competing financial interests.
REFERENCES


Table 1: Radioactivity associated with cells and cell components*

<table>
<thead>
<tr>
<th>Sample description</th>
<th>% Radioactivity</th>
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<tr>
<td>Cell culture media</td>
<td>97.1</td>
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<tr>
<td>Cell lysate</td>
<td>2.9</td>
</tr>
<tr>
<td>NP-40 lysate: pellet</td>
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</tr>
<tr>
<td>NP-40 lysate: soluble frac.</td>
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<tr>
<td>TCA precipitate</td>
<td>2.01</td>
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<tr>
<td>TCA soluble frac.</td>
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</table>

* Cells were cultured in media containing [14C]4F-GalNAc (0.5μCi/ml, 100μM) for 38h. Radioactivity in culture supernatant and cells was measured. Additionally, cells were lysed in 2% NP-40 and fraction of radioactivity in detergent soluble/insoluble fractions was determined. 10% TCA (trichloroacetic acid) was applied to precipitate proteins in the NP-40 soluble fraction and radioactivity associated with TCA precipitate was determined.
FIGURE LEGENDS

Figure 1: Glycan epitopes and monosaccharide analogs. a. Putative structure of selectin-binding glycan located at the N-terminus of PSGL-1 (P-selectin glycoprotein ligand-1). This glycan has a terminal tetrasaccharide sialyl Lewis-X (sLe^X) epitope and binding sites for DSA and AAL lectins. Neuraminidase cleaves sialic acid (NeuAc) residues to expose the terminal trisaccharide Lewis-X (Le^X) epitope. Metabolic inhibition of these glycan structures may reduce leukocyte selectin binding function. b. Structure of per-acetylated compounds GalNAc, 4F-GalNAc, 4F-GlcNAc and [14C]4F-GalNAc.

Figure 2: Effect of monosaccharides on epitope expression and P-selectin binding function. The effect of fully acetylated natural monosaccharide (GalNAc) or monosaccharide analog (4F-GalNAc, 4F-GlcNAc) added to HL-60 cells during their exponential growth phase was assessed in terms of: i) P-selectin binding function (panels a, e, i), ii) CLA/HECA expression (b, f, j), iii) PSGL-1 expression (c, g, k) and iv) DSA binding efficiency (d, h, l). Neuraminidase was used to remove cell surface sialic acids and selectin binding function at t=0h in panels a-h, but not i-l. P-selectin binding (a), CLA/HECA expression (b) and DSA binding (d) returned to baseline levels (denoted by 100%) following removal of neuraminidase. 4F-GalNAc was more effective than 4F-GlcNAc in reducing P-selectin binding (e), CLA/HECA expression (f) and DSA binding (h). Control monosaccharide GalNAc behaved similarly to vehicle (DMSO) control. Similar results as e-h were observed when HL-60 were treated with modified sugars in the absence of neuraminidase treatment at t=0h (i-l). Data are presented as % with respect to HL-60 cells treated with vehicle control and no neuraminidase. n.d. = not done. * p<0.05 with respect to vehicle control.

Figure 3: E-/L- selectin binding. a. E-/L-selectin-IgG fusion protein binding to HL-60 cells was measured at t=38h following protocols identical to Fig. 2i-l, in the absence of prior neuraminidase treatment. In vehicle control runs, selectin binding was blocked by 80-100% upon addition of 3mM sLe^X analog (TBC1269), 5mM EDTA or 30μg/mL anti-selectin Ab (either EP5C7 against E-selectin or
DREG56 against L-selectin). Culturing cells in the presence of 50µM 4F-GalNAc reduced E-/L-selectin binding by >70%. b. Le\(^X\), sLe\(^X\) expression and AAL lectin (binds α1,3/4/6-linked fucose antigen) binding was measured at 38h. 4F-GalNAc reduced Le\(^X\) expression and AAL lectin binding. Data are presented as Mean ± SEM (N ≥3 experiments). * p<0.05 with respect to vehicle control with no blocking reagent.

**Figure 4: Cell adhesion under flow.** 0.4×10\(^6\)/ml HL-60 cells cultured with 50µM 4F-GalNAc or vehicle control for 38h were perfused over either L-E/I (E-selectin) cell monolayers (panel a-c) or reconstituted P-selectin bearing surfaces (d-f) in a parallel plate flow chamber. Fluid shear was step increased from 0.35 dyn/cm\(^2\) to 3 and 6 dyn/cm\(^2\) in the case of E-selectin experiments as indicated in inset to panel a. Wall shear stress was constant at 0.4 dyn/cm\(^2\) in P-selectin runs (inset to d). Representative binned-histograms of rolling velocities in vehicle control (a, d) and 4F-GalNAc treated samples (b, e) are presented. Panels c and f summarize rolling velocity data at indicated shear rates. 4F-GalNAc increased cell rolling velocity by 2.4-fold for E- and 2-fold for P-selectin mediated rolling. * p<0.05 with respect to vehicle control.

**Figure 5: Glycan structures on PSGL-1.** a. HL-60 cells were cultured in the presence of 50µM acetylated monosaccharides (4F-GalNAc, 4F-GlcNAc, GalNAc) for 36h as outlined in Fig. 2a-h. PSGL-1 was immunoprecipitated from 400µg cell lysate onto TB5-beads. Carbohydrate epitopes on this antigen were then probed using HECA-452 and DSA lectin. The number of immobilized PSGL-1 was quantified using anti-PSGL-1 polyclonal mAb H-300. Black-empty and color-filled histograms represent the fluorescence of beads due to Ab/lectin in the absence and presence of cell lysate respectively. Dashed vertical line corresponds to peak fluorescence intensity in vehicle control. Representative histograms are shown. b. Summary of results from panel a for ≥ 3 independent runs. Normalized Geometric Mean Fluorescence Intensity (%)= (MFI of sample – background MFI in the absence of cells)/ (MFI of vehicle control – background MFI in the absence of cells). 4F-GalNAc reduces CLA/HECA expression on
PSGL-1 and the amount of DSA-lectin recognition. * $p<0.05$ with respect to GalNAc treatment. c, d. Representative cytometry-bead histograms (c) and Normalized MFI data (d) show temporal changes in PSGL-1 glycosylation upon 50µM 4F-GalNAc or GalNAc treatment. Here, HL-60 were cultured in the absence of prior neuraminidase treatment (protocol in Fig. 2i-l). CLA/HECA-452 expression, DSA-lectin binding and PSGL-1 amount on beads was quantified after capture of PSGL-1 from same amount of lysate in all samples. * $p<0.05$ with respect to $t=0$h. e. PSGL-1 from HL-60 cells cultured in 50µM 4F-GalNAc, 4F-GlcNAc or GalNAc were immunoprecipitated using Ab H-300 and analyzed using western blot analysis. Protein immunoprecipitated from 300µg lysate was loaded in each lane of two identical 8% PAGE-gels. One blot was probed with anti-PSGL-1 monoclonal antibody (KPL-1) and the other with HECA-452. CLA/HECA expression on PSGL-1 was reduced upon 4F-GalNAc treatment. f. 4F-GalNAc treatment reduced the molecular weight of PSGL-1 by ~10-15%.

Figure 6: $[^{14}C]4F$-GalNAc distribution in culture media and glycoproteins. Cell culture media containing $[^{14}C]4F$-GalNAc (0.5μCi/ml, 100µM) was placed under standard growth conditions either in the presence or absence of HL-60 cells for 38h. Growth media and cells were harvested separately. a-b. Culture media harvested from cultures with (panel a) and without HL-60 (panel b) separated on Biogel P-2 column. c. Samples at the void volume (sample 1 and 4), peak fractions in panel a (samples 2 and 3) and panel b (sample 5) were separated using TLC with Chloroform/methanol (5:1) as mobile phase. Phosphorimaging detected radioactivity on TLC plates. Non-radiolabeled, non-acetylated 4F-GalNAc (lane 6) and per-acetylated 4F-GalNAc (lane 7) standards were run in parallel and developed using ethanol/sulfuric acid spray followed by charring. Addition of acetylated 4F-GalNAc to HL-60 cells leads to de-acetylation as evidenced by prominent peak 3 in the chromatogram. d. PSGL-1 was immunoprecipitated from HL-60 lysates cultured with $[^{14}C]4F$-GalNAc using Ab H-300. This immunoprecipitate and PSGL-1 depleted cell lysate were resolved on an SDS-PAGE gel and blotted onto nitrocellulose membrane. Radioactivity was measured using scintillation counter in panels a and b; and
using phosphorimaging in panels c and d. 4F-GalNAc is incorporated into leukocyte PSGL-1 and other glycoproteins.

**Figure 7: Effect of 4F-GalNAc on selectin binding and leukocyte recruitment to peritoneum.** BMCs were cultured in media containing G-CSF and IL-3, and either 25µM 4F-GalNAc, 25µM GalNAc, or vehicle control (0.125% DMSO). a. After two days in culture, two cell populations (Region R1 and R2) stained positive for nuclear dye LDS-751 in the flow cytometer. Cells in R1 were neutrophils (Gr-1+1A8+). b. PSGL-1 and CD45 antigen expression, and P-selectin IgG binding to Gr-1+ cells was measured. 4F-GalNAc, but not GalNAc or vehicle control, reduced P-selectin fusion protein binding to cells. Data are represented as mean ± SEM with respect to vehicle control. *p<0.05 with respect to all other treatments. c. BMCs cultured for 2 days with 25µM 4F-GalNAc or vehicle control were labeled with either DiL or DiO dyes. Mixed cell populations just prior to injection into C57BL/6 recipient mice contained approximately equal numbers of DiL labeled vehicle control cells ((Vehicle)DiO) and DiO labeled 4F-GalNAc treated cells ((4F-GalNAc)DiL) (or vice versa). d. Peritoneal lavage sample from mice obtained 5h. after injection of thioglycollate ip., shows substantial accumulation of neutrophils in the peritoneum. These cells were positive for LDS-751, Gr-1 and 1A8. DiO/DiL labeled cells were not injected in this experiment. e. Granulocytes from the peritoneal lavage of animals which were tail-vein injected with mixed DiO+DiL BMCs just prior to induction of peritonitis. Number of DiL labeled cells (vehicle control) exceeds DiO labeled cells (4F-GalNAc treated). f. Ratio of 4F-GalNAc to vehicle control cells in infusion and peritoneum lavage sample. Data are presented as Mean ± SEM for 4 animals injected with DiL labeled 4F-GalNAc treated cells along with paired DiO labeled vehicle controls. 4 animals were injected with DiO/DiL labeling switched. 4F-GalNAc treatment reduced cell migration into peritoneum irrespective of whether these cells were labeled with DiO or DiL. *p<0.05 with respect to infusion sample. g. 100mg/kg/day 4F-GalNAc or vehicle control was injected into mice for 4 days prior to induction of peritonitis. Total leukocyte (Total), neutrophil (Neut), eosinophil (Eos) and macrophage
(Mac) count was determined in peritoneal lavage 5h after injection of thioglycollate ip. Data are Mean ± SEM for 4 animals for each treatment. * $p<0.05$ with respect to vehicle control.
Figure 1

(a) Structural representation of the carbohydrate epitope.

- **$sLe^X$/HECA**
- **$Le^X$**
- **AAL lectin**
- **DSA lectin**

Chemical structures:
- NeuAcα2→3 Galβ1→4 GlcNAcβ1→3 Galβ1→4 GlcNAcβ1
- NeuAcα2→3 Galβ1→3 GalNAcα

(b) Chemical structures with modifications:

<table>
<thead>
<tr>
<th>$R^1$</th>
<th>$R^2$</th>
<th>$C^*$</th>
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<td>H</td>
<td>$^{14}$C</td>
<td>$[^{14}$C]4F-GalNAc</td>
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Figure 5

(a) Flow cytometry histograms showing fluorescence distribution for HECA, DSA, and PSGL-1 under different conditions: Vehicle, 4F-GalNAc, 4F-GlcNAc, and GalNAc. The fluorescence is measured on a log scale.

(b) Bar graph showing normalized MFI (mean fluorescence intensity) for HECA, DSA, and PSGL-1 treated with 4F-GalNAc, 4F-GlcNAc, and GalNAc at various time points (9h, 18h, 27h, 36h).

(c) Flow cytometry histograms for HECA, DSA, and PSGL-1 with time points (9h, 18h, 27h, 36h) and fluorescence intensity on a log scale.

(d) Bar graph for normalized MFI with treatments 50 μM 4F-GalNAc and 50 μM GalNAc at time points 9h, 18h, 27h, 36h.

(e) Western blot analysis showing IP (immunoprecipitation) for anti-PSGL-1 (H-300) with bands for HECA, 4F-GalNAc, and 4F-GlcNAc.

(f) Anti-PSGL-1 Western blot with bands for 200KDa and IgG heavy.
Figure 6
Fluorinated per-acetylated GalNAc metabolically alters glycan structures on leukocyte PSGL-1 and reduces cell binding to selectins

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