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Naturally occurring antibodies as next generation immunotherapy for high-risk neuroblastoma

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1 INTRODUCTION

1.1 Neuroblastoma

Neuroblastoma presents a major clinical challenge for pediatric oncology. It is characterized by high clinical and molecular heterogeneity, ranging from spontaneously regressing tumors to aggressive, treatment-resistant forms. Despite significant advances in multimodal treatment approaches, the prognosis for patients with high-risk subtypes remains poor, highlighting the need for novel therapeutic targets and strategies^{1,2}.

1.1.1 Epidemiology

Neuroblastoma, a neuroendocrine tumor of the sympathetic nervous system, is the most common extracranial solid tumor diagnosed in infants and young children. About 90% of cases are diagnosed before the age of five and the median age at diagnosis is 18 months³. Although rare, with a median age-standardized incidence rate of 13.2 per million children per year in Germany⁴, neuroblastoma accounts for 8% to 10% of all childhood cancers and contribute to around 15% of cancer-related mortality in this age group². Over the past years, the 10-year overall survival increased from 46% to 72%⁴. However, patients with a high-risk neuroblastoma have poor prognosis, with a 50-60% relapse rate and an overall survival of only 20%, despite aggressive treatments^{5,6}.

The majority of neuroblastoma occurs spontaneously, as only 1% of cases are familial forms¹. However, potential exogenous risk factors for the development of neuroblastoma could not be identified in epidemiologic studies⁷.

1.1.2 Origin and clinical presentation

Although the exact timing and cell of origin remain debated, neuroblastoma is an embryonal tumor of the developing sympathetic nervous system. It originates during development from immature precursor cells derived from neural crest tissue, which normally differentiate into sympathetic neuroblasts and adrenal chromaffin cells^{8,9}. Neuroblastoma may arise from different cells of origin at various developmental stages, contributing to the heterogeneity and variable clinical behavior of the disease. While some tumors resemble early progenitor cells with an undifferentiated mesenchymal-like phenotype, others align with more differentiated neuroblasts and chromaffin cells¹⁰⁻¹².

The location of neuroblastoma is closely linked to its developmental origin, as tumors can arise anywhere along the sympathetic nervous system. Approximately 65% of primary tumors develop in the abdomen, with the adrenal medulla being the most common site. Other frequent locations include sympathetic ganglia in the neck, chest, and pelvis¹³. At diagnosis, about half of the children present with

localized disease, while 35% already exhibit regional lymph node metastasis. In advanced stages, neuroblastoma most commonly metastasizes to the bone, bone marrow, lymph nodes and liver^{2,14}. Although, some patients present with aggressive, treatment-resistant tumors other cases have a favorable prognosis, even without intensive treatment, as neuroblastoma is associated with the highest rate of spontaneous and complete regression of all human malignancies^{15,16}, further highlighting the high clinical heterogeneity.

1.1.3 Classification and staging

The International Neuroblastoma Staging System (INSS) classifies and diagnoses these heterogeneous tumors into different stages after surgery, based on tumor resection and lymph node involvement^{17,18}. Stage 1 comprises completely resected tumors with no lymph node involvement. Partially resected tumors with no lymph node involvement are categorized as stage 2A, while those with ipsilateral regional lymph node involvement are classified as stage 2B. Stage 3 tumors have either crossed the midline and/or involve regional lymph nodes on the contralateral side. Tumors with distant metastatic spread are classified as stage 4. Additionally, stage 4S is distinguished by a unique metastatic pattern, limited to the liver, skin, and minimal bone marrow involvement. This stage occurs exclusively in infants under 18 months of age and is associated with a favorable prognosis due to its tendency for spontaneous regression¹⁹.

Since the INSS relies on post-surgical findings, it is not ideal for preoperative risk stratification. To address this, the International Neuroblastoma Risk Group Staging System (INRGSS) was developed, which classifies tumors based on imaging and clinical characteristics²⁰. In this system, stage L1 describes a localized tumor without image-defined risk factors, while stage L2 includes localized tumors with risk factors such as spinal involvement or encasement of major blood vessels. Metastasized tumors are classified as stage M or MS, with stage M indicating distant metastasis and stage MS representing a special category for infants with metastatic disease patterns as those described in INSS stage 4S.

In 2000, the Children's Oncology Group (COG) established a risk classification system for neuroblastoma, stratifying patients into low-, intermediate-, and high-risk categories. This definition was based on age at diagnosis, INSS stage and histopathology of the tumor, genetic alterations and the amplification status of the oncogenic driver MYCN. In 2021, the COG classification system was revised to enable more precise risk stratification and treatment assignment²¹. The updated classifier now integrates the INRGSS along with additional genomic biomarkers, including segmental chromosome aberrations. Patients in the low- and intermediate-risk groups have favorable prognosis, with 5-year overall survival (OS) probabilities of $97.9\% \pm 0.5\%$ and $95.8\% \pm 0.8\%$, respectively. However, patients with adverse clinical and molecular characteristics, including age above 18 months^{3,22}, stage L2 or M disease, MYCN amplification²³, poorly differentiated tumors with a high mitosis-karyorrhexis index

(MKI)²⁴ and segmental chromosomal aberrations²⁵, are typically classified as high-risk²¹. These patients have significantly poorer outcomes, with a 5-year OS probability of only 62.5% $\pm$ 1.3% (**Figure 1**)²¹.

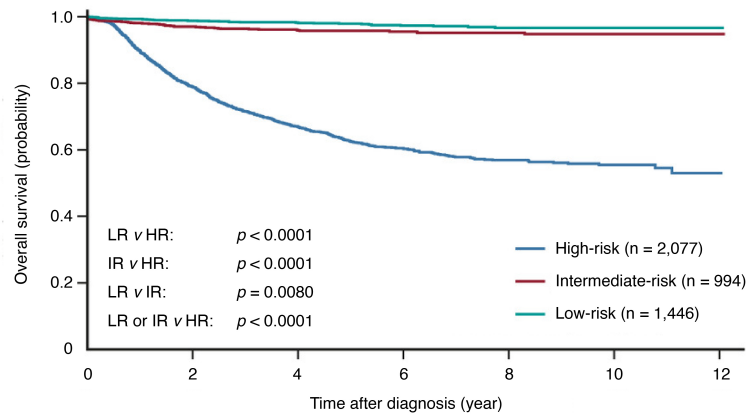


FIGURE 1 | Overall survival probability for low-, intermediate- and high-risk neuroblastoma patients. Kaplan-Meier survival analysis for neuroblastoma patients enrolled in the ANBL00B1 study and diagnosed between 2007 and 2017. Classification into low-, intermediate- and high-risk patients according to the revised Children’s Oncology Group (COG) classifier from 2021. Illustration is adapted from Irwin *et al.*²¹. LR, low-risk; IR, intermediate-risk; HR, high-risk.

1.1.4 Molecular alterations in neuroblastoma

Neuroblastoma generally exhibits a low mutational burden, with only a few genes being recurrently mutated either sporadically or through inheritance²⁶. However, the anaplastic lymphoma kinase (*ALK*) as well as the paired-like homeobox 2B (*PHOX2B*) gene play a crucial role in both familial and sporadic neuroblastoma. *ALK* encodes a receptor tyrosine kinase essential for neural development. In familial cases, gain-of-function *ALK* mutations are the primary genetic drivers, accounting for the majority of hereditary neuroblastoma cases²⁷. In sporadic neuroblastoma, *ALK* mutations are found in 5% to 15% of cases, underscoring its significance as an oncogenic driver in this disease. These activating mutations lead to constitutive activation of the tyrosine kinase domain, driving uncontrolled tumor growth, proliferation, and survival^{28–30}. Additionally, loss-of-function mutations in *PHOX2B* are linked to an impaired transcriptional regulation of cell differentiation and survival. In neuroblastoma, *PHOX2B* mutations are thereby associated with rare hereditary as well as with 2.3% of sporadic cases^{31,32}.

Moreover, neuroblastoma is driven by chromosomal losses and gains, which significantly impact tumor behavior and prognosis. While whole-chromosome gains result in hyperdiploidy, which is generally associated with a favorable prognosis, segmental chromosomal aberrations, including *MYCN* amplification and regional gains or losses, are associated with worse outcomes^{33,34}. Notably, *MYCN* amplification is one of the most significant molecular alterations in neuroblastoma, occurring in 20% to 25% of cases^{35,36}. The *MYCN* proto-oncogene which is located on chromosome 2 encodes a transcription factor that regulates key cellular processes, including cell growth, differentiation and metabolism^{37,38}. Patients with *MYCN*-amplified neuroblastoma typically present with aggressive tumors, rapid disease progression, and poor prognosis²³. *MYCN* amplification is therefore particularly associated with high-

risk neuroblastoma, found in 40% of children over 18 months with stage 4 disease, compared to only 9% of patients with localized tumors⁴.

Another frequent genetic aberration in neuroblastoma is the gain of chromosome 17q, occurring in 53.7% of cases, which is strongly associated with advanced disease, *MYCN* amplification, and chromosome 1p deletion²⁵. The loss of chromosome 1p is thereby associated with the deletion of a tumor suppressor region, contributing to worse outcomes³⁹. Additionally, other chromosomal deletions, such as loss of heterozygosity in chromosome 11q and 14q, which occur in 34% and 22% of neuroblastoma cases, respectively, are also associated with aggressive disease behavior and serve as prognostic markers for survival outcomes^{40,41}.

In the recent years, specific cell surface markers have been identified in neuroblastoma, including the disialoganglioside GD2⁴², the immune checkpoint molecule B7-H3⁴³, and the cell surface heparan sulfate proteoglycan Glypican-2 (GPC2)⁴⁴. GD2 is highly expressed in neuroectodermal tumors, such as neuroblastoma, small cell lung cancer, retinoblastoma, and melanoma, as well as in various sarcomas, including rhabdomyosarcoma and osteosarcoma⁴⁵. While GD2 is normally found in neural and mesenchymal stem cells during development, its postnatal expression is largely restricted to neurons, peripheral nerve fibers and melanocytes^{46,47}. Due to its selective expression in tumors and limited presence in normal tissues, GD2 has become a key immunotherapeutic target in neuroblastoma treatment⁴⁸.

1.1.5 Current treatment strategies and limitations

The treatment of neuroblastoma is highly dependent on patients' stage and risk stratification. While low- and intermediate-risk neuroblastoma typically respond well to surgical resection and chemotherapy, high-risk cases require an intensive multimodal therapy approach to improve survival rates. In infants with stage 4S neuroblastoma, surgical intervention is often unnecessary, as these tumors tend to spontaneously differentiate and regress without aggressive treatment. In such cases, observation alone may be sufficient. However, for advanced-stage neuroblastoma, a comprehensive treatment strategy is required to achieve disease control and long-term remission, including surgery, chemo- and radiotherapy, autologous hematopoietic stem cell transplantation (AHSCT) and immunotherapy^{1,2}.

The standard treatment regimen for high-risk neuroblastoma begins with induction chemotherapy to achieve remission by reducing the tumor burden before surgery. This regimen includes a combination of multiple chemotherapeutic agents, such as cisplatin, vincristine, doxorubicin, and cyclophosphamide, which are administered in alternating cycles. Following induction chemotherapy, surgical resection is performed, although complete removal may be challenging depending on tumor location and invasiveness. After surgery, patients undergo high-dose chemotherapy, which is myeloablative and requires AHSCT to restore bone marrow function. In some cases, radiation therapy is used for local disease control, particularly when residual tumors remain after surgery^{1,49}.

As part of the maintenance therapy, immunotherapy is administered to eliminate minimal residual disease and prevent relapse. The FDA- and EMA- approved chimeric monoclonal antibody Dinutuximab (ch14.18) is used for this purpose. The antibody targets GD2, a ganglioside highly expressed on neuroblastoma cells. Dinutuximab consists of a murine antigen-binding fragment from the murine 14G2a antibody fused to a human constant region of an immunoglobulin G1, enhancing immune system activation⁵⁰. GD2 immunotherapy is often combined with interleukin-2 (IL-2) and granulocyte-macrophage colony-stimulating factor (GM-CSF), as well as with isotretinoin, which promotes differentiation in neuroblastoma.

A phase III clinical trial demonstrated that the addition of Dinutuximab significantly improved event-free (EFS) survival in high-risk neuroblastoma patients, achieving 66% EFS at two years compared to 46% EFS with isotretinoin alone⁵¹. However, long-term follow up studies indicate, that the survival benefit of immunotherapy diminishes over time due to late relapses⁵². Additionally, Dinutuximab therapy is associated with severe side effects in the patients, including fever, neuropathic pain, peripheral neuropathy, capillary leak syndrome and hypotension⁵⁰. Thereby, neuropathic pain is most likely mediated by the binding of Dinutuximab to GD2 on peripheral nerve fibers, leading to complement system activation⁵³. As the pain can be severe, administration of gabapentin and continuous morphine infusion before, during and after treatment are required⁵⁰. Furthermore, the observed GD2 downregulation on neuroblastoma cells during therapy limits Dinutuximab efficacy⁵⁴, highlighting the need for new immunotherapeutic targets.

Although the 5-year overall survival probability for patients with high-risk neuroblastoma has improved due to myeloablative therapy and immunotherapy, prognosis for high risk patients still remains poor^{1,55}. A major challenge is the high relapse rate, as 50% to 60% of high-risk patients experience refractory or relapsed disease, which is associated with worse outcomes^{5,6}. Ongoing research is focused on developing novel targeted therapies, including immunotherapeutic strategies such as chimeric antigen receptor (CAR) T cell therapies⁵⁶, immune checkpoint inhibitors⁵⁷, and targeted treatments against ALK, MYCN, and other oncogenic pathways⁴⁹. Additionally, antibody-drug conjugates targeting ALK or GPC2, which demonstrated anti-tumor activity in preclinical studies, are currently under investigation^{44,58}.

To develop more effective and safer immunotherapeutic strategies, it is crucial to identify tumor-associated antigens that are highly expressed on neuroblastoma cells while being absent or minimally expressed on healthy tissues. The limitations of GD2-targeting therapy, including antigen downregulation as a mechanism of immune escape and severe neurotoxicity due to GD2 expression on peripheral nerves, highlight the urgent need for alternative targets and antibodies.

1.2 Antibodies and their role in immunotherapy

Antibodies, also known as immunoglobulins (Ig), are essential components of the adaptive immune system, playing a critical role in pathogen defense, immune surveillance and tumor immunity. Due to their high specificity to target antigens, they are important agents for targeted cancer therapies⁵⁹.

1.2.1 Structure and isotypes of antibodies

Antibodies are Y-shaped glycoproteins, that consist of four polypeptide chains, two identical heavy chains (~50 kDa each) and two identical light chains (~25 kDa each), that are linked by disulfide bonds (**Figure 2A**). Each antibody consists of two fragment antigen-binding (Fab) regions, which are responsible for antigen recognition, and the fragment crystallizable (Fc) region, that mediates interactions with immune effector cells. The Fab region contains the variable domains of the heavy (V_H) and the light chains (V_L), which include the complementarity-determining regions (CDR), that are highly diverse in their amino acid sequence and determine the antigen specificity of the antibody. The Fc part is responsible for the interaction with immune components, leading to complement activation, binding to Fc receptors (FcR) on various immune cells, and mediating effector functions⁶⁰. There are five different isotypes of antibodies, that differ in their heavy chain sequences. The heavy chains alpha (α), delta (δ), epsilon (ϵ), gamma (γ), and mu (μ) can be found in IgA, IgD, IgE, IgG and IgM antibodies, respectively (**Figure 2B**). IgG, IgA and IgD are characterized by three constant domains of the heavy chain, C_{H1} , C_{H2} and C_{H3} . IgM and IgE have an additional C_{H4} domain. The light chain can either be kappa (κ) or lambda (λ) in all isotypes. While IgD, IgE and IgG antibodies are monomers, IgA and IgM antibodies form dimers and pentamers, respectively⁶¹.

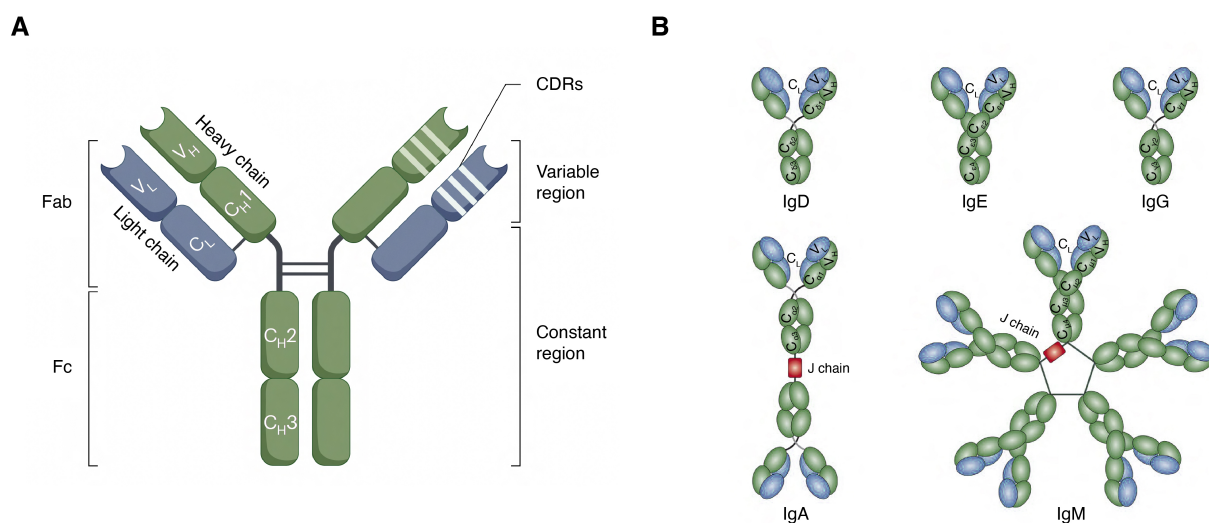


FIGURE 2 | Structure and isotypes of antibodies. **A**, Schematic representation of an antibody structure, highlighting the antigen-binding fragment (Fab), responsible for antigen recognition, and the crystallizable fragment (Fc), which mediates effector functions. The variable region contains the complementarity-determining regions (CDRs), which determines antigen specificity, while the constant region interacts with immune cells. Antibodies consist of two identical heavy and light chains, linked by disulfide bonds. Illustration adapted from Paul *et al.*⁵⁹. **B**, Different immunoglobulin isotypes. IgG, IgD and IgE are monomeric, while IgA forms dimers and IgM forms pentamers. A J chain is involved in multimerizing IgA and IgM. Illustration adapted from Duarte *et al.*⁶². V_H , variable heavy; V_L , variable light; C_H , constant heavy; C_L , constant light.

IgM is typically the first antibody generated during an immune response, and its pentameric structure compensates for its low initial affinity by providing high antigen avidity, which enables pathogen neutralization and activation of the complement system⁶⁰. IgG antibodies are the most abundant antibodies in human serum, responsible for pathogen neutralization or cytotoxicity mediated by activation of the complement cascade or mediated by immune effector cells⁶¹. Moreover, it provides long-term immunity as it is produced in a later stage of immune response and has the longest half-life among all isotypes⁶³. IgA is primarily found in mucosal secretions, providing defense against pathogens in mucosal areas. IgD functions mainly in immune regulation⁶⁴, whereas IgE is primarily involved in the defense against endoparasites and plays a role in allergic reactions⁶⁰.

1.2.2 Generation of antibodies

Antibodies are naturally generated by B cells as part of the adaptive immune response to recognize and neutralize foreign antigens, such as pathogens or tumor-associated antigens. This antibody response can be either T cell-dependent or T cell-independent, based on the antigen. To identify specific antibody sequences for therapeutic applications, several strategies can be used, including hybridoma technology, phage display, and single B cell cloning. These approaches enable the discovery of highly specific antibodies, which can then be further optimized through antibody engineering techniques to enhance their affinity, stability, and therapeutic potential.

1.2.2.1 B cell activation and antibody production in the humoral immune response

The humoral immune response is initiated when an antigen-reactive B cell encounters an antigen in secondary or peripheral lymphoid tissues. Upon antigen binding, B cell receptor (BCR) signaling activates the B cell, leading to upregulated expression of costimulatory molecules, pro-survival factors and cell-cycle regulators. The BCR-antigen complex is internalized and transported via endosomes to lysosomes, where the antigen is proteolytically degraded into short peptide fragments. The peptides are then loaded to MHC class II molecules and delivered back to the cell surface, where they are presented to T helper cells, which are needed for the T cell-dependent B cell activation⁶⁵. T helper cells recognize the antigen-MHC complex and initiate B cell proliferation, differentiation and maturation through CD40-CD40L interactions and IL-4 and IL-21 cytokine stimulation^{66,67}.

Thereby, cells undergo critical modifications to enhance antibody affinity. Somatic hypermutation introduces point mutations into the variable region of the Ig genes, which should increase antigen-binding affinity. Simultaneously, class switch recombination leads to the transition from IgM and IgD-expressing B cells to other isotype-producing B cells, through a DNA rearrangement process of their Ig heavy chain gene. The T cell-dependent B cell response thereby results in the formation of short-lived, antibody-secreting plasma cells, and long-lived memory B cells.

In contrast, T cell-independent B cell activation occurs when thymus-independent antigens, such as polysaccharides or lipopolysaccharides, directly stimulate B cells. The B cell response can thereby result

from strong activation signals, such as engagement of Toll-like receptors (TLRs), or from simultaneously cross-linking of multiple BCRs by repetitive antigenic structures^{68,69}. As the B cell response does not require T cell help, it primarily leads to the production of low-affinity IgM antibodies with limited isotype switching and only minimal B cell memory formation^{70,71}.

The ability of B cells to generate a huge antibody repertoire against a variety of different antigens is mediated by V(D)J recombination, a process driven by recombination activation genes (RAG1 and RAG2). This mechanism enables the rearrangement of variable (V), diversity (D), and joining (J) gene segments to produce an estimated number of 10^{12} unique antibody variants^{72,73}.

1.2.2.2 Antibody generation techniques for the development of therapeutic antibodies

The development of therapeutic monoclonal antibodies (mAb) involves various techniques to generate high-affinity and highly specific antibodies⁷⁴. One approach is hybridoma technology, which combines mouse immunization with B cell-myeloma fusion. Therefore, mice are immunized with a specific antigen, triggering an immune response and leading to the activation and proliferation of antigen-specific B cells. These B cells are then fused with immortal myeloma cells, generating hybridoma cells, that are then screened and selected for antibody production. As hybridoma cells retain the ability to produce antibodies and proliferate indefinitely, large quantities of mAbs can be produced with this method^{75,76}.

Moreover, phage display technology has emerged as an alternative approach for antibody generation⁷⁷. In this method, libraries of human antibody V_H and V_L domains from immunized or infected individuals are genetically inserted into bacteriophage genomes, resulting in the surface expression of diverse antibody fragments. Antibody-displaying phages, that specifically bind to an immobilized antigen of interest, are then selectively enriched and used for further validation. This approach allows for the rapid screening of vast antibody libraries, enabling the identification of high-affinity antibodies without the need for animal immunization^{78,79}. However, the antibodies are generated by random pairing of heavy and light chain and differ from antibodies produced in mammalian cells due to differences in protein folding and post-translational modifications⁸⁰.

Another approach to isolate monoclonal antibodies from humans is the Epstein-Barr Virus (EBV) mediated immortalization of B cells⁸¹. Following EBV-mediated transformation the immortalized, antibody-producing B cells are cloned by limited dilution and then screened for their antigen specificity and reactivity before individual heavy and light chain pairs are cloned and sequenced⁸².

As an alternative, the newly developed technique of single B cell cloning can be used. This technique enables the pre-selection of antigen-specific single B cells from infected or vaccinated donors followed by cloning of the Ig genes and their expression as recombinant monoclonal antibodies⁸³. Following B cell isolation from peripheral blood, they were labeled with antigen-coated magnetic beads^{84,85} or fluorescence-conjugated antigens⁸⁶⁻⁸⁸, which enables the enrichment of antigen-specific B cells over magnetic isolation or fluorescence-activated cell sorting (FACS). After enrichment, the heavy and light

chain sequences are cloned using nested or semi-nested reverse transcription polymerase chain reaction (RT-PCR), with primers specific for the variable leader and constant regions, leading to the amplification of each chain⁸⁹. The V_H and V_L sequences are then cloned into antibody-expression plasmids and were then expressed in cell lines, such as HEK293T, to produce recombinant mAbs⁹⁰. This technique enables the production of mAbs, that are representative of the natural immune response, while maintaining the natural pairing of heavy and light chain.

The Barcode Enabled Antigen Mapping (BEAM) technology from 10x Genomics offers a novel high-throughput method for the identification of antigen-specific B cell clonotypes, enabling the discovery of full-length V(D)J sequences along with an antigen-specificity score. Unlike the traditional single B cell cloning method using fluorescence-conjugated antibodies, BEAM utilizes unique barcoded conjugates loaded with specific antigens to label B cells, allowing for the precise determination of antigen binding to the BCRs of the B cells through single-cell sequencing followed by computational analysis.

Antibodies generated with these methods, can further be optimized using recombinant engineering techniques to enhance the therapeutic efficacy. For example, murine antibodies, that were often immunogenic and show low effector function, can be humanized by grafting murine CDRs onto human antibody frameworks, leading to reduced immunogenicity and longer half-life^{91,92}. To improve stability, extend half-life, or optimize Fc-mediated effector functions, Fc engineering can be applied through site-directed mutagenesis of specific amino acid residues in the Fc region⁹³. Moreover, affinity maturation techniques, including directed evolution and computational modeling, are used to enhance the antigen-affinity for optimization^{94,95}.

1.2.3 Mechanisms of antibodies used for cancer immunotherapy

The therapeutic application of antibodies in immunotherapy relies on effective anti-tumor mechanisms, including the direct inhibition of tumor growth and induction of complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Additionally, antibodies can be used for immune checkpoint inhibition or are engineered for advanced therapeutic strategies, including CAR T cell therapy, antibody-drug conjugates (ADCs), and bispecific T cell engagers (BiTEs)⁹⁶.

Antibodies can be used to disrupt pro-survival signaling pathways in cancer. For example, Cetuximab blocks ligand binding to the epidermal growth factor receptor (EGFR), preventing tumor proliferation⁹⁷, while Trastuzumab inhibits the heterodimerization of the human epidermal growth factor receptor 2 (HER2), leading to tumor cell death⁹⁸.

Moreover, antibodies can mediate cell lysis through CDC, which leads to the activation of the classical complement cascade. Upon binding of IgG or IgM antibodies to the target cells, the Fc region recruits the C1q protein, initiating a sequential proteolytic cleavage of complement proteins that results in the

formation of C3 convertase. This leads to enhanced phagocytosis and immune cell recruitment. Further activation leads to the generation of C5 convertase, which initiates the formation of the membrane attack complex, which forms pores in the target cell membrane, leading to cell lysis⁹⁹. CDC plays a critical role in the therapeutic efficacy of several antibodies, for example of Dinutuximab, that is used for neuroblastoma treatment^{100,101}.

However, the major mode of action of various approved antibodies, including Trastuzumab, Cetuximab and Dinutuximab, is ADCC. The Fab region of IgG antibodies binds to the target cells, marking them for the destruction by immune effector cells, while the Fc region interacts with activating Fcγ receptors (FcγR), such as FcγRI, FcγRIIA and FcγRIIIA, which are expressed on various immune cells, including natural killer (NK) cells, macrophages, neutrophils and dendritic cells¹⁰². Upon FcγR engagement, the effector cells are activated through receptor cross-linking, which triggers downstream signaling. Tumor cells were then lysed by the release of cytotoxic granules, such as perforin and granzyme B from NK cells, which induce the formation of pores in the target cell membrane and induce apoptosis or by Fas/FasL signaling, which leads to caspase-dependent apoptosis in Fas-expressing target cells^{103,104}.

Another class of antibodies, referred to as immune checkpoint inhibitors (ICIs), are used for blocking inhibitory immune checkpoint pathways. This leads to the enhancement of T cell activation and restores anti-tumor immunity¹⁰⁵. A key immune checkpoint molecule, programmed death-ligand 1 (PD-L1), is highly expressed on several malignant cells, allowing tumors to evade immune destruction^{106,107}. Monoclonal antibodies targeting the PD-1/PD-L1 axis, such as Nivolumab and Atezolizumab, restore T cell function by preventing PD-1-mediated T cell exhaustion^{108,109}. Despite the success of ICIs in melanoma and lung cancer^{110,111}, their effectiveness in neuroblastoma is limited, as neuroblastoma is an immunologically “cold” tumor with a highly immunosuppressive environment¹¹². However, combination therapies combining ICIs with other immunotherapeutic strategies have shown promising preclinical results, such as in combination with Dinutuximab^{113,114}.

Moreover, antibodies and their antigen-binding regions can be adapted for advanced immunotherapeutic strategies, including CAR T cell therapy, BiTEs, and ADCs. In CAR T cell therapy, the single chain variable fragment (scFv) of an antibody is incorporated into a CAR, which is linked to intracellular signaling domains, enabling T cells to specifically recognize and eliminate tumor cells¹¹⁵. While CAR T cell therapies have been successfully approved for hematological malignancies^{116,117}, no CAR T cell therapy has yet been approved for solid tumors due to challenges such as the immunosuppressive tumor microenvironment and poor CAR T cell infiltration into solid tumor tissues¹¹⁸. Similarly, BiTEs are composed of two linked antibody fragments, one targeting a tumor-associated antigen and the other binding CD3 on T cells. This leads to a redirection of T cells to the cancer cells¹¹⁹. A key example is Blinatumomab, a BiTE targeting CD19 on B cell malignancies and CD3 on T cells, which has been FDA-approved for the treatment of B cell acute lymphoblastic leukemia (B-ALL)¹²⁰. In case of antibody-drug conjugates, tumor-targeting antibodies were conjugated to cytotoxic drugs, ensuring a targeted cytotoxic effect on the tumor. An example is Brentuximab vedotin, which targets CD30 in

Hodgkin lymphoma and anaplastic large cell lymphoma, where the antibody delivers the potent cytotoxic agent monomethyl auristatin E to the tumor cells¹²¹.

While therapeutic antibodies are primarily designed and engineered for cancer treatment, the immune system naturally produces a diverse repertoire of antibodies that contribute to immune surveillance and tumor recognition. These naturally occurring antibodies can target tumor-associated antigens, altered self-antigens or cancer-specific carbohydrate structures, offering great potential for immunotherapeutic applications.

1.2.4 Naturally occurring antibodies and their role in cancer immunity

Naturally occurring antibodies (nAbs) play a fundamental role in the body's first line defense against pathogens, but they also contribute to immune surveillance by recognizing and eliminating malignant cells^{122,123}. These antibodies, predominantly of the IgM isotype, are constitutively secreted by B1 cells, mainly in the absence of exogenous antigenic stimulation, and are present in the circulation of healthy individuals^{124,125}. Unlike affinity-maturated antibodies, nAbs are germline-encoded and recognize conserved molecular carbohydrate structures present on glycoproteins and glycolipids. These include tumor-associated glycan epitopes and oncofetal antigens^{126–128}.

As malignant cells often fail to trigger strong adaptive immune responses, the immune system relies on other mechanisms for tumor surveillance. nAbs contribute to this defense by inducing apoptosis and CDC^{125,127,129}. Evidence for the immune surveillance function of nAbs was provided by Brändlein *et al.*, who isolated several human monoclonal IgM antibodies from cancer patients that specifically recognized cancer cells with minimal reactivity to healthy cells. These antibodies were effective in inhibiting carcinoma cell proliferation and inducing apoptosis¹³⁰. One example, the antibody PAM-1, was isolated from a patient with gastric carcinoma. It specifically targets a post-transcriptionally modified version of the cysteine-rich fibroblast growth factor receptor 1 (CFR-1), which is overexpressed in epithelial cancer, but is absent in healthy tissue. Binding of PAM-1 triggers apoptosis both *in vitro* and *in vivo*, suggesting its putative role as a therapeutic antibody^{129,131}.

Similarly, nAbs derived from peripheral blood of healthy donors have been shown to mediate complement-dependent tumor cell lysis in human melanoma and neuroblastoma cells. Interestingly, the titer of these cytotoxic antibodies remained stable over a period of 3.5 years in one donor, who had no extraordinary history of infections. This suggests that the antibodies were not induced by infection but are naturally occurring¹³². Interestingly, these cytotoxic antibodies recognized gangliosides, such as GD2 and GD3¹³², that are highly expressed on melanoma and neuroblastoma cells. Moreover, GM3-targeting nAbs were predominantly found in the sera of healthy young individuals, with their levels and cytotoxic effects on GM3-expressing tumor cells decreasing significantly with age. Interestingly, these nAbs were absent or at very low level in patients with non-small cell lung cancer, suggesting a protective role of nAbs secreted by B1 cells against malignant transformation¹³³.

Given their ability to recognize tumor-associated carbohydrate antigens, induce apoptosis and activate CDC, nAbs present a promising approach for cancer immunotherapy. Moreover, nAbs derived from healthy individuals may offer advantages over engineered antibodies, particularly in terms of safety and potential side effects. As gangliosides are among the targets of nAbs, other gangliosides, besides GD2, which is already targeted in neuroblastoma therapy, could serve as promising targets for novel immunotherapeutic approaches.

1.3 Gangliosides as immunotherapeutic targets in neuroblastoma

Glycolipids are ubiquitous components of cell membranes, predominantly localized in the outer leaflet of the plasma membrane across various tissue types. They consist of one or more carbohydrate residues covalently linked to a hydrophobic lipid moiety¹³⁴. A major class of glycolipids, known as glycosphingolipids, contain a ceramide backbone as the hydrophobic lipid tail. Within this group, a specialized subclass containing sialic acid residues, is referred to as gangliosides.

Gangliosides are expressed throughout the human body, but their distribution varies depending on tissue type, developmental stage, and physiological state^{135,136}. They are particularly enriched in neuronal cells in the central and peripheral nervous system¹³⁷, where they play key roles in neuronal differentiation and signal transduction. They can also be found at a lower levels in non-neuronal cells, such as immune and epithelial cells¹³⁸. However, altered ganglioside expression is frequently observed in malignant cells, contributing to tumor progression, metastasis and immune evasion^{136,139}.

1.3.1 Structure and biosynthesis of gangliosides

Gangliosides are composed of a hydrophobic ceramide, that is linked to an hydrophilic oligosaccharide chain, containing different carbohydrate residues and one or more sialic acid residues, such as N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc), or, less commonly, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (Kdn)¹⁴⁰. Thereby, their hydrophobic ceramide structure is anchored in the cell surface lipid bilayer and their hydrophilic carbohydrate moiety extends into the extracellular environment¹⁴¹. Gangliosides are named and classified based on the number of sialic acid residues that are attached to the oligosaccharide moiety. The “G” thereby indicates the belonging to the ganglio-series of glycosphingolipids, whereas the subsequent letters indicate the number of sialic acid residues present: A (asialo-), M (mono-), D (di-), T (tri-), Q (quadri-) and P (penta-). The numerical designation further specifies the structural variations within the ganglioside family. Unlike these, GM4 belongs to the gala-series of glycosphingolipids. Additionally, gangliosides are categorized into 0-, a-, b-, and c-series, based on the number of sialic acid residues attached to the innermost galactose. The 0-series lacks sialic acid, whereas the a-, b-, and c-series contain one, two, and three sialic residues, respectively^{138,142}.

Gangliosides are synthesized in the endoplasmic reticulum (ER) and Golgi apparatus, starting with the formation of ceramide. The enzyme UDP-glucose ceramide glucosyltransferase (UGCG) catalyzed the transfer of a glucose molecule to ceramide, forming glucosylceramide (GlcCer), which is then transported to the Golgi apparatus, where additional sugar residues, such as galactose and N-acetylgalactosamine, are sequentially added by glycosyltransferases. Subsequently, sialyltransferases add sialic acids, leading to the formation of GM3, GD3 and GT3, which serve as precursors for more complex gangliosides. The structural diversity arises through the sequential addition of more sialic acid residues or other sugar moieties, catalyzed by various glycosyltransferases and sialyltransferases (**Figure 3**). For example, the β -1,4-N-acetylgalactosaminyltransferase 1 (B4GALNT1) is responsible for the formation of the gangliosides GM2 and GD2. Once synthesized, gangliosides are transported to the plasma membrane^{138,143}.

The diversity of ganglioside structures arises from variations in glycan composition, sialylation patterns, and ceramide tail modifications, resulting in over 200 distinct ganglioside structures¹⁴⁴. Furthermore, sialic acids undergo biochemical modifications such as acetylation, methylation, and sulfation, further contributing to their structural and functional heterogeneity¹⁴⁵. In mammals, the most common sialic acids are Neu5Ac and Neu5Gc. In humans, only Neu5Ac is synthesized *de novo*, as a mutation in the gene of the enzyme cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMAH) prevents the conversion of Neu5Ac to Neu5Gc¹⁴⁶. However, Neu5Gc is still found in human tissues, including some tumors, likely due to dietary intake¹⁴⁷.

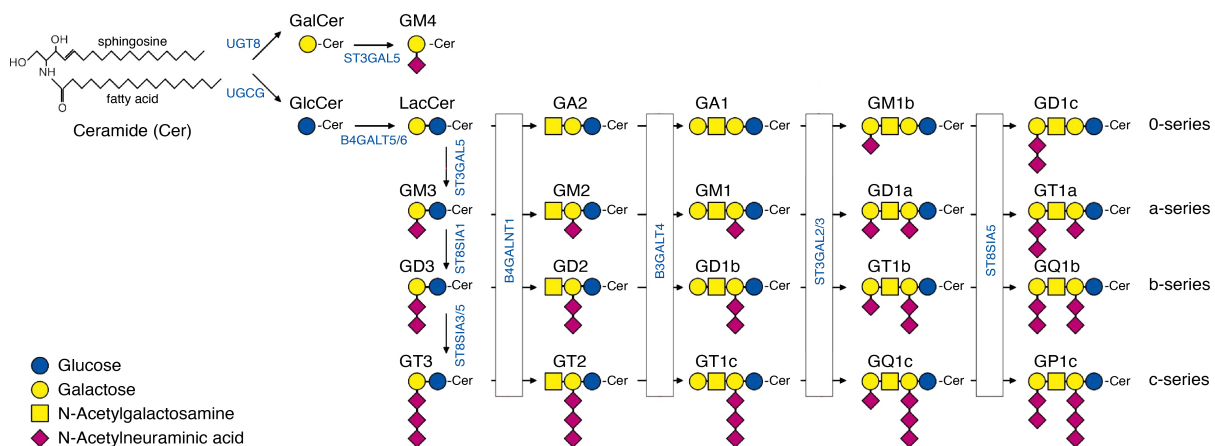


FIGURE 3 | The biosynthesis of gangliosides. The biosynthetic pathway of gangliosides starts from the ceramide backbone. Enzymatic steps catalyzed by glycosyltransferases and sialyltransferases sequentially add sugar residues, leading to the formation of more complex gangliosides from the 0-, a-, b- and c-ganglio-series. Illustration is adapted from Sipione *et al.*¹³⁸.

1.3.2 Role of gangliosides in cellular function and cancer progression

Gangliosides regulate critical cellular processes, including signal transduction, cell proliferation, differentiation, adhesion, and immune modulation^{148–151}. Knockout studies in mice have demonstrated their essential roles in embryonic development and neurogenesis. For instance, deletion of B4GALT5,

an enzyme involved in lactosylceramide (LacCer) synthesis, results in embryonic lethality¹⁵². Similarly, the combined loss of ST3GAL5 and B4GALNT1 leads to severe neurodegeneration in mice¹⁵³. Due to their location in lipid rafts, gangliosides modulate receptor tyrosine kinase (RTK) signaling by directly influencing growth factor receptors, such as the epidermal growth factor receptor (EGFR) and vascular endothelial growth factor receptor (VEGFR)¹⁵⁴. Notably, while GM3 inhibits both the EGFR and the VEGFR^{155,156}, GD1a strongly enhanced VEGF-induced endothelial cell proliferation and migration¹⁵⁷.

During oncogenic transformation, cancer cells often show aberrant glycosylation pattern, including dysregulation of ganglioside expression due to alterations in glycosyltransferase activity¹⁵⁸. This leads to overexpression of tumor-associated gangliosides, such as GD2 and GM2, which are found in many cancers, including neuroblastoma and melanoma^{45,159}. GD2 and GD3, are associated with cell growth and invasiveness in various tumors, including melanoma and small cell lung cancer^{160–162}. GM2 was associated with enhanced proliferation, invasion and metastasis^{163,164}. Interestingly, the expression pattern can change by cancer progression as metastatic tumors tend to have a different ganglioside expression profile compared to the primary tumors^{165,166}.

Additionally, gangliosides, such as GD2, can also be found in the plasma of neuroblastoma patients, suggesting a potential use as biomarker¹⁶⁷. The shedding of gangliosides in the microenvironment can further facilitate tumor progression and actively suppress immune responses^{165,166}. Notably, gangliosides might be considered as checkpoint-like molecules, as they can inhibit the action of multiple cells of the immune system, such as T cells, B cells, NK cells and dendritic cells¹³⁶. For example, GD3 can suppress NK cell cytotoxicity, through interaction with sialic-acid binding immunoglobulin-like lectin 7 (Siglec-7)¹⁶⁸. Moreover, also GM3 and GM2 were capable of inhibit NK cell function and cytotoxicity¹⁶⁹. Additionally, GM2 expression was associated with a downregulation of cytokine production, T cell dysfunction and T cell apoptosis¹⁷⁰ and leads to the inhibition of Ig production in B cells due to an impaired IL-10 and TNF α production¹⁷¹.

Giving their high aberrant expression in cancer cells along with their immunosuppressive properties, gangliosides represent promising targets for cancer immunotherapies.

1.3.3 Ganglioside-based immunotherapies

Due to their overexpression in some cancer cells and their crucial role in tumor progression, gangliosides have been explored as targets for various immunotherapeutic strategies¹⁷². These approaches include active vaccination to prime the immune system against ganglioside-expressing tumors and monoclonal antibodies that mediate direct cytotoxicity.

The most notable example is Dinutuximab, a chimeric monoclonal antibody approved for neuroblastoma treatment⁵¹. But several additional preclinical and clinical trials have been completed or are ongoing to investigate ganglioside-targeting approaches¹⁷³. For instance, patients with small cell lung cancer vaccinated with synthetic Fucosyl-GM1 conjugated to keyhole limpet hemocyanin (KLH) developed

IgM antibodies capable of inducing potent CDC against tumor cells¹⁷⁴. Similarly, GM2 vaccination has been shown to activate T cells, enhance immunogenicity, and improve survival rates in melanoma patients^{175,176}. Moreover, a clinical trial evaluating the efficacy of Racotumomab-alum, an anti-idiotypic vaccine targeting GM3(Neu5Gc), showed tolerability and enhanced overall survival in advanced non-small cell lung cancer patients¹⁷⁷. The application of the anti-GD3 antibody R24 in combination with IL-2 in patients with metastatic melanoma enhanced T cell activation and increased ADCC against GD3 expressing cells¹⁷⁸.

Beyond antibodies and vaccine-based strategies, CAR T cell therapy targeting gangliosides has emerged as a promising approach in cancer immunotherapy. CAR T cells engineered against GD2 and GM2 are currently being tested in clinical trials (NCT03373097, NCT05192174) for relapsed or refractory high-risk neuroblastoma and solid GM2-expressing tumors, respectively^{56,179}.

This highlights the therapeutic potential of ganglioside-targeting immunotherapies, supporting further development also for high-risk neuroblastoma.

1.3.4 Discovery of GM2 as a potential target for immunotherapy in neuroblastoma

Several studies have demonstrated that naturally occurring IgM antibodies, present in the sera of 1.8% to 4.3% of healthy individuals, exhibit cytotoxicity against neuroblastoma cells^{132,180}. Ollert *et al.* demonstrated that these antibodies induce CDC in neuroblastoma cells, with effectiveness ranging from 40% to 95%, while showing no reactivity against normal human fibroblasts, melanocytes, or epidermal keratinocytes. Notably, these nAbs were absent in the sera of neuroblastoma patients^{180–182}. In animal models, injection of purified human IgM from cytotoxic sera into nude rats with solid human neuroblastoma tumors resulted in complement activation, neutrophil granulocyte accumulation, and tumor growth retardation¹⁸³. Furthermore, a phase I/II study using plasma from donors with high *in vitro* cytotoxicity against neuroblastoma cells was conducted in nine children with recurrent, metastatic neuroblastoma. This treatment involved complete plasma exchange over one to ten cycles and showed no severe side effects. Some patients experienced minor tumor responses, while one patient achieved stable disease for 3 years. Notably, another patient, who received ten plasmapheresis cycles, achieved long-term disease-free survival¹⁸². While Ollert *et al.* were not able to identify the specific target of the antibodies, Devarapu *et al.* proposed that the epitopes recognized by these antibodies were GD2 and GD3. This suggestion was based on experiments using *Vibrio cholerae* neuraminidase (VCN), which removes terminal Neu5Ac from sialylated glycoconjugates on cell surfaces. After VCN treatment, the cells expressed only asialoglycosphingolipids, which could no longer be recognized by the serum-derived antibodies. In contrast, previous studies in our working group demonstrated that the cytotoxic effect of these nAbs is independent of GD2 and GD3 expression on neuroblastoma cells. Specifically, the binding and cytotoxicity was not reduced after treatment of neuroblastoma cell lines with VCN. However, further biochemical modifications of glycosylation pathways, using inhibitors, revealed that

the target was an N-glycosylated carbohydrate. Moreover, this target was confirmed to be a low-complexity ganglioside, as shRNA-mediated knockdown of B4GALNT1, the enzyme responsible for the biosynthesis of GA2, GM2, GD2, and GT2, led to reduced cell lysis. Since GM2 could not be hydrolyzed by VCN, likely due to steric hindrance¹⁸⁴, and because it was reported to be expressed in neuroblastoma¹⁵⁹, GM2 was selected as the most convincing target responsible for the observed cytotoxic effect in neuroblastoma cells.

However, efforts to isolate large quantities of these cytotoxic nAbs from serum or to identify the B cells producing them, using techniques such as affinity chromatography and EBV immortalization, have so far been unsuccessful. Therefore, new methods are needed to isolate neuroblastoma-specific B cells, identify the antibody sequence, and produce the antibodies recombinantly on a larger scale for therapeutic use.

1.4 Aim of this work

Given the poor prognosis and high relapse rates despite advances in multimodal treatment strategies, high-risk neuroblastoma remains a major clinical challenge in pediatric oncology. New therapeutic options are urgently needed to improve patient outcomes.

Certain naturally occurring antibodies in healthy individuals have demonstrated great potential in inducing complement-dependent cytotoxicity against various cancer cells. Increasing evidence suggests that these antibodies specifically target gangliosides, such as GM2, on the surface of neuroblastoma cells, making them a promising approach for novel immunotherapies. However, previous attempts to isolate these naturally occurring antibodies and identify their cognate B cell origin have been unsuccessful.

Therefore, this study aims to identify naturally occurring antibodies targeting gangliosides like GM2 in healthy individuals and evaluate the potential of a GM2-targeted immunotherapy for neuroblastoma.

To achieve this, the specific objectives of this study are:

1. Evaluate GM2 as a therapeutic target by analyzing ganglioside-associated gene expression and assessing the surface expression of GM2 and other gangliosides in neuroblastoma cells.
2. Generate GM2-specific antibodies either by hybridoma technology or by identifying naturally occurring GM2-specific antibodies in healthy individuals, followed by the isolation of corresponding B cells for B-cell receptor (BCR) sequence identification.
3. Validate and characterize GM2-specific antibodies through recombinant expression and functional evaluation.

2 MATERIALS AND METHODS

2.1 Materials

All materials and instruments were provided by the laboratory facilities of the Research Institute Children's Cancer Center Hamburg, the Department of Pediatric Hematology and Oncology at the University Medical Center Hamburg-Eppendorf, the Leibniz Institute of Virology and the Single Cell Core Facility at the University Medical Center Hamburg-Eppendorf.

2.1.1 Chemicals, media, reagents and solutions

Table 1 shows the chemicals, media, reagents and solutions used for experimental procedures.

TABLE 1 | Chemicals, media, reagents and solution.

Name	Provider	Cat. No.
Adjuvant MM	Gerbu Biotechnik (Heidelberg, Germany)	3001
Agarose	Life Technologies (Carlsbad, USA)	16500100
Aminoglucitol-biotinylated (HOCH ₂ (HOCH) ₄ (CH) ₂ NH-Biotin)	GlycoNZ (Auckland, New Zealand)	0000-BM
Ampicillin	Carl Roth GmbH (Karlsruhe, Germany)	15140148
Blasticidin solution	Invivogen (San Diego, USA)	ant-bl-1
Bovine serum albumin (BSA)	Carl Roth GmbH (Karlsruhe, Germany)	8076.4
Calcium chloride (CaCl ₂)	Carl Roth GmbH (Karlsruhe, Germany)	HN04.1
CD40-Ligand (CD40L) Multimer Kit, human	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-098-775
Chloroquine diphosphate	Sigma Aldrich (St. Louis, USA)	C6628
DAPI (4',6-Diamidino-2-phenylindole dihydrochloride)	Merck (Darmstadt, Germany)	D9542
Dimethyl sulfoxide (DMSO)	AppliChem (Darmstadt, Germany)	A3672
Dithiothreitol (DTT)	Carl Roth (Karlsruhe, Germany)	6908.1
DNA Loading Dye (5×)	Qiagen (Hilden, Germany)	239901
dNTP-Mix, peqGOLD	Avantor (Radnor, USA)	20-30-11
DreamTaq PCR Master Mix (2×)	Thermo Fisher Scientific (Waltham, USA)	K1071
Dulbecco's Modified Eagle Medium (DMEM)	Gibco (Carlsbad, USA)	41965-039
Dulbecco's Phosphate Buffered Saline (DPBS)	Gibco (Carlsbad, USA)	14190-094
eFluor670 Proliferation Dye	Thermo Fisher Scientific (Waltham, USA)	65-0840-85
Ethanol	Merck (Darmstadt, Germany)	107017
Ethylene diamine tetra acetic acid (EDTA)	Th. Geyer GmbH (Renningen, Germany)	2281.1000
FastDigest BamHI	Thermo Fisher Scientific (Waltham, USA)	FD0054
FastDigest BglII	Thermo Fisher Scientific (Waltham, USA)	FD0083
FastDigest EcoRI	Thermo Fisher Scientific (Waltham, USA)	FD0274
FastDigest NcoI	Thermo Fisher Scientific (Waltham, USA)	FD0574

FastDigest NotI	Thermo Fisher Scientific (Waltham, USA)	FD0596
FastDigest XhoI	Thermo Fisher Scientific (Waltham, USA)	FD0694
FastDigest Buffer (10×)	Thermo Fisher Scientific (Waltham, USA)	B64
Fetal bovine serum (FBS)	Thermo Fisher Scientific (Waltham, USA)	10270106
Ficoll-Paque PLUS	Cytiva (Marlborough, USA)	17144003
GelRed Nucleic Acid Stain	Biotium, Inc. (Fremont, USA)	41003
GeneRuler 100 bp plus DNA Ladder	Thermo Fisher Scientific (Waltham, USA)	SM0321
GeneRuler 1 kb plus DNA Ladder	Thermo Fisher Scientific (Waltham, USA)	SM0311
Gerbu Adjuvant MM	Gerbu Biotechnik (Heidelberg, Germany)	3001
Glycin	Carl Roth (Karlsruhe, Germany)	3908.2
GM2-KLH (GalNAc β 1-4(Neu5Ac α 2-3)Gal β 1-4Glc-NH-KLH)	Elicityl (Crolles, France)	GLY093-KLH
GM2-biotinylated (GalNAc β 1-4(Neu5Ac α 2-3)Gal β 1-4Glc-NAC-CH ₂ -(1.4-Tz)-(CH ₂) ₂ -EG3-NH-Biotin)	Elicityl (Crolles, France)	GLY093-NAC-sp3-Bt
GM2-biotinylated (Neu5Ac α 2-3(GalNAc β 1-4)Gal β 1-4Glc β -NHCOCH ₂ NH-Biotin)	GlycoNZ (Auckland, New Zealand)	0835a-BM
GM2-PAA (Neu5Ac α 2-3(GalNAc β 1-4)Gal β 1-4Glc β -NHCOCH ₂ NH-Poly[N-(2-hydroxyethyl)acrylamide-co-N-acrylamide])	GlycoNZ (Auckland, New Zealand)	0835a-BP
HAT Supplement (50X)	Gibco (Carlsbad, USA)	21060-017
Horse serum (HS)	Sigma Aldrich (St. Louis, USA)	H1270
Human serum albumin (HSA), biotinylated	abcam (Cambridge, UK)	ab8033
Human TruStain FcX (Fc Receptor Blocking Solution)	BioLegend (San Diego, USA)	422302
Hygromycin B Gold	Invivogen (San Diego, USA)	ant-hg5
Interleukin-2 (IL-2), human	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-097-748
Interleukin-21 (IL-21) Recombinant Protein, human	Gibco (Carlsbad, USA)	PHC0215
Iscove's Modified Dulbecco's Medium (IMDM) + GlutaMAX	Gibco (Carlsbad, USA)	31980-030
Isopropanol	Th. Geyer GmbH (Renningen, Germany)	1136.1000
KLD Enzyme Mix	New England Biolabs (Ipswich, USA)	M0554
KLD Reaction Buffer	New England Biolabs (Ipswich, USA)	B0554
L-Glutamine (200 mM)	Gibco (Carlsbad, USA)	25030-024
LB agar	Carl Roth (Karlsruhe, Germany)	X969.1
LB medium	Carl Roth (Karlsruhe, Germany)	X964.2
MACSQuant Tyto Running Buffer	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-107-207
Minimum Essential Medium Eagle (MEM)	Sigma Aldrich (St. Louis, USA)	M8042
NuPAGE MES SDS Running Buffer (20×)	Thermo Fisher Scientific (Waltham, USA)	NP000202
PageRuler Plus Prestained Protein Ladder	Thermo Fisher Scientific (Waltham, USA)	26619
Para-nitrophenyl phosphate (pNPP)	Mabtech (Nacka Strand, Sweden)	3652-P10
Paraformaldehyde (PFA)	Carl Roth (Karlsruhe, Germany)	7398.1

Penicillin (10,000 U/ml)/Streptomycin (10,000 µg/ml)	Gibco (Carlsbad, USA)	15140-122
Phalloidin-iFluor555	abcam (Cambridge, UK)	ab176756
Pierce ECL Western Blotting substrate	Thermo Fisher Scientific (Waltham, USA)	10590624
Polybrene	Sigma Aldrich (St. Louis, USA)	TR-1003-G
Polyethylene Glycol 1500 (PEG 1500)	Sigma Aldrich (St. Louis, USA)	10783641001
Propidium Iodide Solution	BioLegend (San Diego, USA)	421301
Q5 High-Fidelity DNA Polymerase	New England Biolabs (Ipswich, USA)	M0491S
Q5 Reaction Buffer	New England Biolabs (Ipswich, USA)	B9027S
RPMI Medium 1640	Gibco (Carlsbad, USA)	21875-034
SOC Outgrowth Medium	New England Biolabs (Ipswich, USA)	B9020S
Sodium Pyruvate (100 mM)	Gibco (Carlsbad, USA)	11360-039
T4 DNA Ligase	Thermo Fisher Scientific (Waltham, USA)	EL0012
T4 DNA Ligase Buffer	Thermo Fisher Scientific (Waltham, USA)	46300018
Tris-(hydroxymethyl)-aminomethan (Tris)	Carl Roth (Karlsruhe, Germany)	4855.2
Tris acetate-EDTA (TAE) buffer (50×)	PanReac AppliChem (Darmstadt, Germany)	A1691
Trypan blue solution (0.4%)	Carl Roth GmbH (Karlsruhe, Germany)	1680.1
Trypsin-EDTA (0.05%)	Gibco (Carlsbad, USA)	25300-054
Tween-20	Carl Roth GmbH (Karlsruhe, Germany)	8022.0500
ViabilityDye 405/452	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-130-420

2.1.2 Software and (online) tools

Packages used for *in silico* analysis in R are listed in **Table 2**. Software and tools used for this work are listed in **Table 3**. For grammatical refinement and proofreading, the large language model GPT-4 (OpenAI, San Francisco, USA) was utilized.

TABLE 2 | R packages.

R package	Version
clusterprofiler	4.12.6
DOSE	3.30.5
enrichplot	1.24.4
ESTIMATE	1.0.13
fgsea	1.30.0
ggplot2	3.5.1
limma	3.60.6
MCPcounter	1.2.0
pheatmap	1.0.12
rtzne	0.17
survival	3.8.3

TABLE 3 | Software and tools.

Name	Provider	Version
ABodyBuilder2	SABPred tools by Oxford Protein Informatics Group (Oxford, United Kingdom) ¹⁸⁵	-
ANARCI	SABPred tools by Oxford Protein Informatics Group (Oxford, England) ¹⁸⁶	-
CCTop	University of Heidelberg (Heidelberg, Germany) ¹⁸⁷	-
Cell Ranger	10x Genomics (Pleasanton, USA)	7.1.0
Clustal Omega	European Bioinformatics Institute (Hinxton, United Kingdom) ¹⁸⁸	1.2.4
FlowJo	Becton, Dickinson & Company (Franklin Lakes, USA)	10.9.0
GraphPad Prism	GraphPad Software (Bosten, USA)	8.3.0
ImageJ	National Institutes of Health (Bethesda, USA)	2.0.0
Incucyte Software	Sartorius AG (Göttingen, Germany)	2021A
Loupe V(D)J Browser	10x Genomics (Pleasanton, USA)	5.1.0
MACSQuant Poisson Calculator	Miltenyi Biotec (Bergisch-Gladbach, Germany)	-
NEB Ligation Calculator	New England Biolabs (Ipswich, USA)	-
NEB Tm Calculator	New England Biolabs (Ipswich, USA)	-
PyMOL	Schrödinger, Inc (New York, USA)	3.1.1
R	R Foundation for Statistical Computing	4.4.1
RStudio	Posit (Boston, USA)	2024.9.0.375
SkaniIt	Thermo Fisher Scientific (Waltham, USA)	7.1
TIDE	Netherlands Cancer Institute (NKI) (Amsterdam, Netherlands) ¹⁸⁹	-

2.1.3 Instruments

Instruments used for experimental work are listed in **Table 4**.

TABLE 4 | Instruments.

Name	Provider
Countess 3 Automated Cell Counter	Thermo Fisher Scientific (Waltham, USA)
Incubator Heracell 240i CO2	Thermo Fisher Scientific (Waltham, USA)
Incucyte SX5 Live-Cell Analysis System	Sartorius AG (Göttingen, Germany)
MACSQuant Tyto Cell Sorter	Miltenyi Biotec (Bergisch-Gladbach, Germany)
MACSQuant X Flow Cytometer	Miltenyi Biotec (Bergisch-Gladbach, Germany)
Megafuge ST4 Plus	Thermo Fisher Scientific (Waltham, USA)
Nikon Eclipse Ts2R Microscope	Nikon Corporation (Tokyo, Japan)
Nikon Eclipse Ti2 Microscope	Nikon Corporation (Tokyo, Japan)
Odyssey XF Imaging System	LI-COR Biosciences (Lincoln, USA)
Sorvall LYNX 4000 Superspeed Centrifuge	Thermo Fisher Scientific (Waltham, USA)
Thermal Cycler Biometra T Advanced	Analytik Jena (Jena, Germany)
Varioskan LUX Multimode Microplate Reader	Thermo Fisher Scientific (Waltham, USA)

2.1.4 Cell lines

In this work various human neuroblastoma cell lines were used (**Table 5**). HEK293T were used for the antibody production, whereas Sp2/0-Ag4 cells were used for the hybridoma generation. T47D were used for the validation of the Trastuzumab IgM production. All cell lines used were verified by short tandem repeat polymerase chain reaction (STR-PCR) and were mycoplasma-negative.

TABLE 5 | Cell lines, characteristics and corresponding cultivation medium.

Cell line	Characteristics (species, origin, gender, growth properties, morphology, description) *	Cultivation medium	RRID
HEK293T	Human, embryonal kidney, adherent, epithelial-like, expresses the SV40 large T antigen	DMEM complete growth medium	CVCL_0063
Kelly	Human, neuroblastoma (brain), female, adherent, neuronal-like, genomic amplification of MYCN	RPMI complete growth medium	CVCL_2092
LAN-1	Human, neuroblastoma (bone marrow metastasis, stage IV), male, slightly adherent, MYCN amplification	RPMI complete growth medium	CVCL_1827
LS	Human, neuroblastoma (abdominal tumor, stage III), female, adherent, MYCN amplification	RPMI complete growth medium	CVCL_2105
NK-92	Human, lymphoblastic leukemia, non-adherent	NK medium	CVCL_2142
NK-92-pBullet.US2011.GFP (GM2-CAR)	NK-92 expressing a GM2-specific chimeric antigen receptor	NK medium	-
SH-EP	Human, neuroblastoma (bone marrow metastasis), female, adherent, epithelial-like, stably express a GFP reporter and Luciferase gene, clonal subline of SK-N-SH	RPMI complete growth medium	CVCL_0524
SH-SY5Y	Human, neuroblastoma (bone marrow metastasis), female, adherent, neuronal, clonal subline of SK-N-SH	RPMI complete growth medium	CVCL_0019
SK-N-AS	Human, neuroblastoma (bone marrow metastasis), female, adherent, epithelial-like, MYCN amplification	RPMI complete growth medium	CVCL_1700
SK-N-LO	Human, neuroblastoma (bone marrow metastasis), male, adherent, epithelial-like, stably express a GFP reporter and Luciferase gene, amplification of MYCN	RPMI complete growth medium	CVCL_4569
SK-N-SH	Human, neuroblastoma (bone marrow metastasis), female, adherent, epithelial-like	RPMI complete growth medium	CVCL_0531
Sp2/0-Ag14	Murine, suspension, hybrid between BALB/c spleen cells and the myeloma cell line P3X63AG8	DMEM complete growth medium	CVCL_2199
T47D	Human, breast cancer, female, adherent, HER2 expression	DMEM complete growth medium	CVCL_0553

*Cell characteristics were taken from ATCC, Cellosaurus, and DSMZ.

2.1.5 Cell culture media

The media used for cell culturing is detailed in **Table 6**.

TABLE 6 | Cell culture media and composition.

Name	Composition
B cell medium	IMDM + 10% heat-inactivated FBS + 6 U/ml (0.5 µg/ml) crosslinked CD40L + 100 U/ml IL-2 + 0.05 µg/ml IL-21
DMEM complete growth medium	DMEM + 10% heat-inactivated FBS
NK medium	Minimum Essential Medium Eagle + 12.5% heat-inactivated FBS + 12.5% heat-inactivated HS + 1% L-glutamine + 1% penicillin/streptomycin + 100 U/ml IL-2
RPMI complete growth medium	RPMI 1640 + 10% heat-inactivated FBS + 1% penicillin/streptomycin + 0.5% sodium pyruvate + 1% L-glutamine

2.1.6 Buffers

The composition and preparation details of the buffers used are provided in **Table 7**.

TABLE 7 | Composition of buffers.

Name	Composition and preparation
B cell isolation buffer	1× PBS + 0.5% heat-inactivated FBS + 2 mM EDTA
FACS Buffer	1× PBS + 2% heat-inactivated FBS
TBS (10×)	Dissolve 24 g of Tris and 88 g of NaCl in 900 ml H ₂ O. Adjust pH to 7.6 with HCl. Adjust final volume to 1000 ml.
Towbin Buffer (10×)	0.25 M Tris, 1.92 M Glycine in H ₂ O
Towbin Buffer (1×)	0.025 M Tris, 0.192 M Glycine, 20 % methanol H ₂ O

2.1.7 Antibodies

The following antibodies were used for FACS, immunofluorescence or Western blot (**Table 8**).

TABLE 8 | Antibodies.

Category	Name	Clone	Clonality, host species and isotype	Provider	Cat. No.
Primary antibody	Anti-CD19, APC-Vio770	LT19	monoclonal, mouse IgG1κ	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-113-166
	Anti-Biotin, PE	Bio3-18E7	monoclonal, mouse IgG1	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-113-853
	Anti-Biotin, VioBlue	Bio3-18E7	monoclonal, mouse IgG1	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-113-857
	Anti-GM1, unconjugated	-	polyclonal, rabbit IgG	Biorbyt (Cambridge, UK)	Orb312249
	Anti-GM2(Neu5Ac), unconjugated,	E1	monoclonal, mouse IgG1κ	Absolute Antibody Ltd (Cleveland, UK)	Ab01184-1.1

	Anti-GM2(Neu5Ac/ Neu5Gc), unconjugated	KM966	monoclonal, human IgG1κ	Production by Centre for Structural Systems Biology (CSSB), Hamburg	-
	Anti-GM2(Neu5Ac), unconjugated	MK1-16	monoclonal, mouse IgMκ	TCI Chemicals (Tokyo, Japan)	A2576
	Anti-GM3(Neu5Ac), unconjugated	GMR6	monoclonal, mouse IgMκ	TCI Chemicals (Tokyo, Japan)	A2582
	Anti-GD2(Neu5Ac), unconjugated	14G2a	monoclonal, mouse IgG2a	Becton, Dickinson & Company (Franklin Lakes, USA)	554272
	Anti-GD2(Neu5Ac), APC/Fire750	14G2a	monoclonal, mouse IgG2a, κ	BioLegend (San Diego, USA)	357322
	Anti-GD3(Neu5Ac), unconjugated	R24	monoclonal, mouse IgG3κ	abcam (Cambridge, UK)	ab11779
	Anti-Human IgG (H+L)	-	polyclonal, rabbit IgG	abcam (Cambridge, UK)	ab6715
	Anti-Kappa Light Chain	EPR5367-8	monoclonal, rabbit IgG	abcam (Cambridge, UK)	ab124727
Isotype Control	Isotype Ctrl Antibody, APC/Fire 750	MOPC-173	monoclonal, mouse IgG2a, κ	BioLegend (San Diego, USA)	400284
Secondary antibody	Anti-Human IgG + IgM (H+L), Biotin-SP	-	polyclonal, goat IgG	Jackson Immuno Research Laboratories Ltd. (West Grove, USA)	109-065-044
	Anti-Human IgG + IgM (H+L), Alexa Fluor 488	-	polyclonal, goat IgG	Jackson Immuno Research Laboratories Ltd. (West Grove, USA)	109-545-044
	Anti-Human IgG, APC	IS11-3B2.2.3	monoclonal, mouse IgG1κ	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-119-772
	Anti-Human IgM, APC	PJ2-22H3	Monoclonal, mouse IgG1	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-122-915
	Anti-Mouse IgG + IgM (H+L), HRP	-	polyclonal, goat IgG	Thermo Fisher Scientific Inc. (Waltham, USA)	A-10677
	Anti-Rabbit IgG H+L, HRP	-	polyclonal, goat IgG	abcam (Cambridge, UK)	ab205718
	Anti-Human IgM Fc, HRP	-	polyclonal, goat IgG	Novus Biologicals (Wiesbaden, Germany)	NB7439
	Anti-Mouse IgG + IgM (H+L), Alexa Fluor 488	-	polyclonal, goat IgG	Jackson Immuno Research Laboratories Ltd. (West Grove, USA)	115-545-044
	Anti-Mouse IgM, APC	RMM-1	monoclonal, rat IgG2a, κ	BioLegend (San Diego, USA)	406509
	Anti-Mouse IgG, APC	-	polyclonal, goat IgG	BioLegend (San Diego, USA)	405308
	Anti-Rabbit IgG (H+L), Alexa Fluor 647	-	polyclonal, donkey IgG	abcam (Cambridge, UK)	ab150063

2.1.8 Primer

HPLC-purified primers were obtained from Metabion (Planegg, Germany) and supplied pre-dissolved at a concentration of 100 mM (Table 9).

TABLE 9 | Primers for cloning and sequencing. The sequences starting with “sgRNA” were used for the generation of the B4GALNT1-specific guide RNAs and were designed using the CCTop online tool. Primers starting with “Seq” were used for Sanger sequencing, which was performed by Microsynth (Balgach, Switzerland).

Name	Sequence (5' – 3')
sgRNA_B4GALNT1_fw	CACCGTTATCGGCAGCTGCTGAGCG
sgRNA_B4GALNT1_rev	AAACCGCTCAGCAGCTGCCGATAAC
TIDE_B4GALNT1_fw	GTGGGTGGACGACGACTTCG
TIDE_B4GALNT1_rev	TGCAGCCTAGGAGGAGAGGC
Seq_TIDE_B4GALNT1	GCCTTGAACAGGCTAAGGCC
Clonotype02_VH_EcoRI_fw	CTCCGAATTCGAGGTGCAGCTGTTGGAGT
Clonotype02_VH_NcoI_rev	TAGCCCATGGTGAGGAGACGGTGACCAGGG
Clonotype02_VL_BglII_rev	GTACGAGATCTTTTGATCTCCAGCTTGGTCCC
Clonotype02_VL_XhoI_fw	GGTCTCGAGGACATCCAGATGACCCAGTCTCC
Clonotype04_VH_Mut_fw	ATACAGCTCCCCGTGGTACGGGA
Clonotype04_VH_Mut_rev	CCACGTCTGGACCTCGC
Clonotype04_VH_EcoRI_fw	CTCCGAATTCAGGTGCAGCTGGTGCAG
Clonotype04_VH_NcoI_rev	TAGCCCATGGTGAGGAGACGGTGACCG
Clonotype04_VL_XhoI_fw	GGTCTCGAGCAGTCTGCCCTGACTCAGCCTG
Clonotype04_VL_BglII_rev	GTACGAAGATCTAGGACGGTGACCTTGGTCCCA
Clonotype07_VH_EcoRI_fw	CTCCGAATTCGAGGTGCAGCTGGTGGAG
Clonotype07_VH_NcoI_rev	TAGCCCATGGTGAGGAGACGGTGACCAGGG
Clonotype07_VL_XhoI_fw	GGTCTCGAGGAAACTGTGTTGACACAGTCTCCAG
Clonotype07_VL_BglII_rev	GTACGAGAGATCTTGATCTCCACCTTGGTCCCCTC
Clonotype10_VH_EcoRI_fw	CTCCGAATTCGAGGTGCAGCTGGTGGAGTC
Clonotype10_VH_NcoI_rev	TAGCCCATGGTGAGGAGACGGTGACCAGGG
Clonotype10_VL_XhoI_fw	GGTCTCGAGGAAATTGTGTTGACGCAGTCTCCAG
Clonotype10_VL_BglII_rev	GTACGAGATCTTTTGATTTCCACCTTGGTCCCCT
Clonotype19_VH_EcoRI_fw	CTCCGAATTCAGCTGCAGCTGCAGGAG
Clonotype19_VH_NcoI_rev	TAGCCCATGGTGAGGAGACACTGACCAGGGT
Clonotype19_VL_XhoI_fw	GGTCTCGAGCAGCCTGTGCTGACTCAGC
Clonotype19_VL_BglII_rev	GTACGAAGATCTAGGACGGTCAGCTTGGTCC
Clonotype31_VH_EcoRI_fw	CTCCGAATTCAGGTACACCTGAAGGAGTCTGGTCCT
Clonotype31_VH_NcoI_rev	TAGCCCATGGTGAGGAGACGGTGACCGTGGTCCCCT
Clonotype31_VL_XhoI_fw	GGTCTCGAGCAGCCTGTGCTGACTCAGCCACCT
Clonotype31_VL_BglII_rev	GTACGAAGATCTAGGACGGTGACCTTGGTCCCAGTT
Clonotype37_VH_EcoRI_fw	CTCCGAATTCGAGGTGCAGCTGGTGGAGT
Clonotype37_VH_NcoI_rev	TAGCCCATGGTGAGGAGACGGTGACCAGGG
Clonotype37_VL_XhoI_fw	GGTCTCGAGCAGTCTGCCCTGACTCAGCC
Clonotype37_VL_BglII_rev	GTACGAAGATCTAGGACGGTGACCTTGGTCCC
Clonotype41_VH_EcoRI_fw	CTCCGAATTCGAGGTGCAGCTGGTGGAGTC
Clonotype41_VH_NcoI_rev	TAGCCCATGGTGAGGAGACGGTGACCGTGGTC

Clonotype41_VL_XhoI_fw	GGTCTCGAGCAGTCTGCCCTGACTCAGCCTG
Clonotype41_VL_BglII_rev	GTACGAAGATCTAGGACGGTCACCTTGGTCC
Seq_VH_IgG_pViro	AGGACTGTAGGACAGCCGGG
Seq_VH_IgM_pViro	CGTACTTCCCGCCTCTCAGA
Seq_CH_IgG/IgM_pViro	TGGTTTGTCCAACTCATCAATGT
Seq_VL_IgG/IgM_pViro	GAGGCTAATTCTCAAGCCTC
Seq_CL_IgG/IgM_pViro	CGTACGGTGGCGGCGCCATCTGTCTTCATCTTCCCGCCAT
Seq_CAG_pVB	GGCAAAGAATTGGCCACC
Seq_EF1a_pVB	TCAAGCCTCAGACAGTGGTTC
Seq_hlgG1-CH_pVB	CCTCAGGACTCTACTCCCTC

2.1.9 Plasmids

The plasmids listed in **Table 10** were used for cloning, lentiviral particle production, or were generated during this work.

TABLE 10 | Plasmids.

Name	Information
DHC013-pLKO5d.SFFV.hSpCas9.P2A.BSD	Cas9 and blasticidin resistance gene; kindly provided by Dirk Heckl
pL40C-CRISPR.sgRNA_B4GALNT1.EFS.dTomato	sgRNA for B4GALNT1; kindly provided by Laura Rekowski ¹⁹⁰
DHC013-pLKO5d.CRISPR.B4GALNT1.BSD	Modified from DHC013-pLKO5d.SFFV.hSpCas9.P2A.BSD
pMDLg/pRRE	Lentiviral packaging plasmid for gag/pol
pRSV-Rev	Lentiviral packaging plasmid for rev
phCMV-VSV-G	Lentiviral packaging plasmid for VSV-G
pVB_H+L_IgG_KM966/02/04/07/10/19/31/37/41/1846/8258/9543	Antibody heavy and light chain co-expression plasmid, created by VectorBuilder (Chicago, USA); IgG C _H and C _L sequences, kappa or lambda light chains depending on natural isotype (as identified through BCR sequencing of human B cell clonotypes), V _H and V _L of clonotype sequences or KM966 sequence, blasticidin resistance and GFP reporter
pViro_H+L_IgG_KM966/900/10066/11767	Antibody heavy and light chain co-expression plasmid; IgG C _H and C _L sequences, kappa light chain, V _H and V _L of clonotype sequences or KM966 sequence, hygromycin resistance; KM966 plasmid kindly provided by Laura Rekowski ¹⁹⁰
pViro1-Trastuzumab-IgM/κ	Antibody heavy and light chain co-expression plasmid; IgM C _H and C _L sequences, kappa light chain, V _H and V _L of Trastuzumab; Expression of anti-HER2 IgM ¹⁹¹ (Addgene (#61881), Teddington, United Kingdom)
pViro1-Trastuzumab-IgM/κ + restriction sites	Modified from pViro1-Trastuzumab-IgM/κ by integrating restriction sites flanking the V _H and V _L sequences, performed by Laura Rekowski ¹⁹⁰
pViro_H+L_IgM_KM966/02/04/07/10/19/31/37/41/900/10066/11767	Antibody heavy and light chain co-expression plasmid; IgM C _H and C _L , kappa light chain, hygromycin resistance; KM966 plasmid kindly provided by Laura Rekowski ¹⁹⁰

2.1.10 Kits and assays

The following kits and assays were used in this work (**Table 11**).

TABLE 11 | Kits and assays.

Name	Provider	Cat. No.
5' Feature Barcode Kit, 16 rxns	10x Genomics (Pleasanton, USA)	PN-1000541
Anti-ganglioside dot assay	Generic Assays GmbH (Dahlewitz, Germany)	5003
B Cell Isolation Kit II, human	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-091-151
CellTox Green Cytotoxicity Assays	Promega (Madison, Wisconsin, USA)	G8743
Chromium Next GEM Single Cell 5' Kit v2, 16 rxns	10x Genomics (Pleasanton, USA)	PN-1000265
Chromium Next GEM Chip K Single Cell Kit, 16 rxns	10x Genomics (Pleasanton, USA)	PN-1000287
Chromium Single Cell 5' BEAM Core Kit, PE, Set A, 128 rxns	10x Genomics (Pleasanton, USA)	PN-1000539
Chromium Single Cell Human BCR Amplification, 16 rxns	10x Genomics (Pleasanton, USA)	PN-1000253
Dual Index Kit TT Set A, 96 rxns	10x Genomics (Pleasanton, USA)	PN-1000215
Dual Index Kit TS Set A, 96 rxns	10x Genomics (Pleasanton, USA)	PN-1000251
EasySep Human B Cell Isolation Kit	Stemcell Technologies (Vancouver, Canada)	17954
Glycan Array 300	RayBiotech (Peachtree Corners, USA)	GA-Glycan-300-1
Human IgG (ALP) ELISA Flex	Mabtech (Nacka Strand, Sweden)	3850-1AD-6
Human IgM (ALP) ELISA Flex	Mabtech (Nacka Strand, Sweden)	3880-1AD-6
Library Construction Kit, 16 rxns	10x Genomics (Pleasanton, USA)	PN-1000190
NucleoSpin Gel and PCR Clean-Up	Macherey-Nagel (Düren, Germany)	740609.205
NucleoSpin Plasmid EasyPure	Macherey-Nagel (Düren, Germany)	740727.250
NucleoSpin Tissue	Macherey-Nagel (Düren, Germany)	740952.50
NucleoBond Xtra Midi	Macherey-Nagel (Düren, Germany)	740410.100

2.1.11 Consumables

Following consumables were used in this study (**Table 12**).

TABLE 12 | Consumables.

Name	Provider	Cat. No.
BIOFLOAT 96 well cell culture plate	Sarstedt AG & Co. KG (Nümbrecht, Germany)	83.3925.400
Cell strainer 70 µm Nylon	Corning (New York, USA)	352350
LS columns	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-042-401
Filter Filtropur S (0.2 µm/0.45 µm)	Sarstedt AG & Co. KG (Nümbrecht, Germany)	83.1826.001/ 83.1826
MACSQuant Tyto Cartridge	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-106-088
Micro-Fine+ disposable syringes 0.5 ml U-100 29G	Becton, Dickinson & Company (Franklin Lakes, USA)	324824
Microvette 500 LH	Sarstedt AG & Co. KG (Nümbrecht, Germany)	20.1345
Microvette 500 Serum	Sarstedt AG & Co. KG (Nümbrecht, Germany)	20.1343

Nitrocellulose Membrane Amersham Protan 0.45 µm	Cytiva (Marlborough, USA)	10600002
NuPAGE 4-12% Bis-Tris Gel	Thermo Fisher Scientific (Waltham, USA)	NP0322BOX
Pre-Separation Filter 20 µm	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-101-812
Safety Lancets	Sarstedt AG & Co. KG (Nümbrecht, Germany)	85.1018
Safety Multifly 21G	Sarstedt AG & Co. KG (Nümbrecht, Germany)	85.1638.235
S-Monovette EDTA K3E	Sarstedt AG & Co. KG (Nümbrecht, Germany)	04.1901.021
S-Monovette Lithium-Heparin	Sarstedt AG & Co. KG (Nümbrecht, Germany)	01.1608.001
S-Monovette Serum Gel	Sarstedt AG & Co. KG (Nümbrecht, Germany)	02.1388.001
QuadroMACS Separator	Miltenyi Biotec (Bergisch-Gladbach, Germany)	130-090-976
EasySep Magnet	Stemcell Technologies Inc. (Vancouver, Canada)	18000

2.2 Methods

2.2.1 *in silico* analysis of neuroblastoma gene expression data sets

The gene expression data (“GSE49711_SEQC_NB_TUC_G_log2”) and the corresponding clinical data of the neuroblastoma cohort GSE49711 were downloaded from the publicly available Gene Expression Omnibus (GEO) database¹⁹². Similarly, the gene expression data for the dataset GSE89413 (“GSE89413_norm_counts_FPKM_GRCh38.p13_NCBI”) were also downloaded from the GEO database¹⁹³. All analyses were conducted using R within the RStudio environment.

2.2.1.1 Data processing

Patients with an unknown MYCN status in the GSE49711 dataset were excluded from the analysis, resulting in 493 samples available for gene expression analysis. The dataset was filtered to include only protein-coding genes, based on a reference list from the NCBI database. Additionally, genes with low mean expression (< 0.1 across all samples) and low variance (< 0.1 across all samples) were removed to reduce background noise and focus on genes with significant biological variability. In the dataset GSE89413, only the cell lines available in our laboratory were retained for analysis, as they allow for downstream validation of findings through flow cytometry. All other cell lines were excluded from the analysis.

2.2.1.2 Identification of differentially expressed genes

Sample similarity in the GSE49711 dataset was visualized using t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis with the “Rtsne” package. This technique reduces high-dimensional data to a lower-dimensional space, maintaining the relative similarities between data points. Moreover, differentially expressed genes (DEGs) were identified between samples with favorable and worse outcomes using the “limma” package. DEGs were determined based on a log₂ fold change (logFC) threshold of > 0.5 or < -0.5, combined with an adjusted *p*-value < 0.01, using the Benjamini-Hochberg method to control the false discovery rate.

2.2.1.3 Gene set enrichment analysis

To identify key pathways and their associated genes relevant for patient outcomes, a gene set enrichment analysis (GSEA) was performed using the “clusterProfiler”, “enrichplot”, “DOSE” and “fgsea” packages. Thereby, the DEGs were ranked by their fold change, and GSEA was applied using gene sets derived from Kiefer *et al.*¹⁹⁴, that are associated with the Hallmark of Cancer pathways. The “cnetplot()” function from the “clusterProfiler” package was used to create a gene-pathway network plot for immune- and inflammation-related pathways.

2.2.1.4 Immune profiling

To investigate differences in the immune microenvironment associated with worse patient outcomes in the GSE49711 dataset, the ESTIMATE algorithm (Estimate of Stromal and Immune Cells in Malignant Tumor Tissues from Expression Data) was applied¹⁹⁵. This computational method estimates the proportions of stromal and immune cells in tumor samples using gene expression profiles. To compare the infiltration of specific immune and stromal cell populations in tumors of patients with and without survival the “MCPcounter” package was used.

2.2.1.5 Ganglioside-related gene expression analysis

Ganglioside-related gene expression in tumor samples from the GSE49711 dataset was visualized using a heatmap generated with the “pheatmap” package. Gene expression values were row-scaled to normalize expression across samples, and hierarchical clustering was performed using Euclidean distance and complete linkage. The heatmap included annotations for MYCN status, high-risk classification, overall survival, and INSS stage, providing additional clinical context for the observed clustering patterns. The prognostic impact of ganglioside-related genes was assessed through univariate Cox regression analysis using overall survival data. The analysis was conducted using the “survival” package. Only genes with *p*-values below 0.05 were considered statistically significant, and adjustments for multiple testing were applied to control the false discovery rate. To refine the set of prognostic genes, Least Absolute Shrinkage and Selection Operator (LASSO)-penalized Cox regression was performed using the “glmnet” package. The optimal lambda value (λ_{1se}) was identified through cross-validation to achieve a balance between model simplicity and predictive accuracy. Genes with non-zero coefficients from the LASSO model were selected for further investigation due to their significant association with patient outcomes. Genes with positive coefficients were associated with worse survival, indicating that higher expression levels of these genes increased the risk of adverse outcomes. In contrast, genes with negative coefficients were linked to better survival, suggesting that higher expression levels of these genes were protective and associated with improved outcomes.

The ganglioside-related gene expression in neuroblastoma cell lines from the GSE89413 dataset was visualized in a heatmap generated with the “pheatmap” package. Gene expression was row-scaled to normalize expression across cell lines, and hierarchical clustering was performed for genes.

2.2.2 Cell culture

2.2.2.1 Cell cultivation and passaging of cell lines

All cells were grown in their corresponding culture medium within suitable culture flasks and kept in an incubator set to 37 °C with a 5% CO₂ atmosphere. All work on the cells was conducted in a sterile workbench. When adherent cells reached a confluence of 80% to 90%, they were passaged to maintain an optimal density for continued cell proliferation and growth. To passage these cells the medium was initially removed, and the cells were rinsed twice with 1× PBS. Subsequently, Trypsin/EDTA solution was added to detach the adherent cells from the flask's surface enzymatically. The cells were then incubated for 5 min at 37 °C in the incubator. The trypsin activity was stopped by adding three times the volume of the respective serum-containing medium. The resulting cell suspension was transferred to a 15 ml tube and centrifuged at 300 × g for 5 min. Following centrifugation, the cell pellet was resuspended in fresh cell culture medium, and the necessary volume for achieving the desired subculturing ratio was seeded back into the culture flask. The flask was replenished with fresh medium. To subculture suspension cells, a portion of cells was removed from the culture flask, and the remaining cells were diluted to achieve a seeding density suitable for the specific cell line, using fresh cell culture medium. Notably, the NK-92 cells and the CAR-expressing NK-92 cells were stimulated with 100 U/ml IL-2 every two to three days. A monthly check for mycoplasma contamination was performed on all cell lines.

2.2.2.2 Cell cultivation of primary B cells

Primary B cells isolated from healthy donors (see Section 2.2.9.4) were cultured in B cell medium, with activation and stimulation achieved using IL-2, IL-21, and multimerized CD40L. To multimerize CD40L, equal volumes of CD40L and the cross-linking antibody from the CD40L Multimer Kit were pre-incubated for 30 min prior to cell application. Subsequently, up to 0.2×10^6 B cells per ml were stimulated in B cell medium supplemented with freshly added IL-2 (100 U/ml), IL-21 (0.05 µg/ml), and multimerized CD40L (0.5 µg/ml). After 5 days, half of the medium was replaced with fresh B cell medium to maintain optimal culture conditions.

2.2.2.3 Freezing and thawing of cells

To maintain frozen stocks of a cell line, mycoplasma-negative cells were frozen in cryovials and preserved for long-term storage in liquid nitrogen. Initially, adherent cells were detached from the surface of the culture flask using Trypsin/EDTA (see Section 2.2.2.1). After centrifugation, the resulting cell pellet was resuspended in FBS + 10% (v/v) DMSO. Subsequently, 1 ml of the cell suspension was transferred into a 2 ml cryovial. The aliquots were stored at -80 °C for 24 h before they were transferred to liquid nitrogen for long-term storage. If cells from the frozen stocks were needed, the cryovials were initially placed in a 37 °C warm water bath until the cell suspension had thawed. The cells were transferred into a 15 ml tube containing 10 ml of prewarmed medium and centrifuged at 300 × g for 5 min. Subsequently, the resulting cell pellet was resuspended in 12 ml of culture medium. The cell suspension was then transferred into a culture flask and incubated at 37 °C and 5% CO₂ in the incubator.

2.2.2.4 Cell counting

In certain experiments, precise cell quantification was essential. Therefore, the cell suspension was diluted at a 1:1 ratio with a 0.4% trypan blue solution and then filled into a Neubauer chamber. Since trypan blue selectively stains dead cells by permeating the cell membrane, the unstained viable cells were counted by placing the chamber into the Countess 3 Automated Cell Counter.

2.2.3 **Spheroid generation and cultivation**

Neuroblastoma cells cultured in a monolayer were detached from the surface of the cell culture flask using Trypsin/EDTA (see Section 2.2.2.1). After centrifugation, the cells were resuspended in fresh cell culture medium and counted. A total of 6×10^3 neuroblastoma cells were seeded into a 96-well BIOFLOAT cell culture plate, featuring an anti-adhesive surface designed to promote spheroid formation. Within 24 hours, LS cells self-assembled into larger, high-density spheroids, which were then cultured for a total of 3 days in RPMI complete growth medium before the ganglioside expression was analyzed using immunofluorescence (see Section 2.2.5).

2.2.4 **Flow cytometry and cell sorting**

2.2.4.1 Flow cytometry

Flow cytometric analysis was performed to evaluate ganglioside expression on the cell surface of neuroblastoma cells. Before antibody staining, the cells were harvested, washed twice with FACS buffer, and counted (see Section 2.2.2). A total of 10^5 cells were used for each analysis in 50 μ l FACS buffer. To prevent FcR-mediated Ig Fc binding, cells were incubated with Fc Receptor Blocking Solution at 4 °C for 15 min. Cells were stained either directly with fluorophore-conjugated antibodies or indirectly using primary unconjugated antibodies followed by a second incubation step with fluorescence-conjugated secondary antibodies. For direct fluorescence staining, cells were incubated with the antibody for 30 min at 4 °C. For GD2 staining, an APC/Fire750-conjugated anti-GD2 antibody in a dilution of 1:100 was used. The cells were then washed three times with 200 μ l FACS buffer and centrifugated at $300 \times g$ for 5 min. For indirect fluorescence staining, the cells were incubated with the primary antibody for 45 min at 4 °C. For GM2 staining (clone E1, KM966 and MK1-16), as well as for GM1, GD3 and GM3 staining a concentration of 10 μ g/ml was used. After primary antibody incubation, the cells were washed three times with FACS buffer and the secondary antibody was incubated for 30 min at 4 °C. All secondary antibodies were diluted 1:100 in FACS buffer. Before flow cytometry analysis, the cells were washed three times with FACS buffer. To discriminate between live and dead cell populations, the cells were incubated with ViabilityDye 405/452 in 1 $\times$ PBS for 10 min at RT, followed by two additional washing steps. Alternatively, propidium iodide solution was added directly before measurement. Fluorescence intensity was measured using the MACSQuant X flow cytometer. The cell populations were gated based on their forward and side scatter properties. Doublet

discrimination was performed by plotting FSC-H versus FSC-A. Dead cells were excluded using the fluorescence intensities from the viability dye. The gate of interest was set based on the signal from an isotype or secondary antibody-only control, which was implemented in the experiment to determine and exclude non-specific binding. The data were analyzed using the FlowJo software.

2.2.4.2 Cell Sorting

Cells of interest were labeled with fluorophore-conjugated antibodies and resuspended in MACSQuant Tyto Running Buffer. The optimal cell density was determined using the MACSQuant Poisson Calculator to ensure high sorting purity. The labeled cells were then loaded into MACSQuant Tyto Cartridges, and sorting was performed with the MACSQuant Tyto's microchip-based technology, that ensures gentle handling and high-purity isolation of the cells.

2.2.5 **Immunofluorescence**

To stain the gangliosides GD2 and GM2 on the cell surface, coverslips were rinsed with 70% and 95% ethanol, allowed to dry, and then placed in wells of a 48-well plate. Each well was seeded with 10^5 cells and cultured for 48 h in 1 ml of the respective medium. After incubation, the medium was removed, and the cells were washed twice with $1\times$ PBS. The cells were then blocked with a solution of 3% BSA in $1\times$ PBS for 1 h at RT. For GD2 staining, the cells were incubated with 5 $\mu\text{g/ml}$ of anti-GD2 antibody (clone 14G2a), and for GM2 staining 5 $\mu\text{g/ml}$ of anti-GM2 antibody (KM966) was used. Both antibodies were diluted in 0.5% BSA in $1\times$ PBS and incubated for 2 h at RT. Following primary antibody incubation, the coverslips were washed three times with $1\times$ PBS. The secondary antibodies, an anti-mouse IgG APC-conjugated antibody for GD2 and an anti-human IgG AF488-conjugated antibody for GM2, were diluted 1:250 in 0.5% BSA in $1\times$ PBS and applied for 1 h at RT. After incubation, the samples were washed three times with $1\times$ PBS. Counterstaining was performed with 1 $\mu\text{g/ml}$ DAPI, followed by another $1\times$ PBS wash. The coverslips were mounted onto microscope slides using mounting medium. Images were captured using a Nikon DS-Qi2 camera on a Ti2 microscope at $40\times$ magnification.

Cell line-derived spheroids (see Section 2.2.3) were harvested after 3 d of culturing and were transferred into a 1.5 ml tube for staining. The spheroids were washed three times with $1\times$ PBS, then fixed with 4% PFA in $1\times$ PBS for 10 min at RT. Then a blocking solution of 3% BSA and 0.5% Tween-20 in $1\times$ PBS was added for 1 h at RT with gentle shaking. For GD2 and GM2 staining, the spheroids were incubated with 10 $\mu\text{g/ml}$ of anti-GD2 antibody (clone 14G2a) and 10 $\mu\text{g/ml}$ of anti-GM2 antibody (KM966), respectively, diluted in 0.5% BSA and 0.5% Tween-20 in $1\times$ PBS for 2 h at RT while shaking. Following primary antibody incubation, the spheroids were washed three times with 0.5% Tween20 in $1\times$ PBS. Secondary antibodies, diluted 1:100 in 0.5% BSA and 0.5% Tween-20 in $1\times$ PBS, were applied for 1 h at RT with shaking. For GD2 staining, an anti-mouse IgG AF488-conjugated antibody was used, while for GM2 staining, an anti-human IgG AF488-conjugated antibody was applied. After incubation,

2.2.6.2 Detection of anti-GM2 antibodies in mouse serum

After immunization, a blood sample was taken from the submandibular vein to isolate serum (see Section 2.2.9.1). Subsequently, the presence of GM2-specific antibodies in the serum of immunized mice was tested using the anti-ganglioside dot assay (see Section 2.2.10.1). A non-immunized C57BL/6 mouse was used as a control.

2.2.6.3 Production of hybridoma

For the generation of hybridomas, myeloma cells were required as fusion partners for the splenocytes. Therefore, Sp2/0-Ag14 cells were expanded three days before fusion, ensuring they were in the exponential growth phase on the day of fusion. On day 31, the mouse was sacrificed, and the spleen was extracted for fusion. Subsequently, the spleen was cut into two parts and pressed through a 70 μ m cell separation filter. The cell suspension as well as the Sp2/0-Ag14 cells were washed twice in 1 $\times$ PBS and counted. Afterwards, both cell types were centrifuged at 300 $\times$ g for 10 min, and the pellets were resuspended in RPMI + 1% penicillin/streptomycin. The cells were combined in a 50 ml tube at a 1:1 ratio, centrifuged at 300 $\times$ g for 5 min, and the pellet was gently resuspended and placed in a 37 $^{\circ}$ C water bath. Following this, 1.2 ml of PEG was added drop by drop over one minute with gentle stirring. Then, an additional 2 ml of RPMI + 1% penicillin/streptomycin were added dropwise over two minutes, followed by an additional 4 ml over 4 min. Finally, 8 ml of RPMI complete growth medium containing 10% heat-inactivated FBS was added, and the cell suspension was centrifuged at 300 $\times$ g for 5 min. The fragile cells were gently resuspended in RPMI complete growth medium and plated in 24-well plates. The cells were incubated overnight at 37 $^{\circ}$ C in the incubator. On the day after fusion, 1 $\times$ HAT was added to the medium to prevent the growth of unfused myeloma cells and normal spleen cells and support the growth of hybridomas. After 10 days, the supernatant was tested for anti-ganglioside antibodies using the anti-ganglioside dot assay (see Section 2.2.10.1).

2.2.7 **Cloning**

This section describes the methods used for the generation and modification of DNA plasmids in general. Cloning was performed to create a B4GALNT1-specific CRISPR/Cas9 knockout plasmid with blasticidin resistance to generate cell lines with a B4GALNT1-specific knockout (see Section 2.2.8) and to generate and modify antibody expression plasmids for the recombinant expression of antibodies (see Section 2.2.17).

2.2.7.1 Polymerase chain reaction (PCR)

The amplification of specific DNA sequences from DNA templates was performed using polymerase chain reaction (PCR). The components for the PCR reaction are listed in **Table 14**, while the thermocycling conditions are shown in **Table 15**.

TABLE 14 | PCR reaction setup.

Reagents	Final concentration
5× Q5 Reaction Buffer	1×
10 μM forward primer	0.5 μM
10 μM reverse primer	0.5 μM
10 mM dNTPs	200 μM
DNA template	1–50 ng
Q5 High Fidelity DNA Polymerase	0.02 U/μl

TABLE 15 | Thermocycler conditions.

Step	Temperature	Time
Initial denaturation	98 °C	30s
30 cycles	98 °C	10 s
	*50-72 °C	20 s
	72 °C	30 s/kb
Final extension	72 °C	120 s
Hold	4 °C	∞

*Annealing temperature was determined individually based on the primer length and GC content using the NEB Tm Calculator.

2.2.7.2 Gel electrophoresis and DNA purification

The PCR products were mixed with 1× DNA Loading Dye and loaded onto a 1% agarose gel containing GelRed for visualization, along with a 100 bp or 1 kb DNA ladder for size reference. Gel electrophoresis was performed in a chamber filled with Tris-acetate-EDTA (TAE) buffer at 120 V for 30 min. DNA fragments were visualized under UV light, and the desired fragments were excised from the gel using a scalpel. The DNA was then purified using the NucleoSpin Gel and PCR Clean-up Mini Kit, following the manufacturer's instructions. The final elution was performed in 20 μl of H₂O.

2.2.7.3 Restriction enzyme digest and ligation

Restriction enzyme-based ligation cloning was used to insert DNA fragments into vectors by creating compatible sticky ends that enable precise ligation. Inserts, generated by PCR, and the vector were digested with the same FastDigest restriction enzymes for 30 min at RT, following the manufacturer's protocol. Digested inserts were purified using the NucleoSpin Gel and PCR Clean-up Mini Kit, and DNA concentrations were measured with the NanoDrop One/OneC spectrophotometer. Linearized vectors or inserts, that were isolated from a donor plasmid by restriction enzyme digestion, were separated on an agarose gel, and the desired fragments were excised and purified as described before

(see Section 2.2.7.2). The NEB Ligation Calculator was used to determine optimal DNA amounts for ligation, maintaining a 3:1 insert-to-vector ratio. Ligation reactions were performed using the T4 DNA Ligase according to the manufacturer's instructions. The ligated plasmids were transformed into chemically competent *E. coli* cells for amplification (see Section 2.2.7.5).

2.2.7.4 Site-directed mutagenesis of plasmids

The Q5 Site-Directed Mutagenesis Kit was used to introduce single-nucleotide mutations into plasmids following the manufacturer's instructions. In brief, a PCR reaction was performed using final concentrations of 1× Q5 Reaction Buffer, 200 µM dNTPs, 0.5 µM of each forward and reverse primer, 10 ng template DNA, and 0.02 U/µl Q5 High-Fidelity DNA Polymerase, with an annealing temperature of 65 °C. Following amplification, 1 µl of the PCR product was incubated with 1× KLD Reaction Buffer and 1× KLD Enzyme Mix for 5 min. Subsequently, 5 µl of this reaction mixture was transformed into chemically competent cells (see Section 2.2.7.5).

2.2.7.5 Transformation of chemically-competent *E. coli* cells

To transform chemically competent *E. coli* JM109 Mix&Go bacteria for plasmid amplification and replication, 1–5 µl of the plasmid of interest was mixed with the bacteria and incubated on ice for 30 min. A heat shock at 42 °C for 30 s was performed, followed by immediate transfer of the bacteria to ice for 5 min. Next, 200 µl of pre-warmed SOC medium was added, and the mixture was incubated at 37 °C for 60 min with shaking at 250 rpm. Then the bacteria were plated on LB agar plates containing either 100 µg/ml ampicillin or 100 µg/ml hygromycin B, depending on the antibiotic resistance gene encoded by the plasmid. The plates were incubated overnight at 37 °C to allow colony formation.

2.2.7.6 Isolation of plasmid DNA

Colonies from LB agar plates or 1–10 µl of an *E. coli* glycerol stock were used to inoculate 2 ml (for small-scale plasmid isolation) or 100 ml (for high-yield isolation) of pre-warmed LB medium supplemented with either 100 µg/ml ampicillin or 100 µg/ml hygromycin B, depending on the plasmid's encoded resistance gene. The cultures were incubated overnight at 37 °C with shaking. Plasmid DNA was isolated using the NucleoSpin Plasmid EasyPure Kit or the NucleoBond Xtra Midi Kit, following the manufacturer's instructions. The elution was performed in H₂O. DNA concentrations were measured using the NanoDrop One/OneC spectrophotometer, and the plasmid sequences were validated by Sanger sequencing, performed by Microsynth (Balgach, Switzerland).

2.2.8 **Generation of neuroblastoma cells with a B4GALNT1 knockout**

To establish a suitable negative control for anti-GM2 antibody experiments, neuroblastoma cells with a B4GALNT1 knockout (KO) were generated, resulting in the loss of GM2 and GD2 expression.

2.2.8.1 Generation of a B4GALNT1-specific CRISPR/Cas9 knockout plasmid

A B4GALNT1-specific CRISPR plasmid with a blasticidin resistance gene was generated by restriction enzyme and ligation-based cloning (see Section 2.2.7). The recipient plasmid, DHC013-pLKO5d.SFFV.hSpCas9.P2A.BSD contained the Cas9 sequence and a blasticidin resistance gene. The vector was linearized by digestion with NotI and BamHI, which resulted in the removal of the SFFV promoter controlling Cas9 expression. The donor plasmid, pL40C-CRISPR.sgRNA_B4GALNT1.EFS.dTomato, was also digested with NotI and BamHI, to release the desired insert. The insert sequence included a cPPT sequence for facilitated nuclear import during target-cell infection, a B4GALNT1-specific sgRNA under the control of a U6 promoter, and an EFS promoter to drive Cas9 expression. The insert was then ligated into the linearized recipient plasmid. Cloning was performed by Sijarlina Sivanantham. The plasmid map of the generated plasmid DHC013-pLKO5d.CRISPR.B4GALNT1.BSD as well as the cloning strategy is illustrated in the appendix (Appendix, Supplementary Figure 1).

2.2.8.2 Production of lentiviral particles and transduction

To produce stable B4GALNT1 KO neuroblastoma cells, integrase-deficient lentiviral particles were produced in HEK293T cells and used for transduction of selected neuroblastoma cells. HEK293T cells were seeded at a density of 4×10^6 cells per 10 cm culture dish in 10 ml DMEM complete growth medium and incubated overnight at 37 °C. The following day, 10 µg of DHC013-pLKO5d.CRISPR.B4GALNT1.BSD plasmid, along with helper plasmids for the packaging system (10 µg pMDLg/pRRE, 5 µg of pRSV-Rev, and 2 µg of pHCMV-VSV-G), were diluted in H₂O to a total volume of 450 µl. To this solution, 50 µl of 2.5 M CaCl₂ was added. In a separate 15 ml conical tube, 500 µl 2× HBS was prepared, and the plasmid solution was then added dropwise, while blowing air with a pipette. The mixture was incubated for 15 min at RT. During this incubation, the medium of the HEK293T cells was replaced with fresh medium supplemented with 25 µM chloroquine to enhance transfection efficiency. The plasmid-containing mixture was then added dropwise to the cells while gently swirling the dish. The cells were incubated at 37 °C overnight. On the following day, the medium was replaced with fresh DMEM complete growth medium. After 24 h, the supernatant containing the lentiviral particles was collected using a syringe and filtered through a 0.45 µm filter into a 50 ml conical tube. To concentrate the lentiviral particles, the filtered supernatant was centrifuged at $3,000 \times g$ at 4 °C overnight. The supernatant was carefully discarded, leaving 200 µl to resuspend the viral particles. The lentiviral particles were immediately aliquoted and stored at -80 °C.

To transduce the neuroblastoma cells with lentiviral particles, 2×10^4 cells were seeded into a 96-well plate containing RPMI complete growth medium supplemented with 8 µg/ml polybrene to enhance transduction efficiency. Different volumes of viral supernatant (0.1 µl, 1 µl, 10 µl and 20 µl) supernatant were added to the wells. The medium was replaced after 6 h incubation. After 4 days, the medium was replaced again with RPMI complete growth medium containing 8 µg/ml blasticidin to selectively eliminate non-transduced cells and enrich for transduced cells. The following B4GALNT1 KO cells

were generated using this method: Kelly^{ΔB4GALNT1}, LAN-1^{ΔB4GALNT1}, LS^{ΔB4GALNT1}, SK-N-LO^{ΔB4GALNT1}, SH-EP^{ΔB4GALNT1}, SH-SY5Y^{ΔB4GALNT1} and SK-N-SH^{ΔB4GALNT1}.

2.2.8.3 Tracking of Indels by Decomposition (TIDE) analysis

To evaluate the efficiency of the CRISPR/Cas9-mediated B4GALNT1 KO, TIDE (Tracking of Indels by Decomposition) analysis was performed. For this, 10⁶ neuroblastoma wildtype cells and corresponding B4GALNT1 KO cells were seeded into T25 flasks and cultured overnight at 37 °C. The following day, cells were harvested, and genomic DNA was extracted using the NucleoSpin Tissue Kit according to the manufacturer's instructions. PCR amplification of the B4GALNT1 target sequence was performed using the TIDE_B4GALNT1_fw and TIDE_B4GALNT1_rev primers in a 25 µl reaction containing 50 ng of template DNA, 0.5 µM of each primer, and 1× DreamTaq PCR Master Mix. The thermocycler conditions used for amplification are summarized in **Table 16**. The resulting PCR products were sequenced using Sanger sequencing with the Seq_TIDE_B4GALNT1 primer, performed by Microsynth (Balgach, Switzerland). Raw, unprocessed sequencing files from the wildtype sample were used as a baseline reference for the TIDE analysis, while the corresponding CRISPR-edited sample files were loaded to assess insertions and deletions (indels) introduced by the editing process. TIDE analysis allowed for precise quantification of KO efficiency by comparing the indel profiles between the wildtype and the KO samples.

TABLE 16 | Thermocycler conditions for TIDE PCR.

Step	Temperature	Time
Initial denaturation	95 °C	60 s
30 cycles	95 °C	15 s
	62 °C	15 s
	72 °C	60 s
Final extension	72 °C	120 s
Hold	4 °C	∞

2.2.9 Collection, processing and isolation of human blood samples and cells

Experimental procedures involving human materials were approved by the local Ethics Committee in Hamburg, Germany (approval no. 2021-100729-BO-ff). All participants gave their informed consent before participating in the study. To test healthy donors for the presence of anti-GM2 antibodies, serum or plasma was isolated. Additionally, anonymised buffy coats were provided by the Institute for Transfusion Medicine at the University Medical Center Hamburg-Eppendorf (UKE). Furthermore, from

volunteers who tested positive for anti-GM2 antibodies, a larger blood donation was requested to isolate peripheral blood mononuclear cells (PBMCs) and subsequently B cells for further analysis.

2.2.9.1 Serum preparation

Serum was derived from whole blood collected from healthy donors. For smaller quantities, capillary blood from the finger was collected using a sterile lancet. Larger amounts of peripheral blood were drawn from the arm vein. In both cases, a serum separator tube was used to isolate the serum. Subsequently, the blood was allowed to clot for 30 min at RT. After centrifugation at $2,000 \times g$ for 10 min, the serum was immediately transferred into a tube and stored at -20°C for preservation.

2.2.9.2 Plasma preparation

Plasma was extracted from Buffy Coats obtained from healthy individuals. A small portion of the Buffy Coat underwent centrifugation at $1,000 \times g$ for 10 min. The resulting supernatant, designated plasma, was transferred into a tube and stored at -20°C for preservation.

2.2.9.3 Isolation of peripheral blood mononuclear cells

PBMCs were obtained from whole blood collected from healthy individuals using anti-coagulant-treated tubes, such as EDTA or lithium heparin, during standard arm vein blood draws. The whole blood was initially diluted at a 1:1 ratio with $1\times$ PBS. In some instances, PBMCs were isolated directly from Buffy Coats derived from the blood bank, with the Buffy Coats diluted at a 1:5 ratio with $1\times$ PBS. Following dilution, the blood samples were carefully layered onto a Ficoll-Paque density gradient solution. Subsequent centrifugation at $800 \times g$ for 20 min, with the brake turned off, enables the separation of blood components. The resulting upper layer, containing plasma and platelets, was removed. The mononuclear layer at the interface was then transferred into a sterile 50 ml tube and washed twice with $1\times$ PBS. After the final centrifugation step, the cell pellet was resuspended in an appropriate media or buffer for further applications.

2.2.9.4 Isolation and stimulation of B cells from PBMCs

B cells were isolated from PBMCs using either the Human B Cell Isolation Kit II or the EasySep Human B Cell Isolation Kit according to the manufacturer's instructions. After PBMC isolation, the cells were washed with B cell isolation buffer and quantified. The exclusion of non-B cells was achieved through magnetic labeling. The labeled non-B cells were then depleted using LS columns in the QuadroMACS Separator or the column-free EasySep magnet, resulting in the isolation of pure, untouched B cells.

2.2.10 **Detection of anti-glycan antibodies in serum, plasma and cell culture supernatants**

2.2.10.1 Anti-ganglioside dot assay

The detection of ganglioside-specific antibodies was conducted using the anti-ganglioside dot assay, a line immunoassay featuring a membrane with 12 different gangliosides. The incubation periods and washing steps were performed according to the manufacturer's instructions, although the primary and

secondary antibodies used varied depending on the experimental application. For the identification of anti-GM2 antibodies in healthy human individuals, 15 μ l of serum or plasma were applied. In experiments involving mouse immunization with GM2, the efficacy of immunization was evaluated by incubating 15 μ l of mouse serum on the membrane. To detect the production of anti-GM2 antibodies from B cells or antibody-expressing HEK293T cells, 1 ml of cell culture supernatant was used for the initial incubation step. In cases involving human primary antibodies, the kit-supplied secondary antibodies, specific to human IgM or IgG conjugated to HRP, were used. Conversely, if primary antibodies produced by mice were applied, anti-mouse IgG and IgM antibodies conjugated to HRP were used. The presence of anti-ganglioside antibodies was visualized by adding TMB substrate, resulting in distinctive bands on the membrane.

2.2.10.2 Glycan array

Naturally occurring antibodies against glycan structures in human serum were detected using a glycan-specific microarray technology. The Glycan Array 300, which was used for this purpose, contains 300 distinct glycan structures. Serum samples from different donors were applied according to the instructions of the manufacturer. In brief, the glass slide containing immobilized glycans was incubated with human serum. Subsequently, a biotinylated anti-human IgG and IgM antibody was used for the detection of bound antibodies of IgG or IgM isotypes. Following this step, a Cy3-conjugated streptavidin was added, and the glycan array was documented using a Nikon Eclipse Ti2-E imaging system. The mean fluorescence intensity was determined by image analysis with ImageJ. The values were then normalized to a scale of 0 to 100, corresponding to the negative and positive controls included in the assay. Fold changes for all donors were calculated relative to donor D37 using R. The top 25 glycans with the highest fold changes were identified for cytotoxic donors (D25, D78, and D108) compared to non-cytotoxic donor (D37), as well as for D108 compared to D37. The results were visualized as a heatmap with a color gradient representing the fold change, created using the “pheatmap” package in R.

2.2.11 **Detection of serum antibody binding to neuroblastoma cells**

To assess the binding of IgM antibodies in human serum to neuroblastoma cells, 5×10^4 LS and LS ^{Δ B4GALNT1} cells were transferred into pre-chilled 1.5 ml tubes and washed twice with ice-cold 1 $\times$ PBS. The cells were resuspended in 50 μ l FACS buffer containing 1 μ l Fc Receptor Blocking Solution and incubated for 15 min at 4 °C. Subsequently, the cells were incubated in 100 μ l donor serum diluted 1:1 with FACS buffer, with gentle mixing, and incubated for 30 min at 4 °C. After two washes with FACS buffer, the cells were resuspended in 50 μ l FACS buffer and stained with 1 μ l of APC-conjugated anti-human IgM antibody, followed by incubation for 30 min at 4 °C. The cells were washed twice with FACS buffer, and live/dead discrimination was performed using ViabilityDye 405/452, following the manufacturer's instructions. Finally, the IgM binding to neuroblastoma cells was analyzed by flow cytometry.

2.2.12 Complement-dependent cytotoxicity assay

To evaluate the cytotoxic effect of donor serum on neuroblastoma cells, 10,000 cells were seeded into a 96-well plate and incubated overnight at 37 °C. On the following day, the medium was replaced with 50 µl RPMI medium containing CellTox Green at a 1:500 dilution per well. The 0-hour time point was imaged using the Incucyte Imaging system to measure initial levels of dead cells in the wells. Subsequently, 50 µl of treatment solutions were added to the wells in triplicates. For treatment with donor serum, a final concentration of 15% (v/v) was used. When testing the ch14.18 antibody, 15% (v/v) of human non-cytotoxic serum was added as a source of human complement. Controls included a medium-only control to measure spontaneous lysis and a maximum lysis control using 0.1% Triton-X100. Cytotoxicity was measured over 4 hours using the Incucyte Imaging system, with data analysis performed using the associated Incucyte software. The cytotoxicity values were normalized to the 0-hour time point, and specific lysis was calculated using the formula:

$$\text{Cytotoxicity (\%)} = \frac{\text{Lysis} - \text{Spontaneous Lysis}}{\text{Maximum Lysis} - \text{Spontaneous Lysis}} \times 100$$

2.2.13 Proliferation assay

The proliferation of isolated, primary B cells (see Sections 2.2.2.2 and 2.2.9.4) was evaluated using the eFluor670 cell proliferation dye. On the day of isolation, the cells were washed twice with 1× PBS and then mixed at a 1:1 ratio with a 10 µM solution of eFluor670 in 1× PBS while vortexing. The cells were incubated in the dark at 37 °C for 10 min. To stop the labeling reaction, 5 volumes of IMDM medium supplemented with 10% FBS were added, followed by three washes using the same medium. The labeled cells were then cultured in complete B cell medium. The maximum eFluor670 fluorescence intensity was measured immediately after labeling using flow cytometry. Proliferation was subsequently assessed on days 1, 2, 3, 4, 7, and 9, based on the principle that the fluorescence intensity decreases by half with each cell division, as the dye is evenly distributed between daughter cells.

2.2.14 ELISA

Human IgG and IgM, produced by either B cells or by HEK293T cells, transfected with antibody expression plasmids, were quantified using the MABTECH ELISA Flex kits, following the manufacturer's instructions. Nunc Maxisorp strips were used for coating with the kit-provided capture antibody. Detection was performed using the p-nitrophenyl phosphate (pNPP) substrate, which was incubated at RT for 10 min to 15 min. The optical density was measured at 405 nm, with a reference wavelength of 600 nm subtracted to correct for background noise. Additionally, the blank control value was subtracted before calculating antibody concentrations based on a standard curve.

2.2.15 Isolation of antigen-specific B cells using fluorescence-activated cell sorting

Antigen-specific B cells were isolated using a biotinylated GM2 substrate in combination with fluorophore-conjugated anti-biotin antibodies. B cells were obtained from donors by collecting 50 to 100 ml blood (see Section 2.2.9). The isolated B cells were cultured in B cell cultivation medium for 5 to 10 days to ensure sufficient cell numbers for labeling and subsequent isolation. The B cells were washed two times with FACS buffer. For labeling, 2×10^5 B cells per condition were prepared. 1 μ g of biotinylated GM2 was pre-incubated separately with 1 μ l of PE-conjugated anti-biotin antibody and 1 μ l of VioBlue-conjugated anti-biotin antibody in 50 μ l of $1 \times$ PBS, creating two separate complexes: GM2-PE and GM2-VioBlue. Similarly, 1 μ g of biotinylated aminoglucitol was pre-incubated with the same antibodies to generate PE-conjugated aminoglucitol and VioBlue-conjugated aminoglucitol as an off-target control. The labeling mixtures were incubated at RT for 30 min. After incubation, GM2-PE and GM2-VioBlue, as well as aminoglucitol-PE and aminoglucitol-VioBlue, were combined and added to the B cells. As an additional control, a secondary antibody-only condition was prepared by adding 1 μ l of anti-biotin PE and 1 μ l of anti-biotin VioBlue antibody. The cells were incubated with the labeling mixtures for 45 min at 4 °C and then washed three times with FACS buffer. Subsequently, the cells were resuspended in 50 μ l FACS buffer containing 1 μ l of APC-Vio770-labeled anti-CD19 antibody. After a 10 min incubation at 4 °C, the cells were washed three times with FACS buffer. For live/dead cell discrimination, propidium iodide solution was added at a 1:100 dilution. The labeled cells were analyzed using the MACSQuant Analyzer, focusing on double-positive cell populations to minimize false positives. Double-positive cells from donor D78 were sorted using the MACSQuant Tyto (see Section 2.2.4.2).

2.2.16 Barcode Enabled Antigen Mapping (BEAM) technology

The Barcode Enabled Antigen Mapping (BEAM) technology from 10x Genomics (Pleasanton, USA) was used for the identification of antigen-specific B cell clonotypes, including their full-length V(D)J sequences, by matching antigens of interest to their corresponding B cell receptors. The approach thereby combines FACS for the isolation of antigen-specific B cells with subsequent single-cell sequencing. The technology enables the analysis of the immune repertoire, gene expression, and antigen specificity from every single cell. Here, enriched B cells from a donor with GM2-specific antibodies were screened for their binding specificity to GM2. The BEAM technology was performed according to the user guides CG000590 (Rev B), CG000591 (Rev B) and the workbook CG000597 (Rev B).

2.2.16.1 Sample preparation and BEAM reagent assembly

PBMCs were obtained from 450 ml of whole blood collected from donor D78 using Ficoll-Paque density gradient centrifugation (see Section 2.2.9.3). B cells were enriched from fresh PBMCs using the Human B Cell Isolation Kit II (see Section 2.2.9.4). BEAM reagents were assembled by coupling biotinylated antigens of interest and a biotinylated control antigen to barcoded BEAM conjugates, that contain

streptavidin and a PE fluorescent marker. Biotinylated GM2 (Elicityl, Crolles, France) was used as the target antigen, while biotinylated human serum albumin (HSA) (abcam, Cambridge, UK) served as an off-target antigen control. Each antigen was coupled to uniquely barcoded BEAM conjugates based on their molecular weight and stock concentration (**Table 17**). The assembled reagents were sufficient for labeling up to 2×10^7 cells. The assemblies were incubated for 30 min in the dark on ice. Subsequently, 20 μ l Quenching Reagent was added to each assembly, followed by an additional 30 min incubation in the dark on ice. The assemblies were then centrifuged at $2,500 \times g$ for 5 min at 4 °C. The target antigen assembly and the off-target antigen assembly were pooled in a pre-chilled 1.5 ml tube.

TABLE 17 | Assembly of antigens and BEAM conjugates.

Assembly	Component	Volume
Target antigen assembly	1× PBS	131.2 μ l
	BEAM conjugate	8.0 μ l
	Biotinylated GM2 (Stock: 10 ng/ μ l, MW: 1.38 kDa)	20.8 μ l
Off-target antigen assembly	1× PBS	141.6 μ l
	BEAM conjugate	8.0 μ l
	Biotinylated HSA (Stock: 1 μ g/ μ l, MW: 69 kDa)	10.4 μ l

2.2.16.2 B cell labeling

For labeling, 5×10^6 enriched B cells were centrifuged at $300 \times g$ for 5 min at 4 °C and resuspended in 90 μ l pre-chilled 1× PBS + 2% FBS. Subsequently, 50 μ l Fc Receptor Blocking Solution was added and the cells were incubated on ice for 10 min. Following this, 72 μ l of the pooled BEAM assemblies was added to the cells, and the mixture was carefully pipetted up and down to ensure thorough mixing. The cells were incubated on ice for 30 min, with gentle mixing every 10 min. After incubation, 3.5 ml of 1× PBS + 2% FBS was added, and the cells were centrifuged at $300 \times g$ for 5 min at 4 °C. The B cells were further stained by adding 10 μ l of CD19-APC/Vio770 antibody, followed by an incubation for 10 min at 4 °C. After washing the cells with 5 ml 1× PBS, viability staining was performed using ViabilityDye 405/452, according to the manufacturer's instructions. The cells were washed twice with 5 ml 1× PBS + 2% FBS, centrifuged, and resuspended in 10 ml 1× PBS + 2% FBS.

2.2.16.3 Isolation of antigen-specific B cells

Antigen-specific B cells were isolated using the MACSQuant Tyto Cell Sorter (see Section 2.2.4.2). The distinct cell population was identified by gating based on backward and sideward scatter properties, while dead cells were excluded by gating for ViabilityDye 405/452⁻ cells. The purity of the B cell population was confirmed by gating for CD19⁺ cells. B cells binding either GM2-coupled or HSA-coupled BEAM assemblies were then isolated by selecting the PE⁺ population.

2.2.16.4 Gel Bead-in-emulsion generation and library preparation

Isolated B cells are loaded into the 10x Genomics Chromium Next GEM Chip K and were placed into the 10x Chromium Controller to generate Gel Beads-in-emulsion (GEMs) using the Chromium Next GEM Single Cell 5' Kit. Within the GEMs, reverse transcription is performed to generate barcoded cDNA from mRNA as well as from barcodes derived from bound BEAM assemblies. Barcoded cDNA is then collected to generate libraries for 5' gene expression profiling (5' GEX) to assess transcriptomic data, V(D)J sequencing to analyze the sequence of the B cell receptors and the BEAM antigen capture to determine the antigen specificity. For the library construction the Library Construction Kit, 5' Feature Barcode Kit, and the Chromium Single Cell Human BCR Amplification Kit were used. The Dual Index Plate TT, Set A was used for V(D)J and 5' Gene Expression libraries, while the Dual Index Plate TS, Set A was used for BEAM library. The quality of the libraries was assessed using the Agilent TapeStation 4200. The GEM and library generation were performed by the Single Cell Core Facility at UKE.

2.2.16.5 Sequencing of libraries

The libraries were sequenced on Illumina NovaSeq 6000 (NovaSeq PE150) as per the instructions provided in 10x Genomics user guide. The sequencing was performed by Novogene (Cambridge, UK).

2.2.16.6 Data analysis

Raw FASTQ files were processed using 10x Genomics Cell Ranger, a software pipeline for analyzing Chromium Single Cell gene expression data. This software was used to align the raw sequencing reads to a human reference genome dataset (GRCh38-2020-A), generate a BEAM-derived feature matrix to quantify the number of reads or unique molecular identifiers (UMIs) associated with each gene in individual cells, and performed clustering and gene expression profiling to identify distinct cell populations. Data analysis with Cell Ranger was supported by the Bioinformatics Core at UKE. The 10x Genomics V(D)J Browser was used for interactive visualization and exploration of clonotypes and immune receptor sequences.

2.2.17 **Antibody production**

2.2.17.1 Identification of variable heavy and light sequences and sequence alignment

The nucleotide and amino acid sequences of the V(D)J regions of the heavy and light chains from selected BEAM-derived clonotypes were exported from the Loupe V(D)J Browser. Using the amino acid sequences, the immunoglobulin domains of the V_H and the V_L chains were identified based on the IMGT numbering scheme using the ANARCI tool. Sequence alignment was performed using the Clustal Omega tool.

2.2.17.2 Antibody structure prediction

The three-dimensional structures of the V_H and the V_L chains of the BEAM-derived antibodies were modelled using the prediction tool ABodyBuilder2. The chains and the CDRs were visualized using the 3D visualization tool PyMOL.

2.2.17.3 Generation of plasmids for the expression of IgG antibodies

The nucleotide sequences of the V_H and V_L domains were selected for the generation of IgG antibody expression plasmids. The V_H and V_L sequences from clonotypes 02, 04, 07, 10, 19, 31, 37, 41, 1846, 8258, and 9543 were cloned into an antibody heavy and light chain co-expression vector (referred to as pVB_H+L_IgG) using VB UltraStable *E.coli*, which was performed by the VectorBuilder (Chicago, USA). Additionally, the V_H and V_L sequences of the KM966 antibody were incorporated into the same plasmid to serve as a positive control for validating antibody production and GM2-specific binding. The plasmid map is shown in the appendix (**Appendix, Supplementary Figure 2**). The *E. coli* glycerol stocks were used to inoculate LB medium supplemented with ampicillin, and plasmid DNA was isolated from the cultures the following day (see Section 2.2.7.6). Prior to transfection of HEK293T cells for antibody production, the plasmids were verified by sequencing using the Seq_CAG_pVB, Seq_EF1a_pVB, and Seq_hIgG1-CH_pVB primers.

For clonotypes 900, 10066, and 11767, the V_H and V_L sequences were modified to include specific restriction sites for cloning. The V_H sequence was flanked by EcoRI and NcoI restriction sites, while the V_L sequence was flanked by XhoI and BglII restriction sites. These modified sequences were synthesized by BioCat (Heidelberg, Germany). As the pVitro_H+L_IgG_KM966 plasmid was already available in the laboratory, it was used both as a positive control and as a recipient plasmid for exchanging the V_H and V_L sequences of the KM966 with the sequences from clonotypes 900, 10066, and 11767 using restriction enzyme-based ligation cloning (see Section 2.2.7.3). The cloning strategy is visualized in the appendix (**Appendix, Supplementary Figure 3**). After plasmid isolation (see Section 2.2.7.6) the plasmids were verified by sequencing using the Seq_VH_IgG_pVitro, Seq_CH_IgG/IgM_pVitro, Seq_VL_IgG/IgM_pVitro and Seq_CL_IgG/IgM_pVitro primers.

2.2.17.4 Generation of plasmids for the expression of IgM antibodies

To generate antibodies of the IgM isotype, the V_H and V_L sequences of clonotypes 02, 04, 07, 10, 19, 31, 37, 41, 900, 10066, and 11767 were cloned into the pVitro_H+L_IgM_KM966 plasmid. Clonotypes 1846, 8258, and 9543, derived from IgG-expressing B cells, were not cloned into the pVitro_IgM plasmids due to their native IgG isotype. As the pVitro_KM966_IgM plasmid was already available in the laboratory, it served both as a positive control and as a recipient plasmid, where the V_H and V_L sequences were exchanged with the clonotype-specific sequences. The KM966 plasmid's V_H sequence was flanked by EcoRI and NcoI, while the V_L sequence was flanked by XhoI and BglII. Consequently, restriction sites were introduced into the V_H and V_L sequences of each clonotype by PCR amplification using primers containing the necessary restriction sites, enabling restriction enzyme-based ligation cloning (see Sections 2.2.7). For clonotype 04, an internal NcoI restriction site in the V_H sequence

necessitated a mutagenesis step prior to restriction-site amplification. Mutagenesis was performed using the primers Clonotype04_VH_Mut_fw and Clonotype_VH_Mut_rev (see Section 2.2.7.4). The V_H and V_L sequences of clonotypes 900, 10066, and 11767 already contained the appropriate flanking restriction sites, allowing direct restriction enzyme digestion and ligation cloning without additional modification steps (see Section 2.2.7.3). The cloning strategy is visualized in the appendix (**Appendix, Supplementary Figure 4**). Plasmid sequences were verified using the primers Seq_VH_IgM_pVitto, Seq_CH_IgG/IgM_pVitto, and Seq_VL_IgG/IgM_pVitto before being used for antibody expression in HEK293T cells (see Section 2.2.17.5).

2.2.17.5 Antibody production

Antibodies were produced by transient transfection of antibody expression plasmids into HEK293T cells using the jetOPTIMUS transfection reagent. For this purpose, 4×10^6 HEK293T cells were seeded into a T75 culture flask containing DMEM complete growth medium. On the following day, when the cells reached 60% to 80% confluence, the medium was replaced with 8 ml of fresh growth medium. For the transfection, 10 µg of plasmid DNA was diluted with 1 ml jetOPTIMUS buffer, vortexed briefly, and then 10 µl of jetOPTIMUS reagent was added to the solution. After incubation for 10 min at RT, the transfection mix was added dropwise to the cells. After 6 h incubation, the medium was replaced with pre-warmed fresh medium. After 3 days, the supernatant was collected, centrifuged at $4,000 \times g$ for 30 min at 4 °C, and filtered through a 0.22 µm filter. The filtered supernatants were stored at 4 °C until further use.

2.2.18 Western blot

The antibody production (see Section 2.2.17.5) was validated by Western blot analysis. For reducing conditions, antibody-containing supernatants were prepared with 100 mM dithiothreitol (DTT) and 1× sodium dodecyl sulfate (SDS) loading dye, followed by heating at 95 °C for 10 min. For non-reducing conditions, no DTT was added, and the samples were not heated. The prepared samples were loaded onto polyacrylamide gels (4–12% Bis-Tris), and proteins were separated at 150 V in 1× MES buffer for 1 h. Subsequently, the proteins were transferred to a nitrocellulose membrane using wet/tank electrotransfer at 400 mA for 2–3 h at 4 °C in 1× Towbin Buffer. The membrane was blocked for 1 h in blocking buffer, that consists of 5% skimmed milk powder in Tris-buffered saline (TBS), pH 7.6, with 0.1% Tween-20, while shaking. Primary antibodies diluted in blocking buffer were then applied and incubated overnight. For IgG detection, an anti-human IgG H+L antibody was used at a 1:10,000 dilution. For IgM detection, an anti-human IgM antibody, diluted 1:5,000, and an anti-human kappa antibody, diluted 1:10,000, were used. The following day, the membrane was washed three times, and the HRP-conjugated anti-rabbit IgG H+L antibody, diluted 1:1,000 in blocking buffer, was incubated for 2 h at RT. After three washes, antibodies were detected using Pierce ECL Western Blotting Substrate on an Odyssey XF Imager system. Western blot analysis was performed with the support of Marianne Klokow.

2.2.19 Statistical analysis

Statistical analyses were performed using R in RStudio or GraphPad Prism. p -values < 0.05 were considered statistically significant. Statistical significance is indicated as follows: *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.

For parametric data, two-way ANOVA was performed with Benjamini-Hochberg correction applied to control the false discovery rate (FDR) for multiple comparisons. For non-parametric (non-normally distributed) data, the Wilcoxon rank-sum test was used for comparisons between two groups, with Bonferroni correction applied to adjust for multiple testing. Details of the specific statistical tests used for individual analyses are provided in the corresponding figure legends.

3 RESULTS

3.1 Characterization of ganglioside GM2 as a putative target for immunotherapy in high-risk neuroblastoma

3.1.1 *in silico* characterization of the immune microenvironment in neuroblastoma

Neuroblastoma samples from the publicly available dataset GSE49711, comprising 493 neuroblastoma cases, were analyzed using an *in silico* approach to explore the underlying mechanisms associated with disease outcomes. Among the cohort, 78.9% of patients had a favorable prognosis, whereas 21.1% had poor outcomes. The distribution of patients according to INSS stage included: stage 1 (24.3%), stage 2 (15.8%), stage 3 (12.6%), stage 4 (36.7%) and stage 4S (10.5%). Additionally, 35.5% of the patients were classified as high-risk and 18.7% had neuroblastoma, driven by amplification of the oncogenic driver MYCN (Table 18).

TABLE 18 | Patient characteristics of the neuroblastoma cohort GSE49711, including sex, age, MYCN amplification status, INSS stage, risk classification, disease progression and overall survival.

	Overall (n = 493)
Sex	
Male	284 (57.6%)
Female	209 (42.4%)
Age	
≤ 18 months	301 (61.1%)
> 18 months	192 (38.9%)
MYCN status	
Non-Amplified	401 (81.3%)
Amplified	92 (18.7%)
INSS stage	
1	120 (24.3%)
2	78 (15.8%)
3	62 (12.6%)
4	181 (36.7%)
4S	52 (10.5%)
High risk	
Yes	175 (35.5%)
No	318 (64.5%)
Progression	
Yes	180 (36.5%)
No	313 (63.5%)
Overall survival	
Yes	389 (78.9%)
No	104 (21.1%)

A tSNE analysis of the gene expression data revealed distinct clustering of samples based on survival status, clearly separating cases with severe outcomes from those with favorable prognosis (**Figure 4A**). To explore the biological mechanisms underlying these differences, a differential gene expression analysis was conducted, identifying 1560 differentially expressed genes (DEGs), including 741 upregulated and 819 downregulated genes (**Figure 4B**). Among the upregulated genes *MYCN* and *TWIST1* could be found, whereas *NTRK1* was downregulated. To further assess the biological processes associated with the DEGs, a gene set enrichment analysis (GSEA) was conducted using pathways defined in the Hallmarks of Cancer framework^{194,197}. While gene upregulation is linked to genome instability, sustained proliferation, and replicative immortality, gene downregulation is associated with enhanced invasion, metastasis, and, most notably, with avoiding immune destruction and inflammation (**Figure 4C**). To further investigate the genes enriched in immune- and inflammation-related pathways, a gene-pathway network was generated (**Figure 4D**). Patients with poor outcomes demonstrated reduced expression of several HLA class I and class II genes (e.g. *HLA-DQB2*, *HLA-B*, *HLA-DRA*), suggesting impaired antigen presentation within the tumor and its microenvironment, which may hinder immune recognition and facilitate immune evasion. Additionally, genes involved in lymphocyte development or activation (e.g. *IL7R*, *CD44* or *ZAP70*), T cell receptor signaling (e.g. *CD4* or *LCP2*) or NK cell function (e.g. *KLRB1* or *KLRK1*) were also lower expressed. Chemokines and pro-inflammatory cytokines essential for immune cell recruitment and activation were also reduced in expression, including *CCL19*, *CCL2*, *CXCL12*, *IL7*, *IL18* and *IL32*.

These findings highlight a significant suppression of immune- and inflammation-related pathways, which may facilitate immune evasion in patients with poor prognosis. Building on these findings, differences in the immune microenvironment regarding immune and stromal cell infiltration were evaluated. The ESTIMATE (Estimation of stromal and immune cells in malignant tumor tissue using expression data) algorithm was used to quantify the presence of immune cells in the neuroblastoma samples. Patients with favorable prognosis showed significantly higher immune scores compared those with worse outcomes, indicating reduced immune cell infiltration in the tumor (**Figure 4E**). Further analysis using the MCPcounter tool revealed distinct differences in the infiltration of immune and stromal cell subtypes. Specifically, patients with good prognosis showed significantly higher infiltration of NK cells and T cells compared to those with poor outcomes (**Figure 4F**). These findings underscore the critical role of immune cell presence in shaping the tumor microenvironment and thereby influencing neuroblastoma patient outcomes.

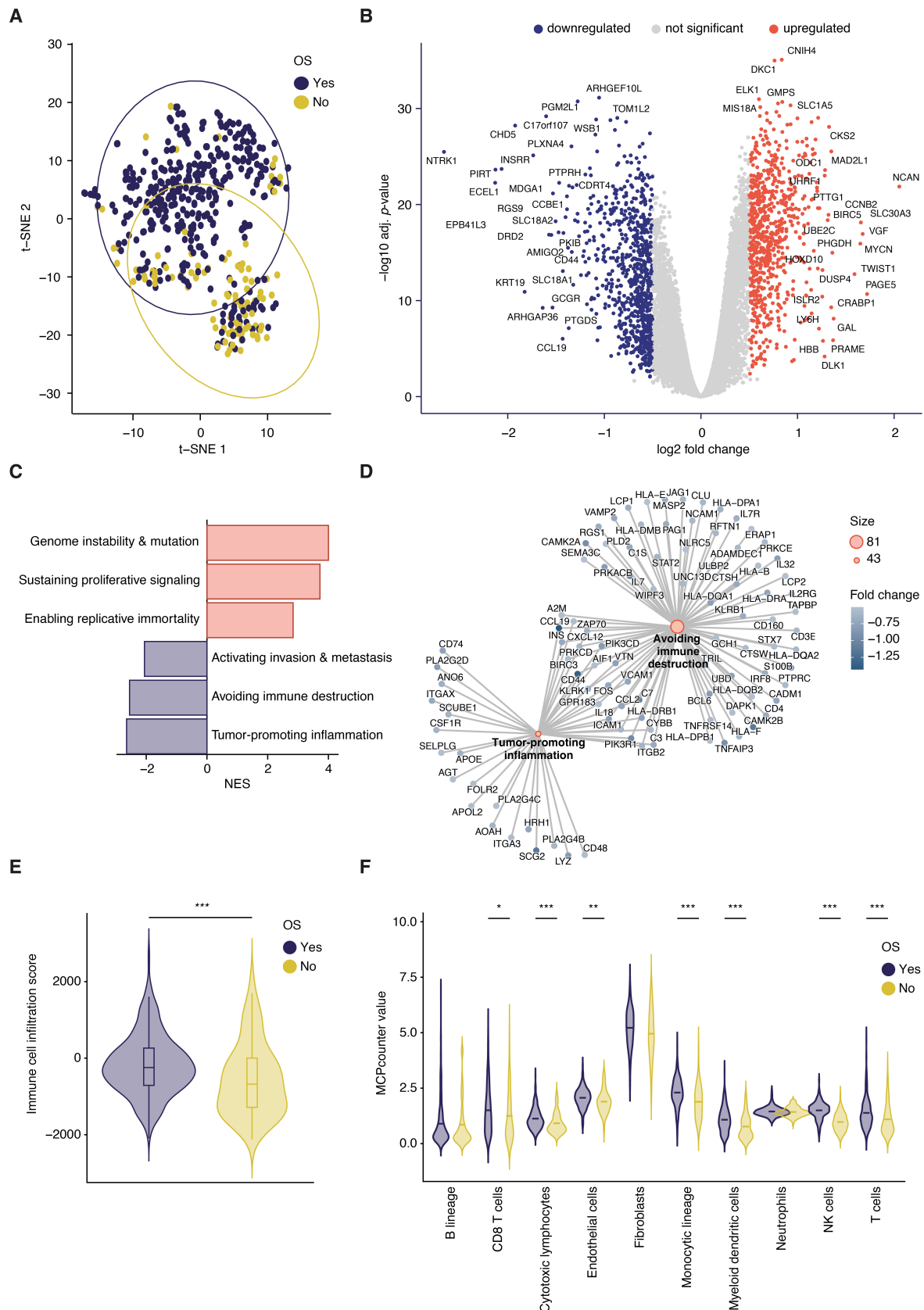


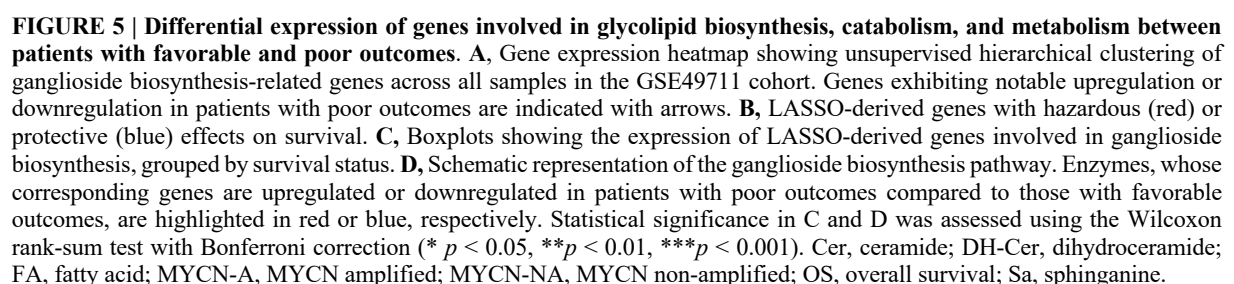
FIGURE 4 | Reduced immune infiltration correlates with poor prognosis in neuroblastoma. **A**, t-SNE plot of 493 neuroblastoma samples from the GSE49711 cohort, annotated by overall survival status. Ellipses represent 95% confidence intervals for each group. **B**, Volcano plot of differentially expressed genes based on survival status. Genes are classified as upregulated ($\log_2 \text{FC} > 0.5$, adj. p -value < 0.01 , shown in red), downregulated ($\log_2 \text{FC} < -0.5$, adj. p -value < 0.01 , shown in blue), or not significant (shown in grey). Highly significant or strongly regulated genes are labeled. **C**, Two-sided bar plot showing significantly enriched pathways associated with the Hallmarks of Cancer, identified by GSEA of DEGs between samples stratified by overall survival status. The normalized enrichment score (NES) represents the magnitude of pathway enrichment,

with positive NES values (red) indicating gene sets that are upregulated and negative NES values (blue) indicating gene sets that are downregulated. **D**, Gene-pathway networks highlighting downregulated genes involved in the Hallmarks of Cancer "Avoiding immune destruction" and "Tumor-promoting inflammation" identified by GSEA. **E**, Immune cell infiltration scores stratified by overall survival status, derived from ESTIMATE analysis. **F**, MCPcounter analysis of samples stratified by overall survival status, showing tumor infiltration by various stromal and immune cell populations. Statistical significance in E and F was assessed using the Wilcoxon rank-sum test with Bonferroni correction (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). OS, overall survival.

3.1.2 Evaluation of ganglioside expression in high-risk neuroblastoma

Given that cell surface glycosylation can impact key biological processes such as cell-cell recognition and immune response¹⁹⁸, and given the clinical success of anti-ganglioside GD2 immunotherapy⁵², the expression of gangliosides and their impact on survival were analyzed in greater detail. Therefore, the expression patterns of genes involved in the biosynthesis, metabolism and catabolism of gangliosides were evaluated. The analysis identified distinct gene expression patterns that clearly stratified patients based on survival outcomes and adverse clinical parameters, including high risk status and MYCN amplification. Unsupervised clustering in the heatmap showed that patients with poor prognosis and high-risk status clustered predominantly on the left side, which suggests a potential link between altered ganglioside biosynthesis and prognosis. Specifically, the genes *B3GNT5* and *ST3GAL6* were upregulated in high-risk neuroblastomas, whereas *HEXA* and *GM2A*, which are responsible for the degradation of ganglioside GM2¹⁹⁹, were downregulated in these. Furthermore, genes encoding key enzymes in the biosynthesis of more complex gangliosides, such as *B3GALT4* and *ST3GAL3*, were also downregulated. Notably, *ST8SIA1*, which is critical for GD2 biosynthesis²⁰⁰, is lower expressed in patients with poor prognosis (**Figure 5A**).

To further assess the prognostic relevance of the ganglioside-associated gene expression, univariate Cox regression analysis was performed, followed by Least Absolute Shrinkage and Selection Operator (LASSO) regression on the ganglioside-related genes. This analysis identified eight genes with a strong prognostic significance. Among these, three genes were associated with hazardous effects on patient outcomes when highly expressed (*B3GNT5*, *ST8SIA3*, *ST6GALNAC4*), while five genes were linked to protective effects when highly expressed (*ST8SIA*, *ST6GALNAC5*, *ST3GAL3*, *ST8SIA2*, *ST3GAL1*), as indicated by their respective LASSO regression coefficients (**Figure 5B**). All hazardous genes are significantly higher expressed in patients with worse survival, while all protective genes have a significantly lower expression in these patients compared to those with favorable outcomes (**Figure 5C**). The production of gangliosides from the (Neo)lacto-series might be elevated in patients with high-risk status and poorer outcomes due to an elevated *B3GNT5* expression. Additionally, patients with worse outcomes displayed reduced expression of genes critical for the biosynthesis of more complex gangliosides, including *B3GALT4*, *ST3GAL2* and *ST3GAL3* (**Figure 5D**). These findings support the hypothesis that structurally simpler gangliosides from the a- and b-series may predominate in tumors associated with lower survival rates, highlighting their potential as targets for therapeutic strategies.



To further explore the association between gene expression patterns and ganglioside expression, the gene expression of ganglioside-related genes was analyzed in the dataset GSE89413, which includes gene expression data for the neuroblastoma cell lines Kelly, SH-SY5Y, SK-N-SH, and SK-N-AS. Reduced expression of *ST8SIA1* was observed in SK-N-SH and SK-N-AS compared to Kelly and SH-SY5Y, indicating lower levels of ganglioside GD2 in these cells. Moreover, *HEXA* was upregulated in SK-N-AS and SK-N-SH, while *GM2A* was additionally upregulated in SK-N-AS cells. These findings suggest a reduced expression of GM2 on the surface of these cells. Interestingly, *B3GALT4*, a key gene involved in the biosynthesis of GM1 and GD1 from GM2 and GD2, respectively²⁰¹, was found to be downregulated in Kelly and SH-SY5Y (**Figure 6A**). To compare these results with ganglioside expression on the cells, a flow cytometric analysis was performed on neuroblastoma cell lines to evaluate the expression of GM2, GD2, GD3, GM3 and GM1. Consistent with the gene expression data, higher levels of GM2 were observed in Kelly and SH-SY5Y cells compared to SK-N-SH and SK-N-AS, which exhibited only weak GM2 expression. This weak expression aligns with the upregulation of the genes critical for GM2 degradation (*HEXA* and *GM2A*) or the biosynthesis of downstream gangliosides from GM2 (*B3GALT4*). Notably, most neuroblastoma cell lines tested expressed high levels of GM2, regardless of the antibody used for detection (**Figure 6B**). Expression of *ST8SIA1* correlated with GD2 expression across the cell lines. Lower expression of *ST8SIA1* in SK-N-SH and SK-N-AS was associated with reduced GD2 levels in these cells (**Figure 6C**). GD3 expression was detected in Kelly, LAN-1, SK-N-AS and SK-N-LO cells (**Figure 6D**). Similar to GM2, GM3 was highly expressed in all neuroblastoma cell lines tested (**Figure 6E**). Conversely, the structurally more complex ganglioside GM1 was barely expressed in neuroblastoma cell lines (**Figure 6F**), despite higher *B3GALT4* expression in SK-N-SH and SK-N-AS, which would suggest increased GM1 levels.

All in all, structurally simple gangliosides, such as GM3, GM2, and GD2, were expressed in neuroblastoma and may serve as promising targets for targeted therapies in high-risk neuroblastoma. To evaluate the level and localization of GD2 and GM2 expression under more physiological conditions, immunofluorescence staining was performed on monolayer cultures of adherent neuroblastoma cells and on cell line-derived 3D spheroids. GM2 surface expression was detected across all neuroblastoma cell lines tested, though levels were notably reduced in SK-N-AS and SK-N-SH cells. In contrast, GD2 expression was completely or nearly absent in three out of eight cell lines, specifically SH-EP, SH-SY5Y, and SK-N-SH (**Figure 7A**). Using LS cells cultured in self-assembling spheroids, high surface expression levels of GM2 and GD2 could also be confirmed under 3D culturing conditions (**Figure 7B**). Notably, LS cell lines with a knockout of the GD2/GM2 synthase *B4GALNT1* (LS^{AB4GALNT1}) did not express GM2 or GD2, further confirming the role of *B4GALNT1* in the biosynthesis of these gangliosides. The generation of these knockout cell lines is described in Section 3.2.2.1.

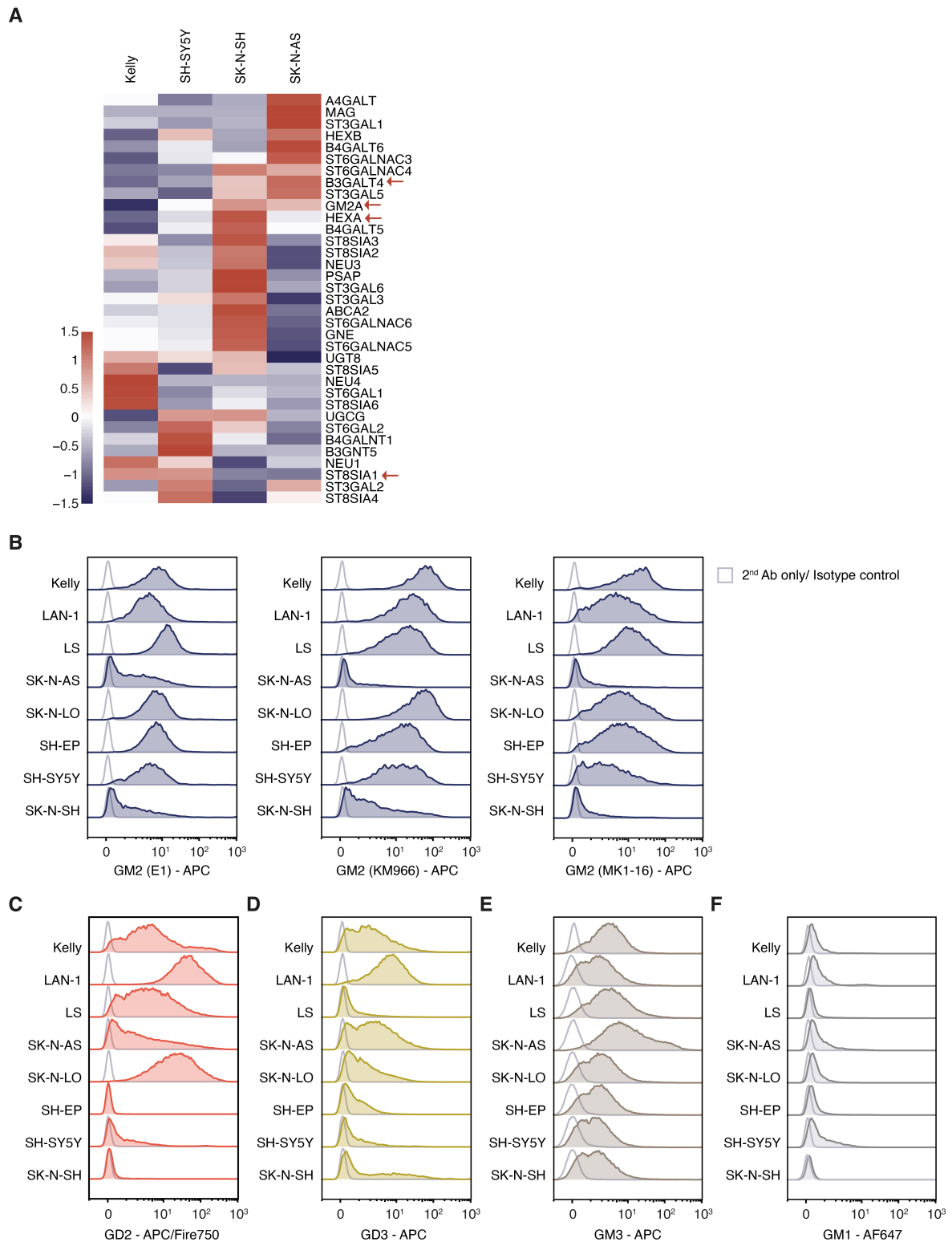


FIGURE 6 | Ganglioside expression patterns of human neuroblastoma cell lines. **A**, Gene expression heatmap of ganglioside biosynthesis-related genes in neuroblastoma cell lines (Kelly, SH-SY5Y, SK-N-SH, and SK-N-AS) from the GSE89413 dataset. **B**, Flow cytometric analysis of GM2 expression using different anti-GM2 antibodies: E1 (Neu5Ac-specific), KM966 (Neu5Ac- and Neu5Gc-specific), and MK1-16 (Neu5Ac-specific) (blue). Appropriate APC-labeled secondary antibodies were used, and a secondary antibody only control was applied (light grey). Flow cytometric analysis of ganglioside expression **C**, GD2 (Neu5Ac) (red), **D**, GD3 (Neu5Ac) (yellow), **E**, GM3 (Neu5Ac) (brown) and **F**, GM1 (grey). GD2 staining was performed using an APC/Fire750-conjugated anti-GD2 antibody. An appropriate isotype control was used (light grey). GD3, GM3 and GM1 staining were performed using specific primary antibodies and the corresponding APC- or AF647-conjugated secondary antibodies, with a secondary antibody only control (light grey). Representative histograms of three independent experiments are shown.

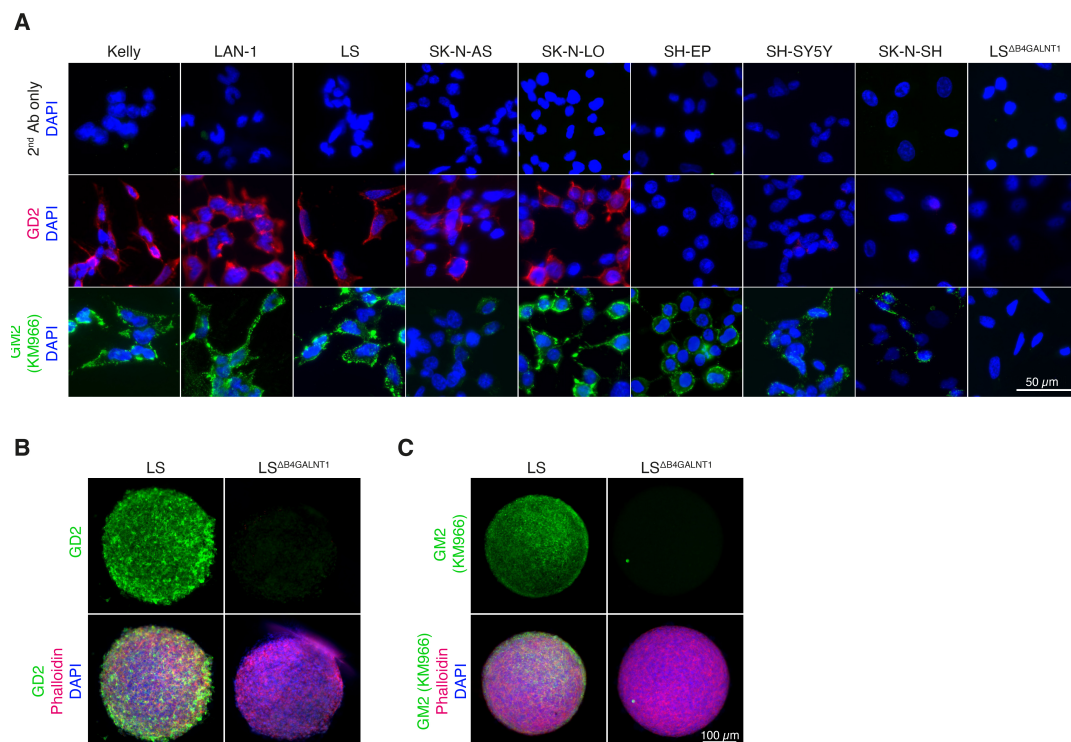


FIGURE 7 | Expression of gangliosides GD2 and GM2 on neuroblastoma cells in monolayer and 3D spheroid models. **A**, Immunofluorescence staining of GD2 and GM2 on several neuroblastoma cells cultured for 2 d in monolayer conditions. GD2 was stained using 5 μ g/ml of anti-GD2 antibody (clone 14G2a) and a secondary anti-mouse IgG APC-conjugated antibody (1:200). GM2 was stained using 5 μ g/ml anti-GM2 antibody (KM966) and a secondary anti-human IgG AF488-conjugated antibody (1:250). Counterstaining was performed with 1 μ g/ml DAPI, and a secondary antibody-only control was included. The knockout cell line LS Δ B4GALNT1, which lacks GD2 and GM2 expression, was also included as a control. Images were acquired using 40 $\times$ magnification. Representative overlay images of DAPI and antibody staining from two independent experiments performed in duplicates are shown. Immunofluorescence staining of GD2 (**B**) and GM2 (**C**) in 3D spheroids derived from neuroblastoma cell lines cultured for 3 d. GD2 was stained with 10 μ g/ml of anti-GD2 antibody (clone 14G2a) and a secondary anti-mouse IgG AF488-conjugated antibody (1:100). GM2 was stained with 10 μ g/ml anti-GM2 antibody (KM966) and a secondary anti-human IgG AF488-conjugated antibody (1:100). Counterstaining was performed with 1 μ g/ml DAPI and Phalloidin-iFluor555 (1:1000). The knockout cell line LS Δ B4GALNT1 was used as a control. Images were acquired using 20 $\times$ magnification. Representative images of antibody staining, as well as overlays of antibody staining, DAPI, and Phalloidin-iFluor555 from two independent experiments performed in triplicates are shown.

In summary, the *in silico* analysis of gene expression data suggests a predominance of structurally simpler gangliosides, particularly in high-risk neuroblastoma associated with reduced overall survival, driven by the downregulation of genes involved in the biosynthesis of more complex downstream gangliosides. Coupled with the universal surface expression across various neuroblastoma cell lines, these findings highlight GM2 as a promising target for immunotherapeutic strategies in neuroblastoma.

3.2 Generation of GM2-specific antibodies

3.2.1 Mouse immunization

To generate GM2-specific antibodies for an immunotherapeutic approach for the treatment of high-risk neuroblastoma, C57BL/6 mice were immunized with GM2 substrates at various time points (**Figure 8A**). Two different GM2 substrates were tested for their immunogenic potential to induce an immune response against GM2. The first substrate consisted of the carbohydrate moiety of GM2

conjugated to KLH to enhance immunogenicity, while the second substrate consisted of multiple GM2 carbohydrate moieties conjugated to a PAA carrier (**Figure 8B**). Six mice were immunized with GM2-KLH, while five mice received GM2-PAA. On day 31, blood samples were collected, and the spleens were harvested for hybridoma generation. Sera isolated from blood samples were analyzed for anti-ganglioside antibody production using the anti-ganglioside dot assay. Notably, no specific immune response against GM2 substrates was detected, as no reactivity to GM2 was observed on the test strips. Interestingly, antibodies targeting structurally related gangliosides were identified. Anti-GM4 antibodies were present in 5 out of 11 mice, and anti-GM3 antibodies were detected in 7 out of 11 mice (**Figure 8C**). The spleens of two mice were further selected for hybridoma generation: mouse M1, which was immunized with GM2-KLH, and mouse M7, which received GM2-PAA. Splenocytes from these mice were fused with Sp2/0-Ag14 cells to produce hybridoma cells. After 10 days of culture, the hybridoma supernatants were tested for anti-ganglioside antibodies using the anti-ganglioside dot assay (**Figure 8D**). Although the immunization with GM2 substrates leads to the generation of structurally similar gangliosides, such as GM3 and GM4 (**Figure 8E**), these antibodies were detectable only in serum. All in all, the hybridoma cells did not produce detectable antibodies against GM3, GM4, or GM2.

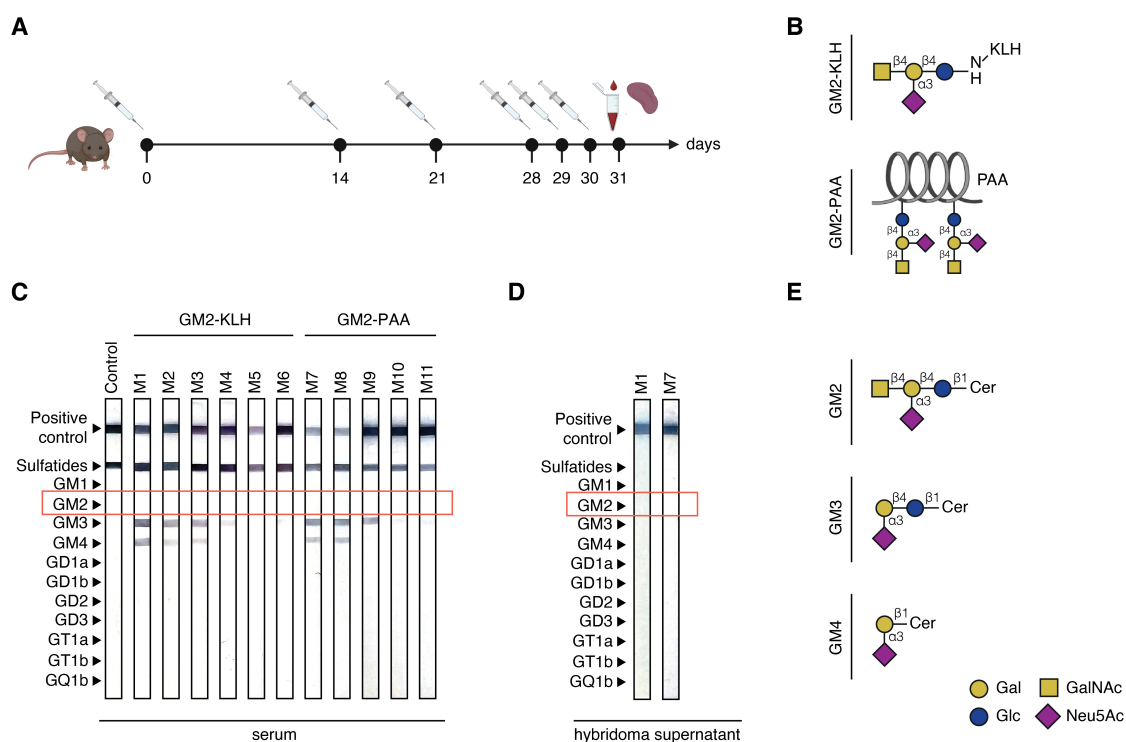


FIGURE 8 | Immunization of C57BL/6 mice with GM2 substrates does not stimulate a GM2-specific immune response. **A**, Schematic representation of the immunization protocol. Mice were immunized with GM2 substrates on days 0, 14, 21, 28, 29, and 30. Blood samples were collected, and spleens were harvested on day 31. **B**, GM2 substrates used for immunization. GM2 conjugated to KLH (keyhole limpet hemocyanin) or multiple GM2 bound to PAA (Poly[N-(2-hydroxyethyl)acrylamide-co-N-acrylamide]) were used to enhance immunogenicity against GM2. **C**, Anti-ganglioside dot assay evaluating anti-ganglioside antibodies in serum samples from mice immunized with GM2-KLH or GM2-PAA, compared to serum from a non-immunized mouse (control). **D**, Anti-ganglioside dot assay evaluating the presence of anti-ganglioside antibodies in hybridoma supernatants generated from spleens of mice immunized with GM2-KLH and GM2-PAA. **E**, Schematic overview of the structures of gangliosides GM2, GM3 and GM4.

3.2.2 Isolation of GM2-specific antibodies from healthy donors

As the mouse immunization experiments with GM2 failed to generate a GM2-specific antibody response, the next approach focused on screening for naturally occurring antibodies with cytotoxic activity against neuroblastoma cells within the healthy population, as reported in previous studies^{132,180,202}. To evaluate cytotoxic effects against GM2- and GD2-expressing neuroblastoma cells, it was first necessary to generate control cell lines lacking GM2 and GD2 expression.

3.2.2.1 Generation of B4GALNT1 knockout neuroblastoma cell lines

Neuroblastoma cell lines with a knockout (KO) of the *B4GALNT1* gene, which encodes the enzyme β -1,4-N-acetyl-galactosaminyltransferase 1 (B4GALNT1), were generated to inhibit the biosynthesis of GA2, GM2, GD2, GT2, and thereby all downstream gangliosides. The CRISPR/Cas9-induced KOs were generated using a guide RNA specifically targeting *B4GALNT1* (**Figure 9A**). The genome editing efficiency of all KO cell lines was assessed using the Tracking of Indels by DEcomposition (TIDE) algorithm, which enabled precise evaluation of the distribution and frequency of insertions and deletions in the DNA of a targeted cell pool. Representative results for the LS ^{Δ B4GALNT1} cell line revealed a total editing efficiency of 94.4%. The most prominent event was a 1 bp insertion, observed in 84.9% of the analyzed sequences (**Figure 9B**). Further analysis identified adenine (A) as the predominantly inserted nucleotide in LS ^{Δ B4GALNT1} (**Figure 9C**). Similar results were observed for the other KO cell lines, including Kelly ^{Δ B4GALNT1}, LAN-1 ^{Δ B4GALNT1}, SK-N-LO ^{Δ B4GALNT1}, SH-EP ^{Δ B4GALNT1}, SH-SY5Y ^{Δ B4GALNT1} and SK-N-SH ^{Δ B4GALNT1}, with all lines showing editing efficiencies above 91.3% (**Figure 9D, Appendix Supplementary Figure 5**). To confirm that the B4GALNT1 KO led to the loss of GD2 and GM2 expression, flow cytometry was performed using GM2- and GD2-specific antibodies. As expected, all B4GALNT1 KO cell lines lacked GM2 and GD2 expression, whereas their respective wildtype controls retained ganglioside expression consistent with prior analysis (**Figure 9E**).

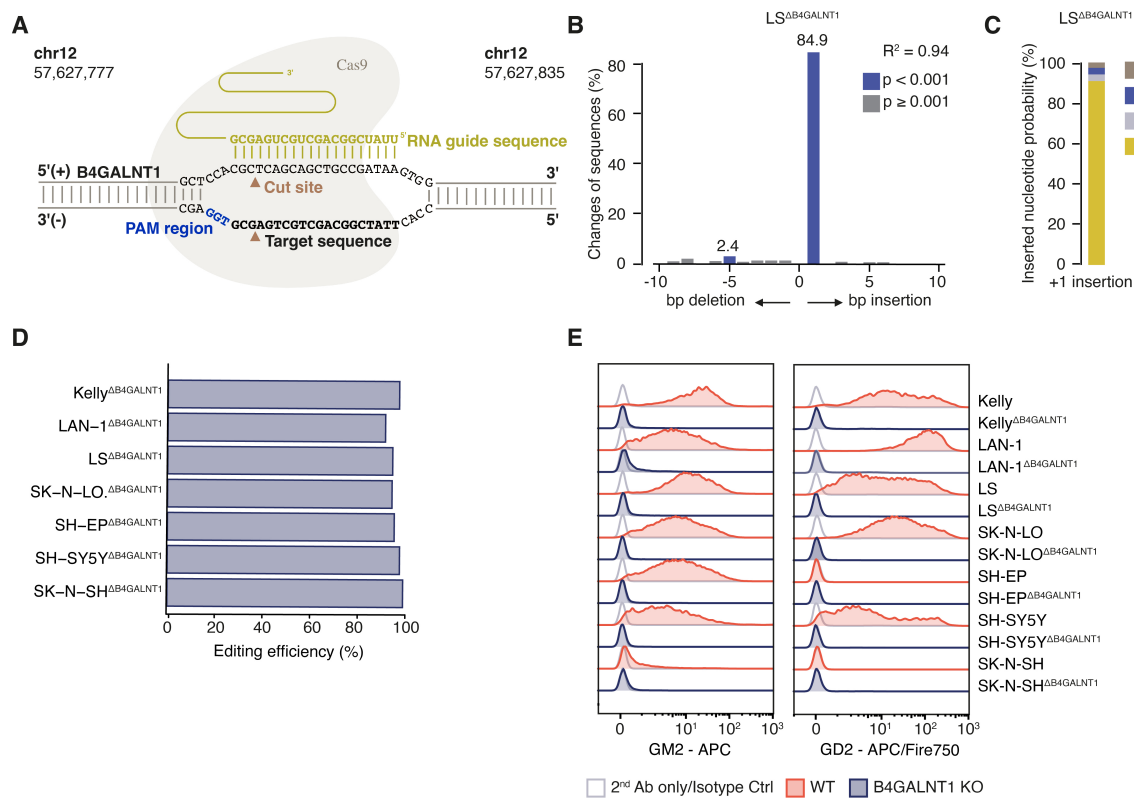


FIGURE 9 | Generation and characterization of B4GALNT1 knockout neuroblastoma cell lines with complete loss of GM2 and GD2 expression. **A**, Schematic representation of a CRISPR/Cas9-induced B4GALNT1 knockout (KO). A guide RNA specific to the B4GALNT1 gene was designed to induce a KO in several neuroblastoma cell lines. The schematic was generated and modified using Syntheo's CRISPR guide. **B**, TIDE analysis of the B4GALNT1 KO, illustrating the distribution and frequencies of insertions and deletions introduced at the target site. Data are representative of the LS^{ΔB4GALNT1} cell line. **C**, Probability of each nucleotide (G, T, A and C) being inserted during repair of CRISPR/Cas9-induced double-strand breaks, representatively shown for LS^{ΔB4GALNT1}. **D**, Editing efficiency of CRISPR/Cas9-induced B4GALNT1 KO across multiple neuroblastoma cell lines. **E**, Flow cytometric analysis of GM2 (left) and GD2 (right) expression in B4GALNT1 KO cells (blue) versus wildtype cells (red). GM2 was detected using 10 µg/ml anti-GM2 antibody (MK1-16), followed by an APC-conjugated anti-mouse IgM antibody. GD2 was stained using an APC/Fire750-conjugated anti-GD2 antibody (1:100). Controls for secondary antibody binding (GM2, grey) and isotype control staining (GD2, grey) were included. Representative histograms of three independent experiments are shown. WT, wildtype.

3.2.2.2 Screening healthy donors for naturally occurring antibodies with cytotoxic activity against neuroblastoma

To identify healthy donors with naturally occurring antibodies (nAbs) cytotoxic to neuroblastoma cells, serum and Buffy Coat-derived plasma samples were collected from 248 healthy donors. These samples were analyzed for their cytotoxic potential using a complement-dependent cytotoxicity (CDC) assay with the neuroblastoma cell line LS. Cytotoxic effects exceeding 50% were observed in 5.65% of the cohort (**Figure 10A**). The serum and plasma samples were further tested for the presence of anti-ganglioside antibodies using the anti-ganglioside dot assay. In most samples, no anti-ganglioside antibodies were detected. Remarkably, anti-GM2 antibodies (GM2-Ab) of the IgM isotype were identified in the serum or plasma of 14 donors (**Figure 10B and C**). In some cases, IgM antibodies recognizing other gangliosides, such as GM1, GM3, GD2 and GD3, could be detected. Additionally, serum from D78 contained GM2- and GD3-Abs of the IgG isotype. Interestingly, samples positive for anti-GM2 antibodies (GM2-Ab^{pos}) induced significantly higher cytotoxicity against LS cells compared

to samples negative for anti-GM2 antibodies (GM2-Ab^{neg}). However, some GM2-Ab^{neg} samples also exhibited high cytotoxicity, suggesting the presence of nAbs targeting also other antigens on the cell surface (**Figure 10D**). Moreover, the cytotoxic efficacy of the samples was also tested using LS^{ΔB4GALNT1} cells. This analysis revealed no induction of CDC, indicating that the cytotoxic antibodies present in the serum specifically target ganglioside structures synthesized by B4GALNT1 or downstream products in the biosynthesis of more complex gangliosides (**Figure 10E**).

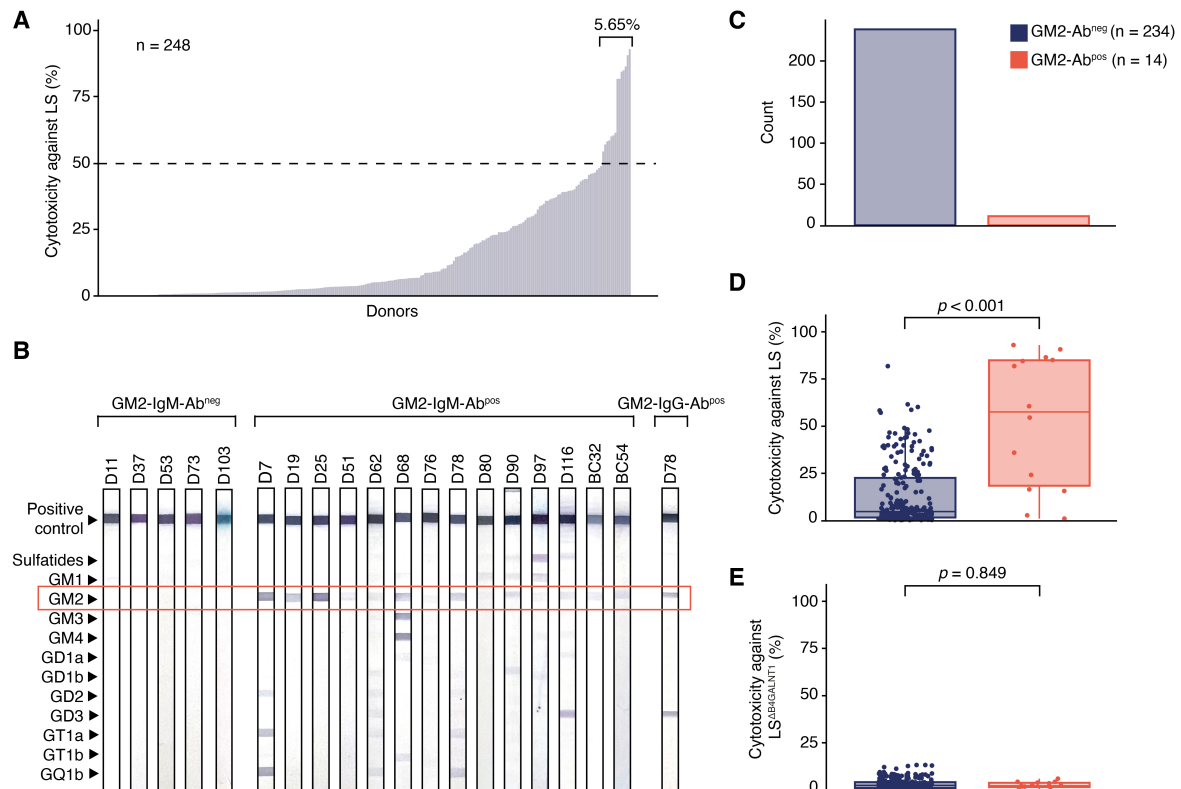
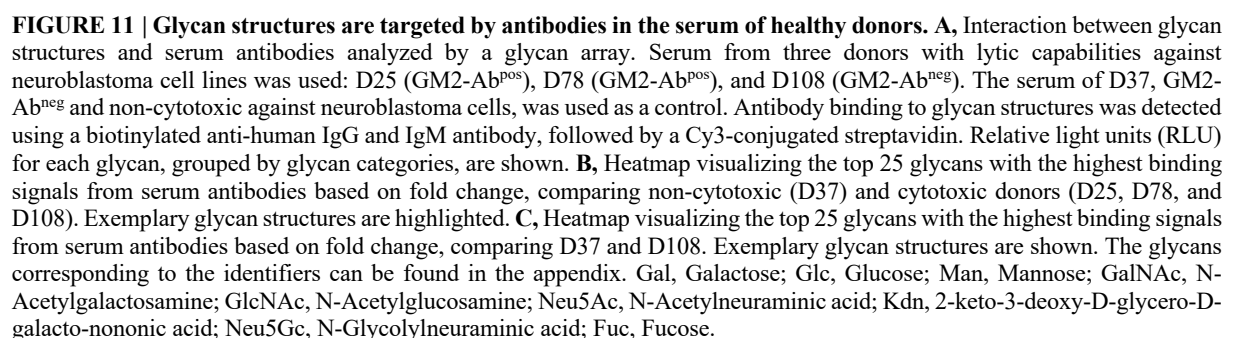


FIGURE 10 | Naturally occurring IgM anti-GM2 antibodies in healthy donors mediate cytotoxicity against human neuroblastoma cells. **A**, Incucyte-based complement-dependent cytotoxicity assay for the screening of 248 serum and plasma samples from healthy donors, evaluating cytotoxic effects on the neuroblastoma cell line LS. Positive responses were defined as reaching or exceeding 50% cytotoxicity. **B**, Anti-ganglioside dot assay detecting anti-ganglioside IgM and IgG antibodies in the serum and plasma of healthy donors. Representative results from donors who tested negative for anti-ganglioside antibodies are shown, while all dot blots from donors with anti-GM2 antibodies are presented. **C**, Anti-GM2 antibody status of donors screened in the anti-ganglioside dot assay. Cytotoxicity mediated by donor serum from anti-GM2 antibody-positive (GM2-Ab^{pos}) and anti-GM2 antibody-negative (GM2-Ab^{neg}) donors, against the neuroblastoma cell line LS (**D**) and the GM2-negative cell line LS^{ΔB4GALNT1} (**E**). Each donor serum sample was screened for cytotoxicity and anti-ganglioside antibody presence once. Statistical analyses included Wilcoxon rank-sum tests for group differences between GM2-Ab^{pos} and GM2-Ab^{neg} donors. BC, buffy coat; D, donor.

3.2.2.3 Screening for putative glycan targets of naturally occurring cytotoxic antibodies

To gain further insight into the antigens targeted by nAbs in the serum of healthy donors, a glycan assay was performed. The assay included three cytotoxic serum samples, one GM2-Ab^{neg} sample (D108) and two GM2-Ab^{pos} samples (D25 and D78), as well as one non-cytotoxic sample (D37). This assay detects antibody binding to 300 different glycan structures, representing various glycan classes, such as mono-

and disaccharides, Lewis antigens, gangliosides, sialylated oligosaccharides, O- and N-glycans, and human milk oligosaccharides. The serum of all donors showed antibodies targeting various human milk oligosaccharides and natural oligosaccharides. Additionally, strong binding was observed to glycans categorized as glycolipid glycans (**Figure 11A**). Next, the top 25 glycans bound by serum antibodies from cytotoxic donors compared to the non-cytotoxic donor were visualized in a heatmap, illustrating the fold change between the cytotoxic and non-cytotoxic donors (**Figure 11B**). Notably, ganglioside GM2 (identifier G0072) displayed a higher binding signal, particularly in serum from D25 and D78, consistent with the findings from the anti-ganglioside dot assay. Additionally, binding to ganglioside GD2 (identifiers G0073 (GD2(Neu5Ac)) and L1218 (GD2 (Kdn))) was detected in D25 and D78, although this was not observed in the anti-ganglioside dot assay. Furthermore, the glycolipids Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc (identifier L2111) and Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc (identifier 2112) emerged as potential antibody targets, particularly for D108-derived antibodies. The analysis of the top 25 glycan structures based on fold change between the non-cytotoxic D37 and cytotoxic GM2-Ab^{neg} D108, mainly revealed antibody binding to glycan structures belonging to the “Tandem epitope” group (**Figure 11C**). Interestingly, D108 also possessed antibodies targeting the ganglioside Lc3 from the globo-series. This may be relevant, as Lc3 is likely overexpressed in high-risk neuroblastoma due to increased expression of *B3GNT5*, the gene encoding the enzyme responsible for globo-series ganglioside biosynthesis, as suggested by the previous *in silico* analysis (**Figure 5**). However, given the association between cytotoxicity and B4GALNT1 activity observed in previous analyses, it is more likely that the cytotoxic effect of these antibodies is related to gangliosides synthesized by the B4GALNT1 enzyme or enzymes involved in subsequent steps of the ganglioside biosynthesis pathway. Since serum antibody binding to ganglioside GM2 was confirmed via glycan array analysis, GM2-specific IgM antibodies from GM2-Ab^{pos} donors were prioritized for further evaluation. No other ganglioside structures associated with B4GALNT1 expression could be identified using the glycan array with the serum from D108. Raw data, as well as glycan names and corresponding identifiers, are available in the appendix (**Appendix, Supplementary Table 1**).



3.2.2.4 Characterization of binding specificity of naturally occurring anti-GM2 IgM antibodies

After identifying donors with anti-GM2 antibodies in their serum, the binding of these nAbs to GM2⁺ LS cells and GM2⁻ LS^{AB4GALNT1} cells was analyzed using flow cytometry. Donor serum was incubated with the cells, and IgM antibody binding was detected using an APC-conjugated secondary anti-human IgM antibody. Serum incubation generally resulted in increased IgM binding to both LS wildtype and KO cells, regardless of the donor serum, suggesting a GM2-independent background signal. However, incubation with GM2-Ab^{pos} serum led to higher IgM binding to LS wildtype compared to KO cells. In contrast, GM2-Ab^{neg} serum showed no difference in IgM binding between the two cell lines, as the fluorescence intensity levels were comparable (**Figure 12A**). When analyzing the percentage of cells bound by IgM antibodies, a higher proportion of LS wildtype cells were IgM⁺ after incubation with serum from GM2-Ab^{pos} donors compared to KO cells. This effect was particularly evident with serum from D7, D19, D25 and D78, showing statistically significant differences. The highest proportions of IgM⁺ cells were observed with serum from D7 (23.8%), D19 (40.5%), D25 (25.7%), D68 (25.8%) and D78 (23.6%). Interestingly, no significant differences in the proportion of IgM⁺ cells were observed between wildtype and knockout cells when incubated with GM2-Ab^{neg} serum (**Figure 12B**). These findings suggest that IgM binding to GM2⁺ LS cells is mediated by anti-GM2 antibodies present in the serum of GM2-Ab^{pos} donors.

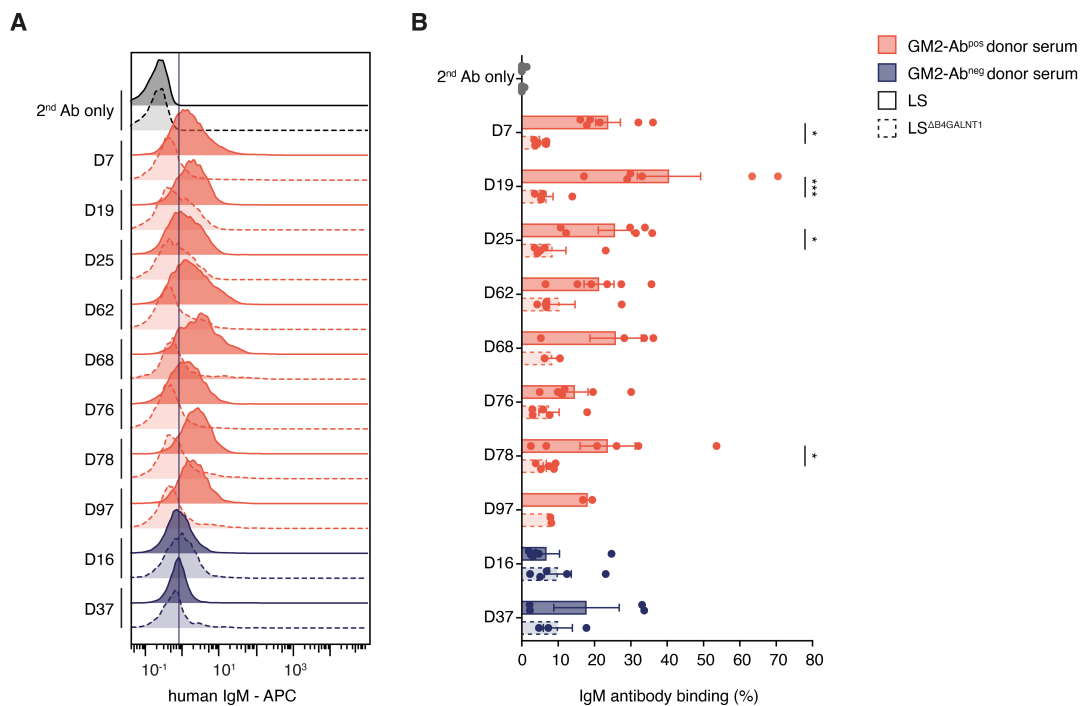


FIGURE 12 | Anti-GM2 antibody-containing sera show enhanced IgM binding to the surface of LS cells compared to LS^{AB4GALNT1} cells. **A**, Flow cytometric analysis of IgM binding to LS wildtype and B4GALNT1 KO cells. Cells were incubated with 25% (v/v) serum from several donors, and the binding of human IgM antibodies to the cell surface was detected using an APC-conjugated anti-human IgM antibody. Representative histograms of at least three independent experiments are shown. **B**, Quantification of IgM⁺ cells from flow cytometry analysis, presented as mean ± SEM from at least three independent experiments. A second antibody only control was included. Statistical analyses were performed using two-way ANOVA with multiple comparisons and FDR correction using the Benjamini-Hochberg method. * $p < 0.05$, *** $p < 0.001$.

3.2.2.5 Characterization of cytotoxicity and target specificity of anti-GM2 IgM antibodies

Since the serum of GM2-Ab^{pos} donors demonstrated GM2-dependent binding to LS cells, the next step was to evaluate the CDC potential and target specificity of these nAbs. To achieve this, GM2-Ab^{pos} sera from D7, D19, D25, D62, D68, D76 and D78 were tested in a CDC assay to assess their cytotoxic effects on neuroblastoma cell lines with differing GM2 expression levels. GM2-Ab^{neg} sera from D16 and D37 were used as a control. LS cells, which express high levels of GM2 on their surface, were lysed by sera from all GM2-Ab^{pos} donors. Notably, a concentration of 8 µg/ml of the anti-GD2 antibody ch14.18 resulted in approximately 33% cytotoxicity, whereas most GM2-Ab^{pos} sera induced lysis rates ranging from 36% to 91%. Remarkably, the cytotoxicity mediated by activation of the complement cascade in the neuroblastoma cells occurred within the first hour of serum incubation. As expected, GM2-Ab^{neg} control sera did not mediate CDC within 4 hours (**Figure 13A**). The cytotoxicity assay with another GM2-expressing cell line, Kelly, showed no cytotoxic effects induced by GM2-Ab^{neg} sera. In contrast, GM2-Ab^{pos} sera induced lysis rates between 15% and 81%, while the anti-GD2 ch14.18 antibody mediated approximately 50% cytotoxicity (**Figure 13B**). In the low-GM2-expressing cell line SK-N-AS, GM2-Ab^{pos} sera were unable to activate the complement cascade. However, cytotoxicity was observed with ch14.18 due to the presence of GD2 on SK-N-AS cells (**Figure 13C**). To confirm that the observed cytotoxic effects were specifically dependent on GM2 expression and mediated by anti-GM2 antibodies, the assay was performed with the LS^{ΔB4GALNT1} cell line, which lacks both GM2 and GD2 expression. Neither GM2-Ab^{pos} sera nor ch14.18 induced any cytotoxicity in these cells, confirming the specificity of the complement-mediated killing (**Figure 13D**).

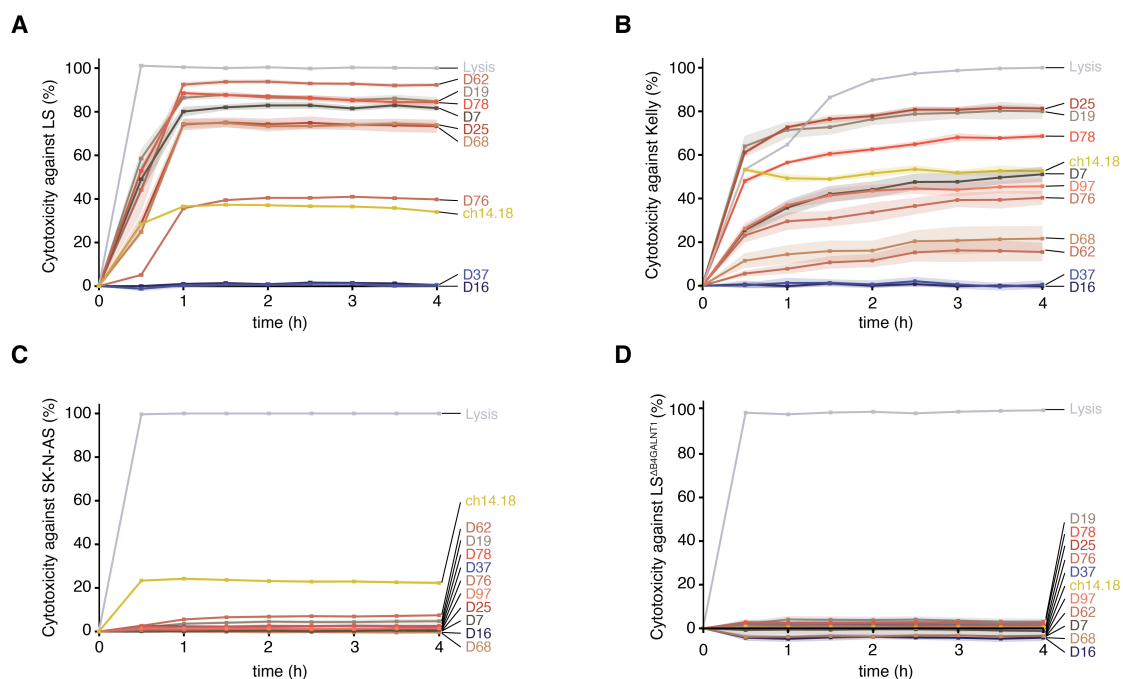


FIGURE 13 | Serum containing anti-GM2 antibodies induces high cytotoxicity against GM2 expressing neuroblastoma cells. Incubate-based cytotoxicity assay using 15% (v/v) serum from GM2-Ab^{pos} donors (reddish colors) and GM2-Ab^{neg} donors (bluish colors) on various neuroblastoma cell lines: GM2^{high}/GD2^{high} LS (A), GM2^{high}/GD2^{high} Kelly (B), GM2^{low}/GD2^{high} SK-N-AS (C) and GM2^{neg}/GD2^{neg} LS^{ΔB4GALNT1} (D). The anti-GD2 antibody ch14.18 (yellow) at a concentration of 8 µg/ml was used as a positive control, while 0.1% (v/v) Triton-X100 served as a total lysis control. Data represent the mean cytotoxicity over time from one experiment performed in triplicates, normalized to 0 h time point. The SEM are depicted as shades.

To evaluate the cytotoxic effect of donor-derived serum on neuroblastoma cell lines with varying GM2 expression levels, further CDC assays were performed. The assays were conducted using Kelly, LAN-1, LS, SH-SY5Y, SK-N-LO and SK-N-SH cells, along with their respective B4GALNT1 KO counterparts, over a 4-hour incubation period (**Figure 14A-F**). SK-N-AS cells, which naturally exhibit low GM2 expression, additionally served as a negative control (**Figure 14G**). The anti-GD2 antibody ch14.18 mediated CDC in GD2-expressing wildtype cell lines, including Kelly, LAN-1, LS, SK-N-LO, and SK-N-AS, with variable cytotoxic effects ranging from 23.3% to 85.6%. In contrast, GD2^{neg} or GD2^{low} cells, such as SH-SY5Y and SK-N-SH, were resistant to lysis by this antibody. Serum from donors lacking anti-GM2 antibodies (D16 and D37) did not exhibit cytotoxic effects on any tested neuroblastoma cell lines. This indicates that the absence of anti-GM2 antibodies renders the serum unable to mediate CDC against GM2-expressing cells. In contrast, GM2-Ab^{pos} sera from most donors induced high cytotoxic effects in GM2-expressing neuroblastoma cell lines, with the highest effects observed in Kelly, LAN-1, LS and SK-N-LO cells. These cell lines had been previously identified as having high GM2 expression levels. Notably, serum from D19, D25 and D78 demonstrated the highest cytotoxic activity against these GM2^{high} expressing cell lines, with cytotoxic effects ranging from 36.0% and 94.2% (D19), 36.1% and 89.4% (D25) and 22.3% and 91.2% (D78). However, sera from some donors, such as D68 and D76, showed generally lower cytotoxic effects. Importantly, B4GALNT1 KO cells, which lack both GD2 and GM2 expression, were resistant to lysis by all serum samples and the ch14.18 antibody, confirming the specificity of anti-GM2 and anti-GD2 antibody-mediated CDC.

As ganglioside GD3 cannot be converted to GD2 in B4GALNT1 KO cells due to the absence of the B4GALNT1 enzyme, GD3 expression in B4GALNT1 KO cells was investigated. Interestingly, flow cytometric analysis confirmed increased GD3 expression in LS^{ΔB4GALNT1} and SK-N-LO^{ΔB4GALNT1} cells (**Appendix, Supplementary Figure 6A**). Furthermore, an anti-GD3 antibody that was unable to induce CDC in wildtype LS and SK-N-LO cells showed cytotoxicity against B4GALNT1 KO cells (**Appendix, Supplementary Figure 6B**), indicating that increased GD3 expression, induced by B4GALNT1 downregulation, renders these cells susceptible to GD3-targeted cytotoxicity as well.

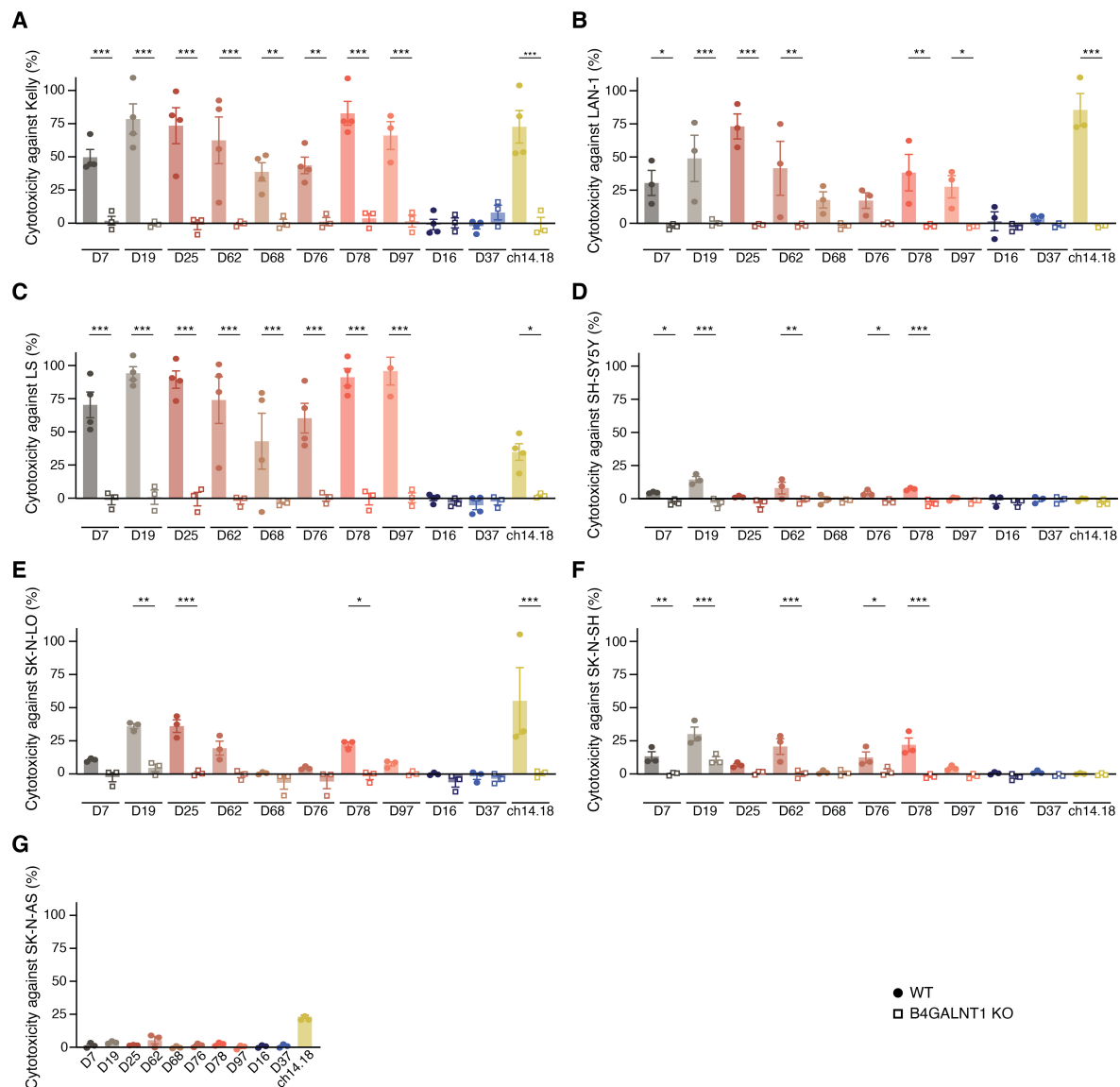


FIGURE 14 | Serum containing anti-GM2 antibodies specifically induce complement-dependent cytotoxicity in various GM2-expressing neuroblastoma cells. Incubate-based cytotoxicity assay using 15% (v/v) serum from GM2-Ab^{pos} donors (red colors) and GM2-Ab^{neg} donors (blue colors) on various neuroblastoma cell lines: GM2^{high}/GD2^{high} Kelly (A), GM2^{high}/GD2^{high} LAN-1 (B), GM2^{high}/GD2^{high} LS (C), GM2^{high}/GD2^{low} SH-SY5Y (D), GM2^{high}/GD2^{high} SK-N-LO (E), GM2^{low}/GD2^{neg} SK-N-SH (F) and GM2^{low}/GD2^{high} SK-N-AS (G). The anti-GD2 antibody ch14.18 (yellow) at a concentration of 8 μ g/ml was used as a positive control. Data represent the mean cytotoxicity $\pm$ SEM after 4 hours, normalized to a medium control, a complete lysis control, and the 0-hour time point from three independent experiments performed in triplicates. Statistical analyses were performed using two-way ANOVA with multiple comparisons and FDR correction using the Benjamini-Hochberg method. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The glycan-array analysis demonstrated a high presence of GM2-specific antibodies in the serum of D25 and D78, along with slight antibody binding to GD2. To further investigate the antigen-specificity of the cytotoxic antibodies, another cytotoxicity assay was performed using KO cells, which have a knockout of the enzyme ST8SIA1, which is responsible for synthesizing GD3. The KO leads to a loss of GD3 and any downstream gangliosides from the b- and c-series, including GD2.

As observed previously, the assay revealed that CDC could be induced in LS wildtype cells when serum containing anti-GM2 antibodies was applied. However, no CDC was observed in LS^{AB4GALNT1} cells, consistent with the earlier findings. Notably, the cytotoxic effect persisted when the cytotoxic serum

was used on LS^{ΔST8SIA1} cells (**Figure 15**). This finding indicates that the cytotoxic effect depends on the expression of gangliosides from the 0- and a-series, as CDC could still be induced in ST8SIA KO cells. As the serum-derived antibodies could not mediate CDC in B4GALNT1 KO cells, the target of the nAbs must be a ganglioside such as GA2, GM2, GA1, GM1, GD1 or GT1. Considering previous results showing that the cytotoxicity is dependent on the level of GM2 expression, it is highly likely that the CDC effect is mediated by anti-GM2 antibodies.

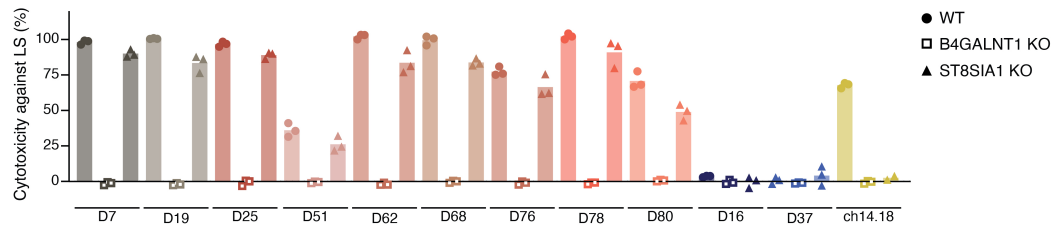


FIGURE 15 | Serum containing anti-GM2 antibodies specifically induce complement-dependent cytotoxicity in LS wildtype and ST8SIA1 knockout cells, but not in B4GALNT1 knockout cells. Incucyte-based cytotoxicity assay using 15% (v/v) serum from GM2-Ab^{pos} donors (reddish colors) and GM2-Ab^{neg} donors (bluish colors) on LS wildtype, LS^{ΔB4GALNT1} and LS^{ΔST8SIA1} cells. The anti-GD2 antibody ch14.18 (yellow) was used at a concentration of 16 μg/ml. Data represent the mean cytotoxicity after 4 hours, normalized to a medium control, a complete lysis control, and the 0-hour time point from one experiment using triplicates.

3.2.2.6 Isolation of GM2-specific B cells using fluorescence-activated cell sorting

To isolate GM2-specific B cells from healthy donors, peripheral blood was collected from GM2-Ab^{pos} and GM2-Ab^{neg} donors as controls. The next step involved evaluating whether the B cells of interest, which produce cytotoxic anti-GM2 antibodies, were present in the peripheral blood of these donors. Therefore, B cell cultivation conditions were established, in which the B cells can proliferate and secrete antibodies into the supernatant. Freshly isolated B cells were stimulated with IL-2, IL-21 and multimerized CD40L. The B cell proliferation was evaluated by eFluor670 staining after isolation and tracking of eFluor signal over a 9-day culture period using flow cytometry. This analysis showed a decrease in signal intensity, particularly from day 3 onwards (**Figure 16A**). Additionally, the culture conditions supported the production of IgG and IgM antibodies, as confirmed by collecting supernatants on days 2, 5, 7, and 9, followed by IgG- and IgM-specific ELISA. Antibody production increased over time, reaching concentrations of 12.8 μg/ml IgG and 19.4 μg/ml IgM after 7 days of culture (**Figure 16B**). B cells isolated from GM2-Ab^{pos} donors (D19 and D78) and GM2-Ab^{neg} donors (D37 and D103) were cultured using the same culturing conditions for 9 days. Supernatants were then analyzed for anti-GM2 IgM or IgG antibodies using an anti-ganglioside dot assay. As expected, B cells from GM2-Ab^{neg} donors did not produce anti-ganglioside antibodies. Surprisingly, despite the high levels of antibodies in the serum of D19, its cultured B cells did not produce anti-GM2 antibodies. Remarkably, B cells from D78 produced anti-GM2 antibodies after 9 days of culture. Further anti-ganglioside dot assay differencing between IgM and IgG isotype antibodies revealed that these antibodies were of the IgM isotype (**Figure 16C**).

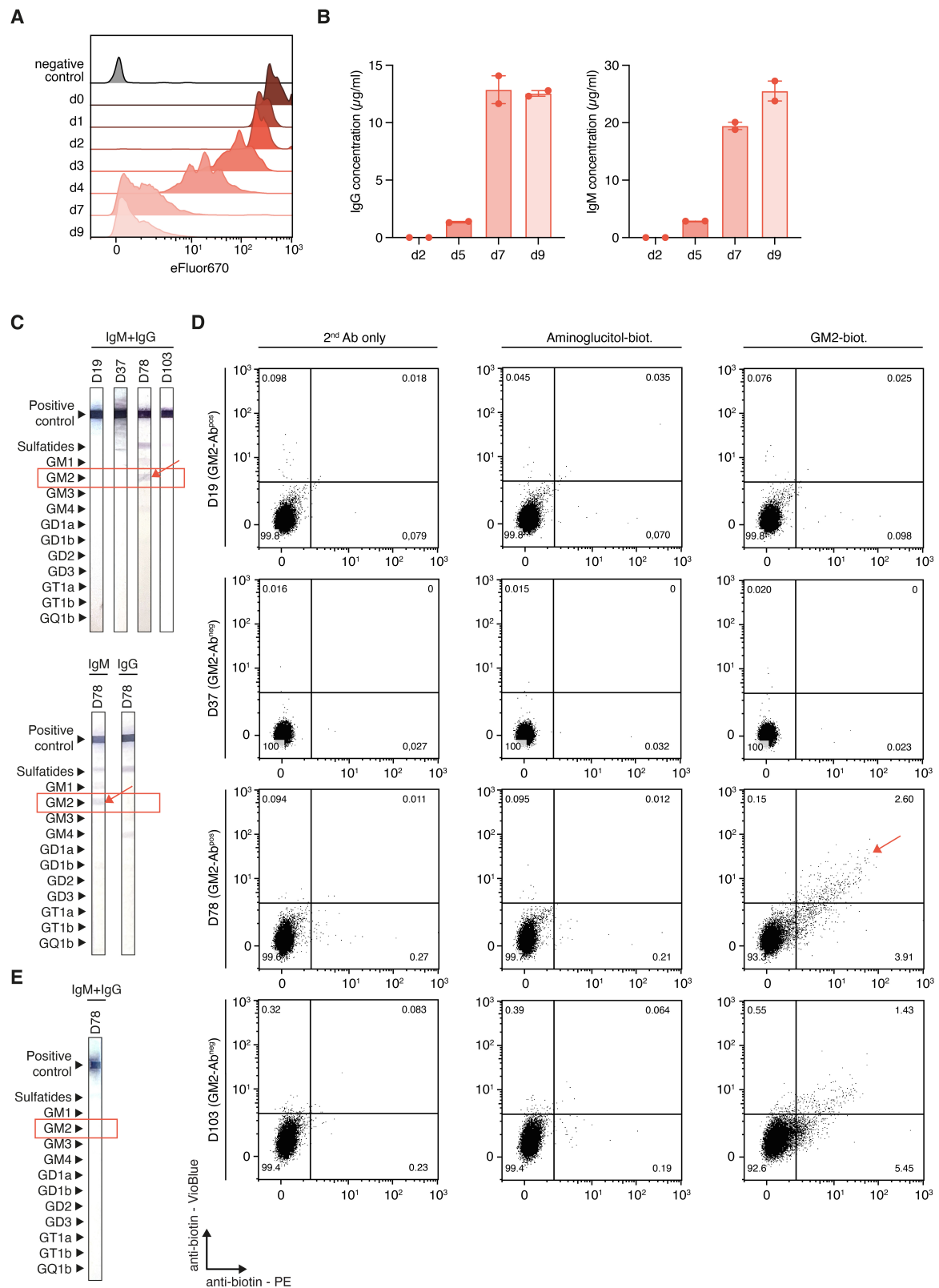


FIGURE 16 | Culturing and GM2-specific labeling of B cells from anti-GM2 antibody positive donors. **A**, Proliferation assay of isolated B cells from an GM2-Ab^{neg} donor labeled with eFluor670. Cells were cultured in the presence of IL-2, IL-21, and multimerized CD40L, and proliferation was tracked by flow cytometry on days 0, 1, 2, 3, 4, 7, and 9. Representative histograms of two independent experiments are shown. **B**, Production of IgG (left) and IgM (right) antibodies by B cells from a GM2-Ab^{neg} donor was quantified in cell culture supernatant using IgG- and IgM-specific ELISA on days 2, 5, 7, and 9. Results are shown as mean concentrations $\pm$ SEM from one representative experiment, with antibody production confirmed in two independent experiments with different donor B cells. **C**, Anti-ganglioside dot assay evaluating the presence of anti-ganglioside IgG and IgM antibodies in the supernatant of B cells from two GM2-Ab^{neg} donors (D37 and D103) and two GM2-Ab^{pos} donors (D19 and D78) after 9 days of culturing (top). The distinction between anti-ganglioside IgM and IgG antibodies in the supernatant of B cells from D78 was evaluated using anti-ganglioside dot assay (bottom). Red arrows mark positive

signals for anti-GM2 antibodies. **D**, Flow cytometric analysis of B cells from different donors incubated with dual-labeled antigen complexes. Biotinylated GM2 was separately pre-incubated with PE-conjugated anti-biotin or VioBlue-conjugated anti-biotin antibodies, forming GM2-PE and GM2-VioBlue complexes, which were then combined and incubated with B cells. As an off-target control, biotinylated aminoglucitol was similarly labeled with PE and VioBlue. Dead cells were excluded using propidium iodide solution, and gating was performed based on the secondary antibody-only control. To minimize false positives, a double-positive gating strategy was applied. Representative dot plots show separate analyses from two independent experiments, each with one sample per condition. D19 and D37 were cultivated for 5 days, while D78 and D103 were cultivated for 10 days before labeling. The red arrow indicates the population sorted using the MACSQuant Tyto. **E**, Anti-ganglioside dot assay of the sorted B cell population from D78 after 14 d of culturing.

To isolate GM2-specific B cells, biotinylated GM2 was conjugated to anti-biotin VioBlue and anti-biotin PE antibodies. Biotinylated aminoglucitol conjugated to the same antibodies served as an off-target control to detect unspecific binding. Cultured B cells from D19, D37, D78 and D103 were incubated with these complexes and afterwards analyzed for GM2 binding using flow cytometry. B cells specific to GM2 should be double-positive ($PE^+/VioBlue^+$) and were expected in the B cell populations from D19 and D78. However, few double-positive cells were observed in B cells from D19 incubated with GM2, but with no significant difference compared to the aminoglucitol control. Unexpectedly, B cells from GM2-Ab^{neg} D103, which did not produce anti-GM2 antibodies in culture, showed a 1.43% double-positive population when incubated with GM2, compared to only 0.064% with aminoglucitol control. Similarly, B cells from D78 exhibited a 2.60% double-positive population with GM2, while 0.012% were double-positive with aminoglucitol (**Figure 16D**). To further investigate, if GM2-specific B cells from D78 were correctly labeled with GM2, 0.01% of the most $PE^+/VioBlue^+$ B cells were sorted using the MACSQuant Tyto and cultured for additional 10 days. However, these sorted cells showed no proliferation and did not produce anti-GM2 antibodies, as confirmed by an anti-ganglioside dot assay (**Figure 16E**).

These findings demonstrate that B cells can proliferate and produce antibodies under the established culture conditions. Moreover, the production of anti-GM2 antibodies by B cells isolated from the peripheral blood of D78 was confirmed, highlighting that GM2-specific B cells of interest can indeed be derived from peripheral blood. However, the combination of the labeling method, sorting, and subsequent culturing showed significant limitations in maintaining proliferative capacity and antibody production necessary for effective screening. These challenges render the method inefficient for isolating and characterizing B cells post-sorting.

3.2.2.7 Isolation of GM2-specific B cells using Barcode Enabled Antigen Mapping technology

As sorting and subsequent culturing of GM2-specific cells failed to isolate cells suitable for PCR amplification of the V(D)J region to identify GM2-specific BCR sequences, an alternative approach was tested, that overcome the need for culturing and subsequent testing due to a flow sorting process combined with single-cell sequencing. The Barcode Enabled Antigen Mapping (BEAM) technology from 10x Genomics was designed for rapid discovery of antigen-specific B cells, providing full-length paired BCR transcripts along with their antigen specificity. PBMCs are first isolated using a Ficoll

density gradient. B cells are then enriched via magnetic negative selection, ensuring untouched and viable B cells. In the labeling step, BEAM conjugates, each containing streptavidin, a PE fluorophore, and a unique molecular identifier (UMI), are assembled with biotinylated antigens of interest and incubated with B cells. To consider unspecific binding, an off-target control is also implemented in the protocol. Additional antibodies, such as anti-CD19, are optionally used to refine selection. Antigen-specific (PE⁺) B cells are then flow-sorted and subsequently encapsulated into Gel Beads-in-Emulsion (GEMs) on a Chromium chip. Within the GEMs, reverse transcription is performed to generate barcoded cDNA from polyadenylated mRNA and BEAM-derived UMIs. Barcoded cDNA is then collected to create libraries for 5' gene expression profiling (5' GEX) to assess transcriptomic data, V(D)J sequencing to analyze the immune repertoire and BEAM antigen capture to determine the antigen specificity. After sequencing, the single-cell sequencing data are linked to individual cells using the unique 10x Genomics barcodes, enabling analysis of gene expression and the V(D)J sequence for each cell. Moreover, the antigen-specificity score can be determined and represents the likelihood of antigen binding to a specific receptor relative to the negative or off-target control (**Figure 17**).

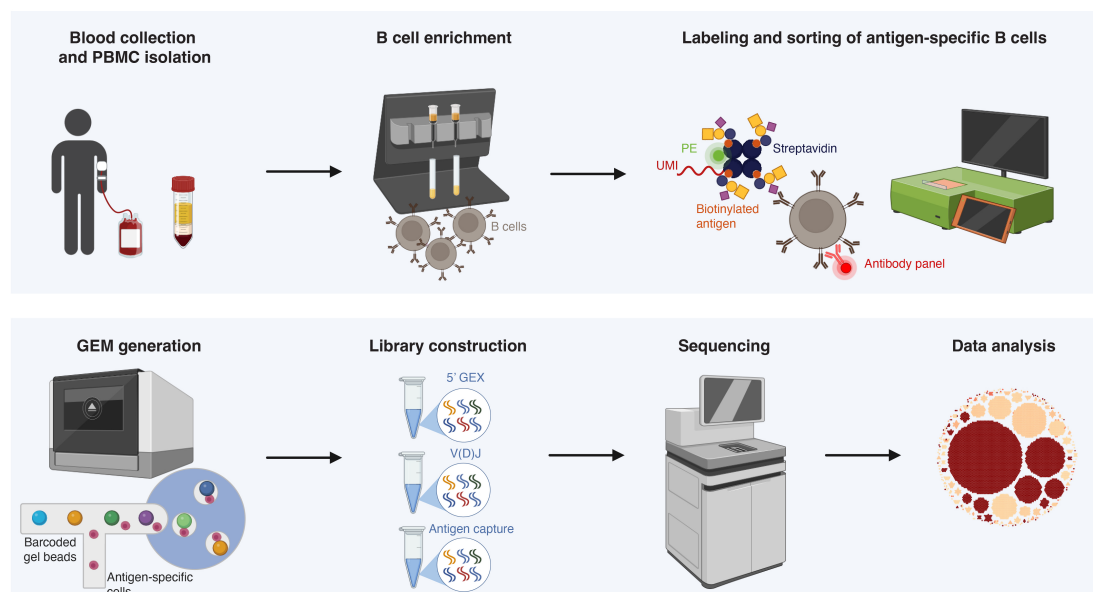


FIGURE 17 | Barcode Enabled Antigen Mapping (BEAM) technology for the identification of antigen-specific B cells. The workflow for discovering antigen-specific BCRs involves assembling biotinylated antigens of interest with uniquely barcoded 10x Genomics BEAM conjugates and labeling B cells with the assembled reagents. Antigen-specific cells are then flow-sorted and processed with Chromium Single Cell 5' Reagent Kits to generate 5' Gene Expression, V(D)J, and Antigen Capture libraries, that are then sequenced and analyzed. PBMC, peripheral blood mononuclear cells; UMI, unique molecular identifier; GEM, Gel Beads-in-emulsion; GEX, gene expression.

To evaluate whether the 10x Genomics BEAM approach is suitable for identifying GM2-specific cells, the technology was first tested using a positive control. NK-92 cells transduced with a GM2-specific chimeric antigen receptor (GM2-CAR) were used to mimic GM2-specific BCRs on the cell surfaces. The CAR consists of an anti-GM2 antibody-derived scFv, a CD28 transmembrane domain, and

CD28/CD3 ζ intracellular signaling domains. An internal ribosome entry site (IRES) enables co-expression of GFP as a reporter protein in these cells (**Figure 18A**).

A



B

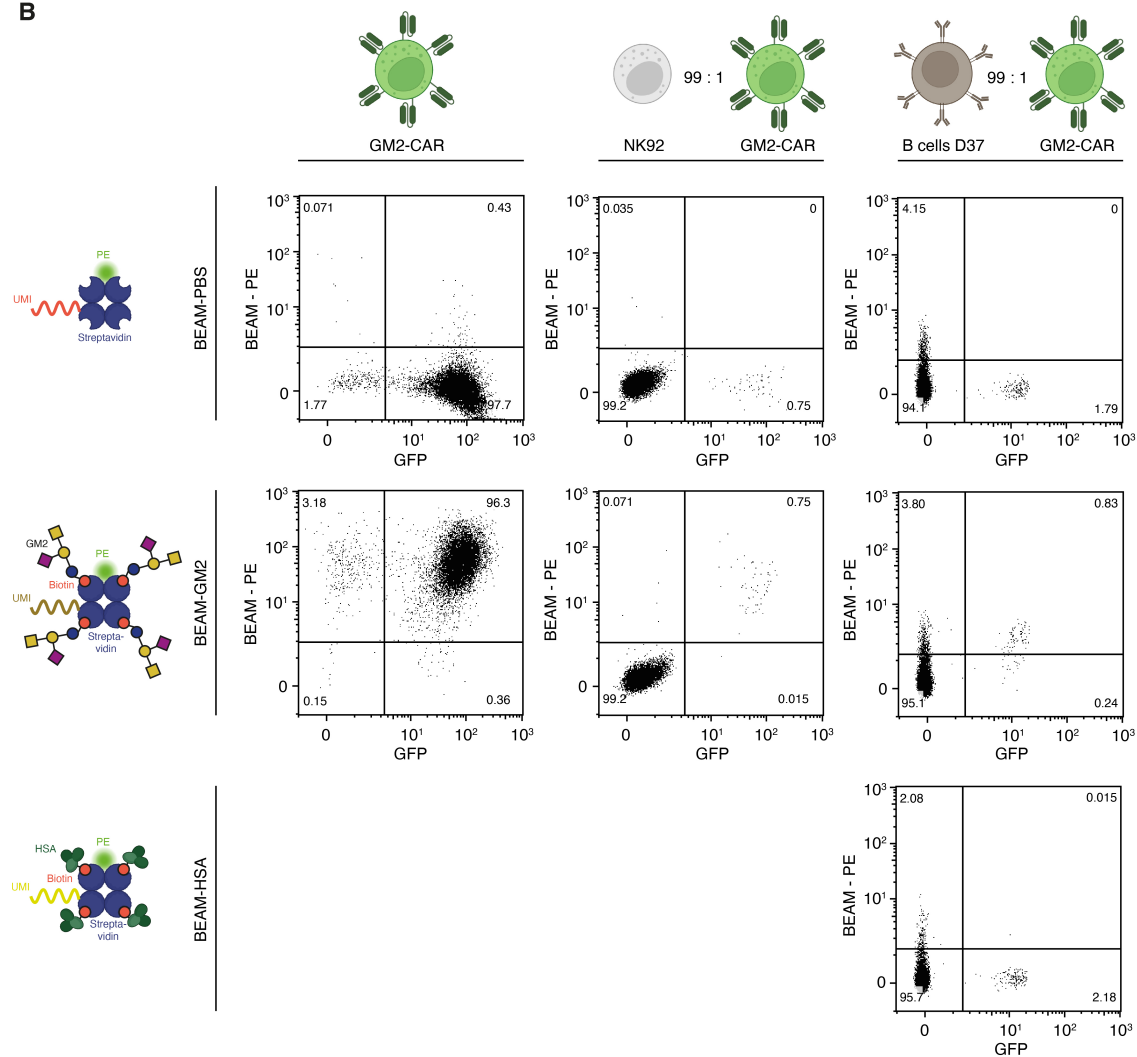


FIGURE 18 | Identification and isolation of GM2-specific CAR NK cells using BEAM technology. **A**, Schematic representation of the GM2-specific CAR construct. The CAR consists of the scFv region of the GM2-specific antibody US2011, followed by a CD28 transmembrane domain, and CD28/CD3 ζ intracellular signaling domains. An IRES enables co-expression of GFP as a reporter. The construct was used to transduce NK92 cells, generating GM2-specific CAR NK cells (GM2-CARs). **B**, Flow cytometric analysis of BEAM-mediated GM2-CAR identification. BEAM technology was conducted to label and thereby identify GFP⁺ GM2-CAR cells. GM2-CARs were analyzed alone or mixed with NK92 cells or GM2-Ab^{neg} B cells from D37 in a 99:1 ratio. BEAM conjugates, consisting of a UMI, a PE fluorophore and a streptavidin, were assembled with a biotinylated GM2 antigen. Control conditions included a BEAM conjugate incubated with PBS and an off-target BEAM conjugate assembled with biotinylated HSA. Dead cells were excluded using ViabilityDye 405/452, and gating was based on BEAM-PBS controls. GM2-CARs were identified as GFP⁺ on the x-axis, while binding of BEAM-assemblies is displayed on y-axis. Representative dot plots from three analyses conducted in different experiments, each with duplicates per condition, are shown. BEAM, Barcode enabled antigen mapping; CAR, chimeric antigen receptor; GFP, green fluorescent protein; HSA, human serum albumin; IRES, internal ribosome entry site; scFv, single-chain variable fragment; TM, transmembrane domain; UMI, unique molecular identifier.

To test the labeling specificity, GFP-expressing GM2-CARs were labeled with either unassembled BEAM conjugate (BEAM-PBS, negative control) or a BEAM conjugate assembled with biotinylated GM2 (BEAM-GM2). Labeling with BEAM-GM2 resulted in a double-positive population (GFP⁺/PE⁺), confirming that all GFP⁺ GM2-CARs bound to the PE-conjugated BEAM-GM2 assemblies. In contrast, labeling with the negative control resulted in a GFP⁺/PE⁻ population, indicating no unspecific binding by the unassembled BEAM-PBS. Next, GM2-CARs were spiked into NK-92 wildtype cells at a 1:99 ratio to simulate a more natural scenario, as the abundance of GM2-specific B cells was expected to be low. When the cell mixture was labeled with BEAM-PBS, 0.75% of the cells were GFP⁺ but PE⁻, representing the GM2-CAR population with no unspecific BEAM-PBS binding. Labeling with BEAM-GM2 again resulted in a double-positive population of 0.75% demonstrating that all GM2-CARs spiked into the mixture could be specifically labeled and identified with BEAM-GM2. To further test the method, GM2-CARs were mixed with B cells isolated from a GM2-Ab^{neg} donor (D37) at a 99:1 ratio. This mixture revealed some non-specific binding by the B cells, as shown by the presence of PE⁺, but GFP⁻ cells, when labeled with BEAM-PBS, BEAM-GM2, or an off-target control (BEAM-Human Serum Albumin, BEAM-HSA). Despite this, GFP⁺ GM2-CARs were successfully labeled and identified by BEAM-GM2, resulting in a double-positive population of 0.83% (**Figure 18B**).

Next, the GM2-specific B cells should be identified from a GM2-Ab^{pos} donor using the BEAM technology. D78 exhibited a high presence of anti-GM2 antibodies in serum, along with strong binding and CDC activity against neuroblastoma cells. Furthermore, previous results confirmed that B cells isolated from this donor produced anti-GM2 antibodies, indicating that the B cells of interest can be derived from peripheral blood. Therefore, 450 ml of blood was collected from this donor. PBMCs were isolated via Ficoll density gradient centrifugation, and B cells were enriched using bead-based magnetic negative selection. To isolate GM2-specific B cells, the cells were labeled with a BEAM conjugate assembled with biotinylated GM2. To account for the non-specific binding of B cells, as detected in earlier findings, an off-target control, BEAM-HSA, was included in the experiment. Both assemblies were conjugated to PE but were distinguishable during data analysis due to UMI sequences specific to either GM2 or HSA antigens. An anti-CD19 antibody was also used to ensure that only B cells were included in the analysis (**Figure 19A**). A total of 20×10^6 B cells were labeled and sorted using the MACSQuant Tyto. Dead cells were excluded using ViabilityDye 405/452 and only CD19⁺ cells were considered. Approximately 0.5% of the living/CD19⁺/PE⁺ cell population were selected for sorting (**Figure 19B**), resulting in 4.763×10^4 B cells, which bound either BEAM-GM2 or BEAM-HSA. The sorted cells were then used for the preparation of 5' Gene Expression (5' GEX), V(D)J, and Antigen Capture libraries. The quality of libraries was confirmed via TapeStation analysis (**Appendix, Supplementary Figure 7**).

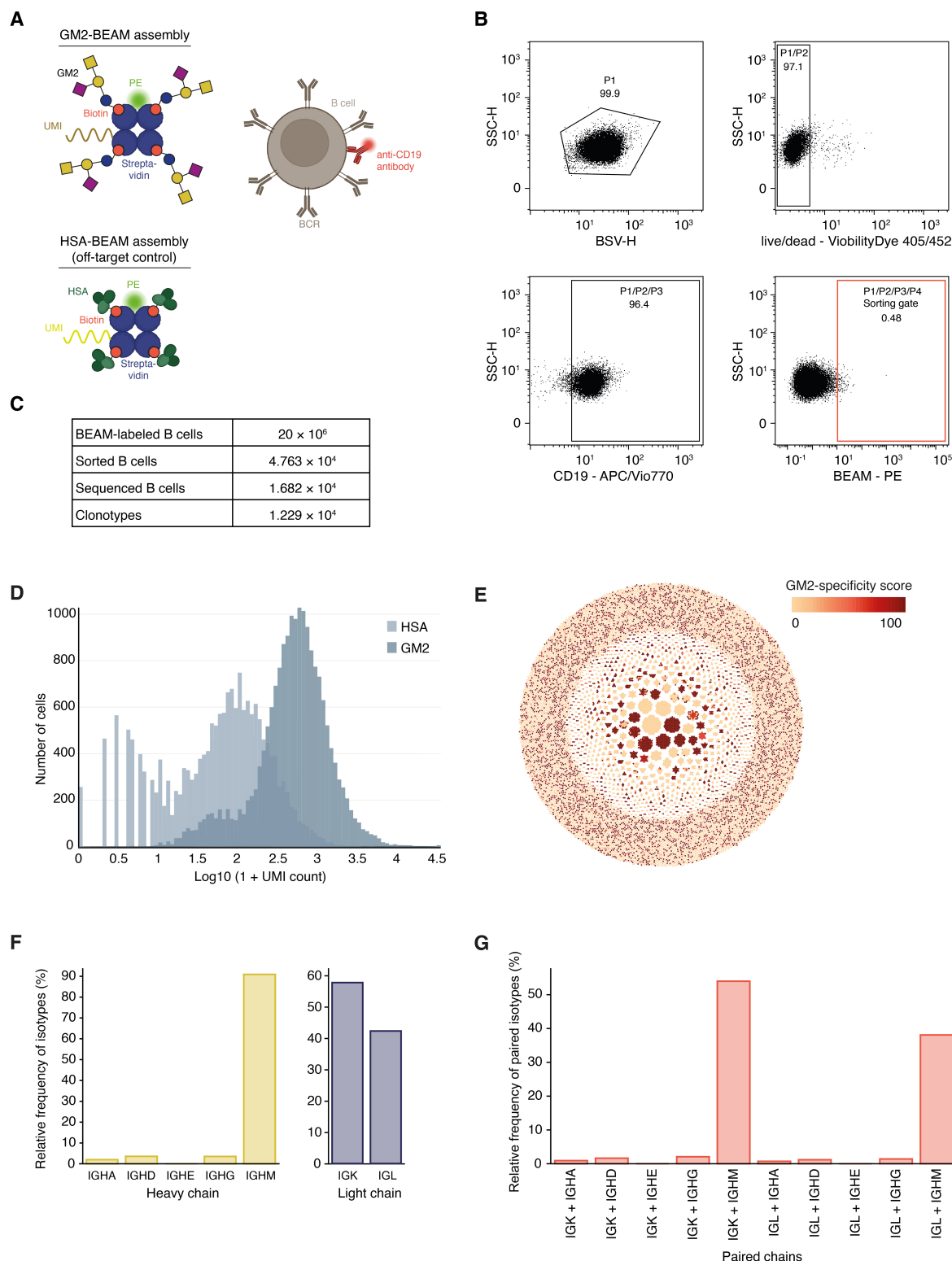


FIGURE 19 | Barcode enabled antigen mapping technology for the isolation of GM2-specific D78-derived B cells. **A**, Schematic of BEAM labeling for GM2-specific B cells. B cells were labeled with BEAM assemblies and anti-CD19 antibodies to isolate GM2-specific B cells. BEAM assemblies consist of a UMI, a PE fluorophore, and streptavidin, conjugated to either biotinylated GM2 (target antigen) or biotinylated HSA (off-target control). BEAM-GM2 and BEAM-HSA assemblies carry distinct UMIs, allowing for differentiation of specific and non-specific binding. **B**, Sorting of BEAM-labeled B cells. CD19⁺ B cells binding to BEAM assemblies (PE⁺) were sorted using the MACSQuant Tyto, with dead cells excluded by ViabilityDye 405/452. **C**, Overview of cell counts, including BEAM-labeled B cells, sorted cells used for library preparation and single cell sequencing, and clonotypes identified during data analysis. **D**, Histogram of antigen counts showing the distribution of antigen-specific UMIs detected in the BEAM experiment. UMI counts are shown on a log scale, plotted against the number of B cells. **E**, Clonotype distribution plot. B cells are grouped by clonotypes, with each dot representing a single cell. Larger clonotypes are positioned in the center, while smaller clonotypes are on the periphery. Clonotypes are colored based on their GM2-specificity score, calculated by comparing GM2-specific UMIs to HSA-specific UMIs. **F**, Isotype

distribution. The frequency of immunoglobulin isotypes is presented for heavy (left) and light chain (right). **G**, Paired isotype and light chain distribution. Bar chart displays the distribution of paired heavy and light chain isotypes, including only clonotypes with exactly one heavy and one light chain. BCR, B cell receptor; HSA; human serum albumin; PE, Phycoerythrin; UMI, unique molecular identifier.

Following sequencing and data analysis using CellRanger software, 1.682×10^4 sequenced cells could be identified. These cells were grouped into 1.229×10^4 clonotypes, with each clonotype comprising cells that share a fully rearranged, unmutated common ancestor (**Figure 19C**). Analysis of antigen binding revealed that GM2 binding occurred more frequently than binding to the off-target control HSA, as demonstrated by higher GM2-specific UMI counts in a larger proportion of cells (**Figure 19D**). To quantify this binding, the antigen specificity score was calculated as the relative enrichment of GM2-specific UMI counts compared to HSA-specific UMI counts. This score, referred to as the GM2-specificity score, measures the likelihood of GM2 binding for each cell. When examining clonotype distribution and the corresponding GM2-specificity scores, most clonotypes were found to consist of only a single cell, represented by individual clusters located at the periphery, showing a wide range of GM2-specificity scores. In contrast, larger clonotypes, containing up to 180 cells, were observed centrally. Among these larger clonotypes, some displayed high GM2-specificity scores, while others exhibited low scores or divergent results, with a mix of high and low specificity within the same clonotype. Notably, cells with high antigen specificity were found in both larger clonotype and single cell clonotypes (**Figure 19E**). Isotype analysis revealed that IGHM was the most abundant heavy chain isotype (90.89%), with low frequencies of IGHG (3.53%) and IGHD (3.59%). The high prevalence of IGHM suggests a predominantly naïve or non-class-switched B cell population. Regarding light chain usage, both kappa and lambda light chains were represented, with a slight preference for IGK (57.72%) (**Figure 19F**). Analysis of paired isotypes showed that IGK+IGHM was the most prominent pairing (54.0%), followed by IGL+IGHM (38.09%), while other paired isotypes are present at much lower frequencies (**Figure 19G**).

3.3 Characterization and validation of isolated antibodies

A total of 14 B cell clonotypes identified in the BEAM experiment were selected for further characterization (**Figure 20A**). Eight clonotypes (02, 04, 07, 10, 19, 31, 37, and 41) were chosen based on their large clonotype size, ranging from 20 to 124 cells, and their high GM2-specificity scores of 100. All these clonotypes were classified as IgM isotype. Additionally, three clonotypes (900, 10066, and 11767) were selected due to their exceptionally high GM2-related UMI counts. Although GM2-specificity scores were less emphasized in this group, only clonotype 900 had a score below 100. These clonotypes also belonged to the IgM isotype. Three additional clonotypes were selected for further characterization based on their antigen specificity in combination with their classification of IgG isotype (**Figure 20B**). IgG clonotypes were included in the analysis based on the previous finding, that the serum of D78 contains IgM and IgG antibodies specific for GM2.

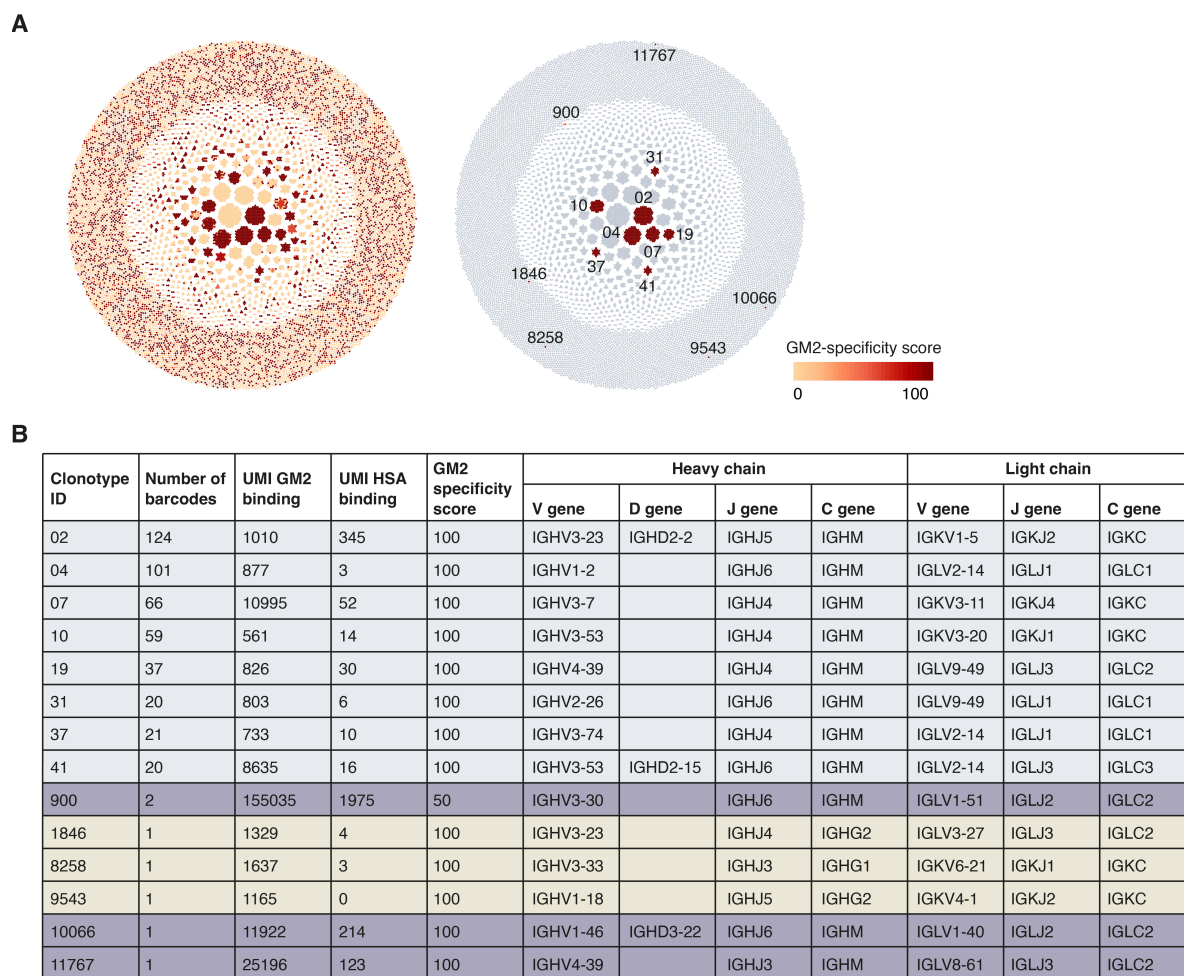


FIGURE 20 | Selection of BEAM-derived B cell clonotypes. **A**, All clonotypes derived from the BEAM experiment (left) and clonotypes selected for downstream analysis (right) are shown. B cells are grouped by clonotypes, with each dot representing a single cell. Larger clonotypes are centrally located, while smaller clonotypes are distributed toward the periphery. Colors represent the GM2-specificity score of each clonotype. A total of 14 clonotypes were selected for further analysis and are labeled. **B**, Characteristics of selected clonotypes. Metrics include the number of 10x barcodes (representing the number of cells in each clonotype), UMI counts for GM2 and HSA antigens, and GM2-specificity scores. For each clonotype, the corresponding V, D, J, and C genes are listed for the heavy chain, and the V, J, and C genes are listed for the light chain. Clonotypes highlighted in blue were selected based on large clonotype size combined with a high GM2-specificity score and IgM isotype. Clonotypes highlighted in purple were chosen for high GM2-related UMI counts and IgM isotype, with less emphasis on GM2-specificity score. Clonotypes highlighted in beige were selected for evaluation based on their antigen-specificity and IgG isotype.

Sequence alignment of the amino acid sequences of the V_H and V_L chains from all selected clonotypes, along with the previously established anti-GM2 antibody KM966 (a chimeric human/mouse IgG1 antibody), was performed using the Clustal Omega tool. The CDRs for each clonotype and the KM966 sequence were predicted and annotated according to the IMGT antibody numbering scheme, as implemented in the AbRSA tool. The alignment revealed high conservation in the framework regions outside the CDRs, indicated by the asterisks marking identical residues across the sequences. In contrast, the CDRs, which are responsible for antigen recognition and specificity, exhibit significant variability across all clonotypes. Among the CDRs, CDR3 displays the highest sequence variability, as it is the most diverse region in both heavy and light chains. Additionally, the lengths of the amino acid sequences defining the CDR3 regions vary among the clonotypes. Moreover, none of the selected clonotypes share

A

B

Additionally, three-dimensional structures of the V_H and V_L chains were predicted using the grafting-based algorithm ABodyBuilder2. The tool enables a template structure selection, CDR loop modeling and the prediction of orientation and side chains for the variable domains of antibodies. All predicted structures displayed a conserved framework of the antibody variable domains, with the characteristic immunoglobulin fold, which consists predominantly of β -sheet structures. The CDR1 and CDR2 regions were similar in their overall positioning across all clonotypes and KM966, despite some structural variations. Notably, the CDR3 regions showed differences in both structure and orientation among the clonotypes, indicating the diversity in antigen or epitope binding specificity. Moreover, none of the predicted CDR structures from the clonotypes were similar to those of KM966 (**Figure 22**).

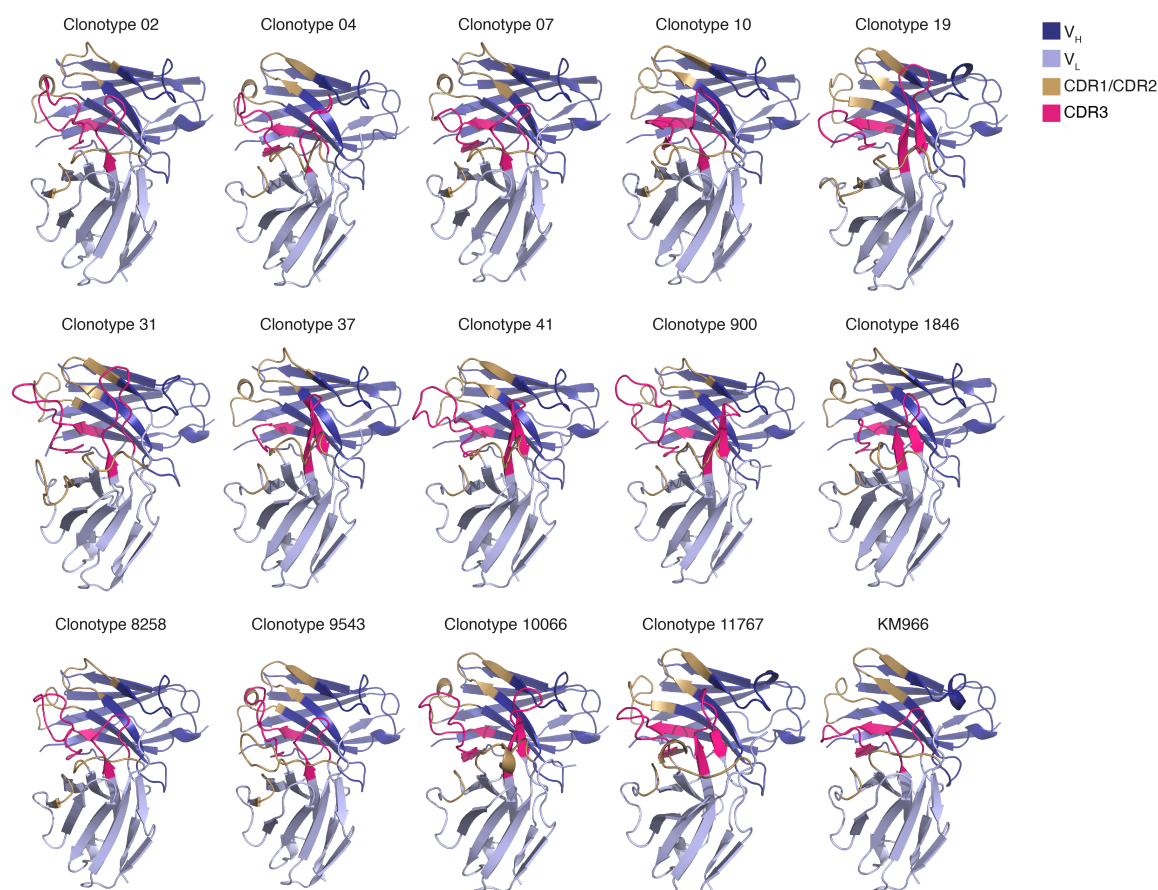


FIGURE 22 | Predicted three-dimensional structures of antibody Fv regions. 3D models of the Fv regions of antibodies derived from BEAM experiment were generated using ABodyBuilder2, based on amino acid sequences of the variable regions. The variable heavy chain (dark blue), variable light chain (light blue), CDR1 and CDR2 (brown), and CDR3 (pink) are highlighted using PyMOL. The annotation of the variable domains is based on the IMGT numbering scheme. CDR, complement-determining regions; Fv, fragment variable; V_H , variable region of heavy chain; V_L , variable region of light chain.

To characterize the identified antibodies and validate their GM2 specificity the V_H and V_L chains were cloned into antibody heavy and light chain co-expression vectors to generate recombinant monoclonal IgG antibodies. The V_H and V_L sequences of KM966 were also cloned into antibody expression plasmids to include a GM2-specific antibody control for validation of antibody production. HEK293T cells were transiently transfected with these plasmids to produce IgG antibodies, which were subsequently secreted into the supernatant.

To confirm successful antibody production, Western blot analysis was performed. Under reducing conditions, the expected bands corresponding to ~50 kDa heavy chains and ~25 kDa light chains were detected (**Figure 23A**). However, some antibodies were produced at lower levels than others, such as clonotype 07, which showed reduced expression, and the light chain of clonotype 1846, which was only faintly detectable. Notably, the KM966 control antibody was consistently produced at lower levels compared to most clonotype-derived antibodies. To assess the correct assembly of heavy and light chains, Western blot analysis was performed under non-reducing conditions, revealing strong signals at ~150 kDa, indicating proper oligomerization of the antibodies (**Figure 23B**). Despite successful IgG antibody production, clonotype-derived antibodies did not exhibit GM2 reactivity in the anti-ganglioside

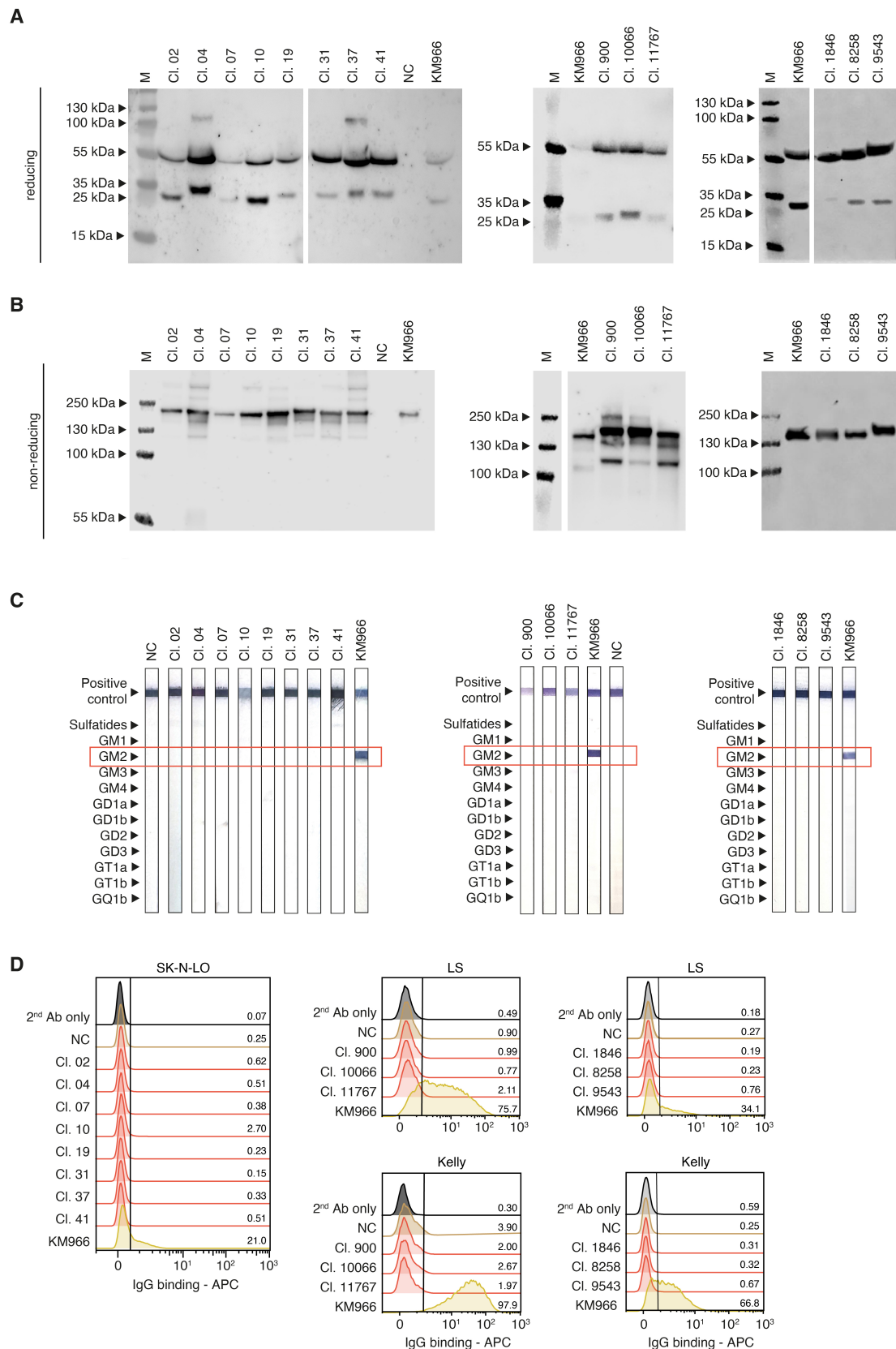


FIGURE 23 | Expression and validation of clonotype-derived IgG antibodies. V_H and V_L sequences from clonotypes 02, 04, 07, 10, 19, 31, 37, 41, 1846, 8258, and 9543 were cloned into the pVB_H+L_IgG heavy and light chain co-expression vector, while sequences from clonotypes 900, 10066, and 11767 were cloned into pVitro_H+L_IgG. As a GM2-specific antibody control, KM966 sequences were cloned into both expression plasmids. HEK293T cells were transiently transfected with these plasmids, and antibody-containing supernatants were harvested after 3 days. A transfection control without plasmid was used as a negative control (NC) to assess unspecific binding from HEK293T-conditioned media. Western blot analysis of the supernatants was performed under reducing conditions (A) and non-reducing conditions (B) to confirm antibody expression.

The antibodies were detected via chemiluminescence using an anti-human IgG (H+L) antibody along with an HRP-conjugated anti-rabbit IgG antibody. **C**, Anti-ganglioside dot assay conducted to evaluate the binding specificity of antibodies in the supernatants to gangliosides. **D**, Flow cytometry analysis. The binding of secreted antibodies in HEK293T supernatants was assessed by flow cytometry using SK-N-LO, LS, and Kelly cells. Antibody binding was detected using APC-conjugated anti-human IgG antibodies. The data shown are derived from independent experiments: antibody production and Western blot for clonotypes 02–41 ($n = 3$), clonotypes 900–11767 ($n = 2$), and clonotypes 1846–9543 ($n = 1$); all antibody productions were verified using an anti-ganglioside dot assay (representative strips shown); and flow cytometry analysis for all clonotype antibodies ($n = 1$).

dot assay. In contrast, despite its lower expression levels, KM966 displayed GM2-specific binding as expected (**Figure 23C**). To further evaluate GM2 specificity of antibodies, flow cytometry analysis was performed using GM2-expressing SK-N-LO, LS, and Kelly cells, which present a more native GM2 epitope compared to the immobilized GM2 on the anti-ganglioside dot strips (**Figure 23D**). However, only KM966 exhibited binding to these cells, while the clonotype-derived IgG antibodies showed no detectable binding, like the negative control (HEK293T-conditioned media). Interestingly, binding intensities of the KM966 antibody varied between independent antibody productions and experiments, likely due to differences in antibody concentrations in the supernatant, highlighting potential variability in expression levels during production.

These findings demonstrate that, although clonotype-derived IgG antibodies were successfully produced and assembled, they did not exhibit GM2 specificity under the tested conditions. Since all clonotype sequences, except for clonotypes 1846, 8258, and 9543, were originally derived from IgM isotype B cells, an IgM isotype format was tested, as IgM antibodies typically have lower affinities but achieve strong antigen binding through high avidity.

After cloning the V_H and V_L sequences into the pVitro_H+L_IgM plasmid, IgM antibodies were produced in HEK293T cells following plasmid transfection. The antibody-containing supernatants were analyzed by Western blot under reducing conditions to assess heavy and light chain production (**Figure 24A**). Heavy chain expression, indicated by ~70 kDa bands, was detected for clonotype 02, 04, 10, 19, 31, 41, and 900 antibodies, whereas light chains (~25 kDa) were only observed in clonotype 02, 10, 900, and KM966 antibodies. Notably, no detectable heavy and light chains were present in the supernatants of clonotypes 07, 37, 10066, and 11767. To determine whether the heavy and light chains were produced but not secreted, a Western blot analysis of HEK293T cell lysates was performed (**Figure 24B**). Heavy chains were detected for all clonotype-derived IgM antibodies, but light chains were hardly detectable and only for clonotype antibodies 02, 04, 10, 19, 41, 10066 and the KM966 antibody. This suggests that IgM antibodies may not be efficiently assembled or secreted by HEK293T cells.

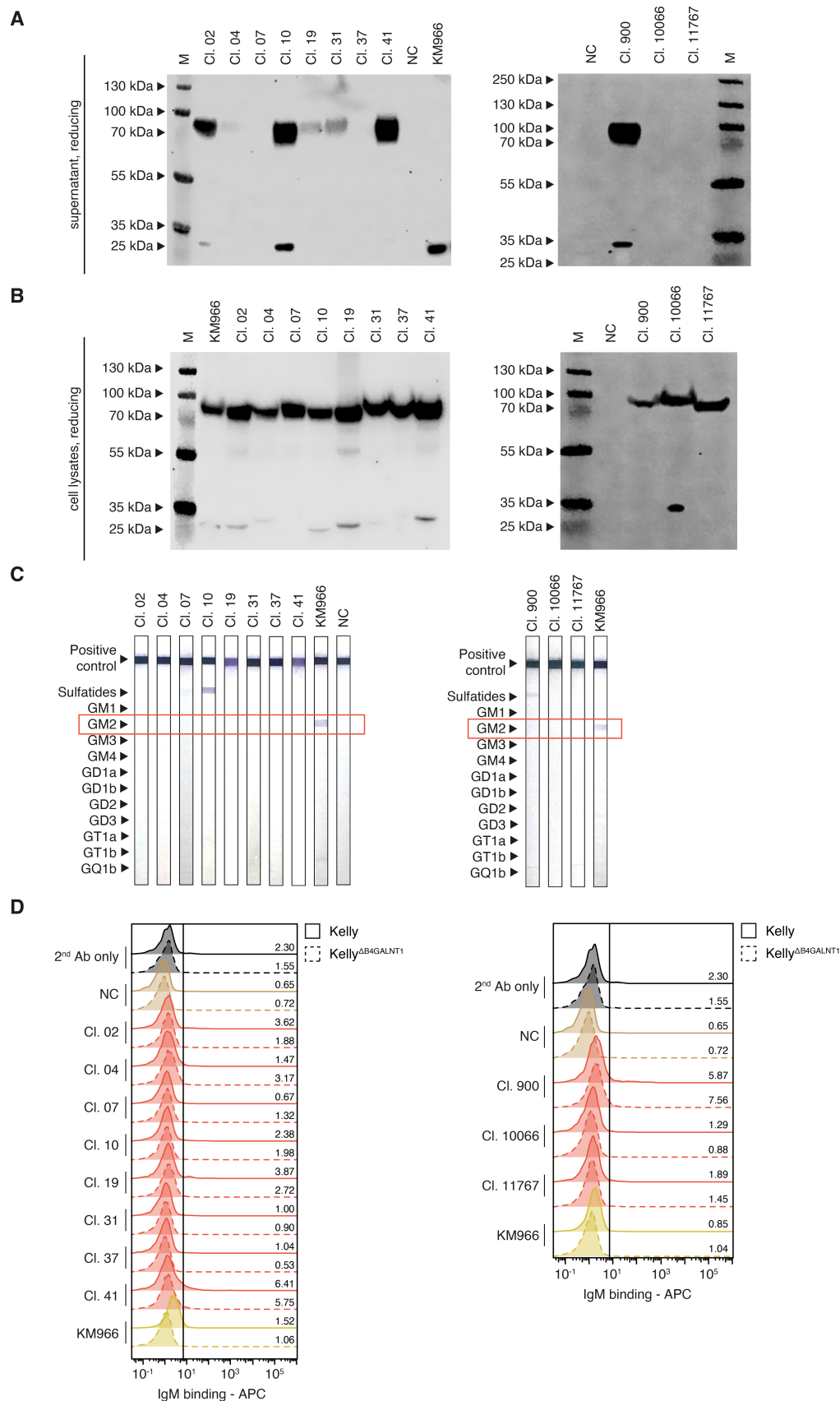


FIGURE 24 | Expression and validation of clonotype-derived IgM antibodies. V_H and V_L sequences from clonotypes 02, 04, 07, 10, 19, 31, 37, 41, 900, 10066 and 11767 were cloned into the pVito_{H+L} IgM heavy and light chain co-expression vector. The pVito_{H+L} IgM_KM966 plasmid was used as a GM2-specific antibody control. HEK293T cells were transiently transfected with these plasmids. Antibody-containing supernatants were harvested after 3 days, and cell lysates were prepared. A transfection control without plasmid was used as a negative control (NC) to assess unspecific binding from HEK293T-conditioned media. Western blot analysis was performed under reducing conditions using supernatants (A) and cell lysates (B)

to confirm antibody expression and secretion. An anti-human IgM antibody and an anti-human kappa antibody, along with an HRP-conjugated anti-rabbit IgG antibody, were used for chemiluminescent detection. **C**, Anti-ganglioside dot assay conducted to assess the binding specificity of antibodies to gangliosides. **D**, Flow cytometry analysis. The binding of secreted antibodies in HEK293T supernatants was assessed by flow cytometry using Kelly and Kelly^{AB4GALNT1} cells. Binding was detected using APC-conjugated anti-human IgM antibodies. The data shown are derived from independent experiments: antibody production and Western blot for clonotypes 02–41 (n = 3) and clonotypes 900–11767 (n = 3); all antibody productions were verified using the anti-ganglioside dot assay (representative strips shown); and flow cytometry analysis for all clonotype antibodies (n = 1).

Despite confirming heavy and light chain production for some antibodies, the anti-ganglioside dot assay did not reveal any GM2-specific antibody binding, except for the KM966 control antibody (**Figure 24C**). Interestingly, although KM966 heavy chains were not detectable by Western blot, the antibody still produced a weak, but positive GM2 signal on the dot assay. This suggests that even small amounts of correctly assembled and secreted KM966 were sufficient to confirm its GM2 specificity. Notably, clonotype 10, expressed in the IgM format, displayed binding to sulfatide on the test strip. However, throughout this study, a high background of anti-sulfatide signals was frequently observed in donor and mouse serum samples tested on these strips, indicating that this signal may not be specific.

To further evaluate IgM binding to GM2-expressing cell lines, flow cytometry analysis was performed using Kelly and Kelly^{AB4GALNT1} cells (**Figure 24D**). No difference in antibody binding was observed between GM2⁺ Kelly cells and their B4GALNT1 KO counterpart, suggesting that the antibody concentration in the supernatant was too low to enable GM2-specific binding under these conditions. This may be due to the inefficient antibody production observed.

Notably, successful IgM antibody production in HEK293T cells was demonstrated using the pVito_H+L_IgM expression system by producing Trastuzumab-IgM with both the original pVito-Trastuzumab-IgM plasmid and the modified version containing additional restriction sites flanking the heavy and light chain sequences required for cloning. The latter construct thereby served as the donor plasmid for all IgM expression plasmids generated in this study. Both Trastuzumab-IgM variants were successfully produced, and strong binding to HER2⁺ T47D cells was confirmed (**Appendix, Supplementary Figure 8A,B**). Additionally, in this experiment, KM966 production using the same protocol was enhanced, as 19.1% of Kelly cells were positive for antibody binding, compared to only 1.16% for Kelly^{AB4GALNT1} cells. This further underscores the variability in IgM production and highlights the need for optimization strategies to enhance antibody yields and achieve higher concentrations for functional assays.

All in all, these findings demonstrate that although IgG and some IgM antibodies derived from clonotypes identified using the BEAM approach were successfully produced, they failed to recognize GM2 under the tested conditions. This suggests that these antibodies may either target different antigens or require alternative, optimized antibody production strategies to confirm their GM2-specificity.

4 DISCUSSION

This work highlights the potential of naturally occurring antibodies (nAbs) targeting the ganglioside GM2 as a promising immunotherapeutic approach for neuroblastoma. The following section will therefore discuss the findings of this work, addressing the challenges and limitations related to ganglioside expression in neuroblastoma, the isolation of nAbs from healthy individuals and the generation of GM2-specific antibodies.

4.1 The immunosuppressive microenvironment in neuroblastoma

High-risk status and amplification of MYCN in neuroblastoma is closely linked to immune escape mechanisms and an immunosuppressive microenvironment, which significantly impacts survival²⁰³. *In silico* analysis of gene expression data from a neuroblastoma cohort revealed distinct gene expression patterns between patients with favorable and unfavorable outcomes. For example, *MYCN* and *TWIST1* upregulation was identified, which promotes cell cycle progression³⁷ and metastasis²⁰⁴, whereas downregulation of *NTRK1* could indicate less differentiated tumors of patients with worse outcomes²⁰⁵. Notably, altered gene expression also included downregulation of genes, responsible for inflammation and immune-related pathways. Moreover, lower immune cell infiltration, especially for NK cells and T cells, could be identified in patients with poor outcomes. Immune evasion mechanisms include the downregulation of MHC class I and II molecules in neuroblastoma cells or their microenvironment, impairing antigen presentation and T cell recognition^{206–208}, or downregulation of *KLRK1*, encoding the activating receptor NKG2D on cytotoxic T and NK cells, reducing the efficacy of NK cell and cytotoxic T cell responses against neuroblastoma²⁰⁹. Moreover, reduced expression of chemokine-related genes, including *CCL2* and *CCL19*, could be identified. Kacher *et al.* identified an impaired immune response due to lack of CCL2 secretion, which is associated with decreased monocyte and dendritic cell (DC) recruitment, leading to decreased CCL19 production by DCs, which in turn limits T cell infiltration²¹⁰. As T cell and NK cell infiltration is positively associated with favorable outcomes in neuroblastoma^{211,212}, these findings highlight the need of immunotherapeutic strategies to overcome immune evasion by enhancing immune cell infiltration and restoring anti-tumor immunity.

4.2 The relevance of gangliosides as targets for immunotherapy

Gangliosides play a key role in physiological processes such as cell-cell communication and signal transduction, and are also considered checkpoint-like molecules due to their ability to inhibit various immune cell functions in various cancer entities¹³⁶. Gangliosides are also associated with neuroblastoma pathophysiology, with distinct ganglioside-related gene expression patterns correlating with adverse clinical characteristics and prognosis. In this study, high-risk patients exhibited downregulation of galactosyl- and sialyltransferase genes, like *B3GALT4*, responsible for complex ganglioside

biosynthesis, which has been linked to increased tumor proliferation, migration, and invasion when knocked down in neuroblastoma cells²¹³. Additionally, genes such as *ST3GAL3* and *ST6GALNAC5*, which are also involved in complex ganglioside biosynthesis, were downregulated in patients with adverse clinical outcomes and could be identified as independent prognostic factors for outcome prediction in this study. Moreover, *B3GNT5* was significantly overexpressed in patients with poor survival and had the highest hazard score for predicting outcomes, suggesting increased production of (neo)lacto-series gangliosides in these patients, which have been shown to sterically hinder MHC class I interactions and impair cytotoxic T cell activation²¹⁴, further contributing to immune evasion. While, *B3GNT5* is linked to cancer stem cell characteristics and aggressive disease in breast cancer²¹⁵, its specific role in neuroblastoma remains to be clarified.

Importantly, *ST8SIA1*, which encodes the key enzyme for GD2 biosynthesis, was downregulated in high-risk patients. This reduction may correlate with lower GD2 levels in advanced tumors, potentially diminishing the efficacy of anti-GD2 immunotherapy. Notably, GD2 downregulation has been observed during adrenergic-to-mesenchymal transition²⁰⁰ and *ST8SIA1* suppression by Yes-associated protein (YAP) has been linked to resistance to GD2-targeted therapies in high-risk and relapsed neuroblastoma²¹⁶, which highlights the need for new immunotherapeutic targets beyond GD2.

The limitation of *in silico* studies on galactosyl- and sialyltransferase gene expression data is that they do not necessarily reflect actual ganglioside expression on the cell surface. However, evidence suggests that certain tumor-associated gangliosides serve as prognostic markers, as ganglioside metabolism differs between tumors with different phenotypes and clinical behavior. For example, b-series gangliosides (e.g. GD3, GD2) predominate in tumors of patients younger than one year, while their prevalence decreases in older patients²¹⁷. Moreover, low or absent expression of complex gangliosides from the b-series (GD1b, GT1b and GQ1b), correlate with aggressive disease²¹⁸. The aggressive phenotype, characterized by cell proliferation and migration, could be inhibited *in vitro* by the expression of complex a- and b-series gangliosides^{219,220}.

Due to the limited availability of primary tumor material, ganglioside expression was assessed in human neuroblastoma cell lines, revealing variability across different cell lines. While GD2 was highly expressed in most cell lines, those with lower *ST8SIA1* expression exhibited absent or reduced GD2 levels. The structurally complex ganglioside GM1 was consistently low or absent. Notably, GM2 and GM3 were universally high expressed. Thereby, GM2 was detected using GM2(Neu5Ac)-specific antibodies, suggesting that neuroblastoma cells predominantly express GM2 containing Neu5Ac rather than Neu5Gc, despite reports that Neu5Gc is the predominant form in other tumor entities²²¹. However, as there was no GM2(Neu5Gc)-specific antibody commercially available, the presence of GM2(Neu5Gc) on neuroblastoma cells was not tested and can therefore not be ruled out. GM2 expression has been linked to promotion of tumor growth, cancer stem cell-like properties and invasion through TGF- β 1 signaling in pancreatic ductal adenocarcinoma¹⁶³. Moreover, it functions as an immune modulator, capable of inhibiting NK cell function¹⁶⁹. Additionally, GM2 expression is associated with

reduced cytokine production, T cell dysfunction, and T cell apoptosis¹⁷⁰, as well as inhibited Ig production in B cells, likely due to impaired IL-10 and TNF α production¹⁷¹.

Given its high and consistent expression across neuroblastoma cells, and its role as regulator of immune evasion and tumor progression, GM2 emerges as a promising target for immunotherapy. As GM2 is not only expressed in neuroblastoma, but is also present in other malignant tissues, such as melanoma²²² or lung cancer¹⁶⁴, an immunotherapeutic approach targeting GM2 may have a broader applicability beyond neuroblastoma.

4.3 Immunization of mice with GM2

Efforts to generate GM2-specific antibodies through immunization of C57BL/6 mice with GM2 substrates did not result in a detectable GM2-specific immune response. This finding is consistent with previous studies that have reported poor immunogenicity of tumor-associated carbohydrate antigens, including gangliosides, due to their structural similarities with self-antigens and their classification as T cell-independent antigens^{223,224}. Despite the absence of GM2-specific antibodies, several mice developed immune responses against structurally related gangliosides, GM3 and GM4. However, these antibodies were only detectable in serum and not in hybridoma supernatants. This suggests a transient or weak immune response, or alternatively, a failure in hybridoma generation.

One possible explanation for the lack of GM2-specific antibodies in mice is the high endogenous expression of GM2 in normal tissues, particularly in the liver²²⁵. Previous studies have reported an inverse correlation between the expression of gangliosides in non-brain tissues and the ability to generate a specific antibody response²²⁶. This indicates that a higher endogenous GM2 expression may lead to immune tolerance. In contrast, human vaccination studies have demonstrated a GM2-specific antibody response following immunization, which could be attributed to lower baseline GM2 expression in normal human tissues²²⁶.

To enhance the immunogenicity of carbohydrate antigens, they are often conjugated to carrier proteins such as KLH, enabling a T cell-dependent immune response through the recruitment of CD4⁺ T cells¹⁷⁶. Moreover, T cell-independent immune responses are typically triggered by highly repetitive carbohydrate structures, which can effectively cross-link B cell receptors⁶⁹. In this study, both GM2-KLH conjugates and a GM2-PAA construct, consisting of multiple GM2 mimicking repetitive antigenic structures, were tested along with an adjuvant. However, neither approach resulted in a GM2-specific immune response. Previous studies have occasionally observed GM2-specific immune responses in mice and also in melanoma patients when GM2 was co-administered with *Salmonella* Minnesota R595 as an adjuvant and the mice or patients were pretreated with the immunosuppressive agent cyclophosphamide^{226,227}, which might be considered for further immunization strategies.

Another limitation is the natural structural diversity of GM2, including variations in sialic acid (Neu5Gc and Neu5Ac) and differences in length, hydroxylation and saturations of the ceramide. In this study, only the carbohydrate moiety of GM2 was used for immunization, coupled to KLH or PAA. As the exact GM2 substrate used in the anti-ganglioside dot assay is unknown, it is possible that GM2-specific antibodies were generated but failed to recognize the epitope presented in the assay. Additionally, GM2 shares structural features with GM3 and GM4, which could contribute to cross-reactivity. However, if cross-reactivity was the main factor, antibodies would likely have recognized both GM2 and related epitopes, making it an unlikely explanation for the observed immune response.

Moreover, given the observed immune responses against GM3 and GM4, it is also possible that GM2 conjugates were unstable or degraded after injection. GM2 might also have undergone *in vivo* degradation, for example by hexosaminidase A (HEXA) or neuraminidase 3 (NEU3)²²⁸.

These findings underscore the challenges of generating a GM2-specific immune response in mice, likely due to immune tolerance, structural diversity or antigen instability. As murine antibodies were often immunogenetic, show low effector function and have shorter half-life^{91,92}, their therapeutic application is limited. Therefore, naturally occurring antibodies derived from healthy individuals may provide a more suitable alternative. Consequently, the main focus was placed on identifying GM2-specific BCR sequences derived from human B cells.

4.4 The origin of naturally occurring cytotoxic antibodies in healthy individuals and their potential for immunotherapeutic approaches

This study confirms the presence of nAbs with cytotoxic potential against neuroblastoma cells in the serum of healthy individuals. Specifically, 5.65% of screened donor sera induced more than 50% CDC in neuroblastoma cells. This prevalence aligns with previous reports identifying cytotoxic nAbs in 1.8% to 4.3% of individuals, with variations depending on the assay used for detection and positivity thresholds used^{132,182}.

Notably, the cytotoxic activity was strongly linked to the presence of anti-GM2 IgM antibodies, as sera containing these antibodies exhibited significantly higher cytotoxicity against GM2-expressing neuroblastoma cells. However, some GM2-Ab^{neg} samples also induced high cytotoxicity, suggesting that additional, yet unidentified tumor-associated antigens may contribute to nAb-mediated tumor cell lysis. While GM2-specific IgM antibodies were the most prevalent, rare cases of antibodies targeting GD2, GD3, GM1 and GM3 were also identified using an anti-ganglioside dot assay, suggesting that multiple ganglioside-targeting nAbs exist naturally within the healthy population. Among these, GM3- and GD2-specific antibodies were particularly noteworthy, as previous studies proposed GM3 and GD2 as targets for nAb-mediated cytotoxicity in neuroblastoma¹³². Furthermore, glycan array analysis revealed a broad repertoire of anti-glycan antibodies in both cytotoxic and non-cytotoxic donors, consistent with findings by Bovin *et al.*, who demonstrated that healthy individuals naturally possess

antibodies capable of binding over 100 mammalian glycans on a glycochip²²⁹. Many of these antibodies recognize short glycan fragments, while failing to bind whole glycans found in human tissue²²⁹.

Despite detecting this broad range of anti-glycan antibodies, the findings in this study indicate that the cytotoxic effect against neuroblastoma must be linked to gangliosides synthesized by the B4GALNT1 enzyme, including GA2, GM2, GD2, and GT2, or subsequent gangliosides in the biosynthesis pathway. This conclusion is supported by the absence of CDC in B4GALNT1 KO cells, which lack these gangliosides but still express GM3 and GD3, allowing GM3 and GD3 to be excluded as primary nAb targets. Importantly, ST8SIA1 KO cells, which lack GD2, remained susceptible to cytotoxic donor serum, indicating that GM2, rather than GD2, is the primary target of these nAbs. Although GA2, GA1, GM1, GD1, and GT1 cannot be completely ruled out as potential targets, it is unlikely that structurally complex gangliosides, such as GM1, GD1, and GT1, serve as major nAb targets, given their low or absent expression in neuroblastoma cells.

The presence of cytotoxic anti-ganglioside antibodies in healthy donors raises the question of their origin. There are several possible explanations for how these antibodies arise, including germline-encoded natural immunity, infection-induced immune responses, and associations with neuropathological diseases. Additionally, there might be a potential link between these antibodies and the spontaneous regression of neuroblastoma, suggesting a role in immune surveillance.

One possibility is that anti-GM2 antibodies are naturally occurring as part of the innate-like immune system. It is well known that nAbs, particularly IgM, are often germline-encoded and capable of recognizing conserved carbohydrate structures found on pathogens and tumors¹²⁷. As gangliosides like GM2 are highly expressed during fetal development²³⁰, but largely absent in normal adult tissues, the immune system may recognize GM2 as a tumor-associated antigen, leading to the production of low-affinity IgM antibodies that contribute to immune surveillance. Supporting this hypothesis, GM2-specific antibodies in this study were predominantly IgM, with only one donor exhibiting IgG-class GM2 antibodies. This suggests that these antibodies were not generated through classical adaptive immune responses requiring T cell help, but rather represent a constitutive, innate-like immune function. Additionally, the persistence of GM2-specific IgM antibodies in some positive donors over three years could be observed during this study, which also aligns with the findings in other studies¹³², suggesting that these antibodies are not transiently induced by external antigenic stimuli, but may persist as part of the baseline immune repertoire.

Additionally, studies have shown that nAbs cytotoxic to neuroblastoma cells are more prevalent in younger individuals and decline with age^{132,181}. The highest prevalence is observed in childhood, with up to 82% of individuals aged 5 to 14 exhibiting cytotoxic antibodies. Moreover, cytotoxic activity in the serum of neuroblastoma patients was found to be significantly lower compared to age-matched healthy children¹⁸¹. This suggests that nAbs may play a crucial role in early-life immune surveillance, helping to eliminate tumor cells before they can establish malignancy.

Furthermore, an intriguing hypothesis is that anti-GM2 antibodies might contribute to spontaneous regression of neuroblastoma, a phenomenon that has been observed in infants with stage 4S disease¹⁵. Large-scale neuroblastoma screening studies have shown that the true incidence of neuroblastoma is higher than previously assumed, with many cases resolving spontaneously without clinical detection^{231,232}. However, the mechanisms underlying this spontaneous regression is poorly understood, though immune-mediated mechanisms have been discussed²³³. Given this, some GM2-Ab^{pos} donors may have unknowingly experienced a spontaneously regressed neuroblastoma in infancy. This could have triggered an immune response against the neuroblastoma-associated ganglioside GM2 early in childhood, which then persisted into adulthood, resulting in the presence of GM2-specific cytotoxic antibodies capable of targeting neuroblastoma cells.

Another hypothesis is that anti-GM2 antibodies were induced by infections, such as cytomegalovirus (CMV) or *Campylobacter jejuni*²³⁴. CMV infection has been linked to autoimmune neuropathies, including Guillain-Barré syndrome (GBS)²³⁵, where molecular mimicry between viral glycoproteins may trigger cross-reactive anti-ganglioside antibodies²³⁶, that lead to severe immune-mediated neurological dysfunctions. Studies have shown that CMV-infected fibroblasts can stimulate anti-GM2 immune responses, likely due to structural similarities between GM2 and CMV-induced antigens²³⁷. This raises the possibility, that some GM2-specific nAbs arise from previous infections. Supporting this, Erttmann *et al.* observed higher frequencies of cytotoxic antibodies in polyinfected African individuals compared to healthy Caucasian controls²³⁸. Therefore, it is also possible that chronic infection may contribute to nAb induction. However, while GBS-associated anti-GM2 antibodies can either be IgM or IgG²³⁹, the donors in this study predominantly expressed IgM antibodies and had no reported active viral infections or neurological diseases, arguing against a transient, infection-induced immune response.

Although the origin of naturally occurring anti-GM2 antibodies remains unclear, it is likely that these nAbs play a role in natural immune surveillance, targeting pathogen- or tumor-associated gangliosides without the autoreactive characteristics observed in neuropathological conditions. The potential link between naturally occurring anti-GM2 antibodies and spontaneous neuroblastoma regression warrants further investigation. Future studies could use cytotoxicity assays with patient sera and age-matched control sera to test this hypothesis.

However, the ability to selectively recognize and eliminate GM2-positive tumor cells highlights the potential of these nAbs as a promising source for novel immunotherapeutic antibodies.

4.5 Isolation of GM2-specific B cells from healthy donors

Despite the promising findings that nAbs from healthy donors exhibit potent and specific cytotoxic activity against neuroblastoma cells through CDC, significant challenges remain in harnessing them for therapeutic applications. One approach, demonstrated in a phase I/II clinical trial by Erttmann *et al.*, involved plasmapheresis, in which plasma from neuroblastoma patients was exchanged with plasma

from healthy donors containing cytotoxic nAbs¹⁸². However, this approach is not feasible as a large-scale treatment for all neuroblastoma patients. Instead, the antibodies responsible for this cytotoxic effect must either be isolated in large quantities or their amino acid sequences must be identified to enable recombinant production.

One method to generate stable B cell lines producing specific antibodies is the immortalization of B cells with Epstein-Barr Virus (EBV)^{81,82}. However, previous attempts using EBV immortalization failed to generate GM2-Ab-expressing cells, highlighting the need for alternative approaches.

4.5.1 B cell culturing and single B cell sorting

As antibody-secreting B cells are typically found in secondary lymphoid tissues²⁴⁰, the presence of GM2-Ab-expressing B cells in the peripheral blood of healthy donors needed to be confirmed before isolation. It was assumed that, if present, their frequency would be very low, as the antibody expression was most likely not induced by an active immune response. To enhance the likelihood of detecting and isolating these cells, a strategy was implemented to first expand and stimulate B cells in culture prior to isolation. Stimulation with IL-2, IL-21, and multimerized CD40L was used to promote proliferation and antibody secretion, as IL-2 and IL-21 have been found to effectively drive B cell proliferation, maturation and differentiation into plasma cells^{241–243}, while multimerized CD40L play a critical role in T cell-dependent B cell activation²⁴⁴. Under these conditions, cultured B cells produced both IgG and IgM antibodies, with antibody levels increasing over time. However, secretion of GM2-specific antibodies from cultured B cells could only be confirmed for donor D78, who had high GM2-IgM and IgG serum antibodies. In contrast, no GM2-specific antibody secretion was detected from the cultured B cells of another GM2-Ab^{pos} donor tested. Notably, the GM2 antibodies produced from D78-derived B cells were exclusively of the IgM isotype. Therefore, an alternative approach optimized for T cell-independent B cell differentiation involving stimulation with CpG, IL-2, and BAFF, which has been shown to drive proliferation, differentiation, and antibody secretion in T cell-independent B cells²⁴⁵ might be tested in the future (along with GM2 stimulation).

However, as GM2-Ab-expressing B cells were successfully identified within the B cell population isolated from peripheral blood, an approach was attempted to isolate these GM2-specific B cells using FACS. As B cells rely on their membrane-bound BCRs for antigen recognition, a dual antigen labeling strategy was employed to specifically detect and isolate GM2-binding B cells. This approach involved labeling B cells with biotinylated GM2, which was conjugated to two distinct fluorophore-labeled anti-biotin antibodies, allowing the identification of B cells that were positive for both fluorescent markers. Dual antigen labeling strategies are commonly used to enhance specificity by reducing background noise, as only B cells that bind to both labeled antigen probes are considered truly antigen specific. Similar strategies has been successfully employed for the isolation of antigen-specific memory B cells in the context of SARS-CoV-2 and Hepatitis B, where biotinylated antigens were combined with fluorophore-conjugated streptavidin^{86,246,247}. However, there is a fundamental difference between these

studies and the present work. In previous B cell isolation approaches, donors likely had a T cell-dependent immune response to protein antigens due to vaccination or recent infection, leading to a higher prevalence of antigen-specific B cells in peripheral blood. Furthermore, the isolation targeted memory B cells that had most probably undergone class switching and affinity maturation, suggesting increased specificity for the antigen used in labeling. In contrast, the GM2-specific B cells in this study most likely secrete the nAbs as a part of T cell-independent immune response or even in the absence of exogenous antigenic stimulation. Additionally, it remains unknown, whether the BCR of GM2-specific B cells can recognize the biotinylated GM2 antigen, as it is only composed of the carbohydrate head group of the GM2 ganglioside, which may not accurately present the native antigenic structure required for specific BCR binding.

Labeling of B cells from one GM2-Ab^{pos} and one GM2-Ab^{neg} donor resulted in no detection of double-positive B cells. Since the B cell culture supernatant from this GM2-Ab^{pos} donor also did not produce GM2-specific antibodies, it is possible that the B cells of interest were not present in peripheral blood of this donor. Notably, double-positive B cells were detected in the B cell population of the GM2-Ab^{pos} donor D78, consistent with the secretion of GM2-specific antibodies from the cultured B cells of this donor. Unexpectedly, double-positive B cells were also observed in the B cell population of one GM2-Ab^{neg} donor. In both cases, there was a notable difference in the number of positive cells when comparing labeling with GM2 to labeling with aminoglucitol, which was used as an off-target control to assess non-specific binding. Despite detecting double-positive cells in D78, sorting and subsequent culturing of these cells did not result in detectable GM2-specific antibody production. This suggests that either the labeling strategy did not successfully enrich for truly GM2-specific B cells, or that the sorted cells failed to survive and proliferate after the sorting process, thereby complicating validation.

4.5.2 Barcode Enabled Antigen Mapping technology

As the isolation of GM2-specific B cells via FACS did not yield promising B cell candidates for further characterization, the BEAM technology was explored as an alternative, promising approach, which enables the simultaneous identification of antigen-specific B cells and their full-length paired BCR sequences through FACS combined with single-cell sequencing. This method successfully isolated and analyzed over 12,000 sequenced cells, that were grouped in clonotypes according to their V(D)J-rearranged sequence. Many clonotypes showed much higher binding to GM2 compared to HSA, which was used as an off-target control, suggesting the identification of GM2-specific B cells. However, some challenges emerged during method establishment, optimization and validation of selected BEAM-derived antibodies.

One major limitation was the unexpected labeling of B cells from GM2-Ab^{neg} donors, indicating non-specific binding of BEAM conjugates, even in the absence of biotinylated antigens. As the BEAM assemblies used for detection consist of PE-conjugated streptavidin, some B cells may possess reactivity toward streptavidin or the fluorescent PE tag, leading to false-positive signals. This type of background

signal has been widely reported in antigen-specific B cell detection studies and highlights the importance of implementing off-target controls²⁴⁸. Notably, in this study the B cells showed much higher GM2 UMI counts compared to the HSA UMI counts, indicating that the binding specificity for GM2 was generally higher. However, it cannot be ruled out, that GM2 may have interacted with surface molecules other than the BCR. Some gangliosides serve as ligands for specific sialic acid-binding lectins (Siglecs)²⁴⁹. Some Siglecs, such as Siglec-2 or Siglec-G, are expressed on B cells²⁵⁰. While a direct interaction between gangliosides, such as GM2, and B cell-expressed Siglecs has not been reported, such a mechanism could theoretically explain GM2 binding by B cells independent of BCR specificity.

Moreover, gangliosides such as GM2 represent challenging antigens for antigen-specific B cell isolation strategies compared to protein antigens. Unlike proteins or peptides, the biotinylated GM2 antigen used in this study consisted of oligosaccharides and a sialic acid and has a low molecular weight of only 1.38 kDa. This small size may limit its ability to engage with BCRs, as steric hindrance or unfavorable conformational changes could prevent proper antigen presentation when conjugated within the BEAM assembly, which includes streptavidin, PE, and a UMI. Notably, 10x Genomics has optimized this method for antigens ranging from 10 kDa to 200 kDa, raising the possibility that the small molecular size of GM2 affected its detectability in this setting. Additionally, the GM2-conjugated BEAM assembly used for labeling may not fully capture the antigenic properties of GM2 in its natural membrane environment¹⁴⁹. Together with other types of lipids, gangliosides typically cluster within lipid rafts²⁵¹. The density of glycosphingolipids present in those clusters can influence the structure and thereby affecting antigen specificity. This was for example seen with M2590, an antibody that selectively reacted with melanoma cells but not with normal tissues due to differences in GM3 density^{252,253}. If the GM2-specific nAbs behave similarly, the GM2 antigen used in BEAM labeling may not fully mimic the clustered organization on cell membranes, potentially leading to the selection of B cells that do not truly recognize native GM2 expressed on neuroblastoma cells.

Importantly, the GM2-specific CAR NK cell control performed during method optimization successfully demonstrated the feasibility of identifying GM2-specific CAR cells using the BEAM labeling method. However, CAR expression is highly uniform, consistently enabling high levels of GM2-binding receptors, as demonstrated in previous studies from our laboratory. In contrast, primary B cells naturally exhibit variability in BCR expression. This variability could also be a limitation in the BEAM approach, as the labeling intensity of antigen-specific B cells depends on BCR expression levels, antigen binding affinity and the overall detection sensitivity, which can be influenced by background noise²⁵⁴.

Given the challenges of distinguishing true GM2-specific B cells from background signals, future studies could optimize the BEAM approach by refining the labeling conditions. For example, pre-blocking with a structurally similar antigen, such as GD2, might help reduce non-specific interactions. Additionally, computational approaches for predicting GM2 binding to identified antibody sequences could have facilitated the selection of promising candidates from the multitude of identified clonotypes

with high GM2-specificity scores. However, most available antigen-antibody interaction prediction tools are limited to protein or peptide antigens, making such analyses currently infeasible. With the rapid advancement of deep learning and machine learning techniques^{255,256}, future developments may enable more accurate predictions of glycan-antibody interactions, improving candidate selection. Notably, some tools have already been developed to predict carbohydrate-binding sites in proteins or to predict glycan structures^{257,258}, providing promising possibilities for future research.

With further optimization, BEAM technology could become a valuable tool for identifying naturally occurring anti-GM2 antibodies, paving the way for their therapeutic application. In this study, a high number of B cell clonotypes with high GM2-specificity scores – indicating strong GM2 binding and absent or minimal HSA binding – were identified using this method. As validation is time-consuming, only the most promising candidates were selected for further analysis. However, other identified clonotype sequences may also hold potential as highly cytotoxic, GM2-specific antibodies suitable for GM2-targeted immunotherapeutic strategies and can be validated in future studies.

4.6 Antibody production and validation

Some B cell clonotypes identified in the BEAM experiment were selected for further characterization based on their high antigen-specificity scores or high GM2-specific UMI counts, suggesting that their BCRs, and consequently, their expressed antibodies, were likely GM2-specific. While IgG and some IgM antibodies from these clonotypes were successfully produced, they did not exhibit GM2 binding, in contrast to the positive control KM966. Several factors could account for this lack of GM2-specificity or the inability to detect antigen specificity under the tested conditions. One possibility is that the B cell isolation using BEAM technology may not have enriched truly GM2-specific B cells, as discussed previously. Another explanation could be that the recombinant expression format used in this study does not support efficient GM2 recognition.

IgG antibodies are the most widely used immunoglobulins in research and therapeutic applications⁷⁴. Therefore, the selected V(D)J sequences derived from promising B cell clonotypes were expressed as IgG-class antibodies. While their production was successful, GM2-specificity could not be demonstrated in this setting. One explanation for this could be, that the selected clonotype sequences were derived from IgM-expressing B cells, suggesting that their antigen recognition might depend on multimerization. Unlike monomeric IgG, IgM forms pentamers or hexamers, where subunits are covalently linked via disulfide bonds²⁵⁹. This multimeric structure enables increased avidity, allowing IgM to compensate for its lower affinity by engaging multiple antigen-binding sites simultaneously. However, not all IgM antibodies equally benefit from avidity effects, as demonstrated in studies comparing Trastuzumab and Pertuzumab in their IgM formats. Trastuzumab IgM demonstrated steric hindrance, which prevents some Fab regions from engaging HER2, thereby reducing its effective avidity. In contrast, Pertuzumab IgM fully utilizes all of its Fab regions to bind multiple HER2 receptors

simultaneously, leading to stronger overall binding and higher functional efficacy in HER2-overexpressing cells²⁶⁰. However, converting IgM antibodies into an IgG format frequently results in a loss of function, as multimerization is often essential for antigen binding^{261,262}. Carbohydrates, including gangliosides, are classified as T cell-independent antigens, typically triggering a B cell response dominated by IgM antibodies that lack affinity maturation. Consequently, anti-carbohydrate antibodies are generally 10^3 to 10^5 times weaker in binding than those specific for proteins or peptides, and also show faster dissociation rates^{223,263}. This suggests that the lack of antigen binding observed using the expressed IgG antibodies may be attributed to their original IgM B cell isotype, as their affinity may be too low in monomeric form and multimerization may be necessary to achieve effective antigen recognition.

However, expressing V_H and V_L sequences in an IgM expression plasmid in HEK293T cells proved to be challenging. Only a small subset of heavy and light chains was successfully secreted following transient transfection, as detected by Western blot analysis. While heavy chains were produced, they appeared to be retained within the HEK293T cells, suggesting potential impairment in antibody assembly or secretion. Additionally, light chain production was barely detectable for most antibodies, further indicating inefficiencies in expression or intracellular transport. Several factors may contribute to these findings. Light chains may be inefficiently transcribed, translated, or transported to the ER. Additionally, the heavy-light chain pairing may be disrupted, leading to misfolding or improper antibody assembly. This could trigger ER retention or proteasomal degradation of unassembled chains. Similar challenges in antibody production have been reported in other studies. For example, Mathias *et al.*, showed that antibody secretion can be impaired due to light chain misfolding and impaired disulfide bond formation, which leads to intracellular retention and thereby reduces overall antibody secretion²⁶⁴. These findings highlight the importance of optimized antibody expression systems, particularly for IgM production, where multimerization and proper folding are essential for secretion and functional activity.

In this study, inconsistent antibody production efficiencies, leading to variable antibody concentrations, limited the ability to confirm the expected high GM2 specificity, even for the KM966 control antibody when expressed in the IgM format. Although the heavy chain was not detectable in the supernatants by Western blot analysis under reducing conditions, it is possible that the weak GM2 signal observed in the anti-ganglioside dot assay and the positive staining of some GM2-expressing cells resulted from small amounts of correctly assembled and secreted KM966 IgM that were sufficient for low-level antigen recognition. However, as the V_H and V_L sequences of the clonotype-derived antibodies differ from KM966 and may exhibit lower affinities, their concentrations in the supernatant may have been insufficient for detection of GM2-specificity. Future studies could therefore optimize the IgM production efficiency to ensure improved antibody yields for functional assays. Successful high-yield antibody production can be enhanced by various factors, including vector design, expression system, and the choice of host cell line.

Here, the pVITRO expression system was used, allowing the co-expression of heavy and light chains under the control of two individual promoters within a single plasmid¹⁹¹. However, a potential limitation using this system could be that all light chains of the clonotype antibodies were expressed with a constant κ chain sequence, regardless of their natural isotype. In general, λ and κ light chains differ in structural flexibility, antigen binding, and stability. λ chains exhibit greater Fab flexibility and a more diverse CDR loop repertoire, while κ chains provide greater structural stability^{265,266}. These differences can impact antibody affinity, specificity, and folding, which may be particularly relevant since clonotypes 04, 19, 31, 37, 41, 900, 10066, and 11767 naturally express λ light chain constant genes (*IGLC1*, *IGLC2*, or *IGLC3*). A study investigating the κ to λ switch in an antibody demonstrated that this exchange affects structural and functional properties, including altered interaction with the variable domain and modified conformation of the antigen-binding pocket. However, despite these structural differences, the reactivity and antigen-binding properties of the antibody remained largely unchanged, suggesting that the constant domain influences stability and conformation but does not necessarily affect antigen recognition.

Additionally, the expression ratio of heavy and light chains plays a crucial role in antibody production. A higher light-to-heavy chain ratio promotes efficient antibody formation, leading to increased titers and stable glycosylation profiles. Conversely, a lower ratio can cause aggregation, altered glycosylation, and reduced stability²⁶⁷. The pVitro IgM expression plasmid currently contains strong promoters for both chains, an mEF1 α promoter for the heavy chain and an rEF1 α promoter for the light chain. To fine-tune expression levels, alternative promoters with varying strengths could be considered. For instance, the SV40 promoter²⁶⁸, which has medium strength, and the CAG promoter²⁶⁹, known for its strong expression, may offer better control over chain balance and optimize antibody yield and stability.

Moreover, the pVitro_H+L_IgM expression plasmid used in this study did not include a J chain, a short polypeptide essential for IgM multimerization. The J chain binds to the C-terminus of the heavy chains, facilitating the formation and stabilization of the pentameric IgM structure²⁷⁰. While studies have demonstrated that the J chain is not strictly required for IgM assembly and secretion, its absence predominantly leads to the formation of hexameric IgM instead of the pentameric form²⁷¹. Interestingly, hexameric IgM exhibits distinct functional advantages. At low antigen densities, hexameric IgM induces the complement cascade more effectively than pentameric IgM²⁷², potentially enhancing immune responses. Therefore, the decision to include or exclude J chain expression should be carefully considered based on the desired properties of the recombinant IgM. However, the incorporation of a J chain could potentially enhance IgM assembly, stability, and secretion efficiency and might therefore be tested for the IgM production of clonotype-derived sequences.

In this study, HEK293T cells were used for antibody production due to their high IgG yields during transient transfection²⁷³. However, IgM molecules are significantly more challenging to express recombinantly than IgG²⁷⁴, which is why exploring alternative expression systems could further enhance production efficiency. For example, studies have shown that Chinese hamster ovary (CHO) cells achieve

high recombinant protein yields²⁷⁵. Additionally, human PER.C6 cells have demonstrated high levels of IgM expression, both in the presence and absence of the J chain²⁷⁶.

While optimizing expression systems is critical for improving antibody yields, another key factor in validating GM2-specificity is the detection method itself. The anti-ganglioside dot assay used for detection of GM2-specificity may not recapitulate the physiological conditions required for binding. The organization of gangliosides within lipid rafts in the membrane differ significantly from their immobilized forms on dot blots, potentially affecting recognition by these antibodies. Studies have shown that antibody-glycosphingolipid interactions are often dependent on a specific density of glycosphingolipids organized in lipid rafts^{149,252}, which may not be mimicked effectively in dot assays. Furthermore, it is unclear whether the nAbs recognize GM2 in a Neu5Ac- or Neu5Gc-specific way. Some anti-ganglioside antibodies recognize only specific variants of gangliosides, such as the monoclonal 14F7, which is specific for the N-glycolyl GM3 (Neu5Gc), but did not recognize the N-acetyl variant (Neu5Ac)²⁷⁷. But as the nAbs in donor serum were capable of binding to the dot blot strips as well as on the neuroblastoma cell lines, a binding of recombinant antibodies derived from the corresponding B cells to them as well can be expected. Therefore, it is unlikely that the GM2 variant used for detection may have impacted the validation.

In summary, challenges in antibody production, particularly IgM expression, limited the validation of promising candidates from the BEAM experiment for GM2-specificity. While IgG production was successful, none of the clonotype antibodies expressed as IgG showed binding to GM2. Importantly, Western blot detection of both heavy and light chains of IgM antibodies from clonotypes 02, 10, and 900 suggests successful expression, even though GM2 binding was not observed. Verifying their correct assembly as multimeric IgM remains a key next step, which can be assessed using methods such as native PAGE²⁷⁸ or size-exclusion chromatography²⁷⁹. For the remaining clonotype antibodies, inefficient expression and secretion may have impacted the ability to assess GM2 binding. Western blot analysis revealed low or undetectable secretion of heavy and/or light chains in several cases, pointing to potential assembly or expression challenges. These findings highlight the importance for further optimization of expression systems, vector design, and host cell selection to improve IgM production efficiency.

Naturally occurring GM2-specific antibodies have been shown to induce strong complement-dependent cytotoxicity, suggesting that fully assembled and functional IgM antibodies in this study could exhibit similar properties. Increasing antibody yields will help eliminate expression-related factors that may affect GM2 recognition, while properly assembled IgM antibodies at higher concentrations may reveal their GM2 specificity. Furthermore, if weak GM2 binding is detected in future studies, affinity maturation strategies could be applied to enhance binding affinity and improve antibody functionality.

5 CONCLUSION AND OUTLOOK

This study explored the potential of naturally occurring antibodies targeting the ganglioside GM2 as a novel immunotherapeutic approach for high-risk neuroblastoma.

The findings demonstrate that aberrant ganglioside biosynthesis is associated with poor clinical outcomes and that the ganglioside GM2 is highly expressed on neuroblastoma cell lines, highlighting its relevance as a therapeutic target. Notably, some healthy donors possess GM2-specific nAbs capable of inducing strong complement-dependent cytotoxicity against GM2-expressing neuroblastoma cells, supporting their potential for targeted immunotherapy. However, despite these promising findings, isolating and characterizing GM2-specific B cell clonotypes from peripheral blood proved challenging. While BEAM technology successfully identified numerous B cell clonotypes with predicted GM2 specificity, inefficient recombinant antibody expression prevented confirmation of their antigen specificity, underscoring the need for further methodological optimization.

Future studies should focus on confirming GM2 expression in primary neuroblastoma samples and investigating its presence in normal tissues to validate the therapeutic potential of GM2-targeted approaches while evaluating potential off-target effects. However, given that GM2-specific nAbs naturally exist in healthy individuals, their therapeutic application is likely to be well tolerated. A critical next step involves optimizing B cell isolation and validation strategies to improve the efficiency and specificity of isolating GM2-specific B cells. While BEAM technology demonstrated potential in identifying promising clonotypes, refinement of this approach is necessary to enhance accuracy and eliminate non-specific interactions.

Several alternative strategies could improve the antigen-specific B cell identification. Human hybridoma technology, which involves fusing B cells with myeloma cells to generate stable hybridomas²⁸⁰, could be tested to identify anti-GM2 expressing hybridoma cells. B cell ELISPOT assay could help to identify the antigen-specific B cells by capturing secreted antibodies on antigen-coated plates and detect them with enzyme- or fluorescent-labeled secondary antibodies²⁸¹. Furthermore, FACS-based methods can be enhanced using magnetic enrichment, where magnetic nanoparticles conjugated to antibodies targeting fluorophore-conjugated antigens selectively enrich antigen-specific B cells prior to flow-sorting, increasing detection sensitivity²⁸². Additionally, improved antigen presentation strategies could enhance the isolation efficiency of truly GM2-specific B cells. One promising approach is the Immudex dCODE Klickmer technology, which uses a dextran backbone with multiple acceptor sites for biotinylated antigens²⁸³, presumably enabling a more physiologically relevant antigen presentation by mimicking the dense organization of gangliosides in lipid rafts. Moreover, Klickmers incorporate a fluorophore, facilitating efficient sorting of antigen-specific B cells, and a unique barcode, allowing for precise determination of antigen specificity after single cell sequencing. Beyond BEAM technology,

other high-throughput B cell receptor sequencing methods could be tested to improve antigen-specific B cell identification. LIBRA-seq (Linking B cell Receptor and Antigen through sequencing), a method similar to BEAM, also uses DNA-barcoded antigens to directly link B cell receptor sequences with antigen specificity, providing another potential approach for identifying GM2-specific B cells²⁸⁴. Moreover, *de novo* antibody sequencing could be promising^{285,286}. It involves enzymatic digestion of antibodies into peptides followed by separation and sequencing using liquid chromatography/tandem mass spectrometry (LC-MS/MS). Bioinformatic algorithms are then used to assemble sequences and pair heavy and light chains. However, this approach would require affinity purification of GM2-specific antibodies from serum in large quantities and might also be challenging in polyclonal IgM settings.

To overcome the challenges in antibody expression, future efforts should optimize expression systems for higher yields and proper IgM folding, secretion, and functionality. Using CHO cells instead of HEK293T and co-expressing the J chain may enhance IgM multimerization and antigen-binding avidity. Additional strategies could include the optimization of promoter strength, heavy-to-light chain ratios, or the generating of stable cell lines for consistent yields. Purification methods like affinity chromatography or size-exclusion filtration could further concentrate antibodies for downstream validation.

In conclusion, while challenges remain, the identification of GM2-specific nAbs represents a promising step toward the generation of a novel immunotherapeutic approach for high-risk neuroblastoma. Further efforts should focus on refining B cell isolation, optimizing antibody expression, and validating the functional potential of GM2-targeting antibodies in preclinical models. If successful, these strategies could lead to the development of effective GM2-based immunotherapies, complementing existing anti-GD2 treatments and enhancing therapeutic outcomes for high-risk neuroblastoma patients.

ABSTRACT

Neuroblastoma, the most common extracranial solid tumor in children, presents a major clinical challenge in pediatric oncology. Despite advances in multimodal treatment strategies, the prognosis for high-risk patients remains poor. Although anti-GD2 immunotherapy has improved overall survival, its severe side effects and limited efficacy in recurrent tumors highlight the urgent need for alternative therapeutic approaches. Naturally occurring antibodies (nAbs), present in healthy individuals, have shown potential in targeting tumor-associated antigens, including gangliosides. This study investigated nAbs that specifically recognize GM2-expressing neuroblastoma cells as a novel immunotherapeutic strategy for high-risk neuroblastoma.

Gene expression analysis of neuroblastoma patient cohorts revealed aberrant ganglioside-related gene expression patterns, with high-risk cases predominantly expressing glycosyl- and sialyltransferase genes involved in the biosynthesis of low-complexity gangliosides such as GM2. Flow cytometry confirmed high GM2 expression on most neuroblastoma cells, further supporting its relevance as a therapeutic target. Remarkably, screening of 248 healthy donors identified GM2-specific nAbs, predominantly of the IgM isotype, in approximately 6% of individuals. These nAbs effectively mediated strong complement-dependent cytotoxicity against GM2-expressing neuroblastoma cells.

To translate these findings into a therapeutic approach, B cells from donors with cytotoxic, GM2-specific nAbs were isolated using antigen-specific B cell sorting combined with single-cell sequencing. This strategy enabled the identification of full-length, paired V(D)J sequences of antigen-specific B cell receptors (BCRs) and revealed several promising B cell clonotypes with high predicted specificity for GM2. However, recombinant expression of these sequences as IgG antibodies has not yet confirmed GM2 binding, indicating the need for optimized IgM expression strategies to preserve the natural antigen specificity of the clonotype-derived IgM sequences.

In conclusion, this study provides strong evidence that GM2-specific nAbs can mediate potent tumor cell lysis, representing a promising novel immunotherapeutic strategy for high-risk neuroblastoma. Given that nAbs occur naturally in healthy individuals and may contribute to natural immune surveillance, their use in immunotherapy could offer reduced toxicity and improved safety profiles compared to current monoclonal antibody treatments. These findings position GM2-targeting nAbs as a promising tool for future immunotherapies, although further research is needed to validate GM2 specificity in identified clonotype antibodies and to identify additional GM2-specific nAb sequences.

ZUSAMMENFASSUNG

Das Neuroblastom ist der häufigste extrakranielle solide Tumor im Kindesalter und stellt eine große klinische Herausforderung in der pädiatrischen Onkologie dar. Trotz bedeutender Fortschritte in multimodalen Behandlungsstrategien bleibt die Prognose, insbesondere für Hochrisikopatienten, ungünstig. Obwohl die Einführung der anti-GD2-Immuntherapie das Gesamtüberleben verbessert hat, wird ihr therapeutischer Nutzen durch schwere Nebenwirkungen und eine begrenzte Wirksamkeit bei rezidivierenden Tumoren erheblich eingeschränkt. Diese Limitationen verdeutlichen den dringenden Bedarf an alternativen, wirksameren Therapieansätzen.

In dieser Studie wurden natürlich vorkommende Antikörper (nAbs, *naturally occurring antibodies*) untersucht, die in gesunden Individuen vorhanden sind und tumorassoziierte Antigene, wie Ganglioside, gezielt erkennen können. Ziel dieser Studie war es, nAbs zu identifizieren, die spezifisch Gangliosid GM2-exprimierende Neuroblastomzellen erkennen, um ihr Potenzial als alternative immuntherapeutische Strategie für die Behandlung von Hochrisiko-Neuroblastomen zu analysieren.

Genexpressionsanalysen einer Patientenkohorte zeigten veränderte Expressionsmuster Gangliosid-assoziiierter Gene. Hochrisikopatienten exprimierten vorwiegend Glycosyl- und Sialyltransferase-Gene, die an der Biosynthese von Gangliosiden mit geringer Komplexität, wie GM2, beteiligt sind. Durchflusszytometrische Analysen bestätigten zudem eine hohe GM2-Expression auf den meisten Neuroblastomzellen, was GM2 als vielversprechende therapeutische Zielstruktur hervorhebt.

Ein Screening von 248 gesunden Spendern identifizierte GM2-spezifische nAbs, überwiegend vom IgM-Isotyp, bei etwa 6 % der Individuen. Diese nAbs vermittelten eine starke komplementabhängige Zytotoxizität gegen GM2-exprimierende Neuroblastomzellen.

Zur Translation dieser Erkenntnisse in einen therapeutisch nutzbaren Ansatz, wurden antigenspezifische B-Zellen von Spendern mit zytotoxischen, GM2-spezifischen nAbs isoliert. Dies erfolgte mittels antigenspezifischer B-Zell-Sortierung in Kombination mit Einzelzell-Sequenzierung. Mit dieser Methodik konnten vollständige V(D)J-Sequenzen der isolierten B-Zellen identifiziert werden, einschließlich der Sequenzen der schweren und leichten Ketten der antigen-spezifischen B-Zell-Rezeptoren. Eine anschließende *in silico*-Analyse führte zur Identifikation mehrerer vielversprechender (teilweise klonaler) B-Zellen mit hoher vorhergesagter Spezifität für GM2.

Allerdings konnte die rekombinante Expression dieser Sequenzen als IgG-Antikörper die GM2-Spezifität bisher nicht bestätigen. Dieses Ergebnis deutet darauf hin, dass optimierte IgM-Expressionssysteme erforderlich sind, um die natürliche Antigen-spezifität der ursprünglich IgM-basierten Antikörper beizubehalten.

Zusammenfassend zeigt diese Studie, dass GM2-spezifische nAbs eine effektive Tumorzell-Lyse vermitteln können und somit ein vielversprechendes Potenzial für einen neuartigen, immuntherapeutischen Ansatz bei Hochrisiko-Neuroblastomen bieten. Da nAbs natürlicherweise in gesunden Individuen vorkommen und möglicherweise eine Rolle in der natürlichen Immunüberwachung spielen, könnte ihr therapeutischer Einsatz mit weniger Nebenwirkungen verbunden sein als die derzeitige anti-GD2-Immuntherapie. Allerdings ist weitere Forschung erforderlich, um die GM2-Spezifität der identifizierten Antikörper zu validieren oder zusätzliche GM2-spezifische nAb-Sequenzen zu identifizieren.

ABBREVIATIONS

°C	Degree Celsius	DMSO	Dimethyl sulfoxide
5' GEX	5' gene expression	DNA	Deoxyribonucleic acid
κ	kappa	dNTP	Deoxyribonucleotide triphosphate
λ	lambda	DTT	Dithiothreitol
μg	Microgram	e.g.	<i>exempli gratia</i> "for example"
μl	Microliter	EBV	Epstein-Barr Virus
μM	Micromolar	ECL	Enhanced chemiluminescence
ADCC	Antibody-dependent cellular cytotoxicity	EDTA	Ethylene diamine tetra acetic acid
AF488	Alexa Fluor 488	EF1α	Elongation factor 1-alpha
AHSCT	Autologous hematopoietic stem cell transplantation	EFS	Event-free survival
ALK	Anaplastic lymphoma kinase	EFS promoter	EF1α short
APC	Allophycocyanin	EGFR	Epidermal growth factor receptor
B-ALL	B cell acute lymphoblastic leukemia	ELISA	Enzyme-linked immunosorbent assay
BCR	B cell receptor	ER	Endoplasmic reticulum
B4GALNT1	β-1,4-N-acetylgalactosaminyl-transferase 1	ESTIMATE	Estimate of stromal and immune cells in malignant tumor tissues from expression data
BEAM	Barcode Enabled Antigen Mapping	FACS	Fluorescence-activated cell sorting
BiTE	Bispecific T cell engager	Fab	Fragment antigen-binding
BSA	Bovine serum albumin	FBS	Fetal bovine serum
BSD	Blasticidin S deaminase	Fc	Fragment crystallizable
CaCl ₂	Calcium chloride	FcR	Fc receptors
CAR	Chimeric antigen receptor	FDA	Food and Drug Administration
CD	Cluster of differentiation	FPKM	Fragments per kilobase million
CDC	Complement-dependent cytotoxicity	FSC-A	Forward scatter area
CDR	Complementarity-determining region	FSC-H	Forward scatter height
Cl.	Clonotype	g	Gram
CMAH	Cytidine monophosphate-N-acetylneuraminic acid hydroxylase	GEM	Gel bead-in-emulsion
CO ₂	Carbon dioxide	GEO	Gene Expression Omnibus
COG	Children's Oncology Group	GFP	Green fluorescent protein
cPPT	Central polypurine tract	GlcCer	Glucosylceramide
CRISPR	Clustered regularly interspaced short palindromic repeats	GM2-Ab	anti-GM2 antibody
D	Donor	GM-CSF	Granulocyte-macrophage colony-stimulating factor
d	Days	GPC2	Glypican-2
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride	GSEA	Gene set enrichment analysis
DEGs	Differentially expressed genes	h	Hour
DMEM	Dulbecco's Modified Eagle Medium	H ₂ O	Water
		HAT	Hypoxanthine-Aminopterin-Thymidine
		HBS	HEPES-buffered saline

HC	Heavy chain	MKI	Mitosis-karyorrhexis index
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	ml	Mililiter
HER2	Human epidermal growth factor receptor 2	mM	Millimolar
HEXA	Hexosaminidase A	nAbs	Naturally occurring antibodies
HLA	Human leukocyte antigens	NC	Negative control
HPLC	High-performance liquid Chromatography	NCBI	National Center for Biotechnology Information
HR	High risk	neg	negative
HS	Horse serum	NES	Normalized enrichment score
HSA	Human serum albumin	NEU	Neuraminidase
ICI	Immune checkpoint inhibitors	Neu5Ac	N-acetylneuraminic acid
Ig	Immunoglobulin	Neu5Gc	N-glycolylneuraminic acid
IL	Interleukin	ng	Nanogram
IMDM	Iscoe's Modified Dulbecco's Medium	NK cells	Natural killer cells
INPC	International Neuroblastoma Pathology Classification	OS	Overall survival
INRGSS	International Neuroblastoma Risk Group Staging System	PAA	Poly[N-(2-hydroxyethyl)acrylamide-co-N-acrylamide]
INSS	International Neuroblastoma Staging System	PBS	Phosphate buffered saline
IR	Intermediate risk	PCR	Polymerase chain reaction
IRES	Internal ribosome entry site	PE	Phycoerythrin
kDa	Kilodalton	PEG	Polyethylene glycol
Kdn	2-keto-3-deoxy-D-glycero-D-galactononic acid	PFA	Paraformaldehyde
KLD	Kinase, Ligase and DpnI	PHOX2B	Paired-like homeobox2B
KLH	Keyhole limpet hemocyanin	PI	Propidium iodide
KO	Knockout	pNPP	Para-nitrophenyl phosphate
L	Litre	pos	positive
LASSO	Least absolute shrinkage and selection operator	RNA	Ribonucleic acid
LB	Luria Broth	RPMI	Roswell Park Memorial Institute Medium
LacCer	Lactosylceramid	RT	Room temperature
logFC	log ₂ fold change	RT-PCR	Reverse transcription polymerase chain reaction
LR	Low risk	RTK	Receptor tyrosine kinase
M	Molar	s	Second
mAb	Monoclonal antibody	scFv	Single chain variable fragment
MEM	Minimum Essential Medium Eagle	SFFV	spleen focus-forming virus
mg	Milligram	sgRNA	Single guide RNA
MHC	Major histocompatibility complex	shRNA	Small hairpin RNA
min	Minute	SOC	Super optimal broth with catabolite repression
		ST8SIA1	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1
		STR-PCR	Short tandem repeat polymerase chain reaction

t-SNE	t-distributed stochastic neighbor embedding
TAE	Tris acetate-EDTA
TBS	Tris-buffered saline
TIDE	Tracking of indels by decomposition
TLR	Toll-like receptor
UMI	Unique molecular identifier
UV	Ultraviolet
V(D)J	Variable, diversity and joining
V	Volt
VCN	<i>Vibrio cholerae</i> neuraminidase
VEGFR	Vascular endothelial growth factor receptor
V _H	Variable heavy
V _L	Variable light

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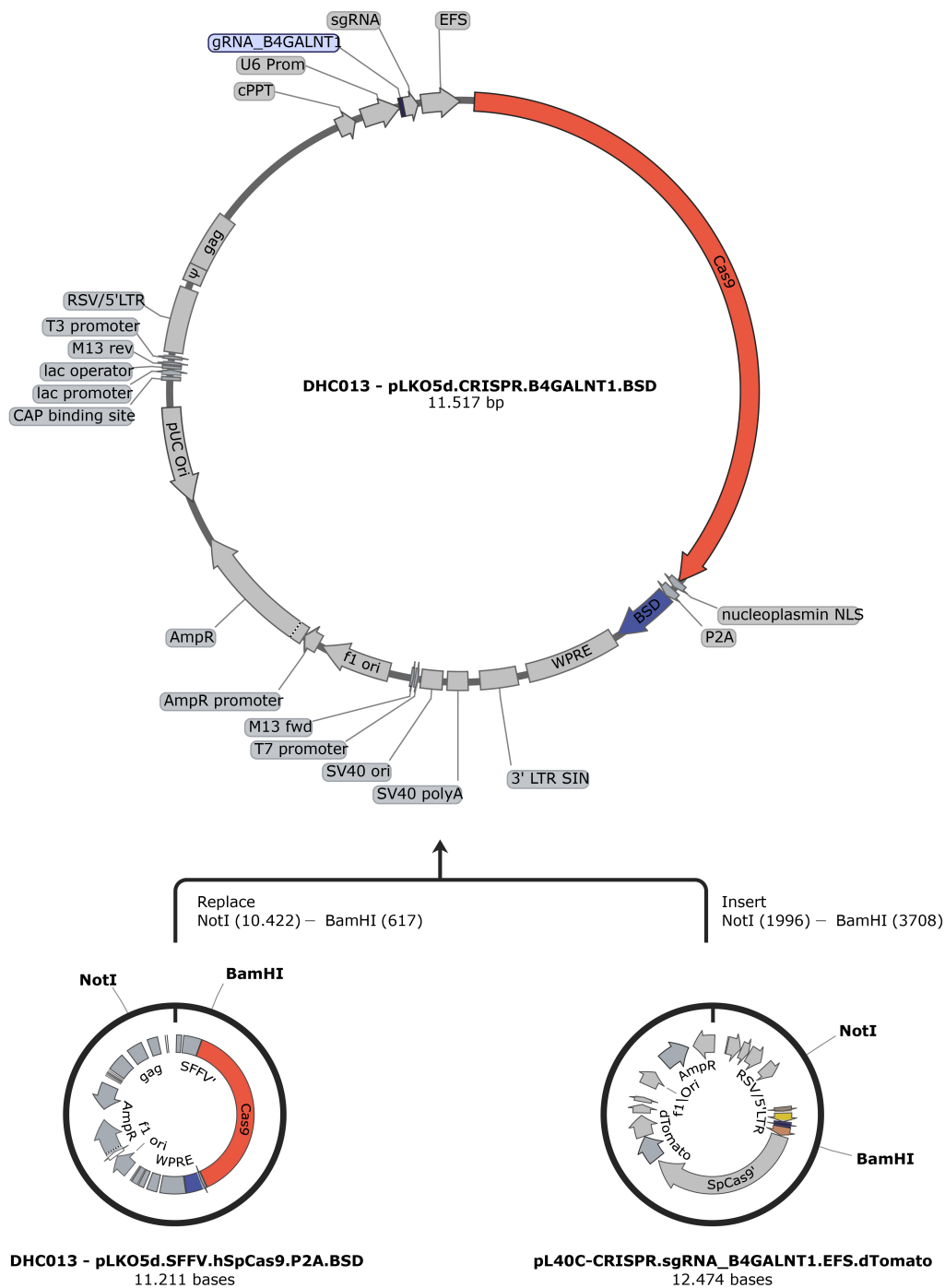
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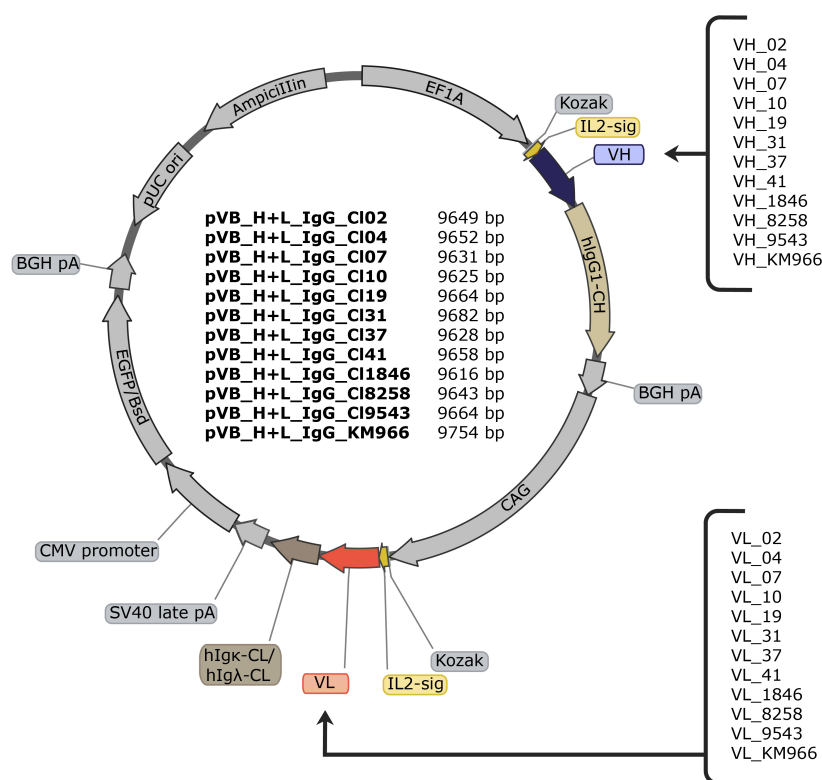
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APPENDIX

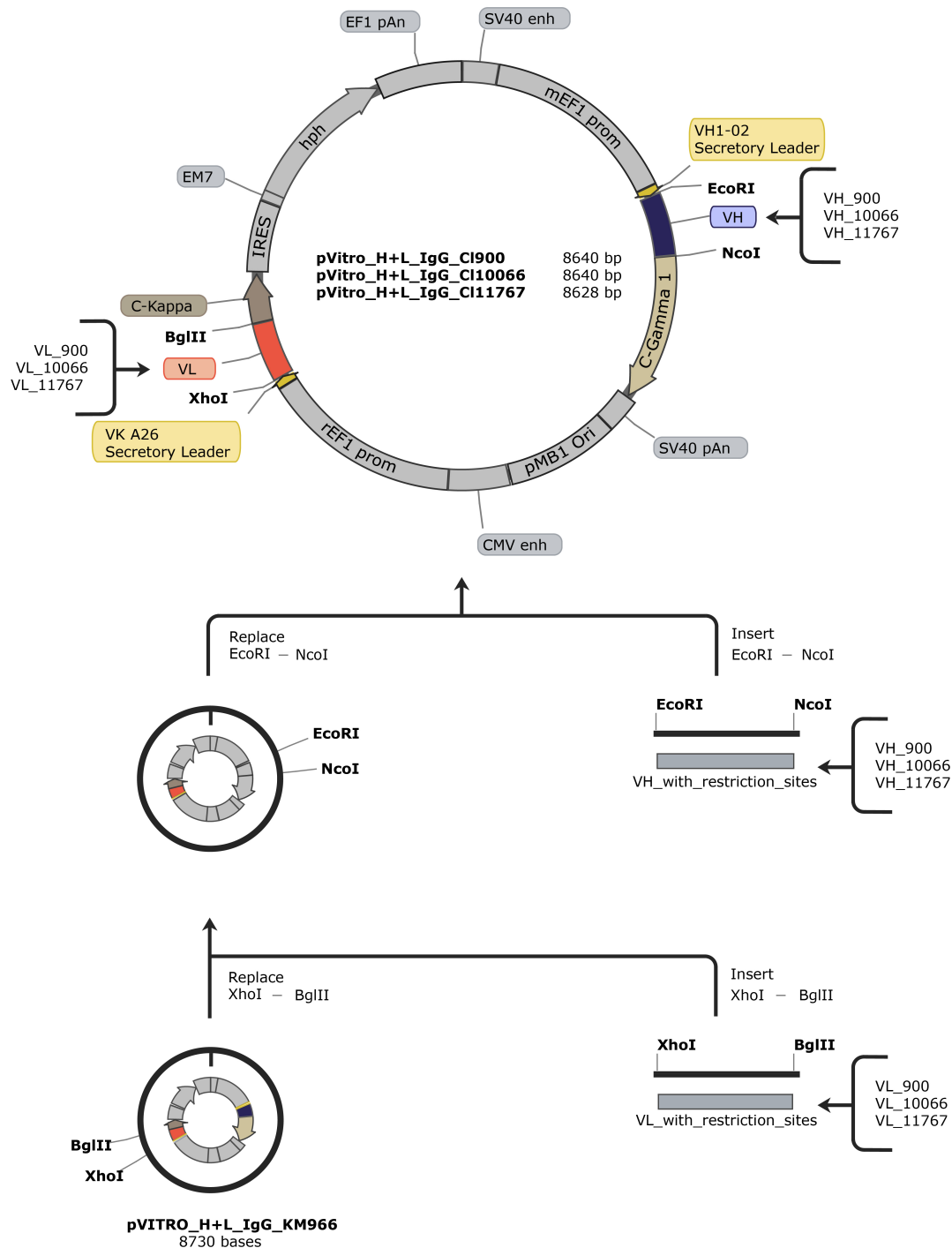
Supplementary Figures



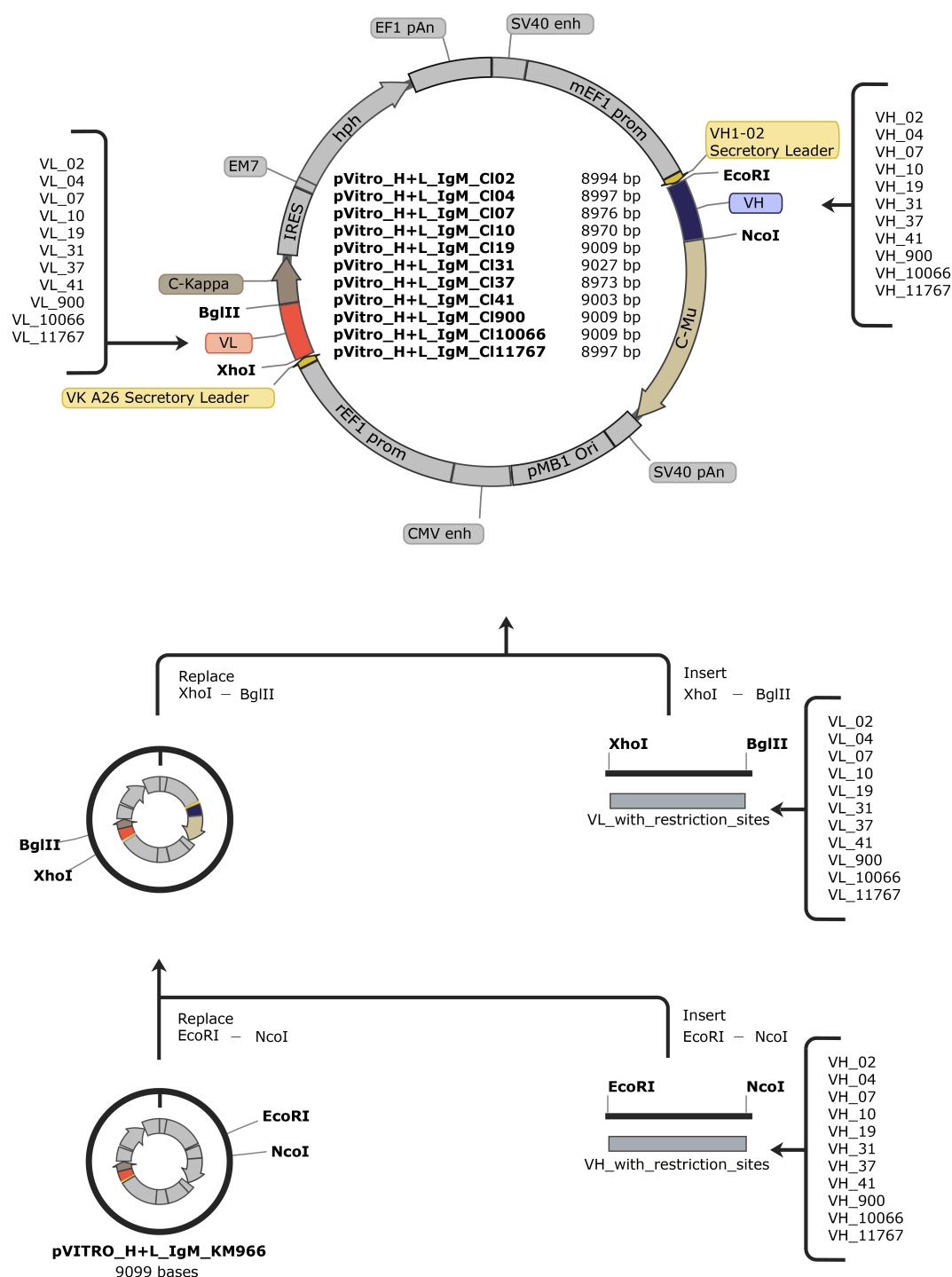
SUPPLEMENTARY FIGURE 1 | Cloning strategy and plasmid map of the B4GALNT1-specific CRISPR/Cas9 plasmid. The B4GALNT1-specific gRNA, including a cPPT and U6 promoter, as well as an EFS promoter for Cas9 expression, was isolated from the pL40C-CRISPR.sgRNA_B4GALNT1.EFS.dTomato plasmid via restriction enzyme digestion with NotI and BamHI. This fragment was inserted into the DHC013 – pLKO5d.SFFV-hSpCas9.P2A.BSD plasmid, which was digested with the same restriction enzymes. The recipient plasmid already contained the Cas9 gene, a Blastocidin resistance gene, and essential elements for lentiviral transduction, enabling stable gene delivery and selection of the recipient cells after transduction. The plasmid map was generated with SnapGene.



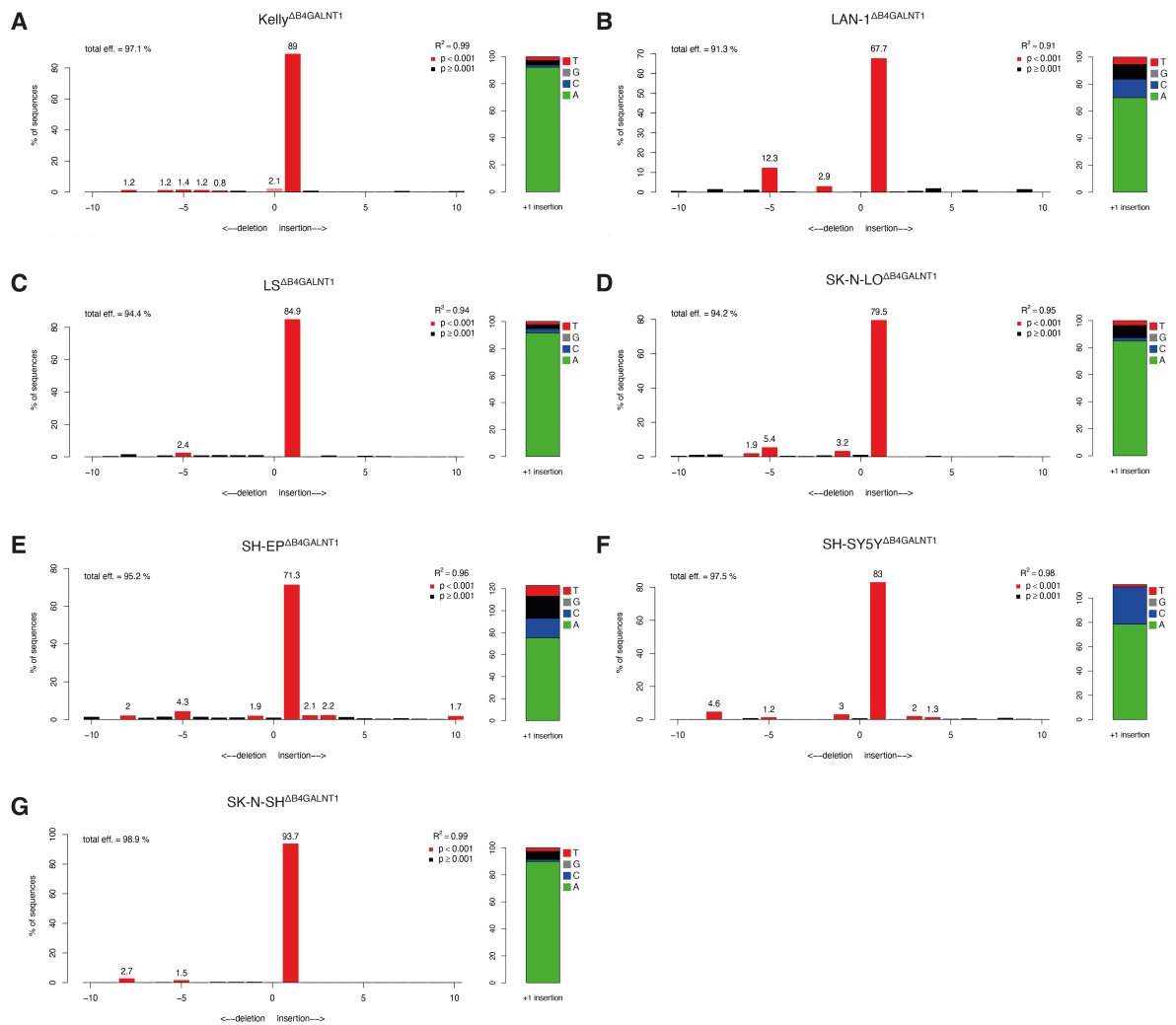
SUPPLEMENTARY FIGURE 2 | Plasmid map of the antibody expression plasmid pVB_H+L_IgG. The variable heavy (V_H) and variable light (V_L) chain sequences of the selected clonotypes were cloned into a heavy and light chain co-expression plasmid, generated by VectorBuilder. The heavy chain, containing an IgG1 constant domain, is expressed under the control of the EF1 α promoter, while the light chain, incorporating either a kappa or lambda constant region (depending on the naturally occurring light chain of the clonotype), is expressed under the CAG promoter. Both chains include IL-2 signal sequences to facilitate proper protein secretion. The plasmid map was generated with SnapGene.



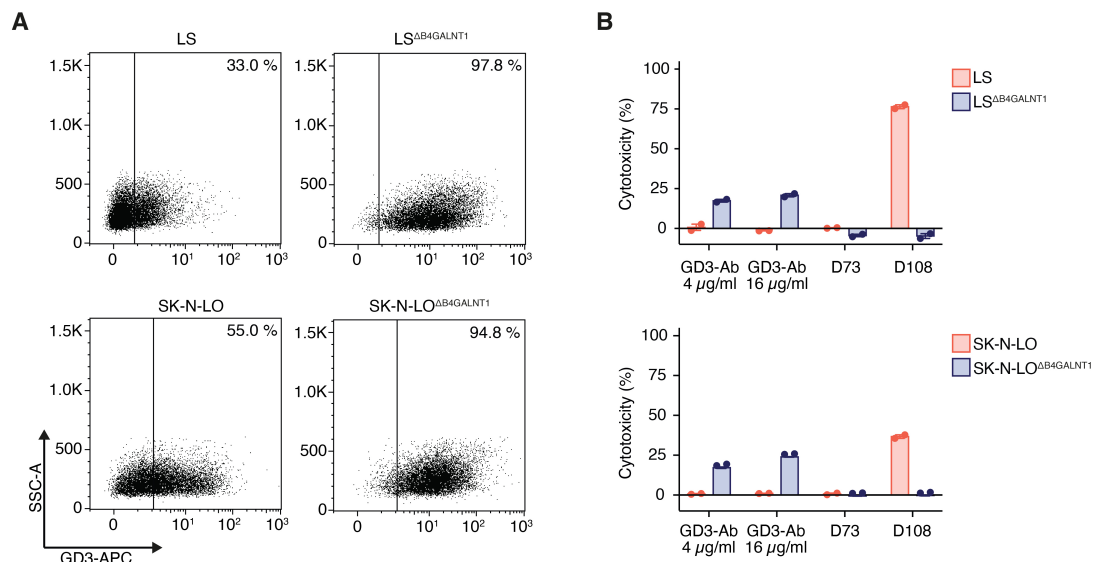
SUPPLEMENTARY FIGURE 3 | Schematic representation of the cloning strategy and plasmid map of the antibody expression plasmid pVITRO_H+L_IgG. The variable heavy (V_H) and variable light (V_L) sequences of clonotypes 900, 10066, and 11767 were synthesized by BioCat with flanking restriction sites and initially cloned into a pUC19 plasmid (not shown). Using restriction enzyme digestion, these sequences were subsequently cloned into the pVITRO_H+L_IgG_KM966 plasmid, where the V_H and V_L sequences of KM966 were sequentially replaced with the clonotype-specific V_H and V_L sequences. The heavy chain, containing an IgG1 constant domain, is expressed under the control of the mEF1 promoter, while the kappa light chain is expressed under the rEF1 promoter. Both chains have a secretory leader to enable protein secretion. The plasmid map was generated using SnapGene.



SUPPLEMENTARY FIGURE 4 | Schematic representation of the cloning strategy and plasmid map of the antibody expression plasmid pVitro_H+L_IgM. The variable heavy (V_H) and variable light (V_L) sequences of clonotypes 02, 04, 07, 10, 19, 31, 37 and 41 were synthesized by PCR using primers with flanking restriction sites. The V_H and V_L sequences of clonotypes 900, 10066, and 11767 were isolated via restriction enzyme digestion from their respective pVitro_H+L_IgG plasmids (not shown). All sequences were subsequently cloned into the pVitro_H+L_IgM_KM966 plasmid using restriction enzyme digestion, replacing the V_H and V_L sequences of KM966 with the clonotype-specific V_H and V_L sequences. The heavy chain, containing the IgM constant domain, is expressed under the mEF1 promoter, while the kappa light chain is expressed under the rEF1 promoter. Both chains include a secretory leader sequence to facilitate protein secretion. The plasmid map was created using SnapGene.

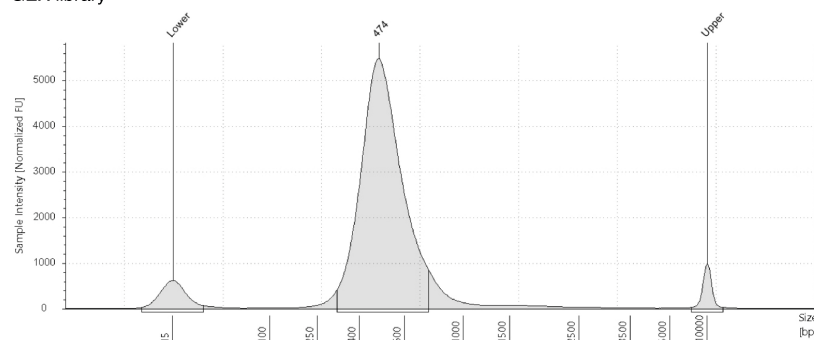


SUPPLEMENTARY FIGURE 5 | Genome editing efficiency after CRISPR/Cas9-based B4GALNT1 knockout analyzed by TIDE. Distribution and frequencies of base pair deletions and insertions at the target site, along with the probabilities of nucleotide insertion during +1 insertion events in Kelly^{ΔB4GALNT1} (A), LAN-1^{ΔB4GALNT1} (B), LS^{ΔB4GALNT1} (C), SK-N-LO^{ΔB4GALNT1} (D), SH-EP^{ΔB4GALNT1} (E), SH-SY5Y^{ΔB4GALNT1} (F), and SK-N-SH^{ΔB4GALNT1} (G).



SUPPLEMENTARY FIGURE 6 | B4GALNT1 knockout enhances GD3 expression, enabling anti-GD3-antibody-mediated cell cytotoxicity. **A**, Flow cytometry analysis of GD3 expression in LS, LS^{ΔB4GALNT1}, SK-N-LO, and SK-N-LO^{ΔB4GALNT1} cells. GD3 was stained using an anti-GD3 antibody, followed by an APC-conjugated anti-mouse IgG secondary antibody. Gating was performed based on secondary antibody only controls. The percentage of GD3⁺ cells is indicated in each plot. Representative histograms of two independent experiments are shown. **B**, Incucyte-based cytotoxicity assay comparing the effect of an anti-GD3 antibody and 15% (v/v) serum from D73 (GM2-Ab^{neg}) and D108 (GM2-Ab^{neg}) on LS and LS^{ΔB4GALNT1} cells (top) and SK-N-LO and SK-N-LO^{ΔB4GALNT1} cells (bottom). The mean cytotoxicity from duplicates of one experiment is shown.

GEX library



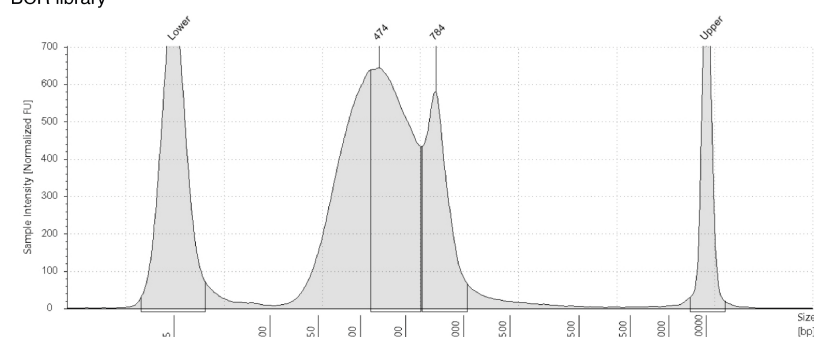
Sample Table

Well	Conc. [pg/ul]	Sample Description	Alert	Observations
E1	4930	D78 GEX Library		

Peak Table

Size [bp]	Calibrated Conc. [pg/ul]	Assigned Conc. [pg/ul]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
15	293	-	30000	-		Lower Marker
474	4930	-	16000	100.00		
10000	180	180	27.7	-		Upper Marker

BCR library



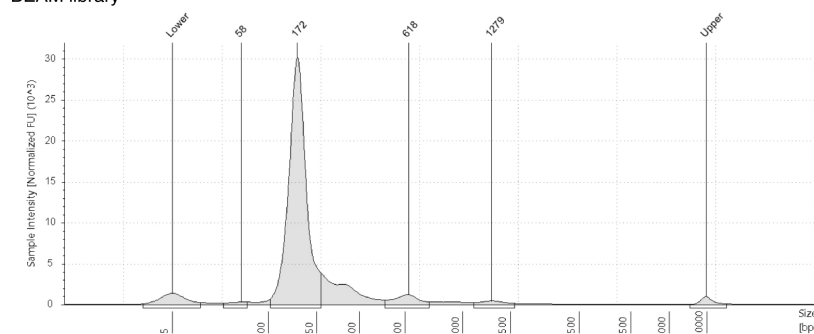
Sample Table

Well	Conc. [pg/ul]	Sample Description	Alert	Observations
G1	751	D78 BCR Library		

Peak Table

Size [bp]	Calibrated Conc. [pg/ul]	Assigned Conc. [pg/ul]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
15	358	-	36700	-		Lower Marker
474	485	-	1580	64.60		
784	266	-	521	35.40		
10000	180	180	27.7	-		Upper Marker

BEAM library



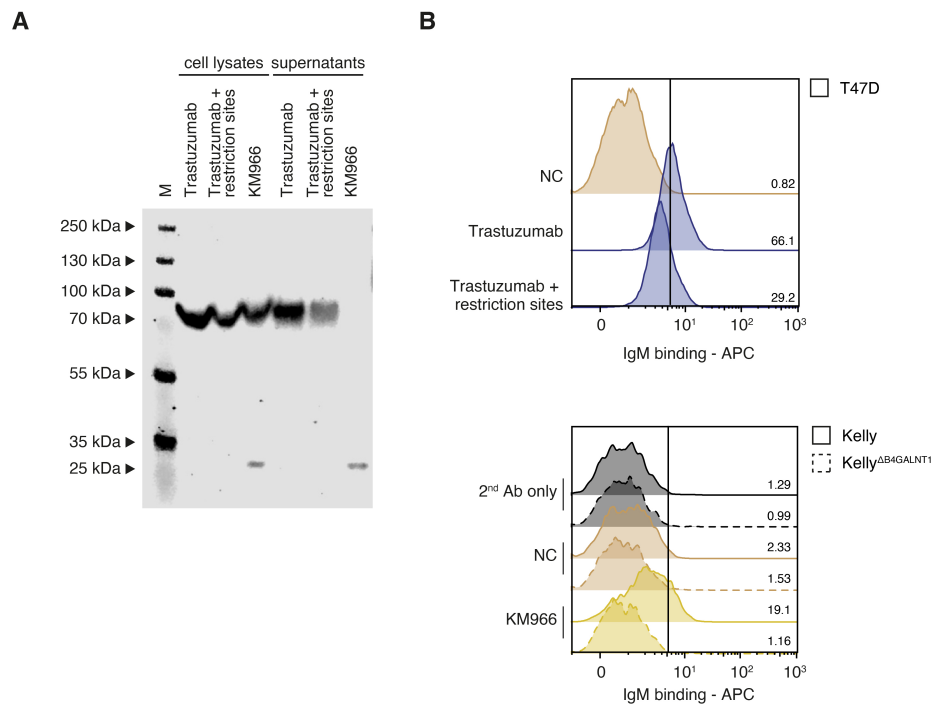
Sample Table

Well	Conc. [pg/ul]	Sample Description	Alert	Observations
H1	10600	D78 BEAM Library		

Peak Table

Size [bp]	Calibrated Conc. [pg/ul]	Assigned Conc. [pg/ul]	Peak Molarity [pmol/l]	% Integrated Area	Peak Comment	Observations
15	476	-	48800	-		Lower Marker
58	103	-	2720	0.97		
172	9730	-	87000	91.89		
618	547	-	1360	5.16		
1279	209	-	251	1.97		
10000	180	180	27.7	-		Upper Marker

SUPPLEMENTARY FIGURE 7 | Quality control of BEAM-experiment-related libraries using D5000 ScreenTape. Electropherograms and corresponding peak tables for the GEX library (top), BCR library (middle) and BEAM library (bottom) derived from the B cell sample of D78 to assess the integrity, size distribution and concentration of the libraries before sequencing. Lower (15 bp) and upper (10,000 bp) markers are used for calibration. Analysis was performed by the Single Cell Core Facility, UKE.



SUPPLEMENTARY FIGURE 8 | Trastuzumab and KM966 IgM antibody production and validation. Trastuzumab and KM966 IgM antibodies were produced by transiently transfecting HEK293T cells with the corresponding antibody expression plasmids. The original pVito-Trastuzumab-IgM/ κ plasmid from Addgene (#61881) and a modified version with V_H and V_L regions flanked by restriction sites for cloning, as well as KM966 plasmid, were used for transfection. Supernatants were harvested after 3 days, and cell lysates were prepared. **A**, Western blot analysis. Western blotting under reducing conditions was performed on both cell lysates and supernatants to confirm antibody expression. **B**, Flow cytometry analysis. The binding of secreted antibodies in the HEK293T supernatants was assessed by flow cytometry. The antibodies were tested for binding to HER2⁺ T47D cells, GM2⁺ Kelly wildtype cells and GM2⁻ Kelly^{ΔB4GALNT1} cells, respectively. Binding was detected using APC-conjugated anti-human IgM antibodies. The data shown are derived from a single antibody production, validated by one Western blot analysis and one flow cytometric analysis.

Supplementary Tables

Supplementary Table 1 | Glycans on the Glycan Array 300 and corresponding identifier. Raw data from Donor 25, 78, 37 and 108.

	Identifier	Glycan	Donor 25	Donor 78	Donor 37	Donor 108
Monosaccharides	G0001	β -Glc-Sp	17.44	16.03	6.36	9.65
	G0002	β -Gal-Sp	17.37	12.98	12.98	29.81
	G0003	α -Man-Sp	6.31	9.33	3.64	5.19
	G0004	α -Fuc-Sp	6.46	9.23	10.18	20.39
	G0005	α -Rha-Sp	2.85	1.92	3.52	7.29
	G0006	β -GlcNAc-Sp	20.89	16.09	7.57	21.78
	G0007	β -GalNAc-Sp	12.36	10.97	4.99	15.66
	G0052	β -D-GlcA-Sp	15.57	12.04	8.14	13.62
Disaccharides	G0075	β -D-Rha-Sp	7.98	17.75	12.28	14.52
	G0009	Gal- β -1,3-GlcNAc- β -Sp	10.28	7.23	8.88	19.22
	G0016	Gal- β -1,4-Glc- β -Sp	11.46	15.07	14.30	12.68
	G0027	GlcNAc- β -1,6-GlcNAc- β -Sp	56.30	19.43	28.46	28.73
	G0028	4-P-GlcNAc-b-1,4-Man-b-Sp	0.90	11.79	5.53	5.51
	G0030	Gal- β -1,3-GalNAc- α -Sp	22.71	14.13	10.45	26.41
	G0031	Gal- β -1,4-GlcNAc- β -Sp	5.95	8.00	8.32	10.99
	G0041	GalNAc- β -1,4-GlcNAc- β -Sp2	19.66	19.58	14.88	23.24
	G0045	GlcNAc- β -1,2-Man- α -Sp	14.93	9.93	9.80	14.09
	G0046	Neu5Ac- α -2,3-Gal- β -Sp1	6.88	4.68	3.36	7.84
	G0048	Gal- α -1,2-Gal- α -Sp	49.19	35.06	11.14	26.12
	G0051	GlcNAc- β -1,4-GlcNAc- β -Sp1	30.96	20.10	22.55	34.64
	G0053	Gal- β -1,4-(6S)GlcNAc- β -Sp	18.82	14.76	9.67	14.34
	G0066	Gal- β -1,3-GalNAc- β -Sp1	22.61	19.21	13.16	23.14
Glyco series, Milk Oligosaccharides and GAGs	G0076	Glc- α -1,4-Glc- β -Sp1	13.67	11.07	12.24	18.94
	G0087	D-cellose- β -Sp1	40.13	14.00	16.95	20.64
	G0088	Gal- α -1,3-Gal- β -Sp1	42.19	17.31	9.13	13.51
	G0018	Gal- α -1,4-Gal- β -1,4-Glc- β -Sp	62.60	10.85	14.60	14.84
	G0019	GlcNAc- β -1,3-Gal- β -1,4-Glc- β -Sp	9.76	10.16	7.15	14.89
	G0020	GalNAc- β -1,3-Gal- β -1,4-Glc- β -Sp	39.82	8.63	10.91	9.33
	G0026	GalNAc- β -1,3-Gal- α -1,4-Gal- β -1,4-Glc- β -Sp	34.43	30.07	17.25	16.58
	G0036	Gal- α -1,4-Gal- β -1,3-GlcNAc- β -Sp	62.44	11.59	12.98	14.75
Blood Groups, Lewis Antigens and Fucosylated Oligosaccharides	G0037	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc- β -[LNnT]-Sp	6.40	7.53	4.13	9.74
	G0038	GlcA- β -1,4-GlcNAc- α -1,4-GlcA- β -Sp	40.86	17.90	19.43	18.68
	G0047	Gal- β -1,3-GalNAc- β -1,3-Gal- β -Sp1	21.06	19.95	14.57	25.82
	G0074	Gal- α -1,4-Gal- β -1,4-GlcNAc- β -Sp1	92.06	27.00	24.26	20.47
	G0081	GlcNAc- α -1,4-GlcA- β -1,4-GlcNAc- α -1,4-GlcA- β -Sp	26.42	21.92	25.53	20.61
	G0015	Gal- β -1,3-(Fuc- α -1,4)-GlcNAc- β -[Lewis A]-Sp	2.82	-0.14	2.29	6.33
	G0025	Gal- β -1,4-(Fuc- α -1,3)-Glc- β -Sp	2.85	3.18	2.74	7.79
	G0032	Gal- β -1,4-(Fuc- α -1,3)-GlcNAc- β -[Lewis X]-Sp	2.67	5.39	4.72	13.27
	G0033	Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3)-GlcNAc- β -[Sialyl Lewis X]-Sp	2.63	0.65	1.01	4.62
	G0034	Neu5Ac- α -2,3-Gal- β -1,3-(Fuc- α -1,4)-GlcNAc- β -[Sialyl Lewis A]-Sp	0.35	1.19	-0.34	2.52
	G0035	Neu5Gc- α -2,3-Gal- β -1,3-(Fuc- α -1,4)-GlcNAc- β -[Sialyl Lewis A]-Sp	60.54	34.57	29.80	49.86
	G0044	GalNAc- α -1,3-(Fuc- α -1,2)-Gal- β -1,4-Glc- β -[Blood A antigen tetraose]-Sp1	3.11	3.86	15.52	11.37
	G0049	Gal- β -1,4-(Fuc- α -1,3)-GlcNAc- β -1,3-Gal- β -Sp1	2.71	0.53	3.80	9.80
	G0050	Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3)-Glc- β -[3-Sialyl-3-fucosyllactose/ F-SL]-Sp1	11.04	5.17	2.73	5.27
	G0057	GalNAc- α -1,3-(Fuc- α -1,2)-Gal- β -[Blood A antigen trisaccharide]-Sp1	22.40	26.62	15.85	29.71
	G0058	Fuc- α -1,2-Gal- β -1,4-GlcNAc- β -[Blood H antigen trisaccharide]-Sp1	4.77	6.03	5.52	6.16
Gangliosides and Sialylated Oligosaccharides	G0059	Gal- α -1,3-(Fuc- α -1,2)-Gal- β -[Blood B antigen trisaccharide]-Sp1	14.12	8.18	15.97	30.60
	G0060	Fuc- α -1,2-Gal- β -1,3-GlcNAc- β -1,3-Gal- β -1,4-Glc- β -[LNFP I]-Sp1	5.81	6.66	4.63	8.46
	G0061	Fuc- α -1,2-Gal- β -1,4-Glc- β -[Blood H antigen trisaccharide]-Sp1	11.94	8.01	11.31	5.90
	G0062	Gal- α -1,3-(Fuc- α -1,2)-Gal- β -1,4-Glc- β -[Blood B antigen tetrasaccharide]-Sp1	8.89	14.53	21.28	26.06
	G0063	(Fuc- α -1,2)-Gal- β -1,4-(Fuc- α -1,3)-GlcNAc- β -[Lewis Y]-Sp1	2.11	6.27	2.27	5.52
	G0064	(Fuc- α -1,2)-Gal- β -1,3-(Fuc- α -1,4)-GlcNAc- β -[Lewis B]-Sp1	2.12	1.90	2.77	5.64
	G0065	Gal- β -1,3-(Fuc- α -1,4)-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,4)-Glc- β -[Lewis A]-Sp1	6.54	6.99	5.79	8.38
	G0011	Neu5Ac- α -2,3-Gal- β -1,3-GlcNAc- β -Sp	3.54	3.86	10.90	21.16
	G0012	Neu5Ac- α -2,6-Gal- β -1,3-GlcNAc- β -Sp	1.06	3.08	8.08	17.49
	G0013	Neu5Gc- α -2,3-Gal- β -1,3-GlcNAc- β -Sp	4.05	7.39	20.98	29.52
Glycans, N-Glycans, Glycans	G0014	Neu5Gc- α -2,6-Gal- β -1,3-GlcNAc- β -Sp	4.69	3.13	9.86	20.13
	G0021	Neu5Ac- α -2,3-Gal- β -1,4-Glc- β -Sp	14.22	5.47	8.91	8.78
	G0022	Neu5Ac- α -2,6-Gal- β -1,4-Glc- β -Sp	3.52	2.10	4.14	6.07
	G0023	Neu5Gc- α -2,3-Gal- β -1,4-Glc- β -Sp	5.63	4.77	10.97	14.88
	G0024	Neu5Gc- α -2,6-Gal- β -1,4-Glc- β -Sp	7.12	1.64	8.83	5.66
	G0040	Neu5Ac- α -2,3Gal- β -1,4-(6S)GlcNAc- β -Sp	7.28	6.86	2.56	5.41
	G0042	Neu5Ac- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-Glc- β -Sp	6.00	1.70	3.50	4.44
	G0043	Neu5Gc- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-Glc- β -Sp	4.30	3.39	1.76	3.22
	G0055	Gal- β -1,3-GalNAc- β -1,4-(Neu5Gc- α -2,3)-Gal- β -1,4-Glc- β -Sp1	36.29	15.91	10.20	23.31
	G0067	Gal- β -1,3-(Neu5Ac- α -2,6)-GalNAc- β -Sp	17.54	9.85	7.23	16.19
	G0068	Neu5Ac- α -2,6-Gal- β -1,3-GalNAc- β -Sp	10.25	12.99	15.08	20.69
	G0069	Neu5Ac- α -2,6-Gal- β -1,3-(Neu5Ac- α -2,6)-GalNAc- β -Sp	3.65	7.90	9.40	20.47
	G0070	Neu5Ac- α -2,3-Gal- β -1,3-(Neu5Ac- α -2,6)-GalNAc- β -Sp	2.31	3.88	4.70	4.28
	G0071	Neu5Ac- α -2,6-(Neu5Ac- α -2,3)-Gal- β -1,3-GalNAc- β -Sp	6.24	12.29	4.18	4.20
	G0072	GalNAc- β -1,4-(Neu5Ac- α -2,3)-Gal- β -1,4-Glc- β -[GM2]-Sp	53.33	21.91	5.66	7.98
	G0073	GalNAc- β -1,4-(Neu5Ac- α -2,8-Neu5Ac- α -2,3)-Gal- β -1,4-Glc- β -[GD2]-Sp	8.07	10.90	2.58	2.91
Natural Oligosaccharides	G0091	KDN- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-Glc- β -Sp	3.18	5.74	8.25	16.51
	G0092	Neu5Ac- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-Glc- β -Sp	3.12	11.07	2.84	6.47
	G0093	Neu5Ac- α -2,8-Neu5Ac- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-Glc- β -Sp3	5.26	33.06	4.84	10.30
	G0094	Neu5Ac- α -2,8-Neu5Ac- α -2,6-Gal-b-1,4-Glc-Sp5	1.83	3.31	4.04	9.10
	G0095	Gal- β -1,3-GalNAc- β -1,4-(Neu5Ac- α -2,3)-Gal- β -1,4-Glc- β -Sp1	57.13	23.18	16.61	41.10
	G0029	Glc- α -1,2-Gal- α -1,3-Glc- α -Sp	78.66	68.11	42.36	66.84
	G0054	GlcNAc- α -1,3-(Glc- α -1,2-Glc- α -1,2)-Gal- α -1,3-Glc- α -Sp	27.36	26.94	31.02	28.77
	G0077	Glc- α -1,6-Glc- α -1,4-Glc- β -Sp1	18.02	12.55	16.41	27.11
	G0078	Maltotriose- β -Sp1	19.22	11.43	16.28	22.28
	G0079	Glc- α -1,6-Glc- α -1,6-Glc- β -Sp1	12.23	12.28	16.03	20.25
Amnoglycosides	G0080	Maltotetraose- β -Sp1	13.62	17.29	15.56	17.95
	G0082	Maltohexaose- β -Sp1	22.54	20.25	16.01	18.43
	G0083	Maltoheptaose- β -Sp1	24.34	26.81	21.35	31.81
	G0084	Acarbose- β -Sp1	34.13	27.59	24.88	35.82
	G0085	D-pentamannuronic acid- β -Sp1	11.68	16.29	11.12	19.85
	G0086	L-pentaguturonic acid- β -Sp1	42.45	16.47	20.01	22.50
	G0089	β -1,4-Xylotetraose-Sp1	15.32	15.94	19.04	14.32
	G0090	Chitin-trisaccharide-Sp1	43.73	32.88	39.66	51.51
	G0010	Gal- α -1,3-Gal- β -1,3-GlcNAc- β -Sp	17.23	6.40	11.03	14.31
	G0017	Gal- α -1,3-Gal- β -1,4-Glc- β -Sp	47.80	21.83	15.60	23.74
Amnoglycosides	G0039	GlcNAc- β -1,6-(Gal- β -1,3)-GalNAc- α -O-Ser-Sp4	19.06	10.56	6.61	15.26
	G0100	SGP	0.14	0.06	4.30	-0.07
	G0008	Tobramycin	15.62	6.63	10.58	6.25
	G0056	Sisomicin Sulfate	29.77	16.81	24.52	22.82
	G0096	Gentamicin Sulfate	2.56	12.78	21.55	3.44
	G0097	Kanamycin sulfate	37.08	26.72	19.55	31.92
	G0098	Genetion Disulfate Salt (G418)	3.63	9.47	19.95	4.48
	G0099	Neomycin trisulfate	0.49	25.35	6.45	43.65

N-Glycans	N010	Man- α -1,6-(Man- α -1,3-)Man- α -1,6-(GlcNAc- β -1,2-Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	6.04	3.34	5.21	7.27
	N011	Man- α -1,6-(Man- α -1,3-)Man- α -1,6-(Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	4.60	3.39	4.62	3.70
	N012	Man- α -1,6-(Man- α -1,3-)Man- α -1,6-(Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	7.01	4.37	5.42	8.58
	N013	Man- α -1,6-(Man- α -1,3-)Man- α -1,6-(Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	6.03	4.41	5.15	6.37
	N014	Man- α -1,6-(Man- α -1,3-)Man- α -1,6-(Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	4.66	2.49	3.51	3.52
	N015	Man- α -1,6-(Man- α -1,3-)Man- α -1,6-(Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	3.71	2.36	1.51	3.66
	N020	GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	22.15	6.89	13.30	15.01
	N021	Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	9.33	10.96	9.11	13.05
	N022	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	4.17	4.10	3.63	3.81
	N023	Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	5.04	4.53	4.83	7.08
	N024	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	3.88	5.52	6.97	7.94
	N025	Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	1.00	3.51	0.76	5.29
	N022G	Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	73.38	29.66	25.48	38.63
	N023G	Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	1.98	2.00	16.53	5.54
	N025G	Neu5Gc- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	4.29	2.70	10.49	4.38
	N026	Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	8.16	2.67	18.75	2.64
	N030	Man- α -1,6-(GlcNAc- β -1,2-Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	18.37	2.46	9.59	7.12
	N040	GlcNAc- β -1,2-Man- α -1,6-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	85.92	26.26	48.30	20.49
	N041	Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	4.61	4.13	5.80	11.13
	N042	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	29.95	13.57	23.21	14.49
	N043	Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	4.75	1.47	4.39	2.72
	N044	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	5.10	0.99	4.99	2.77
	N045	Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	4.34	1.17	6.87	3.18
	N050	GlcNAc- β -1,2-Man- α -1,6-(Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	1.77	7.45	3.82	5.26
	N051	Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-(Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	17.39	7.28	14.06	6.85
	N052	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-(Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	2.07	1.94	1.52	4.95
	N053	Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-(Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	2.51	2.20	1.16	3.72
	N054	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-(Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	2.41	1.54	0.94	3.77
	N055	Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-(Man- α -1,3-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	2.23	1.62	2.29	4.52
	N210	GlcNAc- β -1,2-Man- α -1,6-(GlcNAc(3Ac)- β -1,2-Man- α -1,3-Man- β -1,4-GlcNAc- β -1,4-GlcNAc-Sp5	2.45	5.32	6.61	5.74
Glycolipid Glycans	L1001	Neu5Ac- α -2,3-Gal- β -1,4-Glc-Sp5	12.49	7.28	9.81	14.83
	L1002	Neu5Gc- α -2,3-Gal- β -1,4-Glc-Sp5	12.15	21.33	23.32	12.25
	L1003	Kdn- α -2,3-Gal- β -1,4-Glc-Sp5	1.34	16.69	26.89	35.06
	L1011	Neu5Ac- α -2,3-(GalNAc-b-1,4-)Gal-b-1,4-Glc-Sp5	60.00	25.89	16.77	20.95
	L1012	Neu5Gc- α -2,3-(GalNAc-b-1,4-)Gal-b-1,4-Glc-Sp5	45.08	20.36	12.65	12.47
	L1013	Kdn- α -2,3-(GalNAc-b-1,4-)Gal-b-1,4-Glc-Sp5	18.43	13.13	27.45	25.24
	L1021	Neu5Ac- α -2,3-(Gal-b-1,3-GalNAc-b-1,4-)Gal-b-1,4-Glc-Sp5	53.25	34.76	17.68	36.85
	L1022	Neu5Gc- α -2,3-(Gal-b-1,3-GalNAc-b-1,4-)Gal-b-1,4-Glc-Sp5	51.45	26.41	17.39	32.99
	L1023	Kdn- α -2,3-(Gal-b-1,3-GalNAc-b-1,4-)Gal-b-1,4-Glc-Sp5	47.34	23.52	27.22	30.69
	L1201	Neu5Ac- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-Glc-Sp5	1.18	2.56	4.39	5.49
	L1202	Neu5Gc- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-Glc-Sp5	16.26	6.84	4.29	8.17
	L1203	Kdn- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-Glc-Sp5	1.48	4.86	13.73	19.47
	L1204	Neu5Ac- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-Glc-Sp5	0.24	3.56	2.56	5.30
	L1205	Neu5Gc- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-Glc-Sp5	0.39	2.81	2.51	4.95
	L1206	Kdn- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-Glc-Sp5	0.98	7.62	12.97	20.29
	L1207	Neu5Ac- α -2,8-Kdn- α -2,3-Gal- β -1,4-Glc-Sp5	3.66	4.99	6.38	8.86
	L1209	Kdn- α -2,8-Kdn- α -2,3-Gal- β -1,4-Glc-Sp5	0.93	4.70	23.72	33.71
	L1211	Neu5Ac- α -2,8-Neu5Ac- α -2,3-(GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	1.42	11.20	5.02	5.43
	L1212	Neu5Gc- α -2,8-Neu5Ac- α -2,3-(GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	1.83	8.88	7.18	5.25
	L1213	Kdn- α -2,8-Neu5Ac- α -2,3-(GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	5.96	12.08	23.13	23.60
	L1214	Neu5Ac- α -2,8-Neu5Gc- α -2,3-(GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	3.76	8.54	6.06	8.39
	L1215	Neu5Gc- α -2,8-Neu5Gc- α -2,3-(GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	6.52	10.44	6.26	6.49
	L1216	Kdn- α -2,8-Neu5Gc- α -2,3-(GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	4.37	13.33	23.55	22.10
	L1218	Neu5Gc- α -2,8-Kdn- α -2,3-(GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	29.47	17.70	7.10	10.50
	L1221	Neu5Ac- α -2,8-Neu5Ac- α -2,3-(Gal-b-1,3-GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	25.61	13.83	7.98	14.24
	L1222	Neu5Gc- α -2,8-Neu5Ac- α -2,3-(Gal-b-1,3-GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	23.23	12.68	7.04	11.71
	L1225	Neu5Gc- α -2,8-Neu5Gc- α -2,3-(Gal-b-1,3-GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	31.76	19.31	17.72	27.84
	L1226	Kdn- α -2,8-Neu5Gc- α -2,3-(Gal-b-1,3-GalNAc-b-1,4-)Gal- β -1,4-Glc-Sp5	40.58	16.38	27.32	24.34
	L2000	GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	11.67	10.99	7.36	21.80
	L2100	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	75.79	42.17	31.77	46.83
	L2101	Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	111.05	23.05	40.52	29.22
	L2102	Gal- α -1,4-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	17.75	2.73	2.13	9.28
	L2103	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	6.56	5.37	19.02	9.78
	L2104	Gal- α -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	2.98	6.20	23.39	33.22
	L2111	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	3.61	3.34	1.46	10.76
	L2112	Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	2.18	2.74	1.35	10.25
	L2113	Kdn- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	4.10	2.33	10.33	13.87
	L2121	Neu5Ac- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	53.01	8.24	18.97	24.19
	L2122	Neu5Gc- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	1.43	5.57	2.62	5.28
	L2123	Neu5Ac- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	2.42	1.75	13.94	5.98
	L2124	Neu5Gc- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	0.19	3.93	16.91	21.83
	L2131	Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	2.33	5.36	9.28	9.95
	L2132	Neu5Gc- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	4.70	0.72	25.89	20.23
	L2133	Kdn- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	12.89	7.83	13.71	20.18
	L2200	GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	12.26	15.04	7.88	12.16
	L2201	GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	75.96	32.94	27.36	47.53
	L2300	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	91.87	26.08	37.69	27.91
	L2301	Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	8.18	1.70	0.74	7.87
	L2302	Gal- α -1,4-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	7.75	1.39	14.86	6.46
	L2303	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	24.49	3.53	3.02	5.29
	L2304	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	1.57	1.48	3.57	3.42
	L2311	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	1.05	1.17	2.45	1.68
	L2312	Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	4.49	1.66	27.61	10.08
	L2313	Kdn- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	23.98	19.54	16.28	20.36
	L2900	Gal- β -1,3-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	10.66	2.16	3.23	8.00
	L2911	Neu5Ac- α -2,3-Gal- β -1,3-GlcNAc- β -1,3-Gal- β -1,4-Glc-Sp5	3.12	4.29	13>1	

Tandem Epitopes

TE001	Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc-Man-1,3-(Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	1.78	1.63	2.14	4.61
TE002	Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	2.05	2.61	4.32	8.41
TE003	Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.68	0.63	5.68	2.75
TE004	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.24	0.03	0.38	5.07
TE005	Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	2.00	1.34	15.92	3.95
TE006	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	-0.16	1.95	2.64	5.88
TE007	Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	74.25	33.59	29.48	40.59
TE008	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	60.21	8.14	16.19	34.81
TE009	Neu5Ac- α -2,8-Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,8-Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.56	1.21	1.42	4.38
TE010	Neu5Gc- α -2,8-Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,8-Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.95	0.82	1.94	2.90
TE011	Neu5Ac- α -2,8-Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,8-Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.28	2.72	1.50	3.86
TE012	Neu5Gc- α -2,8-Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,8-Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	-0.13	0.63	0.99	3.47
TE013	Neu5Ac- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.18	1.84	0.67	7.17
TE014	Neu5Gc- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,8-Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	-0.15	2.41	1.15	4.62
TE015	Neu5Ac- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	6.97	1.16	1.49	4.85
TE016	Neu5Gc- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,8-Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	3.61	1.74	1.05	5.21
TE017	Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.26	2.17	1.82	5.16
TE018	Neu5Gc- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	3.92	1.28	17.71	6.17
TE019	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	3.26	0.45	2.27	4.10
TE020	Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	2.02	0.42	13.48	5.06
TE021	Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,3-(Neu5Ac- α -2,6-)Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,3-)Neu5Ac- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.02	1.21	2.07	2.78
TE022	Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,3-(Neu5Gc- α -2,6-)Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,3-)Neu5Gc- α -2,6-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	2.42	0.51	5.23	4.45
TE023	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-(Neu5Ac- α -2,6-)Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-)Neu5Ac- α -2,6-)Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.45	0.56	0.91	5.84
TE024	GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	7.30	4.87	4.82	17.29
TE025	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	5.25	7.11	2.26	7.73
TE026	GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	8.29	8.34	6.66	14.01
TE027	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	17.15	15.28	9.85	16.16
TE028	GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	9.03	7.09	6.91	13.76
TE029	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	9.88	11.44	5.01	11.93
TE030	GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	10.05	10.71	6.46	16.10
TE031	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	8.06	10.15	2.40	7.23
TE032	GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	8.16	5.99	6.20	8.09
TE033	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	6.88	2.92	5.50	4.36
TE034	Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Neu5Ac- α -2,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	2.12	0.84	4.10	3.00
TE035	GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	6.06	5.43	6.46	10.60
TE036	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	22.06	5.25	5.01	8.18
TE037	GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	9.42	6.35	7.37	17.23
TE038	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	12.76	10.15	6.44	9.91
TE039	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	0.72	0.46	5.28	9.30
TE040	GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	8.40	7.91	7.18	9.80
TE041	Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	38.07	4.03	7.91	4.20
TE042	Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	1.62	1.25	6.41	4.92
TE043	Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	74.75	51.00	40.40	54.18
TE044	Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	55.86	41.74	32.19	40.73
TE045	Gal- α -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- α -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	80.82	12.55	21.66	47.95
TE046	Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,3-(Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β -1,4-GlcNAc- β -Asn	60.60	48.59	39.23	72.13
TE047	Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-(Fuc- α -1,3-)GlcNAc- β -1,2-Man- α -1,3-(Gal- α -1,3-Gal- β -1,4-GlcNAc- β -1,3-Gal- β -1,4-GlcNAc- β -1,2-Man- α -1,6-)Man- β -1,4-GlcNAc- β				

Human Milk Oligosaccharides	H0100	GlcNAc-β-1,3-(GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	57.06	40.20	36.89	52.91
	H0101	Gal-β-1,4-GlcNAc-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	38.38	27.21	25.45	41.26
	H0103	Neu5Gc-α-2,6-Gal-β-1,4-GlcNAc-β-1,3-(Neu5Gc-α-2,6-Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	20.30	10.34	23.75	16.17
	H0105	Fuc-α-1,2-Gal-β-1,4-GlcNAc-β-1,3-(Fuc-α-1,2-Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	25.31	16.41	18.73	27.08
	H0106	Fuc-α-1,2-Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-(Fuc-α-1,2-Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	27.38	18.60	18.60	26.93
	H0200	Gal-β-1,4-GlcNAc-β-1,3-(GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	34.75	17.09	18.86	34.80
	H0201	Neu5Ac-α-2,6-Gal-β-1,4-GlcNAc-β-1,3-(GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	30.00	16.26	19.27	30.76
	H0202	Neu5Gc-α-2,6-Gal-β-1,4-GlcNAc-β-1,3-(GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	42.66	19.43	31.50	32.89
	H0203	Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-(GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	36.34	20.27	25.14	42.47
	H0204	Fuc-α-1,2-Gal-β-1,4-GlcNAc-β-1,3-(GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	48.38	27.50	31.67	42.37
	H0205	Neu5Ac-α-2,6-Gal-β-1,4-GlcNAc-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	35.25	20.33	25.41	31.99
	H0207	Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	47.90	24.43	32.20	50.31
	H0208	Fuc-α-1,2-Gal-β-1,4-GlcNAc-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	41.17	26.75	28.04	45.28
	H0209	Fuc-α-1,2-Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-(GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	38.31	23.86	20.43	39.81
	H0210	Fuc-α-1,2-Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-(Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	28.46	20.23	18.77	32.93
	H0300	GlcNAc-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	44.80	25.85	29.21	49.86
	H0301	GlcNAc-β-1,3-(Neu5Ac-α-2,6-Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	36.38	22.49	24.69	47.65
	H0303	GlcNAc-β-1,3-(Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	48.04	27.64	32.87	52.98
	H0304	Fuc-α-1,2-GlcNAc-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	39.68	23.05	25.12	45.66
	H0305	Gal-β-1,4-GlcNAc-β-1,3-(Neu5Ac-α-2,6-Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	24.88	15.40	17.91	25.70
	H0306	Gal-β-1,4-GlcNAc-β-1,3-(Neu5Gc-α-2,6-Gal-β-1,4-GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	24.32	13.44	17.02	25.77
	H0307	Gal-β-1,4-GlcNAc-β-1,3-(Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	41.39	18.90	18.03	37.71
	H0400	Gal-β-1,4-Glc-Sp	39.92	30.01	27.38	37.80
	H0402	GalNAc-β-1,3-Gal-β-1,4-Glc-Sp	41.96	26.20	24.79	43.58
	H0403	Neu5Ac-α-2,3-Gal-β-1,4-Glc-Sp	45.85	21.25	21.82	32.58
	H0404	Neu5Gc-α-2,3-Gal-β-1,4-Glc-Sp	42.50	23.90	37.86	31.19
	H0405	Neu5Ac-α-2,6-Gal-β-1,4-Glc-Sp	32.52	18.30	22.60	27.01
	H0406	Neu5Gc-α-2,6-Gal-β-1,4-Glc-Sp	39.93	24.22	30.93	28.65
	H0407	Gal-α-1,3-Gal-β-1,4-Glc-Sp	79.58	40.23	44.30	52.06
	H0408	Neu5Ac-α-2,8-Neu5Ac-α-2,3-Gal-β-1,4-Glc-Sp	45.63	20.32	22.53	32.32
	H0409	Neu5Ac-α-2,8-Neu5Ac-α-2,6-Gal-β-1,4-Glc-Sp	29.34	16.53	12.65	23.41
	H0410	Neu5Ac-α-2,3-Gal-α-1,3-Gal-β-1,4-Glc-Sp	32.93	24.07	21.59	35.14
	H0411	Neu5Ac-α-2,6-Gal-α-1,3-Gal-β-1,4-Glc-Sp	38.11	21.09	28.42	30.58
	H0500	Gal-α-1,4-Gal-β-1,4-Glc-Sp5	123.82	46.81	47.26	58.67
	H0503	GalNAc-β-1,3-Gal-α-1,4-Gal-β-1,4-Glc-Sp5	75.16	40.11	39.68	43.43
	H0504	Gal-β-1,3-GalNAc-β-1,3-Gal-α-1,4-Gal-β-1,4-Glc-Sp5	75.13	40.64	40.21	46.46
	H0505	Fuc-α-1,2-Gal-β-1,3-GalNAc-β-1,3-Gal-α-1,4-Gal-β-1,4-Glc-Sp5	30.66	52.24	31.01	46.07
	H0600	Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	31.01	26.88	25.62	42.12
	H0601	Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	30.03	16.39	25.33	36.52
	H0602	Fuc-α-1,2-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	26.89	22.50	27.81	36.31
	H0603	GlcNAc-β-1,3-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	37.33	21.17	29.97	44.00
	H0604	Neu5Ac-α-2,3-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	41.72	11.79	17.09	31.83
	H0606	Gal-β-1,4-GlcNAc-β-1,3-(Neu5Ac-α-2,6-)Gal-β-1,4-Glc-Sp5	26.75	14.62	16.73	28.57
	H0608	Fuc-α-1,2-Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	25.01	13.32	12.06	16.29
	H0609	Neu5Ac-α-2,3-Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	36.66	23.59	22.84	32.88
	H0610	GlcNAc-β-1,3-Gal-β-1,4-(Fuc-α-1,3-)GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	50.21	17.95	22.65	27.81
	H0700	Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	63.16	48.41	42.29	64.51
	H0701	GlcNAc-β-1,3-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	39.17	23.97	26.53	43.81
	H0800	Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-GlcNAc-β-1,3-Gal-β-1,4-Glc-Sp5	41.89	30.52	22.16	39.25
	H0900	Gal-β-1,3-GlcNAc-β-1,3-(GlcNAc-β-1,6-)Gal-β-1,4-Glc-Sp5	54.61	36.22	25.68	44.30

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CURRICULUM VITAE

Lebenslauf aus datenschutzrechtlichen Gründen nicht enthalten.

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Hamburg, 05.03.2025

Isabelle Ariane Bley