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Targeting Immune-Mediated Responses to Tackle GNE Myopathy †

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Abstract: GNE myopathy (GNEM) is a rare disease clinically characterized by progressive muscle atrophy and weakness. Besides the rare incidence of the disease, the limited preclinical models are among the major bottlenecks for a better pathophysiological understanding of GNEM. This work aimed then at exploring the immunological context of GNEM in a recently established cell model. Our main findings suggest the involvement of an immune adaptive response, pointing to new alternative biological targets behind GNEM pathomechanisms.

Keywords: GNE myopathy; congenital disorders of glycosylation; hyposialylation; major histocompatibility complex class I; immunomodulation

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1. Introduction

GNE myopathy (GNEM) is an ultra-rare (1 to 9:1,000,000 people worldwide) congenital disorder of glycosylation (CDG) that manifests in early adulthood, causing progressive distal muscle atrophy and weakness. GNEM results from mutations in the *GNE* gene, leading to decreased sialic acid (Sia) production [1]. Even though Sia is known to have immunomodulatory potential [2], immune-mediated responses are not common in GNEM, and inflammatory cell infiltration with increased expression of major histocompatibility complex class I (MHC-I) has only been reported in muscle biopsies of early-stage GNEM patients [3]. MHC-I plays a key role in the adaptive branch of the immune system and sialylation may regulate the complex's stability [4]. Therefore, our work aims to evaluate MHC-I expression in a recently established GNEM cell model, by flow cytometry analysis. The exploitation of an immunological link in GNEM may contribute to identifying new biomarkers that facilitate diagnosis and novel therapeutic approaches.

2. Materials and Methods

2.1. Standards and Reagents

N-Acetyl-D-mannosamine (ManNAc) and N-acetyl-D-mannosamine-6-phosphate (ManNAC-6-P) were purchased from Biosynth Carbosynth® (Compton, UK). Biotinilated *Sambucus nigra* agglutinin (SNA) was obtained from Vector Labs (Burlingame, CA, USA). FITC-conjugated HLA-ABC was acquired from ImmunoTools (Friesoythe, Niedersachsen, Germany). FITC-conjugated peanut agglutinin (PNA), bovine serum albumin (BSA), and trypan blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). PE-conjugated

Streptavidin was obtained from BD Pharmingen™ (San Diego, CA, USA). Polyclonal goat anti-mouse immunoglobulins/FITC goat F(ab')2 was purchased from Dako (Agilent Technologies, Santa Clara, CA, USA). Paraformaldehyde (PFA) was purchased from Polysciences Inc. (Warrington, PA, USA). Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate was acquired from Corning® (Corning, NY, USA). High-protein serum-free medium (DCCM-1) was obtained from Sartorius (Beit Haemek, Israel). Heat-inactivated fetal bovine serum (FBS), trypsin-EDTA (0.05%), and Pen-Strep solution (penicillin 10,000 units mL^{-1} and streptomycin 10,000 μg mL^{-1}) were acquired from Gibco® (Grand Island, NY, USA).

Human embryonic kidney (HEK) 293 cells were kindly provided by Prof. Dr. Rüdiger Horstkorte (Martin-Luther-Universität, MLU, Halle-Wittenberg, Germany). Mouse monoclonal IgG2a antibody 735 against polySia was provided by Prof. Dr. Martina Mühlenhoff (Medical School Hannover, Germany) and Prof. Dr. Rita Gerardy-Schahn (Medical School Hannover, Germany).

2.2. Cell Culture

Wild-type (WT)-HEK 293 cells and GNEM-HEK 293 cells obtained by CRISPR/Cas9 technology (GLCNE CRISPR/Cas9 KO Plasmid (h): sc-406100 and GLCNE HDR Plasmid (h): sc-406100-HDR) with 30–35% *GNE* KO cell population were cultured using DMEM, supplemented with 10% FBS, 1% Pen Strep and 1% L-glutamine, and incubated at 37 °C, in a 5% CO_2 atmosphere. At near-confluent stage, cells were detached with EDTA-trypsin (0.05%) and sub-cultured for the experiments described below.

Cell Culture Treatments

Both WT-HEK and GNEM-HEK cells were seeded in 6-well plates (6×10^5 cells per well), allowed to attach for 24 h, and exposed to ManNAc (100 μM in DCCM-1) and ManNAc-6-P (100 μM in DCCM-1) for another 24 h (37 °C, 5% CO_2). Afterwards, cells were collected for further assays.

2.3. Cell Viability

Cell suspensions were mixed with a 0.4% trypan blue solution (1:1, *v*/*v*), and 10 μL of the mixture was applied to a hemocytometer counting chamber. Cells were counted using an automatic cell counter (EVE™, VWR, USA). The results of cell viability were expressed as the % of viable cells over the total number of cells.

2.4. Cell Staining

Cells (1×10^5 cells) were harvested and washed with PBS and centrifuged (300× *g*, 4 °C, 5 min). SNA solution (1:100 in PBS + 1% BSA) and 735 antibody (1:100 in PBS + 1% BSA) were added to cell pellets and incubated for 20 min, at 4 °C, after which the fluorophore-labeled streptavidin (1:1000 in PBS + 1% BSA) and the FITC-conjugated anti-mouse IgG (1:100 in PBS + 1% BSA) were added, respectively, and incubated for another 10 min (at 4 °C). FITC-conjugated PNA solution (1:100 in PBS + 1% BSA) and MHC I antibody (2:100 in PBS + 1% BSA) were added to cell pellets and incubated for 30 min at 4 °C. After each incubation cell pellets were washed with PBS + 1% BSA and following the surface staining cells were fixated with 2% PFA for flow cytometry analysis.

2.5. Flow Cytometry

Acquisition of data was performed on an Attune Acoustic Focusing Cytometer (Applied Biosystems, Waltham, MA, USA). Data were analyzed using FlowJo software version 10.0.5 (TreeStar, San Carlos, CA, USA) after cell gating and doublet exclusion by height and width parameters, to ensure only single cells were counted. For each staining condition, the respective mean fluorescent intensity (MFI) of the unstained control was subtracted.

2.6. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism 8.0 software (GraphPad Software, La Jolla, CA, USA). A Shapiro–Wilk normality test was employed to check the distribution of the data, and a Grubb's test to determine the presence of outliers. Statistical significance (at $p < 0.05$) was calculated using a two-tailed unpaired *t*-test.

3. Results and Discussion

Among the limited preclinical models of GNEM, cell lines with *GNE* mutations or decreased *GNE* expression have been used to clarify disease pathomechanism and for exploring new therapeutic options. In this work, we used a recently developed HEK293 *GNE* KO cell line obtained by CRISPR/Cas9 technology. Although the *GNE* KO is embryonic lethal in the mouse model, this cell-based model system is extremely important given the large degree of uncertainty in patient reports and the reduced availability of patient cells.

3.1. Sialophenotype of GNEM-HEK cells

The effect of the *GNE* KO in the cell surface profile was evaluated by flow cytometry analysis (Figure 1a).

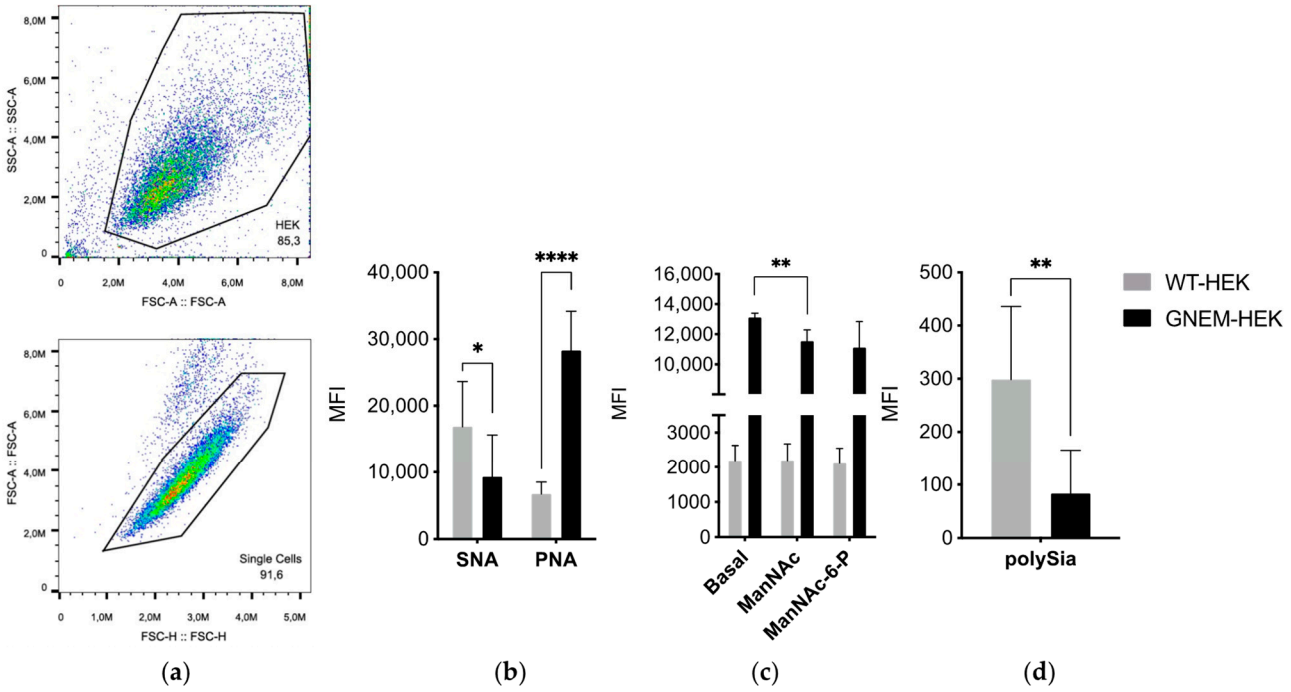

Figure 1. Characterization of sialic acid profile. (**a**) Gating strategy. (**b**) SNA and PNA staining of WT-HEK and GNEM-HEK cells. (**c**) PNA staining of WT-HEK and GNEM-HEK cells supplemented with 100 µM ManNAc and 100 µM ManNAc-6-P. (**d**) polySia staining of WT-HEK and GNEM-HEK cells. Significant differences at * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$ ($n \geq 3$).

3.1.1. Sialic Acid Profile

Whereas a significant decrease in the Sia-binding lectin SNA was observed for GNEM-HEK cells, an inverse trend was observed for staining with PNA, a lectin that binds to nonsialylated structures (Figure 1b). These results confirm the efficacy of the interruption of Sia biosynthesis in the GNEM-HEK cells. Moreover, when WT-HEK and GNEM-HEK cells were supplemented with noncytotoxic concentrations of ManNAc and ManNAc-6-P (data not shown), intermediates in the Sia biosynthesis, an increase in Sia was observed (Figure 1d). Unlike α2,3- and α2,6-sialylated glycans that are easily detected by lectin staining, no lectins are available to detect polySia. Therefore, an anti-polySia antibody, such as the 735 antibody, was selected to assess polySia expression in the cells. As it can

be seen (Figure 1c), polysialylation of NCAM, one of the main polysialylated proteins, is significantly lower in GNEM-HEK cells than in WT-HEK cells. Although the relevance of polySia-NCAM depletion has not been evaluated, loss of polySia in the protein backbone of NCAM was already reported to have a key role in immune regulation [5].

3.1.2. MHC-I Signature

A significant increase in MHC-I expression was found on the GNEM-HEK cell surface (Figure 2a). However, when WT-HEK and GNEM-HEK cells were supplemented with ManNAc and ManNAc-6-P a reduction in MHC-I staining was observed (Figure 2b).

In a previous work by our group [4], Sia shortage was found to lead to increased cell surface expression of MHC-I, which in turn increases antigen presentation and immune potency. Our current findings support the hypothesis that Sia content modulates the presence and stability of the MHC-I complex.

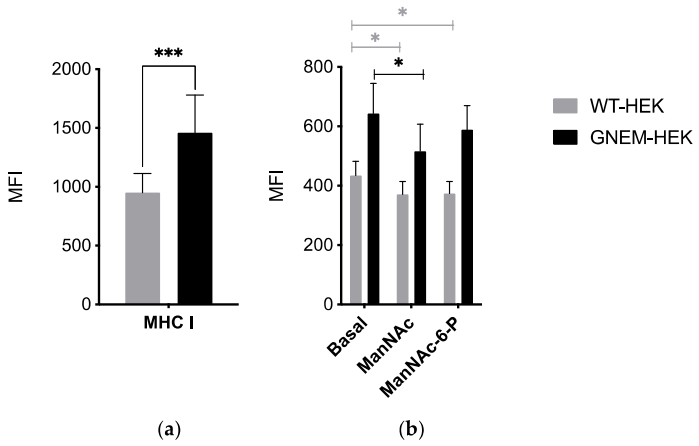

Figure 2. Evaluation of MHC-I expression. (**a**) MCH-I antibody staining of WT-HEK and GNEM-HEK cells. Schemes follow the same formatting. (**b**) MCH-I staining of WT-HEK and GNEM-HEK cells supplemented with 100 μM ManNAc and 100 μM ManNAc-6-P. Significant differences at * $p < 0.05$, and *** $p < 0.001$ ($n \geq 3$).

4. Conclusions

Our work exploring the cellular and molecular context in GNEM sheds light on the possibility of the involvement of a cytotoxic immune response initiated via MHC-I presentation. Sia on MHC-I is suggested to have an important contribution to antigen presentation, opening doors to a novel pharmacological target to tackle GNEM.

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Conflicts of Interest: The authors declare no conflict of interest.

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