Thesis for the degree of Philosophiae Doctor

N-glycolyl GM3 as potential target for cancer immunotherapy

Paula Bousquet

Department of Chemistry
Faculty of Mathematics and Natural Science
University of Oslo
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To Liv and Léon

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Abstract

Gangliosides are sialic acid-containing glycosphingolipids expressed on all vertebrate cells. They are primarily positioned in the plasma membrane, with the ceramide part anchored in the membrane and the glycan part exposed on the surface of the cell. These lipids have highly diverse structures, in particular with respect to their carbohydrate chains, with *N*-acetylneuraminic acid (NeuAc) and *N*-glycolylneuraminic acid (NeuGc) being the two most common sialic acid residues in mammalian cells. Generally, human healthy tissue is considered nearly deficient in NeuGc, but since this molecule is present in tumors and in human fetal tissues, it was earlier classified as an onco-fetal antigen. Gangliosides perform important functions through carbohydrate-specific interactions with proteins, for example as receptors in cell-cell recognition, which can be exploited by viruses and other pathogens, and also by regulating signaling proteins through lateral interaction in the membrane. Through both mechanisms, tumor-associated gangliosides may affect malignant progression, which makes them attractive targets for cancer immunotherapies.

In this thesis, the ganglioside NeuGc GM3 is the focus of attention. Different approaches have been used in order to reveal both its importance in cancer progression but also its potential for cancer immunotherapy. In particular, we investigated the effect of hypoxia on tumor cells, since hypoxic conditions are known to induce a change in carbohydrate determinants correlating with a more aggressive and therapy-resistant phenotype. We studied alterations in protein expression as a response to hypoxia, using stable isotope labeling with amino acids in cell culture (SILAC) in combination with LC-MS/MS. The results obtained from the quantitative proteome analysis uncovered proteins important in hypoxia-induced cancer progression, representing potentially interesting targets in future therapies. The same method (SILAC) was used in order to study the cell death killing mechanism employed by a promising monoclonal antibody (14F7) that specifically targets NeuGc GM3. The identified proteins advance our understanding of this novel mechanism of cell killing. In addition, we studied the molecular interaction between the antibody 14F7 and its antigen NeuGc GM3 or its anti-idiotypic antibody using a

combination of mass spectrometry-based methods and surface plasmon resonance (SPR).

Table of contents

Appendix

List of manuscripts I-IV

I. **Paula A. Bousquet**, Joe A. Sandvik, Magnus Ø. Arntzen, Nina F. Jeppesen Edin, Stine Christoffersen, Ute Krengel, Erik O. Pettersen, Bernd Thiede. Hypoxia strongly affects glycolysis and expression of mitochondrial proteins, as shown by quantitative proteomics of HeLa cells. *In Manuscript.*

II. **Paula A. Bousquet**, Joe A. Sandvik, Nina F. Jeppesen Edin, Ute Krengel. How does hypoxia trigger the expression of NeuGc GM3? *In Manuscript.*

III. **Paula A. Bousquet**, Joe A. Sandvik, Magnus Ø. Arntzen, Bernd Thiede. Ernesto Moreno, Ute Krengel. SILAC analysis of HeLa cells treated with the anti-tumor monoclonal antibody, 14F7. *In Manuscript.*

IV. **Paula A. Bousquet**, Nebiyu Abshiru, Matthiew Tessier, Rune F. Johansen, André van Eerde, Karin Lindkvist, Magnar Bjørås, Bernd Thiede, Robert J. Woods, Ernesto Moreno, Ute Krengel. MS and SPR analysis of the molecular interaction of a unique anti-tumor antibody with its antigen *N*-glycolyl GM3 and an anti-idiotypic antibody. *In Manuscript.*

Abbreviations

Ab2	anti-idiotypic antibody
Ab3	anti-anti-idiotypic antibody
ADCC	antibody-dependent cellular cytotoxicity
CDC	complement-dependent cytotoxicity
CDR	complementarity determining region
CH	constant heavy chain domain
CL	constant light chain domain
CMAH	cytidine monophosphate-N-acetylneuraminic acid hydroxylase-like protein
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ESI	electro-spray ionization
Fab	fragment, antigen binding
Fc	fragment crystallizable
FITC	fluorescein isothiocyanate
Fv	fragment, variable
GSL	glycosphingolipid
HD	Hanganutziu-Deicher
LC	liquid chromatography
mAb	monoclonal antibody
MALDI	matrix-assisted laser desorption/ionization
MSD	meso scale discovery assay
NeuAc	*N*-acetyl neuraminic acid
NeuGc	*N*-glycolyl neuraminic acid
PDB	protein data bank
PI	propidium iodide
SILAC	stable isotope labeling by amino acids in cell culture
SPR	surface plasmon resonance

TOF	time-of-flight
VEGFR	vascular endothelial growth factor receptor
VH	variable heavy chain domain
VL	variable light chain domain

1. Introduction

1.1 Gangliosides

Few lipid species included in biological membranes have received as much attention as glycosphingolipids (GSLs), and especially gangliosides, sialic-acid containing GSLs. They were discovered by Ernst Klenk in the 1940's and later named by Svennerholm in 1956 according to the number of sialic acid and chromatographic mobility (Svennerholm 1956). These molecules are complex, diverse and consist of a ceramide anchor linked to a hydrophilic sugar part, characterized by the presence of one or more sialic acid residues. As an example, the GM3 ganglioside, which is abundant in almost all healthy tissues, is shown in **Figure 1**. The large structural variability is related to developmental stage and cell type, and more than hundreds of gangliosides are known today (Schwarz & Futerman 1996; Yu 2007). This number increases when ceramide lipid variations are taken into account. Accumulating evidence indicates that many cellular events, including differentiation, growth, signaling, interactions and immune reactions are highly influenced by gangliosides, which may lead to malignancies. Positioned in the plasma membrane, gangliosides influence and interact with proteins both laterally in the membrane but also as surface receptors, being perfectly accessible for antibodies or other ganglioside-binding molecules.

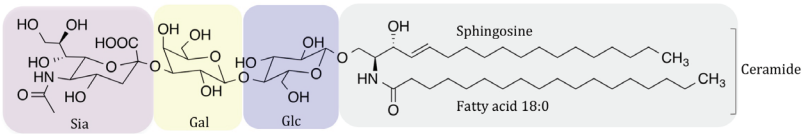

Figure 1. Schematic structure of NeuAc GM3, a common ganglioside in most vertebrate tissues. Carbohydrate symbols follow the nomenclature of the Consortium for Functional Glycomics (www.functionalglycomics.org/static/consortium/Nomenclature.shtml).

1.1.1 Biosynthesis of gangliosides

The biosynthesis of gangliosides is primarily mediated by specific glycosyltransferases which transfer sugar residues to the lipid part. The glycosyltransferases are specific to the sugar they transfer, and are grouped into families according to their specificity. Interestingly, all glycosyltransferase promoters lack the TATA sequence, hence do not have any core promoter element characteristic for house-keeping genes. Although some indications relate their transcription to complex developmental and tissue-specific regulation, very little is known about how glycosyltransferases are regulated (Zeng &Yu 2008). Glycosphingolipids are mainly synthesized in the endoplasmic reticulum (ER), modified in the Golgi apparatus (Kolter et al. 2002; Merrill 2002) by sequential addition of carbohydrate moieties to the lipid molecule, followed by vesicle sorting to fuse with the plasma membrane (Maccioni 2007). Gangliosides are assumed to recycle to the plasma membrane from early endosomes and a degradation process is thought to take place at the late endosomal level (Tettamanti 2004). The biosynthetic pathways of gangliosides are shown in **Figure 2.**

The synthesis of gangliosides starts with the synthesis of ceramide, the common precursor for all glycosphingolipids, in the ER compartment. Aided by a transfer protein, CERT, ceramide is then transferred to the Golgi apparatus, and thereafter converted to glucosylceramide (GlcCer) (Hanada et al. 2003). By addition of a galactose moiety to GlcCer, lactosylceramide (LacCer), the common precursor for almost all gangliosides (except for GM4), is formed. Addition of one sialic acid to LacCer converts this precursor molecule to GM3, a reaction catalyzed by sialyltransferase I (ST-I) or GM3 synthase. In the same manner, GD3 and GT3 can be generated by further addition of sialic acid residues, catalyzed by ST-II or GD3 synthase and ST-III or GT3 synthase, respectively. The number of sialic acid residues linked to the inner galactose residue (0, 1, 2, or 3) classify the ganglioside into asialo, a-, b-, or c-series (**Figure 2**). However, only trace amounts of gangliosides from the asialo- and c-series are found in adult human tissue (Sandhoff & Kolter 2003).

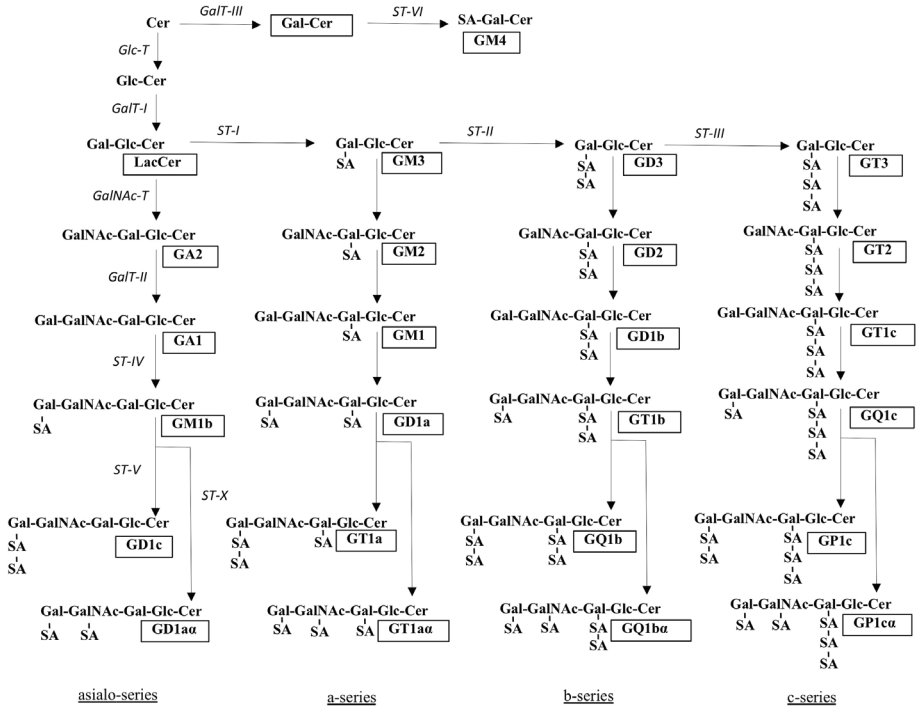

Figure 2. The biosynthetic pathways of gangliosides. Cer, ceramide; SA, sialic acid. Ganglioside nomenclature is shown in boxes. Adapted from (Yu et al. 2011).

1.1.2 Ganglioside functions

While gangliosides are primarily present in the plasma membrane, they have recently been recognized as functionally important constituents of nuclear membranes (Katoh et al. 1993; Saito & Sugiyama 2002; Ledeen & Wu 2004). Knock-out studies in mice have been essential for revealing the functions of gangliosides, especially in embryonic development and differentiation. Yamashita *et al* observed that mouse embryo carrying a knock-out of the glycosylceramide synthase gene did not survive more than 7.5 days (Yamashita et al. 1999). Other examples are studies of mice with knock-down of GM3 synthase or GM2/GD2 synthase. They exhibit increased insulin sensitivity and decreased ability to repair nervous tissues, respectively (Takamiya et al. 1996;

Yamashita et al. 2003). Glycosphingolipids (including gangliosides) are key molecules in recognition and signaling. Because of the strictly packed backbone, gangliosides are able to associate with other types of sphingolipids and cholesterol, therefore forming lipid rafts, to which many proteins can associate (Simons & Ikonen 1997; Simons & Toomre 2000). Organization of gangliosides in membranes will be further discussed in Section *1.1.3.* Since gangliosides have the ability to interact with both sugars and proteins, a large variety of events can be triggered or inhibited by these molecules. Cell growth, migration, differentiation, adhesion and apoptosis are some examples (Bremer et al. 1986; Hakomori & Igarashi 1995). These interactions (mainly) involve the carbohydrate part of the ganglioside, most importantly the sialic acid residue, making gangliosides attractive targets for many pathogens using sialic acids as a way to enter the cell.
The most common ligand for viruses is Neu5Ac, however, Neu5Gc and 9-O-acetylated sialic acids are also specific ligands (Lehmann et al. 2006; Schauer et al. 2011). Pathogens binding to gangliosides can be different types of viruses, with the oldest example being the influenza virus (Suzuki et al. 1986). Other examples are the ganglioside recognition by simian virus 40 (SV40) (Neu et al. 2008) and rotavirus (Delorme et al. 2001). A number of toxins can also directly interact with gangliosides, including the cholera toxin, Shiga toxin and clostridial neurotoxins (Lencer et al. 1999; Stenmark et al. 2008; Sandvig et al. 2014). Gangliosides may also suppress natural killer cell cytotoxicity, through interaction between Siglec-7 (sialic acid-binding immunoglobulin-like lectin 7), which preferentially binds to gangliosides of the b-series, and cells engineered to overexpress GD3 (Nicoll et al. 2003). Melanoma cells have high expression levels of GD3 ganglioside, thus the efficiency of NK cell cytotoxicity against tumor cells may be suppressed.

As gangliosides are localized in the plasma membrane, structural characterization of anchored gangliosides has been difficult to achieve. De Marco & Woods employed an atomic-resolution conformational analysis to study the presentation and conformation of the GM3 head-group and the dynamics associated with membrane anchoring in protein recognition (DeMarco and Woods 2009). Changes in the internal structural properties were not observed for the membrane-bound GM3 compared to the GM3 head-group in solution, however, accessibility of the carbohydrate residues was altered when anchored

(**Figure 3**). Two known GM3-binding proteins (sialo-adhesin and wheat germ agglutinin) were studied in order to determine the importance of carbohydrate accessibility and protein recognition. Based on crystal structures of the head-group and proteins, the interactions were determined to mainly involve the Neu5Ac residue, since the Glc residue had high *B*-factors and was not in contact with the protein (May et al. 1998). The less exposed residues have been suggested to indirectly affect recognition, by ceramide-Glc and Glc-Gal rotations, altering the Neu5Ac presentation. The properties of the hydrophobic ceramide together with the polar Glc residue may also regulate the insertion depth, giving an additional effect on antigen presentation (DeMarco & Woods 2009).

1.1.3 Organization of gangliosides in membranes

There is a great interest in understanding how membrane proteins interact with surrounding lipids in a biological membrane, since a growing number of important recognition events involve interactions between proteins and glycolipids. In the past decade, many studies have focused on the lateral determination of the membrane and it is well established that GSLs are able to create order in biological membranes (Sonnino et al. 2007). Glycerophospholipids, the main components of biological membranes, are highly unsaturated. However, gangliosides are characterized by a rigid and planar structure, composed of saturated acyl chains, which can be more tightly packed. Membrane sphingolipids can segregate and form “clusters” together with cholesterol, also referred to as “lipid rafts”, creating more ordered membrane regions, to which proteins associate (Simons & Ikonen 1997; Simons & Toomre 2000; Simons & Sampaio 2011). **Figure 4** shows the suggested interaction between GSLs and cholesterol, where a tilted conformation is induced (Nyholm et al. 1990; Yahi et al. 2010).

The clustering and density of GSLs can also affect antigen specificity. For example, an antibody established by immunizing mice with syngeneic B16 melanoma, named M2590, reacted only with melanoma and not with healthy tissues (Taniguchi & Wakabayashi 1984). Remarkably, this epitope was later identified as GM3, an abundant ganglioside in membranes of normal cells (Hirabayashi et al. 1985). Further studies showed that a ganglioside density above threshold value was required for reactivity, suggesting that this antibody recognized a cluster of GM3 (Nores et al. 1987). These results indicate that

ganglioside antigens can be differently organized in tumor cells compared to normal cells and that some ganglioside antigens are fully antigenic when organized in clusters, but fail to bind antibodies when the density is under the threshold value (Hakomori et al. 1981; Nores et al. 1987).

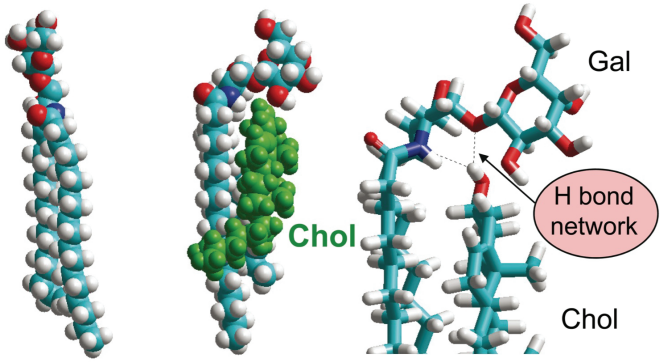

Figure 4. Interaction between glycosphingolipid and cholesterol, important constituents of lipid rafts. Adapted from (Yahi et al. 2010) in an extension of earlier work by (Nyholm et al. 1990).

1.1.4 Gangliosides and membrane protein signaling

It has been suggested that activation of membrane proteins can be influenced by lipid cluster association. As the sphingolipids have unique properties, bearing a carbonyl oxygen, a hydroxyl group and an amide nitrogen, thus able to act as both donors and acceptors for hydrogen bond formation (Pascher 1976), this results in a variety of interaction possibilities. As previously mentioned, more stable interactions can be formed due to a more ordered lipid surrounding. It has been suggested that tightly packed gangliosides, associated to the protein, may cause conformational changes of the protein, influencing the activity. The carbohydrate moiety of the ganglioside may interact directly with amino acids of the extracellular part of the protein or interact with sugar residues of a glycosylated protein.

Most growth factor receptors are known to be regulated by gangliosides (Miljan et al. 2002). Here, I would like to discuss two examples of membrane proteins important in cancer research, EGFR and VEGFR (**Table 1**). A number of cancers have over-expressed or over-activated EGFRs in their membrane (Kuan et al. 2001; Lynch et al. 2004; Walker et al. 2009). Another important factor for tumor progression is the growth of new blood vessels. Tumor cells produce and release VEGF, that will stimulate VEGFR, resulting in proliferation and migration of vascular endothelial cells (Ferrara & Kerbel 2005). The EGFR undergoes dimerization by ligand binding, resulting in an autophosphorylation of tyrosine residues at the C-terminus of the protein (Downward et al. 1984). This initiates downstream signaling, leading to adhesion, cell migration and proliferation (Oda et al. 2005).

Table 1. List of gangliosides affecting the growth factor receptors EGFR and VEGFR.

Ganglioside	Growth factor receptor	References
GM3	EGFR	(Mirkin et al. 2002; Yoon et al. 2006; Kawashima et al. 2009; Coskun et al. 2011)
GM1	EGFR	(Mirkin et al. 2002; Hofman et al. 2009)
GM2	EGFR	(Miljan et al. 2002; Zurita et al. 2004)
GM4	EGFR	(Miljan et al. 2002)
GD3	EGFR	(Zurita et al. 2004; Wang & Yu 2013)
GD1a	EGFR	(Mirkin et al. 2002; Liu et al. 2004)
GT1b	EGFR	(Mirkin et al. 2002)
GM3	VEGFR	(Mukherjee et al. 2008; Chung et al. 2009)
GD1a	VEGFR	(Liu et al. 2006; Mukherjee et al. 2008)
GD3	VEGFR	(Zeng et al. 2000)

The ganglioside GM3 is a well known regultator of the insulin receptor (IR), but also has an inhibitory effect on the epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor (VEGFR), while the ganglioside GD1a strongly induce VEGFR-2 activation (Bremer et al. 1986; Nojiri et al. 1991; Mukherjee et al. 2008). Moreover, the proangiogenic effects of GD1a could efficiently be reduced by GM3

(Mukherjee et al. 2008). The latter has been suggested to inhibit VEGFR-2 activation by blocking both binding of VEGF and dimerization through a direct interaction with the extracellular domain of VEGFR (Chung et al. 2009). The molecular interaction between the EGFR and GM3 is not fully elucidated, although it has been studied extensively. It has been shown that the inhibition of EGFR activation by GM3 involves binding between GlcNAc-terminated *N*-glycans on the EGFR and the GM3 oligosaccharide, suggesting a carbohydrate-to-carbohydrate interaction (CCI) (Yoon et al. 2006; Yoon et al. 2006; Kawashima et al. 2009). Additionally, evidence is accumulating that ganglioside organization in the membrane may affect localization and activation of growth factor receptors. As cellular biological membranes are complex, and the dynamics are difficult to study, Coscun *et al*. reconstituted EGFR into proteoliposomes with defined lipid composition, forming variants with either uniform liquid-disordered (ld) membrane phases or a combination of disordered and ordered (ld/lo) domains, to which gangliosides could be added (Coskun et al. 2011). Interestingly, they show that GM3 had a strong inhibitory effect of EGFR activation, without interfering with ligand-binding, but in ld/lo proteoliposomes only (Coskun et al. 2011).

1.1.5 Gangliosides and cancer

Gangliosides play important roles in many normal physiological processes, such as cell growth, differentiation and embryogenesis (Yamashita et al. 1999), as well as in pathological events such as tumor malignancy and progression (Hakomori 1996). Formation of tumors results from autonomous uncontrolled proliferation of neoplastic cells, while metastasis occurs when tumor cells are released from the primary tumor and continue to proliferate at a distant site. Multiple factors affect these processes, in which gangliosides may serve both as inhibitory and stimulating molecules. For instance, It has been shown that highly metastatic melanoma cells have high expression levels of GD3 in contrast to poorly metastatic cells or the normal counterpart, melanocytes, expressing very low levels of GD3 (Carubia et al. 1984; Ravindranath et al. 1991; Merzak et al. 1994), suggesting a role in transforming melanocytes to melanomas and promoting metastasis. **Table 2** lists some of the gangliosides found in human tumors.

Table 2. Gangliosides expressed in human cancer cells

Ganglioside	Structure	Cancer type	References
NeuAc GM3	αNeu5Ac(2-3)βDGal(1-4)βDGlc(1-1)Cer	Melanoma, NSCLC, breast carcinoma, renal carcinoma	(Pukel et al. 1982; Ravindranath, et al. 1991; Marquina et al. 1996; Morton & Barth 1996; Kudo et al. 2003; van Cruijsen et al. 2009)
NeuGc GM3	αNeu5Gc(2-3)βDGal(1-4)βDGlc(1-1)Cer	Colon cancer, retinoblastoma, melanoma, breast carcinoma, neuroectodermal cancer, Wilms tumor	(Higashi et al. 1985; Marquina et al. 1996; van Cruijsen et al. 2009; Scursoni et al. 2009 ; Scursoni et al. 2011)
GM2	βDGalNAc(1-4)[αNeu5Ac(2-3)]βDGal(1-4)βDGlc(1-1)Cer	Melanoma, neuroblastoma, SCLC, t-ALL, breast carcinoma, renal carcinoma	(Brezicka et al. 1989; Marquina et al. 1996; Morton & Barth 1996; Okada et al. 1996; Kudo et al. 2003; Hettmer et al. 2005; Chung et al. 2009)
GM1	βDGal(1-3)βDGalNAc[αNeu5Ac(2-3)]βDGal(1-4)βDGlc(1-1)Cer	SCLC, renal carcinoma	(Brezicka et al. 1989; Marquina et al. 1996)
GD3	αNeu5Ac(2-8)αNeu5Ac(2-3)βDGal(1-4)βDGlc(1-1)Cer	Melanoma, neuroblastoma, glioma, SCLC, t-ALL, breast carcinoma	(Pukel et al. 1982; Cheung et al. 1985; Mujoo et al. 1987; Ravindranath et al. 1991; Hakomori & Igarashi 1995; Morton & Barth 1996; Okada et al. 1996; Hettmer et al. 2005)
GD2	βDGalNAc(1-4)[αNeu5Ac(2-8)αNeu5Ac(2-3)]βDGal(1-4)βDGlc(1-1)Cer	Melanoma, neuroblastoma, glioma, SCLC, t-ALL	(Pukel et al. 1982; Cheung et al. 1985; Mujoo et al. 1987; Brezicka et al. 1989; Ravindranath et al. 1991; Morton & Barth 1996; Okada et al. 1996; Hettmer et al. 2005)

Gangliosides are also actively shed from the tumor to the microenvironment. These gangliosides can interact with proteins or be incorporated into the membrane of other cells, leading to signaling events (Li & Ladisch 1991; Chang et al. 1997; Rusnati et al. 1999). Tumor cells shed gangliosides in greater quantities than normal cells. Notably, exogenous GD3 added to the culture medium of glioma cells stimulate the release of VEGF (vascular epidermal growth factor) (Koochekpour et al. 1996). Other possibilities to mediate cell-cell interaction and signaling functions are ganglioside (sialic acid) recognition by Siglecs (Crocker et al. 2007). The function of ganglioside as suppressor of the antitumor immune response is supported by many studies. For instance, tumor-associated gangliosides decrease the activity of several immune cells, such as T and B cells, natural killer cytotoxicity and active dendritic cells (Ando et al. 1987; Grayson &

Ladisch 1992; Biswas et al. 2006). T-cell dysfunction is promoted by the ganglioside GM2, however, an antibody targeting GM2 blocked 50-60% of T-cell apoptosis (Biswas et al. 2006). These observations may explain, at least partly, how tumor-associated gangliosides may contribute to malignancy and progression.

Many of the tumor-associated gangliosides are also found in normal healthy tissues, but are over-expressed in tumors. Interestingly, the sialic acid Neu5Gc is found in several tumor types. Among all sialic acid forms, Neu5Ac and Neu5Gc are the most abundant, however, humans are a notable exception. Due to a 92-bp deletion in the gene coding for CMP-Neu5Ac hydroxylase (CMAH), humans lack a functional enzyme required for generation of Neu5Gc. However, Neu5Gc is present in fetal tissues and malignant cells (Hirabayashi et al. 1987; Kawachi & Saida 1992; Marquina et al. 1996). For this reason, Neu5Gc was earlier assumed to classify as an “oncofetal” antigen, being expressed in fetus, suppressed during adult life and re-expressed in malignant cells. As humans lack the putative active site in the enzyme, other explanations must be found for this change in carbohydrate profile. Diet incorporation and hypoxic conditions have been described to increase the levels of NeuGc (Irie et al. 1998; Varki 2001; Tangvoranuntakul et al. 2003; Yin et al. 2006). Interestingly, it has been shown that sera from healthy humans contain antibodies recognizing glycoconjugates containing Neu5Gc. These antibodies are called Hanganutziu-Deicher (HD) antibodies, as they were first (and separately) described in the 1920s by Hanganutziu (Hanganutziu 1924) and Deicher (Deicher 1926) (as cited in (Merrick et al. 1978)). They attract complement molecules to malignant cells (Zhu & Hurst 2002; Ravindranath et al. 2007), and the levels HD antibodies in serum decreases with age, which may correlate with cancer risk increasing with age (Ravindranath et al. 2007). Characteristic for natural antibodies is that they recognize highly conserved antigens (Cojocaru et al. 2009). Importantly, auto-antibodies against tumor-associated antigens can arise and be detected early, before symptoms occur (Zhang et al. 2003; Storr et al. 2006; Chapman et al. 2008).

1.2 Cancer Immunotherapy

Cancer is among the most life-threatening diseases in industrial countries, causing 25%

of mortalities, even though standard therapies, including surgery, chemotherapy and radiation are well established. Current therapies rely on drugs killing dividing cells or blocking cell division, which also has severe effects on normal proliferating cells. An alternative treatment, cancer immunotherapy, has grown significantly during the last decades, and was selected by *Science* as the breakthrough of the year 2013. The immune responses to tumors are mainly specific to tumor antigens and will not injure healthy cells. Approaches applied in immunotherapy are based on complementation activation or stimulation of the immune system *via* a variety of compounds, including cytokines, vaccines, effector cells or antibodies. The intention of cancer immunotherapy is to augment the weak host immune response to the tumor (active immunity) in order to fight the disease. More than 100 years ago, a surgeon in New York (W.B Coley) did pioneering work in immunotherapy by vaccinating sarcoma patients with a mixture of attenuated streptococcal and staphylococcal bacteria, also known as Coley's vaccine, resulting in a febrile reaction and reduction in size of tumor mass (Coley 1991). During the last decades, modern cancer immunology was reinforced mainly as a result of three discoveries. First, Kohler and Milstein (Kohler & Milstein 1975) developed the hybridoma fusion technology and paved way for new, target-specific molecules, monoclonal antibodies. Second, the establishment of molecular biology allowed the characterization of cancer-associated signaling cascades, such as the epidermal growth factor signaling in breast cancer (Cohen et al. 1980). Third, the discovery of cytokines added an extra tool in cancer treatment (Smith et al. 1983).

1.2.1 Passive immunotherapy

Passive immunotherapy involves the transfer of immune effectors, such as antibodies and tumor-specific T cells, into patients. Currently hundreds of different monoclonal antibodies are being considered, either in experimental pre-clinical studies or in human clinical trials, and a few anti-tumor monoclonal antibodies have been approved for clinical use.

In early human trials, anti-tumor antibodies originated from mice resulted in a frequently occurring immune response against the mouse antibody. This reaction, known as the HAMA response, induce human anti-mouse antibodies, resulting in rapid clearance of

circulating therapeutic antibodies, thus abrogating the efficacy of the treatment (Klee 2000). This issue has been partially solved by the use of chimeric or “humanized” anti-tumor antibodies, consisting of a combination of murine variable regions or complementarity-determining regions, (CDRs), with human constant domain (Fc) (Morrison et al. 1984; Riechmann et al. 1988) (**Figure 5**).

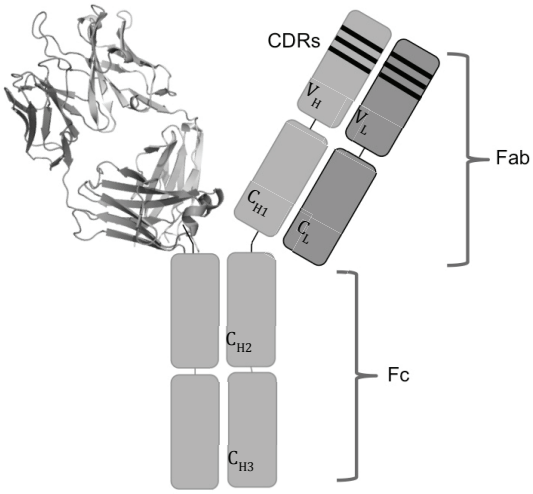

Figure 5. A schematic representation of an antibody.

1.2.1.1 Cell death killing mechanisms

Monoclonal antibodies can cause tumor cell death using different mechanisms. The most common ‘classical’ mechanisms include antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and induction of apoptosis (Liu et al. 2008; Chan & Carter 2010; Weiner et al. 2010). Tumor-cell killing by ADCC is triggered by Fc-Fcγ receptor interaction. The Fcγ receptors are present on immune effector cells, such as macrophages, neutrophils and natural killer cells, mediating lysis or

phagocytosis of the tumor cells. CDC is dependent on the recruitment of the complement component C1q by the antibody. When the complement initiator C1q is bound to the Fc region of the antibody, a proteolytic cascade to activate the complement system will be triggered. This can lead to membrane attack complex formation or trigger cell-mediated tumor-cell lysis or phagocytosis. Although other types of mechanisms are known, they are rarely described in the literature. For example, some killing mechanisms can induce cytotoxicity in an Fc-independent manner, without causing the morphological changes often occurring in apoptosis-related cell death. Such novel mechanisms have been found to be associated with membrane lesions. These lesions were observed when antibodies, in an unaided and non-apoptotic way, killed tumor cells (Hellstrom et al. 1990; Garrigues et al. 1993; Matsuoka et al. 1995; Bhat et al. 1996; Zhang, Xu et al. 1998; Ma, Zhang et al. 2001).

1.2.1.2 Anti-NeuGc GM3

Several antibodies targeting tumor-associated gangliosides are under investigation in pre-clinical or clinical studies, also including molecular vaccines. Two examples are 14F7 and chP3, both specifically recognizing Neu5Gc GM3, but not the abundant Neu5Ac GM3, even though these molecules have highly similar structures. 14F7, discussed in this thesis, is a promising antibody with remarkable features. For instance, it has been tested for a strong anti-tumor effect, comparable to standard chemotherapy treatment (Carr et al. 2002). The binding affinity for this antibody is within the nanomolar range, and it generates an anti-idiotypic antibody, 4G9, which in turn provokes an efficient anti-anti-idiotypic (Ab3) response (Rodriguez et al. 2003).

In order to achieve more advantageous properties of therapeutic antibodies, such as an extended binding specificity or higher affinity, it is essential to characterize the binding interactions between the antibody and its antigen (or anti-idiotypic antibody), thus providing a basis for engineering the interface. The crystal structure of the 14F7 Fab was solved by Krengel *et al*. (Krengel et al. 2004) but to date, no crystal structures of the antibody in complexes with its antigen or anti-idiotypic antibody are available. However, docking and phage display studies have contributed to understand the recognition mechanisms (Agostino et al. 2012; Rojas et al. 2013).

1.2.2 Active immunotherapy

The historic findings by Coley suggested that the immune system is able to attack the tumor cells, provided that the malignant cells are recognized in an immunogenic context (Coley 1991). Early attempts to boost the immune system relied on non-specific immune stimulation. Today, cancer vaccines are targeted because they do not just boost the immune system in general, but they direct it specifically towards the tumor. Examples of different components of cancer vaccines include killed tumor cells, purified tumor antigens, anti-idiotypic antibodies, professional antigen-presenting cells (APCs), DNA or viral vectors. They can also be cytokine- and co-stimulator-enhanced vaccines. In many cases, the vaccines are combined with other substances (adjuvants), in order to boost the immune response further.

1.2.2.1 "GM3 mimic"

As a tool in cancer immunotherapy, idiotypes (Ids) have gained in interest over the last three decades. An idiotype is defined as the set of antigenic determinants close to the antigen-binding site of an antibody (Oudin & Michel 1963; Grey et al. 1965). These determinants are able to elicit an immune response both at the humoral and cellular level. The "idiotypic network theory" was developed by the Danish immunologist Nils Jerne in the 70's (Jerne 1974) and together with Bona's "regulatory idiotope" (Victor-Kobrin et al. 1985) and Couthino's "second generation networks" (Varela & Coutinho 1991) fundamentally impacted the concept of immune regulation. These anti-idiotypic hypotheses are often weakly supported by experimental evidence, but regardless of this, anti-idiotypic antibodies that can "mimic" antigens of protein or non-protein type, are considered attractive in cancer immunotherapy. The antigen-induced antibody (Ab1) recognizes the epitope of the antigen through its variable regions, including the heavy (VH) and light (VL) chains. Anti-idiotypic antibodies (Ab2s) are directed against the variable part of Ab1 (**Figure 5**). Anti-idiotypic antibodies (Ab2s) are classified into different types based mainly on functional properties of the antibody, such as the ability of inhibiting the interaction between Ab1 and the antigen, but also the capability to

induce anti-anti-idiotypic antibodies (Ab3s). For example, the Ab2α type is not able to inhibit the Ab1-antigen interaction, thus should bind to non-variable parts of the Ab1. In contrast, the Ab2γ will compete for the binding site and therefore inhibit interaction, but is incapable of generating Ab3 antibodies when used as an immunogen. As antigen mimicry is ultimately the desired feature when it comes to Ab2, the Ab2β, which are carrying an "internal image" of the original epitope, are considered the most promising vaccine candidates (Jerne 1974; Coutinho 1995). Several anti-idiotypic antibodies targeting gangliosides are under investigation (Bhattacharya-Chatterjee et al. 2000). For example, a vaccine against GD3, using an Ab2β type of anti-idiotypic antibody mimicking GD3, has shown increased survival in small-cell lung carcinoma patients (Chapman 2003). Moreover, B cells derived from a relapse-free neuroblastoma patient treated with an anti-GD2 antibody, were used to generate Ab2 antibodies (GK8) with vaccine potential (Mueller et al. 1990; Uttenreuther-Fischer et al. 2006). 14F7 and P3 are monoclonal antibodies targeting the NeuGc GM3 ganglioside. They both generate several Ab2s (Vazquez et al. 1998; Rodriguez et al. 2003; Lopez-Requena et al. 2007; Rodriguez et al. 2007). The most studied one is Racotumomab (1E10). It was first thought to be an Ab2γ since it is able to inhibit the Ab1 (P3)-antigen binding, but when injected into mice, the Ab3s were unable to recognize the antigen (Vazquez et al. 1998). Later it was proven that this was a species-dependent reaction and Ab3s with the same specificity as P3 (so-called Ab1'), are induced only in species where the antigen (NeuGc) is non-self. Racotumomab is therefore able to "mimic" NeuGc GM3 only in humans and chicken (Hernandez et al. 2005). It is currently in clinical trials (approved in Cuba and Argentina), successfully completed a proof-of-concept in advanced non-small cell lung cancer (Vazquez et al. 2012).

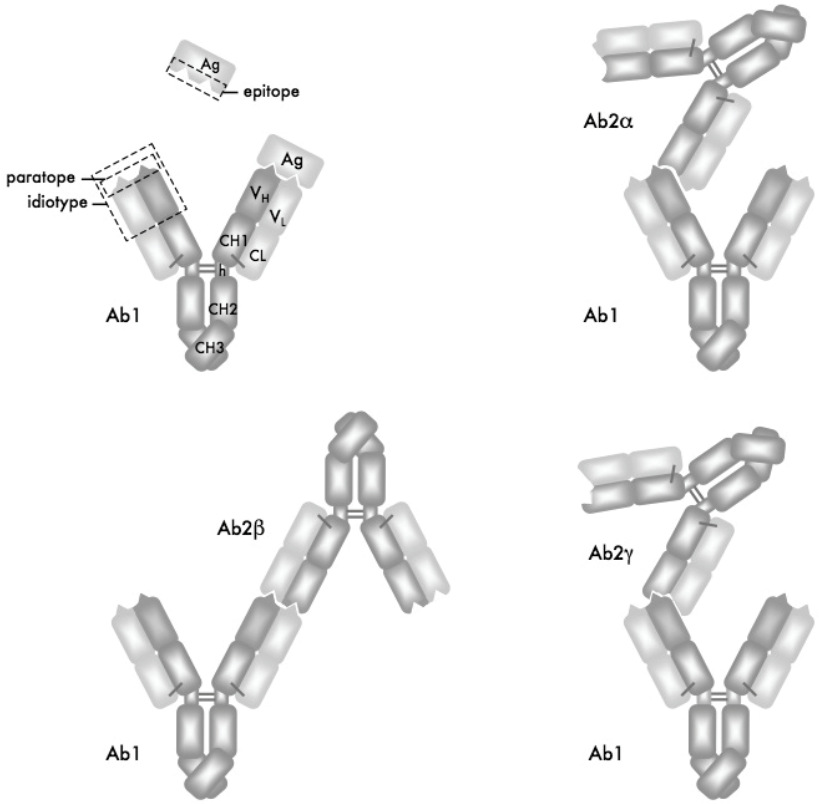

Figure 5. Different types of anti-idiotypic antibodies. Adapted from (López-Requena 2009).

1.3 Cancer and hypoxia

Hypoxia is defined as oxygen levels below 5%, often observed in advanced tumors, but also in embryonic cells, as an essential factor for differentiation and proliferation processes (Vaupel et al. 1989; Lee et al. 2001; Forristal et al. 2010; Prado-Lopez et al. 2010). Approximately 90% of all known cancers are solid tumors (Brown 2000). During the process of tumor progression (proliferation, invasion and metastasis), rapid growth of the tumor is associated with changes in the cellular microenvironment. These changes are mainly due to inadequate oxygen supply, resulting in regions in the tumor where the oxygen concentration is considerably lower than in surrounding healthy tissues. This is

referred to as tumor hypoxia, and is a common characteristic of advanced tumors. Tumor hypoxia is considered an adverse prognostic indicator and a therapeutic problem, making tumors more resistant to ionization radiation and chemotherapy (Burnet et al. 1994; Brown 2000; Shannon et al. 2003). More recently, alteration of intra-tumoral oxygen levels has been associated with malignant progression (Brown & Giaccia 1998; Sutherland 1998; Semenza 2000). Moreover, sustained tumor hypoxia may also trigger cellular alterations, causing a more aggressive phenotype (Brizel et al. 1996; Hockel et al. 1998; Walenta et al. 2000). A well-known character of malignant and invasive cells is an altered glucose metabolism. Glycolysis is inhibited by the presence of oxygen, allowing mitochondria to oxidize pyruvate to CO_2 and H_2O, a process termed the 'Pasteur effect' (Racker 1974). Cancer cells can convert glucose to lactate when oxygen is present, a process referred to as 'aerobic glycolysis' or the 'Warburg effect', after Otto Warburg, who first observed this phenomenon in the 1920s (Warburg et al. 1927). This resulted in the hypothesis that cancer may be caused by mitochondrial dysfunction. Hypoxia induces changes in the tumor proteome, allowing the tumor to successfully adapt to low oxygen levels and survive in a harsh environment. Details about this adaptation, which simultaneously provokes more aggressive tumors, are discussed in this thesis.

2. Experimental techniques

2.1 Mass spectrometry

2.1.1 Instrumentation

Mass spectrometry is a sensitive technique to identify and quantify molecules based on their mass and charge (m/z). Although this technique was developed almost a century ago, ionization of larger molecules, such as proteins was not possible until 1981 (Barber et al. 1981). Macromolecule ionization methods were then developed, such as electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), which enabled scientists to study protein structure and identify unknown proteins by the use of mass spectrometry (Tanaka et al. 1988; Fenn et al. 1989; Chowdhury et al. 1990). Both ESI and MALDI are soft ionization techniques, allowing the analysis of more fragile molecules such as proteins, DNA and carbohydrates. The electrospray ion source is easily connected to liquid chromatography (LC), which simplifies the analysis of complex protein mixtures. An "in-line" system elutes analytes through the column at different rates, which are immediately analyzed by MS. The MALDI technique generates far fewer ions that are multiply charged. Common for all mass spectrometers is that they all have an ion source, a mass analyzer and an ion detector, although these components vary depending on the mass spectrometer type. The instrument must also be connected to a vacuum pump in order to control the pressure within the instrument, as low pressure (10^{-6} to 10^{-8} torr) is necessary to limit the number of ion collisions. In a typical experiment, the sample, which can be in liquid or dry form, is ionized and in some cases vaporized by the ion source, which could be ESI or MALDI. During this process, the molecules receive a charge, allowing acceleration throughout the system. Using an ESI source, the ionization occurs when the inlet stream is released from a capillary that has a voltage applied to it. A spray of charged droplets is formed, which are desolvated through stages of decreasing pressure. MALDI is based on co-crystallization of the sample within an organic matrix, such as α–cyano-4-hydroxycinnamic acid or sinapinic acid on a metal plate. The crystals are then irradiated with laser pulses, resulting in a photoexcited matrix that ionizes the analyte via proton transfer, producing ions in (mostly) singly

charged analyte ions in gas phase. The ions will then be directed to the mass analyzer, which can be of different types (e.g. time-of-flight (TOF), ion traps or quadrupoles). It provides an electrical and/or magnetic field to the charged ions, which deflects, based on the mass and charge, the path of the individual ions. The low pressure is essential to not alter the path or charge of the ions. These will then hit the ion detector, which are often microchannel plates or electron multipliers. As the ions hit the detector (a metal plate), a cascade of electrons is emitted, resulting in a measurable current (Finehout & Lee 2004). MS/MS (also known as tandem MS or MS^2) is a method frequently used in biological MS to reveal structural details or amino acid sequences. Ions with a selected m/z (known as precursor ions) are selected, fragmented and the resulting product ions are measured. This can be achieved using two mass analyzers separated by a collision cell. Commonly used mass analyzers are ion trap, TOF, quadrupole and the Fourier transform-ion cyclotron resonance analyzer. For instance, using a MALDI TOF/TOF, two TOF analyzers are employed and separated by a collision cell. The collision cell is pressurized with a non-reactive gas (e.g. helium), in which the ions are fragmented via collision-induced dissociation (CID). The product ions are thereafter reaccelerated before entering the second TOF analyzer for final analysis.

2.1.2 Protein identification

Protein identification methods include a combination of peptide mass fingerprinting and amino acid sequencing using MS/MS. Since the amino acid sequence is unique for each protein, proteolytically cleaved peptides provide a “fingerprint” of the protein. Typically, proteins are separated prior to digestion, either by LC or gel electrophoresis. In an unknown protein identification experiment, the protein is alkylated and reduced, and a subsequent digestion by a sequence-specific enzyme (e.g. trypsin) is performed. The resulting peptides are measured with MS to obtain a list of peptide masses, which is then entered into a search program (e.g. Mascot). A comparison between the amino acid sequences of proteins obtained from databases such as Swiss-prot or NCBI and the list of peptide masses from the sample result in a list of possible proteins and probability values. In order to increase the probability of a correct match, an MS/MS analysis can be performed. Here, a specific m/z is selected and fragmented. As the bonds between the

amino acids are the weakest, this results in a spectrum with peaks that represent peptides that differ only in the number of amino acids they contain. The mass differences between the peaks can identify the amino acid sequence of the peptide.

2.1.2 Protein quantification

Quantitative proteomics relies on the ability to detect small changes in peptide abundance in response to an altered state (Ong & Mann 2005). Quantitative proteomics can be relative or absolute, where fold changes in different samples and absolute amounts of proteins are determined, respectively. Quantification by proteomics was previously based on separation by 2D gels, where differences could be visualized by the intensity of spots (Bantscheff et al. 2007; Schulze & Usadel 2010). Label-free methods can be used for relative quantification, including spectral counting and peptide peak intensity measurements. These methods are based on comparing the number of spectra from the same protein between different samples and comparing peak intensity belonging to a given protein, respectively. Another approach is stable isotope labeling, which is based on the introduction of a tag with a mass difference that does not affect the chemical properties of the protein but only affects the mass of the protein. The labeling can be metabolic, involving introduction of isotopes to whole cells through the medium, or chemical, where the isotope is introduced by a chemical reaction. A number of chemical labeling tags are available, including isotope-coded affinity tags (ICAT), isotope-coded protein labels (ICPL), and isobaric tags for relative and absolute quantification (iTRAQ). Stable isotope labeling by amino acids in cell culture (SILAC) is a metabolic labeling method, allowing quantitation of very small changes in protein levels. This method is discussed in detail in *Section 2.2.*

2.2 Proteomic analysis

Proteomics has considerably grown as a field over the recent years. Studies in quantitative proteomics have traditionally been performed using 2D gel electrophoresis, recording alterations in the staining pattern derived from two cell populations in different

states. Recently, an isotope labeling approach was developed, so-called 'stable isotope labeling by amino acids in cell culture' (SILAC) (Ong et al. 2002). This is a relative quantification method that compares protein or peptide abundance between samples. In SILAC, two cell populations are grown in identical media in all respects except that one of them contains a natural 'light' and the other a 'heavy' form of a particular amino acid (e.g. $^{12}C_6$ L-lysine or $^{13}C_6$ L-lysine). With each cell doubling, each copy of this particular amino acid (e.g lysine) will be replaced by its isotope-labeled analog. After complete incorporation, the two cell populations are treated differently. Finally, equal amounts of the cell lysate from both cell populations are combined for sample processing and subsequent separation by SDS-PAGE. Trypsin is used to digest the protein for MS analysis and quantitation of isotopic peptide pairs. Quantitative proteomic analyses rely on MS in order to identify and quantify the peptides (as spectrum), although tandem mass spectrometry is essential for identification of the peptide (protein). During the first round (MS1), the precursor ion spectrum represents all ionized peptides produced. In the second round (MS2), selected ions undergo collision-induced fragmentation (CID), resulting in a fragment ion spectrum for each precursor ion. These spectra are thereafter assigned peptide sequences based on database comparison. In this thesis, SILAC analysis was employed to study protein alteration as a response to hypoxic conditions and anti-tumor antibody treatment. Both experiments (manuscript I and III) were performed using HeLa cells grown in Dulbecco's modified Eagle's medium (DMEM) high glucose, supplemented with 10% dialyzed fetal bovine serum (FBS)(F6178-Sigma), 1% pencillin/streptomycin and either $^{13}C_6{}^{15}N_4$ L-arginine (89990-Fisher) and $^{13}C_6$ L-lysine (89988-Fisher) or media containing natural L-arginine (89989-Fisher) and L-lysine amino acids (89987-Fisher). After at least five cell doublings, the two cell populations were subjected to treatment. For the hypoxia experiment (described in manuscript I), one population of HeLa cells was grown under hypoxic conditions (1%O_2) in an InVivo2 400 multi-gas incubator box (Ruskinn Technology, UK), while the other population was grown under normoxic conditions (19%) for 72 h. During the whole experiment, oxygen consumption was measured using an automatized oxygen sensor (Unisense, Denmark) starting measurements of the oxygen concentration above the medium and proceeding until it reached the cell surface. This procedure was repeated every 2 h, giving the real pericellular oxygen concentration from the last point in each measured profile. Combined

with cell density, calculated from pictures taken every 24 h with an inverted microscope (Nikon TS100, Japan) and a Paxcam3 camera (MIS, IL, USA), oxygen consumption per cell was defined. For the antibody treatment experiment (manuscript III), one population of HeLa cells was incubated with monoclonal anti-tumor 14F7 mAb in 37°C for 3 h, kindly provided by the Center of Molecular Immunology (CIM), Havana, Cuba. The other cell population was not treated and used as control. The mass spectrometer was operated to switch between Orbitrap-MS and LTQ-MS/MS acquisition, allowing isolation of up to the seven most intense ions for fragmentation using CID. Protein identification and quantification were performed with MaxQuant (Cox & Mann 2008) employing the Andromeda search engine (Cox et al. 2011) with the IPI human database including common contaminants. For both experiments, at least two quantification events were required per protein, in 2 of 3 replicates. Moreover, proteins with a minimum fold change of 1.5 and a corrected p-value < 0.05 were considered differentially altered.

2.3 Binding studies

2.3.1 Flow cytometry

This technique is a laser-based biophysical technology used in cell sorting, cell counting, biomarker detection and other purposes. It allows characterization of one cell a time. It is commonly performed by staining the cells with fluorescently labeled probes specific for the surface molecule of interest, measuring the quantity of fluorescence emitted by the cells in real time. The number of cells in a suspension expressing the molecule of interest can therefore be determined, by passing the cells one at a time, through a fluorimeter with a laser-generated incident beam. By labeling the different cell populations with the same probe, the relative amounts of a particular molecule can be determined, comparing the amount of fluorescence admitted. The incident laser beam is of a designated wavelength, for instance 488 nm for FITC (fluorescein isothiocyanate) and PI (propidium iodide). The light emerging from the cell suspension is analyzed for forward and side scatter in addition to the wavelength of the fluorochrome labels on the cell surface. A simplified model can be used to describe cell size and internal complexity

in terms of forward and side light scattering, respectively. A flow cytometer can also be used to study the cell cycle, by which the cells are labeled with a dye that becomes fluorescent when bound to DNA. Therefore it is possible to quantify DNA in each cell, as the fluorescence is directly proportional to the amount of DNA. Normally, the cells fall into three categories: Unreplicated complement of DNA (G1 phase), fully replicated DNA complement (G2, twice the amount of DNA in G1 phase) or intermediate amount of DNA (S-phase). This method is important not only for understanding normal cell cycle control but also to understand loss of normal cell cycle progression, often occurring in cancer.
In manuscripts I and II, flow cytometry analysis was employed either to determine cell cycle progression under hypoxic conditions or to measure the relative amount of the antigen NeuGc GM3 in HeLa cells.

2.3.2 Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA is an antibody-based technique used to specifically detect the presence of an antigen or another antibody in a sample. The antigen is most commonly coated to a polystyrene microtiter plate either by adsorption (non-specifically) or via capturing by an already bound antibody. A detection antibody, sometimes enzyme-linked, will form a complex with the immobilized antigen. The detection antibody can also be recognized by a secondary antibody (linked to an enzyme), which forms a covalent link to its target. Different enzymes can be linked to the antibodies, most commonly used are horse radish peroxidase and alkaline phosphatase. In order to produce a signal, representing the quantity of antigen or antibody binding to the antigen, a substrate is added to the plate. This method was used to confirm binding activity of the 14F7 mAb to NeuGc GM3 (manuscripts III and IV) and to study binding between the anti-idiotypic antibody 4G9 and 14F7 (manuscript IV). For antibody-antigen binding, a Nunc Polysorp 96 well plate was coated with NeuGc GM3 and a dilution series of 14F7 mAb was added to the plate, whereas for the antibody-antibody binding a Nunc Maxisorp plate was used to bind the one of the protein before dilution series of the other protein was added. An alkaline phosphatase conjugated goat anti-mouse IgG was used as secondary antibody and a visible signal was generated using the substrate *para*-nitrophenylphosphate (pNPP).

2.3.2.1 Meso scale discovery multi-array (MSD)

MSD assays follow a workflow similar to that of an ELISA. The plate is coated with the capturing agent followed by blocking and adding of samples. Thereafter the detection reagent is added and the plate is analyzed. However, MSD has ultra-low detection limits and requires minimal sample volumes. Proteoliposomes, containing a combination of EGFR and NeuGc GM3 (derived from horse erythrocytes or HeLa cells) were coated on the plate and antibodies specifically recognizing NeuGc GM3 (14F7 mAb) and EGFR (Nimotuzumab) were tested for binding.

2.3.3 Surface plasmon resonance (SPR)

This method is used to determine the binding constant in a real-time and label-free manner. The SPR method has been used to characterize several molecular interactions, including antibody-antigen binding, binding of protein to DNA, small molecules, carbohydrates and other proteins. Proteins can bind to their ligands either permanently or engage in transient interactions, in which they remain associated temporarily. To measure binding, a solution of potentially binding molecules (e.g. proteins) is allowed to flow past a biosensor surface coated with immobilized molecules. As molecules bind to the sensor surface, the refractive index close to the surface changes. When a light beam impinges onto a metal film on the sensor chip at a certain (resonance) angle, the light is absorbed by electrons in the metal film, causing the electrons (also known as surface plasmons) to resonate. This is monitored in real time and reflects the injected molecules' association or dissociation with the immobilized molecules. SPR was used in manuscript IV, both to determine the binding constant of 14F7 to its antigen NeuGc GM3, and to investigate if the anti-idiotypic antibody 4G9 interfered with 14F7 mAb binding to the immobilized NeuGc GM3. For these experiments, NeuGc GM3 was coated onto a Biacore CM5 sensor chip and antibody binding was analyzed with a Biacore 3000 instrument.

2.4 Signaling studies

2.4.1 Western blotting

This method is used to determine the relative quantity of a protein within a sample of tissue homogenate. Western blotting can also be used to detect the phosphorylation state of a protein through the use of phosphotyrosine-specific antibodies. Generally, the mixture of proteins is first analytically separated, typically by SDS-PAGE. The separated proteins are then transferred from the gel to a support membrane (nitrocellulose or polyvinylidene difluoride (PVDF)) by electrophoresis such that the membrane acquires a copy of the array of proteins present in the gel. The protein on the membrane can then be identified using a primary antibody, specific for the protein of interest, and thereafter a species-specific (labeled) secondary antibody, directed against the primary antibody. The secondary antibody is detected by colorimetric, chemiluminiscent, radioactive or fluorescence detection. Western blotting was used in manuscript II to confirm the proteome analysis results, and in previous and ongoing work, studying the effect of NeuGc GM3 on EGFR autophosphorylation.

2.4.1.1 Proteoliposomes

Biological systems are complex and membrane protein and lipid studies in these systems are therefore challenging. In order to bypass cellular complexity when addressing the role of gangliosides for EGFR activity, proteoliposomes were produced. Using a minimal system with defined lipid composition, the lipid environment and its importance in receptor modulation could be investigated. The lipids (1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), N-stearoyl-D-erythro-sphingosylphosphorylcholine (SM) and cholesterol) were mixed in two different compositions (37.5/37.5/25 or 80/15/5 mol%) and dried under a nitrogen stream. The gangliosides NeuAc GM3 and NeuGc GM3 (0.5 mol%) were added to the membrane systems. Multilamellar vesicles were produced by adding HEPES liposome buffer (HLB) and shaking at 58°C. To achieve unilamellar vesicles, freeze-thaw cycles and extrusion were performed. To reconstitute human EGFR into the liposomes, the liposomes were first fully solubilized with CHAPS

(3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and then incubated with the receptor. Samples were shaken and sonicated repeatedly and the detergent was removed by dialysis. The proteoliposomes were incubated with anti-NeuGc GM3 antibodies (14F7, P3) in order to target the possible activator/inhibitor. Western blotting was thereafter performed using antibodies specific for phosphorylated sites or the C-terminus of the EGFR.

2.5 Structural proteomics

Structural proteomics is a combination of mass spectrometry techniques and protein chemistry. It is an approach for fast and efficient molecular characterization of protein-protein interactions. The techniques used in structural proteomics include limited proteolysis, crosslinking, chemical protein modification and hydrogen/deuterium (HDX) exchange, all followed by mass spectrometric analysis. These methods are often used for complementary analysis, in parallel to classical structural biology methods such as X-ray crystallography and NMR allowing the study of protein-protein interactions under native conditions, often of proteins of biomedical importance. Chemical modification, HDX and limited proteolysis can provide direct information about exposed protein surfaces, while chemical cross-linking can provide information on inter- or intra-protein interactions and distances. None of these methods can by themselves provide structural information, but a combination with mass spectrometry is required. Structural proteomics therefore demands a highly inter-disciplinary environment between protein and analytical chemistry, biomedical and biological sciences.

2.5.1 Chemical cross-linking mass spectrometry (CXMS)

Mass spectrometry has become and important tool in both protein and protein complex structure elucidation. Chemical cross-linking mass spectrometry (CXMS) can provide low-resolution analysis and works through covalently binding a linker of a defined size between two amino acids in close proximity to each other. Subsequent digestion (e.g. trypsination) of the protein, followed by MS analysis, gives information about how

proteins interact with each other through the distal restraints set by the linker (Sinz 2003; Singh et al. 2010). Several crosslinkers of varying length and reactive sites are available, including homolinkers (e.g. Lys/Lys) or heterolinkers (e.g Lys/Arg). This method is not going to replace X-ray crystallography as a means to obtain high-resolution structures, but it may give low-resolution structural information of proteins and protein complexes. In manuscript IV, a Bis(sulfosuccinimidyl) suberate (BS3) crosslinker (21580-Thermo Scientific) was used to achieve information about the 14F7/4G9 complex. This is an amine-to-amine crosslinker with reactive sulfo-*N*-hydroxysulfosuccinimide (NHS) esters at both ends. Proteins generally contain a number of primary amines, in the side chains of lysine residues and the N-terminus, representing suitable targets for the BS3 crosslinker.

2.5.2 Oxidative footprinting

This technique is based on hydroxyl radicals that react with proteins to yield stable oxidative modifications. These modifications occur in solvent-accessible amino acid side chains, some more susceptible to oxidation than others. This method has been developed to analyze macromolecules, conformational changes and protein complex interaction (Pappin et al. 1993; Sharp et al. 2003; Nukuna et al. 2004). Proteins and protein complexes are exposed to hydroxyl radicals reacting with amino acids that are both reactive and solvent accessible. Subsequent to oxidation, the proteins are digested with site-specific proteases to generate well-defined peptides followed by MS analysis. Hydroxyl radicals that are generated using Fenton reagents (Heyduk & Heyduk 1994; Sharp et al. 2003) from photo-oxidation of peroxide, radiolysis of water or using electrical discharge, are suitable for footprinting experiments (Maleknia et al. 1999; Nukuna et al. 2004; Sharp et al. 2004; Sharp et al. 2005). Different amino acid side chain products can be generated in footprinting experiments, with the most frequently occurring products being alcohol or aldehyde groups associated to mass increases of +16 Da or +14 Da, respectively. The footprint approach to structural characterization depends on the ability to localize the oxidation site within the target peptide. To achieve this, the peptide is subjected to fragmentation using CID. To localize the oxidation site, masses of the fragments are then analyzed. An ion is dissociated as a result of interaction with a

neutral species, most commonly a gas in a collision cell. During the collision, this dissociation is brought about by conversion of translational energy of the ion to internal energy of the ion. For these experiments, the b-type and y-type fragmentation ions are of special interest, as they retain the N- and C-termini of the peptide, respectively. For instance, a b-ion retaining only the N-terminal residue is named b_1 and the next ion (containing two residues) would be denoted b_2, and so on. The N-terminal residue is identified by the mass of the b_1 ion, whereas the mass difference between the b_2 and the b_1 ions identifies the second amino acid. A comparison is made of two MS/MS spectra, one containing a modification and one unmodified. Ions retaining the modification will show mass shifts relative to the unmodified ion. In protein complex interactions, the oxidative footprinting method provides an accurate and sensitive probe of amino acids and changes occurring upon complex formation. This method was employed in manuscript IV, in order to study antibody-antigen interactions and changes occurring during complex formation. Here, hydroxyl radicals were generated by laser photolysis of hydrogen peroxide-containing samples of 14F7 mAb and 14F7, preincubated with NeuGc GM3 (trisaccharide). The samples were then subjected to digestion and MS analysis by MALDI-TOF and LC-MS/MS.

3. Summary of manuscripts

Manuscript I

Hypoxia strongly affects glycolysis and expression of mitochondrial proteins, as shown by quantitative proteomics of HeLa cells

In this manuscript, we performed a global proteome analysis of HeLa cells in hypoxia. Hypoxia is a common feature in advanced tumors and is associated to radio- and chemotherapy resistance and malignant progression. SILAC, a quantitative proteomic-based approach, combined with LC-MS/MS was used to study hypoxic HeLa cells. Differences in protein expression of the treated (hypoxia) or non-treated (normoxia) cells were analysed by *in vivo* metabolic labeling of the two cell populations with either the 'light' or the 'heavy' form of a particular amino acid, such as L-lysine or L-arginine. This is a powerful method to identify and quantify relative alterations in complex protein samples. The HeLa cells were cultured in the 'light' or 'heavy' medium for at least five cell doublings in order to ensure sufficient label incorporation into proteins, and thereafter subjected to normoxic or hypoxic conditions for 72h. Out of the 3260 proteins identified, 137 proteins were found to be regulated according to our criteria. The proteins were altered in a wide range, from substantial down-regulation to 18-fold up-regulation. Three clusters of proteins were identified, showing significant up-regulation of glycolysis and revealing efficient down-regulation of mitochondrial ribosomal proteins and translocases of the inner and outer mitochondrial membrane (TIMM/TOMM), processes that are most likely synchronized by the 'master regulator' of hypoxia, HIF-1. We also studied the cell cycle of the cells subjected to hypoxia, and found a cell cycle arrest at a restriction point in G1 and a prolonged S phase. This was shown by FACS experiments in combination with a cell cycle analysis. Altered proteins involved in these processes are discussed in the manuscript. This work contributes to a better understanding of the effect of hypoxia on cancer cells and uncovers proteins important for malignant progression, with potential for future therapies.

Manuscript II

How does hypoxia trigger the expression of NeuGc GM3?

In this manuscript, the hypoxia-induced NeuGc GM3 expression is investigated by a combination of FACS and SILAC analysis. NeuGc GM3 not only represents an attractive target in cancer therapy but is also a potential prognostic and diagnostic marker. It is specific for several types of cancer, but due to a deletion in the gene encoding the active site of an enzyme responsible for NeuGc synthesis, this ganglioside is not present in healthy human tissues. However, hypoxic conditions have been shown to trigger NeuGc expression. In order to study the mechanism by which the hypoxia-induction occurs, HeLa cells were placed in normoxic or hypoxic conditions and were thereafter subjected to SILAC analysis. We confirmed that hypoxia also triggers NeuGc GM3 expression in HeLa cells, and identified proteins with potential of being involved in this up-regulation; in particular a protein containing an iron-sulfur cluster was found to be significantly altered. This protein, subunit B of the succinate dehydrogenase complex, representing complex II of the respiratory chain, was in contrast to many mitochondrial proteins, significantly up-regulated. Western blotting was used to confirm up-regulation of SDHB. Interestingly, the active site deletion consists of an iron-sulfur cluster (Rieske domain). Whether this protein could, provided tumor hypoxia, replace the function of the Rieske domain and trigger progression in cancer is yet to be investigated.

Manuscript III

SILAC analysis of HeLa cells treated with the anti-tumor monoclonal antibody 14F7

In this manuscript, we study a novel cell death mechanism by which the antibody 14F7 eliminates cancer cells, using SILAC analysis in conjunction with LC-MS/MS. The antibody 14F7 specifically targets the tumor-associated ganglioside NeuGc GM3 and has been shown to kill cells unaided, causing lesions in the membrane of tumor cells. This novel mechanism does not belong to the ‘classical’ cell death mechanisms, such as

antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or induction of apoptosis (Liu et al. 2008; Chan & Carter 2010; Weiner, Surana et al. 2010). 14F7, a promising IgG antibody with high binding affinity for NeuGc GM3, has a strong anti-tumor effect, comparable to standard chemotherapy treatment (Carr et al. 2002). The mechanism by which it kills the cells is not fully elucidated, but no characteristics of apoptosis have been observed; however, membrane lesions described as 'holes' or 'pores' in combination with cellular swelling, often seen in oncosis, have been described (Roque-Navarro et al. 2008). We found, when we studied the effect of 14F7 treatment on HeLa cells, that 13 proteins had an altered expression compared to the non-treated cells. In particular, down-regulated proteins associated with the cytoskeleton and their possible functions are discussed in this manuscript. Which processes these proteins inhibit or trigger in response to the antibody treatment is yet to be verified, but this work significantly advanced our understanding of this novel cell death mechanism, which may be followed up by super-resolution microscopy.

Manuscript IV

MS and SPR analysis of the molecular interaction of the unique anti-tumor antibody 14F7 with its antigen *N*-glycolyl GM3 and an anti-idiotypic antibody

In this manuscript, we investigated the binding interactions between the antibody 14F7 and its antigen NeuGc GM3 or its anti-idiotypic antibody 4G9. NeuGc GM3 is an attractive antigen for cancer immunotherapy as it is tumor-associated, while the highly similar NeuAc GM3 is present in almost all healthy tissues. The monoclonal anti-tumor antibody 14F7 specifically targets NeuGc GM3 and does not cross-react with the abundant NeuAc GM3. 14F7 has remarkable features. For instance, it belongs to the IgG class and has a strong binding affinity (within the nanomolar range) to its antigen, which is rare for anti-carbohydrate antibodies. Furthermore, it generates an anti-idiotypic antibody, 4G9, reported to elicit an efficient anti-anti-idiotypic antibody (Ab3) response (Rodriguez et al. 2003). An important aspect in the development of therapeutic antibodies is to identify the binding interface between antibody and its antigen or anti-

idiotypic antibody, and to characterize the molecular recognition mechanism based on the structure of the two molecules. For the antibody-antigen interface, a higher affinity or extended binding specificity may be engineered, while for the antibody-anti-idiotypic antibody interaction, it is possible to partly determine to which Ab2-class (more or less promising vaccine candidates) the anti-idiotypic antibody belongs. Since the complexes have so far eluded characterization by crystallography, we use (in this manuscript) mass spectrometry-based approaches to structurally characterize the interactions of 14F7 with antigen or anti-idiotypic antibody. Approaches include SPR, cross-linking and oxidative footprinting, a newly developed method to reveal the structure of protein-ligand interfaces (Gau et al. 2009), where the accessibility of different parts of the protein is probed by oxidation, comparing the free and ligand-bound forms. Mass shifts due to the oxidized amino acids can easily be detected by MS. Structural information can thereafter be obtaine by comparing the levels of oxidation of the protein alone or in complex with the ligand. This method was employed to analyze the binding interface between the antibody 14F7 and its ligand NeuGc GM3 (trisaccharide moiety). To investigate the binding interactions between 14F7 and the anti-idiotypic antibody 4G9, we applied an MS-based cross-linking approach and SPR analysis. The results suggest that not only the heavy chain, but also the light chain may be interacting with the ligand NeuGc GM3, although additional confirmation is needed. It seems that the binding site of the anti-idiotypic antibody extends far beyond the ligand binding site, indicating that 4G9 does not belong to the most promising anti-idiotype class, Ab2β. Interestingly, 4G9 (Fab and mAb) strongly affected the kinetics of NeuGc GM3 binding to 14F7, in addition to blocking antigen binding. Taken together with the MS results, this indicates that 4G9 may either induce a long-range conformational change in 14F7 that is transmitted to the antigen binding site, or dampen its dynamics.

4. Ongoing work and preliminary results

It is known that gangliosides affect membrane receptor activation, such as EGFR and VEGFR (*Section 1.1.4*). However, membrane protein and lipid studies in biological systems are challenging, due to the complexity of cells. To bypass this complexity, a unilamellar membrane system consisting of defined lipids and proteins has been used, and more studies will most likely follow. The proteoliposomal system has either GM3, EGFR or both incorporated in membranes with two different lipid compositions, forming uniform liquid-disordered (ld) membrane phases or liquid-disordered/liquid-ordered (ld/lo) domains. This difference is important, as the lipid environment (not only the presence of gangliosides) has been shown to be important for the activation of the EGFR. Coskun *et al*. showed that NeuAc GM3 inhibits EGFR activation, but only in ld/lo proteoliposomes (Coskun et al. 2011). For this reason, we used ld/lo proteoliposomes, and incorporated a combination of human EGFR, NeuAc GM3 and NeuGc GM3 (comparing gangliosides derived from horse erythrocytes and HeLa cells) into the proteoliposomes. The monoclonal antibodies 14F7 mAb (humanized and murine) and P3 mAb (chimera) were incubated with the proteoliposomes and the EGFR activation was analyzed using Western blotting with phosphotyrosine-specific antibodies. While we could confirm that P3 mAb and 14F7 mAb (murine) slightly inhibited EGFR activation at one phosphorylation site, a more interesting finding was the presence of EGFR dimers in proteoliposomes incubated with these antibodies. Furthermore, we used the sensitive method MSD, to measure binding of an anti-NeuGc GM3 antibody (14F7 mAb, humanized) and an anti-EGFR antibody (Nimotuzumab) to the proteoliposomes. This measurement was repeated twice for anti-NeuGc GM3 and showed that in ld/lo proteoliposomes, the presence of both EGFR and NeuGc GM3 ganglioside significantly enhances antibody binding. This is true for both anti-EGFR and anti-NeuGc GM3 antibodies (**Figure 6**). Apart from the known inhibitory effect of NeuAc GM3 on EGFR activation, we observed that P3 and 14F7 (murine) targeting NeuGc GM3 slightly decreased activation of EGFR. Moreover, EGF-independent EGFR dimerization was observed as a response to the treatment with these antibodies. For the humanized 14F7 mAb and Nimotuzumab, it was clear that an enhanced binding occurs in the presence of both EFGR and NeuGc GM3.

Proteoliposome	0.1 ug/ml	0.1 ug/ml	1 ug/ml	1 ug/ml	10 ug/ml	10 ug/ml	50 ug/ml	50 ug/ml	Antibody
Ld/lo+NeuGc GM3 (horse)	263	728	370	239	1122	2273	6749	6366	14F7
Ld/lo+NeuGc GM3 (HeLa)	380	361	698	707	3007	2941	10850	4807	14F7
Ld/lo+EGFR+NeuGc GM3 (horse)	16189	16703	16128	13317	17952	14099	22620	24359	14F7
Ld/lo+EGFR+NeuGc GM3 (HeLa)	11399	11178	14982	8842	12593	18420	19782	19787	14F7
Ld/lo+EGFR	12211	14356	24400	24937	13620	45752	47567	55913	Nimotuzumab
Ld/lo+EGFR (degl)	25284	18792	34340	34939	56822	48558	56919	65584	Nimotuzumab
Ld/lo+EGFR+NeuGc GM3 (horse)	32561	34849	103797	78266	124432	137238	131038	114971	Nimotuzumab
Ld/lo+EGFR+NeuGc GM3 (HeLa)	48540	41883	91197	95867	138545	116754	110370	139031	Nimotuzumab

Figure 6. Humanized 14F7 (anti-NeuGc GM3) and Nimotuzumab (anti-EGFR) (left column) were used to incubate proteoliposomes consisting of a combination of incorporated human EGFR (in one case, deglycosylated EGFR) and NeuGc GM3 (derived from horse erythrocytes or HeLa cells)(right column). The antibody concentration is showed in the upper row. As seen from the values, for both antibody targets (anti-NeuGc GM3 and anti-EGFR), enhanced binding was observed when EGFR or NeuGc GM3 was present, respectively.

5. Summary and future perspectives

This thesis focuses on the tumor-associated antigen NeuGc GM3 and its potential for cancer immunotherapy. Also discussed are the roles of NeuGc GM3 and hypoxia for tumor progression and malignancy. Although many aspects have been studied and a lot of data have been gathered, many questions, in particular essential questions regarding the mechanism of NeuGc GM3 expression, remain to be answered. How is this antigen, despite a deletion in the (human) gene encoding the enzyme necessary for its synthesis, is nevertheless expressed in cancer cells? We focused on tumor hypoxia and its potential for inducing NeuGc GM3 expression in HeLa cells. SILAC analysis uncovered more than 100 proteins with significantly altered expression levels as a response to hypoxic conditions, with simultaneously induced NeuGc GM3 expression. Some of these proteins were already known hypoxia-induced proteins, such as glycolytic proteins, resulting in up-regulated glycolysis. Many other proteins were newly discovered (as hypoxic-related proteins), among them clusters of translocases of the inner and outer mitochondrial membrane (TIMMs/TOMMs) and mitochondrial ribosomal proteins (MPRs). Proteins with a potential to be involved in the mechanism of NeuGc GM3 expression were also found up-regulated, including succinate dehydrogenase subunit B (SDHB), carrying the protein domain deleted in the enzyme required for synthesizing NeuGc GM3, and a GM3 ganglioside synthase (ST3GAL5/SLC35E1), catalyzing the addition of sialic acid to lactosylceramide. It would be interesting to study these proteins further, using gene silencing methods (e.g. siRNA or CRISPR) to investigate whether knock-down of these genes affect antigen expression. The SILAC and NeuGc GM3 expression studies were performed in HeLa cells. As a follow-up study, it would be interesting to extend these studies to several other cell lines of different origins. This could give a better understanding of tumor-specific response to hypoxia and hypoxia-induced expression of NeuGc GM3. In addition, alteration of membrane lipids as a response to hypoxia is poorly understood, and lipid analyses have been limited to detection by specific antibodies. It would be of great interest to investigate another unilamellar system developed in Kai Simons laboratory (Coskun & Simons 2010), where giant plasma membrane vesicles (GPMVs) (Baumgart et al. 2007) are derived from the membrane of cultured cells. This system would allow the analysis of the entire lipid

content of the plasma membrane of cells grown in normoxia or hypoxia, further advancing our understanding of tumor hypoxia, an important clinical challenge in the treatment of cancer.

The antibody 14F7, specifically targeting NeuGc GM3, has been important for the work discussed in this thesis - from the structural interaction with its antigen or anti-idiotypic antibody to investigations of its novel killing mechanism and finally its potential for altering EGFR activation. As the two complexes have so far resisted crystallization attempts, we employed MS-based approaches to achieve structural information. For the interaction between the antibody and the antigen, an approach based on oxidative footprinting was used, while for the interaction with the anti-idiotypic antibody, methods based on cross-linking mass spectrometry and SPR were employed. These results needs confirmation, in particular the cross-linking results. This can be achieved by studying the binding of Fab and scFv fragments from the two antibodies. Such fragments are currently produced in the lab, in a Master's project that I have co-supervised, and will be tested for binding in the near future. A single-molecule cryo electron microscopy (EM) approach (in collaboration with Caroline Jegerschöld at KTH), has also been initiated and may be followed up. Even though we are operating at the limits of this technique, we hope to gain further insight into how 14F7 binds to its antigen and anti-idiotypic antibody. However, as crystal structures of the complexes would provide information at the atomic level, this remains the ultimate goal, and crystallization attempts are still ongoing, now including the scFvs.

Finally, we investigated the effect of 14F7 on cancer cells by a SILAC study of HeLa cells, targeting the novel cell killing mechanism of 14F7. This work revealed several interesting proteins, with the potential of being involved in the formation of "pores" observed during the cell treatment process. The most promising proteins will be confirmed by Western blotting and is planned to be followed up by the use of super-resolution microscopy (in collaboration with NorMIC).

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