Glycosylation Affects Ligand Binding and Function of the Activating Natural Killer Cell Receptor 2B4 (CD244) Protein

Stefanie Margraf-Schönfeld, Carolin Böhm, and Carsten Watzl
From the Institute for Immunology, University Heidelberg, 69120 Heidelberg, Germany

2B4 (CD244) is an important activating receptor for the regulation of natural killer (NK) cell responses. Here we show that 2B4 is heavily and differentially glycosylated in primary human NK cells and NK cell lines. The differential glycosylation could be attributed to sialic acid residues on N- and O-linked carbohydrates. Using a recombinant fusion protein of the extracellular domain of 2B4, we demonstrate that N-linked glycosylation of 2B4 is essential for the binding to its ligand CD48. In contrast, sialylation of 2B4 has a negative impact on ligand binding, as the interaction between 2B4 and CD48 is increased after the removal of sialic acids. This was confirmed in a functional assay system, where the desialylation of NK cells or the inhibition of O-linked glycosylation resulted in increased 2B4-mediated lysis of CD48-expressing tumor target cells. These data demonstrate that glycosylation has an important impact on 2B4-mediated NK cell function and suggest that regulated changes in glycosylation during NK cell development and activation might be involved in the regulation of NK cell responses.

Natural killer cells are the first lymphoid subpopulation in the defense against tumors and viral infection. Their activity is regulated by the interplay between inhibitory receptors, most of which recognize MHC class I expression on target cells, and activating receptors, which are engaged by various ligands (1). Important inhibitory receptors are members of the killer cell immunoglobulin-like receptor family as well as CD94/NKG2 heterodimers (2). Examples for activating receptors are NKG2D (CD314), DNAM-1 (CD226), and the well characterized natural cytotoxicity receptors (NCRs) Nkp30 (CD337), Nkp44 (CD336), and Nkp46 (CD335) (2, 3). These receptors transduce their activation signal via small adaptor proteins like CD3zeta or DAP10 (4). The human activating NK cell receptor 2B4 (CD244) belongs to the SLAM-related receptor (5) and is a 365-amino acid type 1 transmembrane protein with a calculated molecular weight of 39 kDa and a pI of 9.16 (6). It has a large intracellular domain containing four immunoreceptor tyrosine-based switch motifs that recruit adaptor molecules like SAP (SH2D1A or DSHP) or EAT2 after phosphorylation (7–9). The extracellular part of 2B4 consists of an N-terminal V-set Ig domain, a membrane-proximal C2-set Ig domain, and several N-glycosylation sites (6). The ligand of 2B4 is CD48, a glycosyl-phosphatidylinositol-anchored molecule with broad expression in the hematopoietic system (10, 11). Engagement of 2B4 by its ligand results in the recruitment and clustering of the receptor into lipid rafts and phosphorylation of its immunoreceptor tyrosine-based switch motif domains (12, 13). This initiates a signaling cascade, leading to polarization and the release of cytolytic granules into the contact zone between the NK and target cell (14, 15). Although 2B4 stimulation alone is sufficient to activate IL-2 stimulated NK cells, it also serves as an important coactivatory signal for the stimulation of resting NK cells (16–18). 2B4-mediated activation of NK cells can be blocked by engagement of inhibitory receptors such as CD94/NKG2A or KIR2DL1 (12, 13, 19).

Glycosylation plays an important role in receptor-ligand recognition. It can act as a biochemical signal recognized by lectins but also have structural influence in receptor-ligand recognition because of its bulky shape caused by branched side chains (20). Carbohydrates are linked to proteins by N- and O-glycosidic bonds. The negatively charged sialic acids are the outermost monosaccharides on the glycosidic chains and offer a recognition site for specific lectins (e.g. sialic acid binding immunoglobulin-like lectin [Siglec]) or pathogens. Siglec-7 and Siglec-9 are known inhibitory receptors on NK cells (21). Sialic acids also play an important role in naïve T cells, where they can impair the recognition of MHC class I molecules (22, 23).

Here we show that the NK cell receptor 2B4 is modified by N- and O-glycosylation and that it is highly sialylated. Our data demonstrate that N-linked carbohydrates are essential for the 2B4/CD48 interaction. In contrast, sialylation has a negative impact on the ligand binding of 2B4 and on 2B4-mediated NK cell cytotoxicity.

EXPERIMENTAL PROCEDURES

Cells—Primary NK cells were isolated and cultured as described (7). Cell lines used in this study were NK92CI, NKL (NK cell lines), HEK 293T (HEK) and HeLa. HeLa and HEK cells were stably transfected with CD48 (HEK-CD48, HeLa-CD48) or empty vector as a control.

Antibodies, Enzymes, and Inhibitors—The following antibodies were used: anti-2B4 (C1.7, Beckman Coulter, Germany), IgG1 control (MOPC-21, Sigma), biotinylated anti-phosphotyrosine 4G10 (Upstate Cell Signaling Solutions, VA), anti-CD48...
Glycosylation Affects 2B4 Function

(MEM-102), goat anti-rabbit HRP-conjugated (both Santa Cruz Biotechnology, Germany), goat anti-mouse IgG HRP-conjugated, goat anti-mouse IgG PE-conjugated, and streptavidin HRP-conjugated (all Jackson ImmunoResearch, PA). The anti-isoleucine zipper mAb (ILZ-11) as well as rabbit anti-2B4 antibodies have been described previously (13, 24).

The enzymes used were PNGaseF (peptide-N-glycosidase F, Sigma), Arthrobacter ureafaciens α2–3,6,8,9-neuraminidase (Merck-Calbiochem) and O-glycosidase (endo-α-α-N-acetyl galactosaminidase, New England Biolabs, Franklin, Germany). BADG (benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside, dissolved in dimethyl sulfoxide) and castanospermine (Castanospermum australe, dissolved in water) (both Merck-Calbiochem) were used as inhibitors for O-glycosylation and N-glycosylation, respectively.

Protein Phosphorylation—Protein phosphorylation was induced by using the phosphatase inhibitor pervanadate. Cells were resuspended at $2 \times 10^7$ cells/ml in PBS containing 200 μM Na-orthovanadate activated by 0.3% H2O2 and incubated for 10 min at 37 °C.

Immunoprecipitation—Lysates of up to $2 \times 10^7$ cells were prepared as described previously (7) and incubated for 1 h with 20 μl of Dynabeads Pan Mouse IgG (Invitrogen) coated with 1.6 μg of isotype mAb (MOPC21) for preclarification and subsequently with specific antibody (Cl1.7) to isolate 2B4. Beads were washed in lysis buffer, and proteins were eluted with 20 μl of ASB-14 lysis buffer (7 μM urea, 2 μM thiourea, 1 M Tris-base, 4% (w/v) SDS plus 0.5% (w/v) DTT and then in equilibration buffer with 4.5% (w/v) iodacetamid instead of DTT. Proteins were separated on 4–12% Bis-Tris ZOOM gels (Invitrogen) and transferred to a PVDF membrane (Millipore) and developed as described above. PVDF membranes were blocked using Carbo-Free™ blocking solution (Vector Laboratories, CA). After overnight incubation with appropriate lectins at 5 μg/ml (biotinylated lectin Kit I and II, Vector Laboratories), membranes were washed and subjected to streptavidin conjugated with HRP (Jackson ImmunoResearch Laboratories).

Enzymatic Deglycosylation and Dephosphorylation of Proteins—Immunoprecipitated proteins were treated enzymatically directly on the magnetic beads. For dephosphorylation, 8 × 10^6 beads were resuspended in 20 μl of 1× NEB3 buffer and incubated for 1 h at 37 °C with 2 μl of CIP (New England Biolabs). The same amount of beads were resuspended in 20 μl of 50 mM sodium phosphate buffer (pH 7.4) with 1 μl of one of the following deglycosylation enzymes: PNGaseF for de-N-glycosylation (overnight at 37 °C), neuraminidase plus O-glycosidase for de-O-glycosylation (2 h at 37 °C), and neuraminidase plus O-glycosidase for de-O-glycosylation (2 h of 37 °C).

Fusion Proteins—2B4-ILZ fusion proteins were produced and purified as described previously (24). Prior to protein elution from the agarose beads, samples were divided and left untreated (overnight incubation at 4 °C or at 37 °C) or enzymatically deglycosylated. For desialylation, beads were incubated for 2 h at 37 °C with 17 μl of neuraminidase in PBS. Additional de-O-glycosylation was achieved in a second approach using 10 μl of O-glycosidase together with the aforementioned amount of neuraminidase. For ablation of N-glycosidic bond carbohydrates, the same amount of beads were incubated overnight at 37 °C with 17 μl of PNGaseF in 50 mM phosphate buffer G7 reaction buffer. After treatment, beads were washed twice and proteins were eluted as described in Ref. 24. Concentration was quantified using the EZQ protein quantitation kit (Invitrogen). Purity was controlled via SDS-PAGE and silver staining and successful deglycosylation via 2D gel electrophoresis and Western blot analysis, respectively. For 2D gel electrophoresis, 100 ng of 2B4-ILZ fusion proteins were supplemented with ASB-14 lysis buffer up to 20 μl and processed as described above.

Site-directed Mutagenesis—For 2B4 point mutants of the predicted N-glycosylation sites, appropriate asparagines were replaced by glutamines by site-directed mutagenesis. PCR products were restricted and religated into the 2B4-ILZ plasmid. The following primers were used (point mutations are underlined): N71Q: forward, GTGGGAGCAGGGCTCTTTG and reverse, GACTCA ACTGCTTGACTATAAAACT. N89Q: forward, GTCA N77Q: forward, GTCA ACTGCTTGACTATAAAACT. N89Q: forward, GTCA N89Q: forward, GTCA ACTGCTTGACTATAAAACT. N89Q: forward, GTCA ACTGCTTGACTATAAAACT. N89Q: forward, GTCA ACTGCTTGACTATAAAACT.

Deglycosylation of NK Cells—Desialylation of cells was done using 1 μl of neuraminidase for $1 \times 10^6$ cells/100 μl of PBS, rotating for 2 h at 37 °C with 5% CO2. To inhibit elongation of O-glycosyl chains, cells were incubated with 2.5 mM BADG for 48 h at 37 °C with 5% CO2. N-glycosylation was inhibited by blocking glycosidase I and II using castanospermine (CS). Cells were incubated with 10 μg/ml CS for 24 h at 37 °C with 5% CO2. Cells were washed once with CTL medium (Iscove's modified Dulbecco's medium with 10% FCS and penicillin/streptomycin) and used for cytotoxicity assays as described (7).

RESULTS

To study the glycosylation of 2B4, we immunoprecipitated the receptor from different NK cells and stable 2B4-transfected HEK-293T (HEK-2B4) cells and analyzed it via 2D gel electrophoresis and Western blotting. The 2B4 receptor migrated at an apparent molecular weight of about 70 kDa in all cells analyzed and showed distinct spots over a broad pH range (Fig. 1A). The spot pattern between 2B4 molecules from primary NK cells, the NK cell line NKl, and HEK-2B4 cells were compara-
ble, whereas the NK cell line NK92C1 expressed a more alkaline receptor. To test whether these differences in pH could be due to background phosphorylation of the four cytoplasmic immunoreceptor tyrosine-based switch motif domains, immunoprecipitated 2B4 proteins were dephosphorylated using Calf Intestinal Phosphatase (CIP). Although this treatment led to a complete dephosphorylation of 2B4, as evident by anti-phosphotyrosine blotting (supplemental Fig. 1, A and B), it only resulted in a marginal shift of the whole spot pattern toward a more basic pH (Fig. 1B). In contrast, inhibition of phosphatases using pervanadate led to an overall shift toward acidic pH and a significant phosphorylation of immunoprecipitated 2B4, as proven by phosphotyrosine staining (supplemental Fig. 1C). This indicates that background phosphorylation is not the reason for the diverse spot pattern of the 2B4 receptor.

Human 2B4 contains eight possible N-glycosylation sites, and the calculated molecular weight of the core peptide is about 39 kDa. To test whether 2B4 is N-glycosylated, the immunoprecipitated receptor was dephosphorylated using CIP or additionally de-N-glycosylated using PNGaseF. Removal of N-glycans reduced the mass of 2B4 to the predicted molecular mass of 39 kDa (Fig. 1C). Interestingly, the spot patterns between NK92C1 and the other NK cell lines was comparable after PNGaseF treatment (Fig. 1C). This suggests that N-linked carbohydrates on 2B4 differ between NK92C1 and the other NK cells analyzed. It further shows that N-linked sugars account for the 30-kDa difference between calculated and apparent molecular weight of the 2B4 receptor. However, the 2B4 receptor still migrated as distinct spots covering a broad pH range after the removal of N-glycans. This suggested the presence of additional sugar moieties that are highly charged and of small size.

Sialic acids are highly negatively charged and therefore shift the pH of proteins toward acidic pH with only minor change in molecular mass. To test for the presence of sialic acids on 2B4, we desialylated immunoprecipitated 2B4 using neuraminidase. Desialylation shifted the pH of immunoprecipitated 2B4 to its calculated pI of 9.61 (Fig. 2, A and B). Additional removal of O-linked glycans did not change the pI further (Fig. 2C). Instead, we sometimes observed a slight increase in molecular weight of the 2B4 receptor under these conditions. These data show that the distinct spot pattern of 2B4 is caused by sialic acid residues on N- and O-linked sugars.

Some of the N-glycosylation sites within 2B4 are in close proximity to the binding site of its ligand CD48. According to the structure of murine 2B4 (25), the N-linked carbohydrates may lie outside of the binding interface. However, they could...
still be involved in ligand binding because of their large size and high negative charge. Additionally, O-linked carbohydrates, for which no consensus motifs are known, could have an impact on the function of the 2B4 receptor. To test how these carbohydrates could influence the binding affinity of 2B4 to its ligand CD48, we expressed the extracellular Ig-like domains of 2B4 linked to an isoleucine ILZ motif as a recombinant soluble fusion protein in HEK cells (24). These 2B4-ILZ fusion proteins showed a glycosylation pattern similar to the 2B4 receptor found in stably transfected HEK cells and primary human NK cells (Fig. 3A, top panel). Also, the difference between the apparent molecular weight of 2B4-ILZ of about 55 kDa and the calculated molecular mass of 27 kDa can be explained by the addition of about 30 kDa because of glycosylation of the 2B4 fusion protein. Desialylation as well as de-O-glycosylation of the fusion protein shifted the pl to a more alkaline pH, and removal of N-linked carbohydrates resulted in a mass shift toward the calculated molecular weight (Fig. 3A). This demonstrates that the glycosylation of the 2B4-ILZ fusion protein is comparable with the 2B4 receptor expressed on NK cells.

We next asked how the removal of the different sugars from the 2B4-ILZ fusion protein would affect the binding to its ligand CD48. For this we used the fusion protein to stain HEK and HeLa cells stably transfected with CD48. Removal of N-linked carbohydrates by PNGase treatment severely compromised the specific binding of 2B4-ILZ to HEK-CD48 and HeLa-CD48 cells (Fig. 3, B and C). This suggests that N-linked sugars on 2B4 are essential for its binding to CD48. To further corroborate this, we substituted asparagines (N) in the three predicted N-linked glycosylation sites of the N-terminal V-set Ig domain of the 2B4-ILZ fusion protein by glutamines (Q) using site-directed mutagenesis (Fig. 4A). Two sites (N71Q and N77Q) were in close proximity to the binding site of CD48 according to the murine structure (25), with the third mutation (N89Q) lying outside of this interaction site. The mutated 2B4-ILZ fusion proteins were used in binding studies against CD48 expressing HeLa and HEK cells. Interestingly, all mutated proteins showed diminished binding capacity compared with the WT 2B4-ILZ (Fig. 4B). The fusion proteins with mutations close to the CD48 binding interface (N71Q and N77Q) displayed a somewhat
reduced binding, whereas removing N-glycans at position 89 had a more pronounced effect on binding to CD48. These data support our findings that N-glycosidic-linked carbohydrates are important for 2B4-mediated ligand recognition.

When we removed sialic acid residues or additionally O-linked sugars, we observed a somewhat increased binding of 2B4-ILZ to HeLa-CD48 cells (Fig. 3C). This increase was consistently seen for many different CD48-expressing cells (Fig. 3D) and suggests that sialic acid residues on 2B4 may have a negative impact on its binding to CD48.

To test whether 2B4 glycosylation also has an impact on 2B4-mediated NK cell activation, we used mock- or CD48-transfected HeLa cells as targets for different NK cells. The presence of CD48 clearly enhanced the lysis of HeLa cells by primary human NK cells or the NK cell lines NKL and NK92C1, demonstrating the specific NK cell activation through 2B4 (Fig. 5). We then removed sialic acids from the surface of NK cells by neuraminidase treatment. The analysis of the immunoprecipitated 2B4 receptor from the treated cells by 2D gel electrophoresis followed by Western blotting showed a clear shift toward basic pH, demonstrating the effectiveness of the treatment (supplemental Fig. 2A). Neuraminidase-treated NK cells showed enhanced killing against CD48-expressing target cells (Fig. 5A). Although the extent of the enhanced lysis varied between experiments, we consistently observed better 2B4-mediated killing by neuraminidase-treated NK cells.
To interfere with O-glycosylation, we cultured the NK cells with the inhibitor BADG. BADG competes with endogenous GalNac-O-Ser/Thr for galactosyl transferase and therefore prevents the elongation of O-glycosyl chains. Analysis of 2B4 from the treated cells showed a loss of negative charge as well as a slight decrease in molecular mass because of loss of O-glycosidic-bound carbohydrates (supplemental Fig. 2A). Also, the BADG-treated cells showed enhanced killing activity against CD48-expressing target cells (Fig. 5B). These data demonstrate that 2B4-specific NK cell activation can be enhanced by removal of sialic acid from the surface of NK cells or the inhibition of O-linked glycosylation and correlates nicely with our observation that the removal of these sugars can enhance the ligand binding activity of 2B4.

Because ablation of N-glycans or mutation of predicted N-linked glycosylation sites of 2B4-ILZ fusion proteins interfered with the binding to CD48-expressing cells, we wanted to test if de-N-glycosylated NK cells were impaired in CD48-mediated target cell lysis. PNGase failed to de-N-glycosylate whole NK cells, and the inhibitor of the GlcNAc phosphotransferase, tunicamycin, showed toxic side effects. We therefore cultured expanded human NK cells and analyzed via SDS-PAGE and Western blotting using the lectin PSA (Fig. 7A, top panel) and anti-2B4 antibodies as a loading control. This suggests that N-linked sugars are important for 2B4-mediated NK cell activation.

It is well known that glycans can play a major role in cell-cell communication (26). However, only little is known about the glycosylation of lymphocytes, their changes during cellular activation, and how this might influence the reactivity of these cells. We therefore wanted to examine the glycosylation of NK cells and how this might change upon cytokine-driven expansion and activation of these cells. We compared the lysate of freshly isolated or IL-2 expanded NK cells from the same donor by Western blotting using different lectins. Three of these lectins (Pisum sativum agglutinin (PSA), Lens culinaris agglutinin (LCA), and Datura stramonium lectin (DSL)) showed clear alterations in the carbohydrate spectrum of NK cells after activation (Fig. 7A). To directly investigate possible changes in 2B4 glycosylation, we compared immunoprecipitated 2B4 from freshly isolated and IL-2 expanded NK cells via 2D gel electrophoresis and Western blotting. We detected a clear difference in the migration of 2B4 before and after IL-2 expansion with the 2B4 receptor from freshly isolated NK cells migrating to a more acidic position (Fig. 7B). We also analyzed immunoprecipitated 2B4 from freshly isolated and IL-2 expanded NK cells by Western blotting using different lectins. Although LCA and DSL did not recognize 2B4, PSA was able to stain 2B4 (Fig. 7C). Interestingly, the PSA staining of immunoprecipitated 2B4 was more intense in the sample from IL-2 expanded NK cells, again suggesting changes in 2B4 glycosylation upon NK cell activation.
**Glycosylation Affects 2B4 Function**

**DISCUSSION**

Glycosylation is an important posttranslational modification of membrane proteins and crucial for proper cell function. Our data show that the activating human NK cell receptor 2B4 is expressed as a highly glycosylated protein. We found that the majority of the sugars are attached to 2B4 via N-linked glycosylation, adding about 30 kDa to its molecular mass. Additionally, 2B4 is also O-glycosylated and contains sialic acid residues on both N- and O-linked sugars, which are responsible for shifting the pI of 2B4 to a more acidic pH. 2B4 contains eight possible N-glycosylation sites. However, Zielińska et al. (27) have shown that N-glycosylation is not restricted to the N-!P-(S/T) sequence motif (where !P is any amino acid except proline) or the uncommon N-X-C motif but can also be found on yet unknown positions. Furthermore, only 30% of all available motifs within a protein are glycosylated, with some of them showing only incomplete glycosylation (28). This results in a heterogeneous population of differentially glycosylated proteins as we have found for the 2B4 receptor. The differential glycosylation of 2B4 could be attributed to sialic acid residues on both N- and O-linked sugars, as removal of sialic acids created a population of 2B4 molecules migrating at one distinct pI. Considering the bioinformatic tools of the CBS prediction service databank, the sequence of 2B4 does not reveal any O-glycosylation sites. However, there is no clear consensus sequence for O-glycosylation sites. As we still observed distinct spots after removal of N-glycosylation, we suggest that 2B4 is also modified by O-linked sugars. The enzymatic removal of O-glycosidic-bound carbohydrates requires first a removal of sialic acids, making a specific analysis of O-linked sugars difficult. We observed an increase in the molecular weight of 2B4 after removal of O-glycosidic-bound carbohydrates compared with the removal of sialic acids alone. This rather surprising result might be explained by peptide dimerization because of diminished repulsion after the removal of O-linked sugars.

The structural information available today about the interaction between 2B4 and CD48 did not implicate glycosylation to be important for this binding. Our data demonstrate that N-glycosylation of 2B4 is essential for this interaction, as removal of these sugars from the 2B4 fusion protein almost completely prevented its binding to CD48-expressing cells. Removal of N-linked sugars can affect the three-dimensional structure of proteins. Indeed, we saw that de-N-glycosylation of 2B4-ILZ resulted in reduced recognition of the fusion protein by the conformation-specific anti-2B4 mAb C1.7. This might indicate that N-linked sugars are part of the epitope that is recognized by C1.7 or that N-linked sugars are necessary for the stabilization of this epitope. Additionally, removal of N-linked sugars may also cause protein aggregation. However, we did not observe such aggregates in our Western blot analysis (Figs. 1C and 3A), and the recognition of the treated fusion protein by a polyclonal anti-2B4 antibody was only slightly impaired. Therefore, the reduced binding of the de-N-glycosylated 2B4-ILZ fusion protein to CD48 can only partially be explained by aggregation and is more likely because of an involvement of N-linked carbohydrates in the binding to CD48.

This was further corroborated by our functional analysis of CS-treated NK cell lines and the mutational analysis of 2B4. All three mutants influenced the binding to CD48-expressing cells negatively. Among these, N-linked glycans in close proximity to the CD48 binding interface (Asn-71 and Asn-77) displayed a more intermediate effect in contrast to the carbohydrates nearby the C2-set Ig domain (Asn-89). This rather surprising result can be explained in several ways: Firstly, carbohydrates linked to Asn-89 might be important to stabilize the two Ig-like domains into a defined, rigid structure. Therefore, point mutation of Asn-89 might result in a fusion protein with more flexible domains that is hampered in its binding to CD48. Secondly, glycans linked to Asn-89 could be large enough to directly contribute to the interaction with CD48. Finally, because the structure of human 2B4 has not yet been solved, small alterations in the tertiary structure might bring the Asn-89 glycosylation site closer to the CD48 binding site in comparison to the murine structure. Further mutations and combinations of mutations will be necessary to analyze this in more detail.

Interestingly, the glycosylation pattern of 2B4 from NK92C1 cells differed from that of other NK cells or 2B4-transfected HEK cells. This difference in glycosylation could be attributed to N-linked sugars, suggesting that it could also influence the binding of 2B4 to CD48.

It has been shown that binding by the 2B4 related receptor CD2 to its ligand CD58 is independent of glycosylation (29). CD2 contains only one potential N-glycosylation site in its membrane-distal IgV domain, which lies on the opposite site of the contact area with CD58 (29). Contrary to this, 2B4 contains three N-glycosylation sites in its membrane-distal IgV domain, two of which are in close proximity to the putative CD48 binding site (25). On the basis of the CD2-CD58 interaction, Mathew et al. (30) showed that Lys-68 and Glu-70 of the human 2B4 IgV domain were necessary for ligand binding. However, in our hands, these mutations were not sufficient to abolish ligand binding. Only a 2B4 triple mutant, K54A/H65A/T110A, based on the structure of the murine 2B4-CD48 complex (25), completely abolished binding to CD48. These results show that the human 2B4/CD48 interaction is similar to the one defined for mouse 2B4/CD48.

Our data also show that sialylation of 2B4 affects its binding to CD48. Interestingly, sialic acids seem to hinder this interaction, as we observed increased binding of 2B4 to CD48 after neuraminidase treatment. The negative charge introduced by the addition of sialic acids may lead to some repulsion within the 2B4-CD48 interaction, which would explain the positive effect on binding upon removal of sialic acids. This effect was not only seen in the binding of the recombinant 2B4 fusion protein to CD48-expressing cells but could also be confirmed in NK cells, as neuraminidase treatment of NK cells resulted in enhanced 2B4-mediated lysis of CD48-expressing target cells. Our data show that 2B4 is sialylated on N- and O-linked sugars. This would explain why the inhibition of O-linked glycosylation in NK cells by BADG also enhanced the lysis of CD48-expressing cells and suggest that sialic acids on O-linked sugars are involved in the repulsion during the 2B4-CD48 interactions.

---

3 M. Claus and C. Watzl, unpublished results.
However, BADG seemed to have a greater impact on 2B4-mediated NK cell activation compared with neuraminidase treatment. This could suggest that O-linked sugars also directly interfere with the 2B4–CD48 interactions. Removal of sialic acids by neuraminidase or blocking O-linked glycosylation by BADG treatment of NK cells does not only affect the glycosylation of 2B4 but affects many different surface molecules and can therefore have general effects on NK cell reactivity (31). This explains why the treated NK cells already show some difference in lysis of mock-transfected target cells. However, the lysis of CD48-transfected targets was affected to a much larger extent, arguing for a specific effect on 2B4-mediated NK cell activation.

Glycans are involved in many cellular processes. The binding of CD8 to MHC-I is enhanced upon removal of sialic acids (32), and desialylation increases CD8+ T cell sensitivity to antigen-presenting cell stimulation (33). Interestingly, in contrast to naïve T cells, activated T cells show a reduction in sialylation (34). Both lectins bind core-fucosylated, mono- and biantennary N-glycans but not their tri- and tetra-antennary forms. However, a recent study suggests that they differ in their binding affinity to branched N-glycans (35). LCA binds better to biantennary N-glycans, whereas PSA exhibits a higher affinity to trimannosyl structures. Interestingly, sialylation seems to decreases the binding affinity of both lectins (35). Hence, PSA but not LCA staining of 2B4 indicates different core glycosylation, whereas the stronger PSA staining after NK cell activation would be compatible with a reduction in sialylation, as already suggested by the 2D gel electrophoresis. As sialylation of 2B4 has a negative impact on CD48 recognition, a reduced sialylation would result in enhanced 2B4-mediated NK cell activation. Indeed, we have shown previously that IL-2-stimulated NK cells show better 2B4-mediated NK cell cytotoxicity (36). However, IL-2 stimulation has various effects on NK cells, including the up-regulation of SAP (36), an adapter molecule important for 2B4-mediated NK cell activation. Therefore, although the reduced sialylation of 2B4 might be involved in the better reactivity of the receptor, it is certainly not the only reason for the enhanced 2B4-mediated activation of IL-2-stimulated NK cells.

Acknowledgment—We thank Birgitta Messmer for help in isolation and culturing primary human NK cells.

REFERENCES
1. Lanier, L. L. (2008) Nat. Immunol. 9, 495–502
2. Moretta, L., and Moretta, A. (2004) EMBO J. 23, 255–259
3. Lanier, L. L. (2005) Annu. Rev. Immunol. 23, 225–274
4. Moretta, A., Bottino, C., Vitale, M., Pende, D., Cantoni, C., Mingari, M. C., Biassoni, R., and Moretta, L. (2001) Annu. Rev. Immunol. 19, 197–223
5. Claus, M., Meinke, S., Bhat, R., and Watzl, C. (2008) Front Biosci. 13, 956–965
6. Tangye, S. G., Lazetic, S., Woollatt, E., Sutherland, G. R., Lanier, L. L., and Phillips, J. H. (1999) J. Immunol. 162, 6981–6985
7. Eissmann, P., Beauchamp, L., Wooters, J., Tilton, J. C., Long, E. O., and Watzl, C. (2005) Blood 105, 4722–4729
8. Veillette, A. (2006) Nat. Rev. Immunol. 6, 56–66
9. Latour, S., and Veillette, A. (2004) Semin. Immunol. 16, 409–419
10. Brown, M. H., Boles, K., van der Merwe, P. A., Kumar, V., Mathew, P. A., and Barclay, A. N. (1998) J. Exp. Med. 188, 2083–2090
11. Latchman, Y., McKay, P. F., and Reiser, H. (1998) J. Immunol. 161, 5809–5812
12. Watzl, C., and Long, E. O. (2003) J. Exp. Med. 197, 77–85
13. Watzl, C., Stebbins, C. C., and Long, E. O. (2000) J. Immunol. 165, 3545–3548
14. Bryceson, Y. T., March, M. E., Barber, D. F., Ljunggren, H. G., and Long, E. O. (2005) J. Exp. Med. 202, 1001–1012
15. Chen, X., Trivedi, P. P., Ge, B., Krzewski, K., and Strominger, J. L. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 6329–6334
16. Assarsson, E., Kambayashi, T., Persson, C. M., Chambers, B. J., and Ljunggren, H. G. (2005) J. Immunol. 175, 2045–2049
17. Sivori, S., Parolini, S., Falco, M., Marcenaro, E., Biassoni, R., Bottino, C., Moretta, L., and Moretta, A. (2000) Eur. J. Immunol. 30, 787–793
18. Bryceson, Y. T., March, M. E., Ljunggren, H. G., and Long, E. O. (2006) Blood 107, 159–166
19. Tangye, S. G., Cherwinski, H., Lanier, L. L., and Phillips, J. H. (2000) Mol. Immunol. 37, 493–501
20. André, S., Kozár, T., Koijima, S., Unverzagt, C., and Gabius, H. J. (2009) Biol. Chem. 390, 557–565
21. Yamaji, T., Mitsuki, M., Teranishi, T., and Hashimoto, Y. (2005) Glycobiology 15, 667–676
22. Baum, L. G., Derbin, K., Perillo, N. L., Wu, T., Pang, M., and Uittenbogaart, C. (1996) J. Biol. Chem. 271, 10793–10799
23. Casabó, L. G., Mamalaki, C., Kioussis, D., and Zamoyska, R. (1994) J. Immunol. 152, 397–404
24. Stark, S., Flag, R. M., Sandusky, M., and Watzl, C. (2005) J. Immunol. Methods 296, 149–158
25. Velikovsky, C. A., Deng, L., Chlewicki, L. K., Fernández, M. M., Kumar, V., and Mariuzza, R. A. (2007) J. Biol. Chem. 282, 897–907
26. Ohtsubo, K., and Marth, J. D. (2006) Cell 126, 855–867
27. Zielinska, D. F., Gnad, F., Wiśniewski, J. R., and Mann, M. (2010) Cell 141, 897–907
28. Mellquist, J. L., Kasturi, L., Spitalnik, S. L., and Shakin-Eshleman, S. H. (1998) Biochemistry 37, 6833–6837
29. Davis, S. J., Davies, E. A., Barclay, A. N., Daenke, S., Bodian, D. L., Jones, E. Y., Stuart, D. I., Butters, T. D., Dwek, R. A., and van der Merwe, P. A. (1995) J. Biol. Chem. 270, 369–375
30. Mathew, S. O., Kumaresan, P. R., Lee, J. K., Huynh, V. T., and Mathew, P. A. (2005) J. Immunol. 175, 1005–1013
31. Benson, V., Grobarova, V., Richter, J., and Fiserova, A. (2010) Int. Immunol. 22, 167–177
32. Daniels, M. A., Devine, L., Miller, J. D., Moser, J. M., Lukacher, A. E., Altman, J. D., Kavathas, P., Hogquist, K. A., and Jameson, S. C. (2001) Immunity 15, 1051–1061
33. Crespo, H. J., Cabral, M. G., Teixeira, A. V., Lau, J. T., Trindade, H., and Videira, P. A. (2009) Immunology 128, e621–631
34. Kornfeld, K., Reitman, M. L., and Kornfeld, R. (1981) J. Biol. Chem. 256, 56–66
35. Tateno, H., Nakamura-Tsuruta, S., and Hiranayashi, I. (2009) Glycobiology 19, 527–536
36. Endt, J., Eissmann, P., Hoffmann, S. C., Meinke, S., Giese, T., and Watzl, C. (2007) Eur. J. Immunol. 37, 193–198