

Intact cell adhesion to glycan microarrays

Leonardo Nimrichter^{1,3,4}, Ari Gargir^{1,5}, Monica Gortler⁵,
Rom T. Altstock⁵, Avi Shtevi⁵, Oori Weisshaus⁵, Ella Fire⁵,
Nir Dotan⁵, and Ronald L. Schnaar^{2,3}

³Department of Pharmacology and Molecular Sciences, The Johns Hopkins School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205;

⁴Instituto de Microbiologia Prof. Paulo de Goes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; and ⁵Glycominds, Ltd., Lod 71291, Israel

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A rapid and reproducible method was developed to detect and quantify carbohydrate-mediated cell adhesion to glycans arrayed on glass slides. Monosaccharides and oligosaccharides were covalently attached to glass slides in 1.7-mm-diameter spots (200 spots/slide) separated by a Teflon gasket. Primary chicken hepatocytes, which constitutively express a C-type lectin that binds to nonreducing terminal N-acetylglucosamine residues, were labeled with a fluorescent dye and incubated in 1.3- μ L aliquots on the glycosylated spots. After incubating to allow cell adhesion, nonadherent cells were removed by immersing the slide in phosphate buffered saline, inverting, and centrifuging in a sealed custom acrylic chamber so that cells on the derivatized spots were subjected to a uniform and controlled centrifugal detachment force while avoiding an air–liquid interface. After centrifugation, adherent cells were fixed in place and detected by fluorescent imaging. Chicken hepatocytes bound to nonreducing terminal GlcNAc residues in different linkages and orientations but not to nonreducing terminal galactose or N-acetylgalactosamine residues. Addition of soluble GlcNAc (but not Gal) prior to incubation reduced cell adhesion to background levels. Extension of the method to CD4⁺ human T-cells on a 45-glycan diversity array revealed specific adhesion to the sialyl Lewis x structure. The described method is a robust approach to quantify selective cell adhesion using a wide variety of glycans and may contribute to the repertoire of tools for the study of glycomics.

Key words: CD4⁺/glycomics/hepatocyte/lectins/oligosaccharides

Introduction

Carbohydrate-mediated cell–cell recognition is emerging as an important component in the repertoire of molecular recognition events that underlie the orderly development and functioning of multicellular organisms (Crocker and

Varki, 2001; Taylor and Drickamer, 2003; Vestweber and Blanks, 1999). Lectins (carbohydrate-binding proteins) on one cell surface bind to complementary carbohydrates on an apposing cell to initiate physiologically important cell–cell interactions. Genomic analyses indicate ~100 human lectins, and the diversity of lectin protein folds implies that additional families may yet be discovered (Drickamer and Taylor, 2002). Specific carbohydrate–carbohydrate interactions may also mediate cell–cell adhesion (Iwabuchi *et al.*, 1998; Pincet *et al.*, 2001). A challenge for glycobiology is to develop robust screening methods to identify and quantify the binding specificities of carbohydrate-mediated cell recognition events. The variety and complexity of naturally occurring glycan structures, and the typical multivalent nature of carbohydrate-mediated adhesion (low monovalent site affinity, high polyvalent affinity) add to the complexity of the challenge (Dwek, 1996; Lee and Lee, 2000).

Microarrays have been applied successfully to the quantification of nucleic acid and protein interactions with immobilized complementary binding partners and recently have been extended to the investigation of protein–carbohydrate interactions. Several methods were published in which natural and/or synthetic saccharides were adsorbed or covalently bound to surfaces in a manner suitable for screening binding antibodies and/or soluble lectins (Fazio *et al.*, 2002; Fukui *et al.*, 2002; Houseman and Mrksich, 2002; Schwarz *et al.*, 2003; Wang *et al.*, 2002). These methods have been heralded as the birth of glycomics, the first steps to large-scale screening of glycan recognition in biological systems (Bidlingmaier and Snyder, 2002; Drickamer and Taylor, 2002; Flitsch and Ulijn, 2003; Hirabayashi, 2003; Kiessling and Cairo, 2002; Love and Seeberger, 2002).

Many of the protein–carbohydrate interactions that occur at cell surfaces are intrinsically multivalent (Lee and Lee, 2000). Arrays of lectins (or carbohydrates) on one cell surface interact like Velcro with complementary arrays of carbohydrates on an apposing surface. The best characterized example of the kinetic role of multivalency is binding by the hepatic asialoglycoprotein receptor (Lee *et al.*, 1983). On the intact hepatocyte surface, the site affinity of this lectin for lactose is 300 μ M, whereas its affinity for a trivalent oligosaccharide with appropriately spaced galactose termini is 7 nM, nearly five orders of magnitude higher. Similarly, carbohydrate–carbohydrate binding may have even lower site affinity, requiring highly multivalent interactions for function (Pincet *et al.*, 2001). The off rate of a monovalent carbohydrate-mediated interaction may be so rapid that solid-phase binding to immobilized glycan arrays, which require a wash step to remove unbound protein, may be difficult to detect. This problem has been circumvented to some extent by using multivalent soluble

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed; e-mail: schnaar@jhu.edu

lectins or by generating multivalency using chimeras or secondary binding proteins.

In nature, multivalency is often generated by lectin expression on cell surfaces, where lectin molecules self-associate or cluster in response to multivalent binding arrays on an apposing surface (Weis and Drickamer, 1996; Weisz and Schnaar, 1991). Here we report methods that

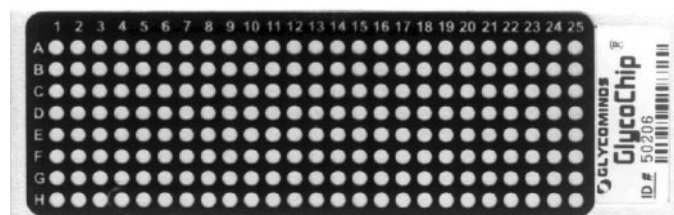


Fig. 1. Glycan microarray (GlycoChip) glass slide patterned with hydrophobic Teflon mask, creating 200 flat wells that can accommodate up to 1.5 μ L of hydrophilic liquid. *p*-Aminophenyl glycosides were covalently attached to the glass surfaces in the wells via an oligomeric 1,8-diamino-3,6-dioxaoctane linker, as described (Schwarz *et al.*, 2003).

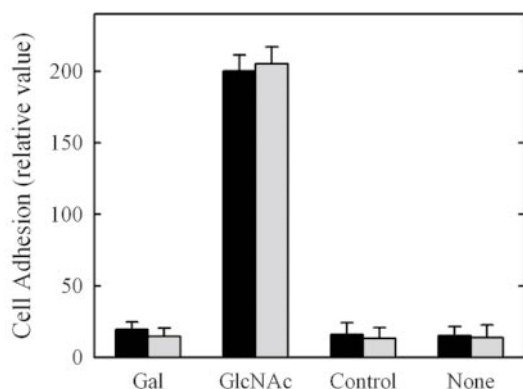
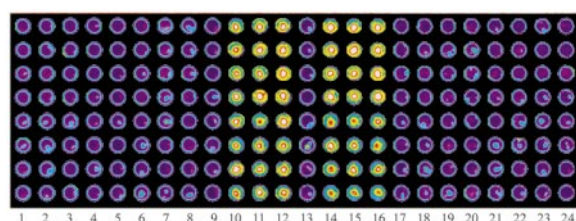


Fig. 2. Selective adhesion of chicken hepatocytes to β -GlcNAc on a glycan microarray slide. DiIC18-labeled primary chicken hepatocytes (6500 in 1.3 μ L) were pipetted onto each spot of a glass slide glycan microarray. Adhesion was quantified by determining fluorescence remaining after removal of nonadherent cells as described in the text. Top: Pseudocolor image of the fluorescence intensity. The spots in each column were uniformly derivatized with β -galactoside (Gal, columns 2–4 and 6–8), β -N-acetylglucosaminide (GlcNAc, columns 10–12 and 14–16), or with control linker (control, columns 18–20 and 22–24). Intervening columns (none, columns 1, 5, 9, 13, 17, and 21), which also received aliquots of labeled cells, were underivatized. In the upper four rows, spots were blocked with and cells suspended in medium containing 5 mg/mL BSA; in the lower four rows 10 mg/mL BSA was used. Bottom: Quantification of the fluorescence intensities. Values are given as mean \pm SD. Black bars represent spots receiving cells suspended in 5 mg/mL BSA and gray bars represent 10 mg/mL BSA.

detect specific adhesion of intact cells to covalent carbohydrate microarrays engineered on glass slides. These methods take advantage of the natural multivalency of cell surface carbohydrate binding to extend the applicability of glycan microarrays.

Results

Glass-slide arrayed carbohydrates

Defined glycosides were covalently arrayed on standard-size glass slides using previously described chemistry (Schwarz *et al.*, 2003). The array consisted of 8 rows, 25 columns of 1.7-mm diameter spots separated by a Teflon gasket (Figure 1).

Adhesion of intact chicken hepatocytes to GlcNAc-terminated glycans

A method for quantifying intact cell adhesion to glass slide glycan arrays was developed and refined using primary chicken hepatocytes, which express the well-defined GlcNAc-specific chicken hepatic lectin on their surface (Drickamer, 1981). Initial experiments used slides with multiple spots derivatized with GlcNAc, Gal, linker arm (control), and no modification (Figure 2). Chicken hepatocytes adhered selectively to spots derivatized with GlcNAc glycosides. Cell adhesion to Gal-derivatized spots and control surfaces was very low. Varying the conditions for blocking nonspecific cell adhesion (5 mg/mL or 10 mg/mL bovine serum albumin [BSA]) did not alter the results. Microscopic examination of the wells (Figure 3) confirmed

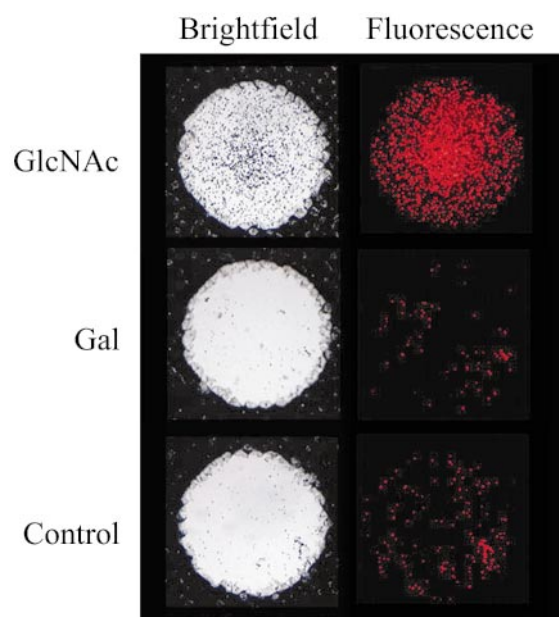


Fig. 3. Chicken hepatocyte adhesion to GlcNAc-derivatized spots on a glycan microarray slide. Representative spots of the slide in Figure 2 were viewed under bright field (left column) and epifluorescent microscopy (right column). Individual fluorescent cells are evident. The nonuniform distribution of adherent cells within the well is due to mounding of the 1.3- μ L droplet of cells added to each spot.

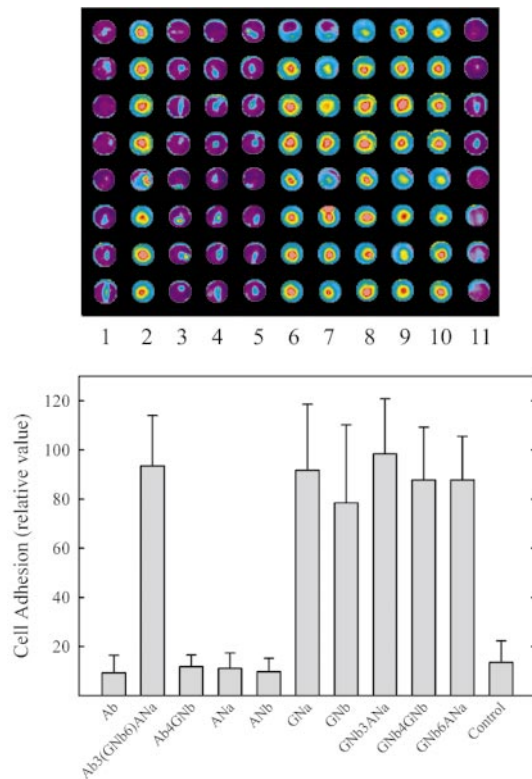


Fig. 4. Primary chicken hepatocyte adhesion to diverse glycosides on a glycan microarray slide. DiIC18-labeled primary chicken hepatocytes (6500 in 1.3 μ L) were pipetted onto each spot on a glass slide arrayed with a variety of Gal-, GlcNAc-, and GalNAc-terminated glycans. Adhesion was quantified by determining fluorescence remaining after removal of nonadherent cells as described in the text. Top: Pseudocolor image of the fluorescence intensity. Columns were uniformly derivatized with (column number): Gal β (1), Gal β 3(GlcNAc β 6)GalNAc α (2), Gal β 4GlcNAc β (3), GalNAc α (4), GalNAc β (5), GlcNAc α (6), GlcNAc β (7), GlcNAc β 3GalNAc α (8), GlcNAc β 4GlcNAc β (9), GlcNAc β 6GalNAc α (10) or control linker (11). Bottom: Quantification of the fluorescence intensity. Data are presented as mean \pm SD.

that fluorescence correlated with the adhesion of intact cells to the GlcNAc- but not the Gal- or control-derivatized spots.

Using slides with a more diverse set of glycans (Figure 4) revealed cell adhesion that was fully consistent with the GlcNAc specificity of the chicken hepatic lectin (Burrows *et al.*, 1997). Glycans with a nonreducing terminal GlcNAc residue, whether straight chain or branched, supported robust cell adhesion, whereas glycans with terminal Gal or GalNAc residues supported only background adhesion. Linkage position and anomeric configuration had little effect on GlcNAc-specific cell adhesion. To further test the specificity of the interaction, replicate spots were seeded with cells in control medium or medium supplemented with 25 mM GlcNAc as inhibitor or 25 mM Gal as control. Whereas addition of Gal to the medium did not reduce GlcNAc-specific cell adhesion, addition of GlcNAc reduced adhesion to at or near background levels (Figure 5). These data demonstrate robust glycan-specific adhesion of intact chicken hepatocytes to glycan arrays bearing nonreducing terminal GlcNAc residues.

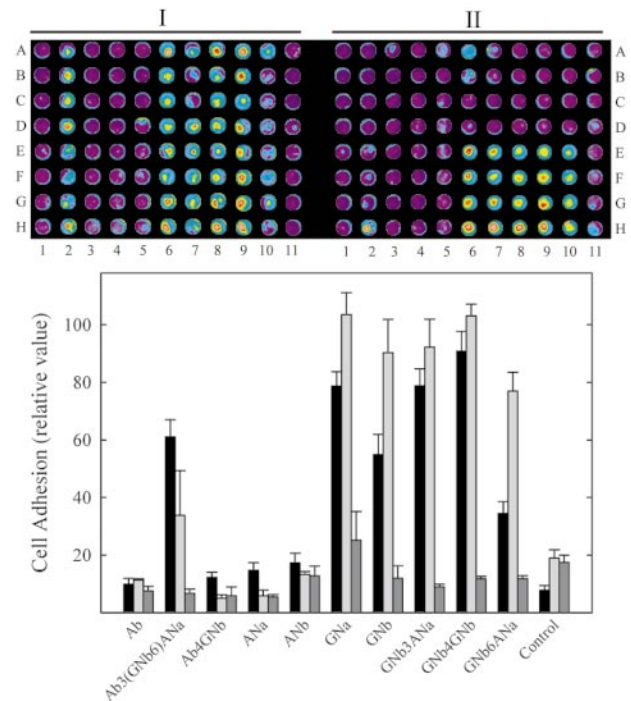


Fig. 5. Inhibition of primary chicken hepatocyte adhesion to glycosides on a glycan microarray slide by soluble GlcNAc. DiIC18-labeled primary chicken hepatocytes (6500 in 1.3 μ L) were pipetted onto each spot on a glass slide arrayed with a variety of Gal-, GlcNAc-, and GalNAc-terminated glycans as in Figure 4. Adhesion was quantified by determining fluorescence remaining after removal of nonadherent cells as described in the text. Top: Pseudocolor image of the fluorescence intensity. In the columns marked (I) cells were added in the absence of soluble sugars. In the columns marked (II) cells were added in the presence of 25 mM GlcNAc (rows A–D) or 25 mM Gal (rows E–H). Column numbers correspond to different glycosides as described in the legend to Figure 4. Bottom: Quantification of the fluorescence intensity. Black bars represent cells suspended in medium without sugar supplementation, light gray bars with 25 mM Gal, and dark gray bars with 25 mM GlcNAc. Data are presented as mean \pm SE.

Selective adhesion of human CD4⁺ T cells to sialyl Lewis x antigen

The described method was used to test human CD4⁺ T-cell adhesion to a panel of 45 different glycans (Figure 6). CD4⁺ T-cells from seven healthy individuals were compared for their adhesive specificities. Robust adhesion of CD4⁺ cells to sialyl Lewis x antigen (Neu5Ac α 3Gal β 4(Fuc α 3)-GlcNAc β) was detected, perhaps via cell surface L-selectin (Camerini *et al.*, 1989). In contrast, the nonfucosylated form of the same glycan (Neu5Ac α 3Gal β 4GlcNAc β) supported little cell adhesion ($p < 0.01$, t -test for the sialylated versus nonsialylated structure). Three other glycans (Man β 4Glc β , Man α 3Man α , GlcNAc β 4GlcNAc β) supported less robust CD4⁺ T-cell adhesion that was nonetheless significantly above background.

Discussion

Biomolecular arrays are powerful tools for determining intramolecular interactions (Bidlingmaier and Snyder, 2002;

procedure is that cells need not be preincubated with fluorescent label, which may alter their adhesive state. We tested CD4⁺ human T-cells using both methods (data not shown) and found that preincubation with dye affected binding, in that CD4⁺ T-cells pre-labeled with DiIC18 lost their mannose binding but retained their sialyl Lewis x binding.

The loss of mannose/GlcNAc binding by CD4⁺ T-cells after preincubation may indicate a lectin of low stability, abundance, and/or affinity. Whereas CD4⁺ T-cells express L-selectin, a lectin capable of binding sialyl Lewis x (Camerini *et al.*, 1989), expression of intrinsic mannose/GlcNAc binding lectins on these cells has not been established. The mannose/GlcNAc-dependent adhesion that was detected, therefore, may represent a novel lectin activity on CD4⁺ T-cells. However, it may also be due to other leukocytes present as minor contaminants in the affinity-purified CD4⁺ cell population (~10% of the cell population, see *Materials and methods*), some of which express mannose/GlcNAc lectins (East and Isacke, 2002; McGreal and Gasque, 2002). The lectin identity and specificity of this binding activity were not analyzed further.

The limitations of cell-adhesion microarray studies are primarily technical. Intact cells are not amenable to long-term storage, cannot be exposed to extreme conditions or detergents, and are less compatible with robotic and high-throughput liquid manipulation systems. Unlike protein-carbohydrate binding assays, cell adhesion requires carefully controlled detachment forces (Schnaar, 1994). As in our previous glycan cell adhesion assays (Collins *et al.*, 2000; Schnaar *et al.*, 1989; Swank-Hill *et al.*, 1987; Yang *et al.*, 1996), centrifugal force was used for this purpose, requiring the fabrication of acrylic centrifuge carriers so that the cells on the adhesive surfaces were subjected to a uniform detachment force in a sealed, fluid-filled compartment. Although these limitations present a challenge to the application of cell-adhesion based glycan microarray studies, they are offset by the advantages for specific applications. Low numbers of cells required, low background adhesion, reproducibility, and highly specific glycan-dependent cell adhesion were demonstrated using this approach, establishing that intact cells can be used to probe the specificity of carbohydrate-based cell adhesion in a robust microarray format.

Materials and methods

Glycan microarray slides

Glycan microarray (GlycoChip) slides were custom made by Glycominds (Lod, Israel). Defined glycosides were covalently arrayed using previously described chemistry (Schwarz *et al.*, 2003).

Chicken hepatocytes

Chicken hepatocytes were prepared by collagenase perfusion of juvenile chicken livers as described previously (Obrink *et al.*, 1977). The resulting cell populations were predominantly single cells and >85% viable by trypan blue exclusion. Cell suspensions were maintained at $\leq 4^{\circ}\text{C}$ until use.

An aliquot containing $\sim 5 \times 10^6$ cells was placed in a 1.5-mL microcentrifuge tube, the cells were collected by centrifugation (15 s, $3000 \times g$), washed once with 0.53 mM EDTA in calcium-magnesium-free Dulbecco's phosphate buffered saline (PBS), and resuspended in 1 mL of the same solution. After counting, the cells were centrifuged as before and then resuspended at a final concentration of 5×10^6 cells/mL in HEPES buffered Dulbecco's minimal essential medium (DMEM-HEPES) containing 3 μM DiIC18 (Molecular Probes, Eugene, OR). After 1 h at 37°C in the dark with constant gentle agitation, the labeled cells were washed three times with DMEM-HEPES and resuspended at 5×10^6 cells/mL in the same medium supplemented with BSA (5 mg/mL or 10 mg/mL as indicated). In some experiments, cells were suspended in medium containing 25 mM Gal or 25 mM GlcNAc (as indicated) immediately prior to use.

Human CD4⁺ T-cells

Ten milliliters of peripheral blood were collected from each of seven healthy volunteers into two 10-ml EDTA-Vaccutainers. The blood was incubated for 4 h at ambient temperature. Immediately after incubation, the blood samples were centrifuged ($230 \times g$; 10 min); after the plasma was removed, the upper 2 mL of buffy coat were transferred to a fresh 15-ml test tube. CD4⁺ cells were then negatively enriched using RosetteSep CD4⁺ enrichment kit (StemCell Technologies), a procedure that yields 90% CD4⁺ cells with the remaining 10% representing the other leukocytes in the starting population. An aliquot of cells were diluted 1:10 in Türk solution (0.01% crystal violet and 3% glacial acetic acid) and counted. Cells were further diluted to a density of 5×10^6 cells/ml in RPMI-1640 medium containing 2% fetal bovine serum and incubated overnight in a humidified incubator (37°C , 5% CO_2 , 95% humidity).

Chicken hepatocyte adhesion to glycan microarrays

Prior to use, glycan array slides were washed with distilled water and air-dried. To reduce nonspecific cell adhesion, an aliquot (1.3 μL) of Dulbecco's PBS containing either 5 mg/mL or 10 mg/mL BSA was placed on each spot, and the slide was incubated in a humid atmosphere (a closed Petri dish with moist filter paper) for 2 h at 37°C . Blocking solution was removed by aspiration and replaced with an aliquot (1.3 μL) of cell suspension (5×10^6 cells/mL). After 30 min at 37°C in a humid atmosphere to allow adhesion to proceed, the slides were gently immersed in a vat of PBS, inverted onto an immersed custom Plexiglas centrifuge carrier (Figure 7), which was then sealed to exclude air. The carrier containing the inverted slides was centrifuged at $800 \times g$ at ambient temperature for 20 min to remove nonadherent cells. The carrier was reimmersed in PBS; the slide was removed while immersed, righted, lifted out of the PBS, and placed gently in a Petri dish containing 2% paraformaldehyde in PBS for 30 min at ambient temperature. The slides were washed by transferring to a Petri dish filled with PBS, sealed under a coverslip, and stored in the dark at 4°C .

Fluorescent images of the glycan array slides were captured using a Fujifilm LAS-1000 image analyzer (Fuji

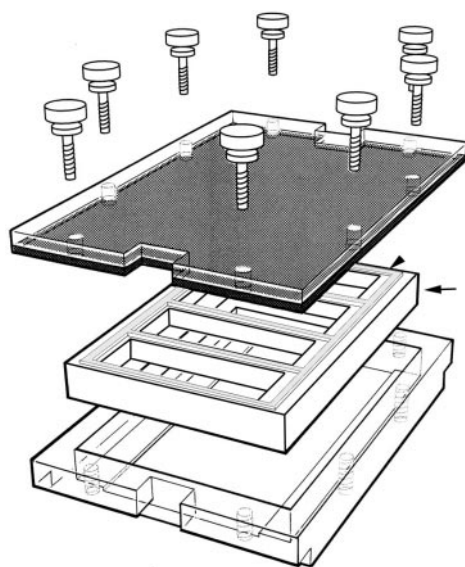


Fig. 7. Custom acrylic adhesion chamber for glycan microarray slide cell adhesion assays. The chamber is a modification of a custom 96-well plate adhesion chamber described previously (Collins *et al.*, 2000). An acrylic insert (arrow) consisted of a $12.7 \times 8.6 \times 0.45$ cm acrylic sheet with four evenly spaced rectangular holes each 5.7×2.2 cm to accommodate four glass slides. The holes were flanked by thin (0.16 cm thick) acrylic spacers (arrowhead) to keep the slides in place. The insert was placed in the chamber, the chamber was immersed in a vat of PBS; slides with adherent cells were gently immersed (cell side up) in the vat, inverted, and placed face down over the rectangular holes. While still immersed, an acrylic top with an attached foam rubber gasket and holes that line up with tapped holes in the chamber was used to seal the chamber using knurled-head polypropylene screws. The fluid-filled chamber was then transferred to a centrifuge carrier, nonadherent cells were removed by centrifugation at $800 \times g$, and the chamber was returned to the PBS-filled vat. While immersed the slides were removed, righted, and then gently lifted from the vat and placed in a Petri dish containing paraformaldehyde in PBS to fix adherent cells in place for subsequent fluorescent detection.

Photo Film, Valhalla, NY), and fluorescent pixel density was quantified using Metamorph image analysis software (Universal Imaging, Downingtown, PA). Adherent cells were also viewed by bright field and fluorescence microscopy using a Nikon T2000 epifluorescent microscope fitted with a Sony CCD camera.

Human CD4⁺ T-cell adhesion to glycan microarrays

Prior to use, the glycan arrays slides were washed with distilled water and air-dried. The adhesion assay was initiated by applying 1.2 μ L human CD4⁺ T-cell suspension (5×10^6 cells/ml) on each well on the slide; cells from each individual were spotted on replicate slides. After an incubation of 60 min in a humidified incubator (37°C, 5% CO₂, 95% humidity) the slides were gently immersed in a vat of PBS, inverted onto an immersed custom acrylic centrifuge carrier (Figure 7), which was then sealed to exclude air. The carrier containing the inverted slides was centrifuged at $50 \times g$ at ambient temperature for 2 min to remove non-adherent cells. The carrier was reimmersed in PBS; the slides gently removed while immersed, then placed in a slide rack. The cells were then fixed to the slides by immersion in PBS/3.7% formaldehyde for 60 min at ambient

Table I. LinearCode abbreviations used in this publication

A[3S]b	(3-O-HSO ₃)Gal β
Aa	Gal α
Aa3(Fa2)Ab	Gal α 3(Fuca2)Gal β
Ab	Gal β
Ab3(GNb6)ANa	Gal β 3GlcNAc β 6GalNAc α
Ab3ANa	Gal β 3GalNAc α
Ab3GNb	Gal β 3GlcNAc β
Ab4Gb	Gal β 4Glc β
Ab4GNa	Gal β 4GlcNAc α
Ab4GNb	Gal β 4GlcNAc β
Ab6Ab	Gal β 6Gal β
ANa	GalNAc α
ANa3(Fa2)Ab	GalNAc α 3(Fuca2)Gal β
ANb	GalNAc β
Fa	Fuca α
Fa2Ab	Fuca2Gal β
Fb	Fuc β
Ga	Glc α
Ga4Ga	Glc α 4Glc α
Ga4Gb	Glc α 4Glc β
Gb	Glc β
Gb3Gb	Glc β 3Glc β
Gb4Gb	Glc β 4Glc β
Gb4Gb4Gb	Glc β 4Glc β 4Glc β
Gb4Gb4Gb4Gb	Glc β 4Glc β 4Glc β 4Glc β
GN[6S]b	(6-O-HSO ₃)GlcNAc β
GNa	GlcNAc α
GNb	GlcNAc β
GNb3ANa	GlcNAc β 3GalNAc α
GNb4GNb	GlcNAc β 4GlcNAc β
GNb6ANa	GlcNAc β 6GalNAc α
Ha	L-Rha α
Lb	GalA β
Ma	Man α
Ma3Ma	Man α 3Man α
Mb	Man β
Mb4Gb	Man β 4Glc β
NNa	Neu5Ac α
NNa3Ab4(Fa3)GNb	Neu5Ac α 3Gal β 4(Fuca3)GlcNAc β
NNa3Ab4GNb	Neu5Ac α 3Gal β 4GlcNAc β
Ra	L-Ara α
Ub	GlcA β
Xa	Xyl α
Xb	Xyl β

temperature. The slides were then washed three times in distilled water and air-dried. The bound cells were fluorescently labeled postfixation by incubation of the slides in propidium iodide solution (50 mg/mL in PBS) for 15 min.

The slides were then washed three times in distilled water and airdried in the dark.

Fluorescent images of the glycan array slides were captured using an Affymetrix 428 laser scanner (Affymetrix, Santa Clara, CA) using an excitation wavelength of 522 nm and an emission wavelength of 665 nm; fluorescent pixel density was quantified using OptiQuant image analysis software (Packard, Boston, MA).

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Abbreviations

BSA, bovine serum albumin; DiIC18, 1,1'-dioctadecyl-3,3,3'-tetramethyl-indocarbocyanine perchlorate; PBS, phosphate buffered saline. The LinearCode for saccharides is fully described (Banin *et al.*, 2002), as indicated in Table I for structures used in the current study.

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