GBS/CIDP foundation International: 4 months report

Introduction

This project aims to understand the mechanisms underlying the genesis and maturation of anti-GM1 antibodies, crucial in the development of multifocal motor neuropathy (MMN). These antibodies target a key component of nerve cell membranes and are believed to be pathogenic. However, the mechanisms underlying their induction, their sustained production, and their association with clonal B-cell expansions and paraproteinemia, remain poorly understood. Identifying and tracking antibody-producing B-cell clones in MMN patients could offer valuable insights into disease mechanisms and guide the development of targeted therapies.

Structure

The project is currently divided into four key phases, with the first two successfully completed and the subsequent phases recently initiated.

The first phase involved achieving IgM production through hybridoma cultures derived from MMN patients to generate positive controls for the remaining experiments.

The second phase focused on the collection and testing of a cohort of MMN patients for anti-GM1 antibody positivity and the presence of paraproteinemia.

For patients identified as positive, the third phase will establish PBMC cultures to facilitate the production of anti-GM1 antibodies and identify the specific population of B-cells responsible for generating the pathogenic antibody via flow cytometry and FACCS.

Finally, the fourth phase will directly test these sera on iPSC-derived sensory and motor neurons to evaluate their direct or indirect pathogenic effects.

Phase 1: Generate IgM in Hybridoma culture

Methods

Hybridoma cells are a fusion of B lymphocytes with myeloma cell, which have the capacity for indefinite expansion and immunoglobulin secretion. These hybridomas, previously derived from an MMN patient exhibiting anti-GM1 antibodies (BO3), were cultured for 26 days. During this period, the production of IgM was monitored and assessed. Following the culture phase, IgM extraction and purification was performed using a HiTrap affinity columm.

Results

The 26-day culture of hybridoma cells successfully yielded supernatants containing IgM, as confirmed in Figure 1. The subsequent purification process using the HiTrap column resulted in the recovery of 6 mL of concentrated IgM, that will be reused as positive control for the following experiments.

Conclusion

This purified IgM can now serve as a reliable positive control in future experiments.

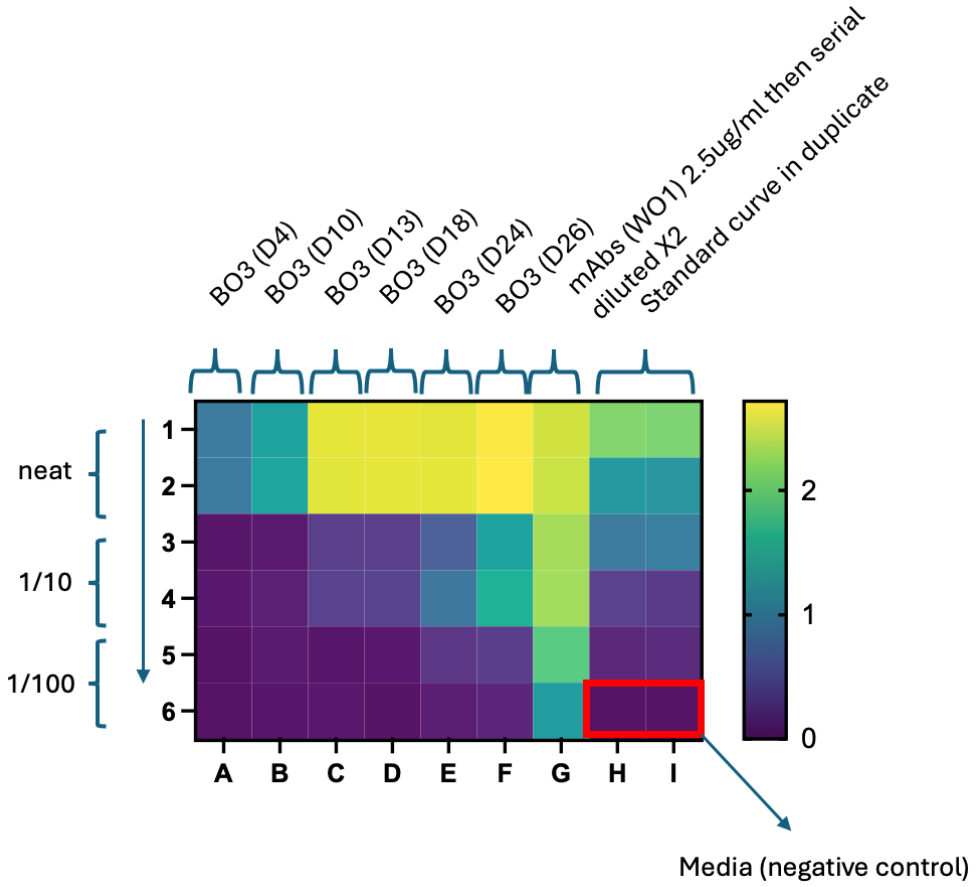

Figure 1: Heatmap illustrating antibody titers from a single hybridoma cell line (BO3) in supernatants over time (Days 4, 10, 13, 18, 24, and 26) across serial dilutions (neat, 1:10, and 1:100). Columns represent different samples, including standard curve duplicates, positive control (W01 at 2.5 µg/mL serially diluted), and negative control (media alone).

The color scale indicates signal intensity, with higher values represented in yellow and lower values in purple. Standard curve range 1,000–15.6 ng/mL.

Phase 2: MMN cohort collection and analysis

Methods

Serum samples from 34 MMN patients were gathered and analyzed to determine the frequency of GM1 antibodies using enzyme-linked immunosorbent assays (ELISA). Serum protein electrophoresis (SPEP) and light chain restriction analysis were performed to investigate the presence of paraproteinemia and assess B-cell clonality.

Results

IgM GM1 antibodies were identified in 14/34 MMN patients (41%). Among them, SPEP analysis revealed IgM paraproteins in 4/14 patients (28,5%). Light chain restriction was observed in 6 GM1-positive patients, with 3/14 (21%) showing kappa, and 3/14 (21%) lambda (Figure 2).

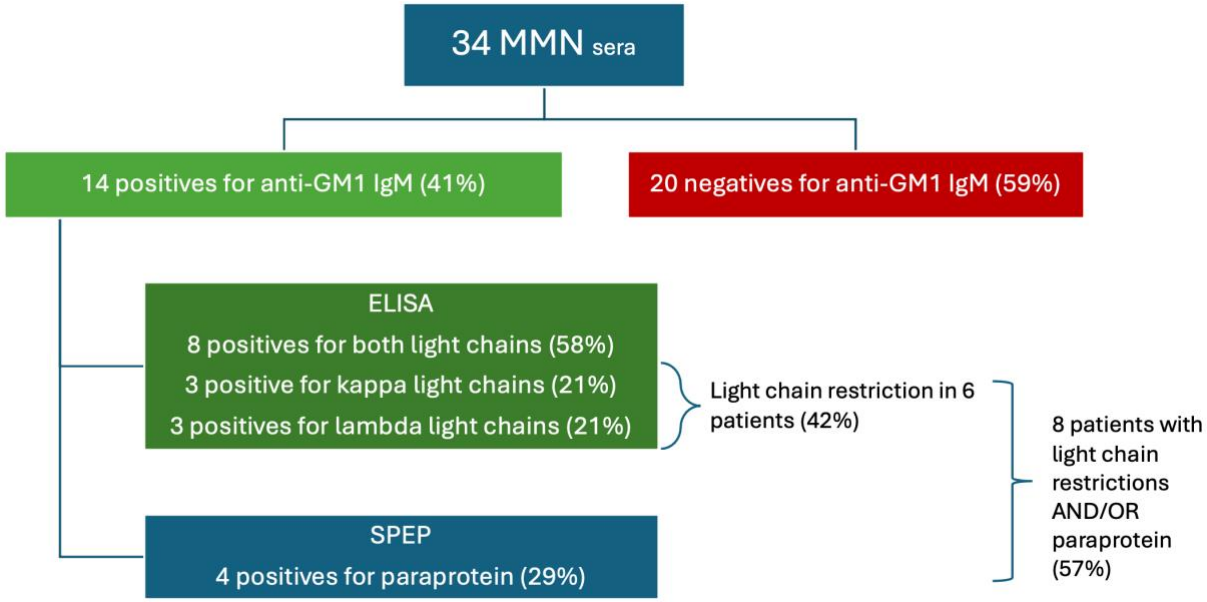

Figure 2. Flowchart of MMN cohort and subsequent ELISA and SPEP results

Conclusions

Our findings demonstrate the presence of GM1 antibodies in a significant subset of MMN patients (41%), with IgM paraproteins and/or light chain restriction consistent with B-cell monoclonal expansions in 8/14 (57%) GM1 positive patients. Higher-sensitivity paraprotein assays are planned for further investigation.

Phase 3: PBMC culture

Method

Peripheral blood mononuclear cells (PBMCs) isolated from one MMN patient (BIO-053) were cultured under cytokine-stimulated (Resiquimod (R848), Interleukin-2 (IL-2), Soluble CD40 ligand (sCD40L), Tumour necrosis factor alpha (TNFa), IL-1β, and IL-21) and unstimulated conditions to evaluate total and GM1 specific antibody production potential.

Results

Cultured PBMCs under specific cytokine stimulation yielded optimal immunoglobulin production when 50,000–100,000 cells were seeded per well in a 24-well plate, achieving IgM concentrations of up to 100 µg/ml (Figure 3 and 4). Further analyses are ongoing to quantify GM1-specific antibodies under these conditions.

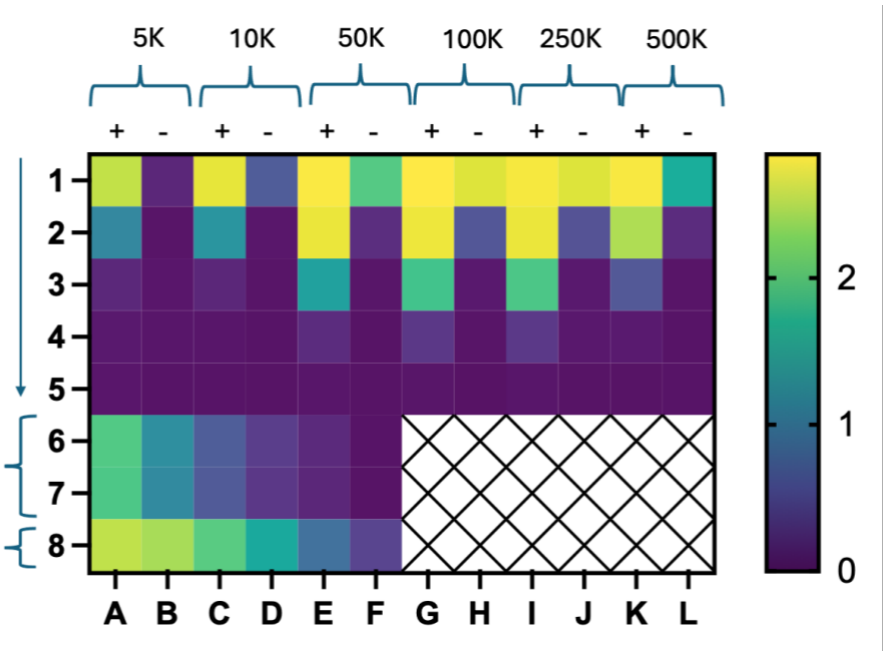

Figure 3: Heatmap showing from row 1 to 5 the produced amount of IgM from PBMC cultures at day 14 at different dilutions (neat, 1/10, 1/100, 1/1000, 1/10000), with "+" indicating PBMC stimulated by a specific cytokine cocktail and "-" indicating unstimulated. PBMC were seeded at progressive increased number starting at 5K per well to 500K per well. Rows 6 and 7 represent the standard curve, while row 8 corresponds to the positive control (hybridoma cell line WO1). WO1 was prepared at an initial concentration of 5 µg/mL and subsequently subjected to a 2-fold serial dilution. The intensity of the color scale indicates corrected optical density (OD), ranging from low (purple) to high (yellow). Standard curve range 1,000–15.6 ng/mL. Wells marked with a crosshatch pattern represent conditions excluded from the analysis.

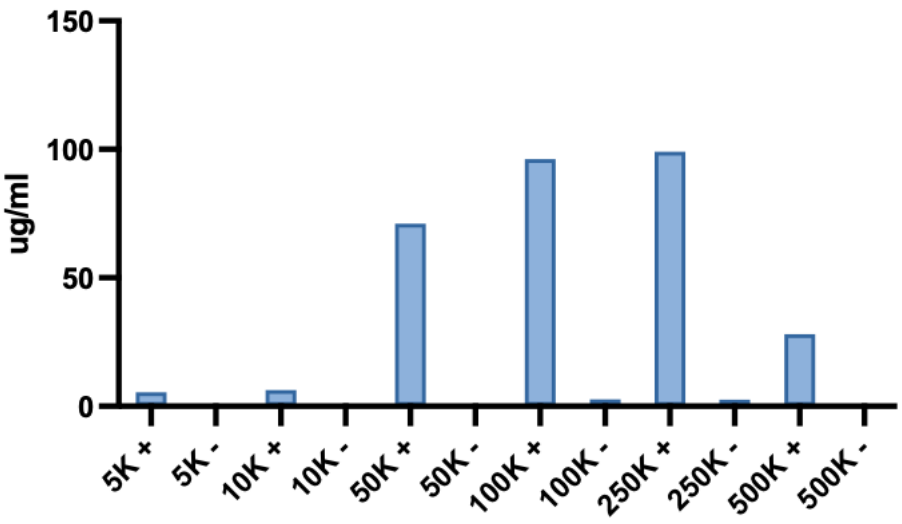

Figure 4: Bar graph showing the IgM production relative to the number of hybridoma cells seeded per well. The graph illustrates the concentration of IgM antibodies (measured in µg/ml) secreted by hybridoma cells seeded at varying densities, ranging from 5,000 to 500,000 cells per well, in 24-well plates. "+" indicates wells supplemented with a specific cytokine cocktail, while "-" represents wells without supplementation.

Conclusion

Optimized PBMC cultures provide a platform for further investigation into GM1-specific antibody production, testing therapeutic strategies, and the later isolation and characterisation of pathogenic clones via flow cytometry and FACCS.

Phase 4: IPSC culture and immunofluorescence

Method

Using an established laboratory protocol, we differentiated induced pluripotent stem cells (iPSCs) into mature motoneurons. A pilot experiment was subsequently conducted by exposing mature motor neurons to serum from anti-GM1 positive patients for one hour. Immunofluorescence staining was then performed using specific antibodies to label axons (NFL) and IgM.

Results

Immunofluorescence staining revealed the presence of IgM antibodies along the axons, supporting the notion that they might exert some axonal effect (Figure 5). Further studies are planned on myelinated motor neurons, utilizing a specific GM1 marker to gain deeper insights into antigen distribution, and to characterize the action of anti-GM1 IgM on myelinated motor neurons.

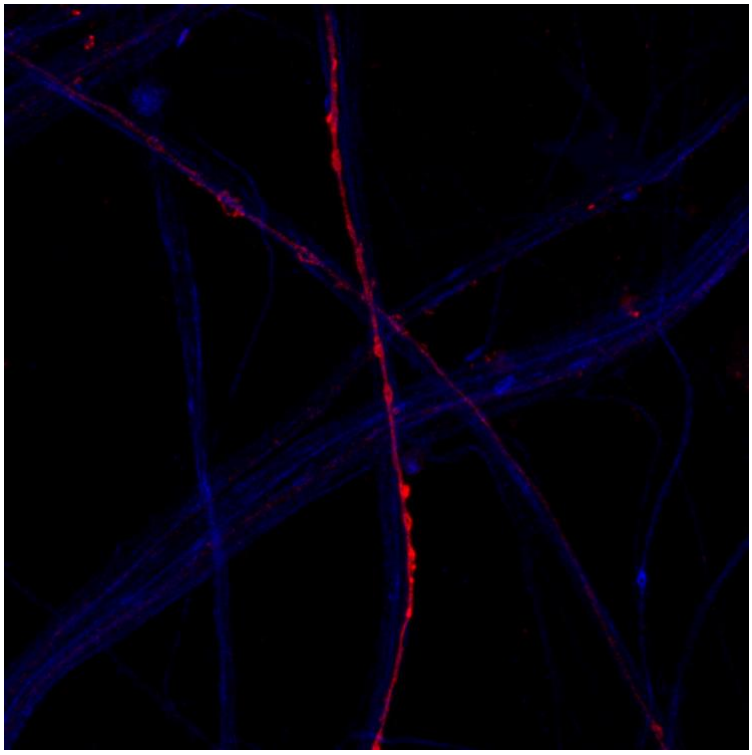

Figure 5: Immunofluorescent staining of motor neuron cultures with axonal marker NFL and IgM marker. Immunofluorescence staining demonstrated that the cultures were effectively producing mature motor neurons with long axons, and that both NFL (blue) and IgM (red) are localized to axons. Pacific blue (blue rounds) staining shows the nuclei.

Conclusion and perspectives

In this four-month progress report, important advancements have been made in understanding GM1 neuropathies and their associated pathogenic mechanisms. Phase 1 successfully established IgM production in hybridoma cultures, providing a reliable positive control for subsequent experiments. Phase 2 revealed that 41% of MMN patients exhibit anti-GM1 antibodies, with evidence of monoclonal B-cell expansions in over half of these cases. Phase 3 optimized PBMC cultures for IgM production, laying the groundwork for isolating and characterizing pathogenic clones. Finally, Phase 4 demonstrated the localization of IgM antibodies to axons in iPSC-derived motor neurons, highlighting their potential axonal effects.

In the next phase of the project, using pre-treatment PBMCs from patients with MMN will identify the lymphocyte populations generating anti-GM1 autoantibodies through FACS and ELISA. Cultivating these lymphocytes will then allow us to monitor their development, while cloning the antibodies of interest will permit a detailed characterization. Their affinities will be assessed using surface plasmon resonance, and their clonal evolution will be studied through IgBLAST. Finally, the impact of the cloned anti-GM1 antibodies on nerve function will be examined in neuronal cultures derived from human stem cells.