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# ABO blood group glycans modulate sialic acid recognition on erythrocytes

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**ABH(O) blood group polymorphisms are based on well-known intraspecies variations in structures of neutral blood cell surface glycans in humans and other primates. Whereas natural antibodies against these glycans can act as barriers to blood transfusion and transplantation, the normal functions of this long-standing evolutionary polymorphism remain largely unknown. Although microbial interactions have been suggested as a selective force, direct binding of lethal pathogens to ABH antigens has not been reported. We show in this study that ABH antigens found on human erythrocytes modulate the specific interactions of 3 sialic acid-recognizing proteins (human Siglec-2, 1918SC influenza hemagglutinin, and *Sambucus nigra* agglutinin) with sialylated glycans on the same cell surface. Using specific glycosidases that convert A and B glycans to the underlying H(O) structure, we show ABH antigens stabilize sialylated glycan clusters on erythrocyte membranes uniquely for each blood type, generating differential interactions of the 3 sialic acid-binding proteins with erythrocytes from each blood type. We further show that by stabilizing such structures ABH antigens can also modulate sialic acid-mediated interaction of pathogens such as *Plasmodium falciparum* malarial parasite. Thus, ABH antigens can noncovalently alter the presentation of other cell surface glycans to cognate-binding proteins, without themselves being a direct ligand. (Blood. 2009; 114:3668-3676)**

## Introduction

The 1930 Nobel Prize in Medicine was awarded to Karl Landsteiner "for his discovery of human blood groups" as the major cause of blood transfusion reactions. The ABO blood group polymorphisms of humans and other primates are now known to be determined by expression of A, B, or H(O) antigens,[1-4] which are terminal neutral glycan sequences found in abundance on glycoproteins and glycolipids (supplemental Figure 1A, available on the *Blood* website; see the Supplemental Materials link at the top of the online article). Almost 110 years after their discovery, the major functions of this evolutionarily conserved allelic polymorphism remain a mystery.[3] The A and B alleles code for a polymorphic glycosyltransferase that adds either *N*-acetylgalactosamine or galactose to the core H antigen structure. It was recently shown that this gene is under balancing selection.[5] Pathogen interactions have been suggested as the evolutionary driving force, and various indirect associations with bacterial and viral infections described.[6] However, direct interactions of microbes with ABH antigen structures are only known for some strains of *Helicobacter pylori*[7] and certain noroviruses.[8] As both of these cause nonlethal diseases, which should not have a major impact on reproductive fitness, they cannot explain the evolutionary fixation of the ABO blood group polymorphisms in the human population, nor their persistence throughout primate evolution.[2] We present evidence for an alternate explanation, showing that ABH antigens can modulate cellular interactions without being a direct ligand themselves, by stabilizing other glycans on the cell surface in clusters, thus making them more (or less) accessible for glycan-binding proteins.

There are 5 major membrane glycoproteins expressed on the cell surface of human erythrocytes (red blood cells [RBCs]): the anion transport protein (band 3); the glucose transport protein (band 4.5); and glycophorins A, B, and C.[9] There are approximately 2 million ABH(O) glycan antigen sites on each RBC, and most are presented on bands 3 and 4.5 (supplemental Figure 1B).[9] Glycophorins are also heavily glycosylated and are the major carriers of sialic acid-containing glycans, with only a minor fraction of their glycans carrying ABH antigens.[10] A minor fraction of ABH antigens is also found on gangliosides containing sialic acids in α2-3 linkage.[11] Sialic acids (Sias) are monosaccharides typically found at outer terminal positions of glycans attached to cell surface glycoconjugates (supplemental Figure 1B), and thus play important roles in a variety of physiologic and pathologic interactions.[12] There are approximately 50 million Sias on each RBC.[13] Sialic acid recognition often has well-defined requirements for the specific linkage and sequences of the underlying glycans.[12,14,15] Sialic acid-mediated binding is therefore a good model for testing the effect of ABH antigen status on interactions involving unrelated adjacent glycans on RBC surfaces.

In this study, we show that 3 Sia-binding proteins, specific for binding Sias in α2-6 linkage, each interact with RBCs in a unique pattern: human Siglec-2 (CD22),[16,17] the pandemic human influenza hemagglutinin (1918-South Carolina),[18] and the plant lectin *Sambucus nigra* agglutinin (SNA; elderberry).[19] These sialic acid-mediated interactions are modulated by the ABH antigen status, although none of these proteins can directly bind A, B, or H antigens. We show that Siglec-2 and SNA bind in distinct clusters that are stabilized by A and B antigens, and propose a model for spatial organization of sialylated glycan clusters on RBC surface, unique for each blood type. By stabilizing these clusters, ABH blood group antigens modulate interactions involving Sias without being direct ligand themselves. Based on our model, we could

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predict the binding preference of the invasive merozoite erythrocyte-binding antigen (EBA)–175 of *Plasmodium falciparum* (the major cause of malaria mortality), which is specific for binding Neu5Acα2-3Gal on glycophorins A.[20]

## Methods

### Erythrocyte-binding assay

COS7 cells were cultured according to ATCC specifications. Cells were transfected with 0.125 μg/well pEGFP and either 0.375 μg/well pfEBA-175[21] or pcDNA3.1 using Fugene 6 reagent. Transfected cells were prepared for binding assays, as previously described.[21] Erythrocytes from 15 volunteers were washed and resuspended to 0.25% hematocrit in Dulbecco modified Eagle medium containing 0.25% bovine serum albumin, and 500 μL was added to the transfected cells for 7 minutes on a rotating plate at 37°C. Nonbound cells were washed extensively with phosphate-buffered saline, and the samples were immediately examined with DeltaVision Real Time fluorescence microscope (Applied Precision). Twenty randomly selected fields were viewed for each sample, and the number of rosettes per green fluorescent protein (GFP)–expressing cells was determined for each image. All human blood samples were collected with approval from the University of California Human Subjects Committee, and informed consent was obtained in accordance with the Declaration of Helsinki.

### Confocal microscopy

RBCs were incubated with Siglec-2-Fc-quantum dot (QD) conjugates (30 μg/mL), 1918SC complex (5 μg/mL), biotinylated SNA (bSNA; 0.2 μg/mL), or Siglec-2-Fc (60 μg/mL) in Alsever solution, for 1 hour at 4°C. Incubations with bSNA or Siglec-2-Fc were followed by 30-minute incubation at 4°C with streptavidin conjugated QDs (SA-QDs) or goat anti–mouse-conjugated QDs, respectively. The 1918SC complex was prepared by preincubation of 1918SC hemagglutinin (kind gift from J. Stevens, Centers for Disease Control and Prevention) with biotinylated mouse-penta-His and SA-QDs at 3.6:1.3:1 ratio for 1 hour at 4°C. Control complex was prepared by incubating biotinylated mouse-penta-His with SA-QDs at 1.3:1 ratio. This control complex did not bind to RBCs. Cells were finally fixed with 0.5% paraformaldehyde in Alsevier solution overnight at 4°C. Control cells were also treated with 25 mU of Arthrobacter *ureafaciens* sialidase (AUS) for 1 hour at room temperature before labeling. Samples were plated on 35-mm culture plates with glass bottom, and examined with Olympus FV1000 confocal microscope. QDs were excited with 488 nm to avoid ultraviolet damage to the cells. Kalman filtering was used as each frame was scanned 3 times to compensate for QD blinking. Z-sections were done at 0.15-μM steps. Image analysis and 3D reconstruction were done with Image Pro Plus software (Media Cybernetics) and ImageJ 1.33K software (National Institutes of Health [NIH]).

### Transmission electron microscopy

RBCs were fixed with 0.5% paraformaldehyde in Alsever 30 minutes on ice, washed, and spun onto 12-mm round glass coverslips precoated with poly(D-lysine) in 24-well plate at 4°C. Unbound cells were removed, and the slides were incubated with bSNA (2 μg/mL), Siglec-2-Fc (180 μg/mL), anti-blood group A antigen (1/25 dilution, Z2A; Santa Cruz Biotechnology), anti-blood group B antigen (1/25 dilution, Z5H-2; Santa Cruz Biotechnology), or anti-blood group H antigen (1/5 dilution, 87-N; Santa Cruz Biotechnology) for 1 hour on ice, followed by incubation with secondary antibodies for 30 minutes on ice: SA-QD655 (for bSNA), goat anti–mouse-conjugated QD655 (for Siglec-2-Fc), and goat anti–mouse immunoglobulin IgM-gold 5 nm (EY Labs). For some experiments, cells were incubated with 5-nm gold-conjugated *Lotus tetragonolobus* lectin (1/2 dilution; EY Labs). RBC plasma membranes were transferred to hexagonal, 200 mesh copper grids, formvar/carbon coated, as previously described.[22] Grids were fixed with 2% paraformaldehyde in Alsever for 30 minutes on ice, 1% glutaraldehyde in 0.1 M sodium cacodylate 10 minutes at room temperature, and stained with 0.5% uranyl acetate for 5 minutes at room temperature. Samples were examined with Philips CM10 transmission microscope equipped with Gatan MultiScan digital camera model 794. Images were analyzed using ImageJ 1.33K software (NIH).

### Flow cytometry

RBCs were incubated with Siglec-2-Fc (15 μg/mL) for 1 hour on ice, followed by goat anti-mouse fluorescein isothiocyanate (FITC) for 30 minutes on ice. The cells were washed and incubated with Retic-COUNT reagent (BD Biosciences), according to manufacturer instructions. Control cells were treated with 25 mU AUS 1 hour at room temperature before labeling. To confirm that Retic-COUNT reagent does not disrupt Siglec-2-Fc interaction with the cells, fractions of each sample were also labeled only with Siglec-2-Fc/goat anti–mouse FITC. Fifty thousand events were collected on a FACSCalibur flow cytometer with CellQuest software. Postcollection analysis was done with FlowJo software (TreeStar). The experiment was repeated 3 times with blood from 9 volunteers.

### Analysis of sialic acid-binding properties

Enzyme-linked immunosorbent assay (ELISA)–like binding assays were performed with slight modification, as previously described.[23] The following proteins were incubated for 16 hours at 4°C with 2 μg/mL biotinylated polyacrylamide probes (PAA probes; GlycoTech): Siglec-2-Fc, SNA-FITC, 1918SC complex (1918SC hemagglutinin preincubated with mouse-penta-His–Alexa-647 at 3.6:1.3 ratio), mock complex (phosphate-buffered saline and mouse-penta-His–Alexa-647 at 3.6:1.3 ratio), anti–blood group A antigen (Z2A; Santa Cruz Biotechnology), and anti–blood group B antigen (Z5H-2; Santa Cruz Biotechnology). The PAA probes used were Neu5Acα2-6lactose, Neu5Acα2-3lactose, blood type A, and blood type B (see supplemental Figure 2B for their structure). Microtiter plate was coated with streptavidin, washed, and blocked with Tris-buffered saline (TBS)–Tween 20. The mixtures were transferred to the microtiter plate and incubated for 2 hours at room temperature in TBS–Tween 20 buffer, and then washed extensively with TBS to remove unbound protein. Interaction was determined in a colorimetric assay, by adding secondary antibodies conjugated to alkaline phosphatase (AP), followed by *p*-nitrophenyl phosphate as a substrate: anti–mouse IgG-AP, anti–mouse IgM-AP, and anti–FITC-AP. In some experiments, Siglec-2-Fc and Siglec-2-Fc-QDs were immobilized on the microtiter plate before the biotinylated PAA probes were added. Interaction in this case was determined by adding streptavidin-conjugated AP, followed by *p*-nitrophenyl phosphate. Background was subtracted from the results.

### Other methods

See supplemental Materials and Methods for production of Siglec-2-Fc-QD probes, blood collection, and enzymatic blood group conversion.

## Results

### ABO blood group status affects Siglec-2 binding to human RBCs

Circulating RBCs are constantly exposed to interactions with immune cells, which express many glycan-binding proteins. One such protein is Siglec-2 (CD22), an Ig-superfamily lectin expressed on B cells, which binds specifically to Sias presented in α2-6 linkage to galactose.[16,17,24] To study the interaction of Siglec-2 with human RBCs at a single molecule level, we conjugated QDs to a recombinant soluble chimera of the extracellular domain of human-Siglec-2 with the Fc region of mouse IgG1 (Siglec-2-Fc), at a ratio of approximately 1 QD/2 Siglec-2-Fc molecules. The unique properties of QDs enable each Siglec-2-Fc-QD complex to be individually detected under a confocal microscope.[25] The previously known binding specificity of Siglec-2-Fc-QDs did not

change significantly upon conjugation, maintaining preferential binding to the Siaα2-6Gal versus Siaα2-3Gal in an ELISA (supplemental Figure 2). Purified RBCs from 10 human volunteers were incubated for 1 hour with 30 μg/mL Siglec-2-Fc-QD probe at 4°C, a temperature at which the cell membrane is not fluid, minimizing lateral diffusion of membrane proteins and lipids. To ensure detection of only high-affinity binding sites and to avoid cell agglutination, the probe was used at subagglutinating concentration, which was separately determined in standard assays.[26] Cells were then washed at 4°C, fixed, and examined with a confocal microscope at a single cell resolution (Figure 1A). Siglec-2-Fc-QD–binding density was calculated by dividing the number of QDs by the surface area of each cell at the focal plane, with 50 to 170 randomly selected cells analyzed from each sample. Siglec-2-Fc-QD density was highest on blood type A, medium on blood type O, and lowest on blood type B RBCs ($P < .0001$; Figure 1A-B). Sialidase treatment abolished Siglec-2-Fc-QD binding for all blood type RBCs, confirming Sia-dependent binding (Figure 1A). No major variations in Sia content have been reported between RBCs of ABO blood groups,[27] and we confirmed this by 1,2-diamino-4,5-methylenedioxybenzene dihydrochloride–high performance liquid chromatography analysis of RBC Sias from our samples (supplemental Table 1). Thus, the differential binding of Siglec-2-Fc-QD cannot be attributed to the Sia content. The remarkable difference in Siglec-2-Fc-QD binding to RBCs with highest density on type A and lowest on type B RBCs is particularly surprising, as Siglec-2-Fc does not directly bind A, B, or H antigens (supplemental Figure 3), and these 2 antigens differ by only a single N-acetyl group (supplemental Figure 1A).

To confirm the involvement of ABO blood type antigens in sialic acid binding, we treated blood type A and B RBCs with specific glycosidases: A-Zyme (*Elizabethkingia meningosepticum* α-*N*-acetyl-galactosaminidase) and B-Zyme (*Bacteroides fragilis* α-galactosidase A), respectively. These enzymes were recently shown to effectively convert RBCs from blood groups A and B to O at neutral pH (supplemental Figure 1A),[28] without changing the Sia content on the cell membrane (supplemental Table 1). As control, RBCs were treated with the conversion buffer alone. The cells were then washed and incubated with Siglec-2-Fc, followed by goat anti-mouse–conjugated QDs. The experiment was repeated 3 times with blood from 9 volunteers, and representative results for a single volunteer from each blood group are shown (Figure 1C). Incubation with the required low salt conversion buffer (3 mM NaCl) resulted in a reduction of the overall binding density of all samples compared with Figure 1B, but did not change the differences between blood groups. Siglec-2-Fc–binding density on type A RBCs was decreased by A-zyme treatment ($P \ll .001$), whereas the binding density on type B RBCs was increased upon B-zyme treatment ($P = .003$), resulting in a density similar to that on type O. This confirms that the ABO blood group antigens directly modulate Sia-dependent interaction of Siglec-2-Fc with RBCs.

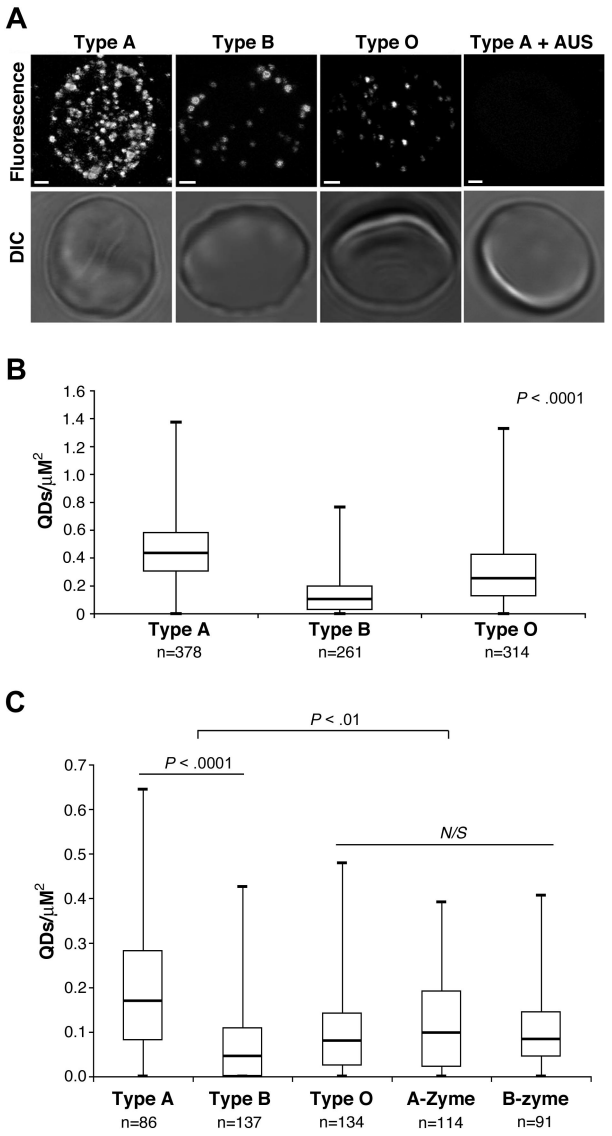


**Figure 1. Blood group status affects Siglec-2-Fc-QDs binding to Sias on RBCs.** (A) Top panel: each Siglec-2-Fc-QD complex appears as a single bright dot on the cell surface. Sialidase treatment (AUS; right panel) abolishes Siglec-2-Fc-QD binding to RBCs; type A RBC is shown as an example. Bottom panel: brightfield image of each cell. Bar represents 1 μM. (B) Box plot of Siglec-2-Fc-QD–binding density on RBCs from 3 type A, 3 type B, and 4 type O volunteers. Binding density was unique to each blood type ($P < .0001$). (C) Representative results for a single volunteer from each blood group are shown. Enzymatic treatment of type A and B RBCs with A-Zyme (α-*N*-acetylgalactosaminidase) and B-Zyme (α-D-galactosidase), respectively, changes Siglec-2-Fc-QD density ($P < .01$) to resemble type O. Statistical significance was determined by an unpaired, 2-tailed *t* test. n = Number of cells in each group; N/S = not significant. Box plot: Box shows the quartile 25 to quartile 75 range, the line represents the median value, and whiskers show the minimum and maximum values. Images were acquired with Olympus FV1000 confocal microscope. Z-sections were done at 0.15-μM steps. Image analysis and 3-dimensional reconstruction were done with ImagePro Plus software (Media Cybernetics) and ImageJ 1.33K software (NIH).

### Siglec-2-Fc–binding density variations within each person do not reflect RBC aging

Similar correlation between Siglec-2-Fc binding and ABO blood group status was found by conventional flow cytometry analysis of RBCs that were incubated with Siglec-2-Fc, followed by secondary antibody (goat anti–mouse FITC). Compared with Siglec-2-Fc-QDs that are tetravalent, this study used bivalent Siglec-2-Fc. Therefore, the concentration of Siglec-2-Fc used in this experiment was higher compared with Siglec-2-Fc-QDs. Regardless, Siglec-2-Fc/FITC binding on type A RBCs was again higher than that measured on type B and type O RBCs (Figure 2A; note the log scale for fluorescence intensity).

Within each person, some of the RBCs had higher density of Siglec-2-Fc-QD binding (quartile 75-max; Figure 1B). As the total Sia content of RBCs decreases during the aging process,[29] we looked for a correlation between RBC age and Siglec-2-Fc-QD binding. Cells were double labeled with Siglec-2-Fc, followed by secondary antibody (goat anti–mouse FITC), and with thiazole orange, which interacts with RNA that is found only in the young

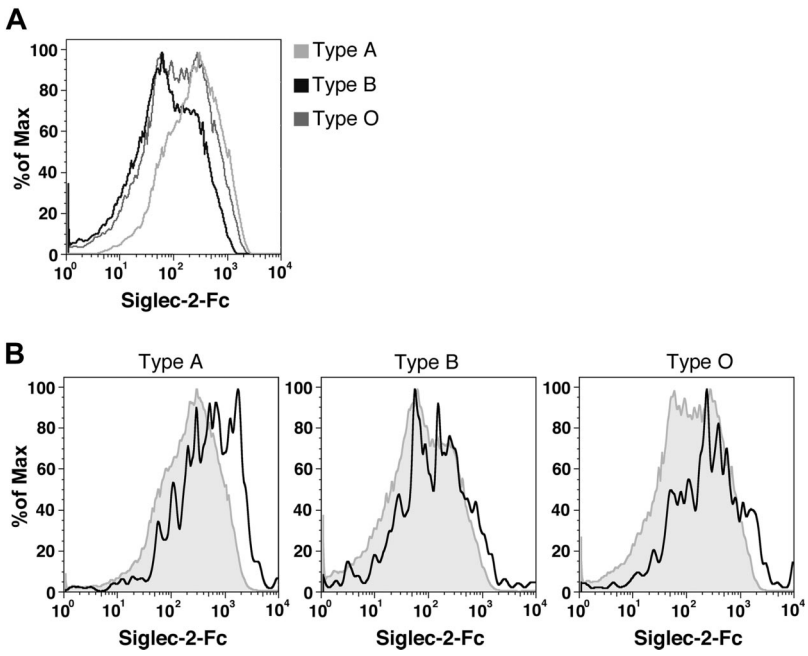


**Figure 2. Differences in Siglec2-Fc binding do not reflect RBC aging.** Human RBCs of A, B, and O blood groups were double labeled with Siglec-2-Fc, followed by secondary antibody (goat anti–mouse FITC), and with thiazole orange, which interacts with RNA that is found only in the young reticulocytes. Cells were analyzed by flow cytometry, and the fluorescence intensity of Siglec-2-Fc labeling is shown. (A) Siglec-2-Fc/FITC binding on type A RBCs (gray) was higher than that measured on type B (black) and type O (dark gray). (B) Thiazole orange–positive cell fraction comprising 1% to 1.5% of total RBCs (reticulocytes, black) was slightly shifted to the right, but the width of the histogram is similar to that of the thiazole orange-negative cells (mature RBCs, gray). Fifty thousand events were collected on a FACSCalibur flow cytometer with CellQuest software. Postcollection analysis was done with FlowJo software (TreeStar). The experiment was repeated 3 times with blood from 9 volunteers.

RBCs (reticulocytes, comprise approximately 1% of total circulating RBCs). Flow cytometry analysis showed that although the reticulocyte fraction was slightly shifted to the right (more strongly positive) in all 3 blood group types, most likely due to their increased Sia content, the histogram width is similar to that of total RBCs (Figure 2B). Because the histogram width reflects the distribution of binding levels within a population of cells, this implies a similar distribution of binding levels in reticulocytes compared with total RBCs. Thus, reticulocytes emerging from the bone marrow have already been imprinted with the range of distinct patterns of Siglec-2 ligands.

### Other Siaα2-6–specific binding proteins show blood group–dependent differences in binding patterns to RBCs

We next studied 2 other Sia-binding proteins believed to have the identical binding specificity as Siglec-2 (for Sias in α2-6 linkage): the pandemic human influenza hemagglutinin (1918-South Carolina)[18] and the plant lectin SNA (elderberry).[19] Despite showing the expected binding similarity when studied by in vitro ELISA-type assays (supplemental Figure 3), they each bound to RBCs in different and unique patterns dependent on the blood group status, distinct from those seen for Siglec-2-Fc-QDs (Figure 3A-C). As before, we performed all studies at subagglutinating concentrations, to avoid cell agglutination and focus only on high-affinity binding sites. Each sample of RBCs was incubated for 1 hour at 4°C with either Siglec-2-Fc-QDs, biotinylated SNA followed by streptavidin-conjugated QDs, or 1918SC hemagglutinin complexed with biotinylated mouse-penta-His and streptavidin-conjugated QDs.[18] Cells were examined using the confocal microscope, and the number of QDs/$\mu m^2$ was determined for 40 to 140 randomly selected cells from each sample. Whereas Siglec-2-Fc and SNA differentially bound to RBCs from each blood type (Figure 3A,C), there was no significant difference in 1918SC hemagglutinin-binding density between the 3 blood groups ($P \gg .1$; Figure 3B). The binding density of both SNA and Siglec-2-Fc was the highest on type A RBCs; however, Siglec-2-Fc–binding density on type B RBCs was dramatically reduced, whereas SNA binding remains high (Figure 3A,C). Interestingly, all 3 proteins bound to blood type O RBCs at a similar density (Figure 3A-C).

Enzymatic treatment of type A and B RBCs with A-Zyme and B-Zyme,[28] respectively, changed the binding pattern of SNA accordingly (Figure 3D). SNA-binding density to A-Zyme–treated type A RBCs was reduced significantly ($P = .002$). The binding density to B-Zyme–treated type B RBCs was also reduced significantly ($P = .0007$), and there was no significant difference between the binding density to the enzymatically treated cells and to type O RBCs ($P = .71$).

Thus, RBCs from different ABO blood group types present the same quantity of Sias on their cell surface, but are distinctly recognized by 3 different α2-6–linked Sia-binding proteins, each with a unique binding preference, affected by the ABH(O) blood type. None of these Sia-binding proteins can directly bind to the ABH antigens (supplemental Figure 3), as they do not contain Sias themselves. However, enzymatic modification of the A and B antigens alters the binding of Sia-recognizing proteins accordingly. These data further indicate that the presence of ABH antigens modulates interactions involving Sias on adjacent glycans, by affecting their presentation on RBC surfaces.

### Siglec-2- and SNA-binding clusters on RBC membranes are modulated by A and B blood group antigens

To understand how ABH antigens modulate the binding to Sias, we first tried to reconstruct Sia/ABH antigen interactions in vitro by mixing different ratios of Neu5Acα2-6lactose and either type A or type B antigens carried on artificial polyacrylamide backbones in an ELISA plate. We then tested the binding of Siglec-2 and SNA to these surface mixtures in an ELISA-like assay. However, we were unable to mimic the ABH effect observed on the cells (data not shown). This suggests that the Sia organization on the cell surface is even more complex, with both specific local concentration and spatial factors being important for binding and recognition. The complexity of the binding structure is also reflected by the differential binding of SNA and Siglec-2 to type B RBCs: SNA binds these cells at much higher density, compared with Siglec-2 (Figure 3A,C).

We thus decided to examine the spatial organization of ABH antigens in relation to Siglec-2 and SNA binding sites on RBCs at a

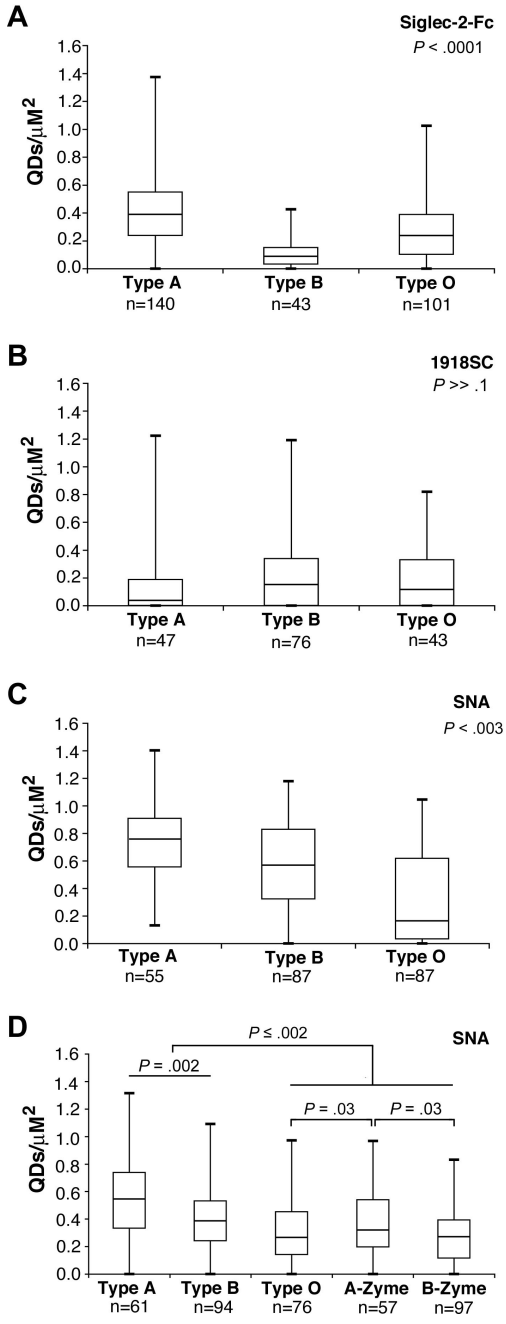


**Figure 3. Differential binding patterns of 3 Siaα2-6–binding proteins to RBCs.** RBCs from 9 human volunteers with blood types A, B, and O (3 from each group) were incubated with subagglutinating concentrations of Siglec-2-Fc-QDs, 1918SC-QD complex, or biotinylated SNA, followed by streptavidin-conjugated QDs. (A,C) Binding density of Siglec-2-Fc and SNA was unique to each blood type ($P < .001$ and $P < .003$, respectively). (B) No significant difference was observed in 1918SC hemagglutinin binding to the 3 blood groups ($P \gg .1$). (D) Enzymatic treatment of type A and B RBCs with A- and B-Zyme, respectively, significantly decreased SNA density ($P < .002$). No significant difference was found between B-Zyme–treated and type O RBCs ($P = .71$). The experiment was repeated 3 times, and representative results for a single volunteer from each blood group are shown. Statistical significance was determined by an unpaired, 2-tailed *t* test. n = number of cells in each group.

single molecule resolution using a transmission electron microscope. Purified RBCs from 8 human volunteers were fixed (0.5% paraformaldehyde on ice for 30 minutes) and then washed and spun down on poly(D-lysine)–coated glass slides at 4°C. The glass slides were washed with buffer to remove unbound cells and incubated for 1 hour on ice with saturating concentrations of either Siglec-2-Fc followed by anti–mouse-IgG–conjugated QD655, or biotinylated-SNA followed by streptavidin-conjugated QD655. In addition, the samples were incubated with antibodies specific for type A, B, or H antigens, followed by anti–mouse-IgM–conjugated gold. The labeled plasma membranes were then transferred to electron microscope grids using a previously described method of sandwiching the cells between a glass slide and electron microscope grid, followed by ripping of the cells.[22] The membranes were further fixed, stained with uranyl acetate to enhance membrane contrast, and examined in a transmission electron microscope (supplemental Figure 4).

Siglec-2-Fc and SNA were found to be localized in distinct clusters on RBC plasma membranes from all 3 blood groups (see QD distribution; Figure 4A). For each blood type, 52 to 108 clusters were analyzed, and the area and center of mass for each cluster were determined using ImageJ 1.33 (NIH) software. Siglec-2 and SNA (QDs; Figure 4A) occupied most of the clusters area, whereas only 2 to 3 anti–blood group antigens were found in each cluster (gold; Figure 4A). The clusters on membranes from blood type A and B RBCs were twice the size of those on type O RBCs, both for Siglec-2-Fc (Figure 4B; $P < .005$) and SNA (Figure 4C; $P < .005$). Enzymatic treatment of type A and B RBCs with A-Zyme and B-Zyme, respectively, reduced the cluster size significantly (Figure 4B-C; $P < .005$). Thus, Siglec-2-Fc and SNA bind to RBCs in distinct clusters whose size depends on ABH status.

The majority of type A and B antigens (gold distribution; Figure 4A) were associated with these clusters both for Siglec-2-Fc (70% ± 0.18, type A; 83% ± 0.18, type B) and for SNA (84% ± 0.10, type A; 78% ± 0.24, type B). The overall binding of anti-H–specific antibody was poorer, and only 27% to 40% of type-H antigens was associated with Siglec-2-Fc and SNA clusters. Type H antigen was also detected with *Lotus tetragonolobus* lectin (Asparagus pea), which is known to specifically bind type H antigen. As with the anti-H antibody, approximately 40% of *Lotus tetragonolobus* lectin gold particles was associated with Siglec-2-Fc and SNA clusters (supplemental Figure 5). To determine whether the blood group antigens are localized in the center of the cluster or at its periphery, the distance of A/B antigens (gold; Figure 4A) from the center was calculated for each cluster. For each cluster, a radius was approximated from the calculated cluster area [r = SQRT(area/2π)]; the circumference determined by that radius includes 50% of the cluster area, leaving out only the periphery. A/B antigens localized within a distance of that radius from the center of mass were considered to be in the cluster center. Blood type A antigens were mostly localized in the periphery of Siglec-2-Fc and SNA clusters, as only 20% to 30% were in the center (filled bars; Figure 4D). Interestingly, whereas the majority of blood type B antigens were localized in the periphery of Siglec-2-Fc clusters, 59 ± 18% were localized in the center in SNA clusters (open bars; Figure 4D). Despite the significantly different distribution of type B antigens, the cluster size of Siglec-2-Fc and SNA on membranes from type B RBCs was similar ($P = .07$; Figure 4, compare B with C).

### Proposed model for the spatial organization of sialic acids and ABH antigens on RBCs

Based on our findings, we propose a model in which Sias and blood group antigens are organized in meaningful clustered "saccharide patches" on RBCs (Figure 5). On blood type A RBCs, the A antigens are localized at the periphery of the cluster, whereas Sias

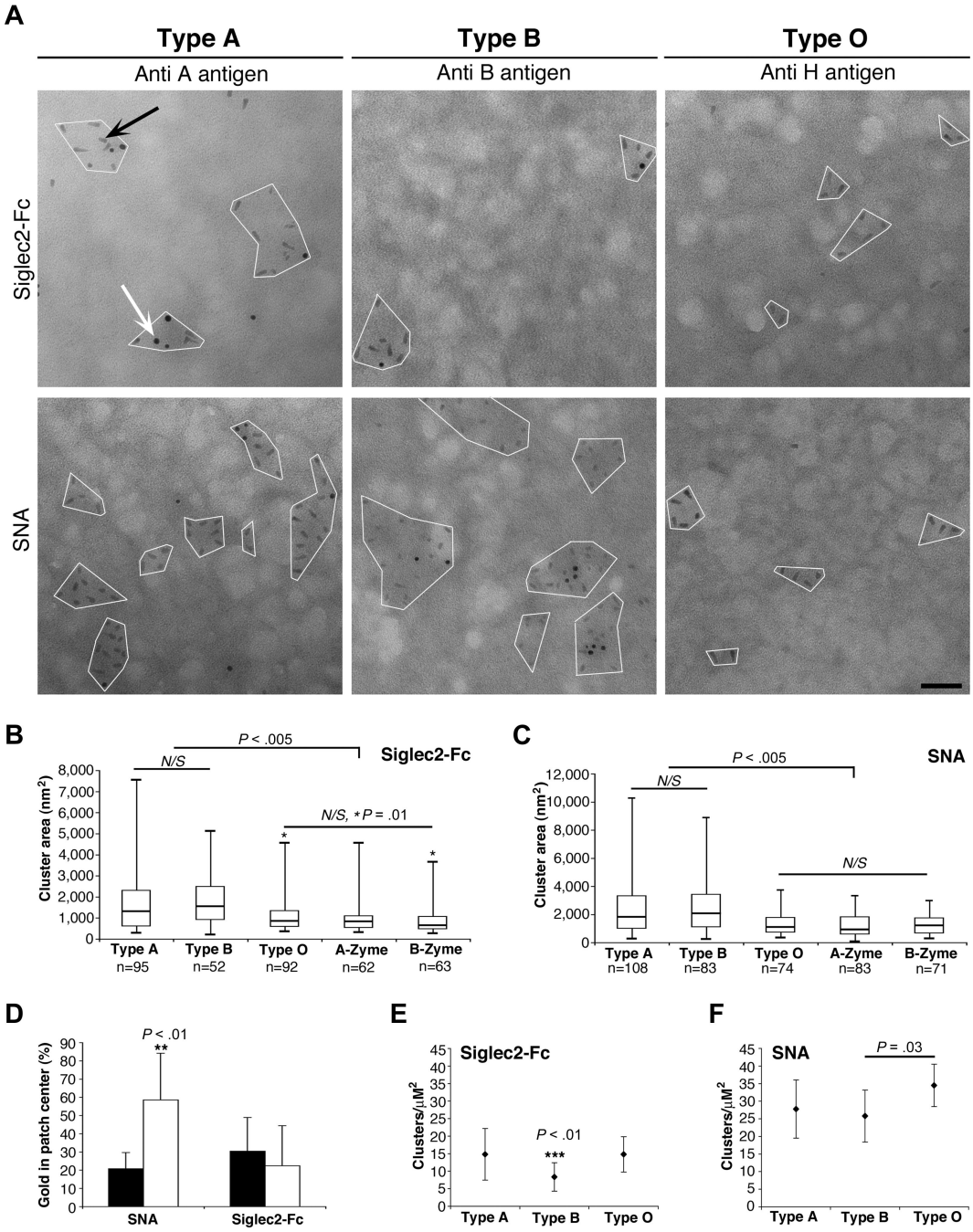


**Figure 4. Siglec-2-Fc and SNA form clusters on RBC membrane.** (A) Electron micrographs of Siglec-2-Fc (top panel) and SNA (bottom panel) on plasma membranes of RBCs. Siglec-2-Fc and SNA were both detected using QD655-conjugated secondary antibody (gray ovals, indicated by black arrow). The membranes were also labeled with A-, B-, and H-specific antibodies, detected with gold 5-nm–conjugated secondary (black dots, indicated by white arrow). Siglec-2-Fc and SNA bound the membranes in defined patches. The area of these patches was calculated using ImageJ 1.33 (NIH) software, by marking a polygon around each patch. The software calculated the area and center of mass for each polygon. The distance between each A and B antigen associated with the cluster center was easily derived. Bar indicates 50 nm. (B-C) Box plot of the area of Siglec-2 and SNA clusters, respectively. Enzymatic treatment of type A and B RBCs with A- and B-Zyme, respectively, reduced Siglec-2 and SNA cluster size significantly ($P < .005$) to resemble type O. (D) The percentage of A antigen (■) and B antigen (□) localized in the patch center. Error bars indicate SD. (E-F) Number of clusters per $\mu M^2$ was calculated for Siglec-2-Fc and SNA, respectively. The number of Siglec-2-Fc clusters on blood type B membranes was reduced by 58% compared with type A ($P = .01$) and 43% compared with type O ($P = .002$). Average ± SD is shown. Statistical significance was determined by an unpaired, 2-tailed *t* test. N/S = not significant. Images were acquired with Philips CM10 transmission microscope.

form a continuous cluster that yields a local increase in the Sia concentration. On blood type B RBCs, the B antigen is localized in the center of the Sia cluster in approximately 60% of the clusters. On blood type O RBCs, the Sias clusters are relatively small and may not include the H antigen (Figure 5). Indeed, on blood type A RBCs, both Siglec-2-Fc and SNA have increased number of high-affinity binding sites compared with B and O (Figures 1 and 3, A,C-D). On blood type B RBCs, the B antigen is localized in the center of the Sia cluster in approximately 60% of the clusters, which may pose a steric interference problem for the Sia-binding proteins. SNA is known to bind Sias in α2-6 linkage at very high affinity.[19] When using subagglutinating concentration of SNA, the density of SNA binding to blood type B RBCs is reduced compared with blood type A ($P = .002$; Figure 3C-D), but still remains high

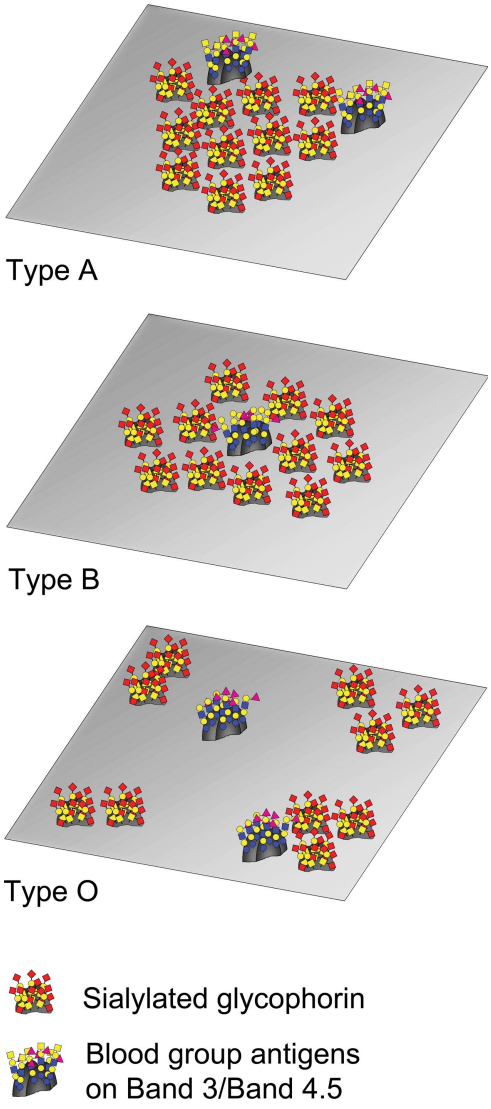


**Figure 5. Model for clustered sialic acid patches on RBCs of different blood groups.** Sialylated glycans and ABH blood group antigens are organized in clusters on RBC membranes. These clusters are stabilized by carbohydrate-carbohydrate interactions with the type A and B terminal saccharides. (Top) On blood type A RBCs, the A antigens are localized at the periphery of the cluster, whereas Sias form a continuous cluster that yields a local increase in the Sia concentration. (Middle) On blood type B RBCs, the B antigen is localized in the center of the Sia cluster in approximately 60% of the clusters. (Bottom) On blood type O RBCs, the Sias clusters are relatively small and may not include the H antigen.

compared with Siglec-2-Fc and 1918SC (Figure 3A-C). Siglec-2-Fc binding to blood type B RBCs is very low compared with type A and O RBCs (Figure 3A), suggesting that the B antigen localized in the cluster interferes with Siglec-2 binding. Interestingly, Siglec-2-Fc cluster size is similar between blood type A and B plasma membranes (Figure 4B). However, the number of Siglec-2-Fc clusters on blood type B membranes was reduced by 58% compared with type A ($P = .01$; Figure 4E) and 43% compared with type O ($P = .002$; Figure 4E). As B antigen was found to be in the center of approximately 60% of the SNA clusters, this may suggest that Siglec-2-Fc binds primarily to the clusters in which B antigen is at the periphery (Figure 4A,D). On blood type O RBCs, the Sias clusters are relatively small and may not include the H antigen (Figure 5). As the clusters are small, there is no significant increase in the local density of Sia, explaining why all 3 Sia-binding proteins bound blood type O RBCs at the same density (Figure 3A-C). In contrast, the pandemic human influenza hemagglutinin, 1918SC, has relatively weak binding affinity for Sias,[18] and thus bound RBCs from all 3 blood types at the same density (Figure 3B). We are of course aware that a minor fraction of ABH antigens is found on glycophorin A. However, they are not likely to account for the differential binding to sialic acids, due to their very low stoichiometry (only 1% of O-glycans on glycophorin A carries ABH antigens[10]).

Our model explains how Sia-binding proteins can interact differentially with RBCs from blood types A, B, and O without binding directly to the ABH antigens. Sialic acids are involved in a variety of pathologic interactions;[12] thus, Sia-mediated pathogen interaction with RBCs may underlay the maintenance of ABO blood group polymorphisms in the human population, that is, the selection pressure on ABO blood group does not act in isolation from other glycans on RBCs.

### Sialic acid-binding protein of *P falciparum* malarial parasite preferentially interacts with type O RBCs

The *P falciparum* malarial parasite has been suggested as an evolutionary force for the ABO blood group polymorphism, as ABO status was correlated with the severity of the disease.[30] Once infected, the RBCs present *P falciparum* erythrocyte membrane protein-1, which is encoded by the parasite genome and presented on the RBCs membrane. *P falciparum* erythrocyte membrane protein-1 binds to blood type A and B antigens on other cells to promote rosetting, but not to blood type O(H) antigen.[30,31] However, there is no evidence for direct interactions of the parasite with the ABH antigens on RBC surfaces during the primary process of infection. Duffy binding-like molecules are one of the main protein families involved in the invasion of *P falciparum* merozoite into RBCs.[32,33] The best studied Duffy binding-like–binding protein is pfEBA-175, which binds to RBCs in a Sia-dependent manner,[21,33,34] by recognizing α2-3–linked Sias on O-linked tri- and tetrasaccharides on the extracellular domain of glycophorin A, the major surface sialoglycoprotein of RBCs. The binding site for pfEBA-175 consists of both glycans and the amino-terminal sequence of glycophorin A.[20] Our model predicts that the highest interaction would be with blood type O RBCs, because the Sias-carrying glycophorins are not aggregated in large clusters, and thus, more readily accessible for pfEBA-175 binding (Figure 5).

To test this prediction, COS7 cells were cotransfected with a membrane-anchored form of pfEBA-175 along with GFP, and allowed to interact for a short time (7 minutes) with RBCs from 15 human volunteers (5 individuals from each blood type). The cells were then washed extensively and imaged using a fluorescence microscope. The rate of transfection was controlled for by monitoring the number of GFP-expressing cells in each sample. The number of RBC rosettes per GFP-labeled cell was calculated in 20 randomly selected fields of each sample[21] (Figure 6). RBCs from blood type O individuals formed 1.6 times more rosettes compared with type A and B RBCs ($n = 6$, $P < .0001$; Figure 6). Enzymatic treatment of type A and B RBCs with A-Zyme and B-Zyme, respectively, resulted in a significant increase in the number of rosettes ($P < .0001$ and $P = .029$ for A and B, respectively) to be the same level as with type O RBCs. Thus, in agreement with our model, pfEBA-175 preferably binds to blood type O RBCs. Blood type O RBCs may be more susceptible for initial binding by the *P falciparum* merozoite; however, postinfection, the type H antigen becomes advantageous, with less severe disease.[30,31]

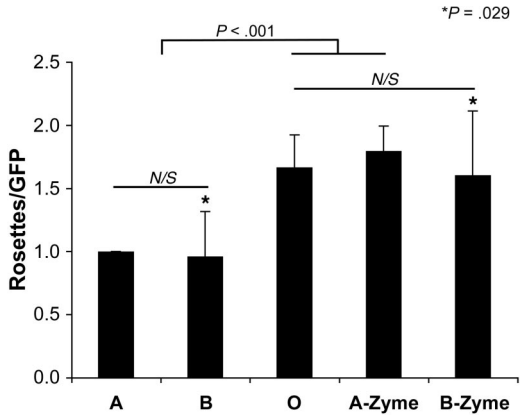


**Figure 6. *P falciparum* EBA-175 preferably binds type O RBCs.** RBCs were allowed to interact with COS7 cells expressing pfEBA-175 along with GFP.[21] Unbound cells were washed, and the percentage of rosettes per GFP-expressing cell was determined for each sample; results were normalized such that rosettes/GFP for type A RBCs equal to 1. Whereas type A and B RBCs formed the same number of rosettes/GFP-positive cell, type O RBCs formed 1.65× more rosettes/GFP (n = 6, $P < .001$). A-Zyme–treated and B-Zyme–treated RBCs formed the same number of rosettes/GFP-positive cell as type O RBCs (n = 3, $P = .4$). Statistical significance was determined by an unpaired, 2-tailed *t* test. Error bars indicate SD.

## Discussion

We show in this study that nonsialylated ABH antigens modulate Sia-specific interactions of human lectin (Siglec-2), plant lectin (SNA), and *P falciparum* erythrocyte-binding molecule (pfEBA-175) with Sias on RBCs. Thus, by stabilizing other glycans on the cell surface in clusters, ABH antigens affect cellular interactions without being a direct ligand themselves. On a more general note, these data demonstrate how one set of glycans (ABH antigens) can modulate specific interactions with another set of glycans (α2-3–linked Sias and α2-6–linked Sias), without themselves being a ligand. We propose a model for formation and modulation of Sias clusters on plasma membranes of blood type A and B RBCs. Enzymatic removal of *N*-acetyl-D-galactosamine from A antigen or galactose from B antigen results in dispersion of the Sia clusters (Figure 4B-C). This indicates that the clusters are stabilized by carbohydrate-carbohydrate interactions with the type A and B terminal saccharides. On blood type O RBCs, the clusters are relatively small and may not include the type H antigen. Alternatively, type H antigen, which is shorter compared with type A and B by a whole monosaccharide (supplemental Figure 1A),[3,4] stabilizes smaller clusters (Figure 4A-C).

The sialoglycoprotein glycophorin A (GPA) and the anion transport protein band 3 (which carries the ABH antigens) are the 2 most abundant proteins in human RBC membranes and have been extensively studied. However, despite more than 40 years of research, the question of band 3 and GPA interactions remains equivocal. There is some evidence that band 3 and GPA may interact,[35-37] but there is also evidence to the contrary.[38] Regardless, in all studies, the proposed GPA-band 3 complex is not stable enough to survive coimmunoprecipitation.[36,37] Such an interaction is also not detectable in our hands by fluorescence resonance energy transfer experiments (data not shown). Taken together, this suggests that if GPA and band 3 form interactions, they are very weak. This supports our finding that it is the glycans on GPA and band 3 that may form carbohydrate-carbohydrate interactions.

Carbohydrate-carbohydrate interactions between certain neutral and acid glycans have been previously documented in the context of cell-cell recognition.[39-41] These interactions occurred in *trans* between 2 cells, or between cells and immobilized glycans. In *cis* carbohydrate-carbohydrate interactions between glycosphingolipids and N-linked glycans were suggested to promote signal transduction.[42] It has also been shown that mixtures of 2 gangliosides can generate novel glycan binding sites in vitro, apparently via glycolipid-glycolipid *cis* interactions that can enhance or attenuate binding.[43,44] In this study, we present novel evidence for the possibility that in *cis* cell surface carbohydrate-carbohydrate interactions can actually alter the presentation of the glycans themselves, significantly modifying recognition by binding proteins. The concept of "clustered saccharide patches"[15] as a way to regulate binding to cells with otherwise similar saccharide content is intriguing. Sialic acids are involved in many physiologic and pathologic interactions;[12] by stabilizing unique sialylated clusters, other glycans, such as ABH antigens, can indirectly affect many of these processes. As cellular interactions mediated by one type of glycans may be subjected to modulation by other set of glycans, such effects could easily be missed when individual glycans are studied in isolation.

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## Authorship

Contribution: M.C. designed and performed research, analyzed data, and wrote the paper; N.H.-Z. performed research; and A.V. designed research, analyzed data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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