Glycosylation regulates NK cell-mediated effector function through PI3K pathway

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Abstract
Aberrant glycosylation, which impairs recognition capability of NK cells or modifies recognition pattern of target cells, is associated with cancer. Synthetic glycoconjugates (GCs), which modulate cell glycosylation, increase the sensitivity of tumor cells to therapy or boost anti-cancer immune response. In the current study, we employed N-acetyl-D-glucosamine-calix[4]arene (GN4C) as a modulator of cell glycosylation of NK cells represented by the NK-92 cell line and fresh human NK cells. For the first time, we have demonstrated that calix[4]arene-based GC down-regulated the expression of glycosyltransferases MGAT3 and MGAT5 in NK-92 and fresh NK cells. GN4C increased the susceptibility of tumor cells to cytotoxicity by purified fresh NK cells or NK-92 cells. This functional activation of NK cells and the NK-92 cell line correlated with an increased expression of NKG2D mRNA. For the first time, we have demonstrated that calix[4]arene-based GC down-regulated the expression of glycosyltransferases MGAT3 and MGAT5 in NK-92 and fresh NK cells. GN4C increased the susceptibility of tumor cells to cytotoxicity by purified fresh NK cells or NK-92 cells. This functional activation of NK cells and the NK-92 cell line correlated with an increased expression of NKG2D mRNA. In the NK-92 cell line, GN4C reduced the rate of proliferation and down-regulated the c-MYC, EGF-receptor 1 and REL-A molecules. In conclusion, the modulation of glycosyltransferases MGAT3 and MGAT5 by synthetic GN4C correlated with the improvement of NK cell effector functions and the augmentation of tumor cells sensitivity to NK cell-mediated cytotoxicity.

Keywords: cytotoxic activity, gene regulation, glycoconjugate, glycosyltransferases, NK cells, NK-92

Introduction
A particular glycan profile on the cell surface is crucial for proper cell function, and aberrant glycosylation is associated with diseases like cancer (1). Changes in glycosylation arise from alterations in the expression level of glycosyltransferases.

N-acetyl-D-glucosamine (GlcNAc) transferases MGAT3 and MGAT5 have major involvement in linking terminating residues on glycans. MGAT5 is responsible for adding β1-6 GlcNAc residues and forming branched structures, which are especially abundant in cancer tissues with high metastatic potential. MGAT3 catalyzes the addition of β1-4 GlcNAc residues and forms a bisecting structure that disables further addition of GlcNAc by other glycosyltransferases like MGAT5. Competition of MGAT3 and MGAT5 in vivo leads to the suppression of cancer metastasis (2). Nevertheless, both enzymes, MGAT3 and MGAT5 tend to be over-expressed in tumor cells (3, 4).

NK cells recognize particular carbohydrate structure as cytolytic signals (5), triggered by inhibitory and activation receptors on the cell surface regulating NK cell-mediated cytotoxicity and production of chemokines and inflammatory cytokines (6).

NKG2D is a functional activation receptor of NK-92 cells, which plays a role in NK cell-mediated tumor recognition and cytosis (7). Stimulation of NKG2D recruits two critical signaling components, the regulatory subunit p85 of PI3-kinase/ERK but not phospholipase C-γ as well. Cellular signaling triggered by GN4C engaged PI3-kinase/ERK and not phospholipase C-γ as well. Cross linking of NK cell receptors like NKG2D results in activation of kinases ERK and JNK. Activation of ERK and JNK triggers the microtubule-organizing center and granule polarization in NKG2D-mediated NK cell cytotoxicity (9). NKR-P1A (CD161) is a representative of human inhibitory receptors. Signaling through this receptor and its function remains unclear. Oligosaccharides such as N-acetyl-D-glucosamine serve as ligands for recombinant NK cell receptor NKR-P1A in rats (10).
Glyco-gene profiling array

The NK-92 cell line was incubated with GN4C for 24 h and total RNA was isolated with RNeasy Mini Kit involving DNAse I treatment as described by the manufacturer (Qiagen, Hilden, Germany). A custom-designed Glyco-gene Chip array (GLYCOv3 Gene Chip) that contains probe sets to monitor the expression of ~2000 human transcripts relevant to the Consortium for Functional Glycomics was developed using Affymetrix technology. The microarray experiments (triplicates of each sample) were performed by The Microarray Core of Consortium for Functional Glycomics, NIH/NIGMS (http://www.functionalglycomics.org). BRB ArrayTools [Biometric Research Branch, NIH/National Cancer Institute (NCI), Bethesda, MD, USA] were used to filter and analyze experimental data sets. Class comparison used a two-sample t-test with a random variance model, and differences with P-value of log-ratio <0.05 were considered significant. Gene Ontology analysis was performed using DAVID software available from NCI (http://david.abcc.ncifcrf.gov).

Reverse transcriptase–PCR and real-time reverse transcriptase–PCR

Total RNA from the NK-92 cell line or fresh NK cells incubated with GN4C for 24 h was used for semi-quantitative expression analysis by real-time reverse transcriptase (RT)–PCR as well. Five micrograms of RNA were transcribed into cDNA using cDNA Archive Kit (Applied Biosystem, Foster City, CA, USA). RT–PCR for detection of cytokine mRNA, epidermal growth factor receptor 1 (EGFR1) and Ki-67 was carried out with HotStarTag DNA Polymerase (Qiagen) and an iCycler5 (Bio-Rad, Philadelphia, PA, USA). Real-time RT–PCR was performed with PowerSybr® Green Master Mix (Applied Biosystem) and an iCycler5. PCR product specificity was checked by melt curve analysis. The primers designed by us with the Primer3 Input software were as follows: MGAT5: F (forward)—5′-CTCTTCTTCACGACCTCAAC-3′ and R (reverse)—5′-ACAGCTGGGAGATGAGTGA-3′; NKG2D: F—5′-CACAGCTGGGAGATGAGTGA-3′ and R—5′-CTACAGCATGAAACAGCAGCAG-3′; NKR-P1A: F—5′-TTTCTCTGGGAAGATGAGTGA-3′ and R—5′-CCTGCTCTGTGATGTGACA-3′.

3H-thymidine proliferation assay

The NK-92 cell line (1.25 × 10⁵, 2.5 × 10⁵, 5 × 10⁵ 200 µl⁻¹ per well) and fresh human NK cells (2.5 × 10⁴–200 µl⁻¹ per well) were plated in triplicates on 96-well plates and cultured in presence of GN4C (10 nM) or IL-2 (100 U ml⁻¹) for 1–3 days. Cells cultured at the same density in the absence of IL-2 or GN4C were used as controls. At the end of the time period, 18.5 kBq per well of 3H-thymidine (GE Healthcare, Amersham, UK) was added to each well and samples were subsequently harvested after 8 h and analyzed as described earlier (11).

Cytotoxicity assay

NK cell-mediated cytolysis was performed as described previously (18). The cell lines and fresh NK cells were incubated with or without GN4C (1 dose, 10 nM) for 30 min. In experiments with a PI3K inhibitor, NK-92 cells and fresh NK cells were incubated with wortmannin (50 nM, Calbiochem, Hilden, Germany). A custom-designed Glyco-gene Chip array (GLYCOv3 Gene Chip) that contains probe sets to monitor the expression of ~2000 human transcripts relevant to the Consortium for Functional Glycomics was developed using Affymetrix technology. The microarray experiments (triplicates of each sample) were performed by The Microarray Core of Consortium for Functional Glycomics, NIH/NIGMS (http://www.functionalglycomics.org). BRB ArrayTools [Biometric Research Branch, NIH/National Cancer Institute (NCI), Bethesda, MD, USA] were used to filter and analyze experimental data sets. Class comparison used a two-sample t-test with a random variance model, and differences with P-value of log-ratio <0.05 were considered significant. Gene Ontology analysis was performed using DAVID software available from NCI (http://david.abcc.ncifcrf.gov).

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Darmstadt, Germany) for 30 min and subsequently with or without GN4C. The NK-92 cell line and fresh NK cells with or without GN4C pre-treatment were used as effectors. HT-29 and K562 cell lines with or without GN4C pre-treatment were used as targets for evaluation of cell-mediated cytotoxicity. The effector:target ratios were optimized for the experiment at 3.5:1 for NK-92 cells and 10:1 for NK cells. Release of $^{51}$Cr was measured after 18 h of incubation.

**Cytokine detection by flow cytometry**

For intracellular detection of cytokines, the NK-92 cell line, PBMC and purified NK cells were incubated with GN4C for 30 min, fixed and permeabilized with BD Cytofix/Cytoperm™ according to the manufacturer’s protocol (BD Bioscience). TNF-α and IFN-γ were detected with anti-human TNF-α-APC (Mab11, eBioscience, San Diego, CA, USA) and IFN-γ-APC monoclonal antibodies (Caltag, Buckingham, UK), respectively. The isotype controls IgG1 (BD Bioscience) and IgG2b (BD Bioscience) were used to determine the non-specific fluorescence of TNF-α and IFN-γ, respectively.

To confirm the results and broaden the panel of tested cytokines, we used BD™ Cytometric Bead Array Human $^{1}$/1$^{2}$ (BD Bioscience). This array consisted of six cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ), which were detected in cell supernatants as well as in cell lysates prepared as described previously (19). The production of cytokines was measured with a flow cytometry BD LSRII and analyzed with FlowJo 7.2.2 software.

**Detection of protein phosphorylation pattern**

To evaluate tyrosine phosphorylation, the NK-92 cell line was incubated with GN4C for 10 min and immediately placed on ice. Cell lysates were prepared as described previously (19). The protein lysates were subjected to SDS-PAGE and blotted on PVDF membranes. Membranes were incubated with specific primary antibodies, which were detected with anti-mouse, anti-rabbit and anti-goat (Jackson ImmunoResearch, West Grove, PA, USA) secondary antibodies and SuperSignal West Pico Chemiluminescent Substrate Kit (Thermo Scientific, Rockford, IL, USA). Blots were subjected to densitometric analysis as described above. 12-O-tetradecanoyl phorbol-13-acetate (TPA, Sigma-Aldrich, Taufkirchen, Germany) served as a positive inducer of MAPK phosphorylation. In experiments with PI3K inhibitor, NK-92 cells and fresh NK cells were incubated with wortmannin (50 nM) for 30 min and subsequently with or without GN4C.

**Statistical analysis**

Statistically significant differences in the parameters tested in NK-92 cells or fresh NK cells cultured in the presence or absence of GN4C were assessed using the non-parametric Mann-Whitney test with a confidence interval of 95%. Values of $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), were considered to be statistically significant.

**Results**

**mRNA expression changes in NK-92 cells involved genes related to cancer progression**

Particular surface glycosylation is essential for NK cell function. We employed transformed cell line NK-92 to evaluate the effect of GN4C on glycosylation-related genes in NK cells. To determine gene expression changes caused by GN4C, we undertook a complex approach analyzing the glyco-gene expression profiling. To identify genes differentially expressed in NK-92 cells, in the presence or absence of GN4C, we used high-density oligonucleotide microarray. We excluded (with BRB Array Gene Filter) all genes in which the percentage of absent data exceeded 50% and $P$-value was $>0.05$. Class comparison revealed 24 genes displaying significantly (2-fold change, $P$-value $< 0.05$) different expressions between control and GN4C-treated cells. The GN4C-responsive genes were functionally categorized according to Gene Ontology classification and then mapped to biological pathways with the use of the KEGG and BioCarta Pathways. The listed genes encoded glycoproteins with N-linked glycosylation site (GlcNac) (46% of filtered genes), growth factors (17%) and mitogens (13%). According to the functional classification, GN4C influenced transfer of hexosyl/glycosyl groups (38% of filtered genes), signal transduction (38%), carbohydrate binding (25%), regulation of cell proliferation (25%) and organ development (25%). Cellular response to GN4C involved glycan biosynthesis, MAPK, JAK-STAT and transforming growth factor (TGF) signaling pathways. Considering disease relationship, 77% of the genes listed were linked to cancer (Gene Card database). A complete list of those differentially expressed genes related to cancer is shown in Table 1.

GN4C GC effected mRNA expression of glycoproteins and enzymes involved in their processing the most. Along with other genes, GN4C-mediated down-regulation of glycosyltransferases MGAT3 and MGAT4B, JUND, TGF-β1 and vascular endothelial growth factor B (VEGF-B). These genes play an important role in cancer progression, and inhibition of their expression is of particular interest. Up-regulated genes included galactosaminyltransferase GaINt4, Siglec-8 and Sialomucin. Groups of glycosyltransferases and positive regulators of proliferation were further examined by real-time RT-PCR.
Glycosyltransferases and cell growth regulators displayed decrease of gene expression

For deeper examination of GN4C effect on glycosylation machinery, we examined mRNA expression of glycosyltransferases, involved in terminal glycan elongation. Housekeeping gene for \( B2M \) exhibited stable expression in our experimental system and was further used as control gene for data normalization. In fresh NK cells as well as in the NK-92 cell line, GN4C down-regulated the expression of both glycosyltransferases \( MGAT3 \) \((P = 0.0001 \text{ and } 0.0316)\) and \( MGAT5 \) \((P = 0.0001 \text{ and } 0.0467)\) (Fig. 1). Down-regulation of \( MGAT3 \) expression in NK-92 cells detected by real-time RT-PCR agreed with the equal result of \( MGAT3 \) expression obtained by glyco-array described above.

The NK-92 is a transformed tumor cell line; thus, we examined if GN4C affected proliferation factors \( c-MYC \), \( Ki-67 \) or \( EGFR1 \) in comparison to fresh NK cells derived from healthy donors. NK-92 cells incubated with GC reduced the expression of positive regulators of cell proliferation, transcription factor \( c-MYC \) \((P = 0.001)\), proliferation antigen \( Ki-67 \) \((P = 0.0032)\) and \( EGFR1 \) \((P = 0.0063)\) (Fig. 2B). Fresh NK cells incubated with GN4C showed decreased mRNA level of \( c-MYC \) \((P = 0.05)\) (Fig. 2A) but did not express \( EGFR1 \) or \( Ki-67 \) genes (Fig. 2C). The decrease of \( c-MYC \) in fresh NK cells was less significant than in the NK-92 cell line. Together, these results demonstrate that GN4C down-modulated the expression of glycosyltransferases in the NK-92 cell line as well as in fresh NK cells and decreased the expression of cell growth regulators \( EGFR1 \), \( Ki-67 \) and \( c-MYC \) primarily in the tumor NK-92 cell line.

The proliferation rate in both fresh NK and NK-92 cells after adding GN4C was checked by \(^3\)H-thymidine incorporation assay. We used recombinant human IL-2 as a positive control of fresh NK cells proliferation. The incubation of fresh NK cells with GN4C exhibited equal increase of proliferation rate as cells incubated with IL-2 but the effect of increased proliferation lasted longer in GN4C-treated cells (Fig. 2D). The NK-92 cell line exhibited comparable rate of proliferation in untreated and GN4C-treated cells for 24 h and then GN4C slightly inhibited further proliferation of the cell line. NK-92 cells incubated in the presence of IL-2 displayed increase in proliferation (Fig. 2E).

### Table 1. Output list of GN4C-responsive genes related to cancer in the NK-92 cell line (determined by online databases Gene Ontology and Gene Cards)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>GI</th>
<th>Biological function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up-regulated genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD164</td>
<td>Sialomucin</td>
<td>34222157</td>
<td>GP, ST, RCP, OD, I</td>
</tr>
<tr>
<td>Down-regulated genes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CAPN1</td>
<td>Calpain 1</td>
<td>12408655</td>
<td>RCP, OD, apoptosis</td>
</tr>
<tr>
<td>CHRD</td>
<td>Chordin</td>
<td>11494372</td>
<td>GP, GF, CB</td>
</tr>
<tr>
<td>EPOR</td>
<td>Erythropoietin receptor</td>
<td>182200</td>
<td>GP, ST</td>
</tr>
<tr>
<td>FLT3LG</td>
<td>fms-related tyrosine kinase 3 ligand</td>
<td>494978</td>
<td>GP, ST, RCP, OD, I</td>
</tr>
<tr>
<td>FLNA</td>
<td>Filamin A</td>
<td>160420313</td>
<td>ST, actin filament binding</td>
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<td>JUND</td>
<td>Jun D proto-oncogene</td>
<td>5177963</td>
<td>TF</td>
</tr>
<tr>
<td>MDK</td>
<td>Midkine</td>
<td>182650</td>
<td>M, T, ST, CB, RCP, OD</td>
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<td>MGAT3</td>
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<td>T</td>
</tr>
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<td>MGAT4B</td>
<td>Beta-1,4-N-acetyl-glucosaminyltransferase</td>
<td>11282</td>
<td>T</td>
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<td>MST1</td>
<td>Macrophase stimulating 1</td>
<td>31543211</td>
<td>GP, GF</td>
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<tr>
<td>TGFB1</td>
<td>Transforming growth factor beta 1</td>
<td>12652748</td>
<td>GP, GF, M, ST, TF, RCP, OD, I</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>Vascular endothelial growth factor B</td>
<td>39725673</td>
<td>GP, GF, M, ST, T, CB, RCP, neovascularization</td>
</tr>
</tbody>
</table>

CB, carbohydrate binding; GF, growth factor; GI, database accession number; GP, glycoprotein; I, immune process; M, mitogen; OD, organ development; RCP, regulation of cell proliferation; ST, signal transduction; T, transferase; TF, transcription factor.
NK-92 and NK cells exhibited up-regulation of NKG2D
NKG2D and NKR-P1A genes encode activation and inhibitory NK cell receptors, respectively, and are involved in a functional regulation of NK cells. Glyco-gene array did not determined mRNA changes in these genes as significant; nevertheless, we involved them in our analysis by real-time RT–PCR that operates with higher sensitivity than high-throughput arrays. GN4C notably increased mRNA expression of C-type-lectin-like receptor NKG2D in the NK-92 cell line (\( P = 0.0069 \)) as well as in fresh NK cells (\( P = 0.009 \)). In fresh NK cells, GN4C repressed NKR-P1A expression (\( P = 0.0075 \)) (Fig. 3). NK-92 cells did not express lectin receptor NKR-P1A either in control or in GN4C-treated cells.

GN4C increased cytotoxic activity of NK cells and susceptibility of tumor cells to NK-mediated cytolysis
Cell-mediated cytotoxicity is a primary effector function of NK cells. We previously found (11) that PBMC incubated with GN4C increased cell-mediated cytotoxicity against K562 cells. To determine if NK cell population was responsible for that cell-mediated cytotoxicity, we investigated effect of GN4C on killing potential of freshly purified NK cells and

**Fig. 2.** Expression of cell growth regulators c-MYC, EGFR1 and proliferation antigen Ki-67 in fresh NK cells (A and C) and the NK-92 cell line (B and C) after incubation with GN4C. Level of specific mRNA was detected by real-time RT-PCR and normalized to the expression of control gene B2M. Quantification of gene expression was performed by Bio-Rad IQ5 2.0 software and results are presented as an average ± SD of triplicates. The HT-29 cell line (C) served as control of gene expression. Presence of GN4C is indicated by plus and absence by minus under the representative gel electrophoresis (C). Proliferation of fresh NK cells (D) and the NK-92 cell line (E) after incubation with GN4C measured by incorporation of \(^{3}H\)-thymidine for 3 days. NTC indicates non-treated control cells, GN4C indicates cells treated with 10 nM GN4C and IL-2 indicates cells treated with 100 U of recombinant IL-2.
the NK-92 cell line against sensitive K562 and resistant HT-29 tumor cells. Fresh NK cells incubated with GN4C displayed increased cytolytic activity against K562 (P = 0.0001) as well as against resistant HT-29 (P = 0.0001) target cells (Fig. 4A). We found that GN4C augmented the cytotoxicity of the NK-92 cell line against K562 (P = 0.005) and HT-29 (P = 0.0045) cells too (Fig. 4B). These results showed that GN4C promoted NK-92 and NK cell-mediated lysis against tumors of both myeloid and adenocarcinoma origin.

Further, we investigated the effect of GN4C on tumor cells, particularly their susceptibility to NK cell-mediated cytotoxicity. We used fresh NK cells as effectors against K562 and HT-29 tumor cells pre-incubated with GN4C. Both GN4C-treated tumor cell lines were more susceptible to fresh NK cell-mediated cytolysis (P = 0.0001 and 0.0383) (Fig. 5A). The same experimental design we applied to NK-92 cells as effectors against HT-29 and K562 target cells incubated with GN4C. In the case of the HT-29 cell line incubated with GN4C, we detected significant increase in cytotoxicity (P = 0.0001), nevertheless in K562 cells, the effect of GN4C was opposite (p = 0.0001) (Fig. 5B).

Pre-treatment with wortmannin effectively suppressed GN4C-mediated increase of cell-mediated cytotoxicity in both NK-92 cells (P = 0.0001 and 0.005) and fresh NK cells (P = 0.03 and 0.0005) (Fig. 5B and C). Wortmannin itself significantly reduced the basal cytotoxicity of fresh NK cells against K562 target cells (P = 0.024) and cytotoxicity of NK-92 cells against HT-29 cells (P = 0.0021). In summary, GN4C increased both the cytotoxic activity of NK cells and sensitivity of tumor cells to cytolysis. However, the cytotoxic activity of NK-92 lymphoma against GN4C-pre-treated tumor cells was not straightforward.

**NK-92 cells and fresh NK cells synthesized IL-2 in response to GN4C**

Besides the cytotoxicity against tumor target cells, activated NK cells also produce cytokines TNF-α and IFN-γ. To evaluate the effect of GC on cytokine synthesis by the NK-92 cell line and fresh NK cells, we measured secreted and intracellular protein levels of cytokines in control and GN4C-treated cells.

We determined the level of secreted cytokines in supernatant of the NK-92 cell line and primary culture of fresh NK cells in response to GN4C using BDTM Cytometric Bead Array. After GN4C incubation, NK-92 cells secreted significantly more TNF-α (P = 0.05), IFN-γ (P = 0.0004) and IL-2 (P = 0.0097) that are involved in Th1 immune response. Changes in secretion of Th2 type cytokines (IL-4 and IL-10) and IL-6 were not significant (P = 0.2, 0.31 and 0.4, respectively) (Fig. 6C). Fresh NK cells secreted significantly more IL-2 (P = 0.0001) after GN4C stimulation than untreated cells. The level of other tested cytokines (TNF-α, IFN-γ, IL-4, IL-6 and IL-10) remained unchanged (P = 0.09, 0.19, 0.8, 0.3 and 0.25, respectively) (Fig. 6D).

Since we found elevated secretion of cytokines by NK-92 and fresh NK cells, we checked their intracellular synthesis. In the NK-92 cell line, GN4C increased protein expression of TNF-α, IFN-γ and IL-2 (Fig. 6A). We found apparently increased mRNA levels for TNF-α and IFN-γ cytokines after
GN4C treatment as well (Fig. 6E). On the other hand, in fresh NK cells, GN4C increased intracellular level of IL-2 but not TNF-α and IFN-γ (Fig. 6B). TNF-α or IFN-γ gene expression did not change either (Fig. 6F).

To evaluate the participation of other cell types, missing in sorted NK cells, for cytokine production, we checked the intracellular TNF-α and IFN-γ synthesis in all major cell populations (CD3+/CD56−, CD3+/CD56+, CD3−/CD56−/CD4−/CD8+, CD3+/CD56−/CD4+CD8−, monocytes) within PBMC by means of polychromatic FACS analysis. We did not detect significant changes in cytokine production in any of tested populations. TPA, used as a positive control, significantly elevated production of both cytokines in lymphocytes (CD3−/CD56− and CD3+/CD56+) and monocytes (data not shown).

**Fig. 5.** Effect of GN4C on susceptibility of tumor cell lines HT-29 and K562 to cytolysis mediated by fresh NK cells (A) and NK-92 cells (B). K562 and HT-29 cells incubated with GN4C were used as targets for fresh NK cells and NK-92 cells. The effector to target (E:T) cell ratios were 10:1 and 3.5:1 for fresh NK and NK-92 cells, respectively. Non-treated tumor cells (NTC) were used as controls of NK-mediated cytotoxicity without the effect of GN4C. Percentage of specific lyses was determined by the ⁵¹Cr-release assay. Results are presented as average ± SD of triplicates.

**Discussion**

Our previous results using PAMAM-based (polyamidoamine) GC with GlcNAc moieties for therapeutical application to tumor-bearing animals (colorectal carcinoma in rats and melanoma in mice) showed a decreased tumor growth and prolonged survival time of treated animals accompanied by enhancement of immune response (cytokine production, cytotoxicity and infiltration of tumor by activated lymphocytes) (20, 21). The introduction of calix[4]arenes in GCs as a novel scaffold bearing sugar units enabled better three-dimensional structural control than PAMAM-based GC (11) and displayed superior binding affinity than the previously tested PAMAM-GlcNAc. In animals, the results indicate structural preferences of the NK cell receptors NKR-P1 and CD69 for the GlcNAc-containing dendrimeric structures with aromatic spacers. The immunomodulatory activity results with the GlcNAc tetramers on calix[4]arene scaffold exhibit stimulation of natural cytotoxicity of human PBMC (11).

Since the GCs-like GN4C showed binding affinity especially to the NK cell receptors, we further focused on the effect of GN4C on NK cells and the NK model cell line NK-92. Glycosylation is an important protein modification changing cell appearance and function, including NK cell recognition. However, alterations of NK-92 and NK cells involving glycosylation are involved in mediating signals resulting in cytotoxicity or cytokine production. In both, fresh NK and NK-92 cells, GN4C elevated level of phosphorylated ERK1/2 but not JNK1/2 kinases (Fig. 7B). Phosphorylation of NF-κB significantly decreased in NK-92 cells treated with GN4C but not in fresh NK cells (Fig. 7B). In conclusion, GN4C modulated signaling pathways in fresh NK and NK-92 cells through protein phosphorylation of important PI3K/ERK1/2 pathway. To support this statement, we performed experiments with PI3K inhibitor wortmannin, which reduced the amount of phosphorylated PI3K and downstream ERK1/2 (Fig. 8A) and abolished GN4C-mediated cytotoxicity in both NK-92 cells and fresh NK cells (Fig. 8B and C).

**Modulation of NK cells by GC**

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Fig. 6. Cytokine production in the NK-92 cell line (A, C and E) and sorted fresh NK cells (B, D and F) revealed with flow cytometry and RT-PCR. Intracellular production of cytokines TNF-α, IFN-γ and IL-2 in NK-92 cells (A) and fresh NK cells (B) examined by flow cytometry. Non-specific fluorescence was controlled by appropriate isotype antibodies. Expressions of cytokines are indicated by solid and dashed lines in non-treated control and in GN4C-treated cells, respectively. Data are representative of three independent experiments. Production of secreted Th1 and Th2 cytokines was measured by BDTM Cytometric Bead Array Human Th1/2 in the NK-92 cell line (C) and fresh NK cells (D). Open columns indicate untreated cells (NTC) and closed columns indicate GN4C-treated cells (GN4C). The amount of cytokines in pg ml⁻¹ ± SD was determined from calibration curve of standards; Ns, not significant. Production of cytokines in NK-92 cells (E) and fresh NK cells (F) was evaluated on the level of mRNA by RT-PCR as well. Gene for B2M was used as a control of template amount (PCR product 150 bp). Particular cytokines are marked, as TNF-α and IFN-γ and PCR products were 166 and 396 bp long, respectively. Each lane is indicated with a plus where GN4C was added and minus where cells remained untreated. Representative figure of three independent experiments is shown.
First, we demonstrated that GN4C interfered with the glycosylation processes of NK-92 and fresh NK cells. This compound suppressed the expression of glycosyltransferases MGAT3 and MGAT5, the key enzymes involved in terminal glycan elongation.

Second, we found functional activation of fresh NK cells and the NK-92 cell line by GN4C, which correlated with increased mRNA expression of activation receptor NKG2D. Third, GN4C increased susceptibility of tumor cells to NK cell-mediated cytolysis in fresh NK cells.

In parallel, GN4C lessened the expression of cellular proto-oncogenes c-MYC, JUND, EGFR1, TGF-β1, VEGF-B and proliferation-associated marker Ki-67 in tumor NK-92 cells. Overall, the present study provides evidence that GN4C is a potent modulator of fresh NK cells as well as the NK-92 cell line.

Glycosyltransferases MGAT3 and MGAT5 are competitors driving terminal glycan elongation, and their activity is controlled by gene expression (22). Therefore, down-regulation of glycosyltransferases by GN4C may promote recognition capability of fresh NK cells and the NK-92 cell line and contribute to enhanced functional activity. The effect of GN4C on NK cell-mediated cytotoxicity and cytokine synthesis was lower in fresh NK cells than in the NK-92 cell line. This difference might be a result of lacking inhibitory receptors in NK-92 cells and thus higher effector function of the cell line. Inhibitory receptor CD161 (NKR-P1A) exhibited reduction of its expression after GN4C treatment (Fig. 3), which supported the activation of NK cells. In contrast to the NK-92 cell line, fresh NK cells exhibit also whole family of KIR inhibitory receptors that may reduce GN4C-mediated activation signal (23, 24).

Human NK cells require IL-2 to activate their antitumor cytotoxic response. Saito et al. (25) described expression of endogenous IL-2 mRNA in NK cells. IL-2 secreted by NK cells after GN4C incubation might serve as the autocrine factor for proliferation and cytolysis enhancement.

Cancer cells surface possesses many glycosylated structures and we supposed that tumor cells could bind the GC
as well. GN4C binding by tumor cells and subsequent modulation of MGAT glycosyltransferases expression may change tumor cells recognition pattern and make them more attractive for immune cells. In functional cytotoxicity assay performed with GN4C-pre-treated tumor targets, we found higher cytolysis than in the experiment with GN4C pre-incubated effector cells. NK cells kill preferentially hematopoietic cells, whereas many tumors derived from other tissues are resistant to NK cells (26). Thus, GN4C increased susceptibility of the HT-29 adenocarcinoma cell line to fresh NK cell-mediated killing is of remarkable importance. Using NK-92 cells as effectors against GN4C-pre-treated HT-29 adenocarcinoma cells, we obtained increase of HT-29 cytolyis. When we applied this approach to GN4C-pre-incubated K562 leukemia cells, we detected decrease of NK-92-mediated cytolyis. We suppose that K562 may express different glycogen pattern that may bind GN4C and trigger intracellular signal or mask ligands for NK-92 recognition and thus protect tumor cells from NK-92 cytolyis.

NK cell activity is triggered by NK cell receptors and further mediated through kinase phosphorylation including PI3K/ERK or PLC-γ/JNK molecules (7, 9). GN4C binds to rat NKR-P1A, which is NK cell rodent activation receptor; nevertheless, in human, NKR-P1A serves as inhibitory receptor (5). Since GN4C resulted in increased NK cell effector functions, another receptor triggering activation signal (NKG2D) has to be involved.

GN4C triggered signal through PI3K/ERK pathway resulting in NK cell cytotoxic activity. This finding is in an agreement with the description of pathway leading to activation of NK cell functions described earlier (9). The decrease of GN4C-induced cytotoxicity in NK-92 cells and fresh NK cells by PI3K inhibitor supported the involvement of PI3K/ERK pathway in GN4C-triggered enhancement of cytotoxicity. We hypothesized that NKG2D was the activation receptor triggered by GN4C because of its increased expression. JNK kinase, which is downstream of PLC-γ, is required for NKG2D-triggered NK cell cytotoxicity (7). In our experiments, GN4C did not enhance JNK phosphorylation, which excludes NKG2D to be GN4C-binding activation receptor. Therefore, the discovery of a GN4C-responsive activation receptor leading to cell cytotoxicity without JNK involvement is a challenge for future experiments. Less abundant changes in protein phosphorylation of fresh NK cells reflect the presence of NK cell inhibitory receptor NKR-P1A or KIRs that may bind GN4C simultaneously and thus reduce the activation signal strength as mentioned above.

Since NK-92 cells are originally lymphoma cells, GN4C-mediated down-regulations of glycosyltransferases MGAT3, MGAT5 and especially proto-oncogenes c-MYC, REL-A, EGRF1, VEGF-B and TGF-β1 were tremendously important. The expression of growth factors often alters in human cancer, and their over-expression correlates with tumor progression. Recently, a new role for EGRF1 in cancer has appeared; in tumors of epithelial origin, EGRF1 associates and stabilizes sodium/glucose cotransporter SGLT1. Downregulation of EGRF1 leads then to a loss of SGLT1 expression and low intracellular glucose levels (13). Given that GN4C interferes with cell glycosylation, we examined the glucose uptake transporter SGLT1/EGRF1 in fresh NK and NK-92 cells. We found decreased expression of important cellular proto-oncogene EGRF1 in NK-92 cells but no detectable SGLT1 gene. So a different transporter than SGLT1/EGRF1 mediated the glucose uptake in fresh NK and NK-92 cells.

Moreover, we found that GN4C GC inhibited expressions of TGF-β1 and VEGF-B, positive regulators of neoangiogenesis, the fundamental requirement for cancer progression (27, 28). Calix[4]arene compounds show inhibition of angiogenic factors in vitro as well as in vivo in mouse models (29). Therefore, our results confirmed and extended previous findings indicating that calix[4]arene-based GCs acted as regulators of neoangiogenesis via inhibition of TGF-β1 and VEGF-B (29).

In our opinion, the down-regulation of proliferation markers by GN4C is independent to increased cytotoxic function of the NK-92 cell line or fresh NK cells. The tested GC consists of two different chemical structures that can trigger different signaling pathways. We suppose that GlcNAc part is responsible for higher cytotoxicity and calix[4]arene core is responsible for the inhibition of expression of Ki-67, EGRF, c-MYC and other tumor markers (VEGF-B and TGF-β1) as we observed in further experiments with different tumor cell lines (V. Benson, V. Grobarova, J. Richter and A. Fiserova, unpublished data).

In conclusion, the modulation of glycosyltransferases MGAT3 and MGAT5 by chemically defined synthetic GC GN4C correlated with the improvement of NK cell effector functions and the augmentation of tumor cell sensitivity to NK cell-mediated cytotoxicity. These results demonstrate the great potential of the GN4C compound as a NK cell-mediated antitumor response modulator.

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